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Research Paper

# Hexapeptide-11 is a novel modulator of the proteostasis network in human diploid fibroblasts

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ABSTRACT

Despite the fact that several natural products (e.g. crude extracts or purified compounds) have been found to activate cell antioxidant responses and/or delay cellular senescence the effect(s) of small peptides on cell viability and/or modulation of protective mechanisms (e.g. the proteostasis network) remain largely elusive. We have thus studied a hexapeptide (Hexapeptide-11) of structure Phe–Val–Ala–Pro–Phe–Pro (FVAPFP) originally isolated from yeast extracts and later synthesized by solid state synthesis to high purity. We show herein that Hexapeptide-11 exhibits no significant toxicity in normal human diploid lung or skin fibroblasts. Exposure of fibroblasts to Hexapeptide-11 promoted dose and time-dependent activation of proteasome, autophagy, chaperones and antioxidant responses related genes. Moreover, it promoted increased nuclear accumulation of Nrf2; higher expression levels of proteasomal protein subunits and increased proteasome peptidase activities. In line with these findings we noted that Hexapeptide-11 conferred significant protection in fibroblasts against oxidative-stress-mediated premature cellular senescence, while at in vivo skin deformation assays in human subjects it improved skin elasticity. Finally, Hexapeptide-11 was found to induce the activity of extracellular MMPs and it also suppressed cell migration. Our presented findings indicate that Hexapeptide-11 is a promising anti-ageing agent.

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## Introduction

Ageing is driven by diverse molecular pathways and biochemical events that are promoted by both genetic and environmental factors [1,2]. Specifically, ageing is defined as a time-dependent decline of stress resistance and functional capacity, associated with increased probability of morbidity and mortality. These effects relate to (among others) age-related gradual accumulation of damaged biomolecules (including proteins) which eventually compromise cellular homeodynamics as they result in failure of most cellular maintenance pathways.

Considering that most (if not all) of the critical cellular functions depend on the functionality of highly sophisticated protein machines [3] it is not surprising that proteostasis (proteome homeodynamics) regulation is critical for cellular functionality and consequently for the overall healthspan and/or longevity of the organism. To maintain proteostasis cells have developed a modular, yet integrated system which ensures general proteome quality control and it is called the proteostasis network (PN) [3–6]. The PN curates the basal functionality of the proteome and it also responds to conditions of proteotoxic stress by addressing the triage decision of *fold*, *hold*, or *degrade*. PN is constituted from several complex protein machines that ensure normal proteome synthesis and recycling, or respond to conditions of proteotoxic stress by launching the proteome damage responses (PDR), which, firstly identify, and then, either rescue or degrade unfolded, misfolded or non-native polypeptides. Additional integrated modules

*Abbreviations:* ALS, autophagy lysosome system; becn1, beclin-1; ctsl, cathepsin-L; ctsd, cathepsin-D; CLU, apolipoprotein J/Clusterin; ER, endoplasmic reticulum; hdac6, histone deacetylase 6; HDFs, human diploid fibroblasts; hsf1, heat shock transcription factor-1; hsp, heat shock protein; Keap1, Kelch-like ECH-associated protein 1; Nqo1, NAD(P)H dehydrogenase, quinone 1; Nrf2, NF-E2-related factor 2; PDR, proteome damage responses; PN, proteostasis network; ROS, reactive oxygen species; Sqstm1, sequestosome 1; Txnrd1, thioredoxin reductase 1; UPS, ubiquitin proteasome system

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of the PN can be considered the regulatory pathways of the cellular stress (e.g. heat or oxidative) responses which mobilize the proteome caretakers; mitotic cells can also dilute proteome damage by mitosis [3,6,7].

Central to the PN functionality and PDR are the two main proteolytic systems namely the autophagy lysosome (ALS) and the ubiquitin-proteasome (UPS) systems. ALS is mostly involved in the degradation of long-lived proteins, aggregated ubiquitinated proteins, as well as in the recycling of damaged organelles [8,9]. On the other hand, UPS curates proteome stability in various subcellular sites including the nucleus, the cytosol, the endoplasmic reticulum (ER) and mitochondria. UPS is the main site of protein synthesis quality control (at the cytosol and the ER) and it is also involved in the recycling of both normal short-lived proteins and of non-repairable misfolded or unfolded proteins [10–12]. The UPS functionality and activity decline during either cellular senescence or in vivo ageing [13–15] indicating that UPS is actively involved in the molecular process that are linked with the appearance and, likely, the progression of the ageing phenotypes. On the other hand, UPS activation has been linked to prolonged efficient removal of damaged and/or dysfunctional polypeptides and it is thus anticipated that this strategy will likely, exert anti-ageing effects [11,12].

A number of small molecules, namely natural products (e.g. crude extracts or purified compounds) isolated from various sources (e.g. plants, microorganisms, marine organisms, etc.) were found to delay either cellular senescence of normal human cells or in vivo ageing of model organisms [1]. Natural compounds represent an extraordinary inventory of high diversity structural scaffolds and seemingly they affect the process of ageing or cellular senescence by either intervening with nutrient sensing pathways (e.g. the INS/IGF-1 pathway) or by activating stress sense/response pathways; namely Sirtuins, the AMP-dependent kinase or the FOXO and Nrf2 transcription factors [1].

Notably, the effects of small peptides on normal human cells viability, cellular senescence and/or modulation of protective mechanisms (e.g. the proteostasis network) remain largely elusive. In few previous studies it was found that extracts from *Saccharomyces cerevisiae* fermentation exert wound healing properties [16–18]. These properties were attributed to improved collagen synthesis [16,18] and to increased cellular oxygen consumption [19,20]. We have studied a hexapeptide (Hexapeptide-11) of structure Phe–Val–Ala–Pro–Phe–Pro (FVAPFP) that was isolated from yeast extracts and later synthesized by solid state synthesis to high purity; this peptide seemed particularly interesting since its amino acid sequence was found in various proteins including stress- or proteostasis-related proteins (e.g. the molecular chaperone, hsp70) (our unpublished data). We report herein the impact of the hexapeptide FVAPFP on normal human cells proteostasis and anti-oxidant modules and its capacity to exert anti-ageing effects in normal human cells.

## Materials and methods

### *Cell lines and cell culture conditions*

Human lung embryonic (IMR90 cells) and human newborn foreskin (BJs) fibroblasts were obtained from the American Tissue Culture Collection and were maintained in Dulbecco's modifies Eagle's medium (Gibco Life Technologies), supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 1% non-essential amino acids in a humidified incubator at 5% $CO_2$ and 37 °C. In all experimental procedures proliferating cells were subcultured at a split ratio 1:2 (when confluent) by using a trypsin/EDTA solution (Gibco Life Technologies).

### *Induction of premature senescence by $H_2O_2$ and SA β-Gal staining*

Stress-induced premature senescence (SIPS) in relatively early passage proliferating cells was induced by exposing subconfluent cell cultures to serial short term oxidative stress as previously described [21] with minor modifications. Briefly, cells were treated with a subcytotoxic concentration of 300 μM $H_2O_2$ (three exposures of 48 h each); also, in this series of experiments cells were treated for the same periods with 5% (v/v) (see below) Hexapeptide-11 or with both 300 μM $H_2O_2$/5% (v/v) Hexapeptide-11. Control cells were kept under the same culture conditions without $H_2O_2$.

