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Improving the passive permeability of macrocyclic peptides: Balancing permeability with other physicochemical properties

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ABSTRACT

A number of methods to improve the passive permeability of a set of cyclic peptides have been investigated using 6- and 7-mer macrocyclic templates. In many cases the peptides were designed by molecular dynamics calculations to evaluate the methods. The aim of this study was not only to improve passive permeability, but also to balance permeability with other physicochemical properties with the goal of understanding and applying the knowledge to develop active cyclic peptides into drug candidates. Evaluation of the methods herein suggest that increasing passive permeability often occurs at the expense of solubility and lipophilicity. Computational methods can be useful when attempting to predict and design features to balance these properties, though limitations were observed.

To improve the likelihood of clinical success, pharmaceutical companies have become increasingly focused in recent years on the discovery and development of innovative medicines.[1] Despite considerable effort, >80% of intracellular targets are believed to be 'undruggable' and new approaches to modulate their activity represent a significant opportunity.[2] One approach which shows promising potential is the development of novel methods to disrupt protein–protein interactions (PPIs).[3] PPIs represent a large proportion of undruggable intracellular targets, and small molecules generally cannot modulate these targets due to the large, flat surfaces of interaction required for binding (1500–3000 A^2).[4,5] While monoclonal antibodies can effectively block these interactions, they are generally not cell penetrant and, consequently, cannot be applied to intracellular targets. In comparison, stabilized peptides[6–12] can mimic the secondary structure of protein surfaces and effectively disrupt PPIs with the potential for improved cell penetrance relative to monoclonal antibodies. Applying this modality to drug discovery and development would allow unprecedented access to a number of previously intractable drug targets in multiple therapeutic areas and add new diversity to the industry's pipeline.

Among stabilized peptides, macrocyclic peptides have recently gained renewed attention for development as therapeutics.[6,13–19] Although there are reportedly over 100 macrocyclic drugs and clinical candidates, some of which are peptides,[6,13] cyclic peptides are scarcely present in drug discovery programs due to their unconventional features compared to traditional drug compounds. Despite their high molecular weight and polar backbone, cyclic peptides such as cyclosporin have been shown to change their structural conformation upon a change of environment,[20–22] allowing them to become more drug-like than predicted and in some cases, even orally bioavailable.[18] As they are peptidic, cyclic peptides also have the ability to interact along the flat surfaces of proteins which could lead to greater specificity and potency for drug targets compared to small molecules. However, the few examples of membrane permeable cyclic peptides[23–31] generally suffer from high lipophilicity and consequently low solubility. The challenging formulation of cyclosporin due to its low solubility is a good example of the potential consequences of working in this physicochemical space.[32–34]

Due to their large size, complex functionality, and proposed conformational flexibility, it can be very difficult to predict the behavior of cyclic peptides with respect to cell permeability, solubility and other physicochemical properties. A major component of the cell membrane is the lipid bilayer and, as a result, compounds with greater lipophilicity tend to be more passively permeable. However, increased lipophilicity often leads to a higher risk of drug attrition[36] due to lower target specificity, increased plasma protein binding, greater volume of distribution, increased toxicity, high clearance, and lower aqueous solubility. Thus, our aim in this study was to try to reduce lipophilicity while maintaining passive

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permeability and good solubility. The existing literature for macrocyclic peptides remains largely focused on the permeability,[23–31] pharmacokinetic profile[28] or activity[35–37] of cyclic peptides. However, to the best of our knowledge, there has not been a systematic study evaluating the interdependent triangle between passive permeability, lipophilicity and solubility for macrocyclic peptides. We therefore focused on evaluating approaches to predict passive permeability while balancing the other two properties which are crucial for transforming peptides into therapeutics.[38–40] Here we report our findings on the synthesis and analysis of hexa- and hepta-peptides based on literature reported scaffolds, with the goal of applying the knowledge to develop novel peptide therapeutics for 'undruggable' intracellular targets.

Our initial work focused on the hexapeptide scaffold reported by Jacobson and Lokey, which displayed a range of permeabilities driven by subtle conformational changes in structure.[27–31] In high dielectric media such as water, the authors postulated that a high dielectric conformer (HDC) in which polar amides pointed towards solvent was dominant; in low dielectric environments, the peptide switched to a low dielectric conformer (LDC), where the polar amides were hidden by intramolecular H-bonding and the peptide was able to cross an artificial membrane.[27,30] N-methylated derivatives were also shown to be more permeable than their non N-methylated counterparts due to the lower desolvation cost.[29] Inspired by these findings, we chose these peptides as a starting point to validate literature strategies for improving passive permeability.

