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Structural Characterization of a Peptoid-Inspired Conformationally Constrained Oligomer (PICCO) Bound To Streptavidin

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Abstract

A screen of a DNA-encoded library of PICCOs was screened against streptavidin (SA) resulting in the isolation of a high affinity ligand. The X-ray crystal structure of this complex is presented, providing the first detailed view of how a PICCO molecule interacts with a protein.

There is considerable interest in developing compounds capable of binding to relatively shallow pockets of protein, for example sites of protein-protein interactions (PPIs). We have developed combinatorial one bead one compound (OBOC) libraries^{1, 2} of PICCOs (peptoid-inspired conformationally constrained oligomers) for this purpose.³ PICCOs are constructed using peptoid-like chemistry,⁴ where amines are used stitch together carboxylic acid building blocks that also contain a good leaving group.^{5–7} The significant molecular “wingspan” of PICCOs, combined with greater chemical diversity in the main chain (compared to peptides, peptoids, etc.) seems to be well suited for challenging targets such as PPI sites, as evidenced by published studies of PICCO ligands for several immune receptors.^{8–13}

OBOC libraries are screened by incubating the beads with a fluorescently labelled target protein in the presence of a large excess of unlabelled, diverse competitor proteins.^{14–16} Beads that retain a high level of fluorescence are separated from the bulk of the library and the compound on the bead is characterized by tandem mass spectrometry after release from the bead.¹⁷ This process has been made far more powerful and efficient by applying the DNA-encoding technology developed by Clark and co-workers¹⁸ to the OBOC platform.¹⁹ The library is created on tiny 10 μ m TentaGel microspheres. This allows ligand-displaying beads that retain a fluorescent target protein to be collected using a fluorescence activated cell sorter (FACS),^{20, 21} after which the encoding tags on these beads are amplified and deep sequenced.

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Conflicts of Interest

T.K. is a co-founder and major shareholder of Deluge Biotechnologies, which creates and screens DNA-encoded libraries.

To fluorescently label the target protein, we were attracted to site-specific biotinylation of an Avi tag.²² Alexafluor-labelled streptavidin (SA), which binds biotin with extremely high affinity, is commercially available in a variety of colors, making detection of beads that have retained a biotinylated protein straightforward. The only downside to this approach is that most OBOC libraries will contain compounds that bind directly to SA, since this protein is known to be highly ligandable.²³ Therefore, it will presumably be necessary to pre-screen OBOC DNA-encoded libraries (DELs) against labelled streptavidin to identify direct ligands to this detection reagent that might be present in the library. We report here such a “pre-screen”, which resulted in the discovery of a high affinity PICCO ligand for SA. We were able to obtain an X-ray crystal structure of the complex, which has provided the first high-resolution view of how this class of molecules interacts with a protein.

About six copies of the library shown in Figure 1 (also see Supplementary Figure 1) were incubated with Alexafluor 647-labeled SA (A647-SA; 100 nM) in the presence of a large excess of diverse competitor proteins (50% Starting Block®) for 12 hours at 37°C. After rigorous washing (see SI for details), the beads were passed through a flow cytometer gated to collect those that showed a high level of red fluorescence. The encoding tags were amplified and deep sequenced. Because of the high false positive rate inherent in bead screening²⁴, only compounds that appeared on more than one bead in the hit pool were considered as potential SA ligands, since these “redundant hits” are far more likely to be bona fide ligands.²⁵ Fluorescein-tagged derivatives of several of these structures (Supplementary Figure S2) were synthesized. Their affinities for SA were evaluated by titration followed by the change in fluorescence polarization (FP). KPM-6 (Fig. 1A) proved to be the highest affinity ligand with an apparent K_D of approximately 30 nM (Figure 1B). KPM-6 did not display detectable affinity for two other proteins, Rpn13 or PAFAH183 (not shown).

Like all of the compounds in the DEL used for this study, KPM-6 has a modestly electrophilic moiety at its terminus (the library was designed to allow for the isolation of covalent ligands to cysteine-containing proteins^{26, 27}). However, we assumed that KPM-6 binds SA reversibly, since the protein lacks a cysteine residue. To test this, fluorescein-labelled KPM-6 was incubated with SA, then the complex was challenged with an excess of biotin (we hypothesized the ligand likely occupied the biotin-binding pocket since this is the most “ligandable” surface of SA). This resulted in a nearly complete loss of the FP signal, indicating disruption of the complex (Fig. 2C). This provides strong evidence that KPM-6 binds to SA reversibly.