Senescent cells were stained with β-galactosidase staining as described previously [21,22]. Briefly, cells were washed with PBS and fixed in 0.2% glutaraldehyde and 2% formaldehyde in PBS for 5 min. Following fixation cells were washed with PBS and were then stained (in the absence of $CO_2$) for 12–16 h at 37 °C in staining solution [150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 40 mM citric acid/sodium phosphate, pH 6.0 containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside]. Cells were viewed under phase contrast optics in a TS-100F NIKON inverted microscope and at least 10 optical fields were used to score positively stained cells.

### *Cell survival assay*

Cells were plated in flat-bottomed 96-well microplates. After 24 h they were treated with different concentrations of Hexapeptide-11 for 24 or 48 h. Following the completion of the treatment the medium was replaced by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) dissolved at a final concentration of 1 mg/mL in serum-free, phenol red–free medium. The reduction of the dye by the living cells was allowed to take place for 3–4 h. The MTT solution was discarded and isopropanol was added to dissolve the formazan crystals. The absorbance of the solution was measured at 570 nm wavelength. Survival of control cells was arbitrarily set to 100%.

### *Yeast (S. cerevisiae) culture and peptide synthesis*

Hexapeptide-11 was initially manufactured by using fermentation biotechnology; the resulting fermentation product was fractionated using proprietary filtration and chromatography to isolate the desired dominant peptide, which was then separated from the crude mixture and isolated in a highly purified state as described previously [23]. The isolated peptide was sequenced and the sequence of Hexapeptide-11 was found to be of the specific amino acid sequence Phe–Val–Ala–Pro–Phe–Pro. The peptide (named as Hexapeptide-11) was later synthesized by solid state synthesis to high purity, as described previously [23], and was then diluted in sterile $H_2O$ at a stock solution concentration of 295 μM. The % (v/v) concentration of Hexapeptide-11 that is indicated in text or figures refers to percent by volume of the sterile 295 μM stock solution of Hexapeptide-11 in the total volume of cell culture medium.

### *RNA extraction, cDNA synthesis and quantitative Real Time PCR (Q-PCR) analysis*

Total RNA was isolated using the TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich) and quantified with BioSpec-nano spectrophotometer (Shimadzu Inc.). Subsequently, 1 μg RNA was converted to cDNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and the PikoReal 96 Real-Time PCR System (Thermo

Scientific). Primers were designed using the primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are described in Supplemental data.

*Immunoblotting analysis*

Cells were lysed in a buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris–HCl, pH 8.0 and phosphatase, protease inhibitors; cell lysates were immediately cleared with centrifugation for 15 min at 19,000*g* (4 °C). After adjustment of protein content with the Bradford method (Bio-Rad Laboratories), samples were mixed with reducing Laemmli buffer and equal protein amounts were fractionated by SDS-PAGE followed by immunoblotting, as described previously [24]. Primary and horseradish peroxidase-conjugated secondary antibodies were applied for 1 h at RT and immunoblots were developed by an enhanced chemiluminescence reagent kit (Santa Cruz Biotechnology).

*Measurement of proteasome enzymatic activities*

Cells were lysed on ice by using a buffer suitable for the isolation of 26S proteasome (0.2% Nonidet P-40, 5 mM ATP, 10% glycerol, 20 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 20 mM Tris, pH 7.6). Lysates were cleared with centrifugation at 19,000*g* (4 °C), and, after protein content adjustment with Bradford, supernatants were immediately used to determine the three proteasome proteolytic activities as described previously [14,25]. Briefly, the chymotrypsin-like (CT-L/LLVY), caspase-like (C-L/LLE) and trypsin-like (T-L/LRR) activities were assayed by recording the hydrolysis of the fluorogenic peptides Suc–Leu–Leu–Val–Tyr–AMC, Z-Leu–Leu–Glu–AMC, and Boc–Leu Arg–Arg–AMC (Enzo Life Sciences, Farmingdale, NY, USA), respectively, at 37 °C for 3 min. The fluorescence was measured at a VersaFluorTM Fluorometer System (Bio-Rad laboratories, Hercules, CA, USA) at excitation and emission wavelengths of 350 and 440 nm, respectively.

*Measurement of cathepsins B, L enzymatic activities*

The cathepsin B, L activity was measured as described previously [26] with minor modifications. Briefly, cells were lysed on ice in 1 mM dithiothreitol and 50 mM Tris, pH 4.0 and the lysates were cleared at 14,000*g* for 20 min at 4 °C. Following protein content measurement with Bradford assay (Bio-Rad), 20 μg of protein were incubated in the reaction buffer (50 mM sodium acetate, 8 mM cysteine-hydrochloride, 1 mM EDTA, pH 4.0) containing the substrate z-FR-AMC (Enzo Life Sciences) for 30 min at 37 °C. The fluorescence was measured (VersaFluorTM Fluorometer System) at excitation and emission wavelengths of 350 and 440 nm, respectively.

*Immunofluorescence staining*

Cells grown on coverslips were fixed in 4% formaldehyde in PBS and permeabilized with 0.2% Triton X-100. Blocked cells (1% BSA in PBS) were then incubated with primary anti-Nrf2 antibody (sc-722, Santa Cruz; diluted 1:100) and secondary antibody (anti-rabbit-IgG, Texas Red, diluted 1:200) for 1 h at RT. For visualizing nuclei cells were counterstained with DAPI (Molecular Probes). Samples were viewed at an Eclipse TS-100F NIKON inverted fluorescent microscope.

*Cell migration assay*

The migration capacity of treated (or control) cells was evaluated by using the in vitro scratch assay as described previously [27]. Cells were plated in 60 mm dishes and left to grow until they reached confluency. Then, the confluent monolayer was scratched down with a 200 μL sterile pipette tip. After scratching, plates were washed once with culture medium to remove floating cells and cells were fed with fresh medium containing (or not) Hexapeptide-11. Cells were allowed to migrate across the wound and micrographs were taken at various time points by using phase contrast optics at an Eclipse TS-100F NIKON inverted microscope.

*Assessment of MMP-2 and MMP-9 activities by gelatin zymography*

The gelatinases MMP-2 and MMP-9 activity in cell-conditioned media was evaluated by gelatin zymography. Cell culture supernatants were subjected to SDS-PAGE on a 10% SDS-polyacrylamide gel impregnated with 1 mg/mL gelatin in the presence of 0.1% SDS (under non-reducing conditions). After electrophoresis, gels were washed in 5 mmol/L $CaCl_2$, 50 mmol/L Tris–HCl, pH 7.4 containing 2.5% Triton X-100 for 1 h at RT, with gentle agitation to remove SDS. Gels were then incubated for 40 h at 37 °C in the reaction buffer containing 5 mmol/L $CaCl_2$, 50 mmol/L Tris–HCl, pH 7.4. Afterwards, gels were stained with a 0.5% Coomassie Brilliant Blue solution containing 20% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid. The gelatinolytic activity was detected as clear bands against the blue background.

