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Optimization of the Magnetic Recovery of Hits from One-Bead−One-Compound Library Screens

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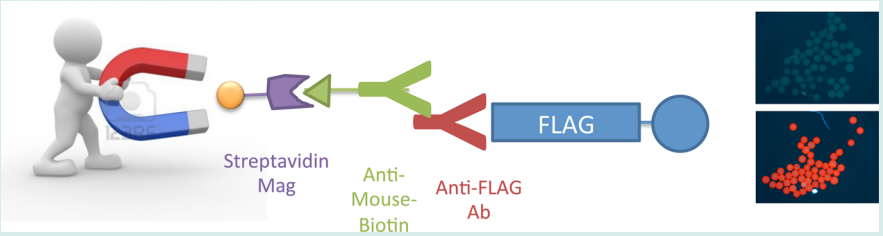

ABSTRACT: On-bead screening of one-bead−one-compound (OBOC) libraries is a useful procedure for the identification of protein ligands. An important aspect of this experiment is the method by which beads that bind the target protein are separated from those that do not. Ideally, such a method would be rapid and convenient and result in the isolation of 100% of the "hits" with no false positives (beads that display compounds that are not good ligands for the target). We introduced a technique in which beads that have bound a labeled target protein can be magnetized, thus allowing their convenient isolation (Astle et al.et al. *Chem. Biol.* **2010** *17*, 38−45). However, recent work in our laboratory and others has shown that magnetic hit recovery can result in the isolation of large numbers of false positives and has also suggested that many true hit beads are missed. In this study, we employ a well-defined model system to examine the efficiency of various magnetic hit isolation protocols. We show that the choice of reagents and the particular operations employed are critical for optimal results.

KEYWORDS: *one-bead−one-compound library, screening, peptides, peptoids, magnetic recovery, antibody*

INTRODUCTION

One-bead−one-compound (OBOC) libraries are readily prepared by split and pool synthesis. While this technology was originally developed for the creation of combinatorial peptide libraries,[1] it has been applied to create large collections of a variety of nonpeptide oligomers[2] and, when various encoding strategies are employed, nonoligomeric small molecules.[3] When these libraries are created on appropriate resins that have a hydrophilic surface layer to discourage nonspecific protein binding, such as TentaGel, they can be used directly in binding screens for the identification of ligands for proteins or other biomolecules.[2b] In such experiments, the bead library is incubated with a labeled target protein in the presence of unlabeled competitor proteins. Beads that display the desired ligands are then separated from those that do not. Finally, the compound is released from the bead and its structure is determined, either directly or indirectly, usually by tandem mass spectrometry.[3b,4]

While this protocol seems simple in theory, in fact there are many technical difficulties that make the rapid and reliable isolation of "hits" somewhat problematic. Of particular relevance to this study is the method that is employed to segregate hits from nonhits. All such protocols rely on the presence of a tag incorporated into the target protein through chemical modification (such as biotinylation) or genetic manipulation (such as expression of an epitope-tagged protein) to attract a second protein that carries a label of some sort. Various schemes have been reported, all of which have advantages and disadvantages.[5] We reported the use of streptavidin-coated quantum dots to detect the binding of biotinylated proteins to compounds displayed on TentaGel beads.[6] Quantum dot fluorescence is easily seen on TentaGel beads, despite the high level of intrinsic autofluorescence of the beads. This is due to the very large Stokes shift of the quantum dots, whereas the Stokes shift of the bead autofluorescence is modest. Another popular technique is to employ commercially available streptavidin-horseradish peroxidase (SA-HRP) as the secondary agent.[5,7] When a soluble dye is added in the presence of a terminal oxidant, the densely colored, oxidized dye product is deposited locally on beads that have attracted the SA-HRP secondary agent.[8] In both cases, the hit beads must then be picked manually using a micropipette under a low power microscope. This is because the beads used commonly in such screens (75−160 μm in diameter) are too large to pass through typical commercial flow sorters, though less commonly available embryo sorters, such as the COPAS instrument have been used in this regard.[9] Manual hit collection is fine for relatively small libraries, but becomes extremely tedious for libraries of hundreds of thousands or millions of beads.

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Thus, there has been interest in the development of methods for the bulk separation of hit beads from the rest of the library. The most common technique is to use a secondary agent that results in magnetization of the hit-displaying beads, for example SA-coated, paramagnetic iron oxide particles. When the bead population is passed over a powerful magnet, in theory those beads that have attracted the target protein, and thus the magnetic particles, will easily be separated from those that have not.[10] This is far more convenient than manual segregation of fluorescent or dye-colored beads from the remainder of the population. While this procedure works, results from screens done in our laboratory[11] and others[7] have indicated that it is far from optimal. For example, we have rarely isolated all of the copies of a given hit in magnet-based screens of libraries containing several copies of each compound, suggesting inefficient retention of hit beads. In addition, it is clear that significant numbers of false positives are pulled along with the true hits in magnetic isolation procedures. In some cases, these can represent the vast majority of the beads isolated. They are comprised of true negatives that appear to be dragged along with the true hits in the magnetic isolation procedure, as well as very weak, low quality ligands that happen to be displayed with unusually high density on the surface of a particular bead (the bead population is heterogeneous with respect to bead capacity).[12] The problem with missing true hits is obvious. The danger of isolating large numbers of false positive beads lies in the huge amount of time and resources that can be wasted on the resynthesis and attempted validation of what prove to be useless compounds. This is especially a problem in screens against serum samples aimed at the identification of ligands for antibodies of potential diagnostic significance,[13] where one does not have a single, known, abundant target that would allow the use of certain high-throughput validation protocols that do not require compound resynthesis.[10,14]

In a previous paper, we showed that the use of redundant libraries facilitates discrimination of high quality hits from very poor ligands in OBOC screens.[12] The latter are dependent on being displayed on rare beads in the heterogeneous population with unusually high density, the former are not. Thus, the odds of a poor ligand being found on more than one of the rare high density beads are low. Clearly, if one intends to rely on the isolation of multiple copies of a hit as a confirmation of its quality, it is important that hit bead capture is highly efficient. In this study we focused on optimizing the various steps of the magnetic harvesting protocol to maximize the retention of high quality ligands while minimizing the isolation of false positives. We chose to use the well-characterized Anti-FLAG antibody and FLAG peptide epitope displayed on TentaGel beads as a model system to optimize the magnetic recovery. The optimization involves the choice of TentaGel bead size, secondary antibodies and magnetic particles, as well as the magnetic isolation technique. We found that both the hit recovery and false positive isolation vary substantially with each of these variables, with the choice of magnetic particles being the most critical.

Once an optimized magnetic recovery protocol was established employing the high affinity model system, we tested its efficiency on a lower affinity antibody-ligand interaction that would be more representative of a typical primary hit from a library screen. Not surprisingly, we found that the hit recovery declines with a lower affinity ligand, yet with the application of redundant libraries it should be possible to isolate multiple copies of true hits quickly and efficiently. To further test this theory we used the Anti-FLAG antibody to screen a random redundant peptoid library magnetically and succeeded in isolating many redundant hits with modest affinity. The establishment of this optimized magnetic screening protocol should facilitate more rapid progress in OBOC screening projects.

