induchem Induchem AG Switzerland | Industriestrasse 8A CH-8604 Volketswil | T +41 44 908 43 33 F +41 44 908 43 30 | info@induchem.com www.induchem.com

RUBIXYL®

THE EQUALIZER OF BEAUTY RECEPTORS

A NEW ANTI-AGING APPROACH BY STIMULATING DELTA OPIOID RECEPTORS

RUBIXYL®

Contents

1. Introduction 3
2. Mode of Action 4
3. Experimental Set Up 8
3.1 Vehicle and Active Ingredients 8
3.2 Application of the product 8
3.3 Reconstituted Human Skin Model Study 8
3.4 Clinical tests 8
4. Efficacy Results 9
4.1 Tests on human reconstituted skin 9
4.2 Clinical tests on human volunteers 12
5. Conclusions 16
6. Characteristics 17
7. References 18
8. Appendix 1 19

RUBIXYL®

1. Introduction

In the communication between cells and the extracellular matrix (ECM), receptors play an important role. A receptor is a molecule most often found on the surface of a cell, which receives signal molecules originating externally from the cell[1]. Through binding to a receptor, an information transport through the cell membrane is initiated. The function of this transport is to direct information flow to the cell nucleus and induce new signals from the cellular DNA to start the synthesis of biological molecules from the outer membrane, which are constitutional parts of the ECM. The restructuring of the connective tissue of the dermis by synthesis of structural proteins, like collagen, is a typical process. This process is controlled by a signal transmission from specific receptors on the membrane of a cell to its DNA in the nucleus and initiates gene expression followed by protein synthesis.

During the last 15 years, several experimental evidences have suggested that skin physiology is regulated through the interaction of the nervous, immune, cutaneous, and endocrine (NICE) systems[2,3]. In order to respond to the nervous system, the skin expresses several receptors, including opioids receptors (OPR)[4,5]. These receptors are G protein-coupled receptors mediating the effects of opioid peptides ligands either of endogenous or exogenous origin[6,7].

The following Opioid Receptors (OPR) were identified
- μ (mu) receptors (MOR)
- δ (delta) receptors (DOR), and
- κ (kappa) receptors (KOR).

Endogenous ligands are respectively:
- Peptides β-endorphines,
- Enkephalines, and
- Dynorphines.

Recent studies suggest that OPR are not only implicated in pain control, but also in the regulation of homeostasis of skin cells[4]. More specifically, delta-opioid receptors (OPRD1, DOR) have a significant role in governing skin cell differentiation, migration and proliferation, thereby establishing these as key biological targets to control the evolution of skin aging[4]. Recently, involvements of opioid receptors in other than nerve functions have been investigated. Among the opioid receptors delta opioid and mu opioid receptors were studied in skin. Deletion of either receptor caused severe effects on skin differentiation, e.g. the thickness of the epidermis was strongly reduced in mice devoid of DOR[5]. We speculate that activation of DOR is improving differentiation, mainly in aged skin. Thus, we investigated effects of a hexapeptide agonist of the Opioid Receptor Delta 1 (DOR) expression, its modulation, its capacity to act downstream on markers of differentiation, and to influence skin properties such as skin barrier and wrinkle depth.

Aging skin is characterized by increased micro-inflammation, water-loss, and slower regeneration. Reducing inflammation, reconstituting the skin-barrier, as well as increasing differentiation, all help fight aging. Induchem has discovered a new anti-aging approach, controlling the side effects of skin's micro-inflammation by stimulating DOR.

2. Mode of Action

DOR are suspected to control some skin renewal processes. Deletion of delta opioid receptors in mice leads to the formation of an abnormal thin epidermis[5]. These results suggest an essential role of DOR in skin differentiation, proliferation, and migration[4].

Delta opioid receptors are also expressed in human epidermis. The expression of DOR is observed in the granular layer of the epidermis, and some dermal cells were also labeled in their cytoplasm. These receptors are stored intracellularly in order to be transported to the cell surface upon stimulation[5].

In vitro, the expression of DOR can be observed in Reconstructed Human Epidermis (RHE). The expression of DOR is strongly observed in the granular layer cell membranes. RHE have been used to investigate the mode of action of DOR during skin aging as they are more reliable (normalized) than regular skin biopsies.

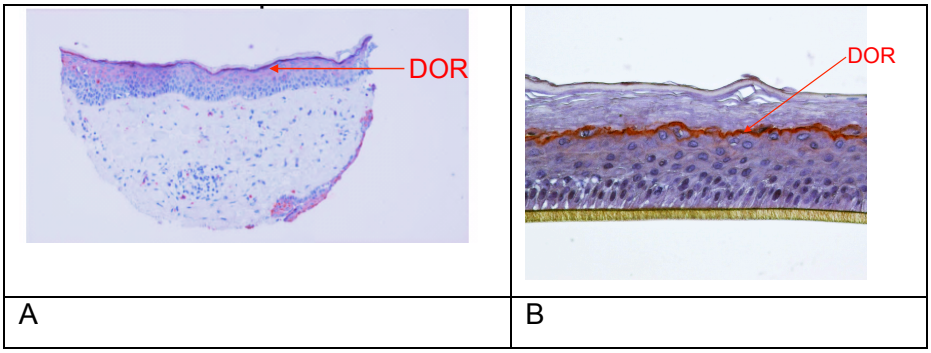


Figure 1: Labelling of delta opioid receptors in human skin (A) and human reconstructed epidermis (B).

Upon aging, the level of cytokines increases in the skin, due to the increase of micro-inflammations. Cytokines are signaling molecules from irritated skins to the immune system (Langerhans Cells), which inform about abnormal conditions. But the over-production of cytokines activates a feedback reaction called heterologous desensitization[8,9,10]. This process inactivates the opioid receptors. The desensitization leads to lower skin differentiation, which is one reason for visible signs of aging.

