Design, synthesis and development of a novel peptide to overcome every front of cellular oxidation and its outcomes

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ABSTRACT

Oxidation as a consequence of external factors, such as radiation and pollution, is one of the main accelerators of skin aging. Oxidative events such as peroxidation of lipid bilayers, generation of Advance Glycation End (AGE) products, DNA oxidation and pigmentation, are highly altered by such mentioned factors. Consequently, there is an urgent need for new active molecules able to act effectively on the consequences of the current contaminated environment.

BLD0001 is a novel peptide, specifically designed to meet the needs that skin under oxidative stress demands, which is able to act on multiple of these oxidative events keeping the skin protected and sustained.

Out of a collection of self-designed peptides with potential antioxidant activity, BLD0001 rendered the best Trolox Equivalent Antioxidant Capacity (94 µM at 0.1 mg/mL). BLD0001 contains a specific sequence of amino acids that makes it an

effective radical scavenger acting on ROS, able to quench unpaired electrons or radicals by supporting protons and to chelate ions with additional high reactivity to hydrophobic PUFAs.

We show the effectiveness of this unique peptide to decrease lipid peroxidation, AGEs levels and DNA oxidation induced by oxidative stress, heavy metals and synthetic smoke, while additionally showing a melanin decrease. Furthermore, we also show the potential of this peptide to modulate the expression of key genes involved in the antioxidative response pathway.

KEYWORDS

Peptide, pollution, oxidative stress, melanin, skin ageing.

INTRODUCTION

Skin ageing is a very complex process resulting from two overlapping types of ageing, intrinsic and extrinsic, which lead to the progressive loss of structural integrity and physiological function. Intrinsic ageing is mostly genetically determined and occurs inevitably as a natural consequence of physiological changes over time while extrinsic ageing, also termed as environmental ageing, is largely preventable and is due to exogenous sources such as pollution, sun exposure, smoking, etc.

Oxidation and oxidative stress are probably the most harmful contributors to skin aging and skin damaging, leading to degradation of the skin extracellular matrix (ECM) and to inflammatory processes among others [1]. The origin of oxidation and oxidative stress is varied and can be induced by, for example, physiological

factors but more importantly by environmental factors such as gases and particulate matter (PM) from anthropogenic sources (pollution), tobacco smoke, UV radiation or other exogenous sources [2]. Nowadays, pollutants have been clearly identified both in urban and indoor pollution, which include the aforementioned particulate matter, gases such as ozone (O_3) and carbon monoxide (CO), Polycyclic Aromatic Hydrocarbons (PAHs), microbial and chemical volatile organic compounds (VOCs); heavy metals and cigarette smoke (a highly complex aerosol composed of thousands of chemical substances, including reactive species, aldehydes, etc., both particulate and/or in the gas phase) among others [3]. These environmental toxicants are inherently oxidants and/or catalyse the production of several damaging reactive species such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive carbonyl species (RCS) in a direct or indirect manner (Figure 1). The production of ROS is a natural consequence of oxygen metabolism and these short-lived entities play integral physiological roles in cell signalling and oxygen homeostasis, however, an excess of ROS causes oxidative damage to biomolecules [4]. Harmful RNSs, such as peroxynitrite ($ONOO^-$) or nitric oxide radical ($NO^{\cdot}$), among others, are produced by reaction of nitric oxide (NO) with other molecules such as oxygen, glutathione and superoxide radicals. Although NO is not very reactive and plays an important role in our body [5], the intermediates produced have high reactivity towards amino acids, glycosaminoglycans, lipids, DNA, just to state some [6]. Finally, RCSs are a very heterogeneous group of reactive low molecular weight carbonyls, both exogenous, due to air contamination, or endogenous formed during biomolecule metabolism, such as 4-hydroxynonenal (4-HNE) an alkenal product of lipid peroxidation, which are able to interact with various biomolecules, such

as proteins, DNA, or phospholipids, resulting in structural distortions and functional impairment [7]. One of the major sensors recognizing several of these environmental chemicals in the skin is the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor able to trigger a xenobiotic response through the expression of CYP1A1, producing ROS and subsequently cell damage and an antioxidative response through the expression of Nrf-2 [8]. Interestingly, hyperpigmentation due to pollutants, such as PAHs in cigarette smoke or cooking oil, has been found to be mediated by AhR [9]. Antioxidants, e.g. enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin (TXN), heme oxygenase-1 (HO-1) or non-enzymatic antioxidants such as ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), glutathione (GSx), among others, are naturally produced in order to counteract oxidative stress generated by an excess of reactive species [10]. However, when environmental stress is high, elevated ROS, RNS and/or RCS levels can overwhelm endogenous cellular antioxidant mechanisms leading to an imbalance in tissue homeostasis [4].

Such pollutants may induce severe interference of normal functions of lipids, DNA and/or proteins of the human skin [11]. Peroxidation of cellular and subcellular polyunsaturated fatty acids (PUFAs) of lipid bilayers can lead to a decreased fluidity, loss of function and even cell death. Malondialdehyde (MDA) is a well-known and characterized RCS and one of the main lipid hydroperoxide-derived bifunctional electrophile from lipid peroxidation which binds covalently to DNA and/or proteins generating adducts seriously damaging cellular metabolism and integrity [12]. Glycation is a non-enzymatic reaction between reducing sugars and proteins, lipids or nucleic acids which is accelerated due to the aforementioned factors. Schiff bases and Amadori products derived from the

reaction between sugars and free amino groups can react irreversibly with other residues of peptides or proteins forming protein adducts or crosslinks or undergo further oxidative reactions yielding Advanced Glycation End (AGE) products [13]. Our genetic fingerprint, the DNA, is also highly susceptible to oxidation [14-15]. Both the sugar phosphate backbone and the nucleobases are direct targets for oxidizing agents. Guanin (G) is especially sensitive to oxidation by a variety of oxidants including hydroxyl radical (·OH), singlet oxygen ($^1O_2$) and derivatives of peroxynitrite ($ONOO^-$). A common oxidized form of Guanine is 8-Hydroxydeoxyguanosine (8-OHdG), which is the major oxidative DNA-damage product that can produce mutations and has been involved in genotoxic and mutagenic lesions [16].

