Phage selection of cyclic peptides for application in research and drug development

Kaycie Deyle, Xu-Dong Kong, and Christian Heinis*

Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

*E-mail address: christian.heinis@epfl.ch

CONSPECTUS

Cyclic peptides can bind to protein targets with high affinities and selectivities, which makes them an attractive modality for the development of research reagents and therapeutics. Additional properties, including low inherent toxicity, efficient chemical synthesis, and the facile modification with labels or immobilization reagents, increase their attractiveness. Cyclic peptide ligands against a wide range of protein targets have been isolated from natural sources such as bacteria, fungi, plants, and animals. Many of them are currently used as research tools, and several have found application as therapeutics, such as the peptide hormones oxytocin and vasopressin and the antibiotics vancomycin and daptomycin, proving the utility of cyclic peptides in research and medicine.

With the advent of phage display and other in vitro evolution techniques, it has become possible to generate cyclic peptide binders to diverse protein targets for which no natural peptides have been discovered. A highly robust and widely applied approach is based on the cyclization of peptides displayed on phage via a disulfide bridge. Disulfide-cyclized peptide ligands to more than a hundred different proteins have been reported in the literature. Technology advances achieved over the last three decades, including methods for generating larger phage display libraries, improved phage panning protocols, new cyclic peptide formats, and high-throughput sequencing, have enabled the generation of cyclic peptides with ever better binding affinities to more challenging targets.

A relatively new cyclic peptide format developed using phage display involves bicyclic peptides. These molecules consist of two macrocyclic peptide rings cyclized through a chemical linker. Compared to monocyclic peptides of comparable molecular mass, bicyclic peptides are more constrained in their conformation. As a result, they can bind to their targets with a higher affinity and are more resistant to proteolytic degradation. Phage-encoded bicyclic peptides are generated by chemically cyclizing random peptide libraries on phage. Binders are identified by conventional phage panning and DNA sequencing. Next-generation sequencing and new sequence alignment tools have enabled the rapid identification of bicyclic peptides.

Bicyclic peptide ligands were developed against a range of diverse target classes including enzymes, receptors, and cytokines. Most ligands bind with nanomolar affinities, with some reaching the picomolar range. To date, several bicyclic peptides have been positively evaluated in pre-clinical studies, and the first clinical tests are in sight. While bicyclic peptide phage display was developed with therapeutic applications in mind, these peptides are increasingly used as research tools for target evaluation or as basic research probes as well. Given the efficient development method, the ease of synthesis and handling, and the favorable binding and biophysical properties, bicyclic peptides are being developed against more and more targets, ever increasing their potential applications in research and medicine.

INTRODUCTION

Cyclic peptides have a number of favorable properties that make them an attractive format for use as research tools and therapeutic agents.[1-3] First, cyclic peptides can bind with high affinities to macromolecular targets. They can interact with flat, featureless surfaces of proteins, whereas small molecules generally need a pocket to bind, and therefore can efficiently target protein-protein interactions. Second, cyclic peptides can be designed to have a high target selectivity; they can distinguish between similar proteins with the same tertiary structure and a high sequence homology. Third, cyclic peptides have a low inherent toxicity due to their amino acid makeup, which is particularly advantageous for therapeutic applications. Fourth, cyclic peptides are relatively stable, particularly when compared to linear peptides that are more easily degraded by proteases. Fifth, cyclic peptides can be efficiently produced by automated solid-phase peptide synthesis. Their modular structure and the commercial availability of hundreds of amino acid building blocks simplifies the rapid development of cyclic peptides with tailored properties. Sixth, cyclic peptides can be easily modified with labels or conjugated to proteins, macromolecular structures, or surfaces, which is useful for research applications and drug development. For example, they can easily be tailored to form imaging reagents or peptide-drug conjugates.

There are numerous cyclic peptides offered by commercial providers as research reagents, and around 40 cyclic peptide drugs are in clinical use. Prominent examples of cyclic peptide research reagents are phalloidin, a bicyclic peptide that binds filamentous actin and is used for staining the cytoskeleton in cell microscopy, and the cyclic RGD peptides that bind integrin receptors and are used for interfering with the interactions of cells and the extracellular matrix. Extensive lists of these commercially available cyclic peptides are found in the product catalogs of common distributors, such as Bachem, IRIS, and Sigma. The approximately 40 approved cyclic peptide drugs are used for a wide range of diseases, including the treatment of cancer, infections, and cardiovascular diseases.[3,4] Examples of widely applied cyclic peptide drugs are the hormones or hormone analogs oxytocin, octreotide, and vasopressin; the antibiotics vancomycin, daptomycin, and polymyxin B; or the immunosuppressant cyclosporine.

A majority of the above-mentioned cyclic peptides are natural products isolated from microorganisms, human hormones, or derivatives thereof. They are obtained either through isolation and purification or chemical synthesis. Only a few of the cyclic peptides were developed de novo by cyclizing natural linear peptide ligands or by rational design based on the linear binding epitopes of protein ligands.[5,6] The invention of phage display in the 1980s[7] and the subsequent development of other display techniques, such as mRNA display[8] or ribosome display,[9] allowed for the creation of ligands from scratch with no natural template.

These display technologies, with phage display at the forefront, have revolutionized the development of cyclic peptide ligands.

The first chapter of this review will discuss the important developments in cyclic peptide phage display and highlight examples of phage-selected cyclic peptide ligands. The next chapter will discuss bicyclic peptide phage display, which is a technique routinely applied in our laboratory that allows for the development of cyclic peptide ligands with particularly high affinities and stabilities. In the final chapter, we will review recently developed bicyclic peptide ligands and discuss their properties and potential applications.

PHAGE SELECTION OF CYCLIC PEPTIDES

The first cyclic peptide phage display libraries consisted of random peptides flanked by two cysteines that could form a disulfide bridge upon oxidation (Figure 1a). O'Neil et al. constructed a phage library of random hexapeptides flanked by cysteines and isolated high-affinity cyclic peptides to the platelet glycoprotein IIb/IIIa.[10] McLafferty et al. developed a library of the form XCX_4CX-phage and isolated disulfide-cyclized peptide ligands for streptavidin and an anti-β-endorphin monoclonal antibody.[11] Following these successes, disulfide-cyclized peptide libraries were panned against a wide range of protein targets or other molecules such as DNA, carbohydrates, and the surfaces of materials. Most libraries had the format $X_lCX_mCX_n$ where m = 4–10 and l, n = 0–4. This included the widely applied Ph.D.™-C7C library with the format CX_7C that is distributed commercially by New England Biolabs.[12] Some phage display peptide libraries were designed to contain only one cysteine in a fixed position that could form a disulfide bridge with a second cysteine appearing in the random region.[13] Similarly, phage libraries containing three cysteines in fixed positions were oxidized and screened to isolate peptides cyclized by two disulfide bridges.[14] The reported dissociation constants of disulfide-cyclized peptides range from millimolar to picomolar, though most fall in the micromolar range, and these affinities tend to be substantially higher than for their linear peptide counterparts. Lists of disulfide-cyclized peptides isolated by phage display are provided in several review articles.[15-19] The targets are highly diverse and include enzymes, receptors, cytokines, transporters, and structural proteins. Two examples for the phage selection of disulfide-cyclized peptides are described in the following.