We can summarize the following known scientific facts:

- Delta opioid receptors (DOR) are involved in the control of skin differentiation.
- A normal activity of DOR is required for a normal evolution of skin (inactivation of DOR leads to undifferentiated thinner skin).
- Upon aging, there is measurable increase in micro-inflammation in the skin.
- Over-production of cytokines inactivates opioid receptors' function.

RUBIXYL®

This leads us to the following questions:

- Does 'aging' (cytokine overproduction) hinder the activity of DOR, thereby blocking the normal differentiation process of skin?
- If so, could a molecule reactivate the DOR activity in order to recover the natural evolution of skin and reverse signs of aging?

The answer is the novel hexapeptide, Rubixyl®, a small molecule that can act as a DOR agonist. An agonist is a molecule that binds to a receptor of a cell and triggers a response by that cell.

Rubixyl® is a **nature inspired bio-mimetic peptide**, with a half maximal effective concentration EC_{50}=3.9 10^{-6} M.

Rubixyl® binds to, and activates, DOR (OPRD1). Increasing concentration of Rubixyl® was tested in a cellular functional OPRD1 stimulation assay (fig. 2).

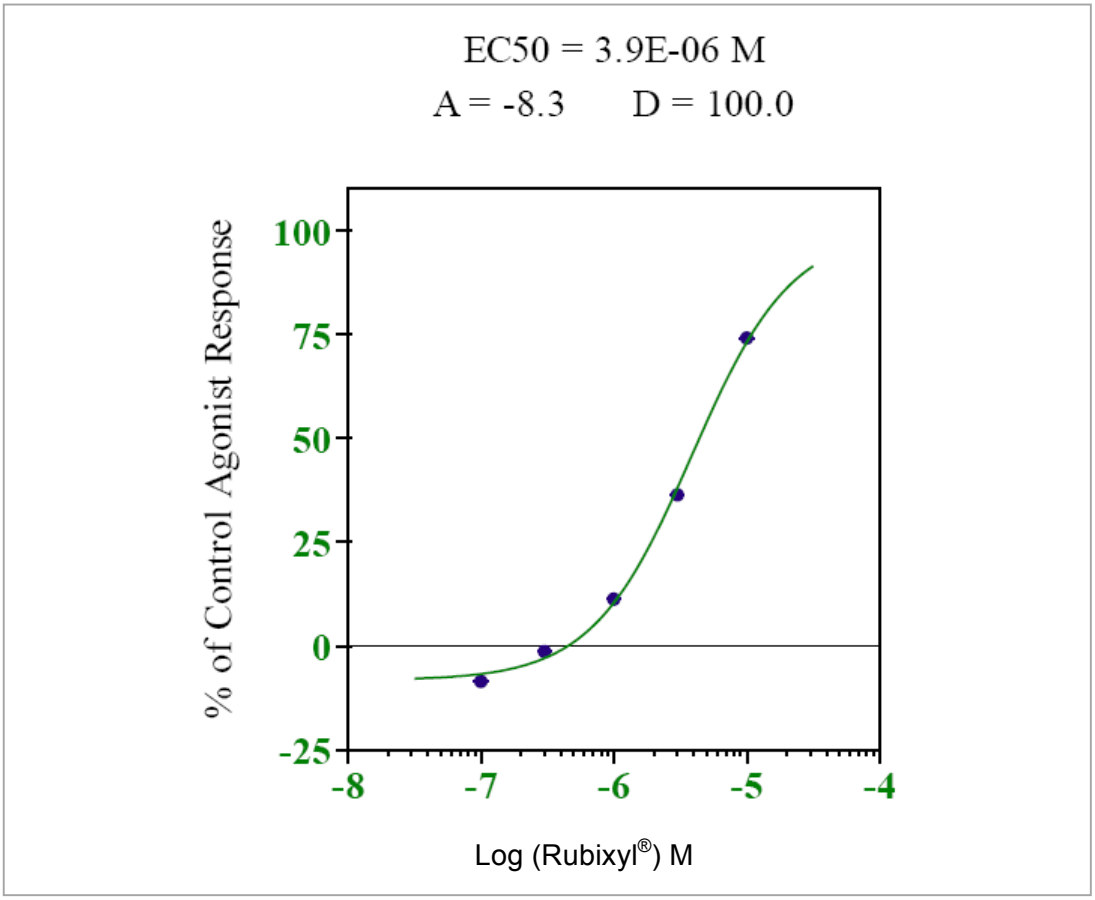
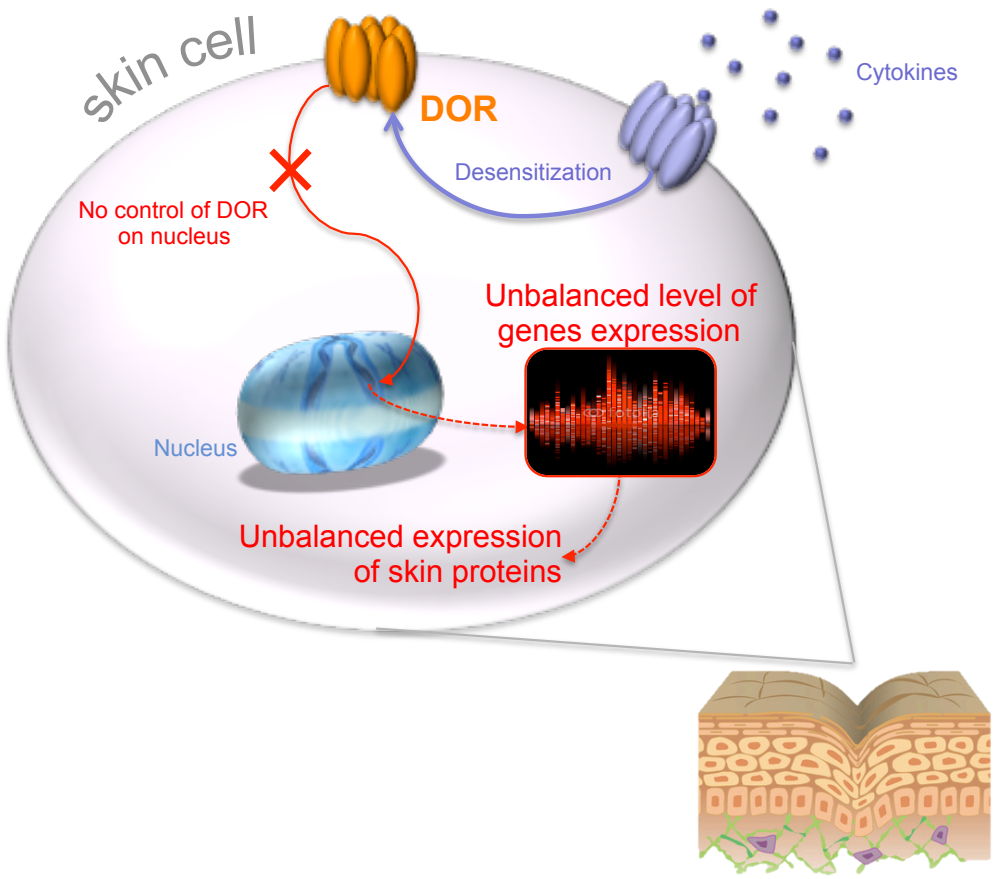


