



The N-Acylethanolamine Acid Amidase Inhibitor ARN077 Suppresses Inflammation and Pruritus in a Mouse Model of Allergic Dermatitis

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N-acylethanolamine acid amidase (NAAA), a cysteine hydrolase highly expressed in macrophages and B lymphocytes, catalyzes the degradation of palmitoylethanolamide. Palmitoylethanolamide is an agonist of PPAR- α and an important regulator of pain and innate immunity. In this study, we investigated the properties of the NAAA inhibitor, ARN077, in a mouse model of allergic contact dermatitis. Acute topical applications of ARN077 attenuated key signs of DNFB-induced dermatitis in a dose-dependent manner. Moreover, ARN077 increased tissue palmitoylethanolamide content and normalized circulating levels of cytokines and immunoglobulin E. No such effect was seen in PPAR- α -deficient mice. Moreover, mice lacking NAAA failed to develop edema or scratching behavior after challenge with DNFB, confirming that this enzyme plays an important role in dermatitis. Consistent with this conclusion, subchronic applications of ARN077 suppressed DNFB-induced inflammation when administered either before or after the DNFB challenge. The effects of subchronic ARN077 were dose dependent and comparable in size to those produced by the steroids clobetasol and dexamethasone. Unlike the latter, however, ARN077 did not cause skin atrophy. The results identify NAAA as a promising target for the development of effective and safe treatments for atopic dermatitis and other inflammatory disorders of the skin.

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INTRODUCTION

Atopic dermatitis is a chronic relapsing disease of the skin characterized by disruption of the epidermal barrier, inflammation, IgE-mediated sensitization to allergens, and persistent pruritus (Bieber, 2008). Its pathogenic mechanism is unknown but is likely to involve complex interactions between the environment (including the skin microbiome) (Kong et al., 2012) and genes encoding elements of the innate and adaptive immune system, such as IL-4 and INF- γ (Yamanaka and Mizutani, 2011), and structural epithelial proteins, such as filaggrin (McLean, 2016). It has been suggested that atopic dermatitis proceeds through three distinct phases (Bieber, 2008). An initial non-atopic stage of the disease, in which a preexisting epidermal barrier dysfunction allows environmental factors to trigger an inflammatory response, is followed in genetically predisposed individuals by the induction of IgE-mediated sensitization to allergens. In the last phase, pruritus-triggered scratching damages skin

cells, causing further release of proinflammatory cytokines and autoantigens (Bieber, 2008).

The prevalence of atopic dermatitis has almost tripled in industrialized countries over the past 40 years. A recent literature survey estimates its current annual costs at approximately \$5.3 billion in the United States alone (Drucker et al., 2017). Despite this growing societal burden, the existing armamentarium of drugs that can be used to treat atopic dermatitis remains limited to two main classes—topical steroids and calcineurin inhibitors—which have only partial therapeutic efficacy and have been linked to multiple potentially serious adverse events (Adams, 2013). Common adverse effects of topical steroids include skin atrophy, rosacea, and epithelial barrier dysfunction at the application site, and adverse effects of calcineurin inhibitors include photosensitivity and increased risk of certain skin cancers (Adams, 2013). Thus, safe and effective therapies that target pathological control points of atopic dermatitis are strongly needed.

A growing body of evidence suggests that macrophages and other host-defense cells of the skin generate biologically active lipids that modulate nociception and inflammation. One important member of this class of protective mediators is palmitoylethanolamide (PEA), an endogenous PPAR- α agonist that displays analgesic and anti-inflammatory activity in animal models (Fu et al., 2003; LoVerme et al., 2005; Piomelli and Sasso, 2014) and in humans (Gabrielsson et al., 2016). The biological actions of endogenously produced PEA are stopped by intracellular hydrolysis, which is

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Abbreviations: NAAA, N-acylethanolamine acid amidase; OEA, oleoylethanolamide; PEA, palmitoylethanolamide

