Development of a Platform To Enable Efficient Permeability Evaluation of Novel Organo-Peptide Macrocycles

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S Supporting Information

ABSTRACT: As more macrocycle structures are utilized to drug intracellular targets, new platforms are needed to facilitate the discovery of cell permeable compounds in this unique chemical space. Herein, a method is disclosed that allows for the efficient synthesis and permeability evaluation of novel organo-peptide macrocycle libraries. Thoughtful library design allows for the collection of crude permeability data using supercritical fluid chromatography mass spectrometry (SFC-MS) (EPSA) by mass-encoding the stereochemistry, ring size, and organic linker of the desired macrocycles. Library synthesis was aided via the development of a new on-resin *N*-arylation reaction. Further insights on the permeation of these organo-peptide macrocycles will be discussed, such as the permeability enhancement when utilizing a 2-substituted phenethyl linker versus a 3-substituted phenethyl linker. Lastly, selected macrocycles were scaled up and tested in the MDCK-II permeability assay, and the results of this assay reiterated the permeability trends from the crude SFC-MS data.

KEYWORDS: Macrocycles, peptides, permeability

Intracellular drug targets with extended binding sites such as protein–protein interactions (PPIs) are commonplace in drug discovery today.^{1–3} This has led to an increased interest in peptidic macrocycles as therapeutics since these compounds are well suited to drug targets with flat extended binding sites while maintaining the potential for cellular permeability. Nonetheless, the deconvolution of structure–permeability relationships (SPRs) in peptidic macrocycles is difficult, making it challenging to access cell-permeable peptidic macrocycles.⁴ To date, much of the work to understand SPRs of peptidic macrocycles has focused exclusively on cyclic peptides bearing natural head to tail amide bonds, with large efforts toward developing model systems with cyclic hexapeptides.^{5,6} However, as peptidic macrocycles possessing unconventional linkages in the ring framework become more prevalent,⁷ methods that allow for the synthesis and permeability evaluation of these types of organo-peptide macrocycles need to be developed.

One approach to access novel organo-peptide macrocycles relies on adding a unique ring system into the macrocycle, which has been shown to enhance permeability in certain studies.⁸ For instance, recent work from the Yudin group detailed the positive effects on permeability from the incorporation of a 1,3,4-oxadiazole motif into peptidic macrocycles (Figure 1A).⁸ Other nonproteinogenic rings are also found in natural products such as the thiazole ring in Sanguinamide A, a permeable peptidic macrocycle with oral

bioavailability.⁹ The inclusion of phenyl rings in organo-peptide macrocycles has also been utilized to generate biologically active macrocycles, such as the oxygen linked compounds in Figure 1A.¹⁰ However, the effect of these phenyl rings on permeation has yet to be studied.

Since understanding permeability trends in organo-peptide macrocycles is important for drug developability, we wanted to gain a deeper understanding on the effects phenyl linkers might have on the permeation of these compounds. Recently, we disclosed a publication highlighting a novel synthesis of organo-peptide macrocycles from the head of an amino acid chain to the phenyl group of a phenylalanine ester or a phenylalanine linker mimic via a Pd-catalyzed macroamination reaction (Figure 1B).¹¹ To understand the effects on permeability from these phenyl linkages, permeability studies of organo-peptide macrocycles bearing these motifs needed to be initiated. Herein, a closer look at the permeability of novel organo-peptide macrocycles is documented, including the disclosure of a method that allows for facile synthesis and permeability evaluation of the desired macrocycles.

As much of the work to study permeation of cyclic peptides has been done on six amino acid model systems, this study was designed to access macrocycles bearing five amino acids with

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Figure 1. Nonproteinogenic rings in macrocycles.

