



Research Article

Effect of Tripeptide-85 (EVF) on Sebogenesis

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Abstract

Excess skin sebum production has been a concern and could result in oily skin and acne. The cosmetic industry's search for compounds that modulate sebogenesis has been increasing. MC5R is an important regulator of this process. In this work, we report an *in silico*-designed peptide (Tripeptide-85) that regulates MC5R. We observed that Tripeptide-85 downregulates several sebocyte development and differentiation markers, such as PLIN2, MYC, and SCD, inhibits the formation of lipid droplets in SEBO662AR cells, and reduces sebum production in a clinical randomized, single-blind, placebo-controlled trial. The tripeptide-85 can modulate sebogenesis and can be used alone or as an adjuvant in the management of conditions related to excess sebum production such as acne and oily skin.

Keywords: Sebum; Cosmetic peptides; Computer modelling; Chemical synthesis; Sebocytes.

Introduction

The sebaceous glands (SG) are multilobular, glandular structures associated with hair follicles and constitute the pilosebaceous units of the skin. Their main function is the production of sebum [1]. Under normal physiological conditions, sebum contribute to the function of the skin, as they reduce trans epidermal water loss, carry antioxidants to its surface, bring innate antimicrobial activity, and affect inflammatory processes [2].

Alterations in normal physiological processes can lead to pathological conditions such as seborrhea, which involve excessive sebum production due to the hyperactivity of SGs, and the variation in sebum composition is affected. This demonstrates that not only can sebum production act as a marker of diseases but

can also play a significant role in its pathogenesis [1,3]. It favors the development of conditions such as acne [4] and seborrheic dermatitis [1], in addition to biofilm formation, thus interrupting the function of the follicular barrier, inducing comedogenesis and promoting subsequent inflammation [3].

One of the most effective substances capable of reducing sebogenesis is isotretinoin. However, it is not exempt from adverse side effects such as dry mucous membranes, dry skin, nosebleeds, and dry eyes [5,6]. The most serious effects related to its use are suicidal ideation, pancreatitis, hepatitis, cataracts, and menstrual disorders, among others [6]. Due to these outcomes, it is important to search for new compounds that can act with the same purpose while reducing the drawbacks and increasing the desired effect. In cosmetics, the current main target for sebum control is 5 α reductase. Clinical trials indicated an inverse correlation between sebum excretion rate and 5 α reductase expression in women only; for men,

no significant correlation was found [6]. A few other clinical trials indicated the likely functionality of 5 α reductase inhibitors in acne treatment [8]. However, a clinical trial has already been reported where a 5 α reductase inhibitor alone was proved ineffective in the treatment of acne vulgaris [9]. For this reason, the identification and exploration of new drug molecules relevant to the control of sebogenesis is necessary. The melanocortin receptors (MCRs) are a family of five neuropeptide hormones derived from the family A (rhodopsin-like) G protein-coupled receptors (GPCRs) [10,11]. These receptors have diverse functions in the body due to their involvement in several homeostatic and physiological processes including energy homeostasis, skin pigmentation, hemodynamics, inflammation, adrenocortical steroidogenesis, exocrine secretion, sexual function, and immunomodulation [10]. Among the five melanocortin receptors, two of them are expressed in human sebocytes: MC1R and MC5R. The former plays an important role in regulating skin and hair pigmentation, in addition to acting on skin homeostasis, while the latter has been associated with sebocyte differentiation and sebum production [4].

The cosmetic industry offers several products intended to clean, promote beauty, improve attractiveness, and even modify appearance. Vitamins, growth factors, antioxidants, polysaccharides, amino acids, and peptides are commonly used to fulfill these objectives [12]. Peptides have increasingly attracted the attention of the cosmetic industry due to their bioactive properties, such as high activity at low dosages, ability to interact with skin cells through various mechanisms, and small molecular size, a property that helps impart moderate penetration into the skin layers [13].

