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PhD Thesis

Identification and characterization of Digoxin as a novel senolytic drug

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Doctoral Programme in Molecular Medicine

DOCTORAL THESIS

IDENTIFICATION AND CHARACTERIZATION OF DIGOXIN AS A NOVEL SENOLYTIC DRUG

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Thesis Title: **Identification and characterization of Digoxin as a novel senolytic drug**

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Identification and characterization of Digoxin as a novel senolytic drug

Mr. Manuel Collado Rodríguez (as director) and Mr. Anxo Vidal Figueroa (as tutor)

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He imaginado mil veces como escribiría los agradecimientos de esta tesis y ahora que llega el momento no sé qué decir, asique no me tengáis en cuenta si me dejo a alguien.

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Just keep swimming…

Cellular senescence is a potent protective response against several potentially dangerous stimuli that prevent the progression of damaged cells by inducing a stable proliferative arrest. In this way, cancer cells need to avoid this response in order to grow and form tumors. Thus, we can consider cellular senescence as an essential antitumor mechanism. Restoring this response in tumor cells has been shown to have a positive impact in cancer treatment, since various therapies can induce senescence in tumors, preventing their growth.

However, in recent years it has been described that senescent cells remain active and secrete a large number of factors to their environment with antagonistic activities. In some cases, these factors may have a reinforcing effect on senescence, but in other cases they may have a protumoral activity.

At the same time, senescent cells accumulate in the organism over time and contribute to the functional deterioration of the tissues, mainly due to the action of these same secreted factors. This contribution to the aging of senescent cells has sparked interest in the identification of compounds capable of selectively eliminating senescent cells, the so-called senolytic compounds.

In this thesis, we performed a high-throughput screening of a chemical library to identify novel senolytic compounds. In this way, we identified the family of Cardiac Glycosides, and in particular Digoxin, as a promising senolytic drug. We also characterized the potential mechanisms behind the senolytic effect of Digoxin.

La senescencia celular es una potente respuesta protectora contra estímulos potencialmente peligrosos que previene la progresión de las células dañadas al inducir una parada proliferativa estable. Por lo tanto, las células dañadas necesitan evitar esta respuesta para crecer y formar tumores. De esta forma, podemos considerar la senescencia celular como un mecanismo antitumoral esencial. Restaurar esta respuesta en las células tumorales ha demostrado tener un impacto positivo en el tratamiento del cáncer ya que diversas terapias pueden inducir senescencia en los tumores deteniendo su crecimiento.

Sin embargo, en los últimos años se ha descrito que las células senescentes permanecen activas y secretan una gran cantidad de factores a su entorno con actividades antagónicas. En algunos casos, estos factores pueden tener un efecto potenciador de la senescencia, pero en otros casos pueden tener actividad protumoral.

Al mismo tiempo, las células senescentes se acumulan en el organismo con el tiempo y contribuyen al deterioro funcional de los tejidos, principalmente por la acción de estos mismos factores secretados. Esta contribución al envejecimiento de las células senescentes ha despertado el interés por la identificación de compuestos capaces de eliminar selectivamente las células senescentes, los denominados compuestos senolíticos.

En esta tesis, realizamos un *high-throughput screening* de una librería química para identificar nuevos compuestos senolíticos. De esta manera, identificamos a la familia de los Glucósidos Cardíacos, y en particular a la Digoxina, como un fármaco senolítico prometedor. También caracterizamos los posibles mecanismos detrás del efecto senolítico de la digoxina.

Mª del Pilar Picallos Rabina

A senescencia celular consiste nunha parada do ciclo celular desencadeada por diferentes estímulos. Dentro da variedade de estímulos que poden inducir senescencia topámonos con estímulos prexudiciais, como a activación oncoxénica, o dano o ADN ou o acurtamento dos telómeros, así como tamén poden ser procesos fisiolóxicos como o peche dunha ferida ou a rexeneración dun tecido.

Aínda que existen certas características comúns a todas as células senescentes, coma o tamaño agrandado, a acumulación de lisosomas con conseguinte aumento na actividade do encima beta-galactosidasa, non se pode dicir que se observen en tódolos tipos de senescencia. Por iso, de entre todos os tipos distintos de células, contextos biolóxicos ou estímulos de estrés, establecéronse catro características como as principais e compartidas que definen as células senescentes: unha detención xeralmente irreversible do ciclo celular, un fenotipo secretor distintivo (SASP, fenotipo secretor asociado á senescencia), signos dano macromolecular e reprogramación metabólica.

En canto ó proceso de parada proliferativa, está mediada por unha variedade de vías de sinalización, destacando a activación das vías reguladas polos supresores tumorais p53 ou p16. Estas vías actívanse en resposta a diversas sinais de dano celular desencadeando a parada do ciclo celular.

Outra característica destacada da senescencia celular é a súa capacidade para modular o microambiente mediante a secreción dunha complexa mestura de factores solubles. Este fenotipo secretor asociado á senescencia, coñecido como SASP, está composto por citocinas e quimiocinas, factores de crecemento e remodelación da matriz, e é responsable dos efectos fisiopatolóxicos da senescencia celular.

Historicamente, nun primeiro momento a senescencia celular describiuse coma un reflexo do proceso de avellentamento a nivel celular. A principios dos anos 60, este concepto de senescencia celular foi acuñado por Leonard Hayflick como resultado de observar que os cultivos de fibroblastos humanos normais entran nun estado non proliferativo despois de varias divisións celulares. Anos máis tarde describiuse a causa desta detención proliferativa observada por

Hayflick como consecuencia do acurtamento dos telómeros. Este acurtamento dos telómeros ocorre despois de divisións celulares consecutivas como resposta protectora das células contra o dano no ADN.

A medida que avellentamos, o noso organismo comeza a perder paulatinamente a súa integridade volvéndose máis susceptible a agresións externas que poden resultar no desenvolvemento de diferentes enfermidades. A dexeneración de tecidos é unha consecuencia da característica perda de función observada en todos os organismos pluricelulares a medida que avellentan. Este declive na integridade dos tecidos maniféstase en diferentes patoloxías asociadas coa idade como a osteoporose, a neurodexeneración e a sarcopenia, entre outras. En consecuencia á perda de integridade do tecido durante o avellentamento, as células son máis propensas a acumular mutacións que poden dar lugar a alteracións proliferativas. Neste caso, as células poden proliferar sen control levando a formación de hiperplasias ou cancro (Campisi, 2013). Neste contexto, a senescencia celular xurdiu como unha resposta protectora ao acurtamento dos telómeros e ao dano no ADN que acontece co paso do tempo, evitando a proliferación de células aberrantes. Non obstante, a relación entre os efectos beneficiosos da senescencia celular e o prexuízo da acumulación de destas células demostrouse máis tarde grazas a o uso modelos in vivo de avellentamento no que a eliminación específica das células senescentes melloraba o fenotipo avellentado e paliaba as enfermidades asociadas a el.

Co paso do tempo, o concepto de senescencia celular ampliouse máis aló do avellentamento, dando como resultado a identificación da senescencia celular non só durante contextos patolóxicos se non tamén en situacións fisiolóxicas. Así, demostrouse que a senescencia celular desempeña papeis esenciais durante o desenvolvemento do embrión, a cicatrización de feridas e a rexeneración de tecidos.

Máis tarde, o SASP foi descrito como un dos principais mecanismos efectores das células senescentes. Gracias a este fenotipo secretor, as células senescentes poden modular o medio que as rodea, reforzar o estado de senescencia, ou actuar sobre o sistema inmune. Sen

embargo, a complexidade de factores que forman o SASP fai que teña diferentes función segundo o contexto, sendo o responsable dos efectos contraditorios da senescencia celular. Os factores do SASP que promoven a inflamación crónica xunto con actividades pro-fibróticas foron reportados en diferentes modelos de enfermidades relacionadas coa idade como a aterosclerose, a artrose ou a diabetes tipo 2, que prexudican a saúde e reducen a esperanza de vida.

Ademais dos efectos da senescencia durante o avellentamento, describiuse a indución da senescencia inducida por oncoxenes. Este programa senescencia inducida por oncoxenes (OIS) actívase en resposta a un estado hiperproliferativo causado pola activación de oncoxenes que conduce ó estrés replicativo, activación de p53 e p16 e detención do ciclo celular. A observación dunha indución repentina de senescencia en células primarias normais despois da introdución dun oncoxene activado levou á noción de senescencia celular como mecanismo supresor de tumores.

A partires deste momento, moitos outros mecanismos de indución de senescencia foron descritos. No contexto do cancro, diferentes terapias enfocadas ó tratamento dos tumores como a quimioterapia ou a radioterapia, foron descritas como non só indutoras de apoptose das células tumorais se non tamén indutoras da senescencia. A pesares dos mecanismos fisiolóxicos que activan a resposta de senescencia para previr a transformación maligna das células, non é un mecanismo infalible. Así, moitos tipos de cancro mostran mutacións nos principais reguladores do proceso de senescencia para así evitalo. Así, aproveitando os beneficios da senescencia celular como é a parada proliferativa, as terapias do cancro capaces de inducir senescencia abriron unha nova modalidade de terapia.

Sen embargo, a pesares de estar detidas no crecemento, as células senescentes son metabólicamente activas cun fenotipo secretor particular coñecido como SASP. Entre as diferentes moléculas liberadas polas células senescentes hai un gran número de citocinas e quimiocinas proinflamatorias (por exemplo IL6, IL8, CXCL1…), encimas de remodelación da matriz (por exemplo, MMP1, MMP3, PAI-1…) e factores promotores do crecemento (por exemplo, HGF,

epiregulina, anfiregulina...). Este SASP relacionouse non só cos efectos prexudiciais da senescencia durante o avellentamento se non que tamén relacionouse cos efectos secundarios das terapias contra o cancro, cas metástases ou cas recaídas.

Desta forma xurdiu unha nova corrente de investigación enfocada en eliminar ou paliar os efectos prexudiciais da senescencia celular sen afectar os beneficios da mesma. Por unha banda, houbo grupos de investigación que se enfocaron na inhibición ou modulación do SASP, xa que é o primeiro causante dos efectos negativos da senescencia. Pola outra banda, xurdiron grupos de investigación enfocados na completa eliminación das células senescentes unha vez cumprida a súa misión, evitando así a súa acumulación ca conseguinte dexeneración dos tecidos.

O uso de fármacos que actúan sobre o fenotipo secretor das células senescentes, os coñecidos coma senomórficos, demostraron ter efectos beneficiosos sobre modelos de avellentamento onde se melloraban non só o fenotipo avellentado se non que tamén se retrasaba o desenvolvemento das patoloxías asociadas ó mesmo. Estes fármacos senomórficos tamén demostraron unha mellora na resposta os tratamentos contra o cancro inhibindo a secreción de citocinas proinflamatorias e previndo así a resistencia fronte á quimioterápia. Algúns destes fármacos son: o inhibidor de mTOR Rapamicina, o antidiabético Metformina, a estatina para o control do colesterol Simvastatina, entre outros. A pesares dos efectos prometedores da modulación do SASP, poder inhibir os factores secretados prexudiciais sen afectares os beneficiosos é aínda un reto.

De maneira complementaria, valorouse a eliminación específica das células senescentes para previr a súa acumulación de forma aberrante. Como se mencionara anteriormente, modelos de ratos demostraron que a eliminación específica das células senescentes dun organismo avellentado melloraba o estado de avellentamento e prevía a aparición de diferentes enfermidades. Este modelo transxénico de rato, coñecido como INK-ATTAFC, se baseaba na expresión dun casete suicida inducible ligado á expresión de p16. Deste xeito, as células

senescentes p16-positivas poden ser inducidas a sufrir apoptose de maneira específica.

Sen embargo, a eliminación das células senescentes non sempre ten un efecto beneficioso, xa que a súa eliminación nun contexto fisiolóxico coma o peche dunha ferida ou a rexeneración dun tecido é prexudicial.

A partires destas observacións usando modelos transxénicos, xurdiu a necesidade de desenvolver fármacos ca capacidade de eliminar especificamente ás células senescentes, os coñecidos coma senolíticos. Estes compostos senolíticos baseanse na inhibición ou bloqueo dos mecanismos de supervivencia das células senescentes. Os primeiros fármacos identificados coma senolíticos foron o Navitoclax e a combinación do Dasatinib ca Quercetina. A eliminación selectiva das células senescentes por estes compostos demostrou melloras non só en modelos de avellentamento se non tamén no tratamento do cancro. Con esta vantaxe de poder beneficiarse da indución de senescencia previndo os efectos prexudiciais xurdiu o concepto do one-two punch contra o cancro. Esta idea baséase nun primeiro tratamento con quimio ou radioterapia para a indución de senescencia no tumor, non só previndo o seu crecemento, se non tamén proporcionando unha fiestra terapéutica para o uso de senolíticos, eliminado o tumor por completo. Deste xeito, mellórase a resposta fronte os tratamentos contra o cancro, redúcese a posibilidade de resistencia as terapias, as metástases e as recaídas. Para facer isto posible, son necesarias novas estratexias e fármacos con efecto senolítico xa que non todos son efectivos en todos os contextos de senescencia nin en todos os tipos de cancro.

Por estes motivos propuxémonos a realización dun cribado de alto rendemento ca finalidade de topar novos compostos senolíticos. Para iso empregamos a liña celular de adenocarcinoma de pulmón A549 modificada para a expresión de GFP ou RFP. As células A549-RFP foron levadas a senescencia tras o tratamento con Bleomicina, un axente quimioterapéutico causante de roturas no ADN. Para a realización do cribado, mesturáronse células proliferantes A549-GFP con células senescentes A549-RFP. Sobre estes co-cultivos ensaiáronse os compostos da librería química de Prestwick, composta de 1280

fármacos e moléculas pequenas aprobadas por diferentes axencias reguladoras para o seu uso en pacientes.

Ó cribado realizouse en 3 etapas. Na primeira etapa os compostos foron testados de maneira única a unha concentración de 10µM durante 24 horas. Os compostos identificados nesta primeira etapa como potenciais senolíticos pasaron á segunda etapa onde foron testados a 1 e 10 µM. Finalmente, os candidatos obtidos desta segunda etapa foron levados a avaliación mediante a realización dun IC50 onde se testaron os compostos a un rango decrecente de concentracións.

Como resultado deste cribado obtivemos como senolítico prometedor a Proscilaridina A, un fármaco pertencente a familia dos Glicósidos Cardíacos. Esta familia de compostos foi descrita vai máis de douscentos anos por William Withering, quen a empregou para o tratamento do edema e diferentes patoloxías cardíacas. Para saber se a capacidade senolítica da Proscilaridina A era unha característica propia do fármaco ou común a súa familia, testamos outros dous Glicósidos Cardíacos, a Ouabaina e a Digoxina. Ámbolos dous compostos mostraron tamén capacidade de eliminar especificamente as células senescentes, e debido a que a Proscilaridina A non se utiliza actualmente na clínica, decidimos continuar a caracterización da Digoxina como potencial senolítico. Para iso, testamos a Digoxina en diferentes liñas celulares tumorais así coma en liñas normais levadas a senescencia con Bleomicina. Tamén, testamos o efecto senolítico da Digoxina en condrocitos derivados de pacientes con osteoartrose, unha patoloxía asociada ó avellentamento e que se caracteriza pola indución de senescencia nestas células.

Unha vez demostrado que a Digoxina semella ter un amplo espectro de acción sobre diferentes tipos celulares e contextos de senescencia (en cancro e nun modelo de avellentamento), testamos o mecanismo polo cal a Digoxina estaba causando morte celular. Para iso medimos marcadores de apoptose, o tipo de morte celular máis común, por citometría de fluxo nos nosos modelos in vitro tras o tratamento con Digoxina. Como ferramenta para confirmares que a Digoxina estaba causando apoptose nas células senescentes por mor da activación da vía

das caspasas, empregamos un inhibidor de pan-caspasas e avaliamos á súa protección contra o efecto senolítico da Digoxina.

Sen embargo, o efecto senolítico da Digoxina non puido ser demostrado en fibroblastos embrionarios de rato senescentes, dado que posúen unha mutación na principal subunidade da ATPasa á que se une a Digoxina.

A metades do século XX describiuse que a diana da Digoxina, e doutros Glicósidos Cardíacos, era a sodio-potasio-ATPasa (NKA) que se topa na membrana celular. Esta ATPasa permite o intercambio activo de sodio e potasio a expensas de ATP, o que xera un gradiente iónico que mantén a homeostase celular. Iso levounos a analizar se o mecanismo senolítico da Digoxina estaba tamén relacionado co seu efecto sobre a NKA. Para iso medimos a concentración intracelular de diferentes ions, coma o sodio, o potasio, o calcio e os protóns. Tamén avaliamos os posibles efectos que podía ter o bloqueo da NKA pola Digoxina sobre o potencial da membrana celular. ensinounos que as células senescentes presentan alteracións no potencial da membrana celular, o que lles confire certa susceptibilidade ó efecto da Digoxina. Ademais, e a consecuencia do desbalance iónico causado pola Digoxina, descubrimos que a acumulación de protóns nas células senescentes fai que teñan un citoplasma máis ácido que as súas compañeiras proliferativas. Todas estas observacións demostráronnos que as células senescentes presentan unha homeostase alterada de forma basal facéndoas máis susceptíbeis do efecto da Digoxina.

