

A synthetic hexapeptide (Argireline) with antiwrinkle activity

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Synopsis

Botulinum neurotoxins (BoNTs) represent a revolution in cosmetic science because of their remarkable and long-lasting antiwrinkle activity. However, their high neurotoxicity seriously limits their use. Thus, there is a need to design and validate non-toxic molecules that mimic the action of BoNTs. The hexapeptide Ac-EEMQRR-NH₂ (coined Argireline) was identified as a result of a rational design programme. Noteworthy, skin topography analysis of an oil/water (O/W) emulsion containing 10% of the hexapeptide on healthy women volunteers reduced wrinkle depth up to 30% upon 30 days treatment. Analysis of the mechanism of action showed that Argireline significantly inhibited neurotransmitter release with a potency similar to that of BoNT A, although as expected, it displayed much lower efficacy than the neurotoxin. Inhibition of neurotransmitter release was due to the interference of the hexapeptide with the formation and/or stability of the protein complex that is required to drive Ca²⁺-dependent exocytosis, namely the vesicular fusion (known as SNARE) complex. Notably, this peptide did not exhibit *in vivo* oral toxicity nor primary irritation at high doses. Taken together, these findings demonstrate that Argireline is a non-toxic, antiwrinkle peptide that emulates the action of currently used BoNTs. Therefore, this hexapeptide represents a biosafe alternative to BoNTs in cosmetics.

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Résumé

Les Botulinum neurotoxins (BoNTS) représentent l'évolution de la science cosmétique grâce à leur remarquables actions anti-rides longue durée. Cependant, leur importante neurotoxicité limite sérieusement leur usage. Il est donc nécessaire d'étudier et de valider des molécules non toxiques qui s'apparentent à l'action des BoNTS. L'Hexapeptide Ac-EEMQRR-NH₂ (Argireline) a été identifié suite à un résultat d'un programme d'étude rationnel. Une analyse de la topographie de la peau des femmes volontaires montre que des applications d'une émulsion (H/E) contenant 10% d'Argireline ont réduit leurs rides jusqu'à 30% sur un traitement de 30 jours. L'analyse du mécanisme d'action montre que l'Argireline, par inhibition du relarguage du neurotransmetteur, produit des effets semblables à celle du BoNT A, bien que, comme attendu, son efficacité soit beaucoup moins importante que celle des neurotoxines. L'inhibition des neurotransmissions est due à l'interférence de l'Argireline avec une formation et/ou la stabilité du complexe de protéines nécessaire à l'exocytose Ca⁺⁺ dépendante, appelé le complexe de fusion vésiculaire (SNARE). Normalement, l'Argireline ne montre pas de toxicité *in vivo*. Mises ensemble, ces découvertes démontrent qu'il s'agit d'un peptide non-toxique et anti-rides qui imite l'action des BoNTS couramment utilisés. L'Argireline représente donc une alternative "bio-safe" aux BoNTS en cosmétique.

Introduction

One of the most striking signs of skin ageing is the relative intensity of frown and wrinkle lines of

the forehead, glabella, lateral periorbital area as well as the intensity of chin, upper lip wrinkling, nasolabial folds, nasal flare and platysma neck bands among others [1]. This can occur naturally over time and is identified by certain biochemical, histological and physiological changes that are enhanced by environmental exposure. There are, however, other secondary factors that can cause characteristic folds, furrows and creases of the face, such as the constant pull of gravity, frequent and constant positional pressure of the skin of the face (e.g. during sleep) or repeated facial movements caused by the contraction of the muscles of facial expression [1, 2].

Physiologically, the formation of wrinkles appears to be due, at least partly, to the excessive stimulation of the muscle fibres in the face, which pull inwards the skin giving rise to the well-known wrinkle [1–3]. Thus, a useful strategy to reduce the intensity of wrinkle lines is to down-regulate muscle action either directly or by attenuating the activity of the innervating neurone [1, 3–5]. In support of this tenet, treatment with botulinum neurotoxin (BoNT) A significantly reduces the intensity of frown and wrinkle lines. BoNTs strongly inhibits the Ca^{2+} -dependent neurotransmitter release in neurones [6, 7]. These proteins are metalloproteases that specifically cleave synaptic proteins essential for regulated neuronal exocytosis [6, 7], specifically the vesicular protein VAMP, and the membrane proteins syntaxin and SNAP-25 [7]. As a result, the critical protein fusion complex assembled by these proteins, known as SNARE complex, is destabilized preventing vesicle fusion with plasma membrane, and consequently abrogating Ca^{2+} -triggered exocytosis [7].