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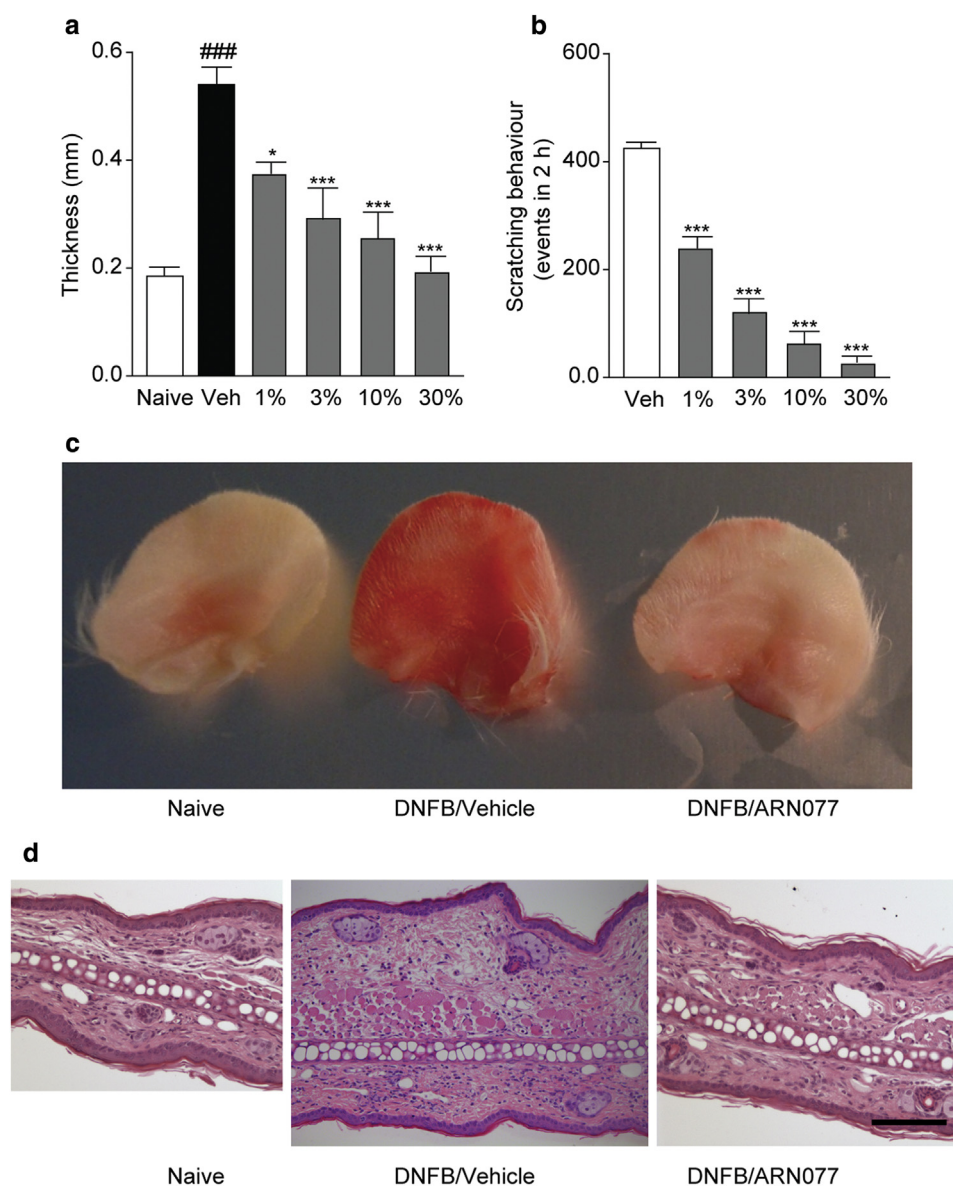


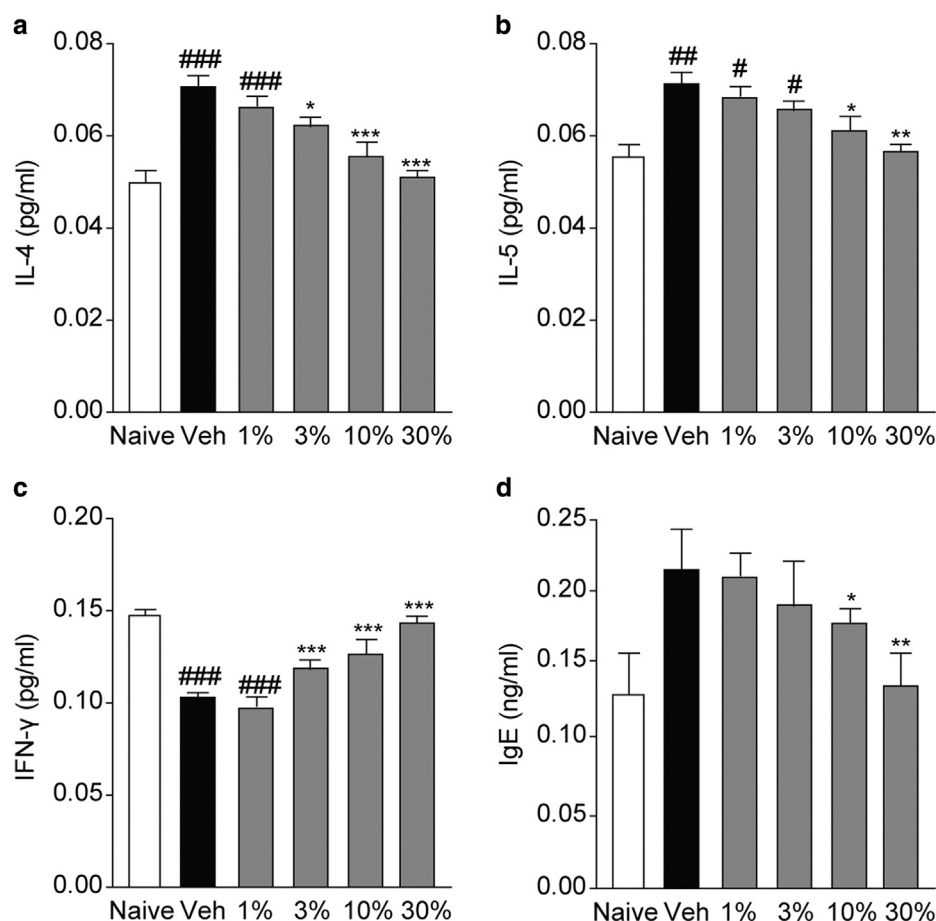
Figure 1. Effects of the NAAA inhibitor ARN077 in the mouse model of DNFB-induced allergic contact dermatitis. Effects of ARN077 (% weight/volume) on (a) ear edema, (b) scratching behavior, (c) ear erythema, and (d) ear tissue structure (hematoxylin and eosin staining, original magnification $\times 10$). Naive mice, open bars; vehicle, closed bars; ARN077, gray bars. Results are expressed as mean $\pm$ standard error of the mean ($n = 9$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Two-way analysis of variance followed by Bonferroni test for multiple comparisons. h, hour; Veh, vehicle.