Xunto con estas observacións, a sobreexpresión da subunidade alfa da ATPasa, tanto a de rato coma de humano, demostrou ter un efecto protector contra a Digoxina, confirmando tamén que a diana senolítica da Digoxina é a NKA.

Ademais do importante papel que ten a NKA no intercambio iónico e mantemento da homeostase, a principios do século XXI describíronse novas funcións da NKA. Así, a NKA tamén desemprega funcións como mecanismo de sinalización, incluíndo a activación da quinasa SRC, a transactivación de EGFR, a activación da vía RAS-MAPK, a modulación de NF-κB ou a xeración de ROS mediante a alteración das

mitocondrias. Por este motivo decidimos caracterizar como esta sinalización se estaba a producir nas células senescentes tras o tratamento con Digoxina. Para iso medimos a expresión de: p-AKT/AKT como proteínas executoras da ruta PI3K-AKT, p-ERK/ERK como proteínas efectoras da ruta RAS/RAF/MAPK, e p-p65/p65 como proteínas executoras da activación de NF-κB.

Ademais dos efectos da Digoxina sobre a sinalización mediada pola NKA, tamén avaliamos os posibles efectos da Digoxina sobre outras rutas de sinalización implicadas no proceso de senescencia como é a expresión de p53 e p21.

Con todo, a pesares de observar unha modulación nestas rutas de sinalización nas células senescentes tratadas con Digoxina non puidemos certificar de forma fidedigna a súa implicación no proceso de senolisis.

Por outra banda, a parte dos efectos da Digoxina sobre a NKA, tamén quixemos avaliar os efectos da Digoxina sobre o proceso de autofaxia, xa que os Glicósidos Cardíacos foron descritos como potenciais moduladores deste proceso. Para iso, comezamos analizando o efecto da Digoxina sobre a activación de TFEB, un factor de transcrición clave na regulación da autofaxia e a biosíntese de lisosomas. Tamén avaliamos a posible modulación por mor da Digoxina na expresión dos xenes implicados nestes procesos e regulados por TFEB, como BECN1, UVRAG, SQSTM1, LAMP1 ou MCOLN1. Estes análises suxeriron que a regulación transcripcional de TFEB é activada pola Digoxina como mostra a regulación positiva de varios destes xenes. Non obstante, non podemos especificar as implicacións destas regulacións transcripcionais na senólise.

Tras observar que a Digoxina actuaba sobre a autofaxia a nivel transcripcional, avaliamos os seus afectos no proceso da autofaxia. Para iso medimos a expresión de diferentes proteínas imprescindíbeis no proceso de autofaxia, como son BECLIN1, LC3B, LAMP1 ou p62, tras o tratamento con Digoxina. Isto mostrounos que as células senescentes presentan alteracións no proceso de autofaxia e que a Digoxina está a prexudicar máis este proceso impedindo a fusión do autofagosoma co

lisosoma. Estas observacións leváronnos a valorar o efecto da Digoxina sobre os lisosomas. O que descubrimos foi que a Digoxina altera a integridade lisosomal, o que podería considerarse unha nova vulnerabilidade das células senescentes.

De maneira complementaria, analizamos o posible efecto da Digoxina sobre outro tipo de organela como a mitocondria, xa que a súa integridade vese comprometida durante a senescencia. Observamos que a Digoxina tamén altera o potencial de membrana mitocondrial, aínda que non puidemos confirmar que estes efectos fosen implícitos ca indución de senólise.

Tras a caracterización dos efectos da Digoxina no noso modelo de senescencia in vitro¸ foi fundamental a validación do efecto senolítico da Digoxina en modelos in vivo. Nun primeiro momento avaliamos o potencial senolítico da Digoxina nun modelo ortotópico de cancro. Para iso inxectamos a liña celular A549 modificada para a expresión da luciferasa por vía subcutánea nos flancos de ratos inmunodeficientes. Unha vez os tumores estivesen formados, tratamos os ratos con Xemcitabina como axente indutor de senescencia, con Digoxina ou a combinación de ambos. O que puidemos observar foi unha case completa eliminación dos tumores na condición da terapia combinada de Xemcitabina e Digoxina gracias a medición do volume tumoral e da emisión de luminiscencia medido por IVIS. Ademais, para confirmar o efecto senolítico da Digoxina neste modelo, procesamos os tecidos dos tumores, realizamos a tinguidura da beta-galactosidasa asociada a senescencia (SABG) e determinación inmunohistoquímica de marcadores de senescencia como p21 ou de proliferación como Ki67. Deste xeito confirmamos que a Xemcitabina estaba a inducir senescencia de maneira apropiada e que o tratamento combinado ca Digoxina eliminaba as células senescentes de forma específica.

Finalmente, puxemos a punto outro modelo in vivo para a realización de explantes derivados de tumores. Neste caso, e como proba de concepto, utilizamos outra liña tumoral, a SK-Mel-103 de melanoma, inxectada por vía subcutánea nos flancos de ratos inmunodeficientes. Como axente indutor de senescencia empregamos Palbociclib, un inhibidor das cinasas dependentes de ciclinas (CDKs) 4

e 5 empregado para o tratamento de diferentes tipos de cancro. Ó final do procedemento, rescindíronse os tumores e deriváronse explantes que foron tratados in vitro con Digoxina. Tras o tratamento, estes explantes tinguíronse para determinar a actividade beta-galactosidasa asociada a senescencia (SABG). Observouse que, por unha banda Palbociclib estaba a inducir senescencia nos tumores dos ratos, e por outra banda que o tratamento in vitro destes explantes con Digoxina eliminaba ás células senescentes. Polo tanto, demostramos en dous modelos de diferentes tipos de cancro in vivo, tratados con distintos fármacos indutores de senescencia, que a Digoxina elimina especificamente ás células senescentes.

Con todo isto, esta tese mostra dunha forma ampla e completa todo o proceso de caracterización dun fármaco senolítico novidoso coma a Digoxina, dende a súa identificación nun cribado de alto rendemento, a súa caracterización do mecanismo de acción in vitro, ata os seus efectos en modelos in vivo.

INDEX

ABBREVIATIONS

DNA	Deoxyribonucleic Acid
SASP	Senescence-associated secretory phenotype
DDR	DNA-damage response
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
H2AX	H2A histone family member X
CHK1	Serine threonine checkpoint kinase 1
CHK2	Checkpoint kinase 2
MDM2	Murine double minute 2
CDK	Cycline-dependent kinase
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
NK-κB	Nuclear factor kappa B
C/EBPβ	CCAAT enhancer-binding protein β
IPF	Idiopatic pulmonary fibrosis
EMT	Epithelial-mesenchymal transition
TIFs	Telomere dysfunction-induced foci
TAFs	Telomere-associated foci
DNA-SCARSs	DNA segments with chromatin alterations reinforcing senescence
PML	Promyelocytic leukemia
CCFs	Cytoplasmic chromatin fragments
ROS	Reactive oxygen species
FASN	Fatty acid synthase
TCA	Tricarboxylic acid cycle
CCL	Chemokine C-C motif ligand
CXCL	Chemokine (C-X-C motif) ligand
MiF	Macrophage migration inhibitory factor
ERK	Extracellular signal-regulated kinase
FAKs	Focal-adhesion kinases
RhoA	Ras homolog family member A
Rac1	Ras-related C3 botulinum toxin substrate 1
Cdc42	Cell division control protein 42 homolog
ER	Endoplasmic reticulum
SAMPs	Senescence-associated morphological profile

BCL	B-cell lymphoma
MCL-1	Myeloid cell leukemia 1
BAX	Bcl-2-associated X protein
BAK	Bcl-2 homologous killer
BOK	Bcl-2 related ovarian killer
BAD	Bcl2-associated agonist of cell death
BID	Bcl-2 interacting domain
BIM	Bcl-2 interacting mediator of cell death
PUMA	p53 upregulated modulator of apoptosis
MOMP	Mitochondrial outer membrane permeabilization
SABG	Senescence-associated beta-galactosidase
DSBs	Double-strand breaks
SIRTs	Sirtuins
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
AMPK	AMP-activated protein kinase
MiDAS	Mytochondrial dysfunction-induces senescence
IL	Interleukin
GDF15	Growth differentiation factor 15
VEGF	Vascular endothelial growth factor
OIS	Oncogene-induced senescence
TIS	Therapy-induced senescence
AI	Artificial intelligence
SBB	Sudan-B-black
BrdU	Bromodeoxyuridine
EdU	5-Ethynyl-2′-deoxyuridine
PCNA	Proliferating cell nuclear antigen
ELISA	Enzyme-linked immunosorbent assay
qPCR	Quantitative polymerase chain reaction
SAHFs	Senescence-associated heterochromatin focis
H3K9me3	Trimethylation of lysine 9 on histone H3
H4K20me3	Trimethylation of lysine 20 on histone H4
Hp1γ	Heterochromatin protein 1 gamma
BubR1	Budding uninhibited by benzimidazole-related 1

RAS	Rat sarcoma virus oncogene
HRAS	Harvey rat sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma virus oncogene homolog
NF1	Neurofibromin 1
PTEN	Phosphatase and tensin homolog
PI3K	Phosphoinositide 3-kinases
PIN	Prostatic intraepitelial neoplasia
VHL	Von Hippel–Lindau
IRIS	Ionizing irradiation-induced senescence
RFP	Red fluorescence protein
GFP	Green fluorescence protein
TK	Thymidine kinase
HSV	Herpes simplex virus
DQ	Dasatinib plus Quercetin
ABT-263	Navitoclax
FOXO	Forkhead box O
PROTAC	Proteolysis targeting chimera
uPAR	Urokinase-type plasminogen activator receptor
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
cDNA	Complementary Deoxyribonucleic Acid
FDA	Food and Drug Administration
EMA	European Medicines Agency
CGs	Cardiac glycosides
IC50	Half-maximal inhibitory concentration
OA	Osteoarthritis
NKA	Na^+-K^+-ATPase
FITC	Fluorescein isothiocyanate
DiBAC4(3)	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol
NHE1	Sodium–hydrogen antiporter 1

EGFR	Epidermal growth factor receptor
TFEB	Transcription factor EB
MCOLN1	Mucolipin-1
LAMP1	Lysosomal-associated membrane protein 1
AO	Acridine orange
CQ	Chloroquine
Baf	Bafilomycin A1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay
LLOME	L-leucyl-L-leucine methyl ester
TMRM	Tetramethylrhodamine methyl ester
IVIS	In vivo imaging system
SQSTM1	Sequestosome-1
UVRag	UV radiation resistance-associated gene
LMP	Lysosomal-membrane permeabilization

INTRODUCTION

1. UNVEILING CELLULAR SENESCENCE

Throughout our lives, the cells of our bodies divide from the womb to the last days of our existence. During this process, our cells work to maintain a healthy, homeostatic environment allowing our organism to function efficiently. However, during this long journey, the cells have to defeat different aggressions and suffer damage, compromising the integrity of our body. Thus, our cells had to develop defense mechanisms against those insults trying to maintain a homeostatic state in the best possible way.

As we age, the amount of damage received in our cells accumulates, and although they have several defense mechanisms, their ability to succeed decreases. For this reason, during aging more pathologies appear, what we know as age-related diseases.

In the early 60s, the concept of cellular senescence was coined by Leonard Hayflick as a result of observing that normal human fibroblasts *in vitro* invariably enter a non-proliferative state after several cell divisions in culture (Hayflick & Moorhead, 1961). The proliferative arrest observed by Hayflick was a consequence of the telomere shortening that happens after consecutive cellular divisions as a protective response of the cells against DNA damage (Harley et al., 1990). With this observation, Hayflick laid the ground for the fundamentals of "cellular aging" demonstrating that there is a limit to cell divisions, what is now known as replicative senescence. From that moment, a new field of research appeared focused on the role of cellular senescence during aging.

Over time, the concept of cellular senescence has been expanded beyond aging resulting in the identification of cellular senescence during different physiological and pathological contexts. Thus, cellular senescence has been shown to play essential roles during embryo development, wound healing, and tissue regeneration. However, as observed during aging, the accumulation of senescent cells or their inefficient elimination has been described associated to the development of different diseases (Gorgoulis et al., 2019; Muñoz-Espín & Serrano, 2014).

1.1. HALLMARKS OF CELLULAR SENESCENCE

Cellular senescence consists of a proliferative arrest triggered by different stimuli, either a response to damage or a physiological process. Among the various cell types, biological contexts, or stress stimuli, four features were established as the main, shared ones defining senescent cells: a generally irreversible cell-cycle arrest, a distinctive secretory phenotype (SASP, senescence-associated secretory phenotype), signs of macromolecular damage, and metabolic reprogramming (**Figure 1**).

An irreversible growth arrest is one of the main characteristics of a senescent cell, independent of the stimuli or the cell type. Different from quiescence, senescent cells cannot re-enter the cell cycle despite subjecting them to proliferative signals.

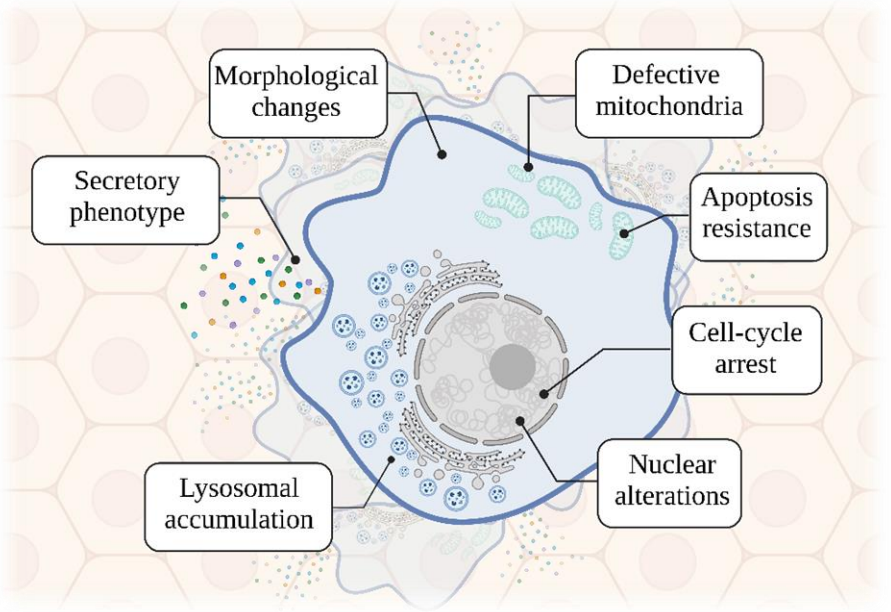

Figure 1. Hallmarks of cellular senescence. The most common features of senescence are the growth arrest, the SASP, macromolecular damage, and metabolic alterations. However, some other markers of senescence are the lysosomal accumulation or the apoptosis resistance. Own authorship created with BioRender.com

The growth detention of senescent cells also differs from that observed in terminally differentiated cells. After, differentiation cells lose their proliferative capacity after acquiring specialized functions, while cellular senescence is triggered as a response to damage (S. He & Sharpless, 2017).

This detention in the proliferative capacity is usually triggered by persistent DNA damage resulting in single or double-strand breaks. This DNA damage activates the DNA damage response (DDR) with the subsequent activation of a signaling cascade that ends in senescence induction. In the case of replicative senescence, the progressive loss of telomeres is read as DNA damage, since the end of the chromosomes are left unprotected, also activating DDR (d'Adda di Fagagna, 2008). Besides, different from telomere attrition, other types of DNA damage can be induced as a response to irradiation, chemotherapeutics, oxidative stress, or oncogene activation leading to senescence (Muñoz-Espín & Serrano, 2014).

As a response to the DDR, ATM and ATR are activated. The activation of ATM recruits γ-H2AX (gamma histone H2AX) to the site of damage to signal and initiate the repair cascade. Among others, CHK1 and CHK2 play an important role acting as effectors of ATR in the DDR. Altogether, the activation of these signaling pathway after DNA damage culminates in the activation of the p53/p21 pathway (Shiloh, 2006; Zou, 2007).

In a physiological context, p53 remains under the control of different regulators like MDM2. The activation of p53 in response to stress drives a complex anti-proliferative signal that can result in either senescence or apoptosis (Kastenhuber & Lowe, 2017).

After irreversible DNA damage, p53 is stabilized and activated leading to its transcriptional activation. One of its targets is the cyclin-dependent kinase inhibitor p21, that by inhibing CDKs results in the hypophosphorylation of the retinoblastoma protein Rb. This culminates in growth arrest (d'Adda di Fagagna, 2008).

