Targeting the Senescence-Associated Secretory Phenotype

By

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Abstract

Age is the single-most significant variable in the loss of normal tissue homeostasis resulting in degenerative disease. Increasing with age is a slight, but significant rise in pro-inflammatory factors in the absence of detectable infection, also known as “sterile inflammation”. Although the source(s) of this low-level chronic inflammation remain unclear, senescent cells have emerged as one potential candidate. Cellular senescence is an essentially irreversible growth-arrested state adopted by mitosis-competent cells, usually in response to potentially oncogenic stimuli. Because the main feature of senescence is growth-arrest, senescence has been described as a tumor suppressor mechanism, but the presence of senescent cells in a variety of physiological contexts, including regeneration during wound healing and embryonic development in addition to age-related pathologies, suggests a more complex role. Indeed the presence of senescent cells in multiple settings becomes more interesting when coupled with the recent discovery that they often express a “Senescence-Associated Secretory Phenotype” (SASP), secreting inflammatory factors into the extracellular milieu including many cytokines and proteases. These factors play important roles in normal tissue homeostasis, however, their chronic expression also has the potential to damage tissues and promote carcinogenesis. Senescent cells and their SASP thus have the potential to affect regenerative processes as well as as degenerative conditions, becoming targets for intervention to influence the cellular environment and maintain tissue integrity.

This thesis asks the question: Can we identify substances able to decrease the pro-inflammatory nature of the SASP and what impact on the development of cancer can this reduction produce?

Preface

This thesis is an original work by Kevin Perrott. No part of this thesis has previously been published except for the publication of a first-author publication by Kevin Perrott in Geroscience, official journal of the American Aging Association. As the primary author, Kevin Perrott designed the research program, performed the experiments and collected and analyzed the data. Special acknowledgement for numerous discussions and mentorship which contributed to the work goes to my co-authors Pierre Desprez, Remi Martin-Laberge, Christopher Wiley and Judith Campisi. Christopher Wiley specifically helped identify the experimental protocol used for determining the activation of the NFκB pathway which led to the confirmation of the mechanism of action of Apigenin in reducing the senescence-associated secretory phenotype. Remi Martin-Laberge provided a starting point for the screening protocol.

Dedication

To all previous and future lights who have dimmed and been quenched.

Dirge without Music

Edna St. Vincent Millay

I am not resigned to the shutting away of loving hearts in the hard ground.
So it is, and so it will be, for so it has been, time out of mind:
Into the darkness they go, the wise and the lovely. Crowned
With lilies and with laurel they go; but I am not resigned.
Lovers and thinkers, into the earth with you.
Be one with the dull, the indiscriminate dust.
A fragment of what you felt, of what you knew,
A formula, a phrase remains, --- but the best is lost.

The answers quick & keen, the honest look, the laughter, the love,
They are gone. They have gone to feed the roses. Elegant and curled
Is the blossom. Fragrant is the blossom. I know. But I do not approve.
More precious was the light in your eyes than all the roses in the world.

Down, down, down into the darkness of the grave
Gently they go, the beautiful, the tender, the kind;
Quietly they go, the intelligent, the witty, the brave.
I know. But I do not approve. And I am not resigned.

Acknowledgments

Without exception, there is no one who embarks upon a journey to gain the skills and expertise associated with an advanced degree that does not do so with a group family and friends wishing them well and supporting them throughout. I would like to acknowledge first and foremost the encouragement and patience of my mother and father who have always looked forward into the future with optimism of a better world. It is largely for them that I became inspired to look at doing something about the aging process as I can't imagine a world without them, as many children feel I'm sure. It is largely because of them that I was able to take the time to investigate the path of science as without their support it would never have been possible for me to work and attend University.

I would also like to acknowledge the support of my husband and best-friend, Darrell Gullion, whose many years of association with a "life-long learner" has indeed almost brought us both to retirement age by the end of the process. His patience and commitment have been the rock I have often turned to when this day seemed so far away and indeed, unattainable. Gratitude can't hope to encompass the feeling of thankfulness I have that he has been there with me and he deserves his own degree in compassion and understanding.

For my fellow Campisi-lab members I'd like to single out Chris Wiley for his always jovial contributions to discussions and his practical help for some key experiments and recognize Remi Martin-Laberge for his assistance as I learned the basics of molecular cell-biology. The other members of the lab who had significant roles in my formative months and years know who they are, and I will be speaking to you all in the future I'm sure!

Finally I'd like to acknowledge my mentors Robert Burrell, Judith Campisi, and Pierre Desprez, all of whom have been instrumental in my achieving this goal. Dr. Burrell is someone who saw in me some capacity to do something different and supported me at every turn while Dr. Campisi has shepherded my intentions with generosity and helped instill in me an appreciation for what dedication to science means. A special thank you to Dr. Desprez who I consider one of my dearest friends. Not only was he instrumental in me completing my publication but he has been a touchstone for my thesis and every other aspect of my work when asked.

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i.vi. List of Abbreviations and Acronyms - in order of appearance

SASP – senescence-associated secretory phenotype

NFκB– nuclear factor kappa B, a key regulator of cellular inflammation

p21 – cyclin-dependent kinase inhibitor that helps regulate cell division

CEBP/b – CCAAT enhanced binding protein b, a transcription factor involved in many processes

p16INKA – cyclin regulating kinase that modulates cell proliferation

KRAS – a cytosolic mediator of mitogenic signals

p53 – a proliferation regulator high in senescent cells

BCL-2 – inhibitor of apoptosis

STAT3 – signal transducer used by inflammatory receptors

NSAIDs – non-steroidal inflammatory drugs, used for analgesia

ROS – reactive oxygen species

IL-6 – a pro-inflammatory cytokine

IL-8 – a pro-inflammatory cytokine

NK cells – a type of immune cell that surveils and eliminates damaged cells

G1 – the first growth phase of cell division

S – the phase of cell division in which the genome is replicated and partitioned

G2 – the second growth phase of cell division

M – the splitting process of a daughter cell from a mother cell

CDK – cyclin-dependent kinase, a class of proteins that regulates cell cycle

CDKC – CDK complexes that transduce signaling events

VEGF – vascular endothelial growth factor, a mitogenic signal

MAPK – mitogen-activated protein kinase, governs cell proliferation and stress response

JNK – mitogen-associated cell stress regulator

p38 – MAPK family

MEFs – mouse embryonic fibroblasts

SAbGal – senescence-associated b galactosidase, a marker of stalled autophagy

DDR – DNA damage response

NAD – nicotinamide riboside

CDKN2A – cyclin-dependent kinase inhibitor

CDKN2B – cyclin-dependent kinase inhibitor

CDKN1A – cyclin-dependent kinase inhibitor

CDK4/6 – cyclin-dependent kinases, drives cell proliferation

DNMT1 – DNA methyltransferase

PRC1/2 – protein regulator of cytokinesis 1 and 2, involved in locomotion

ANRIL – long non-coding antisense RNA that inhibits p16INK

SP1 – promiscuous transcription factor

ETS – transcription factor regulating vascular development

AP1 - transcription factor that integrates mitogenic and cell stress functions

PPARG – peroxisome proliferation-activated receptor gamma, involved in inflammation

ID1 – a transcription factor involved in the determination of cell identity

IRES – internal ribosome entry site

p15 – a kinase involved in the regulation of cell proliferation

PI3K – an intracellular adapter of mitogenic signaling

AKT – an intracellular adapter of mitogenic signaling

FOXO3 – activator of apoptosis in the absence of survival factors

TGFB– transforming growth factor b, an anti-inflammatory factor

TNFB – tumor necrosis factor b, a pro-inflammatory factor

MTOR – mammalian target of rapamycin, a key integrator of cell division

GDF11 – growth-defined factor 11, a hormone involved in aging signaling

UPR – unfolded protein response, a cellular stress program

IMR90 – a fibroblast cell line

NRF2 – nuclear-related factor, a DNA damage response effector

PARP – poly(ADP-ribose) polymerase 1, a DNA repair enzyme

DSBs – double strand breaks, a type of DNA damage

SAHF – senescence-associated heterochromatin foci, a sign of epigenetic remodeling

DNA-SCAR – DNA segments with chromatin alterations reinforcing senescence, another form of epigenetic remodeling associated with senescent cell states

CD4 T cells – a type of adaptive immune cell involved in coordination of inflammation

IL-1A – a pro-inflammatory cytokine

GM-CSF – a pro-inflammatory cytokine that mobilizes myeloid cells

ADAM17 – an extracellular sheddase that releases signaling proteins

IL-10 – an anti-inflammatory cytokine

CD8 T cells – another type of adaptive immune cell involved in inflammation

TME – tumor microenvironment

TIS – treatment-induced senescence, from cell stresses of chemotherapy or other

MMP – matrix metalloproteinase, a class of enzymes that degrades extracellular matrix

Siltuximab – IL-6 blocking antibody

Tocilizumab – anti-IL-6-receptor blocking antibody

Simvastatin – small molecule that lowers endogenous cholesterol production

LDL – low density lipoprotein, an endogenous cholesterol-transport mechanism

HMG-CoA – a key enzyme involved in the internal production of cholesterol

GTPases – any enzyme that hydrolyzes guanosine triphosphate to release phosphate bond energy

RhoA – a kinase involved in cell motility

Fulvestrant – selective estrogen receptor degrader

ER – estrogen receptor

MEK-ERK1/2-RSK – mitogenic signaling pathway upregulated in cancers

Glucocorticoid – a steroidal anti-inflammatory

Type 1 inflammation – acute inflammation dominated by IFNg and IL1A

Rapamycin / sirolimus – small molecule inhibitors of mTOR and thus of cell proliferation

mTORC1 – mTOR complex 1, involved in cell proliferation

mTORC2 – mTOR complex 2, also involved in cell proliferation and stress response

GPCR – G-protein coupled receptor, a common class of cellular receptors

B-cells – a type of adaptive immune cell involved in the production of antibodies

IL-2 – a pro-inflammatory cytokine

COX1/2 – a metabolic enzyme that produces inflammatory metabolites, inhibited by NSAIDs

CAR-T – chimeric antigen receptor in T-cells, a form of cellular immunotherapy for cancer

CIT – checkpoint inhibitor therapy

Navitoclax – anti-apoptosis protein inhibitor

Obatoclax – an improved version of navitoclax

Apigenin – a natural plant-derived flavonoid with intriguing anti-inflammatory properties

VEGFR1/2 – vascular endothelial growth factor receptor 1 and 2

HER2/neu – human epidermal growth factor receptor 2

BJ cells – a cell line of human foreskin-derived fibroblasts

HCA2 cells – fibroblast cell line with markedly high SASP secretion

DMEM – Dulbecco's Modified Eagle Media, a commonly used growth medium

MDA-MB231 – an aggressively metastatic breast cancer cell line

ZR75.1 – human breast epithelial line with high MUC1 expression

FBS – fetal bovine serum

H-RASV12 – a highly oncogenic mutation in the Ras kinase

MKK6EE – a highly oncogenic mutation in the MKK pathway

DMSO – dimethylsulfoxane, a commonly used solvent

PCR – polymerase chain reaction, a method for amplifying DNA sequences

MSCs – mesenchymal stem cells

MUC1 – mucin 1, a glycoprotein involved in cell-cell adhesion

AMPK – an intracellular protein involved in cellular proliferation

CD38 – cell marker involved in the ectodomain synthesis of NAD

PDGFA – platelet-derived growth factor subunit A, a mitogenic signal

HeLa – cervical epithelial cell lines

U2OS – osteosarcoma cell line

IFNg – interferon gamma, a potent pro-inflammatory cytokine

TLR4 – Toll-like receptor 4, an inflammatory innate immune receptor

CXCL10 – a chemokine involved in immune cell motility, invoked by IFNg

TBB – 4,5,6,7-Tetrabromobenzotriazole

DRB – 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole

qPCR – quantitative PCR, a method for determining the levels of specific mRNAs

Calyculin A – a chemical reagent used to "reset" the phosphorylation state of kinases

IRAK4 – an intracellular mediator of inflammatory signaling

MyD88 – an intracellular mediator of TLR4 signaling

IRAK1 – an intracellular mediator of inflammatory signaling

MEKK1 – an intracellular modulator of cell stress and proliferation

IKKA/B – inhibitory complex that antagonizes NFkB-mediated inflammatory signaling

p65-Ac – the acetylated subunit of the NFκB complex, which marks it for degradation

p38-MAPK – a family of mitogen-activated kinases that govern cellular proliferation

ATPase – any enzyme or subunit thereof that utilizes the phosphate bonds of ATP for energy

ATP – adenosine triphosphate, a commonly used energy currency within cells

AKT – intracellular protein involved in metabolism, apoptosis, and proliferation

S6K – signaling molecule whose phosphorylation induces ribosomal protein synthesis

4EBP1 – eukaryotic translation initiation factor 4E-binding protein 1, repressor of protein translocation in cellular stress conditions

GSK3B/A-P – GSK-3 is a promiscuous kinase whose A or B subunits' phosphorylation state determines its effects on pro-proliferative targets

p44-MAPK – family of mitogen-activated kinases involved in cell proliferation

PBS – phosphate-buffered saline

PS – penicillin / streptomycin, commonly-used antibiotics in cell growth media

HSP90 – heat shock protein 90, a cell stress response factor

FDA – Federal Drug Administration

L3P – lentivirual control vector with an empty insert

IR – ionizing radiation

ZO-1 – zonula occludens-1 is a tight junction protein involved in cell adhesion

CM – conditioned media

Rb – retinoblastoma protein, a factor governing cell proliferation

1. Chapter 1 - Senescence and the SASP

At the turn of the 20th century, high infant and child mortality rates gave rise to an average lifespan of only ~40 years (Wegman, 2001). In the decades since, advances in science have eliminated or greatly alleviated the burden previously imposed by child mortality, resulting in extension of current lifespan into the 80s or beyond. As this has progressed, the leading causes of death in mankind have shifted from communicable childhood diseases to the chronic diseases associated with aging, which, despite immense research efforts to improve their treatment, continue to inflict high morbidity and mortality upon increasingly large numbers of people. In fact, for the first time in human history, we will soon have more adults over the age of 60 than children under the age of 5 (United Nations Department of Economics and Social Affairs Population Division 2011). Unfortunately, both developed and developing nations are ill-prepared to meet this seismic demographic shift and current medical and social security infrastructure may ultimately prove inadequate to the task of caring for the elderly and frail among us (Peterson 2001). This looming challenge has led to increasingly urgent calls for reformed medical and drug development practices to mitigate the rising impact of chronic, aging-associated diseases and thus smooth the impact of this demographic tidal wave (Rae et al. 2010). Unfortunately, these calls have all too often fallen upon deaf ears, although glimmers of hope are starting to appear with the increasing visibility of aging research and potential prophylactic or rejuvenative interventions.

Aging manifests as the progressive deterioration of tissue renewal and inflammation resolution processes, which results in compromised organ function, incomplete wound healing, and the accumulation of damage at the cellular, regulatory, and DNA levels. Aging begins at the conclusion of sexual maturation and proceeds unerringly until death. As aging is common to all multicellular, sexually-reproducing organisms, some have suggested that the aging process is an inevitable pleiotropic outcome of the developmental and regulatory processes essential to coordination of cellular function within multicellular organisms (Mian and Rose 2011). Complementarily, others have suggested that aging emerges once peak sexual fitness is passed, an event which coincides with decreased evolutionary pressure for organismal fitness (Libertini 2017). Although the first visible signs of aging which we are aware are usually external changes to the skin and hair, the processes by which it operates are pervasive and operate at every level of biology and within every organ system, where its calling cards are the emergence of

persistent low-grade inflammation, loss of tissue renewal, and decreasing efficiency of metabolic processes. These factors collectively create a milieu conducive to the emergence of several age-related progressive disorders ranging from chronic obstructive pulmonary disorder to arthritis. While the cellular processes underlying such disorders appear to be universal, the resilience of individuals against such stimuli appears to vary widely, as seen in comparison between progeric patients and exceptionally long-lived patients, who suffer from markedly increased and decreased inflammation, respectively. This disparity suggests that the manner in which aging unfolds in a given individual is subject to both genetic and environmental perturbation, where optimal conditions may extend healthspan while adverse conditions hasten aging's effects (Moorad and Walling 2017). These findings show that, contrary to the experienced immutability of the aging process, the trajectory of aging is amenable to modification. Investigation into the mechanisms by which aging processes proceed has revealed a great deal of dysregulated cellular phenomena ranging from perturbed mitochondrial biogenesis to cytosolic accumulation of nuclear chromatin fragments (Mian and Rose 2011). While scientific debate as to the organismal impact of each of these factors has been spirited and productive, these phenomena may perhaps be better unified and understood through the phenomenon of cellular senescence, a non-tissue-specific cell state characterized by an essentially irreversible growth arrest, altered metabolic and secretory profiles, and resistance to normal apoptotic stimuli (Campisi 2012). Senescent cells are found in almost every tissue type and share a metabolic and gene expression profile. Although senescent cells were originally thought to be damaged and have lower metabolic activity, the reverse is actually true with senescence being profoundly more metabolically active than normal cells, affecting neighboring cells as well as distant tissues alike (Hernandez-Segura et al. 2018). The exact nature of senescence in different tissue types, in response to varied environmental factors and stressors, or despite underlying genetic variation is, at present, unknown, but their elimination in a transgenic mouse model and the resulting benefits (Baker et. al. 2011) have led to the development of senolytic therapeutics to reduce the impact of senescent signaling on neighboring cells to slow the aging process, or outright eliminate senescent cells to enable endogenous tissue regeneration (Xu et al. 2018). Much regarding senescent cells or the treatment thereof remains to be established, but we are nonetheless poised to make significant strides in senolytic therapeutics to alleviate the burden of aging and improve the healthspan, life expectancy, and quality of life for an increasingly greying world population.

Since our earliest recorded history aging and its attendant mortal consequences has been a driver of social and individual attempts to prevent or eliminate aging. However, it wasn't until the late 1800s that August Friedrich Leopold Weissman, the Prussian pioneer of the germ theory of developmental biology, put the phenomenon of aging into more scientific terms with observations of reduced proliferative capacity in aged organisms. Although the concept of homeostasis was but crude and emerging, Weissman nonetheless accurately framed aging as a gradual loss of the phenomena governing tissue renewal and homeostasis over time. Eight decades later, this idea was expanded by Leonard Hayflick, an inaugural American gerontologist, with his observation of gradual loss of replicative potential in cultured human cells. Fittingly, the horizon past which cultured cells no longer divide was dubbed the "Hayflick Limit" (Hayflick and Moorhead 1961). These observations led to the theory that all cells contain an innate upper limit on the number of times they may successfully divide. Senescence was proposed early on to be a mechanism prevent cells with damaged DNA from becoming cancerous. All cells were thought to eventually need such restraint but the discovery of stem cells which maintained their telomere length and their corresponding *in situ* niches deposed the idea that all cells must die, and focused attention on the internal cellular mechanisms which regulate when to divide, when to die, and when to wait, again leading us back to the cessation of cell division and the senescent phenotype.

Senescence results from the accumulation of cell stressors that result in the activation of a cell-intrinsic damage repair process that returns the stressed cell to homeostasis or, if that fails, leads to stable senescence (Kuilman et al. 2010). These stressors include hypoxia, sustained mitogenic signaling disruption, ionizing radiation, and telomere signaling (Campisi 2013) and are accompanied by the activation of the unfolded protein response, DNA damage repair effectors, and inhibitors of cell cycle progression. As the exact identity of these effectors may vary by tissue or stressor type, senescence refers to a complex continuum of cell states sharing irreversible growth arrest as a central hallmark. The exact nature of which stressors or which internal effectors mediate distinct senescence profiles remains under active investigation.

While senescent cells were initially thought to drive aging via gradual loss of tissue function and renewal via growth arrest, they have since been found in multiple normal biological roles, which suggests that the effects of the senescent phenotype are pleiotropic and context-dependent. For instance, both normally- and poorly-healing wounds are infiltrated by senescent fibroblasts, whose growth arrest and subsequent removal reduces fibrotic

overgrowth / scarring and helps to maintain tissue function (Clark 2008; Telgenhoff and Shroot 2005). Furthermore, senescent cells are vital in normal embryonic development, where tissue "scaffolds" enter senescence prior to clearance to enable separation of limbs, fingers, and layers of tissues ((Muñoz-Espín et al. 2013; Storer et al. 2013)). Finally, as mentioned previously senescent cells are also associated with the inhibition of cancerous proliferation and serve as an anti-cancer mechanism. There are some hallmarks of senescence, which although not shared by all cells, are recognized as highly indicative that a cell is senescent. Some of these are intracellular effectors of senescence, such as high p21, CEBP/β, or wild-type $p16^{INKA}$, that have been found to be highly active in rapidly proliferating cancer cells indicating that they no longer constrain the cell cycle. Senescent cells, however, have not lost inhibitory onco-gene activity, such as K-RAS or p53, that maintains their irreversible growth arrest phenotype ((Muñoz-Espín et al. 2013; Mosteiro et al. 2016; Storer et al. 2013; Demaria et al. 2014)). This is, nonetheless, a double-edged sword, as senescent cells may represent a pre-cancerous pool of cells that can become oncogenic upon the loss of a constraining oncogene, thus underscoring the importance of reduced environmental exposure to mutagens or ionizing radiation that drive DNA damage and accumulation of mutations.

None of the above, however, eliminates the possibility that senescent cells are intimately involved in aging. Indeed, senescent cells are also characterized by a senescence-associated secretory proteome (SASP), which may drive low-level inflammation in tissues as well as induction of senescent phenotypes in neighboring cells ((Franceschi et al. 2007; Krizhanovsky et al. 2008)). SASP factors include several interleukins, cytokines, and tissue-remodeling factors that act upon both stromal and immune cells to induce low-grade inflammation (Lasry & Ben-Neriah 2015). These factors are directly implicated in the progression of multiple age-related pathologies, such as cancer, neurodegeneration, and decreased immunity and wound healing, where increased abundance of senescence cells has also been found ((Karrasch et al. 2008; Clark 2008; Baker et al. 2004; Campisi 2013)). The role of senescent cells and the SASP in aging has been further shown in several studies that have used experimental techniques to ablate senescent cells or inhibit SASP factors in preclinical models of degenerative disease, such as kidney disease, arthritis, atherosclerosis, cataracts,, and sarcopenia ((Baker et al. 2016; Jeon et al. 2017; Baker et al. 2008; Baker et al. 2011; Childs et al. 2016)). These intriguing findings strongly suggest that modulation of senescence or the SASP profile are promising avenues of

therapeutic development to decrease the morbidity of progressive degenerative diseases associated with aging.

How well manipulation of the senescent phenotype or the SASP will work in a therapeutic context is highly dependent upon the method by which it is achieved. For example, small molecule-mediated inhibition of the anti-apoptotic BCL-2 nexus, which is present in many senescent cells, resulted in off-target apoptosis of non-senescent immune cells and resulted in profound thrombocytopenia ((Rudin et al. 2012)). Alternatively, inhibition of the interleukin-6 and -8 chronic inflammatory pathway downstream of STAT3, a key effector in the SASP, via multi-tyrosine kinase inhibitors, such as dasatinib, disrupt normal processes in other cell types and can result in dangerous cardiotoxicity (Vaidya et al. 2018). While gene-specific and/or reporters may enable more accurate identification and treatment of senescent cells, the means to accurately and safely target them to only senescent cells are, as yet, undeveloped. Although current approaches are compromised by side effect profiles and lack of specificity that demand further drug development efforts, there are, nonetheless, some promising candidates that may affect senescence or the SASP in a manner that alleviates the negative effects of senescence without toxicity or side effects. These candidates, such as chronic use of non-steroidal anti-inflammatory drugs (NSAIDs, discussed further below), are well-characterized but have not previously been clinically studied in the context of aging and senescence. Further investigation of senolytics, both new and re-purposed, is a highly promising area for further development, in which success means reduced burden of age-associated diseases and their attendant morbidity and mortality.

1.1. Origins of Cellular Senescence

When August Weismann proposed "*Death takes place because a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting but finite*." in 1881, he based that statement on his observations of two simple multicellular organisms, *Pandorina morum* (colonial algae) and *Volvox minor* (multicellular colony algae). These organisms, although similar in many ways, differed in one vital feature: one was immortal (*Pandorina morum*) and the other (*Volvox minor*) was not. The former consists of undifferentiated, identical cells each able to individually form another organism while the latter is comprised of 2 distinct specialized cell types, one which replicates indefinitely as a germ line

while another forms the *Volvox* body and experiences aging and cell death. From this, Weissman observed that the process of aging may be inherent to multicellularity itself, as cooperative specialization of differentiated cell types enable more successful germline propagation even if it means that support cells do not pass on their own genetic information.

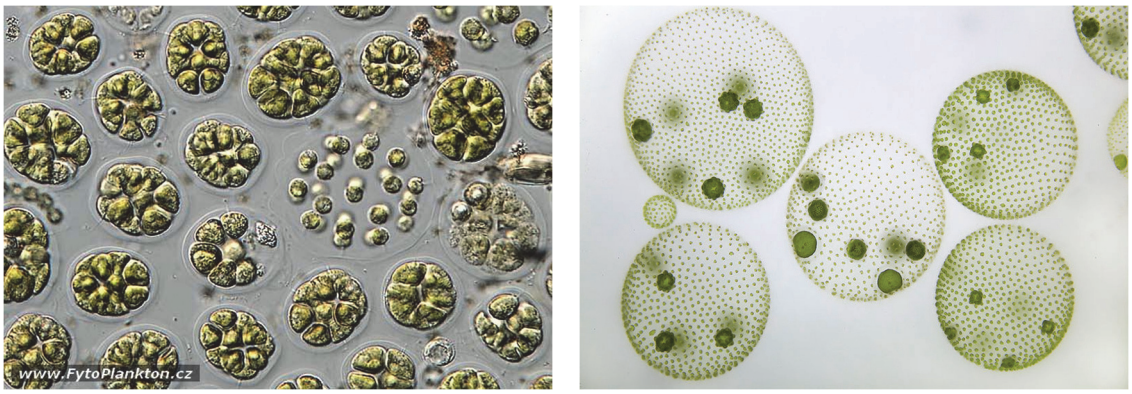


Figure 1: Development, Homeostasis and Terminal Differentiation Pandorina morum Volvox minor (Wikipedia)

Pandorina morum's cell colonies are less like a single multicellular entity than a colony comprising identical, quorum-sensing cells that each bear metabolic, replicative, and damage-repair burdens while participating in the communal benefits of concentrated nutrient and metabolic waste-product processing. However, in more complex multicellular organisms like *Volvox minor*, some cells undergo maturation to specialize into differentiated and mortal "support" cells that execute distinct roles in organismal fitness. As these mortal cells differentiate further, they also become incapable of further cell division, which is eventually followed by cellular aging, death, and biochemical breakdown (Nishii and Miller 2010). When damaged beyond repair, they are "sacrificed" for the benefit of the whole organism, enabling better transmission of the complete genome contained in their cognate, specialized germline cells and thus contributing to greater evolutionary fitness of the multicellular genome through time. These patterns are also seen in "higher" organisms, such as *Dictyostelium discoideum* (slime mold), in which highly-flexible slime mold cells cooperate in complex behaviors, including locomotion, nutrient-sensing, and the formation of terminally-differentiated reproductive structures that support the formation and propagation of specialized germ cells for dissemination of offspring and continuance of the slime mold species. While locomotive, nutrient-sensing, and other functions do not decrease the plasticity or replication capacity of

slime mold cells, the formation of the reproductive stalk does demand terminal cell type differentiation, suggesting that arrest of cellular immortality was co-opted for organismal fitness early in the origins of life itself (Glöckner and Marwan 2017).

As an evolutionary strategy (Hughes et al. 2002), this may be thought of as diminishing the individual fitness of one set of cells in order to boost the individual fitness of specialized reproductive cells beyond the average individual fitness of the remaining cells engaged only in homeostasis. This pattern becomes particularly pronounced upon the introduction of sexual reproduction and its accompanying "safe" meiotic mutagenesis. At a population level, extrapolation of this pattern to game theory suggests compromised cell function enables greater participation in evolutionary selection via exposure of high-fidelity genomes to tightly-controlled mutagenic processes during reproduction, in turn yielding a greater diversity of offspring genotypes and greater odds at thriving in a given niche.

1.2. Features of Cellular Senescence

Senescence can be generally described in terms of irreversible growth arrest, altered metabolism, and altered secretome of the cells it affects (Kuilman et al. 2010). At present, there appear to be multiple distinct types of cellular senescence. The degree to which these senescence sub-types are mutually exclusive and the differences by which they are induced is not yet fully characterized and remains an area of ongoing investigation. Open questions in cellular senescence include the nature of the factors capable of inducing it *in situ*, tissue-specific variance in the senescent phenotype or sensitivity to its induction, and the intracellular means by which a given cell may resist senescence-inducing stimuli.

1.2.1. Types of Cellular Senescence

Cellular senescence is distinguished by the loss of replication capacity, where cells stop responding to signals that would otherwise induce proliferation. The transition to a senescent phenotype is accompanied by conserved dysregulations of homeostatic cellular processes, such as cyclin or p53 activity, as well as epigenetic remodeling, in which chromatin access to transcriptionally active genes is significantly altered (Jakhar et al. 2018; Hoare and Narita 2018). Senescence-associated epigenetic reprogramming is, at present, incompletely characterized, however, in the near term it will be of great interest to further characterize the sites at which

heterochromatin foci preferentially form in senescent cells, how those sites may vary by tissue type, and how senescence-associated cell function varies by cellular neighborhood.

Cellular senescence may take multiple forms, which are not mutually exclusive. These forms include the canonical replicative senescence, and overlapping senescent phenotypes such as developmental senescence, oncogene-induced senescence, and therapy-induced senescence (Kuilman et al. 2010).

Although senescent cells were originally thought to be key drivers of aging, additional research since then has revealed that senescence actually plays a key role in normal developmental and homeostatic processes at a tissue- and organismal-scale. In the both the developing embryo and a healing wound, proliferating fibroblasts may enter a senescent state that marks them for elimination via leukocytes involved in the morphogenesis of complex tissues or excessive fibrosis in wound healing to limit the extent of scarring (Krizhanovsky et al. 2008; Jun and Lau 2010). In the immune system, leukocytes that undergo rapid expansion in response to an inflammatory insult must later be removed in order to restore tissue homeostasis. This is accomplished via entry to a cell stressed and then apoptotic state, where previously-activated leukocytes exhibit the metabolic derangements of senescence and no longer respond to inflammatory or proliferative signals, although apoptotic sensitivity is not decreased as it is in senescent fibroblasts (Allard et al. 2018; Pandiyan et al. 2007). Senescence may also limit the rate of tumorigenesis via arrest of DNA-damaged cells in a senescent phenotype, where oncogenes such as p16 and p53, are upregulated and prevent the proliferation of damaged cells. This growth inhibition is particularly needed in the inflammatory microenvironment of damaged/stressed tissues where both fibroblasts and leukocytes may be undergoing rapid cell division (Gordon et al. 2018; Yu et al. 2018). This cancer interdiction effect may, however, be a double-edged sword, as loss of an inhibitory oncogene can lead to tumorigenesis, which in turn may be more rapid than from non-senescent cells due to the impaired metabolic efficiency, pro-inflammatory cytokine milieu, and deafness to proliferative and apoptotic signals inherent to senescent cells. Senescence may also be an artificially-induced condition, such as in response to environmental conditions or therapies that place cells under acute metabolic, toxic, or inflammatory stressors. This is particularly pronounced as a side-effect of cytotoxic chemotherapies, which act to disrupt rapidly-dividing cancer cells via disintermediation of one or more key factors involved in cell replication (Krutmann et al. 2016; Keeney et al. 2018; Gewirtz et al. 2008). While this leads to curative effects in many cancer

cases, it also leads to the cell death and induction of senescence in other non-cancerous tissues that are also undergoing cell division, such as hair, bone marrow, and the gut epithelium, which gives rise to significant and long-lasting side effects after treatment cessation.

None of this is to say, however, that senescent cells are almost always a net positive for the organism in which they are induced. Just because senescent cells stop proliferating does not mean that they are metabolically or functionally inert. Senescent cells also suffer from impaired mitochondrial biogenesis, stalled lysosomal clearance / autophagy, decreased metabolic efficiency, and degraded intracellular membranes (Madreiter-Sokolowski et al. 2018; Habiballa et al. 2018; Kim et al. 2018), all of which act to derange both signaling reactions and metabolite flux, which, in turn, act upon neighboring cells in a juxtacrine fashion to increase the likelihood of neighboring cells also entering senescence. These metabolites include increased reactive oxygen species (ROS), citrate, malate, and other products of the anaerobic glycolysis that is upregulated in senescence cells. This metabolic program resembles a weaker form of Warburg metabolism, which characterizes glucose-dependent, ROS-producing tumor cells in a wide variety of cancer types (Holloway et al. 2018; Callender et al. 2018; Tanner et al. 2018). Senescent cells also secrete a characteristic set of pro-inflammatory mediators, with interleukins-6 and -8 being two major factors, that act to induce chronic, low grade inflammation ("parainflammation"). This set of inflammatory mediators is called the senescence-associated secretory phenotype (SASP), which also acts to degrade extracellular matrix, alter immune responses, and attract tissue-remodeling effector cells, such as fibroblasts and macrophages (Coppé et al. 2010; Coppé et al. 2011). Metabolite secretion and the SASP may act to attract NK or other cell types to remove senescence cells under normal function (Burton and Stolzing 2018; Brighton et al. 2017), but this is not yet well-understood. Meanwhile, it has been conclusively shown that senescent cells are significantly associated with a number of age-related degenerative pathologies, such as atherosclerosis, rheumatoid arthritis, and decreased immunity (Jeon et al. 2018), thus demonstrating that senescent cells have a vital role in the pathologies associated with aging even if they are not driving aging itself. Even so, preliminary evidence from model organisms in which senescent cells were limited or inhibited by experimental therapies have shown that the removal of senescent cells can extend healthspan and attenuate several age-related degenerative pathologies (Baker et al. 2008; Naylor et al. 2013; Childs et al. 2015), showing that senescent cells at least sustain and amplify aging-related degenerative signals even if they are not the source thereof. While these findings

show that senescent cells are vital in both homeostasis as well as aging morbidity, they may at least be temporarily safely eliminated via senolytic therapies, which, in turn, may finally allow elucidation of whether senescence-extrinsic effects precede or follow the induction of senescent cells in aging (Wei and Ji 2018; Menicacci et al. 2018; Maciel-Barón et al. 2018; Sagiv and Krizhanovsky 2013).

1.2.2. G1 Cell Cycle Growth Arrest

The proliferation of cells is normally regulated by the interplay of environmental factors and endogenous response factors that broadly group into the 4 phase cell cycle, which consists of growth 1 (G1), synthesis (S), growth 2 (G2), and finally mitosis (M). Each of these phases is governed by a set of cyclin-dependent kinases (CDKs), who form heterogenous supramolecular assemblies termed cyclin-dependent kinase complexes (CDKCs) that subsequently regulate the balance of autophagy and anabolism in a proliferating cell (Lim and Kaldis 2013). At each stage, CDKCs are sensitive to environmental influences, such as the presence of growth factors, e.g., vascular endothelial growth factor (VEGF), or the absence of sufficient nutrients, such as glucose and amino acids, where a lack of constituents leads to activation of cell cycle repressors, such as p16 and p53. By progressing through CDKC-governed metabolic checkpoints that are counterbalanced by cell cycle repressors, dividing cells are subject to “quality control” at each stage, ensuring that daughter cells are not left defective or incomplete in a manner that would harm organismal fitness or tissue function (Brown and Geiger 2018; Neurohr et al. 2018). Signals that induce CDKC progression generally include abundant nutrients, growth factor support, and activation of members of the mitogen-associated protein kinase (MAPK) pathway, such as Erk or Ras, which are intracellular “transducers” of signals from growth factors and activating cytokines. Downstream effects include increased protein biosynthesis, upregulated autophagy, and mitochondrial biogenesis that favors sustained oxidative phosphorylation. Signals that inhibit CDKC progression consist of cellular stressors, such as hypoxia, DNA damage, heat shock, or acute inflammation, that disrupt normal cellular microenvironments and associated signaling. These signals lead to activation of an alternative MAPK pathway in which different proteins form regulatory complexes in which c-Jun N-terminal kinases (JNKs) are strongly activated (Barutcu et al. 2018; Girnius et al. 2018). JNK signaling then cooperates with p38, a key clock protein, to activate p53, a highly potent and very promiscuous inhibitor of cell cycle progression that also increases a given cell’s sensitivity to apoptotic stimuli. Together, p53, JNK, p38, and other

effectors of cell cycle arrest such as p16, p21, and CCAAT/enhancer-binding protein beta (CEBP/β), which collectively act to shut down autophagy, halt cell proliferation, and engage damage response pathways, such as the conserved DNA damage response or upregulation of heat shock proteins and other chaperonins (Qin and Gao 2018; Hao et al. 2018; Sottile and Nadin 2017).

Although the macroscopic effects of aging have been apparent since prehistory (Jeyapalan and Sedivy 2008), the more specific phenomenon of cellular senescence was initially discovered looking at the results of studies conducted in cell culture. Early experiments looking at dividing human fetal fibroblasts dividing showed an eventual cessation of cell division when cell cultures achieved greater than 50 doublings (Campisi 2013) coming to be termed "replicative senescence". This discovery by Hayflick went against the current dogma that given the right conditions, primary human fibroblasts would divide indefinitely. Hayflick however showed that with passaging, even though they did not die, cells ceased replicating and entered a state of what was thought to be a 'living death', keeping the damaged cell alive and locked in a state where they were no longer functioning normally. This was initially thought to be a natural adaptation to prevent cancer at a young age, without inhibiting the ability of the organism to reproduce, although ultimately the accumulation of these damaged cells over time invariably led to tissue dysfunction and degeneration, aging and death, in a clear example of antagonistic pleiotropy. Researchers involved in the study of cellular biology have shown that only cancer are truly immortal (Hernandez-Segura et al. 2018). Considering that, with this observation, Hayflick overturned decades of dogma, he is lauded as one of the leading progenitors of biogerontology and accordingly the cessation of growth at high passage numbers is referred to as the "Hayflick Limit" (Ndifon and Dushoff 2016; Gill et al. 2017).

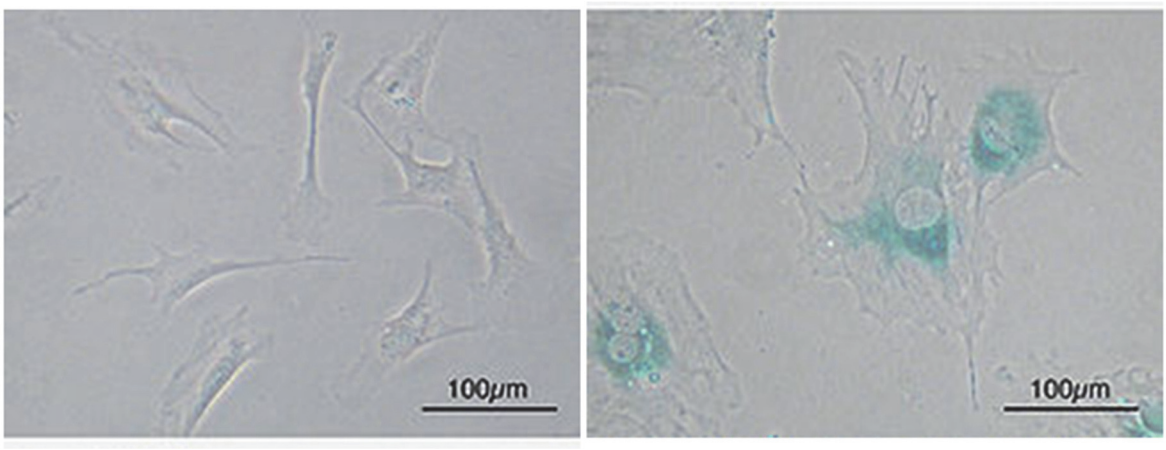


Figure 2: SA-B-Gal staining of mouse embryonic fibroblasts. Left panel: non-senescent, Right Panel: Senescent Source: (Campisi 2013).

Non-senescent MEFs are show in the left panel of Figure 2. The cell bodies have a spindle shape and smooth processes, a hallmark of mitotically-competent fibroblasts. The cells in the panel on the right are replicatively senescent, showing an enlarged and flattened morphology with rough cytoplasmic processes. In the image, senescence-associated β-galactosidase (SAβGal) was used to delineate areas of lysosomal aggregation, a marker of senescent state, in blue via standard immunohistochemical methods (Leeman et al. 2018; Arensman and Eng 2018).

Senescence is generally thought to emerge as a response to cell stress. The different sub-types of senescence may be governed by distinct stressors or cellular contexts; this remains an area of active investigation. Cell stress may include DNA damage, as can occur with acute ionizing radiation, long exposure to ultraviolet radiation, or environmental contaminants with mutagenic properties. DNA damage then provokes a highly conserved DNA Damage Response (DDR) pathway that entails upregulation of polymerase complex subunits and coordination of cellular resources targeted to the cell nucleus to effect genomic repairs, such as mismatched base excision and double-strand break repair (Taylor and Yeeles 2018). While this process is highly effective in most cases, when it fails, it may result in widespread epigenetic remodeling, cell death, degradation of nuclear integrity, and, in the worst case, a precancerous cell with the potential to grow into a malignant tumor (Ma et al. 2018; Gassman and Holton 2018). Even when the DDR is successful, it may leave a cell depleted of key metabolites and cofactors, such as mitochondrial nicotinamide riboside (NAD), and thus impaired. Other stressors also provoke acute damage responses, such as the activation of hypoxic-inducible factor 1a (HIF1a) when a cell is deprived of hemoglobin- or ambiently-delivered oxygen or the upregulation of heat shock

proteins and the unfolded protein response in heat shock conditions (Pastukh et al. 2015). Collectively, such stressors demand that the cell execute a delicate balancing act: repair of damage without impairment of cellular function. While from an organismal perspective, repairs succeeding to return a cell to homeostasis or failing in a manner that induces apoptosis may be ideal, the persistence of senescence as a "damaged but not dead" third path suggests that senescence may exist as a failsafe mechanism that prevents inflammatory necroptosis if neither repair nor apoptosis are viable options for a cell.

The irreversible growth arrest of senescence is governed in part by the induction of three proteins which are members of the Cyclin-Dependent Kinase (CDK) family. These CDKs are CDKN2A (aka p16), CDKN2B (aka p15) and CDKN1A (aka p21). Of these, p16, which inhibits CDK4/6, has been the most used as a marker for senescence *in vivo* and has been used as a marker to therapeutically target senescent cells for elimination. De-repression of the p16 promoter by inhibitors of the methyltransferase, DMNT1, which otherwise keeps p16 levels low, results in expression of p16 and some features of senescence (Venturelli et al. 2013), such as disrupted cell cycle progression and disintermediated CDKCs. Levels of promoter methylation, however, do not always correlate with levels of p16 protein. There are multiple interactions at the p16 promoter which repress p16 expression, including Polycomb repressive complexes (PRC1/2) activity (Li et al. 2011), which in turn is recruited to the p16 locus by ANRIL, a long non-coding RNA generated by reverse transcription of the p16 open reading frame (Yap et al. 2010). Transcription of p16 occurs via cooperation of a number of transcription factors, including Sp1, Ets, AP1 and PPARγ (Wang et al. 2007; Ohtani et al. 2001), while repressive elements such as YBD1, ID1, AP1 and the INK4 transcription silence element (ITSE) offer some balance to the activation (Li et al. 2011; Kotake et al. 2013) . Transcripts and proteins of p16 are stabilized by the RNA-binding proteins hnRNP A1/2 (Zhu et al. 2002), translationally regulated by an internal ribosome entry site (IRES) (Bisio et al. 2015), and ultimately the half-life of the protein itself is regulated by its ubiquitination, which may vary under conditions of cellular stress (Ko et al. 2016).

p15 has not been studied nearly as much as p16 but is known to act downstream of the PI3K/AKT/FOXO3 pathway that mediates sensitivity to proliferative, inflammatory, and apoptotic stimuli (Malumbres et al. 2000). Similar to p16, p15 is epigenetically regulated by PRC complexes through histone alterations while transcription factors Sp1 and the more recently discovered Miz-1 cooperate to produce p15 mRNA (Culerrier et al. 2016). Interestingly, the half-

life of p15 protein itself is positively affected by binding to the protein TGFβ (Sandhu et al. 1997), which suggests that it is involved in promotion of the differentiation and epigenetic remodeling.

The role of p21 in senescence is more complicated in that it is upregulated in response to activation of p53 via direct p53 activity and thus is part of the DDR. However, due to the myriad factors upstream of p21 activity, it cannot be readily used as a senescence-specific marker (Hernandez-Segura et al. 2017). p21 may also be activated independently of p53 state via pathways involving TNF-β and using Sp1 as a transcription factor (Koo et al. 2015).

Although growth arrest and DDR-related signaling are common features of senescence, on their own they are incapable of distinguishing cells that have ceased dividing due to non-senescent stressors or signaling from those that have entered growth arrest due to senescence. The factors which senescent cells produce and secrete into the environment are more useful as markers of senescence.

1.2.3. Beta-Galactosidase

Homeostatic cells engage in a continuous process of protein biosynthesis and recycling, the former via the endoplasmic reticulum and ribosomes and the latter via lysosomes, which are membrane-enclosed organelles embedded with ion pumps that enable them to profoundly lower their pH to induce the proteolysis of encapsulated proteins. This cycle removes damaged or misfolded proteins from the cell cytoplasm and also provides recycled cellular building blocks for continued biosynthesis. The process by which a cell recycles its components is known as autophagy and it is most active in proliferating cells. In senescent cells, however, the normal rate of autophagy becomes disrupted as the engagement of cell damage repair pathways demand use of the resources that would otherwise maintain autophagy (Arensman and Eng 2018). This leads to an accumulation of lysosomes that are full of damaged or misfolded biomolecules, in which the lysosomes fail to mature and process their load. This may result in the entanglement and oxidation of lysosomal constituents, leading the emergence of intracellular aggregates termed lipofuscins, which are a key sign of senescence.

Although senescence was identified in culture as a speculative cause of aging, it was initially thought to be nothing more an artifact of cell culture for many years due to the inability to detect them *in vivo*. It was more than three decades after the demonstration of replicative senescence in culture that one of the gold standards of detecting senescent cells was

discovered. In 1995 it was found that the pH within the lysosomes of senescent cells became more basic and that lysosomal B-galactosidase increased in activity. The increase in this activity was measured by the cleavage of a colorless molecule by the enzyme to form a bright blue product. (Dimri et al. 1995). This color change led to the ability to detect senescent cells *in vivo* as non-senescent cells do not have, in general, the same color change. It was further identified only the activity of vacuolar-b-galactosidase was important and cytoplasmic enzyme was not involved. Thus vacuolar-b-galactosidase became known as SA-B-Gal method of detecting senescent primary cells in tissues. Until recently, there was no other method that was as reliable as this stain to find senescent cells *in vivo.* However, recently, a separate assay utilizing the production of fluorescent molecules via cleavage of fluorescence-quenching moieties upon entry to a more basic lysosome has been developed (Sosińska et al. 2014). This enables assessment of senescence without relying on the complicated environmental variables involved in colorimetric development of the SA-B-Gal immunohistochemistry method. Unfortunately, this new probe can only be used on fixed cells, a process which fixes SA-B-Gal in place. Other types of tissue preparation, such as paraffin, also increase the difficulty of detecting senescence cells.

1.2.4. Enlargement in Morphology

Another major differentiator between normal non-senescent cells and senescent cells is the dramatic increase in size that occurs when a non-senescent cells becomes senescent. Although the exact nature of the cause for this enlargement remains obscure, we do know that activation of the mTOR pathway is required. This intuitively makes sense because mTOR is responsible for integrating many signals of proliferation or environmental damage upstream of both cell cycle progression and senescence. Decline in the levels of growth factors such as GDF11 may be related to the increase in size of cells observed (Loffredo et al. 2013). As an increase in cell size must necessarily involve an increase in the amount of cytoskeleton proteins produced, it is not surprising to see increased levels of vimentin, one of the major components of the filaments that maintain the structure of the cell and which is governed by ATF6α, itself a regulatory of the unfolded protein response (UPR), involved in determining the size of the endoplasmic reticulum (Druelle et al. 2016; Hwang et al. 2009).

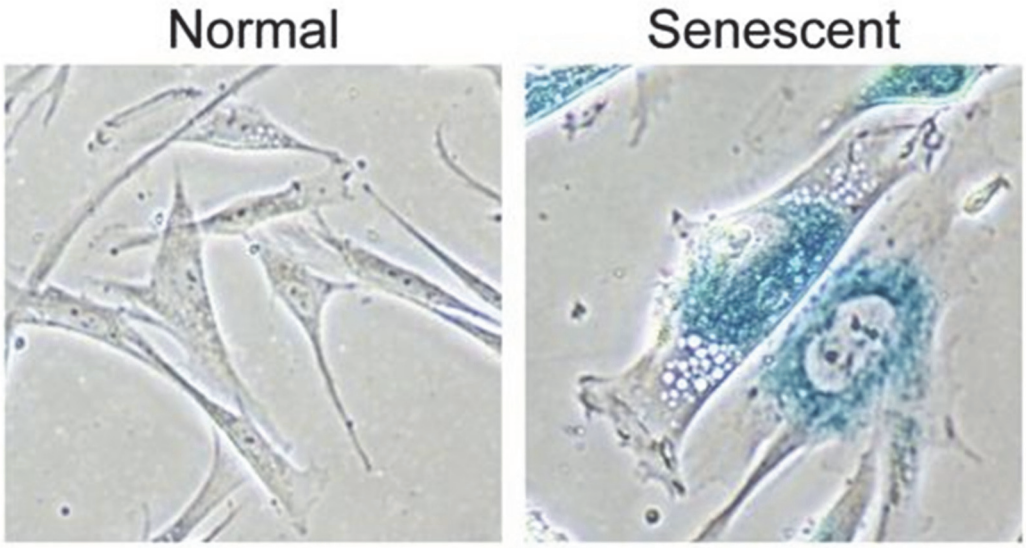


Figure 3: SA-B-Gal staining of non-senescent and senescent primary IMR90 lung fibroblasts. Senescent cells are enlarged and flattened in shape with an increased number of vacuoles and SA-B-Gal enzyme activity compared to non-senescent cells.
Image source: Wikipedia

Measurement of cell size, however, is not a reliable metric for senescence *in vivo* due to the wide range of cellular morphologies and sizes that non-senescent cells may take on in the complete *in situ* environment. Current microscopic and molecular imaging methods, such as intravital microscopy or positron emission tomography, lack the resolution and/or reagents to clearly delineate the cell boundary and thus approximate cell volume, particularly for motile cell types. At present, the degree to which senescence cells enlarge *in situ* is incompletely characterized, giving rise to pressing questions as to the dynamics of cell morphology enlargement, lipofuscin/lysosome accumulation, and eventual clearance of a senescence cell from an otherwise healthy tissue.

