Crash Course in Macroyclic Peptides
Structure, properties, synthesis, challenges

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MacMillan Research Group
Group Meeting
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A Simplified Description of the Pharmaceutical Landscape

small molecule drugs

(cyclic) peptides, macrolides, etc.

biologics

molecular weight

500 Da

5000 Da

high bioavailability

low–moderate selectivity

chemical synthesis

rational design

best of both worlds
(in an ideal scenario...)

poor bioavailability

exquisite selectivity

biological synthesis

high throughput screening

The Development of Macrocyclic Peptides

- synthetic organic chemistry
- conformational analysis
- combinatorial chemistry
- computational chemistry
- medicinal chemistry
- macrocyclic peptides
- chemical biology

I immense progress in the past 30 years
- better high-throughput screening
- improved rational design
- better understanding of pharmacokinetics

Macrocyclic Peptides in the Pharmaceutical Industry

- Over 40 cyclic peptides in clinical use; 7 in clinic trials
- In the past 10 years, nine cyclic peptides approved
- Traditionally inspired by or derived from natural products
- De novo synthesis becoming increasingly more common

Morisson, C. *Nat. Rev. Drug Discov.* **2018**, *17*, 531
Macrocyclic Peptides – Outline

Properties and structure
Why cyclic peptides?
Structural & conformational aspects

Macrocyclization
General considerations
Synthetic methods
- cation-assisted, sulfur reagents,
- ring contraction, click, RCM,
- cross-coupling, C–H activation etc.

Library synthesis
Phage display
Split intein circular ligation
“Split and pool” approach

Challenges
Metabolic stability
Cellular uptake & bioavailability
Roads to achieving lipophilicity
Why Macrocyclic Peptides?

Numerous conformations:
- Structural pre-organization
- Entropic cost built-in
- High potency, high selectivity

Extended structure with both lipophilic and polar elements:
- Intricate and extensive binding to target
- High potency, high selectivity

Functionally a small “large molecule” rather than a large “small molecule”:
- “Functional sub-domains” (like a protein)
- Disrupt protein-protein interactions
- Undruggable targets

What Determines Structure in a Macroyclic Peptide


**basic conformation**

**α-helix**

**β-turn**

**β-helix**

**secondary structure** – can be “frozen” via macrocyclization

**amino acid residue**

**amide geometry**

**amino acid chirality**

**methylation of amide**

number of amino acids

sequence of amino acids

everything really…
List of Conformational Analysis Methods

- VT NMR, IR, H/D exchange, NMR constraints/calculations (solvent shielding, hydrogen bonds)
- E-COSY NMR (x-space)
- ROESY NMR, $^{15}$N HSQC (cis / trans amides)
- $^1$H NMR (ϕ, ψ dihedral angles)
- Circular dichroism (secondary structure)

Global structure:
- Monte Carlo MM
- Molecular dynamics
- Ab initio chemical shift calculations
- X-ray crystallography
- EXSY NMR
- Principal moments of inertia (PMI)

Yudin, A. K. Chem. Sci. 2015, 6, 30
Influencing Geometry and Rigidity via Fluorine Incorporation

1,2-difluoroalkane

fluorinated backbone can strongly influence structure via conformational biasing

unguisin A

gauche conformation preferred

1,2-difluoroalkane

gauche conformation preferred

fluorinated backbone can strongly influence structure via conformational biasing

internal H-bond

syn-difluoro

two internal H-bonds

anti-difluoro

three internal H-bonds

Stabilizing $\alpha$-Helix Structures via Peptide “Stapling”

$\alpha$-helix

motif present at 2/3 of protein-protein interfaces

how to stabilize this structure in peptides?

[i, i+4, i+7, i+11]

amino acids i, i+4, i+7 and i+11 are on same face

“stapled peptides”

incorporate unnatural residues in synthesis

ring-closing metathesis, Click rxn, etc.

staples can provide beneficial binding to lipophilic surfaces

staple interacts with protein-protein surface

double Click staple involved in binding

Aileron
(biotech company)

focused on RCM-stapled peptides

candidate ALRN-692
Phase II clinical trials for lymphoma


Lau, Y. H.; de Andrade, P.; Wu, Y.; Spring, D. R. Chem. Soc. Rev. 2015, 44, 91
Four Possible Ways to Form Peptidic Macrocycle

1. **side-chain to tail**
   - $X$ is a side-chain amino acid.
   - $Y$ is another amino acid.
   - Formation of a macrocycle with $N$-terminus (tail) and $C$-terminus (head).

