CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

UNIVERSITÄT DES SAARLANDES

Faculty of Natural Sciences and Technology

Department of Pharmacy, Pharmaceutical Biology

DIPLOMA THESIS

Hradec Králové 2019 Tereza Andrašková

CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

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CELL SENESCENCE IN AGING, CELL STRESS, AND CHEMORESISTANCE

Diploma thesis

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Hradec Králové 2019 Tereza Andrašková

I hereby declare this diploma thesis to be my original work. All the sources and literature are listed and properly cited in the bibliography section. This work has not been used for obtaining an equivalent or any other academic degree.

In Hradec Králové, ………..……………………

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ABSTRAKT

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Názov diplomovej práce: Bunková senescencia v starnutí, bunkový stres a chemorezistencia

Proces biologického starnutia je spojený so stratou funkcie tkanív, ktorá vedie k mnohým patologickým stavom, vrátane kardiovaskulárnych chorôb a rakoviny. Bunková senescencia, jav charakterizovaný trvalým zastavením bunkového cyklu vyvolaným endogénnym alebo exogénnym stresom, hrá významnú úlohu vo vývoji metabolických ochorení súvisiacich s rastúcim vekom. Senescentné endotelové bunky boli objavené v aterosklerotickom pláte a kvôli faktorom vyvolávajúcim zápal sa podieľajú na jeho vývoji. Napriek tomu, spustením antiproliferatívnej reakcie, môže mať senescencia tiež potenciál ako prostriedok na potlačenie rakoviny. Cieľom tejto práce bolo vyvinutie metódy stanovujúcej bunkovú senescenciu v rozličných bunkových typoch a následne analyzovať zlúčeniny s potenciálom inhibovať starnutie vyvolané H_2O_2, ako aj faktory, ktoré môžu byť vďaka tomuto javu pozmenené. Test aktivity β-galaktozidázy spojenej so senescenciou (SA-β-gal) v primárnych ľudských endotelových bunkách odhalil schopnosť statínov a kurkumínu inhibovať bunkové starnutie na rozdiel od xanthohumolu a metabolitov kurkumínu - kyseliny ferulovej a feruoylacetónu. SA-β-gal esej tiež ukázala zvýšený počet pozitívne sfarbených pečeňových rakovinových buniek, rezistentných voči doxorubícínu (DOXO).

Naše výsledky prezentujú sľubnú úlohu statínov a kurkumínu v inhibícii bunkovej senescencie a senescenciu indukovanú terapiou ako zaujímavý cieľ pre ďalší výskum liečby rakoviny.

ABSTRACT

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Faculty of Pharmacy in Hradec Králové

Department of Pharmacology & Toxicology

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Title of diploma thesis: Cell senescence in aging, cell stress, and chemoresistance

The process of biological aging is connected with a loss of tissue function leading to the various pathologies, including cardiovascular diseases and cancer. Cellular senescence, the phenomenon characterized by permanent cell cycle arrest triggered by endogenous or exogenous stress plays a significant role in age-related metabolic diseases development. Senescent endothelial cells have been found in atherosclerotic plaque and due to the inflammation-inducing factors participate to its progression. Nevertheless, by triggering antiproliferative reaction, senescence may also have a potential as a cancer suppressor. The aim of this work was to establish assay determining senescence with different origins and subsequently, to analyse compounds with a potential to reduce H_2O_2-induced senescence as well as factors, which may be altered in the senescent phenotype. Senescence-associated β-galactosidase (SA-β-gal) assay in primary human endothelial cells revealed the senescence-inhibiting ability of statins and curcumin in contrast to xanthohumol and curcumin metabolites - ferulic acid and feruoylacetone. SA-β-gal assay also displayed elevated positive-stained cells in doxorubicin-resistant liver cancer cells.

Taken together, our results present a promising role of statins and curcumin in cellular senescence inhibition and therapy-induced senescence as an interesting target for further cancer therapy research.

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1 LIST OF ABBREVATIONS

ATOR	atorvastatin
CDK	cyclin-dependent kinase
CDKi	cyclin-dependent kinase inhibitor
CO_2	carbon dioxide
CUR	curcumin
DDR	DNA-damage response
DMSO	dimethyl sulfoxide
DOXO	doxorubicin
cDNA	complementary DNA
DSB	double-strand break
ECG	endothelial cell growth medium
eNOS	endothelial nitric oxide synthase
FEA	ferulic acid
FLA	feruoylaceton
FCS	fetal calf serum
GILZ	glucocorticoid-induced leucine zipper
GILZ KOY	GILZ knock-out young mice
GILZ KOM	GILZ knock-out middle-aged mice
HUVECs	human umbilical vein endothelial cells
NF-κB	nuclear factor κB
PBS	phosphate buffered saline
PBS+	phosphate buffered saline +
PCR	polymerase chain reaction
PRAVA	pravastatin
RNAse	ribonuclease
ROS	reactive oxygen species
SA-β-gal	senescence-associated β-galactosidase
SAHF	senescence associated heterochromatic foci
SASP	senescence-associated secretory phenotype
SIRT1	sirtuin 1
XAN	xanthohumol
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

WTY	wild type young
WTM	wild type middle-aged
WTO	wild type old

2 INTRODUCTION

Virtually every developed western country is facing the population aging due to the low childbirth rate and delayed mortality. According to the latest statistics, by the year 2050, the elderly population over 65 in Europe, where the high age remains the primary risk factor for chronic diseases, cancer, and metabolic disorders, could raise to 25% (United Nations, Department of Economic and Social Affairs, 2019).

Cellular senescence, characterized by irreversible cell cycle arrest, is one of the root causes of aging and age-related diseases. Pro-inflammatory factors secreted by senescent cells are involved in atherosclerotic plaque growth and instability (Childs et al., 2016), and cause chronic inflammation resulting in the tissue impairment (Franceschi et al., 2014), what provides a supportive niche for cancer (Coppé et al., 2010). In order to promote healthy aging, the selective elimination of senescent cells, senolysis, has become a new target in the therapy of age-related diseases (van Deursen, 2019) and results from the first-in-human senolytic drug treatment have already been published (Justice et al., 2019).

However, regarding cancer, chemotherapy-induced senescence appears to act antagonistically, like a tumour suppressor, which provides a new direction in cancer treatment (Childs et al., 2015). Senescence is evolutionary younger than apoptosis and may bring advantages over the therapy-induced cell death, since in senescence remain these cells functional but not able to divide (He et al., 2017).

Taken together, cellular senescence plays an important role in age-related pathologies and cancer, and is undoubtedly an interesting target for the future research.

3 THEORETICAL PART

3.1 Cellular senescence

Cellular senescence is described as a condition, in which cells lose their ability to proliferate and remain in a persistent cell cycle arrest. Nevertheless, senescent cells might show metabolic activity and resist apoptotic death for a long time (Bodnar et al., 1998).

Almost 60 years ago, L. Hayflick and P. Moorhead described the term “cellular senescence” for the first time. Their study on human diploid cells discovered limited ability of fibroblast proliferation after a specific number of divisions (Hayflick et al., 1961). Nowadays, it is known that this kind of senescence is just one specific type named “replicative senescence”. Replicative senescence is caused by telomere shortening during normal aging processes when the cells lose their mitotic activity. The other type of senescence called “accelerated” or “premature senescence” is triggered by DNA-damaging stimuli and the telomere loss is not necessarily observed (Mathon et al., 2001).

3.1.1 Mechanisms of senescence

The variety of internal and external stressors, resulting in cell defect, is vast. If the damage has irreversible character, normally dividing cells undergo either programmed death (apoptosis) or cycle arrest (cellular senescence), to avoid reproduction of defective DNA (Campisi et al., 2007) (*Fig. 1*). It is still unclear, what factors decide about the cell fate (Panneer Selvam et al., 2018), but the cell type and stress level are likely to be the determiners. Furthermore, initial studies have also shown a possible role in Caspase-2 suppression or manipulation of anti-apoptotic Bcl-2 protein expression (Rebbaa et al., 2003; Nelyudova et al., 2007).

<u>Regulation of senescence pathways</u>

Depending on the cell type and incoming signal, two tumour suppressing

pathways - p53 or p16-retinoblastoma protein (pRB), play a key role in growth regulation (Evan et al., 2004). After stimulation by an external or internal factor, the p53 pathway leads to an antiproliferative cascade (Ko et al., 1996). The following reaction causes the transcription of the cyclin-dependent kinase inhibitor (CDKi) $p21^{CIP1}$, which leads to the cyclin-dependent kinase 2 (CDK2) activity blockade. Consequently, the pRB remains in its active, hypophosphorylated form and the cell is unable to proceed in the cell cycle (D'Adda Di Fagagna, 2008). The activation of the p16-pRB pathway results in the same effect. p16, as a CDKi, suppresses the activity of E2Fs, a family of transcription factors essential for further DNA replication (Sherr et al., 2002).

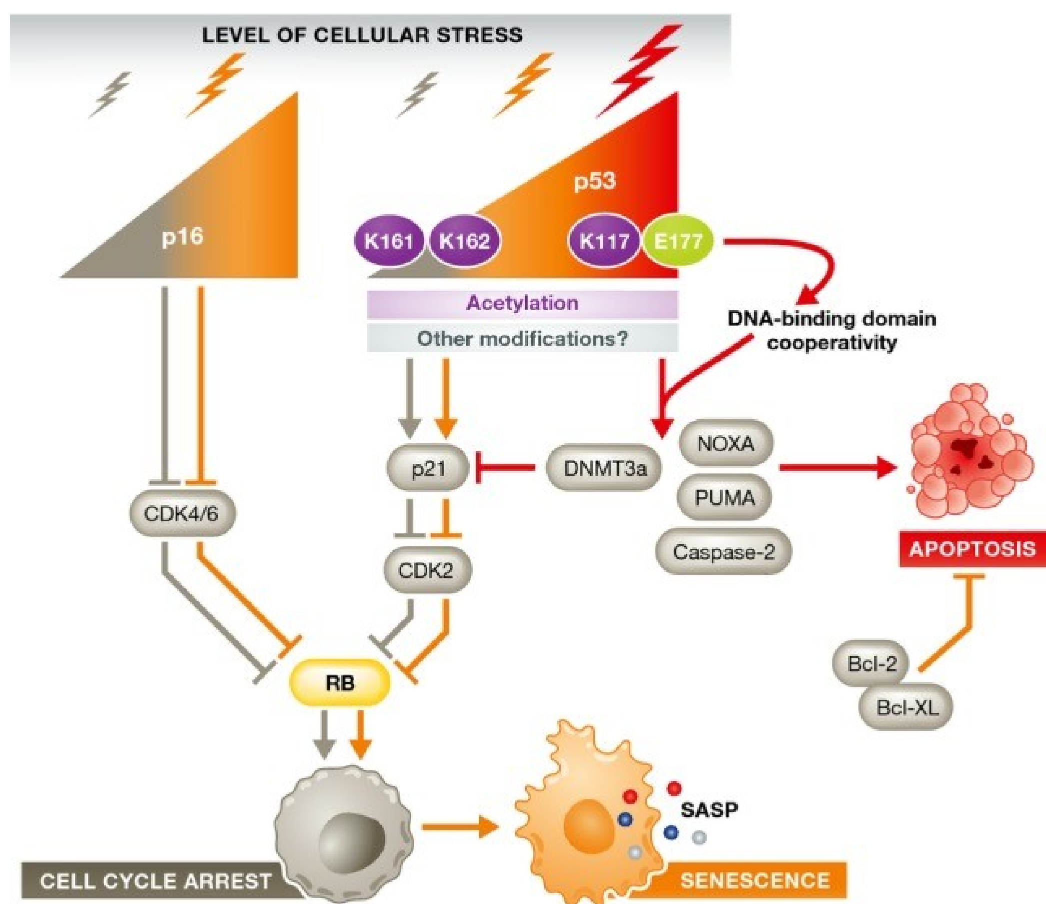


Fig. 1. *Regulation pathways differentiating two possible antiproliferating reactions – cellular senescence or apoptosis, adopted from (Childs et al., 2014). Pathways resulting in cell cycle arrest (grey), in senescence (orange). The decision to activate p16, p53, or both pathways is based on the cell type and stress level. Lower p16 levels support a temporary arrest, whilst higher levels tend to cause senescence. Low levels of p53, with temporary kinetics and K161/K162 acetylation, lead to cell cycle arrest and senescence. High levels of p53, K117*

acetylation, and cooperativity of domains binding DNA within the p53 tetramer lead to the transcription of apoptotic genes and inducing apoptosis, both directly and by blocking pro‐senescence signals. CDK (cyclin-dependent kinase), RB (retinoblastoma), BCL-2 (B-cell lymphoma 2, an important anti-apoptotic protein), BCL-XL (B-cell lymphoma extra-large), DNMT3a (DNA methyltransferase 3a), NOXA (phorbol-12-myristate-13-acetate-induced protein 1), PUMA (p53 upregulated modulator of apoptosis).

Telomere attrition and DNA-damage-initiated senescence

Under normal conditions, the ends of chromosomes in cellular DNA are terminated with telomeres. Besides protecting the chromosome unity (Blackburn, 2001), it has been acknowledged that telomeres are an integral part of chromosomes due to malignancy protection. With the gradual cell replications, telomeres shorten with an exception of cancer of artificial immortalized cells, whose telomeres are considered to achieve a stable length. When the telomere gets too short or dysfunctional, a DNA-damage response (DDR) is evolved, usually as double-strand breaks (DSB), subsequently activating the p53 pathway and causing either apoptosis or cell-cycle inhibition (Fagagna et al., 2003).

Oncogene-induced senescence

Oncogenes are mutated genes characterized by their ability to alter the cell function. Standard living cells protect themselves from this influence by apoptosis or undergoing cell cycle arrest. It has been proven that OIS appears not only *in vitro* but also *in vivo*, where it can possibly restrain the tumour development (Collado et al., 2010).