1.2.5. DNA Damage Response Signalling

One of the most robust hallmarks of senescence is the activation of the DDR. DNA damage is caused by a variety of factors, from endogenous reactive oxygen species generation during mitochondrial metabolism or inflammatory effectors to exogenous ionizing radiation or mutagenic chemical exposure. DNA damage may take many forms, from backbone nicks to thymidine bridges and epigenetic oxidation to double strand breaks. While less-damaging forms of DNA damage, such as base oxidation or pyrimidine dimers, may occur as a natural

consequence of cellular metabolism or environmental exposure, more-damaging forms of DNA damage, particularly double-strand breaks, are much rarer and warrant a greater state of cellular emergency. Constitutive activity by DNA repair enzymes ensures genome integrity against minor damages whereas double-strand breaks and other forms of widespread damage evokes the expression and activity of specialized DNA repair effectors, such as nuclear factor-like 2 (NRF2/NFE2L2) and poly-ADP-ribose polymerase (PARP). From an energetic perspective, this is a rational strategy (Chen et al. 2018; Pietrzak et al. 2018). Non-threatening low-level damage is a constant factor of biochemical activity and does not require special utilization of resources to resolve. Nor does low-level damage generally threaten the sequence or function of multiple genes at once. In serious DNA damage, however, the invocation of the DNA damage response, a form of cellular stress, may be necessary to effectively correct widespread damage that affects the function and/or structure of multiple genes.

In the DDR, genetic damage, especially double-strand breaks (DSBs) attract ATM serine/threonine kinase and stimulate the phosphorylation of neighboring H2AX histone complexes. The DNA repair complex is then assembled with the participation of numerous proteins, some of which are cooperate with epigenetic remodeling processes (Celeste et al. 2002). This methylation is used as a feedback signal for the activation of ATM maintaining the DDR, which also reduces levels of the GLP methyltransferase and thus results in a negative feedback loop eventually restraining ATM (Ayrapetov et al. 2014; Takahashi et al. 2012). Interestingly, this same process also derepresses the promoters of interleukins 6 and 8 (IL-6, IL-8). DDR duration correlates with increasing phosphorylation of p53 on a number of serine residues, which enhances the activity of transcription factor p53 and its antiproliferative target genes (Turenne et al. 2001).

Of particular note is the potential for DNA-modifying viruses or biotechnology gene-editing tools to induce the DDR response, which may increase off-site mutagenesis rates or decrease cellular fitness. While it has been shown that these tools do increase DDR in many cases, the exact nature of that DDR and how it impacts cellular function is currently uncharacterized. This consideration must be taken into account when using such tools or models in the laboratory setting, particularly in the examination of senescence, where upregulated DDR may significantly skew results.

1.2.5.1. DDR Signalling in Telomeric Shortening

Studies examining the mechanisms underlying cellular senescence have been conducted. (Tollefsbol, 2010; Martinez, & Rodier, 2016). One of the major factors contributing to the initiation and evolution of the senescence phenotype, according to (Childs et al. 2015)), is the response to DNA damage (Gomes et al. 2017). Telomeres are complex structures found at the ends of chromosomes (Raices et al. 2008). It is thought that one of the main functions of the telomere is to protect the otherwise exposed free end of the DNA helix both from degradation and from being interpreted by DNA repair machinery as a double-strand break (DSB). As cells divide throughout an organism's lifespan, telomeres become shorter. This is due to the inability of DNA Polymerase to replicate the DNA to which it is directly bound, leaving a small segment of (TTATTG) overhanging every replicated chromosome, which may activate the DNA Damage Response. Consequently, the ends of the chromosomes are protected from enzymes which modify the free ends of the double strand breaks produced by telomeric erosion or damage to DNA. Often we find that fusion and trimming of DNA bases results from emergency DSB repair (Cejka 2015; Longhese et al. 2010), as such damage would catastrophically disrupt chromosomal superstructure and gene expression.

1.2.5.2. DDR Signalling in SAHFs and DNA-SCARS

Added to the hallmark flattened morphology one observes in cells that undergo senescence, we also find a significant modification of the size and shape of the nucleus. Observations of nuclei of senescent cells show senescence-associated heterochromatin foci (SAHF) (Pazolli et al. 2012), in which several punctate points of chromatin condensation may be observed throughout the nuclei. Although the exact function of SAHF is, at present, unknown, they may serve to compact regions of the genome involved in cell proliferation and apoptosis, thus increasing the permanence of the senescence state via decreased transcription factor and polymerase access to non-senescence genes. SAHFs that support continued senescence are referred to as chromatin alterations reinforcing senescence (DNA-SCARS).

SAHFs and DNA-SCARs emergence in senescence cells are also characterized by a loss of nuclear envelope integrity, as seen in cytosolic chromatin and disrupted nuclear transport. While the effects of these structural changes are currently uncharacterized, loss of nuclear compartmental integrity and function can only serve to reinforce senescence or apoptosis in

that the accumulated damage seen once the nuclear membrane begins to weaken may be irreparable no matter what genes are altered.

1.2.6. Senescence-Associated Secretory Phenotype (SASP)

Even though a senescent cell may cease to replicate, it is by no means inert. Senescent cells remain metabolically active, albeit with a significantly altered metabolic profile, and still participate in cell signaling, where senescence-associated alteration in cell signal responses can exert profound effects on the senescent cell itself, its neighboring cells, or even distant cell via circulation of potent signaling factors (Wei and Ji 2018; Mendelsohn and Larrick 2018). These alterations collectively give rise to the senescence-associated secretory phenotype (SASP), which is characterized by increased activation and secretion of inflammatory mediators, including cytokines, metabolites, and altered extracellular matrix components. Although the precise physiological role of the SASP in homeostasis is currently unknown, it has been shown that SASP+ senescent cells accumulate with age and are present in progressively greater numbers in a multitude of aging-related inflammatory pathologies, including type 2 diabetes, rheumatoid arthritis, and atherosclerosis (Young and Narita 2009). It is possible that SASP+ cells take on an inflammatory phenotype to cause a reaction in surrounding tissue-resident immune cells, which may be key to the removal of senescent scaffolding in developmental biogenesis or the clearance of overgrown or damaged fibroblasts in the wound healing response. Some have speculated that, due to the normal major histocompatibility complex type 1 surveillance role of natural killer (NK) cells, the SASP+ phenotype serves as a chemo-attractant to trigger NK-mediated removal of senescent cells for the restored homeostasis of the surrounding tissue. However, like many other immune cell types, NK cell population numbers may increase in number but decrease in effectiveness, trending towards impotent reactionaries with increasing age (Brighton et al. 2017). The gradual decrease in NK cell clearance of SASP+ senescence cells may thus account for their gradual accumulation with age. This remains an area of active investigation that is sure to yield fundamental insights into the nature of aging on the tissue and cellular levels.

At present, the degree of heterogeneity in the SASP profile is incompletely characterized. Differences in SASP profile may arise from cell type origin, tissue context, cell stressors responsible for the induction of senescence, underlying genetic variation, and interactions with tissue-resident or -infiltrating immune subpopulations (Hudgins et al. 2018).

These putative differences aside, however, there are several hallmarks of the SASP profile that have emerged. Chief among these hallmarks is the requirement for the activation of NFκB in the emergence of the SASP profile. NFκB is a pleiotropic transcription factor widely involved in the inflammatory response whose binding partners in a cytosolic supramolecular activation complex, termed the inflammasome, dictate the downstream nature of the inflammatory response evoked by a given stimulus (Freund et al. 2010; Salminen et al. 2012). While alternative activation of NFκB via the relB subunit has been cast as responsible for immunosuppression in other immunology studies, the SASP contradicts the easy dichotomy of pro-inflammatory vs. immuno-suppressive types of immune activation. Furthermore, the SASP does not fit neatly into a pre-existing category of inflammation, such as Type 2 CD4+ helper T-cell responses characterized via high levels of interleukin-4. Instead, the SASP upregulates pro-inflammatory elements such as IL-1, -6, -8, granulocyte- and macrophage-colony stimulating factor (GM-CSF), and ADAM17 (a sheddase responsible for the final step of inflammatory mediator secretion) as well as factors canonically involved in immunosuppression, such as the CEBP/b transcription factor, IL-10, and transforming growth factor beta. Invocation of the SASP also upregulates the unfolded protein and cell stress response pathways, which cooperate with intracellular effectors of inflammatory activation to produce hybrid immune responses specific to senescent cells. These protein factors are accompanied by a profoundly shifted metabolism, in which oxidative phosphorylation and merged mitochondrial membranes are downregulated in favor of anaerobic glycolysis and short-chain fatty acid oxidation. Collectively, SASP protein and metabolic factors act endogenously on the senescent cell to promote senescence-associated signaling and function while also exerting significant signaling effects on neighboring cells, which may increase their sensitivity to the induction of senescence. Although these elements may act to increase tissue defenses in the event of an infectious or injurious event while aiding in the immune-mediated clearance of injured cells, there exists a threshold past which the accumulation of senescent, SASP+ cells in a tissue begin to compromise its homeostatic function and thus negatively impact organismal fitness and wellbeing.

When senescent cells with a strong SASP profile exceed a given tissue's capacity to eliminate and/or function despite senescent cells, homeostasis is compromised. Whether this phenomenon is ultimately responsible for the emergence of aging-related degradation is compelling hypothesis under active investigation. Meanwhile, that senescent cells and the SASP profile are responsible for this degradation of homeostasis is borne out in multiple studies

demonstrating that ablation of senescent cells or inhibition of SASP effectors is capable of restoring tissue function and increasing organismal fitness, such as the rescue of a systemic progeric phenotype upon inhibition of NFκB in a mouse model.

The similarities between senescent and cancer cells are uncanny (Campisi 2013; Mathon and Lloyd 2001; Schmitt 2007). Like senescent cells, cancer cells also engage in secretion of a mixed bag of inflammatory factors and altered metabolites. The key difference, of course, is that cancer cells engage in uncontrolled replication whereas senescent cells are replication-incompetent. This difference may be attributable to the activity of so-called oncogenes, such as p53, p16, and p21, which are intimately involved in the arrest of replication in cancer cells and whose loss-of-function mutations are associated with aggressive, invasive cancers in every tissue. In this light, senescence cells may be considered a potential pool of tumorigenic cells awaiting the accidental silencing of a key cell cycle arrestor before blooming anew as a transformed and hostile force spreading through the body. Both cancerous and senescent cells experience increased hypoxia, extracellular matrix detachment, and invocation of multiple cell stress pathways, which collectively mediate resistance to the body's first lines of apoptotic control, anoikis and CD8+ T-cell-mediated cytotoxicity. Anoikis occurs when a given epithelial cell loses contact-dependent survival and regulation signals with its surrounding cells and extracellular matrix. Evasion thereof is a signature of tumorigenesis, in which cancerous cells survive basement membrane detachment and become competent of tissue invasion. Although the role of anoikis in senescent cells is as-yet uncharacterized, SASP is also characterized via upregulation of matrix metalloproteinases, which mediate extracellular matrix breakdown and cellular detachment--a process mirrored by tumorigenic cells. Furthermore, the SASP and the secretory profiles of growing tumors are also similar in many ways, both in cytokine secretion, such as TGFb, VEGF, IL-6, and TNFa, and in metabolite secretion, such as reactive oxygen species, malate, citrulline, and other mediators of anaerobic glycolysis. These factors act on the surrounding tissues to activate the surrounding stromal cells, which primes them for immune infiltration and remodeling processes, as well as to induce the infiltration of various immune subpopulations. Immune subpopulations may be recruited from the blood, bone marrow, differentiated tissue-resident populations, and distal secondary lymphoid organs. In the tumor microenvironment (TME), an acidic, hypoxic, inflammatory cellular context similar in composition and effect to the SASP, infiltrating immune cells experience a complex set of regulatory effectors, both activating, such as IL-1 and -6, and inactivating, such as nitric oxide

and IL-10. These regulatory elements act to reprogram the infiltrating immune system, which, over time with repeated exposure, begins to skew towards an immunosuppressive state, in which specialized myeloid and lymphoid populations restrict the activation and promote the anergy or apoptosis of a wide array of immune effectors, including inflammatory macrophages and T-cells that would otherwise be capable of eliminating the tumorigenic or senescent insult and resolving the local inflammation. The arrival of these specialized immunosuppressive cells portends tissue invasion and metastasis in cancer; their role in senescence is as-yet uncharacterized. These similarities indicate that, overall, senescence may be a double-edged sword. While senescence can restrain the continued proliferation, and subsequent compromise of tissue homeostasis, of damaged cells while cell cycle inhibitors are active and intact, lesions that impact key cell cycle inhibitors may free formerly replication-incompetent senescent cells to participate in an tumorigenesis process made more aggressive and lethal by the pre-existence of various pro-tumorigenic factors, such as the altered microenvironment and increased tissue detachment (Vincent-Chong et al. 2018; Collado et al. 2007; Terry et al. 2017; Concha-Benavente and Ferris 2017). If this linkage is further borne out by ongoing studies, the elimination of extant senescent populations may, in fact, act as a prophylactic treatment to decrease the incidence of cancer in aging populations.

In a twist of subtle irony, senescence may also be caused by the very treatments used to combat the spread of a tumor. Many of the leading chemotherapies, as well as radiotherapy, place profound pro-apoptotic stressors upon normal, non-cancerous cells in addition to the tumor cells to which they are targeted. This phenomenon, termed treatment-induced senescence (TIS), is understudied and, at present, it is unknown how widespread this phenomenon is in cancer patients (Ewald et al. 2010). Given the many other deleterious and related side effects of chemo- or radio-therapy, investigation into the molecular means by which to inhibit TIS without giving safe haven to cancer cells will also yield dividends for the increased understanding of how senescence can be resolved or reversed in damaged cells or tissues, which may hold the key to more effective regenerative therapies for a wide range of conditions.

1.3. Modulation of the SASP

1.3.1. Review of Field

The previous sections reviewed the origins and features of senescence. The phenomenon of senescence became a lot more complex when it was discovered that far from being "dead" or close to it, senescent cells were very metabolically active, something that came as quite a surprise to most researchers studying the topic. In addition to being metabolically active, it was further shown that these cells were producing many factors which could impact the cell and its surrounding environment and it became of particular interest to understand the nature of the SASP and to determine whether it can be modulated by known substances in beneficial ways.

The SASP can not only have negative effects but also play a beneficial role in maintenance and repair (Tchkonia et al. 2013). Whether the impact is good or bad depends on the environment and the method of senescence induction as well as the temporal cadence of resolution of any stressors. For instance, in the resolution of wound healing some factors of the SASP can lead to aberrant activation of immune cells and can initiate an inflammatory cascade disrupting the local tissue architecture promoting cancer invasion and growth. In a different context, however, these same effects can stimulate immune-mediated clearance of scaffolding- or wound healing-related senescent cells from the tissue to restore its function.

The impact of the SASP is particularly clear under conditions of frailty, obesity, progeric disorders, and advanced age where widespread chronic inflammation results in dysregulation of tissue function and aberrant immune reactivity are obvious. Concurrently and perhaps counter-intuitively, the SASP can be pro-regenerative; with multiple factors able to reduce the tissue degeneration. (Crescenzi et al. 2012). In this context, during wound healing, senescent cells appear to alert the homeostatic mechanisms of the cell that damage has occurred and aid in orchestrating an appropriate healing response. Consequently, the innate immune system becomes activated as it works to eliminate the damaged cells from the site of injury (Crescenzi et al. 2012; Tchkonia et al. 2013) and factors can stimulate local stem cell populations to assist in limited regeneration of tissue.

One can see other positive aspects of the SASP, one being that of MMPs preventing fibrosis. This is particularly visible in the context of liver injury or with wound healing (Tchkonia et al. 2013), where various MMPs present in the SASP of senescent cells prevent the

accumulation of collagen and compromised tissue function that occurs with fibrosis. Two SASP cytokines in particular have been found to help enhance the development of the senescent phenotype and its growth arrest to guard against oncogenesis. The interleukins IL-6 and IL-8 act prevent tumorigenesis and cancer progression (Menicacci et al. 2017) by increasing immunosurveillance and increasing the odds of the endogenous immune system recognizing and eliminating incipient cancer lesions. However, in keeping with the pleiotropic nature of the SASP, the same two cytokines are also capable of increasing the proliferation of cancer via promotion of cancer stem cell signaling and recruitment of immunosuppressive immune cell types. These negative impacts are particularly pronounced in the epithelial-to-mesenchymal transition, a critical event preceding metastatic dissemination in cancer progression.

The powerful nature of the SASP and its capacity to influence the outcome of so many health challenges makes it important and in the case of cancer especially, urgent, to develop effective means to modulate the process. This modulation must be aimed at promoting or at least preserving the positive effects of senescence, while minimizing or eliminating the negative impacts the senescent phenotype has on the body.

There are 3 primary strategies for the modulation of senescence, each with trade-offs and unknown frontiers in the underlying biological landscape.

1) **Senolytics** - Direct ablation of senescent cells offers several benefits to the organism, but is constrained by several key parameters (Xu et al. 2018; Cazzola et al. 2018; Fuhrmann-Stroissnigg et al. 2018). While the induction of apoptosis of senescent cells and the promotion of NK cell-mediated cytotoxicity or macrophage-mediated efferocytosis both offer improved tissue fitness via amelioration of the SASP and restoration of normal tissue architecture and function, the means by which senescent cell clearing is achieved has important biological consequences. Although apoptosis neatly partitions cellular constituents for efficient recycling, other forms of cell death, such as necroptosis or pyroptosis, result in the induction of local damage responses and may ultimately result in greater local senescence and dysfunction as tissue functions are compromised during the damage response. Senolytics are also a nontrivially difficult therapeutic target in that senescence cells must be safely distinguished from their non-senescent neighbors. As the metabolic effectors and immunogenic secretome of senescent cells are also critical to the normal

function of immune and other specialized subpopulations, reliance upon singular metabolic or SASP factors for discrimination of senescent cells is insufficient. Although work in developing effective delivery vectors is ongoing, strategies that rely upon single- or multi-factorial DNA sensors to express a suicide gene upon detection of senescence are a promising senolytic strategy that offers high discriminatory power and is under active investigation.

2) **Anti-SASP Drugs** - Recent progress in immuno-oncology has resulted in the development of an increasingly comprehensive library of targeted immunomodulatory drugs, such as monoclonal antibody inhibitors of programmed death ligand 1 (Raju et al. 2018). These approaches may be repurposed to counteract the effects of the SASP, such as via inhibition of IL-6 or its cognate receptor with siltuximab or tacilizumab, both monoclonal antibody treatments available for the inhibition of the IL-6 signaling axis in other pathologies. However, such approaches are not without drawbacks. Because the immune system regulates itself based upon a complex set of interacting feedback elements, alteration of any given immune signaling component, such as IL-6, can exert profound side effects elsewhere. Indeed, in many cases, perturbation of the immune system with monoclonal antibody therapies does evoke potent off-target effects, such as the decimation of specific immune subpopulations or the shifting of a given signaling threshold in an related pathology (Hryniewicki et al. 2018). These factors do not bar the use of immunomodulatory treatments in antagonizing senescence, however, they must merely be factored in to appropriate study design, including patient selection and monitoring criteria.

3) **Prophylactic Anti-Senescence Treatments** - At present, it appears that the induction of senescence and the accumulation of senescent cells are an invariable fact of aging. That this process is present even in the early developmental stages of organismal biogenesis demonstrates how widely conserved senescence is as a strategy for coping with cellular excess among complex, multicellular life. However, from a human healthspan perspective, an ounce of prevention is ultimately worth a pound of cure. If, instead of eliminating senescent cells driving a progressive chronic disease, such as

rheumatoid arthritis, one could prevent the accumulation of the senescent cells themselves, it would offer substantial benefit to patients and healthcare systems alike. At present, regrettably, too little is known about the conditions that differentiate the rescue of cell homeostasis after insult and the induction of senescence to begin designing prophylactic treatments to inhibit senescence itself. This is, as such, an exciting frontier for future investigators to explore.

1.3.1.1. Simvastatin

Simvastatin is an FDA-approved drug sold using the brand name Zocor, among others (Liu et al. 2015). Like other statins, such as Lipitor, it is primarily used to lower patients' low-density lipoprotein levels to decrease the progression of atherosclerosis and reduce the likelihood of damaging ischemic events. In many patients, decreased LDL leads to increased healthspan, albeit not without side effects.

Statin treatment side effects include altered steroid hormone biosynthesis, protein prenylation distribution, and cytokine secretion patterns. These effects are due to the inhibition of HMG-CoAcetyl-A-reductase, a key enzyme in the biosynthesis of endogenous sterols. While inhibition of HMG-CoA-reductase is effective at stopping internal production of cholesterol, this also leads to a significant decrease in many other steroid hormones and their derivatives, such as testosterone and the immunomodulatory products of the arachidonic acid pathway. These effects filter further downstream to inhibit the production and secretion of cytokine proteins, such as IL-6, IL-8, and MCP-1. These factors are also key to the SASP profile of senescent cells. Due to this similarity, it has been suggested that statins may be an effective means to antagonize the effects of the SASP on surrounding tissues and cells.

The family of Rho GTPases has three members, Cdc42, RhoA and Rac1 which are targets of simvastatin. They have been implicated as being connected with the SASP through the inhibition of isoprenylation of proteins that affects their half-life and autophagic flux (Soto-Gamez and Demaria 2017). Researchers have shown that simvastatin can inhibit the activity of Rac1 and Cdc42, which in turn reduces the expression of IL-6, modulating its myriad of biological effects. The impact of these effects on senescence are, as yet, incompletely characterized, but a subject of ongoing inquiry.

It may be through the above mechanism by which simvastatin reduces the expression of IL-6 along with a large number of other SASP intermediaries that are able to promote

oncogenesis and the progression of cancers throughout the body. The drug can have this negative effect on SASP factor levels without changing the level of beneficial anti-cancer aspects of senescence (Soto-Gamez and Demaria 2017). As an example, simvastatin has the effect of reducing the expression of IL-6 without encouraging the senescent cells to re-enter the cell cycle to themselves become cancerous. Liu (Liu et al. 2015), showed that breast cancer cells exposed to conditioned media from simvastatin-treated senescent fibroblasts showed a pronounced reduction in proliferation. A clue for a possible mechanism for this emerged when it was shown that conditioned media from senescent cells led to decreased response to Fulvestrant (ICI 182780), a selective estrogen receptor degrader used in receptor-positive breast cancers. Simvastatin significantly reduces this resistance.

These results demonstrate that modulation of lipid levels and lipid biosynthesis via statins may represent a viable means of reducing the induction, or impact, of the SASP in senescent cells in both oncological and non-oncological contexts. These benefits emerge from a downstream side effect of the statin mechanism of action, in which a central mediator of lipid biosynthesis is inhibited, leading to widespread reduction in multiple classes of lipid-based signaling molecules, from sex hormones to immunomodulatory cytokines. While the benefit of statin treatment on the SASP profile is both clear and compelling, it remains to be determined whether the other downstream effects of statin treatment play a beneficial or deleterious role in the modulation of the SASP or alteration of senescence biology.

1.3.1.2. Glucocorticoids

Glucocorticoids are a class of commonly-used anti-inflammatory small molecules that act via binding to the glucocorticoid receptor, which then translocates to the nucleus to act as a transcription factor and alter the expression of inflammatory genes (Laberge et al. 2012), Achuthan et al., 2018). As a result, glucocorticoids exert profound anti-inflammatory effects in a wide variety of cell types, which has led to their adoption for the treatment of progressive inflammatory diseases, such as chronic obstructive pulmonary disorder and rheumatoid arthritis. Glucocorticoid treatment inhibits the activation of NFkB, a master regulator of inflammation, but also gives rise to significant side effects, such as edema and eventual treatment resistance. As such, glucocorticoids are primarily recommended for short-term management of inflammatory conditions and their chronic usage is closely monitored.

Glucocorticoids have homology relationships with immune system effectors (Velarde and Demaria 2016). These effectors broadly regulate the feedback network of immune cell interactions, which collectively determine the scope and nature of inflammatory events. They also inhibit cancer cells *in culture* and *in vivo*. Because of this anti-cancer capability, they are often used as a co-treatment to inhibit the proliferation of lymphocytes in the case of treating lymphoma and leukemia as well as minimize the harmful side-effects of inflammation associated with cytotoxic drugs involved in chemotherapy.

The adrenal gland produces different glucocorticoids which have been shown to suppress factors produced by senescent cells. These would include cortisol which is the most common and abundant found in humans as well as cortisol which is the glucocorticoid primarily found in rodents although it can be found in humans as well (Laberge et al. 2012). These two endogenous glucocorticoids are able to reduce the expression levels of many factors known to be part of the canonical SASP. The suppression of the sentinel SASP maker IL-6 can, in turn, help in the mitigation of some of the more harmful effects of SASP, primarily via the compound down-regulating the IL-1α and NFkB pathways, two major pathways closely tied to SASP (Velarde & Demaria, 2016).

1.3.1.3. Rapamycin

Rapamycin, also known as sirolimus, is a potent macrolide inhibitor of mTOR (mechanistic target of rapamycin) first discovered in a Gram-positive soil bacteria species found on Rapa Nui (Easter Island) (Harrison et al. 2009). Rapamycin acts as a potent immunosuppressant and as such is commonly used to prevent transplant rejection and decrease the local inflammatory response to the placement of coronary stents. Rapamycin's primary molecular target is mTOR, which is a key integrator of signals governing cellular proliferation and apoptosis (Anisimov 2013). mTOR is a serine/threonine kinase that, under certain conditions, can also function as a tyrosine kinase. It is the key component of the mTORC1 and mTORC2 supramolecular activation complexes that differentially regulate cellular responses to pro-proliferation signals and thus governs overall cellular behavior. mTORC1 is comprised of mTOR, Raptor, Deptor, and other canonical protein components that forms in response to increased nutrients and growth factors to upregulate overall protein biosynthesis, lipolysis, and increased microtubule dynamics while down-regulating autophagy. mTORC1 is thus favored in conditions of cellular proliferation and activation, where its overactivation is associated with increased

tumorigenesis and is closely tied to the MAPK (mitogen associated protein kinase) pathway. mTORC2 is comprised of mTOR, Rictor, Protor, and others that preferentially respond to PI3K-Akt signaling downstream of insulin or GPCR signaling. mTORC2 activity acts to regulate actin cytoskeleton organization and cell survival under conditions of cellular stress, such as acute environmental damage or chronic inflammation. mTORC2 is essential to normal cell cycle progression. mTORC1 and mTORC2 are linked by IKK, a kinase that inhibits NFκBand in doing so restricts the cellular inflammatory response (Dan et al. 2008). Overall, mTOR serves as a central regulator of cell homeostasis, where its dysregulation can lead to apoptosis, tumorigenesis, or senescence.

The activities of mTOR and its subsequent mTORC1/2 complexes govern proliferative responses in a wide variety of cell types. Interestingly, it was found that the inhibition of mTOR via rapamycin greatly increased average lifespan in a variety of model organisms. Rapamycin exerts these effects in 2 ways. First, rapamycin inhibition of mTOR leads to decreased overall proliferative drive and increased autophagy, which may serve to increase individual cellular fitness and eliminate sub-optimal cellular processes otherwise enabled by continued mTOR activation. Second, mTOR activation sits upstream of the activation of many components of the SASP, so inhibition thereof leads to a reduced SASP expression even in senescent cells (Jahrling and Laberge 2015; Laberge et al. 2015). Rapamycin has been successfully used in the clinic to treat conditions that are driven by overactivation of proliferative signals, such as tuberous sclerosis complex which results in widespread benign tumorigenesis, to great effect, as well as in meditiating decreased transplant reduction via lowering of the sensitivity of T- and B-cells to interleukin-2. These effects have led to the study of rapamycin in other conditions, where studies are ongoing.

Rapamycin's potent cellular effects have also attracted attention from aging researchers (Wilkinson et al. 2012). Due to how mTOR responds to calorie restriction and hypoxia, namely, by stalling cell proliferation and conserving resources, rapamycin treatment exerts myriad cellular effects that closely mirror cell function under calorie restriction. Although research into the exact mechanism by which these altered cellular functions contribute to increased longevity are an ongoing area of research, it has become clear that there is a clear link between effective metabolic regulation and the senescent phenotype. This similarity has led to the initiation of human clinical trials to study the effects of rapamycin on aging-related features, whose results will be eagerly received by the aging community.

While rapamycin has clear clinical promise, it also has significant drawbacks. Because rapamycin depresses immune activation, chronic usage of rapamycin increases patients' risk for infectious disease. Also, due to the effects of rapamycin on mTORC2 and glucose metabolism, prolonged rapamycin treatment can lead to the emergence of insulin insensitivity and Type 2 diabetes. These potent side effects make rapamycin, in its current embodiment, unsuitable for long-term chronic treatment, but do not necessarily bar anti-aging usage thereof. Instead, short-term dosing schedules and/or combination with complementary drugs, such as metform which acts to stabilize mTORC2 even with rapamycin treatment, offer the potential to reap the benefits of rapamycin treatment while limiting its deleterious side effects.

1.3.1.4. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Non-steroid anti-inflammatory drugs (NSAIDs) are among the most-widely used medicines on Earth, commonly taken for their analgesic effect, which ranges from mild in small doses to potent in larger doses, particularly in carefully-designed combinations (such as compounded ibuprofen and acetaminophen). NSAIDs are a diverse molecular class, comprising ibuprofen, acetaminophen, naproxen, diclofenac, celecoxib, piroxicam, and aspirin that act via a common mechanism: the inhibition of cyclooxygenase 1 and 2 (COX1 and COX2). The COX enzymes are an important mediator of inflammation and pain via synthesis of a wide variety of lipid-derived signaling molecules, such as prostaglandins, that activate local inflammatory and pain responses. Different members of the NSAID family have differing affinity for COX1 or COX2, which determines their efficacy and side effect profile. While the risks of stomach ulcers, edema, and dizziness are common to all NSAIDs, which COX enzyme they preferentially inhibit determines the severity of their side effects. For example, ketorolac is more selective for COX1 and accordingly is associated with a higher incidence of debilitating stomach ulcers whereas celecoxib is specific to COX2 and causes very few side effects (Lasry and Ben-Neriah 2015; Gao et al. 2018).

The products of COX lipid metabolism are also intimately involved in the formation and effects of the SASP. By inhibiting the production of inflammatory mediators, NSAIDs may aid in attenuating the SASP and its deleterious effects on surrounding cells and tissue. While this has been directly shown in animal models, it has yet to be proven in human patients. The preponderance of inflammatory comorbidities in human patients chronically taking NSAIDs prohibits straightforward trial design and interpretation while the definition of aging and the

medical means to measure it are a matter of emerging debate. These 2 factors, in conjunction, have meant that most human studies of NSAID effects have been in relation to their acute effects on tissue repair after surgery or infarction instead of aging. As such, this remains a compelling frontier for further investigation, where comparison of which NSAID offers the best anti-senescence effects with the lowest long-term side effect complications (Ruhland et al. 2015).

1.3.1.5. Targeted Immunotherapy

Immunotherapy has recently leapt into public awareness with the high-profile successes of checkpoint inhibitor immunotherapy and chimeric antigen receptor in T-cells (CAR-T) engineered cell therapies in the treatment of various cancers. This progress has been driven by an increasing body of research demonstrating that the manner in which the immune system reacts to a growing tumor determines whether that tumor is eliminated by endogenous effectors, grows to clinical significance, or responds to treatment, including chemo- or radiotherapy (Pardoll 2012; Mellman et al. 2011). Those patients with highly active immune systems face extremely favorable prognosis, where anti-cancer therapies act to activate and strengthen an inflammatory response that cooperatively restrains tumor growth and/or directly mediates its destruction. This phenomenon drives the abscopal effect, where successful immune activation at 1 tumor site can lead to cancer regression at many other sites to which it has spread. However, in patients with highly suppressed immune systems, potent feedback loops between growing tumor cells and reprogrammed immune cells lead to the formation of a varied immunosuppressive microenvironment that inhibits anti-cancer immunity, diminishes treatment efficacy, and facilitates disease spread. Characterization of the molecular means by which these changes occur has led directly to the successful design and testing of engineered cancer immunotherapies (CIT), which act to disturb the pre-existing immunological balance in cancer in a manner that evokes disease stabilization or regression. This has been particularly successful with the application of antibodies that neutralize immune-inactivating factors in skin and lung cancer as well as in the revolutionary use of genetically-reprogrammed T-cells to target and eliminate B-cell leukemias. Additional targets and strategies are the subject of intense therapeutic investment and development efforts, hinting at the emergence of a broad library of targeted CITs enabling rational combination therapeutic strategies to address the specific vagaries of a given patient's cancer.

The success of CIT is notable for aging research in what it has accomplished clinically: precision manipulation of the endogenous immune system to evoke a therapeutic response to a pathological stimulus. Already, researchers are exploring the application of engineering cell therapies and precision neutralizing antibodies to a host of other conditions, such as graft-vs-host disease, rheumatoid arthritis, and Crohn's disease, where the manipulation of the overall immunological state of a given patient offers significant potential to improve their health. While currently speculative, these approaches may find success in the treatment of senescence-associated conditions, via reduction in the effects of the SASP or an increase in immunological surveillance and elimination of senescent cells (Vilgelm et al. 2016). The former may be accomplished with neutralizing antibodies specific to dominant SASP features, such as IL-6 or its receptor, while the latter may be realized through restimulation or engineering of NK cells to better recognize and eliminate senescent cells. These approaches may engender immunological side effects downstream in the complex feedback network of immune communication, but this itself may eventually be harnessed to therapeutic ends via clinically coordinated, deliberate inflammation to remodel damaged or aged tissues and restore them to their juvescent state.

1.3.1.6. Senolytics

Given the accumulating evidence of the deleterious health effects of senescent cell accumulation and function, a compelling therapeutic strategy is the outright elimination of senescent cells from the body, either totally or partially. Preclinical efforts to accomplish this in progeric or aging-related inflammatory disorder mouse models have demonstrated profound therapeutic benefit for the model organism, where reduction in senescent cell population or function has led to renewed tissue function and recovery of homeostasis. However, the clinical translation of previous approaches and the development of new approaches is hindered by a central issue in senolytic therapies: accurate discrimination of sensescent from non-senescent cells. The stakes of this issue are high: off-target elimination of non-senescent cells has the potential to compromise tissue function and evoke potent side effects. Indeed, this is seen in senolytics with poor discrimination of senescent cells, such as the use of navitoclax to inhibit anti-apoptotic proteins upregulated in senescent cells. However, this also affects other cells in which the balance of apoptotic and anti-apoptotic effectors are critical to homeostasis, such as platelets. Although navitoclax's safety profile is an improvement on its predecessor molecule, obatoclax, its side effect of thrombocytopenia greatly limits its clinical use (Zhu et al. 2016).

Other approaches, such as combination kinase inhibitors, similarly offer anti-senescence potential but, again, are compromised by off-target side effects, such as the cardiotoxicity associated with dasatinib. Recognition of these off-target effects has motivated the design and testing of more targeted strategies, such as the novel anti-senescent genetic construct used by Demaria and Campisi to detect and induce apoptosis in $p16^{INK4A}$-overexpressing cells (Kohli et al. 2018). While this approach offers superior discrimination of senescent cells from non-senescent cells, it is complicated by the need for a safe, effective delivery vehicle, a considerable engineering challenge under active investigation by multiple groups. Nonetheless, the field of senolytics remains a promising field for the mitigation of the effects of senescence and holds significant clinical potential in the coming years.

1.3.1.7. Prophylactic Anti-Senescence Treatments

Another promising senescence mitigation strategy is that of prophylaxis, wherein lifestyle changes or medical interventions are undertaken to lessen the entry into senescence, decrease the accumulation of senescent cells, promote the clearance of senescent cells, or blunt the effects of senescent cells on neighboring cells and the organism. This strategy is, however, at present completely speculative, as our understanding of the processes by which senescence is entered or senescent cells are cleared *in vivo* is still rapidly evolving. The identification of circulating biomarkers and the surveillance of stem cell health and activity is, nonetheless, providing vital clues as to how senescence unfolds in human patients and how it may be modulated. These studies are buttressed by genomic epidemiology in which increasingly powerful genetics technologies are brought to bear on human subpopulations that experience exceptional healthspan and/or lifespan. Results have indicated that specific macronutrient ratios and micronutrient availability may potently influence the programming of apoptosis, particularly with chronic exposure over time. Disparate results in the study of metabolism have also shown that periodic periods of mild cellular stress, such as fasting or prolonged endurance exercise, can stimulate autophagy and successful completion of the cellular damage response. Nonetheless, at present, this field is completely uncharted territory that demands careful study of human interventions, whether through innovative trial design or retrospective molecular epidemiology, to identify and characterize the causative factors involved in pro-senescence or pro-longevity at the cellular level.

2. Chapter 2 - Apigenin and the SASP

2.1. Introduction and Literature Review

In the past, we screened the Prestwick Library of 1280 small molecule FDA-approved drugs in order to identify potential candidate compounds able to lower the pro-inflammatory nature of the SASP, which characterized glucocorticoids (Laberge et al. 2012) as potent SASP inhibitors. None of the candidates surveyed were selectively toxic to senescent cells, which was one of the goals of the study, however, a number of other candidate compounds emerged from the screen which warranted further investigation for their possible effects on the pro-inflammatory nature of the SASP as well as on other senescent phenotypes. Among the candidates identified was apigenin, unusual among the library of FDA approved drugs in that it is natural compound. Being derived from foodstuffs with no known toxicity when obtained from the diet (Ross and Kasum 2002), it has the potential to be more readily useful as a possible intervention or therapeutic for conditions associated with the SASP, and therefore represented an attractive focus for further examination.

Apigenin (4',5,7,-trihydroxyflavone; MW: 270.24) is a member of the family of flavonoid compounds, abundant in a variety of plants and herbs such as parsley and chamomile tea (McKay and Blumberg 2006), where it can make up to 1.2% of the plant by weight (Shukla and Gupta 2010). It exists in food sources as various acylated derivatives and apigenin-7-O-glucoside (Svehliková et al. 2004). Pure chemical apigenin itself is hydrophobic and relatively unstable compared to the food borne glycoside, which is also much more soluble and bioavailable (Ross and Kasum 2002).

Apigenin, like other bioflavonoids, inhibits cell proliferation and selectively induces apoptosis in cancer cells *in vivo* (Gupta et al. 2001). This ability, as well as acting as an inhibitor of angiogenesis by binding to VEGFR1/R2 receptors (Seo et al. 2013), has made it a compound of relatively intense study as an anticancer agent. Targets of apigenin that could be involved in this anti-carcinogenic activity include heat shock proteins (Osada et al. 2004), telomerase 30 (Menichincheri et al. 2004), fatty acid synthase (Brusselmans et al. 2005), the aryl hydrocarbon receptor (Reiners 1999), casein kinase 2 alpha (Singh and Ramji 2008), HER2/neu (Way et al. 2004), and matrix-metalloproteinases (Kim 2003), while apigenin is also a weak xanthine oxidase inhibitor alone (Lin et al. 2002). The ability of apigenin to reduce markers of inflammation

produced by immune cells in response to stimulation with lipopolysaccharides or TNF-alpha has been examined (Liang et al. 1999), as well as in the context of allergen-induced airway inflammation (Duarte et al. 2013; Li et al. 2010; Kang et al. 2011). It is clear from all of these studies that, during acute inflammation, apigenin has a potent anti-inflammatory effect; however, it is also clear that there is not yet a consensus on the singular mechanism, or multiple mechanisms, by which apigenin exerts its potent anti-inflammatory effects.

The ability of apigenin to reduce the inflammation profile of the SASP had never before been examined; we therefore investigated whether its mechanism of action might reveal some novel aspects of cellular senescence and the SASP, either in their biological mechanisms or the therapeutic inhibition thereof.

With respect to its pharmacokinetics and safety, apigenin is rapidly converted to its bioavailable glucuroside and sulfate conjugates upon ingestion (Patel et al. 2007), achieving maximum plasma concentrations in humans within 7 hrs (Meyer et al. 2006) and having, in comparison to other flavonoids, a relatively long half-life of 91.8h in rats (Gradolatto et al. 2005). Intake of 100 mg flavonoids results in a plasma concentration of ~410 nM (Cao et al. 2010) so, theoretically, ingestion of 30 g of dried parsley could produce plasma levels of 5 μM of bioavailable apigenin. If we consider its relatively long-half life and that apigenin is a hydrophobic molecule normally associated with lipid in blood plasma, local concentrations of apigenin in lipid-rich tissues could be higher, holding out the potential for dietary apigenin to give rise to concentrations in a range able to reduce the SASP of senescent cells *in vivo*. As adipose tissue-related inflammation, in particular, has been associated with age-related inflammation (Berg and Scherer 2005), this could be a particularly attractive method of addressing the production of SASP factors produced by senescent cells in tissue.

2.2. Methods

2.2.1. Cell Culture

All primary human fibroblasts were cultured as follows (except where noted otherwise). Fetal female lung cells (IMR90); and male foreskin dermal cells (BJ and HCA2) were incubated at 37°C in a 3% O2/10% CO2 atmosphere after being seeded at 10,000 cells/cm2. They were grown in Dulbecco's modified Eagle medium (DMEM) (VWR, Cat# 45000-312), which was

supplemented with glucose (4.5 g/l), glutamine (4 mM), 10% fetal bovine serum (FBS), as well as antibiotics (100 IU penicillin, 100 µg/ml streptomycin). Media were replaced every 48 h.

IMR90 and BJ cell lines are well known in the established literature as they are readily available from ATCC, a major cell line supplier. HCA2 cells, however, are not quite as well known having been given as a gift somewhere in the mists of time to the Campisi Lab. HCA2 cells were used here because, compared to other cell lines they secrete substantially higher level of proinflammatory cytokines upon induction of senescence and as such the effects of drugs able to reduce those levels are more readily discerned when starting with relatively higher levels of secretion. The other two cell lines were used to follow up on and confirm the findings in HCA2 fibroblasts in different tissue types and gender to ensure that observed effects were not specific to the effects of senescence in HCA2 cells. For the cancer cells assays, highly metastatic human breast cancer cell lines (MDA-MB231, ZR75.1) were cultured in 10% FBS (the standard condition) in the same medium at 20% oxygen; although it would be more physiologically accurate to culture them in 3% oxygen, this is not commonly used employed in available literature and, as such, we elected to use standard methods from previous reports rather than risk confounding results via the introduction of an idiosyncratic environmental variable.

2.2.2. Senescence Induction and Treatment with Drugs

2.2.2.1. Irradiation Induced Senescence

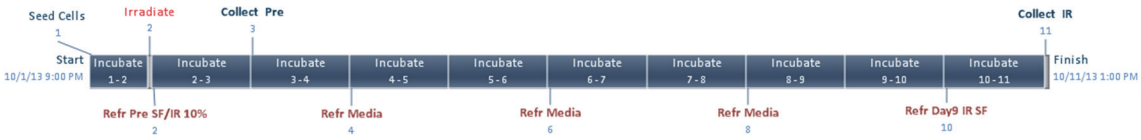


Figure 4: Timeline for standard senescence induction for irradiation-induced senescence and sample collection.

Senescence induction with ionizing radiation used 10 Grays of irradiation in HCA2, BJ, IMR90, MDA-MB231, and ZR75.1 cell lines. This method results in widespread DNA damage, which in turn induces the DDR and senescence-associated cell signaling. In previous reports (Jones et al. 2005; Panganiban et al. 2013), irradiation has been found to induce senescence and its associated secretory phenotype, rendering this method an ideal technique for replicable, high-throughput study of senescent behavior *in culture*.

2.2.2.2. Oncogene Induced Senescence

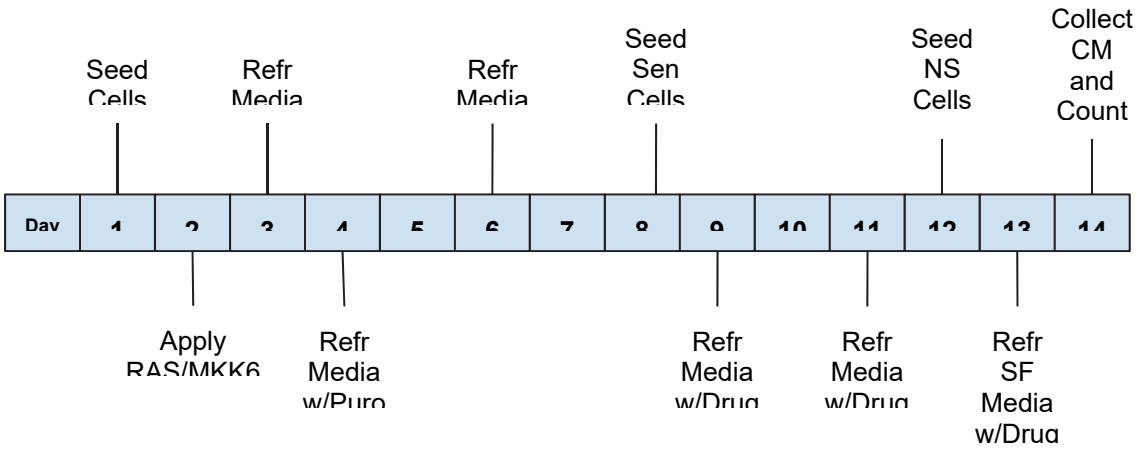


Figure 5: Timeline for treatment of oncogene-induced senescent cells. Human primary fibroblasts are induced to undergo senescence via overexpression via infection using lentivirus-mediated containing RAS or MKK6 oncogenes. The resulting senescent cells are treated with apigenin and conditioned media collected and analyzed.

Primary human HCA2 and BJ fibroblasts were infected with lentiviruses containing H-RASV12 or MKK6EE or control L3P vector and placed under puromycin selection as previously described in (Coppé et al. 2008; Freund et al. 2011). The resulting infected cells were reseeded after selection and provided media containing DMSO or apigenin (10 μM) and the media were refreshed after 48 h. After another 48 h, the cells were given serum-free media containing DMSO or apigenin at 10 μM; conditioned media were then collected 24 h later and IL-6 levels were quantified by AlphaLISA normalized by cell number.

2.2.2.3. Replicative Senescence

IMR90 and BJ primary fibroblasts (PD35) were cultured under standard conditions and passaged until they ceased to have an observable increase in cell number and started exhibiting a large, flattened morphology and stained positive for SA-B-Gal to indicate they had undergone replicative senescence through telomere erosion. IMR90 achieved a relatively high-passage (PD 73.2) while the BJs ceased dividing at 60.2 passages.

2.2.3. SA-B-Gal

Senescence-associated beta-galactosidase (SA-β-gal) activity was determined using the BioVision Senescence Detection Kit (Cat# K320-250) protocol. For each experiment, approximately 100–150 cells were counted.

2.2.4. qPCR - Roche UPL Probe + TaqMan Protocol

The UPL system is a probe-based system for doing quantitative PCR. UPL stands for "Universal Probe Library" and of the 165 prevalidated probes, each of which binds a specific short DNA sequence, there is one that will match a sequence within your amplification region if you use Roche's web tools to design your primers. The probe will only fluoresce once it binds to its target sequence, so even if the primers amplify some other segment as well as your gene, there is a very low chance that it will have the probe sequence as well, making the assay very specific to the genes of interest.

2.2.4.1. Primer design for the UPL system

Primers were designed using Roche's Universal Probe Library Assay Design Center, a web-based primer design tool, via selection of organism, gene of interest, sequence ID, and other information. This system then generates primers specific to the gene of interest. Primers were designed and ordered via this system. Upon arrival, they were reconstituted to 100µM in DNase-free water.

2.2.4.2. Sample preparation - RNA extraction and RT rxn

All mRNA was extracted using the RNeasy Mini Kit (Qiagen #74104) or some other RNA extraction kit/protocol (e.g. the mirVana miRNA isolation kit from Ambion,#AMI 560, which recovers all RNA, not just miRNAs). RNA was then quantified on the NanoDrop and its purity determined using a BioAnalyzer (or an RNA gel if you don't have a bioanalyzer).

Reverse transcription reactions on 2 ug of RNA for each sample was then performed according to the instructions of the vendor whose kit is being used. A 100% conversion from RNA to cDNA (so if you had 2 ug of RNA in a 20 uL reverse transcription, you now have 100 ng/uL of cDNA) was assumed for each sample.