RESULTS

Illustration of the Utility of the Anti-FLAG and FLAG Peptide Model System. To examine the technical issues associated with OBOC library screening, we employed the FLAG peptide (NH_2-DYKDDDDK) and monoclonal mouse anti-FLAG antibody, a well-characterized system. The FLAG peptide was synthesized on 75 μm TentaGel beads following a linker and, as a control, 75 μm TentaGel beads displaying the linker were capped with acetic anhydride. Experiments were designed to test the efficiency with which a known number of FLAG peptide-displaying beads could be recovered from a mock library of TentaGel beads capped with the acetyl group. To establish a "gold standard" for the rapid identification of beads displaying FLAG peptide and those that do not, we employed anti-mouse IgG secondary antibodies conjugated to red quantum dots. As shown in Figure 1, when anti-FLAG

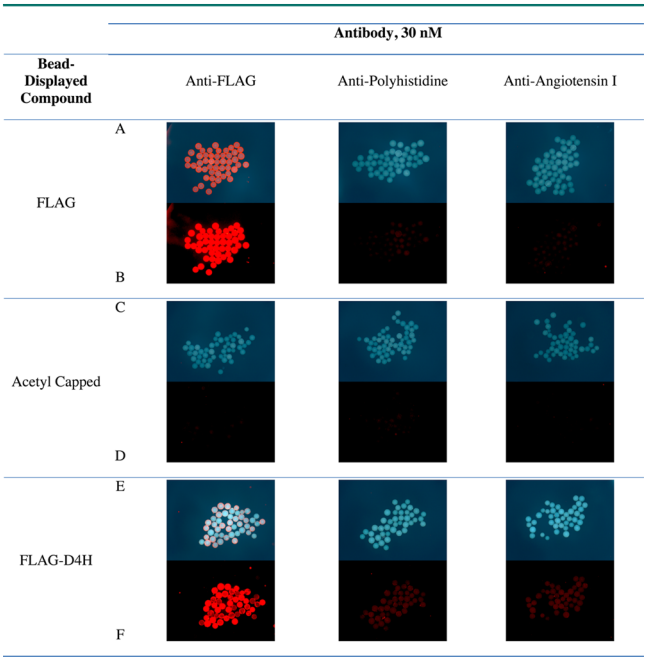

Figure 1. Photomicrographs of 75 μm TentaGel beads displaying the FLAG peptide, an acetyl group, or the FLAG-D4H peptide following incubation with anti-FLAG, anti-PolyHistidine, or anti-angiotensin I antibody at 30 nM and staining with Anti-Mouse IgG 655 Quantum Dots. Rows A, C, and E show the beads viewed through the DAPI filter and rows B, D, and R show the beads viewed through a 655 nm filter.

antibody (30 nM) was incubated with FLAG peptide-displaying TentaGel beads, followed by washing and staining with the secondary antibody-conjugated quantum dots, the beads acquired an intense red fluorescence, as expected. When the same procedure was carried out using acetyl-capped TentaGel beads, no such fluorescence was observed. Likewise, no halo was seen upon incubation of the FLAG-displaying beads with anti-PolyHistidine antibody or an anti-Angtiotensin I antibody, demonstrating the selectivity of the interaction. Figure 2 shows

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ELISA data that confirm high affinity binding of the FLAG peptide to anti-FLAG antibody, but not to the control antibodies.

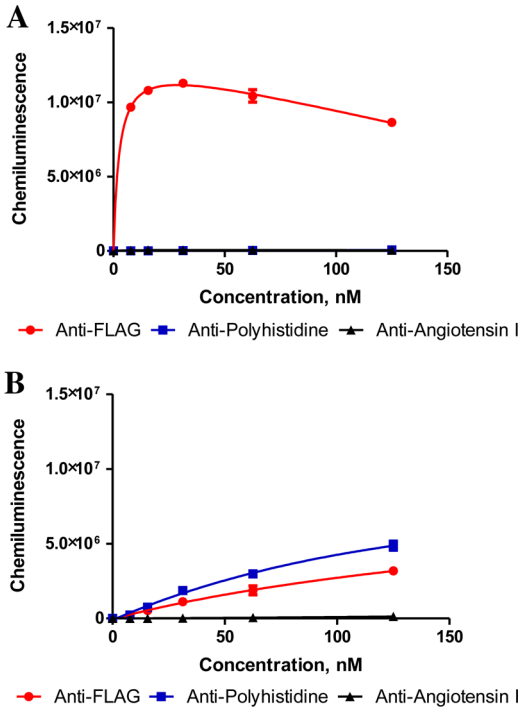

Figure 2. ELISA binding results for FLAG and FLAG-D4H peptides. Each peptide was synthesized with a C-terminal cysteine and coated onto a maleimide-activated ELISA plate. A titration was performed up to 125 nM for the anti-FLAG, anti-PolyHistidine, or anti-angiotensin antibodies and the chemiluminescence from a secondary antibody was measured. Panel A shows the binding of the three antibodies to the FLAG peptide, and panel B shows the binding of the three antibodies to FLAG-D4H.

Optimization of Magnetic Recovery with FLAG Peptide Beads. We carried out experiments in which 50 FLAG peptide-displaying beads were doped into about 50 000 acetyl-capped beads, to simulate a library screening experiment with a hit rate of 0.1%. Various protocols to retrieve these 50 beads using magnetic pullout were examined. First, we evaluated the utility of two different commercial sources of paramagnetic iron oxide particles, Magnabind (goat anti-mouse IgG-conjugated iron oxide particles, Thermo Scientific) and Dynabeads (sheep anti-mouse IgG conjugated to iron oxide, Life Technologies). In each case, the TentaGel beads were incubated with the anti-FLAG antibody (30 nM) in 100% PBS Starting Block for 2 h. The beads were pelleted by brief centrifugation then washed three times with TBST. After resuspension, the magnetic beads were added and allowed to incubate with the TentaGel beads for 2 h. Finally, the Eppendorf tube was placed in a magnetic collection stand that places powerful magnets at the sides of the tubes. Nonmagnetized beads sink to the bottom. These were carefully removed using a pipet. More buffer was then added to the tube, which was removed from the rack and agitated to resuspend the TentaGel beads. It was then placed back in the rack and the nonmagnetized beads were again withdrawn. This procedure was repeated until no more beads appeared at the bottom of the tube. Each experiment was done twice. This is the protocol that one would use to be as careful as possible about eliminating false positives from the final population in a real screening experiment.

Only one and three of the 50 FLAG peptide-displaying beads were isolated in the final magnetized population in the two experiments using the Magnabind beads (Table 1). Most of the beads were acetylated TentaGel particles (28 and 32 beads, respectively, in the two runs) as revealed by quantum dot staining of this population (Table 1). In the two Dynabead experiments, 15 and 19 (30−38%) of the 50 FLAG peptide-displaying beads were recovered along with 45 and 64 acetyl-capped beads (Table 1). Thus, the Dynabeads outperform the Magnabind particles by a wide margin, though in both cases recovery was far from perfect.