Together with the activation of the p53/p21 pathway, the sustained DNA damage signaling can promote the hypophosphorilation of Rb by

activation of *INK4/ARF*. The *INK4/ARF* locus encodes for the $p14^{ARF}$, $p16^{INK4a}$, and $p15^{INK4b}$ tumor suppressors, which are commonly mutated in cancer (Sharpless, 2005). The activation of $p16^{INK4a}$ (hereinafter referred to as p16) inhibits CDK4 and CDK6 while $p14^{ARF}$ stabilizes p53 activation, blocking the cell cycle progression by the dephosphorylation of Rb.

It is worth mentioning that, despite the evidence of the stable growth arrest of senescent cells, some experimental approaches have demonstrated the possibility of senescence escape in some cancer models (Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019).

1.1.1. Senescence-associated secretory phenotype (SASP)

Another prominent feature of cellular senescence is its capacity to modulate the microenvironment through the secretion of a complex mixture of soluble factors. This senescence-associated secretory phenotype, known as SASP, is composed of cytokines and chemokines, growth factors, and matrix metalloproteinases, and is responsible for the pathophysiological effects of cellular senescence (J. P. Coppé et al., 2010; Kuilman & Peeper, 2009).

Through the SASP, senescent cells can reinforce the senescence state in an autocrine manner while they can also induce senescence in neighboring cells in a paracrine manner. The composition of the SASP varies according to the senescence-inducing stimulus, the cell type, and the physiological context in a dynamic manner (Wiley et al., 2017).

The regulation of the SASP depends on the activation and modulation of different signaling pathways, such as the DDR (Malaquin et al., 2015), GATA4 (C. Kang et al., 2015), mTOR (Herranz et al., 2015; Narita et al., 2011; A. R. J. Young et al., 2009), JAK/STAT (Toso et al., 2014), and p38/MAPK (Alspach et al., 2014; Freund et al., 2011). However, these different regulators of the SASP usually converge in the activation of NF-κB and C/EBPβ transcription factors (Ito et al., 2017).

The SASP has been pointed out as responsible of the dual roles of cellular senescence in health and disease. Thus, an acute and controlled SASP production can be beneficial in a physiological context such as

embryo development or wound healing, or in response to oncogene activation (Demaria et al., 2014; T. W. Kang et al., 2011; Muñoz-Espín & Serrano, 2014; Storer et al., 2013; Xue et al., 2007). In the context of development or wound healing, the SASP is essential in recruiting immune cells to promote the clearance of senescent cells once they are no longer useful (Demaria et al., 2014; Muñoz-Espín & Serrano, 2014; Storer et al., 2013). During the tumor suppressor response of senescence, the SASP reinforces the growth arrest of cancer cells and activates the antitumor mechanism of the immune system.

However, a chronic or uncontrolled SASP can be detrimental leading to fibrosis development or to tumor progression. The abnormal accumulation of senescent cells is related to a chronic SASP production of pro-inflammatory cytokines together with pro-fibrotic proteins. This pro-fibrotic SASP has been described during idiopathic pulmonary fibrosis (IPF) and myocardial hypertrophy (Anderson et al., 2019; Schafer et al., 2017). Similarly, numerous evidences have been reported for the protumor contributions of the SASP in many cancers (Angelini et al., 2013; Eggert et al., 2016; Krtolica et al., 2001; Yoshimoto et al., 2013). These protumor effects of the SASP were first described when senescent cells were co-cultured with premalignant cells, leading to the induction of an EMT and their full malignant transformation (J.-P. Coppé et al., 2008).

This double-edged sword of the SASP together with its complexity has generated a new topic of study within the field of senescence to understand its implications in health and disease, and opening a new therapeutic approach focused on its modulation.

1.1.2. Macromolecular damage

As previously mentioned, the first and most common senescence-inducing stimulus is DNA damage, activating the DDR. Although the progressive shortening of telomeres is the triggering event in the first described model of cellular senescence, different insults were described later as senescence inducers.

The maintenance of functional telomeres is carried out by telomerase, an enzyme not expressed in normal somatic cells, but found

overexpressed in most cancer cells (Greider & Blackburn, 1985; N. W. Kim et al., 1994; Shay & Wright, 2019). The lack of telomerase in normal cells is what makes them progressively shorten after consecutive cell divisions triggering in the end signals of damage (Harley et al., 1990; Lindsey et al., 1991). Telomere shortening leads to the destabilization of the telomeric DNA forming TIFs (telomere dysfunction-induced foci), activating DDR, and finally cellular senescence (Takai et al., 2003). Apart from TIFs, the oxidative degradation of the telomeric DNA forms TAFs (telomere-associated foci) that can also promote DDR and senescence (Birch et al., 2016). Furthermore, genetically modified systems to express telomerase to recover telomere length result in a prolonged *in vitro* lifespan (Bodnar et al., 1998; Shay & Wright, 2019).

Persistent DNA damage in senescent cells leads to the formation of DNA-SCARSs (DNA segments with chromatin alterations reinforcing senescence). These DNA-SCARSs have been described as regulators of different features of senescence, such as the SASP. The DNA-SCARSs are associated with PML (promyelocytic leukemia nuclear bodies) and activate DDR through p53 and CHK2. However, since DNA-SCARSs are formed in response to sustained DNA damage signals, they are not common in all types of senescence (Rodier et al., 2011).

Another macromolecular alteration that has been also observed during senescence is cytoplasmic chromatin fragments (CCFs). These CCFs are chromatin fragments released from the nucleus to the cytoplasm, being processed by the autophagic/lysosomal pathway and activating a pro-inflammatory response (Ivanov et al., 2013; Li & Chen, 2018).

Apart from DNA damage, protein integrity is also compromised in senescent cells. The production of reactive oxygen species (ROS) during senescence causes the oxidation of protein residues with the consequent accumulation of misfolded proteins (Höhn et al., 2017).

1.1.3. Metabolic alterations

Senescent cells are highly metabolic, not only because of their secretory phenotype but also because of their energy requirements due

to their increased glycolysis or autophagy (Dörr et al., 2013). These cells display some metabolic alterations probably due to the accumulation of dysfunctional mitochondria and lysosomes.

The defective mitochondrial metabolism during senescence seems to be caused by an accumulation of dysfunctional mitochondria since they cannot be eliminated properly due to impaired mitophagy. One potential player involved in this defective mitophagy could be p53, since it has been reported to interact during senescence with PARKIN, a key regulator of this process (Ahmad et al., 2015; Hoshino et al., 2013).

Senescent mitochondria show a reduction in their membrane potential leading to an overproduction of ROS (Chapman et al., 2019). At the same time, ROS production as a response to cellular stress can induce senescence as a consequence of oxidative DNA damage (Q. Chen et al., 1995; Passos et al., 2007). Moreover, activation of p16, p21, or p53 during senescence has been also linked to an increase in ROS (Macip, 2002; Macip et al., 2003; Takahashi et al., 2006). Thus, ROS production during senescence is linked to the maintenance of DDR for a stable cell-cycle arrest (Passos et al., 2010).

Defective mitochondria show also a reduction in fatty acid oxidation leading to the accumulation of lipids in senescent cells (Ogrodnik et al., 2017). However, the accumulation of lipids can also result from an increase in lipid uptake, the upregulation of lipid biosynthesis, or deregulation in lipid exhaustion (Flor et al., 2017). In this sense, it has been described that in senescent cells lipid biosynthesis is upregulated in a FASN-dependent manner. FASN upregulation was identified at the early stages of senescence induction, and it has been related to the development of different age-related diseases (Borghesan et al., 2019; Fafián-Labora et al., 2019). Moreover, mitochondria are responsible for the degradation of fatty acids through β-oxidation. During senescence, the accumulation of mitochondria allows a high catabolism of lipids through the TCA (tricarboxylic acid) cycle to fulfill the energetic demands, for gene transcription, and SASP production (Hamsanathan & Gurkar, 2022).

The increase in the catabolic process during senescence requires a high demand for lysosomes, the last effectors of the degradative process. Senescent cells are characterized by an enlargement of the lysosomal compartment (Robbins et al., 1970). Regardless of the high metabolic demands during senescence, lysosomes in senescent cells are defective leading to an increase in lysosomal biogenesis as a counterbalancing response. In line with the previously mentioned accumulation of dysfunctional mitochondria, defective lysosomes play an important role in the maintenance of mitochondrial homeostasis. There is an interaction between lysosomal degradation commitment and mitochondrial integrity, since lysosomes have to recycle defective mitochondria. However, in senescent cells, lysosomal degradation seems to be inefficient leading to the accumulation of dysfunctional mitochondria and increasing the levels of ROS, in an infinite feedback loop (Park et al., 2018).

Apart from the degradative function of lysosomes, they are also involved in the profound secretory phenotype of senescent cells, playing an important role in cell-cell communication and signaling. In fact, different SASP components have been identified as part of lysosomes/exosomes (Basisty et al., 2020). Moreover, it has been described that the secretion of some SASP factors, such as CCL2, CCL3/4, CXCL12, MIF, or SERPINE1, depends on RAB27A via lysosomal secretion through extracellular vesicles (Rovira et al., 2022).

1.1.4. Other features of senescence

Some other characteristics of senescent cells are their morphological changes and resistance to apoptosis.

In culture conditions, cells becoming senescent change their morphology acquiring a peculiar shape. Senescent cells are large and flattened, highly vacuolized, with large or even multiple nuclei. Changes in senescent cell morphology are regulated by caveolin-1, a downstream signaling pathway that comprises ERK activation. Also, the interaction between integrins and caveolin-1 allows the activation of different focal-adhesion kinases (FAKs), RhoA, Rac1, and Cdc42 during senescence which leads to cytoskeletal reorganization and morphological changes (Cho et al., 2004). This reorganization of the

cellular membrane and cytoskeleton is also responsible for the cell-cell communication shown by senescent cells since they create cytoplasmic bridges in a CDC42-dependent manner. With these cytoplasmic bridges, senescent cells have been suggested to communicate with immune cells to promote their immunosurveillance (Biran et al., 2015).

Interestingly, the expansion in size causes a dilution of the cytosolic components reducing the DNA:cytoplasm ratio. The dilution of the cytoplasm impairs gene transcription and protein synthesis compromising cell integrity. Thus, cytoplasmic dilution was related to growth arrest and short lifespan in yeast, leading to senescence (Neurohr et al., 2019). Regarding the high vacuolization observed during senescence, it has been described as a consequence of endoplasmic reticulum (ER) stress caused mainly by oncogene activation (Denoyelle et al., 2006).

Recently, the morphological changes that cells undergo when entering senescence have been described using high-content analysis, allowing the generation of a senescence-associated morphological profile (SAMP). These SAMPs demonstrate the heterogeneity in the morphology of senescent cells between models (Wallis et al., 2022).

Although these morphological changes were described *in vitro*, there is recent evidence of morphological changes in senescent cells during aging *in vivo* using a mouse model (Biran et al., 2017).

Another common feature of senescence is the resistance to apoptosis. Even though senescence and apoptosis are two sides of the same coin, being cellular responses to damage, paradoxically senescent cells show resistance mechanisms to apoptosis. One of the mechanisms used by senescent cells to activate resistance to apoptosis is regulated by members of the Bcl-2 family of proteins in response to stress, chemotherapeutics, radiation, or hypoxia (Elmore, 2007). The Bcl-2 family of proteins are multi-BH-domain proteins and can be divided into antiapoptotic proteins or proapoptotic proteins, establishing a balance between the survival of the cells and their suicide. The antiapoptotic part of the family is composed of Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1. The proapoptotic members of the family can be

divided into proteins that contain multiple BH domains similar to Bcl-2, such as Bax, Bak, and Bok, and proteins with only BH3 domains, such as Bad, Bid, Bim, Noxa, and Puma (Basu, 2022). The first evidence of apoptotic resistance of senescent cells was reported in the mid-90s and was shown to be caused by the increased expression of Bcl-2 antiapoptotic protein (E. Wang, 1995). Later, it was described that the main player in apoptosis resistance during senescence is Bcl-xL since its inhibition promotes the death of different types of senescent cells (Chang et al., 2016; Rochette & Brash, 2008; Yosef et al., 2016).

The intrinsic apoptotic pathway is regulated by the release of apoptogenic factors from the mitochondria such as cytochrome c, activating a signaling cascade in which late effectors are caspase-3 and caspase-7 (Singh et al., 2019). After a damage stimulus, BH3 proapoptotic proteins such as Bax and Bad are activated and cause mitochondrial outer membrane permeabilization (MOMP), activating the intrinsic apoptotic signaling.

However, several survival mechanisms were further identified, such as metabolic reprogramming or immune evasion (Soto-Gamez et al., 2019). The identification of this Achilles heel for senescent cells changed the course of the field opening a therapeutic opportunity to eliminate senescent cells efficiently and specifically to improve healthspan.

1.2. CELLULAR SENESCENCE AS A DYNAMIC PROCESS

Contrary to what was determined years ago, cellular senescence is a highly dynamic process that progressively modifies its features, giving rise to the heterogeneity of senescent cell types described in the literature (Herranz & Gil, 2018; S. Lee & Schmitt, 2019; van Deursen, 2014).

The first step of the process is the response to different stressors that promote the transition from light and temporal detention of the proliferation to an irreversible and stable growth arrest. Once the irreversible proliferative arrest is established, the typical features of senescence appear, a phase known as “early senescence”. During this “early senescence” step, we observe the appearance of the different

markers of senescence such as the SASP, the senescence-associated beta-galactosidase (SABG) activity, the loss of nuclear integrity, or chromatin remodeling, among others. This process can keep progressing into a more adaptive stage, known as the "full senescence" step, in which the composition of the SASP can be modified according to the needs of the microenvironment (De Cecco et al., 2013; Hernandez-Segura et al., 2017; Hoare et al., 2016; Ivanov et al., 2013).

However, this adaptive process of senescent cells will depend on their functionality in the organism. If the stimulus that triggers the induction of senescence leads to the repair of acute damage that contributes to the maintenance of tissue homeostasis, an acute senescence program will be activated. Once the damage is fixed, the immune system will remove these senescent cells. That situation is commonly described in the context of tissue repair and wound healing in which a physiological and acute response of senescence is induced. When this induction of senescence is maintained for long periods, and these senescent cells cannot be eliminated efficiently, it is called chronic senescence. This chronic senescence is described during aging or during pathological processes in which the immune system can no longer remove these senescent cells, leading to a detrimental pro-inflammatory environment (Muñoz-Espín & Serrano, 2014).

1.3.TYPES OF CELLULAR SENESCENCE

There are several senescence inducers, some of which could be interconnected (**Figure 2**). The stimulus triggering senescence may vary according to the cell type or the pathophysiological context. However, most of these stimuli converge on the activation of the p53/p21 and p16/Rb pathways.

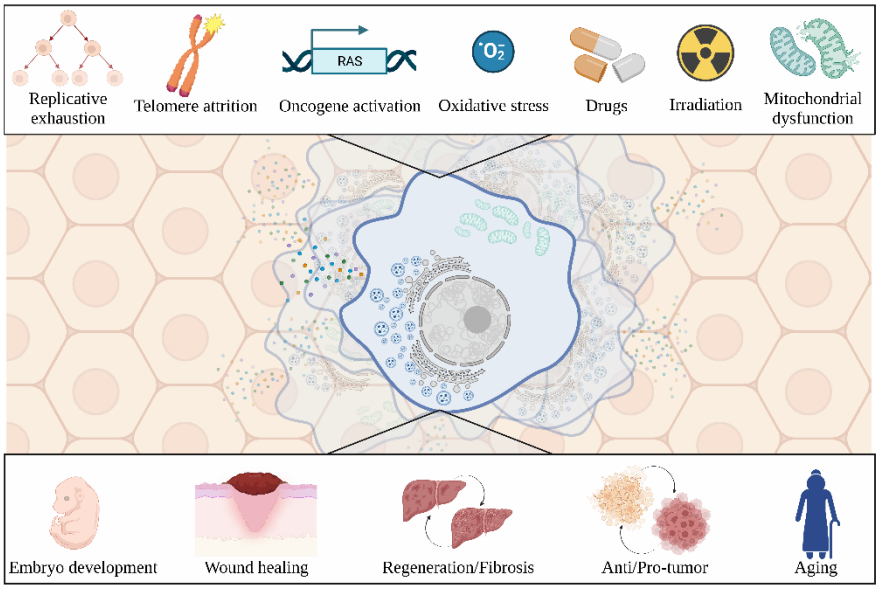

Figure 2. Senescence inducers and implications. Different senescence-inducing stimuli were described in different cell types and conditions. Among the conditions in which cellular senescence was identified are embryo development, wound healing, or aging. Additionally, the opposing roles of senescence induction were reported during fibrosis or cancer. Own authorship created with BioRender.com

1.3.1. Replicative senescence

The first mechanism described as an inducer of senescence was the telomere shortening observed after several passages in culture (Harley et al., 1990). Loss of telomere integrity triggers a damage response

similar to DNA strand breaks activating the DDR and p53 leading to cell-cycle arrest (Hayflick & Moorhead, 1961; Takai et al., 2003).

