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## SNAP-8 SOLUTION C

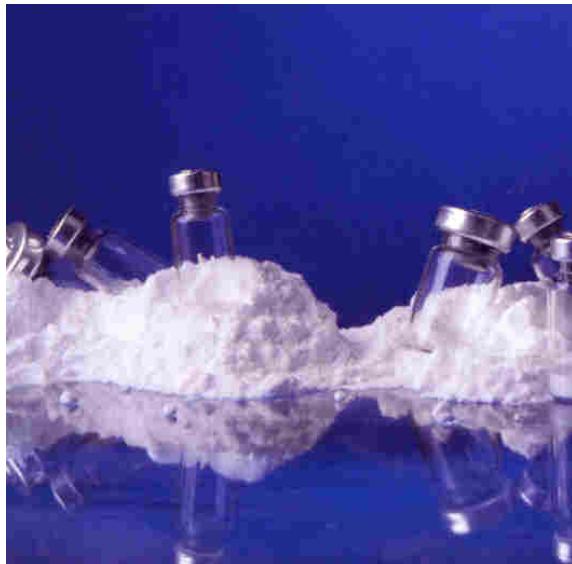
AN ANTI-AGING PEPTIDE

CODE: P06-PD017

Date: July 2008

Revision: 0

### A GMP PEPTIDE FOR COSMETIC APPLICATIONS



### A NEW SYNTHETIC COSMETIC INGREDIENT

## SUMMARY

The anti wrinkle octapeptide SNAP-8 SOLUTION C is an elongation of the famous hexapeptide ARGIRELINE®. The study of the basic biochemical mechanisms of anti-wrinkle activity led to the revolutionary hexapeptide which has taken the cosmetic world by storm. Those same studies have now been applied to bring another addition to the Botulinum Toxin-inspired family of peptides.