We next examined if a different kind of Dynabead-based system might improve recovery. In this case, we employed a biotinylated secondary antibody and streptavidin (SA)-coated Dynabeads. Since there are 3−6 biotin molecules per secondary antibody, this might allow the recruitment of more magnetized particles to the FLAG-displaying TentaGel beads, perhaps improving recovery. Indeed, as shown in Table 1, repeating the same experiment described above resulted in the retention of 44 and 43 of the 50 FLAG peptide-displaying beads in the final magnetized population for the two runs, in addition to 81 and 95 acetyl-capped beads. When the control antibodies (anti-PolyHistidine and anti-Angtiotensin I were used in place of anti-FLAG antibody, 0 and 1 FLAG peptide-displaying beads were pulled out, respectively, along with numerous acetyl-capped beads (Table 1). Clearly, this approach is superior to

Table 1. Magnetic Recovery Statistics using Bracket Magnet

Step 1	Anti-FLAG		Anti-FLAG		Anti-FLAG		Anti-Polyhistidine	Anti-Angiotensin I
Step 2	Anti-Mouse IgG Magnabind		Anti-Mouse IgG Dynabead		Anti-Mouse IgG Biotin		Anti-Mouse IgG Biotin	Anti-Mouse IgG Biotin
Step 3	N/A		N/A		Streptavidin Dynabead		Streptavidin Dynabead	Streptavidin Dynabead
# FLAG beads recovered	1	3	15	19	44	43	0	1
% FLAG recovery	2%	6%	30%	38%	88%	86%	0%	2%
# Capped beads recovered	28	32	45	64	81	95	165	285
Fluorescence Micrograph (DAPI Filter)								

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Table 2. Magnetic Recovery Statistics using Fishing Magnet

Step 1	Anti-FLAG		Anti-FLAG		Anti-FLAG		Anti-Polyhistidine	Anti-Angiotensin I
Step 2	Anti-Mouse IgG Magnabind		Anti-Mouse IgG Dynabead		Anti-Mouse IgG Biotin		Anti-Mouse IgG Biotin	Anti-Mouse IgG Biotin
Step 3	N/A		N/A		Streptavidin Dynabead		Streptavidin Dynabead	Streptavidin Dynabead
# FLAG beads recovered	0	0	13	4	44	50	0	0
% FLAG recovery	0%	0%	26%	8%	88%	100%	0%	0%
# Capped beads recovered	92	49	46	19	14	79	27	56
Fluorescence Micrograph (DAPI Filter)								

that employing Dynabeads conjugated to the secondary antibody directly.

We also examined an alternative type of magnetic pullout protocol that substituted cubic magnets for the magnetic stand. In this "fishing pole" procedure, the magnet is wrapped in a piece of Parafilm and a string is then attached to the film. The magnet is then lowered into an agitated solution of the beads and allowed to incubate for 10 min. The magnet is then removed from the tube, the film is removed and the beads that come along are collected in a tube. As shown in Table 2, the results were fairly similar to those obtained with the magnetic stand protocol, with the exception that fewer numbers of acetyl-capped beads were retained when the SA-Dynabeads and biotinylated secondary antibodies were employed.

Optimization of Magnetic Recovery with FLAG-D4H Beads. The affinity of the anti-FLAG antibody for the FLAG peptide is extremely high ($K_D \approx 1.5$ nM; Figure 3). It is highly unlikely that a primary hit from a naïve library would evince this type of affinity for a target antibody. Therefore, we were interested in further testing the SA-Dynabead-based protocol using a lower affinity antigen–antibody complex that would better model a screening hit. We found that FLAG-D4H binds to anti-FLAG antibody with, at best, a K_D of approximately 130 nM (Figure 3). Note that the ELISA data in Figure 3 reflect significant avidity effects and the intrinsic binding affinity is likely to be at least 10-fold worse than this measured value. Thus, the FLAG-D4H peptide-anti-FLAG antibody complex is a reasonable model for a typical hit in a true screening experiment.

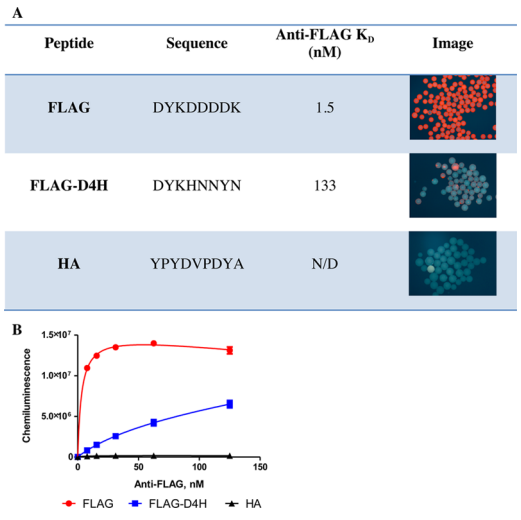

Figure 3. (A) 75 μm beads displaying the three different peptides were incubated with 30 nM of the anti-FLAG antibody followed by anti-mouse IgG 655 quantum dots and viewed through the DAPI filter on a fluorescence microscope to visualize the intensity of red signal from each type of bead. (B) The native FLAG peptide, the FLAG-D4H peptide, and a negative control HA peptide were synthesized with a cysteine residue at the C terminus and coated onto a maleimide-activated ELISA plate. The anti-FLAG antibody was titrated up to 125 nM to determine the K_D value by ELISA.

Table 3 shows the results of repeating the same experiments described above with 50 beads displaying this lower affinity FLAG peptide analogue doped into about 50 000 acetyl-capped beads. Using the collection stand, 75–80% of the FLAG-D4H-displaying TentaGel beads were recovered along with about twice as many acetyl-capped beads. Substitution of anti-Angtiotensin I antibody for anti-FLAG antibody resulted in no FLAG-D4H-displaying TentaGel beads being recovered, as expected. The cubic magnet-based fishing pole method provided lower recovery efficiencies (14 and 16 of the 50 FLAG-D4H-displaying TentaGel beads in the two runs). Nearly identical results were obtained when the anti-FLAG antibody concentration was increased from 30 to 100 nM (Table 4) with the fishing pole method showing 3-fold poorer recovery efficiency.

Magnetic Isolation of Anti-FLAG Antibody-Binding Peptoids from a Redundant Library. Next, we applied the magnetic isolation protocol to a real screen utilizing the redundant peptoid library shown in Figure 4. The library had a theoretical diversity of 292 032 compounds and 1.5 million beads were screened. This means that each compound in the library should be represented on approximately 5 different beads. Additionally, five beads displaying the FLAG-D4H peptide were doped into the library prior to screening to determine how many we could recover during a real screen.

The screen employed serum from normal mice to denude the library of any beads displaying compounds that bind to antibodies present in normal mouse serum. Beads that were isolated from the mouse serum prescreen were removed from the population magnetically. After the proteins were stripped from the denuded library and re-equilibrating the library in buffer, we doped the anti-FLAG antibody into the mouse serum at a concentration of 200 nM and isolated the hits magnetically as described. Approximately, 3000 beads were isolated as hits from the magnetic screen. The hit beads were stripped, re-

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Table 3. Magnetic Recovery Statistics of FLAG-D4H at 30 nM Anti-FLAG Antibody

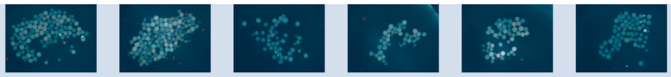
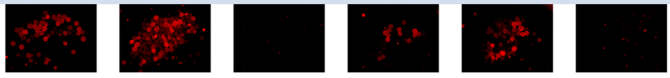
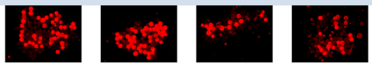
Step 1	Anti-FLAG		Anti-Angiotensin I	Anti-FLAG		Anti-Angiotensin I
Step 2	Anti-Mouse IgG Biotin		Anti-Mouse IgG Biotin	Anti-Mouse IgG Biotin		Anti-Mouse IgG Biotin
Step 3	Streptavidin Dynabead		Streptavidin Dynabead	Streptavidin Dynabead		Streptavidin Dynabead
Recovery Method	Collection Stand		Collection Stand	Cubic Fishing Magnet		Cubic Fishing Magnet
# FLAG-D4H beads recovered	44	37	0	14	16	0
% FLAG-D4H recovery	88%	74%	0%	28%	32%	0
# Capped beads recovered	72	94	48	26	36	66
Fluorescence Micrograph (DAPI Filter)						
Fluorescence Micrograph (655nm Filter)						

