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A General Synthetic Approach for Designing Epitope Targeted Macrocyclic Peptide Ligands

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Abstract: *We describe a general synthetic strategy for developing high-affinity peptide binders against specific epitopes of challenging protein biomarkers. The epitope of interest is synthesized as a polypeptide, with a detection biotin tag and a strategically placed azide (or alkyne) presenting amino acid. This synthetic epitope (SynEp) is incubated with a library of complementary alkyne or azide presenting peptides. Library elements that bind the SynEp in the correct orientation undergo the Huisgen cycloaddition, and are covalently linked to the SynEp. Hit peptides are tested against the full-length protein to identify the best binder. We describe development of epitope-targeted linear or macrocycle peptide ligands against 12 different diagnostic or therapeutic analytes. The general epitope targeting capability for these low molecular weight synthetic ligands enables a range of therapeutic and diagnostic applications, similar to those of monoclonal antibodies.*

Monoclonal antibodies (mAbs) raised against short peptide (ca. 10 amino acids) antigens (epitopes)[1] can target protein regions inaccessible to traditional small-molecule binders of proteins, which bind in well defined binding pockets. This capability has made mAbs the therapeutic platform of choice for any extracellular or membrane targets that they can readily access,[2] as well as the only diagnostic reagents which can detect targets such as enzymes that are phosphorylated at specific residues.[3] Crystal structures of antibody–antigen complexes often reveal direct contacts of the antigen with ca. 10 residues in the variable heavy CDR loop region, while the rest of the antibody acts as a semi-rigid scaffold holding the right residues in the correct orientation.[4] This chemical model guides our approach: we target a relatively small fragment of the protein, but we also screen that fragment against different libraries designed to explore ligand scaffold rigidity.

Our strategy mirrors that used for developing epitope targeted mAbs, but yields peptide-based protein catalyzed capture (PCC) agents[5] with small molecular footprints ($\approx$1 kDa). A key strategy element is in situ click chemistry, which is a powerful tool for target-guided synthesis of small molecules and peptide ligands for proteins.[6] We synthesize a comprehensive one-bead-one-compound (OBOC) library[7] of linear or macrocyclic peptides. We screen the library against a synthetic epitope (SynEp), a 9–30 amino acids long peptide representing a modified variant of the epitope of interest. The library elements are designed to present an azide (or alkyne) click handle, and a complementary alkyne (or azide) presenting amino acid is strategically substituted into the SynEp. During the screen, a library element that interacts with the SynEp in the right orientation undergoes 1,3-dipolar cycloaddition to covalently bond to the epitope. The precise orbital alignment of terminal alkynes and azides required for the cycloaddition increases the entropic penalty of orientation making the un-catalyzed reaction non-spontaneous under ambient conditions. This limitation is exploited here: we rely on specific interactions between the SynEp and the library element to overcome this entropic penalty so that the reaction proceeds un-catalyzed.

We provide a detailed description of the screening process and demonstrate its generality through the identification of 12 epitope targeted PCC agents. These ligands fulfill very challenging targeting aims such as selective detection of a phosphorylated epitope,[8] a single amino acid point mutation,[9] a genus specific sequence in a malarial protein biomarker, and a universally conserved small region of a geographically variable malarial biomarker. The process of development of the PCC agents against malarial biomarker proteins is elaborated to illustrate the technique. Macrocyclic peptide libraries have yielded superior performing PCC agents, and so are described in detail.

The various proteins and epitopes targeted, along with the amino acid sequence of the best PCC binder, are given in Table 1. The SynEp is a 9–30 amino acid long fragment of the

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Table 1: Epitope-targeted PCC agents.[a]