Several research groups have developed cyclic peptide ligands against streptavidin to establish phage selection protocols or test new cyclic peptide libraries.[11,14,20-22] Through these studies, it can be seen that disulfide-cyclized peptides containing the HPQ motif often have higher binding affinities than their linear

counterparts.[11,21] Crystallographic analysis of various HPQ peptides in complex with streptavidin offered interesting insights into the molecular basis by which cyclic peptide ligands are recognized by their targets (Figure 1b) that could help explain this difference in binding.[23] The HPQ motif in the two studied cyclic peptides, cyclo(CHPQGPPC) and cyclo(CHPQFC), and the linear peptide, FSHPQNT, shared the same type I β-turn conformation that formed the same interactions with streptavidin, while the flanking regions were different in all peptides. In the cycles, the cysteines did not form contacts with streptavidin and thus did not directly contribute enthalpically to the binding, demonstrating that the better binding affinity of the cyclic format is largely due to entropic effects.

Disulfide-cyclization has become a popular format for peptide development, and researchers now show that these peptides can be taken into clinical use. An impressive example of this is the development of a small peptide erythropoietin (EPO) mimetic. EPO regulates the proliferation and differentiation of immature erythroid cells by dimerizing the EPO receptor. Phage display was applied to generate a disulfide-cyclized 20-amino acid peptide that served as an EPO receptor-dimerizing agonist.[24] The peptide is much smaller than EPO and has a sequence that is completely unrelated. Crystallographic study showed a 2:2 complex formed between the receptor and the peptide (Figure 1c).[25] To develop an effective therapeutic, the peptide was then chemically dimerized with polyethylene glycol (PEG), increasing the affinity, potency, and circulation time in blood. In clinical studies in dialysis patients, the resulting cyclic peptide-PEG conjugate, named peginesatide, corrected anemia and maintained hemoglobin within the desired target range when administered once a month. Unfortunately, safety concerns led to the withdrawal of the drug from the market only one year after its approval, though this example still shows that disulfide-cyclized peptides with impressive therapeutic effects can be developed.

Several technology advances over the last three decades have enabled the generation of cyclic peptides against more challenging targets and the isolation of ligands with ever better binding affinities. New molecular biology reagents, such as improved DNA polymerases and commercially available electrocompetent cells with high transformation efficiencies, and new cloning methods, such as Gibson assembly, enable the construction of larger phage display libraries. The development of strategies for chemically modifying peptides displayed on phage have expanded the number of cyclic peptide formats that can be screened by phage display.[26,27] Chemical cyclization on phage was first introduced to generate phage-encoded bicyclic peptides, a new ligand format that is described in detail in the next chapter.[27] In subsequent work, peptides displayed on phage were cyclized with other reagents, as, for example, with azobenzene linkers in order to use phage display to select light-responsive cyclic peptide ligands.[20,22]

Phage panning procedures and sequencing methods have also become more efficient through the availability of better selection protocols and reagents. For example, both streptavidin and neutravidin magnetic beads are commercially available, allowing for the alternating immobilization of biotinylated target proteins on different surfaces to reduce the enrichment of binders to streptavidin or neutravidin. Automated magnet-based washing stations, such as the KingFisher™ Flex system, allow for multiple parallel phage selections to be performed under different conditions, and these systems offer the advantage of high reproducibility (Figure 1d). Phage display has recently benefited from the broad availability and reduced cost of next-generation sequencing (NGS) techniques that allow the identification of millions of (poly)peptides after a phage selection.[28-31] This is a huge advancement considering that until recently only a few hundred clones could be de-convoluted by Sanger sequencing. The sequencing of millions of clones through NGS has even been shown to identify binders after only a single round of phage selection.[31,32] Sequence alignment software tools also enable the automated identification of consensus motifs from among thousands of peptides and facilitate the identification of peptides that bind either to different epitopes or to the same site on the target but through different interactions (Figure 1e).[31]

BICYCLIC PEPTIDE PHAGE DISPLAY

Bicyclic peptides contain two connected macrocyclic rings that can both engage in binding. Compared to monocyclic analogs of a comparable size, bicyclic peptides are less flexible. The reduced conformational freedom can limit the entropic penalty upon binding to a target, leading to a higher binding affinity. This reduction in flexibility typically also renders bicyclic peptides more resistant to proteases. Many bicyclic peptides can be found in nature and several of them have found applications as research tools or therapeutics. For example, the above-mentioned natural product phalloidin is broadly applied for staining the cytoskeleton in cells, and there are two approved bicyclic peptide anti-cancer drugs, actinomycin D and romidepsin.[33]

The following phage display approach uses a chemically modified library for the development of bicyclic peptides binding to targets of interest.[27] Linear peptides displayed on phage are chemically transformed into bicycles by reacting the three cysteines in each peptide with a reagent containing three thiol-reactive groups (Figure 2a). Reagents were chosen that allowed efficient cyclization of the peptides via the cysteines,[34] and reaction conditions were established to cyclize the peptide library while sparing phage coat proteins in order to conserve phage functionality. In a first proof-of-principle study, billions of phage, each displaying a

different peptide of the format ACX_6CX_6CG as a fusion to the p3 coat protein, were reacted with 1,3,5-tris(bromomethyl)benzene (TBMB; Figure 2a). Phage were panned against the proteases plasma kallikrein and cathepsin G, and bicyclic peptides with inhibitory constants in the nanomolar range were isolated (Figure 2b).[27]

Bicyclic peptides were subsequently developed to a range of different targets including urokinase-type plasminogen activator (uPA), a protease involved in extracellular matrix homeostasis and signaling.[35] Extensive studies of bicyclic peptides binding to and inhibiting uPA offered a deep insight into the properties of bicyclic peptides, such as binding affinity, target specificity, binding kinetics, and stability. Structural analyses of the uPA ligands by NMR and X-ray crystallography determined the conformation of bicyclic peptides in solution,[36] the size of the interaction interface between peptide and target,[35] and the structural role of the cyclization linkers.[26,36] Figure 3a shows the peptides that were isolated from a peptide library of the format ACX_6CX_6CG that was cyclized with the reagent TBMB.[35] The peptides converge to either of two consensus sequences: a major one showing a conserved peptide motif in the first peptide ring, and a minor one shared by only two peptides that covers the second ring and extends into the first. The identification of one or multiple consensus sequences is typically indicative for the successful isolation of target-specific peptide ligands, whereas the absence of consensus sequences in phage selections is usually a sign that no target-selective ligands could be enriched. Peptides from separate consensus groups form different interactions with the target, often binding to distinctive surface regions, and typically differ more in their properties (e.g. modulation of target, affinity, target selectivity, stability) than peptides within the same consensus family. For this reason, it is of great interest to identify as many consensus motifs as possible. From the peptides isolated against uPA shown in Figure 3a, peptides of both consensus groups bound to the active site and were inhibitors. Peptides of the second consensus group bound more tightly, and the best one, peptide UK18, inhibited uPA with a K_i of 53 nM.[35] In selections against some targets, bicyclic peptide ligands were identified, but they did not modulate the activity of the target proteins. For example, bicyclic peptides isolated against the negative regulatory region (NRR) of the NOTCH1 receptor bound with nanomolar affinity, but they did not inhibit Notch signaling.[37]

The bicyclic peptide UK18 inhibited other trypsin-like serine proteases only weakly (> 1000-fold weaker K_is than for human uPA). Even murine uPA, sharing high sequence similarity with human uPA (71% in the catalytic domain), was not efficiently inhibited (K_i > 100 μM). The crystal structure of UK18 bound to uPA could be solved at a resolution of 1.9 Å (Figure 3b).[35] The structure revealed that both peptide rings of a bicyclic peptide can engage in binding and that the peptides form a perfectly complementary shape to the

surface of the protein target. In this specific example, the interaction surface between the bicyclic peptide UK18 and protein was > 700 Å^2. The numerous non-covalent interactions identified based on the crystal structure explained the high binding affinity and selectivity.