Oxidative stress and other inducers of senescence *in vitro*

Transporting cells form a living organism into the artificial culture conditions is a stressful factor. They have to acclimatize to the new environment containing different growth factors, nutrients, oxygen level and a zero contact with other cell types. Any of mentioned factors or their combination can lead to a cell shock with a stress-induced senescence as a result (Sherr et al., 2000). Among other stress-factors like UV light exposure (Rodemann et al., 1989), *tert*-butylhydroperoxide (Toussaint et al., 1992) or hyperoxia (Honda et al., 2001)

is H_2O_2 the most common inductor (Frippiat et al., 2002).

Oxidative stress caused by H_2O_2 generates reactive oxygen species (ROS) (Colavitti et al., 2005). ROS, metabolic byproducts of mitochondria working also as intracellular signalling molecules (Finkel, 2003), trigger DDR and induce cellular senescence (Pole et al., 2016) (*Fig. 2*). In addition, mitochondrial and telomere dysfunction, hallmarks of aging, have also been reported as the result of ROS activity (Passos et al., 2007).

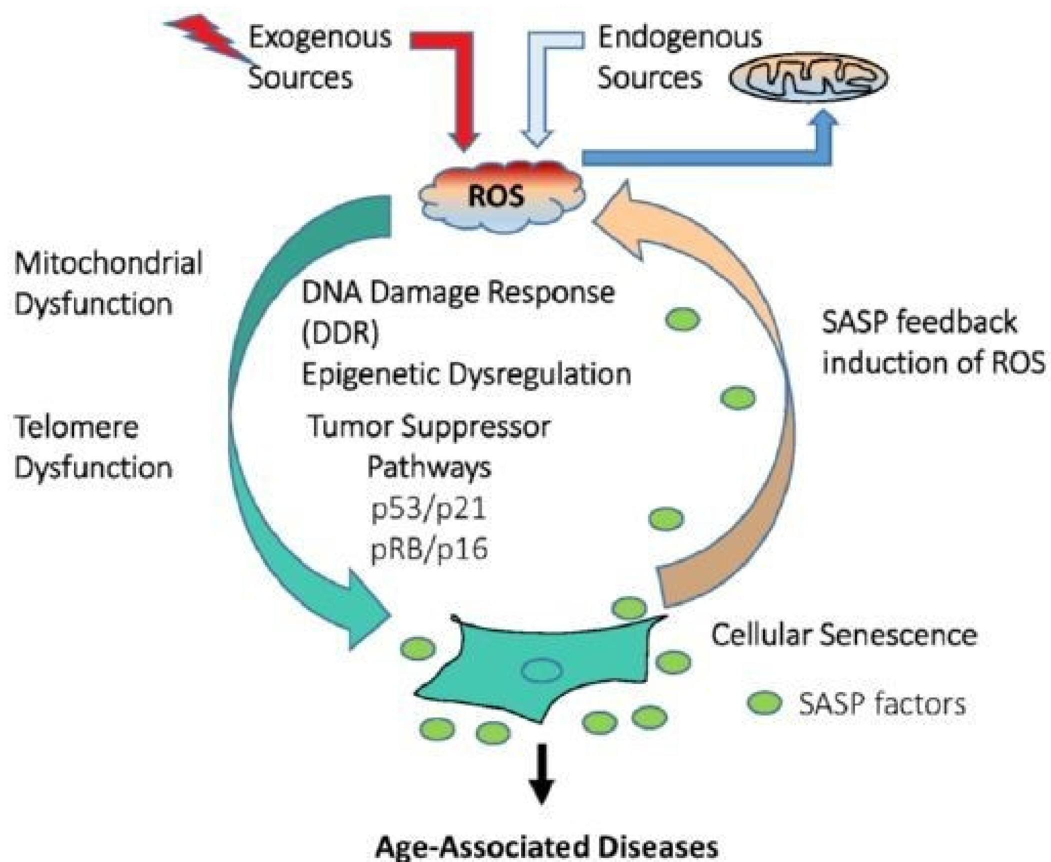


Fig. 2 *Mechanism of ROS – cellular senescence positive feedback loop, adopted from* (Pole et al., 2016). *ROS with exogenous (H_2O_2) or endogenous (mitochondria) origin trigger DDR and upregulates tumour suppressor pathways – particularly p53/p21 and pRb/p16 that lead to onset of cellular senescence. Once the cells become senescent, they start producing senescence-associated secretory phenotype (SASP) factors inducing formation of endogenous ROS, what promotes more cycles of senescence induction.*

Cellular senescence caused by oxidative stress, and consequently, SASP factors play a role in several age-related pathological processes, including neurodegenerative, biliary, cardiovascular diseases and cancer. Cardiovascular

risk factors, such as hypertension, obesity, diabetes, and atherosclerosis are connected with inflammation mediated by interleukins (IL-1α, IL-6, IL-8), and increased cellular senescence (Chandrasekaran et al., 2017). Taken together, the close relationship between aging, inflammation, and oxidative stress forms the oxidation-inflammatory theory of aging: the immune system reaction to oxidative stress is the state of inflammation, which leads to the endless loop with age-related pathologies as a result. However, the precise mechanism of oxidative stress-induced aging is still not fully explained and further investigations are necessary for better understanding (Liguori et al., 2018).

3.1.2 Biomarkers of senescence

Unlike the wide range of senescence-inducing pathways, there are a few specific features of senescent cells, which vary them from other viable cells, also those unable to divide, such as quiescent cells or terminally differentiated cells (Kuilman et al., 2010).

Irreversible cell cycle arrest

In comparison to quiescent cells, which undergo reversible cell cycle arrest but renew proliferation after restoring convenient conditions (Terzi et al., 2016), the cell cycle detention in senescent cells is persistent and they do not react to any growth factor or mitogenic impulse (Campisi et al., 2007).

Senescence-associated β-galactosidase activity

Senescence-associated β-galactosidase (SA-β-gal) has become the most common marker of senescence, because of its simple identification in almost every cell type. This enzyme activity can be detected at pH 6.0 in cultured senescent cells by using the artificial substrate X-gal and be evaluated visually (Dimri et al., 1995). SA-β-gal is derived from a lysosomal enzyme, β-D-galactosidase, which is also present in lysosomes of non-senescent cells. Because of the acidic nature of these organelles, the highest activity is observed at pH 4.0 – 4.5 (Lee et al., 2006) However, also some normal proliferating cells with a high content of lysosomes, such as macrophages, may express β-

galactosidase activity. Nevertheless, in most cell types SA-β-gal activity is a reliable senescence-identifying marker.

Morphological change

Senescent cells *in vitro* show a tendency to become flat, larger, and sporadically contain more than one nucleus. However, the morphology of *in vivo* senescent cells remains unchanged, corresponding to the tissue morphology and physiology (Collado et al., 2006).

Senescence associated heterochromatic foci (SAHF)

Narita et al. (2003) described a clear distinction between the chromatin of normal and senescent cells in their study. SAHF are particular regions of heterochromatin whose function is to eliminate proliferation-stimulating genes in senescent cells.

Senescence-associated secretory phenotype

Even though senescent cells lose their ability to divide, they release a wide range of pro-inflammatory factors and other components (enzymes, growth factors, chemokines, interleukins, and others), likely affecting neighbouring cells. SASP factors can trigger signal pathways through cell-surface receptors and result in an altered tissue microenvironment, increased cell proliferation and motility. Undergoing a short-time SASP factors influence induces a tissue reparation, a persistent secretion stimulates a chronic inflammation, possibly leading to aging or tumorigenesis (Coppé et al., 2010).

3.2 Aging

Aging is a time-depending attribute, occurring in every living organism. The main characteristic is a progressive degeneration or a decline in function, starting on the molecular level and possibly becoming a whole-organism feature. Yet, a wide range of pathways contributing to the aged phenotype, including insulin-like signalling pathway, sirtuins and NAD^+ decrease, DNA damage, telomere erosion, mitochondrial dysfunction, chronic inflammation, protein homeostasis, stem cell depletion, modified intercellular communication, loss of protein homeostasis,

epigenetic adjustment, and cellular senescence, have been described (López-Otín et al., (2013); Campisi et al., (2019)). Besides the fact that aging is a physiological process, it acts as a fundamental contributor to many age-related diseases.

3.2.1 Senescence in age-related disease

There are various aging-associated pathological conditions, in which senescence markers have been observed. Yet, it has not been clearly proven whether they trigger or contribute to the disease development. In some pathologies, such as osteoarthritis (Martin et al., 2004) or glaucoma (Liton et al., 2005), the inhibition of proliferation may play a role in disease development. In other cases, the SASP due to its inflammation-promoting factors may induce diabetes mellitus type 2 (Shimizu et al., 2012), atherosclerosis (Zhou et al., 2006), or cancer (Thangavel et al., 2011).

Atherosclerosis

Atherosclerosis and the following cardiovascular consequences are the main cause of death in developed western countries (Benjamin et al., 2019). Atherosclerosis is a degenerative inflammatory disease, characterized by the formation of plaques in the vascular wall that lead to its thickening and subsequently to a lower blood flow. Atherosclerotic plaques consist of lipids (especially low-density lipoproteins), vascular smooth muscle cells (VSMCs), macrophages and T lymphocytes (Ross, 1993).

Aging has been presented as one of the major risk factors for atherosclerosis (Lakatta et al., 2003) and senescent cells have been recognized in the vessel wall and also in the blood of atherosclerotic patients. Due to the continuous plaque growing, proliferation of smooth-muscle, and decreasing concentration and activity of endothelial nitric oxide synthase, cells experience oxidative stress and their telomeres get shortened (Kawashima et al., (2004), Salpea et al., (2010)). Senescent cells interact with the cells of the immune system, occurring in the plaque, through various signalling pathways, what increase the assumption of senescent cells multitasking in atherosclerosis. As

mentioned above, SASP produces pro-inflammatory factors, which may launch the early attack of circulating monocytes on the vessel wall (Zhou et al., 2006b). Especially interleukins and chemokine MCP1 show proatherosclerotic features and support the plaque growth (Rippe et al., 2012). Another possibility could be the role in reduction of the plaque stability and raising the chance of rupture, what may lead to thrombosis and fatal cardiovascular consequences. Unlike the healthy arteries, macrophages located in atherosclerotic plaques release specific enzymes – metalloproteinases (Finn et al., 2010). They also belong to the SASP products in vascular smooth muscle and cause the extracellular matrix degradation. A fibrous cap, consisting of smooth muscles, works as a protective layer of the stable atherosclerotic plaque. Gradual matrix degradation leads to the cap narrowing and transition to an unstable plaque formation (Coppé et al., 2010). All the scenarios mentioned above have not been scientifically fully confirmed and are based on senescence-associated features occurring in like increased levels of CDKi p16^{Ink4a} and p21, telomere attrition, and SA-β-gal–positive stained cells both – *in vitro* and *in vivo* (Charles et al., 2006).

3.2.2 Senescence in cancer therapy

A standard cancer therapy is based on cytotoxic effect of chemotherapeutic treatment. It has always been assumed that entire tumour cell elimination diminishes mortality. However, anticancer drugs can cause not only grievous side effects but, in some cases, tumour tissue may also become resistant to the treatment so that further tumour growth and metastasis formation can be induced. Another standby mechanism could possibly be cellular senescence induction (Lee et al., 2019). Whether the cancer cells undergo apoptosis or senescence after chemotherapeutic treatment depends partially on the stress intensity. Lower doses cause DNA-damage, that is not too serious to invoke an apoptotic answer (Chang et al., 1999). In comparison to the therapy-induced senescence, which takes a few days to be fully developed, apoptotic damage of the cells typically runs within 24 hours. The potential of cancer cells to choose the senescence pathway as an answer to stress, has been observed in both chemo- and radiotherapy (Roninson, (2003); Gewirtz et al., (2008)). The highest impact on senescence induction was detected in drugs interacting with

DNA such as aphidicolin, cisplatin, and especially DOXO, which induces senescence in 11 of 14 cell lines gained from diverse human tumour types (Chang et al., 1999). Enhancing senescence in cancer cells appears to be a promising approach in tumour treatment. Since the senescence-inducing doses are minimal, cytotoxicity and side effects of chemotherapeutics will be reduced. However, the senescent phenotype can also overproduce factors encouraging tumour cells to grow and other enzymes accompanied with different pathological features (Roninson, 2003).

Besides the beneficial antiproliferative effect on pre-malignant cells, senescence may also have a reverse side in cancer. Regulation pathways of senescence, $p16^{INK4a}$, $p21^{CIP1}$ and p53, also regulate the stem-cell functions named stemness. The study of Prasad et al., (2019) demonstrated that a small population of cancer stem cells shows the ability of self-renewal like normal stem cells and may give a rise to a tumour aggressiveness. The possibility of changing the non-stem malignant cells into cancer stem cells by chemotherapy-induced senescence was studied by Milanovic et al., (2017). Cellular senescence appeared to catalyse *de novo* reprogramming of non-stem leukaemia cells into leukaemia stem cells and therefore, the therapy strategies for targeted removal of senescent cells have previously become a needed approach.

3.2.3 Aging process and glucocorticoid-induced leucine zipper

Glucocorticoid-induced leucine zipper (*GILZ*) was recognized for the first time in 1997 (D'Adamio et al., 1997) and is defined as a protein with anti-inflammatory potential induced by glucocorticoids. *GILZ* binds to the nuclear factor NF-κB and represses pro-inflammatory signalling pathway in many types of cells (Emira Ayroldi et al., 2009). As the process of aging is connected with continuously decreasing function of the immune system, a state termed "inflamm-aging", characterized by a persistent, low-grade inflammation, can be driven (Franceschi et al., 2000). It has been proven that there is a direct proportion between the macrophage activation and "inflamm-aging", called "macroph-aging" (Prattichizzo et al., 2016). Assuming that a substantial role in activation of the macrophages is attributed to *GILZ* (Hoppstädter et al., (2012); Hoppstädter et al.,

(2015); Hoppstädter & Kiemer, (2015)), a recent study has confirmed that its loss accelerates aging and consequently results in building an inflammaged phenotype (Valbuena-Perez et al., n.d.).

3.3 Senescence-influencing compounds

Since the excessive accumulation of senescent cells is associated with age-related diseases development, many recent studies focus on their elimination and optimize the health condition of elderly people (Mária et al., 2017). Several natural substances and synthetic compounds with potential anti-senescence features have already been examined, though the research is still in its infancy and further studies are required for sufficient understanding of the anti-senescence effect *in vivo*.