Protein and description	SynEp (corresponding residues in full length protein)	Binder sequence	EC_{50} or K_D **(bold)** and *selectivity assay (italics)*
I. *Pf*LDH *Plasmodium falciparum* lactate dehydrogenase (malaria diagnostics)	LISDAELEAIFD–Az4–PEG_5–Biotin (amino acids 218–229 of *Pf*LDH)	Cy(HWSAN–click)	23.4 nM (Figure S1A) **40.6 nM** (Figure 2D) *(Figure 2C)*
II. *Px*LDH *Plasmodium* (x = many species) lactate dehydrogenase (general malaria diagnostics)	Biotin–W–PEG_5–GVEQV–Pra–ELQLN (amino acids 297–308 of *Px*LDH)	hevwh (Li)	1.7 μM (Figure S2B)
III. *Pf*HRP2 *Plasmodium falciparum* histidine-rich protein-2 (malaria diagnostics) **epitope A** (C terminus of *Pf*HRP2)	AHHATDAHHAAAHHEAATHC–Pra–PEG_5–Biotin	Cy(GSTEWL–rcm)	20 nM (Figure S3A) **54.2 nM** (Figure 2H) *(Figures 2G and S3C)*
IV. *Pf*HRP2 epitope B (variant of type 2 repeat of *Pf*HRP2)	Biotin–PEG_5–AHHAADAHHA–Pra	Cy(Y–4F–Phe–YRV–click)	538 pM (Figure S4B)
V. *Pf*HRP2 epitope C (type 2 repeat of *Pf*HRP2)	Biotin–PEG_5–AHHAHHAAD–Pra	Cy(YKYYR–click)	218 nM (Figure S5B)
VI. *Pf*HRP2 epitope D (N terminus of *Pf*HRP2)	Biotin–PEG_5–LHETQAHVDD–Pra	Cy(RYKHY–click)	4 nM (Figure S6B)
VII. L1R vaccinia virus L1R myristyl protein (closely related to variola)	Biotin–PEG_5–KALMQLTTKATQIA–Pra–PKQVAGTGVQ	Cy(DARNI–click)	875 nM (Figure S7B) *(Figure S7C)*
VIII. IL17-F (Interleukin member of the highly homologous IL-17 family)	Biotin–PEG_3–FFQ–Az4–PPVPGGS (amino acids 40–54 of IL17-F)	Cy(RRATS—click)	66 nM (Figure S8B) *(Figure S8C)*
IX. *p*-Akt2 (phospho serine/threonine kinase; strategy targets *p*-Ser474 epitope)	Biotin–PEG_5–ITPPDRYDSLGLL ELQRTHFPQF(pS)YSASIRE (amino acids 450–481 of pAkt2)	Cy(YYTYT–click)	122 nM (Figure S9B) *(Figure S9D)*
X. *p*-Akt2 (Protein kinase B2; strategy targets region adjacent to *p*-Ser474)[8]	ITPPDRYDSLGLLELQRTHFPQF [pS-(Zn^2L)]YSASIRE (amino acids 450–481 of pAkt2)	wkvkl (Li)	3.6 μM
XI. $Akt1^{E17K}$ (Protein kinase B with oncogenic point mutation)[9]	Biotin–PEG_5–PEVAIVKEGWLKKRGK Y–Pra–KTWRPRYFLLKNDG	yleaf (Li)	61 nM **54 nM**
XII. BoNT A LC (Botulinum neurotoxin serotype A light chain)[10]	Az4–SFGHEVLNLTRN–PEG_4–Biotin (amino acids 166–179)	Cy(NYRWL–click)	68 nM **70 nM** *(Figure S10)*