Although botulinum neurotoxins, specially BoNT A (BOTOX), has been extensively used to attenuate facial ageing signs, its use is limited because of its high toxicity (human $\text{LD}_{50} \approx 2500$ biologic mouse units) [1, 6]. Paradoxically, BoNT A is the most potent toxin known to humankind and therefore BOTOX treatment has to be under strict medical control [1, 3–5]. To circumvent this limitation, small molecules that mimic the action of BoNTs is being pursued [8–10]. In this regard, synthetic peptides of ≥ 20 mer that emulate the amino acid sequence of the synaptic protein SNAP-25 were shown to be specific inhibitors of neurosecretion at micromolar concentrations [8–10].

The length of these peptides, however, along with a poor membrane permeability significantly limited their cosmetic utility. Accordingly, there is a need to identify new sequences that are shorter while preserving a biological activity. We have used rational

design to address this issue. We report the identification of a 6-mer peptide (Ac-EEMQRR-NH₂), patterned after the N-end of the N-terminal domain of SNAP-25 (aa 12–17), that interferes with the assembly of the SNARE ternary complex, and inhibits Ca^{2+} -dependent catecholamine release from chromaffin cells. The hexapeptide was coined with the name Argireline. This peptide exhibited a significant skin permeation. Noteworthy, topical use of O/W emulsions containing 10% of the peptide reduced the intensity of wrinkles in the lateral preorbital area healthy human volunteers. Toxicological and irritation primary data indicate that Argireline is well tolerated. Collectively, these findings demonstrate the feasibility of the rational strategy to find peptide-based mimetics of BoNTs action, and indicate that these peptides are a biosafe cosmetic alternative to attenuate facial wrinkles.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized by Fmoc chemistries by solid-phase synthesis as described [8]. Briefly, Argireline was synthesized by solid phase on a pMBHA-resin (*p*-methylbenzylamine-resin) with AM-handle, which allows the cleavage of the peptide amide in acid conditions with the concomitant deprotection of the side chains protection. The elongation of the peptide chain was carried out using the Fmoc/tBu strategy. The resulting peptidyl resin was treated at room temperature with a mixture of TFA/thioanisole/ H_2O (95/2.5/2.5, v/v/v, 7 ml g^{-1} resin) for 2 h. The crude peptides were precipitated by filtration into cold diethyl ether and vacuum-dried. The crude product was dissolved in 10% acetic acid for de-*tert*-butylation at 60 °C and treated with DIAION for purification. Characterization was done by ESI/MS and analytical HPLC using Kromasil C8 column (4.6 mm \times 250 mm, 5 μm , 100 Å) flow rate 1 ml min^{-1} , eluent A 0.1% TFA, eluent B 0.07% TFA in CH_3CN . Elution conditions: isocratic 11% and 0–40% in 30-min gradient.

Recombinant SNARE proteins expression and purification

Recombinant VAMP (lacking the transmembrane segment) and cytosolic domain of syntaxin were expressed as GST fusion proteins in the *E. coli* strains BL21DE3 and C43, respectively. Protein expression

was induced with 1 mM IPTG for 5 h at 30 °C, and purified from bacterial extracts by affinity chromatography on glutathione agarose as described [11]. Resin-bound fusion proteins were released by digestion with thrombin protease (Pharmacia) for 2 h at 23 °C [11]. Proteins concentration was assayed with the BCA kit (Pierce), and purity verified by gel analysis.

***In vitro* expression of SNAP-25**

In vitro translation of the cDNA clone coding for SNAP-25 from rat brain in the presence of [³⁵S]methionine involved a transcription–translation-coupled reticulocyte lysate system (Promega) as described [12].