2. **head to tail**
   - $X$ forms the head of the macrocycle.
   - $Y$ is a side-chain amino acid.

3. **side-chain to side-chain**
   - $X$ and $Y$ are side-chain amino acids.
   - Connection through non-native amino acids.

4. **side-chain to head**
   - $X$ is a side-chain amino acid.
   - $Y$ is a functional handle attached to the head of the macrocycle.

*Innate functional handles*:
- $N$-terminus (tail) and $C$-terminus (head) are natural residues.
- Some natural residues are used as innate functional handles.

*Unnatural amino acids*:
- Commonly incorporated for programmed side-chain reactivity.

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Successfully Inducing Macrocyclization

\[
\text{linear peptide} \xrightarrow{\text{amide coupling}} \text{rate} \sim k_{\text{intra}} [\text{pept}] \quad \text{rate} \sim k_{\text{inter}} [\text{pept}]^2
\]

- universally applied strategy – minimize peptide concentration
  - run at high dilution
    - (< 10 mM very common)
  - cyclization on solid support
    - (high pseudo-dilution)
  - biphasic reaction systems
    - (low [pept] in active phase)

- high strain in TS for 8–12 membered rings
- effects level off past ring sizes of 12+
- cyclic peptides with 7+ amino acids are accessible

<table>
<thead>
<tr>
<th>n</th>
<th>(k_{\text{intra}} \text{ (s}^{-1}))</th>
<th>yield (%)</th>
<th>EM (M)</th>
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<tr>
<td>3</td>
<td>0.42</td>
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<td>1.5</td>
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<tr>
<td>4</td>
<td>(6 \times 10^2)</td>
<td>quantitative</td>
<td>(2.1 \times 10^3)</td>
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<td>12</td>
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<td>46</td>
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<tr>
<td>16</td>
<td>(2.1 \times 10^{-3})</td>
<td>73</td>
<td>(7.5 \times 10^{-3})</td>
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<tr>
<td>20</td>
<td>(3.1 \times 10^{-3})</td>
<td>77</td>
<td>(1.1 \times 10^{-2})</td>
</tr>
</tbody>
</table>

\[k_{\text{inter}} = 0.28 \text{ M}^{-1}\text{s}^{-1}\]

\[EM = \frac{k_{\text{intra}}}{k_{\text{inter}}}\]

Centelles-Martí, V.; Pandey, M. D.; Burguete, M. I.; Luis, S. V. *Chem. Rev.* 2015, 115, 8736
Conformational Control Strategies – Pseudoprolines

pseudoproline-containing peptide \[\xrightarrow{\text{PyBOP, DIPEA}}\] cyclic peptide \[\xrightarrow{\text{HCl}}\] threonine residue unmasked

rigid proline motif induces $\beta$-turn

strategy applicable to many other pseudoproline-containing chains

Metal Ion-Assisted Cyclization of Peptides

$\text{Ag}^+ \quad \text{NaOAc} / \text{H}_2\text{O}$

preferential $S$-coordination

pH-dependent pre-organization mediated by $\text{Ag}^+$

$pH > 6$

*internal amine attack*

$pH = 5 - 5.7$

*terminal amine attack*

$pH = 4$

*phenol attack*

Sulfur Reagents and Ring Contractions

head-to-tail thioesterification strategy

cysteine & thioester  \[ \text{S-to-} \text{N acyl migration} \rightarrow \text{amide product} \]

traceless Staudinger ligation

lactamization via ring contraction

White, C. J.; Yudin, A. K. Nat. Chem. 2011, 3, 509
Click Reactions in Cyclic Peptide Synthesis

Click reactions

- applicable to constrained rings
- incorporate triazole heterocyclic motif
- introduce geometrical constraints

