

K. Rodan[†], K. Fields[†], L. Bush[†], L. Zhang[‡] and T.J. Falla[‡]

[†]Rodan + Fields Dermatologists, San Francisco, CA ; [‡] Helix BioMedix Inc., Bothell, WA

Background

LL-37 is a naturally occurring antimicrobial peptide in human skin. Its key role is to protect against infection by killing bacteria, fungi and viruses in the skin and other organs (1). In addition, LL-37 can also produce pro-inflammatory activity (2, 3), anti-inflammatory activity (4), promote chemotaxis (5) and angiogenesis (6), and enhance wound repair (7). Owing to this array of functions, LL-37 has been implicated in a wide variety of inflammatory skin diseases including psoriasis, atopic dermatitis, and rosacea (3, 8, 9). In skin, the various manifestations of LL-37 activity are determined by the presence of proteolytic enzymes that can segment the full length LL-37 into smaller peptide fragments. It is proposed that the result of such proteolytic peptide processing is responsible, at least in part, for the symptoms of inflammatory skin conditions such as rosacea (3).

As with all peptides and proteins, LL-37 is susceptible to processing and degradation by a wide range of proteases. When compromised, such as in dry skin conditions, facial stratum corneum (SC) has been shown to contain increased levels of pro-inflammatory cytokines and proteases. Seasonal variation in SC biophysical and biological characteristics is well described. Importantly, winter weather has been shown to more severely affect the properties of skin exposed to the harsh environmental conditions than those of skin that is routinely covered. Voegeli et al. (10) examined the distribution of key protease activities in different layers of the SC on the cheek and the forearm by analysis of consecutive tape strippings of healthy Caucasian subjects in winter and summer. The activity of proteases was approximately two to four times higher on the cheek than on the forearm. In the case of rosacea, often triggered by such environmental conditions, these proteases can lead to the breakdown of LL-37 into its pro-inflammatory fragments (3). In the case of psoriasis, such proteases can be responsible for accelerating the desquamating cycle of superficial cells, leading to scaling and over-shedding of the skin. These conditions lead to inflammation and other significant clinical symptoms. Even at sub-clinical levels, these processes can lead to dry, reddened skin and a breakdown in barrier function (leading to loss of skin moisture), breakdown of extracellular matrix and premature skin aging.

Yamasaki and Gallo (11) recently proposed that all forms of rosacea are associated with a dysfunction of the innate immune system and that individuals with rosacea have elevated levels of LL-37, LL-37 pro-inflammatory fragments and proteases. The factors that promote LL-37 production and increases in protease levels include microbes and environmental changes, such as sun and UV exposure which lead to the histological changes commonly observed in rosacea.

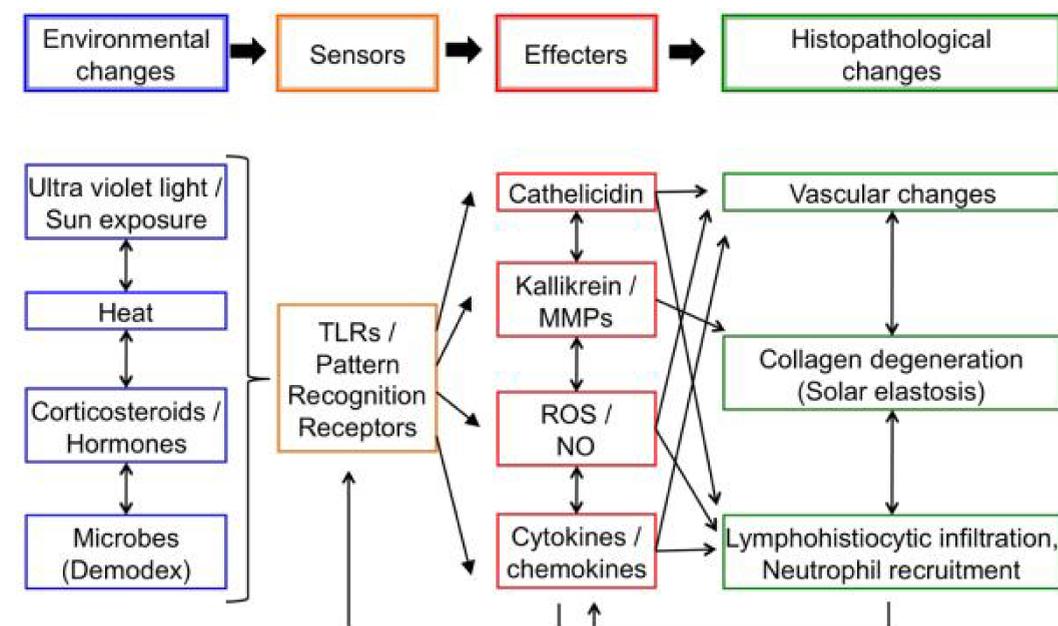
Figure 1: Immunity and rosacea

The possible molecular mechanisms for the pathogenesis of rosacea

The molecular pathology of rosacea.

Yamasaki K and Gallo RL.

J Dermatol Sci. 2009 Aug;55(2):77-81.



Introduction

In healthy individuals, the cathelicidin peptide LL-37 is a key component of skin's innate immunity system. However, in patients with rosacea, increased proteolytic processing of LL-37 is believed to be a factor in the inflammatory process. It has been suggested that down-regulating this proteolytic processing may have benefit to rosacea patients. To test the hypothesis that targeting the production of pro-inflammatory peptide fragments on skin could mitigate the symptoms of rosacea, we designed a complex (RFp3) containing a protease inhibitor and two anti-inflammatory peptides (Oligopeptide-10 and Tetrapeptide-16). This complex was designed around three specific functionalities: (1) the inhibition of surface proteases (2) the delivery of peptides known to reduce elevated cytokine levels and (3) the protection of these exogenous peptides from surface protease attack. The peptides in the complex were designed to target reduction in cytokine levels via two mechanisms of action: interruption of the Toll-like receptor pathway of inflammation and inhibition of bacterial toxins known to be a key inducer of LL-37. The purpose of including a protease inhibitor in the RFp3 technology was two-fold. Firstly, the protease inhibitor slows the breakdown of LL-37 into pro-inflammatory fragments thus reducing one of the key triggers of rosacea. Secondly, its inclusion protects the exogenous anti-inflammatory peptides when applied to the skin's protease rich environment, thus extending the activity of these extremely valuable molecules.

