99 Erie Street Cambridge, MA 02139 USA (617) 492-6977
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ENSEMBLE THERAPEUTICS

Identification and Characterization of Synthetic Small Molecule Macrocycle Antagonists of Human IL-17A

David Livingston, Sethu Alexander, Julian Bond, Timothy Briggs, Andrew Fraley, Stephen Hale, Tanya Landsman, Richard Martinelli, Kelley Shortsleeves, Nick Terrett and Nathan Walsh

Abstract

Background/Purpose: IL-17A has been demonstrated to be a key pro-inflammatory cytokine in human rheumatoid arthritis and in several rodent models of arthritis. Synthetic macrocycles are more amenable to optimization for metabolic stability and oral absorption than biotherapeutics. The aim of this investigation was to identify high-affinity macrocycle binders of human IL-17A, to quantify their inhibitory potency against cytokine production in human cells, and to determine if active compounds could inhibit a delayed-type hypersensitivity response in mice.

Methods: DNA programmed chemistry (DPC) libraries were generated to synthesize in vitro libraries of non-peptidic synthetic macrocycles of molecular weight 600– 1000 kDa. Compounds binding to immobilized IL-17A were identified by PCR and DNA sequencing. Two compounds were resynthesized and characterized by 1) competitive ELISA to determine affinity for human IL-17A, 2) inhibition of IL-17A-driven IL-6 production in human rheumatoid arthritis synovial fibroblasts (RASF) and human HT-29 adenocarcinoma cells, 3) inhibition of other pro-inflammatory human cytokine activities, such as IL-1β, IL-6, IL-22, and TNFα, and 4) efficacy in a delayed-type hypersensitivity (DTH) mouse model. The DTH model used a 1-fluoro-2,4-dinitrobenzene (DNFB) sensitizer, which was applied to the animals at day 0. On day 7, compounds dissolved in DMSO were dosed by intraperitoneal (i.p.) injection at a dose of 10 mg/kg. A second application of DNFB was performed on the left ear 30 min after compound dosing. After 24 hours, left ear edema was measured by change in ear weight compared to the right ear, and levels of INF-γ in ear tissue homogenates were quantified by ELISA.

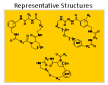
Results: Two synthetic macrocycles identified in this investigation, E-34935 and E-35018, were characterized by a competition ELISA with human IL-17A, and determined to have a dissociation constant (K_d) = 2 nM. E-34935 and E-35018 were found to inhibit IL-17A with EC50 of 2.0 and 2.1 μM in RASF, and 45 and 20 nM in HT29 cells, respectively. Both compounds were inactive (EC_{50} > 25 μM) in a battery of cellular assays for the human cytokines IL-1β, IL-6, IL22, and TNFα. A single i.p. dose of 10 mg/kg of E-34935 or E-35018 in the murine DTH model suppressed edema vs. vehicle control by 50 or 54% respectively ($p < 0.05$ vs. vehicle control). In comparison, a rat anti-mouse IL-17A IgG_1 (5 mg/kg, i.p.) resulted in 76% inhibition of edema. INF-γ levels in tissue homogenates were also suppressed by E-34935, E-35018, or anti-IL-17A Ab vs. vehicle control by 72%, 62% or 75%, respectively ($p < 0.05$ for all groups vs. vehicle control group).

Conclusion: Our data provide evidence that synthetic macrocycles can be identified that bind potently and specifically to human IL-17A, and act as inhibitors of IL-17A-stimulated IL-6 production in RASF and HT29 cells. These compounds are also anti-inflammatory in an IL-17-directed murine DTH model. Prior to this investigation, such specific inhibitors of the IL-17A-IL17receptor interaction were limited to polypeptides.

Synthetically Accessible Macrocyclic Chemical Matter: *Unique Design Elements for Inhibiting Protein-Protein Complexes*

- **Cyclic structure:**
  - Structural and "shape" diversity
  - Potential for high affinity/selectivity
  - "Drug-Like" DMPK; Oral bioavailability
- **Structural variation through:**
  - Macrocycle architecture and ring-closing chemistries
  - Natural and un-natural building blocks
  - N-alkylation
  - Stereochemical variation
- **Synthetically accessible in library and discrete-compound formats**
  - Highly "modifiable" for affinity, specificity, delivery, and "drug-like" properties

