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Rapid Lead Discovery Through Iterative Screening of One Bead One Compound Libraries

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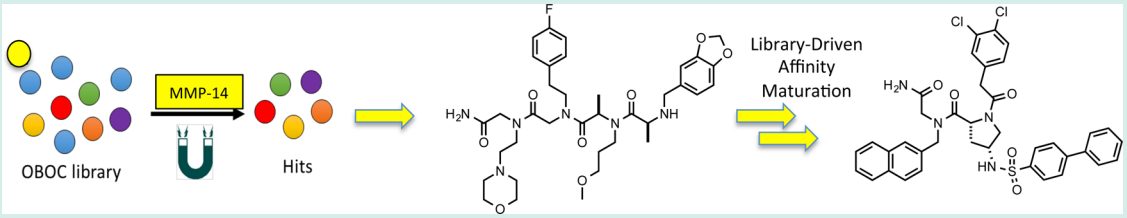
Supporting Information



ABSTRACT: Primary hits that arise from screening one bead one compound (OBOC) libraries against a target of interest rarely have high potency. However, there has been little work focused on the development of an efficient workflow for primary hit improvement. In this study, we show that by characterizing the binding constants for all of the hits that arise from a screen, structure−activity relationship (SAR) data can be obtained to inform the design of "derivative libraries" of a primary hit that can then be screened under more demanding conditions to obtain improved compounds. Here, we demonstrate the rapid improvement of a primary hit against matrix metalloproteinase-14 using this approach.

KEYWORDS: *rapid lead discovery, iterative screening, one bead one compound (OBOC) libraries, structure−activity relationship (SAR)*

INTRODUCTION

In the past two decades, high-throughput screening (HTS) of small molecule libraries or compound collections has become the most common method for the discovery of tool compounds and drug leads.[1] HTS is most commonly done in a highly automated fashion using tens to hundreds of thousands of compounds placed in individual wells of microtiter plates using some kind of functional assay. However, this requires robotics and other sophisticated equipment, as well as specialized staff. Moreover, the primary hits that arise from such screens almost always must be improved significantly to yield compounds of real utility. This is usually done via the synthesis and analysis of many derivatives of the most promising hits, ideally providing structure−activity relationship (SAR) that will guide the development of a more potent or selective lead molecule. This medicinal chemistry phase of such projects requires expert organic chemists and may be quite tedious, depending on the structure of the primary hit. So while current screening and hit optimization methodologies can be quite effective, the development of much faster and cheaper ways to discover bioactive compounds remains an important goal.

An alternative technology that has received attention from several laboratories, including our own, is to employ simple protein binding screens and libraries of bead-displayed compounds.[2] This method employs the split and pool strategy[3] for the solid-phase synthesis of libraries. This provides beads that display many copies of only a single compound. When beads with a hydrophilic surface, such as TentaGel, are employed in the synthesis, the same beads can be used in the screening step. This technique was originally developed in the context of peptide libraries[3] but is being extended to many other classes of compounds with more drug-like properties.[4] One advantage of this screening platform is that all of the beads can be screened in a single vessel by simply incubating them with a labeled target protein and an excess of unlabeled competitor proteins to enforce high selectivity.[2b] The beads that bind high levels of the labeled protein can be isolated and then evaluated for binding to the target protein.

Libraries created by split and pool synthesis are constructed by assembly of readily available building blocks using high yielding reactions. The modular nature of these compounds should, in theory, simplify the improvement of primary hits into leads. This could be done by creating a library of derivatives in which the building blocks used bear some resemblance to those employed to construct the primary hit, but alter the steric or electronic nature of that module somewhat. This derivative library could be screened under more demanding conditions for better ligands. This exercise could be repeated more than once. Such a scheme would be particularly attractive if one could

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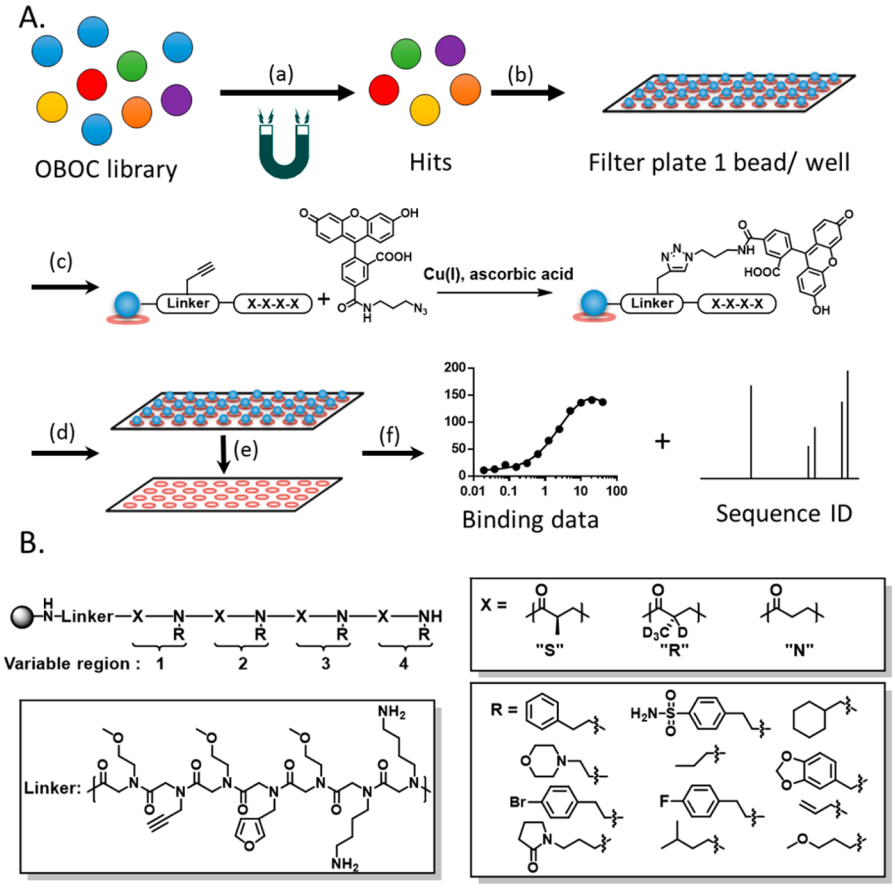

Figure 1. Initial library and screening strategy employed against MMP-14. (A) Workflow for screening and hit characterization. (a) The beads are incubated with MMP-14, then an anti-MMP-14 primary antibody, then a secondary antibody attached to magnetic particles. Beads that contain MMP-14 compounds were separated using a powerful magnet. (b) Hit beads are transferred to 96-well plate, with one bead/well. (c) All hit compounds are labeled with fluorescein by "click" chemistry. (d) Compounds are cleaved from beads by TFA. (e) Compounds are separated from the beads and transferred to a new plate by a vacuum manifold. (f) A titration experiment, monitored by fluorescence polarization spectroscopy is used to determine the binding affinity between each fluorescein labeled compound and MMP-14. Each of the compounds was sequenced by tandem mass spectrometry. (B) Library design. Total theoretical diversity = 1 679 616.

obtain significant SAR information in the process to guide the next round of library design.