Figure 2: DOR activation profile of Rubixyl®. Rubixyl stimulates DOR with an EC50 of 3.9 µM. Reference agonist: DPDPE (a cyclic disulfide containing pentapeptide that selectively binds to the delta opiate receptor).

RUBIXYL®

Delta opioid receptor activation leads to changes in gene expression towards cell differentiation, and reduction of inflammation markers. DOR agonist activity helps to bring back DOR to the cell surface for proper function (fig. 3). Rubixyl®'s function is defined as RUBI™ "**R**eceptors **U**phold against **B**ioaging".

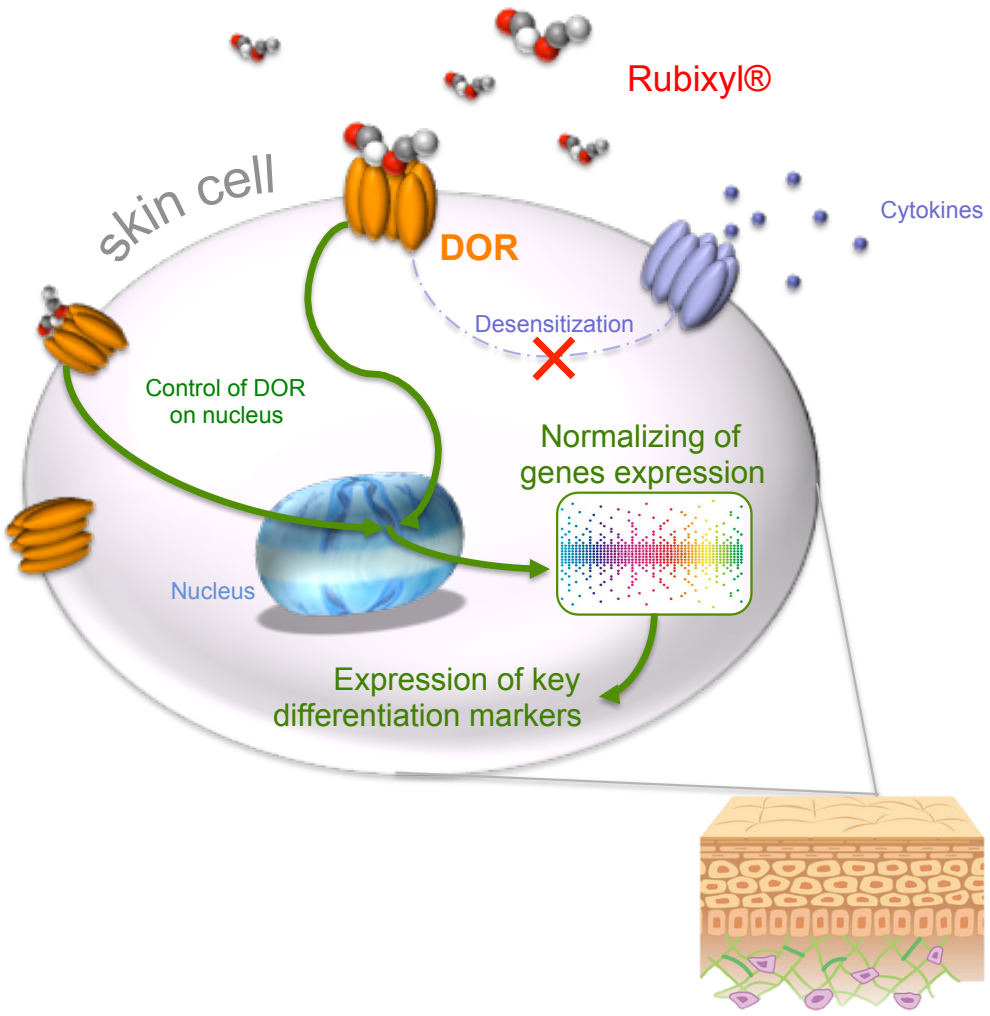
Figure 3: RUBI™ technology: **R**eceptors **U**pholding against **B**ioaging. Rubixyl® upholds DOR.



A: Skin cells without Rubixyl®

Without Rubixyl® Desensitization of delta opioid receptors due to over activation of cytokines receptors in aging skin which leads to accumulation of visible signs of aging.

RUBIXYL®



B: Skin cells with Rubixyl®

With Rubixyl®. Activation and protection of delta opioid receptors' activity by Rubixyl®, leading to a normalizing effect on genes, and proteins, expression goes back to a normal level: This will reverse aging effects, with visible improvement of skin's properties (reduction of trans-epidermal water loss, increased smoothness, and reduction of deep wrinkles).

