Polígon Industrial Camí Ral
C/Isaac Peral, 17
08850 Gavá – Barcelona - Spain
Telf. +34 93 638 80 00
fax. +34 93 638 93 93
Internet: www.lipotec.com
e-mail. commercial@lipotec.com

ARGIRELINE®

AN ANTI-AGING PEPTIDE

CODE: PD010 / PD011

Date: June 2006 Revision: 18

A GMP PEPTIDE FOR COSMETIC APPLICATIONS

A NEW SYNTHETIC COSMETIC INGREDIENT

SUMMARY

The anti wrinkle hexapeptide ARGIRELINE® represents the discovery of a positive hit based on a scientific pathway from rational design to GMP production. The study of the basic biochemical mechanisms of anti-wrinkle activity has led to this revolutionary hexapeptide which has taken the cosmetic world by storm.
Finally, an anti-wrinkle treatment which can compete with the efficacy of Botulinum Toxin A but leaves aside the risks, the injections and the high cost: ARGIRELINE®.

GENERAL DESCRIPTION

One of the most striking signs of skin aging is increased wrinkling of the face. This can occur naturally over time and is identified by certain biochemical, histological and physiological changes that are enhanced by environmental exposure. There are other secondary factors that can cause characteristic folds, furrows and creases of the face. These include 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. In any case and independently of the ultimate physiological pathway, the molecular mechanism involved in face aging is directly related to changes in the conformation of the collagen triple helix, degradation of the elastin polypeptides and certain disorder in the packing of the lipidic matrix of the skin.

It has been clearly established that these conformational changes and the disturbance of the perfect packing of the lipid matrix can be significantly avoided by modulating muscle contraction.

Muscles are contracted when they receive neurotransmitter released from inside a vesicle. The SNARE (SNAp REceptor) complex is essential for this neurotransmitter release at the synapsis (A. Ferrer Montiel et al, The Journal of Biological Chemistry, 1997, 272, 2634-2638). It is a ternary complex formed by the proteins VAMP, Syntaxin and SNAP-25 (SyNaptosomal Associated Protein). This complex is like a cellular hook which captures vesicles and fuses them with the membrane for the release of neurotransmitter.

ARGIRELINE® is a mimic of the N-terminal end of SNAP-25 which competes with SNAP-25 for a position in the SNARE complex, thereby modulating its formation. If the SNARE complex is slightly destabilized, the vesicle can not release neurotransmitters efficiently and therefore muscle contraction is attenuated, preventing the formation of lines and wrinkles (see Fig.1).

