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Abstract

Cellular senescence is an important contributing factor for many age-related diseases and studies accomplished in pre-clinical models showed that the elaboration of procedures of selectively removing senescent cells may be of great clinical importance in preventing or combating negative consequences of aging.

In our study we tested six substances regarding their senolytic effects in human endothelial cells. These substances encompassed the three anti-oxidants: butylatedhydroxytoluene (BHT), a general ROS scavenger, diphenyleneiodonium chloride (DPI), an anti-oxidant inhibiting NADPH-oxidase, and N-acetyl-L-cysteine (NAC), another antioxidant; and three anti-inflammatory compounds. These three NF-κB- inhibitors encompassed, besides the plant-derived plumericin (PL), two synthetic inhibitors, namely BAY11-7082 and PHA 408. We applied these compounds separately or in combination to study their assumed senolytic effects. To this end, we applied these compounds to “young” HUVECs at passage3 (P3), to “old” (senescent) HUVECs at passage 30 (P30) and to human endothelial cells immortalized with hTERT (HUVECs-hTERT). The latter cells were growing relatively slowly in our laboratory in comparison with some published reports.

We found that none of the tested inhibitors alone or in combination was able to selectively/preferentially eliminate endothelial cells in the state of replicative senescence. However, some of the tested anti-oxidant substances had a pronounced proliferation-enhancing effect, specifically NAC alone at concentrations 2, 1 and 0.5 mM on P3 HUVECs, and BHT in combination with PHA-408 in case of HUVECs-hTERT cells. Furthermore, Bay11-7082, commonly used as an anti-inflammatory NF-κB inhibitor, stimulated proliferation of P3 HUVECs and HUVECs-hTERT cells when used at 1.7μM. At the higher concentration of 5μM, Bay11-7082 eliminated the proliferating P3 HUVECs as well as the non-proliferative P30 HUVECs. The highly selective IKK2 inhibitor PHA-408, while being ineffective on its own, synergistically increased the impact of the anti-oxidants NAC or BHT on HUVECs-hTERT cells.

By elaborating a methodology for testing senolytic substances, this study placed a solid ground for examining senolytic properties of natural or synthetic compounds to be tested alone or in combination with other substances in future studies.

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List of abbreviation

BHT, butylated hydroxytoluene; BM-MSCs, bone marrow – mesenchymal stem cells; CAT, catalase; DPI, diphenyleneiodonium chloride; ECGS, endothelial cell growth supplement; EFSA, European Food Safety Authority; FBS, fetal bovine serum;DDR, DNA damage response; GSH, glutathione, HUVECs-hTERT, human umbilical vein endothelial cells- telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; IKK, IkappB kinase, IKK2, IkappaBkinase-2; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8; LPS, lipopolysaccharides; MiDAS, mitochondrial dysfunction-associated senescence; NMDA, N-methyl-D-aspartate; NAC, N-acetylcysteine; NAD+/NADH, nicotinamide adenine dinucleotideoxidized /reduced; NMDA, N-methyl-D-aspartate; P3, passage 3; P30, passage 30, PBS, phosphate buffer saline; PFA, paraformaldehyde; PL, plumericin; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulfur species; SASP, senescence-associated secretory phenotype; SIRT1, silent mating type information regulation 2 homolog 1; SOD, superoxide dismutase; TNFα,tumor necrosis factorα.

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1 Introduction

1.1 Aging

Aging is a major risk factor for many chronic diseases that result in the bulk of illness and are the cause of large health costs worldwide (Goldman et al., 2013, Kirkland, 2016). Many of these diseases such as type 2 diabetes mellitus, blindness, kidney dysfunction, osteoarthritis, osteoporosis, and sarcopenia affect predominantly elderly people (St Sauver et al., 2015). With an increase in age, there is also an increased risk of incontinence, immobility, mild cognitive impairment, and frailty. Aging also fosters development of strokes, age-related macular degeneration, ocular cataracts, presbycusis, benign prostatic hyperplasia and many types of malignant cancer, as well as neurological illnesses such as Parkinson's disease, frontotemporaldementia, Alzheimer's disease, peripheral neuropathies and Lewy body dementia (Driver et al., 2008, Kelly-Hayes, 2010, Martin, 2011). In addition, people who are 60 or above are more susceptible to develop vascular diseases such as arteriosclerosis, microvascular diseases, peripheral arterial disease or coronary artery disease and myocardial infarction, because of the changes in their anatomy and performance of their vascular system (Lakatta, 2002, Brandes et al., 2005, Ungvari et al., 2010, North and Sinclair, 2012). Altogether, age-related diseases are the reason for disabilities leading to productivity loss. According to the United Nation’s report, it is foreseen that in 2050 people who are 60 years and more will represent more than 22% of the world’s population.Such development will not only cause a decline in the productive workforce but also negatively affect the countries’ economy (Wiener and Tilly, 2002). These facts show the need of fostering studies related to age-associated diseases that should aim to elaborate strategies for prevention, delay or better treatment of age-related diseases.

1.2 Aging and cellular senescence

Studies in several last decades showed that genetic factors (Brown-Borg et al., 1996, Sinclair and Guarente, 2006), disturbances in metabolism (Barzilai et al., 2012), unhealthy diet (Wood et al., 2004), oxidative stress (Finkel and Holbrook, 2000) and inflammation (Woods et al., 2012, Tabas and Glass, 2013) heavily influence the progression of aging. In addition, many studies showed that age-related disorders are affected by the phenomenon of “cellular senescence”.

The phenomenon of cellular senescence was discovered in the 1960s by Leonard Hayflick and Paul Moorhead who found out that cultured human fibroblasts have only a limited capacity to proliferate (Hayflick and Moorhead, 1961). After prolonged passaging, cells gained features of “replicative senescence”, a state characterized by the cell-inability to proliferate as well as by changes in cell morphology including e.g., an enlarged cell cytoplasma and visible vacuoles (Hayflick and Moorhead, 1961). Not only cell cultured *in vitro*, but also cells in the living organisms tend to gradually become senescent (Brown-Borg et al., 1996). Cellular senescence is an irreversible cell-arrest which includes different cellular changes, including protein aggregation in the endoplasmic reticulum, enlarged and functional ineffective mitochondria, nonfunctional lysosomes as well as unfolded protein response (Kaufman, 2002, Nuss et al., 2008). In addition to these morphological changes, senescent cells also express various marker proteins, such as senescence-related β-galactosidase (Dimri et al., 1995).

Furthermore, senescent cells may lose or shorten their telomeres, the regions of repetitive nucleotide sequences that cap the ends of chromosome and protect them from decline or from fusion with adjacent chromosomes (Harley et al., 1990, Blackburn, 1991, Kim et al., 1994, Gasser, 2000). Loss of telomeres or their shortening may be caused by a diminished endogenous telomerase transferase activity, an enzyme that adds telomere repeat sequence to the 3' end of telomeres (Morin, 1989). Uncapped chromosomes are susceptible to recombination, degradation or fusion, and this may lead to genetic rearrangement and the loss of genetic information (Blackburn, 1991, Kim et al., 1994, Gasser, 2000). Somatic cells are exposed to telomere shortening with every cell division (Wynford-Thomas, 1999, Kyo and Inoue, 2002).On the other hand, researchers showed that immortalization of cells e.g., by expressing telomerases, can return the cells their replicative capacity. Some studies showed that telomere shortening can also be induced by ionizing radiation or chemotherapeutic drugs. Telomere shortening also plays an important role during replicative senescence where it may contribute to a persistent DNA damage response (DDR), and cause an irreversible loss of division potential of somatic cells (Hewitt et al., 2012). Such reduced telomere-associated DNA damage foci are typical for senescence cells (Hewitt et al., 2012). It was also reported that high concentration of glucose in culture medium or oxidative stress may lead to replicative senescence that can be reverted by overexpression of the telomerase

catalytic subunit (human telomerase reverse transcriptase, hTERT) (Stolzing et al., 2006, Estrada et al., 2013).

At molecular level, senescent cells show a change in gene transcription that involves increased expression of suppressor genes and diminished expression of cell cycle promoter genes (Campisi and d'Adda di Fagagna, 2007, Collado et al., 2007, Munoz-Espin and Serrano, 2014). Senescence cells are also categorized by a specific secretory profile designated as senescence-associated secretory phenotype (SASP), characterized by increased release of pro-inflammatory cytokines, proteases and chemokines (Coppe et al., 2006, Coppe et al., 2010, Xu et al., 2015b). Campisi in 2013 described cellular senescence as an irreversible cellular growth arrest that causes serious phenotypic changes that include modification of chromatin organization, gene expression, and changes in secretory profiles (Campisi, 2013). Moreover, senescent cells produce extensively growth factors and matrix metalloproteinase designated as senescence-messaging-secretome (Acosta et al., 2008, Coppe et al., 2008, Kuilman et al., 2008, Kuilman and Peeper, 2009).

In vivo, accumulating senescent cells in the tissues deteriorate tissue function as well as cell growth and also affect neighboring cells, thus contributing to developing inflammation and age-related pathologies (Ovadya and Krizhanovsky, 2014, van Deursen, 2014). The accumulation of senescence cells in blood vessels may contribute to extracellular matrix deposition and cause functional and structural changes of endothelial- and vascular smooth muscle cells (Gates et al., 2009, Iantorno et al., 2014). Senescence may also affect other tissues such as knee joints and may contribute to the development of osteoarthritis (Xu et al., 2017) and other major human diseases involving Parkinson's disease, Alzheimer's disease, age-related macular degeneration, peripheral neuropathies, type 2 diabetes mellitus, arteriosclerosis and many types of cancer (Martin, 2011, Childs et al., 2017). Therefore, the elaboration of strategies leading to elimination of senescent cells in body tissues seems to be important.

1.3 Reactive oxygen species (ROS) play an important role in cellular homeostasis and affect development of cellular senescence

Tight balance between oxidants and antioxidants is very important for maintaining a healthy cellular microenvironment. A situation in which cells generate

more oxidants as they remove leads to a variety of different pathological conditions (Kurutas, 2016).

Oxidants derive from the metabolic processes in cells and tissues, and their occurrence can be influenced by external stimuli such as pollution, pesticides, microbes, allergens, radiation and cigarette smoking (Hekimi et al., 2011). Oxidative substances include e.g., reactive oxygen species (ROS) involving oxygen-derived radicals (hydroxyl radical, superoxide, nitric oxide) and non-radical oxygen derivatives (hydrogen peroxide, singlet oxygen, and hypochlorite and others) (Pham-Huy et al., 2008, Fransen et al., 2012, Phaniendra et al., 2015). In addition to ROS, also reactive nitrogen species (RNS) and reactive sulfur species (RSS) control redox balance in cells and tissues, by playing a multi-functional and context-dependent role (Kawagishi and Finkel, 2014). ROS are synthesized mainly in the respiratory chain of mitochondria. Excessive generation of free radicals may damage cell membranes and mitochondria, and this process may result in exacerbating aging (Terman et al., 2006).

In immune cells, reactive species participate in combating infections and in controlling redox balance (Popa-Wagner et al., 2013, Zhao, 2009). It was shown that shifting the balance towards increased ROS concentration in the tissues may occur in many pathological conditions such as heart ischemia or chronic inflammatory diseases (Dudda et al., 1996, Spiteller, 1998). Excessive ROS generation may also cause structural degradation and destruction of the inner mitochondrial membrane, thus contribute to premature aging (Harman, 1972, de Grey, 1997). In addition, mutations in mitochondrial DNA were reported to associate with some myopathies and cardiomyopathies (Khrapko et al., 1999, Cao et al., 2001).

Generation of ROS is tightly controlled by antioxidants defined as “any substance that, present in low concentrations compared to oxidizable substrates (carbohydrates, lipids, proteins or nucleic acids), significantly delays or inhibits the oxidation of the mentioned substrates” (Halliwell et al., 1992). Another definition of antioxidants encompasses “any substance that prevents, delays or eliminates oxidative damage of target molecule”(Halliwell and Gutteridge, 1990) or “any substance that can eliminate ROS directly or indirectly, acting as a regulator of the antioxidant defense, or inhibiting the production of those species” (Khlebnikov et al., 2007).

According to their mechanism of action, antioxidants can be classified into three lines of defense (Shetti et al., 2009, Irshad and Chaudhuri, 2002). The first line of defense includes substances that prevent the formation of new free radicals, e.g., enzymes glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), proteins ferritin, ceruloplasmin, and metals Zn, Se, and Cu (Figure 1). The second line of defense includes substances that capture free radicals via their anti-oxidative epitopes, thus preventing oxidative chain reactions (glutathione, albumin, carotenoids, flavonoids vitamins C and E). Finally, the third line of defense plays important role in repairing cell damage that had been caused by free radicals. Important components of this group encompass e.g., DNA repair enzymes, lipases, transferases, proteases, and methionine-sulfoxide reductases.

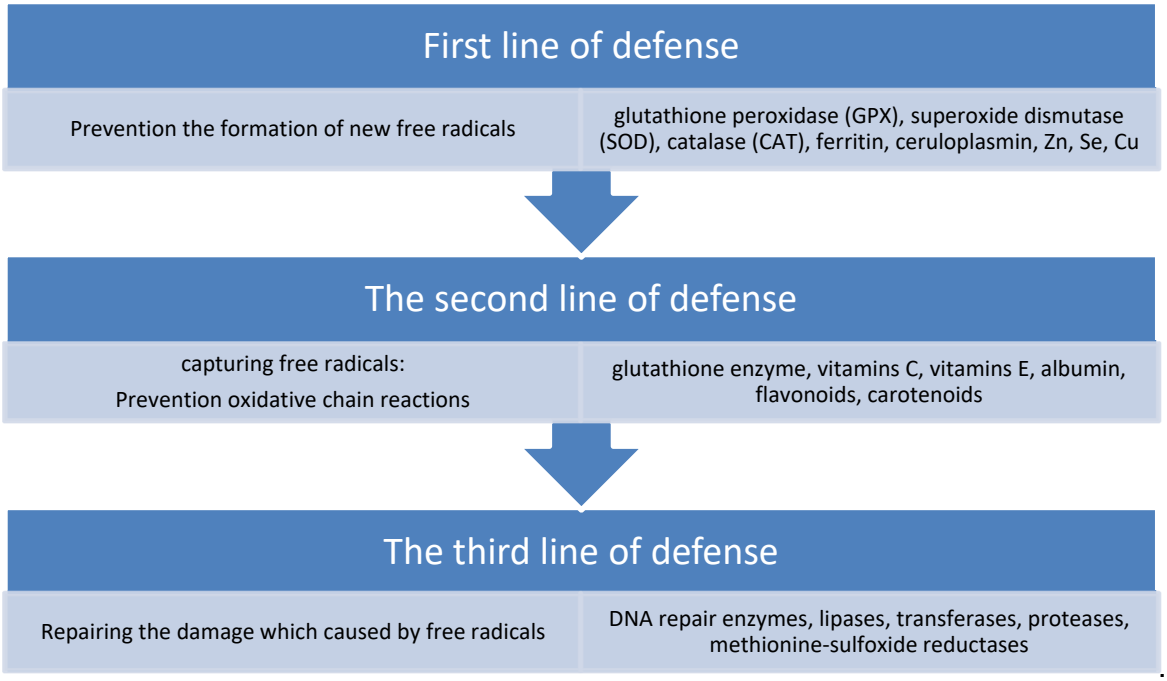


Figure 1. The antioxidant defense lines (Mut-Salud et al., 2016). (Picture not copy-right protected).

The use of antioxidants in clinical settings has been intensively studied in many different areas of biological and medical research. It was shown e.g., that use of antioxidants that exhibit some anti-proliferative and anti-inflammatory effects can diminish development of cancer (Valko et al., 2007, Bennett et al., 2012). Importantly, an increased ROS generation may also participate in the development of cellular senescence (Passos et al., 2009, Tchkonia et al., 2010, Debacq-Chainiaux et al., 2010, LeBrasseur et al., 2015) and numerous studies showed that diminishing of oxidative stress by antioxidants may decelerate the development of cellular

senescence (Chen et al., 1995, Lee et al., 1999, Macip et al., 2002, Munoz-Espin and Serrano, 2014).

1.4 Inflammation mediated by the transcription factor NF-κB contributes to the development of cellular senescence

Inflammatory response can be seen as defense mechanism of the body against different harmful stimuli such as UV- or γ-irradiation, microbial- or viral infection. Also, the presence of damaged cells or malignant cells in tissues or over-activated adaptive immune response promote the inflammatory response (Medzhitov, 2008). Inflammatory signs of the injured tissue include redness, swelling, overheating, pain and functional restriction. While acute inflammation is generally considered as beneficial for body tissues, chronic inflammation may worsen the outcome of many chronic diseases including atherosclerosis, metabolic syndrome and cancer (Medzhitov, 2008).

Inflammation is tightly controlled by the main pro-inflammatory transcription factor NF-κB that plays a crucial role in mediating inflammatory response (Baeuerle and Henkel, 1994, Oeckinghaus and Ghosh, 2009) (Figure 2).

