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TITLE: Targeting Chemerin Receptor CMKLR1 in Multiple Sclerosis

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Therapies that target leukocyte trafficking pathways can reduce disease activity and improve clinical outcomes in multiple sclerosis (MS). Experimental autoimmune encephalomyelitis (EAE) is a widely studied animal model that shares many clinical and histological features with human MS. Chemokine-like receptor-1 (CMKLR1) is a chemoattractant receptor that is expressed by key effector cells in EAE and MS, including macrophages, subsets of dendritic cells, natural killer cells and microglia. CMKLR1-deficient mice develop less severe clinical and histological EAE than wild-type mice. In this study, we identified alpha-naphthoyl ethyltrimethylammonium iodide (alpha-NETA) as a selective CMKLR1 small molecule antagonist that inhibits chemerin-triggered CMKLR1+ cell migration. Prophylactic dosing with alpha-NETA significantly delayed the onset of EAE induced in C57BL/6 mice. In addition, alpha-NETA treatment significantly inhibited accumulation of inflammatory leukocytes within the CNS. This study provides additional proof-of-concept data that targeting CMKLR1:chemerin interactions may be beneficial in preventing or treating MS.
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INTRODUCTION

Therapies that target leukocyte trafficking pathways can reduce disease activity and improve clinical outcomes in multiple sclerosis (MS). Experimental autoimmune encephalomyelitis (EAE) is a widely studied animal model that shares many clinical and histological features with human MS. Chemokine-like receptor-1 (CMKLR1) is a chemoattractant receptor that is expressed by key effector cells in EAE and MS, including macrophages, subsets of dendritic cells, natural killer cells and microglia. We previously showed that CMKLR1-deficient (CMKLR1 KO) mice develop less severe clinical and histological EAE than wild-type mice. In this study, we sought to identify CMKLR1 inhibitors that would pharmaceutically recapitulate the CMKLR1 KO phenotype in EAE. Aim 1 is to identify small molecule antagonists of CMKLR1. We identified α-naphthoyl ethyltrimethylammonium iodide (α-NETA) as a selective CMKLR1 small molecule antagonist that inhibits chemerin-stimulated β-arrestin2 association with CMKLR1, as well as chemerin-triggered CMKLR1+ cell migration. We will also evaluate the toxicology and pharmacokinetic (PK) profiles of α-NETA following in vivo administration in mice. Aim 2 is to define the effects of CMKLR1 inhibitor α-NETA on cell migration and integrin activation. In this Aim, we will evaluate the effects of α-NETA on chemerin-stimulated migration responses. We will also test whether α-NETA inhibits chemerin-triggered integrin activation and cell adhesion. Aim 3 is to determine the effects of α-NETA on immune pathology in a mouse model of MS. In this Aim, we will determine whether treatment with α-NETA ameliorates clinical disease and alters inflammatory cell accumulation in the CNS of mice during EAE. Prophylactic dosing with α-NETA (i.e., prior to the onset of clinical symptoms) significantly delayed the onset of EAE induced in C57BL/6 mice by active immunization with myelin oligodendrocyte glycoprotein amino acids 35-55. In addition, α-NETA treatment significantly inhibited accumulation of inflammatory leukocytes within the CNS. This study provides additional proof-of-concept data that targeting CMKLR1:chemerin interactions may be beneficial in preventing or treating MS.
STATEMENT OF WORK

Note: The timeframes listed below are a rough approximation of when the work will be performed. It is likely that we will perform certain experiments earlier than projected.

Revised: Red text is work to be performed by Co-Investigator Professor Kareem L. Graham in his laboratory at Emory University.

Task 1. Identification of novel small molecule antagonists of CMKLR1 (months 1-12).
Subtask 1a. Screen a 140,000 compound library utilizing high throughput instrumentation at the Stanford High Throughput Bioscience Facility (HTBC). Timeframe: months 1-6
Current Status: We have performed initial quality control experiments in our lab to verify the activity of the chemerin protein used as the agonist. We have confirmed the activity of the CMKLR1/βARR2/CHO cells in chemerin-stimulated enzyme complementation. We have sourced a high quality chemilluminescence β-gal substrate to use in the screen, and acquired sufficient quantities of positive control α-NETA compound. The next step is to run a small scale pilot screen at the HTBC, and then the full screen of the compound library for novel CMKLR1 antagonists.

Subtask 1b. Additional validation of compounds identified in Subtask 1a. Timeframe: months 7-9
Current Status: Not yet completed.

Subtask 1c. Further evaluation of compounds for inhibition of CMKLR1+ cell chemotaxis to chemerin. Timeframe: months 10-12
Current Status: Not yet completed.

Task 2. α-NETA toxicology and PK studies (months 6-12).
Subtask 2a. Determine the maximum tolerated dosage (MTD) of α-NETA in mice in vivo. Timeframe: months 6-7
Current Status: Daily s.c. injection with 10 mg/kg α-NETA in 10% captisol over 30 days is the maximum tolerated dose based on the development of subcutaneous skin adhesions and dermal lesions. All mice in the α-NETA group developed subcutaneous adhesions, making injections during the later stages of the model challenging due to tight skin. Most mice develop a mild skin lesion at one or more injection site; occasionally a severe skin lesion will develop requiring humane endpoint euthanasia (CO2 asphyxiation followed by cervical dislocation to ensure death).

Subtask 2b. Determine the serum PK profile of various doses of α-NETA. Timeframe: months 8-12
Current Status: We used the Mass Spectrometry core facility at Stanford University to assist with this subaim. In initial experiments to establish a method for quantifying α-NETA levels in plasma by quadrupole mass spectrometry, we were unable to detect compound “spiked-in” to normal mouse plasma. We concluded that the parent α-NETA compound may be unstable in
plasma, likely via rapid degradation or covalent modification (perhaps binding to a plasma protein). Nevertheless, α-NETA was active in inhibiting chemerin-dependent CMKLR1 signaling in the presence of serum proteins in vitro (Appendix C Figure 2,3,4). We next identified a solvent compatible with daily s.c. injection that maintains reasonable α-NETA stability: 10% captisol in water. In this formulation, 30% of initial α-NETA was detectable 24 hours later at room temperature. We moved forward with the project based on the MTD value (10 mg/kg) using 10% captisol to formulate the compound for in vivo injection for evaluation in EAE.

**Task 3. Determine effects of α-NETA on chemerin-mediated integrin activation and cell migration (months 4-15).**

**Subtask 3a.** Evaluate effects of α-NETA on chemerin-mediated migration of mouse macrophages in vitro. Timeframe: months 7-10

**Current Status:** Not yet completed.

**Subtask 3b.** Evaluate effects of α-NETA on chemerin-mediated migration of mouse microglia in vitro.

Primary microglial cells will be isolated from the brains of neonatal C57BL/6 mouse pups. Migration to 0.1, 1, and 10 nM chemerin (in the absence or presence of a dose range of α-NETA) will be assessed using the Dunn chemotaxis chamber. Collectively, these studies will require harvesting brains from approximately neonatal 150 C57BL/6 mice (ages 0-2 days old), which will be generated using 25 mouse breeder pairs. Prof. Kareem Graham will assist in performing these in vitro studies in his lab at Emory University.

- **Time frame:** months 11-14
- **Current Status:** Not yet completed.

**Subtask 3c.** Evaluate effects of α-NETA on chemerin-triggered adhesion in flow chamber assays. Timeframe: months 12-15.

**Current Status:** Not yet completed.

**Task 4. Determine the effects of α-NETA on clinical disease in a mouse model of MS (months 13-24).**

**Subtask 4a.** Prophylactic administration of α-NETA to EAE mice. Time frame: months 13-18

**Current Status:** Preliminary data complete; there is a significant reduction in inflammatory cell infiltration into the CNS measured by histology and flow cytometry (see Appendix B, Appendix C Figure 12,13). Next step is to qualitatively evaluate myelin levels by luxol fast blue staining.

**Subtask 4b.** Therapeutic administration of α-NETA to EAE mice. Time frame: months 19-24

**Current Status:** Not yet completed.

**Task 5. Determine effects of α-NETA on antigen-specific lymphocyte activation events (months 22-24).**

**Subtask 5a.** Leukocyte functional assays. Time frame: months 22-24
Current Status: Preliminary data complete; there no difference in IFN\(\gamma\) or IL-17 secretion from splenocytes and lymphocytes between \(\alpha\)-NETA treatment mice vs. controls, and there were no significant differences in T cell proliferation (quantified by \(^{3}\text{H-}\)thymidine incorporation).


Current Status: Preliminary data complete; there is a significant reduction in inflammatory cell infiltration into the CNS measured by histology and flow cytometry (see Appendix B, Appendix C Figure 14). Next step is to qualitatively evaluate myelin levels by luxol fast blue staining.


Current Status: Not yet completed.

Subtask 6c. Determine effects of \textit{in vivo} \(\alpha\)-NETA treatment on inflammatory cell localization within the CNS. Timeframe: months 31-33

Current Status: We have the histological CNS section prepared. Next step is to stain for CD3, F4/80, and CD11c.

Subtask 6d. Determine effects of \(\alpha\)-NETA on inflammatory cell accumulation within the CNS. Timeframe: months 34-36

Current Status: Preliminary data complete; there is a significant reduction in inflammatory cell infiltration into the CNS measured by histology and flow cytometry (see Appendix B).

Subtask 6e. Determine effects of \(\alpha\)-NETA on inflammatory cytokine/chemokine production within the CNS. Timeframe: months 34-36

Current Status: Not yet completed.
KEY RESEARCH ACCOMPLISHMENTS

- In studies evaluating the stability of $\alpha$-NETA in various diluents used for in vivo dosing, $\alpha$-NETA was unstable in water, but relatively stable in 10% captisol (90% water), with 30% of the original concentration remaining in solution after 24 hr incubation at room temperature. For in vivo dosing, we made fresh formulations for daily s.c. injection. The maximum tolerated dose was daily s.c. injection of 10 mg/kg for 30 consecutive days.

- Prophylactic dosing (i.e., prior to the onset of clinical symptoms) with 10 mg/kg $\alpha$-NETA in 10% captisol significantly delayed the onset of EAE induced in C57BL/6 mice by active immunization with myelin oligodendrocyte glycoprotein amino acids 35-55.

- Prophylactic dosing with $\alpha$-NETA significantly inhibited accumulation of inflammatory leukocytes within the CNS, measured either histologically (quantification of inflammatory foci in the meninges and parenchyma) or by flow cytometry of isolated CNS tissue.

- $\alpha$-NETA treatment did not have noticeable effects on T cell recall proliferation or cytokine production (IFN-$\gamma$, IL-17), consistent with the phenotype of lymphocytes from the CMKLR1 KO mouse treated under similar conditions.
REPORTABLE OUTCOMES


- U.S. Patent application submitted March 1, 2012: Graham KG, Zhang J, Butcher EC, Zabel BA, Small Molecule CMKLR1 Antagonist in Demyelinating Disease (Patent application appended)

- VA Merit Review Award application (BLR&D) submitted September 10, 2012: Zabel BA (PI): Role of Chemerin and its Receptors in Pulmonary Fibrosis (proposed to use α-NETA in Aim 2 to test the hypothesis that CMKLR1 inhibition would lead to exacerbated pulmonary fibrosis in the bleomycin-induced mouse model of pulmonary fibrosis).
CONCLUSION

Several features make CMKLR1 an attractive target for therapeutic manipulation in MS. For one, microglial cells and macrophages, key effector cells in MS, express this receptor. Also, given that clinical EAE is significantly reduced in CMKLR1 KO mice during the chronic phase of disease, CMKLR1 may be a target for secondary-progressive forms of MS, for which there are currently no approved therapies. Lastly, chemerin and CMKLR1+ plasmacytoid dendritic cells are present in the perivascular cuffs of MS patients, further demonstrating relevance for these molecules in the pathology of human disease. In this report, we identify α-NETA as a novel and selective small molecule antagonist of CMKLR1. α-NETA inhibits CMKLR1+ cell migration in vitro, as well as chemerin-stimulated β-arrestin association with CMKLR1. Administration of α-NETA in vivo significantly delayed onset of clinical EAE and limited leukocyte accumulation in the CNS.

