

Fingolimod hydrochloride gel shows promising therapeutic effects in a mouse model of atopic dermatitis

Mayurkumar Tamakuwala, Warren Ratna, Amit Joshi and Grazia Stagni

Division of Pharmaceutical Sciences, Arnold and Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, Brooklyn, NY, USA

Keywords

atopic dermatitis; colloidal oatmeal (Aveeno®); Eczema Area and Severity Index score; fingolimod hydrochloride; T-lymphocyte-driven inflammatory skin disease

Correspondence

Grazia Stagni, Division of Pharmaceutical Sciences, Arnold and Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, 75 DeKalb Ave., Brooklyn, NY 11201, USA.
E-mail: gstagni@liu.edu

Received February 28, 2016

Accepted May 14, 2016

doi: 10.1111/jphp.12588

Abstract

Objectives To assess the efficacy of topically applied 2% hydroxypropyl cellulose gels containing 0.5% fingolimod hydrochloride (FNGL) with or without 6% colloidal oatmeal in an *in vivo* model of atopic dermatitis (AD).

Methods AD-like lesions were induced in SKH1/Hr hairless mice and were treated with FNGL gels, non-medicated base gels and Elidel® cream for 6 weeks. The severity/improvement of the lesions was assessed regularly using the Eczema Area and Severity Index (EASI), pH of the skin, transepidermal water loss, g/m²/h (TEWL), humidity and temperature. At the end of the experiments, the plasma levels of cytokines, FNGL and white blood cells were determined.

Key findings The EASI score was almost unchanged for the vehicle-only groups compared to before the treatments, whereas the medicated groups showed a significant decrease in the overall EASI score ($P < 0.01$), although there was non-significant differences among them ($P > 0.081$). Both the FNGL groups also showed a significant ($P < 0.05$) reduction in blood WBC.

Conclusion This study shows that the gels containing 0.50% FNGL and FNGL 0.50% plus 6% colloidal oatmeal have potential for the treatment of AD. The presence of colloidal oatmeal may provide additional benefits.

Introduction

Atopic dermatitis (AD) is a common, chronic and non-curable immune-mediated inflammatory skin disorder. The number of patients suffering from AD has tripled in the last three decades,^[1–4] and current estimates show that AD affects approximately 17% of the population worldwide.^[5] AD clinical symptoms include skin redness, thickening, lichenification and pruritus. Although the complete pathogenesis of AD is not fully understood yet, there is consensus that AD is the results of skin inflammation and hyperproliferative epidermis.^[6,7] The inflammation consists mainly of T cells, antigen-presenting cells like dendritic cells (DC) and macrophages.^[8] Typically, in patients with AD, TH₂ cell response that is responsible for activating B cells overwhelms TH₁ cell response resulting in increased production of IgE immunoglobulin isotype responsible for type I hypersensitivity in the serum.^[9,10] TH₂ cytokines (e.g. interleukin-4; IL-4) are overexpressed, and TH₁ cytokines (e.g. interferon gamma (IFN- γ)) are underexpressed^[11,12]

in acute lesions. This results in an abnormal immunological response and skin barrier malfunction.^[5,7,13] Topical glucocorticoids are still the first-line therapy for patients with AD, although prolonged use of these drugs may cause adverse effects due to its non-specific immune modulation.^[14,15] There is also an increasing number of AD patients with steroid resistant dermatitis. It is therefore vital to find and evaluate new types of therapeutic agents.

Fingolimod (FNGL) is an immune modulator whose mechanism of action differs from those of established immune suppressants like corticosteroid.^[16] FNGL is a synthetic structural analogue of myriocin (ISP-I), a metabolite of *Isaria cinclarii*,^[17,18] and is currently approved for relapsing forms of multiple sclerosis.^[19,20] However, because of its mechanism of action, FNGL has a potential in preventing the development of AD^[21,22] as well as other immunological diseases like rheumatoid arthritis,^[23] myasthenia gravis^[21,24] and type 1 diabetes mellitus.^[25,26] The presence of FNGL in the skin may inhibit the ability of dendritic cells to migrate to draining lymph nodes and present antigens to

naïve lymphocytes, leading to the anti-inflammatory action.^[27,28] FNGL may also inhibit keratinocytes proliferation^[29] addressing the other aspect of AD pathogenesis, which is hyperproliferation of the epidermis. Unfortunately, FNGL has a narrow therapeutic window and has severe adverse effects, such as bradycardia, infections and macular oedema,^[30,31] that limit its systemic administration. Moreover, it was shown that systemic administration of FNGL impaired the initiation but not the elicitation phase in an animal model of AD skin lesions. Therefore, topical administration of FNGL may have superior benefits to systemic administration in the treatment of a skin disease like AD.^[32] The availability of topical products of FNGL is a necessary step in the evaluation of FNGL as a therapeutic agent for AD or other immune-mediated inflammatory skin disorders like acne vulgaris, psoriasis and systemic lupus erythematosus. In a previous study,^[33] we developed a series of 2% hydroxypropyl cellulose gels containing various percentages of fingolimod (0.05%, 0.10%, 0.50% and 1.00%) and of colloidal oatmeal (0%, 1%, 3%, 6% and 10%) as a possible penetration enhancer. *In vitro* permeability and skin retention studies showed that the gel containing 0.50% FNGL had the most promising skin penetration properties over the other strengths and that the presence of colloidal oatmeal at the 6% level further enhanced FNGL penetration across porcine skin of 3.5-fold compared to the base gel.

In this study, we examined the *in vivo* efficacy of topically applied FNGL gels in an animal model of AD. Specifically, we tested the gels containing 0.50% FNGL with or without 6% colloidal oatmeal (Aveeno®) on AD-like lesions induced in SKH1/Hr hairless mice and compared with the effects of non-medicated gels (base gel and base gel with Aveeno®) and of Elidel® cream, a currently state of the art topical treatment for AD.

