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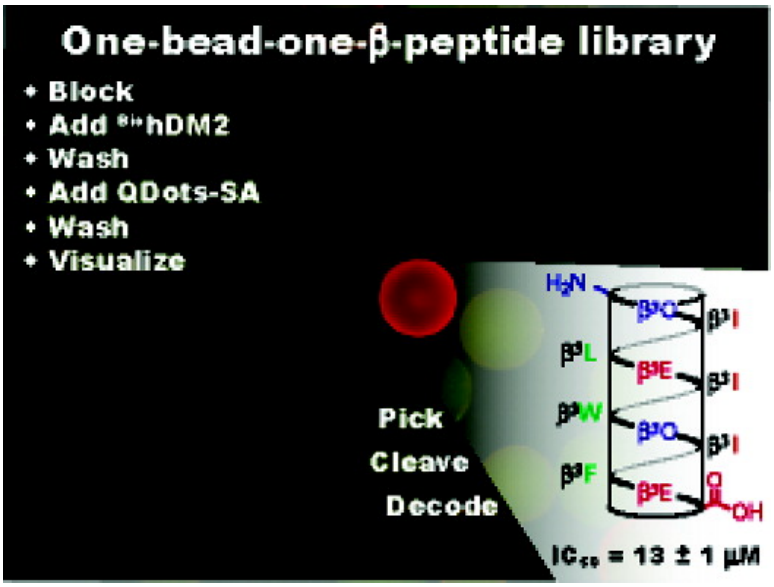

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A Rapid Library Screen for Tailoring β-Peptide Structure and Function

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Recently we described the β-decapeptide **β53-1** (Figure 1), which folds into a 14-helix, binds the oncoprotein hDM2, and inhibits the interaction of hDM2 with a peptide derived from the activation domain of p53 (p53AD).[1] The solution structure of **β53-1** in CD_3OH revealed an unexpected C-terminal unwinding that staggers the side chains comprising the hDM2 recognition epitope to better mimic those of p53AD.[2,3] The structure−function relationship implied by this distortion suggested that a library of **β53-1** analogues with diversity along a *nonrecognition face* might contain more active analogues. Here we describe (1) β-peptide synthesis protocols that produce high-quality one-bead-one-β-peptide (OBOβ) libraries suitable for on-bead screening without purification,[4] (2) a scalable on-bead screen, and (3) a simple tandem mass spectrometry (MS/MS) decoding method. Using this procedure, we identified **β53-1** analogues with improved structural and functional properties.

Previous reports have documented poor yields and low purities in the synthesis of β-peptide oligomers longer than hexamers, making HPLC purification a prerequisite for analysis.[5−8] To evaluate conditions that would produce high-quality β^3-peptide decamers suitable for analysis without purification, we synthesized **β53-1** and βNEG (Figure 1) on Tentagel macrobeads containing a 4-hydroxymethylbenzoic acid (HMBA) handle. Synthesis products were deprotected (96:2:2 TFA/water/triisopropylsilane), and pools of 100−200 beads were cleaved (1.0 M CH_3ONa/MeOH) for bulk purity analysis. We found that a combination of 90-min coupling times, double couplings, and an extra Fmoc deprotection step using DBU after the fourth coupling[7,9] yielded bead-bound β-decapeptides with purities between 80 and 90% as judged by analytical HPLC. The purities of β-peptides isolated from individual **β53-1** and **βNEG** beads were evaluated by MALDI and tandem electrospray (MS/MS) mass spectrometry; in each case a single major product was observed. Overall, this refined protocol produces β-peptide libraries whose quality matches or exceeds those of previously reported peptoid and oligocarbamate libraries[10−15] and equals the purities reported for microwave-assisted β-peptide syntheses.[8]

To establish a procedure for on-bead screening, we used **β53-1** and **βNEG** beads synthesized using the refined protocols as positive and negative controls for $hDM2_{1-188}$ binding. The screen entailed blocking, incubation with biotinylated $hDM2_{1-188}$ (^{Bio}hDM2), washing, incubation with streptavidin-coated quantum dots (QDots-SA605), further washing, and visualization using a fluorescence microscope. Detection using streptavidin-coated quantum dots eliminates false positives due to Tentagel bead autofluorescence.[12,16] Exhaustive testing of buffers and additives indicated that a blocking and washing buffer (BW buffer) composed of 10 mM Tris-HCl (pH 8.0), 2.5 mg/mL gelatin, 0.15 M NaCl, and 0.1% Tween-20 prevented localization of quantum dot fluorescence to βNEG beads while maintaining signal on **β53-1** beads (Figure 2A). Notably, decreasing ^{Bio}hDM2 concentration, increasing wash number and duration, or increasing the concentrations of gelatin, NaCl, and/or Tween-20 in the blocking and washing buffer each attenuated signal intensity.[9] The sensitivity of the screen to multiple parameters indicates that it can be tuned in several independent ways to achieve a desired hit rate.[17]

