UNIVERSIDADE DA CORUÑA

Traballo Fin De Máster

Control de la senescencia celular en cáncer: desarrollo de nuevas estrategias terapéuticas

Control da senescencia celular no cancro: desenvolvemento de novas estratexias terapéuticas

Control of cellular senescence in cancer: development of new therapeutic strategies

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CERTIFICAN QUE:

La presente memoria de Trabajo de Fin de Máster, titulada "Control de la senescencia celular en cáncer: desarrollo de nuevas estrategias terapéuticas" presentada por Federica Corrao, ha sido realizada bajo nuestra dirección en el INIBIC, ha sido revisada y está en disposición de ser presentada,

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RESUMO

O uso de terapias dirixidas xunto co as campañas de detección precoz melloraron o tratamento e manexo do cancro de mama nos últimos 10 anos aumentando a supervivencia e calidade de vida das doentes. Entre as terapias dirixidas, atopamos a os inhibidores CDK4/6 que supuxeron unha revolución no tratamento do cancro de mama metastático subtipo ER+/ HER2- ao inducir un arresto estable da proliferación nas células tumorais (senescencia celular). Ademáis, descubriuse recentemente que estes inhibores actúan tamén sobre o sistema immune favorecendo a eliminación das células tumorais e promovendo a rexeneración tisular, abrindo novas xanelas para mellorar a resposta inmune anti-tumoral. Ademáis, as canles de Cx43 forman parte da sinapse das células immunes e están implicados na presentación de antígenos promovendo unha resposta immune anti-tumoral eficiente. A pesar destes resultados, hoxe en día aínda non se coñece en detalle o mecanismo e as vias de sinalización implicadas na "limpeza" das células tumorais senescentes por parte do sistema immune. Por este motivo, neste proxecto propoñemos identificar novos marcadores e estudar novos compostos que participan na activación da resposta immune en células tumorais tratadas co inhibidor Palbociclib e mediadas por la Cx43, co obxectivo de desenvolver novas terapias combinadas que favorezan a eliminación das células senescentes e promovan a rexeneración tisular. Os resultados deste proxecto van permitir aumentar a eficacia destos inhibidores e expandir a actividade dos inhibidores CDK4/6 a outros tipos tumorais. A identificación de compostos senolíticos permitirá o desenvolvemento de novas estratexias terapéuticas para evitar a resistencia a o tratamento.

RESUMEN

El uso de terapias dirigidas y las campañas de detección precoz ha mejorado el tratamiento y el manejo de las pacientes con cáncer de mama en los últimos 10 años al aumentar la supervivencia y la calidad de vida de estas pacientes. Entre las terapias dirigidas, destacan los inhibidores de CDK4/6 que revolucionaron el tratamiento del cáncer de mama metastático subtipo ER+/HER2- al inducir una detención estable de la proliferación en las células tumorales (senescentia celular). Además, recientemente se descubrió que estos inhibidores también actúan sobre el sistema inmunitario al promover la eliminación de células tumorales senescentes y la regeneración de tejidos, abriendo nuevas ventanas para mejorar la respuesta inmunitaria anti-tumoral. Los canales de comunicación formados por conexina 43 (Cx43) participan en la sinapsis inmune y están involucrados en la presentación de antígenos para activar una respuesta inmune anti-tumoral eficiente. A pesar de estos resultados, el mecanismo y las vías de señalización involucradas en la "limpieza" de las células tumorales senescentes por el sistema inmune aún no se conocen en detalle. Por esta razón, en este proyecto proponemos identificar nuevos marcadores y estudiar nuevos compuestos involucrados en la activación de la respuesta inmune en las células tumorales tratadas con Palbociclib y mediada por la Cx43, con el objectivo de desarrollar nuevas terapias combinadas que favorezcan la eliminación de células senescentes y promueva la regeneración del tejido. Los resultados de este proyecto permitirán de aumentar la eficacia de estos inhibidores y expandir la actividad de los inhibidores de CDK4/6 a otros tipos de tumores. La identificación de compuestos senolíticos permitirá el desarollo de nuevas estrategias terapéuticas para evitar la resistencia al tratamiento.

ABSTRACT

The use of targeted therapies and the early detection campaigns have improved the treatment and management of breast cancer patients in the last 10 years, increasing the survival and quality of life of these patients. Among the targeted therapies, CDK4/6 inhibitors have revolutionized the treatment of ER+/HER2- metastatic breast cancer by inducing a stable cell cycle arrest of tumour cells (cell senescence). In addition, it has recently been demonstrated that these inhibitors also promote the anti-tumour activity by the immune system and promote the elimination of tumour cells favouring tissue regeneration. On the other hand, Cx43 channels are part of the immune synapse and are involved in antigen presentation processes promoting an efficient anti-tumour immune response. Despite these results, the mechanism and signaling pathways involved in the "cleaning" of senescent tumour cells by the immune system are not yet known in detail. For this reason, in this project we propose to identify new markers and study the effect of new compounds involved in activating the anti-tumour immunity under Palbociclib treatment and mediated by Cx43. The aim of this proposal is to develop new combined therapies to promote the elimination of senescent cells and to promote tissue regeneration. The results of this project will also improve these inhibitors' efficacy and will expand the activity of CDK4/6 inhibitors to other tumour types. The identification of senolytic compounds will allow to the development of new therapeutic strategies in order to avoid drug resistance.