Unfortunately, although the literature is replete with reports of primary hits from OBOC libraries, little work along these lines has been reported.[5] One potential reason for this may be the propensity of OBOC library screens to yield large numbers of false positives,[6] which represents one of the major disadvantages of this technology. These are compounds that appear to bind the labeled target protein on bead quite well, but then fail to do so when tested free in solution or in other formats. This is a major problem because a great deal of time and expense can be wasted in the postscreening phase on the resynthesis and characterization of compounds that ultimately prove to be useless. Clearly, this is a disincentive to the analysis of many hits from a derivative library to glean SAR information.

Various strategies have recently been published to deal with this issue. One is to use redundant libraries in which each compound in the library is displayed on several different beads. Compounds isolated multiple times from a redundant library are almost always bona fide ligands.[7] The other general strategy is to evaluate the binding of each of the hits to the target protein without the need to resynthesize each compound.[8] Perhaps the most effective method to accomplish this was described by Auer and co-workers.[9] After separating the beads chosen as hits into individual wells of a microtiter plate, a fluorescent label is affixed to the molecule via functionality present in a conserved linker. The fluorescently labeled compounds are then washed and released form the bead. Using 90 μm TentaGel beads, there is enough compound in solution to allow the semiquantitative determination of the K_D for the target protein by a titration experiment monitored by fluorescence polarization (FP) spectroscopy in a 96-well format. In this way, only the compounds with the highest affinity for the target protein can be selected for resynthesis and more detailed analysis.

In this study, we explore whether the use of a highly redundant library, combined with the postscreening FP analysis protocol can indeed support an efficient, SAR-driven hit improvement effort. Beginning with a library of oligomers comprised of peptoid and peptide tertiary acid (PTA) units, we report the identification of a modest affinity ligand for matrix metalloproteinase 14 (MMP-14), a potentially interesting target for slowing tumor metastasis.[10] We then show that, indeed, OBOC library screening and SAR analysis by semiquantitative FP without hit resynthesis allows one to proceed rapidly through an initial screen and multiple rounds of hit optimization, arriving at a useful lead candidate suitable for more detailed characterization.

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RESULTS AND DISCUSSION

Identification of a Primary Hit from a PTA Library. MMP-14 is a zinc-dependent type-1 transmembrane metalloproteinase involved in pericellular proteolysis, migration and invasion.[10a−d] Its role in collagenolysis appears to be crucial for tumor invasion. While several inhibitors of MMP-14 are known, there remains interest in the development of new compounds since the existing small molecule antagonists have modest selectivity, at best, over other MMP family members.[11] While the focus of this study is solely on establishing methodology to improve screening hits, we are interested in the long run in improved MMP-14 inhibitors, so used this protein as a test case.

The library that was employed for the primary screen is shown in Figure 1B. It consisted of four N-substituted amino acids and was synthesized by split and pool chemistry using the submonomer route developed by Zuckermann and colleagues for peptoid synthesis.[12] However, rather than using 2-bromoacetate as the sole acid submonomer, as is done for the synthesis of simple peptoids, the acid was also made a diversity element by also using the two enantiomers of 2-bromoproprionic acid. The combination of the chiral center at the α-carbon and N-substitution provides significant conformational constraints on the molecule[13] relative to peptides or peptoids, which may allow for higher affinity binding.[14] The building blocks employed are shown in Figure 1B. The theoretical diversity of the library was 1 679 616 compounds. All of the molecules were built off of an invariant linker that included an alkyne moiety for subsequent tagging of the molecules with azidofluorescein.[9] Approximately 400 000 beads were used for the initial screen. To remove unwanted ligands from the library, the beads were first incubated with blocking buffer and primary antibody, and then magnetic particles coated with secondary antibody were added. After incubation and washing, strong magnets were used to remove all beads that bound to the primary or secondary antibody.[8] Approximately 1% of the beads were removed in this way. The denuded library was washed and incubated with blocking buffer again. The library was then incubated first with MMP-14 protein (112Y-541A) and then, after washing, the primary antibody. After another wash, magnetic particles coated with secondary antibody were added. Again, the magnetized beads were removed. About 100 beads were isolated. 96 were picked and placed into the wells of a 96 well filter plate, with one bead per well.

Hit Evaluation without Resynthesis. As mentioned above, false positives are common in bead screening experiments, so the binding of each of the 96 hits to MMP-14 was evaluated. Azidofluorescein was added to each well, along with a copper catalyst, resulting in the attachment of the label to the bead bound molecule via a Huisgen cycloaddition with the unique alkyne in the linker[9] (Figure 1). Trifluoroacetic acid (TFA) was added to release each compound from the bead. The soluble compounds were separated from the bead by filtration and transferred to the corresponding well in a new plate.

Each compound was titrated with MMP-14 and binding was followed by FP spectroscopy to determine the crude binding affinity. This process allowed us to evaluate the binding of all 96 hits to MMP-14 in 2 days without the need for resynthesis.

As anticipated, many of the 96 hits proved to be poor MMP-14 ligands. Only 25 showed binding curves that suggested a K_D below 150 μM (Supporting Information Table S1). On the basis of these data, the best four compounds were chosen for resynthesis. In addition, compound KYG-1 was also included in this group because of its significant sequence similarity to the best hit KYG-2. FP analyses were then carried out with the resynthesized, HPLC-purified compounds to obtain accurate binding constants. As shown in Table 1, KYG-2 (K_D = 22 μM) was found to have the best affinity for MMP-14, so this hit was chosen for further development.

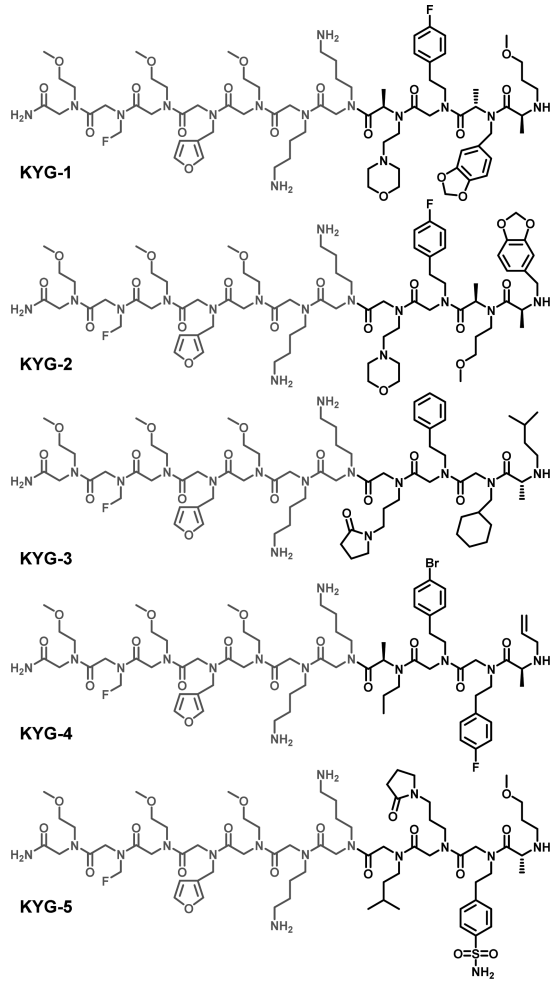

Figure 2. Structures of five highest affinity hits identified from first round of screening. For the sequence of all 96 hits, see Supporting Information Table S1. F = fluorescein tag (see Figure 1 for details). The common linker is shown in gray and the variable elements in black.

