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Screening Bicyclic Peptide Libraries for Protein−Protein Interaction Inhibitors: Discovery of a Tumor Necrosis Factor-α Antagonist

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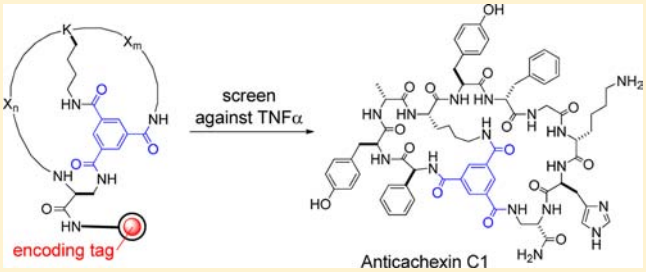
Supporting Information

ABSTRACT: Protein−protein interactions represent a new class of exciting but challenging drug targets, because their large, flat binding sites lack well-defined pockets for small molecules to bind. We report here a methodology for chemical synthesis and screening of large combinatorial libraries of bicyclic peptides displayed on rigid small-molecule scaffolds. With planar trimesic acid as the scaffold, the resulting bicyclic peptides are effective for binding to protein surfaces such as the interfaces of protein−protein interactions. Screening of a bicyclic peptide library against tumor necrosis factor-α (TNFα) identified a potent antagonist that inhibits the TNFα−TNFα receptor interaction and protects cells from TNFα-induced cell death. Bicyclic peptides of this type may provide a general solution for inhibition of protein−protein interactions.



INTRODUCTION

Protein−protein interactions (PPIs) are of central importance in essentially all biochemical pathways, including those involved in disease processes. PPIs therefore represent a large class of new, exciting drug targets.[1] However, PPIs are considered the prototypical "undruggable" or "challenging" targets for the conventional small-molecule approach, because PPIs usually involve large, flat interfaces, with which small molecules usually do not make enough points of contact to impart high affinity or specificity. For some of these PPIs, small-molecule inhibitors have been successfully developed by targeting the so-called "hot spots" at the interaction interface.[1−4] A more general approach is to develop specific antibodies against the PPI interface.[5−7] Nonimmunoglobulin protein scaffolds have also been engineered into specific binders to target proteins through library screening and/or in vitro evolution.[8−11] Antibodies and protein binders possess large binding surfaces of their own and are capable of making multiple contacts with a target surface (e.g., those involved in PPIs). Unfortunately, protein-based drugs are impermeable to the mammalian cell membrane; as such they are generally limited to targeting extracellular proteins and are not orally available. Recently, others[12−18] and we[19,20] have begun a third approach by generating macrocyclic compounds (e.g., cyclic peptides and peptidomimetics) as PPI inhibitors. These macrocycles typically have molecular weights between 500 and 2000 and occupy a largely untapped therapeutic space, often referred to as the "middle space".[21] Because of their relatively large sizes and ability to make multiple points of contact with a flat surface, macrocycles effectively compete with proteins for binding to flat surfaces and yet retain many of the pharmacokinetic properties of small molecules such as membrane permeability.[22−24] Compared to protein drugs, macrocycles have greater metabolic stability, less likelihood of eliciting an immune response, and lower cost of production. To further rigidify the structures and improve the binding affinity/specificity and metabolic stability, bicyclic peptides and peptoids have also been generated.[25−32] Since rational design of macrocyclic inhibitors against PPIs is difficult, a popular approach has involved synthesizing and screening large libraries of bicyclic peptides and peptoids. To date, bicyclic peptide libraries have only been synthesized ribosomally by phage or mRNA display and are largely limited to proteinogenic amino acids (and certain unnatural α-L-amino acids) as building blocks.[27−29]

Tumor necrosis factor-α (TNFα) is a pleiotropic inflammatory cytokine of a variety of functions, many of which are not yet fully understood.[33] TNFα is responsible for cachexia, a wasting in patients with chronic diseases such as cancer and tuberculosis,[34] and is implicated in the development of septic shock and multiorgan failure in severely infected patients.[35] It is also responsible for numerous chronic inflammatory disorders such as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, psoriasis, hidradenitis suppurativa, and refractory asthma.[36] These disorders are currently treated with protein inhibitors, including monoclonal antibodies infliximab (Remicade), adalimumab (Humira), or certolizumab pegol (Cimzia), and a circulating receptor fusion protein etanercept (Enbrel). These proteins bind specifically to TNFα and prevent its interaction with TNFα receptors (TNFRs).