1. Introduction

1.1 Cellular Senescence

Cellular senescence was discovered by Dr. Leonard Hayflick in 1961 (L.Hayflick, P.S. Moorhead, 1962) and is a mechanism that can occur in cells in response to different conditions. Cellular senescence is a special form of cycle arrest, where cells lose their proliferative capacity, but mantain their metabolic activity and their viability. Senescence is due to various causes such as DNA damage, cellular damage, stress (e.g ROS: reactive oxigen species) or oncogenic signaling; however, it is triggered during tissue remodelling and occurs during normal embryonic development (Muñoz-Espin and Serrano, 2014). For example, senescence-positive structures include the mesonephric tubules during mesonephros involution, the endolymphatic sac of the inner ear, the apical ectodermal ridge (AER) of the limbs, the regressing interdigital webs and the closing neural tube (Munoz-Espin, D. *et al*, 2013; Storer, M. *et al.* 2014). Senescent cells differ from other non-dividing cells (such as quiescent or terminally differentiated cells) by several markers and morphological changes. These features include the absence of proliferative markers, senescence-associated β-galactosidase (SA-βGAL) activity, expression of tumour suppressors and cell cycle inhibitors, and often DNA damage markers, nuclear foci of costitutive heterochromatin and prominent secretion of signalling molecules. (D. Muñoz-Espín et M. Serrano, 2014) (**Figure 1**). Senescence occurs if the stressors persist for a long period (>4 days) (Chen *et al.,* 2002; Dai and Enders, 2000), bringing to the upregulation of CDKN2A ($p16^{INK4}$) and in the late phase to the decline of p53 and $p21^{CIP1}$. The durable nature of senescence is helped by the heterochromatinization of E2F target (Narita M. *et al.,* 2003), the effect of the secreted cytokines, the DNA damage signaling, (Acosta et al., 2013; Acosta*et al.,* 2008; Coppe et al., 2008). But it has been reported that this durable state can be reversed by oncogenic events (Souroullas and Sharpless, 2015). Besides, senescent cells secrete many factors to the sourrounding medium, referred as Senescent–Associated Secretory Phenotype (SASP) (**Figure 1**), which improves and reinforces the signaling in an autocrine manner (Acosta, J. C. *et al.* 2008; Kuilman, T. *et al.* 2008). This SASP factors can act also in a paracrine manner (Baz-Martinez, M. *et al.* 2007; Acosta, J. C. *et al.* 2013). SASP factors are mainly form by a complex pro-inflammatory response molecules that includes pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8, chemokines (monocyte chemoattractant proteins (MCPs) and macrophage inflammatory proteins (MIPs)), growth factors such as the transforming growthfactor-β (TGFβ) and granulocyte–macrophage colony-stimulatingfactor(GM-CSF) and proteases (Acosta, J. C. *et al.* 2008; Kuilman, T. *et al.* 2008). Even if the SASP is generally described as a proinflammatory element, its composition may vary

according to cell types and stimuli triggering senescence. Following the secretion of SASP by senescent cells the inflammation response starts and this response can be pivotal for the clearance of senescent cells by phagocytosis (Xue, W. *et al.* 2007; Hoenicke, L. &Zender L., 2012). In fact, the elimination of senescent cells in ageing mice increases the health span and reduces the consequences of age-related pathologies or chemotherapy (Jianhui Chang *et al.*; 2016). Furthermore, it has been demonstrated that a senolytic compound conjugated with galactose-encapsulated nanoparticles releasing cytotoxic cargoes induces senescent cell apoptosis through the inhibition of the BCL-2 regulated pathway (Gualda G. E. *et al.,* 2020). This effect is specific for chemotherapy-induced senescent cells, preserving viability of nonsenescent cells. Another important consequence of this type of senolytic drugs is the reduction on platelet apoptosis preventing thrombocytopenia, that is a main result for the generation of prodrugs that permit a more selective senolytic activity (Gualda G. E. *et al*., 2020), because it is necessary to reduce the impact of the treatment against blood and immune cells, as well as healthy cells.

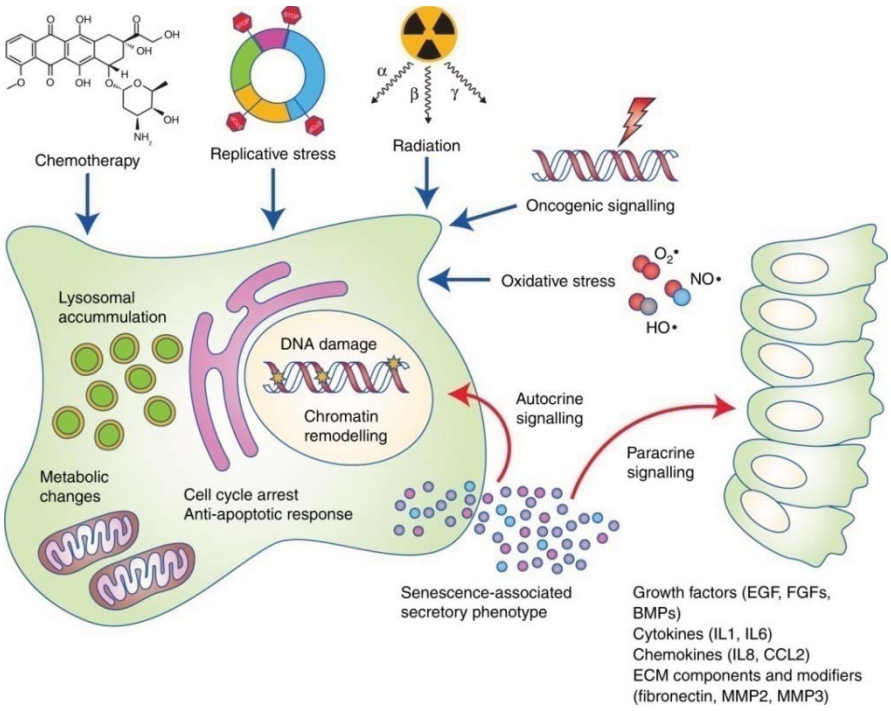

Figure 1.*Overview of senescence inducers, In the image is also shown the changes in cell physiology and morphology and the activation of the senescence-associated secretory phenotype (SASP) (Gonzalez-Meljem J. M. et al., 2018)*

1.2 CDK4/6 inhibitors: new therapeutic strategies in cancer

One characteristic of tumour cells is their ability to escape cellular senescence. The tumour cells have uncontrolled proliferation and the capacity to escape to the cell cycle checkpoints (Hanhan D. and Weinberg R. A., 2011)(**Figure 2**). In the last years, different studies have been conducted in order to find strategies to block tumour progression by inducing a senescence state in tumour cells (Triana-Martínez F., Loza M. I., and Domínguez E., 2020).