The overall affinity of the best binders isolated from a combinatorial library typically increases as the library diversity increases. Towards the generation of larger and structurally more diverse bicyclic peptide libraries, the number of amino acids in the two macrocyclic rings and the chemical linker connecting the three cysteines were varied in addition to the already-variable amino acid sequence. The screening of libraries with variable ring sizes (ranging from three to six random amino acids per ring) yielded bicyclic peptides to a large number of epitopes as evidenced by many different consensus sequences.[38] In order to vary the chemical linker, four new reagents based on bromoacetamide, acrylamide and bromomethylbenzene thiol-reactive groups were developed that fulfill a number of necessary criteria: molecules possess the ability to react efficiently and selectively, are compatible with aqueous solvents, and are symmetric such that a single product is formed (Figure 4a). These linkers efficiently cyclized cysteine-rich peptides and were found to impose different conformational constraints.[36,39] This is evident because peptides isolated in different phage selections performed with different chemical linkers showed entirely different consensus sequences, and swapping the chemical linkers in these peptides led to a large reduction or a complete loss of the binding activity (Figure 4b).[26] This observation suggested that the structural diversity of bicyclic peptide libraries was greatly increased by applying several chemical linkers in parallel. The further observation that the affinities of these differently cyclized bicyclic peptides varied substantially confirmed that screening larger library diversities yields more options, which can lead to better binders. In contrast to the initially applied chemical linker TBMB, the new linkers contained polar groups. Structural analysis of a bicyclic peptide containing the TBAB linker (UK903) by X-ray crystallography revealed that the linker forms hydrogen bonds with amino acids of the peptide (Figure 4c).[26]

APPLICATIONS OF BICYCLIC PEPTIDES

The bicyclic peptide format combines several qualities that make it attractive for the development of therapeutics. The good binding properties allow specific interactions with most protein targets, including the efficient inhibition of protein-protein interactions that can be difficult to impossible for small molecules. Due to their relatively small molecular weight, ranging from one to three kDa, bicyclic peptides can diffuse efficiently in tissues or cross biological barriers that cannot be easily passed by much larger proteins. These properties hold promise for efficient tumor penetration and administration via various topical routes. The small molecular weight further allows for the full chemical synthesis of these peptides, which is important

for several reasons. First, chemical synthesis allows for the production of a uniform product, which is often an issue with biologicals that are expressed and purified from cells. Second, unnatural amino acids can be employed to improve the binding affinity and proteolytic stability. Third, chemical synthesis provides for easily addressable modification tags, permitting the development of bicyclic peptide-drug conjugates and immobilization on surfaces.

Besides the therapeutic applications, bicyclic peptide ligands have great value as research tools. They can be exploited as chemical probes to interfere selectively with the function of specific proteins in biological systems, and can also be applied as labeled ligands for the microscopic imaging of specific proteins. Furthermore, they may be used as robust affinity reagents for pull-down experiments and for protein purification by affinity chromatography. Chemical synthesis allows for the efficient production of peptides for all of these applications. The easily addressable tags that could be appended to assist in therapeutic applications can also enable the facile labeling of these peptides with fluorophores and biotin as well as provide for immobilization onto chromatography resins and surfaces.

Bicyclic peptides have been developed against diverse targets including several proteases, receptors, and cytokines. Published bicyclic peptides, their targets, and binding affinities are summarized in Table 1. Bicyclic peptides to a wide range of additional targets were developed by the spin-off company Bicycle Therapeutics, including a high-affinity ligand of matrix metalloproteinase 14 (MT1-MMP) that was linked to a cytotoxic agent for developing a bicyclic peptide drug conjugate (BT1718).[40]

Potential applications of bicyclic peptide phage display in research and drug development are illustrated in the following paragraphs with two examples. The first example describes bicyclic peptide inhibitors of uPA, a trypsin-like serine protease that is overexpressed in some tumors and is considered a potential target for the inhibition of tumor growth and invasion. Bicyclic peptide inhibitors of uPA (see their development in previous sub-chapter) were evaluated in a human tumor xenograft mouse model to assess if selective inhibition of uPA activity affects tumor growth.[41,42] This set of experiments was the first in which bicyclic peptides were tested in vivo, and valuable information about the bicyclic peptide format, including stability, distribution, plasma half-life, and tumor penetration, was discovered.

Incubation of the uPA inhibitor UK18 in blood plasma showed that a significant fraction of the bicyclic peptide remained intact while monocyclic and linear analogs were rapidly degraded.[41] Interestingly, the two

rings of the bicyclic peptide UK18 were less stable when they were separated (synthesized individually) than when they were linked, suggesting that the two rings in a bicyclic peptide stabilize each other mutually, most likely by sterically hindering the access of proteases or by non-covalent interactions that reduce their conformational flexibility. Later studies with other bicyclic peptides confirmed an overall good proteolytic stability of this peptide format in blood. Intravenous administration of UK18 to mice showed that the bicyclic peptide is stable in vivo, but it is filtered out by the kidney with a relatively short half-life of 30 minutes (Figure 5a).[41] Clearance rates in the same range were later found for bicyclic peptides developed to other targets. While the observed circulation times can be ideal for some applications, like tumor imaging, they are too short for targets that require longer time periods of modulation. The plasma half-life of UK18 could be prolonged substantially by conjugating it to an antibody Fc fragment (70-fold, $t_{1/2}$ = 1.5 days)[43] or an albumin-binding peptide (50-fold, $t_{1/2}$ = 1 day) (Figure 5a).[41]

A biodistribution study with radionuclide- and fluorescence-labeled bicyclic peptides showed that this format diffuses into the tissues of organs and solid tumors.[42] In particular, a bicyclic peptide conjugated to the albumin-binding peptide was delivered in substantial quantities into tissues (e.g. 1.5% of injected dose per gram in tumor tissue 24 hours after i.v. administration). Microscopy studies revealed that the bicyclic peptide was distributed to all of the regions of the tumor tissue, including those that were more distant from the blood vessels (Figure 5b).[42] An analysis of the bicyclic peptide extracted from tumor tissue samples showed that over 90% of the peptide remained intact and functional, though this inhibition of overexpressed uPA in mice bearing solid MDA-MB-231 xenograft tumors did not significantly reduce the tumor growth compared to the controls.[42] While UK18 appears not to be a therapeutic candidate based on the lack of tumor response upon target inhibition, the bicyclic peptide uPA inhibitors are still valuable tools for studying the role of the serine protease in health and disease.

A second application of a bicyclic peptide is illustrated by an inhibitor of the coagulation factor XII (FXII) enzyme (Figure 6a). FXII has emerged in recent years as a potential target to treat or prevent pathological thrombosis, to inhibit contact activation in extracorporeal circulation, and to treat the swelling disorder hereditary angioedema. While several protein-based inhibitors with a high affinity for activated FXII (FXIIa) were developed, the generation of small molecule inhibitors has been challenging. A first attempt yielded a rather weak inhibitor with a K_i of 1.2 μM.[44] Phage selections with peptide libraries cyclized with TATA, one of the new chemical linkers described above (Figure 4a), yielded a 60-fold better inhibitor (FXII618) that had a K_i of 22 nM and a high selectivity (> 2000-fold over other proteases) (Figure 6b).[45] As

desired, the inhibitor selectively blocked the intrinsic coagulation pathway in mice in a dose-dependent manner.