The personal care industry has, for the most part, relegated the application of bioactive peptides to the cosmetic care of aging skin. However, because peptides modulate a wide array of the body's cellular functions, there is a host of skincare opportunities associated with innovative application of peptide technology. Our goal was to offer enhanced benefits of daily facial skincare to individuals presenting with hyper-irritable skin, particularly those prone to flairs of rosacea. A novel formulation was created to comply with the monograph for Skin Protectant Drug Products for Over the Counter Human Use, 21 CFR Parts 310, 347, and 351. The formulation includes the active ingredients allantoin and dimethicone, along with the previously described cosmetic RFp3 technology and optical diffusers as components of a lotion vehicle.

Here we describe the in vitro characterization of the RFp3 complex and the examination of its incremental benefit when compared to a standard formulation in vivo.

In vitro testing

Oligopeptide-10: One of the important triggers, of the innate immune system, is toxins and in particular those that are present on the outside of Gram-positive bacteria. One such toxin, lipoteichoic acid (LTA), causes inflammatory reactions in the skin whether attached to bacteria or released (Figure 2A). Oligopeptide-10 is an amphipathic, alpha-helical peptide specifically designed to mimic the LTA binding and neutralizing capability of innate immunity peptides. The binding affinity of Oligopeptide-10 for LTA was determined using the LTA binding assay described previously by Scott *et al.* (Infect. Immun. 76: 6445-6453, 1999). Dansyl polymyxin B (DPX) was used at a concentration of 2.5 μM to obtain 90 to 100% of the maximum fluorescence when bound to LTA. The DPX and 5 μg of *S. aureus* LTA were mixed in 1 ml of 5 mM HEPES (pH 7.2). Fluorescence was measured by the fluorescence spectrophotometer. Sequential additions of peptide, in 1 μg amounts, were made to reactions mixtures, and the decreases in DPX fluorescence was determined. As seen in Figure 2B 5 μg of Oligopeptide-10 reduces the available LTA, capable of initiating an inflammatory response, by 50%. This continues to be reduced with the further addition of peptide.

Figure 2A: Inflammatory cascade induced by LTA

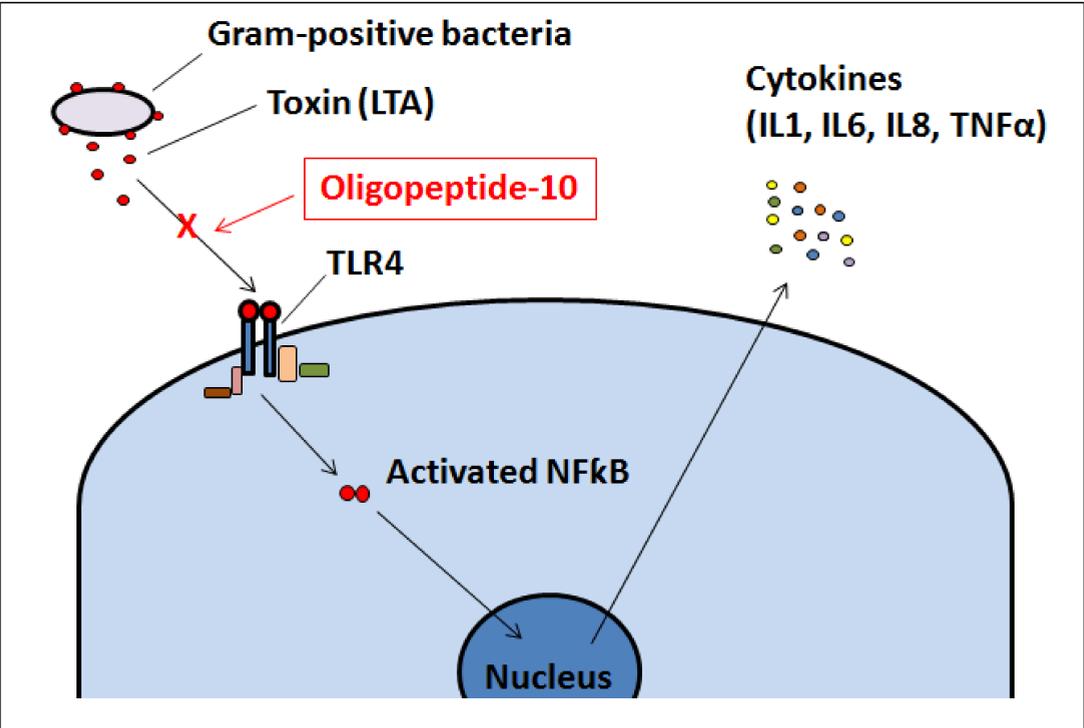
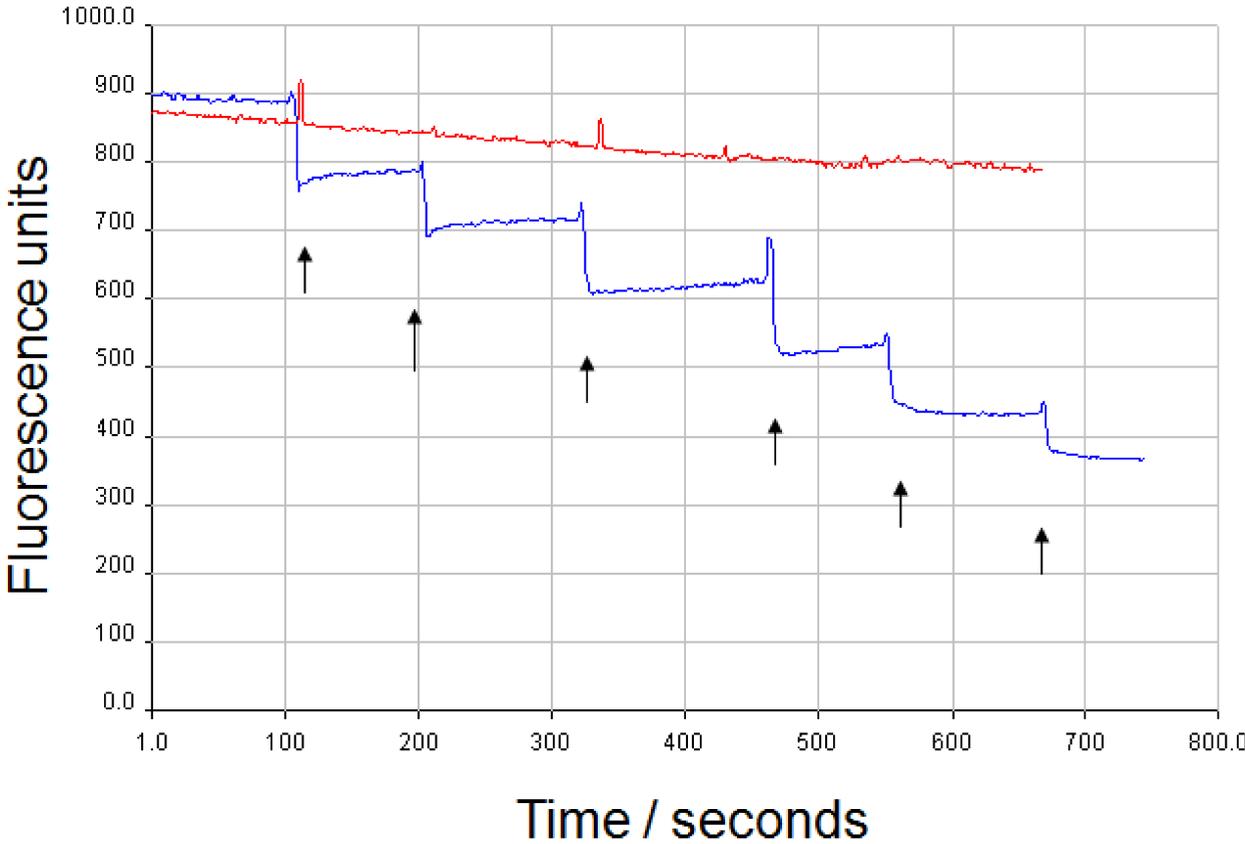