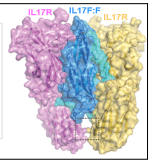
Representative Structures



Molecular Weight Distribution: Library C

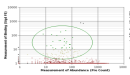
IL-17:IL-17Receptor Complexes Involve Substantial Protein-Protein Surfaces

- IL17:FF—IL17RA X-ray crystal structure published
- Large 2200 Å² Protein:Protein interaction interface
- Proven intractable to "small molecule" discovery efforts



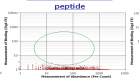
IL-17A Macrocycle Leads Identified: *Good Enrichments and Confirmed Binding to a Functional Site of Human IL-17A*

IL17A selection against a macrocycle library



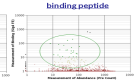
✓Hits identified

IL17A Selection replicate in the presence of an "on mechanism" anti-IL17 peptide



✓Hits are "on mechanism"

IL17A Selection replicate in the presence of non-IL17-binding peptide



✓Hits are "on-mechanism"

Productive Medicinal Chemistry Campaign Produced *High-affinity Human IL-17A Binders*

> 500 Discrete macrocycles prepared by < 3 chemists in 16 months

Original Library Hits Synthesized

K_D = 0.9-5 μM

•"On-Mechanism"

Iterative Medicinal Chemistry Campaign

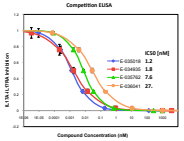
3rd Generation Macrocycle Scaffold

$K_D \approx 3$ nM

- *MW ≈ 750*
- *1-2 Stereocenters*
- *1 simple custom building block*
- *Up to 50% overall synthetic yield*

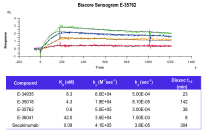
Biochemical Characterization of IL-17A Binders Demonstrates *Competition with IL-17RA and Slow Off-Rates*

Competition ELISA



A competitive ELISA binding assay in which selected anti-IL-17A macrocycles disrupt the interaction of human IL-17A to IL-17RA.

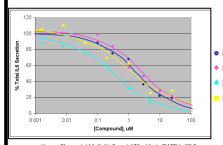
Biacore Sensogram E-35762

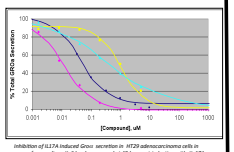


Compound	K_D (nM)	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	Dissoc $t_{1/2}$ (min)
E-34935	6.3	6.8E+04	5.0E-04	23
E-35018	4.3	1.8E+04	8.1E-05	142
E-35762	0.6	5.8E+05	3.0E-04	38
E-36041	42.8	3.9E+04	1.0E-03	8
Secukinumab	0.08	4.1E+05	3.0E-05	384

The binding of anti-IL-17A macrocycles to human IL-17A was investigated using Biacore® SPR techniques. The inhibitors displayed rapid ON-rates of binding and slow OFF-rates of dissociation from the cytokine target.

IL-17A Inhibitors Suppress IL-17A- induced Cytokine and Chemokine Production in Human RASF and HT29 Cells





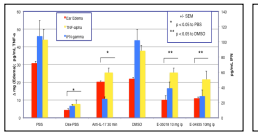
Human Rheumatoid Arthritis Synovial Fibroblasts (RASF) in 1% Serum; IL-6 levels measured by ELISA 20 hrs. post-induction with IL17A. IC_{50} determined were 1.8, 2.6, 0.37 and 1.4 μM for E-34935, E-35018, E-35762 and E036041, respectively.

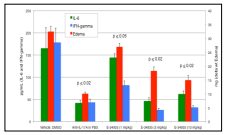
Inhibition of IL17A Induced Groα secretion in HT29 adenocarcinoma cells in serum-free medium; IL-6 levels measured at 48 hrs. post-induction with IL-17A. IC_{50} determined were 0.045, 0.011, 0, 33 and 1.0 μM for E-34935, E-35018, E-35762 and E-36041, respectively.

