Tetrapeptide-111: slowing down the signs of aging with a new synthetic ecofriendly peptide.

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Abstract

Wrinkles are dermal folds formed by muscle contractions during facial expressions. Dynamic wrinkles appear with muscle movement and disappear at rest, while static wrinkles persist and increase with age due to frequent muscle contractions. Botulinic toxin and nicotinic acetylcholine receptor (AChR) antagonists can prevent these wrinkles by inhibiting muscle contractions, though botulinum toxin has limitations due to toxicity and invasiveness. Tetrapeptide-111 (L-prolyl-L-tryptophil-L-lysyl-L-tryptophan) is a novel peptide developed via a green solid-phase peptide synthesis (GSPPS) method, reducing the use of toxic solvents, and increasing sustainability. Tetrapeptide-111 emerged from virtual screening as an AChR antagonist. In vitro tests showed that Tetrapeptide-111 significantly reduced calcium influx in muscle cells, indicating reduced muscle contraction. Clinical trials with 14-day topical application of Tetrapeptide-111 (50ppm) demonstrated significant reductions in crow's feet wrinkles, outperforming a benchmark peptide. Compared to traditional SPPS, GSPPS for Tetrapeptide-111 reduced petrochemical usage by 52.7% and greenhouse gas emissions by 253-fold. These results highlight Tetrapeptide-111's potential as a safer, effective alternative to traditional anti-wrinkle treatments, offering significant cosmetic benefits with reduced environmental impact.

Keywords: Peptide; wrinkles reduction; botox-like effect; GSPPS; AChR Antagonist.

Introduction.

Wrinkles manifest as dermal folds perpendicular to muscle contraction induced by facial expressions. Wrinkles are classified into dynamic and static types. Dynamic wrinkles appear during facial expressions due to muscle contractions and disappear when muscles relax. Static wrinkles persist regardless of muscle relaxation and increase with age. The transition from dynamic to static wrinkles is caused by damage from frequent muscle contractions [1,2].

One of the strategies for mitigating facial expression marks consists of using nicotinic acetylcholine receptor (AChR) antagonist, preventing muscle contraction [3].

The mechanism of myocontraction involves the release of acetylcholine (ACh) in the synaptic cleft, and ACh binding to nicotinic acetylcholine receptor (nAChR), triggering a cascade of events that starts with an influx of sodium ions to muscular cells that will ultimately lead to muscle contraction [4,5].

Botulinum neurotoxin injections, a primary attenuation method that impairs ACh release from the motor neuron to the neuromuscular junction by cleaving SNARE proteins, face limitations due to high toxicity and side effects and by requiring an invasive procedure. However, several substances with action in nAChR are known to paralyze skeletal muscles, indicating the feasibility of this target [6,7,8].

Peptides are short chains of amino acids. Although many peptides may be found in nature, many commercial peptides are obtained by chemical synthesis. Solid-phase peptide synthesis (SPPS) is a well-known protocol where purity is one of its best attributes. In this method, the peptide grows attached to the surface of a solid support, after a series of deprotection, coupling and washing steps, and is finally obtained by cleavage of the peptide from the support and purification. However, traditional SPPS makes use of large amounts of solvents and other toxic or potentially dangerous reagents [9].

In this work, we present Tetrapeptide-111 (L-prolyl-L-tryptophil-L-lysyl-L-tryptophan), a novel skin-permeable peptide capable of substantially reducing skin wrinkles, obtained from a green solid-phase peptide synthesis (GSPPS) protocol.

Materials and Methods.

1. **Molecular modeling:**

A structure-based virtual screening was performed using a nAChR subunit alpha retrieved from a known structure (PDB ID 4ZJS) [10,11] and an in-house built peptide database, using AutoDock Vina [12] as docking software. The best ranked poses were visually inspected, and four peptides were sent to clinical testing (data not shown). The most promising peptide was selected for further investigation.