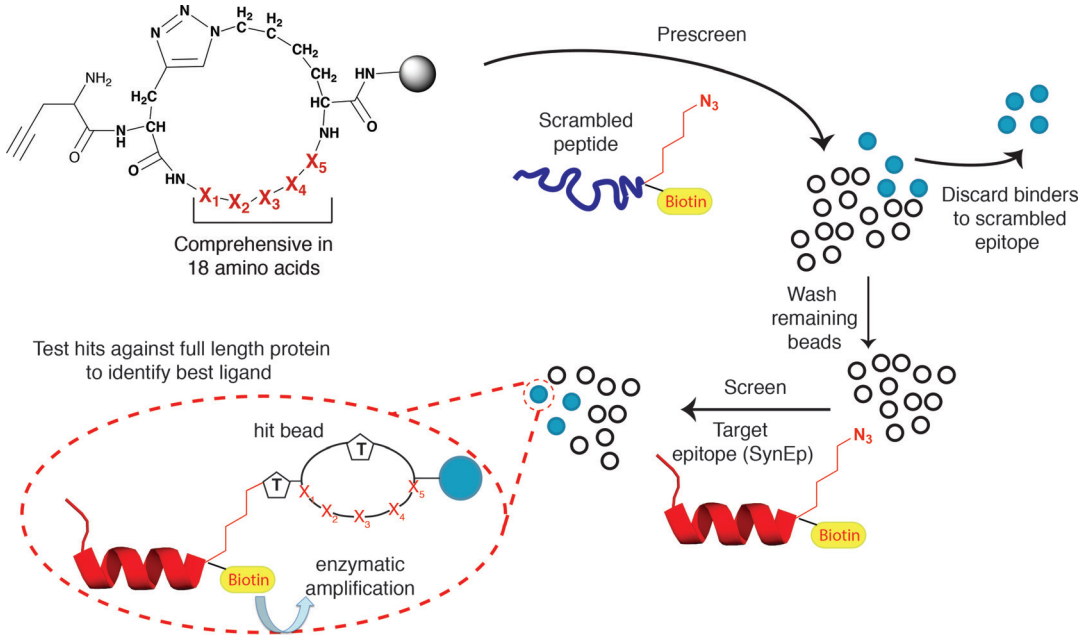
[a] For each entry, the sequence of the synthetic epitope (SynEp) is given, indicating the substitution position for the azide or alkyne "click" handle and the biotin tag, and, in parentheses, the position of the epitope in the full-length protein. Azides are incorporated as azidolysine (Az4) and alkynes are incorporated as propargylglycine (Pra) in the SynEp. Polyethylene glycol (PEG) is used as a spacer between the peptide sequence and the biotin tag in the SynEp. Macrocycle PCCs are indicated by the prefix "Cy", while linear PCCs are indicated by the suffix (Li). Macrocycles closed using Cu-catalyzed azide–alkyne cycloaddition (CuAAC) or Ru^{IV}-catalyzed ring-closing metathesis reaction (RCM) are indicated by "click" or "rcm" respectively. PCC IX is obtained through a variation of the epitope targeting strategy. PCC X was developed by screening against the epitope chelated to a dinuclear zinc ligand tagged with an azide handle and biotin.

target protein. In general, PCCs developed against shorter (9–12-mer) epitopes and longer (20–30-mer) SynEps have similar affinity and selectivity.

The SynEp is prepared with a terminally appended biotin assay label, as well as the click handle substitution, which can be appended at the C or N terminus of the SynEp (Table 1, entries IV–VI), or it can be substituted for specific natural residues.

We have replaced arginine, cysteine and lysine residues with azidolysine (Az4) (Table 1, entries I, VIII, XII) and leucine and isoleucine with propargylglycine (Pra) (Table 1, entries II, III, VII, XI). To develop binders that detect single point mutations (Akt1 E17K, Table 1, entry XI) or a post-translational modification (Akt2 pS474, Table 1, entry X), separating the click handle by 3–4 residues from the key residue is an effective strategy.

The epitope targeted in situ click screen is a single generation screen, with results that are filtered through one or more anti-screens. The OBOC peptide libraries,[7] which are comprehensive in 18 natural or non-natural amino acids ($\approx$2 million sequences), are screened against a biotin tagged scrambled sequence of the same length as the SynEp, or an off-target peptide representing a different epitope of the same protein (Table S1 in the Supporting Information). Non-specific binders from the anti-screen are identified colorimetrically by treatment of the screened library with an anti-



Scheme 1. An epitope-targeted in situ click screen. A peptide library (a macrocycle library is shown) is first screened against a scrambled variant of the target synthetic epitope (SynEp). The library elements that bind to that variant are detected by utilizing the biotin label (yellow) to execute an enzymatic assay that changes the color of reactive beads from clear to turquoise. The remaining library is washed, resuspended in buffer, screened against the target SynEp, and then thoroughly washed to remove non-covalently bound copies of the SynEp. Following treatment with anti-biotin antibody conjugated to alkaline phosphatase and its colorimetric BCIP substrate, hit beads are picked for sequencing. Candidate ligands are tested against the full-length protein to identify the best binder.

