Final Report

2020-EB-00XX

Active Compound evaluation by Quantitative proteomics: skin cell model

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Compound evaluation by Mass Spectrometry

Final Report SAMPLE

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Contents

Example of report with types of results that can be generated. For illustrative purposes.

Abbreviations | Glossary

Biomarker	observable and measurable molecular or cellular change
mRNA	Messenger RNA transient copy of a portion of DNA corresponding to a gene
ROS	reactive oxygen derivatives of oxygen, oxygen ions and peroxides
GSH	intracellular thiol used for the defense and elimination of ROS
OXPHOS	oxidative phosphorylation metabolic pathway for the production of ATP
ATP	adenosine triphosphate complex organic product that provides energy
TCA	metabolic pathway also known as the Krebs cycle releases ATP and produces pyruvate for OXPHOS
UPR	Unfolded Protein Response
Stressor	Treatment used to mimic damage to skin
SOP	Standard Operating Procedure

I. Summary

The aim of this project was to determine the proteomic response of an in vitro cellular model of skin to active compound treatment before and after application of an external stressor (oxidative stress). We used a **quantitative proteomics approach to establish protein profiles and signalling pathways**, from which differentially regulated proteins can be identified.

The project was designed to provide information on the maintenance, restoration and improvement of energy and related intracellular signalling pathways after treatment with a bioactive compound in combination with a stressor. The results suggest the bioactive compound has the ability to correct the vital capacity of the skin, to maintain an optimal supply of energy and/or the stimulation of intracellular signalling pathways necessary to protect the skin cells from oxidative damage.

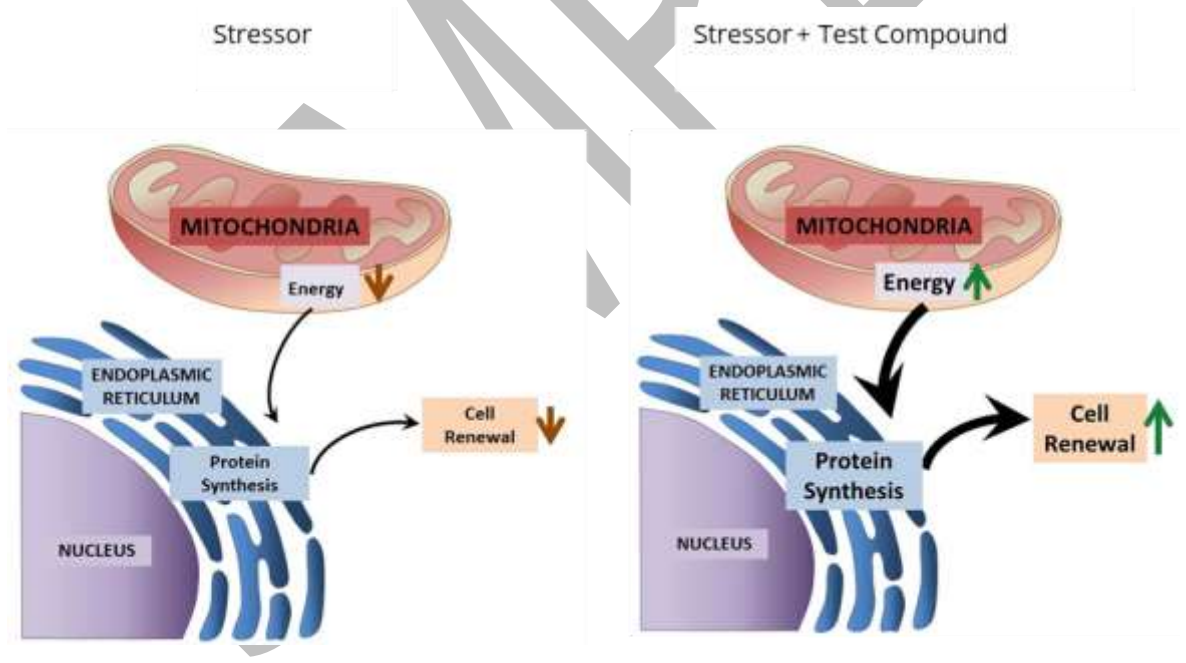


Figure 1. Illustration of positive effects of Active compound on cellular decline induced by Stressor.