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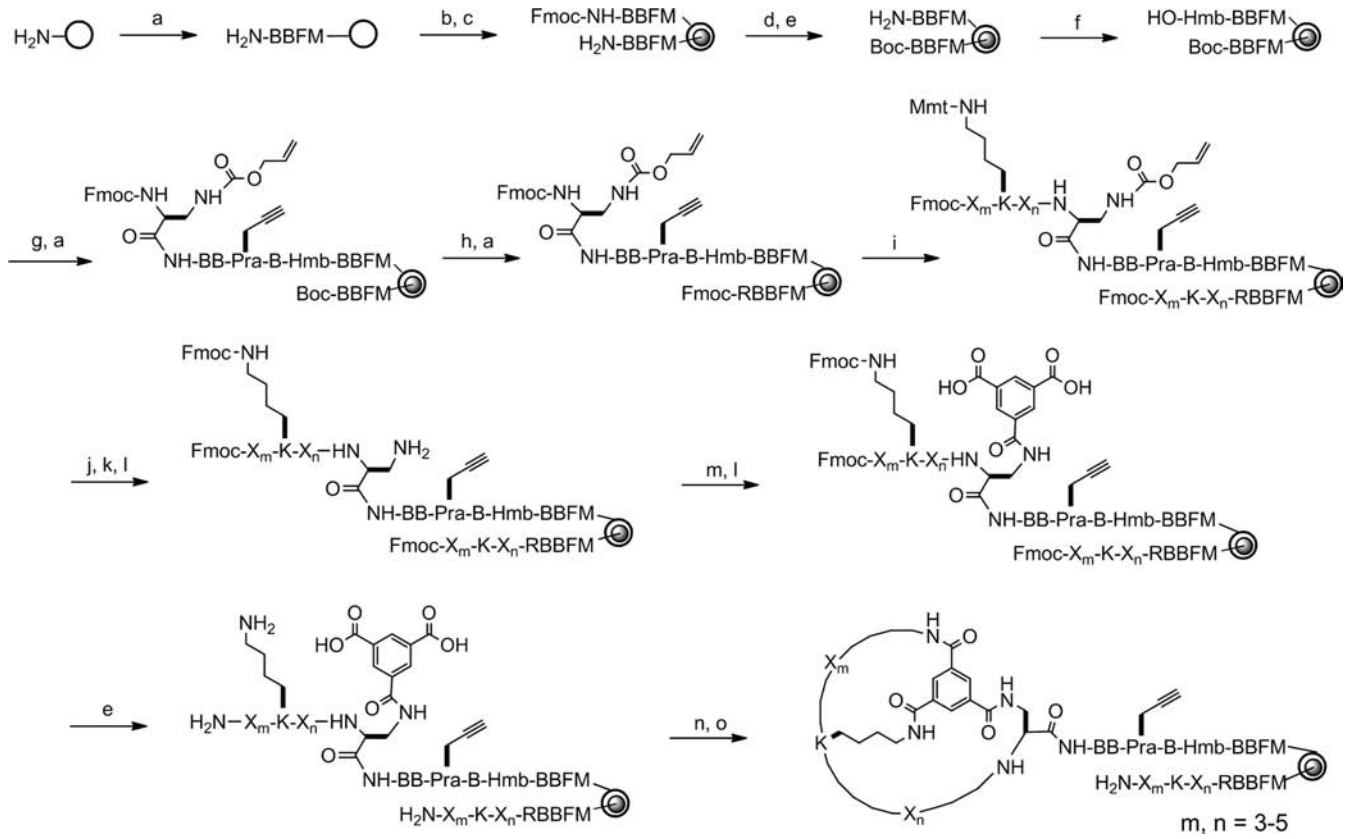

Figure 1. Synthesis of bicyclic peptide library. Reagents and conditions: (a) Standard Fmoc/HATU chemistry; (b) soak in water; (c) 0.4 equiv of Fmoc-OSu in Et_2O/CH_2Cl_2; (d) di-*tert*-butyl dicarbonate; (e) piperidine; (f) 4-hydroxybenzoic acid/HBTU/HOBT; (g) Fmoc-β-Ala-OH/DIC; (h) 50% TFA in DCM; (i) split-and-pool synthesis by Fmoc/HATU chemistry; (j) 2% TFA in DCM (6×); (k) Fmoc-OSu/DIPEA in DCM; (l) $Pd(PPh_3)_4$; (m) diallyl protected trimesic acid/HATU; (n) PyBOP/HOBT/DIPEA; (o) modified reagent K.