*Measurement of skin firmness in human subjects*

The skin firming properties of Hexapeptide-11 were evaluated by conducting an *in vivo* study on 25 volunteers according to standard ethical procedures (Lonza Personal Care, USA). A firming toner containing 2.8% (v/v) Hexapeptide-11 (or a control toner with no Hexapeptide-11) was applied twice daily around the eyes and cheeks for 4 weeks. An estimated concentration of the used preparation was ~2 mg/cm$^2$ of skin (mass/skin area). The firmness of the skin was analyzed using a Cutometer SEM 575 Skin Elasticity Meter; this device measures the rheological properties of the skin, looking for the elastic and viscous response of the skin to externally applied stress (i.e. a gentle twisting of the skin). The initial elastic response of the skin is called Ue and the total deformation response (i.e. the ability of the skin to rebound from the twist) called Uf. A typical measurement only requires a few seconds to complete.

*Antibodies used*

Primary antibodies against human Nrf2 (sc-722), proteasome α7 subunit (sc-100456), proteasome β5 subunit (sc-55009), Nqo1 (sc-16464), beclin-1 (sc-11427), p53 (sc-47698), p21$^{CIP1}$ (sc-6246), and GAPDH (sc-25778), as well as the HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

*Statistical analysis*

Experiments were performed at least in duplicates unless otherwise indicated in figure legends. For statistical analyses MS Excel and the Statistical Package for Social Sciences (IBM SPSS; version 19.0 for Windows) were used. Statistical significance was evaluated using one-way analysis of variance (ANOVA). Data points correspond to the mean of the independent experiments and error bars denote standard deviation (SD.); significance at $P < 0.05$ or $P < 0.01$ is indicated in graphs by one or two asterisks, respectively.

## Results

*Hexapeptide-11 shows no toxicity to human fibroblasts and promotes dose and time-dependent induction of proteasome, autophagy, chaperones and antioxidant responses related genes*

We first set out to examine the effect of Hexapeptide-11 on the viability of human lung IMR90 (Fig. 1A) and skin BJ (Fig. 1B) fibroblasts. To this end, cells were incubated with increasing concentrations of Hexapeptide-11 for 24 or 48 h and, as shown in Fig. 1, up to (at least) the concentration of 5% (v/v), this peptide exerted no significant cytotoxic effect on human fibroblasts; notably at low concentrations in IMR90 cells Hexapeptide-11 seemed to even slightly enhance cell viability (Fig. 1A). The indicated % (v/v) concentration hereafter refers to percent by volume of the stock Hexapeptide-11 solution (295 μM) in the total volume of cell culture medium.

Given the absence of cytotoxicity we then sought to investigate the effect(s) of Hexapeptide-11 on the expression of genes involved in the PN modules. As shown in Fig. 2 Q-PCR analyses revealed that exposure of proliferating IMR90 cells to Hexapeptide-11 induced the expression of 20S and 19S proteasome subunits genes (*α7*, *β1*, *β2*, *β5*, *rpn6, rpn11*); of genes involved in ALS functionality (i.e. *becn1*, *sqstm1* and *hdac6*, along with the cathepsins *ctsl* and *ctsd*) and it also resulted in the upregulation of molecular chaperones genes (*hsf1*, *hsp27*, *hsp70*, *hsp90* and *clu*), as well as of genes that are functionally involved in cellular antioxidant responses, namely *nrf2* and *keap1*, along with downstream transcriptional targets of Nrf2 (i.e. the *nqo1* and *txnrd1* genes). Notably, in most cases upregulation of the studied genes was dose-dependent. Prolonged exposure of IMR90 cells to Hexapeptide-11 for 96 h enhanced significantly the intensity [especially at the concentration of 5% (v/v)] of the studied genes upregulation (Supplementary Fig. S1), indicating the existence of, not only dose-dependent, but also time-dependent effects on gene expression patterns. To exclude the possibility of a cell type-specific effect we also studied the impact of Hexapeptide-11 in human skin fibroblasts (BJ cells). As shown in Supplementary Fig. S2, exposure of BJ cells to Hexapeptide-11 for 24 h also promoted the upregulation of PN related genes, namely proteasome, ALS-related, antioxidant responses and molecular chaperones genes.

*Hexapeptide-11 enhances Nrf2 nuclear accumulation; induces higher expression levels of proteasomal protein subunits and increases proteasome peptidase activities*

Considering the induction of transcriptional targets of Nrf2 (i.e. *nqo1* and *txnrd1*) after cell exposure to Hexapeptide-11, we then investigated the protein expression of Nrf2 along with its subcellular distribution in IMR90 cells exposed for 24 h to either 150 μM $H_2O_2$ or to (2%, 5% v/v) Hexapeptide-11. As shown in Fig. 3 $A_1$ Hexapeptide-11 (mainly at the 5% concentration) induced a mild induction in the expression of the Nrf2 protein, while immunofluorescence studies revealed that (as in the case of $H_2O_2$ treatment) Hexapeptide-11 enhanced the nuclear accumulation of Nrf2; this effect was more prominent in cells treated with 5% (v/v) Hexapeptide-11 (Fig. $3A_2$). Moreover, in support to our aforementioned gene expression analyses, cell treatment with Hexapeptide-11 for 24 h induced higher expression levels of the 20S α7 proteasomal subunit (Fig. 3B).

We then investigated the molecular effects that are induced in IMR90 cells after more prolonged exposures to Hexapeptide-11. As

