Synthesis and Screening of A DNA-Encoded Library of Non-Peptidic Macrocycles

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ABSTRACT: There is considerable interest in the development of libraries of non-peptidic macrocycles as a source of ligands for difficult targets. We report here the solid-phase synthesis of a DNA-encoded library of several hundred thousand thioether-linked macrocycles. The library was designed to be highly diverse with respect to backbone scaffold diversity and to minimize the number of amide N-H bonds, which compromise cell permeability. The utility of the library as a source of protein ligands is demonstrated through the isolation of compounds that bind streptavidin, a model target, with high affinity.

INTRODUCTION

It is generally acknowledged that molecules with some characteristics outside the “rule of 5”, particularly molecular weight, will be required to engage difficult protein targets efficiently. Such target proteins lack deep binding pockets suitable for interactions with more traditional drug-like molecules. Engagement of these protein surfaces requires larger, conformationally stable molecules capable of occupying multiple shallow pockets. To that end, macrocycles are of particular interest. Powerful biological methods, such as phage display and ribosome display exist for the creation of large libraries of macrocyclic peptides, which have been shown to be an excellent source of ligands for many different proteins. However, with a few notable exceptions, macrocyclic peptides exhibit poor cell permeability, spurriing interest in the development of large libraries of non-peptidic macrocycles that would be better able to access intracellular targets. DNA-encoded libraries (DELs) are of particular interest since these can rival the size of phage display libraries. To date, only a few macrocyclic DELs have been reported. The first was created by Liu and co-workers using DNA-templated chemistry, including a Wittig reaction to close the ring. The most recent version of this approach has produced a library of 256,000 macrocycles from which a 40 nM ligand for insulin degrading enzyme was identified. More recently, Gillingham and colleagues reported a seven cycle DEL comprised of approximately 1.4 million macrocycles in which the ring was closed via amide bond formation. Ligands with µM dissociation constants were mined from this library. Both of these DELs were created by solution-phase chemistry.

In this study, we report the development of efficient chemistry for the synthesis of DELs of thioether macrocycles by solid-phase split and pool synthesis. The construction of these libraries on TentaGel® resin allows them to be screened by simply mixing the beads with a fluorescently labeled protein and isolating those that retain the labeled target using a fluorescence activated cells sorter (FACS). This protocol, in turn, facilitates the use of targets and off targets labeled with different colored dyes, allowing ligands with a high level of selectivity for the target protein to be differentiated from those that do not. We demonstrate the utility of this system by isolating macrocyclic ligands for streptavidin.

RESULTS & DISCUSSION

Establishment of Efficient, DNA-Compatible Macrocyclization Conditions. We sought an efficient strategy for the on-resin macrocyclization of peptoid-inspired conformationally-constrained oligomers (PICCOs) that would be compatible with DNA encoding technology. A common strategy used for the creation of macrocyclic peptides is thioether formation, which often relies on a cysteine residue possessing a protected thiol that is then deprotected to initiate ring closure. However, removal of some thiol protecting groups requires treatment with strong acid. This can be highly detrimental to the DNA encoding tags. Therefore, we focused on the S-trimethoxyphenol (STmp) protecting group, which is efficiently removed under mild reducing conditions that are likely DNA-compatible.

A linker designed to facilitate ionization by mass spectrometry (Linker-2) was generated on 90 µm TentaGel-RAM resin (Fig. S1). The linker was then acylated with Fmoc-Cys(STmp)-OH. After removal of the Fmoc group, the amine was acylated with 2-(chloromethyl)oxazole-4-carboxylic acid to generate Compound 1a (Fig. 1A). To build the test PICCO, the beads were subjected to 3 rounds of amination with 3-methoxybutylamine and subsequent acylation with 2-(chloromethyl)oxazole-4-carboxylic acid to generate Compound 2 (Fig. 1A). After synthesis of the linear precursor was complete, the STmp group was removed and the beads were incubated at 37°C for 18 hours to allow for thioether formation. Macrocyclization was then assessed by liquid chromatography/mass
spectrometry (LC/MS) as well as matrix assisted laser desorption ionization (MALDI). The molecular ion expected for the macrocyclic product was evident, while the peak expected from the linear precursor was not detected (Figures 1B, S2).

Figure 1: Assessment of PICCO Macrocyclization (A) Beads displaying a linker capped with Cys-STmp and an oxazole (Compound 1a) were subjected to 3 rounds of amination and acylation to generate a linear PICCO (Compound 2). Cysteine deprotection allows thioether formation, generating the macrocycle (Compound 3).

(B) LC/MS analysis demonstrates complete cyclization of the linear precursor. See supporting information for complete chromatograms (Figure S2).