Figure 2B: Oligopeptide-10 binding of LTA



In vitro testing

Tetrapeptide-16: If the innate immune system is triggered by the environment, by microbes, or by sunlight, specific pathways are activated that lead to inflammation. Tetrapeptide-16 was specifically designed to dampen down these pathways irrespective of the trigger (Figure 3A). Human skin epithelial cells (ATCC CRL-2592), keratinocytes (ATCC CRL-2404) and skin fibroblasts (ATCC CRL-7481) were employed in the study. Cells were seeded into 6-well plates and allowed to grow to >95% confluence in Dulbecco's modified Eagle's medium (DMEM; 4 mM L-glutamine, 4.5 g/L glucose) adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS). For keratinocytes, cells were grown in keratinocyte growth media (without serum) supplemented with 5 ng/mL human recombinant epithelial growth factor (EGF; Invitrogen, Grand Island, NY). After the cell monolayer reached >95% confluence, the cells were serum-starved or EGF-starved for 24 hours in complete medium without serum. UVB was generated using a UVLMS lamp (4-W model, 3UV assembly; Upland, CA) with the irradiation wavelength set at 302 nm. The UV lamp was placed 15 cm above the tissue culture plate. Before UV treatment, the tissue culture media was replaced with PBS, after which the cells were placed under the UVB lamp (450 mW/cm², measured using a radiometer) for 35 seconds (epithelial cells and fibroblasts) or 25 seconds (keratinocytes). After UV treatment, PBS was immediately replaced with complete medium (without serum or EGF) containing either no peptide or peptide at a specified concentration, and the plates were incubated at 37 °C, 5% CO₂ for 15-24 hours. The cell media was then collected and spun down at 15000 rpm for 2 minutes to remove cell debris. IL-6 and MMP-1 levels in the media were measured, respectively, using human IL-6 (DIACLONE, Stamford, CT) and MMP-1 (Calbiochem, San Diego, CA) ELISA kits according to manufacturers' instructions. These measurements served as indicators of cell inflammatory activity in response to UV exposure and the effects of Tetrapeptide-16 can be seen in Figure 3B in which the peptide reduces IL6 induction due to UVB induction by 32%.