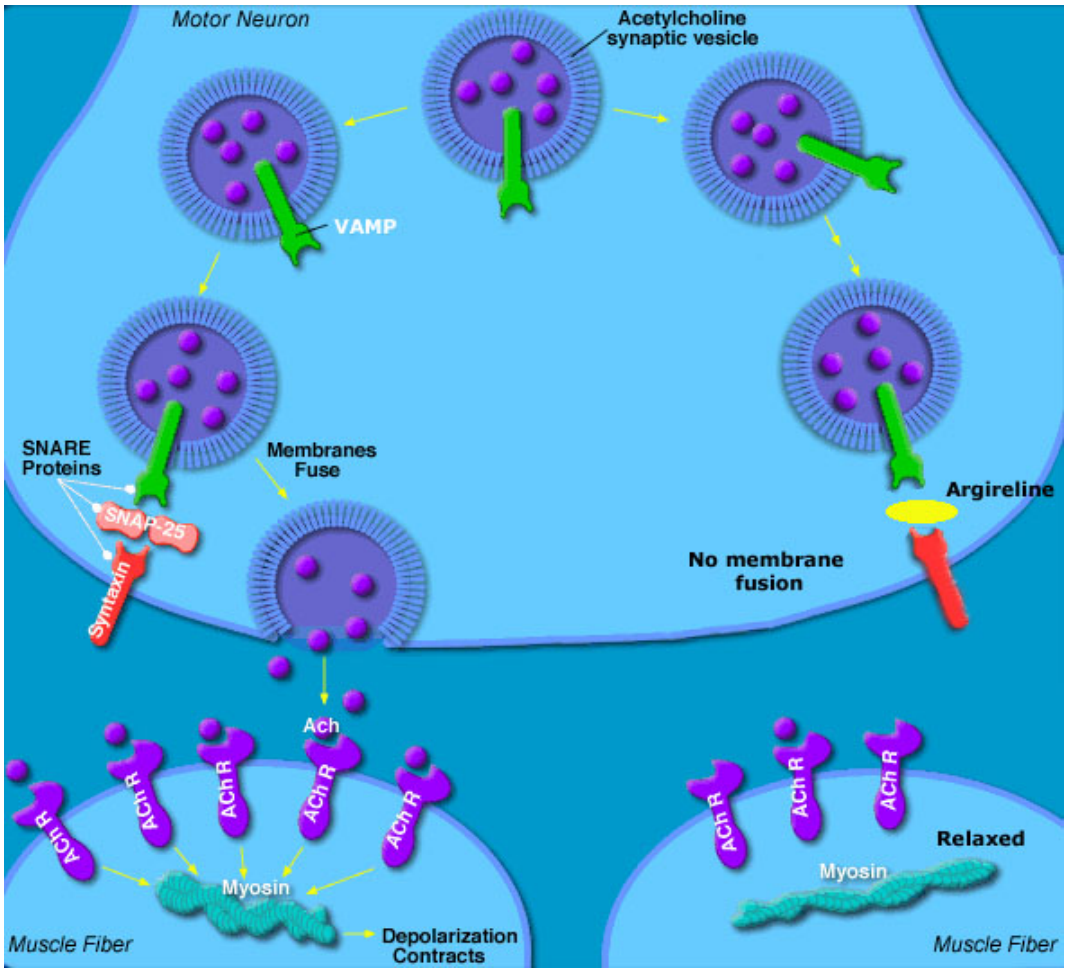

Fig. 1. Argireline® biochemical mechanism

PROPERTIES AND APPLICATIONS

- ARGIRELINE® reduces the depth of wrinkles on the face caused by the contraction of muscles of facial expression, especially in the forehead and around the eyes.
- ARGIRELINE® is a safer, cheaper, and milder alternative to Botulinum Toxin, topically targeting the same wrinkle-formation mechanism in a very different way.

ARGIRELINE® can be incorporated in cosmetic formulations such as emulsions, gels, sera, etc., where removal of the deep lines or wrinkles in the forehead or around the eyes area is desired.

TECHNICAL INFORMATION

PRODUCT SPECIFICATIONS

ARGIRELINE® Powder	
Code:	PD011
INCI name:	Acetyl Hexapeptide-8
Appearance:	White to off white crystalline powder
Mass spectrometry:	$[M+1]^+ = 890.1$
Amino acid analysis:	Glutamic acid: 2.7 – 3.3 Methionine: 0.6 – 1.0 Arginine: 1.8 – 2.2
Peptide purity:	>80 %

ARGIRELINE® Solution	
Code:	PD010
INCI name:	Water, Acetyl Hexapeptide-8
Appearance:	Transparent solution
Contents:	0.05 % ARGIRELINE® Powder
Preservative:	0.3 % Phenonip

The synthesis of ARGIRELINE® is carried out at our factory in Gavà following GMP guidelines and involves a final freeze-drying step. Freeze-dried products are commonly obtained as a polymorphous crystalline powder, which means that locally some aggregates and differences in crystal size may appear. This polymorphism is not associated to chemical differences and extensive work performed by the analytical department has ensured the homogeneity of the product.