biotin antibody (alkaline phosphatase conjugate; anti-biotin-AP) and its substrate 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Scheme 1 illustrates a screen such as that used against *Pf*LDH (Table 1, entry I). The binders against a scrambled version of the epitope (Table S1) are eliminated, and the rest of the library is washed with protein denaturizing solutions. After re-equilibration in the screening buffer, the pre-cleared library is incubated with the SynEp, which, for *Pf*LDH, is based upon a unique *falciparum*-specific sequence at residues 218–229 (Table 1). For this screen, the library is washed with protein denaturizing solution to remove any non-covalently bound SynEp, prior to enzymatic development to identify hit beads. The hit beads (typically about 2–5 per 10^6 screened peptides) are synthesized in bulk without the terminal alkyne, and tested for binding to full-length protein and, in case of *Pf*LDH, to the targeted epitope (without the click handle) and to non-*falciparum* variants of LDH. The best binder (Table 1 and Scheme 1) is selected based on these and similar selectivity and affinity assays.

A result from Table 1, and from previous work,[5] is the superior performance of the macrocycles relative to the linear PCCs. This is anticipated.[11] Macrocycles yield an average $-\log [K_D$ (or $EC_{50})]$ value of >7, while for linear PCCs, that value is <6. The macrocyclic libraries used here are designed for these screens, and so we turn to a discussion of those libraries.

Macrocyclic peptide libraries prepared using phage display are typically cyclized through a disulfide linkage originating from two cysteine amino acids,[12] and are susceptible to a number of physical, chemical and biochemical processes[13] which can confound screening results. Peptide sequences from such libraries are obtained by DNA sequencing. We sought to develop a macrocycle library which is stably cyclized, and which can be sequenced using a standard method such as Edman degradation. We use the Cu^I-promoted alkyne–azide cycloaddition (CuAAC) reaction[14] and the Ru^{IV}-catalyzed ring-closing metathesis reaction (RCM)[15] to create OBOC macrocyclic libraries on TentaGel (TG) beads for sequencing by Edman degradation. CuAAC-cyclized libraries start with the synthesis of a linear library Pra-$X_1X_2X_3X_4X_5$-Az4-TG using standard solid phase peptide synthesis (SPPS). X_1–X_5 comprises the variable region and artificial amino acids are readily incorporated. The library is subjected to the CuAAC reaction (Figure 1). The 4-carbon side chain of Az4 at the C-terminus is optimal for intramolecular cyclization with Pra at the N-terminus. Alkyne containing amino acids at the N-terminus give higher cyclization yields.[16] Washes with dimethylformamide (DMF) solution of sodium diethyldithiocarbamate remove the adsorbed Cu^I from the library. IR spectroscopy is used to verify on bead cyclization for one representative case. The uncyclized peptide has an asymmetric NNN stretch ($\approx 2100\ cm^{-1}$), while the isobaric cyclized peptide does not (Figure S11). The CuAAC reaction typically yields monomer as the major product, with small amounts of dimer and trimer formation (Figure S12).

For RCM stapled peptides, a linear library of the form R_8-$GX_1X_2X_3X_4X_5$-S_5-TG, (where $R_8 = (R)$-2-(7′-octenyl)alanine, $S_5 = (S)$-2-(4′-pentenyl)alanine) is synthesized. On-bead peptides are cyclized using 1st generation Grubbs catalyst[15] in dichloroethane (DCE), twice for 6 hours under argon atmosphere. Excess catalyst is removed by washing with 5%