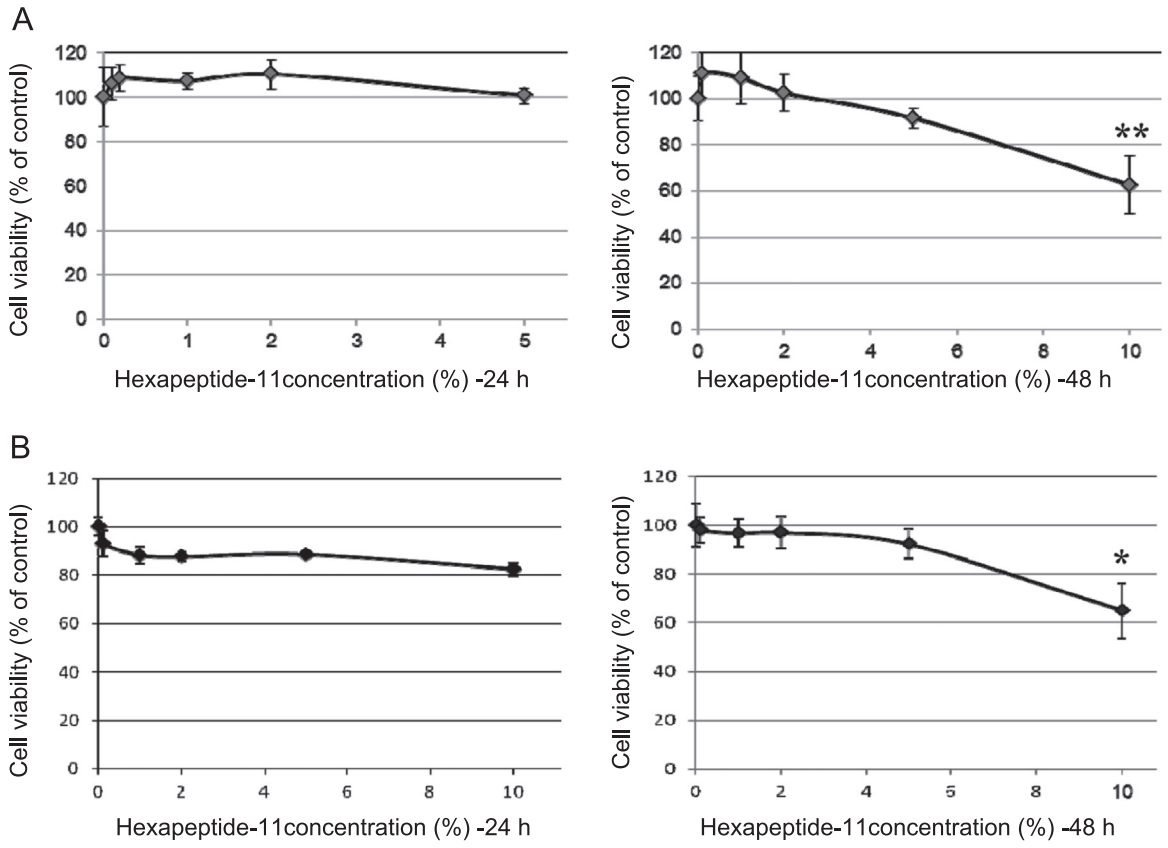


**Fig. 1.** Hexapeptide-11 does not exert any significant toxicity in normal human diploid fibroblasts. (A, B) Relative (%) survival (MTT assay) of proliferating human lung [IMR90, (A)] or skin [BJ, (B)] fibroblasts exposed to the indicated concentrations of Hexapeptide-11 for 24 (left panels) or 48 (right panels) h. The indicated % (v/v) concentration refers to percent by volume of the stock Hexapeptide-11 solution in the total volume of cell culture medium. Bars, ± SD. *, $P<0.05$; **, $P<0.01$ vs. controls set to 100%.

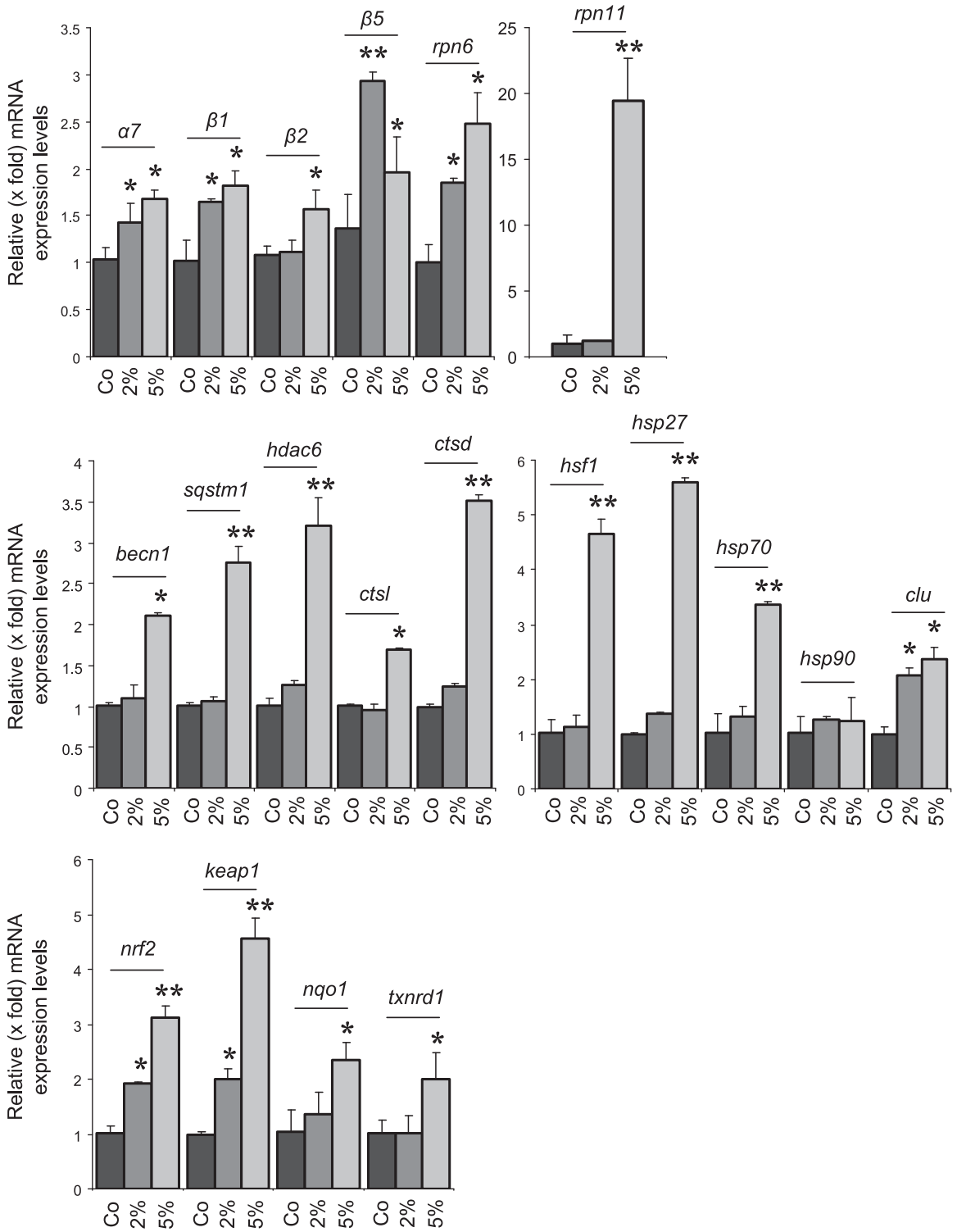


**Fig. 2.** Exposure of IMR90 cells to Hexapeptide-11 for 24 h results in the upregulation (mostly dose dependent) of genes involved in the proteostasis network regulation, as well as in cellular antioxidant responses. Shown Q-PCR gene expression analyses refer to 20S and 19S proteasome genes (*α7*, *β1*, *β2*, *β5*, *rpn6*, and *rpn11*); to genes involved in ALS functionality (*becn1*, *sqstm1*, *hdac6*, *ctsl* and *ctsd*) and in molecular chaperones (*hsf1*, *hsp27*, *hsp70*, *hsp90* and *clu*), as well as to genes involved in antioxidant responses (*nrf2*, *keap1*, *nqo1* and *txnrd1*). The beta-2-microglobulin (*b2m*) gene expression was used as normalizer; shown % v/v) concentration of Hexapeptide-11 is as in Fig. 1. Bars, ± SD. *, $P < 0.05$; **, $P < 0.01$ vs. controls set to 1.

shown in Fig. 4, treatment of cells with Hexapeptide-11 for 48 or 72 h induced higher protein expression levels of the 20S proteasome subunits β5 and α7, as well as of the antioxidant protein Nqo1 (Fig. 4$A_1$); it also promoted a dose- and time-dependent induction of (mainly) the LLVY/β5 proteasome peptidase activity (Fig. 4$A_2$). On the other hand, Hexapeptide-11 either suppressed (at 2%) or had no significant effect on the cathepsins B, L enzymatic activity (Fig. 4$B_1$); in support we noted no significant differences on the expression levels of the autophagy-related protein beclin-1 after cell exposure to 2% or to 5% (v/v) Hexapeptide-11 (Fig. 4$B_2$).

