Beta-peptides as Inhibitors of Protein-Protein Interactions

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This dissertation describes the design and optimization of novel protein-protein interaction (PPI) inhibitors which consist of β-peptides. β-peptides are non-natural molecules which fold in a manner similar to natural peptides and proteins. The propensity for β-peptides to fold into a specific helical conformation, the 14-helix, was analyzed in a systematic way using spectroscopic methods and NMR structural analysis. Insights gained from the biophysical analyses were then applied to the design of inhibitors of the p53•hDM2 interaction, an important target for new cancer therapies. These molecules represent the first β-peptide helices which bind a cellular protein and inhibit a discrete PPI. Finally, the first high-throughput method for the discovery and optimization of β-peptide PPI inhibitors was developed, paving the way for broad evaluation of designed β-peptides as a new and promising class of PPI inhibitors.

Chapter 1 describes the design and optimization of miniature protein ligands for hDM2. A previously optimized miniature protein, p05, was further evolved using monovalent phage display in order to develop more potent hDM2 ligands. Several functional miniature proteins were gleaned from the selection. The most potent of these binds hDM2 and inhibits its interaction with a peptide derived from p53 (p53AD) roughly 8-fold more potently than p53AD itself, and roughly 4-fold more potently than the parent miniature protein p05.
Chapter 2 describes a rigorous host-guest analysis of 14-helix folding propensities of various $\beta^3$-amino acids. This work used a short $\beta$-peptide which is roughly 50% 14-helical in aqueous solution as a sensor for the effects of individual side chains on overall 14-helix structure. The results proved some previous hypotheses regarding 14-helix folding (including that residues branched at the first side chain carbon are potently 14-helix stabilizing) while presenting new hypotheses regarding how $\beta$-peptides fold. Most interestingly, the trends observed among $\beta^3$-amino acid 14-helix propensities differed widely from those observed among $\alpha$-amino acid $\alpha$-helix propensities. Analysis of $\beta$-peptide folding can thus address unresolved questions regarding $\alpha$-helix folding.

Chapter 3 details the rational design and characterization of 14-helical $\beta$-peptide ligands for hDM2, including the potent molecule $\text{\beta53-1}$. Careful controls and the methanol solution structure of $\text{\beta53-1}$ calculated using NMR data clearly demonstrate that the $\beta$-peptide folds into a 14-helix and presents the hDM2-binding epitope in a manner which recapitulates the p53AD functional epitope. Further analysis of the structure and experiments with constrained residues revealed that $\text{\beta53-1}$ and its variants do not resemble idealized or constrained 14-helices but rather have slightly distorted backbones. This distortion serves to make the $\text{\beta53-1}$ 14-helix more like an $\alpha$-helix with regard to presentation of side chains.

Chapter 4 highlights the development and application of a novel one-bead-one-$\beta$-peptide (OBO$\beta$) method for the rapid discovery and optimization of new $\beta$-peptide PPI inhibitors. This method is facile and versatile with respect to synthesis and screening, and can be easily scaled up and automated to test millions of compounds in a single week. The screening is tunable with respect to overall hit rate, which should allow
discovery of novel β-peptide ligands of moderate affinity as well as subsequent refinement of these leads into PPI inhibitors with nanomolar potency. The versatile and tunable nature of the assay should make it an invaluable tool in the effort to evaluate β-peptides as a class of PPI inhibitors for biological research and medicinal therapeutics.
Beta-peptides as inhibitors of protein-protein interactions

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Chapter 1 – Miniature protein inhibitors of the p53•hDM2 interaction

1.1 – The pre-organization principle

The concept of pre-organization as applied to molecular recognition has its roots in host-guest chemistry. In seminal work by Cram and colleagues it was demonstrated that spherands, large macropolycyclic molecules with structured cavities, could be designed to bind alkali metal ions with extraordinary affinity and specificity compared to similar classes of unstructured or partially structured molecules. The directness and elegance of these early host-guest systems allowed the meaningful separation of structure, embodied by rigid macrocycles and polycyclic groups, and function, embodied by appended functional groups which could wholly account for the metal binding activity. Measurement of binding affinities, examination of crystal structures of free hosts and bound complexes, and careful thermodynamics calculations enabled the quantitative comparison of enthalpic contacts between host molecule and guest ion, entropic factors regarding the host conformation, and solvation energies. A huge role was observed for host entropy in overall binding affinity and specificity. Observing the thermodynamic benefits of shaping spherands to interact with metal ions, Cram stated the principle of pre-organization as “the more highly hosts and guests are organized for binding and low solvation prior to their complexation, the more stable will be their complexes.”

This pre-organization principle has since been extended to describe increasingly complex models of molecular recognition. Often, pre-organization is applied through the appending of functional groups onto a molecule whose structure is well-characterized and
relatively rigid. This convenient separation of structure and function emphasizes the rigidity of the scaffold and the exact three-dimensional orientation of appended side chains, but de-emphasizes the identity of the scaffold itself. In a simplified view, then, all that matters is the shape and electronic character of the interaction surface (the \textit{functional epitope}); what is “behind the curtain” in terms of scaffold identity has little effect on binding thermodynamics. Though it broadly simplifies molecular recognition, this paradigm has successfully guided the design of molecules which recapitulate catalysis or binding of a natural functional epitope within a non-native context. These include supramolecular catalysts which orient a catalytic motif in a manner which mimics that of a natural enzyme,\textsuperscript{2} and chimeric proteins which have altered catalytic or recognition function that stems from substituting a portion of the protein with a homologous portion from a related protein.\textsuperscript{3}

In contrast to the case-by-case design of enzyme mimics and chimeric proteins, protein grafting provides a more general and ambitious approach to scaffold-based pre-organization of functional epitopes. Protein grafting is a strategy by which a natural functional epitope is recapitulated on an unrelated protein scaffold, one with similar local secondary structure but different tertiary structure. Perhaps the greatest advantage of protein grafting is the potential to develop molecules which present a large functional epitope using minimal scaffolding. The ideal end result of a protein grafting experiment is thus a small, well-structured molecule that reproduces (or improves upon) native function.

\subsection*{1.2 – Miniature proteins}
Our lab has established a general protein grafting strategy by which functional epitopes presented by an α-helix or a polyproline type II (PPII) helix in their native context are recapitulated on a small, structured scaffold. Capitalizing on the primary advantage of the protein grafting strategy, the scaffold (avian pancreatic polypeptide or aPP, shown in Figure 1.1A) and the resulting functional polypeptides are only 36 residues long. We have termed these molecules “miniature proteins” due to their small size and stable structures. The first attempt at designing an aPP-based miniature protein was performed by Neal Zondlo in our lab. The process involved “grafting” the DNA recognition epitope from the yeast transcription factor GCN4 onto the aPP scaffold. This was accomplished by aligning the sequence of the α-helix of aPP with the basic region of GCN4, an α-helical leucine zipper protein, and merging the sequences. The various mergers, termed PPBR polypeptides, represented various compromises between retaining residues known to be critical for aPP folding and including residues GCN4 uses to contact DNA. This design strategy ensured that, if the PPBR polypeptides folded into an aPP-like structure, the GCN4-derived residues would be positioned in a manner which re-formed the same epitope native GCN4 uses to bind half-site CRE DNA (hsCRE).

The PPBR polypeptide with the most favorable DNA-binding characteristics was termed PPBR4. PPBR4 indeed bound hsCRE with high affinity, but when analyzed by circular dichroism (CD) spectroscopy it showed α-helix structure only when bound to hsCRE. This belied the convenient assumption that structure, embodied by the aPP scaffold, and function, embodied by the residues which form the GCN4 DNA-binding epitope, were completely independent. Still, the high hsCRE affinity and α-helix
propensity of PPBR4 indicated that the GCN4 epitope was indeed being recapitulated on aPP’s α-helix. The lack of tertiary structure in the absence of DNA simply meant that further pre-organization was possible.

Developing a PPBR4 variant with tertiary structure in the absence of DNA meant repacking an aPP-like hydrophobic core. An iterative design process, even one assisted by time-intensive molecular dynamics simulations, would amount to educated guesswork. Instead, Jason Chin in our lab opted to use phage display optimization in an effort to select for folded molecules based upon affinity for target DNA. Not only would this be a quicker route to a true miniature protein, if the selected molecules possessed increased tertiary structure and increased DNA affinity it would validate the application of the pre-organization principle to the design of aPP-based miniature proteins. Several rounds of selection yielded p007, a miniature protein which is well-folded at room temperature and populates a fold nearly identical to that of aPP. In addition, it bound target DNA with extraordinary affinity and specificity, providing proof-of-principle that aPP can be altered to present an α-helix-derived functional epitope while maintaining its overall fold.

Since the development of p007, other miniature proteins based on aPP have been developed by our lab and others. Notably, we have designed and/or optimized miniature proteins which: bind protein targets; recognize specific DNA with only part of a functional epitope; bind an exceptionally flat protein interaction surface; bypass the need for phosphorylation in mediating a transcriptional activation event; distinguish among highly-related paralogs within a common protein family; recapitulate an epitope presented by a PPII helix; tune the specificity of a potent small
molecule kinase inhibitor;\textsuperscript{16} and deconstruct the folding, binding affinity, and binding specificity of p007 on a residue-by-residue basis.\textsuperscript{17} Taken as a whole, our research in the design, optimization, and application of miniature proteins demonstrates the promise of the protein grafting strategy. In the near future, miniature proteins should prove versatile tools in delineating and mediating cellular processes because they have tunable specificities and can be either expressed or made (and modified with useful fluorescent tracers or chemical functionalities) synthetically. In concert with emerging technologies in drug delivery and gene therapy, miniature proteins might eventually prove valuable as specificity agents, gene vectors, artificial transcription factors, or therapeutics in their own right.

1.3 – The p53•hDM2 interaction

After the development of p007, our lab quickly turned to the problem of protein recognition. Recognition of site-specific DNA was a burgeoning field, with several examples of manipulated protein systems\textsuperscript{18, 19} and small molecules\textsuperscript{20, 21} capable of binding specific DNA. However, protein recognition presented a far more difficult challenge. Protein-protein interactions (PPIs) had been recognized as key to nearly all cellular pathways, most notably signal transduction.\textsuperscript{22, 23} But while traditional small molecule drugs often nestle deep within an active site, almost in the interior of the target protein, PPIs occur over broad interfaces at protein surfaces.\textsuperscript{23-25} This difference sets up several opposing requirements for potential PPI inhibitors as therapeutics:\textsuperscript{26-28} such molecules would need to be large enough to present a large interaction surface, but small enough to
be orally bioavailable and cell-permeable; they would need to be hydrophobic enough to exclude water upon binding to the target surface, but hydrophilic enough to be soluble in aqueous solution; and they would need to be rigid enough to take advantage of pre-organization, but flexible enough to allow for universal strategies for PPI inhibition. It was clear a new class of molecules would be needed to target PPIs, and miniature proteins, with their minimal scaffolding and potentially large interaction surfaces, seemed suitable for the task. After careful consideration, we decided to target the oncogene product **human double minute 2** (hDM2) as one of our first forays into PPI inhibition using miniature proteins.

hDM2 is a primary regulator of the tumor suppressor protein p53, and is intimately involved in the proper and pathogenic function of this vital transcription factor. In response to cellular stress and/or DNA damage, the cell up-regulates p53, which then activates transcription of genes responsible for DNA repair, cell cycle arrest, and apoptosis (programmed cell death). This process ensures that cells with damaged or mutagenic DNA do not propagate. p53 levels in normal cells must be kept low – this is where hDM2 comes in. hDM2 down-regulates p53 through at least three known mechanisms: hDM2 binds p53’s N-terminal activation domain (p53AD) and physically blocks its transcription activity; hDM2 shuttles p53 out of the nucleus; and hDM2 ubiquitinates p53, marking it for degradation by the proteasome. hDM2 is transcribed from a p53-dependent promoter, which results in an autoinhibitory feedback loop that keeps cellular p53 levels low and permits normal cell growth; however, in some cancers hDM2 is over-expressed, preventing p53-mediated apoptosis. Molecules which inhibit the p53•hDM2 interaction have been shown to be potent anti-cancer agents in
cells that have wild-type p53. Inhibitors prevent hDM2 from down-regulating p53, and allow damaged cells to undergo cell cycle arrest and apoptosis. Thus, targeting the p53•hDM2 PPI has been a widely recognized avenue for developing new cancer therapies.

Due to its unique structural characteristics, the p53•hDM2 interaction has served an allegory of sorts for the larger problem of PPI inhibition. The crystal structure of hDM2 with p53AD bound was published in 1996 (shown in Figure 1.2), and revealed that the residues Phe19, Trp23, and Leu26 of p53 bury deep into a series of hydrophobic pockets within hDM2. Biochemical evidence backed up the assertion that the side chains of these three residues comprise the essential functional epitope for hDM2 recognition by p53. This raised hopes that small molecule inhibitors could be found, since the deep binding clefts and limited recognition surface render the p53•hDM2 PPI an intermediary between the deeply buried sites small molecules typically target and the broad surface patches more characteristic of PPIs. Non-peptidic inhibitors of the p53•hDM2 interaction have indeed been reported, including substituted benzodiazepines, sulfonamides, the fungal metabolite chlorfusin, and chalcone derivatives. Most recently, cis-imidazoline compounds termed nutlins were identified as the first highly potent small molecule antagonists of hDM2. Cell culture and mouse xenograft experiments testing the nutlins have demonstrated the potential for cancer therapies based on p53•hDM2 inhibition. The vast efforts behind developing small molecule inhibitors of the p53•hDM2 interaction appear to be only now paying off, and though they will hopefully be a great success for the treatment of cancer, they have not yet provided a general route for discovery of PPI inhibitors.
If targeting hDM2 is to be used as a stepping stone to the larger problem of PPI inhibition, studies by Garcia-Echeverria and coworkers are perhaps more informative.\textsuperscript{32, 48, 49} Their experiments capitalized on the fact that short p53AD-based peptides are unstructured in solution, but $\alpha$-helical upon binding. The pre-organization principle suggests that stabilization of the $\alpha$-helix structure of the native peptide should result in a lower entropic penalty upon binding, and thus higher affinity. Along these lines, they substituted the native p53AD peptide with unnatural amino acids and were able to improve its affinity for hDM2 by 1700-fold.\textsuperscript{49} This boost in affinity was a combination of pre-organization of the peptide in an $\alpha$-helical conformation and optimization of the electronic and physical shape of the epitope. Later studies confirmed that the high-affinity peptide was effective at raising p53 levels in tumor cells and promoting apoptosis.\textsuperscript{32} Our approach was analogous to that of Garcia-Echeverria and coworkers; however, instead of stabilizing the $\alpha$-helix structure of the native peptide using artificial amino acids, we sought to design aPP-based miniature proteins which pre-organize the p53AD epitope recognized by hDM2.

1.4 – Development of miniature protein inhibitors of the p53•hDM2 interaction

Our first miniature protein inhibitors of the p53•hDM2 interaction were designed and optimized by Dr. Reena Zutshi, whose work is described in this section. The sequences of p53AD and aPP were aligned and merged in a manner similar to the genesis of the PPBR polypeptides. As shown in Figure 1.1B, residues critical for hDM2 recognition were retained, as were residues critical for aPP folding. Since it was unclear whether
substitution of p53AD-derived residues within aPP would disrupt the tertiary fold, as happened with PPBR4, five aPP-derived residues on the α-helix known not to play a significant role in folding were identified for optimization of the overall fold. Due to their proximity to the p53AD-derived residues, randomizing these five residues also had potential for creating new contacts with hDM2, or otherwise favorably altering the shape of the functional epitope. An M13 phage display library was constructed which displayed the merged sequence, with the selected five residues varied over all 20 naturally occurring amino acids (RZ-lib, Figure 1.1B). After construction and transformation into E. Coli TG-1 cells, the library contained $6 \times 10^7$ unique transformants, ensuring that it would evaluate DNA sequence space with > 83% confidence.

The RZ-lib library was panned for three rounds against GST-hDM2$_{1-188}$ immobilized on the surface of glutathione-coated wells. A 100-fold increase in overall library affinity was observed over the course of the three rounds (as determined by comparing titers of phage retained after each round). Clones from the second and third rounds of panning were isolated and sequenced, and several prominent sequences were synthesized. Peptides were synthesized with a C-terminal cysteine residue to enable labeling with 5-iodoacetamidofluorescein. All synthesized library members bound hDM2$_{1-188}$ as judged by fluorescence polarization (FP) assay, with equilibrium dissociation constants ($K_d$’s) ranging from 2800 to 99 nM. The polypeptide with the highest affinity, p05, had a measured $K_d$ for GST-hDM2$_{1-188}$ of 99 ± 11 nM, which compared well to the measured $K_d$ for the p53AD$_{15-31}$•GST-hDM2$_{1-188}$ complex (261 ± 59 nM; this value matches similar values from the literature$^{39, 40}$). The CD spectrum of p05 was characterized by negative
ellipticity at 208 and 222 nm that was comparable to that of aPP, indicating significant α-helix structure. p05 underwent a cooperative melting transition (T_m) at 47 °C, indicating the presence of a stable tertiary fold. Finally, when dye-labeled p53AD_{15-31} was incubated with GST-hDM2_{1-188} and various concentrations of p05 were added (a FP competition assay), p05 inhibited the p53AD•hDM2 interaction with an observed K_i of 722 nM. This value, while nearly 8-fold higher than the actual K_d, indicates that p05 is indeed a miniature protein PPI inhibitor and recapitulates the epitope by which hDM2 recognizes p53AD.

**Materials and Methods**

Note: This section was adopted from material written by Dr. Reena Zutshi.

*Expression and purification of GST-hDM2*

The GST-hDM2 fusion protein used in the above experiments carried out by Dr. Reena Zutshi consisted of a 26 kDa glutathione S-transferase (GST) protein fused to the N-terminus of residues 1-188 of human MDM2 (hDM2_{1-188}) and was prepared by over-expression of ‘clone G’ reported by Lane and coworkers.\textsuperscript{50} Briefly, *E. coli* BL21 cells were transformed with ‘clone G’, grown at 25 °C, and protein expression induced at log-phase (OD_{600} = 0.8) with 1 mM IPTG. After three hours at 25 °C, the cells were harvested, resuspended in buffer A (0.5 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, 10 mM 2-mercaptoethanol, pH 7.3) and lysed by sonication. GST-hDM2_{1-188} was purified essentially as described\textsuperscript{50} with a glutathione
sepharose 4B column (Pharmacia) and eluted using buffer B (50 mM Tris-HCl, 10 mM reduced glutathione, 0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM 2-mercaptoethanol, pH 8.0). Excess salt was removed from the eluant using a NAP-10 column and the desired product purified further on a Mono Q column (Pharmacia) by FPLC. The purified protein was characterized by amino acid analysis (AAA), SDS-PAGE and MALDI-TOF mass spectrometry. Purified GST-hDM21-188 was flash frozen in liquid nitrogen and stored at -70 °C until use.

Selection of GST-hDM2 ligands from RZ-lib

A 200 µL aliquot of a 5 µg/mL solution of GST-hDM21-188 in PBS was added to each of the wells of a glutathione-coated microtiter plates (Pierce) at 4 °C and the immobilization reaction was incubated overnight. Excess protein was removed by washing each well three times with 250 µL TBST and the remaining binding sites in each well were blocked upon treatment with 3% w/v non-fat dry milk (Carnation) in TBST for 45 minutes at 4 °C. Excess milk protein was removed by washing each well 5 times with TBST. Phage were grown as described. 4, 7, 8 200 µL of phage in TBST was added to each well and incubated at 4 °C for 3 hours. Weakly bound phage were removed by washing the wells 10 times with 250 µL TBST for 1 minute per wash in round 1 and for 5 minutes per wash in rounds 2 and 3. Tightly bound phage were then eluted by incubating the well for 20 min at 4 °C using 200 µL 0.1 N HCl-glycine, pH 2.2 containing 1 mg/mL BSA. The eluted phage were neutralized with 4.5 µL 2 M Tris, pH 9.5 and used to infect TG-1 E. coli cells grown to OD600 = 0.8. After 1 hour, cells were plated on SOB-AG (20 g tryptone, 5 g yeast, 0.5 g NaCl, 2 g MgCl2, 2 g glucose, 100 µg/mL ampicillin) plates at
different serial dilutions. Bacterial colonies were grown overnight, and DNA was purified by alkaline lysis (Wizard Miniprep-Promega) and sequenced.

\[\text{Equation used to determine the affinity of peptide} \cdot \text{GST-hDM2}_{1-188} \text{ or miniature protein} \cdot \text{GST-hDM2}_{1-188} \text{ complexes} \]

The equilibrium dissociation constant of a ligand-protein complex \((LP)\) may be determined by fluorescence polarization (FP) analysis by application of the following equation:\(^{51}\)

\[
F = F_L + \left( \frac{F_{LP} - F_L}{2[L]_T} \right) \left( [L]_T + [P]_T + K_d - \sqrt{([L]_T + [P]_T + K_d)^2 - 4[L]_T[P]_T} \right)
\]

where

- \(K_d\) = the equilibrium dissociation constant of the \(LP\) complex,
- \(F_L\) = the fluorescence polarization of free ligand \(L\),
- \(F_{LP}\) = the observed fluorescence polarization of the \(LP\) complex,
- \([L]_T\) = total concentration of ligand \(L\), and
- \([P]_T\) = total concentration of protein \(P\).

\[\text{Equations used to evaluate the concentration-dependent competition between peptides/miniature proteins and p53AD for GST} \cdot \text{hDM2}_{1-188} \]

The concentration at which inhibition is half its maximum value (the \(IC_{50}\)) may be calculated from a FP competition assay in which the protein \(P\) can alternatively complex with labeled ligand \(L\) or unlabeled inhibitor \(I\), but not both, using the following equation:\(^{52,53}\)
\[ F = F_L + \left( \frac{F_{LP} - F_L}{1 + (IC_{50}/[I])^n} \right) \]

where

\([I]\) = Inhibitor concentration, and

\(n\) = Hill coefficient

The apparent affinity of the inhibitor I for the protein target P \((K_i)\) can be calculated from the same FP competition assay by applying the following equation: \(^{52, 53}\)

\[ K_i = \frac{IC_{50}}{1 + [L]_T/K_{d,L}} \]

where

\([L]_T\) = total concentration of labeled ligand L, and

\(K_{d,L}\) = measured \(K_d\) of the ligand L for the protein P.

1.5 – Development of second-generation miniature protein inhibitors of the p53•hDM2 interaction

Note: This work was performed in conjunction with the launch of the Chem427 course, entitled Chemical Biology Lab, in spring 2004. I designed the project and planned and supervised its execution. Parts of the Kunkel mutagenesis, phage display panning, and HPLC purification steps were performed with the help of a team of four sophomore undergraduates: Fei Ann Ran, Mingtatt Cheah, Taritree Wongjirad, and Rachel Webman.
Library design and construction

Since p05 only bound hDM2\textsubscript{1-188} with a $K_d$ 2.6-fold lower than that of p53AD\textsubscript{15-31}, there was room for improvement. Two phage display libraries were designed to further optimize p05 for hDM2 binding. The first, termed ST-lib, randomized four residues on the polyproline helix portion of p05 (Figure 1.1B). The strategy behind the design of ST-lib was to promote a better overall tertiary fold, in keeping with the general pre-organization strategy. This had worked previously, most notably in the optimization of PPBR4 to yield p007; however, since p05 appeared to be nearly as well-structured as aPP at room temperature (p05 and aPP have $T_m$ values of 47 °C and 60 °C, respectively, as monitored by CD spectroscopy), p05 was not quite analogous to PPBR4 with regards to pre-organization. Still, further pre-organization or fine-tuning of epitope shape by altering contacts between the $\alpha$-helix and the PPII helix was a promising avenue for optimization. The second designed library, termed RG-lib, randomized four residues C-terminal to the recognition motif originally grafted from p53AD (Figure 1.1B). RG-lib was designed to expand the functional epitope, with the expectation that new contacts would lead to higher affinity.

One significant change that was made prior to library construction was a modification to the sequence of p05 which better reflected the protein actually displayed on the phage (and thus the exact sequence being selected). The altered sequence was termed pZut. Note that the sequence for pZut (as coded for in pCANTAB\_pZut and as synthesized from this point forward) is identical to that of p05, except that p05 possesses the sequence ENNVC at its C-terminus while pZut has the sequence TRHRYAAAC at its C-terminus (Figure 1.1B). The pZut C-terminal sequence possesses the TRHRY sequence native to
aPP, as well as the AAA sequence which is appended when the protein is displayed on phage. Previous work in our lab had indicated that inclusion of the AAA after the aPP-like sequence can be beneficial to miniature protein function. Finally, since the THRHY region absent from p05 was being modified in RG-lib, it was necessary to use pZut as the “starting point” for the second-generation optimizations.

The libraries were constructed from the pCANTAB_aPP phagemid originally cloned by Jason Chin. First, pCANTAB_aPP needed to be altered to obtain the starting template, pCANTAB_pZut. This involved changing 17 base pairs in a 33-base pair span, too difficult for Quickchange (Stratagene) mutagenesis commonly used for mutation of inserts. Instead, the phagemid was altered by cloning. Two complementary oligos that reflected the desired sequence were ordered from the Keck facility at Yale, with ends that mimicked restriction by Bgl II and Not I (Figure 1.3A). These were annealed and then incubated with T4 DNA ligase and pCANTAB_aPP that had been doubly digested with Bgl II and Not I. The resulting closed circular DNA was transformed via electroporation into XL1-Blue E. Coli cells. Colonies that grew on LB-Amp plates were picked, miniprepped (Qiagen), and ten were tested by double digestion with Kpn I and Xho I. Xho I makes a single cut at a position far distant from the insert region, while Kpn I is a site found in the pCANTAB_pZut insert region but not in pCANTAB_aPP (Figure 1.3). This facilitated the use of the double digest as a diagnostic of whether the plasmid had re-ligated properly (the Xho I cut), and whether the insert was incorporated (the Kpn I cut). Nine of ten clones were doubly cut (as would be expected if the cloning were successful), so several of these were sent for sequencing. The clone with the cleanest
*pCANTAB_pZut* sequence (in terms of sequencing accuracy and efficiency) was used for library construction.