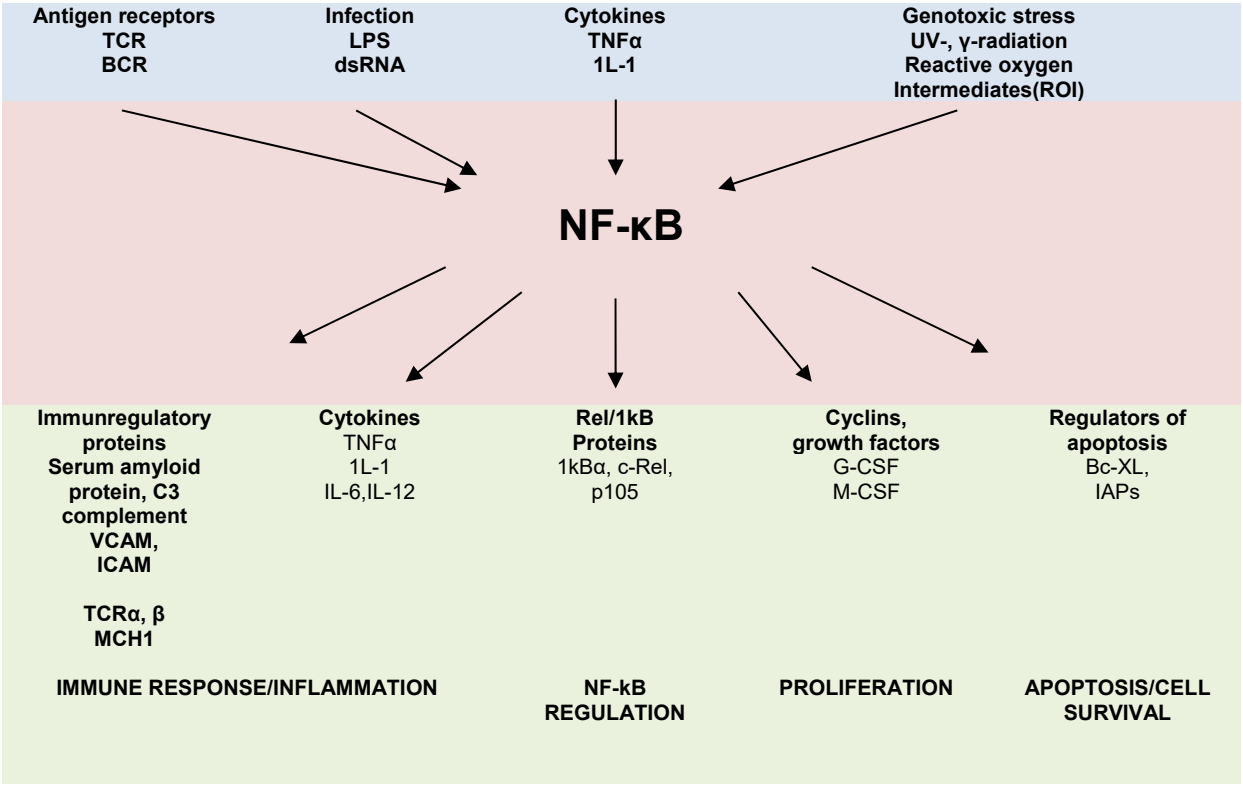


Figure 2. NF-κB acts as a central mediator of immune and inflammatory responses (Hayden et al., 2006).

NF-κB response may be activated by inflammatory mediators produced by immune-cells such as tumor necrosis factor (TNF), chemokines, interleukin-1 (IL-1),

and interleukin-6 (IL-6), lipopolysaccharides (LPS), infection, genotoxic stress and immune-regulatory proteins (Perkins, 2007, Medzhitov, 2010). Activation of NF-κB leads to transcription of chemokines, cell adhesion molecule, cytokines, and acute-phase proteins controlling the inflammatory process.

This central pro-inflammatory transcription factor is very critical for controlling numerous physiological and pathological processes including immunity, inflammation, cell proliferation, apoptosis, survival, and differentiation. NF-κB was shown to mediate pro-inflammatory processes in many different cell-types e.g., in leukocytes and endothelial cells by activating many downstream pathways (Rao et al., 2007, Wissink et al., 1997).

It has been shown that mutations in NF-κB contribute to many human diseases (Courtois and Gilmore, 2006). Accordingly, any dysregulation of NF-κB pathways results in diseases like immunodeficiency, autoimmunity, arthritis, and cancer (Ben-Neriah and Karin, 2011). In addition, NF-κB activity was found dysregulated and over-activated in many types of solid and hematopoietic malignancies including breast cancer and different types of leukemia (Matsumoto et al., 2005), colorectal cancer (Sakamoto and Maeda, 2010), glioblastoma(Nogueira et al., 2011), hepatitis-associated hepatocellular carcinoma (Luedde and Schwabe, 2011), esophageal cancer (Li et al., 2009), uveal melanoma (Triozzi et al., 2008) as well as pancreatic cancer (Vaccaro et al., 2011). Studies of controlling cancer progression and development also showed the importance of the NF-κB signaling pathway as a target for cancer therapy, by inhibiting IkappB kinase (IKK) activity using natural compounds or anti-inflammatory and immunomodulatory drugs (Karin et al., 2004, Karin, 2009).

Under physiological conditions, the NF-κB response is automatically self-limiting by IκBα that masks the nuclear localization sequences in NF-κB proteins (Ben-Neriah and Karin, 2011). Furthermore, regulation NF-κB affecting different cell types and tissues is governed by negative regulators such as IκB proteins and positive regulators such as IκB kinases. Its subunit IκB kinase-2 (IKK2) plays a crucial role in regulation of NF-κB. Phosphorylation of IKK2 causes a deterioration of the inhibitory subunit IκB-α, resulting in nuclear translocation of the NF-κB (Baeuerle and Henkel, 1994, Oeckinghaus and Ghosh, 2009).

Inflammatory drugs inhibit different mediators of NF-κB signaling pathway including IKK I-kappaB and I-kappaB (Gamble et al., 2012, Fakhrudin et al., 2014). It

was shown that suppression of I-kappaB, an inhibitor of NF-κB, promotes apoptosis (Paillard, 1999). NF-κB is also involved in regulation of apoptosis by controlling the expression of the anti-apoptotic Bcl family members and inhibitors of apoptosis (IAPs) as well as involved in regulation of proliferation and cell survival (Chen and Manning, 1995, Kopp and Ghosh, 1995, Hayden et al., 2006). Thus the ability of NF-κB to control inflammation and apoptosis points towards an important role of this transcription factor in controlling cellular senescence.

1.5 Strategies to selectively target senescence

Many attempts to elaborate strategies for prevention or better treatment of age-related diseases have been made in the last couple of decades. To this end, researchers investigated if targeting of certain signaling pathways by certain compounds might delay aging. Treatment of mice with rapamycin, an inhibitor of mTOR, slowed down their aging (Wilkinson et al., 2012, Zhang et al., 2014, Mannick et al., 2014, Bitto et al., 2016) and increased their lifespan (Harrison et al., 2009). Rapamycin administration also alleviated age-dependent cognitive deficits and diminished the expression of inflammatory cytokine IL-1beta (Majumder et al., 2012). Such treatment further stimulated NMDA (N-methyl-D-aspartate) signaling (Majumder et al., 2012). Treatment of cardiac patients with rapamycin, as shown by a group of Sinclair, improved their physical performance, reduced levels of TNFα and interleukin-8 (IL-8), and diminished the activity of senescence-associated beta-galactosidase, a known marker of senescence (Singh et al., 2016). Another substance that showed beneficial anti-aging in mice was resveratrol, reducing inflammation and mimicking effects of calorie restriction (Pearson et al., 2008, Barger et al., 2008). Many of these effects were assigned to enhancing levels of the NAD-dependent deacetylase, known as SIRT1, silent mating type information regulation 2 homolog 1 (Hubbard and Sinclair, 2014, Herranz et al., 2010). Furthermore, uptake of metformin by diabetic patients not only improved insulin resistance, but also enhanced their life span in comparison to a control group (Bannister et al., 2014). The use of nicotinamide riboside, an alternative form of vitamin B3 and nicotinamide mononucleotide, boosted levels of oxidized nicotinamide adenine dinucleotide (NAD+), improved vascular function, and was protective against oxidative stress in aged mice (de Picciotto et al., 2016).

Furthermore, some recent works also showed that selective elimination of senescent cells might significantly delay or alleviate certain pathological states linked to aging e.g., increase adipogenesis and metabolic function in old age (Xu et al., 2015a). It was also shown that such approach might be beneficial for cancer patients, as also this condition is associated with an increase in damaged cells that accumulate in the tissues (Xu et al., 2015a).

Many studies also focused on targeting SASP, by applying different pharmacological drugs. For example, glucocorticoids suppressed IL-6, an important component of SASP (Laberge et al., 2012). Use of metformin interfered with IKK/NF-κB activation (Moiseeva et al., 2013). Rapamycin reduced IL-6 mRNA levels and suppressed translation of the membrane-bound interleukin 1A, IL1A (Laberge et al., 2015). It was also shown that mitochondrial dysfunction affects changes associated with SASP and may result in the absence of IL-1-dependent response (Wiley et al., 2016). Specifically, proliferating human cells with mitochondrial dysfunction exhibited so called “mitochondrial dysfunction-associated senescence (MiDAS)” that was characterized by lower nicotinamide adenine dinucleotide oxidized/reduced (NAD+/NADH) ratios that hindered the IL-1-associated SASP through AMPK-mediated p53 activation (Wiley et al., 2016).

Importantly, in 2015 Zhu et al. published a pivotal paper in which they showed that it might be possible to selectively eliminate senescent cells,and that such treatment may, in fact, improve clinical outcome in living organisms (Zhu et al., 2015). In cell cultures, suppressing of gene expression using siRNA (e.g., against PI3Kδ, p21, anti-apoptotic gene BCL-xL, or plasminogen-activated inhibitor-2, PAI-2), induced selective killing of senescent cells, while proliferating or quiescent, differentiated cells, remained unaffected (Zhu et al., 2015). The authors of this study furthermore showed “senolytic effects” of two compounds: dasatinib that eradicated senescent human fat cell progenitors, and quercetin that was efficient in selectively removing senescent human endothelial cells and senescent mouse bone marrow mesenchymal stem cells, BM-MSCs (Zhu et al., 2015). They also pointed to the fact that a pro-survival pathway in the elder cells might be associated with mitochondrial dysfunction, a finding that also may help to explain why senescence cells are more sensitive against certain drugs in comparison to young cells (Zhu et al., 2015). In a subsequent study, an intermittent oral treatment with dasatinib and quercetin combined reduced senescent cell markers in the aortic media of aged and

hypercholesterolemic mice, and improved their vasomotor function (Roos et al., 2016). These benefits were also associated with an increase in NO production (Roos et al., 2016). Use of the above two substances was also successful in alleviating consequences of a bleomycin-injury induced pulmonary hypertension in mice (Schafer et al., 2017).

A possible strategy of eliminating senescent cells might be their reprogramming to undergo apoptosis. In the year 1995 Wang reported that senescent fibroblast cells show resistance against apoptosis and that this phenomenon encompasses the persistence of pro-survival pathways that protects cells to become apoptotic (Wang, 1995). In such senescent cells a failure to suppress anti-apoptotic bcl2 protein was observed (Wang, 1995). Navitoclax (ABT-263), a specific inhibitor of the anti-apoptotic BCL-2 and BCL-xL proteins, was successfully tested for killing old hematopoietic stem cells (Paillard, 1999, Chang et al., 2016) as well as senescent HUVECs (Zhu et al., 2016). Further substances of interest might include e.g., danazol that has been proposed for the treatment of telomerase-related diseases (Townsley et al., 2016).

Additional information on different drugs with putative senolytic potential can be found in specialized scientific manuscripts or clinical studies, or in recent excellent review (Kirkland et al., 2017, Hudgins et al., 2018).

2 The aim of the work

Senescent cells, as discussed above, contribute to several human diseases involving Parkinson's disease, Alzheimer's disease, age-related macular degeneration, peripheral neuropathies, type 2 diabetes mellitus as well as arteriosclerosis and many types of cancer (Martin, 2011). Recently accomplished *in vitro* and *in vivo* studies in pre-clinical models revealed that it is very important to selectively target/remove senescent cells and spare non-senescent ones. Further identification of compounds with ability to attack senescence cells but spare the non-senescent ones might, therefore, be of immense clinical importance and might have a translation potential.

In our study, we investigatedthe senolytic capacity of two groups of substances, by testing their effects in endothelial cells. These compounds included:

1) Anti-oxidants:butylated hydroxytoluene (BHT/touluedine, a synthetic general ROS scavenger), diphenyleneiodonium chloride (DPI, an anti-oxidant inhibiting NADPH-oxidase enzyme), and the antioxidant N-Acetyl-L-cysteine (NAC).

2) Anti-inflammatory substances:plumericin (PL, an NF-κB inhibitor and IKK2 inhibitor), BAY11-7082 (a synthetic NF-κB inhibitor), and PHA 408 (a NF-κB inhibitor suppressing IKK2 activity).

We applied these compounds to senescent (“old”) HUVECs (at passage 30, P30) and “young” HUVECs passage3, P3), and to human endothelial cells immortalized with hTERT (HUVECs-hTERT) to see if they preferentially eliminate senescent cells and how they affect cell growth of non-senescent cells. Below, the compounds used in our study are briefly described.

2.1 Anti-oxidants used in this study

2.1.1 Butylated hydroxytoluene (BHT)

BHT (butylated hydroxytoluene) is a synthetic phenolic compound (Figure3) that has been shown to be protective against different chemical and physical agents. This compound, together with BHA (butylated hydroxyanisole), is the most widely used chemical antioxidant in the food and cosmetic industry (Carocho and Ferreira, 2013). Via reacting with peroxyl radicals, BHT quenches ROS (Lambert et al., 1996).

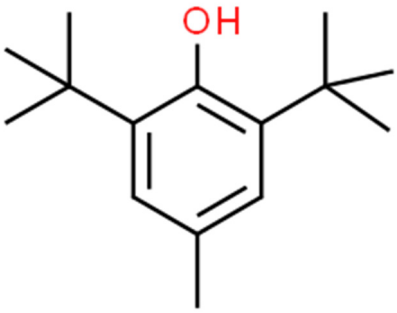


Figure 3: Structural formula of BHT
(http://www.chemspider.com/Chemical-Structure.13835296.html?rid=ad8a63d4-f666-4092-9cd2-71c3dc608b02)

The safety of BHT use was assessed in several studies in the 1980s and the 1990s in pre-clinical studies (Ito et al., 1985, Kahl and Kappus, 1993). BHT was not found to be carcinogenic in rats or mice (Ito et al., 1985). However, when applied in high doses, BHT interfered with blood clotting due to its ability to antagonize vitamin K (Kahl and Kappus, 1993). Nevertheless, it was concluded that BHT used in food, drugs, and cosmetics is probably harmless, and this compound is widely used in cosmetic formulations as an antioxidant at concentrations ranging from 0.0002% to 0.5% (Lanigan and Yamarik, 2002). Oral administration of BHT is regarded safe by the European Food Safety Authority (EFSA) if applied at daily doses less than 1.0 mg/kg/day to adults or children (Carocho and Ferreira, 2013).

2.1.2 Diphenyleneiodonium chloride (DPI)

DPI (Diphenyleneiodonium chloride, structure presented in Figure 4) has been known since decades as a hypoglycemic agent (Holland et al., 1973). Later studies showed that this compound inhibits NADH-ubiquinone-1 and NADH-menadione reductase (Ragan and Bloxham, 1977). DPI (at 3 and 10 μM) also inhibited potassium and calcium currents in pulmonary artery smooth muscle cells as well as in type I cells derived from carotid body of neonatal rats (Wyatt et al., 1994, Weir et al., 1994).

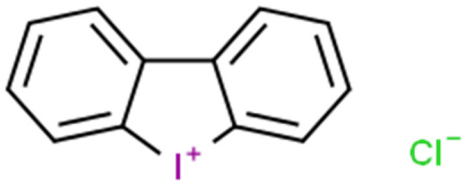


Figure 4. Structural formula of DPI
(http://www.chemspider.com/Chemical-Structure.2015292.html?rid=5ce7cd8d-2748-4286-9e69-ba151fd377fb&page_num=0)

In neutrophils, DPI suppressed NADPH-dependent superoxide production by 36% (Cross and Jones, 1986). Strikingly, some studies also reported opposing effects of DPI, which in N11 glial cell even induced oxidative stress (Riganti et al., 2004), and in zebrafish suppressed DNA repair genes (Osaki et al., 2011). However, a recent study showed that DPI is an anti-oxidant that reduces oxidative stress via inhibiting NADPH-oxidase, an enzyme catalyzing ROS production by mitochondria (Ozsvari et al., 2017). The authors of this study found that DPI suppressed mitochondrial respiration via inhibiting flavin-containing enzymes- flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Furthermore, DPI induced chemo-quiescence phenotype that strongly diminished the propagation of cancer stem cells (IC-50 of 3.2 nM). At 10 nM, DPI suppressed the oxidative mitochondrial metabolism (OXPHOS) by inhibiting mitochondrial ATP production by more than 90%. Even though this effect could be induced only after one hour of DPI treatment, further experiments showed the ability of DPI to potently reduce mitochondrial activity during an extended treatment of cells. Furthermore, the suppressing effects on mitochondrial activity were reversible (Ozsvari et al., 2017).

2.1.3 N-Acetylcysteine (NAC)

NAC (N-acetylcysteine, alternatively designated as N-acetyl-L-cysteine or acetylcysteine, structure presented in Figure 5) is a synthetic N-acetyl derivative from

the amino acid L cysteine. It is a precursor of the antioxidant glutathione and can act as a direct ROS scavenger. NAC structure contains two chelating sites (thiol and hydroxyl) that are responsible for its antioxidant potential as well as for providing the substrate for the generation of intracellular glutathione (GSH) synthesis (Ziment, 1986), as presented in Figure 5. Both NAC and glutathione (GSH) are ROS/RNS scavenging (Ziment, 1986).

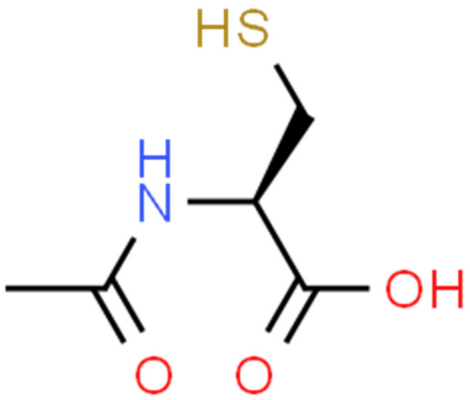


Figure 5. Structural formula of NAC showing two chelating sites (thiol and hydroxyl) and deacetylation leading to the generation of glutathione that is accountable for the NAC antioxidant.
(http://www.chemspider.com/Chemical-Structure.11540.html?rid=927b6f74-4093-4897-9bcc-c629cd16d7b3&page_num=0).

In cultured endothelial cells, NAC was protective against exposure to hyperoxia or treatment with paraquat (Junod et al., 1987). *In vivo*, NAC treatment was chemoprotective in a rat model of cisplatin-induced cytotoxicity and apoptosis (Dickey et al., 2004, Wu et al., 2005). Pretreatment of rabbits with NAC before injecting doxorubicin diminished both apoptosis and oxidative stress in cardiomyocytes (Wu et al., 2014). Beneficial effects of use of NAC were also demonstrated in a rat colitis model (Cetinkaya et al., 2005). NAC supplementation furthermore reduced ROS-mediated hepatocellular tumor growth in rats (Shimamoto et al., 2011). This compound was found beneficial in alleviating chronic obstructive pulmonary disease (Santus et al., 2014, Rushworth and Megson, 2014). Besides anti-oxidative effects, NAC was also shown to inhibit NF-κB activation *in vivo* (Wu et al., 2014). Finally, a treatment of bone marrow-derived mesenchymal stem cells with

NAC or DPI (a NADPH oxidase inhibitor reviewed above), was beneficial in alleviating premature senescence induced by high glucose treatment, as such treatment reduced interleukin-6 production (IL-6) production and suppressed autophagy (Chang et al., 2015). In a recent study, NAC, similarly as the below described inhibitors of the NF-kappaB pathway, PHA-408 and plumericin, prevented development of TNF-α induced premature senescence in HUVECs (Khan et al., 2017).

