Regulation of Sirtuin-dependent skin cell Senescence by dermatology-associated compounds

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Doctoral Thesis

Regulation of Sirtuin-dependent skin cell Senescence by dermatology-associated compounds

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"*Success is stumbling from failure to failure with no loss of enthusiasm*"
Winston S. Churchill

Se podría decir que esta frase me ha acompañado en varios momentos durante el desarrollo de mi tesis. Momentos en los que ves que tus experimentos no van bien y todo es fracaso tras fracaso. Pero como dijo el gran Churchill, el éxito se consigue a través de no perder el entusiasmo, la esperanza, la creencia de que a pesar de los fracasos al final consigues llegar a tu meta del tan ansiado éxito. En mi caso ese éxito es el poder haber conseguido llevar a cabo una tesis doctoral, y no solo finalizarla de cualquier manera, sino finalizarla obteniendo unos buenos resultados a nivel científico que serán aprovechados por la compañía en la que trabajo para mejorar sus productos.
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ABBREVIATIONS

Ac Acetylation
AMPK 5' adenosine monophosphate-activated protein kinase
APS Ammonium persulfate
β-Gal β-Galactosidase
BSA Bovine serum albumin
DSB Double strand breaks
DAPI 4',6-diamidino-2-phenylindole
ddH_2O Distilled water
DMEM Dulbecco's modified eagle medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DTT Dithiothreitol
EDTA Ethylenediaminetetracetic acid
EdU 5-ethynyl-2'-deoxyuridine
FACS Fluorescence-activated cell sorting
FBS Fetal bovine serum
FITC Fluorescein
γH2AX Histone H2AX variant phosphorylated at serine 139
H3K9 Lysine 9 in histone 3
H4K16 Lysine 16 in histone 4
HAS2 Hyaluronan synthase 2
HAT Histone acetyltransferase
HDAC Histone deacetylase
HDF Human dermal fibroblasts
HEK293 Human embryonic kidney 293 cells
IF Immunofluorescence
IL Interleuquin
KO knockout
MMP Matrix metalloproteinase
mRNA Messenger RNA
NAD^+ Nicotinamide adenine dinucleotide
PBS Phosphate buffered saline
PI Propidium iodide
PMSF Phenylmethylsulfonyl fluoride
PTM Post-translational modification
qPCR Quantitative polymerase chain reaction
RNA Ribonucleic acid
ROS Reactive oxygen species
SASP Senescence associated secretrory phenotype
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT Silent mating type information regulation 2 homologs
SOD Superoxide dismutase
SRB Sulforhodamine B
TCA Trichloroacetic acid
TEMED Tetramethylethylenediamine
UVA Ultraviolet type A
UVB Ultraviolet type B
WB Western Blot

INTRODUCTION

1. Epigenetic mechanisms

The term epigenetics was first introduced in 1942 by embryologist Conrad Waddington as the complex processes that take place between genotype and phenotype (Waddington CH 1942). After intensive research and characterization of these processes, we can now define epigenetics as the study of the biochemical processes that regulate the expression of genes without altering its sequence (Deichmann U et al. 2016). Thus, epigenetics study how environmental stimuli can regulate gene expression through specific modifications, which induces a change in phenotype. These modifications are inherited by daughter cells and last for multiple generations. The main mechanisms through which epigenetics modulates gene expression are DNA methylation, histone modification and non-coding RNA.

1.1 DNA methylation

DNA methylation involves the enzymatic addition of a methyl group onto the cytosine ring of DNA. This methylation occurs predominantly in CG dinucleotides (Meng H et al. 2015), and DNA regions that contain a high number of CG dinucleotides are known as CpG islands. These CpG islands play a key role in gene expression regulation as about 70% of gene promoters contain a CpG island (Deaton AM et al. 2011). DNA methylation is also essential during early development and genomic imprinting, where dynamic changes in the methylation pattern occur (Paulsen M et al. 2001, Zeng Y et al. 2019).

DNA methylation/demethylation balance is carried out by DNA methyltransferases (DNMT), which add methyl groups to DNA, and DNA demethylases, which remove DNA methyl groups (Allis CD et al. 2016). Regarding the DNMTs family, the key members involved in DNA methylation regulation are DNMT1, DNMT3A and DNMT3B. DNMT1 plays a key role in maintenance of methylated DNA, having preference for partially methylated DNA, while DNMT3A and DNMT3B regulate de novo DNA methylation (Okano M et al. 1999, Hermann A et al. 2004). DNA demethylation is mainly catalyzed by ten-eleven translocation methylcytosinedioxygenase (TET) family of proteins and takes place in a multi-step reaction, where 5-methylcytosine (5-mC) is converted to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) successively before ending in unmethylated cytosine (Wu H et al. 2014).

In response to environmental factors, the DNA methylation patterns change and alter the expression of genes. While methylation of gene promoters normally represses transcription, DNA methylation occurring in gene bodies is mainly linked to gene activation (Figure 1) (Holliday R et al. 1975, Wolf SF et al. 1984). Despite this inheritability, DNA methylation can be dynamically modified (Wu H et al. 2014). Aberrant DNA hypermethylation and hypomethylation have been associated with a wide range of human diseases. Hypermethylation typically occurs at CpG islands promoter regions and induces the transcription repression of tumor suppressor genes, thus favoring tumorigenesis and cancer development. On the other hand, global hypomethylation typically occurs both in cancer and during aging, and it induces the expression of genes

that would be silenced in normal conditions, including retrotransposons and repetitive sequences, which in turn increase genome instability and contribute to the development of age-related diseases (Wilson AS et al. 2007, Jin Z et al. 2018).

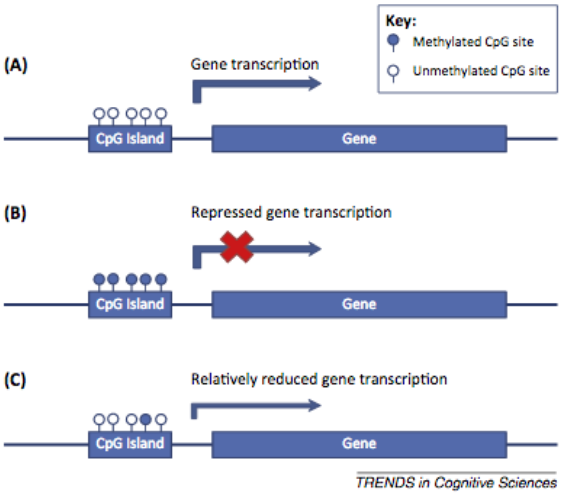


Figure 1. DNA methylation in the CpG islands controls gene expression. High methylation levels in CpG islands of promoter regions are normally associated with repressed gene expression, while low methylation levels are associated with upregulated gene expression (Nikolova YS et al. 2015).

1.2 Histone modifications

Histones are proteins that bind DNA to form a compacted structure called nucleosome (Cheung P et al. 2000). Nucleosome units are formed by a histone octamer core, which contains one H3-H4 tetramer and two H2A-H2B dimers, wrapped around approximately 146 DNA base pairs. These nucleosomes build up together to form chromatin (Figure 2) (Kornberg RD. 1974). Thus, we refer to chromatin as the dynamic structure that packs DNA along with histones playing essential roles in many biological processes including cell division, DNA replication and gene expression regulation.

Histones can be subject of post-translational modifications (PTM) throughout their sequence, but mainly histone N-terminal regions (also known as histone tails) are highly modified. Some of these modifications -mainly acetylation- can directly regulate chromatin state, making chromatin to be more open – relaxed (less binding between histones and DNA and thus increased gene expression) or more closed – condensed (more binding between histones and DNA and thus decreased gene expression) (Bannister AJ et al. 2011). These PTMs have been associated with a wide range of biological functions, including formation of heterochromatin, transcriptional activation/repression and DNA repair, and thus its involvement in many diseases is extensively studied (Shahbazian MD et al. 2007). We can classify the proteins involved in PTMs regulation as writers, readers and erasers. Writers are the proteins that add these PTMs to histones, readers are the proteins that recognize these PTMs and recruit other proteins involved in the previously mentioned biological functions, and erasers are the proteins that remove these PTMs (Biswas S et al. 2018). Regarding the type of modifications, we can find histone acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation.

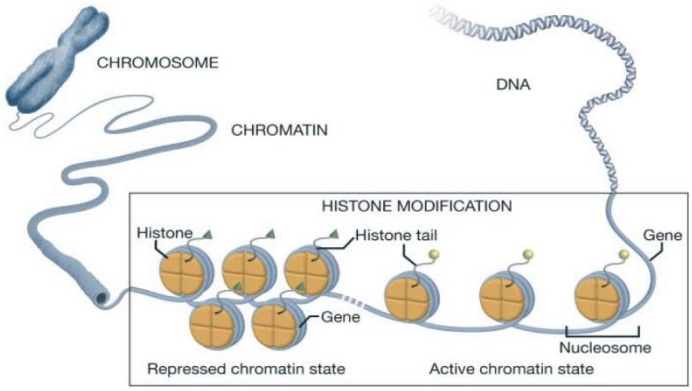


Figure 2. Chromatin structure including DNA wrapped around histone octamers. Post-translational modifications at histone tails can influence chromatin state by increasing or decreasing histone binding to DNA and thus regulate gene expression (Pieterman CR et al. 2014).

1.2.1 Histone acetylation

The addition of an acetyl group to histones is one of the most studied histone modifications, along with histone methylation and phosphorylation. The binding of histones to DNA occurs through the positively charged lysine from the histone with the negatively charged phosphate group in DNA. The acetyl group in the histone lysine removes the positive charge and thus prevents the binding of histones and DNA, producing a more relaxed chromatin state and therefore an increased gene expression (Bannister AJ et al. 2011). Some examples of histone acetylation include acetylation of lysine 5 in histone H2A (H2AK5ac), acetylation of lysine 9, 14, 18, 27 or 36 in histone 3 (H3K9ac, H3K14ac, H3K18ac, H3K27ac and H3K36ac) and acetylation of lysine 5, 8, 12 or 16 in histone 4 (H4K5ac, H4K8ac, H4K12ac and H4K16ac). Histone acetylation is regulated by enzymes that add acetyl groups to histones, named histone acetyl transferases (HATs), and by enzymes that remove acetyl groups from histones, named histone deacetylases (HDACs) (Yang XJ et al. 2007). Thus, HATs induce gene expression while HDACs repress it, but they also play key roles in DNA repair, mitosis, metabolism, apoptosis, cytoskeleton and chaperone regulation, among others (Yang XJ et al. 2007). HATs can be grouped in two major classes, type-A or nuclear and type-B or cytoplasmic, and nuclear HATs can be subdivided in three different families depending on their amino acid sequence homology and structure: Gcn5/PCAF, MYST and p300/CBP (Hodawadekar SC et al. 2007). On the other hand, there are 18 HDACs in humans, and are divided in four different families, named class I-IV. Class I, II and IV are numbered according to their chronological order of discovery (HDAC1 to HDAC11) and are Zn-dependent enzymes (Seto E et al. 2014). Class III HDACs, also known as Sirtuins (SIRT1 to SIRT7), differ from the rest of the family in the fact that they are nicotinamide adenine dinucleotide (NAD+)-dependent enzymes and will be introduced deeply in the next section.

1.2.2 Histone methylation

Histone methylation occurs both in arginine and lysine residues. The methylation of arginine and lysine is performed by different enzymes and regulates an array of different functions.

Histone lysine can be mono-, di- and tri-methylated. This type of methylation occurs mainly in H3 and H4, specifically in the residues H3K4, H3K9, H3K27 and H4K20, among others (Bannister AJ et al. 2011). The most described histone methyltransferases that add methyl groups to these lysines are SUV29H1, G9A, SET8/PR-Set and EZH2, while histone lysine demethylation is performed by LSD1. Histone methylation at the lysine residues has important functions in a wide range of processes including heterochromatin formation, X-chromosome inactivation, transcriptional regulation, cell cycle control and DNA repair, among others (Martin C. 2005).

On the other hand, histone arginines can be mono- and di-methylated, and this modification occurs mainly in H2A, H3 and H4, specifically in the residues H2AR3, H3R2, H3R8, H4R3. The enzymes that catalyze histone arginine methylation are englobed in the protein arginine methylatransferase (PRMT) family, which contains 11 members in humans (named PRMT1-11), while arginine demethylation is carried out by JMJD6 and PAD4 (Zhang J et al. 2019). Histone arginine methylation plays essential roles in signal transduction, gene transcription, translation, RNA processing, cellular proliferation and mRNA splicing, among others (Bedford MT et al. 2009).

1.2.3 Histone phosphorylation

This type of modification occurs in serine, threonine and tyrosine residues of histones and is mainly associated to proliferative genes activation and chromatin condensation during mitosis (Rossetto D et al. 2012). Some examples of histone phosphorylated residues include H2AS1, H2BS36, H3S10, H3S28. Enzymes that phosphorylate histones include Aurora-B kinase and MSK1, while some examples of histone phosphatases include PP2A and PP4 (Hendzel MJ et al. 1997, Soloaga A et al. 2003, Gil RS et al. 2019). Interestingly, a crosstalk relationship has been described for phosphorylation and acetylation of histones. For example, phosphorylation of H3S10 is linked to acetylation of H3K9 and H3K14 in epidermal growth factor (EGF) stimulated cells (Cheung P et al (b). 2000, Lo WS et al. 2000).

1.2.4 Histone ubiquitination

Histone ubiquitination was first discovered in histones H2A and H2B (Goldknopf IL et al. 1975, West MH et al. 1980). H2A is monoubiquitinated in lysine 119 and enriched in the satellite regions, while H2B is monoubiquitinated in lysine 120 and is associated to transcriptional active genes (Minsky N et al. 2008, Zhu Q et al. 2011). Core histones H3 and H4 can also be ubiquitinated (Wang H et al. 2006, Nishiyama A et al. 2013, Zhang X et al. 2017). Examples of histone ubiquitin ligases, which add ubiquitin groups to histones, include RING1B and 2A-HUB, while histone deubiquitinating enzymes (DUBs) include USP-16 and 2A-DUB (Cao J et al. 2012).

1.2.5 Histone sumoylation

Histone sumoylation shows similarities with ubiquitination, as it is carried out by ubiquitin-like proteins and follows an enzymatic cascade similar to that of ubiquitination enzymes (Iñiguez-Lluhí JA 2006). The first paper on this field described how histone H4 sumoylation induced transcription repression (Shiio Y et al. 2003). Sumoylation occurs on lysine residues and to date there are four different sumoylation enzymes described, named small ubiquitin-related modifiers SUMO-1, SUMO-2, SUMO-3 and SUMO-4 (Cubeñas-Potts C et al. 2013). On the other hand, desumoylation reactions are performed by the family of sentrin-specific proteases (SENP) (Wang Y et al. 2009). Interestingly, sumoylation can compete with other PTMS such as acetylation or ubiquitination, thereby switching transcription activation to inhibition (Iñiguez-Lluhí JA 2006).

1.2.6 Histone ADP-ribosylation

Histone ADP-ribosylation has been less studied than the other modifications, but recently significant advances have increased considerably our understanding about this modification. All histones, including the linker histone H1, can be mono- and/or poly-ADP ribosylated in glutamate and arginine residues (Bannister AJ et al. 2011). The enzymes that catalyze this modification are known as ADP-ribosyltransferases (ARTs), from which the PARP subclass is the main responsible for ADP-ribosylation in histones. The PARP family (also known as ARTD family) is formed by 18 members, named PARP1-18. On the other hand, the enzymes that remove ADP-ribose groups from histones are divided in the ADP-ribosylhydrolases (ARHs) class and the PAR glycolhydrolases (PARGs) class (Messner S and Hottiger MO. 2011). Histone ADP-ribosylation correlates with a relaxed chromatin state, and it is involved in DNA damage response and repair, transcription, cell cycle and replication (Martinez-Zamudio R and Ha HC. 2012, Hou WH et al. 2019).

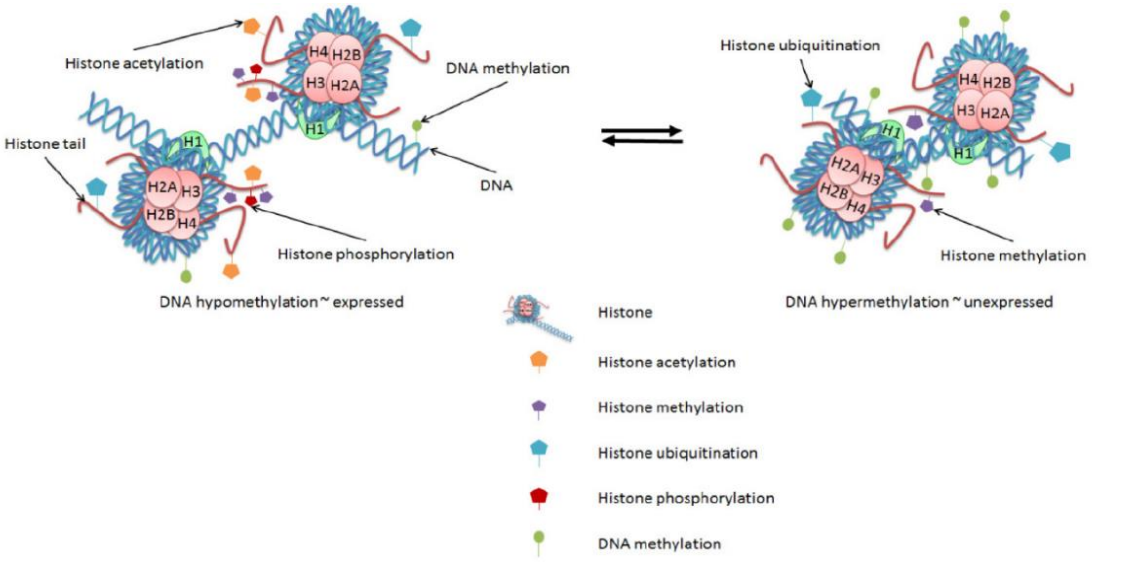


Figure 3. Many of the histone modifications that can influence chromatin structure. These modifications occur mainly at histone tails, modulating chromatin accessibility and thus the expression of genes (Pennington KL et al. 2016).

1.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) are a group of RNA that do not encode for functional proteins and target mRNAs to modulate its transcription and translation, thus regulating gene expression.

ncRNAs can be divided into short chain non-coding RNAs, which include short interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNA (piRNAs), and long non-coding RNAs (lncRNAs).

1.3.1 siRNAs

siRNAs are RNA fragments of 19-24 nucleotides (nt) that arise from double-stranded RNAs (dsRNAs) and are cut enzymatically by Dicer (Carthew RW et al. 2009). These dsRNAs are found in virus replication, transposons, hairpin RNAs or gene duplexes, among others. siRNAs are processed by Dicer and RISC complexes, and once processed, they bind specific mRNAs to promote its degradation, thereby blocking the expression of that specific gene. Besides, siRNAs are specific for a single target mRNA and are fully complimentary to this target (Lam JK et al. 2015).

1.3.2 miRNAs

miRNAs are single-stranded RNAs of 19-24 nt. Unlike siRNAs, which have an exogenous origin, miRNAs are always formed endogenously. Besides, while siRNA is specific for a single target mRNA, miRNA can have multiple targets and can regulate multiple genes, which also means that miRNAs can be partially complimentary to perform its function (Ahmadzada T et al. 2018). However, siRNAs and miRNAs have similar intracellular processing through the Dicer and RISC complexes (Carthew RW et al. 2009). miRNAs also interfere with other epigenetic mechanisms. For example, miRNAs can regulate DNA methylation, as miR-29 family targets DNMTs, or histone acetylation, as miR-34a targets SIRT1 (Fabbri M et al. 2007, Yamakuchi M. 2012).

1.3.3 piRNAs

piRNAs are RNA molecules of 26-31 nt, and its name refers to their ability to bind Piwi proteins (Lin H. 2007). piRNAs are formed from single stranded precursors, meaning that Dicer is not involved in their processing, and they are mainly involved in transposon repression and DNA methylation (Wei JW et al. 2017). Moreover, piRNAs are highly enriched in the germline tissues and regulate fertility in many animal species (Girard A et al 2006, Carmell MA et al. 2007, Houwing S et al. 2007, Das PP et al. 2008).

1.3.4 lncRNAs

lncRNAs are RNA molecules of more than 200 nt. Because of this broad definition, lncRNAs are heterogeneous in their origin, abundance, stability and localization (Ayupe

AC et al. 2015). LncRNAs can arise from different parts of the genome, including exonic, intergenic or non-protein-coding regions of the genome (Dhanoa JK et al. 2018). The first studies on this type of ncRNAs revealed its involvement in genomic imprinting and X chromosome inactivation (Yank PK et al. 2007). Later studies have described new roles of lncRNAs including chromatin remodeling, transcription, post-transcriptional processing and intracellular trafficking (Cao J. 2014). lncRNAs exert their functions through different mechanisms, including mRNA degradation, translation inhibition, recruitment of chromatin modifiers or regulation of protein activity (Akhade VS et al. 2017). For example, lncRNAs can directly interact with histone modifiers to modulate chromatin state and gene expression (Davidovich C et al. 2015).

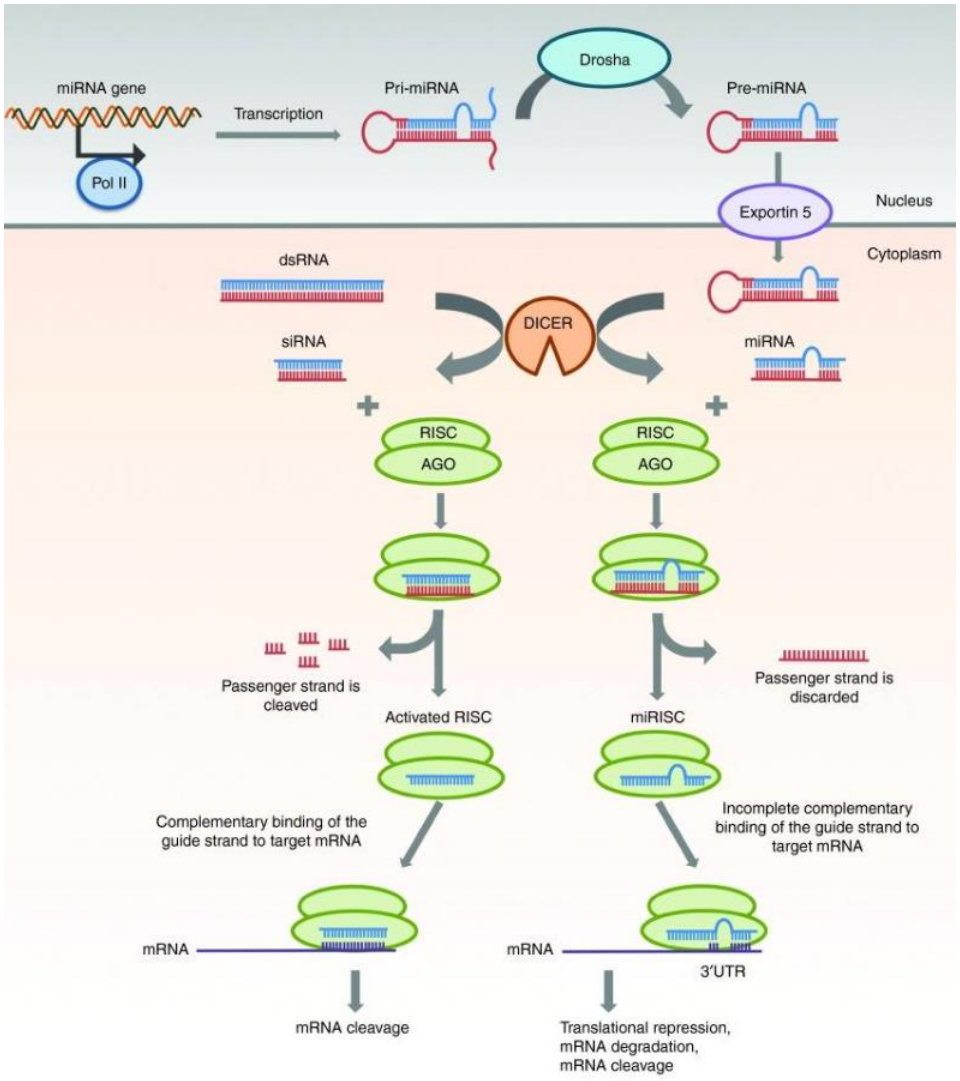


Figure 4. Formation and mechanisms of action of siRNA (left) and miRNA (right). Despite sharing intracellular processing and formation, siRNAs come from exogenous origin, while miRNAs are formed from endogenous precursors. Besides, siRNA shows high specificity for its target mRNA, thus targeting one single mRNA, while miRNA shows incomplete specificity for its target mRNA, thus targeting multiple mRNAs from different genes (Lam JK et al. 2015).

2. Sirtuins

2.1 General introduction of Sirtuins

Sirtuins were first identified in yeast, when the Sir2 protein was described to play a role in transcriptional repression of the *HM* (Homothallic Mating) loci, related to mating type and sterility in this organism (Ivy JM et al. 1986). Four additional Sir2 homologs, also known as sirtuins, were identified later, named Hst1p, Hst2p, Hst3p and Hst4p (Brachmann CB et al. 1995). Some studies proved that Sir2 overexpression could extend yeast lifespan by 30% (Kaeberlein M et al. 1999). Although this effect was only observed in replicative lifespan (the number of daughter cells produced by a mother cell) but not in chronological lifespan (the length of time a yeast cell can survive in a non-dividing state) (Fabrizio P et al. 2005). Years later, it was shown that sirtuins are evolutionarily conserved from bacteria to humans (Frye RA 1999, Vaquero et al. 2009). As well as for yeast, sirtuin overexpression also increases lifespan of the model organisms *C. elegans* and *Drosophila melanogaster* by 50% and 57%, respectively (Tissenbaum HA et al. 2001, Rogina B et al. 2004).

In mammals, there are seven proteins belonging to the sirtuin family, being SIRT1 the closest homolog to Sir2 (Michan S, et al. 2007). Regarding sirtuins structure, sirtuins are defined by a conserved catalytic core region (CD), which is evolutionally conserved from yeast. This CD is formed by a large Rossmann fold domain, characteristic of NAD^+/NADH binding proteins, and a Zn^{2+} binding domain. The Zn^{2+} molecule bound to this domain is not involved in the catalysis, but it plays a structural role and it is essential for the deacetylase activity of sirtuins. Between these two domains we find different flexible loops where the NAD^+ and substrate bind to sirtuins (Moniot S et al. 2012). In the case of SIRT6, it lacks the flexible NAD^+ binding loop, containing instead a stable single helix (Pan PW et al. 2011). Apart from these, SIRT1 also contains an N-terminal domain, which is only present in SIRT1 and Sir2 and contains functional nuclear localisation sequences, and a C-terminal domain, which is also an ATP binding site that can regulate SIRT1 activity (Davenport AM et al. 2014, Kang H et al 2017).

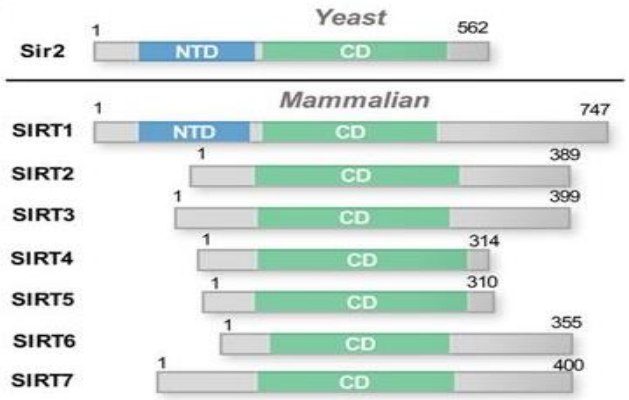


Figure 5. Comparison of the structure domains among the different human sirtuins (SIRT1-7) and yeast Sir2 (Hou X et al. 2016). The N-terminal domain (NTD) is only present in Sir2 and SIRT1, while the catalytic domain (CD) is conserved throughout evolution in all sirtuins.

Throughout evolution, sirtuins are key regulators of the genome-environment crosstalk and the response to different stimuli. By deacetylating many different proteins, including histones and transcription factors, sirtuins are involved in a wide variety of functions, including DNA repair, cell survival, metabolism, lipid and glucose homeostasis, response to stress, genome stability and cell cycle control, among others (Haigis MC et al. 2010).

One of the best studied ways to extend the lifespan in model organisms is calorie restriction (CR). This procedure is defined as the dietary regimen in which calorie intake is reduced by 30-40% (Vaquero A and Reinberg D. 2009, López-Lluch G et al. 2016, Das SK et al, 2017). Multiple studies indicate that sirtuins play a key role in calorie restriction-dependent extension of lifespan (Bitterman KJ et al. 2002, Lin SJ et al. 2004, Guarente L. 2005). The relationship between sirtuins and CR was observed when resveratrol, an activator of Sir2, was able to increase yeast lifespan but it could not extend CR-induced lifespan, proving that resveratrol may use similar pathways than CR to increase lifespan (Howitz KT et al. 2003).

As previously mentioned, sirtuins use NAD^+ as a cofactor, and this dependency on NAD^+ explains the link between sirtuins function and the metabolic status of the cell (McGuinness D et al. 2011, Serravallo M et al. 2013). This NAD^+-dependent deacetylation was first observed in yeast (Imai S et al. 2000). To carry on this enzymatic activity, sirtuins perform a two-step reaction. First, sirtuins cleave NAD^+ into nicotinamide (NAM) and ADP-ribose, releasing the former molecule. Then, an acetyl group is transferred from the substrate to the ADP-ribose moiety of NAD^+, producing as a result the formation of 2'-O-acetyl-ADP-ribose and a deacetylated substrate (Michan S, et al. 2007). Regarding this deacetylase activity, some authors have shown that sirtuins can also remove larger acyl moieties such as palmitoyl, butyryl or succinyl groups, meaning that it is actually a deacylase activity (Bheda P et al. 2016, Kupis W et al. 2016). Furthermore, some authors have also reported an ADP-ribosylation activity in several sirtuins (Liszt G et al. 2005, Hawse WF et al. 2009).

The production of the essential cofactor NAD^+ is controlled by the de novo and the salvage biosynthesis pathways. In the de novo pathway, NAD^+ is synthesised from the amino acid tryptophan (Bender DA 1983), while in the salvage pathway, NAM is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) (Srivastava S. 2016). On the other hand, NAD^+ can be degraded by CD38, which converts NAD^+ into ADP-ribose and NAM. CD38 activity increases during aging, in part caused by chronic inflammation, leading to a decrease in NAD^+ levels and a subsequent decrease in sirtuin activity. NAD^+ levels also decrease during aging due to an increased NAD^+ consumption by PARP1, a DNA repair protein that shows increased activity because of the accumulation of DNA damage with aging (Yaku K et al. 2018).

The different members of the sirtuin family are located in different cellular compartments, indicating the development of new roles (some of them redundant) and the regulation of a wide range of targets. SIRT1 is mostly found in the nucleus, where it regulates key processes like genome organization and stability, gene silencing and heterochromatin structure. SIRT6 is also found in the nucleus, where it is involved in genomic stability and DNA repair. In the case of SIRT7, this sirtuin is enriched in the nucleolus, where it

participates in ribosome biogenesis. On the other hand, SIRT2 is found mainly in the cytoplasm, and regulates cell cycle progression, microtubule organization and metabolism. Finally, SIRT3, 4 and 5 are located in the mitochondria, where they participate in cellular metabolism regulation (Kupis W et al. 2016).

Sirtuin	Activity	Location	Function	Targets
SIRT1	Deacetylase	Nucleus/Cytoplasm	DNA repair, metabolism, chromatin regulation, oxidative stress response, inflammation	H3K9, H4K16, PGC1-α, AMPK, FOXO1, p53, Ku70, NF-kB, PPAR-Y
SIRT2	Deacetylase	Nucleus/Cytoplasm	Cell cycle, metabolism, oxidative stress response	H4K16, H3K56, FOXO1, α-Tubulin
SIRT3	Deacetylase	Nucleus/Cytoplasm/ Mitochondria	Regulation of mitochondrial metabolism, oxidative stress response	H3K9, H4K16, H3K56, LCAD, ACS2, GDH
SIRT4	Deacetylase/ADP -ribosyl- transferase	Mitochondrial	Insulin secretion, regulation of mitochondrial metabolism, DNA repair, amino acid metabolism	GDH, IDE, ANT2, ANT3
SIRT5	Deacetylase	Nucleus/Cytoplasm/ Mitochondria	Urea cycle, fatty acid metabolism, amino acid metabolism	SOD1, CPS1, UOX
SIRT6	Deacetylase/ADP -ribosyl- transferase	Nucleus	Telomere maintenance, DNA repair, metabolism, inflammation	H3K9, H3K18, H3K56, HIFα, PARP1, NF-kB
SIRT7	Deacetylase	Nucleolus/Cytoplasm	Metabolism, transcription	H3K18, H3K36 RNA pol I, FOXO, p53, mTOR

Table 1. Summary of the features of the sirtuin family (Haigis MC et al. 2010, Kupis W et al. 2016, Grabowska W et al. 2017).

Many studies have connected sirtuin activation with prevention of aging and diseases of aging (Guarente L. 2013). Sirtuins have been suggested to play a role in diabetes, cancer, cardiovascular disease, neurodegenerative diseases, pulmonary fibrosis, metabolic

kidney disease, obesity, osteoarthritis, (Morris BJ 2013, Hall JA et al. 2013, Wątroba M et al. 2016). This has led to the search for sirtuin activating molecules with the aim of regulating the aging process and age-related diseases (Milne JC et al. 2007).

In this project, we will focus on SIRT1, 2, 6 and 7 because these are the sirtuins that have been proved to play a role in senescence (Ghosh S et al. 2015, Anwar T et al. 2016). Interestingly, these are in fact the four sirtuins that play a direct role in epigenetic regulation. In contrast, in the case of the mitochondrial sirtuins, the information available is limited and controversial (Jing H et al. 2015).

2.2 SIRT1

SIRT1 is the most studied among the sirtuin family members. It has been mapped to chromosome 10 and it is considered the human orthologue of yeast Sir2. Many protein targets have been described for SIRT1, including histones, transcription factors, metabolic enzymes, signaling proteins and other epigenetic enzymes (Martínez-Redondo P and Vaquero A. 2013), SIRT1 participates in genome stability, metabolic homeostasis and stress survival, among other functions. Interestingly, SIRT1 mainly induces transcription inhibition through the promotion of facultative heterochromatin and regulate many biological functions in human cells.

2.2.1 Chromatin organization

SIRT1 plays a key role in the formation and maintenance of facultative and constitutive heterochromatin by deacetylating lysine residues at positions 9 and 26 of histone H1, lysine 9 of H3, and lysine 16 of H4 (Vaquero A et al. 2004, Shoba et al. 2009, Vaquero A. 2009). SIRT1 not only regulates chromatin through histone deacetylation, but it can also regulate the activity of histone acetyltransferases, histone methyltransferases and DNA methyltransferases. SIRT1 deacetylates and inhibits the acetyl-transferases p300 (Bouras T et al. 2005), hMOF (Lu L et al. 2011) and TIP60 (Peng L et al. 2012), and promotes the deposition of the heterochromatin mark H3K9me3 through the activation of the methyltransferase SUV39H1 (Vaquero A et al. 2007). SIRT1 can also interact with DNA-methyltransferase 1 (DNMT1) modulating its activity through the deacetylation of different residues (Peng L et al. 2011). Thus, SIRT1 plays a key role in epigenetic silencing and chromatin structure and organization (Zhang T et al. 2010).

2.2.2 Metabolism

SIRT1 is involved in the modulation of many metabolic pathways. For instance, SIRT1 is associated with nutrient and energy-sensing pathways such as AMPK (Ruderman NB et al. 2010). SIRT1 also activates PGC-1α, which promotes mitochondrial biogenesis and the expression of antioxidant genes such as glutathione peroxidase, catalase or manganase superoxide dismutase (St-Pierre J et al. 2006, Scarpulla RC. 2011, Merksamer PI et al. 2013), and inhibits PPAR-γ, thereby promoting fat mobilization and inhibiting adipogenesis (Picard F et al. 2004).

2.2.3 Autophagy

Another key metabolic process regulated by SIRT1 is autophagy, a housekeeping mechanism by which cells eliminate aberrant and dysfunctional molecules and organelles. This is performed by the formation of vesicles known as autophagosomes, which englobe these dysfunctional molecules and fuse with lysosomes to induce the degradation of its content.

Many proteins are involved in the regulation of this biological process, including the family of autophagy-related proteins (ATG). The initiation of the process starts with the activation of the ULK1/2 complex (Hurley JH and Young LN. 2017), which phosphorylates and activates different proteins that form the VPS34 complex, including VPS15, VPS34 and Beclin-1 as the core of this complex, and other regulatory proteins such as AMBRA1, VMP1 and UVRAG (Abrahamsen H et al. 2012, Cicchini M et al. 2015). The VPS34 complex then promotes the formation of the phagophore, a double membrane that envelops cytoplasmic contents and will fuse to become the autophagosome in later steps. Once the phagophore is created, other proteins are recruited and induce the elongation of the phagophore, such as ATG5, ATG7, ATG10, ATG12 and ATG16.

During elongation, the protein microtubule-associated protein 1 light chain 3 (LC3) in its inactive form (LC3-I) is converted to its active form (LC3-II) in several steps controlled by ATG3, ATG4 and ATG7. The active form LC3-II is attached to the autophagosome membrane and will bind cargo proteins targeted to degradation through adaptor proteins, thus remaining associated with autophagosomes after their fusion with lysosomes and being degraded with its content (Rubinsztein DC, et al. 2012). Some adaptor proteins that are involved in dysfunctional proteins cargo include p62/SQSTM 1, NIX or NBR1 (Johansen T and Lamark T. 2011). Then, the phagophore closes forming the autophagosome, which will fuse with the lysosome in a process controlled by proteins such as LAMP-2 and proteins from the SNARE and RAB family (Kocaturk M and Gozuacik D. 2018).

In this context, SIRT1 has been described to induce autophagy directly through deacetylating some of these proteins involved in autophagy regulation, such as ATG5, ATG7, and indirectly through the deacetylation of FOXO3, which induces the expression of autophagy genes (Mammucari C et al. 2007, Salminen A et al. 2009, Kroemer G et al. 2010, Ng F et al. 2013).

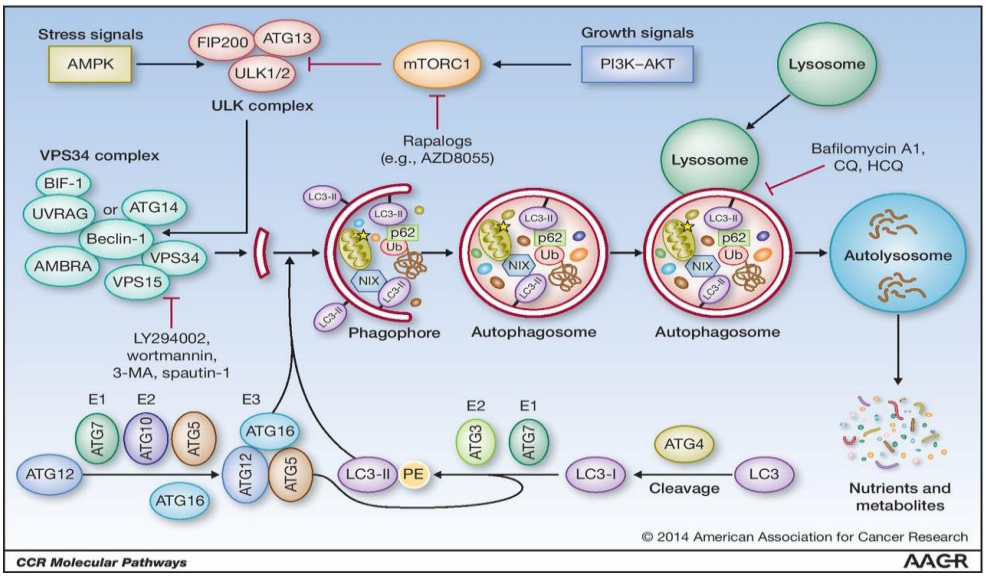


Figure 6. A summary with most of the proteins involved in the autophagy process. After the autophagic stimulus (e.g. AMPK activation), the ULK complex activates and induces the formation of the VPS34 complex, which starts the formation of the autophagosome membrane. This membrane is further elongated by several members of the ATG family, including ATG5, ATG7 and ATG12. During this elongation step, LC3 inactive form (LC3-I) is lipidated to form its active form (LC3-II), which is attached to the autophagosome membrane and acts as a receptor for proteins targeted to be degraded by autophagy (Cicchini M et al. 2015).

2.2.4 Inflammation

SIRT1 is widely known to have an anti-inflammatory effect. SIRT1 deacetylates the RelA/p65 subunit of NF-kB, inhibiting its transcriptional activity and thus the apoptosis and inflammatory pathways (Yeung F et al. 2004). SIRT1 also deacetylates and inhibits c-JUN, leading to transcription inhibition of cell proliferation and inflammation genes, such as matrix metalloproteinase 9 (MMP-9) (Gao Z et al. 2008).

2.2.5 Stress response

SIRT1 participates in the coordination of cellular response to stress. p53 was the first non-histone substrate of SIRT1 to be described, and by deacetylating it, SIRT1 promotes cell survival by inhibiting cellular senescence and apoptosis (Nemoto S et al. 2004, McGuinness D et al. 2011). SIRT1 is also known to deacetylate the members of the FOXO family, which are involved in the cell defence response against oxidative stress. By regulating FOXO proteins, in particular FOXO3a, it promotes the transcription of antioxidant genes (Brunet A et al. 2004, Van der Horst A et al. 2004). Other targets of SIRT1 related to the stress response are Nrf2, a master regulator of the antioxidant response of the cell (Huang K et al. 2015) or HIF1-a, which activates during hypoxia stress (Lim JH et al. 2010).

2.2.6 DNA repair

SIRT1 is involved in the signaling and repair of DNA damage, both in single-stranded and double-stranded breaks. In response to UV-induced damage, SIRT1 deacetylates XPA to promote its interaction with ATR and other proteins to drive the nucleotide excision repair (NER) (Fan W 2010, Jarrett SG et al. 2018). SIRT1 also activates DNA repair through modulating the activity of other DNA repair proteins such as BRCA1 (Lahusen TJ et al 2018), Ku70 (Jeong J et al. 2007), GADD45a (Maeda T et al 2002), KAP1 (Lin YH et al. 2015), NBS1 (Yuan Z et al. 2007) and XPC (Ming M et al. 2010), thus regulating also the processes of homologous recombination (HR) and non-homologous end-joining (NHEJ).

2.2.7 Circadian clock

By sensing cellular metabolic state, SIRT1 also plays a role in circadian rhythms homeostasis by regulating the transcription of specific genes. The key regulators of circadian clock are BMAL1 and CLOCK, which activate the expression of *PER* and *CRY* genes. The PER and CRY proteins, once accumulated to a certain level, block their own transcription through BMAL1-CLOCK repression. In this context, SIRT1 associates with the BMAL1-CLOCK complex and regulates the circadian clock target genes (Asher G et al. 2008, Nakahata Y et al. 2008). NAMPT, the key enzyme of the NAD salvage pathway, is also a target gene controlled by BMAL1-CLOCK and SIRT1 (Nakahata Y et al. 2009).

2.2.8 SIRT1 and disease

A small proportion of SIRT1 knockout (KO) mice survives postnatally, while the majority of them die in the perinatal period. These survivors show developmental defects of the retina and the heart (Cheng HL et al. 2003). SIRT1 KO mice also show decreased insulin production, increased liver steatosis (also known as fatty liver disease) and liver inflammation (Bordone L et al. 2006, Purushotham A et al. 2009, Xu F et al. 2010). SIRT1 has also been described to play a key role in skeletal muscle differentiation and metabolism (Pardo PS et al. 2011), type 2 diabetes (Kitada M et al. 2013), heart function (Xiaosong G et al. 2011), pulmonary fibrosis (Chu H et al. 2018), fatty liver disease (Ding RB et al. 2017), Alzheimer and other neurodegenerative diseases (Kim D et al. 2007, Donmez G et al. 2013) and cancer (Firestein R et al. 2008, Wang RH et al. 2008, Simic P et al. 2013). Interestingly, a point mutation of SIRT1 was identified in a family in which the carrying members developed type 1 diabetes, thus indicating a possible link between SIRT1 and autoimmune diseases (Biason-Lauber A et al. 2013). In the case of cancer, SIRT1 KO mice show accelerated sarcoma and lymphoma development, while SIRT1 overexpression decreases the number of spontaneous carcinomas and sarcomas (Oberdoerffer P et al. 2008, Wang RH et al. 2008, Herranz D et al. 2010). However, SIRT1 plays a dual role in human cancer cells, both promoting and suppressing cancer depending on various factors like tissue and cell type (Bosch-Presegué L et al. 2011, Yuan H et al. 2013). Thus, SIRT1 regulation is a good approach to deal with and prevent the different age-related diseases.

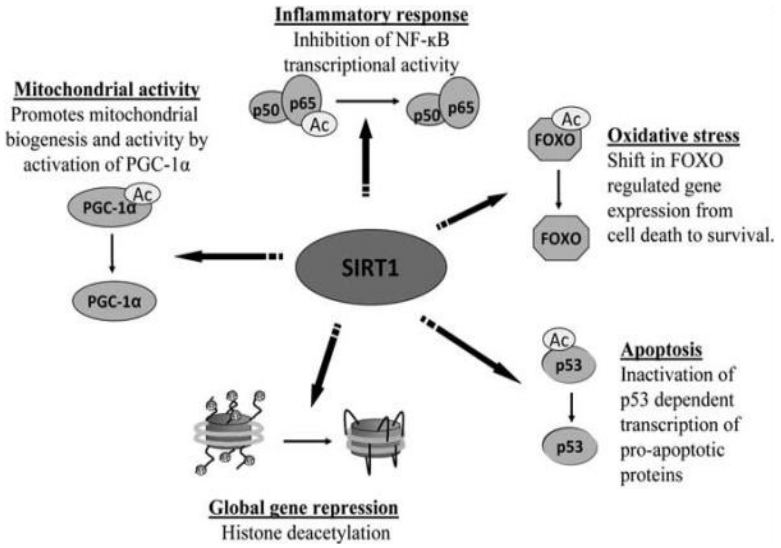


Figure 7. Some of the molecular targets of SIRT1 involved in mitochondrial activity regulation, inflammatory response, oxidative stress activation, apoptosis and gene repression (Morris KC et al. 2011).

2.3 SIRT2

SIRT2 has been mapped to chromosome 19, and its main role seems to be cell cycle regulation, mainly by regulating epigenetics and cytoskeleton dynamics. However, SIRT2 has a wider range of deacetylation targets and participates in diverse functions including metabolism, development, stress response, myelination regulation in the nervous system, synaptic plasticity and memory, cell death and DNA damage signaling, among others. (Gomes P et al. 2015).

2.3.1 Chromatin organization

One of the major substrates of SIRT2 during mitosis is the acetyl lysine 16 of histone 4 (H4K16ac) (Vaquero A et al 2006). The deacetylation of this histone during mitosis inhibits the folding of higher orders of chromatin organization (Shogren-Knaak M et al. 2006), thus being essential for chromosome compaction during metaphase. Other SIRT2 histone substrates include H3K56, by which it regulates DNA repair during S-phase (Vempati RK et al. 2010, Yuan J et al. 2009). Besides, SIRT2 participates also in mitotic exit, by regulating BubR1 (North BJ et al. 2014). As in the case of SIRT1, SIRT2 can also deacetylate the acetyltransferase p300 (Black JC et al. 2008).

2.3.2 Stress response

A role in stress response has been described also for SIRT2. As well as SIRT1, SIRT2 can also deacetylate and inhibit p53 (van Leeuwen IM et al. 2013). In this context, SIRT2 activates FOXO1 and FOXO3, which induce the expression of *catalase*, *MnSOD* and activation of DNA repair pathways (Wang F et al. 2007, Ghosh S et al. 2015). Besides, SIRT2 deacetylates CDK9 and promotes recovery from replication stress arrest (Zhang H et al. 2013).

2.3.3 Metabolism

SIRT2 activates gluconeogenesis and fatty acid oxidation and inhibits lipid biosynthesis in low glucose situations. SIRT2 deacetylates many enzymes involved these metabolic processes, such as LDH-A (Zhao D et al. 2013), PEPCK (Jiang W et al. 2011), ACLY (Lin R et al. 2013) and G6PD (Wang YP et al. 2014). Besides, SIRT2 also deacetylates and activates PGC-1α, which not only induces fatty acid oxidation but also stimulates mitochondrial biogenesis (Krishnan J et al. 2012).

2.3.4 Inflammation

Although not well characterized, SIRT2 regulates the transcriptional activity of the transcription factor NF-κB, master regulator of inflammation and immune system. SIRT2-mediated deacetylation of NF-κB inhibits its transcriptional activity, probably to avoid hyperactivation of the pathway (Rothgiesser KM et al. 2010).

2.3.5 Structural proteins

SIRT2 modulates cytoskeleton organization through α-tubulin deacetylation, which has a major impact in cell motility, intracellular transport, cell shape and cell division (North BJ et al. 2003, Skoge RH et al. 2014).

2.3.6 SIRT2 and disease

A role as a tumor supressor has been described for SIRT2, as mice lacking SIRT2 undergo aberrant cell division and high risk of tumor development (Kim HS et al. 2011, Nakagawa T et al. 2011, Serrano L et al. 2013, Ming M et al. 2014). This higher risk of tumours is gender-specific, as female SIRT2 KO mice developed mainly mammary tumours, while male mice are more prone to develop hepatocellular carcinoma (HCC). SIRT2 KO mice also show increased insulin resistance, cardiac hypertrophy and fibrosis (Tang X et al. 2017, Lantier L et al. 2018). The expression of SIRT2 is increased in cells upon oxidative stress, for example induced by treatment with hydrogen peroxide (Wang F et al. 2007). SIRT2 has a role in preventing neurodegenerative diseases (De Oliveira RM et al. 2012, Donmez G et al. 2013) and adipocyte differentiation (Shoba B et al. 2009). In fact, SIRT2 is the most abundant sirtuin in adipocytes, where it induces lipolysis and inhibits adipocyte differentiation, indicating that SIRT2 activators would be a good approach for metabolic syndrome (De Oliveira RM et al. 2012).

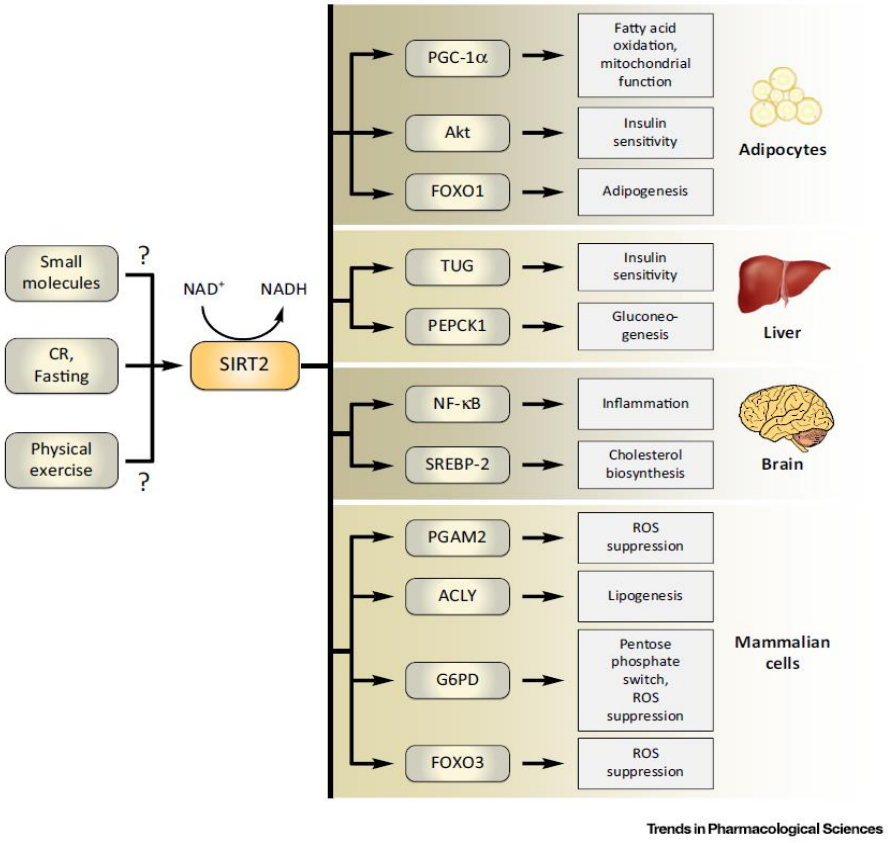


Figure 8. SIRT2 regulation of fat and glucose metabolism, inflammation and oxidative stress response. By deacetylating its targets, SIRT2 activates gluconeogenesis, fatty acid oxidation and the stress response while it inhibits lipid biosynthesis and inflammation (Gomes P et al. 2015).

2.4 SIRT6

SIRT6 has been mapped to chromosome 19 and is mainly involved in genome stability, DNA repair, inflammation and metabolism.

2.4.1 Chromatin organization

SIRT6 plays a key role in telomere stability by deacetylating H3K9, H3K18 and H3K56 during S-phase, which allows the binding of WRN and controls proper telomere maintenance (Michishita E et al. 2008, Michishita E et al. 2009). Other recent studies have described wider roles of SIRT6 in genome stability, such as the inhibition of Long Interspersed Element-1 (LINE-1) transposition and the maintenance of pericentric heterochromatin (Van Meter M et al. 2014, Tasselli L et al. 2016).

2.4.2 DNA repair

As in the case of SIRT1, SIRT6 has an essential role in DNA repair, by recruiting factors to double stranded DNA breaks (DSB) (McCord RA et al. 2009, Toiber D et al. 2013). SIRT6 activates ADP-Ribose Polymerase 1 (PARP1) (Mao Z et al. 2011, Mao Z et al.

2012) and facilitates Ku80 interaction with DNA (Chen W et al. 2017) to promote DSB repair through both homologous recombination (HR) and non-homologous end joining (NHEJ).

2.4.3 Metabolism

SIRT6 plays a key role in metabolic homeostasis, by inhibiting both glycolysis and gluconeogenesis. SIRT6 glycolysis inhibition is mediated both by inhibition of HIF1α activity and glycolytic enzymes gene expression, thereby regulating the Warburg effect in tumor cells (Zhong L et al. 2010, Sebastián C et al. 2012). On the other hand, SIRT6 binds and activates GCN5 to inhibit gluconeogenesis (Dominy JE Jr et al. 2012).

2.4.4 Inflammation

Although in some contexts SIRT6 might appear to induce pro-inflammatory effects (Jiang H et al. 2013), it has anti-inflammatory effects through its inhibition of NF-κB signaling via H3K9 deacetylation. This is proven by the fact that NF-κB, which is normally inhibited by SIRT6, is hyperactivated in SIRT6 KO mice and contributes to the premature aging phenotype in these animals (Kawahara TL et al. 2009).

2.4.5 SIRT6 and disease

In cancer, SIRT6 plays an important role as a tumor suppressor through different mechanisms including DNA repair, genome stability, inhibition of MYC target genes, and glycolysis (Van Meter M et al. 2011, Yuan H et al. 2013, Tasselli L et al. 2017). Besides, SIRT6 is downregulated in many cancers including pancreatic, colorectal cancer, breast, ovarian and lung cancer, among others, in which loss-of-function point mutations of SIRT6 have been identified (Sebastián C et al. 2012, Kugel S et al. 2015). However, in some specific functional contexts SIRT6 could also play a pro-oncogenic role. This has been detected in some types of cancer including thyroid cancer, melanoma and squamous cell carcinoma (Ming M et al 2014 (b), Garcia-Peterson LM et al. 2017, Qu N et al. 2017).

SIRT6 is the only mammalian sirtuin that clearly induces an increase (of 14%) in mice lifespan when overexpressed, although this increase is only observed in male mice (Kanfi Y et al. 2012). Its inhibition causes cell senescence in some cell types, like chondrocytes, endothelial cells and porcine fibroblasts (Xie X et al. 2012, Nagai K et al. 2015). SIRT6 levels, like SIRT1, are also increased in the brain, kidney and heart of mice after a 24hr fasting period, and in white adipose tissue (WAT), heart and brain of rats fed a calorie-restricted diet for a minimum of one year (Kanfi, et al. 2008). Furthermore, SIRT6 activation has shown protective effects in the development of cardiac hypertrophy and failure (Sundaresan NR et al. 2012).

On the other hand, mice lacking SIRT6 develop a degenerative disorder that shows features of accelerated ageing, including small size, lymphopenia, loss of subcutaneous fat, lordokyphosis (excessive inward curvature of the lumbar spine) and metabolic defects

including reduced serum IGF-1 levels and hypoglycemia. These mice eventually die at about 4 weeks (Mostoslavsky R et al. 2006).

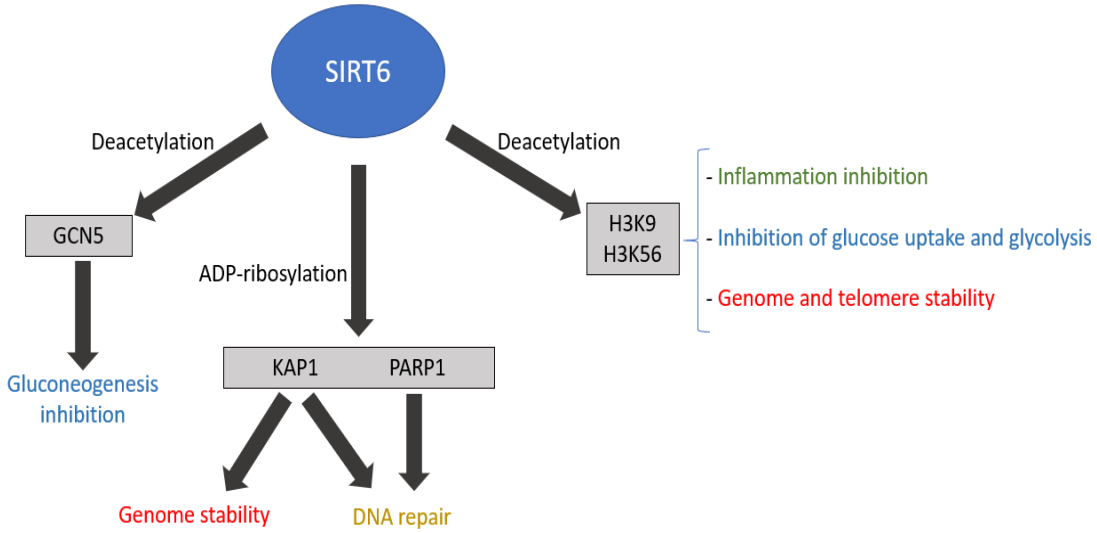


Figure 9. Molecular targets of SIRT6. Both histone and non-histone targets (grey boxes) regulate the main functions of SIRT6: genome stability (red), metabolism (blue), DNA repair (orange) and inflammation (green).

2.5 SIRT7

SIRT7 is nuclear protein that has been mapped to chromosome 17 and is localized mainly in the nucleolus where it controls rDNA transcription, ribosome synthesis and cell proliferation (Kim W et al. 2013, Priyanka A et al. 2016). SIRT7 can both promote and inhibit transcription. Despite being the less studied of the sirtuin family, there has been several biological functions and targets described for SIRT7.

2.5.1 Chromatin organization

SIRT7 is involved in DNA repair and the maintenance of genome integrity through H3K18 and H3K36 deacetylation (Vazquez BN et al. 2016, Vazquez BN et al. 2019, Wang WW et al. 2019). Besides, the deacetylation of H3K18 is involved in ribosome metabolism and oncogenic transformation (Barber MF et al. 2012).

2.5.2 Stress response

SIRT7 plays a key role in the stress response, as SIRT7 deficient cells show decreased resistance to oxidative and genotoxic stress (Vakhrusheva O et al. 2008). This role of SIRT7 in stress response is mediated by deacetylation of SAPK and p53 (Kiran S et al. 2015). Besides, SIRT7 inhibits the activity of HIF1α and HIF2α in response to hypoxia stress (Hubbi ME et al. 2013).

2.5.3 Metabolism

SIRT7 is involved in the regulation of both glucose and lipid metabolic pathways. Unlike SIRT1, SIRT7 induces the expression of genes involved in fatty acid uptake and triglyceride synthesis, thus favouring fat storage (Yoshizawa T et al. 2014, Fang J et al. 2017). On the other hand, SIRT7 inhibits glycolysis through G6PC gene inhibition and gluconeogenesis through PGK1 deacetylation, respectively (Hu H et al. 2017, Jiang L et al 2017).

2.5.4 RNA regulation and ribosome metabolism

SIRT7 interacts with PAF53, a subunit of RNA pol I, thus activating ribosomal DNA (rDNA) transcription (Ford E et al. 2006, Chen S et al. 2013). Furthermore, SIRT7 also regulates RNA pol II through the deacetylation of CDK9, which phosphorylates RNA pol II and promotes transcription elongation (Blank MF et al. 2017). SIRT7 specific deacetylation of H3K18 has shown to regulate the expression of genes related to RNA processing, translation, RNA splicing, and mRNA metabolism (Tsai YC et al. 2014).

2.5.5 Mitochondria homeostasis

A role in regulating protein folding in mitochondria has been described, although the exact mechanisms are still unknown (Liu JP et al. 2015). SIRT7 also deacetylates GABPβ1, controlling mitochondria homeostasis (Ryu D et al. 2014).

2.5.6 DNA repair

SIRT7 is involved in DNA damage response and repair. In fact, SIRT7 depletion impairs the repair of DSB, thus inducing accumulation of DNA damage and increased mutation frequency (Vazquez BN et al. 2016). Specifically, SIRT7 regulates the DNA damage response by deacetylating and activating ATM and modulates NHEJ by favouring the recruitment of 53BP1 to DSB (Vazquez BN et al. 2016, Tang M et al. 2019).

2.5.7 SIRT7 and disease

The first paper on SIRT7 KO mice showed that these mice develop cardiac hypertrophy and inflammatory cardiomyopathy, with increased fibrosis and cell apoptosis under basal and in response to stress conditions (Vakhrusheva O et al. 2008). Later reports showed that SIRT7 KO mice also develop a premature aging phenotype, as in the case of SIRT6, where more than 20% die within the first month, and the remaining ones die significantly earlier (12-20 months) than the WT mice (Vazquez BN et al. 2016). These mice show kyphosis (excessive outward curvature of the thoracic spine), decreased gonadal fat pad content, increased hepatic lipid content, reduced IGF-1 levels in plasma, bone marrow stem cell dysfunction and increase in p16 levels.

There is a progressive loss of SIRT7 expression as the cell enters senescence (Kiran S et al. 2013). Many studies coincide in describing an oncogenic potential of SIRT7, as it is overexpressed in breast cancer, thyroid cancer and hepatocellular carcinoma cells (Frye R et al. 2002, Ashraf N et al 2006, Kim JK et al. 2013). However, SIRT7 expression is downregulated in other cancers like head and neck squamous cell carcinoma (Lai CC et al. 2013). Apart from cancer, SIRT7 has been described to be involved in the pathology of many diseases, including pulmonary fibrosis (Wyman AE et al. 2017), cardiac homeostasis (Vakhrusheva O et al. 2008), neuroinflammation (Burg N et al. 2018) and fatty liver disease (Shin J et al. 2013), among others. Unlike SIRT1, SIRT2 and SIRT6, there are not small molecule activators or inhibitors available for SIRT7. The development of new drugs acting on SIRT7 activity could be used to treat aging related diseases like cancer.

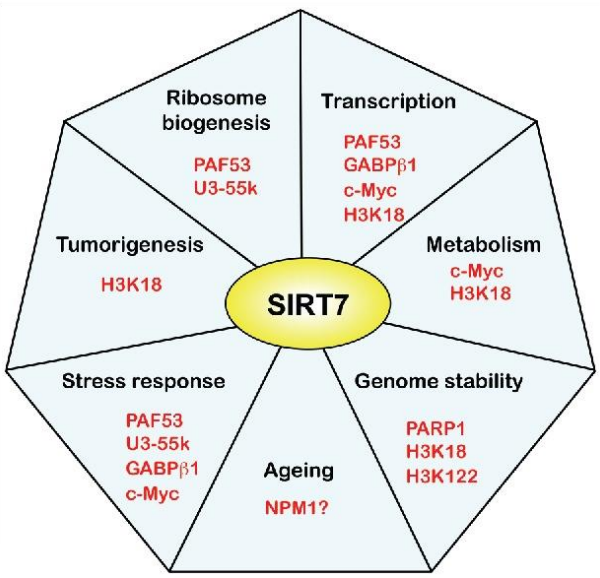


Figure 10. Molecular targets and processes regulated by SIRT7. This sirtuin is involved in stress response regulation, glucose and lipid metabolism, ribosome biogenesis, DNA repair and genome stability and tumorigenesis (Blank MF et al (b). 2017).

2.6 Role of sirtuins in skin

Regarding the skin, sirtuins play essential roles in coordinating the response against cellular stress. They participate both in the response against intrinsic damage (chronological aging) and external damage (photoaging), mainly attributed to the pro-oxidative and genotoxic effects of UV light (see section 4.1 for detailed features of skin aging types) (Garcia-Peterson LM et al. 2017 (b)).

Specifically, SIRT1 has showed to decrease cell senescence and DNA degradation in skin cells (Moreau M et al. 2007). Besides, it promotes differentiation of normal human keratinocytes, a process that is dysregulated in acne or psoriasis (Blander G et al. 2009), and it has antifungal properties (Wurtele H et al. 2010). SIRT1 modulates psoriasis pathology by regulating TNF-α and IL-22 signaling (Sestito R et al. 2011). Besides, SIRT1 plays a crucial role in proper skin wound healing (Qiang L et al. 2017). SIRT1 plays a key role in skin photoaging by different mechanisms. On one hand, SIRT1 inhibits

the expression of the collagen degrading enzyme MMP-1 (Ohguchi K et al. 2010). On the other hand, SIRT1 protects against UVB-induced damage in human dermal fibroblasts (Chung KW et al. 2015). Regarding skin cancers, SIRT1 acts as an oncogene in melanoma and basal cell carcinoma (Garcia-Peterson LM et al. 2017 (b)).

Regarding SIRT2, its tumor suppressor effect has been observed in tumoral keratinocytes (Ming M et al. 2014), while it is upregulated in melanoma cells (Wilking-Busch MJ et al. 2017).

SIRT6 controls collagen metabolism in human dermal fibroblasts, as SIRT6 silencing decreases type I collagen synthesis (Baohua Y et al. 2012). SIRT6 regulation of NF-κB has been studied as a treatment for inflammatory and autoimmune diseases like psoriasis (Kawahara TL et al. 2009). As mentioned before, SIRT6 has also been suggested to be a potential oncogene in squamous cell carcinoma and melanoma (Ming M et al 2014 (b), Garcia-Peterson LM et al. 2017).

SIRT7 is upregulated in skin psoriasis lesions along with SIRT6, while SIRT1, SIRT2, SIRT3, SIRT4 and SIRT5 are downregulated. This might be the result of an internal imbalance caused by the chronic inflammation state in psoriasis (Fan X et al. 2019).

2.7 Regulation of Sirtuins at different levels

Sirtuins not only regulate a wide range of cellular substrates, but also are regulated by a wide variety of signals, that include metabolic, oxidative or inflammatory stimuli, among others (Buler M et al. 2017). This modulation of sirtuins can be performed at a gene expression level, a post-transcriptional level, a post-translational level or at a complex formation or substrate-dependent level.

2.7.1 Sirtuin gene expression and post-trancriptional regulation

- SIRT1: SIRT1 gene expression can be regulated directly by transcription factors that bind its promoter, by proteins that stabilize SIRT1 mRNA or by miRNAs that bind SIRT1 mRNA and inhibit its translation or induce its degradation (Milner J 2009). Hypermethylated in cancer-1 (HIC1) forms a transcriptional repression complex with SIRT1, that binds the SIRT1 promoter to inhibit its transcription (Chen WY et al. 2005). As explained previously, nutrient availability regulates sirtuin activity. This is controlled by the transcription factors cAMP response element-binding protein (CREB) and the carbohydrate response-element-binding protein (ChREBP), that bind the SIRT1 promoter depending on the nutrient status (Noriega LG et al. 2011). PPAR-γ, a master regulator of lipid metabolism, negatively regulates SIRT1 expression by binding SIRT1 promoter (Han L et al. 2010). NF-κB, an essential inflammatory transcription factor, can also bind and activate the SIRT1 promoter (Katto J et al. 2013). APE-1, a protein involved in DNA repair, also binds the SIRT1 promoter to activate it upon oxidative stress (Antoniali G, et al. 2014). The regulation of SIRT1 in oxidative stress is complex, as SIRT1 expression is upregulated in response to mild stress but downregulated after high or prolonged levels of oxidative stress (Santos L et al. 2016). Regarding

the factors that stabilize SIRT1 mRNA, HuR binds the 3' untranslated region of SIRT1 mRNA and stabilizes it, increasing SIRT1 expression (Abdelmohsen K et al 2007). Finally, regarding SIRT1 regulation by miRNAs, one of the best described miRNAs that targets SIRT1 is miR-34a. This miRNA is upregulated by p53 activation, and it binds the 3' untranslated region of SIRT1 mRNA, blocking its translation. Thus, miR-34a forms a regulatory loop with SIRT1 and p53 (Raver-Shapira N et al 2007, Yamakuchi M et al 2008, Lou G et al 2015). A wide variety of miRNAs have been described to regulate SIRT1 expression in different cell types, including miR-29b, miR-132, miR-181a, miR-204 (Buler M et al. 2017, Kosciuk T et al. 2019).

- SIRT2: High glucose levels inhibit SIRT2 gene expression, as well as SIRT1, SIRT6 and SIRT7 (Jiang W et al. 2011, Mortuza R et al. 2013). Other external stimuli such as calorie restriction or oxidative stress increase SIRT2 expression (Wang F et al. 2007). HIF1a can also bind the SIRT2 promoter and inhibit its expression, which has shown to contribute to fat accumulation in adipocytes (Krishnan J et al. 2012).

- SIRT6: p53 transcription factor has shown to directly activate SIRT6 gene expression, and miR-34a, which is upregulated by p53 and targets SIRT1, can inhibit SIRT6 expression (Lefort K et al. 2013, Zhang P et al. 2014). Another miRNA that can inhibit SIRT6 expression is miR-766 (Sharma A et al. 2013). Additionally, the inflammatory and immunologic signals by LPS and TNF-a can suppress SIRT6 expression (Lappas M 2012, Moschen AR et al. 2013).

- SIRT7: MiR-125a-5p and miR-125b have been shown to inhibit SIRT7 expression and thus regulate its oncogenic potential, acting as tumor suppressors (Kim JK et al. 2013). As p53 controls the transcription of miR-125a-5p and miR-125b, a complex regulatory loop has been proposed including p53, SIRT7, SIRT1, and miRNAs 34, 125-5p and 125b (Buler M et al. 2017). Thus, the link between SIRT1 and p53 is much more complex than anticipated, as p53 can downregulate sirtuin expression through the upregulation of several miRNAs. CCAAT-enhancer-binding protein α (C/EBPα) also inhibits SIRT7 expression (Liu GF et al. 2016).

2.7.2 Post-translational regulation

- SIRT1: SIRT1 activity can be modulated by many post-translational modifications. Sumoylation activates SIRT1 deacetylase activity (Yang Y et al. 2007). SIRT1 can be phosphorylated in many residues. Activation of SIRT1 can be achieved for example by phosphorylation at S27 by JNK2, which stabilizes SIRT1 protein (Ford J et al. 2008), at S27, S47 and T530 by JNK1, which increases SIRT1 nuclear localization and enzymatic activity (Nasrin N et al. 2009), or at T280 and T301 by JAK1, which enhances its interaction with STAT3 and suppresses its transcriptional activity, thus inhibiting *IL-6* expression (Wang W et al. 2018). SIRT1 phosphorylation in T344 by AMPK can also induce its deacetylase activity (Lau AW et al. 2014). SIRT1 can be also methylated, which

disrupts SIRT1 binding to p53 (Liu X et al. 2011). Finally, O-GlcNAcylation of SIRT1 at S549 increases its deacetylase activity (Han C et al. 2017).

- SIRT2: SIRT2 activity can be modulated positively by phosphorylation. While S331 phosphorylation through cyclin-dependent kinases inhibits SIRT2 deacetylation activity (Pandithage R et al. 2008), phosphorylation by ERK1/2 enhances SIRT2 protein stability which in turn results in increased activity (Choi YH et al. 2013).
- SIRT6: SIRT6 phosphorylation at S338 by AKT1 favors its interaction and ubiquitination by MDM2, which induces SIRT6 degradation (Thirumurthi U et al. 2014).
- SIRT7: USP7-mediated deubiquitination of SIRT7 at L63 represses its enzymatic activity (Jiang L et al. 2017). Other evidences have reported hyperphosphorylation of SIRT7 during mitosis, but the sites of phosphorylation and functional consequences of this modifications are not defined (Grob A et al. 2009). Under energy starvation, SIRT7 is phosphorylated by AMPK and targeted to degradation, which reduces rDNA transcription (Sun L et al. 2016).

2.7.3 Complex formation and substrate-dependent regulation

A key feature of sirtuin activity is its dependency on NAD+. However, there are other regulators that modulate specifically the different sirtuins.

- SIRT1: The first reported modulator of SIRT1 activity was Active Regulator of SIRT1 (AROS) (Kim EJ et al. 2007). AROS is a nuclear protein that enhances SIRT1-mediated deacetylation of p53, thereby inhibiting its transcriptional activity. This regulation is SIRT1-specific as AROS binds the N-terminus of SIRT1, a non-conserved region among sirtuins. On the other hand, the first cellular suppressor of SIRT1 activity to be described was Deleted in Breast Cancer-1 (DBC1) (Kim JE et al. 2008, Zhao W et al. 2008). This protein forms a specific stable complex with SIRT1, blocking its enzymatic activity. Interestingly, ATP can also bind the C-terminal domain of SIRT1 repressing its enzymatic activity (Kang H et al. 2017). In contrast, cAMP binding to SIRT1 can increase its deacetylase activity (Wang Z et al. 2015). An antagonistic interaction has also been described between SIRT1 and SIRT7, where SIRT7 inhibits SIRT1 autodeacetylation in the control of adipogenesis (Fang J et al. 2017) and Suv39h1 regulation (Kumari P et al. 2018).
- SIRT6: long-chain fatty acids including myristic, oleic and linoleic acid can induce SIRT6 deacetylase activity at physiological concentrations (Feldman JL et al. 2013). Additionally, Lamin A has been described as an endogenous activator of SIRT6 in DNA repair (Ghosh S et al. 2015).