Figure 3A: Tetrapeptide-16 target sites

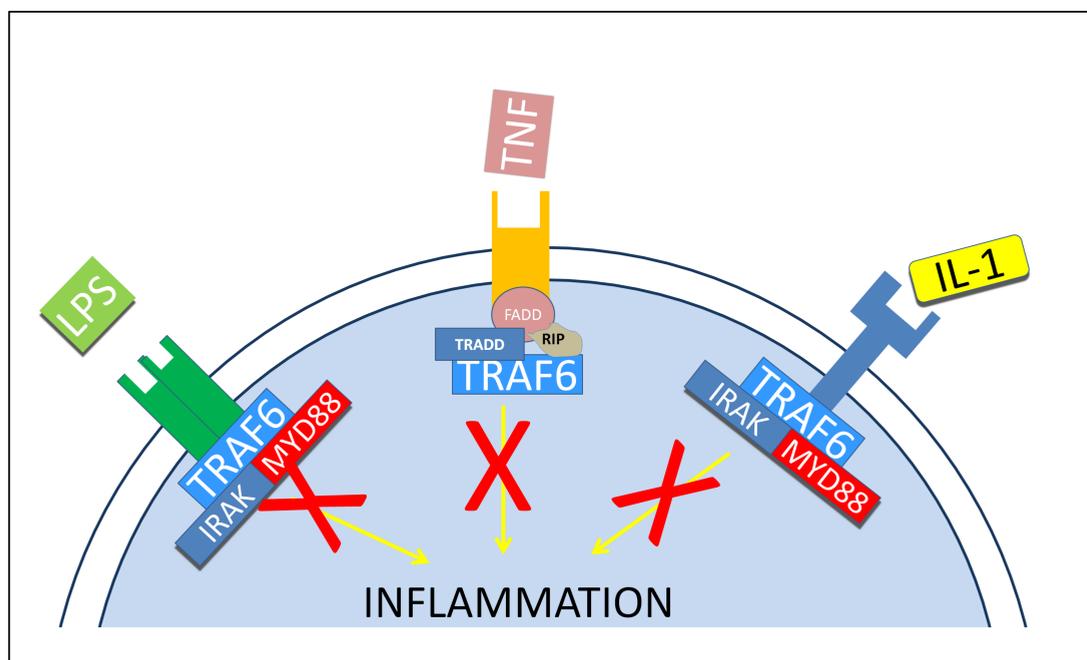
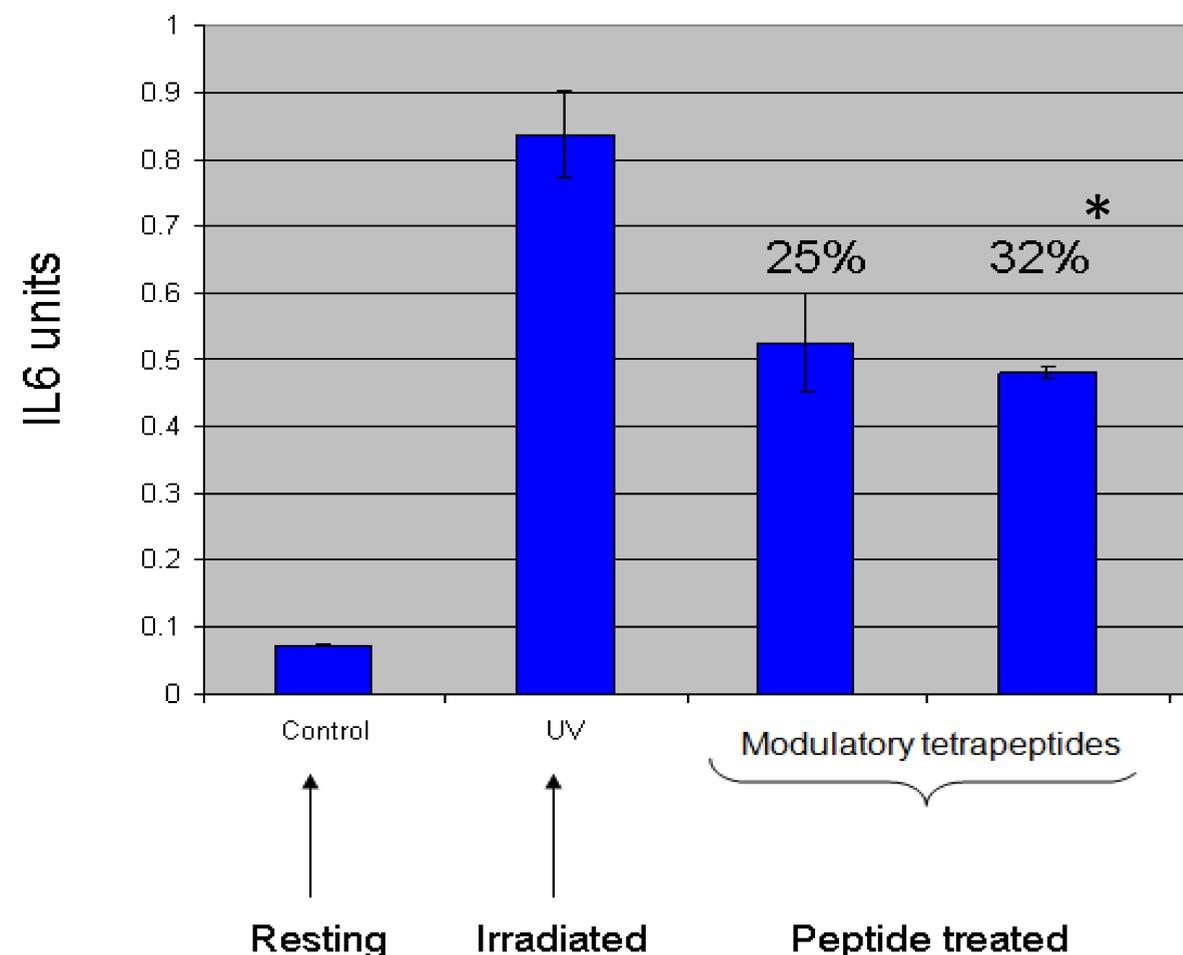


Figure 3B: Tetrapeptide-16* reduction of UV induced inflammation in keratinocytes



In vitro testing

Protease inhibitor: The purpose of including a protease inhibitor in the RFP3 complex was two-fold. Firstly, the protease inhibitor slows the breakdown of LL-37 into pro-inflammatory fragments thus reducing one of the key triggers of rosacea (Figure 4). Secondly, its inclusion protects Oligopeptide-10 and Tetrapeptide-16 when applied to the skin's protease rich environment, thus extending the activity of these molecules (Figure 5).