The complexity and heterogeneity of MS poses challenges for attaining desirable clinical outcomes by targeting a single chemoattractant receptor. Small molecule inhibitors of CCR2 have been shown to ameliorate clinical EAE, but small molecule antagonists of CCR2 have failed in MS clinical trials(1). Our results to date also suggest the existence of CMKLR1-independent pathways to CNS inflammatory pathology, as CMKLR1 KO mice are susceptible to EAE. Therefore, MS treatments that inhibit CMKLR1, CCR2 or other candidate receptors as part of a ‘polypharmacologic’ approach may be more efficacious than targeting a single receptor (2). Nevertheless, the data presented in this report implicate CMKLR1 as a GPCR with important roles in CNS pathology; and also suggest that targeting CMKLR1 with small molecule inhibitors warrants investigation as a possible treatment strategy for certain forms of MS.
REFERENCES

SUPPORTING DATA

None (it is all included in Appendix B and Appendix C)
APPENDICES

Appendix A: FOCIS Abstract
Appendix B: FOCIS Poster
Appendix C: US Patent Application
W.51. Therapeutic Stimulation of Monocytoid Cells Promotes Remyelination
Axinia Doering, Smriti Agrawal, Lorraine Lau, Manoj Mishra, Jan van Minnen, V. Wee Yong. University of Calgary, Calgary, AB, Canada

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS). While the majority of currently used MS medications target the inflammatory aspects of the disease, treatments to induce remyelination and axonal regeneration are lacking. Approaches to stimulate remyelination could lead to recovery from demyelinating injuries and protect axons. Given the increasing evidence that a properly directed inflammatory response can have healing properties, we tested the hypothesis that activating monocytoid cells with clinically used medications would promote remyelination in mice. From a library of 1040 medications, we identified the anti-fungal medication, amphotericin B, as an activator of human and murine monocytoid cells in culture, through engaging MyD88/TRIF signalling. When mice were subjected to lysolecithin-induced demyelination of the spinal cord, systemic injections of non-toxic doses of amphotericin B and another activator, macrophage colony stimulating factor (MCSF), further elevated the local monocytoid activity. Treatment with amphotericin B, particularly with MCSF, increased the number of oligodendrocyte precursor cells and promoted remyelination. This regimen of clinically used medications to stimulate the benefits of inflammation has potential applications to conditions such as MS to facilitate myelin repair.

W.52. Novel CMKLR1 Small Molecule Antagonist Suppresses Autoimmune Demyelinating Disease
Brian Zabel1, Jian Zhang1,2, Susanna Lewén1, Thomas Burke1, Maria Zoudilova3, Raymond Sobel3, Eugene Butcher3, Kareem Graham4. 1Palo Alto Institute for Research and Education & Veterans Affairs Palo Alto Health Care System, Palo Alto, CA; 2Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China; 3Stanford University School of Medicine, Stanford, CA; 4Emory University School of Medicine, Atlanta, GA

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that affects more than two million people worldwide. Experimental autoimmune encephalomyelitis (EAE) is a widely studied animal model that shares many clinical and histological features with MS. Tissue injury in EAE and MS is mediated in part by inflammatory leukocytes that transmigrate across the blood-brain barrier. Therapies that target leukocyte trafficking pathways can reduce disease activity and improve clinical outcomes in MS. Chemokine-like receptor-1 (CMKLR1) is a chemoattractant receptor that binds chemerin, a proteolytically regulated chemoattractant. CMKLR1 is expressed by macrophages, subsets of dendritic cells, natural killer cells and microglia. We previously showed that upon active immunization with myelin oligodendrocyte glycoprotein amino acids 35-55, CMKLR1-deficient (CMKLR1 KO) mice develop less severe clinical and histological EAE than wild-type mice. We sought to identify suitable CMKLR1 inhibitors that would pharmaceutically recapitulate the KO phenotype. Using a live cell functional screening assay, we recently identified a selective CMKLR1 small molecule antagonist that inhibits chemerin-stimulated β-arrestin2 association with CMKLR1 (IC50: 0.4 uM) as well as chemerin-dependent CMKLR1+ cell migration (IC50: 1.2 uM). Daily dosing with this compound (3 or 10 mg/kg s.c.) beginning on the day of immunization significantly delayed the onset of clinical EAE. Moreover, compound treatment significantly inhibited accumulation of inflammatory leukocytes within the CNS. This study provides additional “proof-of-concept” data that targeting CMKLR1:chemerin interactions may be beneficial in preventing or treating MS.

W.53. A Mouse Model to Address the Specific Role of B Cells in MS
Joseph Cantor1, Claude Bernard2, Mark Ginsberg1. 1University of California San Diego, La Jolla, CA; 2Immunology
Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that affects approximately 2 million people worldwide. Tissue injury in MS and experimental autoimmune encephalomyelitis (EAE), its widely used preclinical model, is mediated in part by inflammatory leukocytes that transmigrate across the blood-brain barrier. Therapies that target leukocyte trafficking pathways can reduce disease activity and improve clinical outcomes in MS. Currently approved disease-modifying drugs for MS that function by altering systemic leukocyte migration or distribution (e.g., Tysabri, an anti-CD40 ligand adenylate molecule antibody, or Gilenya, a small molecule sphingosine-1-phosphate receptor modulator) are, however, associated with potentially severe side effects. Agents that selectively target the trafficking of key inflammatory cell subsets involved in the pathophysiology of MS may therefore be superior to current treatment strategies.

Chemokine-like receptor-1 (CMKL1) is a G protein-coupled receptor (GPCR) that binds chemerin, a proteolytically regulated leukocyte chemoattractant. CMKLR1 protein is expressed by macrophages, subsets of dendritic cells, natural killer (NK) cells and microglia. There are several lines of evidence that point to pivotal roles for CMKLR1 in pathogenic CNS inflammation. For one, CMKL1-knockout (KO) mice develop less severe clinical and histological EAE than wild-type (WT) mice. In addition, chemerin co-localizes with intracellular endothelial cells in the brains of MS patients, and CMKLR1+ dendritic cells are present in the leptomeninges and in perivascular cuffs of chronic and active MS lesions. CMKL1 therefore represents a novel target for the treatment of MS. Here we identified a novel small molecule pharmacological inhibitor of CMKLR1 and tested it in a mouse model of autoimmune demyelinating disease.

**Results 1:** ZAB084 selectively inhibits chemerin-stimulated huCMKL1 association with β-arrestin2

We next asked if ZAB084 blocked CMKLR1+ cell migration in an in vitro transwell chemotaxis experiments. CMKLR1(L1.2) pre-B cells) transfectants were pre-incubated for 10 minutes with vehicle control (0.01% DMSO) or various concentrations of ZAB084. Cells were then exposed to 1 mM chemerin for 90 minutes via transwell chamber, and cell migration was quantified by flow cytometry. Mean of duplicate wells ± range is shown for each concentration of ZAB084, representative of n=3 experiments.

ZAB084 inhibited migration of cells expressing mouse CMKL1 with potency similar to cells expressing human CMKL1. The compound had little effect on mouse splenocyte chemotaxis to CCL20 (which acts through a different chemokine receptor, CCαT), providing additional evidence of specificity.

Furthermore, ZAB084 was not acutely toxic to splenocytes, huCMKL1/L1.2 cells, or CMKLR1-CHO cells cultured in vitro in the presence of the compound for 90 min at concentrations up to 30 mM, as determined by PI and annexin V viability staining (not shown).

**Results 2:** ZAB084 selectively inhibits CMKLR1+ cell migration to chemerin

We next tested CMKL1 antagonist ZAB084 in vivo for efficacy in limiting immune pathology and inflammatory cell accumulation in the MOG<sub>35-55</sub>-induced EAE model of MS. C57Bl/6 female mice received daily s.c. injections of 10 mg/kg ZAB084 (n=7) or 10% capstool vehicle (n=8) beginning at the time of disease induction and monitored daily for clinical signs (0 = normal; 1 = mild; 2 = moderate; 3 = severe; 4 = moribund). *p < 0.05 by Mann-Whitney U test.

(A) ZAB084 significantly delayed onset of EAE. Mean clinical score ± SEM vs. time, representative of 3 independent experiments with similar results. *p < 0.05, as determined by Mann-Whitney U test.

(B) Significantly fewer inflammatory foci in the CNS of ZAB084-treated EAE mice. Brain and spinal cord tissues were harvested 16 days after EAE induction and evaluated histologically. Results are pooled from two independent experiments with similar results, each with 4-6 mice per group. *p < 0.05 by t test.

**Results 3:** ZAB084 suppresses clinical and histological EAE

Since chemokine receptors can regulate other cellular functions in addition to cell migration, we assessed possible effects of CMKLR1 antagonist ZAB084 on lymphocyte activation.

MOG<sub>35-55</sub>-immunized mice received ZAB084 or vehicle once daily. Ten days after immunization, splenocyte chemotaxis to CCL20, which activates through a different chemoattractant receptor, CCR7, providing additional evidence of specificity.

(B) ZAB084 significantly delayed onset of EAE. Mean clinical score ± SEM vs. time, representative of 3 independent experiments with similar results. *p < 0.05, as determined by Mann-Whitney U test.

(C) ZAB084 treatment did not have noticeable effects on T cell recall proliferation or cytokine production, consistent with the phenotype of lymphocytes from the CMKLR1 KO mouse treated under similar conditions.

**Results 4:** Recall proliferation and cytokine responses of lymphocytes from ZAB084 treated mice

To confirm that ZAB084 diminishes leukocyte trafficking/accumulation in the CNS during EAE, we quantified the total number of CNS-infiltrating mononuclear cells.

MOG<sub>35-55</sub>-immunized mice received ZAB084 or vehicle once daily. Sixteen days after immunization, mononuclear cells in the (A) spleens and (B) CNS tissues of the mice were enumerated by flow cytometry. Mean ± SEM, n=1 experiment (n=7 mice per group).

Consistent with the histological reduction in inflammatory foci in ZAB084 treated mice, there were significantly fewer CNS-infiltrating mononuclear cells. *p < 0.05 by t test. Although there was an increase in total number of cells in the spleen, this difference did not reach statistical significance.

**Conclusion**

This study provides important preclinical "proof-of-concept" data that targeting CMKLR1: chemerin interactions with small molecule inhibitors may be beneficial in preventing or treating MS.
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This application is a::
> Application One::
Filing Date::

PRIOR FOREIGN APPLICATIONS

Foreign Application One::
Filing Date::
Country::
Priority Claimed::
SMALL MOLECULE CMKLR1 ANTAGONISTS IN DEMYELINATING DISEASE

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[01] This invention was made with government support under Grant No. AI079320 awarded by National Institutes of Health, and Grant No. W81XWH-11-1-0512 awarded by The Department of Defense--Congressionally Directed Medical Research Program. The government has certain rights in the invention.