2.8 Sirtuin activators

Given the role of sirtuin in aging and age-related diseases, a considerable effort has been made to discover new sirtuin activators that could be used to treat these diseases. A group of several polyphenols were the first molecules that were reported to activate SIRT1 and extend the lifespan of Saccharomyces cerevisiae. This group included resveratrol, piceatannol, butein, quercetin and myricetin (Howitz KT et al. 2003).

Resveratrol is a phytoalexin antioxidant present in grapes, berries, peanuts and red wine, and it is produced as a form of defence against harmful environment stimuli (Ren S, et al 1997). It has been shown to improve the general health in mice fed with a high-calorie diet (Baur JA et al. 2006, Lagouge M et al. 2006, Kao CL et al. 2010). Because of these biological properties, resveratrol has been included in some skin care formulations (Baxter RA et al. 2008). *In vitro* experiments on skin cells have demonstrated resveratrol ability to protect against UVA and UVB radiation (Adhami VM et al. 2003, Liu Y et al. 2011, Cao C et al. 2009), downregulate the expression of *MMP-9* (Lee JS et al. 2010) and promote apoptosis in tumor cells (Ahmad N et al. 2001, Niles RM et al. 2003). Human skin equivalent experiments have confirmed induction of extracellular matrix protein synthesis (collagen and elastin), induction of antioxidant genes and inhibition of inflammatory genes by resveratrol (Lephart ED et al. 2014). *In vivo* studies have demonstrated that resveratrol activates SIRT1 in an indirect way, by acting on the cAMP-AMPK-Sirtuin1 pathway (Park SJ et al. 2012, Prince NL et al. 2012), but a new study demonstrated that resveratrol can allosterically activate SIRT1 (Hubbard BP et al. 2013).

However, one drawback of resveratrol use is its effect on apoptosis. Resveratrol can have beneficial effects when use at low doses, while it can induce cell apoptosis at high doses (Shakibaei M et al. 2009). Besides, resveratrol does not only activate SIRT1, but also has many other protein targets, what makes it to have undesirable secondary effects (Pirola L et al. 2008). Resveratrol is a very unstable molecule, it is fast metabolized by human metabolism (poor bioavailability) and presents low skin penetration (Walle T et al. 2011, Scalia S et al. 2015). Resveratrol analogues have been synthesised to avoid these problems. These molecules were found to kill melanoma cells (Moran BW et al. 2009, Wong Y et al. 2010) and inhibit tyrosinase activity (Choi J et al. 2010). Regarding skin research, resveratrol has been incorporated in several formulations for its delivery into the skin (Hung CF et al. 2008). However, to allow resveratrol to incorporate into the creams or oils, microparticles are used that supposedly prolong its release into the skin. The drawback of this approach is that it also reduces the amount of resveratrol available for penetration into the skin. Some authors have tried to solve this problem with nanosuspensions of resveratrol (Kobierski S et al. 2009).

These limitations of resveratrol prompted many pharmaceutical companies to synthesise novel sirtuin specific activators. Until now, several activators have been discovered, named SRT1460, SRT1720 and SRT2183 (Milne JC et al. 2007). These compounds have been reported to have anticancer activity (Chauhan D et al. 2011) and have beneficial effects in the pathology of many diseases, including diabetes, osteoarthritis and renal fibrosis (Ren Y et al. 2017, Nguyen LT et al. 2018, Nishida K et al. 2018), but their mechanism of action is controversial (Huber JL et al. 2010, Pacholec M et al. 2010).

Regarding SIRT6, the fact that it has multiple enzymatic activities, such as deacetylation and ADP-ribosylation, has awaken interest in scientist that want to modulate only one of these activities. A paper described the activation of SIRT6 deacetylase activity but not demyristoylase activity by long fatty acid molecules at physiological doses (Feldman JL et al. 2013). Other authors also described the activation of SIRT6 deacetylation activity by a synthetic compound without affecting the demyristoylation activity (You W et al. 2017).

Indirect activation of sirtuins has also been explored. Iso-nicotinamide prevents nicotinamide inhibition of sirtuins, thus leading to an apparent activation (Sauve AA et al. 2005.). Based on the essential role of NAD^+ in sirtuin enzymatic activity, many companies are also screening for compounds that boost NAD^+ levels to increase sirtuin activation. The NAD^+ precursors nicotinamide riboside (NR) and nicotinamide mononucleotide have been used to for this purpose (Yoshino J et al. 2011). However, these approaches suppose a non-specific stimulation of all the sirtuin isoforms and can affect other processes and proteins regulated by NAD+, such as PARP-1 (Dai H et al. 2018).

2.9 Sirtuin inhibitors

Many sirtuin inhibitors have been identified so far, most of them through a substrate and/or product mimetics approach (Bruzzone S et al. 2013). The main potential application of these agents is as anticancer drugs (Villalba JM et al 2012). One of the first compounds discovered was nicotinamide, the endogenous inhibitor that is produced in the hydrolysis of NAD^+ step during the deacetylation reaction (Tervo AJ, et al. 2004). Nicotinamide can inhibit all sirtuins by binding the nicotinamide binding pocket. However, it has limited therapeutic potential as it has no specificity when inhibiting sirtuins activity (Avalos JL et al. 2005).

An hydroxynaphthaldehyde derivative, sirtinol, targets SIRT1 and SIRT2 and induces cytotoxic effects on cancer cells of different origin (Grozinger CM et al. 2001, Ota H et al. 2006, Peck B et al. 2010, Cea M et al. 2011). Subsequent structure-activity relationship studies have identified other derivatives with improved properties. These derivatives include salermide (Lara E et al. 2009), cambinol (Heltweg B et al. 2006) and splitomicin (Bedalov A et al. 2001). Later on, a group of thiobarbiturates similar to cambinol and with SIRT1/SIRT2 specificity were identified (Uciechowska U et al. 2008). Probably the best-known inhibitor nowadays is EX-527, identified in a high-throughput screening of SIRT1 inhibitors (Napper AD et al. 2005). This compound requires NAD^+ bound to SIRT1 to inhibit the enzymatic activity, and its binding site in SIRT1 is common among the different sirtuins, which makes EX-527 selectivity on SIRT1 dependent on the kinetic features of the enzyme (Gertz M et al. 2013). Interestingly, EX-527 can also weakly inhibit SIRT6 (Kokkonen P et al. 2014).

Based on the idea that sirtuins and protein kinases contain an adenosine binding site, a group of known kinase inhibitors were tested against SIRT2. Of them, Ro31-8220 and GW5074 showed good SIRT1 and SIRT2 inhibitory activites (Trapp J et al. 2006). Interestingly, other inhibitors have been discovered in screenings of sirtuin activators.

This is for instance the case of Suramin, an adenosine receptor antagonist with a potent SIRT1/SIRT2 inhibitor ability (Howitz KT et al. 2003), that has been studied in clinical trials due to its anticancer properties (McGeary RP et al. 2008). The subsequent search for an improved derivative of suramin led to the discovery of NF675, which shows a high selectivity for SIRT1 over SIRT2 (Trapp J et al. 2007). However, all these compounds have bad drug-like properties, especially due to its high molecular weight and its anionic nature, which limits its use as a therapeutic drug. Finally, a specific and potent inhibitor for SIRT2, AGK2, was discovered in 2007 (Outeiro TF et al. 2007). Recently, several papers have been published describing the synthesis of novel selective SIRT2 and SIRT6 inhibitors (Parenti MD et al. 2014, Tatum PR et al. 2014). Among them, OSS128167, a SIRT6-specific inhibitor, has been reported. OSS128167 treatment induces H3K9 acetylation, reduced TNF-alpha secretion and increased glucose uptake (Parenti MD et al. 2014).

2.10 Role of sirtuins in senescence

Although the majority of sirtuins seem to inhibit cell senescence, this seems to be more complex in some family members. For instance, the role of SIRT1, SIRT6 and SIRT7 in preventing senescence is clear, as their protein levels decrease in senescent cells and their overexpression suppresses cellular senescence (Lee N et al. 2014, Lee SH et al. 2019). In contrast, the involvement of SIRT2 in senescence seems to be more complex (Anwar T et al. 2016).

2.10.1 SIRT1

SIRT1 can prevent cellular senescence by deacetylating several proteins involved in different cellular processes. SIRT1 deacetylates p53, a well-known senescence inducer (Langley E et al. 2002, Chung KW et al. 2015). The SIRT1-p53 interaction is regulated by insulin-like growth factor-1 (IGF-1) pathway. A recent study showed that prolonged IGF-1 treatment of cells induces premature cell senescence by activating p53 and inhibiting SIRT1 (Tran D et al. 2014). SIRT1 regulates senescence through the deacetylation of FOXO proteins, which are involved in stress resistance and autophagy, and DNA repair proteins such as Ku70, XPA and NBS1, among others (Jeong J et al. 2007, Fan W. 2010, Lim CJ et al. 2017). By deacetylating NF-κB, SIRT1 inhibits the senescent secretory phenotype, thereby preventing inflammation and reinforcement of senescence (Malaquin N et al. 2019). SIRT1 regulation of heterochromatin maintenance reaffirms its role in senescence, as loss of heterochromatin is one of the hallmarks for premature cellular senescence and accelerated aging (Vaquero et al. 2004, Ghosh S et al. 2015).

2.10.2 SIRT2

As mentioned earlier, the relationship between SIRT2 and senescence is controversial. A role of SIRT2 in senescence has been proposed due to its well demonstrated regulation of FOXO1 and FOXO3 activities, which in turn regulate many aging-related pathways as DNA repair, apoptosis or metabolic regulation (Wang F, et al. 2007, Wang F, et al. 2009). Additionally, SIRT2 regulates BubR1, a checkpoint kinase that inhibits senescence (North BJ et al. 2014). However, a recent paper claims that SIRT2 is upregulated in

different types of cellular senescence (replicative senescence, oncogene-dependent senescence and stress-induced senescence), although it remains unknown if it contributes to senescence development or it is a consequence of senescence induction (Anwar T, et al. 2016).

2.10.3 SIRT6

Like the case of SIRT1, SIRT6 has a clear role in senescence. *In vivo*, SIRT6 knockout mice shows premature aging signs, and when overexpressed in the hole body, SIRT6 increases mice lifespan (Mostoslavsky R et al., 2006). SIRT6 regulation of senescence can be explained by the described roles of this sirtuin in maintaining genome stability, regulating DNA damage repair, telomere maintenance and tumor suppression (Mao Z et al. 2011, Tasselli L et al. 2017). Additionally, SIRT6 deacetylation of H3K9 stabilizes its interaction with WRN, maintains telomeric metabolism (Michishita E et al. 2008) and inhibits NF-κB-regulated genes that induce inflammation and senescence (Kawahara TL et al. 2009). Finally, SIRT6-induced heterochromatin silencing of retrotransposable elements (RTEs) protects against genomic instability (De Cecco M et al. 2013, Van Meter M et al. 2014).

2.10.4 SIRT7

As described earlier, SIRT7 KO mice shows premature aging and reduced lifespan (Vazquez BN et al. 2016). SIRT7 downregulation induces cellular senescence (Gu S et al. 2016), while its overexpression prevents it (Kiran S et al. 2013). SIRT7 also plays a key role in maintaining rDNA heterochromatin, and thus protecting against senescence induced by loss of heterochromatin in the rDNA gene clusters (Paredes S et al. 2018).

3. Cellular senescence

Cellular senescence is characterized by an irreversible growth arrest during the cell cycle and exhibits various phenotypic changes in gene expression, cellular functions, and morphology (Kuilman T et al. 2010, Chung KW et al. 2015), among others. Initially, cellular senescence was described as the cell cycle exit that occurs in cultured cells after a finite number of replications (Hayflick L 1965). Now, it has been demonstrated that senescent cells play many roles involved not only in disease and aging, but also in tissue homeostasis (Van Deursen JM 2014). In fact, cellular senescence can be observed in physiological processes like wound healing or during embryonic development, where it has a beneficial role through the induction of tissue remodelling (Muñoz-Espín D et al. 2013, Demaria M et al 2014).

Despite performing important physiological roles, as preventing cancer-prone cells to divide, senescent cells accumulate in aged tissues, including skin, and alter cellular microenvironment, which refers to all the proteins and growth factors secreted by the cells (Dimri GP et al. 1995, Ressler S et al. 2006, Waaijer ME et al. 2012, Campisi J. 2013, DeMaria M et al. 2015). Specifically, senescent cells induce a pro-inflammatory and detrimental state of the tissue, leading to tissue overall damage, which contributes to cancer development and aging (Toutfaire M et al. 2017). To date, senescence has been implicated in the pathogenesis of many age-related diseases, including osteoarthritis,

pulmonary fibrosis, non-alcoholic fatty liver disease, atherosclerosis, diabetes and Alzheimer disease (Childs BG et al. 2017).

3.1 Causes of cellular senescence

Cellular senescence can be induced in response to a wide range of stimuli, including telomere shortening, genomic damage, strong chronic mitogenic signals or mitochondrial dysfunction (Wei W and Ji S. 2018, Muñoz-Espín D et al. 2014). Telomere shortening, which occurs after each cell replication, leaves the chromosome ends unprotected, exposing and inducing double strand breaks and subsequent activation of the DNA damage response. This in turn increases genomic instability and finally causes cell cycle arrest and cellular senescence (Hezel AF et al. 2005). The DNA damage response has also been shown to induce inflammation through activation of NF-κB, which at the end contributes to tissue degeneration and aging (Kang C et al. 2015). This type of senescence triggered by telomere shortening after a finite number of cell replications is known as replicative senescence (Cristofalo VJ et al, 1993). Abnormal activation of oncogenic genes is a mechanism to prevent cellular transformation into a tumour cell. Oncogenic induced-senescence is characterised by inhibition of DNA repair and thus an increase in genomic damage (Larsson LG. 2011, Tu et al. 2011). Mitochondrial dysfunction also induces cellular senescence by increasing ROS production, creating a feed-forward cycle where the increase in ROS production leads to more damage in the mitochondria (Gallage S et al. 2016).

On the other hand, external insults like ionizing radiation, UV or H_2O_2 induce a cellular state that has been defined as stress-induced premature senescence (SIPS). This type of senescence occurs after several sublethal stresses. After a first stress, the cell enters a transient cell cycle arrest in which p53 pathway, among others, activates and modulates DNA repair to remove the damage. If this first stress is too high, cell enters apoptosis and dies. However, if the cell survives the first insult and receives a second stress that cannot be repaired, the cell enters cellular senescence (Chainiaux F et al, 2002).

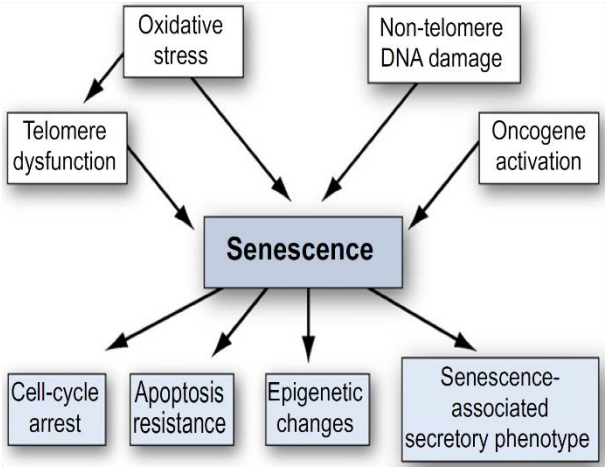


Figure 11. Different types of stimuli can induce senescence and produce a wide range of phenotypic changes, including cell-cycle arrest and pro-inflammatory secretory phenotype (Hoare M et al. 2010).

3.2 Molecular pathways involved in senescence regulation

As explained above, several conditions induce cell senescence, which in turn prevents replication of damaged cells. Several pathways are activated to modulate senescent phenotype under these conditions.

3.2.1 The p53 and Rb pathways

The transcription factor p53 participates in many processes including cell cycle, cellular stress responses and DNA repair. After the induction of cellular damage, p53 becomes activated as a protection mechanism to halt cell proliferation and induce DNA repair. If the damage cannot be repaired, the cell enters in apoptosis or in senescence depending on the intensity of the stress (Childs BG et al. 2014). Many genes of the p53 pathway are involved in the cell cycle arrest and senescence induction, including *p21*, *PML*, *PAI-1* and *DEC1* (Qian Y et al. 2013, Rufini A et al, 2013). p53 activity is antagonized by many proteins such as HDACs (including sirtuins) and methyltransferases (Wang YA et al. 2005, Yi J et al. 2010). p53 plays a key role in the establishment and regulation of cellular senescence induced by different stimuli, such as oncogene-induced, replicative and genotoxic stress-induced senescence (Qian Y et al. 2013). Another key regulator of cell cycle arrest in senescence is the retinoblastoma (Rb) tumor suppressor. This protein is activated in parallel to p53 and binds to E2F proteins to suppress their transcriptional activity, thus inhibiting cell proliferation and inducing cell cycle arrest (Ben-Porath I et al. 2005).

3.2.2 The p38 MAPK pathway

The p38 MAPK pathway regulates several key biological events such as cell proliferation, apoptosis and development. This pathway is activated at the cellular level in response to stress (Cuenda A et al. 2007) and it has been described to trigger inflammation independently of the DNA damage response (Freund A et al, 2011). Additionally, p38 represses human telomerase (*hTERT*) expression during cellular senescence, and p38 inhibition can delay senescence (Harada G, et al. 2014). p38 interacts with p53 to drive cellular senescence (Qian Y et al. 2013) and participates in senescence induced by a wide range of stress conditions (Iwasa H et al. 2003). Many proteins can regulate this pathway upon senescence, including MAPK phosphatases (MKPs), Dual-Specificity Phosphatases (DUSP) or sirtuins (Chung KW et al. 2015, McHugh D et al. 2018).

3.2.3 The NF-κB pathway

This pathway controls a wide range of genes involved in inflammation, cell survival and immune response regulation (Hayden MS et al. 2008). Thus, NF-κB mainly regulates the inflammation induced in senescent cells (Jing H et al. 2014). NF-κB also interacts with p53 to promote senescence (Chien Y et al. 2011), and its activity is inhibited by sirtuins, among others (Yeung F et al. 2004, Salminen A et al. 2008). Besides, NF-κB inhibition delays *in vivo* DNA damage-induced senescence in mice (Tilstra JS et al. 2012).

3.2.4 The mTOR pathway

The mTOR pathway is involved in the response to nutrient availability and growth factor signaling to coordinate cell proliferation, cell growth, apoptosis and inflammation, among other processes (Weichhart T, 2018). mTOR regulates inflammation through NF-κB signaling (Xu S et al. 2013), inhibits autophagy and promotes diverse senescence phenotypes. Interestingly, mTOR does not respond to nutrient signaling during senescence, being constitutively active (Carrol B et al. 2017). Rapamycin, a widely known mTOR inhibitor, can inhibit cellular senescence by upregulating Nrf2 expression and autophagy and downregulating the inflammatory phenotype (Tai H et al. 2017, Wang R et al. 2017). *In vivo*, mTOR inhibition by rapamycin can prevent the increase of many senescence markers (Xu S et al. 2013). Furthermore, many papers have highlighted its involvement in general organisms aging, as it has been described that mTOR inhibition extends lifespan and delays the onset of age-related diseases in different model animals, including mice (Weichhart T, 2018).

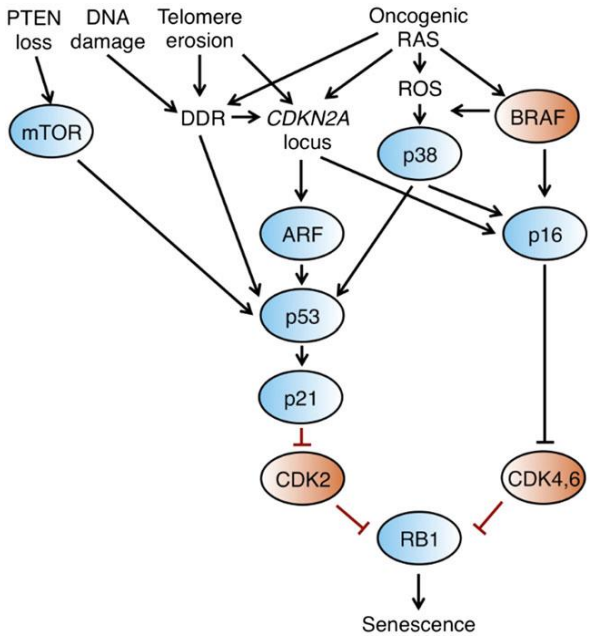


Figure 12. Molecular pathways involved in senescence establishment. Certain stimuli can induce the activation of one or more of these pathways to induce the development of cellular senescence (Lujambio A. 2016).

3.3 Hallmarks of cellular senescence

Senescent cells have many features at the molecular level that are a result of the damage and stress response. These hallmarks are used as biomarkers to identify senescent cells and to study its behavior both *in vitro* and *in vivo* (Hernandez-Segura A et al. 2018). Some of these biomarkers reflect the activation of the aforementioned pathways, while others are triggered as a consequence of the induction of the senescence state itself. Particularly, defined processes and properties of senescent cells are the following.

3.3.1 Morphological change

Senescent cells show typically increased cell size, increased nuclear size, and depending on the cell type, become multinucleated. This is probably due to the increase in RNA and protein content, which is caused by reduced protein degradation and decreased RNA turnover. Senescent cells also show increased number of vacuoles, cytoplasmic microfilaments and large lysosomal bodies (Cristofalo VJ et al. 1993, Nishio K, et al. 2001).

3.3.2 Cell cycle arrest

Senescence is defined as irreversible as there are no physiological stimuli that can reverse cell cycle arrest, meaning that these cells do not respond to mitogenic signals (Beauséjour CM et al. 2003, Kuilman T et al. 2009). Thus, long-term exit from the cell cycle is a central marker for senescent cells. This process is mainly induced by the activation of the p53/p21 and p16 tumour suppressor pathways. P53 exerts its cell cycle arrest effects through the upregulation of p21. It has been suggested that p21 is more involved in the establishment of senescence, while p16 has a role later on in the maintenance of the senescence phenotype (Stein GH et al. 1999, Van Deursen JM. 2014). Thus, p53, p21 and p16 are widely used to detect senescent cells (Atadja T et al. 1995, Serrano M et al. 1997, Bunz F et al. 1998, Campisi J. 2005). However, these changes are not specific for senescence as they can also be activated in an initial response to DNA damage or during transient cell cycle arrest (such as cellular differentiation), so they should be quantified along with other markers when monitoring cellular senescence (Kuilman T et al, 2010).

3.3.3 Induction of SA-β-Gal activity

This is the most common used biomarker for senescence. This activity corresponds to lysosomal β-galactosidase. In non-senescent cells, β-galactosidase activity can be detected optimally at pH 4, but upon senescence, it's also detected at suboptimal pH 6 (Dimri GP et al. 1995, Debacq-Chainiaux F et al. 2009, Lee BY et al. 2006). This is due to the increase in lysosomal content that occurs in senescent cells (Kurz DJ et al. 2000), which is at the same time caused by accumulation of non-degradable intracellular macromolecules and organelles in autophagic vacuoles (Brunk UT et al. 2002). Despite it is used as a biomarker, there is not clear evidence about the involvement of this enzyme in the senescence response (Lee BY et al. 2006). Furthermore, it should always be quantified along with other senescence biomarkers as constitutive β-Gal activity has been observed in nonsenescent cells (Kopp HG et al. 2007, Hall BM et al. 2017). At the skin level, both epidermis and dermis from elderly people shows an increase in SA-β-Gal activity (Dimri GP et al. 1995).

3.3.4 Chromatin rearrangement and nuclear changes

Despite the global loss of heterochromatin that occurs during aging, senescent cells can reorganize their chromosomes, leading to the formation of senescence-associated

heterochromatin foci (SAHF) (Wang J et al. 2016). SAHF are specialized domains of facultative heterochromatin that contribute to silencing of proliferation-promoting genes such as E2F target genes in senescent cells (Aird KM et al. 2013). Thus, the formation of these heterochromatin foci contributes to the characteristic exit of cell proliferation in senescent cells. In the case of SAHF, they are cell-type dependent and are mainly observed in oncogene-induced senescence (Kosar M et al. 2011). SAHF can be detected by the specific immunofluorescence of key factors, like enzymes, histone variants and histone post-translational modifications, such as H3K9me3, macroH2A, HP1-α/β/Y (Aird KM et al. 2013). The reduction of Lamin B1 has also been recently defined as a senescence marker as its downregulation by miR-23a can induce cellular senescence (Dreesen O et al. 2013). Besides, Lamin B1 is downregulated in senescent cells *in vitro* and *in vivo*, and this decrease in Lamin B1 levels is dependent on the p53 and p16 pathways but independent from other pathways previously mentioned like p38-MAPK, NF-kB, DDR and ROS (Freund A et al. 2012). The reduction of Lamin B1 induces chromatin structure changes through its essential interaction with heterochromatin, and thus modulating the expression of genes involved in cell proliferation and senescence (Lukášová E et al. 2017). In fact, mutations in the lamin gene family are associated with premature aging disease, like progeria (Taimen P et al. 2009).

3.3.5 Senescence-associated secretory phenotype (SASP)

Senescent cells also show changes in their transcriptome, which consequently translates in the secretion of many factors, like proinflammatory cytokines, chemokines and growth factors (Campisi J et al. 2005). Proinflammatory cytokines secreted by senescent cells have a negative impact in the microenvironment as they produce chronic inflammation, alter tissue structure and promote malignant cells proliferation (Dilley TK et al. 2003, Yang G et al. 2006). Moreover, some of these cytokines play a role in establishing and maintaining senescence (Kuilman T et al. 2008). All this group of secreted factors by senescent cells is known as the senescence-associated secretory phenotype (SASP). The early steps of SASP production include cytokines that promote cell cycle arrest, such as TGFβ, while late steps of SASP production include cytokines that induce a proinflammatory microenvironment and reinforce cell cycle arrest, such as IL-6 and IL-8. Other members of this secretory phenotype matrix metalloproteinases (MMPs), TNF-α, VEGF and PGE2 (Coppé JP et al. 2010). Many pathways have been identified to regulate SASP, including mTOR pathway, mitogen-activated protein kinase (MAPK) pathway, Notch signaling, the phosphoinositide 3 kinase (PI3K) pathway, GATA4/p62-mediated autophagy and JAK/STAT pathway (Soto-Gamez A and DeMaria M et al. 2017).

3.3.6 Metabolic reprogramming

One observation regarding the metabolic state of senescent cells showed that these cells have increased glycolysis (Bittles AH et al. 1984). This metabolic switch is present also in cancer cells, as Otto Warburg described decades ago (Warburg O 1956). Additionally, this switch is also associated to increased levels of adenosine diphosphate (ADP) and

adenosine monophosphate (AMP) compared to adenosine triphosphate (ATP) (James EL et al. 2015). These altered ratios activate AMP-activated protein kinase (AMPK), which induces cell senescence by activating p53 and inhibiting p16 and p21 degradation (Wiley CD et al. 2016). Despite this established relationship between senescence and glycolysis, it is not clear why senescent cells become more glycolytic (Wiley CD et al. 2016).

3.3.7 Immunogenic phenotype

Stress response induced during cell senescence triggers the expression of immunogenic-related proteins, which are poorly expressed in normal cells. These proteins, including NKG2D ligands, are recognised by immune cell receptors, which in turn induce cytotoxic response over senescent cells (Sagiv A et al. 2016). This phenomenon is known as senescence surveillance.

3.3.8 Activation of pro-survival pathways

Senescent cells are also characterised by having anti-apoptosis pathways upregulated compared to normal cells. These pathways include PI3K and the Bcl-2/Bcl-xL pathway, among others (Zhu Y et al. 2016). Drugs that target these pathways are called senolytics, because of its ability to induce apoptosis selectively on senescent cells (Zhu Y et al. 2015).

3.3.9 DNA damage

Another hallmark of senescent cells is a persistent DNA damage response (DDR) caused by the stress exposure. This persistent response is caused by the accumulation of irreparable damage, which can be monitored by detecting phosphorylation of H2AX at Ser 139 (γH2AX) foci. During the DDR, histone H2AX gets phosphorylated and marks the DNA damage site together with other proteins that participate in DNA repair. After DNA is repaired, the phosphorylation in γH2AX is removed along with the other protein complexes (Sharma A et al. 2012). Thus, γH2AX large foci indicate this persistent DNA damage that can't be repaired in senescent cells. This feature is not senescence-specific, as γH2AX foci are also formed during transient DNA damage (Toutfaire M et al. 2017). Senescent cells also harbor chromosome aberrations and a decline in DNA repair systems (Nucleotide excision repair -NER- and Base Excision repair -BER-) (Tigges J et al. 2014).

3.3.10 Loss of proteostasis and autophagy dysfunction

Proteostasis is the regulation of correct protein folding and the removal of damaged proteins by several systems including the proteasome and the autophagy ones. This process is altered in aged cells. Indeed, impairment of proteostasis induces cell senescence associated to a decrease in protein turnover mechanisms and accumulation of protein aggregates (Tigges J et al. 2014, Höhn A et al. 2017). For instance, proteasome function and autophagy are altered in UV-irradiated dermal fibroblasts (Bulteau AL et al. 2007). The link between autophagy and senescence is complex and not well understood

(Gewirz A. 2013). Some authors suggest that autophagy can activate and inhibit senescence depending on the functional context. Autophagy was initially described to have an anti-senescence role as it removes intracellular stressors that can induce senescence, such as damaged proteins or damaged mitochondria. However, some studies also reported a pro-senescent role of autophagy (Kwon Y et al. 2017). Some studies claim that autophagy activity increases in OIS, while others have shown that inhibition of autophagy facilitates senescence (Herranz N et al. 2018). Interestingly, autophagosome formation and its fusion with lysosomes are decreased in senescent cells (Kuilman T et al. 2010) and autophagy is impaired in oxidative stress-induced senescence (Tai H et al. 2017). One possible explanation to this dual role is that autophagy could degrade equally both activators and inhibitors of autophagy. Therefore, senescence could be favored or prevented depending on many variables like timing, duration or type of inhibition (Kang C et al. 2016, Kwon Y et al. 2017).

Another feature of senescent cells regarding proteostasis is endoplasmic reticulum (ER) stress. The accumulation of proteins that are not properly folded contributes to ER stress and induces an adaptive response named the unfolded protein response (UPR), which operates by decreasing general protein synthesis while inducing chaperones and autophagy machinery expression. The UPR occurs in all types of senescence, but the specific changes in this pathway are different depending on the senescence inducer, meaning that a UPR-related gene might be upregulated in OIS but not in stress-induced senescence. Despite ER stress and UPR are members of the senescent phenotype, it remains controversial if these are causes or consequences of senescence. Arguments in favor of UPR as a driver of senescence include the fact that an overactivity of ER could be a source of oxidative stress, which at the end could induce senescence. On the other hand, arguments in favor of UPR activation as a consequence of senescence include the increased demand of the ER activity to ensure the synthesis of the SASP proteins during senescence (Pluquet O et al. 2015).

3.3.11 Mitochondrial damage and mitophagy

The role of mitochondria in senescence is very complex. Mitochondrial dysfunction can induce cell senescence (Passos JF et al. 2006). Additionally, senescent cells show impaired mitochondria function, increased ROS production, accumulation of mtDNA mutations and altered mitochondria biogenesis (Tigges J et al. 2014). Specifically, a 4977-bd mitochondrial DNA deletion has been identified in senescent cells, and it is used as a marker to study them (Debacq-Chainiaux F et al. 2006). Damaged mitochondria accumulate in senescent cells due to impaired mitochondrial autophagy, named mitophagy (Brunk UT et al. 2002). The accumulation of these damaged mitochondria contributes to the metabolic dysfunction observed in aging (Herranz N et al. 2018).

3.3.12 Senescence-associated microRNAs

Some miRNAs are upregulated or downregulated in senescence and modulate this process. For example, mi-34a is regulates senescence through targeting different proteins such as E2F, MYC or SIRT1 (Abdelmohsen K et al. 2015).

Thus, when studying cellular senescence *in vitro* or *in vivo*, it is essential to monitor many of these molecular markers, as none of them is exclusive and specific for cellular senescence.

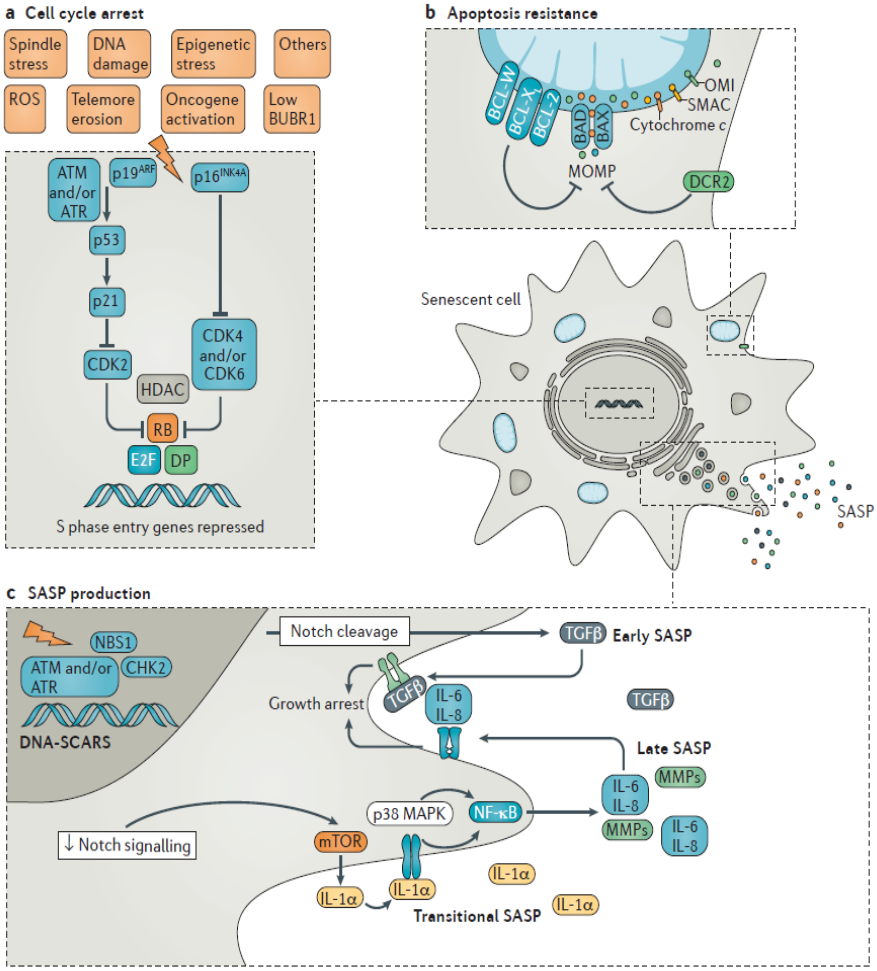


Figure 13. Some of the molecular features of senescent cells. (a) The cell cycle arrest is mediated by the p53 and p16 pathways. (b) The apoptosis resistance in senescent cells is mediated by the BCL-2 protein family. (c) The SASP production is initially regulated by Notch signaling during early steps to promote cell cycle arrest, while mTOR and p38 pathways regulate late steps inducing the secretion of proinflammatory cytokines (IL-6, IL-8) and matrix metalloproteinases (MMPs) (Childs BG et al. 2017).

3.4 Cellular senescence in skin

Regarding skin senescence, UV is the main senescence-inducing stressor in skin cells through direct DNA damage and increasing oxidative stress (Dupont E et al. 2013). However, cellular senescence is also observed in skin protected areas, which suffer chronological skin aging (Dreesen O et al. 2013). Among all the senescence features previously described, skin senescent cells present some of them, including typical senescent morphology, SA-β-gal activity, p16 overexpression, DNA damage foci, the SASP and the 977-bd mitochondrial DNA deletion (Debacq-Chainiaux F et al. 2012, Waaijer ME et al. 2012). Interestingly, cellular senescence is not a synchronized process, meaning that each cell will enter senescence depending on its individual background and experience. Thus, a mosaic of cells can be found in the skin (and other tissues), where

some cells are senescent and others not. In this context, a small number of senescent cells (around 20%) is enough to produce adverse effects in the tissue (Herbig U et al. 2006, Toutfaire M et al. 2017).
At the epidermal level, keratinocytes show features of replicative senescence, leading to an irregular and partial loss of epidermal proliferative capacity that contributes to the detrimental effects observed in aged skin. The epidermis is constantly renewing, so this physiological function, that relies on constant keratinocyte proliferation, is impaired by the increase in cell senescence. (Kohl E et al. 2011, Velarde MC et al. 2016).

At the dermal level, there are physiological situations where transient cellular senescence is induced and has beneficial effects. This is the case of wound healing, where senescence is temporarily induced to prevent excessive fibrosis and contribute to normal wound closure (He S et al. 2017). However, chronic accumulation of senescent fibroblasts presents a detrimental situation for the tissue. Senescent fibroblasts express the SASP, thereby secreting inflammatory cytokines and MMPs, which degrade matrix proteins such as collagen. Besides, these cells also show lower expression of the procollagen I gene, corresponding to the most abundant type of collagen in skin (Toutfaire M et al. 2017). Thus, this fibroblast phenotype induces tissue structure disorganisation and loss of function in the dermis (Kohl E et al. 2011). Moreover, the SASP of senescent fibroblasts contributes to age-associated hyperpigmentation, as senescent fibroblasts tend to accumulate in age-related pigmentation spots and elimination of these cells favours skin lightening (Yoon JE et al. 2018).

Cellular senescence also affects skin stem cells. Several studies have reported that skin stem cells show increased growth arrest, senescence and apoptotic cell death during aging (Zouboulis CC et al. 2008), which impairs the skin regenerative capacity. The main causes of this loss of stem cells during aging are telomere shortening, hormones level fluctuations and exogenous stressors such as UV light (Peng Y et al. 2015).

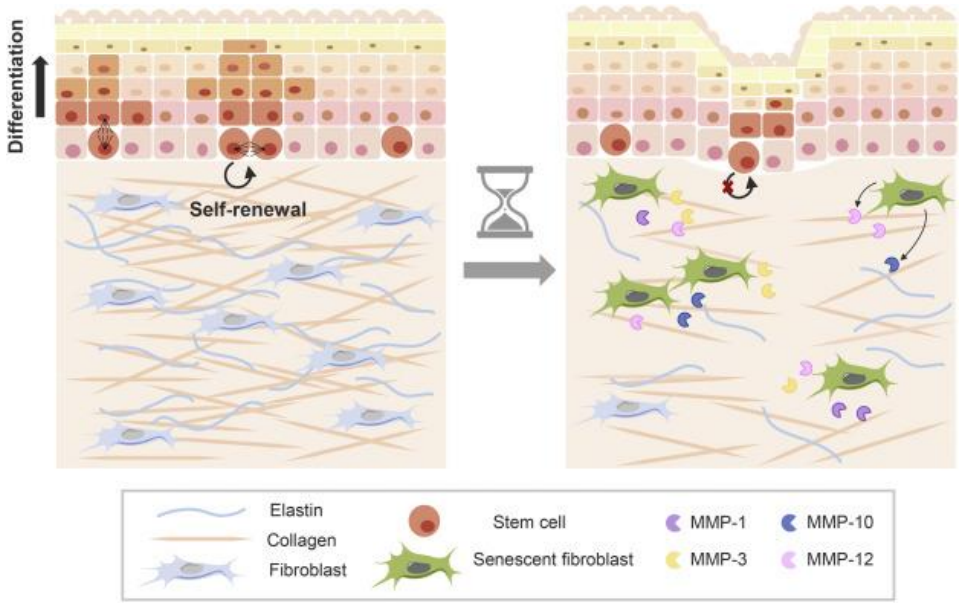


Figure 14. Aged skin shows a decrease in the self-renewal ability of stem cells and an accumulation of senescent fibroblasts, which secrete high levels of MMPs, promoting tissue damage and skin aging (Freitas-Rodríguez S et al. 2017).

3.5 Therapeutic strategies for cellular senescence

Several approaches have been proposed as therapeutic strategies to delay/treat senescence: senescence prevention, rejuvenation of senescent cells, cell removal by apoptosis induction or increased immune function and inhibition of senescence-associated secretory phenotype (SASP) (Malavolta M, et al. 2014).

3.5.1 Prevention of cellular senescence

This approach is based on interfering during the first steps of cellular senescence, aiming to protect the cells from the stressors that induce senescence (Toutfaire M et al, 2017). To this date, many compounds have been shown to prevent stress-induced cellular senescence (Malavolta M et al. 2014). In the case of skin, the use of UV filters and photoprotective compounds is a good option to prevent the emergence of senescence in skin, which clearly contributes to tissue degeneration and aging (Afaq F. 2011, Cavinato M et al. 2017).

3.5.2 Cell removal by apoptosis induction

Although the development of compounds that selectively kill senescent cells has just started, it has already generated some interesting results *in vitro* (Dörr JR, et al. 2013). These compounds are known as senolytics (Tchkonia T et al. 2013). Several pathways have been shown to keep senescent cells survival, including ephrins (EFNB1 or 3), PI3Kδ, p21, BCL-xL, or plasminogen-activated inhibitor-2 (Zhu Y, et al 2015, Wei W et al. 2018). Besides, a recent article shows how targeting FOXO4 and p53 interaction can induce specific death in senescent cells and rejuvenating effects in mice (Baar MP, et al 2017). However, senolytic research is facing several problems. First, senolytics described to date do not show activity against all senescent cell types, and it should be tested for each senescent cell type of interest (Soto-Gamez A et al. 2017). Second, the lack of universal senescence biomarkers and the heterogeneity of senescent cells is a great limitation regarding the search of novel compounds with senolytic activity (Toutfaire M et al, 2017).

3.5.3 Immune function activation

The increase in immune surveillance is also an interesting approach to selectively eliminate senescent cells (Huang MC et al. 2011, Mocchegiani E et al. 2013). A variety of immune cells, such as macrophages, NK Cells and T cells can be recruited to selectively induce death in senescent cells. An example of this approach is through the action of interferons (IFN), which can increase the expression of ligands in the NK cells to selectively recognize and induce the clearance of senescent cells (Katlinskaya YV et al. 2015).

3.5.4 SASP regulation

Finally, another promising approach is based on the modulation of SASP, which is responsible for promoting an aging phenotype in the senescent cell's microenvironment. Although there are not available modulators of SASP, some compounds that regulate pathways upstream NF-κB, such as mTOR, MAPK or PI3K or that interfere in the pro-inflammatory pathway mediated by NF-κB could be used for this purpose (Soto-Gamez A et al. 2017). Some compounds like metformin, resveratrol or cortisol have shown to regulate the SASP, but more studies are needed to clarify its effect *in vivo* (Moiseeva O et al. 2013, Menendez JA et al. 2011). However, a disadvantage of targeting SASP could be the inhibition of the inflammatory response necessary to eliminate senescent cells by the immune system (Xue W et al. 2007).

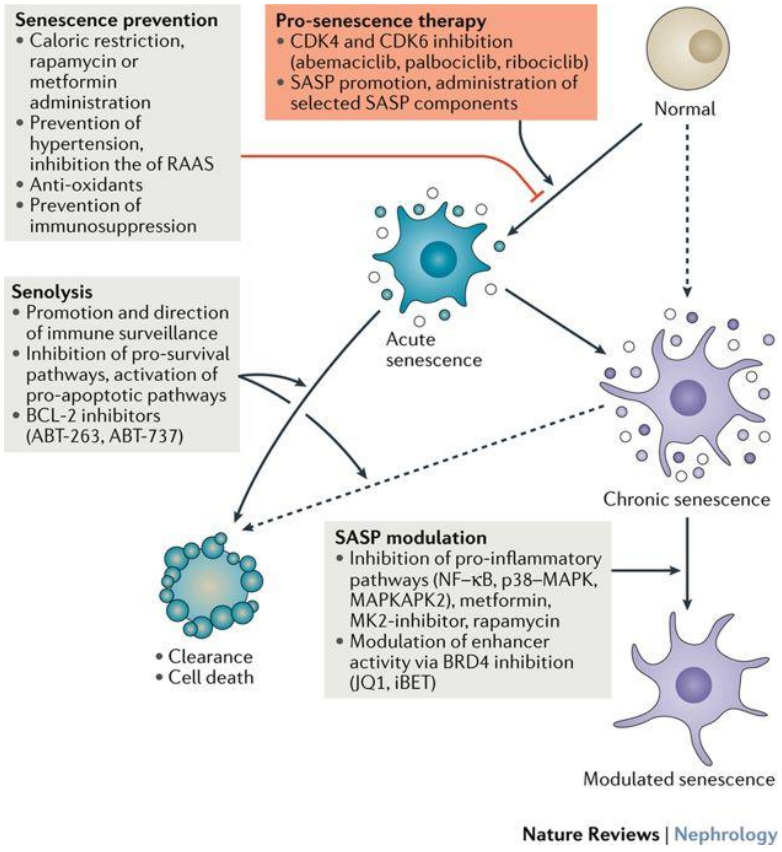


Figure 15. Therapeutic approaches for cellular senescence. Senescence prevention is achieved by protecting against the senescence-inducing stressor. Several drugs, such as rapamycin or metformin, and lifestyle interventions, such as caloric restriction, are included in this approach. Senolysis consists in the induction of senescent cells without affecting normal cells. Several drugs that modulate apoptotic and survival pathways, such as ABT-263 and ABT-737, and the stimulation of the immune system function are included in this approach. Finally, SASP inhibition prevents the chronic proinflammatory and tissue-detrimental effect of senescent cells. Several drugs that modulate NF-κB and p38-MAPK pathways, such as metformin, rapamycin and MK2 inhibitors, are included in this approach (Sturmlechner I et al. 2017).

In this context, sirtuins could play a role in preventing senescence by interfering in many cell processes that include telomere maintenance, autophagy, inflammation, DNA repair and antioxidant machinery. Moreover, the key role of sirtuins in a wide range of signaling pathways, mechanisms and functions of cellular processes and pathways (p53, p38-MAPK, mTOR…) make them very promising targets for the development of new anti-aging treatments for the skin.

Among all the processes mentioned previously, this project will focus on cell senescence induced by UV damage, which resembles the cellular senescence associated to skin aging (Debacq-Chainiaux F et al. 2012). UV-induced senescence not only stops proliferation of skin cells through p53 and p16, but also contributes to the secretion of the previously explained SASP, which induces chronic inflammation in skin leading to a process known as inflammaging (Zhuang Y et al. 2014, Sanada F et al 2018). This pro-inflammatory microenvironment favours skin tissue degeneration, through degradation of collagen and other matrix proteins, which are essential to maintain the skin firmness. This overall process impairs the normal function of skin and increases the number of wrinkles.

4. Skin

The skin is the first line of defence against disease, playing essential roles such as prevention of water loss, immune system function regulation, homeostasis maintenance, regulation of body temperature (Di Meglio P et al. 2011, Chiang A et al. 2012, Romanovsky AA et al. 2014). Reflecting the relevance of this organ, anything affecting skin's integrity can be harmful to the body (Bickers DR et al. 2006). Even non-life-threatening disorders can cause significant social and mental pathologies (Zhang S et al. 2018).

The skin is divided in three layers: the epidermis, the dermis and the hypodermis or subcutaneous tissue. The epidermis is the most superficial layer and it's mainly formed by keratinocytes, an epidermis-specific cell type that contributes to the epidermal structure and renewal process. At the same time, the epidermis can be divided into different sublayers: the basal layer, the spinous layer, the granular layer, the lucidum layer and the stratum corneum (Bennet RG et al. 2002). Keratinocytes proliferate and migrate from the basal layer to the stratum corneum acquiring different features. In the basal layer, the keratinocytes are highly proliferative, and as they reach the most external layer, they start differentiating. In the stratum corneum, the differentiated keratinocytes exert a protective function by preventing water loss. The epidermis also contains melanocytes, a cell type that produces the sun-protecting pigment known as melanin, and Langerhans cells, which are immune cells that recognise external antigens (Montagna W et al. 1979). The dermis is responsible for the thickness of the skin and is formed mainly by collagen fibers. The upper part of the dermis is called papillary dermis, characterised by greater cellularity and low density of collagen fibers. In contrast, the lower part is called reticular dermis, and shows less cellularity and high density of collagen fibers. The main cell type of the dermis is the fibroblast, which synthesises matrix proteins such as collagen, elastin, and degradative enzymes such as MMPs. Fibroblasts also produce glycosaminoglycans,

being hyaluronic acid the most abundant one in the dermis, which are responsible for hydration maintenance in skin. Other cells that can be found in the dermal layer are mast cells, vascular cells and immune cells (Khavkin J et al. 2011). The hypodermis mainly contains adipocytes, which are responsible for fat storage and hormone secretion. It also contains nerve and vascular cells (Baumann L. 2010).

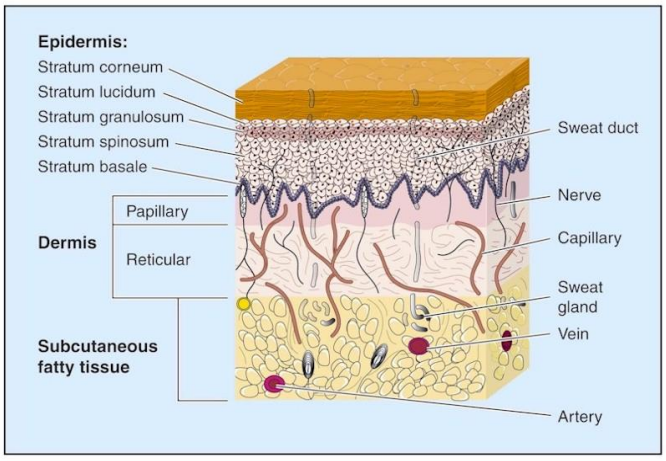


Figure 16. The different layers of human skin (epidermis, dermis and subcutaneous tissue or hypodermis) and its subdivisions.

4.1 Skin aging

Skin aging is defined as the degenerative process in which structural and physiological alterations cause an impairment of skin biological functions (Sjerobabski-Masnec I et al. 2010, Kohl E et al. 2011, Ganceviciene R et al 2012). Regarding skin aging, we can differentiate between two types depending on the origin of the damage that causes aging. On one hand, skin aging that depends on the passage of time and is influenced by the genetic background and hormonal levels is known as intrinsic aging. On the other hand, skin aging caused by external stressors such as UV light, pollution or tobacco smoke is known as extrinsic aging (Sjerobabski-Masnec I et al. 2010). Each of these types has both common and specific features. For example, both types of skin aging show increased levels of oxidative stress, in one case coming from internal damage (intrinsic aging) and in the other case coming from external sources (UV light, pollution). The specific properties of each type of skin aging are described below.

4.1.1 Intrinsic aging

Intrinsic aging, also known as chronological aging, is mostly detectable in areas of the skin that are not sun-exposed. This type of aging is characterised clinically by a thin and smooth skin with light wrinkles and loss of elasticity. The main factors regulating this type of skin aging are genetic background and hormonal levels (Tobin DJ 2017). Morphologic and functional changes include the following:

- Epidermal thinning: the epidermis becomes thinner (by 10% to 50%) due to the reduction in keratinocyte proliferation. There is also an increased heterogeneity in the size of keratinocytes from the basal layer. A reduction of lipids in the stratum corneum and an abnormal cholesterol synthesis contribute to a general dysregulation in skin permeability function (Zouboulis CC et al. 2011).

- Dermal thinning: the dermis also becomes thinner, caused by the decrease in the number of fibroblasts and atrophy of the extracellular matrix. This reduction in dermal thickness is also due to the decrease of skin appendages (sebaceous glands, sweat glands) and their functions (Branchet MC et al. 1990).

- Hormonal decline: Human skin cells produce several hormones, including growth factors, sexual hormones and vitamins, which are responsible of important roles such as wound healing, protection from oxidative stress or pigmentation, among others (Zouboulis CC et al. 2007). These hormones suffer a physiological decline during aging, which in turn compromises skin functions. First, it has been shown that a decline in estrogen levels produces a decrease in skin antioxidant defense and dysregulates all the wound healing phases (inflammatory, proliferative and remodeling phase), causing an increase in inflammation state, delayed re-epithelialization and less collagen deposition. Finally, the pigmentation process is also altered due to the hormonal level decline (Thornton MJ. 2013).

- Vasculature: this type of skin aging is associated to a reduction in the number and size of cutaneous microvasculature and its organization (Chung JH et al. 2007). The reactivity of the vessels to vasodilation or vasoconstriction also declines with age, while vascular stiffness increases (Bentov I et al. 2015).

- Pigmentation: the number of melanocytes decreases by 6-8% per decade. Consequently, skin pigmentation and tanning efficacy is reduced with age in sun-protected areas (Khavkin J et al. 2011).

- Immune cells dysfunction: Some authors have reported that the number of Langerhans cells decreases during aging (Zouboulis CC et al. 2011), but this is a controversial issue as other groups have observed a similar number of cells between young and old skin (Rittié L et al. 2015). However, all these groups have consistently observed less dendrite formation, less phagocytosis and less response to stimulus by TNF-α in these cells (Grewe M. 2001).

- Vitamin D synthesis: Vitamin D3 is produced in the skin through sun exposure and has a clear role in maintaining skin homeostasis, regulating cell proliferation, apoptosis and immune system, among others (Umar M et al. 2018). The capacity of the skin to produce this vitamin decreases with age (MacLaughlin J et al. 1985).

- Skin support system: Bone, cartilage and subcutaneous tissues influence the architecture of the skin. Bone demineralization, decreased fat cell size and impaired fat cell differentiation also contribute to aged appearance of the skin (Sjerobabski-Masnec I et al. 2010).

4.1.2 Extrinsic aging

Extrinsic aging, also known as photoaging, occurs in sun-exposed skin parts, being the face and the hands backside the most affected parts. Clinically, this type of aging is

characterised by an increase in the skin thickness, deeply marked wrinkles and irregular pigmentation (age spots). The main external source responsible for extrinsic aging is UV light, accounting for 80% of facial skin aging (Uitto J. 1997). Among the different types of UV, UVC is stopped by the ozone layer, and both UVA and UVB reach the skin. Particularly, UVB is absorbed in the epidermis and the upper dermis, while UVA penetrates deeply into the dermis. UVA generates reactive oxygen species (ROS) that can react with and damage intracellular proteins, lipids and DNA. On the other hand, UVB mostly affects DNA directly, apart from increasing the levels of ROS, thus inducing the formation of photoproducts such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) (Cadet J et al. 2015, Rinnerthaler M et al. 2015, de Jager TL. 2017). These modifications in DNA contribute potentially to the appearance of skin cancer (Pandel R et al. 2013). Morphologic and functional changes include the following:

- Epidermal hypertrophy: this happens as a protective response to UV-induced damage. Shortly after UV irradiation (mainly UVB), keratinocytes enter apoptosis, which can be identified by their pyknotic nuclei. These cells are known as sunburn cells (Bayerl C et al. 1995). Later, epidermal growth factor signaling increases in response to the apoptotic process, leading to an increase in keratinocyte proliferation. These proliferative keratinocytes accumulate and generate the epidermal hypertrophy, which protects better against UV penetration (D'Orazio J et al. 2013).

- Dermis: At the dermal level there is an accumulation of abnormal elastin fibers (known as solar elastosis), a decrease and more dispersed deposition of collagen fibers and an increased number of dysfunctional glycosaminoglycans (Zouboulis CC et al. 2011).

- Vasculature: As it happens in intrinsic aging, the decrease in dermal vasculature also occurs in extrinsic aging. In addition, the damage in connective tissue may also induce the disorientation of the vessels (Zouboulis CC et al. 2011). However, under acute UV exposure, new blood vessels form, contributing to the initial inflammation process. After chronic UV exposure, these vessels decrease in number.

- Immune cells: As it happens in intrinsic aging, Langerhans cells show impaired function. Moreover, there is increased number of mast cells and neutrophils, which account for local inflammation in photoaging (Zouboulis CC et al. 2011). However, there is a global immunosuppression effect exerted by UV light (Hart PH et al. 2018).

- Pigmentation: melanocytes are irregular and decreased in number, although this number is still high compared to intrinsic aging (Gilchrest BA et al. 1979, Ortonne JP. 1990). However, melanocyte activity is increased because of the UV-induced melanogenesis, which results in irregular pigmentation spots such as freckles and lentigines.

- Tumorigenesis: there is increased susceptibility to skin tumors due to the DNA damage generated directly by UVB and indirectly from ROS created by UVA and UVB.

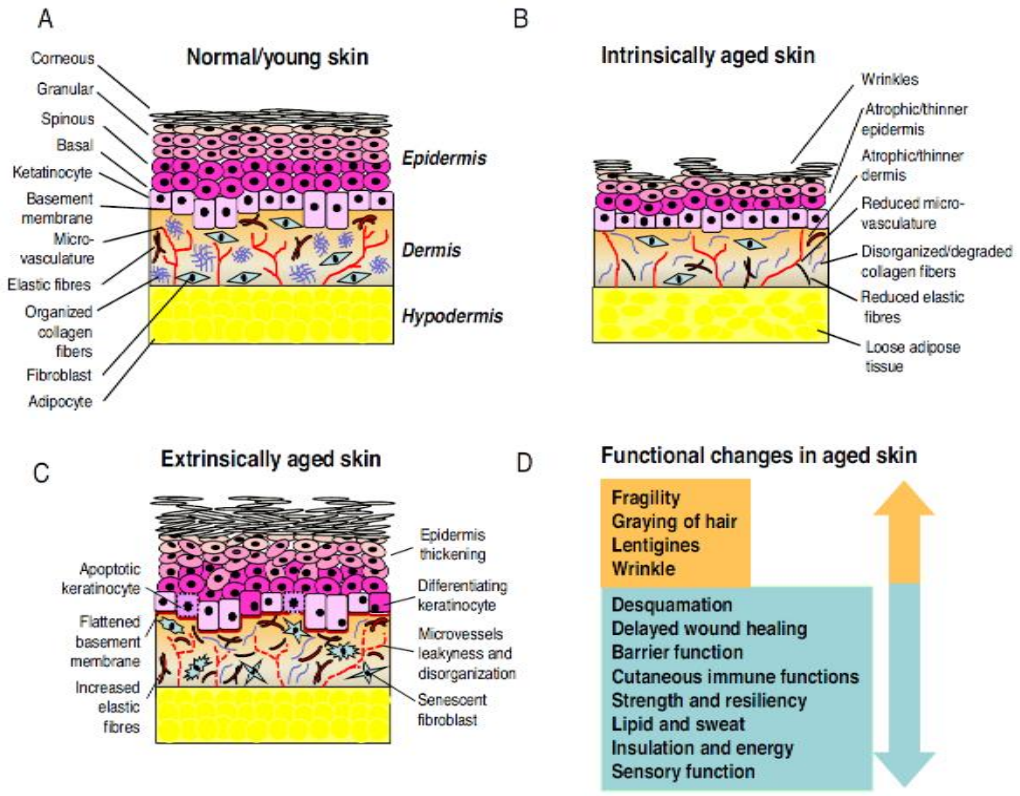


Figure 17. Morphological features of (A) young skin compared to (B) intrinsical (or chronological) and (C) extrinsical (or photoaging) aging, and (D) functional changes in skin aging (Mancini M et al. 2014).

4.2 Molecular hallmarks of skin aging

In this section, the most relevant changes that occur at a molecular level in skin aging are detailed. Some of these molecular features are similar in both intrinsic and extrinsic aging, while others are regulated differently in the two types of skin aging.

4.2.1 DNA damage and genome instability

Many intracellular molecules are altered by UV irradiation. In the case of UVB, it can directly produce rearrangements and modifications in DNA, such as CPDs, mutations and DSB (Rastogi RP et al. 2010). This damage is normally repaired by nucleotide excision repair (NER) and base excision repair (BER). The importance of the DNA repair response to UV is illustrated by diseases such as xeroderma pigmentosum, a pathology associated to NER-deficiency, that leads to bipyrimidine photoproducts accumulation, increasing the risk of skin cancer in up to ten thousand-fold (Cleaver JE et al. 2008).

The DNA repair capacity of the cells decreases during aging, leading to an accumulation of DNA damage and consequently increasing the susceptibility to skin carcinogenesis. One of the best-established markers of DNA damage is the level of phosphorylation of H2AX which accumulates in the damage site when the damage cannot be repaired (Sharma A et al. 2012). Both BER and NER capacity decline during aging (Takahashi Y et al. 2005, Pons B et al. 2010, Gaddameedhi S et al. 2011), and non-homologous end

joining (NHEJ) is less efficient in human fibroblasts aged *in vitro* (Seluanov A et al. 2004).

Telomere shortening has a key role in aging (López-Otín C et al. 2013). However, there is controversy regarding its specific role in skin aging. Some authors claim that telomere length is reduced during aging both in the epidermis and the dermis (Sugimoto M et al. 2006, Buckingham EM et al. 2011, Ikeda H et al. 2014) and that UV radiation can damage DNA leading to telomere loop disruption and telomere shortening (Gilchrest BA et al. 2009, Yin B et al. 2013), thus proving that telomere length is involved in skin aging. On the other hand, some authors showed that telomere lengths in photoaged and photoprotected skin are similar and that active telomerase activity is detected in proliferating basal keratinocytes in an age-independent manner (Härle-Bachor C et al. 1996, Krunic D et al. 2009), thus claiming that telomere shortening is not one of the causal factors in skin aging.

4.2.2 Epigenetic dysregulation

Facultative and constitutive heterochromatin are essential for normal cellular functions, as they maintain proper gene expression and structure of chromatin (Villeponteau B et al. 1997). Aged skin cells show global loss of heterochromatin and consequently an increase in activation of retrotransposons, which contributes to DSB and genome instability (Pal S et al. 2016). There are many features at the epigenetic level that are characteristic of skin aging:

- Histone methylation: Heterochromatin loss occurring in skin aging can be reflected by a decrease in histone 3 lysine 9 trymethylation (H3K9me3) and histone 3 lysine 27 trymethylation (H3K27me3). An important family of histone methyltransferases, the Polycomb Repressor Complex (PRC), is downregulated in skin photoaging (Cordisco S et al. 2010).

- Histone acetylation: Histone acetyltransferases (HATs), such as p300 and CBP, and histone deacetylases (HDACs) are involved in aging and senescence regulation (Orioli D et al. 2018). Among the HDACs, the class III or sirtuin family, can regulate several aspects of skin aging through is involvement in many cellular processes, including stress response, DNA repair and metabolism (further detailed in section 2). A decrease in SIRT1 activity has been described in intrinsically-aged human skin, mainly caused by the decrease of the cofactor that they use for its enzymatic activity, nicotinamide adenine dinucleotide (NAD^+). This reduction in NAD^+ levels is in part due to hyperactivation of the polyADPribosylase PARP-1, in order to counteract the high levels of oxidative stress in aged skin (Massudi H et al. 2012, Mangerich A et al. 2012). SIRT1 and SIRT6 expression levels also decrease in intrinsic skin aging. Regarding extrinsic skin aging, decreased SIRT1 activity has also been observed, together with increased p300 activity and histone H3 hyperacetylation (Ding S et al. 2018). Remarkably, human dermal fibroblasts show downregulation of SIRT1 and SIRT6 after UVB irradiation *in vitro* (Wahedi HM et al. 2016, Joo D et al. 2017).

- DNA methylation: At the skin level, a global hypomethylation of DNA is typically observed during aging, plus the hypermethylation of specific CpG islands (Grönniger E et al. 2010, Hovarth S 2013, Vandiver AR et al. 2015). This change in the DNA methylation pattern that occurs with age is known as epigenetic drift (Teschendorff AE et al. 2013, Jung M et al. 2015). DNMT1, a DNA methyltransferase, is also dysregulated in skin aging. DNMT1 dysfunction is associated with epidermal cell senescence and its expression inversely correlates with p16 in aged skin. Reduced expression of DNMT1 is associated with an increase in miR-377 occurs in photoaging and UVA induced senescent fibroblasts (Xie HF et al. 2017, Orioli D et al. 2018, Yi Y et al. 2018).

4.2.3 Extracellular matrix degradation

The characteristic increase in oxidative stress observed in skin aging activates several intracellular pathways, which include MAPK p38, JNK and ERK signaling pathways (Toutfaire M et al. 2017). These pathways activate, among several key regulators, the transcription factor AP-1, which in turn induces the upregulation of MMP-1, -3 and -9 and inhibits the effects of the collagen-promoting cytokine TGF-β (Kohl E et al 2011). Thus, there is a decrease in collagen content because of an increased degradation by MMPs and a decreased collagen synthesis due to the loss of response and downregulation of the TGF-β pathway (Shin JW et al 2019).

The number of functional elastin fibers is reduced in extrinsic aging, which results in an accumulation of dysfunctional elastin fibers, a process known as solar elastosis. This is due to a combination of overexpression of defective elastin isoforms together with downregulation of canonical elastin generation, and an increased activity of the elastin-degrading protein MMP-12 (Weihermann AC et al. 2017).

Hyaluronic acid (HA), which plays a key role in skin by maintaining matrix interactions and skin hydration, is also altered in aged skin. In extrinsic aging, the expression of hyaluronan synthases (HAS), the enzymes that produce hyaluronic acid, are decreased, while the expression of hyaluronidases (HYAL), the enzymes that degrade hyaluronic acid, is increased (Tzellos TG et al. 2009, Papakonstantinou E et al. 2012). These changes produce a downregulation in the levels of HA in the skin during aging (Röck K et al. 2011). As a consequence of this increased HA degradation, low molecular-weight HA levels increase, which induce inflammatory responses (Noble PW et al. 1996, Termeer C et al. 2002, Cyphert JM et al. 2015). Recent publications have described an important role of miRNAs in organismal aging, and in skin aging specifically (Smith-Vikos T et al. 2012, Mancini M et al. 2014). Particularly, miR23a-3p induces cellular senescence by targeting HAS2, which belongs to the previously mentioned family of enzymes that produce HA. miR-23a-3p expression is increased and HAS2 expression is decreased in aged fibroblasts and in the skin of old mice (Röck K et al. 2015).

Another feature that contributes to altered amounts and function of extracellular matrix proteins is the accumulation of advanced glycation end products (AGEs). AGEs are formed by the covalent binding of sugars, such as glucose or fructose, to proteins, lipids or nucleic acids, which impairs the normal function of the target molecules (Farrar MD. 2016). This process is different from glycosylation, a post-translational modification of

proteins involved in protein folding and stability. Glycated extracellular proteins are resistant to MMPs, which favors its abnormal accumulation in the skin, like in the case of solar elastosis (Yoshinaga E et al. 2012). Thus, in aged skin there is a decrease of functional collagen, due to increased degradation by MMPs and decreased gene and protein expression, and an increase in the accumulation of MMP-resistant and dysfunctional collagen (DeGroot J et al. 2001). AGEs formation is potentiated by UV light from the sun and its accumulation is also caused by a decrease in the enzymes that eliminate AGEs, such as glyoxalase 2 (Radjei S et al. 2016). Besides, AGEs also function as cell signaling molecules, as they can bind to specific receptors and activate many signaling pathways including MAPKs and NF-κB, thereby favoring microenvironment inflammation (Iwamura M et al. 2016).

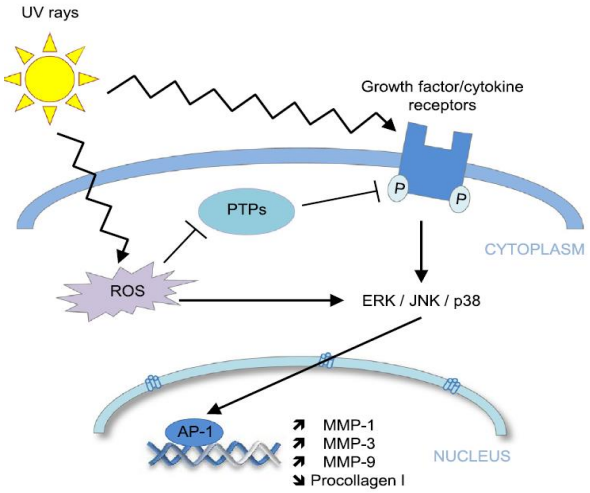


Figure 18. Molecular pathways involved in ROS-induced extracellular matrix degradation during extrinsic aging. UV rays induce the generation of intracellular ROS and activate growth factor/cytokine receptors. This induces the activation of MAPK pathways, including ERK, JNK and p38 pathways, which in turn induce the expression of MMPs and reduce the expression of procollagen I through the activation of the transcription factor AP-1 (Toutfaire M et al. 2017).

4.2.4 Mitochondrial dysfunction

The levels of mitochondrial ROS, the main source of intracellular ROS, are increased in aged dermal fibroblasts (Koziel R et al. 2011). Mitochondrial DNA (mtDNA) is a major direct target of ROS (Kohl et al. 2011). Consistently, mtDNA shows higher mutation rates than nuclear DNA, both because of the increased contact with ROS and the limited repair mechanisms of mtDNA (Wallace DC et al. 1999, Naidoo K et al. 2018). Both extrinsic and intrinsic aging are associated to increased mtDNA mutations, being photoaged skin particularly relevant in photoaged skin, possibly due to UV-associated generation of ROS (Berneburg M et al. 1997, Stout R et al. 2019). A frequent mitochondrial deletion of 4977 bp is found in photoaged skin, termed the "common deletion" (Berneburg M et al. 2004). Depletion of mitochondrial DNA results in oxidative stress and increased expression of MMP-1 (Schroeder P et al. 2008).

Increased mtDNA mutations lead to mitochondrial dysfunction, which stablishes a vicious cycle considering that dysfunctional mitochondria produce higher levels of ROS.

Mitochondrial dysfunction has been linked to other processes in skin aging such as pigmentation and wrinkle formation (Stout R et al. 2019). These dysfunctional mitochondria accumulate during aging, mainly caused by impaired removal by autophagy (Yen WL et al. 2008).

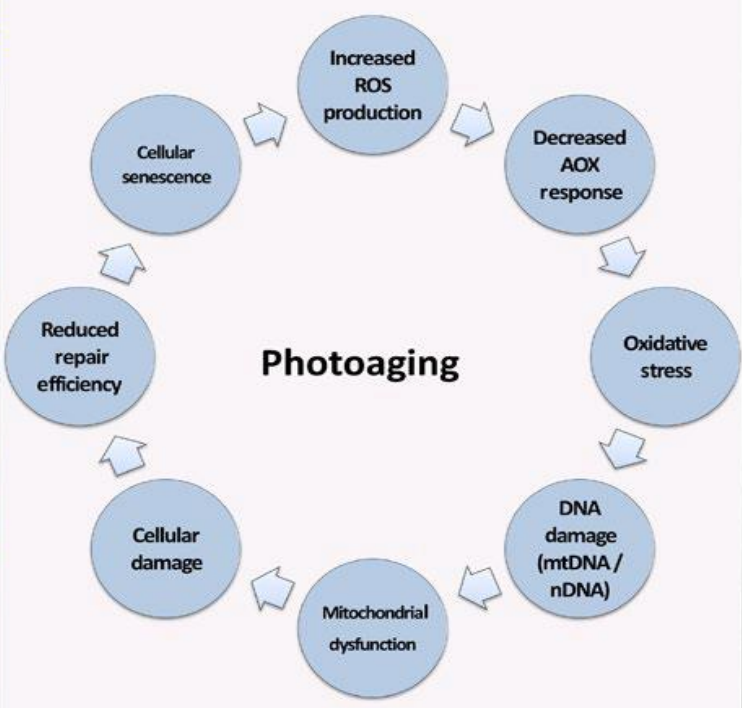


Figure 19. The vicious circle of oxidative stress and mitochondrial dysfunction that occurs in skin photoaging. The increase in ROS production leads to increased nuclear and mitochondrial DNA damage. Damaged mtDNA leads to mitochondria dysfunction, which ultimately promotes cellular senescence and increased ROS production, restarting the cycle (Naidoo K et al 2018).

4.2.5 Inflammaging

A chronic, low-grade level of inflammation has been described as another feature in skin aging. This process is known as "inflammaging", and it does not only play a role in skin aging, but also in many age-related diseases like Alzheimer's disease, cardiovascular diseases and diabetes, among others (Franceschi C et al. 2018). Inflammaging is caused by the increase in oxidative stress, which takes place in both intrinsic and extrinsic aging. Oxidative stress activates different pathways that end up in the activation of NF-κB, a master regulator of inflammation. This inflammation is not only induced by damaged keratinocytes or fibroblasts, but also by immune cells, such as macrophages, that are located in the skin and induced the release if proinflammatory cytokines and prostaglandins upon cellular damage (Zhuang Y et al. 2014). This inflammatory microenvironment contributes to skin tissue degeneration and aging (Zhang S et al. 2018).

4.2.6 Loss of proteostasis and autophagy dysfunction

Proteostasis refer to all the processes that include preservation of the stability of correctly folded proteins, proteolytic systems to eliminate damaged proteins, and regulation of the

aggregation of misfolded proteins. This entire process is impaired in skin aging (Höhn A et al. 2017). Proteasome function decreases in aged dermal fibroblasts (Tigges J et al. 2014), partially caused by lipofuscin, an aggregate of oxidized proteins, lipids and sugars that is resistant to proteases and accumulates in aged cells (Höhn A et al. 2011, Skoczyńska A et al. 2017). Loss of proteasome activity has also been linked to MMP-1 activation (Catalgol B et al. 2009).

Autophagy is a catabolic process through which damaged organelles and macromolecules are degraded and recycled in the cell. This elimination of damaged cytoplasmic contents facilitates cell survival and adaptation during starvation, genotoxic stress, and oxidative stress in normal cells (Kroemer G et al. 2010). The importance of autophagy in aging has been highlighted as (i) overexpression of autophagic genes promotes longevity in many species and (ii) many compounds with pro-longevity properties (metformin, rapamycin, spermidine) act through autophagy activation (De Cabo R et al. 2014).

Autophagy plays many context-dependent roles in UV response (Sample A. 2017). Upon genotoxic stress, autophagy is activated to mitigate the effects of DNA damage. In addition to removing damaged cellular components, autophagy is functionally associated to other pathways including DNA repair and the antioxidant cellular system involved in the response to UV-induced damage (Vessoni AT et al. 2013). This initial activation of autophagy after UV irradiation is dependent on key stress response proteins such as AMPK and p53, and the transcription of autophagy proteins is regulated by the FOXO family of transcription factors. Thus, autophagy inhibits apoptosis and promotes cell survival upon UV damage. However, in later steps, autophagy impairment has been described as an important feature of oxidative stress-induced senescence (Tai H 2016).

The levels of autophagosomes are similar in young and old dermal fibroblasts (Tashiro K et al. 2014, Kim HS et al 2018). Moreover, the expression of autophagy-related genes, like *Beclin-1* and *ATG5*, is similar between young and old dermal fibroblasts. Accordingly, the basal autophagic flux is similar between young and old dermal cells, but the higher speed and production of waste in old cells increases damage accumulation in these cells and contributes to skin aging (Kim HS et al. 2018). Other authors suggest that the decrease in autophagy function during aging is caused by post-translational modifications of autophagy-related proteins (Morselli E et al. 2011, Lapierre LR et al. 2015, Santos AL et al. 2017). The loss of autophagy function in aged dermal fibroblasts decreases the levels of collagen, elastin and hyaluronan, and increases the expression of *MMP-1* (Tashiro K et al. 2014).