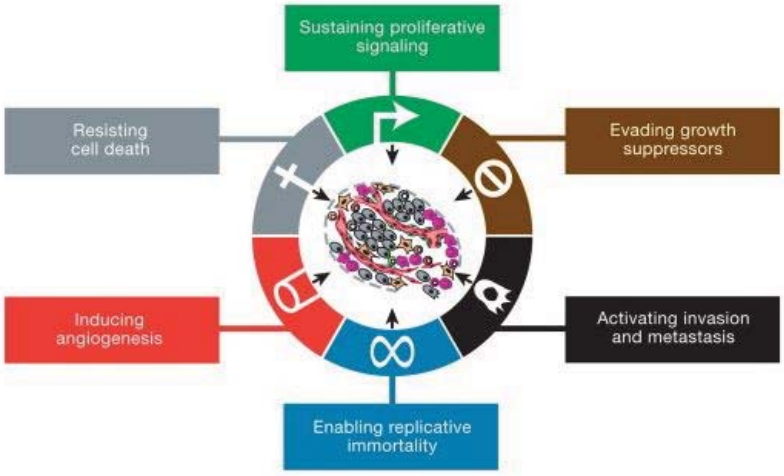

Figure 2. *The Hallmarks of Cancer (Hanhan D. and Weinberg R. A., 2011).*

CDK4/6 inhibitors (Palbociclib, Abemaciclib, Ribociclib), a class of new generation therapeutics has become a revolution in the field of cancer and, in particular, in metastatic breast cancer; being considered a potential anti-cancer strategy which so far has increased the overall survival (Navid Sobhani *et al*.; 2019). Although the three CDK4/6 inhibitors reached phase III clinical trials, Palbociclib has progressed further towards the clinic, having received accelerated approval from the Food and Drug Administration (FDA) in February 2015 (Nicholas C. Turner, *et al*.; 2018). It has been demostrated that these compounds induce cell cycle arrest in tumour cells (senescence), change cellular metabolism, reinforce cytostasis induced by signaling pathway inhibitors, and clearly change tumour microenvironment (Klein M. E. *et al*., 2018). Also, they can promote anti-tumour immunity (Goel S.*et al*.; 2017), reduce NADPH and glutathione levels (Wang H, *et al*.; 2017) or stimulate tumour antigen presentation (Schaer D,A. et al.; 2018). In line with these last findings, CDK4/6 inhibitors activate tumour cell expression of endogenous retroviral elements, thus increasing intracellular levels of double-stranded RNA.This in turn stimulates production of type III interferons and enhances tumor antigen presentation. Besides, CDK4/6 inhibitors markedly suppress the proliferation of regulatory T cells (Tregs) (Goel S. *et al*.; 2017; Schaer D.A. *et al*.; 2018). Mechanistically, the effects of CDK4/6 inhibitors on both tumor cells and

Tregs is associated with reduced activity of the E2F target, DNA methyltransferase 1(DNMT1), these events promote cytotoxic T cell-mediated clearance of tumor cells, which is further enhanced by the addition of immune checkpoint blockade (Schaer D,A. *et al.,* 2018). In fact, it is important to highlight that cells of the immune system (mostly T-cells and B-cells) are able to clear tumour cells, but it also happen that, mainly with aging, immune cells accumulate, in a process known as immunosenescence, so there is an impaired immune surveillance, allowing the tumour to growth (**Figure 3**). Altogether, these findings indicate that CDK4/6 inhibitors increase tumour immunogenicity and provide rationale for new combination regimens comprising CDK4/6 inhibitors and other drugs to promote senescent-tumour cells elimination and tumour surveillance.

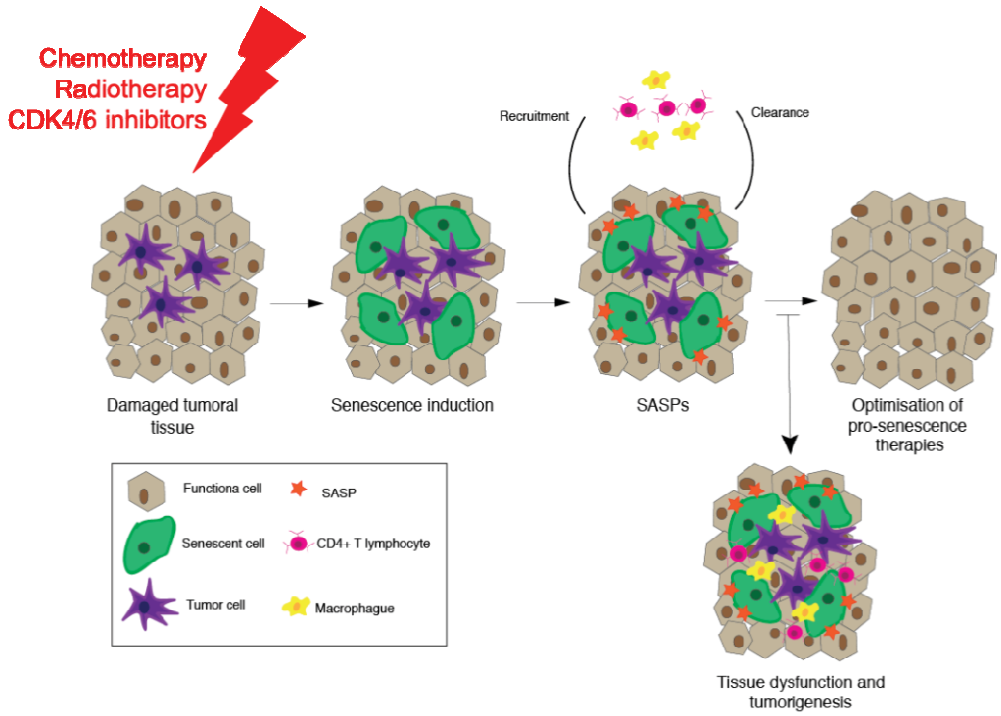

Figure 3*. Optimization of pro-senescence therapies. Under different types of stress (chemotherapy, radiotherapy, CDK4/6 inhibitors) tumours cells (purple) acquire a senescence-like phenotyope (green) accompained by the secretion of SASP factor. The SASP recruits the immune system that will eliminate the senescent-tumour cells and promote tissue regeneration. If senescent-tumour cells are not fully eliminated, they persist in the tissue, accumulate damage causing tissue dysfunction, tumorigenesis and cancer relapse. Adapted from Muñoz-Espin and Serrano (Muñoz-Espin D. and Serrano M., 2014)*