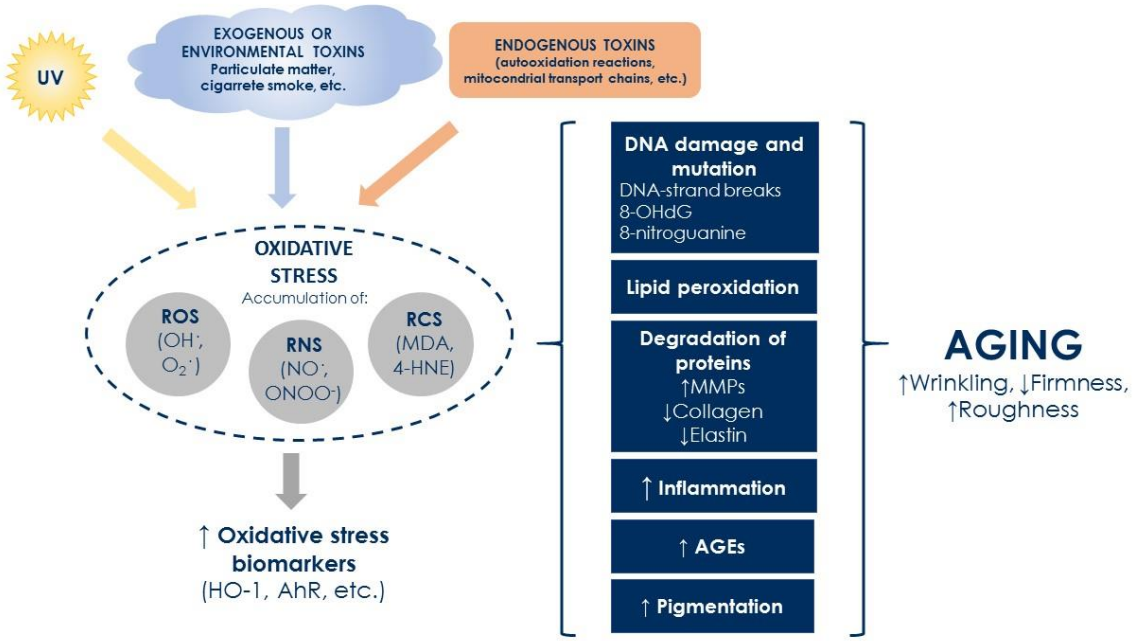

Figure 1: Schematic of the interplay between different endogenous and exogenous sources of oxidative stress and its reactive species. Possible outcomes of oxidative damage to the cells (damage to DNA, proteins and lipids) are shown on the right side of the figure.

The increase in human lifespan and air pollution being one of the world's largest environmental health risk, cause an increase in the aforementioned harmful effects on the skin, which translate in changes in tissue structure and appearance of wrinkles, hyperpigmentation and loss of elasticity, among others [17]. Therefore, it is necessary to find innovative active compounds able to restore homeostasis, protecting and repairing our skin from the harmful consequences of such a predominant contaminated environment.

We present here an innovative cosmetic ingredient, peptide BLD0001, able to act on multiple of these oxidative events keeping the skin protected and sustained. This peptide has been specifically designed to meet the needs that skin under oxidative conditions is currently demanding.

Through its direct antioxidant capacity along with its ability to increase the expression of the antioxidant switch Nrf-1 and the direct expression of key antioxidant genes, this peptide acts as a broad antioxidant amplifier, showing its effectiveness at decreasing lipid peroxidation, glycation and DNA oxidation induced by oxidative stress, heavy metals and synthetic smoke on both acellular and cellular models. This unique peptide is also able to decrease melanin levels on dark pigmented melanocytes with the subsequent general lightening efficacy and decrease of age spots, which have been clinically proven (data not shown).

Since oxidative stress plays a major role in the aging process, this antioxidant peptide also favours skin rejuvenation by improving skin elasticity through restoration of ROS imbalance both at the dermis and the epidermis (18).

EXPERIMENTAL

Peptide synthesis and purification

The peptide was obtained by using standard solid phase Fmoc protocols [19]. The crude peptide was purified using a high-pressure liquid chromatography (HPLC) device from Shimadzu (Kyoto, Japan) on a preparative Waters C18 column using a water/acetonitrile gradient supplemented with 0.1% TFA. The identity of the purified peptide was confirmed by ESI mass spectrometry, and analytical HPLC (Shimadzu, Kyoto, Japan) showed them to be at least 95% pure.

Trolox Equivalent Antioxidant Capacity (TEAC) assay

Antioxidant capacity was measured using the TEAC assay or ABTS Decolorization Assay based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$) compared with a reference antioxidant standard (Trolox, a water-soluble analogue of vitamin E) [20]. In this assay, the pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) generated by oxidation of ABTS with potassium persulfate is reduced in the presence of the antioxidant (BLD0001). The $ABTS^{\bullet+}$ has a blue-green colour, with maximum absorbance values at 650, 734 and 820 nm. The antioxidant compound captures $ABTS^{\bullet+}$ suppressing this colour production and therefore a reduction in absorbance can be detected, to a degree that is proportional to its concentration and antioxidant capacity. Quantification of absorbance was conducted at 734 nm using a Polarstar 35 Omega plate reader (BMG Labtech, Ortenberg, Germany).

Thiobarbituric Acid Reactive Substances (TBARS) assay

The TBARS assay measures lipid peroxidation levels through the detection of malondialdehyde (MDA), a RCS that results from the decomposition of polyunsaturated fatty acid lipid peroxides and it is an indicator of oxidative stress in cells and tissues [21]. Briefly, Small Unilamellar Vesicles (SUVs) from egg yolk were prepared and incubated with the analysed compounds (BLD0001 and the corresponding controls). Then, oxidation of SUVs was induced by the addition of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical able to oxidize different molecules. Oxidative reaction was then stopped with Butylated hydroxytoluene (BHT), a lipophilic organic compound with antioxidant properties. Finally, Thiobarbituric Acid (TBA) was added for the detection and quantification of MDA by fluorescence at 530 nm using a Cytation 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, USA).

Antioxidant Gene expression

Modulation of gene expression profile was assessed by RTqPCR using Primary Human Epidermal Keratinocytes (HEKa) cells. Briefly, HEKa cells were seeded in duplicate in 6-well plates at a density of 4 x 10^5 cells/well and maintained at standard culture conditions (37°C, 95% RH, 5% CO2) for 24 hours. Then, cells were treated with BLD0001 peptide at the non-cytotoxic concentration of 0.05 mg/mL for additional 24 hours. Untreated cells were used as basal control. Cells were then lysed for RNA extraction using Qiagen RNeasy Mini kit following manufacturer's instructions. Purified RNAs were used to generate the corresponding cDNAs which served as templates for amplification. RT-qPCR was performed with a panel of TaqMan assay probes and 2x gene expression Master Mix (Thermo Fisher Scientific) using StepOne plus Real-Time PCR instrument (Thermo Fisher Scientific).