Rubixyl® is controlling the skin cell receptors to reverse aging processes. By binding to DOR, it stimulates the expression of differentiation markers that are directed towards aging skin.

3. Experimental Set Up

3.1 Vehicle and Active Ingredients

Rubixyl® consists of Hexapeptide-48 HCl, containing a high concentration of glycerol to avoid microbial growth (70%). This active ingredient was tested in different concentrations starting at 0.01% to 1% for subsequent studies.

3.2 Application of the product

For in vitro tests and tests on reconstructed human skin models, Rubixyl® was applied alone in different concentrations ranging from 0.01% to 1% of Rubixyl®.

For clinical tests, Rubixyl® was used in a formulation at a final concentration of 1%.

3.3 Reconstituted Human Skin Model Study

Tests were carried out using a reconstituted human skin model at Bioalternatives (Gencay, France). Expression of DOR was studied in several different tissues including brain as positive control, and in reconstituted human skin models. Gene expression was assayed by analysis of DOR expression, and several differentiation markers.

3.4 Clinical tests

In vivo tests were carried out at Farcoderm (San Martino Siccomario, Italy). A double blind and placebo-controlled clinical evaluation of the efficiency of Rubixyl® on wrinkles effect was carried out with 20 volunteers, 50% of them being smokers. Product efficiency was evaluated at days 15, 30, and 60, after daily product use, respectively. Analysis was performed both by dermatological wrinkle depth determination and by self-assessment of subjects (only after 60 days). Dermatologists determined skin profilometry, skin moisturizing, and transepidermal water loss (TEWL).

RUBIXYL®

4. Efficacy Results

4.1 Tests on human reconstituted skin

The presence of DOR was studied by histological analysis in reconstituted human epidermis (RHE). Using a cytokine mix (CTK mix: IL-17, TNF-α and Oncostatin M at 3ng/ml) expression was decreased, which could be reversed by JAK/STAT inhibitor (not shown) or increasing concentrations of Rubixyl® (fig. 4).

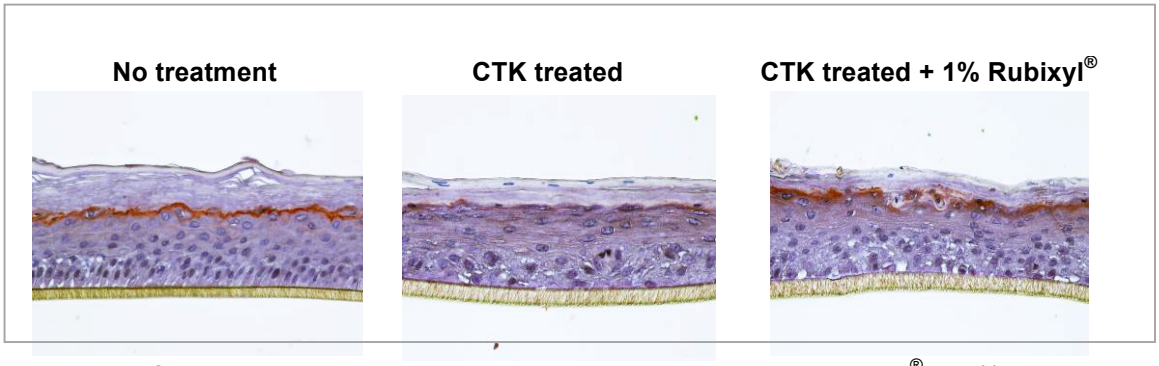


Figure 4: DOR expression (red staining) in the RHE granular layer. Rubixyl® at 1% inhibited the cytokine mix- induced DOR down-regulation. Control: Untreated; CTK treated: Cytokine mix (IL-17, TNF-α, Oncostatin M) treated. + = addition of Rubixyl®.

To test the effects of Rubixyl® on gene expression, skin differentiation markers (64 mRNA (see complete list in appendix 1) and 7 proteins) were studied in reconstituted human epidermis (RHE) treated with pro-inflammatory cytokines in the presence or absence of inhibitors of inflammation pathways, or the DOR peptide agonist (Rubixyl®).

Several marker genes were up-regulated in the cytokine-treated RHE, e.g.: DOR-related genes: such as DOR signalling pathways like Prostaglandine G/H synthase, Suvrivin (Birc5), and Stat-3; or keratinocyte differentiation markers: like Keratin 1 and 10, Involucrin, and the Small proline rich proteins 1A, 1B and 2A.

Most of the upregulated genes (80%) in the inflamed state could be brought back to normal expression levels by RHE treatment with Rubixyl® (fig. 5).