1.3.2. Oncogene-induced senescence (OIS)

Despite the first description of cellular senescence as a mirror of aging, it was also subsequently described as a tumor-suppressor mechanism (Lowe et al., 2004; Serrano et al., 1997). Cellular senescence is induced as a response to activated oncogenes causing an aberrant hyperproliferative state interpreted as a stress signal. When the cellular mechanisms of defense fail, this signal will lead to tumor development. This fate decision of a damaged cell after an oncogenic stimulus takes place at the early stages of tumorigenesis. If the senescence program is activated properly, the potential tumor will enter a proliferative arrest causing a premalignant lesion (Braig et al., 2005; Z. Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005).

1.3.3. Oxidative-stress-induced senescence

ROS production is a result of different stressors, including DDR, oncogene activation, or chemotherapy. So, ROS production can trigger senescence as a stress response but also reinforce the senescence state as ROS levels are increased during this process(Q. Chen et al., 1995; Passos et al., 2007).

1.3.4. Therapy-induced senescence (TIS)

Even though originally antitumor therapies were developed to kill cancer cells, the induction of cellular senescence has been observed as part of the therapeutic effect elicited by current anticancer treatments (Schmitt et al., 2002). Both chemo- and radiotherapy can induce senescence usually by causing DNA double-strand breaks (DSBs) triggering the above-mentioned DDR signaling (Ewald et al., 2010; Shao et al., 2014). However, the recently developed family of CDK4/6 inhibitors are chemotherapeutics that can induce senescence without activating DDR (Fry et al., 2004; Gelbert et al., 2014; S. Kim et al., 2013).

Despite this antitumor activity of senescence induction, the accumulation of senescent tumor cells can be detrimental mainly due to the SASP, since this accumulation has been related with the

development of relapses and metastasis (Demaria et al., 2017). However, the induction of senescence in tumors could provide a therapeutic opportunity for a more efficient elimination of the residual tumor.

1.3.5. Mitochondrial dysfunction-induced senescence (MiDAS)

In the mid-2010s a new senescence phenotype was described triggered by mitochondrial impairment (Wiley et al., 2016). Sirtuins (SIRTs) were identified as a target to induce senescence. The knockdown of different SIRTs leads to mitochondrial dysfunction and alterations of the NAD+/NADH ratio, which in turn induces senescence through the AMPK signaling pathway.

Senescent cells through MiDAS show a distinctive phenotype characterized by no DDR activation and a SASP lacking IL-1/NF-κB regulation (Wiley et al., 2016).

1.3.6. Paracrine senescence

One of the effects of the SASP is the induction of senescence in a paracrine manner. Apart from its opposing roles during tumorigenesis, paracrine senescence induction gained importance for its potential implications during age-related diseases. Different SASP factors, such as activin A, GDF15, VEGF, CCL2, and CCL20 were identified as key paracrine effectors inducing senescence in surrounding tissues (Acosta et al., 2013). However, this paracrine senescence is not effective in all cell types and not all senescent cells display this property. Cells undergoing OIS can induce paracrine senescence in epithelial cells surrounding the premalignant lesion promoting its immune clearance (Acosta et al., 2013). On the other hand, paracrine senescence induced after TIS could be related with the detrimental effects of chemo- and radiotherapy (Demaria et al., 2017).

1.4. SENESCENCE DETECTION MARKERS

Even though the field of senescence was established 60 years ago and has been the subject of intense investigation by researchers worldwide, there is still no unique specific marker for senescence detection. Thus, it is recommended to use a combination of different

markers according to the experimental procedure. In line with this, recently, AI (artificial intelligence) and algorithmic models are being developed for detecting senescent cells based on combining different markers for the hallmarks of senescence (Kohli et al., 2021).

1.4.1. Senescence-associated beta-galactosidase (SABG) staining

One of the most common markers of senescence is based on the characteristic increased lysosomal compartment. The beta-galactosidase enzyme is normally expressed in the lysosomes of all the cells, being detectable at normal lysosomal pH (4.0-4.5). However, due to the increased lysosomal content in senescent cells, the enzymatic activity of the beta-galatosidase is higher than in normal cells, allowing its detection at a suboptimal pH of 6.0. (Dimri et al., 1995; B. Y. Lee et al., 2006). The limitation of SABG staining is that it requires fresh tissue and that it is not specific of senescent cells, since any cell with high lysosomal activity will be also positive for this staining. Therefore, other techniques have been developed as an alternative to SABG, such as Sudan-black-b (SBB) staining. SBB is a marker for lipofuscins, an aggregate of oxidized proteins, lipids and metals, known to accumulate in aged tissues (Georgakopoulou et al., 2012).

1.4.2. DDR markers

Since most of the senescence-induced stimuli alter DNA integrity causing DNA damage and consequently activating DDR, several DNA-damage markers can be used to detect senescence.

DNA-damage foci are identified during replicative senescence by labeling dysfunctional telomeres. OIS or TIS can cause double-strand breaks that can be detected by immunohistochemical γH2AX or 53BP1 staining (Sharpless & Sherr, 2015).

Another marker is the detection of DNA-SCARS, formed as a consequence of prolonged DNA damage. These SCARS are characterized by the accumulation of DDR proteins such as ATM, CHK2, or PML, and differ from transient foci by the absence of RPA and RAD51 (Rodier et al., 2011).

1.4.3. Cell-cycle arrest

A hallmark of senescent cells is their withdrawal from the cell cycle. To identify the proliferative arrest, it is possible to measure the proliferative capacity of the cells using colony-formation assays or by assessing the incorporation of BrdU/EdU, which measures DNA synthesis. Other markers of proliferation are the immunodetection of PCNA or Ki-67. On the other hand, it is possible to measure the levels of CDK inhibitors such as p16 or p21 as markers of growth arrest. One CDK inhibitors that is most frequently used to detect senescence is p16 since it is nearly undetectable in normal conditions while it is highly upregulated during senescence (Hara et al., 1996; Serrano et al., 1997). However, not all types of cellular senescence show an upregulation of this cell cycle inhibitor.

1.4.4. Morphology

Changes in morphology arose as a rough marker of senescence induction but it is useful for *in vitro* detection. The enlarged and irregular shape of senescent cells can be observed by bright-field microscopy (Sharpless & Sherr, 2015).

Recently, measuring the characteristic autofluorescence of senescent cells and the changes in their size by using flow cytometry has been proposed as a fast and simple method to detect cellular senescence (Malavolta et al., 2022).

1.4.5. Secretory phenotype

Another hallmark of senescence is its robust secretory phenotype, which can be used to detect the senescent state.

Different techniques can be used to detect cytokines, chemokines, or growth factors secreted by senescent cells, such as ELISA or immunostaining. Also, the transcription of these factors can be measured by qPCR (J.-P. Coppé et al., 2008; Sharpless & Sherr, 2015).

1.4.6. Nuclear changes

Senescent cells display different nucleus alterations, being enlarged, sometimes multinucleated, with an irregular nuclear envelope, and with heterochromatin foci in their nuclear periphery. The loss of the nuclear lamina protein Lamin B1 is a typical event during

senescence and for this reason it has been proposed as a marker of senescence (Freund et al., 2012).

Alterations in chromatin condensation also allow us to detect senescent cells. SAHFs are formed as part of the process responsible for the stability of the proliferative arrest during senescence, although they cannot be used as a general marker in all types of senescence. These SAHFs can be visualized as DNA condensed foci that typically also present markers of heterochromatin such as H3K9me3, H4K20me3, or Hp1γ (Sharpless & Sherr, 2015).

Recently, changes on the nucleus size analyzed by deep learning have also been reported as a novel technique for senescent cell detection (Heckenbach et al., 2022).

1.4.7. Apoptosis resistance

Finally, another hallmark of senescence that can be used as a marker is the upregulation of antiapoptotic proteins in senescent cells. However, it cannot be used alone and not all the senescent cells regulate the family of Bcl-2 in the same manner (Chang et al., 2016; Rochette & Brash, 2008; Yosef et al., 2016).

2. CELLULAR SENESCENCE AND AGING

As we age, our organism starts to gradually lose its integrity becoming more susceptible to external insults that can give rise to the development of different pathologies. Tissue degeneration is a consequence of the characteristic loss of function observed in all the multicellular organisms as they age. This tissue decline manifests as different age-related pathologies such as osteoporosis, neurodegeneration, and sarcopenia, among others. Overall, these aging features are known as frailty, since the aged organism loses its potential to overcome damage entering a progressive degeneration (Campisi, 2013).

However, the loss of tissue integrity during aging makes cells prone to accumulate mutations that can give rise to proliferative alterations.

In this case, cells can proliferate uncontrollably leading to hyperplasias. Consequently, hyperplasic proliferation and an inefficient response against damage can result in cancer development (Campisi, 2013).

The original description of senescence could be considered as an *in vitro* reflection of the aging process in which cells lose their proliferative capacity after several passages as a response to replicative stress. In this context, cellular senescence emerged as a protective response to telomere shortening and DNA damage, preventing the proliferation of aberrant cells. However, the link between the beneficial effects of cellular senescence and the detrimental development of several diseases related to aging was not fully understood.

The presence of cellular senescence in the context of organismal aging *in vivo* was first demonstrated at the beginning of the 21st century. Despite the lack of a specific marker for cellular senescence, some of the previously described *in vitro* markers, such as SABG, p16, and DDR, were identified in tissues derived from aged mammals suggesting a relationship between cellular senescence and physiological aging (rodents: (Burd et al., 2013; Krishnamurthy et al., 2004; C. Wang et al., 2009). Primates: (Herbig et al., 2006; Jeyapalan et al., 2007). Humans: (Chkhotua et al., 2003; Dimri et al., 1995; Melk et al., 2004)). However, the abundance of senescent cells in an aged organism varies between species and tissues, suggesting that other players must be responsible for the deleterious effects of senescence during aging (Campisi & d'Adda di Fagagna, 2007).

Later, the SASP was described as one of the main players in the pathological effects of senescence in aging by promoting a chronic pro-inflammatory microenvironment, known as inflammaging (Franceschi et al., 2006). SASP factors promoting chronic inflammation together with pro-fibrotic activities have been reported in different age-related diseases such as atherosclerosis, osteoarthritis, or type 2 diabetes, impairing the healthspan and reducing the lifespan in mice late in life (Childs et al., 2016; Jeon et al., 2018; Minamino et al., 2009).

Moreover, exposure to external insults also affects the stem cell pools as they are forced to repair and regenerate the damaged tissue.

This cycle of injuring and repairing leads to stem cell exhaustion, compromising the homeostasis in tissues, and taking part in organismal decline (Sharpless & DePinho, 2007). Thus, the accumulation of senescent cells, their pro-inflammatory SASP, and the defective maintenance of tissue homeostasis have been related with the physiological decline and frailty observed during aging and the development of age-related diseases.

The first *in vivo* evidence of the detrimental effect of senescence during aging was described in a model of premature aging by knocking-down *BubR1*. These progeroid mice were genetically modified to express a fusion protein making p16-positive cells susceptible to a pro-drug that triggers apoptosis. This model is known as the INK-ATTAC. With this model, the specific elimination of p16-positive cells, potentially considered senescent, results in a delay of the aging phenotype of these mice. In addition, this study also showed that the tissue dysfunction observed during aging might be caused not only by the accumulation of senescent cells but also by the detrimental effects of the SASP (Baker et al., 2011). Later, by using the same INK-ATTAC model in physiologically aged mice, the elimination of senescent p16-positive cells was reported to improve the healthspan of these mice (Baker et al., 2016). These observations provide evidence of the detrimental effect of the accumulation of senescent cells during aging and their implication on the onset of different pathologies, such as renal dysfunction, pulmonary fibrosis, sarcopenia, osteoarthritis, and cancer, among others. Interestingly, animal models of these aging diseases showed amelioration of the pathological phenotype after the selective elimination of senescent cells (McHugh & Gil, 2018).

3. CELLULAR SENESCENCE AND CANCER

Although cellular senescence was first described as a tumor suppressor mechanism, years of research provided evidence for the dual role of senescence in cancer. Contrary to its antitumor effects, an aberrant senescence program may also promote neoplastic growth during aging or be responsible for relapses after antitumor therapy.

3.1. ONCOGENE-INDUCED SENESCENCE (OIS)

In 1982, the first human oncogene, *HRAS*, was identified in human bladder carcinoma, and its capacity to transform immortalized cells was reported (Parada et al., 1982; Santos et al., 1982; Taparowsky et al., 1982). Thus, to be able to fully transform normal cells into tumor cells additional genetic alterations, apart from RAS activation, are required (Land et al., 1983). The requirement for an additional genetic mutation opened the question on the effect of the single expression of RAS in normal cells. In this context, in 1997 the induction of oncogene-induced senescence (OIS) after expression of oncogenic mutant *HRAS* (*HRAS*^{G12V}) in normal cells was described (Serrano et al., 1997). This OIS program is activated in response to a hyperproliferative state caused by oncogene activation leading to replicative stress, activation of p53 and p16, and growth arrest. The outcome resulting from RAS activation was described to be dependent on the levels of expression of RAS together with the bypass of senescence checkpoints (Sarkisian et al., 2007).

Following the evidence of the tumor suppressor effects of senescence, several subsequent studies demonstrated the implications of cellular senescence during oncogenic activation *in vivo*. In line with this, the endogenous expression of *Kras*^{G12V} triggered cellular senescence in premalignant lesions of the lung and pancreas preventing tumor formation (Collado et al., 2005). Other members of the RAS family have been described also as inducers of senescence *in vivo*. Low levels of *Hras*^{G12V} in the mammary gland caused hyperplasias, while high levels caused senescence (Sarkisian et al., 2007). Similar results have been reported in human nevi, which are benign melanocytic tumors, that entered senescence, preventing melanoma formation caused by *BRAF* mutations (Michaloglou et al., 2005). The anticancer properties of the senescence program have also been described in response to protumor or hyperproliferative signals under conditions of loss of tumor suppressors. The tumor suppressor NF1 (neurofibromin 1) is a RAS GTPase that inhibits RAS activity. Mutation of *NF1* is associated with neurofibromatosis type 1, a heritable cancer syndrome. Hyperactivation of RAS was reported as a consequence of the loss of *NF1* leading to neurofibroma formation, a type of pre-neoplastic lesion

in which senescence induction was observed (Courtois-Cox et al., 2006). Another example is provided by the loss PTEN, a tumor suppressor gene which counteracts the proliferative signals of PI3K. The absence of PTEN leads to prostate intraepithelial neoplasia (PIN) development in vivo, a lesion composed of senescent cells, and to high levels of p53 and senescence induction in vitro. Combined absence of PTEN together with ablation of p53 bypasses senescence and results in malignant transformation (Z. Chen et al., 2005). Similarly, activation of AKT1, an effector of the PI3K pathway, also caused PIN with senescence induction through $p27^{kip1}$ activation (Majumder et al., 2008). Also, the loss of the tumor suppressor protein von Hippel-Lindau (VHL) was described as a senescence-inducing event in the kidney through the activation of Rb and $p27^{kip1}$ preventing renal carcinomas (A. P. Young et al., 2008).

Finally, once the malignant tumor is formed, the restoration of tumor suppressor gene expression was identified as capable of eliciting an antitumoral response. Using a p53-switchable mouse model in which tumors were developed spontaneously or after irradiation in the absence of p53, restoration of the expression of p53 resulted in apoptosis induction in lymphomas, while it resulted in senescence induction in sarcomas (Ventura et al., 2007; Xue et al., 2007). Also, senescence induced by p53 restoration in tumors seemed to promote the activation of the immune system contributing to tumor clearance (Xue et al., 2007).

3.2. THERAPY-INDUCED SENESCENCE (TIS)

Several therapies currently used in the clinic for the treatment of many cancers have been described as capable of inducing cellular senescence (B. Wang et al., 2020). Apart from a positive effect controlling tumor growth, senescence-inducing cancer therapy has also been associated with potential side effects as well as comorbidities.

3.2.1. Radiotherapy

The discovery of the X-Rays by WC Roentgen at the end of the 19^{th} century, together with the discovery of radium by Marie Curie, revolutionized medicine from diagnoses to treatments. However, in the

20th century, apart from its therapeutic effect in the treatment of cancer, the protumor effect of overexposure to radiation was reported.

The therapeutic benefit of cancer radiotherapy relies on its capacity to induce DNA damage leading to cancer cell death. Also, the lack of a specific therapeutic target makes radiotherapy a broad-spectrum treatment for many cancers like lymphomas, soft tissue sarcomas, and tumors from the central nervous system, among other tissue carcinomas (Baskar et al., 2012). Ionizing radiation can induce senescence (ionizing irradiation-induced senescence, IRIS) in different cancer models *in vitro* and *in vivo* (Demaria et al., 2014; Jones et al., 2005; B. Wang et al., 2020). Interestingly, IRIS response seems to be dependent on p53 activation in some tumors such as breast cancer or glioblastomas, since *in vitro* models of these tumors with mutations in the p53 DNA-binding domain showed no senescence induction or recovered their proliferative potential (Jones et al., 2005; Quick & Gewirtz, 2006).