**synthesis of constrained ring**

$$
\text{Click reactions (CuBr, DBU, Toluene, reflux)}
$$

$$
\text{H}_2, \text{Pd/C, MeOH, CH}_2\text{Cl}_2 (91\%)
$$

**cis-amide bioisostere**

$$
\text{HATU, DIPEA, DMF (0.0005 M), (>95\%)}
$$

**facile cyclization**

Ring-Closing Metathesis in Cyclic Peptide Synthesis

ring-closing metathesis for side-chain tethering

In this case, also stabilizes β-turn structure

White, C. J.; Yudin, A. K. Nat. Chem. 2011, 3, 509
Peptide Stapling via Side-Chain S_NAr or Cross-Coupling

Peptide Stapling via Metal-Catalyzed C–H Activation

\[ R = H \text{ or OAc} \]

\[
\begin{array}{c}
\text{Pd(OAc)}_2 \text{ (cat.)} \\
\text{AgBF}_4, \text{TFA} \\
\text{DMF, 80 °C, MW} \\
15 \text{ min}
\end{array}
\]

1 to 3 amino acids in chain

can tolerate Asn, Arg, Asp, Ser

\[
\text{Pd(OAc)}_2 \text{ (cat.)} \\
\text{Ag}_2\text{CO}_3, \text{oPBA} \\
t\text{BuOH (5–25 mM)} \\
100 °C, 12 \text{ h}
\]

modified side-chains

\text{ring sizes of 11–37 atoms accessible}

cyclophane products
anti-cancer activity (IC\(_{50}\) = 2 μM)

Photoredox-Catalyzed Peptide Cyclization

Photoredox decarboxylative macrocyclization: A selective route – harnessing native C-terminus carboxylate functionality

50% yield, 2:1 dr
82% yield
36% yield, 4:1 dr

53% yield

Glu

50% yield, 2:1 dr

Ty
(Me)Ala

82% yield

36% yield, 4:1 dr

High-Throughput Screening of Cyclic Peptide Libraries

ribosomal synthesis

- highest throughput methods
- least variation (unnatural residues difficult)

phage display
- $10^9$ peptides
- express peptides, then cyclize

mRNA display
- $10^{14}$ peptides
- in vitro method
- combine with genetic reprogramming

split intein circular ligation
- $10^9$ peptides
- spontaneous cyclization (splicing)

chemical synthesis

- quite laborious approaches
- highest control over topology, residues, cyclization

one-bead-one-compound
- $10^6$ peptides
- solid-support synthesis
- “split and pool” diversification

DNA-encoded libraries

- see David Liu’s work
- *JACS* 2008, 130, 15611
- *Nat. Chem.* 2018, 10, 704

randomized DNA sequence encodes peptide

DNA sequence now in phage

ribosomal synthesis in E. Coli

linear peptides displayed on phage surface

macrocyclic peptides attached to DNA that encoded them

Heinis, C.; Winter, G. Curr. Opin. Chem. Biol. 2015, 26, 89
Cyclic Peptide Libraries via SICLOPPS

SICLOPPS = split-intein circular ligation of peptides and proteins

White, C. J.; Yudin, A. K. Nat. Chem. 2011, 3, 509
Tavassoli, A. Curr. Opin. Chem. Biol. 2017, 38, 30
Chemical Synthesis of Libraries via Split & Pool Approach

Challenges in Macrocyclic Peptide Development

**Lipinski’s Rule of 5**

- MW < 500
- cLogP > 5
- HBD < 5
- HBA < 10

*(somewhat) useful for small molecules*

**Linaclotide (2012)**

- MW
- cLogP
- HBD
- HBA

*(inadequate for macrocyclic peptides)*

**straightforward library synthesis**

**very potent molecule**

**bad drug candidate**

**suboptimal understanding of what determines good PK properties**

- few well-established guidelines or rules-of-thumb

Combatting Metabolism – Not the Worst of Problems

Combatting Metabolism – Not the Worst of Problems

Degarelix (prostate cancer drug)

- Numerous non-proteogenic side-chains
- Numerous D-amino acids
- \( t_{1/2} = 40 – 70 \text{ days} \)

Metabolism via proteolytic cleavage is generally avoidable by design

Other pathways should not be discounted (e.g., oxidative degradation via P450)

**Bigger Challenges: Cellular Uptake and Oral Bioavailability**

**challenge: cellular uptake**

cyclic peptides are inherently polar

difficult passive diffusion through cell membrane

- increase peptide lipophilicity
- exploit other mechanisms

![Chemical structure](image)

“Clearly, macrocyclic peptides can be efficiently uptaken by the cell, at least in principle. But at any rate, the general strategy for transforming a bioactive peptide into a cell-permeable compound remains elusive.”