Materials and Methods

Reagents

FNGL·HCL (FNGL; LC Laboratories, Woburn, MA, USA); 1-chloro, 2-4 dinitrobenzene (DNCB) and 1, 4-dioxane

(dioxane; Sigma-Aldrich, St. Louis, MO, USA). HPLC-grade alcohol anhydrous (ethanol), acetic acid, acetone and methanol (J.T. Baker, Philipsburg, NJ, USA); colloidal oatmeal fine powder (Aveeno®; local pharmacy store). Hydroxypropyl cellulose (Klucel®; Hercules Inc., Wilmington, DE, USA). ELISA kit for IgE determination (Gen Way® Biotech, Inc., San Diego, CA, USA), ELISA kits for assaying for cytokine IL-4 and IFN- γ (Cayman® Chemical Company Ann Arbor, MI, USA). Elidel® 1% cream (Valeant Pharmaceuticals North America LLC Bridgewater, NJ, USA) was from a local pharmacy store.

Animals

The Institutional Animal Care and Use Committee (IACUC) at Long Island University, Brooklyn, NY, approved all the animal procedures. Six-week-old female mice (SKH1/Hr type, hairless, immunocompetent, euthymic, inbred, transgenic) were purchased from Charles River (Wilmington, MA). The animals were housed at constant temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) and fed with standard diet and water throughout the experiments. All the experiments in mice were performed in accordance with regulations.

Gel formulations

Klucel® (2%) gel was prepared in batches of 20 g each according to a procedure previously described.^[33] Physical evaluation and stability of the gel formulations were also described in that article. Table 1 shows the actual composition of the gel preparations used in the experiments.

Induction of atopic dermatitis-like skin lesions in mice and study protocol

Mice were divided into six groups of four mice each (Table 1). Four mice did not receive any treatment (group 6: control group). In the remaining five groups, AD lesions were induced according to a method previously described.^[34,35] Briefly, 100 μl of 1% solution of DNCB in acetone was applied daily for 1 week (day 1–7) on the back

Table 1 Composition of gel formulation and investigational groups

	Group 1 Vehicle	Group 2 Vehicle/Aveeno®	Group 3 Positive Control	Group 4 FNGL in Vehicle/Aveeno®	Group 5 FNGL in Vehicle	Group 6 Negative Control
GEL Composition						
Klucel	2%	2%	–	2%	2%	–
Aveeno®	–	6%	–	6%	–	–
FNGL	–	–	–	0.5%	0.5%	–
Ethanol/water	qs ^a 100	qs 100	–	qs 100	qs 100	–
Elidel cream 1%	–	–	100%	–	–	–

^aqs: as much as suffices.

of the hairless mice using a sterile micropipette. The DNCB solutions were freshly prepared just before the application due to the volatile nature of acetone. Care was taken to apply the DNCB solution only to the area of the back where mice cannot reach, to avoid spreading of the reagents to other parts of the body. Then, from day 8 to the end of the study (day 42), the concentration of the DNCB solution was decreased to 0.5%, but the same volume (100 µl) of the solution was applied every other day. Starting on the same day (day 8), the AD-like lesion was treated with 0.5 g of the investigational gels (groups 1, 2, 4, 5) or 0.1 g of Elidel® cream (Valeant Pharmaceuticals North America LLC Bridgewater; group 3). Gel or cream was applied evenly to the back of the mice with mini silicone rubber spatula (Williams-Sonoma, New York, NY, USA) every 24 h, 2 h before the application of the DNCB solution. Figure 1 shows a schematic outline of the procedures.

Assessment of atopic dermatitis-like skin lesions

An Eczema Area and Severity Index (EASI) score was used to measure the severity and extent of atopic eczema induced in the mice. Erythema (redness), scratching (excoriation), thickness (induration, papulation, oedema) and lichenification (lined skin) were scored independently on a scale from 0 to 3: 0 – none; 0.5 – none/mild; 1 – mild; 1.5 – mild/moderate; 2 – moderate; 2.5 – moderate/severe; and 3 – severe. Evaluation of EASI scoring was conducted in double blind by three observers at baseline and twice a week thereafter. Average of the three evaluators was used to determine the average cumulative EASI scoring (0–12). The cumulative EASI score (erythema plus scratching plus thickness plus lichenification) is an index of the severity of inflammation (scale 0–12).^[36,37]

The weight of each animal in grams was measured every week using a properly calibrated weighing balance (Taylor TS5 analogue scale, Las Cruces, NM, USA). The pH of the lesional area of the skin was determined at baseline (0), atopic skin (1) and 6 weeks (end of treatment) using a flat

surface electrode pH meter (Mettler-Toledo Giessen, Columbus, OH, USA). Transepidermal water loss (TEWL), temperature (°C) and humidity of the mice dorsal skin were determined using a vapometer® SWL-3 (Delfin Technologies Ltd., Kuopio, Finland). Each measurement was performed in triplicate. At the end of the 6-week study, blood was collected from the heart and stored in heparinized tubes (BD Vacutainer®, Franklin lakes, NJ, USA) at or below 4 °C. Blood was centrifuged (Beckman Coulter, Allegra® X-22R centrifuge, rotor # 4250) at 3901 g for 20 min to separate plasma from the haematocrit. Plasma was analysed for IgE, IL-4, IFN-γ concentration and also for fingolimod content, whereas haematocrit was analysed for white blood cell (WBC) count.^[38] IgE, IL-4 and IFN-γ were measured by ELISA using commercially available kits according to manufacturer's protocol. WBC count was determined by diluting 10 µl of haematocrit (1 : 20 dilution) with 190 µl of acetic acid (3%) and vortex-mixed. After 5 min, the diluted sample was placed evenly on Neubauer chambers and covered, and after waiting an additional 2 min, the WBCs were counted using a hand-tally counter (Heathrow scientific® counter, Vernon Hills, IL, USA) and an inverted light microscope (Axiovert® 200M; Thornwood, NY, USA) set at 100× optical magnification.