Although radiotherapy acts locally, senescent cells produced after irradiation in mammary stromal cells can promote alterations in the surrounding tissue. The SASP produced by these senescent cancer cells was described to cause malignant transformation and breast carcinomas (Tsai et al., 2005).

Also, radiotherapy of glioblastoma *in vivo* resulted in the induction of senescence in a wide range of nervous cells, with astrocytes as the most affected cells. These senescent astrocytes, through the SASP, promote the proliferation and invasiveness of glioma cells causing recurrences. Interestingly, elimination of these senescent astrocytes by senolytic treatment prevents the detrimental effect of IRIS on the central nervous system (Fletcher-Sananikone et al., 2021).

3.2.2. Chemotherapy

The concept of chemotherapy was born in the 1940s after observing that treatment with DNA alkylating agents, such as nitrogen mustard, caused tumor regression in a patient with non-Hodgkin's lymphoma (Chabner & Roberts, 2005). Further research and medical advances provided more efficient and less toxic new chemotherapeutic drugs such as Cisplatin or Temozolomide. Although the emergence of

chemotherapy treatment was based on the use of agents that caused unrepairable damage to the DNA leading to cell death, lately, it has been reported that they can also induce senescence (Aoshiba et al., 2003; B. Wang et al., 2020; X. Wang et al., 1998; Yang et al., 2012).

Topoisomerase inhibitors appeared as drugs that interfere with the topoisomerase II hindering DNA repair, thus activating DDR signaling and growth arrest. One of the most common compounds from this family is Doxorubicin, used in the treatment of solid tumors, such as breast or lung cancer, as well as hematologic tumors, such as lymphomas and acute lymphocytic leukemia (Thorn et al., 2011). Doxorubicin was reported to induce senescence both *in vitro* and *in vivo* models of different types of tumors. Nevertheless, Doxorubicin was also reported to induce senescence in healthy tissue (Bielak-Zmijewska et al., 2014; F. Chen et al., 2018; B. Wang et al., 2020). Similar effects were described after Etoposide and Camptothecin treatments.

Another example of DNA damaging chemotherapeutic drug is Bleomycin. This compound is used in the treatment of germinal cell tumors, and Hodgkin's and non-Hodgkin's lymphoma (J.-M. Lavoie & Kollmannsberger, 2019). *In vitro,* Bleomycin induces senescence in A549 cells as shown by cell-cycle arrest, SABG staining, p21 upregulation, and SASP production. Bleomycin can also cause pulmonary fibrosis by inducing senescence in the lung, as a consequence of the SASP secreted by senescent cells (Aoshiba et al., 2013).

Different from DNA-damaging agents, taxane drugs such as Paclitaxel, were described as microtubule inhibitors for the treatment of ovarian, breast, and non-small cell lung cancer. These drugs disrupt the microtubule spindle formation during the metaphase-anaphase stages of cell division, arresting the cells (Mikuła-Pietrasik et al., 2019). Similar to the previously described chemotherapeutics, these drugs also triggered senescence induction in tumors but also in healthy tissue (Ota et al., 2009; B. Wang et al., 2020).

Other chemotherapeutic treatments have also been described as inducing TIS. Different senescence markers, such as p16, p21, and

SASP factors, were detected in biopsies from prostate cancer patients after Mitoxantrone therapy (J.-P. Coppé et al., 2008). Similarly, senescence markers were also identified in biopsies from breast cancer patients after neoadjuvant chemotherapy (te Poele et al., 2002). Animal models of cancer chemotherapy have provided evidence of senescence induction in healthy tissue apart from the tumor. This has also been observed in samples derived from cancer patients (Sanoff et al., 2014).

3.2.3. Cyclin-dependent kinase 4/6 inhibitors (CDK4/6i)

More recently, a family of drugs collectively known as CDK4/6i were developed for the treatment of breast cancer. These CDK4/6i target the D-type cyclin-CDK4/6 pathway, which is critical for G1 to S phase cell cycle transition. Prolonged inhibition of the cell cycle at this stage in cancer cells leads to cellular senescence. Palbociclib, the first CDK4/6i described, Ribociclib, and Abemaciclib (Fry et al., 2004; Gelbert et al., 2014; S. Kim et al., 2013) belong to this family. Apart from their clinical indication for breast cancer, CDK4/6i induce senescence in different cancer models such as gastric cancer, melanoma, liposarcoma, and neuroblastoma, among others (B. Wang et al., 2020). However, even though some senescence markers were observed after CDK4/6i treatment in these tumor models, an irreversible proliferative arrest was not always identified.

3.2.4. Other TIS mechanisms

Different epigenetic modulators were reported as senescence inducers in some cancer models. An inhibitor of the DNA methyltransferase, 5-aza (5-Aza-2′-deoxycytidine), induced p16 expression, proliferative arrest, and other features of senescence in osteosarcoma and mesothelioma cells (Amatori et al., 2011; Widodo et al., 2007). In line with this, Vorinostat, a histone deacetylase inhibitor approved for the treatment of cutaneous T cell lymphomas, induced senescence in colon and breast cancer cells, leukemia cells, and urothelial carcinoma cells (Almeida et al., 2017; Elknerova et al., 2011; Kaletsch et al., 2018; W.-S. Xu et al., 2005).

Finally, the development of immunotherapies opened a new opportunity for cancer treatment taking advantage of immune-mediated tumor clearance. In physiological conditions, senescent cells can recruit

immune cells through the SASP for their elimination. This ability to communicate with the immune system could also be exploited in the context of cancer therapy to promote the elimination of senescent tumor cells. In line with this, Rituximab, an anti-CD20 antibody for the treatment of leukemia and lymphoma, promotes senescence induction in B-cell lymphoma with an immunomodulatory SASP (Däbritz et al., 2016).

3.3. NON-CELL AUTONOMOUS SENESCENCE BY SASP

One of the most interesting features of cellular senescence is the pleiotropic effects of the SASP. Evolutionary, the SASP has been proposed as a mechanism by which cellular senescence could contribute to tissue development and regeneration (Muñoz-Espín & Serrano, 2014). Also, during OIS, SASP factors mediate tumor suppressor functions by inducing paracrine senescence, recruiting the immune system, or reinforcing the senescence state (Acosta et al., 2013; Kuilman & Peeper, 2009; Lujambio et al., 2013). These effects of the SASP can act both locally and systematically, resulting in the induction of senescence in stromal cells that surround the tumor promoting tumor relapses (Saleh et al., 2018).

The first evidence of a pro-tumorigenic effect of the secreted factors from senescent cells was reported after observing that co-culture of senescent fibroblasts with preneoplastic epithelial cells promoted the proliferation and invasiveness of these premalignant cells (Krtolica et al., 2001). The SASP from senescent normal fibroblasts can also promote epithelial-to-mesenchymal transition (EMT), mainly through IL-6 and IL-8, in a non-invasive breast cancer cell model (J.-P. Coppé et al., 2008). Similarly, conditioned medium from chemotherapy-induced senescent mesothelioma cells, promotes EMT and chemotherapy resistance of non-treated mesothelioma cells (Canino et al., 2012).

Pro-tumorigenic effects of the SASP are not only produced by an enhancement of the proliferation of preneoplastic lesions, but they can also promote tumor immunosuppression. An example of this was observed in senescent hepatocytes that secreted CCL2 as part of their SASP, recruiting immature myeloid cells. These immature cells turned

into macrophages and promoted hepatocellular carcinogenesis through NK-cell inhibition (Eggert et al., 2016).

Finally, the SASP can mediate resistance to anticancer therapies preventing TIS, promoting tumor relapse, and causing a decreased survival (Chien et al., 2011). Thus, the detrimental effects of the SASP during cancer have been widely reported.

4. SENOTHERAPIES

The first evidence of the detrimental effects of accumulating senescent cells were provided by their specific elimination in a progeroid mouse model. Depleting senescent cells in these mice improved their healthspan and delayed the onset of age-related diseases (Baker et al., 2011). Later, similar results were shown in normally aged mice not only improving the healthspan of the animals, but also extending their lifespan, delaying tumorigenesis, and resulting in less frailty (Baker et al., 2016). However, in different physiological conditions, the elimination of senescent cells could be unfavorable. During wound healing, for example, senescent cells play an essential role in repairing injured tissue. The elimination of these proregenerative senescent cells has been reported to impair wound closure (Demaria et al., 2014).

The promise of modulating or eliminating senescent cells opened new opportunities not only to study the function of senescence in different physiological and pathological conditions, but also for developing novel therapeutic strategies (**Figure 3**).

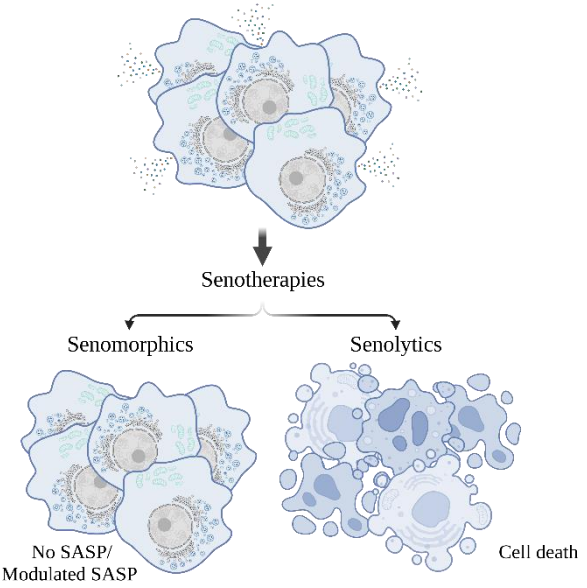

Figure 3. Senotherapies. To prevent the undesirable effects of the accumulation of senescent cells, new therapeutic opportunities give rise. The development of senomorphics for the modulation of the SASP, or senolytics for the elimination of senescent cells, provide evidences of improving different aging conditions. Own authorship created with BioRender.com

4.1.SASP MODULATORS OR SENOMORPHICS

As previously mentioned, SASP factors secreted by senescent cells can have both beneficial and detrimental effects in the surrounding tissue, but also in the whole organism. To overcome the detrimental effects of the SASP, senomorphics, drugs modulating SASP secretion without affecting viability, have been proposed (**Figure 4**).

Since both mTOR and NF-κB signaling pathways are key players in SASP production, targeting these pathways could represent a promising strategy to modulate the SASP. The most famous component of this group of drugs is Rapamycin. Through the inhibition of mTOR, Rapamycin improved the lifespan in different organisms including mice (Harrison et al., 2009). This observation led to the identification of potential effects of Rapamycin on senescence. Consistent with this, Rapamycin has been reported as a SASP suppressor in different models

of senescence alleviating the senescent cell burden and delaying the onset of aging conditions (Selvarani et al., 2021; R. Wang et al., 2017). However, mTOR inhibition can have more effects on cellular senescence than SASP modulation alone, so these approaches should be cautious (Wall et al., 2013).

Similar results were shown with Metformin, an antidiabetic drug that improves aging conditions by inhibiting the SASP through NF-κB targeting (Moiseeva et al., 2013). Thus, pharmacological or genetic inhibition of NF-κB improves progeroid and aging phenotypes, and alleviates senescence through SASP suppression in different models (Tilstra et al., 2012; L. Zhang et al., 2021).

Targeting other pathways involved in SASP regulation also showed an improvement in the deleterious effects of the SASP. Inhibition of the p38/MAPK ameliorates the secretion of SASP factors after different senescence-inducing stimuli and reduces markers of senescence (Freund et al., 2011; Y. Wang et al., 2011). On the other hand, JAK/STAT inhibitors such as Ruxolitinib improved the aging phenotype and alleviated frailty in old mice (M. Xu et al., 2015). In addition, Ruxolitinib has also been shown to reduce the premature aging of progeroid mice and to inhibit progerin-induced senescence through the alleviation of the SASP (Griveau et al., 2020).

Apart from the beneficial effects of modulating the SASP during aging, senomorphic treatment resulted also in a better response to cancer therapies. Despite the positive anticancer effects of SASP in preventing tumor growth and promoting immune clearance, it might also affect normal stromal cells causing resistance to therapies and tumor progression (J. P. Coppé et al., 2010). The proinflammatory SASP produced by senescent thymic cells have been reported to promote chemoresistance of lymphoma cell *in vivo* (Bent et al., 2016; Gilbert & Hemann, 2010). Similar observations were reported in a model of prostate cancer in which senescent fibroblasts cause the chemoresistance of prostate cancer cells. However, Rapamycin treatment of senescent fibroblasts suppressed their proinflammatory SASP allowing the therapeutic response of the prostate cancer cells to chemotherapy (Laberge et al., 2015). Simvastatin, a cholesterol-

lowering drug, was also reported as a SASP modulator preventing breast cancer progression driven by senescent fibroblast (S. Liu et al., 2015).

Therefore, modulating the SASP in aging and cancer represent an efficient opportunity to circumvent the deleterious effects derived from using cellular senescence as a therapeutic strategy. Exploiting senomorphics for modulating the detrimental effects of the SASP without affecting its positive effects represents a critical challenge.

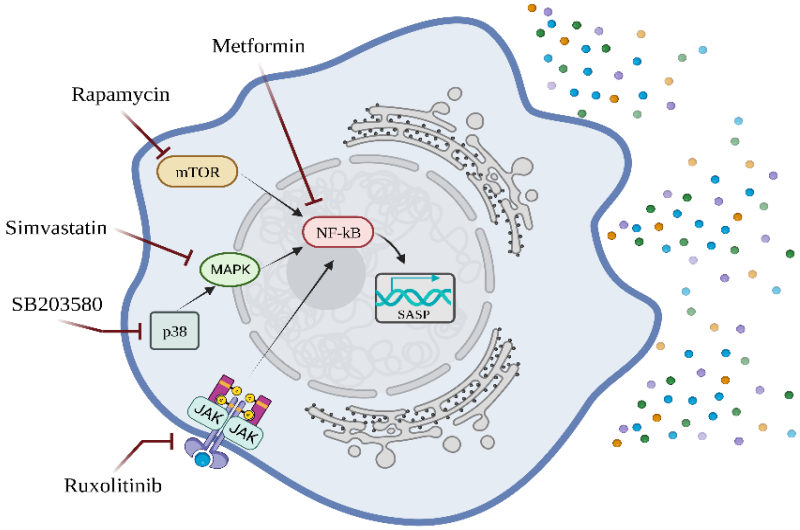

Figure 4. SASP modulators. Different approaches have been described as SASP modulating targets. The most well-known senomorphics drugs are Rapamycin and Metformin. However, other drugs were identified as SASP modulators such as Simvastatin, SB203580, or Ruxolitinib. Own authorship created with BioRender.com

4.2. Elimination of senescent cells with Senolytics

Since cellular senescence could also play positive roles, such as during development and tissue regeneration, the modulation or elimination of these cells must be cautious. Still, depletion of senescent cells in a pathological context could represent a promising therapeutic strategy during aging or cancer.

The first evidence that proved the beneficial effects of the elimination of senescent cells was provided by the generation of the INK-ATTAC mouse model. The INK-ATTAC transgenic model expresses an inducible suicidal cassette linked to p16 expression. In this manner, p16-positive cells can be induced to undergo apoptosis. The elimination of p16-positive senescent cells by using the INK-ATTAC mouse model caused the amelioration of aging conditions and frailty in both progeroid and normally aged mouse models (Baker et al., 2011, 2016). Similar observations were obtained using another transgenic mouse model known as p16-3MR. In this *in vivo* model, p16 promoter drives the expression of a fusion protein between luciferase, RFP and HSV-TK. Luciferase and RFP expression allow the identification of p16-positive cells, while TK causes cell death after ganciclovir treatment. Apart from the involvement of senescent cells in aging observed also with the INK-ATTAC model, the elimination of senescent cells in the p16-3MR mice revealed a positive contribution for these cells during wound healing (Demaria et al., 2014). Although these mouse models demonstrated opposing roles of senescence, a new therapeutic opportunity based on the elimination of senescent cells appeared.

The observations described above of eliminating senescent cells gave rise to the concept of senolysis, the specific elimination of senescent cells. In line with this, senolytics, small-molecules targeting senescent cells by taking advantage of their vulnerabilities to defeat age-related diseases and cancer, were developed. The first senolytics described were the combination of Dasatinib plus Quercetin (hereafter referred to as DQ) and ABT-263 (or Navitoclax) (Chang et al., 2016; Y. Zhu et al., 2015).

Dasatinib, a tyrosine kinase inhibitor, is an approved anti-cancer drug, while Quercetin is a naturally derived flavonoid that seems to target PI3K. DQ eliminate senescent cells induced by different stimuli *in vitro*, and *in vivo*, they improve cardiac function and fitness, increasing the healthspan of old and progeroid mice (Zhu et al., 2015). At the same time, and as a result of a drug screening, ABT-263, a potent inhibitor of the BCL-xL and BCL-2, was identified. The senolytic

activity of ABT-263 was reported as a consequence of the apoptotic resistance of senescent cells due to their overexpression of the BCL-2 family of proteins. This activity was shown in different cellular models but also in total body irradiated mice and normally aged mice, causing a delay on the onset of premature aging and rejuvenating old mice (Chang et al., 2016).