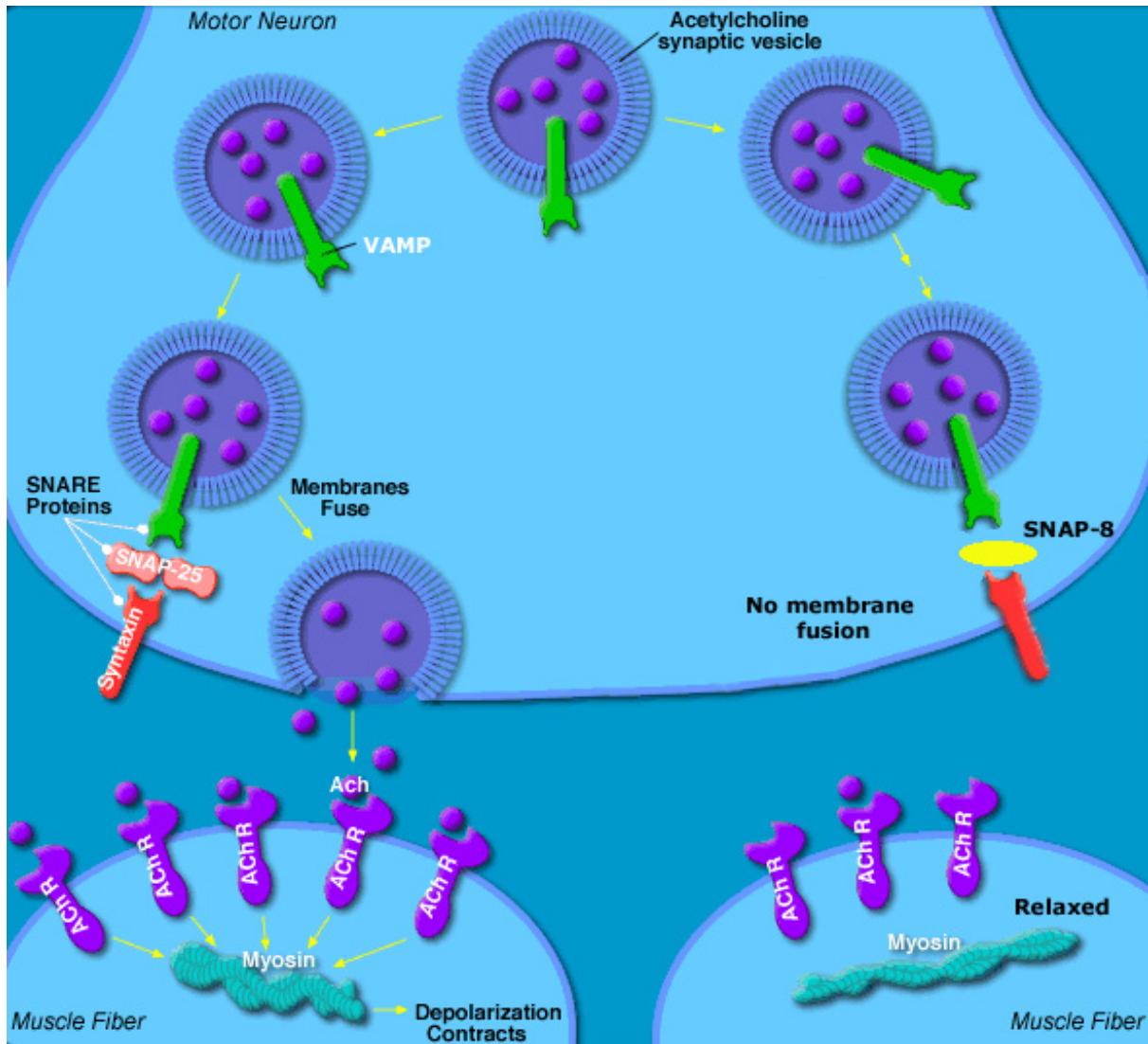
## GENERAL DESCRIPTION

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.

SNAP-8 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.** SNAP-8 biochemical mechanism

## PROPERTIES AND APPLICATIONS

- SNAP-8 SOLUTION C 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.
- SNAP-8 SOLUTION C is a safer, cheaper, and milder alternative to Botulinum Toxin, topically targeting the same wrinkle-formation mechanism in a very different way.

SNAP-8 SOLUTION C 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

<b>SNAP-8 Solution C</b>	
Code:	P06-PD017
INCI name:	Water, Acetyl Octapeptide-3
Appearance:	Translucent solution
Contents:	0.05 % SNAP-8 Powder

The synthesis of SNAP-8 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

SNAP-8 is presented either as **SNAP-8 Powder (Code PD018)**, an octapeptide in powder form which can be easily dissolved in water, or as **SNAP-8 Solution (Code PD017)**, 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 **SNAP-8 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

**SNAP-8 Powder** must be kept in a cool, dark and clean place to ensure a shelf life of thirty months whereas **SNAP-8 Solution** must be kept under the same conditions to ensure a shelf life of, at least, twenty-four months.

**SNAP-8 Powder** and **SNAP-8 Solution** are best kept in the refrigerator. In rare cases, refrigerated storage of **SNAP-8 Solution** can cause precipitation of the preservative. This does not affect the integrity of the product.

## **SAFETY**

The toxicological profile of SNAP-8 for cosmetic purposes was assessed only *in vitro* and in tests on human volunteers (in Italy). A full toxicological report and a summary of all the safety tests performed are available on request.

All tests were performed using solutions of **SNAP-8 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.

#### **3T3 NRU Phototoxicity Test**

The product showed not phototoxicity.

### ***In vivo* tests**

#### **Acute oral toxicity test**

Analysis design allowed to conclude that  $DL_{50}>2500$  mg/Kg body weight in rats and therefore SNAP-8 shows no acute oral toxicity at the dosage tested. This test is compulsory for any new chemical entity according to the Dangerous Substances Directive 67/548/EC.