These biologic drugs are administered by in-hospital intravenous injections. Considerable efforts have been made over the past two decades to develop small-molecule inhibitors against TNFα, which have the potential to be administered orally. However, these efforts have so far led to only a few weak inhibitors.[37−42] In this work, we developed a general methodology for chemical synthesis and screening of large combinatorial libraries of bicyclic peptides displayed on rigid small-molecule scaffolds. Chemical synthesis permitted the incorporation of unnatural amino acids (e.g., D-amino acids) and potentially nonpeptidic building blocks into the bicyclic molecules, increasing their structural diversity and metabolic stability. Screening of a bicyclic peptide library against TNFα identified a compound that inhibits the TNFα−TNFR interaction and protects cells from TNFα-induced cell death.

RESULTS AND DISCUSSION

Design and Synthesis of Bicyclic Peptide Library. Antibodies recognize specific antigens by utilizing six small loops, called the "complementarity determining regions". By grafting two or more flexible loops onto rigid protein scaffolds, other investigators have engineered protein binders of antibody-like affinity and specificity.[8−11] We envisioned that displaying peptidic loops on rigid small-molecule scaffolds should also generate molecules that rival antibodies for binding affinity and specificity. To develop inhibitors against PPIs, we chose a planar structure as the scaffold, which should bias the resulting bicyclic peptides toward an overall "planar" (as opposed to globular) shape. An overall planar geometry would maximize the surface area of the molecules and therefore their ability to interact with flat protein surfaces. To test the validity of this approach, we designed a bicyclic peptide library by "wrapping" a peptide sequence of 6−10 random residues around a trimesoyl group (Figure 1). Peptide cyclization was mediated by the formation of three amide bonds between trimesic acid and the N-terminal amine, the side chain of a C-terminal L-2,3-diaminopropionic acid (Dap), and the side chain of a fixed lysine within the random region. The resulting bicyclic peptides contained 3−5 random residues in each ring. The random sequence was constructed with a 24-amino acid set that included 10 proteinogenic amino acids [Ala, Arg, Asp, Gln, Gly, His, Ile, Ser, Trp, and Tyr], 4 nonproteinogenic α-L-amino acids [L-4-fluorophenylalanine (Fpa), L-norleucine (Nle), L-ornithine (Orn), and L-phenylglycine (Phg)], and 10 α-D-amino acids [D-2-naphthylalanine (D-Nal), D-Ala, D-Asn, D-Glu, D-Leu, D-Lys, D-Phe, D-Pro, D-Thr, and D-Val]. These building blocks were selected on the basis of their structural diversity, metabolic stability, and commercial availability. Although not included here, nonpeptidic building blocks are also compatible with our library synthesis and decoding method.[19] This library has a theoretical diversity of 6.6×10^{13}. In practice, the library size is limited by the amount of resin that can be conveniently employed and typically on the order of 10^7 (vide infra). Despite the fact that only a small fraction of all possible structures can be synthesized, we felt that it is critical to sample a large structural space. Once an active compound is identified, its affinity and specificity for the target protein may be improved by synthesizing and screening a second-generation library consisting of analogues of the initial hit. Inclusion of the unnatural amino acids increases the structural diversity and metabolic

