Peptide Stapling for Better Stability & Oral Delivery

Adrian J. Giovannone[1], Nisar Farhat[1], Jeanette Ampudia[1], Cherie Ng[1], Stephen Connelly[1]

[1] Equillium Inc., La Jolla, CA 92037

Introduction

- The common gamma chain (γc, CD132) receptor subunit is shared by six interleukin members: IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These γc-cytokines play a crucial role in regulating major immune responses and contribute to various immune and inflammatory disorders in humans.
- Among these γc-cytokines, IL-15 functions in dictating T cell response and activating NK cells, which are involved in pathogenesis of various autoimmune and inflammatory diseases. Expression of IL-15 is commonly found on many different cell types (T cells, NK cells, Dendritic cells and Monocytes) and strongly regulated due to their pro-inflammatory nature [1].
- IL-21 has broad effects on multiple cell types including lymphoid, myeloid, and epithelial cells, and plays an important role in B cell differentiation. It is known to promote a range of autoimmune diseases such as psoriasis, inflammatory bowel disease (IBD), and systemic lupus erythematosus[2].
- Monoclonal antibodies typically inhibit a single cytokine and can be insufficient to adequately treat complex disease pathologies; in contrast, JAK inhibitors can lead to broad immuno-suppression. Therefore, selective multi-cytokine inhibitors that target at the receptor level may be advantageous in improving the therapeutic effect for diseases that are implicated by multiple cytokines, see Figure 1.
- One such peptide is EQ102: a selective multi-cytokine inhibitor peptide that targets IL-15 and IL-21 signaling[3] (Figure 2).
- Celiac Disease is an autoimmune disease triggered by consuming gluten resulting in damage to the Gastrointestinal (GI) tract and is driven by both IL-15 and IL-21 signaling (Figure 3). Symptoms include vomiting, diarrhea, abdominal pain, anemia and fatigue. Many patients are non-responsive to strict gluten free diet, experiencing ongoing GI inflammation. Unresolved inflammation leads to malnutrition and GI cancers. Estimated >750,000 non-responsive Celiac Disease patients in United States[4].
- Hydrocarbon stapling is a known method to confer protease resistance, enhance stability, and stabilize the bioactive secondary structure of peptides by introducing covalent linkages between amino acids within a single molecule[5] (Figure 4).
- Here, we sought to create orally deliverable and locally acting form of EQ102 by introducing hydrocarbon stapling to the peptide to treat GI indications such as IBD and Celiac Disease. SAR and computational biology yielded a family of stable, stapled peptides termed the **EQ302** s

FIGURE 1. EQ Peptides Specifically Blocks The Common Receptor

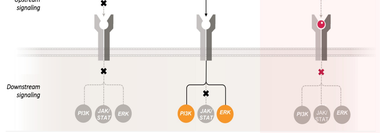


FIGURE 2. EQ102 Cytokine Inhibition Profile

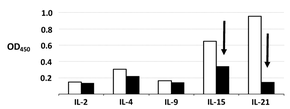


FIGURE 3. Celiac Disease Is Driven By Il-15 & Il-21 Signaling

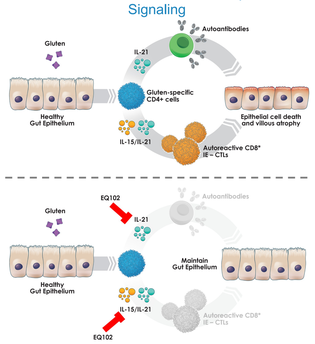
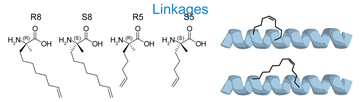


FIGURE 4. Hydrocarbon-stapled Peptides Contain Covalent Linkages



Results

FIGURE 5. Determining Structure Activity Relationship (SAR) by Mutational Walk

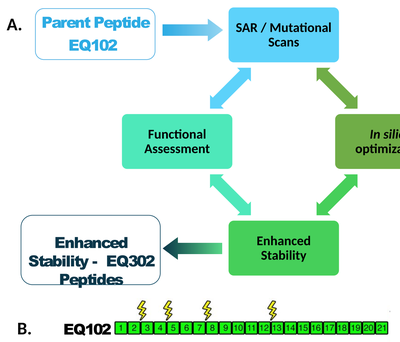


Figure 5. Determining Structure Activity Relationship (SAR) by Mutational Walk.
(A) Diagram of SAR strategy. (B) Sites in the EQ102 parent peptide sequence that are susceptible to hydrolysis during incubation with, indicated by lightning bolt.

