Stereochemistry Balances Cell Permeability and Solubility in the Naturally Derived Phepropeptin Cyclic Peptides

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S Supporting Information

ABSTRACT: Cyclic peptide (CP) natural products provide useful model systems for mapping “beyond-Rule-of-5” (bRo5) space. We identified the phepropeptins as natural product CPs with potential cell permeability. Synthesis of the phepropeptins and epimeric analogues revealed much more rapid cellular permeability for the natural stereochemical pattern. Despite being more cell permeable, the natural compounds exhibited similar aqueous solubility as the corresponding epimers, a phenomenon explained by solvent-dependent conformational flexibility among the natural compounds. When analyzing the polarity of the solution structures we found that neither the number of hydrogen bonds nor the total polar surface area accurately represents the solvation energies of the high and low dielectric conformations. This work adds to a growing number of natural CPs whose solvent-dependent conformational behavior allows for a balance between aqueous solubility and cell permeability, highlighting structural flexibility as an important consideration in the design of molecules in bRo5 chemical space.

KEYWORDS: phepropeptin, cyclic peptide, bRo5, epimer

Research into macrocycles as an emerging class of pharmaceutically relevant molecules has increased in recent years. Advances in combinatorial chemistry and screening have yielded a number of potent macrocycles against challenging protein targets.^{1–9} Although they often exhibit favorable target binding characteristics, with long off rates and high specificity,^{10–13} they generally tend to suffer from an inability to cross cellular membranes and are thus limited to extracellular targets. By contrast, some cyclic peptide natural products or derivatives thereof are cell permeable.¹⁴ While Lipinski's Rule of 5 has provided a framework for predicting oral bioavailability in small molecules,¹⁵ these types of simple two-dimensional descriptors do not predict the exceptional ADME characteristics of many large macrocyclic natural products.^{14,16–18} This observation has led to a surge of interest in understanding the factors that govern ADME characteristics such as cell permeability, solubility, and plasma stability, in medium- and large-ring macrocycles.

In addition to being a rich class of natural products, cyclic peptides are relatively easy to synthesize and diversify, making them a centerpiece in “beyond-Rule of 5” (bRo5) chemical space. A variety of factors have been shown to affect membrane permeability in cyclic peptides, including side chain composition,^{16,19,20} N-methylation,^{21–23} β -branching,^{24,25} and the introduction of nonproteinogenic residues such as peptoid,²⁶ statine, and vinylogous residues.²⁷ All of these modifications

appear to improve permeability at least in part by increasing lipophilicity.

Cyclosporine A (CSA), an uncharged cyclic undecapeptide, has provided a compelling focal point for inquiry in this chemical space. Its solvent-dependent conformational behavior has been invoked as a potential cause of its exceptional passive membrane permeability and oral bioavailability.²⁸ We also observed a very large solvent-dependent conformational effect on aqueous solubility among synthetic analogues of the natural product sanguinamide A.²⁴ Yet although conformational flexibility has been observed in CSA as well as some other natural products,²⁹ the effect of flexibility per se on physicochemical properties has not been studied in these natural systems.

The phepropeptins are cyclic hexapeptides that were isolated from *Streptomyces* in a search for proteasome inhibitors.³⁰ Although the proteasome inhibitory activity of this series was modest, we hypothesized that the phepropeptins were nonetheless likely to exhibit favorable cell permeability. In particular, both the absence of polar or charged side chains as well as calculated octanol/water partition (ALogP)³¹ coefficients between 3 and 5,³² suggested that the phepropeptins should have passive

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Figure 1. Structure, physicochemical, and ADME properties of the phepropeptins and their proline–epimeric isomers, the epiphepropeptins. ^aThermodynamic aqueous solubility pH 7.4. ^bAverage of A–B and B–A transport reported in cm/s $\times 10^{-6}$.

permeabilities close to those observed in similar cyclic hexapeptide systems.

In an effort to determine a structure–property relationship for this class of natural products, we synthesized and tested various ADME properties of a series of phepropeptin analogues as well as a congeneric series of epimers. We found that the natural products all exhibited higher permeabilities than their congeneric epimers, which we attributed to their ability to adopt lipophilic conformations in low dielectric media. We also compared solution structures in both high- and low-dielectric solvents and found that the ability to adopt different conformations in these two media was consistent with their observed permeability and solubility trends.