The data were analyzed using the ΔΔCt method, which provides the target gene expression values as fold changes in the treated sample compared with an untreated basal sample. Both samples were normalized with the relative expression of a housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

Advanced Glycation End products (AGEs) modulation assay induced by heavy metals

The efficacy against AGE generation induced by heavy metals was evaluated in vitro as follows: Primary human epidermal keratinocytes were seeded in 96-well plates in standard culture medium, after a 24-hour incubation, the cells were subjected simultaneously to treatment with heavy metals (standard mixture of Fe, Pb and Cr) and the peptide BLD0001 for 48 hours. Quantification of the resulting AGE product levels were quantified by competitive ELISA assay at 450 nm.

DNA oxidation and lipid peroxidation assay induced by synthetic smoke

The in vitro antioxidant activity of peptide BLD0001 against synthetic smoke was evaluated to determine the efficacy of the abovementioned peptide at decreasing DNA oxidation and lipid peroxidation. Briefly, primary human epidermal keratinocytes were seeded in 96-well plates in standard culture medium and incubated for 24 hours under controlled conditions. The cells were then simultaneously subjected to treatment with synthetic smoke, which contains compounds from the particle phase (Cd and nicotine) and the vapor phase (formaldehyde, FA) of tobacco smoke, and the peptide BLD0001 for 48 hours. After the treatment, cells were lysed and DNA oxidation on cell homogenates was analysed by measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

using a competitive ELISA assay at 450 nm. Additionally, lipid peroxidation was also quantified using the levels of intracellular MDA, the marker of oxidative stress previously mentioned, by the reaction of MDA and N-methyl-2-phenylindole (NMPI) which renders a blue chromophore with an absorption peak at 586 nm.

Lightening efficacy on primary human melanocytes

The lightening efficacy of peptide BLD0001 was evaluated in Human Epidermal Melanocytes darkly pigmented of neonatal origin (HEMn-DP). Briefly, cells were seeded in 6-well plates (200.000 cells/well) and incubated for 72 h before treatment with different concentrations of peptide BLD0001 and Kojic acid (70 µM) which was used as a control for melanin synthesis inhibition. 7 days after treatment, cells were trypsinized, counted and transferred to microtubes for centrifugation at 900 xg (3000 rpm) during 15 min. The obtained pellet was lysed with 1 mL of NaOH 1 N with10% DMSO and incubated for 2h at 80ºC on the thermo-block.

Samples were centrifuged again for 10 min at 100 xg (1200 rpm). Supernatant containing melanin was transferred to a 96-well plate together with a standard curve prepared with synthetic melanin diluted on NH_4OH 1 N-10% DMSO. Absorbance was measured at 450 nm using the microplate reader Multiskan FC (Thermo Fisher Scientific, Massachusetts, USA).

Antioxidant and anti-pollutant capacity on human skin explants

The protection efficacy of peptide BLD0001 against pollution aggression on human living explants was assessed by observation of the general morphology, immunostaining of aryl hydrocarbon receptor and heme oxygenase.

Briefly, human skin explants from a 49-year-old Caucasian woman were put in culture under controlled conditions. A formula containing BLD0001 (P, Product) was topically applied on days D0, D1, D3 and D4. Untreated explants (T, control batch) were used as controls and did not received any treatment. For each condition, half of the explants were exposed to a pollutant mixture containing Benzene, Xylene, Toluene, heavy metals and hydrocarbons. Explant exposure was performed 3 hours after the treatment with BLD0001 using a Pollubox® system and lasted for 1.5 hours. Samples were processed for histology at D0 and D5. The observation of the general morphology was performed after staining of paraffinized sections according to Masson's trichrome, Goldner variant. AHR immunostaining was performed on paraffin sections with a monoclonal anti- AHR antibody (Thermoscientific, ref. MA1-514, clone RPT1) and HO-1 with a monoclonal anti- HO-1 antibody (Novus biologicals, ref. NBP1-97507). Finally, image analyses and quantification were performed using Cell^D software.

Statistical analysis

For all the tests, a minimum of 2 independent experimental sessions were performed (N=2) and each condition was tested at least in duplicate (n=2) unless indicated otherwise. All data was normalized as follows:

$$\%\ vs\ control = \frac{OD\ treated\ wells}{OD\ control\ wells}\ x\ 100$$

Statistical data analysis was performed using Student t-test by comparison of % of oxidation from test substance *vs.* % of oxidation of the damage or untreated control condition wells where *** if $p<0.001$, ** if $p<0.01$ and * if $p<0.05$.

RESULTS

Scavenging efficacy of BLD0001 by TEAC assay

The first indications that peptide BLD0001 was a good antioxidant were obtained when carrying out the TEAC assay (Figure 2). The values of 15 µM, 24 µM and 94 µM of Trolox equivalents were obtained for the three concentrations of peptide tested (0.01, 0.05 and 0.1 mg/mL respectively). As derived from these results, the peptide shows an important antioxidant power, when compared to other antioxidants in the state of the art, such as ascorbic acid, which shows an antioxidant capacity of 252 µM of Trolox at 2839 µM, a concentration 3 times bigger than the highest used of peptide (approx. 880 µM) (data not shown).

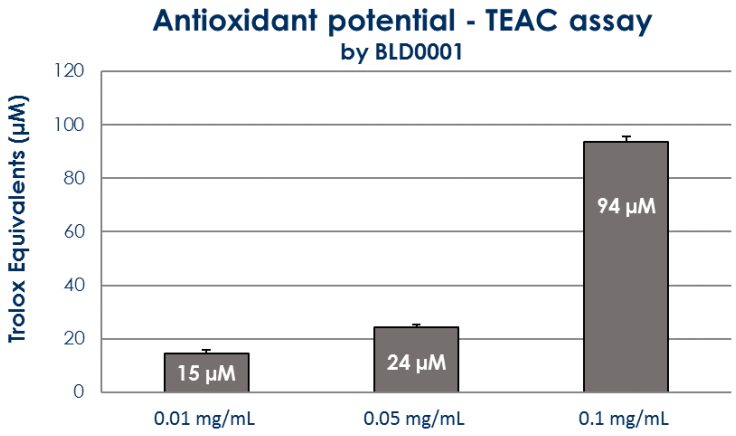

Figure 2: Effect of peptide BLD0001 on the µM of Trolox equivalents determined by the TEAC assay (N=3, n=2).

