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Cyclic Peptides

Cyclic Peptides for Drug Development

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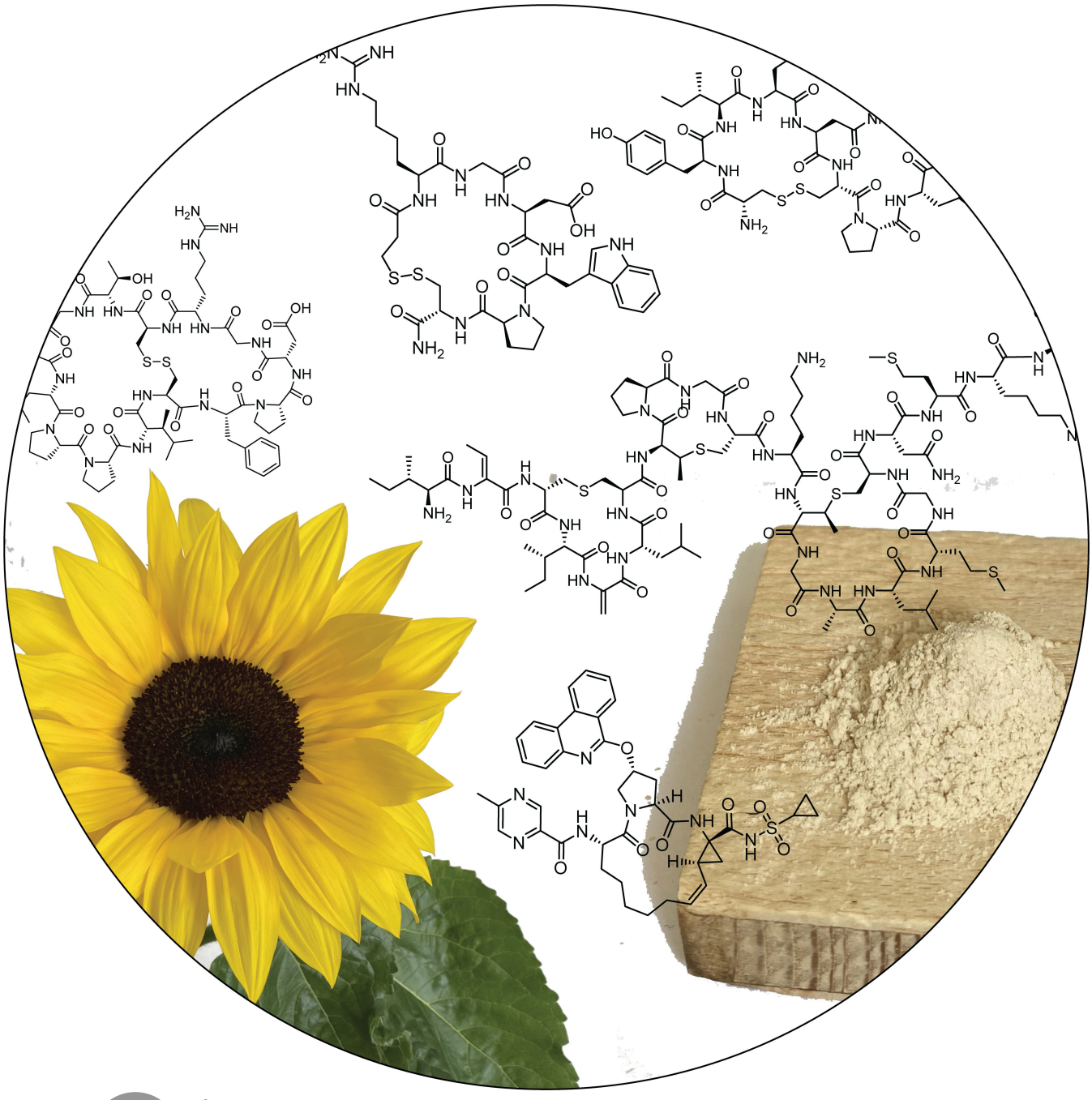

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Abstract: Cyclic peptides are fascinating molecules abundantly found in nature and exploited as molecular format for drug development as well as other applications, ranging from research tools to food additives. Advances in peptide technologies made over many years through improved methods for synthesis and drug development have resulted in a steady stream of new drugs, with an average of around one cyclic peptide drug approved per year. Powerful technologies for screening random peptide libraries, and de novo generating ligands, have enabled the development of cyclic peptide drugs independent of naturally derived molecules and now offer virtually unlimited development opportunities. In this review, we feature therapeutically relevant cyclic peptides derived from nature and discuss the unique properties of cyclic peptides, the enormous technological advances in peptide ligand development in recent years, and current challenges and opportunities for developing cyclic peptides that address unmet medical needs.

1. Introduction

Cyclic peptides are polypeptide chains composed of canonical and non-canonical amino acids that are connected at distant positions to form macrocyclic structures. A wide range of cyclic peptides with diverse shapes, sizes and chemical compositions were found in nature and showcase the enormous structural diversity of cyclic peptides as a molecule class.[1] Many of them display remarkable biological activities, ranging from signaling agents for complex biological processes to chemical weapons for defense, which has shown the enormous functional capabilities of cyclic peptides. More than 40 cyclic peptides from nature or derivatives thereof are used as therapeutics today.[2] Not confined to the clinic, though, the incredible qualities of cyclic peptides also make them excellent research tools, such as for probes that selectively modulate target proteins or high-affinity ligands for biomolecular imaging. To illustrate the diversity and utility of this class of molecules, we will showcase key examples of naturally derived cyclic peptides in a first chapter.

What makes cyclic peptides so suitable as ligands for drug development is likely a combination of multiple favorable properties that are based on both the cyclic shape and the peptidic composition. The cyclic structure of peptides offers improved capabilities compared to linear analogs, such as high binding affinity and specificity, proteolytic stability, and in some cases, improved membrane permeation. The cycle itself is composed of a polypeptide that is modular, allowing for easy, automated synthesis as well as generation of combinatorial libraries. Cyclic peptides are also accessible via cellular machinery, making this molecular format amenable to biological display systems for the generation and screening of billions of different molecules. The strengths resulting from the cyclic shape and peptidic structure, and the ease of access to cyclic peptides by chemical synthesis are covered in further sections of this review.

The vast majority of approved cyclic peptide drugs target extracellular proteins and are administered by injection.[2b,c,3] This rather narrow range of application is related to the membrane permeability of cyclic peptides which is often low, especially for the large and polar ones. However, there are several examples of smaller cyclic peptides that target intracellular proteins and/or are orally available, demonstrating that cyclic peptides can cross membranes and/or be orally available.[4] We will discuss the properties and scope of larger and smaller cyclic peptide therapeutics in a further chapter.

While numerous newly developed cyclic peptide drugs continue to stem from the study and exploitation of naturally derived cyclic peptides, many recent advances are due to the advent of new, powerful methods for generating and screening large combinatorial libraries. Methods such as phage display, introduced in the late 1980s,[5] and mRNA display, introduced about a decade later,[6] have enabled the de novo development of cyclic peptides to targets of interest. Methods for generating and screening cyclic peptide libraries are discussed in a further chapter. Finally, we conclude with a perspective on emerging trends, challenges, and opportunities in the cyclic peptide field.

2. Fascinating Cyclic Peptides from Nature

Nature applies cyclic peptides for important host functions, such as for defense in plants, fungi, or bacteria or for hormone signaling in animals. In fact, for good or for bad, cyclic peptides are found in many aspects of our daily life, although they may not always be recognized as such. For example, the group of microcystins produced by cyanobacterial blooms in freshwater lakes are naturally occurring cyclic peptides that can be harmful to animals (Figure 1a, left).[7] More positively, the pentacyclic peptide nisin is commonly used as an additive in processed cheeses, meat products, canned foods, and beverages to extend shelf life, as it inhibits the growth of a wide range of gram-positive bacteria (Figure 1a, middle).[8] This naturally occurring molecule, produced by certain strains of the bacteria *Lactococcus lactis*, is thus regularly consumed by most people without their awareness that they take in a cyclic peptide (see label E234 on food products).

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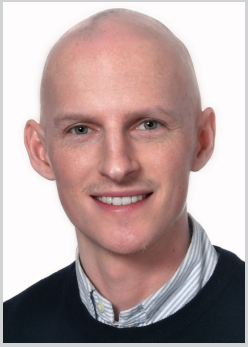
The structures of naturally occurring cyclic peptides can be highly complex. Many contain non-canonical amino acids or other non-amino acid building blocks introduced by post-translational modifications or during non-ribosomal synthesis and may not always be recognized as peptides at first glance, such as the antibiotic vancomycin (Figure 1b, middle). Some contain more than one macrocyclic ring system, affording multicyclic molecules that occasionally fold to three-dimensional structures. For example, sunflower trypsin inhibitor (SFTI) found in seeds of sunflowers forms a bicyclic structure by connecting the N- and C-terminus via an amide bond and a pair of cysteines by a disulfide bridge (Figure 1a, right). Cyclic peptides with even more sophisticated structures are the cyclotides, molecules found in plants in which a cystine knot motif is formed by three disulfide bonds and the backbone is cyclized head-to-tail.[9] This structure is highly stable and compact and confers resistance to heat, pH changes, and enzymatic degradation. An example of this class is the cyclotide kalata B1, an active ingredient of a medicinal tea made from leaves of the plant *Oldenlandia affinis*, which has been used through generations by African women to induce labor and facilitate childbirth.[10]

Many naturally derived cyclic peptides, or derivatives thereof, are used as therapeutics, including prominent drugs like the hormone oxytocin (Figure 1b, left), the antibiotic vancomycin (Figure 1b, middle), and the immunosuppressant cyclosporine (Figure 1b, right). These and several additional cyclic peptides are among the 479 medications that are on the WHO Model List of Essential Medicines (22nd list, 2021),[11] showing the importance of cyclic peptide for medicine. In the following two sections, we highlight naturally derived cyclic peptides exploited as medicines, focusing on the strongly represented classes of human hormones and anti-infectious agents.

