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Synthesis and screening of a cyclic peptide library: Discovery of small-molecule ligands against human prolactin receptor

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Abstract—Prolactin receptor is involved in normal lactation and reproduction; however, excessive prolactin levels can cause various reproductive disorders such as prolactinomas. Small-molecule antagonists against the human prolactin receptor (hPRLr) thus have potential clinical applications and may serve as useful molecular probes in biomedical research. In this work, we synthesized a large, support-bound cyclic peptide library (theoretical diversity of 1.2×10^7) on 90-μm TentaGel beads and screened it against the extracellular domain of hPRLr. To facilitate hit identification, each TentaGel bead was spatially segregated into outer and inner layers, with a cyclic peptide displayed on the bead surface while the bead interior contained the corresponding linear peptide. The identity of a positive bead was revealed by sequencing the linear encoding peptide within the bead by partial Edman degradation/mass spectrometry. Screening of the library resulted in 20 hits, two of which were selected for further analysis and shown to bind to hPRLr with dissociation constants of 2–3 μM.

1. Introduction

Cyclic peptides are widely produced in nature and possess a broad range of biological activities.[1–3] Several cyclic peptides such as cyclosporin A (an immunosuppressant),[4] daptomycin (an antibiotic),[5] and caspofungin (an antifungal agent)[6] are clinically used therapeutic agents. The superior pharmacological properties of cyclic peptides as compared to their linear counterparts are partly due to the reduced conformational freedom of the former, which makes cyclic peptides more resistant to proteolysis and potentially more potent and specific binding ligands to macromolecular receptors. Also important is the fact that many naturally occurring cyclic peptides contain non-proteinogenic amino acids (e.g., D-amino acids, N^{α}-methylated amino acids, and amino acids containing unusual side chains), which further increase their protease resistance as well as structural diversity.[1–3] In recent years, there has been much interest in the development of cyclic peptides of improved or novel biological/pharmacological activities, through modification of natural products[7–10] or de novo design.[11–13] In particular, efforts have been made to synthesize and screen cyclic peptide libraries against various biological targets (e.g., proteins, RNA, and live cells). The reported cyclic peptide libraries can be classified into biological and chemical libraries, on the basis of how they were prepared (ribosomally vs chemically synthesized). Among the ribosomally synthesized cyclic peptide libraries, the most commonly employed method has been phage display and peptide cyclization is mediated by the formation of a disulfide bond between a pair of cysteines, added before and after a random peptide sequence.[14–17] Another powerful and increasingly popular method generates N-to-C cyclized peptide libraries inside living cells by utilizing the protein splicing properties of split inteins.[18,19] Finally, mRNA display has been used to generate linear peptide libraries by in vitro translation and the resulting linear peptides were chemically cyclized between their N-termini and a lysine side chain by using a bifunctional reagent.[20,21] A major advantage of the biological methods is their ability to generate exceptionally high library diversity (10^9–10^{14}), which in turn increases the probability of discovering high-affinity ligands against a molecular target. The biological methods are, however, generally limited to the 20 proteinogenic amino acids.[20]

Keywords: Cyclic peptides; Combinatorial library; Partial Edman degradation; Prolactin; Prolactin receptor.
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In principle, one can chemically synthesize large cyclic peptide libraries by the split-and-pool method[22,23] *and* incorporate any unnatural amino acids into the libraries. However, combinatorial synthesis and screening necessitate post-screening hit identification; for backbone cyclized peptides, this has been a challenge. To avoid this problem, Houghten et al.[24] and others[25] screened cyclic peptide libraries by iterative deconvolution. Unfortunately, this method is laborious and does not always identify the most active component of a library. Parallel synthesis has also been employed to prepare cyclic peptide libraries, but the size of these libraries has typically been small (on the order of 10^2).[7,9] We recently developed a general methodology for the combinatorial synthesis, encoding, screening, and post-screening identification of cyclic peptides.[26] In this method, each resin bead (e.g., TentaGel) is spatially segregated into outer and inner layers, with a cyclic peptide displayed on the bead surface and the corresponding linear peptide restricted to the bead interior. During library screening against a macromolecular target (e.g., a protein), which is too large to diffuse into the bead, only the cyclic peptide on the bead surface is accessible to the target. After a positive bead is selected, the identity of the cyclic peptide on that bead is determined by sequencing the linear peptide within the bead by partial Edman degradation/mass spectrometry (PED/MS).[27]

Prolactin is a proliferation and viability factor for breast epithelial cells, prostate epithelial cells, and various cells of the immune system. It acts by binding to two prolactin receptors (PRLrs) on the surface of target cells. Although first identified as classic endocrine hormone, prolactin has been shown to be produced by tumors of these cells where it functions as a viability factor promoting the growth of the tumor cells.[28–30] Competitive inhibition of prolactin thus provides a potential treatment of these tumors. Many efforts have been made to develop antagonists against the human prolactin receptor (hPRLr). While previous work to develop hPRLr antagonists has focused on various mutant forms of prolactin, this work describes a novel approach for designing and screening a new class of cyclic peptide inhibitors that act upon the prolactin receptor.