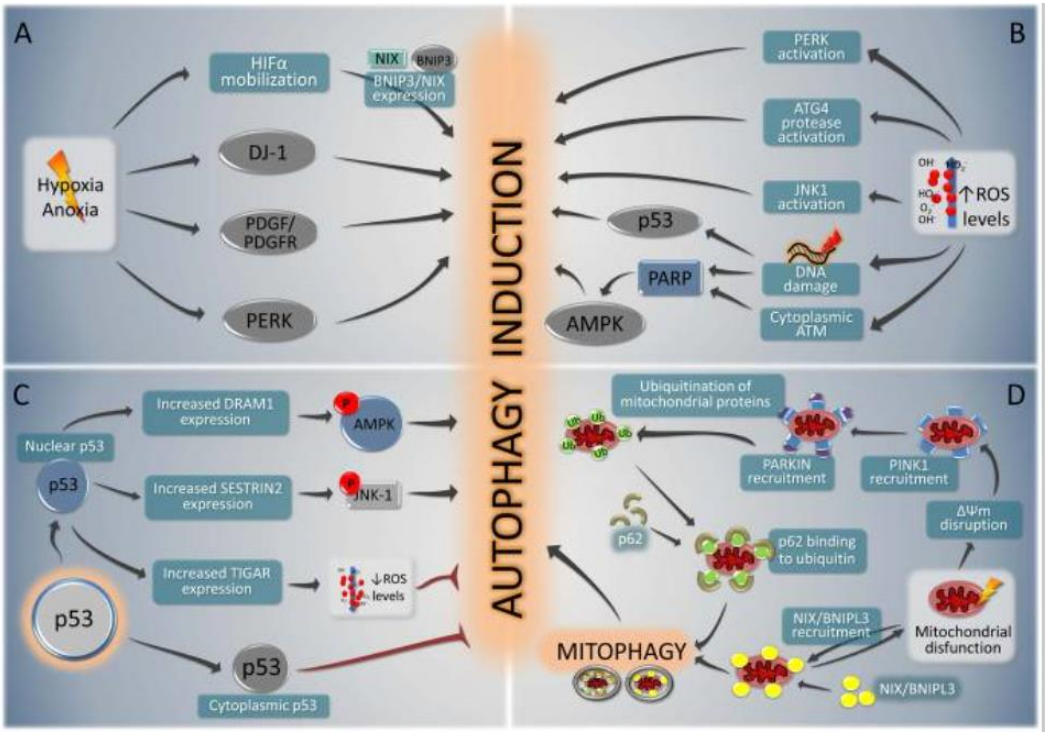


Figure 20. Overview of several stresses that induce autophagy. These stresses include hypoxia or anoxia, increased ROS levels, mitochondrial dysfunction and p53 pathway perturbation, which regulate different signaling pathways that end up inducing autophagy (Kroemer G et al. 2010).

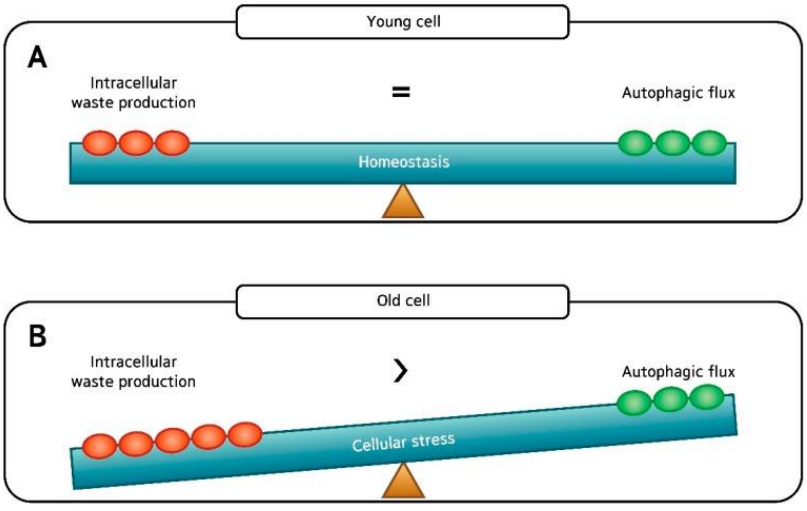


Figure 21. The balance between intracellular waste production and autophagic flux in young and old cells. In old cells, increased waster production with similar autophagic flux compared to young cells increases damage accumulation and impairs cellular homeostasis (Kim HS et al. 2018).

4.2.7 Cellular senescence

Chronic damage stimuli that take place in skin aging finally drive the cells into a state of irreversible growth arrest, known as cellular senescence. Senescent cells not only stop proliferating, but also have a pro-inflammatory phenotype (Van Deursen JM 2014). Senescent keratinocytes and fibroblasts accumulate during skin aging which, contributes to skin degeneration and loss of function (Campisi J 1998).

More detailed information about the inducers, features and pathways regulating senescence as well as the influence of cellular senescence in skin aging is included in section 3.

4.3 Skin anti-aging strategies

4.3.1 UV filters

Sunscreens are used to prevent UV-induced premature skin aging. We can find inorganic (or physical) filters, which block or scatter the UV radiation, and organic (or chemical) filters, which absorb UV radiation, enter an excited state and normally release the energy in the form of heat to return to its normal state (Sambandan DR et al. 2011). Inorganic filters cannot penetrate the skin, while organic filters are skin-permeable. Examples of inorganic filters include titanium dioxide and zinc oxide, while examples of organic filters include cinnamates and aminobenzoates (Mancuso JB et al. 2017).

4.3.2 Antioxidants

Another approach for skin aging is to deal with the reactive oxygen species that have been produced by the UV damage. For this purpose, cells have both enzymatic and non-enzymatic antioxidants to protect themselves against this damage. Enzymatic antioxidants include superoxide dismutase, catalase and thioredoxin, while non-enzymatic intracellular antioxidants include glutathione, carotenoids, coenzyme Q10 and niacinamide (Höhn A et al. 2017). These intracellular antioxidants are depleted after UV-induced oxidative stress, and therefore an exogenous supplementation of antioxidants is necessary to handle this stress (Kohl E et al. 2011). Examples of currently used antioxidants for skin include Vitamin C and E, carotenoids and polyphenols. However, antioxidant concentration must be precisely adjusted. Excessive supplementation of antioxidants has shown adverse effects, considering that small amounts of ROS are necessary due to its role as a cell signaling molecule (D'Autréaux B and Toledano MB. 2007, Zhang J et al. 2016).

4.3.3 DNA repair enzymes

Some authors have suggested that exogenous administration of DNA repair enzymes from animals or bacteria can exert positive effects in repairing CPDs induced by UV irradiation. These DNA repair enzymes can directly repair CPDs but are not present

naturally in humans. Consistently, the bacterial DNA-repair enzyme T4 endonuclease V (T4N5), which recognizes CPDs and removes the dimers, reduced the UV-induced MMP-1 expression in human keratinocytes (Dong KK et al, 2008). Liposomes containing photolyase, another DNA repair enzyme that is not present in humans, decreased the levels of UVB-induced CPDs, erythema and sunburn-cell formation when applied to the skin (Stege H et al. 2000).

4.3.4 Stem cell therapy

Given the key role that stem cells play in skin repair and regeneration (Chu GY et al. 2018.), many studies show promising results in the use of stem cells to prevent skin aging. Both autologous and allogenic adipose tissue transplantation improve many aspects of skin aging, and this action is carried out by adipose-derived stem cells (ADSCs) (Park BS et al. 2008). These cells secrete a plethora of growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF), which reverse skin aging (Kim WS et al. 2008, Choi JS et al. 2019).

4.3.5 Hormones

Hormones play a key role in skin aging as previously described, and hormone levels decrease with age, especially after menopause in women. Thus, it has been proposed that a hormone replacement therapy could work to prevent or treat skin aging (Dunn LB et al. 1997). Supporting this idea, topical application of estrogen has shown positive effects, including prevention of wrinkles, enhanced hydration, elasticity and collagen levels (Wolff EF et al. 2005, Sator PG et al. 2007, Rittié L et al. 2008).

4.3.6 Anti-aging peptides

The ability of peptides to penetrate the upper layer of the skin due to their small size has awaken the interest of the cosmetics industry to include them in skin anti-aging products. These peptides boost the production of collagen, elastin, hyaluronate, fibronectin and other matrix proteins and produce skin wrinkle improvements (Robinson LR et al. 2005, Husein El Hadmed H et al. 2016, Pai VV et al. 2017).

4.3.7 Chemical peelings

Chemical peelings are used to cause chemical ablation of defined skin layers to induce a regenerative and repair response (Ganceviciene R et al 2012). There are different types of chemical peelings, depending on the depth that they reach in the skin. This depth is influenced by the active used, its concentration, the pH of the solution and the duration of the treatment (O'Connor AA et al. 2018). At the level of the epidermis, chemical peelings induce superficial exfoliation and basal layer cellular proliferation and renewal. At the level of the dermis, chemical peelings induce the production of collagen, GAGs and increased skin hydration (Truchuelo M et al. 2017).

4.3.8 Fillers

Dermal fillers are used to fill skin wrinkles and replace the tissue that is lost with aging. They enchance the structural support in aged skin. These fillers restore contractile properties of aged fibroblasts and enhance the production of collagen fibers (Liu MH et al. 2019, Shin JW et al. 2019). Dermal fillers may contain autologous cells (adipocytes or fibroblasts), animal collagen, nutrients and vitamins, hyaluronic acid and synthetic or pseudo-synthetic compounds (poly-l-lactic acid, calcium hydroxylapatite) (Ganceviciene R et al 2012).

OBJECTIVES

In the dermocosmetic and medical aesthetics market, there are many treatment options to delay or prevent skin aging, many of which have been mentioned before. However, these treatments do not always provide a clear improvement. Thus, there is an increasing demand of effective anti-aging products. Importantly, customers pursue treatments supported on solid scientific evidences. Consequently, nowadays companies are not only engaged in screening for new anti-aging molecules but are also interested in understanding the molecular mechanisms that describe the effect of their products.

In this context, epigenetics has increasingly awakened the interest of this industry, as it plays a key dynamic role in the control of cell physiology, gene expression and genome stability, and it is directly associated to adaptation to environmental conditions. Among the wide range of factors that that exert their functions through epigenetic mechanisms, sirtuins stand out for their involvement in a wide range of cellular processes, including stress response, metabolism, inflammation, genome stability, chromatin regulation and cellular senescence, as previously described in the introduction. The accumulation of senescent cells promotes skin aging development, and thus sirtuins prove to be a good target to treat skin aging by protecting the cells against senescence. However, to this day, the number of reported sirtuin activators used in the skin cosmetic industry is very limited.

Therefore, the aim of this doctoral thesis was to identify novel compounds able to activate sirtuins. Based on previous reports, the main driving hypothesis of the PhD was that this activation should prevent skin cellular senescence induced by UV damage, a process that occurs during skin photoaging. The identified sirtuin activators with anti-senescent activity will be characterized and incorporated in the company portfolio or will be used to formulate new products with new scientific claims and improved efficacy, in order to meet the constantly-changing requirements of the cosmetic market. Additionally, all the laboratory techniques and know-how implemented during the development of this project will be a new tool for the company to perform future screenings to continue searching for anti-aging compounds with epigenetic mechanisms.

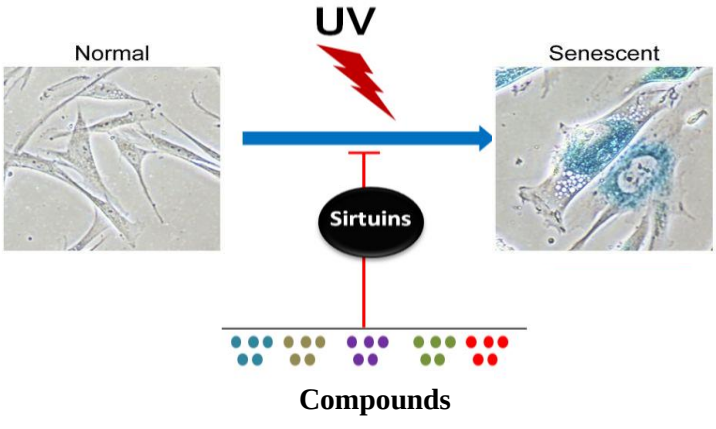


Figure 22. Scheme summarizing the aim of the project.

To develop this main objective, the PhD has been divided in the following specific aims:

- Establishment and optimization of human dermal fibroblasts cell culture.
- Selection of the compounds and determination of the optimal compound concentration in human dermal fibroblasts cell cultures through cell viability assay.
- Screening of compounds that activate sirtuins through (I) an increase of sirtuin gene expression, (II) sirtuin protein levels and/or (III) sirtuin enzymatic activity in human dermal fibroblasts cell cultures.
- Development of an *in vitro* model of UV-induced senescence in human dermal fibroblasts.
- Testing the compounds in the cell senescence assay to select the ones that can protect against UV-induced senescence in human dermal fibroblasts.
- Determination of whether the effect of sirtuin-activating compounds on senescence is sirtuin-dependent.
- Molecular characterization of the most promising compounds through the study of its effect on different cellular processes that are altered in UV-induced senescence (apoptosis, cell cycle and DNA damage accumulation) and signaling pathways related to sirtuins (autophagy, DNA repair and antioxidant enzymes among others).

MATERIALS AND METHODS

1. Cell culture

Human dermal fibroblast (HDF) (Promocell) from 40-year old caucasian women were used as the *in vitro* model for compound viability and efficacy screening. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) Low Glucose (Biowest) supplemented with 10% Fetal Bovine Serum (FBS) (Capricorn Scientific) and 10 U/mL Penicillin/Streptomycin (Biowest) in 150x20 mm culture plates in a humidified incubator at 37ºC and 5% CO_2. For cell passage, cells at 80% confluence were washed twice with phosphate buffered saline (PBS) (Capricorn Scientific) and incubated in 0.05% trypsin/0.025% EDTA (Biowest) for cell detachment. Fresh cell culture media was added to neutralize trypsin and cells were harvested and plated in a ratio of 1:3 plates in a 60% confluence. Cells between passage 13 and 18 were used for the assays.

For the SIRT1 *in vitro* assay, HEK293 -a cell line derived from human embryonic kidney- cells were used for the transfection and immunoprecipitation of SIRT1 protein and HeLa -a cell line derived from human cervical cancer- cells were used for the purification of hyperacetylated histones. These cells were grown in DMEM High Glucose (Gibco) supplemented with 10% FBS in 150x20 mm culture plates in a humidified incubator at 37ºC and 5% CO_2.

2. Compounds purchase and solution preparation

All the compounds used for the screening are detailed in Table 2, along with the purchase information and the solution preparation for cell treatment.

Active	Manufacturer	Solution preparation
Dexpanthenol	Alfa Aesar (#A18499.22)	500 mM in DMEM
Allantoin	Alfa Aesar (#A15571)	100 mM in DMEM
Idebenone	Sigma Aldrich (#I5659)	296 mM in ethanol and dilution to 1 mM in DMEM
Ferulic acid	Alfa Aesar (#A13890)	20 mM in DMEM
Lipoic acid	Sigma Aldrich (#T5625)	40 mM in DMEM
Gallic acid	Sigma Aldrich (#G7384)	5 mM in DMEM
Taurine	Sigma Aldrich (#T0625)	400 mM in DMEM and adjustment with NaOH to neutral pH
Salicylic acid	Sigma Aldrich (#S5922)	30 mM in DMEM
Shikimic acid	Alfa Aesar (#L04848)	100 mM in DMEM and adjustment with NaOH to neutral pH
Ectoine	Sigma Aldrich (#81619)	500 mM in DMEM
3,4-dihydroxybenzaldehyde	Alfa Aesar (#A11558)	5 mM in DMEM
Andrographolide	Tokyo Chemical Industry (#A2459)	250 mM in DMSO and dilution to 250 µM in DMEM
Kinetin	Alfa Aesar (#A13720)	100 mM in DMSO and dilution to 1 mM in DMEM

Table 2. Continued

Active	Manufacturer	Solution preparation
Zeatin	Tokyo Chemical Industry (#Z0012)	250 mM in DMSO and dilution to 4 mM in DMEM
Carnosine	Enzo Life Sciences (#ALX-153-055-G001)	250 mM in DMEM
Damascenone	Sigma Aldrich (#W342017)	Dilution to 250 µM from 45 mM commercial stock solution in ethanol
Betaine	Alfa Aesar (#B24397)	1 M in DMEM
Pyridoxine	Alfa Aesar (#A12041)	50 mM in DMEM
Verbascoside	Carbosynth (#OV08034)	2 mM in DMEM
Hamamelitannin	Extrasynthese (#0958)	41 mM in DMSO and dilution to 500 µM in DMEM
Phloretin	Enzo Life Sciences (#BML-EI154)	74 mM in DMSO and dilution to 300 µM in DMEM
Vanillin	Alfa Aesar (#A11169)	10 mM in DMEM
Sesamol	Sigma Aldrich (#S3003)	10 mM in DMEM
Salicylaldehyde Thiosemicarbazone	Sigma Aldrich (#658774)	200 mM in DMSO and dilution to 3 mM in DMEM
Sclareol	Tokyo Chemical Industry (#S0916)	30 mM in DMSO and dilution to 80 µM in DMEM
Gentiopicrin	Carbosynth (#MG09586)	7.5 mM in DMEM
Irisflorentin	Carbosynth (#FI73921)	50 mM in DMSO and dilution to 200 µM in DMEM

Table 2. Compounds chosen for this project with the manufacturer reference information and the solution preparation for cell culture treatments.

3. Cell viability assays

3.1 WST-1 assay

HDF were plated at a density of $3x10^3$ cells/well in 96-well plates. After 24h, culture medium was removed and replaced with fresh culture medium containing the compounds at different concentrations. After 24h, 10 µl of WST-1 reagent (Sigma Aldrich) were added to each well and the cells were incubated for 4 hours at 37ºC. Finally, the absorbance was measured at 450 nm using a Multiskan^{TM} FC Microplate Photometer (Thermo Scientific). The absorbance of each compound in cell culture media without cells was also measured to consider possible changes in the absorbance induced by the compounds alone. The experiments were performed in triplicate. The percentage of cell viability after compound treatment was calculated using the formula:

$$\frac{(Abs_{\ treatment} - Abs_{compound})}{(Abs_{\ control\ cells} - Abs_{\ culture\ medium})} \text{x}100$$

where:

$Abs_{\ treatment}$ corresponds to the absorbance of cells treated with the compounds

$Abs_{\ compound}$ corresponds to the absorbance of compounds in culture medium without cells

$Abs_{\ control\ cells}$ corresponds to the absorbance of non-treated cells (control)

$Abs_{\ culture\ medium}$ corresponds to the absorbance of cell culture medium without cells

3.2 Sulforhodamine B (SRB) assay

HDF were plated at a density of $3x10^3$ cells/well in 96-well plates. After 24h, culture medium was removed and replaced with fresh culture medium containing the compounds at different concentrations. After 24h, cell culture supernatant was removed by gently tapping onto paper towel. Cellular fixation was performed by adding 100 µl of trichloroacetic acid (TCA) 10% per well and incubating 1 hour at 4ºC. TCA solution was removed by gently tapping onto paper towel and plates were immersed twice in distilled water and tapped in paper towel to remove excess water. Cells were allowed to dry for 1 hour at room temperature. Then, 100 µl of SRB (Sigma Aldrich) solution (0.057% W/V in 1% acetic acid) were added to each well and plates were incubated 30 minutes at room temperature in the dark. The SRB solution was removed by tapping the plates onto paper towel and cells were washed twice with 1% acetic acid. Cells were allowed to dry for 1 hour at room temperature. Finally, 100 µl of Tris 10 mM (pH 10) were added to each well and pipetted up and down to dissolve the bound SRB reagent. Absorbance was measured at 540 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific). The experiments were performed in triplicate. The percentage of cell viability after compound treatment was calculated using the formula:

$$\left(\frac{Abs_{\ treatment}}{Abs_{\ control\ cells}}\right) \text{x}100$$

where:

$Abs_{\ treatment}$ corresponds to the absorbance of cells treated with the compounds

$Abs_{\ control\ cells}$ corresponds to the absorbance of non-treated cells (control)

3.3 Trypan blue assay

HDF were plated at a density of $6x10^4$ cells/well in 6-well plates. After 24h, culture medium was removed and replaced with fresh culture medium containing the compounds at different concentrations. After 24h, cells were washed twice in PBS and incubated in 0.05% trypsin/0.025% EDTA for cell detachment. Fresh cell culture medium was added, cells were harvested and centrifuged. Cells were resuspended in 500 µl of fresh cell

culture medium and 10 µl of cell solution were combined with 10 µl of Trypan blue solution (Sigma Aldrich) in an eppendorf tube. Ten microliters of the mix were pipetted into a hemocytometer. Total viable cells (not blue-stained) were counted under the phase-contrast microscope. The experiments were performed in triplicate.

4. Cell treatment for sirtuin and sirtuin-regulated pathways gene expression, protein levels and sirtuin activity determination

HDF were plated at a density of $4.8x10^5$ cells/plate in 100x20 mm cell culture plates. After 24h, cell culture medium was removed and replaced with fresh cell culture medium containing the compounds at different concentrations. After 24h (and 8h in the case of the autophagy markers), cells were detached using cell scrapers, harvested in 15-mL centrifuge tubes and centrifuged at 300 x g for 5 min. Supernatant was removed and cells were washed in 5 mL of cold PBS. Half of the cells were processed according to the qPCR protocol for gene expression determination and the other half was processed according to the Western Blot protocol for protein levels and histone modifications determination. Cell pellets were stored at -80ºC when not processed immediately.

5. UV irradiation

5.1 UVA irradiation protocol for senescence induction

HDF were plated at a density of $6x10^4$ cells/well in 6-well plates. After 24h, medium was removed and 2 mL of PBS were added in each well. Cells were irradiated at 20 J/cm2 of UVA light (365 nm) in a Bio-LINK Crosslinker BLX-312/365 (Witec ag), PBS was then removed and fresh cell culture medium was added to each well. This process was repeated once a day for 3 more consecutive days. After the last irradiation, cells were incubated in fresh cell culture medium for 3 days and then were trypsinized and reseeded at a density of $6x10^4$ cells/well in 6-well plates. After 2 days, cells were processed according to the β-Gal staining protocol. The experiments were performed in triplicate.

For the non-irradiation control conditions, HDF were plated at a density of $6x10^4$ cells/well in 6-well plates. After 24h, culture medium was removed and replaced by fresh cell culture medium. Cells were washed in PBS and incubated in fresh cell culture medium once a day for 3 more consecutive days. Cells were then trypsinized and reseeded at a density of $6x10^4$ cells/well in 6-well plates with fresh cell culture medium. After 3 days, cells were trypsinized again and reseeded at a density of $6x10^4$ cells/well in 6-well plates. After 2 days, cells were processed according to the β-Gal staining protocol. The experiments were performed in triplicate.

5.2 UVB irradiation protocol for senescence induction

HDF were plated at a density of $6x10^4$ cells/plate in 6-well plates. After 24h, culture medium was removed and replaced with fresh cell culture medium containing the compounds at different concentrations. After 24h, cell culture medium was removed and 2 mL of PBS were added to each well. The cells were irradiated at 25 mJ/cm2 of UVB

light (312 nm) in a Bio-LINK Crosslinker BLX-312/365 (Witec ag), PBS was then removed and fresh cell culture medium with the compound was added to each well. After 3 days, cell culture medium was removed and cells were irradiated again in 2 mL PBS at 25 mJ/cm2 of UVB light. After the irradiation, PBS was removed and cells were incubated in fresh cell culture medium with the compound. After 2 days, cells were processed according to the qPCR, β-Gal staining, apoptosis, cell cycle or immunofluorescence protocols. The experiments were performed in triplicate.

For the non-irradiation control conditions, HDF were plated at a density of $6x10^4$ cells/plate in 6-well plates. After 24h, culture medium was removed and replaced by fresh cell culture medium containing the compounds at different concentrations. After 24h, cells were trypsinized and reseeded at $6x10^4$ cells/plate in fresh cell culture medium containing the compound in 6-well plates. After 3 days, cells were trypsinized again and reseeded at $8x10^4$ cells/plate in fresh cell culture medium containing the compound in 6-well plates. After 2 days, cells were processed according to the qPCR, β-Gal staining, apoptosis, cell cycle or immunofluorescence protocols. The experiments were performed in triplicate

6. Gene expression quantification by qPCR

First, RNA was extracted from cells pellets using the Total RNA Purification Kit (Norgen) according to the manufacturer's protocol. Extracted RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific) and 300 ng of RNA were retrotranscribed to cDNA using the PrimeScript™ RT reagent kit (Takara) by incubating the mixture at 37ºC for 15 minutes (retrotranscription) and 85ºC for 5 seconds (inactivation of reverse transcriptase). cDNA was diluted 1/10 in ultrapure water and qPCR mix was prepared according to Table 3. The list of primers and their respective sequences are included in Table 4, except for the Bio Rad primers for which the sequence is unknown due to commercial purposes.

qPCR reaction mix	Amount of each reagent	Final concentration
1/10 dilution of cDNA	4 µl	12 ng
SYBR green supermix	5 µl	-
Forward+Reverse primer mix	0.2 / 0.5 µl*	200 nM
RNAse-free water	1 µl	-

Table 3. qPCR reaction mix preparation with the amount and final concentration of each reagent.

*For the PCR primers purchased form Sigma, 20 µM working solutions of forward and reverse primer were mixed and diluted 1/2, giving a 10 µM solution from which 0.2 µl were added to each well of the PCR plates. For the PCR primers purchased from Bio Rad, 0.5 µl of the commercial stock were added directly to the PCR plates.

Gene	Forward primer	Reverse primer
ATG5	5’-GTTTTGGGCCATCAATCGGAA-3’	5’-TCTCCTAGTGTGTGCAACTGT-3’
ATG7	5’-ATGATCCCTGTAACTTAGCCCA-3’	5’-CACGGAAGCAAACAACTTCAAC-3’
ATG12	5’-TAGAGCGAACACGAACCATCC-3’	5’-CACTGCCAAAACACTCATAGAGA-3’
Beclin-1	5’-AGCTGCCGTTATACTGTTCTG-3’	5’-ACTGCCTCCTGTGTCTTCAATCTT-3’
β-actin	5′-ACTGGAACGGTGAAGGTGACA-3′	5′-ATGGCAAGGGACTTCCTGTAAC-3′
Catalase	5’-CGTGCTGAATGAGGAACAGA-3’	5’-TTGACCGCTTTCTTCTGGAT-3’
DDB1	5’- TGAAGACCAAGGGCGACTTC-3’	5’- TGGCAGCGCTATCCTTTTGA-3’
Gadd45a	5'-CTGCACTGCGTGCTGGTGAC-3'	5'-TCCATGTAGCGACTTTCCCGGC-3'
HAS2	Bio Rad	Bio Rad
HO-1	5’-GCAGTCAGGCAGAGGGTGATAGAAG-3’	5’- TGGTCCTTGGTGTCATGGGTCAG -3’
IL-6	Bio Rad	Bio Rad
LC3	5’-GATGTCCGACTTATTCGAGAGC-3’	5’-TTGAGCTGTAAGCGCCTTCTA-3’
p21	5′-CTGGAGACTCTCAGGGTCGAA-3′	5′-CCAGGACTGCAGGCTTCCT-3′
p16	Bio Rad	Bio Rad
SIRT1	5’-TGGGTACCGAGATAACCTTCT-3’	5’-TGTTCGAGGATCTGTGCCAA-3’
SIRT2	Bio Rad	Bio Rad
SIRT6	5’-GCAGTCTTCCAGTGTGGTGT-3’	5’-AAGGTGGTGTCGAACTTGGG-3’
SIRT7	5’-ACTTGGTCGTCTACACAGGC-3’	5’-CAGCACTAACGCTTCTCCCT-3’
SOD1	5’-GAAGGTGTGGGGAAGCATTA-3’	5’-ACATTGCCCAAGTCTCCAAC-3’
SOD2	5’-GCGACCTACGTGAACAATCTGAACG-3’	5’-TCAATCCCCAGCAGTGGAATAAGGC-3’
ULK3	5’-GCCACCTTCTCAGGCAGAAT-3’	5’-GGCAGAGTCCCCAAATCTCC-3’
VMP1	5’-ACTCTTTTGCTGGAAGCGGT-3’	5’-GGAACACTGGCAAACCAACT-3’

Table 4. Primers used for qPCR assay.

qPCR was then run in a CFX Connect™ Real-Time PCR Detection System (Bio Rad) using a protocol including one step at 95ºC for 30 seconds and 40 cycles of 95ºC for 10 seconds and 60ºC for 1 minute. The analysis of qPCR results was performed using the Bio-Rad CFX Manager software. β-actin was used as a housekeeping gene for gene expression normalization. The experiments were performed in triplicate.

7. Protein levels quantification by Western Blot

Cells pellets were lysed in 1x Laemmli Buffer (Bio Rad) supplemented with 100 mM DTT by pipetting the cell solution and vortexing. The solution was incubated 10 minutes at 95ºC and centrifuged 10 minutes at maximum speed. The supernatant was placed in new eppendorf tubes and used immediately or stored at -20ºC. Ten microliters of Laemmli Buffer sample were run by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using running buffer (25 mM Tris, 192 mM Glycine and 0.1% SDS) in a PowerPac™ power supply (Bio Rad). The preparation of the separating gel according to its acrylamide content is detailed in Table 5, and the preparation of the stacking gel is detailed in Table 6. The percentage of acrylamide used for each protein analyzed is included in Table 7.

8% acrylamide separating gel	SIRT1, SIRT2, SIRT6, SIRT7, Tubulin
15% acrylamide separating gel	H3K9ac, H3, H4K16ac, H4, LC3B, p-AMPK, AMPK, p62, ATG5, Tubulin

Table 5. Separating gel preparation according to the percentage of acrylamide

Stacking Gel (10 mL)	**4 % acrylamide**
30 % Acrylamide/Bis (37,5:1)	1.33 mL
0,5 M Tris (pH 6,8)	2.5 mL
ddH2O	6 mL
20 % SDS	50 µL
10 % APS	100 µL
TEMED	10 µL

Table 6. Stacking gel preparation

Separating gel (20 mL)	**8% acrylamide**	**15% acrylamide**
30 % Acrylamide/Bis (37,5:1)	5.33 mL	10 mL
1,5 M Tris (pH 8,8)	5 mL	5 mL
Distilled H2O	9 mL	4.7 mL
20% SDS	100 µL	100 µL
10% APS	200 µL	200 µL
TEMED	20 µL	20 µL

Table 7. Percentage of acrylamide used in separating gel depending on the protein of interest

Gels were then transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS and 20% Methanol) at 100 V and 4ºC for 1 hour. Membranes

were blocked in blocking buffer (5% BSA + 0.1% Triton X-100 in 1x PBS) overnight at 4ºC. Membranes were washed twice in PBS-T (0.1% Tween 20 in 1x PBS) and incubated 2 hours in primary antibody (see Table 8 for dilutions of antibodies). Then, membranes were washed 3 times in PBS-T and incubated in secondary antibody for 1 hour. Finally, membranes were washed 6 times, 10 minutes each. For signal detection, membranes were incubated in Luminata™ Forte Western HRP Substrate (Millipore) for 1 minute, exposed to CL-Xposure films (Thermo Scientific) and revealed manually using developer and fixation solutions (Agfa). The quantification and analysis of western blot bands was performed using the ImageJ software.

Antibody	Dilution	Purchase information
Anti-H3K9ac (rabbit)	1:1.000	Cell Signaling (#9649)
Anti-H3 (rabbit)	1:10.000	Cell Signaling (#9715)
Anti-H4K16 (rabbit)	1:1.000	Millipore (#07-329)
Anti-H4 (rabbit)	1:10.000	Abcam (#ab10158)
Anti-SIRT1 (mouse)	1:1.000	Abcam (#ab7343)
Anti-SIRT2 (rabbit)	1:1.000	Abcam (#ab51023)
Anti-SIRT6 (rabbit)	1:1.000	Abcam (#ab62739)
Anti-SIRT7 (rabbit)	1:1.000	Cell Signaling (#5360S)
Anti-Tubulin (mouse)	1:20.000	Sigma Aldrich (#T5168)
Anti-LC3B (rabbit)	1:500	Cell Signaling (#2775S)
Anti-p62 (rabbit)	1:1.000	Abcam (#ab91526)
Anti-ATG5 (rabbit)	1:1.000	Sigma Aldrich (#A0856)
Anti-p-AMPK (rabbit)	1:1.000	Cell Signaling (#2535)
Anti-AMPK (rabbit)	1:1.000	Cell Signaling (#5831)
Anti-IgG rabbit (donkey)	1:3.000	Tebu bio (#611-7302)
Anti-IgG mouse (goat)	1:10.000	Sigma Aldrich (#A9917)

Table 8. Antibodies used during this project with their correspondent dilution and purchase information

8. β-Galactosidase (β-Gal) staining for senescence quantification

Cells were washed twice in PBS and fixed in 1 mL of 10% Formalin (4% formaldehyde) (Sigma Aldrich) for 3 minutes. Cells were washed twice in PBS and incubated in freshly prepared β-Gal solution at pH 6 (40 mM citric acid/sodium phosphate solution (pH 6.0), 150 mM sodium chloride, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride and 1 mg/mL X-Gal) overnight in the dark at 37ºC in a dry incubator (no CO_2). Finally, cells were washed twice in PBS and pictures were obtained

under bright-field microscopy using the 4x objective. Five pictures were taken for each condition, and the experiments were performed in triplicate.

9. Flow cytometry

9.1 Fluorescence-activated cell sorting (FACS) analysis of cell cycle using propidium iodide (PI) assay

Cells were harvested by trypsinization, washed twice in 1 mL of 1% FBS in PBS and centrifuged 1 minute at 500 g. Cells were resuspended in 0.5 mL of 1% FBS in PBS and were fixed by pipetting drop by drop in 5 mL of ice-cold 70% ethanol while slightly vortexing, in order to avoid cell aggregates. Samples were placed at -20ºC for 2 hours (or stored to continue the protocol later on). Cells were centrifuged 5 minutes at 800 g, the supernatant was removed and cells were washed in 1 mL of 1% FBS in PBS. Cells were centrifuged 5 minutes at 800 g and were washed again in 200 µl of 1% FBS in PBS. Finally, cells were resuspended in 430 µl of DNA staining solution (400 µl of 1% FBS in PBS, 25 µl of PI 1 mg/mL and 5 µl of RNAse 10 mg/mL) and incubated for 45 minutes at 37ºC in the dark. Samples were analyzed using a BD FACSCanto™ II cytometer (BD Biosciences). PI fluorescence was quantified by excitation at 488 nm and detection at 585 nm.

9.2 FACS analysis of cell cycle using 5-ethynyl-2′-deoxyuridine (EdU) assay

The EdU assay was performed using the Click-It™ EdU Alexa Fluor 647 Imaging kit (Invitrogen). Cells were harvested by trypsinization, washed twice in 1 mL of 1% FBS in PBS and centrifuged 1 minute at 500 g. Cells were resuspended in 0.5 mL of 1% FBS in PBS and were fixed by pipetting drop by drop in 5 mL of ice-cold 70% ethanol while slightly vortexing, in order to avoid cell aggregates. Samples were placed at -20ºC for 2 hours (or stored to continue the protocol later on). Cells were centrifuged 5 minutes at 800 g, the supernatant was removed, and cells were washed in 1 mL of 1% FBS + 2 mM EDTA in PBS. Cells were washed a second time in 200 µl of 1% FBS + 2 mM EDTA in PBS and centrifuged 5 minutes at 800 g. Cells were permeabilized by resuspending the cell pellet in 1 mL of 1% FBS + 2 mM EDTA + 0.5% Triton X-100 in PBS and incubated 20 minutes at room temperature in continuous agitation. Cells were washed twice in 200 µl of 1% FBS + 2 mM EDTA in PBS and incubated in 500 µl freshly prepared reaction solution (430 µl of Click-iT reaction buffer, 20 µl of $CuSO_4$, 1.2 µl of Alexa Fluor® azide and 50 µl of reaction buffer additive) for 30 minutes at room temperature in the dark. Cells were washed twice in 200 µl of 1% FBS + 2 mM EDTA in PBS and incubated in 500 µl of DNA staining solution (500 µl of 1% FBS + 2 mM EDTA in PBS, 25 µl of PI 1 mg/mL and 5 µl RNAse) for 30 minutes at room temperature. Samples were analyzed using a BD FACSCanto™ II cytometer (BD Biosciences). EdU fluorescence was quantified by excitation at 633/635 nm and detection at 660/20 nm, while PI fluorescence was quantified by excitation at 488 nm and detection at 585 nm.

9.3 FACS analysis of apoptosis using Annexin V assay

Apoptosis detection was performed by using Annexin V-FITC Apoptosis Staining / Detection Kit (Abcam). Cells treated with tert-butyl hydroperoxide (TBOOH) for 1 hour were used as positive control of apoptosis to define the viable cells, early apoptotic and late apoptotic/necrotic areas. Cells were harvested by trypsinization in cold medium. Cells were washed in 1 mL of cold PBS, resuspended in a mix containing 250 µl of cold 1X Binding Buffer and 2.5 µl of Annexin V-FITC and incubated 15 minutes at room temperature in the dark. Finally, 2.5 µl of PI (50 ug/ml) were added to each sample and samples were incubated for 5 minutes and analyzed using a BD FACSCanto™ II cytometer (BD Biosciences). Annexin V was quantified by excitation at 488 nm and emission at 530 nm and PI was quantified by excitation at 488 nm and detection at 670 nm.

10. γH2AX immunofluorescence assay

For this protocol, cells must be seeded in 6-well plates over coverslips for immunofluorescence (Thermo Scientific). Cells were washed twice in 2 mL PBS for 5 minutes each wash and fixed in 1 mL of 10% Formalin for 15 minutes at room temperature. Cells were then washed in 2 mL of PBS 3 times for 5 minutes each wash and permeabilized in 2 mL of 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Cells were washed twice in 2 mL of PBS and incubated in blocking solution (3% Goat serum + 0.2 M glycine + 0.2% Triton X-100 in PBS, filtered using a 0.22 µm filter) for 5 hours at 4ºC. Cells were washed twice in 2 mL of PBS and incubated in 8 µl of anti-γH2AX primary antibody from mouse at 1:500 (Abcam) per coverslip overnight at 4ºC. The next day, the cells were placed 1 hour at room temperature and washed afterwards 3 times in 2 mL of PBS + 0.5 % Tween 20 for 15 minutes each wash. From this point, samples were kept in the dark until the end of the protocol. Cells were then incubated in 8 µl of anti-IgG 488 secondary antibody from goat at 1:1000 (Abcam) per cubre for 1 hour at room temperature. Cells were washed 3 times in 2 mL of PBS + 0.5 % Tween 20 for 15 minutes each wash and incubated in 1 mL of DAPI (1 µg/mL) (Sigma Aldrich) for 4 minutes. Cells were then washed in 2 mL of H_2O MilliQ for 5 minutes and coverslips were fixed in 5 µl of Mowiol (Sigma Aldrich) on slides for several at room temperature until dried. Once the Mowiol was dried, slides were stored at 4ºC until analysis or analysed by confocal microscopy using a Confocal Leica SP5 microscope (Leica Microsystems) with the 63x objective. Five pictures were taken for each condition and experiments were performed in triplicate.

11. *In vitro* assay for SIRT1 activity

11.1 Transfection and immunoprecipitation of SIRT1

HEK293 cells grown in 150x20 mm plates were transfected with pcDNA4TO plasmid (SIRT1-flag) using 35 µg of DNA and 140 µL of polyethylenimine (PEI) 1mg/mL (4 µL per µg of DNA) per plate. Cells were grown at 37ºC, 5% CO_2 with humidity for a further 48 hours. Cells were harvested using cell scrapers and centrifuged at 300 xg for 5 minutes.

Cell pellets were stored at -80ºC or processed immediately by resuspending in 1 mL of Buffer A (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.1 mM PMSF, 0.5 mM DTT, 1 tablet of cOmplete™ Protease Inhibitor Cocktail). Lysates were centrifuged 30 seconds at maximum speed at 4ºC and the pellet, which contains the nuclei, was resuspended in 1mL of Buffer C (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 25% glycerol, 0.2% EDTA, 0.1 mM PMSF, 0.5 mM DTT, 1 tablet of cOmplete™ Protease Inhibitor Cocktail, Sigma Aldrich) plus 5 µL of Benzonase (1.25 U/ µL) (Sigma Aldrich) and incubated 6 hours at 4ºC in continuous agitation. Samples were centrifuged at 7000 xg for 10 minutes at 4ºC and the supernatant was incubated with Flag resin beads (50 µL per plate) overnight at 4ºC in continuous agitation. Then, samples were centrifuged 10 seconds at maximum speed and the resin beads were washed 5 times with BC500 buffer (20 mM Tris, pH 8.0, 500 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40). The SIRT1 protein was then eluted by incubation (in a 1:1 proportion) of the resin beads with 0.4 mg/ml Flag peptide diluted in BC100 buffer (20 mM Tris, pH 8.0, 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40) for 40 minutes at 4ºC in continuous agitation. The samples were finally centrifuged 10 seconds at maximum speed and the supernatant, which contains the eluted SIRT1 protein, was stored at -80ºC or used for the *in vitro* assay.

11.2 Hyperacetylated histone extraction

Hyperacetylated histones were used as the sirtuin substrate in the *in vitro* SIRT1 activity assay. These histones were purified from HeLa cells treated with 2mM Nicotinamide and 1 µM Trichostatin A (TSA) (Sigma Aldrich). After 24 hours of treatment the media was replaced with fresh media containing 1 µM TSA for 2 hours. Cells were washed twice with PBS and lysed in buffer A (10 mM Tris, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, 0.5 mM DTT, 1 tablet of cOmplete^{TM} Protease Inhibitor) by pipetting up and down and incubating for 10 minutes on ice, then centrifuged at 12,000 x g for 1 minute. The supernatant was removed and the pellets containing the nuclear fraction were resuspended with 0.5 M HCl (500 µL/ 10 cm^2 plate), mixed by vortexing and incubated on ice for 15 minutes. After centrifugation at 14,000 x g for 10 minutes at 4ºC, the supernatant was precipitated with TCA 20% (final concentration) and incubated on ice for 1 hour. After centrifugation at 14,000 x g for 10 minutes at 4 ºC, the pellet was resuspended with 100% ice-cold acetone and then centrifuged at 14000 x g for 10 minutes at 4ºC. The pellet was resuspended with BC100 and stored at -80ºC.

11.3 *In vitro* SIRT1 activity assay

The *in vitro* SIRT1 deacetylation assay was performed by incubating 500 ng of the purified SIRT1 enzyme with 500 ng of hyperacetylated histones and with or without 1 mM NAD^+ for 15 minutes at 37ºC. The reaction mixture also included 50 mM Tris pH 7.8, 4 mM $MgCl_2$, 0.2 mM DTT and Shikimic acid at 0-250 µM (dissolved in Tris 50 mM pH 7.8) to a final volume of 30 µl. The reaction was stopped by adding 5x Laemmli sample buffer supplemented with 10% β-Mercaptoethanol to a final concentration of 1x

Laemmli sample buffer. Histone deacetylation was then evaluated by quantification of H4K16ac normalized to H4 by Western blot.

RESULTS

1. Human dermal fibroblasts cell culture implementation

Our first objective was to establish the cell culture of HDF, the working model that we chose for this project. These cells are widely used in the cosmetics and dermatology field as they grow easily in culture and participate in a wide range of biological processes occurring in the skin, including wound healing, extracellular matrix synthesis or inflammation (Tracy LE et al. 2016). In our case, we chose HDF since they are key players in skin aging and are considered to be one of the best cellular models of skin senescence and aging, both at cellular and tissue levels (Kohl E et al. 2011). Specifically, in our studies we have used primary HDF obtained from 40-year-old Caucasian women, which is the main target of anti-aging products by the cosmetics industry. To grow the cells, we chose DMEM with low levels of glucose (1 g/L) supplemented with 10% FBS. We chose low glucose levels to grow these cells, in contrast to the regular 5 g/L used in regular DMEM medium because these conditions are closer to the physiological glucose levels in human blood. We determined that the population doubling time of our cells was approximately 29h. Also, these cells grew slower above passage 18, entering replicative senescence at passages 22-23. Cells in cultured are considered to reach replicative senescence when they are unable to complete one population doubling during a 4-week period that includes 3 consecutive weeks of re-feeding with fresh medium containing 10% FBS (Chen H, et al. 2013). Thus, to avoid the pre-senescent state and replicative cellular senescence, we used passages 13-16 for our experiments.

2. Compound selection for screening

The compounds selected for this project were chosen based on previous reports about their biological effects on sirtuins, sirtuin targets, senescence and/or skin aging. This list includes compounds that i) have been described to induce sirtuin activity or expression in model organisms or human cells; ii) compounds that protect against replicative or stress-induced senescence in other human cell types; iii) and compounds with proven efficacy in the prevention of skin aging but without a described effect on sirtuins and/or senescence, among others (Table 9).

Compound	Biological effect	References
Dexpanthenol	Promotes skin hydration and skin wound healing	Ebnef F et al. 2002
Allantoin	Promotes skin hydration and skin wound healing Induces AMPK activation in muscle cells	Chen MF et al. 2012 Kippel AP et al. 1977
Idebenone	Prevents skin photoaging Prevents oxidative stress-induced senescence in retinal cells Increases NAD^+ levels through NQO-1 in hepatocytes	McDaniel DH et al. 2005 Arend N et al. 2015 Haefeli RH et al. 2011

Table 9. *Continued*

Compound	Biological effect	References
Ferulic acid	Prevents skin photoaging Protects against UVB-induced DNA damage Induces FOXO3a activation in old rats	Saija A et al. 2000 Ambothi K et al. 2014 Choi YJ et al. 2012
Lipoic acid	Protects against PUVA-induced skin senescence Induces SIRT1 activation in liver of rats feed with high fat diet Induces SIRT1 activity in muscle cells	Briganti S et al. 2008 Valdecantos MP et al. 2012 Chen WL et al. 2012
Gallic acid	Prevents skin photoaging Activates AMPK/SIRT1/PGC1α pathway in mice	Hwang E et al. 2014 Doan KV et al. 2015
Taurine	Its depletion accelerates muscle senescence and shortens lifespan in mice	Ito T et al. 2014
Salicylic acid	Activates AMPK	Hawley SA et al. 2012
Shikimic acid	Protects against oxidative stress-induced damage in hepatocytes and neuroblastoma cells	Manna K et al. 2014 Rabelo TK et al. 2015
Ectoine	Prevents UVA-induced skin cells aging Induces cellular stress response pathways	Buenger J et al. 2004 Buommino E et al. 2005
3,4-dihydroxybenzaldehyde	Inhibits activity of JMJD2a histone demethylase and prolongs female *Drosophila* lifespan Inhibits oxidative DNA damage in NIH/3T3 cells	Nakagawa-Yagi Y et al. 2012 Jeong JB et al. 2009
Andrographolide	Inhibits inflammation and oxidative stress in neutrophils and endothelial cells	Shen YC et al. 2002 Lu CY et al. 2014
Kinetin	Prevents skin photoaging Delays aging features in skin fibroblasts	Wanitphakdeedecha R et al. 2015 Rattan SI and Clark BF. 1994
Zeatin	Delays aging features in skin fibroblasts Inhibits UVB-induced *MMP-1* expression in human skin fibroblasts	Rattan SI and Sodagam L. 2005 Yang B et al. 2009
Carnosine	Reduces telomere damage and shortening in lung fibroblasts Delays senescence in lung and foreskin cells	Shao L et al. 2004 McFarland GA and Holliday R. 1994
Damascenone	Prevents skin photoaging in mice	Uddin AN et al. 2012
Betaine	Suppresses age-related activation of NF-kβ in rats and human endothelial cells Enhances mitochondrial respiration	Go EK et al. 2005 Yi EY and Kim YJ. 2012 Lee I. 2015

Table 9. *Continued*

Compound	Biological effect	References
Pyridoxine	Participates in antioxidant defence and glutathione levels maintenance Protects against oxidative stress in rats and several cell types	Hsu CC et al. 2015 Kannan K and Jain SK. 2005 Mahfouz MM and Kummerow FA. 2004
Verbascoside	Shows antioxidant, skin wound healing and anti-inflammatory properties	Korkina LG et al. 2007 Alipieva K et al. 2014
Hamamelitannin	Protects against oxidative stress- and UVB-induced damage in skin cells	Masaki H et al 1995 Masaki H et al 1995 (b)
Phloretin	Increases *Sir2* expression in yeast Promotes lipolysis in mouse adipocytes Inhibits inflammation in human keratinocytes Induces *HO-1* expression and glutathione synthesis in rat hepatocytes	Xiang L et al. 2011 Huang WC et al. 2013 Huang WC et al. 2015 Yang YC et al. 2011
Vanillin	Protects against radiation- and UV-induced chromosomal aberrations in V79 cells Protects against UVB-induced senescence in human keratinocyte stem cells	Keshava C et al. 1998 Lee J et al. 2014
Sesamol	Prevents skin photoaging in mice and human dermal fibroblasts Promotes skin wound healing in rats	Sharma S and Kaur IP. 2006 Ramachandran S et al. 2010 Shenoy RR et al. 2011
Salicylaldehyde thiosemicarbazone	Shows antioxidant properties	Prabhakaran R et al. 2011 Nguyen Dt et al. 2013
Syringic acid	Reduces oxidative stress in rats Shows hepatoprotective, antidiabetic and cardioprotective properties in mice	Cikman O et al. 2015 Itoh A et al. 2010 Wei X et al. 2012 Kumar S et al. 2012
Sclareol	Used as a fragrance in the cosmetic industry Shows anti-inflammatory properties in mouse macrophages	Huang GJ et al. 2012
Gentiopicrin	Promotes wound healing in chicken fibroblasts Shows anti-inflammatory properties in rat chondrocytes	Oztürk N et al. 2006 Zhao L et al. 2015
Irisflorentin	Shows anti-inflammatory properties in mouse macrophages Induces proteasome activation in *C. elegans*	Gao Y et al. 2014 Chen YM et al. 2015

Table 9. Described biological effects on sirtuins, sirtuin targets, senescence and/or skin aging of the selected compounds.

3. Cell viability screening to obtain the optimal concentration of the compounds for cell culture

In order to test the effect of the compounds on sirtuin regulation, we first needed to determine the optimal dose at which we can incubate them in cell culture. We defined this dose as the highest dose of compound that does not compromise cell viability. In order to determine cell viability, we incubated the cells for 24 hours with the compounds at different concentrations and then performed the WST-1 assay. This assay is based on the ability of cells to metabolize the tetrazolium salt WST-1 into a colored dye by the action of mitochondrial dehydrogenase enzymes. Thus, this assay measures metabolic activity of the cells, which is reflective of the number of cells. The doses chosen for each compound were based on previous published reports that used these compounds. Thus, we obtained dose-response graphs for each compound with the effect of each dose on cell viability (Figure 23-24, Annex 1).

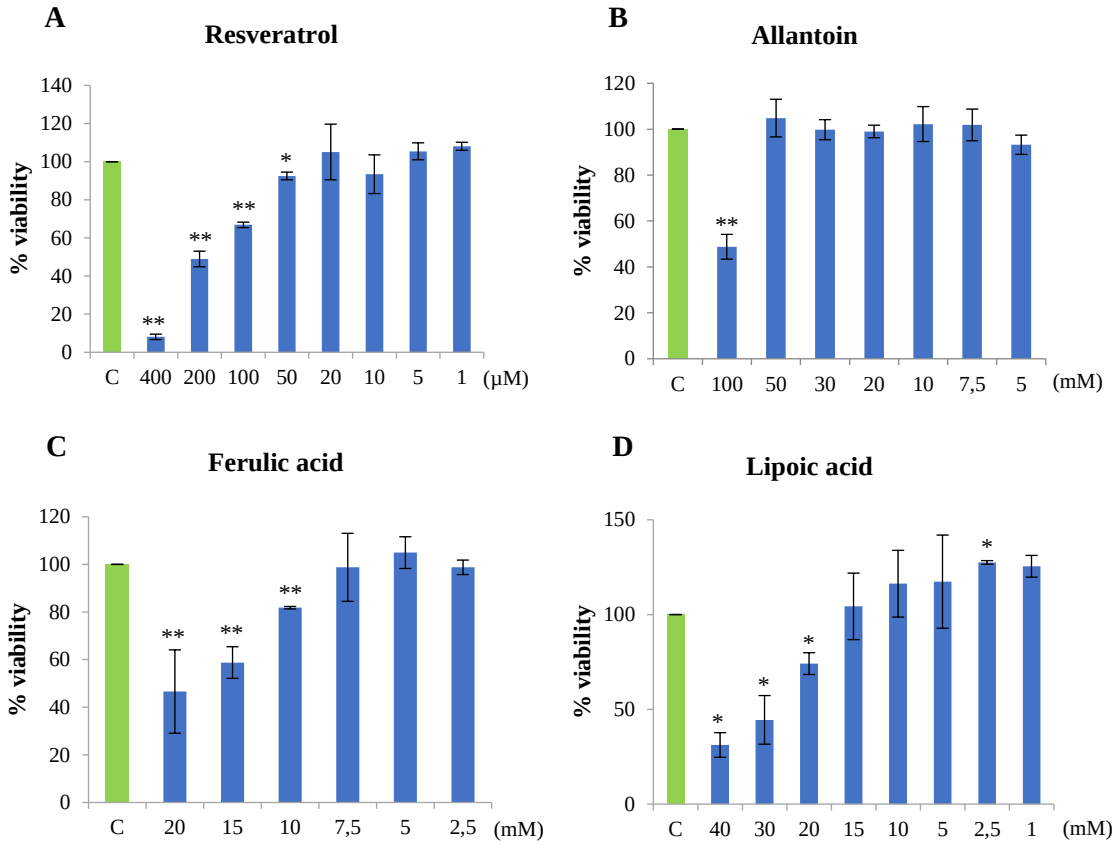


Figure 23. WST-1 assay performed in HDF after 24h treatment with (A) Resveratrol (1, 5, 10, 20, 50, 100, 200 and 400 µM), (B) Allantoin (5, 7.5, 10, 20, 30, 50 and 100 mM), (C) Ferulic acid (2.5, 5, 7.5, 10, 15 and 20 mM), (D) Lipoic acid (1, 2.5, 5, 10, 15, 20, 30 and 40 mM).The results of the rest of compounds can be found in Annex 1. Statistics: Student's T-test. Treated cells were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

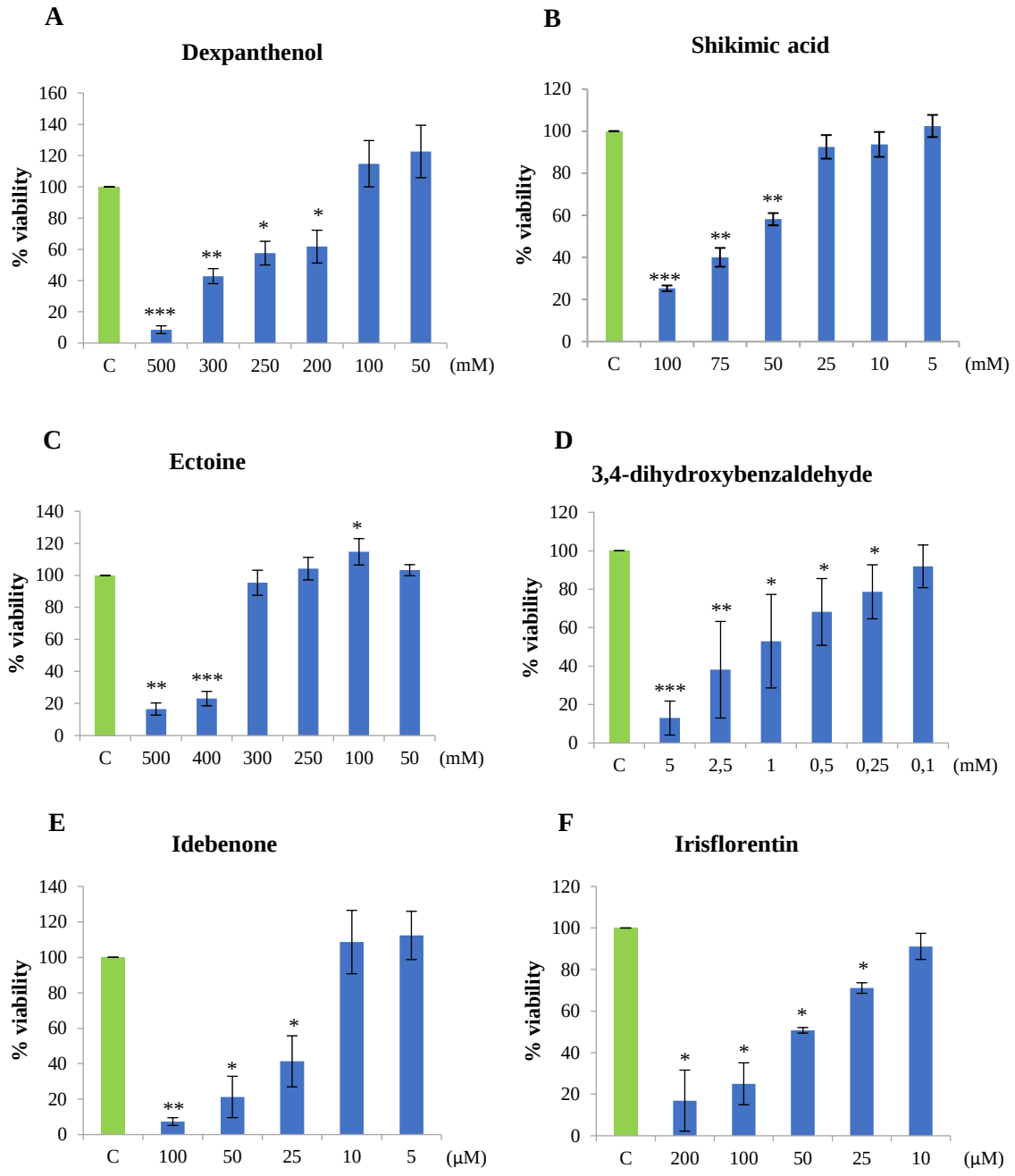


Figure 24. WST-1 assay performed in HDF after 24h treatment with (A) Dexpanthenol (50, 100, 200, 250, 300, 400 and 500 mM) and (B) Shikimic acid (5, 10, 25, 50, 75 and 100 mM), (C) Ectoine (50, 100, 250, 300, 400 and 500 mM), (D) 3,4-dihydroxybenzaldehyde (0.1, 0.25 0.5, 1, 2.5 and 5 mM), (E) Idebenone (5, 10, 25, 50 and 100 µM), (F) Irisflorentin (10, 25, 50, 100 and 200 µM). The results of the rest of compounds can be found in Annex 1. Statistics: Student's T-test. Treated cells were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

This assay provided valuable information, including two important parameters: the lethal dose 50 (LD_{50}) and the maximum non-cytotoxic dose. The LD_{50} is the compound concentration necessary for the reduction of the cell population to 50%, and it is useful to compare the toxicity among the different compounds. The maximum non-cytotoxic dose is the higher dose at which the compound does not cause any significant harm to the cells, and it is the maximum dose at which the compounds will be tested in the efficacy assays. The LD_{50} and the maximum non-cytotoxic dose for each active are represented in Table 10.

Compound	**LD_{50} (mM)**	**Maximum non-cytotoxic dose (mM)**	**Compound**	**LD_{50} (mM)**	**Maximum non-cytotoxic dose (mM)**
Resveratrol	0,173	0,05	Damascenone	0,121	0,05
Quercetin	0,604	0,25	Irisflorentin	0,049	0,01
Allantoin	100,000	50	Betaine	507,810	50
Idebenone	0,021	0,01	Pyridoxine	16,920	5
Ferulic acid	16,500	7,5	Gentiopicrin	13,825	2,5
Lipoic acid	34,690	1	Verbascoside	0,122	0,05
Phloretin	0,166	0,05	Hamamelitanin	0,247	0,1
Gallic acid	0,179	0,1	Sclareol	0,040	0.02
Taurine	396,580	150	Vanillin	5,912	1
Shikimic acid	58,44	25	Syringic acid	3,650	1
Salicylic acid	20,880	1	Sesamol	4,358	0,5
Carnosine	85,460	25	Salicylaldehyde thiosemicarbazone	4,175	0,5
Ectoine	359,120	300	Zeatin	2,462	1
3,4-dihydroxybenzaldehyde	1,080	0,1	Kinetin	1,835	0,5
Andrographolide	0,124	0,01	Dexpanthenol	262,480	100

Table 10. LD_{50} and maximum non-cytotoxic dose of the compounds after 24h treatment in HDF culture.

We observed that these compounds induced very different levels of toxicity in this cell type. Compounds like Dexpanthenol, Ectoine or Shikimic acid were very well tolerated by HDF (LD_{50} above 100 mM), in contrast to compounds like Idebenone, Irisflorentin or Sclareol (LD_{50} below 50 µM).

Previous reports on Dexpanthenol effect on human dermal fibroblasts show that this compound can be used up to 100 mg/ml (500 mM) (Weimann BI and Hermann D. 1999), which is 5 times the maximum amount tolerated in our cell cultures (100 mM).

In the case of Ectoine, previous studies showed a decrease in cell proliferation above 100 mM, while we observed high viability up to 300 mM. However, the quantification of cell viability in this study was performed after 3 days, which is a considerably longer than our incubation time (24h) (Rieckmann T et al. 2019).

Shikimic acid is widely used in cosmetics, as well as Dexpanthenol and Ectoine, and previous reports show no toxicity up to 500 µM in human dermal fibroblasts (Chen YH

et al. 2016), while it has been used at concentrations up to 10 mM in other cell lines (Rabelo TK et al. 2015, Chen X et al. 2018, Kim MJ et al. 2019). In our case, Shikimic acid does not affect cell viability up to 25 mM. Interestingly, despite these results indicated a progressive reduction in cell viability above 25 mM, optical microscopy visualization seemed to indicate that cell viability was not compromised up to 50 mM. This was also the case for 3,4-dihydroxybenzaldehyde, which seems to be viable up to 2 mM according to the optical microscopy visualization, compared to the 0.1 mM indicated by the WST-1 assay. One possible explanation for this difference is that these compounds might be regulating metabolism homeostasis, as WST-1 assay is based on the measurement of mitochondrial activity to assess cell viability (Stepanenko AA and Dmitrenko VV. 2015).

On the other hand, the effect of Idebenone on cell viability matches with other studies, which use this compound in the range of 1-10 μM (Dong KK et al. 2007, Angebault C et al. 2011). Irisflorentin, which also has a low maximum tolerated dose in our cells (10 μM), has not been tested in HDF as far as we know. However, it has been used up to 50 μM in other cell lines (Gao Y et al. 2014). Finally, other studies use Sclareol up to 10 μM in human dermal fibroblast culture (Lee S et al. 2016, Park JE et al. 2016), while we have proven that it can be tolerated up to 50 μM.

4. Compound efficacy screening for regulation of sirtuin gene expression, protein levels and enzymatic activity

4.1 Effect of the compounds on sirtuin gene expression

Once we determined the cell viability of HDF in the presence of each compound, our first approach was to test whether the compounds promoted any alteration in sirtuin expression. For that, we chose the maximum non-cytotoxic dose and 2 more diluted concentrations as the optimal range to test the compounds in the cell cultures. Specifically, we focused our attention on SIRT1, SIRT2, SIRT6 and SIRT7, the four sirtuins with proven roles in aging and epigenetic regulation (Ghosh S et al. 2015, Anwar T et al. 2016). To assess the effect of the compounds on sirtuin gene expression, we used the qPCR assay. As a positive control, we used mild oxidative stress (30 μM) (Figure 24), which has been previously described to induce sirtuin expression (Bosch-Presegué L et al. 2011).

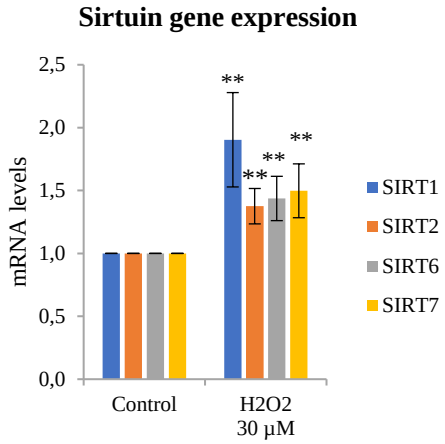
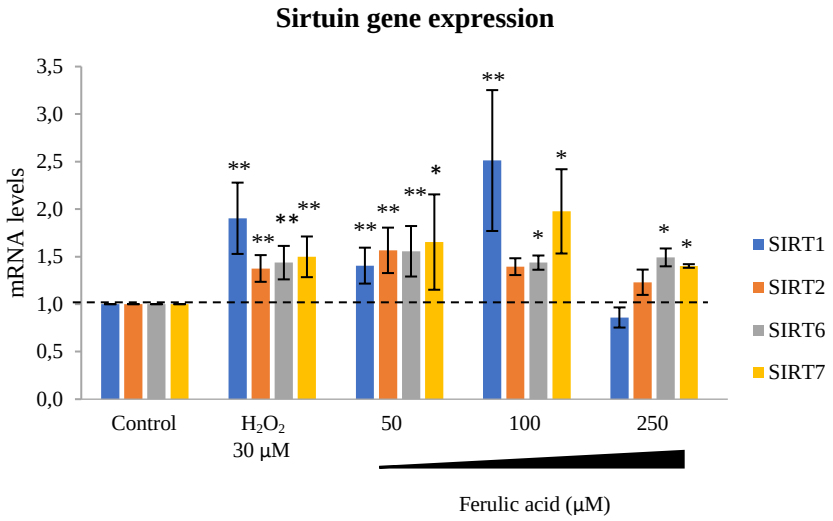


Figure 24. Sirtuin gene expression analysis by qPCR in HDF culture after the treatment with mild oxidative stress (H_2O_2). Cells were treated with H_2O_2 for 1h and then harvested for analysis. Statistics: Student's T-test. Cells treated with H_2O_2 were compared to control cells for each sirtuin gene (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Upon mild oxidative stress, all the sirtuins tested were significantly increased at the gene expression level, being *SIRT1* expression higher than the rest of the sirtuins. Once the technique was implemented, we carried out the screening of the compounds to find out if they could increase sirtuin gene expression. Interestingly, the optimal concentrations previously determined from the WST-1 assay had to be reduced for many compounds. This was produced by false negatives in the WST-1 assay, as the apparent maximum non-cytotoxic dose for many of these actives was indeed producing damage and cell death. For example, Idebenone initial optimal range to test in the cell culture was 10, 5 and 2.5 µM as determined by the WST-1 results, but afterwards it had to be diluted to 5, 2.5 and 1 µM to avoid cell toxicity.

Among the first actives to be tested, two of them, Ferulic acid and Lipoic acid, showed significant increase in sirtuin gene expression (Figure 25). In their case, the optimal range of concentrations had to be highly reduced, from 7.5 mM to 250 µM in the case of Ferulic acid and from 15 mM to 250 µM in the case of Lipoic acid due to cell death. These big differences might be due to an effect of these compounds on cell metabolism and mitochondrial activity, which can alter the WST-1 result, as previously mentioned for Shikimic acid and 3,4-dihydroxybenzaldehyde. Besides, the fact that mitochondria are involved in apoptosis indicates a link between cell death and mitochondria activity that could disrupt the WST-1 results.

A



B

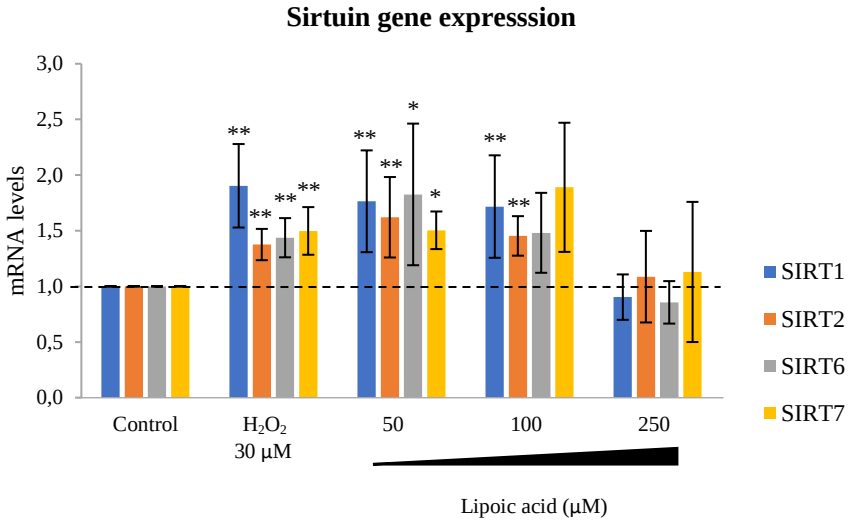


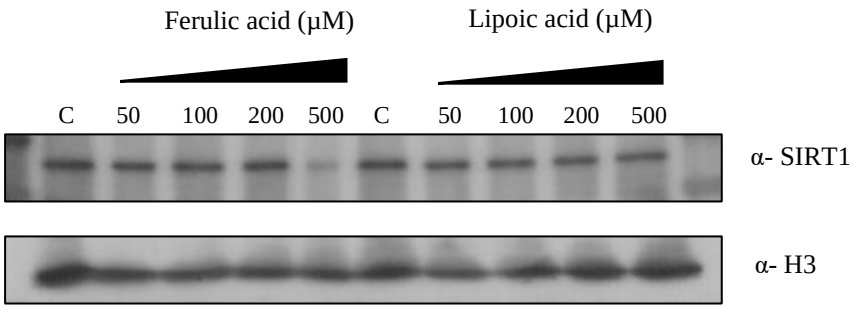
Figure 25. Sirtuin gene expression analysis by qPCR in HDF culture after 24h treatment with (A) Ferulic acid (50, 100, 250 µM) and (B) Lipoic acid (50, 100, 250 µM). H_2O_2 is used as a positive control of sirtuin upregulation. Statistics: Student's T-test. Cells treated with H_2O_2, Ferulic acid or Lipoic acid were compared to control cells for each sirtuin (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Both Ferulic acid and Lipoic acid increased *SIRT1*, *2*, *6* and *7* gene expression in a similar way than the positive control of oxidative stress (H_2O_2) (Figure 25). This increase was mild but statistically significant. Besides, for both compounds, lower doses (50 µM) were more effective than higher doses (250 µM), probably indicating that these higher doses are close to produce a detrimental effect on the cells.

4.2 Effect of the compounds on sirtuin protein levels

While we continued the screening of sirtuin gene expression modulation for the rest of compounds, we decided to test whether the compounds were also increasing sirtuin protein levels, as we planned in our initial aims. We started testing Ferulic acid and Lipoic acid, which induced sirtuin gene expression in our previous analysis (Figure 25). First, we determined SIRT1 protein levels in cells treated with Ferulic acid or Lipoic acid by using the Western blot assay (Figure 26).

A



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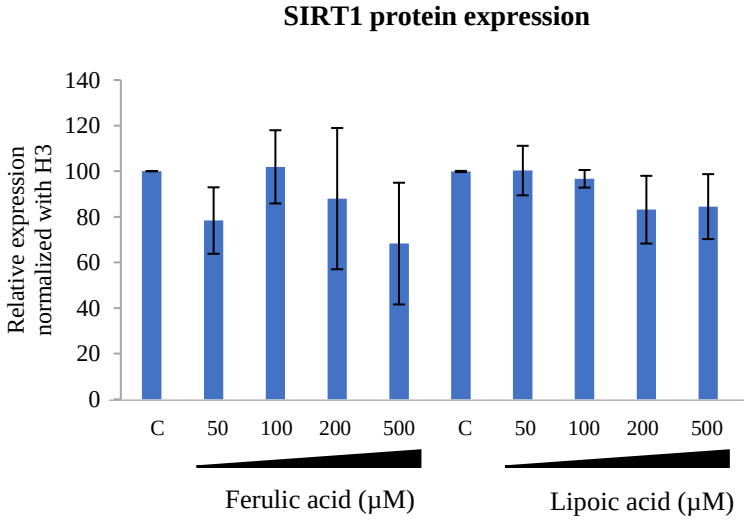


Figure 26. (A) Sirtuin protein levels analysis by Western blot in HDF culture after 24h treatment with Ferulic acid (50, 100, 250 µM) and Lipoic acid (50, 100, 250 µM). (B) Relative expression of SIRT1 in the cells treated with Ferulic acid or Lipoic acid compared to control (MC) and normalized with H3. Statistics: Student's T-test. Cells treated with Ferulic acid or Lipoic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

We observed that neither Ferulic acid nor Lipoic acid upregulated significantly SIRT1 protein levels in HDF after 24h treatment. Therefore, the observed increase in sirtuin gene expression does not result in an increase in protein levels, indicating that sirtuin gene expression was not a reliable technique to screen for novel sirtuin inducers. At this point,

we decided to focus the screening on the third proposed approach, which was to measure the effect of the compounds on sirtuins deacetylation activity.

4.3 Screening of the compounds based on detection of H3K9ac and H4K16ac as markers of sirtuin activity

In order to study the effect of the compounds on sirtuin deacetylation activity, we decided to quantify by Western blot the acetylation of lysine 9 in histone 3 (H3K9ac) and the acetylation of lysine 16 in histone 4 (H4K16ac) in the cellular extracts of HDF treated with the compounds for 24h. These two histone modifications are common targets of sirtuins and functionally relevant to their function (Martínez-Redondo P and Vaquero A. 2013). H3K9ac can be deacetylated by SIRT1 and SIRT6, while H4K16ac is a specific target of SIRT1 and SIRT2. Thus, decreased acetylation levels of any or both of these targets upon incubation with a compound would be a good indication of sirtuin activity.

To set up the assay, we first used previously described regulators of sirtuin activation as positive controls. Many compounds have been described to activate sirtuins *in vitro* (Dai H et al. 2018). Among these, Resveratrol and Quercetin are two of the first known sirtuin activators and have been widely studied (Howitz KT et al. 2003, Bai X et al. 2018). Specifically, Resveratrol and Quercetin target specifically SIRT1. Thus, we first titrated these two compounds to determine whether they could induce global changes in H3K9 and H4K16 acetylation levels.