Table 4. Magnetic Recovery Statistics of FLAG-D4H at 100 nM Anti-FLAG Antibody

Step 1	Anti-FLAG		Anti-FLAG	
Step 2	Anti-Mouse IgG Biotin		Anti-Mouse IgG Biotin	
Step 3	Streptavidin Dynabead		Streptavidin Dynabead	
Recovery Method	Collection Stand		Cubic Fishing Magnet	
# FLAG-D4H beads recovered	42	43	15	19
% FLAG-D4H recovery	88%	86%	30%	38%
# Capped beads recovered	122	52	80	67
Fluorescence Micrograph (DAPI Filter)				
Fluorescence Micrograph (655nm Filter)				

equilibrated in buffer, and then re-exposed to the anti-FLAG antibody doped into mouse serum. After addition of an anti-mouse secondary antibody conjugated to a red quantum dot (Qdot655) the beads were examined under a low power fluorescence microscope. Approximately 100 beads produced a red halo under the DAPI filter of the fluorescent microscope (Figure 4C). These beads were picked, stripped of antibody, then re-exposed to normal mouse serum and Qdot655 secondary antibody (Figure 4D). Less than 10 beads produced a faint red halo and were removed from the population. After cleavage of the compounds from each bead using CNBr, the compounds were sequenced using MALDI-TOF MS/MS (Table 5).

Many highly redundant hits were isolated from the library screen. Furthermore, three of the five FLAG-D4H beads were isolated as hits during the screen. Hits that were represented in three or more copies are listed in Table 5. All the hits shared significant sequence homology. Each contained Nasp, Nlys, and Ntyr in that order at positions R1−R3. Not surprisingly, the residues of the FLAG peptide that are absolutely critical for binding the anti-FLAG antibody include Asp, Tyr, and Lys. In fact, a crystal structure of the antibody has shown that only the first four residues (DYKD) of the peptide are sufficient for binding.[15] Figure 5 shows the hit and the native FLAG peptide.

Characterization of the Binding Properties of the Repeat Hits. To determine the binding affinities of the redundant hits, they were resynthesized with a cysteine-PEG linker at the C-terminus. This linker facilitated the covalent linkage of the hit compounds to Pacific Blue and Pacific Orange encoded, 10 μm TentaGel microspheres for multiplexed analysis on a flow cytometer.[16] Binding to increasing amounts of anti-FLAG antibody or to controls such as normal mouse serum or anti-Angiotensin I antibody was measured using a multiplex flow cytometry assay as described.[16] All of the repeat hits exhibited strong binding to Anti-FLAG antibody, with K_d values between 75 and 150 nM. The binding curve for the best hit, **9**, is shown in Figure 5. The data indicate a dissociation constant of 78 nM. All the compounds exhibited a much lower affinity for normal mouse serum or the control antibody anti-angiotensin I (Figure 5). These data indicate that the repeat hits are high affinity, selective ligands for anti-FLAG antibody.

The structural similarity of the screening hits to the native FLAG peptide, along with the much lower affinity for different antibodies, strongly suggests that they bind to the antigen binding pocket of the Anti-FLAG antibody. To confirm that they bind to the same pocket, a competition experiment with soluble competitor molecules was performed. If the soluble molecule binds to the same site as the bead-displayed molecule, then the mean fluorescence intensity should decrease as the concentration of the competitor increases. The Anti-FLAG Antibody was preincubated with soluble FLAG peptide, soluble FLAG-D4H, hit 9, or a negative control peptoid prior to incubation with flow cytometry beads displaying the FLAG peptide. In the absence of any soluble competitor the mean fluorescence intensity is very high (Figure 5D). As expected, competition with soluble FLAG peptide abolishes the binding to the FLAG peptide displaying microspheres. Preincubation with hit 9 or FLAG-D4H decreases the binding by approximately 80%, while the control peptoid has no effect on the binding of anti-FLAG antibody to FLAG displaying microspheres. These results confirm that the screening hit binds to the same binding pocket as the FLAG peptide.

Determination of the Detection Limit of Magnetic Isolation. After optimizing the magnetic isolation and performing a successful screen, we are confident that we can isolate hits from a redundant library assuming that there are

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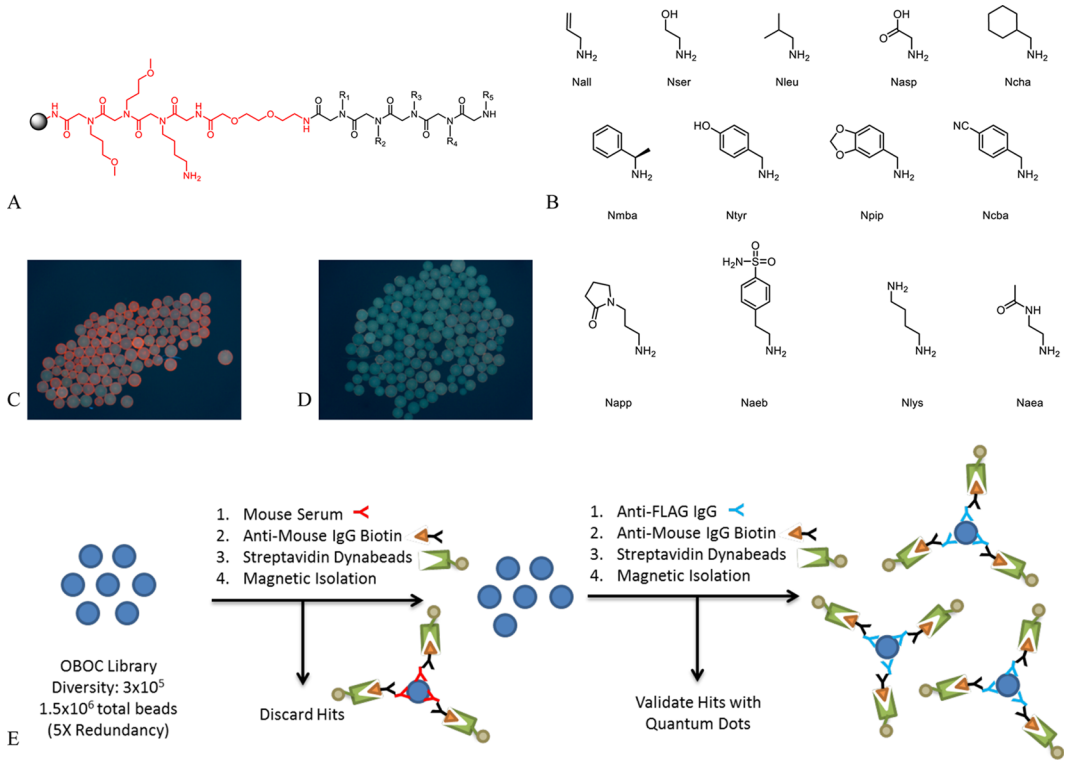

Figure 4. Peptoid library design and anti-FLAG IgG screening schematic. (A) Peptoid library design depicting the invariant region (red) and the diversity region (black). (B) Primary amines used at the diversity positions (R1−R5) of the library. Nlys was only included in positions R2 and R5. (E) Screening was performed on six copies of a 300 000 bead library by first removing hits to IgG in mouse serum. The remainder of the library was incubated with anti-FLAG IgG monoclonal antibody. Hits were isolated magnetically. The hits to anti-FLAG IgG were stripped in guanidine/BME and validated using a red quantum dot labeled anti-mouse IgG secondary antibody. (C) Shows photomicrograph of anti-FLAG magnetic hits labeled with anti-FLAG antibody and red Qdots. (D) Shows photomicrograph of the beads from panel C labeled with normal mouse serum and red Qdots.

