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Targeting Intracellular Protein-Protein Interactions with Macrocyclic Peptides

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Disclosure

D.P. is co-Founder and Shareholder of Entrada Therapeutics

Abstract

We are exploring macrocyclic peptides (including mono- and bicyclic peptides) as a general modality for targeting intracellular protein-protein interactions (PPIs), by leveraging a novel class of highly active cyclic cell-penetrating peptides (CPPs). In the first approach, potent PPI inhibitors such as linear peptides, stapled peptides, cyclic peptides, and proteins, which are generally impermeable to the cell membrane, are rendered cell-permeable and biologically active by conjugating them to a cyclic CPP. In an alternative approach, macrocyclic peptide libraries containing cyclic CPPs are synthesized in the one bead-two compound format and screened for binding to PPI targets of interest, resulting in cell-permeable and biologically active hits directly from library screening. Potent, selective, proteolytically stable, and cell-permeable macrocyclic peptidyl inhibitors have been generated against a variety of intracellular PPIs including the calcineurin-NFAT, β -catenin-TCF, Keap1-Nrf2, and NEMO-IKK interactions.

Current Drugs Are Effective for only ~20% Protein Targets

Three classes of challenging targets shown in red

Solution = Intracellular Biologics

Enzymes

Nucleic acids

CRISPR/Cas9

Antibodies

Peptides

Macrocycles

An Intracellular Biologic Requires Two Components:

- A drug molecule (e.g., a peptide, protein, antibody, or nucleic acid) that effectively modulates (or replaces) the activity of a disease protein; and
- A delivery vehicle that effectively delivers the drug molecule into the cell interior.

Cyclic Cell-Penetrating Peptides as Highly Active Intracellular Delivery Vehicles

- **>100** cyclic CPPs discovered
- Up to **120%** cytosolic delivery efficiency

Tat^{FITC} (3 μ M, 2 h; **2%**):

HeLa cells treated with 3 μ M **CPP9^{FITC}**:

CPPs Enter Cells by Endocytosis and Exit the Endosome by Inducing Vesicle Budding and Collapse

Initially Proposed: *Biochemistry* **2016**, 55, 2601.
Reviewed: *Bioconj. Chem.* **2019**, 30, 273.

Examples of Macrocyclic Peptidyl Inhibitors against Intracellular PPI Targets

Target	Active Modality	Application	Current Status
Calcineurin-NFAT	Linear peptide	Inflammatory diseases, Organ transplantation	In vivo POC
β -Catenin-TCF	Stapled peptide	Cancer	In vitro POC
Keap1-Nrf2	Cyclic peptide	Inflammatory diseases	In vitro POC
NEMO-IKK	Bicyclic peptide	Inflammatory diseases, Cancer	In vitro POC; lead optimization

I. Cytosolic Delivery of Linear Peptides: Inhibitor of Calcineurin-NFAT Interaction

Dr. Patrick Dougherty

Prof. John Christman

Dr. Manjula Karpurapu

Manuscript in preparation

Calcineurin, NFAT, and Immunosuppressants

- Current treatment after **organ transplantation**:

Cyclosporine A (CsA)

FK506

- Cyclophilin-CsA** and **FKBP-FK506 complexes** bind to calcineurin and sterically block the access of substrates (including NFATs).
- Serious side effects** likely due to:
 - 1) inhibition of **all** CN substrates
 - 2) inhibition of immunophilins
 - 3) off-target effects

Calcineurin-NFAT Inhibitor as Safer Immunosuppressant

- The ideal immunosuppressant should:
 - 1) not bind or inhibit immunophilins
 - 2) inhibit CN activity for NFATs only
- CN recognizes NFATs (and a subset of other substrates) by using a substrate-docking site
- Peptide GPHPVIVITGPHEE (**VIVIT**) binds to the NFAT-docking site and inhibits CN activity towards NFATs (L. Cantley, P. Hogan, & A. Rao, *Science* **1999**)
- **R₁₁-VIVIT** is effective in mouse models:
 - 1) Organ transplantation (Noguchi et al. *Nat. Med.* **2004**)
 - 2) Inflammatory bowel diseases (Elloumi et al., *PLoS ONE* **2012**)
- **Problems:**
 - Low potency ($K_D = 500$ nM)
 - Low cell-permeability
 - Poor metabolic stability

G. Wagner, *Structure* **2007**

Optimization of Calcineurin Inhibitor

R₁₁-VIVIT ($K_D \sim 500$ nM; serum $t_{1/2} \sim 1$ h)

CNI103 ($K_D = 16$ nM; serum $t_{1/2} > 24$ h)

Calcineurin Inhibitor CNI103

Calcineurin Binding

Serum Stability

Ex Vivo Inhibition of LPS-Induced Cytokine Production by Mouse Lung Macrophages (n = 4)

- CsA, Cyclosporine A; LPS, lipopolysaccharide
- CPP9-VAVAA, “-” control (no binding to calcineurin)

In Vivo Efficacy in Mouse Model of Acute Lung Injury

- CNI103 administered for 2 h by **nasal insufflation**;
- Challenge animals with 2 mg/kg LPS (**intranasal**);
- Quantitate cytokines in bronchoalveolar lavage fluid (**BALF**) after ≥ 2 h.