2.2.4.3. Quantitative PCR with thc UPL system + TaqMan (Applied Biosystems, #4304437)

Quantitative PCR was then carried out, using tubulin as a control, according to the following protocol:

Each well will have a final volume of 10 uL, with the following reagents:

-cDNA template (10-50 ng in 2.5 uL; I use 50 ng whenever possible)

-Primer pair (final concentration 200 nM, stock from Operon at 100 µM — 500x dilution)

-UPL Probe (final concentration 100 nM, stock at 10 µM — dilution)

-TaqMan 2x mix (final concentration Ix, 5 uL per well)

cDNA:

10-50 ng of cDNA

0.1-0.5 uL of RT rxn per well

bring up to 2.5 uL with water

2.4-2.0 of H2O per well

Make a master mix with enough DNA for all the reactions of a given sample + —10% extra.

E.g. If you have 3 primer pairs per sample, each of which you are running in quadruplicate, that's 12 wells per sample, so make enough for 14 wells.

0.5 uL of (50 ng) = 7 uL cDNA

2.0 uL H2O x14 = 28 uL H20

Primer+Probe+TaqMan:

200 nM final concentration of each primer (100 µM stock)

10 uL(0.2/100) = 0.02 uL of each primer per well

100 nM final concentration of probe (10 µM stock)

10 uL(0.1/10) = 0.1 uL of probe per well

1x final concentration of TaqMan (2x stock)

10 uL(l/2) = 5 uL of TaqMan mix per well

Water up to 7.5 uL

7.5-5-0.1-0.02 = 2.38 uL H20 per well

Make a master mix with enough for all the reactions of a given primer set + —10% extra.
E.g. If you have 8 samples, each of which you arc running in quadruplicate, that's 32 wells per primer set, so make enough for 35 wells,
0.02 uL of each primer x35 = 0.7 uL primer
0.1 uL probe x35 = 3.5 uL probe
5 uL TaqMan mix x35 = 175 uL TaqMan mix
2.38uL x35 = 83.3 H2O

Add the 2.5 uL of cDNA mix to the wells first
Then add 7.5 uL of Primer+Probe+TaqMan mix, changing tips with each well

Seal with LightCycler 480 Sealing Foil (Roche, #04 729 757 001) and use sealing tool to make sure every well is completely sealed, or evaporation will destroy your data

Centrifuge at 1500xg for 2 minutes (use microplate centrifuge)

Run quantitative PCR on Roche 480 LightCycler
-make sure that the correct plate block (96 or 384) is in the machine — if not, remove it and insert the other one
-start the program.
-click "New Experiment"
-in the dropdown box labeled "Detection Format", set to "Monocolor hydrolysis Probe/UPL system"
-set the reaction volume right corner
-add 2 programs using the "+" button, for a total of 3
-under the "Analysis Mode" drop down box, set the 2nd program to Quantitative'
-the first program is 1 cycle, 10 min at 95C
-the second is 40 cycles with three steps
15 sec @ 95C, 5 sec @ 70C, 1 min @ 60C
-under the "Acquisition" drop-down box, set the 60C step to “single"

-the third program is 1 cycle, 1 hour at 37C

-put the plate into the machine using the gray button on the front of the machine to open and close the door. Well A1 goes on the top left.

-click "Start Run", takes about an hour

2.3. Results

Statistical Analysis: All statistical analysis is done with p-values from a normal T-Test after normalization. Unless otherwise indicated, a * indicates $p<0.05$, ** indicates $p<0.01$ and *** $p<0.001$. Unless otherwise noted, statistics were derived from three biological replicates for each condition.

2.3.1. Identification and Verification of Apigenin as a Candidate

2.3.1.1. Initial Candidates from the Prestwick Library

Using the standard method for inducing senescence as described in above, candidate drugs shown to reduce IL-6 secretion in a prior screen of the Prestwick Library were selected for verification of their ability to reduce IL-6. A summary of the result of that experiment are shown in Figure 6.

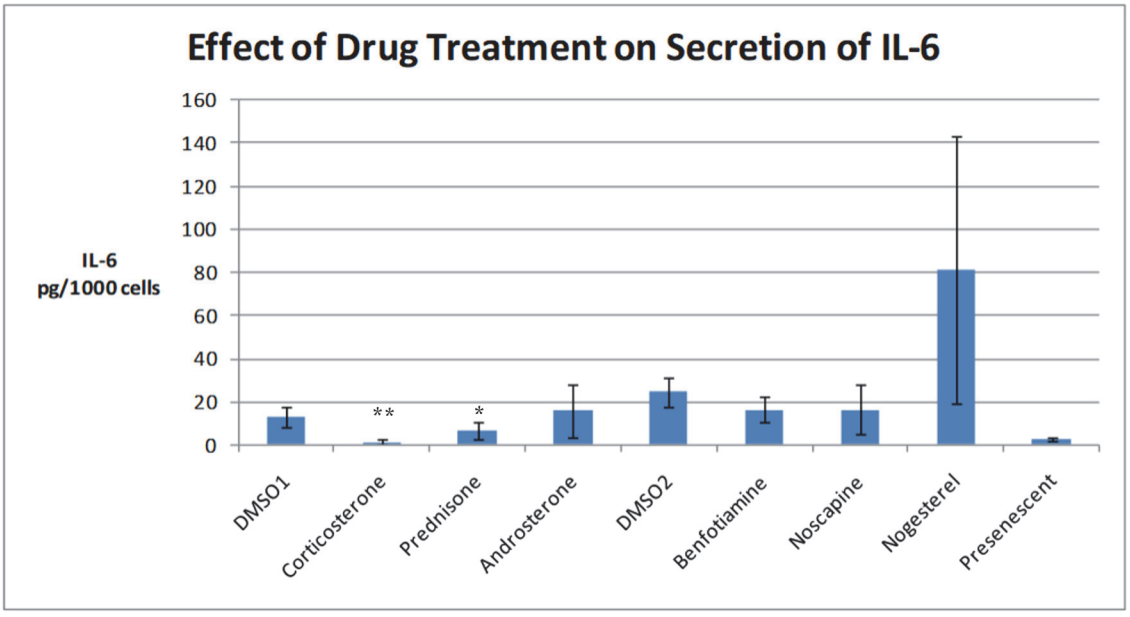


Figure 6: Effect of selected Prestwick Library candidates on IL-6 secretion of senescent WI-38

cells.

In Figure 6 corticosterone was the most effective at reducing secreted IL-6. This major hit went on to be published in (Laberge et al. 2012). The results above showed that the most of the candidates were able to lower IL-6. However, the positive control IL-6 levels were quite low in comparison to historical experiments using this same cell line. Therefore, to test candidate drugs on a cell line with higher background levels of IL-6 secretion upon senescence induction, we decided to try a different cell line, HCA2, primary human fibroblasts from neonatal foreskin. After deciding that HCA2 was the appropriate cell line, we repeated the experiment with another group of potential candidates with the results shown in Figure 7. One of the early candidates which was verified to significantly reduce IL-6 secretion in senescence HCA2 cells was apigenin.

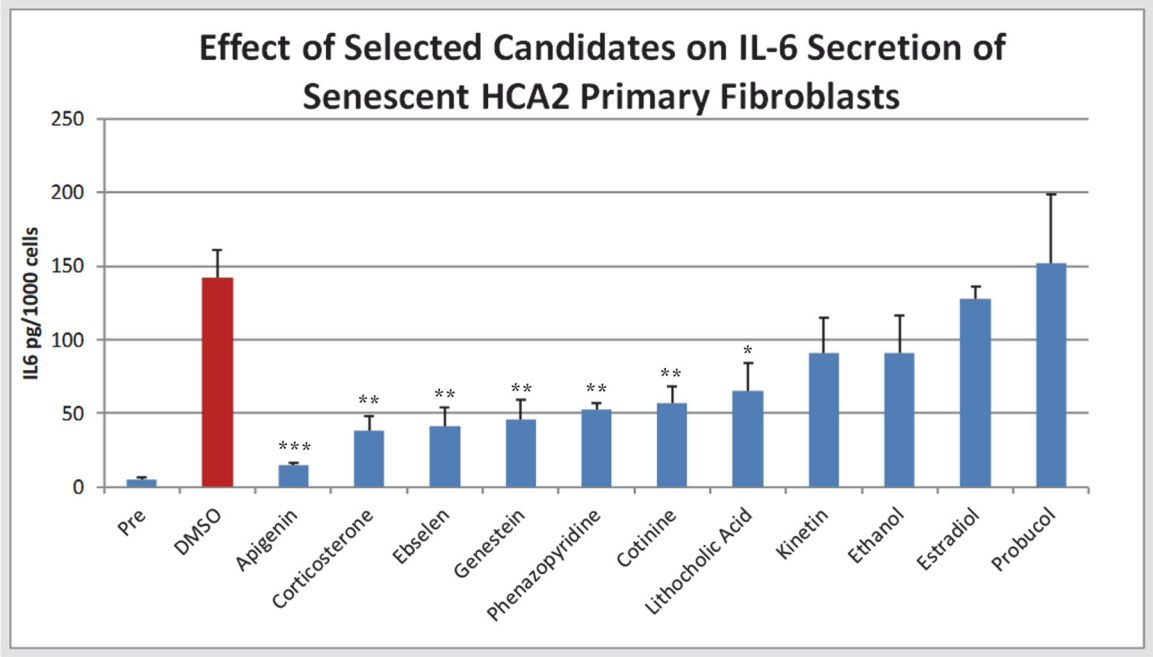


Figure 7: Effects of selected Prestwick Library candidates on IL-6 Secretion of senescent HCA2 primary fibroblasts

After multiple experiments with other selected candidates from the Prestwick Library which produced qualitatively similar results, we identified apigenin as a primary candidate with which to move forward not only because of the magnitude of the suppression of IL-6 but also because it was the only natural compound in the Prestwick Library of FDA-approved drugs and as such would likely have fewer side-effects if it were ever to be used in humans.

We then conducted an initial dose response study which found that apigenin was able to reduce IL-6 secretion in senescent cells to levels close to that of non-senescent HCA2 cells.

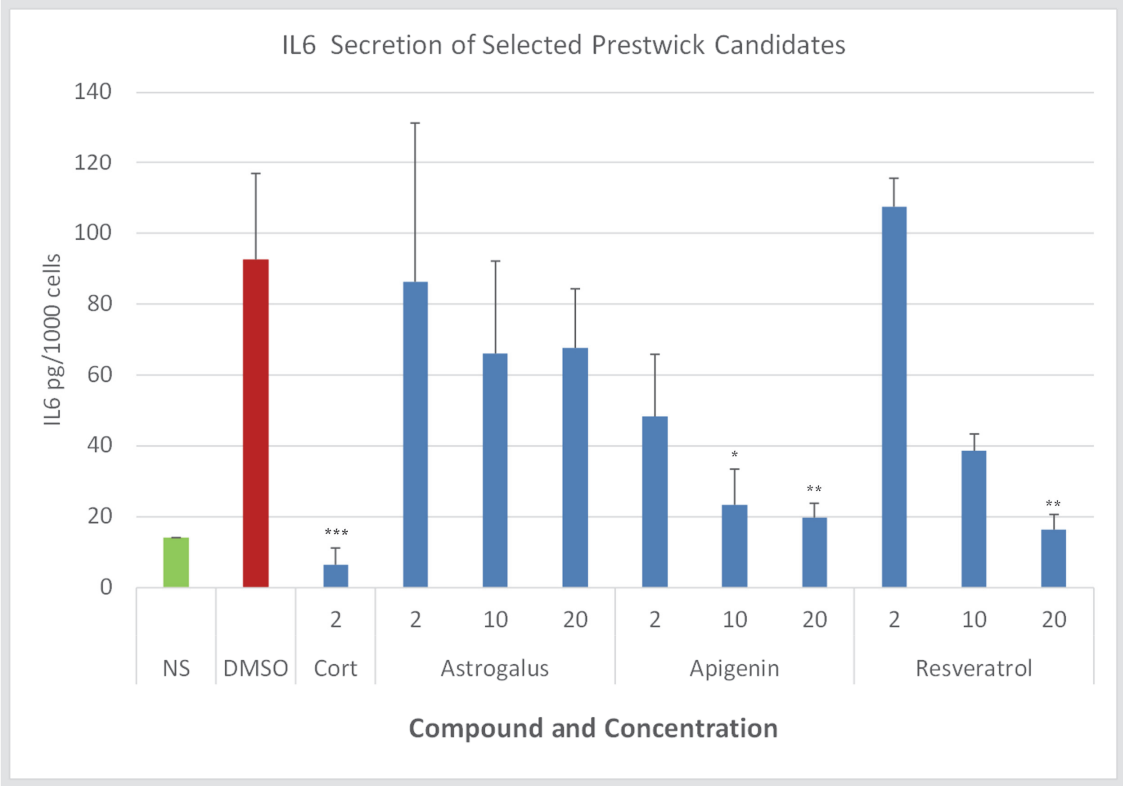


Figure 8: Effect of selected Prestwick Library candidates on IL-6 Secretion of senescent HCA2 primary fibroblasts

These results were quite encouraging. Like resveratrol, but unlike Astrogalus, apigenin treatment exhibited a clear dose-dependent reduction in IL-6 secretion from senescent HCA2 fibroblasts, which indicates that the observed treatment mechanism is due to apigenin treatment and not other, unstudied factors, which would otherwise be suspected of therapeutic effect if no dose-dependency in the therapeutic effect were observed. Therefore, we decided to move forward with Apigenin. We started by examining its effects on the SASP and other aspects of the senescence phenotype with an eye towards identifying the mechanism by which it was able to potently reduce IL-6.

2.3.2. Other Forms of Senescence Induction

We began by looking at the effects of Apigenin on the SASP by exploring the effect of apigenin on SASP effectors using several different mechanisms of inducing senescence with the results below. These experiments were intended to determine whether the anti-SASP effects of

apigenin reported above were specific to the means by which senescence was induced, or instead represent a pan-senescence subtype phenomenon worthy of further therapeutic investigation.

2.3.3. Oncogene-Induced Senescence

Double-strand breaks induced by irradiation (Rodier et al. 2009) and telomere shortening (Serrano et al. 1997) are both recognized as genotoxic stressors able to potently activate the DNA-Damage Response, leading to expression of many markers of senescence and the senescent phenotype. Other types of stress also related to the maintenance of DNA integrity and synthesis, such as the strong mTOR-mediated mitogenic signalling produced by various growth factors from epidermal growth factor to vascular endothelial growth factor and luteinizing hormone to insulin, are also senescence-inducing. It is now well know that even the low concentrations of growth factors in fetal bovine serum (FBS), a commonly used cell culture reagent, can also induce senescence in sensitive cell types, such as mesenchymal stem cells (MSCs) which rapidly enter senescence in the presence of high concentrations of FBS. Primary fibroblasts are more resistant to FBS-induced senescence and continue to rapidly proliferate in the presence of high serum; however, the genes which such factors stimulate can cause oncogene-induced senescence when mutated in even these more robust cell types. An example of these genes which we examined in this project that can stimulate cell proliferation were RasV1042 and MKK6EE, both of which are known to be causal in the development of many types of cancer.

To produce oncogene-induced senescence, primary fibroblasts were infected with lentiviral-mediated oncogenic Ras and MKK6 constructs as referred to in the Methods section. Conditioned media was collected and analyzed with the results shown below.

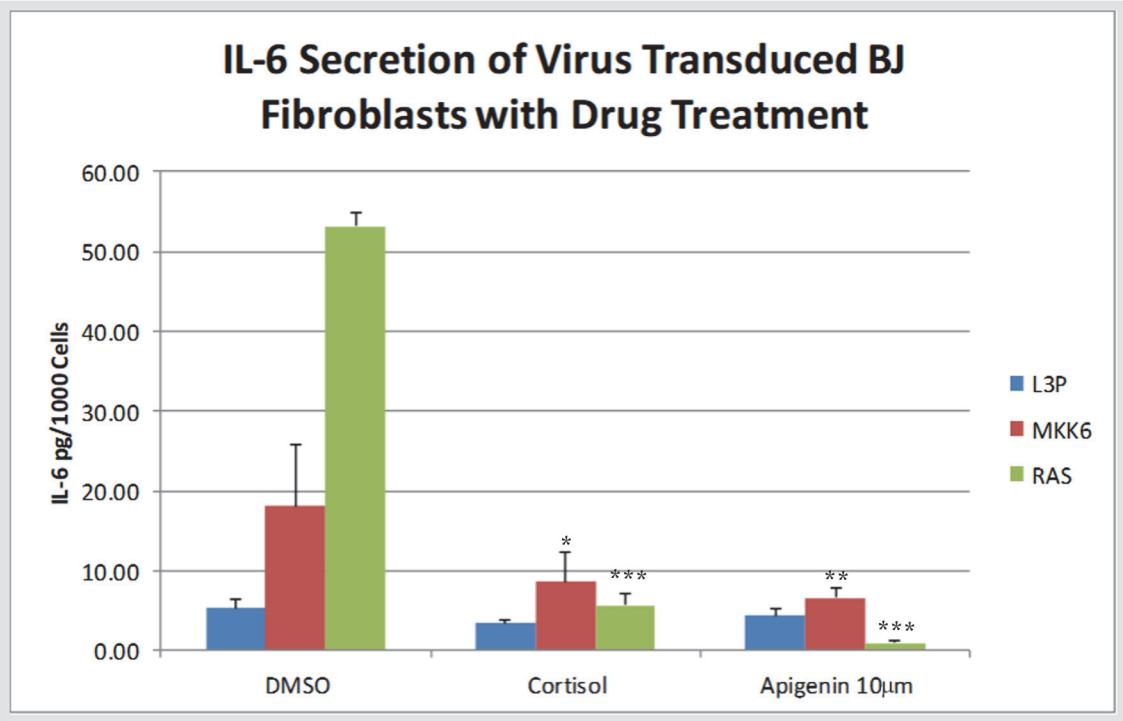


Figure 9: Apigenin reduces IL-6 secretion of oncogene-induced senescent BJ fibroblasts. BJ fibroblasts induced to undergo senescence via lentivirus-mediated overexpression of RAS or MKK6 secreted higher levels of IL-6 when compared L3P vector-only negative controls after treatment with DMSO-vehicle. This increase was potently attenuated by treatment with apigenin or positive control cortisol.

Results obtained showed that both MKK6 and RAS oncogene manipulation increased the levels of secreted IL-6 profile of senescent BJ fibroblasts, an effect which was potently inhibited by both cortisol and apigenin. Results shown above are compared to the L3P negative control genetic construct.

2.3.4. Replicative Senescence

The shortening of telomeres has been shown to be the cause of the growth arrest and loss of proliferation via induction of the DNA Damage Response pathway and subsequent failure to stabilize cellular homeostasis, which is significantly more difficult for cells with degraded telomeres to accomplish. We therefore investigated the effects of apigenin treatment on replicatively senescent BJ cells (PD 60.2) with damaged telomeres to determine whether the anti-SASP effects of apigenin were restricted to senescence induced via irradiation. Results are shown below.

2.3.4.1. IL-6 Secretion

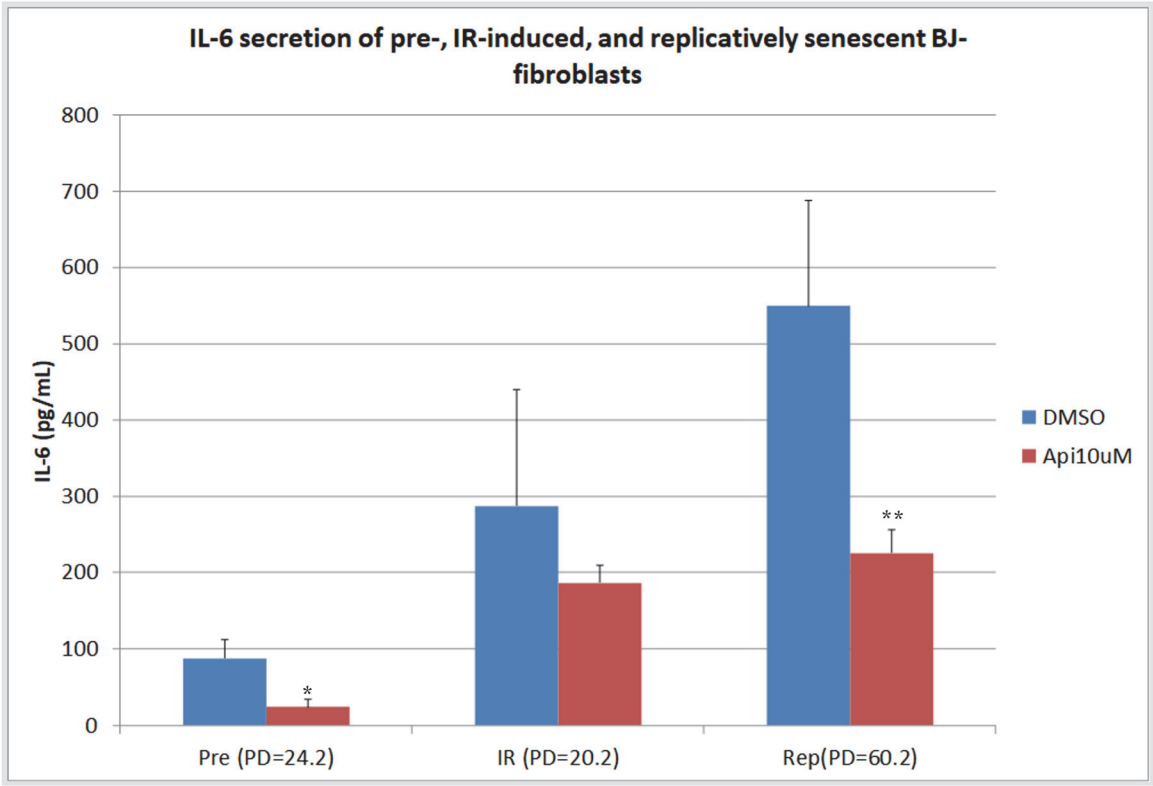


Figure 10: Apigenin reduces IL-6 Secretion of irradiation or replicatively senescent cells.

Results above showed that apigenin treatment was able to reduce the SASP profile of replicatively senescent cells to an even higher degree than irradiation-mediated senescent cells, although the SASP profile of replicatively senescent cells was markedly higher than that of irradiated-senescent cells at baseline.

2.3.4.2. β-Galactosidase Quantitation

SA-β-galactosidase is a marker of stalled autophagy commonly used to differentiate senescent cells *in vitro*. Therefore, we tested the levels of senescence, as shown with the reduction of autophagy and accumulation of SA-b-gal in BJ fibroblasts under DMSO or apigenin. Results are shown below.

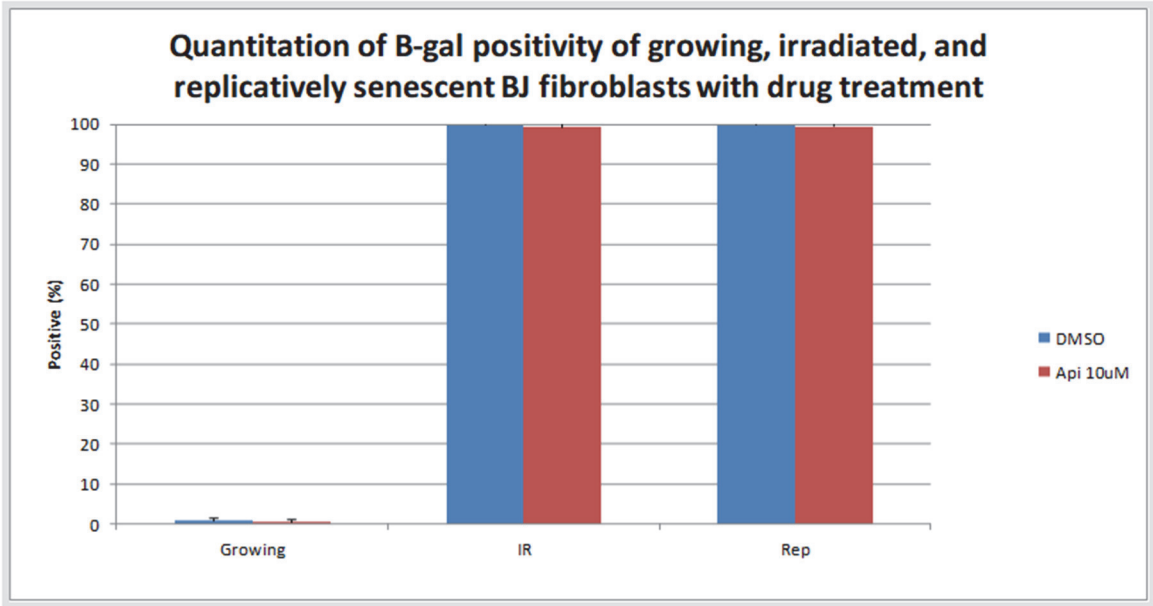


Figure 11: Apigenin treatment has no effect on SA-β-Gal staining of senescent or non-senescent cells

Results obtained demonstrated that apigenin treatment failed to reduce SA-b-gal levels in senescent cells obtained via irradiation and replicative exhaustion. This shows that apigenin treatment may be unable to reverse the accumulation of lipofuscins in senescent endosomes. Alternatively, apigenin may not have been administered at high-enough doses, or cells studied for long-enough time periods, to observe a reduction in SA-b-gal staining in treated senescent cells. These results indicate that the therapeutic effects of apigenin treatment may be specific to the secretion of IL-6 and the associated SASP, but not have a significant therapeutic effect upon the underlying senescent phenotype. Nonetheless, co-treatment via apigenin and another, pro-senolytic or anti-SASP treatment may yet yield even greater reduction in the SASP or associated senescence.

2.3.5. Other Cell Types

To determine whether the effects of apigenin on senescent cells were a unique feature of HCA2 fibroblasts, we repeated the above studies using other cell lines, including primary cells.

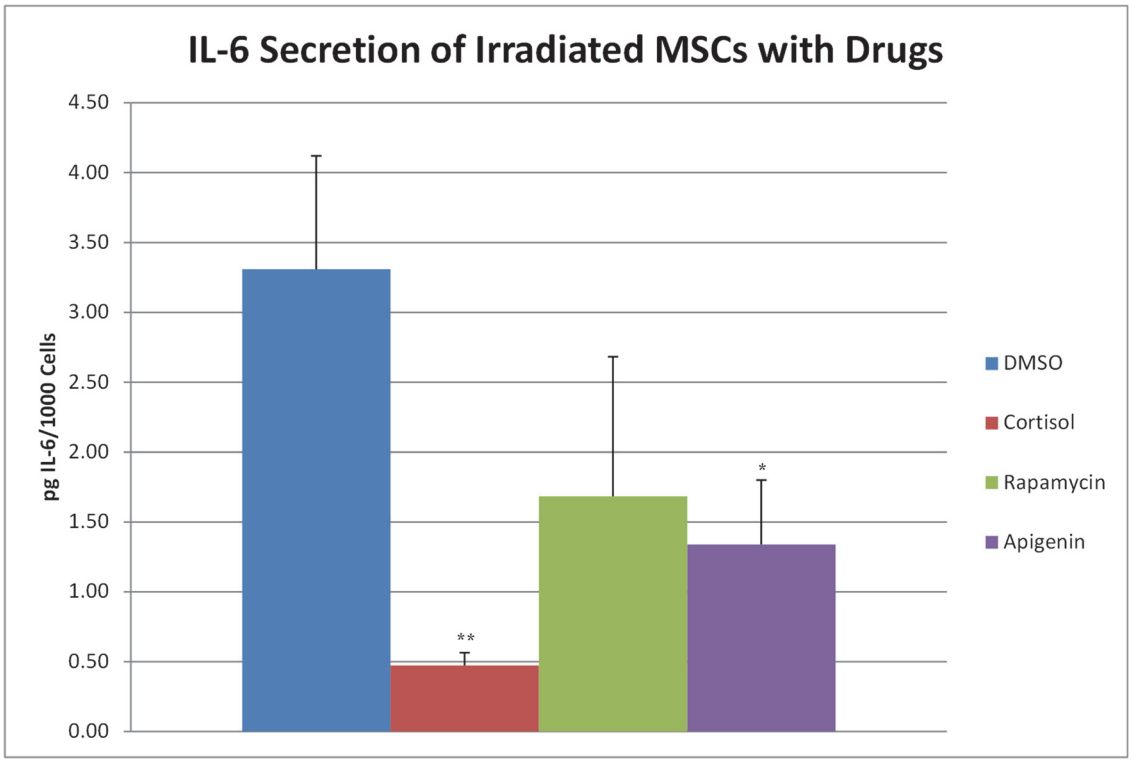


Figure 12: Apigenin reduces secretion of IL-6 by irradiation-induced senescent mesenchymal stem cells with cortisol and rapamycin as positive controls.

Low passage mesenchymal stem cells (MSCs) were obtained from Lonza (Cat# PT-2501) from a 20 year-old black female donor with greater than 90% positive for CD105, CD166, CD29, and CD44 and less than 10% positive for CD14, CD34, and CD45 to indicate the purity of the isolated cells. The cells were cultured in the recommended Lonza MSC Poetics basal media (Cat# PT-3238) supplemented according to the Lonza SingleQuot Kit (Cat# PT-4105) and subjected to senescence-inducing irradiation as previously described. Senescent MSCs were then treated with cortisol, rapamycin, and apigenin at 10µM each. Results obtained showed that apigenin was more potent than rapamycin at reducing the IL-6 SASP, but less potent than cortisol, showing that cell type-specific differences in the efficacy or mechanism of apigenin treatment may exist.

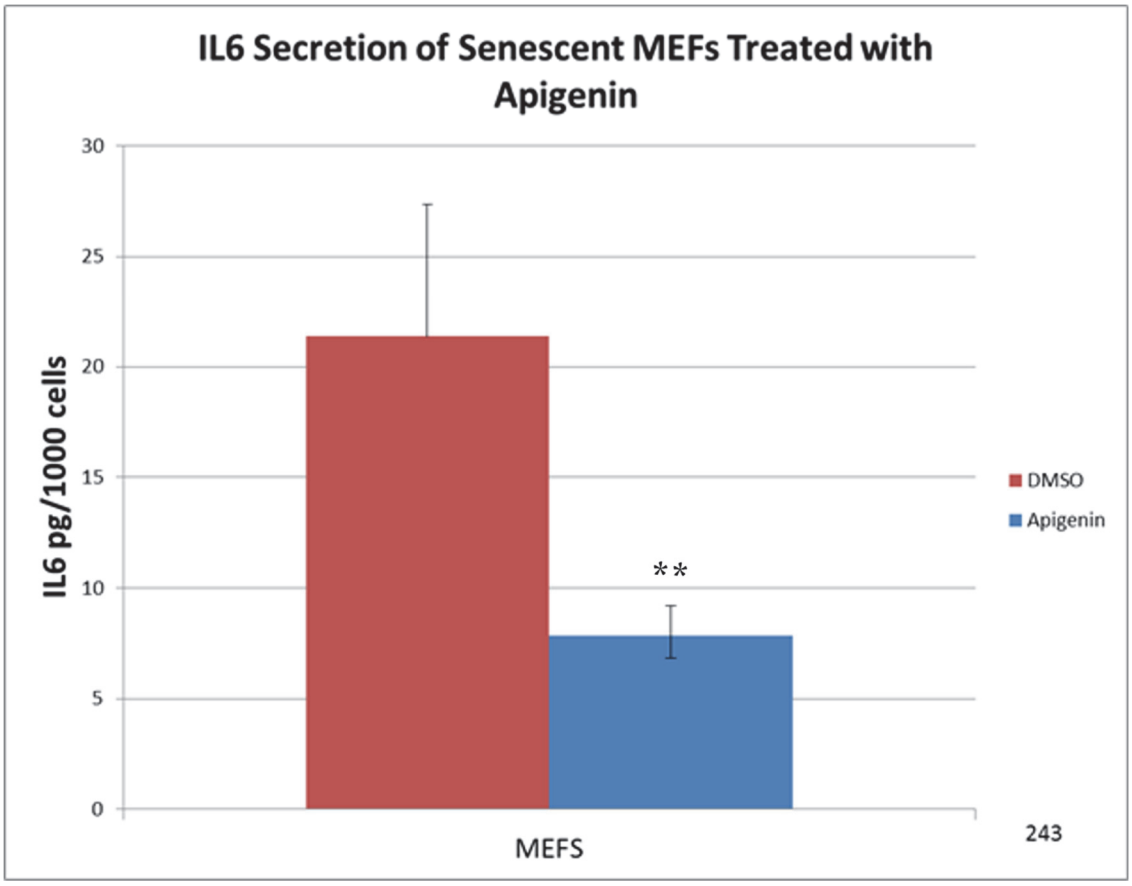


Figure 13: Apigenin (10µM) reduces IL-6 secretion of irradiated senescent mouse embryonic fibroblasts

Primary mouse embryonic fibroblasts (MEFs) were obtained from Marco Demaria who had previously characterized them as producing IL-6 after treatment with doxorubicin. The low passage number (PD 5) MEFs were irradiated as previously described and allowed to become senescent over 10 days treating them with apigenin or vehicle from the day of irradiation. The senescent cells treated with apigenin demonstrated a significant reduction in the SASP, showing that the ability of apigenin to reduce the SASP of these cells and is not restricted to primary human fibroblasts.

2.3.6. Apoptosis of Senescent Cells by Apigenin

The Apo-Tox Glo Assay from Promega was used to determine whether the decrease in IL-6 secretion by senescent cells could have been caused by an increase in cells becoming apoptotic. This assay showed that there was no apoptosis of senescent cells over the 48 hr treatment period where we see a reduction in IL-6, indicating that the attenuation of IL-6

secretion is specific to an alteration of the senescence phenotype and not an artifact of cell viability.

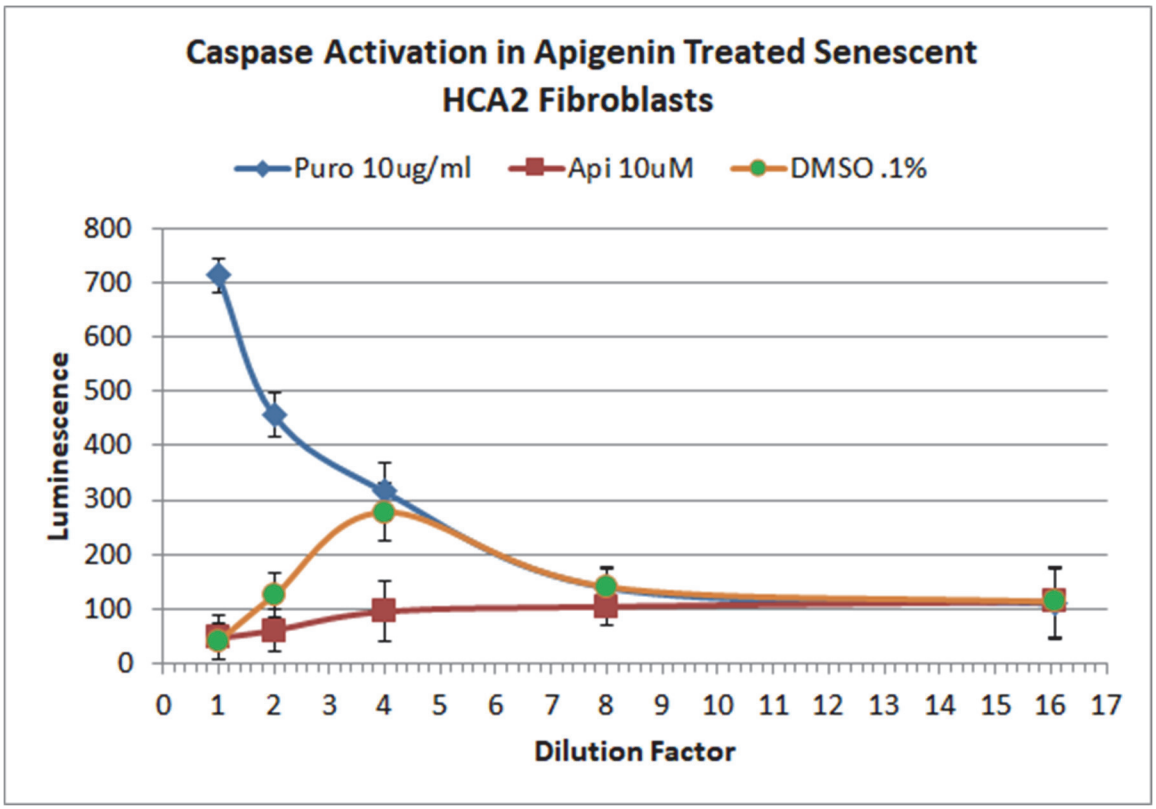


Figure 14: Caspase activation measured via luminescence produced by the cleavage of a substrate by activated Caspase-3 shows a dose-dependent response with the positive control puromycin (blue).

In senescent HCA2 fibroblasts, treatment with apigenin failed to induce apoptosis. Treatment with puromycin did induce apoptosis. These results demonstrate that apigenin's reductive SASP effects are not mediated by an increase in cell death. This effect holds for multiple doses of apigenin, showing that apigenin does not affect apoptotic signaling even at high concentrations

2.3.8. Effects of Apigenin on SA-B-Gal

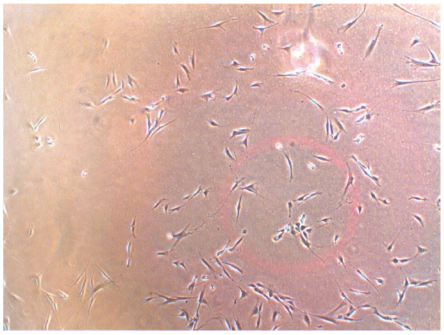
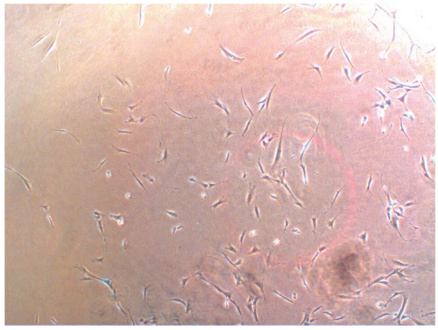
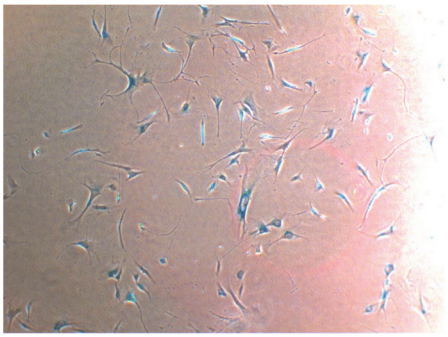
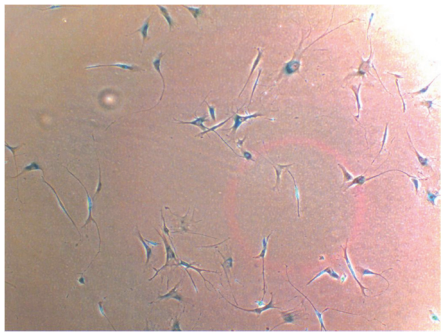
BJ Fibroblasts	DMSO	Apigenin 10µM
Non-Senescent		
Irradiated		

Figure 15: Apigenin has no effect on staining of SA-β-Galactosidase staining of non-senescence or senescent primary BJ fibroblasts.

In irradiated BJ fibroblasts, apigenin treatment at 10um does not exhibit an observable effect on the rates of SA-b-gal staining in senescent cells at multiple time points, consistent with results obtained for senescent HCA2 fibroblasts. Results shown above are representative of data obtained at 48 hours post apigenin treatment.

2.3.9. Comparison to Other Flavonoids

It was found that apigenin some other flavonoids they had been testing were able to prevent protein aggregation in *C. elegans*. They wanted to know if these flavonoids might also be able to reduce the SASP. These protein aggregation-reducing flavonoids were structurally similar to apigenin.

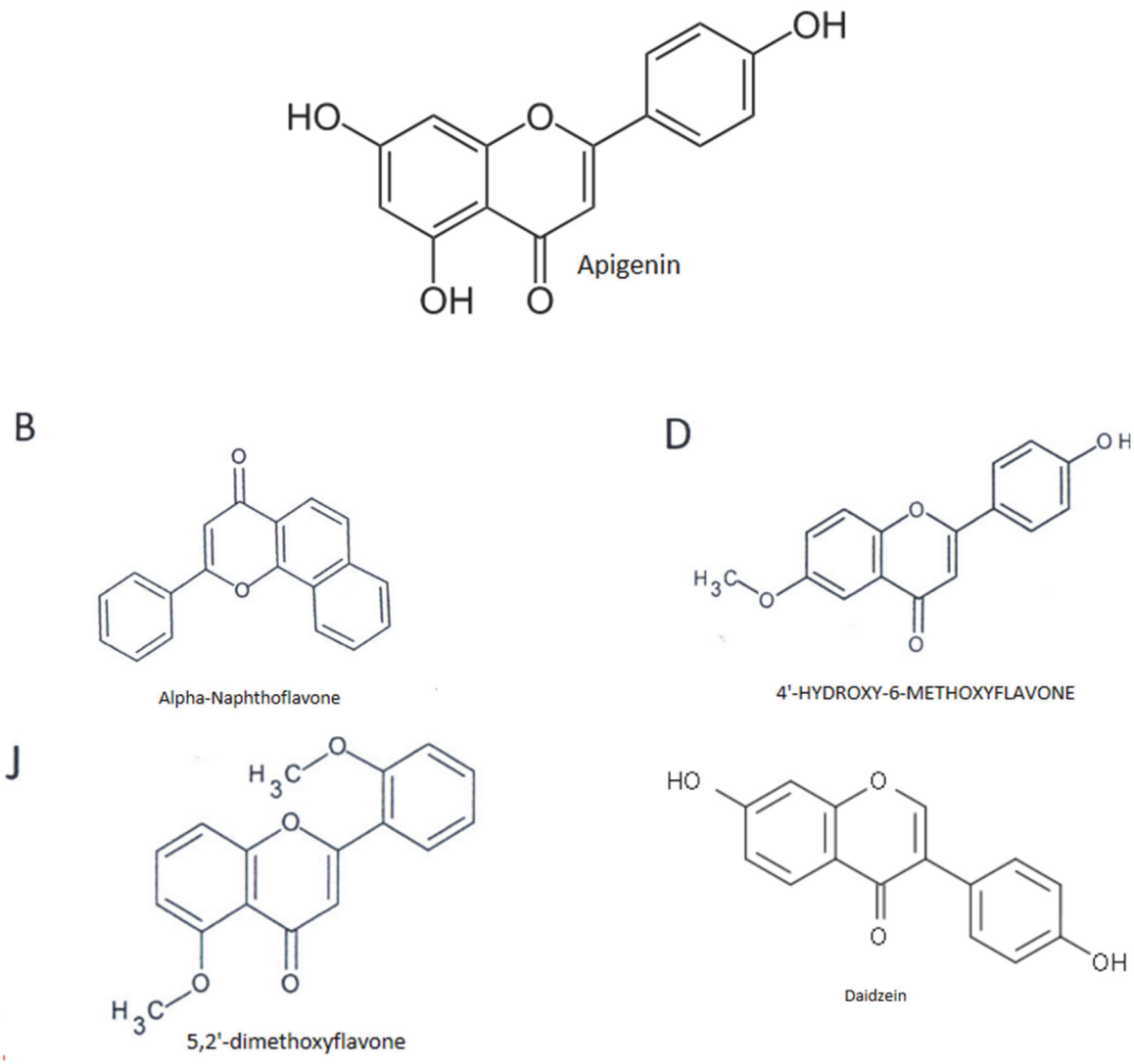


Figure 16: Examples of other flavonoids tested in the Lithgow lab for the ability to reduce protein aggregation in a *C. elegans* model of Huntington's Disease.

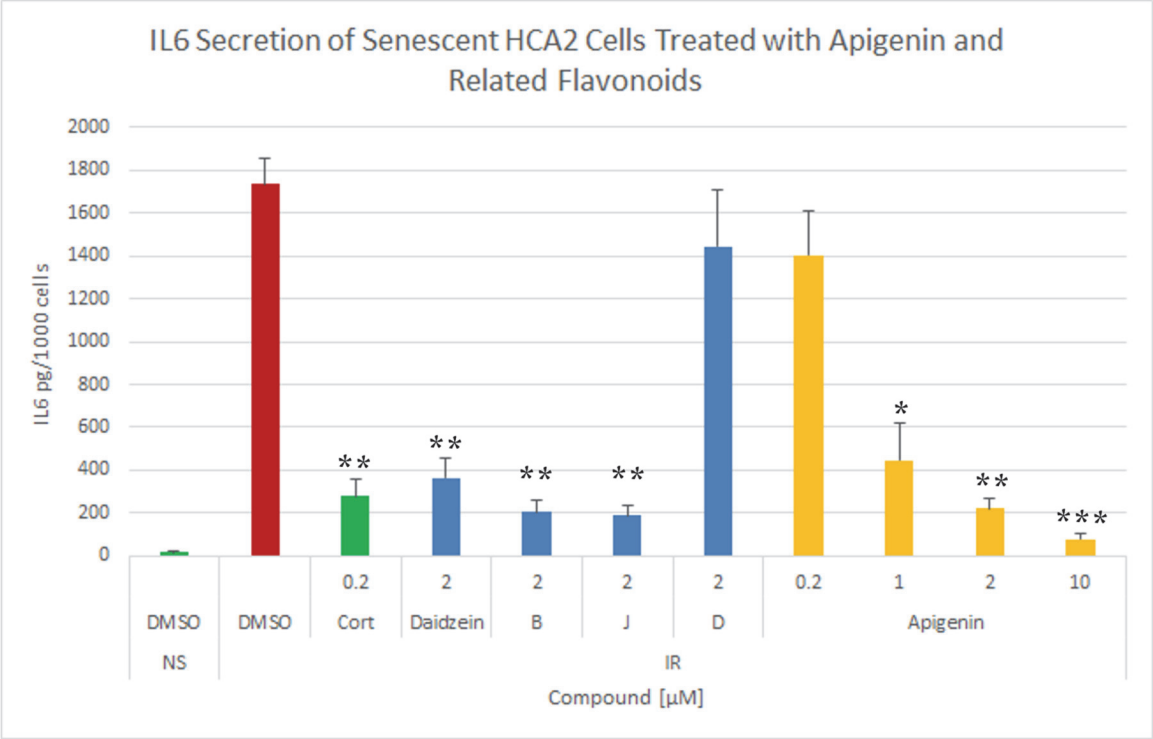


Figure 17 : Apigenin and some structurally related flavonoids reduce IL-6 secretion of senescent HCA2 fibroblasts.

Selected flavonoids B and J demonstrated an ability to reduce IL-6 secretion while D did not exhibit a similar capacity at the chosen concentration, similar to that of apigenin treatment, indicating potential therapeutic activity. This suggests that the structural class to which apigenin belongs may share therapeutic anti-SASP profiles that warrant further investigation.

2.3.10. Effects on Proliferation of Non-Senescent Cells

To examine whether apigenin treatment had any effects on the viability or proliferation of non-senescent cells, we conducted a dose-response study in non-irradiated fibroblasts.

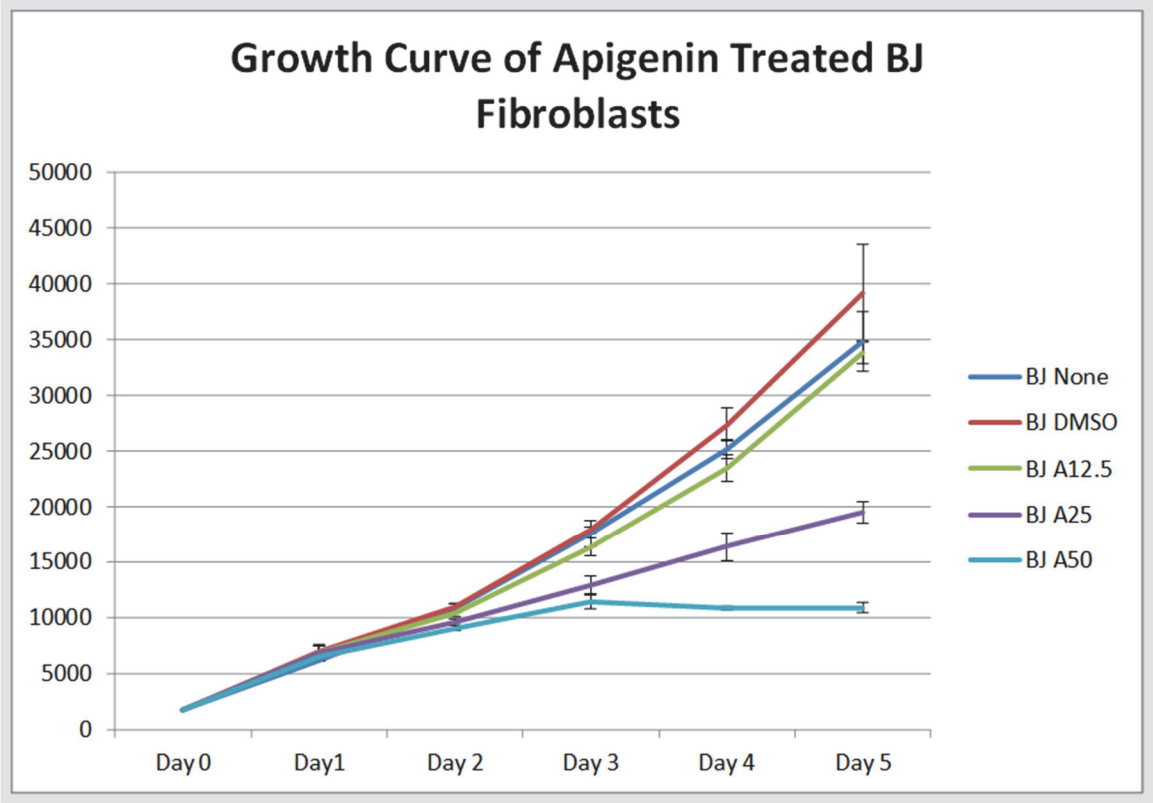


Figure 18: Apigenin treatment slows growth of non-senescent primary human BJ fibroblasts growth in a dose-dependent manner.