The aim of supporting healthy aging can be reached by at least two molecular mechanisms: senescent cells removal by using senolytic compounds triggering apoptosis or SASP suppression. Since the SASP express pro-inflammatory and pro-oxidant factors, it is likely that molecules with antioxidant or anti-inflammatory properties, such as polyphenols and statins that are also the subject of this study, can behave as SASP-suppressive agents (Gurău et al., 2018).

Suggested mechanisms for the health profits of natural substances, including wide range of polyphenols, are following: direct antioxidant activity or elevated expression of antioxidant enzymes; blocking of pro-inflammatory cytokines and transcription factors associated with metabolic diseases; and formation of inflammation-antagonizing transcription factors. However, the exact molecular mechanisms through which food can influence the health status are still insufficiently known (vel Szic et al., 2015).

3.3.1 Curcumin

A natural polyphenol CUR (*Fig. 2*), acquired from the rhizome of *Curcuma longa* (turmeric), is not reviewed only as a food supplement but widely studied for its beneficial activity in human organism. CUR is one of the 12 active compounds of turmeric (Tyagi et al., 2015) and its proportion is not more than 3.14% in the

dry powder (Tayyem et al., 2006). CUR is well known for possessing anti-inflammatory and antioxidant properties (Hewlings et al., 2017), which explain the anti-aging character of CUR, since aging is associated with the persistent low-grade inflammation (Calder et al., 2017). Antioxidant features are manifested by increased sirtuin 1 (SIRT1) expression (Grabowska et al., 2017) and suppression of the pro-apoptotic factors - peroxisome proliferator-activated receptor-γ (PPAR-γ) and Bcl-2-associated X protein (Wenwen Liu et al., 2011; Yang et al., 2013).

CUR degradation process produces a number of metabolites, including ferulic acid (FEA) and feruloylacetone (FLA), vanilline, and vanillylidene acetone (Fig. 3), which have been attracting attention in the past years due to their potential benefitial health effects (Shen et al., 2012).

The major barrier impeding its administration is its very limited bioavailability. Due to its two aromatic rings connected by two unsaturated carbonyl groups is the molecule poorly soluble in water and quickly metabolized and excreted from the organism. Since combining with other phytochemicals, such as xanthohumol (XAN) (*Fig. 4*), possibly inhibiting its metabolism have not increased its bioavailability (Kocher et al., 2015), recent studies focus on the strategies raising the blood concentrations by forming CUR-loaded nanoparticles (Weidong Liu et al., 2016).

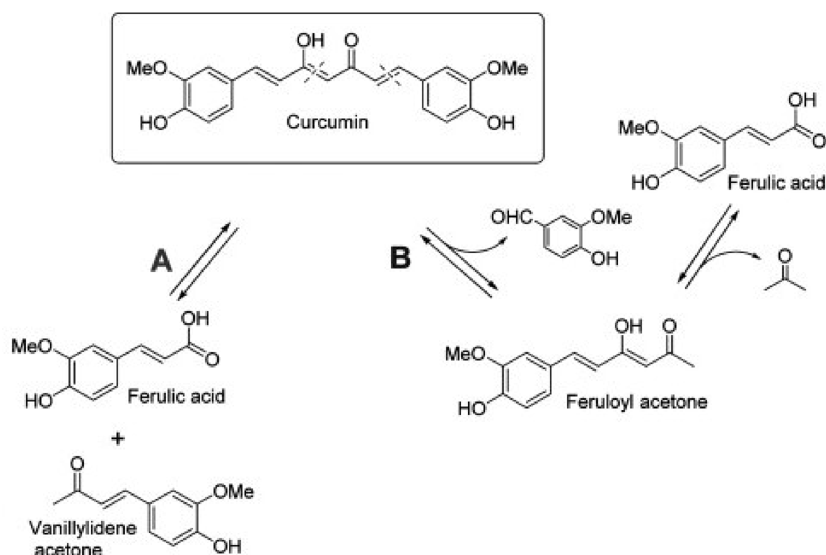


Fig. 3 *Degradation process of curcumin, adopted from (Dahmke et al., 2014). (A) Cleavage of the 1, 3-diketone bridge via retro aldole reaction providing ferulic acid and vanillylidene acetone. (B) Cleavage of the α,β-unsaturated bridge via retro aldole reaction providing feruloylacetone and vanillin.*

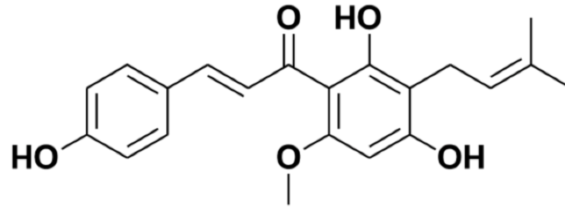


***Fig. 4** Chemical structure of xanthohumol* (M. Liu et al., 2015)

3.3.2 Statins

The HMG-CoA reductase inhibitors, statins, are the most commonly used drugs in hypercholesterolemia treatment due to their effectiveness in reducing the level of low-density lipoprotein cholesterol, which is strongly associated with atherosclerosis development (li et al., 2007). Besides their anti-inflammatory and cholesterol-lowering effects (Hosomi et al., 2015), statins are reported for amelioration or renewal of endothelial function, reduced oxidative stress and elevated activity of endothelial nitric oxide synthase (eNOS), a key enzyme responsible for the proper function of the vascular endothelium (Chatterjee et al., 2008; Blum, 2014). ROS cause the endothelial cells dysfunction and contribute to endothelial senescence, which is involved in atherogenesis (Tohru et al., 2002). Paradisi et al. (2012) have demonstrated senescence-inhibiting as well as proliferation-accelerating properties of statins in endothelial progenitor cells.

4 AIM OF WORK

Senescent cells are described to vary from normal cells by a few specific features, which are in the focus of studying for the past years. Not all the connections in the relationship between the specific senescent markers and aging process or aging-related diseases are fully understood.

The first aim was to establish the senescence-identifying method - SA-β-gal assay and telomere length PCR for senescence evaluation. Afterwards, a few aspects of accelerated senescence were elucidated:

- the impact of senescence on the *GILZ* mRNA expression
- the possible capability of CUR, XAN, FEA, FLA and statins to attenuate senescence

Further, we wanted to test the hypothesis that chemoresistant cancer cells aged-phenotype macrophages become senescent.

5 EXPERIMENTAL PART

5.1 Materials

5.1.1 Technical equipment

CO_2 incubator (Memmert, Beuingen)
Centrifuge Heraeus Megafuge 8R (Thermo Scientific)
Microscope (Axiovert 40 CFL, Zeiss)
NanoDrop Lite Spectrophotometer (Thermo Scientific)
Multiwell reader (GloMax Discover)
Thermal cycler CFX96 Real-Time System (Bio-Rad, Munich, Germany)

5.1.2 Chemical reagents

If not stated otherwise all chemicals were purchased from Sigma-Aldrich (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

Endothelial cell growth medium (Promocell, Heidelberg, Germany)
Earle`s medium 199 (PAA, Cölbe, Germany)
RPMI-1640 medium (PAA, Cölbe, Germany)
Fetal calf serum gold (FCS) (PAA, Cölbe, Germany)
Trypsine EDTA (PAA, Cölbe, Germany)
Phosphate buffered saline (PBS)
Penicilline/streptomycine (PAA, Cölbe, Germany)
Dimethyl sulfoxide (DMSO)
Doxorubicin (Sigma-Aldrich, Taufkirchen, Germany),

DNA and RNA analysis:
EvaGreen® Mix (SolisBioDyne, Tartu, Estonia), RNAseOUT™ ribonuclease inhibitor (Invitrogen, Karlsruhe, Germany), primer sequences (Eurofins MWG Operon, Ebersfeld, Germany)

SA-β-gal assay:
citric acid, sodium phosphate, potassium ferricyanide, potassium ferrocyanide, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), X-gal, formaldehyde, N,N-dimethylformamide,

Other used chemicals:
30% hydrogen peroxide (H_2O_2), curcumin (high purity) (ENZO), xanthohumol, feruloic acid, feruloylacetone, atorvastatin, pravastatin

5.1.3 Assay kits

All kits were obtained from Applied Biosystems, Darmstadt, Germany.

GenElute™ Mammalian Genomic DNA Miniprep Kit
High Pure RNA isolation Kit
High Capacity cDNA Reverse Transcription Kit

5.2 Cell culture

5.2.1 Human umbilical vein endothelial cells (HUVECs)

Endothelial cells cover the inner surface of blood vessels and their degeneration appears to play the key role in vascular dysfunction resulting in different age-related diseases development (Regina et al., 2016). Since there is a number of evidences that senescent endothelial cells are present in pathologies associated with aging (Childs et al., (2016); (Franceschi et al., (2014)), they are an important element of further studying.

HUVECs are primary endothelial cells, which were isolated from the human umbilical cords. Immediately after birth, the umbilical cords were transferred into phosphate buffered saline+ (PBS+) (Na_2HPO_4 1.15 g/l, KH_2PO_4 0.20 g/l, $CaCl_2$*$2H_2O$ 0.10 g/l, NaCl 8.0 g/l, KCl 0.20 g/l, $MgCl_2$*$6H_2O$ 0.10 g/l) containing 1% [v/v] penicillin (10000 U/ml)/streptomycin (10 mg/ml) and stored at 4°C in Klinikum Saarbrücken (Germany). The HUVEC isolation was performed

up to 10 days after childbirth as previously described (Hahn et al., 2014).

5.2.2 Isolation of HUVECs

The isolation of HUVECs was carried out under sterile conditions by digestion of umbilical veins with 0.1 g/l collagenase A at 37°C according to Jaffe et al., (1973). The digestion reaction was stopped by rinsing the veins with Earle`s medium 199. After centrifugation (200 x *g*, 10 min) cells were resuspended in 5 ml endothelial cell growth (ECG) medium and cultivated in the incubator at 37°C and 5% $CO_2$ in a 25 $cm^2$ cell culture flask. Earle`s medium 199 and endothelial cell growth-medium always contained 10% [v/v] FCS Gold, 1% [v/v] penicillin (10,000 U/ml)/streptomycin (10 mg/ml), 0.1% [v/v] kanamycin (50 mg/ml). On the next day cells were washed three times with PBS (NaCl 7.20 g/l, KH_2PO_4 0.43 g/l, Na_2HPO_4 1.48 g/l) and cultivated under the same conditions in ECG medium until reaching the confluence (approximately 4 days). Isolation of HUVECs was performed by Theo Ranßweiler. The use of umbilical vein for the isolation of primary cells was approved by the local ethics committee (permission no. 130/08).

5.2.3 Cultivation of HUVECs

After reaching confluence, cells were passaged and transferred to a 75 cm^2 cell culture flask or seeded for experiments in a defined amount: cells were washed three times with PBS and 1 ml (25 cm^2 flask) or 3 ml (75 cm^2 flask) trypsin-EDTA-solution was added and incubated for 3 min at 37°C and 5% CO_2, trypsin was inactivated with 10 ml Earle`s medium 199. The suspension was centrifuged (10 min, 175 x *g*) and the pellet resuspended in ECG medium. Subsequently, cells were splitted 1:3 or 1:4, and one part seeded into new 75 cm^2 cell culture flasks, in 6-well plates, or in 12-well plates for experiments. A fraction of the cells were frozen in the liquid nitrogen for future use. Experiments were performed in passage two or three.

5.2.4 Thawing of HUVECs

After thawing the cryovials 2-3 min at 37°C, the suspension was quickly transferred into a 25 cm^2 cell culture flask with 5 ml ECG medium. To lower the cytotoxic effect of DMSO, after attachment of the cells, cells were washed with PBS and fresh medium was added.

5.2.5 Huh7

Huh7 cells were cultured in RPMI-1640 medium with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5% CO_2. Resistant cells were established by treatment with increasing concentrations of DOXO over several months according to Schultheiss et al. (2017). In order to maintain the resistance, cells were treated biweekly with 0.2 µg/ml DOXO for 24 h. Establishment was performed by Charlotte Dahlem. Chemoresistance was approved by MTT assay.

5.3 Mice

C57BL6J mice with a body weight of 24–26 g, used for all the experiments, were maintained under controlled temperature and humidity with a constant access to water and regular food supply. Animal operations were performed in accordance with the local animal welfare committee. Mice were bred and housed in open cages in the conventional animal husbandry of the Saarland University.

5.3.1 Murine liver tissue

Three differently aged groups of animals were used for the experiments. The wild-type young (WTY) mice were sacrificed at the age of 10 -12 weeks, wild-type middle-aged animals (WTM) at the age of 40-41 weeks, and the wild type old mice (WTO) were at least 85 weeks old. *GILZ* knock-out young (*GILZ* KOY) and *GILZ* knock-out middle-aged (*GILZ* KOM) mice were at the same age when sacrificed as the wild type mice.

After sacrifice, livers were removed and snap-frozen in liquid nitrogen and stored at -80°C for further experiments. Tissue isolation was performed by Dr. Jessica Hoppstädter.

5.3.2 Murine lung tissue

The lung tissue was extracted from WTO and WTY mice and used freshly, directly after sacrifice. Tissues were scissored with a scalpel and washed with medium to obtain alveolar macrophages. Tissue isolation was performed by Dr. Jessica Hoppstädter.

5.3.3 Alveolar macrophages

A fresh murine lung tissue was cut into tiny pieces in a 6 cm Petri dish and transferred into a test tube (50 ml). The Petri dish was rinsed with 5 ml balanced salt solution (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM HEPES, 5.5 mM glucose, pH 7.4) to collect all the residual particles. The whole volume of the test tube was pushed through the cell strainer to another test tube to obtain pure cells only. After centrifugation (10 min, 250 x *g*), the pellet contained too many red blood cells. Therefore, the washing buffer was collected and the red blood cell hypotonic lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM Na_2EDTA) was added and left in the incubator for 2 min. To stop the lysis, 25 ml of PBS were added, centrifuged again, and the supernatant was fully aspirated. The cell pellet was re-suspended in 200 µl medium (RPMI 1640, 5% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine).