- Result: CNI103 dose-dependently reduces TNF α levels in BALF

In Vivo Efficacy in Mouse ALI Model - Intranasal

3 mg/kg CNI103 or CPP9-ZIZIT for 2 h (intranasal) and then 2 mg/kg LPS (intranasal)

CPP9-ZIZIT is an intermediate with a potency between those of R₁₁-VIVIT and CNI103

In Vivo Efficacy in Mouse ALI Model - Intravenous

- Mice were dosed with 5 mg/kg CNI103 (i.v.) for 4 h and then 2 mg/kg LPS (intranasal)
- Protein and cytokine levels in BALF and serum were analyzed after 16 h.

Conclusion:

- ❖ CNI103 (intranasal or intravenous) reduces inflammatory cytokine production and prevents permeability pulmonary edema;
- ❖ CNI103 penetrates alveolar leukocytes and has excellent PK;
- ❖ CNI103 is well tolerated without major toxicities.

II. Cytosolic Delivery of Stapled Peptides: β -Catenin-TCF Interaction Inhibitor

Dr. Patrick Dougherty

Dr. Jin Wen

J. Med. Chem. **2019**, 62, 10098.

Stapled Peptides

Pioneered by:
Greg Verdine
Loren Walensky

- Enhanced binding affinity and proteolytic stability
- Some stapled peptides are cell-permeable
- Stapled peptide against MDM2-p53 interaction currently in phase II clinical trials (Aileron)
- **Major limitation:** 1) Most are not cell-permeable; 2) Unpredictable cell-permeability

Can cyclic CPPs confer cell-permeability to stapled peptides?

β -Catenin-TCF Interaction and Wnt Signaling

β -Catenin-TCF Interaction Inhibitors

Ax4-1-CPP9 (Peptide 9)

($IC_{50} = 152 \pm 8$ nM)

C. Heinis, *ACS Chem Biol* 2016

MTT Assay (72 h)

β -Catenin-TCF Interaction Inhibitors

Ax4-1-CPP9 (Peptide 9)

($IC_{50} = 152 \pm 8$ nM)

C. Heinis, *ACS Chem Biol* 2016

Effect on Target Genes

Myc Protein

Cytosolic Delivery of Other Stapled Peptides

**Stapled Peptides
(no CPP)**

**CPP9-Peptide
Conjugates**

Peptide 20

Peptide 21

Peptide 22

Peptide 23

Peptide 24

Peptide 25

Conclusion:

- Conjugation to CPP9 increased the cytosolic entry efficiency of stapled peptides by 11- to 200-fold;
- Conjugation to a cyclic CPP represents a general method for cytosolic delivery of stapled peptides.

III. Cytosolic Delivery of Macrocyclic Peptides: Keap1-Nrf2 Interaction Inhibitor

Heba Salim

J. Org. Chem. **2020**, *85*, 1416.

Keap1-Nrf2 Interaction and Stress Response

Macrocyclic Peptidyl Keap1-Nrf2 Inhibitor

Peptide 1 ($K_D = 18$ nM)

Low cell-permeability

Lu et al. *Eur. J. Med. Chem.* 2017

Peptide 2 ($IC_{50} = 153$ nM)

Confocal Microscopy

Flow Cytometry (Naphthofluorescein)

Macrocyclic Peptidyl Keap1-Nrf2 Inhibitor

Western Blots (HEK293T cells)

- Peptide 2 dose-dependently increases the transcriptional activity of Nrf2.

IV. Integration of Cell Penetration and Target Binding: Bicyclic Peptidyl Inhibitors of NEMO-IKK Interaction

Dr. Curran Rhodes

J. Am. Chem. Soc. **2018**, *140*, 12102.

NEMO-IKK Inhibitors for Treating Cancer and Inflammation

- Excessive **NF-κB** causes inflammation and cancer resistance to chemotherapy and radiotherapy
- **NEMO-IKK** interaction activates IKK and NF-κB signaling
- **NEMO-IKK inhibitors** are expected to be effective for treating inflammatory diseases and cancer
- **Antp-NBD** is effective for treating Duchenne muscular dystrophy (DMD) and B-cell lymphoma in mice and dogs:

RQIKIWFQNR**RMKWKKGGTALDWSWLQTE**
Antp **NBD**

- **Problem:** Very low potency ($K_D \sim 50 \mu\text{M}$) and poor pharmacokinetics (serum $t_{1/2} \sim 15 \text{ min}$)

NEMO-IKK α/β Inhibitor from a Bicyclic Peptide Library

Screening
against NEMO

Optimization

NEMO Binding ($IC_{50} = 1.0 \mu M$)

Cell-permeable; Proteolytically stable

★ = Residues important for NEMO binding

Bicyclic Inhibitor against NEMO-IKK α/β Interaction

Canonical NF- κ B Signaling Pathway

Inhibition of Canonical NF- κ B Signaling in HEK293(Luc) cells:

Effect on p-IKK β and I κ B α Levels in HT29 (human colon adenocarcinoma) Cells:

Anti-proliferative Activity of Bicyclic NEMO Inhibitor

Inhibition of Ovarian Cancer Cells (A2780 and CP70) but not Normal Ovarian Surface Epithelial (OSE) Cells:

Conclusion:

- Macrocyclic peptides can be designed so that the same residue(s) serves dual function of cell penetration and target engagement.

Summary

- Highly active cyclic CPPs provide a general vehicle for cytosolic delivery of biologics (e.g., peptides, proteins, and nucleic acids).
- Potent, cell-permeable, and metabolically stable peptidyl inhibitors can now be designed to specifically target intracellular protein-protein interactions (e.g., calcineurin-NFAT, β -catenin-TCF, Keap1-Nrf2, and NEMO-IKK interactions).