#### **Skin sensitisation (Hypoallergenicity)**

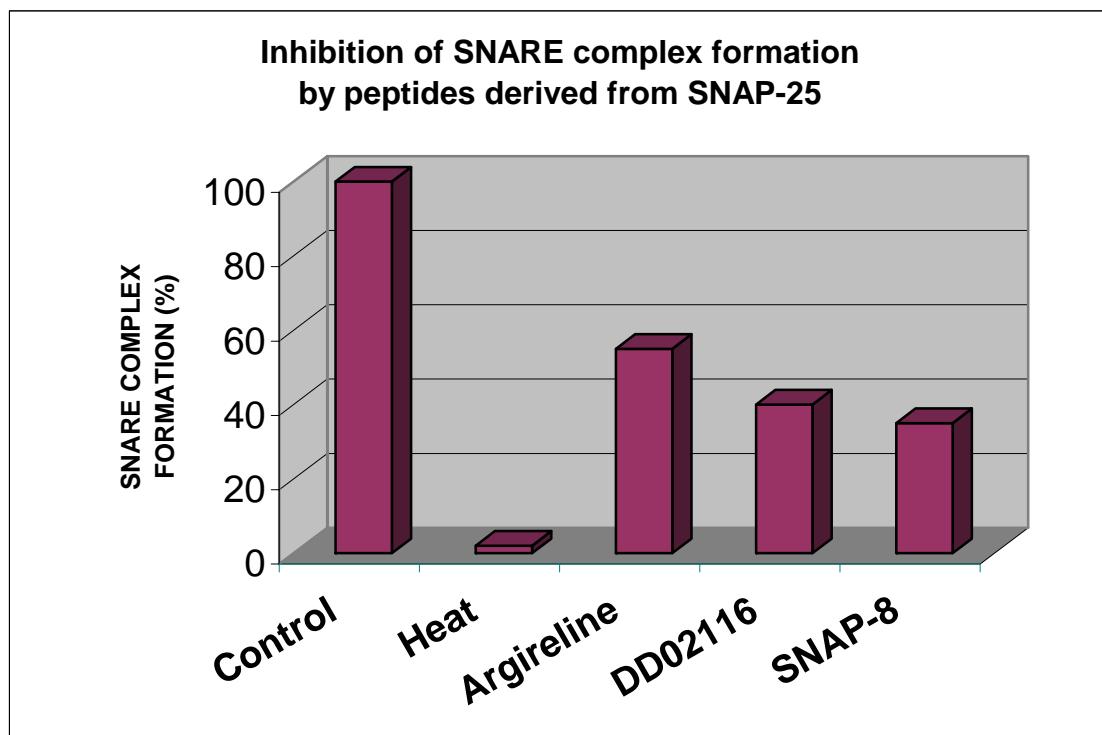
An HRIPT (Human Repeated Insult Patch Test) was performed on 50 volunteers aged 18 to 70. SNAP-8 Solution 0.05% did not cause sensitisation in any volunteer so it can be classified as Low Sensitisation.

# EFFICACY DATA

## *In vitro* tests

### Inhibition of SNARE complex formation

To assay the efficacy of small peptides on the stability of the SNARE complex we developed an *in vitro* method that enabled us to follow the formation and thermal stability of the reconstituted SNARE protein complex. The rational of the method evaluates the antagonistic competitive efficacy of small peptides patterned after the SNAP-25 N-terminal domain with the wild type protein on its capacity to assemble with syntaxin and synaptobrevin forming the SNARE complex



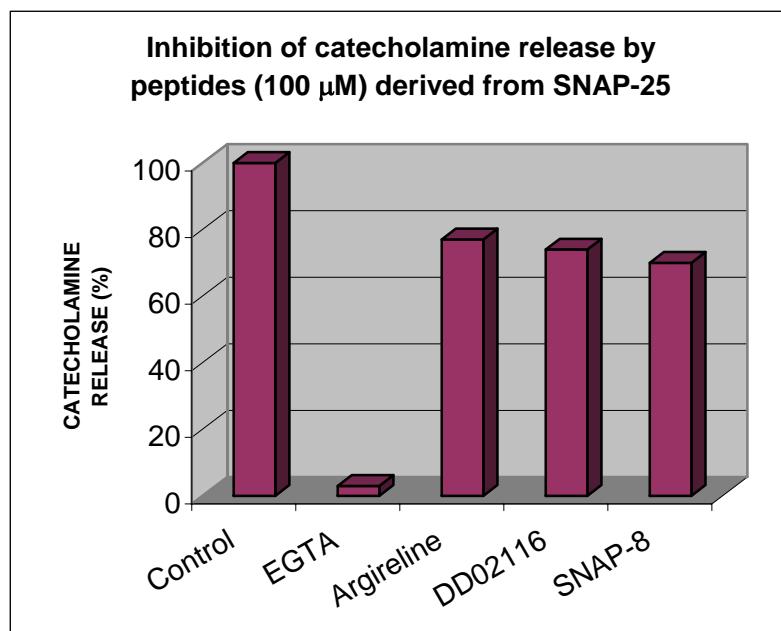
**Fig 2.** Peptides derived from SNAP-25 modulate the formation of the SNARE complex *in vitro*

The results prove that short peptides from the N terminal end of SNAP-25 compete with the native protein and inhibit the formation of the SNARE complex by affecting its stability.

