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Permeability of Cyclic Peptide Macrocycles and Cyclotides and Their Potential as Therapeutics

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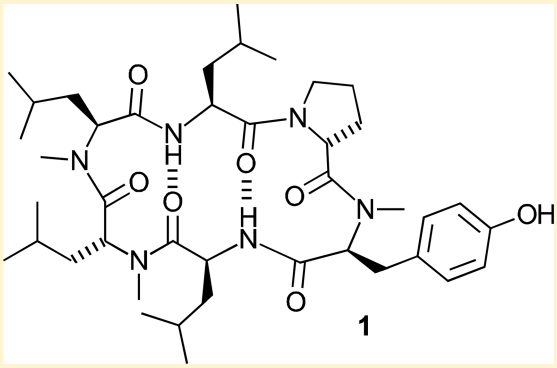
ABSTRACT: Macrocycles have emerged as a viable approach for the modulation of tough targets in drug discovery. In this Innovations article we discuss recent progress toward the design of cell permeable and orally bioavailable peptide macrocycles and cyclotides and provide a perspective for their potential as therapeutics. We highlight design concepts that may be broadly relevant to drug discovery efforts beyond the rule of five.



KEYWORDS: *Peptides, macrocyclization, cell permeability, intramolecular hydrogen bond networks, cyclotides*

With recent advances in genetic and genomic sciences, many of the emerging disease-relevant biological targets are characterized by low drug discovery do-ability (low druggability), as defined by the rule of 5 (Ro5) and other design principles for small molecule drug discovery.[1] This has led to the emergence of new therapeutic modalities, beyond small molecules, which expanded the treatment options for numerous diseases and patients. As a modality, however, small molecules retain several attractive attributes including membrane permeability, tissue targeting, among others. Consequently, medicinal chemists sought to couple small molecule design principles with innovative strategies to enable the exploration of new biology and increase the druggability of difficult targets. Difficult targets, such as protein−protein interactions, class B GPCRs can be now explored by a variety of strategies including protein degradation,[2] selective protein synthesis inhibition,[3] allosterism,[4,5] and protein−protein interaction interface disruption.[6] Protein−protein interaction interface disruption poses a significant challenge for medicinal chemists (or opportunity for innovation depending on one's perspective). Protein−protein interfaces are typically larger that binding pockets for enzyme or receptor targets.[7] Hence, the challenge has become to design molecules that are capable of disrupting these interactions with high efficiency while retaining appropriate physicochemical characteristics for cell permeability and bioavailability.[8−10] Reasonably, many in the drug discovery community turned to inspiration from macrocyclic natural products, including peptides, to deliver molecules with these characteristics. The potential of this science sparked a great deal of investment in both academic research and venture-backed startups. In this Innovations article we will offer a perspective on the therapeutic potential for two classes of naturally inspired macrocycles; cyclic peptides and cyclotides, and comment on key molecular attributes that affect permeability such as macrocyclization and intramolecular hydrogen bond networks. The focus of this Innovations article will be on passive transcellular permeability (energy-independent processes). We will highlight progress to date, discuss the scope and limitations of these modalities as potential therapeutics, and comment on key enabling technologies that could expand the therapeutic utility of these modalities.

WHY PEPTIDES? INSPIRATION FROM CYCLOSPORINE A AND OTHER PEPTIDE NATURAL PRODUCTS

Several important breakthroughs in peptide design, chemistry, and biological characterization influenced the resurgence of interest in macrocyclic peptides as a potentially membrane penetrant modality for the modulation of difficult targets.[11−13] The promise of achieving high affinity and selectivity for biological targets with peptide macrocycles was a key factor for the scientific and business investment in the area. Cyclic peptides can adopt biologically relevant conformations for binding to proteins, and interactions can be optimized to achieve high potency and selectivity given the chemical similarity between targets and ligands.

Synthetic accessibility was also a key factor. The formation of amide bonds has been studied extensively, and an array of

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methods is currently available to synthesize cyclic peptides with high throughput technologies and high efficiency.[14,15] Furthermore, effective synthetic methodologies for the macrocyclization of peptide sequences via side chain functionalization and coupling have been enabled. Highly efficient multicomponent reactions that employ small heterocycles in a cascade synthetic strategy have been applied to the synthesis of peptide macrocycles with great success.[16−18] The synthesis and efficient incorporation of unnatural amino acids with increased structural diversity has emerged as an attractive element as well.[19] In addition to progress in chemistry, several phage display and synthetic biology-based technologies have been developed to produce massive libraries of cyclic and precursor acyclic peptides.[20,21] Biosynthetically produced linear peptides have been converted synthetically to bicyclic products with high yielding chemistry, affording novel adducts with preselected properties.[22] A significant breakthrough in this area has been the incorporation of unnatural amino acid tRNAs in ribosomal biosynthesis of peptides.[23] In all, it is now technically possible to generate peptide macrocycles via a combination of synthetic and biosynthetic methods and to deliver molecules that could address several issues that have plagued this modality including proteolytic stability and immunogenicity.