In the process of testing novel molecules, the biological activities of peptide sequences are being predicted by computer aided drug design techniques, prior to experimental analysis [14]. In this approach, molecules can be evaluated for interaction with specific receptors, providing evidence of binding affinity, pattern, and conformation, and thereby minimizing the number of tests to be performed in a wet lab. Thus, it is a more economical and sustainable process while reducing the time required for predicting the biological activity of small molecule candidates [15]. Most current commercial peptides in the market today are produced by chemical methods, often by solid phase peptide synthesis (SPPS). SPPS is a simple and fast method for obtaining peptides and has a clear advantage over other methods because of its capability of generating high-purity peptides, especially short ones. The SPPS protocol employs a series of deprotection and coupling reactions with washing steps in between, starting from a resin-linked amino acid that later becomes a resin-peptide entity. The peptide is then cleaved from the resin and easily purified. The present study reports a novel peptide (L- α -glutamyl-L-valyl-L-phenylalanine (EVF), Tripeptide-85) synthesized by SPPS, that presents *in vitro*

and *in vivo* MC5R inhibitory activity.

Materials and Methods

In silico studies

The melanocortin receptor 5 (MC5R) was previously investigated as a molecular target for the discovery of novel ligands regulating sebum production. In that study, an MC5R model was built using homology modeling, and a structure-based virtual screening was performed, using a molecular database extracted mostly from the free database ZINC [16], totalizing about 300,000 entries. The molecules were ranked according to their docking scores.

Peptide synthesis

The EVF peptide (tripeptide-85) was synthesized by the standard SPPS methodology using Wang resin and standard Fmoc (9-fluorenylmethyloxycarbonyl) protocols as described by Assane and co-workers, 2021 [17]. The peptide was cleaved from the resin using a mixture of trifluoroacetic acid, water, and triisopropylsilane. The crude peptide was lyophilized and was purified by semipreparative reversed-phase liquid chromatography using a C18 25 \times 10 mm column, and a gradient elution (5 mL/min) with 0.045% trifluoroacetic acid aqueous solution (solvent A) and 0.036% trifluoroacetic acid in acetonitrile (solvent B) from 10% to 40% B in 90 minutes, with UV detection at 220 nm. The fraction containing the peptide was collected with the aid of a fraction collector and lyophilized. The molecular weight of the peptide was confirmed by mass spectrometry.

Cytotoxicity assay

For the definition of the highest non-cytotoxic concentration of Tripeptide-85, a standard MTT reduction assay was performed in a SEBO662AR (human) sebocyte cell line. Cells were grown at 37°C, 5% CO₂ in Keratinocyte-SFM medium supplemented with 25 μ g/mL gentamycin. The peptides were diluted in a stock solution of 100 mM in dimethylsulfoxide (DMSO), and from this solution, concentrations between 0.457 μ M to 1000 μ M were tested for a period of 48 and 120 hours. At the end of the treatment, the cells were incubated with MTT reduced into blue crystals of formazan by succinate dehydrogenase. After cell dissociation and solubilization of formazan crystals using DMSO, the optical densities (OD) of the extracts at 540 nm were recorded with a spectrometer (VERSAmax, Molecular Devices).

Culture and treatment for lipid content analysis

SEBO662AR sebocytes were seeded in 96-well plates and cultured in culture medium. After 24 hours, the medium was replaced by assay medium containing Tripeptide-85 or reference (cerulenin tested at 10 μ M) and the cells were pre-incubated for 4 hours. Cells in assay medium without either Tripeptide-85 or

cerulenin were treated as the control. Then, a lipogenic mix containing calcium, insulin, vitamin C and vitamin D3 was added, and the cells were incubated for 7 days. At mid-term, a part of the medium was removed, and the cells were treated again with Tripeptide-85 or cerulenin, and also with the lipogenic mix; in parallel, a non-stimulated control was also carried. All conditions were ran in triplicates, except for the six replicates performed for the stimulated control conditions.

Lipid content analysis (Bodipy® labeling)

After incubation, the sebocytes were washed, fixed and permeabilized. The neutral lipids in the sebocytes were marked using a Bodipy® fluorescent lipid probe, and the nuclei were stained using Hoescht 33258 solution. Ten images per well were acquired using a 20x objective on the INcell Analyzer TM 2200 (GE Healthcare). Labeling was quantified by fluorescence, and the intensity, analyzed only in the lipid droplets by object segmentation-based image analysis, was normalized to the cell count (numerical data integration with Developer Toolbox 1.5, GE Healthcare).