Measurement of FNGL in plasma

Fingolimod was quantified in plasma using an HPLC method adapted from Andreani *et al.*^[39] Standards were prepared by spiking 900 µl of mouse plasma (GeneTex, Irvine, CA, USA) with standard solutions of FNGL·HCl in water to obtain final concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78 and 39 ng/ml. Fifty microlitres of plasma (standard or sample) was transferred into a 2 ml sterile microcentrifuge tube and extracted with 200 µl of 1M NaCl, 0.25 ml of methanol and 50 µl of 18.5% hydrochloric acid (6.25 N). After vortexing (Fisher vortex genie 2™, Bohemia, NY, USA) for 2 min, 0.5 ml of chloroform was added, vortex-mixed for 5 min and centrifuged at 13225 g for 10 min. The lower chloroform phase was transferred

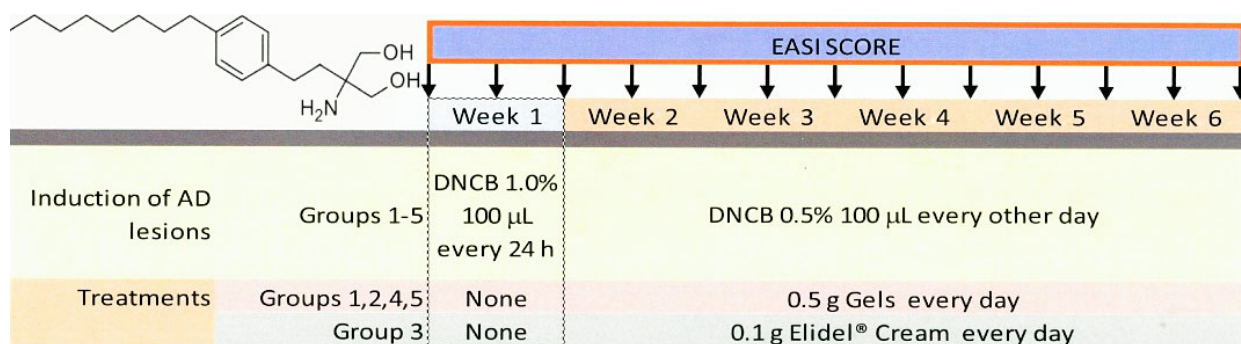


Figure 1 Schematic outline of the protocol procedures and molecular structure of fingolimod.

into a new plastic centrifuge tube. After repeating lipid extraction with another 0.5 ml of chloroform, the two chloroform phases were combined and chloroform was evaporated under nitrogen gas at 50 °C (PIERCE Reacti-Therm® Heating module; Champaign, IL, USA). The samples were reconstituted by subsequent addition of 200 µl of dioxane, 200 µl dipotassium hydrogen phosphate (70 mM, pH 9.00) and 200 µl of the derivatizing agent Fmoc-Cl (18 mg in 5 ml dioxane). Finally, the resulting solution was filtered through BD precision glide® filter needle with 5 µm filter (1.1 × 40 mm, Mettaw, IL, USA) and 10 µl was injected onto the HPLC column, a reversed phase C18 column (250 × 4.6 mm Kromasil™ 100-5 C18) equipped with a guard column (Phenomenex® security guard™, Torrance, CA, USA). Separation was performed at room temperature (25 °C) with a mobile phase consisting of methanol, 70 mM K₂HPO₄ buffer (pH: 9) and water (90 : 5 : 5 v/v). Flow rate was 1.5 ml/min. Excitation wavelength was $\lambda = 263$ nm, and emission wavelength was $\lambda = 316$. Typical retention time was 20 min. Peak height was used to determine FNLG concentration. The assay was linear in the range 5–0.039 µg/ml ($r^2 \geq 0.99$), and the lower limit of quantification was 0.039 µg/ml. The CV (%) for interday assays at 39 ng/ml (lowest limit of quantification, LLOQ) and 5000 ng/ml was 8.67 and 0.1, respectively. As Fmoc-Cl is highly light sensitive, all Fmoc-Cl standard solutions and samples were always freshly prepared and protected from light with aluminium foil or amber-coloured glassware. Plasma extraction efficiency was determined by comparing the extraction from plasma and water. The absolute recovery was in the range (91.68 ± 1.37 to 105.64 ± 17.93), and average relative recovery (extraction efficiency) was in the range of (90.72 ± 2.66 to 95.5 ± 2.81).

Statistical analysis

Mean, standard deviation (SD), standard error mean (SEM), coefficient of variation (CV%), percentage error (E %) were calculated with Microsoft Excel 2013 (Microsoft, Seattle, WA, USA). One-way analysis of variance was performed, followed by the *post hoc* Dunnett's multiple comparison tests (IBM-SPSS for Windows, Armonk, NY), with $P < 0.05$ defined as statistically significant.

Results

General assessment of mice conditions during the study

Mice weight

At the beginning of the study, the average mice weight was 15.8 ± 0.5 g, and at the end of the study, it was

22.4 ± 0.6 . Overall, there was no significant difference in the average weight of the mice ($P > 0.71$) across the study groups. However, reduction in weight and internal bleeding was found in one mouse at week 4 (group 5) and in one mouse at week 5 (group 4). Those mice were immediately euthanized.

Lesion area pH

Figure 2 shows the baseline pH (week 0, no treatment), pH at week 1 (DNCB treatment only) and at week 6 (end of treatment) for each group. For all groups, with the exception of the negative control (group 6), there was a significant difference in pH between the time periods ($P < 0.001$). ANOVA showed that the pH increased significantly with DNCB treatment (week 1) and decreased significantly ($P < 0.05$) following the topical treatments (week 6) compared to baseline pH (week 0) except for group 1 (vehicle only; $P > 0.052$). Within each time period, there was no significant difference between treatment groups ($P > 0.071$).

Transepidermal water loss (g/m²/h)

The TEWL increased significantly ($P < 0.001$) at the end of the first week (DNCB treatment only) compared to baseline TEWL (week 0) and decreased significantly ($P < 0.05$) at the end of the study for all the groups including the vehicle group (group 1; Figure 3).

Temperature and humidity

Both temperature and humidity were not significantly different from the baseline levels (week 0) at the end of the study (humidity: $P > 0.49$; temperature $P > 0.99$). Interestingly, humidity increased significantly ($P < 0.01$) at week

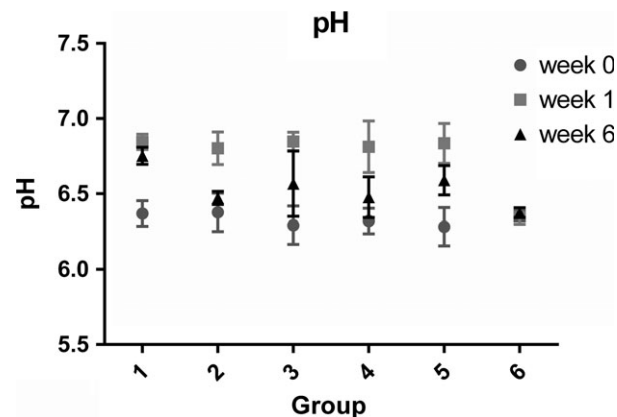


Figure 2 Average pH measured for each group before induction of AD-like lesion (week 0), after 1 week of treatment with DNBC (week 1), and at the end of the study (week 6). All values are mean ± standard error mean (SEM).