PROCESSING AND DOSAGE

ARGIRELINE® is presented either as **ARGIRELINE® Powder**, a hexapeptide in powder form which can be easily dissolved in water, or as **ARGIRELINE® Solution**, an aqueous solution containing 0.5 g/L of the powder version. It can be incorporated at the final stage of the manufacturing product, provided the temperature is below 40 °C. Taking into consideration the concentration of peptide in **ARGIRELINE® Solution**, it is recommended that 3 to 10% of the solution is present in the final formulation in order to obtain significant anti-wrinkle activity.

STORAGE AND SHELF LIFE

ARGIRELINE® Powder and **ARGIRELINE® Solution** must be kept in a cool, dark and clean place to ensure a shelf life of twenty-four months.

ARGIRELINE® Solution is best kept in the refrigerator. In rare cases, refrigerated storage of **ARGIRELINE® Solution** can cause precipitation of the preservative. This does not affect the integrity of the product.

SAFETY

The toxicological profile of ARGIRELINE® for cosmetic purposes was assessed only “in vitro”. Several "in vivo" tests were carried out for the potential registration of the product as a pharmaceutical ingredient. The tests listed below are only a selection of those performed: a full toxicological report and a summary of all the safety tests performed are available on request.

All tests were performed using solutions of **ARGIRELINE® Powder** at the desired concentrations.

In vitro tests

Citotoxicity test on human dermal fibroblasts
No signs of citotoxicity were observed.

Citotoxicity test on human epidermal keratinocytes
The results showed no signs of citotoxicity at the concentrations assayed.

Genotoxicity test (Ames test)
The results showed no genotoxicity under the conditions assayed.

Ocular Irritation (NRU - Neutral Red Uptake test)
The product is potentially not irritating for the eyes.

In vivo tests

Primary skin irritation test
No signs of irritation redness or edema were observed in albino male rabbits after 7 days from the removal of the tested compound (Argireline® Solution 0.05%).

Acute oral toxicity test
Analysis design allowed to conclude that DL_{50}>2500 mg/Kg body weight in rats and therefore ARGIRELINE® shows no acute oral toxicity at the dosage tested.

Skin sensitisation (Hypoallergenicity)
An HRIPT (Human Repeated Insult Patch Test) was performed on 50 volunteers aged 18 to 70. Argireline® Solution 0.05% did not cause sensitisation in any volunteer so it can be classified as Low Sensitisation.

EFFICACY DATA

In vitro tests

Modulation of SNARE complex formation by ARGIRELINE®

The test readily allows us to follow the formation and thermal stability of the reconstituted SNARE complex. Since ARGIRELINE® is patterned after the N-terminal domain of the protein SNAP 25, it can compete with it for a place in the SNARE complex. This test evaluates the antagonistic competitive efficacy of ARGIRELINE® with the wild type SNAP-25 in its capacity to assemble with syntaxin and synaptobrevin forming the SNARE complex. For this purpose, the concentration of SNAP25 is limiting to allow efficient peptide-SNAP-25 competition.

A significant improvement on the method has been to use in vitro translated [^{35}S]-SNAP-25, that has remarkably increased the sensitivity level of the assay. The *in vitro* reconstitution and modulation of SNARE complex involves the expression and purification of recombinant syntaxin and synaptobrevin proteins, the translation of [^{35}S]-SNAP-25 and the SNARE complex assembly and modulation by peptides. Equimolar amounts of recombinant syntaxin and synaptobrevin were incubated in the absence or presence of ARGIRELINE® at 4ºC for 2 h. Thereafter, 4 µl of [35S]-SNAP-25 were added and mixture further incubated at 4ºC for 12 h. SNARE complex assembly was analysed by SDS-PAGE on 12% gels, followed by fluorographic detection on Kodak X-OMAT AR x-ray films. Temperature-dependent disassembly of SNARE complex was used to identify recombinant SNARE. ARGIRELINE® was tested at mM concentrations. This test was developed at our collaborating group from University Miguel Hernandez in Elche (Spain).