RUBIXYL®

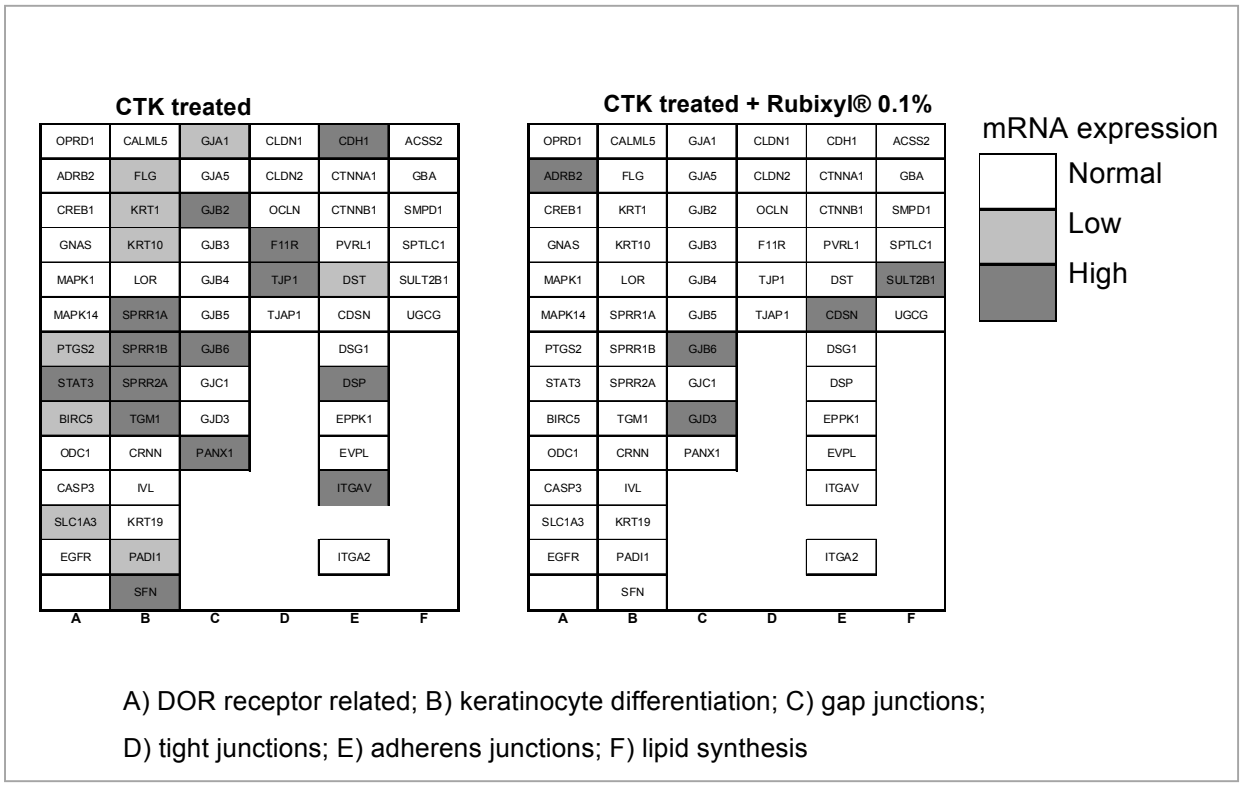


Figure 5: Differentiation markers mRNA expression from cytokine treated RHE in the presence or absence of Rubixyl®. The DOR agonist Rubixyl® inhibited the "pro-inflammatory" phenotype induced by CTK, restoring the normal differentiation status. CTK: cytokine mix (IL-17, TNF-α, Oncostatin M). Normal: Control = 100%; Low = <50% Control; High= >200% Control.

In addition, several protein markers were tested, by induction with inflammation markers, with or without, Rubixyl®. As for the genes, most of the deregulated protein markers in the inflamed state were brought back to a normal level of expression.

Among these markers are several proteins that are involved in: skin barrier function e.g. Corneodesmosin, Keratin 10 and Involucrin, skin homeostasis e.g. Calmodulin like 5, and Occludin, or markers involved in lipid synthesis, e.g. Acyl-CoA-synthetase and Sulfotransferase 2B (fig. 6).

RUBIXYL®

PROTEIN	ROLE IN SKIN	PROTEINS EXPRESSION LEVEL IN HUMAN EPIDERMIS (RHE)		
		Normal conditions	Stimulated conditions with cytokines	
			No Rubixyl®	1% Rubixyl®
Corneodesmosin	**Barrier function** (adh. junc. & corneocytes)	100%	147%	84%
Involucrin	**Barrier function** (kerat. diff. & cornified envelope)	100%	122%	99%
Calmodulin like 5	**Homeostasis** (kerat. diff. & Ca2+ storage)	100%	40%	100%
Occludin	**Homeostasis** (thight junctions)	100%	50%	97%
Keratin 10	**Robustness** (kerat. diff. & epidermis integrity)	100%	120%	104%
Acyl-CoA synthetase	**Energy production** (mitochondria)	100%	33%	65%
Sulfotransferase 2B	**Lipids synthesis** (cholesterol sulfate)	100%	200%	100%

EXPRESSION LEVEL OF PROTEINS

Low (-35%) Normal High (+20%)

Figure 6: Protein expression from CTK treated RHE in the presence or absence of the hexapeptide. The hexapeptide inhibited the “pro-inflammatory” phenotype induced by CTK, restoring the normal differentiation status. Stimulated conditions: cytokine mix (IL-17, TNF-alpha, Oncostatin M at 3 ng/ml).

4.2 Clinical tests on human volunteers

To support the assumption that Rubixyl® is interfering with micro-inflammation induced aging in human skin, a double blind clinical study has been performed, comparing a placebo cream with a cream containing 1% of Rubixyl®, when applied on human volunteers' faces (n=20, age: 40-65, 50% of smokers).

4.2.1 Instrumental evaluation

After 15, 30, and 60 days of treatment, skin properties were measured, including skin barrier and wrinkle depth by trans-epidermal-water-loss (TEWL) and Primo's 3D analysis, respectively. Statistical analysis of the data was then performed.

Rubixyl® significantly reduced TEWL (at day 15, 30, and 60 days after application, respectively), and wrinkles depth when compared to Placebo (at day 60 after application), (fig. 7 and 8).