Inflammatory SASP factors secreted by senescent cells can promote various aspects of tumorigenesis in a nonautonomous manner, including cancer cell proliferation, migration, invasiveness, angiogenesis, and epithelial–mesenchymal transition (EMT) as well as immune-mediated clearance (Wang B., Kohli J., Demaria M., 2020) (**Figure 4**). These aspects indicate that

senescence can act as an anti-tumorigenic mechanism (**Figure 5**) based on the microenvironment of the tumour and the immune system response. In fact, it has been demonstrated that SASP can be used as an anticancer therapeutic strategy (Collado M. *et al.*, 2005; Poele R. H. *et al.*, 2002). However, senescent cells within the tissue, may also act as precursors of tumorigenic signals (**Figure 5**). For instance, several issues exist with the generation and persistence of therapy-induced senescent cells; first because SASP factors can play detrimental roles by protecting tumours from immune clearance, providing growth factors, and enhancing angiogenesis (Wang B., Kohli J., Demaria M., 2020). Second, because most anticancer treatments are administered via systemic routes and many senescent cells are generated in nontumor areas (Ewald J. *et al.*, 2010). Third, because cancer cells are genomically unstable, they can bypass the senescence growth arrest and restore aggressive and uncontrolled proliferation (Wang B., Kohli J., Demaria M., 2020). For these reasons, the function of CDK4/6 inhibitors needs to be analyzed properly, due to the side effect that can cause inducing cell senescence in a tumour microenvironment.

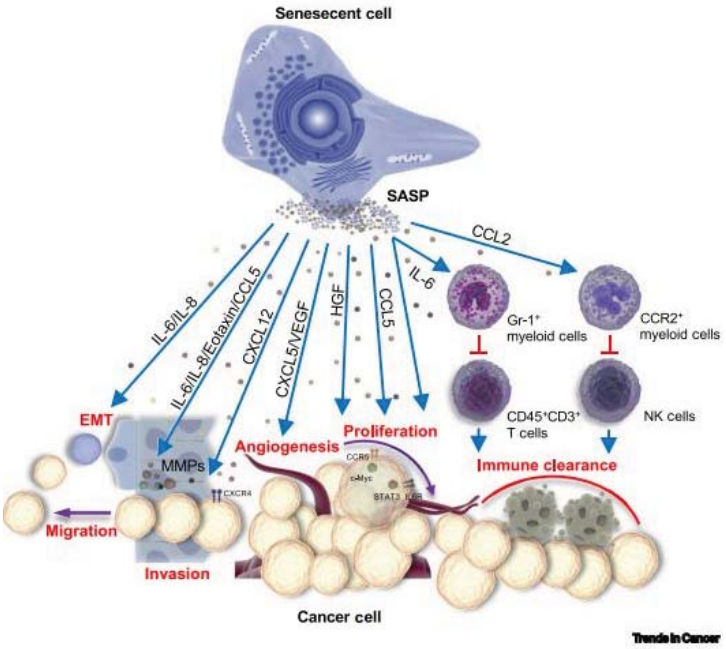

Figure 4.*Multiple effects of senescent cells. The arrows indicate the processes where senescent cells can be participating (Wang B., Kohli J., Demaria M., 2020)*

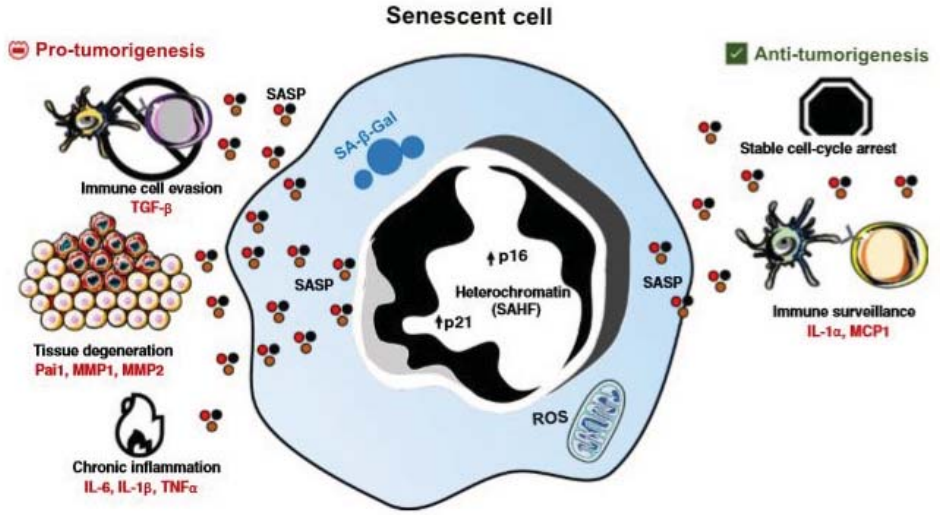

Figure 5. *Senescence has both anti-tumorigenic (right panel in green) and pro-tumorigenic effects (left panel in red)(Prieto L. I., Baker D. J., 2019)*

1.3 Connexins and anti-tumour immunity

Different reports describe the implication of connexin channels (hemichannels and gap junctions (GJs)) as modulators of key immunological processes including anti-tumour responses (Aasen T. *et al*.; 2019). Several evidences strongly indicate that gap junction intercellular communication (GJIC) participates in Antigen (Ag)-presenting processes. GJs are formed by two hemichannels, each one on the membrane of two adjacent cells. A hemichannel is also called 'connexon' and it is formed by six transmembrane protein subunits, termed connexins (Cxs) (**Figure 6**). Connexins are transmembrane proteins implicated in cell-to-cell communication, there are 21 types of connexins in humans but the most expressed is connexin 43 (Cx43) (Aasen T., *et al.* 2016). They mediate intercellular communication and connection, ensuring homeostasis, thanks to the exchange of different factors, metabolites, ions and other molecules (Loewenstein W.R. *et al.,* 1981; Saez J.C. *et al.*, 1989).

