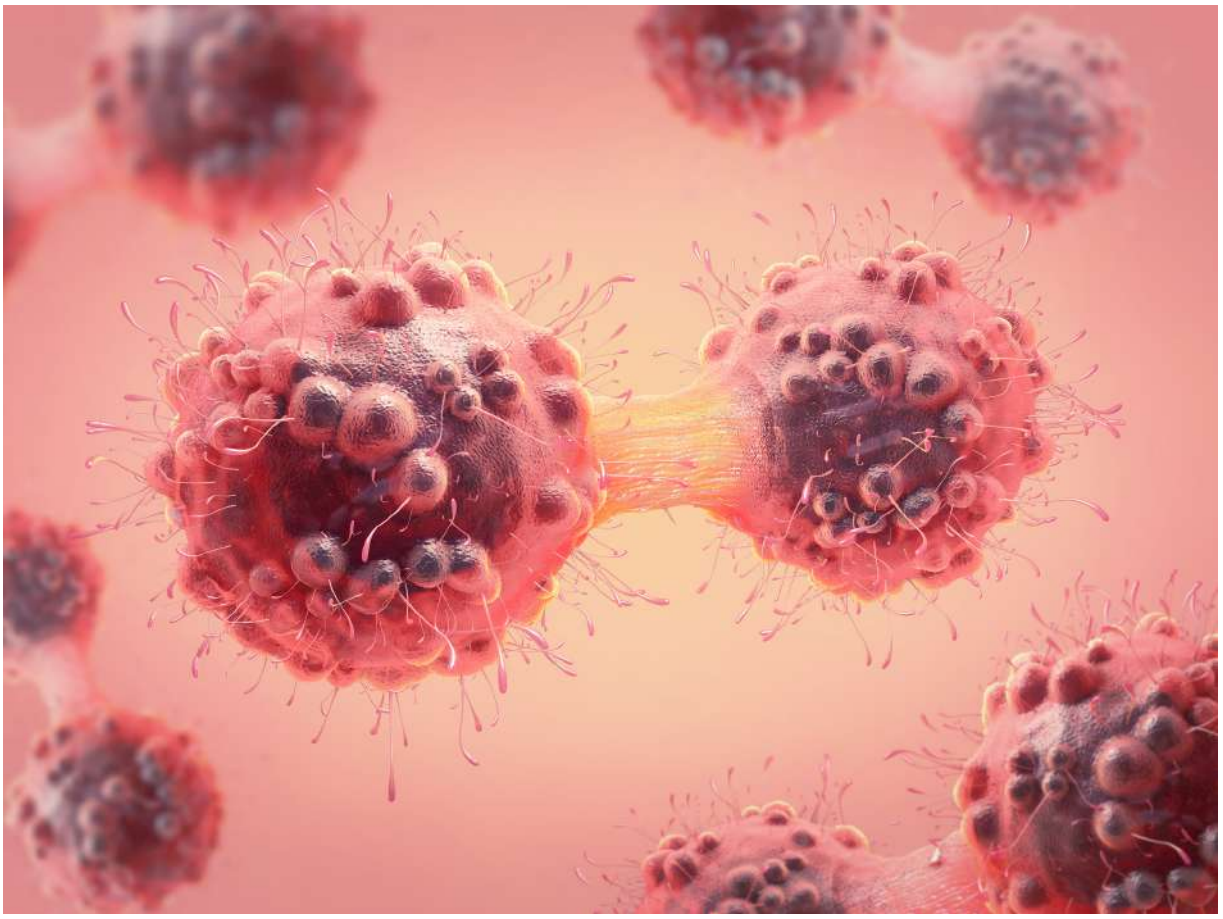
CELLULAR SENESCENCE AND ITS ROLE IN CANCER RESEARCH



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Abstract

Cellular senescence, one of the complex and relatively less known cellular processes, is a permanent state of cell cycle arrest. It promotes tissue remodeling during development and after injury, but can also contribute to the decline in regenerative potential and function of tissues, inflammation, and tumorigenesis in aged organisms. Therefore, the identification, characterization, and pharmacological elimination of senescent cells have become increasingly important in cancer research.

This project is based on investigating whether the two chemotherapeutic drugs (doxorubicin and palbociclib) and radiation therapy induce senescence in vitro and in vivo. Furthermore, to investigate the effective use of senolytics (navitoclax), drugs that remove senescent cells in order enhance and eliminate cancerous processes. For this reason, during the practical session, melanoma cancer cells (in vitro) and tissue sections of subcutaneous tumors from mice (in vivo) were treated with these drugs and with irradiation. It's very difficult to study what senescent cells are doing inside the body, so for any senescence experiment models are needed that can be studied in the lab.

In order to verify the results, the technique of senescence-associated β-galactosidase staining is described and used to identify senescent cells in vitro and in vivo. In this way we have tested various drug compounds for their ability to target senescent cells.

The analysis of results at the end indicates that the chemotherapy and radiation therapy used in the experiment are successful in inducing senescence both in vitro and in vivo. The senolytic agent used also shows its positive effect by reducing the number of senescent cells in different samples used. However the results obtained are not exact or significant in some cases. Hence, it is necessary to repeat the experimental procedures to be able to verify the information already published in other scientific texts. All the details related to the senescence must be continued investigating, it is a very specialized field and that, at the moment, generates more doubts than answers.

Resum

La senescència cel·lular, un dels processos cel·lulars complexos i relativament menys coneguts, és un estat permanent d'aturada del cicle cel·lular. Afavoreix la remodelació dels teixits durant el desenvolupament i després de lesions, però també pot contribuir a la disminució del potencial regeneratiu i de la funció dels teixits, la inflamació i la tumorogènesi en organismes envellits. Per tant, la identificació, caracterització i eliminació farmacològica de cèl·lules senescents ha esdevingut cada cop més important en la investigació del càncer.

Aquest projecte es basa en investigar si dos fàrmacs quimioterapèutics (la doxorubicina i el palbociclib) i la radioteràpia indueixen la senescència in vitro i in vivo. A més a més, també s'investiga l'ús efectiu de fàrmacs senolítics (navitoclax), és a dir, fàrmacs que eliminen cèl·lules senescents, amb la finalitat de millorar i eliminar processos cancerígens. Per aquest motiu, durant la sessió pràctica, es van tractar cèl·lules canceroses de melanoma (in vitro) i seccions de teixit de tumors subcutani de ratolins (in vivo) amb aquests fàrmacs. És molt difícil veure què fan les cèl·lules senescents dins del cos, de manera que per a qualsevol experiment de senescència es necessiten models que es puguin estudiar al laboratori .

Per tal de verificar els resultats, es descriu la tècnica de tinció anomenada β-galactosidasa associada a la senescència, la qual és utilitzada per identificar cèl·lules senescents in vitro i in vivo. D'aquesta manera s'han provat diversos compostos farmacèutics per la seva capacitat d'actuar sobre cèl·lules senescents.

L'anàlisi dels resultats al final indica que la quimioteràpia i la radioteràpia utilitzades en l'experiment tenen èxit a l'hora d'induir la senescència tant in vitro com in vivo. L'agent senolític utilitzat també mostra el seu efecte positiu en la reducció del nombre de cèl·lules senescents en diferents mostres utilitzades. Tanmateix, els resultats obtinguts no són exactes ni significatius en alguns casos. Per tant, cal repetir els procediments experimentals per poder verificar la informació ja publicada en altres textos científics. Cal seguir investigant tots els detalls relacionats amb la senescència, és un camp molt especialitzat i que, de moment, genera més dubtes que respostes.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Introduction

Through this project of research, it is tried to enter into the concept of senescence and its relation with cancer. The particular experiments were performed to determine the effect of senescence on tumors.

There is an attempt to indicate the general relationship between the theoretical knowledge and the experimental part performed. In this way a discussion about the characteristics of senescent cells, which relate them with the tumor suppression process, was made.

Apart from this, specific hypotheses were proposed in each experiment, which later on were tried to be confirmed by experimental techniques detailed in various sections of this project.

1.2 Justification

From the beginning of high school, it was clear that I wanted my research project to be related to science, in particular, to scientific research, as it is to what I would like to dedicate myself in the future.

I have always been curious about cancer studies and research. My interest grew even more when I understood how a benign tumor turns into a malignant tumor known as cancer. I got the opportunity to do research by being part of the program"Crazy about Biomedicine" organized by IRB Barcelona in collaboration with Fundació Catalunya La Pedrera. For this reason I decided to do this project, which is implicitly connected to biomedical research.

1.3 Objectives of the project

- Learning and understanding the procedures and techniques of the laboratory to perform adequate experiments with the use of cell culture.
- Inducing senescence in cells with different inducers (DNA damage to cells by irradiation, use of chemical agents with different concentrations of doxorubicin and palbociclib) and studying the results.
- Understanding the role of senescent cells in the organisms and their identification in vivo using the staining technique of senescence-associated β-galactosidase (SA -β-gal).
- Investigating whether the use of different concentrations of the senolytic agent in these samples kills the zombie cells rather than the normal cells.

2. THEORETICAL FRAMEWORK

2.1 Introduction to cellular senescence

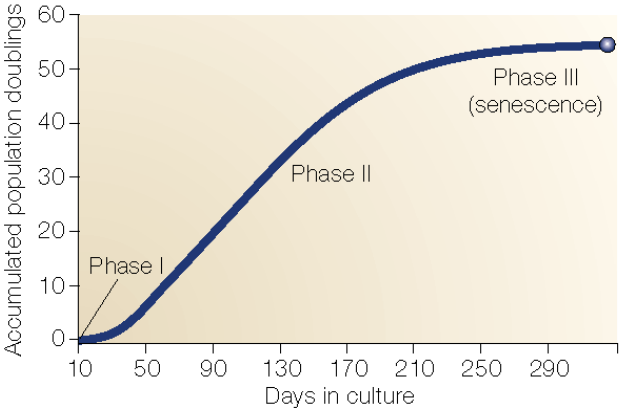
Cellular damage is something that happens to our bodies all the time every day. We are exposed to outside influences, toxins in food, infections, radiation, environmental pollution, etc. The cells in our body are constantly being damaged. These cells are not functioning and can lead to a problem.

Tumor suppressor genes are the genes that respond to damage to protect us from cancer. And the background is that the tumor suppressor genes actually respond to the damage, regardless of the consequences of that damage.

Sometimes the damage is not repaired. Sometimes this happens because the damage is chronic, because we are exposed to constant damage, or sometimes because we get old. The problem with unrepaired damage is that these cells that should be eliminated remain for long periods of time, constantly causing an inflammatory response. This inflammatory response is unsuccessful because it does not repair the tissue, but instead damages it. In this way the state of cellular senescence is reached.

2.1.1 Discovery of cellular senescence by Moorhead and Hayflick

The state of cellular senescence is reached after cells have proliferated in excess or under abnormally strong stimuli, and consists of an **irreversible loss of the ability to divide.** Normal cells are known to have a limited replicative lifespan commonly known as the Hayflick limit. After normal cells enter cell cycle arrest, they enter a senescent phase where they remain metabolically active without undergoing cell death processes.This phenomenon has fascinated researchers for 40 years when it was first described by Moorhead and Hayflick in the early 1960s. It was first observed in fibroblasts from skin and lung in in vitro cultures where it was found that the cells were dividing, but as the culture aged, the cells stopped dividing. They found that the cultures stopped dividing after an average of 50 divisions, which is known as the **Hayflick limit** or Phase III (Figure 1) .



Nature Reviews | Molecular Cell Biology

Figure 1: "Hayflick Limit"

https://www.nature.com/articles/35036093

As the cell divides, the telomeres at the end of a linear chromosome become shorter. Eventually, the telomeres are no longer present on the chromosome (Figure 2) . This terminal stage is the concept that links the deterioration of telomeres to the aging process.

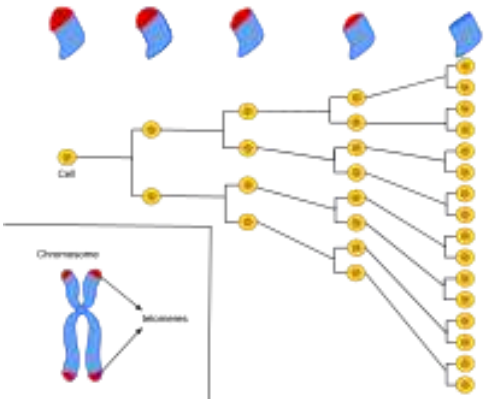


Figure 2: Telomere shortening

https://en.wikipedia.org/wiki/Cellular_senescence

2.1.2 Senescent cells called zombie cells

Zombie cells, formally called senescent cells, are so named because they are damaged and refuse to die. As we age, these damaged cells start to accumulate and cause sterile inflammation[1] that can alter the metabolism and function of stem cells, promoting aging and often related diseases such as Alzheimer's disease, arthritis, cancer, osteoporosis, hypertension, type 2 diabetes, etc. These zombie cells are formally called senescent cells (Figure 3, 4) .

[1] **Inflammation**: It refers to your body's process of fighting against things that harm it, like infections, injuries, and toxins, in an attempt to heal itself.

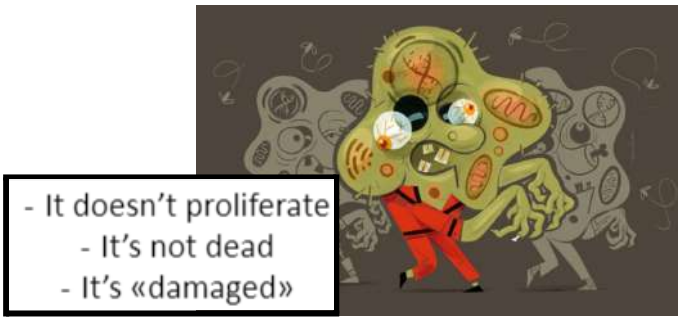


Figure 3: Zombie cells
https://www.nature.com/articles/550448a

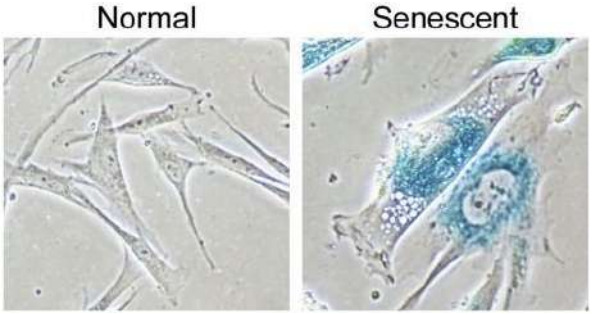


Figure 4: Senescent cells
https://www.manchester.ac.uk/discover/news/new-method-to-detect-ageing-cells--and-aid-rejuvenation-therapies---developed-by-researchers/

2.1.3 Sources of cellular stress/damage

Cellular senescence can be initiated by a wide variety of stress inducing factors (Figure 5). These stress factors include both environmental and internal damaging events, abnormal cellular growth, oxidative and genotoxic stress[2], autophagy[3] factors, oncogene[4] activation, mitochondrial dysfunction, irradiation or chemotherapeutic agents.

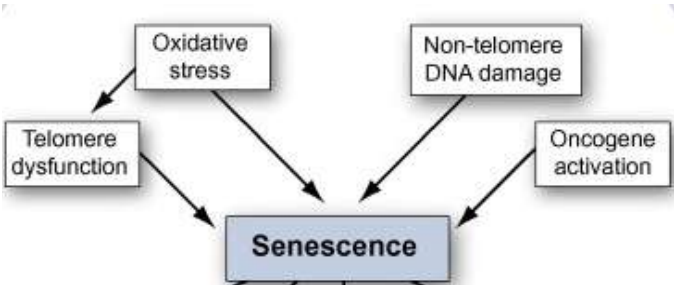


Figure 5: Stress inducing factors

[2] **Genotoxic stress:** Exposure to DNA-damaging agents and subsequent energy expenditures by a cell to repair DNA damage. Genotoxic stress induces biochemical responses that either enhance cell survival or lead to cell death.
[3] **Autophagy:** It is the natural, conserved degradation of the cell that removes unnecessary or dysfunctional components through a lysosome-dependent regulated mechanism.
[4] **Oncogene:** An oncogene is a gene that, when mutated or expressed at high levels, helps turn a normal cell into a tumor cell.

As a result, there are three different possible cellular responses to stress/damage, as shown in the figure below (Figure 6): the ability to resume proliferation, undergo cell death, or entering the stage of senescence. Therefore, senescence is one of the ways to respond to damage and limit cell division.

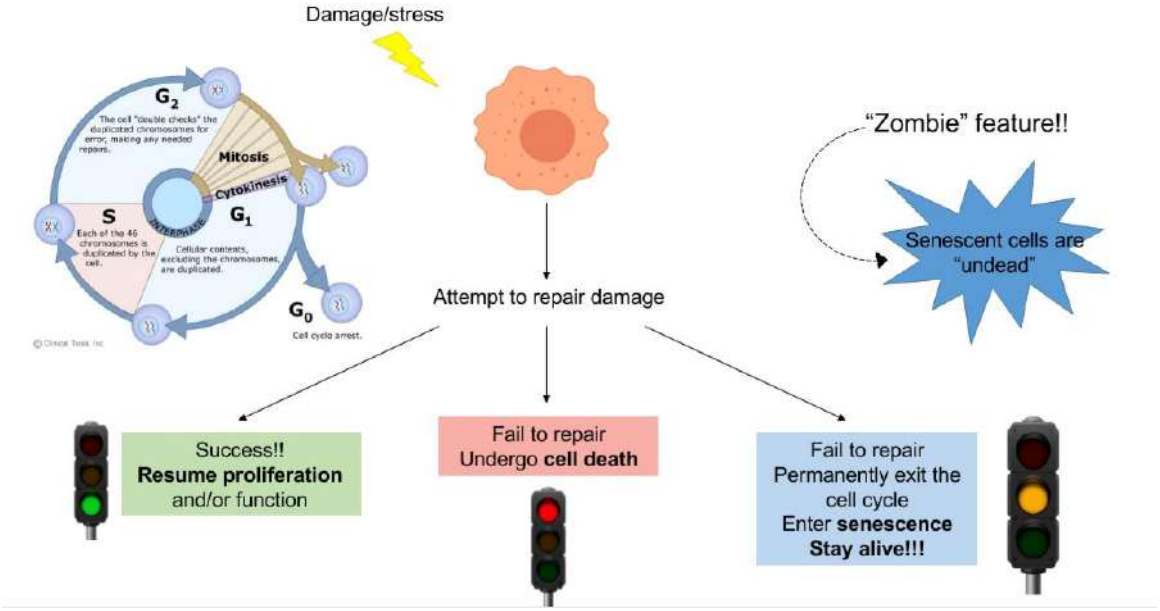


Figure 6: Possible cellular responses to stress/damage

2.1.4 DNA damage response

Senescence is associated with a persistent DDR that results in irreparable DNA damage. The senescence growth arrest triggered by a persistent DNA damage response (DDR) is caused by either intrinsic (oxidative damage, telomere[5] weakening, hyperproliferation) or external insults (ultraviolet, γ-irradiation, chemotherapeutic drugs). For instance, during replicative senescence of human fibroblasts, progressive telomere shortening occurs which leads to DDR. Also in cells undergoing oncogene induced senescence (OIS), DDR can be induced which is associated with telomere dysfunction.

2.1.5 Senescence- associated secretory phenotype (SASP)

Senescent cells have the ability to signal and influence their environment. Senescent cells produce a complex mixture of soluble and insoluble factors, collectively known as the senescence-associated secretory phenotype (SASP) or senescence message secretome. SASP is the general term for the combination of cytokines, chemokines, extracellular matrix proteases, growth factors, and other signaling molecules secreted by senescent cells. Recent evidence suggests that the functionality of

[5] **Telomere:** A telomere is a region of repetitive nucleotide sequences associated with specialized proteins at the ends of linear chromosomes.

senescent cells in vivo is strongly dependent on the effects of SASP on the environment and associated immune responses[6].

However, the effects of SASP are difficult to predict. On the one hand, the composition of secretome (SASP) of senescent cells depends not only on the stage of senescence but also on the affected cell type and the nature of the inducing stressor. The efficiency and kinetics of elimination of senescent cells may vary depending on the organ in which they accumulate or on their overall ability to mount an effective immune response. For example, while cells undergoing oncogene-induced senescence (OIS) in the liver are efficiently eliminated by the immune system, senescent cells in melanocytic nevi[7] often manage to evade elimination by the immune system and survive. The SASP can have beneficial or harmful effects (Figure 7).

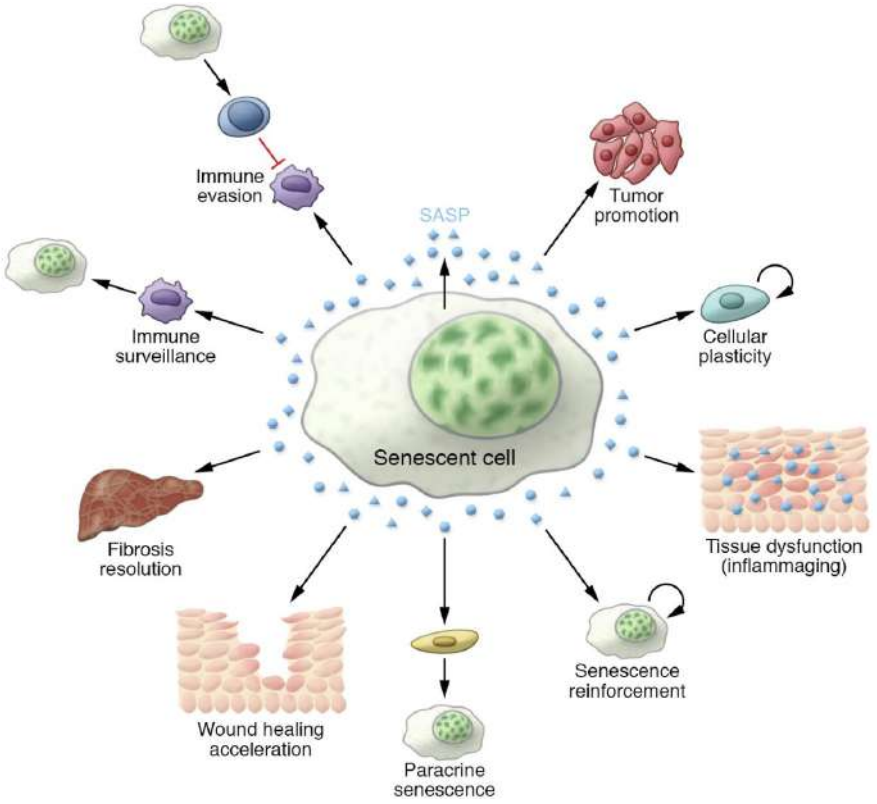


Figure 7: The SASP is an important mediator of the pathophysiological functions of senescent cells. Scheme summarizing some of the functions associated with the SASP.
https://www.jci.org/articles/view/95148/pdf

[6] **Immune response:** The reaction of the cells and fluids of the body to the presence of a substance which is not recognized as a constituent of the body itself.

[7] **Melanocytic nevi :** It is a skin condition characterized by an abnormally dark, noncancerous skin patch (nevus) that is composed of pigment-producing cells called melanocytes.

The SASP enhances growth arrest of senescence in vitro through an autocrine positive feedback loop. This autocrine loop contributes to the tumor-suppressive function of senescence. Interestingly, the SASP can also induce nonmalignant proliferating neighbor cells to undergo senescence termed as paracrine senescence (Figure 8). This suggests that senescent cells could also amplify the antitumoral response by limiting the proliferation of nearby cells exposed to similar stressors.

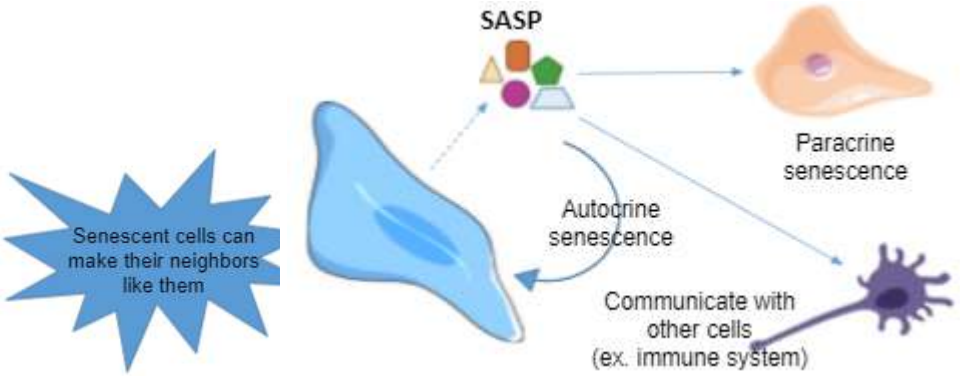


Figure 8: Paracrine senescence

2.1.6 Senescence as a dynamic program

Senescence has been traditionally considered as a defined, static cell fate. However, it is now recognized that senescence is a dynamic multistep process. A simplified model suggests that although the initial senescence-inducing signals are sufficient to initiate cell cycle exit[8], this is only an early step in the senescence process. Senescent cells progressively remodel their chromatin[9] and start to successively implement other aspects of the senescence program, such as the SASP, to enter into a second step of “full senescence.” If these senescent cells persist over time, they will continue to develop and can be classified as entering into a third stage of "late senescence," which can involve adaptation and diversification of the senescent phenotype. It is tempting to suggest that the concept of senescence progression may help explain the heterogeneity of senescent cells and associated phenotypes in vivo (Figure 9).

[8] **Cell cycle exit:** The stage when cells stop dividing and enter a quiescent state, also named G0.
[9] **Chromatin**: It is a complex of DNA and protein found in eukaryotic cells in condensed form.

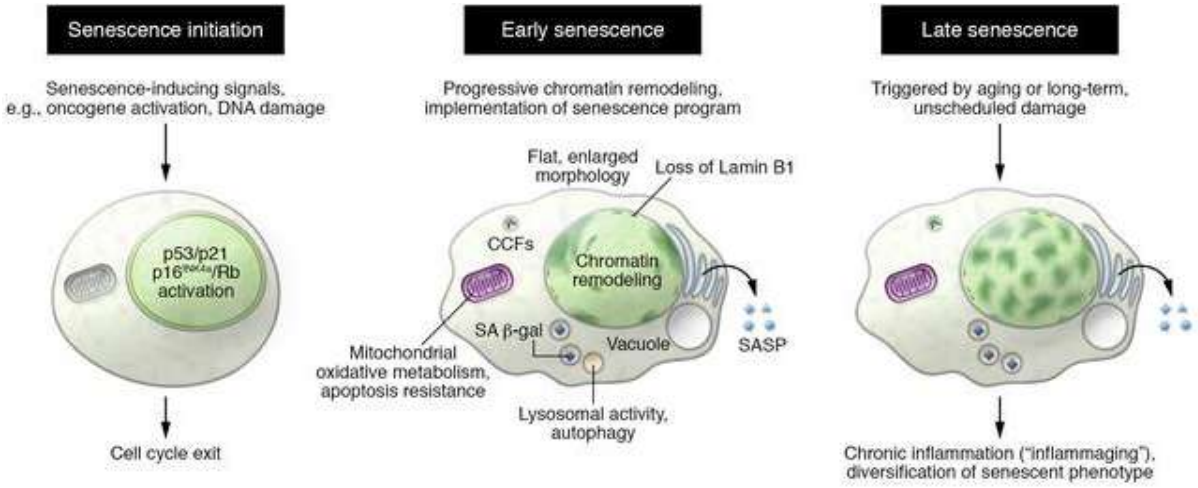


Figure 9: Phenotypic characteristics of senescent cells. Diagram depicting some of the phenotypic alterations associated with senescence initiation, early senescence, and late phases of senescence.
https://www.jci.org/articles/view/95148/figure/1

Indeed, the senescent responses occurring in vivo can be categorized into two types: acute and chronic. Acute senescence seems to be a programmed process that is triggered in response to single stressors, develops rapidly, and normally contributes to tissue homeostasis[10]. In contrast, chronic senescence may result from

long-term unscheduled damage, and it is often associated with detrimental processes such as aging. Only chronic senescence is associated with the presence of cells in a state of late, deep senescence.

2.1.7 Cell cycle arrest & concept of cellular aging

One of the characteristic features of senescent cells is their stable cell cycle arrest. Unlike quiescent cells[11], senescent cells do not respond to mitogenic or growth-promoting stimuli; therefore, they are unable to resume the cell cycle even under favorable growth conditions.
Senescent cells differ from terminally differentiated cells[12], which are also irreversibly eliminated from the cell cycle. While terminal differentiation is the result of a defined developmental program that transforms undifferentiated progenitor cells into

[10] **Tissue homeostasis:** A property of cells, tissues, and organisms that allows the maintenance and regulation of the stability and constancy needed to function properly.

[11] **Quiescent cells:** The cells that enter in a reversible cell state in which it does not divide but retains the ability to re-enter cell proliferation.

[12] **Terminally differentiated cells:** The cells that, in the course of acquiring specialized functions, have irreversibly lost their ability to proliferate.

specialized effector cells, senescence is mainly implemented as a cellular stress response.

This cell cycle exit is controlled by activation of the tumor suppressor pathways p53/p21CIP1 and p16INK4a/Rb that lead to cellular senescence, as shown below (Figure 10). Both pathways are activated in response to cellular stressors and result in cell cycle arrest. p53[13] activates p21[14], which deactivates cyclin-dependent kinase 2 (Cdk 2)[15]. In the absence of Cdk 2, the retinoblastoma protein (pRB)[16] remains in its active, hypophosphorylated form and binds to the transcription factor E2F1, a key cell cycle regulator. This represses the transcriptional targets of E2F1, leading to cell cycle arrest after G1 phase[17].

p16Ink4a[18] also activates pRB, but by inactivating cyclin-dependent kinase 4 (Cdk 4) and cyclin-dependent kinase 6 (Cdk 6). p16Ink4a is responsible for the induction of premature stress-induced senescence. This is not irreversible; silencing of p16Ink4a by methylation of the promoter or deletion of the p16Ink4a locus allows the cell to resume the cell cycle once senescence has been initiated by activation of p16Ink4a.

[13] **p53:** P53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein that in humans is encoded by the TP53 gene.

[14] **p21:** $p21^{Cip1}$ (also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1), is a cyclin-dependent kinase inhibitor (CKI) that is capable of inhibiting all cyclin/CDK complexes, though is primarily associated with inhibition of CDK2.

[15] **Cyclin-dependent kinase 2 (Cdk 2):** It is an enzyme that in humans is encoded by the *CDK2* gene. It is also known as cell division protein kinase 2.

[16] **Retinoblastoma protein (pRB):** It is a tumor suppressor protein that is dysfunctional in several major cancers. One function of pRb is to prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide.

[17] **G1 phase:** It is the first of four phases of the cell cycle that takes place in eukaryotic cell division.

[18] **p16Ink4a:** p16Ink4a is a protein involved in the regulation of the cell cycle. Currently, p16Ink4a is considered a tumor suppressor protein because of its physiological role and downregulated expression in a large number of tumors.