These observations have led to clinical trials for the treatment of different aging pathologies. DQ showed amelioration of the pathogenesis in patients with diabetic kidney disease and idiopathic pulmonary fibrosis in which a reduction in senescent cell burden and circulating SASP factors were reported (Wissler Gerdes et al., 2020). However, despite the great senolytic potential of ABT-263, patients treated with this drug in a clinical trial of cancer manifested severe thrombocytopenia precluding further clinical development (Schoenwaelder et al., 2011).

Exploiting the vulnerabilities of senescent cells as a promising senolytic target led to the development of other drugs (**Figure 5**). Thus, due to the importance of p53 activation during senescence, p53 pathway inhibitors were developed. FOXO-DRI inhibits FOXO4-p53 interaction leading to apoptosis in senescent cells. This was shown to alleviate aging conditions and prevent chemotoxicity derived from Doxorubicin treatment (Baar et al., 2017). Similarly, UBX0101, an inhibitor of MDM2-p53 interaction, showed senolytic effect in mouse models of osteoarthritis (Jeon et al., 2017).

The therapeutic benefits provided by senolytics, together with senescence-inducing anticancer treatments, opened the opportunity of combining both approaches to develop more efficient anti-cancer therapies. This combination therapy of senescence induction to arrest tumor growth together with senolytics is what is now known as “the one-two punch strategy” against cancer. In line with this, ABT-263 showed senolytic activity in different *in vitro* and *in vivo* cancer models in combination with TIS. Combination treatment of aurora kinase inhibitors, which were reported as senescence inducers by its activation of the MAPK pathway, with ABT-263 showed complete elimination of melanoma and lung cancer cells (L. Wang et al., 2017). Also, ABT-263

combined with Etoposide or Doxorubicin showed total tumor remission in lung and breast cancer models (Saleh et al., 2020). However, although ABT-263 has a quite broad-spectrum senolytic activity, side effects derived from platelet toxicity hinder its clinical use.

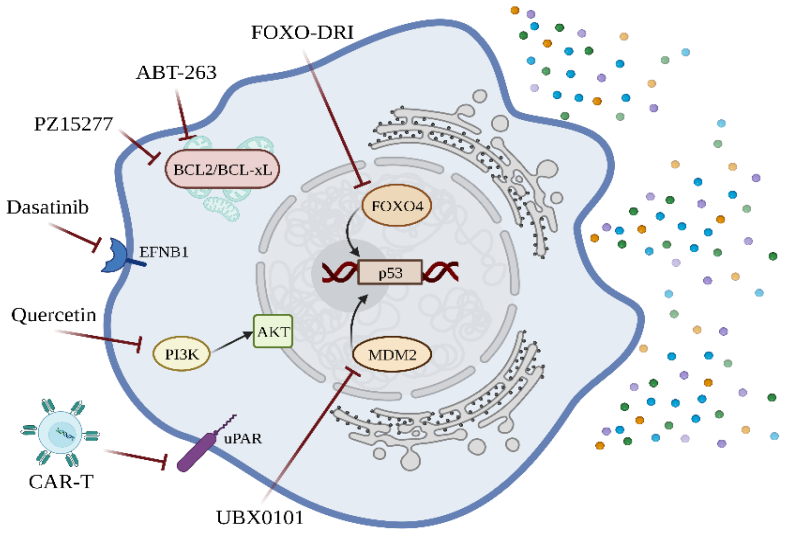

Figure 5. Senolytic therapies. The evidences of the negative effects of the accumulation of senescent cells lead to the development of novel strategies to efficiently eliminate these cells. The first and well-characterized senolytics were ABT-253 and DQ. However, to further exploit the senolytic therapy as broad spectrum, other senolytic drugs were developed such as FOXO-DRI, UBX0101, the PROTAC PZ15277, or CAR-T cells. Own authorship created with BioRender.com

All these beneficial effects of the "one-two punch" strategy motivated a great research effort for the identification of new mechanisms to improve senolytic treatment and boosted high-throughput screenings to find novel senolytics. For example, new delivery mechanisms were developed for improving the treatment with ABT-263. In line with this, nanoencapsulation of ABT-263 as a prodrug that can be activated by beta-galactosidase has been shown to represent an efficient and less toxic approach to target senescent cells (González-

Gualda et al., 2020). Similarly, to overcome the platelet toxicity of targeting BCL-xL, the creation of a PROTAC (proteolysis-targeting chimaera) drug PZ15277 promoting the proteasomal degradation of BCL-xL has been recently reported (Y. He et al., 2020).

Finally, using a different approach, the use of CAR-T cells was proposed as a senolytic strategy by targeting uPAR (urokinase-type plasminogen activator), an abundant protein at the surface of many senescent cells, extending cancer-free survival in mouse models (Amor et al., 2020).

In summary, the “one-two punch” strategy offers a new exceptional opportunity in the treatment of cancer and age-related diseases.

OBJECTIVES

Cellular senescence is a powerful antitumor response, even though negative side effects have also been proven. A chronic senescence-inducing stimulus and/or a failure to efficiently remove these cells can lead to the accumulation of senescent cells in tissues with detrimental functional consequences. This process underlies aging and is the basis of some age-related diseases, but it also operates during chemo/radiotherapy of cancer favoring relapse and metastatic growth. Furthermore, chemo/radiotherapy of cancer can damage normal healthy cells by inducing senescence and this is also the basis of the negative secondary effects observed in cancer patients.

For all these reasons, our main goal is **to identify compounds with senescence-specific cytotoxic activity, known as senolytics.**

In order to carry out this main goal, we propose the following specific objectives:

1. Design a novel system for the development of high-throughput screening for senolytic discovery.
2. Perform a high-throughput screening of chemical libraries to identify compounds with senolytic properties in an *in vitro* model of lung adenocarcinoma and chemotherapy-induced senescence.
3. Validate the compounds identified as potential senolytics *in vitro* and *in vivo*.
4. Characterize the mechanism of action of the senolytics identified.

METHODS

1. CELLULAR CULTURES

1.1. CELL LINES

Throughout this work, different cellular cultures were used, although our main tool for *in vitro* experimentation was the human lung adenocarcinoma cell line A549. To test the potential broad-spectrum senolytic effect of Digoxin (Kern Pharma & Selleckchem), we used different human cancer cell lines: non-small cell lung cancer cell lines H1299 and H1755, spontaneously transformed keratinocytes HaCaT cells, astrocytoma U373 cell line, and breast cancer cell line MCF-7.

Similarly, we used normal human cell lines: normal foreskin fibroblast BJ cells, normal primary human umbilical vein endothelial cells (HUVEC), inmortalized retinal pigmented epithelial ARPE-19 cells, and human chondrocyte T/C-28 cell line.

All cell lines were maintained in DMEM high glucose (4500 mg/L) (Sigma) supplemented with 10% FBS (Corning), 1% penicillin/streptomycin solution (Sigma), and 1% glutamine (Sigma), except for HUVEC, which were provided with a special endothelial culture medium.

In addition, cells were subjected to periodic mycoplasma testing and were used only if negative.

1.2. GENERATION OF MEFS

Apart from human cell lines, we used mouse embryonic fibroblasts (MEFs) extracted from wild-type mouse embryos from 13.5 days post coitum (dpc) pregnant mice. These pregnant mice were sacrificed by cervical dislocation, and the uterus with the embryos was removed. Embryos were maintained in cold phosphate-buffered saline (PBS, Merck) supplemented with 1% of penicillin/streptomycin solution (Merck). After the removal of the head and entrails, the bodies of the embryos were broken up with scalpels, trypsinized with 1X Trypsin-EDTA solution (Merck), and incubated for 10 minutes at 37ºC and %5 CO_2. Once the tissue was disintegrated into its cellular fraction, it was resuspended in high-glucose DMEM (4500 mg/L) (Sigma) supplemented with 10% FBS (Corning), 1% penicillin/streptomycin

solution (Merck), and 1% glutamine (Merck) and seeded into a 100 mm plate (Corning). The following day, the cells were washed with PBS to remove tissue debris and the medium was replaced. Once the cells reached confluence, they were trypsinized and split into 150 mm plates (Corning) for cellular expansion to finally freeze them in aliquots of 4 × 10^6 cells per vial.

1.3. PATIENT-DERIVED CHONDROCYTES

Chondrocytes derived from patients with or without osteoarthritis were a kind gift from María Mayán (INIBIC, A Coruña, Spain). Before starting the experimental procedures, the chondrocytes were tested for mycoplasma and were found to be negative.

These patient-derived chondrocytes were at passage 14 and were cultured in DMEM high-glucose (4500 mg/L) (Sigma) supplemented with 10% FBS (Corning), 1% penicillin/streptomycin solution (Sigma), and 1% glutamine (Sigma).

1.4. GENERATION OF QUIESCENT CELLS

To test the senolytic potential of Digoxin in non-proliferative cells, we serodeprived A549 cells to prevent their active growth.

For this, we used DMEM high-glucose (4500 mg/L) (Sigma) supplemented with 0.05% FBS (Corning), 1% penicillin/streptomycin solution (Sigma), and 1% glutamine (Sigma), for 3 days until the proliferative capacity of the cells stopped. After this period of serodeprivation for 3 days, growth arrest was analyzed by flow cytometry to measure cell cycle phases with propidium iodide.

1.5. TREATMENT FOR SENESCENCE INDUCTION

To induce cellular senescence in our cell cultures, we used Bleomycin, a chemotherapeutic agent that causes single- and double-strand breaks in DNA. The general protocol for inducing senescence with Bleomycin was based on 5 days of treatment at 20 µM. However, non-tumoral cells were treated with 10 µM of Bleomycin for 5 days since the previous protocol implied more toxicity in these primary cells.

1.6. OTHER TREATMENTS

- Bapta-AM (Invitrogen™) was used as a calcium chelator.

- Amiloride (Selleckchem) was used as the proton channel inhibitor.
- Chloroquine (MedChem) was used to inhibit autophagy by blocking the binding of autophagosomes to lysosomes by altering the acidic environment of the lysosomes.
- Vacuolin-1 (MedChem) was used to inhibit late-stage autophagy by impairing lysosomal maturation.
- Torin1 (MedChem) was used as an inhibitor of both mTORC1 and mTORC2, and as an autophagy activator.
- Bafilomycin (MedChem) was used as an inhibitor of autophagy and a lysosomal disruptor impairing autophagosome-lysosome fusion.
- NH_4Cl (Fisher Scientific) was used as a tampon solution to protect the lysosomes from basification.
- Leu-Leu-OMe (Selleckchem) was used as a lysosomal membrane permeabilization agent since induces endolysosomal stress.

2. LENTIVIRAL TRANSFECTION

2.1. BACTERIAL VECTORS

We used *E. coli* strain Stbl3 to grow lentiviral plasmids. Bacteria containing the plasmid of interest were grown in LB media (Luria-Broth) containing 1% tryptone (Becton Dickinson, BD), 0.5% NaCl (Merck), 0.5% yeast extract (BD), in distilled water. After sterilizing the media, 100 µg/mL ampicillin (Merck) was added for bacterial selection. These transformed bacteria were cultured overnight.

2.2. PLASMID ISOLATION

For the isolation of the plasmid DNA from the bacteria, we used the commercial kit Genopure Plasmid Midi (Roche) to perform midi preparations starting from 250 mL of bacterial culture. Once the DNA was isolated, it was quantified using a Nanodrop 2000c spectrophotometer (Fisher Scientific) by measuring the absorbance at 260 nm/ 280 nm.

2.3. PLASMIDS

Plasmid name	Reference
pLP1	ViraPowerTM Lentiviral Packaging Mix (Invitrogen)
pLP2	ViraPowerTM Lentiviral Packaging Mix (Invitrogen)

pLP-VSVG	ViraPowerTM Lentiviral Packaging Mix (Invitrogen)
FUGW	Addgene #14883
pLJM1-EGFP	Addgene #19319
pLJM3-RFP	Generated by cloning derived from pLJM1 to express GFP. A gift from Alejo Efeyan, CNIO, Madrid, Spain
pLOC-ATP1A1-IRES-tGFP	Generated by cloning. A gift from Sean Morrison, UTSW, Dallas, USA
pLOC-Atp1a1-IRES-tGFP	Generated by cloning. A gift from Sean Morrison, UTSW, Dallas, USA
pLOC-SLC9A1-IRES-tGFP	Generated by cloning. A gift from Sean Morrison, UTSW, Dallas, USA
pCDH-CMV-Nluc-P2A-copGFP-T2A-Puro	Addgene #73037

Table 1. List of plasmids used.

2.4. CELLULAR TRANSFECTION AND TRANSDUCTION

We used human embryonic kidney epithelial HEK293T cells for plasmid transfection by using polyethylenimine (PEI; Polysciences) to generate viral particles. To do this, we first plated 5×10^6 HEK293T cells in 100 mm plates (Corning). The transfection protocol was based on a mixture of packaging plasmids pLP1, pLP2, and pLP-VSVG with the plasmid of interest in a 1:1 ratio. On the other hand, we mixed non-supplemented media with PEI (1 mg/mL) in a 1:6 ratio. Finally, the two mixtures were combined and incubated at room temperature for 10 min. Once the cells were attached, the final mixture was added to HEK293T cell plates. After 12-16 hours, the cells were washed with PBS (Sigma) and fresh complete media was added.

For transduction, cells of interest were plated at a subconfluent level the day before starting the transduction protocol. Then, media containing viral particles from HEK293T was collected after 36 hours and it was filtered through 0.45 µm filters (Jet Biofil). The collected media was supplemented with 8 µg/mL of polybrene (Hexadimethrine bromide, Merck), and was added to the target cells. HEK293T cells were replenished with fresh complete media, and the previous process was repeated 3 times. Finally, the media from our target cells was replaced with fresh, complete media.

If needed, the target cells were subjected to antibiotic selection to obtain a homogeneous population of cells containing the plasmid of interest. For antibiotic selection, Puromycin at 1 µg/mL or Blasticidin

at 5 µg/mL was used, depending on the plasmid. Antibiotic treatment was performed every 72 hours 3 times.

3. HIGH-THROUGHPUT SCREENING

The high-throughput screening was performed by Francisco Triana-Martinez, post-doctoral researcher from our lab, in collaboration with Innopharma at CiMUS in Santiago de Compostela, Spain.

For this, we used the human lung adenocarcinoma A549 cell line lentivirally transduced to express GFP or RFP, and RFP cells were induced to senescence with Bleomycin following the above-mentioned protocols. Co-cultures of both proliferative A549-GFP and senescent A549-RFP cells were prepared in a 1:3 ratio, and 4000 cells per well were seeded into 384-well plates (Perkin-Elmer).

3.1. PRIMARY SCREEN

The first step was to perform a primary screening to detect candidates with senolytic potential. For this, we treated A549 co-cultures with Prestwick Chemical Library ® at 10 µM for 24 h, using Navitoclax (AbbVie) at 1 µM as a positive control for senolysis. To analyze cell viability, we stained the cells with Hoechst (Fisher Scientific) prepared in phenol-red free complete media at a 1:5000 ratio and incubated for 10 minutes at 37ºC and %5 CO_2 to detect cell nuclei. Stained nuclei were measured with Operetta® CLS™ High-Content Imaging System (Perking Elmer) by capturing images of 5 different regions of each well. Harmony ® High-Content Imaging and Analysis Software (Perkin Elmer) was used for picture processing. To measure and quantify viable cells, we set up the configuration of the software for detecting our cells considering the roundness and size of the nuclei.

To detect potential hits, we previously calculated the Z' score for each condition with Navitoclax as a reference for senolysis. The Z' score is the number of standard deviations from the mean value of the reference population and is calculated using the following formula (J.-H. Zhang et al., 1999):

$$Z = 1 - \frac{3\,SD\ of\ sample + 3\,SD\ of\ control}{mean\ of\ sample - mean\ of\ control}$$

After this calculation, we considered potential hits those compounds with cell viability values above the threshold established as the average of the whole compounds plus 3 standard deviations.

3.2. COMPOUND VALIDATION

The potential hits detected during the primary screen were subjected to a validation process. For this, A549 co-cultures were seeded under the same conditions as previously described and were treated with the hits identified at 1 and 10 µM in triplicate for 24 h. As a positive control of senolysis, Navitoclax at 1 µM was used. At the end of the treatment, cell viability was analyzed according to the criteria described above. Briefly, cells were stained with Hoechst, image acquisition was performed using the Operetta® CLS™ High-Content Imaging System (Perking Elmer), and analysis was performed using Harmony® High-Content Imaging and Analysis Software (Perkin Elmer).

3.3. IC50 VALIDATION

We measured the IC50, which is the half-maximal concentration of a compound that shows effectiveness, of Proscillaridin A (MedChemExpress), Digoxin (Selleckchem), and Ouabain (Selleckchem). For this, proliferative and Bleomycin-inducing senescence A549 and BJ cells were seeded into 384-well plates, and treated with decreasing concentrations (1:2 dilutions) of each compound. For Proscillaridin A treatment the starting concentration was 9 µM, for Ouabain was 45 µM, and for Digoxin was 18 µM.