2, 3 and 4 for groups 1–5 compared to the negative control group (group 6). However, humidity was non-significantly different among the groups ($P > 0.67$), and at the end of the study, it is known that higher humidity and TEWL are common pathological features of the AD disease.^[40]

EASI score

Figure 4 shows the average cumulative EASI score observed for each group during the 5 weeks of treatment. The column 'before treatment' shows the average cumulative score for all the mice treated with DNCB before they received any topical treatment. The DNCB treatment successfully induced an AD-like lesion as the average cumulative EASI score was significantly ($P < 0.0001$) higher compared to that recorded before the beginning of the treatment or to the negative control group. The Dunnett's *post hoc t*-tests show that at the end of the 5-week treatment, the average cumulative EASI score was almost unchanged for the vehicle-only groups (groups 1 and 2) compared to before the treatments, whereas the medicated groups (3, 4 and 5) showed a significant decrease in the overall EASI score ($P < 0.048$ for group 3 and $P < 0.010$ and $P < 0.019$ for groups 4 and 5, respectively); however, there was non-significant difference among them ($P > 0.081$).

White blood cell count

Figure 5 presents the WBC count/ μ l detected in the blood of each group. For all groups, the WBC count was within the normal range for SKH1 hairless mice (5000–12000/ μ l)^[41]. However, there was a significant decrease in WBC count for the FNGL treatment groups, group 4 ($P < 0.05$) and group 5 ($P < 0.001$) compared with the control group (group 6) even though the two groups were non-significantly ($P > 0.78$) different one from the other. A reduction in the WBC count was observed in the positive control

group (Elidel[®] cream); however, the decrease was non-significant ($P > 0.074$).

Plasma IgE level

Figure 5 shows the plasma IgE levels detected in the six groups. Group 1 (gel vehicle) shows a very significant increase in IgE level ($P < 0.001$) up to $1.73 \pm 0.056 \mu\text{g/ml}$

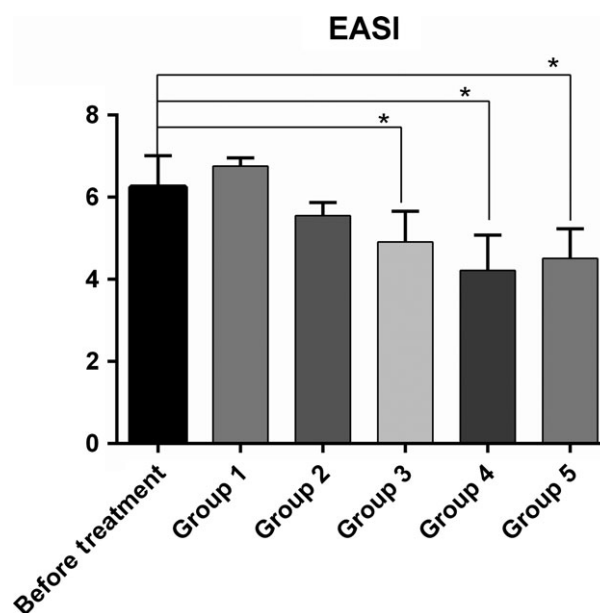


Figure 4 Average cumulative Eczema Area and Severity Index (EASI; mean $\pm$ SEM, $n = 4$) score (0–12) vs time (weeks) in normal mouse (group 6) and mice suffering from DNCB-induced AD-like skin lesion (groups 1–5), which were treated with vehicle (group 1), vehicle/colloidal oatmeal (group 2), positive control (Elidel[®] cream, group 3), FNGL containing 6% colloidal oatmeal gel (group 4) and FNGL containing no colloidal oatmeal (group 5). * $P < 0.05$ compared to the 'before treatment' group with Dunnett's test. EASI score for the control group (group 6) was always 0.

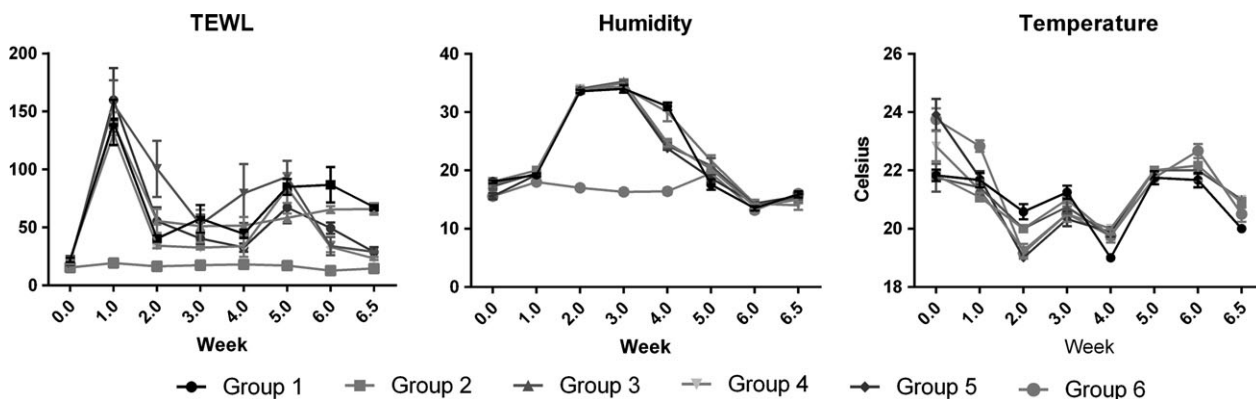


Figure 3 Average TEWL ($\text{g/m}^2/\text{h}$), temperature ($^{\circ}\text{C}$) and humidity of mice atopic dermatitis-like skin lesions ($n = 4$). All values are mean $\pm$ SEM.