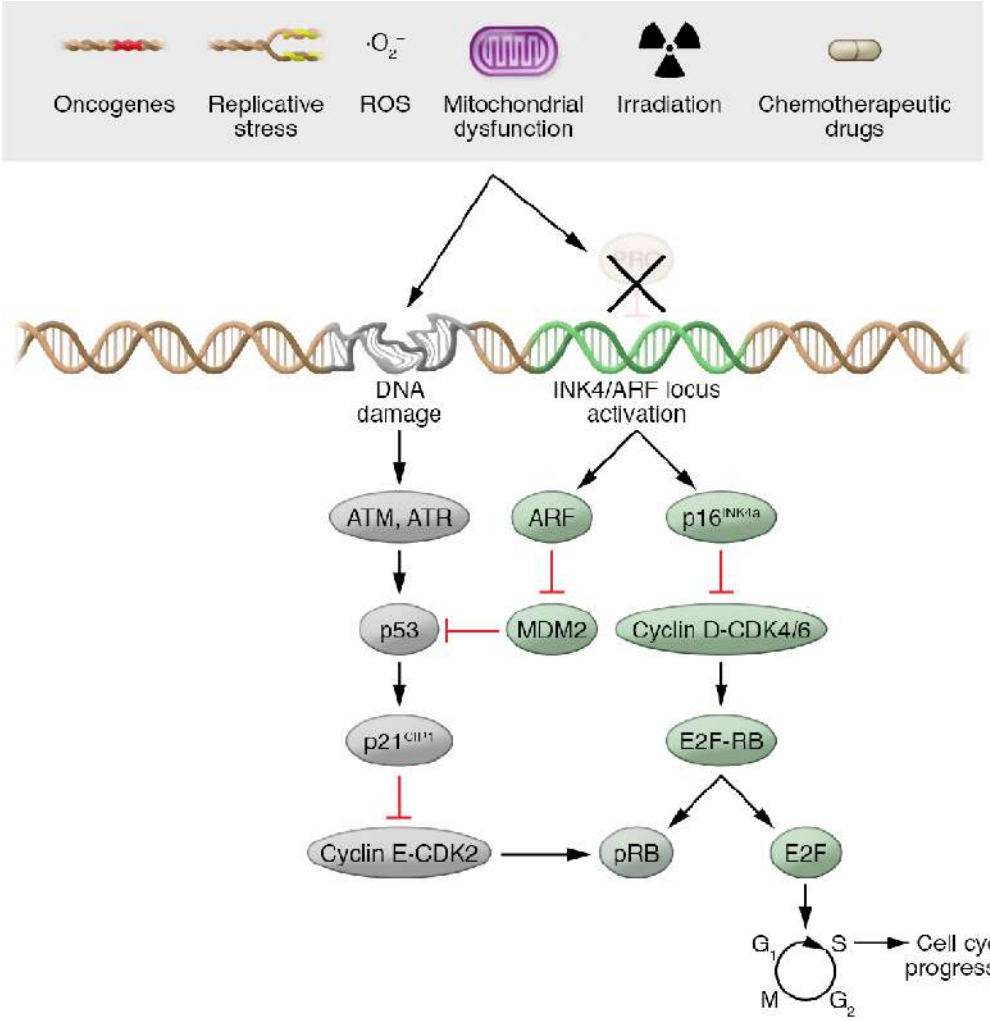


Figure 10: Molecular pathways controlling growth arrest during senescence. A variety of stressors induce senescence-associated growth arrest. Cell cycle exit is regulated by induction of the p16INK4a/Rb and p53/p21CIP1 pathways.
https://europepmc.org/article/med/29608137

Concept of cellular aging

In modern usage, the term senescence implies a permanent cell cycle arrest. In other words, even if other changes have occurred previously, a cell is not considered senescent until permanent cell cycle arrest has been achieved. However, because changes in primary cells occur gradually and continuously long before cell cycle arrest, a new term is needed to encompass both these early changes and what happens after the onset of permanent cell cycle arrest. The term "**cellular aging**" describes a gradual decrease in cell function and an increase in the likelihood of cell death, "**replicative aging**" refers to dividing cells prior to the induction of senescence, while "**cellular senescence**" is a cellular program initiated by the induction of permanent cell cycle arrest that increases cell inflammation and prevents cell proliferation (Figure 11,(a),(b)).

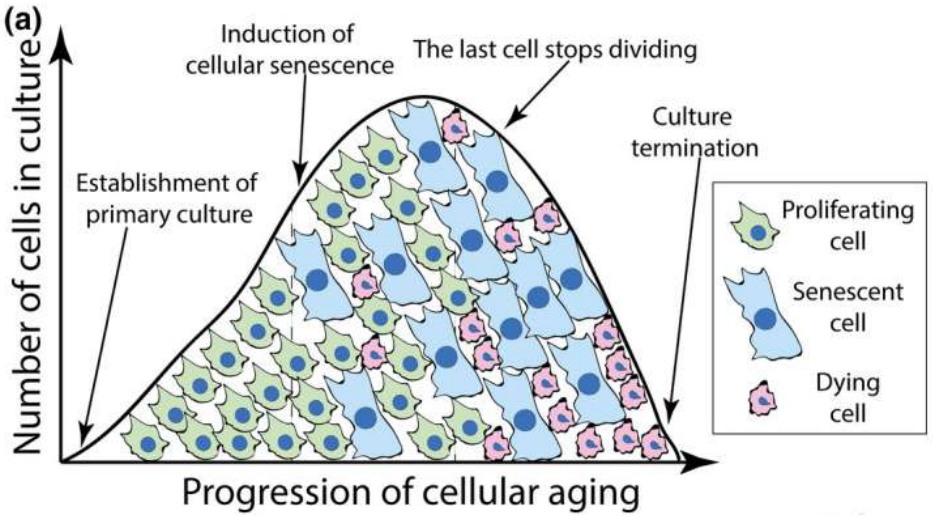


Figure 11 (a): The lifespan curve of primary cells in culture. After the culture establishment, cells enter into a phase characterized by exponential growth, which is followed by induction of senescence and a decline in growth rate. The final stage is a post-senescence continuation of cellular aging and degradation of cell population. The graph simplifies cellular aging and does not show the transition gradient.

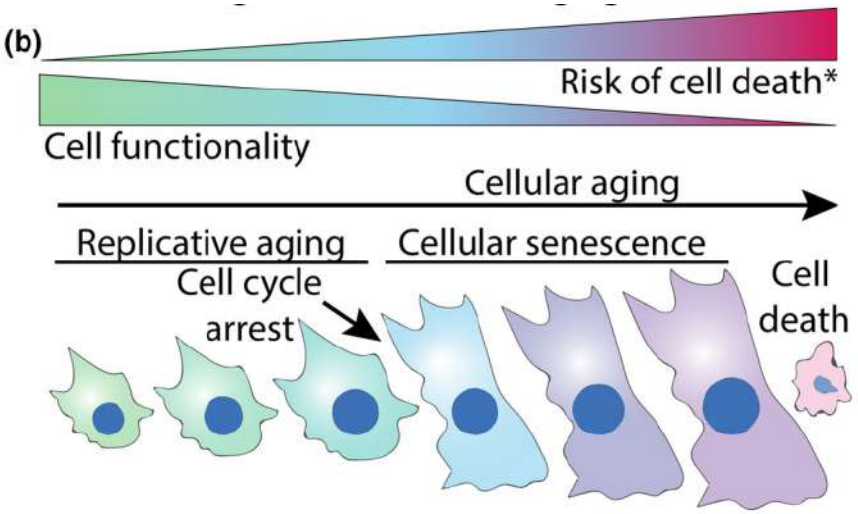


Figure 11(b): Cellular aging is a gradual process, which reduces cell functionality and increases **risk of cell death** over time. (*) The changes in the risk of death are unlikely to be linear, for example, cells shortly after senescence induction might be less prone to death than younger cells.
https://onlinelibrary.wiley.com/doi/10.1111/acel.13338

2.1.8 Metabolism, autophagy, and mitochondrial function,apoptosis resistance in senescent cells

Senescent cells are highly metabolically active as there are cellular processes still going on in the cytoplasm. They exhibit metabolic changes, such as an increase in

glycolysis[19], mitochondrial metabolism, and autophagy. The high production of components of SASP is due to increased ATP production mediated by mitochondrial metabolism and glycolysis. In addition, senescent cells activate several prosurvival acting factors and become resistant to apoptosis[20].

2.1.9 Morphological alterations in senescent cells

Senescent cells develop characteristic morphological changes, which include increased lysosomal content[21], enlarged and often irregular nuclei and chromatin reorganization. They have large but dysfunctional mitochondria that produce high levels of reactive oxygen species (ROS)[22], and their plasma membrane (PM) changes composition. Lamin-B1 is a nuclear intermediate filament protein that exhibits effects on chromatin structure and gene expression[23] and acts as a senescence effector. Therefore, it was examined that the Lamina B1 expression decreases during cellular senescence, which also leads to the appearance of cytoplasmic chromatin fragments (CCFs)[24] (Figure 12).

[19] **Glycolysis:** Glycolysis is the metabolic pathway that converts glucose $C_6H_{12}O_6$, into pyruvic acid, $CH_3COCOOH$. The free energy released in this process is used to form the high-energy molecules adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH).

[20] **Apoptosis**: It is a form of programmed cell death that occurs in multicellular organisms.

[21] Lysosomal content: It refers to a wide variety of hydrolytic enzymes (that break down macromolecules) present inside the membrane-bound cell organelle called lysosome.

[22] **Reactive oxygen species (ROS):** Oxygen reactive species or ROS are molecules that are produced in cells resulting from incomplete oxidation-reduction reactions in which oxygen is involved. Examples of ROS include peroxides, superoxide, hydroxyl radical,etc.

[23] **Gene expression:** It is the process by which the instructions in our DNA are converted into a functional product, such as a protein.

[24] **Cytoplasmic chromatin fragments (CCFs):** Senescent cells shed fragments of nuclear chromatin into the cytoplasm, so called cytoplasmic chromatin fragments (CCF).

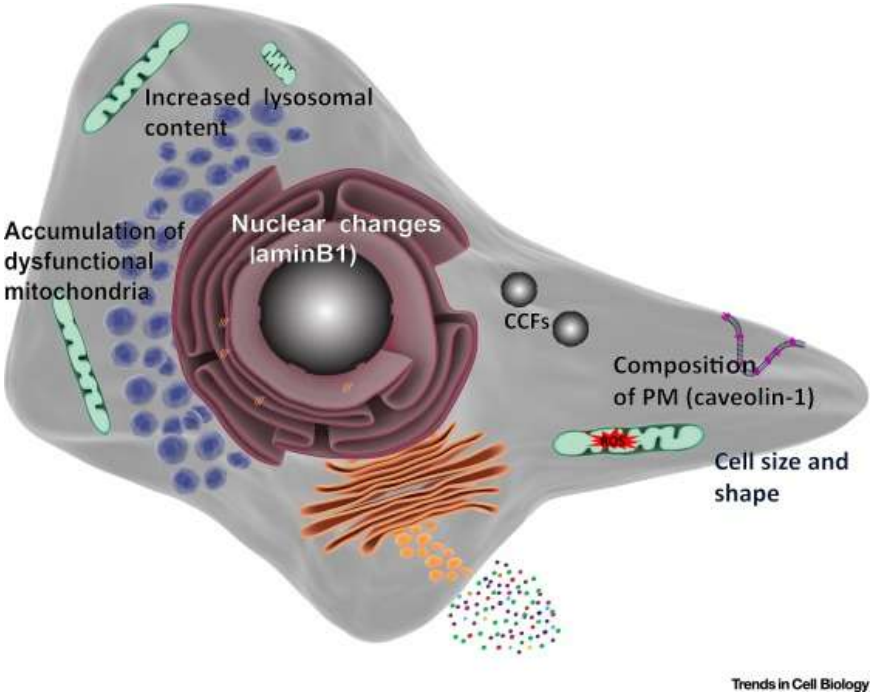


Figure 12: The molecular pathways of senescence result in morphological alterations.
https://www.cell.com/trends/cell-biology/comments/S0962-8924(18)30020-5#relatedArticles

2.1.9.1 Cell size and shape

A major feature of in vitro senescence is the enlarged and irregularly shaped cell body. A contribution to the altered cell shape associated with senescence is the rearrangement of the cytoskeleton[25], mainly of vimentin[26] filaments.

However, the changes in size and morphology are easily measured by normal or fluorescence microscopy, but are difficult to detect and quantify in vivo or in situ.

2.1.9.2 Composition of plasma membrane

The plasma membrane (PM) plays a central role in communication with neighboring cells and the extracellular space. The most consistent change in the composition of

[25] **Cytoskeleton:** It is a complex, dynamic network of interlinking protein filaments present in the cytoplasm of all cells
[26] **Vimentin:** It is one of the fibrous proteins that form the intermediate filaments of the intracellular cytoskeleton.

the PM in senescent cells is the upregulation of caveolin-1[27] which is a structural protein component of caveolae[28].

2.1.9.3 Increased lysosomal content

The state of senescence is characterized by upregulation of many lysosomal proteins and increased lysosomal content. The activity of the lysosomal enzyme[29] senescence-associated beta-galactosidase (SA -gal) is used as a surrogate marker for the increased lysosomal content of senescent cells.

Increased lysosomal content during senescence could be the result of accumulation of old lysosomes or increased lysosomal biogenesis.

2.1.9.4 Accumulation of mitochondria

Senescent cells have an increased number of mitochondria. However, the membrane potential of these mitochondria is decreased, resulting in the release of mitochondrial enzymes, such as endonuclease G (EndoG), and increased production of ROS (reactive oxygen species).

The main source of the extra mitochondrial content is the accumulation of old and dysfunctional mitochondria due to decreased mitophagy[30]. This is at least partly a consequence of decreased mitochondrial fission and increased fusion, possibly as a mechanism to protect mitochondria from mitophagy and senescent cells from apoptosis.

2.1.9.5 Nuclear changes

A common feature of senescent cells is the loss of LaminB1, a structural protein of the nuclear lamina. The destabilization of nuclear integrity caused by the reduction of LaminB1 leads to other nuclear changes, such as the loss of condensation of

[27] **Caveolin-1:** It is a structural protein component of caveolae which functions as a scaffolding protein, regulating different signal transduction pathways and interacting with various molecules, such as cholesterol.

[28] **Caveolae:** These are omega-shaped plasma membrane invaginations.

[29] **Enzyme**: It is a substance that acts as a catalyst in living organisms, regulating the rate at which chemical reactions proceed without itself being altered in the process.Enzymes are known as biocatalysts.

[30] **Mitophagy:** Mitophagy is the selective degradation of mitochondria by autophagy. It often occurs due to defective mitochondria following damage or stress.

constitutive heterochromatin[31] and the appearance of cytoplasmic chromatin fragments (CCFs) enriched in epigenetic marks associated with DNA damage.

2.1.10 Methods of inducing senescence

2.1.10.1 Hayflick's model: Replicative Senescence (RS)

Normal somatic cells are capable of only a limited number of divisions. This phenomenon, known as replicative senescence or Hayflick's limit, depends on the shortening of telomeres, which are repetitive DNA sequences at the end of linear chromosomes. In cells that naturally lack pathways to maintain telomere length, telomere length shortens each time the cell divides (Figure 13). When telomere length reaches a critically short size, senescence or apoptosis is triggered by DNA damage response pathways. The rate of telomere weakening is influenced by several factors, including the level of genotoxic stress in the cell and the presence of reactive oxygen species (ROS).

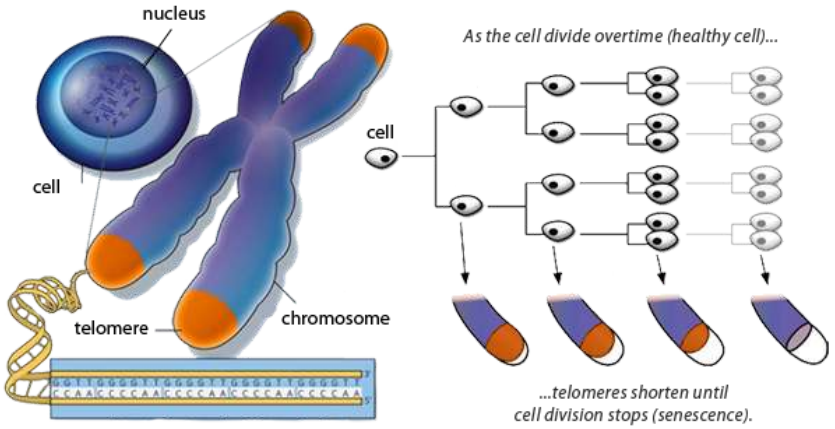


Figura 13: Replicative senescence (RS)
https://tmhome.com/wp-content/uploads/2015/12/telomeres-telomerase-increase-meditation.jpg

2.1.10.2 Oncogene Induced Senescence (OIS)

Oncogene-induced senescence (OIS) is a robust and sustained antiproliferative response brought about by oncogenic signaling resulting from an activating

[31] **Heterochromatin** : It is a tightly packed form of DNA or condensed DNA, which comes in multiple varieties and is the second structural degree of condensation of chromatin, after euchromatin.

mutation of an oncogene or inactivation of a tumor suppressor gene[32]. The pathways mediating OIS are complex and incompletely described, but proliferation arrest involves activation of both the RB and p53 signaling pathways[33].

2.1.10.3 Therapy Induced Senescence (TIS)

Senescence can also be induced by chemotherapy, radiation therapy, targeted therapies, etc (Figure 14).

Figure 14: Therapy Induced Senescence (TIS)

In addition, senescence can be induced by other harms such as:

- Environmental damage (UV light, pollution, cigarette smoke, etc.).
- Metabolism/metabolic by-products.
- Metabolites[34] from our gut microbiota
- Reactive oxygen species (ROS)

2.1.11 Methods of detecting senescence

The detection of senescent cells has become an important area of research in the cancer field. The ways to detect them include senescence associated beta-galactosidase (SA-beta-gal), proliferation arrest, chromatin changes & senescence-associated secretory phenotype (SASP).

Proliferation arrest involves the proliferation markers which are used for detecting senescence. If a cell “in vitro” is found to be negative for a marker of proliferation, it is likely this cell is arrested in cell cycle and possibly senescent. Another approach to

---

[32] **Tumor suppressor gene:** It is a normal gene that slows down cell division, repair DNA errors, or tell cells when to die (a process known as *apoptosis* or *programmed cell death*). When tumor suppressor genes do not function properly, cells can grow out of control, which can lead to cancer.

[33] **Signaling pathway:** It describes a group of molecules in a cell that work together to control one or more cell functions, such as cell division or cell death.

[34] **Metabolites:** They are small molecules produced as intermediates or end products of microbial metabolism.

detect cell cycle arrested cells involves assessing the expression levels of cell cycle inhibitors such as p16 or p21(Figure 15).

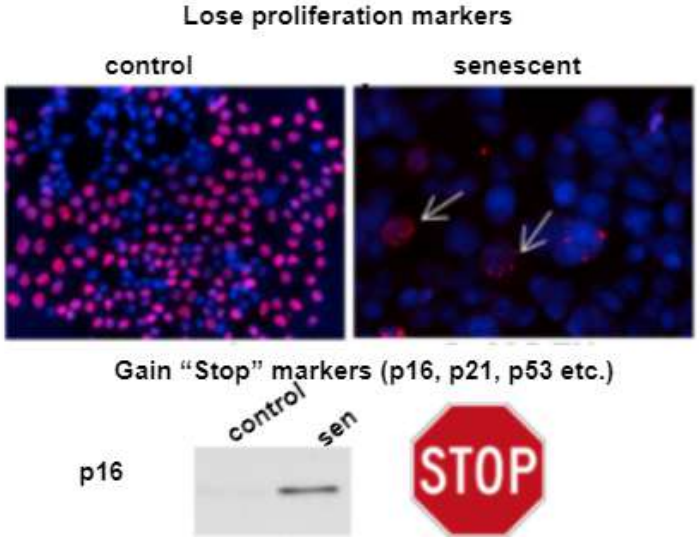


Figure 15 :Proliferative arrest

The chromatin changes in senescent cells involve DNA damage, histone methylation[35] and changes in gene expression (Figure 16).

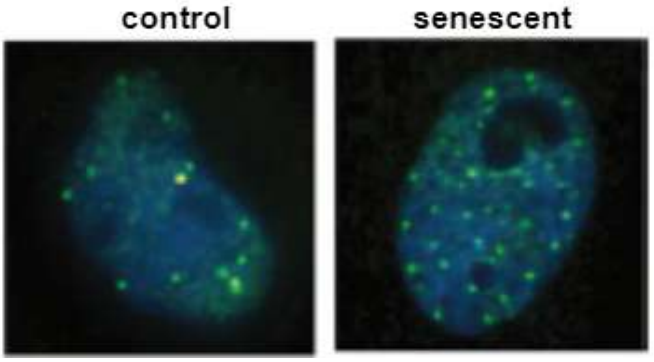


Figure 16: Chromatin changes

2.1.11.1 Senescence-associated β-galactosidase staining (SA-β-gal)

The most widely used senescence marker is senescence-associated β-galactosidase (SA β-gal) activity. This enzymatic activity, which is found in many normal cells under physiological conditions (pH 4.0–4.5), is significantly amplified in senescent cells as a result of increased lysosomal content. Because of this, histochemical detection of β-gal activity at pH 6.0 (suboptimal for normal cells) allows specific identification of senescent cells. The method to detect SA-β-gal is a convenient, single cell-based assay, which can identify senescent cells even in heterogeneous cell populations, aging tissues and cancer tissues. Because it is easy

[35] **Histone methylation:** It is the modification of certain amino acids in a histone protein by the addition of one, two, or three methyl groups.

to detect, SA-β-gal is used as a biomarker of senescence in the practical sessions of my project (Figure 17).

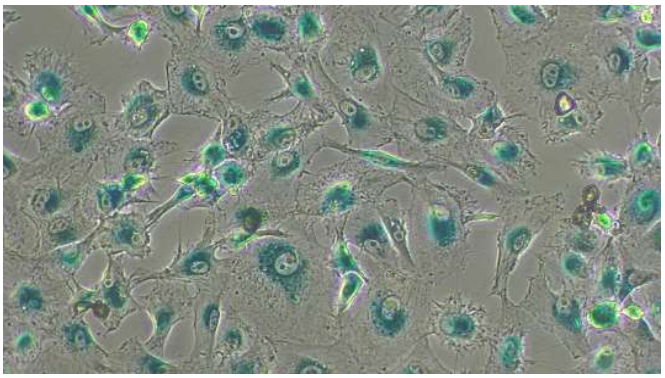


Figure 17 : Senescence-associated β-galactosidase staining
From: Institute of Research Barcelona, 2021

2.2 Bright side of senescence

Senescent cells have several positive functions for the organism. Senescence can be a protective stress response.

2.2.1 Cellular senescence is a critical barrier to carcinogenesis (tumor suppression)

Genomic instability[36] increases during aging and is considered one of the main features of cancer cells. By preventing proliferation of aged and DNA-damaged cells, cellular senescence is considered a strong protection against tumorigenesis[37]. Similar to non-tumor cells, senescent cancer cells adopt SASP and develop modified receptor expression that alters their signaling pathways.

Cellular senescence is also a critical barrier to transformation that plays a role in malignant progression from benign tumors such as colon adenoma to carcinoma and melanocytic nevi to melanoma. For example, the process of senescence prevents melanocytic nevi, a common benign skin lesion caused by the local proliferation of melanocytes containing melanin pigment, from growing into malignant cells and thus transforming into melanoma. As can be seen in the figure below (Figure 18), the senescent cells are present in melanocytic cells (nevi), whereas they are not present in normal skin.

[36] **Genomic instability:** The increased tendency for DNA mutations (changes) and other genetic changes to occur during cell division.
[37] **Tumorigenesis:** It refers to the initial formation of a tumor in the body.

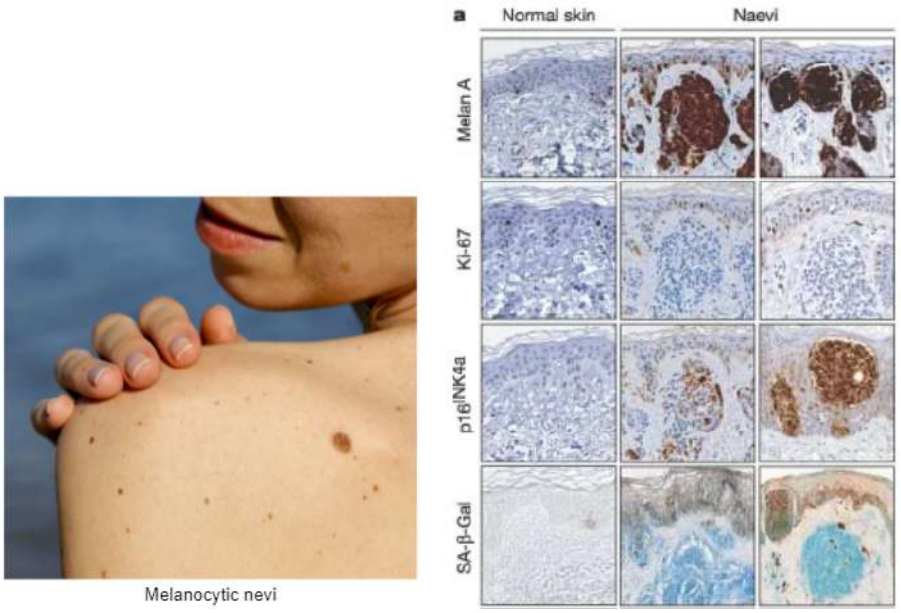


Figure 18: Human melanocytic naevi display the hallmarks of senescent cells

https://www.nature.com/articles/nature03890

2.2.2 Cellular senescence is critical for normal development and tissue repair

Tissue repair is necessary for all life. Although it rarely results in complete regeneration, the process prevents exsanguination[38] and infection and supports the structural and functional recovery necessary for survival. Tissue repair involving multiple cell types and overlapping processes that broadly include hemostasis[39], inflammation, cell proliferation, and skin remodelling.

During embryogenesis[40], cellular senescence is tightly regulated, induced at specific times and locations, and plays a role in morphogenesis[41], tissue remodeling[42], and cell population balance.

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[38] **Exsanguination:** Death caused by loss of blood.

[39] **Hemostasis**:It is the natural process in which blood flow slows and a clot forms to prevent blood loss during an injury.

[40] **Embryogenesis:** It is the complex generative process that leads to the formation of a multicellular organism, plant or animal, from the zygote.

[41] **Morphogenesis:** It is a biological process that causes a tissue or organ to develop its shape by controlling the distribution of cells during embryonic development.

[42] **Tissue remodeling:** It is broadly defined as the reorganization or restoration of existing tissues. Tissue remodeling processes are responsible for directing the development and maintenance of tissues, organs, and overall morphology of an organism.

Cellular senescence plays an important role in tissue protection and regeneration. When cells are exposed to damage, they enter the stage of senescence, followed by the production of the senescence-associated secretory phenotype (SASP). Specific components of the SASP factors secreted by senescent cells attract and activate the immune system and immune cells[43] (macrophages, lymphocytes) with the aim of eliminating the senescent cells and regenerating the tissue (Figure 19).

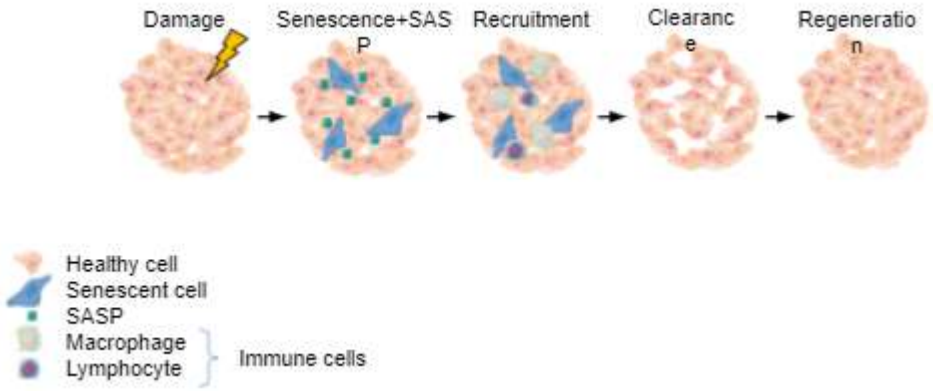


Figure 19: Role of cellular senescence in tissue protection and regeneration

2.2.3 Cellular senescence role in wound healing

Induction of cellular senescence is observed in the initial stages of tissue repair, suggesting that wound healing and tissue regeneration rely on a similar mechanism of cellular senescence to that observed during embryonic development. Following injury, senescent fibroblasts and endothelial cells secrete a variety of proteins that promote myofibroblast[44] differentiation and granulation tissue production[45] to initiate wound healing.

As shown below, senescent cells accumulate in wounds over time as a result of healing of that wound. Whereas, in the case where senescent cells are not present, the wound takes more time to heal (Figure 20).

[43] **Immune cells:** The cells that are part of the immune system and help the body fight infections and other diseases. The types of immune cells include lymphocytes (found in the blood and lymph tissue) and macrophages (large white blood cells that reside in tissues that specialize in engulfing and digesting cellular debris, pathogens and other foreign substances in the body).
[44] **Myofibroblast:** It is a differentiated cell type essential for wound healing which are typically activated fibroblasts.

[45] **Granulation tissue production:** Granulation tissue is produced during the repair phase. This is a complex of fibroblasts, vascular endothelial cells, and macrophages within a matrix of collagen and fibrin.

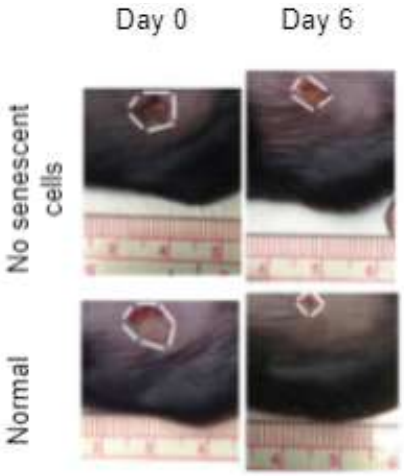


Figure 20: Wound healing

2.3 Cancer

2.3.1 The Definition of Cancer

Cancer is a disease in which some cells of the body grow uncontrollably and spread to other parts of the body. Cancer is characterized by the fact that cells in the human body are constantly multiplying and cannot be controlled or stopped (Figure 21).

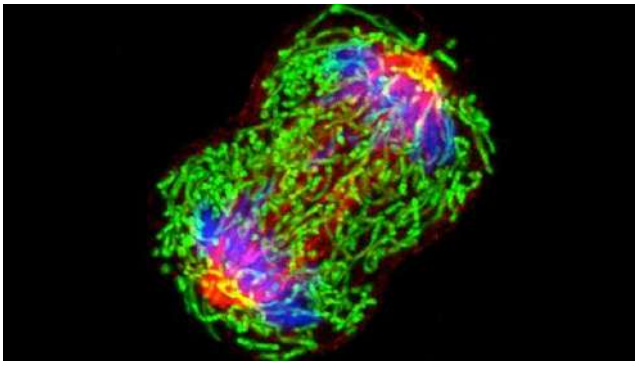


Figure 21: A dividing breast cancer cell.