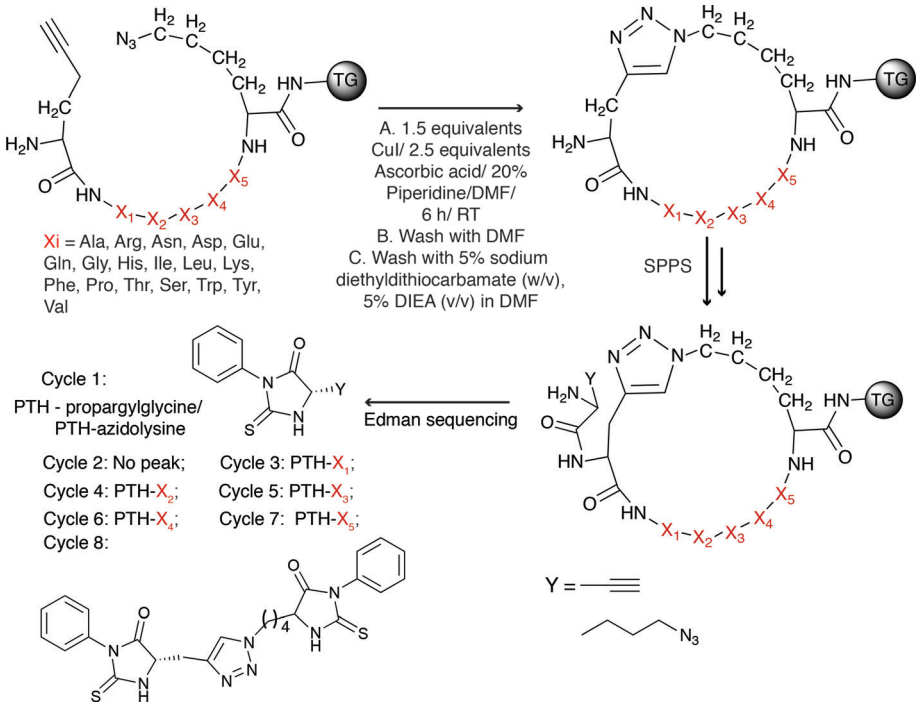


Figure 1. Synthesis and sequencing of a Cu-catalyzed azide–alkyne cycloaddition (CuAAC) cyclized peptide library. A linear peptide library (top left) is subjected to CuAAC reaction. An azide- or alkyne-containing amino acid is incorporated after the cycloaddition and its protecting group removed, following standard solid phase peptide synthesis protocol. The library is treated with a trifluoroacetic acid cocktail to remove side-chain protecting groups and stored in buffer. For sequencing, a single bead is loaded into a cartridge of an Edman Peptide Sequencer. The first cycle yields the phenylthiohydantoin (PTH) derivative of the azide- or alkyne-handle-containing amino acid. The next Edman cycle yields no peak as it cleaves the amide bond forming the cycle but the PTH derivative is bound to the resin. Successive cycles yield PTH derivatives of component amino acids.

triphenyl phosphine in dichloromethane (DCM) and 5% oxine solutions in DMF (Figure S13). The amino acid pair R_8 and S_5 are chosen based upon a literature report.[17] Following RCM cyclization of the linear peptides, high-pressure liquid chromatography (HPLC) purification shows the major peak either as the monomer (Figure S14A), or as the dimer with traces of monomer (Figure S14B). This is confirmed by mass spectrometry analysis. The relative yields of the cyclic monomer or dimer appear to be dependent upon the position of the PEG linker and biotin handle (Figure S14C). Single beads from the libraries are sequenced by Edman degradation (Figures S15 and S16), each yielding the characteristic pattern described in Figure 1 (bottom left).

We describe two PCCs developed using SynEp targeting against *Plasmodium falciparum*-specific malarial biomarker proteins *Pf*HRP2 and *Pf*LDH. Precise epitope targeting can significantly help to identify reagents for the universal, selective capture of these two proteins. For example, *Pf*LDH has high sequence homology with *Px*LDH, where *x* represents malaria species other than *falciparum*. *Pf*HRP2 is an unstructured protein that exhibits broad sequence variations across geographic regions in which the parasite is found.[18] The few regions of *Pf*HRP2 that are conserved are represented by short sequences.