Table 1 – A set of 27 DNA-encoded PICCO macrocycles was synthesized in individual wells of a filter plate, then assayed by DNA sequencing and MALDI. Verified sequences and masses are highlighted.

| Well | DNA Barcode | Macroyclic Mass |
|------|-------------|----------------|
| A1   | 130124011501260117012801 | 1815 |
| A2   | 130124011501260117012802 | 1824 |
| A3   | 130124011501260117012803 | 1762 |
| A4   | 130124011501260117012804 | 1782 |
| A5   | 130124011501260117012805 | 1771 |
| A6   | 130124011501260117012806 | 1709 |
| A7   | 130124011501260117012807 | 1711 |
| A8   | 130124011501260117012808 | 1722 |
| A9   | 130124011501260117012809 | 1658 |
| B1   | 130224021502260217022801 | 1723 |
| B2   | 130224021502260217022802 | 1737 |
| B3   | 130224021502260217022803 | 1675 |
| B4   | 130224021502260217022804 | 1658 |
| B5   | 130224021502260217022805 | 1667 |
| B6   | 130224021502260217022806 | 1605 |
| B7   | 130224021502260217022807 | 1700 |
| B8   | 130224021502260217022808 | 1709 |
| B9   | 130224021502260217022809 | 1646 |
| C1   | 130324031503260317032801 | 1618 |
| C2   | 130324031503260317032802 | 1627 |
| C3   | 130324031503260317032803 | 1565 |
| C4   | 130324031503260317032804 | 1660 |
| C5   | 130324031503260317032805 | 1669 |
| C6   | 130324031503260317032806 | 1607 |
| C7   | 130324031503260317032807 | 1669 |
| C8   | 130324031503260317032808 | 1678 |
| C9   | 130324031503260317032809 | 1616 |

To explore this macrocyclization strategy more broadly and to assess the DNA-compatibility of the reaction conditions, a DNA-encoded, 27-molecule “mini-library” was generated. These compounds follow the general design depicted in Figure 2 and represent a diverse set of PICCO scaffolds (Fig S3A). As above, Cys-STmp-OH and 2-(chloromethyl)oxazole-4-carboxylic acid were added.
to 90 µm TentaGel RAM resin displaying Linker-2. As an attachment point for DNA barcode ligation, azido-PEG headpiece DNA (HDNA)\textsuperscript{26,33} was added to the linker by copper-catalyzed click chemistry.\textsuperscript{34} The beads were then aliquoted into 27 wells of a 96-well filter plate. Parallel synthesis was then carried out, specifically three cycles of amine side-chain/backbone unit addition, deprotection of the cysteine, and cyclization. After each round of amination/acylation, an encoding DNA was enzymatically ligated to build the barcode.\textsuperscript{26} Following cyclization, the DNA barcodes were amplified by PCR, purified and sequenced. From the 27 test compounds, 22 showed PCR products of the expected size (Fig. S3B). Of these, 20/22 provided the expected DNA sequences (Table 1). Additionally, MALDI analysis revealed the expected masses of the macrocycles for 23 of the 27 compounds (Table 1). These data suggest that thioether formation is broadly suitable for PICCO macrocyclization and that the Cys-STmp deprotection conditions are DNA-compatible.

**Library Design and Synthesis.** With these preliminary quality control experiments completed satisfactorily, a one-bead, one-compound (OBOC) DEL of approximately 580,000 compounds (Fig. 2A) was constructed on 10 µm TentaGel resin displaying Linker-1 (Fig S1), modified with HDNA, Cys(STmp), and 2-(chloromethyl)oxazole-4-carboxylic acid (Compound 1). This was followed by three cycles of split and pool amination and acylation. After each round of amination/acylation, an encoding DNA was enzymatically ligated onto the encoding chains, then the beads were pooled together and redistributed into a new filter plate. Finally, the cysteine was deprotected allowing macrocyclization.

Because 6-8 different carboxylic acid building blocks were employed at the three “main chain” diversity positions, the library has exceptional scaffold diversity. Moreover, a “null” row was included at the second PICCO position (X2 in Fig. 2), where no carboxylic acid was added to the bead. This results in the omission of the X2 and N-R3 elements shown in Fig. 2A (for these beads only) and generates a library that includes both “2.5-mer” and “3.5-mer” macrocyclic compounds (Fig S4). Twelve different amines were employed at each diversity position.

**Figure 2: General Library Design.** (A) The library was synthesized by solid-phase split-and-pool synthesis on 10 µm resin possessing a linker (Linker-1) modified with HDNA, Cys(STmp), and an oxazole (compound 1). The linear precursors were formed after 3 rounds of amination/acylation/enzymatic ligation using the backbone units shown (B). The STmp group was then removed to allow thioether formation. (C) After macrocyclization, aliquots (about 10,000 beads) were stained with a thiol-reactive fluorescent dye (mBBr) to confirm that the majority of the library completed cyclization.