Figure 4A:
LL-37 exposed to protease for 1 minute

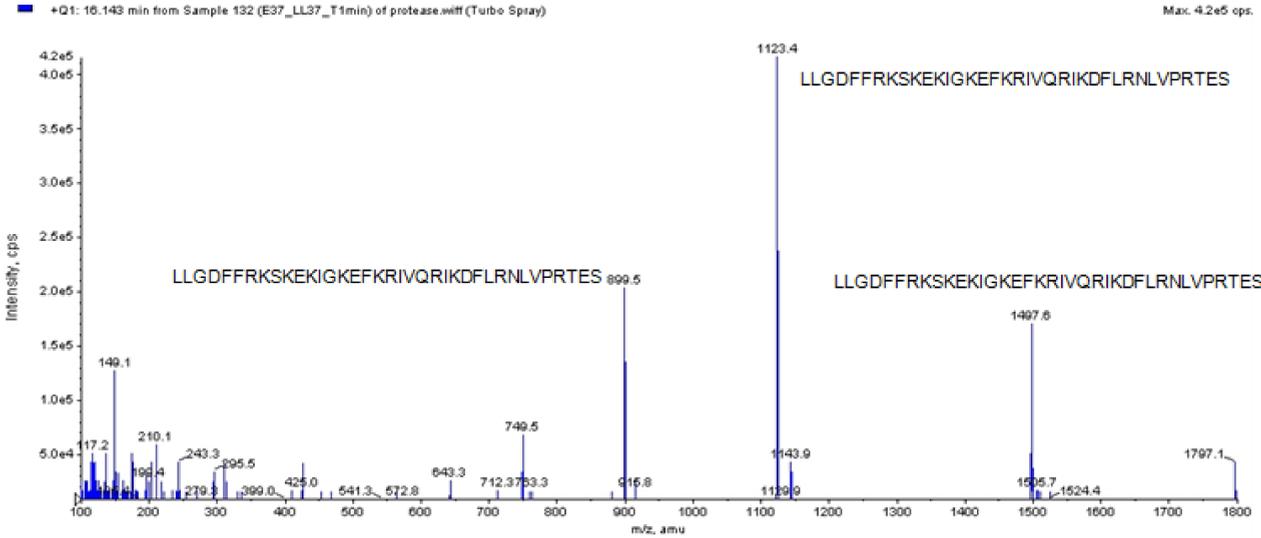


Figure 4B:
LL-37 exposed to protease for 1 hour

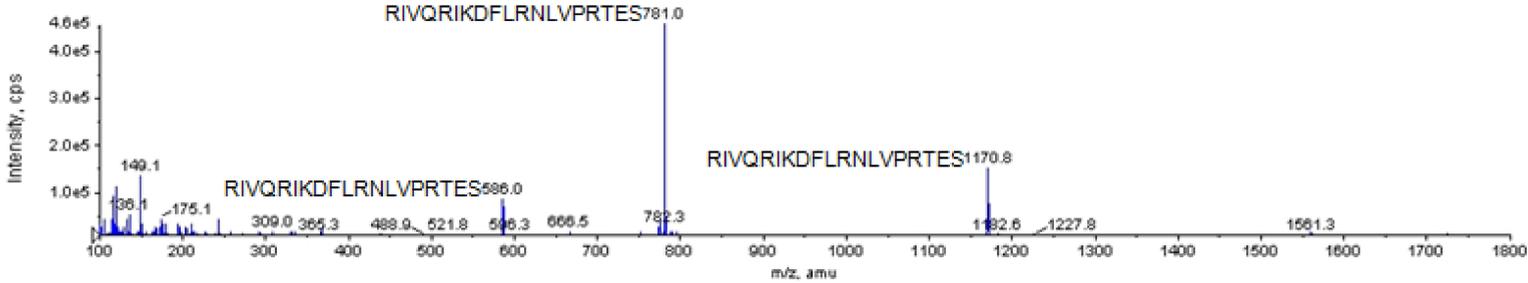
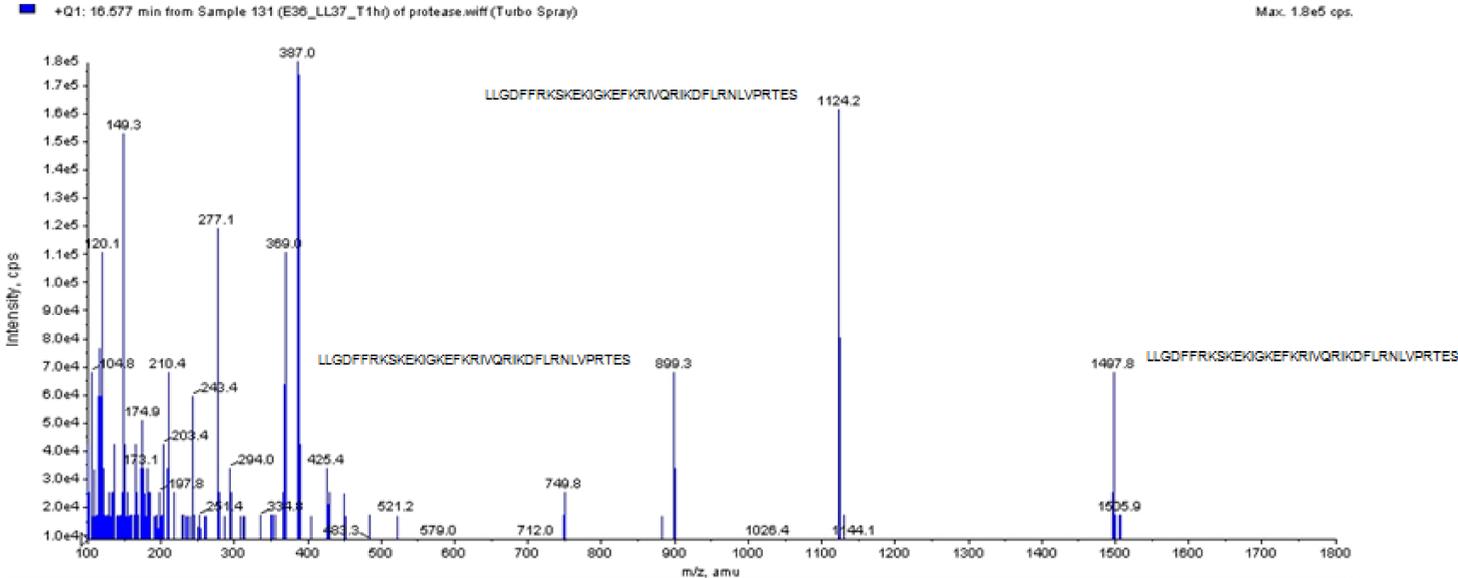