Culture and treatment for gene expression analysis

The SEBO662AR sebocytes were seeded in 24-well plates and cultured for 24 hours in culture medium, in assay medium for further 24 hours, and then in medium containing Tripeptide-85 for 4 hours. Cells in assay medium without the test compound were treated as control. Lipogenic mix was then added to the cells which were incubated for 7 days (half of the medium was removed and the treatments were performed again after 2 days of incubation). The sebocytes were immediately frozen at -80°C after the incubation period and medium removal. All experiments were performed in triplicate.

RNA extraction and Reverse Transcription

Total RNA was extracted using TriPure Isolation Reagent® following the supplier's protocol. The RNA was qualitatively evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) and was quantified using a spectrophotometer (Synergy H1, BioTek Instruments). The cDNA was synthesized by reverse transcription of total RNA using Transcriptor Reverse Transcriptase (Roche) and oligo(dT), and the resulting cDNA quantities were adjusted prior to PCR.

Quantitative PCR (qPCR)

For each experimental conditions, the expression of selected biomarkers was analyzed using qPCR on total mRNA extracted from the SEBO662AR monolayers. The analysis of transcripts was performed in duplicate using a PCR array comparing three sebocyte development-related genes (PLIN2, MYC and SCD)

(Table S1 – Supplementary Material). qPCR analysis was executed using the LightCycler® system (Roche Molecular System Inc.) as per the manufacturer's instructions. The reaction mix (10 µL final volume) was prepared as follows: 2.5 µL of cDNA; forward and reverse primers (0.4 µM each); reagent mix (Ozyme) containing Taq DNA polymerase, SYBR Green I and MgCl₂. The relative amount of mRNA was calculated as previously described [18].

Clinical and experimental assessment

A clinical randomized, single-blind, placebo-controlled trial was conducted with two groups of female volunteers (18-50 years old, oily skin, sebumetric indexes ≥ 100) totaling 16. Half of them received a placebo topical formulation, which consisted of 12% self-emulsion system Uniox C (Cetearyl Alcohol [and] Polysorbate 60), 0.5% Phenoxyethanol as preservative, 1,3-propanediol as solubilizer, and water as the solvent, and the remaining eight received a placebo formulation to which was added 0.01% (w/w) Tripeptide-85 (eight volunteers). The volunteers applied enough amounts of topical formulation (either placebo or Tripeptide-85 0.01%) on the tested area (whole forehead skin), gently spreading it twice a day (morning and evening) for 28 days. Specific inclusion and non-inclusion criteria were evaluated by a dermatologist and are described in Appendix S1.

The clinical effects were determined by the quantification of sebum levels, evaluated by a technician and a dermatologist prior to treatment (Day 0), and at 14 and 28 days after treatments [19]. Prior to all measurements, the volunteers rested for 20 minutes in a room with controlled conditions.

Analysis of facial sebum

Sebum in the forehead was extracted on a special 64 mm², 0.1 mm thick plastic strip which was supported by a mirror and pressed against the skin with a fixed pressure of 10 N by means of a spring for 30 seconds. The strip transparency was photometrically evaluated (Sebumeter® SM 815, Courage+Khazaka, Cologne, Germany) which was correlated with the amount of sebum per area, expressed in absolute values (µg sebum/cm²). Each formulation was applied on the right hemiface; therefore, the opposite side was considered the control area. Measurements were performed in triplicate.

Statistical analysis

For lipid content analysis inter-group comparisons were performed using an unpaired Student's test. Gene expression data were statistically analyzed using unpaired Student's t test. $P < 0.05$ values were considered statistically significant. Finally, data from facial sebum evaluation were statistically analyzed using paired Student's t test. $P < 0.05$ values were considered statistically significant.

Results and Discussion

In silico studies

Based on a previous study on the structure-based virtual screening for MC5R antagonists, Tripeptide-85 was the best ranked peptide and was selected for further investigation.