The graph above shows that increasing apigenin concentration results in a dose-dependent reduction in cellular proliferation under normal culture conditions. We found that lower doses of apigenin, at 12.5µM, resulted in very little inhibition of cellular proliferation, whereas much higher doses of apigenin at levels difficult to obtain from diet or therapy, such as 25 and 50µM, resulted in significant reduction of cellular proliferation in non-senescent, non-irradiated fibroblasts.

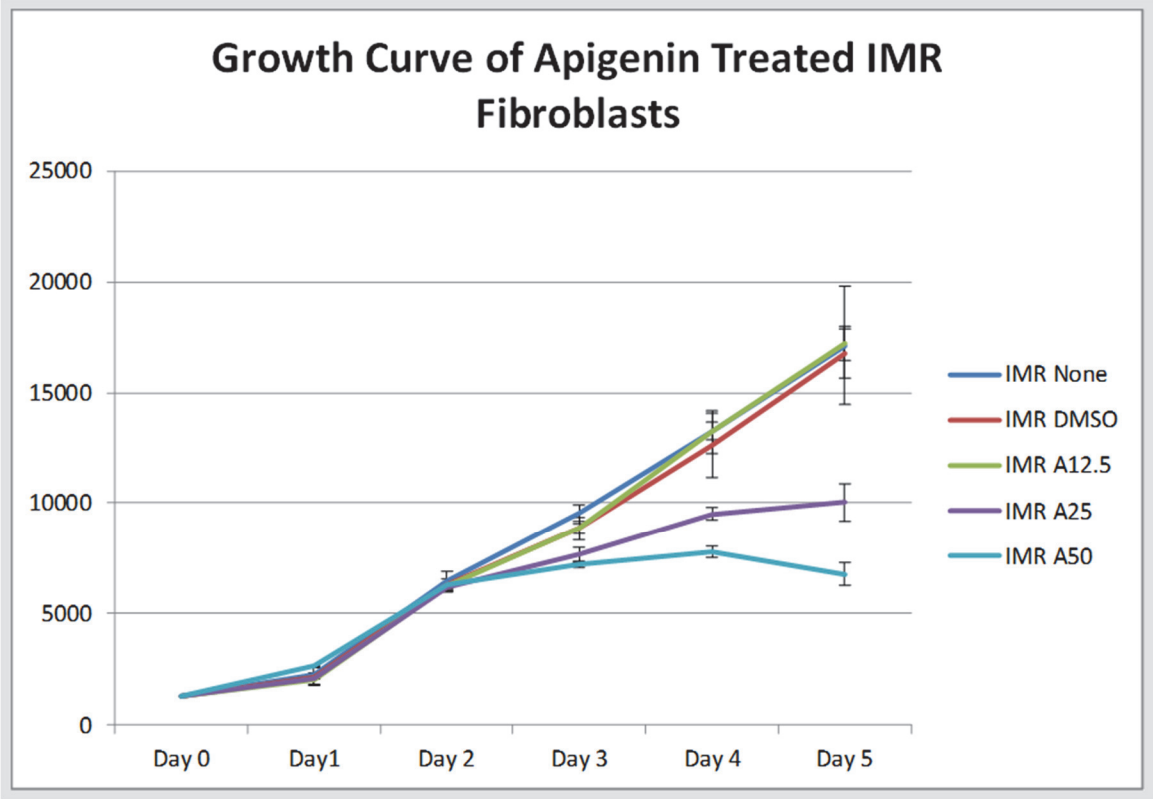


Figure 19: Apigenin inhibits growth of IMR90 fibroblasts in a dose-dependent manner.

Similar results were obtained for non-irradiated, non-senescent IMR90 fibroblasts, showing that the observed proliferation effects of apigenin are not restricted to a particular cell line.

2.4.1. Luminex Characterization of Conditioned Media

Four p100 culture dishes of each condition (Non-Senescent - DMSO, Non-Senescent - Apigenin, Senescent - DMSO, Senescent - Apigenin) were prepared for each cell line. Supernatant from each was then collected to provide protein for Western blots and the conditioned media were sent for analysis to Stanford Human Immune Monitoring Core for analysis by Luminex.

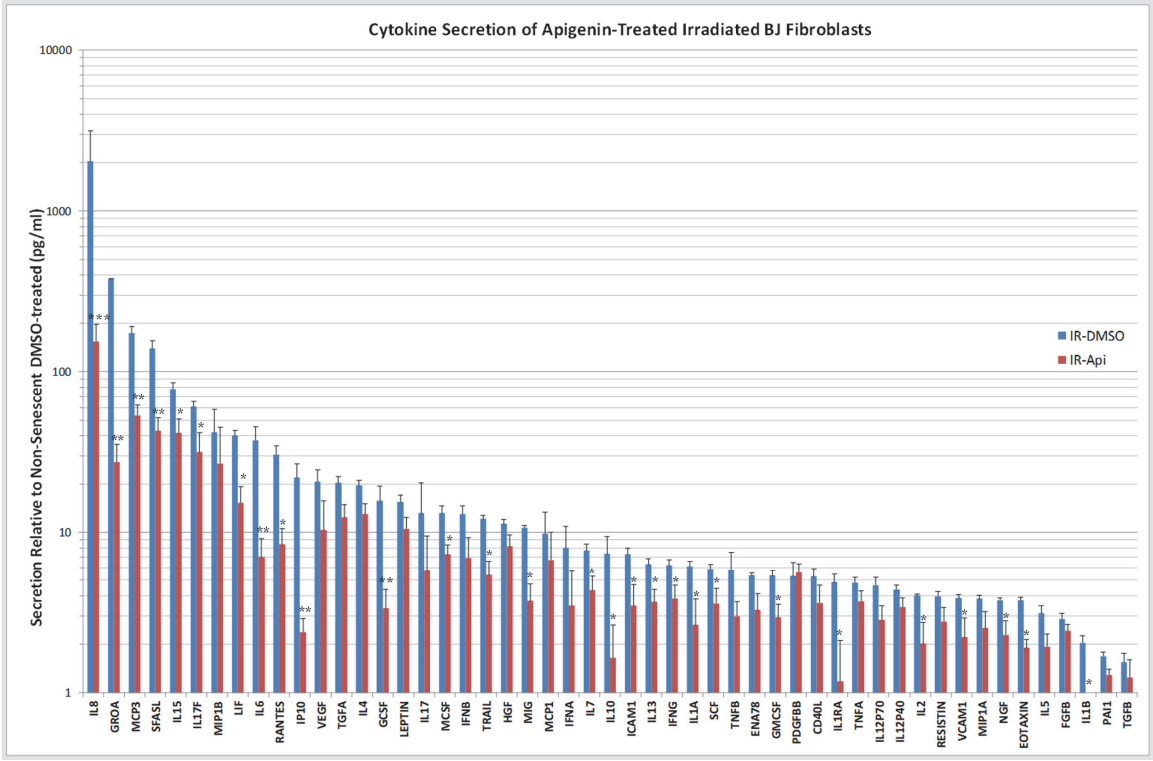


Figure 20: Results from 51-plex cytokine Luminex panel analysis of SASP factors secreted by non-senescent and senescent BJ fibroblasts treated, or not, with apigenin. Secretion is relative to DMSO-treated non-senescent controls.

Apigenin treatment resulted in widespread, significant reduction of other SASP components, such as GROA, IL-15, IL-17, IFNa, TRAIL, CD40L, SCF, IL-2, MMP1A, and TGFb, indicating that its anti-SASP effects are not restricted to IL-6. This finding illustrates the breadth of apigenin's anti-inflammatory effects across the full SASP spectrum, indicating that it may be of therapeutic importance in acute or chronic inflammatory conditions as well as in senescence. These results are shown as absolute reduction in cytokine secretion above and fold change relative to DMSO treatment below.

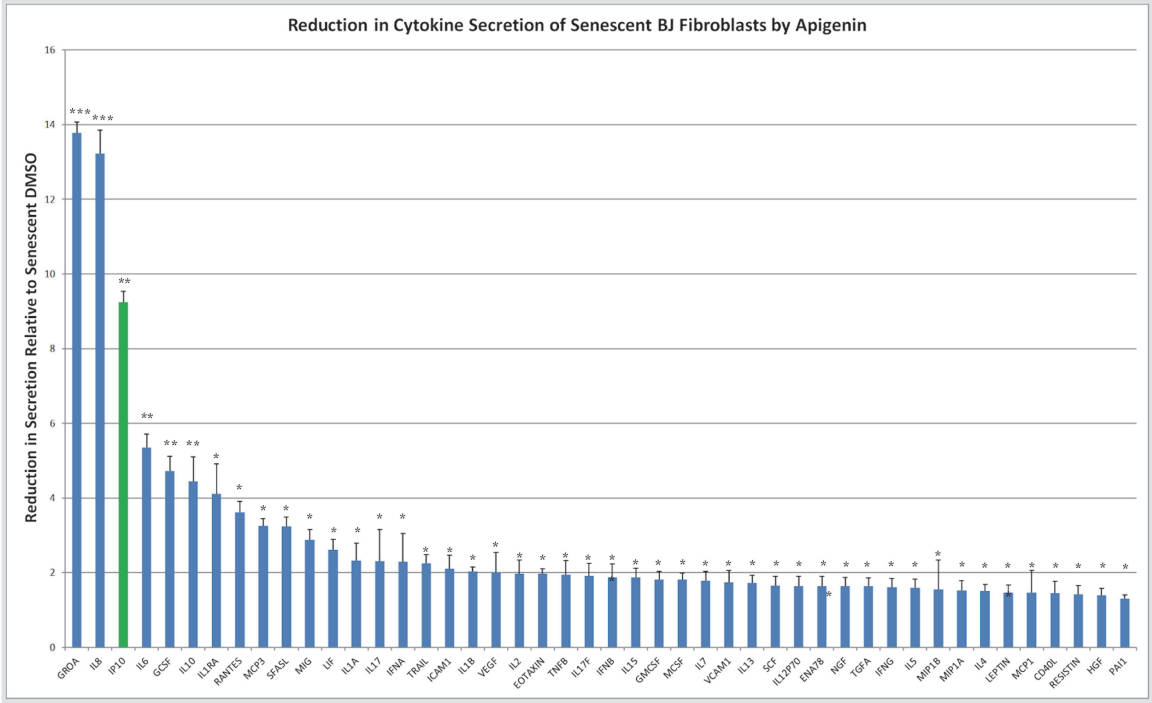


Figure 21: Apigenin significantly blunts the secretion of SASP factors whose expression is elevated in senescent BJ fibroblasts.

These results demonstrate that apigenin is a potent anti-inflammatory treatment that results in widespread reduction of many SASP factors that are associated with chronic inflammation. Via abrogation of the SASP, apigenin treatment may be able to blunt its effects on surrounding tissue and thus slow the degenerative decline associated with the presence of metabolically-active senescent cells.

2.4.1.1. Example of Variability Between Luminex Samples

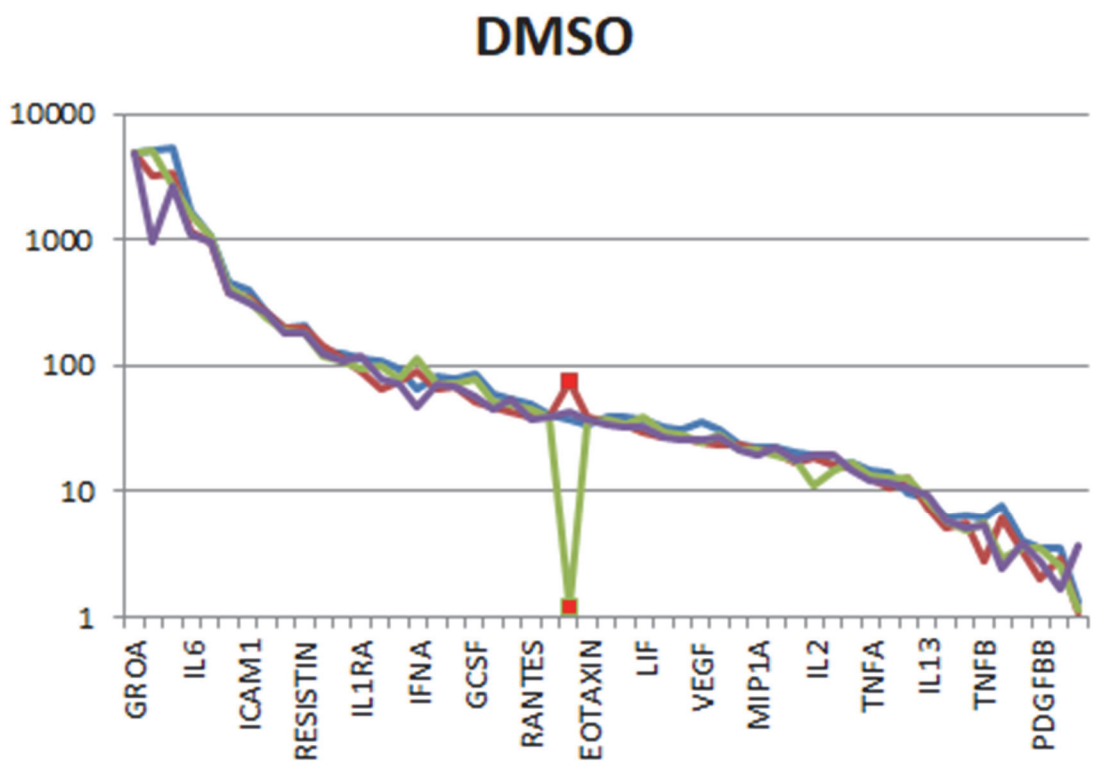


Figure 22: Variability of cytokine levels from four Luminex conditioned media samples collected from senescent BJ fibroblasts.

As shown above, Luminex assays yielded very little variance between different samples. Above are results from BJ fibroblasts, in which only RANTES and EOTAXIN, 2 cytokines involved in paracrine signaling, significantly diverge between replicate samples. Shown below are the same metrics for IMR90 fibroblast samples. These data indicate that the Luminex assays measure robust biological effects in response to apigenin treatment, with little cell signaling noise that would indicate either a faulty assay or a highly promiscuous and inconsistent method of action for the effects of apigenin treatment.

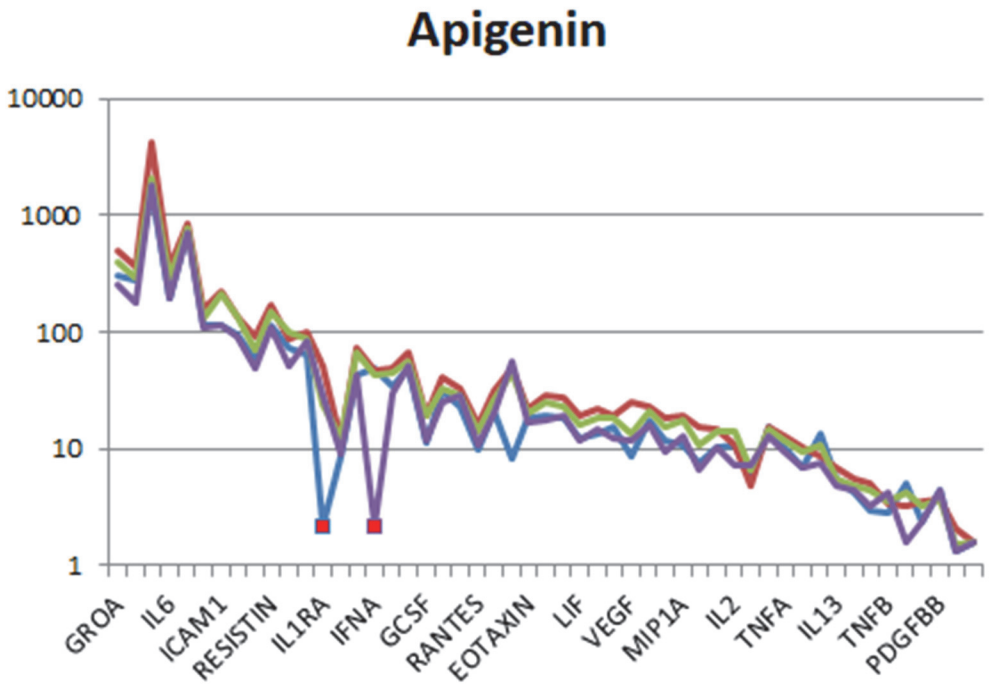


Figure 23: Variability of cytokine levels from four Luminex conditioned media samples collected from senescent IMR90 fibroblasts.

2.4.2. Dynamics of IL-6 Secretion with Timing of Apigenin Treatment

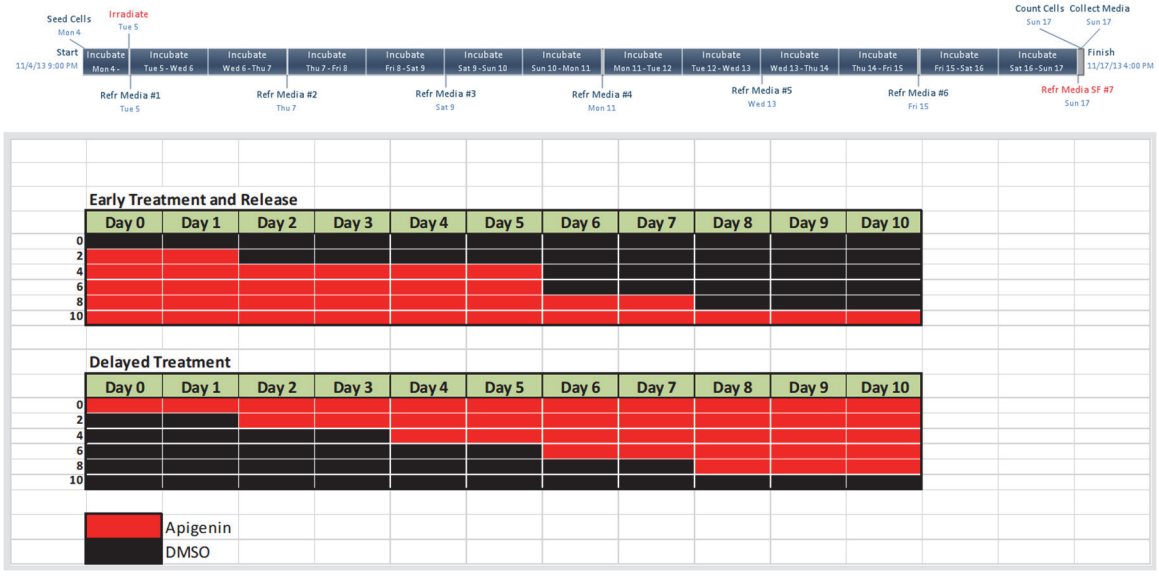


Figure 24: Timeline for experiments that vary timing of initiation and duration of apigenin treatment on IL-6 secretion of senescent fibroblasts to investigate dependency on temporal factors.

The dynamic response of senescent cells to apigenin treatment over time was investigated. According to the schedule above, we characterized two distinct apigenin treatment regimens, including an early treatment schedule in which treatment began immediately following irradiation as well as a delayed treatment regimen in which treatment was withheld until two days post-irradiation. By varying the window of apigenin treatment after irradiation, we can thus observe how it dynamically affects the SASP in senescent cells.

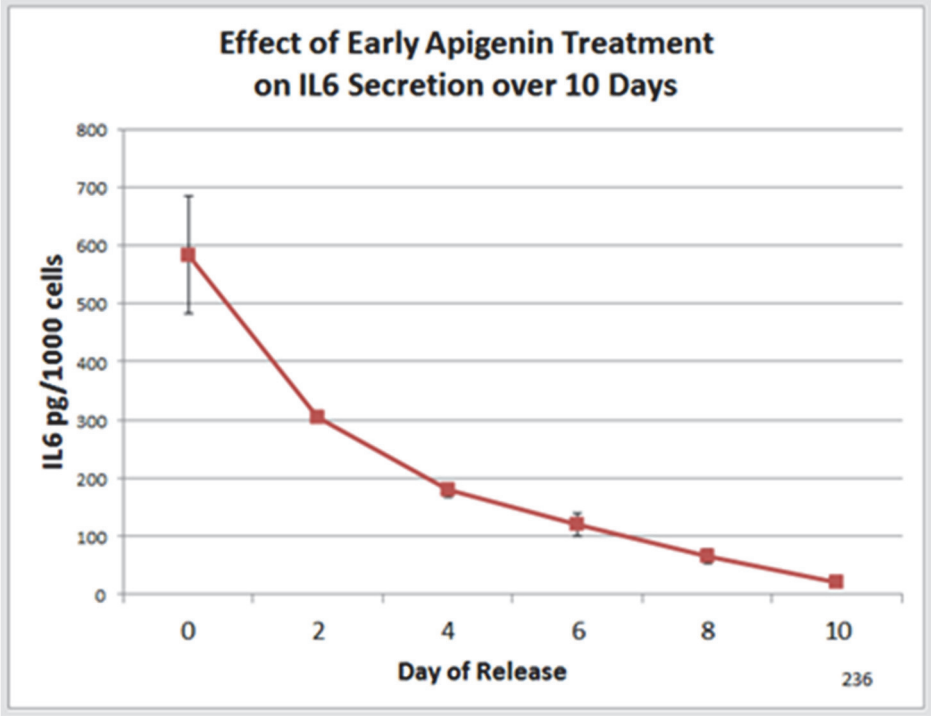


Figure 25: Release of irradiated HCA2 cells from treatment of apigenin allows levels of IL-6 to rebound. Untreated cells (Day 0) had the highest level of secretion after 10 days while cells treated for the full 10 days of the experiment had the lowest (Day 10).

As expected untreated cells (Day 0 “release”) had maximal secretion of IL-6 at time of collection Day 10, while cells treated for the full ten days (Day 10 “release”) had the lowest secretion. There was a progressive “rebound” of IL-6 secretion dependent on how long the cells were free of apigenin treatment. Results above are representative of experiments using 10µM apigenin with 4 replicates per time point.

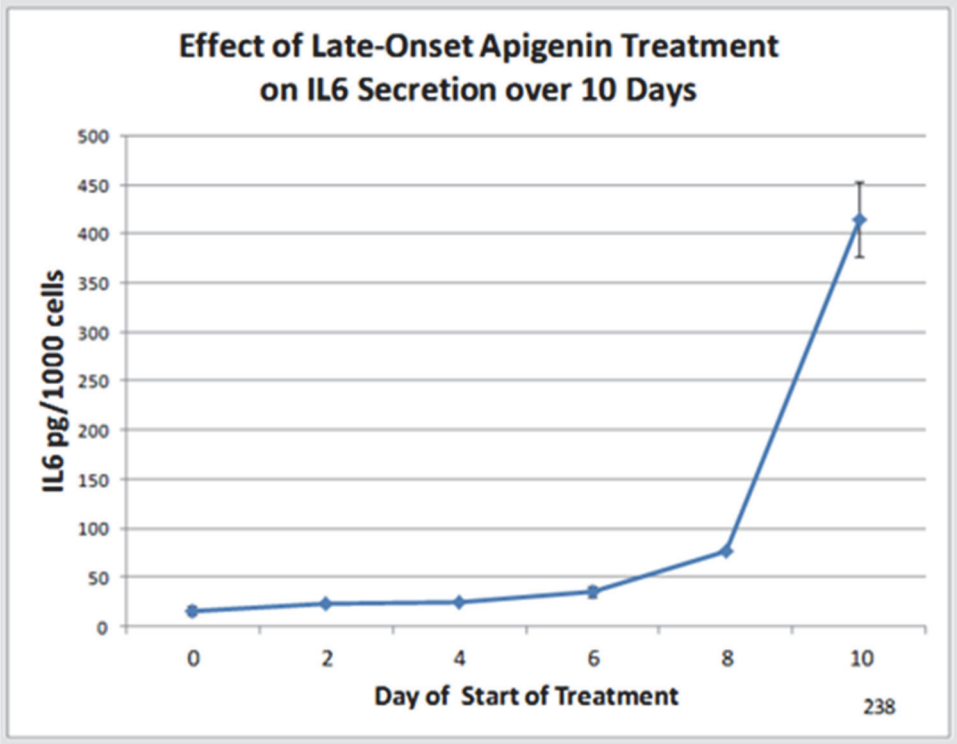


Figure 26: Delayed treatment indicates most of the reduction of IL-6 secretion occurs within the first 48 hrs. Cells treated for the full 10 days (Day 0) had the lowest secretion of IL-6 while those with only two days of treatment starting at Day 8 showed that most of the reduction in IL6 secretion occurs in the first 48 hours of treatment.

In contrast, late-onset apigenin treatment shows that even four days of apigenin treatment starting on Day 6 was enough to reduce IL-6 secretion to close to minimal levels. Interestingly, most of the reduction in IL-6 secretion occurs in the first 48 hrs of treatment as shown by the level from samples that were treated for only two days starting at Day 8. The levels found in the release vs. delayed treatment are not directly comparable as treatment with apigenin early may affect the actual establishment of the senescent phenotype and not just the secretion of factors by cells which are already senescent.

IL-6 secretion steadily rose in all groups of cells that experienced a delay in treatment of a release for any length of time indicating that the continuous presence of apigenin is necessary for SASP suppression, unlike rapamycin which binds its target so strongly that only one treatment is necessary for prolonged effects. Due to the anti-inflammatory nature of apigenin treatment, it will be vital to determine whether these same dynamics are obtained when other anti-inflammatory treatments, such as NSAIDs or similar, are used. Determination of these dynamics, and the variability of the sensitivity of senescent cells to therapeutic intervention, will

be essential to the effective design and characterization of in vivo anti-SASP or senolytic strategies.

2.4.3. Initial qPCR of SASP Markers

SASP marker RNA levels were determined after apigenin treatment. Normal and IR-treated IMR90 fibroblasts were seeded at the same density and cultured in 0.1% DMSO or 10μM apigenin-containing media. 24 hours thereafter, media was removed and replaced with serum-free media. 24 hours later, cells were collected and lysed to extract RNA. RNA was then reverse-transcribed and quantified with qPCR as described above in 3.2.4 above. Levels of mRNA expression after apigenin treatment are shown below.

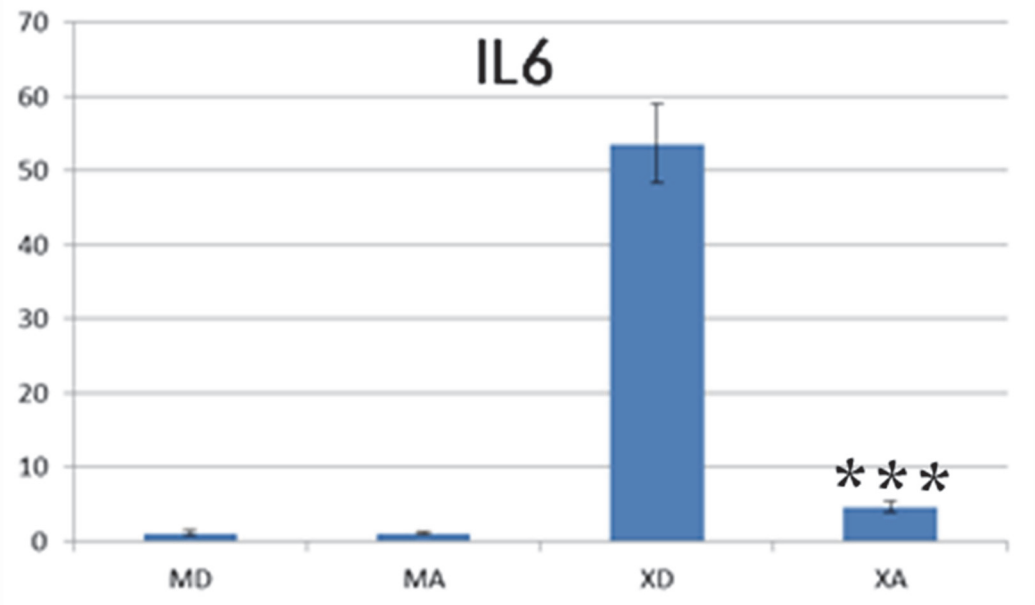


Figure 27: Levels of IL-6 mRNA in senescent IMR90 fibroblasts (XD) are reduced by apigenin (XA) relative to mock-treated fibroblasts (MD and MA).

IL-6 mRNA was greatly increased in irradiated, DMSO-treated cells (XD), relative to mock-treated MD or MA cells. Treatment with apigenin (XA) significantly reduced IL-6 mRNA expression, although it did not restore it to un-irradiated, non-senescent expression levels. This indicates that apigenin treatment can greatly blunt the inflammatory effects of irradiation-induced senescence, but cannot wholly ameliorate the effects of irradiation.

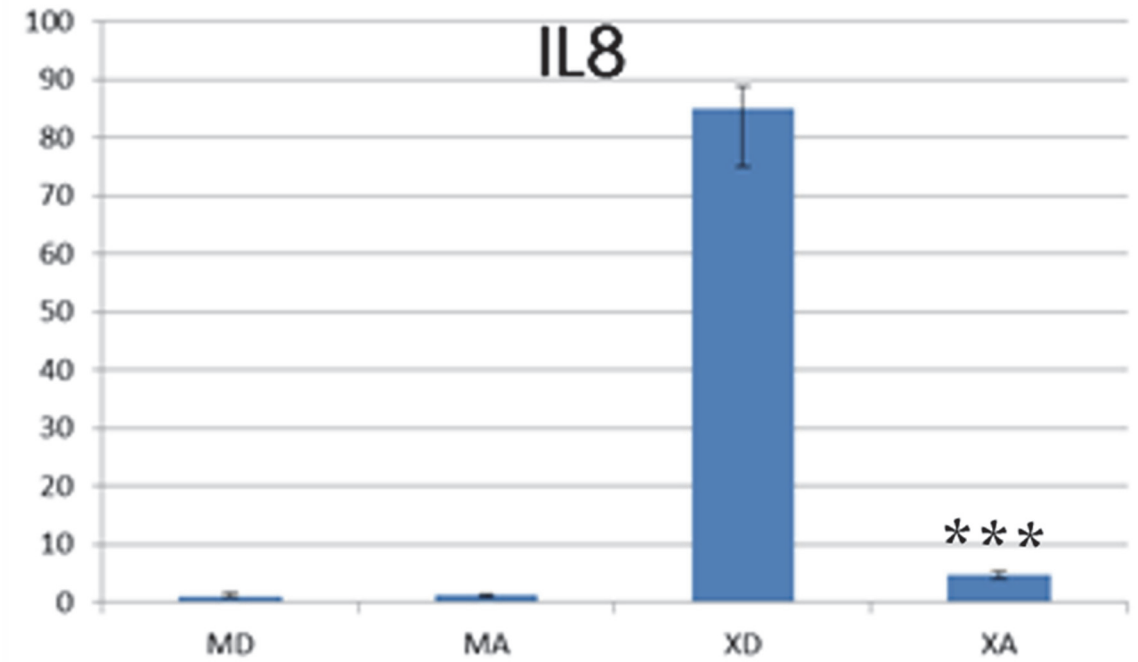


Figure 28: Levels of IL-8 mRNA in senescent IMR90 fibroblasts (XD) are reduced by apigenin (XA) relative to mock-treated fibroblasts (MD and MA).

Senescence-inducing irradiation and apigenin treatment yielded an identical pattern for interleukin-8 (IL-8). IL-8 is a potent inflammatory chemokine involved in the senescence-associated secretory phenotype that induces inflammatory responses in neighboring cells and infiltrating immune cells. Like IL-6, IL-8 levels were greatly increased by irradiation (XD) and significantly reduced by apigenin treatment (XA).

2.5. Search for Mechanism

Understanding the molecular mechanism by which apigenin exerts its anti-SASP effects is crucial to the effective design of therapeutic strategies and further investigation. To define the mechanism by which apigenin acts, we undertook extensive characterization and experimentation as shown below.

2.5.1. Mucin-1

The first mechanism that was investigated involves Mucin-1 (MUC1). The oncoprotein MUC1 is a membrane-bound glycoprotein expressed on apical borders of secretory epithelial cells, where it participates in cell-cell adhesion and serves as an anchor for growth factor

receptors (Pillai et al. 2013). With the loss of polarity which comes with oncogenic transformation, MUC1 cytosolic abundance increases and its distribution widens to cover the surface of the entire cell, thus increasing cancer cell motility and invasive potential via detachment from surrounding cells and the local basement membrane. MUC1 is synthesized as a single polypeptide chain cleaved in the endoplasmic reticulum to form a homodimer. It was found that the cytoplasmic portion of MUC1 (MUC1-C) associates with p53 to enhance p21-mediated growth arrest in ZR-75-1 cells (Wei et al. 2005) induced by genotoxic stress (Figure 29) and that apigenin interfered (Figure 30) with this association (Zhou et al. 2011). It was hypothesized that this might be the mechanism by which apigenin interfered with the SASP through prevention.

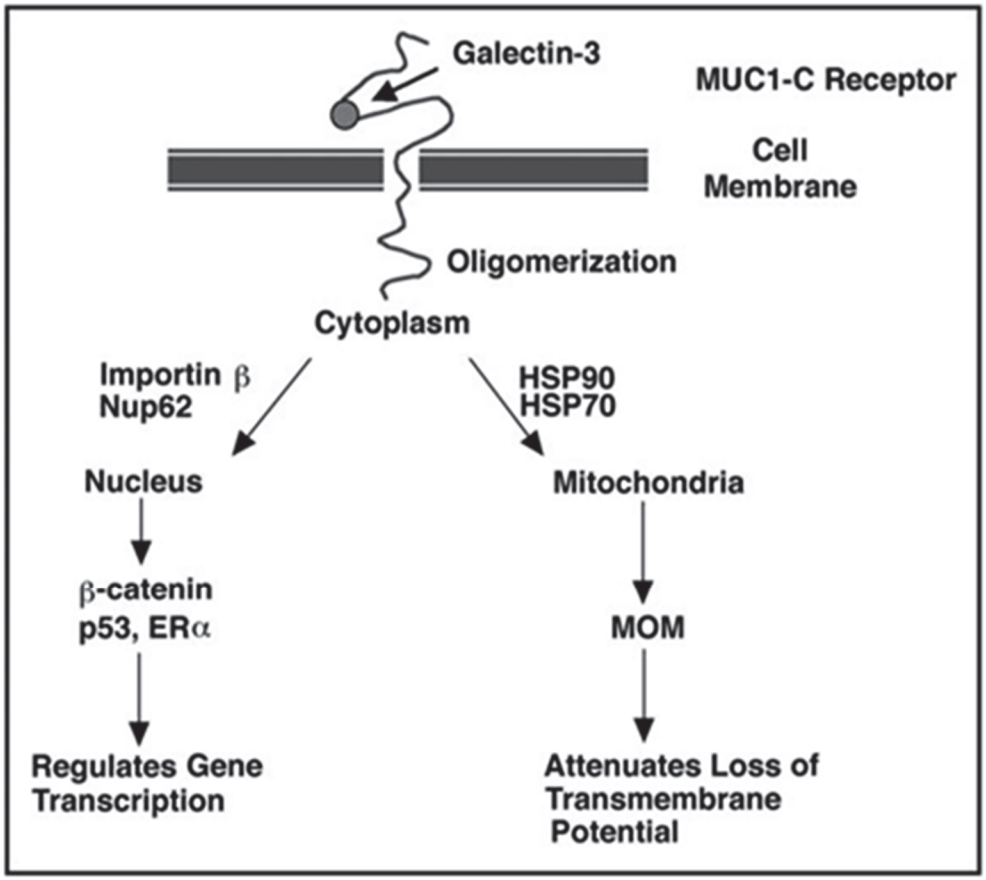


Figure 29: MUC1 regulation of p53 responsive genes (Wei et al. 2005)

As shown in Figure 29, MUC1 activity lies upstream of p53 signaling, wherein MUC1 signaling can activate the translocation of various transcription factors involved in regulation of cellular proliferation and stress responses via modulation of PI3K, AKT, and AMPK (adenosine monophosphate-activated kinase) pathways, which in turn regulate metabolic flux, cell cycle

progression, and protein biosynthesis. As a central integrator of metabolic, cell stress, and proliferative functions, MUC1 was hypothesized to be a prime candidate for the mechanism of action of apigenin in reducing IL-6 secretion of senescent cells and experiments were designed to investigate this possibility.

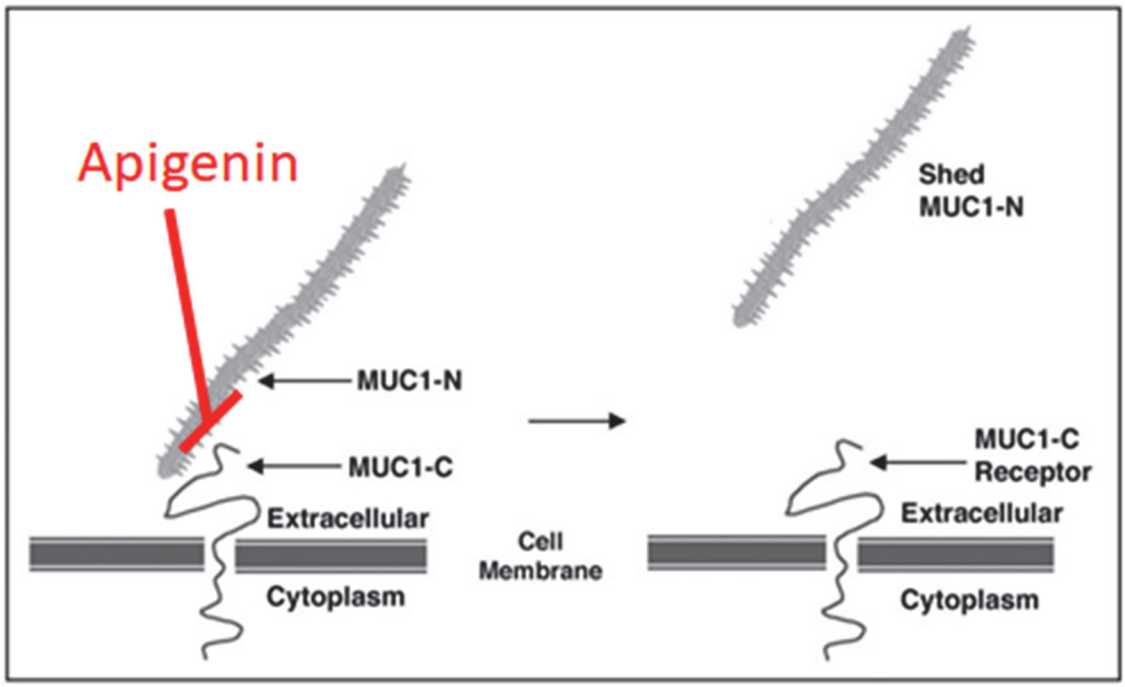


Figure 30: Model for apigenin inhibition of MUC1-C mediated p53-dependent SASP.
(Zhou et al. 2011)

The first question to answer was to discover whether or not senescent HCA2 cells expressed MUC1, as it is usually only found in epithelial cells and not expressed at significant levels in primary fibroblasts. It would have been surprising and novel to discover that MUC1 was involved in a senescence response in fibroblasts, so a qPCR was performed. It was found that levels did not increase significantly in response to irradiation (Figure 31), and remained too low to suggest that enough suggest that MUC1 could be playing a role in senescence, or be a target for apigenin's effects in senescent fibroblasts.

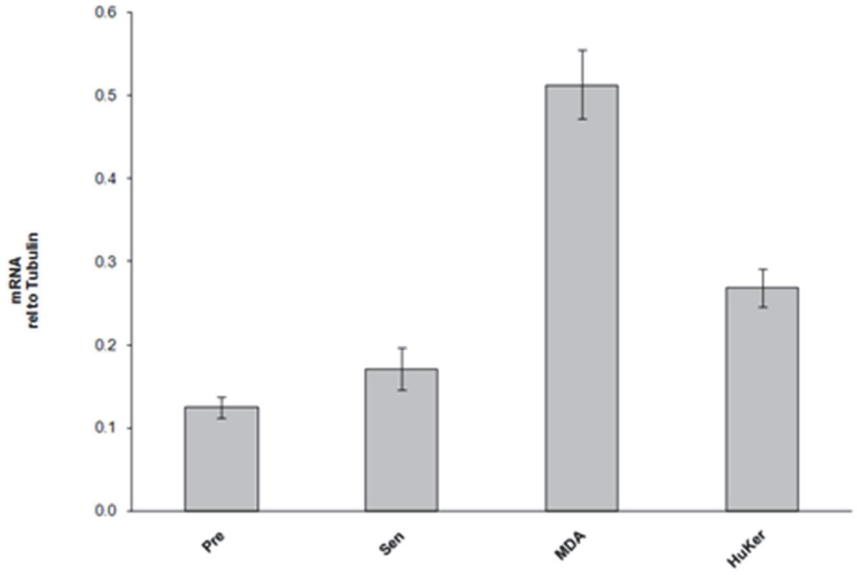


Figure 31: MUC1 mRNA expression in non-senescent (Pre) and senescent (Sen) primary human HCA2 fibroblasts, immortalized epithelial breast cancer cells (MDA) and human keratinocytes (HuKer). Levels of MUC1 are relative to tubulin.

However, although the qPCR (above in Figure 31) showed that mRNA levels of MUC1 did not change, it did not necessarily mean that protein levels couldn't increase with senescence. This was tested with a Western blot (below in Figure 32), which revealed a similar story. Unsurprisingly, MUC1 protein (migrating as double-band due to cleavage of its glycosylated residues) showed up in the positive controls but was not found in fibroblasts, senescent or normal.

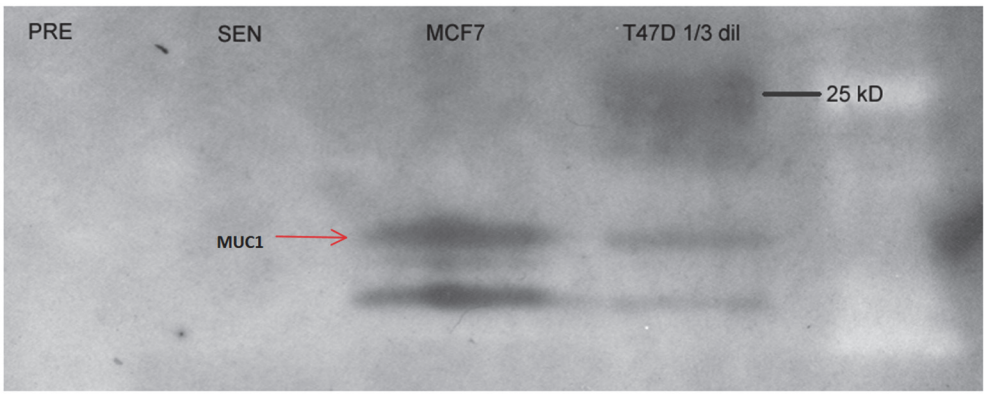


Figure 32: Western blot of MUC1 protein in pre-senescent and senescent fibroblasts, MCF7 and T47D cancer cells showing MUC1 is not present in fibroblasts and is unlikely to be involved in the effects of apigenin on the reduction of secretion of IL-6 of senescent fibroblasts.

Given the relatively low abundance of both MUC1 mRNA and protein in senescent and non-senescent fibroblasts it was decided to look for a different mechanism.

2.5.2. Autophagy

It is known that senescent cells are metabolically active, in many ways more so than non-senescent cells, and as such they produce a lot of reactive oxygen species which presumably would increase the overall amount of proteins or DNA that have experienced oxidative damage and thus increase the amounts of aggregated misfolded proteins as well as other cellular constituents. In a *C. elegans* model of Huntington's disease, pathogenic protein aggregates are detectable using the A11 antibody (Santa Cruz cat# AB9234). It was hypothesized that apigenin may increase the levels of autophagy, helping clear protein aggregates, leading to lower stress, and thus lowering the SASP. This hypothesis was tested using a western blot (Figure 33) to determine whether apigenin treatment reduces protein aggregate formation in senescent cells. The results of that experiment indicate that protein aggregate formation is not decreased by apigenin treatment in either senescent or non-senescent cells at 10 or 50µM.

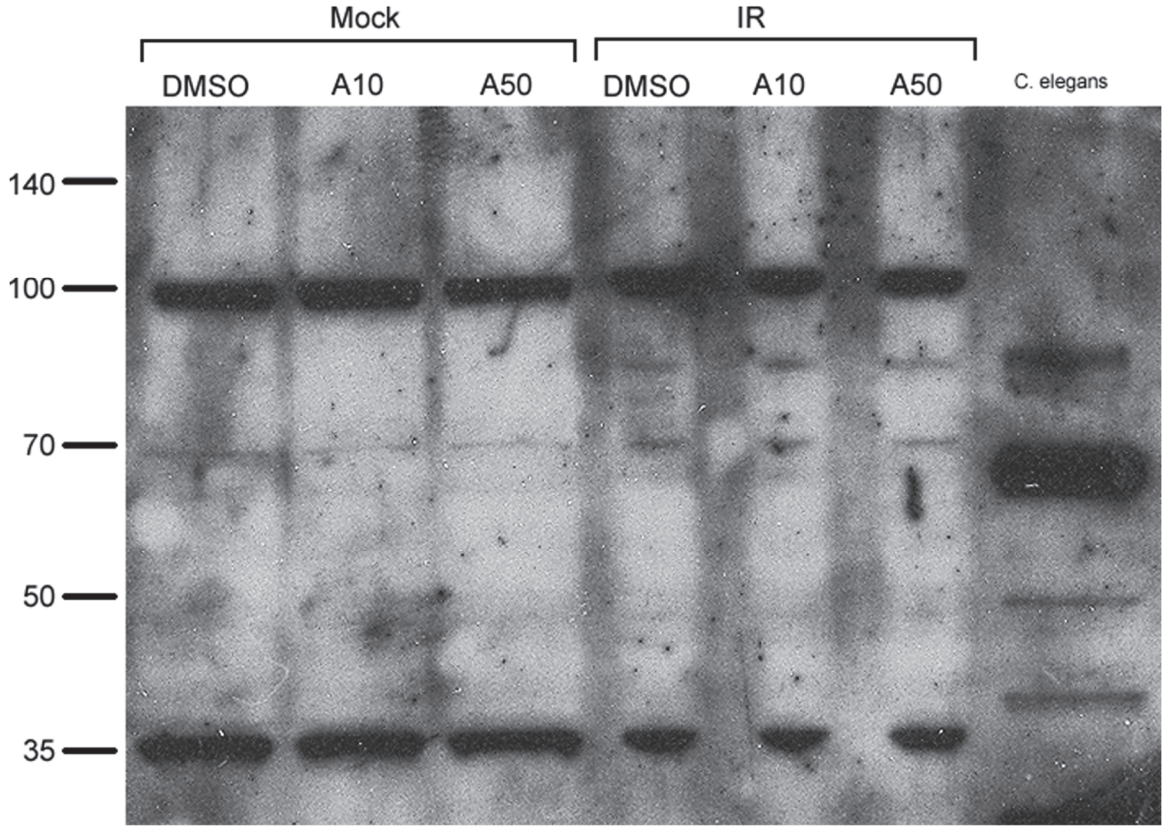


Figure 33: Western blot looking at levels of β-sheet aggregates as detected by antibody A11 in untreated and treated senescent fibroblasts at two concentrations 10 and 50uM of apigenin and compared to positive control. No change in the bands at either concentration was observed.

2.5.3. Other Biomarkers - CD38, PDGFA and IL1A

Decreased levels of nicotinamide riboside (NAD) are associated with aging, where the various enzymes that mediate DNA damage repair and mitochondrial biogenesis deplete intracellular pools of NAD+ (Wiley et al. 2016). Failure to replenish these intracellular NAD+ pools then contributes to a breakdown of coordination between nuclear and mitochondrial function, thus accelerating senescent transition. CD38 is an ectodomain protein involved in the biosynthesis and regulation of NAD levels. Aging is associated with an increase in CD38 levels, which may be driven by inflammation, senescence, or reduced NAD levels themselves (Camacho-Pereira et al. 2016) and experiments were designed to test whether CD38 is involved in the anti-SASP effects of apigenin treatment.

To investigate the role of CD38 in apigenin treatment, senescent IMR90 fibroblasts were prepared using the standard senescence induction with irradiation. qPCR of mRNA from treated cells showed that irradiation potently increased CD38 expression in 0.1% DMSO-treated fibroblasts while apigenin treatment significantly decreased these CD38 levels. HeLa cervical epithelial cells were used as a positive control for CD38 levels in this experiment. However, non-senescent fibroblasts express CD38 only weakly; use of U2OS osteosarcoma with notably high CD38 expression would have been a better positive control. Unfortunately, U2OS cells were unavailable at the time of this experiment.

From this experiment, it was determined that CD38 expression increases with the induction of senescence and decreases with apigenin treatment, consistent with anti-inflammatory, anti-senescence treatment effects but not indicative of a specific mechanism of action for apigenin treatment.

