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Transformation of Low-Affinity Lead Compounds into High-Affinity Protein Capture Agents

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Summary

A simple and potentially general approach to the isolation of high-affinity and -specificity protein binding synthetic molecules is presented. A modest affinity lead compound is appended to the end of each molecule in a combinatorial library of oligomeric compounds, such as peptides or peptoids. The library is then screened under conditions too demanding for the lead to support robust binding to the protein target. It was anticipated that this procedure would select for bivalent ligands in which the oligomer library provides both a second binding element as well as an appropriate linker between this element and the lead compound. We report here synthetic ligands for the Mdm2 protein and ubiquitin able to capture their target proteins from dilute solutions in the presence of a large excess of other proteins.

Introduction

Synthetic compounds that bind proteins are of great utility in biology and medicine. Most are obtained by screening combinatorial libraries or compound collections. Generally, ligands obtained from screening naïve libraries form protein complexes with equilibrium dissociation constants (K_Ds) in the micromolar range. Such modest affinity ligands are suitable for some applications [1–3], such as chemical genetic studies, but not others. For example, there is considerable interest in the development of protein-detecting microarrays based on small molecule capture agents [4, 5], an application that will demand much higher affinity binding. Therefore, a central problem in this area is to develop a general strategy for the maturation of a low-affinity lead compound into a high-affinity protein binding agent. To do so by traditional medicinal chemistry methods is too time consuming and labor intensive to be applied on a proteomic scale. A more modern alternative is to construct libraries of derivatives using split and pool synthesis [6] or other combinatorial methods and screen these for higher affinity derivatives. This is particularly effective with peptide libraries in which consensus residues identified in a primary screen can be held constant in a secondary library [7].

An alternative that has been explored by many laboratories [8–12] is to create bivalent protein binding agents by coupling two or more noncompetitive ligands with an appropriate linker that allows cooperative binding. While this strategy can be quite effective, there remains a need to increase the throughput of the discovery of such ligands.

An alternative to linking together separate binding elements was introduced by Combs et al. in a study targeting the Src SH3 domain [13]. These workers started with a low-affinity peptide (PLPPLP) that occupies only part of the structurally characterized binding pocket of this domain. The chain was extended by diversity-oriented synthesis, resulting in a library of peptides all containing the shared PLPPLP element. This approach was designed to take advantage of the known binding pocket immediately adjacent to the PLPPLP binding site. Screening of this library resulted in the isolation of molecules with much higher affinity for Src SH3 domain than PLPPLP. To the best of our knowledge, little more has been done to develop this "extension" strategy, possibly due to the perceived requirement of structural knowledge of the protein binding pocket and the availability of a ligand that fills only part of it.

We describe here a study that probes whether this type of approach can be applied more generally to the isolation of high-affinity protein ligands, even in the absence of any structural information. Indeed, the SH3 domain study mentioned above is merely a special case of a protein target with two "bindable" sites that can be targeted by a bivalent ligand; in that case these sites happen to be immediately adjacent to one another. Even for proteins where this is not the case, one might be able to mine high-affinity ligands from extension libraries if the library built off of the lead compound has enough "reach" to find nonadjacent sites. For example, the left side of Figure 1 depicts an arbitrary case in which the putative second binding site can be recognized by a three-unit element of the oligomer library. In a large library this unit will be present in many different molecules, with various linkers of disparate length and geometry between it and the lead. Thus, it should be possible to identify this putative secondary binding element and a relatively optimal linker in the same screen, leading to a bivalent ligand that would be expected to exhibit greatly enhanced affinity for the target protein relative to the lead molecule. Of course, one would expect that some protein targets would simply not contain a second binding pocket within reach of the lead molecule binding pocket for a library of the type shown in Figure 1. Nonetheless, we imagined that it might still be possible to isolate a binding agent with greatly enhanced affinity relative to the lead through avidity effects. On a densely functionalized surface or bead it might be possible for the lead compound from one molecule and a library-encoded element from a second molecule to collaborate to form a high-affinity bivalent capture agent (right side of Figure 1). In this case, the prediction would be that the solid-phase and solution binding properties of the derived ligand would be very different since high-affinity binding would be dependent on the support acting as

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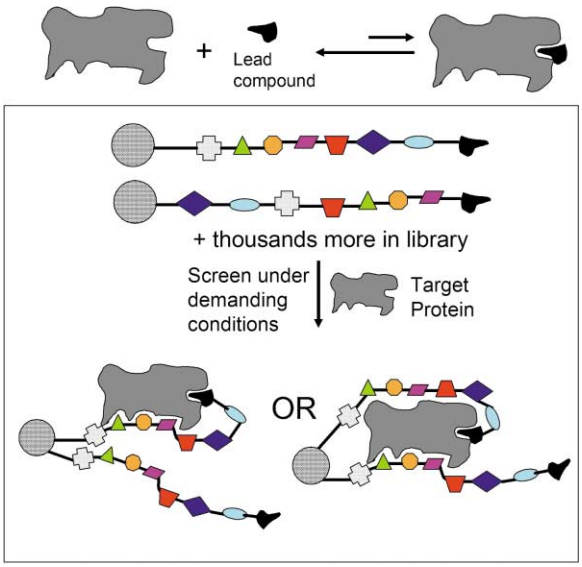


Figure 1. Schematic Representation of the Strategy Employed to Isolate High-Affinity Protein Binding Agents

It was anticipated that molecules which bind the target protein via a 1:1 or 1:2 (protein:ligand) stoichiometry could be isolated. See text for details.

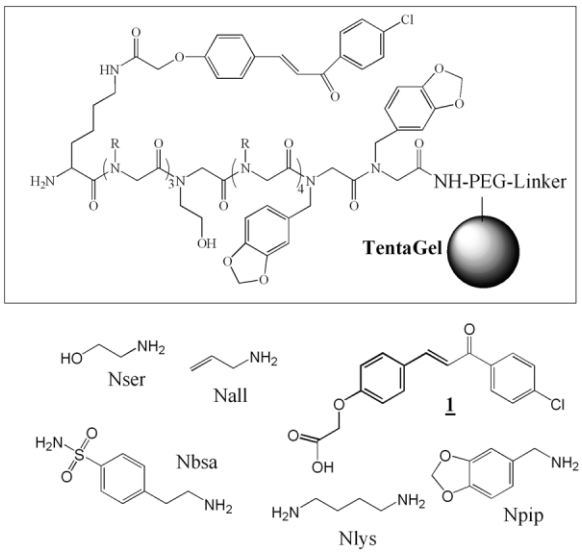


Figure 2. General Structure of the Chalcone-Capped Peptoid Library and the Amines Used for the Synthesis of the Library

Top: structure of the chalcone-capped peptoid library. The linker consisted of a long polyethylene glycol chain to minimize nonspecific protein binding. Bottom: compounds used for the synthesis of the library along with their designations. The library was of the form NH_2-(chalcone)Lys-X_3-Nser-X_4-Npip-Npip, where X = peptoid monomers derived from any of the amines shown at the bottom of the figure. The theoretical diversity of the library was 78,125 compounds.

a linker. Even compounds that evince high affinity only when attached to a surface are of great interest as potential protein capture agents for the construction of protein-detecting microarrays.