No other factor, however, has been as important for the renewed interest and investment in cyclic peptides as the detailed study of peptide natural products, especially cyclosporine A (CSA).[24] CSA is an 11 amino acid macrocyclic natural product that has been described as a "molecular chameleon". CSA contains a combination of natural and modified amino acid side chains and a distinct *N*-methylation pattern. This pattern allows CSA to adopt different conformations in aqueous and organic media as evidenced by NMR studies. It is this conformational flexibility that enables the molecule to transition across membranes. Though there may be many factors that potentially aid the permeability of CSA, passive transcellular permeability is a major contributor to its overall profile based on numerous reports of *in vitro* permeability values. As CSA transitions from a hydrophilic to a hydrophobic environment, the molecule changes shape by utilizing an energetically favorable intramolecular hydrogen bond network. The biggest impact of CSA in medicinal chemistry design may very well be the "rediscovery" of three-dimensional properties as molecular design principles. The Ro5, and other design principles that followed, continue to serve as fundamental design guides in classical small molecule space. The factors, however, which underpin the Ro5 and most small molecule design principles, are shapeless and dimensionless. Consequently, the permeability and oral bioavailability of CSA could not have been rationalized based on most rules that apply to classical small molecules within the Ro5 space. Three dimensional properties such as 3D PSA and radius of gyration have been highlighted as key factors that could rationalize the passive permeability of macrocyclic compounds outside of the Ro5 space, including CSA. These three-dimensional properties are more reflective of relevant attributes for passive diffusion through membranes such as molecular volume, size, and polarity.[25,26] Based on a large computational study, it was suggested that compounds with radius of gyration values ≤7 Å and 3D PSA values ≤100 Å^2 are more likely to permeate cells independently of other factors such as molecular weight and hydrogen bond donors or acceptors (HBD, HBA). CSA has been reported to have a TPSA value of 278.8 Å^2. The 3D PSA value for a permeability favorable conformation of CSA has been calculated as 119 Å^2. Similarly, the radius of gyration for the same conformation has been measured as 6.5 Å, thus providing a rationale for its observed permeability.[27]

MACROCYCLES AND PERMEABILITY

There is good agreement in the literature that peptide macrocycles display better properties such as proteolytic stability relative to their acyclic precursors.[28] The literature has been less conclusive on whether macrocyclization of peptides impacts permeability favorably. An earlier report by Kodadek et al.[29] suggested that macrocyclization of peptides did not improve cell permeability. The peptides in the report, unlike CSA, which is characterized by lipophilic side chains, display side chains with amide substituents. It is also not clear from the study that intramolecular hydrogen bond networks are optimized upon cyclization to impact properties such as 3D PSA or radius of gyration. Another important study compared directly acyclic precursors and macrocycles for a series of hexapeptides.[30] In this study it was observed that the cyclic products displayed better permeability than the acyclic analogs. It was postulated that the macrocycles were generally more lipophilic because they adopted secondary structures that masked hydrogen bonding capabilities of the amide backbone to water. A direct study on the impact of cyclization was reported recently.[27] In this study, the pharmacokinetics of CSA, a truncated ten amino acid structural analog of CSA, and its acyclic precursor were compared. The three compounds were iso-lipophilic based on the amino acid side chain composition and experimental polar surface area (EPSA) values.[31] The EPSA values suggested similar ability to produce conformations, which can form intramolecular hydrogen bond networks and mask polarity in hydrophobic environments. However, based on measured RRCK[32] values, the permeability of the acyclic decapeptide was significantly reduced relative to the macrocyclic decapeptide and cyclosporine (RRCK for CSA is 5.6×10^{-6} cm s^{-1}, for the cyclic decapeptide 5.4×10^{-6} cm s^{-1}, for the acyclic 0.6×10^{-6} cm s^{-1}) despite the physicochemical similarity. The calculated values for 3D PSA and radius of gyration for all three compounds, based on conformations predicted to be permeable, were found in the desired range. It was suggested through this study that the acyclic decapeptide occupies significantly larger conformational space in aqueous media, and thus, the entropy loss for adopting conformations favorable to permeability is higher that the cyclic analog. NMR experiments in polar solvents confirmed that the acyclic decapeptide showed greater conformational flexibility than the macrocycle and reduced ability to form intramolecular hydrogen bonds. Overall, this study supports the notion that macrocyclization positively impacts the permeability of compounds, which can form intramolecular hydrogen bond networks. The study also suggests that, while much progress has been made in computational methods for predicting permeability, the combination of in silico and biophysical methods yields a deeper understanding of the factors that govern permeability.