2. **Peptide synthesis:**

The peptide was synthesized using GSPPS, derived from the standard SPPS protocol [13], with a Wang-type resin pre-liked to Fmoc-Trp(Boc), and Fmoc-protected amino acids. For the deprotection scheme and for the coupling, respectively, a green base and ethyl cyano(hydroxyimino)acetate were used. 2-methyltetrahydrofuran (2THF) was used as solvent. The cleavage was done with trifluoroacetic acid and triisopropylsilane (TIS) as scavenger. The peptide was purified by liquid chromatography, using water and a renewable solvent, both acidified with acetic acid as mobile phase and a C18 column, and the target peptide fraction was lyophilized. The peptide was characterized by LC/MS and FT-IR, and its purity was assessed by HPLC.

3. **Muscle contraction ability in vitro assessment:**

In order to evaluate the effect of Tetrapeptide-111 on blocking calcium influx into skeletal muscle cells treatment, as an indicator of muscle contraction capacity, Human Skeletal Muscle Cells (hSkMC) cells were cultured for 2 hours in the presence of Tetrapeptide-111 50ppm, previously demonstrated as non-cytotoxic. Calcium release influx was induced with 10 µM

ionomycin at the end of the treatment period. Immediately after the addition of ionomycin, calcium levels were quantified by fluorescence (λex = 485 nm/λem = 535 nm) emitted by Ca2+-bound Fluo-4. Fluo-4 is a fluorescent calcium indicator dye widely used in intracellular calcium signaling studies. Data were analyzed using unpaired Student's t-test (Control + Ionomycin, normalized to Control) and Ordinary one-way ANOVA test (data normalized to Control + Ionomycin). Statistical significance was set at $p<0.05$.

4. Antiwrinkles clinical assessment:

Formulas containing Tetrapeptide-111 50ppm, benchmark (Acetyl Hexapeptide-8) 50ppm and Placebo were compared in a study conducted for 14 days. The randomized clinical trial was conducted with 21 ± 1 volunteers per group, aged between 37 and 68 years, presenting moderate to very severe signs of facial aging (grade 2-4 on the Eiben-Nielson photonumeric scale for wrinkles). The products were applied twice a day on face.

Periorbital wrinkles were evaluated by 3D topography analysis using the AEVA-HE V4 system (FoV, S) equipment. High resolution macroscopic pictures were obtained using camera Nikon D5600 installed in the HeadScan Bench Light Face, including complete professional photographic setup. Data recorded from every individual at the timepoint (D14) were normalized versus baseline values (D0) for the whole group and statistically analyzed for each parameter. In addition, participant's subjective perception of the product efficacy was assessed with an individual questionnaire answered at each of the timepoints.

The study protocol is in accordance with the Scientific Committee on Consumer Safety (SCCS) guidance. It meets all international standards for research studies involving human subjects, the Good Clinical Practices (ICH-GCP), and World Medical Association. It has been conducted pursuant to the Declaration of Helsinki (1864), with the amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), and Seoul (2008). All subjects had given their informed consent to participate of the study.

Results.

Tetrapeptide-111 (L-prolyl-L-tryptophil-L-lysyl-L-tryptophan) emerged as a potential antagonist of AChR as a result of virtual screening. Tetrapeptide-111 is a synthetic peptide formed by the amino acids proline, tryptophan and lysine, and valine, which is obtained by the optimized solid phase peptide synthesis (GSPPS) method. The optimization of traditional synthetic process (SPPS) was focused in reducing or even suppressing the use of toxic or controversial materials, making it more attractive and less polluting, while guaranteeing high purity ingredient. As result it was possible to achieve a reduction of the use of raw materials and reagents from petrochemical sources (from initial 74,7% of petrochemical derived components, to 22%) and an increase of more than 280% in the use of raw materials and reagents from renewable sources. Also, the GSPPS for Tetrapeptide-111 demonstrated a remarkable 253-fold reduction in greenhouse gas emissions.

Tetrapeptide-111 was submitted to *in vitro* test to evaluate muscle contraction ability by measuring the intracellular calcium influx. The exposure of hSkMC to ionomycin resulted in a significant 445.6% increase in intracellular calcium levels ($p<0.01$). However, pretreatment with Tetrapeptide-111 at 50ppm led to a 21.4% reduction in calcium influx compared to the ionomycin-treated control ($p<0.05$).