The electrophoresis in Fig. 2 shows a top band that corresponds to formation of the SNARE complex and a bottom band that shows presence of SNAP-2. The left lane is a control with intact SNARE complex, lane number 2 shows SNARE complex destroyed by heat, so no band is detected for the complex, only the SNAP-25 band is present. The rest of the lanes show how ARGIRELINE® inhibits formation of the SNARE complex.

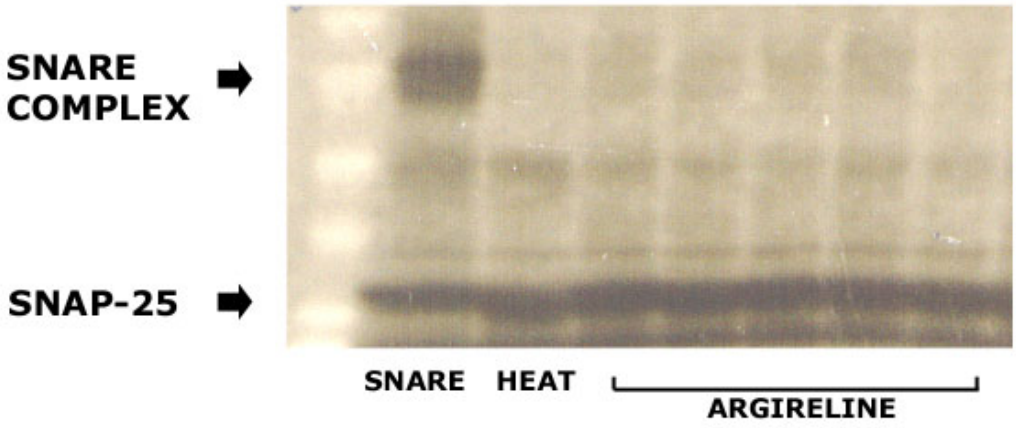

Fig. 2. Modulation of SNARE complex formation by ARGIRELINE®

Modulation of catecholamine release in chromaffin cells

Inhibition in the release of catecholamines was determined by monitoring the neurotransmitters Adrenaline and Noradrenaline. Chromaffin cells were incubated with tritiated noradrenaline/adrenaline and ARGIRELINE®. The release of catecholamines, as well as the total cell content, was determined by liquid scintillation counting. The significant modulation of both neurotransmitters at nM concentrations of ARGIRELINE® is a clear indicator of the potent anti-wrinkle activity of this hexapeptide.

Note: catecholamines are not directly involved in muscle contraction, a function performed by the neurotransmitter acetylcholine being secreted from nerve cells. However, nerve cells are hard and expensive to culture, while chromaffin cells, that secrete catecholamines, are equivalent in all respects, and are easier to manipulate. Chromaffin cells are often used as models for study of neural properties and processes, for instance, exocytosis. (Chromaffin cells as models of endocrine cells and neurons, Tischler AS, *Ann N Y Acad Sci*, 2002, 971: 366 – 70)