Peptide synthesis

The molecular weight of the obtained peptide was in accordance with the theoretical prediction (Figure S1), demonstrating that the peptide was successfully synthesized. Data from analytical HPLC of the crude and pure peptides (Figure S2 and Figure S3) reveal that the process yielded a 99% pure peptide, suitable for *in vitro* and *in vivo* tests.

Cytotoxicity Assay and Effect on Lipogenesis

The highest non-cytotoxic concentration found was 500 μM , and no morphological alterations were observed in the entire tested range.

The formation of lipid droplets (LDs) under non-stimulated conditions was very limited and hardly detected in SEBO662AR sebocytes. A strong stimulus for the formation and accumulation of LDs was noticed in the cells treated with the lipogenic mixture (in the absence of androgens) for 7 days. The reference compound cerulenin (an inhibitor of fatty acid synthase used in this study as a negative control), tested at 10 μM , significantly inhibited the lipogenic mix-induced droplet formation (24% mean inhibition). Under the experimental conditions of the assay, Tripeptide-85 treatment decreased lipid labeling with the Bodipy[®] probe, at a concentration of 500 μM . This indicates that the peptide efficiently reduced sebum production (Figure 1) and inhibited the formation of LDs that were induced by the lipogenic mixture in SEBO662AR sebocytes (Table 1).

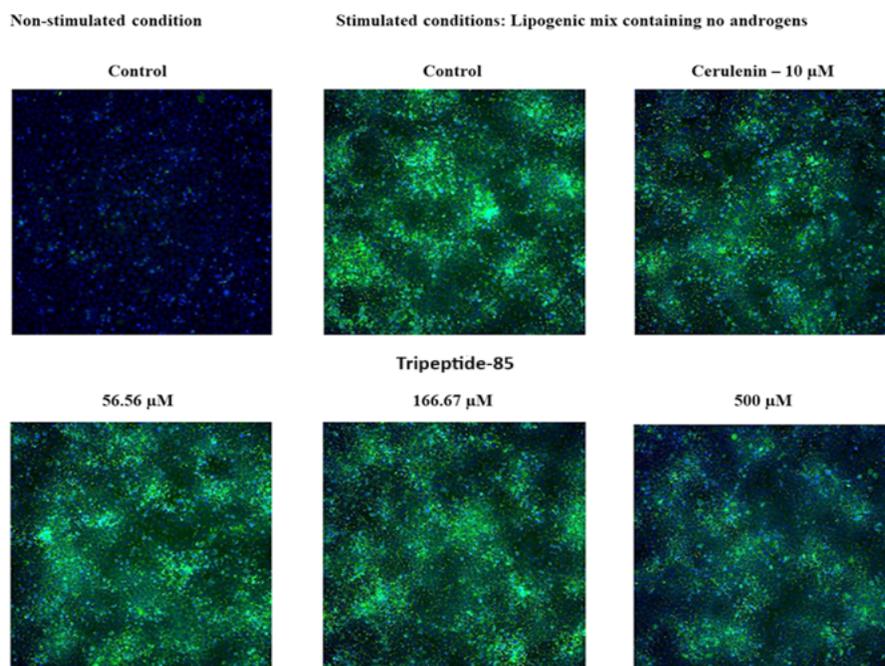


Figure 1: Images of the effect of Tripeptide-85 on the formation of LDs in SEBO662AR sebocytes stimulated with a lipogenic mixture without androgens. Evaluation of the lipogenic inhibition in SEBO662AR cells where nuclei were labeled with Hoechst 33258 solution and LDs were labeled with a Bodipy[®] specific fluorescent probe, which mainly stains neutral lipids (20x-magnification).

Treatment		Normalized data		
Test compound	Concentration (µM)	inhibition (%)	sem (%)	p ⁽¹⁾
Non-stimulated control	-	100	1	***
Control	-	0	6	-
Cerulenin	10	26	3	-
Tripeptide-85	500	28	5	*

Table 1: Effect of Tripeptide-85 on the formation of LDs in SEBO662AR sebocytes stimulated with a lipogenic mixture without androgens; ⁽¹⁾ Threshold for statistical significance; ns: >0.05, Not significant; *: 0.01 a 0.05, Significant; **: 0.001 a 0.01, Very significant; ***: <0.001, Extremely significant. sem = standard error of the mean.