2.1. Cyclic Peptide Hormones Used as Drugs

Several human hormones are based on cyclic peptides, and synthetic versions of them can thus be used to regulate various body processes and functions. For example in medical conditions in which the body does not produce sufficient hormone, supplementing with the affected hormone can restore balance and alleviate associated symptoms.[12] The first cyclic peptide hormones exploited as drugs were oxytocin and vasopressin, introduced to the clinic in 1960s. Initially identified and synthesized in the 1950s by du Vigneaud, both hormones are nonapeptides that feature a six-amino-acid ring closed by a disulfide bridge. Their development was traditional for cyclic peptides, wherein peptides were isolated from animal tissues and purified, their activity was characterized, their primary structure was determined, and identical molecules were produced as pharmaceuticals. Oxytocin is primarily used to begin or improve contractions during labor or to reduce bleeding after childbirth (Figure 1b, left). Vasopressin is used to manage antidiuretic hormone deficiency or to treat diabetes insipidus. Oxytocin is the cyclic peptide that is, probably, most widely applied: in the US alone, it is estimated that half of the more than 3 million women giving birth every year receive oxytocin.[13]

Xinjian Ji obtained his PhD in chemical biology from Fudan University in 2020 under the supervision by Prof. Qi Zhang. During his doctoral studies, he specialized in natural product biosynthesis and investigated the catalytic mechanism of radical SAM enzymes. After completing his PhD, he joined the lab of Prof. Christian Heinis at EPFL as a postdoctoral researcher. Currently, his research focuses on developing cyclic peptide therapeutics for addressing intracellular protein-protein interactions.



Alexander L. Nielsen holds a PhD in medicinal chemistry from University of Copenhagen, which he obtained under the supervision by Prof. Christian A. Olsen. His studies focused on the synthesis, and downstream characterization of selective inhibitors towards sirtuin enzymes. After graduating in 2021, he joined the lab of Prof. Christian Heinis at EPFL as a postdoctoral researcher, where his work encompasses the miniaturization of chemistry for establishing synthesis and screening procedures for designing diverse macrocyclic peptide libraries in high throughput.

Christian Heinis has studied biochemistry/chemistry at the ETH Zurich. After a PhD in the research group of Prof. Dr. Dario Neri at ETH, he did two post-docs, the first one with Prof. Dr. Kai Johnsson at the EPFL and the second one with Sir Gregory Winter at the LMB-MRC in Cambridge, UK. In 2008 he started as Assistant Professor at EPFL (supported with an SNSF professorship) and was promoted in 2015 to Associate Professor. Christian is a co-founder of Bicycle Therapeutics (BCYC) and Orbis Medicines.

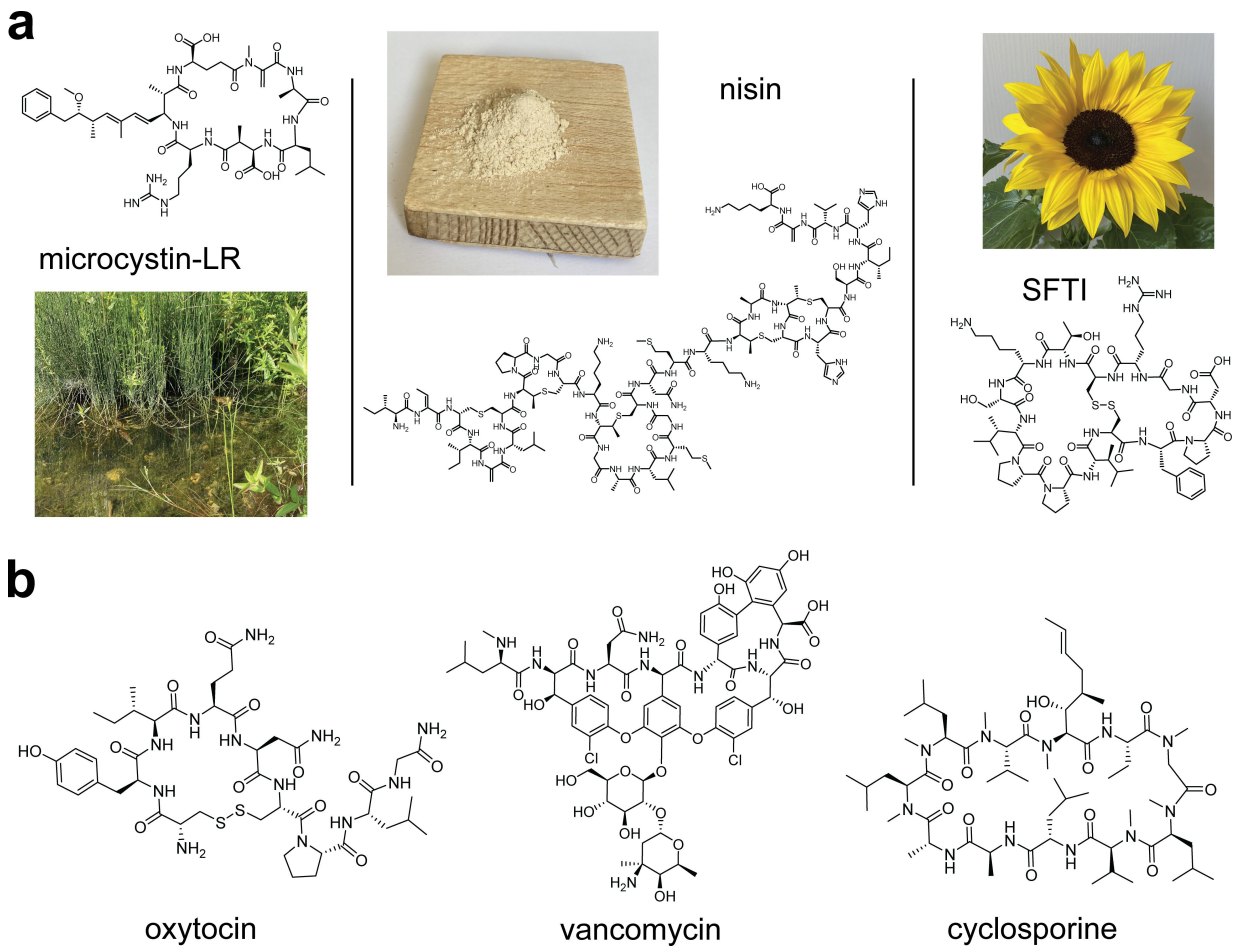


Figure 1. Nature is a treasure trove of cyclic peptides. (a) Examples of natural cyclic peptides with remarkable chemical structures, biological activities, and properties. From left to right: microcystin-LR produced by cyanobacteria is toxic for many animals, nisin powder used in food preservation, and the bicyclic peptide sunflower trypsin inhibitor (SFTI) found in seeds of sunflower. Photos were taken by the authors. (b) Examples of natural cyclic peptides exploited as therapeutics that have tremendous impact on our society. From left to right: the hormone oxytocin applied to induce or hasten contractions during labor or to reduce bleeding after childbirth, the antibiotic vancomycin, and the immunosuppressant cyclosporine.

The modular structure of peptides has enabled the facile generation of analogs of the natural peptide hormones to create compounds with improved activity, specificity, stability, or other properties of interest. For example, several vasopressin analog drugs have been developed, including the more stable and widely used synthetic analogue desmopressin, wherein the N-terminal cysteine is deaminated and the non-natural stereoisomer D-Arg replaces a protease-labile L-Arg residue. Another natural cyclic peptide hormone that was modified for improved properties is somatostatin, a 14-amino acid peptide regulating a variety of bodily functions by hindering the release of other hormones.[14] Truncation to an 8-amino-acid cyclic peptide and mutation for improved stability and pharmacological activity yielded the drug octreotide, which was approved in 1988 and has since achieved annual sales of over a billion dollar and thus reached blockbuster status. Two similar cyclic peptides analogs, lanreotide and pasireotide, vary in their pharmacological activity and were introduced in 2007 and 2012, respectively.

Two recently developed hormone-based cyclic peptide drugs, approved in 2019 and 2020, are the melanocortin 4 receptor agonists bremelanotide, developed for application in hypoactive sexual desire,[15] and setmelanotide, used to treat genetic obesity caused by a single-gene mutation.[16] The natural precursors of these two drugs are linear human peptide hormones that were cyclized to improve stability and activity.[16a]

2.2. Cyclic Peptides for Treating Infectious Diseases

Over millions of years, microorganisms have evolved an abundance of cyclic peptides with antimicrobial activity to serve as chemical weapons that help them compete in their natural environments. These molecules have undergone extensive testing through evolution for their effectiveness in killing or inhibiting the growth of bacteria, fungi, and other microorganisms and their safety for the host. Several such

cyclic peptides were found to be highly suited as antibiotics or antifungal agents, as discussed in the following.[17]

Widely used cyclic-peptide-based antibiotics are vancomycin and its derivatives telavancin, dalbavancin, and oritavancin. Vancomycin is a glycopeptide produced by non-ribosomal synthesis in *Amycolatopsis orientalis* that is applied to treat severe gram-positive bacterial infections (Figure 1b, middle). Derivatives of vancomycin are produced semi-synthetically and differ in their pharmacological effects due to variations in pharmacokinetic properties and activity across bacterial strains. A newer antibiotic introduced in 2003 against gram-positive bacteria is daptomycin, a cyclic lipopeptide derived from a *Streptomyces* strain that interacts with, and disrupts, the bacterial membrane. Cyclic peptides used against gram-negative bacteria include polymyxin B and colistin (polymyxin E), which are polycationic peptides. Their hydrophilic and hydrophobic regions interact with the outer membrane, resulting in disruption and leakage.

Antifungal drugs based on cyclic peptides include caspofungin, micafungin, and anidulafungin, used for treating yeast infections, such as from *Candida* spp. All three drugs belong to the class of echinocandins and are cyclic hexa-lipopeptides that function by inhibiting 1,3-β-glucan synthase to impair cell wall synthesis.[18] The semi-synthetic drugs were developed by modifying natural non-ribosomal cyclic peptides derived from different fungi species and were approved in 2001, 2005, and 2006, respectively.