primarily catalyzed by the cysteine hydrolase N-acylethanolamine acid amidase (NAAA) (Ribeiro et al., 2015; Tsuboi et al., 2005). The preferential localization of this enzyme in macrophages and B lymphocytes is suggestive of a role in the regulation of innate and adaptive immune responses (Ribeiro et al., 2015; Tsuboi et al., 2005). Biochemical and pharmacological studies support this possibility. In both experimental animals and humans, acute and chronic inflammatory states are accompanied by a dramatic decrease in PEA levels (Ribeiro et al., 2015; Richardson et al., 2008; Sasso et al., 2013b; Solorzano et al., 2009), resulting from a combination of impaired biosynthesis and heightened NAAA-mediated degradation (Zhu et al., 2011). That these changes might be functionally significant is suggested by the finding that administration of the systemically active NAAA inhibitor, ARN726, counters the inflammatory responses elicited by bacterial lipopolysaccharide or complete Freund's adjuvant in mice (Bonezzi et al., 2016; Ribeiro et al., 2015). Local application of exogenous PEA also attenuates skin

inflammation in animals (Lo Verme et al., 2005; Noli et al., 2015) and humans (Eberlein et al., 2008) through a mechanism that requires PPAR- α activation (Lo Verme et al., 2005). However, this lipid-derived molecule has limited therapeutic utility owing to its relatively low potency, rapid enzyme-mediated hydrolysis, and poor bioavailability.

This outlined evidence suggests that NAAA blockade may provide an unknown mechanistic approach to treating dermatitis. As a test of this idea, in this study we examined whether topical administration of the compound ARN077 (5-phenylpentyl N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl] carbamate), a β -lactone-based NAAA inhibitor that is rapidly deactivated in blood and other tissues (Duranti et al., 2012; Sasso et al., 2013a), suppresses the allergic reaction elicited by DNFB in mice (Bae et al., 2004; Inagaki et al., 2006; Katayama et al., 1997). We found that acute or sub-chronic administration of ARN077 substantially decreases DNFB-induced symptoms including edema, erythema, pruritus, and epidermal hyperplasia. Confirming a key role for

Figure 2. Effects of ARN077 on circulating cytokines and IgE. Effects of ARN077 (% weight/volume) on DNFB-induced changes in (a) IL-4, (b) IL-5, (c) IFN- γ , and (d) IgE. Results are expressed as mean $\pm$ standard error of the mean ($n = 9$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle; # $P < 0.05$, ### $P < 0.001$ vs. naïve. Two-way analysis of variance followed by Bonferroni test for multiple comparisons. Veh, vehicle.



NAAA in allergic dermatitis, we further show that the DNFB response is markedly reduced in mutant mice that constitutively lack this enzyme. The results implicate NAAA in cutaneous immunity and suggest a potential application of NAAA inhibitors in the treatment of atopic dermatitis and other inflammatory diseases of the skin.

RESULTS

Acute topical treatment with ARN077 attenuates DNFB-induced dermatitis

As shown in Figure 1, application of DNFB to the ear pinnae of sensitized mice produced an acute allergic reaction characterized by localized edema, scratching, erythema, and epidermal hyperplasia accompanied by infiltration of inflammatory cells. A single topical treatment with ARN077 (1–30% weight/volume), applied on the ear pinna at the same time as the DNFB challenge, resulted in a marked attenuation of this response, whereas treatment with vehicle alone had no such effect (Figure 1). ARN077 produced a profound dose-dependent reduction of edema (Figure 1a) ($P < 0.001$, $n = 9$) and scratching behavior (Figure 1b) ($P < 0.001$, $n = 9$), achieving maximum effect at the 3% dose. The same dose also prevented erythema (Figure 1c) and tissue hyperplasia (Figure 1d).

In addition to its local inflammatory and pruritogenic (itch-inducing) actions, DNFB caused marked changes in the levels of circulating cytokines: 24 hours after the DNFB

challenge, plasma concentrations of IL-4 and IL-5 were significantly higher than those measured in plasma obtained from control mice ($P < 0.001$, $n = 9$) (Figure 2a and b), whereas the concentrations of IFN- γ were lower ($P < 0.001$, $n = 9$) (Figure 2c). Moreover, there was a trend toward increased plasma IgE levels, which did not reach statistical significance (Figure 2d) ($P = 0.061$, $n = 9$). Topical application of ARN077 to the ear pinnae (1–30%) dose-dependently reduced these responses, restoring normal plasma levels of IL-4 (Figure 2a), IL-5 (Figure 2b), and IFN- γ (Figure 2c) and significantly lowering the levels of IgE (Figure 2d).