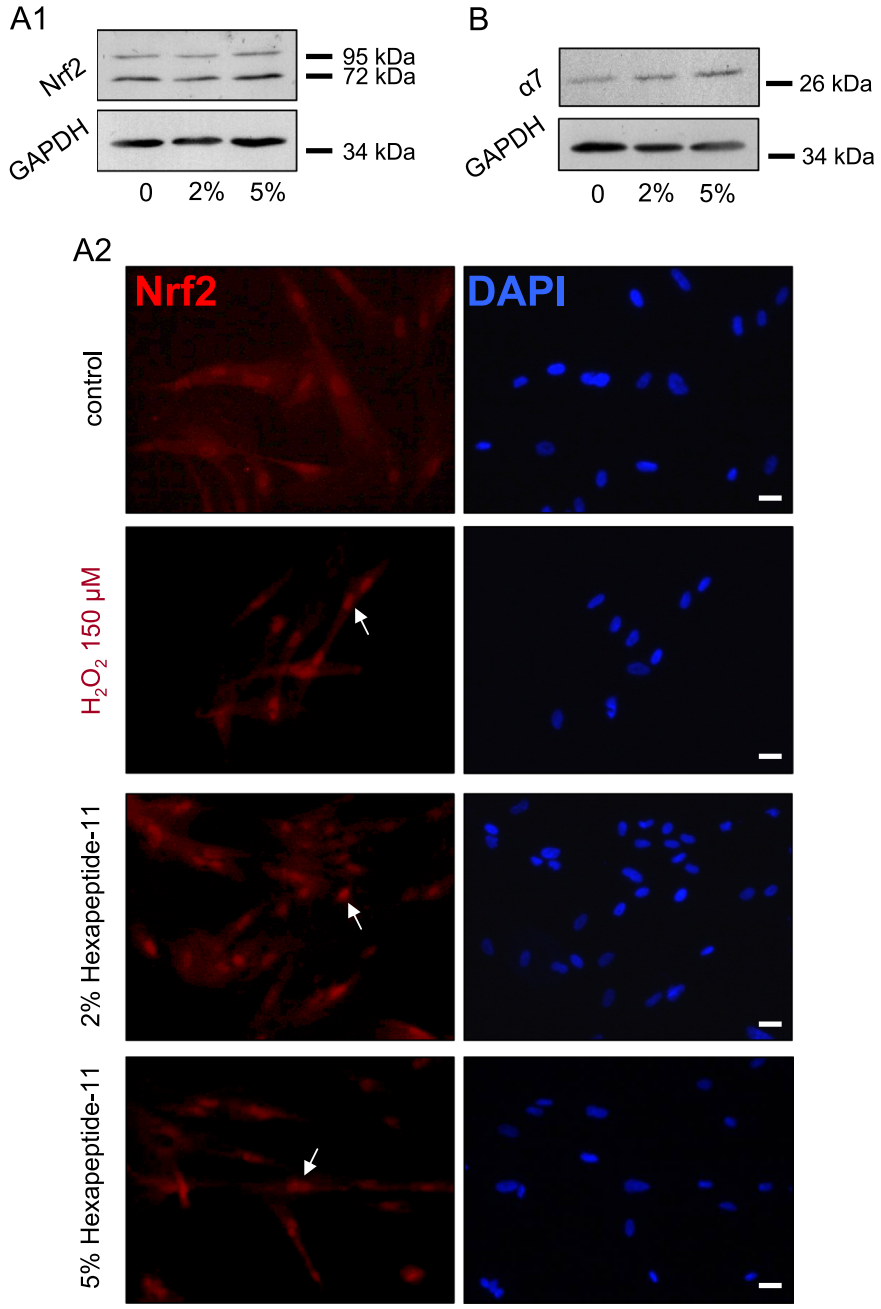


**Fig. 3.** Treatment of IMR90 cells with Hexapeptide-11 for 24 h enhances Nrf2 nuclear accumulation and upregulates the protein expression levels of proteasomal subunits ($A_1$). Representative immunoblot analyses of Nrf2 expression in cells treated with 2% or 5% (v/v) Hexapeptide-11 ($A_2$). Immunofluorescence images following Nrf2 localization in early passage IMR90 fibroblasts treated with the indicated concentrations of $H_2O_2$ or Hexapeptide-11; cells nuclei were counterstained with DAPI. Nrf2 is increasingly distributed in the nucleus (arrows) in $H_2O_2$ or Hexapeptide-11 treated cells. (B) Immunoblot analysis of the 20S proteasome subunit α7 expression after cell exposure to the indicated concentrations of Hexapeptide-11. GAPDH probing ($A_1$, B) was used as reference for total protein input. Molecular weight markers (in kDa) are indicated on the right of each blot; % (v/v) concentration of Hexapeptide-11 is as in Fig. 1. Bars, in ($A_2$) 10 μM.

*Hexapeptide-11 suppresses oxidative-stress-mediated premature senescence in normal human fibroblasts, and improves skin elasticity at in vivo skin deformation assays*

Given our previous findings, we then addressed the question whether Hexapeptide-11 could protect cells from oxidative stress mediated premature senescence. To this end, we triggered SIPS by treating (three exposures of 48 h each) young proliferating IMR90 cells with 300 μM $H_2O_2$; in parallel cells were treated with either 5% (v/v) Hexapeptide-11 or with 300 μM $H_2O_2$/5% (v/v) Hexapeptide-11. As shown in Fig. 5A, SA-β-GAL staining revealed that Hexapeptide-11 conferred significant protection against $H_2O_2$-mediated SIPS. Interestingly, via a currently unknown mechanism, it also suppressed the $H_2O_2$ and SIPS-related p53 and, its transcriptional target $p21^{CIP1}$ upregulation in IMR90 cells (Fig. 5B).

On the basis of these findings, and in order to explore the potential skin firming properties of Hexapeptide-11 we conducted an in vivo half-face study on 25 volunteers (see Materials and methods). As shown in Fig. 5C we observed that the application of a toner containing Hexapeptide-11 resulted in a significant improvement (vs. a control toner) in Ue (the initial elastic response of the skin) but not of Uf (total deformation response) after 4 weeks of treatment.