For Kunkel mutagenesis, I ordered and PAGE-purified randomized oligos representing the complementary sequence to the portion to be varied for each library (Figure 1.3B). The oligos were doped as to lessen the proportion of G’s, because previous libraries had shown an excess of C’s in the coding strand. The template was prepared by first transforming *pCANTAB_pZut* into CJ236 *E. Coli* cells (a *duf* *ung* strain), then grown in the presence of deoxyuridine. The cells were then infected with VCSM13 helper phage and allowed to grow overnight at 37 °C. The following morning, phage were harvested and the phagemid purified using a Qiagen spin M13 kit, yielding a single-stranded deoxyuridine-containing DNA template (ss-dU-DNA) suitable for Kunkel mutagenesis. The purified oligos were separately phosphorylated using T4 polynucleotide kinase, and each was annealed to the ss-dU-DNA template. T7 DNA polymerase and T4 DNA ligase were used to extend the oligo primers, yielding a double-stranded plasmid. This plasmid was then transformed into XL1-Blue cells, inside which the parent strand was destroyed due to its incorporation of dU, leaving only the mutagenized strand. Transformations of the mutagenized library phagemids were extremely efficient, with 2 x 10^8 independent transformants from a single transformation. Statistical analysis of library coverage employed the following relation: confidence level = 1 – exp(-actual library size / theoretical library diversity). By this measurement, both libraries represented the complete range of theoretical diversity (that is, all 32^4 possible combinations among the four NNS codons within each plasmid) with 100% confidence.
In fact, even one tenth of the amount obtained for each library covered theoretical diversity with virtual certainty.

**Library evaluation, panning, and analysis of selected sequences**

The library was evaluated by plating the cells, picking ten colonies from each library at random, miniprepping (Qiagen) them and sending the resulting DNA for sequencing. The sequences were all sufficiently random at the DNA level, with an almost ideal distribution of each of the four residues – this validated the doping scheme and ensured that we were starting with a diverse collection of sequences. We (the undergraduates and I) proceeded to pan the library against GST-hDM2 on glutathione-coated plates as described in materials and methods later in this section. The progress of the selections was monitored by titering input and output phage after each round of panning, and calculating the overall proportion of phage retained for each round (phage retained / total phage added to well = percent retention). These were compared to a negative control, namely aPP-displaying phage produced and panned along with each library at each round. The percent retentions calculated for each round of panning, along with specific round-by-round panning conditions, are shown in Figure 1.4.

Thirty clones were picked from each library for sequencing. Clones were picked primarily from round 3 for the ST-lib selection and from round 4 for the RG-lib selection, because the fourth round of ST-lib panning yielded only four colonies. The sequences for ST-lib were dominated by a single sequence that did not resemble the original pZut-based library. A BLAST search\(^{54}\) revealed that the sequence is actually that of the p3 protein of M13 itself. The most likely explanation for the dominance of this sequence is that a
recombination event resulted in the removal of the insert region but retention of the primer region. This would explain why the stringent conditions of round 4 resulted in so few colonies, because by round 4, despite the selective growth and amplification advantage the recombined phage would have possessed, they did not stick to the plates under the more stringent conditions and thus got washed away. Of the remaining sequences, some were members of RG-lib, while others were pCANTAB_aPP (which was panned alongside the libraries as a negative control), revealing contamination (the bane of any good phage display experiment!). In the end, only one genuine ST-lib library member was recovered from later panning rounds (ST1, sequence shown in Figure 1.1B). The RG-lib selection also had some pCANTAB_aPP among the sequenced clones, as well as several members that did not sequence very well. The predominant trend among the sequenceable RG-lib library members was the presence of stop codons, which in hindsight was to be expected. Since pZut is already a good ligand for hDM2, the selection and amplification pressures could either evolve higher affinity using the randomized portion, or cut the protein there and “settle” for a mostly intact pZut. In all, six sequences were identified as being RG-lib library members with no stop codons. The sequences are shown in Figure 1.1B and include polypeptides RG1 through RG5. Notably, one of the six sequences pulled from the RG-lib library was pZut itself, implying that pZut and its derivatives might be nearly fully optimized for hDM2 recognition, at least with respect to their C-terminal portions.

Initial examination of the sequences of the selected second-generation hDM2-binding miniature proteins led to some closer insights. The sequence of ST1 was interesting if only due to the large residues and different functionalities selected. The sequences pulled
from later round of *RG-lib* panning, by contrast, showed some beginning of consensus among them. For instance, among RG1 through RG4, we observed tyrosine or serine in the first position, histidine or valine or leucine in the second, arginine, tyrosine, or asparagine in the third, and primarily asparagine in the fourth position. The final novel miniature protein, RG5, possesses alanine in three of four positions, yielding a C-terminal cap of seven alanines within a ten-residue span (and ten residues that are either alanine or leucine within a twelve-residue span). This implies that RG5 may have been selected due to overall stabilization of the alpha-helix and not due to specific contacts made with hDM2.

The seven sequences pulled from the *ST-lib* and *RG-lib* selections (ST1, RG1-5, and pZut) were synthesized with the terminal AAA sequence present and a cysteine residue on each C-terminus for later labeling with 5-iodoacetamidofluorescein (Figure 1.1B). Unfortunately, after extensive HPLC and MALDI analysis of HLPC fractions, I was unable to isolate significant amounts of fluorescein-labeled RG2. The remaining fluorescein-labeled miniproteins, plus some older aPPflu, were analyzed by CD, tested for direct binding to hDM2 by fluorescence polarization (FP) analysis and for inhibition of the p53AD15-31•hDM21-188 interaction. Graphs of the data and relevant $K_d$ and $IC_{50}$ values are given in Figure 1.5. All second-generation miniproteins except ST1 bound hDM2 better than the parent peptide, pZut, indicating that the *RG-lib* selection appeared to have worked as planned. The parent scaffold, aPP, has no detectable affinity for hDM2 in the nanomolar-low micromolar concentration range. The best miniature protein hDM2 ligand we have developed so far, RG4, has a $K_d$ of $35 \pm 3$ nM for hDM2 binding and an $IC_{50}$ of $1.6 \pm 0.2$ µM for p53AD15-31•hDM2 inhibition, which are roughly 6-fold.
more potent than similar values for p53AD_{15-31}. The magnitude of this difference compares favorably to work in which artificial amino acids were used to stabilize the α-helix structure of p53AD-derived peptides.\textsuperscript{49}

**Outlook**

Future experiments with these miniature proteins will involve testing their ability to inhibit the p53•hDM2 interaction \textit{in vivo}. Due to the versatility of miniature proteins, this could be done in three ways. Miniature proteins could be added to cultured tumor cells using lipophilic protein transfection agents (Gene Therapy Systems, Inc.) or peptidic carriers,\textsuperscript{55} they could be synthesized with a peptide transducing domain covalently attached,\textsuperscript{56} or they could be introduced genetically by cloning their sequences into a plasmid and transfecting the plasmid into the cell line. In any of these cases, the \textit{in vivo} effects of miniature proteins such as pZut and RG5 could be compared with those of p53AD_{15-31} and other known inhibitors of the p53•hDM2 interaction. Specifically, as a first step, their ability to induce apoptosis could be measured using a multitude of fluorescence-based assays,\textsuperscript{57-59} and their ability to up-regulate p53 and p53-transcribed gene products could be measured by Western blots. If they are able to inhibit the p53•hDM2 interaction in cell culture, previous studies\textsuperscript{32-35} imply that they should be able to rescue p53 function in a dose- and time-dependent manner.

**Materials and Methods**

*Generation of pCANTAB pZut from pCANTAB_aPP*
The insert regions for plasmids \textit{pCANTAB\_aPP} and \textit{pCANTAB\_pZut} are shown in Figure 1.3A. The same figure shows the sequences of the oligonucleotides used to alter \textit{pCANTAB\_aPP} in order to furnish \textit{pCANTAB\_pZut}. These oligonucleotides were synthesized and PAGE purified by the Keck facility. They were annealed by mixing in a 1:1 ratio 200 pmoles of each oligo in a 30 \( \mu \text{L} \) total volume. The annealing reaction was heated to 95 \(^\circ\text{C}\) for 10 minutes, then slow cooled for 1 hour. 1 pmole was run on a 3% agarose gel to verify annealing. \textit{pCANTAB\_aPP} was restricted by adding to 1 \( \mu \text{g} \) plasmid 2 units each of Bgl II, Pst I, and Not I in NEB 3 buffer with BSA added. (Note: the Pst I was included to minimize re-annealing of the cut-out insert after restriction) After 1 hour, the reaction was cleaned up using a Qiagen PCR purification kit and run on a 1% agarose gel to verify restriction. Finally, \( \sim 0.3 \) \( \mu \text{g} \) (\( \sim 0.1 \) pmole) of the restricted plasmid was added to 1 pmole annealed insert in the presence of T4 DNA ligase in T4 DNA ligase buffer, in a total reaction volume of 20 \( \mu \text{L} \). Ligation was allowed to proceed for 15 minutes at room temperature, after which the reaction was cleaned up using the PCR purification kit. Ligation was assessed using a 1% agarose gel. The ligated plasmid was transformed by electroporation into electrocompetent \textit{E. Coli} XL1-Blue cells, and subsequent plating showed a multitude of colonies. Eight were sent for sequencing; all showed successful ligation. The transformed strain with the cleanest sequencing result was used in subsequent experiments.

\textit{Kunkel mutagenesis and library preparation}

The oligonucleotides used for Kunkel mutagenesis were ordered with the doping scheme indicated in the figure so as to reduce the proportion of G’s in the randomized
positions, since previous work had shown a bias towards C in the final coding strand. Also, 15 to 20 base pair overhangs were incorporated to ensure easy annealing. The oligos *rgknnk* and *stknk* were purified on 12% PAGE gels and bands were cut conservatively to minimize *n*-1 contaminants. The cut gel was crushed, eluted in TE overnight, desalted on NAP columns, and lyophilized. Single-stranded deoxyriboeuridine-containing *pCANTAB_pZut* (the ss-dU-DNA template) was produced by transforming purified *pCANTAB_pZut* into *E. Coli* CJ236 cells, which lack the *dut* and *ung* genes required to prevent synthesis and incorporation of deoxyriboeuridine. A single colony was picked from the subsequent plating, and grown at 37 °C with shaking for 6 hours in 1 mL 2XYT with 50 mg/mL carbenicillin and 10 µL 1 mg/mL chloramphenicol. Roughly 1 x 10^{11} cfu/mL of VCSM13 helper phage was added, and the cells were incubated with the phage for 10 minutes at 37 °C, shaking. 250 µL of the phage/CJ236 mixture was then transferred to 30 mLs 2XYT with 0.01 mg/mL carbenicillin and 0.33 µg/mL uridine. Phage were grown in the 30 mL culture overnight at 37 °C, shaking. Cells were then spun down and the supernatant removed. Phage were precipitated by adding 1/5 volume PEG/NaCl solution and incubating for 15 minutes on ice. The precipitated phage sample was spun down and the pellet was resuspended in 0.5 mL PBS. The phage sample was re-spun in a tabletop microcentrifuge and supernatant retained, to remove any remaining cell debris. Phage DNA (ss-dU-*pCANTAB_pZut*) was purified using the Qiagen M13 Purification kit.

The mutagenesis was performed in a series of three steps. First, each oligo was phosphorylated (0.6 µg oligo, 2 µL 10x TM buffer, 2 µL 10 mM ATP, 1 µL 100 mM DTT, sterile water to 20 µL total; incubated one hour at 37 °C). Next, the phosphorylated
oligo was annealed to the ss-dU-template (20 µg ss-dU-pCANTAB_pZut and 25 µL 10x TM buffer added directly to the phosphorylation reaction, sterile water to 250 µL) by heating to 90 °C for 2 minutes, then cooling at 50 °C for 3 minutes, then cooling at room temperature for 5 minutes. Finally, the closed circular double-stranded DNA library was synthesized directly from the annealed oligo•template (10 µL 10 mM ATP, 10 µL dNTP solution containing 25 mM each of ATP, GTP, CTP, and TTP, 15 µL 100 mM DTT, 30 units T4 DNA ligase and 30 units T7 DNA polymerase; added directly to annealing reaction and incubated 3-4 hours at room temperature). The double-stranded library phagemid was purified using the QIAquick PCR purification kit using a final elution volume of 35 µL sterile water. 1 µL of the final library DNA was then added to 40 µL of E. Coli XL1-Blue MRF’ supercompetent cells, incubated on ice 10 minutes, and transformed by electroporation (2.5 kV resistance, 1.38 kV charging voltage, 129 Ω resistance timing, 50 µF capacitance). Immediately after charging was over, 1 mL 2XYT with 5% v/v of a 40% glucose solution (2XYT-G) was added. An additional 4 mLs of the 2XYT-G medium was added upon transfer to a 15 mL tube, and the cells were incubated at 37 °C for 30 minutes with shaking. Cells were plated in dilutions up to 10^-8 on SOB-CG plates to determine transformation efficiency. Individual clones were also picked and sent for sequencing to verify that the randomization was unbiased at the amino acid level and at the DNA level. Stocks of the initial transformation (panning round zero of libraries ST-lib and RG-lib) were frozen at -80 °C in 33% glycerol.

*Phage display panning*
Glycerol stocks of the previous round’s output of each library (ST-lib or RG-lib) as well as a single colony of pCANTAB_apP for a negative control were added to 10 mL 2XYT-CG and incubated at 37 °C, shaking, until OD_{600} was between 0.3 and 0.6 (early to middle log phase). Cells were spun down and resuspended in 2XYT-C, and helper phage were added to a final concentration of roughly 1 x 10^{11} pfu/mL. Cells were then incubated overnight at 37 °C, with shaking. Also, wells of glutathione-coated 8-well strip plates (Pierce) were washed three times for two minutes each wash with 200 µL TBST. 200 µL of a 4.6 µg/mL GST-hDM2 solution was added to each well and wells were incubated overnight at 4 °C, with shaking.

The next morning the cells were pelleted and the supernatant filtered through a 0.45 µm filter to remove cells and cell debris. 2 mLs PEG/NaCl solution were added and the solution was mixed by inversion, then incubated 10 minutes at room temperature. Phage solution was then divided among microcentrifuge tubes and spun at maximum speed for 10 minutes. Supernatant was discarded into bleach, tubes were dried by tapping upside-down on lab mat, and phage pellets were dissolved in 0.5 mL TBST. Phage concentration was estimated by UV absorption at 268 nm using a conversion factor of A_{268} = 1.0 indicative of 5 x 10^{12} phage per mL. Also, wells were further prepared for panning by washing 3 x 2 minutes with 200 µL TBST, adding 200 µL 2% w/v blocking agent (alternating BSA and dry milk) and incubating at 4 °C for 60 minutes, then washing another 3 x 2 minutes with 200 µL TBST. Phage were diluted to a concentration of 5 x 10^{12} phage per mL, then 200 µL (1 x 10^{12} phage) were added to each panning well. Figure 1.4C lists the round-by-round panning conditions used (panning temperature, duration of phage incubation with target protein, blocking agent, and number and
duration of washes following incubation with target). After washes, phage were eluted by adding 200 µL 0.1 M glycine buffer, pH 2.2, and incubating for 20 minutes. Eluted phage solutions were pH neutralized by adding 5 µL 2 M Tris buffer, pH = 9.2, and typically stored at 4 °C for two days until the next lab session. At that time, they were titered by making serial dilutions of each phage stock (library input, library output, aPP input, and aPP output) and incubating 20 µL of each dilution with 1 mL log-phase *E. Coli* XL1-Blue MRF’ cells for 1 hour at 37 °C, shaking. Each cell solution was then plated onto SOB-CG plates, where only infected (and thus carbenicillin-resistant) cells would thrive. The following day, cells were counted and titers back-calculated to determine total phage input and output.

**Buffers, solutions, and recipes**

| 2XYT (750 mL): 12 g tryptone | TAE: (50x, 500 mL) 121 g Tris base | TBST solution: Add 0.1% v/v Tween-20 to TBS. | CHCA matrix: 10 mg CHCA 400 µL water 500 µL CH₃CN 100 µL 3% TFA Vortex 1 min. Spin 1 min. Use supernatant. |
| 7.5 g yeast extract | 28.6 mL glacial acetic acid | 2 M MgCl₂ solution: 40.66 g MgCl₂•H₂O | Carbenicillin or kanamycin solution: 0.5 g carbenicillin or kanamycin sulfate. Water to 10 mL. Sterile filter. Aliquot in 1mL and 100 µL amounts. Store at -20°C. |
| 3.75 g NaCl | 50 mL 0.5 M EDTA | Water to 1L. Autoclave. | |
| Water to 1L. Autoclave. | Water to 1L. Autoclave. | |
| 50% glycerol solution: 100 mL glycerol. 100 mL water. Autoclave. | 2 M MgCl₂ solution: 40.66 g MgCl₂•H₂O | Water to 100mL. Autoclave. | |
| 1 M Tris solution (1L): 121.14 g Tris base. pH to 7.5, autoclave. | TBS solution (1L): 10 mL 1M Tris 8.74 g (150 mM) NaCl | Glycine buffer: 1.5 g glycine Water to 90 mL. pH to 2.2 with HCl. Raise to 100 mL, sterile filter, store at 4°C. | |
| pH to 7.5, autoclave. | Autoclave. | | |
**TM buffer (10x, 50 mL):**
25 mL 1 M Tris
2.5 mL 2M MgCl<sub>2</sub>
Water to 50 mL. St. filter.

**PEG/NaCl solution:**
40 g PEG 8000
29.2 g NaCl
Water to 200mL. Autoclave.

**40% glucose solution:**
200 g dextrose.
Water to 500 mL. Heat and stir to dissolve. Sterile filter. Store at 4°C

**SOB-CG plates (1 L):**
20 g tryptone
15 g agar
5 g yeast extract
0.5 g NaCl
Water to 950L. Autoclave.
Cool to ~60°C.
Add: 1 mL 1000x Car.
5 mL 2M MgCl<sub>2</sub>
50 mL 40% glucose.
Pour in petri plates, let solidify. Store inverted at 4°C.

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**Optimized GST-hDM2 and hDM2 overexpression/purification protocols**

The same clone of GST-hDM2 used by Dr. Reena Zutshi in the experiments detailed in section 1.4 was also used in these experiments. However, the overexpression and purification was streamlined. *E. Coli* BL21 cells transformed with the GST-hDM2 plasmid<sup>50</sup> were grown at 37 °C until OD<sub>600</sub> reached 0.4 – 0.6. IPTG was added to a final concentration of 0.2 mM and cells were incubated for a further 3-4 hours at 37 °C. Cells were then harvested, spun down, frozen, thawed, resuspended in 50 mL per 1 L culture of cold hDM2 buffer (0.5 M NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, 2 mM DTT, pH 7.3) and sonicated. Lysate was added to 2 mL glutathione agarose slurry (Pharmacia, prepared as per manufacturer’s instructions) per 100 mL lysate and incubated with gentle agitation at 4 °C for 1-2 hours. A fritted disposable polypropylene column was used to filter the agarose and wash 3 times with 5-10 mLs cold hDM2 buffer, then twice with 5-10 mLs of room-temperature hDM2 buffer. Then, if the GST-hDM2<sub>1-188</sub> fusion protein was desired, manufacturer-supplied glutathione buffer was used to elute the protein as per manufacturer’s instructions. If hDM2<sub>1-188</sub> without the GST was desired, as for *in vitro* binding experiments, 8 units of biotinylated thrombin (Pharmacia) were added directly to the beads in 1.0 mLs hDM2 buffer and
incubated at room temperature for 2 hours, with gentle agitation. Thrombin was then removed by incubation with streptavidin-agarose (Pharmacia) as per manufacturer’s instructions. FPLC was found to be unnecessary for further purification of both GST-hDM2 and hDM2 prepared in this manner. The purified protein was characterized by amino acid analysis (AAA), SDS-PAGE and MALDI-TOF mass spectrometry. Purified GST-hDM2 and hDM2 were flash frozen in liquid nitrogen and stored at -70 °C until use.

**Fluorescence polarization (FP) binding and inhibition assays**

Fluorescence polarization experiments were performed at 25 °C in 384-well plates (MJ Research, Waltham, MA). For direct binding measurements, 10 µL serial dilutions of hDM2 were made in PBS buffer, pH 7.2, and an aliquot of fluorescently labeled peptide was added to a final concentration of 5 nM (for p53ADflu) or 25 nM (for fluorescein-labeled miniature proteins), to a total volume of 12 µL. The binding reaction was incubated for 30 min at room temperature, then read on an Analyst AD instrument (Molecular Devices). Thirty minutes was a sufficient length of time for the binding reaction to reach equilibrium, as judged by an absence of change in observed polarization values after 60 and 90 minutes. For competition experiments, serial dilutions of short peptide molecules like p53AD15-31flu were incubated with 0.5 µM hDM2 and 25 nM fluorescein-labeled p53AD for 30 min at room temperature, in a total volume of 12 µL. Miniature protein inhibitors were incubated at various concentrations with 0.5 µM hDM21-188 and 25 nM p53AD15-31flu in PBS, pH 7.4 with 1 mM DTT and 2% DMSO until equilibrium was reached. Selectivity of miniature proteins was explored by incubating fluorescein-labeled KID119-148 (phosphorylated on Ser133) with His-tagged KIX586-672 at
final concentrations of 27 nM and 1.5 µM, respectively, in the presence of: β53-1 (orange), β53-3 (blue), and a phosphorylated miniature protein ligand developed in our lab, PPKID4P (green) as a positive control. His-KIX586-672, PPKID4P, and KID119-148 were obtained as described and were a generous gift from Heather Volkman.
Figure 1.1 – (A) X-ray structure of aPP.\(^5\) Backbone ribbon (gray) is shown along with residues substituted with the p53AD recognition epitope (red) and residues varied in \(RZ\)-\(lib\), \(ST\)-\(lib\), and \(RG\)-\(lib\) (purple, blue, and orange, respectively). (B) Sequences of polypeptides and phage-displayed libraries used in this study. aPP secondary structure in each region is indicated at the top. Synthesized peptides have free N-terminals and amidated C-terminals. Residue coloration is the same as in (A).
Figure 1.2 – Crystal structure of p53AD_{15-31} (red) bound to hDM2 (blue; the deep p53AD-binding cleft is shown in yellow).\textsuperscript{39} Image generated using PYMOL.\textsuperscript{61}
Figure 1.3: (A) Insert regions from pCANTAB_aPP and pCANTAB_pZut, and the oligonucleotides used in the generation of pCANTAB_pZut from pCANTAB_aPP via cloning. (B) Insert regions from pCANTAB_pZut and the two phagemid libraries based thereon (ST-lib and RG-lib), with the doping scheme used in the generation of the libraries via Kunkel mutagenesis.
**Figure 1.4**: Panning retentions for each round of panning for libraries *ST-lib* (blue bars) and *RG-lib* (green bars). aPP-displaying phage were panned alongside each library during each round of panning as a control (retentions shown as red bars). Panning conditions for each round are shown.

<table>
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<th>Round</th>
<th>Temperature</th>
<th>Incubation</th>
<th>Blocking Agent</th>
<th>Washes</th>
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<td>2 hrs</td>
<td>Milk</td>
<td>2 x 2 mins</td>
</tr>
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<td>Round 4</td>
<td>22 °C</td>
<td>30 mins</td>
<td>BSA</td>
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Figure 1.5 – (A) Direct binding of fluorescein-labeled miniature proteins to hDM2\textsubscript{1-188} as monitored by fluorescence polarization (FP). (B) Inhibition of p53AD\textsubscript{15-31}\textsuperscript{flu}•hDM2\textsubscript{1-188} complexation by unlabeled peptides as monitored by FP. (C) CD spectra of engineered 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. (D) K\textsubscript{d}, IC\textsubscript{50}, and MRE\textsubscript{222} values derived from the data and curve fits shown in (A-C).
Table 1.1 – Theoretical and MALDI-TOF MS-observed molecular weights for each polypeptide studied.

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<th>Mass Found</th>
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Chapter 2 – Biophysics of $\beta^3$-peptide 14-helix Folding

2.1 – Control over secondary structure in $\beta$-peptide foldamers

Folded polymers are used in Nature for virtually every vital process, from catalysis to information storage, from cellular signaling to molecular transport. Non-natural folded polymers, or foldamers,\(^{62}\) have the potential for similar versatility, and the design and refinement of such molecules is of considerable current interest.\(^{62-65}\) Decades of biophysical analysis of natural polymers – peptides, proteins, and nucleic acids – have shown that polymer folding is an extremely subtle process, governed by countless interactions among the backbone, side chains, and solvent. Nevertheless, control of foldamer structure is crucial for these molecules to realize their full potential as tools in biology and medicine.

A key element of foldamer design is therefore the ability to predict the folded structure of a given backbone, and to modulate structure by altering the backbone and/or side chains.\(^{62, 64, 65}\) Of the many and diverse foldamers currently being pursued, $\beta$-peptides best exemplify the interplay between chemical structure and folding. $\beta$-peptides with different backbone substitution patterns preferentially populate a wide array of secondary structures including helices, sheets, and reverse turns.\(^{65, 66}\) Short $\beta$-peptides are particularly adept at forming helical structures in organic solvents such as methanol and pyridine. There are several well-characterized $\beta$-peptide helices, which are named for the characteristic number of atoms in a hydrogen-bonded ring; these include the 10-helix, the 10/12-helix, the 12-helix, and the 14-helix (Figure 2.1A). Helix type is largely
determined by choice of β-amino acid monomers (Figure 2.1B): cyclic ring constraints within the monomer of 4 atoms, 5 atoms, or 6 atoms promote the 10-helix, 12-helix, and 14-helix, respectively, \(^{67-70}\) while acyclic, monosubstituted residues (β\(^2\)- and β\(^3\)-residues) tend to fold into 14-helices, or 10/12 helices if patterned as alternating β\(^2\)/β\(^3\) residues.\(^{71-76}\) Thus, control over preferred helical secondary structure can be achieved via judicious choice of substitution pattern along the backbone.