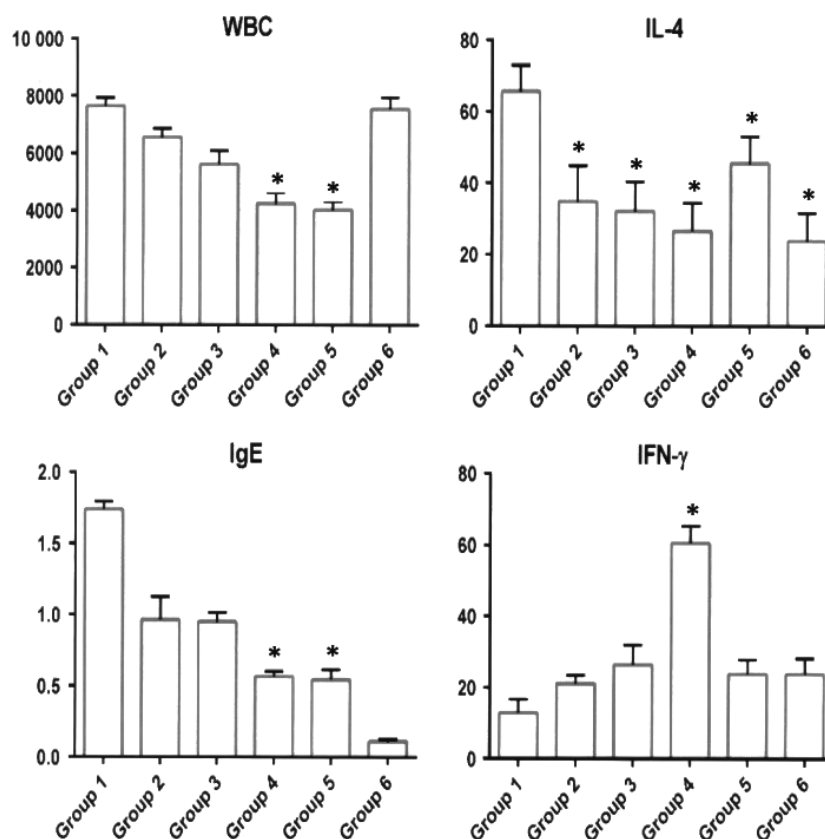


Figure 5 Blood WBC count, plasma IgE, IL-4, IFN- γ and FNGL level in normal mouse (group 6) and mice suffering from DNCB-induced AD-like skin lesion (group 1–5), which were treated with vehicle (group 1), vehicle with colloidal oatmeal (group 2), positive control (Elidel[®] cream, group 3), FNGL containing 6% colloidal oatmeal gel (group 4) and FNGL containing no colloidal oatmeal (group 5). Values are mean $\pm$ SEM ($n = 4$ for groups 1, 2, 3, 6; $n = 3$ for groups 4 and 5). * $P < 0.05$ compared to group 1 with Dunnett's test.

compared with the negative control group (group 6). The increase in IgE levels was partially offset in the other groups (groups 2, 3, 4 and 5). Indeed, all of them show a significant decrease ($P < 0.05$) compared to group 1, although their levels of IgE were still significantly higher than in the normal mice (group 6). The FNGL gels (group 4 and 5) displayed the maximum effect with a non-significant difference between the two groups ($P > 0.32$). Interestingly, the colloidal oatmeal gel (group 2) and Elidel[®] cream (group 3) were equally effective in reducing plasma IgE level.

Plasma IL-4 expression

Figure 5 shows IL-4 expression detected in the six groups at the end of the experiment. Only the group treated with the gel vehicle only (group 1) exhibited a statistically significant increase of IL-4 ($P < 0.01$), whereas the medicated groups are non-significantly different from the untreated group (group 6). The degree of reduction due to the presence of FNGL, Aveeno[®] or pimecrolimus compared to the vehicle alone (group 1; $P < 0.05$) in IL-4 expression was in

the order: group 4 (FNGL + Aveeno[®]) > group 3 (Elidel[®]) > group 2 (Aveeno[®]) > group 5 (FNGL).

Plasma IFN- γ expression

Figure 5 shows IFN- γ expression in the six groups. When the hairless mouse was treated with DNCB, the amount of IFN- γ decreased as shown with the comparison of group 1 with the control (group 6). IFN- γ expressions increased upon the application of the medicated gels with maximum effect for group 4 (60.52 ± 4.91 pg/ml). The increasing rank order of IFN- γ expression was group 4 > group 3 > group 5 > group 2. The rank order of IFN- γ expression was inverse that of IgE levels and that of IL-4 expression.

Plasma FNGL level

FNGL plasma levels were determined to evaluate possible systemic effects. A slight but non-significant ($P > 0.27$) difference was observed in the plasma concentration of FNGL:

30.99 ± 1.4 and 37.91 ± 4.47 $\mu\text{g/ml}$ for the groups 4 and 5, respectively.

Discussion

This study investigates the hypothesis that FNGL, an immune modulator formulated as a topical gel, may reduce the clinical manifestations of AD, an immunological skin disease. Several gels were previously formulated and tested *in vitro* to find out the FNGL strength and concentration of the penetration enhancer (colloidal oatmeal) that exhibited the most promising skin permeability and retention characteristics.^[33] The gels selected for *in vivo* experiments consisted of 0.5% FNGL in a 2% Klucel[®] hydroalcoholic gel base with or without addition of 6% colloidal oatmeal. Colloidal oatmeal, also known as Aveeno[®], was selected because it has its own dermatological benefits.^[42] Indeed, several OTC products containing Aveeno[®] have recognized anti-inflammatory (soothing/calming) and antioxidant properties and improve skin moisture and barrier function.^[43] The gels were tested in an animal model of AD where AD-like skin lesions were induced with a strong sensitizer (DNBC).^[34,44,45] It has been reported that following DNBC challenge, the mice skin expression of both TH₁ and TH₂ immune responses as well as the production of IgE and TH₂ cytokines is similar to that observed in human patients of AD.^[5,34,46,47] On the macroscopic level, the skin of mice treated with DNBC exhibits an AD-like lesion; that is, it appears dry, red and rough: topical signs of a compromised barrier function.^[46,48,49] The results of this study show that the AD-like lesions were successfully induced in the mice models as all the indexes of skin AD lesions (pH, TEWL and EASI) remarkably increased after 1 week of DNBC application compared with both the measurements performed before the application and the negative control group (group 6).

The EASI score was developed by the European Task Force on Atopic Dermatitis to evaluate the severity of the disease as objectively as possible.^[36,37] We used the same criteria for the mouse model; however, we did not account for the area of the lesion, being the area approximately the same in all mice. Analysis of EASI score data from groups 4 (0.5% FNGL gel, 6% Aveeno[®]) and 5 (0.5% FNGL gel) shows that the presence of FNGL substantially reduced the visual symptoms of AD compared to the vehicle-only groups (group 1 and group 2) and resulted in a score similar to that of the positive control (group 3, Elidel[®] cream). Although the presence of Aveeno[®] alone (group 2) moderately reduced the EASI score when added to the base gel (group 2), it did not substantially increase the effect of FNGL (Figure 4).