Acute topical treatment with ARN077 normalizes PEA signaling in DNFB-treated mice

As previously shown with other inflammatory stimuli, such as carrageenan (Solorzano et al., 2009), lipopolysaccharide (Ribeiro et al., 2015), and complete Freund's adjuvant (Bonezzi et al., 2016), local application of DNFB decreased PEA content in mouse ear tissue (Figure 3a) ($P < 0.001$, $n = 9$). By contrast, no change was observed in the levels of oleoylethanolamide (OEA), another lipid amide agonist of PPAR- α (Figure 3b) (Fu et al., 2003). Topical administration of ARN077 (3%) restored normal PEA content in DNFB-treated mice (Figure 3a) ($P < 0.001$, $n = 9$), suggesting that the drug attenuates the allergic reaction elicited by DNFB through a normalization of PEA-mediated signaling. In support of this conclusion, we found that ARN077 did not affect

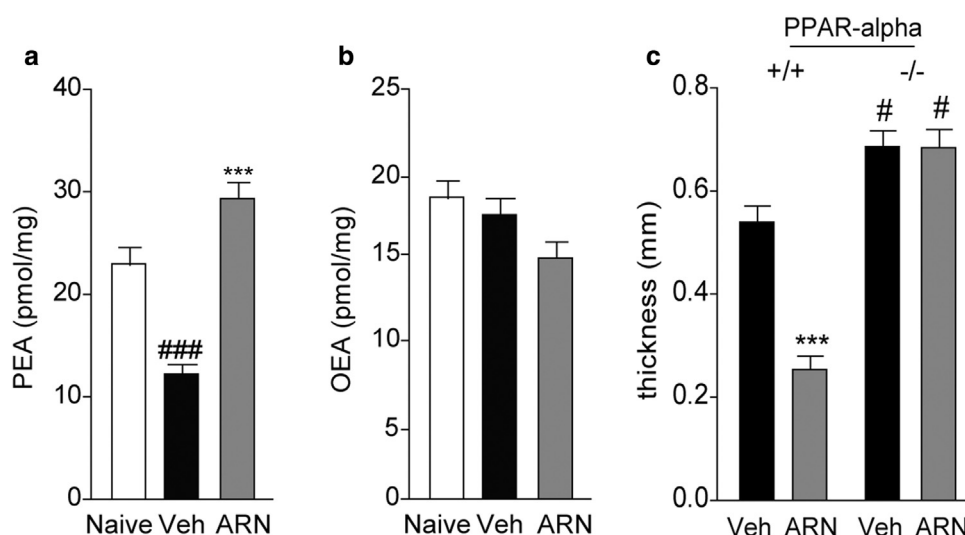


Figure 3. Effects of local application of DNFB. Levels of (a) PEA and (b) OEA in mice challenged with DNFB treated (grey bars) or not (black bars) with ARN077 (3%). (c) Edema in PPAR- $\alpha^{-/-}$ mice and wild-type controls challenged with DNFB treated (grey bars) or not (black bars) with ARN077 (3%). *** $P < 0.001$, # $P < 0.05$, ### $P < 0.001$. Two-way analysis of variance followed by Bonferroni test for multiple comparisons. ARN, ARN077; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; Veh, vehicle.

DNFB-induced edema in genetically modified mice lacking PPAR- α , the primary molecular target for PEA in rodent skin (Lo Verme et al., 2005) (Figure 3c). Consistent with previous findings (Petrosino et al., 2010), PPAR- α -deficient mice displayed a stronger response to DNFB than did wild-type animals (Figure 3c) ($P < 0.001$, $n = 9$).

Genetic NAAA deletion protects mice against DNFB-induced dermatitis

To further probe the role of NAAA in the response to DNFB, we used genetically modified mice that do not express this enzyme. First, we asked whether NAAA ablation affects tissue PEA content in inflamed ear tissue. We sensitized NAAA $^{-/-}$ mice and their wild-type littermates, challenged them with DNFB, and excised their ear pinnae for lipid extraction and liquid chromatography/mass spectrometry analysis. PEA levels in ear tissue of DNFB-treated NAAA $^{-/-}$ mice were elevated relative to DNFB-treated wild-type controls (Figure 4a) ($P < 0.001$, $n = 9$). In contrast, OEA levels were unaltered (Figure 4b). Next, we tested the ability of DNFB to trigger inflammation and pruritus in NAAA $^{-/-}$ mice. When challenged with DNFB, and in the absence of any drug, NAAA $^{-/-}$ mice showed a strongly reduced inflammatory reaction, compared with wild-type animals, with no detectable edema (Figure 4c) or scratching (Figure 4d).