BLD0001 decreases oxidation of lipids induced by synthetic smoke

We next wanted to determine the capacity of peptide BLD0001 to decrease lipid peroxidation. To do so, two experiments were performed, the TBARS assay, on an acellular model, and an additional cellular experiment. Figure 3 shows the results obtained in the TBARS assay where the percentage of lipid peroxidation normalized on the basis of the control sample, that is, setting the percentage of maximal lipid peroxidation as 100% and then performing the comparison with the rest of the samples, can be observed. Peptide BLD0001 causes a decrease in lipid

peroxidation in a dose-dependent manner, reaching a decrease of 13% at the highest tested concentration (0.1 mg/mL). Additionally, lipid peroxidation was also determined cellularly by means of a colorimetric assay where the quantity of MDA was determined after incubation with the peptide and later induction of oxidation with synthetic smoke. The results are shown in Figure 4, where we can observe a significant decrease in lipid peroxidation at the two highest concentrations (0.05 and 0.1 mg/mL) of 12% and 14%, respectively. The two experiments are complementary and demonstrate that peptide BLD0001 is able to decrease lipid oxidation in the presence of oxidative agents.

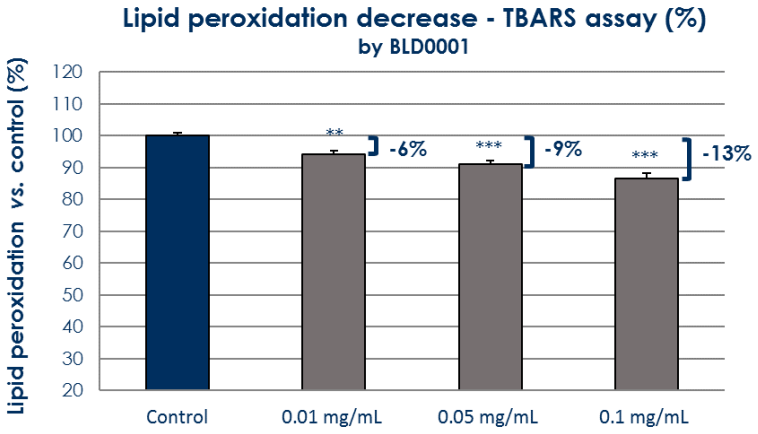

Figure 3: Lipid peroxidation decrease by peptide BLD0001 determined by the TBARS assay. *** if p<0.001, ** if p<0.01 and * if p<0.05 (N=3, n=2).

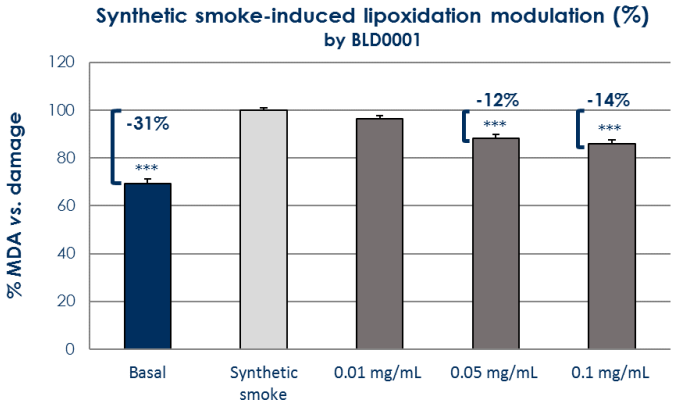

Figure 4: Modulation by peptide BLD0001 of lipid peroxidation induced by synthetic smoke. *** if p<0.001, ** if p<0.01 and * if p<0.05 (N=2, n=3).

BLD0001 increases the expression of key antioxidant genes

It is well known that oxidative stress initiates an adaptive response involving the upregulation of stress response genes and antioxidants that are important in protecting cells from injury. These antioxidants include, among others, enzymes that are key players in detoxifying or limiting the production of ROS. A coordinate regulation of the expression of these genes is essential to overcome cell damage [22]. To evaluate the effect of peptide BLD0001 on gene expression profile, a panel of antioxidant related genes were analysed by RTqPCR on skin HEKa cells. Cells treated with 0.05 mg/ml of BLD0001 show, when compared to basal cells, the upregulation of key genes involved in cellular protection to oxidative stress (Figure 5). These regulated genes encode for Thioredoxin (TRX), Glutathione peroxidase (GPX1), Glutathione synthetase (GSS), Glutathione S-transferase P (GSTP1), Heme oxygenase-1 (HMOX-1 or HO-1), Nuclear factor-like 2 (NFE2L2) and Vitamin D 25-hydroxylase (CYP2R1) proteins, upregulated with a fold change (treated sample *vs* basal sample) of 1.34, 1.22, 1.10, 1.13, 1.48, 1.23 and 1.44, respectively.

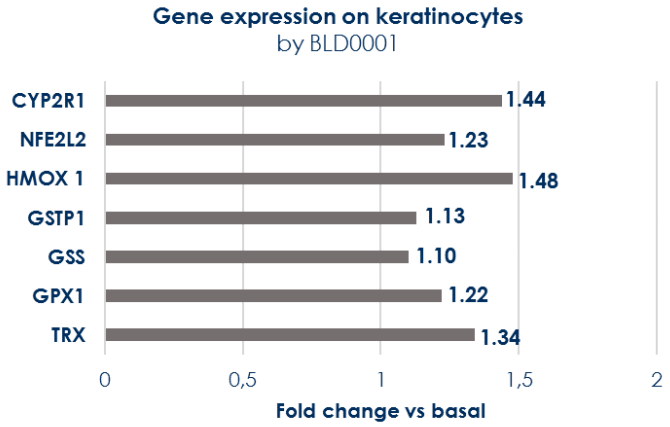

Figure 5: Gene expression on keratinocytes after treatment with peptide BLD0001 (N=1, n=2).

The antioxidant peptide decreases the generation of AGEs after exposure to heavy metals

The capacity of peptide BLD0001 to modulate AGE products induced by heavy metals, greatly present in pollution was determined. As it can be seen in Figure 6, all concentrations tested of peptide BLD0001 produced a statistically significant decrease in the quantity of AGEs of 50%, 57% and 62% and the three tested concentrations (0.01, 0.05 and 0.1 mg/mL), reducing it to levels near those observed in the basal state (quantity or level in control sample not treated with heavy metals.

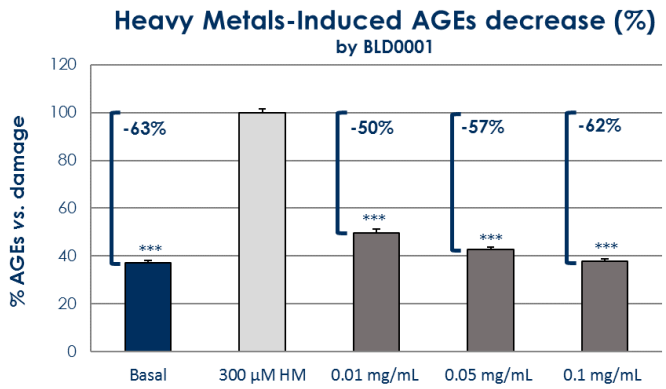

Figure 6: Decrease in Advanced Glycation End Products by peptide BLD0001 induced by heavy metals. *** if p<0.001, ** if p<0.01 and * if p<0.05 (N=3, n=3).