Credit: National Cancer Institute / Univ. of Pittsburgh Cancer Institute

Cancer can develop almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and multiply (through a process called cell division) to make new cells when the body needs them. When cells age or become damaged, they die and new cells take their place. Sometimes this orderly process breaks down, and abnormal or damaged cells grow and multiply when they should not (Figure 22). These cells can form tumors, which are lumps of tissue.

Tumors can be cancerous (malignant) or noncancerous (benign).

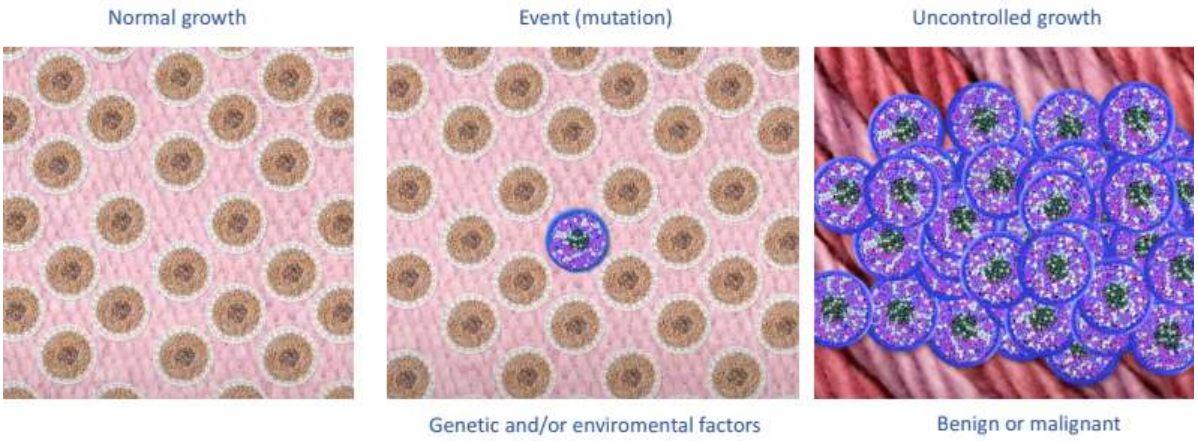


Figure 22: Transformation of normal growth into uncontrolled growth

Cancerous tumors or malignant tumors spread or invade nearby tissues and may travel to distant sites in the body to form new tumors (a process called metastasis). Many cancers form solid tumors, but cancers of the blood, such as leukemias, generally do not. By definition, the term "cancer" refers only to malignant tumors.

Benign tumors do not spread or invade nearby tissue. Therefore, they grow only locally. When removed, benign tumors usually do not grow back, while malignant tumors sometimes do. However, benign tumors can sometimes be quite large. Some can cause serious symptoms or be life-threatening, such as benign tumors in the brain (Figure 23).

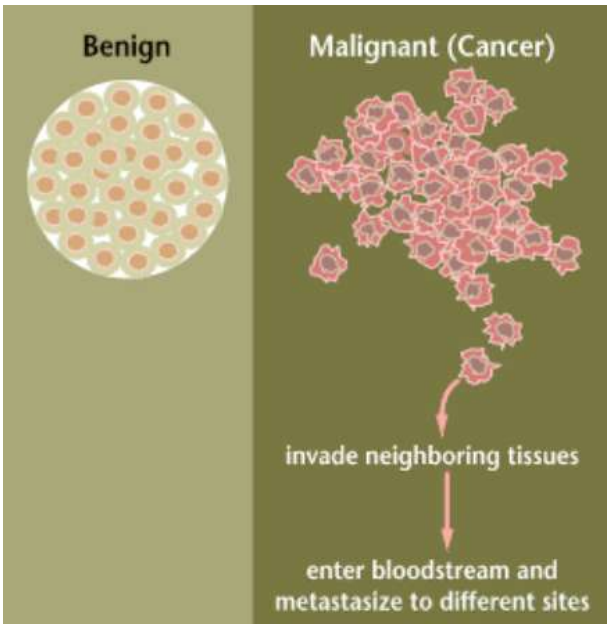


Figure 23: Benign & malignant Tumor

From: National Cancer Institute: http://press2.nci.nih.gov/sciencebehind/cancer/cancer00.htm

Normal cells can become cancer cells. Before cancer cells form in the tissues of the body, the cells undergo abnormal changes called hyperplasia and dysplasia. In hyperplasia, there is an increase in the number of cells in an organ or tissue that appear normal under a microscope. In dysplasia, the cells look abnormal under the microscope but are not cancerous. Hyperplasia and dysplasia may or may not become cancer.In general, the more abnormal the cells and tissue look, the more likely it is that cancer will form (Figure 24).

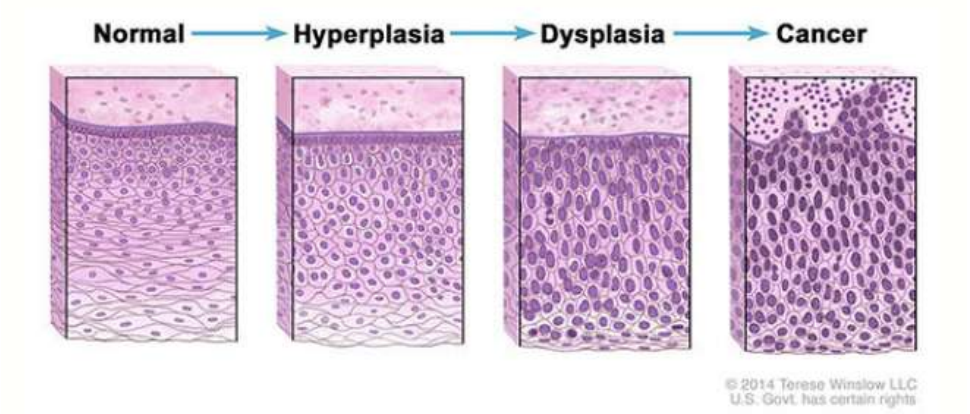


Figure 24: Abnormal tissue changes before cancer cell formation.
https://www.cancer.gov/about-cancer/understanding/what-is-cancer

2.3.2 Differences between cancer cells and normal cells

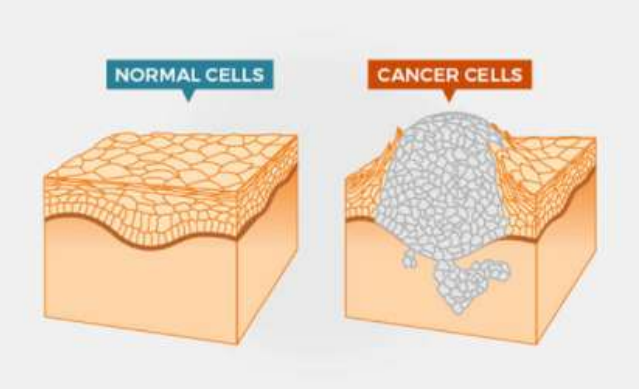


Figure 25: Difference of cancer cells from normal cells
https://www.cancer.gov/about-cancer/understanding/what-is-cancer

Cancer cells differ from normal cells in many ways (Figure 25). For instance, cancer cells:

- They grow in the absence of signals that tell them to grow. Normal cells grow only when they receive such signals.
- Ignore signals that normally tell cells to stop dividing or to die (a process known as programmed cell death or apoptosis).

- Invade nearby areas and spread to other parts of the body. Normal cells stop growing when they encounter other cells, and most normal cells do not migrate throughout the body.
- Cause blood vessels to grow toward tumors. These blood vessels supply tumors with oxygen and nutrients and remove waste products from tumors.
- Hide from the immune system. The immune system normally eliminates damaged or abnormal cells.
- Trick the immune system into helping cancer cells stay alive and grow. For example, some cancer cells convince immune cells to protect the tumor instead of attacking it.
- Accumulate multiple changes in their chromosomes, such as duplications and deletions of chromosomal segments. Some cancer cells have twice the number of chromosomes as normal (Figure 26).

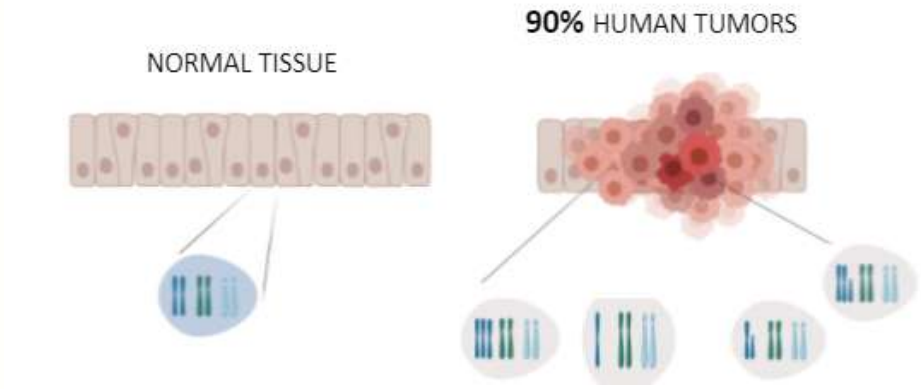


Figure 26: Changes in the chromosomes of human tumor cells

- Rely on different kinds of nutrients than normal cells. In addition, some cancer cells make energy from nutrients in a different way from most normal cells. This makes cancer cells grow faster.

Often, cancer cells rely so heavily on these abnormal behaviors that they cannot survive without them. Researchers have taken advantage of this fact and developed therapies that target the abnormal characteristics of cancer cells. For example, some cancer therapies prevent blood vessels from growing toward the tumor, essentially starving the tumor of needed nutrients.

2.3.3 How does cancer develop?

Cancer is a genetic disease, that is, it is caused by changes to genes present in DNA that control how our cells work, especially how they grow and divide. These changes are also called genetic changes.

Genetic changes that cause cancer can happen because:

- of errors that occur during cell division.
- of damage to DNA caused by harmful substances in the environment, such as the chemicals in tobacco smoke and ultraviolet rays from the sun.
- they were inherited from our parents.
- of bad lifestyle habits.

Normally, the body eliminates cells with damaged DNA before they become cancerous. But the body's ability to do this decreases as we age. This is part of the reason why there is a higher risk of cancer later in life.
Each person's cancer has a unique combination of genetic changes. As the cancer continues to grow, additional changes occur. Even within the same tumor, different cells may have different genetic changes.

Normally, **the cell division process** is regulated by a series of control mechanisms that tell the cell when to start dividing and when to stop. When cell damage occurs that cannot be repaired, cell self-destruction occurs that prevents the damage from being inherited by descendant cells (Figure 27).

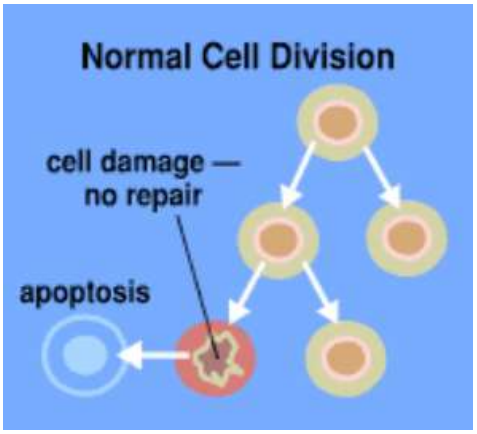


Figure 27: In normal tissues, the rates of new cell growth and old cell death are kept in balance. Apoptosis, or "cell suicide," is the mechanism by which old or damaged cells normally destroy themselves.
From: National Cancer Institute : http://press2.nci.nih.gov/sciencebehind/cancer/cancer00.htm

When these control mechanisms are disturbed in a cell, it and its descendants **initiate uncontrolled division**, eventually leading to a benign tumor or **the ability to invade surrounding tissues and organs causing cancer.** (Figure 28).

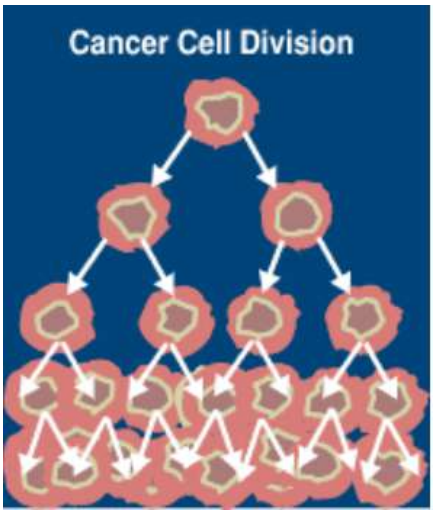


Figure 28: In cancer this balance is disturbed. This disruption can result from uncontrolled cell growth or the loss of a cell's ability to undergo "apoptosis".
From: National Cancer Institute : http://press2.nci.nih.gov/sciencebehind/cancer/cancer00.htm

2.3.4 Types of genes that cause cancer

The genetic changes that contribute to cancer usually involve three main types of genes - proto-oncogenes, tumor suppressor genes, and DNA repair genes. These changes are sometimes referred to as the "drivers" of cancer.

Proto-oncogenes are genes that normally help cells grow. When a proto-oncogene mutates (changes) or there are too many copies of it, it becomes a "bad" gene that can be permanently turned on or activated when it should not be. When this happens, the cell grows out of control, which can lead to cancer. This bad gene is called an oncogene (Figure 29).

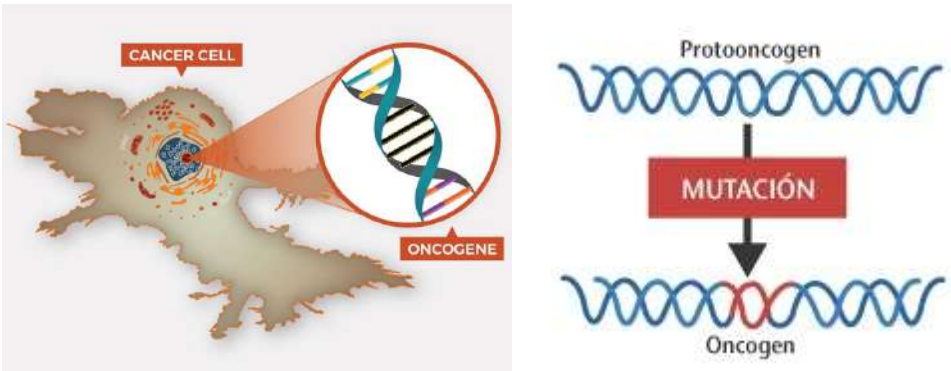


Figure 29: Oncogene
https://www.cancer.gov/about-cancer/understanding/what-is-cancer

It can be helpful to think of a cell like a car. For it to function properly, there must be ways to control how fast it goes. A proto-oncogene usually functions in a way that resembles a gas pedal. It helps the cell grow and divide. An oncogene could be compared to a gas pedal that is depressed, causing the cell to divide uncontrollably.

A few cancer syndromes are caused by inherited mutations of proto-oncogenes that cause the oncogene to be turned on (activated). However, most cancer-causing mutations of oncogenes are acquired which generally activate oncogenes through chromosome rearrangements[46] and gene duplication[47].

Tumor suppressor genes are normal genes that slow down cell division, repair DNA errors, or tell cells when to die (a process known as *apoptosis* or *programmed cell death*). When tumor suppressor genes do not function properly, cells can grow out of control, which can lead to cancer (Figure 30).

A tumor suppressor gene is like the brake pedal on a car. Normally, it keeps the cell from dividing too fast, just like a brake keeps a car from going too fast. If something goes wrong with the gene, such as a mutation, cell division can get out of control.

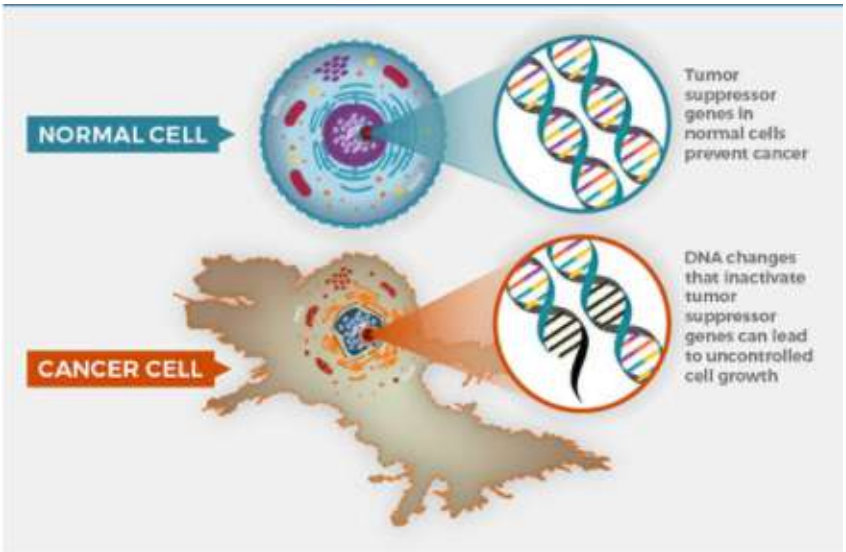


Figure 30: Tumor suppressor genes
https://www.cancer.gov/about-cancer/understanding/what-is-cancer

An important difference between oncogenes and tumor suppressor genes is that oncogenes result from the *activation* (turning on) of proto-oncogenes, but tumor suppressor genes cause cancer when they are *inactivated* (turned off).

[46] **Chromosome rearrangements:** Changes in chromosomes that place one gene next to another, allowing one gene to activate the other.

[47] **Gene duplication:** Extra copies of a gene, which can cause it to make too much of a particular protein.

Inherited abnormalities of tumor suppressor genes have been found in some cancer syndromes. However, most mutations of tumor suppressor genes are acquired, not inherited.

DNA repair genes are involved in repairing damaged DNA. Cells with mutations in these genes tend to develop additional mutations in other genes and changes in their chromosomes, such as duplications and deletions of chromosomal segments. Together, these mutations can cause the cells to become cancerous.

2.3.5 Tumor microenvironment & tumor cell formation

Within a tumor, cancer cells are surrounded by a variety of immune cells, fibroblasts, molecules, and blood vessels-the so-called tumor microenvironment. Cancer cells can alter the microenvironment, which in turn can affect how the cancer grows and spreads (Figure 31).

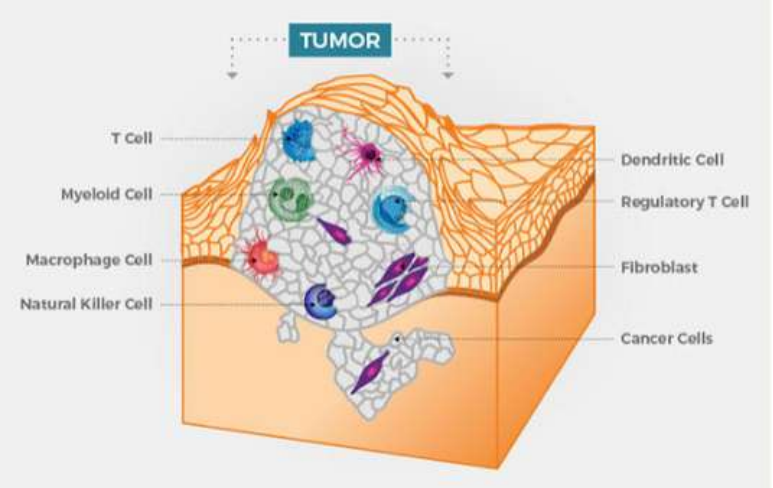


Figure 31: Tumor microenvironment
https://www.cancer.gov/about-cancer/understanding/what-is-cancer

Tumor cell formation involves 3 main steps:

- Loss of Cell Cycle Regulation at checkpoints[48]
- Increased growth rate & escape from apoptosis.

[48] A **checkpoint** is a stage in the eukaryotic cell cycle at which the cell examines internal and external cues and "decides" whether or not to move forward with division.

- Accumulation of DNA damage, errors in replication[49], introduction of mutations, chromosomal translocations[50], aneuploidies[51].

2.3.6 How does cancer develop and spread?

The process of cancer development is called **carcinogenesis** (Figure 32) . Carcinogenesis lasts for years and passes through various stages. The substances responsible for this transformation are called carcinogens. An example is ultraviolet radiation from the sun, asbestos or the human papilloma virus.

The **first phase** begins when these substances act on the cell by changing its genetic material (mutation). An initial mutation is not enough to develop cancer, but it is the beginning of the process. The essential condition is that the altered cell is capable of division. As a result, the **damaged cells begin to multiply** at a slightly higher than normal rate and pass the mutation on to their offspring. This is called the tumor initiation phase and the cells involved in this phase are called initiated cells. The change produced is irreversible, but not sufficient to develop cancer.

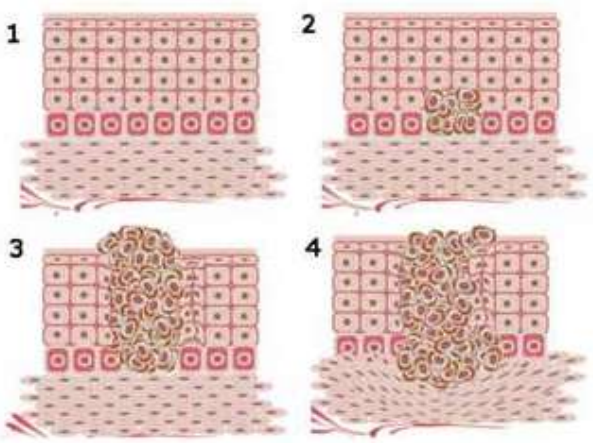


Figure 32: Carcinogenesis
https://www.aecc.es/es/todo-sobre-cancer/que-es-cancer/origen

When carcinogens act again and repeatedly on initiated cells, cell proliferation begins to be more rapid and the likelihood of new mutations occurring increases.

[49] **Replication**: Replication is the process by which a double-stranded DNA molecule is copied to produce two identical DNA molecules.

[50] **Chromosomal translocations:** These are a type of chromosomal abnormality in which a chromosome breaks and a portion of it reattaches to a different chromosome.

[51] **Aneuploidies:** The presence of an abnormal number of chromosomes in a cell, for example a human cell having 45 or 47 chromosomes instead of the usual 46.

This is called the promotion phase and the cells involved in this phase are called promoted cells. Currently, we know many factors that affect this phase such as tobacco, improper diet, alcohol, etc.

Eventually, the initiated and promoted cells undergo new mutations. Each time they become more abnormal in their growth and behavior. They acquire the ability to invade, both locally, by infiltrating the surrounding tissue, and remotely, by forming metastases. This is the progression phase (Figure 33).

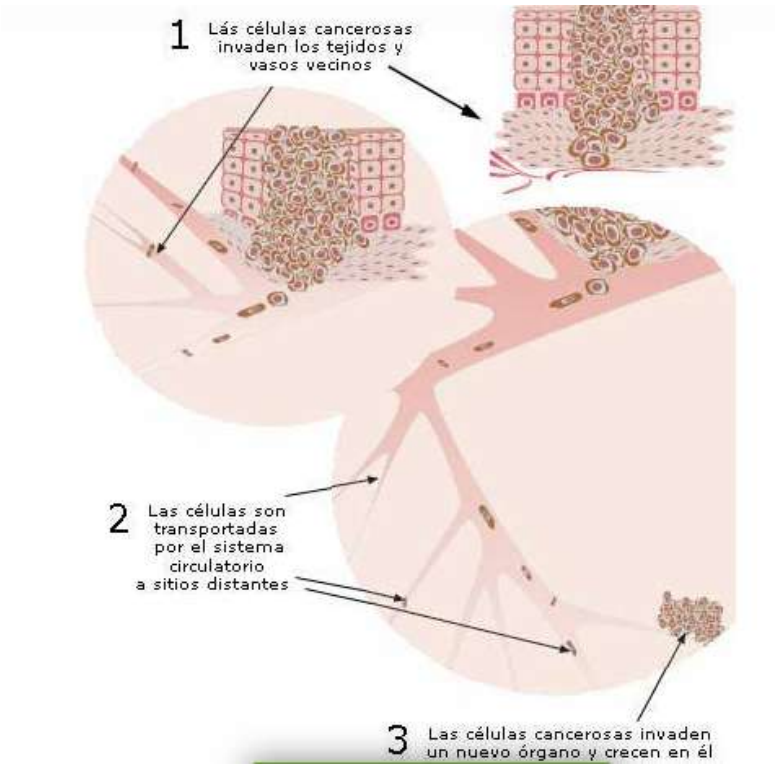


Figure 33: Spreading of cancerous cells
https://www.aecc.es/es/todo-sobre-cancer/que-es-cancer/origen

A cancer that has spread from the place where it first formed to another place in the body is called a metastatic cancer. The process by which cancer cells spread to other parts of the body is called metastasis (Figure 34). **Metastasis** - which literally means "new place" - is one of the final stages of cancer. At this stage, cancer cells enter the bloodstream or lymphatic system and travel to a new location in the body, where they begin to divide and lay the groundwork for secondary tumors. Not all cancer cells can metastasize. To spread in this way, cells must have the ability to penetrate the body's normal barriers, allowing them to both enter and leave the blood or lymph vessels. Even traveling metastatic cancer cells face challenges when they try to grow in new areas.

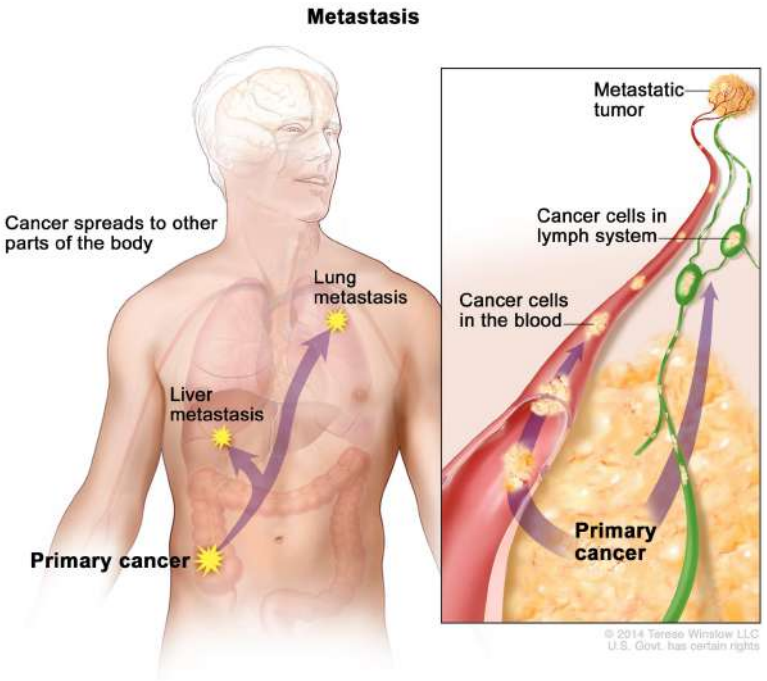


Figure 34: In **metastasis**, cancer cells break away from the site where they first formed (primary cancer), travel through the blood or lymphatic system, and form new tumors (metastatic tumors) in other parts of the body.
https://www.cancer.gov/publications/dictionaries/cancer-terms/def/metastasis

2.3.7 Types of cancer

Cancers are usually classified according to the type of cell in which they begin. There are five main categories of cancer: Carcinoma **(**the most common type of cancer that begins in epithelial tissue such as the skin), sarcoma (begins in connective or supportive tissues such as bone or muscle), leukemia (begins in blood-forming tissue such as bone marrow), lymphoma and myeloma (start in the cells of the immune system- lymphomas start in lymphocytes whereas myeloma begins in plasma cells) and brain and spinal cord cancers (known as cancers of the central nervous system).

2.3.8 Cancer Research

Cancer is a heterogeneous group of diseases that involve abnormal cell growth with the potential to invade or spread to other parts of the body (metastasis). Carcinogenesis is a multistep process that involves the interaction of mutations in signaling, cell cycle, cell death, and metabolic pathways, as well as the interaction between the tumor and its microenvironment. The process of carcinogenesis can be conceptually divided into four steps: 1) tumor initiation; 2) tumor promotion; 3) malignant conversion; and 4) tumor progression and metastasis (Figure 35).

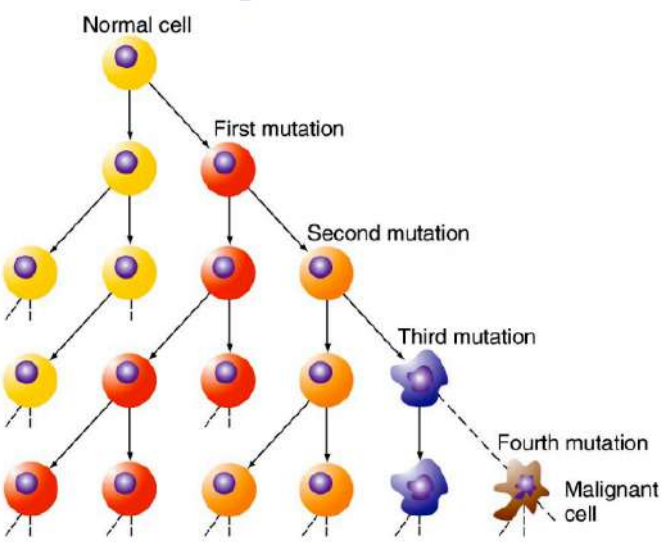


Figure 35 : Multistep Nature of Cancer

The development of human cancer is a complex disease caused by the action of one or a combination of genetic and environmental (chemicals) insults to the cells. A seminal work in the field of cancer research sought to explain that the complexity of cancer can be understood in a simpler way by considering a small number of underlying principles or hallmarks (Figure 36).These hallmarks or characteristics include the following: the ability to maintain chronic proliferation, the ability to evade growth suppressors, the ability to resist cell death, the ability to invade local tissues, and the potential for metastasis. More recently, the same authors have suggested new hallmarks that should be considered, such as genome instability, inflammation, and induction of angiogenesis[52] (to irrigate the tumor mass).

[52] **Angiogenesis:** It is the physiological process through which new blood vessels are formed from pre-existing vessels.