2.2 – Promoting water-stable helix structure in short β-peptides

Initial designed β-peptides possessed helical structures in organic solvents but were insoluble or poorly structured in aqueous solution. Gellman and co-workers addressed this problem by introducing an additional amino group into the 14-helix-promoting cyclic residue trans-2-aminocyclohexanecarboxylic acid (ACHC, Figure 2.1B) to produce trans-2,5-diaminocyclohexanecarboxylic acid (DCHC).\(^{77}\) Oligomers containing ACHC and/or DCHC can be soluble and helical in water, but lack versatility because the cyclic β-amino acids are not easily functionalized.\(^{77, 78}\) It has been shown that ACHC residues can promote 14-helix formation within a larger β-peptide of acyclic residues (Figure 2.2A).\(^{70, 79-81}\) However, it remains to be seen whether peptides containing cyclic residues can tolerate a wide variety of substitutions and still remain both soluble and structured in aqueous solution.

β-peptides consisting solely of the more synthetically accessible and diverse β\(^3\)-residues present a different obstacle: while these oligomers are generally 14-helical in methanol and soluble in water, they are poorly structured in aqueous solution.\(^{76, 82, 83}\) A
14-helical turn is characterized by hydrogen bonding between an amide proton at residue \((i)\) and a backbone carbonyl at residue \((i+2)\),\(^{66}\) positioning side chains three residues apart directly in line when viewed along the helix axis (Figure 2.1A). This unique side chain alignment was exploited by two different groups to increase the extent of secondary structure of 14-helical \(\beta^3\)-peptides in water. Seebach reported in 2001 that a \(\beta^3\)-heptapeptide (Figure 2.2B) containing two pairs of oppositely charged residues (\(\beta^3\)-homoornithine and \(\beta^3\)-homoglutamic acid) at adjacent \((i,i+3)\) positions was completely 14-helical in methanol, as evidenced by CD spectroscopic data and NMR structural data.\(^{84}\) This oligomer was also water-soluble, and, while previous \(\beta^3\)-peptides had shown little evidence of structure in aqueous solution, the salt-bridge-stabilized \(\beta^3\)-heptapeptide showed an appreciable 14-helical CD signature in water. Also in 2001, Cheng and DeGrado independently reported a 15-mer \(\beta^3\)-peptide (Figure 2.2C) patterned with oppositely charged residues in an analogous way.\(^{85}\) Cheng and Degrado’s \(\beta^3\)-peptide was longer than Seebach’s design and contained a greater number of salt-bridges, so it is not surprising that the former possessed greater 14-helical structure as judged by CD spectroscopy. Both \(\beta^3\)-peptides contained oppositely charged residues, and thus the potential to form salt-bridges, on two of the three helical faces, and it was demonstrated in each case that screening of the charges (by varying salt concentration or pH of the buffer) reduced overall structure. Taken together, these studies highlight the powerful stabilizing effects that tailored electrostatic interactions can have within a 14-helix.

More recently, Dr. Scott Hart in our lab showed that the extent of 14-helix structure for \(\beta^3\)-peptides in water can be enhanced in a complementary way by neutralization of the 14-helix macrodipole.\(^{86}\) A \(\beta^3\)-undecapeptide with charged side chains placed to minimize
the overall 14-helix macrodipole (Figure 2.2D and β-peptide 1, Figure 2.3A) showed increased 14-helix structure in water relative to analogous β-peptides that lacked such design elements. Enhancing 14-helix stability in this way restricts residue choice on only one of the three helical faces, allowing the rest of the molecule to be used for the recognition of macromolecular targets. This work also demonstrated that β3-homophenylalanine, β3-homoserine, and β3-homoisoleucine are well-tolerated in several positions within 1. Of particular note was the observation that β3-homoisoleucine was 14-helix-stabilizing relative to β3-homoalanine, a direct confirmation of previous observations that residues branched at the first side chain carbon are 14-helix stabilizing.87-89

2.3 – Host-guest analysis: rationale and scaffold refinement

As a prelude to molecular recognition studies, we chose to explore more rigorously the 14-helix propensities of β3-amino acids with proteinogenic side chains. β-peptides can form stable helical structures with many fewer residues than α-peptides, yet it has been suggested that the intrinsic helical propensities of β3-amino acids are weak relative to those of α-amino acids.66, 85 Thus, a broad, single-substitution host-guest study of the kind used to delineate the α-helix propensities of α-amino acids90-92 seemed ideal for the exploration of the relative 14-helical propensities of β3-amino acids. In addition, since the intrinsic α-helical propensities of α-amino acids has been probed so thoroughly, it was logical to think a meaningful comparison could be made across these two similar
polymers and secondary structures. Finally, knowledge of intrinsic 14-helix propensities was crucial to the incorporation of tailored function into our 14-helical designs.

The host-guest analysis was guided by pioneering host-guest studies of the intrinsic $\alpha$-helical propensities of $\alpha$-amino acids.$^{91,92}$ In these studies, a reference or “host” peptide was designed with several important features, including high water solubility, easy and accurate concentration determination, availability of an appropriate site or sites for substitution of “guest” side chains, control of side chain-side chain interactions within the host, and elimination of potential side chain-side chain interactions between the host peptide and the guest side chain.$^{92}$ To ensure sensitivity the reference and test peptides should possess helical contents between 20 and 80%.$^{90}$ The $\alpha$-helix propensities of natural and non-natural $\alpha$-amino acids have been evaluated in this way using various reference peptides, including one containing mostly alanine,$^{91}$ a known helix-promoting residue, one that uses side chain-side chain salt-bridges to stabilize the helix,$^{93,94}$ and one that uses both alanine residues and side chain-side chain interactions for helix stabilization.$^{95,96}$

$\beta$-peptide 1 contains salt bridges oriented to minimize the 14-helix macrodipole (Figure 2.3A).$^{86}$ Initially, circular dichroism (CD) spectroscopy was used to characterize its structure. While CD data on $\beta$-peptides must be interpreted carefully,$^{97}$ it is reasonable to assume that changes in intensity of the 14-helical signature correlate to relative changes in overall mean 14-helical population.$^{66,80,98-100}$ The CD spectrum of 1 in aqueous buffer (Figure 2.3B) is indicative of 14-helix structure, with a characteristic minimum near 214 nm and a maximum near 195 nm. The secondary structure of 1 in CD$_3$OH was further explored using 2-D NMR spectroscopy (performed by Jade Qiu in
our lab), and all backbone NOEs consistent with 14-helix structure were observed, except those obscured by resonance overlap. No NOEs inconsistent with 14-helix structure were observed.

Although 1 populated a 14-helical structure in CD$_3$OH, CD spectroscopy in aqueous solution suggested an overall helical content between 27 and 37%, falling short of the roughly 50% helical level required for an ideal host peptide. This estimate assumes a mean residue ellipticity at 214 nm ($\Theta_{214}$) of zero for an unstructured $\beta$-peptide chain and a $\Theta_{214}$ between -20,000 and -28,000 deg cm$^2$ dmol$^{-1}$. These values are gleaned from previous work, especially experiments with constrained peptides which imply that short, fully 14-helical $\beta$-peptides may have mean residue ellipticity minima at 214 nm as low as $\Theta_{214} = -20,000$ deg cm$^2$ dmol$^{-1}$, whereas longer $\beta$-peptides (about 15 residues) may have minima as low as -28,000 deg cm$^2$ dmol$^{-1}$. Note that the large negative Cotton effects at 214 nm observed by CD spectroscopy for $\beta$-peptides comprised of L-$\beta^3$-amino acids (substituted carbon in the $S$ configuration) are typical for the left-handed 14-helices. $\beta$-peptides comprised of D-$\beta^3$-amino acids ($R$ configuration at the substituted carbon) show equal Cotton effects, but opposite in sign.

A refined scaffold, $\beta$-peptide 2 (Figure 2.3A), was designed with two changes: the $\beta^3$-homoglycine residue, which we surmised destabilizes the 14-helix, was replaced with a potentially more stabilizing $\beta^3$-homovaline residue and the $\beta^3$-homotyrosine residue was moved to the C-terminus following common practice for $\alpha$-peptidic host peptides. The CD spectra of $\beta$-peptides 1 and 2 in water (Figure 2.3B) demonstrate the magnitude of the effect of these small alterations, with a 78% increase in the mean residue ellipticity intensity at 214 nm ($\Theta_{214} = -7,454$ and -13,321 deg cm$^{-2}$ dmol$^{-1}$ for 1 and 2, respectively).
β-peptide 2 was an excellent reference peptide for a more extensive host-guest analysis for several reasons. First, 2 has roughly 48-67% overall helical content, within the most sensitive region for detecting net changes. Second, the β\(^3\)-homoalanines at the 3, 6, and 9 positions of 2 provided excellent points for side chain substitution. The methyl side chain is ideal for substitution, and because each substituent’s \((i+3)\) and/or \((i-3)\) neighbors are restricted to methyl groups, guest side chain-host side chain interactions are minimized. Third, by substituting into these three positions individually, three independent assessments of helix propensity can be made: one near the N-terminus, one internal, and one near the C-terminus.

2.4 – Host-guest analysis: circular dichroism (CD)

Host-guest analysis

β-peptide 2 was used as a reference peptide for a host-guest analysis of the 14-helix propensities of a wide variety of β\(^3\)-amino acids. The 28 β\(^3\)-peptides used for the analysis represent the host peptide and 27 variants in which each of three positions was substituted with each of nine different proteinogenic side chains. These side chains varied widely in functionality, and included charged, aliphatic, polar, and aromatic groups. In the following discussion, the terms "stabilizing" and "destabilizing" will be used in reference to β\(^3\)-homoalanine (the residue present in the host peptide 2 at all substituted positions) unless otherwise noted, and percent changes in 14-helicity will be calculated based on intensity of CD minima near 214 nm relative to that of the host peptide. Each peptide was analyzed by CD in PBC buffer (1 mM each phosphate, borate, and citrate, pH-
adjusted with sodium hydroxide) at pH 7.0, at 25 °C, over a range of concentrations. A subset of nine peptides was also characterized by analytical ultracentrifugation (AU) to determine oligomeric state. The molecules chosen for AU analysis represent a variety of substitutions among the three positions, while also including those peptides found to have slightly concentration-dependent CD spectra. The AU data, as well as material balance calculations based on the AU data, are shown in Figure 2.4. While curve fits for monomer and dimer molecular weights were equally valid, a characteristic of low molecular weight compounds, the material balance calculations\textsuperscript{101} show that all peptides tested by AU were monomeric at concentrations ranging from 80 to 400 µM.

*Charged side chains modulate 14-helix stability in a position-dependent way*

We have recently shown that, as with α-helices, interactions with the helix macrodipole are a significant factor in 14-helix stability.\textsuperscript{86} Thus, one might expect the relative 14-helix propensities of charged residues to be position-dependent, with positively charged residues exerting a stabilizing effect near the N-terminus and a destabilizing effect near the C-terminus, and *vice versa* for negatively charged residues. To test these predictions, β\textsuperscript{3}-homolysine and β\textsuperscript{3}-homoglutamic acid were substituted individually at each of the three positions within 2. The CD spectra for peptides 2-K3, 2-K6, 2-K9, 2-E3, 2-E6, and 2-E9 are shown in Figure 2.5A-B. In agreement with expectation, when located near the N-terminus (position 3), β\textsuperscript{3}-homolysine increases the extent of 14-helix structure by 20% and β\textsuperscript{3}-homoglutamic acid decreases the extent of 14-helix structure by 31%. When located near the C-terminus (position 9), β\textsuperscript{3}-homolysine diminishes the extent of 14-helix structure by 13% and β\textsuperscript{3}-homoglutamic acid increases
the extent of 14-helix structure by 21%. In a more central position (position 6), β³-homolysine is moderately stabilizing (15% increase) and β³-homoglutamic acid is neutral to slightly destabilizing.

**Aliphatic side chains**

The effects of aliphatic side chains on the 14-helix stability of 2 were examined next. It has been observed in other contexts that β³-homovaline and β³-homoisoleucine tend to increase the extent of 14-helix structure, whereas β³-homoleucine decreases the extent of 14-helix structure.β⁶-β⁹ β-peptides 1 and 2 were each designed with three β³-homovaline residues for this reason. Our host-guest study directly addresses the question of whether side chains branched at the first side chain carbon are 14-helix stabilizing. We individually substituted the iso-propyl (2-V³, 2-V⁶, 2-V⁹), iso-butyl (2-I³, 2-I⁶, 2-I⁹), and sec-butyl (2-L³, 2-L⁶, 2-L⁹) side chains to examine the effects of branching on overall 14-helicity.

The CD spectra of 2-V³, 2-V⁶, 2-V⁹, 2-I³, 2-I⁶, 2-I⁹, 2-L³, 2-L⁶, and 2-L⁹ are shown in Figure 5C-E. In accord with previous observations, the sec-butyl side chain of β³-homoleucine is either 14-helix destabilizing (by 12% at position 3) or neutral (at positions 6 and 9). However, the iso-propyl and iso-butyl side chains of β³-homovaline and β³-homoisoleucine are 14-helix-stabilizing at all positions. The effect is greatest in central and N-terminal positions, where a β³-homovaline or β³-homoisoleucine residue lowers the mean residue ellipticity minimum from -13,320 deg cm² dmol⁻¹ (for the host β-peptide 2) to -17,440 and below, as low as -19,130 deg cm² dmol⁻¹ for 2-V⁶. These values represent increases in mean 14-helix structure of 31 to 44% relative to the host
peptide. The consistently large increases observed for individual substitutions of aliphatic side chains branched at the first side chain carbon directly confirm that these residues are particularly 14-helix-promoting.

**Polar side chains**

To further explore 14-helix propensities of β³-amino acids, two polar residues were chosen for substitution. The hydroxymethyl side chain of β³-homoserine and the 1-hydroxyethyl side chain of β³-homothreonine seemed suitable, and had the added benefit of testing the effect of branching at the first side chain carbon within a different context. The CD spectra for peptides 2-S₃, 2-S₆, 2-S₉, 2-T₃, 2-T₆, and 2-T₉ are shown in Figure 5F-G. β³-homothreonine is stabilizing at all three positions, lowering the mean residue ellipticity minimum near 214 nm as low as -18,720 deg cm⁻² dmol⁻¹ for 2-T₃, corresponding to a 41% increase in 14-helical structure. The changes upon substitution with β³-homothreonine are similar to those seen for other side chains branched at the first carbon, providing further evidence that these side chains are particularly 14-helix-promoting. Unexpectedly, the β³-homoserine substitution is stabilizing at positions 6 and 9 (by 34% and 24%, respectively), but neutral at position 3. Since the unbranched hydroxymethyl side chain appears to be stabilizing to an equal or greater extent than the branched side chain, and since this effect is position-dependent, there may be another stabilizing interaction present involving the hydroxyl group.

**Aromatic side chains**
Extensive analyses of known protein-protein interfaces have shown that large hydrophobic residues occur with higher frequency in surface recognition patches. These residues are also commonly involved in important pairwise interactions that stabilize protein-protein complexes. Thus, the ability to incorporate large hydrophobic residues is critical to the use of β-peptides as peptide and protein mimics. Three large aliphatic residues had already been examined, so to test the 14-helix propensity of large aromatic residues β<sup>3</sup>-homophenylalanine and β<sup>3</sup>-homotryptophan were substituted into β-peptide 2. The CD spectra for the β-peptides with aromatic substitutions are shown in Figures 5H-I. The benzyl sidechain (analogous to that of phenylalanine) was 14-helix neutral in positions 3 and 9, and destabilized 14-helix structure by only 10% in the central position. The methyl-indole sidechain (analogous to that of tryptophan) was neutral at position 3, and slightly destabilizing in the other positions (up to 17%). These results provide optimism that even bulky aromatic side chains will be tolerated within the context of a reasonably stable β-peptide 14-helix.

**Materials and Methods**

**General**

Fmoc-protected α-amino acids, PYBOP®, HOBt, and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), N-methyl morpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). All other reagents were purchased from Sigma-Aldrich. Certain Fmoc-β<sup>3</sup>-(L)-amino acids were
purchased from Peptech Corp. (Cambridge, MA), although most were synthesized from enantiomerically pure $\alpha$-amino acid precursors via the Arndt-Eistert procedure.\textsuperscript{76}

$\beta^3$-peptide synthesis, manual procedure

Some $\beta^3$-peptides were synthesized manually, in a glass peptide synthesis vessel with fritted glass at the top and bottom and a sidearm for addition of reagents (Ace Glass, Vineland, NJ). Peptides were synthesized on a 30 or 50 µmole scale using Wang resin and Fmoc-protected $\beta^3$-amino acid monomers, using standard Fmoc strategy. Wang resin was loaded as described.\textsuperscript{86} Peptide elongation and cleavage were performed as described.\textsuperscript{86}

$\beta^3$-peptide synthesis, semi-automated procedure

Some $\beta^3$-peptides were synthesized on a Symphony/Multiplex automated peptide synthesizer (Protein Technologies, Tuscon, AZ) using standard Fmoc strategy. Peptides were synthesized on a 25 µmole scale using Wang resin loaded as described.\textsuperscript{86} One cycle of peptide elongation consisted of the following steps: resin was washed with N-methyl-2-pyrrolidone (NMP) (3 x 30 sec), deprotected with 20% piperidine/DMF (1 x 2 min, 2 x 8 min), washed with NMP (6 x 30 sec), coupled for 30 mins with 3 equiv. of the appropriate $\beta^3$-amino acid and 3 equiv. PYBOP\textsuperscript{®}, 3 equiv. HOBt, and 8 equiv. DIEA, washed once with NMP (1 x 30 sec), capped for 20 mins with 6% v/v acetic anhydride, 6% v/v NMM in NMP, then washed with NMP (2 x 30 sec). The $\beta^3$-amino acid (3 equivalents, 75 µmol), PYBOP\textsuperscript{®} (39.0 mg, 75 µmol), and HOBt (11.4 mg, 75 µmol) were weighed out previously, and dissolved in 5 mL NMP immediately prior to coupling.
Diisopropylethylamine (DIEA) (8 equivalents, 35 µL) was added immediately prior to adding the reagents to the resin through the peptide synthesis vessel sidearm. Cleavage of peptides synthesized semi-automatically was performed as follows: the resin was washed with NMP (8 x 30 sec), washed with methylene chloride (8 x 30 sec), dried 20 mins under N₂, cleaved for two hours with cleavage reagent (3% v/v water, 3% v/v triisopropylsilane in TFA), then washed once with cleavage reagent. The cleaved peptide was collected and concentrated by rotary evaporation, reconstituted in H₂O/CH₃CN (1:1), and analyzed for purity by HPLC and MALDI-TOF mass spectrometry.

**β-peptide purification and characterization**

Peptides were purified by reverse-phase HPLC. Identity and purity of compounds was assessed by analytical HPLC and MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) mass spectrometry on a Voyager (Applied Biosystems) MALDI-TOF spectrometer with a 337 nm laser using the α-cyano-4-hydroxycinnaminic acid matrix. The instrument was calibrated using adrenocorticotropic hormone (ACTH) (M + H⁺ = 2093.1) and angiotensin I (M + H⁺ = 1296.7). Following purification, peptides were immediately lyophilized, kept at -20 °C, and reconstituted just prior to use. Theoretical and observed molecular weights for each peptide are listed in Table 2.1. β-peptide 1 was characterized as described.86

**Circular dichroism**

Circular dichroism (CD) spectra between 195 and 260 nm were acquired with an Aviv 202 CD spectrometer at 25 °C using a 2 mm path length quartz cell (Hellma,
Plainview, NY). Samples were prepared by dissolving lyophilized, HPLC-purified peptide in PBC buffer (1 mM each phosphoric acid, boric acid, and citric acid, pH adjusted with NaOH to 7.0). The concentration of the sample was determined by measuring absorbance at 280 nm; this analysis assumes that the extinction coefficient of a β-peptide containing a single β3-homotyrosine is equal to that of an α-peptide containing a single α-tyrosine (1300 M⁻¹ cm⁻¹ at 280 nm), as used previously to estimate β-peptide concentration.⁸⁵, ⁸⁹, ¹⁰³ Peptide was diluted to 80 µM in PBC buffer, and serial dilutions were then made to generate solutions of 40, 20, and 10 µM. Three scans of each peptide solution were taken, with 2 sec averaging times and 2 nm bandwidth. These scans were averaged, and a blank buffer spectrum was subtracted to generate the corrected spectra. This full procedure was performed three independent times to ensure accuracy and repeatability, and these three spectra were averaged to generate the final spectrum for each peptide at each concentration. No data smoothing was used at any step. CD signal was converted into mean residue ellipticity ([Θ], deg cm² dmol⁻¹) using the equation:

\[
[\Theta] = \Psi / (100 \text{ res} \cdot \text{l} \cdot \text{c})
\]

where Ψ is raw ellipticity in millidegrees, res is the number of residues, l is path length in decimeters, and c is molar concentration. The concentration of each CD sample was verified after analysis by repeating the UV absorbance measurement. Aromatic side chains are known to contribute to the far-UV CD of model α-helical peptides ¹⁰⁴ in a manner highly dependent on local backbone conformation.¹⁰⁵ It is not yet known whether these contributions are sizable for β³-peptide 14-helices, though any future
corrections for the C-terminal $\beta^3$-homotyrosine will likely be small and uniform within the series. Contributions from the $\beta^3$-homophenylalaine and $\beta^3$-homotryptophan residues used as guest side chains might more significantly affect our conclusions regarding $\beta$-peptides containing these residues, but any future corrections are unlikely to be of a magnitude of more than $\pm 5\%$.104,105

Analytical ultracentrifugation

Note: The experiments and data treatment described in this section were performed by Dr. James Lear, Department of Chemistry, University of Pennsylvania.

Measurements were made using an Optima XLI analytical ultracentrifuge from Beckman-Coulter (Fullerton, CA). Samples were prepared by dissolving HPLC-purified peptide in PBC buffer and were centrifuged to equilibrium at 25 °C at 50,000 RPM in six-channel, carbon-epoxy composite centerpieces supplied by Beckman. Equilibrium was assessed by the absence of significant change in radial concentration gradients in scans at 14 and 16 hours. Data were analyzed by fitting the data to the equation for a single ideal species using Igor-Pro® (Wavemetrics, Lake Oswego OR). The equation is:

$$C(r) = C(r_o) \exp \left( \frac{(1 - \bar{v} \rho) \omega^2 M_n}{2RT} \left( \frac{1}{r^2} - \frac{1}{r_o^2} \right) \right)$$

where:

$C(r, r_o) =$ concentration (any units) of sedimeting species at radial positions $r, r_o$ cm from the center of rotation.

$\bar{v} =$ partial specific volume of sedimeting species (cc/gm)

$\rho =$ density of supporting buffer (gms/cc)
\( \omega \) = angular velocity of rotor (radians/sec)

\( M_n \) = “Molar” molecular weight of sedimenting species (gms/mole)

\( R \) = Gas constant (8.315 \times 10^7 \text{ ergs} \cdot \text{K}^{-1} \cdot \text{mol}^{-1})

\( T \) = Temperature (K)

Peptide partial specific volumes were assumed for simplicity to be the same as the average value calculated for previously studied \( \beta \)-peptides: 0.785 cm\(^3\)/gm.\(^86\) Because of cross-correlation of molecular weight with baseline values, curve-fits were insensitive to variations in this value. In fact, equally good curve-fits could be obtained by assuming either monomer or dimer molecular weights. This ambiguity is an unavoidable consequence of the low curvature exhibited in the concentration profiles of the low molecular weight compounds. To distinguish monomers from higher order aggregated species as the dominant population, curve fits were performed using integral multiples of the sequence-calculated molecular weights. This fixes the molecular weight of the presumed species, which also fixes the baseline (absorbance at zero concentration). Then, one can integrate over the net absorbance profile to obtain the calculated average concentration in the cell. Here, the average concentrations predicted by fixing the molecular weights to those of the monomers are larger and more consistent with known concentrations than those predicted by fixing the molecular weights to dimer values. This is because the higher molecular weights predict higher curvature which, for the same data set, can only be attained by increasing the baseline values and hence reducing the calculated average concentrations. This represents a useful material balance criterion previously developed to study binding of low and high molecular weight compounds.\(^{101} \) Error in the calculated values (shown as error bars in Figure 2.4B)
represents the sum of uncertainties in the determination of the outer radius of the cell compartment and uncertainties arising from baseline values as reported from the curve-fitting algorithm.

2.5 – Host-guest analysis: calculations and simulations

Note: The calculations and simulations described in this section were performed by Dr. Julian Tirado-Rives in Prof. Bill Jorgensen’s laboratory in the Yale Chemistry Department.

Conformational analysis of $\beta^3$-amino acids

The CD data presented above provides a glimpse into the propensities of individual $\beta^3$-amino acids for stabilizing 14-helix structure. We wondered whether the observed helix propensities could be accounted for by the conformational preferences of each individual $\beta^3$-amino acid. Using gas phase energy optimizations and the BOSS 4.5 program (a program for semi-empirical statistical mechanics and energy minimization calculations), we analyzed seven dipeptides of the general form acetyl-$\beta^3$X-NHCH$_3$, where $\beta^3$X was one of $\beta^3$-homoglycine ($\beta^3$G), $\beta^3$-homoalanine ($\beta^3$A), $\beta^3$-homoleucine ($\beta^3$L), $\beta^3$-homoisoleucine ($\beta^3$I), $\beta^3$-homovaline ($\beta^3$V), $\beta^3$-homoserine ($\beta^3$S), or $\beta^3$-homothreonine ($\beta^3$T). The minimizations were performed using starting conformations corresponding to all possible combinations of values of the three backbone dihedral angles $\phi$ (N-C$_3$), $\theta$ (C$_3$-C$_2$), and $\psi$ (C$_2$-C’), each spanning the -180 to 180° range in 30° intervals. From each of these 1,331 conformations, the molecules were allowed to
optimize their geometry to find local energy minima. The calculated relative energies for low-energy conformations of $\beta^3$G and $\beta^3$A, the only residues studied previously, agree well with published \textit{ab initio} results at the MP2/6-31G(d) level,\textsuperscript{107, 108} even though the quantum mechanical calculations used a terminal amide cap instead of a methyl amide.