***In vitro* reconstitution of SNARE complex and modulation by Argireline**

SNARE complex was reconstituted using the recombinant VAMP and syntaxin proteins and *in vitro* translated [³⁵S]SNAP-25. Briefly, equimolar amounts of VAMP and syntaxin were incubated in the absence or presence of Argireline for 2 h at 4 °C. Thereafter, SNAP-25 was added to the mixture and the reaction proceeded for 3 h at 4 °C. Complex assembly was stopped by addition of SDS-PAGE sample buffer. Samples were analysed by SDS-PAGE on 12% gels, followed by fluorographic detection on Kodak X-Omat AR X-ray films.

Chromaffin cell cultures and secretion assays

Chromaffin cell cultures were prepared from bovine adrenal glands by collagenase digestion and further separated from debris and erythrocytes by centrifugation on Percoll gradients as described [8–10]. Briefly, cells were maintained in monolayer cultures at a density of 625 000 cells cm⁻² and were used between the third and sixth day after plating. All the experiments were performed at 37 °C. Secreted [³H]noradrenaline was determined in digitonin-permeabilized cells as described [8–10]. The CPM released from control cells under basal conditions were ~3000, and they were increased to ~11 000, when stimulated with 10 μM Ca²⁺. The total number of counts obtained from detergent-permeabilized cells was ~110 000. Thus, the normalized basal release represents the 3.5% of the total secretion, and the Ca²⁺-evoked ~10%. Statistical significance was calculated using Student's *t*-test with data from four or more independent experiments.

Stratum corneum assay

Human skin was obtained from different donors who underwent cosmetic surgery. All fat was removed from fresh or frozen skin pieces with a scalpel. The skin was then submitted to ammonia vapours in a closed recipient at room temperature for 30 min. The skin was placed, stratum corneum side uppermost, on a glass surface and the epidermis was teased gently away from the underlying dermis using the tip of a gloved finger. To prepare stratum corneum samples, the epidermal membranes were floated overnight stratum corneum side up, on an aqueous solution of trypsin solution (0.01%, pH = 8–8.6). To remove digested matter, membranes were squeezed between two filter papers, placed on the filter paper with nucleated tissue side uppermost, and any remaining digested material was removed by washing with water and gentle swabbing. The SC pieces were floated on 0.001% aqueous NaN₃ solution for 10 min before drying inserted between filter paper sheets in a desiccator. Immediately before use, membranes were floated with the stratum corneum side up, on 0.002% aqueous sodium azide for 1 h.

Permeation experiments were carried out using a static cell manually sampled as described [13]. The cell was fabricated of glass and had two chambers, an upper chamber (donor chamber), and a lower one (receptor chamber); the average diffusion area was 1.3 cm². The receptor chamber volume was 4 ml included that of the outflow tubing. Receptor reservoir was continuously stirred and thermostated through its connection to a circulating bath maintained at 37 °C. A disk of filter paper (to act as rigid support) was located between both chambers and skin disks of about 2 cm² were mounted on them. Isotonic phosphate buffer, pH = 7.4, with 0.01% sodium azide as preservative, was used as the receptor fluid. Samples (0.5 ml of an aqueous solution of Argireline) were poured softly in the donor chamber and 100 μl aliquots of receptor fluid were periodically withdrawn for analysis and replaced with an equal volume of fresh receptor fluid. Argireline concentration in the receptor fluid was quantified by HPLC on C-18 columns, working in isocratic way 11% ACN (0.05% TFA).

Antiwrinkle test on healthy humans

Skin topography analysis for measuring the effectiveness of an O/W emulsion containing 10% of Argireline (solution presentation) was performed obtaining silicon imprints from the lateral preorbital region of

10 healthy women volunteer who apply the emulsion twice a day. Volunteers apply the emulsion containing 10% of Argireline in one lateral preorbital area, and the emulsion alone in the contralateral side. Silicon imprints were obtained after 0, 15 and 30 days, and analysed by confocal laser scanning microscopy to assess the evolution of the skin surface before and after treatment. Confocal Microscopy in reflection mode and three-dimensional analysis to assess the different parameters of roughness was used.