Table 5. Sequences of Hit Compounds from Anti-FLAG Screen

hit	R1	R2	R3	R4	R5	no. copies
1	Nasp	Nlys	Ntyr	Nall	Naea	4
2	Nasp	Nlys	Ntyr	Ncba	Nser	4
3	Nasp	Nlys	Ntyr	Naea	Naea	4
4	Nasp	Nlys	Ntyr	Ntyr	Naea	3
5	Nasp	Nlys	Ntyr	Nall	Naeb	3
6	Nasp	Nlys	Ntyr	Ntyr	Naeb	4
7	Nasp	Nlys	Ntyr	Naeb	Naeb	3
8	Nasp	Nlys	Ntyr	Nser	Ntyr	3
9	Nasp	Nlys	Ntyr	Nall	Nasp	4
FLAG-D4H						3

sufficient amounts of target antibody present in our screening solution and that the library of interest contains molecules capable of binding to the target antibody. One major application of this technology is to isolate ligands to disease-specific antibodies in blood (antigen surrogates) for diagnostic purposes.[13a] Unfortunately, the relative concentration of these disease-linked antibodies, or even the identity of these antibodies, is generally unknown. We used our model screening system to test the detection limit of magnetic isolation. We employed normal mouse serum at 100 μg/mL and doped anti-FLAG antibody into the serum at known concentrations. We started at a very high concentration such that the anti-FLAG antibody represented 60% of the total IgG present in the mixture and titrated down to an anti-FLAG antibody concentration representing only 0.15% of the total IgG present (absolute concentrations from 200 to 0.2 nM). We used the model screening protocol where 50 FLAG peptide-displaying beads were doped into 50 000-capped beads and isolated hits at each dilution of Anti-FLAG antibody magnetically. The hit beads were stripped and restained with Qdots for visually counting under the fluorescent microscope.

We were able to isolate greater than 90% of the FLAG-peptide beads at all the Anti-FLAG concentrations we tested and also recovered between 60 and 160 capped beads in each pullout (Table 6). These data are very promising but, as mentioned previously, we are unlikely to recover such a high affinity ligand in a typical screen. Nevertheless, if we are lucky enough to have a low nanomolar ligand in our library, we should have no trouble isolating it with even trace amounts of target antibody.

We repeated the experiment with FLAG-D4H peptide displaying beads and the results were quite different. With the lower affinity FLAG-D4H the percent recovery dropped off sharply with decreasing anti-FLAG antibody concentrations. No beads were recovered at the lowest concentration (Table 6) and to achieve a percent recovery greater than 50%, the anti-FLAG antibody must be present at greater than 10% of the total IgG. The results were slightly better with hit 9 displaying beads, which have a slightly higher affinity for the anti-FLAG antibody. Even at the lowest concentration of anti-FLAG antibody, we were able to recover 30% of the hit 9 displaying beads. However, as the target antibody concentration is decreased many more capped beads are pulled out along with the true hits (Table 6).

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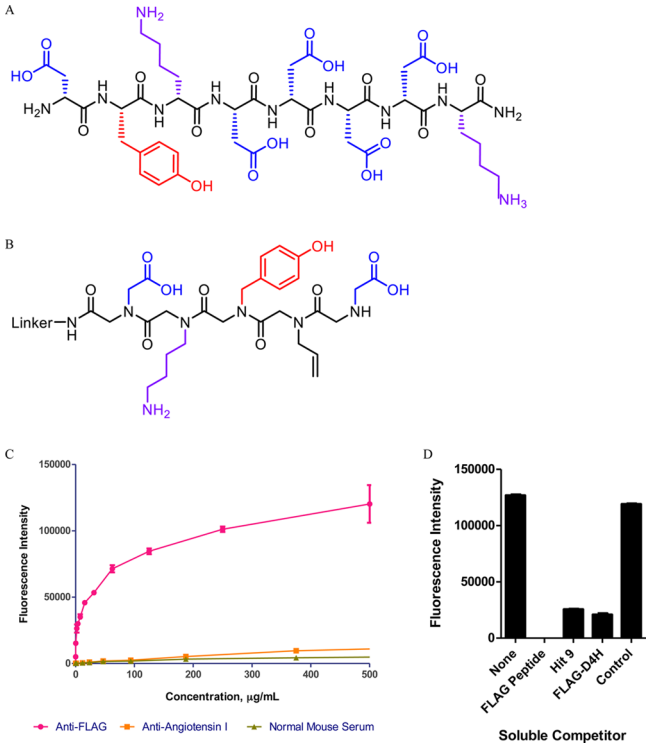

Figure 5. Characterization of Anti-FLAG antibody ligands. Structure of the native FLAG peptide (A) and hit 9 (B) with identical side chain residues highlighted blue, red, and purple. (C) Saturation binding curve for hit 9 against the anti-FLAG antibody, anti-angiotensin I antibody, and normal mouse serum. (D) Competition assay for FLAG-peptide displaying microspheres after preincubation with soluble competitors.

We also examined the magnetic recovery utilizing smaller 30 μm TentaGel beads displaying the FLAG-D4H peptide. One advantage of using smaller beads is that there are approximately ten times more 30 μm beads than 75 μm beads per gram thus facilitating more diverse, yet still redundant libraries. With smaller beads, more compounds can be screened using the same volume of target protein. The downside is that MS/MS-based identification of the compound on the resin can be challenging given the much-reduced amount of compound relative to 75 μm beads. The results in Table 6 show that the 30 μm beads gave higher percent recoveries at lower concentrations of anti-FLAG antibody at the cost of very high numbers of capped beads recovered.

CONCLUSION

In summary, we have validated an optimized protocol for the recovery of a small number of beads displaying antibody-binding ligands from a large collection of those that do not. The procedure recovers 80−90% of the desired hits, along with about twice as many false positives. It is unclear why the acetyl-capped beads are pulled out along with the desired hits, but this happens consistently in all of the experiments we have performed. While this is undesirable, it is not a serious issue. All of the beads collected by the magnet can easily be stripped and stained with secondary antibody-quantum dot particles to distinguish true hits from beads that do not display an antibody-binding ligand, as shown here. Using this magnetic pull-out/strip/quantum dot staining procedure and focusing only on hits isolated more than once from a redundant library, it is possible to almost entirely eliminate false positives or extremely low quality ligands from the more labor-intensive follow-up studies that require hit resynthesis and characterization. This represents a major technical advance that will greatly accelerate the discovery of interesting synthetic molecule-antibody complexes of potential utility in medical diagnostics.