Declined levels of DNA oxidation by BLD0001 after exposure to synthetic smoke

The next natural step was to check the capacity of peptide BLD0001 to reduce the levels of DNA oxidation induced by synthetic smoke. This oxidative state was determined by the detection of the oxidative marker 8-OHdG by means of an ELISA assay. We can observe that at the two highest concentrations the peptide showed a high (18% and 22%), statistically significant, effect leading to an almost complete inhibition of the effects of synthetic smoke at 0.1 mg/mL (Figure 7).

These results are similar to those obtained for the cellular test on lipid peroxidation.

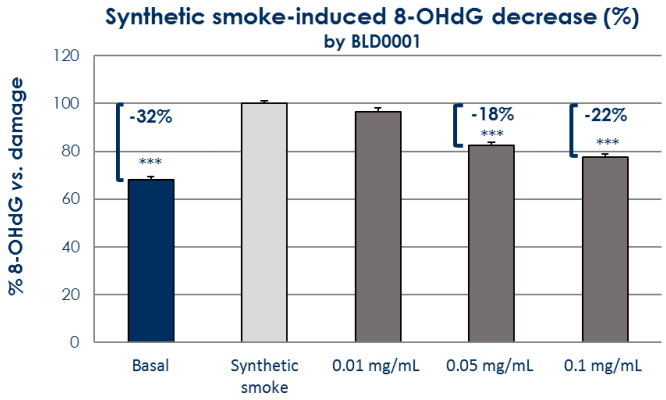

Figure 7: Decrease in 8-OHdG by peptide BLD0001 induced by synthetic smoke. *** if p<0.001, ** if p<0.01 and * if p<0.05 (N=3, n=3).

The novel antioxidant peptide significantly decreases melanin levels

Pigmentation disorders are related to both intrinsic and extrinsic aging, combining multiple factors associated to an increase oxidative stress such as sun exposure, inflammation, hormonal changes, lifestyle and environmental pollutants (which can be triggered by AhR activation). Therefore, the lightening efficacy of peptide BLD0001 was also determined (Figure 8), where it was observed that BLD0001 induces a decrease on melanin synthesis of 41%, 45% and 42% at the three concentrations tested (0.01, 0.05 and 0.1 mg/mL, respectively). Such values clearly compete with those obtained by the know benchmark kojic acid at 0.01 mg/mL, used as the positive control [23].

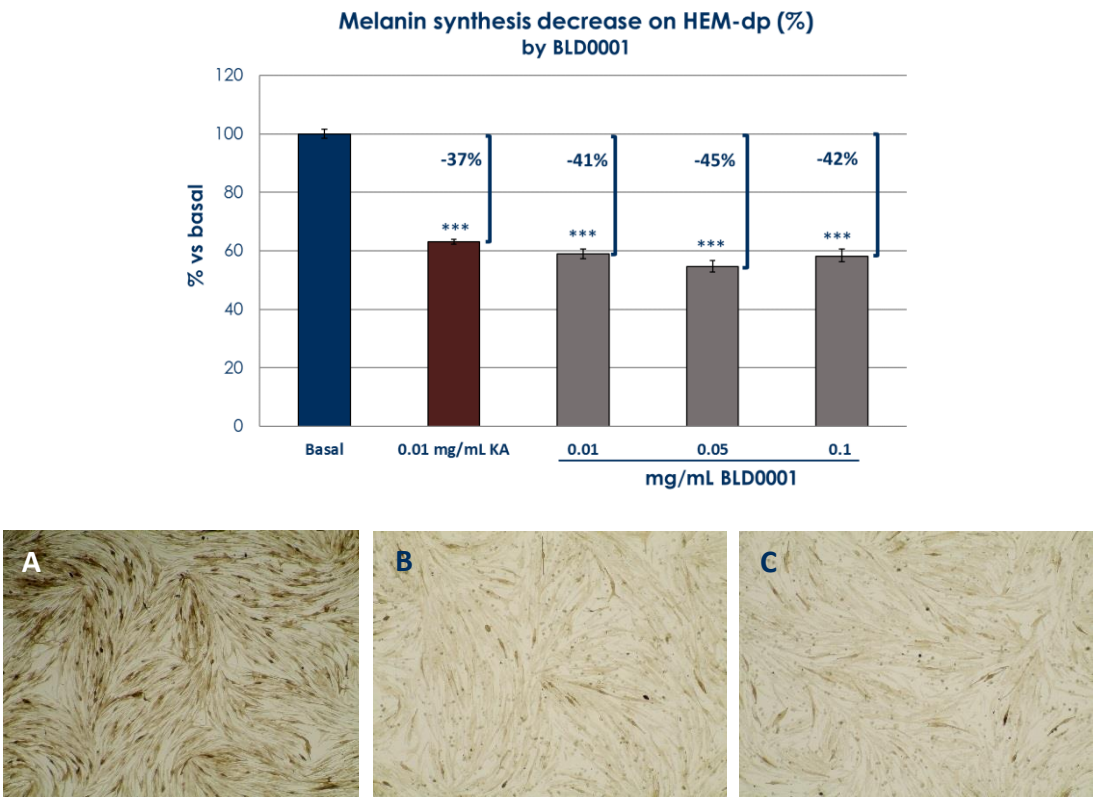

Figure 8: (Upper) Percentage of decrease in melanin content after treatment of HEMn-DP cells with different concentrations of peptide BLD0001 and Kojic acid (KA) control. *** if p<0.001, ** if p<0.01 and * if p<0.05 (N=3, n=3). (Lower) Representative images (10x magnification) of (A) HEMn-DP cells showing melanin basal production, (B) HEMn-DP cells after 7 days of treatment with 0.01 mg/mL Kojic acid and (C) HEMn-DP cells after a 7-day of treatment with peptide BLD0001 at 0.1 mg/mL.

BLD0001 effectively decreases the levels of AhR and HO-1 activated by pollution and increases HO-1 levels directly on human skin explants

Finally, the antioxidant efficacy of peptide BLD0001 against pollutants was confirmed on human skin explants treated with the peptide in a formulation by determining the amount of aryl hydrocarbon receptor (AhR) and heme oxygenase (HO-1) increased after pollution aggression. As observed in Figure 9, peptide BLD0001 showed an anti-pollution activity by inducing a decrease in AhR (16%) and OH-1 (23%) compared to the levels expressed with pollutants.