5.4 H_2O_2 treatment

HUVECs were seeded at a density of 1 x 10^4 cells per well in 96-well plates and cultured until attachment in 200 µl of ECG medium. Afterwards, cells were treated with different concentrations of H_2O_2 (50, 100, 250, 400, 500, 750, and 1000 µM), as well as a control line where the H_2O_2-free medium was used. Cells

were incubated for 24, 48, 72 or 96 hours. Cytotoxicity and proliferation were detected by MTT-assay.

5.4.1 MTT-assay

The MTT assay is a colorimetric reduction assay determining the cell viability. Detection of living cells is based on their ability to convert the water-soluble, yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the water-insoluble, purple formazan. Dead cells do not have the ability to reduce MTT into formazan anymore, thus colour formation serves as a useful marker of only vital cells. MTT assay was provided in order to determine the cytotoxicity degree of different concentrations of H_2O_2, CUR, XAN, FLA, FEA, atorvastatin (ATOR) and pravastatin (PRAVA) in 96 well-plate.

MTT solution (10 ml):

MTT stock solution (5 mg/ml in PBS)	1 ml
medium (ECG medium)	9 ml

At desired time point (24, 48, 72 or 96 hours), the medium was carefully aspirated with a glass pipette, cells were washed with PBS and 150 µl of MTT solution were added into each well. Plates were placed in the incubator (37°C, 5% CO2) until the blue-violet formazan crystals were formed (2 hours). Medium was aspirated again with a glass pipette and 80 µl of DMSO were used to lyse the cells and dissolve the crystals. Absorbance was measured in multiwell reader (GloMax Discover Multiwall Reader, Promega) at 560 nm. Cytotoxicity and proliferation, respectively, was determined by comparison of the absorbance of the treated cells with the untreated control.

5.4.2 Cellular senescence induction

According to the results from the MTT assay, an H_2O_2 concentration, which was not toxic but stopped cell proliferation, was chosen.

HUVECs were seeded at a density of 5 x 10^4 cells per well in 12-well plates and cultured overnight in complete ECG medium in a humidified atmosphere with 5% CO_2 at 37°C. On the next day, cells were treated with H_2O_2 (100 µM, 250 µM) and cultivated in the incubator for 96 hours. The impact of H_2O_2 on cells was proven by SA-β-gal assay.

5.5 Treatment with statins, curcumin and its derivates

5.5.1 Cell proliferation assay

HUVECs were seeded in 96-well plates at a density of 1 - 2 x 10^4 cells per well in 200 µl ECG medium. After cultivation overnight, cells were treated with different final concentrations of ATOR and PRAVA (50, 100, 200 µM), CUR (10, 25 µM), XAN (2.5; 5 µM), FLA (10, 25 µM) and FEA (6.25; 12.5 µM) diluted in ECG medium. All the compounds were stored in stock solutions at −20°C in following concentrations: CUR (34 mM), FLA, FEA (25 mM each), XAN, ATOR, PRAVA (20 mM each) and dissolved in medium prior to cell treatment.

A DMSO control contained an equal amount of DMSO corresponding to the highest concentration of the treatment with compounds. The proliferation was checked by MTT assay.

5.5.2 Cellular senescence induction

Cellular senescence was induced with H_2O_2 treatment as described previously. HUVECs were seeded in 12-well plates at a density of 10^5 cells per well in 0.5 ml ECG medium and cultivated overnight. On the next day, cells were pre-treated with ATOR and PRAVA (50, 100, 200 nM), CUR (10, 25 µM), XAN (2.5; 5 µM), FLA (10, 25 µM) and FEA (6.25 µM; 12.5 µM) diluted in ECG medium. After 24 hours, cells were washed 2 times with PBS and treated with H_2O_2 (100 µM) for 1 h. Then, the compounds were added again to the treated cells and incubated for 96 hours. The effect on cellular senescence was observed with SA-

β-galactosidase assay.

5.6 Senescence associated β-galactosidase assay

SA-β-gal is a hydrolase enzyme considered to be one of the biomarkers of cellular senescence. In senescent cells, an active enzyme catalyses the hydrolysis of β-galactosides into monosaccharides. By using the artificial substrate 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), the enzyme activity can be easily detected at pH 6.0. β-galactosidase cleaves the glycoside bond in X-gal and forms galactose and 5-bromo-4-chloro-3-hydroxyindole which oxidises and dimerises to an insoluble blue compound 5,5'-dibromo-4,4'-dichloro-indigo, which can be identified and quantified under the microscope.

Reagent setup:

Fixation solution: 4% PBS-buffered formaldehyde

Table 1: Composition of the staining solution

Component	Stock solution	Amount for 10 ml	Final concentration
Citric acid/sodium phosphate buffer (pH 6.0)	5x	2 ml	1x
NaCl	5 M	0.33 ml	150 mM
MgCl2	1 M	20 µL	2 mM
Potassium ferricyanide	50 mM	1 ml	5 mM
Potassium ferrocyanide	50 mM	1 ml	5 mM
X-gal	20 mg/ml	0.5 ml	1 mg/ml
H_2O	-	5.2 ml	-

X-gal - 5-bromo-4-chloro-3-indolyl-β- d-galactopyranoside

5.6.1 Cell staining

Briefly, treated HUVECs (H_2O_2, statins, curcumin and its derivatives treatments) were washed twice with PBS, fixed with neutral buffered 4% formaldehyde for 3-5 minutes at room temperature and washed again twice with PBS. Afterwards, the freshly prepared staining solution was added (0.5 ml per well) and cells were incubated overnight. When the blue colour was fully developed (usually within 18 hours), cells were washed twice with PBS. Senescent cells were identified as blue-stained cells and pictures of 3 different randomly chosen fields under the microscope (magnification 100x) were taken to determine the proportion of SA-β-gal positive cells by using the cell counter Fiji software.

DOXO-resistant and sensitive Huh7 cells (Schultheiss et al., 2017) (10^5 cells/well, 200 µl RPMI medium) were seeded in 12 well-plate and on the next day, the SA-β-gal assay was performed as described above by HUVECs.

Freshly isolated alveolar macrophages were seeded into 96-well plates, young and old samples separately. After attachment of the cells (2 hours), galactosidase staining was performed as described above.

5.7 DNA analysis

5.7.1 DNA isolation

DNA was isolated from murine liver tissues of different ages and HUVECs treated with H_2O_2:

WTY	wild type young
WTM	wild type middle-aged
WTO	wild type old
KOY	knock-out *GILZ* (glucocorticoid-induced leucine zipper) young type
KOM	knock-out *GILZ* middle-age type

HUVECs:

- untreated cells
- 100 μM H_2O_2 after 24 and 96 hours
- 250 μM H_2O_2 after 24 and 96 hours

The DNA isolation was performed using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. In the final step, the amount of DNA was measured using Nanodrop (Peqlab, Germany) in every sample and stored in -80 °C.

5.7.2 Telomere PCR

DNA samples and PCR reagents were thawed on ice. All the DNA samples were diluted with H_2O to a final concentration of 7 ng/μl and measured in triplicates. 3 μl of DNA and 17 μl of Mastermix were added into each well after being vortexed and centrifuged properly. PCR plates were covered with a foil, vortexed, centrifuged, and inserted to the CFX Real-Time System (Bio-Rad, Munich, Germany). To determine the initial quantity of cDNA, the iCycler iQ5 software (Bio-Rad, Munich, Germany) was used. The telomere length was calculated using 36B4 gene as a reference housekeeping gene (T/R ratio). Primer sequences (Eurofins MWG Operon) as well as the housekeeping gene were chosen according to the study of Wang et al. (2013).

PCR protocol:

1:	denaturation	95°C	15:00 min	
2:	denaturation	95°C	00:15 min	80 cycles (steps 2–4)
3:	annealing	60°C	00:30 min	
4:	elongation	72°C	00:30 min	

Melting curve 60°C to 95°C: Increment 0.5°C every 0:05 min

Standard curve

A DNA dilution series was used as a standard curve to confirm the reaction efficiency. DNA dilution series was prepared from one of the DNA samples, composed of 7 dilutions ranging from 100% to 1.56% with a constant factor 1:2

Reaction mixture (one sample):

- Mastermix
    - EvaGreen® mix 10 µl
    - forward primer (10 µM) 0.5 µl
    - reverse primer (10 µM) 0.5 µl
    - H_2O 6 µl
- DNA template 3 µl

Table 2: Primer sequences used for telomere length PCR

mRNA	**primer forward, 5'→3'**	**primer reverse, 5'→3'**
36B4	ACTGGTCTAGGACCCGAGAAG	TCAATGGTGCCTCTGGAGATT
TELO	CGGTTTGTTTGGGTTTGGGTTT GGGTTTGGGTTTGGGTT	GGCTTGCCTTACCCTTACCCT TACCCTTACCCTTACCCT

5.8 RNA analysis

Used laboratory equipment was decontaminated under the UV lamp. Reaction tubes were treated with chloroform before use to avoid RNA degradation by RNAses.

5.8.1 RNA isolation

RNA was isolated from HUVECs treated with 100 µM and 250 µM H_2O_2 for 24 and 96 hours. The isolation was performed using the High Pure RNA isolation Kit (Roche, Germany) according to the manufacturer's protocol. The amount of RNA in every sample was measured with Nanodrop (Peqlab, Germany) and samples were stored in −80°C.

5.8.2 Reverse transcription

The RNA samples were diluted in RNase-free H_2O to a final volume of 14 µl containing 400 ng of RNA and transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) with RNAseOUT^{TM} ribonuclease inhibitor (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. A negative control without enzyme was included in every real-time RT-PCR run.

<u>Reaction mixture (one sample):</u>

- Mastermix
  - 10x RT buffer — 2 µl
  - 25x dNTPMix (100 mM, 25 mM each) — 0.8 µl
  - 10x RT Random Primer (10 µM) — 2 µl
  - Ribonuclease Inhibitor RNAseOUT^{TM} (40 U/µl) — 0.25 µl
  - Reverse transcriptase (4 U/µl) — 1 µl
- RNA sample — 14 µl

5.8.3 Real-time RT-PCR

GILZ mRNA levels in HUVECs were analysed by real-time RT-PCR. 3 µl of diluted cDNA samples (1:5) and 17 µl of Mastermix along with standard dilution series and negative control were transported in triplicates into a 96-well reaction plate, vortexed properly and run in CFX96 cycler (Bio-Rad, Munich, Germany). Absolute mRNA amounts were normalized to the housekeeping gene β-actin

(Eurofins MWG Operon, Ebersfeld, Germany). The initial quantity of cDNA was calculated using the iCycler iQ5 software (Bio-Rad, Munich, Germany). B-actin belongs to the standard housekeeping genes used at the department of Pharmaceutical Biology, Universität des Saarlandes. Because of the time reasons, only one housekeeping gene was used for RT-PCR. If the experiment was repeated, one should test at least one additional housekeeping gene.

Table 3: Primer sequences used for real-time RT PCR

mRNA	primer forward, 5'→3'	primer reverse, 5'→3'
GILZ	TCCTGTCTGAGCCCTGAAGAG	AGCCACTTACACCGCAGAAC
β-actin	TGCGTGACATTAAGGAGAAG	GTCAGGCAGCTCGTAGCTCT

Reaction mixture (one sample):

- Mastermix
    - primer forward (100 μM) 0.05 μl
    - primer reverse (100 μM) 0.05 μl
    - EvaGreen® mix 4 μl
    - H_2O ad 17 μl
- cDNA sample 3 μl

Real-time RT-PCR protocol:

- denaturation 95°C 15:00 min
    - denaturation 94°C 00:20 min
    - annealing 60°C 00:20 min
    - elongation 72°C 00:20 min

    } 40 cycles
- final elongation 95°C 00:10 min

Standard line

In order to quantify target mRNAs in a cDNA sample and verify the RT-PCR efficiency, standard dilution series (starting point: 200 attomol with a dilution factor 1:10) were run concurrently with the samples to form the standard curve. The standards were isolated from plasmids.

5.9 Statistical analysis

All data are presented as mean values ± SD. If not stated otherwise, statistical analysis was provided using two-sample student’s t-test in Microsoft Excel. Data were considered statistically significant when p values were smaller than 0.05.

6 RESULTS

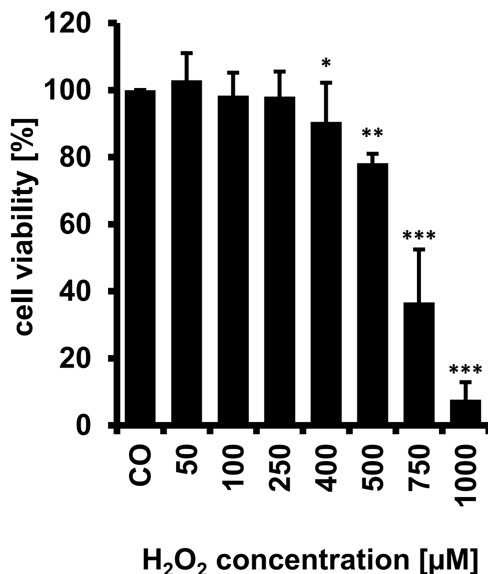
6.1 Establishment of senescence assays

6.1.1 Senescence associated β-galactosidase assay

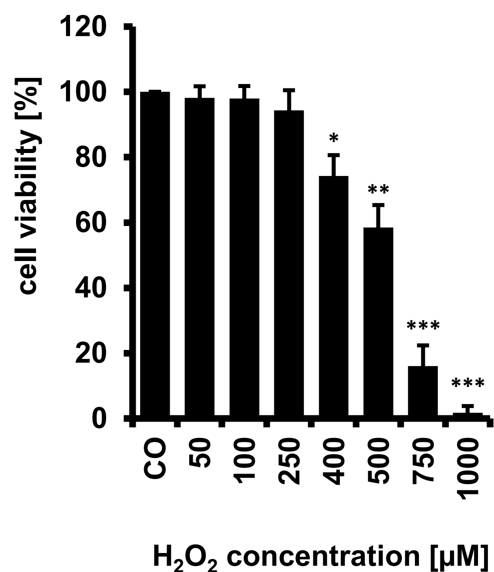
HUVECs

Initial treatment with H_2O_2 was performed in order to find out the concentration, which is not toxic for the cells but stops proliferation. According to the results, 100 µM and 250 µM concentrations were chosen and used in further senescence-inducing experiments, as there was no statistical significant difference after 24 and 48 h treatment corresponding to cytotoxicity. After 96 h treatment, which rather indicates the proliferation halt, also lower H_2O_2 concentrations induced statistically significant effects, in comparison to the control.