3. Favorable Properties of Cyclic Peptides

It is not surprising that nature uses cyclic peptides as ligands for performing important biological functions, considering their numerous advantageous properties, including excellent binding, good stability, flexible evolvability, ease of preparation, and in some cases membrane permeability. Several of these qualities, such as the binding affinity, specificity, and resistance to proteases, are largely because of the circular structure of the peptides. For some peptides, cyclization has also been found to contribute to improved membrane permeability. The beneficial effects of peptide cyclization and the molecular basis of these are highlighted in the following.

3.1. Binding Affinity and Specificity

Cyclized peptides can bind to targets with higher affinity than linear analogs, as demonstrated by many examples comparing both forms.[19] Perhaps the clearest evidence for the superior binding of cyclic peptides comes from screenings of phage display libraries, where cyclic peptides generally outperform linear library members. This could already be seen in one of the first phage display selections, presented by Devlin et al. in 1990. Here, a random library screen of linear 15-mer peptides against streptavidin produced many peptides containing two cysteines in the randomized amino acid positions, suggesting that they were likely isolated in the disulfide-cyclized form.[20] Similar observations were made in many in vitro selection experiments that followed, including phage display selections of bicyclic peptides against a wide range of targets, where incomplete chemical cyclizations on phage produced mixtures of cyclic and linear peptides, but the vast majority of isolated ligands were bicyclic peptides.[21]

The higher affinity upon cyclization may be explained by entropic effects. Because cyclic peptides are less flexible than linear precursors in solution, the entropic penalty upon target binding is lower (Figure 2a). One can also understand the higher binding affinity of cyclic peptides when considering that they can adopt fewer conformations and that therefore the conformations required for the bound state are more occupied than for linear peptides that adopt many more conformations. Thus, a binding event is more probable for cyclic peptides. This becomes particularly evident in peptides that bind as folded structures, such as α-helices or β-strands, where a cyclization linker stabilizes the conformation for a higher population of the secondary structure in solution. This effect was strategically exploited to make inhibitors of protein-protein interactions (PPIs) that involve α-helices, wherein peptides based on the helical sequences were synthesized and stabilized by cyclization in a process now often termed "stapling".[22]

Additionally, most high-affinity cyclic peptides evolved by nature, or developed with display techniques, have high target selectivity approaching levels seen for monoclonal antibodies. The selective binding can be explained by the restricted conformation, as the less flexible backbone has fewer conformations accessible for binding to non-targets. The high target selectivity of cyclic peptides can be appreciated when looking at X-ray structures of protein-peptide complexes, that often show a perfect complementarity in shape and charge across rather large interaction surfaces often exceeding 500 $Å^2$.[23]

3.2. Stability

One of the notable properties of cyclic peptides is their enhanced protection against enzymatic degradation compared to linear peptides.[24] The limited degree of flexibility and strain in the peptide backbone often hinders binding to the active sites of proteases (Figure 2b). For example, the serine proteases trypsin and chymotrypsin dominating peptide degradation in the intestine or the coagulation proteases in the blood require their substrates to adapt to the proteolytic active site to optimize their position relative to the catalytic site for stabilizing the transition state. Based on their resistance to these proteases, it is likely much more challenging and thus less frequent for cyclic peptides to adopt these conformations. Additionally, peptides cyclized via their terminal amino acids are protected from exopeptidases that break down peptides from the ends.

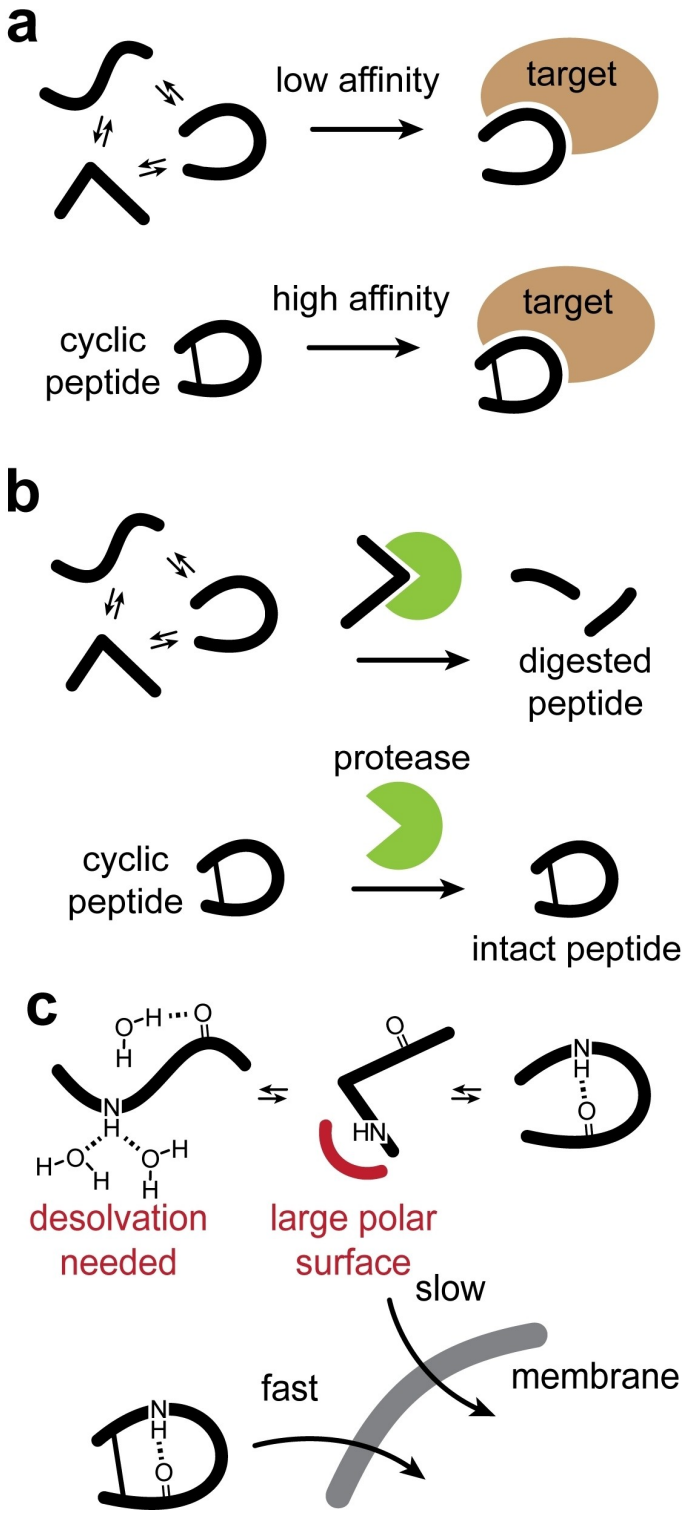


Figure 2. Effects of cyclization on (a) binding affinity, (b) stability, and (c) membrane permeability.

3.3. Membrane Permeability

For crossing the apolar region of a membrane, the polar groups of peptides such as hydrogen (H)-bond donors of peptide bonds, need to be desolvated, which poses an energy barrier. Interestingly, nature adapted for this too, as cyclization can facilitate the formation of intramolecular H-bonds within peptides, reducing solvation and at the same time burying some of the polar surface and thus increasing the likelihood of membrane permeability (Figure 2c). In linear peptides, intramolecular H-bonding is possible too, but is entropically less favored.

The effects of intramolecular H-bond shielding were observed and studied in detail for cyclosporine, an 11-amino-acid cyclic peptide drug that, despite its large size, is cell permeable and orally available. For efficiently crossing membranes, cyclosporine is N^{α}-methylated on 7 out of the 11 amide bonds, and the remaining 4 amide bond H-bond donors form intramolecular H-bonds to hide the donor and acceptor groups from the apolar environment upon entering the lipid bilayer.[25] For target binding, cyclosporine alters its conformation and uses some of the polar group for H-bonding interactions. When linearized, the membrane permeability of cyclosporine is reduced, indicating that the cyclic shape is required for forming the intramolecular hydrogen bonds.[26] The polar group hiding behavior of cyclosporine has been observed in other natural cyclic peptides, and intramolecular H-bonding has been strategically exploited to generate other cyclic peptides with improved membrane permeability and oral availability.[4a]

While cyclization may improve the membrane permeability, there is no universal rule regarding the permeability of cyclic versus linear peptides, and the relative permeability of a particular peptide depends on its specific structural and physicochemical properties. For instance, by systematically comparing the permeability of linear and cyclic peptides, the Kodadek group showed that cyclization does not automatically make peptides membrane permeable.[27] Most recently, computational methods were applied to design cyclic peptide structures that hide amide H-bond donor and acceptors through intramolecular hydrogen bonds. Many of the peptides, that were designed for membrane permeation (and not yet for target binding), displayed good membrane permeability in parallel artificial membrane permeability assays (PAMPA), in live cells, and showed oral bioavailability in rodent models.[28]

4. Chemical Synthesis: Nearly as Simple as LEGOs

Since the synthesis of the first peptide by Emil Fischer in 1901,[29] the methods for peptide synthesis have steadily been improved. Key innovations and developments include solid-phase peptide synthesis (SPPS), protection and deprotection strategies, efficient coupling agents, automation and miniaturization, and high-yielding cyclization strategies. Today, fully automated and hands-off methods for synthesizing peptides generate diverse sequences and structures from hundreds of commercially available amino-acid building blocks. This capability is largely due to the modular and repetitive polymeric structure and the highly efficient coupling between amine and carboxylic acid functional groups. While the synthesis of many peptides, and in particular short sequences, are typically straightforward,

there remain major synthetic challenges in peptide chemistry, such as longer peptides, sequences with challenging building blocks as for example *N*-methylated amino acids,[30] or the synthesis of peptides with peptide bond surrogates.[31]

4.1. Modular and Automated

Nature has evolved ingenious strategies and machineries for making polymeric structures such as peptides, along with DNA, RNA, proteins, and polyketides. For peptides specifically, nature has evolved two different methods for polymerizing the amino acid monomer: ribosomal and non-ribosomal synthesis. Ribosomal synthesis polymerizes the 20 canonical amino acids using a DNA template, and non-ribosomal synthesis employs enzyme complexes to also connect non-canonical amino acids for even more diverse structures.[32] The efficient coupling of amino acids to long polymers could be replicated in the lab using chemical reactions.[33] While nature synthesizes peptides from the N-terminus in the direction of the C-terminus, the opposite direction is generally more efficient for chemical peptide synthesis.