Table 1. Binding Affinity of Top Five Compounds from First Two Rounds of Screening[a]

compound	K_D (μM)	std. dev.	compound	K_D (μM)	std. dev.
KYG-1	110	8.1	KYG-97	2.7	0.3
KYG-2	22	1.9	KYG-98	0.7	0.1
KYG-3	60	7.9	KYG-99	1.8	0.3
KYG-4	35	6.5	KYG-100	1.1	0.2
KYG-5	30	3.0	KYG-101	1.3	0.1

[a] K_D values measured by fluorescent polarization. Std. Dev.: Standard deviation of K_D in μM. For complete K_D list, see Supporting Information Tables S1 and S3.

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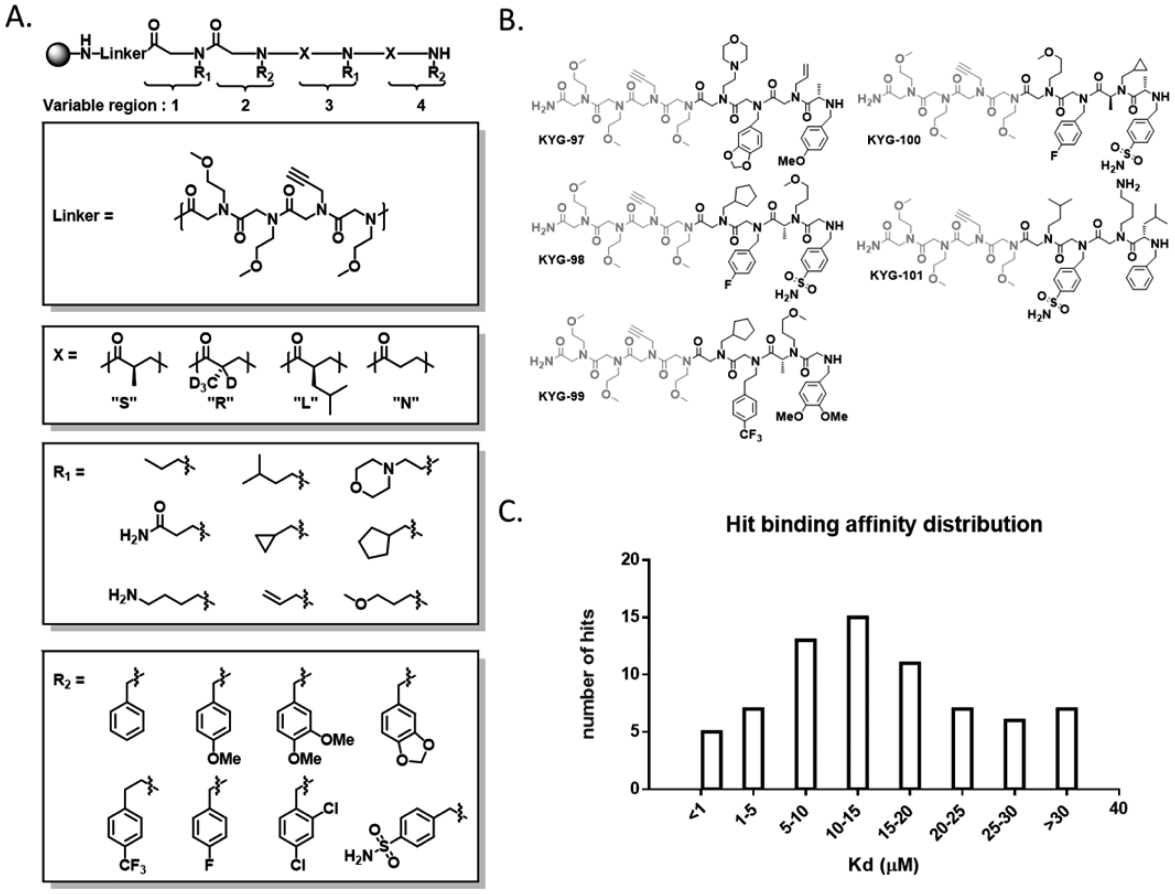

Figure 3. Derivative library screening. (A) Derivative library design. Three PTA submonomers and bromoacetic acid were used at the third and fourth position of the variable region. Theoretical diversity = 82 944. (B) Structures of the five highest affinity hits determined by the fluorescent polarization assay using compound from a single bead. (C) K_D distribution of all 71 hits identified from derivative library screening. F = fluorescein tag (see Figure 1 for details).

Hit Improvement by Derivative Library Screening. To guide the design of a KYG-2 derivative library, the structures of all 25 of the hits that exhibited an apparent K_D of below 150 μM were examined. This revealed that the side chains at the second and fourth variable positions were dominated by aromatic residues, while aliphatic residues were mostly found as the side chains in the first and third variable positions (see Supporting Information Table S1). Furthermore, most of the chiral centers were found in the third and fourth variable position. On the basis of this, the library design shown in Figure 3 was chosen. Aliphatic amines were employed at positions 1 and 3, while benzylic or homobenzylic amines were used at positions 2 and 4. Unfortunately, while anilines would also have been of interest as submonomers and have been incorporated into peptoids,[15] this chemistry is not efficient enough for creation of high quality libraries. With respect to the acid submonomers, only 2-bromoacetate was employed at positions 1 and 2, while this compound and three chiral 2-bromoacids were employed at positions 3 and 4. The theoretical diversity of this library was 82,944 compounds.

This library was screened against MMP-14 as described above. However, thousands of beads were pulled out as hits under the original screening conditions, which is not surprising, since many of the compounds in the library resemble KYG-2. This is too many to handle. Therefore, the screen was repeated using half of the original MMP-14 concentration and a shortened incubation time. From the 250 000 beads employed, 71 were pulled out as hits magnetically and transferred to a 96-well plate in a one bead per well fashion. These were labeled with fluorescein, released from the bead and tested for binding to MMP-14, again using the multiwell FP assay. All 71 compounds were found to bind MMP-14 with K_D values from 0.5 to 32 μM (Figure 3C). This is in stark contrast to the high preponderance of false positives in primary library screens. More than half were found to have a K_D equal to, or better than, the original hit KYG-2. The five exhibiting the best apparent affinity for MMP-14 in this assay were resynthesized, purified and accurate binding constants were determined by FP analysis. Compounds KYG-97, KYG-98, KYG-99, KYG-100, and KYG-101 showed K_D values of 2.7, 0.7, 1.8, 1.1, and 1.3 μM, respectively (Table 1). However, in the course of working with these compounds in different buffered aqueous solutions, we observed that KYG-98, KYG-100, and KYG-101 exhibited poor solubility in some, particularly those with significant Zn^{2+} and Ca^{2+} levels that are used to assess enzyme activity,[16] whereas KYG-97 and KYG-99 displayed excellent solubility in all of the buffers tested. Therefore, we chose to focus on these compounds.