In clinical trial (Figure 1), a 14-day topical application of Tetrapeptide-111 (50ppm) exhibited substantial reductions in crow's feet wrinkles area (19.7%, $p<0.01$), number (17.8%, $p<0.05$), perimeter (17.3%, $p<0.01$), and volume (19.9%, $p<0.01$), compared to initial measurements (basal). Benchmark (50ppm) treatment showed reductions of 13.9% ($p<0.05$), 9.1% (ns), 12.9% ($p<0.05$), and 12.2% (ns) in the corresponding parameters. Placebo group displayed non-significant reductions (ns) of 1.2%, 2.6%, and 3.2% in wrinkles area, number, and perimeter, with a volume increase of 1.6%.

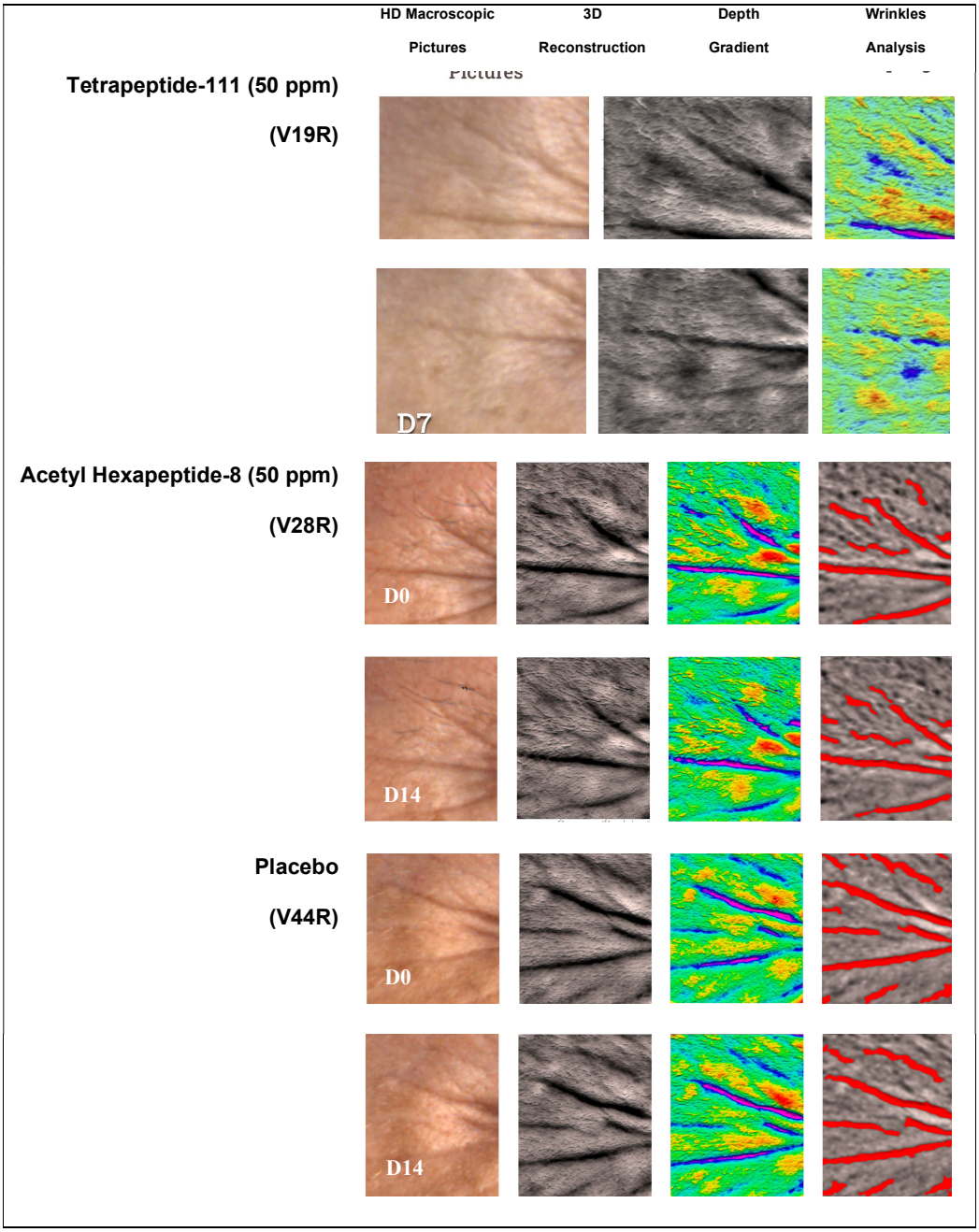

Figure 1: Tripeptide-111 (50ppm), Benchmark (50 ppm) and Placebo HD macroscopic images, 3D reconstruction, dept gradient and wrinkles analysis in periorbital area, obtained with AEVA-HE system; before (D0), after 14 days of treatment (D14).