2. Results and discussion

2.1. Library design, synthesis, and evaluation

A cyclic octapeptide library containing five random residues, cyclo($AX_1X_2X_3X_4X_5VE$)BBRM-resin (Fig. 1; B is β-alanine and X_1–X_5 represents the random residues), was designed. Each of the random positions contained 26 amino acids including 12 proteinogenic α-L-amino acids [Arg, Asp, Gln, Gly, His, Ile, Lys, Pro, Ser, Thr, Trp, and Tyr], four non-proteinogenic α-L-amino acids [L-4-fluorophenylalanine (Fpa), L-norleucine (Nle, used as a replacement of Met), L-ornithine (Orn), and L-phenylglycine (Phg)], six α-D-amino acids [D-Ala,

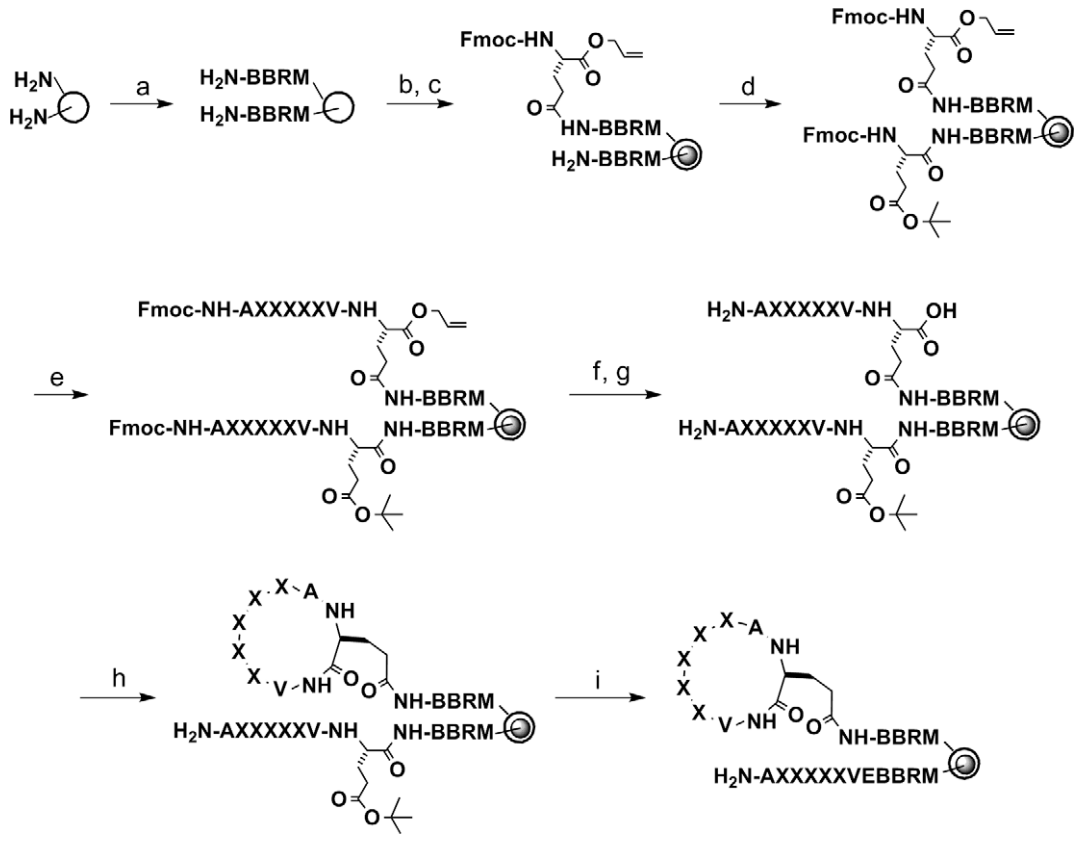

Figure 1. Synthesis of cyclic peptide library. Reagents: (a) standard Fmoc/HBTU chemistry; (b) soak in water; (c) 0.5 equiv. N^{α}-Fmoc-Glu(δ-NHS)–O– $CH_2CH{=}CH_2$ in Et_2O/CH_2Cl_2; (d) excess Fmoc-Glu(tBu)-OH, HBTU; (e) split-and-pool synthesis by Fmoc/HBTU chemistry; (f) $Pd(PPh_3)_4$; (g) piperidine; (h) PyBOP, HOBt; and (i) TFA.

