

In vitro approaches to pharmacological screening in the field of atopic dermatitis

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Summary

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Accepted for publication

25 April 2014

Funding sources

This work was supported by Pierre Fabre Dermo-Cosmétique, France.

Conflict of interest

All of the authors are employees of Pierre Fabre Dermo-Cosmétique.

DOI 10.1111/bjd.13106

In vitro models are valuable for evaluating potential active ingredients and other molecules used in medications for atopic dermatitis (AD). However, finding appropriate *in vitro* models can be problematic. Our strategy was to set up different *in vitro* models that would mimic the pathomechanisms of AD. We describe five such models – the AD keratinocyte model, the AD reconstructed human epidermis model, the adaptive immunity model, the innate immunity model and the pruritus model – which we have used to evaluate a new ingredient for emollients derived from a biological extract. The models chosen provide useful data for the pharmacological characterization of active ingredients in adjunctive treatments for AD.

Atopic dermatitis (AD) is a chronic, complex inflammatory disease with multiple pathogenic pathways that interact with one another. The aetiology of the disease is not fully understood, but it is known that the initial immune response begins with the T helper (Th)2 response and is then shifted to Th1 in the chronic phase.¹ A defective skin barrier is an additional risk factor for developing AD.² Barrier dysfunction exposes immune system cells to external allergens, eliciting an immune response.

The use of emollients for alleviating symptoms of AD is recognized as being part of a proactive therapeutic approach and has been integrated into the current guidelines.^{3,4} There are many emollient products on the market, typically consisting of water-in-oil ointments or oil-in-water creams. Most of these products do not contain a pharmaceutically licensed active ingredient, and as such are licensed as cosmetic preparations.

Although animal models have been very useful in understanding the pathogenesis and molecular mechanisms in AD, and in validating the safety and effectiveness of medical agents before a clinical trial,⁵ regulatory controls and ethical considerations surrounding the development of cosmetic products prohibit the testing of emollient ingredients in animal models. Animal models also pose problems because the results obtained may not always translate exactly to human disease.⁶ Researchers must therefore rely on *in vitro* models when screening new molecules for inclusion in emollients.

Generally, *in vitro* models are valuable in assessing active ingredients and in exploring the pathophysiological basis of AD. Therefore it is vital that validated, sophisticated models based on cell and tissue cultures are developed. However, finding an appropriate *in vitro* model to validate potentially beneficial new molecules in dermocosmetic research remains problematic.

Epidermal keratinocytes express toll-like receptors (TLRs) and thymic stromal lymphopoietin (TSLP), which enhance dendritic-cell-driven Th2-mediated cell differentiation.⁷ They are important in the pathophysiology of AD, as patients with AD are known to have keratinocytes with reduced TLR function and a reduced output of granulocytes that defend against infection.⁷ Much of the research that has provided us with our knowledge of the immunological responses in AD comes from *in vitro* experimentation with cell cultures. However, existing models are limited by the fact that they do not readily show us the overall picture of AD. It is important therefore to have working, validated *in vitro* cell culture and tissue models with which to explore AD.

It is known that Avène thermal spring water can attenuate the effects of inflammatory cytokines in patients with AD.⁸ Recently an active macromolecule complex, an extract of *Aquaphilus dolomiae* bacteria derived from the Avène aquifer (ESO, I-modulia; Pierre Fabre, Castres, France) has been identified.⁹ An extensive and complex research programme has been established to evaluate this extract using validated *in vitro* models. The aim of this manuscript is to describe the strategy and

experimental approaches used to evaluate the pharmacological potential of new active ingredients for adjunctive treatment of AD employing in vitro models. The results generated provide insight into the suitability of these models for use in AD research. The evaluation of *A. dolomia* extract is presented here for exemplary purposes only, and the complete data obtained will be published elsewhere.

Experimental approaches

The atopic dermatitis keratinocyte model

This model is designed to mimic a keratinocyte inflammatory condition resembling that found in AD (Table 1). It is known that proinflammatory cytokines and Th2 cytokines combine synergistically to induce expression of TSLP,¹⁰ which in turn polarizes the immune response towards the Th2 allergic response.⁶ The cytokine interleukin (IL)-18 has also been associated with the severity of AD.¹¹

The AD keratinocyte model involves stimulation of cultured keratinocytes using a cocktail of proinflammatory agents, including Poly(I:C) and tumour necrosis factor (TNF)- α (Th1 cytokine) or Poly(I:C), IL-4 and IL-13 (Th2 cytokines).¹² Thereafter, quantitative polymerase chain reaction of 19 genes is used to analyse expression of differentiation factors, inflammation receptors, antimicrobial peptides (AMPs), chemokines and cytokines. This stimulation of keratinocytes with proinflammatory cytokines mimics the effects of inflammatory cytokines in AD. Examining the role of differentiation factors is also crucial to the understanding of cell differentiation in AD disease.