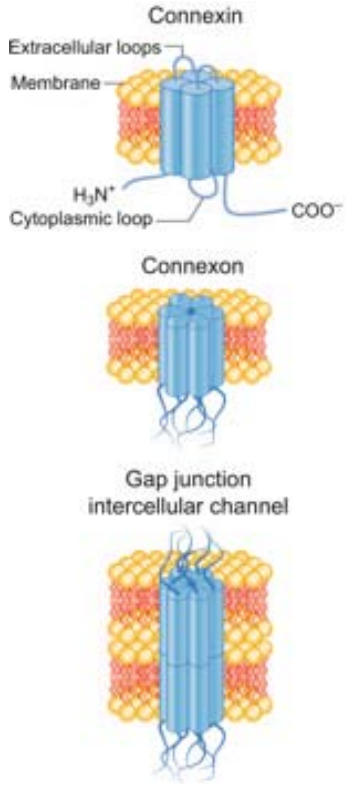

Figure 6. *Gap Junction, hemichannels and connexins structure. In blue, six connexin subunits oligomerize in the plasma membrane to form a connexon. When two connexons from adjacent cells get in touch a gap junction structure is formed (Aasen T. et al., 2019)*

In tumour environment, GJIC can have a doble role: tumour suppressor or tumor enhancer, depending on the interaction between cancer cells and stromal or immune cells (Varela-Vázquez A. *et al.,* 2020). Previously, it was shown that heterocellular GJIC between malignant and non-malignant cells can interrupt tumour growth (Mehta P.*et al.,* 1986), but it is important to highlight its role triggering anti-tumour immunity. In fact, it has been proposed that GJIC is necessary in the cross-presentation of the antigen (Ag) between immune cells. Particularly, considering that GJs are a main component in immune synapses, this junction have been demonstrated to be essential for enhance Ag-dependent dendritic cell (DC)- mediated T cell activation and DC-mediated activation of natural killer (NK) cells (Elgueta, R. *et al.,* 2009; Mendoza-Naranjo, A., *et al.,* 2011; Tittarelli, A., *et al.,* 2014). Studies conducted on melanoma cells demonstrated that Cx43 is fundamental for the activity of granzime B and cytotoxic T limphocytes (CTLs)-mediated cell death (Hofmann, F., *et al.,* 2019); furthermore, the activation of T cells by presentation of melanoma cells Ag is mediated by GJ containing Cx43 (Mendoza-Naranjo *et al.*, 2007); in addition, also the elimination of melanoma cells by CTLs requires the presence of GJIC constitued of Cx43 (Houssem *et al.,* 2009). Another Cx43-mediated action in the anti-tumour immunity involves two communication structure, that are tunneling nanotubes (TNTs) and extracellular

vescicles (EVs) (**Figure 7**), because they present connexin-constituting channels that support the tumour Ag cross-presentation, enhancing tumour immune response (Varela-Vázquez A. *et al.,* 2020).

Neverthless, Cx43 plays impontant roles in a non-channel and GJ structure. First of all, it has been demonstrated that connexins can suppress the growth of cancer cells without forming channels and also have an important function in cancer steam cells (CSCs) biology, for example in some subtypes of breast cancers (Aasen T. *et al,* 2019). Moreover, it has been shown that connexin can influence treatment response in different cancer cells, in fact its overexpression enhances the sensitivity to some chemotherapeutic drugs, such as doxorubin, paclitaxel, fluoroacil and etoposide (Liu D. *et al.,* 2015; Huang R.P. *et al*.,2001); at the same time this protein can suppress tumour angiogenesis, probably regulating the expression of the VEGF (vacular endothelial growth factor) and HIF1-α (Hypoxia-inducible factor 1-alpha) (Wang W.K. *et al.,* 2014). In this context, it has been also shown that Cx43 enhances diapedesis into endothelial cells in model of breast cancer and melanoma metastasis (Villares G.J. *et al*., 2009). However, and on top of all these functions the role of connexins under CDK4/6 inhibitors treatment and their implication to promote tumour surveillance has not been yet elucidated.

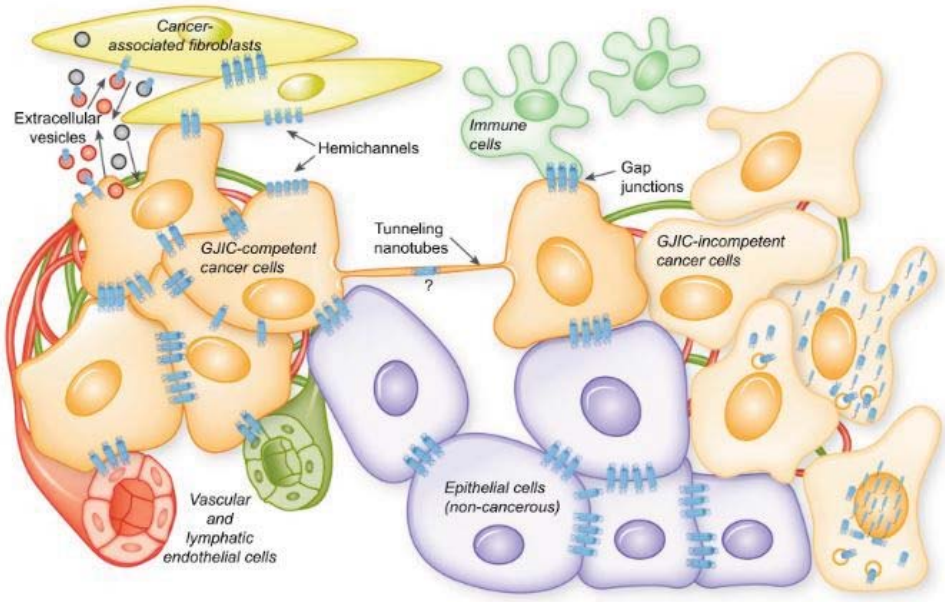

***Figure** 7. Localisation of Cx 43 and GJIC between cancer cells, non-cancer cells, stromal cells and Immune cells in a tumour environment, also displaying the presence of Cx43 and GJIC in TNTs and EVs (Aasen T. et al.,2019).*

2. Hypothesis

Clearance of senescent-tumour cells by using senolytic drugs contributes to tissue repair and regeneration. Role of Cx43 in promoting the elimination of senescent cells and the antitumour immunity under CDK4/6 treatment.