INTRODUCTION


[03] While CMKLR1 does not bind to chemokines, it has been reported that resolvin E1 (RvE1), a bioactive lipid generated upon aspirin-triggered enzymatic processing of omega-3 fatty acids, is a lipid ligand for CMKLR1 [Hasturk, et al. FASEB J. (2006) 20(2):401-3; Serhan Prostaglandins Leukot Essent Fatty Acids. (2005) 73(3-4):141-62].
Relevant literature


[05] The sequence of chemerin (retinoic acid receptor responder 2 (RARRES2) II; tazarotene induced gene 2 product (TIG2)) may be found in Genbank, accession number NM_002889. The sequence of CMKLR1 may be found in Genbank, accession number Y14838, and is described by Samson et al. (1998) Eur J Immunol. 28(5):1689-700. The sequence of a CMKLR1 ligand, mammalian chemerin, may be found in Genbank, accession number NM_002889. Methods of modulating adipogenesis by interfering with the activity of CMKLR1 are described in U.S. Patent Publication 2007-0286863, herein specifically incorporated by reference.

SUMMARY

[06] The present invention is drawn to compositions and methods for interfering with the biological processes associated with CMKLR1 signaling, which processes include, without limitation, regulation of adipogenesis and demyelinating inflammatory disease. In some embodiments, inhibitors of CMKLR1 are provided, which inhibitors are useful for the treatment or prevention of MS and other diseases, e.g. experimental animal models such as experimental autoimmune encephalomyelitis (EAE). Blocking CMKLR1 signaling may also decrease fat accumulation and adipocyte metabolism.

[07] Inhibitors of CMKLR1 include, but are not limited to, agents that interfere with the interaction of CMKLR1 with its natural ligands, agents that reduce CMKLR1 expression (e.g., by reducing transcription or by inducing cell surface receptor desensitization and/or internalization), agents that reduce expression of endogenous ligands of CMKLR1, and agents that inhibit intracellular signals initiated by the binding of CMKLR1 with its ligands. Inhibitors include small molecules. In certain embodiments, the inhibitor is 2-(alpha-naphthoyl)ethyltrimethylammonium iodide or an analog or derivative thereof.

[08] In some embodiments a pharmaceutical formulation of a CMKLR1 inhibitor is provided, where the formulation comprises an effective dose of the inhibitor and a pharmaceutically acceptable excipient. The formulation maybe prepared for the desired route of administration, e.g. oral, parenteral, topical, etc., usually parenteral. The inhibitor may be 2-(alpha-naphthoyl)ethyltrimethylammonium iodide or an analog or derivative thereof.

[09] The present invention is also drawn to methods of screening for agents that can decrease demyelinating inflammatory disease when administered to a subject. In general, the screening
method is designed to determine whether an agent can antagonize CMKLR1 activity in a cell. In certain embodiments, a cell expressing CMKLR1 (e.g., cells that normally express CMKLR1 or those that are genetically engineered to express CMKLR1) is contacted to a candidate agent and its response to a CMKLR1 ligand(s) is evaluated (e.g., chemotaxis, receptor/ligand binding, target gene expression, signaling responses, etc.). In certain other embodiments, a cell expressing CMKLR1 or a ligand is contacted to an agent and the expression level of CMKLR1 or its ligand is evaluated. Candidate compounds include, without limitation, analogs and variants of 2-(alpha-naphthoyl)ethyltrimethylammonium iodide.

BRIEF DESCRIPTION OF THE DRAWINGS

[10] Figure 1 shows a schematic of events in receptor internalization with ligand-triggered association with beta-arrestin2 and CMKLR1 expression by the CHO/bARR cell line used to screen compounds for CMKLR1 antagonists.

[11] Figure 2 shows a graph illustrating inhibition of chemerin-triggered huCMKLR1 association with βARR by aNETA.

[12] Figure 3 shows a graph illustrating inhibition of chemerin-triggered human CMKLR1/L1.2 cell migration.

[13] Figure 4 shows a graph illustrating inhibition of chemerin-triggered mouse CMKLR1/L1.2 cell migration.

[14] Figure 5 shows a graph illustrating specificity in which aNETA does not inhibit CXCR4-dependent MOLT-4 migration to CXCL12.

[15] Figure 6 shows a graph illustrating specificity in which aNETA does not inhibit CCR9-dependent MOLT-4 migration to CCL25.

[16] Figure 7 shows a graph illustrating specificity in which aNETA does not inhibit CXCR4-dependent mouse splenocyte migration to CXCL12.

[17] Figure 8 shows a graph illustrating specificity in which aNETA does not inhibit CCR7-dependent mouse splenocyte migration to CCL19.

[18] Figure 9 shows a graph illustrating specificity in which aNETA does not inhibit FPR-dependent mouse BM neutrophil chemotaxis to fMLP.

[19] Figure 10 shows a graph illustrating specificity in which aNETA does not inhibit C5aR-dependent mouse BM neutrophil chemotaxis to C5a.

[20] Figure 11 shows graphs of cytotoxicity assay results of aNETA.

[21] Figure 12 shows a graph illustrating the result of administration of two different doses of aNETA and controls and the onset of EAE in days post-administration.
Figure 13 shows a graph illustrating the result of administration of three different doses of aNETA and controls and the onset of EAE in days post-administration, as well as the disease incidence.

Figure 14 shows graphs illustrating that aNETA suppresses leukocyte infiltration of CNS in EAE.

**Detailed Description**

As summarized above, the present invention is drawn to methods for inhibiting the activity of CMKLR1. In some embodiments such methods include treating demyelinating inflammatory disease in a subject by administering an agent that antagonizes the activity of chemokine-like receptor 1 (CMKLR1) and/or a CMKLR1 ligand (e.g., chemerin or other endogenous CMKLR1 ligands. As such, the methods of the invention find use in treating EAE or MS in a subject. Methods of screening for agents that regulate demyelinating inflammatory disease are also provided.

Before the present invention is described in greater detail, it is to be understood that this invention is not limited to particular embodiments described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, representative illustrative methods and materials are now described.
All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

Terms

"Activity" of CMKLR1 shall mean any signaling or binding function performed by that protein.

"Comparable cell" shall mean a cell whose type is identical to that of another cell to which it is compared. Examples of comparable cells are cells from the same cell line.

"Inhibiting" the onset of a disorder shall mean either lessening the likelihood of the disorder’s onset, or preventing the onset of the disorder entirely. In the preferred embodiment, inhibiting the onset of a disorder means preventing its onset entirely. As used herein, onset may also refer to deterioration in a patient that has chronic/progressive disease, or relapse in a patient that has ongoing relapsing-remitting disease.

The methods of the invention may be specifically applied to individuals that have been diagnosed with an autoimmune disease, e.g. a chronic/progressive or relapsing-remitting disease such as MS or EAE. Treatment is aimed at the treatment or prevention of relapses, which are an exacerbation of a pre-existing condition.
"Inhibiting" the expression of a gene in a cell shall mean either lessening the degree to which the gene is expressed, or preventing such expression entirely.

"Specifically inhibit" the expression of a protein shall mean to inhibit that protein's expression or activity (a) more than the expression or activity of any other protein, or (b) more than the expression or activity of all but 10 or fewer other proteins.

"Subject" or "patient" shall mean any animal, such as a human, non-human primate, mouse, rat, guinea pig or rabbit.

"Suitable conditions" shall have a meaning dependent on the context in which this term is used. That is, when used in connection with an antibody, the term shall mean conditions that permit an antibody to bind to its corresponding antigen. When this term is used in connection with nucleic acid hybridization, the term shall mean conditions that permit a nucleic acid of at least 15 nucleotides in length to hybridize to a nucleic acid having a sequence complementary thereto. When used in connection with contacting an agent to a cell, this term shall mean conditions that permit an agent capable of doing so to enter a cell and perform its intended function. In one embodiment, the term "suitable conditions" as used herein means physiological conditions.

"Treating" a disorder shall mean slowing, stopping or reversing the disorder's progression. In the preferred embodiment, treating a disorder means reversing the disorder's progression, ideally to the point of eliminating the disorder itself. As used herein, ameliorating a disorder and treating a disorder are equivalent.

The term "immune" response is the development of a beneficial humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against CMKLR1 in a recipient patient. Such a response can be an active response induced by an "immunogen" that is capable of inducing an immunological response against itself on administration to a mammal, optionally in conjunction with an adjuvant.

"Alkyl" refers to monovalent saturated aliphatic hydrocarbyl groups having from 1 to 10 carbon atoms and preferably 1 to 6 carbon atoms. This term includes, by way of example, linear and branched hydrocarbyl groups such as methyl (CH₃-), ethyl (CH₃CH₂-), n-propyl (CH₃CH₂CH₂-), isopropyl ((CH₃)₂CH-), n-butyl (CH₃CH₂CH₂CH₂-), isobutyl ((CH₃)₂CHCH₂-), sec-butyl ((CH₃)(CH₂CH₃)CH-), t-butyl ((CH₃)₃C-), n-pentyl (CH₃CH₂CH₂CH₂CH₂-), and neopentyl ((CH₃)₃CCH₂-).

The term "substituted alkyl" refers to an alkyl group as defined herein wherein one or more carbon atoms in the alkyl chain have been optionally replaced with a heteroatom such as -O-, -N-, -S-, -S(O)ₙ- (where n is 0 to 2), -NR- (where R is hydrogen or alkyl) and having from 1 to 5
substituents selected from the group consisting of alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, acyl, acylamino, acyloxy, amino, aminoacyl, aminoacyloxy, oxyaminoacyl, azido, cyano, halogen, hydroxyl, oxo, thioketo, carboxyl, carboxylalkyl, thioaryloxy, thioheteroaryloxy, thioheterocyclooxy, thiol, thioalkoxy, substituted thioalkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, heterocyclcyl, heterocyclooxy, hydroxymino, alkoxyamino, nitro, -SO-alkyl, -SO-aryl, -SO-heteroaryl, -SO₂-alkyl, -SO₂-aryl, -SO₂-heteroaryl, and -NR¹R², wherein R¹ and R² may be the same or different and are chosen from hydrogen, optionally substituted alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, heteroaryl and heterocyclic.

[43] "Alkoxy" refers to the group –O-alkyl, wherein alkyl is as defined herein. Alkoxy includes, by way of example, methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, t-butoxy, sec-butoxy, n-pentoxy, and the like. The term "alkoxy" also refers to the groups alkenyl-O-, cycloalkyl-O-, cycloalkenyl-O-, and alkynyl-O-, where alkenyl, cycloalkyl, cycloalkenyl, and alkynyl are as defined herein.

[44] The term "substituted alkoxy" refers to the groups substituted alkyl-O-, substituted alkenyl-O-, substituted cycloalkyl-O-, substituted cycloalkenyl-O-, and substituted alkynyl-O- where substituted alkyl, substituted alkenyl, substituted cycloalkyl, substituted cycloalkenyl and substituted alkynyl are as defined herein.

[45] “Acyl” refers to the groups H-C(O)-, alkyl-C(O)-, substituted alkyl-C(O)-, alkanyl-C(O)-, substituted alkanyl-C(O)-, alkynyl-C(O)-, substituted alkynyl-C(O)-, cycloalkyl-C(O)-, substituted cycloalkyl-C(O)-, cycloalkenyl-C(O)-, substituted cycloalkenyl-C(O)-, aryl-C(O)-, substituted aryl-C(O)-, heteroaryl-C(O)-, substituted heteroaryl-C(O)-, heterocyclyl-C(O)-, and substituted heterocyclyl-C(O)-, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein. For example, acyl includes the “acetyl” group CH₃C(O)-.

