HHS Public Access

Author manuscript

ACS Chem Biol. Author manuscript; available in PMC 2016 April 27.

Published in final edited form as:

ACS Chem Biol. 2013 March 15; 8(3): 488–499. doi:10.1021/cb300515u.

Getting in Shape: Controlling Peptide Bioactivity and Bioavailability Using Conformational Constraints

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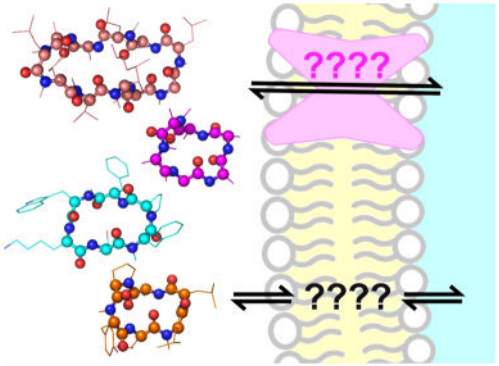
Abstract

Chemical biologists commonly seek out correlations between the physicochemical properties of molecules and their behavior in biological systems. However, a new paradigm is emerging for peptides in which *conformation* is recognized as the primary determinant of bioactivity and bioavailability. This review highlights an emerging body of work that directly addresses how a peptide's conformation controls its biological effects, cell penetration, and intestinal absorption. Based on this work, the dream of mimicking the potency and bioavailability of natural product peptides is getting closer to reality.

Abstract



Discovering new probes and lead compounds for specific biological targets is a complex endeavor – so many molecules, so little time! To reduce this complexity and to promote bioactivity and bioavailability, chemical space can be filtered using parameters such as size, hydrophobicity, and hydrogen-bonding propensity.[1,2] Applying these guidelines to small molecules can be informative, but there are no comparable guidelines for understanding the suitability of peptides as drugs. However, a growing body of work is revealing that, for peptides and their analogues, *conformation* plays a predominant role in determining their biological effects. Increasingly, previous "exceptions" to physicochemical rules are being

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Notes

The authors declare no competing financial interest.

evaluated as general scaffolds for highly bioactive and bioavailable molecules. In this Review, we highlight recent findings from a variety of fields that are converging on a new understanding of how conformation controls peptide bio-activity and bioavailability. This research is galvanizing academic and industrial chemists alike and driving a new wave of research into modified peptides as probes and therapeutics.

Bioactivity is the ability of a molecule to have a specific biological effect when introduced to a living system such as cultured cells or a whole organism. The bioactivity of most compounds is dictated by their specific targets and binding modes, but overarching trends can be observed for specific "privileged" compound classes such as benzodiazepines and certain natural product macrocycles.[3–5] As a class, peptides can be bioactive against diverse targets because they can assume a variety of conformations. However, this flexibility can be a drawback, leaving peptides vulnerable to proteolytic degradation. *Bioavailability* is the extent to which a molecule, once introduced to a living organism, reaches its physiological target and persists long enough to have an effect. The major components of bioavailability through oral administration are breakdown in the gastrointestinal tract, gut absorption, distribution into circulation and physiological compartments, metabolism, and elimination. For peptides, each of these is a formidable obstacle.

Controlling biology with peptides is a century-old field,[6,7] and great strides have been made in the fields of peptidomimetics and peptide drug development.[8,9] Though we have come far, we have not yet matched the activities of orally bioavailable natural products. Only recently have studies directly addressed the factors that govern peptide bioactivity and bioavailability, and the emerging results indicate that modern tools for controlling conformation may finally unlock the full potential of peptides as biological agents.

TWO LESSONS FROM NATURAL PRODUCTS

Medicinal chemistry can be seen as the short-term effort of a few chemists to imitate the long-term efforts of an army of microbes; this is certainly true for the field of peptide design. α-amanitin, for example, is an astonishingly potent, bioavailable (and poisonous) natural product (Figure 1).[10,11] α-amanitin acts by potently inhibiting RNA polymerase II, and its striking bioavailability (oral LD_{50} is less than 0.1 mg/kg; see Table 1) has prompted detailed investigations into its pharmacokinetics.[12] Its mode of gut absorption is unknown, but once absorbed it concentrates in the liver because of facilitated diffusion by bile acid transporters.[13] Structure–activity relationships reffect the known structure of the amanitin/RNA polymerase complex, solved by Kornberg in 1970,[14] but also hint at specific conformational effects on bioactivity and bioavailability.[12] Solution and crystal structures indicate that amanitin's bicyclic scaffold is relatively rigid, and large perturbations to its rigidified structure, such as opening one of the rings, leads to complete loss of bioactivity and bioavailability. More subtle structural alterations, such as reduction, oxidation, or changes within the cross-link, have more subtle effects on overall toxicity that are not easily attributed to changes in target binding. Thus, α-amanitin's locked conformation appears to promote not only potent, selective RNA polymerase binding but also gut absorption and facilitated transport into hepatocytes.