Sequence comparisons of the peptide with protein-based FXIIa inhibitors revealed that one of the macrocyclic rings of the evolved bicyclic peptide mimics the combining loop of corn trypsin inhibitor (CTI), a natural inhibitor of FXIIa commonly used to inhibit contact activation in coagulation assays.[45] As it was known how CTI binds to the active site of FXIIa, the binding mode of the first ring of bicyclic peptide FXII618 could be deduced (Figure 6b).[45] The binding affinity of the bicyclic peptide FXII618 was improved by small modifications that were introduced by incorporating unnatural amino acids.[46,47] A resulting bicyclic peptide FXIIa inhibitor with picomolar binding affinities and high target selectivity (Figure 6c) and stability is currently being evaluated for their pharmacologic activity in three animal disease models.

OUTLOOK

Phage display is a highly robust technique, and cyclic peptide ligands with functions ranging from biological tools to therapeutic leads can be isolated in most panning experiments, though some challenges remain. One of these challenges is the generation of ligands with sufficiently good binding affinities for "difficult" targets. For example, while proteases are rather easy targets as most of them contain binding pockets for peptides, proteins with featureless surfaces are more difficult to target. For some protein targets, the solution to this problem could be close, with the future trending towards the generation and screening of ever larger and structurally more diverse cyclic peptide libraries that will have better chances of targeting varying surface structures. A wide range of chemical reactions can also be used to post-translationally modify peptides on phage in order to generate libraries with even higher diversities that can also contain modalities not found in nature. Another remaining challenge in phage display is that the panning experiments cannot easily be monitored as the phage are not visible to our eyes. The success of a phage selection is typically only seen after 2–3 weeks of work with the characterization of the isolated peptides. Routine application of high-throughput sequencing to monitor the enrichment of millions of clones over the iterative rounds of phage selection, which is now a possibility due to the recent advances in this technology, may solve this problem. Information about the enrichment of these individual clones during the successive rounds of phage selection could then be used to change the protocol when needed, such as for an adjustment of the selection stringency. With these and additional technical improvements, there is no doubt that phage display technology will deliver many more cyclic peptides with new activities and exciting applications in the future.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation, grants no. 123524 and 157842, and the NCCR Chemical Biology.

References

(1) Driggers, E. M.; Hale, S. P.; Lee, J.; Terrett, N. K. The Exploration of Macrocycles for Drug Discovery - an Underexploited Structural Class. *Nat. Rev. Drug Discov.* **2008**, *7*, 608-624.

(2) Waldmann, H.; Valeur, E.; Gueret, S. M.; Adihou, H.; Gopalakrishnan, R.; Lemurell, M.; Grossmann, T. N.; Plowright, A. T. New Modalities for Challenging Targets in Drug Discovery. *Angew. Chem., Int. Ed.* **2017**.

(3) Zorzi, A.; Deyle, K.; Heinis, C. Cyclic Peptide Therapeutics: Past, Present and Future. *Curr. Opin. Chem. Biol.* **2017**, *38*, 24-29.

(4) Giordanetto, F.; Kihlberg, J. Macrocyclic Drugs and Clinical Candidates: What Can Medicinal Chemists Learn from Their Properties? *J. Med. Chem.* **2014**, *57*, 278-295.

(5) Robinson, J. A.; DeMarco, S.; Gombert, F.; Moehle, K.; Obrecht, D. The Design, Structures and Therapeutic Potential of Protein Epitope Mimetics. *Drug Discov. Today* **2008**, *13*, 944-951.

(6) Walensky, L. D.; Bird, G. H. Hydrocarbon-Stapled Peptides: Principles, Practice, and Progress. *J. Med. Chem.* **2014**, *57*, 6275-6288.

(7) Smith, G. P. Filamentous Fusion Phage - Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. *Science* **1985**, *228*, 1315-1317.

(8) Wilson, D. S.; Keefe, A. D.; Szostak, J. W. The Use of mRNA Display to Select High-Affinity Protein-Binding Peptides. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 3750-3755.

(9) Hanes, J.; Pluckthun, A. In Vitro Selection and Evolution of Functional Proteins by Using Ribosome Display. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 4937-4942.

(10) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N. S.; Mousa, S. A.; Degrado, W. F. Identification of Novel Peptide Antagonists for Gpiib/Iiia from a Conformationally Constrained Phage Peptide Library. *Proteins* **1992**, *14*, 509-515.

(11) Mclafferty, M. A.; Kent, R. B.; Ladner, R. C.; Markland, W. Bacteriophage-M-13 Displaying Disulfide-Constrained Microproteins. *Gene* **1993**, *128*, 29-36.

(12) Noren, K. A.; Noren, C. J. Construction of High-Complexity Combinatorial Phage Display Peptide Libraries. *Methods* **2001**, *23*, 169-178.

(13) Bonnycastle, L. L. C.; Mehroke, J. S.; Rashed, M.; Gong, X.; Scott, J. K. Probing the Basis of Antibody Reactivity with a Panel of Constrained Peptide Libraries Displayed by Filamentous Phage. *J. Mol. Biol.* **1996**, *258*, 747-762.

(14) Chen, S. Y.; Rebollo, I. R.; Buth, S. A.; Morales-Sanfrutos, J.; Touati, J.; Leiman, P. G.; Heinis, C. Bicyclic Peptide Ligands Pulled out of Cysteine-Rich Peptide Libraries. *J. Am. Chem. Soc.* **2013**, *135*, 6562-6569.

(15) Joo, S. H. Cyclic Peptides as Therapeutic Agents and Biochemical Tools. *Biomol. Ther.* **2012**, *20*, 19-26.

(16) Krumpe, L. R. H.; Mori, T. The Use of Phage-Displayed Peptide Libraries to Develop Tumor-Targeting Drugs. *Int. J. Pept. Res. Ther.* **2006**, *12*, 79-91.

(17) Ladner, R. C. Constrained Peptides as Binding Entities. *Trends Biotechnol.* **1995**, *13*, 426-430.

(18) Molek, P.; Strukelj, B.; Bratkovic, T. Peptide Phage Display as a Tool for Drug Discovery: Targeting Membrane Receptors. *Molecules* **2011**, *16*, 857-887.

(19) Szardenings, M. Phage Display of Random Peptide Libraries: Applications, Limits, and Potential. *J. Recept. Sig. Transd.* **2003**, *23*, 307-349.

(20) Bellotto, S.; Chen, S. Y.; Rebollo, I. R.; Wegner, H. A.; Heinis, C. Phage Selection of Photoswitchable Peptide Ligands. *J. Am. Chem. Soc.* **2014**, *136*, 5880-5883.

(21) Giebel, L. B.; Cass, R. T.; Milligan, D. L.; Young, D. C.; Arze, R.; Johnson, C. R. Screening of Cyclic Peptide Phage Libraries Identifies Ligands That Bind Streptavidin with High Affinities. *Biochemistry* **1995**, *34*, 15430-15435.

(22) Jafari, M. R.; Deng, L.; Kitov, P. I.; Ng, S.; Matochko, W. L.; Tjhung, K. F.; Zeberoff, A.; Elias, A.; Klassen, J. S.; Derda, R. Discovery of Light-Responsive Ligands through Screening of a Light-Responsive Genetically Encoded Library. *ACS Chem. Biol.* **2014**, *9*, 443-450.

(23) Katz, B. A. Binding to Protein Targets of Peptidic Leads Discovered by Phage Display - Crystal-Structures of Streptavidin-Bound Linear and Cyclic Peptide Ligands Containing the Hpq Sequence. *Biochemistry* **1995**, *34*, 15421-15429.