We demonstrate the efficacy of this approach in two cases by identifying high-affinity capture agents for the Mdm2 protein and ubiquitin. As anticipated, binding studies suggest that both types of ligands pictured in Figure 1 can be obtained through this procedure. The protocol developed in this study is semiautomated and optimized to reduce or eliminate the isolation of false positives or poorly selective, hydrophobic ligands. Thus, we believe that it will be suitable for high-throughput work in the future. We also demonstrate that the Mdm2-targeted compound functions efficiently in a microarray format of the type suitable to carry out complex protein profiling experiments.

Results and Discussion

Semiautomated Screening of a Chalcone-Capped Peptoid Library

Stoll et al. reported that the chalcone **1** (Figure 2) associates with the p53 binding domain of the protooncoprotein Mdm2 weakly (K_D = 220 μM) [14]. Some of the data presented in this study suggested that the carboxylate group of the chalcone was oriented away from the protein and could be utilized for attachment to other moieties. Therefore, the chalcone was appended to the end of each molecule in a combinatorial library of oligomeric compounds, in this case peptoids (Figure 2) [15–17] synthesized by split-and-pool solid-phase synthesis [6]. The resultant "capped" library (78,125 compounds) was then screened against a labeled Mdm2 derivative under conditions too demanding for the chalcone alone to capture the protein (high-salt and detergent-containing buffer). The expectation was that the peptoid library (see ref. [18] for the characterization of this library) would provide both a second binding element as well as a suitable linker to allow this second element and the lead chalcone to bind Mdm2 cooperatively.

Approximately 78,000 beads were presorted using a fluorescent bead sorter to remove beads that exhibited intense autofluorescence [18, 19]. The remaining beads were then incubated with fluorescein-labeled Maltose binding protein (MBP)-Mdm2 fusion protein (500 nM) in the presence of a 10,000-fold excess of unlabeled *E. coli* proteins in a buffer containing 1 M NaCl and 1% Tween 20. As will be reported in detail elsewhere, the large excess of unlabeled bacterial proteins is important in eliminating nonspecific binders (L. Troitskaya, M.M.R. and T.K., unpublished data). After thorough washing, the beads were poured into the bead sorter and separated by fluorescence intensity. Only four beads (0.006% of the library) exhibited fluorescence well above the bulk population. This low hit rate suggested that the conditions employed had indeed selected stringently for the best ligands in the library. One of the "hits" was placed on a microscope slide along with several of the "negative" beads from the sort, and a fluorescence micrograph of part of this field was taken (Figure 3). The high contrast between the hit and the negatives validates that the sorter indeed identified the brightest beads. Automated Edman sequencing of the hits showed that two of the four were identical, having the structure NH_2-Lys(chalcone)-*Nlys*-*Npip*-*Nlys*-Nser-*Nlys*-*Nlys*-*Nlys*-*Nlys*-Npip-Npip (residues that were varied in the library are

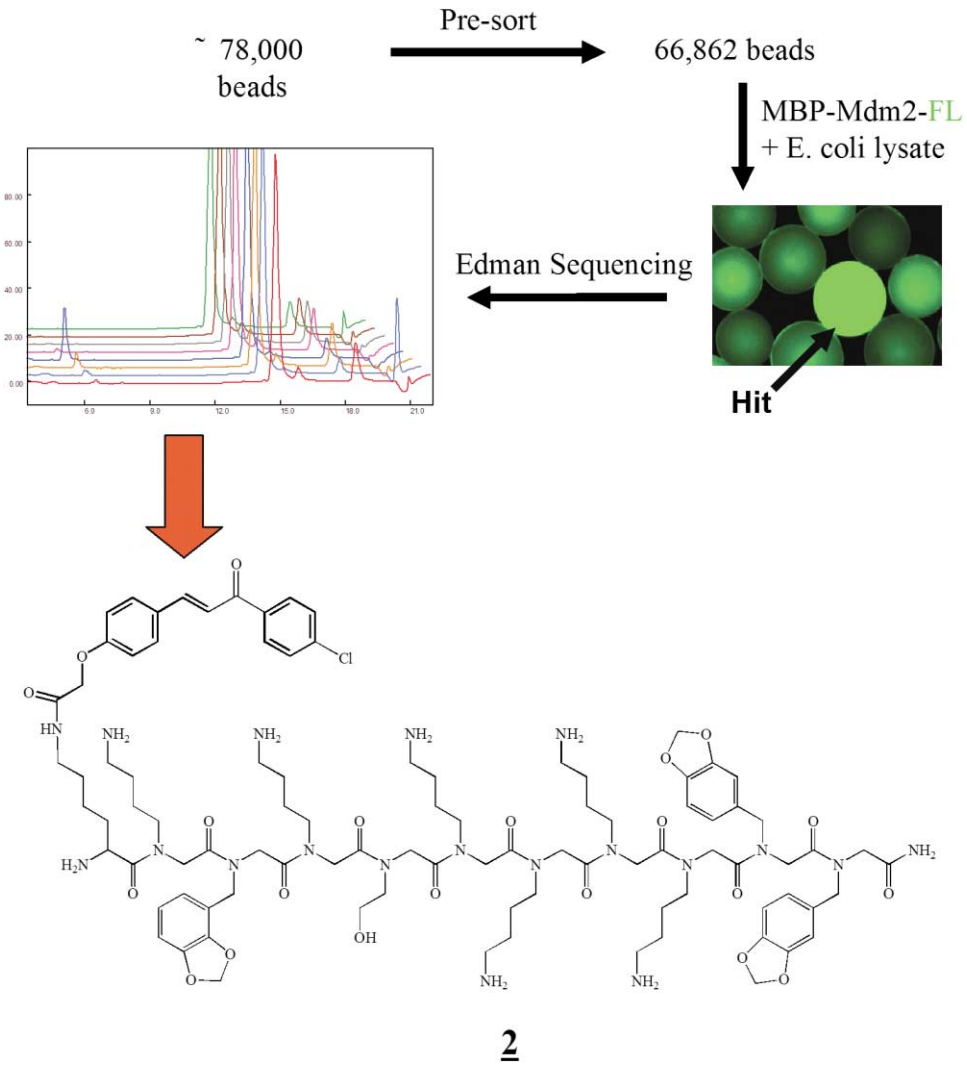


Figure 3. Sequence of Experiments Leading to the Identification of a High-Affinity MBP-Mdm2 Ligand

The fluorescence micrograph of a "hit" identified by the bead sorter mixed with several beads that were scored as negatives. The Edman trace is of the bright bead in the photomicrograph, revealing the structure shown.

in italics) (Figure 3). The other two hits were also Nlys-rich, indicating that a highly basic peptoid facilitates higher affinity binding to the target protein.