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MANAGING HYDROGEN BOND NETWORKS TO DESIGN PERMEABLE PEPTIDE MACROCYCLES: *N*-METHYLATION, HYDROPHOBIC SHIELDING, AND OTHER APPROACHES

The significance of intramolecular hydrogen bonding in medicinal chemistry design, especially for beyond rule of five molecules (BRo5), has been appreciated by the drug discovery community.[33,34] Intramolecular hydrogen bonding has been identified as a key design element, which affects properties like permeability and solubility. For peptide macrocycles specifically, the natural product pool has provided significant insights on strategies to promote intramolecular hydrogen bonding and minimize hydrogen bonding of amides to water.[35] These strategies include *N*-methylation of backbone amides, introduction of heterocyclic amide isosteres, incorporation of steric bias, and hydrophobic shielding among others. *N*-Methylation of backbone amides is a characteristic structural feature of many peptide natural product macrocycles. *N*-Methylation has been employed as a strategy by many research groups in order to influence both biological function and pharmacokinetic properties of synthetic peptide macrocycles including absorption and distribution.[36] The ability to rationally enforce stereochemical bias via *N*-methylation, hydrophobic shielding, and other approaches, to preserve or promote the formation of intramolecular hydrogen bonds, and to ultimately deliver compounds with improved permeability has emerged as a pivotal strategy in the field.[37] We highlight below a few key examples that illustrate the effectiveness of the strategy.

A reference compound that captures the essence of this strategy is a partially *N*-methylated cyclic hexapeptide designed by the Lokey group at UCSC, in the literature referred to as 1NMe3 (**1**, Figure 1).[38] 1NMe3 serves as a reference compound for several reasons discussed below. A key design concept that led to the identification of this compound was that intramolecular hydrogen bond networks could be employed rationally to direct the selective *N*-methylation of solvent exposed backbone amides. This was experimentally validated as under typically nonselective *N*-methylation conditions only the amides that were not engaged in strong intramolecular bond networks were alkylated. Hydrogen−deuterium exchange studies were used to confirm intramolecular hydrogen bonding. The study also confirmed that exhaustive *N*-methylation was counterproductive to permeability and bioavailability, again highlighting the significance of intramolecular hydrogen bonding in improving permeability. Further, a highly effective computational methodology was developed and applied to predict permeability of peptide macrocycles. Physics-based calculations were utilized to identify macrocyles with improved permeability, specifically calculation of the energetic cost from desolvation as a compound is transferred from water to the membrane. This method has been applied to guide the design of compound libraries with improved permeability. Equally, a significant contribution from this research was the detailed pharmacokinetic evaluation of 1NMe3. Based on an i.v./p.o. cross over study the absolute oral bioavailability of the compound was determined as 28%. The *in vivo* clearance of the compound in rat was low, especially when taking into consideration a moderate intrinsic clearance rate in rat liver microsomes. Given the lipophilic content of the compound, it is reasonable to conclude that the low *in vivo* clearance rate may be in large part due to high protein binding. The observed pharmacokinetics, coupled with the lipophilic features apparently required for permeability, define the scope and potential limitation for this modality. While 1NMe3 was purely utilized as a pharmacokinetic probe, its overall attributes suggest that extreme biological potency will be required for molecules of this class to become therapeutically relevant given the overall lipophilicity. The pharmacokinetic characterization of 1NMe3 generated additional interest for developing a biophysical understanding of the behavior of molecules with similar structure and attributes. Toward this end, a NMR-based methodology was reported to enable the rational design of permeable peptide macrocycles by identifying solvent exposed amides with NMR amide temperature coefficients prior to capping with methyl groups.[39,40] The 1NMe3 study also provided hints that it may be possible to balance free clearance and permeability with the incorporation of less lipophilic amino acids with the caveat that the position of such residues in the scaffold is an important consideration. An earlier report by Kessler and co-workers suggested that modest oral bioavailability and incorporation of polar and ionizable residues may be compatible in the context of partially methylated peptides with high aromatic residue content. This finding stands out as a very useful outlier.[41] A follow-on study to 1NMe3 suggested that the incorporation of polar and ionizable residues improved clearance rates with concomitant decrease in permeability. In the same study, it was shown that the incorporation of unnatural amino acids could serve as an effective approach toward balancing *in vitro* clearance rates and permeability, thus pointing to the significance of such fragments in the design of cyclic peptides with more conventional drug-like properties.[42] Similar results regarding the impact of incorporating polar amino acid side chains have been published by the Kessler group.[43]