The applied **stress leads to defects in energy production and produces unfolded proteins**. The **bioactive compound restores a process of cellular quality control through the stimulation of mitochondrial biogenesis** necessary for correct protein synthesis, skin renewal. Stimulation of mitochondrial quality control and protein synthesis are markers showing the beneficial effect of the bioactive compound on cell vitality.

II. Background

The skin is the largest and most visible organ of the body. Cosmetic products are applied to the skin to induce positive changes to its appearance, to protect it from external elements, or to keep it in good condition. Today, there is a growing consumer demand for cosmetics containing natural and/or organic ingredients. Numerous plants and their active compounds are actively researched and evaluated as ingredients in cosmetics: lipids, phenolics, polyphenolics, terpenoids, selenium, polysaccharides, vitamins, and volatile organic compounds are some examples. These compounds show excellent antioxidant, anti-aging, anti-wrinkle, skin whitening, and moisturizing effects, which make them ideal candidates for cosmetics products. However, evaluation of their potential can be difficult.

In general, **cells seek to maintain an optimal environment which is preserved by maintaining an optimal production of metabolic energy**. **High-throughput approaches like proteomics are recognized globally in the study of skin and they represent an innovative tool in the era of precision cosmetics**. Proteome analysis can be performed on excised intact human epidermis and on keratinocytes cultured in model systems that mimic normal human epidermis.

Aim of the Experiment

1. To assess an active compound by establishing a functional skin proteome profile using an in vitro cellular model, in response to a stressor (and/or compared to a reference compound).

2. To produce a protein profile describing the protein networks underlying the biological processes and changes after treatment with the active compound; to determine the potential beneficial effects of active compound as a cosmetic.

Traceability

Item Chemical (Information available)

1. Type:
2. Provenance:
3. Batch code:
4. Chemical identity:
5. Physical form:
6. MW:
7. Characterisation and purity of the chemical, including isomer composition whenever relevant for safety assessment:
8. Characterisation of the impurities or accompanying contaminants:
9. Solubility:
10. Homogeneity and stability:
11. Further physical and chemical properties if relevant for evaluation: (Active pure compound, compound mix, chemical preparation)
12. Test items storage conditions at Elysia Bioscience:

Test samples

1. Type: Cells (fibroblasts, keratinocytes, melanocytes, airway epithelium, other) primary or immortalized according to objective
2. Provenance: ATCC
3. Growing conditions:
4. Storage conditions at Elysia Bioscience:
5. Related other or background information.

Test Treatment(s)

1. Concentration(s) and times tested:
2. Storage at the sponsor:
3. Reference Item: Comparative chemical, active ingredient or standard, commercial reference at XX concentrations.
4. Related other or background information: for the choice of test conditions

Identification of test items and samples

After receipt at Elysia Bioscience, test items and test samples will be stored according to the sponsor's specification. Test samples will be individually noted in an identification sheet. This identification number is used throughout the experiments to follow samples. After testing its biological activity, test items and test samples will not be further analysed outside of this study (cf. Terms and Conditions).

Handling of Test items and test samples

According to the sponsor's specifications remaining test items and test samples will be stored or sent back at the end of the experiments. The sponsor remains responsible for information provided about test items and test samples (cf. Research Contract Agreement). Raw data is provided according to the sponsor's specifications.