D-Asn, D-Glu, D-Leu, D-Phe, and D-Val], and four N^{α}-methylated α-L-amino acids [L-N^{α}-methylalanine (Mal), L-N^{α}-methylleucine (Mle), L-N^{α}-methylphenylalanine (Mpa), and sarcosine (Sar)]. The non-proteinogenic amino acids, many of which are frequently found in naturally occurring nonribosome-synthesized peptides, were included to increase the structural diversity of the library and the stability of library members against proteolytic degradation. Other criteria used during the selection of the building blocks included minimization of mass degeneracy and commercial availability/cost. The invariant glutamate served as a convenient anchor for attachment to the solid support via its side chain, while allowing peptide cyclization at its α-carboxyl group. An alanine was added to the N-terminus of the random sequence to give more uniform peptide cyclization yields, as we anticipated that peptides with N-terminal N^{α}-methylated amino acids (which are less reactive toward a carboxyl group) might be problematic during the cyclization reaction. The L-valine residue C-terminal to the random sequence was arbitrarily chosen to complete the cyclooctapeptide, although L-valine is frequently found in naturally occurring cyclic peptides.[1–3] The BBRM sequence was added to provide a flexible linker to facilitate protein binding (β-Ala-β-Ala), peptide release (cleavage at Met with CNBr), and MS analysis (Arg provides a fixed positive charge). The theoretical diversity of the library was 26^5 or 1.19×10^7.

The cyclic octapeptide library was synthesized on TentaGel microbeads (90 μm, $\sim 2.86 \times 10^6$ beads/g, ~100 pmol peptides/ bead). Each bead was spatially segregated into outer and inner layers by using the method of Lam (Fig. 1).[31] Subsequently, peptide synthesis was carried out as previously described[26] so that the outer layer of each bead displayed a unique cyclic peptide (~50 pmol), whereas the inner core of the bead contained a linear peptide of the same sequence (~50 pmol). The random region was constructed by the split-and-pool method[22,23] to give a biphasic, one bead-one compound (OBOC) library. To assess the quality of the library, 70 beads were randomly selected from the library and the peptides were released by treatment with CNBr and analyzed by MALDI-TOF MS. Out of the 70 beads, 65 (93%) produced a pair of peaks separated by 18 mass units in the expected mass range for the library members (*m*/*z* 966–1611 for cyclic peptides) (data not shown). We assigned the *m*/*z* M peaks to cyclic peptides and the *m*/*z* (M+18) peaks as the corresponding linear peptides. For each of the 65 beads, the molar ratio of cyclic/linear peptide was estimated from the relative abundance of the *m*/*z* M and *m*/*z* (M+18) peaks, assuming that cyclic and the corresponding linear peptides had equal ionization efficiency in the MS. The molar ratio of the 65 beads varied from 0.004 to 4.0, but had an average value of 0.36 (the theoretical value was 1.0). The remaining five beads each produced only one peak in the expected *m*/*z* range; it was not possible to determine whether the signal was derived from the linear or cyclic peptide.

Finally, we tested whether the library members, which contained non-standard amino acids such as N^{α}-methylated amino acids, could be effectively sequenced by PED/MS. Thus, 40 beads were randomly selected from the library and subjected to PED/MS. Out of the 40 beads, 33 (82%) gave MS spectra of sufficient quality to permit unambiguous sequence assignment. This success rate was somewhat lower than what we typically obtain with peptide libraries consisting of only the 20 proteinogenic amino acids (~95%). This is primarily due to the poor reactivity of N^{α}-methylated amino acids toward the capping agent (Fmoc-OSU[27]), generating weak and sometimes invisible MS signals when PED reaches these amino acids.

2.2. Library screening against hPRLr

To identify peptide ligands against hPRLr, the extracellular domain of hPRLr (hPRLr-ECD) was labeled with a biotin or fluorescent group (Texas-red) and used for library screening in a two-stage process. During the primary screening, 400 mg of the cyclic peptide library (~1 million beads) was incubated with 600 nM Texas-red labeled hPRLr-ECD overnight and the 83 most colored beads were removed from the library under a fluorescence microscope. The choice of Texas-red was crucial, as some of the TentaGel beads showed strong background fluorescence at shorter wavelengths. The colored beads were washed extensively with 8 M guanidine hydrochloride to denature/remove the bounded proteins and subjected to a second round of screening, during which the beads were incubated with the biotinylated hPRLr-ECD and a streptavidin-alkaline phosphatase conjugate. Binding of hPRLr-ECD to a bead would recruit alkaline phosphatase to the bead surface. Upon subsequent addition of an alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate, the