**Bigger Challenges: Cellular Uptake and Oral Bioavailability**

**Challenge: Cellular Uptake**
- Cyclic peptides are inherently polar
- Difficult passive diffusion through cell membrane
- Increase peptide lipophilicity
- Exploit other mechanisms

**Challenge: Oral Bioavailability**
- 1) Survive acidic stomach and proteases
- 2) Absorption via diffusion through intestine

Recent efforts mostly dedicated to improving lipophilicity


**Cyclosporin – Remarkable Example of Cellular Uptake and Bioavailability**

- **cyclosporin** *(immunosuppressant)*
  - seven N-Me amides
  - lipophilic side-chains

30% oral bioavailability
(0–5% standard for peptides)

readily diffuses through cell membranes
(10^{-7} vs 10^{-5} to 10^{-6} cm/s for small molecules)

in water – hydrophilic

- 6 conformations
- intermolecular H-bonding of amides
- considerable amount of exposed polar surface

in non-polar media – lipophilic

- compact, folded into self
- four internal hydrogen bonds
- exposed polar surface minimized via intramolecular interactions

**amphiphilic, flexible structure allows for distinct but optimal behavior in both aqueous and lipophilic media**

Effect of Degree of N-Methylation on Lipophilicity

D- and L-amino acids
several degrees of N-methylation

Complex relation between membrane permeability and (degree + positions) of N-methylation

Effect of Degree of N-Methylation on Lipophilicity

analysis for scaffold A
best and worst with 3 x N-Me

analysis for scaffold B
relatively linear correlation

analysis for all scaffolds
relatively no correlation

experimental measurement for synthesized isomers

rat pharmacokinetics – comparable to cyclosporin!

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<th>Intravenous administration</th>
<th>Oral administration</th>
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<tr>
<td></td>
<td>CL (ml min⁻¹ kg⁻¹)</td>
<td>V_{dss} (l kg⁻¹)</td>
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<tr>
<td>Me₃A cyclosporin</td>
<td>4.5</td>
<td>1.1</td>
</tr>
<tr>
<td>cyclosporin</td>
<td>3.5</td>
<td>1.2</td>
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</table>

Effect of Degree of N-Methylation on Lipophilicity

ability to form intramolecular H-bonds can improve permeability by reducing exposed polar surface while in membrane

N–methylation inherently modulates permeability by eliminating N–H bonds

N–methylation modulates macrocyclic structure, leading to intramolecular H bonding

number of N-Me, position of N-Me and chirality of amino acids are critical

a priori predictions still difficult...

A Disconnect in Macro cyclic Peptide Development

straightforward library synthesis

high-throughput screening for potency and selectivity

very potent molecule

bad drug candidate

suboptimal understanding of what determines good PK properties

few well-established guidelines or rules-of-thumb

analysis – 16 targets – three most potent ligands for each (discovered via RaPID mRNA or phage display screen)

interpretation: we know the correct “recipe” for potency but pharmacokinetics are an afterthought…
High-Throughput Screening with Genetically Reprogrammed Library

<table>
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<th>Second position</th>
<th>Third position</th>
</tr>
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<tr>
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<td>Glu</td>
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<tr>
<td>Ala</td>
<td>Gly</td>
<td>Gly</td>
</tr>
</tbody>
</table>

ΔmiLogP: -1.04 to 2.43

- replace charged & polar amino acids
- translation with 23 relatively non-polar amino acids

![RaPID (mRNA display)](image)

interleukin-6 receptor ligand

- $K_D = 350 \text{ nM}$
- $c\text{LogP} = 0.2$

“hydrophobic” cyclic peptide library

(10^{12} members)

Outlook

- genetically reprogrammed libraries?
- multi-layered screening?
- leveraging other diffusion mechanisms?
- better understanding of cellular uptake?
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