As in similar studies of $\alpha$-amino acids, most of the stable conformations located, including the global minimum of each molecule, were stabilized by intramolecular hydrogen bonds. These interactions close a six- or eight-membered hydrogen-bonded ring, to form the C6 or C8 conformation, respectively.\textsuperscript{109} Local energy minima were found at backbone dihedral angles $(\phi, \theta, \psi)$ near (-100°, -60°, -90°) for all dipeptides, which is close to the canonical 14-helix conformation.\textsuperscript{66, 110} However, these conformations possessed energies 5 to 10.5 kcal•mol$^{-1}$ above the global minimum. To obtain better resolution of the conformational space, we built three-dimensional Ramachandran plots\textsuperscript{111} for each dipeptide listed above. In this set of calculations, the three backbone dihedrals were scanned from -180 to 180° in 15° intervals (12,167 different starting points for each dipeptide) and fixed during energy minimizations, allowing all other degrees of freedom to vary. Despite the increased resolution of this analysis, no other minima were found. Taken together, these data imply that the experimentally observed 14-helix propensities cannot be explained by the different conformational preferences of individual $\beta^3$-amino acids.

\textit{Conformational analysis of $\beta^3$-oligopeptides}

We focused next on $\beta^3$-oligopeptides, in order to assess the role of inter-residue interactions in 14-helix stabilization. To this end, we energy-minimized 25 $\beta^3$-
oligopeptides with the general sequence acetyl-$(\beta^3A)_m$-$\beta^3X-$(\beta^3A)_n$-NHCH$_3$, m+n=11, where $\beta^3X$ was one of $\beta^3$A, $\beta^3$L, $\beta^3$I, $\beta^3$V, $\beta^3$S, or $\beta^3$T at positions 3, 6, 9, or 12. The relative energies of these $\beta^3$-oligopeptides were examined using a variety of starting conformations. The three backbone dihedral angles ($\phi$, $\theta$, $\psi$) for all residues were initially set to values corresponding to the C6 (110°, 60°, 180°), C8 (-66°, -45°, 95°), $\beta$-sheet (180°, 180°, 180°), 12-helix (-90°, 90°, -110°) or 14-helix (-155°, 60°, -135°) conformations. These starting points were all minimized in the gas phase, and their energies of solvation in water were calculated using the Generalized Born / Surface Area (GB/SA) model in a manner that differentiates native folds from decoys, although previous applications of this technique employed an equivalent formulation of the same principle. Table 2.2 lists the minimized $\beta$-peptide energies relative to each individual $\beta$-peptide’s lowest-energy conformation ($\Delta E$). These energies predict that the 14-helix should be the lowest-energy conformation for $\beta^3$-oligopeptides, followed by the 12-helix, which matches experimental observations.

The results from energy minimizations report on the lowest-energy structures of each $\beta$-peptide at 0 K. While they can predict relative energies for conformations of a single $\beta$-peptide, the energies are not truly comparable across different peptides and different substitutions. In order to obtain a more general basis for comparison, Monte Carlo (MC) simulations at 25 °C using the GB/SA solvation model were performed, using each of the minima obtained through energy minimization as starting conformations. For each simulation the equilibration period spanned 8 million configurations, after which data was collected and averaged for 2 million configurations more. The average energies for each MC ensemble, shown in Table 2.3, confirm that the 14-helix is the most stable
conformation for all $\beta^3$-oligopeptides studied. The 12-helix is, on average, 33 kcal\(\cdot\)mol\(^{-1}\) higher in energy, but still stable, while all the other starting conformations (C6, C8 and $\beta$-sheet) folded to other, more compact structures. Comparing energies for substituted $\beta$-peptides with those of the simulated host, acetyl-(\(\beta^3\)A)\(_{12}\)-NHCH\(_3\), reveals that the $\beta^3$S and $\beta^3$T substitutions are most 14-helix stabilizing in these simulations, followed by $\beta^3$V and then $\beta^3$I, $\beta^3$A and $\beta^3$L.

**Structural properties of the simulated $\beta^3$-oligopeptides**

The 14-helix content of each simulated $\beta^3$-oligopeptide can be estimated directly from the MC simulation data by quantifying structural properties, specifically the hydrogen bond populations and average torsional angles. Significantly, the average population of the N-terminal hydrogen bond over all the simulations is only 39%, while the average population of the C-terminal hydrogen bond is 77%. On this basis it would be predicted that stabilizing effects would be more pronounced at positions near the N-terminus. This prediction matches our CD results, with the sole exception of $\beta^3$-oligopeptides containing a $\beta^3$S residue.

We also compared the hydrogen bond populations near each substitution site in each of the $\beta^3$-oligopeptides studied, calculated as the average population of the \((i-2 \rightarrow i)\), \((i-1 \rightarrow i+1)\), and \((i \rightarrow i+2)\) hydrogen bonds surrounding a substitution at position \((i)\) over 2 million configurations (Figure 2.6). In all cases there are only small effects at the middle positions, presumably because at these positions the hydrogen bond populations are already high in the all-\(\beta^3\)A peptide. Even so, several trends can be discerned by comparing the relative hydrogen bond populations. Substitution of $\beta^3$L within an all-\(\beta^3\)A
peptide has little or no effect on hydrogen bond populations at positions 3, 6, and 9, and a slight decrease at the C-terminal position 12. By contrast, single substitutions of $\beta^3$I, $\beta^3$V, and $\beta^3$S show large increases in hydrogen bond population at the N-terminus and moderate increases at the C-terminus, while $\beta^3$-T moderately increases hydrogen bond populations at both termini. These observations match the CD data very well in terms of predicting relative 14-helix propensities of the $\beta^3$-amino acids.

Interestingly, the Monte Carlo simulations indicate that, within a fully 14-helical conformation, the side chain hydroxyl groups of $\beta^3$S and $\beta^3$-T can hydrogen bond to the $(i+2)$ carbonyl oxygen when available. When substituted at internal positions 6 and 9, $\beta^3$S and $\beta^3$-T formed these side chain hydrogen bonds in 68% and 35% of the configurations sampled, respectively. This observation implies $\beta^3$S is more likely to form these stabilizing H-bonds within a fully 14-helical context, and may explain the unexpected stabilizing effects observed by CD upon $\beta^3$S substitution.

Another noticeable structural difference among the MC simulations of the 25 peptides is in the average backbone $\theta$ torsion (N-C$^3$-C$^2$-C') at the substituted position. Within the context of a $\beta^3$-oligopeptide, the average $\theta$ torsional angles for $\beta^3$L (48°) and $\beta^3$S (49°) residues are very similar to values for $\beta^3$A in the same positions (52°), and these three deviate significantly from the optimal gauche torsion found for energy-minimized dipeptides (60°). $\beta^3$I (60°) and $\beta^3$V (59°) promote torsions very close to optimal, while $\beta^3$T has a torsional angle that is intermediate (54°). The distorted torsions appear to allow better interaction of the $(i, i+3)$ side chains at the expense of a slight distortion of the helical axis, which does not seem to affect the hydrogen bond distances. Overall, the hydrogen bond populations and torsional geometries of singly substituted oligo-$\beta^3$-
homoalanine help to explain the general trends observed in the CD data, as well as confirm that 14-helix propensities appear to arise from inter-residue interactions.

**Materials and Methods**

All the dipeptide minimizations were done with the BOSS 4.5 program,\textsuperscript{114} modified so that the bonding list was taken from the input Z-matrix and not recalculated based on interatomic distances. All the calculations for oligo-\(\beta\)-peptides were carried out with the development version of MCPRO 1.68\textsuperscript{115} which includes the GB/SA treatment for solvent. The OPLS-AA force field\textsuperscript{109} was used throughout this study, augmented with backbone torsional parameters developed specifically for \(\beta\)-peptides in our laboratories based on high level \textit{ab initio} calculations.\textsuperscript{116} In all cases the torsion angles around the peptide bond (\(\omega\)) were initially set to 180° but allowed to vary freely. All possible degrees of freedom were allowed to vary in all calculations, with the exception of the dipeptide three-dimensional Ramachandran plots where the backbone dihedrals were fixed. Due to convergence problems, particularly with conformations that have very close non-bonded contacts, the dipeptide minimizations were done in triplicate with the BFGS, Fletcher-Powell, and Powell algorithms, and the resulting conformer with the lowest energy was used. The oligopeptide optimizations were started with 250 steps of steepest descent minimization before switching to a conjugate gradients algorithm until convergence was reached. This treatment was not sufficient for 5 of the cases, which had to be optimized using 5000 steepest-descent steps before switching to conjugate gradients. No cutoff was used (\textit{i.e.} all non-bonded pairs evaluated), and convergence criterion was set to 0.1 cal
mol$^{-1}$ for all minimizations. MC simulations were done using W. C. Still’s GB/SA solvation model.$^{112}$

### 2.6 – 14-helix unfolding

*Unfolding by electrostatic screening*

Debye-Hückel theory states that the energy of ion-ion electrostatic interactions should scale negatively with the square root of the salt concentration in molality.$^{117}$ Previously, β-peptides with 14-helical structure stabilized by electrostatic interactions have been shown to unfold in the presence of high salt, in accordance with this principle.$^{85}$ To provide additional evidence that electrostatic interactions contribute to the stabilities of the β-peptides studied here, we monitored the effects of increasing concentrations of NaCl on the structure of peptides 2 and 2-16. Mean residue ellipticity at 214 nm (θ$^{214}$) of these peptides at salt concentrations from 0 to 2.25 M are plotted in Figure 2.7A. The somewhat sigmoidal shape of the curves has been observed in other salt-bridge-stabilized β-peptides,$^{85}$ and suggests that screening of electrostatic interactions destabilizes the 14-helix. Interestingly, the curves for peptides 2 and 2-16 are roughly parallel, implying that the methyl to iso-butyl substitution stabilizes the 14-helical fold in a manner unrelated to electrostatics, and that even when electrostatic interactions are highly screened, the iso-butyl side chain can stabilize 14-helical structure. Notably, the effects of salt-bridging and branched side chain substitution are independent. This is consistent with a model in which 14-helix folding is noncooperative, so that destabilization of one portion of the helix does not affect the stability of the rest of the helix.
To more directly probe the stability of our designed 14-helical β-peptides in water, we used CD to monitor the effect of temperature on the structural stability of β-peptide 2 and two variants, 2-E3 and 2-I6. 2-E3 is destabilized relative to 2 whereas 2-I6 is stabilized relative to 2, so that these three peptides cover the range of overall stabilities observed within the host-guest analysis. The full CD spectrum of each peptide between 195 and 260 nm was monitored as the temperature was raised from 4°C to 98°C, then lowered back to 4°C. Results are summarized in Figures 7B-C. All three peptides had 14-helical signatures at all temperatures, with the 14-helical signature persisting even at 98 °C. All three sets of spectra were also characterized by isodichroic points near 202 nm, a powerful indicator that no other regular structure is significantly populated at any temperature. β-peptides 2, 2-E3, and 2-I6 all show fully reversible unfolding transitions with little to no cooperativity, which is consistent with previous observations of 14-helix unfolding. Because the unfolding appears virtually noncooperative, stability of each characteristic hydrogen bond is roughly independent of the state of the others. Thus, stabilizing a region of the 14-helix via substitution of a helix-promoting residue has little effect on the stability of other regions, and unfolding of other regions has little effect on the stabilized portion. This noncooperative folding model explains the ability of a single substitution to stabilize or destabilize 14-helical structure even at high temperatures or high salt concentrations.

Materials and Methods
The effects of electrostatic screening were monitored by acquiring CD spectra of 80 µM β-peptide at concentrations of NaCl from 0 to 2.25 M. Salt concentration was varied by stepwise addition of high-salt buffer to a sample of peptide within the CD cell, followed by mixing by aspiration and a 20 minute equilibration. Spectra were taken at 25 ºC using a 2 nm bandwidth and 5 sec averaging time, and results were adjusted for cumulative changes in salt concentration, peptide concentration and total volume.

The thermal unfolding transition was monitored by acquiring CD spectra from 260 nm to 195 nm at eleven selected temperatures from 4 ºC to 98 ºC. Reversibility was then examined by acquiring spectra at the same temperatures while cooling back down from 98 ºC to 4 ºC. Spectra were taken using a 2 nm bandwidth, a 20 min equilibration time, and a 5 sec averaging time.

2.7 – Biophysics of 14-helix folding

The overall results from the host-guest analysis are summarized in Figure 2.8. The wide range of CD intensities observed indicates strong thermodynamic preferences among β3-amino acids for or against 14-helix structure. One of the goals of this study was to compare 14-helix propensities of β3-amino acids to the α-helix propensities of the corresponding α-amino acids, and the breadth of the host-guest analysis allows a meaningful and direct comparison. In addition, the results confirmed existing observations, such as the stabilizing effects of side chains branched at the first carbon,
and informed new hypotheses, such as the possibility of 14-helix stabilization via side chain hydrogen bonding for β-peptides containing β\(^3\)-homoserine or β\(^3\)-homothreonine.

Comparison of the helix propensities of α- and β-amino acids

As observed for α-helices over 15 years ago,\(^1^1\) charged residues can stabilize or destabilize the 14-helix depending on their location relative to the N- and C-termini. These effects result from electrostatic interactions that diminish or intensify the 14-helix macrodipole. A similar effect is also observed when charged groups are located at helix termini: α-helices are stabilized by capped (uncharged) termini,\(^1^1\), \(^1^2\) while 14-helices are stabilized by free (charged) termini.\(^8\), \(^6\) The extent of structure stabilization observed herein due to charge-macrodirole interactions is similar in magnitude (stabilization or destabilization of ~10% relative to uncharged sidechains near termini when measured by CD at pH = 7.0) to that seen for α-helices,\(^1^1\), \(^1^2\) suggesting that the magnitude of the 14-helix macrodipole (which has not yet been measured experimentally) may be similar to, or greater than, that of the α-helix macrodipole.\(^1^2\)

Our simulations agree with this prediction, with calculated dipole moments of 45.4 and 56.9 D for oligoalanine- and oligo-β\(^3\)-homoalanine-based peptides, respectively. Because short, isolated α-helices are not well-folded under the conditions used to study 14-helices (25 °C in aqueous buffer), a more quantitative comparison of charged effects is difficult. Overall, it appears that charge-macrodirole interactions play similar roles in α-helix and 14-helix stabilization, and would be predicted to have a similar effect on other foldameric, amide-based hydrogen-bonded helices. It is interesting to note that β\(^3\)-homolysine is more 14-helix-stabilizing than β\(^3\)-homoglutamic acid in the center of the β-
peptide 14-helix. It is unclear whether this difference is due to electrostatics, or to some other intrinsic side chain property, though in α-helical models lysine has been consistently more helix-stabilizing than glutamic acid.92

Among non-charged side chains the 14-helix propensities of β₃-amino acids contrast starkly to the α-helix propensities of their α-amino acid counterparts. The methyl side chain (alanine) is among the most α-helix-promoting, but is one of the least 14-helix-stabilizing side chains (β₃-homoalanine, present in host peptide 2) in the present study. The same is true for the sec-butyl side chain (leucine and β₃-homoleucine). Meanwhile, the iso-butyl side chain of isoleucine, which has moderate α-helix propensity, the iso-propyl side chain of valine, which has very low α-helix propensity, and the 1-hydroxyethyl side chain of threonine, which also has very low α-helix propensity, are all highly 14-helix stabilizing relative to the methyl side chain. The broad discrepancies among individual side chain propensities for α-helices and 14-helices imply that the folding of these secondary structures may be governed by very different biophysical forces, and speak to the mechanism underlying intrinsic helix propensities. While the rank order for α-helix propensities of α-amino acids is generally agreed upon,92 the rationale for this ranking remains controversial. Burial of hydrophobic surface area against the side of the helix,122 shielding of backbone hydrogen bonds from solvent,123-125 backbone electrostatics,126 and side chain conformational entropy,127, 128 among other principles, have been advanced as the dominant basis for intrinsic α-helix propensities. Because aliphatic and small polar side chains show such radical differences in their observed α-helical and 14-helix propensities, the underlying cause of intrinsic propensities must be due primarily to interactions with the helix backbone and/or other
sidechains and not due to attributes such as conformational entropy or steric effects, whose scope is largely intraresidue. This hypothesis is borne out by the computational result that recapitulates 14-helix propensities of β\textsubscript{3}-amino acids only within the context of oligomers. Further, because the 1-hydroxyethyl, \textit{iso}-propyl, \textit{iso}-butyl and \textit{sec}-butyl side chains do not follow the same pattern for both helices, a closer look must be taken at some recent theories.\textsuperscript{122-125} If propensity indeed scales with buried hydrophobic surface area upon helix formation, then calculations should reveal that the 1-hydroxyethyl, \textit{iso}-propyl and \textit{iso}-butyl side chains bury more surface area than the \textit{sec}-butyl side chain in a 14-helix context, but less surface area in an α-helix context. A similar test could be performed for determining the extent of backbone hydrogen bond shielding afforded by these four side chains. It will be interesting to see whether further host-guest analysis, coupled with more rigorous simulations of β-peptide 14-helices, will be able to shed some light on this continuing debate.

\textit{The stabilizing effect of side chains branched at the first carbon}

Observations from as far back as 1999 imply that β\textsubscript{3}-amino acids branched at the first carbon are 14-helix-stabilizing.\textsuperscript{87, 88} A recent study has examined the relative effects of multiple β\textsubscript{3}-homoleucines or β\textsubscript{3}-homovalines on the 14-helicity of a β\textsubscript{3}-decapeptide, albeit one with potentially repulsive \((i,i+3)\) side chain-side chain interactions.\textsuperscript{89} The present study examines the relative effects of methyl, \textit{iso}-propyl, \textit{iso}-butyl, and \textit{sec}-butyl side chains on 14-helicity within the context of an already 14-helical scaffold, and directly demonstrates that a single side chain branched at the first carbon can have a large stabilizing effect on overall 14-helicity. Calculations on singly substituted oligo-β\textsubscript{3}-
homoalanine peptides also predict this effect, while no such effects are seen upon an extended conformational analysis of monomeric $\beta^3$-amino acids. Thus, the stabilizing effects of side chains branched at the first carbon are not due to an intrinsic preference for the 14-helix torsional conformation within the monomer, but instead are dependent upon interactions (direct or indirect) that involve other residues along the 14-helix.

*Large hydrophobic side chains are well-tolerated*

The ability to incorporate large hydrophobic side chains is crucial for the development of functional, 14-helical $\beta$-peptides for two reasons. First, natural protein-protein interfaces contain a relatively high proportion of large, hydrophobic residues.\(^2\)\(^3\)\(^\text{129}\) Second, positioning these residues at $(i, i+3)$ positions within a 14-helical context would generate an extensive, continuous hydrophobic surface with the potential to bind a target with high affinity.\(^1\)\(^3\)\(^\text{0}\) $\beta^3$-homovaline and $\beta^3$-homoisoleucine are 14-helix promoting, and substitutions of $\beta^3$-homoleucine, $\beta^3$-homophenylalanine and $\beta^3$-homotryptophan reveal that these large hydrophobic $\beta^3$-amino acids are neutral or only slightly destabilizing relative to $\beta^3$-homoalanine. This finding highlights the robustness and versatility of the 14-helical host peptide, and gives a first inkling of its suitability as a scaffold for molecular recognition.

*Simulations reproduce the effects of single $\beta^3$-amino acid substitutions*

If the differences in the CD spectra of the peptides are proportional to their 14-helix content, increases of up to 30% can be observed upon substitution of a single $\beta^3$-amino acid. For a two-state equilibrium the difference in free energy between structures that are
50% and 80% helical is only 0.82 kcal·mol\(^{-1}\) at 25 °C, a rather challenging goal for computer simulations. While such resolution could be achieved by free energy perturbation methods,\(^{131}\) the analysis would require a large number of very time-intensive simulations to reproduce the structural trends observed. Instead we attempted to discern through careful simulation if computational models can produce qualitative trends in secondary structure preferences among the β\(^3\)-amino acids studied by CD spectroscopy.

Energy minimization of dipeptides (capped monomers) did not reproduce the experimental trends in 14-helix propensities. The energy minima calculated for dipeptides imply that the monomers do not even intrinsically prefer the 14-helical conformation (as is the case with α-amino acids and the α-helix\(^{109}\)). Moreover, the gaps between the 14-helical local minima and the global minima are actually largest for residues for which 14-helix stabilization (relative to β\(^3\)A) was observed by CD. Thus, the observed trends in 14-helix propensity cannot be explained by examining relative energies of different conformations of monomeric β\(^3\)-amino acids.

It is only when medium-range interactions are taken into account that our calculations are able to reproduce the trends we observe experimentally. Energy minimization of a set of 25 β\(^3\)-peptide 12-mers made up of all β\(^3\)A and one substituted β\(^3\)-amino acid clearly indicates that the 14-helix is stable even \textit{in vacuo}. It was the lowest-energy minimum for all the molecules tested, being an average of 16 kcal·mol\(^{-1}\) below the next lowest minimum, the 12-helix. The effect of solvent increases this gap to 37 kcal·mol\(^{-1}\), which makes sense since, although the 12-helix is longer than the 14-helix, it is calculated to have a smaller dipole moment (42 D on the average versus 57 D for the 14-helix) presumably due to having fewer unfulfilled hydrogen bonds at each end. Monte Carlo
simulations using these lowest-energy conformations as a starting point provided a more realistic look at \(\beta^3\)-peptide energetics. The ensemble average energies of the substituted oligo-\(\beta^3\)A-peptides ranked the \(\beta^3\)-amino acids in the following order, from most 14-helix-stabilizing to least: \(\beta^3T > \beta^3S > \beta^3V > \beta^3I \geq \beta^3A > \beta^3L\). This ranking roughly fits the CD data, and indicates that careful simulation can indeed reproduce the experimentally observed 14-helix propensities of \(\beta^3\)-amino acids.

While equilibrium theory dictates that the relative populations of different conformations should be a function of their relative free energies, a comprehensive comparison of energetics would require exhaustive sampling of the relevant conformational space. By contrast, the overall 14-helix content of each individual \(\beta\)-peptide can be estimated by examining its structural fluctuations over the 2 million Monte Carlo configurations. For instance, for all the \(\beta^3\)-oligopeptides simulated, the N-terminal hydrogen bond is only about half as populated as the C-terminal hydrogen bond, implying that stabilizing effects should be diminished at the C-terminus. This prediction is supported by the CD data (Figure 2.8). More generally, 14-helix propensities of the \(\beta^3\)-amino acids can be ranked by hydrogen bond population as follows: \(\beta^3I > \beta^3V \approx \beta^3S \geq \beta^3T > \beta^3A \geq \beta^3L\). This ranking matches the CD data well. Average torsional angles for the substituted oligo-\(\beta^3\)A-peptides indicate that distortions from the optimal 14-helix geometry, which give the helix axis a slight bend, may aid inter-residue interactions without compromising hydrogen bonding. Overall, the experimental evidence supports our simulations, which appears to accurately model the inter-residue interactions involved in 14-helix formation and side chain-mediated stabilization.
Simulations explain the effects of polar substitutions

Unexpectedly, the CD data indicate that $\beta^3$-homoserine is at least as stabilizing as $\beta^3$-homothreonine, despite a lack of side chain branching. However, serine is not particularly $\alpha$-helix-stabilizing, and threonine is among the least $\alpha$-helix-stabilizing $\alpha$-amino acids. It has been proposed that polar groups can promote or detract from secondary structure, depending upon the relative energetics of hydrogen bonding in the unfolded and folded states.\textsuperscript{92} If this is the case, the 1-hydroxyethyl and hydroxymethyl side chains must make more favorable contacts in the 14-helical context relative to an unfolded $\beta$-peptide (or fewer unfavorable contacts in the unfolded state relative to the folded state) than they make in the $\alpha$-helical context relative to an unfolded $\alpha$-peptide. Interestingly, in our Monte Carlo simulations the side chain hydroxyl groups of $\beta^3S$ and $\beta^3T$ appeared to make hydrogen bond contacts to the $(i+2)$ carbonyl oxygen when available. When substituted at internal positions (positions 6 and 9), $\beta^3S$ and $\beta^3T$ formed these side chain hydrogen bonds in 68% and 35% of the 2 million configurations sampled, respectively. The fact that $\beta^3S$ may have a high propensity to form 14-helix-stabilizing hydrogen bonds may explain its unexpectedly high propensity for promoting 14-helix structure. More high resolution structural data will undoubtedly shed light on the mechanism behind the unexpected 14-helix propensities of polar $\beta^3$-amino acids.