## 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 SNAP-8. The release of catecholamines, as well as the total cell content, was determined by liquid scintillation counting. The significant modulation of both neurotransmitters at  $\mu\text{M}$  concentrations of SNAP-8 is a clear indicator of the potent anti-wrinkle activity of this octapeptide.

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 difficult 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

EGTA is a metal chelator used as a negative control, because it captures  $\text{Ca}^{2+}$  which is essential for vesicle fusion and catecholamine release.

## **Antiwrinkle Activity Units (AAUs)**

One of the problems of working with peptides with similar effects but different potency is to compare their activities.

Several catecholamine release tests at different concentrations of peptide were performed, in order to plot the dose-response curves needed to calculate the IC<sub>50</sub> values for the different peptides. We can therefore quantify and compare the exocytosis-blocking activity, which is directly related to the antiwrinkle power.

The IC<sub>50</sub> is the 50% inhibitory concentration, in this case, the concentration of active that inhibits 50% of catecholamine secretion. The lower the IC<sub>50</sub>, the smaller the amount needed to inhibit secretion 50%, and the more active the compound is.

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).

$$\text{AAU}_{\text{SAMPLE}} = [\text{IC}_{50}]_{\text{ESUP E}} / [\text{IC}_{50}]_{\text{SAMPLE}}$$

COMPOUND	IC <sub>50</sub>	AAU
BoNT A	~0.0260 µM	12
ESUP E	0.310 µM	1 (by definition)
SNAP-8	55 µM	0.0056
ARGIRELINE®	110 µM	0.0028

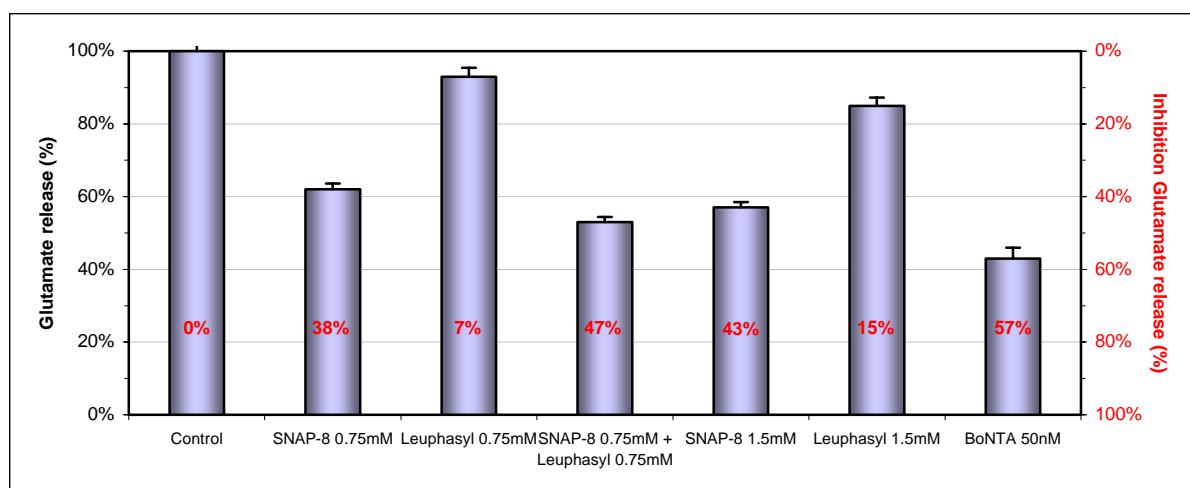
ESUP-E is the most potent synthetic peptide in terms of catecholamine inhibition. It is a long peptide, not suitable for cosmetic application, but is used as a reference to define AAUs.

## Modulation of glutamate release in a neuron cell culture

Inhibition of glutamate release by depolarized neuron cells is a validated cell assay for measuring the potential activity of compounds on the inhibition of neuronal exocytosis. The K<sup>+</sup>-induced depolarization of hippocampal cultures in the presence of extracellular Ca<sup>2+</sup> results in the release of glutamate, which is the most abundant excitatory neurotransmitter in the nervous system.