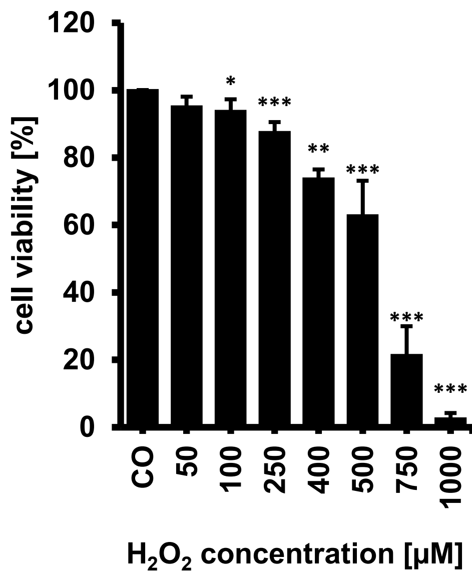
A **24 h**



B **48 h**



C **72 h**



D **96 h**

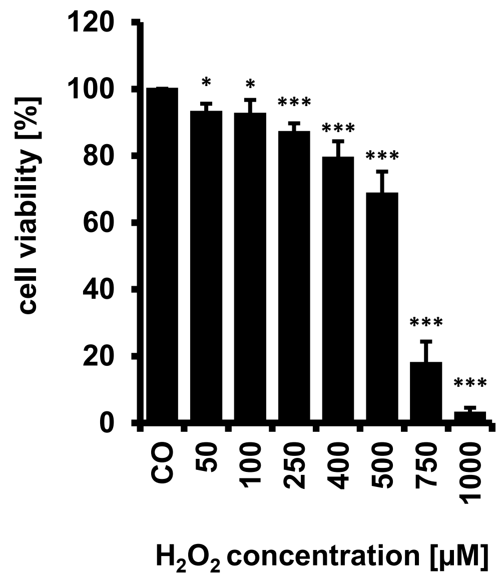
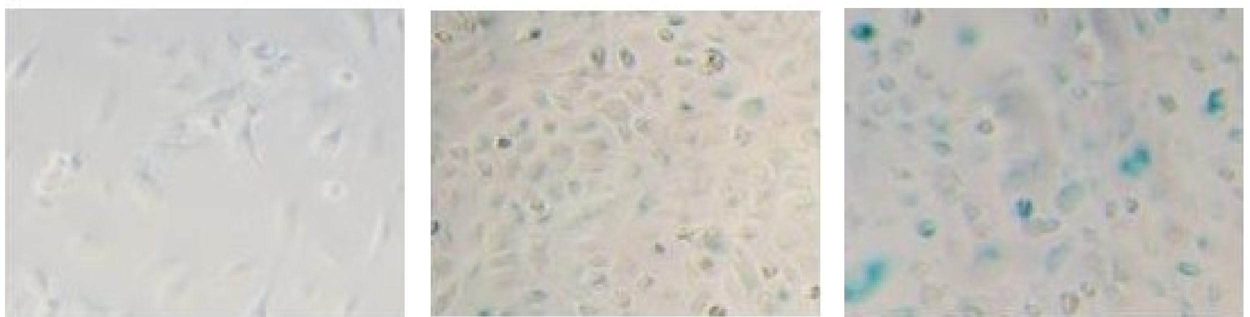


Fig. 5: *Cell viability after 24 h (A), 48 h (B), 72 h (C) and 96 h (D) treatment with raising H_2O_2 concentration compared to untreated cells (CO). Data show means ± SD of 5 independent experiments, (*p<0.05, **p<0.01, ***p<0.001; each concentration - 6 replicates). Statistical analysis was provided using two-sample student's t-test.*

The proportion of blue-stained HUVECs significantly increased after 96 h treatment with 100 and 250 µM H_2O_2 in comparison to untreated cells (*Fig. 6 A, B*).

A



CO **100 µM H_2O_2** **250 µM H_2O_2**

B

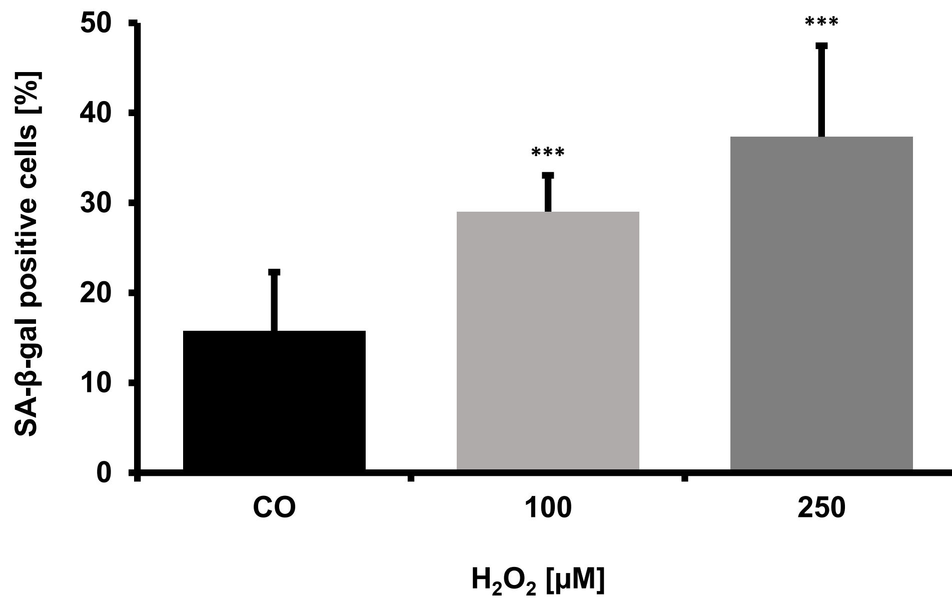
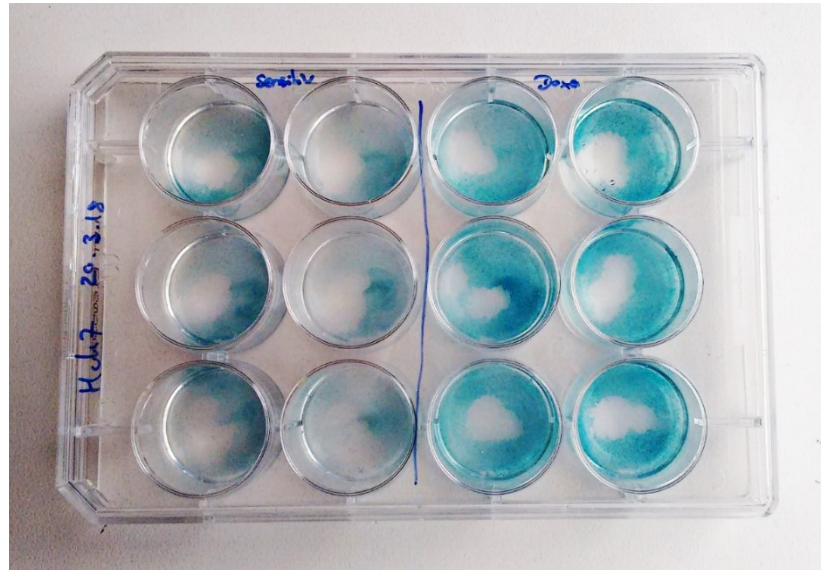


Fig. 6: *H_2O_2–induced cellular senescence after 96 h treatment compared to untreated cells (CO). The pictures A, B, C were taken with a Zeiss Axiovert 40CFL phase contrast microscope (magnification 100x), (***$p<0.001$; n=4; triplicates). Data show means ± SD, statistical analysis was provided using two-sample student's t-test.*

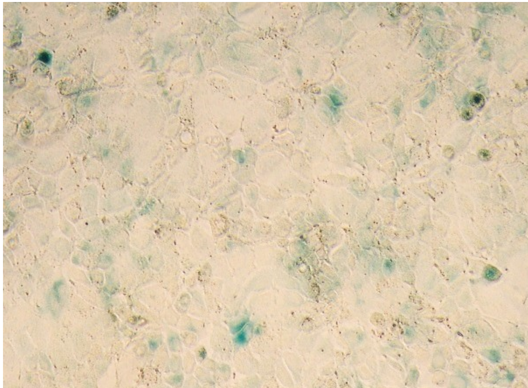
Huh7 cell line

The SA-β-gal assay was also performed on the hepatocarcinoma cell line Huh7, in order to observe the difference in senescence between DOXO-resistant and sensitive cells. The staining in Huh7 was much more intense and clearly visible with a bare eye. Therefore, the difference between resistant and sensitive cells was not counted under the microscope (*Fig. 7A, B, C*). The cells in the middle of the wells (*Fig. 7A*) were accidently washed away during the staining process.

A



B **C**



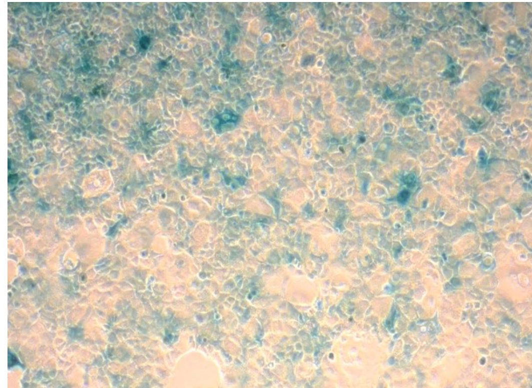


Fig. 7: *Huh7 cell line – DOXO-resistant cells (B) and sensitive cells (C) after 12 h spent in the staining solution inside of the incubator. The pictures B and C were taken with a Zeiss Axiovert 40CFL phase contrast microscope (magnification 100x).*

Alveolar macrophages

Alveolar macrophages isolated from old mice did not show any significant difference in staining in comparison to young mice. Cells were isolated from 2 young (10 weeks old) and 2 old subjects (85 weeks old) and counted after the staining in 3 different microscopic fields by each sample.

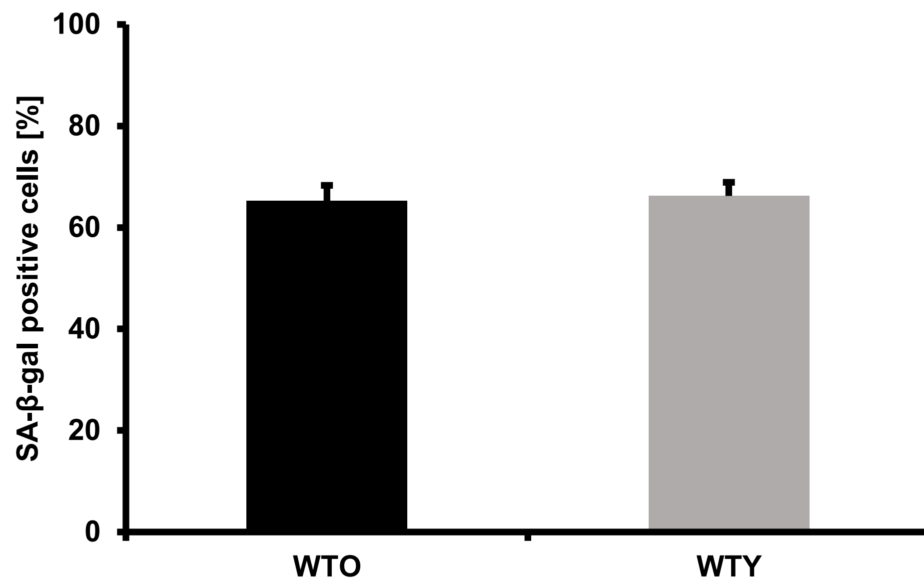


Fig. 8: *Percentage of positive stained macrophages isolated from old (WTO) and young (WTY) mice, (n = 1; triplicates of each subject). Data show means ± SD, statistical analysis was provided using two-sample student's t-test.*

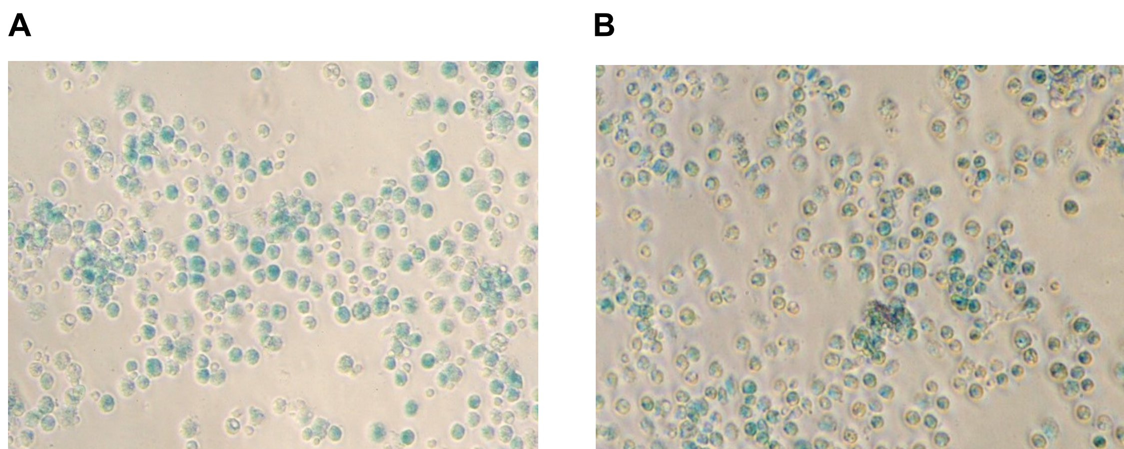


Fig. 9: *Freshly isolated alveolar macrophages from WTO (A) and WTY (B) murine lung tissues after 12 h spent in the staining solution inside of the incubator. The images were taken with a Zeiss Axiovert 40CFL phase contrast microscope (original magnification 100x).*

6.2 Telomere PCR

As cellular senescence occurred in HUVECs after H_2O_2 treatment, also the telomeres were expected to get shortened, since it is one of the senescence biomarkers. Surprisingly, the values from different donors were overly diverse and none of them show any relationship between senescence and shortened telomeres.