Decreasing excessive sebum production is a feasible approach in the management of the diseases caused by it, such as acne, rosacea, seborrheic dermatitis, cicatricial alopecia, and may reduce the involvement of skin eutermia [20,21]. While Tripeptide-85 was designed as an MC5R antagonist, and it was shown that it can reduce sebum production and release onto the skin, it is still to be determined if its mechanism of action in sebogenesis modulation in fact involves MC5R inhibition. Taken together, however, the results obtained in this work are coherent with previous studies that linked MC5R and sebogenesis [20].

Gene expression

The effects of Tripeptide-85 on gene expression modulation of several markers of sebocyte development and differentiation from SEBO662AR cell line culture are presented in Table 2. A large amount of lipids accumulates in LDs during sebocytes differentiation, and LDs, which can be described as a neutral lipid vesicle delimited by a phospholipid monolayer with embedded proteins, are implicated in several biological processes [22,23]. They are composed of a core of neutral lipids enclosed by a phospholipid monolayer with embedded proteins [24]. Among these proteins are perilipins which have structural and regulatory functions [25]. PLIN2 is the second most abundant perilipin during sebocyte differentiation. PLIN2 downregulation clearly impairs lipid accumulation in sebocytes. Previous work with PLIN2-deficient mice links this protein with SG size, number of sebocytes and sebocytes proliferation. Also, a study with PLIN2-deficient SZ95 sebocytes suggest that PLIN2 may have other roles in cell functions besides LD metabolism [26]. In the present study, 0.01% of Tripeptide-85 significantly reduced PLIN2 gene expression by 15% compared to the control group.

Gene	Tripeptide-85 / Stimulated control	p-value	Statistics
PLIN2	-15%	0.0063	very significant.
MYC	-20%	0.0005	extremely significant.
SCD	-14%	0.0961	not quite significant or tendency

Table 2: A representation of the gene expression levels in human sebocyte cell line treated with Tripeptide-85 under lipogenic factors without androgens, compared to the lipogenic factors group (Stimulated control).

c-Myc is a crucial transcription factor encoded by the gene MYC, regulates sebocyte differentiation and SG growth. Using SG organoids, it was discovered that c-Myc is not expressed in differentiated cells, but exclusively in proliferating cells in the SG proliferative zone (SGPZ); while c-Myc super-expression resulted in augmented SG expansion, inhibition of c-Myc activity led to a significant reduction in cell proliferation and in the organoid size and c-Myc conditional deletion reduced SG size, cell proliferation and sebocyte differentiation [27]. We experimentally found that 0.01% of Tripeptide-85 significantly reduced MYC expression by 20%.

Stearyl-CoA desaturase (SCD) is a membrane-bound enzyme that catalyzes the insertion of a double bond between C9 and C10 in saturated acyl-CoA derivatives, which is the rate limiting step in the monounsaturated fatty acid biosynthesis [8 28]. Besides being important components of cell membranes, and the main constituents of adipose tissue, unsaturated fatty acids also take part in the energy metabolism and hormone receptor activation, and influence apoptosis [29,30]. SCD is expressed in SG undifferentiated cells and is critical in sebocyte development [31]. In studies with mice, the absence of SCD in skin drastically reduces the production of sebaceous lipids such as triglycerides and cholesterol esters [32]. Our tests showed that SCD gene was downregulated after treating cells with 0.01% Tripeptide-85.

Taken together, the downregulation of PLIN2, MYC, and SCD, which impair cell proliferation, sebocyte differentiation, and lipid synthesis may explain the reduction of the size of LDs in the presence of Tripeptide-85 *in vitro*.