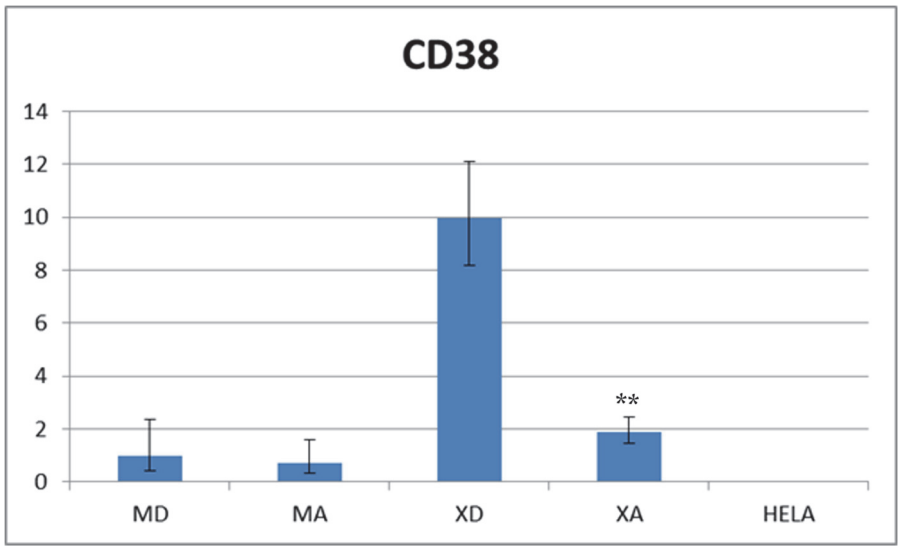


Figure 34: qPCR revealed elevated transcriptional levels of CD38 mRNA in senescent HCA2 cells (XD) are reduced by apigenin (XA) as compared to negative non-senescent untreated controls (MD).

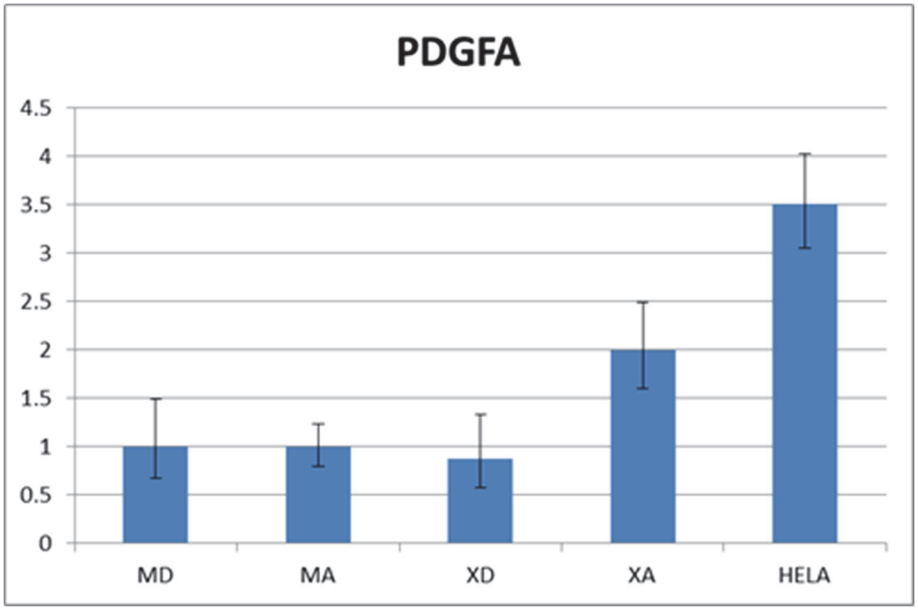


Figure 35: qPCR shows transcription levels of PDGFA mRNA in senescent and non-senescent HCA2 fibroblasts are not significantly changed by apigenin with PDGFA expression in Hela cells as a positive control.

The same experimental conditions and qPCR methods were also used to investigate other putative mechanisms of apigenin treatment. Next, expression levels of platelet-derived growth factor subunit A (PDGFA) were quantified, PDGFA is a potent mitogenic stimulus in mesenchymal cells and whose activity is necessary in senescent fibroblasts associated with wound healing via induction of myofibroblast differentiation (Demaria et al. 2014). Results

demonstrated that apigenin treatment does not reduce PDGFA expression levels in fibroblasts, suggesting that apigenin may reduce the pro-inflammatory nature of the SASP while maintaining the expression of beneficial wound-healing senescence-associated factors.

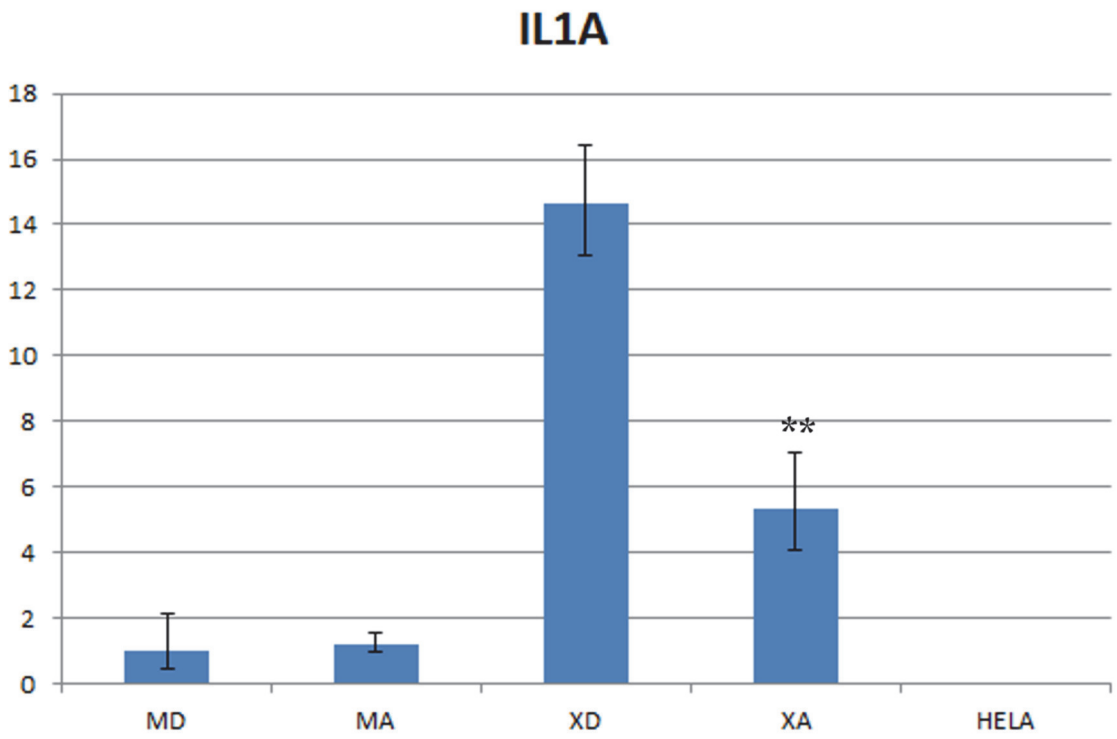


Figure 36: qPCR shows transcription levels of IL-1A mRNA in senescent and non-senescent HCA2 fibroblasts are significantly reduced by apigenin. HeLa cells were used as a negative control.

The same treatment and assay conditions were also used to quantify the expression levels of IL-1A in senescent IMR90 fibroblasts. IL-1A is a potent pro-inflammatory cytokine capable of autocrine signaling and induction of the IL-6, TNFa, and IFNg inflammatory pathways. Blockade of IL-1A signaling has demonstrated total abrogation of ongoing chronic inflammatory disorders, such as systemic onset juvenile idiopathic arthritis (Pascual et al. 2005), suggesting that factors capable of reducing IL-1A expression may yield myriad downstream anti-inflammatory, or anti-SASP, effects. As expected, IL-1A levels rose markedly upon irradiation (XD) but fell significantly upon subsequent apigenin treatment (XA), suggesting that the therapeutic mechanism of apigenin is at least partially mediated through the IL-1A signaling axis. Previous work showing a positive feedback loop was necessary for maintenance of strong SASP profile (Orjalo et al. 2009) lends more strength to his hypothesis which was investigated further.

2.5.4. NFκB Activity

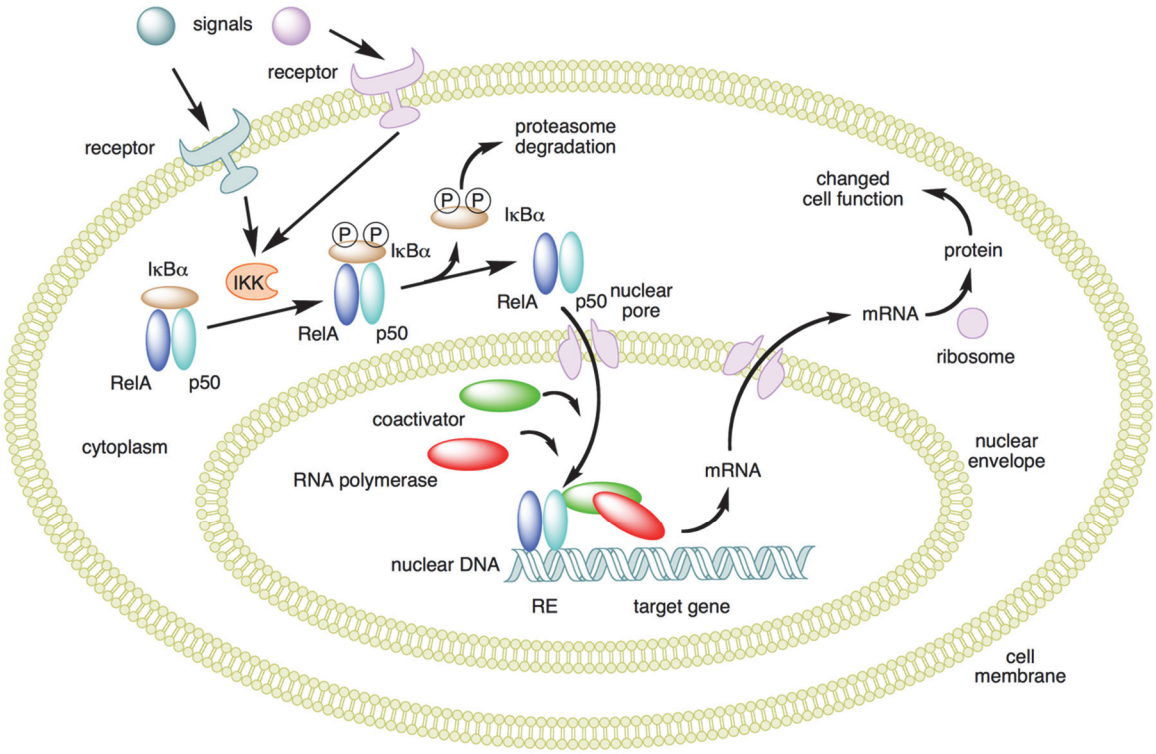


Figure 37: The canonical NFκB pathway (Wikipedia)

NFκB is a central intracellular mediator of inflammatory responses central to the activation, execution, and resolution of both acute and chronic inflammation as may occur in environmental damage or aging, respectively. Many immune and stress response signaling pathways lay upstream of NFkB, with the general canonical activation pathway shown in Figure 37. Depending on the nature of the current signaling inputs, NFκB participates in complex intracellular supramolecular complex formation, wherein an "inflammasome" comprising multiple proteins integrates disparate cell signals and assembles a coordinated transcriptional program that ultimately governs a given cell's response to local inflammatory stimuli.

NFκB activity sits upstream of the SASP, wherein canonical activation of NFκB via its RelA-dominated inflammasome is required for the upregulation of IL-6 and other SASP factors, such as IL-1A, GROA, and CXCL10 (Chi en et al. 2011). Because of this importance to stress responses, the effect of apigenin on NFκB activity was investigated. To do so, HCA2 fibroblasts were transfected with a lentivirus carrying a firefly luciferase reporter paired to the NFκB

promoter according to the supplier's protocol. After selection for successful transfection, cells were seeded at 10,000 cells per square centimeter, induced to senescence by X-ray irradiation, and cultured for 10 days wherein media was replenished every 48 hours. On Day 8, non-senescent cells were seeded at 5,000 cells per square centimeter. On day 9, all cultures were serum-starved via serum-free media containing either apigenin at multiple doses or 0.1% DMSO as a control. 24 hours later, cells were lysed and the luciferase activity of their cytosol was measured using the Promega Luciferase Assay System. Results are shown below in Figure 38 .

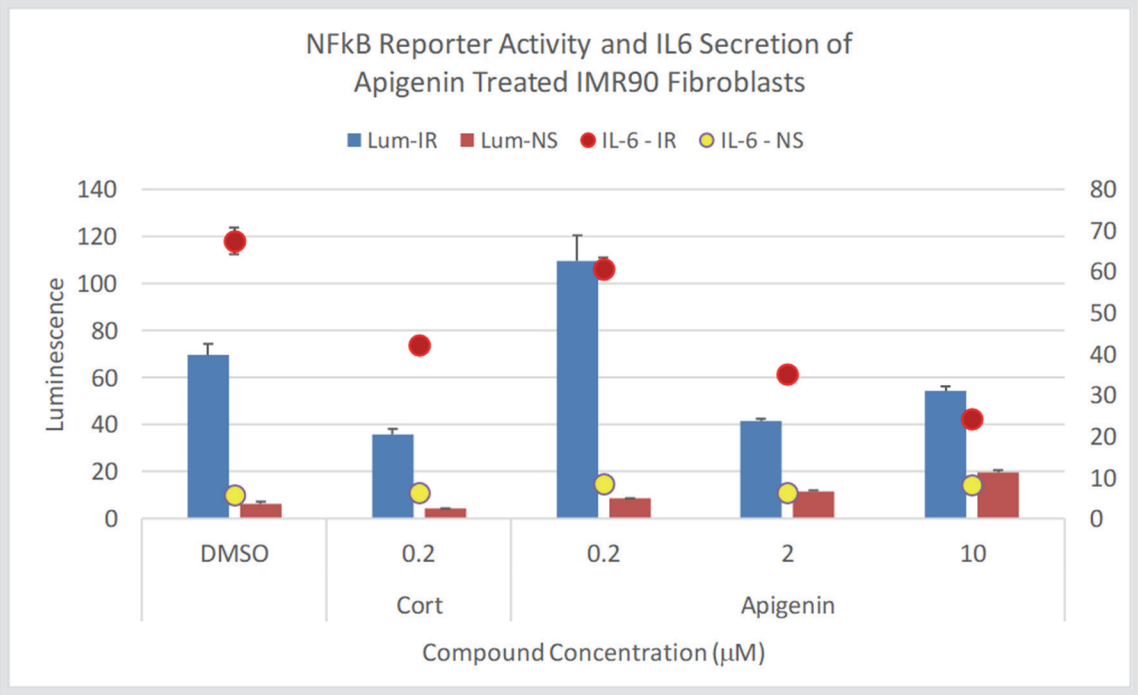


Figure 38: NFκB Reporter Activity and IL6 Secretion of Apigenin Treated IMR90 Fibroblasts

Apigenin treatment was found to significantly reduce NFκB activity and IL-6 secretion in senescent IMR90 fibroblasts in a dose-dependent manner, showing that apigenin acts upon a central mediator of inflammation and exerts broad cellular anti-inflammatory effects. However, this does not prove that NFκB is the direct target of apigenin treatment, merely that the signals feeding into its activation are decreased under apigenin treatment.

2.5.5. Inhibition of p38 Activity

Previous work by Adam Freund in the Campisi laboratory (Freund et al. 2011) showed that p38 activity is upregulated in senescent cells. p38 is a central mediator of the MAPK mitogen signaling pathway that regulates cellular proliferation or the inhibition thereof. It was

hypothesized that because apigenin reduced the rate of growth that it was affecting mitogen signaling and thus inhibition of p38 activation could be involved in apigenin's ability to reduce the SASP and an experiment was done to check the levels of phosphorylated p38. Results are shown below in Figure 39.

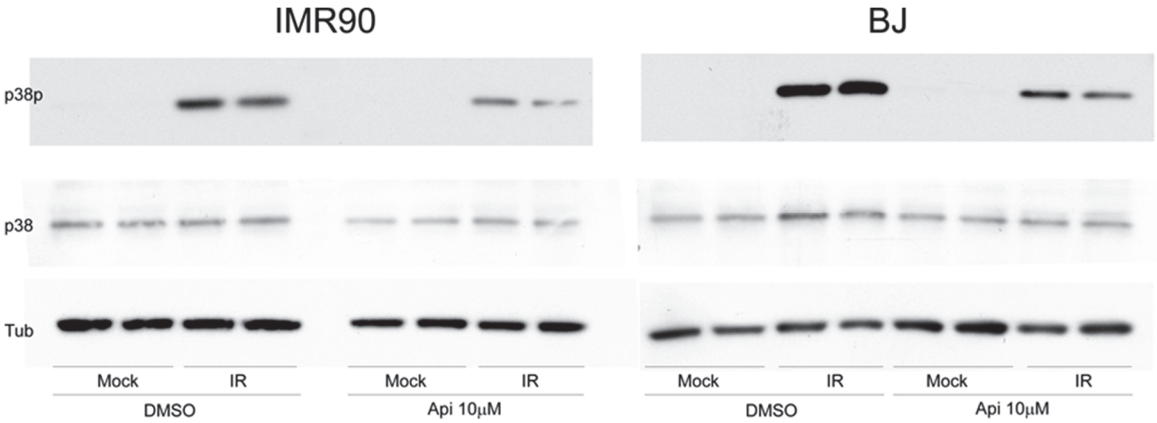


Figure 39: Western blot of total and phosphorylated p38 (T180) in treated and untreated primary human non-senescent (Mock) and senescent (IR) IMR90 and BJ fibroblasts. Each condition is was performed with two biological replicates. There is no phosphorylation of p38 in the non-senescent samples while the elevated phosphorylation of senescent fibroblasts was reduced by apigenin.

p38 is a mitogen-activated kinase whose phosphorylated form is a marker of cellular stress. p38 cooperates with MAPK kinases, central regulators of cell proliferation and stress responses, to coordinate the activity of various cellular effectors. Apigenin treatment reduces phosphorylated p38 in irradiated cells (IR DMSO; and IR Api), demonstrating a potent reduction in intracellular damage signaling in comparison to mock-treated cells (Mock DMSO; and Mock Api). This effect concords with apigenin's other anti-SASP effects and suggests that, with reduction in IL-1A and IL-6, this decrease in phosphorylated p38 is indicative of a holistic anti-senescent change in cell state.

2.5.6. New SASP Factor CXCL10

Interferon-gamma (IFN-γ/g) shown by (Freund et al. 2010) displays a < 2-fold increase in oncogene-induced senescence, increases 12-fold in secretion in irradiated IMR90 fibroblasts and 6-fold in similarly treated BJ primary fibroblasts according to the Luminex data produced earlier. IFN-γ stimulates the expression of IP10/CXCL10 which was also increased dramatically in the Luminex results. CXCL10 is normally secreted in response to IFN-γ and previously was thought

to be secreted only by CD4/8 T-cells so seeing it appear as part of the SASP was a novel finding and warranted further investigation.

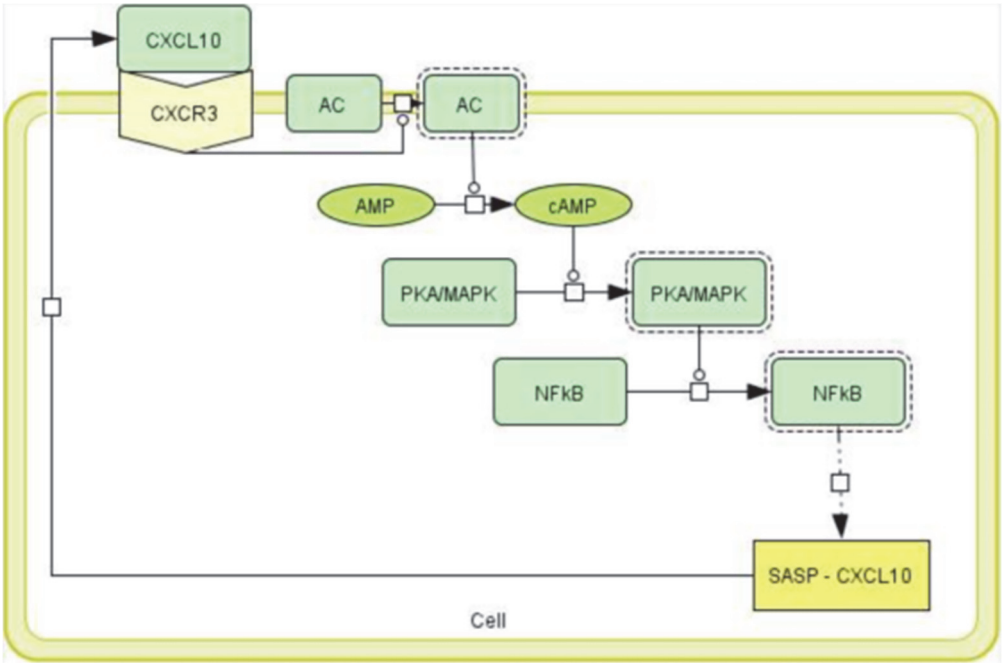


Figure 40: Hypothetical CXCL10/IP10 feedback loop. The promoter of CXCL10 includes an NFkB binding site, while activation of CXCR3 by CXCL10 can result in the downstream activation of NFkB transcription leading to self-reinforcement of CXCL10 expression.

Similar to the IL-1A positive feedback loop, CXCL10 is capable of stimulating it NFkB activity and its own expression in a manner show in the figure above. The question as to the role of this possible feedback loop in maintaining the SASP by IFN-γ was an interesting one and a new hypothesis was formulated that interfering with IFN-γ would reduce IL-6 expression through inhibition of its contribution to CXCL10 production. If this effect was seen, it would suggest that one might be able to deconvolute the complexity of the SASP with one module potentially involving interferon-inducible CXCL10.

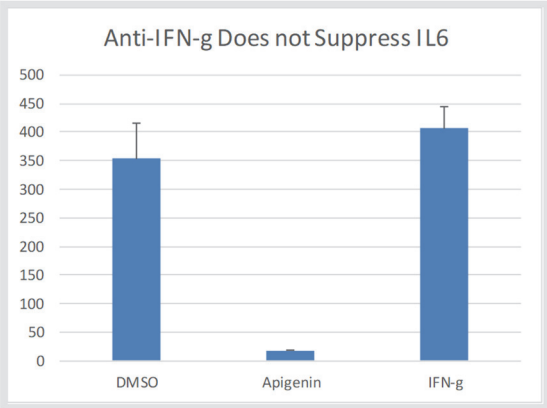


Figure 41: IL-6 expression of senescent HCA2 fibroblasts treated with 10uM apigenin and 1uM Anti-IFN-g (labelled IFN-g). Blockade of IFN-g stimulated expression of CXCL10 through CXCR3 has no effect on IL-6 secretion.

Figure 41 to the left shows the effect of early and continuous inhibition of IFN-γ activity through the use of an anti-IFN-γ antibody on IL-6 secretion of senescent HCA2 fibroblasts. Treatment did not result in reduced IL-6 secretion, suggesting that SASP-associated production of CXCL10 likely results from direct transcription via NFkB-directed activity and not from IFN-γ signaling pathways. In comparison, however, apigenin treatment was able to reduce IL-6 secretion, suggesting that the mechanism of apigenin treatment operates primarily upon the IL-1A or IL-6 axes and not on the IFN-γ signaling pathways.

3. Chapter 3 - Phosphorylation Inhibition by Apigenin

3.1. NFκB Pathway

In reviewing the literature, apigenin has been reported to be able to potently reduce inflammation, although the mechanism has been controversial. Notably, CK2A, a constitutively active kinase and a nexus for many stress-related signals, has been reported to be involved with inflammation through the activation of NFκB transcription (Huang et al. 2017) and has also been reported to be inhibited by apigenin (Pagano et al. 2008). Considering this an investigation of whether other inhibitors of CK2A (such as 4,5,6,7-Tetrabromobenzotriazole [TBB] or 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole [DRB]) was undertake to determine if they might also reduce the SASP and found that, although DRB could reduce the inflammation produced by senescent cells, the well-recognized CK2A inhibitor TBB did not result in a significant down-regulation of IL-6. Therefore, CK2A could not be the target of apigenin, at least when it comes to mediating the decrease in IL-6 associated with the SASP. With respect to the effects of DRB, even though DRB was able to reduce the SASP significantly, it is also known to inhibit RNA Polymerase II, albeit at a much higher concentration of 100uM, and thus the decrease was

ascribed to decreased overall transcription and not studied further. However, returning to an examination of the effects of DRB at 10uM is likely as, qPCR showed the transcripts of the usual genes used to normalize, actin and tubulin, were unaffected by treatment with DRB leaving one to consider that the decrease in IL-6 may not have been due to inhibition of RNA Pol II, which occurs at greater concentrations, but through inhibition of phosphorylation by other kinases. Regardless, because TBB did not reduce the SASP, the effects of DRB were unlikely to be exerted through the inhibition of CK2A and it seemed clear that apigenin must be acting in some other way to decrease the SASP.

With the data indicating that p38 might be involved in the reduction of NFκB transcription, the next logical step was to look at where in the NFkB pathway apigenin's effects were targeted and a common method for examining phosphorylation state became the workhorse for the following results yielded by IL-1A stimulation.

3.1.1. Methods

3.1.1.1. Phosphate stabilization of IL1A stimulated cells by Calyculin A

Primary human HCA2 fibroblasts were incubated at 37 °C in a 3% O2/10% CO2 atmosphere after being seeded at 500,000 cells per plate in each of eight p100 plates in the afternoon. They were cultured overnight in Dulbecco's modified Eagle medium (DMEM) (VWR, Cat# 45000-312) supplemented with glucose (4.5 g/l), glutamine (4 mM), 10% fetal bovine serum (FBS), and antibiotics (100 IU penicillin, 100 µg/ml streptomycin).

After 24hrs, media was refreshed with 10ml of media containing apigenin or vehicle (DMSO) and the cells were then allowed to expand four days to confluency with the media refreshed every 48 hours. On Day 4, the media is exchanged for serum-free media containing apigenin or DMSO to reduce the growth factor signalling to a basal state.

On Day 5, media was refreshed with the same serum-free media containing DMSO or apigenin. Three of the four DMSO/Apigenin plates were treated with 250pg/ml of recombinant IL-1A (Abcam #ab124962). 15 minutes prior to treatment with IL-1A, Calyculin A (Cell Sig#9902S, 10nm final concentration), a pan-phosphatase inhibitor to "reset" the phosphorylation state of cell signaling proteins, was added to DMSO- and Apigenin-treated plates. After adding the IL1A-containing media to the six plates, a DMSO- and apigenin-treated plate was immediately lysed and the protein collected to comprise time point 0. At fifteen minutes after the addition of IL1A

and fifteen minutes prior to collection of the 30 minute time point, two more plates were similarly treated with Calyculin A and at 30 minutes after IL-1A stimulation the cells were lysed and protein collected. Similarly, at 45 minutes, 15 minutes before the 60 minute time point, Calyculin A was added to one DMSO and one apigenin IL-1A-stimulated plate and protein was collected at 60 minutes. This ensures that all time points have been exposed to the phosphatase inhibitor for similar periods of time and the levels of phosphorylation will be maximally comparable. After all time points were collected, the unstimulated plates that were not exposed to either IL-1A or Calyculin A were harvested for baseline phosphorylation states.

The method above removes the activity of phosphatases from the equation and allows one to more easily see the dynamics of kinases and the subsequent phosphorylation of their downstream targets without the confounding consideration of prior phosphatase activity. Although phosphatases can remove phosphate groups from proteins at a relatively rapid rate, this method can reveal increased occupancy of phosphates at various cell signaling proteins to more readily capture the activation state of said proteins. This method was chosen due to its merits in capturing the actual activity of proteins at a rapid time scale, which more closely mimics the manner in which cells regulate their state in real-time. These studies were undertaken in the aim of revealing the precise biological activity of putative apigenin targets before and after treatment to better define its therapeutic mechanism of action.

3.1.2. Results

3.1.2.1. Inhibition of p38 Phosphorylation at Tyr180

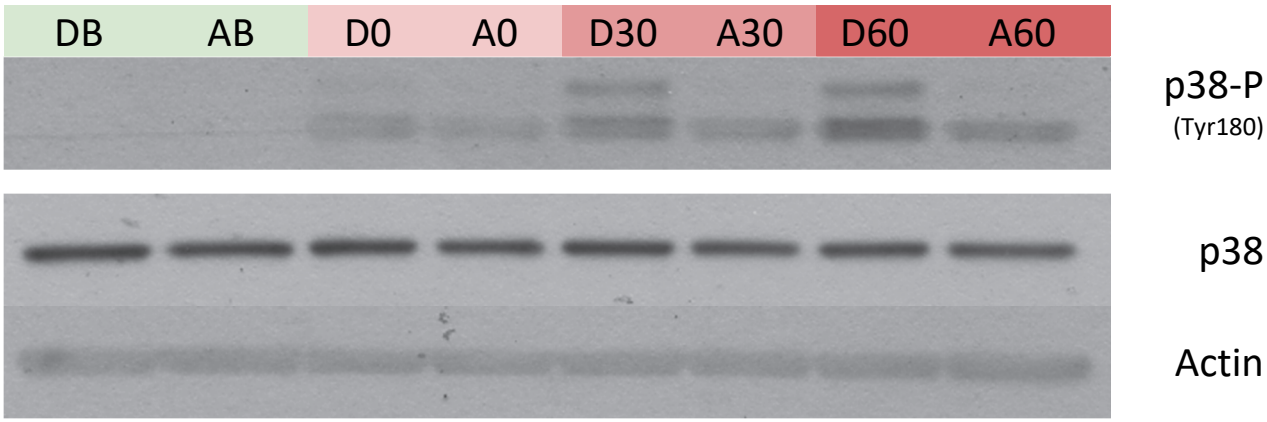


Figure 42: Apigenin suppresses IL-1A-stimulated p38 phosphorylation at Tyr180. Baseline levels were measured in non-senescent IMR90 cells treated with DMSO (DB) or apigenin (AB). Similar DMSO (D0, D30, D60) or apigenin (A0, A30, A60) treated samples were stimulated for 0', 30', or

60’ with recombinant IL-1A. 15’ prior to collection each timepoint was subjected to pan-phosphatase inhibitor Calyculin A to stabilize the phosphorylation state.

In the above, DB and AB lanes were controls unstimulated with IL-1A, where AB was also given 10μM apigenin. D0, D30, and D60 lanes represent IL-1A-stimulated cells whereas A0, A30, and A60 represent IL-1A-stimulated cells also treated with 10μM apigenin. The numbers 0, 30, and 60 represent the times at which samples were taken after IL-1A treatment. All time-dependent samples were treated for 15 minutes prior to collection with Calyculin A to “reset” phosphatase state. All results shown above were gathered using non-senescent HCA2 fibroblasts. The same IL-1A stimulation, sequential time points, Calyculin A treatment, and labeling schema is used below.

Activated p38 is thought to mediate the irreversible nature of cellular senescence, where stressor stimuli and internal cellular reactions lead to high p38 phosphorylation that persists even once stressful stimuli are resolved (Freund et al. 2011; Iwasa et al. 2003). These findings suggest that p38 is a central maintainer of senescence state, where its activity inhibits all mitogenic signaling and promotes low-level inflammation (Schieven 2005).

As it was demonstrated in previous experiments that p38 phosphorylation at Tyr180 was suppressed by apigenin, recapitulating that result with this new protocol was important. These results agrees with previous work in that it clearly demonstrate that apigenin treatment sharply reduces p38 phosphorylation at all timepoints of IL-1A-stimulation, demonstrating that apigenin potently antagonizes a central mediator of the SASP and the senescent phenotype in stark contrast to DMSO-treated cells, in which phosphorylated p38 steadily rose at each time point.

3.1.2.2. IRAK4

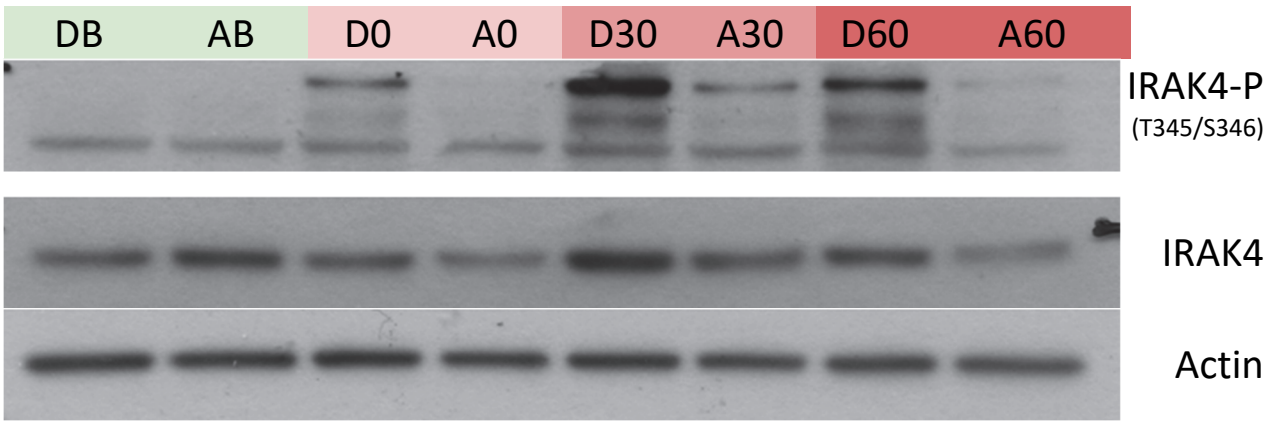


Figure 43: Apigenin suppresses Il1A-stimulated phosphorylation of IRAK4. Baseline levels were measured in non-senescent IMR90 cells treated with DMSO (DB) or apigenin (AB). Similar DMSO (D0, D30, D60) or apigenin (A0, A30, A60) treated samples were stimulated for 0', 30', or 60' with recombinant IL-1A. 15' prior to collection each timepoint was subjected to pan-phosphatase inhibitor Calyculin A to stabilize the phosphorylation state.

IRAK4 is a central mediator of inflammation via cooperation with MyD88. MyD88 enables successful transduction of inflammatory stimuli from both Toll-like receptors, involved in innate immunity, and IL-1AR, involved in increasing and sustaining inflammatory responses. IRAK4 complexes with activated MyD88 to form an activation complex, recruiting TRAF6 and others to increase activation of the MEK and c-Jun pathway, noted above to coordinate the SASP. IRAK4 activity also increases p53 activation, leading to growth arrest and senescence (Salminen et al. 2012; De et al. 2018). Due to its activity in the SASP, the effects of apigenin on IRAK4 activity was assayed.

Fibroblasts were irradiated and treated as described above to yield a time-series of IRAK4 phosphorylation under control DMSO treatment (D0, D30, and D60) in comparison to apigenin treatment (A0, A30, and A60). These results demonstrated that IRAK4 is significantly increased in irradiated fibroblasts and reduced upon apigenin treatment thereof. This shows that apigenin treatment is capable of rapidly reducing inflammatory mediators and antagonizing the SASP.

3.1.2.3. IRAK1 and MEKK1

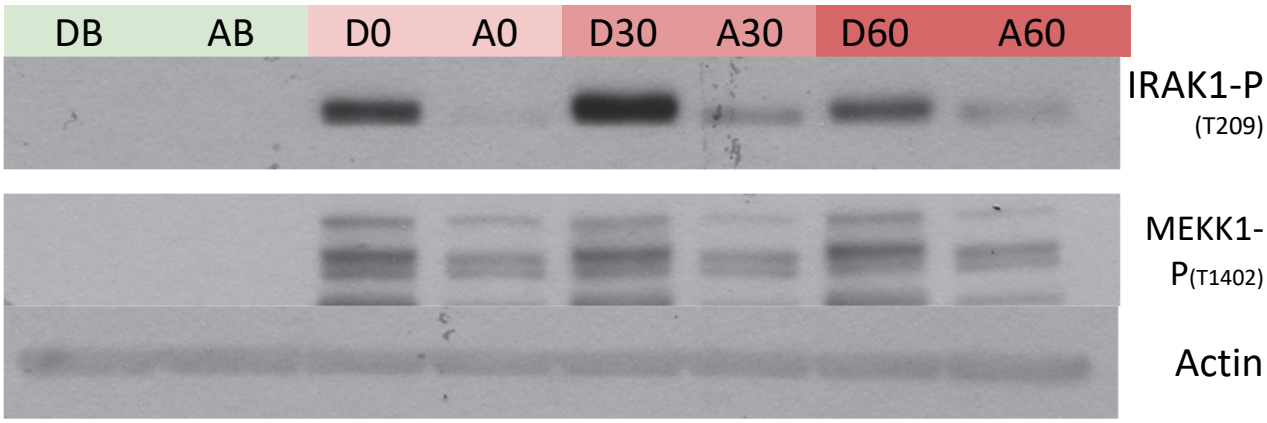


Figure 44: Apigenin suppresses Il1A-stimulated phosphorylation of IRAK1 and MEKK1. Baseline levels were measured in non-senescent IMR90 cells treated with DMSO (DB) or apigenin (AB). Similar DMSO (D0, D30, D60) or apigenin (A0, A30, A60) treated samples were stimulated for 0', 30', or 60' with recombinant IL-1A. 15' prior to collection each timepoint was subjected to pan-phosphatase inhibitor Calyculin A to stabilize the phosphorylation state.

Like IRAK4 above, IRAK1 is a central mediator of IL-1AR signaling and carries activating signals into the cell to promote p53 and MEK activity upstream of senescence and the SASP. IRAK1 has been found as a heterodimer with IRAK4, where their combined activity transduces inflammatory stimuli and helps to mediate cell stress responses. Their activity also contributes to the function of MEKK1, a kinase intimately involved in cell cycle progression via coordination of several mitogen-associated kinases, including c-Jun and MEK as described above (Fuchs et al. 1998). Because of these activities, the effect of apigenin treatment upon IRAK1 and MEKK1 activity in irradiated fibroblasts was assessed.

Similar to IRAK4, IRAK1 activation is significantly decreased by apigenin treatment at all time points. In contrast, MEKK1 phosphorylation was relatively unaffected by apigenin treatment, although slightly decreased. This suggests that MEKK1 activity in senescent cells proceeds independently of apigenin's treatment effects and may be peripheral to the SASP itself, as MEKK1 participates in intracellular complex formation that regulates cellular proliferation overall. This suggests that apigenin acts primarily to decrease inflammatory signaling, but may not directly alter senescence-state-associated signaling in the MEKK pathway. Further study will be needed to conclude whether this effect also holds true for the other effectors involved in these pathways.

3.1.2.4. IKKA/B

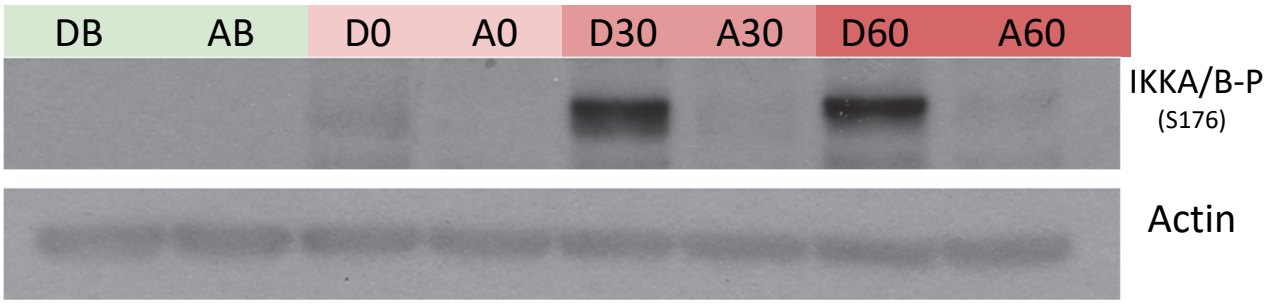


Figure 45: Apigenin suppresses IL-1A-stimulated phosphorylation of IKKA/B.Baseline levels were measured in non-senescent IMR90 cells treated with DMSO (DB) or apigenin (AB). Similar DMSO (D0, D30, D60) or apigenin (A0, A30, A60) treated samples were stimulated for 0', 30', or 60' with recombinant IL-1A. 15' prior to collection each timepoint was subjected to pan-phosphatase inhibitor Calyculin A to stabilize the phosphorylation state.

IKKA/B are inhibitory kinases central to the regulation of the NFκB pathway, itself the central regulator of cellular inflammatory responses. IKKA and IKKB are a cytosolic heterodimer whose activity is required for the repression of IKBA, a constitutive inhibitor of NFκB activation via promotion of its degradation. Once IKBA is repressed via IKKA/IKKB phosphorylation, the rate at which it binds to and promotes the degradation of NFκB is decreased, permitting activation of inflammation. IKKA and IKKB are activated downstream of IRAK1/4 via the TAK1/TAB complex, suggesting that they act to coordinate cellular inflammation at multiple levels (Helenius et al. 2001). Due to these inflammation-mediating activities, the effect of apigenin treatment on IKKA and IKKB phosphorylation was investigated.

As above, apigenin treatment decreased the activation of inflammatory mediators IKKA and IKKB, where apigenin was capable of preventing the irradiation-induced increase in IKKA/IKKB phosphorylation seen in DMSO-treated cells.

3.1.2.5. P65-Ac (K310)

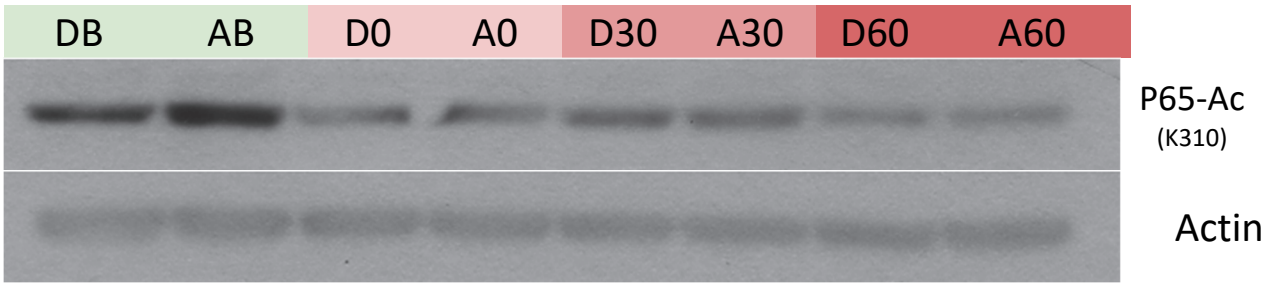


Figure 46: Apigenin has no effect on the acetylation of p65 at K310. Baseline levels were measured in non-senescent IMR90 cells treated with DMSO (DB) or apigenin (AB). Similar DMSO (D0, D30, D60) or apigenin (A0, A30, A60) treated samples were stimulated for 0', 30', or 60'

with recombinant IL-1A. 15' prior to collection each sample was subjected to pan-phosphatase inhibitor Calyculin A to stabilize the phosphorylation state.

Like many other transcription factors, the window in which NFκB activity is active is carefully regulated. The downstream pro-inflammatory effects of NFκB depend on its translocation into the nucleus, where it can then bind to the promoter regions of several inflammatory genes. Within the nucleus, acetyltransferases such as p300 and PCAF, involved in epigenetic regulation, act upon the p65 subunit via acetylation of lysine residues. Increased acetylation decreases NFkB's ability to bind to the promoter regions of inflammatory genes and thus limits its pro-inflammatory activity. Acetylated NFκB is then exported from the nucleus via IKBA, its canonical repressor, for degradation. Therefore, acetylation of NFκB is a key regulator of its activity within cells, where the activity of countermanding p300 and others serves as a strict limiter of NFκB pro-inflammatory function and thus ensures that cellular reactions to inflammatory stimuli are moderated. Without this regulation, unrestrained NFκB activation could lead to runaway positive feedback signaling with deleterious effects on cellular homeostasis and tissue health (Kiernan et al. 2003). Due to these regulatory effects, the impact of apigenin treatment on p65 acetylation was examined.

The results shown above are inconclusive, in which apigenin treatment does not yield a clear difference in p65 acetylation at any time point. This is an interesting result in contrast to overall levels of NFkB, p65, and IRAK1/4, which were all reduced by apigenin treatment. In light of this, apigenin treatment may either also inhibit acetyltransferase activity or merely operate on a longer time-scale than was surveyed here. Therefore, it will be of immense interest to repeat the above experiment with longer time points and comparison with p300 and other acetyltransferase activity.

3.1.2.6. Combined IL-1A Pathway Activation

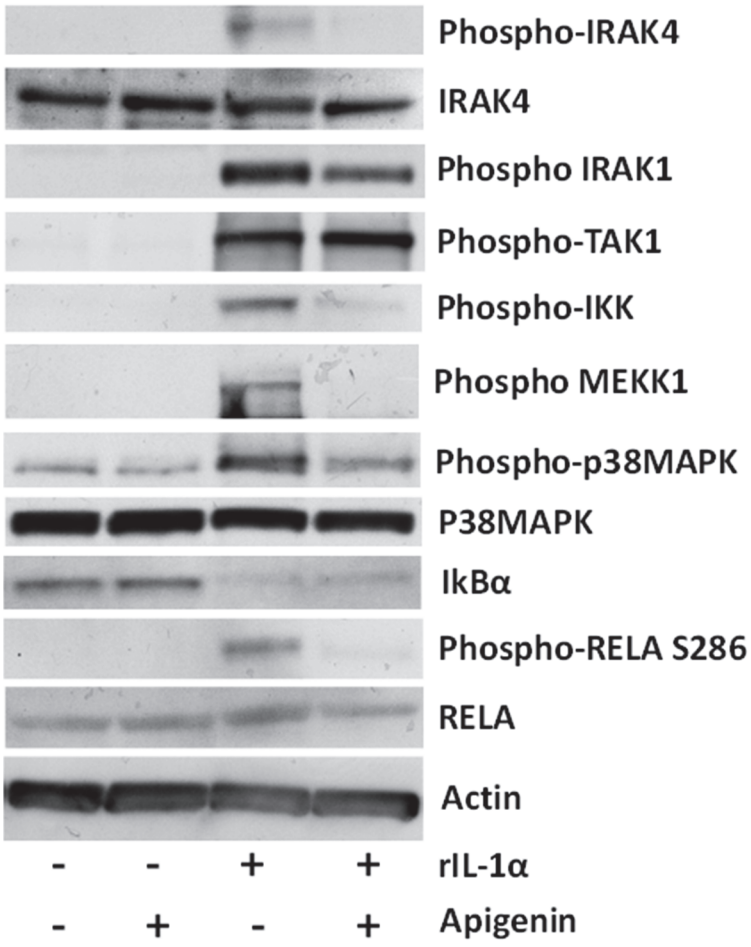


Figure 47: Effects of apigenin treatment on phosphorylation of factors involved IL-1A mediated inflammation after 30' of treatment with recombinant IL-1A/rIL-1α similar to previous experiments, except with one 30' time point. As seen previously, IRAK4, IRAK1, MEKK1, IKK and p38 phosphorylation are suppressed by apigenin. Degradation of IKBA is not protected or restored by apigenin while TAK1 phosphorylation seems to increase. p65/RelA phosphorylation decreases as expected.

Activity in the IL-1A pathway is key to the SASP, where positive feedback from IL-1A signaling elements underpins IL-6, IL-8, and other inflammatory mediator activation. Given these facts a survey of IL-1A-related factors in response to stimulation with recombinant IL-1A and treatment with apigenin undertaken. Non-senescent IMR90 primary human fibroblasts were treated using the same method for the stabilization of phosphorylation previously described. Protein was collected and western blots were run according to standard protocols.

In the above, we found that apigenin treatment alone had little effect on IL-1A-related effectors (2nd column) whereas rIL-1A stimulation significantly upregulated phosphorylated IRAK4, IRAK1, TAK1, IKK, MEKK1, p38-MAPK, and RelA, suggesting increased inflammatory signaling. These increases were, in turn, antagonized by apigenin treatment, revealing the broad anti-inflammatory effects of apigenin, although apigenin treatment did not significantly restore IkBa levels in the time surveyed. Notably, the absolute levels of RelA, the canonical subunit of NFκB involved in acute inflammation, were not significantly altered by any treatment condition, although its phosphorylation was significantly increased by rIL-1A stimulation and decreased by apigenin treatment. This bolsters the argument for apigenin's primary therapeutic effects being mediated by NFkB-related signaling, where a decrease in NFκB activity would explain apigenin's potent anti-IL-6 and other inflammatory cytokine effects.

p65 is one of the two proteins that make up canonical NFkB. The other is p50. These two proteins form the canonical NFκB heterodimer that serves as a nucleation point for supramolecular activation complex formation in inflammatory contexts. IKKA and IKKB phosphorylate IKA/B, which binds and inhibits p65 activity in an unphosphorylated state. (Laveti et al. 2013). There is a decrease in phosphorylation of p65 at S529 with apigenin treatment, a key residue for p65 activation, and also a decrease in the total levels of IKKA and IKB. This suggests that apigenin's mechanism of action is centered around NFkB, where reduction in p65 activity explains the observed decrease in IL-6 and other inflammatory cytokine secretion under multiple conditions as shown above in previous chapters. This mechanism is striking, as it offers wide-ranging reduction in inflammatory mediators without acting upon cyclooxygenase or other NSAID-targeted pathways. A non-NSAID means of reducing inflammation via a central mediator thereof is of great clinical significance, both for the treatment of senescence as well as for the treatment of other chronic inflammatory disorders, which themselves may be mediated by the metabolic and SASP activity of accumulated senescent cells. Additional investigation into this mechanism of action will be necessary to fully confirm that apigenin acts principally upon NFkB, but this is nonetheless compelling evidence that it does act to inhibit NFκB and subsequent SASP activity.

3.2. Apigenin as an ATP-Mimetic

Apigenin has been called "promiscuous" because, like many naturally occurring substances derived from plants and vegetables, it has numerous biological effects. As mentioned earlier, it has been studied in numerous disease contexts, including its roles in inflammation, autoimmunity and cancer among others and has been shown to affect many tenuously connected biological pathways. In many biological settings, apigenin and related flavonoids have been shown to exert potent effects of a diverse nature, from anti-inflammatory to metabolic effects. Because of this wide-range of effects, apigenin's mechanism of action has defied definitive explanation and consequently hindered its usefulness as a potential therapeutic. It was this difficulty in pinning down the mechanism of action throughout the work of this thesis that I began to see patterns emerging, which could form the basis of a new hypothesis on an underlying general mechanism by which apigenin and related compounds affect multiple aspect of cellular biology at once. This unified mechanism suggests that apigenin acts as an ATP mimetic, which could then bind to and affect a wide variety of ATPase-domain-containing proteins. Not only would this mechanism explain how apigenin is able to influence so many cellular functions at once, but such a general mechanism is in keeping with the data collected while investigating its ability to reduce the SASP, itself comprised of myriad cellular functions.