The normal pH of the human skin is slightly acidic (4.5–6.0), whereas the pH of the lesion in patients suffering

from AD is in the range of 5.5–6.5.^[50] The reported pH of stratum corneum surface in SKH1 hairless mice is 6.0.^[51] In this study, we observed a baseline pH of 6.37 ± 0.041 . Treatment with DNBC significantly increased pH to 6.8 ± 0.054 , whereas FNGL treatment significantly reduced the pH of the DNBC-treated skin back to 6.47 ± 0.02 . Reduction in the pH towards baseline pH was observed also for the vehicle/colloidal oatmeal formulation (group 2) and not for the vehicle-only gel (group 1). This suggests that Aveeno[®] alone may have pH normalization properties. The pH of the gel formulations ranged from 5.02 to 5.73.^[33]

TH cells are central to the pathogenesis of AD. T helper cells are classified as TH₁ or TH₂ based on a variety of characteristics including the profile of cytokines produced by the respective cells. TH₁ cells are involved in cell-mediated inflammatory DTH reactions and produce large quantities of IFN- γ , TNF- α and IL-2. Several TH₁ cytokines activate cytotoxic, inflammatory and delayed hypersensitivity reactions and act to promote macrophage activation. TH₂ cells typically produce IL-4, IL-5 and several other cytokines, such as IL-6, IL-9, IL-10 and IL-13. TH₂ cells produce antibodies/immunoglobulins, of which immunoglobulin IgE is responsible for allergic reactions or type I hypersensitivity. A biphasic profile of cytokine expression occurs in AD, with activation of IL-4-producing TH₂ cells predominating over INF- γ -producing TH₁ cells in the early acute phase and vice versa in the chronic phase.^[52,53] The TH₁/TH₂ balance may be fundamental to the pathogenesis of AD.^[38] In our model of AD, we measured the concentration of cytokines in peripheral blood and not directly in skin, because there is a reported good correlation between plasma and skin cytokines levels in AD.^[54,55] Plasma IgE increased by 15-fold (1.73 ± 0.056 $\mu\text{g/ml}$ as compared to 0.11 ± 0.017 $\mu\text{g/ml}$) after DNBC treatment and reduced to more than one-third (0.55 ± 0.068 $\mu\text{g/ml}$) following 5 weeks of topical medication. This effect was quite similar across the FNGL groups (groups 4 and 5), the positive control group (pimecrolimus cream, group 3) and the colloidal oatmeal alone gel (group 2). The decrease in IFN- γ due to treatment with DNBC is possibly due to the increase in IL-4 in response to DNBC treatment, which causes the differentiation of TH₀ cells into TH₂ cells, which produce IgE, and inhibits IFN- γ production by inhibiting the differentiation of TH₀ cells into TH₁ cells that secrete IFN- γ . This was reversed, when treated with FNGL or the positive control due to reversal of the above process. IgE is responsible for type I hypersensitivity reactions resulting in allergic responses, while TH₁ cells are responsible for delayed type hypersensitivity (DTH) reactions (type IV hypersensitivity). IFN- γ production greatly increased with the 0.5% FNGL formulation containing 6% colloidal oatmeal (Figure 4), whereas the

IL-4 plasma level observed for the same group exhibited maximum reduction. This suggests that the presence of 0.5% FNGL and colloidal oatmeal causes an increase in IFN- γ production, most likely by increasing TH₁ activity and by decreasing the levels of IL-4 possibly through inhibition of basophils, which is required for TH₂ cell differentiation.^[56] INF- γ is predominantly produced by TH₁, NK NKT and T_{CTL} cells and IL4 by TH₂ cells.^[57]

Interestingly, the gel containing the FNGL and colloidal oatmeal (group 4) was able to bring the IL-4 to a level closer to the baseline (group 6) than the gel containing no colloidal oatmeal (group 5). The addition of Aveeno[®] in group 4 treatment as compared to group 5 treatment showed a greater reduction in DNCB-induced increase in IL-4; the reverse was observed with regard to INF- γ . IL-4 presumably secreted by basophils causes the differentiation of naïve TH₀ to TH₂ cells that bring about B-cell activation and differentiation. IL-4 is also involved in the differentiation mature B cells into plasma cells that produce IgE. The differentiation of TH₀ into TH₁ cells results in the reduction in the secretion of INF- γ . It appears that the inclusion of Aveeno[®] potentiates the activity of TH₁ cells. This may be due to the presence in colloidal oatmeal of phenolic avenanthramides^[58,59] that may exert additional inhibitory action on plasma cytokine level. IL-4, a TH₂ cytokine, is reported to play a key role in the overproduction of IgE; IL-4 expression and together with IgE level can be used as a measurement to assess the capability of the investigational gels to modulate DNCB-induced immune response.^[12] In all likelihood, it appears that DNCB initiates an allergic reaction caused by IgE-mediated type I hypersensitivity, which is then superseded by TH₁-mediated DTH reaction, a type IV hypersensitivity reaction brought about by the treatment with FNGL and Aveeno[®]. Based on the results of IgE production, IL-4 expression and IFN- γ expression, it is evident that 0.5% FNGL containing 6% colloidal oatmeal, among the preparations tested had the greatest inhibitory effect on the development of AD-like skin lesion due to allergic reactions.