Binding Studies Confirm High-Affinity Mdm2 Binding

NH_2-Lys(chalcone)-Nlys-Npip-Nlys-Nser-Nlys-Nlys-Nlys-Nlys-Npip-Npip (**2**) was resynthesized and purified by reverse-phase HPLC. Titration experiments using MBP-Mdm2 monitored by isothermal calorimetry (ITC) (Figure 4A) revealed a solution equilibrium dissociation constant of 1.3 $\pm$ 0.4 μM. We were unable to determine the K_D of the chalcone **1**•MBP-Mdm2 complex under the same conditions by ITC due to insufficient solubility of the small molecule. The chalcone was therefore linked to a dipeptoid constructed from two glycine residues, providing a much more soluble dianionic compound, but even at the highest protein concentration employed, no binding could be detected by ITC (data not shown). Thus, we unfortunately cannot provide an accurate estimate of the level of improvement in the affinity of chimera **2** for MBP-Mdm2 relative to the parent compound **1**. Based on the literature K_D of 220 μM, this is a 170-fold improvement, but this is probably a lower limit since we cannot detect binding of **1** or its more soluble diglycine conjugate derivative to MBP-Mdm2. The K_D of the complex of MBP-Mdm2 and the peptoid NH_2-Nlys-Npip-Nlys-Nser-Nlys-Nlys-Nlys-Nlys-Npip-Npip lacking the chalcone cap was 378 μM (Figure 4B), demonstrating that neither piece of the chimeric ligand is itself a high-affinity binding agent. An ITC experiment was also done with **2** and MBP lacking the Mdm2 fusion. Only the heat of dilution of the titrant was observed in this experiment (Figure 4C), demonstrating little or no binding ($K_D \geq$ 6 mM). These data demonstrate that interactions between **2** and MBP contribute little or nothing to the observed binding affinity and that the chimeric ligand is specific for Mdm2. It is also interesting to note that the

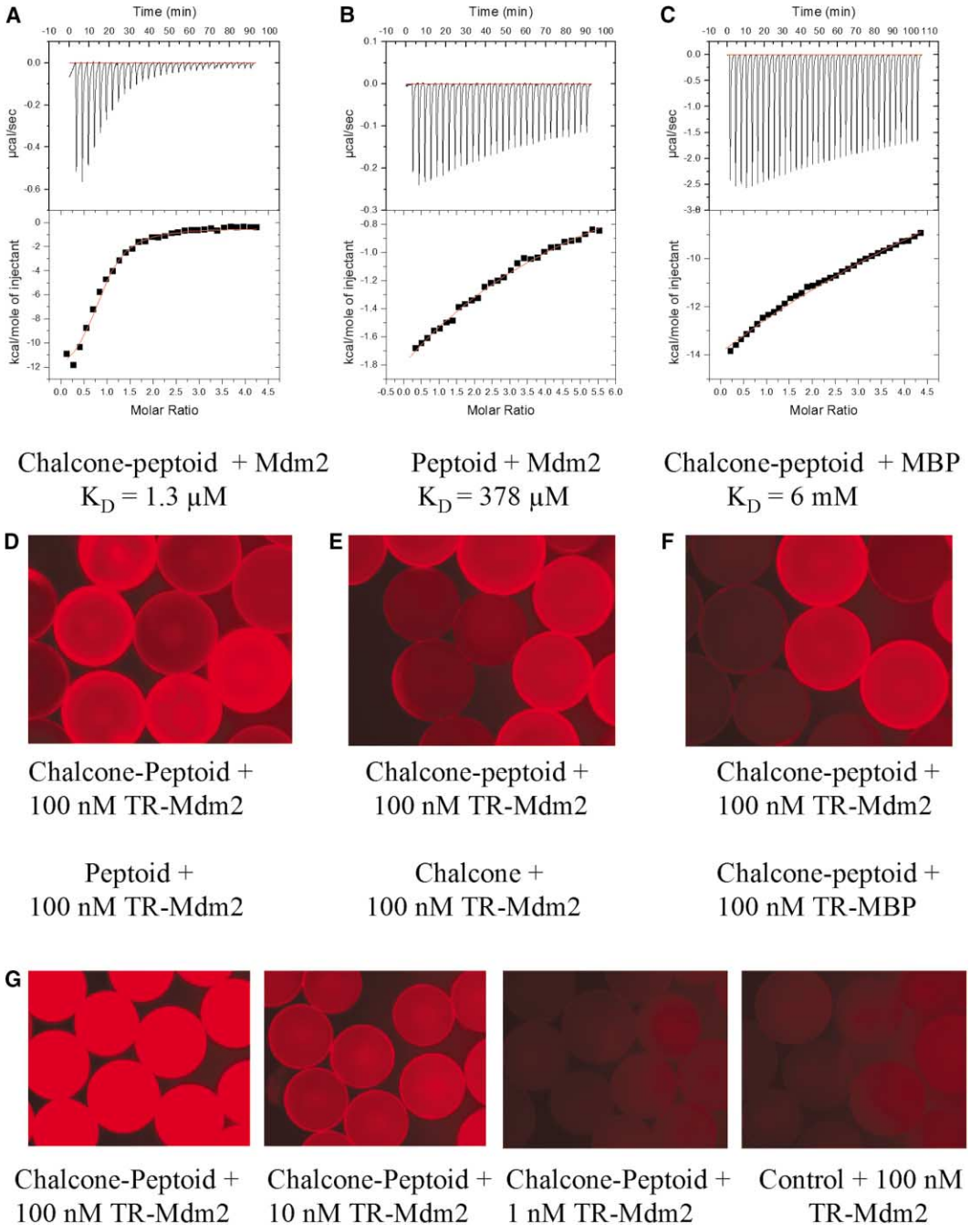


Figure 4. Characterization of the Binding Properties of **2** and Related Compounds in Solution and Immobilized on TentaGel Beads

(A–C) Isothermal titration calorimetry data for the titration of (A) chalcone-peptoid **2** and (B) the peptoid NH_2-Nlys-Npip-Nlys-Nser-Nlys-Nlys-Nlys-Nlys-Npip-Npip lacking the chalcone cap with MBP-Mdm2 and (C) chalcone-peptoid **2** with MBP alone. The equilibrium dissociation constants derived from these data are shown.

(D–F) TentaGel beads displaying the compound indicated were incubated with the Texas red-labeled proteins indicated, and, after washing, the beads were mixed and photographed under a fluorescence microscope.

(G) Photomicrographs of TentaGel beads displaying **2** after incubation with the indicated concentration of Texas red-labeled MBP-Mdm2 protein followed by thorough washing.

same library employed above, but lacking the chalcone-Lys cap, was screened against MBP-Mdm2 under less demanding conditions and a completely different set of peptoid sequences was isolated [18], consistent with the idea that ligand **2** is a unique species that is greater than the sum of its parts.