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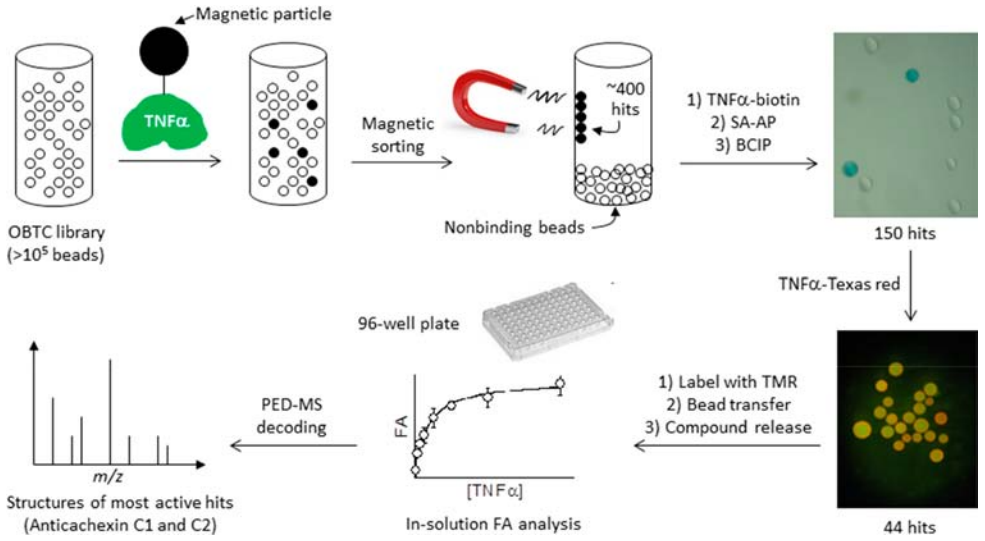

Figure 2. Scheme showing the steps involved in peptide library screening against TNFα.

stability of the library compounds but necessitates chemical synthesis of the library.

The main challenge associated with screening chemically synthesized bicyclic peptide libraries is the structural determination of hit compounds. To overcome this difficulty, we synthesized the bicyclic peptide library in the one bead–two compound (OBTC) format on 2.0 g of TentaGel microbeads (90 μm; ~100 pmol peptide/bead; 2.86×10^6 beads/g). Each library bead was topologically segregated into two different layers, with the outer layer displaying a unique bicyclic peptide and the inner layer containing the corresponding linear peptide as an encoding tag (Figure 1). This was achieved by quickly suspending wet beads in 1:1 (v/v) DCM/Et_2O containing 0.5 equiv of *N*-(9-fluorenylethoxycarbonyloxy)succinimide (Fmoc-OSu).[43,44] Because the organic solvent is immiscible with water, only peptides on the bead surface were exposed to and reacted with Fmoc-OSu. The beads were washed with DMF, and the remaining free N-terminal amines inside the beads were protected with a Boc group. After removal of the Fmoc group, a *p*-hydroxymethylbenzoic acid (Hmb) linker was added (for selective release of the bicyclic peptide), followed by the addition of β-Ala, L-propargylglycine (Pra), two β-Ala, and Fmoc-L-Dap(Alloc)-OH. The Pra residue serves as a handle for selective labeling of the bicyclic peptide via click chemistry (vide infra). The Dap residue permits attachment of the bicyclic peptide to the solid support as well as providing a side chain for peptide cyclization. The N-terminal Boc group was then removed from the inner peptides by treatment with trifluoroacetic acid (TFA), and an arginine residue was added to provide a fixed positive charge, which facilitates later peptide sequencing by mass spectrometry. The random region was synthesized by the split-and-pool method,[45−47] and an N^{ε}-4-methoxytrityl (Mmt)-protected lysine was added in the middle of the random sequence to provide a side-chain amine for peptide cyclization. Following completion of the linear peptide synthesis, the Mmt group was removed using 2% TFA in DCM and replaced with an Fmoc group (the Mmt group was partially removed during deprotection of the Alloc group). The Alloc group on the C-terminal Dap was removed by treatment with $Pd(PPh_3)_4$, and the exposed side chain amine was acylated with diallyl trimesic acid. Finally, the allyl (on the trimesoyl moiety) and Fmoc protecting groups (on the N-terminus and the lysine side chain) were removed, and the surface peptides were cyclized by treatment with benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP). The peptides inside the beads were unaffected by the cyclization procedure due to lack of the Dap residue and remained in the linear form to serve as encoding tags. Note that macromolecular targets (e.g., proteins) cannot diffuse into the bead interior, and thus the linear encoding peptides do not interfere with library screening. The symmetry of the trimesoyl unit ensured that a single bicyclic product was formed on each bead.