2.2 Anti-inflammatory compound used in this study

2.2.1 BAY11-7082

BAY11-7082 [(E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile, CAS 19542-67-7), a compound with a structure presented in Figure 6, is a synthetic NF-κB inhibitor (Pierce et al., 1997).

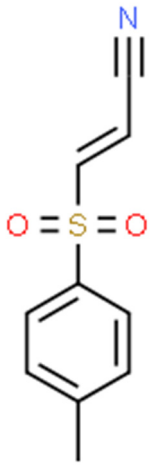


Figure 6. Structural formula of BAY11-7082
(http://www.chemspider.com/Chemical-Structure.4510086.html)

Numerous studies presented below showed the anti-inflammatory effects of this substance. BAY11-7082, by inhibiting IKK, increased apoptosis in neutrophils (Dai et al., 2003) as well as in adult T-cell leukemia cells infected with human T-cell leukemia virus type I, HTLV-I (Mori et al., 2002) and in gastric cancer cells (Chen et al., 2014). Treatment with BAY11-7082 of uveal melanomas in which NF-κB signaling pathway is highly activated, decreased dose-dependently nuclear translocation of NF-κB, suppressed cell proliferation, migration, and stimulated apoptosis without affecting the cell cycle (Hu et al., 2012). In HUVECs stimulated with LPS, BAY11-

7082 suppressed ICAM-1 expression (Lee et al., 2004). In human colon cancer HT29 cells, BAY11-7082 induced the expression of a protective enzyme heme oxygenase-1 (Min et al., 2011). This compound also inhibited proliferation of lung cancer cells (Gastonguay et al., 2012). It also was shown that BAY11-7082 exhibits abroad-spectrum of inhibitory activity against numerous signaling pathways, including release of inflammatory signaling molecules (TNF-α, nitric oxide, NO, and prostaglandin E2, PGE2 (Lee et al., 2012). Furthermore, use of BAY11-7082 induced apoptosis by reducing expression of anti-apoptotic protein Bcl-2, and increasing activity of an apoptotic gene caspase 3, but not affecting the expression of the pro-apoptotic Bax (Hu et al., 2012).

2.2.2 PHA-408

PHA-408 (structure presented in Figure 7) is a highly selective inhibitor that tightly binds IκB kinase-2 (IKK-2) and competes with ATP binding (Mbalaviele et al., 2009, Sommers et al., 2009). Systemic oral or local delivery of PHA-408 suppressed LPS-induced cytokine production and cell infiltration in rats (Sommers et al., 2009). PHA-408, similarly as NAC and plumericin, prevented development of TNF-α induced premature senescence in HUVECs (Khan et al., 2017).

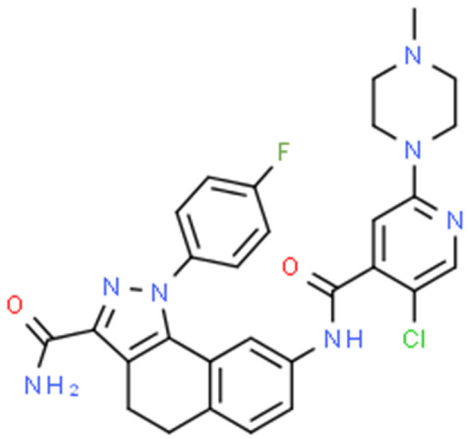


Figure 7. Structural formula of PHA-408
(http://www.chemspider.com/Chemical-Structure.11321741.html?rid=c249e6c5- c44c-479a-b072-bf223c3ec438)

2.2.3 Plumericin (PL)

Plumericin, a compound with the structure presented in Figure 8, was firstly isolated from stem bark extracts of an evergreen tree *Himatanthus sucuuba* (Waltenberger et al., 2011), traditionally used for treatment of many inflammation-associated diseases such as cough, pain, tumors, arthritis and gastritis especially in the nascent population in South America (Amaral et al., 2007). Fakhrudin *et al*. found that plumericin inhibits NF-κB pathway by selectively inhibiting IKK-mediated phosphorylation and degrading IκB (Fakhrudin et al., 2014). Yet, PL in a recent study was shown to interact with antioxidant glutathione, depleting its reserves (Heiss et al., 2016). As mentioned above, use of PL, similarly as NAC and PHA-408, prevented the development of TNF-α induced premature senescence in HUVECs (Khan et al., 2017).

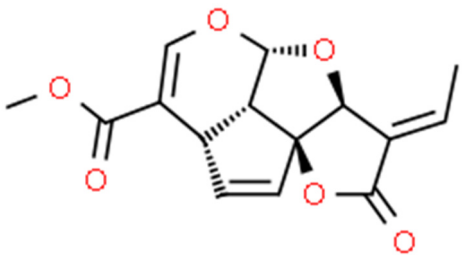


Figure 8. Structural formula of plumericin
(http://www.chemspider.com/Chemical-Structure.4444877.html)

3 Material and Methods

3.1 Material

In our experiments, we used following reagents listed in Table 1.

Name	Suppliers
PL(Plumericin)	Kindly provided by Dr. Stuppner, Institute of Pharmacy/ Pharmacognosy, University of Innsbruck, Innsbruck, Austria
PHA 408	Axon Medchem BV (Groningen, Netherland)
NAC (N-acetyl-L-cysteine)	Sigma-Aldrich, Saint Louis, USA
Triton X-100	Sigma-Aldrich
M199 medium	Sigma-Aldrich
Paraformaldehyde (PFA)	Sigma-Aldrich
FBS (Fetal bovine serum)	Sigma-Aldrich
Gelatin	Sigma-Aldrich
Goat serum	Sigma-Aldrich
DPI (Diphenyleneiodonium chloride)	Sigma-Aldrich
BHT (Butylated hydroxytoluene)	Sigma-Aldrich
(BAY11-7082) [(E)-3-(4-Methylphenylsulfonyl) -2-propenenitrile]	Sigma-Aldrich
Trypsin-EDTA	LONZA, Visp, Switzerland
Penicillin	LONZA
Streptomycin	LONZA
ECGS (Endothelial cell growth supplement)	Promocell (Heidelberg, Germany)
DAPI	Life Technologies, Columbus, USA

Table 1 List of materials used

3.2 Tested anti-oxidative and anti-inflammatory substances

We used following six substances:the anti-oxidants BHT, DPI and NAC, and the anti-inflammatory compounds PL, BAY11-7082 and PHA 408. We tested the

senolytic capacity of these compounds used alone or in combination in primary human umbilical vein endothelial cells (P30 and P3 HUVECs) as well as in an immortalized endothelial cell line, HUVECs-hTERT, as specified below. To this end, we followed the changes in cell counts of treated or control cells over 8 days.

3.3 Cell cultures

We grew primary HUVECs starting from passage 2 or passage 23 (Crampton et al., 2007) as well as immortalized HUVECs-hTERT (kindly provided by Prof. Hannes Stockinger, Medical University of Vienna), prepared by transfection of primary HUVECs with human telomerase reverse transcriptase, in flasks coated with 1% gelatin. Cells were cultured in sterile conditions in an incubator at 37°C, 5% CO_2 and 95% humidity, using M199 medium containing 20% FBS, 3mg/ml endothelial cell growth supplement (ECGS), 2 mM L-glutamine, streptomycin (100ug/ml), penicillin (100 U/ml). To restrict microbial growth, all media, supplements, and reagents for cell culture were filter-sterilized. Within cell passaging, the media were changed every other day.For the experiments, we used HUVECs at passage3 (P3), HUVECs at passage 30 (P30) as well as immortalized HUVECs-hTERT (at passage more than 70) cultured in 384 well plates. Cell count was determined using an automated BIO-RAD cell counter TC10 (Biorad, Vienna, Austria).

For preparing P3 of HUVECs used for the experiments, we chose P2 of HUVECs stored at liquid N2, thawed them quickly at 37°C, added 5 ml of complete M199 medium, and centrifuged them at 400 g for 5min. We removed the supernatant, resuspended cells in M199 complete medium, placed them into T25 flask and cultivated them in the incubator. After three days, when cells have become confluent, we removed the medium and rinsed the cells with Hanks' Balanced Salt Solution (HBSS). We exposed cells for 3 to 5 minutes to pre-warmed Trypsin-EDTA until they started releasing from the bottom of culture flasks. Subsequently, to inactivate Trypsin-EDTA, we added the excess of a complete M199 medium, and after centrifugation at 1700 rpm for 5min, we removed the supernatant and resuspended cells in M199 complete medium. We determined the cell count using an automated cell counter (BIO-RAD TC10) and transferred ~ 1000 cells per selected wells of 384 well plates.

In order to obtain HUVECs at passage P30 used for the experiments as "senescent cells", we thawed the P23 of HUVECs stored at liquid N2. We kept

cultivating them in T25 flask in complete M199 media until cells have become confluent. Then we split them by transferring into a middle-size flask (T75). We daily checked the cells by phase-contrast microscopy. Passaging of cells at 1: 3 ratio was repeated until we obtained HUVECs of passage 30 (P30). Already at P25 and P26, we observed deceleration of the growth rate: Transferred cells became semi-confluent only after 5 to 6-day cultivation and at the same time they became enlarged, as assessed by microscopy (data not shown). For experiments, we used P30 of HUVECs by transferring of ~ 1000 cells into selected wells of 384 well plates.

Concerning HUVECs-hTERT, we thawed them, cultivated in T25 flasks and after two to three days split them to T75 flask. Similarly, as for the HUVECs, we transferred ~ 1000 cells into selected wells of 384 well plates and exposed them subsequently to senolytic substances or to a control complete medium.

We left the cells grow in 384 well plates for 48 hours in the incubator at 37°C and 5% CO_2. At this time-point, we aspirated the old M199 complete medium and added new medium pre-mixed with the substances to be tested. We recorded images every 12 hours using the Olympus imaging microscope system equipped with a cell-counting software described below.

3.4 Microscopy

Microscopy of endothelial cells was performed using the live-cell imaging microscope Olympus IX83 cellVivo (Olympus, Austria). This microscope system equipped with a cellSens 1.18 life science imaging software enabled the determination of cell counts of P3 and P30 HUVECs as well as immortalized cell line HUVECs-hTERT cultured in 384 well plates. The scheme of the experimental protocol is outlined in Table 2.

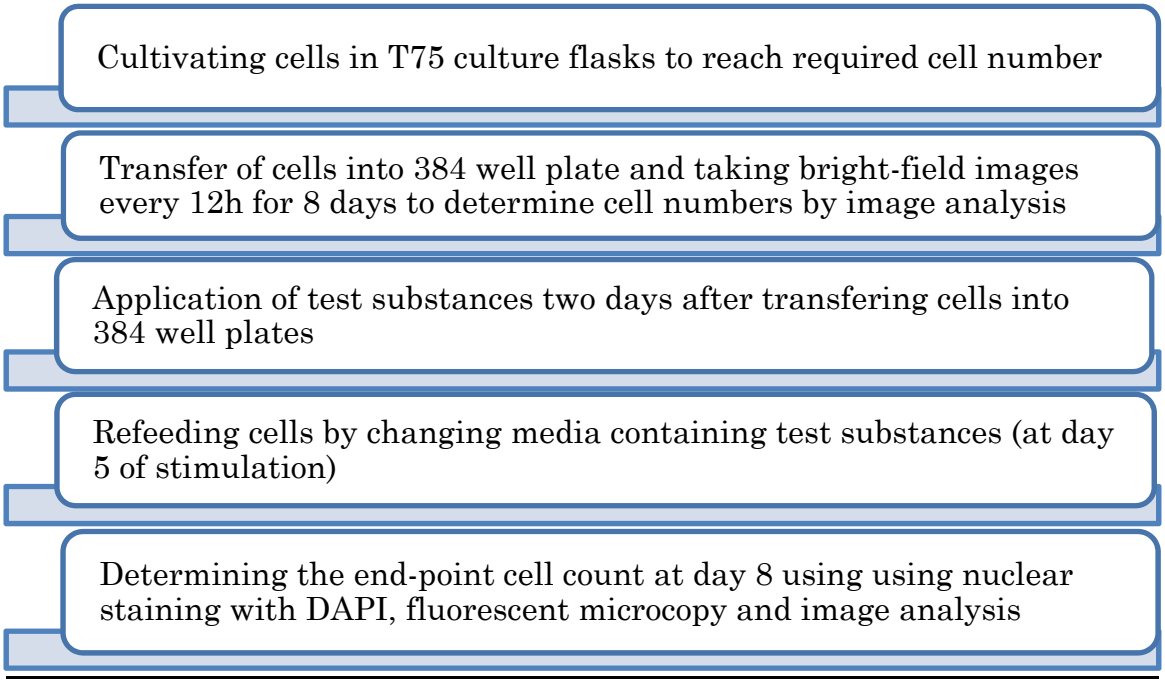


Table 2 The scheme of the experimental protocol for the analysis of cell count of stimulated and control endothelial cells cultured for 8 days

3.5 Statistical analysis

Statistical analysis was done using t-tests and p values <0.05 were considered significant.

4 Results

In our study, we tested the above mentioned six selected substances regarding their senolytic effects in human endothelial cells. These substances encompassed two groups: three anti-oxidative and three anti-inflammatory substances BHT (butylated hydroxytoluene, a general ROS scavenger), DPI (diphenyleneiodonium chloride, anti-oxidant inhibiting NADPH-oxidase) and NAC (N-acetyl-L-cysteine, antioxidant) and the anti-inflammatory compounds PL (plumericin, a NF-κB- and IKK2 inhibitor), BAY11-7082 (a synthetic NF-κB inhibitor), and PHA 408 (a very selective synthetic NF-κB inhibitor). We used these compounds separately or in combination with other substances to determine their putative synergistic or antagonistic effects. We applied these compounds to “young” HUVECs at passage3 (P3), to old (senescent) HUVECs at passage 30 (P30) and to human endothelial cells immortalized with hTERT (HUVECs-hTERT).

We tested these substances alone or in combination with other substances in triplicates.The results encompassing mean value and standard deviation are presented in Figures 9 to 21. For the evaluation of statistical significance, we used t-test.

4.1 Testing senolytic properties of antioxidants applied alone or in combination with selected NF-κB inhibitors

The following section describes how single tested antioxidants BHT, DPI, and NAC affected proliferation of P3 and P30 HUVECs as well as HUVECs-hTERT cells. In addition, it presents effects of these compounds when used in combination with selected anti-inflammatory substances (PHA-408 and plumericin).

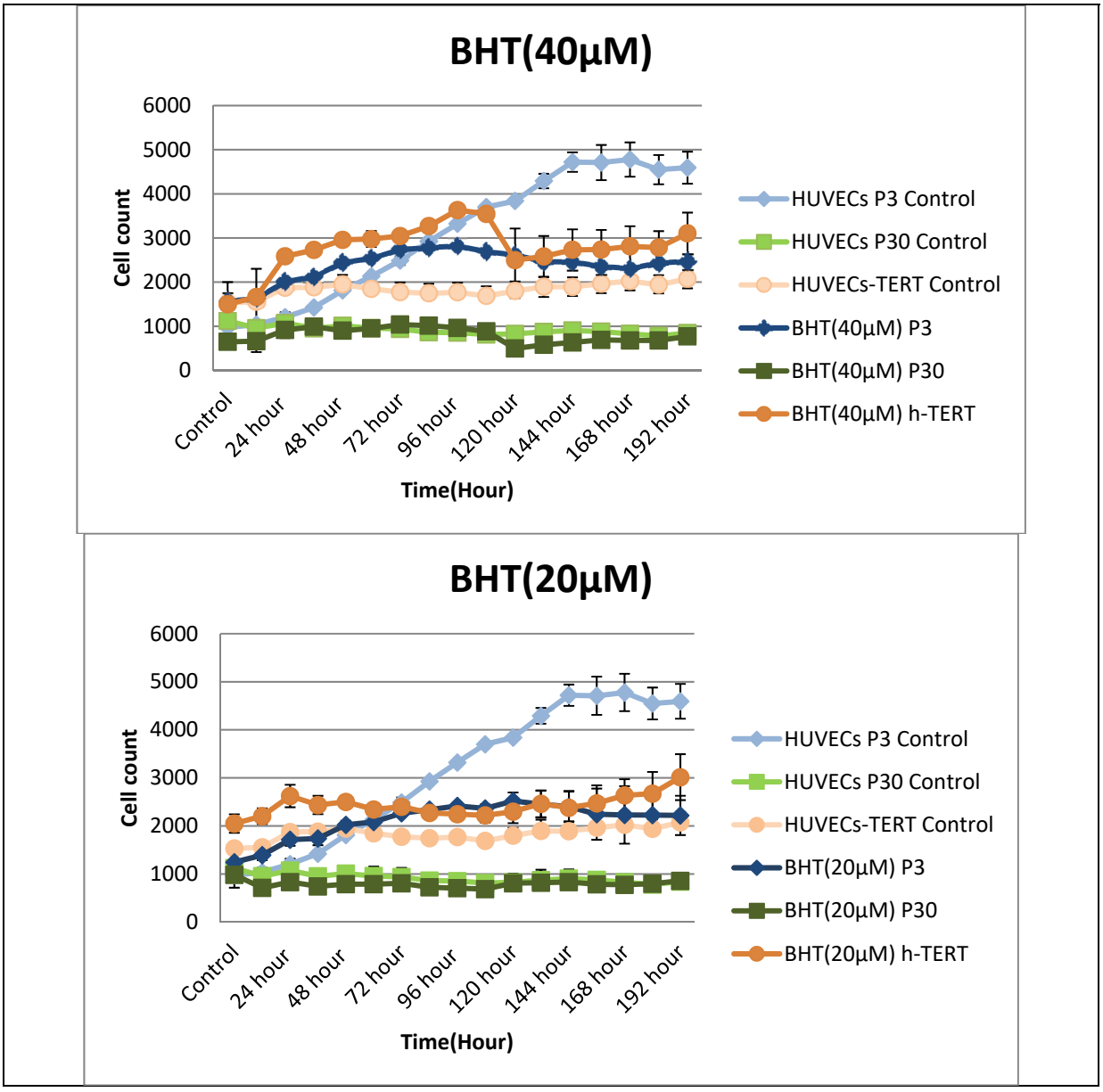
4.1.1 BHT does not exhibit senolytic activity, but tends to stimulate growth of HUVECs-hTERT

We exposed primary HUVECs at P3 and P30 as well as HUVECs-hTERT cells to the general ROS scavenger BHT using different concentrations (40, 20 and 10 µM) for 8 days and determined the cell number every 12 hours (Figure 9).