To probe the binding chemistry of ligand **2** on a solid support, which is more relevant to the issue of creating high-affinity protein capture agents, TentaGel beads were prepared that display either chalcone **1** alone, the 10-mer peptoid lacking the chalcone cap, or the chimeric chalcone-peptoid ligand **2**. In the experiment shown

in Figures 4D–4F, the beads and the Texas Red-labeled protein (100 nM) indicated in the figure were incubated under the demanding conditions similar to those used in the screening experiment, then the beads were washed thoroughly. Two populations of beads were mixed in a 1:1 ratio and photographed under a fluorescence microscope to provide a direct comparison. In each case, two distinct populations were observed, one bright and one dim. Edman sequencing of the bright and dark beads showed that in each case, the bright beads displayed chimera **2**. These data agree qualitatively with the ITC results (Figures 4A–4C) in that they show the chalcone-peptoid chimera has a higher affinity for Mdm2 than does either individual component of the chimera and that it does not recognize MBP.

To better judge the apparent affinity of the immobilized chalcone-peptoid chimera **2** for labeled Mdm2, the experiment shown in Figure 4G was conducted. In this case, a more typical biochemical buffer (150 mM NaCl and 0.1% detergent) was employed and the indicated concentration of Texas Red-labeled MBP-Mdm2 was mixed with 100-fold excess of *E. coli* proteins. After thorough washing, the beads were photographed in the fluorescence microscope using identical settings in each case. Capture of Mdm2 was apparent down to a protein concentration of 10 nM. The image at 1 nM Mdm2 was similar to that of a control bead displaying a different ligand selected randomly from the library.

To evaluate the chalcone-peptoid chimera for MBP-Mdm2 in a more physiologically relevant context, we conducted a "pull-down" protocol that simulates an immunoprecipitation experiment, except that the synthetic molecule **2** is used in place of an antibody. Native MBP-Mdm2 protein (i.e., lacking a fluorescent label) was mixed with HeLa nuclear extract such that it represented 0.1% of the total protein present. This mixture was incubated with Tenta Gel beads displaying the chalcone-peptoid chimera **2**. SDS-PAGE followed by Western blot analysis using anti-MBP antibodies revealed that MBP-Mdm2 was retained by beads (Figure 5A). Neither Tenta Gel beads displaying a control peptoid or the beads alone retained detectable protein. This demonstrates that the chalcone-peptoid chimera **2** is capable of capturing MBP-Mdm2 in the context of a complex mix of mammalian proteins. Importantly, this experiment also demonstrates that the chimera **2** binds the native protein and that the previous binding results were not dependent on an unexpected effect of the Texas Red label.

A High-Affinity Ubiquitin Capture Agent

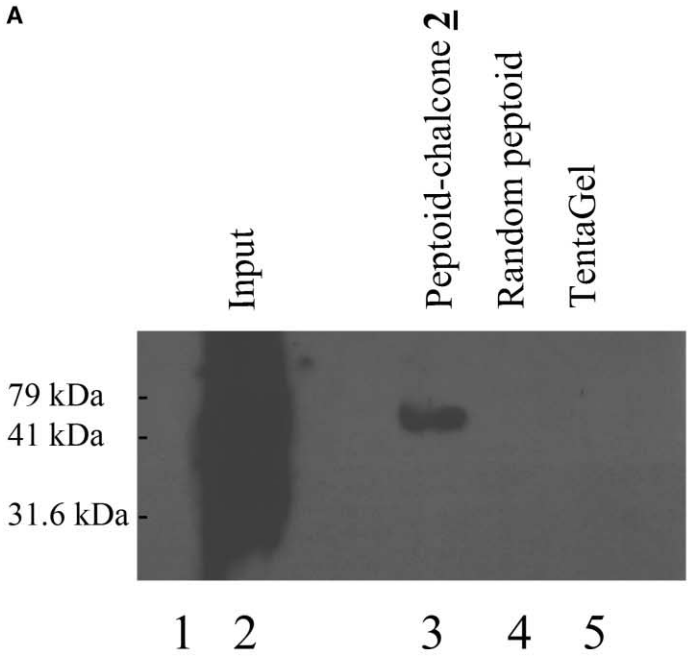
For many proteins, even modest affinity lead compounds are not available. Therefore, we attempted to generate a high-affinity ubiquitin capture agent from scratch using this methodology. A peptide library was first screened under relatively mild conditions to provide a lead compound NH_2-RWDRYYF [19]. Titration experiments monitored by ITC revealed a K_D of 33 $\pm$ 5 μM for the peptide•ubiquitin complex (Figure 5B). A new peptide library was then constructed on TentaGel beads by split and pool synthesis of the form NH_2-X_7-S-RWDRYYF, where X represents a randomized position using the amino acids A, E, G, H, K, L, N, R, T, and W. A fraction of this library (≈250,000 beads) was then incubated with fluorescently labeled ubiquitin (200 nM) in the presence of a 10,000-fold excess of unlabeled bacterial proteins in a buffer containing 0.5 M NaCl and 0.5% Tween 20 detergent. In addition, a 100-fold excess of synthetic lead peptide (NH_2-RWDRYYF) was also included in the buffer to block capture of ubiquitin by molecules that represent only a modest improvement over the lead peptide. This strategy to demand higher affinity ligands is different than that employed in the chalcone case, which used a high salt and detergent buffer. This may be a more general protocol for proteins that do not tolerate high salt and detergent. Under these conditions, only three beads (0.0012% of the library) fluoresced well above background. The structure of the peptides was deduced by Edman sequencing. One of them (NH_2-WGLRALESRWDRYYF) was resynthesized and purified. ITC experiments revealed that this putative high-affinity ligand exhibited only a 3-fold improvement over the lead in terms of its solution affinity for ubiquitin (K_D = 12 $\pm$ 4 μM); Figure 5B).

However, NH_2-WGLRALESRWDRYYF evinced a much-improved apparent affinity for ubiquitin when displayed on TentaGel. Beads displaying the 15-mer, a control peptide, or no peptide at all, were incubated with the indicated concentration of unlabeled ubiquitin in the presence of a 1000-fold excess of *E. coli* proteins (Figure 6). After thorough washing, the beads were then probed with Texas red-labeled anti-ubiquitin polyclonal antibodies to visualize the bound protein. Binding of ubiquitin by the 15-mer was very strong at 4 nM ubiquitin and easily detectable above the background even at 0.8 nM ubiquitin. When the experiment was repeated with ubiquitin omitted from the solution, all of the beads exhibited the same low-level background signal (data not shown), ruling out the possibility that NH_2-WGLRALESR WDRYYF binds the labeled antibody rather than ubiquitin itself. While these binding assays are only semi-quantitative in nature, they argue that the functional dissociation constant of the immobilized 15-mer for ubiquitin is at least in the low nanomolar range.