Cyclosporine is another defining example of a peptide natural product with surprising bioactivity and bioavailability (Figure 1).[15] After 40 years of research, we still lack definitive understanding of the mechanisms of gut uptake and cell penetration by cyclosporine. However, there is a great deal of indirect evidence that indicates a unique model for these processes. In aqueous solvent, cyclosporine assumes a conformation similar to that observed in the co-crystal structure of the cyclosporine/cyclophilin complex.[16–21] In organic solvent, however, it inverts to form internal backbone hydrogen bonds, with its N-methylated amino acids pointing outward.[22] This ability to assume a different conformation in hydrophobic environments is hypothesized to promote passive diffusion across plasma membranes.[23] Practicable strategies for mimicking this "shapeshifting" capability have lagged due to the synthetic challenges of peptide cyclization and N-methylation, and the need to apply rigorous models of cell penetration and tissue absorption. Only recently have these obstacles been overcome, allowing for design and screening for model peptides that appear to diffuse through membranes in a cyclosporine-like manner (see below).[24,25]

Our current understanding of α-amanitin and cyclosporine provide contrasting examples of how conformation controls peptide bioactivity and bioavailability (Figure 1). In the case of cyclosporine, a flexible structure allows for a large, hydrophilic binding interface that can be conformationally "hidden" in order to promote passive diffusion. In α-amanitin's case, nature has evolved a single rigid structure that avoids metabolic modification and degradation, takes advantage of existing transporters, and binds its target with high affinity. As we describe below, chemical biologists have taken both approaches to developing peptide probes, and either approach may be used to maximize bioactivity and bioavailability.

PEPTIDE ENGINEERING WITH SMALL EPITOPES

Given the critical role of conformation in molecular recognition, peptide chemists have long sought to improve natural peptides using conformational constraints. Throughout the late 1970s and 1980s, pioneers such as Hirschmann, Hruby, and Kessler used conformational constraints to recapitulate the active conformations of potent peptide hormones.[26–31] In doing so, they revealed critical information on the binding modes of oxytocin, enkephalin, somatostatin, melanocortin, and other hormones and on the mechanisms of their associated receptors. Constrained analogues of these hormones became important probes for understanding endocrine signaling pathways and in some cases were developed as pharmaceuticals. A particularly illustrative example is the development of somatostatin analogues, recently reviewed by Ovadia et al.[32] Somatostatin, discovered in 1972, is a hormone that counteracts the effects of human growth hormone.[33] Subsequent analysis of the structural and conformational requirements for its activity by Veber, Hirschmann, and colleagues led to the design of potent constrained peptide analogues.[27,34,35] This paved the way for the development of somatostatin analogues

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development of somatostatin analogues as treatments for acromegaly and other conditions that arise from an excess of growth hormone activity.[33] These drugs are minimized forms of somatostatin that contain a disulfide-mediated macrocycle and artificial amino acids including a critical, turn-promoting D-tryptophan. These modifications promote a bioactive conformation and prolong the half-life in the blood, which allows for intravenous administration. Recent work has continued this progress, with a structure-guided N-methyl scan of a backbone-cyclic analogue resulting in a triply N-methylated version with substantial oral bioavailability (Figure 2a and Table 1).[32,36] While the ability of the N-methylated peptide to undergo conformational change in hydrophobic environments was not addressed, the authors noted that the specific N-methylations that promoted bioavailability also appeared to stabilize the conformation responsible for potent bioactivity. Thus, evidence to date implies that, like α-amanitin, stabilized somatostatin analogues derive both target binding and bioavailability from a single, rigidified conformation.

Similar optimization methodologies have been applied to other peptide hormones and small binding epitopes. Peptide ligands for melanocortin receptors were developed by cyclizing fragments of natural hormones to improve bioactivity, then N-methylating to enforce selectivity for specific receptor 37–40 One cyclic peptide that mimics α-melanocyte-stimulating hormone was shown to be orally bioavailable and effective as an antiobesity drug in rats.[41] Another important example is the development of cyclic peptides that incorporate the RGD sequence from fibronectin as potent and selective integrin ligands.[42,43] Several series of analogues were explored that first varied ring size and the placement of a D-amino acid and then later exhaustively searched for N-methylated analogues with improved selectivity and bioavailability.[44–47] RGD-derived cyclic peptides are now standard tools for imaging, targeting, and manipulating integrins, and the RGD peptide Cilengitide is currently being tested as an antiangiogenic agent for the treatment of glioblastoma.[48,49]

All together, these studies share a long-term methodology: first cyclize the backbone to stabilize a biologically relevant conformation, then make rational substitutions with natural and unnatural amino acids to introduce distinct conformational preferences that promote target affinity and desired physical properties. Historically, N-methylation was applied in later stages, perhaps due to the difficulty of the chemistry involved, though thanks to the work of Kessler and others, synthesis of N-methylated peptides is more accessible than ever before.[26,50–52] The effects of cyclization, substitution and N-methylation are typically non-additive and highly cooperative, making it difficult to predict or rationalize the results of even simple series of analogues. Even so, these “scanning” methodologies have established that conformational control of peptide bioavailability is possible, and indeed may be possible in most cases in which a small, contiguous epitope is found to mediate bioactivity.