As stated before, in addition to *N*-methylation, hydrophobic shielding of backbone amide bonds from solvation may also be an effective strategy for improving permeability and oral bioavailability. This concept was well demonstrated in a study that compared the pharmacokinetics of a cyclic penta-leucine

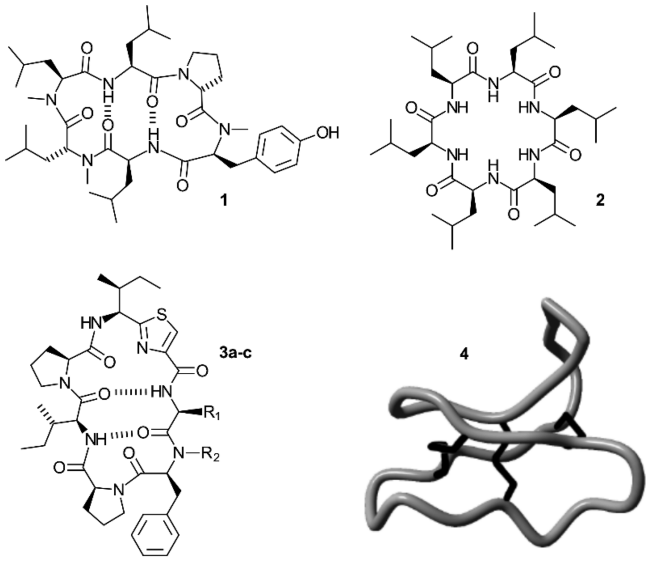

Figure 1. Structure of **1**, 1NMe3; **2**, hexaleucine peptide; **3a**, R_1 = Me, R_2 = H = sanguinamide A; **3b**, R_1 = *t*-Bu, R_2 = Me; **3c**, R_1 = *t*-Bu, R_2 = H; **4**, kalata b1.

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peptide with two diastereomeric cyclic hexa-leucine macrocycles.[44] As the results of the study suggested, the pentamer peptide is characterized by low permeability and bioavailability. The compound assumes a preferred conformation that promotes intermolecular hydrogen bonding and aggregation. Alternatively, the conformation assumed by the hexamer leucine macrocycle (**2**, Figure 1) displays the leucine side chains in a way that prevents intermolecular hydrogen bonding and aggregation. At the same time, the leucine residues provide hydrophobic shielding to the backbone amides and reduce their ability to form a hydrogen bond with water, thus making the compound more permeable. *In vitro*, both hexamer compounds display better permeability than CSA, as measured by RRCK. The increased permeability of the hexamer leucine translated into reasonable bioavailability (17% *F*). Interestingly, the hexamer leucine diastereomeric pair shows markedly different *in vivo* clearance rates in rat suggesting a conformational influence on factors that govern total clearance.