3. Objectives

1. Identification of senolytic drugs that contribute to the elimination of senescent cells under CDK4/6 inhibitors treatment.

2. Characterization of the pathways involved in the senescent cell-clearance.

3. Study of connexins and cell cycle arrest as a complementary strategy for senescent cells removal.

4. Methodology

4.1 Objective 1.

In order to identify how senescent cells respond to Palbociclib, in this project we will use a panel of23 tumoral cell lines currently available in the lab. In order to induce proliferation arrest and senescence *in vitro,* cancer cells we will be treated with different concentrations of Palbociclib as previously described (Rapisarda V. *et al.,* 2017). Proliferation (colony formation assay)and senescence markers will be analysed using immunofluorescence. The five cancer cell lines presenting higher reduction in proliferation and best senescent phenotype will be further used in the project. We will use 23 tumoral cell lines as shown in the **Table 1**.The use of these different cell lines is useful to determine which ones respond to Palbociclib treatment. Each cell line will be cultured with the appropriate medium supplemented with 10% FBS (Foetal Bovine Serum) and 1% of P/S (Penicillin and Streptomycin).

Table 1. *23 tumoral cell lines*

Cell lines	Type of Cancer
HT-1197	Bladder
HT- 1376	Bladder
MCF7	Breast
U118MG	Central Nervous System
U87MG	Central Nervous System
HCT-1116	Colon
HT29	Colon
SNU-387	Liver
HepG2	Liver
SK MEL-2	Melanoma
SK MEL-28	Melanoma
SK MEL-5	Melanoma
A549	Non-Small Cell Lung
NCI H23	Non-Small Cell Lung
OVCAR-3	Ovarian
SK OV 3	Ovarian
CAPAN 2	Pancreatic
Panc1	Pancreatic
DU-145	Prostatic
PC-3	Prostatic
A498	Renal
ACHN	Renal

Some of the experiments proposed in this project will be run in 96-well plate format. For this reason, we will determine the number of cells that will be plated/well depending on the duration of the experiment. For that, we will plate 250, 500, 750, 1000 and 1500 cells per well of each of the cell lines and leave them to grow during 7 and 10 days. The goal is to reach confluency at the end of the experiment. Crystal violet staining (colony formation) will be used to asses proliferation state and the plating number of each cell line will be decided based on the result of 3 independent experiments.

Palbociclib will be first diluted in DMSO at a stock concentration of 10 mM and kept at -20°C. At the same time, an intermediate stock will be prepared, diluting Palbociclib in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10%FBS and 1% P/S at 50μM concentration and kept at -20°C. The final concentration to be used (200nM, 500nM and 1μM) is based on previously reports (Rapisarda V. *et al.*, 2017) for 7- and 10-days treatment.

Cancer cell lines will be divided in two groups: (i) 12 cell lines (ii) 11 cell lines, in order to run the proliferation and senescence experiments. Each cell line will be plated in a 96-well plate (**Figure 8**),according to the results obtained in the previous experiments run in the same conditions, and treated with different concentrations of Palbociclib (200nM, 500nM and 1μM) prepared in DMEM supplemented with 10%FBS and 1% P/S during 7 and 10 days. Medium will be replaced every other day. At the end of the experiment cells will be fixed using 4% paraformaldehyde (PFA) and washed with PBS twice. Crystal violet staining will be used to stain each well of the 96-well plate and to assess proliferation (colony formation) status. Plates will be imaged, and crystal violet will be diluted using 30% acetic acid and quantified at 570nm in a plate reader (NanoQuant). Quantification results will elucidate the cell lines that arrest their proliferation in response to Palbociclib and also the best concentration to be used. We will select the 5 cancer cell lines that better responded to Palbociclib (3 independent experiments) to test senescence activation.

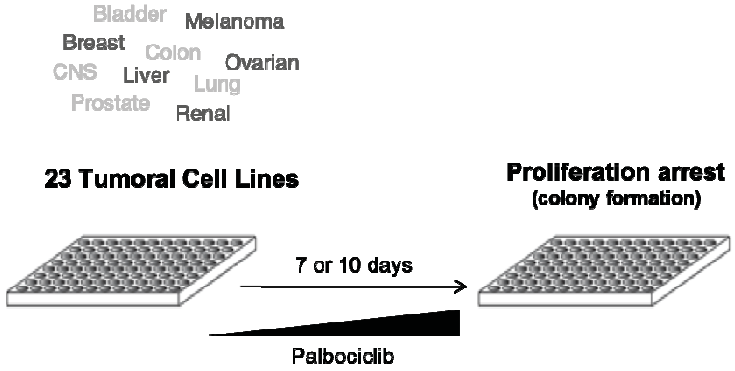

Figure 8. *Schematic representation of proliferation experiments. Cell lines will be plated in 96-well plate format, treated with different concentrations of Palbociclib for 7 and 10 days and colony formation assay will be measured by crystal violet staining.*

Immunofluorescence will be used to test the activation of senescence in the 5 cancer cell lines selected based on proliferation assay results. For immunofluorescence experiments, cells will be plated onto coverslips and we will analyse the activation of senescence markers such as, p21 and IL8. Moreover,we will use BrdU incorporation as a marker of proliferation to further confirm the results of colony formation. Finally, we will study SA-β-galactosidase activity by adding C_{12}FDG compound overnight. Pictures will be taken by using Olympus Fluorescent microscope.