*Pf*LDH is distinguished from the 90% homologous *Pv*LDH (v = vivax) at residues 218–229.[19] The epitope (highlighted in blue, Figure 2A), when screened according to Scheme 1, yields six ligand sequences (Table S2). The six cyclic sequences are tested for binding against the epitope (Figure S17) and the full-length *Pf*LDH (Figure S18), and **1** emerges as the best binder. The molecular structure of **1**, and associated single point ELISA assay demonstrating selectivity, are provided in Figure 2B and Figure 2C. Biotin tagged **1** is titrated against 100 nM recombinant glutathione-S-transferase (GST) tagged *Pf*LDH, *Pv*LDH and hLDH (h = human), to test its selectivity. Subsequent treatment with an anti-GST antibody (horse radish peroxidase conjugate; anti-GST-HRP) and a chromogenic substrate shows selective binding of **1** to the *Pf*LDH. The fluorescence polarization (FP) of fluorescein tagged **1** against *Pf*LDH yields a K_D of 40.6 ± 9.3 nM (Figure 2D). The K_D value is close to the EC_{50} value of 23.39 ± 6.37 nM (Figure S1A).

*Pf*HRP2, a *Pf* specific biomarker for both acute and chronic *falciparum* infection,[20] is localized in the cytoplasm and the cell membrane of infected erythrocytes, and is secreted into the serum by erythrocytes.[21] *Pf*HRP2 consists of varying numbers of repeats of alanine and histidine-rich motifs (Figure 2E).[18] The type 6 repeat (sequence AHHATD) and the type 12 repeat (sequence AHHAAAHHEAATH) of *Pf*HRP2 are near universally conserved.[18] Thus, we develop a PCC against a SynEp representing the C-terminus of the protein, containing both conserved regions (Table 1, entry III). The nine cyclic hits have significant sequence homology and a preponderance of hydrophobic amino acids (Table S3), and all were tested for binding to *Pf*HRP2 (Figure S19). Three promising hits, when titrated against varying concentrations of full-length *Pf*HRP2 (Figure S20), yield EC_{50} values in the nM range, and the binder with the lowest EC_{50} is chosen as the best binder **2**. The molecular structure of **2** is depicted in Figure 2F. The biotin tagged ligand shows no cross-reactivity with a related LDH protein from the same *Plasmodium falciparum* species (Figure 2G), and binds selectively to recombinant *Pf*HRP2 in 1% serum (Figure S3C). The ligand was titrated with varying concentrations of the GST-tagged *Pf*HRP2 and treated with anti-GST-HRP to obtain an EC_{50} value of 20.5 ± 2 nM (Figure S3A), close to the K_D value (54.3 ± 12.1 nM) determined by FP (Figure 2H).

The reported epitope targeting strategy is conceptually similar to the approach used for developing monoclonal antibodies, in that PCCs can apparently target most parts of