To examine this more carefully and quantitatively, an ELISA-like assay was performed (Figure 6C). This involved incubating the bead-bound peptide NH_2-WGLRA LESRWDRYYF or the lead peptide with unlabeled ubiquitin and, after washing, quantifying the amount of ubiquitin captured via a colorimetric sandwich assay using an anti-ubiquitin antibody and an HRP-labeled secondary (see Experimental Procedures for details). The apparent K_D of the chimeric peptide NH_2-WGLRALESRW DRYYF•ubiquitin complex was 6 nM (Figure 6C). The striking difference between this apparent affinity of the immobilized ligand for ubiquitin and the modest solution K_D strongly suggests that avidity effects are operative in the on-resin experiments (vide infra). In this assay, the apparent affinity of the lead peptide for ubiquitin was much lower, as expected.

A Model Protein-Detecting Microarray

To determine if the ligands described here would function in the context of a protein-detecting microarray platform [3, 20, 21], a three-feature array was made as a model system. Cysteine-containing derivatives of the chalcone-peptoid **2**, the random peptoid, and fluores-



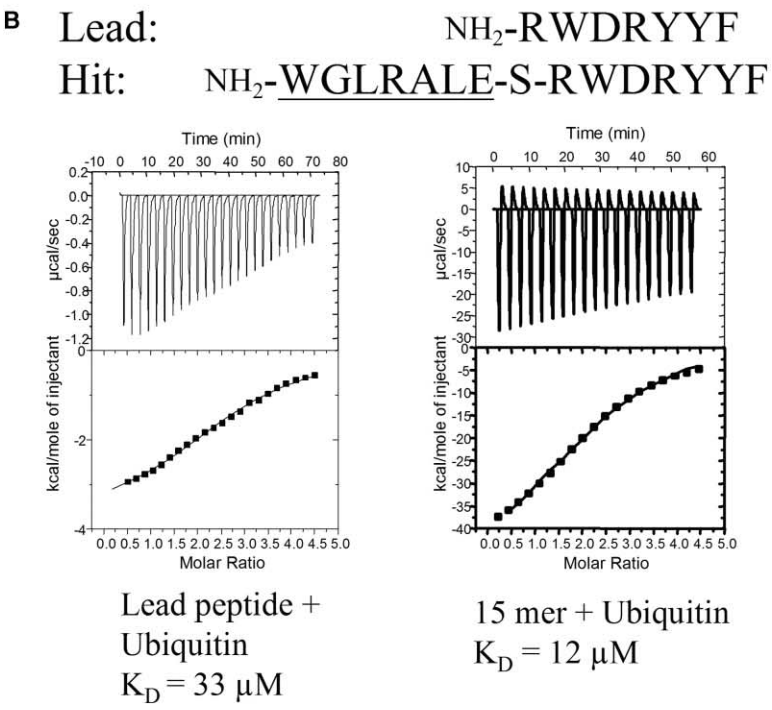


Figure 5. Retention of MBP-Mdm2 from HeLa Extract and Determination of the Dissociation Constant of Ubiquitin Ligands

(A) MBP-Mdm2 in HeLa extract was incubated with TentaGel beads displaying chalcone-peptoid chimera 2. After washing, the bead-bound protein was analyzed by SDS-PAGE and Western blotting with anti-MBP antibody. Lane 1: molecular mass standards. Lane 2: input protein. Lane 3: protein retained by chalcone-peptoid chimera 2. Lane 4: protein retained by control peptoid. Lane 5: protein retained by only TentaGel beads.

(B) Solution binding affinities of the peptide•ubiquitin complexes as determined by isothermal titration calorimetry.

cein-labeled random peptoid were synthesized and purified by HPLC. They were then arrayed using a pin-spotting robot onto a chemically modified glass microscope slide coated with a thiol-reactive group, allowing for covalent attachment. A three-line pattern was printed, with each line consisting of six spots of one of the three synthetic molecules. Imaging of the slide prior to exposure to protein revealed the expected line of fluorescent dots representing the fluorescein-labeled control peptoid (Figure 7A). This confirmed efficient covalent attachment of the ligands. The slide was then incubated with fluorescein-labeled MBP-Mdm2 (100 nM) in the presence of a 100-fold excess of unlabeled bacterial proteins. After a 2 hr incubation followed by

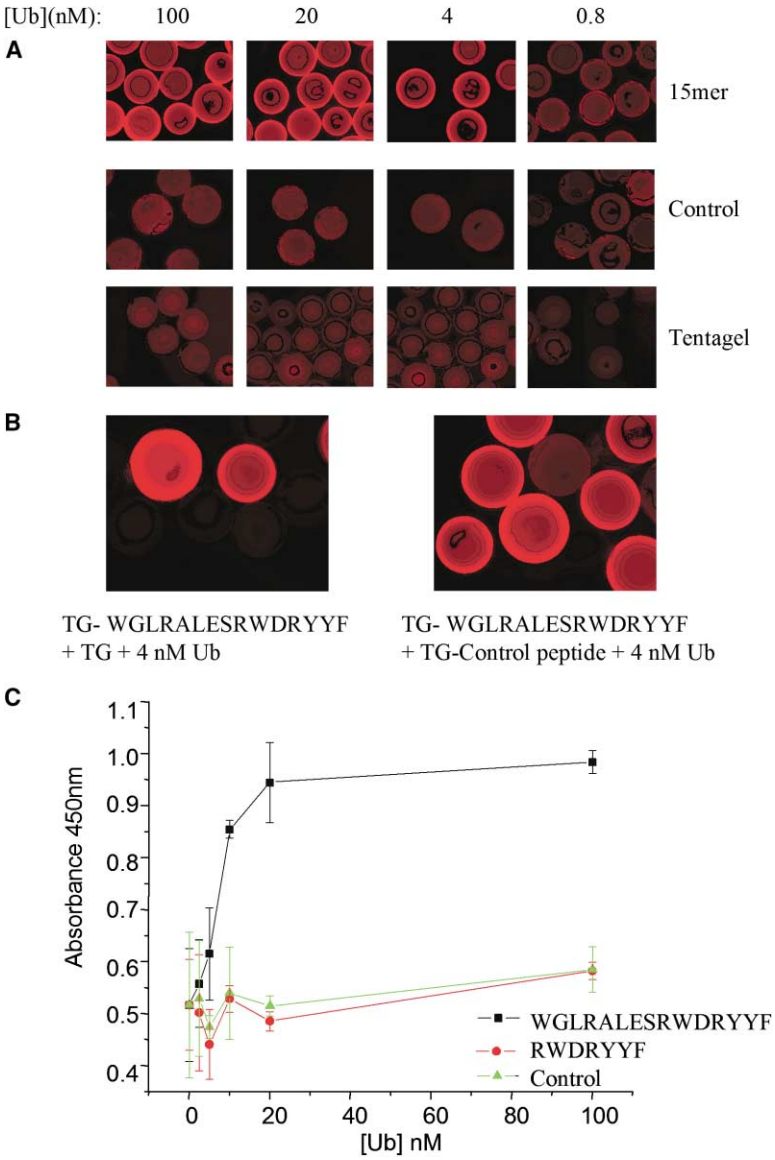


Figure 6. Characterization of the Solid-Phase Binding Properties of the Ubiquitin-Targeted Peptide

(A) Determination of the apparent affinity of the indicated immobilized peptides (and naked TentaGel beads) for ubiquitin. The beads were incubated with native ubiquitin then Texas red-labeled anti-ubiquitin antibody and photographed under a fluorescence microscope.