A

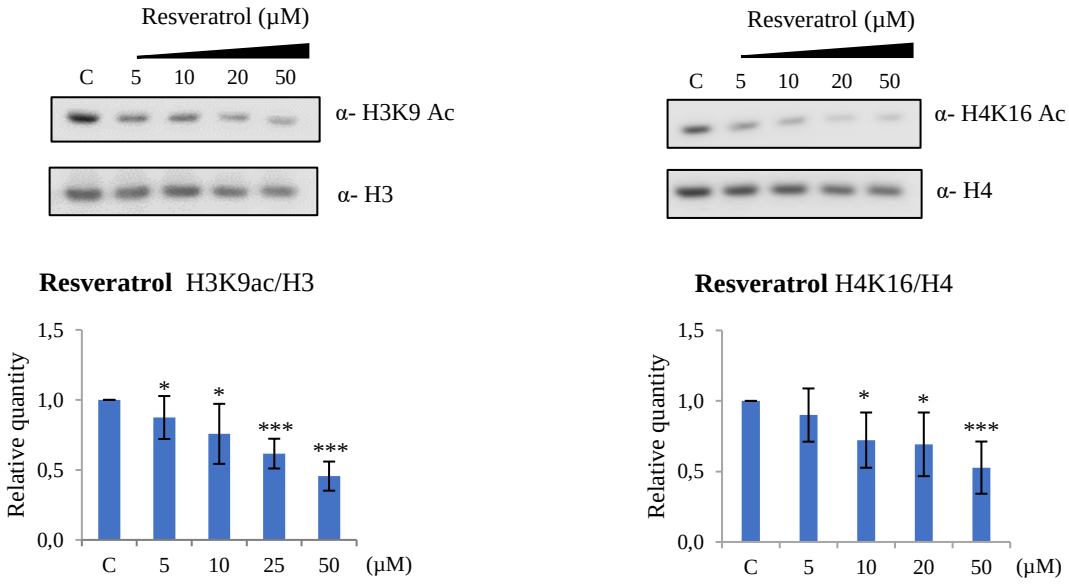


Figure 27. *Continued*

B

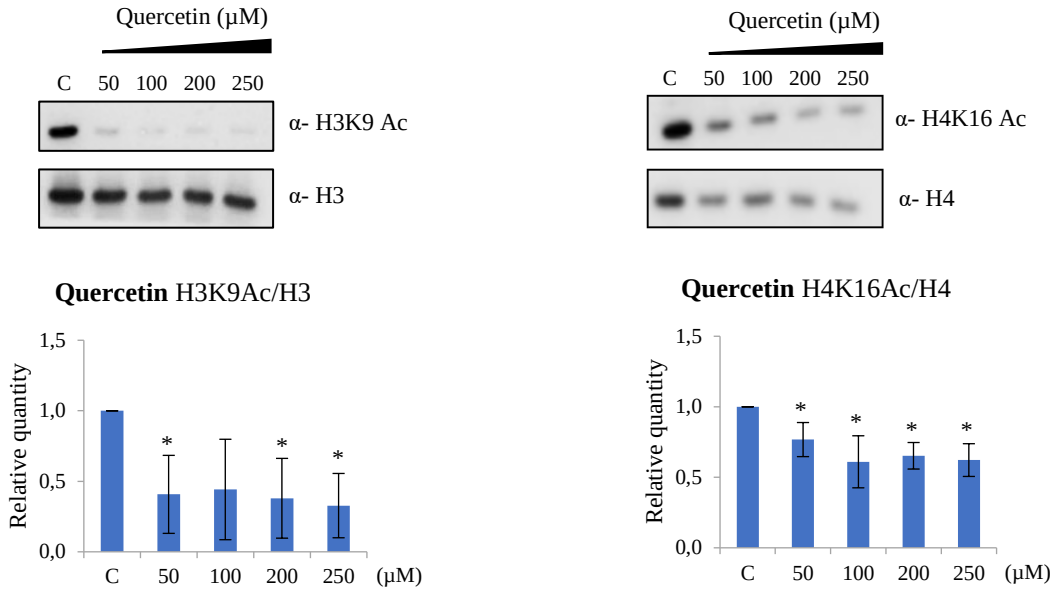


Figure 27. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A, B) Resveratrol (5, 10, 25 and 50 µM) or (C, D) Quercetin (50, 100, 200 and 250 µM), and then cells were harvested and lysed in loading buffer for histone extraction. Statistics: Student's T-test. Cells treated with Resveratrol or Quercetin were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

As expected, Resveratrol and Quercetin induced a decrease in both H3K9 and H4K16 acetylation levels (Figure 27), validating our technical approach. Once the conditions with the positive controls Resveratrol and Quercetin were established, we then proceeded to the screening and tested the rest of the compounds (Figures 28-33 and Annex 2).

A

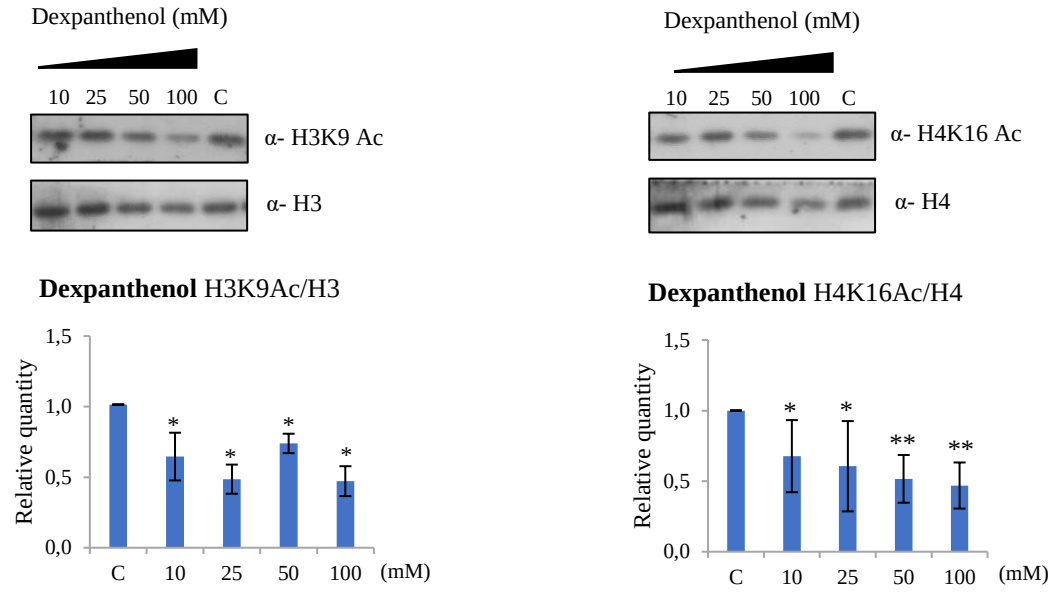
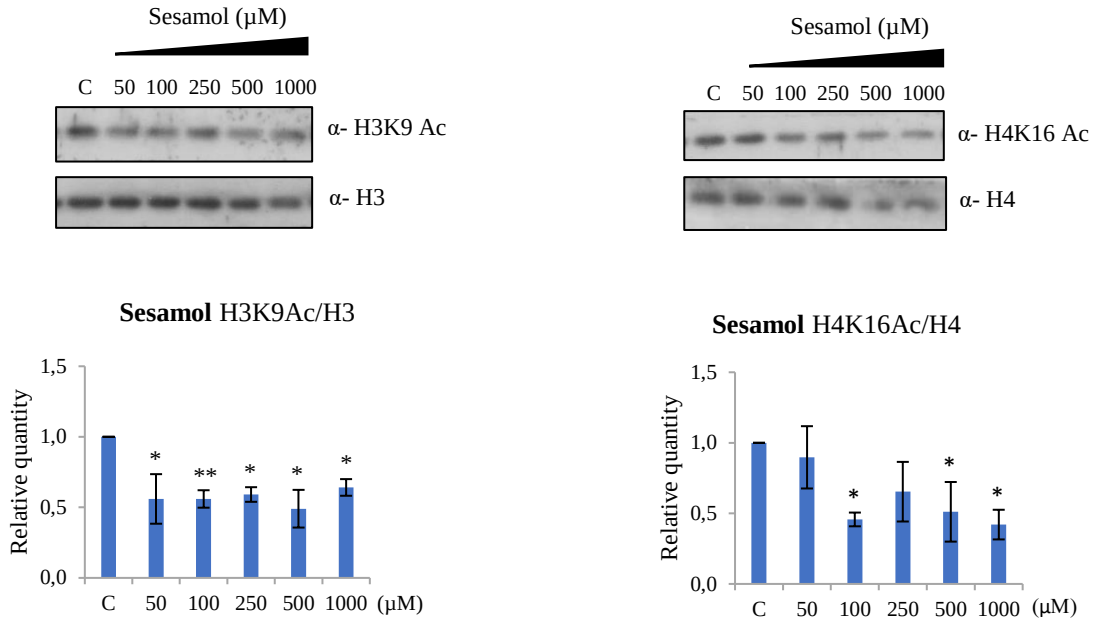


Figure 28. *Continued*

B



C

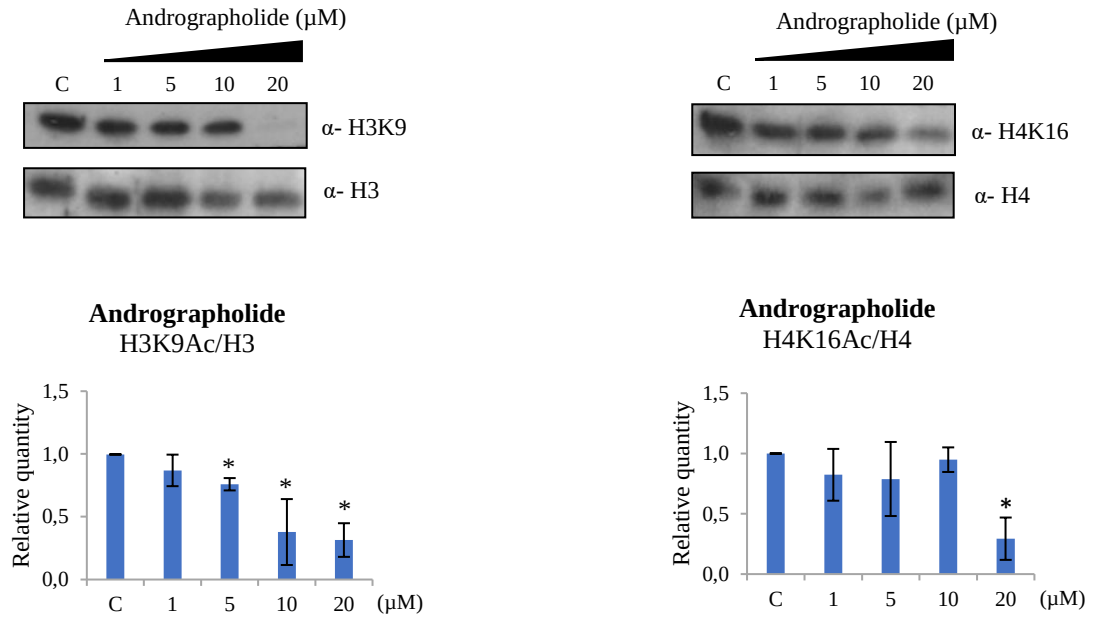


Figure 28. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Dexpanthenol (10, 25, 50 and 100 mM), (B) Sesamol (100, 250, 500 and 1000 µM) and (C) Andrographolide (1, 5, 10 and 20 µM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

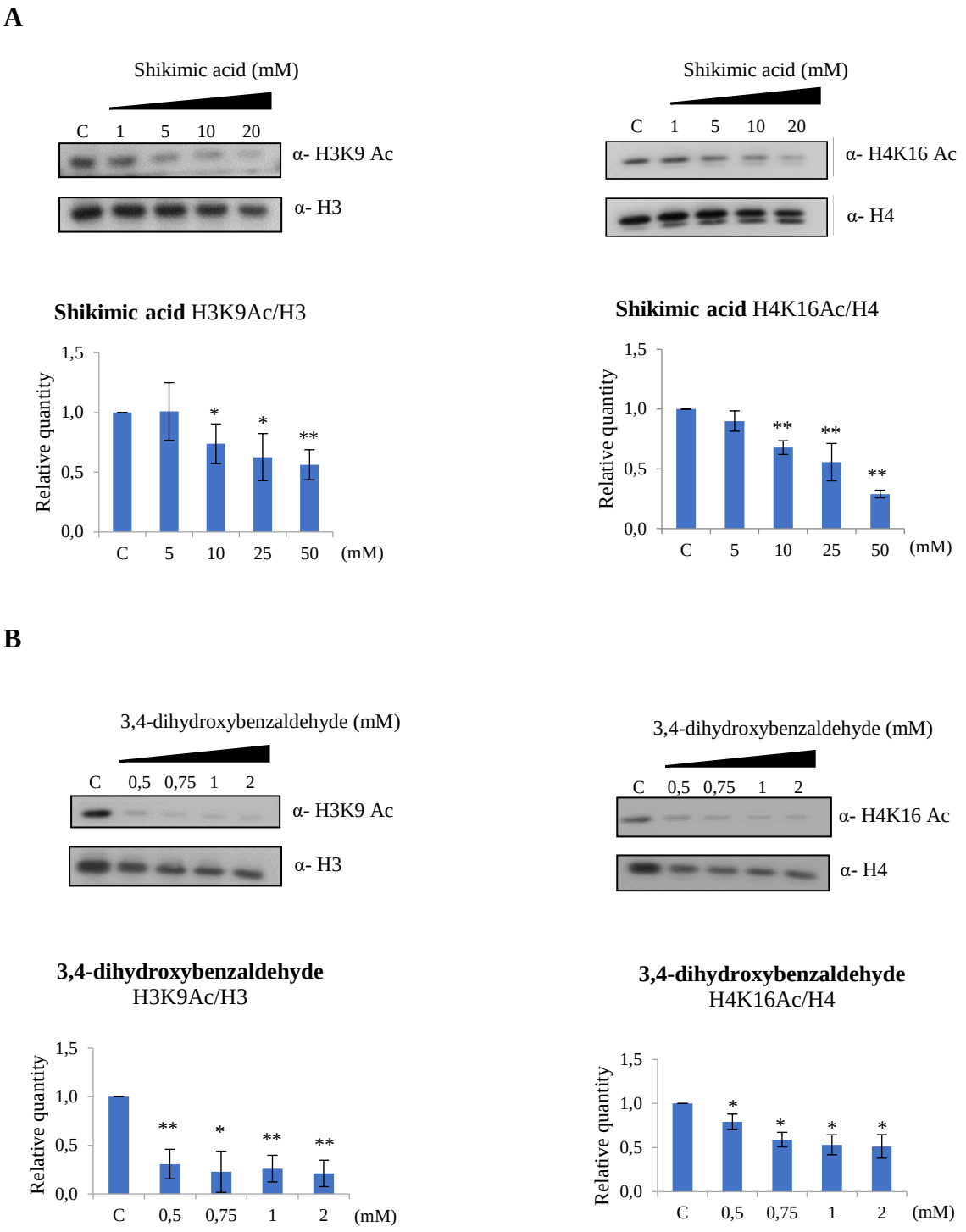
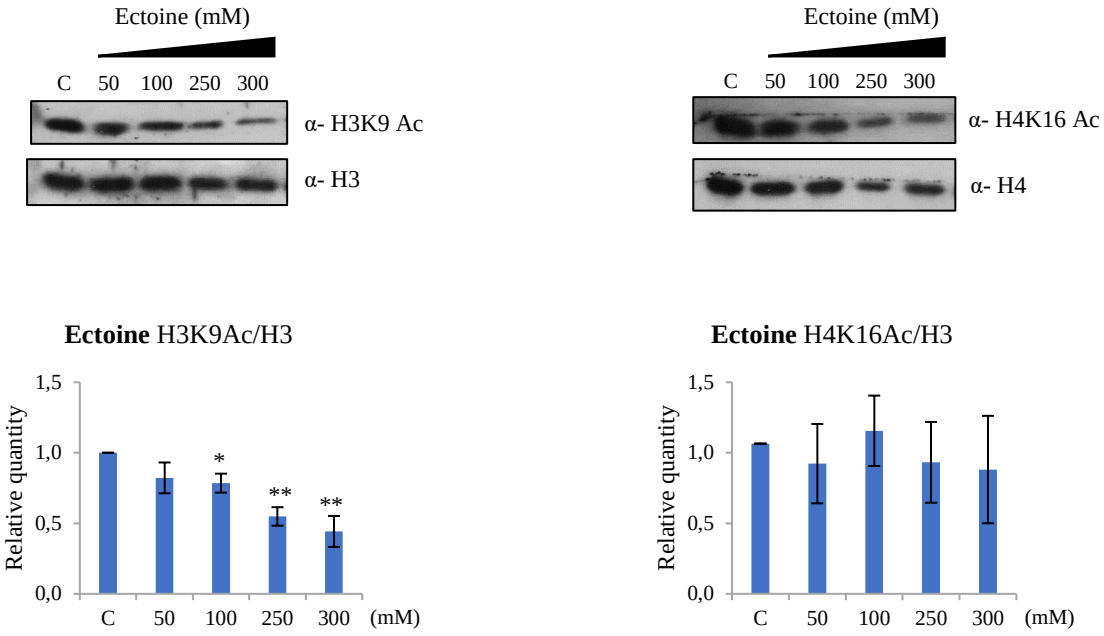


Figure 29. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Shikimic acid (5, 10, 25 and 50 mM) and (B) 3,4-dihydroxybenzaldehyde (0.5, 0.75, 1 and 2 mM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A



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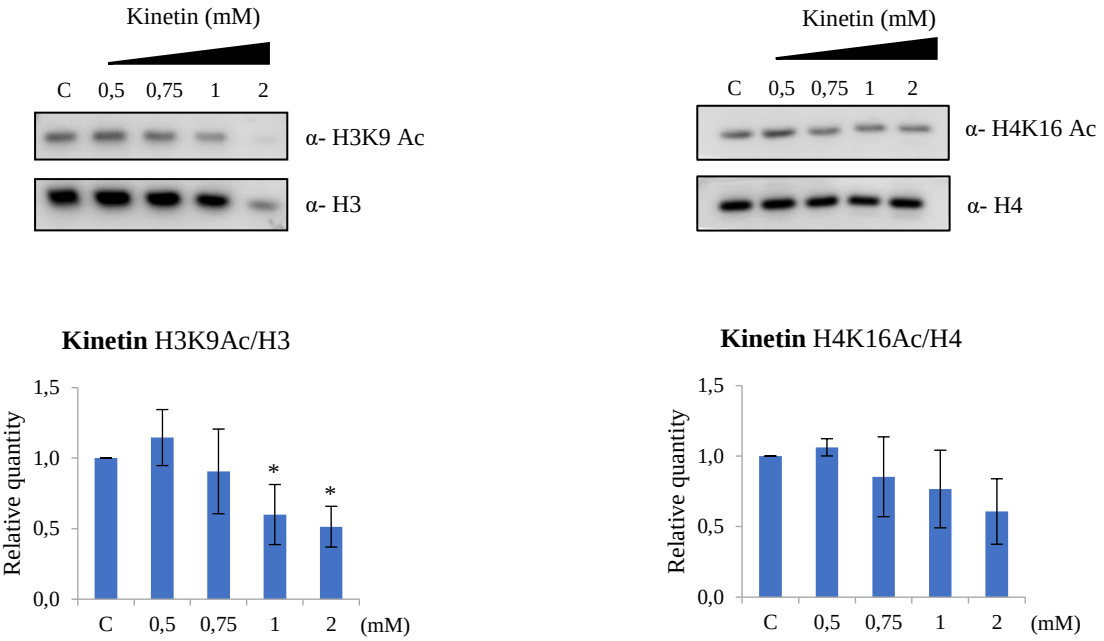


Figure 30. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Ectoine (50, 100, 250 and 300 mM) and (B) Kinetin (0.5, 0.75, 1 and 2 mM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

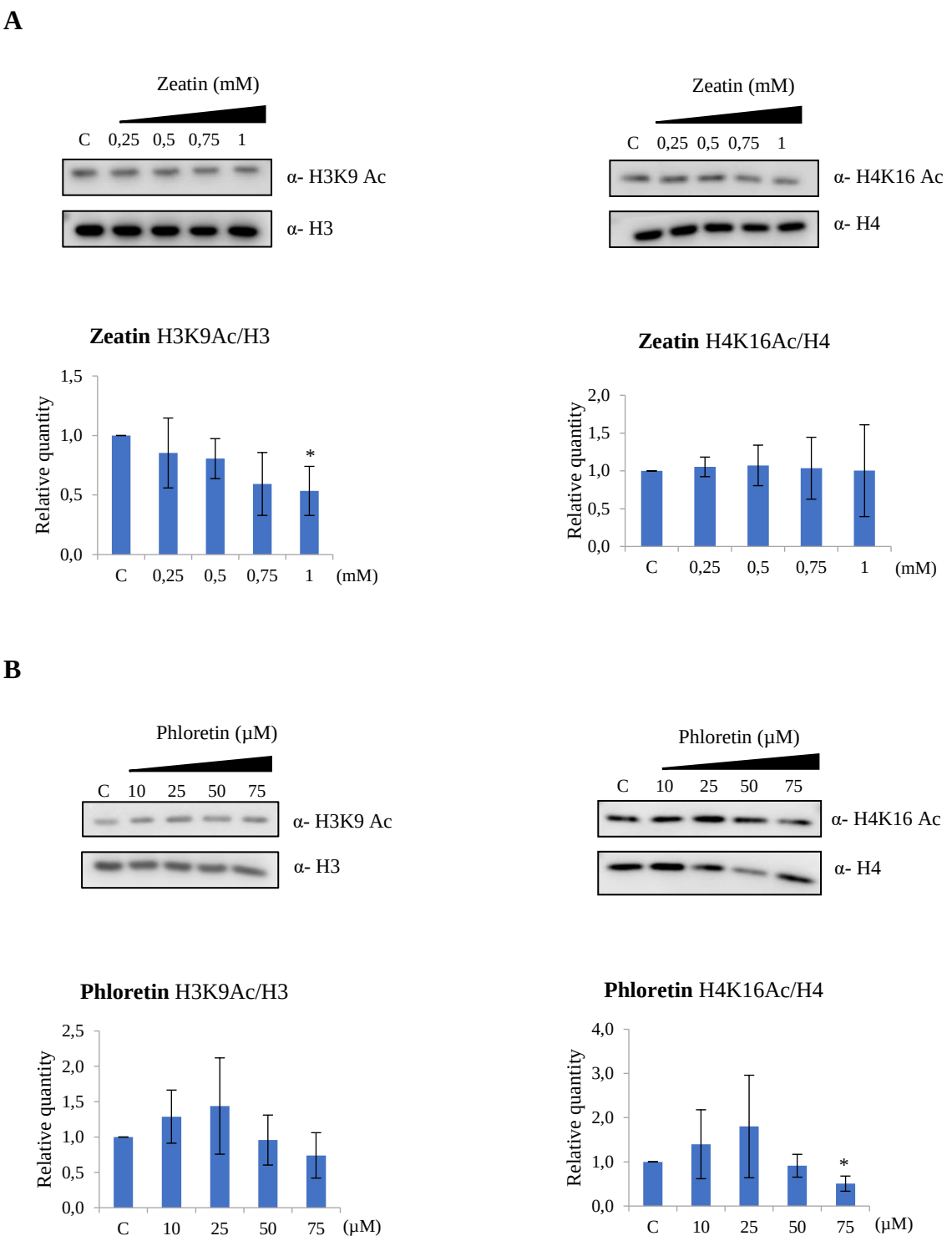


Figure 31. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Zeatin (0.25, 0.5, 0.75 and 1 mM) and (B) Phloretin (10, 25, 50 and 75 µM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

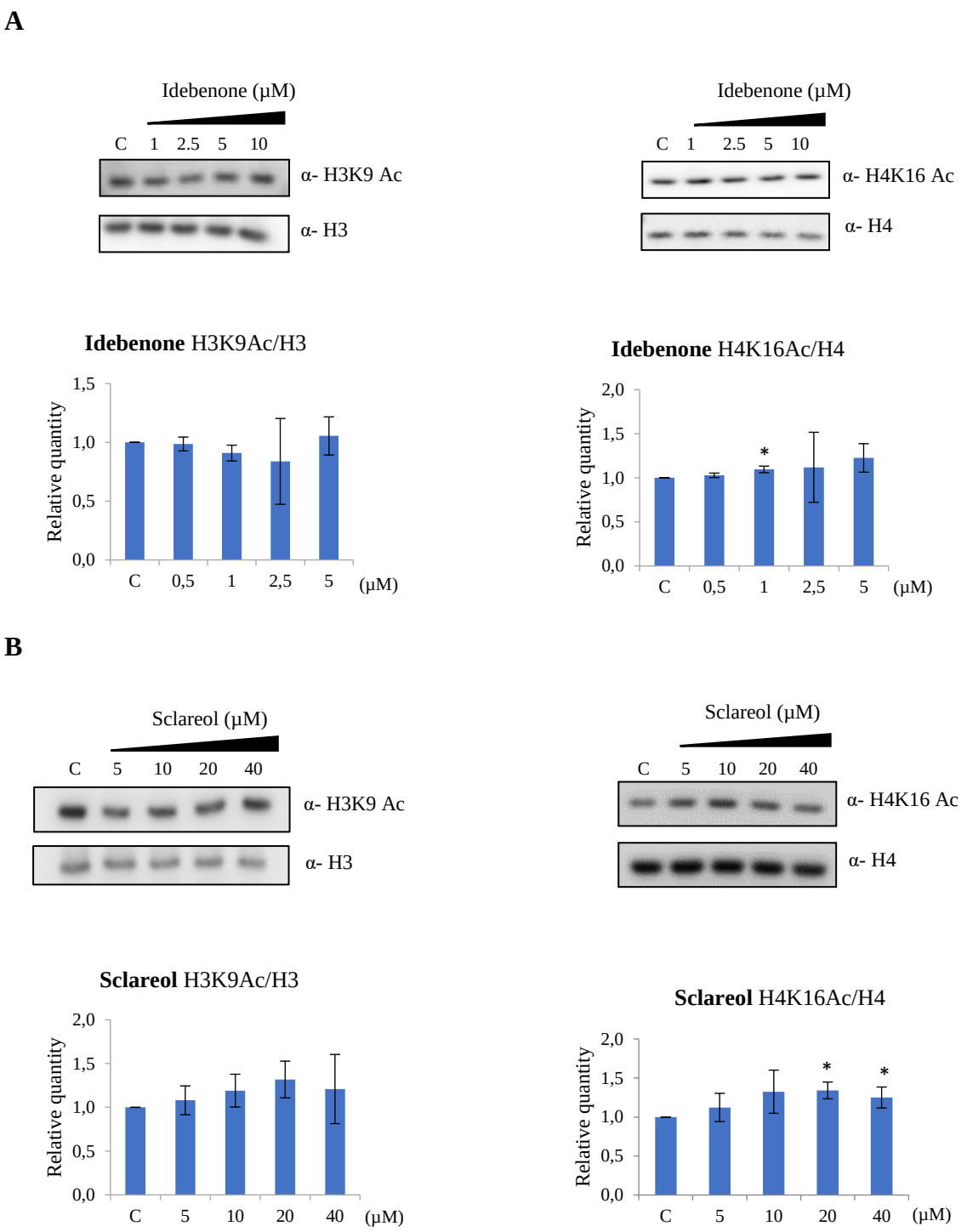
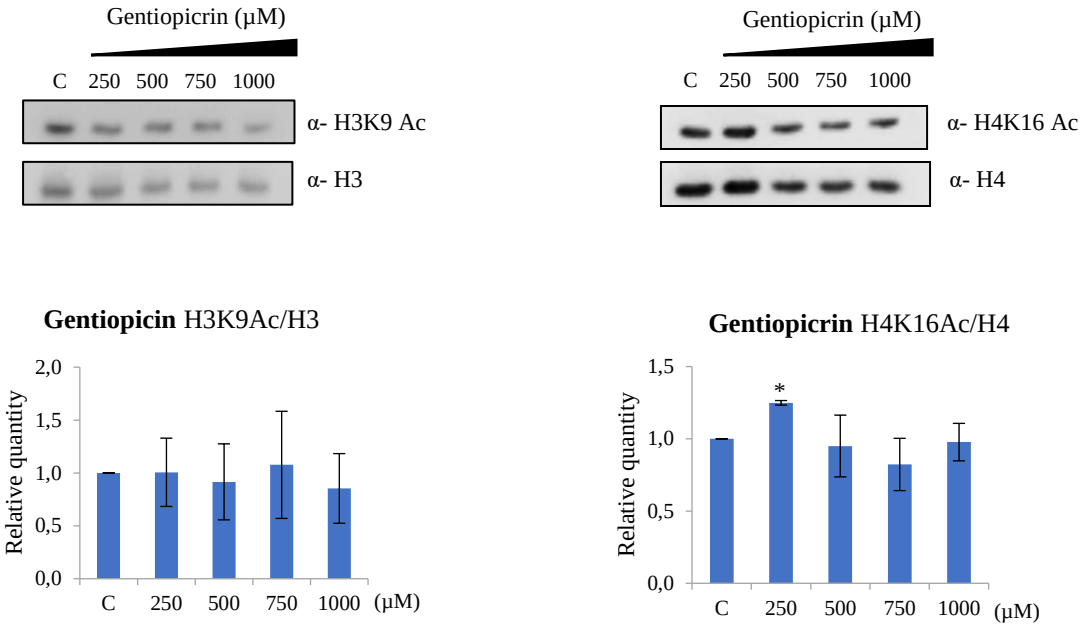


Figure 32. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Idebenone (0.5, 1, 2.5 and 5 µM) and (B) Sclareol (5, 10, 20 and 40 µM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A



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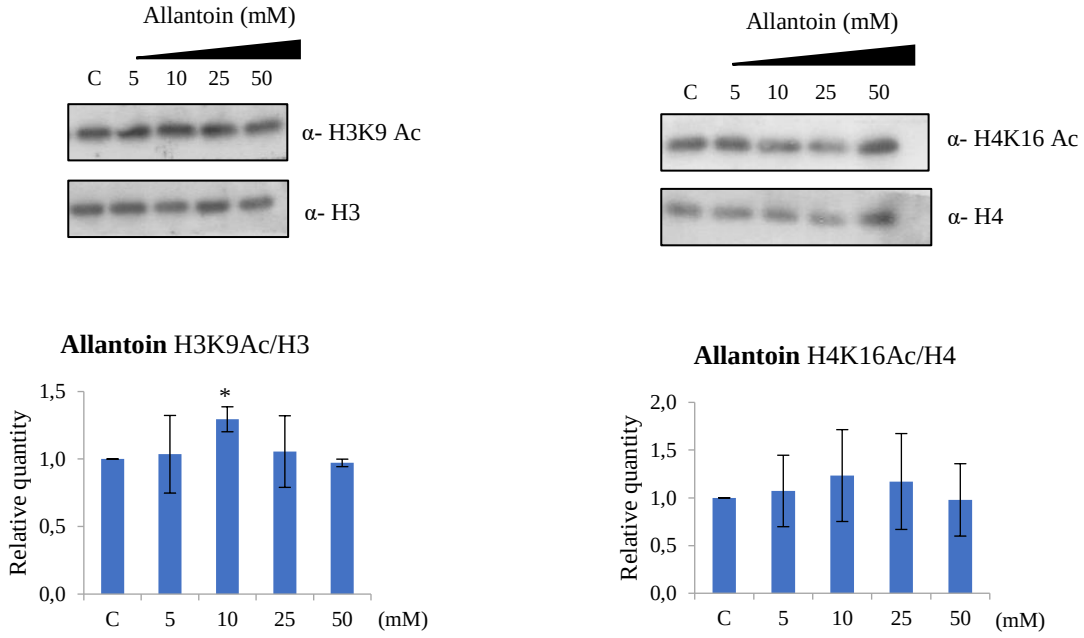


Figure 33. Western blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Gentiopicrin (250, 500, 750 and 1000 µM) and (B) Allantoin (5, 10, 25 and 50 mM). Cells were then harvested and lysed in Loading Buffer for histone extraction. The results of the rest of compounds can be found in Annex 2. Statistics: Student's T-test. Cells treated with the compounds were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Among the different compounds tested, several induced a significant decrease in these marks. Five compounds (Dexpanthenol, Sesamol, Andrographolide, Shikimic acid and 3,4-dihydroxybenzaldehyde) clearly decreased both H3K9 and H4K16 acetylation (Figures 28-29). The combined effect on both histone marks suggests that they exert their effect on SIRT1 (Vaquero A et al. 2004). The way these compounds may be activating SIRT1 could be direct, by binding to SIRT1 and boosting its catalytic activity, or indirect, by modulating signaling pathways that end up inducing SIRT1 activity (Buler M et al. 2016). Moreover, three more compounds (Ectoine, Kinetin and Zeatin) decreased the levels of H3K9ac but not H4K16ac, while another compound (Phloretin) decreased H4K16ac but not H3K9ac (Figures 30-31). In this case, these compounds might be targeting SIRT6 (first case), which deacetylates H3K9ac but not H4K16ac, or SIRT2 (second case), which deacetylates H4K16ac but not H3K9ac. Instead, they could also be targeting other HDACs. For example, HDAC3 and HDAC11 can deacetylate H3K9, while HDAC1 and HDAC2 can deacetylate H4K16 (Miller KM et al. 2010, Seto E et al. 2014). The case of Phloretin was interesting regarding the tendency of the different concentrations in the acetylation of both histones, as the acetylation increases in the lower doses but decreases in the higher doses.

On the other hand, several compounds showed mild increases in H3K9 and H4K16 acetylation. Idebenone, Sclareol and Gentiopicrin (H4K16ac) and Allantoin (H3K9ac) induced small but significant increases in histone acetylation (Figures 32 and 33). Besides, Lipoic acid also increased H3K9 acetylation although the result is not statistically significant (see Annex 2). One possible explanation for these results is the inhibition of one or several sirtuins by these compounds. It is also possible that these increases in histone acetylation are caused by inhibition of other previously mentioned HDACs. However, these increases were very small, hence the effect might not be biologically relevant.

5. Compound efficacy screening for the protecion against UV-induced senescence

5.1 Implementation of the UV-induced senescence protocol

In parallel to the previously described screening of histone acetylation, we established and optimized the UV-induced senescence assay. Our main purpose was not only to find compounds that induce sirtuin targets deacetylation (H3K9 and H4K16), but also to select those compounds with an active effect on the protection of cells against stress. As we are interested in skin aging, we chose UV damage as a source of cellular stress, which is widely used to simulate solar radiation damage and the main factor regulating skin photoaging (Uitto J 1997, Pandel R et al. 2013). Among the different types of UV light, we first chose UVA to induce damage, as it is the most abundant UV radiation in solar light (80%) and it reaches all the dermal layer (Poljšak B et al. 2012, Battie C et al. 2014). In order to induce senescence, UVA irradiation had to be performed once a day for several days, as previously described (Naru E et al. 2005, Shin J et al. 2012, Yi Y et al. 2018). Thus, after trying different number of irradiation days and UVA dosages, we achieved a protocol in which we could induce clearly cellular senescence, detected by the β-galactosidase (β-Gal) staining (Figure 35). For this, we irradiated the cells once a day

with 20 J/cm^2 for 4 consecutive days and left the cell culture several days for recovery before proceeding with the analysis (Figure 34).

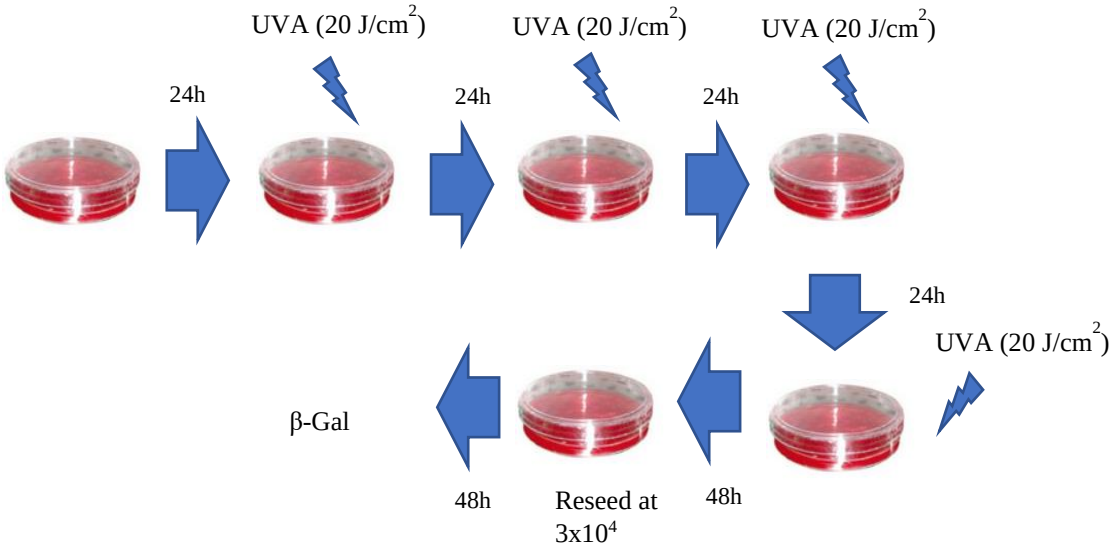


Figure 34. Schematic representation of the UVA-induced senescence assay protocol. Cells at passage 14-16 are seeded in 6-well plate dishes at a concentration of 3x10^4 cells/mL (6x10^4 cells/well). At 24h after seeding, cells are irradiated in PBS with 20 J/cm^2 of UVA and incubated in fresh medium (DMEM +10% FBS). This process is repeated once a day for a total of 4 days. After the last irradiation, cells are incubated in fresh medium for 2 days for recovery and then reseeded at 3x10^4 cells/mL, incubated for 2 more days and finally stained to quantify the senescence marker SA-β-Gal.

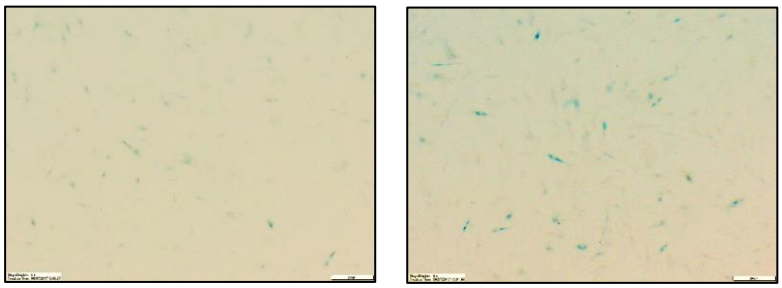


Figure 35. Bright field microscope images of β-galactosidase stained cells showing non-irradiated cells (left) and UVA-irradiated cells (right).

However, practical setbacks appeared in this assay. First, the device that we used for cell irradiation required a long time to irradiate cells (2 hours to irradiate a single 6-well plate). Second, we needed at least 4 days of consecutive irradiations plus 2 days of recovery to induce senescence in these cells, as lower number of days or lower UVA dosage produced cellular damage but did not induce senescence. These reasons, combined with the fact that we aimed to screen 30 compounds at different concentrations, made the assay unsuitable to test all the compounds in the time that we had established.

Thus, at this point we decided to change the UV source to UVB. UVB is higher in energy than UVA, meaning that it takes much less time to induce the same damage. After trying different protocols, we set up an UVB-induced senescence assay, where the cells were irradiated just twice in 5 days (with 2 days of resting between irradiations), and with irradiation times in the scale of seconds for each 6-well plate (25 mJ/cm^2 for each irradiation) (Figure 36). As a senescence readout, we chose two widely used techniques to quantify cellular senescence: the previously mentioned β-Gal staining and the gene expression quantification of *p16* and *p21* by qPCR (Figure 37).

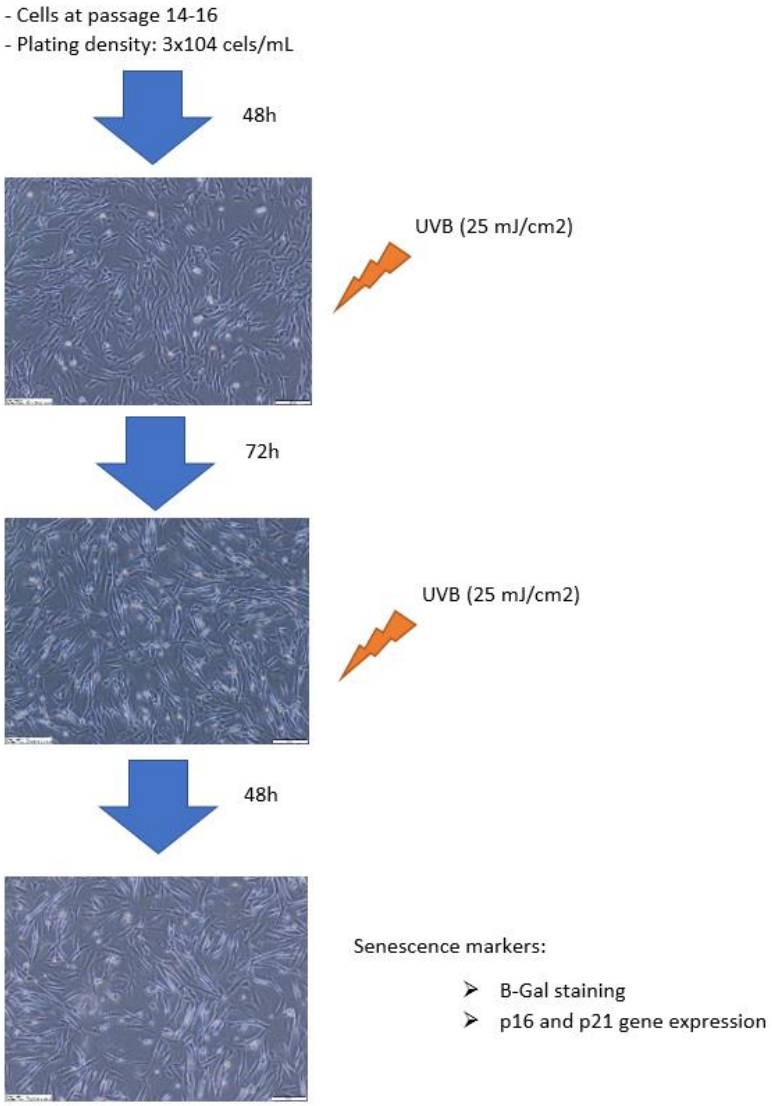


Figure 36. Schematic representation of the UVB-induced senescence assay protocol. Cells at passage 14-16 were seeded in 6-well plate dishes at a concentration of 3x10^4 cells/mL (6x10^4 cells/well). At 48h after seeding, cells were irradiated in PBS with 25 mJ/cm^2 UVB and incubated in fresh medium (DMEM +10% FBS). After 72h, cells were irradiated again in PBS with 25 mJ/cm^2 UVB and incubated in fresh medium (DMEM + 10% FBS). At 48h after the second irradiation, cells were analyzed to quantify the senescence markers SA-β-Gal staining and *p16* and *p21* gene expression.

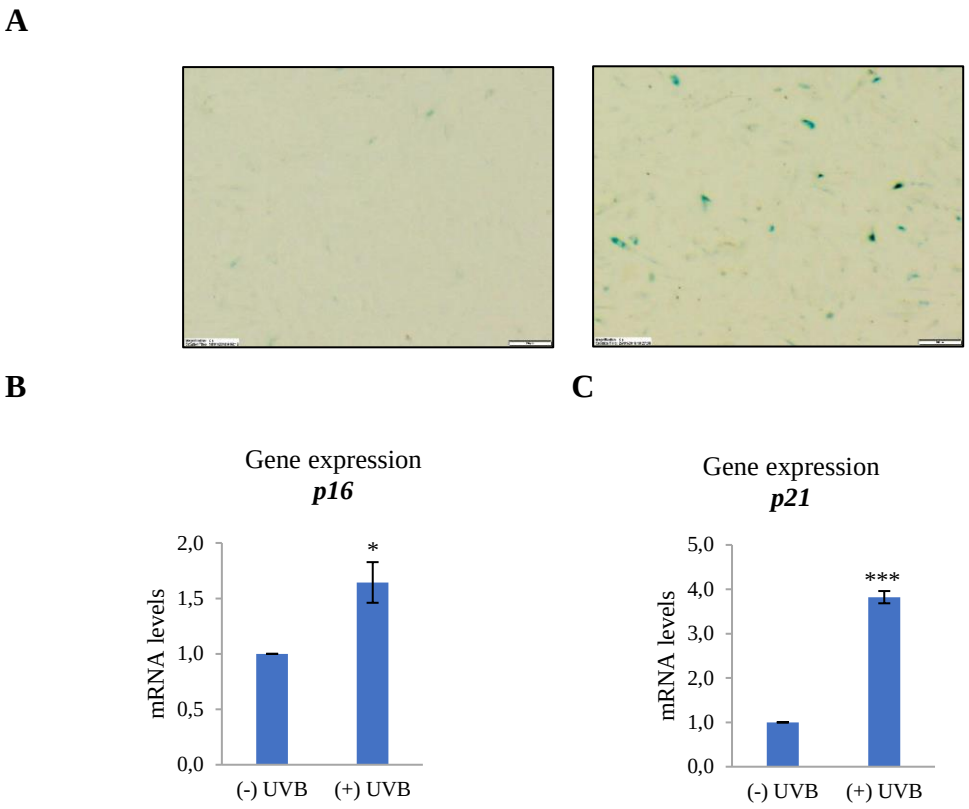


Figure 37. (A) Bright field microscope images of β-Gal staining showing non-irradiated cells (left) and UVB-irradiated cells (right), and qPCR analysis of *p16* (B) and *p21* (C) gene expression in non-irradiated cells (left) and UVB-irradiated cells (right). Statistics: Student's T-test. UVB-irradiated cells were compared to non-irradiated cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

5.2 Screening of the compounds effect on UVB-induced senescence

Thus, once we established the conditions to induce senescence efficiently and in relatively short times, we started screening the compounds to figure out which ones could protect the cells against UVB-induced senescence. We tested the positive controls, Resveratrol and Quercetin, and the first compound that showed a positive result in the histone deacetylation screening, Andrographolide. Unfortunately, for all these three compounds, the same concentrations that induced histone deacetylation also induced cell death after UVB irradiation (Figure 38).

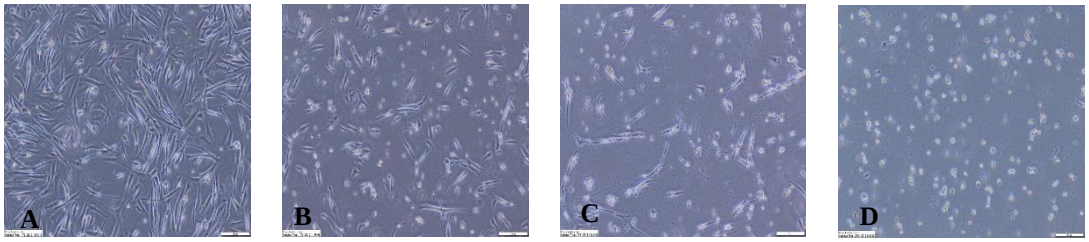


Figure 38. Phase-contrast microscope images of UVB-induced senescent cells. (A) Non-treated cells, (B) cells treated with 100 µM Quercetin, (C) cells treated with 50 µM Resveratrol and (D) cells treated with 20 µM Andrographolide.

Thus, the concentrations of these actives had to be decreased for the UVB-induced assay, and these lower concentrations did not protect against UVB-induced senescence (see Annex 3). One of the first compounds to show protective effects on UVB-induced senescence was Gallic acid. Surprisingly, prevention of UVB-induced senescence was observed at the level of β-Gal staining, but not in the expression levels of *p16* and *p21* (Figure 39).

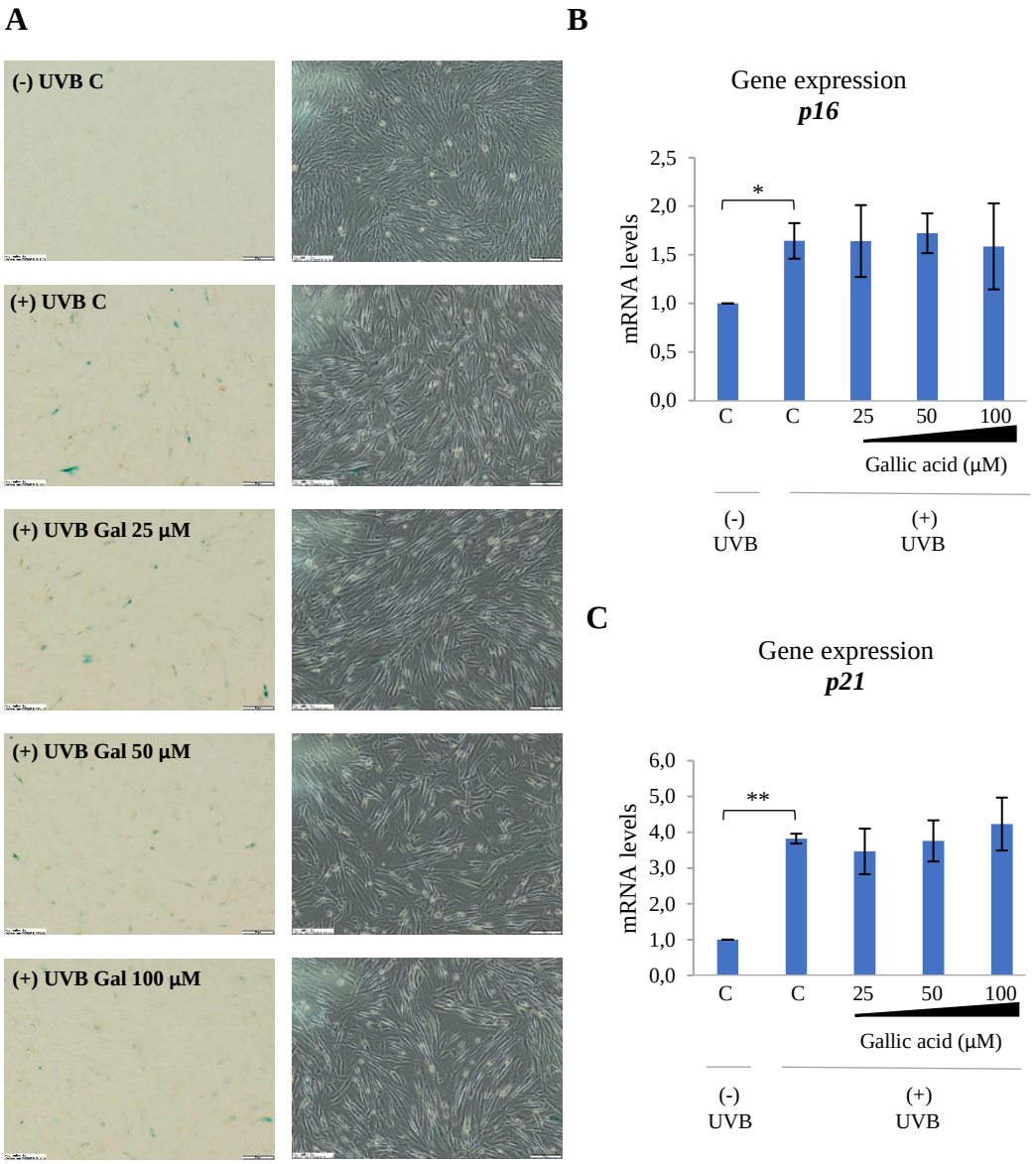


Figure 39. (A) Bright field (left) and phase-contrast (right) microscope images of β-Gal staining showing non-irradiated cells, UVB-irradiated cells and UVB irradiated cells treated with Gallic acid at 25, 50 and 100 µM. qPCR analysis of (B) p16 and (C) p21 gene expression in non-irradiated cells, UVB irradiated cells and UVB-irradiated cells treated with Gallic acid at 25, 50 and 100 µM. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with Gallic acid were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Although p16 and p21 are used as generic markers for senescent cells identification, many *in vitro* studies that test the efficacy of compounds on protecting against senescence do

not use these markers. Instead, these studies use cell-specific genes along with β-Gal staining to determine whether the compound is protecting the cell against senescence or not (Cho EJ et al. 2008, Wang YN et al. 2010, Bae JT et al. 2012). Thus, these markers might not be reliable to study the protection of a compound against skin fibroblasts senescence. In order to overcome this issue, we decided to look for cell-specific senescence markers that could confirm the validity of β-Gal staining, and that could be also included in the screening process.

Reviewing the bibliography on senescence markers, we found a report that described how skin cell senescence is induced by the upregulation of miR-23a-3p, which in turn downregulates hyaluronan synthase 2 (*HAS2*) (Röck K et al. 2015). Thus, we considered the possibility that the downregulation of *HAS2* could be used as a reliable marker in our system to identify senescent cells in skin aging. To prove this hypothesis, we checked the gene expression levels of *HAS2* in the samples of UVB-irradiated cells treated with Gallic acid (Figure 40).

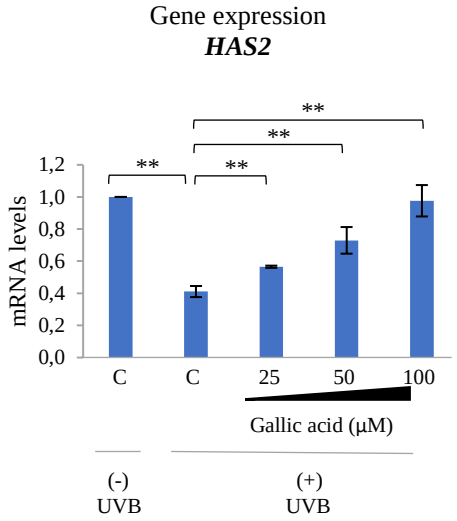


Figure 40. pPCR analysis of *HAS2* gene expression in non-irradiated cells, UVB irradiated cells and UVB-irradiated cells treated with Gallic acid at 25, 50 and 100 µM. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with Gallic acid were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

According to Röck K et al. experiments, *HAS2* was downregulated in replicative senescent fibroblasts, but our results suggested that this was also the case in UVB-induced senescent cells. Furthermore, Gallic acid could reverse this downregulation in a dose-dependent manner (Figure 40), as it happened with the previously described effect on β-Gal staining (Figure 39A). This positive result lead us to use the quantification of *HAS2* as another screening tool to better characterize the effect of the compounds on UVB-induced senescence. Thus, we performed the β-Gal staining and the *HAS2* gene expression quantification for the rest of compounds to see if they could protect against UVB-induced senescence (Figure 41 and Annex 3).

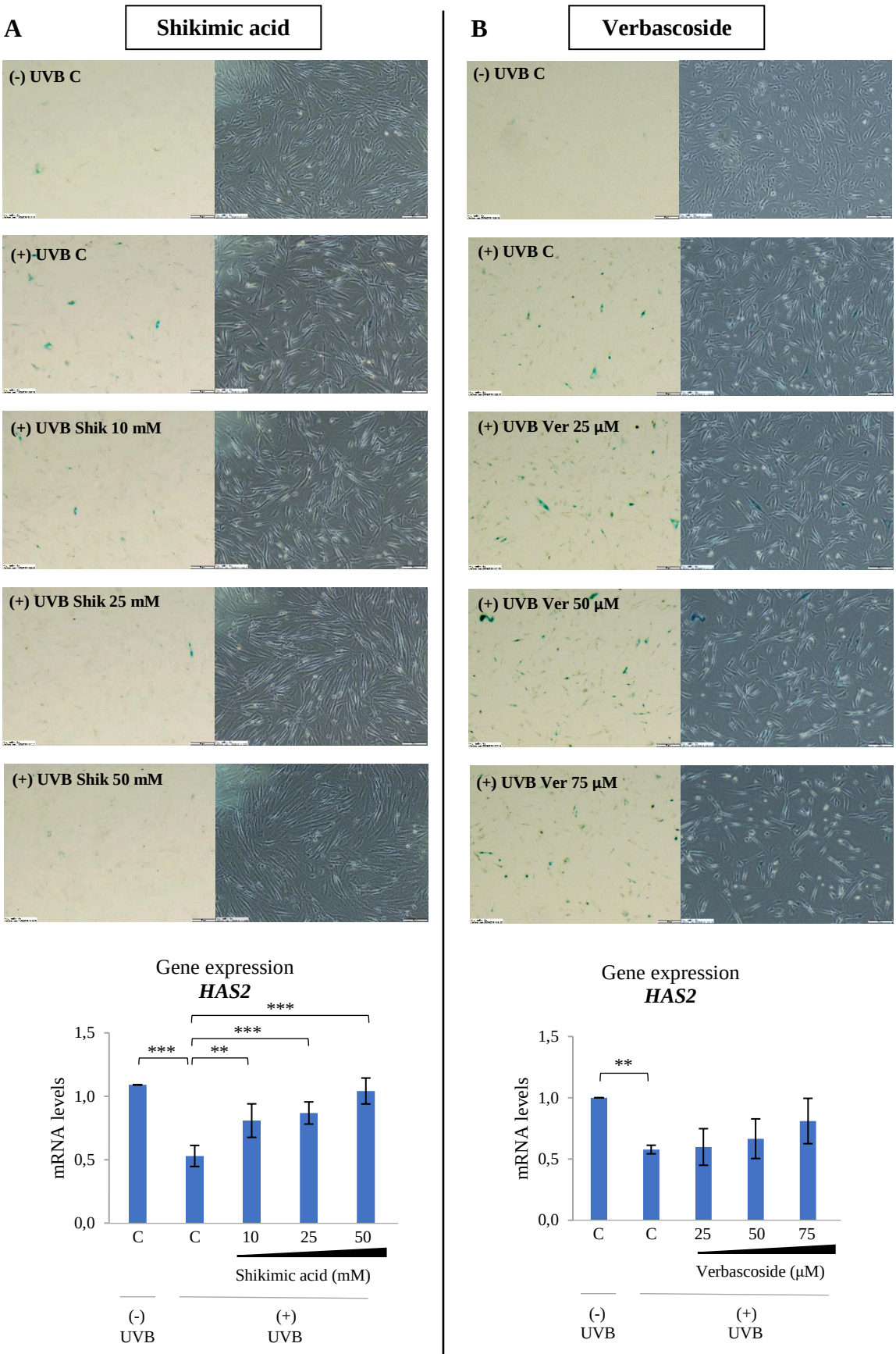


Figure 41. *Continued*

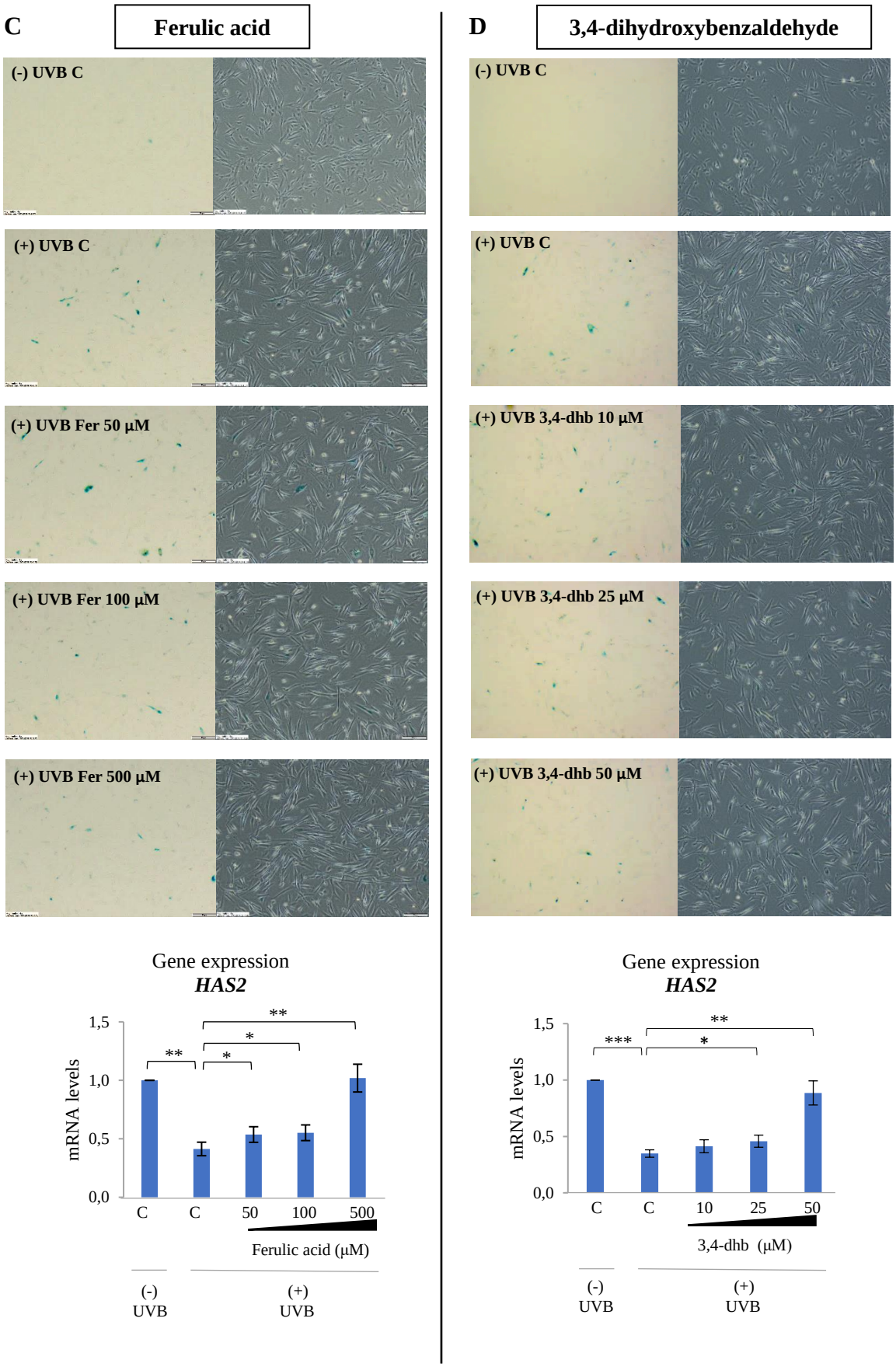


Figure 41. Bright field (up-left) and phase-contrast (up-right) microscope images of β-Gal staining showing non-irradiated cells, UVB-irradiated cells and UVB irradiated cells treated with (A) Shikimic acid (10, 25 and 50 mM), (B) Verbascoside (25, 50 and 75 µM), (C) Ferulic acid (50, 100 and 500 µM) and (D) 3,4-

dihydroxybenzaldehyde (10, 25 and 50 μM). qPCR analysis (bottom) of *HAS2* gene expression in non-irradiated cells, UVB irradiated cells and UVB-irradiated cells treated with with (A) Shikimic acid (10, 25 and 50 mM), (B) Verbascoside (25, 50 and 75 μM) and (C) Ferulic acid (50, 100 and 500 μM). The results of the rest of compounds can be found in Annex 3. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with the compounds were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

As occurred with the positive controls Resveratrol and Quercetin (Figure 38), many of the compounds that induced a decrease in the acetylation of histone markers H3K9 and H4K16 also induced cell death when cells were irradiated with UVB and treated with the compounds. Therefore, the concentration of these compounds had to be lowered for the senescence screening. At lower doses, where the viability of cells was not altered by the combination of the compound and the irradiation, some of these compounds did not show protection against UVB-induced senescence. This was the case for Dexpanthenol, Andrographolide, Sesamol, Ectoine (Annex 3) and 3,4-dihydroxybenzaldehyde (Figure 41D).

Interestingly, in the case of Resveratrol, Sesamol and 3,4-dihydroxybenzaldehyde, these lower doses, that did not induce cell death after UVB irradiation, did increase β-Gal staining in UVB-induced senescent cells, suggesting an increase in cellular senescence rather than a decrease. However, in the case of 3,4-dihydroxybenzaldehyde, *HAS2* expression was also increased in UVB-induced senescent cells. This was also the case for Verbascoside, which increased both *HAS2* expression and β-Gal staining (Figure 41B).

Another group of compounds, which included Carnosine, Quercetin, Taurine, Ferulic acid and Lipoic acid, also showed interesting results, as they increased *HAS2* expression without affecting β-Gal staining (Figure 41C and Annex 3). On the other hand, Phloretin and Irisflorentin decreased HAS2 expression at their maximum concentrations without affecting β-Gal staining, possibly due to cell toxicity (Annex 3).

Finally, only two compounds, Shikimic acid and Gallic acid, decreased β-Gal staining and increased *HAS2* expression in UVB-irradiated cells (Figure 41A, Annex 3). This reversal of β-Gal and *HAS2* markers from the levels in UVB-irradiated cells to the levels of non-irradiated cells is the expected outcome of a compound that protects against UVB-induced senescence. In the case of Gallic acid, which did not show an effect on the acetylation levels of H3K9 and H4K16, the compound might be protecting against senescence through inhibition of senescence pathways, such as p38, mTOR or NF-kβ, or through activation of protective mechanisms that do not involve sirtuins, such as DNA repair proteins or cellular antioxidant machinery. In the case of Shikimic acid, this protection against cellular senescence could be performed through sirtuin activation, as this compound also showed decreased acetylation of H3K9 and H4K16 (Figure 29A).

Thus, at this point we decided to focus on characterizing the molecular mechanisms that involved Shikimic acid protection in UVB-induced senescence. Besides, we also decided to focus on characterizing the compound 3,4-dihydroxybenzaldehyde, as it was the compound with the clearest impact on H3K9 and H4K16 deacetylation (Figure 29B) and a different effect than Shikimic acid on UVB-induced senescence.

6. Molecular characterization of Shikimic acid

6.1 Effect of Shikimic acid on cell viability

Our first step in the characterization of Shikimic acid was to understand the effect of Shikimic acid on cell viability.
During the cell viability testing of the compounds, we used the WST-1 assay in order to assess the maximum non-cytotoxic dose. This assay is an improved version of the widely used MTT assay, as it provides the same information in a faster and less complex protocol. According to the results for this assay, Shikimic acid showed cellular toxicity above 25 mM, although examination under the microscope suggested that Shikimic acid at 50 mM was not inducing cell death. Additionally, Shikimic acid at 50 mM showed positive effects in the previous assays, by inducing the deacetylation of H3K9 and H4K16 and protecting against cellular senescence induced by UVB. As WST-1 measures mitochondrial activity, we wanted to contrast this result with other assays that quantify other cellular properties to assess cell viability. We decided to use (I) the SRB assay, which is based on the binding of the SRB reagent to proteins under acidic conditions (Skehan P et al. 1990, Vichai V et al. 2006), and (II) cellular counting in the hemocytometer using Trypan Blue, in which the Trypan Blue reagent is only permeable in death cells through their damaged membranes and allows to count manually the total number of viable cells (Strober W 2001). These 2 assays can measure more precisely the exact number of viable cells compared to mitochondrial activity-based assays such as MTT or WST-1. Therefore, we incubated Shikimic acid for 24h in cell culture and measured cell viability through SRB assay and Trypan Blue counting (Figure 42).

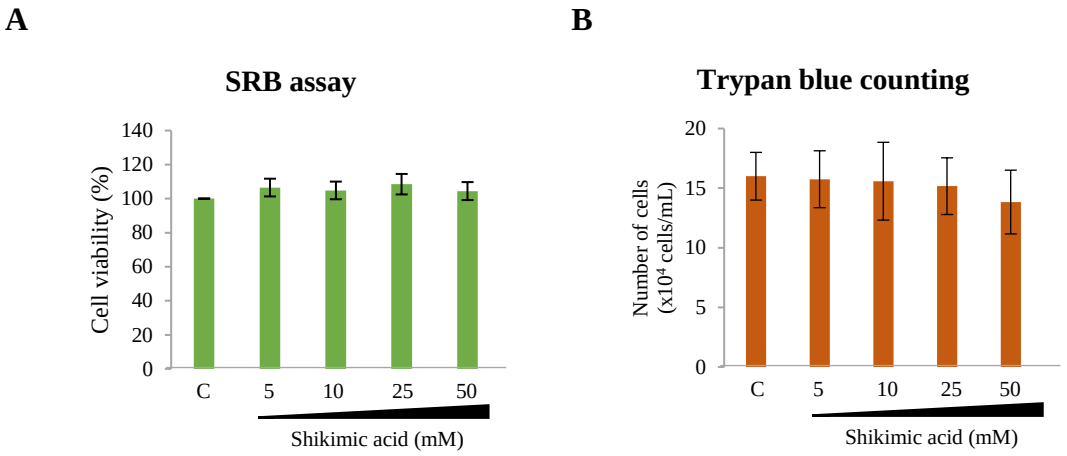


Figure 42. (A) SRB and (B) Trypan blue counting assays performed in cells treated for 24 hours with Shikimic acid at different concentrations. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

According to these results, Shikimic acid does not induce cell toxicity at 50 mM. Thus, a possible explanation for the difference between the WST-1 and SRB/Trypan blue assays is that Shikimic acid might be inhibiting mitochondrial activity (reduced WST-1 signal compared to control cells) without affecting cellular viability (same signal than control cells), which may be related to the well-established role of sirtuins in metabolism.

6.2 Effect of Shikimic acid on apoptosis, cell cycle and DNA damage in UVB-induced senescent cells

6.2.1 Effect of Shikimic acid on apoptosis in UVB-induced senescent cells

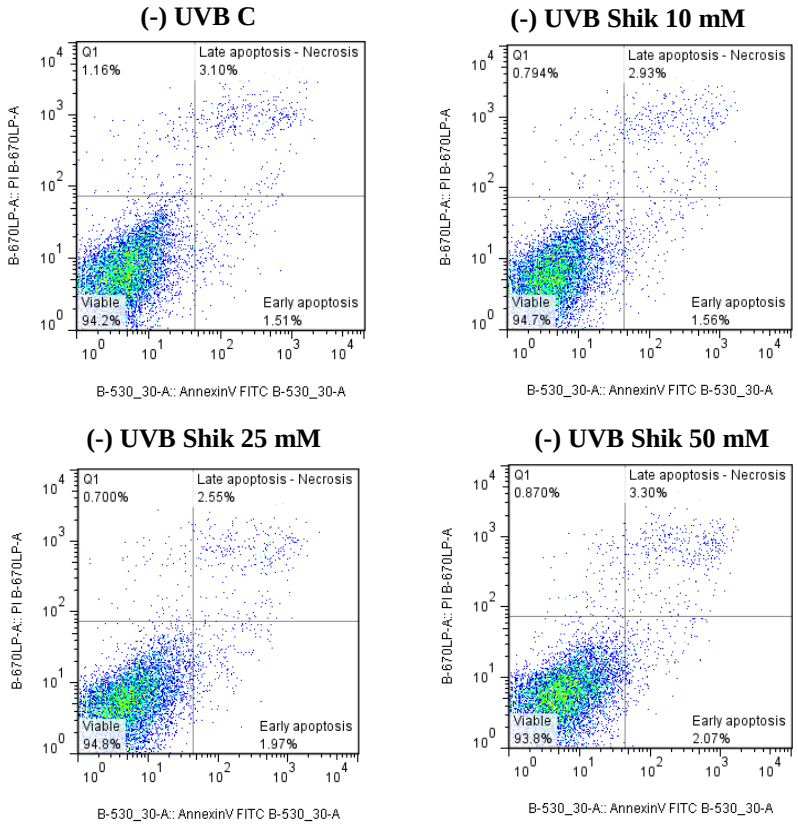
Senescent cells are characterized to be apoptosis-resistant, among other things by maintaining pro-apoptotic pathways inhibited. However, under certain stimuli, senescent cells may enter apoptosis (DeJesus V et al. 2002, Childs BG et al. 2014, Wang D et al. 2016). Thus, when studying senescence, it is important to evaluate whether cells are undergoing apoptosis or not. In our case, we wanted to confirm that the decrease in senescence phenotype observed with Shikimic acid treatment was not caused by a decrease in the cell number due to increased apoptosis, but because Shikimic acid helps protecting these cells against senescence.

For this purpose, we used the Annexin V assay, which allows the quantification of apoptotic cells. Specifically, this assay allows us to quantify both apoptotic and necrotic cells. While apoptosis is a programmed cell death triggered by physiological stimuli that shows intact cell membrane integrity and depends on specific intracellular signaling pathways (Caspases), necrotic cells suffer cell lysis caused by an external insult and trigger an inflammatory reaction (Nikoletopoulou V et al. 2013). The difference in the cell permeability is what allows PI to enter the cell and thus differentiate between apoptotic and necrotic cells. When apoptotic cells in an inital state (early apoptosis) persist due to overload of dying cells and/or impairment of phagocytosis, these cells progress to late apoptosis, which present cell lysis as well as necrotic cells. Thus, the Annexin V assay can differentiate between early apoptosis and late apoptosis/necrotic cells.

Using the UVB protocol to induce senescence (Figure 32), we measured the apoptosis levels in cells irradiated with UVB and treated with Shikimic acid (Figure 44). We also quantified the apoptosis levels in non-irradiated cells treated with Shikimic acid, to determine if Shikimic acid induces apoptosis in the absence of stress (Figure 43).

According to our results, Shikimic acid induced a small dose-dependent increase in early apoptotic cells, although this increase was not significant (Figure 43). Thus, Shikimic acid does not induce apoptosis *per se* in non-irradiated cells. On the other hand, we observed that UVB irradiation increased significantly early apoptotic (7%) and late apoptotic (6%) cells (Figure 44). However, these increases were very mild, indicating that the majority of the cells undergo senescence instead of apoptosis in our model. There is also a significant increase in late apoptosis (5%) in UVB-irradiated cells treated with Shikimic acid 50 mM, although an increase of this degree might not be biologically relevant. Altogether, our evidences suggest that the beneficial role of Shikimic acid in UVB-induced senescence is a protective effect and not due to an increase in apoptosis of UVB-irradiated cells.