Subchronic topical treatment with ARN077 suppresses DNFB-induced dermatitis

Next, as a further means to assess the therapeutic potential of ARN077, we used a protocol designed to mimic clinical treatment of an established eczema flare-up. The compound was administered subchronically to wild-type mice that had been first sensitized and then challenged with DNFB. One group of mice was exposed to ARN077 (0.0001–0.1%) for 7 consecutive days, starting on the same day as the DNFB challenge. Another group of animals was treated in a similar way with either of two standard topical steroids, dexamethasone (0.05–1%) and clobetasol (0.05%). This treatment regimen was highly effective in attenuating the inflammatory response to DNFB. ARN077 reduced DNFB-induced edema in a dose-dependent manner, achieving maximal effect at a

dose of approximately 0.1% (Figure 5a) ($P < 0.001$, $n = 9$). The effects of ARN077 were comparable in size to those produced by topical application of dexamethasone (0.05–1%) ($P < 0.001$, $n = 9$) or clobetasol (0.05 or 0.1%) (Figure 5a) ($P < 0.001$, $n = 9$).

Because prevention of inflammatory flare-ups is an important goal of eczema therapy, in a subsequent experiment we subjected wild-type mice to repeated administration of ARN077 (0.0001–0.1%) for 7 days before the DNFB challenge. This preventive regimen also resulted in a substantial reduction of DNFB-induced edema, with significant activity already apparent at the lowest drug dose tested (Figure 5b) ($P < 0.001$, $n = 9$). Comparable effects were seen after topical application of dexamethasone (0.1%).

Subchronic topical treatment with ARN077 does not cause skin atrophy

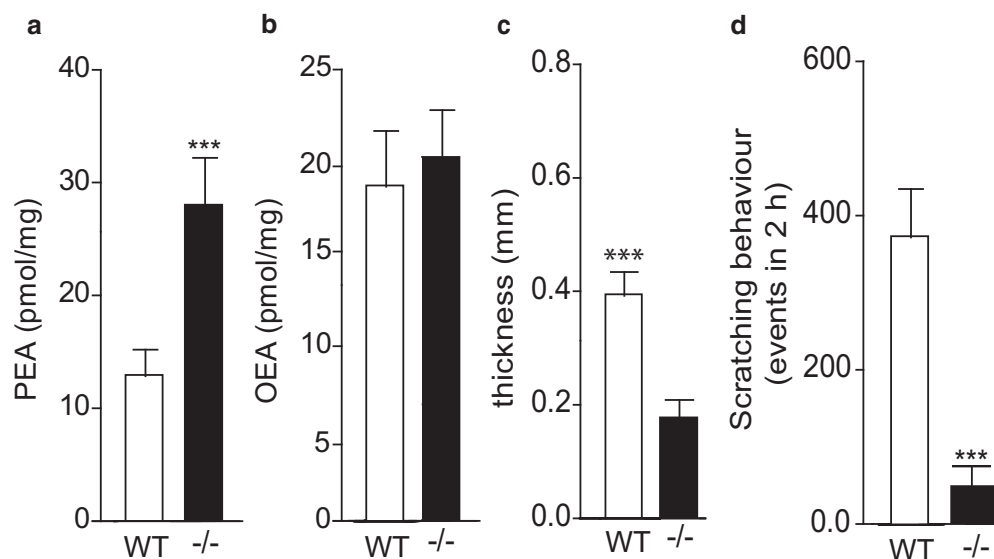
Skin atrophy is a major adverse event associated with prolonged topical steroid therapy (Hengge et al., 2006). We reproduced this response in wild-type mice by once daily applications of dexamethasone (1%). The steroid reduced ear thickness by approximately 50% compared with vehicle (Figure 6a and b). In contrast to dexamethasone, ARN077 (3%) exerted no detectable effect on skin thickness or structure even after 2 weeks of daily treatment (Figure 6a and b).

DISCUSSION

The main finding of this study is that pharmacological inhibition or genetic ablation of the intracellular cysteine hydrolase NAAA suppresses allergic contact dermatitis evoked by DNFB in mice. We found that acute or subchronic topical administration of ARN077, a potent and selective NAAA inhibitor that undergoes rapid hydrolytic degradation in blood and other tissues (Duranti et al., 2012; Sasso et al., 2013a), attenuates DNFB-induced inflammation and pruritus. This response requires the nuclear receptor PPAR- α , the main molecular target for PEA in rodent skin (Lo Verme et al., 2005), and is accompanied by an overall normalization of PEA, cytokine (IL-4, IL-5, and IFN- γ), and IgE levels. Similar to treatment with ARN077, NAAA deletion protects PEA from degradation and prevents DNFB-induced dermatitis.

Figure 4. Effects of DNFB in NAAA-deficient mice.

Levels of (a) PEA and (b) OEA and (c) edema and (d) scratching in NAAA^{-/-} mice (closed bars) and wild-type littermates (open bars) challenged with DNFB. ****P* < 0.001, **P* < 0.05. Two-way analysis of variance followed by Bonferroni test for multiple comparisons. h, hour; NAAA, N-acyl ethanolamine acid amidase; PEA, palmitoylethanolamide; WT, wild type.



Although highly effective at countering inflammation, subchronic ARN077 does not cause skin atrophy, a common adverse event associated with long-term therapy with topical steroids (Adams, 2013). These results suggest that NAAA may offer a new molecular target for the development of effective and safe treatments for atopic dermatitis and other inflammatory disorders of the skin.