Table 1. Peptide sequences selected against hPRLr-ECD

Bead No.	Sequence[a]				
	X_1	X_2	X_3	X_4	X_5
1	**D-Leu**	**L-Tyr**	**L-Ser**	**L-Tyr**	**L-Arg**
2	**D-Asn**	**L-Tyr**	**L-Ser**	**D-Phe**	**L-Lys**
3	L-Tyr	D-Phe	L-Gln	L-Fpa	L-Lys
4	**L-Arg**	**L-Tyr**	**L-Gln**	**L-Ser**	**L-Arg**
5	L-Lys	L-Fpa	D-Ala	Gly	L-Arg
6	L-Arg	L-Thr	L-Tyr	D-Ala	L-Arg
7	L-Lys	D-Asn	L-Tyr	L-Arg	L-Arg
8	L-Trp	L-Orn	D-Val	Gly	L-Phg
9	D-Phe	L-Lys	L-Arg	L-Pro	L-Tyr
10	**L-Gln**	**L-Arg**	**D-Phe**	**L-Tyr**	**L-Phg**
11	**L-Thr**	**L-Arg**	**L-Fpa**	**Gly**	**L-Sar**
12	L-Lys	L-Lys	L-Arg	L-Phg	L-Nle
13	L-Gln	D-Ala	L-Arg	L-Phg	D-Val
14	L-Arg	L-Gln	L-Lys	L-Tyr	D-Val
15	L-Orn	L-Tyr	Gly	L-Thr	D-Phe
16	L-Orn	L-Gln	L-Ile	D-Phe	L-His
17	**Gly**	**L-Mle**	**L-Gln**	**L-Arg**	**D-Phe**
18	L-Mle	L-Gln	L-Sar	L-Arg	L-Arg
19	L-Lys	L-Thr	L-Ser	D-Leu	L-Pro
20	**L-Trp**	**L-Tyr**	**L-Ser**	**L-Arg**	**L-Sar**

[a] Sequences in boldfaced letters were derived from intensely colored beads during secondary screening, whereas the rest were from lightly colored beads.

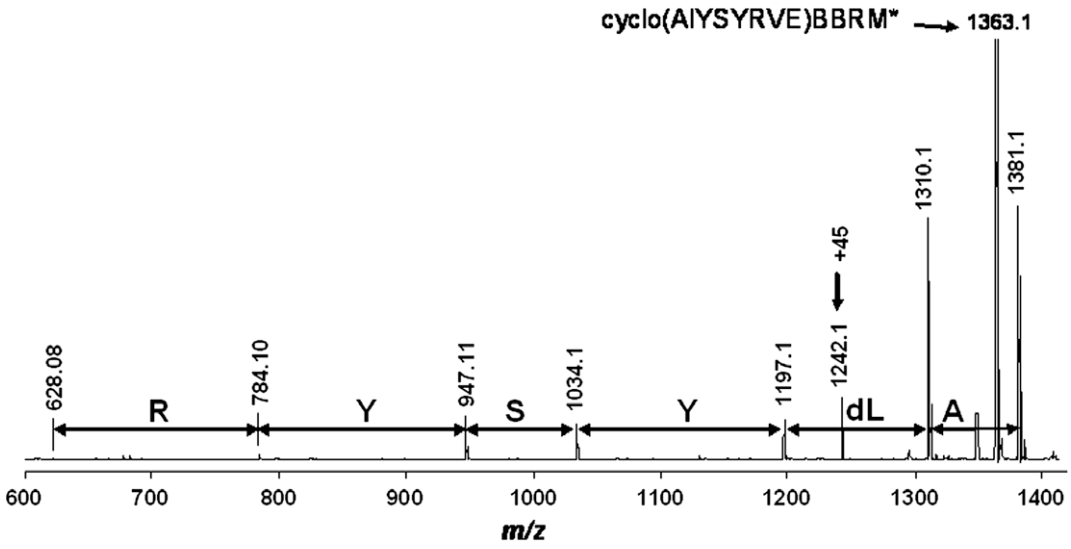

Figure 2. MALDI-TOF mass spectrum of a selected peptide, cyclo(AlYSYRVE)BBRM*, the linear encoding peptide, and its PED products (derived from bead No. 1 of Table 1). The presence of the +45 peak indicates the presence of D-Leu (instead of L-Ile or L-Nle). dL or l, D-leucine; M*, homoerine lactone.

bead became turquoise colored.[32] The secondary screening resulted in 11 strongly colored and 24 weakly colored beads, which were individually sequenced by PED/MS to give 7 and 13 complete sequences, respectively (Table 1). Figure 2 shows the MALDI-TOF mass spectrum of one of the intensely colored beads, after 6 rounds of PED and CNBr cleavage (for peptide release from the bead). The sequence of the cyclic peptide was unambiguously assigned as cyclo(AlYSYRVE)BBRM-resin (where the lower-case letter represents a D-amino acid). As a control, 100 colorless beads were randomly picked from the primary screening and subjected to the secondary screening under identical conditions. None of the 100 beads showed any color during the secondary screening.