A



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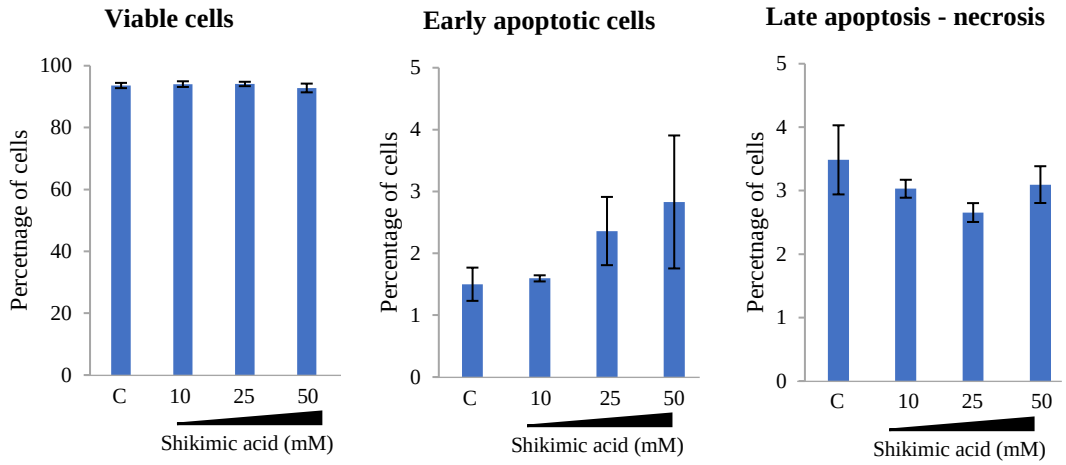
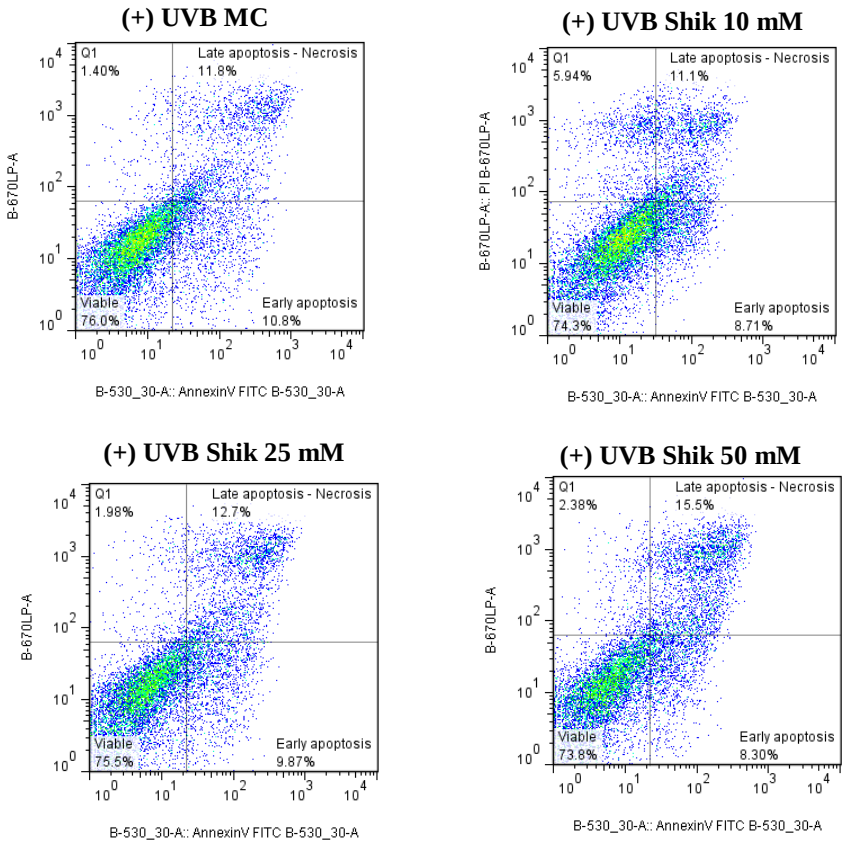


Figure 43. (A) Apoptosis detection using the Annexin V-FITC/PI double staining assay followed by flow cytometry in non-irradiated cells. Cells were cultured for 6 days with or without Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (B) The average percentages of viable, early apoptotic and late apoptotic/necrotic cells. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A



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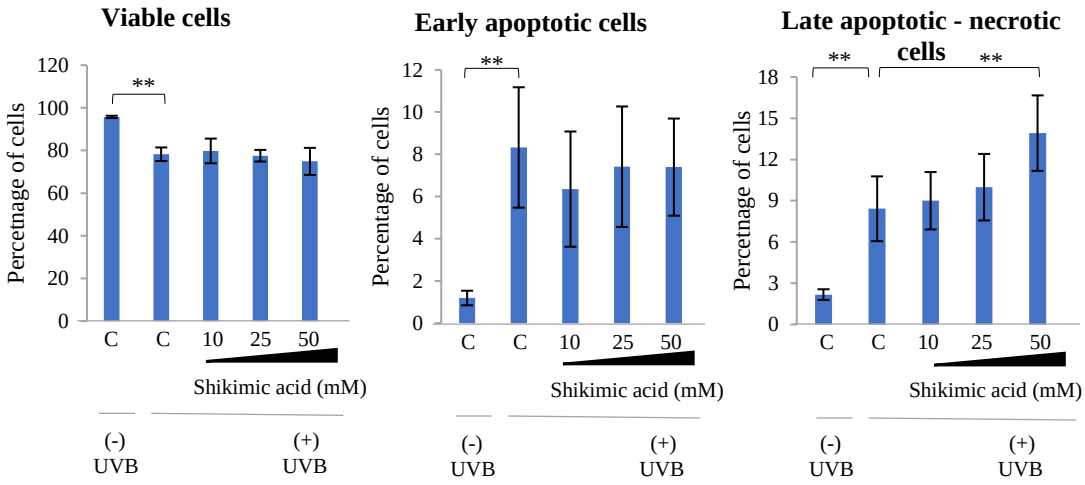

Figure 44. (A) Apoptosis detection using the Annexin V-FITC/PI double staining assay followed by flow cytometry in UVB-irradiated cells. Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (B) The average percentages of viable, early apoptotic and late apoptotic/necrotic cells. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with Shikimic acid were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

6.2.2 Effect of Shikimic acid on cell cycle in UVB-induced senescent cells

One of the main features of senescent cells is the cell cycle arrest (Kuilman T et al. 2010). Here, we wanted to test if Shikimic acid could prevent the cell cycle inhibition of UVB-irradiated cells. For that purpose, we used the detection of PI by flow cytometry. In this assay, PI intercalates in dsDNA and emits fluorescence. The detection of this fluorescence allows us to differentiate 4 different groups of cells according to their DNA content, and therefore their cell cycle phase. First, cells in G1 phase have one copy of the DNA, and appear in one peak. Then, as these cells replicate and enter S phase, the DNA content increases and therefore more fluorescence is detected, and finally another peak is generated with the cells at G2/M phase, which have 2 full copies of DNA (4n). Besides, abnormal cells which have more than 2 copies of DNA (poliploid cells) can also be detected in this assay. Hence, we treated UVB-irradiated cells with Shikimic acid following the senescence induction protocol (Figure 32) and analyzed the cells by FACS PI (Figure 46). In parallel, we also treated the cells with Shikimic acid without irradiation to see the effect of the compound in the cell cycle in the absence of stress (Figure 45).

A

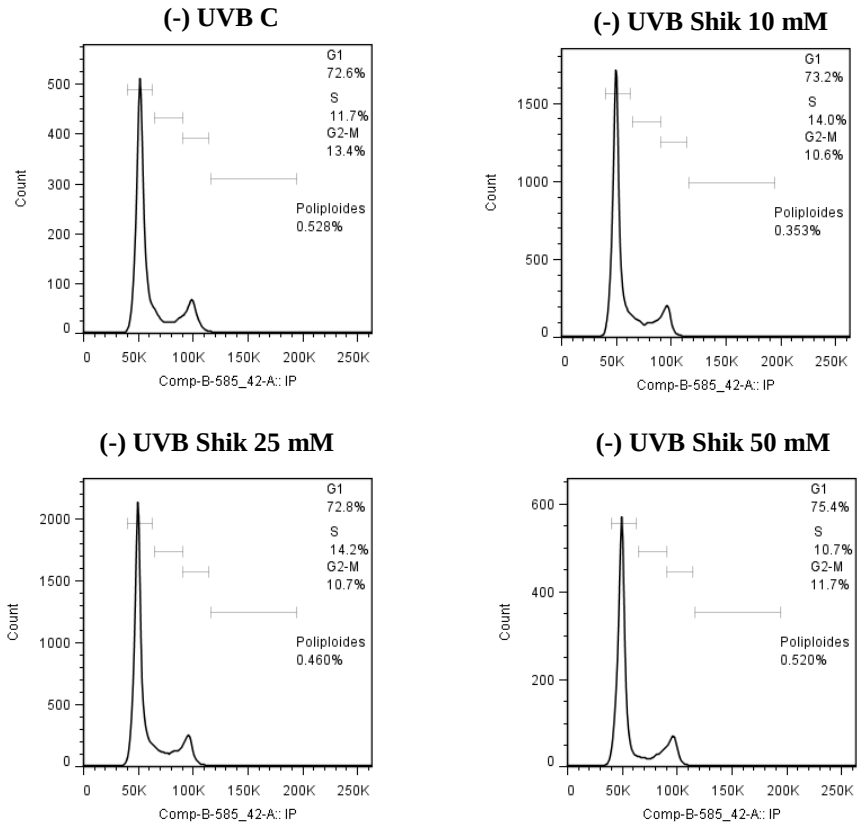

Figure 45. *Continued*

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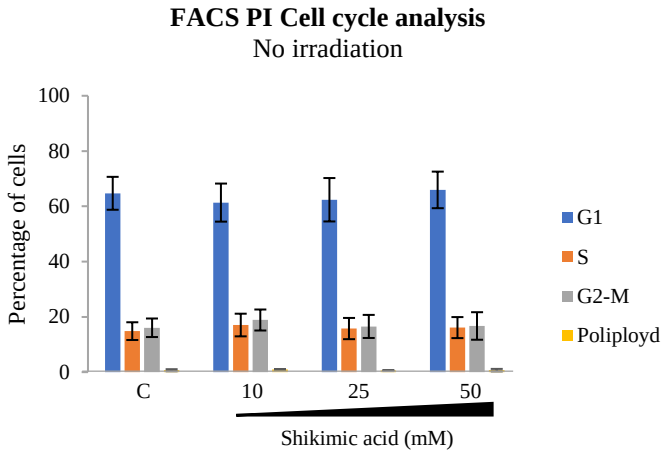

Figure 45. (A) Cell cycle analysis using PI staining followed by flow cytometry. Cells were cultured for 6 days with or without Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (B) The average percentages of G1, S, G2/M and polyploid cells. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

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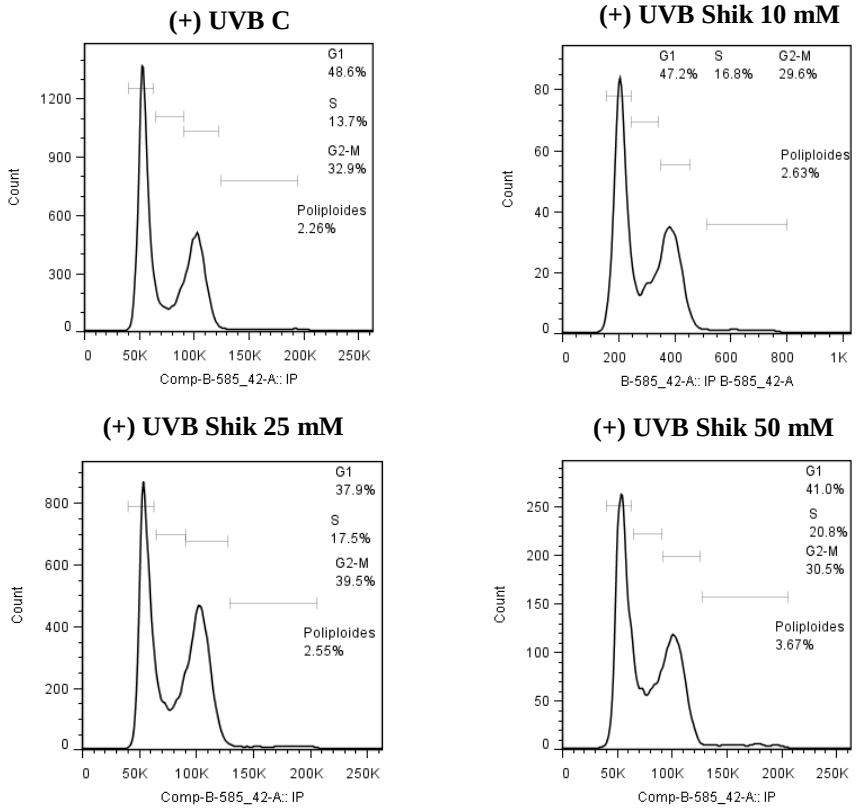

Figure 46. *Continued*

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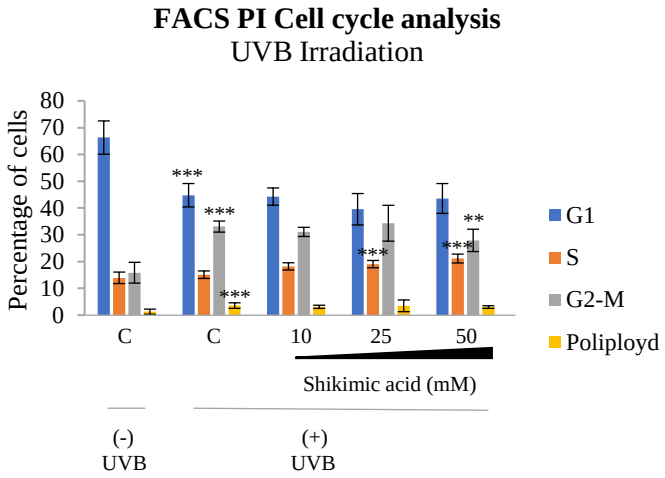

Figure 46. (A) Cell cycle analysis using PI staining followed by flow cytometry. Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (B) The average percentages of G1, S, G2/M and polyploid cells. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with Shikimic acid were compared to UVB-irradiated control cells for each cell cycle phase (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

First, we observed that Shikimic acid does not affect cell cycle when incubated alone, in the absence of stress (Figure 45). On the other hand, UVB-irradiated cells show a decrease in the number of G1 cells and an increase in the number of G2/M phase, which reflects the cell cycle arrest of cells before mitosis(Figure 46). Regarding Shikimic acid treatment, there is a dose-dependent accumulation of cells in S phase, which could indicate that these cells are either proliferating at a lower rate through S-phase or that are arrested in S phase.

Moreover, we also observed an increase in the number of polyploid cells in UVB-irradiated cells, which is a described feature in senescent cells (Kuilman T et al. 2010). The amount of polyploid cells was similar between UVB-irradiated cells and UVB-irradiated cells with Shikimic acid.

In order to confirm whether Shikimic acid is inducing or not proliferation in UVB-irradiated cells, we performed the EdU assay. In this assay, the thymidine analog EdU is incorporated in cells during DNA replication, meaning that only active proliferating cells will uptake EdU. EdU is fluorescent and allows us to further quantify the number of actively proliferating cells by flow cytometry. Thus, we performed the EdU assay on UVB-irradiated cells treated and non-treated with Shikimic acid to determine the proliferative state of cells (Figure 47). In parallel, we tested also cells incubated in Shikimic acid alone as a control to see the effect of Shikimic acid in cell proliferation in the absence of stress.

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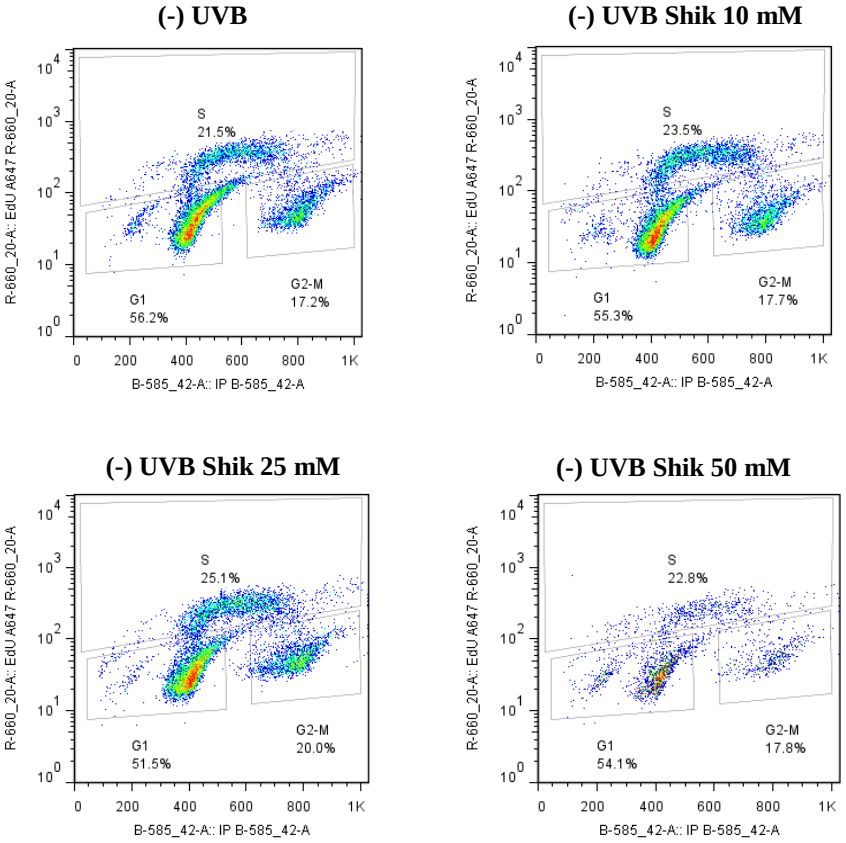

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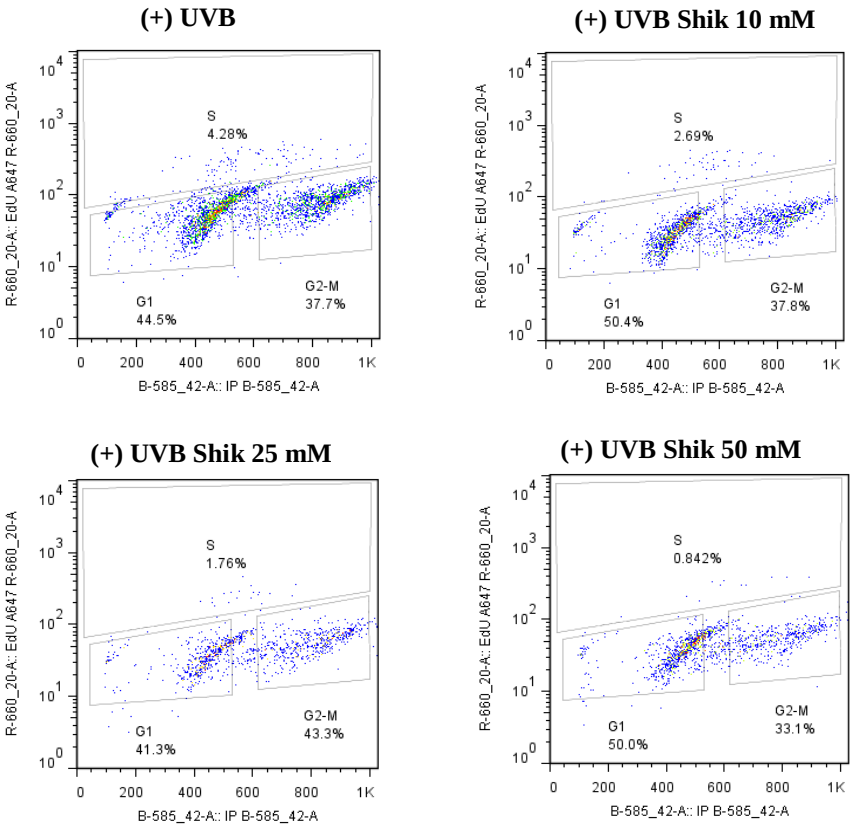


Figure 47. *Continued*

C

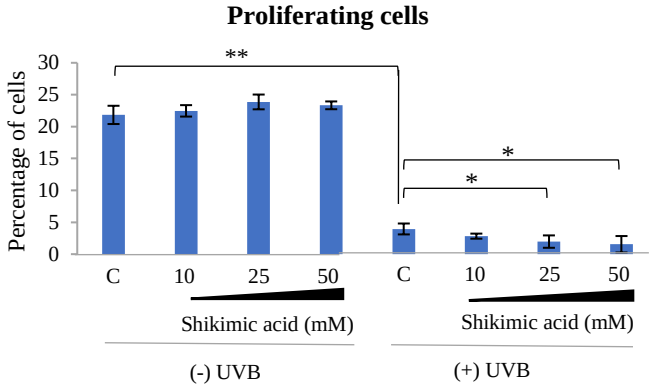


Figure 47. Cell cycle analysis using EdU staining followed by flow cytometry. (A) Cells were cultured for 6 days with or without Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (B) Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of Shikimic acid (10, 25 and 50 mM) and then harvested for analysis. (C) The average number of proliferating cells in cells irradiated and non-irradiated with UVB and in cells treated and non-treated with Shikimic acid. Statistics: Student's T-test. Non-irradiated cells treated with Shikimic acid were compared to non-irradiated control cells, while non-irradiated control cells and UVB-irradiated cells treated with Shikimic acid were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

According to our results, the amount of proliferating cells in control conditions (20%) decreases significantly when cells are irradiated with UVB (4%), as expected for senescent cells (Figure 47C). Moreover, Shikimic acid not only did not increase cell proliferation in UVB-irradiated cells, but decreased cell proliferation (2% and 2.5% for 25 and 50 mM respectively) compared to UVB-irradiated control cells. This decrease might be explained by the small increase in late apoptosis/necrosis (Figure 40). Thus, we can conclude that Shikimic acid favours cell cycle arrest in G1/S phase when these cells are irradiated with UVB. This could be explained by a possible regulation of the G1/S phase checkpoint by Shikimic acid. A plausible option is that after the DNA damage generated by UVB irradiation, Shikimic acid favours the activation of the G1/S phase checkpoint, which recognizes the damage and arrests cell cycle to prevent the proliferation of damaged cells (Iyer DR and Rhind N. 2017).

6.2.3 Effect of Shikimic acid on DNA damage in UVB-induced senescent cells

Another key feature in senescent cells is the persistent DNA damage that cannot be repaired. This damage can be identified by the presence of γH2AX foci. This histone variant is phosphorylated in the DNA damage sites, and dephosphorylated once the damage is repaired. In the case of senescence, the DNA damage is not repaired and thus large foci of γH2AX are present in the cell. Here, we wanted to determine whether Shikimic acid is able to prevent or repair the DNA damage that occur in UVB-induced senescence. For that, we detected γH2AX by immunofluorescence after UVB irradiation and Shikimic acid treatment to assess the presence of foci (Figure 48).

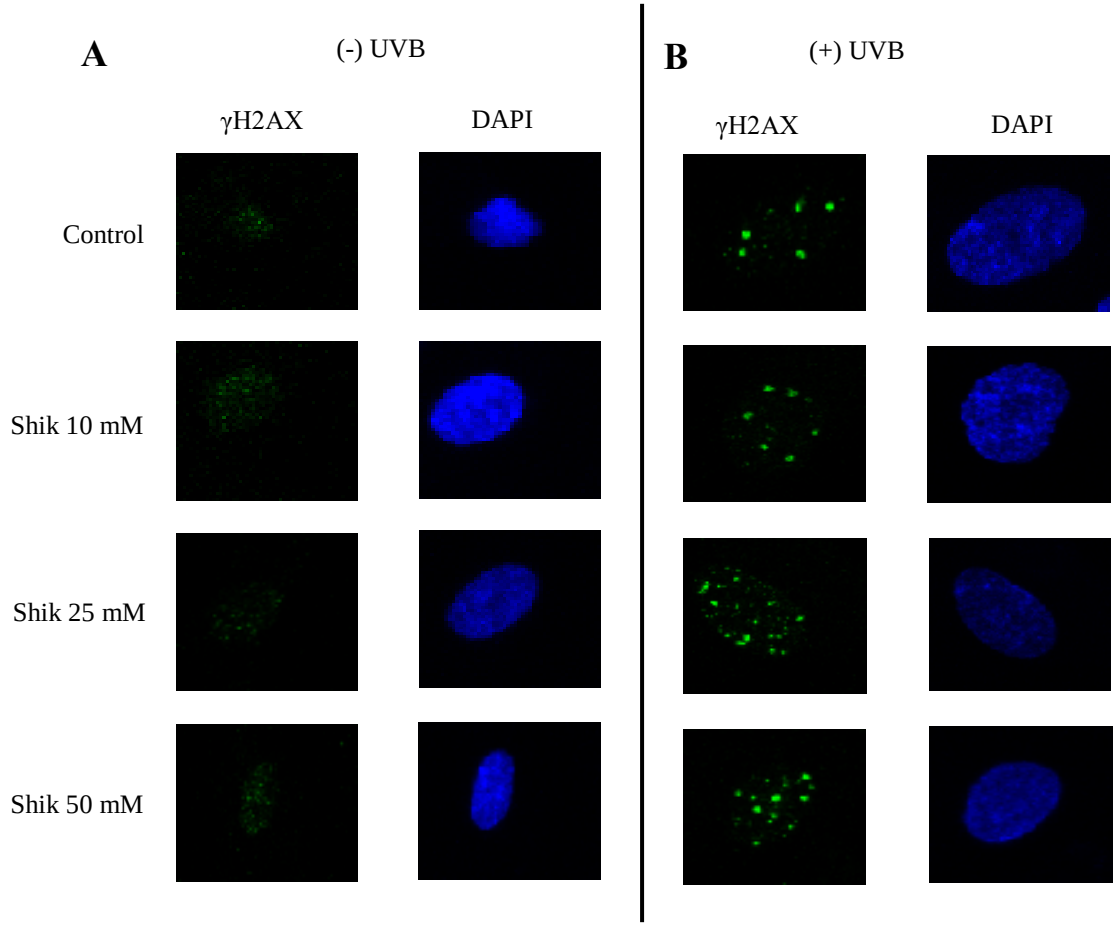


Figure 48. Immunofluorescence of γH2AX in HDF after senescence induction using UVB. (A) Cells were cultured for 6 days with or without Shikimic acid (10, 25 and 50 mM) and then stained for analysis. (B) Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of Shikimic acid (10, 25 and 50 mM) and then stained for analysis.

First, we observed that the amount of γH2AX signal was low in non-irradiated cells, indicating the absence of DNA damage, as expected (Figure 44A). On the other hand, we observed the presence of γH2AX foci characteristic of unrepaired DNA damage in UVB-irradiated cells, also expected for senescent cells (Figure 44B). However, we did not noticed changes in the γH2AX foci with Shikimic acid treatment in UVB-irradiated cells. Thus, Shikimic acid does not prevent the DNA damage that occurs in UVB-induced senescence. This accumulation of unrepaired damage would be in line with the previous results on the cell cycle arrest in S phase, as an unrepaired DNA damage would trigger the S phase checkpoint to avoid cell proliferation.

6.3 Effect of Shikimic acid on generic senescence markers and SASP

In order to better characterize the effect of Shikimic acid on senescence, we first quantified other marker genes also involved in senescence. Regarding the generic senescence markers p16 and p21, although not being suitable as skin senescence markers, we decided to focus on p21, as it has been described to be involved in DNA damage-induced senescence (including UVB damage, as in our case) through p53 activation in early steps of senescence, while p16 has a role in later steps in senescent phenotype maintenance (Mirzayans R et al. 2008, Mirzayans R et al. 2012). On the other hand, we also focused on interleukin-6 (IL-6), which is one of the key members of the SASP, the proinflammatory phenotype of senescent cells that contributes to tissue degeneration (Kuilman T et al. 2010).

In parallel to the quantification of these genes in UVB-induced senescent cells treated or non-treated with Shikimic acid (Figure 50), we also quantified the expression of these genes, along with HAS2, in cells cultured during the same number of days (6 days) than the UVB-induced senescent cells but without being irradiated (Figure 49). This was used as a control to see the effect *per se* of Shikimic acid on the senescence markers without the damage stimulus.

A

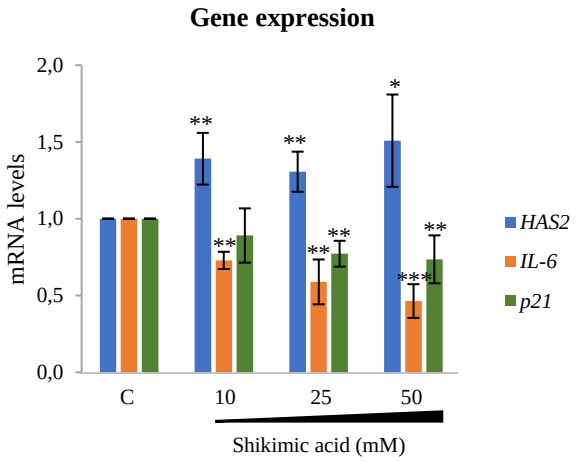


Figure 49. (A) Bright field (left) and phase-contrast (right) microscope images of β-Gal staining showing non-treated cells and cells treated with Shikimic acid at 10 mM, 25 mM and 50 mM for 6 days in non-irradiation conditions. (B) *HAS2*, *p21* and *IL-6* gene expression levels of cells treated or non-treated with Shikimic acid (10, 25 and 50 mM) for 6 days. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

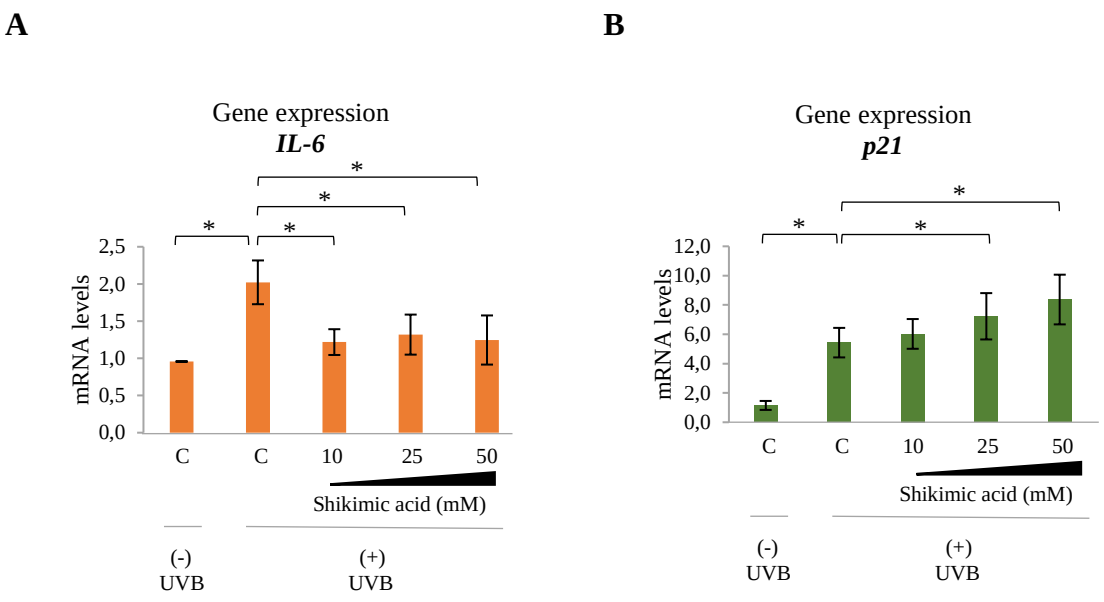


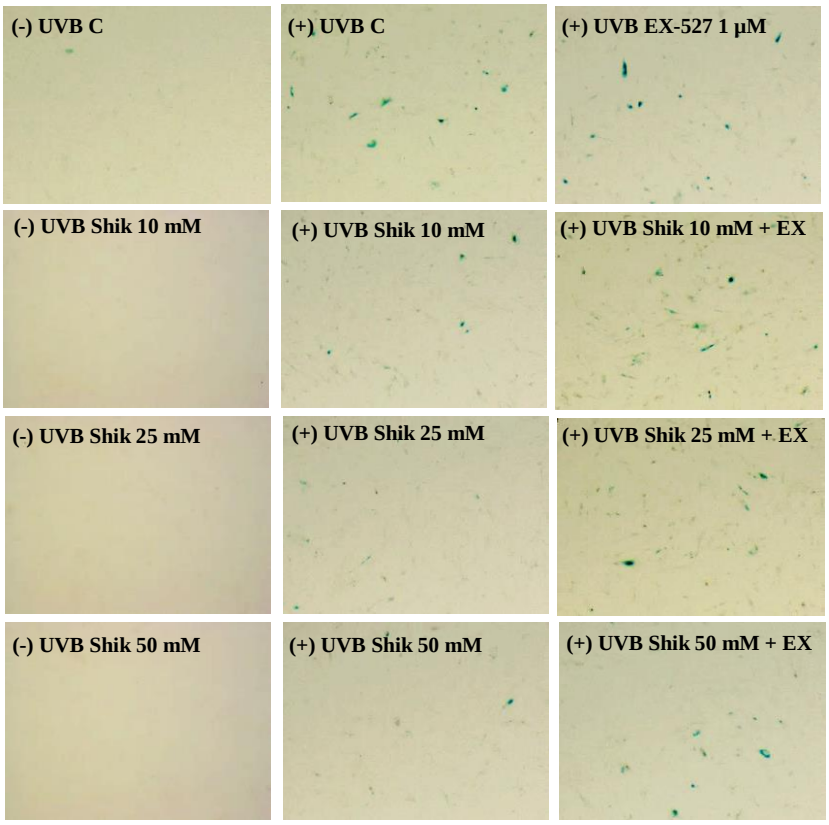
Figure 50. (A) *IL-6* and (B) *p21* gene expression levels of UVB-irradiated cells treated or non-treated with Shikimic acid (10, 25 and 50 mM). Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with Shikimic acid were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

As shown in Figure 49, Shikimic acid increases *HAS2* and decreases *IL-6* and *p21* gene expression in the absence of irradiation, meaning that Shikimic acid could be regulating the signaling pathways of senescence in non-damaging conditions. Besides, in UVB-irradiated cells, Shikimic acid prevented *IL-6* upregulation (Figure 50A). However, also in UVB-irradiated cells, *p21* gene expression is slightly upregulated in Shikimic acid treated cells compared to non-treated cells (Figure 50B).

6.4 Evaluation of sirtuins involvement in UVB-induced senescence effect by shikimic acid

One of our initial aims was to test whether the effect on senescence exerted by the positive compounds was sirtuin-dependent. In this context, SIRT1, SIRT2, SIRT6 and SIRT7 are the sirtuins which have been clearly involved in senescence regulation, as mentioned in the introduction (Ghosh S et al. 2015, Anwar T et al. 2016). For that purpose, we used specific sirtuin inhibitors to determine the involvement of each sirtuin individually in this process. Unfortunately, there are not specific inhibitors for SIRT7 commercially available, so we focused on SIRT1, SIRT2 and SIRT6. The inhibitors selected for this assay were EX-527, AGK2 and OSS128167. EX-527 is a selective sirtuin inhibitor for SIRT1 and to a lesser extent to SIRT6 (Napper AD 2005), AGK2 is a selective SIRT2 inhibitor (Outeiro TF et al. 2007) and OSS128167 is a selective SIRT6 inhibitor (Parenti MD et al. 2014). Hence, we incubated Shikimic acid with EX-527 (Figures 51-52), OSS128167 (Figures 53-54) or AGK2 (Annex 4) during the induction of senescence with UVB damage and measured β-Gal staining and *HAS2*, *IL-6* and *p21* gene expression.

A



B

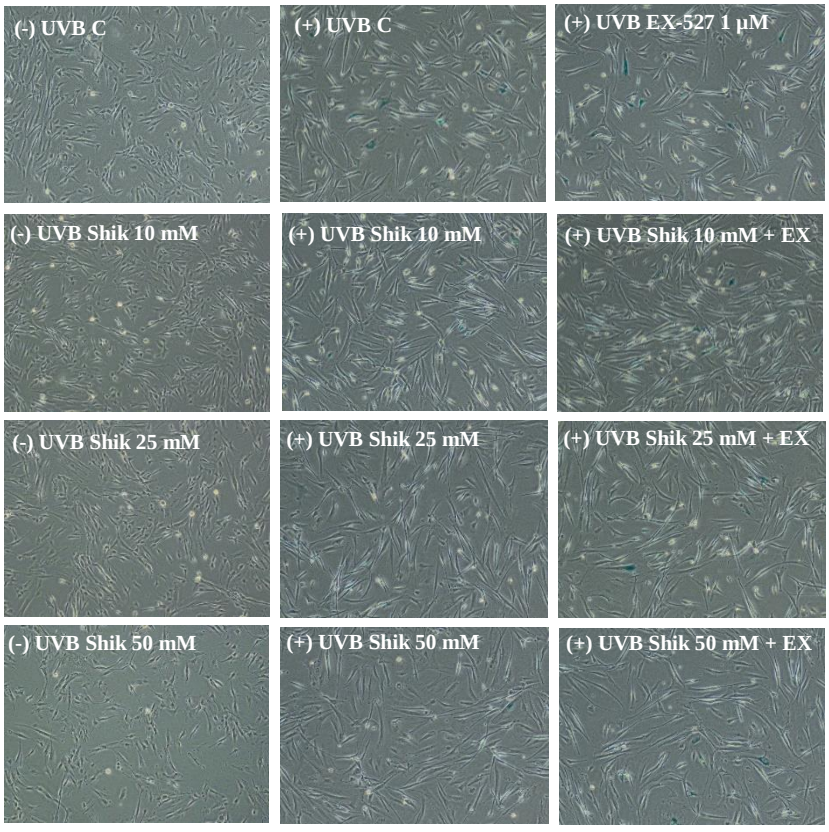


Figure 51. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with Shikimic acid (left column), irradiated cells treated or non-treated with Shikimic acid (center column) and irradiated cells treated or non-treated with Shikimic acid plus EX-527 1 µM (right column).

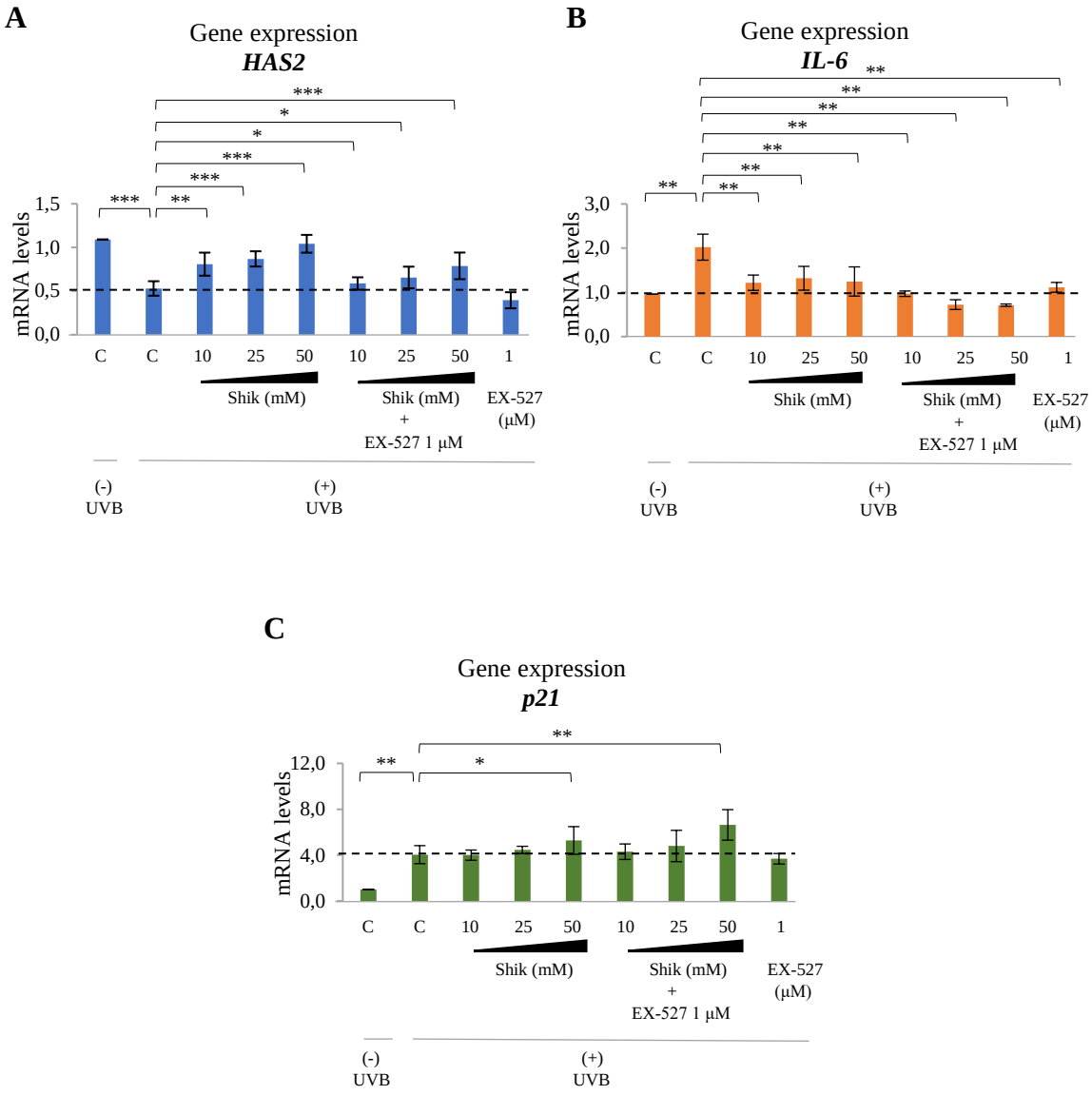


Figure 52. (A) *HAS2*, (B) *IL-6* and (C) *p21* gene expression levels of UVB irradiated cells treated or non-treated with Shikimic acid plus EX-527 1 µM. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with Shikimic acid +/- EX-527 and UVB-irradiated cells treated with EX-527 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

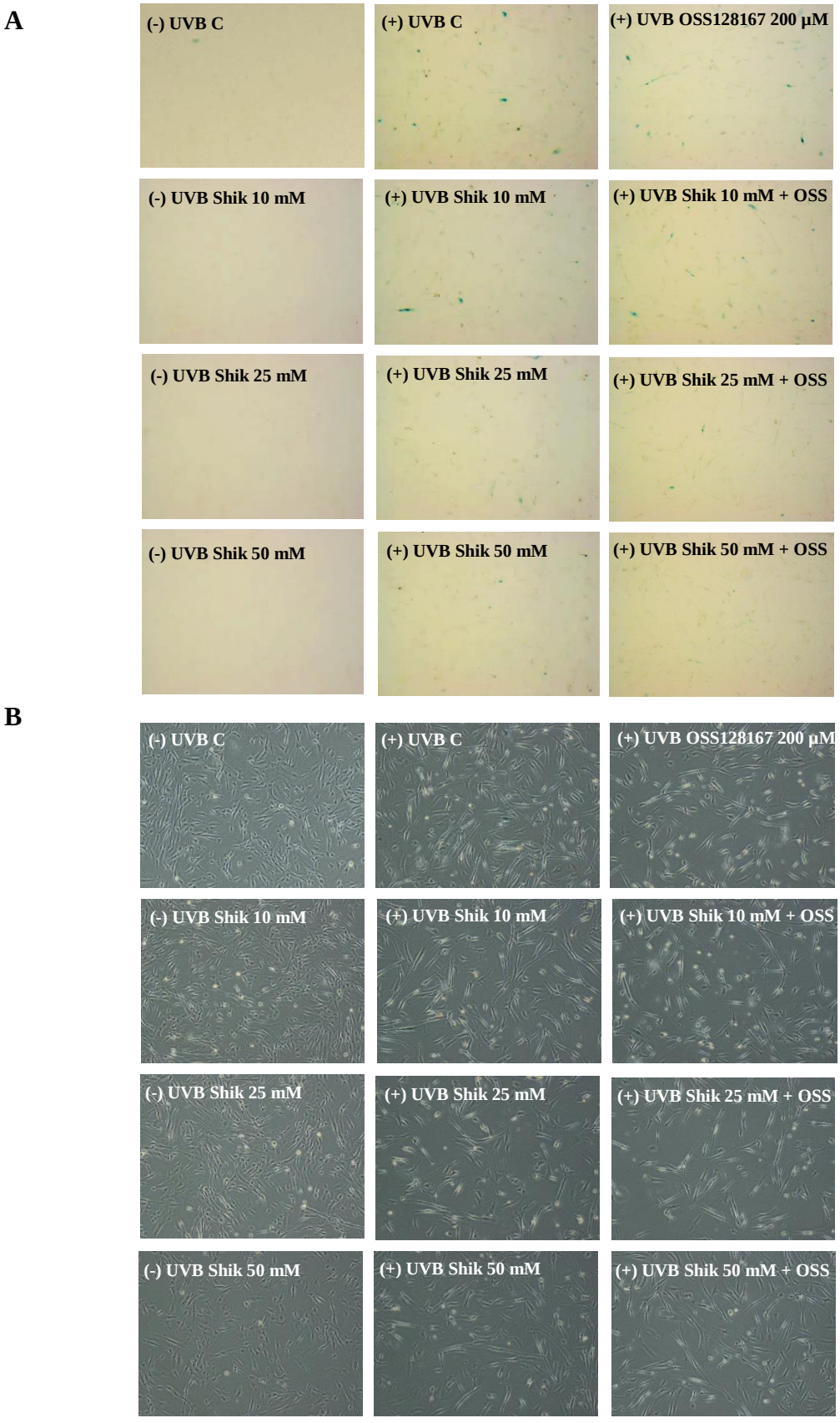


Figure 53. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with Shikimic acid (left column), irradiated cells treated or non-treated with Shikimic acid (center column) and irradiated cells treated or non-treated with Shikimic acid plus OSS128167 200 µM (right column).

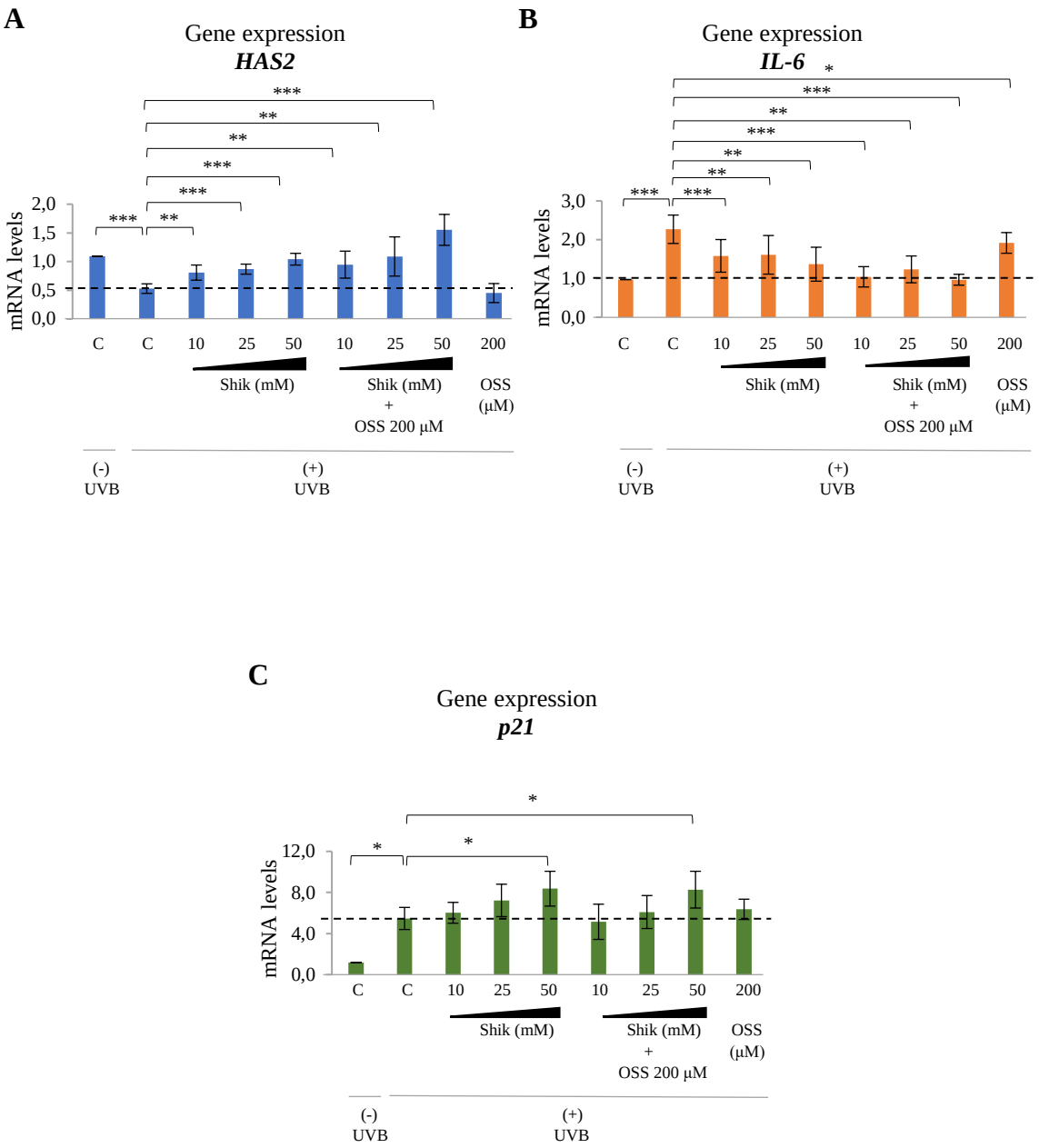


Figure 54. (A) *HAS2*, (B) *IL-6* and (C) *p21* gene expression levels of UVB irradiated cells treated or non-treated with Shikimic acid plus OSS128167 200 µM. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with Shikimic acid +/- OSS128167 and UVB-irradiated cells treated with OSS128167 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

First, we observed that Shikimic acid did not modify the β-Gal staining in non-irradiated cells (Figure 51A, left column). Regarding EX-527, we observed that this SIRT1 inhibitor was able to revert the protective effect of Shikimic acid in UVB-induced senescence. EX-527 incubation with Shikimic acid reversed β-Gal staining decrease (Figure 51A, right column) and *HAS2* upregulation induced by Shikimic acid (Figure 52A). Given the specific effect of EX-527 on SIRT1 activity, this evidence strongly suggested that the protective effect of Shikimic acid was SIRT1-dependent.

Regarding the expression of *p21*, mRNA levels were similar between cells treated with Shikimic acid and cells treated with Shikimic acid plus EX-527 (Figure 52C). On the other hand, *IL-6* gene expression levels were reduced in cells treated with Shikimic acid plus EX-527 compared to cells treated with Shikimic acid or EX-527 alone (Figure 52B), indicating an additive effect when these two compounds are combined. Interestingly, in UVB-irradiated cells, *IL-6* gene expression levels were reduced in cells treated with EX-527 alone compared to non-treated cells. This indicates that the anti-inflammatory action of Shikimic acid is independent of SIRT1. Surprisingly, although SIRT1 is widely known to act as a negative regulator of inflammation, SIRT1 inhibition by EX-527 induces an anti-inflammatory effect.

On the other hand, SIRT6 inhibition by OSS128167 did not influence Shikimic acid decrease in β-Gal staining (Figure 53). p21 mRNA levels did not change in the presence or absence of OSS128167 in cells treated with Shikimic acid (Figure 54C), while *HAS2* mRNA levels were slightly increased in cells treated with Shikimic acid at 50 mM plus OSS128167 compared to cells treated with Shikimic acid at 50 mM (Figure 54A). Interestingly, as in the case of EX-527, OSS128167 treatment alone induced a downregulation of *IL-6* expression in UVB-irradiated cells, and *IL-6* gene mRNA levels were reduced in cells treated with Shikimic acid plus OSS128167 compared to cells treated with Shikimic acid or OSS128167 alone (Figure 54B), indicating an additive effect between these two compounds. Other authors have reported that this inhibitor can reduce TNF-α expression (Parenti MD et al. 2014), indicating a possible anti-inflammatory effect of this compound that would explain the downregulation of IL-6 observed in our model.

Considering that EX-527 can inhibit both SIRT1 and to a lesser extent SIRT6, and OSS128167 specifically inhibits SIRT6, the comparison of the results between EX-527 and OSS128267 strongly highlights the specific role of SIRT1 on Shikimic acid mechanism of action.

Finally, UVB-irradiated cells treated with Shikimic acid and UVB-irradiated cells treated with Shikimic acid plus AGK2 showed similar levels of β-Gal staining (Annex 4), meaning that SIRT2 does not influence the protection against senescence by Shikimic acid. *p21* mRNA levels were also similar in the presence or absence of AGK2, while *HAS2* mRNA levels of cells treated with Shikimic acid plus AGK2 were slightly decreased compared to cells treated with Shikimic acid. Considering that the β-Gal staining was similar in the presence or absence of AGK2, this decrease in HAS2 expression caused by AGK2 might not be related to senescence but caused by the previously described toxicity of this inhibitor (Li Y et al. 2013). Interestingly, AGK2 alone reduces the levels of *IL-6* gene expression in UVB-irradiated cells. This might be explained by the anti-inflammatory effect of SIRT2 inhibition previously described by other authors (Orecchia A et al. 2011, Harrisson IF et al. 2018).

6.5 Effect of Shikimic acid on sirtuin gene expression and protein levels

The previous assays showed that Shikimic acid induced the deacetylation of sirtuin histone targets (Figure 29A) and that SIRT1 activity was involved in Shikimic acid protective effect on senescence (Figure 51 and 52). Here, we wanted to determine, as we did previously with Lipoic and Ferulic acids (Figures 25 and 26), whether Shikimic acid not only activates sirtuins but also regulates sirtuin gene expression and protein levels. For that purpose, we performed qPCR (Figure 55) and Western blot (Figure 56) assays with the samples of cells treated for 24 hours with Shikimic acid to assess the gene and protein levels of sirtuins, respectively.

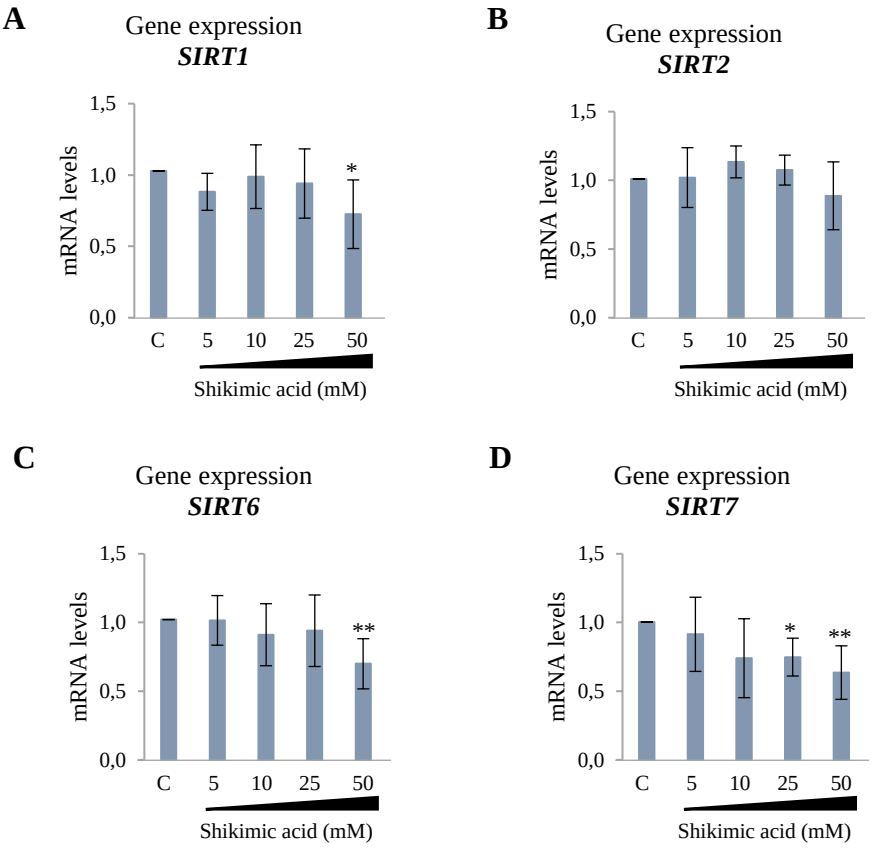


Figure 55. (A) *SIRT1*, (B) *SIRT2*, (C) *SIRT6* and (D) *SIRT7* gene expression levels in HDF treated with Shikimic acid (5, 10, 25 and 50 mM) for 24 hours. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

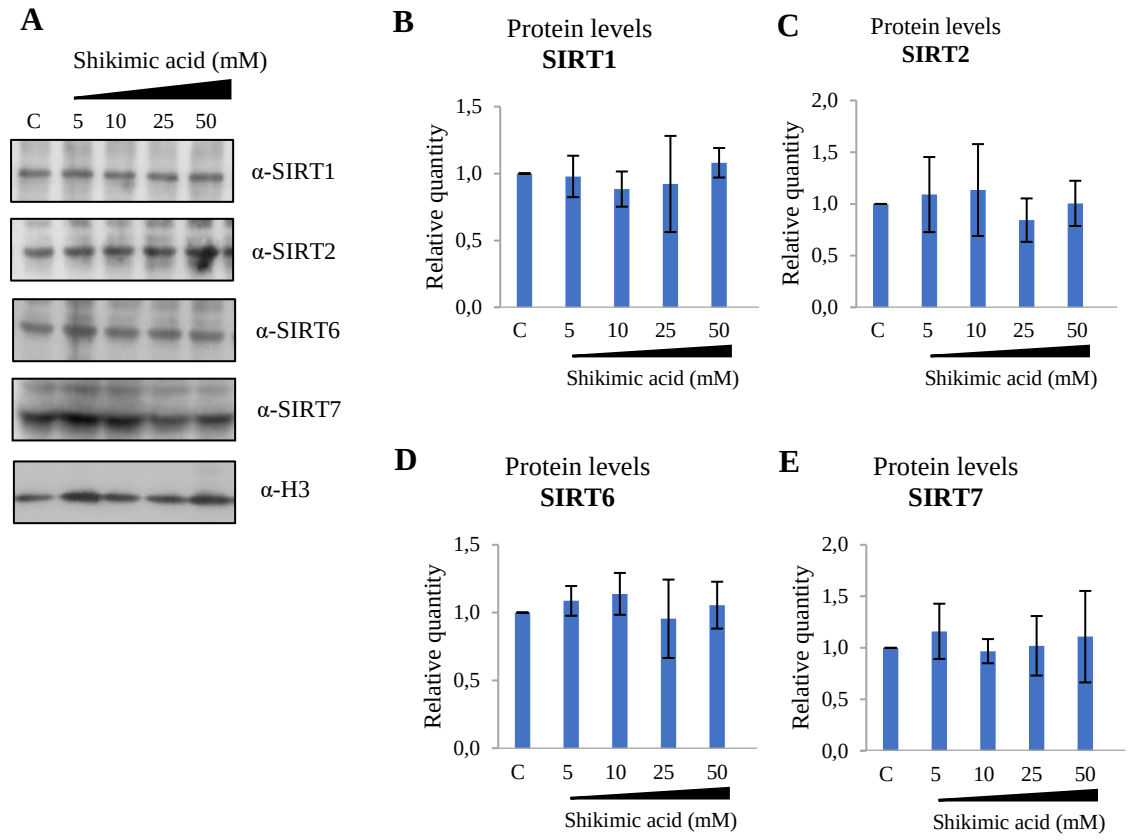


Figure 56. (A) Western blot analysis of SIRT1, SIRT2, SIRT6 and SIRT7 protein levels in HDF treated with Shikimic acid (5, 10, 25 and 50 mM) for 24 hours. Quantification of SIRT1 (B), SIRT2 (C), SIRT6 (D) and SIRT7 (E) Western blot bands. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

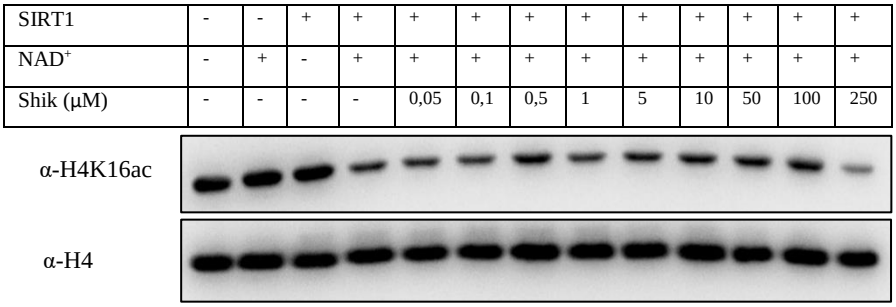
We observed a significant downregulation in *SIRT1*, *SIRT6* and *SIRT7* in the cells treated with Shikimic acid at the higher dose (50 mM) (Figure 55A, C and D). However, the protein levels of all sirtuins in Shikimic acid treated cells were similar to control cells (Figure 56). This decrease in the mRNA levels at the highest dose might be observed because the 50 mM dose of Shikimic acid is close to induce cytotoxicity and might be causing dysregulation on gene expression. Thus, Shikimic acid induces sirtuins activity without regulating sirtuin gene expression or protein levels.

6.6 Evaluation of Shikimic acid direct effect on SIRT1 enzymatic activity

Given that Shikimic acid does not increase neither sirtuin gene expression nor protein levels but induces SIRT1 activity, we wanted to determine whether Shikimic acid can directly activate SIRT1. For that purpose, we transfected HEK293 cells with a SIRT1 plasmid and purified the SIRT1 enzyme to perform an *in vitro* enzymatic assay. In this assay, the purified SIRT1 is incubated with NAD^+ (cofactor) and hyperacetylated histones (substrate) along with different doses of Shikimic acid. After the reaction, SIRT1 activity,

which is proportional to histone deacetylation, is evaluated by quantifying H4K16ac levels through Western blot. Thus, we can monitor SIRT1 *in vitro* activity by detecting the changes in histone acetylation. The doses chosen for Shikimic acid were lowered to the range of µM as if there is a direct activation by the compound in an *in vitro* assay, it should be detected within this range. At first, different reaction times (from 15 to 90 minutes) were tested to define an optimal timing in which we observe a 60% decrease in H4K16 acetylation, so we make sure that the enzyme is active and a possible activation by the compound can be clearly detected. The optimal reaction time was defined at 15 minutes.

A



B

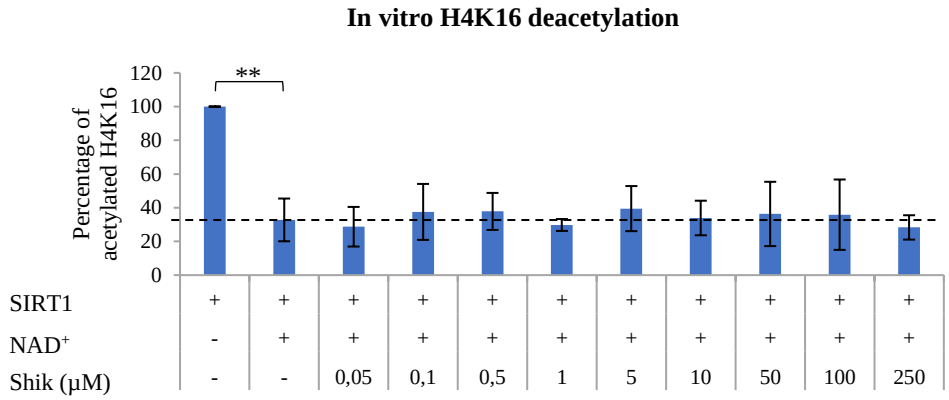


Figure 57. (A) *In vitro* enzymatic activity of SIRT1 in the presence or abscence of Shikimic acid. SIRT1 was purified from transfected HEK293 and was incubated with NAD^+, hyperacetylated histones and Shikimic acid (0.05, 0.1, 0.5, 1, 5, 10, 50, 100 and 250 µM) for 15 min. The reaction was then stopped using loading buffer and H4K16ac and H4 were quantified using Western blot. (B) Quantification of the Western blot bands indicating the amount of deacetylation of H4K16, which is proportional to SIRT1 activity. Statistics: Student's T-test. +SIRT1/-NAD^+ and +SIRT1/+NAD^+ plus Shikimic acid at the different concentrations were compared to +SIRT1/+NAD^+ (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

First, two negative controls for the experiments were used, -SIRT1/-NAD^+ and -SIRT1/+ NAD^+, were as expected no deacetylation of H4K16 occurred (Figure 57A, first and second columns, respectively). Besides, another control included was +SIRT1/-NAD^+. In this condition H4K16 deacetylation was not detected neither, highlighting the dependency of sirtuins on NAD^+ to carry on their enzymatic activity. The

+SIRT1/+NAD$^+$ condition induced a decrease of 30% in H4K16 acetylation, and the addition of Shikimic acid did not influence the levels of H4K16 acetylation (Figure 57B). Thus, Shikimic acid does not seem to activate SIRT1 directly, and we hypothesize that it is possibly acting indirectly through the signaling mechanisms that regulate SIRT1 activity.

6.7 Evaluation of Shikimic acid effect on sirtuin-regulated pathways

According to our initial plan, another point in the characterization of sirtuin activators was to assess the effect of these compounds on several pathways regulated by sirtuins. Among these pathways, we chose the DNA repair, antioxidant machinery and autophagy pathways, as these show a growing interest in the dermocosmetic market. For this purpose, we incubated the cells with Shikimic acid for 24h and quantified the mRNA levels of several members of the DNA repair and antioxidant machinery (Figure 58). Autophagy-related genes were also tested, and the results are detailed in the next section.

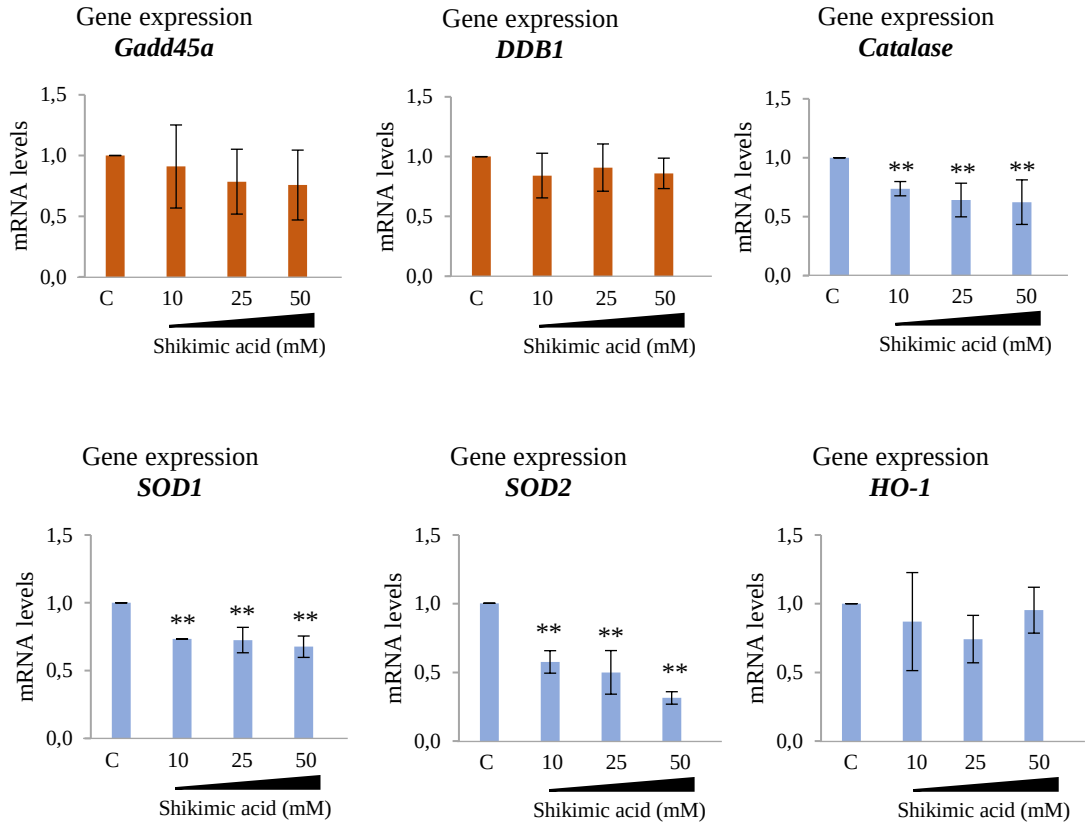


Figure 58. mRNA levels of DNA repair genes, (A) *Gadd45a* and (B) *DDB1*, and antioxidant machinery-related genes, (C) *Catalase*, (D) *SOD1*, (E) *SOD2* and (F) *HO-1*, in HDF treated with Shikimic acid (10, 25 and 50 mM) for 24h. Statistics: Student's T-test. Cells treated with Shikimic acid were compared con control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

As these genes belong to stress-response pathways regulated by SIRT1 (Kobayahsi Y et al. 2005, Olmos Y et al. 2013), we were expecting an upregulation of these genes by Shikimic acid. Surprisingly, none of the genes were upregulated with Shikimic acid treatment (Figure 58). Interestingly, *Catalase*, *SOD1* and *SOD2*, which belong to the

family of intracellular antioxidant enzymes, were downregulated by Shikimic acid. *HO-1*, the other gene of this family that was tested, also showed downregulation but only at the 25 mM dose. This result was surprising, as SIRT1 has been described to increase the expression of antioxidant genes as part of the cellular stress response (Olmos Y et al. 2013). However, a possible explanation for these observations could be the transcription repression effect exerted by SIRT1 through the deacetylation of histones (Zhang T and Kraus WL. 2010, Morris KC et al. 2011). In fact, H3K9 deacetylation has been associated with decreased expression of SOD2 (Pollack BP et al. 2009). Moreover, antioxidant genes such as *Catalase* and *SOD1-SOD2* are normally upregulated in the context of oxidative stress conditions (Espinosa-Diez C et al. 2015), thus meaning that Shikimic acid treatment does not induce oxidative stress in these cells.

6.8 Evaluation of Shikimic acid effect on the autophagy pathway

Along with the gene expression analysis of DNA repair and antioxidant machinery-related genes, we also quantified the autophagy markers *ATG5*, *Beclin-1*, *LC3* and *VMP1* after 24h incubation with Shikimic acid (Figure 60A). Here, we saw that while *ATG5*, *Beclin-1* and *LC3* showed decreased expression, *VMP1* expression was upregulated by Shikimic acid in a dose-dependent manner. Previous reports showed that *VMP1* upregulation activates autophagy in its early steps (Molejon MI et al. 2013), indicating that Shikimic acid is probably inducing autophagy activation.

At this point, given this positive result, we decided to focus our efforts in the characterization of Shikimic acid effect on autophagy. First, we decided to test other genes with key roles in autophagy (*ATG7*, *ATG12* and *ULK-3*) to see if they were also upregulated as in the case of *VMP1* (Figure 60A) (Young AR et al. 2009, Rubinsztein DC, et al. 2012). Second, in order to determine whether the previous observed gene upregulation was indeed activating autophagy, we performed Western blot assays of the main autophagy markers that are widely used to monitor the autophagy state of the cell. The most frequently used marker for autophagy monitorization is the quantification of the ratio between LC3 active form (LC3-II) and LC3 inactive form (LC3-I), in which a higher amount of LC3-II is associated with a higher autophagy flux (Orhon I and Reggiori F. 2017). The autophagy receptor p62 is also widely used to determine the later steps of autophagy, as p62, which is a carrier of proteins targeted to be eliminated within the autophagosome, is expected to be degraded during active autophagy. Furthermore, other participants in the autophagy pathway, such as ATG5, are also quantified to assess differences in their protein amount which could suggest autophagy modulation. Thus, we quantified LC3, p62 and ATG5 by Western blot to determine if Shikimic acid was indeed increasing autophagy in HDF (Figure 60B). Besides, given that AMPK activates autophagy and it has been described to be regulated by SIRT1 and viceversa (Ruderman NB et al. 2010, Herzig S and Shaw RJ. 2018), we also measured the levels of phosphorylated AMPK (p-AMPK), which corresponds to activated AMPK, and total AMPK (Figure 60B). Finally, as autophagy is a very dynamic process, we decided to quantify the gene expression and protein levels of the previously mentioned autophagy markers also at 8h (Figure 59) in addition to 24h (Figure 60).