Treatment of P30 HUVECs with the highest concentration of BHT (40µM) did not significantly reduce cell number. On the other hand, P3 HUVECs subjected to this treatment grew slower than the non-treated P3 HUVECs. The number of HUVECs-hTERT cells that were treated with BHT (40µM), started to considerably

increase within 24 hours. After that, the cell count continued to rise albeit more slowly. After a media change, the cell count dropped, probably due to a physical stress during media exchange, but 12 hours later cells recovered and proceeded growing slowly.

Reduced concentrations of the anti-oxidant BHT at 20µM and 10µM also led to a diminished growth rate of P3 HUVECs when compared to non-treated cells and later on, it even led to a reduction in cell number. In addition, there was no discernable effect on the cell count of the senescent cells (HUVECs P30, Figure 9).



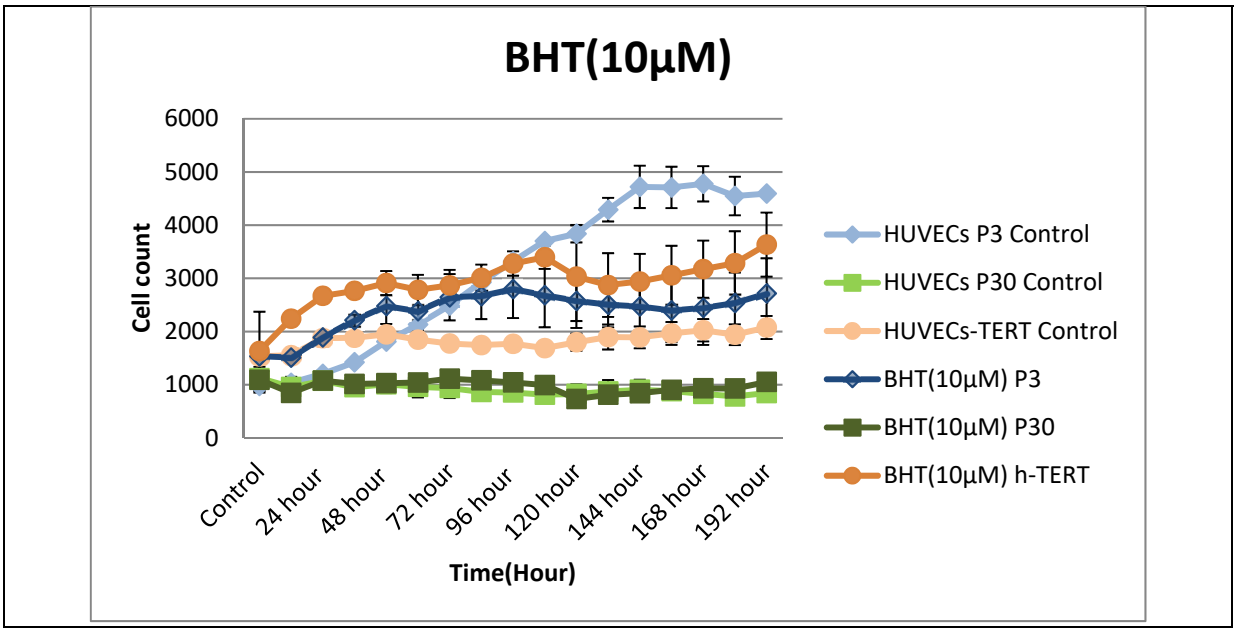


Figure 9: Effect of exposure of P3, P30, and –hTERT- HUVECs for 8 days to BHT (40, 20, 10µM) on their cell count assessed every 12 hours. No selective elimination of P30 was found compared to controls. The final cell number of P3 was lower upon BHT-treatment. Remarkably, there was an increase in proliferation of the very slowly growing of HUVECs-hTERT after treatment with BHT 40, 20 and 10µM.

4.1.2 BHT, in combination with PHA or PL, fosters growth of HUVECs-hTERT

We further tested if the effects of the anti-oxidant BHT could be modulated by the additional use of an anti-inflammatory substance. To this end, different concentrations of BHT (40, 20, 10 µM) in combination with PHA (2µM), a highly specific NF-κB inhibitor, were used to study effects on the cell count on P3 and P30 HUVECs, and HUVECs-hTERT (Figure 10). The combination of BHT and PHA did not selectively eliminate senescent P30 HUVECs. In addition, such combinations were detrimental for the growth/survival of the young P3 HUVECs, and did not show the activity profile looked for in a “senolytic screen”. On the other hand, HUVECs-hTERT benefitted from the treatment, and increased their capacity to proliferate over the whole observation period of 8 days (Figure 10).

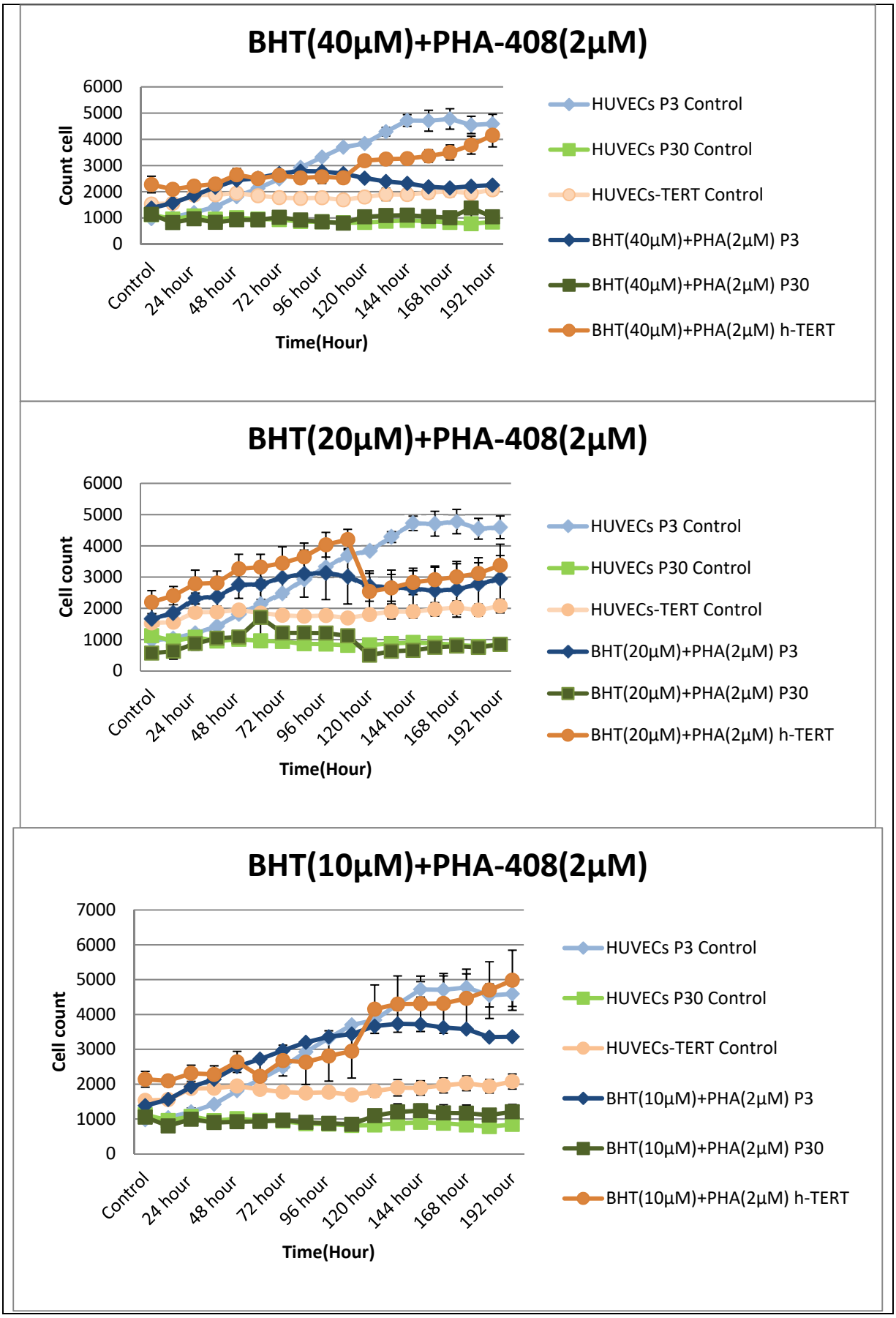
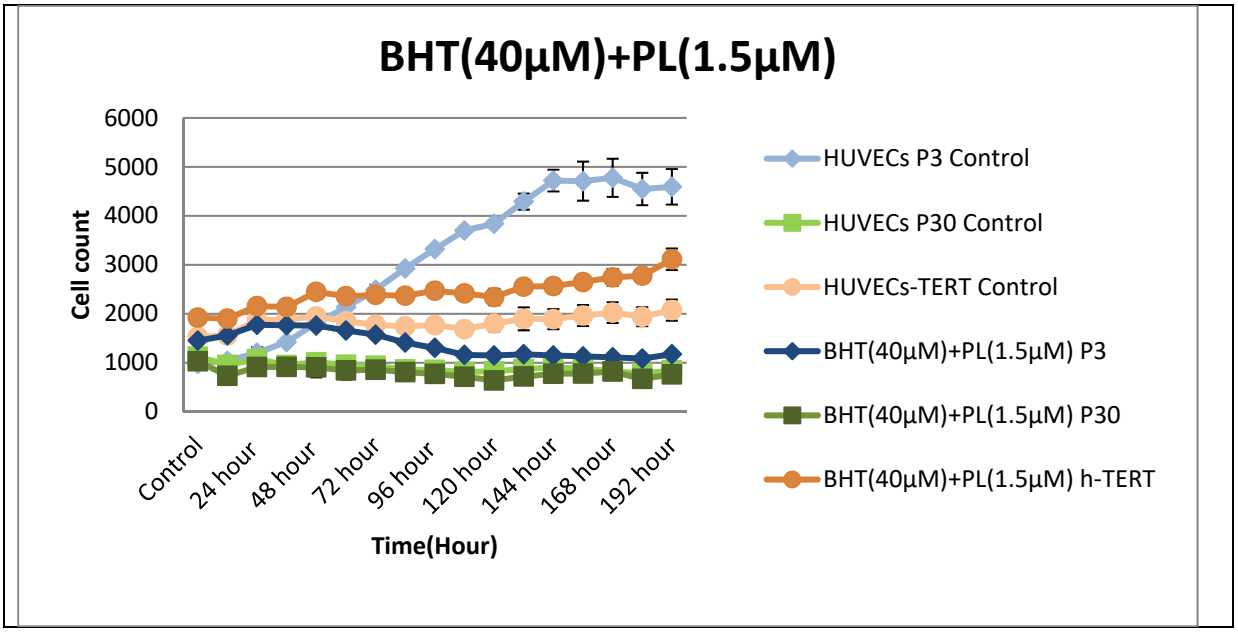

BHT(40µM)+PHA-408(2µM)
Count cell
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BHT(40µM)+PHA(2µM) P3
BHT(40µM)+PHA(2µM) P30
BHT(40µM)+PHA(2µM) h-TERT
BHT(20µM)+PHA-408(2µM)
Cell count
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BHT(20µM)+PHA(2µM) P3
BHT(20µM)+PHA(2µM) P30
BHT(20µM)+PHA(2µM) h-TERT
BHT(10µM)+PHA-408(2µM)
Cell count
7000
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BHT(10µM)+PHA(2µM) P3
BHT(10µM)+PHA(2µM) P30
BHT(10µM)+PHA(2µM) h-TERT

Figure 10. Effect of BHT (40, 20 and 10μM) combined with PHA-408 (2μM) on the cell count of P3, P30 and HUVECs-hTERT assessed every 12 hours. There was no selective elimination of P30 but a noticeable reduction in the proliferation of the P3 HUVECs and a strong increase in proliferation of HUVECs-hTERT.

Further, we studied the effects of a combined treatment of the antioxidant BHT and IKK2 inhibitor PL. The use of different concentrations of BHT (40, 20, 10μM) in combination with PL (1.5μM) resulted again in increased cell proliferation of HUVECs-hTERT, but not in selective elimination of P30 senescent HUVECs (Figure 11). The P3 HUVECs showed a dramatic decrease in cell number after treatment, especially in case of 40μM concentration of BHT. Altogether, the treatment of cells with BHT, in combination with PL (Figure 11), yielded results overall similar as in the case of a combined treatment of BHT with an NF-kB inhibitor PHA-408, presented in the previous section (Figure 10).



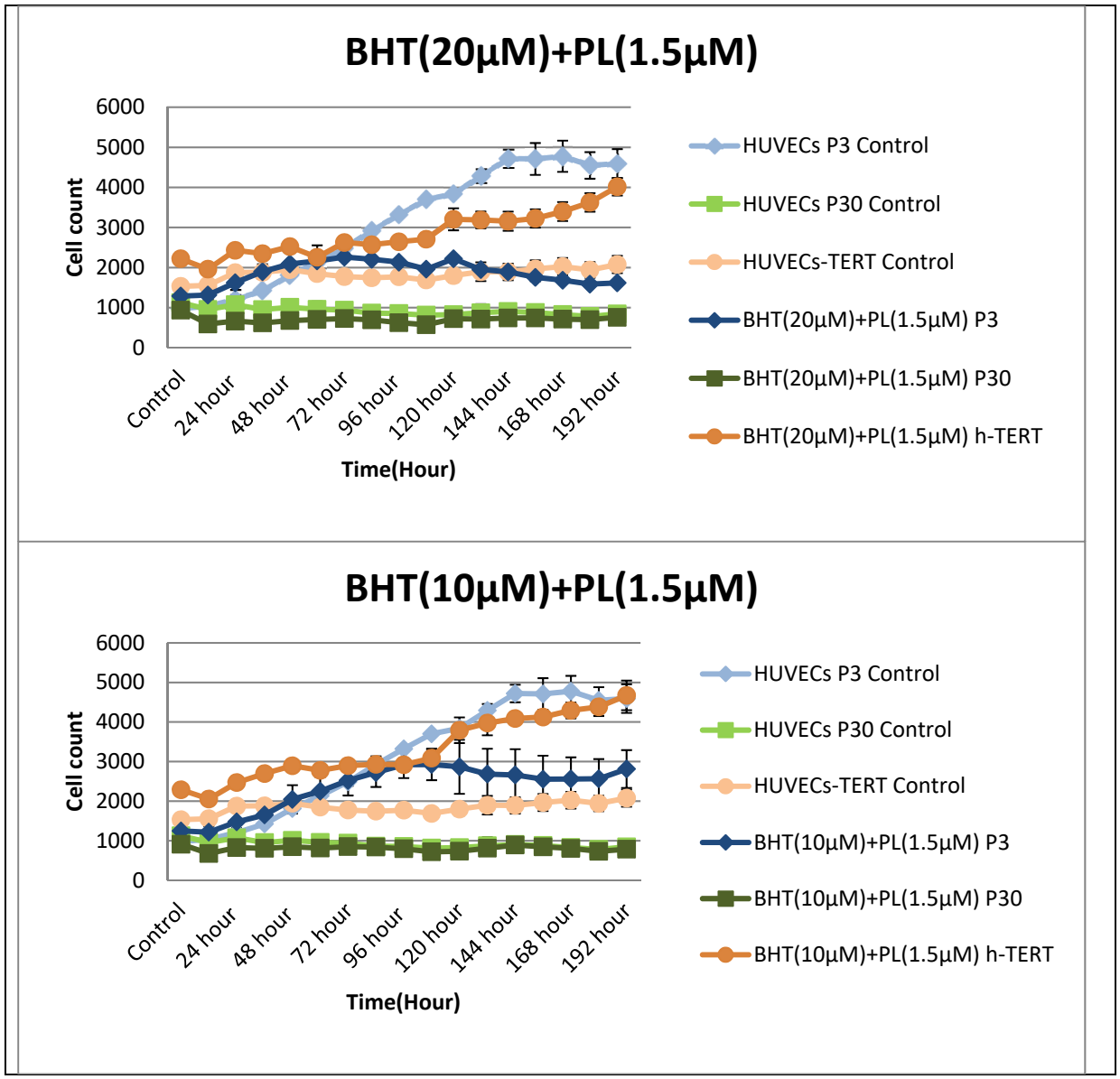
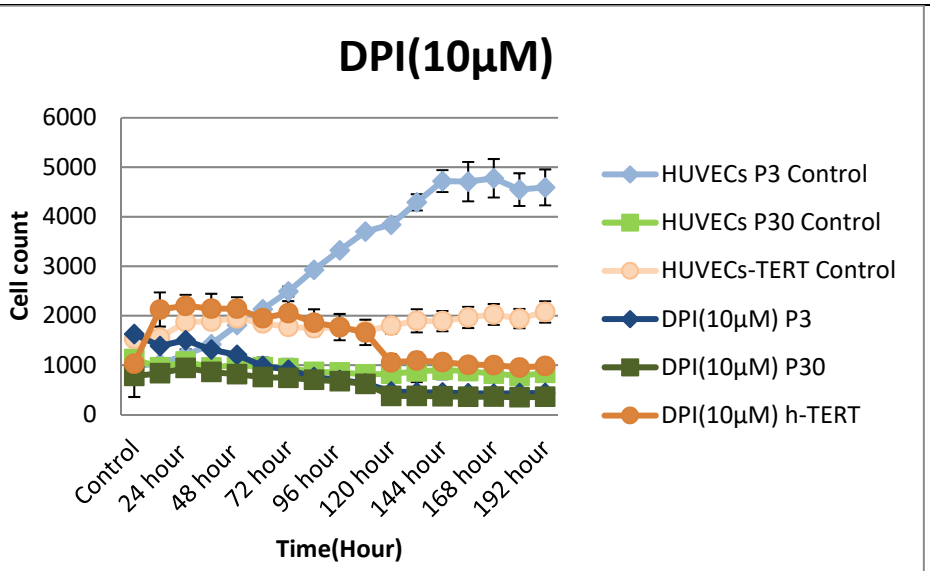
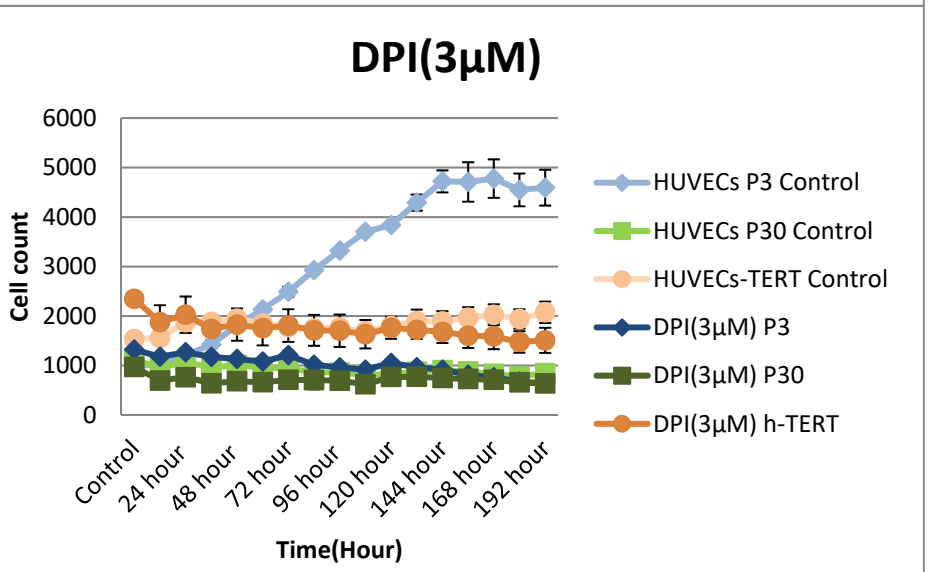


Figure 11. Effect of BHT (40, 20 and 10µM) combined with PL (1.5µM) on the cell count of P3, P30, and HUVECs-hTERT. The data show no selective elimination of P30 HUVECs, decrease in cell number of P3 HUVECs and an increase in cell number of HUVECs-hTERT.