HIGHLY CONSTRAINED NATURAL PRODUCT PEPTIDES: LOCKED AND LOADED

Recent developments using highly constrained natural products have provided a striking counterpoint to the previous work on smaller cyclic peptides.[53–55] In a startling report in 2010, Craik and co-workers reported that the α-conotoxin Vc1.1, a venom peptide produced

by predatory cone snails, can be engineered to be orally active in rats using backbone macrocyclization.[56] Vc1.1 is a 16-residue peptide with a short internal α-helix, an amidated C-terminus, and two intramolecular disulfide bonds. It is a potent analgesic that acts through $GABA_B$ receptors, though its specific mode of action is unclear. In designing the cyclic conotoxin, Craik and co-workers used previously published work on protein and peptide cyclization to derive a linear relationship between the distance between termini and the number of residues in a successful linker.[56] On the basis of this empirical correlation, they tested cyclic Vc1.1 analogues with simple five-residue and six-residue linkers composed of glycine and alanine and found the six-residue linker afforded a molecule with optimal activity (Figure 2d). This cyclic Vc1.1 was more potent than linear Vc1.1 as a $GABA_B$-mediated calcium channel blocker and was also more selective for this effect over inhibition of nicotinic acetylcholine receptors. Most importantly, cyclic Vc1.1 showed dose-dependent relief of neuropathic pain in rats when administered orally. Its activity at 1.3 mg/kg (see Table 1) was similar to the activity of gabapentin, a commonly prescribed oral analgesic, at 30 mg/kg, highlighting the potency and bioavailability of the cyclic conotoxin.

While the most advanced applications of natural product peptides are targeting their native receptors, significant efforts are being made to adapt these peptides as designer scaffolds for inhibition of other targets. Example scaffolds include highly disulfide-bonded scaffolds such as animal defensins and plant cyclotides,[53–61] as well as naturally knotted “lasso” peptides.[62] In a striking example, Tam and co-workers recently described an orally active bradykinin receptor antagonist based on the highly constrained peptide kalata B1.[63] Kalata B1 is a member of the cyclotide family of natural products, which are plantderived head-to-tail cyclic peptides with multiple disulfides in a “cystine knot” topology.[55,64] Kalata B1 is itself orally bioavailable; it is the active ingredient responsible for the oxytocin-like activity of an herbal tea prepared by native Congolese for inducing labor.[65] Key sequences from established bradykinin receptor antagonist peptides were “grafted” within a loop of the 29-residue kalata B1. The most successful analogue (Figure 2e) showed pain relief in mice when orally administered at 1 mg/kg (Table 1). Equal effects were observed for intraperitoneal injection and oral administration, demonstrating that digestive tract stability and intestinal absorption were not the primary limiting factors for the observed pain relief. Taken together with studies on α-conotoxin work and other venom peptides and cyclotides, it is clear that larger, highly constrained cyclic peptides can be as potent and bioavailable as smaller cyclic peptides.

There are a few clues to explain the exceptional properties of these highly constrained peptides. Backbone cyclization is clearly important, but the effects of masking and restricting the termini cannot be understood solely through estimates of hydrophobicity or hydrogen-bonding potential. These cyclic peptides appear to be genuine “privileged structures,” because the structure of the cyclic Vc1.1 peptide was revealed to be nearly identical to that of the linear form,[56] and NMR chemical shift data indicate that the grafted kalata analogue has a fold similar to that of Kalata B1.[63] In fact, greater overall rigidity of the bioactive peptide epitope seems to affect binding in a receptor-specific way, allowing for selectivity tuning based on conformational restriction.[56] It is also likely that, even for highly constrained peptides, further rigidification can reduce dynamic “breathing” of the structure, resulting in a longer overall halflife. This was observed directly for cyclic conotoxins, which

had reduced inactivation due to disulfide shuffing in simulated intestinal fluid.[56,58,66,67] Further investigations on these and related scaffolds will reveal how unique these privileged structures are and how well they can be used to target various extracellular proteins.

PLEASE RETURN YOUR HELICES TO AN UPRIGHT, LOCKED POSITION

The field of α-helix stabilization is well-understood with respect to biophysics and design, and so this field has also recently converged on understanding how conformational restriction affects activity in biological systems.[68,69] This aspect has always been part of the study of constrained helices. Some of the earliest work in this area was Lerner and colleagues' demonstration in 1988 that a simple peptide derived from malarial sporozoites could be "chemically shaped" to a more structured, immunogenic form.[70] By the early 1990s, stabilization of α-helix structure was generalized by several groups through the formation of side chain lactams, disulfides, metal ion coordination complexes, and a variety of other linkages.[71–75] Several reviews have described the biophysics and structure–activity relationships of these stabilized α-helical peptides.[76–78] Because of their synthetic accessibility, helices stabilized by (i,i+4) lactam constraints have been extensively analyzed for bioactivity and bioavailability. For example, empirically optimized lactam cross-links increased the potency of N-terminal fragments of parathyroid hormone related protein (PTHrP) *in vitro* and in cell-based assays and were shown to alter transit times following subcutaneous administration.[79–82] Analgesics derived from the peptide hormone nociceptin were successfully stabilized using two lactam bridges, resulting in a very potent antagonist for the associated opioid receptor whose effects lasted up to 1 h after injection.[83,84] These and other examples have shown that, even without demonstration of oral bioavailability, lactam bridges clearly alter the distribution and degradation of helical peptides in whole organisms.