E-34935 IL-17A Inhibitor Specifically Blocks IL-17A-dependent Induction of Cytokines and Chemokines

Cytokine	Cell Type	Endpoint	EC_{50} (μM)
Human IL-17A	Human HT29	Groα	0.045
Human IL-17A	Human RASF	IL-6	1.8
Murine IL-17A	Mouse 3T3	IL-6	6
Human IL-1β	Human RASFs	IL-6	>>25
Human IL-2	Mouse HT2	Cell proliferation	>>25
Human IL-6	Human HeLa	STAT3 phosphorylation	>>25
Human IL-15	Mouse HT2	Cell proliferation	>>25
Murine IL-15	Mouse HT2	Cell proliferation	>25
Human IL-22	Human HT29	CXCL-1 (Groα)	>> 30
Human TNFα	Human HT29 or RASFs	IL-6	>>25

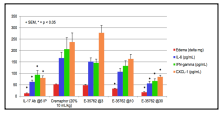
Murine Delayed-type Hypersensitivity (DTH) Model with DNFB Sensitizer: *Intraperitoneal Administration of IL-17A Inhibitors Suppresses Disease*

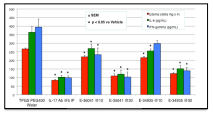




Male Balbic mice were administered a 0.5% 1-fluoro-2,4-dinitrobenzene (DNFB) solution to the shaved abdomen on Day 0 and Day 1. On Day 7, the mice were dosed i.p. with inhibitor, and 30 minutes later a 0.2% DNFB solution was applied to the shaved left ear, and an inactive solution applied to the right ear. 24 hours later, the mice were euthanized and ear edema (left – right ear weight) was determined. Cytokine/chemokine levels were determined by ELISA in ear tissue homogenates. CRO = Washington Biotechnology Inc., Baltimore, MD USA.

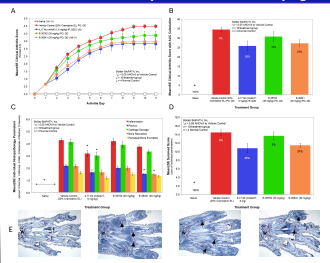
IL-17A Inhibitors Administered by Oral Gavage Suppress Edema and Cytokine Production in Murine DTH Model





DTH protocol as described in prior panel, except that in these experiments the inhibitors were dosed by oral gavage. Inhibitors were formulated in self-emulsifying solutions of Cremophor/Water (20:80) or TPGS/PEG-400/Water (20:60:20). Anti-IL-17-A antibody (affinity purified rat anti-mouse IgG_1 κ isotype, BioLegend) was dosed i.p. in PBS.

Orally Administered IL-17A Inhibitor E-36041 Is Anti-inflammatory and Disease-modifying in Murine CIA Model



Methods

IL-17A inhibitors were evaluated in a murine CIA model. In this study (CRO = Bolder BioPATH, Inc. Bolder, CO USA). DBA-1 mice (10/group) were injected intradermally with 150 μL of Freund's Complete Adjuvant containing bovine type II collagen (2 mg/ml) at the base of the tail on day 0 and again on day 21. On study days 24–25, onset of arthritis occurred and mice were randomized into treatment groups. Randomization into each group was done after swelling was obviously established in at least one paw (score of 1), and attempts were made to ensure approximately equal mean arthritis scores of 0.25 across the groups at the time of enrollment. Treatment was initiated after enrollment and continued as indicated (see below) through arthritis day 10. Mice were terminated on day 11. Clinical scores were given for each of the paws (right front, left front, right rear, left rear) on arthritis days 1–11.

Oral delivery of anti-IL-17A inhibitors inhibits chronic inflammation in the murine CIA model. A) Summed clinical arthritis scores – All Paws (scored 0-5), B) Clinical arthritis score with AUC calculation – All Paws, C) Individual histopathology parameters (All Joints), D) Histopathology sum (All Joints), E) Photomicrographs of forepaws (left to right) naïve, vehicle treated, 5 mg/kg IP anti-IL-17A antibody, 30 mg/kg E-36041, p.o.

Conclusions

- Ensemble has identified and optimized inhibitors of human and murine IL-17A using its proprietary integrated macrocycle drug discovery platform.
- These inhibitors bind IL-17A with nM affinity, compete with IL-17A binding to its cellular receptor, inhibit specifically IL-17A induction of cytokines and chemokines in cell assays , and are selective for IL-17 induced cellular responses vs. responses induced by other pro-inflammatory cytokines.
- The IL-17A inhibitors are active in vivo in murine models of acute inflammation when dosed intraperitoneally or by oral gavage in self-emulsifying solution vehicles.
- One inhibitor described (E-36041) is orally active in a murine CIA model. This compound displays similar activity as an anti-IL-17A antibody. The compound suppresses in paw inflammation, pannus formation, and bone resorption.
- Efforts are underway to further improve the compounds to oral potency and bioavailability and to examine the compounds in other chronic models of human autoimmune/inflammatory disease.