Inspection of the selected sequences reveals that there is no clear consensus among the peptides, although some of the selected sequences are highly similar to each other. For example, two peptides derived from the intensely colored beads, cyclo(AlYSYRVE) and cyclo(AnY-SfKVE) (Table 1, bead No. 1 and 2), are highly homologous. Likewise, peptides 6 [cyclo(ARTYaRVE)] and 7 [cyclo(AKnYRRVE)] are structurally similar. Overall, the selected sequences are rich in hydrophobic aromatic amino acids (e.g., L-Tyr, D-Phe, L-Phg, and L-Fpa) and positively charged residues (e.g., L-Arg, L-Lys, and L-Orn), whereas acidic residues are completely absent. Interestingly, the interaction between hPRLr-ECD and human growth hormone are primarily mediated by aromatic and positively charged residues.[33,34] Lack of a clear consensus sequence may indicate that the selected peptides bind to different sites on hPRLr-ECD. This is not unexpected, since binding to anywhere on hPRLr-ECD would result in a positive bead in our two-stage screening. However, it is also possible for structurally diverse ligands to bind to the same site (or overlapping sites) on a protein, as exemplified by some Src homology 2 domains.[32]

2.3. Binding analysis of selected peptides by surface plasmon resonance (SPR)

Three of the selected peptides (Table 1, peptides 1, 4, and 17) were individually synthesized on a larger scale and analyzed for binding to hPRLr-ECD by SPR. The cyclic peptides contained a flexible linker, bis(8-amino-3,6-dioxaoctanoyl) [$(PEG)_2$], attached to the side chain of the invariant glutamate and a biotin at the side chain of a C-terminal lysine (Fig. 3a). The peptides were purified by reversed-phase HPLC and immobilized to a streptavidin-coated SPR biosensor chip. Increasing concentrations of hPRLr-ECD protein were passed over the chip, resulting in increasing amount protein bound to the surface (Fig. 3b). Secondary plot of the amount of bound protein (in response units) as a function of protein concentration and nonlinear regression curve fitting gave dissociation constants (Fig. 3c). Peptides 1 and 4 bound to hPRLr-ECD with K_D values of 2.9 and 2.0 μM, respectively (Table 2). Surprisingly, peptide 17 showed no significant binding to the receptor protein under the experimental conditions. One possible explanation is that binding of peptide 17 to hPRLr-ECD may also involve the BBRM linker, which was replaced by a $(PEG)_2$ moiety during SPR analysis (Fig. 3a).

To determine whether peptides 1 and 4 bind to the prolactin-binding site, the peptides were tested for their ability to inhibit the interaction between hPRLr-ECD and a M158C mutant prolactin by SPR. The M158C mutation has no effect on its binding affinity to hPRLr,[35] but permitted the immobilization of the mutant protein to a CM5 biosensor chip through a disulfide bond at cysteine-158. Different concentrations of the peptides (0–100 μM) were preincubated with hPRLr-ECD (0.5 μM) for 1 h and the mixtures were passed over the biosensor chip. Even at 100 μM, the peptides did not significantly inhibit the binding of the receptor protein to the immobilized prolactin, indicating that the

