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Masato Tamari  
*Mount Sinai School of Medicine*

Lydia Zamidar  
*Mount Sinai School of Medicine*

Aaron M Ver Heul  
*Washington University School of Medicine in St. Louis*

Kristine Nograles  
*Cara Therapeutics*

Joana Goncalves  
*Cara Therapeutics*

See next page for additional authors

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Difelikefalin suppresses itch and reduces scratching independent of inflammation in a murine model of atopic dermatitis

Masato Tamari, MD, PhD,b,* Lydia Zamidar, BS,b,* Aaron M. Ver Heul, MD, PhD,b Kristine Nogales, MD, MSc,c Joana Goncalves, MD, c Emma Guttman-Yassky, MD, PhD,c Mark Lebwohl, MD,a and Brian S. Kim, MD, MTRa New York, NY; St Louis, Mo; and Stamford, Conn

Background: Therapies specifically targeting nonhistaminergic pruritus are largely lacking. Difelikefalin (DFK) has been found to reduce itch in various chronic pruritic conditions, including atopic dermatitis (AD).

Objective: We sought to investigate the ability of DFK to impact scratching behavior, inflammatory mediators, and neuronal signaling in a murine model of AD.

Methods: The ears of C57BL/6 mice were topically treated with MC903 for 12 consecutive days to induce AD-like inflammation and itch. Before MC903 treatment, mice were treated with either DFK (0.5 mg/kg, intraperitoneal injection twice daily) or vehicle (saline). Skin ear thickness, histological analysis, flow cytometry, RNA-sequencing, and differential gene expression analyses of mouse ear skin were used to examine the effect of DFK on skin inflammation. Scratching behavior was quantified to measure itch behavior in mice that were topically treated with MC903 for 6 consecutive days; then, mice received a single injection of either DFK (1.0 mg/kg, intraperitoneal injection) or saline. Calcium imaging and single-cell RNA-sequencing were used in mouse dorsal root ganglia neurons to determine the size of the neurons activated with DFK treatment. Statistical significance was determined by Mann-Whitney test, unless otherwise noted.

Results: DFK rapidly suppressed itch without altering AD-like skin inflammation in MC903 (calcipotriol)-treated mice. In vitro Ca2+ influx trace of dorsal root ganglia suggested that a major target for DFK is the larger-diameter mechanoreceptors (eg, Aβ-fibers), rather than small-diameter pruriceptive C-fibers. These studies support a potential neuromodulatory role of DFK for reducing itch associated with AD in mice. (J Allergy Clin Immunol 2023;152:927-32.)

Key words: atopic dermatitis, difelikefalin, itch, kappa-opioid receptor agonist, pruritus

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by barrier defects, allergic inflammation, rash, and both acute and chronic pruritus. It is increasingly appreciated that immune cell–derived AD-associated cytokines, such as IL-4, IL-13, and IL-31, can promote itch by acting directly on itch-sensory neurons (pruriceptors). The therapeutic efficacy of agents that inhibit or block these cytokines has been firmly established in recent phase 2 and 3 clinical trials in moderate to severe AD. However, the degree to which their antipruritic efficacy derives from effects on immune cells versus sensory neurons remains debated. Furthermore, whether primary neuromodulation, exclusive of neuroimmune interactions, is a viable therapeutic approach in AD remains unexplored.

Difelikefalin (DFK) is a selective κ-opioid receptor (KOR) agonist. Intravenous DFK was first approved by the US Food and Drug Administration in 2021 for the treatment of moderate to severe pruritus in adults undergoing hemodialysis. In this issue, we have published data from a phase 2 clinical trial evaluating the efficacy and safety of oral DFK for moderate to severe pruritus in subjects with AD. In the current preclinical study, we sought to determine the antipruritic mechanism of action of DFK on molecular, cellular, and behavioral changes in a translational mouse model of AD. We found that DFK had rapid and potent antipruritic efficacy independent of effects on skin inflammation. Our findings suggest that DFK may be effective in treating mildly inflammatory, itch-predominant AD in patients through its selective targeting of neurons.