Figure 2. Library design for 20–23-membered organo-peptide macrocycles.

an organic linker in place of the sixth amino acid (Figure 2). In this chemical space, interpretation of SPRs for these macrocycles would be difficult due to the multifactorial effects that the stereoisomers (16 possible stereoisomers), ring size, and the organic linker will have on permeation of these macrocycles. Therefore, an efficient process to deconvolute all components and their relationships to permeability would be necessary.

Inspired by recent efforts from the Lokey group,⁶ a method was devised to carry out an on-resin library synthesis of novel organo-peptide macrocycles to test the crude macrocycle mixtures for permeability (Figure 2). Key differences in this work include mass encoding the stereochemistry of the crude macrocycles,¹² development and use of an on-resin *N*-arylation

reaction to access nonamide bonds in the macrocycles, and use of the EPSA assay for crude permeability measurements.¹³ As shown in Figure 2, the final macrocycles start from L-Pro on 2-chlorotrityl resin, and the first three splits and pool strategy would mass encode the stereochemistry by careful choice of amino acids (i.e., L-4-fluorophenylalanine vs D-phenylalanine in the first split).¹⁴ The fourth split in the library was γ -amino butyric acid or glycine, which allows for the ring length to be mass encoded in these libraries as well (20-, 21-, 22-, and 23-membered rings). The last split involves an on-resin *N*-arylation reaction to attach the organic linkers to the amino acid chain, followed by a resin cleavage and macrolactamization reaction to access the desired macrocycles. By selecting closely related organic linkers for this last split, we hoped to

test the robustness of the EPSA measurements to predict permeabilities that would be hard to do *a priori*. Lastly, a difference between this work and previous cyclic hexapeptide studies is the fact that no *N*-methylated amino acids were used in this work.^{6,12} *N*-Methylated amino acids were purposely kept out of this library to understand if our novel organic linkers alone could enhance the permeation of these organo-peptide macrocycles.

To allow for a successful synthesis of this library, conditions to *N*-arylate amino acids on-resin needed to be developed, as the on-resin arylation of amino acids had yet to be disclosed in the literature. Initially, conditions for the arylation of amino acids in solution were tested on substrate **S1a** for the desired on-resin *N*-arylation reaction (Table 1).¹⁵ However, no desired

Table 1. Optimization of On-Resin *N*-Arylation^a

entry	substrate	solvent	catalyst %	conversion % ^b
1	S1a	THF	100	0
2	S1a	ACN	100	trace
3	S1a	^t AmOH	100	0
4	S1a	DMF	100	>95
5	S1a	DMF	20	>95
6 ^c	S1b	DMF	10	(44)
7 ^d	S1c	DMF	10	(60)

^aConditions: 1.0 equiv. **S1a–c**, 6 equiv. Cs₂CO₃, % precatalyst, solvent [0.03 M], 40 °C, 15 h. Reactions were conducted on a 0.05 mmol scale. ^bConversion determined by amount of starting material left in LCMS. Numbers in parentheses refer to isolated yields. Yields and conversions were determined after resin cleavage by TFA. ^cBromobenzene was used as the aryl halide. ^d*t*BuXphos was used as the catalyst and phenyl triflate was used as the aryl halide.

product was formed under these conditions even with stoichiometric palladium (entry 1). A further solvent screen showed that both acetonitrile and ^tAmOH gave no reaction for our desired transformation (entries 2–3). However, the use of DMF (entry 4) as the solvent in this reaction gave full conversion to the desired product **1a**. Further reduction of the amount of palladium from 100 mol % to 20 mol % still led to complete conversion to **1a** (entry 5). To further test the utility of this method, the on-resin *N*-arylation reactions of substrates **S1b** and **S1c** bearing substitution adjacent to the nitrogen were attempted. In both reactions, the desired products **1b** and **1c** were afforded in moderate yields (entries 6–7). With on-resin *N*-arylation conditions now in hand, the synthesis of the library was commenced.