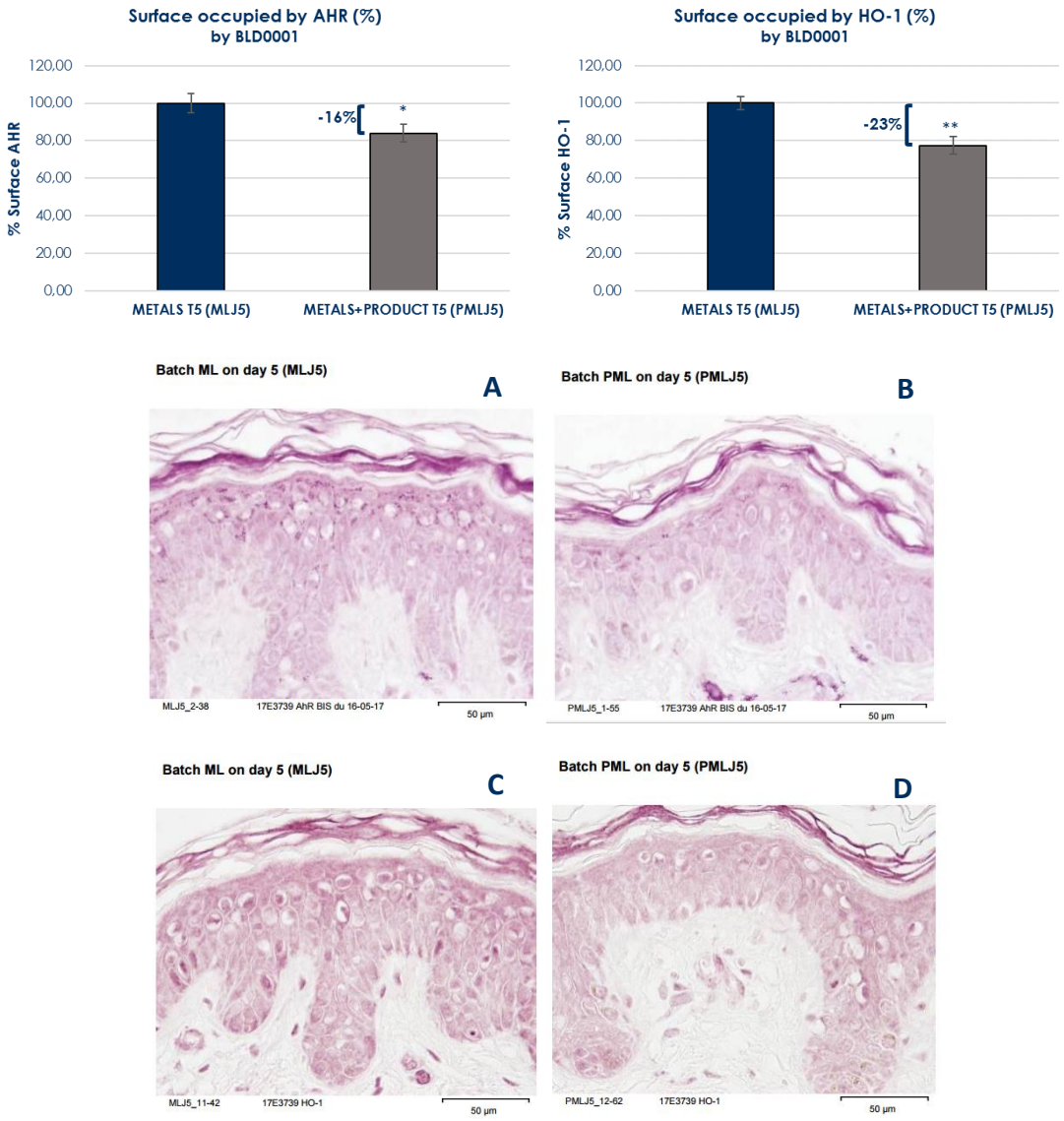

Figure 9: (Upper) Percentage of epidermal surface occupied by AhR (left) and HO-1 (right) on human skin explants, previously treated with peptide BLD0001, after exposure to a mixture of pollutants (heavy metals and hydrocarbons). *** if $p<0.001$, ** if $p<0.01$ and * if $p<0.05$ (N=1, n=9). (Lower) Histological images of the treated skin explants. Immunostaining of AhR: (A) non-treated explants, (B) treated with a formulation containing BLD0001. Immunostaining of HO-1: (C) non-treated explants, (D) explants treated with a formulation containing BLD0001.

Additionally, it was confirmed the efficacy of BLD0001 at increasing the expression on HO-1 for further prevention of oxidative damage. After treatment of skin explants with the peptide in a final formulation, the levels of HO-1 significantly increased by 69% (Figure 10).

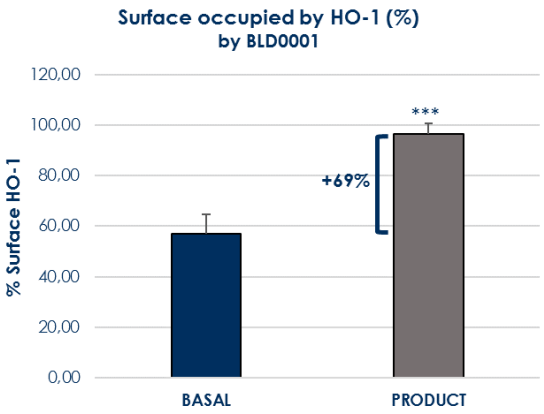

Figure 10: Percentage of epidermal surface occupied by HO-1 on human skin explants, after treatment with a formulation containing peptide BLD0001, before exposure to pollutants. A boost of the natural antioxidant HO-1 can be clearly observed. *** if $p<0.001$, ** if $p<0.01$ and * if $p<0.05$ (N=1, n=9).

DISCUSSION

In the last years, there has been a growing interest in the design and posterior commercialization of products to prevent, reduce or eliminate skin imperfections and damage caused by endogenous and environmental stressors, in order to maintain the skin with a healthy and youthful appearance. Oxidation as a consequence of external factors (for example, UV radiation or pollution) is, nowadays, one of the main accelerators of skin aging and the appearance of skin imperfections. As already stated before, oxidation and oxidative stress, among other consequences, generates multiple radicals which, in turn produce, for example:

- Lipid Peroxidation: Levels of lipid peroxidation products are increased with age which lead to multiple cellular responses, such an increase in antioxidative enzymes, activation of inflammatory processes, among others [12].

- Advanced glycation products: They modify the biomechanical and functional properties of biological molecules such as extracellular matrix (ECM) proteins, one of the major target for glycation, by for example inducing collagen cross-linking and an increase in metalloproteinases (MMPs 1, 2 and 9) [13].
- Other DNA damage. As already stated above, DNA is highly susceptible to oxidation [14-16] but besides the modification of DNA bases, ROS also induces single-strand breaks and to a lesser degree double strand breaks [24].
- Hyperpigmentation: As already introduced, pigmentation can be due to multiple factors associated to an increase oxidative stress such as UV radiation, inflammation, hormonal changes, lifestyle and environmental pollutants. In the case of the latter, AhR is of special interest as it has been described that it plays a role in pigment formation by regulating the expression of genes coding for enzymes of the melanogenic pathway [9], consequently, its activation by pollutants, such as those contained in tobacco smoke, causes hyperpigmentation [25].