We observed markedly increased expression of six chemokines, five cytokines and one cytokine receptor in normal human epidermal keratinocytes (NHEKs). These markers are normally upregulated in AD. We also observed markedly decreased expression of three keratinocyte differentiation markers that are normally downregulated in AD, and an increase in the levels of four antimicrobial peptides (Fig. 1). We used this experimental model to study the effect of *A. dolomia* extract on these key inflammatory mediators and keratinocyte differentiation markers.

Results from our studies demonstrated clearly that *A. dolomia* extract inhibits the production of TSLP and/or IL-18, among other factors, when added to keratinocytes in this model (data on file). The macromolecule complex severely inhibited IL-8 expression, which was highly expressed in our AD model compared with normal keratinocytes. Finally, an increase in involucrin expression was observed in our AD model following incubation with *A. dolomia* extract, showing its beneficial effect on epidermal barrier integrity (data on file). These results show that the model is a useful mimic of AD in vitro and are consistent with what might be expected in AD.

Atopic dermatitis reconstructed human epidermis model

Whole-tissue models allow for more in-depth analysis of expression patterns consistent with AD, thus providing

researchers with more information from which data can be extracted and interpreted. The biology of whole-tissue models would also be expected to be more closely aligned with in vivo modelling than cell culture alone.

The role of TSLP in driving dendritic-cell enhancement of Th2 cell differentiation in AD has already been described in this paper. It has previously been reported that addition of TNF- α or IL-1 α to cultured cells from skin-punch biopsies induces production of TSLP.¹⁰ Stimulation of cultured abdominal keratinocytes obtained by skin punch with IL-4/IL-13 has also been shown to induce TSLP production.

The methods by which epidermal keratinocytes are isolated from skin and cultured have been described previously.¹³ Briefly, after 14 days of air-liquid interface culture, reconstructed human epidermis (RHE) cells are stimulated with the Th2 cytokines IL-4 and IL-13, and the TLR ligands Poly(I:C) and Pam3CKS4. The samples are then analysed histologically to investigate their effect on structural protein organization. The culture supernatants are analysed by enzyme-linked immunosorbent assay (ELISA) and the messenger RNAs are analysed to determine whether gene expression patterns are consistent with known AD keratinocyte responses.

The cultured cutaneous tissue (RHE) upon stimulation showed disruption of structural proteins and, in particular, reduced levels of filaggrin and marked alteration of membrane staining of claudin-1 in histological samples (Fig. 2). The ELISA results demonstrated marked increases in TSLP and other markers of AD, while gene expression analysis showed that the expression profiles were in agreement with known keratinocyte responses to AD.

These results indicate that the RHE model may be relevant for AD and suitable for studying the impact of active ingredients, or *A. dolomia* extract in particular, by systemic exposure or incorporation into formulation for topical application.

Adaptive immunity model (T helper 2 response)

The Th2 allergic response is important in the development of AD. Reducing Th2 cytokine activity may therefore be an effective way of controlling AD. As such, study of the adaptive immune response is important. The bacterium *Staphylococcus aureus* is often found in AD lesions. The *S. aureus* protein product enterotoxin B (SEB) is considered a superantigen responsible for the dysregulated cytokine production by keratinocytes observed in AD.

One method used to analyse the adaptive immune response is a model that involves purifying human CD4⁺ lymphocytes from circulating peripheral blood mononuclear cells isolated from healthy donors. This method is illustrated in Fig. 3a and has been described in detail by Hauk et al.¹⁴ Briefly, a CD4⁺ lymphocyte population is purified and stimulated with SEB, with or without the anti-inflammatory agent dexamethasone. It is clear that experiments conducted using this model can be validated using both positive and negative controls when attempting to determine the anti-inflammatory activity of a

Table 1 Summary of *in vitro* models used for analysis of atopic dermatitis (AD)