III. Results of Analysis

Cytotoxicity test and determination of safe test concentration

Viability and cytotoxicity of active compound at 1mM, 10mM and 100mM was evaluated on a primary adult keratinocyte cell line by means of Trypan blue exclusion cell count and LDH release assay, respectively (See Material and Methods). As shown in **Figure 1A**, the percent of live cells with respect to the total cell number were similar between the control and the active compound treatment at 1mM and 10mM but viability was affected at the highest concentration of 100mM. Comparable results were obtained by LDH release (**Figure 1B**), indicating that 24h treatment with the active compound at 100 mM may have a cytotoxic effect on the test keratinocyte cell system. The highest apparent non-toxic concentration of 10 mM was chosen for proteomic analyses.

(A) (B)

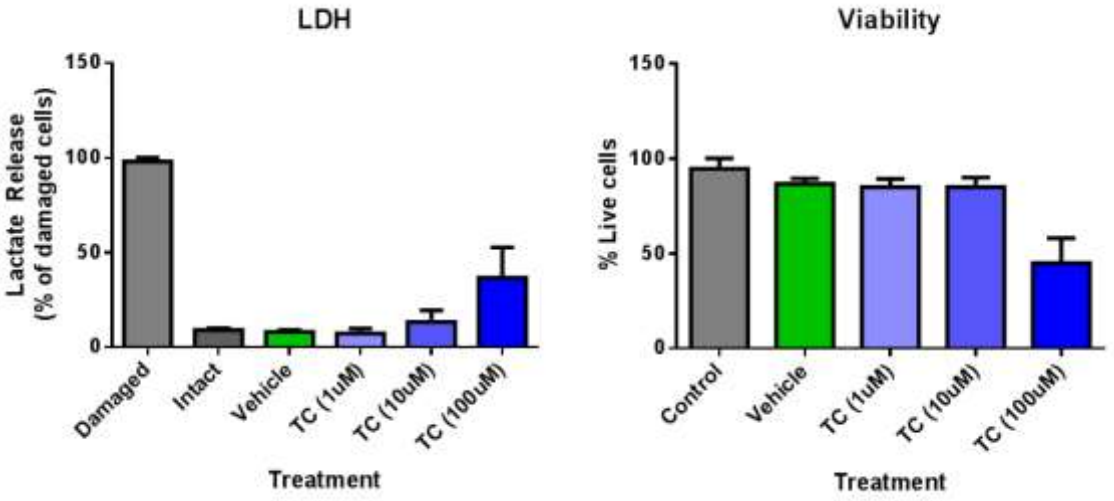


Figure 1. Example test results. Wide range of Test Compound (TC) doses do not show any cytotoxic effect at 1mM and 10mM on the test keratinocyte cell system as measured by Trypan Blue staining

(A) and LDH release assay (B). Viability was affected at the highest concentration tested of 100mM, verified by release of LDH ($p \leq 0.01$, n=3, mean + SD).

Proteomic analysis

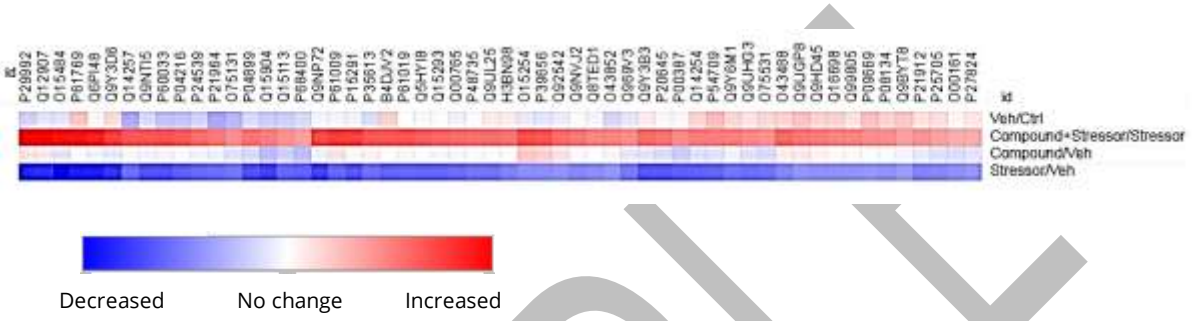
A total of 4357 proteins were identified according by mass spectrometry. The following thresholds were applied to filter only high-quality identifications: Confidence score ≥ 40 and Unique Peptides ≥ 3. A total of 3665 proteins were selected for advanced analyses. Statistical analysis identified differentially expressed proteins under the experimental conditions. Identified proteins were assigned to main functional categories and interrogated against 1371 metabolic signalling pathways, including but not limited to: carbohydrate and energy metabolism, nucleotides and amino acid metabolism, signal transduction and cellular process, folding and degradation, redox and homeostasis, biosynthetic process and transcription/translation. The list of proteins and associated signalling pathways is listed in Annexe 2. Results of differentially expressed proteins (non-exhaustive, for examples purposes) detected using proteomics analyses for each treatment are discussed below.