The determination by which apigenin reduces the SASP served as the starting point for a wide variety of experiments to delineate the limits of apigenin's biological activities.

As described before, the enzyme CK2A was known to have a role to play in inflammation (Singh and Ramji 2008; Huang et al. 2017; Lin et al. 2015). Prior research largely examined the role of CK2A in immune cells, and here we examined whether CK2A could be a target of apigenin and could thus mediate its anti-inflammatory effects in senescent cells. Subsequent investigation comparing the ability of known inhibitors of CK2A (data not shown) did not show a significant decrease in IL-6 secretion similar to that seen with apigenin treatment, so it was clear that, although apigenin may be able to reduce the activity of CK2A and phosphorylation of its downstream targets, it was not this ability that conferred its ability to reduce the SASP.

During the literature review, it was found that apigenin had already been studied and shown to inhibit a wide variety of kinases *in vitro.* The chart below highlights some of the most significant members of this set of kinases.

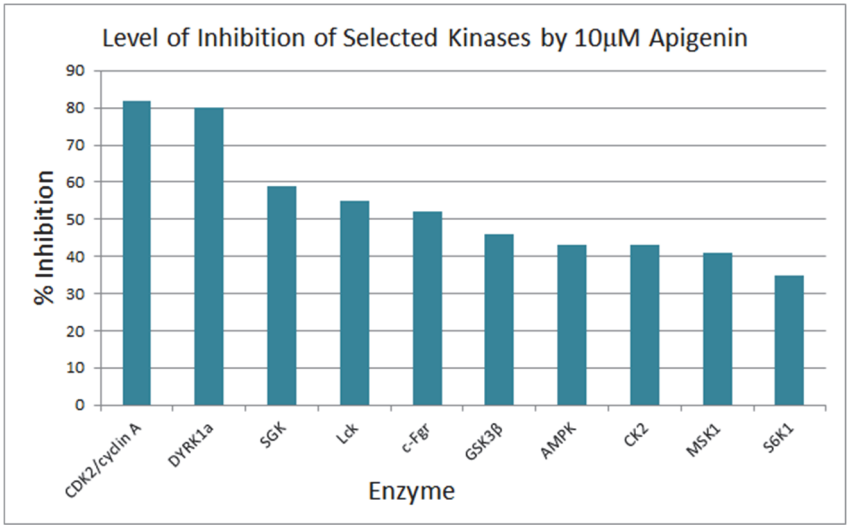
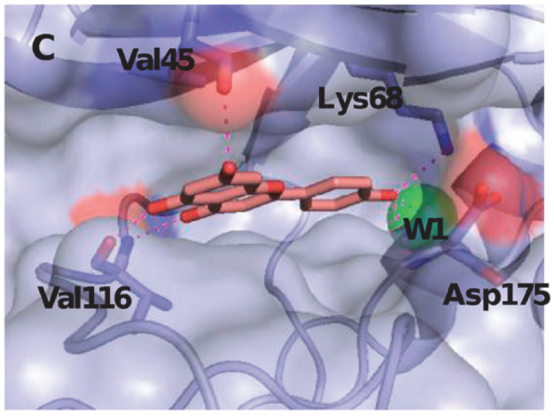


Figure 48: Apigenin significantly inhibits a number of kinases including S6K1 whose target S6 has been shown to have its phosphorylation state inhibited by rapamycin, a drug that has demonstrated capacity to extend both maximum and median healthy lifespan in mice.

The above data from (Pagano et al. 2008) was obtained by testing the *in vitro* capacity of apigenin to inhibit the baseline activity of a panel of kinases as described elsewhere. (Bain et al. 2007). These results in combination with the experimental observation that apigenin reduced phosphorylation of most of the members of the NFκB pathway (as described above), led to the hypothesis that apigenin could be exerting its effects through inhibition of many kinases at the same time. A publication dealing with the structure-function relationship of CK2 and its inhibition by apigenin (Sarno et al. 2003) served as the basis of future investigation.



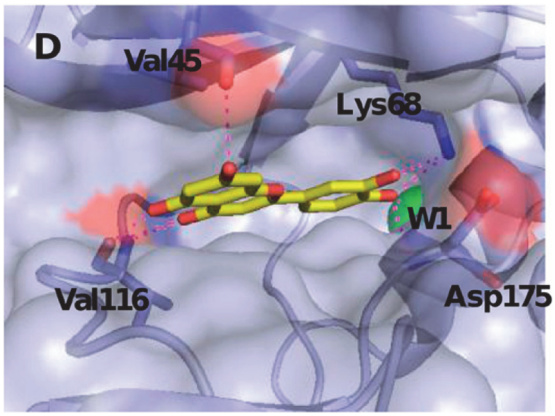


Figure 49: The above images are from (Lolli et al. 2012) where panels C and D show the orientation and hydrogen bonding of apigenin and related luteolin in the ATP binding pocket of CK2A

That apigenin and related molecules were able to bind to the pocket normally occupied by ATP could be thought of as mechanism by which apigenin and other flavonoids could compete with ATP and therefore inhibit the activity of ATP-dependent enzymes, essentially acting as a competitive inhibitor and a bona fide ATP-mimetic.

The inhibition profiles of different flavonoids would naturally tend to vary, with some flavonoids being more able to inhibit a given enzyme than others given that this inhibition would vary depending on the structure. Thus some flavonoids would preferentially alter the activity of some pathways, leading to the conclusion that it might be possible to create a custom mixture of flavonoids capable of differentially inhibiting a given set of signaling pathways to influence a biological system to perform in a particular way. This extends as well to the use of ATP not only in the utilization of the energy stored in the phosphate bonds of the various forms of AMP, ADP, ATP, but also for those enzymes which utilize ATP for the ribose moiety such as PARP and others, in which ATP acts as an allosteric modulator of protein function. This suggests that apigenin could exert its anti-SASP effects via direct or allosteric modulation of the various proteins that are involved in the NFκB signaling pathway, decreasing their activity and thus decreasing the secretion of IL-6 in the SASP.

3.2.1. Methods - Phosphorylation of Proteins in Apigenin Treated Cells

Primary human BJ fibroblasts were seeded at a density of 500k cells per plate into three p100 plates and allowed to incubate overnight under standard culture conditions at 37 °C in a 3% O2/10% CO2 atmosphere in Dulbecco's modified Eagle medium (DMEM) (VWR, Cat# 45000-312) supplemented with glucose (4.5 g/l), glutamine (4 mM), 10% fetal bovine serum (FBS), as well as antibiotics (100 IU penicillin, 100 μg/ml streptomycin).

After 24hrs, the cells were irradiated with 10Gy of ionizing radiation, the media refreshed and the cells allowed to become senescent over a period of six days with a media change every 48 hrs.

On the fifth day non-senescent BJ fibroblasts from the original culture were seeded into an additional three plates as described above and allowed to attach overnight. On the following day, the media for two plates of both the non-senescent and senescent cells was exchanged for serum-free media containing apigenin while the third plate was refreshed with serum-free media containing DMSO.

The plates were then allowed to sit for 24 hours, whereupon the protein for that time point (labelled 24 in the following tables) from the DMSO containing plates as well as one each of the non-senescent and senescent apigenin-treated plates was collected according to previously described methods and and stored in the -80C freezer.

The following day, protein from each of the senescent and non-senescent apigenin-treated plates comprising the 48 hour time point (labelled 48 in the tables below) was collected and the following western blots were performed (representative of three separate experiments).

3.2.2. Results

3.2.2.1. AKT

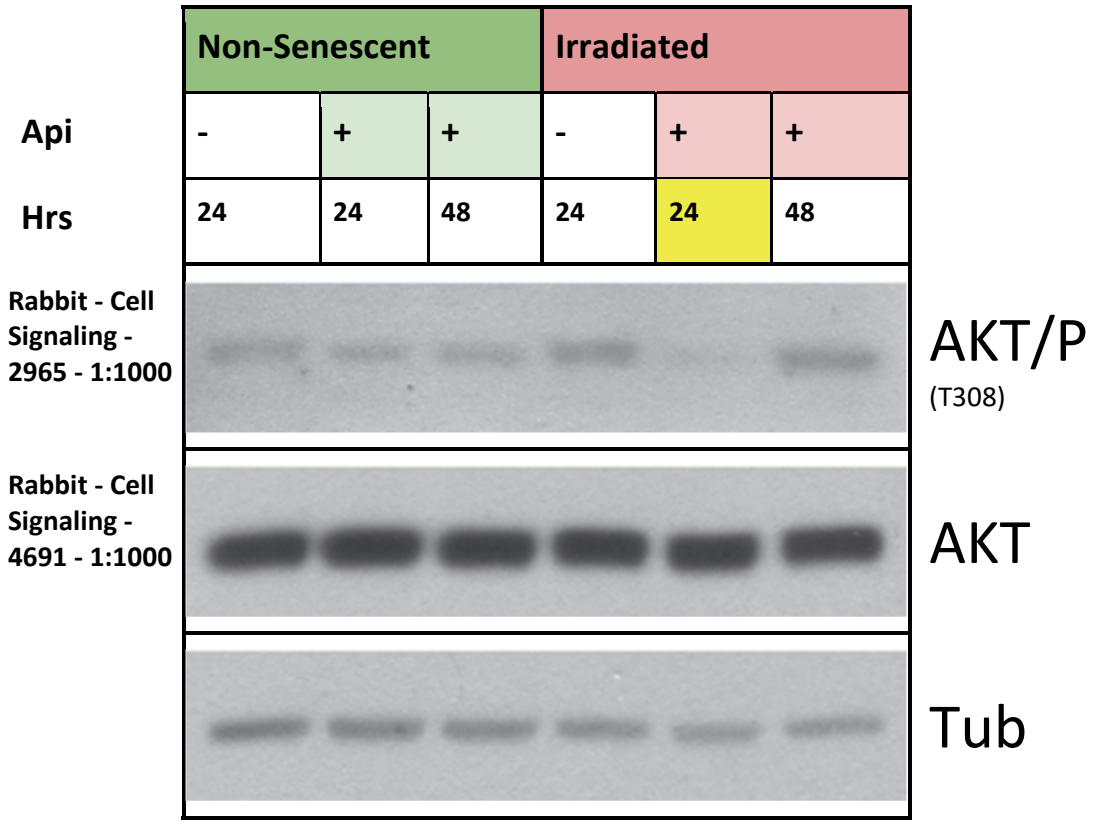


Figure 50: Apigenin reduces AKT phosphorylation at T308 by inhibition of PDK1 in irradiated IMR90 at 24 hrs of treatment but the effect is temporary. The loss of suppression could be due to the half-life of apigenin in culture quite short at about 36 hours.

AKT is a prominent cell signaling protein involved in the activation of mitogenic behavior in many cell types, where it cooperates with PI3K and other effectors to regulate proliferation

within tissues. AKT is also a prominent ATPase, in which the ATP:ADP ratio regulates AKT activation potential and activity within the cell (Vadlakonda et al. 2013). Therefore, temporal AKT phosphorylation by upstream kinase PDK1 with apigenin treatment was investigated.

Polyclonal rabbit antibodies against phosphorylated AKT (Cell Signaling Technology #2965) and unphosphorylated AKT(Cell Signaling Technology #4691) were used in Western blots of treated cells as described above. These results demonstrate that apigenin treatment of irradiated cells significantly reduces AKT phosphorylation (lane #5) at 24 hours, although this effect disappears at 48 hours. This suggests that the window of time in which apigenin exerts therapeutic effects is relatively constrained, where metabolism or excretion of apigenin can limit its overall effects on signaling proteins. Another reason could be the degradation of apigenin in culture media over time does not leave enough apigenin to inhibit AKT phosphorylation by PDK1.

3.2.2.2. mTOR

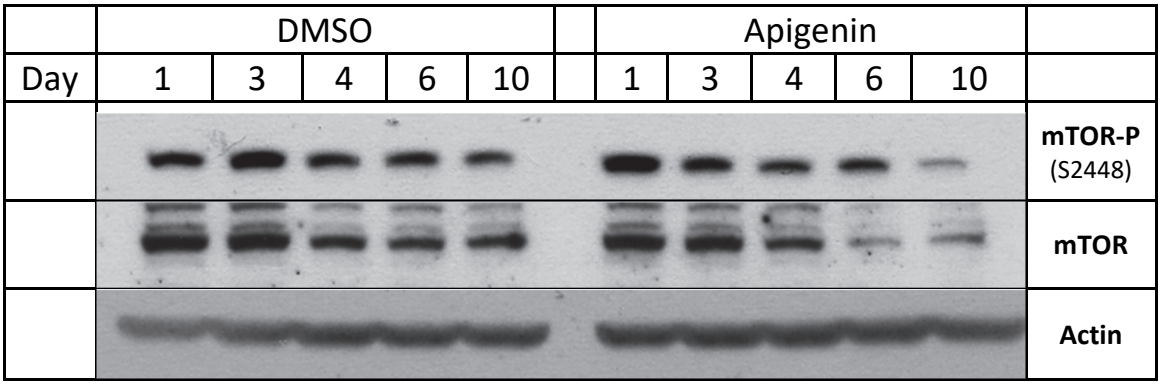


Figure 51: mTOR activation in irradiated HCA2 fibroblasts treated with apigenin over the 10 Day establishment of senescence.

Using the standard protocol for senescence induction and treatment with apigenin over the course of 10 days, we examined the status of mTOR and its phosphorylation at serine 2448, the target of its upstream kinase PI3K. We observed a marked attenuation of S2448 phosphorylation and abundance of mTOR in apigenin treated cells compared to DMSO-treated. This is in keeping with the overall hypothesis that apigenin is inhibiting upstream kinases and reducing growth factor signalling overall. Similarly we went on to examine another member of the AKT/PI3K/mTOR/S6 pathway.

3.2.2.3. S6K

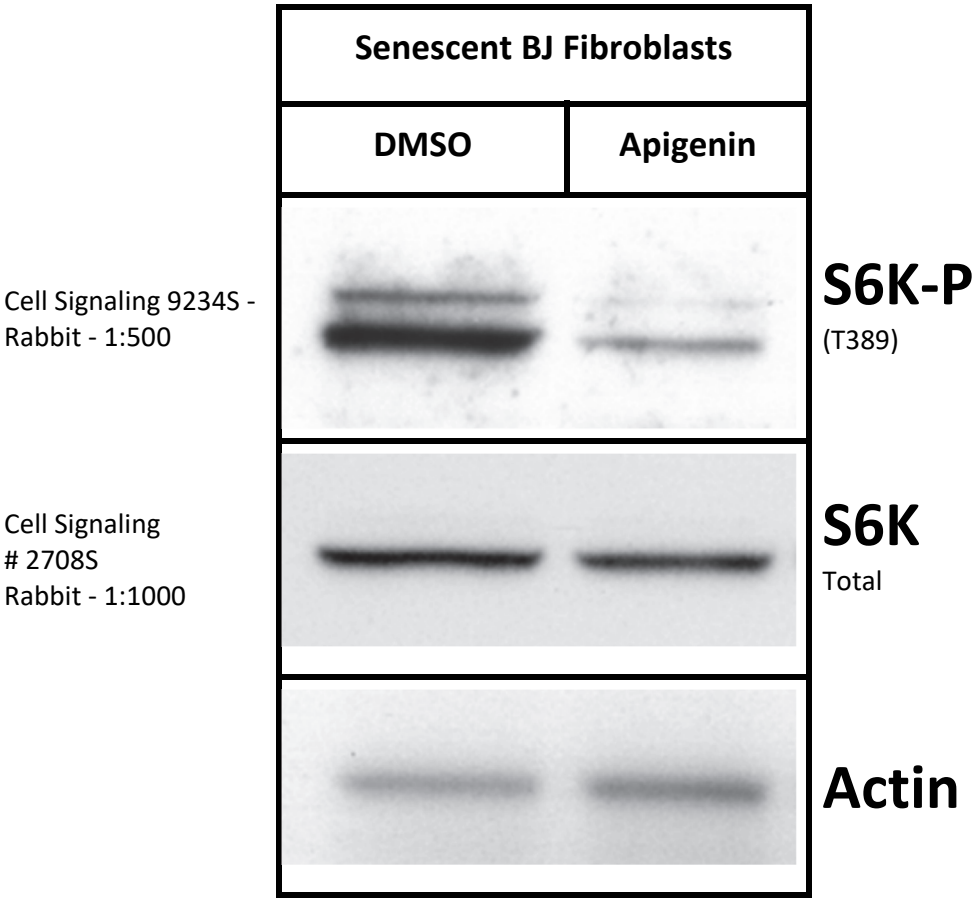


Figure 52: Apigenin attenuates phosphorylation of S6K in senescent BJ fibroblasts.

S6 kinase (S6K) is a central mediator of the mTORC1 pathway that regulates cell proliferation. S6 kinase is capable of phosphorylating the S6 ribosomal subunit to modulate its activity, thereby governing, in part, cell size and proliferative capacity. Inactivation of S6K via antibody inhibition or rapamycin (indirectly via mTOR inhibition) yields cell cycle arrest as well as enlargement of cell size, indicating that activated S6K may play a key role in the adoption of a senescent phenotype and its associated SASP (Takata et al. 2013; Ito et al. 2017). Therefore, the effects of apigenin on S6K phosphorylation examined, In this experiment, using senescent fibroblasts, the phosphorylation of S6K markedly decreased upon apigenin treatment independently of total S6K levels.

3.2.2.4. S6

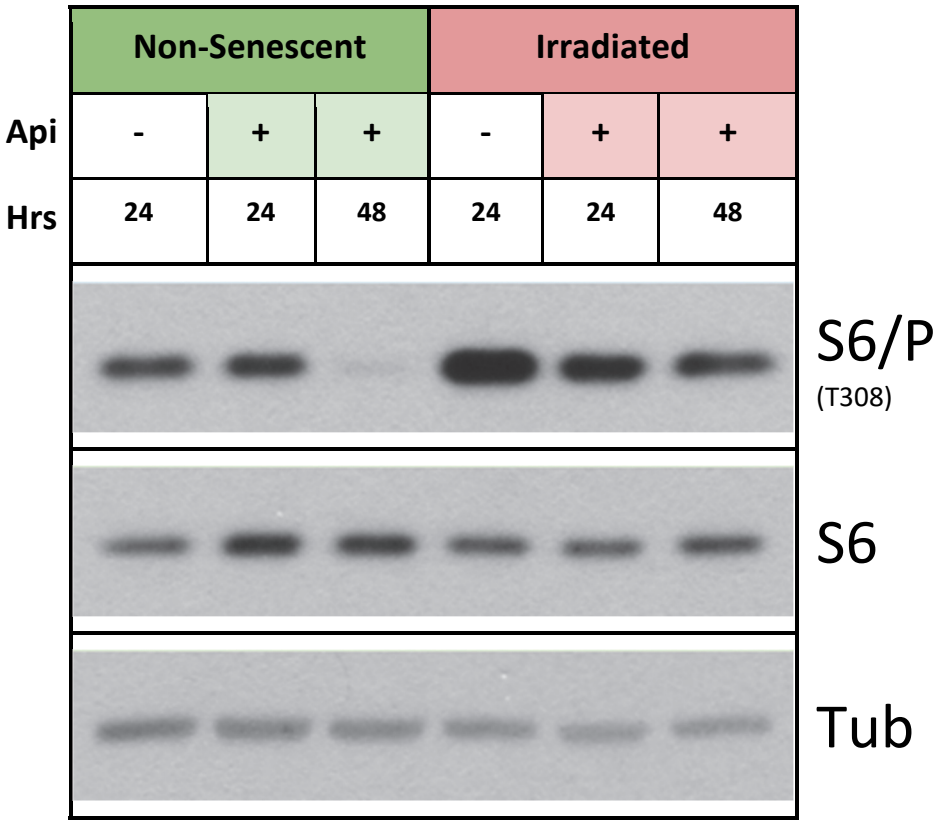


Figure 53: Phosphorylation of S6 in senescent and non-senescent IMR90 fibroblasts is reduced by apigenin

S6 is the target of S6K. S6 is a ribosomal subunit that makes up the eukaryotic p70 ribosomal complex critical to the successful translation of mRNAs to polypeptides. Alteration of S6 activity via phosphorylation state is associated with regulation of protein biosynthesis, cell size, and glucose homeostasis (Magnuson et al. 2012). Therefore, S6 levels were investigated to determine if they were significantly affected by either senescence-inducing irradiation or apigenin treatment or both.

In the above, senescence increases phosphorylation of S6 at all timepoints and in all conditions without alteration of underlying S6 protein levels. Interestingly, apigenin treatment was capable of significantly inhibiting S6 phosphorylation at 48 hours in non-senescent fibroblasts while qualitatively lessening S6 phosphorylation in senescent fibroblasts.

3.2.2.5. 4EBP1

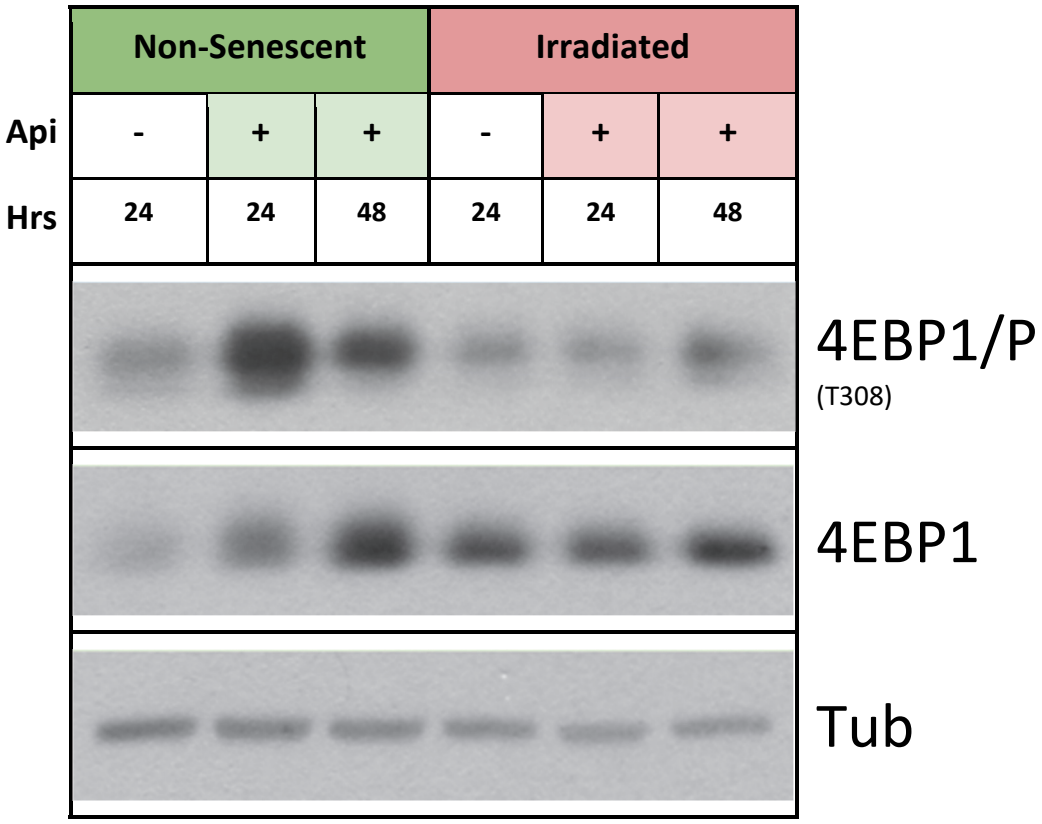


Figure 54: 4EBP1 phosphorylation of senescent IMR90 cells is blunted by apigenin.

Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) is a canonical repressor of protein translation in times of cellular stress. 4EBP1 acts opposite of S6K, wherein mTORC1 signaling leads to inhibition of 4EBP1 activity, thereby de-repressing eukaryotic translation initiation factor 4 (eIF4E), itself required for successful recruitment of the 40S ribosomal complex during translation. Therefore, inhibition of 4EBP1 via phosphorylation is a central means by which mTOR signaling increases cellular biosynthesis and proliferation (Chao et al. 2011). However, as senescent cells do not cease biosynthesis but do exhibit decreased 4EBP1, it was critical to examine the effect of apigenin on 4EBP1 over time.

In the results above, apigenin treatment significantly increases 4EBP1 phosphorylation, and thus inhibition, in non-senescent cells while exhibiting only a qualitative time-dependent increase in senescent cells. This suggests that apigenin treatment may negatively regulate overall protein biosynthesis and thus account for differences in SASP factor secretion. However, given results in S6, S6K, and AKT signaling above, wherein apigenin treatment reduces pro-proliferative effects, it may be the case that apigenin treatment only exerts an indirect effect on

4EBP1 and overall protein biosynthesis while its primary mechanism lies with NFκB activation. Indeed, activation of NFkB is also associated with increased protein biosynthesis to cope with increased cellular secretion of cytokines (Blackwell and Christman 1997), so by acting to restrict NFκB activation and subsequent upregulation of protein biosynthesis, apigenin's mechanism may inhibit the SASP at multiple levels simultaneously.

3.2.2.6. GSK3B/A-P

GSK-3 has been identified as a kinase regulating over forty different proteins in a variety of different pathways (Jope and Johnson 2004). It is a unique kinase in that its phosphorylation can decrease, rather than increase, its activity. Specifically, phosphorylation of GSK-3 at serine-9 in GSK-3β or serine-21 in GSK-3α significantly decreases the availability of the ATP-binding pocket and reduces GSK3 activity towards its targets.

When apigenin reduces phosphorylation at these residues, one can assume that the the activity of GSK3 could increase, however, GSK3 targets need to be "primed" first by having a serine residue four positions c-terminal to the target residue phosphorylated. This priming phosphorylation allows a positively charged region on GSK3 to bind the priming phosphate group in order for GSK3 to become competent of phosphorylating its myriad targets.

Due to the necessity for a priming phosphorylation, just increasing binding pocket availability would not be enough for GSK3 to phosphorylate its downstream targets and thus one cannot assume that the pathway would be upregulated unless those priming phosphates were present. It is possible that whatever priming phosphorylation existed would be maintained, and the activity of GSK3 would then increase. However, it is perhaps more probable, given that apigenin generally reduces phosphorylation, that phosphorylation at those sites would be also downregulated and thus it would be hard to predict the actual biological effect of apigenin in this pathway, in contrast to the IL-1A pathway where inhibition of phosphorylation throughout the pathway results in downregulation of transcription of NFκB targets.

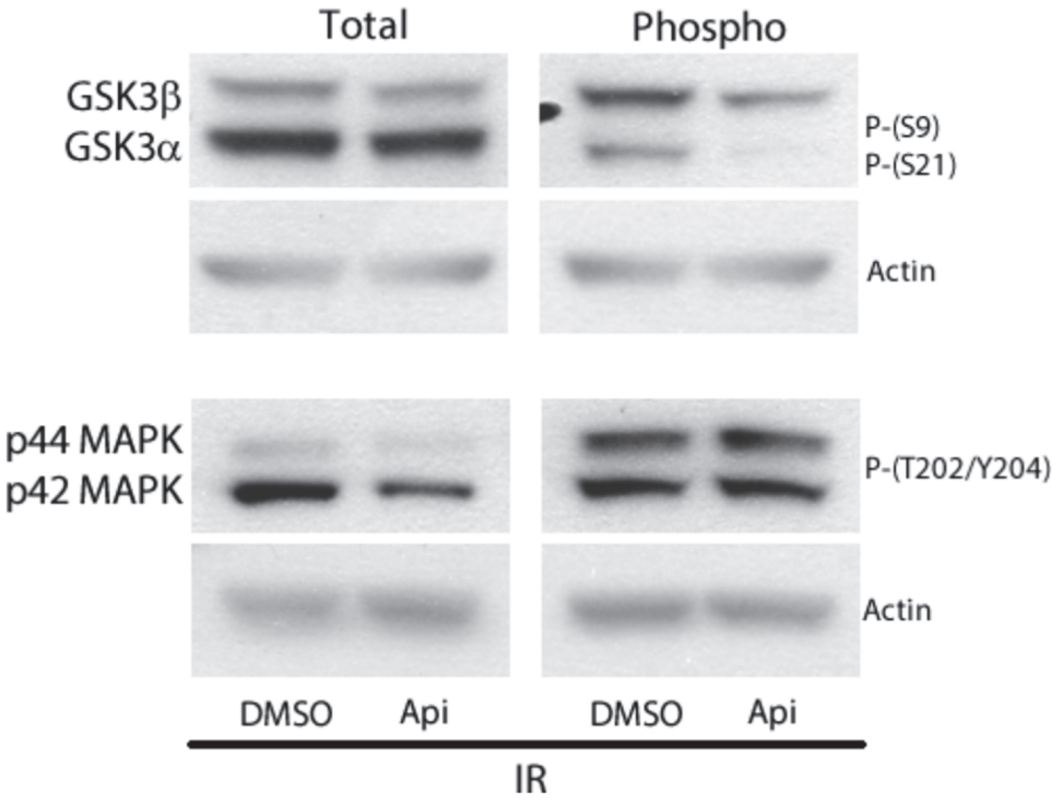


Figure 55: Phosphorylation of GSK3a/b but not p44/42 is reduced in senescent IMR90 fibroblasts

Glycogen synthase 3 beta and alpha (GSK3a and GSK3b) are serine/threonine kinases involved in the regulation of 40+ proteins involved in innate immunity, metabolism, and cell proliferation (Doble and Woodgett 2003). Upon growth factor withdrawal, GSKs bind to and inhibit the activity of cyclin kinases that promote cell proliferation, thereby arresting cell cycle progression (Wang et al. 2011). However, unlike most kinases, GSKs require phosphorylation via beta-catenin or other signaling factors at the C-terminal end, thereby exposing its active sites for further function. GSKs act in concert with the MAPK pathway regulating cell proliferation, where phosphorylation state dictates activity. Therefore, the effects of apigenin treatment on GSK and MAPK constituents, as well as their phosphorylation state was investigated.

As shown above, apigenin treatment had little effect on overall levels of GSK3a, GSK3b, p44-MAPK, or p42-MAPK. However, the levels of phosphorylated p44-MAPK were greatly increased in both DMSO- and apigenin-treated cells, indicative of increased cytokine signaling and cellular activation. Apigenin treatment (far right column) resulted in a significant reduction in GSK3A phosphorylation, suggesting inhibition of mitogenic signaling, with a qualitative reduction in the same for GSK3B phosphorylation, thus bolstering these effects. This

demonstrates that apigenin may reduce activation in the GSK3 pathway, which is involved in the pathogenesis of diverse chronic inflammatory disorders, such as Alzheimer's or atherosclerosis disease. This suggests that apigenin inhibition of GSK3, and its wide-ranging anti-SASP effects, may be an effective intervention in progressive degenerative disorders associated with the accumulation of senescent cells (Jope et al. 2007).

3.3. Summary

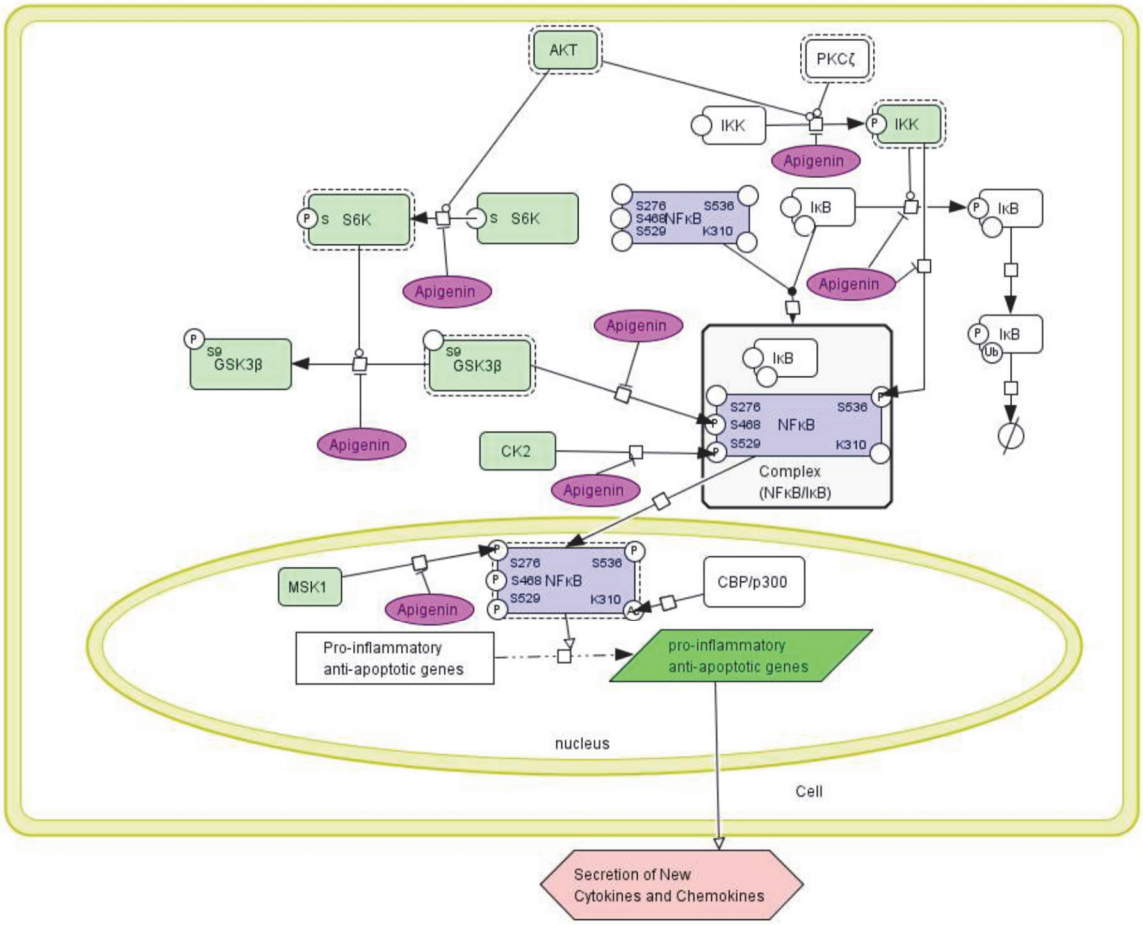


Figure 56: Summary figure of phosphorylation inhibition by apigenin

In summary, apigenin exerts inhibitory effects on multiple proteins whose activity is associated with NFκB activity, ranging from IKK to GSK3b. These broad inhibitory effects are consistent with the observed decrease in NFkB-associated SASP factors, such as IL-6 and many other cytokines. However, the breadth of these effects suggests that apigenin does not act via direct inhibition of each protein at its active site(s). Instead, it is proposed could suggest that

apigenin exerts its inhibitory effects via allosteric modulation of ATPase sites on each of the proteins surveyed or their binding partners. If this is borne out in future studies, it will confirm apigenin as a safe, pleiotropic anti-SASP therapeutic worthy of translational evaluation.

4. Chapter 4 - Biotica Library Screen

4.1. Introduction

The company, Biotica, created a library of polyketide compounds similar to rapamycin using a proprietary technology. They took naturally occuring polyketide compounds and modified them using bacterial enzymes to create new compounds with novel and/or significant activities. The company had an interest in testing them for biological activities in hopes of developing them into pharmaceutical candidates and, given the similarity to rapamycin, they approached the Buck Institute and the lab of Dr. Brian Kennedy, an expert on rapamycin and mTOR, to see if he was willing to help develop the intellectual property. The Buck Institute and the company had negotiated an agreement for work on their library of compounds that allowed their investigation, including how these compounds impact senescence and the SASP.

The library consisted of 267 compounds dissolved in DMSO at a concentration of 10μM split over four 96 well plates. As it turned out, many of the compounds were not actually proprietary, but were indeed natural polyketide compounds with known biological activities, interspersed with compounds which had been derived from them. During the testing phase the the identities of the compounds were blind to investigators. In some cases, although the identity was unknown, the general class of biological activity was already understood.

Experiments were designed that would allow for the testing of all of these compounds at once in a medium-throughput experiment that would allow the interrogation of the potential effects of these compounds on senescence. The experiments were designed to allow a reproducible 'in-plate' dilution scheme that preserved and conserved the precious compounds in the source plate while reducing the number of times they were warmed while also ensuring that each refresh was as similar as possible to the previous over the course of the experiment. Unlike many other pharmaceutical assays, which look for effects over short periods of time, this experiment was designed to handle multiple replicates over the length of the the evolution of senescent-phenotype while remaining efficient about use and exposure.

4.2. Methods

4.2.1. Timeline

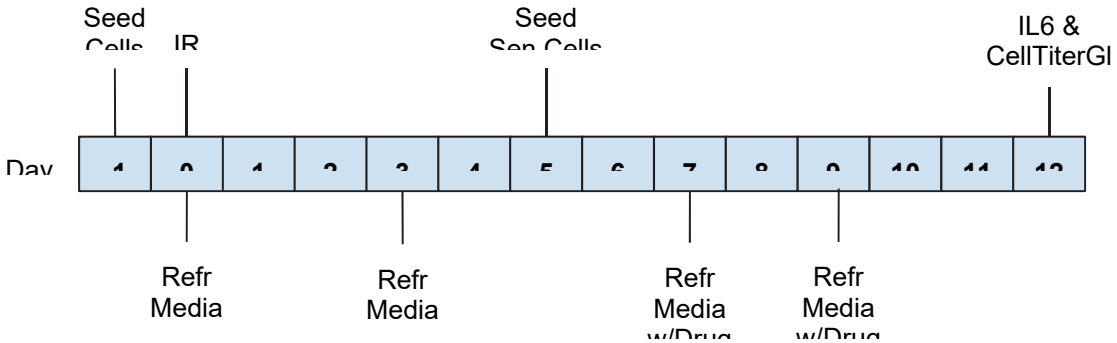


Figure 57**: Timeline for Biotica Experiment**

Day -1 - Seed Four T75s with 2M HCA2 Fibroblasts in 10% FBS

Day 0 - IR 10 Gy - refresh media

Day 1 - Incubation

Day 2 - Incubation

Day 3 - refresh media

Day 4 - Incubation

Day 5 - Seed senescent cells into 96 well plates @ 20k / well

Day 6 - Incubation

Day 7 - Refresh media with Drug

Day 8 - Incubation

Day 9 - Refresh media with Drug

Day 10 - Incubation

Day 11 - Incubation

Day 12 - Collect CM and perform IL6 AlphaLisa and CellTiterGlo

4.2.2. Set-Up

Ultimately the final approach was designed to be performed by hand with a multichannel pipette whereas the Prestwick screen, had been done on a robot. There were four source plates containing compounds.

Due to limited resources, it was decided that having two biological replicates of the treatment would be good enough to identify compounds with robust effects and the format of the experiment plate is shown below. The outer wells of the plate were filled with PBS to reduce edge effects and DMSO and Rapamycin (12.5 nM) were chosen as negative and positive controls for the first and last columns of each plate to provide six replicates per plate. The interior wells were used for treatments with the Biotica compounds. Duplicates were arranged such that rows B and C , D and E, as well as F and G were identical to produce the treatment plate format below.

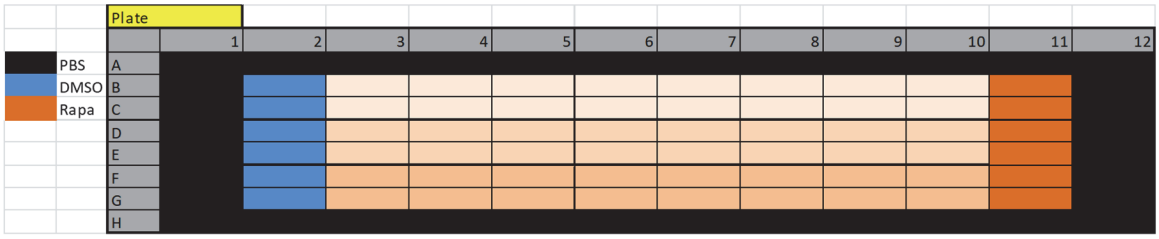
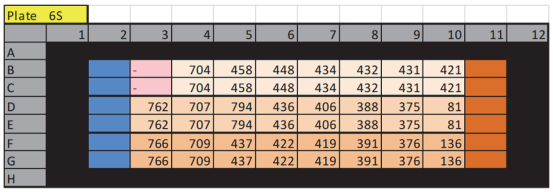
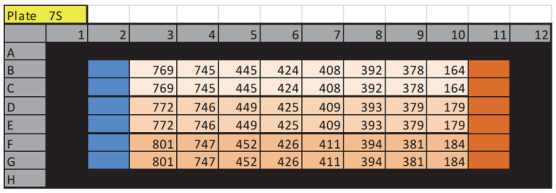


Figure 58: Sample Treatment Plate Format

In order to make refreshing the plate as simple as possible the eight vertical rows of the source plate were mapped onto the destination plate by rotating the source plate 90 degrees clockwise, thus Plate 4 of the Biotica Library below gives rise to four treatment plates (6S-9S).

Plate	4											
	1	2	3	4	5	6	7	8	9	10	11	12
A	421	81	136	164	179	184	185	188	274	343	371	374
B	431	375	376	378	379	381	382	384	385	386	390	387
C	432	388	391	392	393	394	395	397	398	400	404	405
D	434	406	419	408	409	411	412	413	415	416	417	418
E	448	436	422	424	425	426	427	428	429	430	791	435
F	458	794	437	445	449	452	455	456	690	693	705	706
G	704	707	709	745	746	747	748	749	753	754	755	761
H	-	762	766	769	772	801	407	433	792	793	795	796
	Plate 6S			Plate 7S			Plate 8S			Plate 9S		





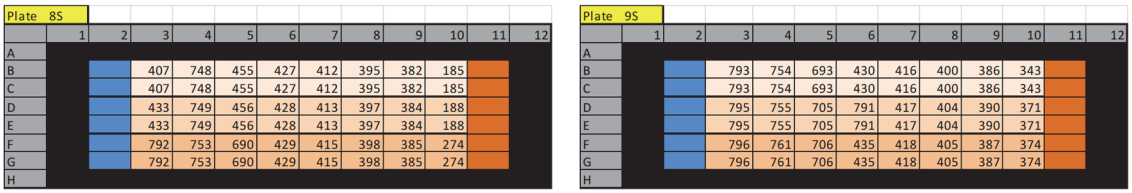


Plate 8S	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			407	748	455	427	412	395	382	185		
C			407	748	455	427	412	395	382	185		
D			433	749	456	428	413	397	384	188		
E			433	749	456	428	413	397	384	188		
F			792	753	690	429	415	398	385	274		
G			792	753	690	429	415	398	385	274		
H												

Plate 9S	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			793	754	693	430	416	400	386	343		
C			793	754	693	430	416	400	386	343		
D			795	755	705	791	417	404	390	371		
E			795	755	705	791	417	404	390	371		
F			796	761	706	435	418	405	387	374		
G			796	761	706	435	418	405	387	374		
H												

Figure 59: Example of source plate of compounds and resulting assay plates.

4.2.3. Media Refreshing

Each well was refreshed with a volume of 150ul of standard media. To this volume was added 50ul of media containing a 4x concentration of the drug to eventually arrive at a total media volume of 200ul of media containing 1x drug concentration. This allowed for the preparation of aliquants to take into account systematic pipetting error.

The above allowed, from one single thaw of the source plate, the use of enough drug for the entire experiment according to the dilution strategy outlined below. This conserved precious source compound and limited their freeze-thaw exposure and also provided for the easy mapping of the source plate through the multiple dilution plates that would be required to ultimately map the destination plates in which the cells were actually being treated.

The final collection would be the standard media (DMEM+10%FBS+PS) instead of serum-free media as would be normal in a larger format assay. The reasons for was the endpoint measurement was IL-6 secretion, which was shown not to be dependent on the use of serum-free media. Further, the use of use of serum-free media mitigated the confounding influence of factors in FBS that could interfere with assays for analytes other than IL-6.

Another consideration which drove the dilution strategy used was a need to at least generally compare results from this screen to the results of the Prestwick Screen. A final concentration of 2µM for all drugs in the Biotica library was chosen as the screening concentration based on personal communication with Remi Martin-Laberge.

4.2.3.1. Dilution Strategy

The dilution strategy required the preparation of enough media from one freeze-thaw cycle from a 10mM stock plate for two media refreshes of 50ul of 8µM compound concentration. It needed to use serial dilutions of multiple compounds in parallel with the final destination being a plate containing over 200ul of compound-containing media per well to refresh two sample wells that contain 150ul of standard media. Efficiency was gained using 4x

containing media delivered from 250ul final volume in a V-Bottom 96-well plate with a maximum of 320ul per well. The serial dilutions as shown below.

4.2.3.2. Drug Preparation

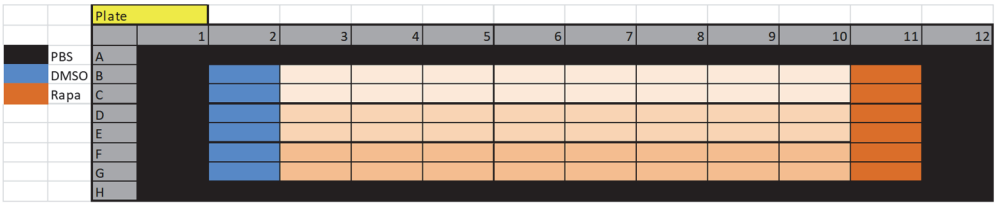
- **First dilution - "Dilution 1" - Cf = 250 ul of 40µM in V-bottom plate**
    - Calculations
        - Ci*Vi=Cf*Vf
        - 10000*1=Cf*250
        - 10000 µM * 1 uL/ 250 uL = 40µM ==> Vi * 40µM = 2µM * 1ml ==> 2/40=0.05
    - Add 200 ul of media to each well
    - Add 1 ul of each sample to each well
    - Add final 50 ul of media to make 40µM sol'n and help mix the 1ul
    - (An alternative would have been to put the 1ul in the plate and add the full 250ml, but with such a small volume, it was felt that delivery into fluid might prevent any adherence of compound to plastic)
- **Second Dilution - "Dilution 2" - Cf = 200 ul of 8µM**
    - Calculations
        - 40µM * Vi = 8µM * 200ul
        - Vi= 200*8/40 = 40ul dil1 + 160ul of media
    - Need to make 200 ul of 8µM in a second V-Bottom plate
    - Add 160ul of media to each well of a 96-well V-Bottom plates.
    - Add 40ul from Dilution 1 to each well using a multichannel pipette
- **4x Rapa and DMSO Controls**
    - Will need to be added to "Dilution 2-1" and "Dilution 2-2" for 200ul / well
    - Need 1.2 ml/ plate * 4 plates = 4.8 ml of 4x solution - make 6 ml
    - Ci * Vi = Cf * Vf
    - 12500 nM * Vi = 50nm * 6000ul
    - 50*6000/12500=24ul 12.5µM Rapa and DMSO into 6ml of media

4.2.3.3. Steps

1. **Rapamycin and DMSO Control Solutions**

1. Put 6ml media into 2-15 ml falcon tubes
2. Add 24ul of 12.5 µM rapamycin to one and 24 ul of DMSO to the other

1. **Preparation of Dilution Plates**
    1. Label one plate "Dilution 1" - it will match the format of the drug source plate in 1-1 correspondence
        1. Fill each well with 200ul of DMEM
    2. Label another two plates "Dilution 2-1" and "Dilution 2-2" which will take on the format of the destination sample plates, except that each row is not repeated in duplicate and will serve to dilute two rows of the destination plate.
        1. Fill each well A3:H3-10 with 160ul media for Dilution 2-1 and A3:D3-10
        2. Fill wells with PBS



3. **Preparation of Dilution 1**
    1. Dilution 1 plate has a 1-1 correspondence with drug plate
    2. With multichannel pipette fill each with 200ul of DMEM + FBS/ps
    3. Thaw drug plate # 4, ensure you have a sterile replacement foil
    4. Using multichannel add 1ul of each drug to each well
    5. Cover drug plate with sterile replacement foil, place in -20 immediately
    6. Add 50ul of media to each well to help homogenize the solution for a total volume of 250ul
4. **Preparation of Dilution 2**
    1. Add 200 ul of DMSO control to A2:H2 and Rapa control to A10:H10 of "Dilution 2-1" and "Dilution 2-2"
    2. To "Dilution 2-1" add 160 ul of media to wells A3-10:H3-10 with multichannel pipette
    3. To "Dilution 2-2" add 160 ul of media to wells A3-10:D3-10 with multichannel
    4. Using a multichannel pipet, transfer 40ul from Dilution 1 so that samples A1:H1 are transposed along

5. A3-A10, pipet up and down once to mix, dispose tips and repeat for each column of Dilution 1 into the
6. corresponding rows of "Dilution 2-1" and "Dilution 2-2"

5. **Application of Samples to Plates 6S-9S**
    1. In groups of two plates aspirate the media to minimize plate drying time
    2. Add 150 ul of DMEM to each sample well
    3. Using a multichannel pipet, transfer 50 ul of A2-10 from "Dilution 2-1" into well B2-10 of plate 6S and create a
    4. duplicate by taking another 50ul from A2-10 of "Dilution 2-1" and transfer into wells C2-10.
    5. Repeat for each sample from Dilution 2 plates.

4.3. Results

4.3.1. Overview

All four source plates containing 241 compounds were tested on senescent HCA2 primary fibroblasts, measuring IL-6 secretion and cell viability via luminescence. The results are in the graph below which is meant to give one an idea as to the variability of the results.