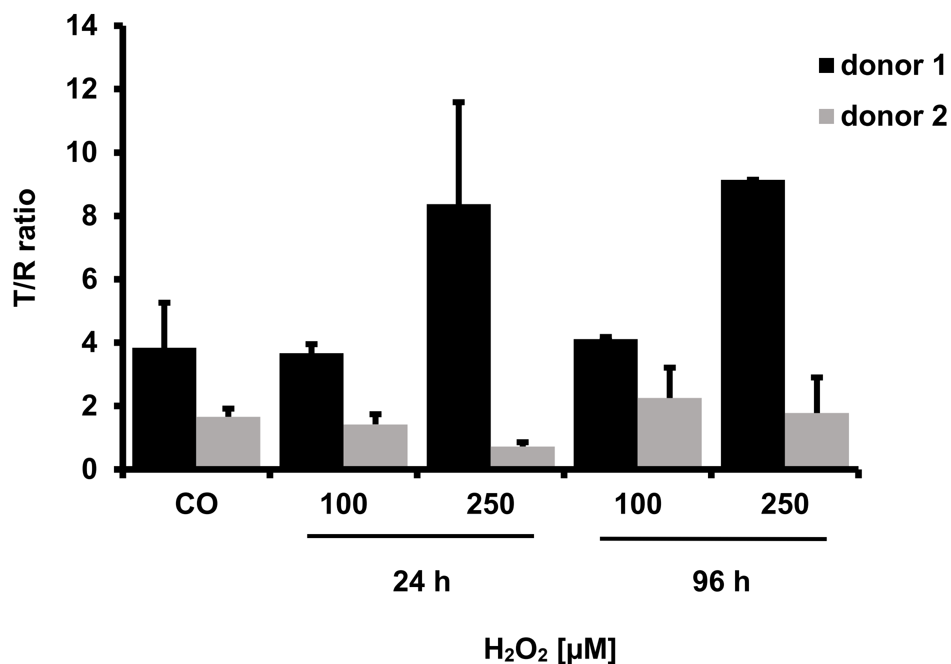


Fig. 10: *Telomere length expressed as T/R ratio measured from DNA of untreated (CO) and senescent HUVECs. Senescence was induced by H_2O_2 treatment (100 and 250 µM) for 24 and 96 h. Data were obtained from two independent experiments performed in triplicates. Data show means ± SD, statistical analysis was provided using Mann-Whitney-U-test.*

As an alternative approach, telomere length was measured in DNA isolated from murine liver tissue obtained from mice at different age and phenotype. No significant difference was found regarding telomere length with respect to age or genotype of these mice (*Fig. 11*).

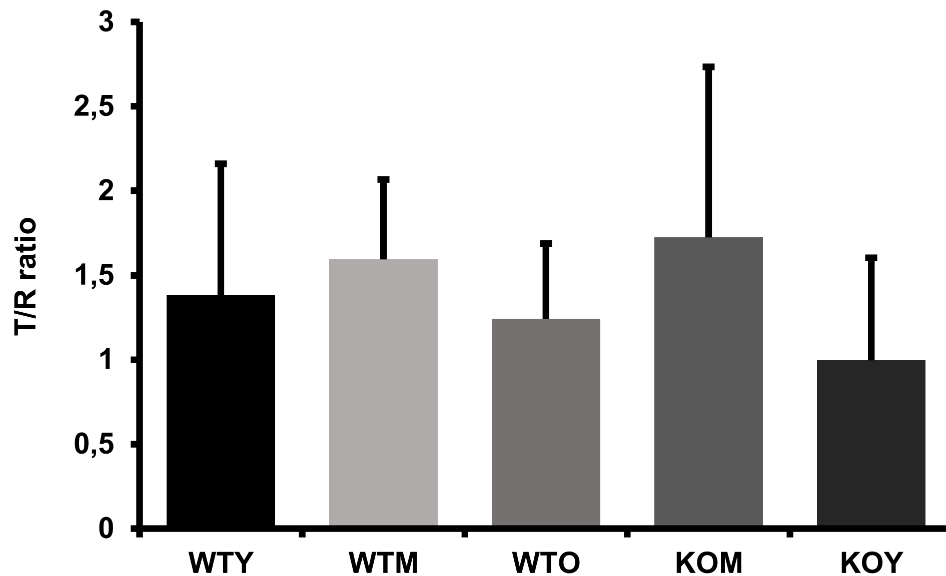


Fig. 11: *Telomere length expressed as T/R ratio measured from murine liver DNA – wild type young (WTY (10-12 weeks old)) n = 15, wild type middle aged (WTM (40-42 weeks old)) n = 12, wild type old (WTO (85 weeks old)) n = 12, GILZ knock-out middle aged (KOM (40-42 weeks old)) n = 10, GILZ knock-out young (KOY (10-12 weeks old)) n = 7. Data were obtained from three independent experiments performed in triplicates. Data show means ± SD, statistical analysis was provided using Mann-Whitney-U-test.*

6.3 Compound treatments

6.3.1 Statins

MTT assay was also carried out to investigate, whether any of the used statin concentrations influence cell viability. DMSO was added as a vehicle control. The results did not show any sign of cytotoxicity or decreased cell proliferation after treating cells for up to 96 h with different concentrations of statins or DMSO.

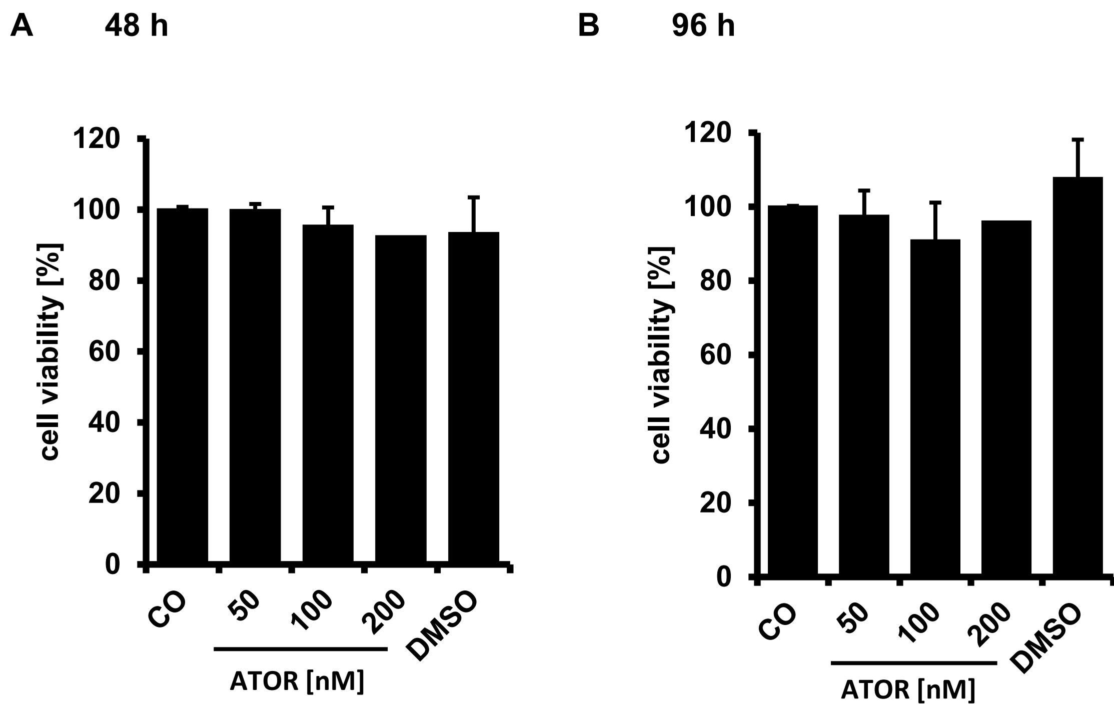


Fig. 12: Cell viability after 48 h (A) and 96 h (B) of atorvastatin [nM] compared to untreated cells (CO), (n = 2, each experiment in six technical replicates). Data show means ± SD, statistical analysis was provided using student´s t-test.

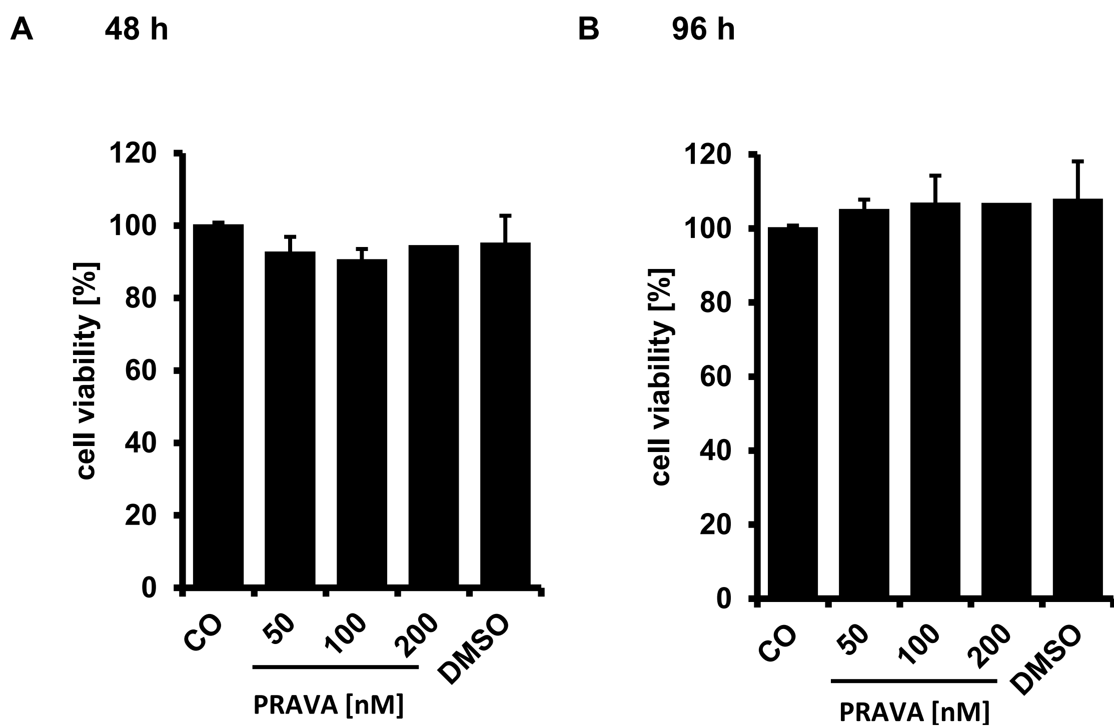


Fig. 13: Cell viability after 48 h (A) and 96 h (B) of pravastatin [nM] treatment compared to untreated cells (CO), (n = 2, each experiment in six technical replicates). Data show means ± SD, statistical analysis was provided using two-sample student's t-test.

Treatment with both, atorvastatin (50, 100, 200 nM) and pravastatin (100, 200 nM) showed a significant inhibition of cellular senescence induced by simultaneous treatment with 100 µM H_2O_2 for 96 hours.

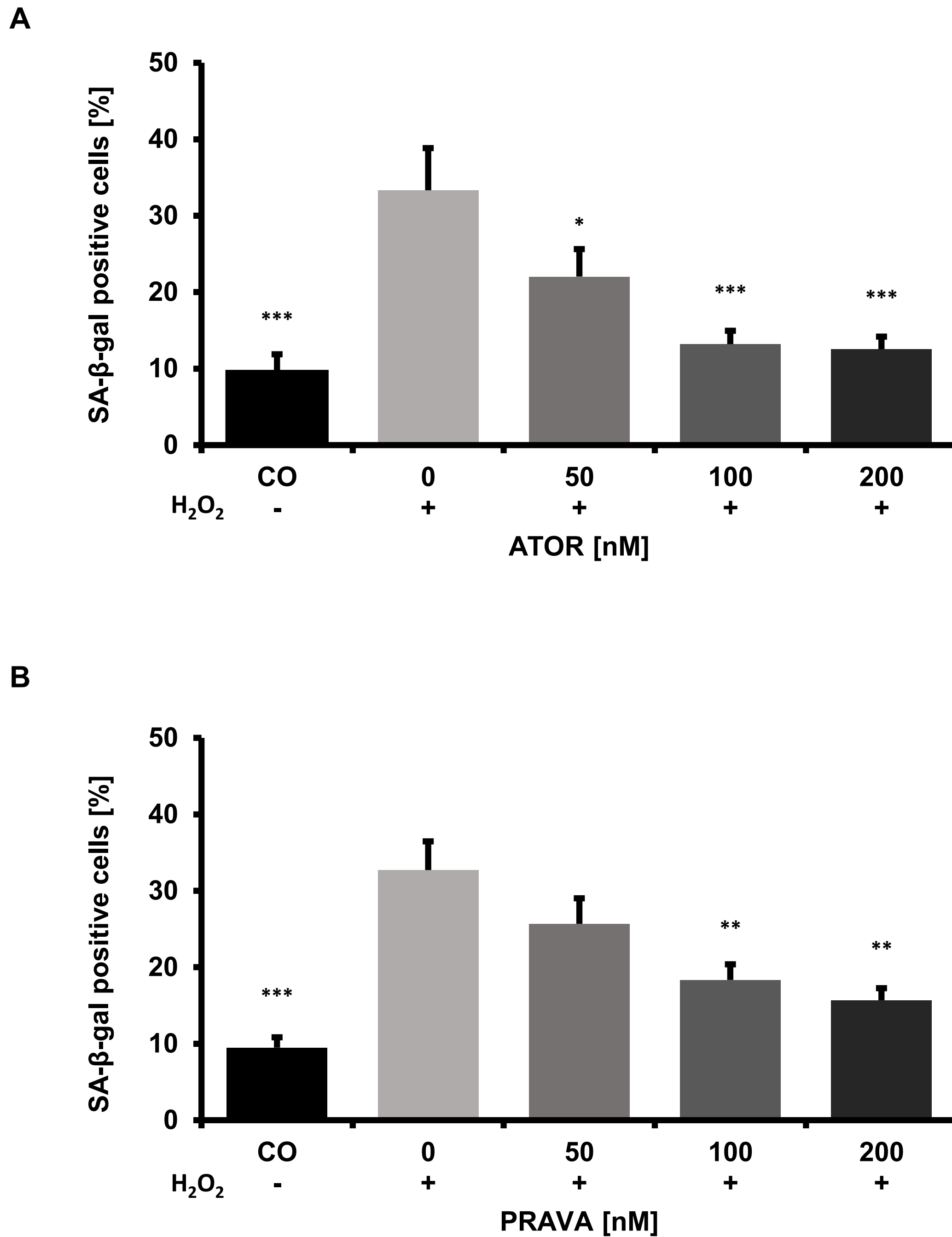


Fig. 14: *Atorvastatin (A) and pravastatin (B) inhibited H_2O_2–induced (100 µM) cellular senescence (+) evaluated by SA-β-gal staining after 96 h treatment. In untreated cells (CO -) neither H_2O_2 nor statins were added to the medium, (*p<0.05, **p<0.01, ***p<0.001; (A) n = 4, (B) n = 6). Data for statins were analysed by Mann-Whitney-U-test. Significance was calculated between respective treatment and H_2O_2 treatment only.*

6.3.2 Curcumin and its derivatives

Cell viability was checked also by curcumin and its derivatives. The results showed that except for the highest concentration of curcumin (25 µM), none of them was either cytotoxic nor proliferation stopping. Therefore, in further experiments the highest concentration of curcumin was excluded and only 5 and 10 µM concentrations were used. For feruoylacetone no proliferation tests were made, since this compound was included in the experiments later.