Despite steady progress using small peptide macrocycles and helices, there was one aspect of natural product peptides that still seemed like it might remain out of reach: cell penetration. While some highly constrained natural product peptides appear to enter cells,[85] most work on cell-penetrating peptides was focused on endocytosis stimulated by polycationic peptides and proteins.[86–88] Addition of arginines to cyclic peptides and protein surfaces has been shown to promote cell penetration,[88–90] and recent work has demonstrated a key effect of peptide conformation on arginine-mediated internalization and endocytic escape.[91] While these works have opened up one front on the problem of cell penetration, it has been more challenging to access additional mechanisms, especially those employed by amanitin, cyclosporine, and other potent natural products.

The idea that peptide conformation can control cell penetration was recently rekindled with reports that helices stabilized through hydrocarbon cross-links can indeed enter cells. Such helices were first described by Blackwell and Grubbs and later refined by Schafmeister and Verdine.[92–94] They incorporate amino acids with olefinic side chains in order to enable an intramolecular metathesis reaction to form the "stapled" helix. A watershed report in 2004 by Walensky *et al.* demonstrated that these stapled helices can possess some striking biological properties.[95] In this work, the BH3 helix of Bid, a commonly used tool for inducing apoptosis *in vitro*, was modified with an all-hydrocarbon "staple". Stapled

analogues possessed enhanced helicity in aqueous solution, improved target affinity, and superior resistance to degradation, mirroring results from other systems that used lactam bridges and other cross-link chemistries. However, the stapled Bid-BH3 helices were also able to penetrate living cells *via* an energy-dependent endocytosis mechanism and could trigger apoptosis *via* interactions with BCL-2 family members present in the cytosol and at the mitochondrial surface. The most potent stapled helix, shown in Figure 2f, increased median survival from 5 days to 11 days in leukemia xenograft mice (Table 1), demonstrating potential as an anticancer therapeutic.[95] This strategy was subsequently applied to other protein–protein interactions relevant to cancer, including additional BCL-2 family members, p53, Notch, and estrogen receptor.[96–101] One report described how addition of two hydrocarbon staples within the known HIV-fusion inhibitor enfuvirtide (Fuzeon) conferred substantial protease resistance and oral bioavailability.[102] Taken together, these exciting results demonstrate that peptides as large as 36 amino acids can penetrate cells, and even be made orally bioavailable by controlling conformation. The effects of hydrocarbon stapling are, to date, best understood as a combination of favorable physicochemical properties (increased hydrophobicity and amphipathic patterning) and favorable conformational properties.[103,104] However, stapled peptides are not universally endowed with these favorable properties, and there are intensive ongoing efforts to figure out how conformation controls the behavior of stapled helices in biological systems.

EMERGING STRATEGIES FOR CONFORMATIONAL CONTROL

Additional work has hinted that other methodologies for controlling peptide conformation may similarly boost bio-activity and bioavailability. Arora and co-workers have developed a complementary strategy for covalently constraining helical peptides that involves replacing the N-terminal (*i,i*+4) hydrogen bond with an isosteric covalent bond, an approach termed "hydrogen-bond surrogate" (HBS).[105,106] This approach uses the same ring-closing metathesis chemistry as helix stapling but involves appending the olefins directly to the backbone rather than incorporating them within side chains (Figure 2g). This approach has been applied to a number of targets, producing helical inhibitors of BCL-X_L, p53, gp41, Hif-1, and Ras.[107–111] Several HBS-stabilized helices were reported with improved metabolic stability compared to that of non-stabilized controls, reduced cytotoxicity, effective cell penetration, and/or potent cellular activity consistent with selective inhibition of their intended targets.

Encouraged by the successes of helix stapling and HBS strategies, a number of other strategies for constraining peptides are emerging. Many of these have yet to be evaluated in vivo but show promise in model systems and cultured cells. Existing strategies for conformational constraint, such as (*i,i*+4) lactamization, are being pushed to new limits. Fairlie and co-workers have recently reported a variety of peptides with multiple (*i,i*+4) lactam bridges, all with impressive aqueous helicity and serum stability despite a variety of primary sequences and binding partners.[112] Copper-catalyzed azide– alkyne 1,3-dipolar Huisgen cycloaddition has been used to constrain (*i,i*+4) side chains within peptides derived from PTHrP and from the transcriptional coactivator BCL9.[113–115] Tetrazole-enone photocycloaddition led to cell permeability and modest cellular activity for a peptide inhibitor of Mdm2 and MdmX, and these were further optimized by linking (*i,i*+7) cysteine

residues with a hydrophobic bisarylmethylene group.[116,117] Introducing a novel metal coordination chemistry for (*i,i*+4) side chain linkages, Ball and co-workers reported the stabilization of helical structure using interactions between acid side chains and rhodium cations to yield biocompatible protein ligands.[118,119] New covalent constraints are also being applied to non-helical peptides. Heinis and Winter reported a strategy for the covalent macrobicyclization of peptides using the reaction between symmetrical tris-bromomethylbenzene and three cysteine side chains.[120,121] Smaller peptide bicycles were recently reported by Quartararo *et al.* that take advantage of lactam bridges across a peptide macrocycle to generate peptide bicycles that act as potent, selective protein ligands.[122] These and similar strategies are reaching a critical stage where their bioactivities and bioavailabilities are being evaluated. Within a decade, there will likely be multiple synthetic platforms for the preparation of constrained peptides suitable for biological probes and pharmaceutical lead compounds.