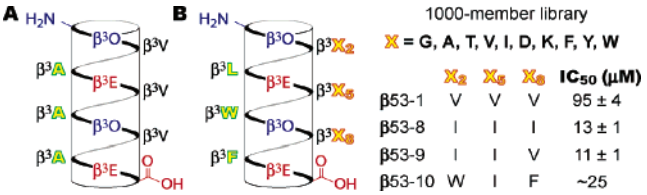

Figure 1. Helical net representations of (A) β**NEG** and (B) the 1000-member library, β**53-1**, and β**53-8**−**10**. β^3X denotes a β^3-homoamino acid; *X* is the common one-letter code for the analogous α-amino acid. Side chains on the recognition, variable, and salt-bridge faces are colored green, orange, and blue/red, respectively. IC_{50} values are derived from curve fits to the p53AD·hDM2 inhibition data in Figure 3A.

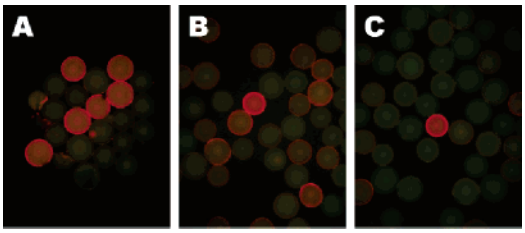

Figure 2. Representative fluorescence micrographs of OBOβ screens. (A) Mixture of **β53-1** and **βNEG** beads treated with 500 nM ^{Bio}hDM2 and 5 nM QDots-SA605 in BW buffer. (B) Beads from the 1000-member library treated as in (A) in BW+ buffer or (C) with 200 nM ^{Bio}hDM2 and 5 nM QDots-SA605 in BW+ buffer and washed extensively.[9]

As a mock screen, we chose one high-intensity bead from a mixture of five **β53-1** beads and a vast excess (>1000) of **βNEG** beads. The bead was washed and cleaved, and the cleaved products were desalted,[9] theoretically yielding up to 0.7 nmol β-peptide. Robust MALDI spectra revealing a single major product were obtained using only 5% of this material. MS/MS spectra were obtained using less than half of the remaining material,[9] highlighting the ease with which high-quality mass spectral data is obtained from β-peptides cleaved from individual beads. Both algorithm-based and manual sequencing confirmed the β-peptide on the bright bead was indeed **β53-1**. Notably, while β^3-peptides have been subjected to MS/MS fragmentation before,[18] our results demonstrate the feasibility of de novo MS/MS decoding of β-peptide identity from single beads.

To address our initial hypothesis regarding optimization of a nonrecognition face of **β53-1**, we synthesized a 1000-member library in which the β^3-homovaline (β^3V) residues at positions 2, 5, and 8 of **β53-1** were replaced with one of 10 β^3-amino acids (Figure 1).[19] The library was initially screened under conditions optimized for the control beads (Figure 2B), and the 25 brightest beads from a pool of ∼8000 were isolated manually.[9] MALDI and

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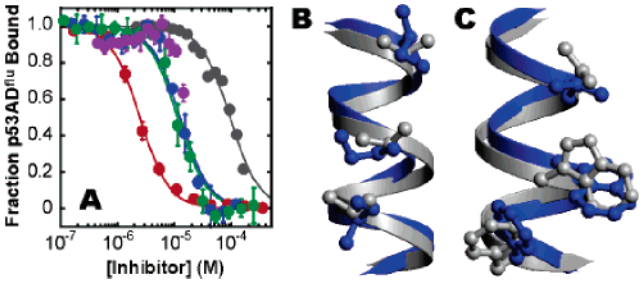