A scaffold for molecular recognition

The host-guest analysis as a whole validates peptide 2 as an excellent folded scaffold for molecular recognition. At its present length it is able to present up to four side chains in a linear arrangement, though it is yet unknown whether the helix propensity effects of
multiple substitutions will be cooperative or simply additive. The scaffold might easily be extended in increments of six residues (to retain the favorable salt-bridging and charge-macrodipole interactions) or be further substituted on the $\beta^3$-homovaline-bearing face, yielding an even larger surface area for incorporation of function. Even so, three residues may be enough to recognize some important protein targets, especially considering $\beta^3$-amino acids have few limitations on side chain functionality. Future work will target important cellular proteins using this scaffold in order to rigorously evaluate 14-helical $\beta$-peptides as $\alpha$-helix mimetics.\textsuperscript{130}

The present study has revealed $\beta^3$-peptides with unprecedented overall 14-helicities in water. These $\beta$-peptides are made from the synthetically accessible and diverse $\beta^3$-amino acids, and require no ring constraints to achieve folding. The data belie the assumption that unconstrained $\beta^3$-amino acids cannot form highly structured 14-helices in water, and demonstrate that diverse functionality can be incorporated into a stable 14-helix through simple substitution. The most 14-helical $\beta$-peptide generated, 2-V6, has a mean residue ellipticity minimum at 214 nm (MRE\textsubscript{214}) of $-19,130 \pm 340$ deg cm$^2$ dmol$^{-1}$ in aqueous solution, indicating mean helix structure of up to 95%. This rivals the values observed for the most 14-helical $\beta$-peptides observed to date in methanol or in micelles, even those with cyclic residues.\textsuperscript{68, 88, 132} The 14-helix propensities of $\beta^3$-amino acids observed in this study contrast sharply with the $\alpha$-helix propensities of corresponding $\alpha$-amino acids. This data, along with other evidence, demonstrates that 14-helix folding is governed by radically different biophysical forces than is $\alpha$-helix folding.
Figure 2.1 – (A) Comparison of secondary structures formed by α- and β-amino acid oligomers. Carbon atoms are shown in black, nitrogen atoms in blue, oxygen atoms in red, and amide hydrogen atoms in white. Other hydrogen atoms are omitted for clarity. (B) β-amino acid monomers that promote the formation of unique helices and their primary proponents.
Figure 2.2 – β-peptides that populate 14-helix structures in water. Coloring has been used to accentuate \((i,i+3)\) salt-bridges. (A) A β-dodecamer utilizing cyclic and acyclic residues reported by Raguse et al.\(^{80}\) (B) A salt-bridge-stabilized \(\beta^3\)-heptapeptide with appreciable 14-helical CD signature in water reported by Arvidsson et al.\(^{84}\) (C) Salt-bridge-stabilized 15-mer \(\beta^3\)-peptide reported by Cheng and DeGrado.\(^{85}\) (D) Macrodipole-stabilized \(\beta^3\)-undecapeptide reported by Hart et al.\(^{86}\)
Figure 2.3 – (A) Helical net diagrams of β-peptides 1 and 2. β^3^X refers to a β^3^-amino acid with side chain analogous to the α-amino acid with the common one-letter code X. Coloring has been used to emphasize electrostatic stabilization features. (B) Circular dichroism spectra of β-peptides 1 (red) and 2 (black) at 100 μM and 80 μM, respectively, in PBC buffer at 25 ºC.
Figure 2.4 – (A) Analytical ultracentrifugation data for: 2-S9 (pink), 2-E3 (red), 2 (black), 2-I6 (blue), 2-V9 (cyan), 2-T3 (green), 2-S6 (brown), 2-L3 (orange), and 2-V3 (gray). (B) Calculated concentration based on material balances for a monomer fit (mon) or a dimer fit (dim) to the AU data. The observed concentration is shown for comparison (obs).
Figure 2.5 – Circular dichroism spectra of variants of 2 containing substitutions at position 3 (red), 6 (green), or 9 (blue) with: (A) $\beta^3$-homolysine, (B) $\beta^3$-homoglutamic acid, (C) $\beta^3$-homoleucine, (D) $\beta^3$-homoisoleucine, (E) $\beta^3$-homovaline, (F) $\beta^3$-homoserine, (G) $\beta^3$-homothreonine, (H) $\beta^3$-homophenylalanine, or (I) $\beta^3$-homotryptophan. Spectra were acquired in PBC buffer at 25 ºC and a $\beta^3$-peptide concentration of 80 µM.
Table 2.1 – Theoretical and MALDI-TOF MS-observed molecular weights for each β₃-peptide studied.

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<th>β-peptide</th>
<th>Mass Calculated (M + H⁺)</th>
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Table 2.2 – Minimized $\beta^3$-oligopeptide energies in vacuo and in aqueous solution.

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<th>Substituted Position</th>
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<th>12</th>
<th>14</th>
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<td></td>
<td></td>
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<td>C6</td>
<td>$\beta$</td>
<td>C8</td>
<td>12h</td>
<td>14h</td>
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<td>acetyl-((\beta^3)A)$_{12}$-NHCH$_3$</td>
<td>Gas Phase$^a$</td>
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<td></td>
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<td>77.0</td>
<td>54.5</td>
<td>37.9</td>
<td>0.0</td>
<td>64.2</td>
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</table>

$^a$Energies are calculated in gas phase in kcal·mol$^{-1}$ ($E_{\text{int}}$) and are normalized to the lowest-energy conformation for each individual $\beta$-peptide.

$^b$Energies are the sum of $E_{\text{int}}$ and $\Delta G_{\text{GB/SA}}$ and are normalized to the lowest-energy conformation for each individual $\beta$-peptide.
Table 2.3 – β^3-oligopeptide average energies in aqueous solution in MC simulations.

<table>
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<tr>
<th>Substituted Residue</th>
<th>Starting Conformation</th>
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<th>6</th>
<th>9</th>
<th>12</th>
<th>14h</th>
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^aEnergies are calculated as E_{int}+ΔG_{GB/SA} in kcal·mol^1 over 2 million configurations.
Figure 2.6 – Hydrogen bond populations near substitution sites in Monte Carlo simulations of 14-helical oligo-β³-homoalanine peptides individually substituted at the indicated position with: β³-homoalanine (gray), β³-homoserine (yellow), β³-homothreonine (green), β³-homoisoleucine (cyan), β³-homoleucine (blue), or β³-homovaline (navy blue). Hydrogen bond population near a substitution site was calculated as the average population of the \((i-2 \rightarrow i)\), \((i-1 \rightarrow i+1)\), and \((i \rightarrow i+2)\) hydrogen bonds (when available) surrounding substitution position \((i)\) over 2 million configurations.
Figure 2.7 – (A) $\Theta_{214}$ of 80 $\mu$M 2-I6 (blue) and 2 (red) plotted against square root of salt concentration in molality. (B) CD spectra of 80 $\mu$M 2-I6 (blue), 2 (red), and 2-E3 (green) at 4 °C (squares), 60 °C (circles), and 98 °C (diamonds). (C) $\Theta_{214}$ of 80 $\mu$M 2-I6 (blue), 2 (red), and 2-E3 (green) from 4 °C to 98 °C (open squares) and back down to 4 °C (open circles).
Figure 2.8 – Summary of the host-guest analysis of $\beta^3$-amino acid 14-helix propensities.

Plot shows the negative mean residue ellipticity at 214 nm of variants of $\beta$-peptide 2 (shown at left, and in chart at right in gray) substituted at positions 3, 6, and 9 with: $\beta^3$-homoglutamic acid (red), $\beta^3$-homolysine (orange), $\beta^3$-homoserine (yellow), $\beta^3$-homothreonine (green), $\beta^3$-homoisoleucine (cyan), $\beta^3$-homoleucine (blue), $\beta^3$-homovaline (navy blue), $\beta^3$-homophenylalanine (purple), and $\beta^3$-homotryptophan (pink). Circular dichroism was performed at a $\beta$-peptide concentration of 80 $\mu$M in PBC buffer, pH 7.0, at 25 °C.
Chapter 3 – β-peptide Ligands for hDM2

3.1 – β-peptides as inhibitors of protein-protein interactions (PPIs)

Nature uses a finite number of chemical building blocks to generate molecules with stunningly diverse molecular functions. The key to this economical but powerful strategy is the construction of linear chains that fold independently into complex three-dimensional structures. Indeed, proteins assembled from natural α-amino acids are among the most potent and diverse ligands for cellular macromolecules, especially other proteins that contain large, shallow binding surfaces.\(^\text{133}\) Despite the attractiveness of well-folded proteins as ligands for protein surfaces \textit{in vitro} and as research tools \textit{in vivo}, their use as therapeutics is currently limited by low cell permeability, high proteolytic sensitivity, and poor pharmacodynamics.\(^\text{134}\) By contrast, β-peptides – a quintessential example of the class of molecules known as non-natural folding oligomers, or foldamers,\(^\text{62, 64}\) – consist of linear chains of β-amino acids, and are thus virtually invulnerable to proteases.\(^\text{76, 135}\) Early results suggest β-peptides have favorable pharmacodynamics.\(^\text{136}\) Moreover, β-peptides can fold into stable secondary structures without the need for tertiary interactions,\(^\text{62, 64}\) allowing an extended, variable protein-binding surface to be presented by a relatively short oligomer. This may translate into potent ligands with favorable cell permeability and tunable pharmacodynamics. For these reasons we became interested in exploring whether β-peptide foldamers could bind protein surfaces and inhibit protein-protein interactions, and if so, whether their affinities
and specificities would compare favorably with those of natural\textsuperscript{23} or miniature proteins.\textsuperscript{4, 6-8, 10-12, 137}

Previous work (outlined in the Chapter 2 and in references therein) had armed us with unique insight into the folding of $\beta^3$-peptide 14-helices. This insight had enabled us to design a scaffold $\beta^3$-peptide that was roughly 50\% folded overall in aqueous solution (as estimated by CD).\textsuperscript{86} We had also used this scaffold as a host peptide, substituting a variety of side chains in order to assess the relationship between side chain structure and 14-helix propensity for the $\beta^3$-peptide 14-helix. One of the general conclusions reached in the host-guest analysis was that scaffold $\beta$-peptide 2 (Figure 2.3A) is highly amenable to substitution. In fact, we inferred that 2 can remain partially folded while restricting only four of eleven residues (to introduce charged groups which form ion pairs and diminish the overall 14-helix dipole upon folding), or four of ten residues if the terminal $\beta^3$-homotyrosine (included for rapid and accurate concentration determination) was omitted. With this structured and versatile scaffold in hand, we returned to one of our primary reasons for designing water-stable, folded $\beta$-peptides: the rational design of helical $\beta$-peptide PPI inhibitors.

We were encouraged by the earlier work of Seebach, who demonstrated that $\beta$-peptide hairpins could bind somatostatin receptors with high affinity and specificity,\textsuperscript{138, 139} and by work of Seebach,\textsuperscript{140} DeGrado,\textsuperscript{88, 141} and Gellman\textsuperscript{142-144} showing that amphipathic $\beta$-peptides could perform a variety of functions including inhibition of cholesterol and fat uptake,\textsuperscript{140} potent antibacterial activity,\textsuperscript{88, 141-143} and RNA binding.\textsuperscript{144} We chose the complex between hDM2 and p53 as a first target,\textsuperscript{39} knowing the importance of p53 as a transcriptional activator critical for stress-induced cell cycle arrest.
and apoptosis.\textsuperscript{30} In the absence of stress, hDM2 down-regulates p53 activity by sequestering p53’s activation domain (p53AD), exporting p53 from the nucleus, and directly ubiquitinating p53.\textsuperscript{29} Cancerous cells often overexpress hDM2, resulting in a loss of the cell’s primary response to stress and leading to unchecked cell growth.\textsuperscript{29, 30} In addition, we had previously targeted hDM2 using miniature proteins (with some success; see Chapter 1 for details) and were eager to put our β-peptide designs to the same task.

3.2 – Rational design of β-peptides that inhibit the p53AD•hDM2 protein-protein interaction

Our design began with closer structural comparison of the β-peptide 14-helix and the hDM2-bound conformation of p53AD’s α-helix (Figure 3.1). Although the dimensions of a 14-helix differ from those of an α-helix,\textsuperscript{65, 66, 110} we hypothesized that the p53AD functional epitope would be recapitulated if the side chains of F19, W23, and L26 were presented at successive positions three residues apart on a stabilized 14-helix (Figure 3.1B). Four β\textsuperscript{3}-peptides were designed in which these side chains are appended in both possible orientations on each of the two available 14-helix faces (β\textsuperscript{53-1–4}, Figure 3.2).

We compared the circular dichroism (CD) spectra of β\textsuperscript{53-1–4} in aqueous buffer to estimate their 14-helix content (Figure 3.3A). The CD signature of a 14-helix is clearly evident, and the relative minima at 214 nm suggest helical contents between 30 and 50\% for β\textsuperscript{53-1}, 3, and 4 (β\textsuperscript{53-2} was only soluble to ~75 µM). Two-dimensional NMR spectroscopy in CD\textsubscript{3}OH confirmed the presence of 14-helix structure in β\textsuperscript{53-1}: ROESY spectra showed four of seven possible C\textsubscript{α}H(\textit{i}) \rightarrow C\textsubscript{β}H(\textit{i}+3) ROEs and two of six possible
$C_N(i) \rightarrow C_{\beta(i+3)}$ ROEs characteristic of the 14-helical conformation (Figure 3.3B). Additional ROEs may be present but were obscured by resonance overlap; no ROEs inconsistent with 14-helical structure were observed. Analytical ultracentrifugation revealed that $\beta_{53-1}$, 3 and 4 were monomeric at concentrations between 80 and 400 µM (Figure 3.4), confirming that these 14-helices are stabilized by intramolecular interactions.

We designed a competition fluorescence polarization (FP) assay using $hDM2_{1-188}$ (hDM2) and a fluorescein-labeled p53AD$_{15-31}$ peptide (p53AD$^\text{Flu}$) to monitor inhibition of p53AD•hDM2 complexation by $\beta_{53-1–4}$. The $K_d$ of p53AD$^\text{Flu}$•hDM2 measured by direct FP analysis was $233 \pm 32$ nM (Figure 3.5B), consistent with previous work. $p53AD_{15-31}$ inhibited the p53AD$^\text{Flu}$•hDM2 interaction with an IC$_{50}$ of $2.47 \pm 0.11$ µM, as expected (Figure 3.5A). Two $\beta^3$-peptides, $\beta_{53-1}$ and $\beta_{53-3}$, inhibited p53AD$^\text{Flu}$•hDM2 complexation with IC$_{50}$ values of $94.5 \pm 4.4$ µM and $1589 \pm 104$ µM, respectively (Figure 3.5A), but only $\beta_{53-1}$ failed to inhibit formation of the unrelated CREB KID•CBP KIX complex (Figure 3.6B); $\beta_{53-3}$ was not studied further. To ensure the observed inhibition was due to direct binding, two fluorescein-conjugated variants of $\beta_{53-1}$ were synthesized. $\beta_{53-1}^\text{Flu}$, labeled on the C-terminus, bound hDM2 with a $K_d$ of $583 \pm 88$ nM, while $\text{Flu} \beta_{53-1}$, labeled on the N-terminus, bound hDM2 with a $K_d$ of $368 \pm 76$ nM (Figure 3.5B). The similarity of the two $K_d$ values, as well as the inability of other fluorescein-$\beta$-peptide conjugates to bind hDM2 (including $\beta_{53-5}^\text{Flu}$ and $\beta_{53-6}^\text{Flu}$), implies that the dye contributes little to the binding free energy. The affinity of $\beta_{53-1}$ for hDM2 is only 1.6 to 2.5-fold lower than that of p53AD.
**Investigating the basis for specific recognition of hDM2 by β53-1**

Next we prepared a series of β3-decapeptides to assess whether the affinity of β53-1 for hDM2 required all or part of the functional epitope composed of p53AD side chains F19, W23, and L26. β3-peptides β53-W6, β53-F9, and β53-L3, in which two of these three side chains were changed to β3-homoalanine, inhibited p53AD•hDM2 complexation with IC50 values of 198.1 ± 10.0, 1701 ± 163, and > 7000 μM, respectively (Figure 3.6A). 6 β53-W6, which retained β3-homotryptophan (β3W), was the most potent inhibitor, with an IC50 value 2-fold higher than that of β53-1, whereas β53-F9, which retained β3-homophenylalanine (β3F), was moderately potent. Importantly, the relative arrangement of β3W and β3F was critical: β3-peptides containing different arrangements of these residues, β53-2 and 4, showed no inhibition at 70 and 700 μM, respectively, and others with a single β3F residue (β53-F3, β53-F6) showed no inhibition at 1 mM. In addition, β3-peptides with a single β3L residue (β53-L3, β53-L6, β53-L9) showed no inhibition at concentrations as high as 20 mM, and those with a single β3I residue (β53-I3, β53-I6, β53-I9) showed no inhibition at concentrations as high as 1 mM. A sequence-unrelated β3-tetrapeptide containing β3W (β53-7) and β3W itself were poor inhibitors (IC50 > 1 mM, Figure 3.5C). These data indicate that β53-1 interacts with hDM2 with specific contributions from two of three residues comprising the p53AD functional epitope, β3W and β3F. The relative importance of β3W, β3F, and β3L in the context of β53-1 is consistent with data for p53AD-based α-peptides.41, 50

We next determined if β53-1 itself could be minimized while retaining high affinity and selectivity for hDM2. β53-5 and β53-6 (Figure 3.2) lack residues 1-2 or 1-4
(including the $\beta^3$L at position 3) of $\beta_{53}$-1, respectively and are less structured in aqueous solution (Figure 3.3A). $\beta_{53}$-5 inhibited the p53AD$^{\text{Flu}}$•hDM2 interaction slightly more potently than did $\beta_{53}$-1 (IC$_{50}$ = 80.8 ± 3.2 µM, Figure 3.5C) but the $\beta^3$-hexapeptide $\beta_{53}$-6 inhibited the p53AD$^{\text{Flu}}$•hDM2 interaction poorly, with an IC$_{50}$ of 250 ± 12 µM. Both $\beta_{53}$-5 and $\beta_{53}$-6 inhibited CREB KID•CBP KIX complexation with potencies similar to those for p53AD•hDM2 inhibition (60.1 ± 5.3 µM and 150.6 ± 15.0 µM, respectively; Figure 3.6B). As a whole, these data imply that the well-defined 14-helical structure of $\beta_{53}$-1 is a prerequisite for selective recognition of hDM2.

Materials and Methods

General

Fmoc-protected $\alpha$-amino acids, PYBOP®, HOBt, and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), N-methyl morpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). CD$_3$OH (99.5% d$_3$) was obtained from Cambridge Isotopes (Andover, MA). Biotinylated thrombin and streptavidin agarose were from Novagen (Madison, WI). Competent E. coli BL21 cells were from Stratagene (La Jolla, CA). Glutathione sepharose was purchased from Amersham Pharmacia (Piscataway, NJ). Hard shell 384-well microplates (black wells) were used for fluorescence polarization experiments (MJ Research, Waltham, MA). All other reagents were purchased from Sigma-Aldrich. Mass spectra were acquired with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City,
Reverse-phase HPLC was performed using a Rainin Dynamax HPLC and Vydac analytical (C18, 300 Å, 5 µm, 4.6 mm x 150 mm) or semipreparative (C18, 300 Å, 5 µm, 10 mm x 250 mm) columns, using water/acetonitrile gradients with 0.1% TFA. β³-peptides and p53AD were synthesized using a Symphony/Multiplex peptide synthesizer (Protein Technologies, Tuscon, AZ). Fluorescence polarization experiments were performed with an Analyst AD (Molecular Devices, Sunnyvale CA) spectrofluorimeter. Analytical ultracentrifugation was performed using a Beckman XLI instrument (Beckman, Fullerton, CA). Amino acid analyses were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine.

β³-peptide synthesis

Fmoc-protected β³-amino acids were prepared following methods described by Seebach. β³-peptides were synthesized on a 25 µmole scale using standard Fmoc chemistry and Wang resin loaded with β³-homoglutamic acid as described. One cycle of peptide elongation consisted of the following steps. The loaded resin was first washed with N-methyl-2-pyrrolidone (NMP) (3 x 30 sec) and the terminal Fmoc protecting group removed with 20% piperidine/DMF (1 x 2 min, 2 x 8 min). The deprotected resin was then washed with NMP (6 x 30 s) and treated for 30 min with a cocktail containing 3 equiv of the appropriate β³-amino acid, 3 equiv PYBOP®, 3 equiv HOBT, and 8 equiv diisopropylethylamine (DIEA). The coupled resin was then washed once with NMP (1 x 30 s), unreacted amino groups acetylated upon treatment with 6% v/v acetic anhydride and 6% v/v NMM in NMP (20 min), and the capped resin washed with NMP (2 x 30 s). These steps were repeated until the β-peptide sequence was complete. Once the final
Fmoc protecting group had been removed, the resin was washed with NMP (8 x 30 s) and methylene chloride (8 x 30 s), dried 20 min under N₂, and then treated for 90 min with a cleavage cocktail composed of 3% v/v water and 3% v/v tri-isopropylsilane in trifluoroacetic acid (TFA). The cleaved resin was washed once with the cleavage cocktail (1 x 30 s) and the cleaved β-peptide collected, concentrated by rotary evaporation and reconstituted in H₂O/CH₃CN (1:1).

β-peptide purification and analysis

The success of each synthesis was assessed first by HPLC and MALDI-TOF analysis of the crude reaction mixture. β-peptides were then purified to homogeneity by reverse-phase HPLC. The identities and purities of purified β-peptides were assessed by analytical HPLC and mass spectrometry (Table 3.1). Following purification, β-peptides were lyophilized, kept at -20 °C, and reconstituted in PBC (for CD) or PBS buffer (for FP) immediately prior to use.

p53AD and p53AD^Flu synthesis

p53AD, containing residues 15 – 31 of p53 with a cysteine residue appended to the C-terminus (¹⁵SQETFSDLWKLLPENN³¹C) was synthesized on a 25 µmole scale using standard Fmoc chemistry and Wang resin as described previously, and was acetylated on the N-terminus and amidated on the C-terminus. p53AD^Flu was generated by reaction of a HPLC-purified sample of p53AD with a 25-fold molar excess of 5-iodoacetamidofluorescein (Molecular Probes) in a 1:9 mixture of dimethylformamide:phosphate-buffered saline (DMF:PBS), pH = 7.4. Labeling reactions
were incubated with rotation for 2 h at RT in the dark. The crude fluorescein-labeled peptide was purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry and amino acid analysis (Table 3.1).

**Preparation of fluorescein-labeled β53-1 variants**

β53-1 was prepared with an N-terminal fluorescein label by synthesizing the β53-1 β3-peptide sequence on-resin as described above, then coupling (as described above) with 4 equiv. β3-glycine, commonly known as β-alanine (NovaBioChem). Two full cycles of deprotection, coupling, and capping were performed with β3-glycine to provide a well-behaved 8-atom linker identical to the β-peptide backbone. Finally, fluorescein-5-succinimidyl ester (5g, from Molecular Probes) was added in 1.5 mL NMP and allowed to mix with bubbling N2 for 4-6 hours. The C-terminally-labeled β53-1 variant was prepared by using an α-cysteine-loaded Wang resin (NovaBioChem) and performing two full coupling cycles using β3-glycine (to provide a linker) prior to coupling the β53-1 sequence. After cleavage from the resin and HPLC purification, the cysteine thiol was alkylated with 5-iodoacetamidofluorescein (30-fold excess in 10% DMF, 90% PBS pH 7.4). The reaction went to completion after 4 hours in the dark, rotating at room temperature. The labeled product was isolated by HPLC purification and identity verified by MALDI-TOF MS.

**Overexpression of hDM21-188**

A fusion protein consisting of residues 1-228 of glutathione-S-transferase, a 13-residue linker (SDLGGGGLVPRGS) containing a thrombin cleavage site, and residues 1-
188 of human MDM2 (GST-hDM2\textsubscript{1-188}) was prepared by over-expression of ‘clone G’ reported by Lane and co-workers.\textsuperscript{41} Competent \textit{E. coli} BL21 cells were transformed by electroporation and a single colony was used to inoculate a 1 L culture of 2XYT media containing 0.1 mg/mL carbenicillin. The culture was incubated at 37 °C with shaking at 220 rpm until the optical density at 600 nm reached 0.7 absorbance units. Protein expression was then induced by adding isopropyl \( \beta \)-D-thiogalactoside (IPTG) to 200 µM final concentration. Cells were harvested after 3 h by centrifugation for 20 min at 6000 g, resuspended in buffer A (0.5 M NaCl, 2.7 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.8 mM KH\textsubscript{2}PO\textsubscript{4} (pH = 7.4), 1 mM EDTA, and 2 mM DTT), and lysed by sonication. Cell debris was pelleted by centrifugation for 30 min at 37000 g and the supernatant was incubated with glutathione sepharose at 4 °C for 1 hr to immobilize the fusion protein. The resin was washed with three bed volumes of chilled Buffer A before being treated with 8 U biotinylated thrombin for 2-4 h at RT to release hDM2\textsubscript{1-188} while leaving GST bound to the solid phase. The biotinylated thrombin was removed from hDM2\textsubscript{1-188} upon incubation with streptavidin-agarose and subsequent filtration. The resulting solution was characterized by SDS-PAGE, MALDI-TOF mass spectrometry, and amino acid analysis to verify identity, purity and protein concentration. Pure hDM2\textsubscript{1-188} was distributed into aliquots, flash-frozen in liquid nitrogen and stored at -70 °C until use.

\textit{Analytical ultracentrifugation}

Note: The experiments and data treatment described in this section were performed by Dr. James Lear, Department of Chemistry, University of Pennsylvania.
Samples of $\beta_{53-1-4}$ used in analytical ultracentrifugation experiments were prepared by dissolving HPLC-purified $\beta$–peptide in PBC buffer (1 mM each phosphoric, boric, and citric acids, adjusted to pH = 7.0 with NaOH) to the desired concentration (80 µM – 100 µM) and then centrifuged to equilibrium at 25 °C at the maximum allowed speed (50,000 RPM) in six-channel, carbon-epoxy composite centerpieces supplied by Beckman. Absorbance was monitored at 275 nm. Data were collected with a 0.03 cm step size. Equilibrium was assessed by the absence of significant change in radial concentration gradients in scans at 14 and 16 h. The data was fit (Figure S1) using Igor-Pro® (Wavemetrics, Lake Oswego OR) to the following equation:

$$C(r) = C(r^0)\exp\left(\frac{(1 - \bar{v}\rho)\omega^2 M_n}{2RT} (r^2 - r_o^2)\right)$$

In this equation, $C$ is the concentration (any units) of the sedimenting species at radial positions $r$ and $r_o$ cm from the center of rotation; $\bar{v}$ is the partial specific volume of the sedimenting species in cc/gm; $\rho$ is the density of supporting buffer in g/cc; $\omega$ is the angular velocity of the rotor (radians/s); $M_n$ is the “Molar” molecular weight of sedimenting species (g/mole); $M_b$ is the “buoyant” molecular weight, equal to $M_n (1 - \bar{v} r)$; $R$ = Gas constant (8.315 x $10^7$ ergs K$^{-1}$ mol$^{-1}$) and $T$ = Temperature (K).