SHAPE SORTERS: SYSTEMATIC STUDIES USING MODEL SYSTEMS

There are now many diverse examples of peptides whose bioactivity and bioavailability are controlled by conformation. However, understanding the underlying mechanisms was historically limited by the need for meaningful *in vitro* models of bioavailability. Throughout the late 1980s and 1990s, groundbreaking work by Borchardt promoted *in vitro* models for investigating bioavailability, most notably the Caco-2 gut epithelial cell line for the measurement of intestinal permeability.[123,124] Following extensive applications of peptides and proteins to these systems, these authors concluded that desolvation of amide bonds was the primary barrier to passive transport of peptides through biological membranes (transcellular permeability) and that electrostatic charge and molecule size limits transport of peptides through junctions between cells (paracellular permeability).[125–129] To understand specific effects of conformation on peptide uptake in gut epithelium, explicit comparisons were made among sets of cyclic peptides, turn peptides, and N-methylated peptides using Caco-2 cells.[126,127,130] These studies showed that conformation can powerfully alter permeability in ways that cannot be fully attributed to physicochemical factors. Later development of artificial membranes, including the parallel artificial membrane permeability assay (PAMPA),[131] has helped to further deconvolute transcellular, paracellular, and active transport mechanisms and better explain peptide uptake. Throughout these works, investigators have often noted that conformation plays a fundamental role in peptide permeability but often explained this by correlating increased permeability to hydrophobicity, size, or other physical parameters.[127,129,132,133]

In the past few years, two distinct efforts have used model systems to go beyond physiochemical correlations and address specific conformations that control cell penetration, gut absorption, and peptide degradation. Both involve systematic screening of model peptides, followed by detailed structural analysis of unusually bioavailable compounds. As part of the first effort, Hoffman, Gilon, and co-workers examined a series based on the model hexapepetide Phe-$(Gly)_4$-Phe to determine the effects of several backbone modifications on various aspects of bioavailability. The authors independently addressed the effects of cyclization, ring size, N-methylation, and C-methylation (Gly to Ala substitutions) within this relatively hydrophilic model peptide using a diverse panel of assays including *ex*

vivo penetration of rat intestine, Caco-2 permeability, liposome bilayer penetration, PAMPA, and degradation in brush border membrane vesicles.[130] They found that the absorption of these hydrophilic peptides is paracellular and remains so despite all backbone modifications tested. They also found that cyclization was the only backbone modification that improved paracellular transport. Using data from high-resolution size exclusion chromatography and NMR, they argued that cyclization reduced overall size and stabilized a single conformation, leading to the observed tissue penetration.

Continuing this effort, recent work by the Kessler and Hoffman groups examined the effects of N-methylation on intestinal permeability using the model hexapeptide cyclo-[D-Ala-$(Ala)_5$].[134] Fifty-four peptides comprising all possible N-methylation patterns were analyzed using an extensive panel of permeability assays. Some highly penetrant peptides were also conformationally homogeneous in aqueous solution, and these were selected for structural analysis by NMR. This led to the identification of two specific structural templates for highly penetrant cyclic, N-methylated peptides (one is shown in Figure 2c and Figure 3c; data given in Table 1).[135] One of their template structures overlaid well with a turn motif from cyclosporine, a turn motif also shared by the orally bioavailable analogue of somatostatin (Figure 3).[36] In contrast to cyclosporine, however, transcellular penetration was judged unlikely for the model cyclic hexapeptide due to poor penetration observed with model membranes. Instead, the authors argued that a receptor-mediated mechanism was likely and would also explain the dependence on a specific conformation. This would match the example of α-amanitin in which a single, locked conformation mediates absorption and bioactivity. However, several of the highly penetrant peptides discovered in these works were conformationally heterogeneous, and specific alternate modes of cell penetration were not exclusively ruled out. Further work with this set of peptides, particularly on variants substituted with diverse side chains, will clarify whether “bottom-up” design using these cyclic peptide templates will promote bioactivity and bioavailability in the resulting inhibitors.