**On-Resin Macrocyclization Assay.** To assess the level of macrocyclization in the library, a FACS-based macrocyclization assay recently developed in our laboratory was employed. Briefly, a thiol-reactive fluorescent dye, Monobromobimane (mBBr), is added to the beads 18 hours after deprotection of the thiol, which should trigger macrocyclization. Any linear starting material remaining at this time will be stained by mBBr, but the thioether product will not be. The fluorescence intensity on each bead, reflecting the amount of uncyclized material. When this assay was carried on an aliquot of beads from the library synthesis, the results shown in Fig. 2C were obtained. The library beads (purple peak) displayed only a low level of fluorescence, barely greater than that observed when beads displaying a methionine (thioether) unit were stained with mBBr (red peak). In contrast, control beads displaying cysteine (free thiol) displayed a dramatically higher level of fluorescence after staining with mBBr (green peak). These data indicate that the vast majority of library compounds efficiently formed thioether macrocycles (Fig 2C).

**FACS-Based Screening.** The DEL shown in Fig. 2 was used in a two-color, FACS-based screen to identify macrocycles that selectively bind to a target protein, streptavidin, over an unrelated off-target, human IgG. Ten copies of the library (almost six million beads) were incubated with differentially labeled streptavidin (the target) and human IgG (the off-target) as well as a large excess of
unlabeled, diverse competitor proteins, then FACS was used to isolate highly fluorescent beads. To minimize the possibility that the fluorophores would affect the outcome of the experiment, the screen was done in duplicate, but with the labels swapped. Specifically, one screen employed AlexaFluor 647 (A647)-conjugated streptavidin (SA-A647) and AlexaFluor 488 (A488)-labelled human IgG, while the second screen used A488-conjugated streptavidin and A647-labelled human IgG.

The DNA barcodes on the beads displaying a high level of fluorescence in the channel represented by the streptavidin dye were then amplified by PCR and deep sequenced. Because bead screening has a significant false positive rate, we focused solely on compounds that appeared in the hit pool on at least three different beads (the encoding tag includes a bead-specific barcode35). We have found that these so-called “redundant hits” are usually bona fide target protein ligands while “singletions” are usually false positives.35 The redundant hits that appeared in both of the screening experiments were then decoded to generate a list of putative ligands for streptavidin (Table S1). Of these 31 hits, the vast majority (28/31) were the smaller, “2.5-mer” macrocycles, where the X2 and N-R3 elements (Fig. 2A) are absent, indicating that the binding site on streptavidin prefers more compact ligands. This is perhaps not surprising since streptavidin is a biotin-binding protein. Of these 28 compounds, 100% include a pyridine side chain at the second amine position that is almost always (24 out of 28 compounds) adjacent to a thiazole, suggesting that this grouping is important for complex formation (Fig. 3D). Indeed, the other four hits contained a closely related oxazole at this position (Fig. 3D). Almost half of the screening hits contain the difluorobenzylamine-derived side chain at position N-R1 and there is always an aromatic ring at this position in all of the hits. Finally, at position X3, the vast majority of the hits (25/28) have a 1,3-substituted aromatic ring, suggesting that the influence of this spacing and regiochemistry on the conformation of the macrocycles is important for high affinity binding.

Validation of Screening Hits. To determine if the redundant screening hits are indeed bona fide streptavidin ligands, 22 of them were re-synthesized by solid-phase parallel synthesis on both 10 µm and 160 µm beads, in the absence of DNA. The 160 µm beads were treated with benzyl bromide after allowing enough time for macrocyclization to go to completion. This modifies any free thiols, while also while also improving MS ionization, thus making it easier to spot unreacted starting material in the mass spectrometer. The compounds were then released from the beads by treatment with trifluoroacetic acid (TFA) and analyzed by MALDI mass spectrometry. The MALDI data indicate the presence of the correct macrocyclic complex formation (Fig. 3D). Indeed, the other four hits contained a closely related oxazole at this position (Fig. 3D). Almost half of the screening hits contain the difluorobenzylamine-derived side chain at position N-R1 and there is always an aromatic ring at this position in all of the hits. Finally, at position X3, the vast majority of the hits (25/28) have a 1,3-substituted aromatic ring, suggesting that the influence of this spacing and regiochemistry on the conformation of the macrocycles is important for high affinity binding.

Figure 3. Summary of screening experiments. (A) Library beads were incubated with fluorescently-labeled streptavidin (SA) and an orthogonally labelled off-target, then sorted by FACS. (B,C) FACS plots showing data from the SA-A647/IgG-A488 (B) and IgG-A647/SA-A488 replicate screens. Beads that shifted into the 647+ or 488+ gates were collected, and the encoding DNAs deep sequenced for structure determination. (D) “Bottom-up” analysis of 28 “2.5-mer” streptavidin hits, indicating the frequency of each chemical unit at each position. This illustrates a conserved pyridine side-chain (green) that is almost exclusively adjacent to a thiazole backbone unit (blue).