FIGURE 6. Computational Biology for Molecular Prediction

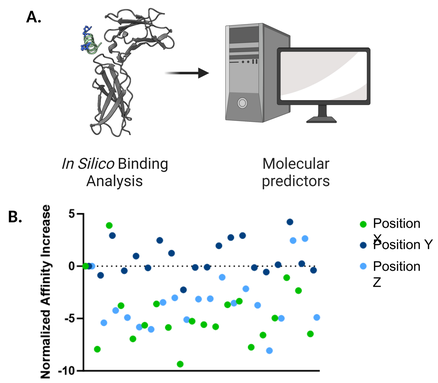


Figure 6. Computational Biology for Molecular Prediction. (A) Diagram of *in silico* modelling. (B) Data analysis of three unique positions in EQ102 predicted to increase biological activity. Variants above the dashed line are predicted to improve receptor binding.

FIGURE 7. Functional Screen Assessment of EQ302 Molecules

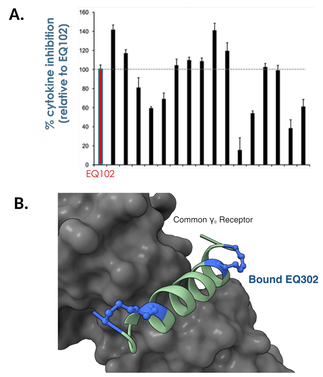


Figure 7. Determining Optimal Molecule from Functional and *in silico* Data. (A) Cytokine inhibition activity of EQ302 candidates compared to parent peptide EQ102. Dashed line represents EQ102 activity level. (B) Model predicting binding location of representative stapled EQ302 peptide to CD132.

FIGURE 8. Measuring Gastric Stability and Inhibitory Activity

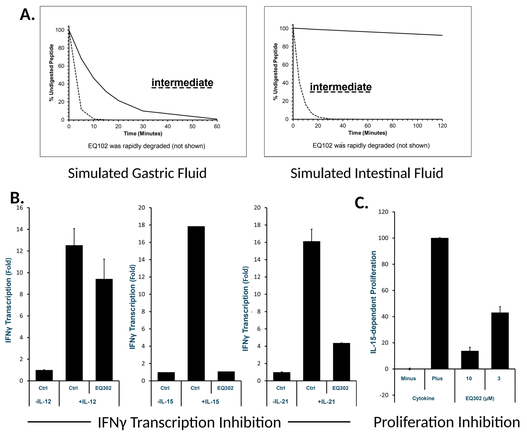


Figure 8. Measuring Gastric Stability and Inhibitory Activity of Representative EQ302 peptide. (A) Graph of undigested EQ302 peptide or intermediate peptide when incubated in Simulated Gastric Fluid or Simulated Intestinal Fluid for 60 or 120 minutes, respectively. (B) Functional assay using NK-92 cells demonstrates the inhibitory characteristics of EQ302 are specific for IL-15 and IL-21. Interferon-gamma (IFNγ) is a known downstream product of cytokine signaling. (C) Inhibition of IL-15-induced proliferation in NK-92 cells shows EQ302 inhibits in a dose-dependent manner.