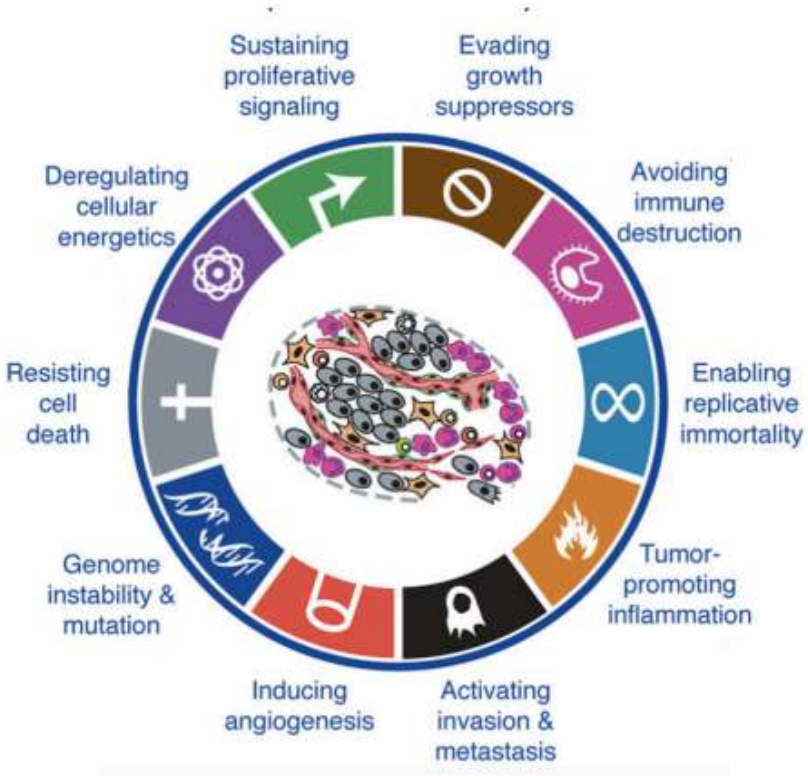


Figure 36: Hallmarks of cancer
https://pubmed.ncbi.nlm.nih.gov/21376230/

Because cancer is a tissue-level disease, some researchers think that looking at it at the cellular level may be misleading. Although studies using cultured cells have provided important information, they have some limitations, such as the inability to consider the interaction between the tumor and the microenvironment.

2.4 Relationship between senescence and cancer

As the interest in defining senescent cells has grown, so has the interest in deciphering why they exist in the first place. The leading hypothesis for why senescent cells exist is that they serve as a preventative measure against cancer. The best option would be for a cell to successfully repair any damage that occurs, but if this is not possible (and the damage has not reached a critical level to induce apoptosis), a cell becomes senescent to try to avoid becoming cancerous by going through permanent cell cycle arrest.

Recent studies have shown that the human organism is able to prevent the growth of potentially cancerous cells by paralyzing them. This antitumor mechanism, which acts like a brake on the malignant process, was already known "in vitro" in laboratory studies, but has now been verified "in vivo" in mice and in tissue samples from cancer patients. This mechanism is known as cellular senescence, an emergency defense system for cells that are on their way to becoming cancerous, i.e. a

response to stimulation of an oncogene. These cells are condemned to "life imprisonment", preventing the progression of premalignant lesions.

Senescence is a double-edged sword that can work in opposite directions. Early-stage senescence can protect cells from transformation, whereas prolonged senescence often promotes cancer development. Age is a strong prognostic marker for reduced survival in many cancers. Therefore, the increased number of senescent cells present in aged tissues may contribute to the increased incidence of cancer with age by altering the cellular microenvironment through a senescence-associated secretory phenotype (SASP) (Figure 37). Although senescence provides a barrier to tumorigenesis and senescent cells are often dormant in vivo for months and even years, once these dormant cancer cells re-enter the cell cycle, tumorigenesis can resume and cancer can relapse.

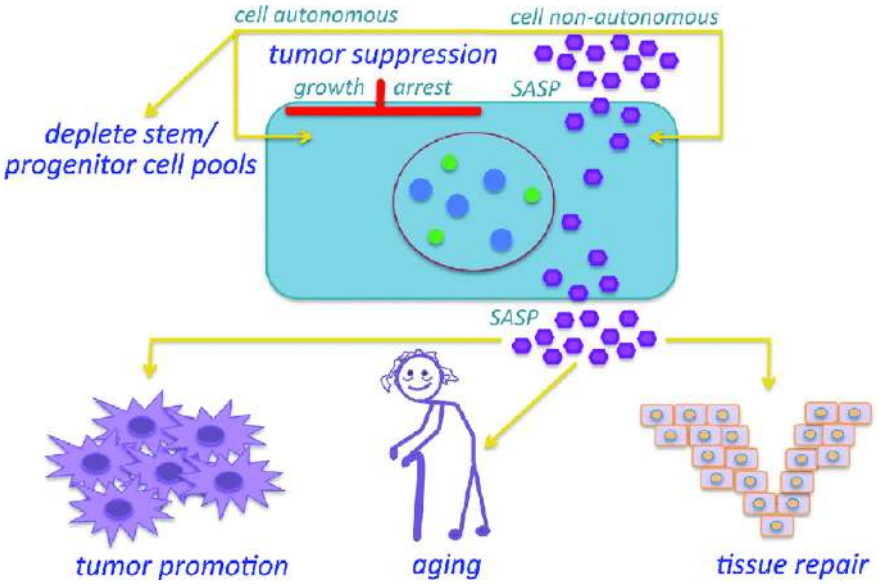


Figure 37: Biological activities of cellular senescence
https://www.researchgate.net/publication/49836091_Four_Faces_of_Cellular_Senescence

2.5 Dark side of senescence

So far it seems that cellular senescence is a great mechanism in our bodies, but as with many cellular processes, too much of a good thing can become a bad thing. Senescent cells are great for the initial prevention of cancer, but remember that SASP I mentioned before? Well, it turns out that some of those inflammatory proteins and molecules that make up SASP can have negative effects on other cells and tissues.

2.5.1 SASP can mediate the protumorigenic effects of cellular senescence

SASP can induce tumor progression and recurrence, underscoring the complex nature of cellular senescence and SASP. Induction of cellular senescence within the tumor microenvironment can occur by several mechanisms, including cancer therapy-induced senescence of tumor and non-tumor cells, via SASP-associated spread of cellular senescence programming, etc.

Effects of cellular senescence in the microenvironment of brain tumors
Induction of replicative or stress-induced senescence in resident brain cells promotes the development of age-associated neurodegeneration[53]. For example, increased numbers of senescent microglial cells[54] are thought to contribute to poorer outcomes after traumatic brain injury in aged individuals due to an exaggerated microglial response and subsequent neuroinflammation.
It is also thought to mediate a range of effects, including reduced proliferation of neural progenitor cells[55], poor antitumor immune response, and reduced myelination[56] by oligodendrocytes[57], further complicating the potential impact of cellular senescence in the brain tumor microenvironment. Additional effects caused by exposure of non-senescent cells to senescent cell-derived SASP factors include induction of cancer cell proliferation, tumor recurrence, and angiogenesis (Figure 38).

[53] **Neurodegeneration**: It is the progressive loss of structure or function of neurons, which may ultimately involve cell death.

[54] **Microglial cells :** These are a specialized population of macrophages that are found in the central nervous system (CNS). They remove damaged neurons and infections and are important for maintaining the health of the CNS.
[55] **Neural progenitors cells:** These are cells that are capable of dividing a limited number of times and have the capacity to differentiate into many of the glial (or neuroglial are non-neuronal cells in the central nervous system and the peripheral nervous system that do not produce electrical impulses) and neuronal cell types that populate the CNS.

[56]**Myelination:** The process of generating myelin which is a lipid-rich substance that surrounds nerve cell axons (the nervous system's "wires") to insulate them and increase the rate at which electrical impulses are passed along the axon.

[57] **Oligodendrocytes :** These are a type of glial cells that help in myelin formation in the central nervous system (CNS; brain, spinal cord and optic nerve).

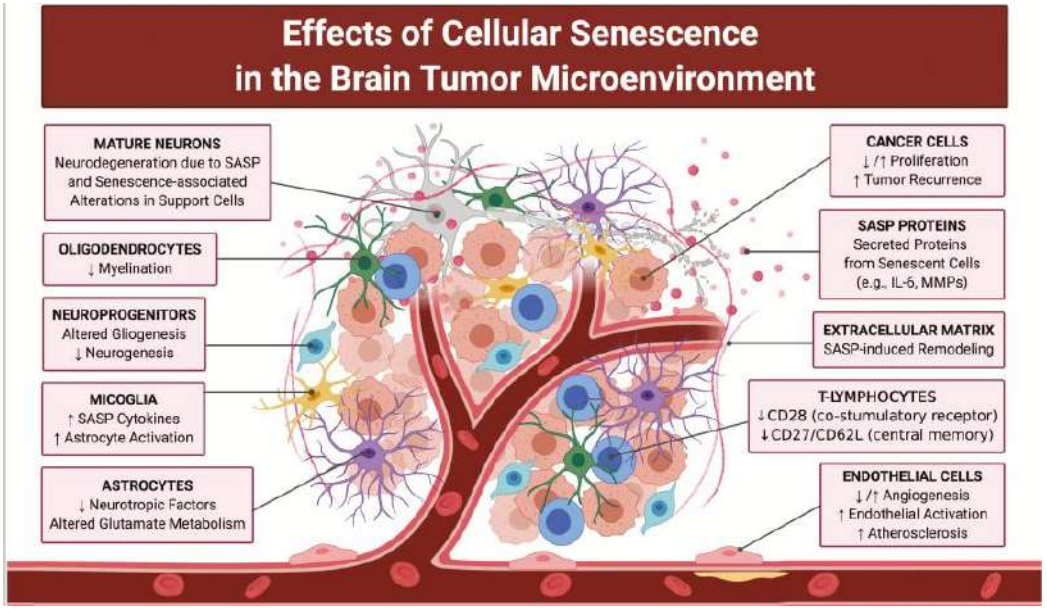


Figure 38: Effects of cellular senescence in the microenvironment of brain tumors

https://academic.oup.com/carcin/article/41/8/1017/5867012

2.5.2 Increased senescent cell burden contributes to disease in physiological and premature aging

Pathological changes induced by the persistence of senescent cells may not be limited to cancer and cancer treatment-associated effects, but may also be promoted in tissues during physiological or accelerated aging. This may be exacerbated by mechanisms that enhance the accumulation of cellular senescence or inhibit its resolution. For example, the application of cellular stress, such as that induced by radiation therapy, can induce DNA damage, oxidative stress, and inflammation, dramatically increase a tissue's senescent cell burden and overwhelm the tissue's ability to target and remove senescent cells. However, there is an inappropriate senescence accumulation due to defective immune surveillance[58] and clearance (Figure 39).

[58] **Immune surveillance:** The monitoring process by which cells of the immune system (such as natural killer cells, cytotoxic T cells, or macrophages) detect and destroy premalignant or malignant cells in the body.

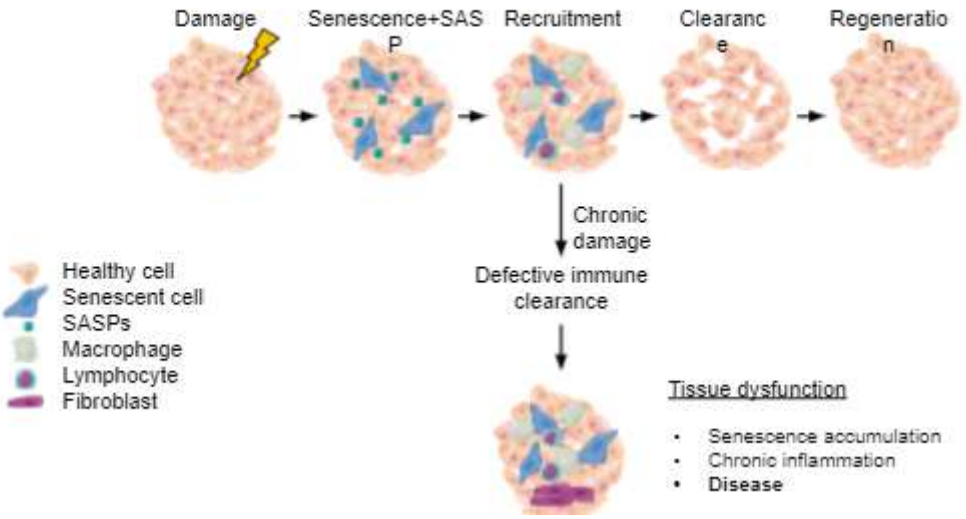


Figure 39: Inappropriate accumulation due to defective immune surveillance

Moreover, cellular senescence has been described as contagious and shown to mediate a "bystander effect" by which senescence programming can spread from cell to cell through the paracrine effects of SASP secretory factors. These mechanisms of rapid accumulation, persistence and amplification may underlie the ability of senescent cells to affect organ function and lead to pathological changes in the aging process.

2.5.3 Cellular senescence promotes age-related diseases including neurodegeneration

A major feature of aged organisms is the accumulation of cellular senescence (Figure 40). Excessive and abnormal accumulation of senescent cells in tissues can negatively affect regenerative capacity and create a proinflammatory milieu that promotes the onset and progression of various age-related diseases.

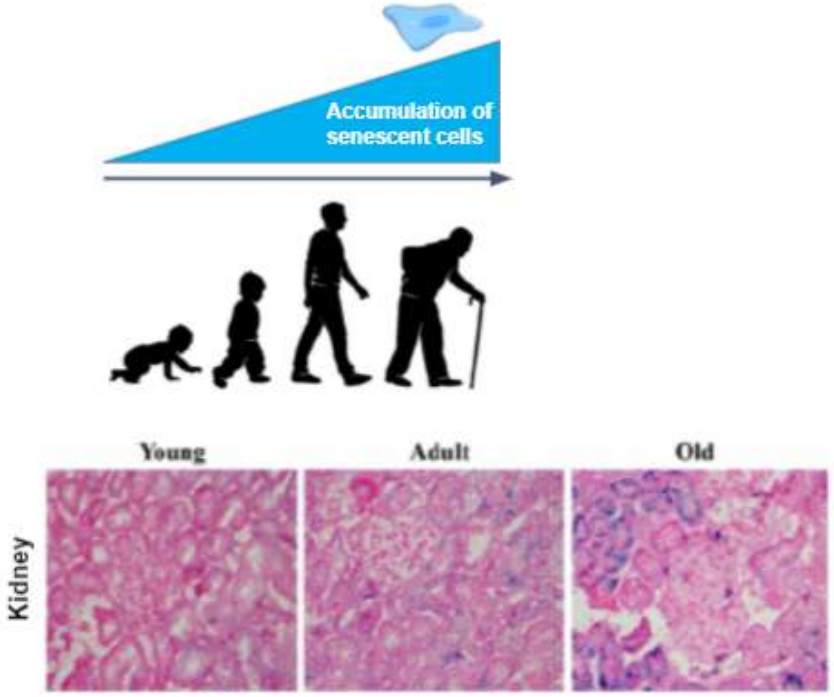


Figure 40: Senescent cells accumulate as we age

Cellular senescence and SASP contribute to organ dysfunction and tissue pathology in a variety of age-related diseases, including pulmonary fibrosis (lung disease), osteoarthritis[59], atherosclerosis[60], and Alzheimer's disease (AD)[61] (Figure 41). Cellular senescence has also been observed in various neurodegenerative diseases.

[59] **Osteoarthritis:** It is a type of degenerative joint disease that results from breakdown of joint cartilage and underlying bone.

[60] **Atherosclerosis:** It is the accumulation of fat, cholesterol and other substances within the arteries and on their walls.

[61] **Alzheimer's disease(AD):** It is a progressive neurologic disorder beginning with mild memory loss and possibly leading to loss of the ability to carry on a conversation and respond to the environment.

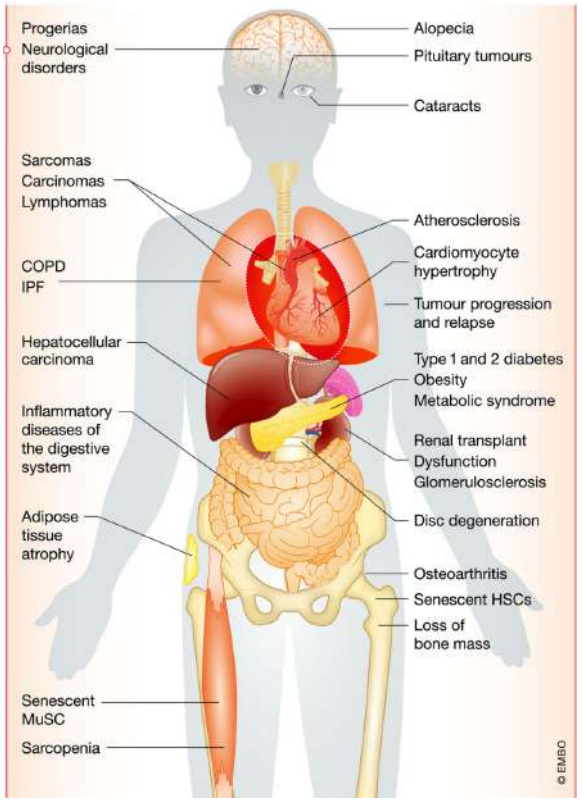


Figure 41: Cellular senescence lead to multiple diseases
https://www.embopress.org/doi/full/10.15252/emmm.201810234

2.6 Strategies to alleviate the detrimental aspects of senescent cells

2.6.1 Senotherapies (senostatic and senolytic drugs)

Throughout the life of an organism, cells are exposed to a number of stresses which induce senescence. With advancing age, senescent cells accumulate. If they are not cleared by the immune system, the pro-inflammatory secretory profile that is characteristic of these cells can induce senescence/damage to surrounding cells. This can promote a chronic inflammatory microenvironment which has the capacity to cause tissue dysfunction. From a therapeutic perspective, **senostatic therapies** alleviate SASP factors but do not remove the tumour suppressive aspect of senescence. **Senolytics,** on the other hand, selectively eliminate senescent cells, however, this may impact on tissue integrity if compensatory proliferation does not occur (Figure 42).

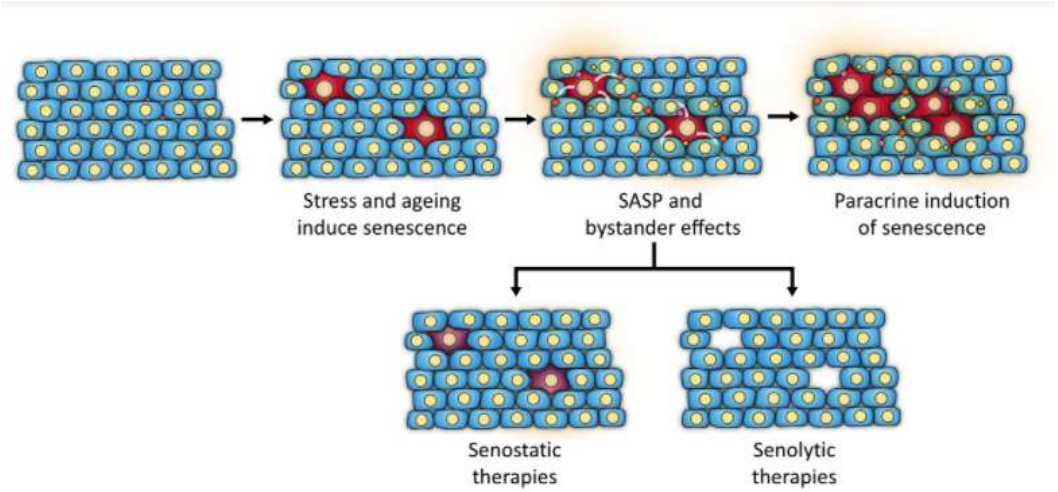


Figure 42: Strategies to alleviate the detrimental aspects of senescent cells.

https://www.researchgate.net/publication/333858190_Mitochondrial_dysfunction_and_cell_senescence_deciphering_a_complex_relationship

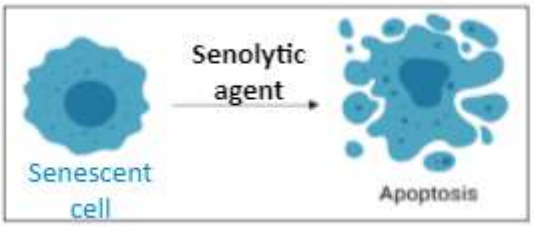


Figure 43: Senolytics: Compounds that specifically kill senescent cells

Of the two drug groups (senostatic & senolytic), senolytics have been more extensively studied and show promise of therapeutic value. **Senolytic agents** (Figure 43) are the pharmacological agents which target characteristic cellular mechanisms and molecular features of senescent cells. Identification of senescent-cell anti-apoptotic pathways (SCAPs) has allowed the development of specifically targeted senolytics for each of these. The most deleterious senescent cells (SC) are resistant to apoptosis and have up-regulation of anti-apoptotic pathways (SCAPs) which defend SC against their own inflammatory senescence-associated secretory phenotype (SASP), allowing them to survive, despite killing neighbouring cells. Senolytics transiently disable these SCAPs, causing apoptosis of those SC with a tissue-destructive SASP. As a result of senolytic therapy there is a reduction in tissue damage and age related diseases (Figure 44).

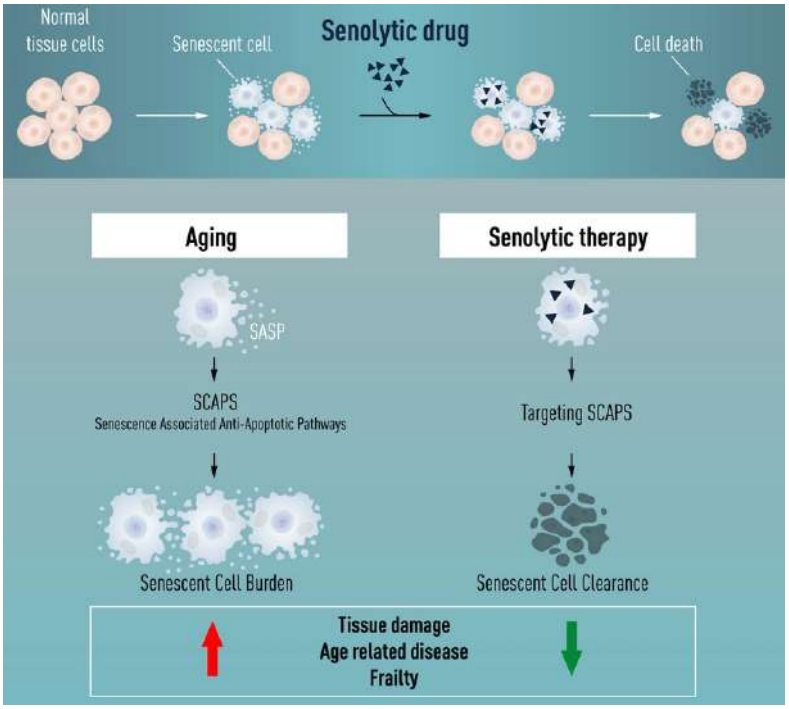


Figure 44: Use of senolytic drugs reduces tissue damage and age related diseases
https://pubmed.ncbi.nlm.nih.gov/32686219/

Senolytic drugs include quercetin, navitoclax, fisetin, etc.

Navitoclax
By far, the most successful senolytic in preclinical cancer models has been navitoclax. Navitoclax has shown a remarkable capacity to eliminate tumor cells induced into senescence by a variety of therapies. Navitoclax interacts with the BCL-2 pathway[62] and prevents it from inhibiting apoptosis, causing the senescent cells to undergo apoptosis. The rationale is that the chemotherapy drug causes cells to undergo either apoptosis or senescence and the navitoclax then induces the senescent cells to undergo apoptosis by inhibiting BCL-2. This should therefore enhance cell killing.

There is widespread evidence that navitoclax potentiates the effect of a range of anticancer therapies in vitro and in vivo, but efficacy in clinical studies has been limited by toxicity.

2.6.2 Eliminating and reprogramming of senescent cells as therapeutic strategies

With unprecedented population aging, it is critical to develop therapeutic strategies to improve and treat cancer- and age-associated disease. These anti senescence

[62] **BCL-2 pathway:** B-cell lymphoma-2 family proteins play an important role in regulating the induction of apoptosis

therapies have been developed to target senescent cells in age-associated diseases and have been shown to hold promise in cell culture studies, in mouse model experiments and in human clinical trials .Methods that are being investigated include inducing apoptosis in senescent cells, enhancing immune-mediated senescent cell clearance, targeting of functional or morphologic senescence phenotypes such as SASP (senomorphics), and cellular reprogramming to inhibit cellular senescence and restore homeostatic cellular functions (Figure 45).

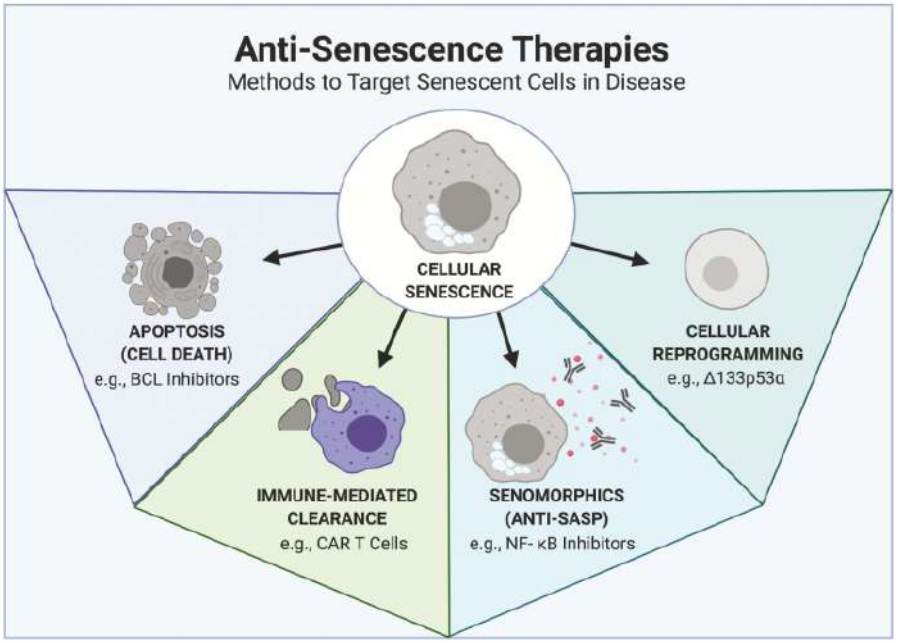


Figure 45: Anti-senescence therapies: methods to target senescent cells in disease.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7422622/

2.6.3 Role of senolytics in cancer and age-related diseases

Senolytics represent a group of mechanistically diverse drugs that can eliminate senescent cells, both in tumors and in several aging-related pathologies. It is observed that there is a delayed onset in tumor formation when senescent cells are eliminated. Senolytic therapy also reduces the incidence of metastasis, the leading cause of cancer-related deaths. Critically, removal of senescent cells in aging mice delays tumorigenesis further underscoring the tumor-promoting functions of persistent cellular senescence and SASP.

Scientists have experimented that removing senescent cells rejuvenates mice. In an experiment by eliminating senescent cells in 12 month old mice, it was demonstrated that there were 27% higher chances of survival of the non senescent mouse in

comparison to a control mouse (Figure 46).

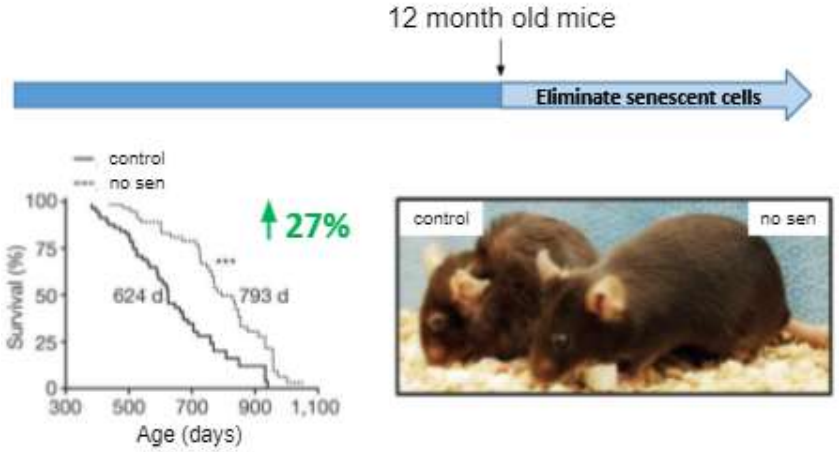


Figure 46: Removing senescent cells rejuvenates mice

2.7 Agents Capable of Inducing Senescence in Cancer

The tumor cells have the ability to undergo senescence in response to stress and damage with both radiation and chemotherapeutic drugs. The anti cancer chemotherapeutic drugs used in the experiments include doxorubicin ($C_{27}H_{29}NO_{11}$) & palbociclib ($C_{24}H_{29}N_7O_2$) due to their efficacy in fighting a wide range of cancers. These are drugs widely used in cancer chemotherapy. The mechanism of action of doxorubicin is complex and not yet fully elucidated, although it is thought to act by intercalation in DNA. It is known that by intercalating it inhibits the biosynthesis of nucleic acids, as it hinders the advance of an enzyme that develops DNA in transcription.

Most chemotherapeutic drugs induce DNA damage and activate the DDR. If the extent of damage is severe, the cell may die—e.g., through apoptosis. If the damage is sub-lethal, the cell may enter a state of senescence. This senescence response may represent a mechanism of inducing clinical tumour stasis (growth arrest) but in some situations such growth-arrested cells may re-enter the cell cycle and cause disease recurrence. In addition, the release of SASP by senescent cells may contribute to tumour recurrence as well as having an immune stimulatory function. The balance of these various processes will vary, depending on the host tissue and the type and degree of stimuli (Figure 47).

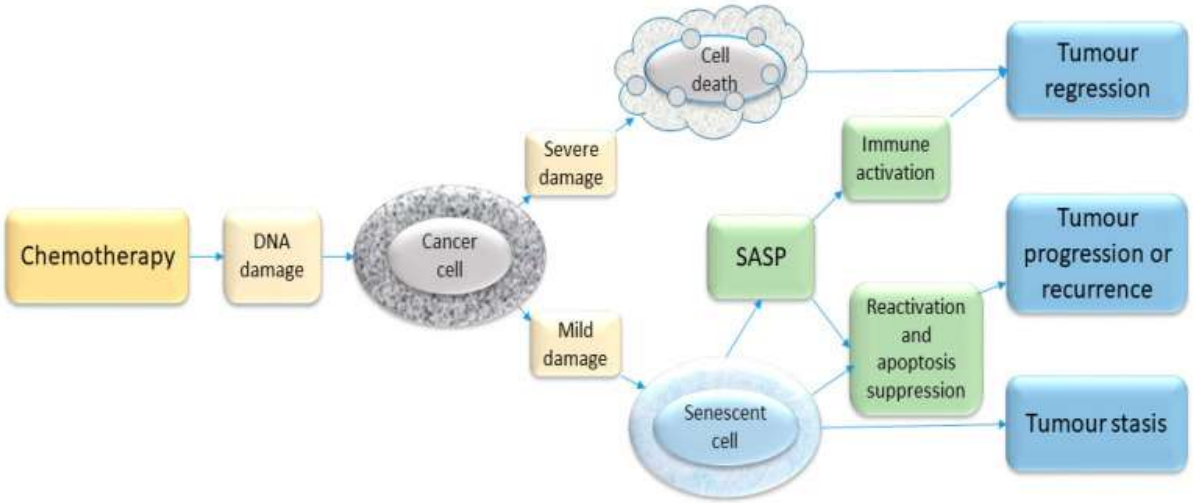


Figure 47: Schematic illustration of some chemotherapy-triggered responses in cancer cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7464619/

3. PRACTICAL FRAMEWORK

The practical part of this research work can be divided into two groups:
in vitro experimentation and in vivo experimentation. Both experiments were carried out in the facilities of the Institute of Biomedical Research of Barcelona. The experiments were coordinated by the laboratory of Cellular Plasticity and Disease, and their purpose is to demonstrate the efficiency of biological models in cancer research.