[46] "Aminoacyl" refers to the group -C(O)NR²₁R²², wherein R²¹ and R²² independently are selected from the group consisting of hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic and where R²¹ and R²² are optionally joined together with the nitrogen bound thereto to form a heterocyclic or substituted heterocyclic group, and wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, substituted cycloalkenyl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.
cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

“Amino” refers to the group –NH$_2$.

The term "substituted amino" refers to the group -NRR where each R is independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, substituted alkynyl, aryl, heteroaryl, and heterocyclic provided that at least one R is not hydrogen.

“Carboxyl,” “carboxy” or “carboxylate” refers to –CO$_2$H or salts thereof.

“Carboxyl ester” refers to the groups -C(O)O-alkyl, -C(O)O-substituted alkyl, -C(O)O-alkenyl, -C(O)O-substituted alkenyl, -C(O)O-alkynyl, -C(O)O-aryl, -C(O)O-cycloalkyl, -C(O)O-substituted cycloalkyl, -C(O)O-cycloalkenyl, -C(O)O-substituted cycloalkenyl, -C(O)O-heteroaryl, -C(O)O-substituted heteroaryl, -C(O)O-heterocyclic, and -C(O)O-substituted heterocyclic, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein.

“Cyano” or “nitrile” refers to the group –CN.

“Halo” or “halogen” refers to fluoro, chloro, bromo, and iodo.

“Hydroxy” or “hydroxyl” refers to the group –OH.

“Nitro” refers to the group –NO$_2$.

“Sulfonyl” refers to the group SO$_2$-alkyl, SO$_2$-substituted alkyl, SO$_2$-alkenyl, SO$_2$-substituted alkenyl, SO$_2$-cycloalkyl, SO$_2$-substituted cycloalkyl, SO$_2$-ary, SO$_2$-substituted aryl, SO$_2$-heteroaryl, SO$_2$-substituted heteroaryl, SO$_2$-heterocyclic, and SO$_2$-substituted heterocyclic, wherein alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocyclic, and substituted heterocyclic are as defined herein. Sulfonyl includes, by way of example, methyl-SO$_2$-, phenyl-SO$_2$-, and 4-methylphenyl-SO$_2$-.

“Thiol” refers to the group -SH.

In addition to the disclosure herein, the term “substituted,” when used to modify a specified group or radical, can also mean that one or more hydrogen atoms of the specified group or
In addition to the groups disclosed with respect to the individual terms herein, substituent groups for substituting for one or more hydrogens (any two hydrogens on a single carbon can be replaced with =O, =NR$_7^6$, =N-OR$_7^6$, =N$_2$ or =S) on saturated carbon atoms in the specified group or radical are, unless otherwise specified, -R$_7^{60}$, halo, =O, -OR$_7^7$, -SR$_7^7$, -NR$_8^8$R$_8^8$, trihalomethyl, -CN, -OCN, -SCN, -NO, -NO$_2$, =N$_2$, =N$_3$, =SO$_2$R$_7^7$, =SO$_2$O$^-$M$^+$, -SO$_2$OR$_7^7$, -OSO$_2$R$_7^7$, -OSO$_2$O$^-$M$^+$, -OSO$_2$OR$_7^7$, -P(O)(O)$^-$M$^+$, -P(O)(OR)$_7^7$, -P(O)(OR)$_7^7$O$^-$M$^+$, -P(O)(OR$_7^7$)$_2^2$, -C(O)R$_7^7$, -C(S)R$_7^7$, -C(NR$_7^7$)R$_7^7$, -C(NR$_7^7$)R$_7^7$, where R$_7^{60}$ is selected from the group consisting of optionally substituted alkyl, cycloalkyl, heteroalkyl, heterocycloalkylalkyl, cycloalkylalkyl, aryl, arylalkyl, heteroaryl and heteroarylalkyl, each R$_7^7$ is independently hydrogen or R$_8^8$; each R$_8^8$ is independently R$_7^7$ or alternatively, two R$_8^8$'s, taken together with the nitrogen atom to which they are bonded, form a 5-, 6- or 7-membered heterocycloalkyl which may optionally include from 1 to 4 of the same or different heteroatoms selected from the group consisting of O, N and S, of which N may have -H or C$_1$-C$_3$ alkyl substitution; and each M$^+$ is a counter ion with a net single positive charge. Each M$^+$ may independently be, for example, an alkali ion, such as K$^+$, Na$^+$, Li$^+$; an ammonium ion, such as $^+$N(R$_7^{60}$)$_4$; or an alkaline earth ion, such as [Ca$_{2+}$]$_{0.5}$, [Mg$_{2+}$]$_{0.5}$, or [Ba$_{2+}$]$_{0.5}$ ("subscript 0.5 means that one of the counter ions for such divalent alkali earth ions can be an ionized form of a compound of the invention and the other a typical counter ion such as chloride, or two ionized compounds disclosed herein can serve as counter ions for such divalent alkali earth ions, or a doubly ionized compound of the invention can serve as the counter ion for such divalent alkali earth ions). As specific examples, -NR$_8^8$R$_8^8$ is meant to include -NH$_2$, -NH-alkyl, N-pyrrolidinyl, N-piperazinyl, 4$^+$N-methyl-piperazin-1-yl and N-morpholinyl.

In addition to the disclosure herein, in a certain embodiment, a group that is substituted has 1, 2, 3, or 4 substituents, 1, 2, or 3 substituents, 1 or 2 substituents, or 1 substituent.

It is understood that in all substituted groups defined above, polymers arrived at by defining substituents with further substituents to themselves (e.g., substituted aryl having a substituted aryl group as a substituent which is itself substituted with a substituted aryl group, which is further substituted by a substituted aryl group, etc.) are not intended for inclusion herein. In
such cases, the maximum number of such substitutions is three. For example, serial substitutions of substituted aryl groups specifically contemplated herein are limited to substituted aryl-(substituted aryl)-substituted aryl.

[61] Unless indicated otherwise, the nomenclature of substituents that are not explicitly defined herein are arrived at by naming the terminal portion of the functionality followed by the adjacent functionality toward the point of attachment. For example, the substituent "arylalkyloxycarbonyl" refers to the group (aryl)-(alkyl)-O-C(O)-.

[62] As to any of the groups disclosed herein which contain one or more substituents, it is understood, of course, that such groups do not contain any substitution or substitution patterns which are sterically impractical and/or synthetically non-feasible. In addition, the subject compounds include all stereochemical isomers arising from the substitution of these compounds.

**Representative Embodiments**

[63] The present invention provides methods for treating autoimmune disease, including inflammatory demyelinating diseases, such as multiple sclerosis; etc. These methods comprise administering to the subject having an autoimmune condition, e.g. a demyelinating condition; an effective amount of an inhibitor of CMKLR1.

[64] In some embodiments, a method is provided for inhibiting autoimmune diseases in a subject, the method comprising administering to the subject a prophylactically effective amount of a small molecule that specifically reduces levels of CMKLR1. In other embodiments, a method is provided for inhibiting inflammatory demyelinating disease in a subject, the method comprising administering to the subject a therapeutically effective amount of a small molecule.

[65] In other embodiments, the method comprising administering to said subject an agent that downregulates the expression, or inhibits the activity of, a ligand of CMKLR1, which ligand includes, without limitation, chemerin. In these methods, the CMKLR1-expressing cell can be, without limitation, a macrophage; a dendritic cell; or a microglial cell.

**Small molecule compounds**

[66] This disclosure concerns compounds which are useful as small molecule CMKLR1 antagonists in demyelinating disease and are thus useful for treating a variety of diseases and disorders that are mediated or sustained through the activity of CMKLR1. This disclosure also relates to pharmaceutical compositions comprising these compounds, methods of using these
compounds in the treatment of various diseases and disorders, processes for preparing these compounds and intermediates useful in these processes.

The following substituents and values are intended to provide representative examples of various aspects and embodiments. These representative values are intended to further define and illustrate such aspects and embodiments and are not intended to exclude other embodiments or to limit the scope of this invention. In this regard, the representation that a particular value or substituent is preferred is not intended in any way to exclude other values or substituents from this invention unless specifically indicated.

These compounds may contain one or more chiral centers and therefore, the embodiments are directed to racemic mixtures; pure stereoisomers (i.e., enantiomers or diastereomers); stereoisomer-enriched mixtures and the like unless otherwise indicated. When a particular stereoisomer is shown or named herein, it will be understood by those skilled in the art that minor amounts of other stereoisomers may be present in the compositions unless otherwise indicated, provided that the desired utility of the composition as a whole is not eliminated by the presence of such other isomers.

The present embodiments provide a compound of formula (I):

\[
\begin{align*}
Q \quad \text{Q is selected from \(-NR_4^+\), \(-NH_4^+\), \(-NH_2\), \(-NHR\), \(-NR_2\), \(-OH\), \(-SH\), and lower alkyl; wherein } R^Q \text{ is lower alkyl; and wherein } Q \text{ is selected from } \(-NR_4^+\) \text{ or } \(-NH_4^+\), \text{ then } X^- \text{ is present and is a counterion;} \\
R^1 \text{ and } R^2 \text{ are independently selected from hydrogen, alkyl, substituted alkyl, hydroxy, alkoxy, substituted alkoxy, amino, substituted amino, carboxyl, carboxyl ester, cyano, halogen, acyl, aminoacyl, nitro, and sulfonyl; and } \\
n \text{ is a number from one to four.}
\end{align*}
\]

In formula (I), \(Q\) is selected from \(-NR_4^+\), \(-NH_4^+\), \(-NH_2\), \(-NHR\), \(-NR_2\), \(-OH\), \(-SH\), and lower alkyl; wherein \(R^Q\) is lower alkyl; and wherein \(Q\) is selected from \(-NR_4^+\) or \(-NH_4^+\), then \(X^-\) is present and is a counterion;

In certain embodiments, \(Q\) is \(-NR_4^+\), wherein \(R^Q\) is lower alkyl; and wherein \(X^-\) is present and is a counterion. In certain embodiments, \(X^-\) is selected from fluoride, bromide, chloride, and fluoride.
In certain embodiments, Q is selected from $-\text{NR}_4^+$ and $-\text{NH}_4^+$, wherein $R^O$ is lower alkyl; and wherein $X^-$ is present and is a counterion. In certain embodiments, $X^-$ is selected from fluoride, bromide, chloride, and fluoride.

In certain embodiments, Q is selected from $-\text{NH}_2$, $-\text{NHR}^O$, and $-\text{NR}_2^O$; wherein $R^O$ is lower alkyl. In certain embodiments, Q is $-\text{NH}_2$. In certain embodiments, Q is $-\text{NHR}^O$; wherein $R^O$ is lower alkyl. In certain embodiments, Q is $-\text{NR}_2^O$; wherein $R^O$ is lower alkyl.

In certain embodiments, Q is selected from $-\text{OH}$, $-\text{SH}$, and lower alkyl. In certain embodiments, Q is $-\text{OH}$. In certain embodiments, Q is $-\text{SH}$. In certain embodiments, Q is lower alkyl.

In formula (I), $R^1$ and $R^2$ are independently selected from hydrogen, alkyl, substituted alkyl, hydroxy, alkoxy, substituted alkoxy, amino, substituted amino, carboxyl, carboxyl ester, cyano, halogen, acyl, aminoacyl, nitro, and sulfonyl.