METHODS

Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me; catalog 000664), aged 8 to 12 weeks, were used for all experiments. Mice were housed in a specific pathogen-free, environmentally controlled animal facility with a 12-hour light-dark cycle. Animals were given unrestricted access to food and water. All animal protocols and experiments were preapproved by the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai and at Washington University School of Medicine and conformed to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

AD-like chronic skin inflammation model in mice

Mice ears were treated topically with MC903 (also referred to as calcipotriol; Tocris Bioscience, Bristol, UK) based on a
modified version of a protocol previously described. For the 12-day protocol, mouse ears were treated bilaterally (ventral side only) with topical application of 1 nmol of MC903 diluted in 10 μL of ethanol once daily for 12 consecutive days. For the modified 6-day protocol, mouse ears were treated with 2 nmol of MC903 diluted in 10 μL of ethanol once daily for 6 consecutive days. Ear thickness was measured daily using a dial thickness gauge (PEACOCK OZAKI MFG. Co., LTD, Tokyo, Japan). Percentage change in ear thickness was calculated by comparing the average bilateral ear thickness to the baseline (day 0) measurements. Mice were humanely euthanized the day after the final application of MC903 for harvesting and analysis of ear tissue. One ear was used for flow cytometric analysis and the other was preserved in 4% paraformaldehyde for histologic analysis or preserved in RNAlater for RNA-sequencing. Histology slides and digital images were created by HistoWiz, Inc (Brooklyn, NY). Flow cytometry was performed on the basis of a modified protocol previously described (see this article’s Supplementary Methods section and Table E1 in the Online Repository at www.jacionline.org).

Intraperitoneal injection of DFK

In the 12-day AD-like skin inflammation model, mice were injected with 100 μL of either 0.5 mg/kg DFK dissolved in saline or vehicle (saline) intraperitoneally twice daily beginning 2 days before the start of MC903 treatment. At specified times, itch was measured by recording and scoring scratching behavior. On recording days, injections were given 30 minutes before recording.

The 6-day AD-like skin inflammation model was used similarly to the 12-day model, except that mice were injected with 1.0 mg/kg DFK or vehicle once before recording on day 6.

Itch behavior recording

Mice were placed in a clean test chamber for observation. Each mouse had its own chamber for the duration of the experiment. Two days before the initial recording, mice were habituated in the test chamber for at least 90 minutes per day. On behavior recording days, mice were allowed to acclimate to the test chamber for 15 minutes before video recording. Mice were recorded every 4 days. Itch behavior was manually scored by counting the number of scratching bouts per 30-minute period and per 15-minute period. Scorers were blinded to treatment groups.

Bulk RNA-sequencing and differential gene expression analysis

Bulk RNA-sequencing alignment is described in this article’s Supplementary Methods section in the Online Repository at www.jacionline.org. Differential expression analysis was performed using the gene level read counts and the DESeq2 (v1.34.0) R package. Genes with fewer than 5 reads in total across all samples were filtered as inactive genes. A gene is considered differentially expressed if the adjusted P value is less than .05 and the absolute log2 fold change is greater than 1. The differential expression likelihood ratio test was performed using the DESeq2 and DEGReport R package (v1.30.3) (http://lpantano.github.io/DEGreport/). The Fisher test was used to determine statistical significance in overlap of differentially expressed genes (P < .05).

Calcium imaging

Calcium imaging experiments were performed as previously described. Additional details for the calcium imaging experiments are described in this article’s Supplementary Methods section in the Online Repository at www.jacionline.org. Neuronal response was defined as a more than 10% change in fluorescence intensity ratio from baseline. Cell diameter was calculated as the diameter of the cross-sectional area of an imaged neuron. Neurons were classified as small- (<18 μm), medium- (18-25 μm), or large-diameter (>25 μm).