We first wished to validate the importance of backbone internal hydrogen bonding and its relationship with passive permeability. Three hexapeptides **1a**–**c** were chosen which were reported to have different PAMPA permeabilities[29,30] despite being diastereomers (Table 1). In our hands, the three peptides were similar in terms of lipophilicity (ChromLog*D*, ±0.25) but notably different in terms of AMP permeability (±30 nm/s) and CLND solubility (±30 μM, see Supporting information for full descriptions of each assay).

This trend was in line with Jacobson and Lokey's findings that a higher degree of internal hydrogen bonding gave greater passive permeability for this set of cyclic peptides. Deuterium shaking ^{1}H NMR experiments in deuterated chloroform[41] were also in agreement with the authors[29,30] and showed that greater numbers of rapidly exchanging amide NHs (those not participating in internal H-bonding) resulted in less permeability. These results were consistent with the conclusion that passive permeability was related to a cyclic peptide's degree of masking polarity through internal hydrogen bonding. The relationship was also rationalized by in silico computational molecular modeling which correctly predicted the order of permeability.[27,30,42] While these trends hold true, there are other features that may affect permeability such as size, shape and polar surface area,[38,42–48] which complicate the ability to draw a direct relationship between masking polarity and passive permeability for these compounds.[48]

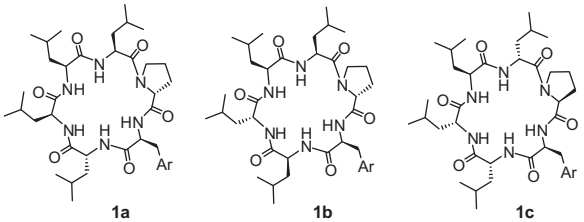
Preliminary NMR studies of NH exchange rates in water were conducted for these three peptides (see Supporting information), where backbone assignments could be made despite relatively low aqueous solubilities. Inter-peptide comparisons indicated that the relative exchange rates in water do not correspond exactly to those found in chloroform. It may be that changes in the conformational equilibria between the two solvents were shifted, perhaps only subtly. This could lead to differences in relative NH solvent exposures between the HDC and LDC environments which, consequently, could affect the observed solubilities and permeabilities. Further work in this area could aid greater understanding of the disparities in physicochemical properties observed for small cyclic peptides.

N-methylation has been a well reported methodology for improving the passive permeability of cyclic peptides,[19,49,50] but few comments have been made on the resulting increase in lipophilicity and, in turn, the effect on solubility. In order to investigate this in more detail we prepared a series of cyclic peptides with an increasing degree of N-methylation to compare their permeability and physicochemical properties in a systematic manner (Fig. 1).[23,24] Non-N-methylated **1b** was chosen as a starting point with the more rapidly exchanging NH protons selected as positions for N-methylation.

The synthesis of tri-methylated derivative **2c** had been previously reported[29] via an on-resin N-methylation protocol. However, the syntheses of mono- and di-methylated derivatives **2a** and **2b** were undescribed. A solvent screen based on a modified literature on-resin N-methylation protocol[29,31] identified acetonitrile as a suitable methylation solvent to synthesize mono-methylated hexapeptide **2a**. It was not possible to find a suitable solvent to access the di-methylated derivative directly and, thus, **2b** was synthesized through microwave SPPS (see Supporting information).

With the N-methylated peptides in hand their individual properties were studied (Table 2). Compared to the parent **1b**, each individual N-methylation increased the ChromLog*D* by approximately one log unit, which corresponded to a 10-fold drop of the compound in the aqueous layer. Along with increased lipophilicity there was a clear trend in decreased solubility across the series

Table 1
Effect of backbone H-bonding on hexapeptide diastereomers[a]



Compd[b]	ChromLog*D*[c]	Solubility[c] (μM)	AMP[c] (nm/s)	No. of Fast NHs[d]
Cyclo(lLLLpY) (**1a**)	5.1	324	41	3
Cyclo(LlLLpY) (**1b**)	5.5	170	111	2
Cyclo(llLlPY) (**1c**)	5.6	249	415	0

[a] Ar represents *para*-phenol.
[b] L = leucine; l = D-leucine; p = D-proline; Y = tyrosine; P = proline.
[c] Average of 2–3 runs; uncertainty limits: ChromLog*D* ±0.25, Solubility ±30 μM, AMP ±30 nm/s.
[d] Rapidly exchangeable NHs as determined by ^{1}H NMR in $CDCl_3$ at ambient temperature, see Supporting information.