FIGURE 9. Development of Pre-clinical Oral Formulation

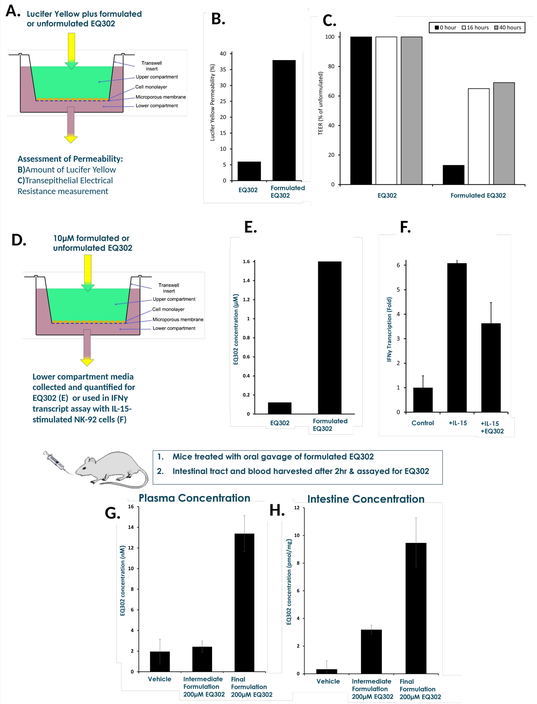


Figure 9. Development of Pre-clinical Oral Formulation for Delivery of Representative EQ302 peptide. (A) Caco-2 cells were cultured on porous membrane allow formation of tight junctions and brush border establishing an epithelial barrier similar to the lining of the intestine. Lucifer yellow was added to the upper compartment with various formulations and EQ302. (B) Media from the lower compartment was assayed for lucifer yellow, indicating increased permeability of the epithelial barrier. (C) Epithelial barrier integrity was assayed using Transepithelial Electrical Resistance (TEER) at times after having new media replaced post incubation with EQ302 or EQ302 plus oral formulation for 2h. (D) EQ302 or EQ302 plus oral formulation was added to the upper compartment and after 2h media from the lower chamber was collected. (E) Amount of EQ302 from the media of the lower chamber was quantified. (F) Media from the lower chamber was incubated with NK-92 cells prior to stimulation with IL-15 to demonstrate that post-epithelial barrier transit EQ302 retains cytokine inhibition activity. (G) The oral formulation was administered to mice via gavage in mice to test how much of EQ302 was absorbed into the plasma. An intermediate formulation was also used to demonstrate the effectiveness of the final formulation. (H) Two hours post oral gavage of EQ302 plus final formulation or intermediate formulation, small intestine was harvested and assayed to quantify the amount of EQ302 was present.

FIGURE 10. Pharmacokinetics of EQ302 in C57BL/6 Mice

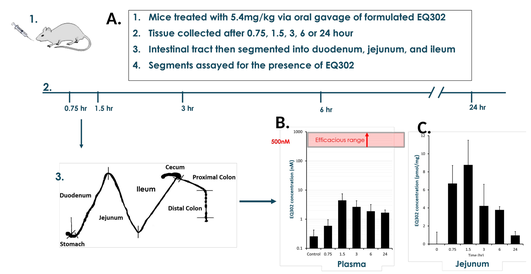


Figure 10. Pharmacokinetics of Representative EQ302 peptide in C57BL/6 Mice.
(A) Experimental methods of determining PK of EQ302 in C57BL/6 mice. (B) Concentration versus time graph of EQ302 in plasma. Estimated therapeutic threshold is at 500nM.
(C) Concentration versus time graph of EQ302 in small intestine.

FIGURE 11. Pharmacodynamics of EQ302 in C57BL/6 Mice

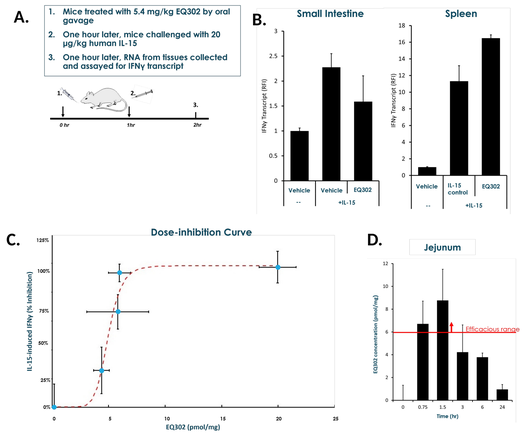


Figure 11. Pharmacodynamics of representative EQ302 peptide in C57BL/6 Mice. (A) Experimental methods of determining PD of EQ302 in C57BL/6 mice. (B) IFNγ transcript used as read-out of IL-15 signaling in small intestine (decrease) and spleen as surrogate for plasma (no effect). (C) Dose inhibition curve of IL-15-induced IFNγ versus EQ302 concentration in small intestine. (D) Concentration of 100% inhibition of IFNγ from dose-inhibition curve (6 pmol/mg) marked on concentration versus time graph of EQ302 in jejunum signifying that concentrations in the small intestine surpass the therapeutic threshold.

Conclusions

- Currently Equillium's peptide-based therapeutics are delivered parenterally.
- In GI tract-based indications (e.g. Celiac Disease and IBD) oral drug delivery is an attractive route of administration.
- Hydrocarbon stapling is a well-known method of conferring peptide stability.

Here, we demonstrate that:

1. Hydrocarbon stapling technology can be applied to EQ102 to confer increased stability in the GI tract while retaining its cytokine inhibitory properties.
2. Computational analysis and *in silico* modeling can predict which residues of the peptide are involved with binding to the target receptor to enhance the binding interaction.
3. Utilizing a pre-clinical formulation for enhanced epithelial permeability, a representative EQ302 peptide achieved meaningful concentration levels for localized cytokine inhibition in the small intestine.
4. A representative EQ302 peptide delivered to mice via oral gavage can inhibit IL-15-induced IFNγ transcription locally in the GI tract indicating potential utility for Celiac Disease and IBD.