Effect of Tripeptide-85 on sebum levels *in vivo*

The 0.01% Tripeptide-85 preparation tested in the clinical randomized, single-blind, placebo-controlled trial improved sebum production on face, after treatment for 14 and 28 days (Figure 2). It promoted a significant reduction of sebum levels on the forehead

by 31.5% (**p<0.001) and 29.7% (**p<0,001), after treatment for 28 and 56 days, respectively, whereas placebo formulation reduced sebum production by 7.4% and 12.3% (*p<0.05) after the same treatment periods (Figure 2). This reduction in sebum production might be linked to It is acknowledged that acne does not occur when sebum production is low [33]. According to the Global Burden of Diseases, acne vulgaris is the eighth most prevalent disease in the world, affecting 9.4% of the population [34]. Psychological and socioeconomical threats are possible outcomes for individuals affected by such condition; hence, the importance of controlling sebum production is beyond aesthetics.

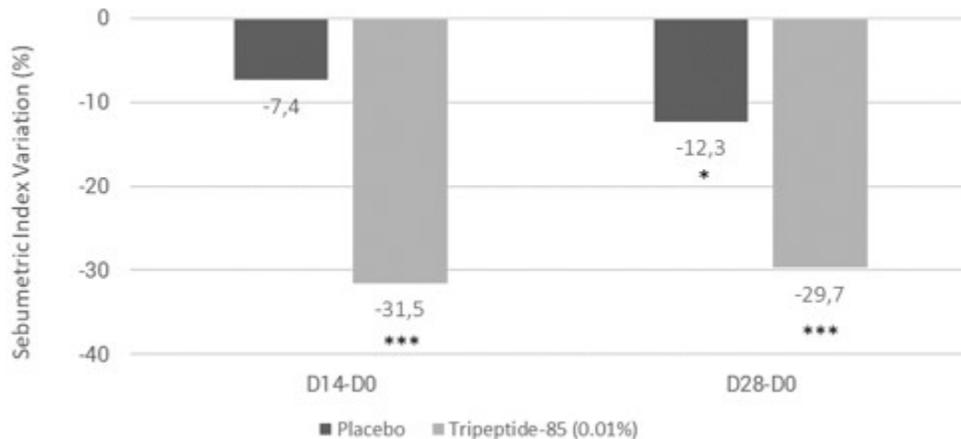


Figure 2: Sebumetric index variation (%) for Tripeptide-85 administration and placebo per 14 days (D14-D0) and 28 days (D28-D0). *p < 0.05 and *** p < 0.001 in relation to the initial time (D0).

The treatment of oily skin lacks options, especially those that do not act on traditional pathways such as 5-alpha reductase. High levels of testosterone are related to increased activity of the sebaceous glands [35] due to their ability to bind to androgen nuclear receptors expressed in sebocytes [36]. The enzyme 5-alpha reductase converts testosterone into dihydrotestosterone (DHT) which has a much higher affinity for the androgen receptor than its precursor [37]. The inhibition of the enzyme is therefore considered a useful strategy for controlling oiliness. However, the use of 5-alpha reductase inhibitors does not seem to be fully effective for all cases of hyperactivity of the sebaceous glands [9].

To the best of our knowledge, no clinical trials were conducted till date, using an MC5R inhibitor intended for cosmetic use as a sebum production regulator. Since Tripeptide-85 reduces sebum production *in vivo* and acts in a non-canonical pathway, we believe that a myriad of combinations are possible with Tripeptide-85 and other ingredients, including 5 α reductase inhibitors, in order to obtain synergistic effects, envisioning a new era in the cosmetic management of sebogenesis.

Conclusion

Excess sebum production is closely related to several skin conditions, such as acne and seborrheic dermatitis. In this study, we showed that Tripeptide-85 substantially reduces sebum production

by sebocytes, both *in vitro* and *in vivo*. Tripeptide-85 suppressed PLIN2, MYC, and SCD in SEBO662 sebocytes, suggesting that the observed effect on LDs *in vitro* and decreased sebogenesis *in vivo* result from impaired cell proliferation, sebocyte differentiation and lipid synthesis due to MC5R antagonism. These findings support Tripeptide-85 as a novel bioactive substance for use in several cosmetic and dermatological applications, especially in formulations intended to mitigate excessive sebum production.

Ethical Approval

Authors declare human ethics approval was not needed for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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