Using this strategy with automated SPPS, many different peptides can be produced quickly and in parallel. For instance, several commercial peptide synthesizers offer the parallel production of around 50 different peptides in 5-mL columns at a 50-µmol scale, which yields around 50 mg of crude product for a 10-amino-acid peptide. Even more peptides can be synthesized in parallel using 96-well reactor plates at a reduced 5-µmol scale (Figure 3a). With modern LC–MS systems, the crude peptides can be purified in a fully automated fashion. At the current largest scale available, thousands of peptides at a time can be synthesized in arrays on surfaces such as membranes and are used for rapidly mapping protein epitopes.[34]

Chemical peptide synthesis also expands beyond the peptides that can be found in nature due to a nearly endless availability of amino acid building blocks. The growth of the field has led to an impressively large number of commercially available amino acids and has reduced prices, which in turn offers new possibilities in peptide research and development.[35] A look into the product catalogs of three major providers shows that over a thousand different Fmoc amino acids are offered as products in stock, with more than 550 available for the affordable price of <100 Euro per gram (Figure 3b and Supporting Information Table 1).

Even more commercial building blocks are available for peptoids, whose side chains are connected to the nitrogen atom instead of the α-carbon. Peptoids are synthesized by a two-step strategy in which two small chemical fragments, named sub-monomers, are sequentially coupled to the solid support to form one amino acid. The second fragment used is a primary amine, of which thousands of different building blocks are available for purchase.[36] This enormous diversity in commercially available building blocks enables the rapid generation of peptide variants for performing iterative cycles of functional fine-tuning various properties, such as binding

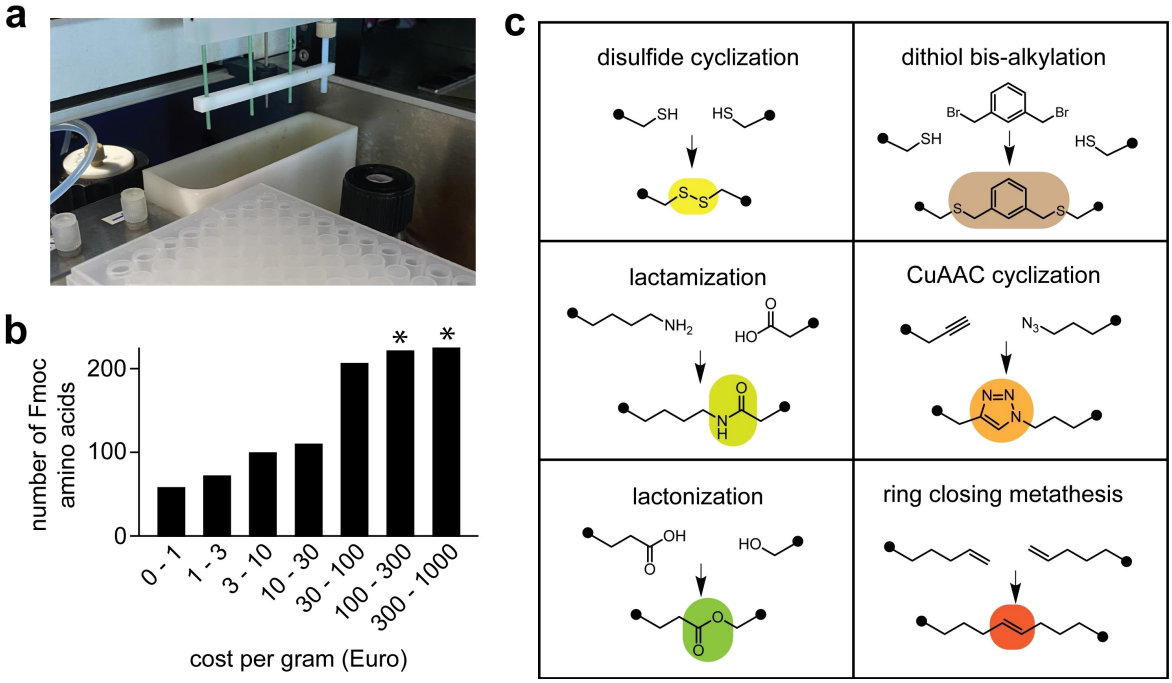


Figure 3. Chemical peptide synthesis and cyclization. (a) Automated SPPS in 96-well plates. Reagents are transferred to wells by syringe and needle and are removed by vacuum through a membrane at the bottom of the wells. Photo by the authors. (b) Commercial availability of amino acid building blocks showing the number of Fmoc amino acids offered by major providers as products in stock. Data with an asterisk (*) are estimates. (c) Cyclization reactions frequently applied in chemical peptide synthesis.

affinity, specificity, stability, solubility, or membrane permeability.

4.2. Cyclization

Natural cyclic peptides are most often cyclized via disulfide bridges between distant cysteines; macrolactamization connecting either the termini (head-to-tail) or a terminus with the side chain of Lys, Glu, or Asp; or macrolactonization in which ester bonds are formed (depsipeptides). Examples of natural peptides cyclized in these ways are the hormones oxytocin, vasopressin, and somatostatin (all disulfide bridges); the sunflower trypsin inhibitor (head-to-tail and disulfide; Figure 1a); and the anti-cancer drug romidepsin (lactone and disulfide). Regardless, a wide range of other natural macrocyclization strategies involving different functional groups and reactions have also been discovered and characterized.[37] Most natural peptide cyclization reactions are catalyzed by enzymes, which have the advantage that they are often fast and selective, the latter enabling their application in also complex environments such for cyclizing peptide fused to proteins or even inside cells.[38]

Numerous chemical strategies have been developed to efficiently cyclize synthetic peptides, as described in depth in other reviews.[39] The most frequently applied chemical reactions are disulfide cyclization, macrolactamization, thiol alkylation, copper-catalyzed azide-alkyne cycloaddition (CuAAC), and ring-closing metathesis (RCM) (Figure 3c). In general, the macrocyclization of peptides is not easy because the peptide must adopt an entropically unfavorable pre-cyclization conformation before the ends can be chemically joined. For short peptides, conformational constraints may additionally hinder or prevent cyclization. Another challenge is that peptides can react intermolecularly, especially at high concentrations. To minimize unwanted oligo- and polymerizations, it is therefore recommended to perform cyclization reactions at low- or sub-millimolar concentrations.

The efficiency of the cyclization reaction is strongly dependent to the type of chemistry and occasionally also to the peptide length and sequence. The highest yielding cyclization reactions connect the thiol groups of cysteines using either oxidation to readily generate disulfide bridges or linkers containing electrophilic groups, such as maleimides, α-halocarbonyls, benzylhalides, acrylamides, and vinylsulfones.[39c] Two strengths of cyclization reactions via thiols is that they often proceed rather independent of the peptide sequence and can be applied to short peptides in which the cysteines are only one or two amino acids apart. Widely applied macrolactamization reactions can also be efficient, though the yields can vary strongly across peptides. A wide range of synthetic strategies, including approaches based on native chemical ligation, are available for macrolactamization, both in solution and on solid support.[40] Other frequently applied macrocyclization reactions are the CuAAC and RCM, which tend to have lower cyclization yields though are more tolerant to other functional groups.

5. Drug Development: Membrane Permeable or Non-Permeable

Over the last three decades, an average of around one cyclic peptide per year has been approved for medical use, which today gives over 40 available drugs in this molecular class (Figure 4a and Supporting Information Table 2).[2,41] For counting these drugs, we considered cyclic peptides and macrocyclic compounds containing between 3 and 50 amino acids and having at least two peptide bonds. Of these new approvals, there are several peptides that have similar structures and have the same mechanism of action. We highlighted the cyclic peptides with entirely new structures or mechanisms of action as red dots (Figure 4a) and present their structures below (Figure 4b), which shows nine cyclic peptides with completely different formats, sizes and shapes, introduced in the last 30 years. For three of these approved drugs, the structures of the cyclic peptide bound to their targets were recently solved by X-ray diffraction or cryomicroscopy and they nicely show the perfect shape complementarity between peptide and target which is the basis for the good binding properties (Figure 4b).

The discovery of new bioactive cyclic peptides in nature as well as new methods for developing variants with improved properties have constantly fueled the development of new cyclic peptide drugs or drug candidates over the years. In the last three decades, powerful methods for creating cyclic peptide ligands de novo have additionally contributed. In fact, much of the growth seen over the last years in pre-clinical and clinical cyclic peptide drug development is based on technologies such as phage- or mRNA display, which are described in the following chapter. Numerous existing pre-clinical and clinical cyclic peptide programs of biotech and pharma companies as well as the strong technology platforms of new spin-off companies suggest an increase of the annually approved cyclic peptide drugs in the coming years.[2b,45]

Cyclic peptide drugs can be broadly divided into membrane permeable and impermeable ones, that differ strongly in their application range with the first group being able to target also intracellular proteins (Figure 5). The membrane permeable cyclic peptides typically have rather small size (most often below 1 kDa), limited polar surface area, and are often composed of many unnatural amino acids. The non-permeable cyclic peptides are typically larger, containing more than 10 amino acids (>1 kDa), and are usually polar; these are developed for binding to extracellular targets. Most approved cyclic peptide drugs belong to the second group of membrane impermeable drugs, as discussed in the following section. While the first group is currently represented by only a few approved drugs, impressive recent developments in understanding and developing membrane-permeable cyclic peptides position this group for strong growth in the coming years, as is described in the following. The last section of this chapter discusses orally available cyclic peptide drugs.