The same skin areas were selected before the processing (Day 0) and after the processing (Days 15 and 30), by means of observation under magnifying glass (Leica M LZIII, augment 10×) with outer white light. Three sample areas (2.25 mm²) were measured for each replica ($n = 30$, for each time point). The selected regions were clean, free of any strange particle, without zones of fissure or pores in silicone. Later, a fragment of the selected wrinkles was cut to and mounted on a microscope slide. The observation was made by means of a confocal microscope TCS SPII adapted to a motorized microscope Leitz DMIRB (lens Leitz 10×, NA 0,3 s, $n = 1$). The pick up was made in mode of reflection with an only photomultiplier. The configuration of filters was: reflection:excitation:488-nm, Beam Splitter RT30/70. The exploration surface was of 2.25 mm². In route in z was of 500 μm. Each series of images consists of 201 optical sections separated among them 2.5 μm. Final measures of observation of each field (x, y, z): 1500 μm × 1500 μm × 500 μm.

Roughness parameters were calculated according to the UNE EN ISO 4287 normative and roughness differences for each volunteer were assessed by calculating the decrease percentage of the Pa (arithmetic roughness, equivalent to the Ra, DIN 4768) between days 0 and 15 or 30. Roughness differences between groups are expressed as a mean of the Pa decrease percentages. Comparisons between control and treated areas were evaluated with the *F*-Fisher's test with statistical significance set at $P < 0.075$.

Three-dimensional reconstructions

For each sample the following three observations of macro-relief were made:

Topographic image (depth-coded image)

The topographic image obtained from the series of sections is a real map of the structure of superficie of the sample. It examines the points of the sample (voxel) that are superposed throughout z -axis of all the series of optical sections.

Three-dimensional graph

It was obtained from the topographic image. It provides a three-dimensional image of the map of surface of the sample. They have been made graphical three-dimensional from the topographic image of the retort and the real topographic image of the skin (inverted process) in different angles.

Measures of roughness parameters

The measurement of the parameters of roughness (Ra) was performed with Leica TCS SPII software, following the UNE EN ISO 4287 norm (geometric product Specification).

Results and discussion

Rational design of Argireline

Sequence and structure analysis of the N-terminal of SNAP-25 revealed the sequence EEMQRR (aa 12–17) that display a high propensity to acquire an α -helical structure along with a pronounced coiled-coil probability (Fig. 1A). AGADIR, a programme that estimates helical propensity of peptides, predicted a remarkable 12% probability for this small peptide. These properties suggest that a peptide patterned after this sequence may modulate Ca²⁺-dependent exocytosis, similar to those peptides derived from the C-terminal of SNAP-25 [8–10]. The acetylated and amidated peptide was coined with the term Argireline.

Argireline interferes with the formation of the SNARE complex

To evaluate the potential antiwrinkle activity of the hexapeptide, we first determined if the peptide prevents or destabilizes the formation of the SNARE complex *in vitro*. For this task, we used recombinant synaptic proteins VAMP, syntaxin and *in vitro* transcribed and translated [³⁵S]SNAP-25. As depicted in Fig. 1B, incubation of the three synaptic proteins led to the formation of a protein complex of 75 kDa that was resistant to the chaotropic detergent SDS (lane 2), but sensitive to heat (lane 3), two well-known properties of the SNARE complex [14]. When the proteins were incubated with Argireline, the formation of the SNARE complex was prevented in a dose-dependent manner (lanes 4 and 5). Note that at 2 mM the 75 kDa band was undetectable, suggesting complete abrogation of complex formation by the small peptide. These results demonstrate that Argireline can prevent the assembly of the protein complex that drives Ca²⁺-dependent exocytosis in