4.1.3 DPI decreased the proliferation rate of HUVECs P3, P30 as well as HUVECs-hTERT

DPI, a substance known for its potent anti-oxidative properties due to inhibiting NADPH-oxidase and thus diminished ROS generation, was investigated in P3 HUVECs, P30 HUVECs and HUVECs-hTERTat three different concentrations (10, 3 and 1µM). We found that this substance decreased the cell number not only in the case of the senescent P30 HUVECs, HUVECs-hTERT, but especially also of the P3 HUVECs, at all three tested concentrations (Figure 12). While HUVECs-hTERTsuccumbed at the two higher concentrations (10 and 3 µM), they survived the exposure to 1µM DPI.


DPI(10μM)
Cell count
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
DPI(10μM) P3
DPI(10μM) P30
DPI(10μM) h-TERT


DPI(3μM)
Cell count
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
DPI(3μM) P3
DPI(3μM) P30
DPI(3μM) h-TERT

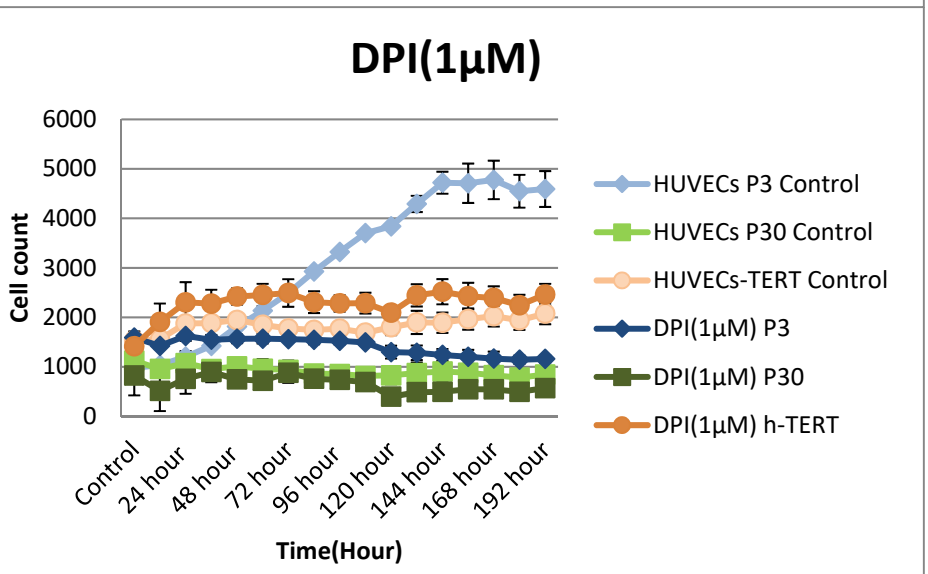

DPI(1μM)
Cell count
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
DPI(1μM) P3
DPI(1μM) P30
DPI(1μM) h-TERT

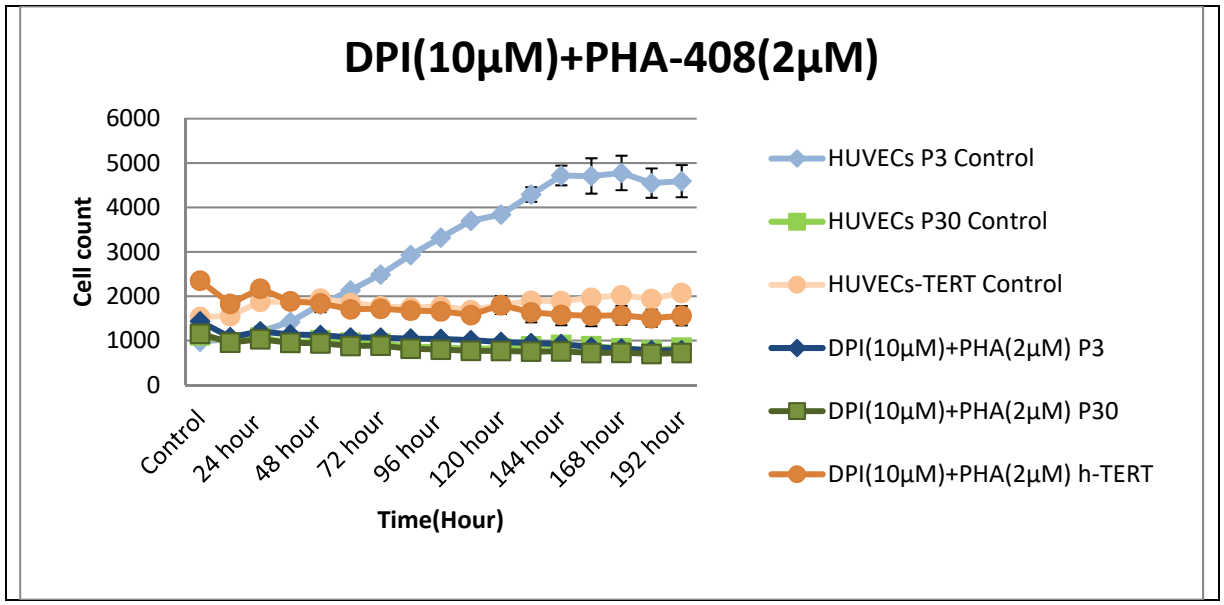
Figure 12: DPI decreases cell counts of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT at all used concentrations (10, 3 and 1μM) compared to controls.

4.1.4 DPI, in combination with PHA or PL, of HUVECs P3, reduced the number P30 as well as HUVECs-hTERT

In order to further examine effects of combinations of anti-oxidative/anti-inflammatory substances on cell elimination/cell proliferation, next we used the anti-oxidant DPI at different concentrations in combination with NF-κB inhibitor PHA-408. The results showed that such the combination of different concentrations of DPI (10μM, 3μM, and 1μM) with PHA-408 (2μM) decreased cell number in all tested cell-types (P3, P30, and HUVECs-hTERT cells) when assessed at day 8. However, neither of these combinations selectively eliminated P30 HUVECs (Figure 13).

Use of different concentration of DPI (10μM, 3μM, and 1μM) in combination with the NF-κB inhibitor PL (1.5μM) also decreased cell number in all tested cell-types (P3, P30, and HUVECs-hTERT cells). However, similarly as in the case of combination of DPI with PHA presented above, also the combination of DPI with PL did not preferentially eliminate P30 HUVECs (Figure 14).

Altogether, these results show that treatments of cells with DPI at concentrations 10μM, 3μM, and 1μM, used alone or in combination with PHA or plumericin, leads to cell loss in cultures of all three cell-types without showing selectivity for senescent cells.



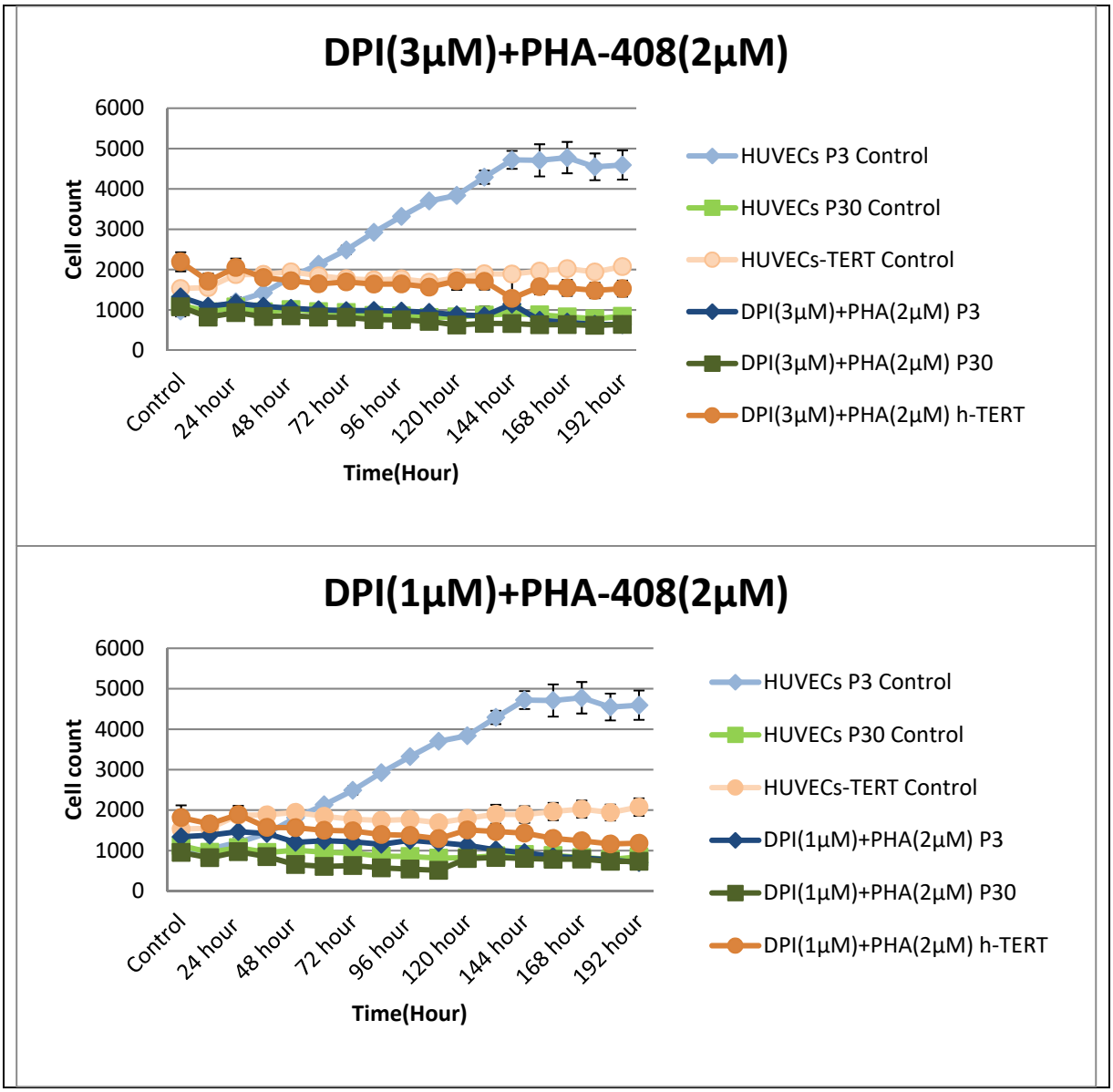


Figure 13. Effects of DPI (10, 3, 1μM) in combination with PHA (2μM) on cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. DPI potently impaired proliferation of P3 HUVECs and even decreased cell numbers of P30 HUVECs and HUVECs-hTERT.

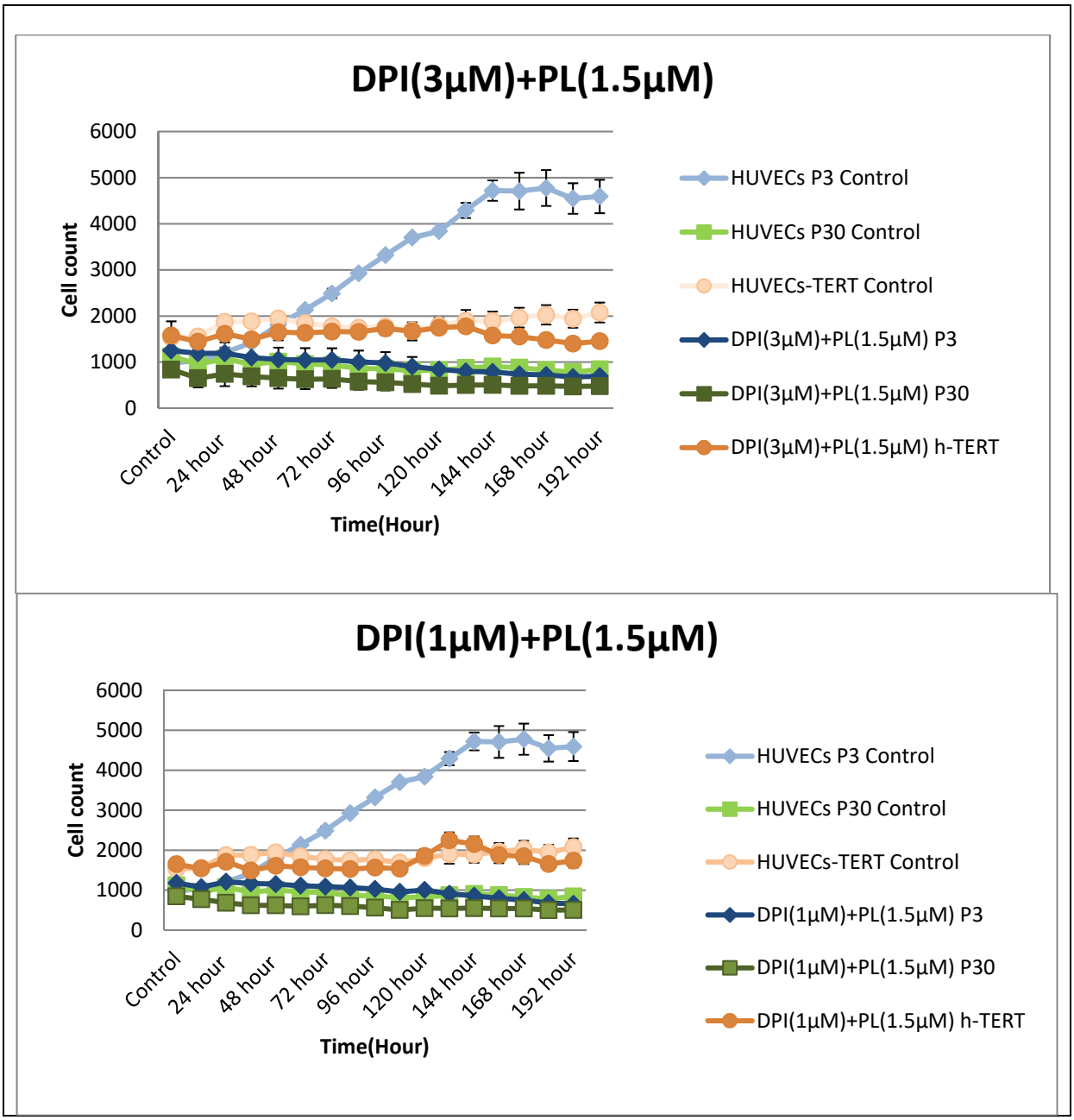


Figure 14. Effects of DPI (10, 3, 1µM) in combination with PL (1.5µM) on cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. DPI in combination with PL potently blocked proliferation of P3 HUVECs and even decreased their cell numbers.

4.1.5 NAC induced proliferation of HUVECs at passage 3 and of HUVECs-hTERT and did not deplete the senescent cells

Next we evaluated consequences of use of antioxidant NAC at 2mM and 1mM concentrations. While NAC did not significantly affect proliferation of P30 HUVECs, its use unexpectedly stimulated cell proliferation in P3 HUVECs. Similarly, we found a gradual (steady) increase in the proliferation of HUVECs-hTERT cells (Figure 15).