The phepropeptins and analogues thereof were synthesized through automated solid phase peptide synthesis followed by solution phase cyclization. The 1D ¹H NMR spectra of the synthetic phepropeptins A, C, and D were identical to those reported for the natural products, confirming their assigned structures.³⁰ In addition to the natural phepropeptins, a series of epimers were synthesized incorporating a D-Pro instead of the naturally occurring L-Pro (“epiphepropeptins” 5–8). The ¹H NMR spectra of the epimers differed substantially from those of the natural compounds (Figure 3; SI pages 31 and 32), indicating a major change in the conformation of the macrocycles. The epiphepropeptins were also poorly soluble in CDCl₃, suggesting they are less lipophilic than their natural epimers.

To quantify the effect of this stereoinversion on ADME properties, we measured experimental LogD_{7.4} (octanol–water), solubility, cell permeability, and plasma stability for both series. As seen in Figure 1 and the accompanying table, all four of the side chain variants of the phepropeptins were rapidly permeable in an MDCK monolayer permeability assay, with permeation rates of 30–40 $\times 10^{-6}$ cm/s. The corresponding epiphepropeptins showed 2–4 fold slower permeability than the natural epimers, and, in contrast to the natural products, their permeabilities showed a $\sim$ 2-fold variation among side chain variants. Although absorption is a complex process that is dependent on multiple factors, MDCK permeability has been shown to be a reasonable indicator of human absorption.³³ While the net impact of increasing MDCK permeability on oral absorption will depend on a given compound and the rate limiting steps for its absorption, the present work offers a potential strategy to modulate permeability toward probing the in vivo impact for a given drug discovery program. None of the

compounds showed statistically significant degradation on incubation with human plasma for 30 min. Overall, thermodynamic aqueous solubility pH 7.4 ranged from 0.165 to 0.011 mg/mL for the compounds assessed. However, comparable solubility was observed for the matched pairs of the phepropeptin and epiphepropeptin series (Figure 2).

Figure 2. *In vitro* cell permeability and solubility for the phepropeptins and epimeric analogues.

Regression-based two-dimensional descriptors such as ALogP³¹ (an atomistic version of the more familiar, group-based calculated octanol–water partition coefficient, cLogP), are necessarily the same for any pair of stereoisomers. Each phepropeptin analogue and its epimer share the same ALogP value and are therefore predicted to have identical lipophilicities based on this simple 2-dimensional metric (Figure 1). Thus, the observed differences between the two series must be due to three-dimensional (e.g., conformational) effects. There was also little difference in *experimental* octanol–water partition coefficients (LogD_{7.4}) between these two compound sets; therefore, in this series neither calculated nor experimental octanol–water partition coefficients were predictive of the permeability differences observed between epimers.

To gain insight into the possible conformational basis for these observed differences, we turned to NMR in CDCl₃ and DMSO, solvents selected to mimic the lipid bilayer^{34,35} and water, respectively. NMR temperature shift coefficients (Tc) have been used to probe intramolecular hydrogen bonding in cyclic

peptides.^{17,23,24,36} As temperature increases, solvent-exposed amide NH protons shift upfield, leading to large negative Tc values (<4 ppb/K being considered solvent-exposed).³⁷ The number of solvent-exposed NH groups has been shown to negatively correlate with cell permeability in a number of model systems.^{17,23,24,35,36}

To compare two matched pairs we conducted temperature coefficient experiments on **1**, **3**, **5**, and **7** in chloroform. For both compounds with natural stereochemistry, **1** and **3**, only one or two amide protons showed significant solvent exposure. In the unnatural epimers, **5** and **7**, at least three amide protons showed significant exposure (Figure 3). Interestingly **1** and **3** differ only

Figure 3. Temperature shift experiments for **1**, **3**, **5**, and **7** in chloroform. Using the cutoff of -4 ppb/K peaks that are solvent exposed are noted in red, while those that are hidden from solvent are noted in green. Peaks that disappear and reappear are highlighted with a red line. Note: only four peaks were observable for compounds **5** and **7**, with the fifth amide NH likely hidden under the phenylalanine side chain protons at 7.2–7.3 ppm.

by a valine to isoleucine substitution at position 6, but this leads to shielding of the Ile6 amide proton in compound **3**. Based on the solution structure of **3** presented below, the Ile γ -methyl may shield its own NH. Spectral overlap and limited solubility in chloroform prevented further characterization of the unnatural epimers. Nonetheless, the temperature shift data is consistent with the greater degree of passive permeability observed for the natural phepropeptins compared to their epimers.