The manipulation of the hexapeptide marine natural product sanguinamide A (**3a**, Figure 1) represents a powerful example of applying a combination of strategies to promote intramolecular hydrogen bonding and to minimize solvation of amides in order to improve permeability.[45] Sanguinamide A is a thiazole containing hexapeptide that contains two proline residues in its scaffold and displays four amides with the potential to solvate with water. The compound shows low permeability based on RRCK measurements (0.7×10^{-6} cm s^{-1}) and bioavailability (7% *F*). Through a combination of NMR and hydrogen−deuterium exchange studies, the residues mostly exposed to solvent were identified. The incorporation of large aliphatic substituents (*t*-butyl glycine) proximally to solvent exposed amides and the selective *N*-methylation of solvent exposed amides delivered analogs with improved permeability. Incorporation of single *t*-butyl glycine mutation into the scaffold, in combination with the *N*-methylation of a solvent exposed amide delivered a compound (**3b**) with 10-fold higher permeability (9.6×10^{-6} cm s^{-1}), relative to the natural product and improved oral bioavailability (21% *F*). Incorporation of just a single *t*-butyl glycine mutation without *N*-methylation of solvent-exposed amides delivered a compound (**3c**) with the strongest intramolecular hydrogen bond network as measured by both NMR and hydrogen−deuterium exchanges studies, which showed a 2-fold increase in permeability (1.2×10^{-6} cm s^{-1}) and a significant increase in bioavailability (51% *F*). The incorporation of hydrophobic shielding and *N*-methylation showed measurable improvement in total clearance rates, relative to the natural product, again likely attributable to increased protein binding as a result of conformational manipulation through stronger intramolecular hydrogen bond networks and increased lipophilicity due to *N*-methylation. A follow-on paper on sanguinamide, however, suggested that the observed impact of sterically bulky side chain substitutions on permeability maybe in part due to increasing aliphatic content.[46] The impact of hydrogen bonding, stereochemistry, and hydrophobic shielding was also reported in the context of a large library design and synthesis ,which revealed numerous scaffolds with increased permeability, albeit again with highly lipophilic amino acid side chains. This study also highlighted the progress in computational predictions of permeability of macrocycles. Calculated 3D solvent accessible surface area (SASA) along with calculated desolvation energy values (ΔG_{desolv}) were highly correlated with permeability as measured in a Caco-2 screen.[47]

The incorporation of nonpeptide fragments into peptide macrocycles, as replacements of canonical amino acids, has been explored by the scientific community.[48] Natural product and peptoid fragments have been utilized to explore the impact on intramolecular hydrogen bonding and stereochemistry, permeability, solubility, and biological properties of the macrocycles. Obviously, the strategic incorporation of nonpeptide fragments can produce macrocycles with increased structural diversity. The incorporation of γ-amino acids (statines) into the backbone of peptide macrocycles delivered molecules that retained the ability to form intramolecular hydrogen bonds and showed good permeability and oral bioavailability.[49] Most importantly, the resulting macrocycles yielded some interesting insights about the impact of stereochemistry on key molecular properties. Specifically, the incorporation of statines into the peptide backbone produced compounds with improved intrinsic clearance rates (measured by human liver microsomes) and solubility relative to 1NMe3. Subtle stereochemical variations within the class produced compounds with markedly different intrinsic clearance rates and solubility values. These findings clearly underline the complex influence of stereochemistry and conformation on the overall drug likeness of macrocycles. Peptoids have been used to effectively mask amides from solvation.[50] In a study aimed at the discovery of orally bioavailable macrocyclic modulators of CXCR7, strategic placement of peptoids delivered macrocycles that possessed the desired intramolecular hydrogen bonding properties supportive of permeability. Furthermore, in the same study it was shown that strongly ionizable amino acids, which hindered permeability, could be replaced by neutral unnatural amino acids, again highlighting the significance of these synthons. Overall, it was convincingly demonstrated that large potency gains and improvements in permeability and oral bioavailability could be achieved in parallel in the context of a lead optimization campaign, albeit in highly lipophilic molecular space.[51]

CYCLOTIDES

Overlooked in many reviews of macrocyclic peptides, much has nonetheless been written about the promise of cyclotides as peptide therapeutics.[52,53] It is worth noting that, while there is strong evidence that passive permeability can be achieved with designed peptide macrocycles, natural cyclotides do not appear to permeate membranes through passive processes. However, the compelling architecture of cyclotides and the reported biological activities with these compounds merit discussion in this Innovations article. Even though no cyclotide has reached the clinic yet, several aspects favor their progression. Cyclotides are naturally occurring plant-based peptides with a cyclic cysteine knot (CCK) structure that imparts a high degree of metabolic, thermal, and chemical stability. These 28−37 amino acid circular peptides with their complex disulfide interconnections can be prepared by solid-phase synthesis and pharmacologic variation is possible by sequence substitution within exposed loops.[54] More promising and further supporting their prospects is the ability to rapidly generate diverse libraries of fully folded cyclotides by in cell expression.[55] The ability to rapidly synthesize and test analogues built around the conserved cyclotide core and to optimize activity for a given target is a major advantage for the progression of cyclotides. As with other peptides therapeutics, cyclotides currently have limitations that still need to be addressed. Chief among these limitations are oral bioavail-