Active compound reverses low cellular energy levels induced by stressor.

The respiratory pathways of glycolysis, the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain are omnipresent in nature. They are essential both for the supply of energy to cells and for many of the synthetic and degrading biochemical reactions required for cellular homeostasis. Protein complexes located in the mitochondria synthesize cellular energy, while consuming oxygen. Mitochondria produce reactive oxygen species (ROS) through these reactions and ROS can, in turn, modify the function and expression of proteins used for other biochemical reactions of synthesis and degradation. Optimal functioning of these pathways is therefore important for cell homeostasis (Caito and Aschner, 2015).

Maintenance of active healthy mitochondria requires both biogenesis and clearance of damaged mitochondria. The maintenance of this quality control is considered a crucial factor to counteract ageing process (Ploumi et al., 2017). **Under the experimental conditions, the stressor affected**

mitochondria biogenesis and cellular energy production (Figure 2). These effects were reversed by treatment with the active compound at the tested concentration.



Pathway name	Proteins found	Total Proteins	p Value	FDR	Affected reactions	Total reactions
Mitochondrial translation initiation	14	88	3.57E-11	6.25E-9	3	4
Mitochondrial translation elongation	14	88	3.57E-11	6.25E-9	5	8
Mitochondrial translation	14	94	8.38E-11	1.1E-8	13	17
Translation	20	294	6.01E-9	6.17E-7	29	99
Complex I biogenesis	10	55	7.09E-9	6.17E-7	12	13
Respiratory electron transport	12	101	2.31E-8	1.64E-6	15	19
Respiratory electron transport, ATP synthesis by	13	124	2.52E-8	1.64E-6	18	26
The citric acid (TCA) cycle and respiratory electron	15	175	2.86E-8	1.66E-6	19	62

Figure 2. Example Proteins and Pathways identified showing decreased levels under Stressor treatment and showing restored levels under Stressor + Active compound, grouped by main functional categories.

Table 1. Example list of proteins found **decreased** by Stressor and restored by treatment with Active Compound. Listed is the Log2 of fold change in protein levels in samples treated with Stressor alone or Stressor + Active Compound with Control condition as base value.