Figure 4C:
LL-37 exposed to protease for 1 hour in the presence of protease inhibitor



In vitro testing

Figure 5A: Protease degradation of target peptide Oligopeptide-10 is degraded by a protease as seen at 1 hour and 23 hours.

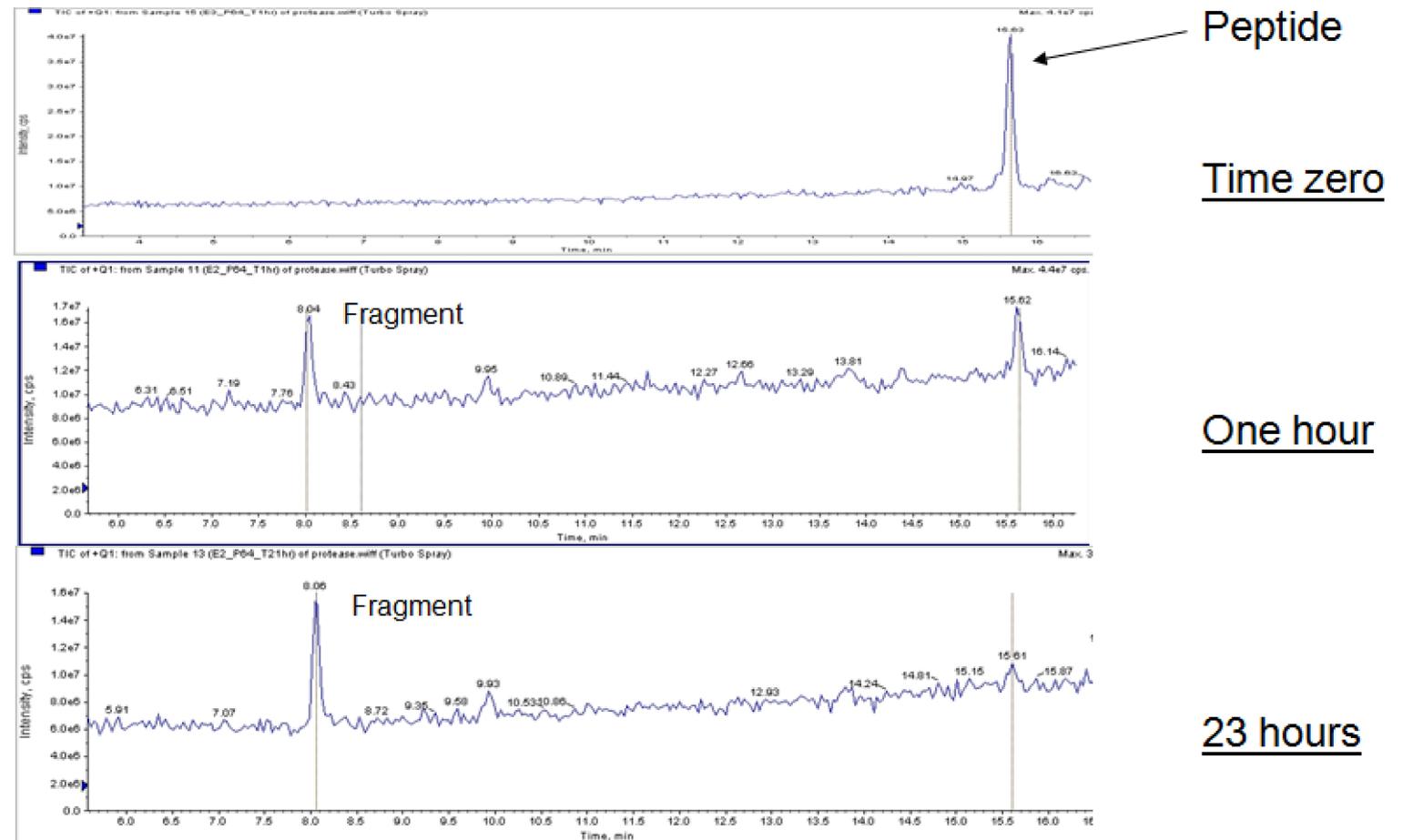
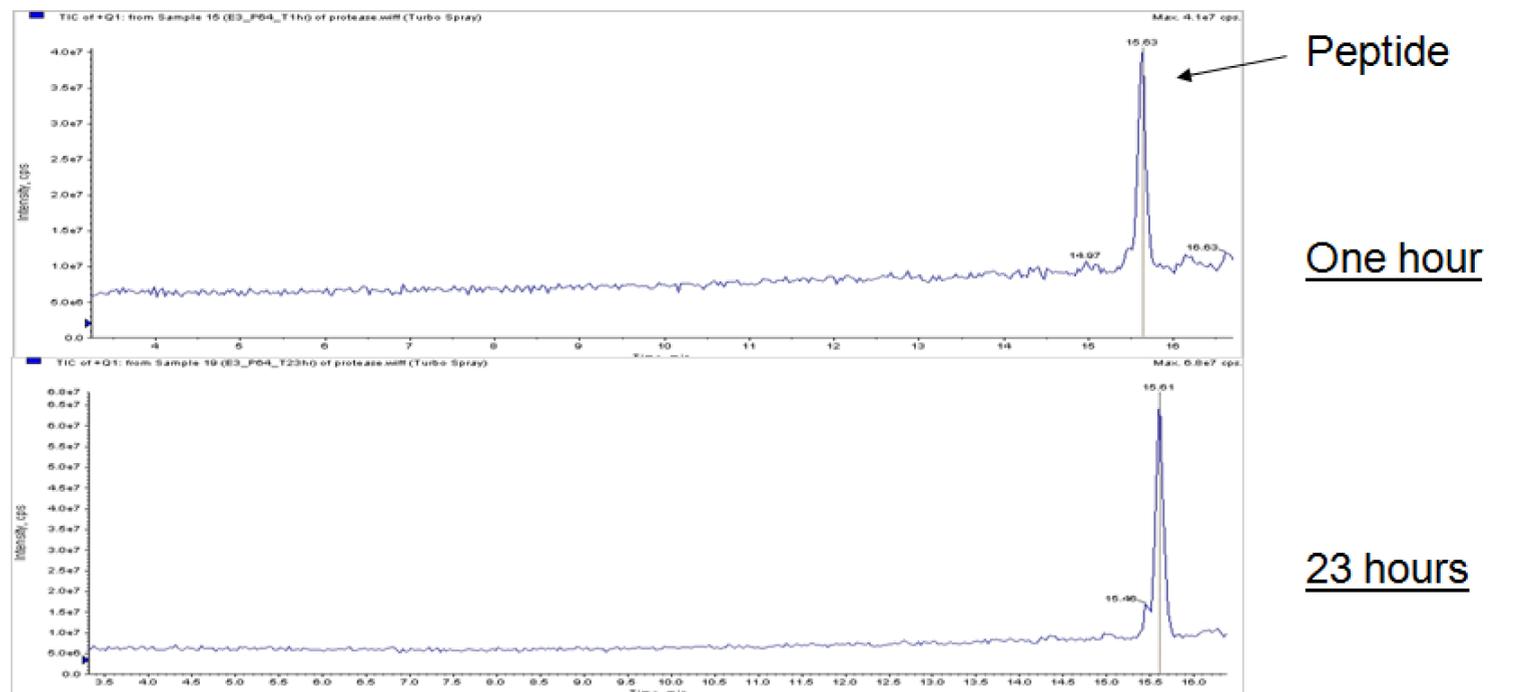