Using standard Fmoc chemistry and the on-resin *N*-arylation reaction with the split and pool strategy outlined in Figure 2, 79 of the 80 desired macrocycles were synthesized. The mass encoding of the crude libraries allowed for easy correlations between the crude EPSA values and various parameters of the macrocycles such as stereochemistry, organic linker, ring size,

or combinations of these parameters. Key relationships uncovered from the crude macrocycle EPSA values are displayed in the graphs in Figure 3.¹⁶ Based on previous

A) Average EPSA vs. stereochemistry

B) Average EPSA vs. sublibrary

Figure 3. EPSA trends from the crude library. (A) Plot of all macrocycles synthesized and their crude EPSA values arranged by stereochemistry. (B) Average crude EPSA values correlated to the type of organic linker used (all with glycine).

literature, EPSA values below 80 show the highest likelihood for good permeability. However, trending EPSA values lower toward 80 should also correlate to a higher probability of achieving good permeability as well.¹³

The relationships between the crude macrocycle EPSA values and stereochemistry are denoted in Figure 3A. The only single parameter in this data that correlated to an average EPSA below 80 was the LLDL amino acid configuration (average EPSA = 76.7). However, a multifactorial combination that led to the second lowest EPSA average for this library of macrocycles was also discovered. The combination of a glycine as the fifth amino acid and 2-phenethylamine as the organic linker afforded an average EPSA of 82.9 (Figure 3B, SL4). Interestingly, when compared to the combination of glycine and 3-phenethylamine as the linker, the 2-phenethylamine macrocycles had a lower EPSA value in every single stereochemical configuration.¹⁶ This finding indicates that the organic linkers can be used to alter permeability and that this method can uncover SPRs that would be hard to predict *a*

priori (i.e., that 2-linked macrocycles would have lower EPSA values than 3-linked macrocycles).

Also, increasing the lipophilicity of these organo-peptide macrocycles with the gem-dimethyl (SL 2) or methylation of the NH (SL 3) showed no improvements in the average EPSA compared to the 2-phenethylamine linker (SL4). This data suggest that the conformation change induced by the 2-phenethyl linker has a larger effect on the EPSA values than blocking a polar NH group or increasing the overall lipophilicity of the compounds.¹⁷

To test the above hypothesis, the crude EPSA values needed to be correlated with permeability values from a cellular permeability assay. Therefore, the MDCK-II assay was used to test the permeability of a set of selected macrocycles. To start, the data trend of the 2-phenethyl linked macrocycles with glycine having lower EPSA values compared to the 3-phenethyl macrocycles was addressed. Three matched pairs of macrocycles from our data with either 2- or 3-phenethyl as the organic linker (each pair had the same amino acid configuration) were selected for synthesis and permeability evaluation. For each matched pair of macrocycles, **1d** and **1e** (LLDL), **1f** and **1g** (LDDL), and **1h** and **1i** (LLDD), the isolated 2-phenethyl linked compounds had a higher Papp than the 3-phenethyl compounds (Table 2, entries 1–6).¹⁸ To

Table 2. Correlation of Crude EPSA Values to Cellular Permeability Data of Purified Macrocycles

entry	product	EPSA (crude)	Papp (10 ⁻⁶ cm/s ²)
1	1d	76	5.7
2	1e	74	12.9
3	1f	88	7.6
4	1g	81	14.5
5	1h	87	4.0
6	1i	82	5.5
7	1j	96	2.9
8	1k	93	3.9

1d: R₁=L-Phe(4-F), R₂=D-Leu, R₃=L-Ile; n=1, y=1
1f: R₁=D-Phe, R₂=D-Leu, R₃=L-Ile; n=1, y=1
1h: R₁=L-Phe(4-F), R₂=D-Leu, R₃=D-Val; n=1, y=1
1j: R₁=L-Phe(4-F), R₂=L-Cha, R₃=L-Ile; n=3, y=0
1k: R₁=L-Phe(4-F), R₂=L-Cha, R₃=L-Ile; n=1, y=0