Multiple strategies and compounds have been developed in order to fight against these undesirable ageing processes, such as topical antioxidants (vitamins, retinoids, flavonoids, contained in plant extracts, or anti-inflammatory actives) [26]. However, some of these compounds are unstable and just able to act on one of these extrinsic factors, or are not compatible with sun exposure, making it necessary to use multiple products in order to obtain a complete effect on the skin. Hence, it is desirable to find stable active ingredients with a wide-spectrum antioxidant activity, which can be used to prevent or treat skin imperfections appearing as the result of environmental oxidative stress.

Out of a collection of self-designed peptides with potential antioxidant activity, peptide BLD0001 rendered one of the best results due to the multiple properties that turn it into an excellent candidate as an amplifier antioxidant for cosmetic applications: It is relatively small (<700 Da) and water soluble, properties that help the diffusion throughout the skin [27], which can additionally be improved by using better delivery systems in formulations such as emulsions, vesicular systems or conditioning agents [28]. BLD0001 contains an aromatic amino acid, which are generally considered effective radical scavengers, acting on for example reactive oxygen species, because they have the ability to donate protons easily to electron deficient radicals while maintaining their stability via resonance structures. The presence of multiple hydrogen donor amino acids makes the peptide able to quench unpaired electrons or radicals by supporting protons. Additionally, BLD0001 contains a non-polar aliphatic group which has high reactivity to hydrophobic polyunsaturated fatty acids (PUFAs) which seem to be the substrate of lipid peroxidation, which eventually leads to loss of the functional integrity of cell membranes [29]. Finally, the presence of amino and carboxyl groups, due to acidic and basic amino acids in the sequence, makes the peptide able to chelate metal ions [30].

The specific sequence and positioning of the amino acids in BLD0001 seem to be important for the high activity, as also seen in [30] and previously in [31], where the equimolar mixture of constituent amino acids rendered not active while the peptide with its characteristic sequence was responsible for activity.

Peptide BLD0001 not only showed a high intrinsic antioxidant activity, as seen in the TEAC assay but, through the increase in the expression of key antioxidant genes such as Nrf-1 and HO-1, it was also able to protect several biological

structures, such as proteins, lipids and DNA against multiple stressors, as heavy metals and tobacco smoke. Tobacco smoke is one of the most important extrinsic factor of premature ageing because it contains more than 3800 constituents, including numerous water-insoluble polycyclic aromatic hydrocarbons (PAHs) that trigger aryl hydrocarbon receptor signalling pathways with the subsequent oxidative levels increase. Additionally, it has been reported that synthetic smoke induces cell damage through the induction of lipid peroxidation [32] and the break of DNA single strands by free radicals [33]. Smoke content also promotes de destruction of endogenous antioxidants (vitamins and enzymes) reducing the vital role of cellular antioxidant defences [34]. Heavy metals have been widely studied, and although some are essential in multiple metabolic processes, their specific chemical properties and excess, as they are present in environmental pollution by itself or contained in other pollutants such as cigarette smoke, make them an important environmental pollutant. They have shown to generate multiple reactive species, which consequently cause modifications in DNA, enhanced lipid peroxidation and general tissue damage [35].

To cope with the different sources of oxidative stress (intrinsic or environmental) the skin has developed sophisticated antioxidative mechanisms, which are mainly concentrated in the exposed epidermis. We have demonstrated that peptide BLD0001 upregulates some of the most prominent enzymes that can handle ROS: GSS, GPX1, GSTP1, NFE2L2, CYP2R1, HO-1 and TRX, protecting skin from accelerated aging.

The results obtained by all the in vitro and ex vivo tests performed indicate that peptide BLD0001, is not only a great antioxidant compound but it is also able to

boost the endogenous antioxidant defences of our skin. Of special interest is the enzyme HO-1 (HMOX-1), detected both at the genetic level and in skin explants, which is known to be an enzyme activated via Nrf-2 (also detected in the smart data gene panel), known as the antioxidant switch, which is itself a transcription factor that binds to antioxidant-responsive elements (AREs), enhancer DNA sequences that initiate the transcription of a battery of genes encoding potent antioxidant enzymes [36]. BLD0001 also acts as a modulator of the increase expression of AhR induced after pollutants exposure, as observed on human skin explants, therefore preventing the oxidative damage associated with AhR activation and the subsequent hyperpigmentation, also observed in vitro through inhibition of melanin synthesis.

All these results demonstrate that peptide BLD0001 has not only a potent antioxidant activity but also that said activity is wide-spectrum. This is, in all the oxidative conditions tested and for all the parameters tested, the peptide has shown a significant and important activity, acting at many levels of environmental aging. The peptide has shown its effectiveness to decrease lipid peroxidation, the formation of AGEs and DNA oxidation induced by oxidative stress, heavy metals and synthetic smoke on both acellular and cellular models, while also showing a decrease in pigmentation making it a perfect compound to prevent, reduce and/or remove skin imperfections related with oxidation and oxidative stress.

CONCLUSION

There is an urgent need for new active molecules able to both protect and repair from the noxious consequences of this prevailing contaminated environment.

The results shown here demonstrate that peptide BLD0001 has not only a potent antioxidant activity but also an antioxidant amplifying response and that said activity is wide spectrum. That is, in all the oxidative conditions tested and for all the parameters tested the peptide has shown a significant and important activity. Hence, these experimental results demonstrate the feasibility of this peptide to be used in cosmetic compositions to prevent, reduce and/or remove skin imperfections related to oxidation and oxidative stress.

REFERENCES

[1] *Bickers, D.R. and Athar, M.,* Oxidative Stress in the Pathogenesis of Skin Disease, J. Inv. Dermatology, **126** (2006) 2565-2575.

[2] *Farage, M.A., Miller, K.W., Elsner, P. and Maibach, H.I.*, Intrinsic and extrinsic factors in skin ageing: a review, Int J Cosmet Sci., **30** (2008) 87-95.

[3] *Baudouin, C., Charveron, M., Tarroux, R. and Gall, Y*. Environmental pollutants and skin cancer, Cell Biology and Toxicology, **18** (2002) 341-348.

[4] *Kammeyer, A. and Luiten, R.M.*, Oxidation events and skin aging, Ageing Res. Rev., **21** (2015) 16-29.