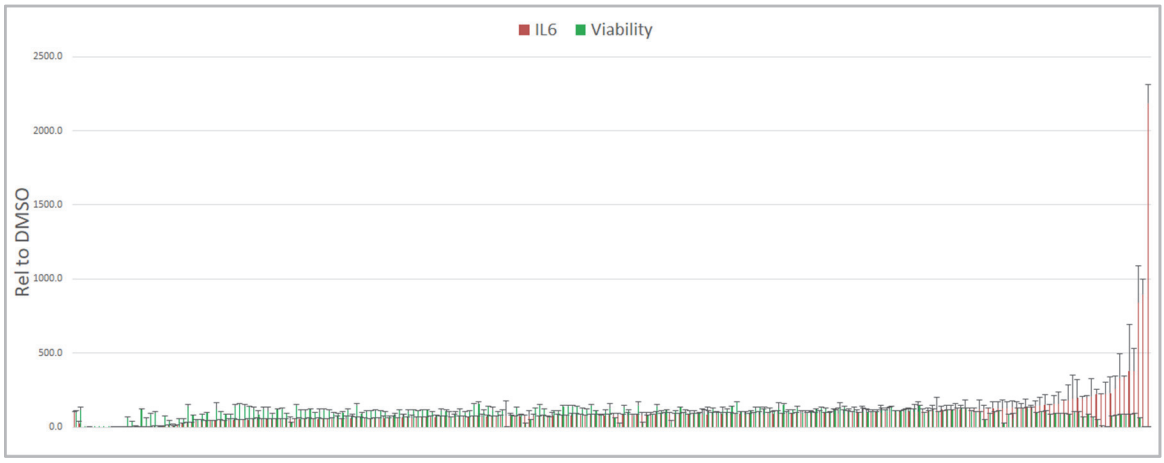


Figure 60: Percent IL-6 secretion and viability (luminescence) of Biotica Compound-treated senescent HCA2 primary fibroblasts compared to DMSO-treated cells.

There were compounds that lowered IL-6 by being toxic to the cells and there were some that lowered IL-6 without overt toxicity. The focus of this research was on compounds

that were able to lower IL-6 without killing normal cells. In future work compounds that killed senescent cells over non-senescent cells will be investigated to determine if there might be any differential toxicity indicating the compound could function as a senolytic.

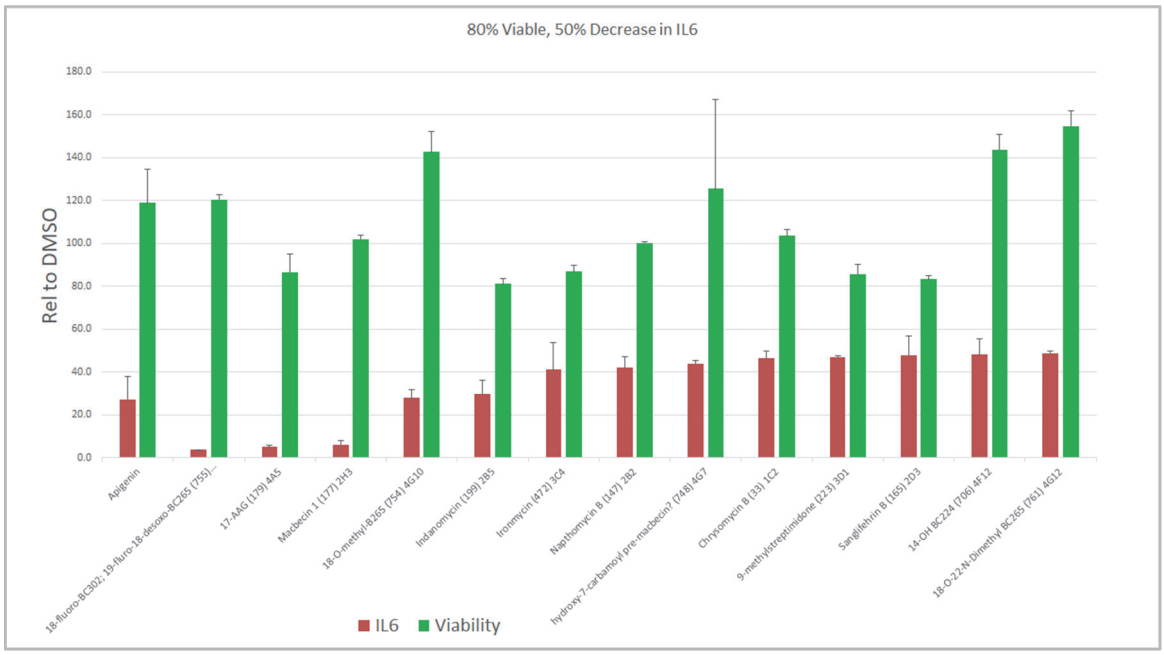


Figure 61: Biotica library compounds able to reduce IL6 with low toxicity

Column1	IL6	Viabilit	il6 std	via std
Apigenin	27.2	118.9167	10.80859	15.87689
18-fluoro-BC302; 19-fluro-18-desoxo-BC265 (75	3.8	120.3422	0.016246	2.466465
17-AAG (179) 4A5	5.0	86.54045	0.990088	8.650499
Macbecin 1 (177) 2H3	6.2	102.0826	1.988213	1.901547
18-O-methyl-B265 (754) 4G10	27.8	142.6358	3.803377	9.544145
Indanomycin (199) 2B5	29.9	81.05579	6.042065	2.658925
417 (417) 4D11	40.1	125.0183	6.964699	1.215359
Ironmycin (472) 3C4	41.1	86.87461	12.66119	3.110934
Napthomycin B (147) 2B2	42.0	100.1812	5.220051	0.472516
not spec (705) 4F11	42.4	160.7335	1.104706	3.967791
791 (791) 4E11	43.6	132.7528	4.080229	0.786409
400 (400) 4C10	43.6	118.6993	2.108402	11.36718
hydroxy-7-carbamoyl pre-macbecin? (748) 4G7	43.9	125.5466	1.358188	41.82266
409 (409) 4D5	44.1	88.94169	0.388764	1.46558
795 (795) 4H11	44.5	146.9327	0.311406	4.468233
386 (386) 4B10	45.2	133.3847	1.104497	4.468233
390 (390) 4B11	45.5	120.4686	1.609615	1.858785
Chrysomycin B (33) 1C2	46.4	103.4086	3.523534	2.88451
9-methylstreptimidone (223) 3D1	47.0	85.81292	0.716853	4.503583
404 (404) 4C11	47.3	116.3233	4.073842	2.50221
BC556 (693) 4F10	47.5	135.4574	3.707782	16.90779
Sanglifehrin B (165) 2D3	47.8	83.23566	9.113287	1.639859
14-OH BC224 (706) 4F12	48.2	143.6468	7.278975	7.041935
18-O-22-N-Dimethyl BC265 (761) 4G12	48.7	154.7431	0.88956	7.292156
371 (371) 4A11	49.0	123.704	4.228236	1.358343
430 (430) 4E10	49.3	130.756	6.116468	13.54768
387 (387) 4B12	49.5	106.7437	5.353269	4.468233

Table 1: Numeric data of compounds in Figure 61

The data above shows selected values for relative suppression of IL-6 secretion of the compounds tested in the table to the left. These are the compounds that retained 80% or greater viability while also delivering at least a 50% decrease in IL-6 secretion.

What the screen revealed was that the natural compound Macbecin and some other compounds were able to dramatically suppress IL-6 secretion without demonstrating overt toxicity to senescent cells, marking these compounds as potentially potent anti-inflammatories.

4.3.2. HSP90 Inhibitors

Although their identity was kept secret, some of the early hits that were identified from Plate #4 were revealed to be HSP90 inhibitors. These compounds were able to reduce IL-6 effectively without high toxicity to senescent cells.

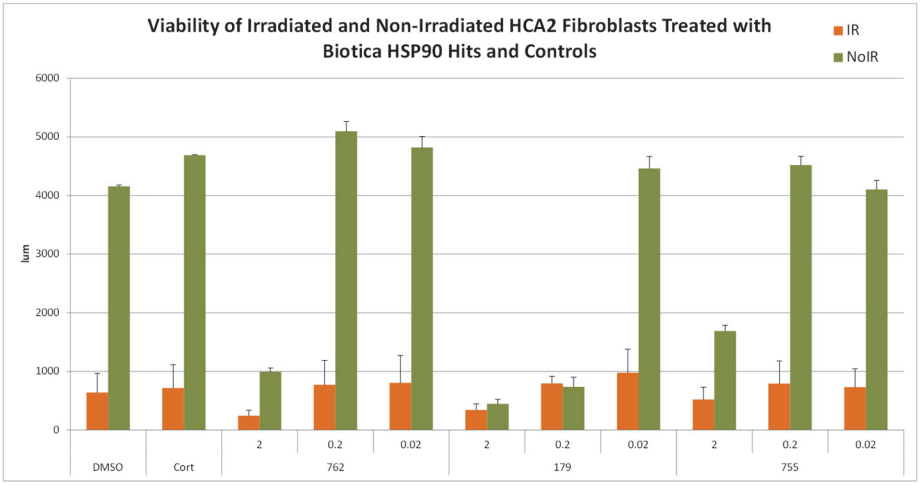


Figure 62: Unknown compounds classified as HSP90 inhibitors reduce IL6

These data showed that several HSP90 inhibitors, such as compounds 762, 179, and 755, were capable of reducing IL-6 secretion in a dose-dependent fashion to a similar degree as cortisone.

4.3.3. Other Polyketide SASP Inhibitors

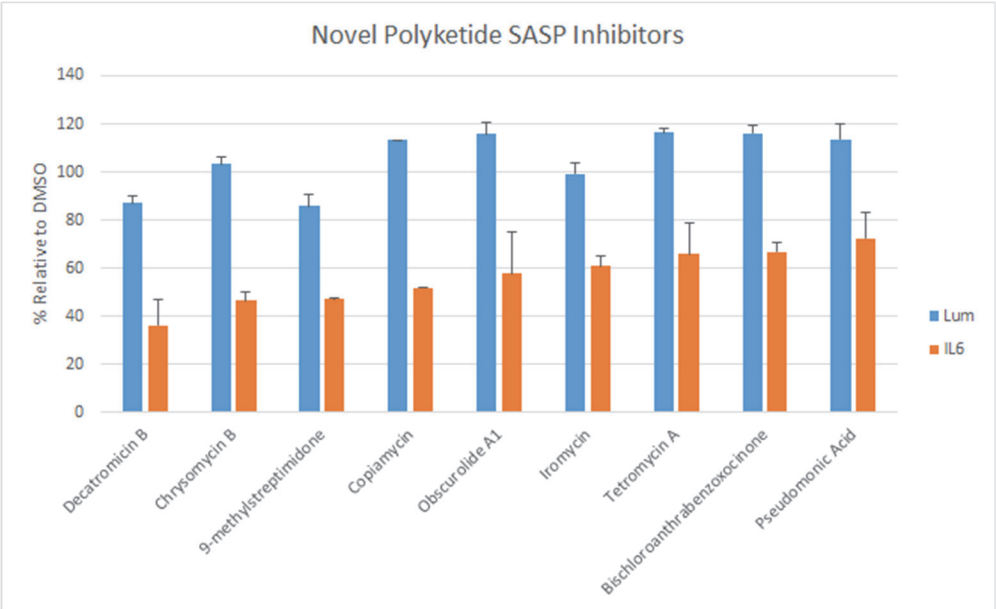


Figure 63: Non-HSP90 inhibitors of IL6 secretion

Polyketides are a diverse class of biological active secondary metabolites produced and utilized in a wide range of living organisms. The Biotica library screen included several polyketide compounds that exhibited potent anti-SASP effects as shown above, where many polyketides significantly reduced IL-6 secretion in senescent HCA2 fibroblasts. While some of these compounds, such as iromycin A or pseudomonic acid, have previously been characterized in other contexts in the literature, many of these compounds have had little investigation and thus this result is an exciting finding for potential anti-SASP therapeutics to complement apigenin.

4.3.4. Possible Senolytics

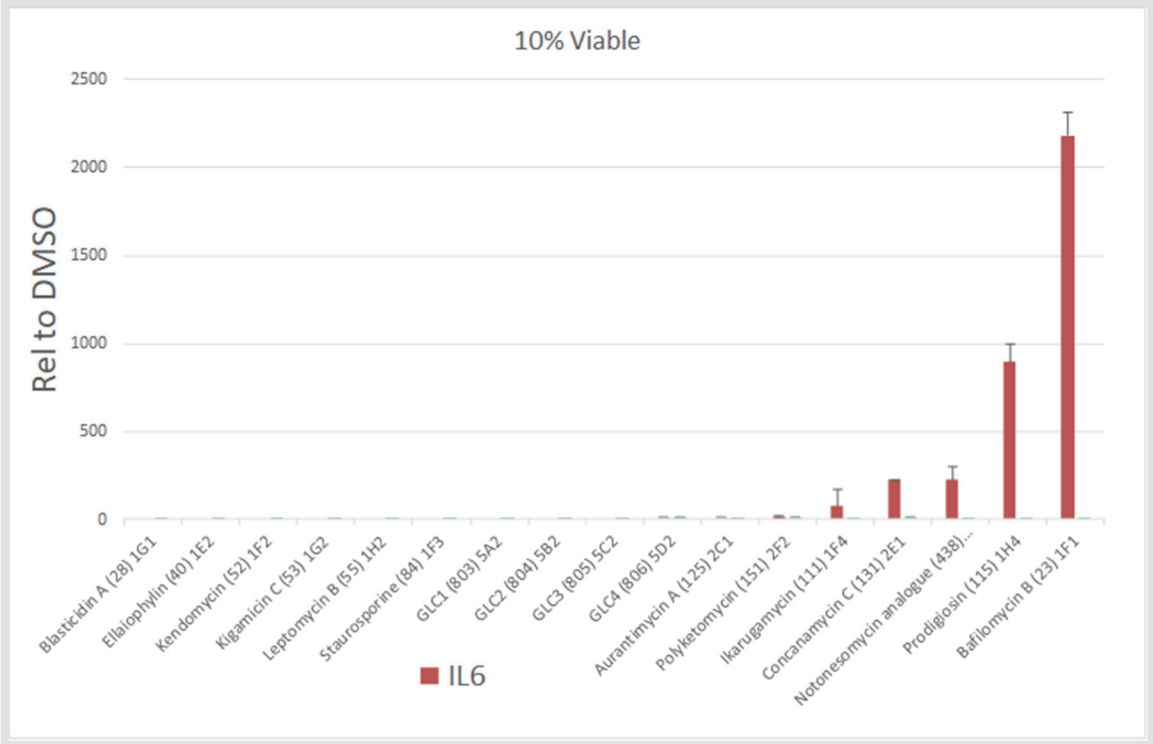


Figure 64: Biotica library potential senolytics

Several of the Biotica compounds, such as Concanamycin C and Bafilomycin B, exhibited cytotoxicity in senescent HCA2 fibroblasts. Although additional study will be necessary to determine whether these compounds exhibit differential toxicity in senescent vs non-senescent cells, these initial results are promising.

Column1	IL6	Viabilit	il6 std	via std
Blasticidin A (28) 1G1	0	0.048033	0	0.129231
Ellaiophylin (40) 1E2	0	0.528366	0	0.162368
Kendomycin (52) 1F2	0	0.346777	0	0.021539
Kigamicin C (53) 1G2	0	0.052719	0	0.079527
Leptomycin B (55) 1H2	0	0.292886	0	0.034793
Staurosporine (84) 1F3	0	0.276484	0	0.107693
GLC1 (803) 5A2	0	0.328556	0	0.43512
GLC2 (804) 5B2	0	0.128725	0	0.63135
GLC3 (805) 5C2	0	0.129951	0	0.48631
GLC4 (806) 5D2	3.01498068	4.308009	4.263827	2.004964
Aurantimycin A (125) 2C1	6.29564471	1.779648	1.361581	0.344543
Polyketomycin (151) 2F2	13.0282947	4.79948	5.396743	2.24609
Ikarugamycin (111) 1F4	72.8078018	0.381277	102.9658	0.37881
Concanamycin C (131) 2E1	224.756587	5.873766	1.408758	0.674319
Notonesomycin analogue (438) 3G3	225.958587	1.76121	77.43981	0.430292
Prodigiosin (115) 1H4	897.078888	1.240357	99.2223	0.303731
Bafilomycin B (23) 1F1	2183.64321	1.351961	129.8562	0.008284

Table 2: Numbers for Figure 64

This table summarizes the IL-6 and cell viability results presented above, showing that many Biotica compounds had potent anti-IL-6 effects without significantly affecting cell viability while other compounds exhibited clear cytotoxic effects without significant anti-IL-6 activity.

4.3.5. Cell-Type Specific Differences in Viability to Some Compounds

Viability of BJ and HCA2 was within 20% of each other in >70% of compounds tested. Some differed substantially.

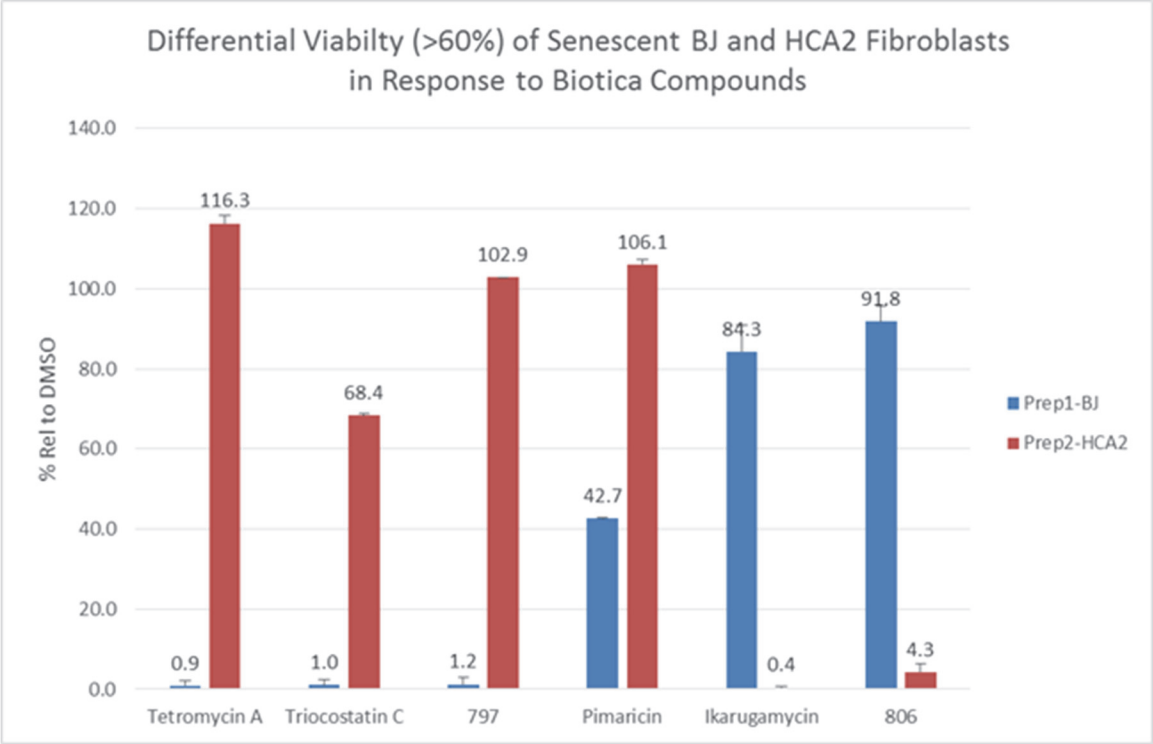


Figure 65: Interesting differential toxicity of compounds towards different cell lines

Several of the Biotica compounds exhibited differential cytotoxicity in BJ vs HCA2 irradiated fibroblasts, indicating that there may be cell line-specific differences in each compound's effects. Of these, the compounds above exhibited the greatest differential effects in BJ vs HCA2 cell types, indicating that they may be excellent candidates for differential cytotoxicity in senescent cells worth further study.

5. Chapter 5: Apigenin Reduces Breast Cancer Cell Aggressiveness by Suppressing the Secretory Phenotype of Senescent Cells

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5.1. ABSTRACT

Apigenin (4',5,7,-trihydroxyflavone) is a flavonoid found in certain herbs, fruits and vegetables. Apigenin can attenuate inflammation, which is associated with many chronic diseases of aging. Senescent cells – stressed cells that accumulate with age in mammals -- display a pro-inflammatory senescence-associated secretory phenotype (SASP) that can drive or exacerbate several age-related pathologies, including cancer. Flavonoids, including apigenin, were recently shown to reduce the SASP of a human fibroblast strain induced to senesce by bleomycin. Here, we confirm that apigenin suppresses the SASP in three human fibroblast strains induced to senesce by ionizing radiation, constitutive MAPK (mitogen-activated protein kinase) signaling, oncogenic RAS or replicative exhaustion. Apigenin suppressed the SASP in part by suppressing IL-1a signaling through IRAK1 and 4, p38-MAPK and NF-kB. Apigenin was particularly potent at suppressing the expression and secretion of CXCL10 (IP10), a newly identified SASP factor. Further, apigenin-mediated suppression of the SASP substantially reduced the aggressive phenotype of human breast cancer cells, as determined by cell proliferation, extracellular matrix invasion and epithelial-mesenchymal transition. Our results support the idea that apigenin is a promising natural product for reducing the impact of senescent cells on age-related diseases such as cancer.

5.2. KEYWORDS

Flavonoids; human fibroblasts; proliferation; invasion; IL-6; IL-1A; IRAK1/4; NF-kB

5.3. INTRODUCTION

Aging is the largest risk factor for a host of age-related diseases, both degenerative and hyperplastic, raising the possibility that interventions into basic aging processes hold promise for ameliorating multiple age-related diseases, including cancer (Kennedy, Berger et al. 2014). Nine basic aging processes – or hallmarks of aging – were recently defined as targets for interventions

to extend the health span (years of healthy aging) and, possibly the life span, of mammalian organisms (López-Otín, Blasco et al. 2013). Among these hallmarks is cellular senescence.

Cellular senescence is a multifaceted stress response adopted by cells that have the ability to undergo cell division (Campisi 2013). The senescence response entails an essentially irreversible arrest of cell proliferation, coupled to a complex senescence-associated secretory phenotype (SASP) that includes numerous cytokines, chemokines, growth factors and proteases (Coppe, Patil et al. 2008). The growth arrest is a potent tumor suppressive mechanism. However, because senescent cells accumulate with age, their chronic presence – most likely through the SASP – can drive age-related pathologies, including, ironically, cancer (Campisi 2013, Muñoz-Espín and Serrano 2014, Baker, Childs et al. 2016). This activity of senescent cells is likely attributable to the pro-inflammatory nature of the SASP (Freund, Orjalo et al. 2010, Lasry and Ben-Neriah 2015).

To identify novel SASP regulators and small molecules capable of suppressing the SASP, we screened a library of FDA-approved drugs for ability to suppress the secretion of IL-6, a robust and common SASP component (Coppe, Patil et al. 2008, Coppe, Patil et al. 2010) without compromising cell viability (Laberge, Zhou et al. 2012). This screen identified glucocorticoids as SASP inhibitors. However, a number of other candidates emerged from the screen that warranted further investigation. Among these candidates was apigenin (4',5,7,-trihydroxyflavone; MW: 270.24), a naturally occurring flavonoid present in several plants and having little or no toxicity when obtained from the diet (Ross and Kasum 2002, Shukla and Gupta 2010).

Apigenin belongs to the flavone subclass of flavonoids (Kim 2003, Osada, Imaoka et al. 2004) and is abundant in a variety of fruits, vegetables and herbs. It often exists in food sources as a glycoside, which improves its solubility and bioavailability (Ross and Kasum 2002). Like other flavonoids, apigenin has a variety of biological activities, including the ability to inhibit proliferation and induce apoptosis in several cancer cell lines (Reiners, Clift et al. 1999, Gupta, Afaq et al. 2001, Way, Kao et al. 2004, Brusselmans, Vrolix et al. 2005), as well as an ability to inhibit angiogenesis (Kim 2003, Osada, Imaoka et al. 2004). Targets of apigenin that could contribute to these anti-cancer activities include heat shock proteins (Osada, Imaoka et al. 2004), fatty acid synthase (Brusselmans, Vrolix et al. 2005), the aryl hydrocarbon receptor (Reiners, Clift et al. 1999), HER2/neu (Way, Kao et al. 2004), and matrix metalloproteinases (Kim 2003). Apigenin can also reduce markers of inflammation in response to lipopolysaccharides,

TNF-a and allergens, and was shown to suppress activities of the inflammatory mediators cyclooxygenase and NF-kB (Liang, Huang et al. 1999, Ruiz and Haller 2006, Li, Pang et al. 2010, Kang, Lee et al. 2011, Duarte, Arango et al. 2013).

Apigenin was recently shown to reduce the secretion of several SASP factors in a human fibroblast strain induced to senesce by the clastogen bleomycin via the IRAK1/IkBa signaling pathway (Lim, Park et al. 2015). To determine how broadly apigenin suppresses the SASP, we analyzed its effects using several human fibroblast strains and senescence inducers, multiplex ELISAs and several indicators of IL-1/IRAK/NF-kB signaling. Importantly, apigenin suppressed the ability of senescent fibroblasts to stimulate aggressive phenotypes of human breast cancer cells, supporting the idea that apigenin has potential as an anti-cancer agent.

5.4. MATERIALS AND METHODS

5.4.1. Cell culture

Except where noted otherwise, primary human primary fibroblasts (IMR90, BJ, HCA2) were seeded at 10,000 cells/cm^2 and incubated at 37˚C in a 3% O_2/10% CO_2 atmosphere and Dulbecco's Modified Eagle Medium (DMEM) (VWR, Cat# 45000-312) supplemented with glucose (4.5 g/l), glutamine (4 mM), 10% fetal bovine serum (FBS) and antibiotics (100 I.U. penicillin, 100 µg/ml streptomycin). Media were replaced every 48 hrs. HCA2 cells were used because of their higher SASP when compared to other fibroblast cell strains. The other two cell strains were used to confirm and extend our results in a different tissue type and gender. Human breast cancer cells (MDA-MB231, ZR75.1) were cultured under standard conditions in the same medium in 20% oxygen.

5.4.2. Induction of senescence by ionizing radiation and apigenin treatment of cells

Human fibroblasts were seeded and incubated overnight. The following day (day 0), cells were induced to senesce by ionizing irradiation (10 Gy X-ray), and immediately given media containing DMSO or apigenin, and incubated. Non-senescent cells were mock-irradiated (they were handled in exactly the same way as the irradiated samples except for not being exposed to irradiation), then given serum-free media containing DMSO or apigenin, and incubated. 24 hrs

later (day 1), conditioned media were collected from non-senescent cells; senescent cells were left to incubate, with media replaced every 48 hrs. On day 9, senescent cells were given serum-free media with DMSO or apigenin, and conditioned media were collected 24 hrs later.

5.4.3. Quantification of IL-6 in conditioned media

IL-6 levels in conditioned media were quantified using the Perkin-Elmer AlphaLISA protocol (Cat# AL322F).

5.4.4. SA-β-gal assay

Senescence-associated beta-galactosidase (SA-β-gal) activity was determined using the BioVision Senescence Detection Kit (Cat# K320-250) protocol. For each experiment, approximately 100-150 cells were counted.

5.4.5. EdU proliferation assay

DNA synthesis was assessed by incorporation of EdU and visualization by fluorescence microscopy using the Invitrogen Click-iT Edu HCS Assay (Cat# C10350/C10351) protocol. For each experiment, approximately 100-150 cells were counted.

5.4.6. Invasion assay

MDA-MB231 cells (80,000 cells/well) were plated atop a layer of Matrigel in the upper chambers of Transwells (BD Biosciences). The lower chambers were filled with conditioned media (lacking apigenin) from non-senescent or senescent HCA2 fibroblasts previously treated with DMSO or apigenin for 10 days. After 18 hrs, cells that migrated to the underside of the upper chamber filter were stained and counted by light microscopy, as described (Coppé, Desprez et al. 2010).

5.4.7. H-RASV12 and MKK6EE-induced senescence

HCA2 and BJ fibroblasts were infected with a control lentivirus (L3P) or lentiviruses expressing H-RAS^{V12} or MKK6EE, and selected as described (Coppe, Patil et al. 2008, Freund, Patil et al. 2011). After selection, infected cells were reseeded and given media containing DMSO or apigenin (10 μM), and the media were refreshed after 48 hrs. After another 48 hrs, the cells

were given serum-free media containing DMSO or apigenin, conditioned media were collected 24 hrs later, and IL-6 levels were quantified by AlphaLISA, normalizing to cell number.

5.4.8. NF-kB reporter assay

HCA2 fibroblasts were infected with a lentivirus carrying a NF-kB-firefly luciferase reporter (SA Biosciences, Cat# CLS-013L) according to the supplier's protocol. After selection, the cells were seeded at 10,000 cells/cm^2, induced to senesce by X-irradiation, then given media containing DMSO or apigenin and cultured for 10 days, with media refreshed every 48 hrs. On day 8, non-senescent cells were seeded at 5,000 cells/cm^2. On day 9, all cultures were given serum-free media with DMSO or apigenin. 24 hrs later, cells were lysed and luciferase activity measured using the Promega Luciferase Assay System (Cat# E1500).

5.4.9. IL-1 alpha stimulation of cytokine secretion

Fibroblasts were seeded at 10,000 cells/cm^2 in two 12-well plates, irradiated to induce senescence, given media containing DMSO or apigenin, and incubated for 10 days. On day 8, two additional plates were seeded with non-senescent cells at 5,000 cells/cm^2 and incubated overnight. On day 9, non-senescent cells were given media containing DMSO or apigenin, and incubated for 48 hrs. On day 11, all the cultures were washed, given serum-free media containing DMSO or apigenin plus the indicated amounts of recombinant IL-1 alpha (R&D Systems, Cat# 200-LA-010), and incubated overnight. The following day, conditioned media were collected and analyzed for IL-6 using an AlphaLISA, normalizing to cell number.

5.4.10. Western blotting

Cell lysates (25 µg protein) were resolved using Life Technologies NuPage 4-12% Bis-Tris Gels (Cat# NP0336) and transferred onto PVDF membranes. The membranes were blocked using 5% BSA, and incubated with primary antibodies in blocking buffer overnight at 4˚C. The membranes were then incubated with the appropriate secondary antibody (BioRad Cat# 172-1011, anti-mouse; or #170-6515, anti-rabbit) followed by detection using a chemiluminescence ECL kit (Amersham LifeSciences Inc.). To control for protein loading, the membrane was stripped and probed with an antibody against actin or tubulin. Primary antibodies were as follows: p38 Thr180/Tyr182 phosphorylation – PhosphoSolutions, Cat# 190-1802; total p38 –

Cell Signaling, Cat# 9212; IRAK4 Thr345/Ser346 phosphorylation – Cell Signaling, Cat# 7652; total IRAK4 – Santa Cruz, Cat# SC34770; IRAK1 Thr387 phosphorylation – Cell Signaling, Cat# 4365; Actin – Chemicon International, Cat# MAB3128; ZO-1 – Santa Cruz, Cat# sc10804; Cytokeratin 18 – Santa Cruz, Cat# sc28284; Vimentin – Millipore, Cat# ab1620; Tubulin – Sigma, Cat# T8203.

5.4.11. Immunofluorescence

Cells were cultured in 8-well chamber slides, fixed in 4% formaldehyde (Sigma) for 10 min at 4° C and permeabilized in PBS + 0.5% Triton for 10 min at 4°C. Slides were blocked for 30 min in 4% goat serum (Invitrogen). Primary antibody (anti-ZO1 (described above) was diluted in blocking buffer at 1:100 dilution and incubated with cells for 1 hr at room temperature. Cells were washed, incubated with secondary antibody (Invitrogen AlexaFluor 488 goat:anti-rabbit, Cat# A-11008) 1:1000 for 30 min at room temperature, washed and mounted with Slow-Fade Gold (Molecular Probes). Images were acquired using an Olympus BX20 fluorescence microscope with Spotfire software (Diagnostics Instruments) and processed with Photoshop CS (Adobe).

5.4.12. Statistical analysis

Results presented are representative of at least three separate experiments using three biological replicates. We used the Student's T-test to compare the results from treated to untreated samples normalized to DMSO-treated non-senescent controls where applicable. * indicates significance of $p<0.05$; ** indicates significance of $p<0.01$; *** indicates significance of $p<0.005$.

5.5. RESULTS

5.5.1. Suppression of IL-6 expression upon apigenin treatment

Upon screening a library of FDA-approved compounds for ability to regulate the secretion of IL-6 (Laberge, Zhou et al. 2012), a prominent component of the human and mouse SASP (Coppe, Patil et al. 2008, Coppe, Patil et al. 2010), we identified apigenin as significantly more active than the vehicle (DMSO) control. Apigenin reduced IL-6 secretion by primary

human fibroblasts (HCA2, from neonatal foreskin) made senescent by ionizing radiation (IR; 10 Gy X-irradiation) to a greater extent than several other compounds in the library, with activity similar that of the most active compound in the library (corticosterone) (Fig. 1A).

We determined 10 μM to be the lowest dose at which apigenin maximally attenuated IL-6 secretion by senescent HCA2 fibroblasts (Fig. 1B), and showed a similar dose response for BJ fibroblasts (Fig. S1A), also from neonatal foreskin, as described (Lim, Park et al. 2015). We used 10 μM apigenin for subsequent experiments.

5.5.2. Apigenin does not cause apoptosis and moderately reduces fibroblast proliferation

To better understand how apigenin suppressed senescence-associated IL-6 secretion and determine whether it had deleterious effects on non-senescent cells, we asked whether it induced apoptosis or inhibited cell proliferation. Apigenin was reported to induce apoptosis of cancer cells (Jayasooriya, Kang et al. 2012). To determine whether this was the case for normal cells, we treated non-senescent and IR-induced senescent HCA2 cells with DMSO or 10 μM apigenin and assessed apoptosis by activated caspase-3 levels (Fig. 1C). Apigenin failed to increase caspase-3 activity in both cell types, whereas 1 μM staurosporine (positive control) increased activity both types of cells. In addition, 10 μM apigenin moderately reduced proliferation (Fig. 1D). We treated proliferating HCA2 cells with 0, 5, 10 and 20 μM apigenin for 5 days. Over this period, untreated cells increased in number approximately 4-fold, whereas 5, 10, and 20 μM apigenin reduced this cell number by approximately 5, 25, and 50 percent, respectively.

5.5.3. Apigenin does not affect SA-β-gal expression, cell morphology or growth arrest

In addition to arresting growth, senescent cells develop an enlarged morphology and express a neutral senescence-associated β-galactosidase (SA-β-gal) (Dimri, Lee et al. 1995). Apigenin did not significantly alter SA-β-gal expression by non-senescent or senescent populations of three human fibroblast strains (BJ, HCA2 as well as IMR-90 from female fetal lung) (Fig. 2A). In addition, apigenin had no significant effect on the senescence growth arrest, as measured by incorporation of the fluorescent thymidine analogue EdU into newly

synthesized DNA over a 24-hr period (Fig. 2B). Finally, the enlarged morphology of senescent cells remained unchanged by apigenin (data not shown).

5.5.4. Apigenin suppresses oncogene- and replication-induced IL-6 secretion

In addition to the genotoxic stress caused by IR, other stressors induce a senescence response and SASP, including activated oncogenes or signaling kinases and telomere erosion caused by repeated replication (Coppe, Patil et al. 2008, Rodier, Coppé et al. 2009, Freund, Patil et al. 2011). To determine whether apigenin was capable of suppressing IL-6 secretion by human fibroblast strains induced to senescence by activated oncogenes or signaling kinases, we infected HCA2 cells with an insertless lentivirus (L3P) or lentiviruses expressing oncogenic RAS (H-RAS^{V12}) (Beausejour, Krtolica et al. 2003) or a constitutively active MAP kinase kinase 6 mutant (MKK6EE) (Freund, Patil et al. 2011), both of which induce a strong senescence response. After selection, the infected cells were reseeded and given DMSO or 10 μM apigenin. Four days later, we measured secreted IL-6 levels (Fig. 2C). Whether cells senesced in response to oncogenic H-RAS^{V12} or MKK6EE, apigenin (as well as the positive control, cortisol) significantly suppressed IL-6 secretion (Fig. 2C). Similarly, apigenin reduced the level of IL-6 secreted by replicatively senescent HCA2 fibroblasts (60 population doublings) by almost 60% (Fig. 2D).

5.5.5. Kinetics of apigenin suppression of IL-6 secretion

To determine the kinetics with which apigenin reduces IL-6 secretion by senescent HCA2 fibroblasts, we conducted three experiments.

First, we treated cells with DMSO or apigenin immediately after IR (day 0) (Fig. 3A). On each following day, for 10 consecutive days, we replaced the apigenin-containing media with DMSO-containing media, thus releasing the cells from the treatment. On day 10, we collected conditioned media and quantified IL-6. As expected, senescent cells that were never exposed to apigenin secreted the highest levels of IL-6. IL-6 secretion progressively decreased with increasing time of apigenin exposure, reaching maximal reduction after 6 days of treatment.

In the second 10-day experiment (Fig. 3B), we delayed the initiation of apigenin treatment after IR for varying intervals, and then quantified IL-6 secretion. As expected, continuous treatment (no treatment delay) reduced IL-6 secretion maximally. No treatment

(day 10) yielded the highest IL-6 secretion level. Interestingly, treatment at day 8 for 48 h was sufficient to produce a robust decrease in IL-6 secretion (>4 fold), indicating that apigenin can suppress a fully formed SASP, which takes about 7 days to develop (Coppe, Patil et al. 2008).

Finally, we examined the recovery of IL-6 secretion after apigenin treatment. We treated senescent cells with apigenin or DMSO continuously for 10 days, then washed the cells and added standard culture medium (Fig. 3C). Over the next 10 days we collected conditioned media and then quantified IL-6 levels. The ability of apigenin to suppress IL-6 secretion lasted 4-5 days after removal, reaching levels achieved by DMSO-treated cells in 5-6 days. Interestingly, IL-6 secretion by DMSO-treated cells also rose during this period, possibly due to nutrient replenishment and mTOR signaling, which drives the SASP ((Laberge, Sun et al. 2015).

5.5.6. Secretion profile of apigenin-treated senescent cells

To obtain a broader view of how apigenin suppressed the SASP, we used a bead-based ELISA assay (Luminex) to quantify the levels of 51 cytokines, some, but not all, of which were shown to be SASP factors by semi-quantitative antibody arrays. We used IMR90 and BJ cells to compare their responses and determine whether effects were cell of origin or sex specific (Fig. S1B; Fig. S2).

Ten days after IR, both fibroblast strains developed a typical SASP compared to sham-irradiated controls, showing increased expression and secretion of several pro-inflammatory cytokines, including IL-8, IL-6 and GROA (Fig. 4A; Fig. S2A-B, S3) as described (Coppe, Patil et al. 2008, Lim, Park et al. 2015). This increase was reduced by apigenin in both strains to similar extents.

As expected (Coppe, Patil et al. 2008), certain markers of acute inflammation, such as TGFb, were relatively low in the secretory profile of senescent IMR90 and BJ cells, whereas many other markers of inflammation, such as IL-6 and IL-8, were markedly upregulated (Fig. S2A, S2B). In addition to known SASP factors, we identified a novel SASP factor, the chemokine interferon-g-inducible protein 10 (IP10/CXCL10). This chemokine was secreted at high levels by senescent fibroblasts, equaling the level of IL-6. Similar to other prominent SASP factors, IP10/CXCL10 secretion was robustly reduced by apigenin (Fig. 4B).

5.5.7. Apigenin reduces NF-kB activity stimulated by IL-1A

The NF-kB transcription factor is a major positive regulator of inflammatory responses (Baker, Hayden et al. 2011), including much of the SASP (Freund, Orjalo et al. 2010, Freund, Patil et al. 2011). Further, apigenin reduced NF-kB activity and improved survival after an acute, otherwise lethal, inflammatory stimulus (Nicholas, Batra et al. 2007). To determine whether apigenin reduced the SASP by suppressing NF-kB activity, we used a lentiviral-delivered reporter consisting of NF-kB transcriptional response elements driving firefly luciferase. We expressed this reporter in HCA2 fibroblasts, induced the cells to senesce by IR, and then measured IL-6 secretion and luciferase activity. As expected, senescent cells showed a robust increase in IL-6 secretion, which was abolished by apigenin (Fig. S4A). In parallel, senescent cells showed a 4- to 5-fold increase in NF-kB activity, which was reduced to <2-fold by apigenin (Fig. S4B).

The NF-kB activity in senescent cells is driven in large measure by an IL-1A-NF-kB positive feedback loop: the cells express cell surface-bound IL-1A, which acts in a juxtacrine manner to induce several genes, including IL-1A itself (Orjalo, Bhaumik et al. 2009). To determine whether apigenin's ability to reduce the SASP depended on this IL-1A feeback loop, we treated non-senescent and senescent HCA2 cells with DMSO or apigenin, added increasing concentrations of recombinant IL-1A, then analyzed conditioned media for IL-6. Apigenin reduced IL-1A-stimulated IL-6 secretion in both non-senescent and senescent cells at all IL-1A concentrations tested (Fig. S4C), consistent with its ability to suppress NF-kB activity. Thus, the anti-inflammatory effects of apigenin are likely mediated in part by its ability to suppress NF-kB activity initiated by IL-1A.

5.5.8. Apigenin reduces IRAK1/4 and p38MAPK phosphorylation

IL-1A signaling stimulates the SASP owing to phosphorylation the signal transduction proteins IRAK 1 and 4, then ultimately of p38MAPK on Thr180 (Freund, Patil et al. 2011). Apigenin was shown to inhibit p38MAPK phosphorylation in prostate cancer cells, albeit at a higher concentration (40 μM) (Shukla and Gupta 2007). To determine whether apigenin alters IRAK1/IRAK4/p38MAPK phosphorylation in normal cells at a concentration that inhibited the SASP (10 μM), we stimulated non-senescent BJ fibroblasts with IL-1A in the presence of the phosphatase inhibitor calyculin. Using western blotting, we then analyzed for the

phosphorylated forms of IRAK1 (Thr387), IRAK4 (Thr345/Ser346) and p38MAPK (Thr180/Tyr182) (Fig. 4C). Phosphorylation of the three proteins was substantially reduced by apigenin.

Noting the reduction of IRAK4 phosphorylation, proximal to the start of the IL-1A cascade, we sought to examine the effects in senescent cells without exogenous stimulation by IL-1A. We therefore made primary BJ fibroblasts senescent by irradiation and showed they also exhibited a decrease in IL-1A-mediated IRAK4 phosphorylation with apigenin treatment (Fig. 4D). This decrease suggests that NF-kB activity in senescent cells could at least in part be reduced by apigenin through inhibition of IRAK4.

5.5.9. Apigenin suppresses ability of the SASP to stimulate breast cancer cell aggressiveness

The SASP can disrupt epithelial organization and promotes premalignant epithelial cell proliferation and invasion in culture and in vivo (Krtolica, Parrinello et al. 2001, Coppe, Patil et al. 2008, Coppe, Patil et al. 2010, Laberge, Sun et al. 2015). We therefore determined the ability of apigenin to counter-balance the effects of the SASP on breast cancer cell aggressiveness. We treated senescent (IR-induced) HCA2 cells with vehicle or apigenin for 10 days, washed the cells, then collected conditioned media (CM) over the next 24 hrs. We added the CM to relatively aggressive MDA-MB231 and non-aggressive ZR75.1 human breast cancer cells, and monitored their ability to proliferate (Fig. 5A). As expected, the SASP-containing CM from senescent cells stimulated the proliferation of both breast cancer cell lines to a significantly greater extent than CM from non-senescent cells. Importantly, apigenin significantly reduced the ability of the SASP to stimulate cancer cell proliferation.

Apigenin also suppressed the ability of the SASP to stimulate MDA-MB231 cells to invade a basement membrane in Boyden chambers (Fig. 5B). SASP-containing CM from senescent fibroblasts stimulated 3.5-fold more invasion than CM from non-senescent fibroblasts, and apigenin reduced this stimulation to non-senescent levels. Consistent with this finding, apigenin suppressed the ability of the SASP to induce an epithelial-mesenchymal transition (EMT) and confer on epithelial cells their invasive and metastatic properties, which is an important step during cancer progression (Laberge, Awad et al. 2012). By immunofluorescence (Fig. 5C) and/or western blotting (Fig. 5D), control, non-aggressive, ZR75.1 cells expressed the tight junction protein ZO-1 and epithelial cytoskeletal protein keratin (K)-18,

as well as detectable levels of the mesenchymal cytoskeletal protein vimentin. As expected (Coppe, Patil et al. 2008, Coppe, Patil et al. 2010), SASP-containing CM reduced ZO-1 and K-18 expression and increased vimentin expression, consistent with inducing an EMT. However, CM from apigenin-treated senescent fibroblasts reestablished the higher expression of ZO-1 and K-18 and lower expression of vimentin (Fig. 5C, 5D). Thus, apigenin can indirectly reduce the aggressive phenotype of breast cancer cells stimulated by the SASP.

5.6. DISCUSSION

Discovering new molecules that can prevent or attenuate the deleterious effects of the SASP, and also have low or non-existent negative side-effects, hold promise for the development of safe therapeutic interventions into the many age-related diseases in which the SASP has been implicated. We followed up on earlier results from a high content screen to identify compounds that either eliminate senescent cells or suppress the SASP (Laberge, Zhou et al. 2012). Among the compounds that robustly reduced the secretion of IL-6, a sentinel SASP factor, was apigenin, one of the few natural products included in the library (the Prestwick Library of FDA approved compounds). Here, we examined the effects of apigenin on several aspects of the senescent phenotype of normal human fibroblasts.

Apigenin reduced IL-6 secretion by two independent strains of senescent human fibroblasts (HCA2 and BJ) in a dose dependent manner. At 10 μM, apigenin significantly and near-maximally reduced IL-6 secretion by these normal cells. Because apigenin was shown to induce apoptosis and inhibit cell proliferation in cancer cell lines (Reiners, Clift et al. 1999, Gupta, Afaq et al. 2001, Way, Kao et al. 2004, Brusselmans, Vrolix et al. 2005, Shukla and Gupta 2007, Jayasooriya, Kang et al. 2012), we tested it for these activities in normal human fibroblasts. At 10 μM, apigenin did not induce apoptosis in non-senescent or senescent normal cells and, while robustly reducing IL-6 secretion, only decreased proliferation of non-senescent cells by ~25% while the flavonoid was present. We saw significant decreases in IL-6 secretion with concentrations as low as 1 μM. These data suggest that a plasma concentrations of ~1.5 μM apigenin, which is achievable through dietary sources (Gradolatto, Basly et al. 2005), can be an effective suppressor of the SASP.

One proposed mechanism for the ability of apigenin to suppress inflammation is inhibition of NF-kB activity. At 100 μM, apigenin suppressed TNF-a activated NF-kB activity in

transformed mouse intestinal cells at the level of phosphorylation and degradation of the inhibitory network (Ruiz and Haller 2006). By contrast, at 20 μM, apigenin reduced TNF-a stimulated NF-kB-reporter activity without affecting the phosphorylation or degradation of NF-kB pathway components, DNA binding activity or translocation of NF-kB to the nucleus in NIH-3T3 immortalized fibroblasts (Funakoshi-Tago, Nakamura et al. 2011). Thus, apigenin can act on NF-kB activity by different mechanisms in different cell types. We found that apigenin inhibited NF-kB activity at 10 μM in normal human fibroblasts primarily by inhibiting signaling through IL-1A, an important upstream regulator of the inflammatory arm of the SASP (Orjalo, Bhaumik et al. 2009). Thus, apigenin did not alter the phosphorylation state of MSK1, which phosphorylates the activation domain of NF-kB (Reber, Vermeulen et al. 2009).

However, apigenin was shown to inhibit the phosphorylation state of p38MAPK in cancer cell lines (Noh, Sung et al. 2010), and we showed p38MAPK is important for the SASP of normal human cells (Freund, Patil et al. 2011). Indeed, here we show that apigenin inhibited p38MAPK phosphorylation on thr380, a critical residue for its activation, in two normal human fibroblast strains. Accordingly, apigenin suppressed the expression and secretion of several SASP factors. These factors included IL-6 and IL-8, which are important pro-inflammatory cytokines, and GROA, a potent growth factor; all three were shown to promote aggressive phenotypes in human breast cancer cells (Coppe, Patil et al. 2008, Coppe, Patil et al. 2010).

Of interest, we also identified IP10 as a SASP factor and target of suppression by apigenin. As its name suggests, IP10 is induced by interferon-gamma, along with CXCL9 and CXCL11, which together promote a Th1 response to viral infection, in essence a response to cellular damage. Interestingly, serum levels of IP10 increase during normal aging (Antonelli, Rotondi et al. 2005), and may promote abnormal immune responses in the elderly. The receptor for IP10, CXCR3, activates NF-kB which has a binding site in the promoter of IP10. Thus, IP10 can stimulate its own transcription, creating a feedback loop that has the potential to reinforce the SASP in a fashion to that of IL-1A (Orjalo, Bhaumik et al. 2009).

In flavones such as apigenin, the ABC three-ring backbone is similar in structure to ATP (Funakoshi-Tago, Nakamura et al. 2011). Flavones are known to inhibit ATP-hydrolysis, therefore apigenin could compete with ATP for the binding site of p38MAPK, and other kinases as well, and thus inhibit multiple downstream targets simultaneously. The pleotropic nature of this inhibition could make determining the precise mechanism(s) of action of apigenin challenging. However, as a potential intervention into cancer and possibly aging, having

multiple targets involved in inflammation and growth could result in a synergy that would make it a promising compound for regulating several pathways involved in healthy longevity.