1e: R₁=L-Phe(4-F), R₂=D-Leu, R₃=L-Ile
1g: R₁=D-Phe, R₂=D-Leu, R₃=L-Ile
1i: R₁=L-Phe(4-F), R₂=D-Leu, R₃=D-Val

Since achieving good permeability ($>10 \times 10^{-6}$ cm/s²) is challenging with peptidic macrocycles without resorting to *N*-methylation,²⁰ it is noteworthy that some of the macrocycles disclosed herein have good permeability without methylating any of the five free nitrogen atoms.²¹ To better understand the unique conformations leading to the higher permeabilities and to understand the trends in EPSA data, computational modeling and calculations were run to gain additional insight into the low energy conformations of these compounds.

First, the free energies of insertion (ΔG_i)²² for **1d**–**1i** were calculated to see how well this parameter correlated with the Papp values in Table 2. In each pair of macrocycles (Figure 4A), the ΔG_i was lower for the 2-linked vs the 3-linked

Figure 4. Membrane bound conformations and ΔG_i . (A) Calculated ΔG_i for macrocycles **1e**–**1i**. (B) Overlay of the membrane bound conformation of **1e** and **1d**. (C) Low energy structure of the membrane bound conformation of **1e**–**1g**. Distance between phenethylamine NH and the glycine carbonyl are noted in angstroms.

macrocycles, which is in agreement with the EPSA and Papp data. Moreover, the membrane bound conformations of macrocycles **1d**–**1e** (Figure 4B) reveal large differences in the two conformations. Detailed examination of these structures (Figure 4C) exposed a key change in the matched pairs **1d**–**1e** and **1f**–**1g**, namely, a hydrogen bond between the phenethyl amine nitrogen and the glycine carbonyl (shown in dashed lines Figure 4C). The combination of this intramolecular bond along with the 1,2-phenyl substitution on the 2-linked macrocycles affords a structure that places the amides off the phenyl ring closer together. In the overlaid structure (Figure 4B), this conformational change appears to have the benefit of further projecting the isoleucine over the center of the macrocycle, which shields the polar amine residues (a similar projection of isoleucine is noted in **1g**). In contrast, the lack of the same intramolecular hydrogen bond for the 3-linked macrocycles combined with the 1,3-phenyl substitution leads

further test the crude EPSA data that was gathered, macrocycles **1j** and **1k**, which both had EPSA values above 90, were synthesized to discern if these compounds actually had a lower Papp than macrocycles **1d**–**1i**. The Papp values for macrocycles **1j** and **1k** were both lower than the other macrocycles (entries 7 and 8). In all, this data demonstrates that the EPSA values from the crude library trended with the isolated permeability values for these organo-peptide macrocycles.¹⁹ It is worth noting that based on the seminal literature report, the EPSA values do not necessarily correlate perfectly with the magnitudes of the Papp values. However, the EPSA values are good for detecting polarity trends that can affect permeability based on minute changes in series of related macrocycles.^{13,19}

to a more open structure, and the isoleucine does not project over the center of the macrocycle, which could lead to a more exposed polar surface in that area and thus lower observed permeabilities for the 3-linked macrocycles.²³

The conformations in Figure 4 also give insight into what could be aiding the low EPSA values for the LLDL stereochemical configuration (Figure 3A). This configuration appears to favor projecting the isoleucine residue up in both macrocycles **1d** and **1e** (and to differing extents over the macrocycle) instead of projecting it away from the macrocycle core. In turn, this favorable configuration should help to sterically shield the polar nitrogen atoms in these macrocycles, leading to lower EPSA values.