3.1 Experiment 1 “in vitro”:

Counting the melanoma cancer cells (SK Mel 103 cell line) in the Neubauer chamber and inducing senescence in cells by different inducers: DNA damage to cells by irradiation, use of chemical agents with different concentrations of doxorubicin & palbociclib (Therapy induced senescence). Also investigating whether the use of different concentrations of the senolytic agent navitoclax in these samples kills the zombie cells rather than the normal cells.

Research question:
Can we create senescent cells by inducing senescence “in vitro”?
What is the possible effect of a senolytic agent?

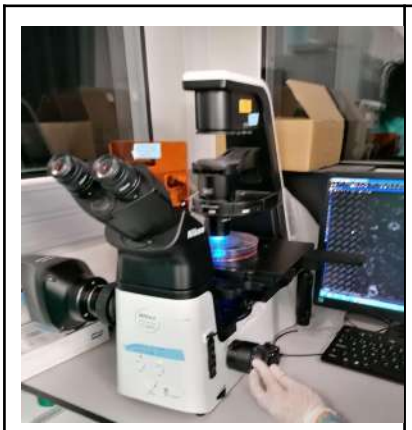
3.1.1 Hypothesis and definition of the experimental approach

Our **hypothesis** is that maybe by inducing DNA damage or by using chemical agents we can create senescent cells “in vitro”. Maybe a senolytic drug is capable of eliminating senescent cells.The **independent variables,** the one which we are able to control, include methods of inducing senescence and the concentration of chemical agents and senolytic agents used in different cases.
The **dependent variable** is if senescence is induced with DNA damage and chemical agents and whether senescent cells number is reduced with the use of senolytic agent. The **controllable variables** include the same temperature conditions, same medium of culture, etc. We have used the **control** which are the cells without any DNA damage, without use of any chemical agent and without use of senolytic agent.

The cell line used in “in vitro” experiments is named as SK-MEL-103 (category -cancer cell line) affected by melanoma disease and its species of origin is *Homo sapiens* (Human). (Consult the picture in the annex on page no. 91 for the reference of the cells used in vitro)

3.1.2 Material

Because we work with cell cultures, the laboratory must have certain apparatus and material. The necessary appliances are freezers, refrigerators and cryogenic[63] facilities and carbon dioxide incubator, to keep cells alive; vacuum cleaner, cabin of laminar flow, Neubauer chamber, cover glass, Dilution buffer/PBS and automatic pipettes, to work with the culture to the laboratory and optical microscope, to make the corresponding observations (Figure 48 (a),(b)).



Optical microscope

Cabin of laminar flow

Carbon dioxide incubator

Figura 48 (a): Material (Source: own).

Figure 48 (b). Necessary equipment for performing cell count with Neubauer chamber

https://www.emsdiasum.com/microscopy/technical/datasheet/68052-14.aspx

The reagents include chemical agents doxorubicin (200nM, 600nM) and palbociclib (25 μM, 5 μM) , senolytic agent navitoclax (0.1 M, 1 M) & irradiation.

[63] Cryogenic: It is the production and behaviour of materials at very low temperatures. The cryogenic facility can be used for storage of cell lines, tissues, and patient specimens etc.

3.1.3 Experimental method

Preparation of culture

It is necessary to prepare the culture with the cell line we use, the SK-MEL-103, which will later be treated with chemotherapeutic agents and irradiation, that might induce senescence.

First of all, we took out the Petri dish (containing SK-MEL-103 cells) found in a carbon dioxide incubator, in order to work on it (Figure 49 (a) (b)).

(a) **(b)**

Figure 49: (a) Petri dish containing SK-MEL-103 cancer cell line (b) The melanoma cells look large and have irregular shape and size when seen under the microscope

(Source: own)

Cell counting with Neubauer's camera

To be able to sow the cells in the plates and perform the experiments correctly we need to know the number of cells in culture we need. This amount depends on the cell concentration set in the protocol of the experiment. To quantify the number of cells the Neubauer camera was used (Figure 50). The steps for introducing samples into the Neubauer camera are shown in the picture in the annex (page no. 94).

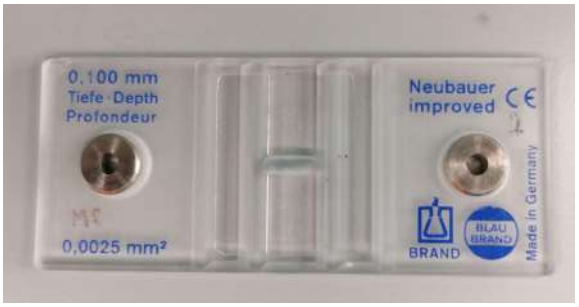


Figure 50: Neubauer camera

(Source: own)

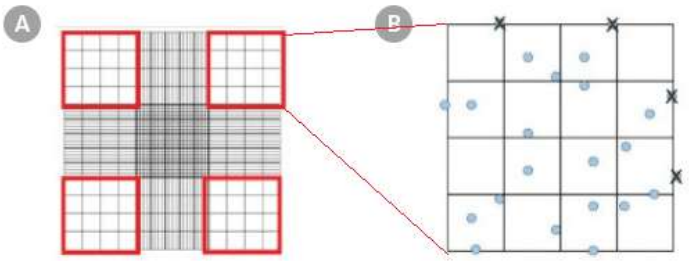


Figure 51: Illustration of the camera Neubauer.
(Source: own)

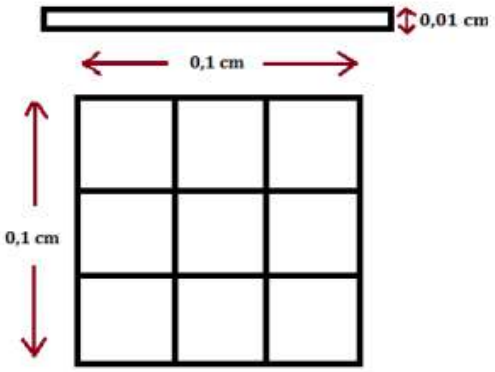


Figure 52: Volume of a square of the camera Neubauer. Volume 0.1 cm x 0.1 cm x 0.01 cm = 10-4 mL or cm3.
(Source: own)

Finally, we look at the camera Neubauer through the optical microscope and, guiding us through the grid of the instrument, we count the observed cells (Figure 53). From the count of the four squares of Neubauer's chamber we get a total of 164 cells.
To calculate the number of cells per milliliter we must multiply the number obtained by 10,000. Thus, the initial concentration of the cells is 41 x 10^4 cell / mL. 10^4

CALCULATION TO OBTAIN THE NO. OF CELLS

$$No\ of\ cells\ in\ 4\ squares\ =\ 164\ cells$$

$$\frac{164\ cells}{4\ squares} =\ 41\ cells/\ square$$

$$41\ cells\ \times\ 10^4 =\ 41\ \times\ 10^4\ cells\ /\ mL$$

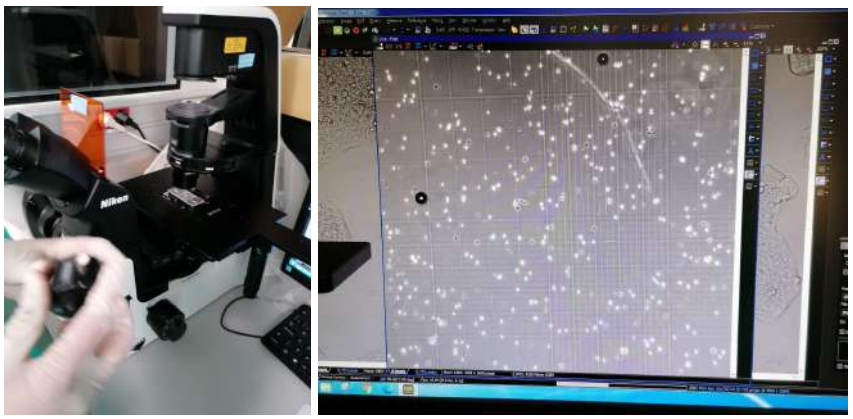


Figure 53: (a)Cell count in optical microscope.(b) each white dot seen is one cell.
(Source: own).

Initial volume calculations for each well of 25000 cells:
We have 1 mL (1000 µL) of initial liquid (dissolution of cells in suspension) and we know the number of cells it contains: 41 x 10^4 cells / mL. Also knowing that the number of cells needed to carry out our experiment are 25000 cells in each well.
So we do a proportion to get the initial volume of liquid that we will deposit inside each well of the plate and then we will dilute with the corresponding medium in order to reach the concentration of desired cells (25000 cells / well):

CALCULATION OF THE REQUIRED VOLUME OF INICIAL LIQUID IN WELLS

$$410000\ CELLS\ IN\ 1\ mL$$

$$25000\ CELLS\ IN\ X\ mL$$

$$X = \frac{25000}{410000} = 0,0609\ mL$$

$$0,0609\ mL \times \frac{1000\mu L}{1\ mL} = 60,9\ \mu L$$

We have to introduce 60,9 µL of dissolution in each well.

Sowing cells in wells

Once the necessary calculations have been made, it is time to start sowing cells in plates. Proper culture techniques and procedures are an essential part of ensuring successful transfection. First of all, subculturing[64] is done and the cell

[64]**Subculturing**: It is also referred to as passaging, it is the removal of medium and transfer of cells from a culture into fresh growth medium, in order to propagate the cells.

growth medium used is 500 ml of DMEM[65], 50 ml of FBS[66] and 5 ml (1%) Penicillin streptomycin (antibiotic) in order to avoid contamination (Figure 54).

Figure 54: Subculturing and cell growth medium is used
(Source: own)

Finally, we put the suspension of cells in the four multiwell plates (Figure 55).

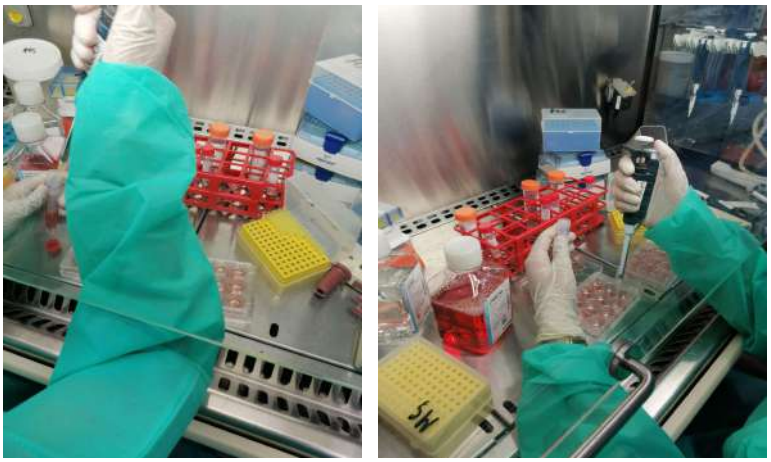


Figure 55: With the pipette I added 60.9 µL of culture medium previously prepared in each well of the plate.
(Source: own)

To make a replica of the experiment I planted four plates, twelve wells each, with the same number of cells following the previous procedure. Finally, when the cells were seeded in the different wells, we cover the plates and place them in the incubator at 37°C so that the cells recover, adapt to the new environment correctly and proliferate for a few days to obtain an optimal amount to carry out the experiment.

---

[65] **DMEM:** Gibco Dulbecco's Modified Eagle Medium (DMEM) is a widely used basal medium for supporting the growth of many different mammalian cells.

[66] **FBS :** Fetal bovine serum (FBS) is a byproduct of harvesting cattle for the meatpacking industry–it offers essential growth factors for the maintenance and growth of cultured cells. FBS is used as a supplement to basal growth medium in cell culture applications.

INDUCING SENESCENCE BY DNA DAMAGE

However, we carry one of the plates for radiotherapy[67] in order to induce senescence by DNA damage on the same day (Figura 56). The current dose of radiation which lasts for 3' 30" (time) is 20 Gy (Grey)[68](Figura 57).

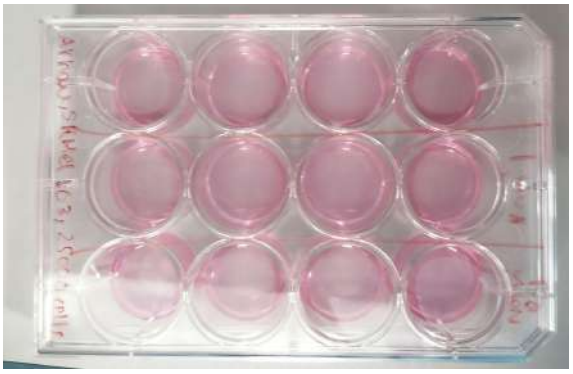


Figura 56 : In this plate, there are 4 wells of control cells, 4 wells with navitoclax 0.1M and 4 wells with navitoclax 1 M.

(Source: own)

Figure 57: DNA damage by radiation to induce senescence

(The time depends on the calibration of the machine, it is not always the same)

(Source: own)

TREATMENT WITH CHEMICAL AGENTS AND SENOLYTIC AGENTS

In order to observe and study senescence I used the two chemotherapeutic agents named doxorubicin and palbociclib with different concentrations and also a senolytic agent named navitoclax. The three multiwell plates (excluding the fourth plate affected by radiation) are tracted with these agents. In order to make a replica we

[67] **Radiotherapy**: It is one of the mainstays of cancer therapy, acts by causing direct DNA damage and has wide ranging impacts on cancer cells mediated by reactive oxygen species. Sometimes when the DNA damage response is triggered and if repair is not possible, cells either die if the damage is severe or enter senescence if less severe.

[68] **Gray (Gy):** It is a unit of absorbed dose and reflects an amount of energy deposited into a mass of tissue (1 Gy = 100 rads).

added the agents in such a way that 3-4 wells were used for the same sample (Figure 58).

Figure 58 : Multiwell plates are divided according to the chemical agent and senolytic agent the cells are treated with. In each well we have different samples with different drugs from less to more concentration.

(Source: own)

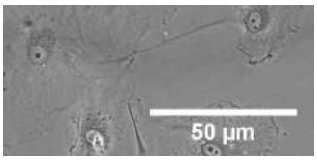
List of the samples (independent variables) used in the experiment (SK-Mel cell line) specifying type of chemical agent and senolytic agent used with specific concentration of each :

- Control cells
- Irradiation + Navitoclax (0.1 μM)
- Irradiation + Navitoclax (1 μM)
- Palbociclib 2 μM
- Palbociclib 2 μM + Navitoclax 1 μM
- Palbociclib 5 μM
- Palbociclib 5 μM + Navitoclax 1 μM
- Doxorubicin 200 nM
- Doxorubicin 200 nM + Navitoclax 1 μM
- Doxorubicin 600 nM
- Doxorubicin 600 nM + Navitoclax 1 μM

Now the samples were placed in the incubator at 37°C so that the cells recover, adapt to the new environment correctly and proliferate for a few days to obtain an optimal amount to carry out the experiment.

3.1.4 Results

Observation of photographs with an optical microscope identifying the morphological features that characterize these samples. All the pictures of experiment 1 are taken with scale bar [69]of 50 µm.



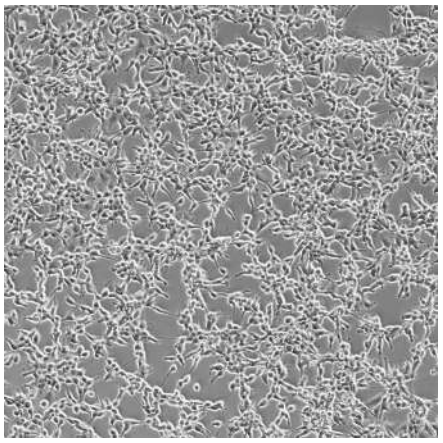


Figure 59 : Control cells are proliferating and have more regular dimensions.

(Source: own)

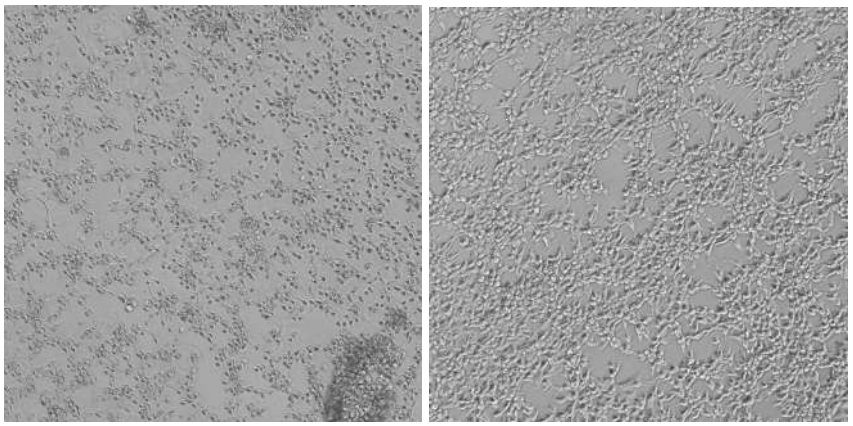


Figure 60 : (a) These are the proliferating cells treated with more concentration (1 µM) of navitoclax and have more quantity of cells as compared to picture (b) (b) Proliferating cells treated with normal concentration of navitoclax (0,1 µM))

(Source: own)

[69] **Scale bar:** Magnification can be calculated using a scale bar. This is a line drawn near the photograph or drawing which has a label showing the actual length of the bar before being magnified.

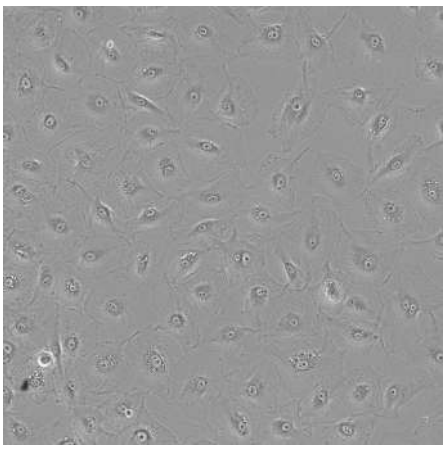


Figure 61 : Cells treated with palbociclib and have irregular shape.
(Source: own)

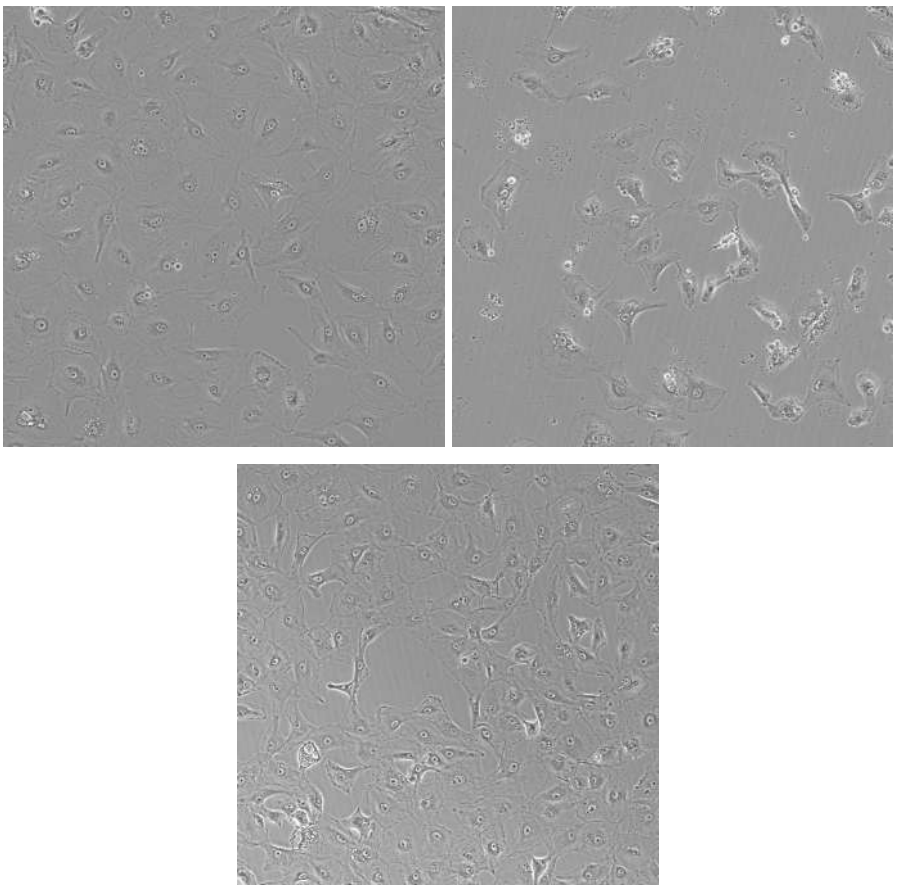


Figure 62 : (a) (upper left) Cells treated with more concentration (5 µM) of palbociclib and have more irregular dimensions
(b) (upper right) Cells treated with palbociclib (5µM) and concentration of navitoclax (1 µM)
(c)Cells treated with palbociclib (5µM) and concentration of navitoclax (0.1 µM)
(Source: own)

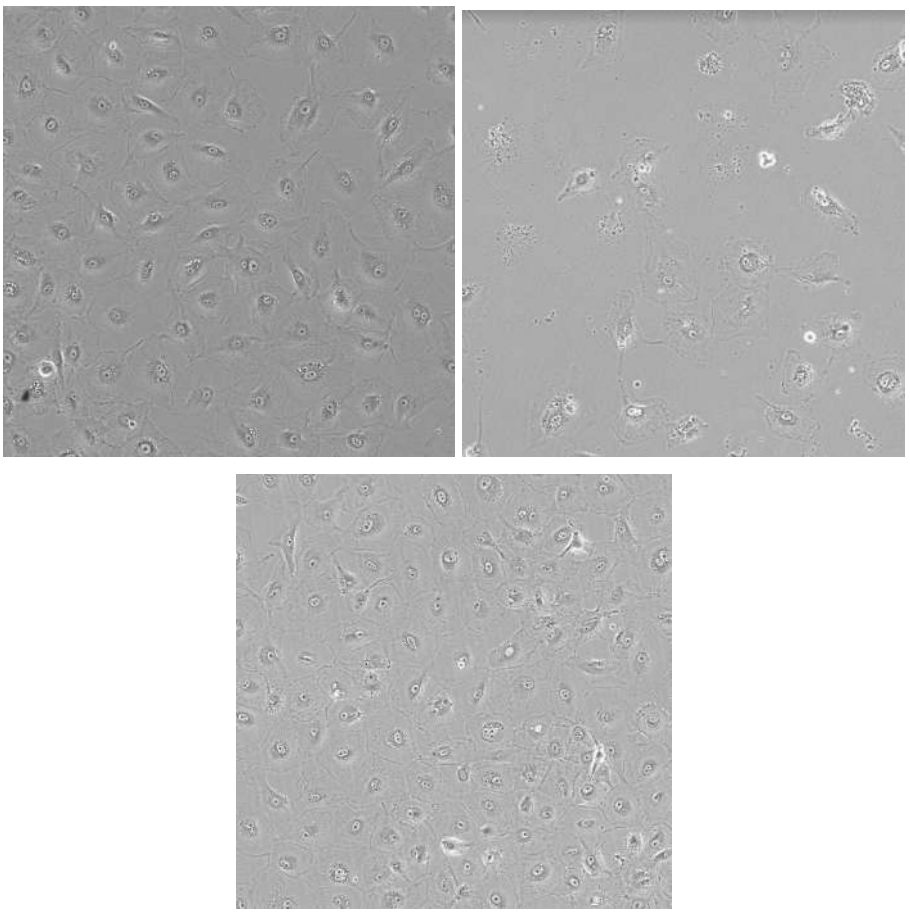


Figure 63 : (a) (upper left) Cells treated with normal concentration (2 μM) of palbociclib and have more irregular dimensions
(b) (upper right)Cells treated with palbociclib (2μM) and concentration of navitoclax (1 μM)
(c)Cells treated with palbociclib (2μM) and concentration of navitoclax (0.1 μM)
(Source: own)

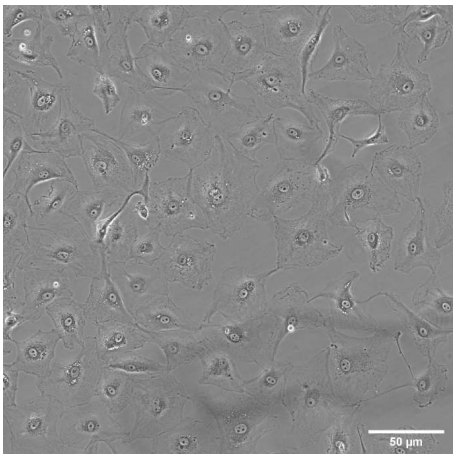


Figure 64 : Cells treated with irradiation and have irregular shape.
(Source: own)

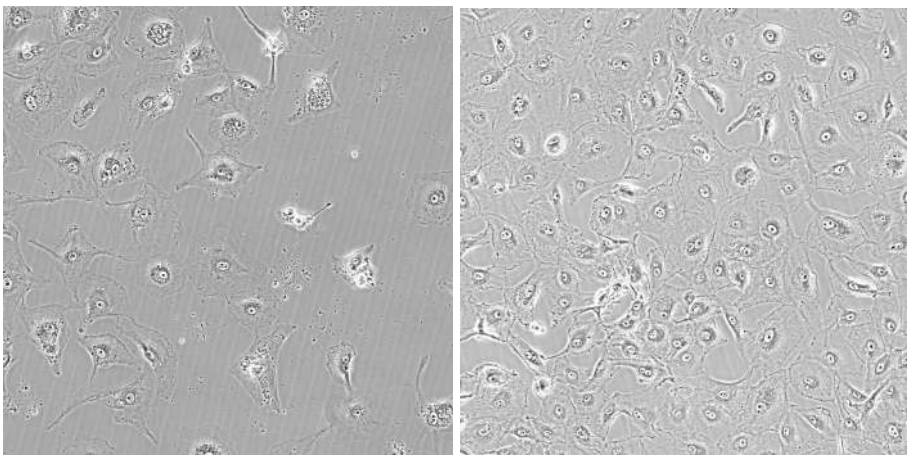


Figure 65 : (a) Cells treated with irradiation and concentration of navitoclax (1 µM)
(a) Cells treated with irradiation and concentration of navitoclax (0.1 µM)
(Source: own)

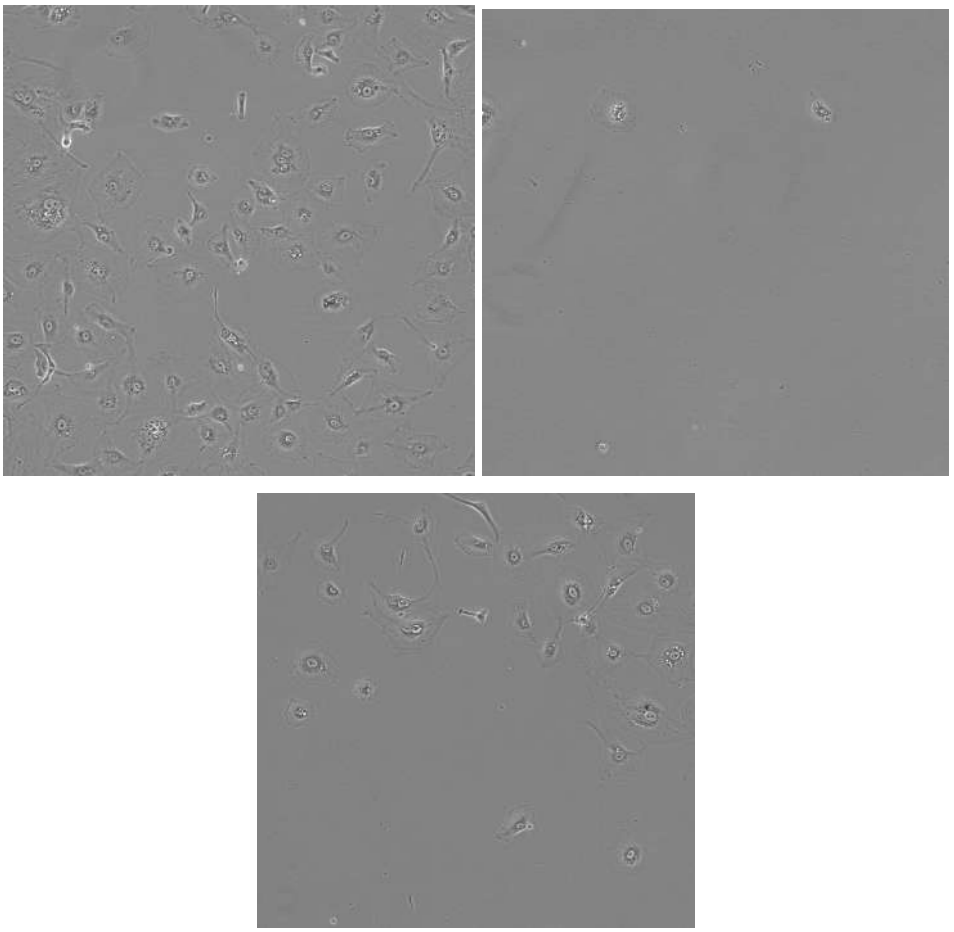


Figure 66 : (a) Cells treated with high concentration (600 nM) of doxorubicin and have more irregular dimensions
(b) Cells treated with doxorubicin (600nM) and concentration of navitoclax (1 µM)
(c)Cells treated with doxorubicin (600nM) and concentration of navitoclax (0.1 µM)
(Source: own)

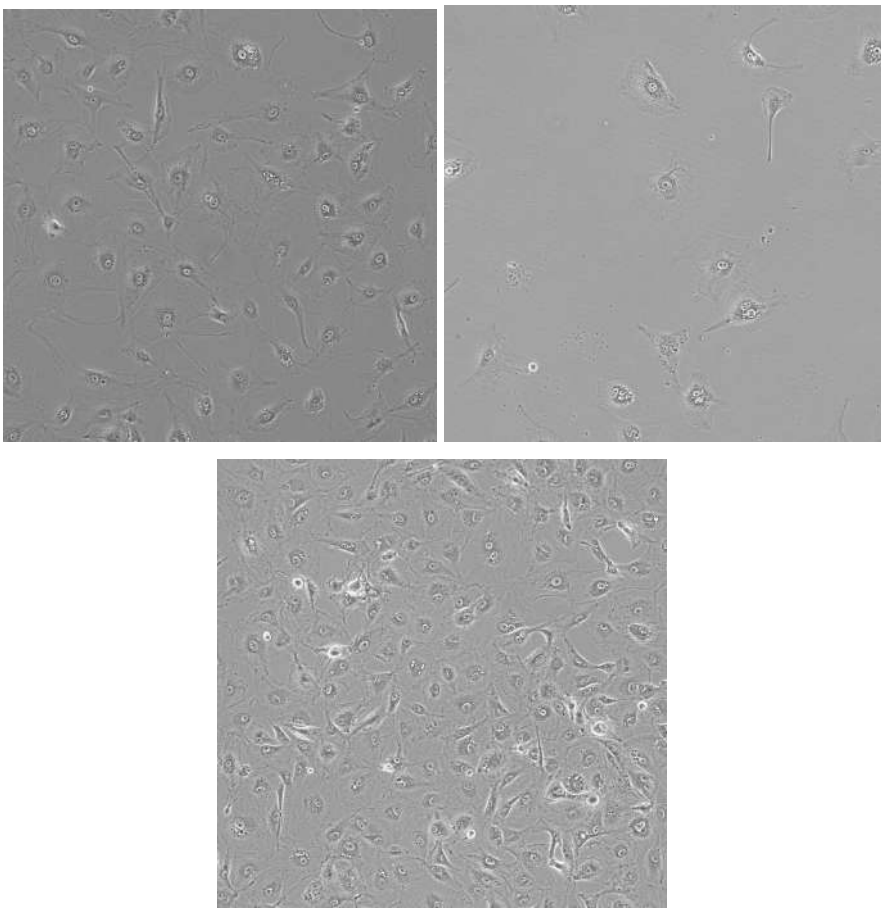


Figure 67 : (a) Cells treated with high concentration (200nM) of doxorubicin and have more irregular dimensions
(b)Cells treated with doxorubicin (200nM) and concentration of navitoclax (1 μM)
(c)Cells treated with doxorubicin (200nM) and concentration of navitoclax (0.1 μM)
(Source: own)

3.1.5 Analysis of results and conclusions

After analysing the pictures we have observed that the DNA damage induces senescence because the morphology of the cells treated with irradiation is very similar to senescent cells which are enlarged cells with irregular shape.