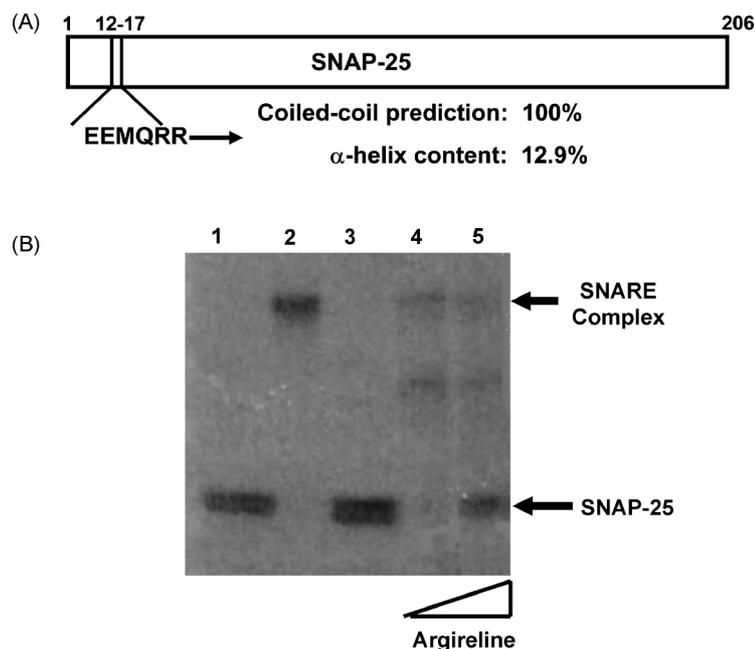


Figure 1 Rational design (A) and *in vitro* activity (B) of a 6-mer peptide derived from the N-terminal domain of SNAP-25. (A) Amino acid sequence of SNAP-25 was analysed for coiled-coil propensity (ExPaSy) and α -helical content (AGADIR). The sequence Ac-EEMQRR-NH₂ (aa 12–17), coined Argireline, showed a significant probability for both properties. (B) *In vitro* reconstitution of the SNARE complex. Assembly of the complex was performed using recombinant VAMP and syntaxin, and *in vitro* translated [³⁵S]SNAP-25. (1) [³⁵S]SNAP-25; (2) SNARE complex; (3) SNARE complex + 100 °C for 5 min; (4) SNARE complex + 1 mM Argireline; (5) SNARE complex + 2 mM Argireline.

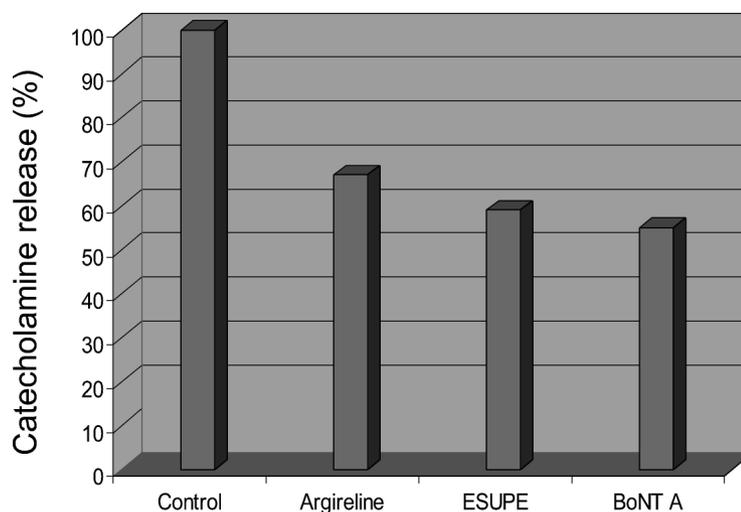
secretory cells, thus implying that this peptide may modulate neurotransmitter release from these cells.

Argireline inhibits catecholamine release from chromaffin cells

To test the cellular activity of the hexapeptide, we measured the inhibitory activity of this peptide on

Ca²⁺-evoked neurotransmitter release from digitonin-permeabilized chromaffin cells. This is a reliable assay that allows a rapid assessment of the biological activity of toxins and peptides [8–10]. As illustrated in Fig. 2, detergent-permeabilized chromaffin cells release both noradrenaline and adrenaline in response to a raise in intracellular Ca²⁺. Catecholamine release was inhibited up to 60% by 20 nM BoNT

Figure 2 Argireline inhibited Ca²⁺-dependent exocytosis from permeabilized chromaffin cells. Digitonin permeabilization lasted 5 min and [³H]noradrenaline secretion was evoked in the absence (5 mM EGTA) or presence of 10 μ M Ca²⁺. Shown is the effect on the basal and Ca²⁺-stimulated release of 100 μ M of the hexapeptide. Data are mean from three different experiments. Data for ESUPE were taken from [10] and for BoNTA from [9].