Identification of Residues Important for MMP-14 Binding. The ~10-fold improvement in MMP-14 affinity in going from the primary hit, KYG-2 to the secondary screening hit KYG-99 was gratifying, particularly since we had no structural information to guide the design of the derivative library. However, it was of interest to determine if further improvement

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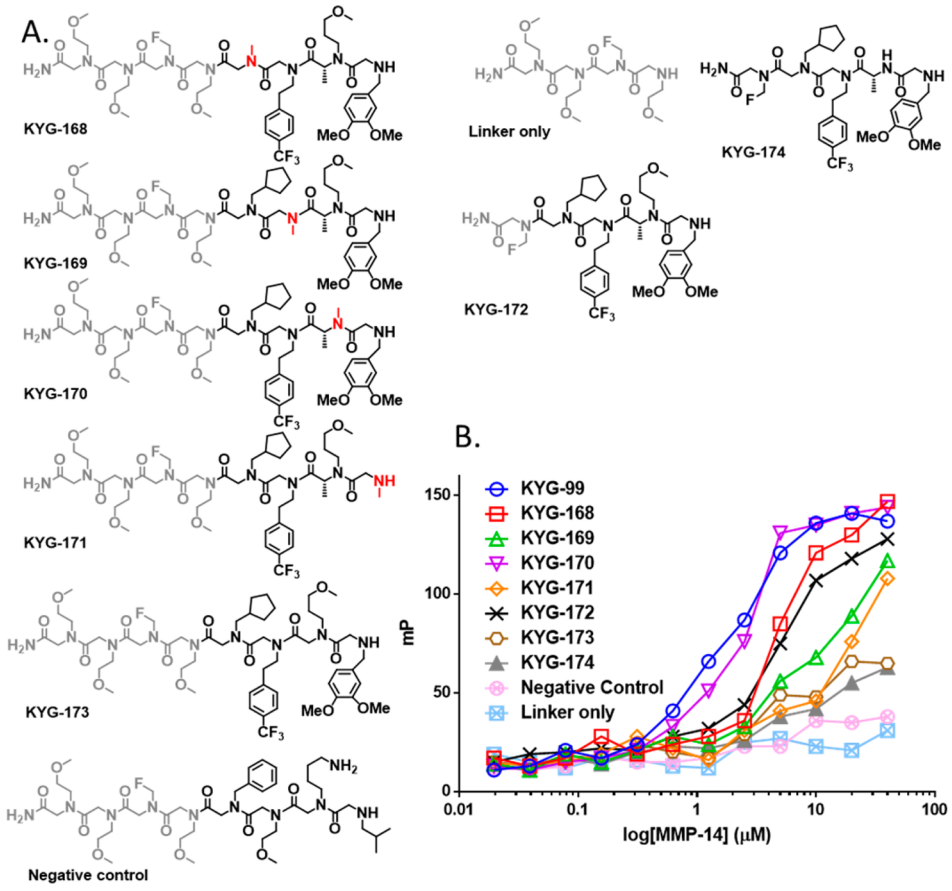

Figure 4. Identification of the pharmacophore of KYG-99. (A) Structure of KYG-99 derivatives. F = fluorescein tag (see Figure 1 for details). (B) Fluorescent polarization assay of KYG-99 derivatives. Points are linked for clearer visualization, these are not curve-fitted lines.

could be achieved. To do so, we decided to first identify the moieties in KYG-99 important for binding to MMP-14, with the intent of reducing the mass of the molecule by eliminating irrelevant parts before moving on. Four derivatives of KYG-99 were synthesized in which each side chain, in turn, was replaced by a methyl group (KYG-168, KYG-169, KYG-170, and KYG-171). As shown in Figure 4, substitution of either of the aromatic side chains (KYG-169 and KYG-171) resulted in at least a 10-fold drop in affinity. However, elimination of the cyclopentyl ring in the side chain at position 1 had only a modest effect while substitution of a methyl group for the ether side chain at position 3 did not affect binding at all.

To assess the importance of the chiral center in KYG-99, the peptoid derivative KYG-173 was created. This resulted in a drastic loss of affinity for MMP-14 (Figure 4). A nearly identical result was observed when the nonessential ether side chain at the third position was replaced with a hydrogen atom (KYG-174; see Figure 4). These two data points strongly suggest that the chiral methyl group and the adjacent *N*-alkyl group are likely to be important for stabilizing a favorable conformation of the molecule for binding MMP-14 through allylic 1,3 interactions.[14]

Finally, to ask if the invariant linker used in the second round of library screening might play a role in MMP-14 binding, compound KYG-172 was synthesized and characterized. This derivative did display slightly weaker binding than KYG-99 (K_D values of approximately 7.6 μM and 1.8 μM), possibly due to the loss of nonspecific, hydrophobic interactions, since the fluorescein-conjugated linker alone did not bind detectably to MMP-14 (Figure 4).

Optimization of the Core Structure. These data show that the most critical elements in KYG-99 for binding MMP-14 are the aromatic residues at the second and fourth positions, which are likely positioned appropriately by the conformationally biased PTA residue at position 3. There are likely weaker hydrophobic interactions between the C-terminal region of the molecule and the protein, as evidenced by the modest effects of deleting the cyclopentyl group at position 1 or the linker. We therefore hypothesized that the minimal binding unit would be represented by compound KYG-175 (Figure 5), in which the first unit of KYG-99 is eliminated entirely and the side chain at the (formerly) third position is methyl. This small molecule, with a molecular mass of less than 600 Da (excluding the fluorescein probe) indeed bound to MMP-14 with a K_D of 11 μM, almost identical to the value measured for KYG-172 (Table 2).

Before creating and screening a library of KYG-175 derivatives, a few more individual compounds were synthesized and analyzed to provide further guidance. Since electron-rich aromatic rings and methyl ethers can sometimes be a metabolic liability due to the action of cytochrome P-450s, we asked if the dimethoxyphenyl ring could be replaced with the sterically similar, but electronically different, dichloroarene. This was indeed the case. As shown in Figure 5 and Table 2, KYG-175 and KYG-177 had an almost identical affinity for MMP-14. Thus, we utilized KYG-177 as the starting point for further work.