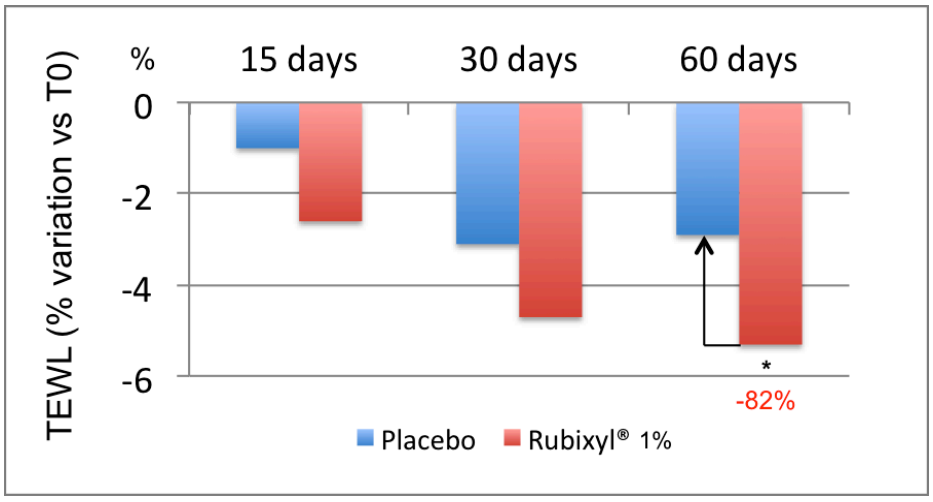
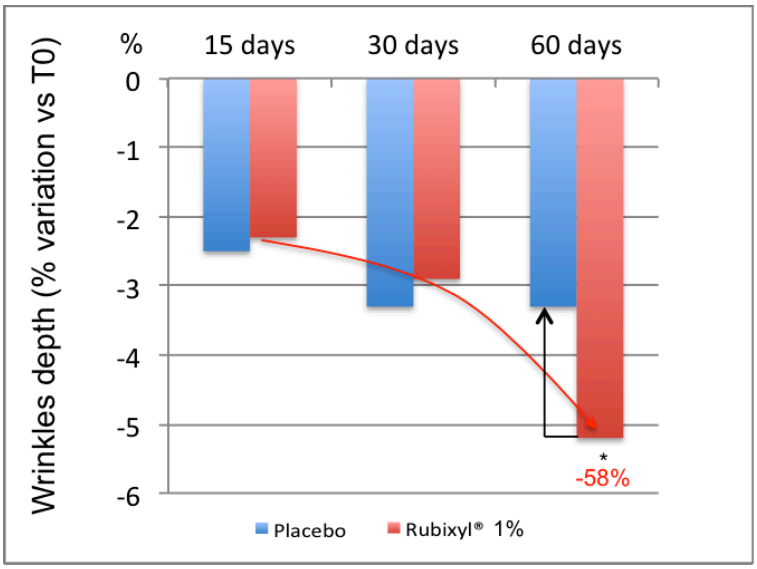


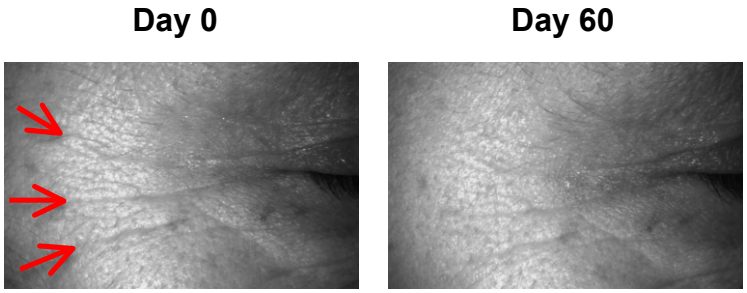
Figure 7: Treatment of 20 human volunteers with a cream containing Rubixyl® at 1%, significantly reduced TEWL: -82% after 60 days, with visible effects in two weeks (* $p<0.05$ Student's t test in comparison to placebo).

RUBIXYL®

A Measurement of the reduction of wrinkles depth



B Crows feet wrinkles evolution with Rubixyl®



C Crows feet wrinkles evolution with the placebo

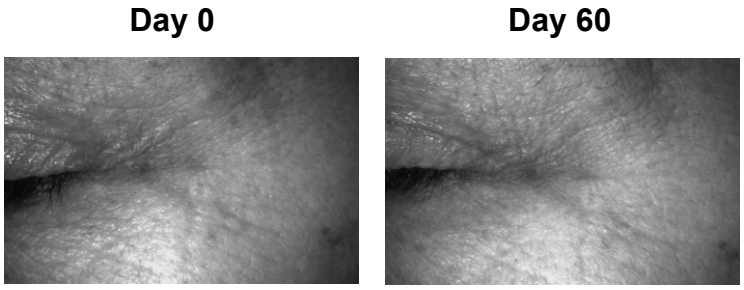


Figure 8: (A) Treatment of 20 human volunteers with a cream containing Rubixyl® at 1%, significantly reduced wrinkle depth (-58%) when compared to a Placebo cream. *p<0.05, (Student's t test vs Time 0). (B) Example of volunteer showing reduced wrinkles after 60 days of treatment with a cream containing 1% of Rubixyl®. (C) Example of a volunteer showing no reduction of wrinkles after 60 days of treatment with a placebo cream.

4.2.2 *Dermatologist's evaluation*

In addition, a dermatologist evaluated the smoothness of the volunteers' skin. **At each evaluation date the skin of those volunteers applying a cream with 1% Rubixyl® were found significantly smoother compared with the volunteers applying the placebo cream** (fig. 9).