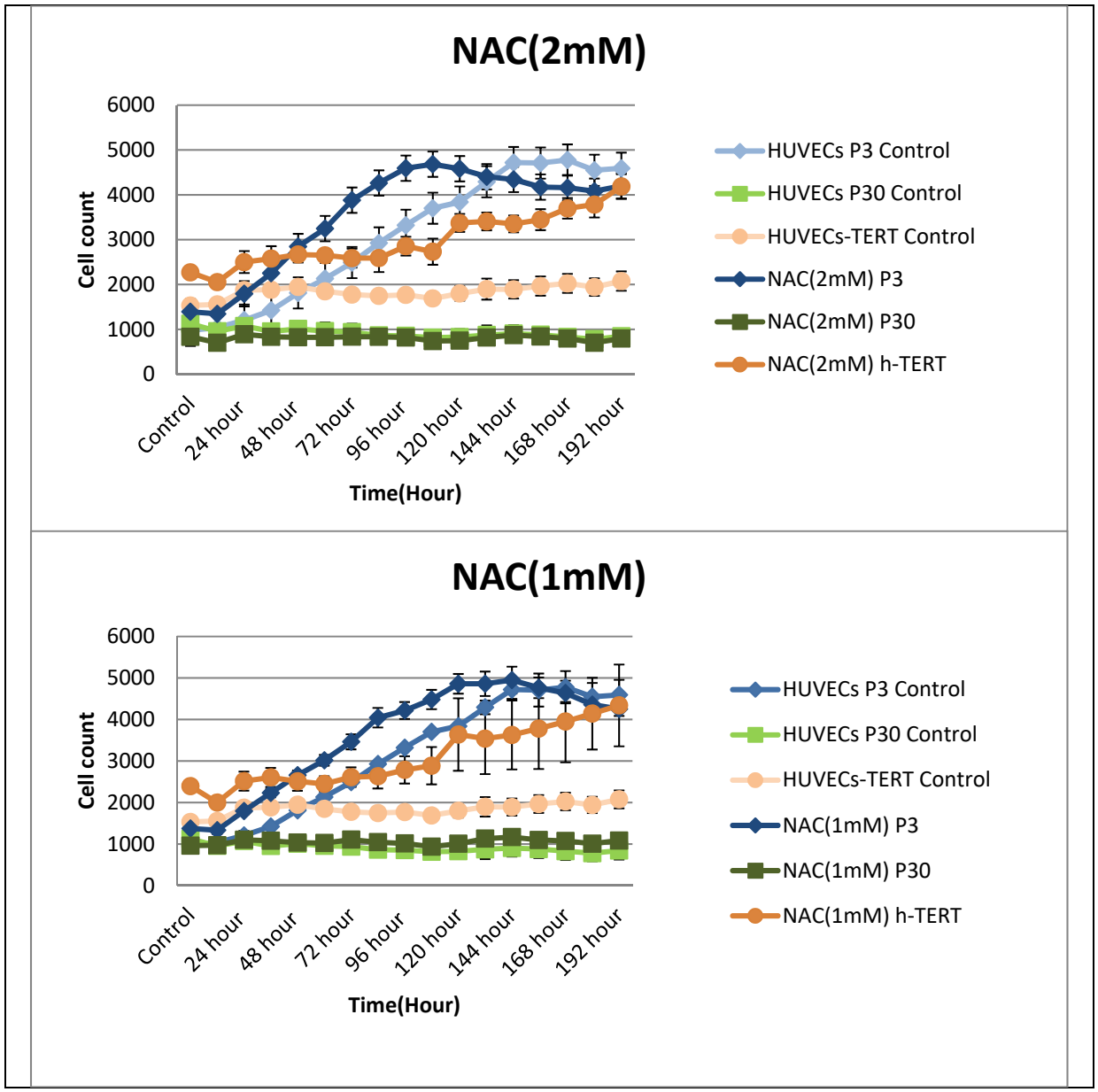


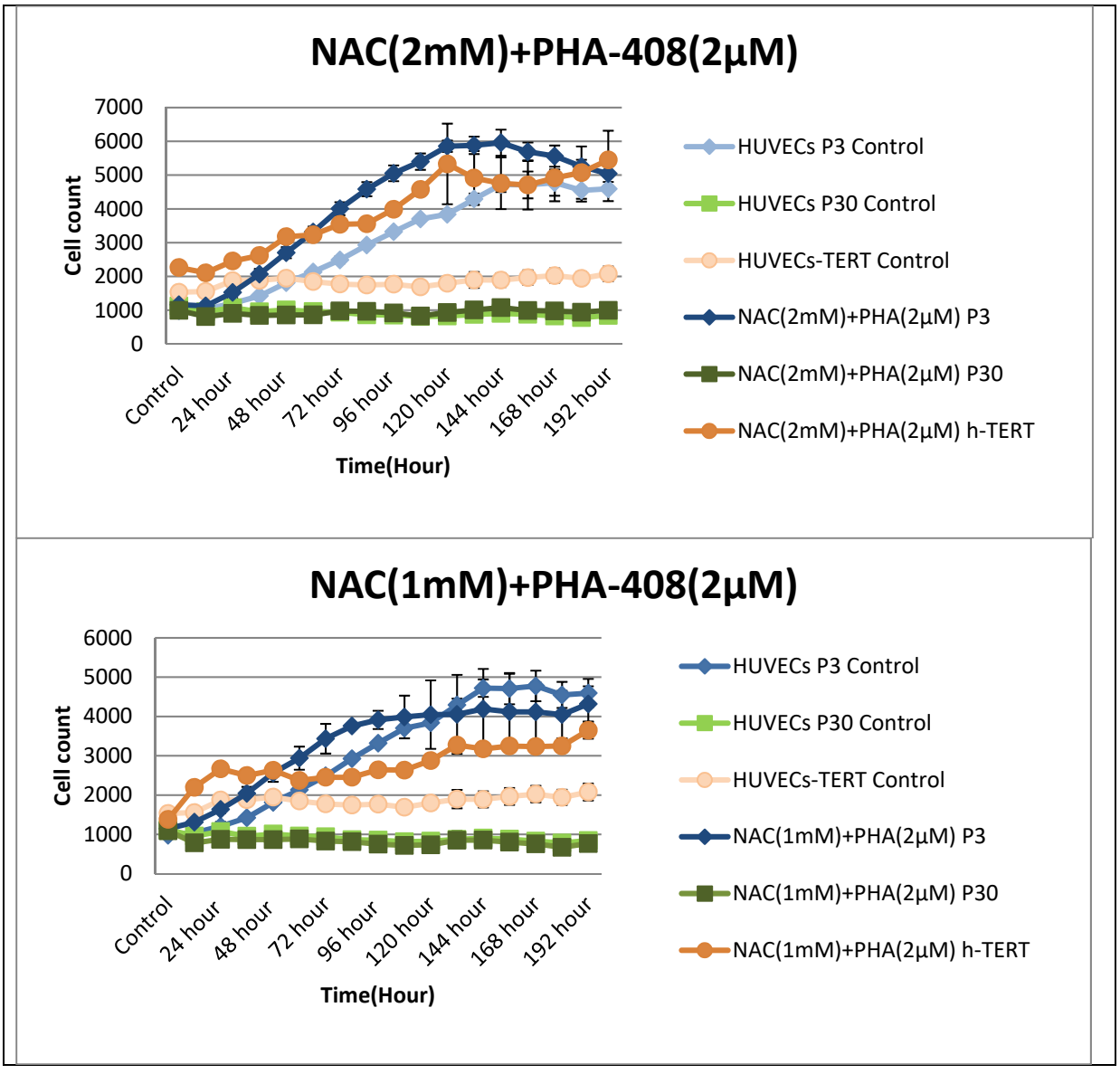
Figure 15. Effect of NAC (2 and 1mM) on the cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. NAC induced proliferation of P3 HUVECs as well as of HUVECs-hTERT cells, but did not significantly affect survival of P30 HUVECs. The increase in cell number within 8 days was for non-treated cells 43%±21,1versus 84% ± 11.9 in treated cell, $p \leq 0.01$.

4.1.6 NAC, in combination with PHA-408 or plumericin, induced proliferation of HUVECs at passage 3 and of HUVECs-hTERT and did not deplete the senescent cells

Because of the relatively strong stimulatory effects of NAC on cell growth observed above, we tested effects of this compound in combination with PHA-408 or PL. In this case we also used NAC at concentration of 0.5mM, in addition to the previously used concentrations at 2mM and 1mM.

While the combination of NAC (0.5mM, 1mM,and 2mM) with PHA-408 (2μM) did not show any senolytic activity towards senescent endothelial cells (Figure 16), NAC at all tested concentrations, in combination with PHA-408, strongly stimulated proliferation of P3 HUVECs and HUVECs-hTERT, and these effects were highest at 2mM NAC.

Again, NAC in combination with PL did not exhibit any selective senolytic activity towards senescent P30 HUVECs (Figure 17). However, a combination of NAC at the highest 2.0mM with PL (1.5μM), significantly enhanced proliferation of P3 HUVECs while it only minimally improved proliferation of HUVECs-hTERT (Figure 17).



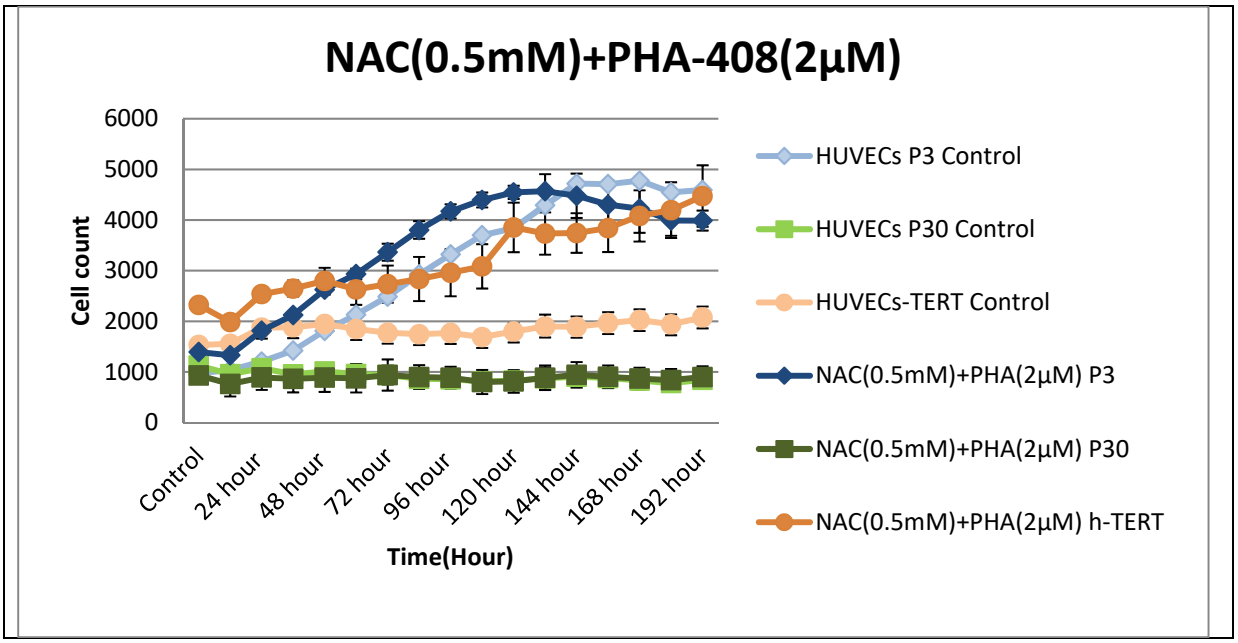
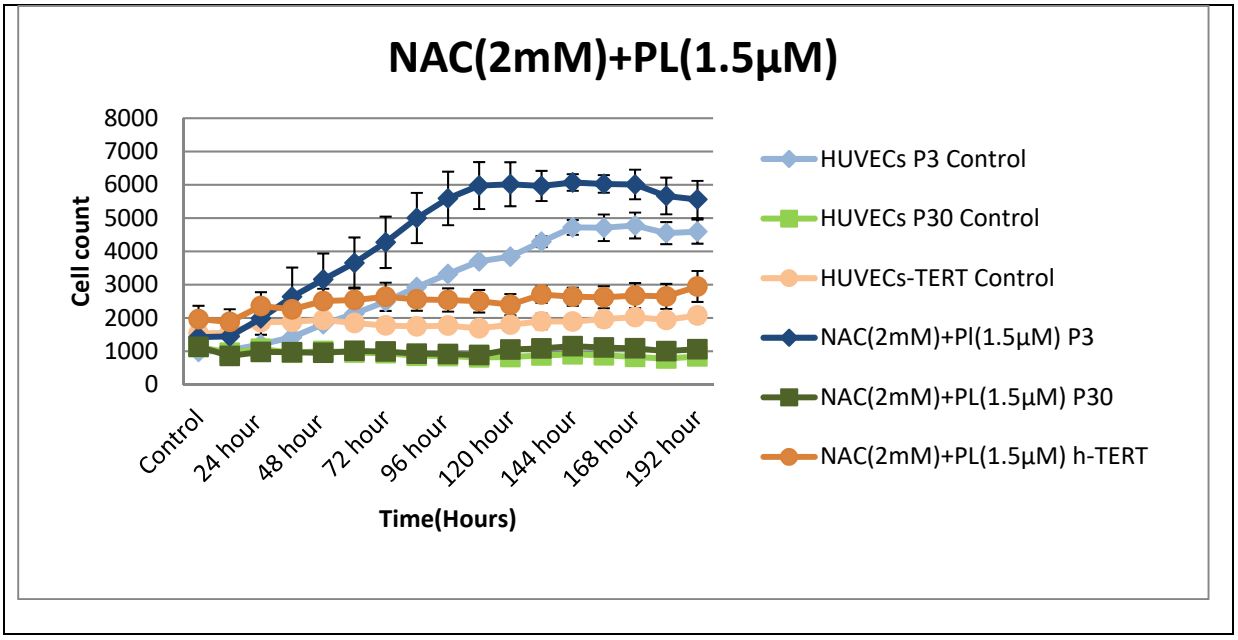


Figure 16. Effect of NAC (2, 1 and 0.5mM) in combination with PHA-408 (2µM) on cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. Combined use of these substances strongly enhanced proliferation of P3 HUVECs and markedly stimulated proliferation of HUVECs-hTERT. No preferential elimination of P30 HUVECs was found.



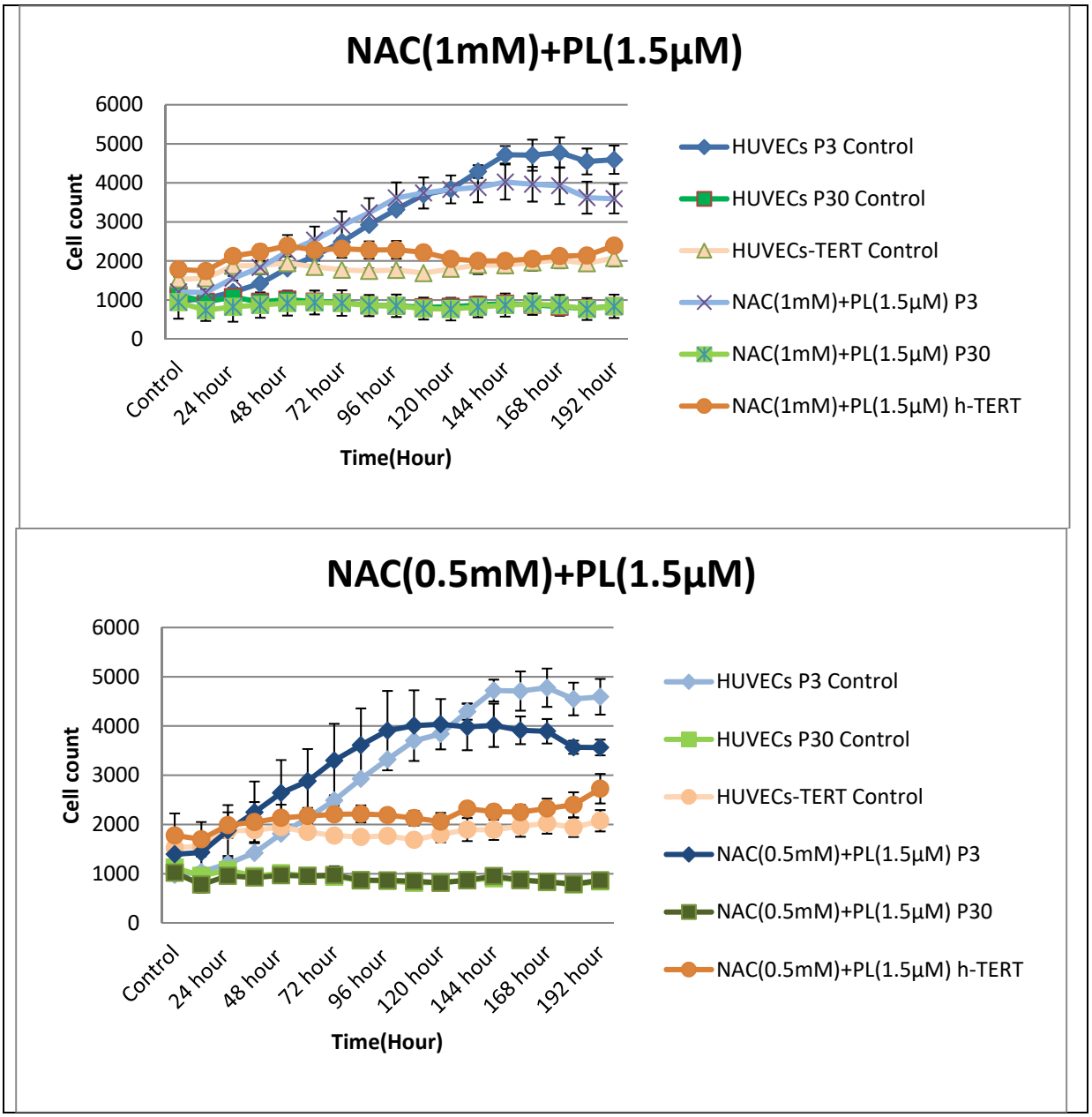


Figure 17. Effect on the cell count of NAC (2, 1 and 0.5mM) in combination with PL (1.5µM) of P3, P30, and HUVECs-hTERT. 2 mM NAC in combination with PL significantly enhanced proliferation rate of P3 HUVECs. The increase in cell number within four days was for non-treated cells 239%±23.9 versus 292% ± 66.7 for treated cells, $p \leq 0.006$. Such treatment only minimally affected proliferation of HUVECs-hTERT cells. No preferential elimination of P30 HUVECs was found.

4.2 Testing anti-inflammatory compounds regarding their senolytic properties

The following section describes the results of testing putative senolytic effects of the anti-inflammatory substances BAY11-7082, PHA and plumericin, used alone or in combination. For this, we studied the degree of cell elimination of P30 HUVEC and their influence on proliferation of P3 HUVECs and HUVECs-hTERT cells.

4.2.1 BAY11-7082 did not selectively deplete P30 HUVECs but was dose-dependently cytotoxic and at lower concentrations increased the proliferation rate of HUVECs-hTERT

First, we assessed effects of BAY11-7082 used alone at 5 μM or 1.7μM concentration. At the higher 5μM concentration BAY11-7082 was rather toxic, decreasing the cell number of P3 HUVECs as well as P30 HUVECs and HUVECs-hTERT, where by this effect was most obvious in P3 HUVECs (Figure 18). However, at the lower 1.7μM concentration, BAY11-7082 not only did not exhibit such cytotoxic effects, but even more, it significantly induced proliferation of P3 HUVECs as well as HUVECs-hTERT while P30 HUVECs seemed unaffected (Figure 18).