A primary cell culture of neurons was incubated with tritiated glutamine during 3h in order to load them with radiolabelled glutamate. Afterwards, the excess of glutamine was rinsed off, and they were incubated with the test items for 1h at 37°C. The release of radiolabelled glutamate is made by depolarization with buffered 75mM KCl/2mM CaCl<sub>2</sub> in physiologic buffer for 10min at 37°C. The culture media was collected and the quantity of radiolabelled glutamate was quantified using a scintillation counter. Values obtained are the average of 6 determinations. Untreated neuron cultures were used as a negative control and cultures treated with Botulinum Toxin A (BoNT A) were used as positive controls.

The release of glutamate from the neurons is measured in order to compare the *in vitro* activities of the anti expression-wrinkle peptides SNAP-8 and Leuphasyl®.

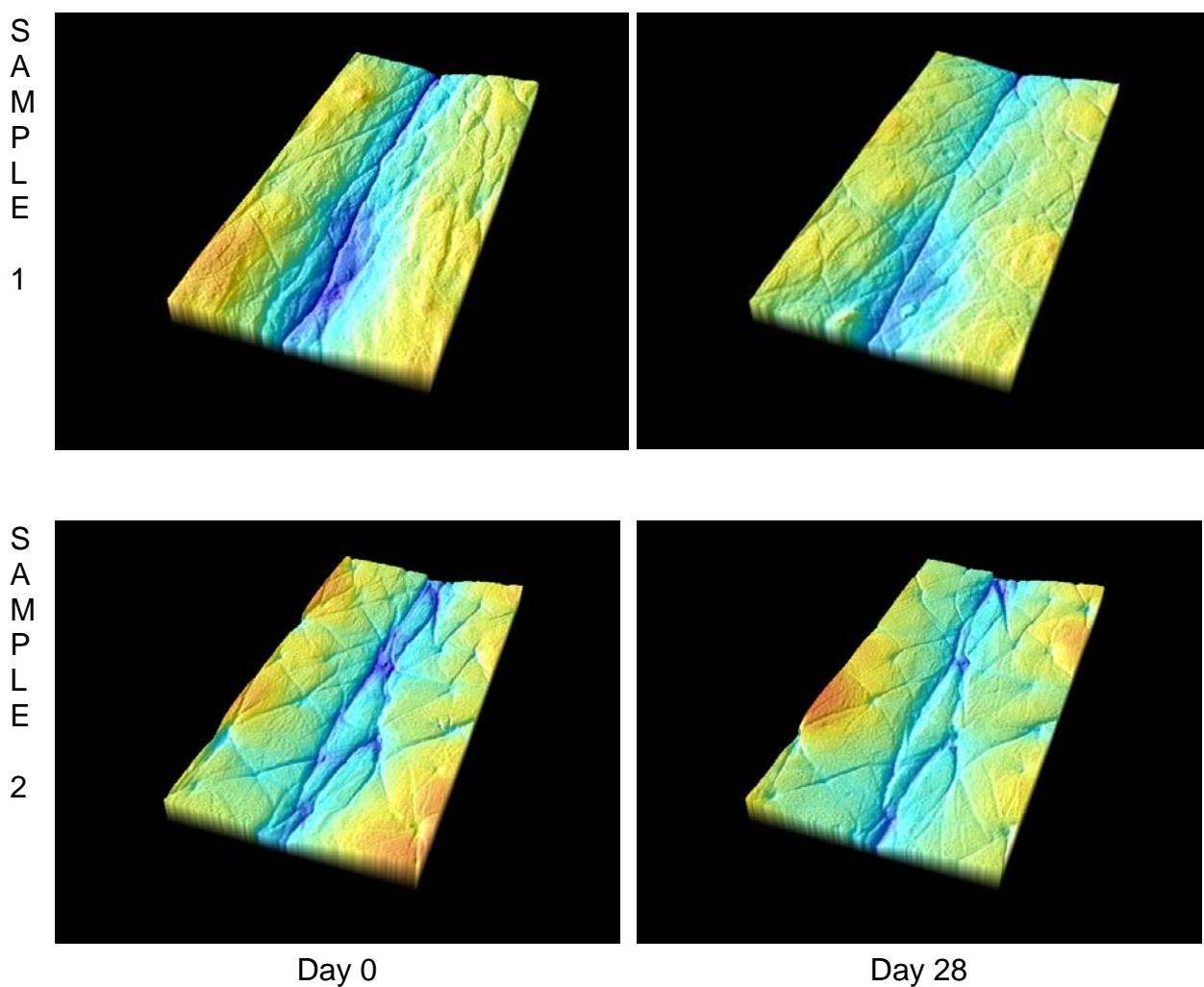


The combination of SNAP-8 with Leuphasyl® showed a higher inhibitory potential of glutamate release than the inhibitory potential resulted from the addition of each single product, which means that both peptides show a synergistic effect *in vitro*, their mechanisms are independent and their effects can be added.