[5] *Koshland, D.E.*, The molecule of the year, Science, **5090** (1992) 1861.

[6] *Martinez, M.C. and Andriantsitohaina, R.,* Reactive Nitrogen Species: Molecular Mechanisms and Potential Significance in Health and Disease, Antioxidants & Redox Signaling, **11** (2009) 669-702.

[7] *O'Brien, P.J., Siraki, A.G. and Shangari, N.,* Aldehyde Sources, Metabolism, Molecular Toxicity Mechanisms, and Possible Effects on Human Health, Critical Reviews in Toxicology, **35** (2005) 609-662.

[8] *Dietrich, C.*, Antioxidants of the Aryl Hydrocarbon Receptor, Stem Cells International, **2016** (2016) 1-10.

[9] *Jux, B., Kadow, S., Luecke, S., Rannug, A., Krutmann, J. and Esser, C.*, The Aryl Hydrocarbon Receptor Mediates UVB Radiation-Induced Skin Tanning, J. of Inv. Dermatology, **131** (2011) 203-210.

[10] *Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J.*, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. of Biochemistry & Cell Biology, **39** (2007) 44-84.

[11] *Drakaki, E., Dessinioti, C. and Antoniou, C.V.*, Air pollution and the skin, Frontiers in Environmental Science, **2** (2014) 1-6.

[12] *Ayala, A. Muñoz, M.F. and Argüelles, S.,* Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal, Oxidative Medicine and Cellular Longevity, **2014** (2014) 1-31.

[13] *Gkogkolou, P. and Böhm, M.,* Advanced glycation end products. Key players in skin ageing? Dermato-Endocrinology, **4** (2012) 259-270.

[14] *Barzilai, A. and Yamamoto, K-I.,* DNA damage responses to oxidative stress, DNA Repair, **3** (2004) 1109-1115.

[15] *Risom, L., Moller, P. and Loft, S.*, Oxidative stress-induced DNA damage by particulate air pollution, Mutation Research, **592** (2005) 119-137.

[16] *Valavanidis, A., Vlachogianni, T. and Fiotakis, C.,* 8-hydroxy-2'-deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis, Journal of Environmental Science and Health Part C, **27** (2009) 120-139.

[17] *Langton, A.K., Sherratt, M.J., Griffiths, C.E.M. and Watson, R.E.B.,* A new wrinkles on old skin: the role of elastic fibres in skin ageing, Int. J. of Cosmetic Science, **32** (2010) 330-339.

[18] *Baumann, L.,* Skin ageing and its treatment, Journal of Pathology, **211** (2007) 241-251.

[19] *Fields, G.B. and Noble R.L.*, Solid-phase peptide synthesis utilizing 9-fluorenylmethozycarbonyl amino acids, Int. J. of Peptide and Protein Res., **35** (1990) 161-214.

[20] *Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C.*, Antioxidant activity applying an improved ABTS radical cation decolourisation assay, Free Radical and Biology Medicine, **26** (1999) 1231–1237.

[21] *Lefèvre, G., Beljean-Leymarie, M., Beyerle, F., Bonnefont-Rousselot, D., Cristol, J.P., Thérond, P. and Torreilles, J.* Evaluation of lipid peroxidation by measuring thiobarbituric acid reactive substances, Annales de Biologie Clinique, **56** (1998) 305-319.

[22] *Zheng R, Heck DE, Mishin V, Black AT, Shakarjian MP, Kong AN, Laskin DL, Laskin JD.,* Modulation of keratinocyte expression of antioxidants by 4-hydroxynonenal, a lipid peroxidation end product, Toxicol. Appl. Pharmacol., **275** (2014) 113-21.

[23] *Curto, E.V., Kwong, C., Hermersdörfer, H., Glatt, H., Santis, C., Virador, V. Hearing, V.J. and Dooley, T.P.,* Inhibitors of Mammalian Melanocyte Tyrosinase: In Vitro comparisons of Alkyl Esters of Gentisic Acid with Other Putative Inhibitors. Biochemical Pharmacology, **57** (1999) 663-672.

[24] *Caldecott, K.W*. Single-strand break repair and genetic disease. Nat. Rev. Genet., **9** (2008) 619–631.

[25] *Nakamura, M., Ueda, Y., Hayashi, M., Kato, H., Furuhashi, T. and Morita, A.*, Tobacco smoke-induced skin pigmentation is mediated by the aryl hydrocarbon receptor, Experimental Dermatology, **22** (2013) 554-563.

[26] *Pillai, S., Oresajo, C., Hayward*, J. Ultraviolet radiation and skin ageing: roles of reactive oxygen spices, inflammation and protease activation, and strategies for prevention of inflammation induced matrix degradation – a review, Int. J. Cosmet. Sci., **27** (2005)17-34.

[27] *Antosova, Z., Mackova, M., Kral, V. and Macek, T., Therapeutic application of peptides and proteins: parenteral forever?* Trends Biotechnology, **27** (2009) 628-635.

[28] *Patravale, V.B. and Mandawgade*, Novel cosmetic delivery systems: an application update, International Journal of Cosmetic Science, **30** (2008) 19-33.

[29] *Rajapakse, N., Mendis, E., Jung, W-K., Je, J-Y. and Kim, S-K.*, Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties, Food Research International, **38** (2005) 175-182.

[30] *Suetsuna, K., Ukeda, H. and Ochi, H.*, Isolation and characterization of free radical scavenging activities peptides derived from casein, J. Nutr. Biochem., **11** (2000) 128-131.

[31] *Tsuge, N., Eikawa, Y., Nomura, Y., Yamamoto, M. and Sugisawa, K.,* Antioxidative activity of peptides prepared by enzymatic hydrolysis of egg-white albumin, Nippon Nogei Kagaku Kaishi, **65** (1991) 1635-1641 (in Japanese).

[32] *Frei, B., Forte, T.M., Ames., B.N. and Cross, C.E.,* Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma, Biochem. J., **277** (1991) 133–138.

[33] *Leanderson, P., and Tagesson, C.,* Cigarette smoke-induced DNA damage in cultured human lung cells: role of hydroxyl radicals and endonucleases activation, Chem. Biol. Interact., **81** (1992) 197-208.

[34] *Cross, C.E., Traber, M., Eiserich, J., and van der Vliet, A.,* Micronutrient antioxidants and smoking, Br. Med. Bull, **55** (1999) 691-704.

[35] *Valko, M., Morris, H. and Cronin, M.T.D.,* Metals, Toxicity and Oxidative Stress, Current Medicinal Chemistry, **12** (2005) 1161-1208.

[36] *Nguyen, T., Nioi, P., and Pickett, C.B.,* The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress, J. Biol. Chem., **284** (2009) 13291-13295.