(B) Direct comparison of beads displaying the 15-mer or no peptide at all (top) and beads displaying the 15-mer and a control peptide (bottom). The same protocol described in (A) was employed.

(C) Determination of the apparent affinity for the TentaGel-displayed peptides using an ELISA-like assay (see Experimental Procedures). NH_2-WGLRALESRWDRYYF (black); RWDRYYF (red); control (green).

thorough washing, the slide was imaged. Figure 7B shows that the spots containing the chalcone-peptoid **2** were much brighter than the random peptoid features. We conclude that the Mdm2-targeted chimeric ligand **2** acts as an effective protein capture agent in the context of a viable protein-detecting microarray platform.

A General Strategy for Capture Agent Discovery?

The results reported above demonstrate that a straightforward and semiautomated screening protocol can provide high-affinity protein capture ligands. We imagine that the protein ligands obtained act as bidentate ligands, with the library-derived binding element recognizing a site other than that bound by the lead compound. However, this model remains to be tested by direct spectroscopic or other types of structural studies. It is important to note that all of the binding experiments reported here were conducted in the presence of a large excess (100- to 10,000-fold) of bacterial proteins derived from a cleared *E. coli* lysate, thus simulating the recognition of a target protein in a crude cellular extract, serum sample, or other complex environment. In addition, Figure 5A demonstrates that MBP-Mdm2 can be retained from a HeLa extract by bivalent ligand **2**. Many reports of protein binding synthetic molecules have employed only purified proteins, a far less rigorous test of ligand quality.

The peptide- and peptoid-based molecules derived from this screening protocol are quite simple, making this approach to synthetic protein ligands accessible even to biological laboratories lacking sophisticated chemical synthesis capabilities. Of course, the basic concept could be extended to libraries containing more elaborate building blocks if desired. A report by Li and Roberts that appeared while this work was in progress also suggests that this capped library approach could be applied to extremely large DNA-encoded mRNA display peptide libraries [22].

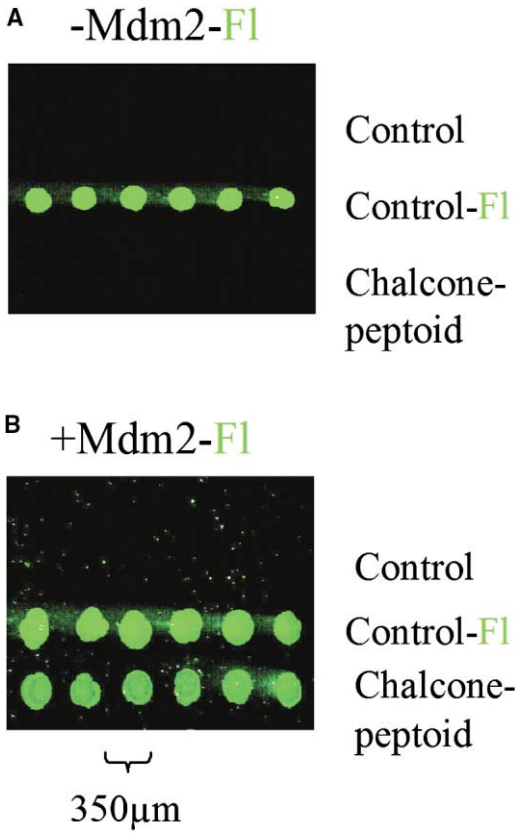


Figure 7. A Model Protein-Detecting Microarray

(A) An image of the glass slide before it was probed with conjugated protein. The spots that are seen in the second row represent the fluorescein-conjugated random (control) peptoid that was spotted along with the unlabeled random peptoid and an unlabeled Cys-containing derivative of **2** (chalcone-peptoid).

(B) A scanned image of an identical slide to that shown in (A), but after incubation with fluorescein-conjugated Mdm2 and a large excess of unlabeled bacterial proteins. Association of the labeled Mdm2 protein is >10-fold higher to the chalcone-peptoid molecule than to the control peptoid.

In both of the cases reported here, the apparent binding affinity of the ligands derived from the capped libraries were considerably better when these compounds were immobilized than was the case free in solution. Of course, the fluorescence-based bead binding assays are only semiquantitative, and in any case, one cannot compare apparent affinities to true solution K_Ds rigorously. It is common to observe enhanced binding of a soluble analyte to a resin-bound compound since once the target molecule associates with the capture agent, it finds itself in a local environment of very high ligand concentration, making escape from the environment of the bead unlikely. It may be that this kinetic effect can explain much of the apparent differences in binding affinity between the solution and immobilized Mdm2 ligand. In addition, since the chimera **2** has a much higher affinity for MBP-Mdm2 in solution that the peptoid lacking the chalcone cap or the chalcone alone, we believe that **2** is a true bivalent ligand of the type shown at the bottom left of Figure 1.

The ubiquitin binding peptide behaves differently. In this case, there is an enormous difference (approximately 2000-fold) between the affinities of the peptide for ubiquitin in solution and when immobilized on TentaGel. Furthermore, whereas **2** shows a large enhancement in its solution affinity over the lead chalcone **1**, the ubiquitin binding 15 residue peptide is only ≈3-fold better in solution than the lead heptamer. Thus, we argue that the immobilized peptide binds ubiquitin tightly because two surface-bound molecules collaborate in an avidity-based binding event as pictured at the bottom right of Figure 1, a mechanism that we have demonstrated previously can lead to high-affinity capture agents [23, 24]. Since avidity effects can be highly dependent on the density and geometry of ligands on a surface, this raises the issue of whether the ubiquitin binding peptide or similar ligands will act as a high-affinity capture agent on other supports. Studies to probe this point are underway.

Significance

The construction of protein-detecting microarrays will require the isolation of many high-affinity and -specificity protein capture agents. If synthetic molecules, rather than antibodies or nucleic acid aptamers, are to be used in this application, an efficient method for the isolation of suitable molecules will be required. The method reported here appears to be a potential route to these agents, as it provides high-affinity protein capture agents quickly and efficiently. The capped peptoid or peptide libraries described here are straightforward to synthesize and screen using the protocol provided. Furthermore, since the sequence of the hits can be determined directly from a single bead, no encoding of the library is necessary. This approach should be within the capabilities of almost any laboratory interested in the isolation of such compounds. While the ligands obtained are only short oligomers, they are capable of capturing the target protein from a complex mixture even when it is present at nanomolar or even subnanomolar concentrations.