The skin in AD lesion, as well as the blood of the patients with AD, presents high levels of WBC, in particular, the T lymphocytes. One of the major systemic effects of FNGL is a remarkable reduction in circulating T lymphocytes that are sequestered in secondary lymphoid organ such as lymph nodes,^[38] an effect, which is similar to that caused by steroidal anti-inflammatory agents. Therefore, the mice blood was analysed for WBC counts to determine systemic effects of the investigational gel after topical administration. WBC count was markedly reduced (43.70% and 46.68% for group 4 and group 5, respectively) with ($P < 0.01$), whereas it was unchanged in the atopic mice (1.32%; group 1). These data suggest that FNGL was absorbed into the systemic circulation from the topical application as confirmed

by the fact that a substantial concentration of FNGL was detectable in plasma at the end of the study. This also may explain why two mice in the groups receiving FNGL gels exhibited signs of severe toxicity and died before the end of the study. The hairless nude mice used in this experiment have very thin skin compared to humans; thus, the transdermal delivery may have been far greater than in an actual clinical setting. Eventually, the strength/frequency of applications of the gels must be optimized in translational clinical studies. Once in the blood, FNGL is phosphorylated to the active moiety, *S*-enantiomer fingolimod-P, and fingolimod-P is dephosphorylated back to the inactive form fingolimod. At steady state, fingolimod and fingolimod-P are in dynamic equilibrium.^[29] Comparable to our results, a recent study demonstrated that relatively high concentrations of topically administered FNGL can reduce dendritic cells migration in secondary lymphoid organ accompanied by a reduced allergic inflammatory response in AD.^[27,28,32] One study showed that fingolimod induced apoptosis at the high dose of 1–10 $\mu\text{g/ml}$ after topical application.^[60] In this study, the strength of the FNGL gels was selected based on the maximum permeability properties observed *in vitro* in intact porcine skin. The present study shows that a substantial amount of FNGL reached the systemic circulation from the topically applied 0.5% gels with or without penetration enhancer. This result may also be because the skin of the hairless mice offers less resistance to transdermal permeability compared to normal skin. Further studies are necessary to optimize the gel strength to minimize systemic effects.

Conclusion

In conclusion, the results of this study show that topical application of 0.5% FNGL gel with or without the addition of 6% colloidal oatmeal is effective in reducing the appearance of DNCB-induced AD-like skin lesion in hairless mice. In addition, FNGL gels were able to reduce specific plasma cytokine levels as well as other inflammation markers that DNCB induce. However, further studies need to be conducted to optimize the gel strength and avoid the systemic toxicity. Based on these results, FNGL topical formulation might have promising effects on other immune-mediated inflammatory skin disorders like disorders such as psoriasis.

Declarations

Conflict of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of this article.

Acknowledgements

These studies were supported by the Division of Pharmaceutical Sciences, Long Island University, Brooklyn

Campus, NY. The authors would like to thank Ms Cora Kaiser and Dr Bruce Scharf, DVM, for their valuable advice in animal procedures and care.

References

- Williams HC. Epidemiology of atopic dermatitis. *Clin Exp Dermatol* 2000; 7: 522–529.
- Leung DY *et al.* New insights into atopic dermatitis. *J Clin Invest* 2004; 5: 651–657.
- Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 2011; 1: 233–246.
- Shaw TE *et al.* Eczema prevalence in the United States: data from the 2003 National Survey of Children's Health. *J Invest Dermatol* 2011; 1: 67–73.
- Matsumoto K *et al.* Establishment of an atopic dermatitis-like skin model in a hairless mouse by repeated elicitation of contact hypersensitivity that enables to conduct functional analyses of the stratum corneum with various non-invasive biophysical instruments. *Skin Res Technol* 2004; 2: 122–129.
- Cork MJ *et al.* Epidermal barrier dysfunction in atopic dermatitis. *J Invest Dermatol* 2009; 8: 1892–1908.
- Guttman-Yassky E *et al.* Contrasting pathogenesis of atopic dermatitis and psoriasis—part I: clinical and pathologic concepts. *J Allergy Clin Immunol* 2011; 5: 1110–1118.
- Nestle FO *et al.* Psoriasis. *N Engl J Med* 2009; 5: 496–509.
- Smart JM, Kemp AS. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. *Clin Exp Allergy* 2002; 5: 796–802.
- Packard KA, Khan MM. Effects of histamine on Th1/Th2 cytokine balance. *Int Immunopharmacol* 2003; 7: 909–920.
- Dieckhoff K *et al.* Deficient translocation of c-Rel is associated with impaired Th1 cytokine production in T cells from atopic dermatitis patients. *Exp Dermatol* 2005; 1: 17–25.
- Qi XF *et al.* Effects of Bambusae caulis in Liqamen on the development of atopic dermatitis-like skin lesions in hairless mice. *J Ethnopharmacol* 2009; 2: 195–200.
- Seidenari S, Giusti G. Objective assessment of the skin of children affected by atopic dermatitis: a study of pH, capacitance and TEWL in eczematous and clinically uninvolved skin. *Acta Derm Venereol* 1995; 6: 429–433.
- Kakinuma T *et al.* Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001; 3: 535–541.
- Furukawa H *et al.* Enhanced TARC production by dust-mite allergens and its modulation by immunosuppressive drugs in PBMCs from patients with atopic dermatitis. *J Dermatol Sci* 2004; 1: 35–42.
- Brinkmann V *et al.* FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation. *Trends Pharmacol Sci* 2000; 2: 49–52.
- Fujita T *et al.* Fungal metabolites. Part 11. A potent immunosuppressive activity found in *Isaria sinclairii* metabolite. *J Antibiot (Tokyo)* 1994; 2: 208–215.
- Kiuchi M *et al.* Synthesis and immunosuppressive activity of 2-substituted 2-aminopropane-1,3-diols and 2-aminoethanols. *J Med Chem* 2000; 15: 2946–2961.
- Brinkmann V *et al.* The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* 2002; 24: 21453–21457.
- Mandala S *et al.* Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 2002; 5566: 346–349.
- Kohno T *et al.* A novel immunomodulator, FTY720, prevents spontaneous dermatitis in NC/Nga mice. *Biol Pharm Bull* 2004; 9: 1392–1396.
- Tsuji T *et al.* Therapeutic approach to steroid-resistant dermatitis using novel immunomodulator FTY720 (Fingolimod) in combination with betamethasone ointment in NC/Nga mice. *Biol Pharm Bull* 2012; 8: 1314–1319.
- Tsunemi S *et al.* Effects of the novel immunosuppressant FTY720 in a murine rheumatoid arthritis model. *Clin Immunol* 2010; 2: 197–204.
- Yoshida Y *et al.* Relapse of experimental autoimmune encephalomyelitis after discontinuation of FTY720 (Fingolimod) treatment, but not after combination of FTY720 and pathogenic autoantigen. *Biol Pharm Bull* 2011; 6: 933–936.
- Tsuji T *et al.* Therapeutic approach for type 1 diabetes mellitus using the novel immunomodulator FTY720 (fingolimod) in combination with once-daily injection of insulin glargine in non-obese diabetic mice. *J Diabetes Invest* 2012; 2: 132–137.
- Tsuji T *et al.* Oral therapy for type 1 diabetes mellitus using a novel immunomodulator, FTY720 (fingolimod), in combination with sitagliptin, a dipeptidyl peptidase-4 inhibitor, examined in non-obese diabetic mice. *J Diabetes Invest* 2012; 5: 441–448.
- Gollmann G *et al.* Sphingosine-1-phosphate receptor type-1 agonism impairs blood dendritic cell chemotaxis and skin dendritic cell migration to lymph nodes under inflammatory conditions. *Int Immunol* 2008; 7: 911–923.
- Reines I *et al.* Topical application of sphingosine-1-phosphate and FTY720 attenuate allergic contact dermatitis reaction through inhibition of dendritic cell migration. *J Invest Dermatol* 2009; 8: 1954–1962.
- Herzinger T *et al.* Sphingosine-1-phosphate signaling and the skin. *Am J Clin Dermatol* 2007; 6: 329–336.