Model name	Tissue/cells used	Reason for use	Physiological modelling	Strengths	Weaknesses	Key findings
AD keratinocyte model	Normal human epidermal keratinocytes	Keratinocyte cells are important in AD	Mimics inflammatory responses in AD	Use of a single cell type (keratinocytes) allows evaluation of the specific keratinocyte response	Not a whole-tissue model, does not allow evaluation of the influence of other skin cells	Reproduction of the targets modulated in AD
AD reconstructed human epidermis model	Three-dimensional epidermal keratinocytes	Whole-tissue model allowing epidermal response	Mimics environmental conditions of AD	Fully differentiated stratified epidermis, better understanding of gene expression patterns in AD	Contains only keratinocytes, free of immune cells	Stimulation with Th2 cytokines IL-4 and IL-13 and TLR ligands Poly(I:C) and Pam3CKS4 resulted in reduced levels of flaggrin and increased levels of claudin-1. Production of TSLP and overall expression profiles agreed with known keratinocyte responses to AD
Adaptive immunity model	CD4 ⁺ lymphocytes	Examination of immune response	Mimics immune response in AD	Model can be validated easily using positive and negative controls	Lacks the thoroughness of <i>in vivo</i> models. Not a whole-tissue sample	Stimulation of CD4 ⁺ lymphocytes with SEB results in production of specific cytokines found in the skin of patients with AD
Innate immunity model	Normal human epidermal keratinocytes	To determine the role of innate immunity and the importance of AMPs	Stimulation of innate immune system in AD	Use of a single cell type (keratinocytes) allows the evaluation of specific keratinocyte response	Lacks the thoroughness of <i>in vivo</i> models, not a whole-tissue sample	Evaluates the efficiency of an active ingredient to stimulate the production of AMPs, which are not sufficiently induced in the skin of patients with AD
Pruritus model	HaCaT keratinocyte cell line	Itch is a major symptom of AD and is partly mediated by the PAR-2 receptor	Mimics one mechanism of itch on cells in AD	Can be used in a dermocosmetic setting, unlike animal models	Difficult to model cutaneous innervation adequately	Incubation with SCTE, an epidermal enzyme, has been shown to induce PAR-2 expression

AMP, antimicrobial peptide; IL, interleukin; PAR, protease-activated receptor; SCTE, stratum corneum tryptic enzyme; SEB, *Staphylococcus aureus* enterotoxin B; TLR, Toll-like receptor; TSLP, thymic stromal lymphopoietin.

Fig 1. Modulation of gene expression in an *in vitro* model of normal human epidermal keratinocyte (NHEK) cells stimulated by Poly (I:C) and cytokines [tumour necrosis factor (TNF)- α + interleukin (IL)-4+ IL-13]. Induction (>2) or inhibition (<0.5) factors are given for the expression of 19 genes in the *in vitro* model of stimulated vs. nonstimulated NHEK cells. TSLP, thymic stromal lymphopoietin; IFN, interferon; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; MDC, macrophage-derived chemokine.