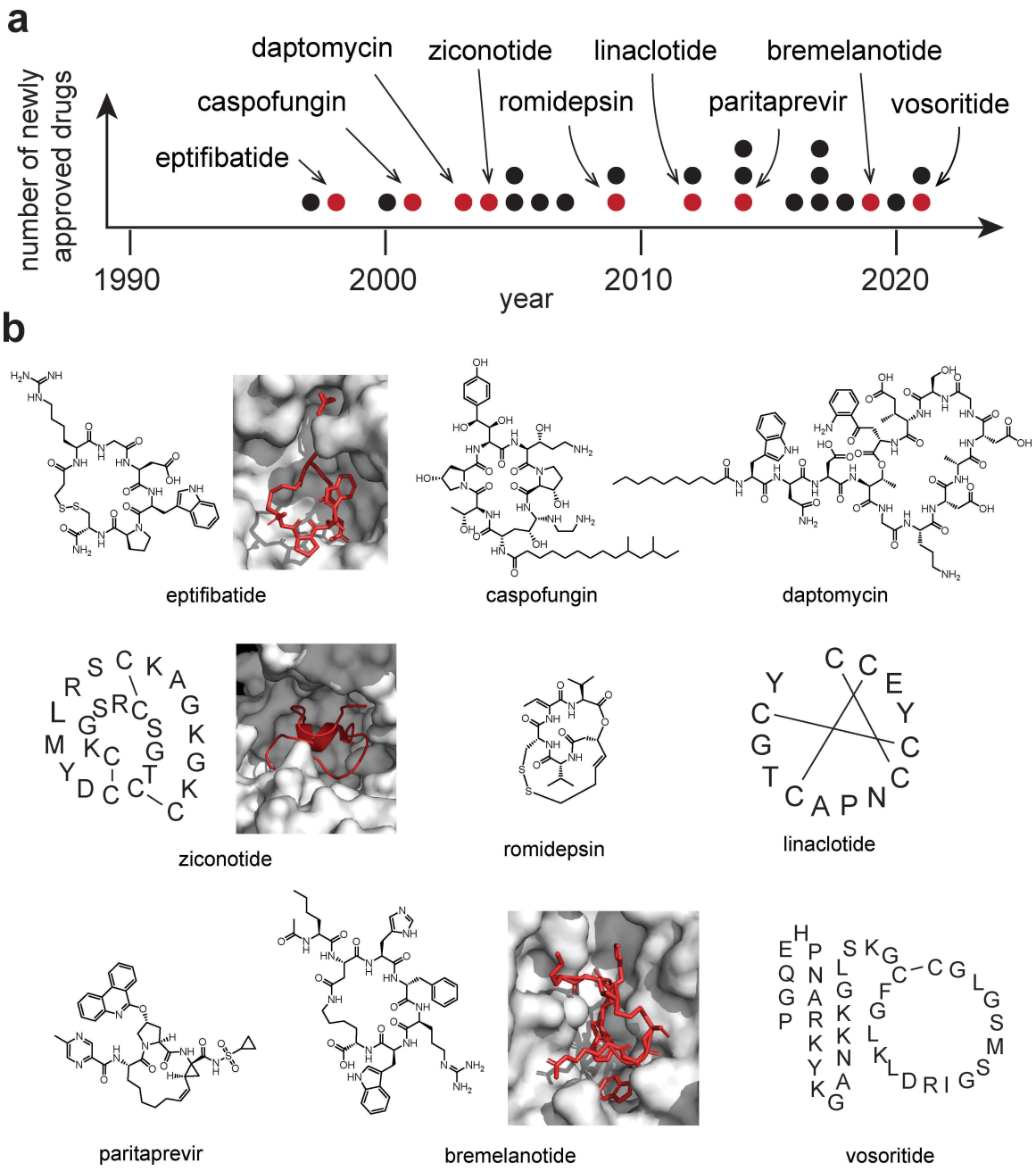


Figure 4. Cyclic peptide therapeutics. (a) Cyclic peptide drugs approved for medical use in the last 30 years. Shown in red are peptides with entirely new structures or mechanisms of action that do not resemble previously approved drugs. (b) Structures of the nine cyclic peptide drugs shown in red in panel a. For longer peptides containing only canonical amino acids, the amino acid sequence and disulfide bridges are shown. For three of the cyclic peptide drugs, structures were reported recently and are shown (eptifibatide: PDB 7THO,[42] ziconotide: PDB 7MIX,[43] bremelanotide: PDB 7F55[44]).

5.1. Peptides for Extracellular Targets

As mentioned above, most of the >40 approved cyclic peptide drugs are rather large in size, composed of 10 or more amino acids. These peptides typically contain several amino acids with polar side chains that render them soluble and easy to handle but also membrane impermeable, thus relegating them mainly to extracellular targets. Being limited to extracellular targets can be an advantage, though, in that properties such as membrane permeability or resistance to digestive enzymes do not need to be considered, reducing the complexity of drug development. Regardless, the vast majority of cyclic peptide drugs act on extracellular targets,[46] and most are applied by injection, though a few exceptions can be orally applied (see section 5.3). A huge opportunity for large cyclic peptides in the extracellular target space lies in the de novo development of high-affinity ligands to tumor markers for either imaging or

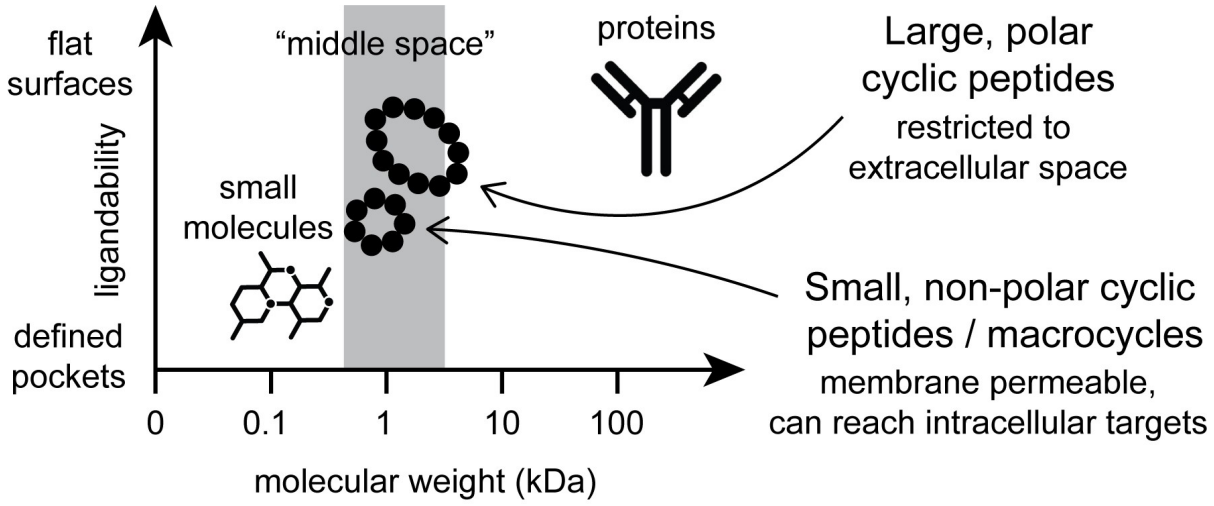


Figure 5. Comparison of cyclic peptide therapeutics to classical small molecules and biologics. Peptides in the "middle space" can be divided into two groups of therapeutics that have different properties and completely different properties and application ranges. The y-axis ligandability refers to the ability of the indicated molecules to bind challenging targets such as proteins with flat, featureless surfaces.

the selective delivery of cytotoxic drugs. Their good tissue penetration and fairly rapid renal clearance allow for selective enrichment at target sites, which is currently being intensively exploited by several biotech and pharmaceutical companies.

While large cyclic peptides are more suited for extracellular targets, strategies are being developed to deliver them to the intracellular space. In the 1990s, several peptides rich in positively charged amino acids were found to penetrate membranes and were thus exploited to increase the delivery of cargo into cells. Such cell-penetrating moieties were later also integrated with target-binding peptides into cyclic peptides.[47] Another approach for increasing membrane permeability lowers the peptide polar surface area by cloaking the amide protons via stapling α-helical peptides using all-hydrocarbon linkers.[22a,b] While the various strategies have considerably improved peptide membrane permeability, they currently do not reach the levels seen for passively diffusing compounds, such as classical small molecule drugs or small apolar cyclic peptides.

5.2. Cell-Permeable Peptides

Small cyclic peptides capable of crossing the membrane are gaining interest due to their ability to address challenging intracellular targets currently deemed "undruggable" via classical small molecules.[4b] Such targets, including protein-protein interactions,[48] are often characterized by rather flat, featureless surfaces that lack defined pockets for small-molecule binding. Cyclic peptides could potentially offer a solution for many of these targets due to their good binding properties in exactly these types of interactions. However, to act on intracellular targets, cyclic peptides need to cross the plasma membrane, which is not trivial due their size and polar surface. To date, there are only a few clinically approved cyclic peptides against intracellular targets, including the immunosuppressant cyclosporine, the anti-cancer agent romidepsin, and the hepatitis C virus (HCV) drugs partitaprevir, grazoprevir, voxilaprevir, and glecaprevir. The latter four HCV protease inhibitors are usually classified as peptidomimetics or macrocycle drugs as they all contain only three amino acids as well as several non-amino acid building blocks.[3,49]

Cyclic peptides can enter cells by various paths and mechanisms, though passive diffusion is by far the most efficient. For diffusing across the nonpolar region of membranes, peptides must have a limited polar surface area, ideally <200 Å^2. At the same time, they must not be too apolar to ensure solubility in the aqueous environment required for diffusion and target binding. The factors dictating cell permeability were studied in depth by comparing the physicochemical properties of many membrane permeable and non-permeable cyclic peptides and macrocyclic compounds in general.[50] Key parameters are the molecular weight, partition coefficient for *n*-octanol/water (logP), polar surface area, number of H-bond donors and acceptors. To reduce the polar surface area for developing membrane-permeable cyclic peptide drugs, amino acids with charged and polar side chains are typically omitted, and the number of peptide bonds are limited. To generate peptides that are still large enough to form sufficient contacts for binding to challenging targets, a common strategy eliminates the H-bond donating effect of amide bonds using *N*-methylated or *N*-alkylated amino acids. Another strategy is based on engineering chameleonic cyclic peptides that can adapt different shapes and surfaces suited for both, good aqueous solubility and target binding, and membrane permeation.

For testing their cell permeability, new methods have been developed for reliably quantifying the proportion of cyclic peptide that crossed the membranes of live cells. Of particular remark is the now widely applied chloroalkane penetration assay (CAPA) by the Kritzer group, in which a

small chloroalkane tag is incorporated into molecules of interest that is sensed in the cell cytosol by a HaloTag protein.[51] In contrast to microscopy based methods that sometimes cannot easily distinguish between peptide in cytosol and compartments such as endosomes, and that may be not fully quantitative, CAPA allows estimating rather precisely the concentration of peptides in the cytosol of cells.