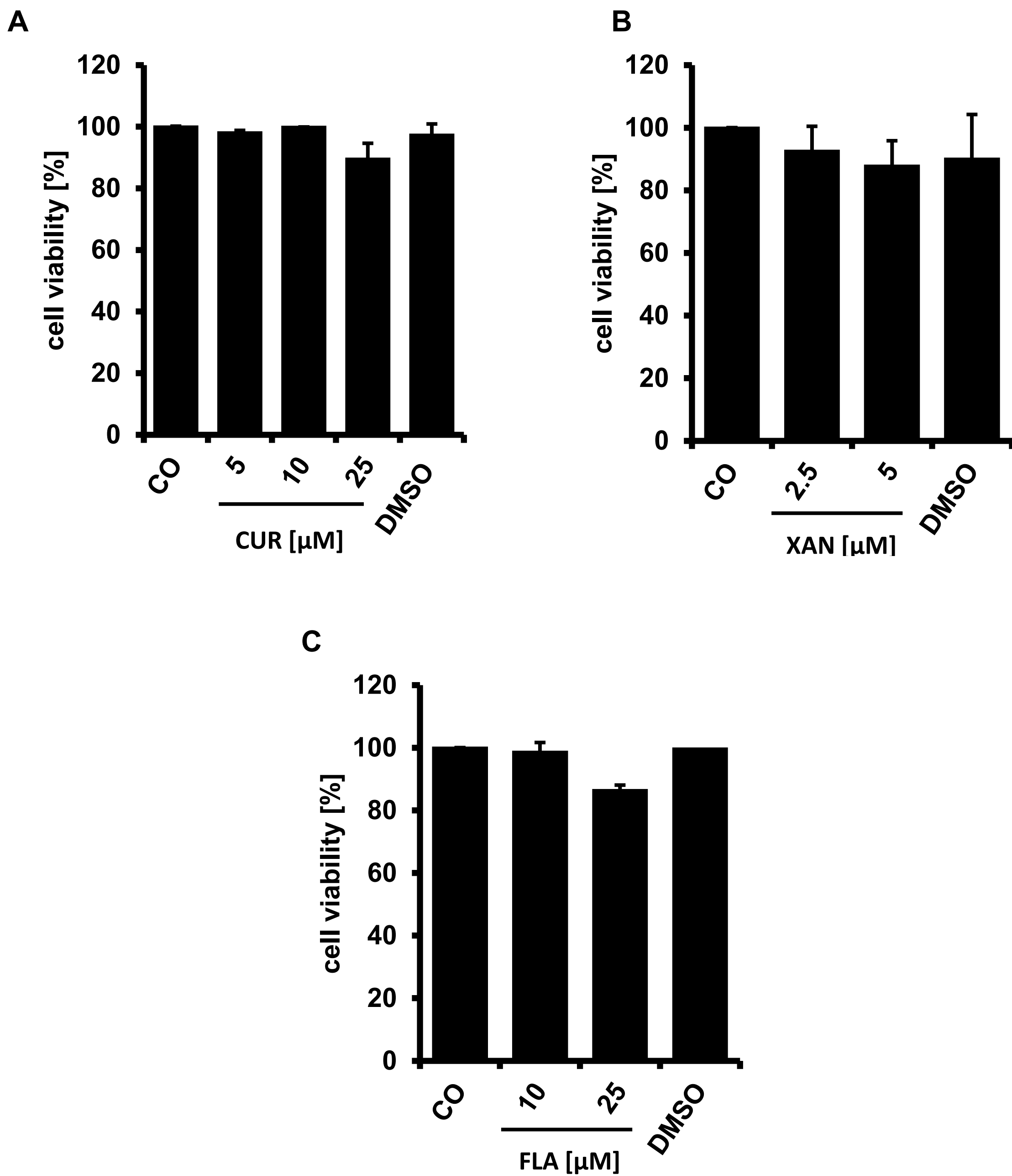


Fig. 15: *HUVEC viability after 48 hours of CUR (A), XAN (B) and FLA (C) treatment compared to untreated cells (CO), (n = 2, 6 replicates).*

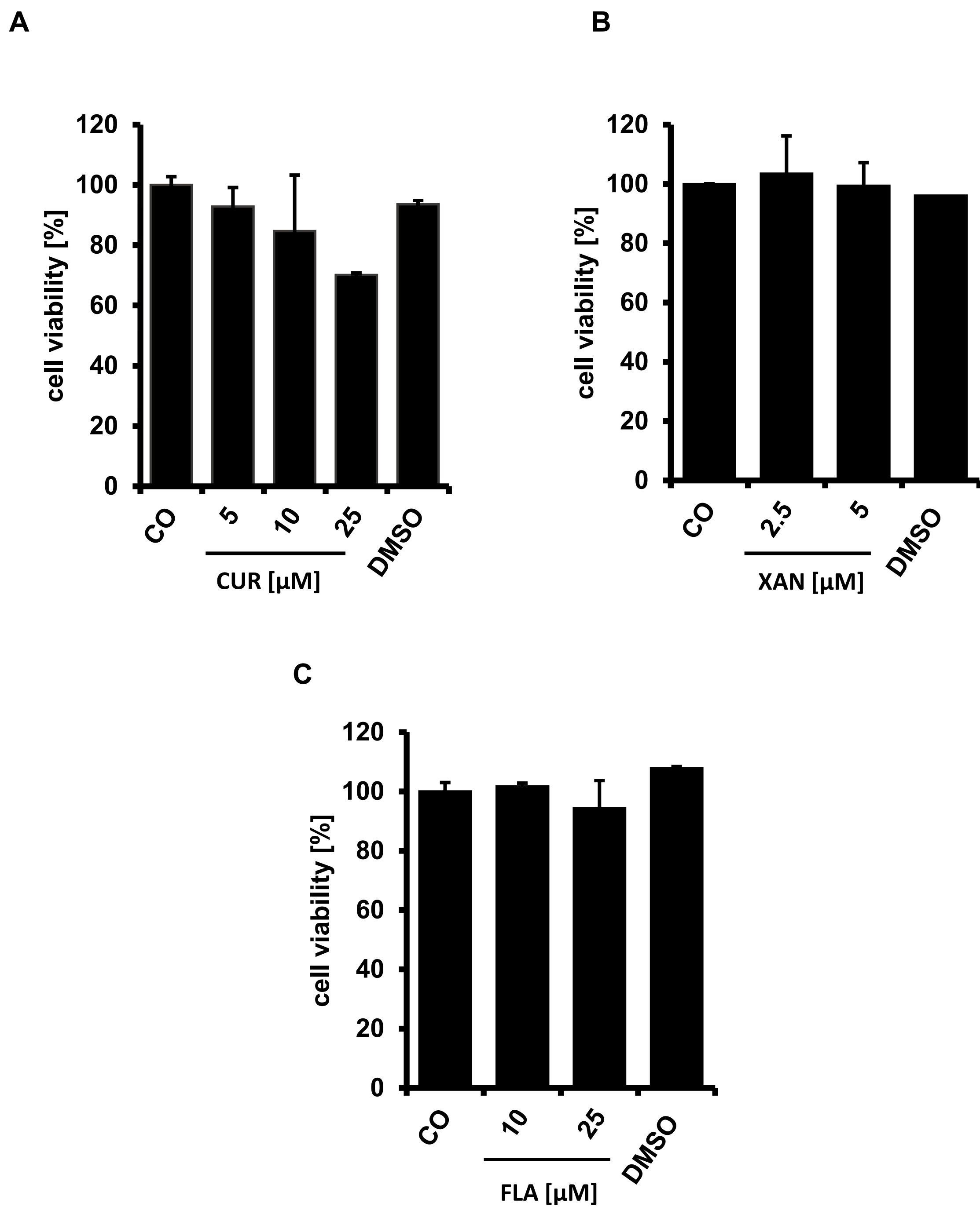


Fig. 16: *HUVEC viability after 96 hours of CUR (A), XAN (B) and FLA (C) treatment, (n = 2, 6 replicates).*

Only curcumin significantly attenuated the H_2O_2-induced senescence determined by SA-β-gal positive cells. The treatment with the curcumin derivatives FLA and FEA did not reveal a significant difference, whereas XAN even caused an increase in senescence (*Fig. 17 A-D*).

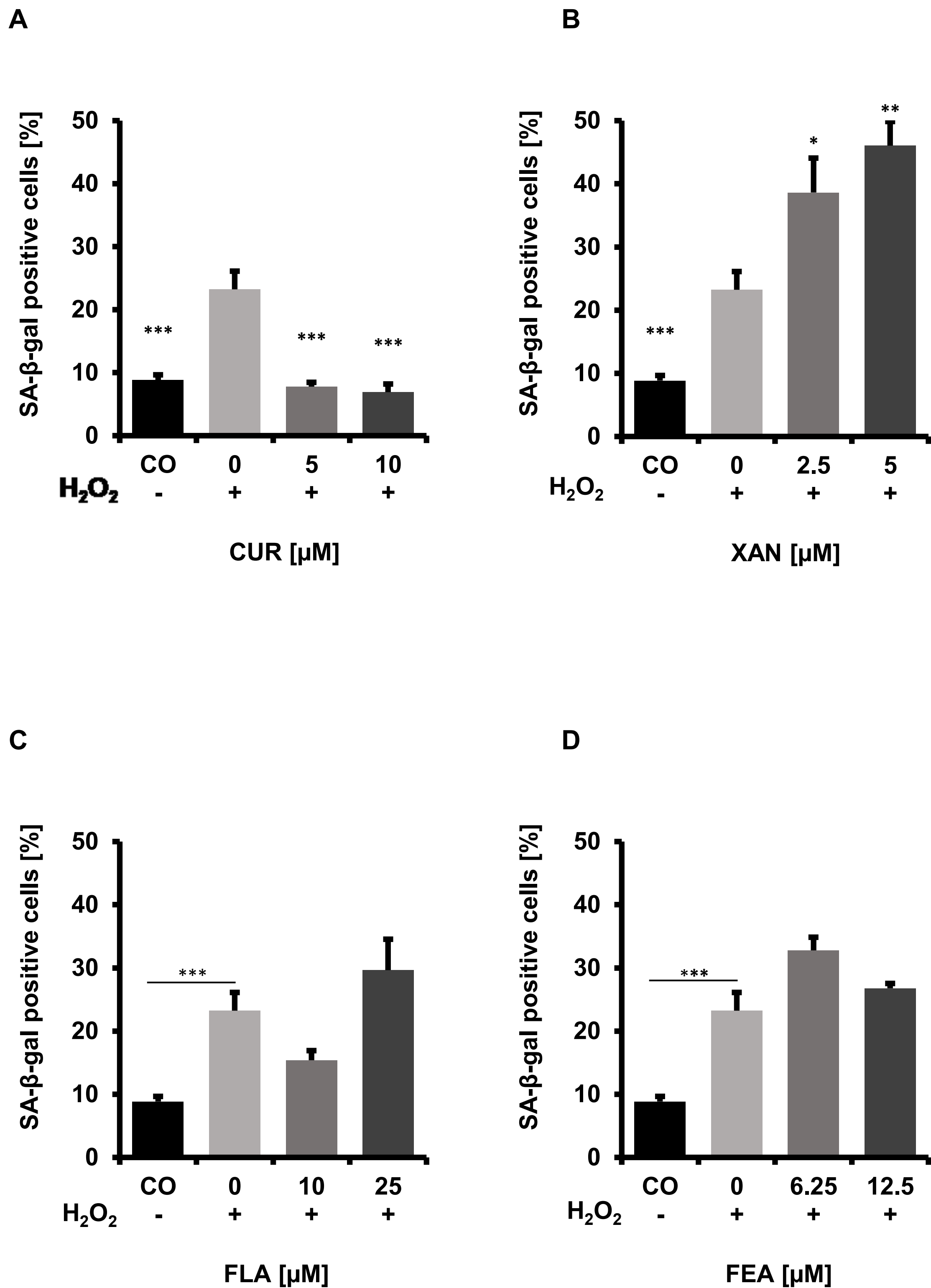


Fig. 17: *Impact of CUR (A), XAN (B), FLA (C) and FEA (D) on senescence inhibition induced by* H_2O_2 *(100 µM) after 96 hour-simultaneous treatment, (*p<0.05, **p<0.01, ***p<0.001 (A, B, C) n = 3, (D) n = 2). Data show means ± SD, statistical analysis was provided using two-sample student's t-test.*

6.4 Effect of senescence on *GILZ* mRNA expression

In order to investigate whether the senescence phenomenon has also an impact on *GILZ*, RNA was isolated from normal and senescent HUVECs and RT-qPCR was performed. Senescence was evaluated by SA-β-gal staining. Senescent HUVECs showed a decrease in *GILZ* expression after 96 h treatment with 250 µM H_2O_2. The results provide data only of one experiment since the senescence induction was not always successful. Due to lack of time, it was not possible to repeat this experiment.