Therefore our hypothesis is confirmed that senescent cells can be created in vitro by DNA damage. However, in order to confirm whether the chemical drugs also induce senescence we have to do the second experiment of SA -β-gal to verify our hypothesis .

On the whole, we also observe less quantity of cells proliferating where there is a major concentration of senolytic drug (navitoclax). Therefore it is confirmed that senolytic agents promote the elimination of senescence. We can identify that sample with irradiation that contains navitoclax in less concentration,has more quantities of

cells. Moreover in the case of samples treated with palbociclib (2 μM and 5 μM) and doxorubicina (600 nM nad 200 nM) ,that also contain navitoclax in more concentration,has less quantity of cells and vice versa.
Therefore, navitoclax acts as a senolytic agent that successfully kills the senescent cells thus reducing the number of cells in the sample.

3.2 Experiment 2 "in vitro" Verify which cells are senescent using the technique of senescence-associated β-galactosidase staining (SA -β-gal) "in vitro" in melanoma cancer cell samples (SK Mel 103 cell line) previously treated with chemical agents and DNA damage to induce senescence.

Research question: Which is the better senescence inducing agent, DNA damage or chemical drugs?

3.2.1 Hypothesis and definition of the experimental approach

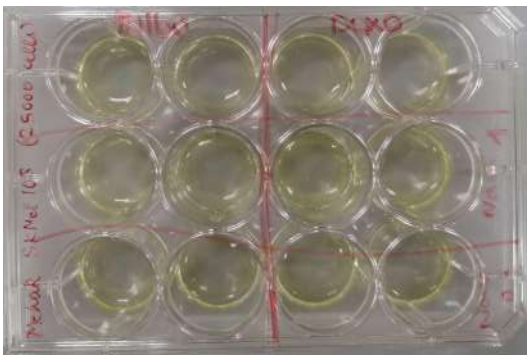
Our **hypothesis** is that maybe the chemical agents are more efficient than DNA damage for inducing senescence. The **independent variables** include cell sample used for detection of senescence (control, irradiation, Doxorubicin 200 nM, Doxorubicin 600nM, Palbociclib 2 μM, Palbociclib 5 μM) with or without senolytic agent. The **dependent variable** is the number and % age of β-gal positive blue senescent cells in each sample. The **controllable variables** include the same temperature conditions, same medium of culture, same quantity of X-gal used for SA -β-gal staining,etc. We have used the **control** which are the cells without any DNA damage, without use of any chemical agent and without use of senolytic agent.

3.2.2 Kit Components for β-gal assay

The kit components, composition, amount and storage conditions are listed in the table in the annex (page no. 94).

3.2.3 Experimental method

Detection of senescence-associated β-galactosidase (SA-β-gal) activity was performed according to the protocol in the annex (page no. 95).



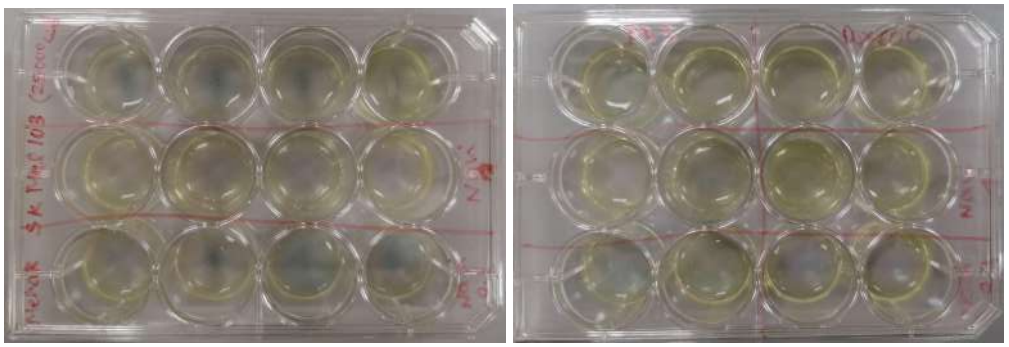
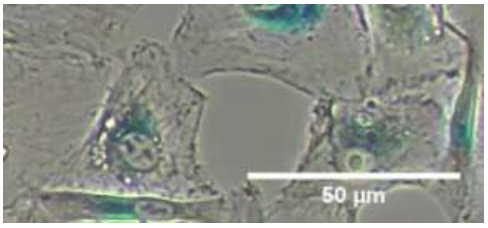
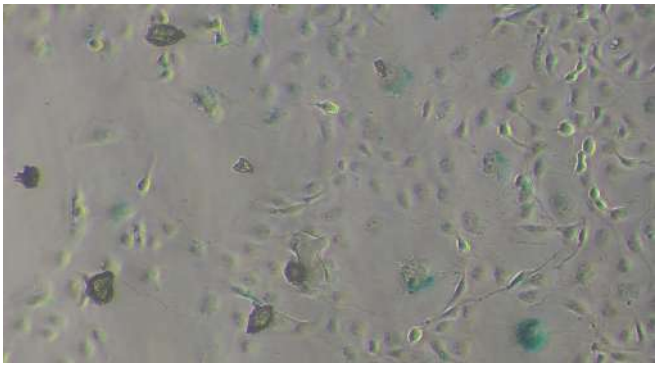


Figure 68 : The multiwell plates of the experiment after being treated with the solution of fixation and staining.
(Source: own)

3.2.4 Results

Observation of photographs with the optical microscope identifying the -gal positive senescent cells in these samples. All the pictures of experiment 2 are taken with a scale bar of 50 μm.





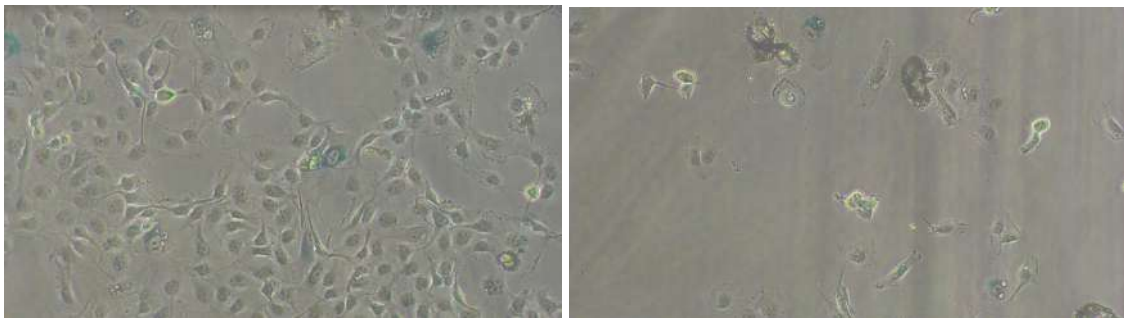


Figure 69: Control cells pic 1, control cells pic 2, control cells pic 3. It is normal for there to be a few blue cells in the control group, but the difference is still quite clear by eye in comparison to senescence induced cells.
(Source: own)

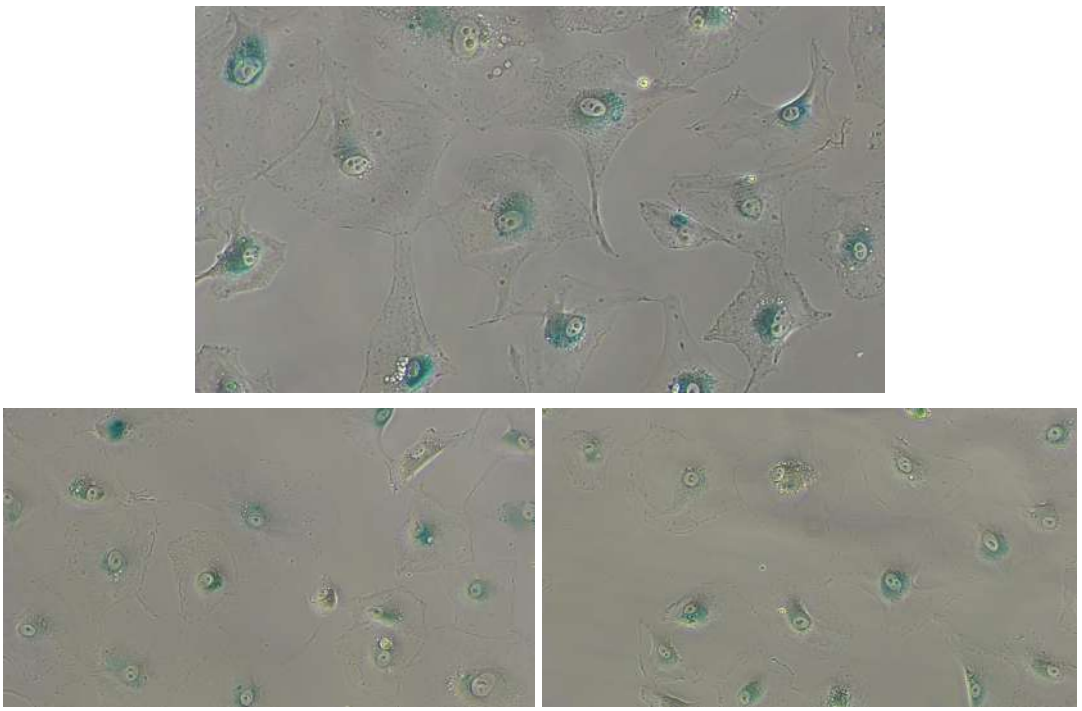


Figure 70: Irradiation pic 1, irradiation pic 2, irradiation pic 3.
There are few senescent cells in irradiation sample, therefore we can say that senescence is induced by DNA damage
(Source: own)

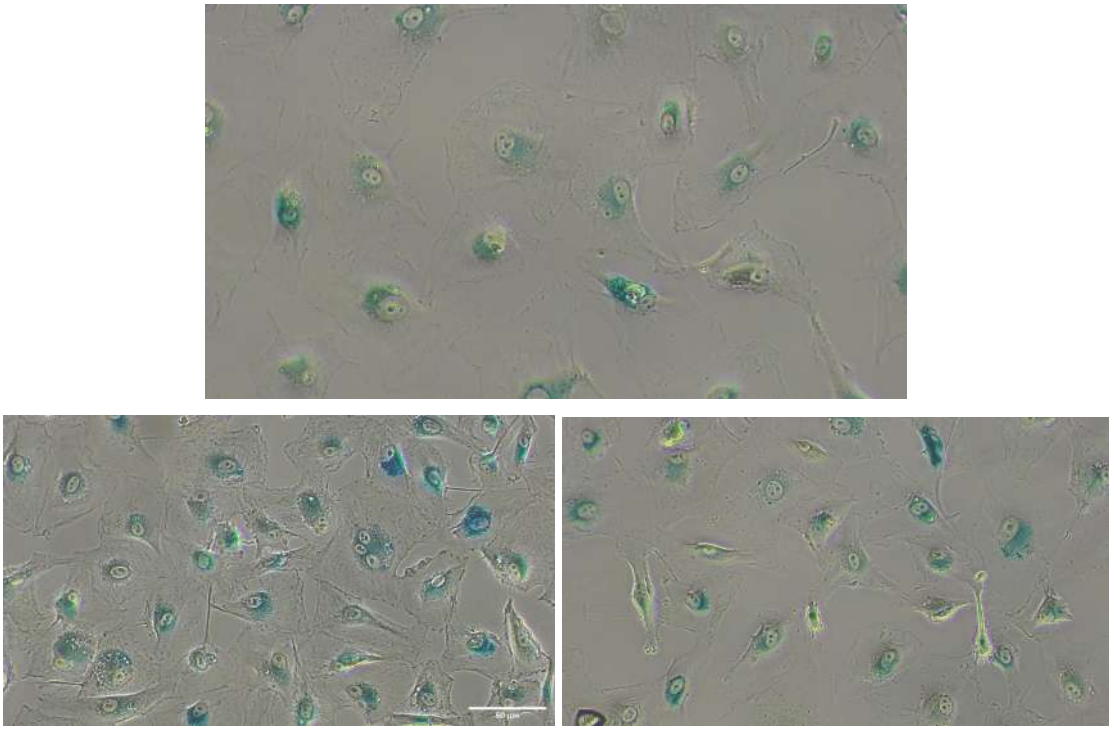


Figure 71: Irradiation navi 0,1 µM-pic 1, pic 2, pic 3
In this case, although we have used the senolytic agent in order to eliminate senescent cells but because of its low concentration it has no effect and we can observe the senescent cells in the sample.
(Source: own)

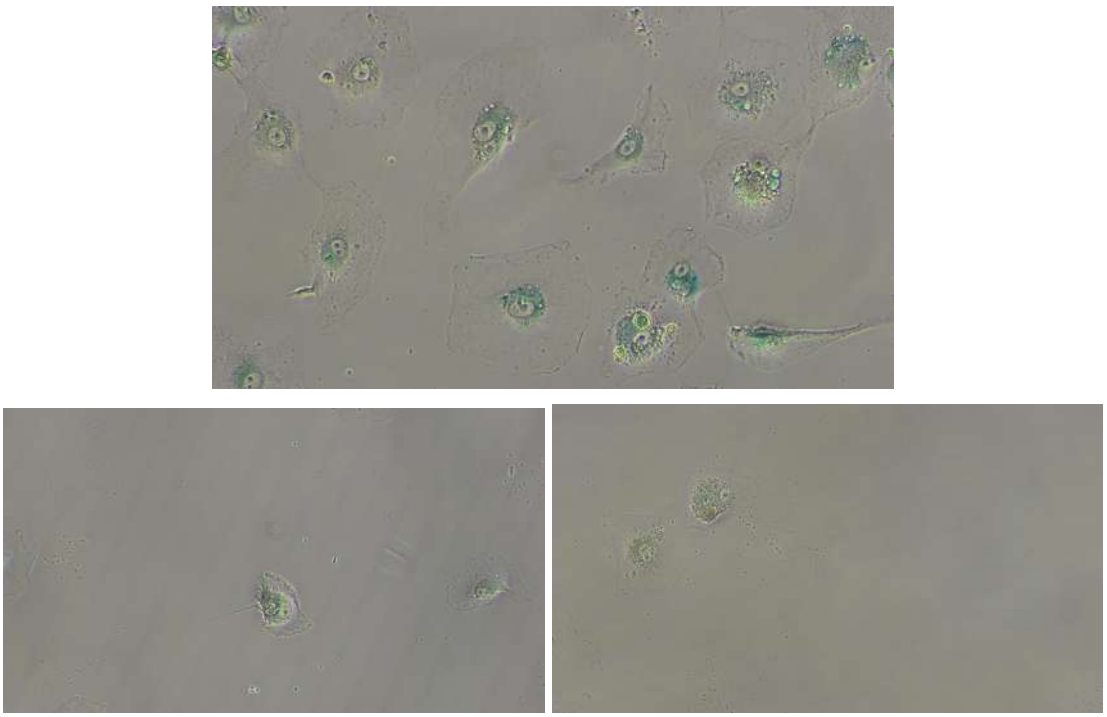


Figure 72: Irradiation navi 1 µM-pic 1, pic 2, pic 3
With the use of senolytic agent in high concentration, nearly no senescent cells were left in these samples.
(Source: own)

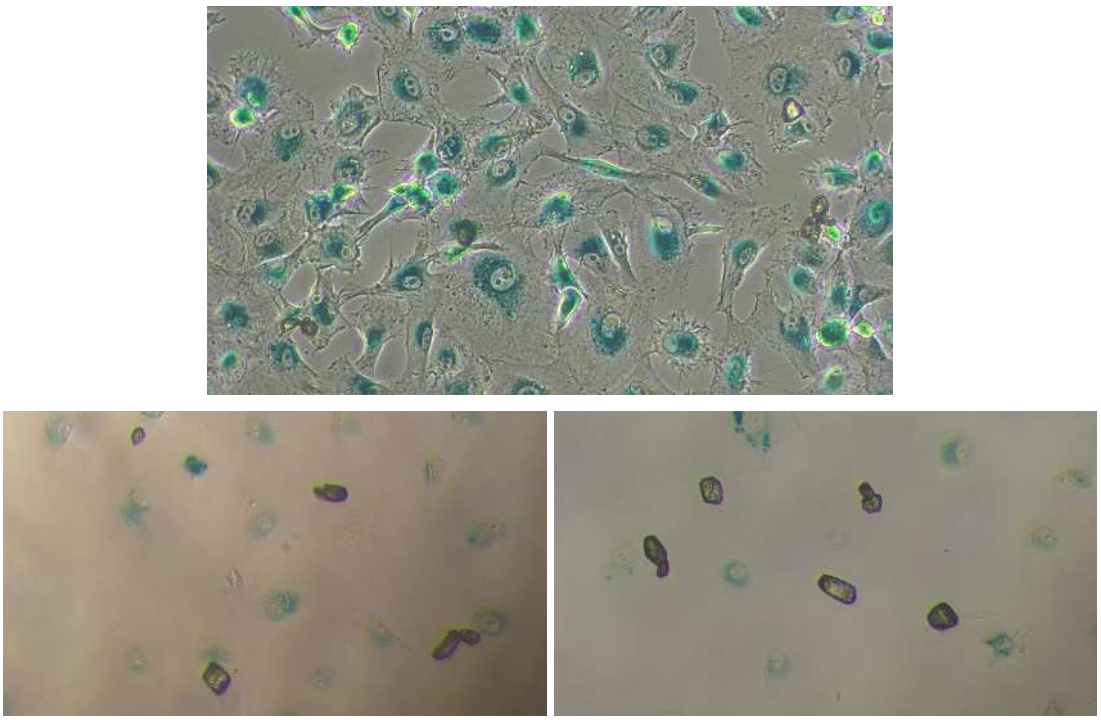


Figure 73: Palbo 2 µM-pic 1, pic2, pic 3
With the normal concentration of chemical agent palbociclib, we can observe a lot of -gal positive blue cells, therefore palbociclib induces a higher level of senescence.
(Source: own)

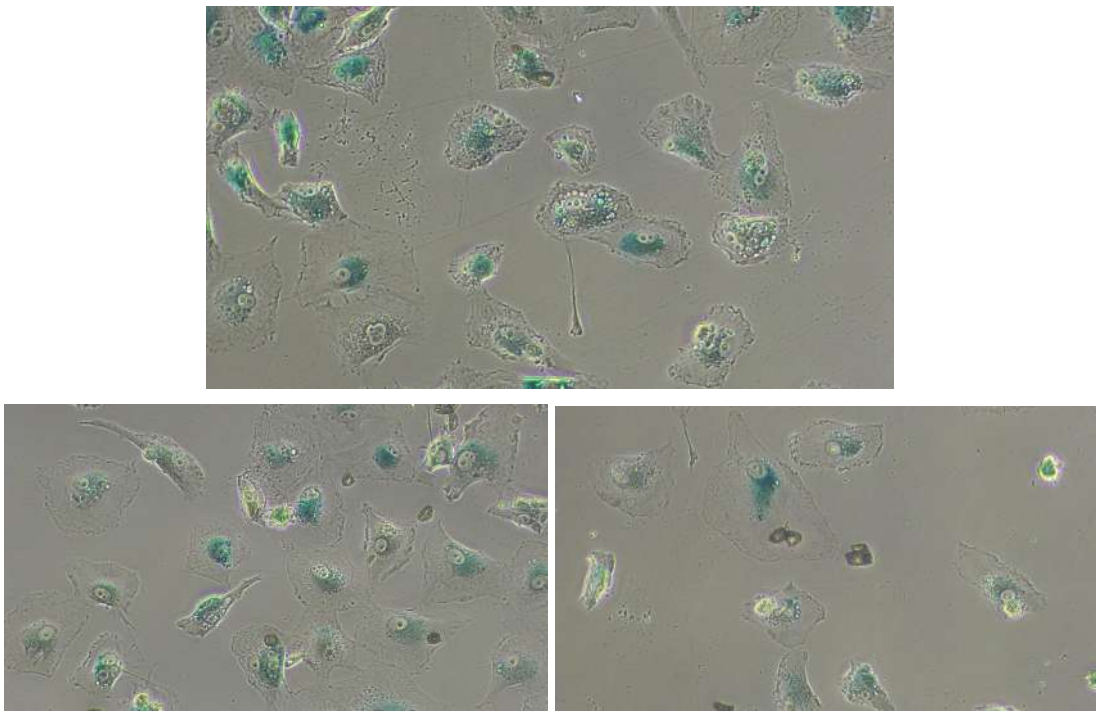


Figure 74: Palbo 2µM-navi 1µM-pic 1, pic 2, pic 3
With the use of senolytic agent, in normal concentration of palbociclib sample, there is a tendency of reduccion in the number of senescent cells.
(Source: own)

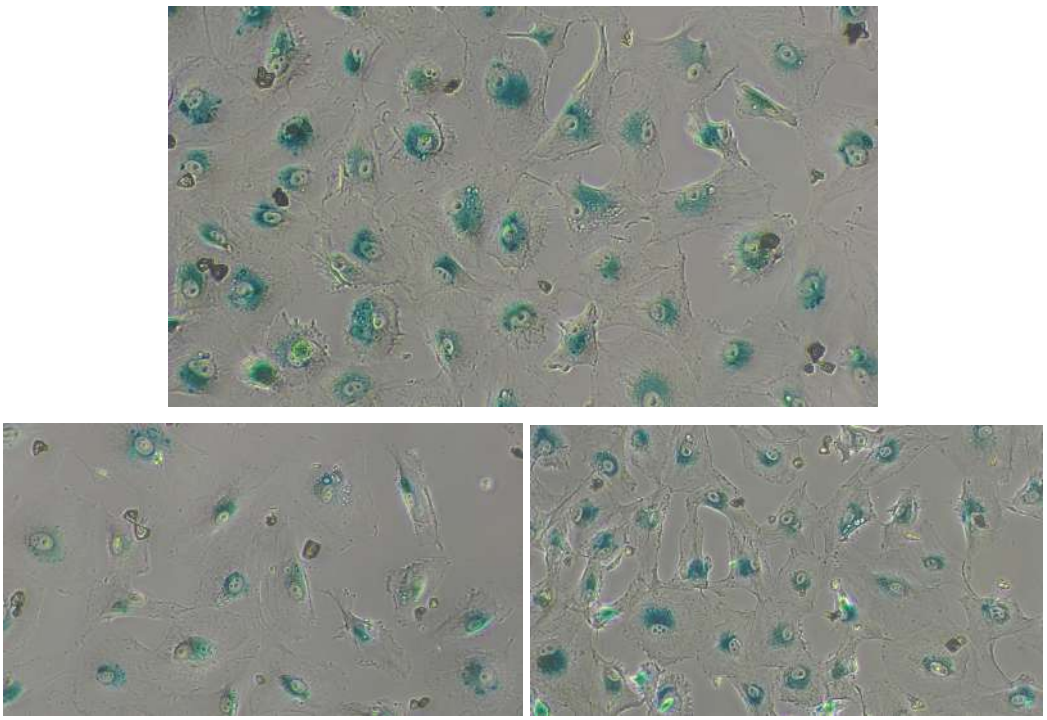


Figure 75: Palbo 5µM-pic 1, pic 2,pic 3
With high concentration of palbociclib, there is a huge number of -gal positive cells in comparison to the samples with its normal concentration.
(Source: own)

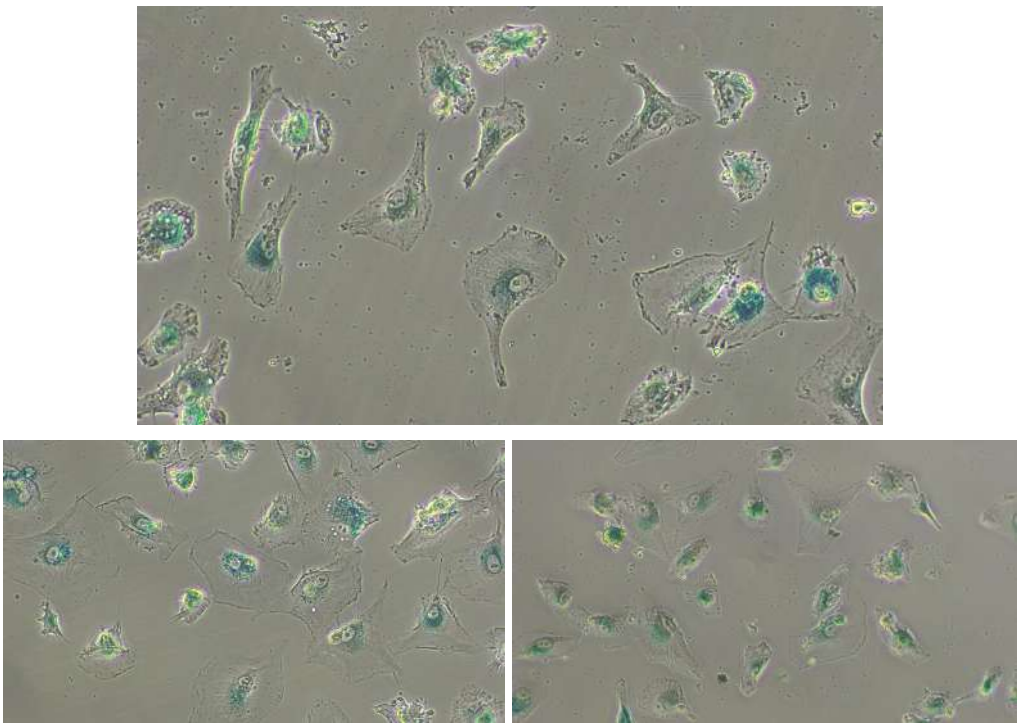


Figure 76: Palbo 5µM-navi 1µM-pic 1, pic 2, pic 3
With the use of senolytic agent, in higher concentration of palbociclib sample, we can also observe that there is reduccion in the number of senescent cells.
(Source: own)

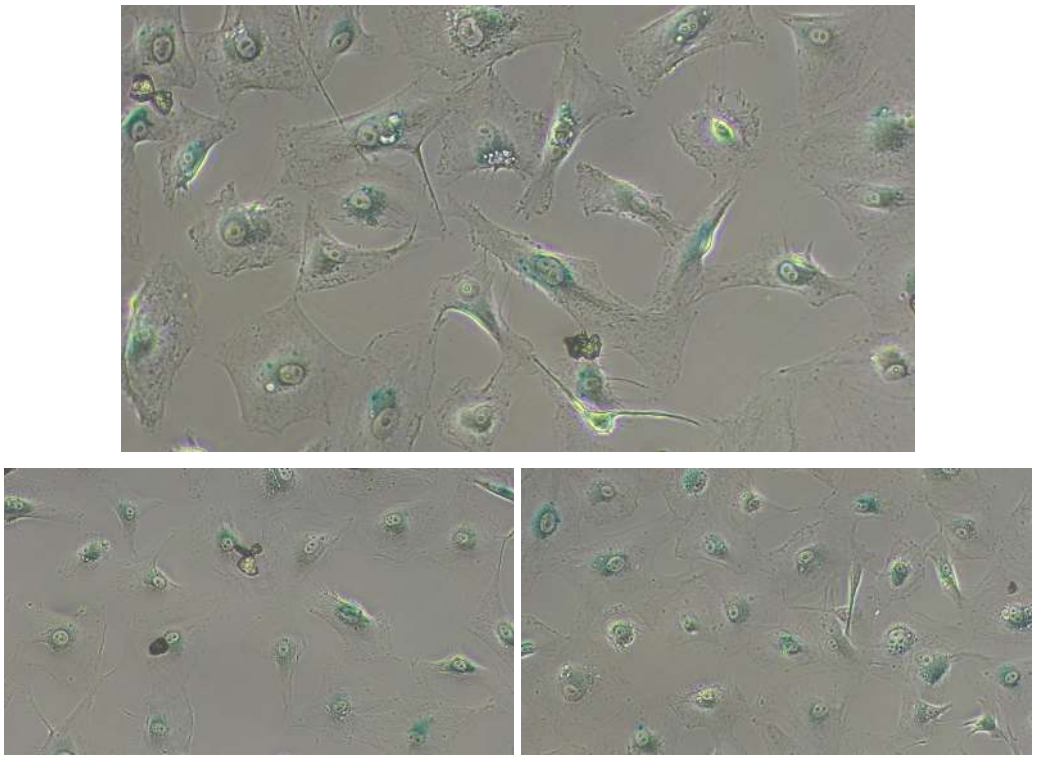


Figure 77: Doxo 200nM-pic 1, pic 2, pic 3
With the normal concentration of doxorubicin, senescence is induced as there are some senescent cells observed in this sample.
(Source: own)