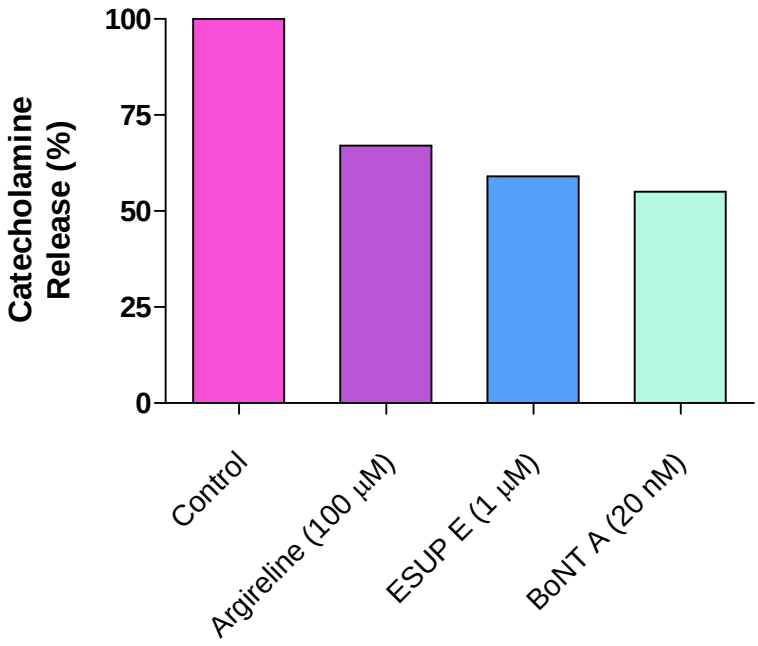

Fig 3. Modulation of catecholamine release (notice different concentrations)

Antiwrinkle Activity Units (AAUs)

Dose-response curves obtained from the catecholamine release tests yield IC_{50} values for the different peptides. We can therefore quantify and compare the exocytosis-blocking activity, which is directly related to the antiwrinkle power. This facilitates the definition of an Antiwrinkle Activity Unit (AAU) (A synthetic hexapeptide – Argireline- with antiwrinkle activity, Blanes-Mira C, *Int.Journal Cosm Sci*, 2002, 24, 303 – 310).

$$AAU_{\ SAMPLE} = [IC_{50}]_{\ ESUP\ E} / [IC_{50}]_{\ SAMPLE}$$

COMPOUND	**IC_{50}**	**AAU**
BoNT A	~0.0260 µM	12
ESUP E	0.310 µM	1 (by definition)
ARGIRELINE®	110 µM	0.0030

In vivo tests

Anti-wrinkle test on healthy volunteers (10% ARGIRELINE® Solution)

Skin topography analysis for measuring the effectiveness of an O/W emulsion containing 10% of **ARGIRELINE® Solution** were performed obtaining silicon imprints from around the eyes from 10 healthy women volunteers. Silicon imprints were obtained pre-test and after 30 days of twice a day applications. Analyses of the imprints were performed by confocal laser scanning microscopy to assess the evolution of the skin surface before and after the treatment. Skin topography images from the three dimensional reconstruction of optical sections are shown in Fig 4. It can be observed that the depth of the wrinkle has significantly decreased after 30 days of treatment which confirms the validation of the biochemical mechanism hypothesis.