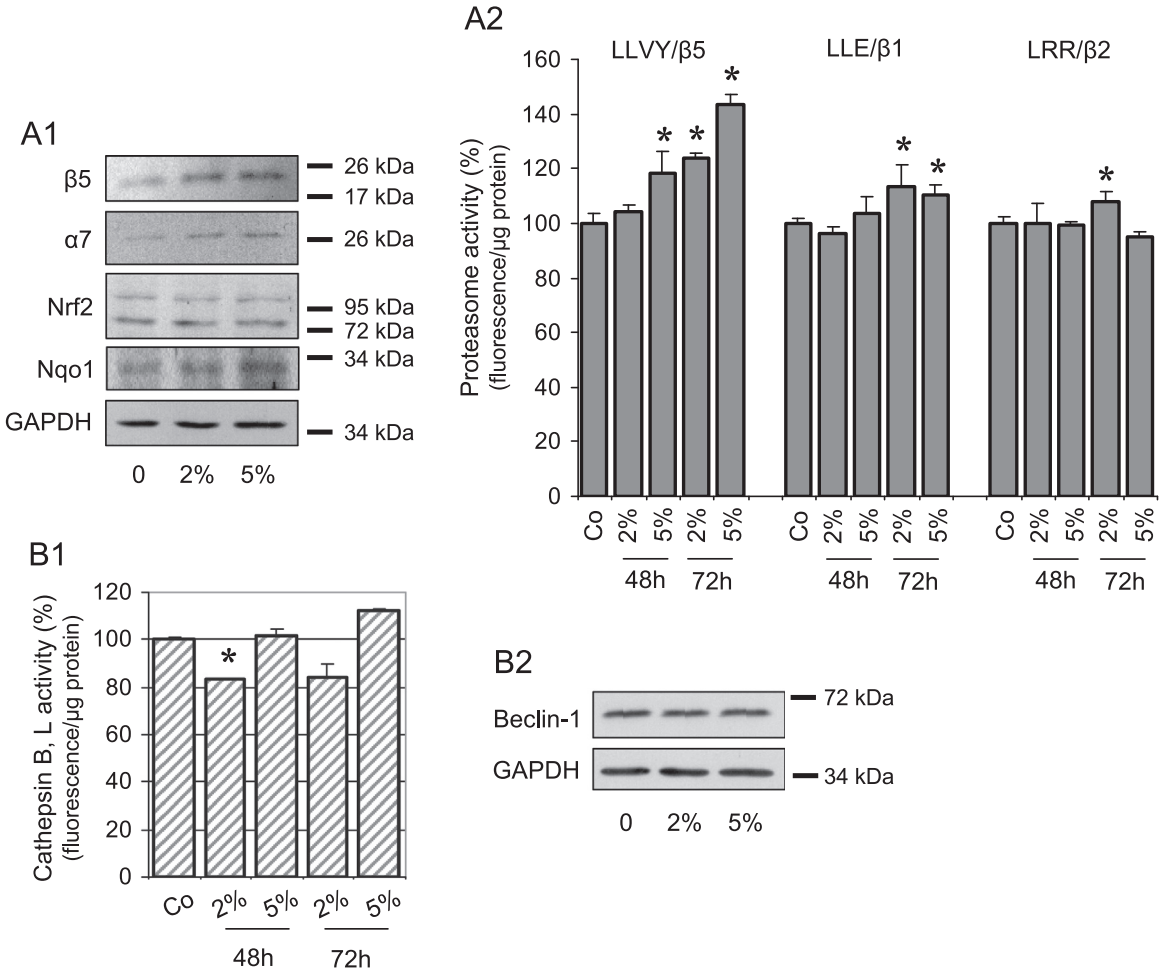


**Fig. 4.** Prolonged exposure of IMR90 fibroblasts to Hexapeptide-11 upregulates proteasome protein subunits and the antioxidant protein Nqo1 and induces the chymotrypsin-like (LLVY/β5) proteasome peptidase activity in a dose-dependent manner ($A_1$). Immunoblot analyses of the 20S proteasome protein subunits β5 and α7, as well as of Nrf2 and Nqo1 in IMR90 cells treated for 48 h with the indicated concentrations of Hexapeptide-11 ($A_2$). Enzymatic activities of the three (LLVY/β5, LLE/β1 and LRR/β2) proteasome peptidases activities in early passage IMR90 cells treated for 48 and 72 h with 2% or 5% (v/v) Hexapeptide-11 ($B_1$). Relative (%) cathepsin B, L activities in sample preparations from IMR90 exposed to 2% or 5% (v/v) Hexapeptide-11 for 48 and 72 h ($B_2$) immunoblot analyses of the autophagy related protein Beclin-1 in IMR90 cells treated for 48 h with 2% or 5% (v/v) Hexapeptide-11. Proteasome and cathepsin activities were expressed in fluorescence units per μg of input protein vs. controls set to 100%. GAPDH probing in ($A_1$, $B_2$) was used as reference for total protein input. Molecular weight markers (in kDa) are indicated on the right of each blot; shown % (v/v) concentration of Hexapeptide-11 is as in Fig. 1. Bars, ± SD ($n=2$). *$P < 0.05$ vs. controls set to 100%.

*Hexapeptide-11 activates extracellular MMPs and suppresses the rate of human fibroblasts migration*

Given the fact that Hexapeptide-11 has been found to influence a number of genes that functionally relate to extracellular matrix function (our unpublished findings), we then investigated the effect of this peptide on the enzymatic activity of the gelatinases MMP-2 and MMP-9. To this end, IMR90 cells were treated with Hexapeptide-11 and after 24-h the conditioned media were subjected to gelatin zymography. As it can be seen in Fig. 6A, cell treatment with Hexapeptide-11 promoted a dose-dependent increase in the enzymatic activity of both the MMP-2 and MMP-9 gelatinases.

Given the fact that this finding indicated that Hexapeptide-11 could exert wound healing effects or even affect the migratory activity of the IMR90 cells, we also performed an in vitro scratch assay in order to investigate the impact of Hexapeptide-11 on IMR90 migration. As it is shown in Fig. 6B we found that treatment of IMR90 cells with Hexapeptide-11 rather decreased the IMR90 cells migratory activity.

## Discussion

Despite the cell-type specific differences that characterize the manifestation of cellular senescence in mitotic and post-mitotic human cell lineages, it is evident that, independently of the cell type, the appearance of cellular senescence is marked by the accumulation of damaged/dysfunctional biomolecules [28–30]. In support, the rate of damaged biomolecules accumulation has been found to increase in aged animal tissues and to correlate with chronological ageing [15,25,31–33]. We report herein that a hexapeptide of structure Phe–Val–Ala–Pro–Phe–Pro (originally isolated from yeast) could promote the generalized activation of genes involved in the PN regulation, namely antioxidant responses, UPS, ALS and molecular chaperones. Moreover, it enhanced the expression of proteasomal protein subunits and also of proteasome peptidase activities (but not of lysosomal enzymes; e.g. cathepsins). Loss of proteostasis is a common feature of ageing and of age-related diseases (e.g. neurodegeneration or cancer) and is characterized by the appearance of non-native proteins or protein aggregates in various tissues. Likely, this relates to the fact that, independently of the triggering event, senescent cells are