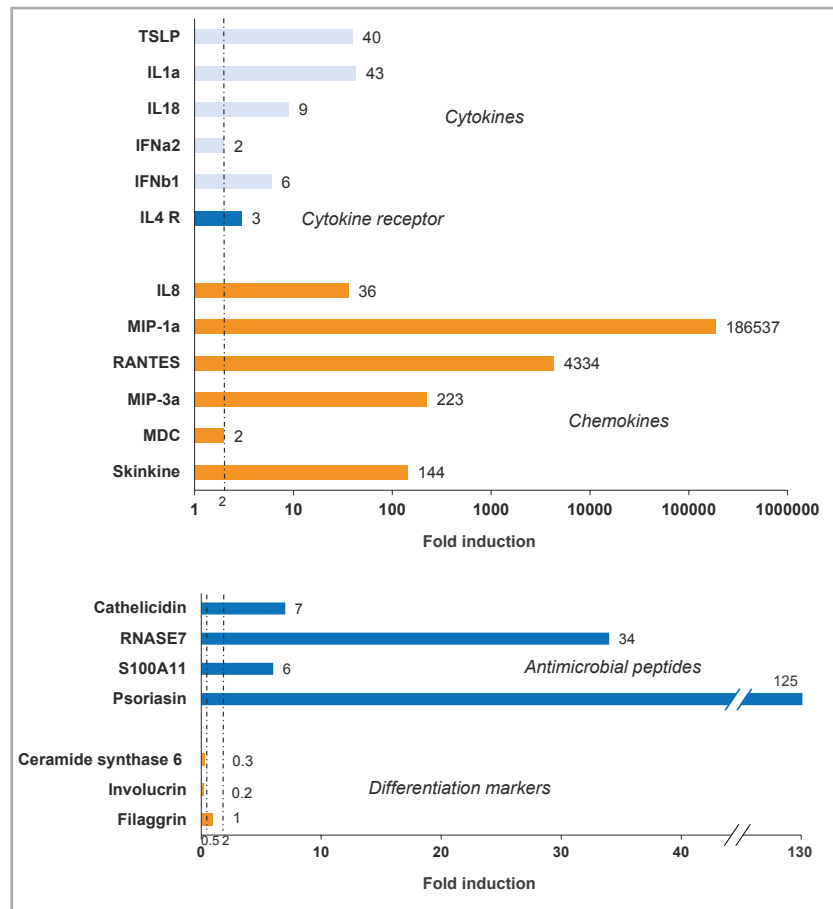
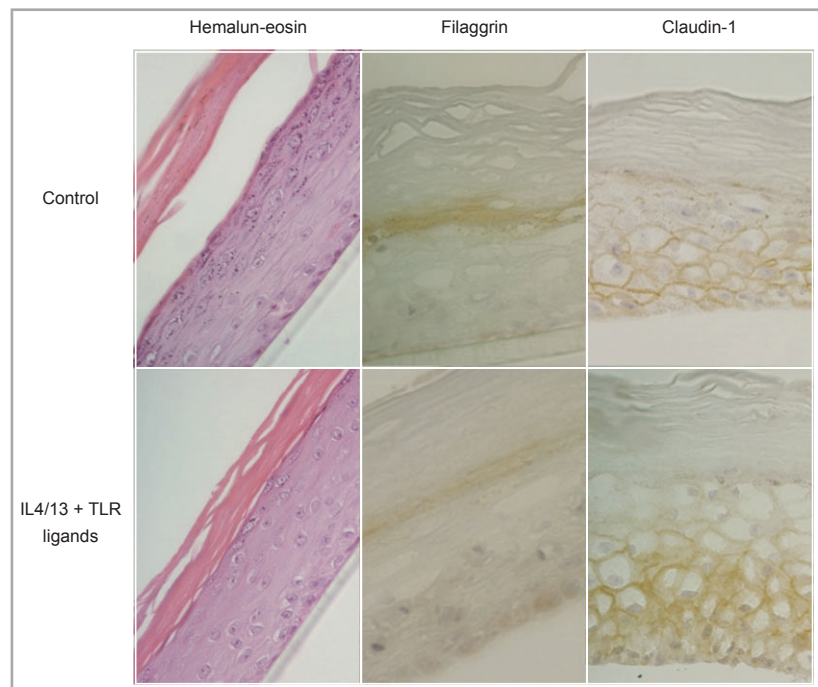


Fig 2. Histology demonstrating effects of incubation of reconstructed human epidermis with T helper 2 cytokines/Toll-like receptor (TLR) ligands. IL, interleukin.



new molecule across a number of adaptive immunity T-cell phenotypes.¹⁴

Figure 3b shows the results of multiplex analysis of CD4⁺ lymphocytes stimulated with SEB with or without dexametha-

sone. There is a distinct reduction of cytokine response across the Th1, Th2 and Th17 phenotypes.

By means of this assay, we were able to show that *A. dolomieu* extract inhibited Th1, Th2 and Th17 cytokines produced

by CD4⁺ lymphocytes in a dose-dependent manner. At the highest concentration tested, the expression of all target cytokines analysed was significantly reduced (data on file).

Innate immunity model (antimicrobial peptides)

The innate immune system is also important in AD. AMPs are naturally occurring compounds, which are sometimes referred to as defensins to describe their role in innate immunity. *In vivo* experimentation using mouse knockout models has demonstrated the importance of AMPs in immunity and resistance to skin infections. *In vitro* experiments with human keratinocytes have shown that AMPs are an important defence against bacterial infection.

AMPs such as cathelicidin antimicrobial peptide LL-37 (CAMP) and human β -defensin (hBD)2 are produced in the skin and are particularly abundant in inflammatory skin conditions.¹⁴ However, expression of LL-37 and hBD-2 is not up-regulated in subjects with AD.^{15,16}

We found that IL-1 β highly induced the expression of hBD2 and CAMP after incubation with NHEK cells (Fig. 4). Parallel experiments using *A. dolomieu* extract gave similar results. The effect of any immunomodulatory agents on AMP expression is of interest, as a low induction of several AMPs

in AD has been related to a higher susceptibility to *S. aureus* colonization, correlating with the severity of atopic lesions.¹⁵

Pruritus model

Itch is one of the major symptoms of AD and has a strong negative impact on patients' quality of life. Therefore, antipruritic treatment should play a central role in AD. The use of emollients that specifically target itch seems particularly promising as an adjunctive treatment for the daily management of AD, but testing for antipruritic activity has proven particularly difficult.