Figure 3. (A) Fluorescence polarization analysis of the inhibition of $p53AD_{15-31}{}^{Flu}$•$hDM2_{1-188}$ complexation by $p53AD_{15-31}$ (red), ***β*53-1** (gray), ***β*53-8** (blue), ***β*53-9** (green), or ***β*53-10** (purple). (B,C) Superposition of the solution structures of ***β*53-1** (gray) and ***β*53-8** (blue) highlighting the varied nonrecognition face (B) and the recognition face (C).[20]

MS/MS spectra confirmed that the selected beads possessed among them at least 10 different sequences out of the possible 1000, a hit rate of at least 1%.[19] The screen was repeated using 200 nM ^{Bio}hDM2 (instead of 500 nM) and five 5-min washes at each washing step (instead of two 2-min washes).[9] The second screen (Figure 2C) yielded 35 high-signal beads from ~16 000 total beads screened, with only a small proportion of nonhit beads showing even dim Qdots-SA605 fluorescence. MALDI and MS/MS spectra confirmed that these 35 beads possessed among them only three sequences: ***β*53-8** (16 beads), ***β*53-9** (18 beads), and ***β*53-10** (1 bead). Significantly, each of these sequences was identified multiple times among the 25 beads culled from the first screen, confirming that the screen had been successfully tuned to reduce the hit rate from over 1 to 0.3%.

The three ***β*53-1** analogues ***β*53-8**, ***β*53-9**, and ***β*53-10** (Figure 1) were synthesized and characterized for their ability to inhibit formation of the $p53AD^{Flu}$•hDM2 complex.[1] ***β*53-8** and ***β*53-9**, which were well-represented among the hit sequences, both competed with $p53AD^{Flu}$ for hDM2 binding, with half-maximal inhibitory concentrations (IC_{50}'s) of 13 ± 1 and 11 ± 1 μM, respectively (Figure 3A). These values represent a roughly 8-fold improvement in inhibitory potency over the parent β-peptide β**53-1**. By contrast, β**53-10**, which was identified only once in the second, more stringent screen, was less soluble in competition assays but appeared to possess an IC_{50} of ~25 μM.

Overall secondary structure of the novel analogues was assessed by circular dichroism (CD) spectroscopy.[1,21] β**53-8** and β**53-9** (but not β**53-10**) possessed surprisingly intense 14-helix CD signatures in aqueous solution, with mean residue ellipticities at 214 nm of −13 700 and −14 900 deg•cm^2•dmol^{-1}, respectively.[9] These minima are roughly 50% more intense than that of β**53-1**. The implied increase in 14-helix structure observed for β**53-8** and β**53-9** is not unexpected for replacement of β^3V with β^3-homoisoleucine (β^3I).[21] To obtain higher resolution information on the differences between β**53-1** and β**53-8**, we determined the NMR solution structure of β**53-8** in methanol in a manner analogous to previous structural work on β**53-1**.[2] The NMR structures of β**53-8** and β**53-1** are strikingly similar, with one major difference: inclusion of three β^3I residues in place of β^3V expands the corresponding 14-helix face, pushing the C_β atoms of residues 2 and 8 from under 9.5 Å apart to over 10.4 Å apart (Figure 3B). This expansion effectively compresses the recognition face: the C_β atoms of residues 3 and 9 are at least 10.1 Å apart in β**53-1**, but under 9.3 Å apart for β**53-8** (Figure 3C). The structure of β**53-8** thus provides a clear rationalization for the observed improvement in activity. Furthermore, because the structural differences between β**53-8** and β**53-1** are so subtle, combinatorial techniques such as those described herein may be especially fruitful for the discovery and refinement of β-peptides as inhibitors of protein−protein interactions.

In sum, we have reported a versatile new method for synthesizing and screening large β-peptide libraries. Our techniques for synthesis, screening, and decoding are scalable and immediately compatible with fluorescence-based bead sorters, high-throughput mass spectrometry, and de novo peptide sequencing algorithms.[9] The output signal can be tuned in multiple ways to achieve a desired hit rate, which should allow for rapid discovery of high-affinity ligands for a wide variety of protein targets.

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Note Added in Proof: A 100-member solution-based library of β-peptide octamers was recently reported by Gellman and coworkers (Murray J. K., Farooqi, B., Sadowsky J. D., Scalf M., Freund W. A., Smith L. M., Chen J., and Gellman S. H. *J. Am. Chem. Soc.* **2005**, *127*, 13271−13280).

Supporting Information Available: β-peptide synthesis and screening protocols, CD spectra, and NMR structure determination details. This material is available free of charge via the Internet at http://pubs.acs.org.

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