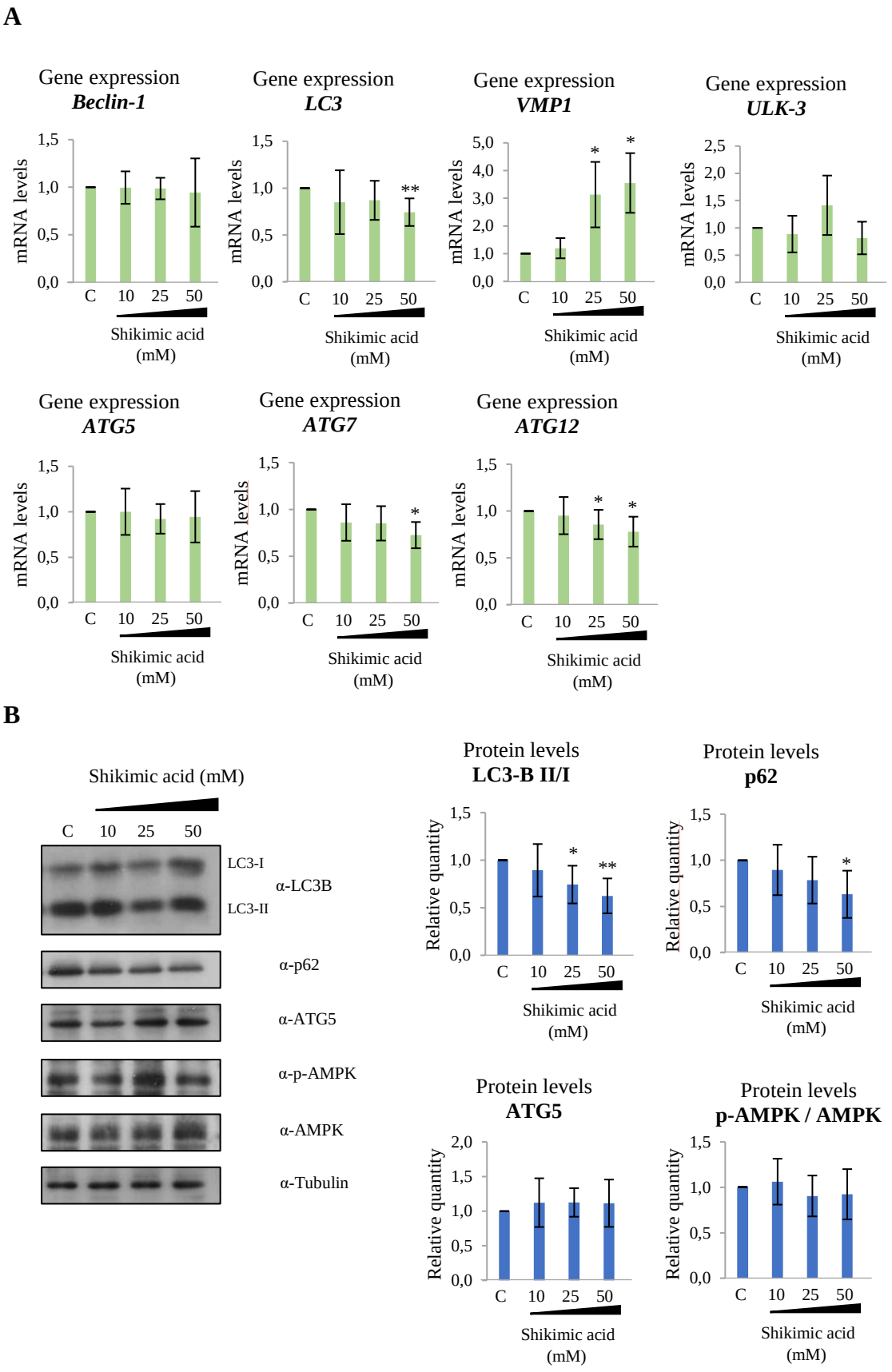


Figure 59. (A) Gene expression and (B) protein levels analysis of autophagy markers in HDF treated with Shikimic acid (10, 25 and 50 mM) during 8h. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

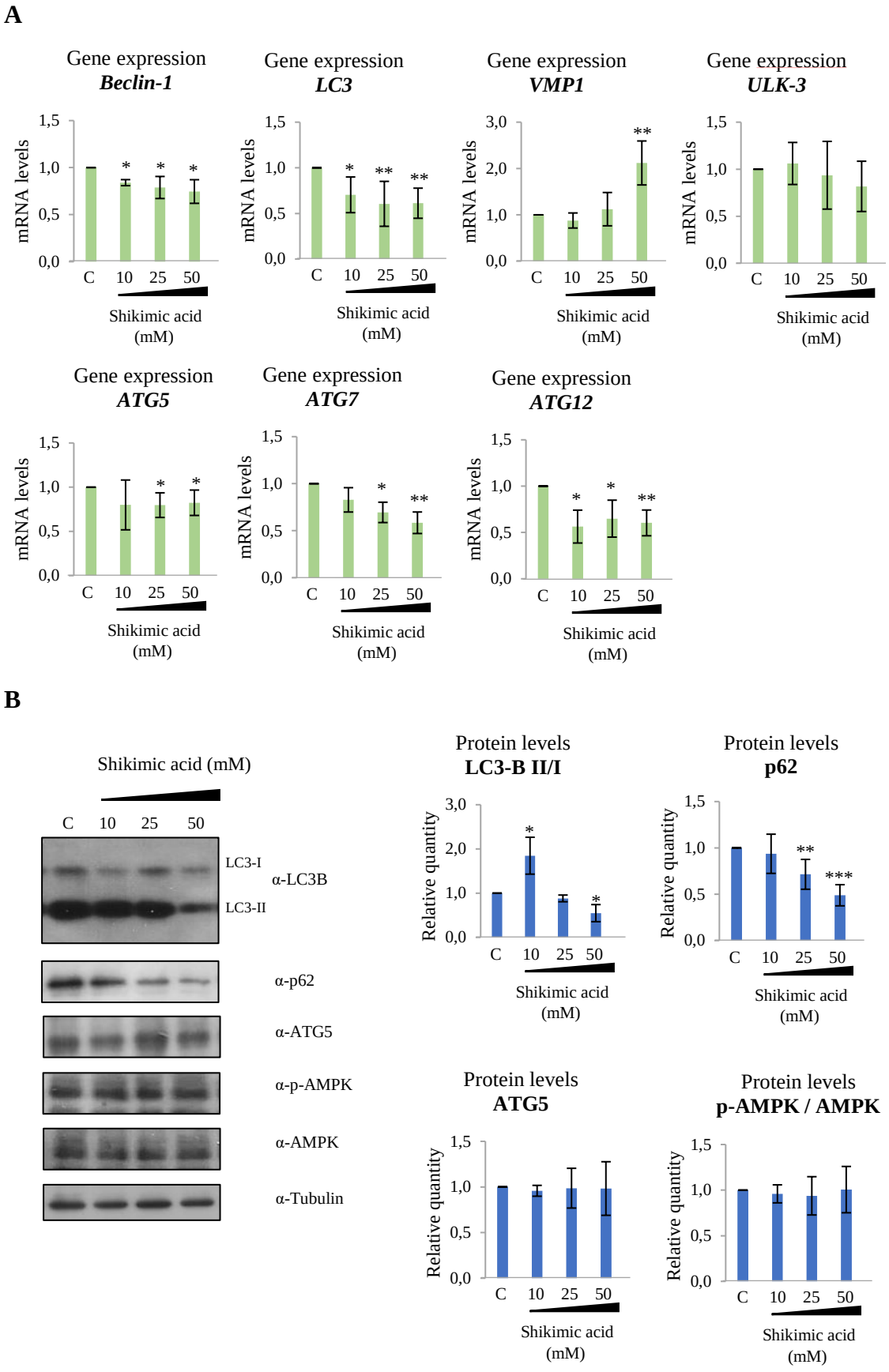


Figure 60. (A) Gene expression and (B) protein levels analysis of autophagy markers in HDF treated with Shikimic acid (10, 25 and 50 mM) during 24h. Statistics: Student's T-test. Cells treated with Shikimic acid were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

According to the qPCR results, we observed that *VMP1* was also upregulated at 8h (Figure 59A) and that this upregulation was higher than the one observed at 24h (Figure 60A). On the other hand, a clear downregulation of *LC3*, *ATG5*, *ATG7*, *ATG12* and *Beclin-1* gene expression was observed at 24h (Figure 60A), while only *LC3, ATG7* and *ATG12* showed smaller decreases in gene expression at 8h (Figure 59A). This downregulation in gene expression is similar to the one observed in the antioxidant machinery genes (Figure 58) and thus it could be associated with the transcription repression effect of SIRT1, while the upregulation of *VMP1* both at 8h and 24h suggests an activation of autophagy by Shikimic acid.

Regarding the Western blot results, neither AMPK nor ATG5 showed significant changes both at 8h and 24h (Figure 59B and 60B). However, the LC3 ratio and p62 protein amount were downregulated both at 8h and 24h, with the exception of Shikimic acid 10 mM at 24h, which increased the LC3 ratio (Figure 59B and 60B). Although LC3 ratio is expected to increase in active autophagy, previous reports have shown that LC3 ratio could be decreased during autophagy activation due to the fact that LC3-II is recruited to the autophagosome and is degraded with its content, as it happens with p62 (Orhon I and Reggiori F. 2017). Thus, the decrease in LC3 ratio and p62 protein amounts along with the VMP1 gene upregulation and the absence of AMPK activation, suggested that Shikimic acid is activating autophagy in an AMPK-independent way.

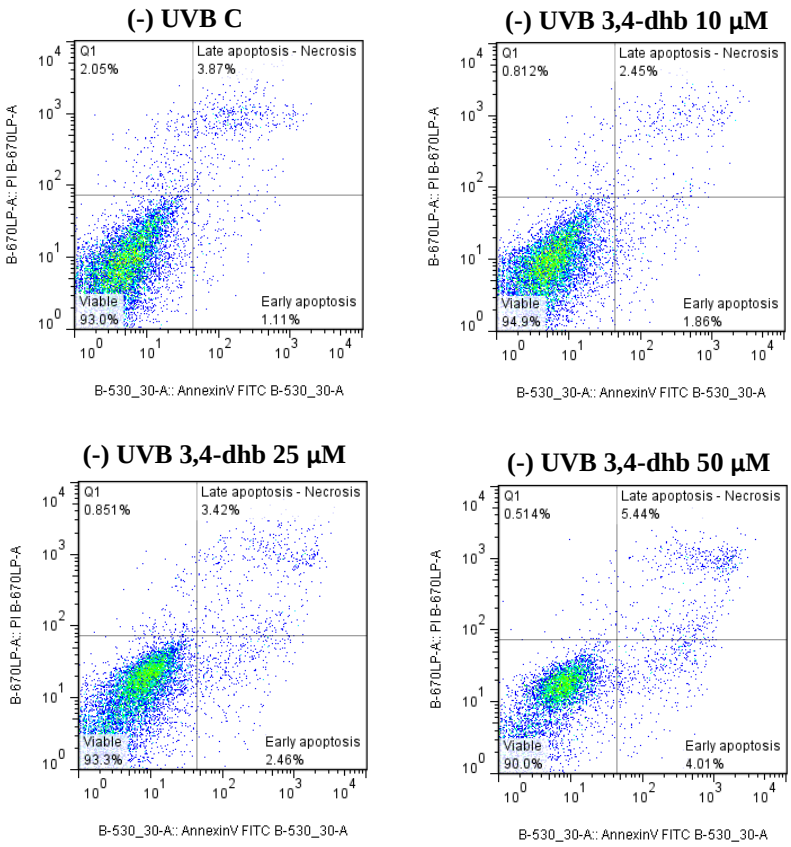
7. Molecular characterization of 3,4-dihydroxybenzaldehyde

7.1 Effect of 3,4-dihydroxybenzaldehyde on apoptosis and cell cycle in UVB-induced senescent cells

7.1.1 Effect of 3,4-dihydroxybenzaldehyde on apoptosis in UVB-induced senescent cells

As previously mentioned, 3,4-dihydrozybenzaldehyde doses had to be lowered from 0.5-2 mM to 10-50 μM for the senescence screening assay, as doses higher than 50 μM induced cell death when cells were irradiated with UVB. According to this senescence assay, these doses of 10-50 μM increased β-Gal staining in UVB-irradiated cells compared to non-treated UVB-irradiated cells (Figure 41D), suggesting an increase in senescence induced by the compound. In order to determine the apoptotic state of these cells, HDF were treated with 3,4-dihydroxybenzaldehyde (10-50 μM) and irradiated with UVB as regarded in the senescence-induction protocol (Figure 32) and harvested for analysis by the Annexin V assay (Figure 62). Besides, HDF treated with 3,4-dihydroxybenzaldehyde in the absence of UVB were analyzed to determine the effect of 3,4-dihydroxybenzaldehyde alone on apoptosis (Figure 61).

A



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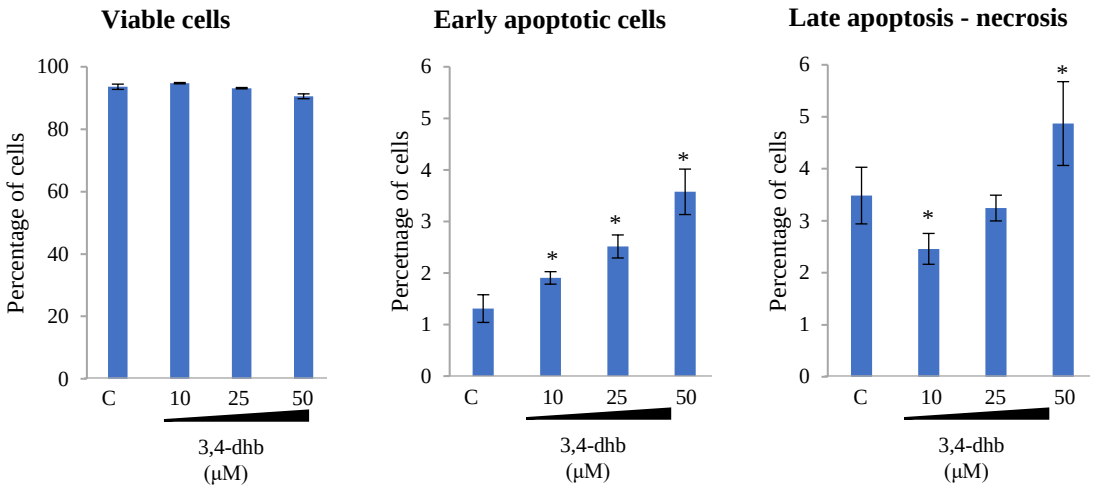
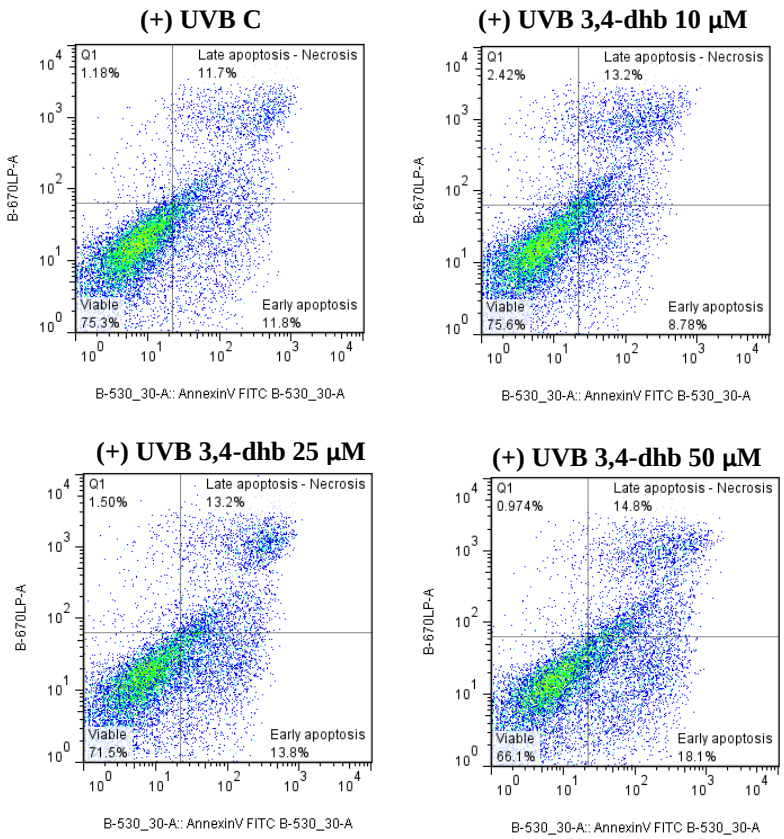


Figure 61. (A) Apoptosis detection using the Annexin V-FITC/PI double staining assay followed by flow cytometry. Cells were cultured for 6 days with or without 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM) and then harvested for analysis. (B) The average percentages of viable, early apoptotic and late apoptotic/necrotic cells. Statistics: Student's T-test. Cells treated with 3,4-dihydroxybenzaldehyde were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A



B

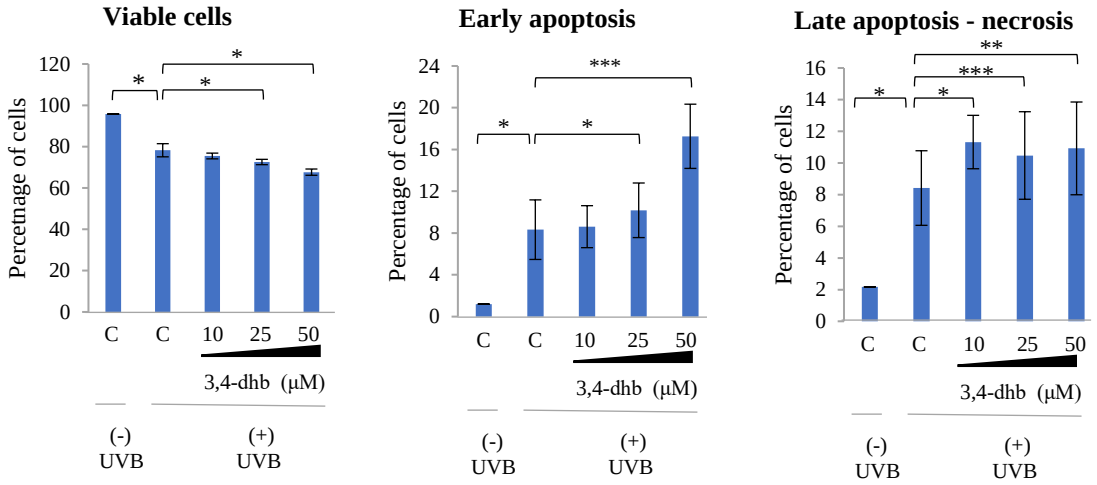


Figure 62. (A) Apoptosis detection using the Annexin V-FITC/PI double staining assay followed by flow cytometry. Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM) and then harvested for analysis. (B) The average percentages of viable, early apoptotic and late apoptotic/necrotic cells. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

In non-irradiated cells, there was a dose-dependent increase in early apoptotic cells induced by 3,4-dihydroxybenzaldehyde treatment (0.6%, 1.2% and 2.3% for 10, 25 and 50 μM, respectively) (Figure 61). On the other hand, there was a 2% increase in late apoptosis/necrosis after the treatment with 3,4-dihydroxybenzaldehyde at 50 μM, while the 10 μM dose decreased late apoptotic/necrotic cells by 1%. These small changes might not be biologically relevant, although they reflect the damaging effects of 3,4-dihydroxybenzaldehyde.

Regarding UVB-irradiated cells, 3,4-dihydroxybenzaldehyde treatment induced a significant increase in early apoptosis (2% and 9% for 25 and 50 μM, respectively) and late apoptosis/necrosis (3% for 10, 25 and 50 μM) (Figure 62). Thus, according to these results, along with the previously observed increase in senescence, 3,4-dihydroxybenzaldehyde also induces apoptotic cell death in UVB-irradiated cells.

7.1.2 Effect of 3,4-dihydroxybenzaldehyde on cell cycle in UVB-induced senescent cells

Considering that cell cycle arrest is a key feature of senescent cells, here we wanted to study the effect of 3,4-dihydroxybenzaldehyde on cell cycle both in non-irradiated and UVB-irradiated cells. Consequently, on one hand, we cultured HDF with 3,4-dihydroxybenzaldehyde in the absence of irradiation and analysed them by FACS to determine the effect of the compound *per se* on cell cycle (Figure 63). On the other hand, we incubated HDF with 3,4-dihydroxybenzaldhyde, irradiated the cells with UVB to induce senescence and analysed them by FACS to determine the effect of the compound on cell cycle changes induced during senescence (Figure 64).

A

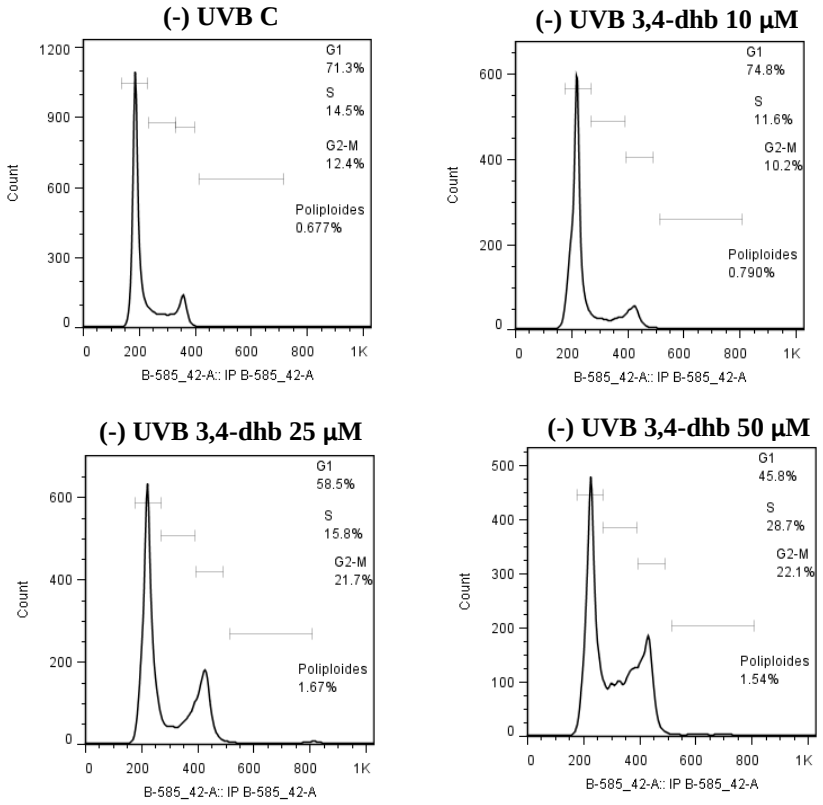


Figure 63. *Continued*

B

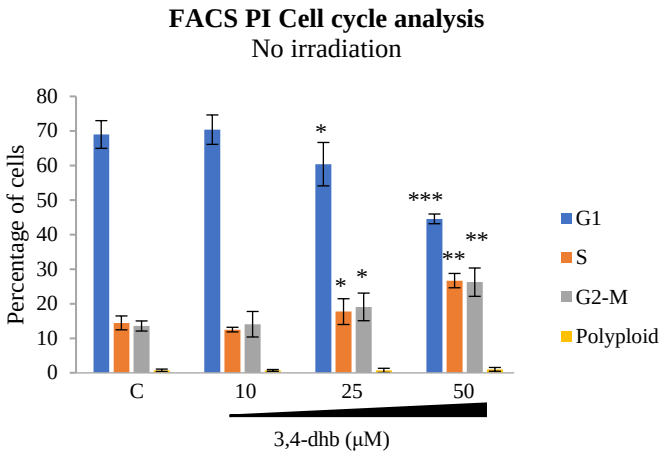


Figure 63. (A) Cell cycle analysis using PI staining followed by flow cytometry. Cells were cultured for 6 days with or without 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM) and then harvested for analysis. (B) The average percentages of G1, S, G2/M and polyploid cells. Statistics: Student's T-test. Cells treated with 3,4-dihydroxybenzaldehyde were compared to control cells for each cell cycle phase (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A

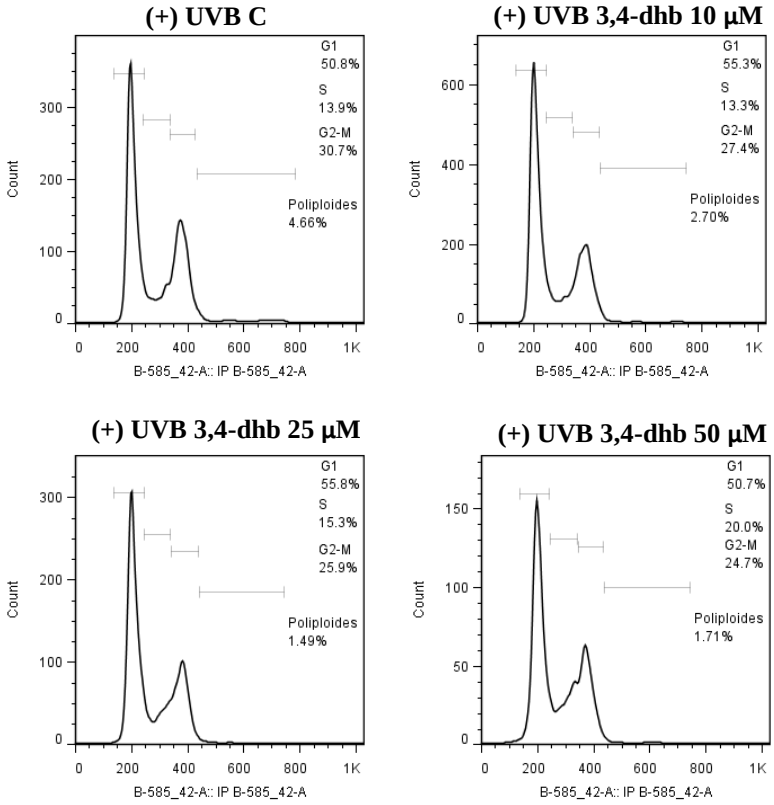


Figure 64. *Continued*

B

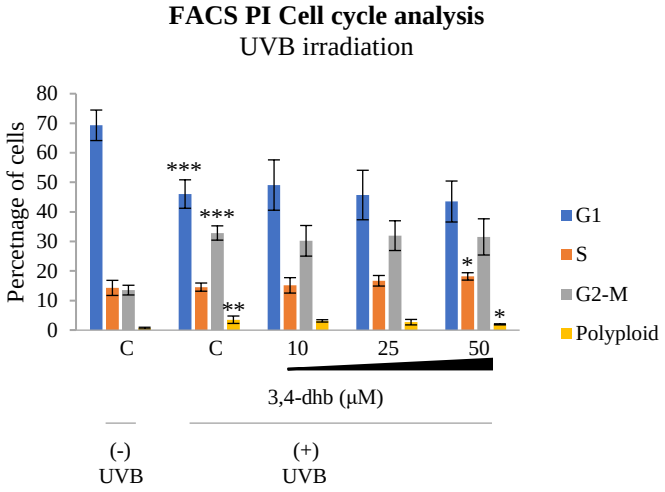


Figure 64. (A) Cell cycle analysis using PI staining followed by flow cytometry. Cells were irradiated twice in 6 days to induce senescence (as described in Figure 9) in the presence or absence of 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM) and then harvested for analysis. (B) The average percentages of G1, S, G2/M and polyploid cells. Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde were compared to UVB-irradiated control cells for each cell cycle phase (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

In non-irradiated cells, we observed that 3,4-dihydroxybenzaldehyde increased the number of cells in S and G2/M phases and decreased the number of cells in G1 phase (Figure 63). This result, combined with the previous observations that 3,4-dihydroxybenzaldehyde increases cellular senescence, indicated that this compound induces a cell cycle arrest in S and G2/M phases. On the other hand, in UVB-irradiated cells, there was an increase in S phase cells and a decrease in polyploid cells after the treatment with 3,4-dihydroxybenzaldehyde at 50 µM (Figure 64). Thus, non-irradiated cells treated with 3,4-dihydroxybenzaldehyde showed a similar pattern in the cell cycle analysis compared to the UVB-induced senescent control cells, with the exception of the increase in S phase at the highest dose of 3,4-dihydroxybenzaldehyde. This might be due to differences in the damage inducing mechanism between the compound and UVB irradiation, in which one case induce a clear G2/M cell cycle arrest while in the other case the cell cycle arrest occurs both at S phase and G2/M phase. The decrease in the number of UVB-induced polyploid cells after 3,4-dihydroxybenzaldehyde treatment at 50 µM might be explained by the cytotoxicity of the compound.

7.2 Effect of 3,4-dihydroxybenzaldehyde on generic senescence markers and SASP

In order to better characterize the effect of 3,4-dihydroxybenzaldehyde on senescence, we focused on the quantification of the generic senescence marker p21 and the marker of SASP IL-6. For that purpose, we first tested the effect of 3,4-dihydroxybenzaldehyde on *HAS2*, *IL-6* and *p21* gene expression levels after 6 days of treatment without irradiation (Figure 65), in order to see the effect *per se* of the compound without stress. Second, we

also tested the effect of this compound on *IL-6* and *p21* gene expression levels in UVB-irradiated cells (Figure 66).

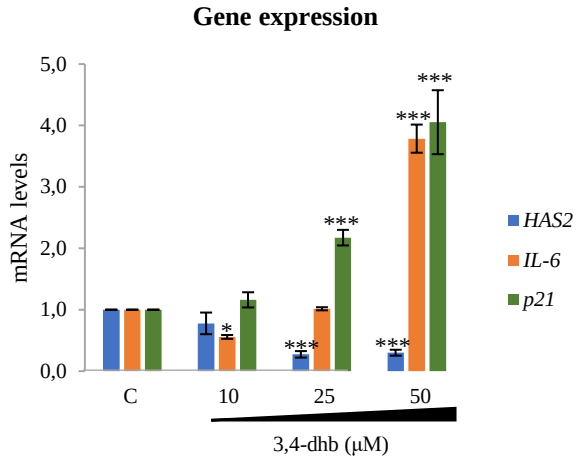


Figure 65. *HAS2*, *p21* and *IL-6* gene expression levels of cells treated with 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM) for 6 days in non-irradiation conditions. Statistics: Student's T-test. Cells treated with 3,4-dihydroxybenzaldehyde were compared to control cells (* p<0.05, ** p<0.01 and *** p<0.001).

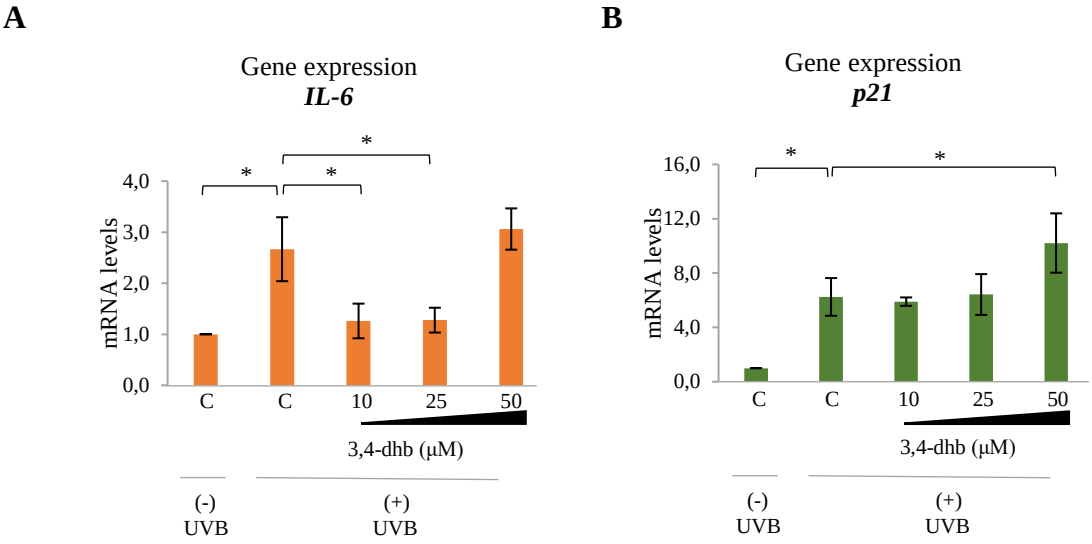


Figure 66. (A) *IL-6* and (B) *p21* gene expression levels of UVB-irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (10, 25 and 50 µM). Statistics: Student's T-test. Non-irradiated cells and UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde were compared to UVB-irradiated control cells (* p<0.05, ** p<0.01 and *** p<0.001).

As observed in Figure 65, 3,4-dihydroxybenzaldehyde induced a downregulation of *HAS2* and an upregulation of *p21* in non-irradiated cells in a dose-dependent manner. In the case of *IL-6*, there is also a dose-dependent upregulation of mRNA levels, but the lower dose (10 µM) of 3,4-dihydroxybenzaldehyde downregulated the expression of this gene. These results suggested that 3,4-dihydroxybenzaldehyde induces senescence *per se*. Interestingly, 3,4-dihydroxybenzaldehyde seems to exert an hormetic effect regarding

the inflammation process, as lower doses are anti-inflammatory while higher doses are pro-inflammatory.

On the other hand, in UVB-irradiated cells, 3,4-dihydroxybenzaldehyde shows a decrease in *IL-6* expression levels at 10 and 25 µM, while inducing *p21* expression levels at 50 µM (Figure 66). Thus, despite inducing senescence by itself, 3,4-dihydroxybenzaldhyde also shows anti-inflammatory effects both in non-irradiated and UVB-irradiated cells.

7.3 Evaluation of sirtuins involvement in UVB-induced senescence effect by 3,4-dihydroxybenzaldehyde

Next, as in the case of Shikimic acid, we wanted to determine the influence of specific sirtuin inhibition on 3,4-dihydroxybenzaldehyde effect on senescence. For these, we used the previously mentioned inhibitors EX-527 (Figures 67-68), OSS128167 (Annex 5) and AGK2 (Annex 6).

A

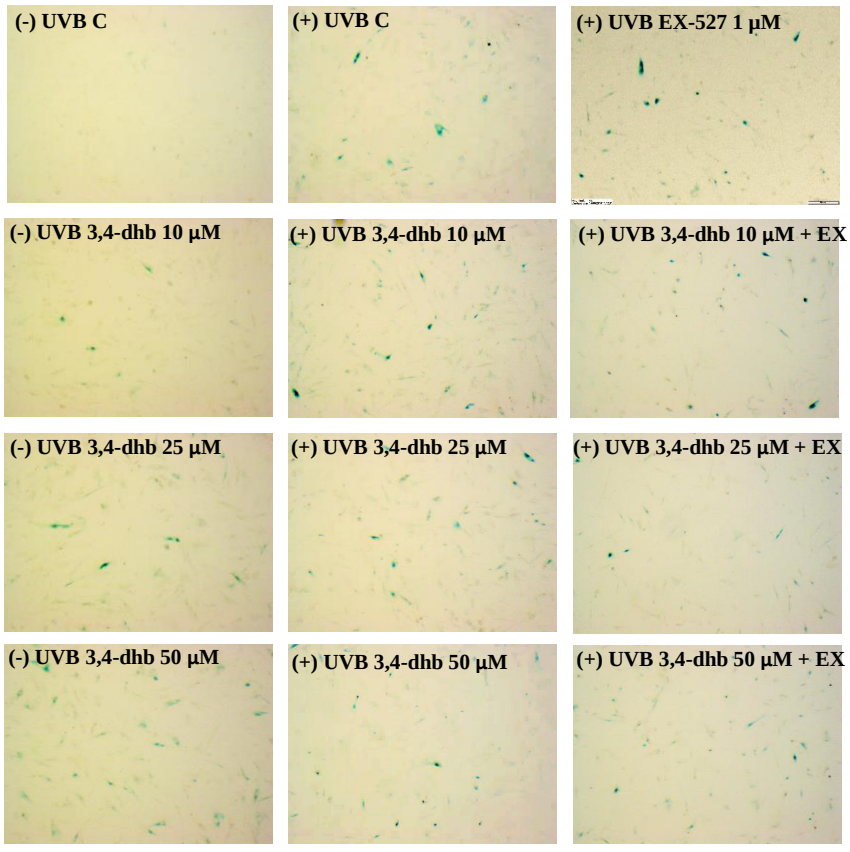


Figure 67. *Continued*

B

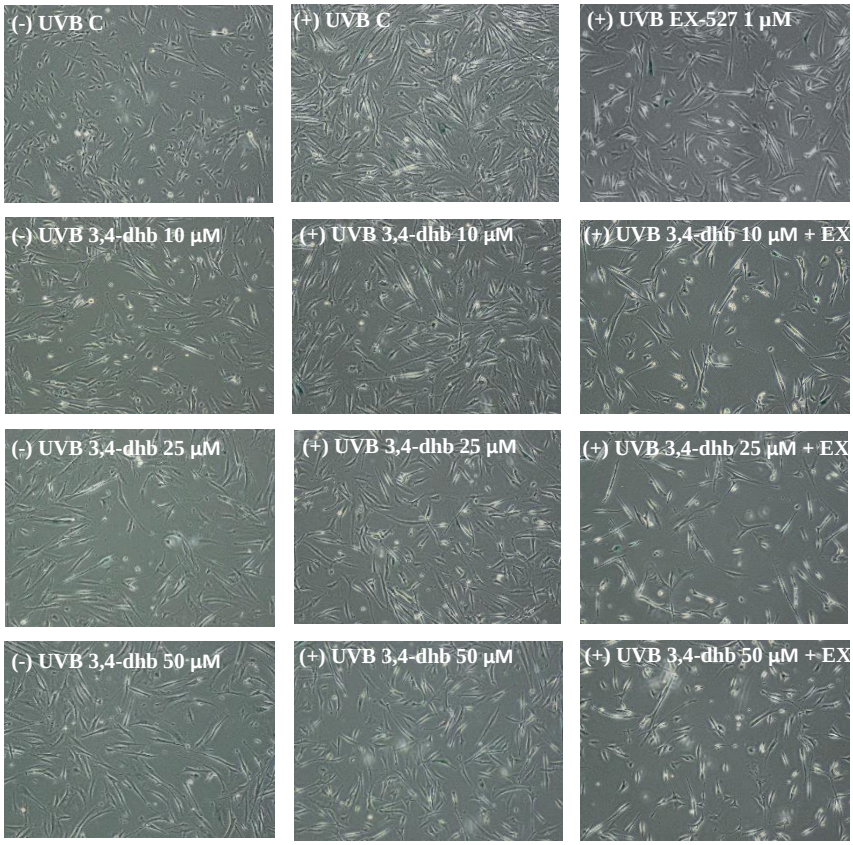
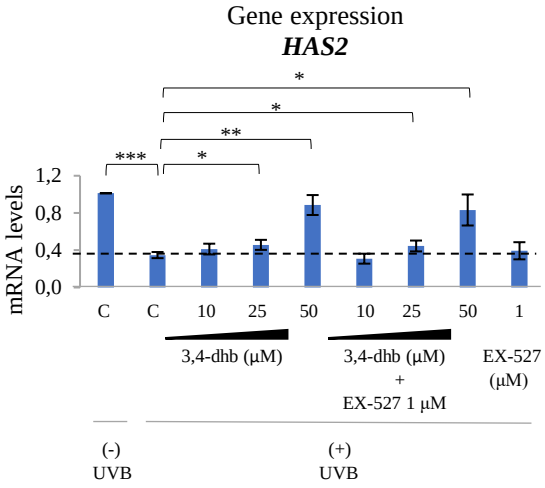


Figure 67. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (left column), irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (center column) and irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus EX-527 1 µM (right column).

A



B

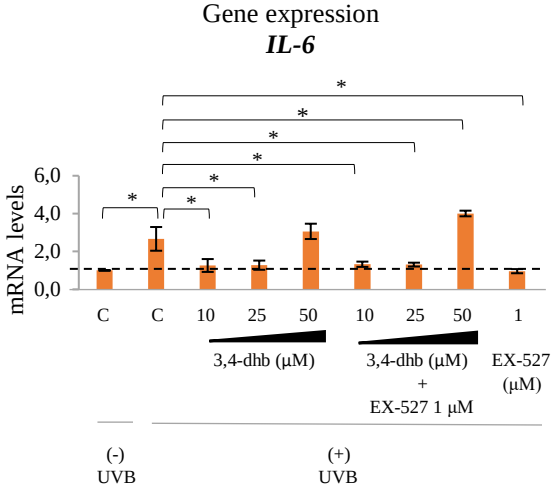


Figure 68. *Continued*

C

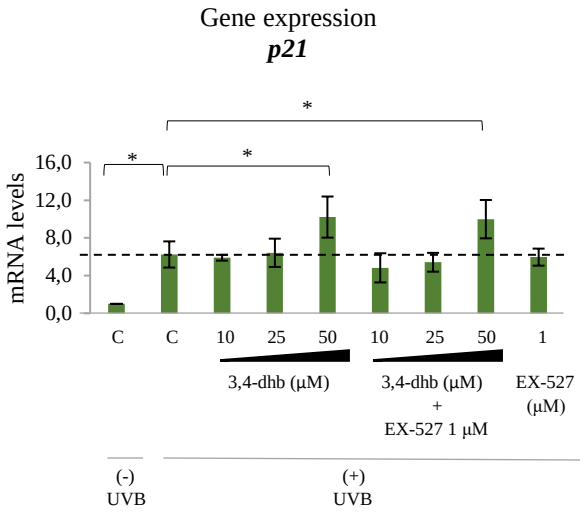


Figure 68. (A) *HAS2*, (B) *IL-6* and (C) *p21* gene expression levels of UVB irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus EX-527 1 µM. The results of UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde plus OSS128167 or AGK2 are shown in Annex 5 and 6, respectively. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde +/- EX-527 and UVB-irradiated cells treated with EX-527 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

First, we observed that 3,4-dihydroxybenzaldehyde increased β-Gal staining in the absence of UVB irradiation (Figure 67), corroborating the previous results that suggested an increase in senescence after 3,4-dihydroxybenzaldehyde treatment.

Regarding EX-527 and OSS128167, SIRT1 or SIRT6 inhibition in UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde did not change neither β-Gal staining nor *HAS2*, *IL-6* and *p21* gene expression levels compared to UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde alone (Figure 67-68, Annex 5). In the case of AGK2, there are neither changes in the senescence markers, except for *IL-6* expression levels, in which there is an additive effect of 3,4-dihydroxybenzaldehyde and AGK2, as their combination produces a higher decrease in gene expression levels compared to 3,4-dihydroxybenzaldehyde or AGK2 alone (Annex 6). Thus, the downregulation of *IL-6* and the upregulation of *HAS2* and *p21* by 3,4-dihydroxybenzaldehyde in UVB-irradiated cells is independent of SIRT1, SIRT2 and SIRT6.

7.4 Effect of 3,4-dihydroxybenzaldehyde on sirtuin gene expression and protein levels

As shown in the previous experiments, 3,4-dihydroxybenzaldehyde clearly deacetylated H3K9 and H4K16, thus suggesting an induction of sirtuin activation (Figure 29B). Here, we wanted to determine whether 3,4-dihydroxybenzaldehyde not only promotes sirtuin activation but also increases sirtuin gene expression and/or protein levels. For that purpose, we performed qPCR (Figure 69) and Western blot (Figure 70) assays on cell cultures treated with 3,4-dihydroxybenzaldehyde for 24 hours to quantify the gene expression and protein levels of sirtuins.

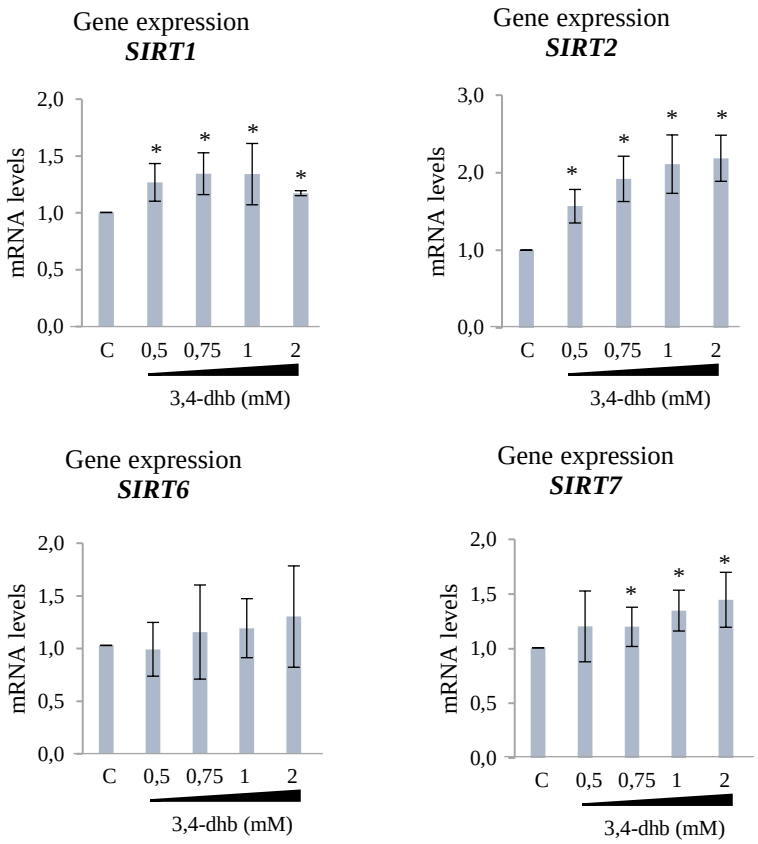


Figure 69. *SIRT1*, *SIRT2*, *SIRT6* and *SIRT7* gene expression levels in HDF treated with 3,4-dihydroxybenzaldehyde (0.5, 0.75, 1 and 2 mM) for 24 hours. Statistics: Student's T-test. Cells treated with 3,4-dihydroxybenzaldehyde were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

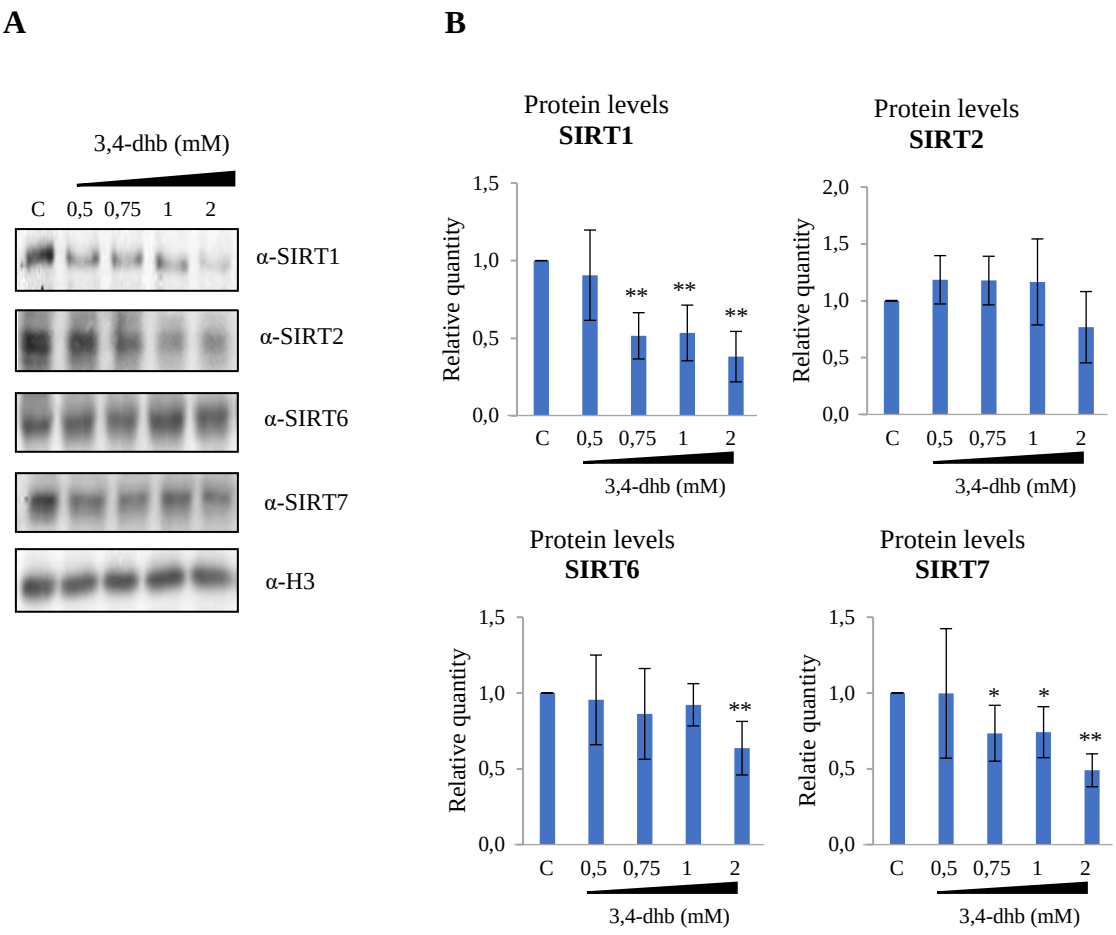


Figure 70. (A) Western blot analysis of SIRT1, SIRT2, SIRT6 and SIRT7 protein levels in HDF treated with 3,4-dihydroxybenzaldehyde (0.5, 0.75, 1 and 2 mM) for 24 hours. (B) Quantification of SIRT1, SIRT2, SIRT6 and SIRT7 Western blot bands. Statistics: Student's T-test. Cells treated with 3,4-dihydroxybenzaldehyde were compared to control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Regarding the qPCR results, we observed that 3,4-dihydroxybenzaldehyde increased significantly *SIRT1*, *SIRT2* and *SIRT7* gene expression, being *SIRT2* upregulation higher than *SIRT1* and *SIRT7*, while *SIRT6* gene expression remained unchanged (Figure 69). Surprisingly, according to the Western blot results, sirtuin protein levels were not increased but decreased after 3,4-dihydroxybenzaldehyde treatment (Figure 70). This protein decrease was observed mostly in SIRT1 and SIRT7, although it occurred also in SIRT6 at the highest dose of 3,4-dihydroxybenzaldehyde. This mismatch between gene and protein levels could be due to the fact that the compound is inducing damage at these doses, and although stress-response pathways activate and induce sirtuin gene expression, an increase in protein degradation in these damaged cells might be inducing a decrease in sirtuin protein levels.

8. Direct impact of the results in the dermocosmetic products of the company

As a project that has been developed partially in a pharmaceutical company, one of the final purposes was to include the compounds with positive results in the dermocosmetic products of the company, or develop new dermocosmetic products with this compounds. Therefore, considering the positive results of Shikimic acid on sirtuin activation and protection against UVB-induced cellular senescence, the company decided to develop a new line of anti-aging products based on epigenetic mechanisms including Shikimic acid as one of the main ingredients (Figure 71). Gallic acid, which was the other active found to protect against UVB-induced cellular senescence in our cells (Figures 39 and 40), was also considered to be included in the company products, but it was discarded after several tests due to product formulation issues.

On the other hand, considering the key role of hyaluronic acid in skin hydration and the continuous desire of cosmetic companies to find compounds that increase hyaluronic acid production in the skin (Papakonstantinou E et al. 2012), the three compounds (Ferulic acid, Lipoic acid and Carnosine) that increased *HAS2* expression (one of the key enzymes in hyaluronic acid synthesis in skin cells) might be considered to be included in the company products aimed to increase hyaluronic acid levels in skin.

Finally, another interesting result for the company is the deacetylation of H3K9 induced by Ectoine. This compound is already included in one company product, based on the compound's ability to active heat shock response. Thus, the possible involvement of sirtuins in the mechanism of action of Ectoine could be further studied to improve the knowledge about the anti aging effects of this compound.

Figure 71. The different creams and solutions that form the new anti-aging line of products including Shikimic acid.

DISCUSSION

Skin aging is the degenerative process that occurs in skin causing detrimental alterations of its biological functions (Kohl E et al. 2011). The main factors that contribute to this process are external stressors, being the UV from solar light the main contributor for skin aging. The UV-induced skin aging is known as photoaging, and it includes many features at a molecular level, such as increased DNA damage, epigenetic dysregulation or increased extracellular matrix degradation, among others. One of these features includes the accumulation of senescent cells, which are cells that do not longer proliferate and increase inflammation and matrix proteins degradation, thereby decreasing tissue regeneration and proper execution of its biological processes. Many approaches have been proposed to deal with this issue. Among them, sirtuins have proven to be a good target to prevent UV-induced senescence by its regulation of cellular stress response pathways and protective mechanisms.

In this context, we aimed to find novel compounds with the ability to activate sirtuins and protect against UV-induced cellular senescence. In the first part of the project, we used the quantification of histone markers H3K9 and H4K16 acetylation in HDF as a screening method for sirtuin activity. We found that 9 compounds (Dexpanthenol, Sesamol, Andrographolide, Shikimic acid, 3,4-dihydroxybenzaldehyde, Ectoine, Kinetin, Zeatin and Phloretin) out of the 30 compounds tested induced the deacetylation of one or both marks, suggesting an increase in sirtuin activity.

On the other hand, a UVB-induced senescence assay was implemented to study the effect of the compounds on senescence. From all the compounds tested, only Gallic acid and Shikimic acid protected the cells against UVB-induced senescence. At this point, we focused on the characterization of Shikimic acid and 3,4-dihydroxybenzaldehyde. The former was selected as the only compound that induced sirtuin activation and protected against UVB-induced senescence. The latter was the compound with the higher impact on sirtuin acetylation targets.

In the last part of the project, Shikimic acid characterization showed that its protective effect against senescence was dependent on SIRT1. Besides, Shikimic acid was able to induce the activation of the autophagy pathway, which is one of the cellular stress-related pathways regulated by SIRT1. On the other hand, 3,4-dihydroxybenzaldehyde induced a cell cycle arrest in S phase and an increase in senescence markers in non-irradiation conditions, suggesting that it might be inducing damage *per se.*

A summary of each compound results for the screening of histone acetylation and senescence assays is shown in Table 11. The compounds that only induce histone deacetylation are marked in blue, the compounds that only protect against UVB-induced senescence are marked in red, and the compounds that both induce histone deacetylation and protect against UVB-induced senescence are marked in yellow.

	Sirtuin levels		Histone acetylation		UVB-induced senescence	
Compound	**mRNA**	**Protein**	**H3K9**	**H4K16**	**β-Gal**	***HAS2* expression**
Resveratrol	na	na	↓	↓	↑	-
Quercetin	na	na	↓	↓	-	↑
Dexpanthenol	na	na	↓	↓	-	-
Allantoin	na	na	↑	-	-	-
Idebenone	na	na	-	↑	-	-
Ferulic acid	↑	-	-	-	-	↑
Lipoic acid	↑	-	-	-	-	↑
Gallic acid	na	na	-	-	↓	↑
Taurine	na	na	-	-	-	↑
Salicylic acid	na	na	-	-	-	-
Shikimic acid	↓	-	↓	↓	↓	↑
Ectoine	na	na	↓	-	-	-
3,4-dihydroxybenzaldehyde	↑	↓	↓	↓	↑	↑
Andrographolide	na	na	↓	↓	-	-
Kinetin	na	na	↓	-	-	-
Zeatin	na	na	↓	-	-	-
Carnosine	na	na	-	-	-	↑
Damascenone	na	na	-	-	-	-
Betaine	na	na	-	-	-	-
Pyridoxine	na	na	-	-	-	-
Verbascoside	na	na	-	-	-	↑
Hamamelitannin	na	na	-	-	-	-
Phloretin	na	na	-	↓	-	↓
Vanillin	na	na	-	-	-	-
Sesamol	na	na	↓	↓	↑	-
Salicylaldehyde Thiosemicarbazone	na	na	-	-	-	-
Syringic acid	na	na	-	-	-	-
Sclareol	na	na	-	↑	-	-
Gentiopicrin	na	na	-	↑	-	-
Irisflorentin	na	na	-	-	-	↓

Table 11. A summary of the screening results of the compounds for the histone acetylation and senescence assays, including sirtuin mRNA and protein levels, histone marks acetylation, senescence markers and the induction of cell death in combination with UVB at the concentrations otherwise viable in non-stress conditions. The results are shown by arrows that indicate increase/decrease (↑, ↓), hyphens that indicate no effect (-) and non-applicable in the case of non-tested compounds (na).

It is interesting to highlight the fact that only 1 out of the 30 compounds tested is able to activate sirtuins and protect against UVB-induced senescence (Shikimic acid). This fact might be explained by the experimental design and the assay conditions. This and other aspects of our research are discussed in the next sections.

1. Defining an optimal viability screening for skin cells

In order to determine the optimal concentration of compounds for the cell culture treatments, we initially selected the WST-1 assay as the most appropriate cell viability assay to identify the maximum non-cytotoxic dose of each compound, which was the dose to be used in the rest of planned studies. The WST-1 assay is based on the ability of mitochondrial dehydrogenases to metabolize tetrazolium salts (including WST-1) into a colored dye that can be quantified using the spectrophotometer. This assay is an improved version of the widely used MTT, as the WST-1 assay lacks the step of dye solubilization present in the MTT protocol that increases the complexity and time of the latter. Thus, the quantification of the amount of WST-1 metabolized by the cells seems to correlate with the number of cells, which in turn should allow to calculate the viability of cells after a specific treatment.

However, we observed that in many cases the value of the WST-1 assay for certain concentrations suggested a decrease in cell viability, while optical examination by microscopy suggested intact cell viability. This was the case for example for Shikimic acid at 50 mM (Figure 23). In fact, previous studies have reviewed the advantages and disadvantages of the most common used cell viability assays, including WST-1 (Stepanenko AA and Dmitrenko VV. 2015, Präbst K et al. 2017). Regarding the assays that depend on mitochondrial activity, both MTT and WST-1 can over/underestimate the effect of a specific treatment on cell viability, as an induction or repression of mitochondrial activity can modify the results of these assays without altering cell viability.

In our case, in order to overcome this issue, we searched for alternative cell viability assays that did not depend on metabolic activity of the cells, and thus with a higher correlation with the real number of viable cells. Among these alternatives, we found that the SRB assay could be a good choice for cell viability testing as it is based on the binding of the SRB to cellular proteins in acidic conditions (Vichai V et al. 2006), and thus providing a more accurate approach to the determination of cell viability. According to our results, Shikimic acid at 50 mM did not reduce cell viability in the SRB assay (Figure 42A), correlating with the previous observations in optical microscopy which suggested that cell viability was unaffected at this dose. These results indicate that WST-1 should not be used alone in the determination of cell viability, as alterations in the resultant absorbance may arise from metabolic changes instead of viability changes. This is important from the company point of view, as the cell viability assay is the first technique used when we aim to characterize a new compound of interest. Thus, SRB has proven to be a better choice to study cell viability than its counterpart WST-1.

Nevertheless, the combination of both assays, SRB and WST-1, could be interesting from the point of view to test the viability of the cell after a certain treatment through the SRB assay and at the same time determine any metabolic changes in the cell through the WST-1 assay, and thus to be the starting point in the characterization of the effect of a specific compound on cell metabolism. An increase in the metabolic activity without a modification of cell viability could indicate an increased mitochondrial biogenesis, which has been described to be regulated by SIRT1 (among others) and to promote cell longevity (Tang BL. 2016, Gureev AP. Et al. 2019), while a decrease in metabolic activity without a modification of cell viability could indicate an inhibition of mitochondrial activity, which has been described to be part of the mechanisms of action of compounds with anti-aging properties, such as Metformin and Berberine (Wang H et al. 2017). However, this would only be an approximation, as these changes in mitochondrial activity could also be related to increased damage and/or apoptosis (Wang C and Youle RJ. 2009).

On the other hand, Trypan blue assay was also tested as an alternative to WST-1 and gave similar results than the SRB assay when testing the effect of Shikimic acid on cell viability (Figure 42B). However, we did not consider this assay to be a good approach for cell viability testing as the Trypan Blue protocol, including cell harvesting and manual counting, would be very laborious to be applied for the study of the effect of many compounds at many concentrations on cell viability. Instead, it might prove to be useful in certain cases regarding the study of a small number of compounds or concentrations.

2. A proper screening for sirtuin activity

In order to find compounds that could enhance sirtuin activity, the levels of H3K9 and H4K16 acetylation were quantified. Among the wide range of described sirtuin targets, these two histone modifications were selected given their functional relevance, high specificity and conserved relationship with sirtuins through evolution (Martínez-Redondo P and Vaquero A. 2013). Several compounds chosen for this project were previously described to induce sirtuin expression and/or activation in other human cell types and model organisms, such as Lipoic acid or Gallic acid (Chen WL et al. 2012, Valdecantos MP et al. 2012, Doan KV et al. 2015). However, neither of these two compounds decreased the acetylation of H3K9 or H4K16 in our cells. In the case of Lipoic acid, we did observe a significant increase in *SIRT1*, *SIRT2*, *SIRT6* and *SIRT7* gene expression upon treatment (Figure 25B), although sirtuin protein levels and histone marks remained unchanged (Figure 26, Annex 2). These results suggest that in our cells, Lipoic acid effect on sirtuins is very mild, as opposed to the observed effect by other authors in other cell types (Chen WL et al. 2012, Valdecantos MP et al. 2012).

Some of these authors and others have used other approaches to determine sirtuin activity, including the Fluor de Lys assay, which is a fluorescence *in vitro* assay that uses a small peptide of the human p53 protein as a sirtuin substrate. However, a lot of controversy emerged around the use of this assay when Resveratrol, a SIRT1 activator according to this assay, was not able to catalyze the deacetylation of the peptide by SIRT1 in the absence of the fluorophore used in the reaction (Borra MT et al. 2005, Kaeberlein M et al. 2005, Beher D et al. 2009). Thus, this assay does not prove to be a good approach for testing the effect of compounds on sirtuin activity. Moreover, some of these reports have

quantified the effect of the compounds in non-physiological conditions, such as in the presence of damage or high-fat conditions (Valdecantos MP et al. 2012, Doan KV et al. 2015), while we have studied the effect of the compounds in cultured cells without type of stress. In the case of non-physiological conditions, specific pathways might be activated that regulate sirtuins, which could explain why we don't observe a regulation of sirtuins using the same compounds in normal culture conditions. All these observations highlight (I) the importance of the cell-specific effects of sirtuins and the need to test the efficacy of the compounds in the specific cell type of interest and (II) the importance of the conditions in which sirtuins activity and/or expression are determined, being essential to differentiate between physiological and specific pathological conditions.

On the other hand, 9 compounds (Dexpanthenol, Sesamol, Andrographolide, Shikimic acid, 3,4-dihydroxybenzaldehyde, Ectoine, Kinetin, Zeatin and Phloretin) induced the deacetylation of one or both marks, proving to be potential sirtuin activity inducers. Interestingly, the effect of these compounds on one or both marks indicates the specific sirtuin that they are regulating. Thus, as Ectoine, Kinetin and Zeatin only deacetylate H3K9, they could be activating SIRT6, while Phloretin, as it only induces H4K16 deacetylation, it could be activating SIRT2. On the other hand, Dexpanthenol, Sesamol, Andrographolide, Shikimic acid and 3,4-dihydroxybenzaldehyde are probably activating in one way or another SIRT1, which deacetylates both marks (Martínez-Redondo P and Vaquero A. 2013). Unfortunately, the concentration of many of these compounds had to be lowered in the UVB-induced senescence assay as they induced cell death when combined with UVB (discussed in the next section). One possibility is that some of these compounds are activating sirtuins through the production of intracellular damage, which is one way to induce sirtuin activation (Santos L et al. 2016, Shrishrimal S et al. 2019). However, from a company's perspective, this approach is not optimal as compounds that induce cellular damage would be difficult to place in the dermocosmetic market. Another possibility is that these compounds are targeting other proteins involved in cell survival and death apart from sirtuins. In fact, it is very common for natural compounds (all these 9 compounds are) to have multiple cellular targets (Lin JK. 2007, Carvalho D et al. 2017). Finally, the use of specific sirtuin inhibitors would be interesting to validate the involvement of sirtuins in the histone deacetylation effect induced by these compounds and to decipher which sirtuin/s are activated in each case.

In conclusion, the quantification of H3K9 and H4K16 acetylation by Western Blot proved to be a good first approach for the screening of sirtuin activators. However, complementary assays should be used to confirm that the compounds are not inducing sirtuin activity by increasing cellular damage.

3. A robust model to study UV-induced senescence in skin cells

Regarding the senescence-inducing stimulus for our cells, we first considered UVA irradiation (320-400 nm) as the optimal source of damage as it makes up to 90% of solar light and is the main contributor of skin aging (Kohl E et al. 2011). However, due to practical setbacks, we had to switch the UV source to UVB irradiation (290-320 nm), which despite being up to 10% of solar light, it also contributes significantly to the skin aging process (Cadet J et al. 2015). UVA light induces damage by increasing the amount

of ROS levels, which target and oxidize the different cellular macromolecules (DNA, lipids and proteins). On the other hand, in addition to increasing ROS levels, UVB can also directly target DNA by inducing the formation of DNA photoproducts, including cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP), which can indirectly cause DSB after the induction of replication fork collapse (Rastogi RP et al. 2010, Cadet J et al. 2015). Thus, UVB produces higher intracellular damage than UVA and allows us to induce cellular senescence in less time and with a smaller number of irradiations. Specifically, this allowed us to obtain a robust model to study UV-induced cellular senescence in a few days interval (6 days) with two UVB irradiations of 25 mJ/cm^2.

The fact that we only needed two UVB irradiations to induce cellular senescence, in contrast to the four irradiations needed for UVA to induce senescence, suggested that these two irradiations were producing a high amount of damage. This could explain why we only observed protection against senescence with 2 compounds out of the 30 compounds tested, considering that many of the chosen compounds protect against senescence in other cell types and/or induced by other stressors (McFarland GA and Holliday R 1994, Rattan SI and Clark BF. 1994, Buenger J et al. 2004, Rattan SI and Sodagam L. 2005, Briganti S et al. 2008, Lee J et al. 2014, Arend N et al. 2015). These compounds that protect against senescence in other circumstances might not be able to do so when the damage stimulus is too high. Therefore, considering that the skin photoaging process occurs after a long time of small damage accumulation produced by UV irradiation, our model could be improved by including more than two irradiation steps of less intensity, which would distribute the damage in a similar way that occurs in skin photoaging *in vivo* (Figure 72).

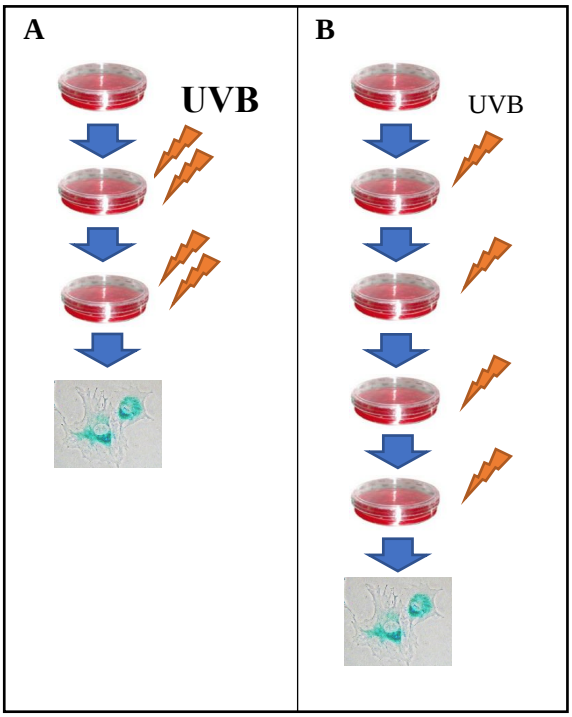


Figure 72. (A) Our current model of UVB-induced senescence where 2 high dosage UVB irradiations are performed to induce senescence. (B) An alternative model to induce senescence using less UVB intensity and distributed in a higher number of irradiations.

Another important issue in our studies was the selection of the markers used for the quantification of senescence. Our first choice was to combine the commonly used β-Gal staining and the determination of *p16* and *p21* gene expression. The first compound we observed that induced protective effects against senescence was Gallic acid. Although this compound reversed the increase in β-Gal staining induced by UVB, it did not reverse the increase in *p16* and *p21* gene expression (Figure 39). This observation could be explained by many reasons: first, Gallic acid could be regulating β-Gal activity through a senescence-independent pathway, and thus be a false positive senescence regulator. Another possibility is that Gallic acid could be exerting a partial effect on senescence, and thus we only observe the protective effect on senescence in some but not all the markers. This could imply that Gallic acid is regulating senescence signaling pathways other than p16 and p53/p21 pathways, such as p38, PI3K/mTOR or NF-kB (Iwasa J et al. 2003, Jing H et al. 2014, Weichhart T. 2018). In fact, senescence is a cellular state where different pathways activate to produce different phenotypes that converge in some cases (Lujambio A. 2016). Finally, it is also possible that regardless the senescence state, the amount of damage induced by UVB irradiation could be high enough to maintain the upregulated levels of p16 and p21, which play key roles in DNA damage response, to counteract the UVB-induced damage.

On the other hand, although p16 and p21 are widely used in senescence studies due to their upregulation associated to cell cycle arrest, a feature of senescence, these markers are not specific for senescence and thus many authors already use alternative cell-type specific markers (Cho EJ et al. 2008, Wang YN et al. 2010, Bae JT et al. 2012). In fact, Herbig U et al. reported that p16 was upregulated in replicative senescent cells but in a telomere shortening and DNA damage-independent manner (Herbig U et al. 2004). Besides, the type of senescence-inducing stimulus (oxidative stress, oncogene activation, telomere damage, among others) also influences the pathways that are activated during the development of senescence (Zhang H. 2007, Mirzayans R et al. 2012, Van Deursen JM. 2014, Hernandez-Segura A et al. 2017), and different cell types undergoing the same type of senescence have shown significant differences in their gene expression senescent-related changes (Zhang H et al. 2003, Hernandez-Segura A et al. 2017). Hernandez-Segura A et al. identified a group of genes whose regulation was shared among different cells undergoing different types of senescence-inducing stimuli and in different time points. Interestingly, p16 and p21 were also analyzed but were not included in this group of reliable senescence markers.

Consequently, the selection of the optimal markers is an essential step to study senescence depending on the cell type and type of stimuli used, as none of the currently used markers is strictly specific for senescence (Kuilman T et al, 2010). In our case, we found that *HAS2* gene expression, one of the genes responsible for hyaluronan synthesis in human dermal fibroblasts, correlated with β-Gal staining results and proved to be a reliable marker of UVB-induced senescence in human skin fibroblasts, in agreement with previous reports (Röck K et al. 2015).

Regarding the screening of the compounds on the senescence assay, first we observed that the compounds with positive results in the histone marks Western Blot assay (Figures 28-31) were inducing cell death when irradiated with UVB (except for Shikimic acid). Interestingly, this cell death occurred at the same concentrations that the compounds

induced histone deacetylation in the Western Blot assay in the absence of UV damage. This result led us to hypothesize that these compounds may be inducing sirtuin activity indirectly by generating high levels of damage in the cells, and thus when this damage was combined with the one from the UVB irradiation, the cells died. Another possibility is that these compounds, apart from targeting sirtuins, were also inhibiting mTOR. This was in accordance to previous reports that suggest that upon DNA damage, mTOR inhibition could switch the cell fate from senescence to apoptosis (Figure 73). From all the compounds that induced the histone marks deacetylation, the positive controls Resveratrol and Quercetin and Andrographolide have shown to inhibit mTOR (Bruning A. 2013, Wu Y and Liu F. 2013, Li J et al. 2015), and thus could fit in this hypothesis. In this case, the cellular outcome for the treatment with these compounds wouldn't be bad at all, as although they are not protecting against cellular senescence, they are inducing the death of damaged cells that would otherwise accumulate and cause detrimental changes to the skin.

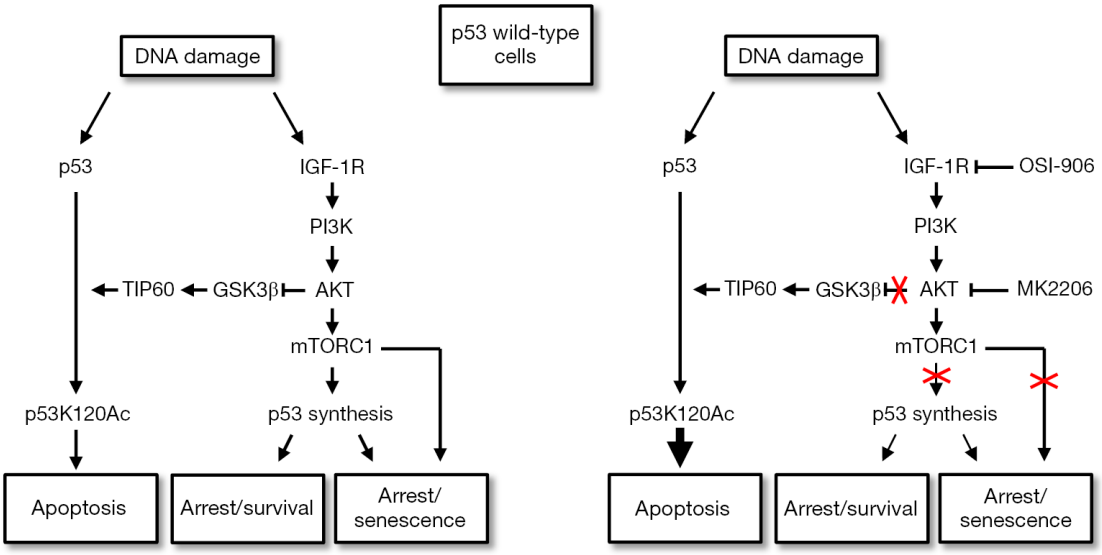


Figure 73. IGF-1R/AKT/mTORC1 signaling inhibits p53-dependent apoptosis and promotes p53-dependent senescence. After DNA damage, both p53 and the IGF-1R pathway activate. AKT inhibits p53-dependent apoptosis through GSK3β inhibition and thus favours senescence development. However, in the presence of IGF-1R and AKT inhibitors (OSI-906 and MK2206, respectively), mTOR activity is blocked and p53-dependent apoptosis is enhanced (Duan L and Maki CG. 2016).

Another interesting result of this screening emerged with the compounds Sesamol and 3,4-dihydroxybenzaldehyde. These two compounds were identified as possible sirtuin activators in the H3K9/H4K19 deacetylation activity screening. Interestingly, they induced cell death when cells were irradiated with UVB. However, when the doses were lowered to non-cytotoxic levels, these two compounds increased β-Gal staining in UVB-irradiated cells, compared to non-treated UVB-irradiated cells (Annex 3). In fact, subsequent experiments showed that 3,4-dihydroxybenzaldehyde increased senescence markers in the absence of UVB (Figures 65). These results would support our hypothesis that these compounds activate sirtuins indirectly by inducing damage in the cell. In this context, the damage produced by both the high doses of the compounds and by UVB irradiation would drive the cells to apoptosis, while a mild damage induced by low doses of the compounds combined with the UVB damage would favor senescence development

instead of apoptosis. Interestingly, 3,4-dihydroxybenzaldehyde not only increased β-Gal staining but also *HAS2* gene expression in UVB-irradiated cells (Annex 3). This increase was also observed with the compound Verbascoside (Figure 41). Although this seems a controversial result, as an increase of *HAS2* expression is associated with less senescence, it could be explained by previous observations of other authors where the *HAS2* gene is induced under other types stress conditions independently of the senescence process, such as the exposure to the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α (Campo GM et al. 2006).

Finally, Carnosine, Ferulic acid and Lipoic acid increased *HAS2* expression without modifying β-Gal staining in UVB-irradiated cells (Figure 41 and Annex 3). In this case it seems that the regulation of *HAS2* gene by these compounds is senescence-independent, and this result could be indicating that these compounds are activating signaling pathways that induce *HAS2* gene expression, including TGF-β and TNF-α pathways (Campo GM et al. 2006, Campo GM et al. 2009). In fact, both Carnosine and Lipoic acid have been described to activate the TGF- β pathway (Tsuji-Naito K et al. 2010, Caruso G et al. 2019).

4. Senescence regulation by Shikimic acid and involvement of SIRT1

Shikimic acid is a natural compound found in plants and microorganisms as an intermediate of the shikimate pathway, by which aromatic aminoacids and folates are synthesized in these organisms (Lingens F. 1968, Herrmann KM and Weaver LM. 1999). It is also found in considerable amounts in many plants, mostly in the *Illicium* genus and in other plants including *Terminalia arjuna*, *Pistacia lentiscus* or *Cocos nucifera*, among others (Bochkov DV et al. 2012). Previous reports have shown many properties of Shikimic acid in a wide range of cells types and organisms (Table 12).

Biological effect	**Cell type**	**References**
Anti-inflammatory	Mouse macrophages	Rabelo TK et al. 2016
Anti-lipogenic	Human hepatocytes Mouse adipocytes	Kim MJ et al. 2019
Neuroprotective	Human neuronal-like cells	Rabelo TK et al. 2015
Wound healing promotion	Human dermal fibroblasts	Antognoni F et al. 2017
Osteoclastogenesis inhibition	Mouse macrophages and monocytes	Chen X et al. 2018
Skin whitening effect	Mouse melanoma	Chen YH et al. 2016
Antioxidant	Mouse hepatocytes Cell-free assay	Manna K et al. 2014 Chen YH et al. 2016
Anti-thrombotic	Human blood samples	Veach D et al. 2016
Anti-bacterial	Several Gram-negative and Gram-positive species	Tripathi P et al. 2015
Anti-diabetic	Rat (whole organism)	Al-Malki AL. 2019

Table 12. A summary of the described biological effects of Shikimic acid by other authors.

Particularly, Shikimic acid is used in the cosmetics field due to its antibacterial and bacterial lipase inhibition properties in products for acne treatment (Rawat G et al. 2013). However, to date there are no reports on the effect of Shikimic acid on sirtuins and/or senescence. In this project, we have described that Shikimic acid induces the deacetylation of the sirtuin targets H3K9 and H4K16 after 24h treatment in human dermal fibroblasts (Figure 29A), and that it can prevent UVB-induced cellular senescence in a SIRT1-dependent manner (Figure 51-52).