(24) Wrighton, N. C.; Farrell, F. X.; Chang, R.; Kashyap, A. K.; Barbone, F. P.; Mulcahy, L. S.; Johnson, D. L.; Barrett, R. W.; Jolliffe, L. K.; Dower, W. J. Small Peptides as Potent Mimetics of the Protein Hormone Erythropoietin. *Science* **1996**, *273*, 458-463.

(25) Livnah, O.; Stura, E. A.; Johnson, D. L.; Middleton, S. A.; Mulcahy, L. S.; Wrighton, N. C.; Dower, W. J.; Jolliffe, L. K.; Wilson, I. A. Functional Mimicry of a Protein Hormone by a Peptide Agonist: The Epo Receptor Complex at 2.8 Angstrom. *Science* **1996**, *273*, 464-471.

(26) Chen, S. Y.; Bertoldo, D.; Angelini, A.; Pojer, F.; Heinis, C. Peptide Ligands Stabilized by Small Molecules. *Angew. Chem., Int. Ed.* **2014**, *53*, 1602-1606.

(27) Heinis, C.; Rutherford, T.; Freund, S.; Winter, G. Phage-Encoded Combinatorial Chemical Libraries Based on Bicyclic Peptides. *Nat. Chem. Biol.* **2009**, *5*, 502-507.

(28) Ernst, A.; Gfeller, D.; Kan, Z. Y.; Seshagiri, S.; Kim, P. M.; Bader, G. D.; Sidhu, S. S. Coevolution of PDZ Domain-Ligand Interactions Analyzed by High-Throughput Phage Display and Deep Sequencing. *Mol. Biosyst.* **2010**, *6*, 1782-1790.

(29) Matochko, W. L.; Chu, K. K.; Jin, B. J.; Lee, S. W.; Whitesides, G. M.; Derda, R. Deep Sequencing Analysis of Phage Libraries Using Illumina Platform. *Methods* **2012**, *58*, 47-55.

(30) Ravn, U.; Gueneau, F.; Baerlocher, L.; Osteras, M.; Desmurs, M.; Malinge, P.; Magistrelli, G.; Farinelli, L.; Kosco-Vilbois, M. H.; Fischer, N. By-Passing in Vitro Screening-Next Generation Sequencing Technologies Applied to Antibody Display and in Silico Candidate Selection. *Nucleic Acids Res.* **2010**, *38*, e193.

(31) Rebollo, I. R.; Sabisz, M.; Baeriswyl, V.; Heinis, C. Identification of Target-Binding Peptide Motifs by High-Throughput Sequencing of Phage-Selected Peptides. *Nucleic Acids Res.* **2014**, *42*, e169.

(32) 't Hoen, P. A. C.; Jirka, S. M. G.; ten Broeke, B. R.; Schultes, E. A.; Aguilera, B.; Pang, K. H.; Heemskerk, H.; Aartsma-Rus, A.; van Ommen, G. J.; den Dunnen, J. T. Phage Display Screening without Repetitious Selection Rounds. *Anal. Biochem.* **2012**, *421*, 622-631.

(33) Baeriswyl, V.; Heinis, C. Polycyclic Peptide Therapeutics. *ChemMedChem* **2013**, *8*, 377-384.

(34) Timmerman, P.; Beld, J.; Puijk, W. C.; Meloen, R. H. Rapid and Quantitative Cyclization of Multiple Peptide Loops onto Synthetic Scaffolds for Structural Mimicry of Protein Surfaces. *ChemBioChem* **2005**, *6*, 821-824.

(35) Angelini, A.; Cendron, L.; Chen, S. Y.; Touati, J.; Winter, G.; Zanotti, G.; Heinis, C. Bicyclic Peptide Inhibitor Reveals Large Contact Interface with a Protease Target. *ACS Chem. Biol.* **2012**, *7*, 817-821.

(36) Chen, S. Y.; Morales-Sanfrutos, J.; Angelini, A.; Cutting, B.; Heinis, C. Structurally Diverse Cyclisation Linkers Impose Different Backbone Conformations in Bicyclic Peptides. *ChemBioChem* **2012**, *13*, 1032-1038.

(37) Urech-Varenne, C.; Radtke, F.; Heinis, C. Phage Selection of Bicyclic Peptide Ligands of the Notch1 Receptor. *ChemMedChem* **2015**, *10*, 1754-61.

(38) Rebollo, I. R.; Angelini, A.; Heinis, C. Phage Display Libraries of Differently Sized Bicyclic Peptides. *Medchemcomm* **2013**, *4*, 145-150.

(39) van de Langemheen, H.; Korotkovs, V.; Bijl, J.; Wilson, C.; Kale, S. S.; Heinis, C.; Liskamp, R. M. Polar Hinges as Functionalized Conformational Constraints in (Bi)Cyclic Peptides. *ChemBioChem* **2016**, *18*, 387-395.

(40) Bennett, G.; Harrison, H.; Campbell, S.; Teufel, D.; Langford, G.; Watt, A.; Bonny, C. Development of BT1718, a Bicycle Drug Conjugate (Bdc) Targeting Mt1-Mmp for Treatment of Solid Tumours. *Eur. J. Cancer* **2016**, *69*, S21-S21.

(41) Angelini, A.; Morales-Sanfrutos, J.; Diderich, P.; Chen, S. Y.; Heinis, C. Bicyclization and Tethering to Albumin Yields Long-Acting Peptide Antagonists. *J. Med. Chem.* **2012**, *55*, 10187-10197.

(42) Pollaro, L.; Raghunathan, S.; Morales-Sanfrutos, J.; Angelini, A.; Kontos, S.; Heinis, C. Bicyclic Peptides Conjugated to an Albumin-Binding Tag Diffuse Efficiently into Solid Tumors. *Mol. Cancer Ther.* **2015**, *14*, 151-161.

(43) Angelini, A.; Diderich, P.; Morales-Sanfrutos, J.; Thurnheer, S.; Hacker, D.; Menin, L.; Heinis, C. Chemical Macrocyclization of Peptides Fused to Antibody Fc Fragments. *Bioconjugate Chem.* **2012**, *23*, 1856-1863.

(44) Baeriswyl, V.; Calzavarini, S.; Gerschheimer, C.; Diderich, P.; Angelillo-Scherrer, A.; Heinis, C. Development of a Selective Peptide Macrocycle Inhibitor of Coagulation Factor XII toward the Generation of a Safe Antithrombotic Therapy. *J. Med. Chem.* **2013**, *56*, 3742-3746.

(45) Baeriswyl, V.; Calzavarini, S.; Chen, S.; Zorzi, A.; Bologna, L.; Angelillo-Scherrer, A.; Heinis, C. A Synthetic Factor XIIa Inhibitor Blocks Selectively Intrinsic Coagulation Initiation. *ACS Chem. Biol.* **2015**, *10*, 1861-70.

(46) Middendorp, S. J.; Wilbs, J.; Quarroz, C.; Calzavarini, S.; Angelillo-Scherrer, A.; Heinis, C. Peptide Macrocycle Inhibitor of Coagulation Factor Xii with Subnanomolar Affinity and High Target Selectivity. *J. Med. Chem.* **2017**, *60*, 1151-1158.

(47) Wilbs, J.; Middendorp, S. J.; Heinis, C. Improving the Binding Affinity of in-Vitro-Evolved Cyclic Peptides by Inserting Atoms into the Macrocycle Backbone. *ChemBioChem* **2016**, *17*, 2299-2303.