## In vivo test

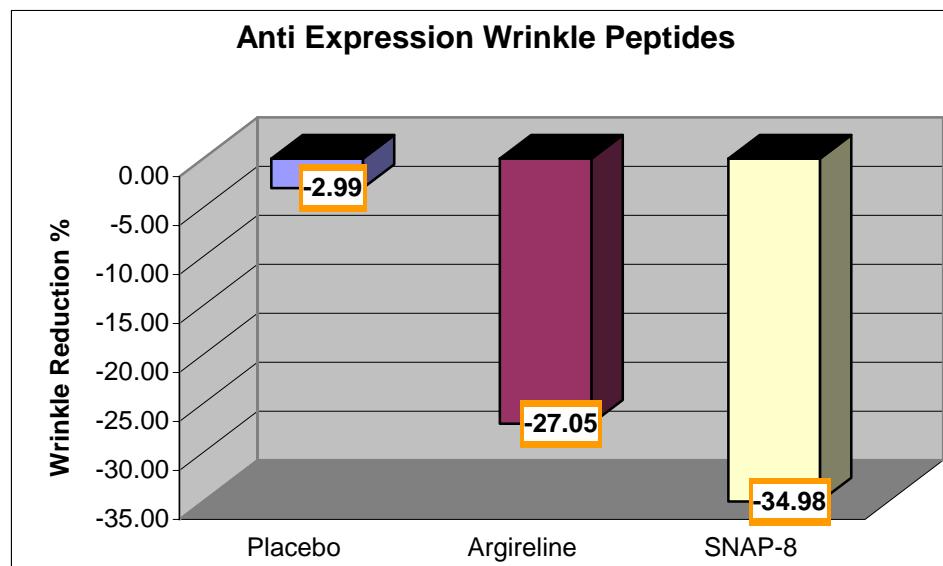
### Anti-wrinkle test on healthy volunteers

Skin topography analysis for measuring the effectiveness of a cream containing 10% of **SNAP-8 Solution** were performed obtaining silicon imprints from around the eyes from 17 healthy women volunteers. Silicon imprints were obtained pre-test and after 28 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 3. It can be observed that the depth of the wrinkle has significantly decreased after 28 days of treatment which confirms the validation of the biochemical mechanism hypothesis.



**Fig. 4.** Skin topography images before (left) and after (right) a 28 day treatment with a cream containing **10% SNAP-8 Solution**. The top pictures show one of the volunteers and the bottom pictures show another volunteer.

These results are summarised in the following graph.



Results are compared to a previous test (October 2001) performed by the same company using the same technique, testing a cream with **10% Argireline® Solution** on 10 women volunteers.

The maximum reduction value found for **10% SNAP-8 Solution** was – 63.13%.

As observed *in vitro*, the two extra amino acids from the SNAP-25 sequence seem to moderately increase the anti-wrinkle activity.

## GENERAL PRODUCT INFORMATION

Trade name	SNAP-8 SOLUTION C
Product code	P06-PD017

## INGREDIENTS

INCI name	CAS No	EINECS No
WATER (AQUA)	7732-18-5	231-791-2
ACETYL OCTAPEPTIDE-3	868844-74-0	-
CAPRYLYL GLYCOL	1117-86-8	214-254-7

Trade name	SNAP-8 POWDER
Product code	PD018

## INGREDIENTS

INCI name	CAS No	EINECS No
ACETYL OCTAPEPTIDE-3	868844-74-0	-

*Note: Graphs and photographs are available for customer use provided that the final product contains the same concentration of active as the formulations in our tests. Customers must request written permission for use of the graphic material and/or ingredient tradenames to Lipotec. Customers are responsible for compliance with local and international advertising regulations*