5.3. Orally Available Cyclic Peptides

A range of bioactive cyclic peptides, mostly derived from nature, were found to be orally available, demonstrating the feasibility of applying these molecules by the oral route.[4a,52] Oral administration offers obvious advantages over injection-based strategies, such as convenience for patients, more flexible dosing, and a longer exposure and lower peak concentration due to a slower release from the gastrointestinal tract. However, drugs administered orally must overcome many hurdles, such as resisting the high protease pressure in the stomach and intestine, crossing the epithelial barrier to enter the bloodstream, and resisting first-pass metabolism.

Currently, only a few cyclic peptide drugs are orally available. These include the cyclosporine that can passively cross membranes despite its large size (1203 Da), the just-described membrane-permeable HCV protease inhibitors, and the human hormone drug desmopressin. The latter drug has an oral bioavailability of less than 1 % that is unusually low for a drug (plasma levels when administered orally compared to equivalent dose injected intravenously), but it still achieved therapeutic effects because of the high potency of the cyclic peptide. A wide range of strategies are available to improve the oral availability of peptides, most of them reducing the size and polar surface and improving the proteolytic stability.[53] Additional strategies for enhancing the oral availability of peptide drugs are based on formulations and involve approaches such as enteric pill coatings, protease inhibitors, or permeation enhancers. For example, a tricyclic peptide binding with low picomolar affinity to PCSK9 (a key regulator of plasma LDL-cholesterol), recently developed in an impressive engineering effort at Merck, achieved an oral availability of 2.9 % in cynomolgus monkeys when dosed with a permeation enhancer.[54]

6. Cyclic Peptide Ligand Generation

With their chemically and structurally diverse building blocks and nearly endless possibilities of three-dimensional architectures, cyclic peptides offer a unique format for engaging some of the most challenging targets, including protein-peptide interactions.[55] However, as nature has not evolved a ligand for every desirable target, it is now up to us to take what we have learned from nature and apply those principles to generate cyclic peptide ligands to new proteins. Efforts in this area can be broadly divided into rational design strategies, screens of vast numbers of random cyclic peptides, or combinations thereof.[56]

6.1. Designing Cyclic Peptides

Luckily, nature has provided us with a large selection of cyclic peptides from which to learn best practices for designing our own. Design strategies take advantage of the already vast amount of existing sequence, structural, and functional data of known peptide ligands, and this data is growing by the day. By considering this data on existing binders as well as design ideas, rational design strategies usually require the synthesis of no more than a few peptides to discover a binder.

As a starting point in this cyclic peptide discovery process, most design approaches use short peptide motifs of natural proteins or peptides that bind to the target of interest. The linear sequences are flexible in solution and tend to have a low binding affinity, so they are cyclized to force them into protein-like bioactive conformations, such as strands, helices, or turns.[19] For example Robinson and co-workers cyclized peptides with a D-Pro-L-Pro template which perfectly mimics a type II' β-turn and stabilizes the peptides in β-hairpin structures,[57] or as mentioned above, many α-helical peptides were "stapled" by introducing conformation-stabilizing linkers.[22a,b] A key step in the design process is finding suitable cyclization positions and linkers that impose the desired conformation onto the peptide motifs. The resulting cyclic peptides can exhibit protein-like biological activities and potencies, enabling their use as biological probes and leads for therapeutics, diagnostics, and vaccines. As an additional strategy, the target-binding regions of peptides have been grafted onto larger peptide scaffolds that possess well-defined structures, such as cyclotides. Such folds better impose a defined conformation onto the binding motif to provide a good binding affinity, and these systems were shown to yield peptides highly resistant to proteases.[58]

Design strategies have recently received a boost from innovative computational methods such as de novo design and in silico screening. Powerful tools such as Rosetta software, developed for computational modeling and protein analysis, have been successfully applied to the design of cyclic peptide ligands. For example, cyclic peptide inhibitors of a metallo-β-lactamase were created based on the weak D-Cys-L-Pro dipeptide ligand to generate 50-fold more potent inhibitors.[59] Additionally, inhibitors of histone deacetylases were designed from small-molecule zinc chelators and by sampling various cyclic peptide conformations.[56] However, current design methods are dependent on binding motifs as starting points and cannot yet propose motifs de novo. While the design of binding properties remains challenging, there has been major progress in computationally designing peptides that are membrane permeable,[28] as discussed above.

6.2. Biological Screening: Phage and mRNA Display

Phage display revolutionized the development of cyclic peptide ligands, making it suddenly possible to generate and screen millions of peptides to obtain ligands to any target of interest.[5] For phage-selecting cyclic peptides, peptides displayed on the tip of phage and genetically encoded by DNA packed inside the virion, are cyclized prior to affinity selections. In early work, peptides were cyclized by disulfide bridges that formed spontaneously between a pair of cysteines by oxidation in air. Later, peptides were cyclized by thiol-reactive chemical linkers that bridged cysteines, which afforded redox stability and allowed for more complex structures such as bicyclic peptides.[21] Bicyclic peptides that bind with nanomolar or even picomolar affinity were developed to a wide range of targets, including tumor markers. Several of these peptides were used to generate bicyclic peptide-drug conjugates (BDCs) that are being evaluated in clinical trials.[60]

Another powerful display technology for generating cyclic peptide ligands is mRNA display.[6] In mRNA display, peptides are covalently linked to their encoding mRNA during ribosomal translation. In contrast to phage display, wherein the library size is limited to a few billion peptides due to DNA transformation limitations during library construction, the in vitro nature of mRNA display allows for substantially larger libraries. The cell-free nature of this system also enables the facile incorporation of non-canonical amino acids. Taking advantage of this, Suga and co-workers have developed ingenious methods for efficiently incorporating structurally diverse non-natural amino acids, including those containing a chloroacetamide group that can react with a cysteine of the same peptide for efficient macrocyclization.[61] Their system also allows for the incorporation of *N*-methylated amino acids for generating membrane-permeable cyclic peptides.[62] Chugai Pharmaceutical recently developed a related mRNA display approach that allows efficient incorporation of larger numbers of *N*-methylated amino acids, which yielded a cyclic peptide inhibitor of the important oncology target KRAS that could be engineered into the picomolar binder LUNA18 displaying membrane permeability and an impressive oral availability of 21–47 % in animal species.[63]

Yet another biological high-throughput screening method for cyclic peptides is split-intein circular ligation of peptides and proteins (SICLOPPS).[64] This method expresses random peptides as a fusion with intein fragments, taking advantage of the self-splicing reactions to form cyclic peptides in bacterial cells. The activity of the cyclic peptides is directly tested inside the bacterial cells using two-hybrid-type reporter systems.

6.3. Chemical Screening: OBOC and DNA-Encoded Libraries

For screening libraries containing non-canonical building blocks, split-and-pool solid-phase synthesis principles were developed in the 1990s.[65] The first strategies were based on one-bead-one-compound (OBOC) libraries, where individual beads carry many copies of a unique peptide. OBOC libraries are synthesized by dividing beads into separate reaction vessels, coupling a unique amino acid building block in each well, pooling the beads of all wells, and splitting the beads to new wells to couple the next amino acid. The combinatorial libraries can be screened on bead or after release, and hits are typically identified by mass spectrometry.[66]

A newer approach for screening cyclic peptides or macrocycles containing non-canonical amino acids is based on DNA-encoded libraries (DEL), wherein each compound carries a DNA tag that enabled its identification after affinity selection from a library pool.[67] In a pioneering work, Liu and co-workers used DNA-templated chemistry for generating cyclic peptide DELs.[68] In later studies, several groups developed cyclic peptide DELs using split-and-pool principles wherein both an amino acid and a segment of the DNA code were appended in each cycle of the combinatorial synthesis. DELs offer the great advantage of allowing for large screens exceeding a billion compounds. A challenge of DEL specifically for cyclic peptides is that the synthesis requires many conjugation reactions, including the challenging macrocyclization, which can compromise library quality and complicate the deconvolution of enriched ligands.

6.4. Nano-Scale Synthesis and Functional Screening

A conceptually and technically simple and robust approach for screening cyclic peptides with non-natural amino acids is based on the high-throughput synthesis of such compounds at low scale in separate wells of microtiter plates and screening of crude, non-purified products. At first glance, this approach is limited by the rather small libraries that can be screened, though recent technology developments have enabled the generation and screening of libraries comprising ten-thousands of different cyclic peptides. For example, automated and high-throughput synthesis techniques can provide thousands of mostly pure peptides,[69] and novel methods allow for the combinatorial cyclization and diversification of libraries by coupling chemical fragments at a nanomole scale using contact-less acoustic dispensing.[70] Screening such libraries has produced ligands to several model targets, such as proteases, as well as more difficult targets, including protein-protein interactions.[70b]

7. Summary and Outlook

Cyclic peptides have captivated researchers and scientists due to their remarkable properties. Their three-dimensional structures offer a diverse range of unique shapes that enable unprecedented interactions with biological targets. As a drug modality, cyclic peptides are experiencing a surge of interest based on past successes in developing powerful therapeutics from natural cyclic peptides, the ongoing development and clinical assessment of de novo-generated cyclic peptides, innovative new methods that may help

overcome long-accepted barriers to cell permeable and orally available cyclic peptides, and future visions for integrating computational and experimental methods for peptide drug development. This outlook will be fully dedicated to the latter two points that were not yet sufficiently covered in this review, the recent innovations in developing membrane-permeable and orally available cyclic peptide drugs and to visions of the future in cyclic peptide drug development.