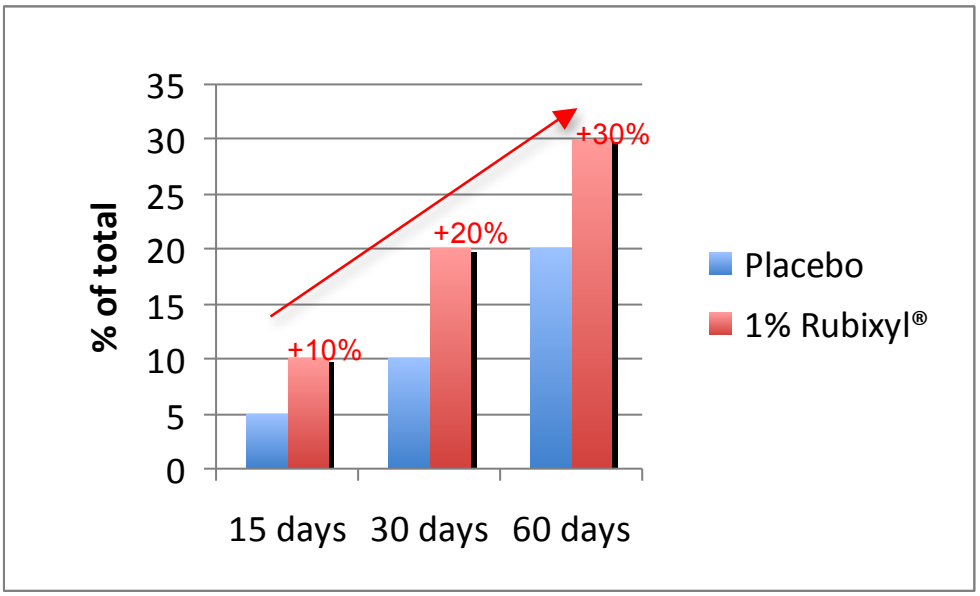


Figure 9: Dermatologist's evaluation on skin smoothness. After day 15, 30, and 60, dermatologist's analysis of skin smoothness revealed a higher instance of skin smoothness after every measurement date.

4.2.3 *Self-evaluation by volunteers*

Finally, after 60 days of application, the volunteers were asked to fill in a self-evaluation form. The premise that Rubixyl® reduces wrinkles and water loss, and increases smoothness of the skin, was confirmed by the self-evaluation of the volunteers 60 days after usage of the Rubixyl®-containing cream (fig. 10).

A significant improvement in the appearance of the skin, along with reduction of wrinkles and increased sensation of hydration were the result of the self-evaluations (fig. 10).

This supports the data we received in RHE, confirming the reinstatement of inflamed skin back to its normal state.

RUBIXYL®

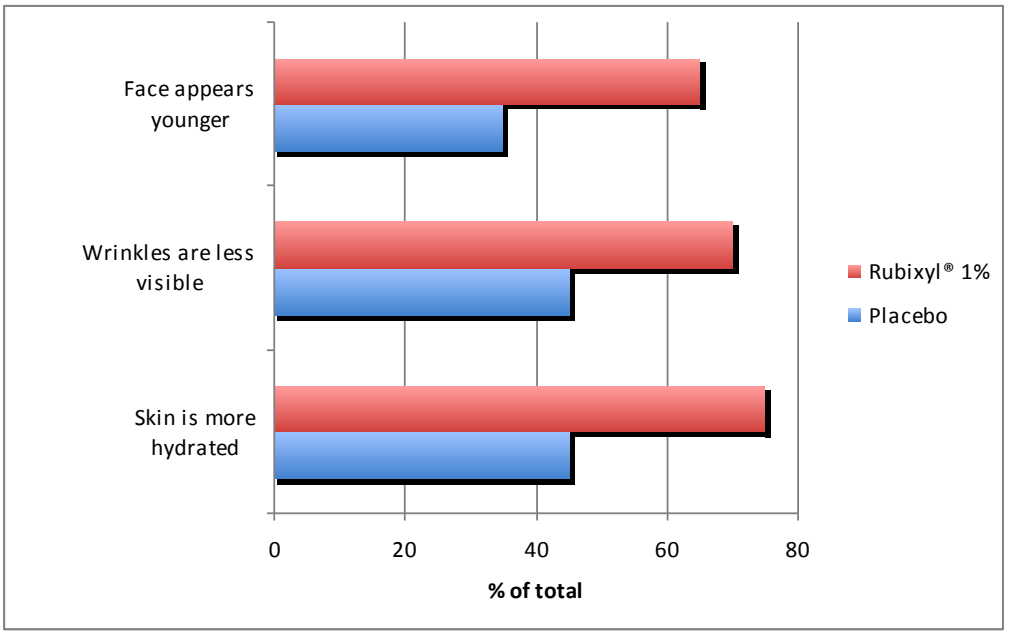


Figure 10: Volunteers' self-evaluation show a significant difference between the skin feeling of the cream containing 1% Rubixyl® versus the placebo cream (evaluation after 60 days of use of cream). Skin was judged as better hydrated, and wrinkles were reduced in the double blind study.

In summary, Rubixyl® acts on DOR and changes gene expression of inflamed cells back to their normal state. It fights against signs of aging, and leads to smoother skin, reduction of wrinkles, and better hydration.

5. Conclusions

The aging process is associated with an increased level of micro-inflammation in the skin. This is due to accumulation of the effect of external aggressions and the diminished aptitude of skin cells to face these aggressions[11]. This chronic inflammation generates feedback responses, one of them being a reduced level of expression of DOR, which leads to a lower differentiation capability of skin cells and a weaker skin structure.

We have shown that cytokine-induced RHE inflammation leads to DOR decrease (fig. 4) and reduced differentiation markers at the mRNA (fig. 5) and at the protein level (fig. 6). The presence of a DOR peptide agonist (Rubixyl®) counteracted the effect of the cytokines, maintaining a proper differentiation status of normal skin. When the hexapeptide was tested clinically, it reduced TEWL and wrinkle depth, suggesting proper maintenance of skin differentiation as a pre-requisite for good skin barrier function and aging reversal.

These data highlight the role of DOR in the skin and promote a new approach for treating aged, inflamed skin by targeting this receptor with a DOR peptide agonist.

Future anti-aging initiatives should take the role that DOR plays in controlling skin differentiation, and ultimately skin barrier and wrinkles formation, into consideration. Rubixyl® is the first active compound with RUBI™ technology, "**R**eceptor **U**phold against **B**ioaging" meaning that receptors that are targets for anti-aging compounds are down-regulated during aging. Compounds targeting such receptors are stimulating the remaining receptors, resulting in their up-regulation. By transporting them back to the cell surface the skin is able to act, and appear, like a younger.