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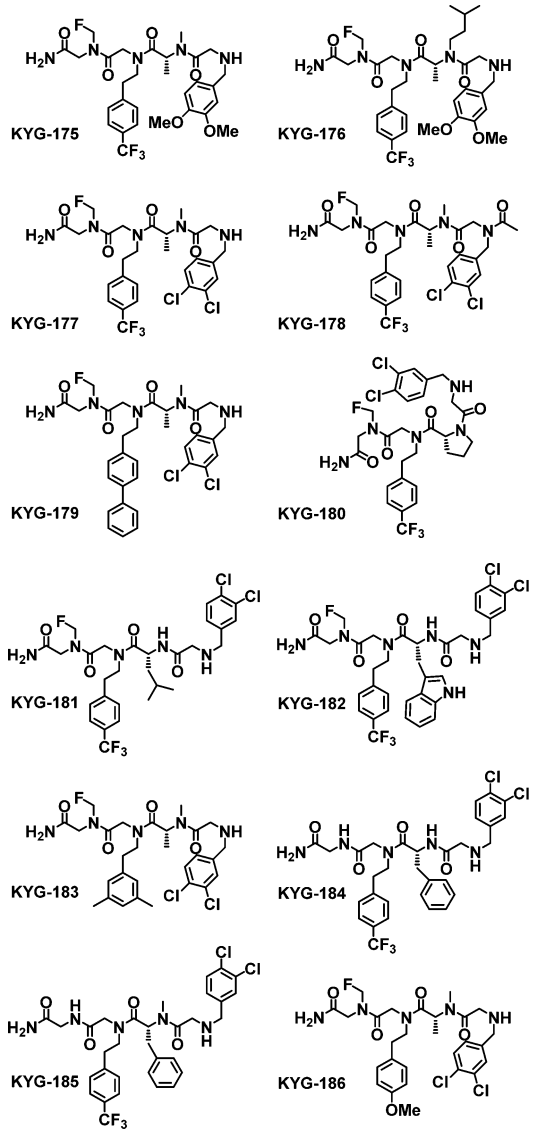

Figure 5. Structures of the KYG-99 derivatives examined in the course of core structure improvement. F = fluorescein tag (see Figure 1 for details).

Table 2. Binding Affinity of Compounds Synthesized for Core-Structure Optimization As Shown in Figures 4 and 5[a]

compound	K_D (μM)	std. dev.	compound	K_D (μM)	std. dev.
KYG-168	6.3	0.63	KYG-178	150	14
KYG-169	NA	NA	KYG-179	76	9.9
KYG-170	2.1	0.17	KYG-180	13	1.1
KYG-171	NA	NA	KYG-181	137	23
KYG-172	7.6	0.66	KYG-182	>200	NA
KYG-173	NA	NA	KYG-183	65	3.8
KYG-174	NA	NA	KYG-184	>200	NA
KYG-175	11	1.8	KYG-185	67	2.2
KYG-176	24	4.1	KYG-186	28	3.4
KYG-177	15	1.2			

[a]Std. Dev.: Standard deviation of K_D in μM.

Although it might be possible to further optimize the side chains of KYG-177, we instead envisioned expanding the core unit represented by this molecule in the hopes of picking up additional favorable interactions with the protein. Thus, a small series of compounds was created in which larger groups were introduced into various positions of KYG-177 to probe which regions of the molecule were tolerant of this sort of manipulation. As shown in Figure 5 and Table 2, none of these alterations resulted in improved binding. Acetylation of the N-terminus (KYG-178) was strongly deleterious to binding. Enlargement of the aromatic group at the first position (KYG-179, KYG-183, and KYG-186) also reduced affinity, though it should be noted that these substitutions also made the rings less electron poor, which may also have played a role. Changing the PTA unit from an *N*-methyl alanine to *N*-methyl phenylalanine (KYG-185) had a detrimental effect. We also examined the substitution of amino acids for the PTA unit, but as predicted by the poor binding of KYG-174 (Figure 5), all of these compounds (KYG-181, KYG-182, and KYG-184) failed to bind well to MMP-14. The only exception was the placement of proline at this position, which makes sense since this is an N-substituted amino acid. KYG-180 and KYG-177 had similar affinities for MMP-14 (Table 2).

Second Round of Derivative Library Screening. The tolerance of a proline insertion into the main chain was of interest, since 4-azidoproline is readily available from 4-hydroxyproline as an inexpensive starting material. The azido group can be coupled to alkynes via Click chemistry, or reduced, allowing electrophilic units to be added to this position, thus allowing expansion of the molecule in a way that was not explored in the molecules shown in Figure 5. We also speculated that while acetylation of the N-terminus of KYG-177 was not tolerated, perhaps alkylation or other processes that preserve the basic nitrogen might be, allowing extension of the molecule in this direction.

With these ideas in mind, derivative libraries based on KYG-180 were created (Figure 6). We used both *cis*- and *trans*-4-azidoprolines in the scaffold. The azido group was either subjected to a copper-catalyzed Huisgen cycloaddition or reduced and used as an amine. To facilitate the downstream hit identification process by mass spectrometry, we divided this library into four sublibraries. A peptoid unit containing 7 different residues was synthesized at the first position, following the linker (Figure 6, position X). The beads were then split and *cis*-4-azidoproline was added to one portion and *trans*-4-azidoproline to the other. These were kept separate. For both bead populations, the second peptoid unit was then added to the pyrrolidine ring nitrogen (Figure 6, position Z). We used 13 different amines (including six amino acids) and 5 aromatic hydrazines to achieve a high diversity at this position. Finally, Fmoc-Cl was used to protect the terminal secondary amines.

The beads were split again, now creating four pools that we will refer to as libraries A–D. In libraries A and C, the azide group of the 4-azidoproline was reduced to an amine on-bead using TCEP and a peptoid unit constructed with 11 different amines and three acylhrdrazines was then added to this amino group.

Libraries B and D were split into 21 portions. In 17 of these, the azido group was reduced to the amine and then condensed with various electrophiles to generate 17 different amides and sulfonamides (position W, Figure 6). The remaining four portions were treated with an alkyne and a copper catalyst to generate 4 different 1,2,3-triazole residues (position W, Figure 6). The theoretical diversity of all four libraries was 8,820 compounds.

All four sublibraries were then screened against MMP-14 as described above, using the same conditions that were employed

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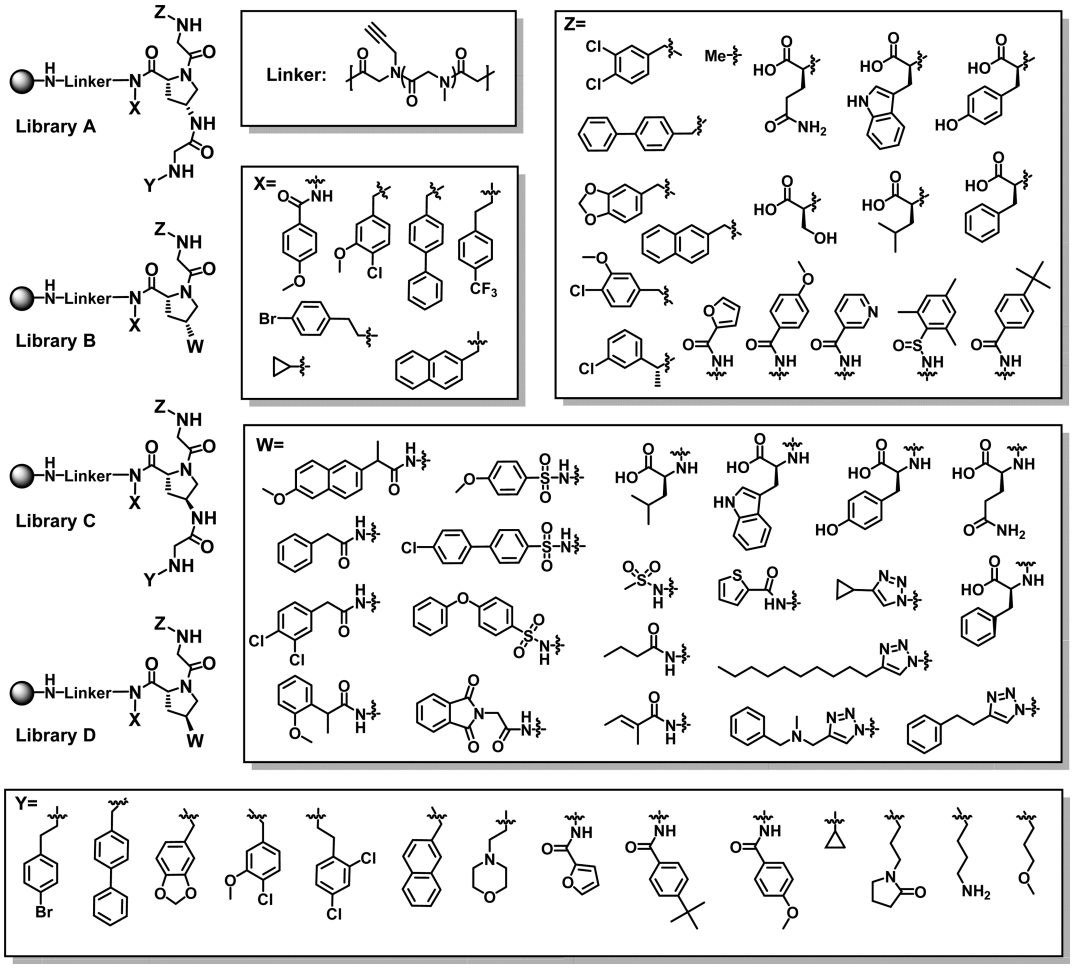