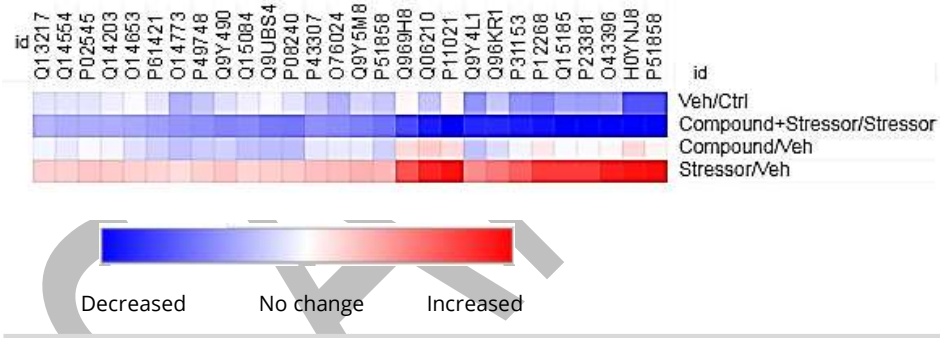
Uniprot	Description	Stress alone	Stress +Compound
Q16698	2,4-dienoyl-CoA reductase 1, mitochondrial	-3.36	-0.74
O15254	acyl-CoA oxidase 3, pristanoyl(ACOX3)	-2.49	-0.82
O43488	aldo-keto reductase family 7 member A2	-2.20	-0.83
P00387	cytochrome b5 reductase 3(CYB5R3)	-1.82	-0.63
Q8TED1	glutathione peroxidase 8 (putative)(GPX8)	-1.71	-1.00
P48735	isocitrate dehydrogenase (NADP(+)) 2, mitochondria	-1.63	-0.64
Q9UHG3	prenylcysteine oxidase 1(PCYOX1)	-1.62	-0.85
P21912	succinate dehydrogenase complex iron sulfur	-1.61	-0.67
P25705	ATP synthase, H+ transporting, mitochondrial	-1.45	-0.98
Q15904	ATPase H+ transporting accessory protein 1	-1.36	-0.28
P54709	ATPase Na+/K+ transporting subunit beta	-1.34	-0.77
P04216	Thy-1 cell surface antigen(THY1)	-1.32	-0.43
P15291	beta-1,4-galactosyltransferase 1	-1.25	-0.67
P61769	beta-2-microglobulin(B2M)	-1.25	-0.58
O43852	calumenin(CALU)	-1.22	-0.95
Q12907	lectin, mannose binding 2 (LMAN2)	-1.18	-0.55
P20645	mannose-6-phosphate receptor	-1.17	-0.57
Q969V3	nicalin(NCLN)	-1.15	-0.17
Q92542	nicastrin(NCSTN)	-1.12	-0.29
Q9UHG3	prenylcysteine oxidase 1 (PCYOX1)	-1.10	-0.23
Q15113	procollagen C-endopeptidase enhancer (PCOLCE)	-1.10	-0.91

The proteins associate or tightly interact in metabolism and energy production. The proteins found have more interactions among themselves than what would be expected for a random set of proteins of similar size. **Such enrichment indicates that the proteins are biologically connected as a group** (Figure 3).

Active compound alleviates ER stress and may restore protein folding

The endoplasmic reticulum (ER) is a sub-compartment network present in all cells. The ER is a main site of protein synthesis and maturation. Correct protein folding is essential for cell survival and function, and for normal healthy skin. Environmental insults often lead to protein misfolding, known as endoplasmic reticulum stress, and to the activation of a response to restore normal protein synthesis.

One important signalling pathway in normal protein synthesis is the IRE1α-XBP1 pathway (Wang and Kaufman, 2016). **Under the experimental conditions, stressor increased ER stress by misfolded proteins. These effects were alleviated by treatment with the active compound at the test concentration, which may help restore normal protein synthesis.**



Pathway name	Proteins found	Total Proteins	p Value	FDR	Affected reactions	Total reactions
XBP1(S) activates chaperone genes	19	48	1.11E-16	9.66E-15	19	47
IRE1alpha activates chaperones	20	50	1.11E-16	9.66E-15	20	53
Unfolded Protein Response (UPR)	20	92	1.11E-16	9.66E-15	23	94
Metabolism of proteins	30	2012	2.24E-11	1.46E-09	59	873
Post-translational protein phosphorylation	5	107	1.08E-04	0.006	1	1
Transport to the Golgi and subsequent modification	5	186	0.0013	0.046	23	59
SRP-dependent cotranslational protein targeting to	4	113	0.0015	0.046	3	5

Figure 5. Example Proteins and Pathways identified showing increased levels under Stressor treatment and restored lower levels under Stressor + Active compound, grouped by main functional categories.

Table 2. Example list of proteins found **increased** by Stressor and restored by treatment with Active Compound. Listed is the Log2 of fold change in protein levels in samples treated with Stressor alone or Stressor + Active Compound with Control condition as base value.