EXPERIMENTAL PROCEDURES

Peptide and Capped Bead Synthesis. TentaGel beads (0.25 g, 75 μm, ~1.14 × 10^6 beads, 0.5 mmol/g, catalog no. HL12902, Rapp-Polymere, Tuebingen, Germany) were swelled in dimethylformamide (DMF) for 2 h before use. DMF was used as the solvent unless otherwise mentioned. For synthesis of the peptides, fmoc amino acids (0.375 mmol) were coupled to beads using HBTU (0.375 mmol), HoBT (0.375 mmol), and *N*,*N*-diisopropylethylamine (DIEA) (0.375 mmol) for 3 h. Fmoc was cleaved by 20% piperidine for 30 min. Beads were washed thoroughly with DMF after each step. The final sequences for the three types of peptides FLAG, FLAG-D4H, and HA were DYKDDDDKM, DYKHNNYNM, and YPYDVPDYAM respectively. Twelve beads of each type were placed in individual wells of a 96 well plate and the peptide was cleaved off the bead using 30 mg/mL CNBr in 5:4:1 acetonitrile/acetic acid/water overnight. The cleavage solution was evaporated and the bead contents were resuspended in 1:1 acetonitrile/water and spotted onto a MALDI plate using α-cinnamic acid matrix. The expected peak mass was observed for each peptide using MALDI mass spectrometry. The capped beads were prepared with 20% acetic anhydride, 10% DIEA in DMF for 2 h. Beads were washed thoroughly with DMF after each step. All beads were washed extensively with water, incubated in water overnight, then washed and equilibrated in TBST prior to use.

The FLAG-D4H peptide on 30 μm beads was prepared as follows. TentaGel beads (0.3 g, 30 μm, 0.25 mmol/g, catalog no. M30352, Rapp-Polymere, Tuebingen, Germany) were swelled in dimethylformamide (DMF) for 2 h before use. DMF was used as the solvent unless stated otherwise. For synthesis of the peptides, fmoc amino acids (8 equiv) were coupled to beads using oxyma (ethyl 2-cyano-2-(hydroxyimino)acetate, 8 equiv) and DIC (*N*,*N*′-Diisopropylcarbodiimide, 8 equiv) for 1 h at RT. Fmoc was deprotected by 20% piperidine via an initial 5 min incubation that was followed with a DMF wash and an additional 20 min deprotection with 20% piperidine. Beads were washed thoroughly with DMF after each amino acid coupling. Beads were placed in individual wells of a 96 well plate and the peptide was cleaved off the bead using 30 mg/mL CNBr in 5:4:1 acetonitrile/acetic acid/water overnight. The cleavage solution was evaporated and the bead contents were resuspended in 1:1 acetonitrile/water and spotted onto a MALDI plate using α-cinnamic acid matrix. The expected peak mass was observed for the peptide using MALDI mass spectrometry. For preparation of the 30 μm acetyl-capped beads, TentaGel beads (0.3 g, 30 μm, 0.25 mmol/g, catalog no. M30352, Rapp-Polymere, Tuebingen, Germany) were swelled in DMF for 1 h at RT, then incubated in 20% acetic anhydride and 10% DIEA in DMF for 2 h at RT. Beads were washed with DMF and a fresh batch of 20% acetic anhydride and 10% DIEA in DMF was added to the beads, which were incubated for another hour at RT. Beads were extensively washed with DMF. All beads were washed extensively with water, incubated in water overnight, then washed and equilibrated in TBST for at

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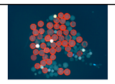
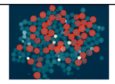
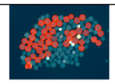
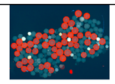
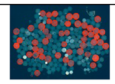
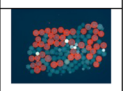
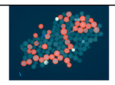
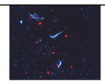
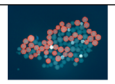
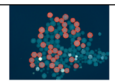
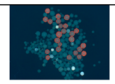
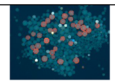
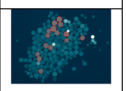
Table 6. Magnetic Recovery Statistics of Decreasing Target Antibody Concentrations in Normal Mouse Serum

Anti-FLAG IgG Concentration, nM		200	50	12.5	3.125	0.781	0.195
% of Total IgG		60	27	8.6	2.3	0.58	0.15
FLAG Peptide	% Recovery (75 μm)	92	98	94	96	100	92
	# Capped Beads Recovered (75 μm)	59	134	76	106	159	78
	Re-stained Image						
FLAG-D4H Peptide	% Recovery (75 μm)	90	78	22	16	2	0
	# Capped Beads Recovered (75 μm)	80	88	76	145	80	77
	Re-stained Image						
	% Recovery (30 μm)		73	45	33		
	# Capped Beads Recovered (30 μm)		>500	>500	>500		
	Re-stained Image						
Hit 9	% Recovery (75 μm)	88	86	76	48	66	30
	# Capped Beads Recovered (75 μm)	63	68	112	154	302	161
	Re-stained Image						

least 1 h at RT prior to use. To determine the number of capped beads per microliter, 1:10, 1:100, and 1:1000 serial dilutions were prepared in TBST and the number of beads counted in 10 μL of the 1:1000 dilution under a light microscope.

Antibody Incubations. Fifty FLAG peptide-displaying beads were doped into approximately 50 000 of acetyl-capped beads suspended in 50 μL of buffer. The bead mixture was incubated with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, catalog no. F3165 from mouse), monoclonal anti-PolyHistidine antibody (Sigma-Aldrich, catalog no. H1029 from mouse), or monoclonal anti-angiotensin I antibody (Thermo Scientific, catalog no. MA1-82995, from mouse at 30 nM in 100% PBS StartingBlock (Thermo Scientific, catalog no. 37538) plus 0.1% Tween 20 for 2 h. The TentaGel beads were washed three times with TBST by centrifugation and decanting of the supernatant solution after each incubation step. Biotin-XX Goat Anti-Mouse IgG (Life Technologies, catalog no. B-2763) was diluted 1:200 in 50% StartingBlock in TBST and incubated with the appropriate TentaGel beads for 2 h at 4° C with rotation. This antibody was only required when using streptavidin magnetic particles. MagnaBind Goat Anti-Mouse IgG (Thermo Scientific, catalog no. 21354), Dynabeads M-280 Sheep Anti-Mouse IgG (Life Technologies, catalog no. 11201D), and Dynabeads MyOne Streptavidin C1 (Life Technologies, catalog no. 65001) were each washed prior to use by diluting 10 μL of magnetic particles in 1 mL of TBST, collecting the magnetic particles on the side of the tube using the magnetic collection rack, removing the solution with a pipet and resuspending the particles in 50% StartingBlock in TBST. The TentaGel beads were incubated with the appropriate magnetic particles for 2 h.

Magnetic Collection of Hits. The hit beads from the magnetic screen were collected using two different methods. The first method utilized a magnetic collection stand (MagneSphere, catalog no. Z5342). After incubation with magnetic particles, the 1.5 mL microcentrifuge tube was placed into a slot on the magnetic collection stand. After 5 min, the magnetic particles and any magnetized TentaGel beads collected on the side of the tube, and the nonmagnetized TentaGel beads settled to the bottom. The beads at the bottom of the tube were slowly removed with a pipet. More TBST was

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added to the tube to replace the volume that had been removed with a pipet and the tube was inverted multiple times and placed back onto the magnetic collection stand. This process was repeated until no TentaGel beads settled to the bottom of the tube. The TentaGel beads held to the side of the microcentrifuge tube by the magnet were kept as "hits".