Partial specific volumes for $\beta$-peptide $\beta_{53-1-4}$ were assumed for simplicity to equal the average value calculated for previously studied $\beta$-peptides: 0.785 cm$^3$/gm.$^{86}$ Because of cross-correlation of molecular weight with baseline values, curve-fits were insensitive to variations in this value. In fact, equally good curve-fits could be obtained by assuming either monomer or dimer molecular weights. This ambiguity is an unavoidable consequence of the low curvature exhibited in the concentration profiles of the low
molecular weight compounds. To distinguish monomers from higher order aggregated species as the dominant population, curve fits were performed using integral multiples of the sequence-calculated molecular weights. A material balance criterion previously developed to study binding of low and high molecular weight compounds was used to choose the better model (Figure 3.4, lower panels).

**Competition fluorescence polarization assays**

Fluorescence polarization experiments were performed at 25 °C in 384-well plates (MJ Research, Waltham, MA). For direct binding measurements, serial dilutions of hDM2 were made in PBS buffer, pH 7.2, and an aliquot of fluorescently labeled peptide (p53AD, β53-1, or β53-1) was added to a final concentration of 25 nM, to a total volume of 12 µL. The binding reaction was incubated for 30 min at RT. Thirty minutes was a sufficient length of time for the binding reaction to reach equilibrium, as judged by an absence of change in observed polarization values after 1 h and 90 min. For competition experiments, serial dilutions of p53AD, β-peptide, or β3-homotryptophan were incubated with 0.5 µM hDM2 and 25 nM fluorescein-labeled p53AD for 30 min at room temperature, in a total volume of 12 µL. Selectivity was explored by incubating fluorescein-labeled KID(119-148) (phosphorylated on Ser133) with His-tagged KIX(586-672) at final concentrations of 27 nM and 1.5 µM, respectively, in the presence of: β53-1 (orange), β53-3 (blue), and a phosphorylated miniature protein ligand developed in our lab, PPKID4P (green) as a positive control. His-KIX(586-672), PPKID4P, and KID(119-148) were obtained as described. Binding reactions containing 1.5 µM CBP KIX, 27 nM of a fluorescein-labeled high affinity CBP ligand (KIDABflu), and serially diluted β53-1
(blue), β53-3 (pink), β53-5 (brown), β53-6 (purple), or the high affinity ligand\(^5\) PPKID4\(^p\) (gray). Reactions were incubated for 30 min at 25 °C; equilibrium was assessed by the absence of a change in polarization after this time. Each point represents the average polarization of three independent samples; error bars denote standard error. Observed polarization values were converted to fraction of peptide\(^{\text{flu}}\) bound using \(P_{\text{min}}\) and \(P_{\text{max}}\) values derived from the best fit of the polarization data to the equation \(\theta_{\text{obs}} = ((\theta_{\text{max}} - \theta_{\text{min}})/(1 + ([\text{competitor}]/IC_{50}^{\text{slope}}))) + \theta_{\text{min}}\).

**NMR spectroscopy of β53-1**

β53-1 was dissolved in CD\(_3\)OH to ~3 mM for NMR analysis. All data were acquired at 20 °C on a Varian INOVA 600 MHz spectrometer (Varian, Palo Alto, CA) with a 5 mm triple resonance (HCN) probe equipped with triple axis (XYZ) pulsed magnetic field gradients. All pulse sequences were part of the Varian ProteinPack user library. Homonuclear two-dimensional (2D) NMR spectra were acquired with spectral widths of 8000 Hz in both dimensions, a 3 second recycle delay between successive scans, and acquisition times of 0.256 and 0.032 s along F2 and F1, respectively. For the z-filtered\(^{147}\) 2D TOCSY\(^{148}\) NMR experiment, isotropic mixing was applied for 100 ms using an ~11 kHz DIPSI-2\(^{149}\) subsequence. Similarly, spin-locking during the 2D ROESY\(^{150}\) NMR experiment was achieved using a 5 kHz continuous radiofrequency field applied during the 300 ms ROE mixing period. The solvent resonance was suppressed in both experiments using WET\(^{151, 152}\) subsequences containing 5 ms selective sinc pulses. Acquisition required a total of 15 hours and 5.5 days due to signal averaging of 32 and 256 scans for the TOCSY and ROESY experiments, respectively. The resulting spectra
were processed using NMRpipe and analyzed using the Sparky software package. In addition to those depicted in Figure 3.3B, there were many potential ROEs corresponding to 14-helical structure that may have been present but were obscured by resonance overlap. Additionally, a few ROEs were not observed due to the proximity of their chemical shifts to solvent resonances.

3.3 – Binding mode of β53-1

*Binding of β53-1 to hDM2 in high salt conditions*

Since β-peptides based on scaffold 2 were known to unfold in the presence of high salt (Figure 2.7), we hypothesized that, if folding and binding were independent events and β53-1 folds prior to binding, testing β53-1 affinity for hDM2 at high salt concentration could provide evidence as to whether it binds as a 14-helix. To this end, the CD spectra of β53-1 at different salt concentrations were obtained, demonstrating that β53-1 also unfolds at high salt concentrations (Figure 3.7A). The p53ADflu•hDM2 interaction was also unaffected by high salt (Figure 3.7B). However, when β53-1flu was incubated with hDM2 at different salt concentrations, no change was seen (Figure 3.7B), allowing few clear conclusions.

*Competition between β53-1 and p53AD for hDM2 binding site(s)*

Because the IC₅₀ for β53-1 seemed higher than it should be considering the measured K₄ for the β53-1flu•hDM2 interaction (compared to the relative K₄’s and IC₅₀’s of p53AD and miniature protein ligands reported in Chapter 1), the binding mode was investigated
next. Unlabeled $\beta_{53-1}$ and p53AD were incubated with 25 nM p53AD$^{\text{flu}}$ in the presence of hDM2, and also with 50 nM $\beta_{53-1}^{\text{flu}}$ in the presence of hDM2. This allowed assessment of self-competition, examining the possible role of the fluorescein label in the observed discrepancies, as well as assessment of how each molecule displaces the other in competition for hDM2’s binding site(s). Results are shown in Figure 3.8A. All competition experiments furnished data which agreed with the measured $K_d$’s except for the competition between $\beta_{53-1}$ and p53AD$^{\text{flu}}$. This indicates that $\beta_{53-1}$ does compete with p53AD for the known binding site; however, there may be another site at which they both bind with lower affinity.

Stoichiometry of the p53AD•hDM2 and $\beta_{53-1}$•hDM2 interactions

To examine the possibility of a second binding site, a Job plot was performed (Figure 3.8B). At 2 $\mu$M total concentration, the Job plots for $\beta_{53-1}^{\text{flu}}$ and p53AD$^{\text{flu}}$ have some scatter, but essentially agree with the 4 $\mu$M Job plots shown in Figure 3.8B. These indicate that there are two p53AD$^{\text{flu}}$ molecules per hDM2, but only one $\beta_{53-1}^{\text{flu}}$ molecule per hDM2. However, at 8 $\mu$M total concentration analogous Job plots imply that there are two $\beta_{53-1}^{\text{flu}}$ molecules per hDM2. The evidence as a whole points to there being two binding sites for p53AD on hDM2$_{1-188}$, with one binding site for $\beta_{53-1}^{\text{flu}}$ or two binding sites with only one at sub-micromolar affinity. This explanation fits the Job plot data and the competition data, and it also explains why the IC$_{50}$ for $\beta_{53-1}$ inhibiting the p53AD$^{\text{flu}}$•hDM2 interaction is unexpectedly high.

3.4 – Solution structure of $\beta_{53-1}$ in methanol
Obtaining assignments and distance restraints from two-dimensional NMR data

To further validate our strategy for mimicking \( \alpha \)-helices using \( \beta \)-peptide 14-helices, and to provide some structural basis for our model of how \( \beta^{53-1} \) binds hDM2, we solved the solution structure of \( \beta^{53-1} \) in CD\(_3\)OH at 10 °C. A \( \beta^{53-1} \) sample suitable for NMR was prepared as follows: \( \sim 3 \) mgs of \( \beta^{53-1} \) were dissolved in 400 \( \mu \)L of CD\(_3\)OH (Cambridge Isotopes) with 75 \( \mu \)M DSS as a chemical shift standard. This sample was put into a methanol-matched Shigemi Advanced Microtube (Shigemi, Inc.). The sample was analyzed using TOCSY, ROESY, and \( ^1\)H\(^{13}\)C HSQC (using natural abundance \(^{13}\)C) NMR experiments. Spectra were obtained using the 800 MHz NMR in the Chemistry Dept., with great assistance from Prof. Michael Hodsdon from the Department of Laboratory Medicine at Yale School of Medicine. At 20 °C, the water resonance hid the \( \beta^3 \)-homotryptophan’s alpha proton, so the solvent resonance was shifted over by performing the experiments at 10 °C. Water suppression was achieved through the use of a WET pulse throughout each experiment. Spectra were assigned using the free program Sparky. With respect to nomenclature, the names for specific atoms within each residue were kept identical to those commonly used for \( \alpha \)-amino acids. The new atom names CME, HAX, and HEQ were added; these correspond to the additional backbone carbon, methylene proton that points axially in the 14-helical conformation, and methylene proton that points equatorially in the 14-helical conformation, respectively. An illustration of the nomenclature used is given in Figure 3.9. The complete assignments of \( \beta^{53-1} \) in CD\(_3\)OH at 10 °C are given in Table 3.2.
With confident assignments it was now possible to use NOE data to obtain structural restraints. Three separate ROESYs were performed (similar to NOESYs but performed in the rotating frame), using mixing times of 200, 350, and 500 ms, to ensure that the ROEs observed were sufficiently built up to observe, but not allowed so much time as to allow spin-transfer or to saturate the signals (that is, to ensure the signals were being obtained on the linear part of the “buildup curve”). The ROESY peaks observed at 350 ms were integrated using SPARKY and compiled, yielding a list of 449 intensities for inter-proton ROEs. These generally represented two peaks per ROE observed, since ideally there is perfect symmetry across the diagonal (in reality, some ROEs had only one integratable peak due to overlap of one of the ROEs with a noise “stripe” or the water or methanol proton resonance). SPARKY was used to format an assignment table for direct import into the DYANA program.

_Torsional dynamics and simulated annealing using DYANA_

Before using DYANA, a torsional dynamics program that keeps bond angles and lengths constant and varies only torsions to fit restraint data, the program needed to be re-parameterized to accept $\beta^3$-amino acids and to recognize the extra methylene group as part of the backbone. This took a little work, but was successfully done using standard bond lengths and angles, and trigonometry to calculate the (x,y,z) coordinates of all atoms relative to the amide nitrogen atom at (0,0,0). DYANA automatically derives restraints from the parameter files outputted by SPARKY using the CALIBA program, as follows: the peak volumes for backbone protons are compared and the average set to a predefined value _avedis_; then peak volumes for side chain and methyl protons are
calibrated as functions of the backbone volumes; then duplicate distance restraints for a single ROE are compared and the higher value is retained (thus taking the more conservative value); then all values that are unnecessary (such as an ROE between two protons attached to the same carbon atom) are discarded. This yielded a set of 151 restraints for structure calculation. It is important to note that all default values and calibration functions were used except for the value of \( \text{avedis} \), which was relaxed from 3.4 to 3.5 Å to eliminate a single restraint violation encountered when using the lower value. This change is reasonable because it represents a relaxing of the default value, and may reflect a broader range of backbone inter-proton distances due to the additional methylene unit.

These 151 restraints can be classified as intraresidual (101 of 151), sequential (10 of 151), or medium-range (40 of 151). The sequential and medium-range restraints are given in Table 3.3. Qualitative examination of the restraints reveals three general trends. First, the sequential restraints mainly reflect the \( \text{HAX}_n\text{HN}_{n+1} \) ROEs that were used to help sequential assignment. Second, medium-range restraints include the \( \text{HN}_n \rightarrow \text{HA}_{n+3} \), \( \text{HN}_n \rightarrow \text{HA}_{n+2} \), and \( \text{HB}_n \rightarrow \text{HA}_{n+3} \) distances characteristic of 14-helices. Finally, many of the remaining medium-range restraints are among the \( \beta^3 \)-homovaline residues or among the \( \beta^3 \)-homoleucine, \( \beta^3 \)-homotryptophan and \( \beta^3 \)-homophenylalanine residues, indicating that these proton-rich faces are well-structured and a great aid in obtaining high-resolution NMR structures of \( \beta^3 \)-peptides.

After calibration, the GRIDSEARCH program was used to refine the search space and speed calculations. This program analyzes local torsional space within each residue to rule out forbidden combinations of torsions, eliminating many disallowed
conformations form being accessed in the eventual structural determination. Then, ANNEAL, a simulated annealing program, was used to perform simulated annealing molecular dynamics (MD) simulations on 100 starting structures with randomized torsional angles. This resulted in a preliminary ensemble of low-energy structures. Note that the initial work was performed with no assumptions about the stereochemistry of protons attached to the same carbon (such as the two beta protons of β3-homoleucine, or the two backbone methylene protons in each residue). The GLOMSA program was used on preliminary structures to compare potential stereospecific assignments, and conservative judgments were made as to whether and how to assign protons stereospecifically. In the end, clear evidence was found to justify the assignments of all backbone methylenes as HAX and HEQ, as well as the beta protons of residues 1, 3, 6, and 10, and the β3-homovaline methyl groups. The beta protons of residues 4, 7, and 9, gamma protons of residues 1, 4, 7, and 10, and the delta protons of residues 1 and 7 were not stereospecifically assigned. Using these inputs, the final iteration of the CALIBA, GRIDEARCH, and ANNEAL programs yielded an ensemble of structures with no reported restraint violations; these are shown in Figure 3.10B.

Analysis of the solution structure of β53-1 in methanol

The ensemble of calculated solution structures of β53-1 (Figure 3.10B) shows a 14-helix with an average backbone atom RMSD from the mean structure of 0.17 ± 0.07 Å. The backbone torsions of individual structures deviate little from the mean, even at the termini (Figure 3.10C). This level of agreement among the 20 structures illustrates the robustness of the β53-1 14-helix in methanol. The helix is characterized by
approximately 1.61 Å rise per residue and 3.0 residues per turn for residues 1-6, with a slight unwinding to approximately 1.49 Å rise per residue and 3.3 residues per turn for residues 7-10. This unwinding appears unique to the structure of β53-1, as it was not observed in previous NMR structures of unrelated β3-peptides with and without side chain ion pairing.83, 84, 158 Side chains are also well-defined among the lowest-energy structures, with an overall average heavy atom RMSD from the mean of 0.60 ± 0.10 Å.

β53-1 contains four charged side chains arranged to favor formation of helix-stabilizing salt bridges on one 14-helix face.86 In all 20 low energy structures, the terminal nitrogen of β3O7 and the nearest terminal oxygen of β3E10 are characterized by a consistent separation of 5.5 ± 0.6 Å. The relative positions of the remaining two ion pairs fall into two sub-populations (Figure 3.10D). In 17 structures, the terminal nitrogen of β3O1 and the nearest terminal oxygen of β3E4 are closer (5.4 ± 0.9 Å) than the equivalent atoms of β3E4 and β3O7 (6.8 ± 0.9 Å). By contrast, in the remaining 3 structures, the terminal nitrogen of β3O7 and the nearest terminal oxygen of β3E4 are closer (3.6 ± 0.4 Å) than the equivalent atoms of β3O1 and β3E4 (7.7 ± 1.3 Å). This interplay among potential ion pairs suggests that the central salt bridge is weaker than those near the termini, and supports the hypothesis that multiple interconnected ion pairs play a key helix-stabilizing role.86, 130

Another feature incorporated into the design of β53-1 was the inclusion of β3-homovaline (β3V) residues at positions 2, 5, and 8. It was long surmised86-89 and recently proven159 that β3-amino acids branched at the first side chain carbon are particularly 14-helix-stabilizing, in stark contrast to the effects of such side chains on α-helix stability.92 The β53-1 structure provides a clear rationale for these observations. In all 20 low
energy structures one observes a unique arrangement of $\beta^3$-homovaline side chains in which one methyl group of a $\beta^3$V side chain nestles into a cleft formed by the two methyl groups of another $\beta^3$V side chain (Figure 3.10E). These interactions are especially noticeable between the side chains of $\beta^3$V5 and $\beta^3$V8, which are in VDW contact in 19 of 20 low energy structures. Overall, interactions among the three $\beta^3$V side chains bury $155 \pm 13$ Å$^2$ of hydrophobic surface area from water (24% of the surfaces of these side chains). The observed packing interactions may explain why these and other branched residues are 14-helix-stabilizing, and suggest new avenues for the design of 14-helix bundles.

The remaining 14-helix face consists of hydrophobic residues that comprise the hDM2-binding epitope, namely $\beta^3$-homoleucine ($\beta^3$L3), $\beta^3$-homotryptophan ($\beta^3$W6), and $\beta^3$-homophenylalanine ($\beta^3$F9). We originally hypothesized that the side chains of these residues would form an extended hydrophobic surface that might mimic that of p53AD. Interestingly, the $\beta^3$F9 side chain can access two specific conformations within the restraints used; the fact that this has been observed in another 14-helix structure implies that the side chain may indeed preferentially populate these rotamers within a 14-helix. The side chains of $\beta^3$W6 and $\beta^3$L3 are in VDW contact in all 20 structures, while the side chains of $\beta^3$W6 and $\beta^3$F9 are in VDW contact in the context of only one of $\beta^3$F9’s two preferred conformations (present in 6 of 20 low energy structures). On average the side chains of $\beta^3$L3, $\beta^3$W6, and $\beta^3$F9 comprise a continuous, solvent-exposed hydrophobic surface area of 520 Å$^2$. This value is comparable to the contact areas measured at the interfaces of transient homo- and heterodimeric protein complexes.
As a consequence of the unexpected unwinding near the C-terminus of β53-1, the β3F9 side chain is not aligned perfectly with the side chains of β3L3 and β3W6 along the helix axis (Figure 3.10B). This subtle distortion may avoid steric repulsions between the large side chains of β3F9 and β3W6. In fact, it is unclear whether the unwinding near the C-terminus, which is unique to β53-1, is due to more favorable ion pairing, more favorable β3V nesting interactions, or the need to avoid steric clashes on the recognition face containing large hydrophobic residues. As structures of other short, stable 14-helices are determined, it will be interesting to note what factors lead to similar distortions in the “ideal” 14-helix geometry.

Importantly, this subtle distortion allows the side chains comprising the β53-1 recognition face to better mimic those on the p53AD α-helix. Overlays between β53-1 in an idealized 14-helical conformation and p53AD bound to hDM239 revealed an imperfect alignment between the two ligands (Figure 3.1B) – while the β3L3, β3W6, and β3F9 side chains of β53-1 could superimpose with their counterparts on p53AD, the 14-helix backbone could not completely fit within hDM2’s binding groove.130 The comparable overlay with the solution structure of β53-1 (produced by superimposing all side chain heavy atoms of hDM2-bound p53AD39 with those of β53-1’s recognition face; the overlay is shown in Figure 3.11) shows no such conflict. In its solution conformation β53-1 can access all three of hDM2’s hydrophobic pockets while occupying the same binding groove as p53AD with no steric clashes. This fit demands subtle unwinding near the β53-1 C-terminus that staggers the side chains, producing a β3-peptide that is uniquely suited for α-helix mimicry.
NMR experiments of $\beta^{53-1}$ in aqueous solution

To ascertain whether structure persists in water, a D$_2$O-based sample of $\beta^{53-1}$ was prepared. This sample had 10% D$_2$O, 75 µM DSS, 10 mM PBS, pH 6.8, and 100 mM NaCl in water. Unfortunately, the peptide was poorly soluble under these conditions, necessitating filtration with a syringe filter (first flushed well to remove trace glycerol). The filtered sample (~420 µL) was used in a water-matched Shigemi tube in initial TOCSY and 350 ms ROESY experiments at 10 °C. The TOCSY yielded some assignable resonances; however, the overall dispersion of the resonances was much poorer, so that overlap became a big problem. The poor dispersion of the resonances may indicate less structure is present relative to $\beta^{53-1}$ in methanol. The ROESY failed to generate ROEs even from protons on adjacent carbon atoms, implying a problem with the pulse sequence for water, or, more likely, a peptide concentration too low to observe ROEs. To boost concentration of the peptide in water, the sample was purified and re-prepared with 10% CD$_3$OD / 10% D$_2$O / 80% H$_2$O, with the hope that the 10% methanol would be enough to boost solubility (but would still enable conclusions to be made about $\beta^{53-1}$’s structure in water). This sample also had 75 µM DSS as a standard, and the water component was 10 mM phosphate buffer, pH 6.8, with 100 mM NaCl. NMR analysis of the 10% methanol sample revealed broadened amide peaks at lower temperatures (10 °C to 20 °C). These peaks narrowed at higher temperatures (25 °C to 40 °C), and this transition occurred in a reversible manner. Rough estimations of peak widths at half maximum led to the hypothesis that soluble aggregates or a gel phase might be forming. A ROESY experiment run at 25 °C on this sample revealed no amide-aliphatic ROEs (due to exchange with solution protons), but did show more aliphatic-
aliphatic ROEs than in the all-water sample. This was an indication that the concentration was not too low to see a signal and that the experiment seemed to have executed properly. Taken together, it appears that \( \beta \text{53-1} \) may be much less well-folded in water compared to methanol. However, without the amide proton ROEs it is not clear whether any nascent structure is present.

**Materials and Methods**

\( \beta \text{53-1} \) (~3 mg) was dissolved in CD\(_3\)OH (~420 \( \mu \)L) for NMR analysis. 75 \( \mu \)M DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) was used as a reference standard. All data were acquired at 10 °C on an 800 MHz Varian Inova NMR spectrometer (Varian, Palo Alto, CA) with a 5 mm triple resonance (HCN) probe equipped with triple axis (XYZ) pulsed magnetic field gradients. All pulse sequences were part of the Varian Biopack user library. Homonuclear two-dimensional (2D) NMR spectra were acquired with spectral widths of 8000 Hz in both dimensions, a 3 second recycle delay between successive scans, and acquisition times of 0.256 and 0.032 second along F2 and F1, respectively. For the z-filtered\(^{163} \) 2D TOCSY\(^{164} \) NMR experiment, isotropic mixing was applied for 100 ms using an \( \sim \)11 kHz DIPSI-2\(^{165} \) subsequence. Similarly, spin-locking during the 2D ROESY\(^{166} \) NMR experiments was achieved using a 5 kHz continuous radiofrequency field applied during the ROE mixing period. The solvent resonance was suppressed in both experiments using WET\(^{167,168} \) subsequences containing 5 ms selective sinc pulses. Acquisition required a total of 15 and 52 hours due to signal averaging of 32 and 128 scans for the TOCSY and ROESY experiments, respectively. Assignments were
aided by the collection of natural abundance $^1$H,$^{13}$C-HSQC NMR spectra with a 2 second recycle delay between scans, and acquisition times of 0.205 and 0.022 second for the F2 and F1 dimensions, respectively. Total acquisition time required for each HSQC (one for aliphatic-region carbons, one for aromatic-region carbons) was 20.3 hours due to signal averaging of 64 scans. All spectra were processed using NMRpipe$^{169}$ and analyzed using the Sparky$^{170}$ software package. Note that some ROEs observed were not integratable due to proximity to other peaks. Four hundred forty-nine integrated peak volumes were exported to DYANA, where they were used to calibrate one hundred fifty-one non-redundant upper-distance restraints for structure analysis using the CALIBA program. The only non-default parameter varied in this calculation was the backbone average distance parameter $avedis$, which was relaxed from 3.4 Å to 3.5 Å. The effect of this change is propagated throughout the calibration and serves to relax the pre-assumed calibration distances, making them more conservative.

A new residue library file was generated for use with DYANA, specifying connectivities and torsional parameters for each $\beta$-amino acid in $\beta_{53}$-1. The upper-distance restraints were then used in the GRIDSEARCH algorithm to exclude disallowed torsions from the simulated annealing calculation. Then ANNEAL was used to perform molecular dynamics simulated annealing from 100 random initial structures. A total of 12,000 steps was used, followed by 2,000 steps of conjugate gradient minimization. The GLOMSA algorithm was then used to score individual hydrogen resonances based on relevant restraints within the new structure, in an attempt to stereospecifically assign them. This process was iterated until no more obvious stereospecific assignments could be made. Only the most evident stereospecific assignments were accepted, including the
β-protons (HB’s) of residues 1, 3, 6, and 10, and the methyl groups (QG’s) of residues 2, 5, and 8. The 20 lowest-energy structures were visualized and analyzed using MOLMOL.\textsuperscript{171}

Assignment nomenclature is analogous to standard NMR nomenclature for α-peptides and proteins, except for the extra backbone methylene unit. The extra backbone carbon atom is denoted \( C_{ME} \), for methylene carbon, and the extra hydrogen atoms are denoted \( H_{\text{AX}} \) (for the pro-R hydrogen atom, which is typically axial within a 14-helix conformation) and \( H_{\text{EQ}} \) (for the pro-S hydrogen atom, which is typically equatorial within a 14-helix conformation). Note that methyl protons are indistinguishable and were represented for structure determination by pseudo-atoms (\( Q_{G1} \) and \( Q_{G2} \)) as is common practice in α-peptides and proteins.

3.5 – Variants of \( \beta 53-1 \) containing cyclic residues

Rationale

Despite the observed irregularity of the \( \beta 53-1 \) 14-helix, we reasoned that conformational restriction of the β-peptidic backbone of \( \beta 53-1 \) could further pre-organize the molecule in its presumed binding conformation. This would be predicted to lower the entropic penalty upon binding and increase the favorability of the binding interaction. Similar reasoning led to incorporation of α-aminoisobutyric acid and 1-amino-cyclopropanecarboxylic acid within p53AD\textsubscript{19-26}, which resulted in conformational restriction significant enough to generate NOEs consistent with α-helix formation in water as well as a 4-fold boost in hDM2 affinity.\textsuperscript{49} Restricting portions of the \( \beta 53-1 \)
backbone to a 14-helix conformation also explicitly tests the model in which \( \beta53-1 \) binds its target as a 14-helix, since other conformations would be inaccessible.