In order to find new compounds (senolytics) that activate an apoptosis response in tumour cells after Palbociclib treatment we will use a library of kinases inhibitors in this project. The 5 cancer cell lines selected in the previous objective will be used in the screening. We will use a FDA approved library (Kinase Screening Library, Cayman Chemicals) that consists of 160 selective and non-selective kinase inhibitors. As a positive control we aim to use the previously described BCL-2/BCL-xL inhibitor (ABT263) senolytic drug (Chang, J. H. *et al.* 2016).The selected cancer cell lines will be plated in a 96-well plate format and treated with the selected Palbociclib concentration for 7 days. After that, kinase inhibitors will be added for 72h. The kinase inhibitor library will be tested at three different concentrations. At the end of the experiment cells will be fixed with 4% PFA, washed with PBS and kept at 4ºC until immunofluorescence is performed. The output of the screen will be viability, measured by DAPI and cell count staining, and apoptotic markers (annexin V and cleaved caspase 3) using an automated fluorescent microscope in collaboration with CIMUS (Santiago de Compostela) (**Figure 9**). We will select the top candidates based on the percentage of cells undergoing apoptosis, and number of inhibitors affecting the same pathway.

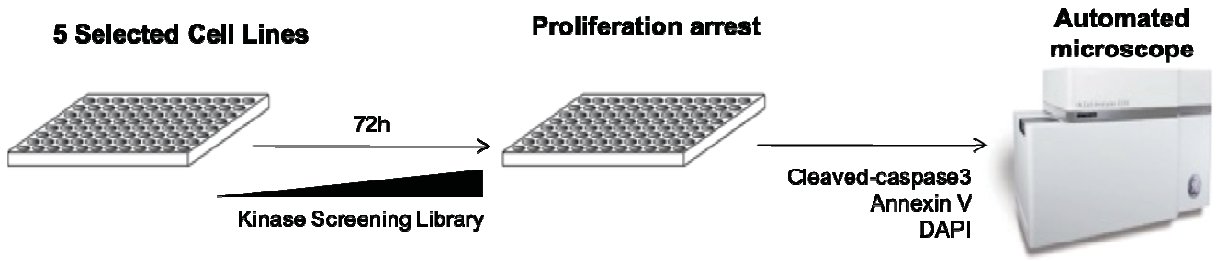

Figure 9. *Kinase library screening workflow*

4.2 Objective 2.

Identifying the pathways that might be abnormal in senescence cells will provide clues as to how senescent cells can be removed. We will use different inhibitors targeting the same pathway to confirm non-target effects of the inhibitors identified in the screening. The concentrations and time of treatment will be decided based on the inhibitors to be used according to the literature. Same as in previous objectives, the cell lines to be used will be the selected from objective 1.

We will further use RNAi (short hairpin and small interference RNA) and genome-editing technology such as CRISPR/Cas9 (already set up in the lab) for loss of function studies targeting at least two different genes of the selected pathway. We will order lentiviral vectors in order to knockdown different genes of the selected pathway from objective 1.Also, we will use the CRISPR technology to perform knockout experiments for the selected genes. Once the knockdown/knockout is confirmed, the selected cell lines will be treated with Palbociclib to induce senescence and the phenotype obtained will be checked by colony formation assay and senescence/apoptosis markers by using immunofluorescence.

The relevance of the identified pathway will be confirmed in other models of senescence (oncogene and DNA damage-induced) in addition to using primary cell cultures. From the 23 cancer cell lines used at the beginning (**Table 1**), we will make a selection of 5 cell lines as explained in objective1. These 5 cell lines will be treated with different drugs to induce senescence, such as etoposide and doxorubicin. These two drugs are both chemotherapy medications and in particular they act in two different ways:(i) etoposide is an inhibitor of topoisomerase II (Topo II), fundamental during cell cycle replication, so its use cause cell cycle arrest and induce apoptosis; (ii) doxorubicin is a DNA intercalant, inhibits molecular biosynthesis

and the progression of topoisomerase II (Topo II), because it stabilizes the complex between TopoII and cutted DNA, stopping the replication, causing a disequilibrium in the machinery of replication, resulting in impaired DNA and cells replication, DNA-damage response, inducing senescence/apoptosis. Besides, we will induce senescence by inserting a vector to overexpress RAS, an important kinase often overexpressed in lots of tumour types. After senescence induction in the different models, we will test the selected inhibitors to check if the induction of apoptosis depends on the CDK4/6 senescence induction or if it depends only in the adquisition of a senescence phenotype.

To fully validate the data, we will isolate human primary fibroblast from skin and we will induce them to senescence by using all the drugs. At the end, we will check if it is possible to reproduces the experiment and obtain the same previous results.

4.3 Objective 3.

Connexins has been described as tumour suppressors but they can also promote cell cycle arrest by decreasing the phosphorylation of the Rb protein. Here, we propose to identify the association between connexin expression and cell cycle arrest induced during therapy-induced senescence by looking for common regulatory pathways that could be altered in non-cleared senescent cells. The output will be to specifically design drugs that effectively remove senescent cells from damaged tissues.

The selected 5 cancer cell lines will be treated with Palbociclib for 7 days, using the concentrations selected in objective 1 and we will check connexin expression (connexin43) by using real-time PCR. For that, the extraction of RNA will be carried out using Tryzol. Later, 1µg of RNA will be retrotranscribed using Superscript VILO cDNA synthesis kit. cDNA will be diluted 1:10 in free-nucleases water. Finally qPCR will be carried out using 5µL of cDNA, 5µL of primers mixture (0.5µL forward, 0.5µL reverse, 4µL water) and 10 µL of SYBR-Green.

To check Cx43 localization, we will perform immunofluorescence experiments using specific antibodies. Cells will be plated onto coverslips , treated with Palbociclib for 7 or 10 days and finally cells will be fixed with 4%PFA and incubated with Cx43 antibody. Images will be taken and analysed in order to check Cx43 localization and expression by using a fluorescent microscope (Olympus).

Moreover, to confirm protein expression, we will perform Western- Blot (WB) experiments. In brief, we will carry out the WB by using home-made gels (SDS and Acylamide/Bis-acrylamide) and nitrocellulose membrane using BioRad System for gel running and transference. Primary antibody will be incubated overnight followed by 1 hour room temperature incubation the day after for the secondary antibody. After that, the membrane will be washed with a wash buffer and protein band detection will be visualized by using Pierce^{TM} ECL Western Blotting Substrate in a LAS-300 Imager168 (Fujifilm). As loading control we will use an α-tubulin antibody and Ponceau-red staining.