Figure 6. Derivative libraries based on the proline core structure. Four sublibraries were created that differed in the aza-proline stereochemistry (see text for details) to facilitate hit identification. Total theoretical diversity = 8820.

in the previous derivative library screen. Seventeen hits were identified in total (KYG-187 to KYG-203, see Figure 7). Interestingly, all of these were from sublibraries C and D, in which trans-4-azidoproline was used, suggesting that the cis-proline scaffold was disfavored. All 17 hits were characterized by mass spectroscopy and their affinity for MMP-14 was measured using the high-throughput FP assay (Table 3). On the basis of these data, the five best hits were resynthesized, purified and their affinity for MMP-14 was remeasured by FP assay. KYG-187 was identified as the best compound after resynthesis, with a K_D value of 0.77 μM. Within the structures of all 17 hit compounds, 3,4-disubstituted aromatic rings and residues bearing biphenyl structure were found multiple times in all 17 hits, showing a clear binding preference for these motifs. In total, 11 out of 17 compounds have a 3,4-disubstituted aromatic ring at the Z position, 8 have biphenyl structure at X, Y, or W position.

While the focus of this study is improvement of binding, these five compounds were nonetheless tested for their ability to inhibit MMP-14 using an assay in which the enzyme cleaves a collagen-like substrate.[16] The results are shown in Table 3. The best compound in the binding assay, KYG-187 showed an IC_{50} value of 16 μM in the in vitro enzymatic assay, while KYG-188 (K_D = 1.6 μM) showed the best potency, with an IC_{50} of 7 μM.

Comparison of Hits From Each Screening Round. To obtain an overview of the utility of each step in the affinity maturation process, the dissociation constants of the best compounds from each round of screening were compared. To allow a fair comparison, the linker region of KYG-2 was eliminated to provide KYG-204, which was compared in the same experiment to KYG-172, KYG-180, and KYG-187, the best compounds from each round of optimization. As shown in Figure 8, KYG-204 did not show saturated binding in the protein concentration range explored (0.02−160 μM), indicating that the linker region in KYG-2 played a significant role in binding. Compound KYG-172, which is the variable region of the best hit from the first derivative library screening, bound to MMP-14 with a K_D value of 7.6 μM, an enormous improvement over the affinity of KYG-204 (Figure 8). KYG-180, the compound to come out of the nonlibrary-driven phase of improvement has about the same affinity for MMP-14 as KYG-172 (K_D values of 11 and 7.6 μM, respectively), but is significant smaller in size and perhaps possesses more drug-like features. It bound to MMP-14 with a K_D value of 11 μM. The best compound from the last round of derivative library screening, KYG-187 showed the best binding curve with a K_D value of 0.77 μM. Overall, a significant improvement in binding was achieved through two rounds of derivative library screening and one round of "pharmacophore trimming".

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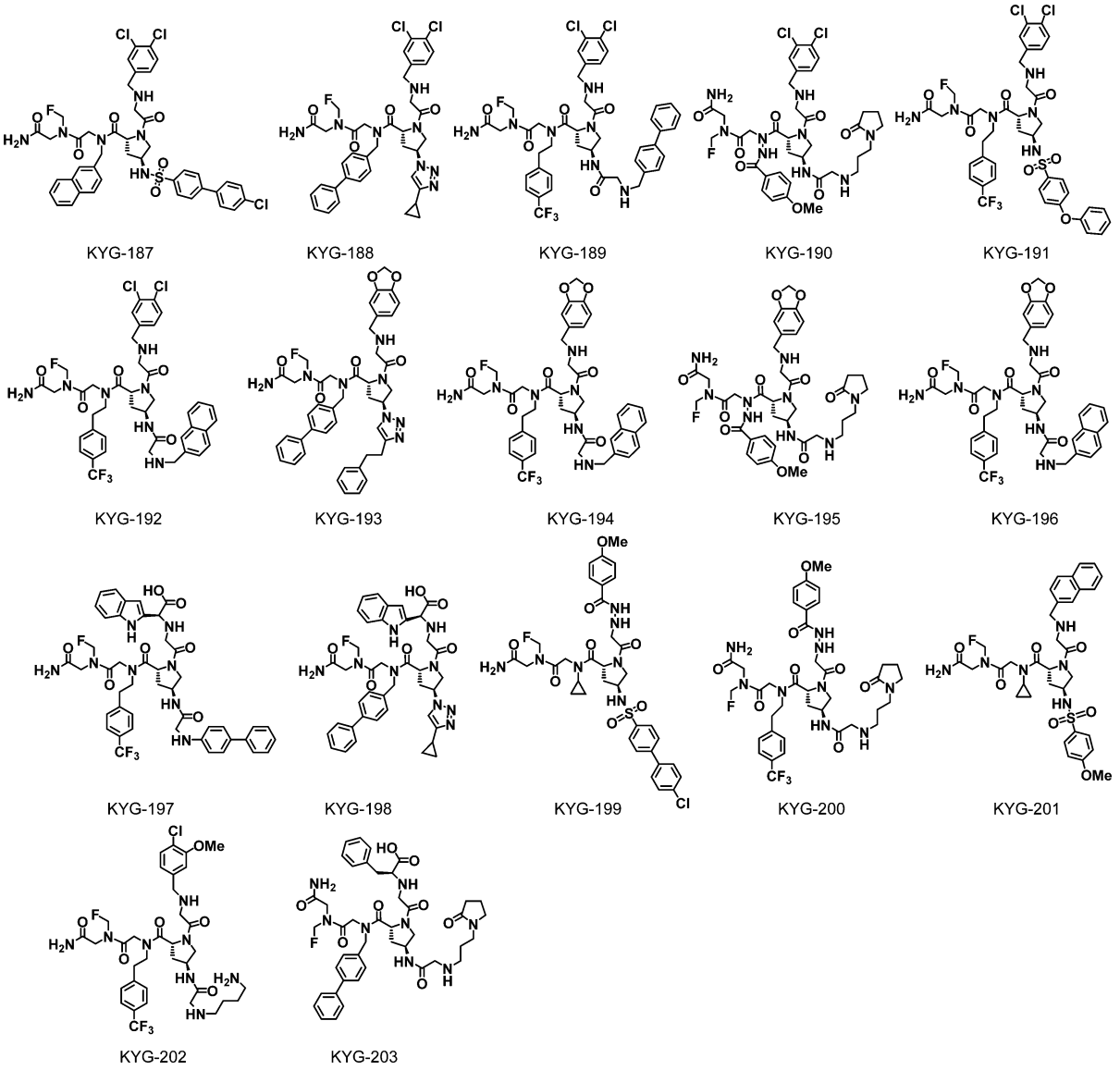