Uniprot	Description	Stress alone	Stress +Compound
P61421	ATPase H+ transporting V0 subunit d1(ATP6V0D1)	0.89	-0.52
Q9UBS4	DnaJ heat shock protein family (Hsp40) member B11(D	0.90	0.25
Q13217	DnaJ heat shock protein family (Hsp40) member C3(DN	0.93	0.03
P08240	SRP receptor alpha subunit(SRPRA)	0.94	-0.91
Q9Y5M8	SRP receptor beta subunit(SRPRB)	1.01	-0.73
P49748	acyl-CoA dehydrogenase, very long chain(ACADVL)	1.02	0.47
Q14203	dynactin subunit 1(DCTN1)	1.03	0.53
Q06210	glutamine--fructose-6-phosphate transaminase 1(GFPT	1.05	0.87
O14653	golgi SNAP receptor complex member 2(GOSR2)	1.07	0.13
P11021	heat shock protein family A (Hsp70) member 5(HSPA5)	1.08	0.59
P51858	hepatoma-derived growth factor(HDGF)	1.10	0.00
Q9Y4L1	hypoxia up-regulated 1(HYOU1)	1.15	0.47
P02545	lamin A/C(LMNA)	1.20	-0.38
Q969H8	myeloid derived growth factor(MYDGF)	1.29	-0.03
Q14554	protein disulfide isomerase family A member 5(PDIA5)	1.30	-0.16
Q15084	protein disulfide isomerase family A member 6(PDIA6)	1.31	-0.22
P43307	signal sequence receptor subunit 1(SSR1)	1.33	0.28
Q9Y490	talin 1(TLN1)	1.38	1.79
O14773	tripeptidyl peptidase 1(TPP1)	1.79	-0.87
O76024	wolframin ER transmembrane glycoprotein(WFS1)	2.51	1.00