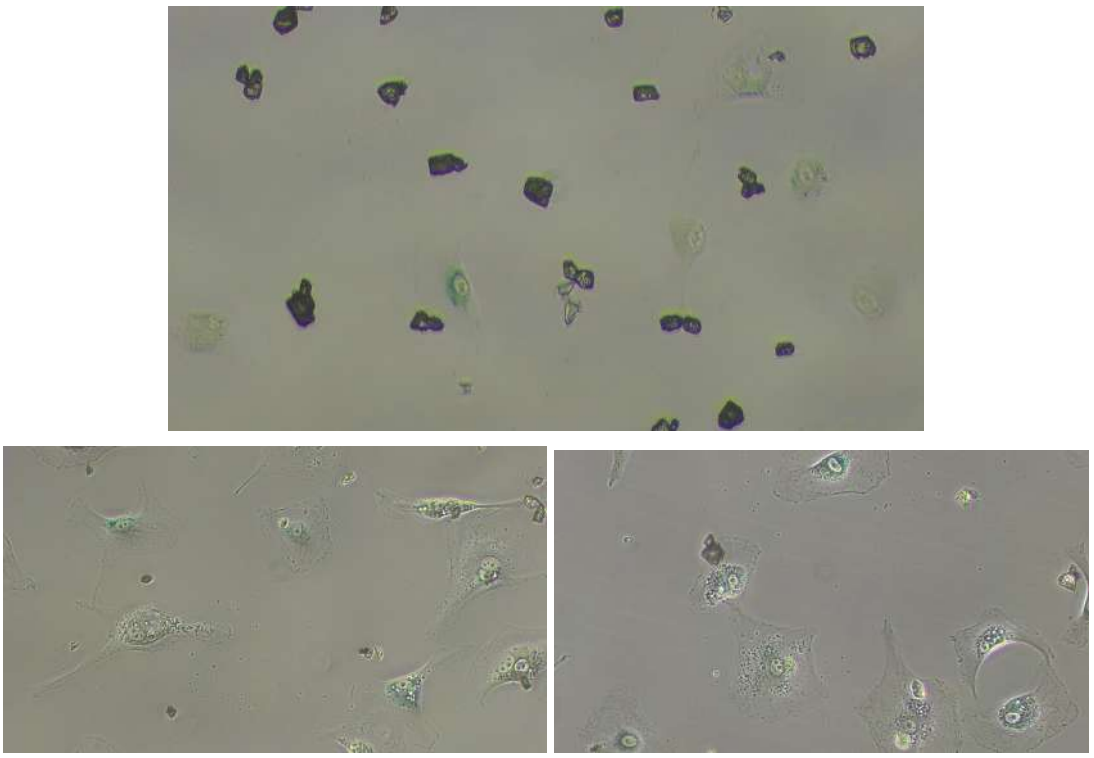


Figure 78: Doxo 200 nM- navi 1µM-pic 1, pic 2, pic 3
With the use of senolytic agent, in normal concentration of doxorubicin sample, nearly we cannot observe any senescent cell left.
(Source: own)

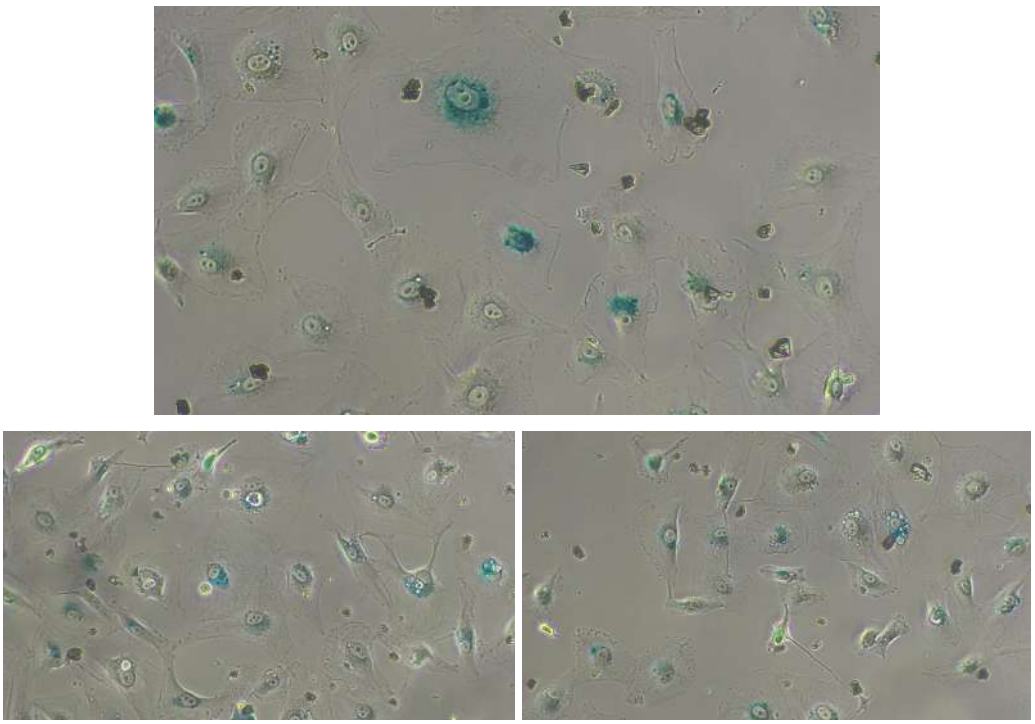


Figure 79: Doxo 600nM-pic 1, pic 2, pic 3
With high concentration of doxorubicin, there is a high number of -gal positive cells in comparison to the samples with its normal concentration.
(Source: own)

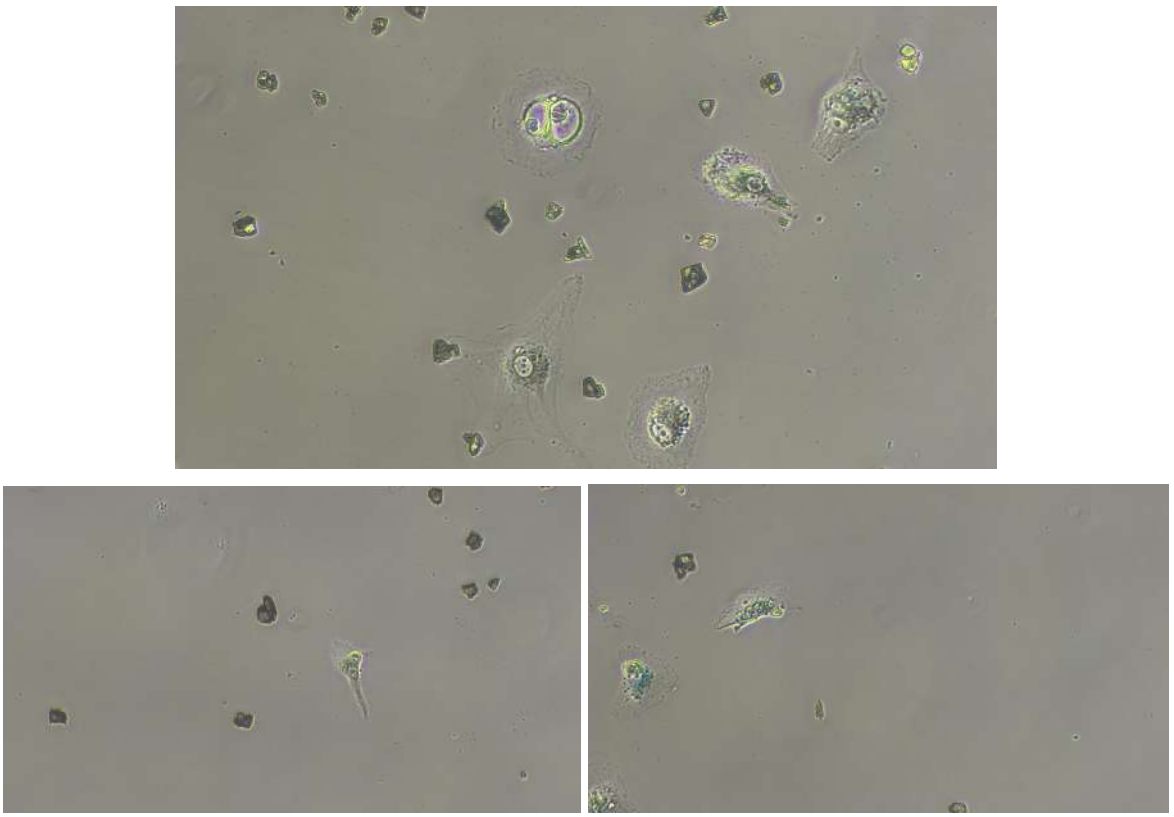


Figure 80: Doxo 600nM- navi 1µM-pic 1, pic 2, pic 3
With the use of the senolytic agent, in high concentration of doxorubicin sample, nearly all the senescent cells are eliminated.
(Source: own)

First of all, three images of each sample were taken and in order to count the number of senescent cells (blue cells), a program named FIJI was used. First we counted the total number of cells, then only the blue cells. The dead cells and the cristalls were not counted (consult the picture in the annex (page no. 96).

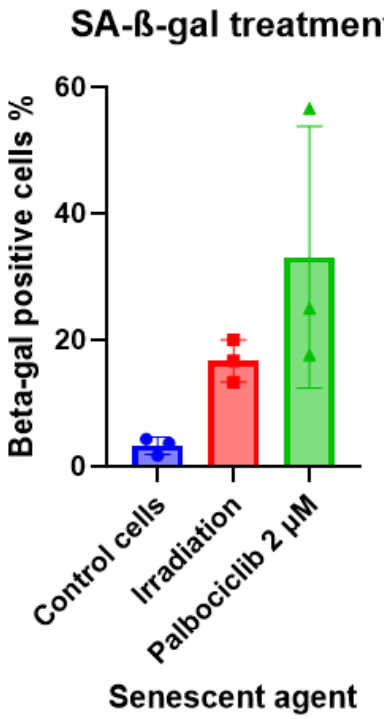
Afterwards, the percentage of SA-β-gal-positive cells was calculated in each sample as shown in the table below:

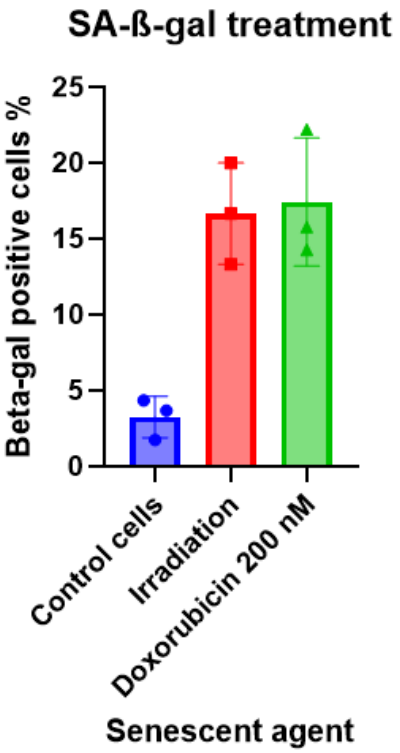
Table 1: Counting -gal positive senescent cell % age

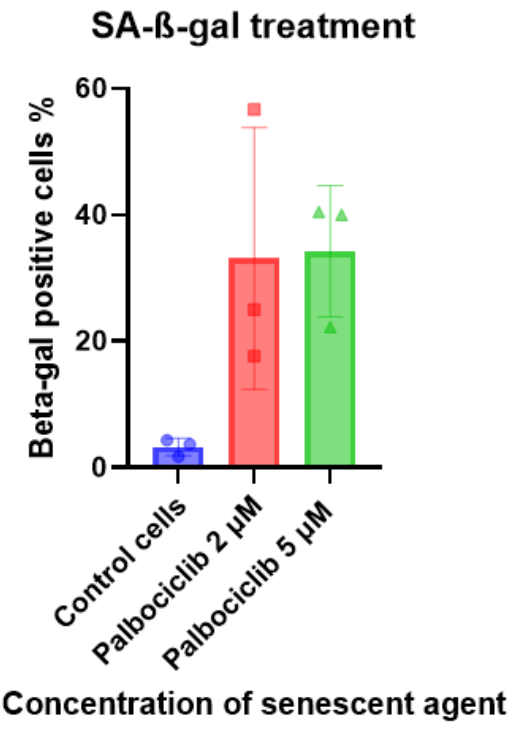
Senescent cells	Senolytic	Concentration of senolytic	Name of cells	Total no. of cells	No. of senescent cells	% age of β-gal positive senescent cells	Average of senescent cells %
Control cells	-		control cells-pic 1	136	5	3,676470588	3,25280024
	-		control cells-pic 2	173	3	1,734104046	
	-		control cells-pic 3	23	1	4,347826087	
Irradiation	-		irradiation-pic 1	15	3	20	16,66666667
	-		irradiation-pic 2	18	3	16,66666667	
	-		irradiationpic 3	15	2	13,33333333	
Irradiation	Navitoclax	0.1 μM	irradiation- navi 0.1μM-pic 1	17	4	23,52941176	17,1865716
			irradiation- navi 0.1μM-pic 2	44	5	11,36363636	
			irradiation- navi 0.1μM-pic 3	24	4	16,66666667	
Irradiation	Navitoclax	1 μM	irradiation- navi 1μM-pic 1	12	1	8,333333333	2,777777778
			irradiation- navi 1μM-pic 2	2	0	0	
			irradiation-navi 1μM-pic 3	2	0	0	
Palbociclib 2 μM	-		palbo 2μM-pic 1	60	34	56,66666667	33,10457516
	-		palbo 2μM-pic 2	17	3	17,64705882	
	-		palbo 2μM-pic 3	8	2	25	
Palbociclib 2 μM	Navitoclax	1 μM	palbo 2μM-navi 1μM-pic 1	28	8	28,57142857	21,19047619
			palbo 2μM-navi 1μM-pic 2	20	3	15	
			palbo 2μM-navi 1μM-pic 3	5	1	20	
Palbociclib 5 μM	-		palbo 5μM-pic 1	42	17	40,47619048	34,23280423
	-		palbo 5μM-pic 2	18	4	22,22222222	
	-		palbo 5μM-pic 3	35	14	40	
Palbociclib 5 μM	Navitoclax	1 μM	palbo 5μM-navi 1μM-pic 1	18	2	11,11111111	13,8047138
			palbo 5μM-navi 1μM-pic 2	18	3	16,66666667	
			palbo 5μM-navi 1μM-pic 3	22	3	13,63636364	
Doxorubicin 200 nM	-		doxo 200nM-pic 1	27	6	22,22222222	17,43247006
	-		doxo 200nM-pic 2	19	3	15,78947368	
	-		doxo 200nM-pic 3	28	4	14,28571429	
Doxorubicin 200 nM	Navitoclax	1 μM	doxo 200 nM- navi 1μM-pic 1	6	1	16,66666667	14,02116402
			doxo 200 nM- navi 1μM-pic 2	9	1	11,11111111	
			doxo 200nM- navi 1μM-pic 3	7	1	14,28571429	
Doxorubicin 600 nM	-		doxo 600nM-pic 1	26	4	15,38461538	13,46153846
	-		doxo 600nM-pic 2	28	4	14,28571429	
	-		doxo 600nM-pic 3	28	3	10,71428571	
Doxorubicin 600 nM	Navitoclax	1 μM	doxo 600nM- navi 1μM-pic 1	5	0	0	0
			doxo 600nM- navi 1μM-pic 2	1	0	0	
			doxo 600nM- navi 1μM-pic 3	3	0	0	

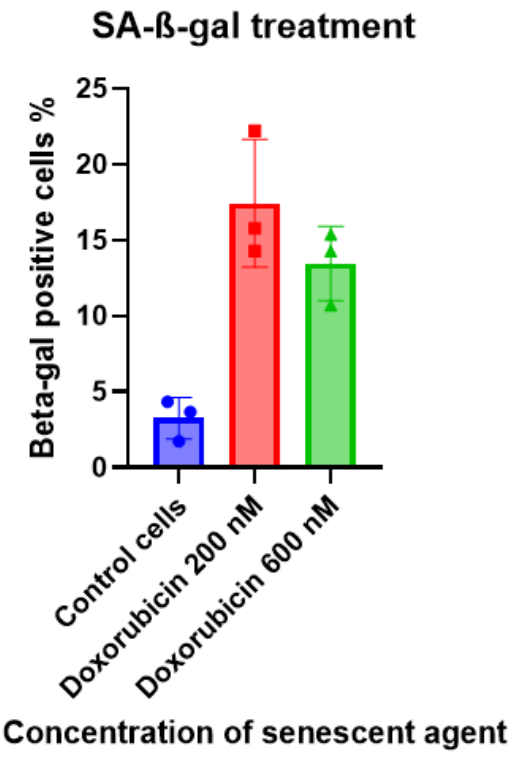
3.2.5 Analysis of results and conclusions

By the excel calculation, we can quantify the percentage of senescent cells in each population to get an estimate of how well our senescence induction worked. Accordingly we have obtained these graphs in order to compare different samples.





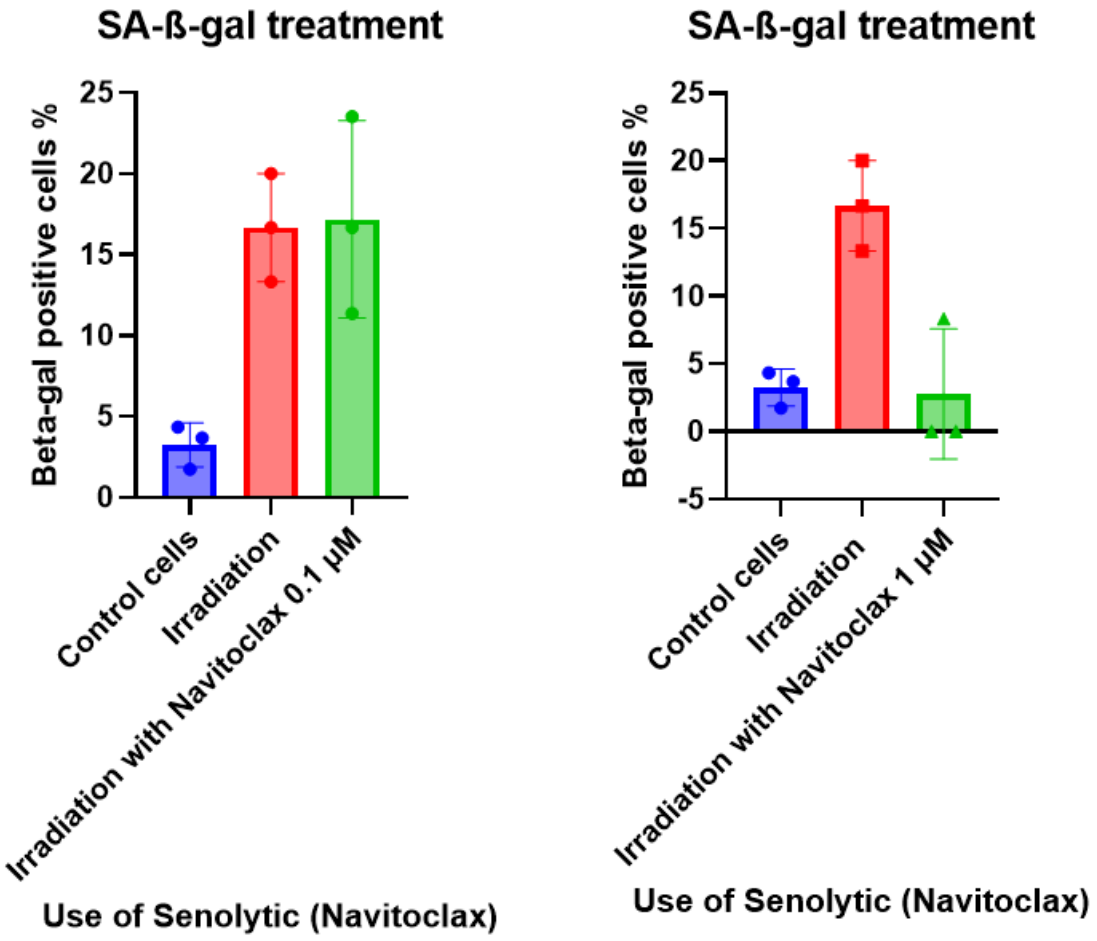




LAYOUT 1: In the upper two graphs I see more -gal positive cells with palbociclib (33.10%) than with irradiation (16.66%). Also with doxorubicin (17.43%) the % age of senescent cells is higher than with irradiation (16.66%) but the difference is low.

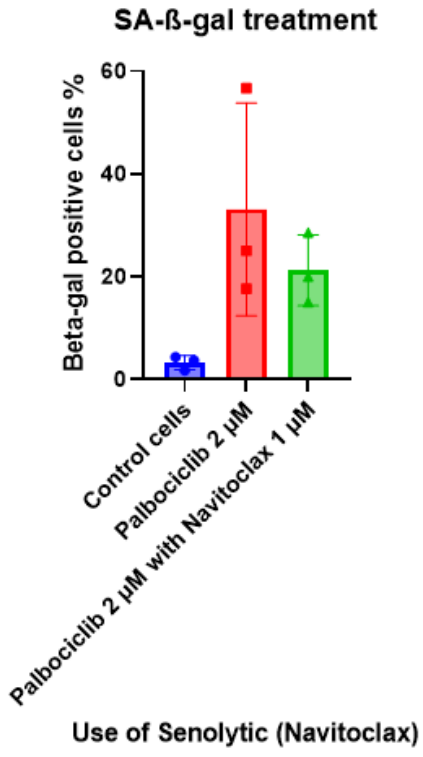
Therefore we can conclude that the palbociclib is the better senescence inducing agent than irradiation and doxorubicin.

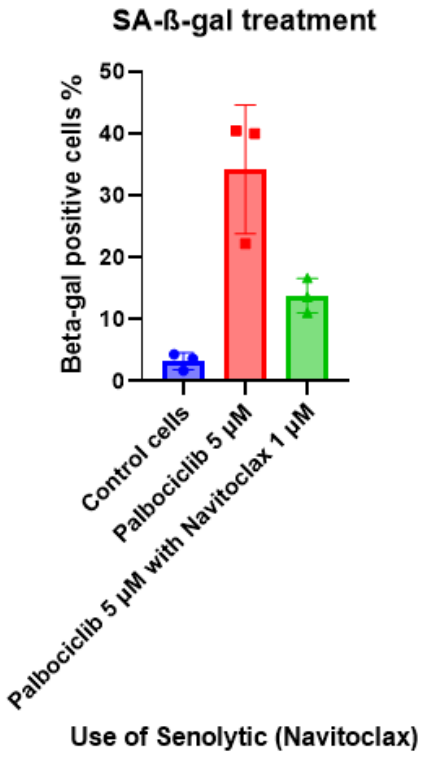
Moreover in the lower two graphs we can interpret that with high concentration of palbociclib 5 µM (34.23%), the % age of beta-gal positive cells is higher than with low concentration 2µM (33.10%). But in the case of doxorubicin this process goes opposite. With high concentration of doxorubicin 600 nM (13.46%), the % age of beta-gal positive cells is lower than with low concentration 200 nM (17.43%). This is not statistically significant since the difference is quite clear compared to control cells and there is also an error to take into account (the standard deviation of the mean).

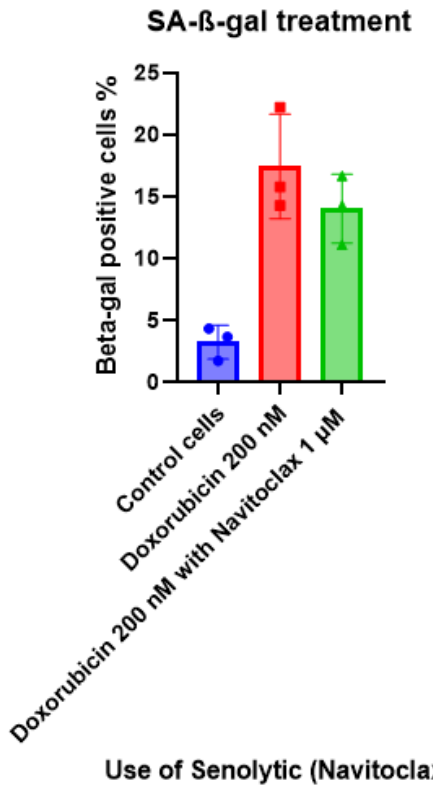


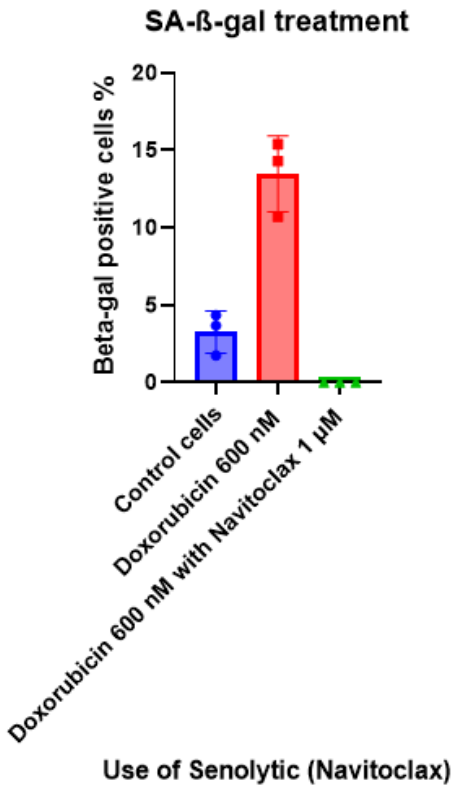
LAYOUT 2

If we compare the irradiation sample with or without navitoclax, we can see that the low concentration of navitoclax has negligible effect but its high concentration leads to high reduction of senescent cells thus reducing the % age of beta-gal positive cells from 16.66% upto 2.77%.









LAYOUT 3:

In the upper graphs while comparing the sample of palbociclib with or without navitoclax, there is a tendency that navitoclax leads to reduction in the percentage of

beta-gal positive cells. In this case the difference in the % age of -gal is lower in the sample of palbociclib 2 µM (11.9%) than palbociclib 5 µM (20.43%). Therefore navitoclax shows more effect when the concentration of palbociclib is lower.
In the lower graphs while doing the comparison of doxorubicin with or without navitoclax, we observe that the navitoclax shows its effect and leads to reduction in the percentage of beta-gal positive cells. However, in the case of 600 nM doxorubicin sample the difference in the % age of -gal is 13.4% which is higher in comparison to the 200 nM (3.4%) doxorubicin sample. Therefore navitoclax shows more effect when the concentration of doxorubicin is higher.

In order to check whether senescence is induced or not, we used the technique of SA -β-gal. In this experiment, on the whole we have observed that neither the cells were dying nor were proliferating. In the control we have a lot of cells and when these cells are treated with different agents, the cells change in size but do not die. This is one of the reasons why we selected the method of -gal in order to detect senescence.

When we compare the presence of the blue dye in our senescent and non-senescent cell populations, we observe a significantly greater number of blue cells in the senescent group than in the control. After analysing the pictures we have observed that in the irradiation samples there are -gal positive senescent cells but in less number in comparison to the samples treated with chemical agents palbociclib and doxorubicin. Therefore our hypothesis that the chemical agents are more efficient than irradiation for inducing senescence is confirmed.

3.3 Experiment 3 “in vivo”: Verify which cells are senescent using the technique of SA -β-gal “in vivo " in tissue sections of subcutaneous tumors from mice treated with chemotherapy. The tissue sample is named as TXK1-2035770L. For our experiment, we choose to use a chemical called palbociclib, which is commonly used in the field to induce senescence in tumors. Moreover we have already seen the positive effect of palbociclib in inducing senescence previously.

Research question: The drug palbociclib is producing the same effects & also working in a real tumor “in vivo” that what we found “in vitro”?

3.3.1 Hypothesis and definition of the experimental approach

Our **hypothesis** is that maybe the drug palbociclib produces the same effects “in vivo” as produced in “in vitro”. The **independent variable** is the use of the chemical agent palbociclib or not. The **dependent variable** is the % age of β-gal positive blue senescent cells in control and palbociclib used sample. The **controllable variables** include the same temperature conditions, same quantity of X-gal used for SA -β-gal staining, same medium of culture,etc. We have used the **control** as the tissue section of the tumor without use of palbociclib.

3.3.2 Material & experimental method

Figure 81: First of all, we take the cryopreserved tumor tissue.
(Source: own)

Figure 82: We took 3 tumor tissue slides (2 control slides and 1 slide treated with palbociclib) and placed it inside the recipient.
(Source: own)

Figure 83 : The media from the cells was removed and washed with PBS.
(Source: own)

Figure 84: Fixation of cells was done with fixative solution

(Source: own)

Figure 85: The sample before and after staining

(Source: own)

Figure 86: Ready to take picture with optical microscope.

(Source: own)

For the protocol of this experiment go to annex on page no 97.

3.3.3 Results

Observation of photographs with the optical microscope identifying the -gal positive area in these tissue samples.

First of all, the images of each sample were taken with different objectives (4x, 10x, 40x) and in order to measure the -gal positive blue area in each sample, a program named FIJI was used to measure the tissue area and the senescence induced blue area. We also found the minimum and maximum color threshold[70] value.
(Consult the picture in the annex page no. 98)

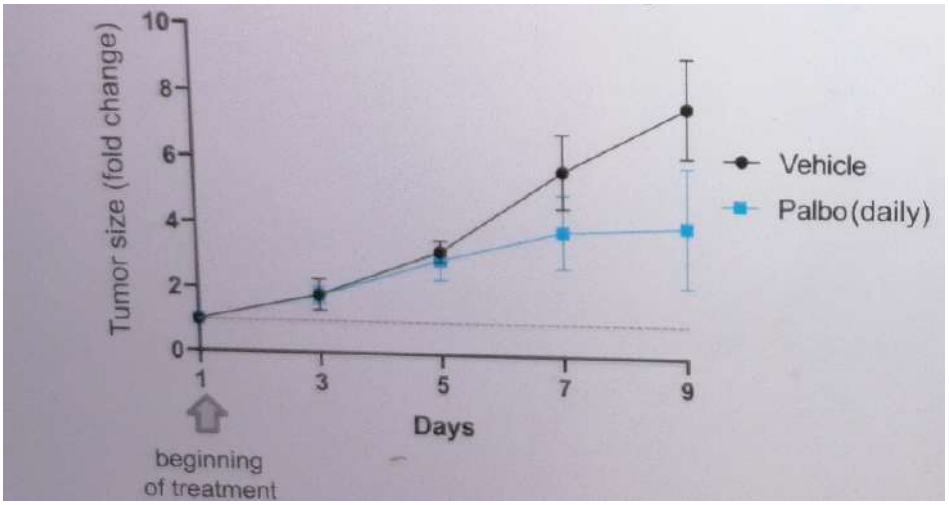
Afterwards, the percentage of SA-β-gal positive cells was calculated in each sample (with or without palbociclib) as shown in the table below:

Table 2: Measuring % age of -gal positive senescent area

Name of the picture		Area	% of b-gal	Average	Color threshold value	
					Min	Max
Tumor sample_4x.tif	B-gal	0,092	0,1025389537		139	142
	Tissue	89,722				
Tumor sample_10x.tif	B-gal	0,695	0,7375100811		147	151
	Tissue	94,236				
Tumor sample_40x.tif	B-gal	0,552	0,5870466872		180	187
	Tissue	94,03				
Tumor sample_palbo_4x.tif	B-gal	4,209	4,556822242		142	147
	Tissue	92,367				
Tumor sample_palbo_10x.tif	B-gal	4,499	4,770741432		139	142
	Tissue	94,304				
Tumor sample_palbo_40x_1.tif	B-gal	4,331	4,587097662		121	136
	Tissue	94,417				
Tumor sample_palbo_40x_2.tif	B-gal	3	2,834492559	4,146832476	132	144
	Tissue	93,597				
Tumor sample_palbo_40x_3.tif	B-gal	4,725	5,018907206		128	140
	Tissue	94,144				

We observed that in the control (tumor sample) the % age of -gal positive area ranged between 0 and 1. Whereas in the tumor sample treated with palbociclib, the % age of -gal positive area lie between 3 and 5.

[70] **Color threshold:** The Color Threshold module is used to remove parts of the image that fall within a specified color range. This module can be used to detect objects of consistent color values.