Figure 7. Structures of all 17 hits identified from screening the aza-proline-containing libraries.

Table 3. K_D and IC_{50} of All 17 Hits from Final Round of Derivative Screening[a]

compound	K_D (μM)	IC_{50} (μM)	compound	K_D (μM)	IC_{50} (μM)
KYG-187	0.23 (0.77)	16	KYG-196	17.4	
KYG-188	2.1 (1.6)	7	KYG-197	16.6	
KYG-189	8.4		KYG-198	5.9	
KYG-190	12		KYG-199	3.5 (1.2)	15
KYG-191	31		KYG-200	29	
KYG-192	1.1 (1.2)	57	KYG-201	4.8	
KYG-193	2.7 (4.1)	28	KYG-202	13	
KYG-194	30		KYG-203	9.8	
KYG-195	15				

[a]Numbers in parentheses are K_D values measured after re-synthesis. IC_{50} measured by in vitro enzymatic assay for re-synthesized hits.

CONCLUSION

In conclusion, we have demonstrated an efficient process for the improvement of primary hits that arise from screens of OBOC libraries. Critical to this process is the use of the protocol developed by Auer and co-workers[9] that allows rough K_D values of all the hits for the target protein to be evaluated without the need for resynthesis. Whereas this process has been used to weed out false positives in primary screens, this is the first example of which we are aware where it has been used to generate the SAR data needed to drive derivative library design and secondary hit evaluation. Significant improvements in affinity and other important properties such as the molecular mass were achieved at each step (Figure 8). We anticipate that this workflow will allow OBOC library screening campaigns to more readily produce truly useful tool compounds and drug candidates.

EXPERIMENTAL PROCEDURES

General Experimental information. All the Fmoc-protected amino acids were purchased from AnaSpec (Fremont, CA). TentaGel resin was purchased from Rapp Polymere GmbH (German). All other reagents were purchased from Sigma-Aldrich or Alfa Aesar, unless otherwise specified. All of the chemical reagents and solvents from commercial sources were used without further purification. Five mL and 10

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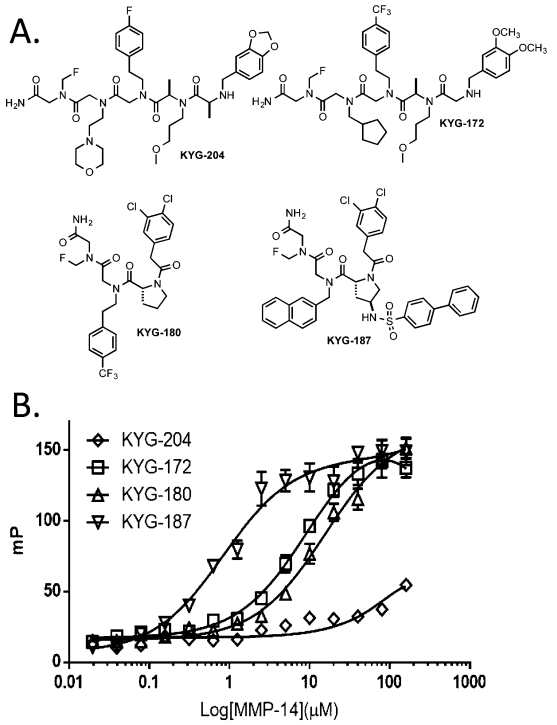

Figure 8. Comparison of the compounds identified from each round of screening/optimization. (A) Structures of KYG-204 from initial screening (KYG-2 with the linker region removed), KYG-172 from the first round of derivative library screening (linker region removed), KYG-180 from the pharmacophore optimization and KYG-187 from the final round of derivative library screening. (B) Binding affinity measured by titration with MMP-14, monitored using FP spectroscopy.

mL disposable reaction columns (Intavis AG) were used as reaction vessels for solid-phase synthesis. Syntheses of peptiods under microwave conditions were performed in a 1500 W microwave oven (GE model JE 1860BH04) with 10% power. HPLC was carried out on Waters systems equipped with Waters 1525 binary HPLC pumps and a 2487 dual λ absorbance detector, or a 2998 photodiode array detector. The mobile phase comprised of buffer A (H_2O containing 20% CH_3CN and 0.1% trifluoroacetic acid (TFA)) and buffer B (CH_3CN containing 0.1% TFA). Analytical HPLC was conducted using a Vydac C-18 column (5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL) at a flow rate of 1.0 mL/min with UV detection at 220 nm. MS and MS/MS (MALDI-TOF) were performed on a 4800 Proteomics Analyzer (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. All steps involving water utilized distilled water filtered through a Barnstead Nanopure filtration system (Thermo Scientific)

MMP Proteins. The plasmid construct for producing soluble MMP-14 (MMP-14 without the transmembrane domain, designated ΔTM-MMP-14) was described previously.[17] ΔTM-MMP-14 was transfected transiently in COS-1 cells to generate soluble MT1-MMP (S. Pahwa, S. Amar, M. Bhowmick, J. Cao, A.Y. Strongin, R. Fridman, S.J. Weiss, and G.B. Fields, manuscript in preparation). ProMT1-MMP was activated by using trypsin-3 at a final concentration of 0.1 ug/mL and incubating for 1 h at 37 °C. The reaction was stopped by addition of AEBSF (at a final concentration of 1 mM) and incubation for 15 min at room temperature. The concentration of active MMP-14 was determined by titration with recombinant TIMP-2.[18]

ΔTM-MMP-14 activation. ΔTM-MMP-14 (80 nM) was incubated with trypsin (final concentration = 5 μg/mL) at 37 °C for 1 h. After incubation, trypsin was then inactivated by adding 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (final concentration = 1 mM) for at least 15 min at room temperature.