We will study the functional relationship between p21(senescence marker) and Cx43 by using real-time qPCR and immunofluorescence in order to establish a common mechanism between senescent activation and connexin activity in tumour-induced senescent cell. Flow cytometry assays (FACSCalibur, BD) will be used in order to validate that connexins are playing an important role in the cell cycle arrest induced by Palbociclib. Finally, we will try to modulate connexins expression using overexpression/downregulation experiments to verify that the expression of connexins could be implicated in senescent cells removal.

The results obtained will be confirmed using cancer cells isolated from primary tumours from patients (Private collection Dr. Mayán). We will also, isolate fibroblasts from human primary tumours, we will induce senescent using Palbociclib and we will confirm senescent phenotype using SA-β-galactosidase. Besides, connexin gene expression will be checked using convectional techniques (real-time qPCR and immunofluorescence).

5. Working Plan

This project will be developped in a three years-period. The time schedule we will follow is represented in the chornogram below.

Table 2. *Chronogram and project plannification*

OBJECTIVE	**CHRONOGRAM**							
	1st Year		**2nd Year**		**3rd Year**			
	M 1-6	**M 7-12**	**M 1-6**	**M 7-12**	**M 1-3**	**M 4-6**	**M 7-9**	**M 9-12**
Cell cycle arrest and senescence phenotype induced byPalbociclib treatment								
Screen for kinase library inducing apoptosis in senescent cells								
Inhibitors validation experiments								
Validation in other models of senescence								
Connexin expression								
Senescence activation and connexin activity								
Validation using human samples								

6. Ethical implications

We will use available cancer cell lines implying that there will be no sampling from human patients. To the development of objective 3 we will need human samples from BiobancoACoruña and from the private collection of human biological samples from Dr. Mayan (C.0003333 - Rexistro Nacional de Biobancos do ISCIII). Ethical approval will be in compliance with Galicia/Spain laws as well as with European Directive 2004/23/EC on the setting of the standards of quality and safety for donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. Ethical approval from local commitee need to be sent. *Personal data*: the oncologist or member of staff involved in patient consenting procedures will hold patient information related to tumor samples and liquid biopsy. Team members will only be provided with information on patient age, sex, disease status, surgery performed, medications and medical history. Further details or clinical outputs will be available if required. This information is confidential and is only consultable by the scientific research team. Data will be treated anonymously and confidentially in compliance with respective legislation regarding protection of privacy and patient rights.

7. Impact

Even though cancer targeted therapies have improved disease-free survival in the last years, 30% of cancer patients will eventually develop resistance and metastasis. Current therapies are mainly based in the use of chemotherapy and radiotherapy, very aggressive treatments with high secondary effects to the patients. Although these therapeutic strategies are able to reduce mortality, there is still a need to develop targeted therapies according to patient and tumor characteristics. In the last years, CDK4/6 inhibitors have become a revolution in the field by reducing tumor cell proliferation in a less invasive manner and so far, improving patient survival from 18 to 27 months in metastatic breast cancer patients.

Based on recently research results, CKD4/6 inhibitors are used in metastatic luminal ER+/HER2- but the only factor known to be involved in the effectiveness is based on the presence of the protein Rb in tumor cells. For this reason, this proposal will expand the knowledge on CDK4/6inhibitors mechanisms and pathways involved which will allow to span their use to different subtypes of cancer. Since mechanisms leading to tumor evasion are diverse, it is necessary to develop new strategies to trigger tumor death by stimulating the patient´s natural defenses to recognize and destroy tumor cells. In this project we will include the study new molecular marks in response to CDK4/6 inhibitors and other drugs to efficiently eliminate senescent tumor cells. The results obtained include the development of a combinational therapy based on the activation of common pathways linking the senescent phenotype induced by CDK4/6 inhibitors and connexins. Besides, the finding of new molecular marks will allow the study the efficacy of CDK4/6i which will allow us to investigate and to target drug resistance pathways. The finding of new molecular marks and compounds, alone or in combination with current therapies, will solve one of the biggest challenges oncologists are facing today in cancer patients. We are hopeful that this research will eventually help patients to cure the disease or extend survival and increase their quality of life, bringing down the cost of cancer patient care, saving lives and averting costs.

8. Plan for the dissemination of research findings to the society

The results of this proposal will be disseminated at scientific conferences focused on cancer (European Association of Cancer, American Association for Cancer Research Congress, ASEICA International Congress), drug discovery, senescence (e.g. International Senescence Association), gap junctions (International Gap Junction Congress 2021). The results will be published in peer-review international high-impact factor journals. Also, the results of this proposal will be disseminated at local workshops organized by Universidade de A Coruña, or INIBIC. In this case, the target audience of the workshops will be medical doctors, cancer researchers and potential industrial customer interested in the results or in their future development.

Communication strategy to the society include presence on social media, social networks such as twitter or the CellCOM webpage (www.mayan-lab.com), reports to press and divulgation through specific networks and societies such as (ASEICA, ASEICA-mujer, SEBBM, SEOM, AECC or CorBi Foundation).Also, we will participate in outreach and divulgation activities in collaboration with museums (MUNCYT, DOMUS), patient associations (AECC-Coruña) and private foundations (Fundación CorBi, Fundación Barrié). Local radio (e.g. Radio-Coruña, Cadena SER) and local press (La Voz de Galicia, La Opinion de A Coruña, El ideal Gallego) will be use as well in order to report impact results to the society.

9. Resources needed

Table 3 shows the costs and resources needed to carry out the project.

Table 3. *Costs and resources*

	2020	2021	2022	TOTAL
- Human Resources		15.691,66	17.391,66	**33.083,32**
- Small inventory equipment and consumable materials	8.286,66			**8.286,66**
- Conferences and research societies costs	250	1200	2000	**3.450**
- Courses and seminars for laboratory members impliated in the development of the project	300	2400		**2.700**
- Travel costs for invited speakers and colaborators	560	800	1000	**2.360**
- Publications and outreach activities	320	500	500	**1.320**
- Facility costs	450	6700	4400	**11.550**
- Audit costs	500	875	875	**2.250**
- Indirect costs of the project	3333,34	5833,34	5833,34	**15.000,02**
Total amount	14.000	34.000	32.000	**80.000**

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