Experimental Procedures

General

All chemicals were purchased from commercial suppliers and used as received unless otherwise noted. The ^{1}H NMR and ^{13}C NMR were recorded on Varian Inova-400 and Mercury-300 magnetic resonance spectrometer respectively and are reported on δ scale using tetramethylsilane as internal reference. TentaGel macrobeads (140–170 μm diameter, 0.51 mmol/g capacity, Rapp Polymere) were used to synthesize library. Resynthesis of hits was performed on Rink Amide MBHA resin (0.66 mmol/g capacity, Nova Biochem). Voyager-DE PRO biospectrometry workstation (Applied Biosystems) was used to record MALDI-TOF MS. HPLC was performed with C18 reverse-phase column (vydac, 5 μM, 4.6 mm i.d. × 250 mm) on Biocad sprint system using 10%–50% B in 20 min followed by 50%–80% B in 5 min at a flow rate of 1 ml/min (solvent A: H_2O + 0.1% TFA; solvent B: CH_3CN + 0.1% TFA). All peptoids were synthesized on 1000 W Whirlpool microwave oven (model MT113SG) at 10% power. The photomicrographs were acquired using Nicon Eclipse TE300 fluorescence microscope equipped with CCD camera, chroma 61002 triple band filter set, and Metamorph software. Isothermal titration calorimetry (ITC) measurements were performed on MicroCal VP-ITC instrument with Origin software. Peptoid library was screened on COPAS SELECT 500 (Union Biometrica, Inc) machine using 488

nm line filter. The sequencing of hits was performed on ABI 476A protein sequencer (Applied Biosystems).

Synthesis of Chalcone

To a stirred solution of 4-Formylphenoxyaceticacid (6 mmol) and 4[1]-chloroacetophenone (6 mmol) in methanol (18 ml) was added an aqueous solution of sodium hydroxide (0.6 ml of a 50% w/v) drop wise over 15 min. After addition was complete the reaction mixture was stirred for 15 hr at room temperature. The solid was filtered, washed with 1 N hydrochloric acid, and dried to yield the chalcone (76%) as a cream color solid. ^{1}H NMR (400 MHz, DMSO, δ ppm): 8.17 (d, 2H, J = 8.6 Hz); 7.86 (d, 2H, J = 8.7 Hz); 7.81 (d, 1H, J = 15.6 Hz); 7.73 (d, 1H, J = 15.6 Hz); 7.63 (d, 2H, J = 8.6 Hz); 7.01 (d, 2H, J = 8.7 Hz); 4.78 (s, 2H). ^{13}C NMR (300 MHz, DMSO, δ ppm): 188.6, 170.5, 160.6, 145.0, 138.5, 137.1, 131.5, 131.0, 129.5, 128.3, 120.1, 115.6, 65.1. MS m/z 317 $[M-H]^{+}$.

Conjugation of Chalcone to Library Beads

150 mg of library beads in 25 ml peptide synthesis reaction vessel were allowed to swell for 2 hr in 6 ml of N, N-dimethylformamide (DMF). The DMF was drained and a solution (1 ml) of HBTU (75.8 mg, 0.2 mmol) in 0.4 M N-methylmorpholine in DMF and 1 ml of BOC-Lys (Fmoc)-OH (0.2 M) in DMF were added. The reaction vessel was stirred at 220 rpm on a shaker for 1.5 hr at room temperature. The beads were washed six times with DMF. To this a 20% solution (2 ml) of piperidine in DMF was added and stirred for 15 min at room temperature. After a thorough wash with DMF, a solution (1 ml) of 1-hydroxybenzotriazole (27.2 mg, 0.2 mmol), in 0.4M N-methylmorpholine in DMSO and chalcone (0.2M) in DMSO were added and incubated for 15 min, whereupon 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (0.2 mmol, 39.4 mg) was added. The reaction was further continued for 15 hr at room temperature. The beads were washed thoroughly with DMSO followed by DCM. After a brief drying, a solution (3 ml) of 95% TFA, 2.5% anisole, 2.5% water (cleavage cocktail) was added and stirred for 2 hr. The solution was drained and library beads were washed with dichloromethane (6 $\times$ 2 ml). The beads were neutralized with 10% diisopropylethylamine (2 ml) in DMF. Finally, beads were washed with dichloromethane (4 $\times$ 2 ml) and dried.

Protein Purification

Ubiquitin-His_6 was purified in our laboratory from the construct pTK378 which has the ubiquitin gene cloned into the pQE-31 parent vector from Qiagen, expressed in the *E.coli* strain BL21-RIL. The cells were monitored for their growth at OD_{600} till 0.8, wherein they were induced with 1 mM IPTG. After further growth at 37°C for 3 hr, the cells were harvested, sonicated, and centrifuged at 22,000 rpm. The cleared lysate was then incubated with Ni-NTA beads, thoroughly equilibrated with sonication buffer (300 mM NaCl, 50 mM NaH_2PO_4 [pH 8.0] + 0.1% Tween 20), at 4°C for 1 hr. The beads were then extensively rinsed with 10–12 vol of wash buffer (500 mM NaCl + 50 mM NaH_2PO_4 [pH 8.0] + 25 mM Imidazole + 0.1% Tween 20) and packed into a column. Ubiquitin-His_6 bound to the beads was eluted with 400 mM Imidazole, fractions collected and analyzed on a 12% SDS-PAGE. The pure fractions were pooled and dialyzed against PBS + 10% glycerol. The protein concentrations were estimated using Coomassie Plus Protein Assay Reagent Kit with BSA employed for obtaining the standard curve. MBP-Mdm2 was purified in the laboratory as described previously [18].

Labeling of Protein with Fluorescein/Texas Red

To a protein solution (1 ml of 2.6 mg/ml) in 0.2 M $NaHCO_3$ buffer (pH 8.3) was added a solution of 5-(and-6)-carboxyfluorescein/Texas Red succinimidyl ester (4 μl of 50 mg/ml) in DMF. This mixture was incubated for 1 hr at room temperature. The reaction was terminated by adding 1.5 M hydroxylamine. The labeled protein was separated from free dye using desalting column. Degree of labeling (D.O.L) was calculated by measuring absorbance of dye-conjugated protein at 280 nm and 494/594 nm for Fluorescein and Texas red, respectively.

Library Presorting

150 mg of library beads ($\sim$78,000) were swollen for 2 hr in DMF. After washing with 1$\times$ TBST (6 $\times$ 2 ml), beads were transferred onto COPAS machine. The library beads were sorted using 488 nm line filter at the flow rate of 10 beads/s. 66,862 (85.7%) beads were collected for screening and remaining beads (14.3%) with high intrinsic fluorescence were discarded.

Library Screening

Presorted library beads (66,862 beads) were first swollen in DMF for 2 hr and washed with 1$\times$ TBST (6 $\times$ 4 ml). The beads were then equilibrated with 1$\times$ TBST for 1 hr. After a brief washing with 1$\times$ TBST, *E. coli* lysate was added and beads were incubated with tumbling for 1 hr at room temperature. The *E. coli* lysate was removed and library beads were incubated in 3 ml of fluorescein labeled MBP-Mdm2 (500 nM) in TBST (1 M NaCl + 1% Tween 20) supplemented with 10,000-fold excess of *E. coli* lysate for 2 hr at room temperature. The beads were washed with 1$\times$ TBST (6 $\times$ 2 ml) and screened using a COPAS SELECT 500 machine. The bright beads collected by COPAS machine were washed with 1% SDS for 30 min and sequenced. The protocol for screening and labeling protein with Texas red for ubiquitin-His_6 are as described elsewhere [19].

