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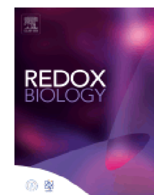

Research Paper

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

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