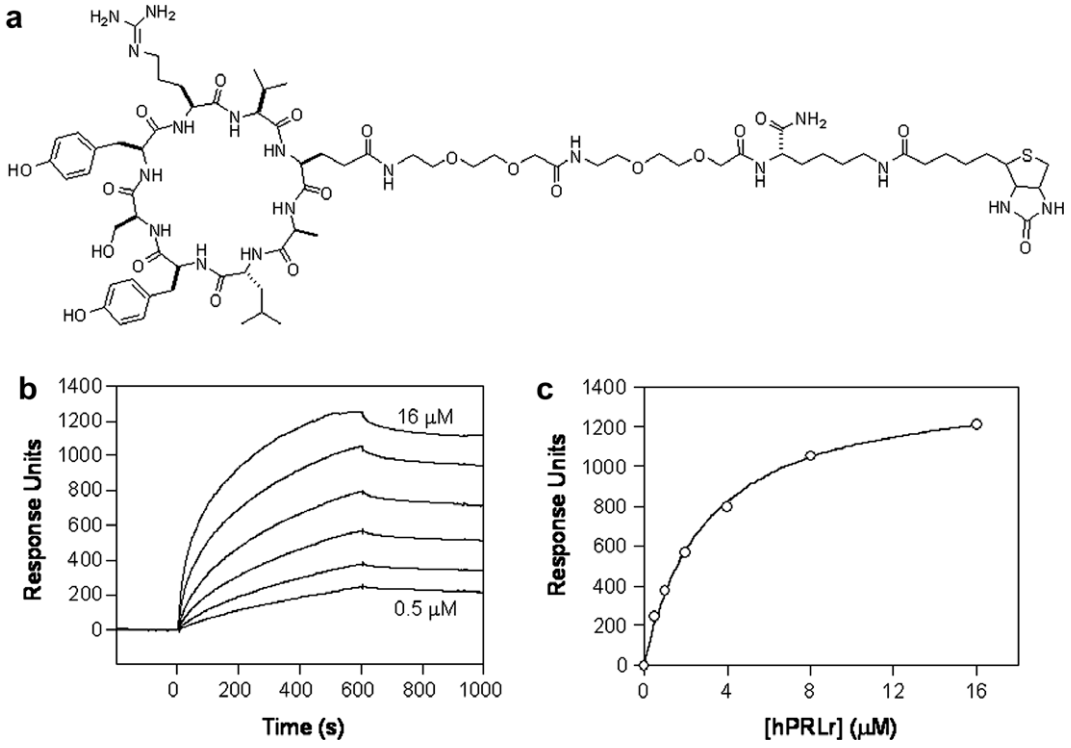

Figure 3. SPR analysis of the interaction between hPRLr-ECD with immobilized peptide cyclo(AlYSYRVE). (a) Structure of the biotinylated peptide; (b) overlaid SPR sensograms showing increasing amount of bound hPRLr-ECD as the concentration of hPRLr-ECD increased (0.5, 1.0, 2.0, 4.0, 8.0, and 16 μM); (c) Secondary plot of the response units from part b as a function of hPRLr-ECD concentration. The curve was fitted to the data according to equation $RU_{eq} = RU_{max} \times [\text{hPRLr-ECD}]/(K_D + [\text{hPRLr-ECD}])$.

Table 2. Dissociation constants (K_D) of selected peptides against hPRLr-ECD

Peptide No.	Peptide sequence	K_D (μM)
1	Cyclo(AlYSYRVE)	3.0 ± 0.2
4	Cyclo(ARYQSRVE)	2.0 ± 0.4
17	Cyclo[AG(Mle)QRfVE]	No binding

peptides bind to another site(s) on the receptor surface. This is consistent with the observation that the selected sequences did not show a clear consensus. In order to select for ligands that bind to the prolactin-binding site, one may have to employ a tertiary screening, during which excess prolactin is added to the screening reaction and colorless beads would be selected (negative screening). Such studies are currently underway in our laboratories.

3. Conclusion

A large OBOC library of natural product-like cyclic peptides has been synthesized by using D-, N^{α}-methylated, as well as proteinogenic amino acids as building blocks. Screening of the library against the ECD of hPRLr identified cyclic peptide ligands of low-micromolar dissociation constants against the protein receptor. Thus, we have developed a general methodology for synthesizing, screening, and decoding large combinatorial cyclic peptide libraries to discover small-molecule ligands against any macromolecular target.

4. Experimental

4.1. Materials

Reagents for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), or NovaBiochem (La Jolla, CA) except for 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), which was from GenScript (Piscataway, NJ). N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSU) was from Advanced ChemTech. Phenyl isothiocyanate (PITC) was purchased in 1-mL sealed ampoules from either Sigma-Aldrich or Pierce (Rockford, IL) and a freshly opened ampoule was used in each experiment. *N*-Hydroxysuccinimidyl ester of Texas Red-X was from Invitrogen (Carlsbad, CA), while (+)-biotin *N*-hydroxysuccinimide was from Sigma. Streptavidin (SA)-coated biosensor chips and HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20) were purchased from BIAcore (Piscataway, NJ). Synthesis of N^{α}-Fmoc-Glu(δ-*N*-hydroxysuccinimidyl)–O–$CH_2CH{=}CH_2$ has previously been reported.[26] Recombinant M158C human prolactin and hPRLr-ECD were expressed in *Escherichia coli* and purified as previously described.[35,36]

4.2. Synthesis of cyclic peptide library

The peptide library was synthesized on 5.0 g of TentaGel S NH_2 resin (90 μm, 0.26 mmol/g, ~100 pmol/bead).