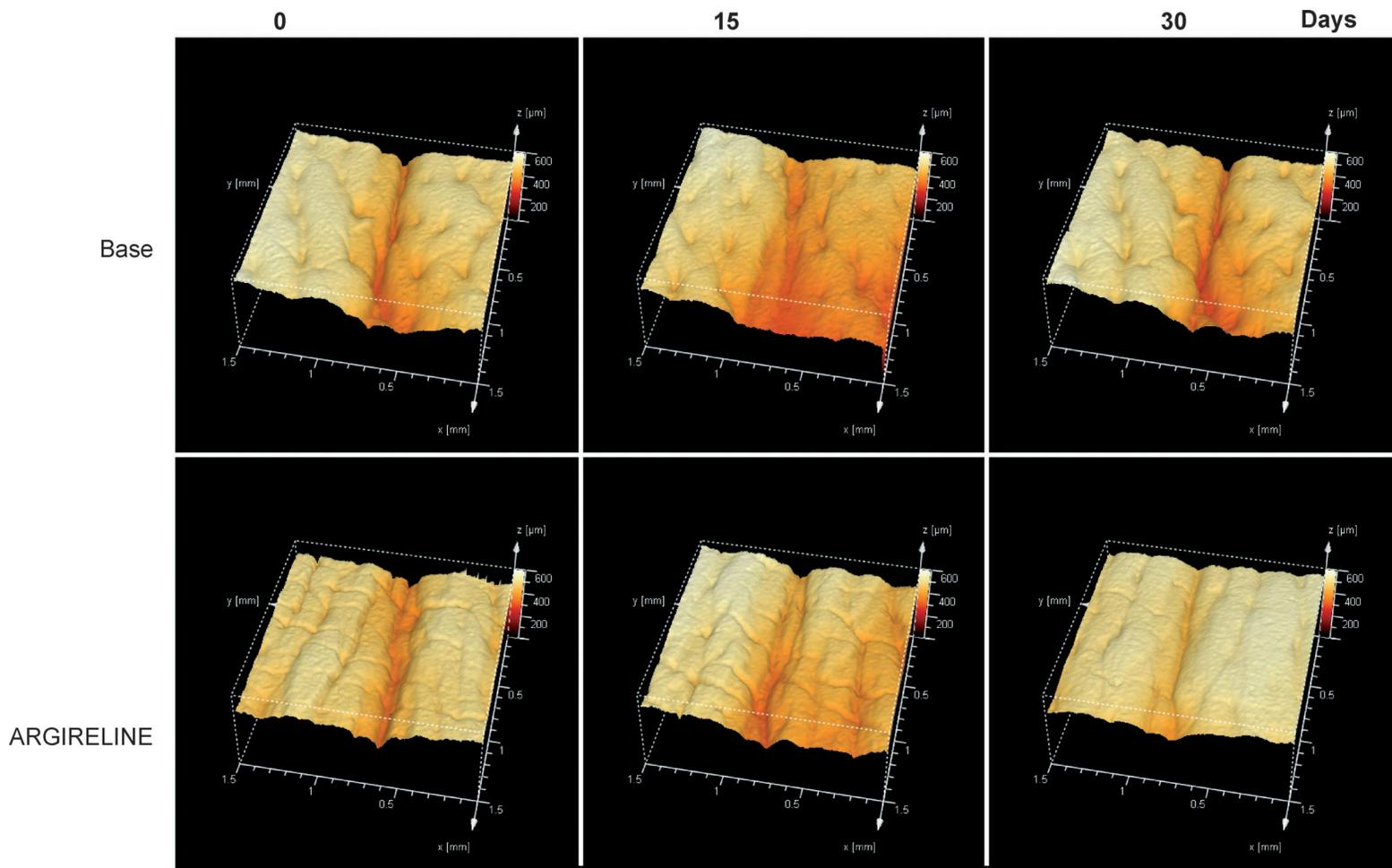


Figure 3 Argireline exhibits *in vivo* activity. Skin topographic imprints of the preorbital region of a healthy healthy volunteer (age 38) treated with and O/W emulsion without (base) or with 10% the hexapeptide. Silicone imprints were taken before the onset of treatment, after 15 days treatment and after 30 days treatment. Imprints were processed by confocal microscopy. Three-dimensional reconstructions were obtained as described in methods.