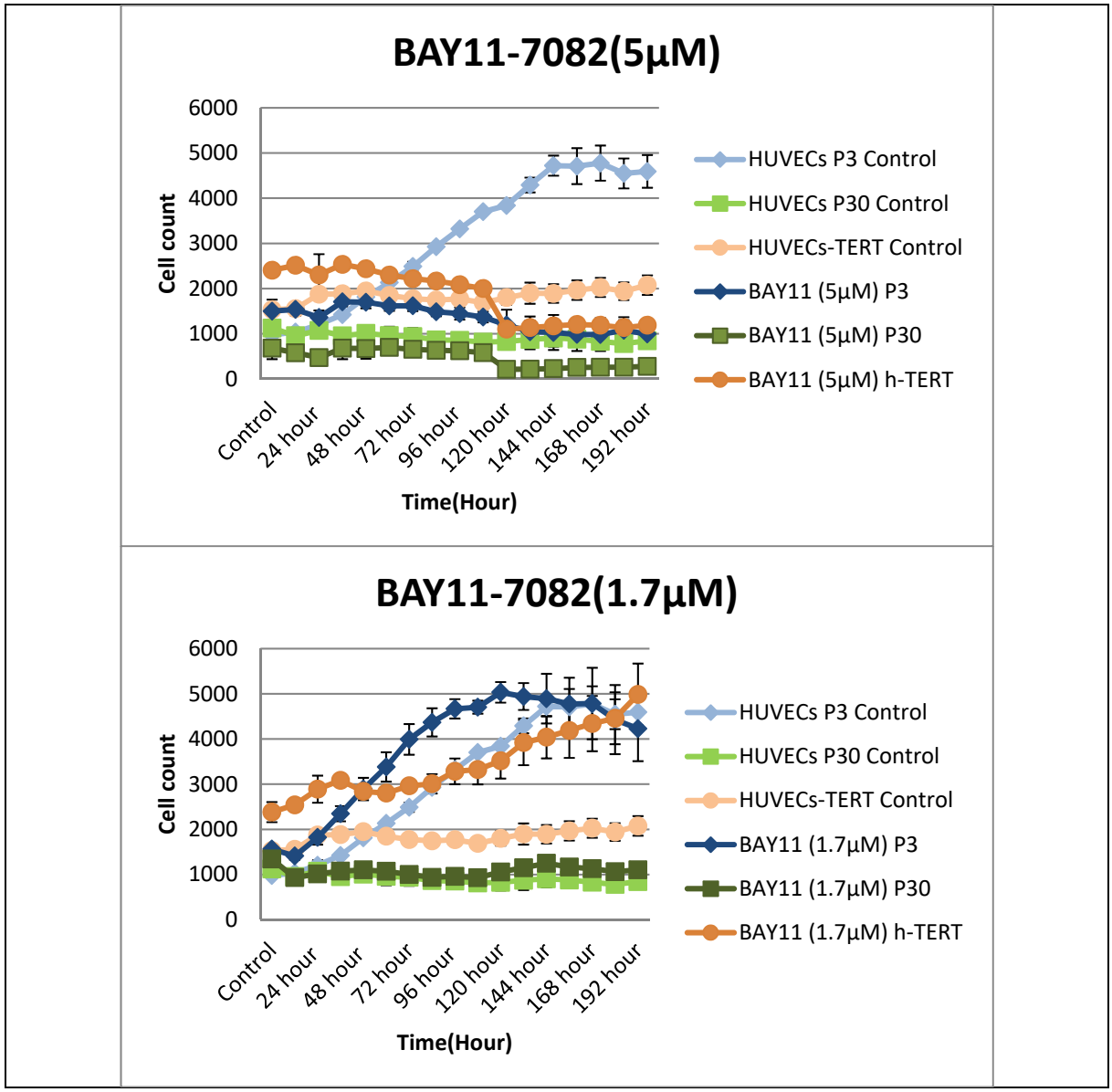
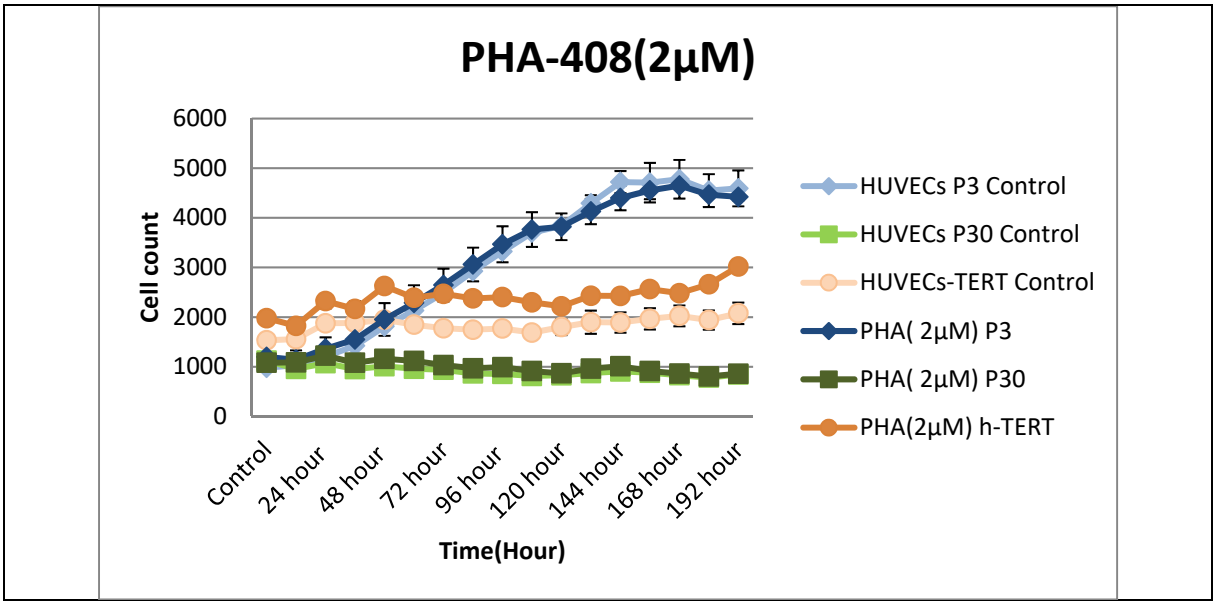


Figure 18. Effects of BAY11-7082 at 5μM and 1.7μM on the cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. Treatment of the cells with BAY11-7082 at 5μM was

cytotoxic. In contrast, at 1.7μM BAY11-7082 significantly enhanced proliferation of P3 HUVECs and stimulated HUVECs-hTERT growth. The increase in cell number over 8 days for non-treated HUVECs-hTERT cells was 43%±21 versus 116% ±41 in treated cell, $p < 0.01$.

4.2.2 PHA-408 or plumericin did not selectively deplete P30 HUVECs, plumericin was cytotoxic at later time-points and both compounds increased proliferation of HUVECs-hTERT

For the assessment of these two other NF-kB inhibitors, we treated P3 HUVECs, P30 HUVECs, and HUVECs-hTERT with PHA-408 at 2μM concentration or PL at 1.5μM. Such separate treatment of P30 HUVECs with PHA-408or PL did not exhibit overt senolytic effects in P30 HUVECs. In P3 HUVECs treatment with PHA-408 did not significantly affect growth over the whole eight-day time course, while treatment with PL became growth-inhibitory after 96 hours. The largely impaired growth of HUVECs-hTERT seemed unaffected by the treatment with PHA-408, while PL clearly had a rescuing effect on their proliferation (Figure 19).



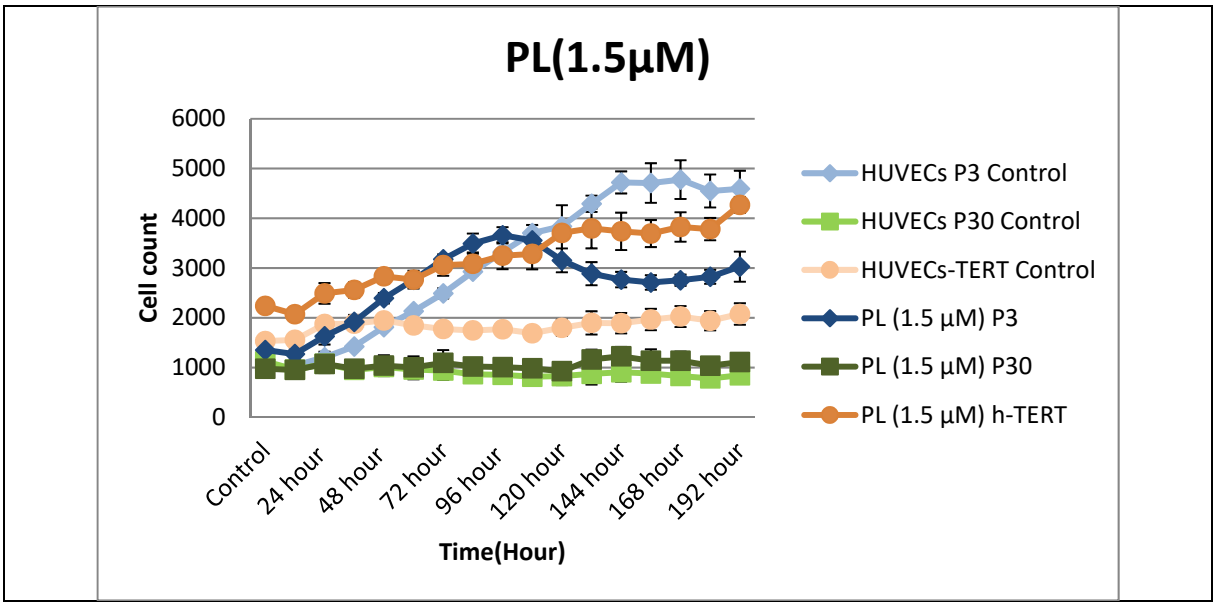


Figure 19. Effect of PHA-408 (2µM) and PL (1.5µM) on cell count of P3 HUVECs, P30 HUVECs, and HUVECs-hTERT. PL applied as a single substance decreased proliferation of P3 HUVECs after 120 hours but did not affect significantly cell count of P30 HUVECs. Its use significantly ($P<0.001$) increased growth rates of HUVECs-hTERT compared to non-treated HUVECs-hTERT. PHA-408, a highly specific IKK2 inhibitor, did not exhibit any proliferation modulating effect on any of these three cell-types.

4.2.3 Combined use of BAY11-7082 with PHA-408 or plumericin, did not improve outcome over the use of BAY11-7082 alone

We here assessed effects on proliferation when BAY11-7082 was used in combination with the other two NF-κB inhibitors, PHA-408 (2µM) or PL (1.5 µM), as presented in Figures 20 and 21, respectively. In these combination treatments, we used BAY11-7082 at concentrations of 5µM, 1.7µM, and additionally at 0.5µM.

While the combined use of BAY11-7082 with PHA-408 (2µM) showed no discernable effect on senescent HUVECs, it reduced proliferation of the young HUVEC (P3) in a Bay11-7280 dependent manner, leading to cell loss after 5 days. The rescuing effect of such combined treatment on HUVEC-hTERT growth was inversely correlated to the concentration of BAY11-7082 used in combination with 2µM PHA-408.

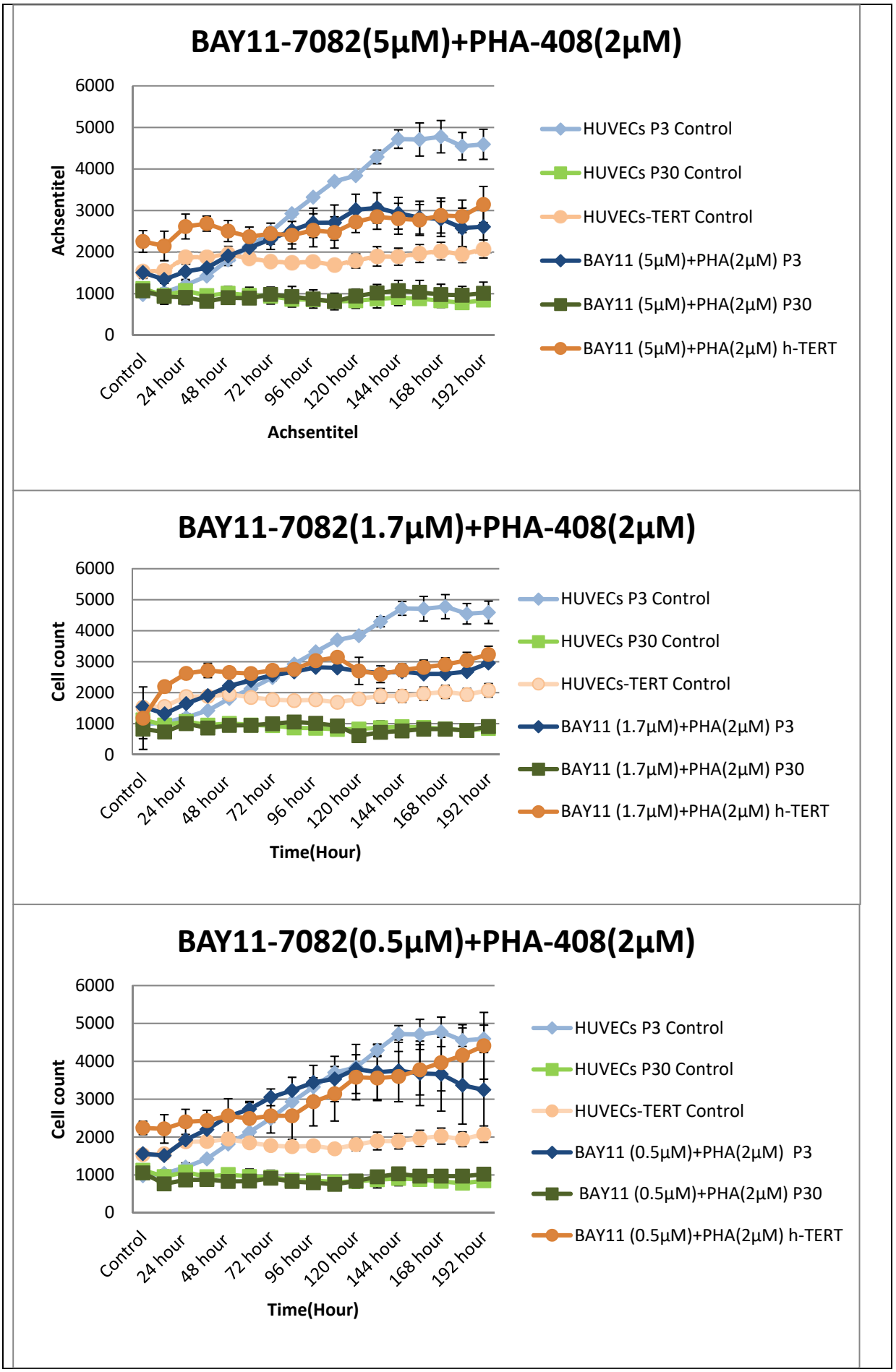
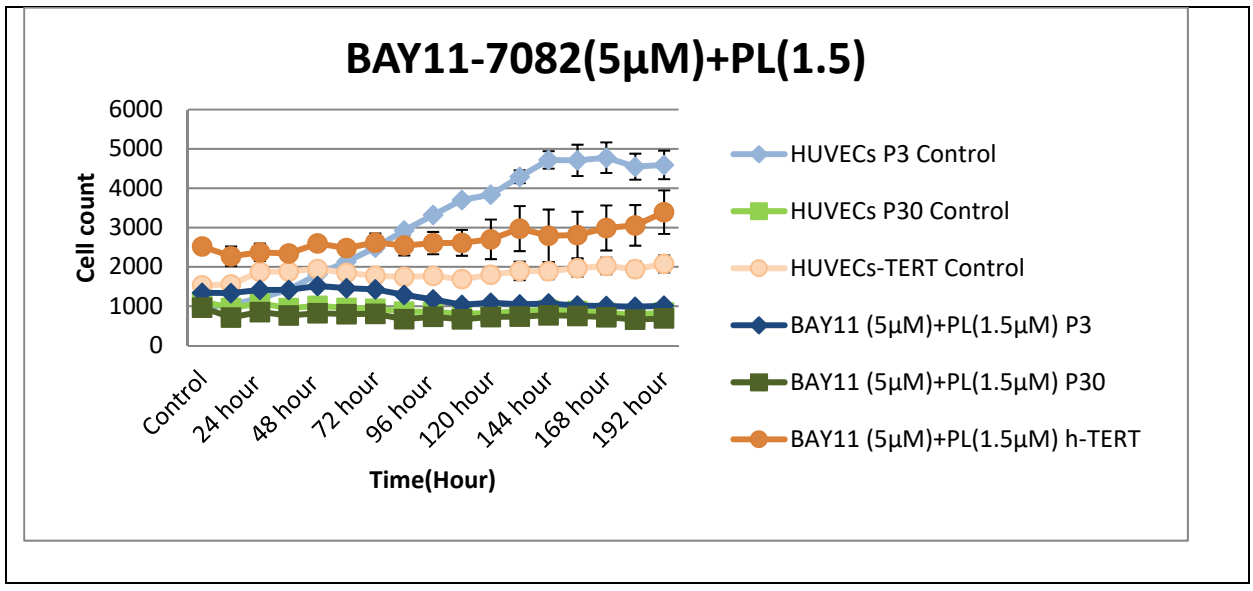

BAY11-7082(5μM)+PHA-408(2μM)
Achsentitel
6000
5000
4000
3000
2000
1000
0
Control
24 hour
48 hour
72 hour
96 hour
120 hour
144 hour
168 hour
192 hour
Achsentitel
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BAY11 (5μM)+PHA(2μM) P3
BAY11 (5μM)+PHA(2μM) P30
BAY11 (5μM)+PHA(2μM) h-TERT
BAY11-7082(1.7μM)+PHA-408(2μM)
Cell count
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BAY11 (1.7μM)+PHA(2μM) P3
BAY11 (1.7μM)+PHA(2μM) P30
BAY11 (1.7μM)+PHA(2μM) h-TERT
BAY11-7082(0.5μM)+PHA-408(2μM)
Cell count
Time(Hour)
HUVECs P3 Control
HUVECs P30 Control
HUVECs-TERT Control
BAY11 (0.5μM)+PHA(2μM) P3
BAY11 (0.5μM)+PHA(2μM) P30
BAY11 (0.5μM)+PHA(2μM) h-TERT

Figure 20. Effect of BAY11-7082 at 5µM, 1.7µM, and 0.5µM in combination with PHA-408 (2µM) on the cell count of P3, P30, and HUVECs-hTERT. BAY11-7082 at all three used concentrations in combination with PHA-408 (2µM) significantly reduced cell proliferation of P3 HUVECs and led even to cell loss after 120 hours of treatment. None of the three used concentrations of BAY11-7082 in combination with PHA-408 affected cell numbers of P30 HUVECs significantly, while cell growth of HUVECs-hTERT was promoted significantly by BAY11-7082 applied at lower concentrations (0.5µM) in combination with PHA-408.The increase in cell number after 8 days was for non-treated cells 43%±21.1 versus 95.6% ± 24.7 in treated cell, $p \leq 0.05$.