An important current goal for cyclic peptides is to exploit their excellent binding properties for targeting challenging intracellular proteins and for generating orally available drugs. Limited membrane permeability and stability have remained the two major hurdles in peptide drug development. Over the last years, significant progress has been made in understanding the properties that govern membrane permeability and oral availability as well as the mechanisms and strategies used by cyclic peptides and macrocycles to enter cells.[4a,50a,53b] These insights are now helping design libraries for developing cyclic peptides for challenging intracellular targets and oral application. The development of membrane-permeable peptides is being greatly advanced by new methods for generating and screening large libraries of cyclic peptides with small size and limited polar surface area suitable for cell permeation. For instance, recent progress in incorporating multiple *N*-methylated amino acids in mRNA display libraries enable creating ligands in which a more substantial fraction of bonds do not have a polar H-bond donor group and thus high chances of crossing cell membranes.[63] OBOC and DNA-encoding technologies can flexibly incorporate non-natural amino acids such as *N*-alkylated building blocks and may aid in the generation of membrane permeable ligands.[67] New screening technologies such as affinity selection mass spectrometry (AS-MS) were recently applied for screening peptides containing non-natural amino acids[71] and may be applied successfully to cyclic peptides too.[72] Over the past years, our laboratory has developed methods for synthesizing ten-thousands of probable-membrane-permeable cyclic peptides at a nanomolar scale and has identified ligands to challenging intracellular targets, such as protein-protein interactions.[70b] While these various technological innovations alone promise important advances, we anticipate that the combination of them will give a major boost to the field, such that the number of membrane-permeable and orally available cyclic peptide drugs will not remain so small for long.

Our dream for the field involves an explosive development in cell permeable and oral drugs to ever more challenging targets and diseases. While the size of a cyclic peptide screen is physically limited by the number of compounds that can be synthesized and screened, there is realistic hope that computational methods could access a much larger chemical space. This would look similar to virtual screenings currently performed for developing small-molecule drugs. Such computational support may be particularly promising for pre-screening huge numbers of theoretically possible cyclic peptides, with subsequent physical testing of only the most promising ones. For example, with the current number of commercially available building blocks for automated peptide and peptoid synthesis, we estimate that more than a quintillion (10^{18}) different cyclic hexamers can combinatorially be formed, that can obviously never be generated and screened. But computational tools and machine-learning methods may be able to pre-screen such theoretical libraries to identify those, for example, 100,000 cyclic peptides that may fulfill desired properties, such as high membrane permeability or proteolytic stability, for subsequent synthesis and screening. Overall, the powerful methods for synthesizing and testing large numbers of cyclic peptides may soon combine with computational and artificial intelligence methods for sampling an enormous chemical space to develop highly potent cyclic peptide drugs in the future.

Source of Photos

Taken by the authors (C.H.).

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Conflict of Interest

C.H. is a co-founder of Bicycle Therapeutics and Orbis Medicines

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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[1] a) L. Thorstholm, D. J. Craik, *Drug Discovery Today Technol.* **2012**, *9*, e13–e21; b) K. Bajaj, in *Studies in Natural Products Chemistry*, Elsevier, Amsterdam, **2019**, pp. 343–376.
[2] a) A. Zorzi, K. Deyle, C. Heinis, *Curr. Opin. Chem. Biol.* **2017**, *38*, 24–29; b) M. Muttenthaler, G. F. King, D. J. Adams, P. F. Alewood, *Nat. Rev. Drug Discovery* **2021**, *20*, 309–325; c) H. Zhang, S. Chen, *RSC Chem. Biol.* **2022**, *3*, 18–31.
[3] G. J. B. Philippe, D. J. Craik, S. T. Henriques, *Drug Discovery Today* **2021**, *26*, 1521–1531.
[4] a) M. R. Naylor, A. T. Bockus, M. J. Blanco, R. S. Lokey, *Curr. Opin. Chem. Biol.* **2017**, *38*, 141–147; b) L. K. Buckton, M. N. Rahimi, S. R. McAlpine, *Chemistry* **2021**, *27*, 1487–1513.

[5] G. P. Smith, *Science* **1985**, *228*, 1315–1317.
[6] R. W. Roberts, J. W. Szostak, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12297–12302.
[7] N. Bouaicha, C. O. Miles, D. G. Beach, Z. Labidi, A. Djabri, N. Y. Benayache, T. Nguyen-Quang, *Toxin Rev.* **2019**, *11*, 714.
[8] J. Wu, M. Zang, S. Wang, B. Zhao, J. Bai, C. Xu, Y. Shi, X. Qiao, *Food Microbiol.* **2023**, *111*, 104207.
[9] S. J. de Veer, M. W. Kan, D. J. Craik, *Chem. Rev.* **2019**, *119*, 12375–12421.
[10] O. Saether, D. J. Craik, I. D. Campbell, K. Sletten, J. Juul, D. G. Norman, *Biochemistry* **1995**, *34*, 4147–4158.
[11] "World Health Organization model list of essential medicines: 22nd list (2021),".
[12] V. P. Martinovich, K. U. Baradzina, *Russ. J. Bioorg. Chem.* **2022**, *48*, 221–232.
[13] E. R. Declercq, C. Sakala, M. P. Corry, S. Applebaum, A. Herrlich, *J. Perinat. Educ.* **2014**, *23*, 9–16.
[14] L. Sun, D. H. Coy, *Curr. Drug Targets* **2016**, *17*, 529–537.
[15] S. Dhillon, S. J. Keam, *Drugs* **2019**, *79*, 1599–1606.
[16] a) M. Cai, V. J. Hruby, *Biopolymers* **2016**, *106*, 876–883; b) T. Montero-Melendez, T. Boesen, T. E. N. Jonassen, *Semin. Immunol.* **2022**, *59*, 101603.
[17] S. Lai, Q. Zhang, L. Jin, *Antibiotics* **2022**, *12*, 42.
[18] M. Rautenbach, A. M. Troskie, J. A. Vosloo, *Biochimie* **2016**, *130*, 132–145.
[19] T. A. Hill, N. E. Shepherd, F. Diness, D. P. Fairlie, *Angew. Chem. Int. Ed.* **2014**, *53*, 13020–13041.
[20] J. J. Devlin, L. C. Panganiban, P. E. Devlin, *Science* **1990**, *249*, 404–406.
[21] K. Deyle, X. D. Kong, C. Heinis, *Acc. Chem. Res.* **2017**, *50*, 1866–1874.
[22] a) L. D. Walensky, G. H. Bird, *J. Med. Chem.* **2014**, *57*, 6275–6288; b) P. M. Cromm, J. Spiegel, T. N. Grossmann, *ACS Chem. Biol.* **2015**, *10*, 1362–1375; c) D. A. Guarracino, J. A. Riordan, G. M. Barreto, A. L. Oldfield, C. M. Kouba, D. Agrinsoni, *Chem. Rev.* **2019**, *119*, 9915–9949.
[23] A. K. Malde, T. A. Hill, A. Iyer, D. P. Fairlie, *Chem. Rev.* **2019**, *119*, 9861–9914.
[24] B. Khatri, V. R. Nuthakki, J. Chatterjee, *Methods Mol. Biol.* **2019**, *2001*, 17–40.
[25] K. M. Corbett, L. Ford, D. B. Warren, C. W. Pouton, D. K. Chalmers, *J. Med. Chem.* **2021**, *64*, 13131–13151.
[26] D. A. Price, H. Eng, K. A. Farley, G. H. Goetz, Y. Huang, Z. Jiao, A. S. Kalgutkar, N. M. Kablaoui, B. Khunte, S. Liras, C. Limberakis, A. M. Mathiowetz, R. B. Ruggeri, J. M. Quan, Z. Yang, *Org. Biomol. Chem.* **2017**, *15*, 2501–2506.
[27] Y. U. Kwon, T. Kodadek, *Chem. Biol.* **2007**, *14*, 671–677.
[28] G. Bhardwaj, J. O'Connor, S. Rettie, Y. H. Huang, T. A. Ramelot, V. K. Mulligan, G. G. Alpkilic, J. Palmer, A. K. Bera, M. J. Bick, M. Di Piazza, X. Li, P. Hosseinzadeh, T. W. Craven, R. Tejero, A. Lauko, R. Choi, C. Glynn, L. Dong, R. Griffin, W. C. van Voorhis, J. Rodriguez, L. Stewart, G. T. Montelione, D. Craik, D. Baker, *Cell* **2022**, *185*, 3520–3532.
[29] E. Fischer, E. Fourneau, *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 2868–2877.
[30] a) A. I. Fernandez-Llamazares, J. Spengler, F. Albericio, *Biopolymers* **2015**, *104*, 435–452; b) K. Nomura, S. Hashimoto, R. Takeyama, M. Tamiya, T. Kato, T. Muraoka, M. Kage, K. Nii, K. Kotake, S. Iida, T. Emura, M. Tanada, H. Iikura, *J. Med. Chem.* **2022**, *65*, 13401–13412.
[31] A. Choudhary, R. T. Raines, *ChemBioChem* **2011**, *12*, 1801–1807.
[32] S. Mordhorst, F. Ruijne, A. L. Vagstad, O. P. Kuipers, J. Piel, *RSC Chem. Biol.* **2023**, *4*, 7–36.
[33] C. A. G. N. Montalbetti, V. Falque, *Tetrahedron* **2005**, *61*, 10827–10852.
[34] L. C. Szymczak, H. Y. Kuo, M. Mrksich, *Anal. Chem.* **2018**, *90*, 266–282.
[35] J. L. Hickey, D. Sindhikara, S. L. Zultanski, D. M. Schultz, *ACS Med. Chem. Lett.* **2023**, *14*, 557–565.
[36] R. N. Zuckermann, T. Kodadek, *Curr. Opin. Mol. Ther.* **2009**, *11*, 299–307.
[37] H. Lee, W. A. van der Donk, *Annu. Rev. Biochem.* **2022**, *91*, 269–294.
[38] M. Schmidt, A. Toplak, P. Quaedflieg, J. H. van Maarseveen, T. Nuijens, *Drug Discovery Today Technol.* **2017**, *26*, 11–16.
[39] a) C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509–524; b) H. Y. Chow, Y. Zhang, E. Matheson, X. Li, *Chem. Rev.* **2019**, *119*, 9971–10001; c) C. Bechtler, C. Lamers, *RSC Med. Chem.* **2021**, *12*, 1325–1351; d) R. Wills, V. Adebomi, M. Raj, *ChemBioChem* **2021**, *22*, 52–62.
[40] A. Parenty, X. Moreau, J. M. Campagne, *Chem. Rev.* **2006**, *106*, 911–939.
[41] A. T. Bockus, C. M. McEwen, R. S. Lokey, *Curr. Top. Med. Chem.* **2013**, *13*, 821–836.
[42] F. Y. Lin, J. Li, Y. Xie, J. Zhu, T. T. Huong Nguyen, Y. Zhang, J. Zhu, T. A. Springer, *Cell* **2022**, *185*, 3533–3550.
[43] S. Gao, X. Yao, N. Yan, *Nature* **2021**, *596*, 143–147.
[44] H. Zhang, L. N. Chen, D. Yang, C. Mao, Q. Shen, W. Feng, D. D. Shen, A. Dai, S. Xie, Y. Zhou, J. Qin, J. P. Sun, D. H. Scharf, T. Hou, T. Zhou, M. W. Wang, Y. Zhang, *Cell Res.* **2021**, *31*, 1163–1175.
[45] L. Wang, N. Wang, W. Zhang, X. Cheng, Z. Yan, G. Shao, X. Wang, R. Wang, C. Fu, *Signal Transduct. Target Ther.* **2022**, *7*, 48.
[46] S. Taguchi, H. Suga, *Curr. Opin. Chem. Biol.* **2021**, *62*, 82–89.
[47] P. G. Dougherty, A. Sahni, D. Pei, *Chem. Rev.* **2019**, *119*, 10241–10287.
[48] M. Buyanova, D. Pei, *Trends Pharmacol. Sci.* **2022**, *43*, 234–248.
[49] P. Ermert, A. Luther, P. Zbinden, D. Obrecht, *Methods Mol. Biol.* **2019**, *2001*, 147–202.
[50] a) B. Over, P. Matsson, C. Tyrchan, P. Artursson, B. C. Doak, M. A. Foley, C. Hilgendorf, S. E. Johnston, M. D. T. Lee, R. J. Lewis, P. McCarren, G. Muncipinto, U. Norinder, M. W. Perry, J. R. Duvall, J. Kihlberg, *Nat. Chem. Biol.* **2016**, *12*, 1065–1074; b) D. Garcia Jimenez, V. Poongavanam, J. Kihlberg, *J. Med. Chem.* **2023**, *66*, 5377–5396.
[51] a) L. Peraro, Z. Zou, K. M. Makwana, A. E. Cummings, H. L. Ball, H. Yu, Y. S. Lin, B. Levine, J. A. Kritzer, *J. Am. Chem. Soc.* **2017**, *139*, 7792–7802; b) L. Peraro, K. L. Deprey, M. K. Moser, Z. Zou, H. L. Ball, B. Levine, J. A. Kritzer, *J. Am. Chem. Soc.* **2018**, *140*, 11360–11369.
[52] a) C. K. Wang, D. J. Craik, *Biopolymers* **2016**, *106*, 901–909; b) D. S. Nielsen, N. E. Shepherd, W. Xu, A. J. Lucke, M. J. Stoermer, D. P. Fairlie, *Chem. Rev.* **2017**, *117*, 8094–8128.
[53] a) A. F. B. Rader, F. Reichart, M. Weinmuller, H. Kessler, *Bioorg. Med. Chem.* **2018**, *26*, 2766–2773; b) A. F. B. Rader, M. Weinmuller, F. Reichart, A. Schumacher-Klinger, S. Merzbach, C. Gilon, A. Hoffman, H. Kessler, *Angew. Chem. Int. Ed.* **2018**, *57*, 14414–14438; c) A. M. Mathiowetz, *Methods Mol. Biol.* **2019**, *2001*, 1–15.
[54] a) T. J. Tucker, M. W. Embrey, C. Alleyne, R. P. Amin, A. Bass, B. Bhatt, E. Bianchi, D. Branca, T. Bueters, N. Buist, S. N. Ha, M. Hafey, H. He, J. Higgins, D. G. Johns, A. D. Kerekes, K. A. Koeplinger, J. T. Kuethe, N. Li, B. Murphy, P. Orth, S. Salowe, A. Shahripour, R. Tracy, W. Wang, C. Wu, Y. Xiong, H. J. Zokian, H. B. Wood, A. Walji, *J. Med. Chem.* **2021**, *64*, 16770–16800; b) D. G. Johns, L. C. Campeau, P. Banka, A. Bautmans, T. Bueters, E. Bianchi, D. Branca, P. G. Bulger, I. Crevecoeur, F. X. Ding, R. M. Garbaccio, E. D. Guetschow, Y. Guo, S. N. Ha, J. M. Johnston, H. Josien, E. A. Kauh, K. A. Koeplinger, J. T. Kuethe, E. Lai, C. L. Lanning,