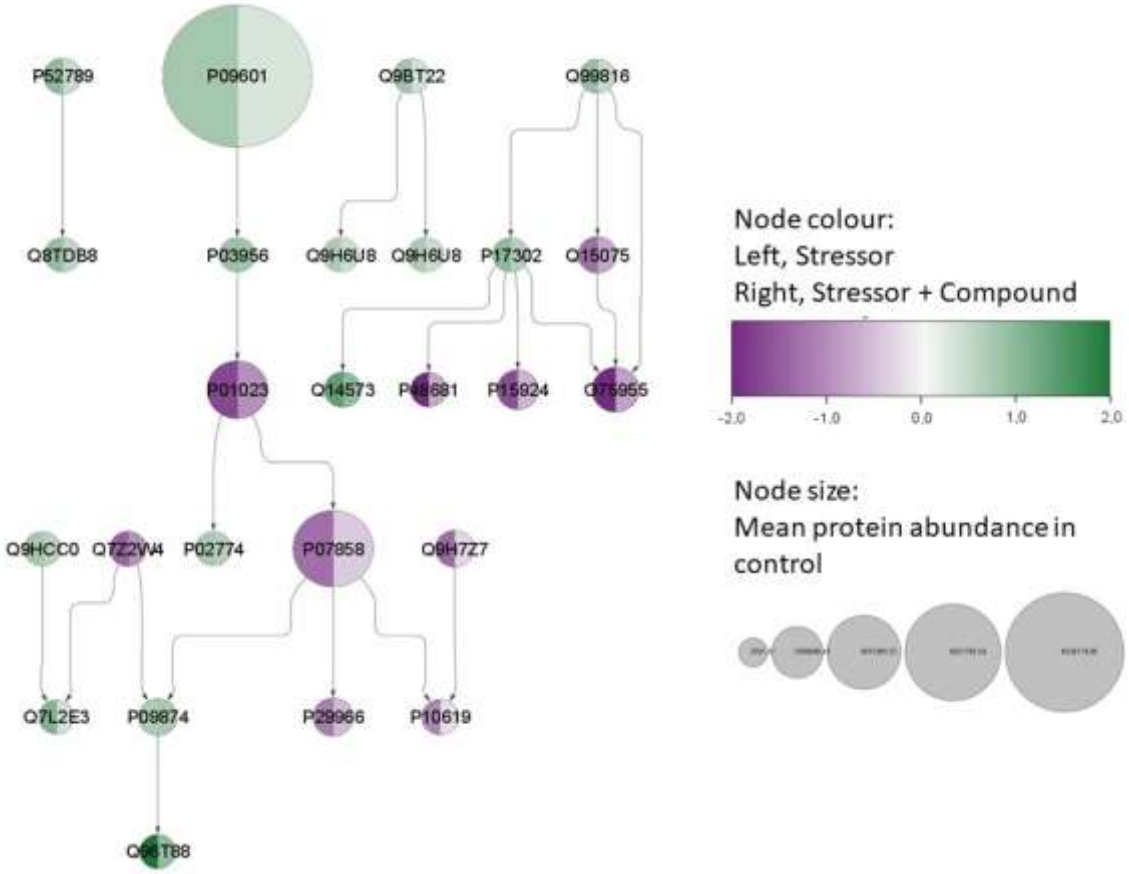


Figure 6. Example protein-protein interaction networks. Proteins are represented with colour nodes (upregulated in green and downregulated in magenta, white denotes no change). Differentially expressed proteins in Stressor (left half of circle) vs. Stressor + Active compound (right half of circle). The size on the node denotes the relative abundance of the protein under normal conditions (control values). The direction of the interactions is shown by arrows. Log2 scale. Shown are proteins with $p \leq 0.05$ differential expression in stressor conditions.

IV. Conclusion

Individual lifestyle and external environmental stressors such as pollution, UV radiation, can have a biological impact on the energy levels of the skin. Cellular energy levels are tightly linked to the firmness, regeneration, elasticity, smoothness and tone of the skin. It is essential to protect, or maintain good metabolic functioning of skin cells (keratinocytes, Langerhans cells, melanocytes, fibroblasts) so that they can naturally counteract the effects of an aggressive environment. **The active compound seems capable of protecting skin against oxidative damage, and to restore levels of correct protein folding and energy supply, necessary for skin renewal.**

V. Recommendations

Several techniques are available for quantification and technical validation of specific and differentially regulated proteins. The biomarkers and the mechanisms identified in this experiment can be further analysed through targeted studies. The increase in proteins involved in energy production suggests that ATP production and mitochondrial respiration may be used to directly verify the effect of active compound. Microscopy analysis could also be used to screen for mitochondria network changes, after application of active compound.

Finally, potential biomarkers identified that can be verified and monitored by Western Blot and RT-qPCR to screen the active compound at other concentrations and/or against other stressors.

VI. References

Caito, S.W., and Aschner, M. (2015). Mitochondrial Redox Dysfunction and Environmental Exposures. Antioxid Redox Signal *23*, 578–595.

Käll, L., Canterbury, J.D., Weston, J., Noble, W.S., and MacCoss, M.J. (2007). Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat. Methods *4*, 923–925.

Ploumi, C., Daskalaki, I., and Tavernarakis, N. (2017). Mitochondrial biogenesis and clearance: a balancing act. FEBS J. *284*, 183–195.

Wang, M., and Kaufman, R.J. (2016). Protein misfolding in the endoplasmic reticulum as a conduit to human disease. Nature *529*, 326–335.

VII. Annexes

Annexe 1: Materials and Methods

Cell culture and growing conditions

Culture conditions are described in detail here. Cells were treated with test item or active compound dissolved in vehicle (composition of vehicle) at a concentration of XX to XX mM serial dilutions. A control Vehicle is included. Vehicle is any mixture used to dilute or dissolve the active compound may consist of a mixture of an amphiphilic substance and distilled water. Cells were treated for 24 hours with control vehicle and XX concentrations of test item (active compound or mix) before or after treatment with the stressor.