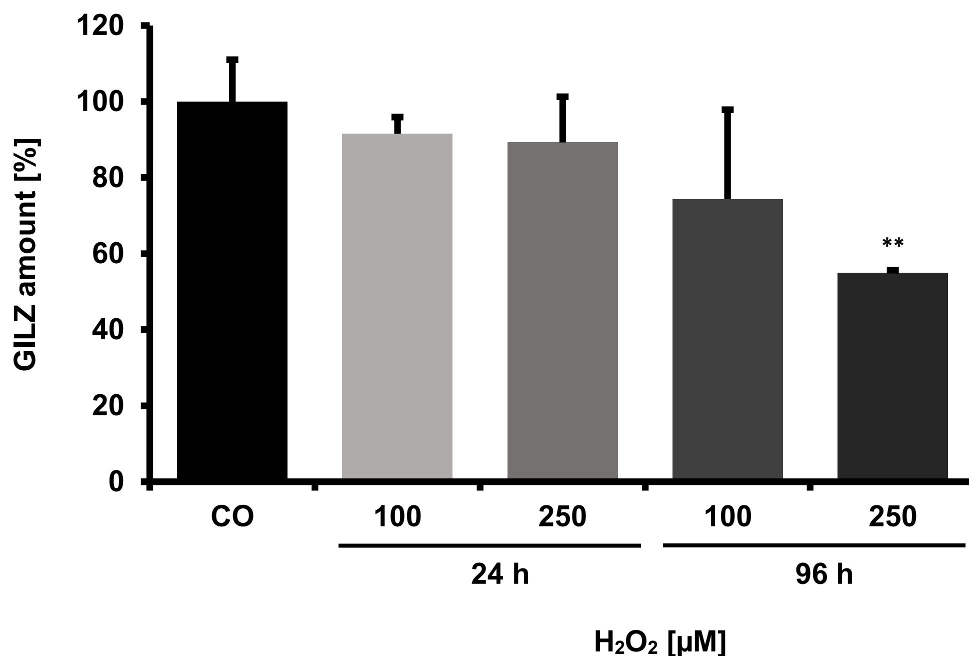


Fig. 18: *Effect of senescence on GILZ. Untreated cells (CO) were set as one hundred percent and GILZ amount was compared with respective treatment, (**P<0.01, n = 1). Data were normalized to β-actin.*

7 DISCUSSION

7.1 Senescence in (inflamm-) aging

In the past decades, the relationship between age-related diseases, cellular senescence and ROS has been studied intensely (Davalli et al., 2016). The aim of this master thesis was to establish a reliable assay to test for cellular senescence and subsequently screen promising compounds for an inhibitory effect on senescence.

Senescence in HUVECs was induced by subcytotoxic concentrations of H_2O_2 and the SA-β-gal assay was performed according to the protocol by Itahana et al. (2013). Although there were some limitations, i.e. the variability of the primary isolated HUVECs due to different donors regarding sensitivity to H_2O_2 and staining intensity, the SA-β-gal assay was successfully established. The SA-β-gal assay was then used to test compounds, which are known to have anti-senescent or anti-inflammatory actions: statins, curcumin, curcumin-derivates, and xanthohumol.

Statins are widely known as one of the most effective drugs for hypercholesterolemia and cardiovascular diseases treatment. Besides lowering the LDL levels, recent studies have shown that statins also appear to inhibit oxidative stress-induced endothelial senescence (Ota et al., 2010). In line with this study, our results demonstrated that both, ATOR and PRAVA, effectively inhibit oxidative stress-induced endothelial senescence in HUVECs in non-toxic concentrations (*Fig. 14*). Ota et al. (2010) described the senescence-inhibiting mechanism of statins: statins trigger the phosphatidylinositol 3-kinase/Akt pathway *via* isoprenylation leading to enhanced eNOS expression and activation. Furthermore, statins upregulate SIRT1, which plays a crucial role in senescence prevention in HUVECs through p53 acetylation. Given that statins have been related with a highly beneficial pleiotropic effect, it is interesting to see that their senescence-inhibiting mechanism could also contribute to the treatment of atherosclerosis, where oxidative stress-induced senescence plays also a significant role in its development (Katsuumi et al., 2018).

Another compound reported to have anti-aging effects is CUR (Grabowska et al., 2016). However, the study of Grabowska et al. (2016) attributes the anti-aging effect to the elevated levels of sirtuins and not to the decreased senescence. Although CUR is one of the best examined plant-derived polyphenols, the research in the cellular senescence influence remains limited. To our knowledge, only a few studies in the literature investigated this issue and the results appear to be contradictory. Our study is in concordance with the study of Sun et al. (2015), in which concentrations of CUR not influencing HUVEC proliferation remarkably decrease H_2O_2-induced ratio of SA-β-gal positive cells in comparison to the cells without CUR (*Fig. 17A*). In addition, Sun et al. (2015) also observed an improved expression and activity of SIRT1, decreased production of ROS, occurrence of apoptosis and cell cycle arrest. Even though, CUR-induced senescence in cancer cells was demonstrated lately, neither SASP, influencing surrounding cells, nor DNA damage, even after using high cytotoxic CUR concentrations, were observed (Mosieniak et al., (2012), Korwek et al., (2013), Bielak-Zmijewska et al., (2010). All these findings support the notion that CUR has a potential in age-related diseases treatment and tumour-suppression.

Surprisingly, none of the CUR derivates showed the same effect (*Fig. 17B-D*). CUR-related compounds and CUR degradation products have attracted attention in the last years due to their proven bioactivities (Srinivasan et al., 2007; Shen et al., 2012). FEA and recently also FLA were both confirmed to belong to CUR degradation products (Schneider et al., 2015; Typek et al., 2019). Given that CUR effectively suppressed H_2O_2-induced senescence in HUVECs, we expected similar potency of its metabolites. SA-β-gal staining showed that neither FEA nor FLA had any senescence-inhibiting influence. Feng et al. (2011) emphasized the relationship between the structure and antioxidant effect of the half-curcumins, which may be associated with this phenomenon. Results of the latter study describe decreased ROS-scavenging activity by half-curcumins with enolic and phenolic-OH groups connected by conjugation. Moreover, compounds with a single phenolic-OH group in the structure demonstrated lower antioxidant activity than those containing more.

XAN shares the 3-(4-hydroxyphenyl) acrylaldehyde skeleton with CUR, both belong between flavonoids and are examined for their beneficial

clinical pleiotropic effects. XAN anti-inflammatory, chemoprotective, antioxidant features and its activity in aging and age-related diseases has been further examined (M. Liu et al., 2015). In our study, XAN, in comparison to CUR, significantly increased the amount of senescent cells during simultaneous treatment with H_2O_2 (*Fig. 17B*). Blanquer-Rosselló et al. (2013) tested a number of XAN concentration regarding the antioxidant function *in vitro*. Low concentrations (below 0.01 μM) showed expression of SIRT1 and inhibition of ROS production. Conversely, higher doses (5 μM and above) caused ROS levels to increase and significant reduction of SIRT1, what could theoretically assist, in our case, to the H_2O_2-senescence induction. On the other hand, there is no evidence that HUVECs would react the same way after the higher doses of XAN as the cancer cells used in the study of Blanquer-Rosselló et al. (2013). However, there is a literature gap in the relationship between normal cells, XAN and senescence.

The second approach of this thesis was to establish an additional method to detect aged cells. A major hallmark of aging in cells is the telomere shortening, which can be detected by telomere length PCR. Even though, shortened telomeres are one of the senescence biomarkers and oxidative stress is linked to their attrition (Ahmed et al., 2018), our PCR experiments did not show any difference in their length between senescent and normal cells (*Fig. 10*). Concordantly, a recent study suggested that telomeres may not get shortened in ROS-induced senescence (Darío et al., 2019). Fumagalli et al. (2012) described the phenomenon, that telomere dysfunction can occur also independently on their length. Their sensitive stress-receptors respond to the acute stress, such as H_2O_2 treatment, by forming telomeric DDR. The unceasing DDR signalling subsequently triggers an antiproliferative cascade regardless the telomere length. Moreover, natural antioxidant enzymes, peroxiredoxins and preferentially peroxiredoxin 1, safeguard the telomeres from ROS-induced damage by reducing H_2O_2 to H_2O and thereby lowering its cellular concentrations (Perkins et al. (2015); Aeby et al. (2016)).

However, due to the lack of differences in PCR results in young, middle-aged, and old mice (*Fig. 11*), the protocol according to Wang et al. (2013) is probably not suitable for DNA isolated from whole tissues or a cell bulk. One approach to solve this problem could involve the use of DNA

isolated from single cells, as they did in the study (Callaghan et al., 2011).

Another setting, in which the cellular senescence was investigated, were freshly isolated alveolar macrophages from old and young mice. Unexpectedly, macrophages from both young and old mice showed a high percentage of positive stained cells without a significant difference between them (*Fig. 8, 9A, B*). Hall et al., (2016) reported in their study that the expression of SA-β-gal and $p16^{Ink4a}$ in macrophages is more likely gained as a part of a normal physiological reaction to the immune stimuli than through senescence. SA-β-gal and $p16^{Ink4a}$ are considered as common senescence biomarkers (Debacq-Chainiaux et al., (2009); Ohtani et al., (2004)). Macrophages producing these factors were found in the adipose tissue of old mice but also in young animals, which were implanted with senescent cells. In their following study, they observed that unlikely the senescent cells, SA-β-gal and $p16^{Ink4a}$ in macrophages were induced independently of p53 – the main mediator of the cell cycle arrest in senescence (Rufini et al., 2013), and their expression was modifiable by physiological stimuli (Hall et al., (2017). These results may question the theory of elimination of $p16^{Ink4a}$-positive cells in senolytic therapy (Baker et al., 2011), since also non-senescent cells may be characterized by its expression.

A feature we assumed to be diminished while senescence-induction in HUVECs is the amount of *GILZ*, referring to the fact that *GILZ* KOY mice imitate aging phenotype (Valbuena-Perez et al., n.d.). Due to time reasons our experiment consists only of one donor. Still, *GILZ* mRNA levels were significantly decreased in the senescent HUVECs (*Fig. 18*), which also supports the hypothesis that *GILZ* is downregulated in aging. Nevertheless, the experiment will have to be repeated.

The recent study of Hoppstädter et al., (2016) examined the relationship between CUR and *GILZ*. The anti-inflammatory feature of both, CUR and *GILZ* is connected with its ability to reduce the signalling of pro-inflammatory nuclear factor κB (NF-κB) (Soetikno et al., (2011); Ayroldi et al., (2001)). It has been observed that CUR causes *GILZ* up-regulation and that *GILZ* induction considerably contributes to the inhibition of NF-κB by curcumin, what increases its anti-inflammatory effect (Hoppstädter et al.,

2016).

7.2 Senescence in cancer

Contemporary cancer therapy is based on the assumption that the absolute tumour cells damage, caused by apoptosis, increases the patient chance of survival. However, such cancer cells acclimatize quickly to the environment containing a chemotherapeutic drug, which leads to treatment resistance and possibly generating of metastasis. Therapy-induced senescence could be an effective method to prevent tumour cells from further dividing and avoid the high-dosage-related side effects. In the field of cellular senescence, there are growing attempts for a detailed explanation of relationship between therapy-induced senescence and cancer. DOXO is a very potent antineoplastic drug from the class of anthracyclines. However, little is known about senescence in liver cancer, which has a high intrinsic chemoresistance. Therefore, we tested senescence in DOXO-sensitive and DOXO-resistant Huh 7 cells. As expected according to the previous studies (Ewald et al., 2010), resistant Huh7 cells showed a high amount of positive-stained cells (*Fig. 7C*). Previous studies have emphasized, that two main regulation pathways involved in cellular senescence are p53-p21^{Cip1} and p16^{INK4a}-Rb protein (Campisi, 2013). Many cancer cell lines have a lack of one or more of the proteins involved in these pathways as they also have tumour-supressing functions. The Huh7 cell line is able to express both – p16 and p53 (containing mutation) (Kaino, 1997). In the recent study of Fallah et al., (2019) have shown in their study that senescent cells treated with subcytotoxic DOXO concentrations were characterized by increased mitochondrial instability as well as the expression of SA-β-gal, p53, and pro-inflammatory cytokines, what contributes to elucidation of the possible chemotherapy-treatment induced senescence mechanism.

Even if the chemotherapy treatment does not result in senescence of all cancer cells, due to SASP expressing factors also the non-senescent tumour cells may stop growing.

Moreover, recently observed fact that senescent cells are able to implement stemness into normal cancer cells, the targeted senolysis appears to be a critical therapeutic need (Milanovic et al., 2017).

8 CONCLUSION

The aim of this thesis was to establish senescence-identifying methods for different types of cellular senescence in various cell types and to evaluate a few compounds possibly influencing oxidative stress-induced senescence as well as some specific features that may be influenced by the senescent phenotype. On this basis, we observed that:

- ATOR, PRAVA and CUR belong to the compounds being able to inhibit oxidative stress-induced cellular senescence, while CUR metabolites, FLA and FEA, have no significant impact and XAN even elevated the SA-β-gal positive stained cells ratio;
- alveolar macrophages showed no significant difference in senescence between young and old mice;
- telo-PCR did not provide any difference in the telomere length neither between the senescent and non-senescent HUVECs and did not work for tissue bulk;
- ROS-induced cellular senescence decreases the amount of *GILZ* mRNA in HUVECs;
- the ratio of SA-β-gal positive stained cells was higher in therapy-induced cellular senescence in the hepatocarcinoma cell line Huh7 resistant to DOXO in comparison to the sensitive Huh7.

Undoubtedly, the future research will provide a more detailed view on the role of *in vivo* senescence and the mechanisms behind it. Regarding clinical aspects, a deeper understanding of cellular senescence and its role in human age-related diseases and cancer could provide promising novel treatment options.

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