A. Y. H. Lee, L. Li, A. G. Nair, E. A. O'Neill, S. A. Stoch, D. A. Thaisrivongs, T. J. Tucker, P. Vachal, K. van Dyck, F. P. Vanhoutte, B. Volckaert, D. G. Wolford, A. Xu, T. Zhao, D. Zhou, S. Zhou, X. Zhu, H. J. Zokian, A. M. Walji, H. B. Wood, *Circulation* **2023**, *148*, 144–158.

[55] a) P. Wojcik, L. Berlicki, *Bioorg. Med. Chem. Lett.* **2016**, *26*, 707–713; b) P. G. Dougherty, Z. Qian, D. Pei, *Biochem. J.* **2017**, *474*, 1109–1125.

[56] X. Li, T. W. Craven, P. M. Levine, *J. Med. Chem.* **2022**, *65*, 11913–11926.

[57] a) N. Srinivas, P. Jetter, B. J. Ueberbacher, M. Werneburg, K. Zerbe, J. Steinmann, B. Van der Meijden, F. Bernardini, A. Lederer, R. L. Dias, P. E. Misson, H. Henze, J. Zumbrunn, F. O. Gombert, D. Obrecht, P. Hunziker, S. Schauer, U. Ziegler, A. Käch, L. Eberl, K. Riedel, S. J. DeMarco, J. A. Robinson, *Science* **2010**, *327*, 1010–1013; b) K. Zerbe, K. Moehle, J. A. Robinson, *Acc. Chem. Res.* **2017**, *50*, 1323–1331.

[58] a) S. E. Northfield, C. K. Wang, C. I. Schroeder, T. Durek, M. W. Kan, J. E. Swedberg, D. J. Craik, *Eur. J. Med. Chem.* **2014**, *77*, 248–257; b) D. J. Craik, J. Du, *Curr. Opin. Chem. Biol.* **2017**, *38*, 8–16.

[59] V. K. Mulligan, S. Workman, T. Sun, S. Rettie, X. Li, L. J. Worrall, T. W. Craven, D. T. King, P. Hosseinzadeh, A. M. Watkins, P. D. Renfrew, S. Guffy, J. W. Labonte, R. Moretti, R. Bonneau, N. C. J. Strynadka, D. Baker, *Proc. Natl. Acad. Sci. USA* **2021**, *118*.

[60] G. E. Mudd, H. Scott, L. Chen, K. van Rietschoten, G. Ivanova-Berndt, K. Dzionek, A. Brown, S. Watcham, L. White, P. U. Park, P. Jeffrey, M. Rigby, P. Beswick, *J. Med. Chem.* **2022**, *65*, 14337–14347.

[61] Y. Goto, A. Ohta, Y. Sako, Y. Yamagishi, H. Murakami, H. Suga, *ACS Chem. Biol.* **2008**, *3*, 120–129.

[62] a) Y. Huang, M. M. Wiedmann, H. Suga, *Chem. Rev.* **2019**, *119*, 10360–10391; b) Y. Goto, H. Suga, *Acc. Chem. Res.* **2021**, *54*, 3604–3617.

[63] M. Tanada, M. Tamiya, A. Matsuo, A. Chiyoda, K. Takano, T. Ito, M. Irie, T. Kotake, R. Takeyama, H. Kawada, R. Hayashi, S. Ishikawa, K. Nomura, N. Furuichi, Y. Morita, M. Kage, S. Hashimoto, K. Nii, H. Sase, K. Ohara, A. Ohta, S. Kuramoto, Y. Nishimura, H. Iikura, T. Shiraishi, *J. Am. Chem. Soc.* **2023**, *145*, 16610–16620.

[64] A. Tavassoli, *Curr. Opin. Chem. Biol.* **2017**, *38*, 30–35.

[65] a) K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82–84; b) K. S. Lam, M. Lebl, V. Krchnák, *Chem. Rev.* **1997**, *97*, 411–448.

[66] Z. Qian, P. Upadhyaya, D. Pei, *Methods Mol. Biol.* **2015**, *1248*, 39–53.

[67] L. Plais, J. Scheuermann, *RSC Chem. Biol.* **2022**, *3*, 7–17.

[68] Z. J. Gartner, B. N. Tse, R. Grubina, J. B. Doyon, T. M. Snyder, D. R. Liu, *Science* **2004**, *305*, 1601–1605.

[69] a) Z. Bognar, G. K. Mothukuri, A. L. Nielsen, M. L. Merz, P. M. F. Panzar, C. Heinis, *Org. Biomol. Chem.* **2022**, *20*, 5699–5703; b) S. Habeshian, G. A. Sable, M. Schuttel, M. L. Merz, C. Heinis, *ACS Chem. Biol.* **2022**, *17*, 181–186.

[70] a) G. Sangouard, A. Zorzi, Y. Wu, E. Ehret, M. Schuttel, S. Kale, C. Diaz-Perlas, J. Vesin, J. Bortoli Chapalay, G. Turcatti, C. Heinis, *Angew. Chem. Int. Ed.* **2021**, *60*, 21702–21707; b) S. Habeshian, M. L. Merz, G. Sangouard, G. K. Mothukuri, M. Schuttel, Z. Bognar, C. Diaz-Perlas, J. Vesin, J. Bortoli Chapalay, G. Turcatti, L. Cendron, A. Angelini, C. Heinis, *Nat. Commun.* **2022**, *13*, 3823.

[71] F. Touti, Z. P. Gates, A. Bandyopadhyay, G. Lautrette, B. L. Pentelute, *Nat. Chem. Biol.* **2019**, *15*, 410–418.

[72] J. M. Mata, E. van der Nol, S. J. Pomplun, *J. Am. Chem. Soc.* **2023**, *145*, 19129–19139.

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