The 10 µm beads displaying resynthesized hits were individually incubated with 100 nM A647-streptavidin and analyzed by FACS for retention of the protein. As a positive control, a previously reported linear PICCO that binds streptavidin (KPM6)36 was included. As a negative control, beads displaying only the linker were also tested. The results are shown in Fig. 4A. All of the beads displaying the various screening hits evinced a much higher brightness than the linker only control, demonstrating that these compounds are bona fide streptavidin ligands. This interaction is selective. When one of the ligands (DEK1; Fig. 4B) was exposed to 200 nM fluorescently labeled IgG, no binding above background was observed (Fig. 4D). The ease of hit validation using this methodology is notable. Dozens of compounds can be re-synthesized on a small scale, using exactly the same conditions employed to create the library, and tested for on-resin binding by FACS in just a few days at little expense.
Figure 4: FACS-based validation of screening hits. (A) FACS histogram showing the relative fluorescence of beads displaying resynthesized hits, or a previously identified PICCO that binds streptavidin (KPM6) after incubation with SA-A647 (100 nM). (B) Structures of DEK1 and DEK2, the two screening hits with the highest apparent affinity for SA-A647. (C) Titration of beads displaying DEK1 or DEL2 with the indicated concentration of SA-A647 followed by FACS analysis. FACS histograms are shown. (D) Assessment of binding selectivity for DEK1. Left: FACS histograms resulting from the analysis of beads displaying either linker only or Linker-DEK1 with 2 nM SA-A647. Right: The same experiment except that 200 nM IgG-A647 was substituted for SA-A647.

The amount of fluorescently labeled protein captured by a compound displayed on 10 µm TentaGel beads at a given concentration of the target correlates directly with the $K_D$ of the ligand-protein complex. In other words, if an immobilized 100 nM ligand is exposed to 10 nM soluble protein, very little of the protein will be retained on the bead but robust binding in the FACS will be detected at 100 nM target protein and the beads will be nearly saturated with protein at 1 µM protein. Therefore, the Fig. 4A data suggest that all of the resin-displayed ligands tested likely have an “effective $K_D$” (including avidity effects since streptavidin is a tetramer) well below 100 nM. Two of the macrocyclic compounds, DEK1 and DEK2, which are nearly identical, differing only by one atom (oxazole vs. thiazole rings at position X1) captured slightly more streptavidin in this analysis than KPM6. These data show that DEL1 and DEK2 are equal, or even superior, to KPM6 as streptavidin ligands. To examine this further, a titration experiment was done in which resin-displayed DEK1 or DEK2 were exposed to different concentrations of A647-streptavidin. The results are shown in Fig. 4C. Robust, above-background binding of the labeled streptavidin is observed even at 1 nM protein concentration for both ligands. Indeed, the results are similar to those obtained using immobilized KPM6, which binds streptavidin in solution with a “true $K_D$” (no contribution from avidity) of 30 nM, suggesting that DEK1 and DEK2 have a similar affinity for streptavidin.

Nature of the macrocycle-streptavidin interaction. As discussed above, all the hits identified in this screen include an oxazole or a thiazole followed by an aminomethyl pyridine (Fig. 3), strongly suggesting that these residues are pivotal for streptavidin binding. To test this, a series of DEK1 analogues were synthesized on 10 µm resin and exposed to 100 nM SA-A647. As shown in Fig. 5, substitution of the pyridine ring with a simple phenyl group essentially abolished binding of the macrocycle to streptavidin. Modification of the oxazole ring in the main chain also had catastrophic consequences for binding. DEK1-II, an isomer of DEK1 in which an isoxazole ring replaces the oxazole moiety, is barely distinguishable from the linker only control. This is also the case for DEK1-III in which a 1,4-substituted phenyl ring replaces the oxazole. These data show clearly that the highly conserved elements in the hit pool are critical to high affinity streptavidin binding.
CONCLUSIONS

An efficient method for the solid-phase synthesis of DNA-encoded libraries of non-peptidic macrocyclic thioethers has been established. A library of more than half a million macrocycles was screened against a model protein, streptavidin, resulting in the identification of dozens of novel ligands. Resynthesis of 22 of these compounds validated that they are indeed bona fide streptavidin ligands with an affinity comparable to a mid-nanomolar PICCO ligand identified previously. An oxazole or thiazole ring in the main chain and a pyridine side chain were shown to be essential for binding, as suggested by their high level of conservation in the hit pool. This work sets the stage for the creation of larger and even more diverse libraries of PICCO macrocycles and the evaluation of these DELs as a source of ligands for difficult targets such as transcription factors and other proteins that function solely through macromolecular interactions.

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ASSOCIATED CONTENT

Supporting Information is provided as a separate file. This includes detailed methods and supplementary data.

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