Statistical analysis

Data are presented as the mean with individual plots, unless otherwise indicated. Data from independent experiments are representative of at least 2 independent replicates, or pooled data if possible. No data were excluded from statistical analyses unless subjected to technical errors. Statistical significance was determined by Mann-Whitney test unless otherwise noted. Statistical evaluations were performed using GraphPad Prism 8.0 software (GraphPad Software, Boston, Mass).

RESULTS

DFK rapidly reduces itch, but not AD-like inflammation, in mice

The effect of DFK on AD-associated itch and inflammation was studied using the well-established MC903 murine model (Fig 1, A). MC903-treated mice were injected with DFK 0.5 mg/kg twice daily for 14 days, and we found no effect on skin inflammation based on clinical appearance (Fig 1, B), histopathologic analysis (Fig 1, C), ear thickness (Fig 1, D; see Table E2 in the Online Repository at www.jacionline.org), and number of total CD4+ immune cells (Fig E1, A and Table E3 in the Online Repository at www.jacionline.org), and number of total CD45+ immune cells (see Fig E1, A, and Table E3 in the Online Repository at www.jacionline.org), CD4+ T cells (Fig E1, B, and Table E3), and group 2 innate lymphoid cells (Fig E1, C, and Table E3). The level of CD4+ T cell and group 2 innate lymphoid cell activation, as determined by inducible costimulatory expression, was also unaffected by DFK treatment (Fig E1, D and E, and Table E3). There were only slight transcriptional changes in the ear skin via bulk RNA-sequencing analysis, indicating that DFK is unlikely to promote meaningful transcriptional alterations (Fig E1, F). Strikingly, despite the lack of any reduction in inflammation (Fig 1, B-E; see Fig E1, A-F, and Tables E2 and E3), scratching behavior was significantly suppressed in DFK-treated mice compared with vehicle-treated control mice (Fig 1, E, and Table E2). Furthermore, itch suppression was observed at day 0, demonstrating that DFK is capable of reducing baseline itch behavior before the induction of AD-like disease (see Fig 1, E, and Table E2). These findings suggest that DFK primarily targets itch pathways independently of the onset of or effect of cutaneous inflammation.

To evaluate the anti-itch effect of DFK more directly, we induced AD-like inflammation with MC903 (Fig 2, A) and subjected mice to a single injection of DFK on the establishment of skin disease. DFK significantly reduced scratching bouts 30 minutes after injection in mice, which was not observed in vehicle-treated mice (Fig 2, B; see Table E4 in the Online Repository at www.jacionline.org). The rapid effect strongly suggested that DFK acts in a neuromodulatory fashion, rather than as an anti-inflammatory agent, which would typically require
continuous dosing and more time to see an effect. Thus, we hypothesized that DFK acts directly on the sensory neurons.

DFK activates medium-to-large diameter sensory neurons

Sensory neurons may be functionally classified either by size or by the genes and proteins they express. Sensations evoked by mechanical stimuli, such as pressure and touch, are primarily mediated by large-diameter, highly myelinated A-fibers (Aα, Aβ, and Aδ neurons; Fig 3, A). In contrast, C-fibers are smaller in diameter, unmyelinated, and mediate sensations related to temperature, pain, and itch. Classification by gene expression, a technique made possible by the advent of single-cell RNA-sequencing data set, has increased our understanding of functional specificity. For example, examination of itch-related gene expression (ie, Nppb, Sst, Cysltr2, Hrh1, Il4ra, Il13ra, and Il31ra) allows for subclassification of itch-sensory neurons (pruriceptors) into NP1, NP2, and NP3 C-fiber groups. Gene expression data sets also allow for the subclassification of various touch neurons, such as NF1-5 A-fiber low-threshold mechanoreceptors. Although it has been reported that KORs, the target receptors for DFK, are expressed on C-fibers, our investigation of a previously published single-cell RNA-sequencing data set identified KOR only in NF2 and NF3 A-fiber low-threshold mechanoreceptors, which are Aβ-fibers (Fig 3, B). In contrast, the μ-opioid receptor was found to be highly enriched within the pruriceptor populations NP1-3 (Fig 3, B). These findings suggest that the primary targets of DFK are mechanoreceptors rather than pruriceptors.