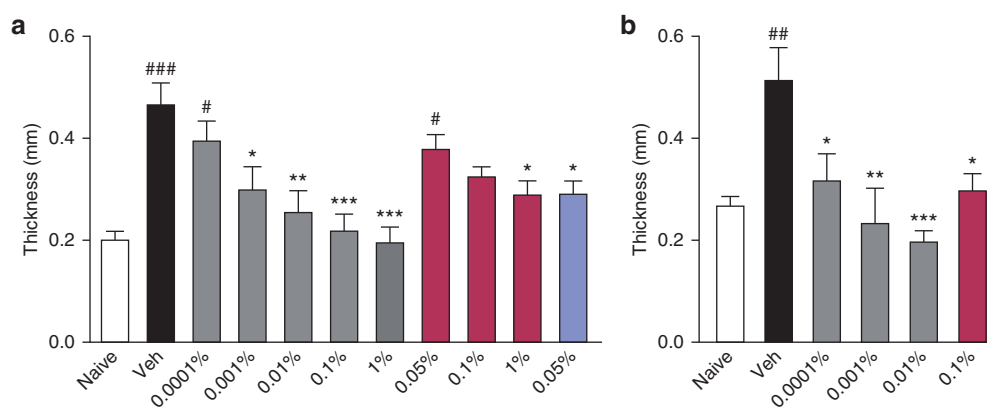
The hydrolytic degradation of PEA into palmitic acid and ethanolamine may be catalyzed by either of two enzymes: the serine hydrolase fatty acid amide hydrolase, which is expressed in many cell types including skin fibroblasts and keratinocytes (Sasso et al., 2016), and the cysteine hydrolase NAAA, which is preferentially expressed in macrophages and B lymphocytes but is also found in T lymphocytes (Ribeiro et al., 2015; Tsuboi et al., 2007). NAAA belongs to the N-terminal nucleophile hydrolase family of enzymes (Tsuboi et al., 2005) and, like other members of this class, is synthesized as an inactive precursor that is converted into a shorter active form by proteolysis (Zhu et al., 2011). The enzyme was first identified in human megakaryoblastic cells (Ueda et al., 1999), and its cDNA was subsequently cloned from human, rat and mouse (Tsuboi et al., 2005). NAAA shows no structural homology

with fatty acid amide hydrolase but is 33–35% identical to acid ceramidase, a cysteine amidase that hydrolyzes ceramides into fatty acid and sphingosine (Zhu et al., 2011). Similar to the latter, NAAA may be predominantly localized to lysosomes (Zhu et al., 2011).

Several classes of NAAA inhibitors have been reported in the scientific and patent literature (Bandiera et al., 2014). These include β -lactone, β -lactam, and isothiocyanate derivatives, which react covalently with the catalytic cysteine of NAAA (Alhouayek et al., 2015; Armirotti et al., 2012; Solorzano et al., 2009, 2010), and benzothiazole piperazine derivatives, which inhibit NAAA through a reversible noncovalent mechanism (Migliore et al., 2016). The β -lactone derivatives N-[(3S)-2-oxo-3-oxetanyl]-3-phenylpropanamide (i.e., (S)-OOPP) and ARN077 inhibit rat NAAA with half-maximal potencies of 420 nmol/L and 50 nmol/L, respectively (Solorzano et al., 2009, 2010). These agents are potent and selective but are quickly hydrolyzed in plasma (half-life time < 1 minute). Despite their rapid clearance, (S)-OOPP and ARN077 have been shown to suppress macrophage activation in vitro and to attenuate local inflammatory and nociceptive responses in vivo (Sasso et al.,

Figure 5. Effects of subchronic treatment with ARN077 on DNFB-induced dermatitis.

Effects of (a) therapeutic administration (7 days after DNFB challenge) and (b) preventive administration (7 days before DNFB challenge) of ARN077 (grey bars), dexamethasone (green bars), or clobetasol (blue bar) on DNFB-induced edema; vehicle, closed bars; naïve mice, open bars. Doses are expressed in percentage, weight/volume. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. vehicle; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. naïve. Two-way analysis of variance followed by Bonferroni test for multiple comparisons. Veh, vehicle.