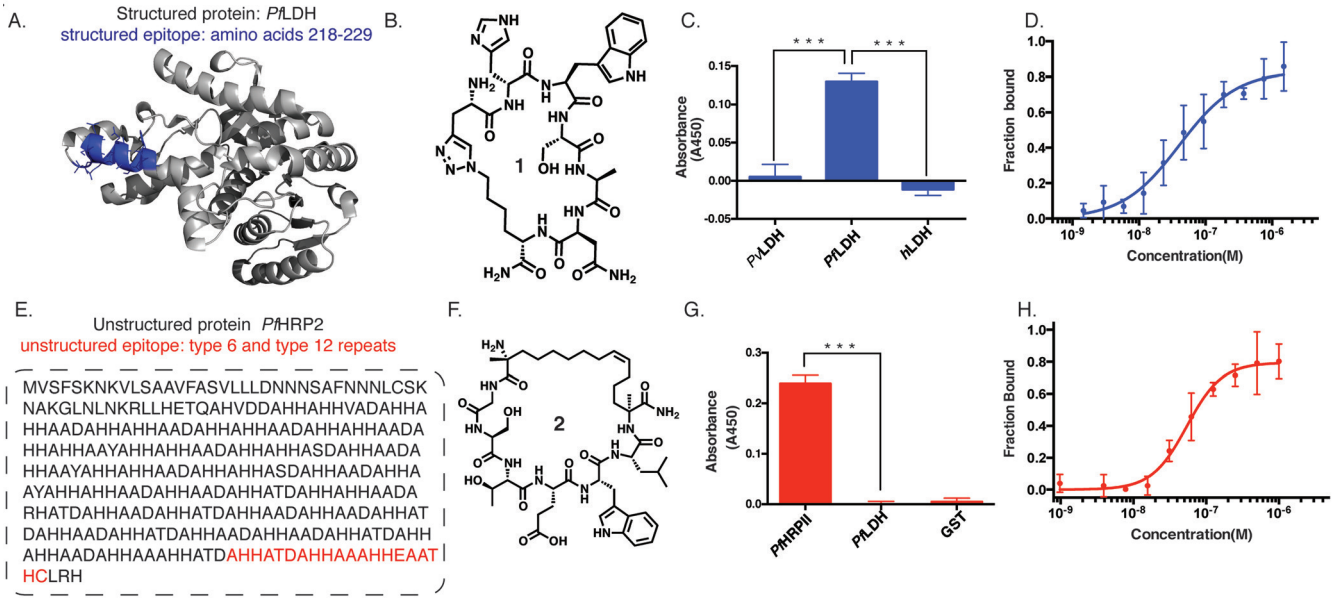


Figure 2. Two distinct cases of epitope targeting. A) A region from the structured *Pf*LDH protein that uniquely distinguishes *Pf*LDH relative to other LDH variants, is highlighted in blue. B) The molecular structure of **1**, the consensus PCC developed to bind to the blue highlighted epitope of *Pf*LDH. C) Peptide **1** selectively distinguishes *Pf*LDH from *Pv*LDH (v = vivax) and hLDH (h = human) in single point ELISA (proteins spiked into buffer solution at 100 nM concentration). D) Fluorescein tagged **1** is titrated with recombinant *Pf*LDH and the fluorescence polarization is measured, yielding a K_D of 40.63 ± 9.27 nM. E) Targeting a universally conserved epitope of intrinsically unstructured *Pf*HRP2 protein. One recombinant (r) *Pf*HRP2 amino acid sequence is shown. The epitope targeted is highlighted in red. F) Molecular structure of **2**, the consensus PCC developed to bind to the highlighted epitope from *Pf*HRP2. G) Selective binding of **2** to 20 nM GST-*Pf*HRP2, showing negligible cross-reactivity with 20 nM GST-*Pf*LDH, a related target from *Plasmodium falciparum* species, and with 20 nM GST protein; H) Peptide **2** binds to recombinant *Pf*HRP2 with a K_D of 54.26 ± 12.05 nM, as measured by fluorescence polarization.

the protein. Thus, there is no requirement for a hydrophobic binding pocket. PCC reagents, however, maintain many advantages of small molecules. A primary consideration in developing the PCCs of Table 1 is the value of the targeted epitope within the context of the ultimate application. For example, the ability to target conserved regions of otherwise genetically varying proteins, or unique regions of protein targets that differentiate them from close analogues, makes this approach a compelling strategy for many challenging diagnostic targets. Depending upon the final required metrics, the PCCs of Table 1 may be viewed as starting point, similar to how an initial small molecule drug candidate is viewed. Both types of ligands can be improved via chemical iterations to improve avidity and/or stability characteristics.[22]

A limitation of this PCC approach is that libraries with more than a few million unique sequences are difficult to screen manually. Other "artificial antibody" approaches allow screening of libraries with 10^8–10^{16} unique linear or cyclic sequences.[23,24] However, the synthetic flexibility that permits incorporation of non-natural amino acids, is tough to achieve with biological methods. This flexibility enables the in situ click screen in which hits are identified via the formation of an on-bead reaction product (rather than a simple binding assay). This advantage is significant and yields viable binders from a single generation screen. However done, the general ability to target specific epitopes on specific proteins is enabling. For example, the broad flexibility of the epitope targeting strategy is already permitting exploitation of the tertiary structure of the drug target as a scaffold for developing highly potent inhibitors.[10]

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