To this end, we synthesized four \( \beta53-1 \) variants that include one or more residues of \( \text{trans-(S,S)}-2\text{-aminocyclohexanecarboxylic acid} \) (ACHC, Figure 3.12A), which promotes left-handed 14-helix structure by constraining the central C-C backbone torsion within a cyclohexane ring.\(^{69, 70, 80, 172}\) Three of the \( \beta53-1 \) variants possess ACHC in place of a single \( \beta^3 \)-homovaline residue (\( \beta53-AC2 \), \( \beta53-AC5 \), and \( \beta53-AC8 \), substituted at the 2, 5, and 8 position, respectively) and the fourth, \( \beta53-ACtrip \), possesses ACHC in place of all three \( \beta^3 \)-homovaline residues (Figure 3.12B). By systematically substituting ACHC within \( \beta53-1 \), we hoped to explore the interplay among local conformation, overall secondary (and possibly tertiary) structure, and target affinity within a functional \( \beta \)-peptide.

**Structure and function of \( \beta53-1 \) variants with cyclic residues**

The ACHC-containing \( \beta \)-peptides were characterized by CD spectroscopy to examine their secondary structure. Incorporation of ACHC within a \( \beta^3 \)-peptide typically increases the intensity of the 14-helix CD signature (particularly the characteristic mean residue ellipticity minimum near 214 nm), implying increased structure.\(^{80, 172}\) \( \beta53-1 \) and its variants are no exception (Figure 3.13A). Interestingly, incorporating a single ACHC residue increases intensity of the minimum by 40-50% independent of position. Incorporation of three ACHC residues increases the intensity of the minimum in a purely additive fashion, leading to an overall 245% increase over \( \beta53-1 \). CD-based thermal denaturation analysis of \( \beta53-1 \) and its ACHC-containing variants (Figure 3.13B) reveals
reversible 14-helix unfolding, with no other detectable secondary structure at any temperature. Intriguingly, even at 98 °C β53-1 and its constrained variants show characteristic 14-helical CD signatures with minima at 214 nm at 45-75% of the intensity at 4 °C. This effect is not seen when unfolding 14-helices by other means, such as neutralization of the stabilizing salt bridges by extremes of pH or high salt. Thus, the persistence of the 14-helix signature at high temperatures may indicate extraordinary thermostability within this series of 14-helices. This stability may derive from the predominance of helix-stabilizing electrostatic forces, enthalpic effects that recent calculations have shown are largely independent of temperature. The CD data as a whole support a model in which 14-helix folding is noncooperative and vastly more temperature-stable than α-helix folding. In agreement with this model, we find that overall extents of helix formation in water by variants of β53-1 are dependent on the proportion, but not location, of conformationally constrained residues.

Next, we characterized the ACHC-containing β-peptides in terms of their affinities for hDM2 and their potencies in inhibiting the p53AD•hDM2 interaction. Fluorescein-conjugated versions of the ACHC-containing β-peptides were incubated with various concentrations of hDM21-188 and monitored by fluorescence polarization (FP) to detect binding, as described. A FP competition assay in which various concentrations of unlabeled β-peptide was incubated with p53AD15-31Flu and hDM21-188 was employed to measure the β-peptides’ inhibitory potencies, as described. Results from the FP assays are shown in Figure 3.14. Incorporation of a single ACHC residue as in β53-AC2, β53-AC5, and β53-AC8 marginally increases apparent hDM2 affinity. The position-independent nature of this increase and the nearly identical CD spectra of β53-AC2, β53-
AC5, and β53-AC8 imply that this effect stems from an increase in overall extent of structure and not on local changes in geometry. Compared to β53-1, all ACHC-containing variants are more potent inhibitors of the p53AD•hDM2 interaction. However, β53-AC5 appears to be a slightly poorer inhibitor than the other three. This may be due to possible self-association of this peptide at high concentrations (as described below). Importantly, the fact that incorporation of ACHC at any of several positions within β53-1 slightly improves apparent binding affinity implies that β53-1 and its variants bind hDM2 in a 14-helical conformation.

β53-ACtrip, the most constrained β-peptide in this study, possesses the same marginal improvements in apparent affinity and inhibitory potency as the variants with a single constrained residue. This indicates that a highly rigidified 14-helix does not perfectly mimic p53AD’s α-helix. However, distortions in the 14-helix backbone, as have been observed for β53-1 in methanol,174 may render a short, unconstrained 14-helix more α-helix-like in shape and in positioning of side chains. While ACHC incorporation should stabilize the overall 14-helix conformation, it locks the central C-C torsion to 60°, which deviates significantly from the values observed for positions 5 and 8 within the β53-1 methanol solution structure (64.8 ± 5.9°, 41.9 ± 3.7° and 78.1 ± 4.0° for C-C torsion angles at positions 2, 5, and 8 respectively).174 These discrepancies imply ACHC induces local and/or global changes in conformation relative to β53-1’s preferred solution structure. The present results seem to indicate a trade-off inherent in ACHC incorporation: while the overall fold may be stabilized, important features of local conformation that account for hDM2 recognition may be altered.


\( \beta_{53-1} \) variants with cyclic residues form oligomers at high micromolar concentrations

Analytical ultracentrifugation (AU) is commonly used to determine whether peptides and proteins oligomerize and to estimate the nature of the self-association equilibrium.\(^{175}\) Previously, AU had shown that \( \beta_{53-1} \) is monomeric at concentrations exceeding 1 mM.\(^{130}\) The ACHC-containing \( \beta_{53-1} \) variants were all analyzed by AU as described previously.\(^{86, 159}\) AU data are shown in Figure 3.15. \( \beta_{53-AC2} \) showed no evidence of oligomerization, while \( \beta_{53-AC8} \) showed slight curvature in the radial distance data that could indicate a monomer-dimer equilibrium at low millimolar concentrations. By contrast, \( \beta_{53-AC5} \) and \( \beta_{53-ACtrip} \) showed striking gaps in the radial distance data, with curvatures consistent with formation of discrete higher-order oligomers, likely hexamers or heptamers. Both data sets suggested apparent \( K_d \)'s for oligomer dissociation in the 100 to 500 \( \mu \)M range. Self-association by ACHC-containing \( \beta \)-peptides has been previously detected by AU\(^{162}\) and by NMR.\(^{176}\) The current data highlight the exquisite sensitivity of 14-helix self-assembly to the presence and placement of conformationally constrained residues, and provide a starting point for the rational design of 14-helix bundles.

Our observations of the effects of conformational restriction on \( \beta_{53-1} \) show there may be limits to the benefits of conformational restriction for development of 14-helical \( \alpha \)-peptide mimics. Indeed, there may be an inherent trade-off between lowering binding entropy via conformational restriction and allowing the \( \beta \)-peptide to assume a more \( \alpha \)-helix-like geometry. In addition, experiments using cyclic residues have provided further evidence that \( \beta_{53-1} \) and its variants unfold noncooperatively and reversibly, and bind hDM2 in a 14-helix conformation. We have also uncovered novel determinants of 14-
helix self-assembly. The data as a whole validate the design strategy whereby residues on three successive turns of an α-helix can be mimicked by a 14-helical β-peptide.

3.6 – More potent β53-1 variants

The subtle contributions of pre-organization and epitope shape to β53-1 affinity led us to re-examine the β3V-bearing 14-helix face. The β3V residues were originally included to boost structure and as placeholders for other, more varied functionalities. In the methanol solution structure of β53-1, we observed that the isopropyl side chains interdigitate, burying 24% of their overall surface area (Figure 3.10E). These interactions may account for the stabilizing effects of residues branched at the first side chain carbon. More importantly, interactions along the β3V face of β53-1 may influence the overall shape of the 14-helix and, in turn, the recognition epitope. Thus, when we were ready to test a method for combinatorial optimization of a β-peptide PPI inhibitor (detailed in the Chapter 4), we generated a library which varied the three β3V residues within β53-1. The most potent hits gleaned from the library, β53-8 and β53-9 (Figure 3.16A), differ from β53-1 only by the addition of three and two methyl groups, respectively, in the form of β3I-for-β3V substitutions. These alterations greatly increase overall 14-helix structure in water as measured by CD, as much as incorporation of a single ACHC residue (Figure 3.16B; compare to Figure 3.13A). In contrast to ACHC incorporation, however, replacement of β3V with β3I results in roughly 7-fold increases in hDM2 affinity and inhibitory potency as judged by FP assays (Figures 3.16C-D). The fact that these subtle changes affect β53-1 structure and target affinity reinforces several
guiding principles: a combination of pre-structuring and epitope shape determine target affinity for PPI inhibitors that use a folded scaffold, the biophysics of helix folding is intimately involved in optimizing functional characteristics, and β-peptides based on 2 are highly tunable with respect to structure and, in turn, epitope shape.
Figure 3.1 – (A) Crystal structure of the p53AD•hDM2 complex. (B) Model of a theoretical β-peptide 14-helix•hDM2 interaction built by overlaying alpha and beta carbons of Phe19, Trp23, and Leu26 on p53AD with the alpha and beta carbons of $i$, $i+3$, and $i+6$ residues on a minimized model of poly-$\beta^3$-homoalanine. Images generated using INSIGHT 2000.
Figure 3.2 – Sequences and helical net diagrams of $\beta^3$-peptides used in the evaluation of helical $\beta$-peptide hDM2 inhibitors. $\beta^3X$ denotes a $\beta^3$-homoamino acid where X is the one-letter code for the corresponding $\alpha$-amino acid. Red and blue accentuate electrostatic features; residues that comprise the p53AD epitope are in yellow.
Figure 3.3 – (A) CD spectra of $\beta_{53-1-6}$ in PBC buffer (pH 7.0) at 25 °C. 6 $\beta$-peptide concentrations were 160 µM, except $\beta_{53-2}$, which was 22 µM. (B) Unambiguous backbone ROEs observed for $\beta_{53-1}$ in CD$_3$OH at 20 °C.
Figure 3.4 – Analytical ultracentrifugation analysis of (A) β53-1; (B) β53-2; (C) β53-3; and (D) β53-4. Points on upper charts represent absorbance values at 275 nm as a function of radial distance in cm. Lines on upper charts represent the best fit of the data to a monomer model; residuals are shown for this fit. Bars on lower charts represent the average concentrations of the indicated β-peptide in the cell compartment before centrifugation (obs.) and after centrifugation at equilibrium (calc.) as calculated by integration over the radial concentration profile for either a monomer or dimer model. 101
Figure 3.5 – (A) Data from competition FP assays detailing inhibition of p53AD\textsuperscript{Flu}\textbullet{}hDM2 complexation by p53AD and β53-1–4. (B) Data from FP binding assays detailing direct binding of β53-1\textsubscript{flu} and \textsuperscript{flu}β53-1 binding to hDM2. (C) Data from competition FP assays detailing inhibition of p53AD\textsuperscript{Flu}\textbullet{}hDM2 complexation by p53AD, β53-1, control β-peptides β53-5–7, and β\textsuperscript{3}W. FP assays were performed as described in sections 1.4 and 1.5, in PBS, pH = 7.4, at 25 °C.
Figure 3.6 – (A) Specificity of protein surface recognition by β53-1 measured by competition fluorescence polarization analysis using β-peptides retaining only one of the three putative recognition residues and changing the others to β3-homoalanine. (B) Selectivity of β53-1, β53-3, β53-5, and β53-6, measured by competition FP analysis with an unrelated peptide•protein pair, KIDABflu and the KIX domain of CBP. ppKid4P is shown as a positive control.12, 15
Table 3.1 – Mass spectrometry data for hDM2_188 and β- and α-peptides used in this study.

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Figure 3.7 – (A) Effect of salt on mean residue ellipticity at 214 nm (MRE$_{214}$) of β53-1. (B) Effect of salt on binding of p53AD$^{flu}$ (circles, dashed curve fits) and β53-1$^{flu}$ (squares, solid curve fits) to hDM2.
Figure 3.8 – (A) Competition of β53-1 and p53AD against p53AD\textsuperscript{flu} and β53-1 and p53AD against β53-1\textsuperscript{flu}. (B) Job plot at 4 μM total concentration.
Figure 3.9 – An illustration of nomenclature used to assign the resonances of the $\beta^3$-homovaline residues of $\beta53$-1. The convention used here is indicative of the general convention used.
Table 3.2 – Chemical shift assignments relative to DSS for β53-1 in CD$_3$OH at 10 °C.

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Figure 3.10 – (A) Chemical structure of β53-1. (B) Solution structure of β53-1 in CD$_3$OH at 10 °C, shown as a bundle of 20 lowest-energy structures, with C-terminus at left. (C) Ribbon representation of the backbones of 20 lowest-energy structures. (D) The two sub-populations of ion pairing configurations. Superposed at left are 17 structures in which β$^3$O1 and β$^3$E4 are proximal; superposed at right are 3 structures in which β$^3$E4 and β$^3$O7 are proximal. (E) Conformations of β$^3$-homovaline residues illustrating the “wedge into cleft” packing found in all 20 lowest-energy structures.
Figure 3.11 – Overlay of the methanol solution structure of β53-1 (red ribbon and side chains) with the crystal structure of a p53AD-derived peptide (gold ribbon and side chains) bound to hDM2 (gray surface). Side chains of β53-1 not implicated in recognition have been omitted, and part of the hDM2 surface has been cut away for clarity.
**Figure 3.12** – (A) trans-(S,S)-2-cyclohexaneamino acid, or ACHC. (B) Helical net diagrams of $\beta_{53}$ and its ACHC-containing variants. Coloration is used to illustrate electrostatic stabilization features. $\beta^3X$ denotes a $\beta^3$-amino acid with a side chain identical to that of the $\alpha$-amino acid with the one-letter abbreviation X. A six-membered ring denotes ACHC.
Figure 3.13 – Circular dichroism analysis of β53-1 and its ACHC-containing variants.

(A) CD spectra of 80 μM β-peptides at 25°C, plotted as mean residue ellipticity (MRE).

(B) MRE at 214 nm of 80 μM β-peptide at temperatures increasing linearly from 4 °C to 95 °C (circles), then decreasing linearly from 95 °C to 4 °C (squares). All CD measurements were acquired on samples prepared in 1 mM sodium phosphate/borate/citrate buffer, pH 7.0.
Figure 3.14 – Plots illustrating the hDM2 affinities of $\beta 53-1$ and ACHC-containing variants and controls as determined by (A) direct fluorescence polarization analysis of fluorescein-labeled ligands and (B) competition fluorescence polarization analysis with p53AD$_{15-31}$ flu. (C) $K_d$ and $IC_{50}$ values derived from curve fits from data shown in (A) and (B), respectively.
Figure 3.15. Raw AU data plotted as absorbance at 280 nm versus radial distance. Curve fits (IGOR-PRO) are shown, with residuals to the fit shown above each plot.
Figure 3.16 – Our most potent β-peptide hDM2 ligands to date. (A) Helical net diagram depicting the sequences of β53-8, β53-9 and β53-10. Legend shows colors used for each β-peptide in (B-D). (B) CD spectra of β53-1 and β53-8–10. (C) Data from a direct FP assay detailing binding of β53-8flu, β53-9flu and β53-10flu to hDM2. (D) Data from a FP competition assay detailing inhibition of p53AD15-31flu•hDM21-188 by β53-8, β53-9 and β53-10.
4.1 – A combinatorial approach to ligand discovery and optimization

Despite our success in designing β53-1 and providing a model for β53-1·hDM2 recognition, a general rational design strategy for developing protein-protein interaction (PPI) inhibitors based on β-peptide scaffold 2 remained a more long-term goal. However, as with most other chemical systems that can be broken down into a modular synthesis, β-peptides are highly amenable to combinatorial chemistry. We recognized that discovery of novel β-peptide PPI inhibitors and refinement of known PPI inhibitors such as β53-1 could be most rapidly performed using combinatorial methods. After consideration of the readily adaptable methods available, the one-bead-one-compound (OBOC) approach was chosen for several reasons: it has been widely applied to peptides,179, peptoids,181 and other peptidomimetics182 with great success, it involves small-scale syntheses relative to other techniques, it involves simple split-and-pool methodology which requires no additional reaction steps, and a variety high-throughput screening and decoding techniques are available. We termed our approach the “one-bead-one-β-peptide” method, or OBOβ.

4.2 – Synthesis of positive and negative control beads and a small OBOβ library

In designing a synthetic scheme for β-peptide libraries, the first and foremost concern was overall yield and purity. As before, the standard solid-phase peptide synthesis
techniques would need to be tweaked to use β-amino acids as sparingly as possible. To be useful for assay development, a small control library would need members with significantly different binding affinities for hDM2. Thus, an eight-member control library was synthesized in which the central and critical β3-homotryptophan of β53-1 was replaced by one of: β3-homovaline, β3-homothreonine, β3-homoisoleucine, β3-homoornithine, β3-homoglutamic acid, β3-homophenylalanine, β3-homotyrosine, or β3-homotryptophan (Figures 4.1 and 4.2). The library was synthesized on a 45 µmole scale using TentaGel HMB resin (~150 µm, ~0.4 mmol/g; Rapp Polymere) that was pre-loaded with β3-homoglutamic acid. In parallel, beads bearing β53-1 and βNEG (Figures 4.1 and 4.2) were synthesized on a 25 µmole scale in the same manner. These would serve as positive and negative controls, respectively, for use in assay development.

The OBOC method involves dividing the solid-phase synthesis prior to coupling at the position to be varied into several separate reaction vessels. Different chemical groups can then be coupled to completion, followed by re-pooling of the solid phase. This generates an exponentially increasing number of unique library members with every split-and-pool coupling step. For the small OBOβ test library, there was a single split-and-pool coupling at the sixth position (β3-homotryptophan in β53-1). At the re-pooling step following coupling at the sixth position, a portion of each reaction was retained and synthesis was finished independently. This provided eight independent pools representing the eight library members. In all, the synthesis yielded 11 different bead populations: β53-1 beads, βNEG beads, the 8-member library synthesized by split-and-pool methodology, and eight individual pools representing library member. Ninhydrin tests were performed before and after the fifth, sixth, and eighth couplings (chains of six,
seven, and nine residues, respectively). The test was positive (blue) prior to the sixth coupling for all pools, and negative after the sixth coupling. However, the test was negative prior to the eighth coupling for all pools. Despite this, most pools showed minimal amounts of \(n-1\) or \(n-2\) products. Thus, folding of the \(\beta\)-peptide 14-helices on the resin may prevent a positive ninhydrin test, but still allow efficient deprotection and coupling under the described conditions.

**Materials and Methods**

Synthesis was performed with a few alterations to optimize purity and yield while still minimizing the amounts of \(\beta\)-amino acids consumed. Synthesis was performed semi-automatically, with the Symphony automated synthesizer performing all steps except coupling. Extra deprotection was used (1 x 2 min 20% piperidine/DMF, 1 x 8 min 20% piperidine/DMF, 1 x 8 min 2% piperidine/2% DBU/DMF, 1 x 8 min 20% piperidine/DMF) at every deprotection step. Capping and washing were as described.\(^{86, 130, 159}\) Couplings proceeded using two equivalents of \(\beta\)-amino acid, HoBt, and PYBOP, and 5.5 equivalents of DIEA, and were allowed to react for 90 minutes or longer. This procedure was repeated twice for each position (double coupling). At the split point, the resin was split into ten equal portions using the following procedure. A Pasteur pipet was marked at ~0.3 to 0.5 mLs, and ten aliquots of this volume of NMP were added to the RV containing the library beads. Then, the same marked pipet was used to mix the beads, forming a homogeneous slurry, then to quickly transfer one aliquot to another RV before the beads could settle. In this way, one tenth of the beads were distributed into nine other
RV’s, with the tenth portion reserved in the original. Ten independent double couplings (at the 4.5 μmole scale) were then performed, after which the resin and RV’s were washed very thoroughly with NMP. Half of each pool of beads was then transferred back into the original RV, giving a library on the 22.5 μmole scale and ten individual stocks of library members on the 2.25 μmole scale. Each of these pools was then finished independently to yield the full-length library members.

After the final coupling, the beads were Fmoc-deprotected using the deprotection procedure described above, and side chains were Boc- and OtBu-deprotected using standard cleavage solution (2% each water, phenol and TIPS in TFA) shaking on a tabletop shaker for 90 minutes. Beads were then rigorously washed using dichloromethane and methanol and dried in silanized (SigmaCote) glass vials. They were stored dry at 4 °C until use.

4.3 – Quality assessment of library synthesis

Rigorous quality assessment was performed on the control beads to evaluate the synthetic protocol and to ensure their utility in developing a OBOβ assay. Roughly a spatula tip’s worth of beads (~75 to 200 beads) were taken from each of the 11 pools and subjected to quality control analysis. Beads were removed from stock vials by first swelling them in methanol for 5 minutes, then removing desired beads and drying out unused beads prior to re-storage at 4 °C. Washing of beads in eppys was accomplished using flat-tip gel-loading pipette tips, which are too narrow to allow passage of swelled beads. Beads were washed twice in methanol prior to addition of 100 μL of saturated
sodium methoxide in methanol, the highly basic conditions recommended for cleavage of the 4-hydroxymethylbenzoic acid (HMB) handle linking the peptides to the beads. Cleavage was allowed to go for 30 minutes at room temperature, shaking vigorously. 50 µL of water was then added, and the solutions were incubated another 30 minutes, shaking vigorously. Then liquid was removed to a clean tube, after which 18 µL 20% formic acid in water was added to acidify the resulting peptide. Samples were desalted using a ZipTip (C18-packed 10 µL ZipTip, Millipore) and used for MALDI TOF MS and RP-HPLC using an analytical C8 column.

MALDI and HPLC results showed that the desired product was present, and indeed was the greatest single component, of every cleaved solution except β53-1-W6F. The β53-1-W6F beads appeared to have failed, showing a single product at low molecular weight. This is not due to the β3-homophenylalanine stock since it was used at the ninth position (the first on-machine coupling) for each bead pool. Thus, this is likely due to human error (β53-1-W6F beads were successfully synthesized at a later date for completeness of the controls). HPLC results showed exceptional purity (>80%) for beads displaying β53-1, βNEG, and β53-1-W6E, acceptable purity (50 – 80%) for beads displaying β53-1-W6W and β53-1-W6Y, and decent purity (30 – 50%) for beads displaying β53-1-W6T, β53-1-W6O, and β53-1-W6I. The synthesis quality matches or exceeds those of previously reported peptoid and oligocarbamate libraries. When the library itself was analyzed by HPLC, with major peaks collected and analyzed by MALDI, each individual member (except β53-1-W6F) was observed (Figure 4.3). MALDI mass spectra obtained from material cleaved from single split-and-pool library
beads showed a single major product in the expected mass range over 95% of the time for this and all subsequent library syntheses.

4.4 – Development of a bead-screening assay

Assay design and materials

Previous work screening OBOC libraries primarily relied on three types of signals: a colorimetric readout, a primary fluorescent readout, or a secondary fluorescent readout. The colorimetric assay involves using a biotinylated target protein and a streptavidin-linked enzyme to recruit the enzyme to those beads which bind the target protein. Then, a substrate is added which both changes color and becomes insoluble upon chemical reaction (catalyzed by the enzyme). This produces a color change which is isolated to beads which bind the target protein. The overall methodology is analogous to an ELISA. While this appeared to work for mixtures of \( \beta\text{53-1} \) and \( \beta\text{NEG} \) beads (Figure 4.4), it was also apparent that it would not be easy to pick out hits since the \( \beta\text{53-1} \) beads showed non-uniform signal intensities (Figure 4.4). Thus, we turned to fluorescence-based readouts which promised greater sensitivity and more uniform and reliable signals.

Initially testing focused on traditional organic dyes including Cascade Yellow and Cy3, each chosen for their large Stokes shifts. This was appealing because a color change would be much more readily detected than an intensity change, both by eye and by machine. This is especially true for Tentagel-bead-based assays, since these beads autofluoresce and about 0.1% appear to have unpredictably bright autofluorescence.
This was a problem for Kodadek’s peptoid OBOC libraries that was overcome through the use of sharp-eyed graduate students and, later, automated bead sorters.\textsuperscript{181} He also addressed this problem using a more nontraditional marker, namely quantum dots.\textsuperscript{187} Quantum dots are tiny fluorescent chunks of semiconductor with broad absorption spectra but narrow emission spectra, and their emission spectra are independent of absorption wavelength (Figure 4.5). The primary advantage of using quantum dots is thus the possibility of using blue light excitation and observing a signal in the orange-red range of the spectrum (as opposed to the green range, in which the beads themselves emit strongly).\textsuperscript{187} Thus, using the Quantum Dot 605-streptavidin conjugate (QSA605, Quantum Dot Corp.), one can excite the QSA605 at 490 nm, and the emission should be exclusively near 605 nm, not near 520 nm as would most dyes that absorb at 490 nm and as do the Tentagel beads themselves.

hDM2 was biotinylated as described in materials and methods. Biotinylation was performed both on frozen stocks and on freshly prepared hDM2 prior to flash freezing. The biotin:protein ratio was on average 4.4 biotins per molecule of hDM2.\textsuperscript{188}

\textit{Blocking conditions}

Previous studies using OBOC methods with peptoids have reported a formidable problem with background signal and false positives.\textsuperscript{181} Great measures had to be taken to cut the hit rate, to the point that concentrated \textit{E. Coli} lysate was used as a blocking agent, along with 1.0\% detergent and 1.0 M salt.\textsuperscript{181} However, other OBOC experiments with $\alpha$-peptides and small molecules report few similar problems.\textsuperscript{179, 180} It was clear that blocking agent and buffer composition would likely be critical for a usable assay. TBST
buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20) was used as a starting point for optimization of the blocking and washing buffer. The assay development protocol, detailed in materials and methods, involved screening a 50:50 mixture of β53-1 and βNEG beads in wells of 96-well PVC plates. Conditions tested in this development assay included various combinations and concentrations of the detergents Tween, NP40, Triton-X, and IGEPAL, and the blocking agents BSA, gelatin, L-tryptophan, and E. Coli lysate. An informative subset of the results is presented in Figure 4.6. After extensive testing, gelatin (which is dissolved in TBST at 37 °C prior to use in the assay) was the only agent that successfully suppressed signal on the βNEG beads, but maintained signal on the β53-1 beads. E. Coli lysate tended to damp all signal, while L-tryptophan seemed to allow bright β53-1 signal but didn’t eliminate βNEG signal. Another appealing aspect of the gelatin blocking was the observed attenuation in signal as higher concentrations were used (Figure 4.6). At 2 mg/mL, a bright signal was evident, while at 4 and 8 mg/mL increasingly faint signals were observed. At 10 mg/mL signal was fully attenuated. This reproducible attenuation could be used when screening a library to lower or raise the hit rate to a desired level. Signal was also attenuated by increasing salt concentration or by increasing Tween-20 concentration (Figure 4.7A).