The second method for magnetic collection of TentaGel beads utilized small magnetic cubes (5 mm in size). Four of these magnetic cubes were wrapped in Teflon tape as tightly as possible, with enough additional tape to form a 1−2 in. long nonmagnetic handle. After incubation of TentaGel beads with magnetic particles, the wrapped magnetic cubes were placed inside the microcentrifuge tube and incubated for 10 min. During this time all the free magnetic particles and any magnetized TentaGel beads collected on the Teflon tape coating the magnetic cubes. Nonmagnetized TentaGel moved freely around the tube. Using the nonmagnetic handle, the cubes were removed from the microcentrifuge tube and the magnetic particles and TentaGel beads were released into a larger tube by spraying with TBST. The Teflon tape was removed from the magnetic cubes and washed with TBST to transfer any remaining beads to the larger tube. The TentaGel beads that were recovered by the magnetic cubes were kept as hits.

Identification of FLAG Beads Recovered from Magnetic Screening. The hits selected from the magnetic screen were stripped of all proteins and antibodies by incubation of the beads with 4 M guanidine hydrochloride pH 7.0, 50 mM 2-mercaptoethanol for 1 h, then washed five times with TBST, and incubated in TBST for 1 h. The TentaGel beads were then incubated with 50 nM anti-FLAG antibody in 100% StartingBlock for 2 h. The beads were washed three times with TBST then incubated with 1:200 dilution of goat anti-mouse IgG 655 QuantumDot (Life Technologies, catalog no. Q-11021MP) in 50% StartingBlock in TBST for 2 h. The beads were washed three times with TBST then viewed under a fluorescence microscope. TentaGel beads displaying the FLAG peptide produced a bright red signal when viewed through a DAPI filter, which could be easily distinguished from the capped beads that appeared blue.

FLAG Peptide ELISA. The cysteine peptides FLAG and FLAG-D4H were coated onto wells of white maleimide activated 384 well plate (Pierce, catalog no. NCI530) at 100 μM in PBS (Fisher catalog no. IC-N2810307) containing 200 μM TCEP (Fisher, catalog no. PI-77720) for 2 h at room temperature. The plate was washed three times with wash buffer (PBS containing 0.05% Tween20) to remove excess peptide solution. The ELISA plate was quenched and preblocked with PBS StartingBlock containing 143 mM 2-mercaptoethanol (Sigma, catalog no. M6250−250 mL) for 2 h at room temperature. The plate was washed three times with wash buffer. A titration up to 125 nM of the appropriate antibody was prepared in 100% StartingBlock, added to the appropriate wells of the ELISA plate, and incubated for 1 h at room temperature. The plate was washed three times with wash buffer. Goat anti-mouse IgG HRP (Abcam, catalog no. ab97040) was diluted 1:50 000 in 25% StartingBlock in TBST, added to the appropriate wells of the ELISA plate, and incubated for 30 min at room temperature. The plate was washed three times with wash buffer, then the Supersignal ELISA Pico Chemiluminescent Substrate (Thermo, catalog no. 37070) was prepared by adding a 50/50 (v/v) mixture of Lumino and stable peroxide and added to the appropriate wells of the ELISA plate. The plate was incubated for 1 min at room temperature, then immediately the chemiluminescence was measured using an Envision Plate Reader. The data were plotted and the binding constants calculated using GraphPad PRISM.

Magnetic Screening of FLAG-D4H Peptide. Fifty TentaGel beads displaying FLAG-D4H were doped into 50,000 capped beads. The magnetic screening protocol described for the native FLAG peptide was followed. To determine the number of FLAG-D4H beads recovered from the screen all the hit beads were labeled with Goat-Anti-Mouse IgG 655 QuantumDots. The total number of beads recovered from the magnetic screen was quantified using the DAPI filter. The FLAG mutant 17 beads do not produce a very bright red signal when viewed through the DAPI filter. To count the number of FLAG-D4H beads recovered, the QuantumDot labeled beads were viewed under a fluorescent microscope with a red quantum dot filter. FLAG-D4H beads show a bright red signal when viewed through this filter and the capped beads are extremely dim or cannot be visualized. For screening of the 30 μm FLAG-D4H peptide beads, the same protocol was followed.

Peptoid Library Synthesis. The peptoid library was synthesized using conventional split and pool synthesis. TentaGel Macrobeads (size 75 μm) were swelled in DMF for 2 h prior to use. Methionine was the first amino acid loaded onto the resin as described previously.[10,17] The beads were washed three times with DMF. The Fmoc protecting group was removed with 20% piperidine and the beads were washed with DMF. A short invariable linker region was synthesized. To install peptoid subunits, the growing chain was bromoacetylated using 1 mL of 2 M 2-bromoacetic acid (BAA) and 1 mL of 2.5 M diisopropylcarbodiimide (DIC). The mixture was shaken at 37 °C for 10 min and washed thoroughly with DMF. The beads were split evenly into 13 separate reaction vessels. Primary amines were added to the bromoacetylated resin as 1 M solutions in DMF and shaken at 37 °C for 1 h. The beads were pooled together and the steps were repeated a total of 6 times for an oligomer length of 6. The library was washed with DMF and DCM. The side chains were deprotected by treatment with TFA/TIPS/H_2O at a ratio of 95/2.5/2.5 for 2 h at room temperature. The library was then washed extensively with DMF.

Screening for Anti-FLAG IgG Ligands. The one-bead−one-compound (OBOC) library was washed 10 times with water and equilibrated in water overnight. The beads were washed in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) three times and equilibrated in TBST for 1 h. Five beads displaying FLAG-D4H were doped into the library beads. The beads were blocked with 100% PBS StartingBlock (Thermo) containing 0.1% Tween-20.

First, normal mouse serum was diluted to 100 μg/mL in 100% PBS StartingBlock containing 0.1% Tween-20. Five milliliters of this solution was applied to the OBOC library and incubated with slow rotation overnight at 4 °C. The library was washed three times with TBST followed by addition of 5 mL of a 1:200 dilution of biotin-XX goat anti-mouse IgG in 100% PBS StartingBlock containing 0.1% Tween-20 and incubated for 2 h at 4 °C. The library was washed three times with TBST followed by addition of 5 mL of a 1:100 dilution of prewashed Dynabeads MyOne Streptavidin C1 in 50% PBS StartingBlock in TBST. The library was incubated with the magnetic particles for 2 h at 4 °C. Hit beads were collected as described above using the magnetic collection stand. The library was stripped of

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all proteins as described using 4 M guanidine hydrochloride pH 7.0, 50 mM 2-mercaptoethanol for 1 h, then washed five times with TBST, and incubated in TBST for 1 h. The beads were blocked in 100% PBS StartingBlock containing 0.1% Tween-20 for 1 h.

Next, 200 nM of anti-FLAG IgG (Sigma-Aldrich) was doped into 100 μg/mL of normal mouse serum (Sigma-Aldrich) in 100% PBS StartingBlock containing 0.1% Tween-20. Five milliliters of this solution was applied to the OBOC library and incubated with slow rotation overnight at 4 °C. The library was washed three times with TBST followed by addition of 5 mL of a 1:200 dilution of biotin-XX goat anti-mouse IgG in 100% PBS StartingBlock containing 0.1% Tween-20 and incubated for 2 h at 4 °C. The library was washed three times with TBST followed by addition of 5 mL of a 1:100 dilution of prewashed Dynabeads MyOne Streptavidin C1 in 50% PBS StartingBlock in TBST. The library was incubated with the magnetic particles for 2 h at 4 °C. Hit beads were collected as described above using the magnetic collection stand. The hit beads were stripped of all proteins using 4 M guanidine hydrochloride pH 7.0, 50 mM 2-mercaptoethanol for 1 h, then washed five times with TBST, and incubated in TBST for 1 h.