Pruriceptive transmission is a complex process that is not yet fully understood. Animal models have been developed,¹⁷ but because of legislation they are not suitable for dermatocosmetic testing. Cutaneous nerve fibres are present at higher densities in the epidermis of patients with AD.¹⁸ The development of a working validated pruritus model has been complicated by the difficulty of adequately mimicking cutaneous innervation.¹⁹ Indeed, there is no available *in vitro* model for cutaneous neurons to work with.

Keratinocyte-derived nerve growth factor (NGF) is one of the mediators determining skin innervation density in AD.²⁰ The intrinsic inhibitor semaphorin 3A is believed to prevent

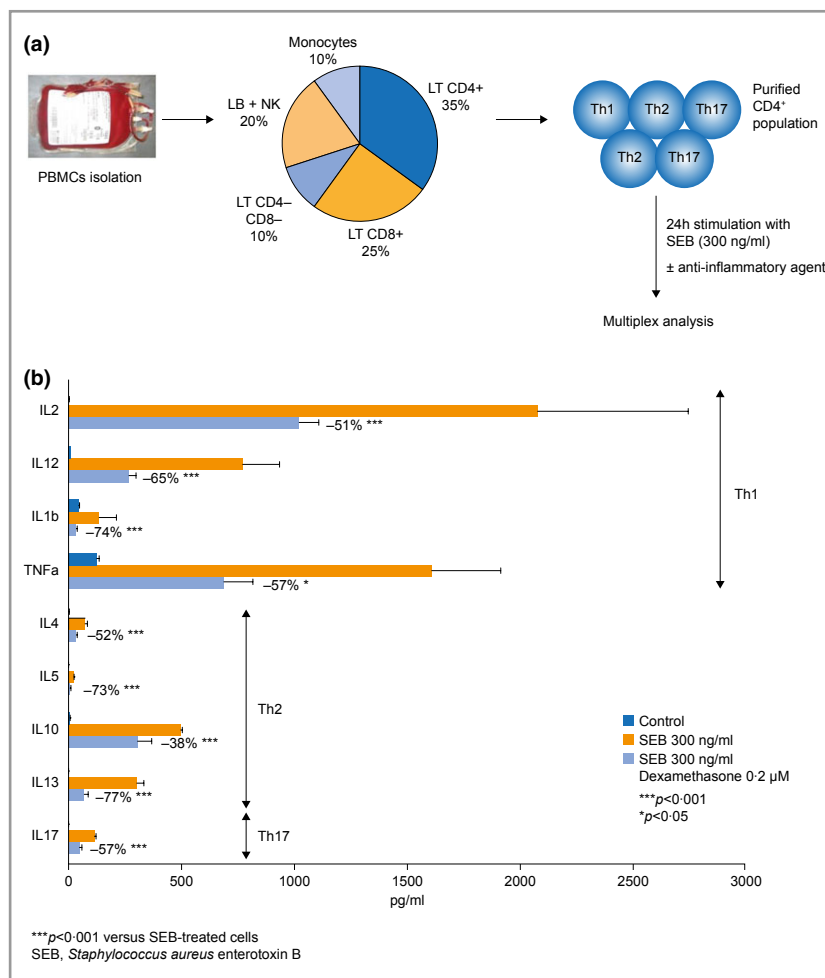


Fig 3. (a) Model to study the influence of an anti-inflammatory ingredient on the cytokine repertoire of activated CD4⁺ lymphocytes.

(b) Characterization of the production of T helper (Th)1, Th2 and Th17 cytokines of activated CD4⁺ lymphocytes. IL, interleukin; LB, B cell; LT, T cell; NK, natural killer cell; PBMC, peripheral blood mononuclear cell; SEB, *Staphylococcus aureus* enterotoxin B; TNF, tumour necrosis factor.