To further investigate the conformation of **3** in a low dielectric environment we sought to obtain a solution structure. We used ROESY and 3J couplings obtained from 2D NMR experiments in CDCl_3 , together with unrestrained molecular dynamics (MD) simulations. The conformation from the unrestrained MD simulations that showed the least deviation from experimental values is shown in Figure 4b. In this conformation, only the amide NH of the Phe residue is exposed to solvent, consistent with the temperature shift data (highlighted in Figure 4c as a dashed circle). Although previous work in our lab and others has shown non-*N*-methylated cyclic hexapeptides can be cell permeable,^{20,38,39} no other examples of all-amide, non-*N*-

Figure 4. (a) Table of NMR data for the NH protons of compound **3** in CDCl_3 ; (b) solution structure of **3** as found by molecular dynamics, ROESY, and 3J HNHA; (c) surface showing solvent accessible polarity of the structure in b, with the solvent exposed NH of phenylalanine 2 highlighted with a dashed circle.

methylated cyclic peptide natural products have been reported to show passive membrane permeability.

Typically, by shielding polar atoms (either sterically or via intramolecular hydrogen bonding), one would expect to see an increase in lipophilicity, which would in turn lead to increased membrane permeability at the expense of decreased aqueous solubility.⁴⁰ Hence, it was interesting to note that the natural epimers with increased shielding of their NH groups maintained similar aqueous solubility as their corresponding unnatural epimers. Based on these observations, we hypothesized that in addition to adopting a sufficiently membrane-permeable lipophilic conformation in a low dielectric environment, the phepropeptins may adopt a more hydrophilic conformation (or conformational ensemble) in a higher dielectric. This phenomenon has been well described for cyclosporine A and has been attributed to other natural products by inference.²⁹ Although it is often discussed in reference to the behavior of CSA and other bRo5 compounds,²⁸ the effect of conformational flexibility on ADME properties has been studied in only a handful of model systems.^{16,24,41} To explore the conformation of **3** in a polar environment we performed NMR experiments in DMSO, which was chosen because of its high dielectric constant and its ability to effectively solubilize lipophilic peptides.

While NMR temperature coefficient experiments for **3** in chloroform indicated a single, solvent-exposed NH, in DMSO three NH groups appeared to be solvent exposed (Figure 5a). After performing a complete spectral assignment followed by MD simulations in an implicit, high-dielectric solvent model, we obtained the solution structure of **3** in DMSO. The lowest energy structure from the high-temperature MD simulation that was also consistent with the NH temperature coefficient data (which was second-lowest conformation in energy overall) was selected as the starting point for the rt-MD simulation.

To our surprise, the solution structure in DMSO (Figure 5b) showed more intramolecular hydrogen bonds and a lower calculated polar surface area than the structure in chloroform (SI Figure 4). Although the amide protons of Phe2 and Leu5 are

Figure 5. (a) Table of NMR data for the NH protons of compound 3 in DMSO; (b) two views of the solution structure of 3 in DMSO; (c) box plots of solvation energy of the 10 best NMR structures in DMSO and chloroform.

involved in gamma turns and engage in exocyclic hydrogen bonds, they are significantly solvent-exposed in accordance with the temperature shift data. Indeed, using a more refined model for calculating solvation energy,⁴² the high-dielectric conformation had a greater energy of solvation than the CDCl₃ conformation (Figure 5c). Given that solvent-dependent conformations have been implicated in increased solubility in the past,^{24,40} one can speculate that 3 possesses a favorable balance of membrane permeability and water solubility because of its ability to equilibrate between polar and nonpolar conformations depending on its environment.

As the number of bR05 molecules grows, so does our understanding of the properties that contribute to favorable PK in this space. For conformation-dependent effects, simple 2-D metrics are limited in their predictive power, while more in-depth conformational analysis may be required to probe the physiochemical factors underlying the properties of large molecules. As with typical small molecules, polarity may need to be adjusted to generate favorable solubility and permeability profiles in a drug discovery context.⁴³ Inspiration from natural products has led to cyclization, *N*-methylation and unnatural stereochemistry as the first tools for increasing the permeability and stability of peptides. In the same way, the solvent-dependent behavior of some natural products may provide inspiration for future efforts in macrocyclic design. Indeed, thousands of cyclic peptide natural products are known, comprising hundreds of unique scaffolds, which upon further study may reveal new insights into the relationship between structure and properties in this interesting chemical space. In summary, with this small set of compounds we present an approach for focusing on the natural stereochemistry of cyclic peptides as a way to increase permeability. Further studies on cyclic peptide natural products might help to reveal additional key features for understanding structure–property relationships in this chemical space.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00100.

Additional information (PDF)

Solution structures (MOL)

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Notes

The authors declare the following competing financial interest(s): R.S.L. is a founder of Circle Pharma.

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