Whereas the most frequently isolated screening hits (see Supplementary Figure 2), including KPM-6, had an oxazole unit in the second and/or third positions in the main chain (counting from the C-terminus or the left side of the molecules as drawn in Figure 2), none had an isoxazole building block at these positions, although these compounds are present in the library. This is somewhat surprising, since the oxazole and isoxazole rings would display the side chains preceding and following them with almost identical geometry. To probe this issue more directly, fluorescein-tagged analogues of KPM-6 containing an isoxazole at the second and third main chain positions, or in all three positions, were synthesized and evaluated for binding to SA. As shown in Figure 2B, substitution of the third oxazole with

the isomeric heterocycle resulted in a catastrophic loss of affinity of at least 1000-fold, as did substitution of all three oxazoles with isoxazoles. Replacement of the central oxazole with an isoxazole also had a major effect on binding, with the curve indicating an approximately 100-fold reduction in affinity relative to KPM-6. These data highlight the critical importance of the main chain units in association of the PICCO with SA.

To better understand the nature of binding, we solved the co-crystal structure of the KPM-6•SA complex (PDB 6AVK). As anticipated, KPM-6 sits in the biotin-binding pocket of the protein with the C-terminus protruding into the solvent (Fig. 3A). As predicted from the biotin competition experiment, the electrophilic unit in KPM-6 is intact (Fig. 3A). The chlorine atom is clearly present in the complex. It does not appear to play a critical role in the ligand-protein association, with the possible exception of hydrogen bonding to a structured water. To evaluate the possible role of the chlorine atom, a derivative of KPM-6 in which the chlorine atom was swapped for a methyl group (KPM-6 (ethyl)) was synthesized with a fluorescein tag and its affinity for SA measured. As shown in Fig. 2B, KPM-6 and KPM-6(ethyl) had very similar affinities for SA, indicating that the chlorine atom does not play a critical role.

Globally, there is apparent hydrogen bonding between KPM-6 and several side chains in streptavidin, including Asn23, Ser27, Tyr54, Arg84 and Thr90 (Fig. 3B). Of the two tertiary amide bonds in KPM-6, one is in the trans configuration and the other is cis. The general mode of interaction of SA with KPM-6 and previously identified peptide ligands, such as the “Strep-Tag”²⁸ share a few similarities, such as hydrogen bonding to residues Arg84 and Thr90. But in general, the PICCO and peptide ligands employ quite different mechanisms of binding (compare Figures 3B and 3C).

Since this structure represents the first detailed view of a PICCO-protein complex, a particularly interesting feature is the role played by the main chain oxazoles. KPM-6 adopts a relatively compact binding pose in the structure (Figure 4). The structure reveals clear stacking interactions^{29, 30} between the second and third oxazole rings and the electron-rich aromatic side chains (distances of 4.2 Å and 3.8 Å). In both cases, the aromatic rings are roughly parallel with one another. In contrast, the C-terminal oxazole unit is not stacked against an aromatic ring, but only approaches within 5.2 Å of the acetal moiety in one of the side chains. Without the tight stacking interactions of the second and third main chain oxazole units and the electron-rich aromatic side chains, KPM-6 would be unlikely to maintain the binding pose seen in the structure, explaining why substitution of the electronically very different isoxazole units at either of these positions is highly deleterious for association with SA (Figure 2). Overall, these data highlight the critical importance of the interaction of the main chain units in a PICCO with the protein, as well as with other moieties in the ligand to support the fold of the molecule.

Finally, it is perhaps worthwhile to mention that the DEL from which KPM-6 was isolated was designed as a source of covalent ligands for cysteine-containing proteins. It is interesting that when screened against a protein lacking this residue, it nonetheless yielded a high affinity ligand. As the interest in protein ligands bearing weakly reactive electrophilic units increases, this study is a useful reminder that such molecules have the potential to bind

reversibly and with high affinity to protein pockets lacking a cysteine. Such interactions would not be scored using typical chemoproteomic profiling methods^{31, 32} but nonetheless could contribute important off-target effects in a cell. Thus, for electrophilic molecules of high interest, it will likely be useful to supplement proteome-wide analysis of covalent engagement with other methods capable of detecting reversible interactions, such as the cellular thermal shift assay (CETSA)³³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
General structure of the DEL employed in this study. See Supplementary Figure 1 for full details.

Figure 2.

Structure and binding properties of SA ligands. A. Structure of KPM-6 and several analogues (see text for details). F = Fluorescein. B. Binding of fluorescein-conjugated molecules to SA. C. Biotin competition. Fluorescein-KPM-6 and 100 nM SA were mixed, the FP reading was measured, then an excess of biotin was added, and the reading was taken again.

Figure 3.

Crystal structure of the KPM-6•SA complex. A. KPM-6 occupies the biotin-binding pocket of SA with the electrophilic unit intact. The C-terminal amide of the PICCO is solvent-exposed. B. A ribbon depiction of SA with hydrogen bonds to KPM-6 highlighted. C. A ribbon depiction of SA with hydrogen bonds to the StrepTag peptide highlighted.

Figure 4.

The binding pose of KPM-6 in the crystal structure shows two strong stacking interactions between the oxazole backbone units and the aromatic side chains, with distances of 3.8 Å and 4.2 Å, respectively. The C-terminal oxazole is not stacked and approaches only to within 5.2 Å of the acetal unit in the side chain.