Figure 5B: Protease inhibitor protection of target peptide. After 23 hours of exposure to proteases, Oligopeptide-10 remains present and intact in presence of protease inhibitor.



Clinical testing

To validate incremental skincare benefits associated with the addition of RFp3 technology to a traditional emulsion containing allantoin and dimethicone, a double blind clinical study was performed on thirty subjects presenting with hyper-irritable skin, including subjects with mild to moderate rosacea. Subjects were graded (0-none, 1-minimal, 2-mild, 3-moderate and 4-severe) for skin redness, peeling, drying and overall irritation by clinical graders and divided into two groups. One group received a formulation with RFp3 added to a vehicle containing the OTC ingredients, a second, control group, received the OTC vehicle formulation alone. Subjects cleansed their skin with a bland emulsion cleanser and then applied either the RFp3 formulation or the OTC treatment vehicle only. All subjects were reassessed for signs of irritation after 5 minutes, two weeks and four weeks. Subjects in both arms of the study showed a statistically significant ($p < 0.05$) improvement in overall irritation at all three time points. However, at 5 minutes, two weeks and four weeks, the RFp3 group showed a greater improvement ($p < 0.001$) compared to the control arm ($p = 0.033$). An example of the data can be seen in Figure 6.

Figure 6A:

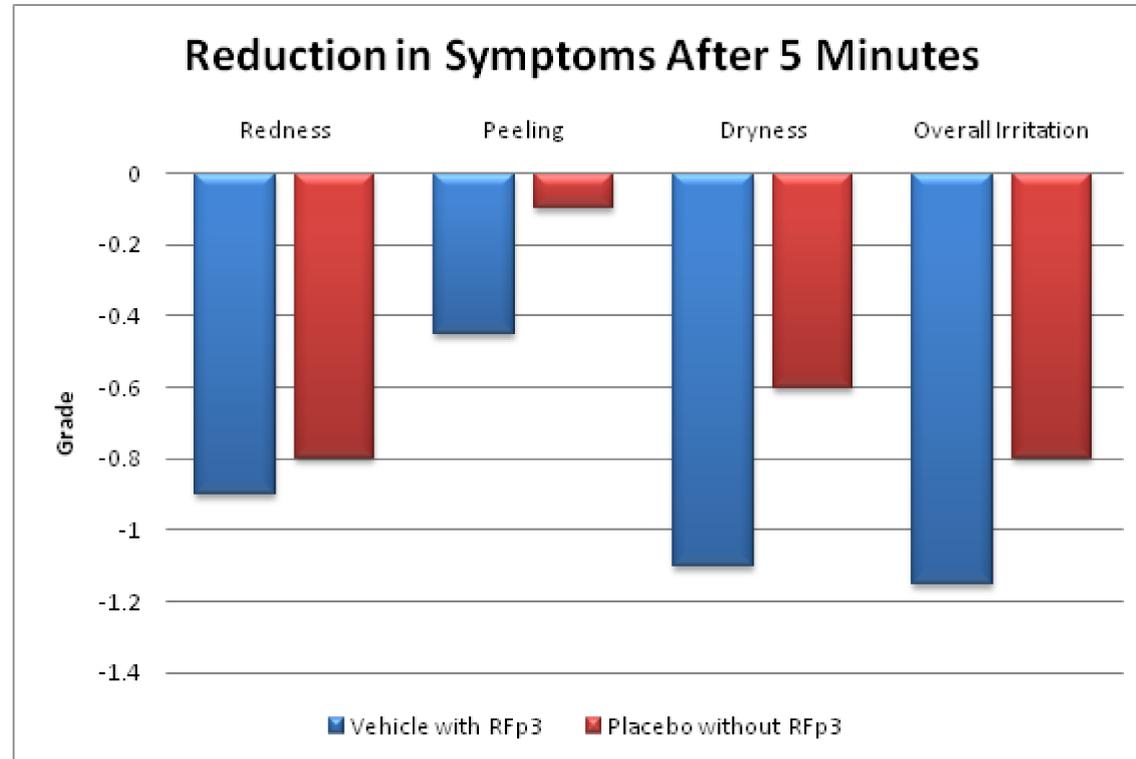
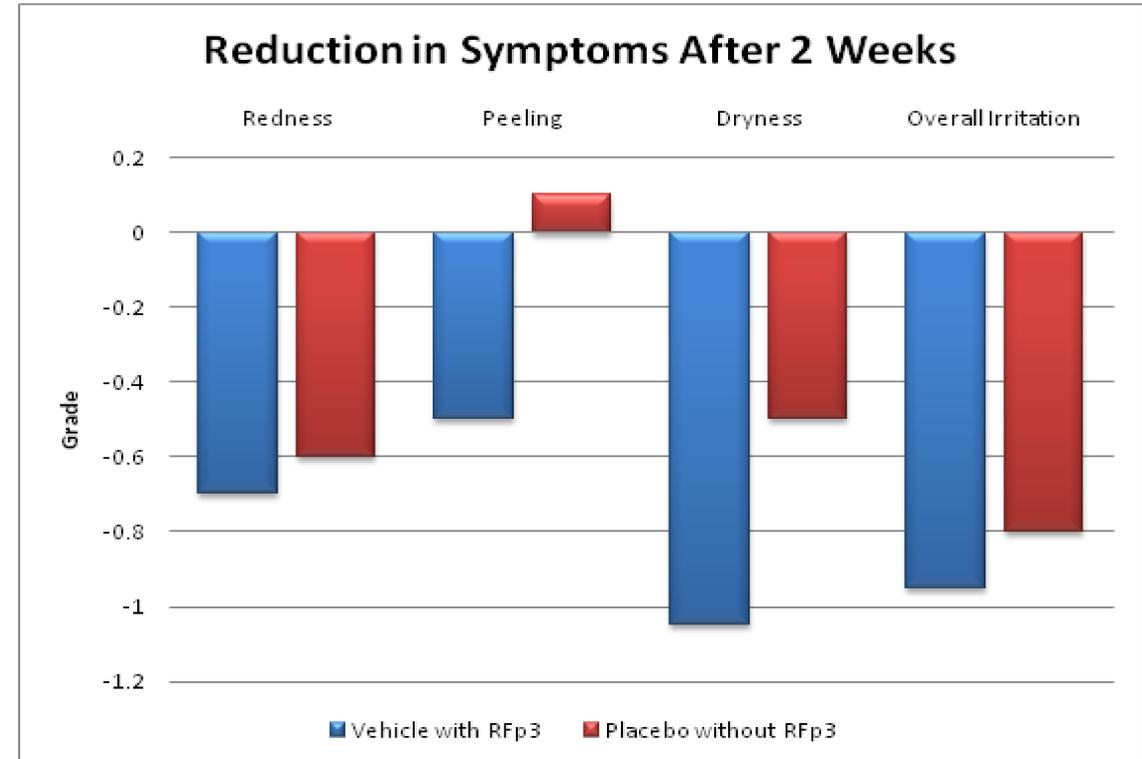


Figure 6B:



CONCLUSION

The opportunity to safely and effectively enhance the benefits of an OTC skin protection drug product without application of topical corticosteroids is advantageous for daily care of both acute and chronic hyper-irritable skin conditions. Bioactive peptides help block inflammatory triggers on skin's surface. The introduction of protease inhibitors further interrupt pro-inflammatory changes in skin while supporting the bioavailability of exogenous peptides. Together, bioactive peptides with a protease inhibitor are proven to provide safe and effective incremental benefits to traditional OTC skin protectant formulations.

REFERENCES

1. M. Zanetti, R. Gennaro et al., *Curr Pharm Des.*, **8**, pp. 779-93 (2002).
2. M.H. Braff, M.A. Hawkins et al., *J Immunol.*, **174**, pp. 4271-4278 (2005).
3. K. Yamasaki, A. Di Nardo et al., *Nat. Med.*, **13**, pp. 975-80 (2007).
4. N. Mookherjee, K.L. Brown KL, et al., *J. Immunol.*, **176**, pp. 2455-2464 (2006).
5. D. Yang, Q. Chen et al., *J. Exp Med.*, **192**, pp. 1069-1074 (2000).
6. R. Koczulla, G. von Degenfeld et al., *J. Clin. Invest.*, **111**, pp.665-72 (2003).
7. S. Tokumaru, K. Sayama et al., *J. Immunol.*, **175**, pp. 4662-4668 (2005).
8. P.Y. Ong, T. Ohtake et al., *N. Eng.I J. Med.*, **347**, pp. 1151-60 (2002).
9. R. Lande, J. Gregorio et al., *Nature*, **449**, pp. 564-569 (2007).
10. R. Voegeli, A.V. Rawlings et al., *Int. J. Cosmet. Sci.*, **29**, pp. 191-200 (2007).
11. K. Yamasaki, R.L. Gallo, *Dermatol. Sci.*, **55**, pp. 77-81 (2009).