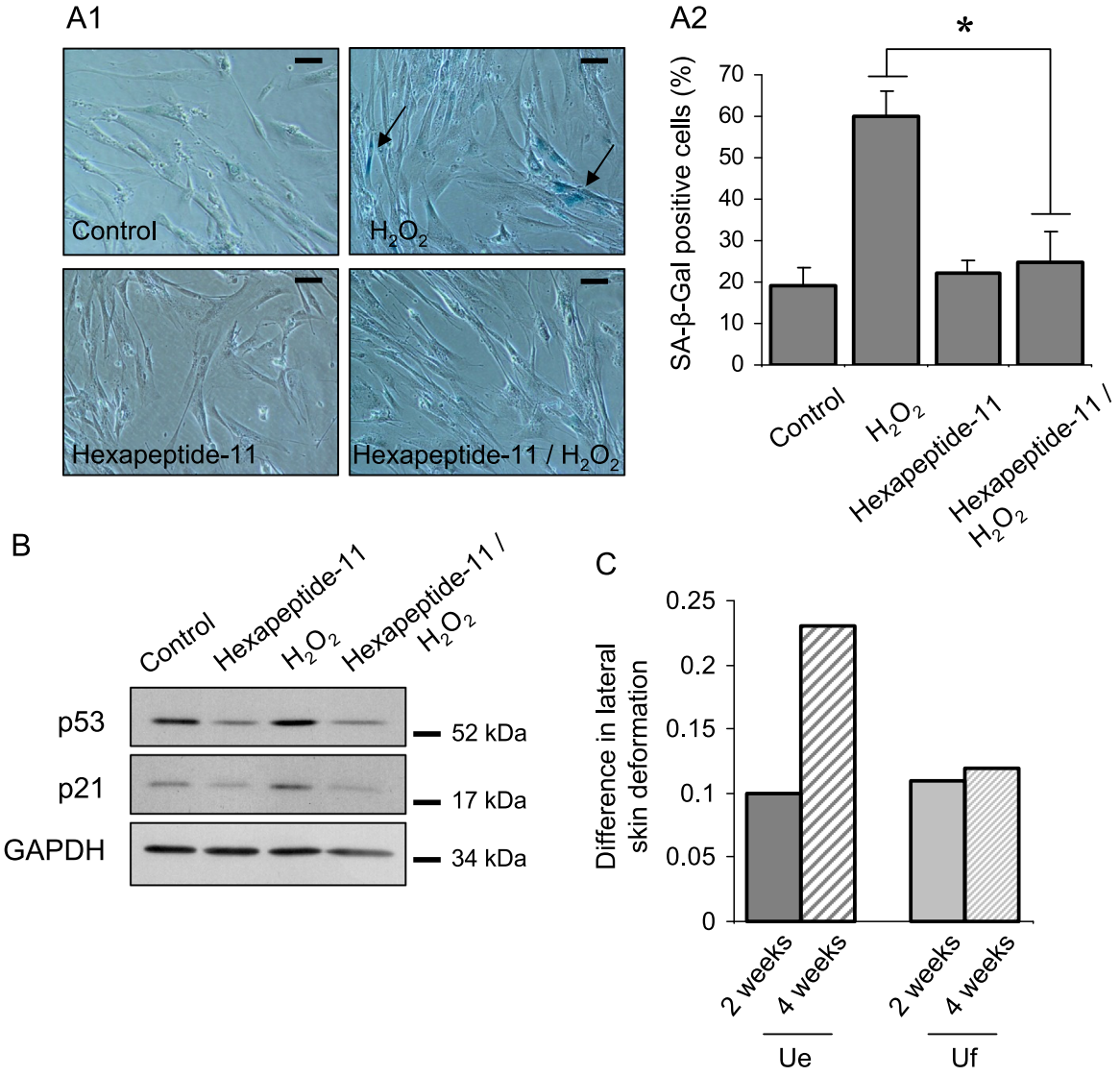


**Fig. 5.** Hexapeptide-11 protects IMR90 fibroblasts from oxidative stress mediated premature senescence, while at in vivo skin deformation assays in human subjects it improves skin elasticity ($A_1$). Representative light field images following SA-β-Gal staining of control IMR90 cells or cells treated (three exposures of 48 h each) with 300 μM $H_2O_2$; 5% (v/v) Hexapeptide-11 or 300 μM $H_2O_2$/5% (v/v) Hexapeptide-11 ($A_2$). Relative (%) number of SA-β-Gal positive cells (mean of 10 optical fields) following treatment of cells exactly as in ($A_1$). (B) Immunoblot analyses of p53 and $p21^{CIP1}$ expression levels in cells treated as in (A); Hexapeptide-11 can inhibit the $H_2O_2$-mediated induction of p53 and its transcriptional target $p21^{CIP1}$. (C) Difference in lateral skin deformation (Ue, Uf parameters) after conducting an in vivo study on the eyes and cheeks of 25 volunteers for 4 weeks. The study was performed by using a preparation containing 2.8% (v/v) of Hexapeptide-11 (vs. control); a significant improvement was found in Ue (initial elastic response of the skin) but not in Uf (total deformation response) after 4 weeks of treatment. GAPDH probing (B) was used as reference for total protein input. Shown % (v/v) concentration of Hexapeptide-11 is as in Fig. 1; molecular weight markers (in kDa) are indicated on the right of each blot. Bars, ±SD ($n=2$). *$P<0.05$ vs. controls set to 100%. Bars, in ($A_1$) 10 μM.

characterized by accumulation of oxidative stress, reduced proteasome activity and high rates of genome/proteome instability [3,11,12]; this was also evident in a recent comparative meta-analysis of ours in human transcriptomics data during cellular senescence and in vivo tissue ageing [34]. In line with these findings it has been proposed that enhanced activity of the PN could suppress the rate of ageing progression indicating that, the PN functional modules may represent potential therapeutic targets in the delay of ageing and/or the prevention of age-related pathologies [3,6,7,35]. In support, we have observed that Hexapeptide-11 could protect normal human diploid fibroblasts from oxidants-mediated SIPS; the beneficial effects of this peptide were also evident in the studies in human volunteers showing an improvement in the elastic response of the more superficial layers of the skin. Modulation of the PN is a rather novel property of peptides from yeast cell derivatives, since (in the limited existing studies) yeast peptides have been found to (mainly) stimulate wound healing [16,18]; it was also reported that peptides are active in nanomolar amounts and are ~600 times more active than the initial extract [17]. On the other hand, a number of natural compounds (isolated from various sources including marine organisms, microorganisms or plants) have been found to activate the PN modules and exert anti-ageing effects both in vitro and in vivo [1]. For instance, Rapamycin was (among others) found to enhance longevity of *Caenorhabditis elegans* in an SKN-1/Nrf2 dependent fashion [36], while oral administration of rapamycin in adult *Drosophila* flies resulted in lifespan extension that was associated with increased resistance to both starvation and oxidative stress. Moreover, Quercetin enhanced proteasome activity, conferred resistance to oxidative stress and extended the replicative lifespan of human fibroblasts [37], while Icariside II when tested in *C. elegans* increased thermo and oxidative stress tolerance, decreased the rate of locomotion decline in late adulthood and extended worms' lifespan [38]; it was postulated that the