In order to better characterize the effect of this compound on senescence, we determined the effect of Shikimic acid on cell cycle, apoptosis and DNA damage in UVB-induced senescent cells. We observed that while both the apoptotic levels (Figure 44) and the γH2AX foci (Figure 48) were similar between UVB-irradiated cells treated and non-treated with Shikimic acid, this compound induced a cell cycle arrest in S phase in UVB-irradiated cells compared to non-treated UVB-irradiated cells (Figure 47). Therefore, although Shikimic acid prevented the UVB-induced changes of the senescence markers β-Gal staining and *HAS2* expression, these results suggest that Shikimic acid is not directly involved in the repair of DNA damage induced by UVB, and that Shikimic acid favored the S-phase cell cycle arrest probably triggered by this unrepaired DNA damage. Interestingly, the fact that Shikimic acid increases S-phase arrest without increasing DNA damage suggests that this compound could be modulating other pathways that can activate the G1/S phase checkpoint, such as the TGF-β pathway or nutrient-sensing pathways upon nutrient restriction (Foster DA et al. 2010, Mukherjee P et al. 2010). In fact, among the different nutrient-sensing pathways, SIRT1 is involved in the glucose-dependent metabolic checkpoint (Liu T et al. 2014), which could mean that the activation of SIRT1 by Shikimic acid in this context could favor the S-phase arrest.

In subsequent experiments where we tested the effect of Shikimic acid in other senescence markers, we observed that *p21* mRNA levels showed a slight upregulation in UVB-irradiated Shikimic acid-treated cells (Figure 50B). As mentioned in the previous section, this could be explained by the fact that p21 is not a reliable senescence marker in our model. However, in this context, as p21 also plays roles in DNA damage response and repair (Perucca P et al. 2006, Cazzalini O et al. 2010), the damage induced by UVB may be too high to be repaired and thus p21 is still upregulated as a participant of the DNA repair pathway. In fact, DNA repair proteins such as BRCA1 can induce *p21* gene expression (Mullan PB et al. 2006). In conclusion, the presence of DNA damage, determined by the appearance of γH2AX foci, might induce *p21* expression and activate the G1/S checkpoint, which consequently induces the cell cycle arrest in S phase.

Apart from *p21*, we also studied the effect of Shikimic acid on *IL-6* expression in UVB-induced senescence. IL-6 is part of the SASP, which includes all the cytokines, chemokines and growth factors released by senescent cells that contribute to tissue damage and paracrine induction of senescence in neighbor cells (Coppé JP et al. 2010). As shown in Figure 50A, Shikimic acid prevented *IL-6* upregulation in UVB-induced senescent cells, thus inhibiting the SASP, the main responsible of the detrimental changes caused by senescent accumulation during aging.

After characterizing the effect of Shikimic acid on senescence, we aimed to determine whether sirtuins were involved in Shikimic acid regulation of senescent markers.

Interestingly, SIRT1 inhibition by EX-527 reversed β-Gal staining and *HAS2* expression changes induced by Shikimic acid (Figure 47A and C), indicating that the protection of Shikimic acid on senescence is SIRT1-dependent. On the other hand, considering the widely described role of SIRT1 as an inhibitor of inflammatory pathways (Yeung F et al. 2004, Salminen A et al. 2008), the fact that UVB-irradiated cells treated with EX-527 alone inhibited *IL-6* upregulation was an unexpected result (Figure 47D). This result could reflect the dual effect of sirtuins in many processes, such as the oncogene/tumor suppressor duality (Bosch-Presegué L and Vaquero A. 2011). In fact, previous reports have shown that EX-527 can reduce inflammation in mice with endotoxemia (Huang J et al. 2017).

Interestingly, we observed that Shikimic acid did not activate directly SIRT1 in an *in vitro* assay (Figure 57). Thus, the effect of Shikimic acid on SIRT1 activity is probably caused by indirect mechanisms. Many authors have reported several proteins and/or PTM that can regulate SIRT1 activity. For example, AMPK is widely known to induce SIRT1 activity through phosphorylation (Lau AW et al. 2014). However, Shikimic acid did not activate AMPK in our cells (Figure 59B-60B), and thus AMPK activation does not explain SIRT1 activation in this case. Similarly, phosphorylation carried out by other proteins, such as JNK1, and other post-translational modifications, including sumoylation and O-GlcNAcylation, have also shown to induce SIRT1 activity (Yang Y et al. 2007, Nasrin N et al. 2009, Han C et al. 2017). On the other hand, other authors reported the induction of SIRT1 activity by the natural compound Ursolic acid in a similar way than the endogenous SIRT1-activator AROS (Bakhtiari N et al. 2018). Therefore, SIRT1 post-translational modifications including phosphorylation, sumoylation and O-GlcNAcylation, or AROS-like activation of SIRT1, could be plausible mechanisms of Shikimic acid action on SIRT1.

Considering all the results we obtained on the effect of Shikimic acid on sirtuins and senescence, we have established a model including the regulation of this compound in the different steps of UVB-induced senescence through SIRT1 (Figure 74). Thus, in our model, UVB induces mainly senescence and to a lesser extent apoptosis through the generation of oxidative and DNA damage. In this context, Shikimic acid prevents β-Gal upregulation and HAS2 downregulation through SIRT1 activation while reinforcing cell cycle arrest caused by the unrepaired DNA damage.

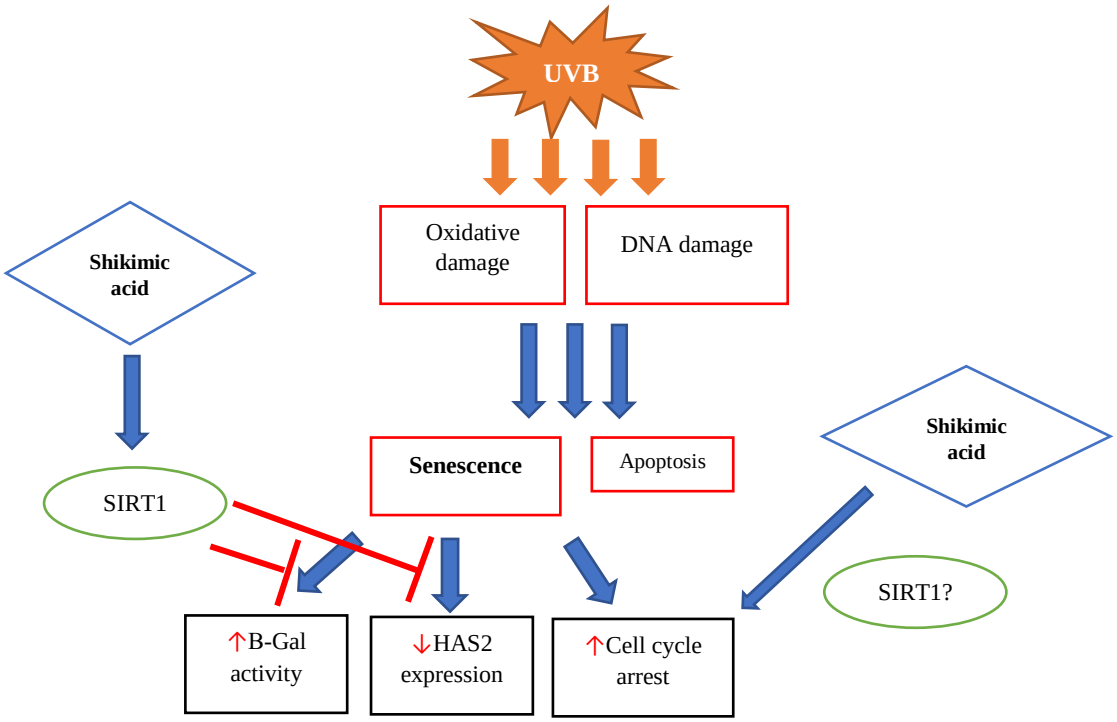


Figure 74. A model including the regulation of senescence by Shikimic acid through SIRT1. UVB light induces oxidative and DNA damage, which in a chronic and mild pattern promotes mainly senescence and to a lesser extent apoptosis. Cellular senescence is characterized by an increase in β-Gal staining, cell cycle arrest and a decrease in HAS2 expression, among other features. In this context, Shikimic acid prevents β-Gal staining upregulation and HAS2 expression downregulation through the activation of SIRT1, while reinforcing the cell cycle arrest due to unrepaired DNA damage. Additional experiments could confirm if SIRT1 is also involved in the cell cycle arrest effect induced by Shikimic acid.

5. Shikimic acid regulation of autophagy

Autophagy is a catabolic pathway that promotes the degradation of intracellular damaged organelles and macromolecules in the context of low nutrient availability, and its activation has been associated with increased longevity (Yen Wl et al. 2008, Salminen A and Kaarniranta K. 2009). Sirtuins, mostly SIRT1, have been described to regulate autophagy (Lee IH et al. 2008, Ng F et al. 2013). Therefore, as one of our final goals in this project was to study sirtuin-regulated pathways, we determined the effect of Shikimic acid on the regulation of autophagy.

As autophagy is a very dynamic process, we studied the effect of Shikimic acid on autophagy markers at two time points (8h and 24h) to detect possible differences between early and late time points. Indeed, we did observe differences in the autophagy markers between the two time points. Regarding the gene expression analysis, we observed that *VMP1* upregulation induced by Shikimic acid was higher at 8h (3-fold and 3.5-fold for 25 and 50 mM, respectively) compared to 24h (2-fold for 50 mM) (Figures 59A-60A, respectively). Besides, at 8h we only observed a mild downregulation of *LC3*, *ATG7* and *ATG12* gene expression, while at 24h we observed a higher downregulation of *LC3*, *ATG5*, *ATG7*, *ATG12* and *Beclin-1* gene expression (Figures 59A-60A, respectively). Thus, these results suggested that Shikimic acid was inducing a time-dependent repression of autophagy genes.

On the other hand, regarding the autophagy markers analysis by Western Blot, we observed a downregulation of LC3 and p62 at 8h, while at 24h these changes were more pronounced (Figures 59B-60B, respectively). As LC3 and p62 are autophagy receptors that bind the proteins that are targeted for degradation, they are expected to increase when autophagy is activated but to decrease once the content of the autophagic vacuole is degraded (Orhon I and Reggiori F. 2017). The fact that we don't see this decrease in the levels of ATG5 (Figures 59B-60B) corroborates our hypothesis, as ATG5 dissociates from the autophagosome once it is formed and therefore it is not degraded with its content (Rubinsztein DC et al. 2012).

Thus, the downregulation of LC3 and p62 along with the increased *VMP1* gene expression suggested that Shikimic acid activates the autophagy pathway. However, unexpectedly, most of the autophagy-related genes quantified were downregulated (Figure 54A). A possible explanation for this event could be the relationship between H4K16 acetylation and autophagy. As proposed by other authors (Füllgrabe J et al. 2013), H4K16 deacetylation suppresses the expression of autophagy genes, which would fit with our results, as Shikimic acid induces H4K16 deacetylation as we previously reported (Figure 29A). However, the epigenetic regulation of autophagy is more complex and has been proposed to include 3 main events that occur in 3 different time points (Pietrocola F et al. 2013, Füllgrabe J et al. 2014, Füllgrabe J et al. 2016, Di Malta C et al. 2019):

- First, the post-translational modification of cytosolic proteins (ATG5, ATG7, LC3), induced by epigenetic modulators such as SIRT1, initiates the autophagy pathway.
- Second, the activation of certain transcription factors (TFEB, FOXO, Jun, CREB, among others) induces the expression of genes that sustain and regulate the autophagy pathway.
- Finally, after a prolonged stimulation of autophagy, global histone deacetylation (including H4K16) acts as a negative feedback regulator.

According to this model, the decrease that we observe in the autophagy-related genes expression would belong to the third phase, where H4K16 deacetylation is a late event that avoids excessive autophagy activation. The fact that *VMP1* is more expressed at 8h than at 24h our could be also explained by this decrease in H4K16 acetylation in the last phase.

In order to see if our results fit completely with the proposed model, it would be interesting to quantify the H4K16 acetylation levels at 8h and earlier time points (minutes to several hours). We would expect that H4K16 acetylation after Shikimic acid treatment would be higher at 8h and earlier than at 24h (Figure 29A), thus correlating with the smaller gene repression that we observe at 8h (Figure 53A) compared to 24h (Figure 54A). Furthermore, by determining the effects of Shikimic acid at these earlier time points, we would expect the post-translational modification of cytosolic autophagy proteins and the increase in autophagy-related genes, which correspond to the first and second phases of the proposed model.

Interestingly, we have observed that the induction of one autophagy-related gene (*VMP1*) is sufficient to induce the autophagy pathway, in agreement with the results of Ropolo A

et al. 2007. VMP1 is a key regulator of the early steps of autophagy in mammalian cells, being required for the formation of autophagosomes, and it is the only ATG protein with no homolog in yeast and other lower eukaryotes (Molejon MI et al (b). 2013). In fact, other studies have reported similar results for other autophagy genes. For example, Eisenberg T et al. reported that Spermidine, a known activator of autophagy pathway, induces the expression of *ATG7*, *ATG11* and *ATG15* genes despite inducing a global histone deacetylation and gene repression (Eisenberg T et al. 2009).

According to our results and the proposed events for the epigenetic regulation of autophagy, we have established a model to explain the mechanism of action of Shikimic acid (Figure 75). During early steps, Shikimic acid induces *VMP1* upregulation, thereby activating autophagy. Considering that Shikimic acid activates SIRT1 and *VMP1* gene expression can be induced by starvation and other types of stress (Ropolo A et al. 2007, Rodríguez ME et al. 2017), SIRT1, which plays a key role in the stress response, might be involved in *VMP1* upregulation. Afterwards, in later steps, the decrease in H4K16 deacetylation -mainly driven by SIRT1- induces the autophagy-related genes repression in order to prevent an excessive autophagic response.

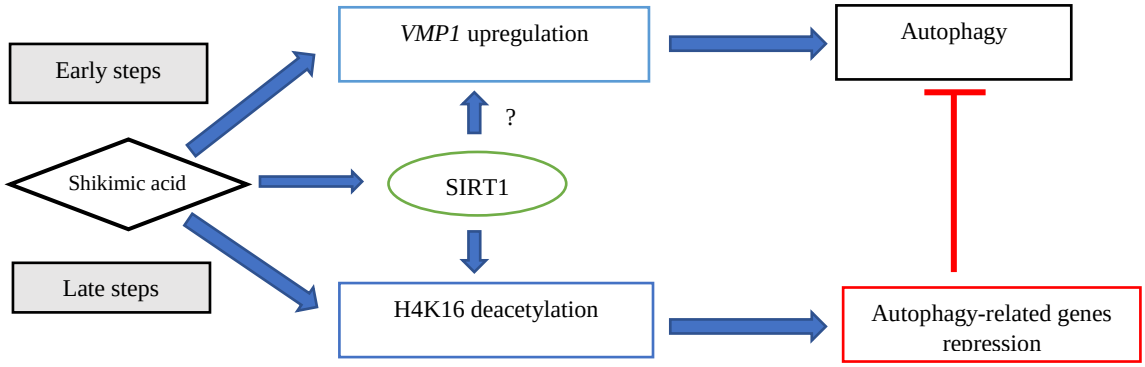


Figure 75. Proposed mechanism of action of Shikimic acid involving SIRT1, H4K16 acetylation and autophagy. In early steps, Shikimic acid induces autophagy through *VMP1* upregulation. SIRT1 might be involved in this process considering that Shikimic acid activates SIRT1 and *VMP1* gene expression is modulated by nutrient availability, a process in which SIRT1 is a key regulator. In late steps, SIRT1 activation by Shikimic acid induces H4K16 deacetylation, which represses autophagy-related gene expression, thereby acting as a negative feedback to prevent excessive autophagy activation.

In order to complete our model, it would be interesting to determine whether the upregulation of *VMP1* and the increase in the autophagic flux (decrease in LC3 and p62 levels) are mediated by SIRT1. For this, the SIRT1 inhibitor EX-527 could be used to stablish a solid relationship between the SIRT1 and autophagy activation by Shikimic acid.

6. 3,4-dihydroxybenzaldehyde regulation of sirtuins and senescence

3,4-dihydroxybenzaldehyde is a natural compound that can be found in significant amounts in many plants, including *Salvia miltiorrhiza*, *Vitis Vinifera* and *Hordeum*

vulgare (Weber B et al. 1995, Etoh H et al. 2004, Shen J et al. 2014). Previous reports have shown that 3,4-dihydroxybenzaldehyde is a strong antioxidant and has a wide range of biological effects (Table 13).

Biological effect	**Cell type**	**References**
Antioxidant	Mouse embryonic fibroblasts Cell-free assay	Jeong JB et al. 2009 Chang Z, et al. 2011
Protection against cardiac hypertrophy	Rat (whole organism)	Fang X et al. 2018
Anti-thrombotic	Rat vascular smooth muscle cells	Moon CY et al. 2012
Anti-apoptotic	Human endothelial cells Mouse embryonic fibroblasts	Xing YL et al. 2012 Jeong JB et al. 2009
Anti-inflammatory	Rat (whole organism) Mouse macrophages	Wei G et al. 2013 Chang Z, et al. 2011
Anti-aging	*Drosophila* (whole organism)	Nakagawa-Yagi Y et al. 2012
Anti-cancer	Human colorectal cancer cells	Jeong JB and Lee SH et al. 2013
Anti-lipogenic	Mouse preadipocytes	Kim JE et al. 2014

Table 13. A summary of the described biological effects of 3,4-dihydroxybenzaldehyde by other authors.

Interestingly, 3,4-dihydroxybenzaldehyde has been shown to inhibit HDAC2 (Jeong JB and Lee SH et al. 2013), but no effect on sirtuins has been reported to date. No effects have been reported in senescence neither, although this compound has been reported to regulate histone demethylase activity in Drosophila, extending the lifespan of female, but not male flies (Nakagawa-Yagi Y et al. 2012).

Our first experiments using 3,4-dihydroxybenzaldehyde indicated a clear decrease in H3K9 and H4K16 acetylation (Figure 29B), suggesting that this compound induced sirtuin activity. Afterwards, we observed that 3,4-dihydroxybenzaldehyde treatment combination with UVB irradiation induced cell death at the concentrations that induced histone deacetylation, and lower concentrations did not induce cell death but increased senescence compared to UVB-irradiated non-treated cells (Figure 41). At this point, we considered the possibility that 3,4-dihydroxybenzaldehyde mechanism of action was mediated by the induction of cellular damage. In fact, later experiments showed that this compound induced cell cycle arrest (Figure 63-64), the increase of senescence markers β-Gal, *p21* and *IL-6* and the decrease of *HAS2* (Figures 65 and 67) in non-irradiated cells, indicating that 3,4-dihydroxybenzaldehyde can induce senescence *per se*. Interestingly, other authors have described the damaging effects of aldehydes, which can even induce senescence (Flor AC et al. 2016). Particularly, these authors use an aldehyde scavenger to prevent the damage induced by aldehydes. In our case, we could apply a similar approach to prove that the mechanism of action of 3,4-dihydroxybenzaldehyde is mediated by causing cellular damage.

Furthermore, we observed that 3,4-dihydroxybenzaldehyde increased apoptosis in non-irradiated (Figure 61) and in UVB-irradiated cells (Figure 62). This result is conflicting compared with the previously observed anti-apoptotic effect of 3,4-dihydroxybenzaldehyde by other authors (Jeong JB et al. 2009, Xing YL et al. 2012). However, we found many differences between these reports and our experimental setup, which could explain our results. In the case of Xing YL et al., they are using a different cell type (human endothelial cells) and inducing apoptosis through lipopolysaccharide (LPS) exposure, which differs from UVB damage in the stimulated signaling pathway that drives apoptosis (Kulms D and Schwarz T. 2000, Bannerman DD and Goldblum SE 2003). On the other hand, in the case of Jeong JB et al., they quantified apoptosis 24h after compound incubation and one H_2O_2 single stress, while we quantified apoptosis 48h after the last of two UVB stresses and a total incubation time with the compound of 6 days. These observations, along with the positive effect of 3,4-dihydroxybenzaldehyde on histone deacetylation after 24h, suggests that this compound is probably inducing positive effects in short term but detrimental effects in long term.

Despite the negative effects of 3,4-dihydroxybenzaldehyde on apoptosis and senescence, we observed that lower doses of this compound (10 and 25 μM) prevented *IL-6* upregulation induced by UVB irradiation (Figure 66A). In fact, the 10 μM dose significantly decreased *IL-6* expression in non-irradiated cells (Figure 65). Thus, moderate amounts of 3,4-dihydroxybenzaldehyde appear to have a protective effect against upregulation of SASP during senescence, while high amounts seem to be detrimental.

Finally, with the aim of better characterizing the effect of 3,4-dihydroxybenzaldehyde on sirtuin levels after 24h treatment, we observed that this compound slightly increased *SIRT1*, *SIRT2* and *SIRT7* gene expression (Figure 69), while the protein levels were decreasing, mostly in SIRT1 and SIRT7 (Figure 70). Interestingly, this is also supported by the observed decrease of SIRT1 protein levels induced by aldehydes through post-translational modifications on SIRT1 (Caito S et al. 2010), which would explain our results.

Integrating all our results, we have described the possible mechanism of action of 3,4-dihydroxybenzaldehyde in Figure 76. We propose that 3,4-dihydroxybenzaldehyde exerts beneficial effects in short term by activating sirtuins through mild damage and inducing H3K9 and H4K16 deacetylation. However, when this damage persists, 3,4-dihydroxybenzaldehyde induces senescence, or apoptosis when it is combined with UVB irradiation.

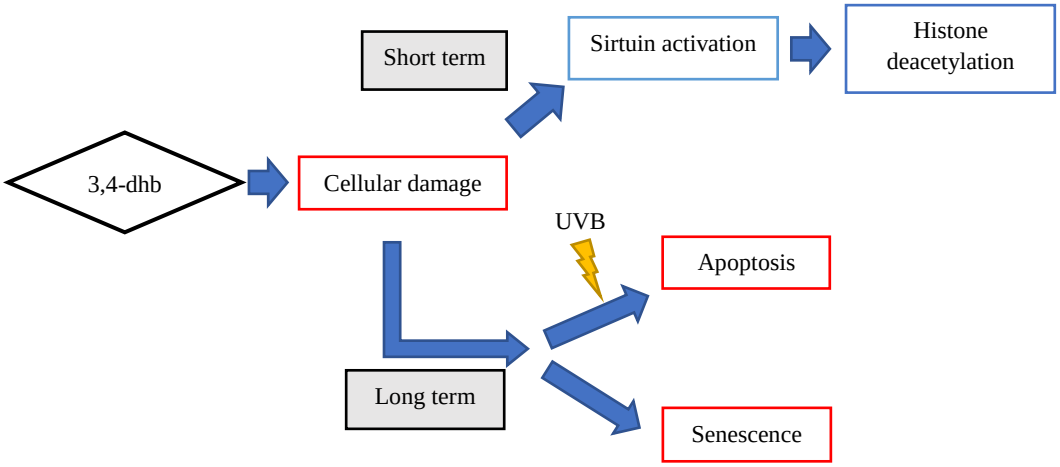


Figure 76. Proposed mechanism of action of 3,4-dihydroxybenzaldehyde involving sirtuins, cellular damage and senescence/apoptosis regulation. 3,4-dihydroxybenzaldehyde generates cellular damage, which exerts beneficial effects after acute exposure (short term) of the compound by inducing H3K9 and H4K16 deacetylation through sirtuin activation. On the other hand, chronic exposure (long term) of this compound induces cellular senescence, while the damage caused by this chronic exposure combined with the damage produced by UVB light drives the cell into apoptosis.

CONCLUSIONS

- Five compounds (Andrographolide, Dexpanthenol, Sesamol, Shikimic acid and 3,4-dihydroxybenzaldehyde) out of the 30 compounds tested induce the deacetylation of both sirtuin targets H3K9 and H4K16 in HDF, proving to be potential sirtuin activators.

-Three compounds (Ectoine, Kinetin and Zeatin) induce the deacetylation of H3K9, and one compound (Phloretin) induces the deacetylation of H4K16, indicating a possible activation of sirtuins.

- A UV-induced senescence assay was used as a screening test to study the ability of compounds to protect against senescence and two compounds (Shikimic acid and Gallic acid) out of the 30 compounds tested, show protection against UVB-induced senescence in HDF.

- Shikimic acid protection against UVB-induced senescence is SIRT1-dependent.

- Shikimic acid does not activate SIRT1 directly, indicating that it induces SIRT1 activation through an indirect signaling mechanism.

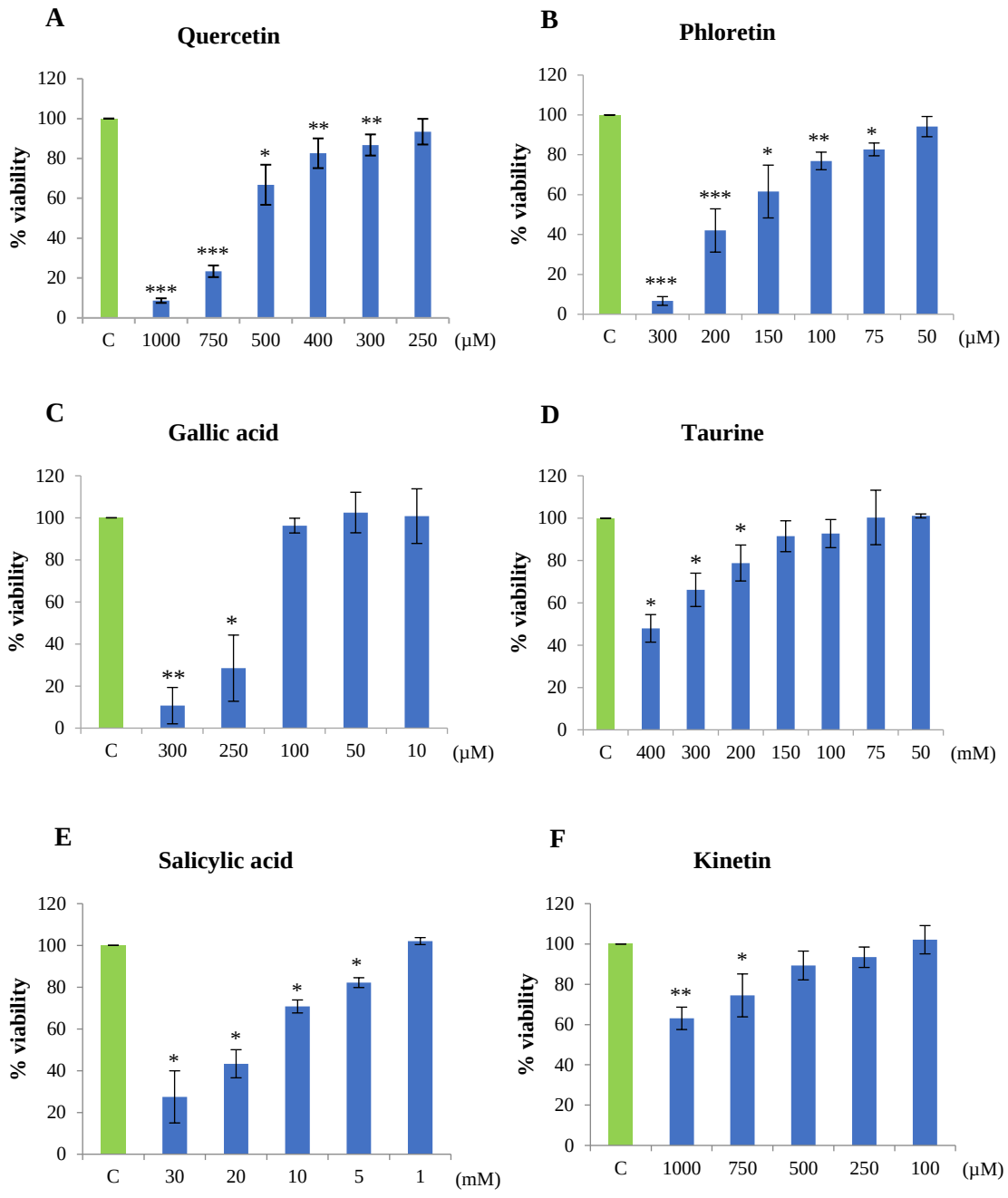
- Shikimic acid activates autophagy in HDF, a cellular stress response pathway regulated by SIRT1 and associated with increased longevity.

- 3,4-dihydroxybenzaldehyde induces cell damage and cell cycle arrest in the absence and presence of UVB irradiation. This mechanism could explain why this compound induces the deacetylation of sirtuin histone targets H3K9 and H4K16.

- Shikimic acid has been included in a new line of anti-aging products of the company, which will be supported with the results of Shikimic acid on sirtuin activation and senescence protection.

-A set of screening techniques have been implemented during this project and will be useful in a near future for the company to find novel compounds with the ability to regulate sirtuins, senescence and/or autophagy.

ANNEXES



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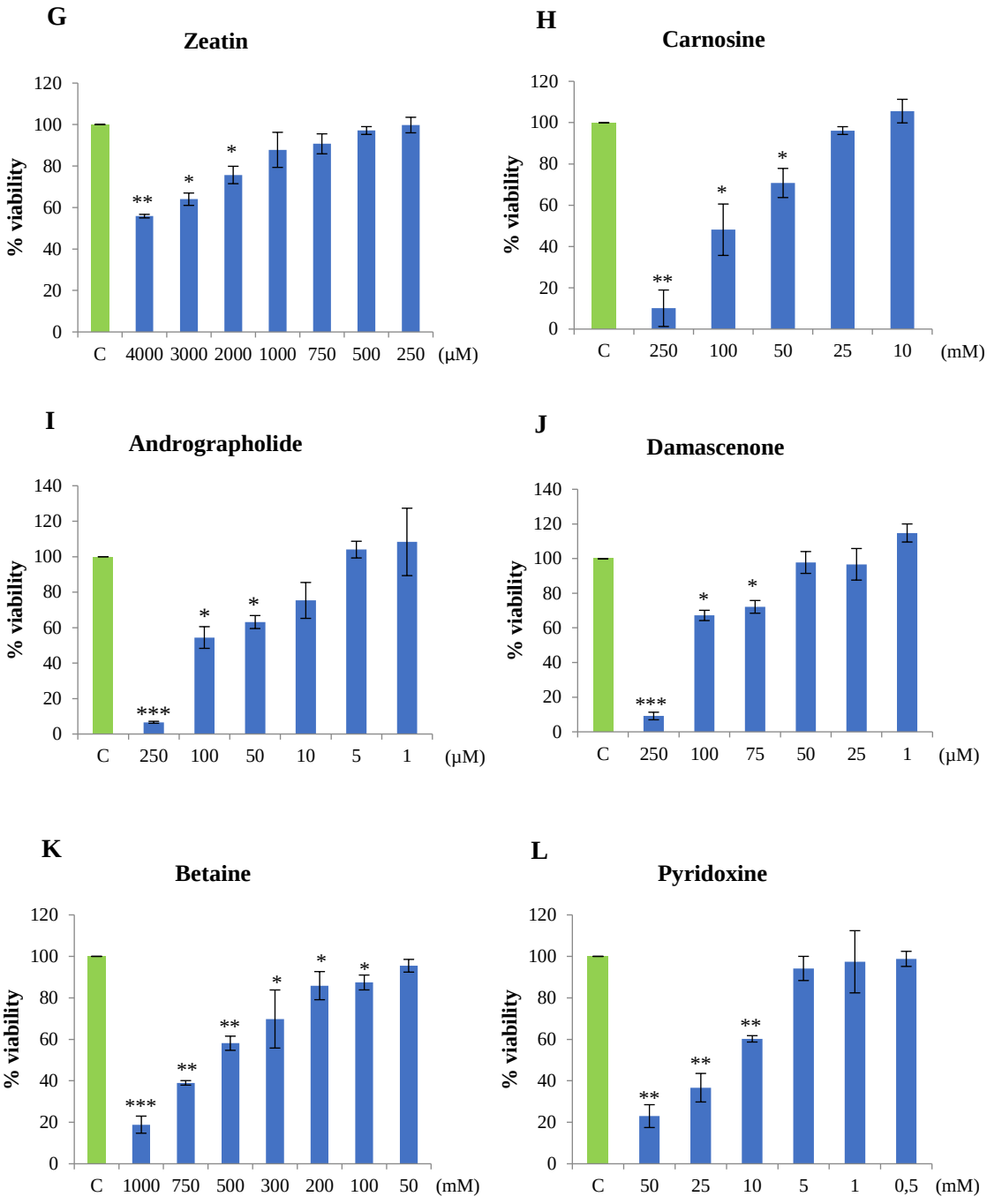

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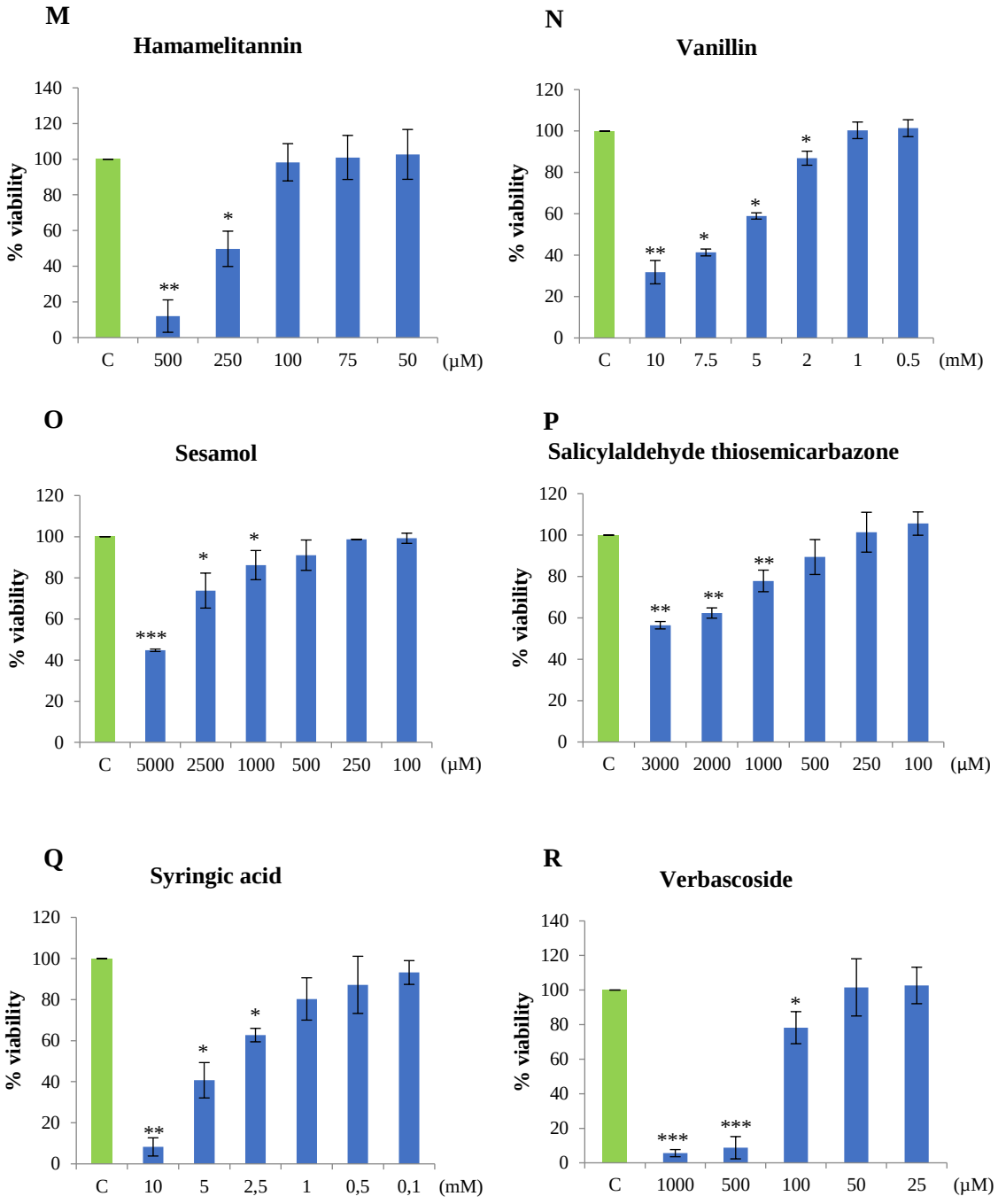

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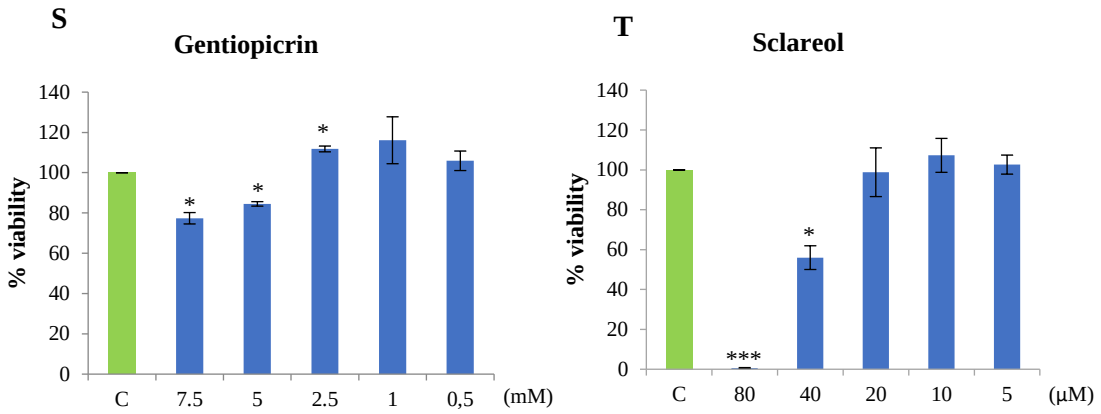
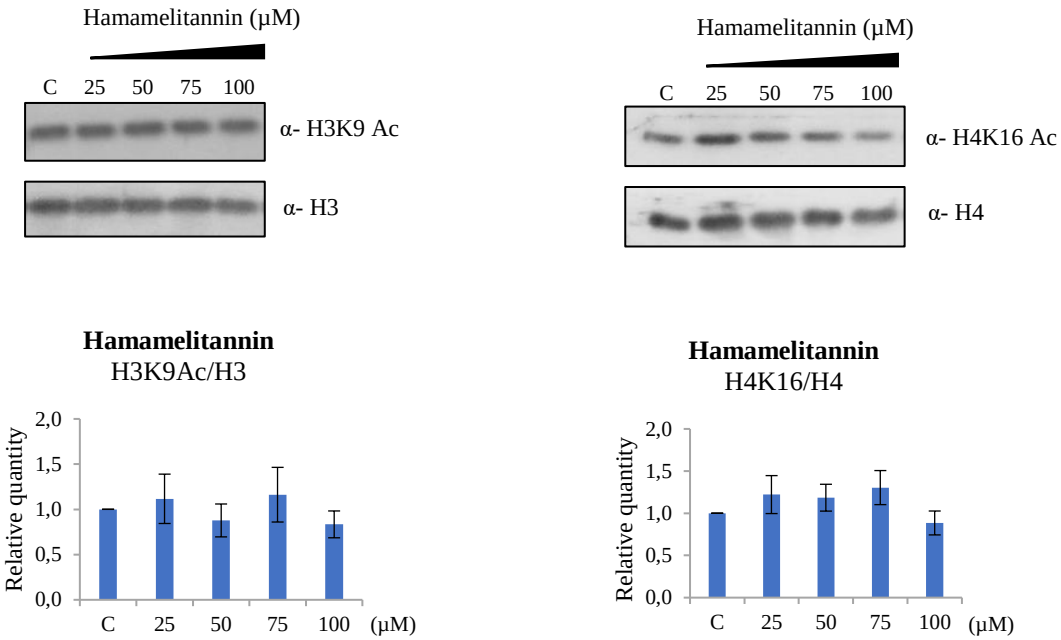


Annex 1. Cell viability of HDF after 24h treatment with (A) Quercetin (50, 100, 200, 250, 300, 400, 500, 750 and 1000 µM), (B) Phloretin (50, 75, 100, 150, 200 and 300 µM), (C) Gallic acid (10, 50, 100, 250 and 300 µM), (D) Taurine (50, 75, 100, 150, 200, 300 and 400 mM), (E) Salicylic acid (1, 5, 10, 20 and 30 mM), (F) Kinetin (100, 250, 500, 750 and 1000 µM), (G) Zeatin (250, 500, 750, 1000, 2000, 3000 and 4000 µM), (H) Carnosine (10, 25, 50, 100 and 250 mM), (I) Andrographolide (1, 5, 10, 50, 100 and 250 µM), (J) Damascenone (1, 25, 50, 75, 100 and 250 µM), (K) Betaine (50, 100, 200, 300, 500, 750 and 1000 µM), (L) Pyridoxine (0.5, 1, 5, 10, 25 and 50 mM), (M) Hamamelitannin (50, 75, 100, 250 and 500 µM), (N) Vanillin (0.5, 1, 2, 5, 7.5 and 10 mM), (O) Sesamol (100, 250, 500, 1000, 2500 and 5000 µM), (P) Salicylaldehyde thiosemicarbazone (100, 250, 500, 1000, 2000 and 3000 µM), (Q) Syringic acid (0.1, 0.5, 1, 2.5, 5 and 10 mM), (R) Verbascoside (25, 50, 100, 500 and 1000 µM), (S) Gentiopicrin (0.5, 1, 2.5, 5 and 7.5 mM) and (T) Sclareol (5, 10, 20, 40 and 80 µM). Statistics: Student's T-test compared to control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A



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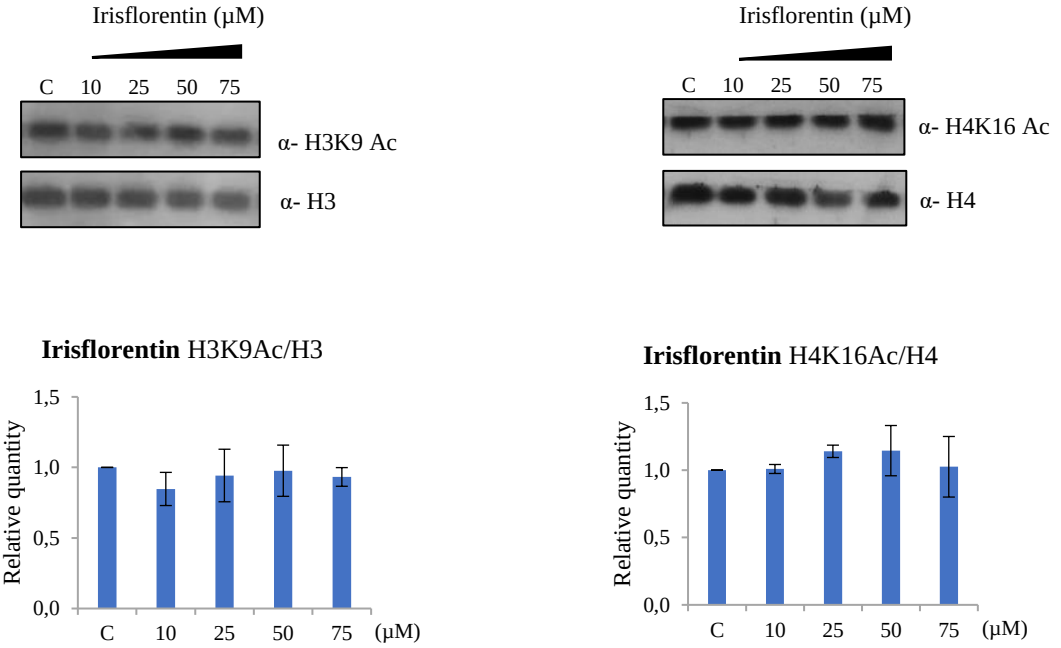

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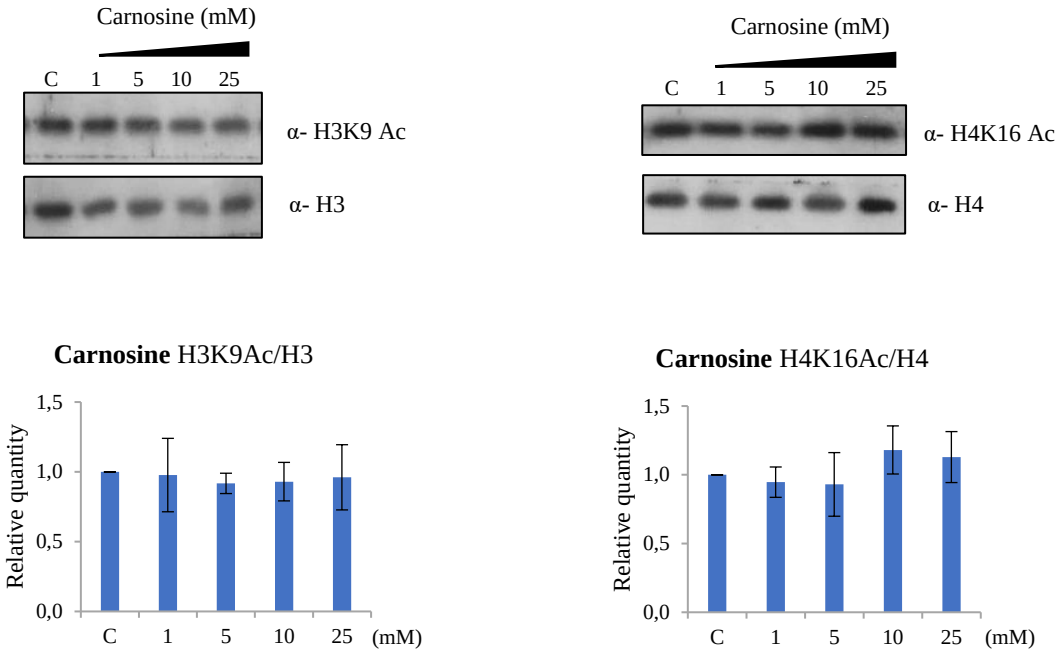

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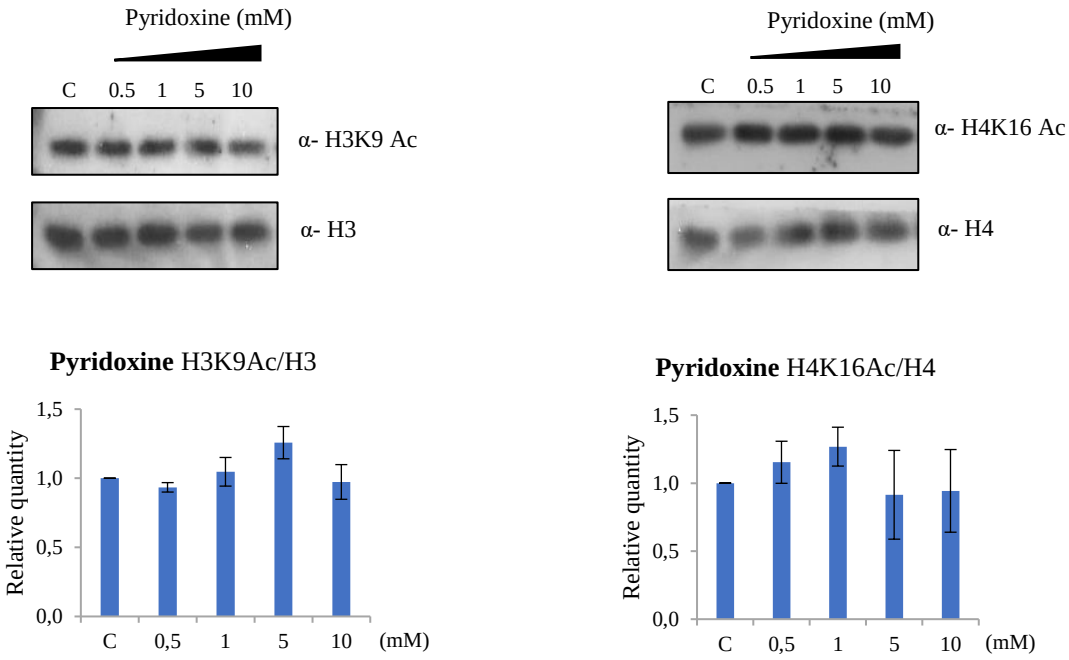

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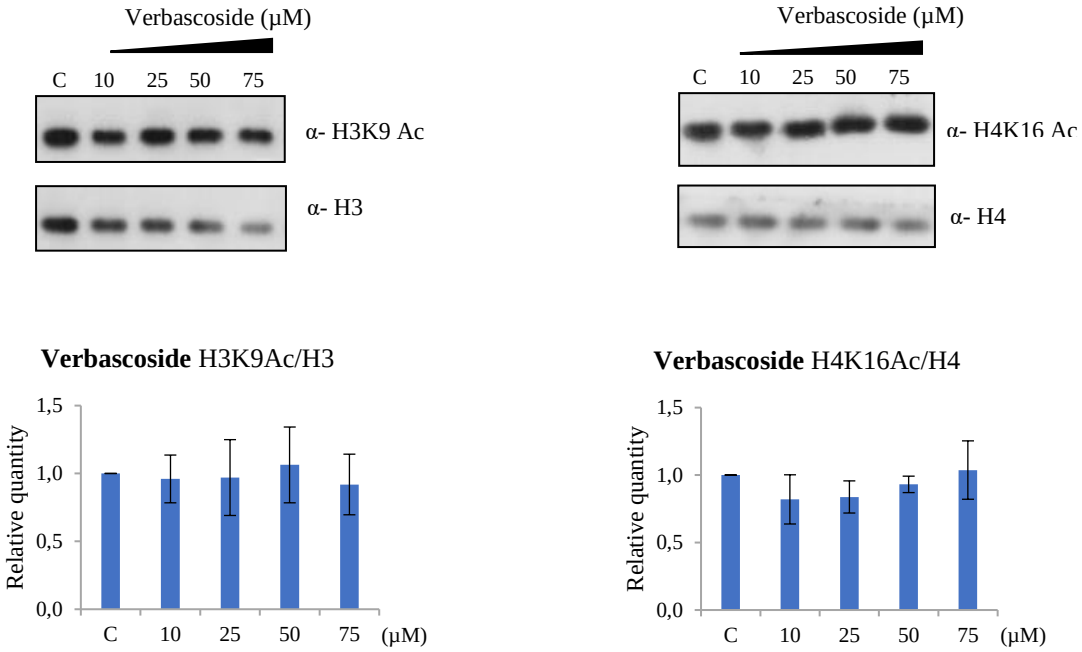

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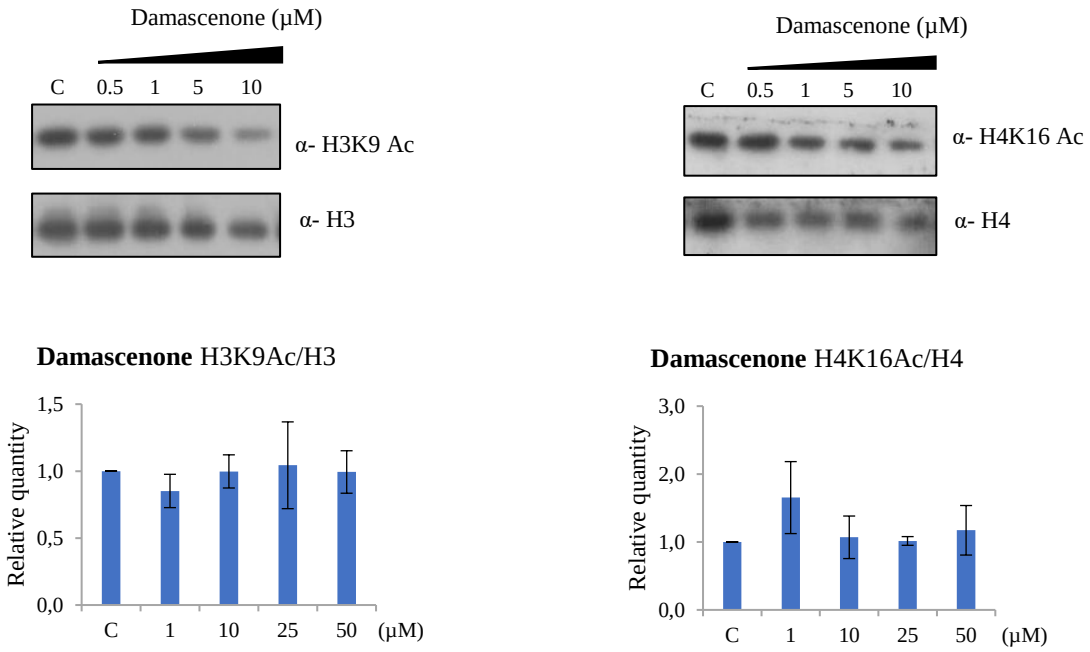

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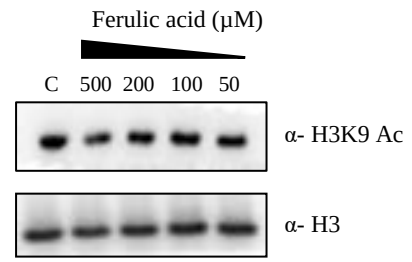

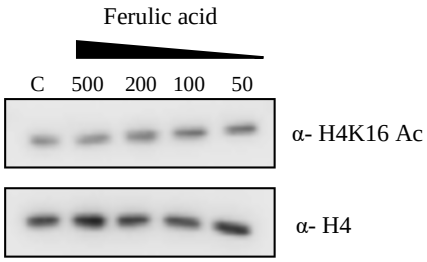

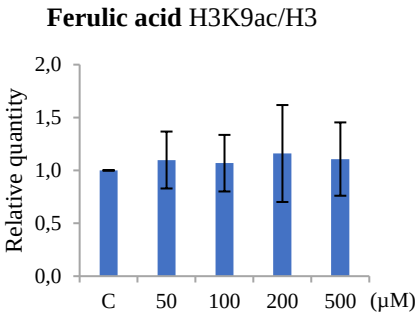

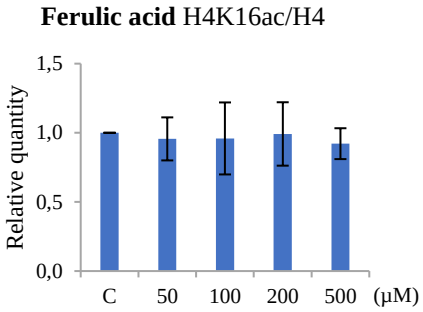

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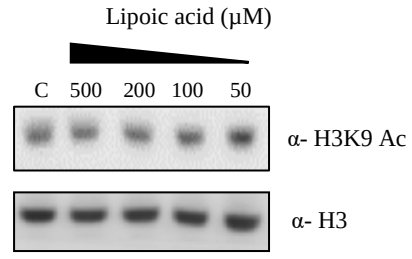

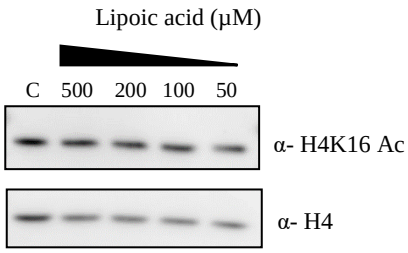

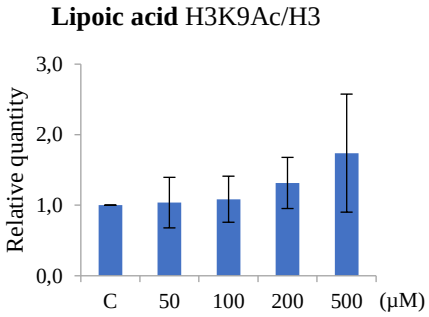

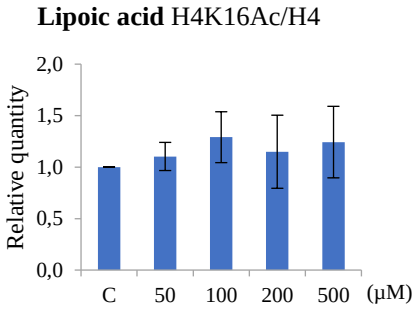

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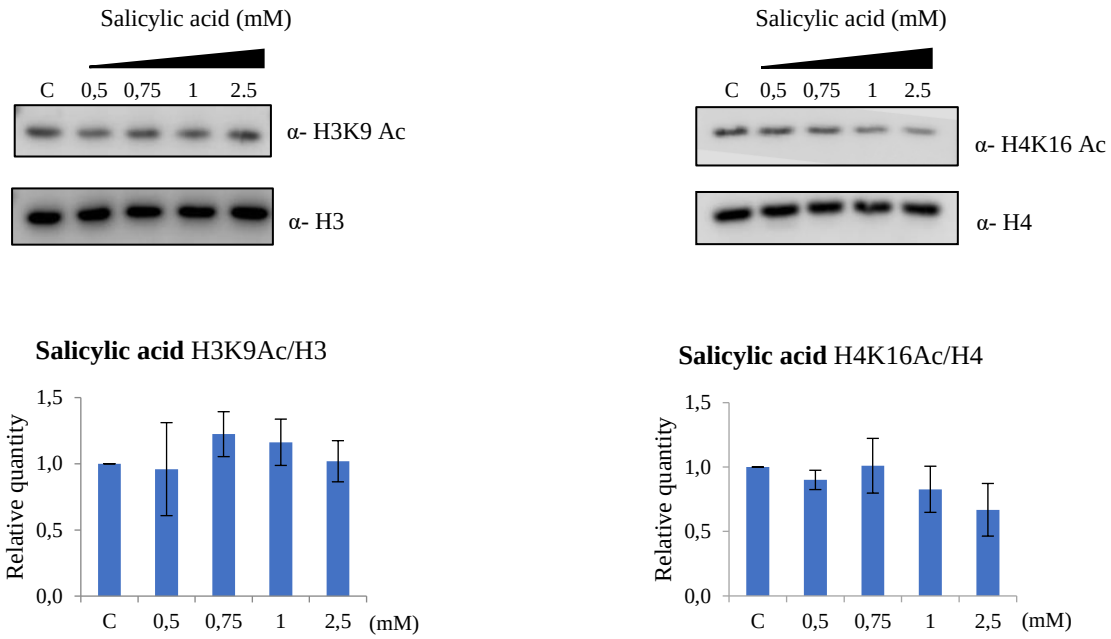

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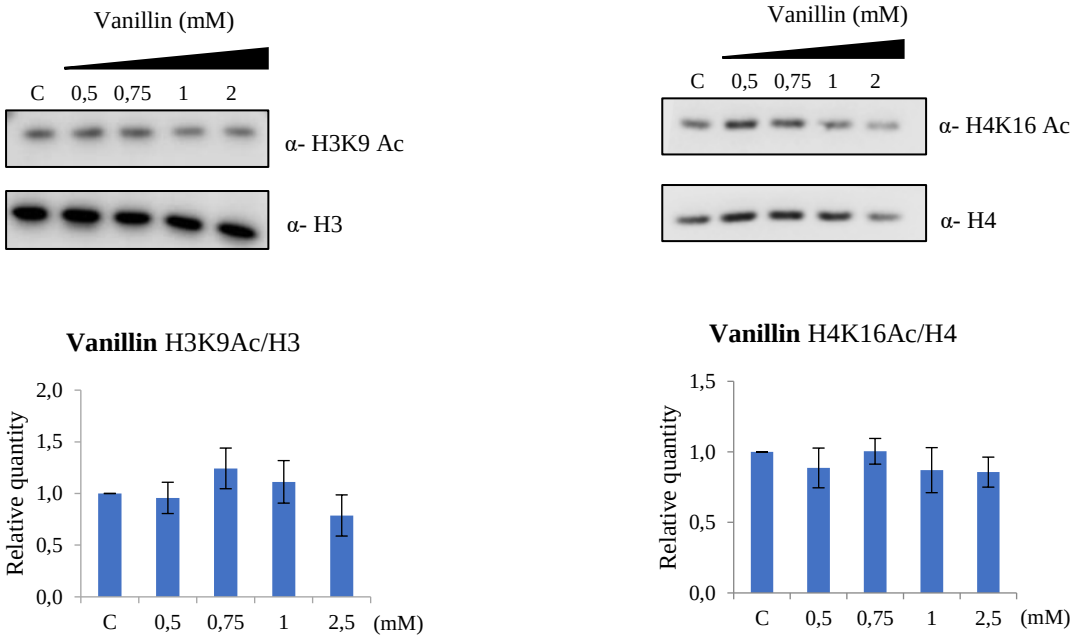

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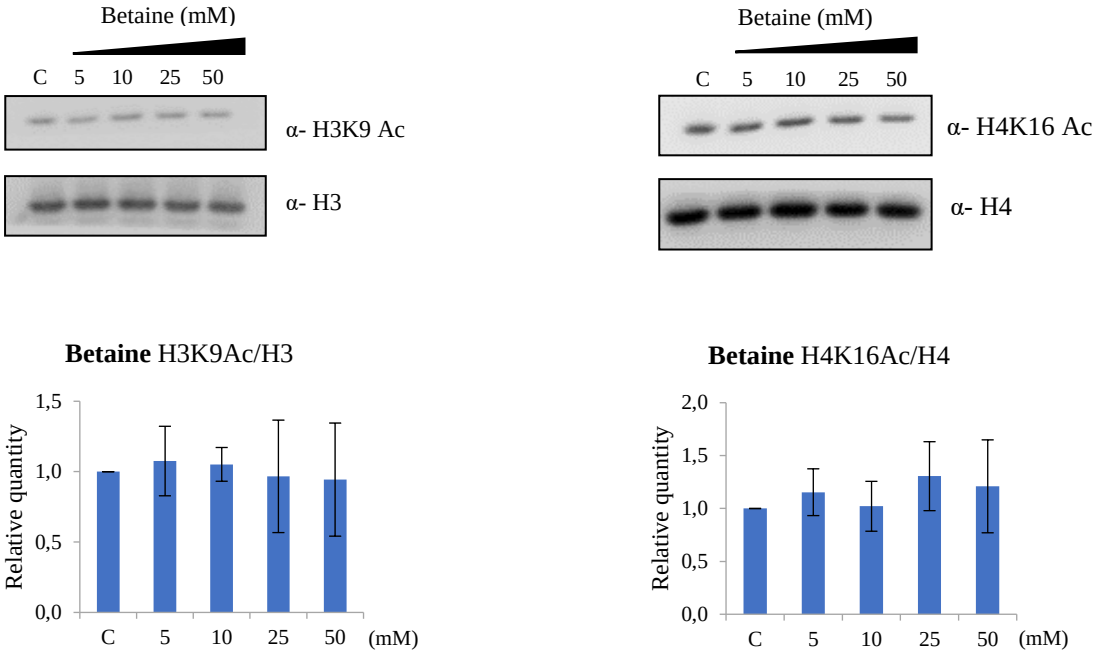

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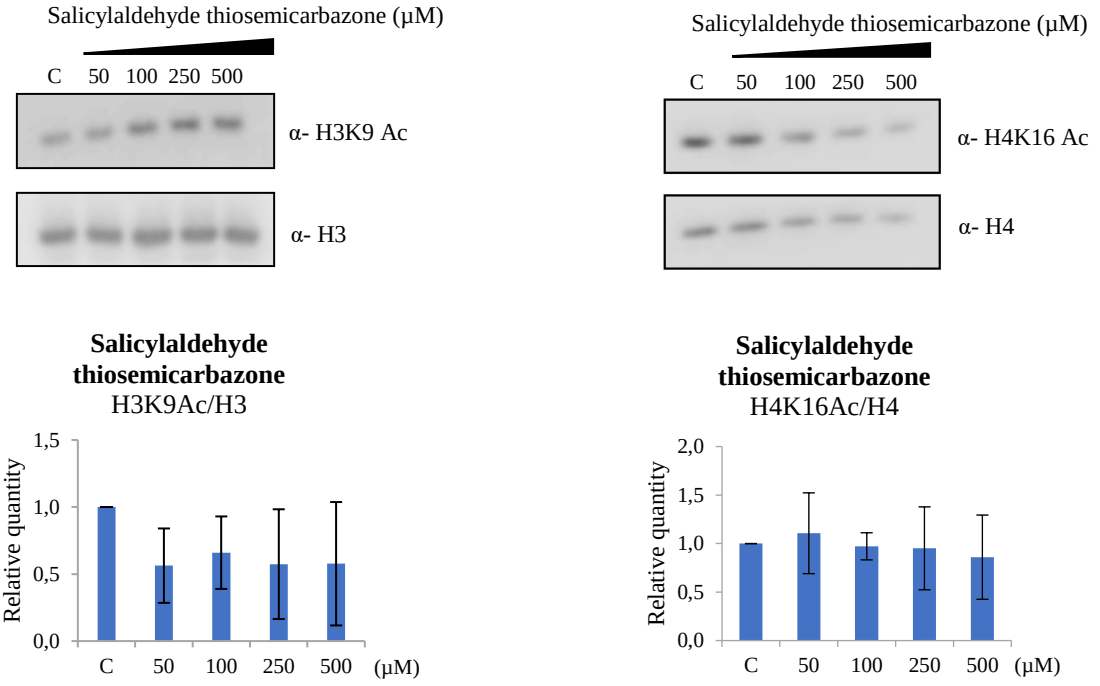

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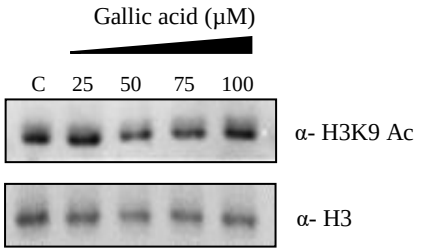

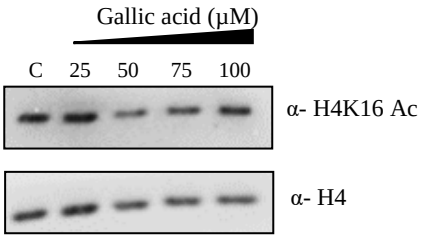

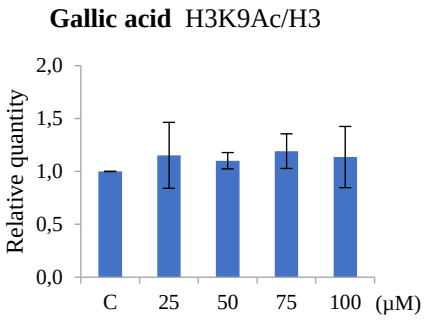

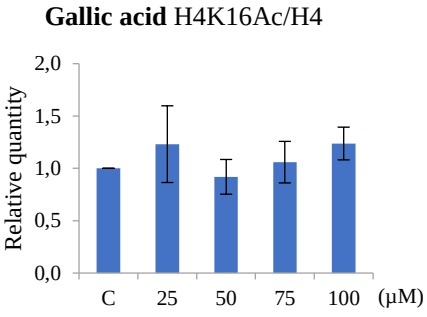

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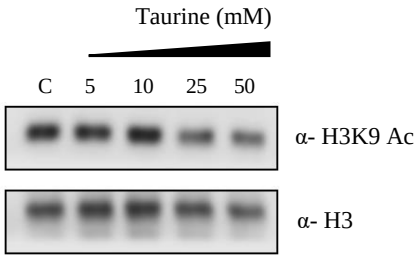

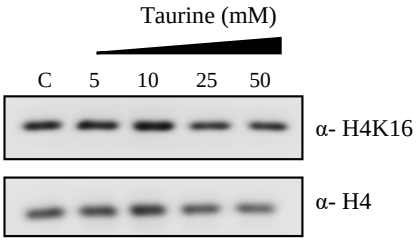

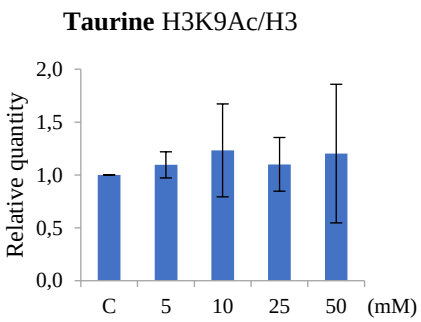

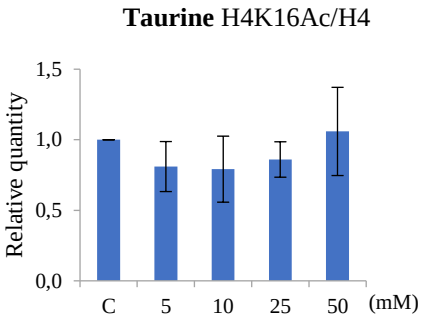

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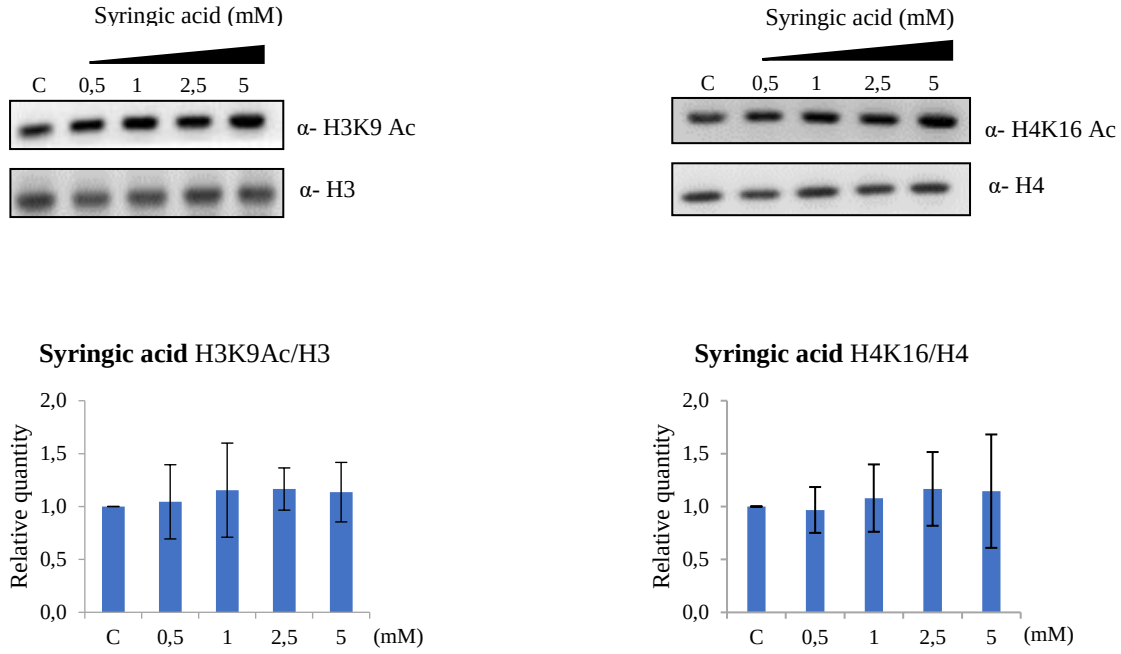
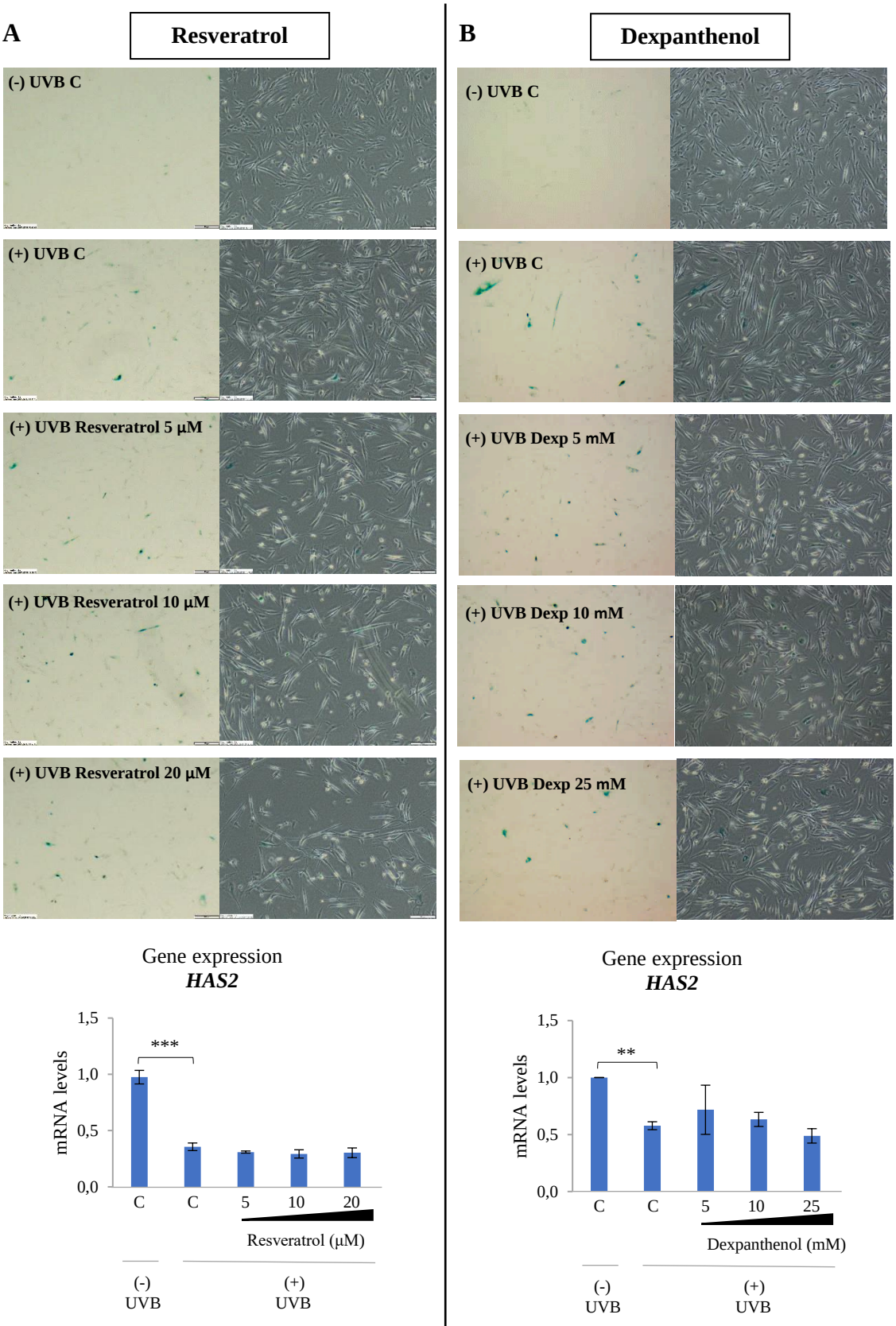


Annex 2. Western Blot analysis of H3K9 and H4K16 acetylation. Human dermal fibroblasts were treated for 24h with (A) Hamamelitannin (25, 50, 75 and 100 µM), (B) Irisflorentin (10, 25, 50 and 75 µM), (C) Carnosine (1, 5, 20 and 25 mM), (D) Pyridoxine (0.5, 1, 5 and 10 mM), (E) Verbascoside (10, 25, 50 and 75 µM), (F) Damascenone (1, 10, 25 and 50 µM), (G) Ferulic acid (50, 100, 200 and 500 µM), (H) Lipoic acid (50, 100, 200 and 500 µM), (I) Salicylic acid (0.5, 0.75, 1 and 2.5 mM), (J) Vanillin (0.5, 0.75, 1 and 2.5 mM), (K) Betaine (5, 10, 25 and 50 mM), (L) Salicylaldehyde thiosemicarbazone (50, 100, 250 and 500 µM), (M) Gallic acid (25, 50, 75 and 100 µM), (N) Taurine (5, 10, 25 and 50 mM), (O) Syringic acid (0.5, 1, 2.5 and 5 mM) and then cells were harvested and lysed in loading buffer for histone extraction. Statistics: Student's T-test. Cells treated with the different doses of compound were compared to control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).