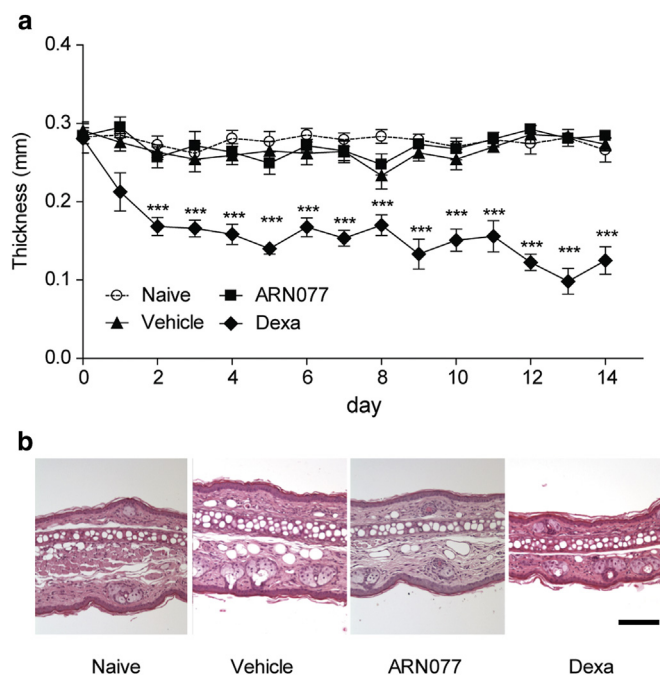


Figure 6. Effects of subchronic treatment with ARN077 on mouse skin thickness. (a) Effects of vehicle (closed triangles), ARN077 (3%, closed squares), or dexamethasone (1%, closed diamonds). Naïve mice, open circles. Skin thickness is expressed in mm. (b) Histological sections (hematoxylin/eosin) of ear pinnae excised from naïve mice and from mice treated with vehicle, ARN077 or dexamethasone. *** $P < 0.001$. Two-way ANOVA followed by Bonferroni test for multiple comparisons.

2013b; Solorzano et al., 2009). NAAA inhibitors with greater metabolic stability, such as the β -lactam ARN726 and isothiocyanate AM9053, display significant anti-inflammatory activity after systemic administration in mouse models of lung (Ribeiro et al., 2015), joint (Bonezzi et al., 2016), and colon inflammation (Alhouayek et al., 2015).

In this study, we show that topical applications of ARN077 inhibit DNFB-evoked dermatitis in mice. This effect is likely due to the normalization of PEA-mediated signaling at PPAR- α , because (i) it is absent in mice lacking this nuclear receptor, and (ii) it is accompanied by a restoration of normal PEA levels in inflamed ear tissue. In further support of this conclusion, previous studies have shown that topical PEA administration attenuates phorbol ester-induced skin inflammation by activating PPAR- α (Lo Verme et al., 2005). Our experiments did not investigate the cell types implicated in the effects of ARN077. Nevertheless, we found that the drug normalizes circulating levels of the Th2 cytokines, IL-4, and IL-5, as well as IFN- γ , which points to T lymphocytes as a possible site of action. The abundant expression of NAAA in macrophages and B lymphocytes (Ribeiro et al., 2015; Tsuboi et al., 2007), which also play key roles in the pathogenesis of dermatitis (Egbuniwe et al., 2015; Kasraie and Werfel, 2013) suggests that the response to ARN077 might be mediated by a crosstalk among multiple lineages of innate immune cells of the skin.

The two drug classes most commonly used in the treatment of atopic dermatitis, steroids and calcineurin inhibitors, do not fully control disease symptoms and produce a variety of adverse effects (Adams, 2013). These limitations have prompted a search for safer and more effective therapies,

which has recently led to the discovery of promising agents such as crisaborole (a small-molecule phosphodiesterase IV inhibitor for topical use) and dupilumab (an anti-IL-4 monoclonal antibody) (Lauffer and Ring, 2016). Our results identify NAAA as a mechanistically distinct target for the treatment of atopic dermatitis. Two lines of evidence support this idea. First, the anti-inflammatory effects of ARN077 are comparable in size to those produced by potent topical steroids such as dexamethasone and clobetasol. Second, ARN077 does not cause any overt impairment in skin structure, a critical problem that hinders prolonged steroid treatment (Adams, 2013). Additionally, the “soft drug” properties of ARN077—that is, its very rapid clearance after systemic absorption—are expected to further contain any potential toxicity of this compound.

In conclusion, this report shows that topical applications of the NAAA inhibitor ARN077 attenuate all primary signs of DNFB-induced dermatitis, including edema, erythema, pruritus, and tissue hyperplasia. Although comparable in efficacy to potent topical steroids, ARN077 does not cause skin atrophy, pointing to a potential use in the treatment of atopic dermatitis and other inflammatory diseases of the skin.

MATERIALS AND METHODS

Chemicals

ARN077 was synthesized as described (Duranti et al., 2012). Clobetasol, dexamethasone, and DNFB were from Sigma-Aldrich (Milan, Italy). Drug solutions were prepared immediately before use in a vehicle consisting of 100% PEG 400.

Animals

Male CD1 mice weighing 25–30 g (Charles River, Calco Italy) were used. Ppara^{tm1jGonz}-targeted mutation (PPAR- $\alpha^{-/-}$) mice were purchased from Charles River. B6N-A^{tm1Brd}Naaa^{tm1a(KOMP)wtsti}/WtsiH mice were acquired from MRC Harwell, The Mary Lyon Centre (Harwell Science and Innovation Campus, Oxfordshire, UK) through the EMMA-European Mouse Mutants Archive. This mouse line was generated as a knockout first, reporter-tagged insertion with conditional potential allele. The strategy relies on the identification of a critical exon common to all transcript variants that, when deleted, creates a frame-shift mutation that abolishes the expression of protein. Founder animals were mated to C57BL6/N to expand the colony. Naaa^{-/-} mice were obtained by mating animals heterozygous for the Naaa^{tm1a(KOMP)wtsti} allele. To detect the presence of the knockout first allele, PCR analyses were performed using primers hybridizing within the first intron of the NAAA gene (NaaaF: 5'-TCTGGCACATCTGAAGACACC-3') and a primer hybridizing within the Naaa^{tm1a(KOMP)wtsti} allele (CAS_R1_term: 5'-TCGTGGTATCGTTATGCGCC-3'). The presence of the wild-type Naaa allele was detected using the NaaaF primer and a reverse primer upstream from the exon 2 (NaaaR: 5'-CTAACCCGTCCTCCCACTATGC-3'). All PCR reactions were performed with PCR SUPERMIX (#10572014; Invitrogen, Waltham, MA). NAAA knockout was verified by reverse transcriptase-PCR, Western blot analysis, and enzyme activity measurements, as detailed in a forthcoming publication (S Pontis et al., unpublished data). Animal procedures were performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain and approved by Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) and with European Economic Community