Cell viability was evaluated following the previously mentioned protocol of Hoechst staining, which was performed using the Operetta® CLS™ High-Content Imaging System (Perking Elmer), and analysis was performed using Harmony® High-Content Imaging and Analysis Software (Perkin Elmer).

The IC50 calculations was performed using GraphPad Prism® software.

4. APOPTOSIS DETECTION

4.1. ANNEXIN V AND CLEAVED-CASPASE-3 DETECTION BY FLOW CYTOMETRY

Apoptosis induction was measured after Digoxin treatment using two different commercial kits: FITC Annexin V Kit (ANXVF-200T, Immunostep) and PE Active Caspase-3 Apoptosis Kit (AB_393957, BD Pharmingen™).

The Annexin-V marker takes advantage of the exposure of phosphatidylserine residues out of the membrane when the cell is dying. The interaction of Annexin-V with these residues can be measured by fluorescence microscopy or flow cytometry since it is labeled with FITC. For this, cells were trypsinized and counted to get 1 x 10^6 cells for each reaction following the manufacturer's protocol. The cells were resuspended in 1 mL binding buffer containing 5 µL probe. The binding buffer was not provided in the commercial kit, and it had to be made using: NaCl 10 mM, Hepes/NaOH 10 mM at pH 7.4, and $CaCl_2$ 2.5 mM. The probe was incubated for 15 min at room temperature in the dark. At the end of the incubation, 400 µL of binding buffer was added, and the samples were analyzed using FACScan (BD Biosciences).

On the other hand, Caspase-3 is a key protease that is activated during the early stages of apoptosis, and its measurement could be used to detect cells undergoing apoptosis. Following the manufacturer's protocol, we trypsinized the cells and counted to get 1 x 10^6 cells for each reaction. The cells were subjected to a series of centrifugations and washed with cold PBS prior to fixation with BD Cytofix/Cytoperm™ and incubating the cells on ice for 20 min. Then, the cells were washed and incubated with the antibody previously prepared in BD Perm/Wash™ buffer for 30 min at RT and protected from light. Finally, the cells were washed, resuspended in BD Perm/Wash™ buffer, and analyzed using FACScan (BD Biosciences).

4.2. CELL DEATH INHIBITION

We used different chemical inhibitors for different mechanisms of cell death: Z-VAD-FMK (Santa Cruz Biotech.) as a pan-caspase inhibitor, Ferrostatin-1 (Sigma) as an inhibitor of ferroptosis, and Necrostatin-1 (Sigma) as an inhibitor of necroptosis.

The Z-VAD-FMK inhibitor was used alone or in combination with Digoxin at 10 µM for 24 hours, and cell viability was evaluated following the previously mentioned protocol of Hoechst staining, which was performed using the Operetta® CLS™ High-Content Imaging System (Perking Elmer), and analysis was performed using Harmony® High-Content Imaging and Analysis Software (Perkin Elmer).

Ferrostatin-1 is an active radical-trapping antioxidant that traps peroxyl radicals, and thus serves as a potential inhibitor of ferroptosis. Similar to what we did with the pan-caspase inhibitor, we treated A549 senescent cells with Ferrostatin-1 at 10 µM alone or in combination with Digoxin for 24 hours, and cell viability was evaluated following the previously mentioned protocol.

The necroptosis inhibitor Necrostatin-1 was used at 20 µM alone or in combination with Digoxin for 24 hours, and cell viability was evaluated following the previously described protocol.

5. SENESCENCE-ASSOCIATED B-GALACTOSIDASE STAINING

5.1. CELL CULTURE STAINING

We performed the SABG staining as a marker of senescence. For this, attached cells were washed with PBS twice and fixed using a mixture of paraformaldehyde (15713-S, EMS) at 2 % with glutaraldehyde (Fisher Scientific) at 0.2% prepared in PBS for 15 minutes at RT. Next, the cells were washed with PBS and stained with the X-Gal staining solution made with: a buffer solution of citric acid (Fisher Scientific) with sodium phosphate dibasic (Fisher Scientific) at 40 mM and pH 6.0; $K_3Fe[CN]_6$ (Sigma) solution at 5 mM; $K_4Fe[CN]_6$

(Sigma) solution at 5 mM; NaCl solution at 150mM; $MgCl_2$ solution at 2mM; and a solution of 5-bromo-4-chloro-3- indolyl β-D-galactoside (Fisher Scientific) prepared in N, N-dimethylformamide (Sigma) at 20 mg/mL. The staining solution was incubated for 16 hours at 37ºC without CO_2, then it was removed and cells were washed to be photographed using the AxioVert.A1 Microscope (Zeiss).

5.2. TISSUE STAINING

Tumor tissues and tumor-derived explants were whole-mount stained for SABG determination. For this, fresh tissue was washed with PBS twice and fixed as previously described using a mixture of paraformaldehyde (15713-S, EMS) at 2 % with glutaraldehyde (Fisher Scientific) at 0.2% prepared in PBS for 20 minutes at RT in a wheel. After fixation, the tissues were washed three times with PBS for 5 min in a wheel. Then, tissues were submerged in staining solution following the previously described protocol and were incubated for 16 hours at 37 ºC without CO_2 in a wheel.

These tissues were then dehydrated in 50º ethanol for 30 minutes at RT in a wheel. These tissues were maintained in 70º ethanol at 4 °C until they were embedded in paraffin for histological processing.

6. RNA EXPRESSION ANALYSIS

6.1. RNA PURIFICATION

To determine gene expression at the mRNA level, we first purified RNA from our cell cultures using the commercial Kit NucleoSpin® RNA (Macherey-Nagel) following the manufacturer's protocol.

Once the RNA was purified from the cells, it was quantified with the Nanodrop 2000 (Fisher Scientific) taking as reference the 260/280 nm ratio of absorbance.

6.2. REVERSE TRANSCRIPTION OF RNA

Purified and quantified RNA was subjected to reverse transcription to obtain cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer’s protocol.

Reverse transcription was performed in a MultiGeneTM OptiMax (Labnet) thermal cycler.

6.3. qRT-PCR

For quantitative RT-PCR, the NZYSpeedy qPCR Green Master Mix (2X) and ROX (NZYTech) reagent were used, and the reaction took place in the AriaMx Real-Time PCR system (Agilent Technologies) thermal cycler following the protocol shown below (**Figure 6**):

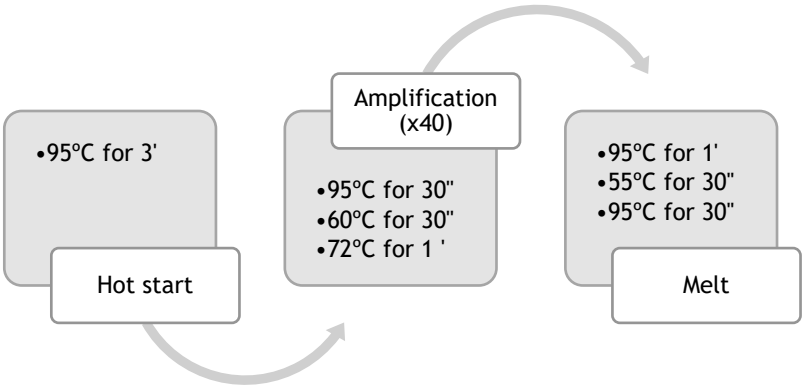

Figure 6. Thermal cycler program for qRT-PCR.

Each reaction was composed of 33 ng of cDNA, a mix of oligonucleotides at 0.25 µM, 5 µL of SYBR™ Green (Applied Biosystems), and nuclease-free water up to a total volume of 10 µL.

Oligonucleotides	Sequence 5'-3'
GAPDH	F: TCCATGACAACTTTGGCATCGTGG-3' R: GTTGCTGTTGAAGTCACAGGAGAC-3'
ATP1A1	F: ACAGACTTGAGCCGGGGATTA R: TCCATTCAGGAGTAGTGGGAG
Atp1a1	F: ACATTCCGGAAATCACCCCC R: CCCGTACACGGTGTTTCTCA
GFP	F: AGTCCGCCCTGAGCAAAGA R: TCACGAACTCCAGCAGGACC
SLC9A1	F: ACCACGAGAACGCTCGATTG R: ACGTGTGTGTAGTCGATGCC
BENC1	F: CCATGCAGGTGAGCTTCGT R: GAATCTGCGAGAGACACCATC
UVRAG	F: CTTGGGTCAGCAGATTCATGC R: CATCGTAAGAATTGCGAACACAG
LC3	F: GAGTGGAAGATGTCCGGCTC R: CCAGGAGGAAGAAGGCTTGG
SQSTM1	F: AAGCCGGGTGGGAATGTTG R: CCTGAACAGTTATCCGACTCCAT
LAMP1	F: CACGAGAAATGCAACACGTTAC R: GGGTGCCACTAACACATCTGTAT
MCOLN1	F: TTCGCCGTCGTCTCAAATACT R: CTCTTCCCGGAATGTCACAGC

Table 2. Oligonucleotides used.

The oligonucleotides (Eurofins Genomics) used in this thesis are listed in the previous table (**Table 2**).

The qRT-PCR results were analyzed using AriaMX software (version 1.0; Agilent Technologies). GAPDH was used as a housekeeping gene, and the expression of different genes was normalized to the expression values obtained for GAPDH. Each reaction was performed in triplicate.

7. PROTEIN EXPRESSION ANALYSIS

7.1. PROTEIN PURIFICATION

7.1.1. Total protein

For the purification of protein extracts from cell cultures, culture media was removed and cells were washed twice with PBS (Merck) while plates were maintained on ice. Cells were scraped and collected

in tubes for their centrifugation at 3000 rpm for 5 minutes and at 4 ºC by using the microcentrifuge Eppendorf 5415R (Eppendorf).

After centrifugation, the supernatant was removed and the pellet was resuspended in RIPA buffer 1X (Radio-Immunoprecipitation Assay Buffer) made with: 150 mM of NaCl, 10 mM of Tris-HCl solution at pH 7.5, 0.1% of SDS, 1% of Triton X-100, 5 mM of EDTA solution at pH 8.0, and 1% of sodium deoxycholate solution with protease inhibitors (1 mM of Sodium Ortovanadate, 1 mM of PMSF, 1 mM of DTT, 4 mM of NaF, and 1X of protease inhibitor cocktail). The pellet resuspended in this mix was incubated on ice for 20 minutes, and then centrifuged at 14.000 rpm for 15 minutes at 4 ºC. The supernatant was collected into new sterile tubes for their later use.

7.1.2. Protein fractions

To measure protein expression in the cytoplasmic and nuclear fractions, we isolated different fractions of the cell by serial centrifugation.

First, the cells were scraped, as described previously, and centrifuged. After removing the supernatant, the cellular pellet was resuspended in 900 µL of cold NP40 (Merck) at 0.1% in PBS to disrupt the cell membrane, and centrifuged for 15 seconds. This supernatant was the cytoplasmic fraction and was collected into new sterile tubes. The pellet left was resuspended in 1 mL of the same cold NP40 at 0.1% in PBS, in this case for disrupting the nuclear membrane, and spin for 15 seconds. The supernatant obtained was discarded and the pellet, which contained the nuclear protein fraction, was resuspended in 180 µL of RIPA Buffer 1X (recipe described above) containing 1X SDS Buffer.

7.2. PROTEIN QUANTIFICATION

Protein extracts were quantified by Bradford's method using the DC Protein Assay Kit (Bio-Rad) following the manufacturer's instructions. The samples were placed in 96-well plates in triplicate, together with a calibration line made with BSA (Merck). The absorbance of the samples was measured at 750 nm using the plate reader spectrophotometer Epoch2 (BioTek).

7.3. WESTERN BLOT

Protein expression was analyzed by western blotting. For this, we used approximately 20 µg or 30 µg of protein extracts mixed with loading buffer (made with 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 125mM Tris-HCl at pH 6.8, in distilled water at a concentration of 1X). Protein samples with loading buffer were heated at 95 ºC for 5 minutes in the Thermomixer Compact (Eppendorf).

The protein samples were loaded into commercial polyacrylamide gels Bolt™ 4 a 12 %, Bis-Tris Protein Gel (Invitrogen) for electrophoresis separation of the proteins based on their size following the Laemmli method (Laemmli, 1970). For this, we used the XCell SureLock™ (Invitrogen) system for vertical electrophoresis. We also used the pre-stained protein ladder PageRuler Plus (Thermo Scientific).

After electrophoresis, the proteins were transferred to a 0.45 µm PVDF membrane (Millipore) by wet blotting using the same XCell SureLock™ (Invitrogen) system. The membrane containing the proteins was blocked with non-fat milk prepared in TTBS (made with 20 mM Tris-HCl at pH 7.5, 150mM NaCl, and 0.05% Tween-20 in distilled water) at 5% w/v for 1 hour at room temperature. The incubation with the following primary antibodies (**Table 3)** was performed at 4 °C overnight in a shaker. Next, the incubation with the following secondary antibodies (**Table 4**) was at RT for 1 hour in a shaker. These primary antibodies were conjugated to horseradish peroxidase (HRP). Finally, the immunodetection was performed by quimioluminescence using the commercial ECL (enhanced chemioluminiscence) SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and the detection was performed using the ChemiDoc™ MP Imaging System (BIO-RAD).

Antibody	Brand	Reference	Host	Dilution
p-AKT	Cell Signaling	#9271S	Rabbit	1:1000
AKT	Cell Signaling	#4685S	Rabbit	1:1000
p-ERK	Cell Signaling	#4370	Rabbit	1:2000
ERK	Cell Signaling	#4695	Rabbit	1:2000
p-p65	Cell Signaling	#3033	Rabbit	1:500
p65	Cell Signaling	#8242	Rabbit	1:1000
p53	Santa Cruz	sc-126	Mouse	1:1000
p21	Santa Cruz	sc-6246	Mouse	1:1000
H3ac	Thermo Fisher	06-599	Rabbit	1:500
TFEB	Cell Signaling	#4240	Rabbit	1:1000
BENC1	Cell Signaling	#3738	Rabbit	1:1000
LC3B	Cell Signaling	#2775	Rabbit	1:1000
LAMP1	Cell Signaling	#9091	Rabbit	1:1000
p62	Cell Signaling	#5114S	Rabbit	1:1000
GAPDH	Santa Cruz	sc-32233	Mouse	1:2000
ACTIN	Santa Cruz	sc-8432	Mouse	1:1000

Table 3. Primary antibodies used

Antibody	Brand	Reference	Dilution
HRP-Anti-Rabbit	Santa Cruz	sc-2004	1:5000
HRP-Anti-Mouse	Santa Cruz	sc-2005	1:2000

Table 4. Secondary antibodies used

8. IMMUNOFLUORESCENCE

We performed an immunofluorescence assay to determine the cellular localization of TFEB. For this, cells were seeded in 4-well glass chamber slides (Lab-Tek® II) and treated or not with Digoxin for 8 hours.

At the end of the treatment, the medium was removed and cells were washed twice with PBS (Merck) for their following fixation with paraformaldehyde (Electron Microscopy Science) prepared at 2% in PBS for 20 minutes. The cells were then washed with PBS 3 times for 5 min each. Permeabilization was performed using 0.25% Triton X-100 (Merck) in PBS for 30 min. We repeated the wash process as previously described for the subsequent blockade with BSA (Merck) at 2% in PBS for 20 minutes at room temperature. Next, the cells were incubated with the primary antibody (TFEB, #4240, Cell Signaling, 1:200) for 1 h at room temperature, followed by washing with PBS. The cells were

incubated with the secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody; Thermo Fisher Scientific; 1:500) for 45 min at room temperature, followed by washing with PBS. Finally, the nuclei were stained with DAPI (1 µg/mL) for 10 min and washed with PBS. ProLong Diamond Antifade Mountant (Invitrogen) was used to mount the slide, let it dry, and kept at 4 °C until it was photographed using a Leica SP8 confocal microscope.

9. ION DETERMINATION

9.1. SODIUM

For sodium ion determination, we used the fluorescent probe CoroNa™ Green, AM (Invitrogen™). Fluorescence emission was measured using confocal microscopy and flow cytometry.

For confocal microscopy, cells were seeded in 4-well glass chamber slides (Lab-Tek® II) and treated or not with Digoxin overnight (approximately 16 hours). The cells were then treated with 10 µM CoroNa ™ Green, AM following the manufacturer's protocol. Images were captured using the Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor, Oxford Instruments). Since image acquisition was performed on living cells, an OKO-lab incubator was used to keep cells at 37 °C during all the experiment. Images were taken with different magnification objectives (20x, 60x, 100x). All the images were processed with ImageJ®.

For flow cytometry analysis, cells were treated in the same conditions as previously in p12-well plates and incubated with CoroNa™ Green, AM at 10 µM following the manufacturer's protocol. Then, cells were trypsinized and fluorescence emission was measured in 96-well plates with the Guava® easyCyte flow cytometer (Millipore®).