Isothermal Calorimetry

Isothermal calorimetry (ITC) experiments were done at 20°C using MicroCal VP-ITC with a 250 μl injection syringe at 310 rpm stirring. Proteins (20–40 μM depending on dissociation constant) were dialyzed against Tris buffer (20 mM Tris-HCl + 200 mM NaCl + 1 mM EDTA, pH 7.4) and degassed for 3 min before loading into the calorimeter cell. The peptoid was dissolved in dialysis buffer to the required concentration and degassed for 3 min before use. A typical binding experiment involved 8 μl injections of peptoid ligand (400 μM) solution into the ITC cell containing 1.43 ml of protein (20 μM). ITC experiments for the ubiquitin lead and the 15-mer were performed at a protein (ubiquitin-His_6) concentration of 40 μM with 12 μl injection vol of 15-fold excess of the respective peptide ligands in phosphate-buffered saline. To correct for heats of dilution, control experiments were performed by injection of ligand into buffer and buffer into protein under same conditions. The ITC binding data were then fit to a single-site binding model using the standard MicroCal ORIGIN software to determine binding constants.

Isolation of the Ubiquitin Binding Lead Peptide

A 7-mer peptide library was synthesized on TentaGel beads using Asp, Phe, Gly, His, Leu, Lys, Arg, Ser, Trp, and Tyr as monomers. 1,000,000 beads from this library were saturated with *E. coli* lysate for 4 hr at 4°C, subsequently they were washed and incubated with 100 nM Texas red-labeled ubiquitin-His_6 in the presence of 1000-fold excess of *E.coli* proteins supplemented with 1 M NaCl and 1% Tween 20 for 20 min at RT. The beads were thoroughly washed with 1$\times$ TBST (6 $\times$ 10 ml) and visualized under the microscope for potential binding of ubiquitin-His_6 on beads. Eleven bright beads were manually isolated from the screen and each one of them given a hot 1% SDS wash prior to Edman sequencing. Of the 11 hits, the one with the sequence RWDRYYF emerged as the best binder and since is referred to as the ubiquitin lead peptide.

Ubiquitin screening involved synthesis of a capped library wherein the above lead peptide was synthesized on TentaGel beads, subsequently capped by a 7-mer peptide library (monomers used were Ala, Glu, Gly, His, Lys, Leu, Asn, Arg, Thr, and Trp) spaced by a single Ser residue. For screening used 250,000 beads, which were preincubated with *E. coli* lysate at 4°C, 4 hr, and then rinsed with 1$\times$ TBST. Prior to exposing the bead library to Texas red-labeled ubiquitin-His_6, the protein was saturated with 100-fold excess of the ubiquitin lead peptide at 4°C, 30 min. Following which, the library was incubated with 200 nM of the above ubiquitin-His_6 in the presence of 10,000-fold excess of *E. coli* proteins in addition to 0.5 M NaCl and 0.5% Tween 20 at RT, 20 min. The beads were extensively washed with 1$\times$ TBST (6 $\times$ 10 ml) and visualized under the microscope for bright "hit" beads.

Bead "Pulldown" Assays

TentaGel beads displaying chalcone-peptoid, peptoid, and chalcone were preblocked with *E. coli* lysate at 4°C. After 1 hr the lysate was removed and beads were incubated with labeled protein (see Figure 4 for concentrations) for 2 hr at 4°C. The beads were then washed with 1× TBST (1 ml × 6) and photographed using Nicon Eclipse TE300 fluorescence microscope equipped with CCD camera.

On-Resin Sandwich Assay

TentaGel resin displaying the 15-mer, random, or no peptide were incubated with 100, 20, 4 nM, and 800 pM ubiquitin-His_6 in the presence of 1000-fold excess *E. coli* proteins for 2 hr, 4°C. Subsequently the beads were thoroughly rinsed with 1× PBS and incubated with 200 nM Texas red-labeled anti-ubiquitin antibody for 2 hr, RT. The resin was then washed extensively, visualized, and photographed.

Pull-Down Experiment

Tenta Gel beads (5 mg) displaying the chalcone-peptoid were incubated with 0.1% MBP-Mdm2 (1 μM) in the presence of HeLa nuclear extract in a total volume of 5 ml at RT for 4 hr. The beads were washed thrice with TBST (20 mM Tris buffered saline + 0.1% Tween 20). 10 μl of 2× SDS-PAGE loading dye was then added directly to these beads and boiled for 10 min. The entire supernatant was loaded onto a 12% denaturing polyacrylamide gel and analyzed by Western blot using anti-MBP antibody.

ELISA on Beads

2 mg resin of each type displaying 15-mer, the 7-mer lead peptide, or a control peptide was soaked overnight in TBST and subsequently blocked with 1 mg/ml *E. coli* lysate for 2 hr at room temperature. Ubiquitin was added (2.5, 5, 10, 20, or 100 nM) and incubated overnight at 4°C. The resin was washed extensively with TBST after which affinity-purified anti-ubiquitin antibody was added at a 1:1000 dilution. After a 1 hr incubation at room temperature, the beads were washed extensively with TBST. The secondary antibody (goat anti-mouse IgG conjugated to horse radish peroxidase [HRP]) was then added and the solution was incubated for 1 hr at room temperature. After washing extensively, 100 μl of freshly prepared HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) in 10 mM citrate phosphate buffer (pH 6.2) was added along with 30% hydrogen peroxide. The color-developing reaction was allowed to proceed for 10 min, after which it was quenched with 100 μl of 500 mM sulfuric acid. The absorbance at 450 nm was recorded.

Printing of Glass Slides

Peptoid (random: NH_2-Nall-Nbsa-Npip-Nbsa-Nlys-Cys, random-fluorescein: NH_2-fluor-Nall-Nbsa-Npip-Nbsa-Nlys-Cys, and chalcone-peptoid: NH_2-Lys (chalcone)-Nlys-Npip-Nlys-Nser-Nlys-Nlys-Nlys-Nlys-Npip-Npip-Cys) solutions (5 mM) were printed on to maleimide functionalized glass slides using GENETIX QARRAY lite (GENETIX) microarrayer. After printing, the glass slides were allowed to stand for 15 hr at room temperature and washed thoroughly with DMF, 1× TBST, and deionized water and dried.

Detection of Protein Binding on Glass Slides

The slide was equilibrated with 1× TBST for 15 min and blocked with *E. coli* lysate at 4°C. After 1 hr, fluorescein-labeled MBP-Mdm2 (100 nM) with 100-fold excess of *E. coli* lysate in 1× TBST was added and incubated for 2 hr at 4°C. The slide was washed four times with 1× TBST and dried by centrifugation. The slide was then scanned using ScanArray ExpressHT Microarray scanner (Perkin Elmer precisely) at 10 μm resolution using Blue 488 nm Excitation Laser.

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