(48) Baeriswyl, V.; Rapley, H.; Pollaro, L.; Stace, C.; Teufel, D.; Walker, E.; Chen, S. Y.; Winter, G.; Tite, J.; Heinis, C. Bicyclic Peptides with Optimized Ring Size Inhibit Human Plasma Kallikrein and Its Orthologues While Sparing Paralogous Proteases. *ChemMedChem* **2012**, *7*, 1173-1176.

(49) Chen, S. Y.; Gfeller, D.; Buth, S. A.; Michielin, O.; Leiman, P. G.; Heinis, C. Improving Binding Affinity and Stability of Peptide Ligands by Substituting Glycines with D-Amino Acids. *ChemBioChem* **2013**, *14*, 1316-1322.

(50) Diderich, P.; Heinis, C. Phage Selection of Bicyclic Peptides Binding Her2. *Tetrahedron* **2014**, *70*, 7733-7739.

(51) Rentero Rebollo, I.; McCallin, S.; Bertoldo, D.; Entenza, J. M.; Moreillon, P.; Heinis, C. Development of Potent and Selective S. Aureus Sortase a Inhibitors Based on Peptide Macrocycles. *ACS Med. Chem. Lett.* **2016**, *7*, 606-11.

(52) Bertoldo, D.; Khan, M. M. G.; Dessen, P.; Held, W.; Huelsken, J.; Heinis, C. Phage Selection of Peptide Macrocycles against β-Catenin to Interfere with Wnt Signaling. *ChemMedChem* **2016**, *11*, 834-839.

Biographical Information

Kaycie Deyle studied biochemistry at the Indiana University of Pennsylvania (USA) before joining the lab of Prof. James Heath at the California Institute of Technology (USA) for her Ph.D. In 2015, she joined the lab of Prof. Christian Heinis at EPFL as a postdoctoral researcher. Her current research involves developing methods to optimize phage display panning against non-enzyme targets.

Xu-Dong Kong studied biochemical engineering and protein crystallography under the supervision of Professor Jian-He Xu at East China University of Science and Technology (China) and Professor Jiahai Zhou at Shanghai Institute of Organic Chemistry (China) during his Ph.D. He joined Prof. Christian Heinis' research group at EPFL as a postdoctoral researcher in 2015. Currently, his research focuses on the development of orally bioavailable bicyclic peptide therapeutics by phage display.

Christian Heinis studied biochemistry and received a Ph.D. under the guidance of Prof. Dario Neri at the ETH Zurich. He did post-docs in the laboratories of Prof. Kai Johnsson at EPFL in Lausanne and Sir Greg Winter at the LMB-MRC in Cambridge. In 2008, he became SNSF Professor at the Institute of Chemical Sciences and Engineering at EPFL and was promoted in 2015 to Associate Professor. He is a scientific co-founder of the start-up Bicycle Therapeutics and co-director of the NCCR Chemical Biology.

Table

Table 1. Target, medical indication, affinity of the best bicyclic peptide ligand (K_d, K_i for inhibitors), and relevant publication(s).

Target	Indication	Affinity	References
Plasma kallikrein (PK)	Hereditary angioedema	300 pM (K_i)	[27,48]
Urokinase-type plasminogen activator (uPA)	Cancer	28 nM (K_i)	[35,49]
Coagulation factor XIIa (FXIIa)	Contact activation in extracorporeal circulation, thrombosis, hereditary angioedema	840 pM (K_i)	[45-47]
Human epidermal growth factor receptor 2 (Her2)	Cancer	300 nM (K_d)	[50]
Sortase A	Infectious disease	1.1 μM (K_d)	[51]
β-catenin	Cancer	3.8 μM (K_d)	[52]
NOTCH1 receptor	Cancer	150 nM (K_d)	[37]

Figures

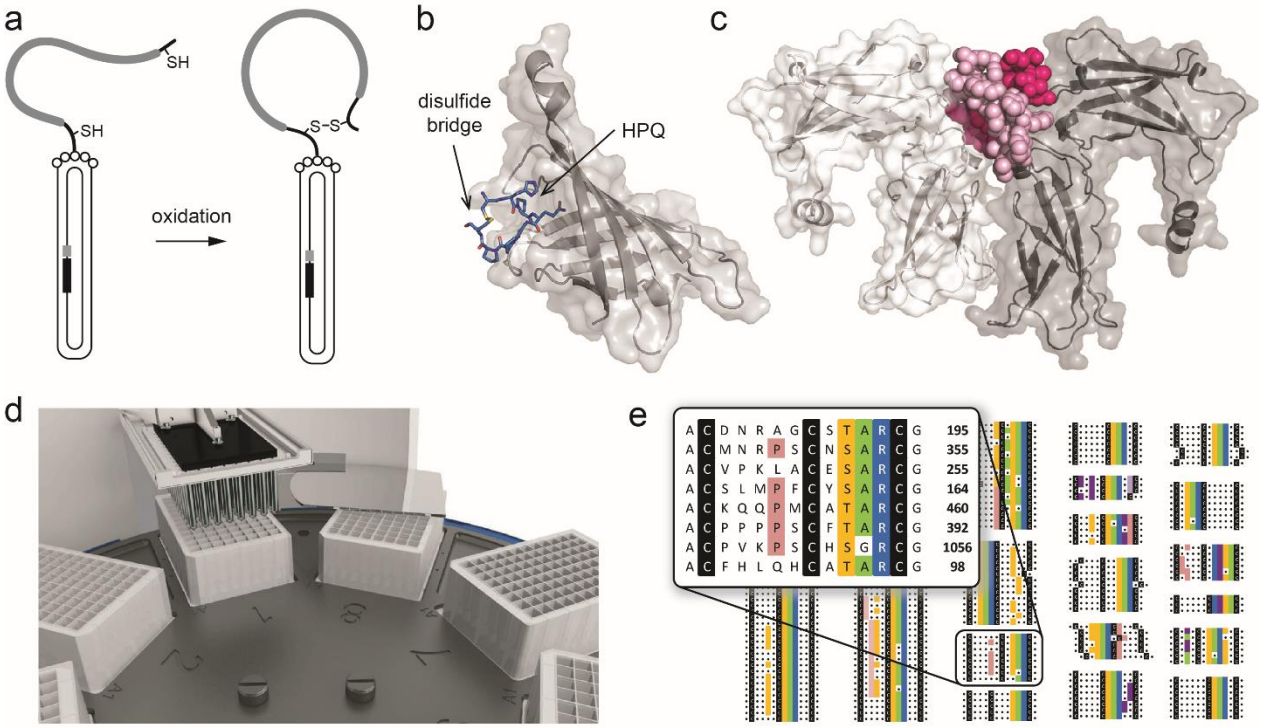


Figure 1. (a) Phage selection of disulfide-cyclized peptides. (b) Crystal structure of disulfide-cyclized peptide cyclo(CHPQGPPC) bound to streptavidin (PDB structure 1SLE). Only one monomer of the tetrameric streptavidin is shown. (c) Crystal structure of disulfide-cyclized agonist peptide EMP1 bound to the extracellular domain of the EPO receptor (PDB structure 1EBP). Monomers of the dimeric EPO receptors are shown in light and dark gray. The two EMP1 peptides are shown as space-filled models in pink and rose. (d) Phage panning by automated, magnetic liquid handling system. 96 magnetic pins can capture paramagnetic beads containing an immobilized protein target to perform many phage selections in parallel. (e) Consensus-finding software identifies preferred binding motifs of phage-selected peptides sequenced by NGS. The example shows 17 consensus groups that were identified among bicyclic peptides isolated against the bacterial target sortase A.[51] One group formed by eight peptides is enlarged. Colors highlight sequence similarities among the peptides and the number indicates how often each peptide was found by NGS.