30. Forrest M *et al.* Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther* 2004; 2: 758–768.
31. Sanna MG *et al.* Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* 2004; 14: 13839–13848.
32. Nakashima D *et al.* Impaired initiation of contact hypersensitivity by FTY720. *J Invest Dermatol* 2008; 12: 2833–2841.
33. Tamakuwala M, Stagni G. Fingolimod hydrochloride gel for dermatological applications: optimization of formulation strength, and effect of colloidal-oatmeal (Aveeno®) as penetration enhancer. *AAPS PharmSciTech* 2015; in press.
34. Zhang EY *et al.* Mechanism of dinitrochlorobenzene-induced dermatitis in mice: role of specific antibodies in pathogenesis. *PLoS One* 2009; 11: e7703.
35. Kwon TK, Kim JC. *In vitro* skin permeation and anti-atopic efficacy of lipid nanocarriers containing water soluble extracts of *Houttuynia cordata*. *Drug Dev Ind Pharm* 2014; 10: 1350–1357.
36. Hanifin JM *et al.* The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. *Exp Dermatol* 2001; 1: 11–18.
37. Schram ME *et al.* EASI (objective) SCORAD and POEM for atopic eczema: responsiveness and minimal clinically important difference. *Allergy* 2012; 1: 99–106.
38. Hanifin JM, Chan S. Biochemical and immunologic mechanisms in atopic dermatitis: new targets for emerging therapies. *J Am Acad Dermatol* 1999; 1: 72–77.
39. Andreani P, Graler MH. Comparative quantification of sphingolipids and analogs in biological samples by high-performance liquid chromatography after chloroform extraction. *Anal Biochem* 2006; 2: 239–246.
40. Nilsson GE. Measurement of water exchange through skin. *Med Biol Eng Comput* 1977; 3: 209–218.
41. McGarry MP *et al.* *Cell Differential Assessment to Peripheral Blood Films*. USA: Cold Spring Harbor, NY Laboratory Press, 2010.
42. Cerio R *et al.* Mechanism of action and clinical benefits of colloidal oatmeal for dermatologic practice. *J Drugs Dermatol* 2010; 9: 1116–1120.
43. Kurtz ES, Wallo W. Colloidal oatmeal: history, chemistry and clinical properties. *J Drugs Dermatol* 2007; 2: 167–170.
44. Jin H *et al.* Animal models of atopic dermatitis. *J Invest Dermatol* 2009; 1: 31–40.
45. Beriat GK *et al.* Is pimecrolimus cream (1%) an appropriate therapeutic agent for the treatment of external ear atopic dermatitis? *Med Sci Monit* 2012; 4: BR135–BR143.
46. Imokawa G *et al.* Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol* 1991; 4: 523–526.
47. Inoue Y *et al.* The combined effect of topical CX-659S, a novel diaminouracil derivative, with topical corticosteroid on the three types of allergic responses in mice or guinea pigs. *J Pharmacol Sci* 2003; 1: 71–78.
48. Loden M *et al.* Friction, capacitance and transepidermal water loss (TEWL) in dry atopic and normal skin. *Br J Dermatol* 1992; 2: 137–141.
49. Kim DW *et al.* Correlation of clinical features and skin barrier function in adolescent and adult patients with atopic dermatitis. *Int J Dermatol* 2006; 6: 698–701.
50. Knor T *et al.* Stratum corneum hydration and skin surface pH in patients with atopic dermatitis. *Acta Dermatovenerol Croat* 2011; 4: 242–247.
51. Hanson KM *et al.* Two-photon fluorescence lifetime imaging of the skin stratum corneum pH gradient. *Biophys J* 2002; 3: 1682–1690.
52. Hamid Q *et al.* Differential *in situ* cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994; 2: 870–876.
53. Suarez-Farinas M *et al.* Residual genomic signature of atopic dermatitis despite clinical resolution with narrow-band UVB. *J Allergy Clin Immunol* 2013; 2: 577–579.
54. Austin LM *et al.* The majority of epidermal T cells in Psoriasis vulgaris lesions can produce type 1 cytokines, interferon-gamma, interleukin-2, and tumor necrosis factor-alpha, defining TC1 (cytotoxic T lymphocyte) and TH1 effector populations: a type 1 differentiation bias is also measured in circulating blood T cells in psoriatic patients. *J Invest Dermatol* 1999; 5: 752–759.
55. Ezzat MH *et al.* Serum measurement of interleukin-31 (IL-31) in paediatric atopic dermatitis: elevated levels correlate with severity scoring. *J Eur Acad Dermatol Venereol* 2011; 3: 334–339.
56. Romagnani S. Th1/Th2 cells. *Inflamm Bowel Dis* 1999; 4: 285–294.
57. Nakayamada S *et al.* Helper T cell diversity and plasticity. *Curr Opin Immunol* 2012; 3: 297–302.
58. Liu L *et al.* The antiatherogenic potential of oat phenolic compounds. *Atherosclerosis* 2004; 1: 39–49.
59. Fowler JF. Colloidal oatmeal formulations and the treatment of atopic dermatitis. *J Drugs Dermatol* 2014; 13: 1180–1183.
60. Brunkhorst R *et al.* Fingolimod for the treatment of neurological diseases: state of play and future perspectives. *Front Cell Neurosci* 2014; 8: 283.