In certain embodiments, $R^1$ and $R^2$ are hydrogen.

In certain embodiments, $R^1$ and $R^2$ are independently selected from hydrogen, alkyl, and substituted alkyl. In certain embodiments, $R^1$ and $R^2$ are independently selected from hydrogen, hydroxy, alkoxy, and substituted alkoxy. In certain embodiments, $R^1$ and $R^2$ are independently selected from hydrogen, amino, and substituted amino. In certain embodiments, $R^1$ and $R^2$ are independently selected from hydrogen, carboxyl, and carboxyl ester. In certain embodiments, $R^1$ and $R^2$ are independently selected from hydrogen, cyano, halogen, acyl, aminoacyl, nitro, and sulfonyl.

In formula (I), $n$ is a number from one to four. In certain embodiments, $n$ is one. In certain embodiments, $n$ is two. In certain embodiments, $n$ is three. In certain embodiments, $n$ is four.

The present embodiments provide a compound of formula (II):

\[
\begin{align*}
\text{O} & \quad \text{Q} \\
\text{(II), wherein} \\
\text{Q is selected from } -\text{NR}_4^+ \text{, } -\text{NH}_4^+ \text{, } -\text{NH}_2 \text{, } -\text{NHR}^O \text{, } -\text{NR}_2^O \text{, } -\text{OH} \text{, } -\text{SH} \text{, and lower alkyl; wherein } R^O \text{ is lower alkyl; and wherein } ; \text{Q is selected from } -\text{NR}_4^+ \text{ or } -\text{NH}_4^+ \text{, then } X^- \text{ is present and is a counterion; and } \\
\text{n is a number from one to four.}
\end{align*}
\]

In formula (II), Q is selected from $-\text{NR}_4^+$, $-\text{NH}_4^+$, $-\text{NH}_2$, $-\text{NHR}^O$, $-\text{NR}_2^O$, $-\text{OH}$, $-\text{SH}$, and lower alkyl; wherein $R^O$ is lower alkyl; and wherein ; Q is selected from $-\text{NR}_4^+$ or $-\text{NH}_4^+$, then $X^-$ is present and is a counterion;
In certain embodiments, \( \text{Q} \) is \(-\text{NR}_4^+\), wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{X}^- \) is present and is a counterion. In certain embodiments, \( \text{X}^- \) is selected from fluoride, bromide, chloride, and fluoride.

In certain embodiments, \( \text{Q} \) is selected from \(-\text{NR}_4^+\) and \(-\text{NH}_4^+\), wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{X}^- \) is present and is a counterion. In certain embodiments, \( \text{X}^- \) is selected from fluoride, bromide, chloride, and fluoride.

In certain embodiments, \( \text{Q} \) is selected from \(-\text{NH}_2\), \(-\text{NHR}_2\), and \(-\text{NR}_2^+\); wherein \( \text{R}^0 \) is lower alkyl. In certain embodiments, \( \text{Q} \) is \(-\text{NH}_2\). In certain embodiments, \( \text{Q} \) is \(-\text{NHR}_2\); wherein \( \text{R}^0 \) is lower alkyl. In certain embodiments, \( \text{Q} \) is \(-\text{NR}_2^+\); wherein \( \text{R}^0 \) is lower alkyl.

In certain embodiments, \( \text{Q} \) is selected from \(-\text{OH}, -\text{SH}, \) and lower alkyl. In certain embodiments, \( \text{Q} \) is \(-\text{OH} \). In certain embodiments, \( \text{Q} \) is \(-\text{SH} \). In certain embodiments, \( \text{Q} \) is lower alkyl.

In formula (II), \( n \) is a number from one to four. In certain embodiments, \( n \) is one. In certain embodiments, \( n \) is two. In certain embodiments, \( n \) is three. In certain embodiments, \( n \) is four.

The present embodiments provide a compound of formula (III):

\[
\text{O} \quad \text{Q}
\]

(III), wherein

\( \text{Q} \) is selected from \(-\text{NR}_4^+, -\text{NH}_4^+, -\text{NH}_2, -\text{NHR}_2, -\text{NR}_2^+, -\text{OH}, -\text{SH}, \) and lower alkyl; wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{Q} \) is selected from \(-\text{NR}_4^+\) or \(-\text{NH}_4^+\), then \( \text{X}^- \) is present and is a counterion.

In formula (III), \( \text{Q} \) is selected from \(-\text{NR}_4^+, -\text{NH}_4^+, -\text{NH}_2, -\text{NHR}_2, -\text{NR}_2^+, -\text{OH}, -\text{SH}, \) and lower alkyl; wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{Q} \) is selected from \(-\text{NR}_4^+\) or \(-\text{NH}_4^+\), then \( \text{X}^- \) is present and is a counterion;

In certain embodiments, \( \text{Q} \) is \(-\text{NR}_4^+\), wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{X}^- \) is present and is a counterion. In certain embodiments, \( \text{X}^- \) is selected from fluoride, bromide, chloride, and fluoride.

In certain embodiments, \( \text{Q} \) is selected from \(-\text{NR}_4^+\) and \(-\text{NH}_4^+\), wherein \( \text{R}^0 \) is lower alkyl; and wherein \( \text{X}^- \) is present and is a counterion. In certain embodiments, \( \text{X}^- \) is selected from fluoride, bromide, chloride, and fluoride.

In certain embodiments, \( \text{Q} \) is selected from \(-\text{NH}_2, -\text{NHR}_2, \) and \(-\text{NR}_2^+\); wherein \( \text{R}^0 \) is lower alkyl. In certain embodiments, \( \text{Q} \) is \(-\text{NH}_2\). In certain embodiments, \( \text{Q} \) is \(-\text{NHR}_2\); wherein \( \text{R}^0 \) is lower alkyl. In certain embodiments, \( \text{Q} \) is \(-\text{NR}_2^+\); wherein \( \text{R}^0 \) is lower alkyl.
In certain embodiments, Q is selected from $-\text{OH}$, $-\text{SH}$, and lower alkyl. In certain embodiments, Q is $-\text{OH}$. In certain embodiments, Q is $-\text{SH}$. In certain embodiments, Q is lower alkyl.

Particular compound of interest is shown below:

![Chemical Structure]

$X^-$ is selected from iodide, bromide, chloride, and fluoride.

Particular compound of interest is 2-(alpha-naphthoyl)ethyltrimethyl ammonium iodide, shown below:

![Chemical Structure]

**General Synthetic Procedures**


Compounds as described herein can be purified by any of the means known in the art, including chromatographic means, such as HPLC, preparative thin layer chromatography, flash column chromatography and ion exchange chromatography. Any suitable stationary phase can be used, including normal and reversed phases as well as ionic resins. Most typically the disclosed compounds are purified via silica gel and/or alumina chromatography. See, e.g., Introduction to Modern Liquid Chromatography, 2nd Edition, ed. L. R. Snyder and J. J. Kirkland, John Wiley and Sons, 1979; and Thin Layer Chromatography, ed E. Stahl, Springer-Verlag, New York, 1969.

During any of the processes for preparation of the subject compounds, it may be necessary and/or desirable to protect sensitive or reactive groups on any of the molecules concerned. This may be achieved by means of conventional protecting groups as described in standard works, such as J. F. W. McOmie, "Protective Groups in Organic Chemistry", Plenum Press,
The subject compounds can be synthesized via a variety of different synthetic routes using commercially available starting materials and/or starting materials prepared by conventional synthetic methods. Exemplary synthetic methods for the compounds described herein are described below.

In certain embodiments, 2-(alpha-naphthoyl)ethyltrimethyl ammonium iodide, shown below:

![Chemical Structure](image)

is commercially available. In certain embodiments, counterion exchanges can be performed with ion exchange chromatography. Modification of the naphthyl ring with substituents can be performed with standard chemical reactions known to one skilled in the art. Suitable reactions for modification of the naphthyl ring include electrophilic aromatic substitution. For example, the naphthyl ring can react with chlorine to form a chloro-substituted naphthyl ring. Further reaction of chloro-substituted naphthyl ring can occur with appropriate substituents. The naphthyl ring can also be alkylated using Friedel-Crafts reactions.

In certain embodiments, a Friedel-Crafts acylation can be performed on naphthylene. Further reaction of carbonyl group of the acylated naphthyl ring can be performed to obtain compounds of Formula (I)-(III).

Administration

In a certain embodiment, the present invention is drawn to methods for treating demyelinating inflammatory disease in a subject by administering an agent that antagonizes the activity of chemokine-like receptor 1 (CMKLR1) and/or a CMKLR1 ligand (e.g., chemerin or other endogenous CMKLR1 ligands).
In a certain embodiment, relapse of an autoimmune disease in a subject is inhibited or prevented by administering to the subject a prophylactically or therapeutically effective amount of an agent of the invention.

Determining a therapeutically or prophylactically effective amount of the CMKLR1 inhibitor compositions can be done based on animal data using routine computational methods.

In this invention, administering the instant compositions can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, intravenously, orally, via implant, transmucosally, transdermally, intramuscularly, intrathecally, and subcutaneously. The following delivery systems, which employ a number of routinely used pharmaceutical carriers, are only representative of the many embodiments envisioned for administering the instant compositions.

Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprylacontes and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylacontone. Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrilodone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Dermal delivery systems include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer.

Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as suspending agents (e.g., gums, xanthans, cellulosics and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol), surfactants (e.g., sodium
lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and Jun. 2,2005 antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating agents, and chelating agents (e.g., EDTA), captisol, etc.

Conditions for Analysis and Therapy

[108] The compositions and methods of the invention find use in combination with a variety of demyelinating autoimmune conditions, including chronic/progressive and relapsing demyelinating autoimmune diseases. Generally patients for the methods of the present invention are diagnosed as having an autoimmune condition, e.g. a relapsing-remitting autoimmune condition, prior to treatment. The inhibition of CMKLR1 decreases the severity or incidence of relapses in such patients.

[109] Multiple sclerosis (MS) is characterized by various symptoms and signs of CNS dysfunction, with remissions and recurring exacerbations. The most common presenting symptoms are paresthesias in one or more extremities, in the trunk, or on one side of the face; weakness or clumsiness of a leg or hand; or visual disturbances, e.g. partial blindness and pain in one eye (retrobulbar optic neuritis), dimness of vision, or scotomas. Other common early symptoms are ocular palsy resulting in double vision (diplopia), transient weakness of one or more extremities, slight stiffness or unusual fatigability of a limb, minor gait disturbances, difficulty with bladder control, vertigo, and mild emotional disturbances; all indicate scattered CNS involvement and often occur months or years before the disease is recognized. Excess heat may accentuate symptoms and signs.

[110] Clinical data alone may be sufficient for a diagnosis of MS. If an individual has suffered two separate episodes of neurologic symptoms characteristic of MS, and the individual also has consistent abnormalities on physical examination, a diagnosis of MS can be made with no further testing. Magnetic resonance imaging (MRI) of the brain and spine is often used during the diagnostic process. MRI shows areas of demyelination (lesions) as bright spots on the image. A substance, called Gadolinium, can be injected into the spinal column to highlight active plaques and, by elimination, demonstrate the existence of historical lesions not associated with clinical symptoms. This can provide the evidence of chronic disease needed for a definitive diagnosis of MS. Testing of cerebrospinal fluid (CSF) can provide evidence of chronic inflammation of the central nervous system. The CSF is tested for oligoclonal bands, which are immunoglobulins found in 85% to 95% of people with definite MS. Combined with MRI and clinical data, the presence of oligoclonal bands can help make a definite diagnosis of MS. Lumbar puncture is the procedure used to collect a sample of CSF.
The brain of a person with MS often responds less actively to stimulation of the optic nerve and sensory nerves. These brain responses can be examined using visual evoked potentials (VEPs) and somatosensory evoked potentials (SEPs). Decreased activity on either test can reveal demyelination which may be otherwise asymptomatic. Along with other data, these exams can help find the widespread nerve involvement required for a definite diagnosis of MS.