All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (BBRM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/*N*-methylmorpholine (NMM) as the coupling reagents. The coupling reaction was typically allowed to proceed for 2 h and the beads were washed with DMF (3×) and DCM (3×). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min) and the beads were exhaustively washed with DMF (6×). To spatially segregate the beads into outer and inner layers, the resin was treated with 20% piperidine in DMF (5 + 15 min), washed with DMF and water, and soaked in water overnight. The resin was drained and suspended in a solution of N^{α}-Fmoc-Glu(δ-*N*-hydroxysuccinimidyl)–O–$CH_2CH{=}CH_2$ (0.50 equiv) and diisopropylethylamine (2.0 equiv) in 30 mL of 55:45 (v/v) DCM/diethyl ether. The mixture was incubated on a rotary shaker for 30 min at room temperature. The beads were washed with 55:45 DCM/diethyl ether (3×) and DMF (8×) to remove water from the beads and then treated with 2 equiv of Fmoc-Glu(tBu)-OH and HBTU/HOBt/NMM in DMF (30 min). Next, Val was added to the resin using standard Fmoc/HBTU chemistry. For the synthesis of random positions, the resin was split into 26 equal portions and each portion (~190 mg) was coupled with 4 equiv of a different Fmoc-amino acid. After the addition of each random residue, the four portions of resin that contained N^{α}-methylated amino acids at their N-termini were combined, mixed, and re-split into 26 portions and a different Fmoc-amino acid (12 equiv) was coupled to each portion with HATU/NMM as the coupling reagents. The coupling reaction was repeated until the vast majority of beads showed negative chloranil test,[37] and any remaining free amines were acetylated by treatment with acetic anhydride. The other 22 portions were combined, mixed, and split into 26 equal portions, each of which was coupled twice with a different Fmoc-amino acid using standard HBTU/HOBt/NMM conditions. To differentiate isobaric amino acids during MS sequencing, 5% (mol/mol) of CD_3CO_2D was added to the coupling reactions of D-Ala, Lys, D-Leu, and Orn, while 5% $CH_3CD_2CO_2D$ was added to the Nle reactions.[27] After the five random positions were synthesized, an Ala was added to the N-terminus of all peptides to facilitate the cyclization reaction. Next, the allyl group on the C-terminal glutamate was removed with a solution containing tetrakis(triphenylphosphine)palladium (1 equiv), triphenylphosphine (3 equiv), formic acid (10 equiv), and diethylamine (10 equiv) in anhydrous THF overnight at room temperature. The beads were sequentially washed with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3×), DCM (3×), and DMF (3×). The N-terminal Fmoc group was then removed in 20% piperidine and the beads were washed with DMF (6×), 1 M HOBt in DMF (3×), DMF (3×), and DCM (3×). For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a rotary shaker for 3 h. The resin was washed with DMF (3×) and DCM (3×) and dried under vacuum for >1 h. Side chain deprotection was carried out with a modified reagent K (6.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 2 h. The resin was washed with TFA and DCM, and dried under vacuum before storage at −20 °C.

4.3. Labeling of prolactin receptor with biotin and Texas-Red

To label hPRLr-ECD (amino acids 25–234) with biotin, 1.2 mg of the protein was diluted into 400 μL of a buffer containing 50 mM $NaHCO_3$ (pH 8.5) and 50 mM NaCl and mixed with 2 molar equivalents of *N*-hydroxysuccinimidyl biotin dissolved in DMSO (10 mg/mL). The mixture was incubated for 30 min at room temperature and any remaining biotin activated ester was quenched by treatment with 50 μL of 1 M Tris buffer (pH 8.5) for 5 min. The reaction mixture was passed through a Sephadex G-25 column (GE Healthcare, Piscataway, NJ) eluted with 30 mM HEPES, pH 7.4, 150 mM NaCl to remove any free biotin. Protein concentration was determined by both Bradford protein assay and UV absorbance at 280 nm. The extinction coefficient for hPRLr was calculated to be 66,000 M^{-1} cm^{-1} using Protoparam.[38] The concentrations obtained by the two methods were within 2-fold difference. Labeling of the protein with Texas-Red was similarly carried out but with the following modifications. The reaction mixture was always kept in dark and the amount of labeling was determined by comparing the absorbance at 280 nm and 595 nm (absorption maximum for Texas-Red; ε = 80,000 M^{-1} cm^{-1}). Typically, ~70% of the protein population was labeled.