In a second major approach to this problem, the Lokey group has examined cyclic peptide permeability in a slightly different manner. Their approach was similar to that of Kessler and Hoffman, but their initial focus was solely on transcellular penetration as judged by PAMPA, and significant allowances were made to accommodate a conformation-switching model as has been proposed for cyclosporine. Initial studies of cyclic hexapeptide diastereomers revealed that PAMPA permeability correlated with the capacity for the peptide to form intramolecular backbone hydrogen bonds.[136] A computational algorithm was developed in collaboration with the Jacobsen group that could rationalize this result by finding a low-energy conformation for the cyclic peptide in a low-dielectric environment and then calculating the energy difference that would occur when this conformation was transferred to a high-dielectric environment.[25] This energy difference was used to predict whether a given cyclic peptide could rapidly partition between aqueous and nonpolar environments, like cyclosporine does, thus promoting passive cell penetration. Ongoing efforts are rapidly improving these and other computational models of membrane penetration by small molecules and peptides.[137–141] These models, especially ones based on predictive physical simulation rather than empirical correlations,[142] will be critical for understanding whether the effects of peptide conformation can be understood using traditional physicochemical

descriptors such as partition coefficients, or whether new descriptors will need to be developed to accurately describe these effects.

More recently, the Lokey group showed that mild N-methylation chemistry, applied to solid-phase peptide synthesis, does not methylate amides that are internally hydrogen-bonded in organic solvent. This was used to screen a library of cyclic peptide diastereomers for those that produced N-methylation patterns indicative of internal structure.[24] The predicted backbone–backbone hydrogen bonds were confirmed using hydrogen–deuterium exchange and NMR solution structures. According to their previous work, the selectively N-methylated peptides were predicted to more readily penetrate membranes by passive diffusion. A selectively N-methylated scaffold (Figure 3d) was indeed shown to possess superior passive membrane permeability, metabolic stability, and oral bioavailability (Table 1), lending further credence to this “cyclosporine-like” approach.

The Kessler/Hoffman/Gilon and Lokey/Jacobsen approaches each provide a long-awaited glimpse into how conformation controls bioavailability, and both use systematically varied collections of cyclic hexamers to uncover scaffolds with surprising oral bioavailability. Both have provided scaffolds for future development, and both remarkably “re-confirmed” that the same orally active somatostatin analogue represents a uniquely bioavailable scaffold among variants of N-methylated, cyclic hexapeptides.[24,36,135] However, the differences between these lines of inquiry serve to highlight their different conclusions. The Kessler/Hoffman groups used alanine-based cyclic peptides and focused on cell-based penetration assays, leading them to isolate single structures that promote paracellular or receptor-mediated transport (as hypothesized for α-amanitin). The Lokey group used more hydrophobic, leucine-rich cyclic hexapeptides and focused on passive membrane diffusion, explicitly looking for a cyclosporine-like mode of transport. This clearly makes a difference, since alteration of even a single leucine to serine diminished, but did not abrogate, cell penetration within their best scaffold.[24] Even so, whether looking for an amanitin-like or cyclosporine-like mechanism, both groups have found what they sought: highly bioavailable scaffolds based on conformationally constrained backbones. This fact bodes very well for the future of peptide drug design.

CONCLUSIONS

After decades of envy directed at natural products such as α-amanitin and cyclosporine, chemical biologists can now point to a critical mass of research demonstrating that peptide bioactivity and bioavailability can be controlled by conformation. These recent insights were catalyzed by advances in synthetic methodology and by systematic adoption of standard *in vitro* and organism-based assays for peptide bioavailability. Model systems have provided new frameworks and atomistic models for understanding and improving the metabolic stability, gut uptake, and cell penetration of constrained peptides. Meanwhile, researchers from diverse subfields of peptide engineering – hormone mimicry, natural products, helix stabilization, non-helical peptide scaffolds, computational design, and model systems – are all converging on the same question: how can peptides with intrinsically high bioactivity and bioavailability be rationally designed? In the next decade, researchers will answer this question by evaluating new structural scaffolds and expanding model systems to incorporate

the interplay between backbone conformation and substituted side chains.[143] The answers may be different depending on cellular target, tissue of interest, or transport mechanism. In all cases, it has become clear that control over peptide conformation is critical for the advancement of these exciting tools and therapeutics.

ACKNOWLEDGMENTS

The authors thank the Lokey and Kessler laboratories for supplying structure files and helpful comments. J.G. is supported by NIH K12GM074869.

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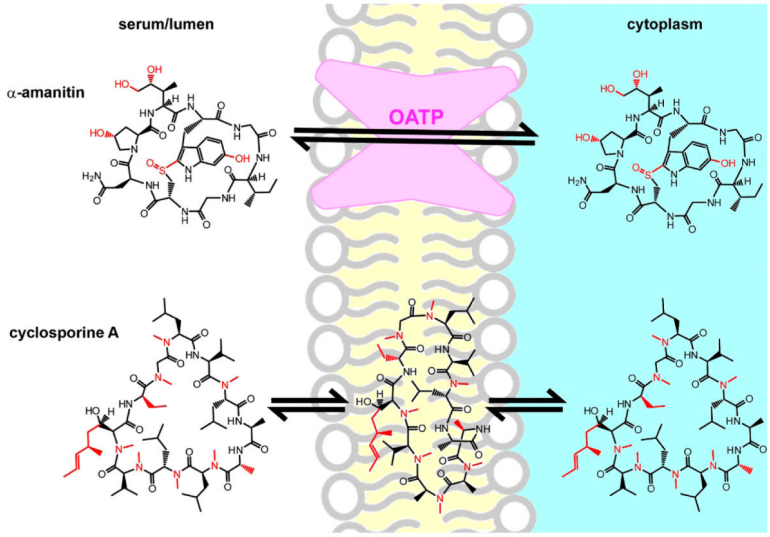

Figure 1.