Library Screening against TNFα. The bicyclic peptide library was subjected to four rounds of screening against recombinant TNFα that contained an N-terminal ybbR tag[48] (MVLDSLEFIASKL) and had been specifically labeled at the ybbR tag with a biotin or fluorescent dye molecule. During the first round, 100 mg of the bicyclic peptide library (~3×10^5 beads) was incubated with biotinylated TNFα (0.8 μM) and streptavidin-coated magnetic particles (Figure 2). The resulting magnetic beads (~400 beads) were isolated from the library by magnetic sorting,[49,50] during which the positive beads were attracted to the wall while the negative beads settled to the bottom of the container. The ~400 beads were washed, incubated again with the biotinylated TNFα (1.5 μM), and subjected to a second round of screening using an on-bead enzyme-linked assay and a streptavidin–alkaline phosphatase (SA-AP) conjugate. Binding of TNFα to a bead recruited SA-AP to the bead surface and,upon the addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), produced a turquoise-colored precipitate on that bead. This procedure resulted in 150 intensely colored beads, which were manually isolated with a micropipet and incubated with Texas-red-labeled TNFα (0.3 μM). The 44 most fluorescent beads were selected under a fluorescence microscope.

Finally, the 44 beads were treated with tetramethylrhodamine (TMR) azide in the presence of Cu(I), resulting in selective labeling of the bicyclic peptides at the Pra residue (Figures 2 and 3a). The beads were then placed into individual microcentrifuge tubes (1 bead/tube), and the TMR-labeled bicyclic peptide was released from each bead by treatment with 0.1 M NaOH, which selectively hydrolyzed the Hmb ester linkage associated with the bicyclic peptide. After neutralization, the released bicyclic peptide from each bead was tested for binding to TNFα in