In conclusion, a new platform to elucidate SPRs in organo-peptide macrocycles was developed. To accomplish this, a novel library of compounds was constructed with mass encoded stereochemistry, ring size, and organic linkers. To allow for the successful synthesis of this library, the first example of an on-resin *N*-arylation reaction was developed to access the desired macrocycles. Once synthesized, the SPRs of these macrocycles (such as the importance of the 2-phenethyl linker or LLDL stereochemistry) were delineated using the EPSA values from the crude macrocycles. Further analysis in the MDCK-II assay of the isolated macrocycles then corroborated the EPSA data trends of the crude macrocyclic compounds. Modeling of these organo-peptide macrocycles also yielded further insight into the structural nuances of these macrocycles and should help to aid in future compound design. Ultimately, as more and more hit compounds for discovery programs come from organo-peptide macrocycles, platforms such as this one will be extremely useful tools to efficiently uncover SPRs in this increasingly important class of compounds.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmmedchemlett.9b00036](https://doi.org/10.1021/acsmmedchemlett.9b00036).

Experimental procedures, crude EPSA data from SFC-MS, and LCMS spectra for isolated macrocycles (PDF)

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Author Contributions

All authors helped design experiments. B.A.H. and H.L. carried out macrocycle synthesis. B.A.H. and H.L. analyzed EPSA data. L.N. and S.M. set up SFC-MS to acquire EPSA data and helped run SFC experiments. S.H. did all the modeling and computational work. B.A.H. wrote the manuscript, and all authors have given approval for the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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- (14) We matched similar constructs for the stereochemical mass encoding, i.e., D-Val and L-Ile together (both branched), L-CyAla and D-Leu, and L-4-FPhe and D-Phe. The split and pool strategy refers to splitting the resin into two equal synthesis vessels each time, coupling each vessel with 1 of the 2 amino acids (i.e., one gets D-Val, the other gets L-Ile). After coupling goes to completion, the resin is mixed and

then split again into two equal amounts for the next split. See SI page 4 for procedure.

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(16) See SI page 18 for all data from the crude library. The trends discussed in this Letter were the best trends found in this library. Other factors such as ring size did not have a correlated EPSA value.

(17) There was no trend between EPSA value and AlogP, suggesting as originally thought that EPSA picks up on additional interactions like internal hydrogen bonding and NH shielding, and it is not just a factor of lipophilicity. See SI page 19.

(18) We also synthesized the pair of 2- vs 3-phenethyl macrocycles from the LLLD library where the 2-phenethyl from SL4 had one of the lowest EPSA values (see SI page 18). Unfortunately, these compounds failed the MDCKII assay due to low recovery, so a comparison could not be made.

(19) The EPSA assay was ran once for each set of macrocycles with a set of standards each time to correct for any differences (see ref 13). The Papp values are an average of three measurements.

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(21) It is likely that the linkers help impart permeability and that it is not just due to the amino acids chosen as we synthesized related cyclic peptides with these amino acids and they showed no permeation enhancements compared to SL4 data (see SI page 20). It should also be noted that decent permeabilities are noted for many of these macrocycles, which suggest that these flexible linkers likely allow for favorable conformations in these macrocycles.

(22) The free energy of insertion is a parameter that was found to correlate well with permeability; for further explanation please see the following reference, or page 25 of the SI: Rezai, T.; Bock, J. E.; Zhou, M. V.; Kalyanaraman, C.; Lokey, R. S.; Jacobson, M. P. Conformational Flexibility, Internal Hydrogen Bonding, and Passive Membrane Permeability: Successful in Silico Prediction of the Relative Permeabilities of Cyclic Peptides. *J. Am. Chem. Soc.* **2006**, *128*, 14073–14080.

(23) This intramolecular hydrogen bond is not noted in the 2-linked macrocycle **1i**, which along with the stereochemical configuration of the amino acids in that macrocycle leads to less of a shielding effect from the side chains leading to very similar Papp values for **1h** and **1i** (see SI page 25). Also, we are not suggesting here that this intramolecular hydrogen bond alone is responsible for the observed permeability. We are suggesting that it is part of a conformation (along with the 1,2-substitution on the phenyl ring) that leads to an overall benefit of shielding polarity in the macrocycles.