4.4. Library screening

The cyclic peptide library was subjected to two rounds of screening to minimize false positives due to nonspecific binding. Primary screening was based on direct binding of Texas-red labeled hPRLr-ECD to beads bearing binding ligands. Briefly, 400 mg of the library resin (~1 million beads; ~10% of the library) was swollen in DCM, washed extensively with DMF, doubly distilled H_2O, and HBST buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), and blocked overnight at 4 °C with 10 mL of blocking buffer (HBST buffer containing 0.1% gelatin) in a 9.5-mm Petri dish. Texas-red labeled hPRLr-ECD was added to a final concentration of 600 nM and the library was incubated overnight at 4 °C with gentle shaking. The resulting library was viewed under a Olympus SZX12 Research stereo microscope equipped with a fluorescence illuminator (Olympus America, Center Valley, PA) and positive beads (red colored) were manually removed from the library with the aid of a micropipette. The positive beads (83 total) were washed extensively with HBST buffer, ddH_2O, and 8 M guanidine hydrochloride to remove the bound proteins and subjected to a second round of screening. The 83 beads were washed with 1 mL doubly distilled H_2O three times, then with 1 mL HBST buffer three times and incubated in the blocking buffer in a microcentrifuge tube overnight at 4 °C. After removing the blocking buffer with a pipette, the beads were

suspended in 1 mL of 100 nM biotinylated hPRLr-ECD in the blocking buffer and incubated overnight at 4 °C. The beads were then transferred into 1 mL of blocking buffer containing streptavidin-alkaline phosphatase conjugate (1 μg/mL final concentration) and incubated at 4 °C for 10 min. The beads were quickly washed with 1 mL of the blocking buffer (3×) and 1 mL of staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$) (3×). Finally, 1 mL of the staining buffer and 100 μL of a stock solution of 5-bromo-4-chloro-3-indolyl-phosphate (5 mg/mL) were added to the beads and intense turquoise color developed on positive beads in 15 min. The turquoise colored beads were manually removed under a dissecting microscope, separated into ''intensely colored'' and ''lightly colored'' categories, and individually sequenced by PED/MS.[27] A control experiment was carried out by randomly selecting ~100 colorless beads from the primary screening and subjected to the same secondary screening procedure. None of the control beads became turquoise colored.

4.5. Synthesis of individual peptides

Each peptide was synthesized on 100 mg of Rink Resin LS (0.2 mmol/g) in a manner similar to that employed for the library construction except that spatial segregation was not necessary. Prior to cyclization, the allyl group on Glu was first removed followed by removal of the Fmoc group. Peptide cyclization was carried out in the same manner as library construction and the progress of cyclization was monitored by ninhydrin tests of any remaining amines. The peptides were cleaved off the resin and deprotected by the modified reagent K and triturated three times with Et_2O. The crude peptides (1.5 mg each) were dissolved in 10 μL of DMSO and treated with 3 molar equivalents of *N*-hydroxysuccinimidyl biotin in 1 M sodium bicarbonate (pH 8.5) (total reaction volume of 100 μL). The mixture was incubated for 30 min at room temperature and any remaining biotin ester was quenched by treatment with 5 μL of 1 M Tris buffer (pH 8.5) for 5 min. Biotinylated cyclic peptides were purified by reversed-phase HPLC on a C_{18} column and the identity of the peptides was confirmed by MALDI-TOF mass spectrometric analyses.

4.6. Measurement of binding affinities of individual peptides by SPR

SPR measurements were carried out on a BIAcore 3000 instrument. A streptavidin-coated biosensor chip was conditioned with 1 M NaCl in 50 mM NaOH according to manufacturer's instructions. Biotinylated cyclic peptides were loaded onto the chip by passing the peptide solution over the surface at a flow rate of 15 μL/min until a constant response unit was obtained (~500 RU). A blank flow cell (no immobilized peptide) was used as a control. Next, varying concentrations of hPRLr-ECD (0.5–16 μM) were passed over the chip for 10 min at a flow rate of 15 μL/min. Higher flow rates (up to 50 μL/min) did not change the binding kinetics. In between two runs, the sensorchip was regenerated by injecting a strip buffer (10 mM NaOH, 200 mM NaCl, 0.05% SDS) for 3 s at a flow rate of 100 μL/min. The equilibrium response unit (RU_{eq}) at a given protein concentration was obtained by subtracting the response of the blank flow cell from the flow cell that contained the peptide. The dissociation constant (K_D) was obtained by nonlinear regression fitting of the data to the equation:

$$RU_{eq} = RU_{max} \times [\text{hPRLr-ECD}]/(K_D + [\text{hPRLr-ECD}]),$$

where RU_{eq} is the measured response unit at a given protein concentration and RU_{max} is the maximum response unit.

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