Stressor treatment

Oxidative damage may be used to simulate environmental or internal stress. Stress may be induced by oxidant drugs commonly used to induce cellular senescence. Cells can also be treated with H_2O_2 or doxorubicin to mimic oxidative damage. tBHP, Menadione or Cumene hydroperoxyde (ROS inducers) with or without N-Acetyl-Cysteine or Vitamin E (antioxidant reference compounds) may also be used.

Cell Viability and LDH release assay

LDH Cytotoxicity Assay is a colorimetric assay that provides a simple and reliable method for determining cellular cytotoxicity. Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types that is released into the cell culture medium upon damage to the plasma membrane. Viability studies can be performed 24 h after treatments LDH release assay. Details will be provided. All tests are performed at least in triplicate and assay are repeated three times independently. Viable and nonviable cells are recorded, and the means of three independent counts are pooled for analysis and expressed as percent of live cells with respect to total cell number.

Label-free quantitative proteome analysis

Label-free quantitative Mass Spectrometry methods are described in detail here, including nLC-MS/MS analysis, Database search and results processing, Label-Free Quantitative Data Analysis, Data processing includes the following steps: (i) Features detection, (ii) Features alignment across the samples, (iii) Volume integration for 2-6 charge-state ions, (iv) Normalization on total protein abundance, (v) Import of sequence information, (vi) ANOVA test at peptide level and filtering for features p <0.05, (vii) Calculation of protein abundance (sum of the volume of corresponding peptides), (viii) ANOVA test at protein level and filtering for features p <0.05. Only non-conflicting features and unique peptides are considered for calculation at protein level. Quantitative data are considered for proteins quantified by a minimum of 2 peptides.

Statistical Analysis

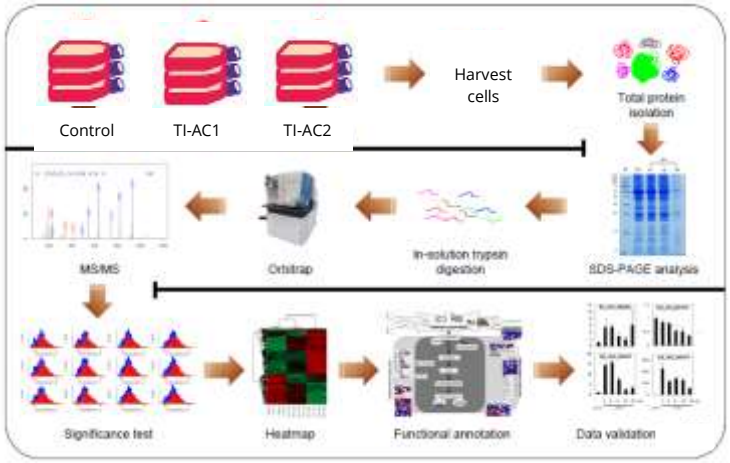
A statistical hypergeometric distribution test is applied to determine whether certain metabolic pathways are enriched in the submitted data. This test answers the question "Does my list contain more protein for the metabolic pathway X than we would expect by chance? ». This test produces a probability score, which is corrected to account for false positives.

A list of interactors (IntAct Molecular Interaction Database) may be included to increase the power of the analysis. The inclusion of interactors significantly increases the detail of metabolic pathways explored, maximizing the likelihood of matching data submitted to the metabolic pathways and proteins investigated, but which may produce interactions that are not biologically relevant to the context of the study. The results are processed and filtered to display only results relevant to the model studied.

The STRING database of known and predicted protein-protein interactions is used to determine associations between proteins and pathways. The interactions may include direct (physical) and indirect (functional) associations; they stem from direct experimental evidence and eventually from computational prediction, information available from other organisms, and/or from interactions aggregated from other (primary) databases.

Observations

Below is an example flow chart of methodology steps used for the identification of effects of Test Compound by label-free quantitative proteomics.



Annexe 2: Supplementary Data

Raw Data of proteomic analysis.

Any other relevant data as requested by sponsor.