RUBIXYL®

6. Characteristics

Composition	Blend of glycerol, water and hexapeptide-48 HCl.
Appearance	colorless liquid.
Analytical data	See specifications.
Solubility	Soluble in water.
Shelf life	2 years at room temperature.
Safety	See safety data sheet.

Selectivity

To exclude any potential interactions of Rubixyl® with other receptors several agonist and antagonist activities of Rubixyl® were tested. 30 different GPCR were among the receptors analyzed, including delta opioid receptor as positive control. No significant stimulatory effect of Rubixyl® on others than delta opioid receptor was found. In addition, binding of Rubixyl towards receptor of melanin concentrating hormone, MCH1, and melanocortin receptors, 1, 3, and 4, respectively, were tested. No significant effect was found on either of these receptors.

Dosage	0,1-2 %
Storage	5°C - 25°C (see safety data sheet)
Shelf life	2 years (see specification)

Identification

INCI name	**CAS No.**
Aqua (US: Water)	7732-18-5
Glycerin	56-81-5
Hexapeptide-48 HCl	n.a.

7. References

1. Mills, IG *Seminars in Cell and Developmental Biology* **23**:2 165-171, 2011

2. O'Sullivan RL, Lipper G, Lerner EA. *Arch Dermatol* **134**: 1431-5, 1998

3. Roosterman D. *Physiol Rev* **86**: 1300-79, 2006

4 Bigliardi PL, Tobin DJ, Gaveriaux-Ruff C, Bigliardi-Qi, M. *Exp Dermatol* **18**: 424-30, 2009

5. Bigliardi-Qi M, Gaveriaux-Ruff C, Zhou H, Hell C, Bady P, Rufli T, Kieffer B, Bigliardi P *Differentiation* **74** 4:174-185 2006

6. Bie B and Pan ZZ, *Molecular Pain* **3**:37

7. Teschemaker H. *Curr Pharm Des* **9**: 1331-44, 2003

8. Adler MW, Geller EB, Chen X, Rogers TJ *AAPS Journal* **7** 4: E865-E870, 2006

9. Hosey MM *American Journal of Physiology. Cell Physiology* **277:** 5: C856-C858, 1999

10. Zang N, and Oppenheim JJ *Journal of leukocyte biology* **78** 6: 1210-1214, 2005

11. Thornfeldt CR. *J Cosmet Dermatol* **7**: 78-82, 2008

8. Appendix 1

GPCR profile

Testing of other GPCR was performed to exclude potential side effects of Rubixyl® on other key receptors, than the delta opioid receptor. 30 different GPCR were tested towards Rubixyl® agonist or antagonist activity. In addition, binding of Rubixyl towards receptor of melanin concentrating hormone, MCH1, and melanocortin receptors, 1, 3, and 4, respectively, were tested. No significant effects were found on either of these receptors.

Name of tested receptors: A2A, A2B, A3, a1A, a1B, a2C, b1, b2, CCK1, D1, D3, D4.4, H1, H2, M1, M2, M3, M4, M5, NK1, d2, k, m, 5-HT1D, 5-HT2A, 5-HT2B, 5-HT3C, 5-HT4e, 5-HT6, 5-HT7, MCH1, MC1, MC3, MC4.

Gene expression

List of the 64 genes tested on reconstructed human epidermis with or without a cytokine mix, and with or without Rubixyl®.

Housekeeping	RPS28	Ribosomal protein S28
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Opioid receptor delta 1 related markers	OPRD1	Opioid receptor, delta 1
	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
	BIRC5	Baculoviral IAP repeat-containing 5
	ODC1	Ornithine decarboxylase 1
	CASP3	Caspase 3, apoptosis-related cysteine peptidase
	SLC1A3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3
Keratinocyte differentiation	CALML5	Calmodulin-like 5
	FLG	Filaggrin
	KRT1	Keratin 1
	KRT10	Keratin 10

RUBIXYL®

	LOR	Loricrin
	SPRR1A	Small proline-rich protein 1A
	SPRR1B	Small proline-rich protein 1B (cornifin)
	SPRR2A	Small proline-rich protein 2A
	TGM1	Transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)
	CRNN	Cornulin
	IVL	Involucrin
	KRT19	Keratin 19
	PADI1	Peptidyl arginine deiminase, type I
	SFN	Stratifin
Gap Junctions	GJA1	Gap junction protein, alpha 1, 43kDa
	GJA5	Gap junction protein, alpha 5, 40kDa
	GJB2	Gap junction protein, beta 2,
	GJB3	Gap junction protein, beta 3, 31kDa
	GJB4	Gap junction protein, beta 4, 30.3kDa
	GJB5	Gap junction protein, beta 5, 31.1kDa
	GJB6	Gap junction protein, beta 6, 30kDa
	GJC1	Gap junction protein, gamma 1, 45kDa
	GJD3	Gap junction protein, delta 3, 31.9kDa
	PANX1	Pannexin 1
Tight junctions	CLDN1	Claudin 1
	CLDN2	Claudin 2
	OCLN	Occludin
	F11R	F11 receptor

RUBIXYL®

	TJP1	Tight junction protein 1 (zona occludens 1)
	TJAP1	Tight junction associated protein 1 (peripheral)
Adherens junctions	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
	CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa
	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa
	PVRL1	Poliovirus receptor-related 1 (herpesvirus entry mediator C)
	DST	Dystonin
	CDSN	Corneodesmosin
	DSG1	Desmoglein 1
	DSP	Desmoplakin
	EPPK1	Epiplakin 1
	EVPL	Envoplakin
	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
Lipid synthesis	ACSS2	Acyl-CoA synthetase short-chain family member 2
	GBA	Glucosidase, beta; acid (includes glucosylceramidase)
	SMPD1	Sphingomyelin phosphodiesterase 1, acid lysosomal
	SPTLC1	Serine palmitoyltransferase, long chain base subunit 1
	SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1
	UGCG	UDP-glucose ceramide glucosyltransferase