Enzymatic Assay. The triple-helical substrate fTHP-9 [(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Cys(Mob)-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2] was synthesized using methods described previously.[19] fTHP-9 stocks were made in 100% dimethyl sulfoxide (DMSO), diluted to 50 μM (150 μM based on raw UV−vis data as triple helix, ε_{363} = 15900) with tryptic soy broth (TSB) and left overnight at 4 degrees to refold. Serial dilution of the inhibitor was made with TS buffer (50 mM Tris-HCl, pH = 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% Brj35) and dispensed in a 384-well plate. Activated MMP-14 solution and fTHP-9 stock were then added sequentially resulting in 8 nM MMP-14 and 15 μM fTHP per well. Whole plate was incubated at 37 °C for 1 h and measured for fluorescence (λ_{Ex} = 324 nm and λ_{Em} = 405 nm)

Library Synthesis. All three libraries were synthesized on Tentagel Rink-amide beads using protocols previously described. See Supporting Information for detailed synthesis of each library used in this study.

Library Screening. *Prescreening.* All beads of the synthesized library were first swelled in DMF for 1 h and washed thoroughly with 1× TBST five times and then equilibrated in 1× TBST overnight under mild shaking. The library was then washed again with PBS five times and then incubated with Starting Block to block possible nonspecific binding for 2 h. A 1:1000 solution of primary antibody, rabbit anti MMP-14, was then used to incubate with all the beads for 2 h at room temperature. After gentle washing, the magnetic beads coated with goat-anti rabbit secondary antibody were then added, made a final concentration of 1 mg magnetic beads/mL (TBS buffer). The whole system was allowed for a further an hour incubation at room temperature with mild shaking. A rare earth 0.5 cm × 0.5 cm × 0.5 cm magnet was used to remove beads that bound to it, which were hits against primary and secondary antibodies. All magnetic beads were then removed by rare earth magnet and the beads were washed with 1× TBST five times. The library was then incubated with Starting Block again and incubated overnight.

Screening with First Library (Figure 1B). After prescreening, all beads were gently washed with TBST, a solution containing 100 nM MMP-14 protein in Starting Block was incubated with all the beads under mild shaking for overnight at 4 °C. After incubation, beads were gently washed with TBS and incubated again with 1:500 rabbit anti-MMP-14 antibody in TBS for 2 h. The beads were washed again and incubated with 1 mg/mL magnetic beads coated with goat antirabbit secondary antibody. After a 2 h incubation, hit beads were isolated with a strong magnet. These beads were collected and stripped of bound protein by incubating in 50% acetonitrile/water (3 × 5 mL) for 30 min, followed by incubation in acetonitrile for 60 min. Each bead was then isolated into a single well of a 96-well filter plate (Millipore) and washed with water.

Screening with Second Library (Figure 3). For the first round of screening, we used exact condition as described above. However, too many hits were separated and made it impossible

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to handle. All bead were stripped off bound protein using a 4.0 M magnesium chloride tris buffer (pH = 7.0). The beads were then washed with 50% acetonitrile/water and equilibrated with TBST for 2 h. After 2 h, all beads were gently washed with TBST, a solution containing 50 nM MMP-14 protein in Starting Block was used to incubate with all the beads under mild shaking for 2 h. After incubation, beads were gently washed with TBS and incubated again with 1:500 rabbit anti-MMP-14 antibody in TBS for 2 h. The beads were washed again and incubated with 1 mg/mL magnetic beads coated with goat antirabbit secondary antibody. After 2 h incubation, hit beads were isolated with a strong magnet. These beads were collected and stripped of bound protein by incubating in 50% acetonitrile/water (3 × 5 mL) for 30 min, followed by incubation in acetonitrile for 60 min. Each bead was then isolated into a single well of a 96-well filter plate (Millipore) and washed with water.

Screening with Third Library (Figure 6). After prescreening, all beads were gently washed with TBST, a solution containing 10 nM MMP-14 protein in Starting Block was used to incubate with all the beads under mild shaking for 2 h. After incubation, beads were gently washed with TBS and incubated again with 1:500 rabbit anti-MMP-14 antibody in TBS for 2 h. The beads were washed again and incubated with 1 mg/mL magnetic beads coated with goat antirabbit secondary antibody. After 2 h incubation, hit beads were isolated with a strong magnet. These beads were collected and stripped of bound protein by incubating in 50% acetonitrile/water (3 × 5 mL) for 30 min, followed by incubation in acetonitrile for 60 min. Each bead was then isolated into a single well of a 96-well filter plate (Millipore) and washed with water.

Single-Bead Fluorescent Polarization Assay. For single-bead labeling with fluorescein azide, 130 μL of a 1:1 mixture of *t*-BuOH and water was first added to each well. A fluorescein azide (structure shown above) methanol solution (0.3 mM) was dispensed to each well, 10 μL per well. Ten microliters of freshly prepared aqueous solution of $CuSO_4$ (0.3 mM) and ascorbic acid (0.6 mM) was then dispensed into each well, and the whole plate was sealed and left on the shaker for 24 h. Final concentrations were: fluorescein azide (20 μM), $CuSO_4$ (20 μM), ascorbic acid (40 μM). The beads were then thoroughly washed with water and then ACN. Compound was cleaved from the bead by incubating each bead in 20 μL of a 50 mg/mL solution of cyanogen bromide (CNBr) dissolved in AcO-H:ACN:water (5:4:1) overnight at room temperature. The next day, the CNBr solution was evaporated by vacuum centrifugation. After it was dried under under vacuum, the cleaved compounds were dissolved in 60% acetonitrile/water, the final concentration of all the compounds were normalized to 100 nM by UV absorption at 495 nm (ε_{495} = 78 000 M^{-1} cm^{-1}). Serial dilution of the streptavidin (homotetramer) in PBS buffer was dispensed in 384-well plate, 10 μL each, and then the dissolved compounds in PBS were added in each well. The whole plate was then mounted on EnVision plate reader (PerkinElmer), and FP data was taken as direct read outs of each well in mP using 450 excitation and 515 nm emission filters. Fitting of the saturation curves to obtain K_D values was accomplished using Prism (GraphPad Software, Inc.) with a one-site specific saturation binding model. For resynthesized compounds, after purification, each compound was dissolved in PBS buffer to obtain a 100 nM solution. Serial dilutions of streptavidin were prepared in a 96-well plate, 50 μL per well. Fifty microliters of compound solution was added to each well and K_D values were obtained as described above.

Structure Identification by MALID-MS and MALDI-MS/MS. For hit sequence identification, all solution was first evaporated by vacuum centrifugation and the dry compound in each well was dissolved in 20 μL of a 75:25 mixture of water/CH_3CN containing 0.1% TFA. 0.6 μL from each well was cospotted with a 10 mg/mL solution of CHCA dissolved in 50% acetonitrile in water containing 0.1% TFA. The spot was dried, and the MALDI plates were analyzed on the 4700 Proteomics Analyzer TOF-TOF mass spectrometer (Applied Biosystems/MD Sciex, Foster City, CA). MS spectra of the intact ions were measured in positive ion reflector mode in a mass range from m/z 800 to 2600 with a time resolution of 0.5 ns, a sensitivity of 50 mV/div and 750 laser shots per sample spot. The instrument parameters (laser position, voltages and times) were optimized for maximal signal intensity and resolution. MALDI-MS spectra were analyzed using the Data Explorer software (version 4.6, AppliedBiosystems,CA, USA). The candidate structures were identified by comparison of all MS- with a table of theoretical fragments for all individual library members.

■ ASSOCIATED CONTENT

Supporting Information

Further experimental details as well as supplementary data tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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