To test which sensory neurons are activated by DFK, we measured the size of the DFK-responsive neurons in the dorsal root ganglia using calcium imaging. We found that the median diameter of DFK-responsive neurons was significantly larger than that of the neuronal subsets that responded to classic pruritogens (ie, histamine and chloroquine) (Fig 3, C-E; see Fig E2 and Table E5 in the Online Repository at www.jacionline.org), indicating that the activity of DFK is skewed toward larger Aβ-fiber neurons rather than smaller C-fibers. Furthermore, when examining rare, individual neurons that responded to both DFK and the

FIG 1. DFK significantly decreases scratching behavior without altering disease-like inflammation in murine AD model. A-E, Combined data from 2 experiments. Each experiment had n = 10 per each treatment group. A, Experimental schematic showing daily treatment of MC903 model, and BID i.p. injections of DFK or vehicle. Figure adapted from image created with BioRender.com. B, Day-12 ears from MC903-treated mice given DFK or vehicle. C, Representative hematoxylin and eosin staining of histopathology from day-12 ear skin taken from MC903-treated mice given DFK or vehicle. D and E, Data were analyzed using Mann-Whitney test. D, Daily percent change in ear thickness compared with baseline (day 0). Line is representative of each treatment group’s average. E, Scratch counting or number of scratch bouts per 30 minutes from DFK and vehicle groups. Line is representative of the treatment group average per day. Scratch counting data were recorded every 4 days from day 0. BID, Twice daily; i.p., intraperitoneal.
DFK reduces scratching in murine AD model. A and B, Combined data from 3 experiments. Experimental schematic showing daily treatment of MC903 model with a single i.p. DFK or vehicle injection halfway through scratching behavior recording on day 7. A, Figure adapted from image created with BioRender.com. B, Data were analyzed using Mann-Whitney test between the different group (vehicle vs DFK) or Wilcoxon matched-pairs test in the same group (vehicle or DFK). For the vehicle group, n = 9. For the DFK group, n = 15. Scratch counting or number of scratch bouts from 15 minutes preinjection and 15 minutes postinjection. B, Individual bouts for the DFK and vehicle-injected groups. i.p., Intraperitoneal.

DISCUSSION

It is increasingly appreciated that agents that block type 2 cytokines in AD derive their antipruritic efficacy, in part, from disrupting the neuroimmune interface on sensory neurons.13 These observations have provoked the hypothesis that neuromodulation via broad suppression of itch may represent an anti-pruritic therapeutic strategy. In this issue, we presented the results from a phase 2, randomized, placebo-controlled, dose-ranging trial with the selective KOR agonist DFK.5 These results demonstrated that DFK reduced itch and exhibited antipruritic efficacy specifically in patients with itch-dominant AD (mild to moderate AD with moderate to severe pruritus). They also demonstrated a reduction in pruritus- and, to a lesser extent, inflammatory-related biomarkers in subjects with AD and moderate to severe pruritus. In the present study, we examined the effects of DFK on inflammation and itch in vivo using a mouse model of AD. In mice, we observed rapid anti-itch efficacy in the absence of any effect on skin inflammation. Together, our findings support a role for DFK as a neurmodulatory antipruritic agent that is uniquely suited for patients with itch-dominant AD who may be disproportionately bothered by itch rather than skin lesions.