Results for the use of BAY11-7082 at 5µM, 1.7µM and 0.5 µM in combination with PL (1.5µM) are presented in Figure 20. Use of a 5µM concentration of BAY11-7082 in combination with PHA-408 (2µM) proved toxic for the P3 HUVEC, while its effects on P30 HUVEC on HUVEC-hTERT were rather inconspicuous. Such anti-proliferative effects of this combined treatment were also observed at 1.7µM and 0.5µM of BAY11-7082, and led to cell counts at day 8 that lower than those of untreated P3 HUVECs. In case of P30 HUVECs, all three used concentrations of BAY11-7082 in combination with PL did not significantly affect cell numbers, while cell proliferation of HUVECs-hTERT was improved by the BAY11-7082/PL combination treatments. The increasement in the cell number for non-treated cells 43%±21.1 versus 244.3% ± 139.1 in treated cell, $p \leq 0.01$ after 8 days (Figure 21).



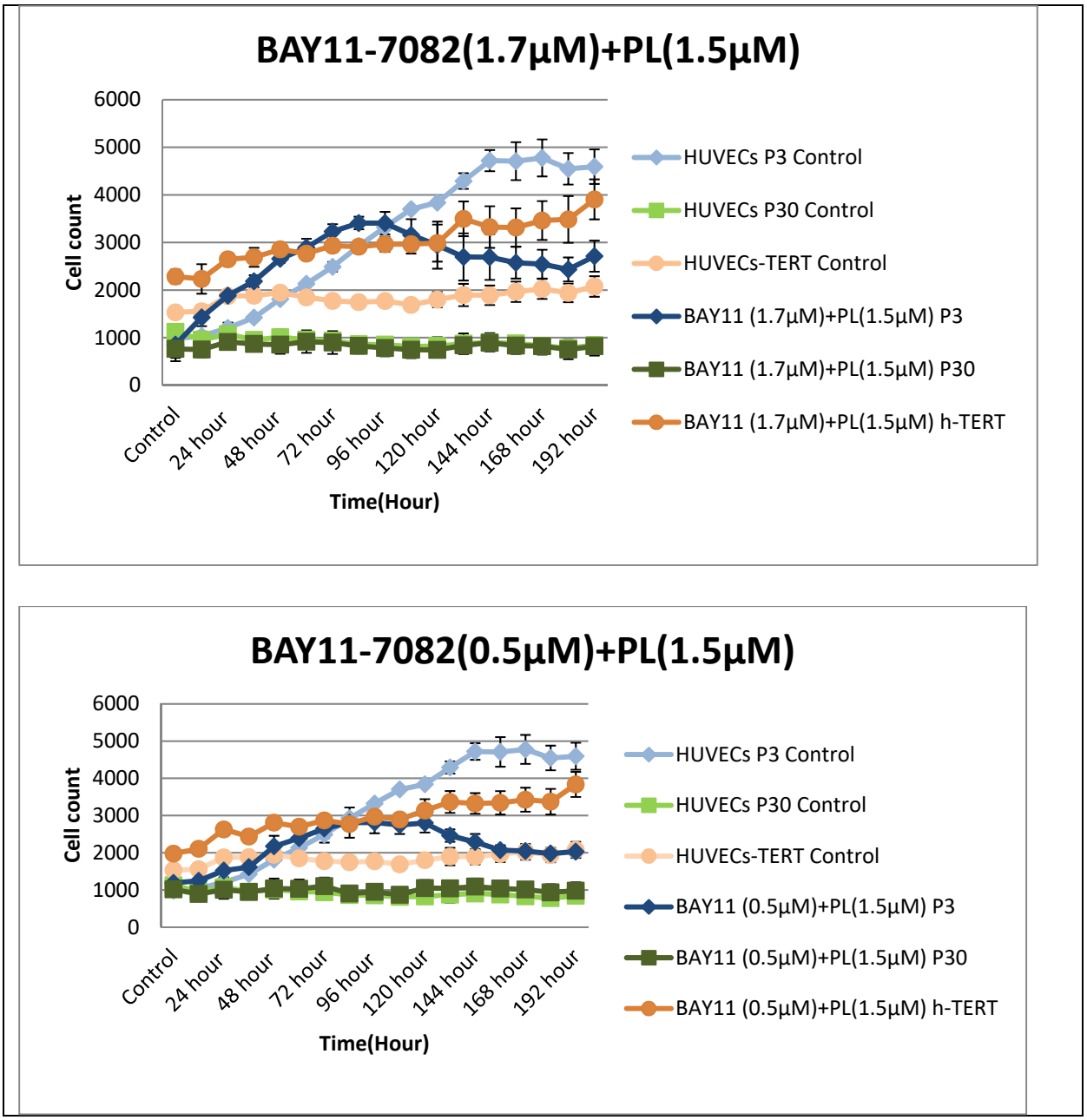


Figure 21. Effect of BAY11-7082 (5, 1.7 and 0.5µM) in combination with PL (1.5µM) on the cell count of P3, P30, and HUVECs-hTERT. BAY11-7082 at the highest concentration (5µM) in combination with PL lead to loss of P3 HUVECs early on. At the concentrations of 1.7µM and 0.5µM BAY11-7082 in combination with PL leads to the reduction in the cell count occurred at later time-points. All three used concentrations of BAY11-7082 in combination with PL did not significantly affect cell numbers P30 HUVECs. Cell growth of HUVECs-hTERT was promoted by BAY11-7082 applied at the two lower concentrations (1.7µM and 0.5µM), in combination with PL.

5 Discussion

Recent scientific works showed that cellular senescence is an important contributing factor for many age-related diseases (Martin, 2011). In addition, studies accomplished in pre-clinical models showed that the elaboration of procedures of selectively removing senescent cells may be of great benefit and clinical importance in preventing or combating negative consequences of aging.

In our study, we tested effects of selected anti-oxidative and inflammatory compounds regarding their senolytic activity in human endothelial cells. The previous work from the laboratory of Prof. Breuss showed that prolonged treatment of HUVECs with TNF-α induces cellular senescence in the exposed cells and that co-treatment with the substances NAC, PL and PHA-408 prevented the development of premature senescence (Khan et al., 2017). Based on this finding on senescence prevention, we were asking whether also the preferential elimination of senescent endothelial cells might be achievable by the use of NF-kB inhibitors and/or the use of anti-oxidative compounds. To this end, we applied these compounds to HUVECs having reached the state of senescence upon extensive propagation (replicative senescence, P30 HUVECs) and for comparison to proliferating early passage P3 HUVECs. In our study, we additionally used also the immortalized endothelial cell-line HUVECs-hTERT, which was growing only very slowly and exhibited a high percentage of cells positive for β-galactosidase, a marker used to assess cellular senescence (Dimri et al., 1995, Kurz et al., 2000). In these three cell types, we studied the elimination/proliferation rates with special attention to the induction of cell loss.

However, in none of the experiments performed here could we find overt preferential elimination of senescent versus non-senescent endothelial cells. In those experimental conditions where senescent endothelial cells were in fact reduced in number, also non-senescent ECs and HUVEC-hTERT cells succumbed to cell death. This non-selective induction of cell death occurred under substance concentrations obviously toxic for HUVECs, such as DPI 1-10 µM and Bay11-7082 5µM.

Of note, though, at least two of the tested compounds elicited responses that might reflect senescence preventing the capacity of these compounds. These were the anti-oxidant NAC and the NF-κB inhibitor Bay11-7082, which rescued proliferation of the only slowly growing HUVEC-hTERT cells used in our experiments,

and additionally enhanced growth of the proliferating HUVECs (at passage 3) as discussed below in more detail.

In our study, N-acetyl cysteine, when used as single substance, strongly enhanced proliferation of both, the P3 HUVEC and the HUVEC-hTERT cells. This compound is known to scavenge ROS/RNS and increase glutathione concentrations (Ziment, 1986), and it was protective in cultured endothelial cells exposed to hyperoxia or paraquat (Junod et al., 1987). Here, N-acetyl cysteine, when used alone at 2 and 1mM concentrations, fostered proliferation. Such a proliferation promoting effect has been reported earlier for adipose-derived stem cells (Xiong et al., 2012). The proliferation stimulating effect on young HUVECs (P3) could be further enhanced by concomitant addition of either of the two NF-κB inhibitors, PHA-408 or plumericin, to NAC used at 2 μM. In the case of the rescuing effect on HUVEC-hTERT, the situation differed. Here the addition of PHA-408 neither furthered nor hindered the positive effect of NAC, while strikingly, the addition of plumericin to the treatment with NAC completely abolished the proliferation rescuing effect of NAC. The reason, why these two IKK2 inhibitors differed so distinctly in their effect on the immortalized HUVECs is unclear, but the fact supports the notion that PHA-408 might be highly specific for IKK2, while plumericin (with its anti-fungal activity) might have a broader interaction spectrum (Kumar et al., 2013, Sharma et al., 2011, Singh et al., 2011).

The use of the two other antioxidants, BHT and DPI, showed a more limited proliferation enhancing effect than NAC. BHT, a compound known to scavenge radicals thus preventing peroxide formation, is widely used as a food preservative (Carocho and Ferreira, 2013). This substance, however, not only failed to selectively eliminate senescent P30 HUVECs, but its use, in addition, hampered proliferation of P3 HUVECs at all concentrations used, further disqualifying it as senolytic substance. Interestingly though, in the case of HUVECs-hTERT we observed an increase in the proliferation after treatment with BHT at 40, 20 and 10 μM (Fig. 9) possibly indicating some senescence preventing capacity. Use of BHT (40, 20 and 10μM) in combination with PHA-408 (2μM) did not selectively eliminate P30 cells either, and furthermore reduced proliferation of P3 HUVECs (Figure 10). On the other hand, such combination increased the proliferation-rate of HUVECs-hTERT (Figure 10) even more than BHT alone and this in spite of the fact that PHA-408 itself did not enhance HUVEC-hTERT proliferation, thus indicating synergistic activity of this substance combination. Similarly, a combined treatment of BHT (at 40, 20 and 10

μM) with PL (1.5μM) fostered proliferation of HUVECs-hTERT, more strongly than BHT alone, pointing in this case to an additive effect of these two compounds (Figure 11), since both can, on their own, enhance HUVEC-hTERT proliferation. Use of higher concentrations of BHT (20μM and 40 μM) had stimulatory effect only during the initial 24-48 hour of treatment (Figure 11), and at later time-points, upon prolonged exposure, such combination might have become toxic to the cells, in agreement with other studies reporting that that prolonged use of BHT at high concentration may be toxic to the cells (Lanigan and Yamarik, 2002). In our study, we did not test consequences of a combined treatment of BHT with BAY11-7082.

The anti-oxidant DPI is widely known to inhibit NADPH-oxidase, an enzyme catalyzing ROS production by mitochondria (Ozsvari et al., 2017). Use of DPI at concentrations (10μM, 3μM, and 1μM), led in all three endothelial cell types to cell loss. The combinations of DPI with the anti-inflammatory compounds PL (1.5μM) or PHA-408 (2μM) yielded comparable results. Altogether, it seems that the concentration of DPI we used in our study might have been too high and thus toxic to the cells, and further research should be conducted to study effects of DPI when used in a lower concentration or when used for a shorter time for exposure to endothelial cells.

The second strongest proliferative effect as a single substance had the anti-inflammatory synthetic NF-κB inhibitor, BAY11-7082 (Pierce et al., 1997). Although treatment of the cells with BAY11-7082 at the high concentration of 5μM caused massive cell death of younger P3 HUVECs, senescent P30 HUVECs, and HUVECs-hTERT cells, this compound, when used at a third of this concentration (at 1,7μM), strongly and significantly enhanced proliferation of P3 HUVECs and HUVECs-hTERT ($p<0.05$) (Figure 18). P30 HUVECs were not overtly affected, or might even have increased in number slightly. This striking effect on EC proliferation was not shared though by the two other NF-κB pathway inhibitors. PHA-408, a highly selective inhibitor that tightly binds IκB kinase-2 (IKK-2), and competes with ATP binding (Mbalaviele et al., 2009, Sommers et al., 2009), had no discernable effect on the proliferation of P3 HUVECs over 8 days and no rescuing effect on the weakened proliferation of the HUVEC-hTERT (Figure 21). Plumericin, on the other hand, acompound derived from *Himatanthus sucuuba* (Fakhrudin et al., 2014), showed no effect on P3 HUVEC growth for the initial 3-4 days when applied as a single substance, but there after caused cell loss. Its rescuing effect on HUVEC-hTERT on

the other hand was quite similar to that of Bay11-7082. Thus, these three NF-κB inhibitors clearly differ in their proliferation-enhancing profiles. Combining the use of BAY11-7082 (1.7μM) with either the very specific PHA-408 (2μM) or PL (1,5μM) hampered the proliferation enhancing impact of BAY11-7082. The reason might be that at the used concentrations the combined anti-NF-κB activity might have already been too high. In favor of this explanation would be the fact, that when the concentration of BAY11-7082 in these treatment combinations was only 0.5μM (instead of 1.7μM), the rescuing effect on proliferation of HUVEC-hTERT was less affected by 2μM PHA-408. These data show, that it might be worth while testing NF-κB inhibition for rescuing of non-proliferating cells at lower inhibitor concentrations in future studies.

A possible limitation of the experimental setup applied in this study might be that P3 HUVECs seeded at cell counts of ~ 1,000 cells to 384 well plates reached a confluent state already at days 4-5 after their transfer to 96-well plates with a maximal cell number of 5,000 to 6,000 cells. On the other hand, seeding of P30 HUVECs at cell-counts of ~ 1,000 might have been rather low for precise assessment of cell loss. Thus the experimental setup of this study might hampered seeing rather small senolytic effects. For example, the observed rescuing effect of BAY11-7082 on slow growing cells discussed above might also enhance the growth of few remnants “not yet senescent” cells in P30 HUVEC cultures, which might still be susceptible to a proliferation rescuing treatment in the P30 cultures. The resulting gain in cell numbers could possibly obscure small cell losses induced by e.g., NF-κB inhibition induced loss of IAP gene expression and subsequent apoptotic cell death.

In summary, none of the utilized anti-oxidative or anti-NF-κB compounds, used as single substances or in combination, elicited the anticipated senolytic response. The anti-oxidant N-acetyl cysteine and the NF-κB inhibitor Bay11-7082 though, showed a strong proliferation enhancing effect possibly reflecting their senescence preventing/delaying capacity. The underlying mechanisms should be addressed in future studies.

6 Outlook

In spite of the fact that none of the tested substances exhibited overt senolytic properties, our study provided valuable preliminary information about effects of these compounds on rapidly proliferating P3 HUVECs, senescent P30 HUVECs as well as HUVECs-hTERT. It also brought information about the experimental conditions that should be used in future studies, e.g., the information about the respective cell numbers to be cultivated in 384 well plates and/or concentration and time range that should be tested in future studies in combination with other potentially senolytic substances.

Future questions to be cleared include e.g., clarification if the rescuing effect of some of the tested compounds on HUVEC-hTERT-proliferation are in fact lasting effects. For how long should the substances be applied in order to sustain the improved growth and how would these cells behave after cessation of treatment with respective substances? As the NF-kB inhibitors, BAY11-7082 and plumericin exhibited NF-kB-independent enhancing effects on proliferation of the “pre-senescent” HUVEC-hTERT, but PHA-408 did not, it seems that some of the effects of these two compounds might not directly be NF-kB-dependent. Furthermore, it will also be very important to determine effects of these compounds on the expression of cell-cycle inhibitors (e.g., p16 and p21), and whether and how these substances affect apoptosis e.g., by determining the expression of anti-apoptotic genes such as BCL-2 and BCL-xL.

7 References

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8 Zusammenfassung

Zelluläre Seneszenz ist ein wichtiger Faktor in der Entstehung von altersbedingten Erkrankungen. Rezente Studien in Tiermodellen haben gezeigt, dass die selektive Elimination von seneszenten Zellen (Senolyse) einen wesentlichen Beitrag zur Bekämpfung solcher Krankheiten leisten könnten.

In unserer Forschung haben wir unter verwendung von humanen Endothelzellender Nabelschnurvene, die senolytische Wirkung von sechs verschiedenen Substanzen untersuchet. Diese Substanzen umfassten einerseits drei Anti-oxidantien; butylatedhydroxytoluene (BHT), diphenyleneiodoniumchloride (DPI), N-acetyl-L-cysteine (NAC) und andrerseits entzündungshemmende Substanzen, die NF-κB- Inhibitoren Plumericin (Naturstoff), BAY11-7082 und PHA 408 (synthetisch). Dabei wurden diese Substanzen separat oder in Kombination angewendet und die Wirkung auf Zelltod und Proliferation von "jungen" nicht-seneszenten von “alten” (seneszenszten) so wie immortalisierten HUVECs-hTERT (Telomerase-transfizierten, HUVECs-hTERT) untersucht.

Wir haben gefunden, dass keiner der Substanzen alleine oder in Kombination verwendet dazu geeignet war, selektiv/präferentiell seneszente HUVECs eliminieren. zu können. Andrerseits, zeigten manche der getesteten Anti-oxidantien einen deutlichen mitogenen Effekt auf nicht seneszente oder auf immortalisierte HUVECs besondere stimulierte N-acetycystein in Konzentrationen von 2, 1 und 0.5 mM die Zellproliferation von jungen HUVECs und BHT stimulierte in Kombination mit PHA-408, die Proliferation von HUVECs-hTERT

Des Weitern hat Bay11-7082, welches oft als anti-entzündlich wirkender NF-κB- Inhibitor verwendet wird, bei einer Konzentration von 1.7µM, eine Proliferations verstärkend Wirkung auf junge HUVECs und HUVECs-hTERT. Bei der höheren Konzentrationen 5µM, war Bay11-7082 toxisch für seneszente HUVECs-allerdings auch für junge, noch proliferierenden Endothelzellen. Der hochselektive NF-κB-Inhibitor PHA-408 ist zwar alleine nicht effektiv, führt jedoch zu einer (synergistischen) Verstärkung der mitogenen Wirkung der Anti-oxidantien NAC und BHT auf HUVECs-hTERT Zellen.

Durch die Ausarbeitung einer Methodik zum Testen senolytischer Substanzen wurde eine solide Grundlage für die Untersuchung der senolytischen Eigenschaften

von natürlichen oder synthetischen Verbindungen geschaffen, die in zukünftigen Studien allein oder in Kombination mit anderen Substanzen getestet werden sollen.