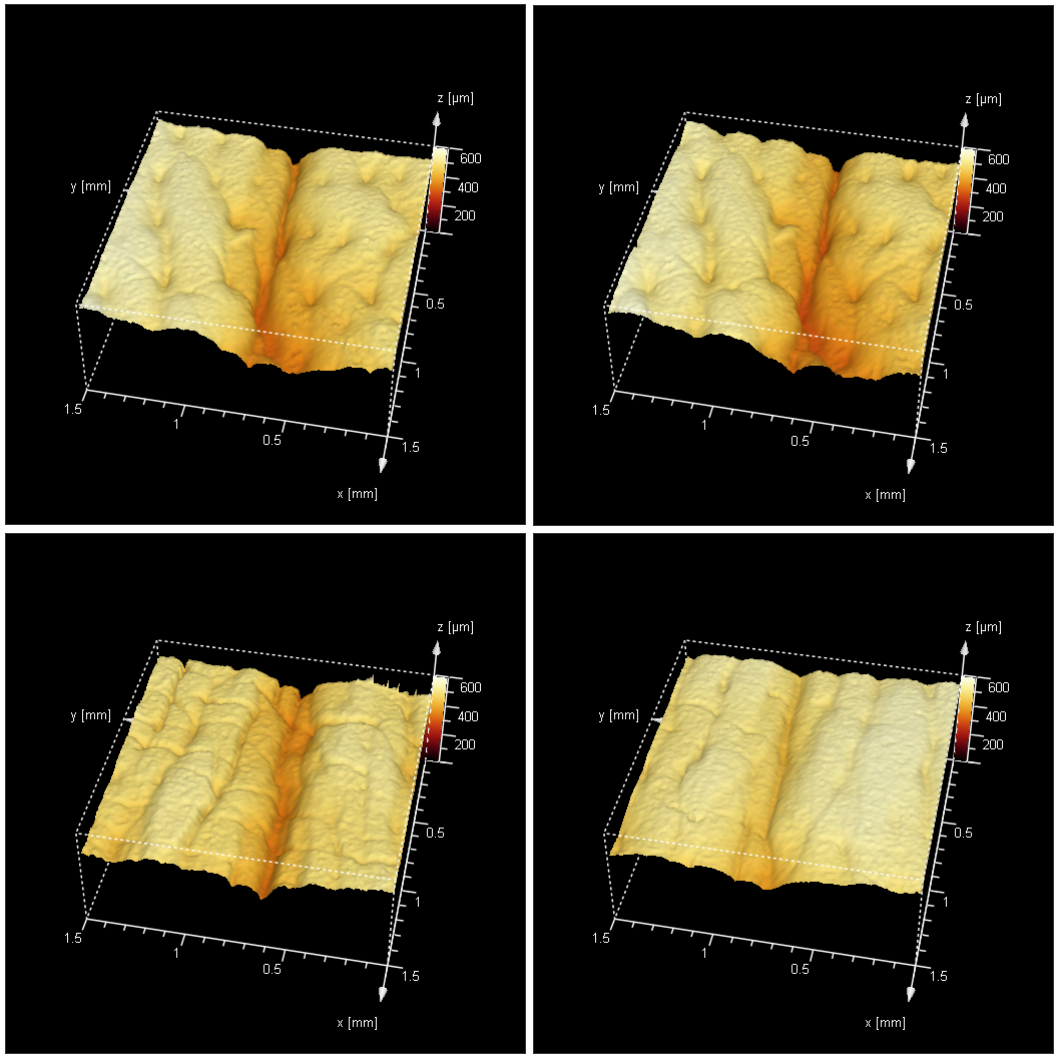

Fig. 4. Skin topography images before (left) and after a 30 day treatment with a cream containing **10% ARGIRELINE® Solution** (right). The top pictures show an ordinary cream used as a negative control. The bottom pictures show a cream with the same formulation containing ARGIRELINE®.

Anti-wrinkle test on healthy volunteers (5% ARGIRELINE® Solution)
A cream containing 5% ARGIRELINE® SOLUTION (0.05%) was applied twice daily around the eyes of 14 volunteers, aged 39 to 64, for 28 days. Silicon imprints of the treated areas were obtained before the test and after 28 days, and roughness was measured by confocal profilometry. A decrease of 16.26% in wrinkle depth was accomplished, with maximum values up to –31.80%.