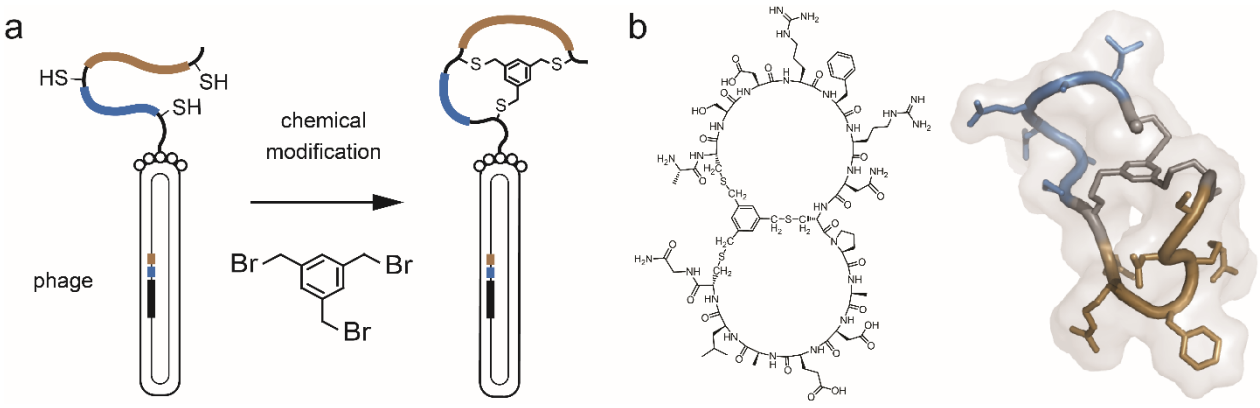


Figure 2. Phage selection of bicyclic peptides. (a) Random peptides containing three fixed cysteine residues are displayed on phage and cyclized with the chemical reagent TBMB. (b) Chemical structure and 3D structural model of the bicyclic peptide PK15 isolated against plasma kallikrein.[27]

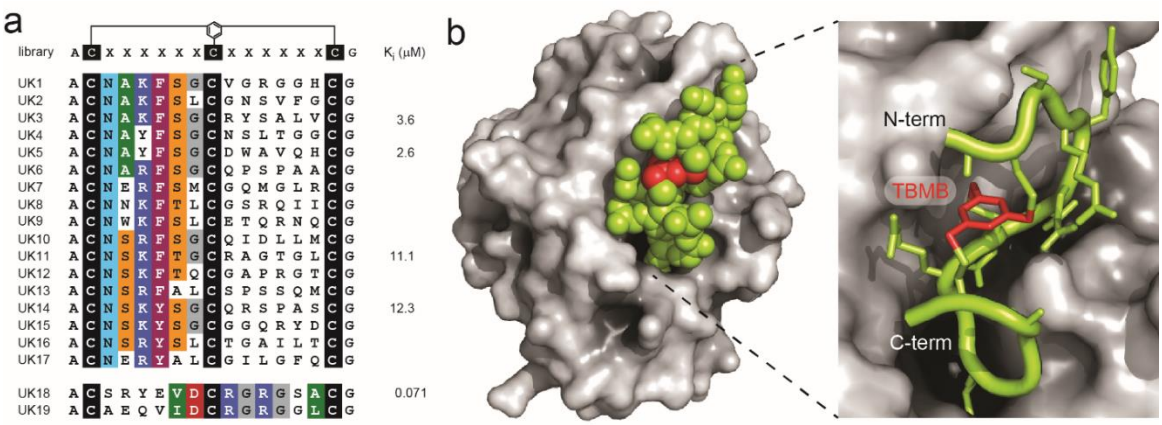


Figure 3. Bicyclic peptides selected against the trypsin-like serine protease uPA. (a) The library format is shown on the top. Isolated peptides share either of two consensus sequences. Similar amino acids are highlighted with colors. (b) X-ray structure of UK18 bound to the serine protease uPA (1.9 Å resolution; peptide in green, TBMB linker in red).

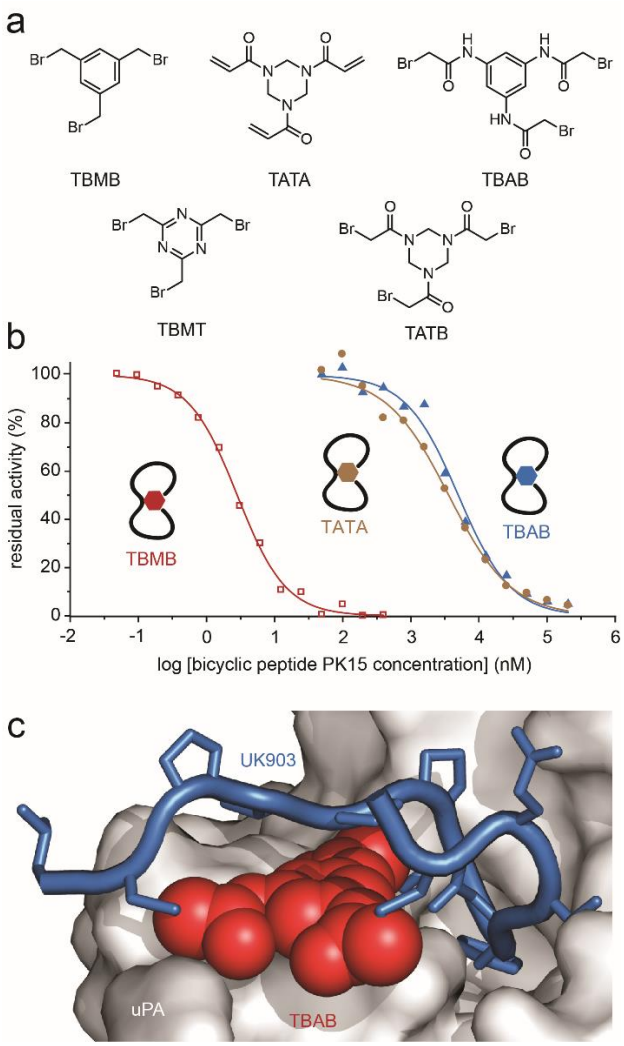


Figure 4. Bicyclic peptide library diversity. (a) Diverse chemical linkers that were developed to cyclize peptides via three cysteines. The upper three reagents were applied in phage selections. (b) Bicyclic peptide PK15 cyclized with the chemical linker TBMB (red) was selected by phage display and inhibits plasma kallikrein with a K_i of 2 nM. Replacement of the linker in PK15 to TATA (brown) or TBAB (blue) reduces its binding affinity more than 1000-fold. (c) Co-crystal structure of a bicyclic peptide UK903 and its protein target uPA (in gray). The peptide (blue) folds around the chemical linker TBAB (red, shown as space-filling model) and forms three hydrogen bonds with the linker.