Controls were also performed using non-biotinylated hDM2, biotinylated BSA, or no protein (all with subsequent incubation with QSA605; Figure 4.7B). These controls showed no bead fluorescence, as would be expected for an assay which reads out specific recognition between bead-immobilized β-peptide and target protein.

Materials and Methods
Enzyme-linked bead screening

Methanol-wetted beads were picked and distributed into wells of a 96-well PVC plate containing 100 µL TBST. This buffer was removed and beads were incubated 2 x 15 min in TBST. They were then incubated 30 min in 100 µL TBST or TBST/2.5% BSA. After the blocking solution was removed, beads were incubated 1 hr in 75 µL biotinylated hDM2 at 250 nM in TBST or TBST/BSA. Beads were washed 2 x 5 min in TBST or TBST/BSA. Streptavidin-alkaline phosphatase conjugate was added at 1:100 dilution and incubated 60 mins. Beads were washed 2 x 5 min in TBST or TBST/BSA, this time with 2.4 mM MgCl₂ added to keep the AP active. The substrate, BCIP (5-bromo-4-chloro-3'-indolyolphosphate p-toluidine salt), was added in TBST with MgCl₂, at a concentration of 1.65 mgs per 10 mL. Substrate was incubated 30 mins – 2 hrs (until beads were seen to be blue by eye). Reaction was halted with a drop or two of 1M HCl and beads were further visualized using a light microscope.

Biotinylation of hDM2₁-188 and BSA

1.0 mg of BSA was dissolved in 100 µL hDM2 buffer (see section 1.5 for composition) to obtain a 150 µM solution. 100 µL of hDM2 aliquots (109 µM by amino acid analysis) was defrosted from the -70 °C freezer. 2.0 mgs of NHS-PEO₄-biotin (Pierce) was dissolved in 200 µL H₂O, making a 17 mM solution. 12 µL NHS-PEO₄-biotin solution was added to each 100 µL of ~150 µM protein and reaction was allowed to proceed for 30 min at room temperature. A NAP-10 column (Amersham Pharmacia) was used to remove excess biotin reagent. Fractions of 4 to 5 drops each were collected
and assayed using a Coomassie dot blot to determine where protein was in abundance; high-concentration fractions were pooled. Concentration of the biotinylated product was 79 µM by AAA. Biotinylated proteins were flash-frozen in liquid N₂ and kept at -70 °C until use.

The initial time protein biotinylation was performed, several different concentrations of biotinylation reagent were used to determine optimal conditions. A dot Western blot using QSA605 as the detection agent was performed to verify the presence of biotinylated protein. While the blot itself did not image well, there was noticeable signal from dots corresponding to 8-fold excess of biotinylation reagent or higher. Thus, a 12:1 biotinylation reagent to hDM2 ratio was used throughout the following work, yielding hDM2 with, on average, 4.4 biotins per protein molecule (determined using a HABA-avidin biotin detection reagent from Sigma).

**Tips for screening and working with Tentagel beads**

Removing beads from a glass container is simple when beads are first swelled in methanol. This ensures that they will be free-flowing, will not stick to glass or polypropylene, and are easily visible. Also, they can be dried following removal of beads using a dessicator with a benchtop aspirator. The beads used in this chapter were swelled and dried up to 10 times or more over the course of four months with no discernible loss in bead integrity, peptide quality or assay signal.

Polystyrene is sticky for beads and for QSA605. Polyvinylchloride (PVC) plates worked much better for assaying small amounts of beads in wells or for visualizing large amounts of beads by pipetting them onto a PVC plate lid. Polypropylene (ie eppendorf
tubes and larger columns) didn’t seem particularly sticky either but this was not visualized directly.

Relatively low concentrations (2 nM, which is a 1:500 dilution of the 1 µM stock) of QSA605 seemed optimal. In some cases, this could be lowered but intensity seemed to scale for bright and weak signals alike. That is, overall intensity appeared to decrease but sensitivity seemed unaffected.

Flat-tip gel-loading pipette tips work well for removing buffers/solvents from beads with minimal loss of actual beads. This was done with micropipettors as well as with a tip attached to a serological pipette attached to an aspirator, for greater speed. This was performed to remove aqueous buffers from beads, as well as methanol or NMP (though NMP will start to dissolve the tip after 30 seconds, necessitating frequent replacement).

Isolating single beads is easiest when they are in methanol. By “streaming” the methanol down the side of a vessel (ie an eppy), one can observe the bead as it also moves down the wall. The bead will typically move more slowly than the solvent, and will be very visible, allowing one to isolate a single bead as well as remove it from the methanol (since the bead stays on the wall while the methanol pools at the bottom). After washing beads with NMP, beads are spun down (2500 rpm for 5 seconds on a tabletop centrifuge), all but ~25 µL of NMP is removed, then MeOH is added and the solvents are mixed. Then the “streaming” method can be used to remove the remaining NMP with the methanol.

When imaging beads using the fluorescence microscope, it is best to take pictures manually. Note that most of the early assay development was imaged using automatic settings. Automatic exposure times tend to brighten the intense portions of an image, but
often increase the contrast in dim portions, making low-intensity signals look more intense. Using manual settings, an exposure time of 1/8 or 1/10 second worked best for capturing what was actually being observed through the fluorescence microscope.

4.4 – Testing the assay

Screening the 8-member library

The 8-member library (Figures 4.1 and 4.2) was screened using the method developed in the previous section. Each isolated library member was also screened in parallel, with the expectation that each individual population would have a uniform and predictable signal intensity, while the library would show varied intensities. Inspection of the screened beads under a fluorescence microscope yielded varied signal intensities among the bead populations, with intensity scaling roughly with the size and hydrophobicity of the residue (Figure 4.8). There were at least four distinct levels of orange fluorescence in the library (Figure 4.8B). Comparing the screened library to the individual pools of screened library members (Figure 4.8C), the four intensity levels are easily accounted for: the $\beta_{53-1}$-W6W beads (which should be identical to the $\beta_{53-1}$ beads) and the $\beta_{53-1}$-W6Y beads show extremely intense signal, the $\beta_{53-1}$-W6I beads show very intense signal, the $\beta_{53-1}$-W6F and $\beta_{53-1}$-W6T beads show intense signal (though $\beta_{53-1}$-W6T beads were consistently splotchy), and the $\beta_{53-1}$-W6K and $\beta_{53-1}$-W6V beads show weak signal (note that some $\beta_{53-1}$-W6E beads were accidentally mixed into the $\beta_{53-1}$-W6V bead pool; this shows up here as some dim beads among the weakly orange $\beta_{53-1}$-W6V beads). Finally, the $\beta_{53-1}$-W6E beads showed no orange fluorescence.
**Mock screens**

Two mock screens were performed: one using the 8-member library and one using a pool of $\beta$NEG beads (>100) with a few (<5) $\beta_{53-1}$ beads added. Also, two different blocking conditions (1 or 2.5 mg/mL in TBST) and two different concentrations of QSA605 (10 nM or 2 nM) were employed in an effort to optimize conditions. Again, several different intensity levels were clearly distinguishable for the library, while the $\beta_{53-1}$ beads were easily distinguishable from the $\beta$NEG beads (as in Figure 4.8A). Single beads were picked using a 0.1 – 10 $\mu$L pipette tip and transferred into separate eppendorf tubes, including a bright bead from the 8-member library, a dark bead from the 8-member library, and a bright bead from the $\beta$NEG/$\beta_{53-1}$ mixture to test by MS/MS sequencing.

**MS/MS sequencing of samples derived from single beads**

Seebach had analyzed fragmentation patterns of $\beta^3$-peptides before, and MS/MS fragmentation of $\beta$-peptides had been performed in our lab as well – in both studies, $\beta^3$-peptides were observed to fragment essentially as natural peptides do. Thus, there were two novel features of this aspect of the assay: obtaining amounts of $\beta$-peptide from a single bead appropriate for MS/MS sequencing, and determining whether fragmentation patterns of single beads would be enough to sequence, essentially *de novo*, a given bead-derived peptide.

Peptides were cleaved and processed as described in materials and methods, and were then analyzed by MALDI-TOF MS and ESI-MS/MS. There was sufficient peptide from
a single cleaved bead for both these assays. MS/MS analysis was performed with the help of Eugene Davidov at the Center for Functional Genomics at Yale and Erol Gulcicek at the Keck Biotechnology resource at Yale. The MALDI and raw ESI-MS spectra showed the major product in each case to be a β-peptide whose mass corresponded to an expected sequence, and observed MS/MS fragments corresponded to predicted fragments of that sequence. The high-signal library bead had a primary mass of 1380, the expected mass of the β\textsuperscript{3}-homotyrosine-containing library member (the methyl ester of that β-peptide; cleavage of single beads tended to produce methyl esters despite adding water and acidifying the resulting solution). The low-signal library bead had a primary mass of 1346, the expected mass of the β\textsuperscript{3}-homoglutamate library member. The bright bead picked from an excess of β\textsuperscript{NEG} beads produced the expected mass of the β53-1 methyl ester, 1403. MS/MS of each of these major products showed fragmentation patterns consistent with the suspected parent ion. Finally, a demo version of the PEAKS program (Bioinformatics Solutions, Inc.) was re-parameterized to use only β\textsuperscript{3}-residues. The MS/MS spectra were then processed with the MaxEnt 3 algorithm (MicroMass, specially designed for processing MS/MS spectra for automated sequencing) and imported into PEAKS. In each case, the PEAKS automated sequencing algorithm outputted the correct sequence in ~30 seconds with high confidence. Alternatively, since only one residue was varied (and, in later libraries, only three residues were varied), careful examination of the peaks in the MaxEnt 3 processed spectra also permitted definitive sequencing of the β-peptides.

**Materials and Methods**
The following protocol was used to perform screens on the 8-member library and on individual library members and mock screens. Beads were first swelled 5 minutes in MeOH. Roughly 20-30 beads were removed to a well of a 96-well PVC plate containing 100 µL block/wash buffer (TBST + 2.5 mg/mL gelatin). Liquid was removed with a flat-tip gel-loading pipette tip and 200 µL fresh buffer was added. Beads were then incubated on a tabletop shaker, highest setting, for 30-60 min. Liquid was removed from each well and biotinylated protein (2 µM, pre-diluted in blocking buffer and pre-incubated at room temperature at least 10 minutes) was added in 50-75 µL total volume. Beads were incubated with protein on the shaker for 60 min. Two 5 minute washes (room temperature on the shaker) with 200 µL buffer were then applied. Qdots605-streptavidin conjugate (QSA605) was applied at 1:500 dilution (2 nM; these were also pre-diluted and pre-incubated at room temperature at least 10 minutes) in 25-50 µL total volume. After 30 minutes of incubation, beads were washed twice, 5 minutes each wash, on the shaker with 200 µL blocking buffer. Finally, 100 µL blocking buffer was added and beads were visualized with a fluorescence microscope (Olympus).

Single beads picked in the mock screens were processed in the following way (adapted from a procedure of Dr. Nathan Luedtke, whose help in experiment design was especially valuable). Beads were transferred to an eppy and wash/block buffer was removed from beads using a flat-tip gel-loading pipette tip. 200 µL MeOH was added and the solution was mixed by aspiration or by gentle vortexing. MeOH was removed from the bead by “streaming” the MeOH down the side of the eppy. This had the benefit of allowing separation of single beads from other beads (if there were others present),
ensuring there was only one bead present per tube, and also removing the bead from the MeOH. Beads were washed down with 200 µL NMP and mixed. Epps were then spun for 5 sec at 2500 rpm on a tabletop centrifuge. All but ~25 µL of the NMP was removed. 200 µL MeOH was added and solutions were mixed and streamed to remove bead from solvents. These MeOH and NMP washes were repeated, followed by one more MeOH wash (three MeOH washes and two NMP washes altogether). Then all MeOH was removed and beads were pushed to the bottom of the eppy. This was to prevent the bead from getting visually lost on the side of the eppy since they are much harder to see once they shrink. Beads were dried then by leaving the open epps in a hood for ~10 mins. 2 µL of fresh sodium methoxide in methanol (3 mg/mL) was then added, and the solution was pipetted up and down to visually confirm bead was in the cleavage solution (can see bead clearly in the barrel of a 0.1 – 10 µL pipette tip). Cleavage proceeded for 30-60 mins, after which 2 µL of 0.5% TFA in MeOH was added, followed by 8 µL H₂O. ZipTip (Millipore) mini-C18 columns were used to remove salts, bead fragments, and other undesired byproducts from peptide mixture. The ZipTip was affixed to a P10 micropipettor (all subsequent volumes are 10 µL) and primed 3 times with acetonitrile, 3 times with water. Then the peptide solution was loaded by pipetting it up and down through the C18 column 5 times. The column was then washed twice with water/0.1% TFA, twice with 20% methanol in water with 0.1% TFA, then twice with water/0.1% TFA. Peptide was eluted with 95% acetonitrile in water with 0.1% TFA. 0.5 µL of the resulting 10 µL was used for MALDI-TOF MS. Typically only ~10-20% of the remainder was needed for MS/MS analysis.
4.5 – Synthesis and screening of the first large library

Design and synthesis

The first 1,000-member library was synthesized using 120 mgs of β³-homoglutamate-loaded Tentagel-HMB resin (~34 µmole scale, >60,000 beads). Protocol was similar to that of the 8-member library except this library was synthesized entirely manually. Three positions were varied (positions 3, 6, and 9, corresponding to the first, fourth and seventh couplings. At split points, ten arbitrary volumes (measured by a marked Pasteur pipet) of NMP were added to the resin, then nine aliquots distributed to nine vials (leaving the tenth in the RV). NMP was removed using a flat-tip gel-loading pipette tip and coupling reagents dissolved in NMP were added to the beads. After 90 minutes, the solvent was removed and another coupling was performed. Finally, the beads were transferred back to the RV prior to the capping step.

The library (shown in Figure 4.9A) was designed to create a novel binding surface to screen against hDM2. The three residues that make up the “recognition face” of β53-1 were varied. The residues chosen as library components included the three side chains that β53-1 possesses (β³-homophenylalanine, β³-homoleucine, and β³-homotryptophan) as well as charged side chains (β³-homoaspartic acid and β³-homolysine), polar side chains (β³-homothreonine and β³-homotyrosine), and differently shaped hydrophobic side chains (a β³-homoamino acid with a styrene-like side chain, β³-homo-2-naphthylene, and β³-homo-CF₃-phenylalanine). Ten components at three possible positions results in a theoretical diversity of 1,000.
Screening and hit verification

As might be expected after the results from the 8-member library, screening the first 1,000-member library led to a continuum of observed intensities, from completely dark to extremely intense. Defining hits required setting an arbitrary intensity threshold and picking beads judged by eye to exceed the threshold. In this subjective way, ~20 hits were isolated. The “hit” beads were processed as described in the previous section and subjected to MALDI and MS/MS analysis. In the end, 17 of the 20 hits yielded clean sequences. I also picked and processed some beads randomly chosen from the library itself, without screening them, to assess overall synthesis quality and bias (termed L1-L10; seven of these yielded clean sequences). Sequences of β-peptides gleaned from the H-series and L-series peptides are shown in Figure 4.9B.

Five β-peptides representative of the patterns seen in the selected sequences were synthesized and tested by FP methods for hDM2 binding affinity (Figure 4.9C). Unfortunately, none of these had higher affinity for hDM2 than $\beta_{53-1}$; in fact, they all showed quite poor affinity. In retrospect, it was probably unwise to allow such large changes in the recognition motif, since our model posits that the side chains of the $\beta^3L$, $\beta^3W$, and $\beta^3F$ make up an epitope which is specific for hDM2 recognition. In the end, the data implies two useful points. First, replacement of $\beta_{53-1}$’s hDM2 recognition motif with similar (but not isosteric) large hydrophobic residues abolishes tight binding, lending further credence to our model that $\beta_{53-1}$ pre-organizes a specific hDM2 recognition epitope. Second, optimization of a binding interface might best be performed in a series of small, targeted libraries.
Materials and Methods

Dried beads were retrieved from storage at 4 °C and swelled in methanol. A portion of beads (later estimated at ~3,000 for the first screen, ~5,000 for the repeat screen) were removed for screening. Remaining beads were dried and stored at 4 °C. The beads were transferred into a 1.5 mL eppendorf with blocking buffer (10 mM Tris pH 8.0, 2.5 mg/mL gelatin, 0.1% Tween-20, and 0.5 M NaCl). Buffer was removed (flat-tip gel-loading pipette tip) and 1 mL fresh blocking buffer was added. Beads were incubated at room temperature on a slow rotisserie for 30 min (all wash and binding steps were performed at room temperature on the slow rotisserie). Buffer was then removed and 250 µL QDots605-SA (2 nM, pre-diluted in blocking buffer and pre-incubated for 10 minutes) was added and beads were incubated for 30 minutes. Beads were then washed 2 x 5 min with 500 µL blocking buffer and visualized using a fluorescence microscope to check for beads which bind the quantum dots, streptavidin, gelatin, or anything else other than the target protein. Since no orange fluorescence at all was seen, the library was judged to be amenable to screening.

After the above control step, beads were washed 1 x 5 min with 500 µL blocking buffer. Another 500 µL was added, and beads were re-blocked 30 minutes. Then, 250 µL of 0.8 µM biotin-hDM2 (pre-diluted in blocking buffer and pre-incubated for 10 minutes) was added and beads were incubated 45 min. Beads were washed 3 x 5 minutes with 500 µL blocking buffer. Buffer was removed and 250 µL QDots605-SA (2 nM, pre-diluted in blocking buffer and pre-incubated for 10 minutes) was added and beads
were incubated 30 minutes. Beads were then washed 2 x 5 min with 500 µL blocking buffer and visualized using fluorescence microscope.

For visualization, 500 µL blocking buffer was added to beads, and slurry was pipetted out onto a PVC 96-well plate lid. Hits were removed manually by aspirating the bead using a P10 micropipettor into a 0.1 – 10 µL tip, then releasing into a separate drop of blocking buffer elsewhere on the plate lid. This method allowed screening of this pool of beads, estimated using the microscope field as ~3,000 beads, in under an hour. Thus, >15,000 beads could easily be screened in one day in this manner, depending on the library’s overall hit rate.

4.6 – Synthesis and screening of a second large library

In a second attempt at demonstrating the utility of the OBOβ assay, we designed a library which did not alter the recognition epitope (Figure 4.10). Instead, it replaced each β3-homovaline residue in β53-1 (positions 2, 5, and 8) with one of ten β3-amino acids, varying the only face with no proven function (the other two faces provide helix stabilization\textsuperscript{86} and hDM2 recognition\textsuperscript{130}). Further, the NMR solution structure of β53-1 in methanol shows that the β3-homovalines interact most favorably when the 14-helix is distorted, making the presentation of the recognition face more like that of an α-helix\textsuperscript{174}. We surmised that optimizing these interactions would lead to even better α-helix mimicry by the β53-1 14-helix.

First, the larger library was screened using conditions identical to those used for the previous libraries, except that more beads were used and screening was done in a 4 mL
volume in a polypropylene column. A representative image from the first screen is shown in Figure 4.11A. In this screen, 25 beads were picked from ~8,000, a raw hit ratio of 0.3%. MALDI and MS/MS spectra confirmed that these represented at least 10 different sequences, yielding an adjusted hit ratio of ≥ 1.0%. In an effort to lower the proportion of hits, the screen was re-performed with less target protein and more stringent washing steps (Figure 4.11B). The second screen yielded 35 hits from ~16,000 beads, a raw hit ratio of 0.2%, with much lower signal among non-hit beads. MALDI and MS/MS spectra confirmed that these 35 beads represented only three different sequences: β53-8 (16 beads), β53-9 (18 beads), and β53-10 (1 bead). The adjusted hit ratio for the more stringent screen is thus 0.3%. Significantly, each of these hits was identified multiple times among the 25 hits from the first screen, confirming that increased washing had narrowed the field of hits considerably.

The three hits (sequences in Figure 4.10B) were synthesized and characterized as previously described. Their overall secondary structure was estimated by CD spectroscopy, their hDM2 affinity was measured by using a fluorescein-labeled variant in a fluorescence polarization (FP) assay with hDM21-188, and their potency for inhibition of the p53AD15-31flu•hDM21-188 interaction was measured in a competition FP assay (Figure 4.11C-E). β53-8 and β53-9 are particularly potent, with binding affinities (Kd’s) of 55 ± 8 nM and 89 ± 20 nM, respectively, and half-maximal inhibitory concentrations (IC50’s) of 13 ± 1 and 11 ± 1 µM, respectively. This can be rationalized by observing that they are 50% more structured than β53-1 as measured by CD, a result not unexpected for replacement of β3-homovaline with β3-homoisoleucine (according to the host-guest analysis described in Chapter 2). Comparing the CD spectra and hDM2 affinities of β53-
and β53-9 with those of conformationally constrained variants of β53-1 (see section 3.5) suggests the newly optimized face contributes not only by pre-organizing the β-peptides into 14-helices, but by subtly altering the helical geometry to make the recognition epitope more closely approximate that of an α-helix.

**Materials and Methods**

Screening of the second larger library was performed in a manner analogous to previous screens. Blocking buffer consisted of 10 mM Tris, pH 8.0, with 0.1% Tween-20, 0.5 M NaCl, and 5 mg/mL gelatin. Methanol-wetted beads were scooped into an empty polypropylene column, washed once (2 min) with 4 mL blocking buffer, and incubated for 30 minutes with 4 mL fresh blocking buffer. Incubation was at room temperature, with end-over-end slow rotisserie mixing. The blocking buffer was drained and the beads were incubated with 0.5 µM biotinylated hDM21-188 in blocking buffer for 30 minutes. Beads were washed (2 x 2 min), then incubated with 5 nM QdotsSA605. Beads were then washed (2 x 2 min), and pipetted onto 96-well PVC plate lids (Falcon 353913, Becton Dickinson) and visualized as before. The second screen was identical to the first except more beads were used, 0.2 µM biotin-hDM2 was used, and more stringent washing (5 x 5 min) was applied at each washing step.

**4.7 – Summary and outlook**
We have developed a method for synthesizing and screening large OBOβ libraries. Our techniques for synthesis, screening, and decoding are all highly scalable, through the use of fluorescence-based bead sorters, high-throughput mass spectrometry, and de novo peptide sequencing algorithms. Specifically, we tested the control beads with a COPAS automated bead sorter (Union Biometrica) and with the PEAKS de novo sequencing algorithm (BioInformatics Solutions) and found both to be ideal for future applications. The assay signal can be tuned in multiple ways to achieve a desired hit rate, which should allow for rapid discovery of modest affinity ligands for a wide variety of protein targets, as well as subsequent refinement of those hits into nanomolar affinity ligands. This bodes well for the future development of β-peptides as a versatile source for bioactive molecules, including inhibitors of protein-protein interactions that are notoriously difficult to target using small molecules.¹⁹⁰
Figure 4.1 – Helical net diagrams of $\beta_{53-1}$, $\beta_{\text{NEG}}$, and the small library used in the development and proof-of-principle of OBO$\beta$ libraries.
Figure 4.2 – Chemical structures and molecular weights of βNEG, β53-1, and the 8-member library used to develop the OBOβ assay.
Figure 4.3 – HPLC trace of a cleaved and processed sample from the 8-member library. Note seven of the eight library members can be uniquely identified simply from MALDI mass spectrometry of the major peaks.
Figure 4.4 – (A) Results from an ELISA-type screen of $\beta$NEG, $\beta_{53-1}$, and a combined mixture of $\beta_{53-1}$ and $\beta$NEG beads.
Figure 4.5. Absorption and emission spectra of quantum dot-streptavidin conjugates (© Quantum Dot Corp., 2003).
Figure 4.6 – Fluorescence images (upper panels use filters which allow blue light excitation, lower panels used filters which allow green light excitation) of beads screened under various blocking conditions. Beads are 1:1 mixtures of β53-1 and βNEG beads.
Figure 4.7 – (A) Fluorescence images of beads screened under conditions which demonstrate attenuation of signal with higher concentrations of gelatin, salt, and detergent. (B) Fluorescence images of beads screened with the indicated protein (or no protein), with subsequent detection using QSA605. Beads are 1:1 mixtures of β53-1 and βNEG beads.
Figure 4.8 – (A) Fluorescence image of a mixture of βNEG and β53-1 beads under optimized screen conditions. (B) Fluorescence image of the 8-member library screened under optimized screen conditions. (C) Fluorescence images of the individual pools of library members screened under optimized screen conditions.
Figure 4.9 – (A) Chemical structure depicting the first 1,000 member library synthesized and screened. (B) Sequence analysis of hits from two separate screens of the first 1,000 member library (H1-H20), and beads randomly picked from the library (L1-L20). (C) Raw FP data examining for direct binding of selected hits to hDM2.
Figure 4.10 – (A) Chemical structure of the second 1,000 member library synthesized and screened. (B) Helix net diagram showing sequences of the library and resulting hits.
Figure 4.11 – (A) Representative fluorescence micrograph of library beads screened under initial, less stringent conditions. (B) Representative fluorescence micrograph of library beads screened under the second, more stringent set of conditions. (C) CD spectra of β-peptide hits β53-8–10. Spectra were obtained in PBC buffer, pH = 7.0, at 25 °C using 80 µM β53-1, β53-8 and β53-9, and 15 µM β53-10. (D) FP binding data and curve fits for incubation of varying concentrations of hDM21-188 with 25 nM of each dye-labeled peptide. (E) FP competition data and curve fits for incubation of 0.5 µM hDM21-188 with 25 nM p53AD15-31flu and varying concentrations of unlabeled competitor. β53-10 was only soluble to 18 µM and did not generate interpretable competition data.
Table 4.1 – Calculated and MALDI-observed masses spectrometry data for β-peptides described in this section.

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