Activation of the KOR pathway suppresses itch not only in the peripheral nervous system but also within the central nervous system. Although it is possible for KOR agonists to suppress itch via their effect in the spinal cord and brain, the uniquely hydrophilic nature of DFK significantly limits its bioavailability in the central nervous system.14 Thus, we speculate that the antipruritic effects of DFK are derived from activity in the periphery. Our analysis of published single-cell RNA-sequencing data sets and preclinical modeling of AD in mice suggest that a primary mechanism of action of DFK is via peripheral neuromodulation. Furthermore, our in vitro studies suggest that a major target for DFK is likely the larger-diameter mechanoreceptors (eg, Aβ-fibers), rather than small-diameter C-fibers (which are activated by pruritogens and μ-opioid receptor agonists such as morphine).15 Activation of larger-diameter neurons by DFK may suppress ascending pruritic signals from these C-fibers within the spinal cord. Thus, DFK may act as a form of chemical “scratching,” which inhibits the ability of itch fibers to relay their signal. However, whether the process occurs in vivo remains to be determined. Recent positive results from a phase 2 study of oral DFK in notalgia paresthetica, a bona fide neuropathic itch disorder, support the hypothesis that DFK acts as a neuromodulatory agent.15 However, a more direct role for DFK in suppressing pruritic signaling cannot be ruled out, because, in contrast to our findings, previous studies have shown that KORs are expressed in both A- and pruriceptive C-fibers.12

Although this study did not find an impact of DFK on visible skin inflammation or immune cell infiltrate in the MC903 mouse model of AD, a potential anti-inflammatory role for DFK should still be considered. It is known that activation of KORs can suppress chemokines, chemokine receptors, and proinflammatory cytokines.17-19 DFK treatment has previously been shown to decrease levels of inflammatory markers both in vitro and in human subjects with pruritus undergoing hemodialysis.20 Indeed, in this issue we report an impact of DFK treatment on inflammatory markers in subjects with AD. This effect of DFK on inflammation may be indirect (eg, secondary to scratching-related trauma) or may be specific to humans, and therefore not observable in the mouse model, or dependent on the underlying pathophysiology.

In conclusion, these data demonstrate a previously unrecognized role for neuromodulation as a therapeutic strategy for pruritus in AD. Despite the variety of pruritogenic pathways triggered by inflammation in AD, our findings demonstrate that DFK can suppress itch both in humans9 and in mice, implicating the KOR pathway as a broadly itch-suppressive circuit. Although most emerging treatments for AD derive their efficacy from acting on the immune system to block immune cell–derived pruritogens from acting in pruriceptors, KOR activation may be...
unique in that it activates an endogenous pathway to suppress itch. DFK represents a novel antipruritic therapy with a primarily neuropeptide and neuromodulatory mode of action in AD best suited for patients with mildly inflammatory, itch-predominant AD. Larger studies will be required to confirm the efficacy and safety of DFK for pruritus in AD.

**DISCLOSURE STATEMENT**

This study was sponsored by Cara Therapeutics. Employees of Cara Therapeutics were involved in the study design, the collection, analysis, and interpretation of data, the review of the manuscript, and the decision to submit for publication.

Disclosure of potential conflict of interest: K. Nograles was employed with Cara Therapeutics, Inc, at the time of study conduct. J. Goncalves is employed with Cara Therapeutics, Inc. E. Gutman-Yassky received research funds (grants paid to institution) from AbbVie, Almirall, Amgen, AnaptyxBio, Asana Biosciences, AstraZeneca, Boehringer Ingelheim, Cara Therapeutics, Celgene, Eli Lilly, Galderma, Glenmark/Ichnos Sciences, Innovaderm, Janssen, KAO, Kiniksa, Kyowa Kirin, LEO Pharma, Novan, Novartis, Pfizer, Ralexar, Regeneron Pharmaceuticals, and UCB and worked as a consultant with AbbVie, Almirall, Amgen, Arena, Asana Biosciences, Aslan Pharmaceuticals, AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Cara Therapeutics, Celgene, Connect Pharma, Eli Lilly, EMD Serono, Evidera, Galderma, Ichnos Sciences, Incyte, Janssen Biotech, Kyowa Kirin, LEO Pharma, Pandion Therapeutics, Pfizer, RAPT Therapeutics, Regeneron Pharmaceuticals, Inc, Sanofi, SATO Pharmaceutical, Siolta Therapeutics, Target Pharma...
Key messages

- This murine study found that DFK reduced AD-like itch, independent of skin inflammation.
- In vitro, DFK preferentially activates larger-diameter neurons over small-diameter C-fibers, which include pruriceptive neurons.
- These results support that DFK, in part, acts as a neuromodulator to reduce AD-like itch.

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