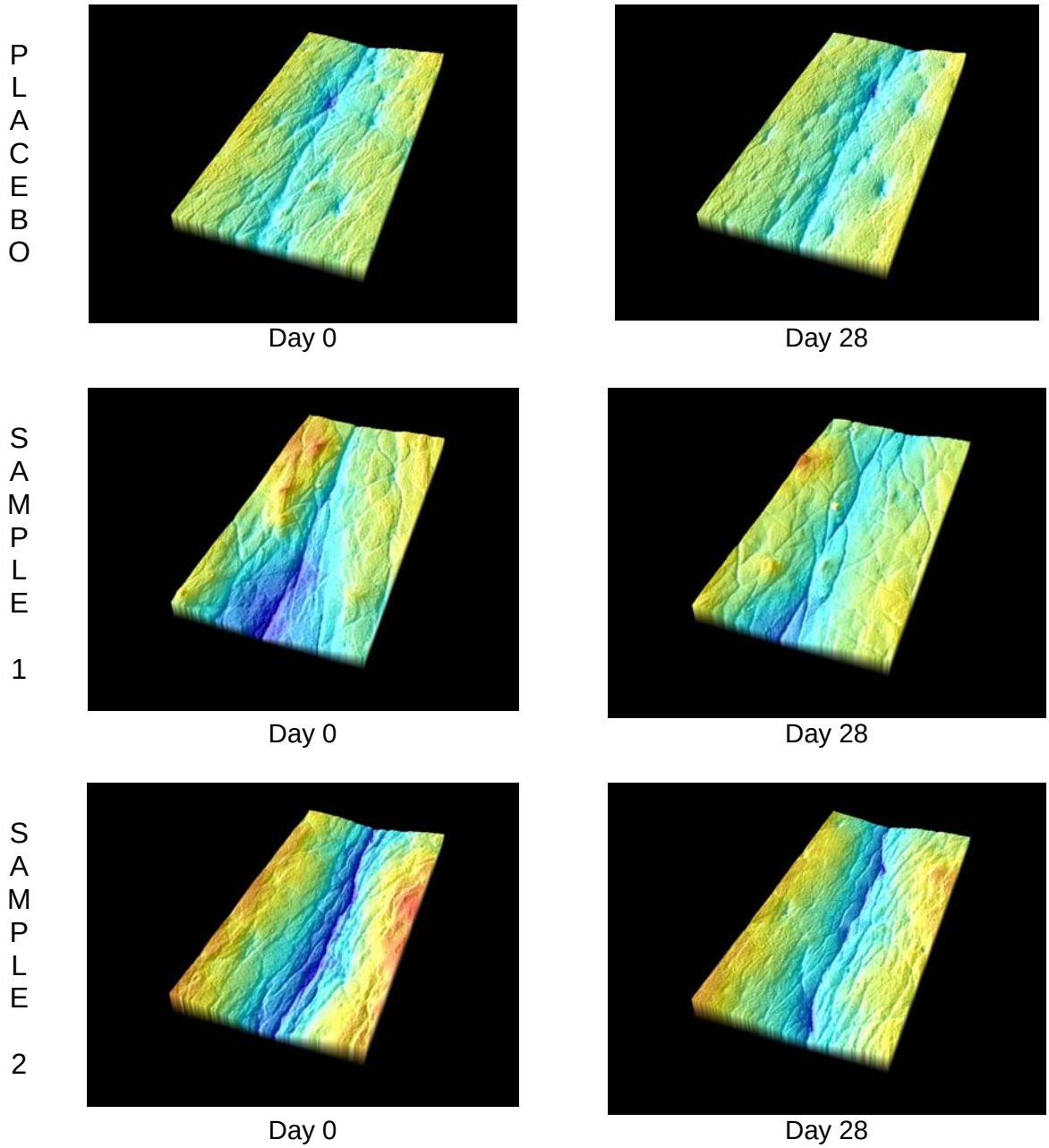

Fig. 5. Representative images of the silicon replicas before (left) and after a 28 day treatment with a cream containing **5% ARGIRELINE® Solution** (right). Dark blue colour indicates maximum depth – bright red colour indicates max height. The top pictures show and ordinary cream used as a negative control.

GENERAL PRODUCT INFORMATION

Trade name	ARGIRELINE® SOLUTION
Product code	PD010

INGREDIENTS

INCI name	CAS No	EINECS No
WATER (AQUA)	7732-18-5	231-791-2
ACETYL HEXAPEPTIDE-8	616204-22-9	Exempt
PHENOXYETHANOL	122-99-6	204-589-7
METHYLPARABEN	99-76-3	202-785-7
ETHYLPARABEN	120-47-8	204-399-4
PROPYLPARABEN	94-13-3	202-307-7
BUTYLPARABEN	94-26-8	202-318-7
ISOBUTYLPARABEN	4247-02-3	224-208-8

Trade name	ARGIRELINE® POWDER
Product code	PD011

INGREDIENTS

INCI name	CAS No	EINECS No
ACETYL HEXAPEPTIDE-8	616204-22-9	Exempt