In 1996 the United States National Multiple Sclerosis Society standardized the following four subtype definitions (see Lublin and Reingold (1996) Neurology 46(4):907-11, herein specifically incorporated by reference) as relapsing-remitting; secondary progressive; primary progressive; progressive relapsing. The methods of the invention find particular use in the treatment of ongoing disease, and particularly in treating relapsing forms.

Relapsing-remitting describes the initial course of 85% to 90% of individuals with MS. This subtype is characterized by unpredictable attacks (relapses) followed by periods of months to years of relative quiet (remission) with no new signs of disease activity. Deficits suffered during the attacks may either resolve or may be permanent. When deficits always resolve between attacks, this is referred to as "benign" MS.

Secondary progressive describes around 80% of those with initial relapsing-remitting MS, who then begin to have neurologic decline between their acute attacks without any definite periods of remission. This decline may include new neurologic symptoms, worsening cognitive function, or other deficits. Secondary progressive is the most common type of MS and causes the greatest amount of disability.

Primary progressive describes the approximately 10% of individuals who never have remission after their initial MS symptoms. Decline occurs continuously without clear attacks. The primary progressive subtype tends to affect people who are older at disease onset.

Progressive relapsing describes those individuals who, from the onset of their MS, have a steady neurologic decline but also suffer superimposed attacks; and is the least common of all subtypes.

Peripheral neuropathies may also have a relapsing remitting course, and may include Miller Fisher syndrome; chronic inflammatory demyelinating polyneuropathy (CIDP) with its subtypes classical CIDP, CIDP with diabetes, CIDP/monoclonal gammopathy of undetermined significance (MGUS), sensory CIDP, multifocal motor neuropathy (MMN), multifocal acquired demyelinating sensory and motor neuropathy or Lewis-Sumner syndrome, multifocal acquired sensory and motor neuropathy, and distal acquired demyelinating sensory neuropathy; IgM monoclonal gammopathies with its subtypes Waldenstrom's macroglobulinemia, myelin-associated glycoprotein-associated gangliopathy, polyneuropathy, organomegaly,
endocrinopathy, M-protein, skin changes syndrome, mixed cryoglobulinemia, gait ataxia, late-onset polyneuropathy syndrome, and MGUS.

[118] An inhibitory agent may inhibit the activity of CMKLR1 by a variety of different mechanisms. In certain embodiments, the inhibitory agent is one that binds to the protein CMKLR1 and, in doing so, inhibits its activity. In other embodiments, the inhibitory agent prevents expression of CMKLR1.

[119] An inhibitory agent may act on CMKLR1 mRNA to inhibit the activity of the target CMKLR1 by reducing the amount of CMKLR1 RNA present in the targeted cells, where the target cell may be present in vitro or in vivo. By "reducing the amount of" is meant that the level or quantity of the target CMKLR1 in the target cell is reduced by at least about 2-fold, usually by at least about 5-fold, e.g., 10-fold, 15-fold, 20-fold, 50-fold, 100-fold or more, as compared to a control, i.e., an identical target cell not treated according to the subject methods.

Methods of Screening for CMKLR1 Antagonists

[120] Agents that can regulate demyelinating inflammatory disease in a subject can be identified by detecting the ability of an agent to antagonize the activity of CMKLR1. Antagonizing agents include, but are not limited to, agents that interfere with the interaction of CMKLR1 with its natural ligands, agents that reduce CMKLR1 expression (e.g., by reducing transcription or by inducing cell surface receptor desensitization, internalization and/or degradation), agents that reduce expression of endogenous ligands of CMKLR1, and agents that inhibit intracellular signals initiated by the binding of CMKLR1 with its ligands. In some embodiments the screening methods are performed on analog or variants of 2-(alpha-naphthoyl)ethyltrimethylammonium iodide.

[121] In certain embodiments, agents that can reduce demyelinating inflammatory disease in a subject can be identified by detecting the ability of an agent to interfere with (e.g., block) the interaction of CMKLR1 with its cognate ligand (e.g., chemerin). For example, a screening assay may be used that evaluates the ability of an agent to bind specifically to CMKLR1 (or its ligand) and prevent receptor:ligand interaction. Assays to determine affinity and specificity of binding are known in the art, including competitive and non-competitive assays. Assays of interest include ELISA, RIA, flow cytometry, etc. Binding assays may use purified or semi-purified protein, or alternatively may use primary cells or immortalized cell lines that express CMKLR1. In certain of these embodiments, the cells are transfected with an expression construct for CMKLR1. As an example of a binding assay, CMKLR1 is inserted into a membrane, e.g. whole cells, or membranes coating a substrate, e.g. microtiter plate, magnetic beads, etc. The
candidate agent and soluble, labeled ligand (e.g., chemerin) are added to the cells, and the unbound components are then washed off. The ability of the agent to compete with the labeled ligand for receptor binding is determined by quantitation of bound, labeled ligand. Confirmation that the blocking agent does not cross-react with other chemoattractant receptors may be performed with a similar assay.

CMKLR1 protein sequences are used in screening of candidate compounds (including antibodies, peptides, lipids, small organic molecules, etc.) for the ability to bind to and modulate CMKLR1 activity. Agents that inhibit or reduce CMKLR1 activity are of interest as therapeutic agents for decreasing demyelinating inflammatory disease in a subject whereas agents that activate CMKLR1 activity are of interest as therapeutic agents for increasing demyelinating inflammatory disease in a subject. Such compound screening may be performed using an *in vitro* model, a genetically altered cell or animal, or purified protein corresponding to chemerin-like chemoattractant polypeptides or a fragment(s) thereof. One can identify ligands or substrates that bind to and modulate the action of the encoded polypeptide.

Polypeptides useful in screening include those encoded by the CMKLR1 gene, as well as nucleic acids that, by virtue of the degeneracy of the genetic code, are not identical in sequence to the disclosed nucleic acids, and variants thereof.

CMKLR1 ligands (e.g., chemerin or resolvin) may be used in screening of candidate compounds for the ability to bind to and modulate the ligands ability to activate CMKLR1. Agents that inhibit or reduce the ability of a CMKLR1 ligand to activate CMKLR1 are of interest as therapeutic agents for decreasing demyelinating inflammatory disease in a subject whereas agents that increase the ability of a CMKLR1 ligand to activate CMKLR1 activity are of interest as therapeutic agents for increasing demyelinating inflammatory disease in a subject. Such compound screening may be performed using an *in vitro* model, a genetically altered cell or animal, or purified protein corresponding to chemerin-like chemoattractant polypeptides or a fragment(s) thereof. One can identify ligands or substrates that bind to and modulate the action of the encoded polypeptide.

Polypeptides useful in screening include those encoded by a CMKLR1 ligand gene (e.g., chemerin), as well as nucleic acids that, by virtue of the degeneracy of the genetic code, are not identical in sequence to the disclosed nucleic acids, and variants thereof.

Transgenic animals or cells derived therefrom are also used in compound screening. Transgenic animals may be made through homologous recombination, where the normal locus corresponding to chemerin-like chemoattractant is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome. Vectors for stable integration include
plasmids, retroviruses and other animal viruses, yeast artificial chromosomes (YACs), and the like. A series of small deletions and/or substitutions may be made in the coding sequence to determine the role of different exons in receptor binding, signal transduction, etc. Specific constructs of interest include antisense sequences that block expression of the targeted gene and expression of dominant negative mutations. A detectable marker, such as lac Z or GFP, may be introduced into the locus of interest, where up-regulation of expression will result in an easily detected change in phenotype. One may also provide for expression of the target gene or variants thereof in cells or tissues where it is not normally expressed or at abnormal times of development, for example by overexpressing in neural cells. By providing expression of the target protein in cells in which it is not normally produced, one can induce changes in cell behavior.

Compound screening identifies agents that modulate CMKLR1 activity or function. Of particular interest are screening assays for agents that have a low toxicity for normal human cells. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. Screening for the activity of G-protein coupled receptors (or GPCRs, of which CMKLR1 is a member) is well known in the art, and includes assays for measuring any of a number of detectible steps, including but not limited to: stimulation of GDP for GTP exchange on a G protein; alteration of adenylate cyclase activity; protein kinase C modulation; phosphatidylinositol breakdown (generating second messengers diacylglycerol, and inositol triphosphate); intracellular calcium flux; activation of MAP kinases; modulation of tyrosine kinases; modulation of gene or reporter gene activity, integrin activation, or chemotaxis inhibition. A detectable step in a signaling cascade is considered modulated if the measurable activity is altered by 10% or more above or below a baseline or control level. The baseline or control level can be the activity in the substantial absence of an activator (e.g., a ligand) or the activity in the presence of a known amount of an activator. The measurable activity can be measured directly, as in, for example, measurement of cAMP or diacylglycerol levels. Alternatively, the measurable activity can be measured indirectly, as in, for example, a reporter gene assay. Knowledge of the 3-dimensional structure of the encoded protein (e.g., CMKLR1 or a ligand, e.g. chemerin), derived from crystallization of purified recombinant protein, could lead to the rational design of small drugs that specifically inhibit activity. These drugs may be directed at specific domains and sites.

The term "agent" as used herein describes any molecule, e.g. protein or pharmaceutical, with the capability of modulating the physiological function of CMKLR1 or its ligand. Generally a
plurality of assay mixtures is run in parallel with different agent concentrations to obtain a
differential response to the various concentrations. Typically one of these concentrations serves
as a negative control, i.e. at zero concentration or below the level of detection.

[129] Candidate agents encompass numerous chemical classes, though typically they are
organic molecules, preferably small organic compounds having a molecular weight of more than
50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary
for structural interaction with proteins, particularly hydrogen bonding, and typically include at
least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional
chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic
structures and/or aromatic or polyaromatic structures substituted with one or more of the above
functional groups. Candidate agents are also found among biomolecules including peptides,
saccharides, fatty acids, lipids, steroids, purines, pyrimidines, derivatives, structural analogs or
combinations thereof.

[130] Candidate agents are obtained from a wide variety of sources including libraries of synthetic
or natural compounds. For example, numerous means are available for random and directed
synthesis of a wide variety of organic compounds and biomolecules, including expression of
randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in
the form of bacterial, fungal, plant and animal extracts are available or readily produced.
Additionally, natural or synthetically produced libraries and compounds are readily modified
through conventional chemical, physical and biochemical means, and may be used to produce
combinatorial libraries. Known pharmacological agents may be subjected to directed or random
chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce
structural analogs. Test agents can be obtained from libraries, such as natural product libraries
or combinatorial libraries, for example.