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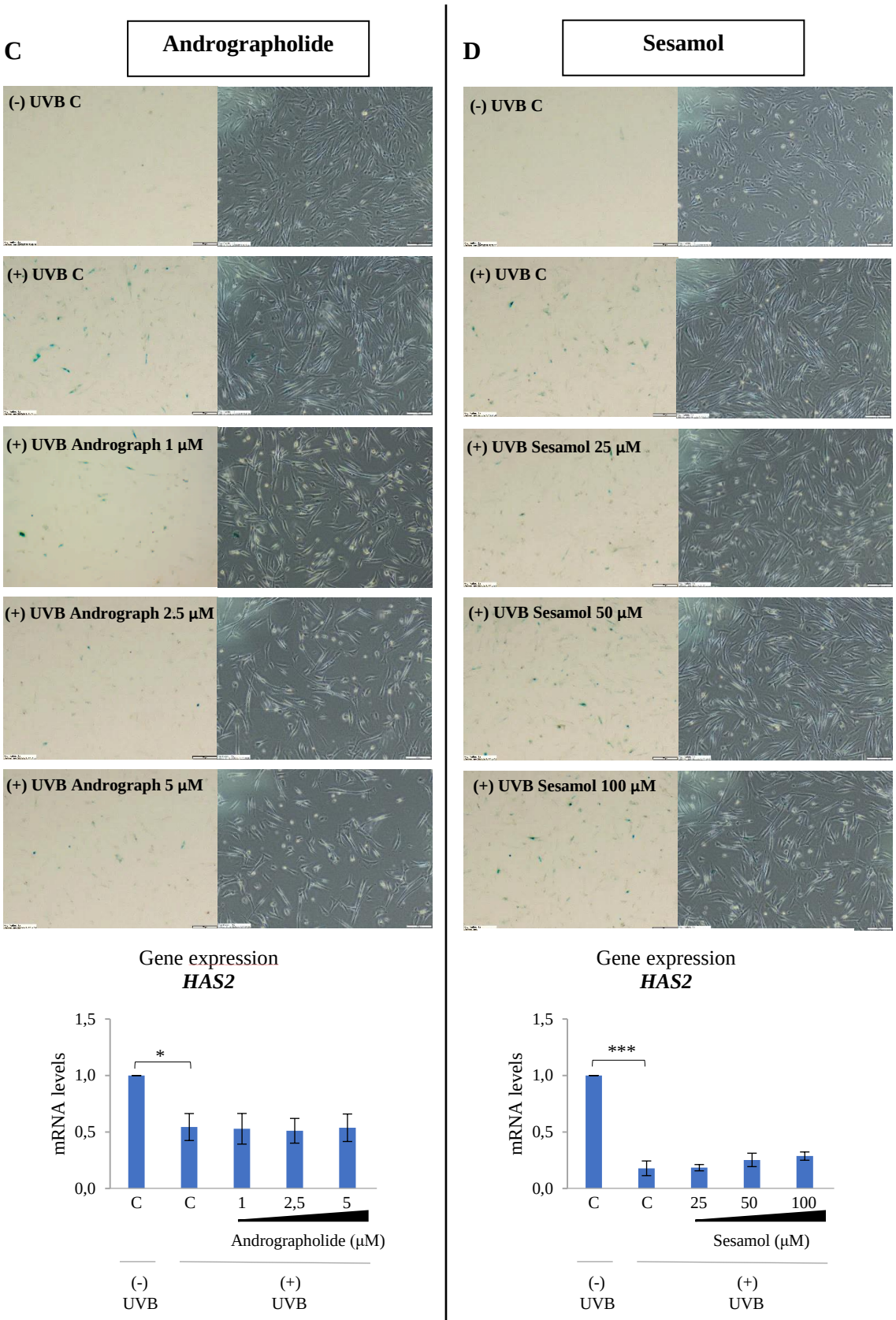

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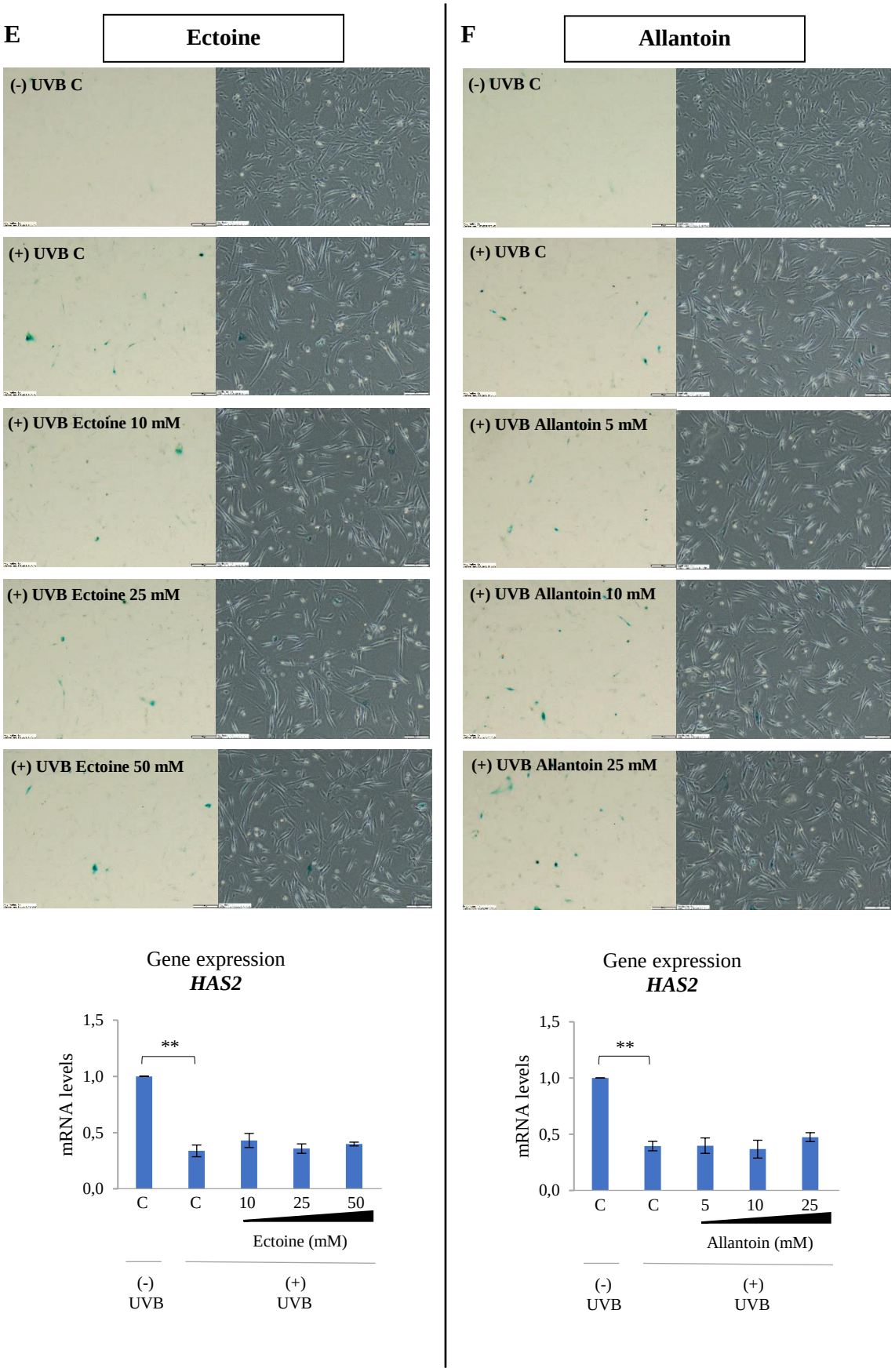

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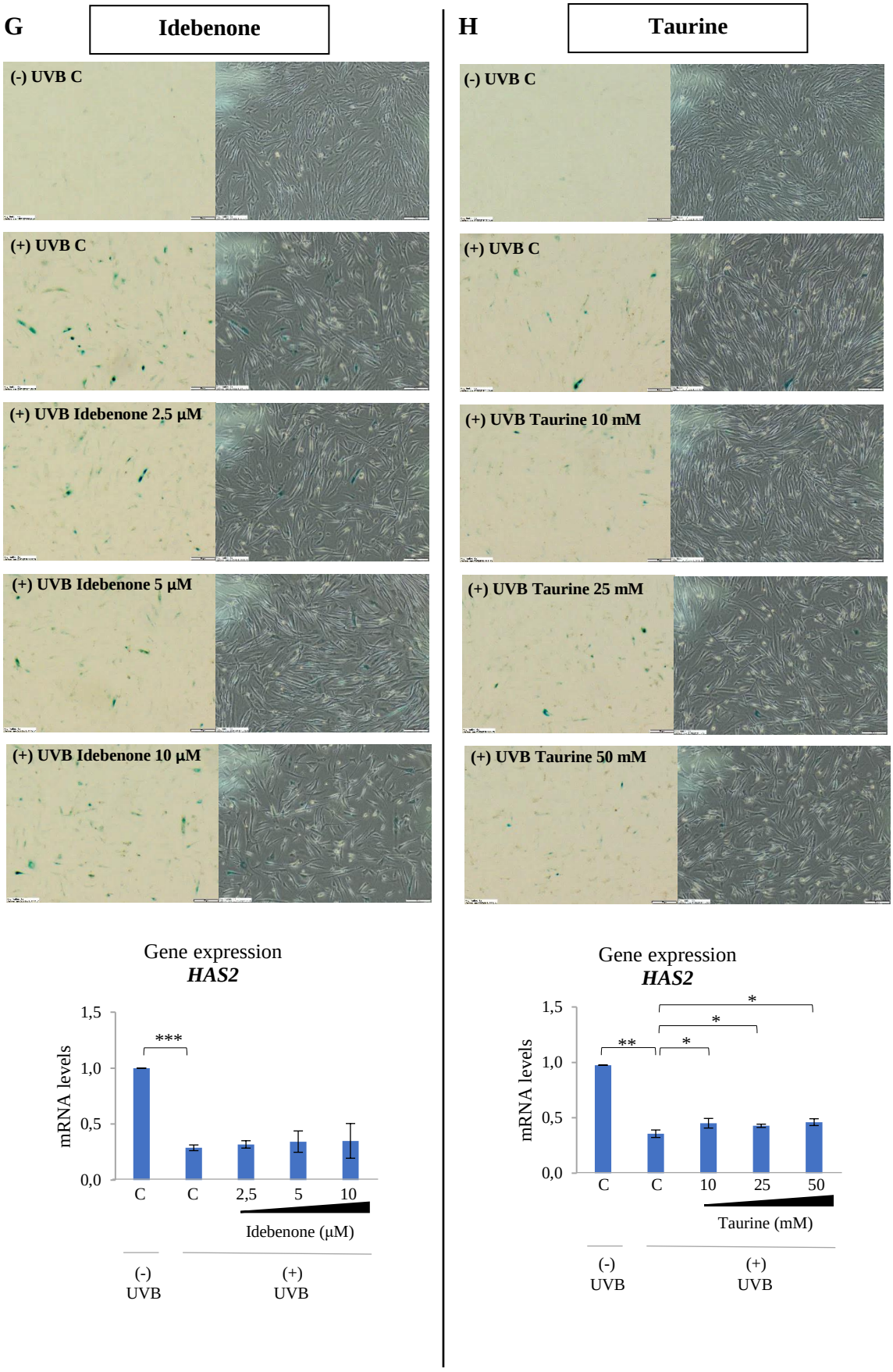

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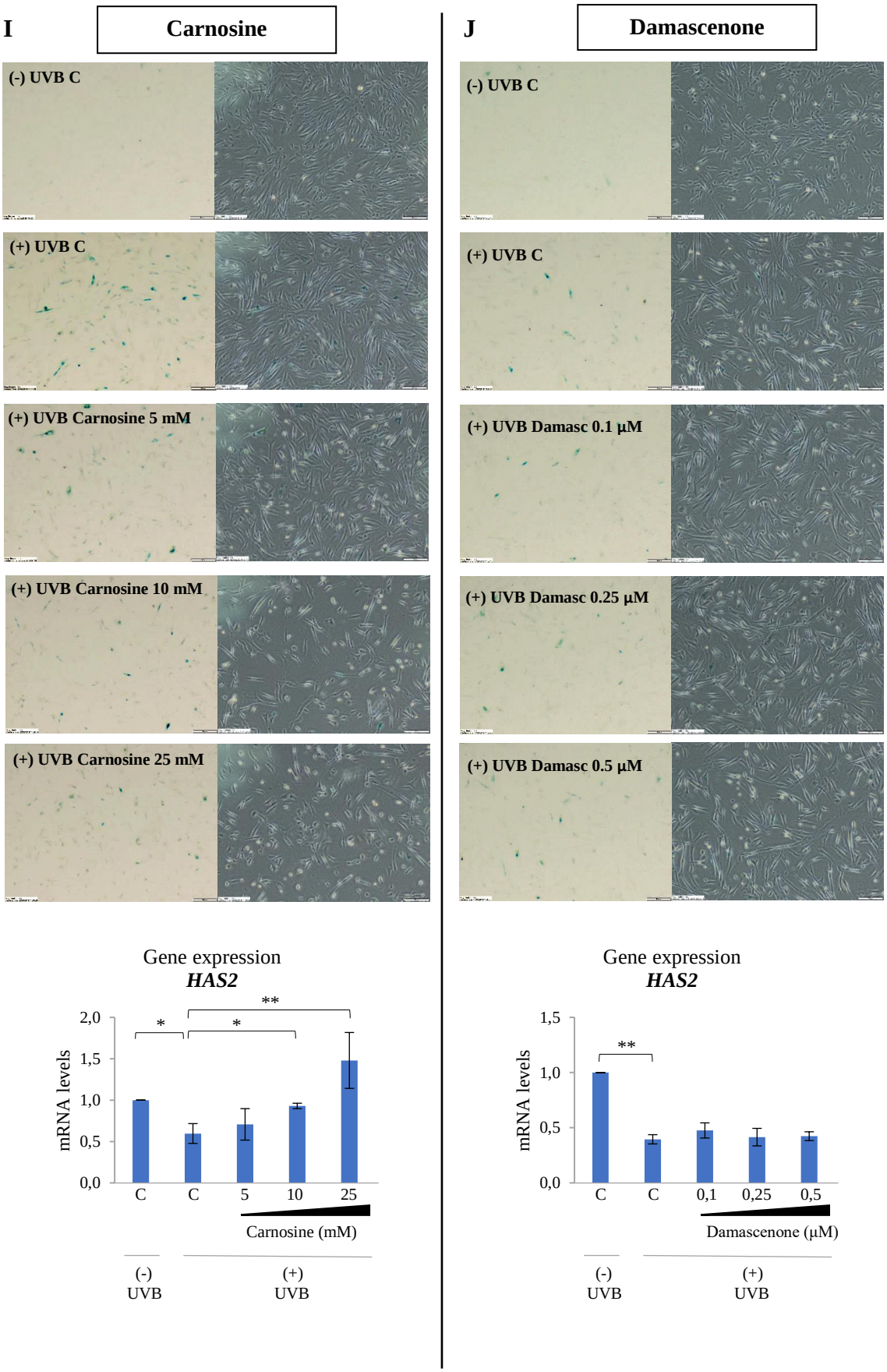

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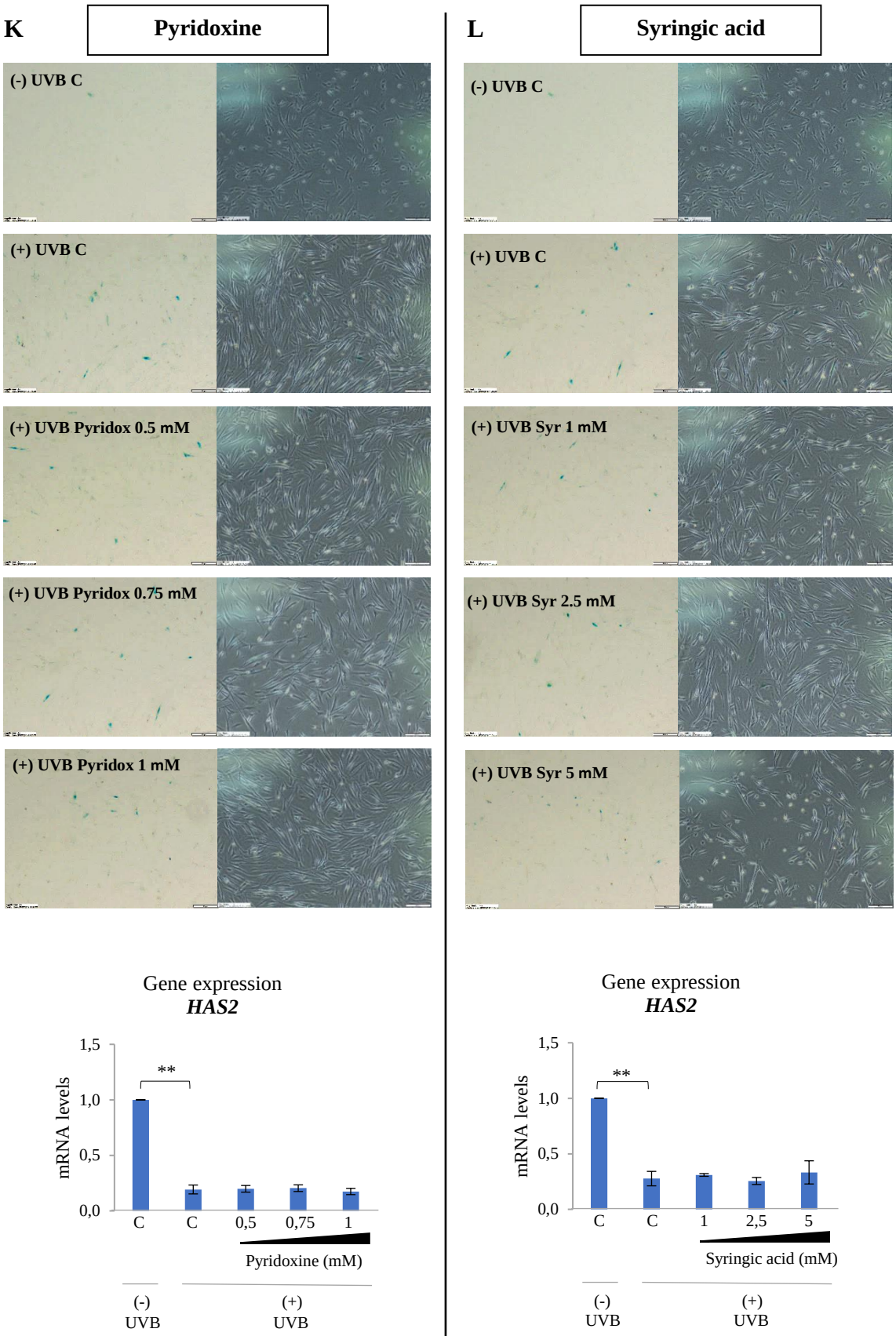
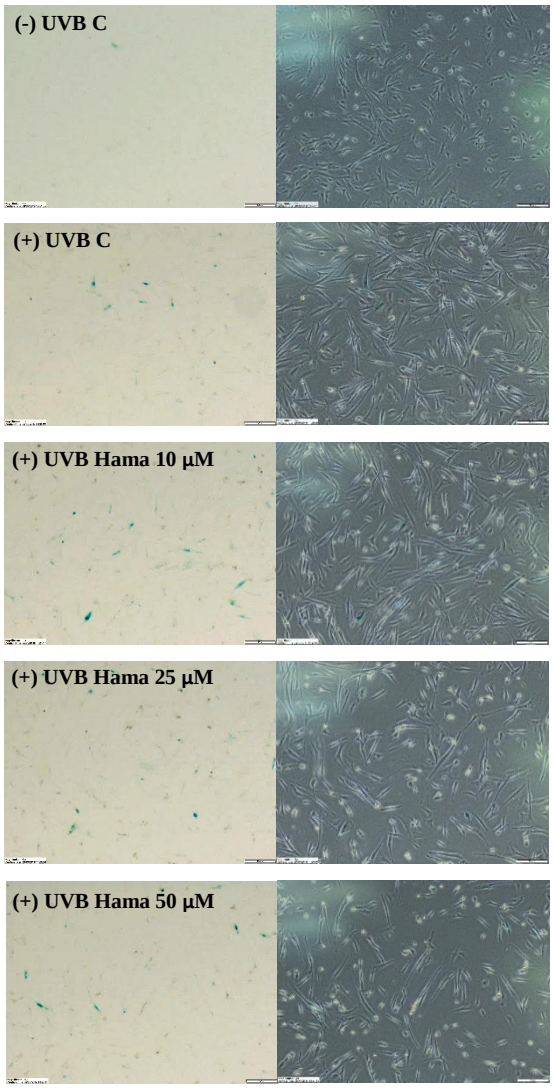
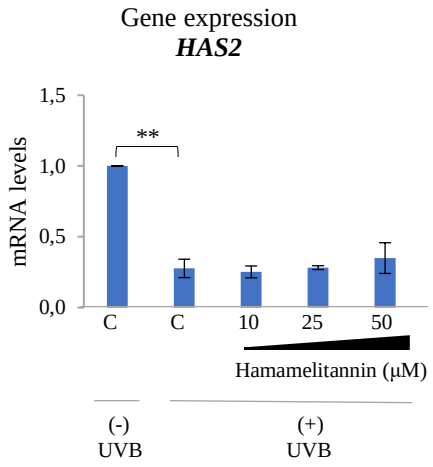


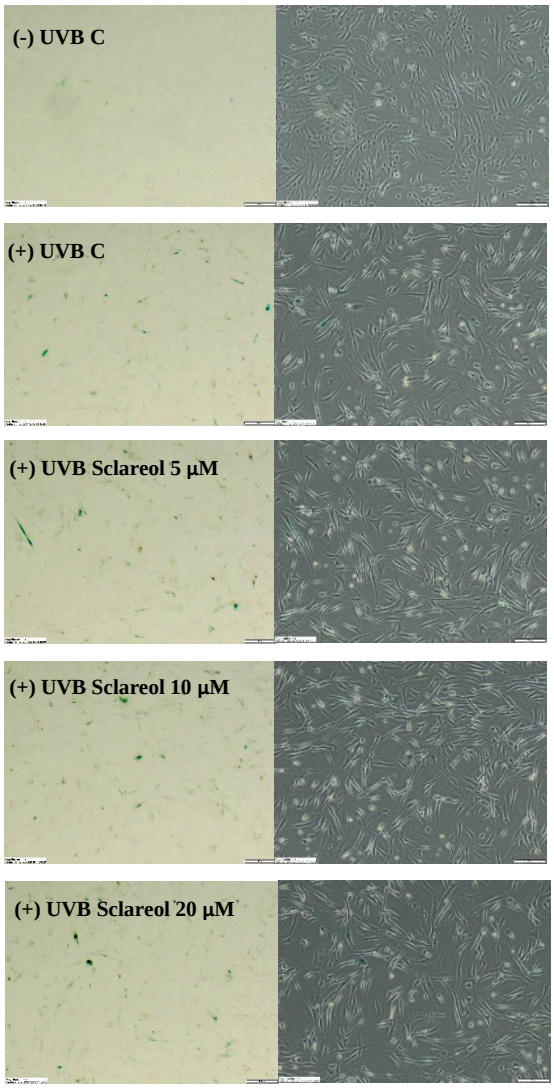
Annex 3. *Continued*

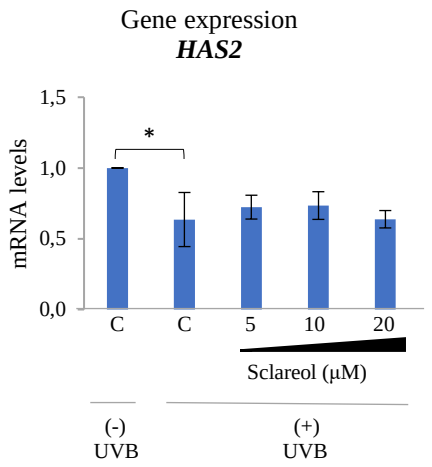
M **Hamamelitannin**





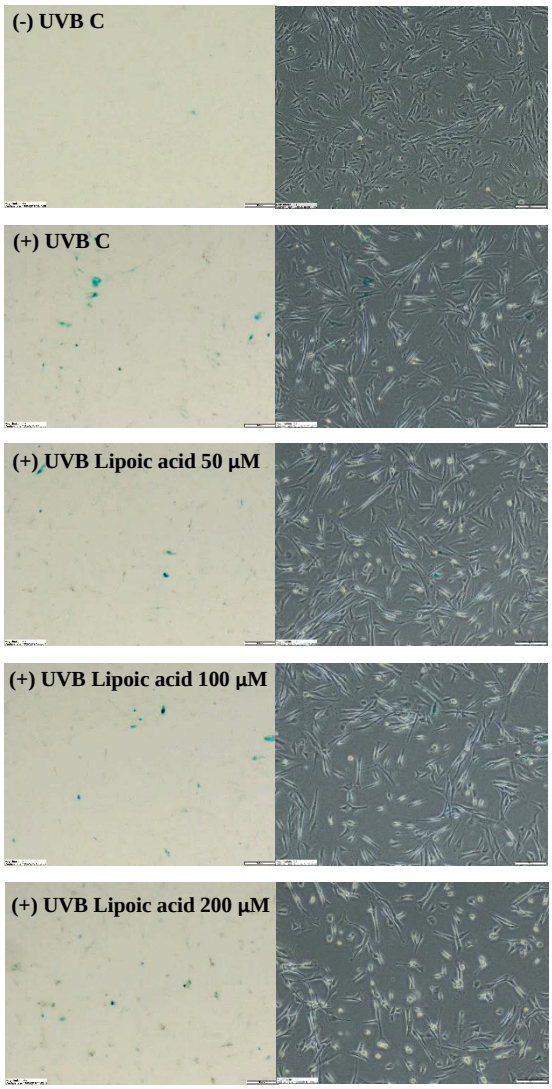
N **Sclareol**

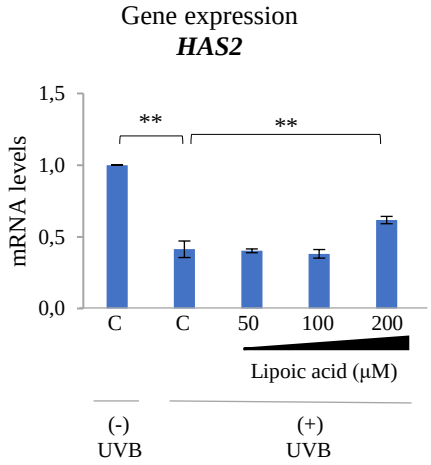




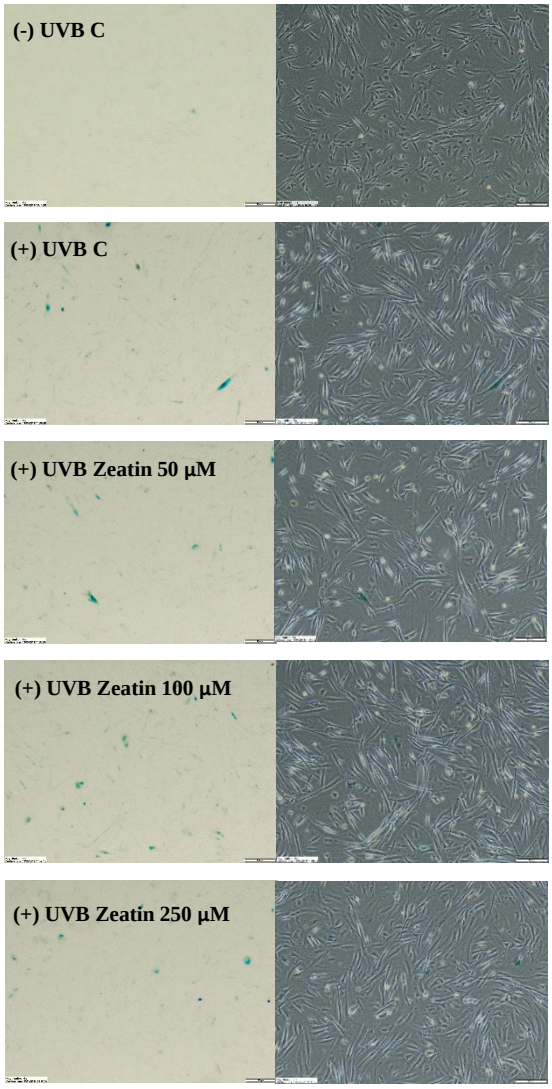
Annex 3. *Continued*

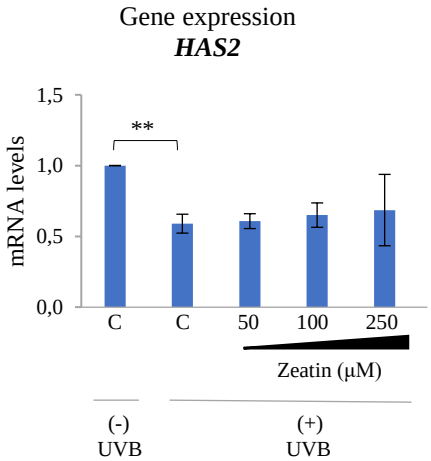
O **Lipoic acid**



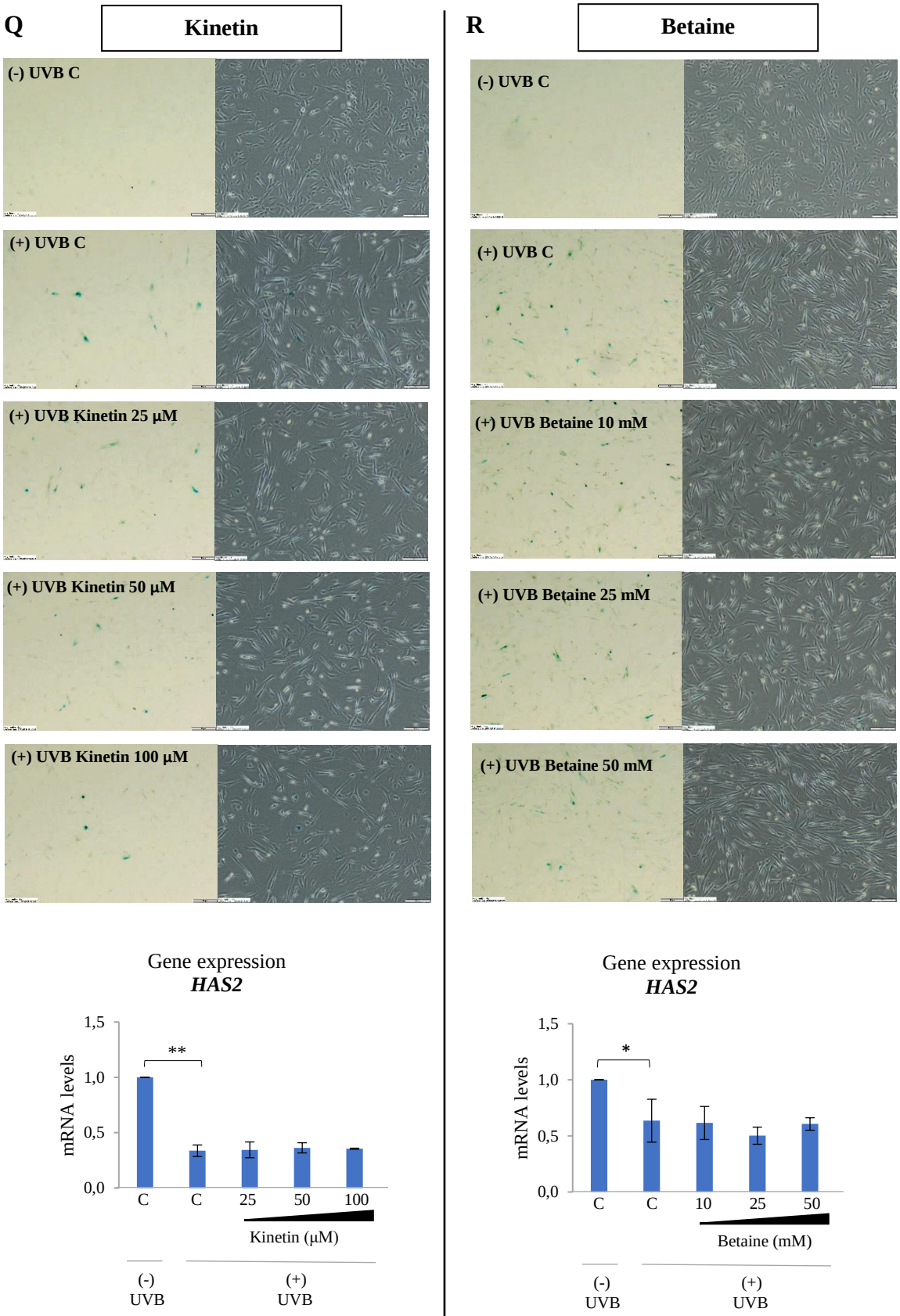


P **Zeatin**



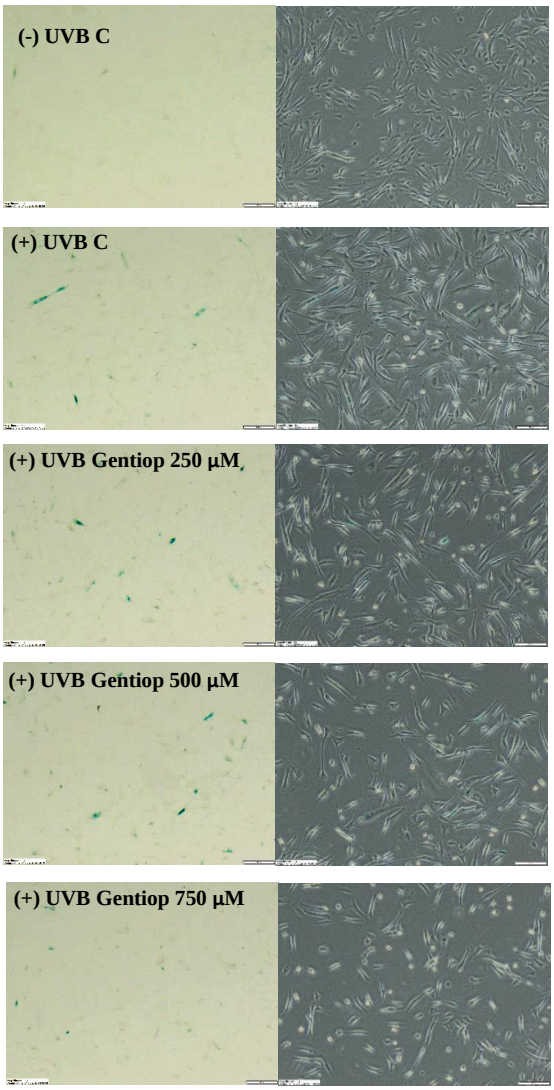


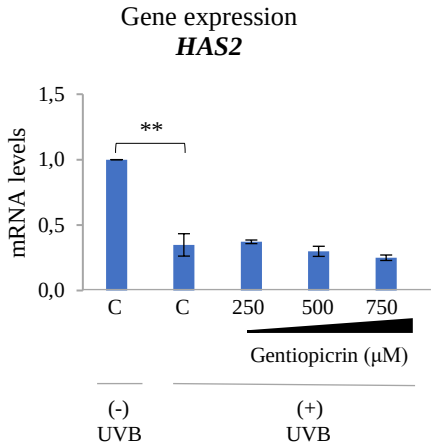
Annex 3. *Continued*



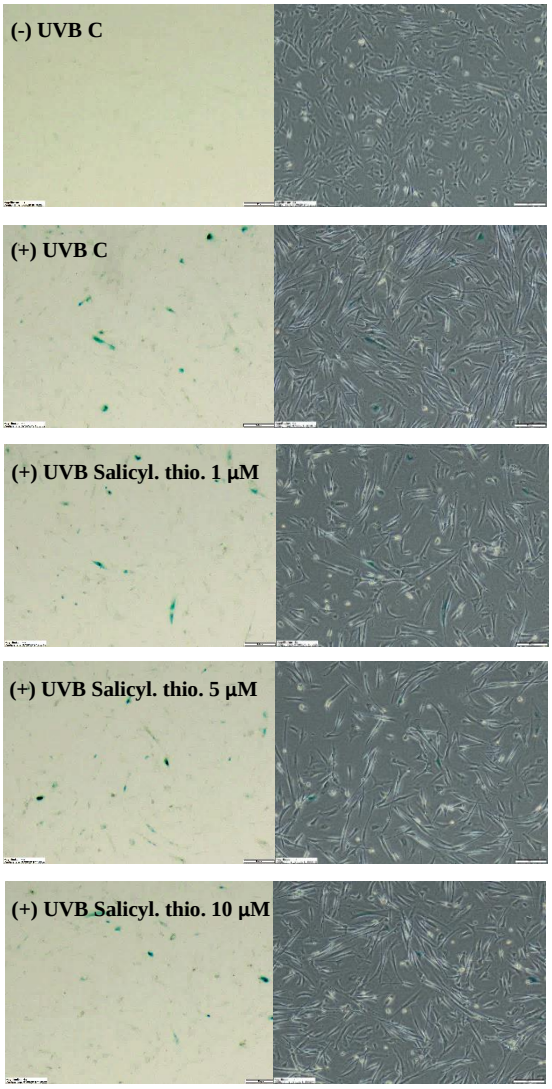
Annex 3. *Continued*

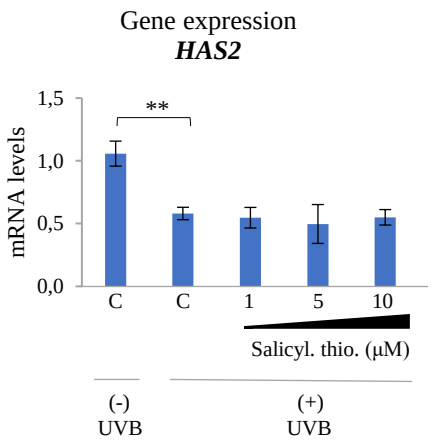
S **Gentiopicrin**





T **Salicylaldehyde thiosemicarbazone**





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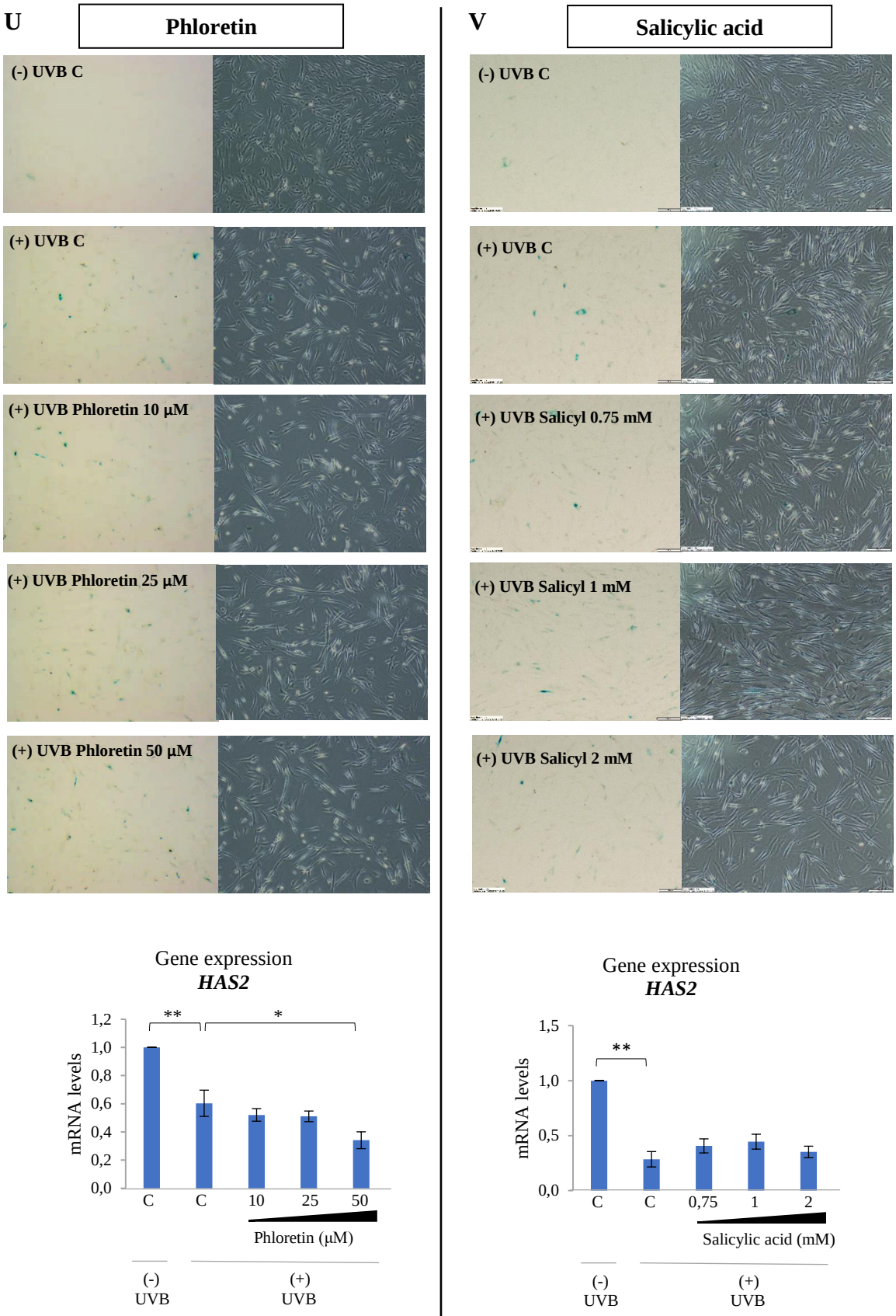

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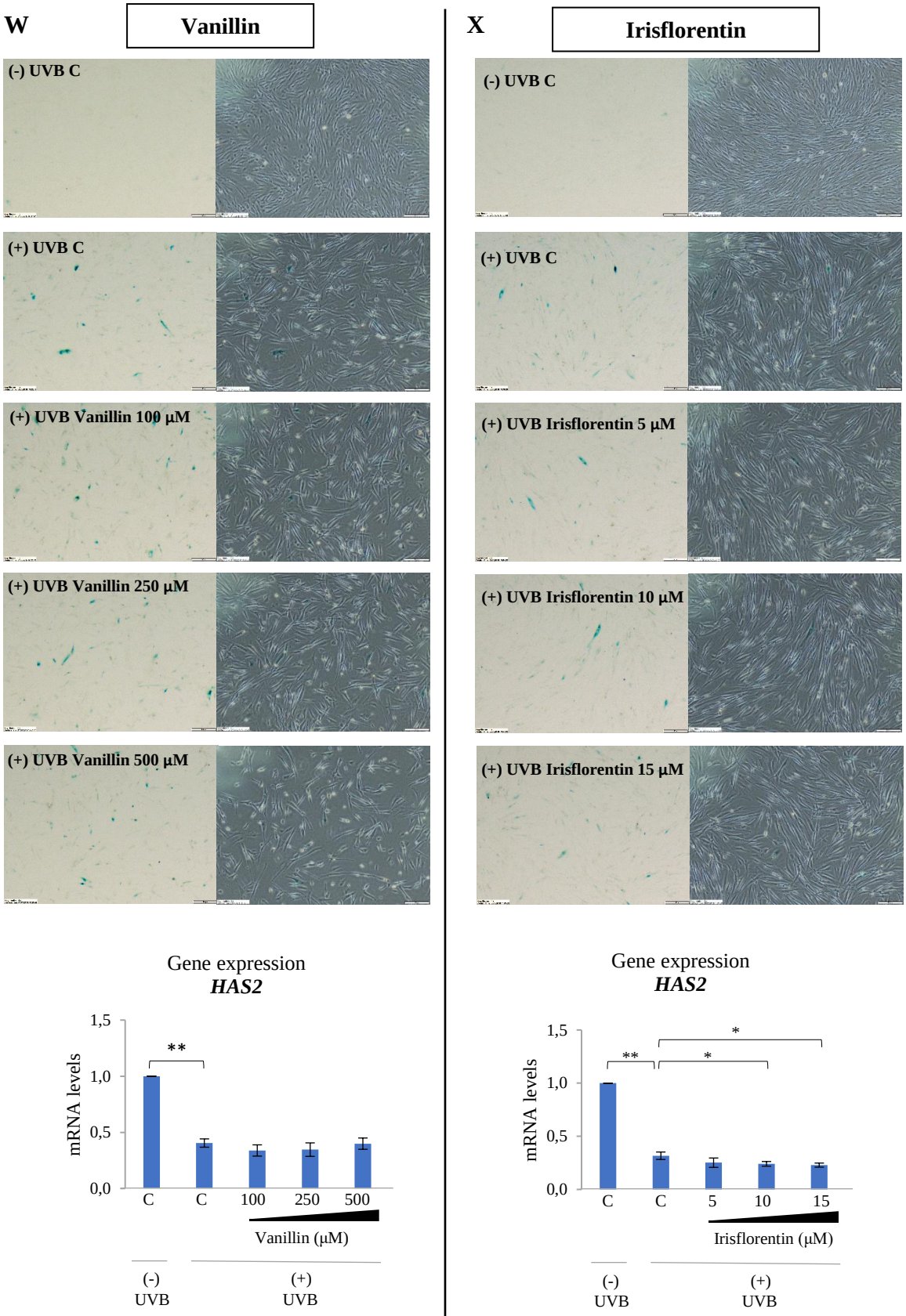
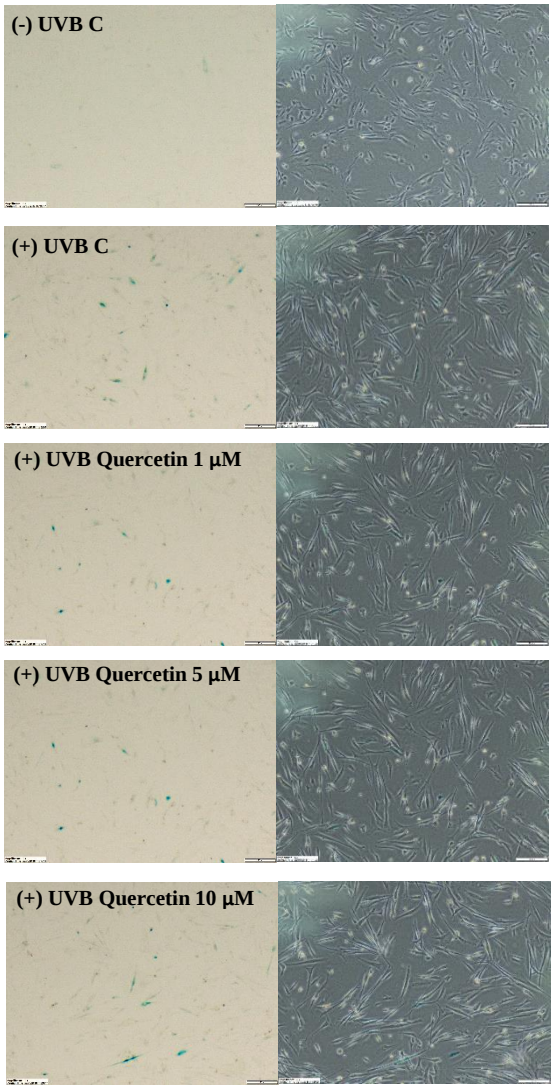
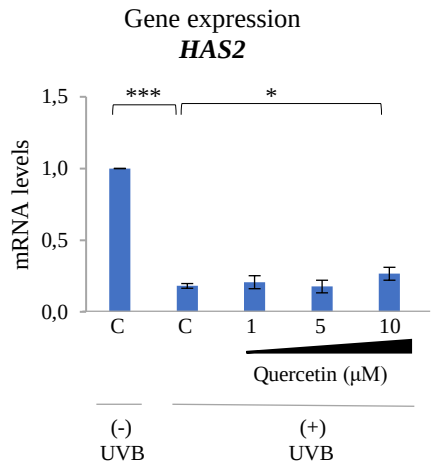


Annex 3. *Continued*

Y **Quercetin**



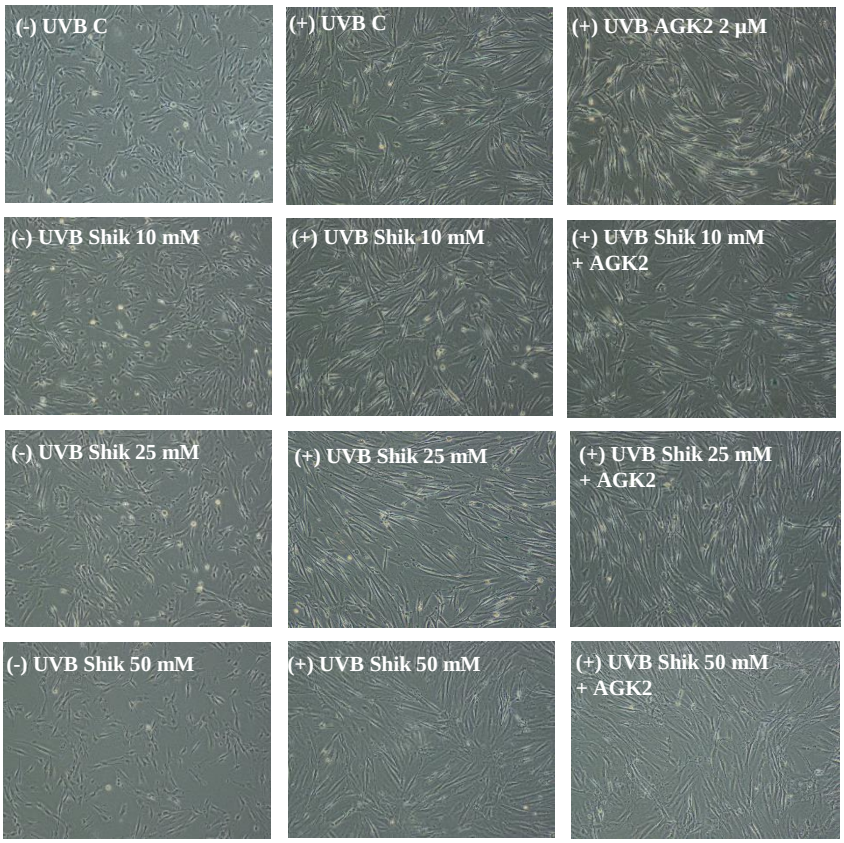


Annex 3. *Continued*

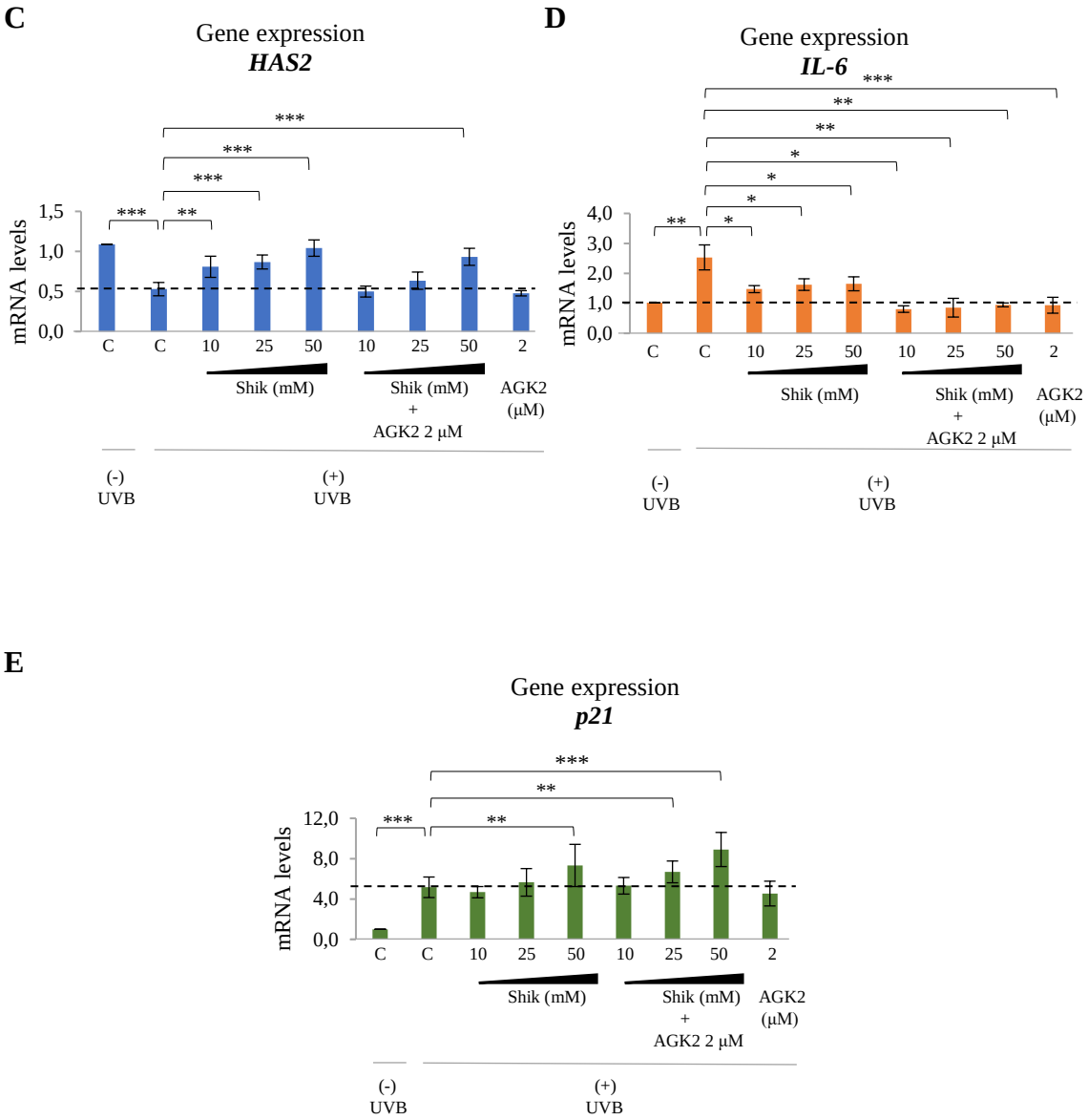
Annex 3. Bright field (up-left) and phase-contrast (up-right) microscope images of β-Gal staining showing non-irradiated cells, UVB-irradiated cells and UVB irradiated cells treated with (A) Resveratrol (5, 10 and 20 μM), (B) Dexpanthenol (5, 10 and 25 mM), (C) Andrographolide (1, 2.5 and 5 μM), (D) Sesamol (25, 50 and 100 μM), (E) Ectoine (10, 25 and 50 mM), (F) Allantoin (5, 10 and 25 mM), (G) Idebenone (2.5, 5 and 10 μM), (H) Taurine (10, 25 and 50 mM), (I) Carnosine (5, 10 and 25 mM), (J) Damascenone (0.1, 0.25 and 0.5 μM), (K) Pyridoxine (0.5, 0.75 and a mM), (L) Syringic acid (1, 2.5 and 5 mM), (M) Hammamelitannin (10, 25 and 50 μM), (N) Sclareol (5, 10 and 20 μM), (O) Lipoic acid (50, 100 and 200 μM), (P) Zeatin (50, 100 and 250 μM), (Q) Kinetin (25, 50 and 100 μM), (R) Betaine (10, 25 and 50 mM), (S) Gentiopicrin (250, 500 and 750 μM), (T) Salicylaldehyde thiosemicarbazone (1, 5 and 10 μM), (U) Phloretin (10, 25 and 50 μM), (V) Salicylic acid (0.5, 1 and 2 mM), (W) Vanillin (100, 250 and 500 μM), (X) Irisflorentin (5, 10 and 15 μM) and (Y) Quercetin (1, 5 and 10 μM). qPCR analysis (bottom) of *HAS2* gene expression in non-irradiated cells, UVB irradiated cells and UVB-irradiated cells treated with the previous mentioned compounds. Statistics: Student's T-test. All the samples were compared to (+) UVB control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

A

B

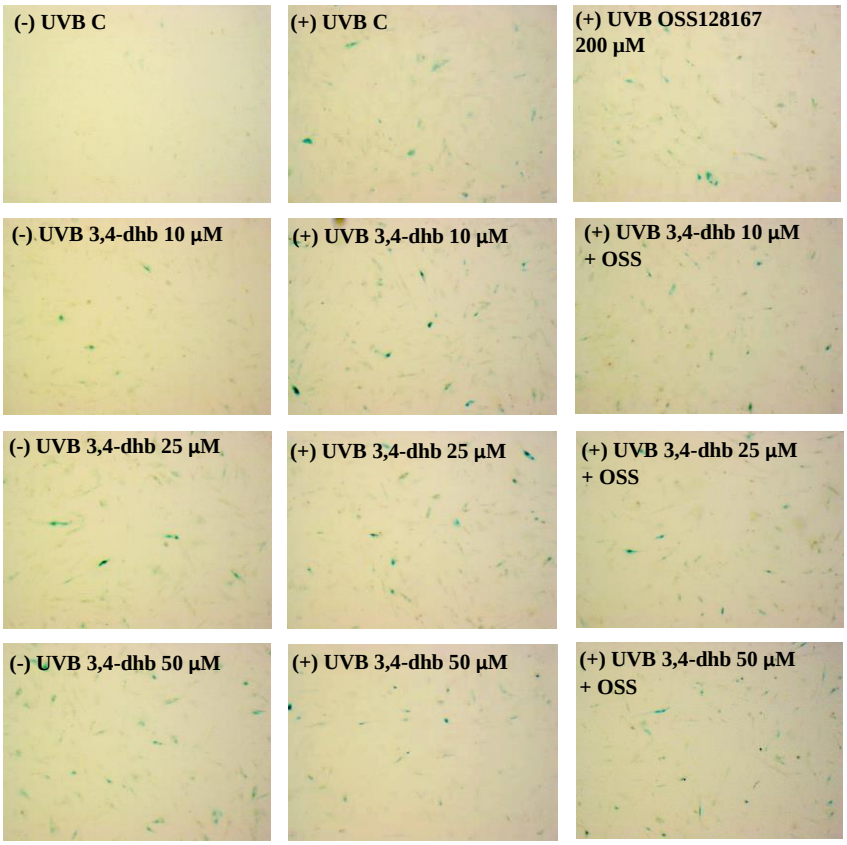


Annex 4. *Continued*

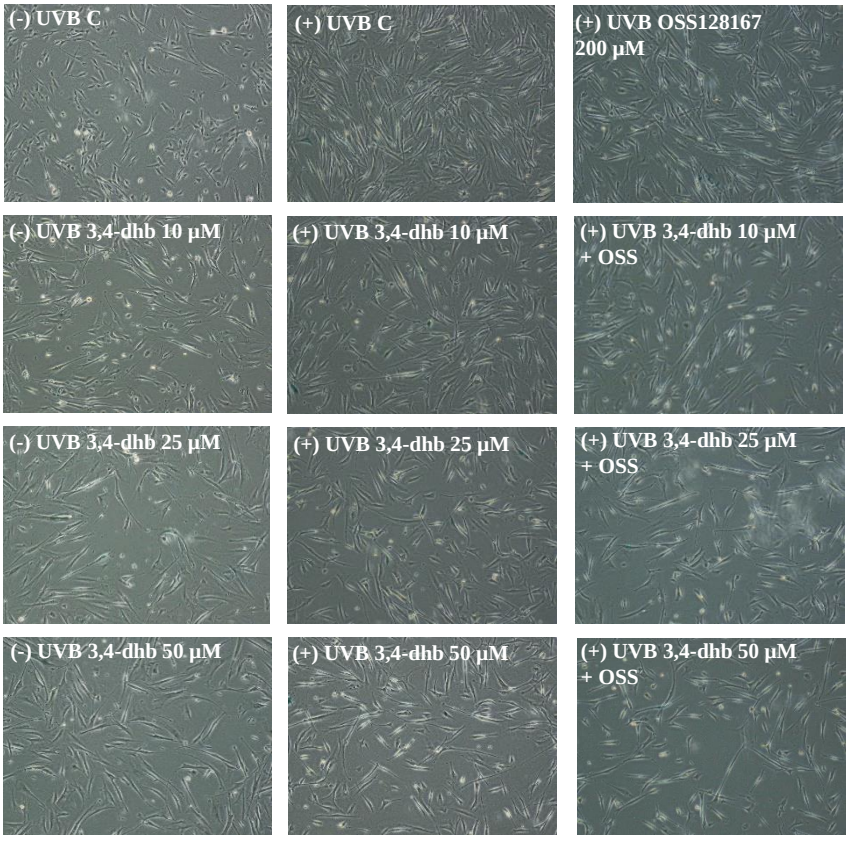


Annex 4. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with Shikimic acid (left column), irradiated cells treated or non-treated with Shikimic acid (center column) and irradiated cells treated or non-treated with Shikimic acid plus AGK2 2 µM (right column). (C) *HAS2*, (D) *IL-6* and (E) *p21* gene expression levels of UVB irradiated cells treated or non-treated with Shikimic acid plus AGK2 2 µM. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with Shikimic acid +/- AGK2 and UVB-irradiated cells treated with AGK2 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

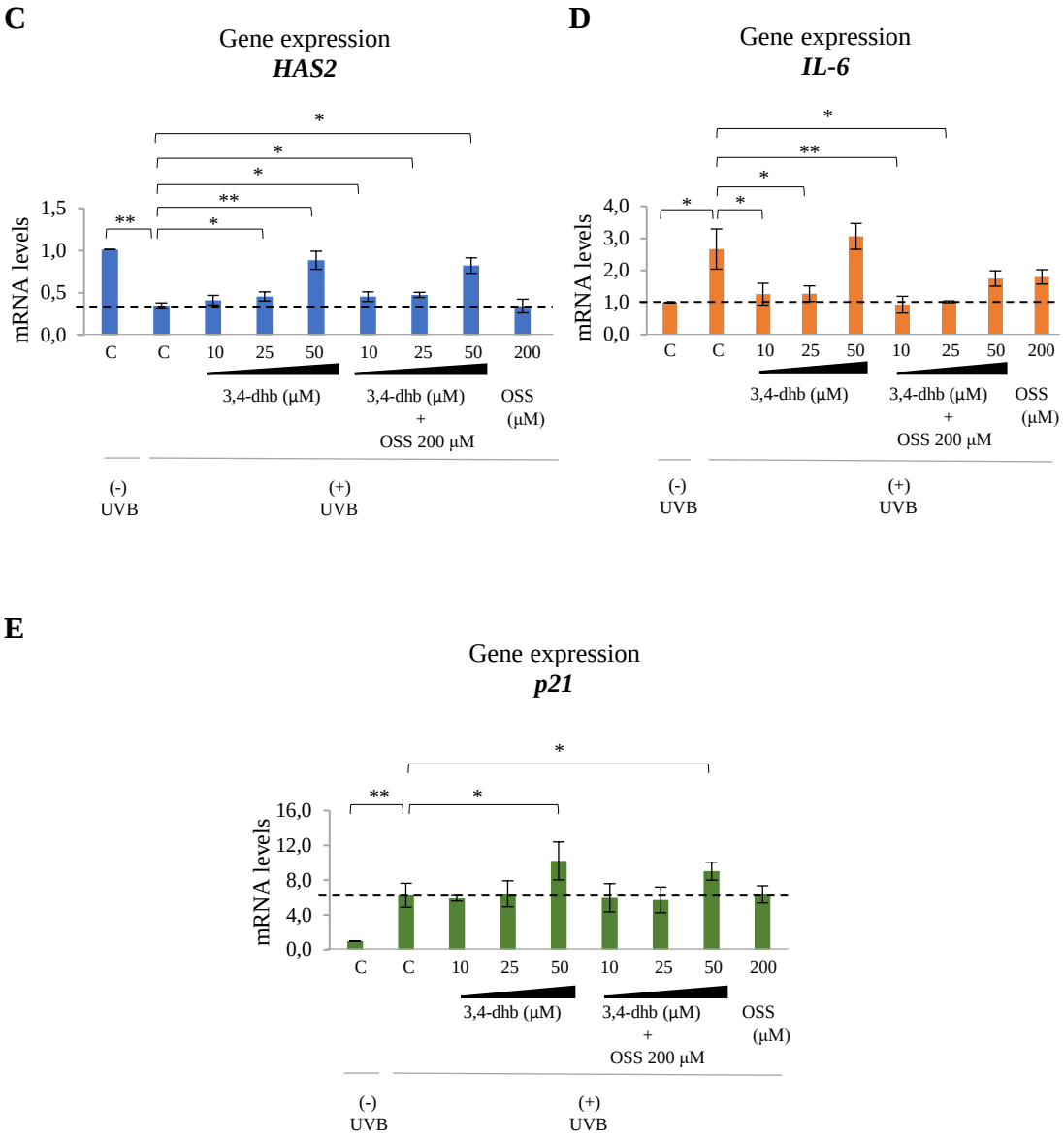
A



B

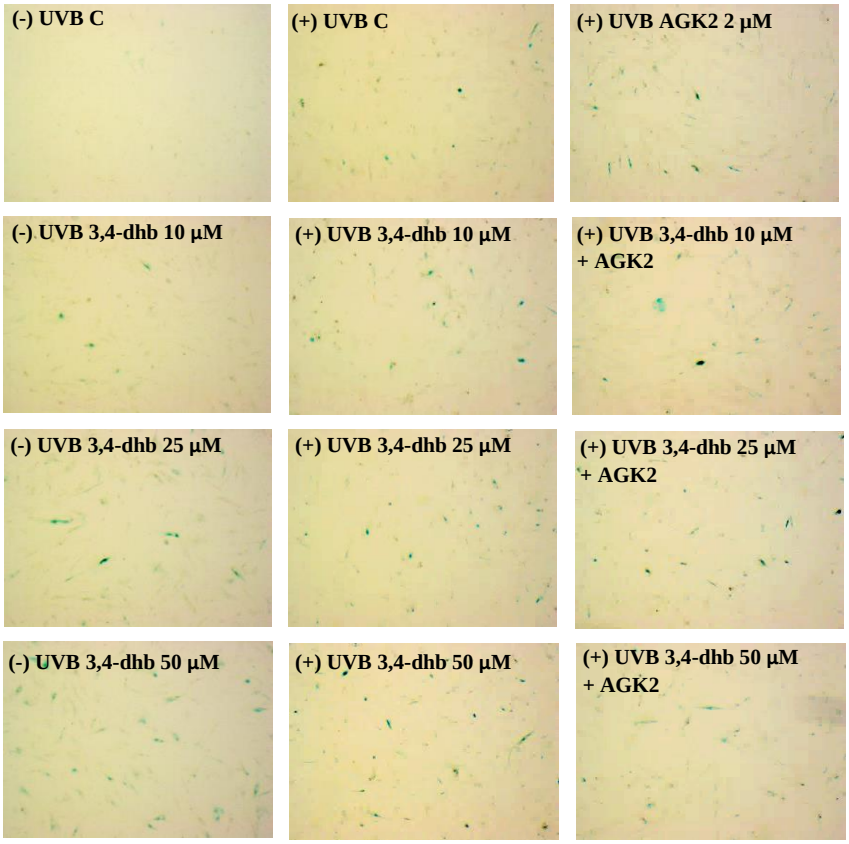


Annex 5. *Continued*

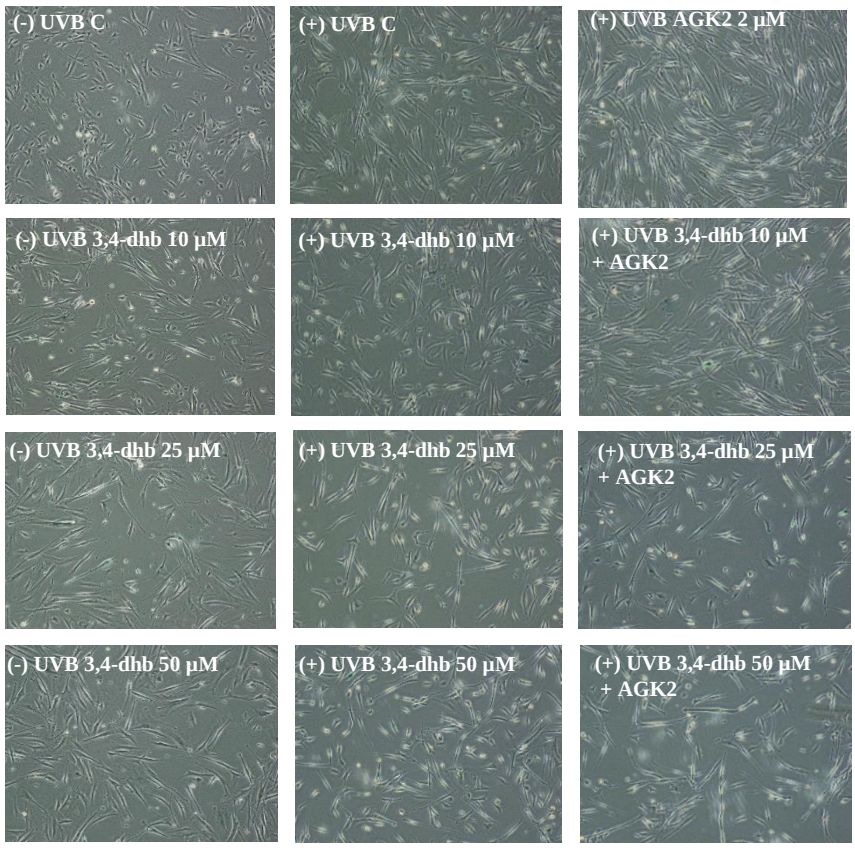


Annex 5. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (left column), irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (center column) and irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus OSS128167 200 µM (right column). (C) *HAS2*, (D) *IL-6* and (E) *p21* gene expression levels of UVB irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus OSS128167 200 µM. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde +/- OSS128167 and UVB-irradiated cells treated with OSS128167 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

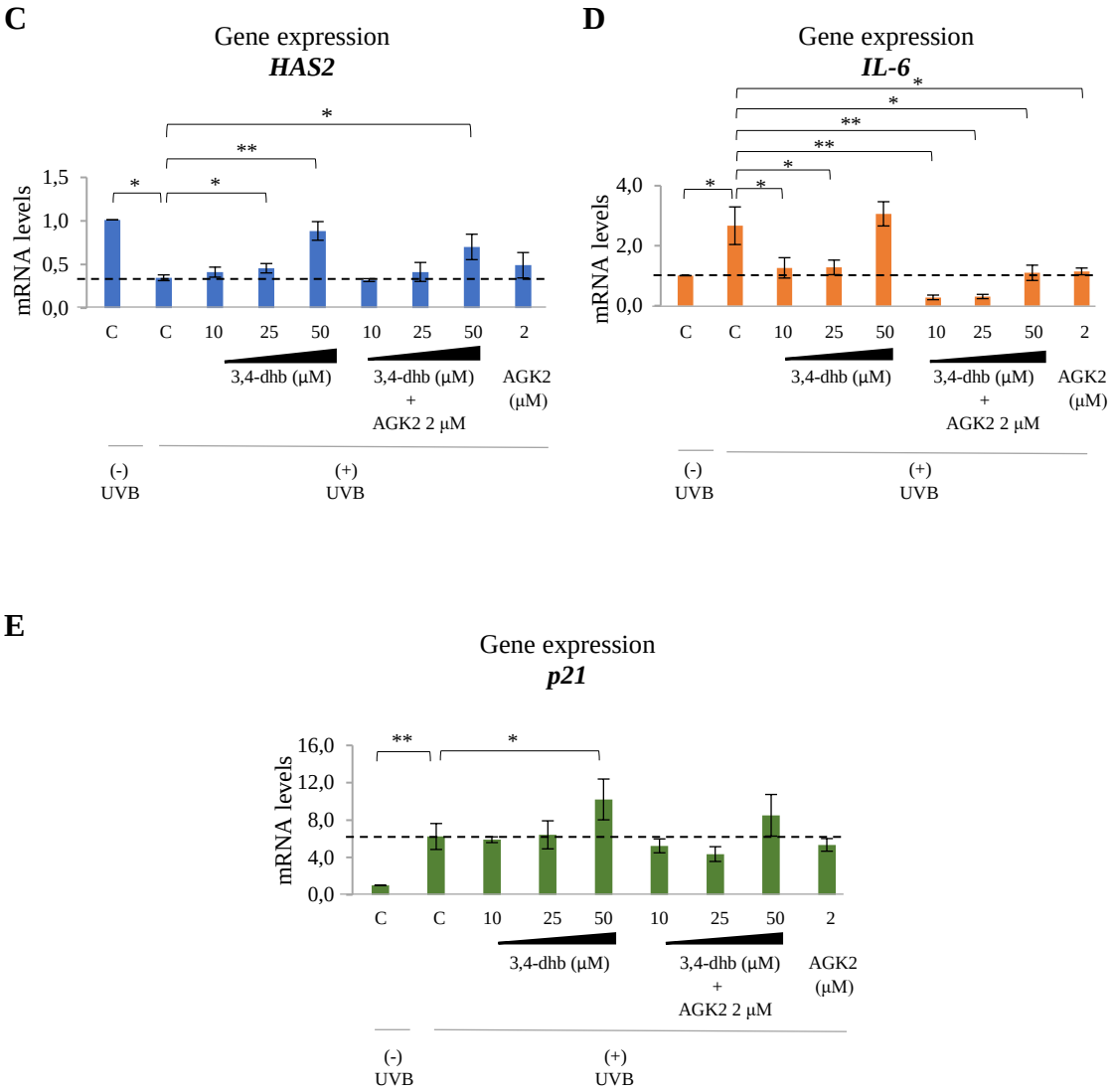
A



B



Annex 6. *Continued*



Annex 6. (A) Bright field and (B) phase-contrast microscope images of β-Gal staining showing non-irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (left column), irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde (center column) and irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus AGK2 2 μM (right column). (C) *HAS2*, (D) *IL-6* and (E) *p21* gene expression levels of UVB irradiated cells treated or non-treated with 3,4-dihydroxybenzaldehyde plus AGK2 2 μM. The results of UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde plus OSS128167 or AGK2 are shown in Annex 5 and 6, respectively. Statistics: Student's T-test. Non-irradiated cells, UVB-irradiated cells treated with 3,4-dihydroxybenzaldehyde +/- EX-527 and UVB-irradiated cells treated with EX-527 were compared to UVB-irradiated control cells (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

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