A and up to 55% by 1 μM of a 26-mer peptide derived from the C-terminal end of SNAP-25 (ESUP-E). Remarkably, 100 μM Argireline inhibited 30% of the total catecholamine exocytosis. Dose–response curves indicated an IC_{50} of 110 μM for Argireline, which is 5000 \times higher than the characteristic of BoNT A, and 400 \times higher than that of ESUP-E. To homogenize the potential antiwrinkle activity of synthetic peptides that mimic the action of BoNT A, we propose to define the antiwrinkle activity unit (AAU) as the ratio of the IC_{50} of ESUP-E, the most potent synthetic peptides, divided by the IC_{50} of the desired peptide. Accordingly, Argireline would have 0.003 AAU. Because this parameter refers to the activity blocking exocytosis from detergent-permeabilized cells, it may be modified depending on the ability to permeate through cell membranes. Nonetheless, the AAU provides an operational observable to classify the family of synthetic peptides that emulate the activity of naturally occurring botulinum neurotoxins.

Argireline attenuates wrinkle depth *in vivo* in healthy volunteers

To assess the antiwrinkle activity of Argireline, we first evaluated the skin permeation ability of the peptide. For this purpose, we investigated *in vitro* its ability to permeate through stratum corneum samples from human skin. The hexapeptide was placed into the donor chamber, and the content of peptide was determined in the receptor reservoir 2 h after placement. The total content of peptide in the receptor reservoir was a significant 30% of the amount deposited onto the membrane in the donor chamber. This result indicates that Argireline has the capability to permeate through the skin and, therefore, may exhibit *in vivo* antiwrinkle activity.

Accordingly, we next performed skin topography analysis to determine the effectiveness of an O/W emulsion containing 10% of Argireline using silicon imprints from the lateral preorbital area in healthy women volunteers. Subjects applied the O/W emulsion containing Argireline in one lateral preorbital side, although administered O/W emulsion alone in the contralateral side. All subjects applied twice daily the emulsion for 30 days. Silicone imprints were analysed at days 0, 15 and 30 by confocal microscopy. As illustrated in Fig. 3, topical application of the O/W emulsion containing the hexapeptide resulted in a significant attenuation of the depth and roughness

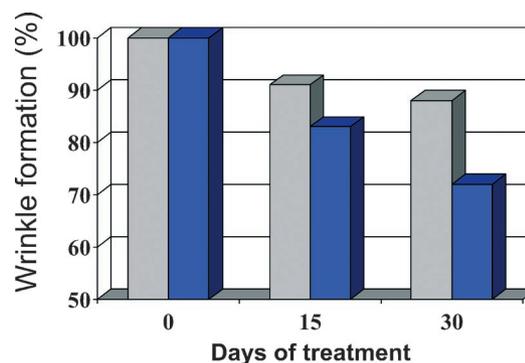


Figure 4 Argireline attenuates wrinkle intensity. The percentage of wrinkle formation as a function of day of treatment with O/W emulsion without or with 10% the hexapeptide. Wrinkle formation was obtained by measuring the depth of the wrinkle, and normalizing the values to those obtained before initiating the treatment. Data are mean of 10 healthy women volunteers.

of the wrinkles. Use of the O/W emulsion for the same period of time did not result in significant changes in the skin topography. Quantitative analysis and normalization of the silicon replicas show that, whereas the O/W base emulsion reduced by 10% the depth of skin wrinkles, the O/W emulsion containing the peptide decreased them by 30% (Fig. 4). These findings demonstrate a significant antiwrinkle activity for Argireline, in agreement with its *in vitro* and cellular activities.

Conclusion

A 6-mer peptide patterned after the N-end of the SNAP-25 protein that mimics the activity of BoNTs in terms of inhibiting Ca^{2+} -dependent exocytosis, display also remarkable antiwrinkle activity when applied topically. Although much less potent than BoNT A (12 vs. 0.003 AAUs), this small peptide exhibits the great advantage of its insignificant acute toxicity ($\geq 2000 \text{ mg kg}^{-1}$) as compared with BoNT A (20 ng kg^{-1}). Furthermore, the hexapeptide does not exhibit primary skin irritation in an intracutaneous test nor genotoxicity as determined by the AMES test (data not shown), thus making its use highly safe and physician-independent. Therefore, peptides that mimic the action of BoNTs, such as Argireline, represent the next generation of biosafe products with antiwrinkle activity which could be extensively used in cosmetic preparations.

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