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ability and a general demonstration of membrane permeability, which would enable the pursuit of intracellular targets. Bioactivity has been reported for the archetypal cyclotide kalata B1 (**4**, Figure 1), as well as "grafted" bradykinin analogues, but oral bioavailability data supported by intravenous and oral exposure data have not been disclosed.[56] Pharmacologic activity after oral dosing without support from exposure by both routes of administration cannot be characterized as orally bioavailable and is better described as orally active. Furthermore, the cellular uptake by macropinocytosis reported for MCoTI-II[57] is an observation that may not be possible to systematically reproduce for novel cyclotides. In addition, the demonstrated membrane interactions of cyclotides such as kalata B1 and subsequent cell membrane disruption and leakage of cell content suggests the need to distinguish membrane transit from cellular toxicity based on cyclotide-mediated pore opening. With cell permeability, an unproven aspect of cyclotides, their initial application may be best suited for parenteral administration for cell surface targets. In addition, the claims[58] that cyclotides are less prone to immunogenicity due to their greater stability are also premature. Unlike peptide therapeutics that are mimetics of endogenous proteins, optimized cyclotides will have xenobiotic characteristics. While cyclotides may require disulfide bond cleavage as a prelude to their susceptibility to proteases,[59] cellular processing and the presentation of unique peptide fragments on antigen presenting cells remain likely for cyclotides. Consistent with this outcome are preclinical pharmacokinetic data that do not support a clearance mechanism entirely based on intact cyclotide.[60] Neither of these hurdles is necessarily insurmountable and the overriding benefit of this class of peptides remains the ability to rapidly prepare and screen in cell. Investing in this approach will require a compelling target choice, coupled with differentiation from other modalities such as antibodies and oligonucleotides. Opportunities in this regard are more likely to exist with extracellular targets where agonist pharmacology or partial modulation of the target is preferred.

PERSPECTIVE

The complexity of biological targets today requires new innovations in drug discovery, and the chemistry community is responding to the challenge. Macrocycles have emerged as one potential solution in the pursuit of difficult drug discovery targets. It is evident that much progress has been made toward understanding the behavior of molecules that reside beyond classical small molecule property space, typically in molecular weight between 500 and 1200 amu. Although the field is still evolving, some potentially useful design principles have emerged.[61] Within the broader concept of macrocycles, peptide macrocycles have received a lot of attention by researchers in academia and industry. No doubt, peptide macrocycles furnish valuable biology and chemical biology tools. Collaborative research on this topic has helped to scope out the therapeutic potential and challenges with this modality. As stated earlier, there are numerous advantages with peptide macrocycles including the potential for high potency and selectivity and synthetic and biosynthetic accessibility. To date, a key limitation for the modality is the high lipophilic content required for permeability and oral bioavailability. This potentially limits the utility of this modality to targets where extreme potency can be achieved. In the end, the choice to employ this modality must be determined by the intrinsic value of the biological target. For difficult targets, though highly validated by human biology, this modality has a place in the armory of potential solutions. In such circumstances where the high value target is intractable to small molecule and not accessible to antibodies or where the pharmacologic profile lies between complete inhibition or activation, this modality could be evaluated. Unlike classical small molecules, drug discovery research in this area is far less mature, and therefore, there are no unifying rules to guide the design of drug-like molecules. Most likely the knowledge base will have to be built by focusing on outlier compounds, especially with respect to pharmacokinetics and disposition. Consequently, the drug discovery journey is likely to be longer. The drug discovery community is responding to the emergence of disease relevant biological targets by moving Bro5. The systematic approach taken toward defining the potential of macrocyclic peptides as cell permeable and orally bioavailable therapeutics has generated capabilities that will be relevant to other BRo5 drug discovery campaigns (e.g., protein degradation). The systematic evaluation of 3D molecular properties and factors that influence molecular shape and volume, the development of physics-based computational methodologies for predicting permeability, the exploration of biophysical methods for rationally designing permeable macrocycles, and the development of high throughput analytical methods for assessing permeability potential are a few examples of scientific breakthroughs that will aid drug discovery efforts in BRro5 space. With respect to cyclotides specifically, more needs to be done to elucidate the differences between bioactivity and bioavailability. There is not strong evidence currently that cyclotides achieve appreciable oral bioavailability. The intriguing architecture of cyclotides, which results in their remarkable stability, can be the basis for designing novel therapeutics, equivalent to mini-antibodies with long duration. Fine tuning the biological activity of cyclotide-inspired molecules against specific targets while retaining their stability attributes will be an area of productive research. In closing, it is once again important to highlight the value of collaboration in this space. To systematically expand the small molecule knowledge base, BRo5 requires the integration of basic and applied research principles, thus making academic-industrial partnerships extremely valuable.

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Notes

The authors declare no competing financial interest.

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