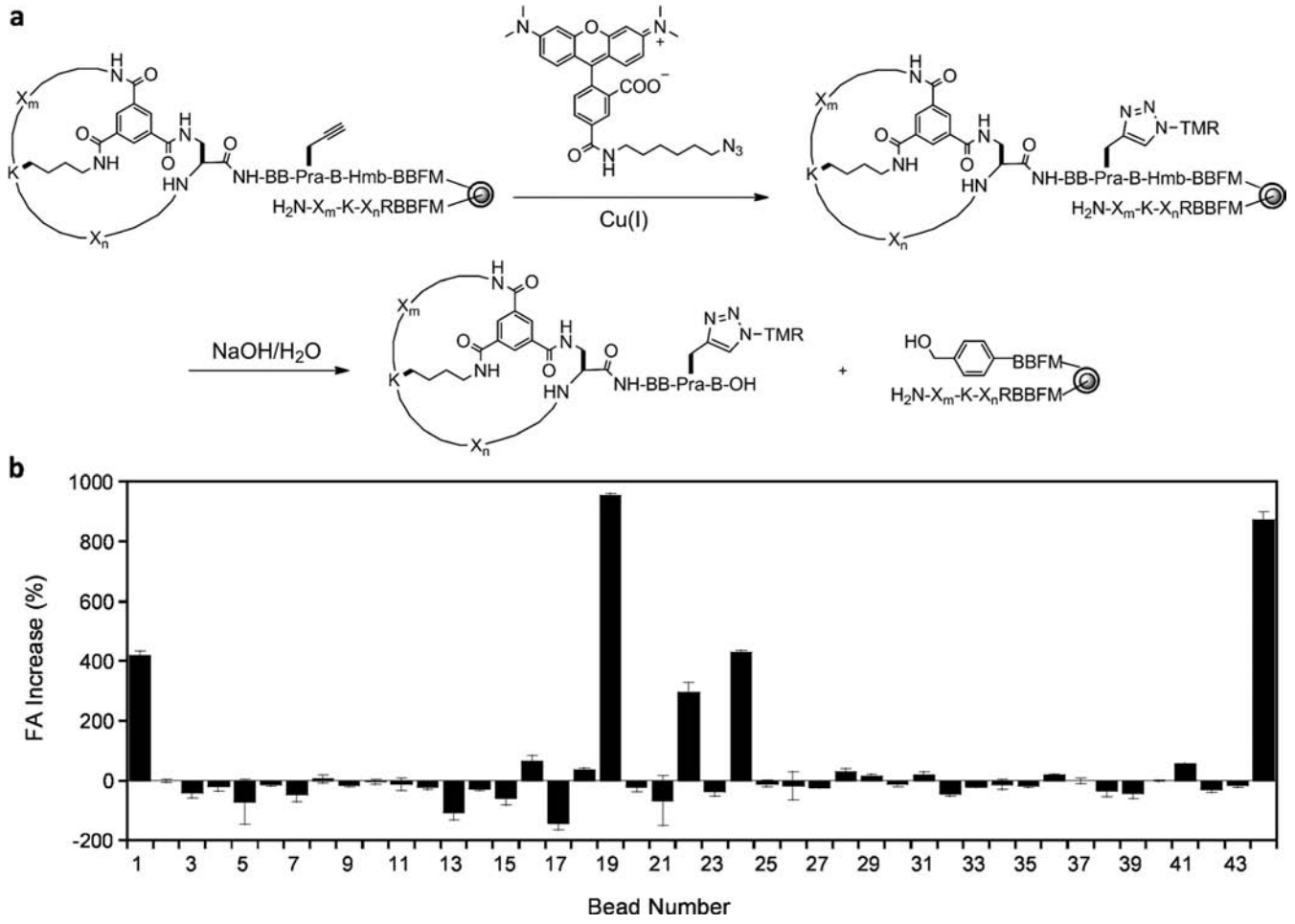

Figure 3. Solution-phase screening of initial hits (fourth round). (a) Selective labeling of bicyclic peptides with tetramethylrhodamine (TMR) and their release from individual beads by base hydrolysis. (b) Evaluation of the 44 released bicyclic peptides for binding to TNFα in solution by fluorescent anisotropy using a fixed concentration of TNFα (5 μM) and TMR-labeled bicyclic peptide (100 nM).

solution by fluorescence anisotropy (FA).[51,52] Each of the 44 bicyclic peptides (~100 nM) was incubated with 5 μM TNFα, and the 12 bicyclic peptides that showed ≥15% anisotropy increase (relative to the control without TNFα) (Figure 3b) were further analyzed at varying concentrations of TNFα (0−18 μM) to determine their dissociation constants (K_D). Six peptides (beads No. 1, 16, 22, 24, 36, and 41) had K_D values ranging from 0.8 to 7.8 μM (Figure S1 in Supporting Information [SI]), while the other peptides showed no significant TNFα binding (bead No. 18, 19, 28, 29, 31, and 44). Next, for the 6 binding peptides, their corresponding beads containing the linear encoding peptides were retrieved from the microcentrifuge tubes and subjected to partial Edman degradation-mass spectrometry (PED-MS) analysis.[53] Two of the beads (hits No. 1 and 36) produced mass spectra of sufficient quality, from which unambiguous, complete sequences of bicyclo(Phg-Tyr-D-Ala-Lys-Tyr-D-Phe-Gly-D-Lys-His-Dap) and bicyclo(Ala-D-Phe-Trp-D-Thr-Gln-Lys-Nle-D-Leu-Ala-His-Dap) were obtained (Figure 4a and Figure S2 in SI). These compounds are named as anticachexins C1 and C2 thereafter, respectively.

The fact that only a relatively small number of the hits derived from on-bead screening (6 out of 44 beads) showed strong binding to TNFα in solution suggests that most of the initial hits were weak binders or false positives, a problem commonly associated with on-bead screening. Most likely, the high ligand density on the library beads (~100 mM) resulted in multidentate interactions (i.e., simultaneous interaction of a single TNFα molecule with two or more resin-bound bicyclic peptides) and high avidity.[54] False negatives are also possible as a result of several factors (e.g., poor aqueous solubility of a bicyclic peptide, inefficient release of a bicyclic peptide from resin by 0.1 M NaOH due to its strong noncovalent binding to the hydrophobic TentaGel resin, and/or strong binding of a bicyclic peptide to bovine serum albumin which was present in all FA assays). Elimination of these false negative compounds at this stage is actually desirable, as they are likely very hydrophobic and may bind nonspecifically to many proteins. This highlights the importance of our library design, which permits selective release of the bicyclic peptide and therefore solution-phase binding analysis and avoids the need to individually resynthesize all 44 initial hits.