"To be steady as a rock and always trembling."[144] α-Amanitin and cyclosporine A provide contrasting lessons from natural products. Both are head-to-tail cyclic peptides, and deviations from the 20 proteinogenic amino acids are shown in red. α-Amanitin is locked into a single conformation by virtue of a sulfone-indole intramolecular cross-link. This protects it from proteolytic degradation despite having a largely unmodified peptide backbone and appears to promote gut absorption and transport into liver cells by organic anion transport proteins (OATPs).[12] Cyclosporine, by contrast, survives digestive proteases by virtue of its highly N-methylated backbone. It can change conformations in order to form intramolecular hydrogen bonds in nonpolar environments.[23] This is hypothesized to promote passive diffusion through plasma membranes.

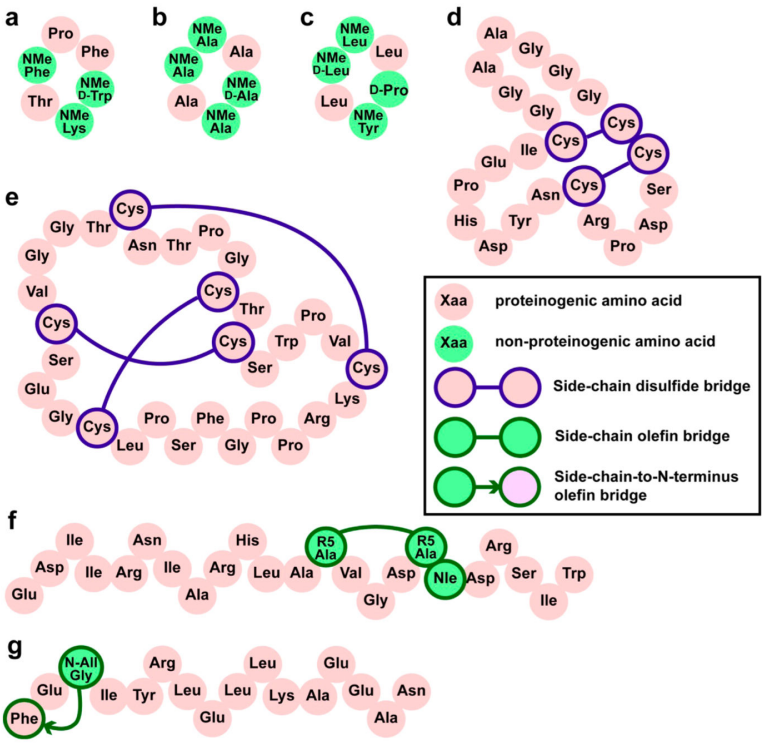

Figure 2.

Engineered peptides with high bioactivity and/or bioavailability. These constrained peptides vary greatly in size and hydrophobicity and employ different chemical cross-links, cyclizations, and folding topologies. (a) Somatostatin mimic with ~60 nM binding affinity to human somatostatin receptors sst2 and sst5. This compound permeates Caco-2 monolayers and has 7% oral bioavailability in rats.[36] (b) Caco-2-penetrant cyclic peptide scaffold found by Kessler, Hoffman, and co-workers.[135] (c) Cyclic peptide scaffold found by Lokey and co-workers to have 28% oral bioavailability in mice.[24] (d) Cyclized α-conotoxin that targets $GABA_B$ receptors and acts as an analgesic. Head-to-tail cyclization resulted in oral bioavailablility as judged by effects on pain-related phenotypes in rats.[56] (e) Grafted analogue of kalata B that acts as an orally bioavailable analgesic that targets bradykinin receptors.[63] (f) Stapled helix of Bid that is able to slow proliferation of leukemia xenografts.[95] (g) Hydrogen-bond-surrogate helix that can penetrate cells and inhibit Ras.[109]

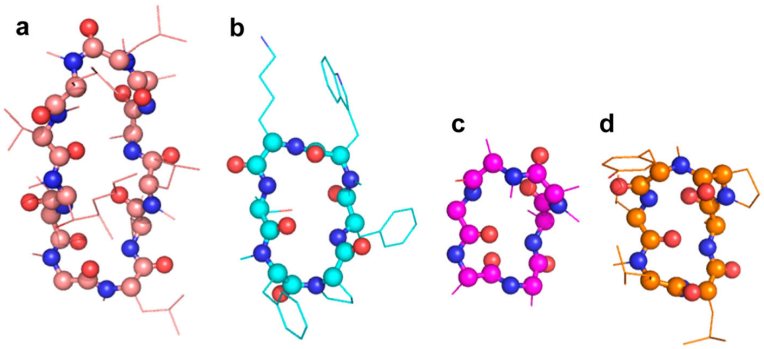

Figure 3.