[131] Libraries of candidate compounds can also be prepared by rational design. (*See generally,*
12:597-604, 1998); each incorporated herein by reference in their entirety). For example,
libraries of phosphatase inhibitors can be prepared by syntheses of combinatorial chemical
libraries (*see generally DeWitt *et al.,* *Proc. Nat. Acad. Sci. USA* 90:6909-13, 1993; *International
116:373-74, 1994; *Ohlmeyer *et al.,* *Proc. Nat. Acad. Sci. USA* 90:10922-26, all of which are
incorporated by reference herein in their entirety.)
A "combinatorial library" is a collection of compounds in which the compounds comprising the collection are composed of one or more types of subunits. Methods of making combinatorial libraries are known in the art, and include the following: U.S. Patent Nos. 5,958,792; 5,807,683; 6,004,617; 6,077,954; which are incorporated by reference herein. The subunits can be selected from natural or unnatural moieties. The compounds of the combinatorial library differ in one or more ways with respect to the number, order, type or types of modifications made to one or more of the subunits comprising the compounds. Alternatively, a combinatorial library may refer to a collection of "core molecules" which vary as to the number, type or position of R groups they contain and/or the identity of molecules composing the core molecule. The collection of compounds is generated in a systematic way. Any method of systematically generating a collection of compounds differing from each other in one or more of the ways set forth above is a combinatorial library.

A combinatorial library can be synthesized on a solid support from one or more solid phase-bound resin starting materials. The library can contain five (5) or more, preferably ten (10) or more, organic molecules that are different from each other. Each of the different molecules is present in a detectable amount. The actual amounts of each different molecule needed so that its presence can be determined can vary due to the actual procedures used and can change as the technologies for isolation, detection and analysis advance. When the molecules are present in substantially equal molar amounts, an amount of 100 picomoles or more can be detected. Preferred libraries comprise substantially equal molar amounts of each desired reaction product and do not include relatively large or small amounts of any given molecules so that the presence of such molecules dominates or is completely suppressed in any assay.

Combinatorial libraries are generally prepared by derivatizing a starting compound onto a solid-phase support (such as a bead). In general, the solid support has a commercially available resin attached, such as a Rink or Merrifield Resin. After attachment of the starting compound, substituents are attached to the starting compound. Substituents are added to the starting compound, and can be varied by providing a mixture of reactants comprising the substituents. Examples of suitable substituents include, but are not limited to, hydrocarbon substituents, e.g. aliphatic, alicyclic substituents, aromatic, aliphatic and alicyclic-substituted aromatic nuclei, and the like, as well as cyclic substituents; substituted hydrocarbon substituents, that is, those substituents containing nonhydrocarbon radicals which do not alter the predominantly hydrocarbon substituent (e.g., halo (especially chloro and fluoro), alkoxy, mercapto, alkylmercapto, nitro, nitroso, sulfoxy, and the like); and hetero substituents, that is, substituents which, while having predominantly hydrocarbyl character, contain other than
carbon atoms. Suitable heteroatoms include, for example, sulfur, oxygen, nitrogen, and such substituents as pyridyl, furanyl, thiophenyl, imidazolyl, and the like. Heteroatoms, and typically no more than one, can be present for each carbon atom in the hydrocarbon-based substituents. Alternatively, there can be no such radicals or heteroatoms in the hydrocarbon-based substituent and, therefore, the substituent can be purely hydrocarbon.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 3 hours will be sufficient.

Preliminary screens can be conducted by screening for compounds capable of binding to CMKLR1 or its ligand; compounds so identified are possible modulators. Compounds capable of binding to CMKLR1 are inhibitors if they do not activate the receptor and activators if they do. The binding assays usually involve contacting CMKLR1 or its ligand with one or more test compounds and allowing sufficient time for the protein and test compounds to form a binding complex. Any binding complexes formed can be detected using any of a number of established analytical techniques. Protein binding assays include, but are not limited to, methods that measure co-precipitation, co-migration on non-denaturing SDS-polyacrylamide gels, and co-migration on Western blots (see, e.g., Bennet, J.P. and Yamamura, H.I. (1985) “Neurotransmitter, Hormone or Drug Receptor Binding Methods,” in Neurotransmitter Receptor Binding (Yamamura, H. I., et al., eds.), pp. 61-89.

Certain screening methods involve screening for a compound that modulates the expression of CMKLR1 or its ligand. Such methods generally involve conducting cell-based
assays in which test compounds are contacted with one or more cells endogenously expressing CMKLR1 or its ligand and then detecting a modulation in expression (e.g., at the mRNA and/or protein level). In certain screening methods, a target cell has a reporter gene (e.g., GFP) under the control of the CMKLR1 promoter (or promoter of its ligand). The level of expression can be compared to a baseline value. The baseline value can be a value for a control sample or a statistical value that is representative of expression levels for a control population. Expression levels can also be determined for cells that do not express the CMKLR1 or its ligand, as a negative control. Such cells generally are otherwise substantially genetically the same as the test cells. Various controls can be conducted to ensure that an observed activity is authentic including running parallel reactions with cells that lack the reporter construct or by not contacting a cell harboring the reporter construct with test compound.

Certain screening methods involve screening for a compound that modulates gene expression normally regulated by CMKLR1 signaling. In certain embodiments, a cell-based assay is conducted in which a cell expressing CMKLR1 is contacted to a candidate agent (e.g., a CMKLR1 binding agent) and monitored for changes in gene expression that are similar, or substantially similar, to those induced by a natural ligand for CMKLR1. In certain other embodiments, a cell-based assay is conducted in which a cell expressing CMKLR1 is contacted to its natural ligand and a candidate agent and monitored for perturbations in gene expression. By “perturbations in gene expression”, it is meant that the gene expression changes induced by a CMKLR1 ligand binding to CMKLR1 is altered when the candidate agent is present.

Certain screening methods involve screening for a compound that modulates CMKLR1 signaling events when contacted to a cell expressing CMKLR1. These assays can be carried out in the presence or absence of a natural ligand for CMKLR1. Such methods generally involve monitoring for modulation of downstream signaling events as described above, e.g., protein phosphorylation, GDP/GTP exchange, etc. Including the b-ARR assay.

Compounds can also be further validated as described below.

Compounds that are initially identified by any of the foregoing screening methods can be further tested to validate their apparent activity. The basic format of such methods involves administering a lead compound identified during an initial screen to an animal that serves as a model for humans. The animal models utilized in validation studies generally are mammals. Specific examples of suitable animals include, but are not limited to, primates, mice, and rats.

Active test agents identified by the screening methods described herein that modulate CMKLR1 activity can serve as lead compounds for the synthesis of analog compounds. Typically, the analog compounds are synthesized to have an electronic configuration and a
molecular conformation similar to that of the lead compound. Identification of analog compounds can be performed through use of techniques such as self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available. See, e.g., Rein et al., (1989) Computer-Assisted Modeling of Receptor-Ligand Interactions (Alan Liss, New York).

[144] A functional assay that detects leukocyte chemotaxis may be used for confirmation. For example, a population of cells that demonstrate chemerin chemotaxis (e.g., dendritic cells or monocyte/macrophages) may be stimulated with chemerin and/or the candidate modulating agent. An agent that antagonizes CMKLR1 activity will cause a decrease in the locomotion of the cells in response to chemerin. An agent that potentiates CMKLR1 activity will act as a chemotaxis factor in the absence of chemerin and/or increase the chemotactic response induced by chemerin. Chemotaxis assays of that find use in these methods are known in the art, examples of which are described in US Patent Application serial number 10/958,527, entitled "Family of Cystatin-Related Chemoattractant Proteins" (incorporated herein by reference in its entirety). An agent that is a chemoattractant inhibitor will decrease the concentration of cells at a target site of higher concentration of chemerin.

EXPERIMENTAL

Example 1

[145] A small molecule CMKLR1 antagonist is identified as 2-(alpha-naphthoyl)ethyltrimethyl ammonium iodide (aNETA), shown below:

![Chemical structure of aNETA](image)

and has the following chemical formula: C_{16}H_{20}O (MW=242 Da, free base). aNETA inhibits chemerin mediated beta-arrestin2 association with CMKLR1 in an in vitro cell-based bioassay with an IC_{50} of about 350 nM. In vivo, daily s.c. dosing with 10 mg of aNETA/kg mouse body weight for 8 days (followed by every other day dosing for the duration of the experiment) significantly delayed the onset of experimental autoimmune encephalomyelitis (EAE).
Example 2

Primary Screen: Inhibition of Chemerin-Triggered huCMKLR1 Association with beta-Arrestin2

With reference to Figure 1, early event in receptor internalization is ligand-triggered association with beta-arrestin2. This process turns off receptor signaling. Enzyme complementation can be used to measure CMKLR1 association with beta-arrestin2. In this assay β-Galactosidase is split into two complementary enzyme fragments, one is attached to CMKLR1, the other to beta-arrestin2. If ligand interaction with CMKLR1 promotes its association with β-arrestin2, the enzyme fragments join and become active.

An assay, such as PathHunter® CHO-K1 CMKLR1 β-Arrestin GPCR Assays (DiscoverX, Inc., Fremont, CA) can provide a method for monitoring activation of the CMKLR1 GPCR receptor through arrestin recruitment in live cells. CMKLR1 cells are engineered to co-express a ProLink (PK)-tagged GPCR and the Enzyme Acceptor (EA)-tagged β-Arrestin fusion proteins. Upon GPCR activation, the two enzyme fragments are brought together, forming an active β-Gal enzyme through Enzyme Fragment Complementation (EFC).

Example 3

Inhibition of Chemerin-Triggered huCMKLR1 Association with βARR by aNETA

A LOPAC (Library of Pharmacologically Active Compounds) library (Sigma-Aldrich, St. Louis, MO) was screened for inhibition of chemerin-triggered huCMKLR1 association with βARR. Three hits were obtained from a primary screen of LOPAC library. One hit was reproducible: 2-(alpha-naphthoyl)ethyltrimethyl ammonium iodide (aNETA). huCMKLR1/ βARR CHO cells and 7 nM chemerin were used. The assay was performed in both buffer (10% FBS) or 100% human serum. The results from buffer and human serum for aNETA were of similar IC50s, as shown in Figure 2. The IC50 result using buffer was 350 nM. The IC50 result using human serum was 620 nM.

Example 4

Inhibition of Chemerin-Triggered Human CMKLR1/L1.2 Cell Migration

2-(alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of chemerin-triggered human CMKLR1/L1.2 cell migration. huCMKLR1/L1.2 ctx and 1 nM chemerin were used. The assay was performed in 0.5% BSA, buffer (10% FBS), or 100% human serum. The results from 0.5% BSA, buffer (10% FBS), and 100% human serum were of similar IC50s, as shown in Figure 3. The IC50 result using 0.5% BSA was 1.2 μM. The IC50 result using buffer was 1.2 μM. The IC50 result using human serum was 1.3 μM.
Example 5
Inhibition of Chemerin-Triggered Mouse CMKLR1/L1.2 Cell Migration

2-(alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of chemerin-triggered mouse CMKLR1/L1.2 cell migration. mCMKLR1/L1.2 ctx and 1 nM chemerin were used. The assay was performed in 0.5% BSA, buffer (10% FBS), or 100% human serum. The results show that aNETA seems to cross to the mouse receptor. As shown in Figure 4, the IC50 result using buffer was 1.1 \( \mu \text{M} \). The IC50 result using human serum was 1.2 \( \mu \text{M} \). The IC50 result using 0.5% BSA was 9.6 \( \mu \text{M} \).

Example 6
Specificity: aNETA Does Not Inhibit CXCR4-dependent MOLT-4 Migration to CXCL12

MOLT4 cell ctx (human T-cell line) and 1 nM CXCL12 were used. As shown in Figure 5, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of CXCR4-dependent MOLT-4 migration to CXCL12. aNETA does not inhibit CXCR4-dependent MOLT-4 migration to CXCL12.