Binding Affinity and Specificity of Hit Compounds for TNFα. Anticachexins C1 and C2 and the linear and monocyclic variants of anticachexin C1 were resynthesized with a fluorescein isothiocyanate (FITC) label (Figure S3 in SI), purified by HPLC, and assayed against TNFα by FA analysis. Anticachexin C1 and C2 bound to TNFα with K_D values of 0.45 and 1.6 μM, respectively (Figure S3 in SI). The linear C1 variant exhibited weak binding to TNFα (K_D > 10 μM), whereas the monocyclic peptide showed no significant binding affinity. Bicyclo(Arg-Arg-Arg-Arg-Nal-Phe-Dap-Ser-D-Val-Pro-pTyr-His-Dap), a control peptide unrelated to TNFα, also showed no detectable binding

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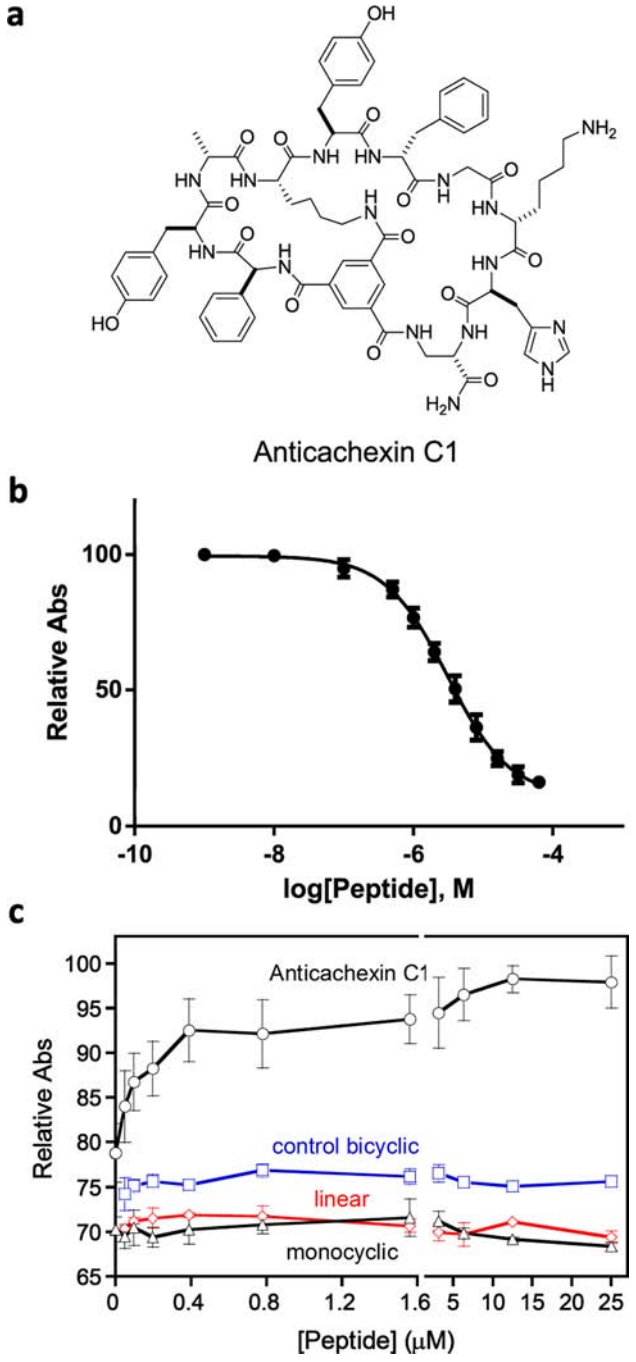

Figure 4. (a) Structure of anticachexin C1. (b) Inhibition of TNFα–TNFR1 interaction by anticachexin C1. The absorbance values on the y axis, which reflect the amount of TNFR1 bound to immobilized TNFα in the presence of increasing concentrations of anticachexin C1, are relative to that in the absence of peptide inhibitor (100%). (c) Protection of WEHI-13VAR cells against TNFα 0.04 ng/mL-induced cell death by anticachexin C1 (0–25 μM). The absorbance values on the y axis, which reflect the number of live cells, are relative to that of DMSO control (no TNFα, no peptide).