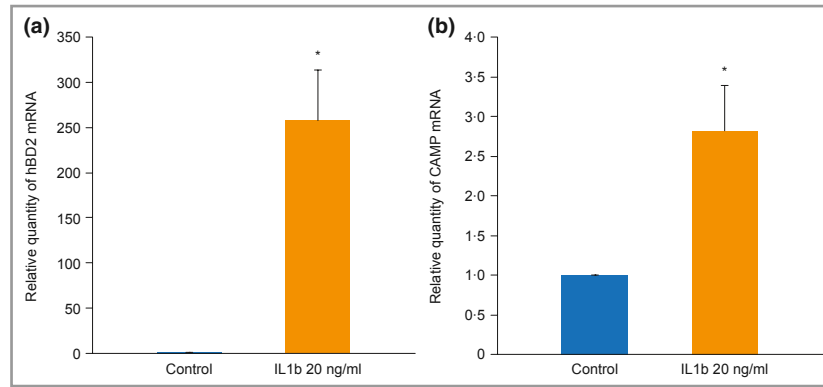


Fig 4. Upregulation of the antimicrobial peptides (a) human β -defensin (hBD)2 and (b) cathelicidin antimicrobial peptide (CAMP) by interleukin (IL)-1 β in normal human epidermal keratinocyte cells. * $P < 0.05$ vs. control cells.

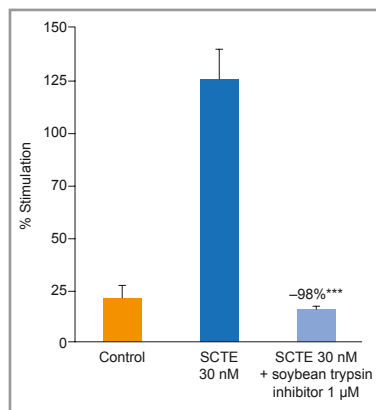


Fig 5. Stimulation of protease-activated receptor-2 receptors by stratum corneum tryptic enzyme (SCTE) in HaCaT keratinocyte cells and inhibition by soybean trypsin inhibitor. * $P < 0.05$, *** $P < 0.001$ vs. cells treated with SCTE alone.

the elongation of epidermal nerve fibres in normal skin, and its decreased expression accelerates epidermal nerve growth in AD.¹⁸

Mast cells are also involved in the inflammation via Th2 cytokines. There is release of pruritogenic substances by mast cells, including histamine and NGF, in response to IgE cross-linkage and nonimmunological stimuli, such as stress.^{21,22} NGF, in turn, has also been shown to stimulate platelet-associated mast cell activation in response to inflammatory stimuli.²³

In addition, histamine has been shown to affect itch sensation via the H4 receptor (expressed on the surface of sensory neurons, keratinocytes and fibroblasts in the skin).^{24,25}

The neurogenic pathways involved in pruritus are partly mediated by the protease-activated receptor (PAR)-2 receptors, which are located on neurons and keratinocytes.²⁶ Therefore, looking for PAR-2 receptor antagonists in keratinocytes is a promising way to look for molecules that can influence itch.²⁷

Preincubation of the tracer Fluo-4 acetoxymethyl ester with the HaCaT keratinocyte cell line, followed by stimulation with stratum corneum tryptic enzyme (SCTE) and subsequent measurement of the enzyme kinetics using fluorescence, has been

used to show that SCTE can activate the PAR-2 signalling pathway. This activation is abolished by adding a reference molecule, a soybean trypsin inhibitor (Fig. 5). The same model and approach have been used to evaluate the potency of *A. dolomiae* extract to interfere with one of the mechanisms involved in itch.

Conclusions

With the help of modern gene technology, different kinds of knockout and knock-in mouse strains generating an AD-like phenotype have been generated, and offer a vast array of possibilities to study the importance of different genetic factors in the pathomechanisms of AD. However, these models are not suitable for dermocosmetic research and the search for alternative methods has been a challenge for industry research.

Our strategy was then to select and develop the appropriate approaches outlined here through different *in vitro* models that cover the different alterations encountered in AD pathology such as inflammation, immune dysfunction, disturbed barrier function and pruritus. In particular, they have proved useful in defining the biological activities of *A. dolomiae* extract (data on file).

Complementary to the cell culture models, RHE models will be useful for testing active ingredients incorporated into formulations for topical application. Such *in vitro* models could be seen as the next step for dermocosmetic testing of active ingredients for adjunctive treatment of AD.

Acknowledgments

The authors would like to thank Dr Alan Murphy and MedSense Ltd, High Wycombe, U.K. for providing editorial assistance, which was funded by Pierre Fabre Dermo-Cosmétique, France.

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