Example 7
Specificity: aNETA Does Not Inhibit CCR9-dependent MOLT-4 Migration to CCL25

MOLT4 cell ctx (human T-cell line) and 250 nM TECK were used. As shown in Figure 6, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of CCR9-dependent MOLT-4 migration to CCL25. aNETA does not inhibit CCR9-dependent MOLT-4 migration to CCL25.

Example 8
Specificity: aNETA Does Not Inhibit CXCR4-dependent Mouse Splenocyte Migration to CXCL12

Splenocyte ctx and 100 nM CXCL12 were used. As shown in Figure 7, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of CXCR4-dependent mouse splenocyte migration to CXCL12. aNETA does not inhibit CXCR4-dependent mouse splenocyte migration to CXCL12.

Example 9
Specificity: aNETA Does Not Inhibit CCR7-dependent Mouse Splenocyte Migration to CCL19
Splenocyte ctx and 100 nM CCL19 were used. As shown in Figure 8, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of CCR7-dependent mouse splenocyte migration to CCL19. aNETA does not inhibit CCR7-dependent mouse splenocyte migration to CCL19.

Example 10
Specificity: aNETA Does Not Inhibit FPR-dependent Mouse BM Neutrophil Chemotaxis to fMLP

Neutrophil ctx and 100 nM fMLP were used. As shown in Figure 9, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of FPR-dependent mouse BM neutrophil chemotaxis to fMLP. aNETA does not inhibit FPR-dependent mouse BM neutrophil chemotaxis to fMLP.

Example 11
Specificity: aNETA Does Not Inhibit C5aR-dependent Mouse BM Neutrophil Chemotaxis to C5a

Neutrophil ctx and 100 nM C5a were used. As shown in Figure 10, (alpha-Naphthoyl)ethyltrimethyl ammonium iodide (aNETA) was tested for inhibition of C5aR-dependent mouse BM neutrophil chemotaxis to C5a. aNETA does not inhibit C5aR-dependent mouse BM neutrophil chemotaxis to C5a.

Example 12
aNETA Not Cytotoxic in vitro

The in vitro cytotoxicity of aNETA can be determined with an annexin V FITC assay kit. One of the early hallmarks of early stage of apoptosis is that membrane phospholipids such as phosphatidylserine and phosphatidylethanolamine redistribute from the inner to outer leaflet of the membrane bilayer where they are exposed on the cell surface. Externalization of phosphatidylserine residues to the outer plasma membrane leaflet allows their detection via their high affinity for annexin V, a phospholipid binding protein. Apoptotic cells bound with fluorochrome-labeled annexin V can be visualized using fluorescence microscopy, flow cytometry, or a plate reader capable of fluorescence measurements. The analysis can be performed with a FITC-conjugated annexin-V, which has a strong affinity for extracellular phosphatidylserines, and the fluorescent intercalating agent propidium iodide (PI).

With reference to Figure 11, cell lines (huCMKLR1/βARR CHO and huCMKLR1/L1.2) and splenocytes were used. aNETA was tested with 90 minute incubation at 37°C. Over the duration of the in vitro functional assays, aNETA does not appear to be acutely toxic.
Example 14

aNETA Suppresses Clinical EAE in vivo

EAE was previously examined in CMKLR1 null mice immunized with MOG 35-55 in CFA (see, for example, U.S. Patent no. 8,038,992, herein specifically incorporated by reference). These mice are resistant to the development of EAE relative to wild-type mice. EAE is driven by pathogenic immune responses against myelin proteins and lipids. CMKLR1 may have an inflammatory role. We next asked whether treatment with agents that inhibit CMKLR1, including aNETA, prevent and/or improve clinical EAE. To test this, WT mice with EAE are treated with aNETA.

Methods

**EAE induction.** EAE is induced in 8-12 week old female null and WT animals via subcutaneous immunization with 100 µg myelin oligodendrocyte glycoprotein (MOG) peptide, amino acids 35-55 (MOG 35-55) in an emulsion mixed (volume ratio 1:1) with Complete Freund's Adjuvant (containing 4 mg/ml of heat-killed Mycobacterium tuberculosis H37Ra). Mice are also injected intravenously with 250-400 ng of *Bordetella* pertussis toxin (BPT) in PBS at the time of, and two days following immunization. MOG 35-55 peptide is synthesized by the Stanford Protein and Nucleic Acid Facility and purified by high performance liquid chromatography (HPLC). Mice (n=8-10 per group) were examined daily for clinical signs of EAE and were scored as followed: 0=no clinical disease, 1=limp tail, 2=hindlimb weakness, 3=complete hindlimb paralysis, 4=hindlimb paralysis plus some forelimb paralysis, and 5=moribund or dead.

**Histopathology.** Brains and spinal cords are dissected from mice, fixed in 10% formalin in PBS and embedded in paraffin. Seven micron thick sections are stained with haematoxylin and eosin to detect inflammatory infiltrates and luxol fast blue for demyelination. Inflammatory lesions in brain, thoracic and lumbar spinal cord sections are counted by an examiner masked to the treatment status of the animal.

**Drug Dosing.** WT mice are induced with EAE using MOG 35-55 and pertussis toxin. aNETA is administered on the day of EAE induction in a 10% captisol formulation at 1, 3, or 10 mg/kg (subcutaneous injection) and every day thereafter for 8 days, and then every other day until the conclusion of the study.

Results

**aNETA suppresses clinical EAE in vivo.** With reference to Figure 12, daily administration of 1 mg/kg of aNETA resulted in onset of EAE of 17.8 days post-immunization. Daily
administration of 10 mg/kg of aNETA resulted in onset of EAE of 26 days post-immunization. In comparison, untreated mice resulted in onset of EAE of 12.7 days post-immunization. Administration of vehicle resulted in onset of EAE of 13.3 days post-immunization.

With reference to Figure 13, with daily administration of 3 mg/kg of aNETA, 19% (3/16) of the animals developed EAE. With daily administration of 10 mg/kg of aNETA, 7% (1/16) developed EAE. In comparison, with administration of the vehicle, 94% (15/16) developed EAE.

Example 15
aNETA Suppresses Leukocyte Infiltration of CNS in EAE

With reference to Figure 14, aNETA suppresses leukocyte infiltration of CNS in EAE. Daily administration aNETA at 10 mg/kg and 3 mg/mg were compared to administration of vehicle. In the spleen, administration aNETA at 10 mg/kg and 3 mg/mg resulted in more leukocytes present. However, with regard to leukocyte infiltration of the CNS, administration aNETA at 10 mg/kg and 3 mg/mg resulted in fewer leukocytes present.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.
WHAT IS CLAIMED IS:

1. A method of decreasing demyelinating inflammatory disease in a subject, the method comprising:

   administering to said subject an effective amount of a compound of formula (I):

   ![Chemical Structure](image)

   where

   - \( Q \) is selected from \(-\text{NR}_4^+, -\text{NH}_4^+, -\text{NH}_2, -\text{NHR}_2, -\text{OH}, -\text{SH}, \) and lower alkyl; wherein \( R^0 \) is lower alkyl; and wherein \( Q \) is selected from \(-\text{NR}_4^+, -\text{NH}_4^+, \) then \( X^- \) is present and is a counterion;

   - \( R^1 \) and \( R^2 \) are independently selected from hydrogen, alkyl, substituted alkyl, hydroxy, alkoxy, substituted alkoxy, amino, substituted amino, carboxyl, carboxyl ester, cyano, halogen, acyl, aminoacyl, nitro, and sulfonyl; and

   - \( n \) is a number from one to four.

2. The method of Claim 1, wherein \( Q \) is \(-\text{NR}_4^+, \) wherein \( R^0 \) is lower alkyl; and wherein \( X^- \) is present and is a counterion.

3. The method of Claim 2, wherein \( X^- \) is selected from fluoride, bromide, chloride, and fluoride.

4. The method of Claim 1, wherein \( R^1 \) and \( R^2 \) are hydrogen.

5. The method of Claim 1, wherein \( n \) is two.

6. A method of decreasing demyelinating inflammatory disease in a subject, the method comprising:

   administering to said subject an effective amount of a compound of formula (II):

   ![Chemical Structure](image)

   (II), wherein
Q is selected from –NR$_4^+$, –NH$_4^+$, –NH$_2$, –NHR$^Q$, –NR$_2^Q$, –OH, –SH, and lower alkyl; wherein R$^Q$ is lower alkyl; and wherein Q is selected from –NR$_4^+$ or –NH$_4^+$, then X$^-$ is present and is a counterion; and
n is a number from one to four.

7. A method of decreasing demyelinating inflammatory disease in a subject, the method comprising:
   administering to said subject an effective amount of a compound of formula (III):
   \[
   \text{Q} \quad \text{(III), wherein}
   \]
   Q is selected from –NR$_4^+$, –NH$_4^+$, –NH$_2$, –NHR$^Q$, –NR$_2^Q$, –OH, –SH, and lower alkyl; wherein R$^Q$ is lower alkyl; and wherein Q is selected from –NR$_4^+$ or –NH$_4^+$, then X$^-$ is present and is a counterion.

8. The method of Claim 1, wherein the compound is
   \[
   \text{X}^- \quad \text{, wherein}
   \]
   X$^-$ is selected from iodide, bromide, chloride, and fluoride.

9. The method of Claim 1, wherein the compound is
ABSTRACT OF THE INVENTION

Compounds and methods are provided for decreasing demyelinating inflammatory disease in a subject by inhibiting the activity of chemokine-like receptor 1 (CMKLR1).
Figure 1
Figure 2

huCMKLR1/β-ARR CHO cells
7nM Chemerin

IC₅₀

Log [α-NEA, M]
Figure 3

The graph shows the effect of different conditions on the migration of cells. The x-axis represents the log of [α-NET, M], and the y-axis represents the percent input migration. The graph includes the following conditions:

- (-) no chem
- DMSO
- CTX Buffer
- Serum
- 0.5% BSA

The IC₅₀ values for these conditions are:

- (-) no chem: 1.2 uM
- DMSO: 1.3 uM
- CTX Buffer: 1.2 uM
- Serum: 1.4 uM
- 0.5% BSA: 1.2 uM
Figure 5
Figure 6
Figure 7
Figure 8

Splenocyte ctx
100 nM CCL19

% input migration

Log [α-NETA, M]

(-) no CCL19
DMSO
Figure 9
Figure 10

Neutrophil ctx
100 nM C5a

% input migration

Log [α-NETA, M]

(-) no C5a

DMSO
Over the duration of our in vitro functional assays, α-NETA does not appear to be acutely cytotoxic.

Figure 11
Data presented as mean ± s.e.m.

Figure 12

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of onset (sem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.7 (1.3)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>13.3 (1.4)</td>
</tr>
<tr>
<td>1 mg/kg α-NETA</td>
<td>17.8 (3.4)</td>
</tr>
<tr>
<td>10 mg/kg α-NETA</td>
<td>26 (1.7)**</td>
</tr>
</tbody>
</table>
Disease incidence (by d16 pi)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>15/16 (94%)</td>
</tr>
<tr>
<td>3 mg/kg α-N ETA</td>
<td>3/16 (19%)*</td>
</tr>
<tr>
<td>10 mg/kg α-N ETA</td>
<td>1/14 (7%) *</td>
</tr>
</tbody>
</table>

*P < 0.0001 (Compared to Vehicle)
Figure 14

Spleen

CNS

* P<0.05

Red = Clinical EAE