5.7. ACKNOWLEDGMENTS

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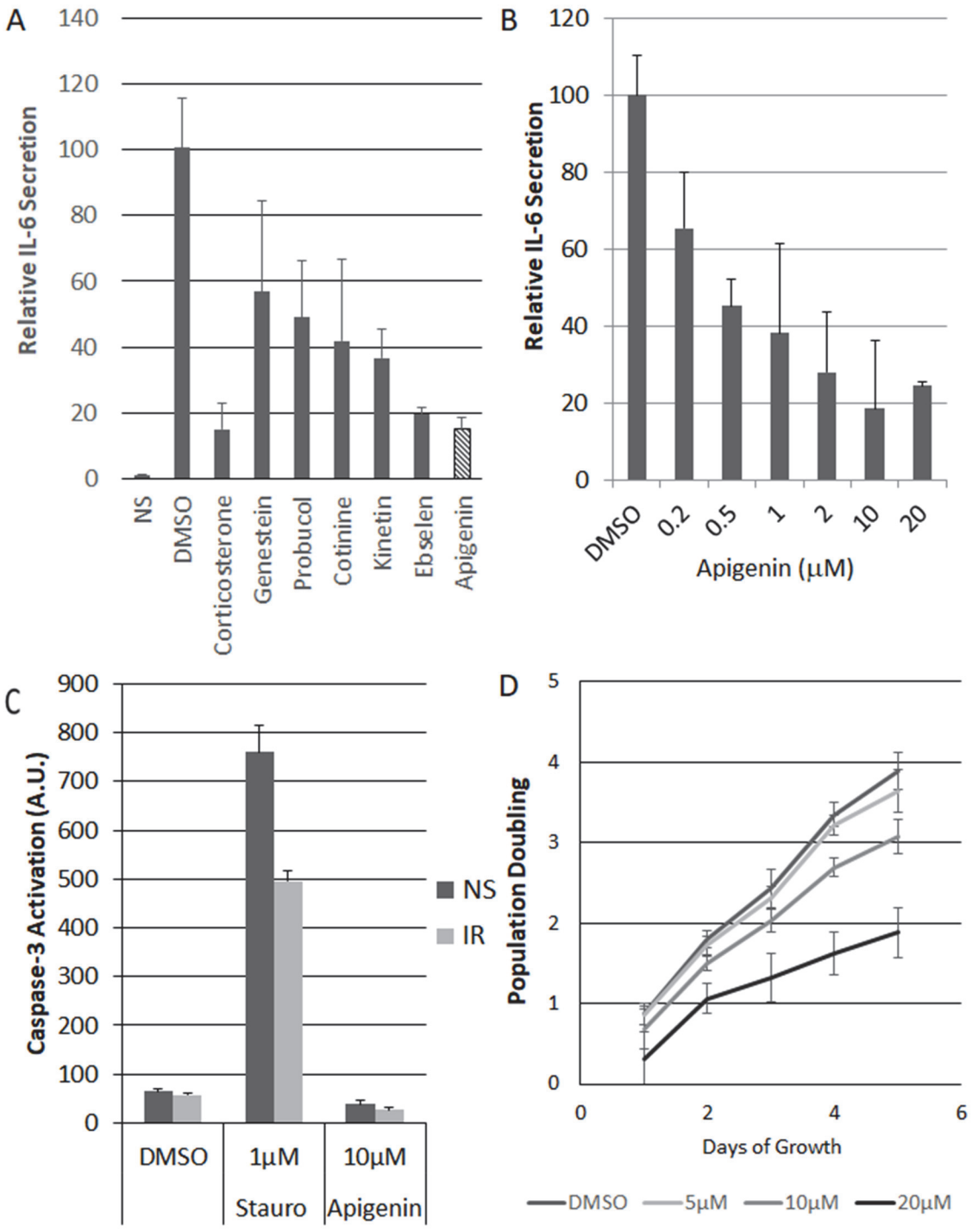
5.8. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

5.9. FIGURES


A
Relative IL-6 Secretion
140
120
100
80
60
40
20
0
NS
DMSO
Corticosterone
Genestein
Probucol
Cotinine
Kinetin
Ebselen
Apigenin
B
Relative IL-6 Secretion
120
100
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20
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DMSO
0.2
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2
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Apigenin (µM)
C
Caspase-3 Activation (A.U.)
900
800
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300
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100
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NS
IR
DMSO
1µM Stauro
10µM Apigenin
D
Population Doubling
5
4
3
2
1
0
0
2
4
6
Days of Growth
DMSO
5µM
10µM
20µM

Figure 1. Apigenin down-regulates IL-6 secretion, moderately reduces fibroblast proliferation but does not induce apoptosis. (A) The indicated compounds from the Prestwick Library were used at 2 μM to treat primary human HCA2 fibroblasts immediately following X-irradiation at 10 Gy. Cells were treated for 10 days, after which conditioned media (CM) were collected and analyzed using the Perkin-Elmer IL-6 AlphaLISA assay. CM from non-senescent (NS) cells treated with vehicle (DMSO) provided a baseline for IL-6 secretion. Senescent cells were also treated with DMSO (negative control) and corticosterone (100 nM) (positive control). (B) HCA2 fibroblasts were induced to senesce (10 Gy X-irradiation) and immediately treated with increasing concentrations of apigenin for 10 days. CM were collected and analyzed for IL-6 secretion. IL-6 levels secreted by senescent cells were set at 100%. (C) HCA2 cells were induced to senesce as above and 7 days later were reseeded in 96-well plates (10,000 cells/well) and allowed to recover for 24 hrs. In parallel, non-senescent cells were seeded at 2,500 cells/well. After 24 hrs, both senescent and non-senescent cells were treated with DMSO, 1 μM staurosporin or 10 μM apigenin, incubated for 48 hrs, and then caspase-3 activity was measured by luminescence using the Promega ApoTox Glo kit and arbitrary units. (D) HCA2 fibroblasts were seeded at 5,000 cells/well in five 12-well plates, one for each day of a five-day treatment with DMSO, 5, 10, or 20 μM apigenin. Three samples corresponding to each concentration were counted from one plate daily to determine cell number.

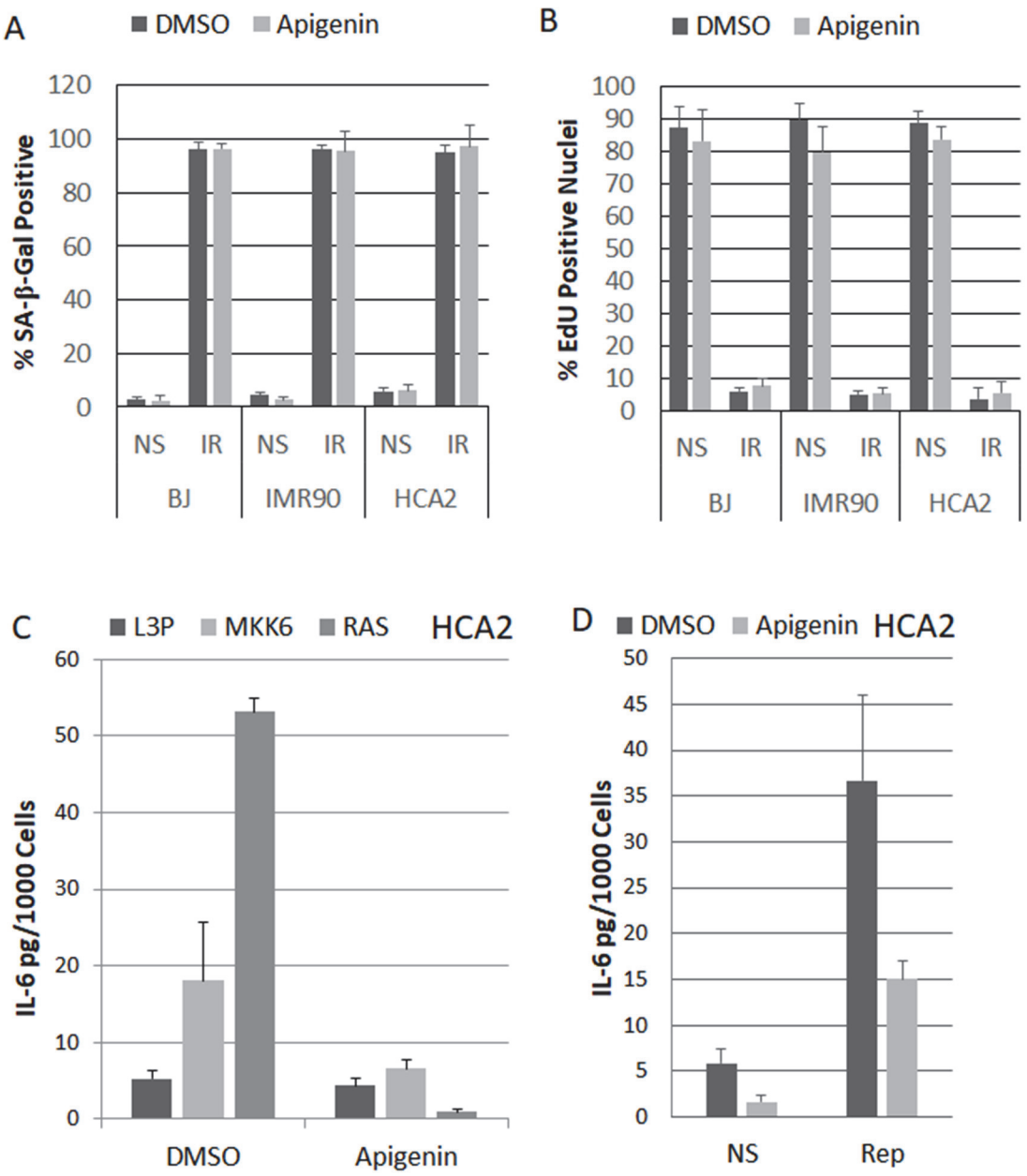

Figure 2. Effects of apigenin treatment on senescence-associated phenotypes. (A) Human BJ, IMR90, and HCA2 fibroblasts were non-senecent (NS) or induced to senesce as described in the legend to Fig. 1 (IR), and treated with DMSO or apigenin for 10 days. 36 hrs before collection of senescent samples, NS cells were plated at 10,000/cm^2, allowed to recover overnight, and media on NS and IR cultures were replaced with serum-free media containing DMSO or apigenin. The following day, cells were stained for SA-β-gal activity and 100-150 cells were counted. (B) Cells were prepared as in (A) except 10,000/cm^2 of senescent or non-senescent cells were seeded into each well of a 8-well slide, and EdU was added to the serum-free media and 100-150 cells

were counted. (C) HCA2 fibroblasts were infected with L3P (insertless vector), MKK6EE, or H-RAS^{V12} containing lentiviruses. Infected cells were selected with 1μg/ml puromycin for 24 hrs, allowed to recover for 4 days, and reseeded. Five days later, all media were replaced with serum-free media containing apigenin or DMSO for 24 hrs. Then, CM was collected and analyzed for IL-6 secretion. (D) Replicatively senescent (PD 60.2) HCA2 fibroblasts were seeded at 10,000/cm^2 onto 12-well plate and allowed to recover for 48 hrs. Cells were then treated with DMSO or apigenin (media refreshed every 48 hrs). On day 8, non-senescent cells were seeded onto 6-well plates at 10,000/cm^2 and allowed to recover overnight. On day 9, all samples were given serum-free media supplemented with DMSO or apigenin, and collected 24 hrs later (day 10) and analyzed for IL-6 secretion.

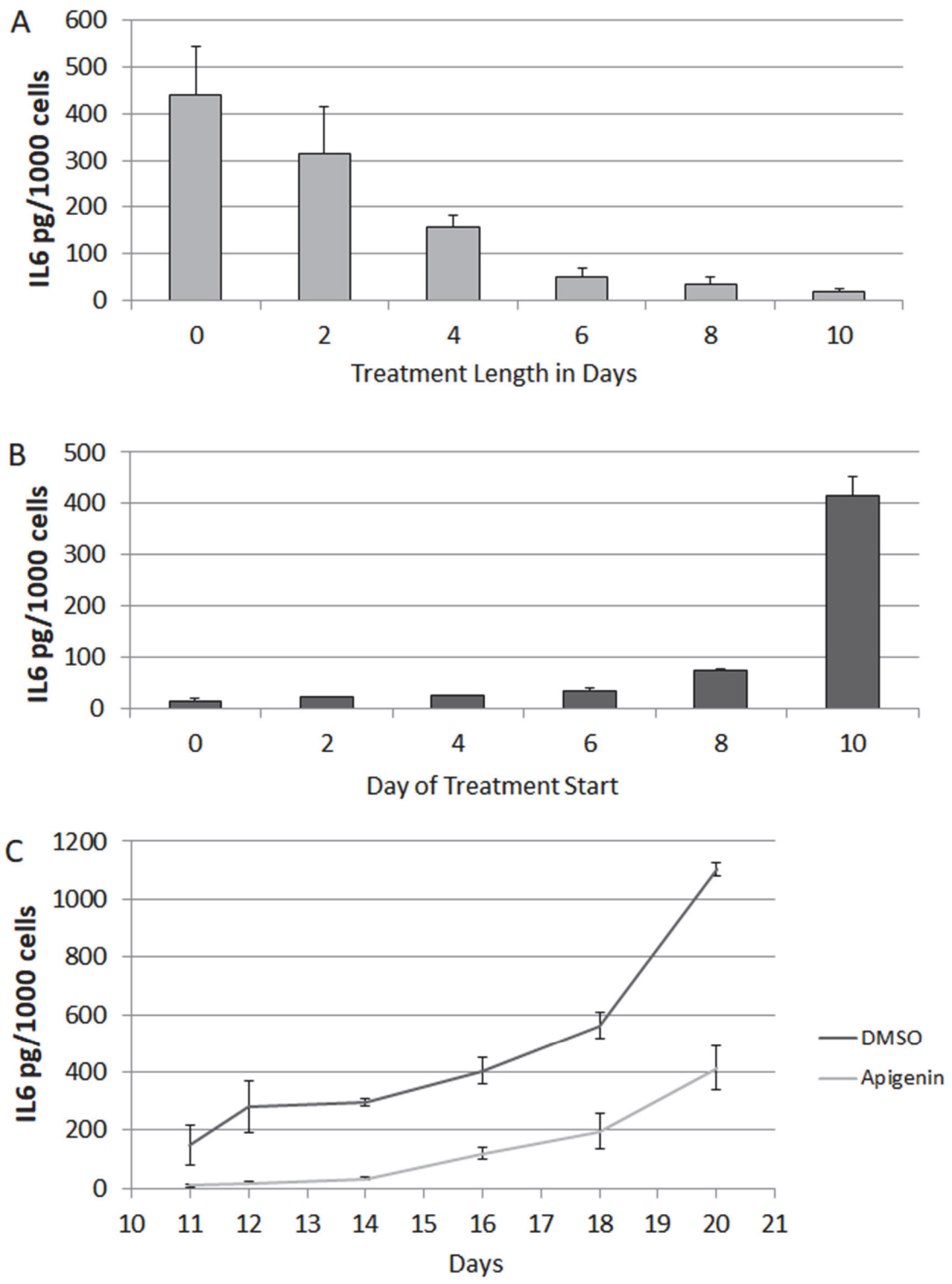

Figure 3. Effect of timing of apigenin treatment on IL-6 secretion. (A, B) HCA2 fibroblasts were seeded at 10,000/cm^2 into two 24-well plates and induced to senesce by IR. 10-days later, we varied the length of continuous treatment with apigenin starting immediately after IR in one

plate (A), and in the other plate we varied the day of initiation of treatment with apigenin after IR (B). Duplicate samples were treated with media containing apigenin or DMSO, and refreshed every 48 hrs. On day 9, media were replaced with serum-free media containing apigenin or DMSO, and, 24 hrs later, cells were counted and conditioned media analyzed for IL-6 secretion. (C) HCA2 fibroblasts were seeded at 10,000/cm^2 into a 24-well plate and induced to senesce by IR. Immediately following IR, media were refreshed with DMSO or apigenin, and incubated for 10 days (media refreshed every 48 hrs with DMSO or apigenin). On day 10, cells were washed and incubated with serum-containing media except for the first sample (day 11) that was replaced with serum-free media. Samples for subsequent time points were similarly washed and media replaced with serum-free media 24 hrs before collection. After the final time point on day 20, CM for all time points were analyzed for IL-6.

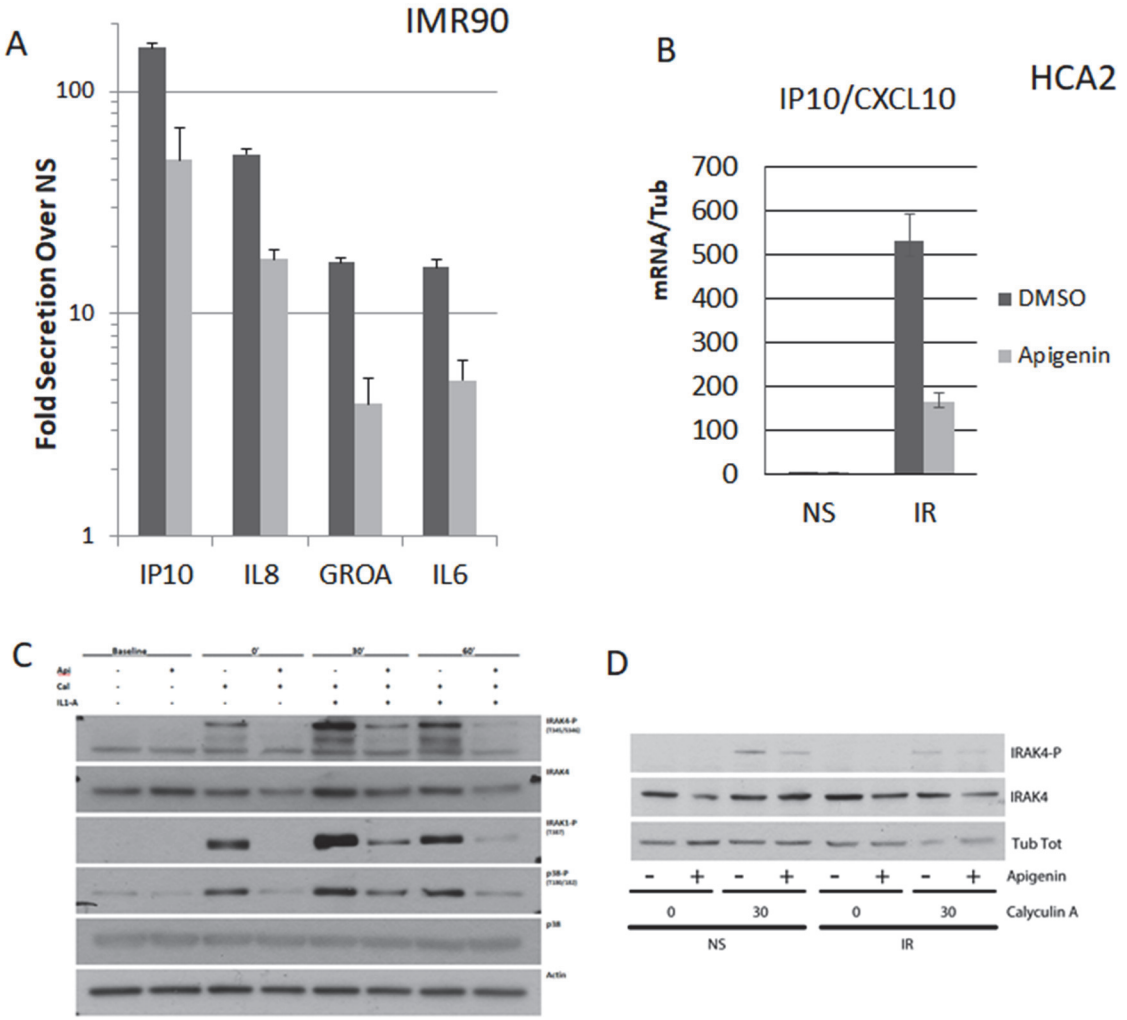

Figure 4. Effects of apigenin on the SASP and its regulation. (A) We compared the levels of selected cytokines (from the Luminex screening) secreted by treated (apigenin) and untreated (DMSO), NS or IR senescent IMR90 fibroblasts. Levels of IP10, IL-8, GROA and IL-6 in the CM from senescent cells are shown relative to NS. (B) Using qPCR, we determined the mRNA level of IP10 in DMSO- and apigenin-treated non-senescent (NS) and senescent (IR) BJ fibroblasts. (C) The kinetics of phosphorylation of IRAK4, IRAK1 and p38MAPK in non-senescent BJ fibroblasts stimulated with IL-1A, and treated or not with apigenin, was examined using stabilization of phosphorylation by Calyculin A (Cal). (D) IRAK4 phosphorylation in response to apigenin treatment was compared between senescent and non-senescent BJ fibroblasts.

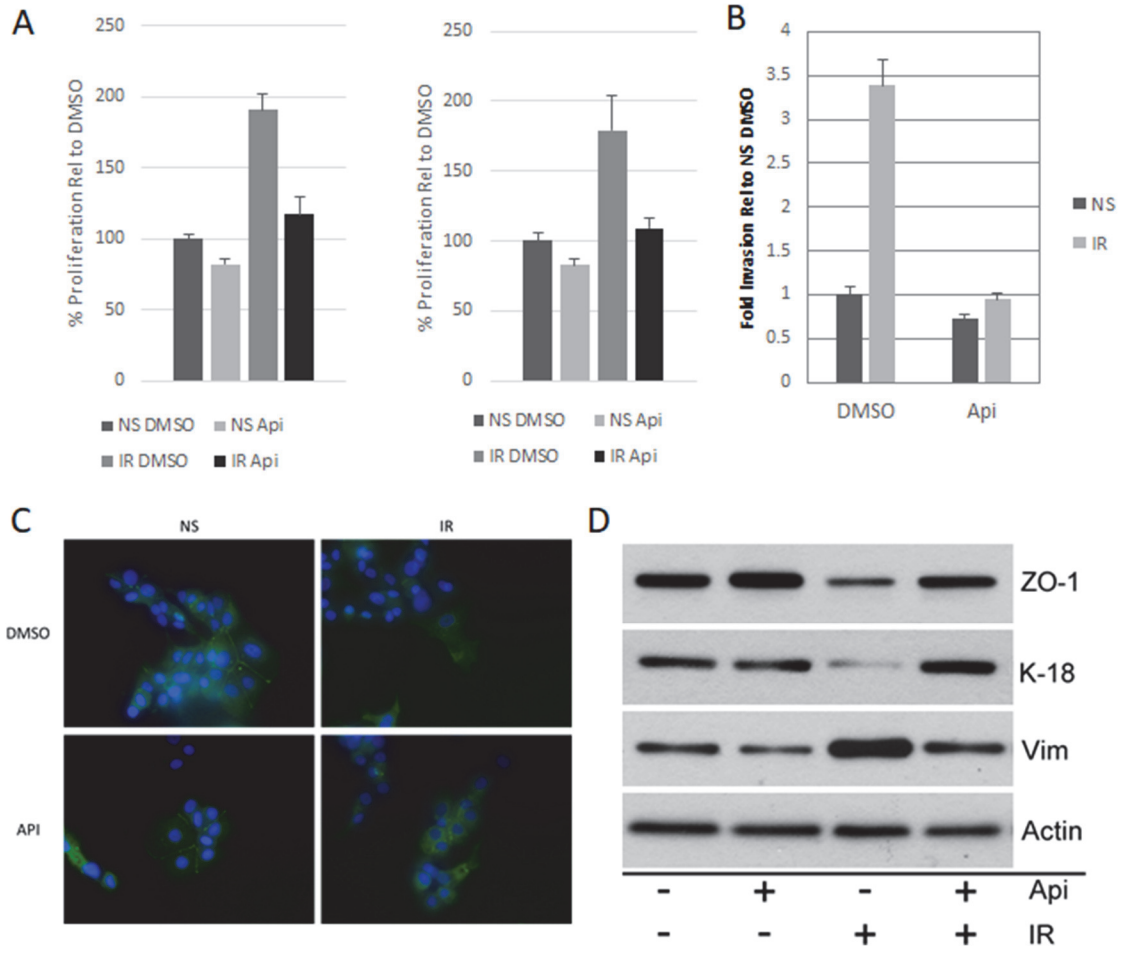

Figure 5. Apigenin suppresses the ability of the SASP to induce cancer cell aggressiveness. (A) MDA-MB231 (left panel) and ZR75.1 (right panel) breast cancer cells were cultured in presence of conditioned media (CM) from DMSO- or apigenin (Api)-treated fibroblasts, and monitored for proliferation over 3 days. (B) CM were prepared from non-senescent (NS) cells or senescent (IR)

cells, treated or not with apigenin (Api). CM were assayed for ability to stimulate MDA-MB231 human breast cancer cells to invade a basement membrane, as described in the Materials and Methods. Invasion stimulated by NS CM was given a value of one, and other conditions were normalized to this value. Error bars indicate the standard deviation around the mean. (C) ZR75.1 cells were incubated with the indicated CM for 3 days, and immunostained for the tight junction protein ZO-1. (D) Using western blotting, we analyzed the expression of ZO-1, the epithelial marker cytokeratin 18 (K-18) and the mesenchymal marker vimentin (Vim) in ZR75-1 cells. The effect of CM from apigenin-treated NS and IR fibroblasts on the expression of these three markers is presented on lanes 2 and 4, whereas lanes 1 and 3 show the expression in cells cultured in CM from DMSO-treated NS and IR fibroblasts, respectively.

5.9.1. SUPPLEMENTARY FIGURES

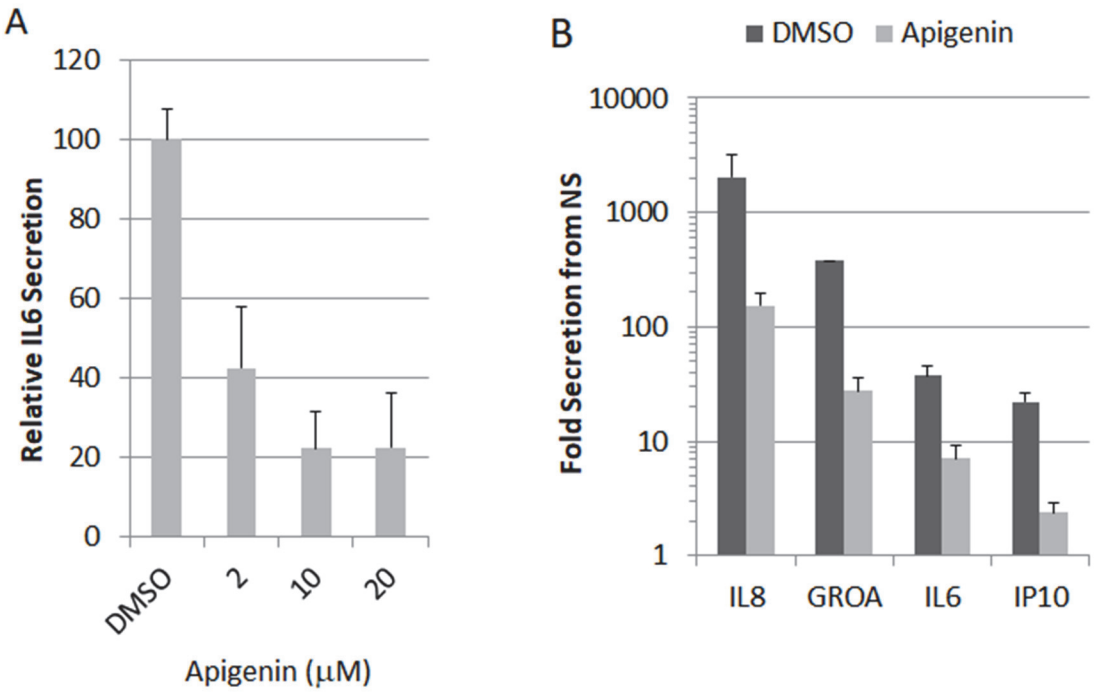

Figure S1. Apigenin down-regulates the SASP in BJ fibroblasts. (A) Primary human BJ fibroblasts were induced to senesce (10 Gy X-irradiation) and immediately treated with increasing concentrations of apigenin for 10 days. CM were collected and analyzed for IL-6 secretion. (B) We investigated the levels of IL-8, GROA, IL-6 and IP10 secreted by treated (apigenin) and untreated (DMSO), NS or senescent BJ fibroblasts. Samples were in

quadruplicate and levels of individual cytokines in senescent samples were normalized to the levels in DMSO-treated NS cells.

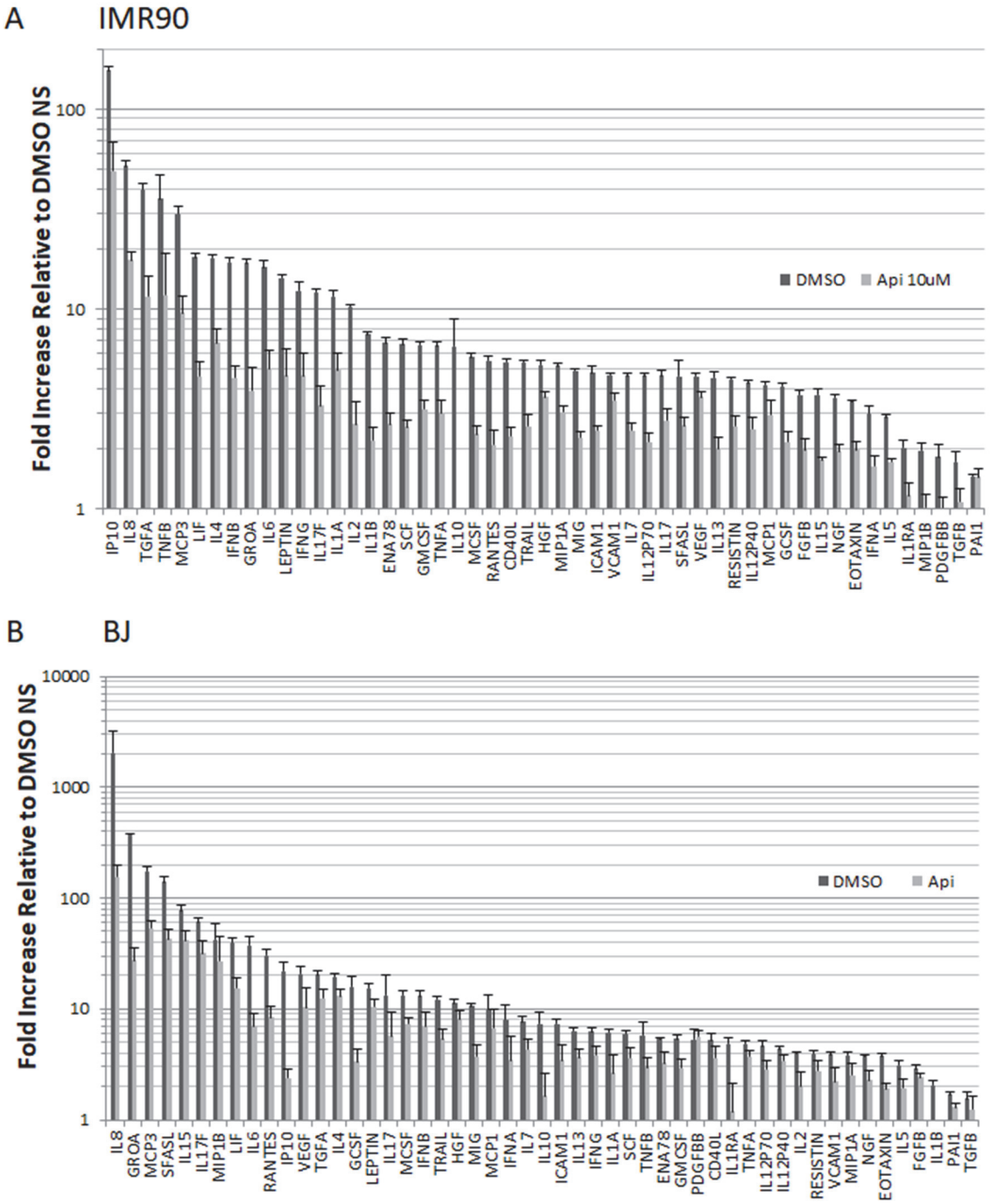

Figure S2. Apigenin reduces cytokine secretion of senescent IMR90 and BJ fibroblasts. (A) Primary IMR90 (PD 37) and (B) primary BJ (PD 34) human fibroblasts were induced to senesce by IR, and treated with apigenin or DMSO. CM were collected and the levels of 51 cytokines were

analyzed by Luminex. Samples were in quadruplicate and levels of individual cytokines in senescent samples were normalized to the levels in DMSO-treated NS cells.

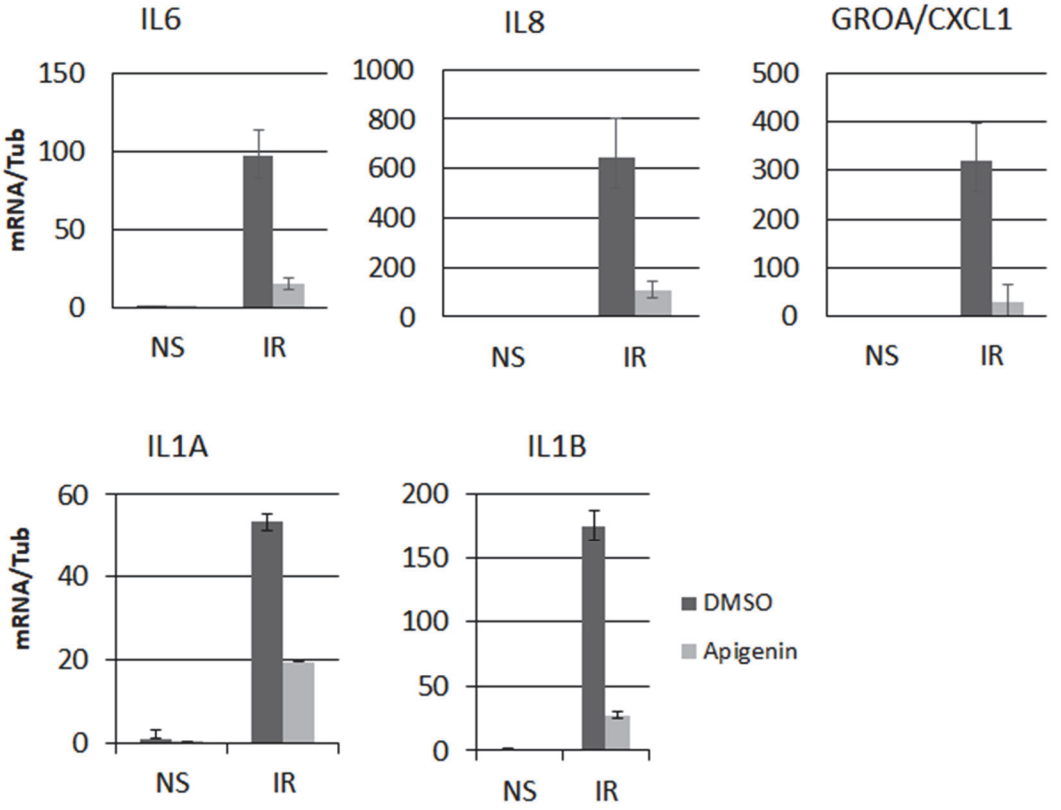

Figure S3. Effect of apigenin on the expression of major SASP factors in BJ fibroblasts. Using qPCR we determined the mRNA levels of major SASP factors in DMSO- and apigenin-treated non-senescent and senescent BJ fibroblasts (IL-6, IL-8, and GROA). IL-1A and IL-1B were also investigated at the mRNA level.

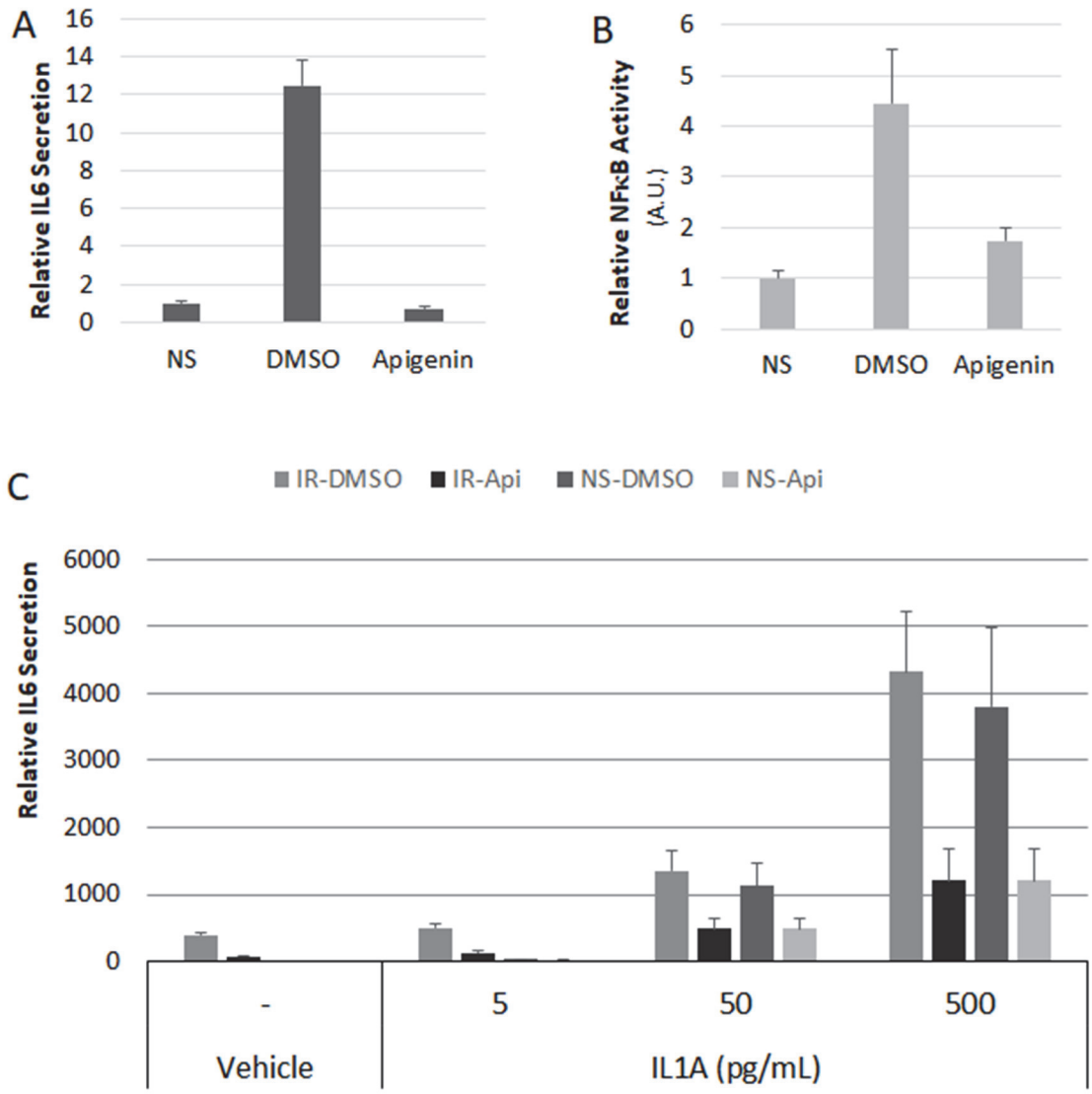

Figure S4. Apigenin reduces the senescence-induced inflammatory pathway activation. (A) IL-6 secretion of cells infected with a lentivirus NF-kB-luciferase reporter construct, induced to senesce by IR in the presence of apigenin, is shown. (B) NF-kB reporter activation in the senescent cells in (A) was measured by luminescence using the Promega Luciferase Assay System. (C) Induction of IL-6 secretion in NS and senescent HCA2 cells upon administration of three concentrations of recombinant IL-1A was measured by AlphaLISA.

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6. Chapter 6 - Conclusion

6.1. Summary of Findings

The findings presented above initially grew out of a high-throughput screen of small molecule compounds aimed at identifying and characterizing compounds capable of exerting potent anti-senescent effects without the deleterious side effects inherent to other powerful inflammation-suppressing drugs, such as corticosteroid. This screening process identified apigenin, a novel flavonoid abundant in leafy cruciferous vegetables and potentially highly bioavailable from diet with no previously reported cytotoxic or side effect profiles (Miean and Mohamed 2001).

Apigenin has been previously reported to reduce inflammation in normal and senescent cells (Liang et al. 1999), which may underlie its reported anti-metastatic effects via inhibition of the inflammation-promoted epithelial-to-mesenchymal transition in cancer cells (Funakoshi-Tago et al. 2011; Comalada et al. 2006). These functions may also hinge upon apigenin's ability to prevent inflammation-induced oncogenesis, wherein previously senescent cells with profoundly altered metabolism and the senescence-associated secretory proteome (SASP) are potentially much more oncogenic upon loss of a tumor-suppressing gene, such as p53 or p21. Due to these effects, I was curious to investigate the exact effects of apigenin treatment on senescent cell biology and the SASP to determine its therapeutic utility as an anti-senescence treatment in reducing the impact of the SASP on many chronic, progressive conditions associated with human aging.

First, I replicated and extended previous reports showing that apigenin potently reduces the secretion of inflammatory cytokines. These effects were shown in multiple senescent cell lines, in which a variety of senescence-inducing methods and apigenin treatment schemas were

used, confirming that the anti-SASP effects of apigenin are more potent in senescent than non-senescent cells. Furthermore, the concentrations at which apigenin significantly reduced the SASP were not cytotoxic to either senescent or non-senescent cells, showing that apigenin's mechanism of action is not due to increased cytotoxicity and therefore fewer cells able to secrete inflammatory mediators. With this finding, I became curious to determine the mechanism by which apigenin treatment exerts its anti-SASP effects.

To investigate the mechanism by which apigenin exerts its anti-senescence effects, I conducted a series of studies characterizing the effects of apigenin under a wide variety of stimuli and conditions. Through these screens, I found that apigenin primarily exerts its effects through the NFκB and related IL-1A pathways, which are prominently featured in all acute and chronic inflammatory conditions. In non-senescent cells, NFκB and IL-1A mediate most inflammatory responses by governing the transient elevation, and eventual decrease in, pro-inflammatory cytokines that govern the inflammation process. In senescent cells, however, NFκB and IL-1A mediate a potent autocrine and paracrine feedback loop that perpetuates chronic inflammation and the SASP. I found that apigenin treatment potently reduced the expression levels of NFκB and IL-1A at multiple points, reducing mRNA expression levels, decreasing phosphorylation of key proteins, such as IRAK1/4 and p65, involved in signal transduction, and inhibiting the secretion of a huge range of SASP factors including those previously described as well as identifying some new factors including CXCL10.

The above investigations also revealed non-SASP-mediated, metabolic effects of apigenin treatment, wherein apigenin was able to potently reduce the activation of the AKT/PI3K/mTOR pathways that canonically drives cellular responses to mitogenic signals, such as highly available glucose. This same pathway, however, also mediates cellular responses to potent stressors, such as DNA damage, hypoxia, and nutrient starvation. While apigenin treatment did not rescue senescent cells from the irreversible growth arrest that accompanies p53, p16, p21, and Rb coordination of senescence-associated epigenetic reprogramming, it did reduce mTORC1 signaling. Although additional studies would be necessary to confirm this, it is likely this reduction in mTORC1 signaling is intimately linked to apigenin's effects on the SASP via downregulation of metabolic flux in senescent cells. This shows that apigenin's therapeutic effects may not be protein-specific, but instead operate via a broader mechanism capable of altering the activity of a broad range of proteins simultaneously. Due to the breadth of apigenin's effects in these screens, it was hypothesized that apigenin was acting as an allosteric

modulator via alteration of ATPase binding and thus able to effect the broad changes observed. Apigenin exhibits structural similarity to ATP at the binding site of select ATPases and has been implicated in other reports as a modulator of ATPase activity, indicating that this theory for its mechanism of action may be borne out by further study.

Lastly, an additional screen on polyketide compounds provided by Biotica identified a few candidates that may be capable of selectively inducing apoptosis in senescent cells. These compounds represent a broad class of naturally-occurring, highly metabolically-active compounds that exert diverse biological side effects. Additional studies will be necessary to confirm these effects, but the initial results are nonetheless promising and represent a strong foundation for further exploration.

6.2. Limitations

The pleiotropic nature of apigenin's effects on a plethora of pathways, similar to that of other natural molecules, is so broad that it is nearly impossible to determine the exact mechanism by which any phenotype resulting from its application emerges. Whether this is due to weak affinity for a wide variety of protein targets or due to an allosteric modulation thereof remains to be determined through application of deeper characterization methods, such as high-throughput phosphorylation state screening or whole-transcriptome survey. Nonetheless, apigenin's anti-SASP effect on many models and in many conditions was highly replicable and undeniable, suggesting that apigenin's anti-senescence effects are very real.

Additionally, the effects of conditioned media from treated senescent cells were not exhaustively tested on various cancer cell lines or immune cell populations, in which inflammatory mediators are crucial, and it is therefore plausible that some cancers or immune subpopulations may react poorly to the reduction of SASP factors. These effects could be surveyed by testing apigenin on a broader range of cancer cell lines and leukocytes and characterizing the change in proliferation, invasion, and migration in response to metabolic and/or cytokine stressors.

In testing how apigenin treatment affects the activation state of multiple inflammation and metabolic signaling proteins, it is possible that the reduction in the phosphorylation of individual targets could have resulted indirectly from reduced activation of the pathway through inhibition of upstream kinases. One way to measure this would involve the creation of mutant

kinases which would be apigenin-insensitive, which if apigenin is acting as a ATP-mimetic, would be difficult as any mutants that would be insensitive to apigenin would probably also have altered affinity for ATP. If that were the case, one could potentially mutate downstream kinases individually, making them non-phosphorylatable or constitutively active and examine the effects of apigenin treatment on the phosphorylation of the targets of the kinase. Another potential means to investigate this potentiality would be to use high-throughput CRISPR-A/I screens, which enable flexible upregulation and downregulation of targeted genes, such as the kinases involved in inflammation and metabolic signaling, to more accurately determine which proteins are sufficient and necessary for apigenin's effects and which are not.

Additionally, we did not yet look at the effects of apigenin on the activity of enzymes that use ATP for reasons other than phosphorylation, where there may be a significant contribution of those activities to the effects of apigenin. For instance, as a donor of the ribose moiety used by enzymes such as PARP, or CD38, apigenin is able to affect the DNA damage . Apigenin is known to inhibit CD38 activity, which, although it is only upregulated 2-fold in senescent cells, is a constitutively active enzyme and can over time have a significant effect on the levels of NADH, which may play a role in the pathogenesis of accumulating senescence in tissues.

Lastly, the scope of the above studies was limited to the *in culture* behavior of senescent cell line models of senescence in response to apigenin treatment and did not yet include study of apigenin's effect on senescence or the SASP in an *in vivo* model of senescence. These studies do, however, provide a compelling mechanistic basis for apigenin treatment that readily justifies the increased research expenditures of *in vivo* studies in aged mice. Completion of such studies will prove highly interesting and may provide strong proof of apigenin's benefit in a complex, living model of distributed senescent cells. These results will be critical to fully assess apigenin's potential as a human treatment for the deleterious accumulation of senescent cells. If possible, such studies will be designed to yield as much data as possible about the effects of apigenin on the phosphorylation state, protein dynamics, and transcription levels of the signaling molecules involved in the IL-1A/NFkB/IL-6 and AKT/PI3K/mTOR pathways that apigenin has been shown to affect. Such study design will permit direct comparison of the *in culture* and *in vivo* mechanisms of apigenin treatment in senescence.

6.3. Future Directions

The next steps in the study of apigenin on senescence can be broadly divided into 2 classes: first, the investigation of the effects and mechanism of related bioactive flavonoids and, second, the study of apigenin in translational models of aging.

There are abundant and diverse flavonoids present in many of our food sources that have potent biological effects, yet we understand the mechanisms by which they act and the effects they wreak upon our biology very poorly. This gap has previously been due to the sheer range of flavonoids and the relatively labor-intensive means of studying their effects. However, with the recent rise of high-throughput, multi-modal, functional biological screens, the means by which many different flavonoids exert their effects can be more efficiently investigated than ever before. To that end, a future direction for characterizing the cellular effects of apigenin and other flavonoids in cancer, immune, and tissue cells will involve continued advancement of mass spectrometry methods capable of capturing ever-larger sets of the phospho-proteome and other post-translational modifications. Utilization of such a method, in combination with a variety of senescence-inducing stimuli, will provide an extremely rich dataset revealing new targets critical to the deleterious effects of senescence in addition to elucidation of the mechanisms of action of many flavonoids. This dataset could then be used to more appropriately design therapeutic interventions, both pharmacological and nutriceutical, to stymy the effects of aging in human populations.

Finally, the further study of apigenin in more complex, and expensive, models of senescent cell biology will yield valuable translational insight into the therapeutic potential of apigenin or its derivatives. For example, in the above, we also observed significantly reduced S6 phosphorylation upon apigenin treatment, a biological shift that is also seen with metformin treatment in the extension of animal health- and lifespan. The study of animal models such as aged, otherwise healthy mice as well as transgenic or experimental models of aging-related pathologies, will enable deep characterization of apigenin's effect on aged and chronically-inflamed tissues, where elucidation of the boundaries of the SASP's paracrine effects on surrounding cells will be of particular interest for the effective design and study of senolytic drugs in the future. Such studies will likely also demonstrate that apigenin itself has beneficial effects on senescence and associated parainflammation *in vivo*, where a reduction therein will restore tissue function and stem cell niche activity. Collection of these endpoints will comprise a

valuable translational foundation for the formulation of apigenin as an anti-SASP therapeutic, whose completion will also illuminate the clinical map for additional anti-aging therapies yet to come.

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