Secondary Validation Screen. The magnetic hits were blocked in 100% PBS StartingBlock containing 0.1% Tween-20 for 1 h. 200 nM of Anti-FLAG IgG (Sigma-Aldrich) was doped into 100 μg/mL of normal mouse serum (Sigma-Aldrich) in 100% PBS StartingBlock containing 0.1% Tween-20. One milliliter of this solution was applied to the magnetic hits and incubated with slow rotation overnight at 4 °C. The magnetic hits were washed three times with TBST followed by addition of 1 mL of a 1:200 dilution of anti-Mouse IgG conjugated to red Qdots (Life Technologies) and incubated for 2 h at 4 °C in the dark. The magnetic hits were washed three times with TBST and visualized under an inverted fluorescent microscope using a DAPI excitation and emission filter. Beads that exhibited a red halo were manually removed from the mixture for further validation. The Qdot-validated hits were stripped of all proteins using 4 M Guanidine Hydrochloride pH 7.0, 50 mM 2-mercaptoethanol for 1 h, then washed five times with TBST, and incubated in TBST for 1 h.

Next the Qdot-validated hits were blocked in 100% PBS StartingBlock containing 0.1% Tween-20 for 1 h. Normal mouse serum (Sigma Aldrich) was diluted to 100 μg/mL in 100% PBS StartingBlock containing 0.1% Tween-20. One milliliter of this solution was applied to the Qdot-validated hits and incubated with slow rotation overnight at 4 °C. The Qdot-validated hits were washed three times with TBST followed by addition of 1 mL of a 1:200 dilution of anti-Mouse IgG conjugated to red Qdots (Life Technologies) and incubated for 2 h at 4 °C in the dark. The Qdot-validated hits were washed three times with TBST and visualized under an inverted fluorescent microscope using a DAPI excitation and emission filter. Beads that exhibited a red halo were manually removed from the mixture and discarded. The beads that did not display a red halo were stripped of all proteins as described using 4 M guanidine hydrochloride pH 7.0, 50 mM 2-mercaptoethanol for 1 h, then washed five times with TBST, then five times with 50% acetonitrile in water.

Sequence Identification of Validated Hits Using Mass Spectrometry. Individual beads were separated into individual wells of a 96-well plate and the compounds were cleaved from the beads by overnight incubation with 25 μL of 5:4:1 acetonitrile/acetic acid/water mixture containing 30 mg/mL CNBr. The solution was removed using a vacuum centrifuge and the cleaved compounds were dissolved in 5 μL of 50% acetonitrile in water. 0.5 μL of this solution was cospotted on a MALDI plate with 0.5 μL of 10 mg/mL CHCA in 50% acetonitrile/water containing 0.1% TFA. The spot was dried, and the mass spectra and tandem mass spectra of these compounds were collected using MALDI-TOF mass instrument. Compounds that were isolated more than one time from the screen were selected for postscreening validation efforts.

Synthesis of Validated Hits. Oligomers were synthesized on Rink Amide resin (0.55 mmol/g) using previously described peptoid synthesis procedures.[2b,18] After synthesis, the resin was washed in DCM and incubated with TFA/TIPS/H_2O (95/2.5/2.5) for 2 h are room temperature, which removed protecting groups and liberated the oligomer from the solid support. The TFA solution was evaporated under argon and the oligomer was precipitated in cold ether. The precipitate was pelleted by centrifugation, washed in cold ether and then dissolced in 50% acetonitrile in water. Crude compounds were purified using a Vydac reverse-phase C18 column (Grace) fitted onto a Waters 1525 binary high performance liquid chromatography (HPLC) pump equipped with a Waters 2487 Dual Wavelength Absorbance Detector. Compounds were purified with a linear gradient from 5 to 95% acetonitrile in water over 60 min. Peptoid identity was confirmed by MALDI-TOF MS using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems) using CHCA as a matrix. The oligomers were lyophilized and stored at 4 °C without further modification.

Multiplexed Microsphere Assay. Ten micrometer TentaGel microspheres (Rapp Polymere GmbH) were topologically segregated using the method of Lam and coworkers[3a] and encoded with varying ratios of Pacific Blue and Pacific Orange dyes using the method of Doran.[16] After encoding the microspheres, a methionine residue was coupled to the beads to allow for CNBr cleavage. The beads were incubated with 20% piperidine in DMF to deprotect the Fmoc from the methionine. The terminal amines were bromoacetylated by incubation with 150 μL of BAA (2 M in DMF) and 150 μL of DIC (2.5 M in DMF) for 10 min at 37 °C. After it was washed with DMF, the ligand of interest (3 equiv) containing a terminal cysteine was dissolved in 1:1 mixture of DMF/PBS and applied to the bead suspension. Thioether formation occurred overnight at room temperature. The beads were washed with DMF and quenched with 150 mM 2-mercaptoethanol in 1:1 DMF/PBS for 1 h at room temperature. The beads were washed extensively with DMF and a small amount of beads (50 μg) was removed from each encoded population for CNBr cleavage and mass spectrometry identification of the correct ligand as described previously. The uncleaved beads were washed ten times with water, equilibrated in water overnight, then washed three times with TBST, and equilibrated in TBST for 1 h.

The beads from each encoded population were mixed together in a single tube and then distributed to wells of a 96-well filter bottom plate. The beads were blocked with 100% PBS StartingBlock containing 0.1% Tween-20 for 1 h at 4 °C. Serial dilutions of anti-FLAG IgG, anti-angiotensin IgG, and normal mouse serum were prepared in 100% PBS StartingBlock containing 0.1% Tween-20. The blocking solution in the filter bottom plate was drained using a vacuum and the antibody or serum solutions were applied to the wells of the plate and incubated with gentle shaking overnight at 4 °C. The solutions were drained by vacuum and the beads were washed three

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times with TBST. An Alexafluor 647 conjugated Anti-Mouse IgG secondary antibody (Life Technologies) was diluted 1:200 in 50% PBS StartingBlock in TBST and incubated with the beads for 2 h at 4 °C. After washing the beads three times with TBST, the Alexafluor 647 mean fluorescence intensity (MFI) was monitored by fluorescence activated cell sorting (FACS).

FACS was performed on an LSRII (BD Biosciences) using violet and red lasers. Emission intensities were monitored at 450 (Pacific Blue), 500 (Pacific Orange), and 655 nm (Alexafluor 647). At least 1000 microspheres were collected for each analysis and the MFI of the Alexafluor 647 emission intensity was calculated using FlowJo Software (Tree Star, Inc.). Data reported are representative of two independent experiments. GraphPad Prism (GraphPad Software, Inc.) was used to fit the saturation curves to obtain K_D values using a one-site saturation model.

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Author Contributions

L.C. performed the experiments with the 30 μm beads. J.M.N. synthesized the libraries employed in this paper, as well as resynthesized many of the hits. K.M. conducted all of the other experiments and wrote the paper. T.K. helped to design the study, oversaw the project, and edited the paper.

Notes

The authors declare the following competing financial interest(s): K.M. and J.M.N. are employees of Opko Health, Inc., which funded most of this work. T.K. is a significant stockholder in Opko Health, Inc.

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