regulations (O.J. of E.C. L 358/1 12/18/1986). Groups of five mice were housed in ventilated cages (Tecniplast, Buguggiate, Italy) with autoclaved cellulose paper as nesting material and free access to food and water. Animals were maintained under a 12-hour light/dark cycle (lights on at 8:00 AM) at controlled temperature ($21 \pm 1^\circ\text{C}$), and relative humidity ($55 \pm 10\%$). Behavioral testing was performed between 9:00 AM and 5:00 PM under blinded conditions. Mice were used once and were killed by cervical dislocation at the end of the experiment.

Lipid extraction

Fatty acid ethanolamides were quantified in ear tissue as previously described (Astarita et al., 2009). Briefly, ears were removed with scissors and quickly frozen in liquid nitrogen. Frozen ears were weighed (~ 15 mg) and homogenized in methanol (1 mmol/L) containing [$^2\text{H}_4$]-PEA and [$^2\text{H}_4$]-OEA (Cayman Chemical, Ann Arbor, MI) as internal standards. Lipids were extracted with chloroform (2 volumes) and washed with water (1 volume). Organic phases were collected and dried under nitrogen. The organic extracts were fractionated by open-bed silica gel column chromatography (Astarita et al., 2009). PEA and OEA were eluted with chloroform/methanol (9:1, volume/volume). Organic phases were evaporated under nitrogen and reconstituted in 60 μL of methanol. Levels of PEA and OEA were measured by liquid chromatography/mass spectrometry as described in the next section.

Liquid chromatography/mass spectrometry analyses

Liquid chromatography/mass spectrometry analyses were conducted on a Xevo TQ UPLC-MS/MS system (Waters, Milford, MA) equipped with a reversed-phase BEH C18 column, using a linear gradient of acetonitrile in water. Quantification was performed monitoring the following multiple reaction monitoring transitions in positive ion mode (parent $m/z \rightarrow$ daughter m/z , collision energy in eV): OEA, $326 \rightarrow 62.20$; [$^2\text{H}_4$]-OEA, $330 \rightarrow 66.20$; PEA, $300 \rightarrow 62.20$; [$^2\text{H}_4$]-PEA, $304 \rightarrow 66.20$. Analyte peak areas were compared with a standard calibration curve (1 nmol/L to 10 $\mu\text{mol/L}$).

DNFB treatment

Mice were sensitized with DNFB as described (Igawa et al., 2001). A solution of DNFB in acetone (0.5% volume/volume; 20 μL), prepared immediately before use, or acetone alone was applied onto the shaved abdomen of mice once daily for 2 consecutive days. Six days after the last application, an additional 20 μL of DNFB (0.2% in acetone) was applied onto the internal and external aspects of the ear pinnae, making sure that drug did not enter the ear canal. Drugs or vehicle were applied immediately after DNFB.

Ear edema

Ear thickness was measured before and 24 hours after DNFB challenge using an electronic thickness gauge (no. 7326; 2Biological, Varese, Italy). Results are expressed in millimeters.

Scratching

Scratching behavior was evaluated as described (Yamaoka and Kawana, 2007). Hind paw scratching directed toward the DNFB application site was evaluated for 2 hours after the DNFB challenge. Results are expressed as number of scratching events in 2 hours.

Histology

Mice were anesthetized with isoflurane, and ears were removed and immersed in 10% formaldehyde for 48 hours, embedded in paraffin, and cut into 5-mm-thick sections using a microtome (SM2000R,

Leica, Jena, Germany). The sections were stained with hematoxylin and eosin.

Cytokine and IgE measurements in plasma

At 24 hours after DNFB application, cardiac blood was collected with an insulin syringe containing heparin sulfate. Plasma was prepared by centrifugation as described (Sasso et al., 2015). Cytokine and IgE levels were measured using ELISA kits (R&D Systems, Italy) following the manufacturer's instructions.

Statistical analyses

All values are expressed as mean $\pm$ standard error of the mean. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Data were compared using two-way analysis of variance followed by Bonferroni test for multiple comparisons. The significance of differences for PEA and OEA levels was determined using one-way analysis of variance followed by Bonferroni test. $P < 0.05$ was considered significant.

CONFLICT OF INTEREST

DP is founder and equity holder, NeoKera LLC. The other authors state no conflict of interest.

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