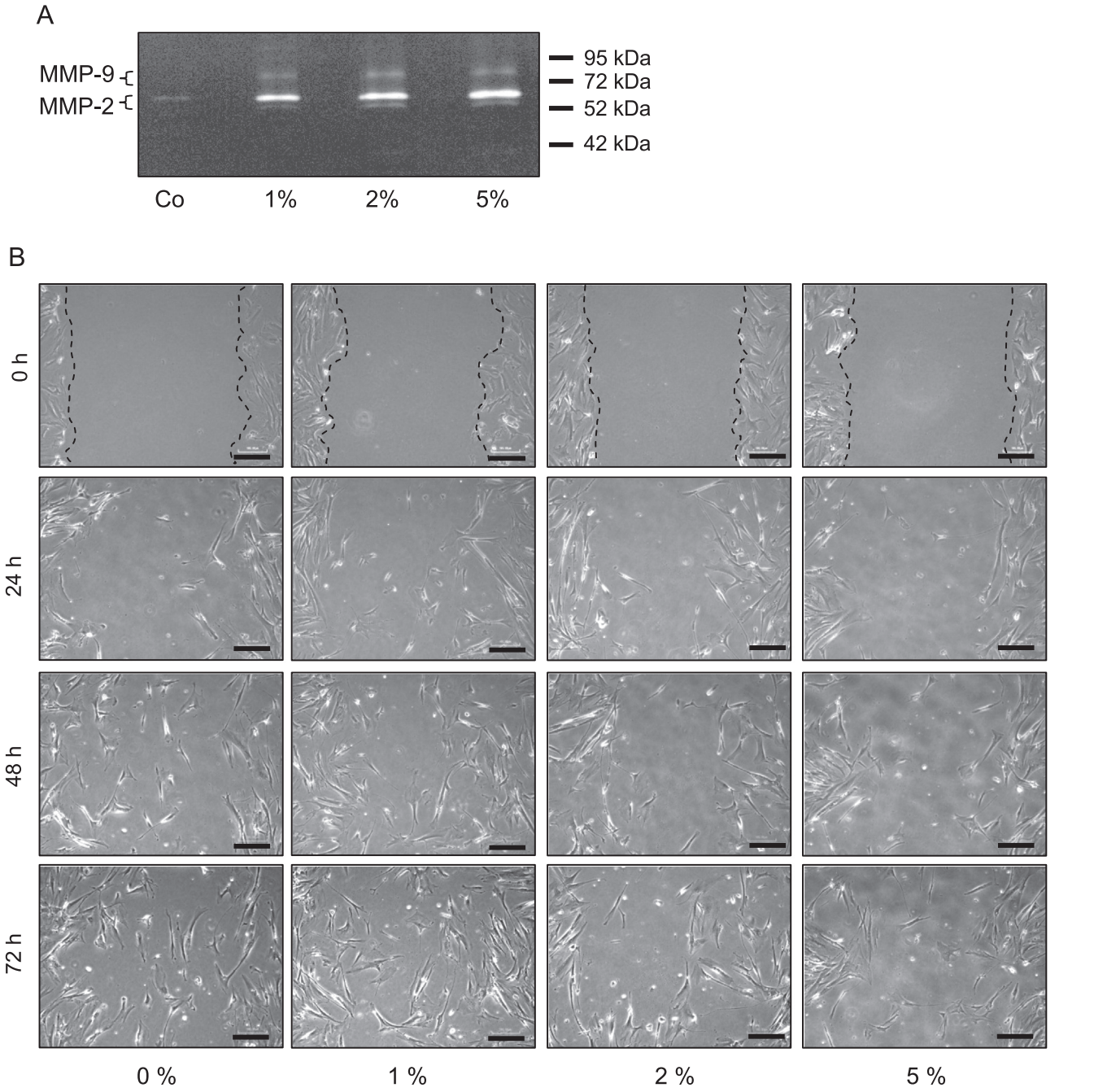


**Fig. 6.** Treatment of IMR90 cells with Hexapeptide-11 activates the MMPs −2 and −9 and it also suppresses cellular migratory activity. (A) Assessment (by gelatin zymography) of the gelatinases MMP-2 and MMP-9 enzymatic activity following cell treatment with 1%, 2% or 5% (v/v) Hexapeptide-11 for 24 h. (B) Representative phase contrast images showing cellular migratory activity 24–72 h after a scratch assay in confluent IMR90 cells; cells were treated with 1%, 2% or 5% (v/v) Hexapeptide-11 as indicated. Shown % (v/v) concentration of Hexapeptide-11 is as in Fig. 1; bars, in (B) 100 μM.

lifespan extension caused by icariside II was dependent (among others) on the hsf1 signaling pathway. Finally, Oleuropein, the major constituent of *Olea europea* leaf extract, when studied for its cell protective effects in human fibroblasts was found to suppress oxidative stress, reduce protein oxidation, activate the proteasome and delay the appearance of cellular senescence [39].

Intriguingly, we found that Hexapeptide-11 activated the MMP-2/MMP-9 metalloproteinases and suppressed fibroblasts migration. In previous studies increased secretion of tissue inhibitors of metalloproteinases 1 and 2 (TIMPs −1 and −2) in fibroblasts were found to represent early indicators of oral sub-mucous fibrosis and ageing [40]; this finding indicated that MMPs activation may represent an anti-ageing adaptation. In support, low-level laser improved tendon healing via an increase of MMPs activity and collagen synthesis [41], while resveratrol induced MMP-9 and cell migration via p38 kinase and PI−3K pathways in human fibrosarcoma cells [42]. On the other hand, it has been found that a subfragment of the carboxyl-terminal propeptide of type I collagen dramatically augmented extracellular matrix production in subconfluent fibroblasts [43], while the pentapeptide Lys–Thr–Thr–Lys–Ser is the minimum sequence necessary for potent stimulation of collagen and fibronectin production in a variety of mesenchymal cells [19]. Overall, MMPs activation by Hexapeptide-11 may indicate wound healing properties which are, however, contradicted by the inhibitory effect that this peptide exerted on cells migratory activity.

Mechanistically, the mode of Hexapeptide-11 action remains elusive. Our finding that it suppressed the $H_2O_2$-mediated p53 and p21 upregulation corroborates a recent study showing that Hexapeptide-11 downregulated ataxia telangiectasia mutated (ATM) and p53 protein expression in human dermal fibroblasts [23]. Interestingly, BLAST alignment analyses (in order to match this peptide sequence against the entire proteome dataset) revealed that the unique sequence of amino acids that comprise Hexapeptide-11 appear in a number of proteins of *S. cerevisiae* including a major module of the PN, namely hsp70. Thus, this peptide may, at least in part, exert its effects by acting as a pseudo-signal of chaperones activation which then triggers a cascade of a more generalized activation (in an alarm state) of additional PN modules. Alternatively, Hexapeptide-11 may function as a "low-dose stressor" that pre-condition cells to resist more severe stress (i.e. by exerting a hormetic effect) [44].

In summary, and despite a number of critical questions in relation to the mechanistic aspects of Hexapeptide-11 action (that certainly need further extensive investigations) this peptide represents a molecule which, likewise a novel hsf1 activator [45], can activate the cellular PN machineries in a dose- and time-dependent fashion. Thus, Hexapeptide-11 is a promising anti-ageing agent for further testing which may play a role in improving human skin health and integrity.

## Conflict of interest

ADS, IP, ALS and IPT declare no conflict of interest; MD is currently an employ of Lonza Personal Care, USA and MR an employ of Korres S.A. Natural Products, Greece.

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## Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.04.010.

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