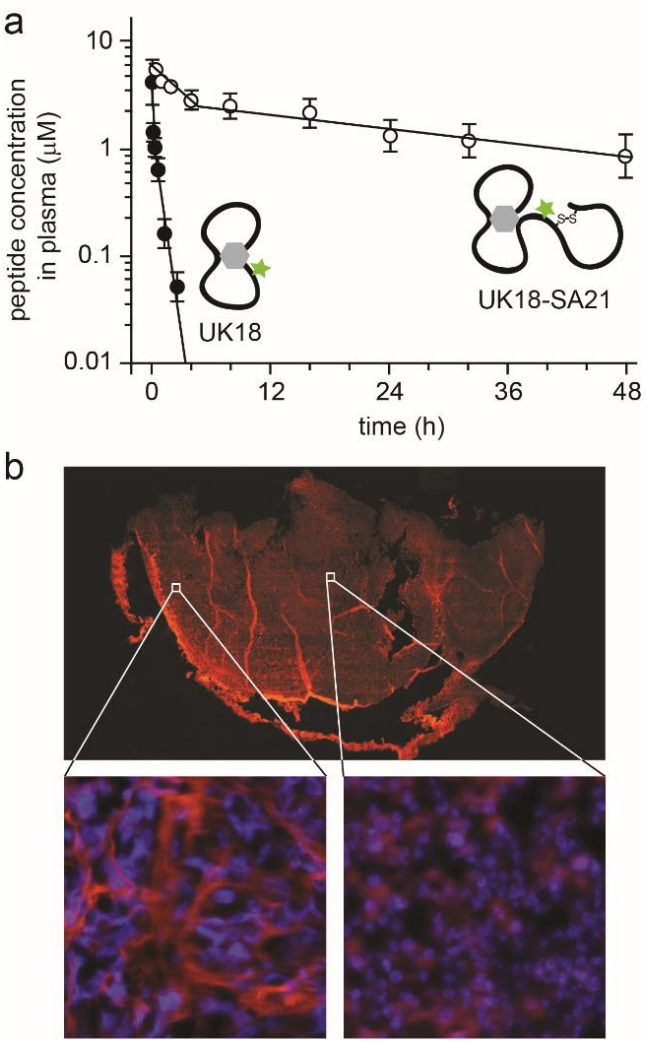


Figure 5. (a) Pharmacokinetics of fluorescein-labeled (green star) bicyclic peptide (UK18) alone and conjugated to the albumin-binding peptide SA21 (UK18-SA21). Concentration of bicyclic peptide in plasma after i.v. administration to mice is shown. The bicyclic peptide UK18 (filled circles) is rapidly cleared by the kidney. The same peptide conjugated to an albumin-binding peptide has a 50-fold extended half-life (24 hours; open circles). (b) Penetration of the bicyclic peptide into tumor tissue (nude mice with s.c. MDA-MB-231 solid tumors in flank). Mice were i.v. injected with fluorescein-labeled bicyclic peptide, then perfused after 24 hours to clear the peptide from the blood circulation before tumor removal. Tissue sections were analyzed by confocal microscopy. Significant fluorescence intensity was found in close proximity to nearly every cell. The upper panel shows the whole tumor section with two small regions enlarged below. Peptide fluorescence is shown in red. DAPI-stained nuclei are shown in blue.

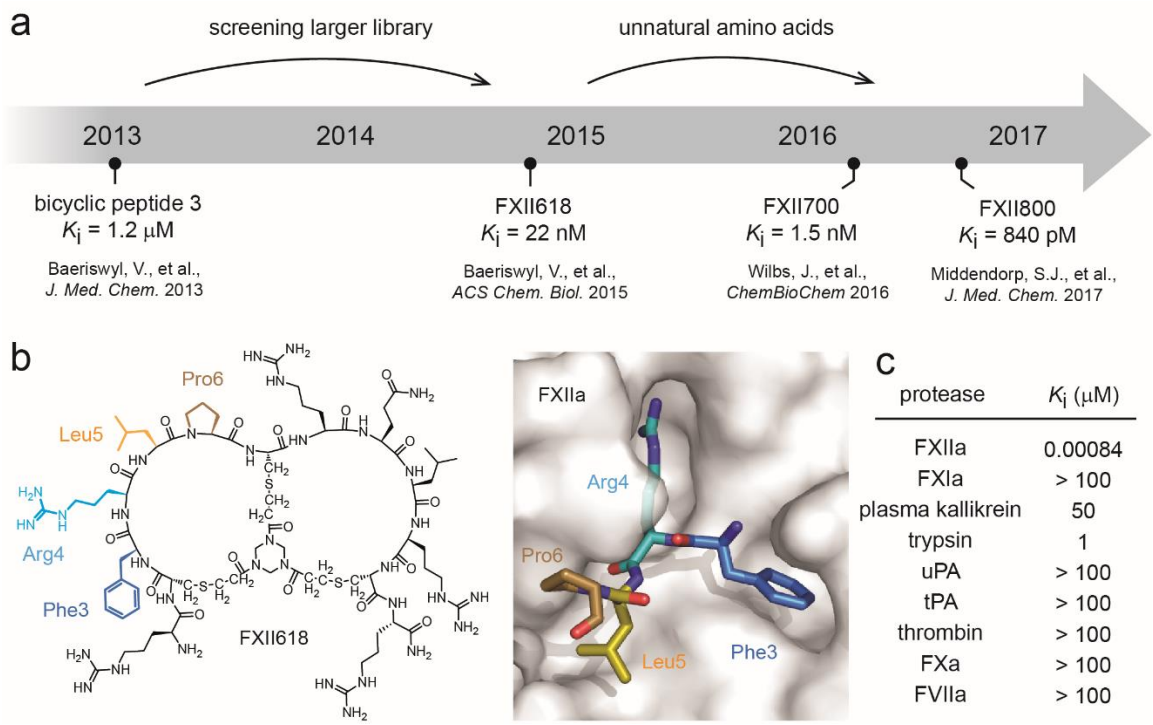
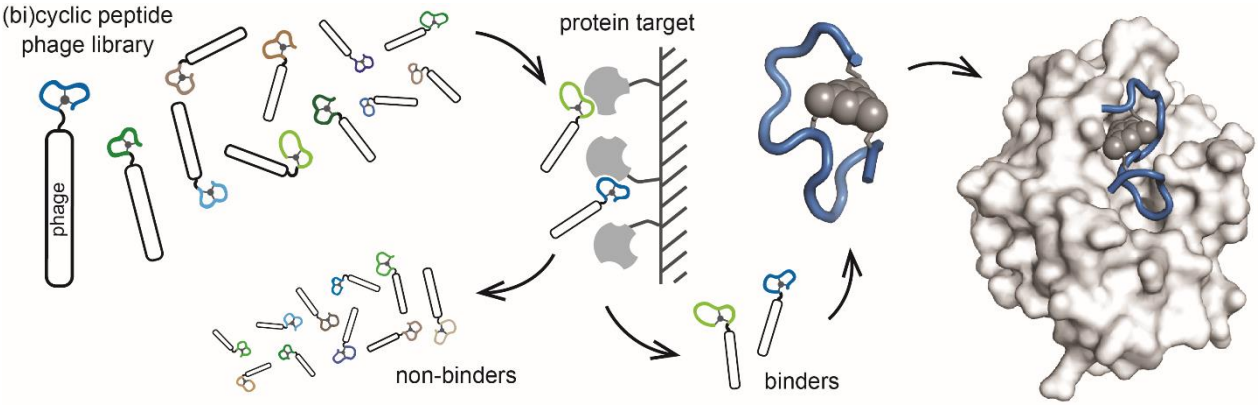


Figure 6. Bicyclic peptide inhibitor of FXIIa. (a) Development timeline and important milestones. (b) Chemical structure of FXII618 and structural model for the key amino acids Phe3, Arg4, Leu5 and Pro6 bound to human FXIIa (gray). The structure is a predictive model developed based on the binding mode of the FXIIa inhibitor CTI that has a binding loop with high sequence homology to the first ring of FXII618. (c) Activity and specificity profile of FXII801.


(bi)cyclic peptide
phage library
phage
protein target
non-binders
binders