Discussion.

The widespread use of botulinum toxin for cosmetic purposes is complex, driven by its significant outcomes as well as shifts in society and culture, advances in technology, and the increasing favor towards less invasive treatments. Botulinum toxin, derived from botulinum exotoxin produced by *Clostridium botulinum*, has been increasingly used in humans since the late 20th and early 21st centuries. Its application in cosmetic procedures stemmed from the observation that treating blepharospasm also improved periocular lines. Subsequent research confirmed its effectiveness in reducing wrinkles like glabellar folds and crow's feet, solidifying its role as a proven method for aesthetic enhancement [14,15]. In addition to the classical botulinum neurotoxin, anti-aging products acting on the facial muscle contractility include several peptides such as the acetyl hexapeptide-8, acetyl octapeptide-3, pentapeptide-3.

Botulinum toxin acts by reducing ACh release from motor neurons to the neuromuscular junction by cutting SNARE proteins. It has limitations like toxicity and invasiveness, but other substances acting on nAChR can also paralyze skeletal muscles, showing potential for this approach. In adults, nAChR is a pentamer, with two alpha subunits, one beta, one gamma and one epsilon, and each nAChR subunit has an ACh binding site [4]. Besides the availability of the receptor structure, the choice of targeting the subunit alpha was also due to statistical reasons; the more binding sites, more likely these can be affected by the small molecule and ultimately paralyze target muscles. Addressing this, we explore a safer alternative through molecular modeling and Tetrapeptide-111 (L-prolyl-L-tryptophil-L-lysyl-L-tryptophan) emerges as a potential AChR antagonist.

The traditional SPPS protocol involves extensive use of DMF during the deprotection, coupling and washing steps, and most purification protocols are done using acetonitrile as organic mobile phase. Other dangerous reagents are also common, such as 1-hydroxybenzotriazole (HOBt) which may pose an explosion threat, and the carcinogenic 4-methylpiperidine. In this work, our group employed a GSPPS protocol, with the substitution of petrochemical solvents

and harmful reagents by sustainable, safer alternatives, achieving 10 of 12 principles of green chemistry to obtain Tetrapeptide-111 molecule [16].

In an *in vitro* study, Tetrapeptide-111 showed significant reduction in calcium influx compared to the ionomycin-treated control. Ionomycin is one of the most widely used ionophores for validation and calibration of calcium change analysis. Specifically, it possesses the ability to bind to calcium ions within the cell and allow their release into the cytoplasm. In the context of skeletal muscle cell studies, when ionomycin is added to cells in culture, it binds to calcium ions present in the extracellular medium and facilitates their entry into cells. This results in an increase in intracellular calcium concentration by activating various proteins and signaling pathways involved in muscle contraction, which can lead to contraction of skeletal muscle cells in culture [17].

In addition, Tetrapeptide-111 also showed significant reduction in area, number, perimeter and volume of periorbital wrinkles. The results indicate a superior efficacy compared to Acetyl Hexapeptide-8 (benchmark). Therefore, the results achieved with Tetrapeptide-111 could be considered of interest in the cosmetic field.

Conclusion.

Through the substitution of synthesis and purification materials, a substantial reduction in environmental and hazard impacts was achieved within this pathway, resulting in Tetrapeptide-111, identified through virtual screening. In vitro testing revealed Tetrapeptide-111's ability to significantly reduce calcium release in hSkMC. The intricate process of vertebrate skeletal muscle contraction, initiated at the acetylcholine receptor (AChR) and dependent on Ca2+ ion release for actin and myosin interaction, was influenced by Tetrapeptide-111. Moreover, Tetrapeptide-111 exhibited a more potent reduction in wrinkles and signs of expression compared to Acetyl Hexapeptide-8, offering a novel option to mitigate age-related signs effectively.

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Conflict of Interest Statement.

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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