References

1. Budagian, Vadim et al. "IL-15/IL-15 receptor biology: A guided tour through an expanding universe", Cytokine & Growth Factor Reviews, Volume 17, Issue 4: 259-280 (2006) https://doi.org/10.1016/j.cytogfr.2006.05.001.
2. Spolski, Rosanne & Leonard, Warren J. "Interleukin-21: A Double-edged Sword With Therapeutic Potential" *Nature Reviews drug Discovery* Volume 13: 379-395 (2014) https://doi.org/10.1038/nrd4296
3. Ciszewski, C., Discepolo, V. et al. "Identification of a c Receptor Antagonist That Prevents Reprogramming of Human Tissue-resident Cytotoxic T Cells by IL15 and IL21" Gastroenterology, Volume 158: 625-637. (2020) https://doi.org/10.1053/j.gastro.2019.10.006
4. Lebwohl et al. "Predictors of Persistent Villous Atrophy in Coeliac Disease: A Population-Based Study" Alimentary Pharmacology and Therapeutics, Volume 39: 488-495. (2014) https://doi.org/10.1111/apt.12621
5. Walensky, Loren D. & Bird, Gregory H. "Hydrocarbon-Stapled Peptides: Principles, Practice, and Progress" Journal of Medicinal Chemistry, Volume 57, 15: 6275-6288 (2014) https://doi.org/10.1021/jm4011675

Disclosures

This study was funded by Equillium, Inc.
A Giovannone, N Farhat, J Ampudia, C Ng, are currently employees and stockholders of Equillium.

S Connelly is currently employee, stockholder, and officer of Equillium.

Additional Information

For questions, comments, or to ask any questions about the poster, please contact medinfo@equilliumbio.com.

Methods

Generation of Hydrocarbon Stapled EQ302

Multiple EQ102 peptides were synthesized with α,α-disubstituted hydrocarbon staples, S5-S5 or R8-S5. Introducing these hydrocarbon staples at each position in the sequence, effectively performs a "staple walk". First, stapled peptides were tested for solubility. Second, soluble peptides were tested for cytokine inhibition properties by performing a cytokine proliferation assay. Briefly, NK-92 cells are incubated with or without inhibitory peptide for 15 minutes prior to having IL-15 added for 24 hours. After 24 hours, cells were harvested and processed with CellTiter-Glo (Promega) per manufacturer's instructions. Positions of staples were compiled illustrate the amenability of a position for stapling and retention of IL-15 inhibitory characteristics. Similar experiments were performed for IL-21.

Computational Biology / *in silico* modeling

Performing in silico saturation mutagenesis of four sites in EQ102, we calculated relative binding free energies toward CD132 normalizing the affinity of the unmutated EQ102 peptide. Residue scanning was performed using MOE (Molecular Operating Environment), which computes the individual amino acid residue impact after substitution. Two parameters dAffinity and dStability were considered while calculating the effect of amino acid substitutions. High positive dAffinity and dStability implied a highly significant substitution. This approach showed significant agreement upon validation against experimental data.

Oral drug delivery formulation development

Stability was assessed by incubating peptides in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF), both produced according to USP, at 37C. Fluids were analyzed for peptide concentration by RP-HPLC. Digested products were analyzed by LC/MS to identify patterns in the degraded products.

IFNγ transcript inhibition was performed by incubating NK-92 cells with or without EQ peptide prior to adding IL-15. After 2 hours, cells were harvested for total RNA, cDNA was synthesized, and RT-qPCR was performed with primers for IFNγ and GAPDH as control. IFNγ values were normalized to GAPDH.

Transwell assays employed Caco-2 cells to establish tight junctions and create an epithelial barrier on the porous membrane. TEER measurements were performed using an EVOM2 (World Precision Instruments) and an EndOhm chamber.

Pharmacokinetic / Pharmacodynamic experiments in mouse

Mice were given different formulations of a representative EQ302 peptide via oral gavage and the concentration of the peptide was measured in the blood and intestinal tissue using an ELISA two-hours post-dosing of the drug. To measure the concentration of EQ302 in the intestine, we washed out the intestinal content and collected multiple sections of the duodenum for EQ302 concentration analysis by ELISA. A similar ELISA was performed on the plasma.

IL-15 activation results in the transcriptional upregulation of IFNγ in resident immune cells of the GI tract, thus we employed a "cytokine challenge" model to evaluate the functionality of EQ302 concentrations observed in the small intestine. Intestinal tissue was isolated 2 hours after EQ302 oral administration and 1 hour after IL-15 challenge. Tissues were processed for total RNA, cDNA was synthesized, and

© Equillium Inc.