to TNFα. These results demonstrate that both the peptide sequence and the overall bicyclic structure are critical for binding to the target protein. To determine whether anticachexin C1 and C2 are specific ligands of TNFα, we tested them for binding to five arbitrarily selected proteins, including bovine serum albumin (BSA), a glutathione-S-transferase-PLCγ SH2 domain fusion (GST–SH2), protein phosphatase PTP1B, HIV capsid protein, and a GST–BRCT fusion protein. Anticachexin C1 showed weak binding to BSA ($K_D \approx 34$ μM), but not to any of the other proteins, while anticachexin C2 was less selective and showed substantial binding to BSA and GST–SH2 proteins (K_D values of 7.4 and 1.5 μM, respectively; Figure S4 in SI). Finally, unlabeled anticachexins C1 and C2 inhibited the binding of FITC-labeled anticachexin C2 to TNFα in a concentration-dependent manner (IC_{50} values of ~1 and ~4 μM, respectively) (Figure S5 in SI), suggesting that both compounds bind to the same (or overlapping) site on TNFα. Because of its higher affinity and specificity for TNFα, anticachexin C1 was selected for further biological tests.

Inhibition of TNFα–TNFR Interaction by Anticachexin C1. TNFα signaling begins with the binding of the TNFα trimer to the extracellular domain of TNFR1, triggering the release of an inhibitory protein, silencer of death domains, from the intracellular domain of TNFR1.[33] To test whether anticachexin C1 inhibits the interaction between TNFα and TNFR1, biotinylated TNFα was immobilized onto a Neutravidin-coated 96-well microtiter plate. The plate was incubated with 0.5 nM horseradish peroxidase (HRP)-conjugated TNFR1 in the presence of varying concentrations of anticachexin C1. After the wells were washed, the amount of HRP–TNFR1 bound to each well was quantitated by ELISA.[55] Anticachexin C1 inhibited the TNFα–TNFR1 interaction in a concentration-dependent manner, with an IC_{50} value of 3.1 ± 0.3 μM (Figure 4b).

Protection against TNFα-Induced Cell Death. The ability of anticachexin C1 to protect cells against TNFα-induced cell death was tested with cultured WEHI-13VAR fibroblasts, which are highly sensitive to TNFα in the presence of actinomycin-D.[56] The cells were treated with a fixed concentration of TNFα (0.04 ng/mL) and varying concentrations of anticachexin C1 (0–25 μM), and the fraction of live cells was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Anticachexin C1 protected the cells from TNFα-induced cell death in a concentration-dependent manner, whereas the corresponding monocyclic and linear peptides did not (Figure 4c). As expected, the control bicyclic peptide that does not bind TNFα had no protective effect. The MTT assay was also conducted at a fixed concentration of anticachexin C1 (50 μM) but varying concentrations of TNFα (0–250 ng/mL). TNFα exhibited an LD_{50} value of 0.46 ng/mL in the absence of TNFα inhibitor; in the presence of 50 μM anticachexin C1, the LD_{50} value was shifted to 1.8 ng/mL (Figure S6 in SI).

■ CONCLUSION

In this study, we report the first *chemical* synthesis and screening of a large combinatorial library of bicyclic peptides against a macromolecular target of biomedical significance. Compared to previous methods for bicyclic peptide library synthesis,[27−29] which involve ribosomal peptide synthesis followed by chemical cyclization, our method has the advantage that it allows the incorporation of any unnatural amino acid or nonpeptidic building blocks, greatly increasing the structural diversity and metabolic stability of the cyclic peptides. Chemical synthesis also allows for the use of orthogonal protecting groups, which in turn permits more "forcing" reaction conditions to drive the desired cyclization reaction to completion and prevents any undesired cyclization reaction from occurring. We demonstrate that bicyclic peptides displayed on a rigid planar scaffold are effective for binding to protein surfaces such as PPI interfaces. With a K_D value of 0.45 μM, anticachexin C1 is the most potent nonprotein TNFα inhibitor reported to date. The bicyclic peptide library may be readily screened against other protein and nucleic acid targets.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional data. 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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