Graph 1: Growth (size) of tumor with time.
(Source: own)

It was found that in vehicle (control) cells were growing. Whereas the cells treated with palbociclib grow abnormally as after the 7th day the tumor stops proliferating. The possible reasons behind this may be that the cells start dying or maybe they are becoming senescent and stop proliferating.

3.3.4 Analysis of results and conclusions

By the excel calculation, we can quantify the percentage of senescent cells in each population to get an estimate of how well our senescence induction worked in vivo.

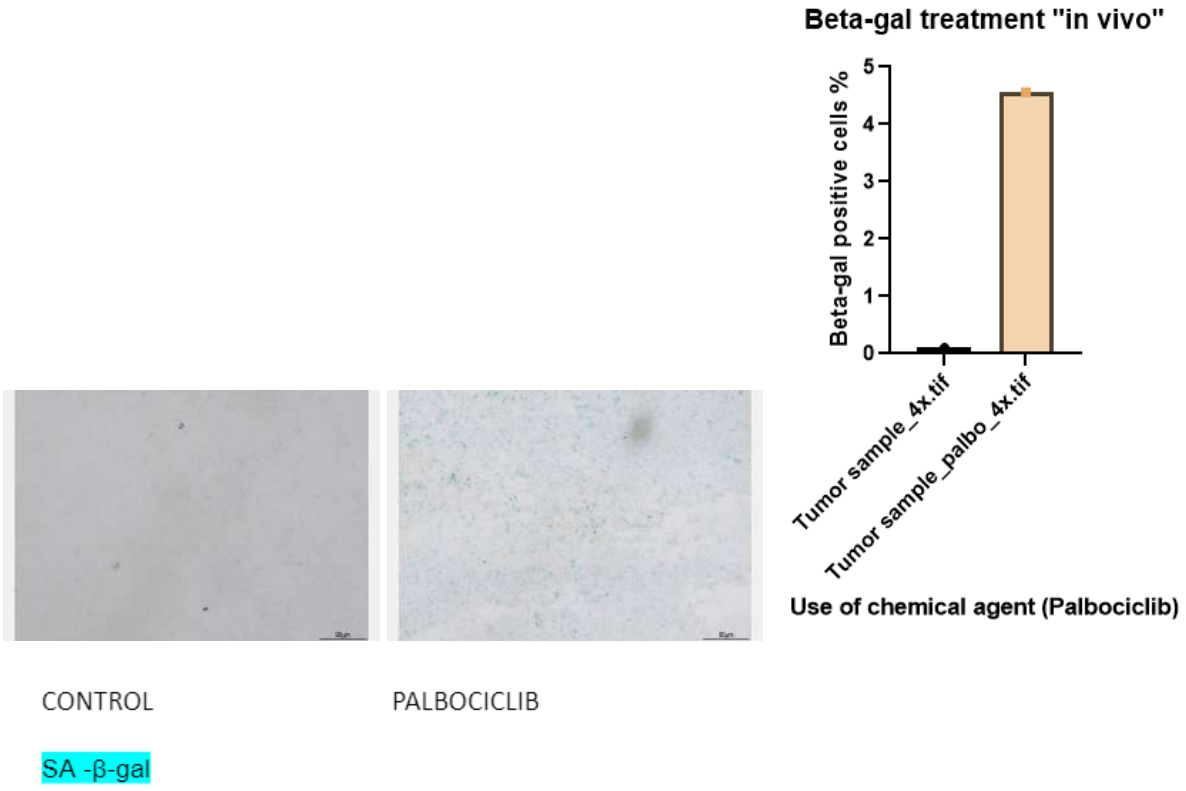
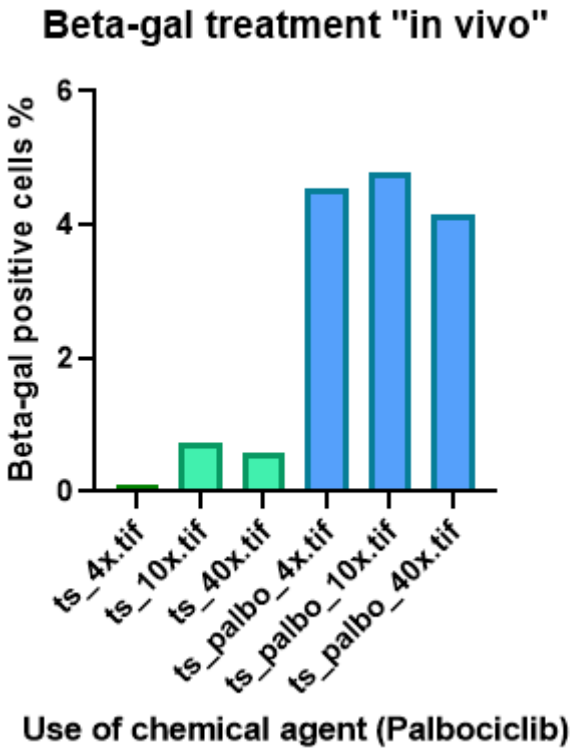


FIGURE 87: Showing area of the tumor tissue with elevated SA-β-gal activity in case of treatment with palbociclib (Source: own)

In the tumor tissue sample treated with palbociclib we can identify the blue area which is positive for β-gal activity whereas in the control we don't see the blue area. This indicated that palbociclib induces senescence “in vivo”. According to the graph, the % age of β-gal positive area in case of palbociclib (4.55%) is much higher in comparison to control (0.10%).



ts- tumor sample

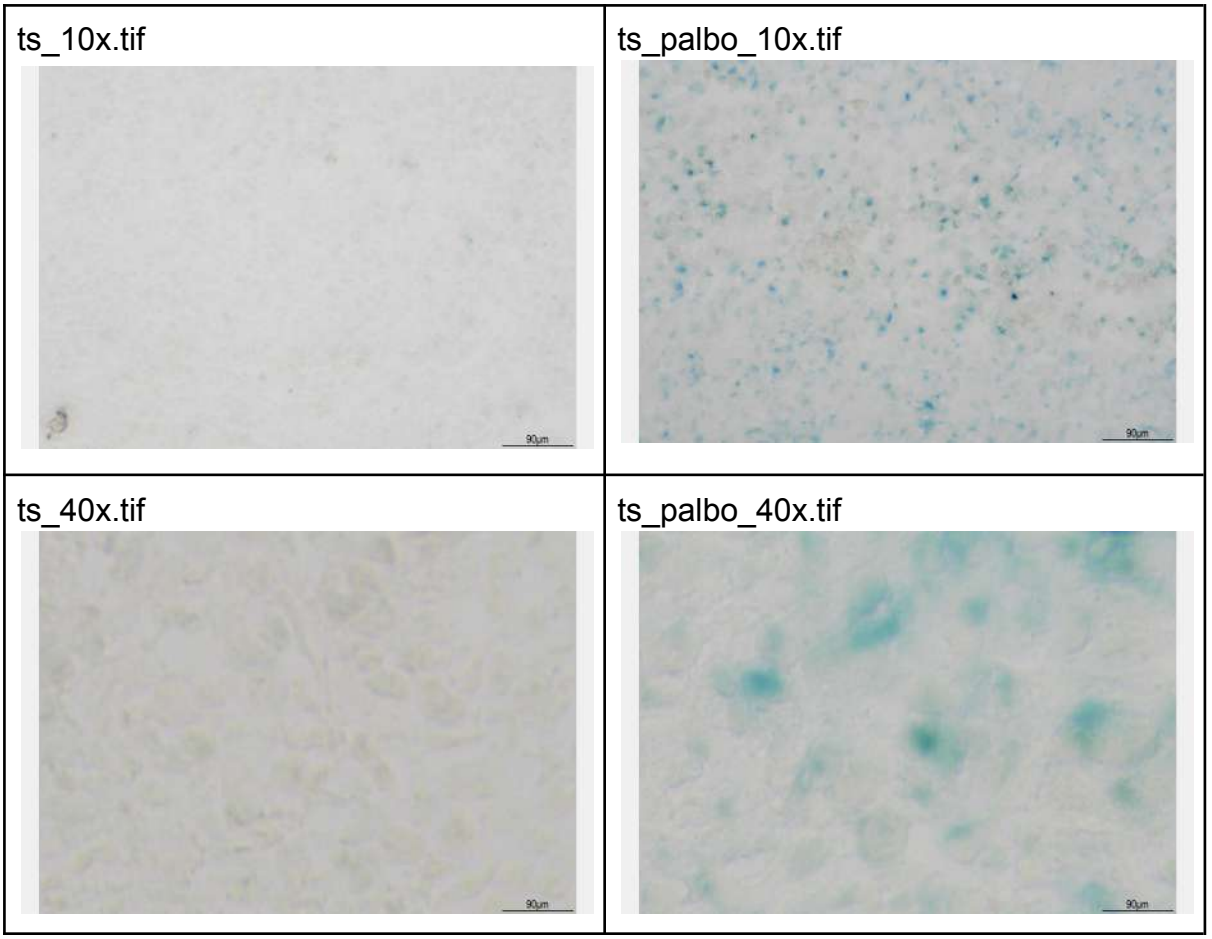


FIGURE 88: Demonstration of senescence induced area of the tumor tissue in case of treatment with palbociclib

(Images taken with different objectius as mentioned)

(Source: own)

In the graph as well in the pictures we can clearly differentiate the control form palbociclib (blue) tumor tissue.The green bars in the graph indicate the low percentage of β-gal positive areas which refer to the control. The blue bars show a high percentage of senescent cells which refer to palbociclib.

After analysing the pictures we have observed a greater area of senescent cells in the palbociclib sample than in the control. Therefore our hypothesis is confirmed that the drug palbociclib produces the same effects “in vivo” as produced in “in vitro”.

4. CONCLUSIONS

4.1 General conclusions and assessment of the project

Throughout this project we have come to know a wide range of aspects related to senescence that we did not know. We have understood that chemotherapy and radiation therapy are capable of inducing senescence whereas the use of senolytic agents reduces the number of senescent cells.

During the experiment in vivo we found that the control tumor cells continue growing normally with time. However, the cells treated with palbociclib grow abnormally as after a certain period of time the tumor stops proliferating. The possible reason behind this is that the cells are becoming senescent and start losing their capability of proliferation thus helping in tumor suppression. That's why we consider cellular senescence as an emergency defense system for cells on the way to becoming cancerous, i.e., a response to the stimulation of oncogene. Unfortunately, sometimes cellular damage can lead to cancer, which happens when the cell's DNA is altered in a way that leads to increased proliferation.

We have concluded that chemotherapy is more efficient than radiation therapy for inducing senescence. Also that senolytic agents promote the elimination of senescence.

While we might consider that our hypotheses in the experiments were directly related to the results obtained, we can say that it is necessary to repeat all these procedures to reach more concrete conclusions.

On a personal level this project has been a challenge. I have faced certain difficulties that have ultimately forced me in some way to organize myself, to work well and not give up on getting the results that may not have suited at first what we expected. This work has given me the opportunity to get to know the scientific world and laboratory life more closely.

I had to accept that this project was complex. The information provided was not easy to understand and write. However, I am happy to have learned how to relate concepts easily and to have been able to summarize and explain scientific processes that at first might seem meaningless.

In conclusion, doing this project has not only provided me with a great deal of knowledge but also a new vision in reference to my day-to-day life which I will apply from now onwards.

4.2 Acknowledgements

First of all, I would like to thank my tutor for her indispensable help in guiding this project.

I would also like to thank a professional researcher at IRB Barcelona for her collaboration. She allowed me to get to know her laboratory, giving me the opportunity to do an experimental part that would not have been possible without her.

Finally, to the Fundació Catalunya La Pedrera and to Crazy about Biomedicine, for getting me closer to the world of science and showing me that knowledge and study are essential tools for education and for daily life.

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6.ANNEX

Interesting facts about cancer:

- **The word "cancer" comes from the Latin word for "crab"-just like the sign of the zodiac.**
  Early doctors, when describing certain tumours that had veins or extensions from the main body, called them crab-like or "cancerous".

- **Cancer was first described by the ancient Egyptians**
  The earliest description of cancer was found in Edwin Smith Papyrus dating back to 1600 BC. The document describes breast tumours that were removed with a tool called a fire drill. However, it states that there is "no treatment".

- **There are more skin cancer cases due to indoor tanning than lung cancer cases due to smoking**
  A recent study has shown that there are more than 419,000 new skin cancer cases each year in the U.S. alone that are due to indoor tanning.

- **More than half of all cancers are preventable**
  Researchers believe that more than half of all cancers - and up to half of all cancer deaths - are preventable. That means there are between 2.4 million

and 3.7 million preventable deaths each year, 80% of which occur in low- and middle-income countries.

- **There are more than 200 types and subtypes of cancer**
  Cancer is not just one disease. In the last 10 years we have come to realise that there are more than 200 different types and subtypes of cancer. This has triggered a shift away from a one-size-fits-all approach to "tailored therapy."

- **Naked mole rats are immune to cancer**
  The body of these subterranean African mammals is rich in a substance called hyaluronan, which acts as a lubricant in the body and prevents the growth of cancer. This discovery could lead to treatments for cancer in the future.

- **There are 28 million cancer survivors worldwide**
  Thankfully, cancer is not always a death sentence - especially with the advances that have been made in recent decades. Although the incidence of cancer is increasing, more people in many countries are surviving cancer than ever before.

- **Only 5-10% of all cancers are entirely hereditary**
  Most cancers develop from a combination of hereditary and environmental factors, including smoking, alcohol, obesity, and diet.

- **Breast cancer occurs more frequently in the left breast than in the right breast**
  The left breast has a 5-10% higher risk of developing cancer than the right breast. The left side of the body is also 10% more prone to melanoma (a type of skin cancer). No one is exactly sure why this is.

Reference of the cells used in vitro:

Cellosaurus SK-MEL-103 (CVCL_6069)

Cell line name	SK-MEL-103
Synonyms	SK-Mel-103; SKMEL-103; SkMel103; SKMEL103
Accession	CVCL_6069
Resource Identification Initiative	To cite this cell line use: SK-MEL-103 (RRID:CVCL_6069)
Comments	From: Memorial Sloan Kettering Cancer Center; New York; USA. Omics: SNP array analysis. Omics: Transcriptome analysis.
Sequence variations	Mutation; HGNC; 7989; NRAS; Simple; p.Gln61Arg (c.182A>G); ClinVar=VCV000013900; Zygosity=Unspecified (PubMed=21725359; PubMed=24576830).
Disease	Melanoma (NCIt: C3224)
Species of origin	Homo sapiens (Human) (NCBI Taxonomy: 9606)
Category	Cancer cell line

Publications	PubMed=7747814 Gruis N.A., Weaver-Feldhaus J., Liu Q., Frye C., Eeles R., Orlow I., Lacombe L., Ponce-Castaneda V., Lianes P., Latres E., Skolnick M., Cordon-Cardo C., Kamb A. Genetic evidence in melanoma and bladder cancers that p16 and p53 function in separate pathways of tumor suppression. Am. J. Pathol. 146:1199-1206(1995) PubMed=17308088; DOI=10.1158/0008-5472.CAN-06-3311 Shields J.M., Thomas N.E., Cregger M., Berger A.J., Leslie M., Torrice C., Hao H.-L., Penland S., Arbiser J.L., Scott G., Zhou T., Bar-Eli M., Bear J.E., Der C.J., Kaufmann W.K., Rimm D.L., Sharpless N.E. Lack of extracellular signal-regulated kinase mitogen-activated protein kinase signaling shows a new type of melanoma. Cancer Res. 67:1502-1512(2007) PubMed=21343389; DOI=10.1158/0008-5472.CAN-10-2958 Rose A.E., Poliseno L., Wang J.-H., Clark M., Pearlman A., Wang G.-M., Vega y Saenz de Miera E.C., Medicherla R., Christos P.J., Shapiro R., Pavlick A., Darvishian F., Zavadil J., Polsky D., Hernando E., Ostrer H., Osman I. Integrative genomics identifies molecular alterations that challenge the linear model of melanoma progression. Cancer Res. 71:2561-2571(2011) PubMed=21725359; DOI=10.1038/onc.2011.250 Xing F., Persaud Y., Pratilas C.A., Taylor B.S., Janakiraman M., She Q.-B., Gallardo H., Liu C., Merghoub T., Hefter B., Dolgalev I., Viale A.J., Heguy A., de

	Stanchina E., Cobrinik D., Bollag G., Wolchok J.D., Houghton A.N., Solit D.B. Concurrent loss of the PTEN and RB1 tumor suppressors attenuates RAF dependence in melanomas harboring (V600E)BRAF. Oncogene 31:446-457(2012) PubMed=24576830; DOI=10.1158/0008-5472.CAN-13-2625 Nissan M.H., Pratilas C.A., Jones A.M., Ramirez R., Won H., Liu C., Tiwari S., Kong L., Hanrahan A.J., Yao Z., Merghoub T., Ribas A., Chapman P.B., Yaeger R., Taylor B.S., Schultz N., Berger M.F., Rosen N., Solit D.B. Loss of NF1 in cutaneous melanoma is associated with RAS activation and MEK dependence. Cancer Res. 74:2340-2350(2014)
Cross-references	
Cell line databases/resources	Lonza; 1337
Gene expression databases	GEO; GSM555127 GEO; GSM555180 GEO; GSM784512 GEO; GSM3039513
Other	Wikidata; Q54953761
Polymorphism and mutation databases	Cosmic; 685207 Cosmic; 1054850 Cosmic; 1122258 Cosmic; 1507613 Cosmic; 1669161 Progenetix; CVCL_6069
Entry history	
Entry creation	04-Apr-2012
Last entry update	20-May-2021
Version number	18

PROTOCOLS OF THE EXPERIMENTS:

EXPERIMENT 1 : In order to introduce the sample in the Neubauer camera, these steps were followed:

Step 2 – Introducing the sample into the Neubauer chamber

Take 10 µl of dilution prepared in step 1 with the micropipette.

1. Put the glass cover on the Neubauer chamber central area. Use a flat surface to place the chamber such as a table or workbench.
2. Put a disposable tip at the end of the micropipette.
3. Adjust the micropipette to draw 10 µl. You can adjust it by turning the upper plunger roulette to select the required pipetting volume.
4. Introduce the micropipette tip on the dilution previously prepared in step 1.
5. Push the pipette plunger slowly until you feel it has arrived to the end of its travel.
6. Remove the pipette tip from the dilution and bring it to the Neubauer chamber. When the pipette is loaded, it must always be held in vertical position
7. Place the pipette tip close to the glass cover edge, right at the center of the Neubauer chamber.
8. Release the plunger slowly, watching how the liquid enters the chamber uniformly, being absorbed by capillarity. See Figure 5.
9. In case of the appearance of bubbles or that the glass cover has moved, repeat the operation.

The Neubauer chamber has been loaded, and is ready to perform the cell count!

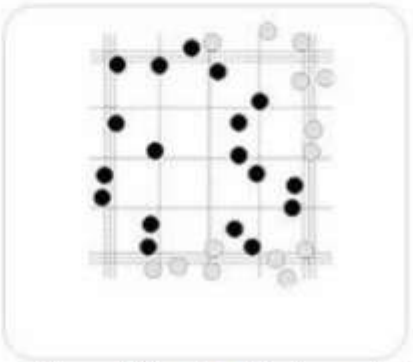


Figure 5. Sample filling a Neubauer chamber; Figure 6. Count in a Neubauer chamber big square

EXPERIMENT 2: The **kit components** composition, amount and storage conditions **for β-gal assay** are listed in the table below. Enough reagents are included for one hundred 800 µl reactions or four hundred 200 µl reactions (equivalent to four 96-well microtiter plates).

Component	Composition	Amount	Storage
10X PBS (Phosphate Buffered Saline)	0.017 M KH_2PO_4 0.05 M Na_2HPO_4 1.5 M NaCl, pH 7.4	60 ml	Room temperature or +4°C
1X Lysis Buffer	0.25 M Tris, pH 8.0	10 ml	Room temperature or +4°C
10X Cleavage Buffer	0.6 M Na_2HPO_4-$7H_2O$ 0.4 M NaH_2PO_4-H_2O 0.1 M KCl 0.01 M $MgSO_4$-$7H_2O$ pH 7	25 ml	+4°C
β-mercaptoethanol	14.3 M	750 µl	Room temperature or +4°C
ONPG	4 mg/ml ONPG in sterile water	5 x 2 ml	-20°C
Stop Buffer	1 M Sodium Carbonate	55 ml	Room temperature or +4°C

EXPERIMENT 2: Detection of senescence-associated β-galactosidase (SA-β-gal) activity was performed according to the following protocol:

β-galactosidase Assay

Before Starting

Be sure to have cells transfected with a *lacZ* construct and prepare the following solutions:

- Dilute the 10X PBS and 10X Cleavage Buffer to make 1X solutions by adding 90 ml distilled, deionized water to 10 ml of stock solution.
- Add 270 µl β-mercaptoethanol to 100 ml 1X Cleavage Buffer before use.
- Unused 1X solutions may be stored at +4°C for 6 months for use in future assays.

Sample Preparation

1. Starting with transfected cells, remove the growth medium from the cells and wash transfected cell monolayers once with 1X PBS.
2. Harvest cell monolayers with trypsin/EDTA or by scraping cells into 1 ml 1X PBS.
3. Centrifuge cells at 250 x g for 5 minutes. Aspirate the supernatant.
4. Completely resuspend the pellet in 1X Lysis Buffer. Keep sample at +4°C.

   Note: The amount of Lysis Buffer used varies depending on the size of the cell pellet. For a pellet harvested 48-72 hours posttransfection from a 60 mm plate, use 50 µl 1X Lysis Buffer. For a 100 mm plate, use 100 µl.
4. Freeze the sample on dry ice and thaw in a 37°C water bath. Repeat 2 times.
5. Pellet the insoluble cell material by centrifugation at maximum speed at +4°C for 5 minutes. Transfer the supernatant to a new microcentrifuge tube.

β-galactosidase Assay

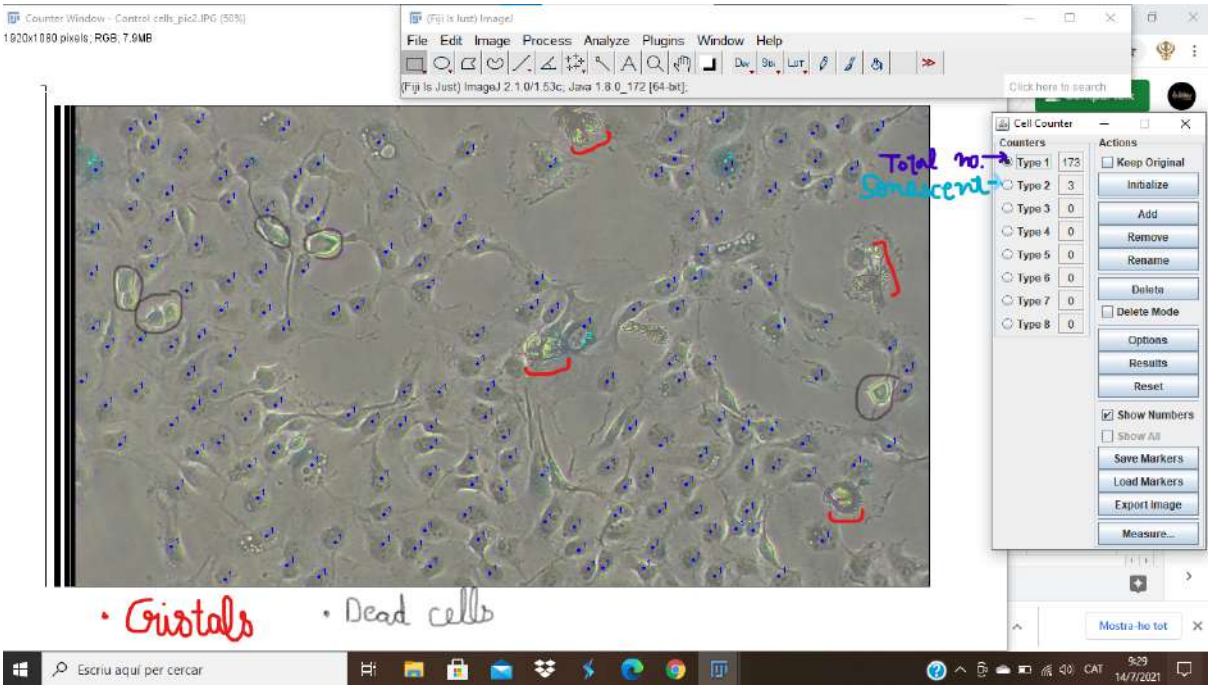
1. For each sample (see Step 5 above), take 1-10 µl of cell lysate and transfer to a fresh microcentrifuge tube.
2. Bring to a final volume of 30 µl with distilled, deionized water.
3. Add 70 µl of ONPG and 200 µl 1X Cleavage Buffer with β-mercaptoethanol. Mix by gently flicking the tube and centrifuging briefly.
4. Incubate the tube at 37°C for 30 minutes. You should see a faint yellow color develop if β-galactosidase is present.
5. To stop the reaction, add 500 µl of Stop Buffer. There may be some intensifying of the color. Final volume is 800 µl.
6. Read the absorbance at 420 nm against a blank containing ONPG and Cleavage Buffer without lysate. Be sure to assay a sample of the untransfected cell lysate as a control.
7. Assay at least three different volumes of lysate (i.e. 1, 5, and 10 µl). Changes in absorbance should be linear with respect to the amount of lysate assayed. If it is not, you will not get an accurate determination of activity.
8. Once you have obtained an accurate reading of your lysate, determine the protein concentration of the lysate, and calculate the specific activity of the lysate using the following formula:

Specific activity = nmoles of ONPG hydrolyzed/t/mg protein

$$\text{nmoles of ONPG hydrolyzed} = \frac{(OD_{420})\ (8 \times 10^5 \text{ nanoliters})}{(4500 \text{ nl/nmoles-cm})(1 \text{ cm})}$$

where 4500 is the extinction coefficient, t = the time of incubation in minutes at 37°C (i.e. 30 minutes), and mg protein is the amount of protein assayed, which can be determined using the BCA assay (Pierce Chemical). Be sure to subtract the background activity of the untransfected cell lysate.

EXPERIMENT 2: Using the FIJI Program, the number of senescent (blue) cells were counted in order to obtain the results.



Cell counting using FIJI (IMAGE; control cells pic 2.)
(Source: own)

EXPERIMENT 3:

Notes: Days are numbered from day of drug addiction. Example: drug was added on Day 0. Day 3 is 3 days following the drug. Day -1 is one day before adding drug. A regular 2-well chamber slide is set up with control untreated cells on the left and drug-treated on the right. Room temperature PBS are used unless noted otherwise. All washes are performed by quickly aspirating after adding 1 mL.

Preparation and treatment of cells:

1. On day -1, plate 2.5 × 10^4 cells into the right chamber of a 2-well chamber slide in 2 mL media.
2. On the next day (Day 0), aspirate media and replace with 2 mL media containing 1 μM PD (1 μM of 10 mM drug stock into 10 mL media). Replenish drug media every 3-4 days as required.
3. On day before fixing, plate 5 × 10^4 control cells into the left chamber of the same slide.

Fixation and staining:

4. Thaw staining kit in the 37°C water bath for at least 1 min before use.
5. On the last day of treatment, remove media from cells and wash 2X 1 mL PBS.
6. Fix the cells with 1 mL 1X Fixative Solution(dilute 1:10 in PBS from 10X stock) for 15 min RT.
7. Prepare Staining Solution
    A. 930 μL Staining solution (93 μL 10X Stain + 837 μL PBS)
    B. 10 μL Staining Supplement A
    C. 10 μL Staining Supplement B
    D. 50 μL X-gal in DMF

8. Wash 2X with 1 mL PBS9.
9. Add 1 mL Staining Solution. Incubate overnight at 37° C (back in tissue culture incubator)
10. Remove Staining Solution. Wash 2X with 1 mL PBS.
11. Add 2 μg/mL DAPI (1:500 dilution from 1 mg/mL stock) 5 min RT in dark.
12. Wash 2X 1 mL PBS.
13. Mount cells for microscopy. Sign up for microscopy time slot, preferably AxioVision#2.
14. Take at least 10 fields at 10X magnification in both brightfield and blue filter settings. Optimal microscope settings is lens II (versus 1/HC for immunofluorescence)

EXPERIMENT 3: Using the FIJI Program, the β-gal positive area was measured in order to obtain the results.

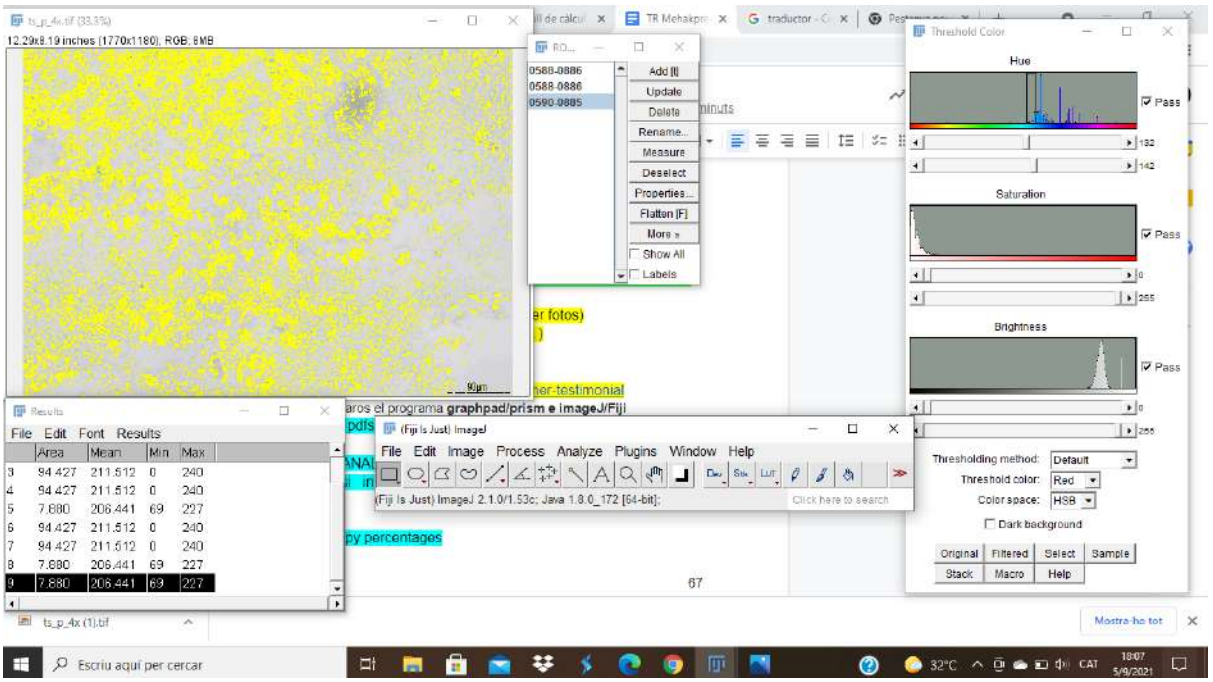


Figure 87: β-gal positive area measurement using FIJI
(Source: own)

Additional image of the workplace :

Laboratory at IRB Barcelona