New exceptions or new rules? Four head-to-tail cyclic peptides with significant oral bioavailability. Peptide backbones are shown in ball-and- stick, with side chains shown as thin lines; oxygens are in red, nitrogens are in blue, and hydrogens are omitted for clarity. All four peptides share similar turn conformations, despite having been developed via different strategies. (a) Three-dimensional structure of cyclosporine A in chloroform, thought to be representative of its structure when passing through lipid membranes.[22,23,136] (b) NMR structure of the optimized, orally bioavailable somatostatin mimic shown in Figure 2a.[36] (c) NMR structure of an orally bioavailable scaffold found by Hoffman, Kessler, and co-workers, similar to the peptide shown in Figure 2b.[135] (d) NMR structure of an orally bioavailable scaffold found by Lokey and co-workers, shown in Figure 2c.[24]

Table 1

Bioactivity and Bioavailability of Selected Constrained Peptides and Controls[a]

name	no. of amino acids	constraint	*in vitro* activity	cell penetration	activity/bioavailability	refs
α-amanitin	8	backbone cyclic, indole-sulfone cross-link	$K_i = 2 \times 10^{-8}$ M for RNA polymerase II	ND	$LD_{50} \approx 0.1$ mg/kg p.o.	10, 12
cyclosporine A	11	backbone cyclic	NA	1.1×10^6 cm/s in RRCK cells	oral bioavailability 27% in rat	24
cyclo(-Leu-NMeDLeu-NMe-Leu-Leu-Pro-NMeTyr-)	6	backbone cyclic	NA	4.9×10^6 cm/s in RRCK cells	oral bioavailability 28% in rat	24
Veber–Hirschmann peptide cyclo(-Pro-Phe-DTrp-Lys-Thr-Phe-)	6	backbone cyclic	$K_d = 9.8$ nM (hsst2), 15.1 nM (hsst5)	5×10^5 cm/s in Caco-2 cells	oral bioavailability in rat negligible	36
cyclo(-Pro-Phe-NMeDTrp-NMeLys-Thr-NMeDPhe-)	6	backbone cyclic	$K_d = 61.7$ nM (hsst2), 60.3 nM (hsst5)	4×10^6 cm/s in Caco-2 cells	oral bioavailability 9.9% in rat	36
cyclo(-NMeDAla-Ala-Ala-Ala-NMeAla-Ala-)	6	backbone cyclic	NA	~$2–3 \times 10^5$ cm/s in Caco-2 cells and in *ex vivo* rat intestine	ND	134
α-conotoxin Vc1.1	16	two disulfides	$IC_{50} = 1.7$ nM for inhibition of Ca^{2+} channel currents in rat neurons	ND	some activity in 0.36 and 3.6 μg i.m. boluses in rat models of neuropathic pain	145
cyclic Vc1.1	22	two disulfides, backbone cyclic	$IC_{50} = 0.3$ nM for inhibition of Ca^{2+} channel currents in rat neurons	ND	1.3 mg/kg p.o. as effective as 30 mg/kg gabapentin in rat model of neuropathic pain	56
des-Arg^{10}-[Leu^{10}]-kallidin	9	none	ND	ND	no effect p.o., 38% inhibition i.p. (1 mg/kg) in a rat model of visceral pain	63
Ckb-kal	31	four disulfides, backbone cyclic	$IC_{50} \approx 0.2–0.4$ μM for competition at the bradykinin receptor	ND	49% inhibition p.o., 42% inhibition i.p. (1 mg/kg) in a rat model of visceral pain	63
gp41-derived variant $T649V_{626-662}$	37	none	$IC_{50} = 2.9$ nM and >3000 nM for *in vitro* HIV infectivity of HXBc2 and YU2 strains, respectively	NA	None detected in plasma after 10 or 20 mg/kg p.o.	102
“stapled” gp41 variant $T649V_{626-662}$	37	two side chain olefin cross-links	$IC_{50} = 2.5$ nM and 87 nM for *in vitro* HIV infectivity of HXBc2 and YU2 strains, respectively	NA	1.5 μg/mL and 2.3 μg/mL detected in plasma after 10 or 20 mg/kg p.o., respectively	102
Bid BH3 helix	24	none	$K_d = 269$ nM for Bcl-X_L binding *in vitro*	no cell penetration or effects on leukemia cells *in vitro*	ND	95
“stapled” Bid BH3 helix	24	one side chain olefin cross-link	$K_d = 39$ nM for Bcl-X_L binding *in vitro*	cell penetration observed with dye-labeled analogues; $IC_{50} = 1.6–10.2$ μM for	median survival increased from 5 to 11 days in mouse leukemia model, i.v. 10 mg/kg	95

name	no. of amino acids	constraint	*in vitro* activity	cell penetration	activity/bioavailability	refs
				killing leukemia cells *in vitro*		
HBS3	16		K_d = 28 μM for nucleotide-free Ras	cell penetration observed with dye-labeled analogues; 75 μM peptide reduced Ras activation 5-fold in HeLa cells	ND	109

[a] Abbreviations: p.o. (peroral administration), i.v. (intravenous injection), i.m. (intramuscular injection), i.p. (intraperitoneal injection). NA = not applicable. ND = not determined. hsst2 and hsst5 refer to different human somatostatin receptors.