This determination was performed by Enrica Soprano at CIQUS, in Santiago de Compostela, Spain.

9.2. POTASSIUM

For potassium ion determination, we used the fluorescent probe PBFI, AM (Invitrogen™). Fluorescence emission was measured using confocal microscopy and flow cytometry.

For confocal microscopy, cells were seeded in 4-well glass chamber slides (Lab-Tek® II) and treated or not with Digoxin overnight (approximately 16 hours). Then cells were treated with PBFI, AM at 3 μM following the manufacture's protocol. Images were captured using the Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor, Oxford Instruments). Since image acquisition was performed on living cells, an OKO-lab incubator was used to keep cells at 37 °C during all the experiment. Images were taken with different magnification objectives (20x, 60x, 100x). All the images were processed with ImageJ®.

For flow cytometry analysis, cells were treated in the same conditions as previously in p12-well plates and incubated with PBFI, AM at 3 μM following the manufacturer's protocol. Then, cells were trypsinized and fluorescence emission was measured in 96-well plates with the Guava® easyCyte flow cytometer (Millipore®).

This determination was performed by Enrica Soprano at CIQUS, in Santiago de Compostela, Spain.

9.3. CALCIUM

For calcium ion determination, we used the fluorescent probe FLIPR® Calcium 6 Assay Explorer Kit (Molecular Devices), following the manufacturer's protocol, in cells treated or not with Digoxin for 6 hours. Fluorescence emission was measured and quantified using the Hamamatsu FDSS® 7000EX (Hamamatsu Photonics).

This determination was performed by Francisco Triana-Martinez at Innopharma's facilities (CiMUS) in Santiago de Compostela, Spain.

9.4. PROTONS

For proton ion determination, we used the fluorescent probe pHrodo™ Red and Green AM (Invitrogen™), following the

manufacturer's protocol, in cells treated or not with Digoxin for 6 hours. Fluorescence emission was measured using the Operetta® CLS™ High-Content Imaging System (Perking Elmer) and image processing was performed with the Harmony® High-Content Imaging and Analysis Software (Perkin Elmer)

This determination was performed by Francisco Triana-Martinez at Innopharma's facilities (CiMUS) in Santiago de Compostela, Spain.

10. MEMBRANE POTENTIAL

For membrane potential determination, we used the fluorescent probe DiBAC4(3) (Invitrogen™), following the manufacturer's protocol, in cells treated or not with Digoxin, KCl, or the combination, for 6 hours. Fluorescence emission was measured and quantified using the Hamamatsu FDSS® 7000EX (Hamamatsu Photonics).

This determination was performed by Francisco Triana-Martinez at Innopharma's facilities (CiMUS) in Santiago de Compostela, Spain.

11. LYSOSOMAL DETERMINATION

11.1. ACRIDINE ORANGE

For lysosomal viability determination, we used Acridine Orange (AO), a fluorescent dye that changes the spectrum of fluorescence emission depending on pH. If the vacuole has acidic pH, the fluorescence will be red, and it will turn green as the pH becomes neutral. Fluorescence emission was measured using confocal microscopy and flow cytometry.

For confocal microscopy, the cells were seeded in 4-well glass chamber slides (Lab-Tek® II). Once attached, the cells were treated with vehicle, Digoxin, Bafilomycin A1, or Chloroquine for 5 hours. AO was added to each condition at a concentration of 1 µg/mL 1 hour before the end of treatment. Then, the medium was removed, cells were washed with PBS, and maintained with PBS supplemented with 2%

FBS for image acquisition. Images were captured using the Leica SP8 confocal microscope. The samples were excited with a wavelength of 488 nm and the emission was captured at 500-550 nm and 660-750 nm.

For flow cytometry analysis, cells were treated in the same conditions as previously and incubated with AO at 1 µg/mL at 37ºC and %5 CO_2. Then, cells were trypsinized and fluorescence emission was measured in 96-well plates with the Guava® easyCyte flow cytometer (Millipore®). This determination was performed by André Pérez Poti at CIQUS, in Santiago de Compostela, Spain.

12. MITOCHONDRIAL DETERMINATION

12.1. MITOTRACKER AND TMRM

We used Mitotracker and TMRM for mitochondrial viability determination. MitoTracker Green FM (Invitrogen™), that fluorescently marks all mitochondria, while Image-iT™ TMRM (Invitrogen™) fluorescently marks mitochondrial with intact membrane potential. For this, cells were seeded into p12-well plates and treated or not with Digoxin for 8 hours. Then, a mixture of Mitotracker and TMRM was used following the manufacturer's protocol. Pictures were taken with the AxioVert.A1 Microscope (Zeiss).

For flow cytometry analysis, cells were treated in the same conditions as previously and incubated with the mixture of Mitotracker and TMRM following the manufacturer's protocol. Samples were analyzed using FACScan (BD Biosciences) in triplicate.

13. ANIMAL MODELS AND PROCEDURES

All experimental procedures in which animals have been used have the approval of the Bioethics Committee of the University of Santiago de Compostela (USC) respecting the Laws for the Protection of Animals used for experimentation and other scientific purposes at the state and European levels. The procedures were carried out under authorization 15010/17/001, for which Manuel Collado Rodríguez is

the responsible investigator according to the current and valid RD 53/2013. The experimental procedures were carried out by Manuel Collado, Francisco Triana-Martinez, Sabela Da Silva-Álvarez, and myself, all trained for functions B and C.

13.1. STABULATION

The experimental animals used were kept stabled in SPF (Specific Pathogen Free) rooms at CEBEGA (Center for Experimental Biomedicine, USC) with registration number: ES150780292901, under controlled photoperiod conditions (12 hours of light/12 hours of darkness). and temperature (20-24 °C).

13.2. NMRI-FOXN1$^{NU/NU}$ MICE

We used athymic nude mice for tumor formation assays. These mice are characterized by an autosomal recessive mutation on chromosome 11 in the *Foxn1* locus that causes aplasia in the thymus and immunodeficiency due to the lack of T cells. These mice are also characterized by keratinization defects in the hair follicles and epidermis, which causes the absence of hair (nude).

For our experiments, we used 8-week-old females supplied by Janvier Laboratories.

13.3. SUBCUTANEOUS INJECTION OF CANCER CELLS

For the validation of the senolytic potential of Digoxin *in vivo*, we used A549 cells lentivirally transduced to express luciferase (A549-Luc). From these A549-Luc, 1 x 10^6 cells were subcutaneously injected into the flanks of nude mice in a 200 µL volume of complete DMEN (Merck) using 25G needles. Mice were anesthetized using an isoflurane inhalation anesthesia machine (Esteve).

For the experiment of tumor-derived explants, we subcutaneously injected 1 x 10^6 SK-Mel-103 cells into the flanks of nude mice in a mixture of 80:20 DMEM:Matrigel (Corning™) using 25G needles. Mice were anesthetized using an isoflurane inhalation anesthesia machine (Esteve).

13.4. DRUG TREATMENTS

Drug treatments started when tumors were detectable.

For the A549-Luc experiment, treatments with Gemcitabine (25 mg/Kg; Hospira UK), Digoxin (2 mg/Kg; Kern Pharma), and PBS (Merck) were administered intraperitoneally twice weekly for 2 weeks.

For the tumor-derived explants experiment with SK-Mel-103, treatment with Palbociclib (100 mg/Kg; Selleckchem) was performed via oral gavage daily for 1 week. For this, Palbociclib was prepared in 50 mM sodium lactate (DL-Lactate solution 60% w/w syrup; Merck) at a final concentration of 12.5 mg/mL.

13.5. TUMOR VOLUME DETERMINATION

Tumor progression was evaluated twice a week throughout the treatments by direct measurement with a caliper and by bioluminescence signal (in the case of A549-Luc). Tumor volume determination with caliper was calculated using the formula $V = (W^2 \times L)/2$.

The bioluminescence signal determination was performed with the IVIS Spectrum In Vivo Imaging System (PerkinElmer) after the injection of 15 mg/mL of D-Luciferin (PerkinElmer). Exposure time ranged from 30 seconds to 3 minutes to ensure optimal setting of fluorescence detection with Living Image Software V4.5.2 (PerkinElmer). For the quantification of the signal, the same Living Image Software V4.5.2 (PerkinElmer) was used.

13.6. TUMOR EXPLANTS ISOLATION

From the SK-Mel-103 *in vivo* experiment, tumors were resected at the end of the procedure after the mice were euthanized by CO_2 inhalation. To derive tumor explants, tumors from mice treated or not with Palbociclib were cut into ~2 mm pieces to obtain several replicates of the same tumor. These explants were cultured in 96-well plates with DMEM (Sigma) and treated with vehicle (DMSO; Merck), 1 µM Navitoclax (ABT-263; Selleckchem), or 0.1 µM Digoxin (Selleckchem) for 24 hours. At the end of the treatments, explants were processed for immunohistochemical analysis.

14. HISTOLOGY

14.1. PARAFFIN EMBEDDING

Whole-mount stained tissues with SABG were fixed at 4°C overnight in ethanol. Then, stained tissued were dehydrated for the embedding in paraffin.

For the dehydration process, the tissues were processed in a series of different alcohols: 96° ethanol for 1h, 96° ethanol for 45 minutes, 100° ethanol for 45 minutes, 100° ethanol for 45 minutes, isopropanol for 45 minutes, 65°C isopropanol for 45 minutes, 1:1 mixture of isopropanol and 65° paraffin C for 8h and paraffin 65°C for 2h. All the steps were carried out in agitation and those that required 65°C were carried out in a thermostatic bath with agitation.

14.2. IMMUNOHISTOCHEMICAL STAINING

The immunohistochemical staining was performed at the Pathological Anatomy Service of the Hospital Clínico Universitario of Santiago de Compostela, and the Pathological Anatomy Service of the National Cancer Research Center (CNIO, Madrid).

First, nuclei from tissues were stained with nuclear fast red. Antibodies against p21 and Ki67 were used as markers of senescence and proliferative growth arrest. Anti-cleaved-caspase-3 antibody was used as a marker of apoptosis. Finally, antibodies against two cytokeratin (CK) clones AE1 and AE3 were used as a marker for epithelial cancer cells.

14.3. IMAGE PROCESSING

After the histological processing of the tissues, slides were scanned and images were analyzed with Zen Blue Edition software (Zeiss) for the quantification of positive cells for each marker.

15. STATISTICAL ANALYSIS

Data analysis was performed from triplicates. The data in the graphs was represented as mean +/- standard deviation. The statistical significance of the data obtained was analyzed using the two-tailed

Student's t-test: *** p <0.001; ** p < 0.01; *p < 0.05; n.s. not significant. This test, which checks whether the means of two normally distributed populations are equal, is the appropriate one to compare in our case whether two samples are significantly different in terms of a single variable.

RESULTS

1. THE IDENTIFICATION OF NEW SENOLYTICS[1]

Apart from the positive role of cellular senescence as a tumor suppressor mechanism, the accumulation of senescent cells has been shown to promote different age-related diseases and, paradoxically, cancer.

Different cancer treatments have been described as senescence inducers, causing a proliferative arrest of the cancer cells and preventing tumor progression. However, the inappropriate elimination or the over-production of these senescent tumor cells leading to their accumulation has been shown to contribute to relapses, more aggressive tumors, or tumors that are resistant to therapies (Demaria et al., 2017).

To overcome the detrimental effects of using therapy-induced senescence while keeping the advantage of its ability to prevent tumor progression, senolytics have been considered as potential new anticancer drugs. Senolytics were developed to take advantage of the vulnerabilities of senescent cells to promote their specific elimination without affecting healthy tissue. Different approaches were taken to eliminate senescent cells, from genetically modified mouse models, massive drug discovery, and repurposing, to the most recent PROTACs.

Drug discovery platforms allow us to screen libraries composed of thousands of small molecules, derived by synthetic chemistry or from natural extracts, with senolytic potential. Also, some of these libraries are composed of drugs already in use in the clinic, potentially allowing us to repurpose them for clinical trials quickly and efficiently. For this reason, we decided to perform a high-throughput screening to find new senolytics.

[1] *The results from this chapter have already been published as Triana-Martínez F., Picallos-Rabina P., et al. Identification and characterization of Cardiac Glycosides as senolytic compounds. Nat Commun 10, 4731 (2019). https://doi.org/10.1038/s41467-019-12888-x

1.1. HIGH-THROUGHPUT SCREENING

As a first step to performing our screening, we set up our experimental model to screen the compounds. We developed a cell system based on the use of human lung adenocarcinoma A549 cells modified to express GFP or RFP. To do so, we lentivirally transduced these A549 cells with two different vectors: pLJM1-EGFP and pLJM3-RFP. From both cell lines, we derived single-cell clones to obtain a 100% pure culture of A549-GFP or A549-RFP cells. We induced senescence in the A549-RFP cells by treating them with Bleomycin, an antineoplastic drug that causes single and double-strand DNA breaks leading to senescence (Aoshiba et al., 2003), at 20 µM for 5 days.

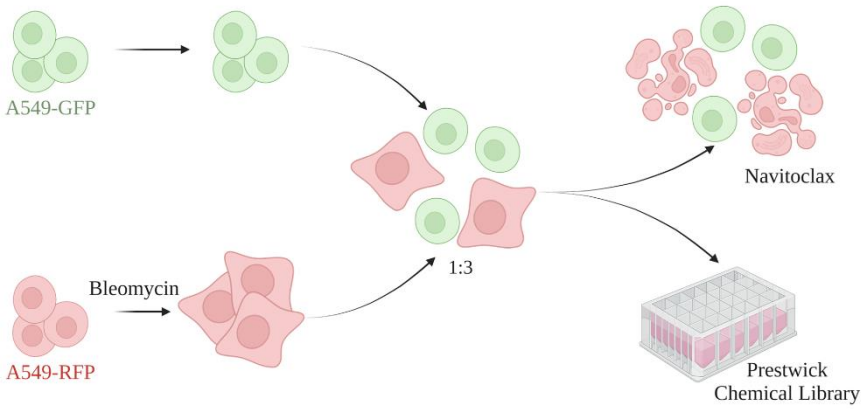

Figure 7. High-Throughput Screening scheme. Schematic representation of the high-throughput screening process. Own authorship created with BioRender.com

For the screening, we made co-cultures of A549-GFP proliferating cells with A549-RFP senescent cells in a 1:3 ratio in 384-well plates. We screened the Prestwick chemical library®, composed of 1280 small molecules approved by different drug agencies (FDA, EMA, and JAN), at 10 µM for 24 hours. For the cell viability determination, we used the Operetta® CLS™ High-Content Imaging System, a high-throughput microplate imager that can obtain and manage fluorescence and brightfield images from each well of the plate. As a positive control of senolysis, we used the well-characterized senolytic Navitoclax (ABT-

263, AbbVie) at 1 µM for 24 hours (**Figure 7**). Our positive control was used to calculate the Z' score of each compound tested to measure the percentage of cell viability, and to identify the potential hits. We established the threshold as the average for the whole population plus 3 standard deviations, and those compounds that fell above our threshold were considered potential hits. We found 9 compounds that fulfilled these criteria, showing a potential high senolytic activity. These 9 compounds were subjected to a validation process, in this case testing them at 10 and 1 µM and comparing their senolytic potential with Navitoclax. From this validation, we found Proscillaridin A as the most promising drug since it is a previously approved drug for use in patients.

1.2. VALIDATION OF CARDIAC GLYCOSIDES AS POTENTIAL SENOLYTICS IN DIFFERENT IN VITRO MODELS

1.2.1. Analysis of the senolytic potential of CGs in different cell lines

Proscillaridin A is a drug belonging to the Cardiac Glycosides (hereinafter referred to as CGs) family of compounds. CGs are cardiotonic steroids derived from the foxglove plant and were described in 1785 by William Withering for the treatment of different cardiac diseases (Withering, 1785). Since their discovery, CGs have continued to be used in the treatment of heart failure, arrhythmias, and atrial fibrillation (Ziff & Kotecha, 2016). Over the years, however, CGs were further characterized as potential drugs for the treatment of other diseases different from cardiac diseases. Several research described the potential therapeutical effect of CGs in models of cystic fibrosis, ischemic stroke, and different types of cancer, among others (Prassas & Diamandis, 2008).

To investigate if the senolytic potential of Proscillaridin A was common to other CGs or it was an exclusive property of this compound, we tested the senolytic potential of three CGs: Proscillaridin A, Ouabain, and Digoxin. We tested a range of concentrations of the three compounds in A549 tumor cells and BJ primary cells non-treated (proliferating) or after the induction of senescence with Bleomycin. Using Operetta®, we analyzed cell viability in our models by counting cells stained with Hoechst and obtained the IC50 values of the different

conditions. We observed that in both, tumor A549 cells and primary BJ cells, the three compounds showed lower IC50 values in the senescent condition, that is, they showed a greater specificity to kill senescent cells than proliferating ones (**Figure 8**).

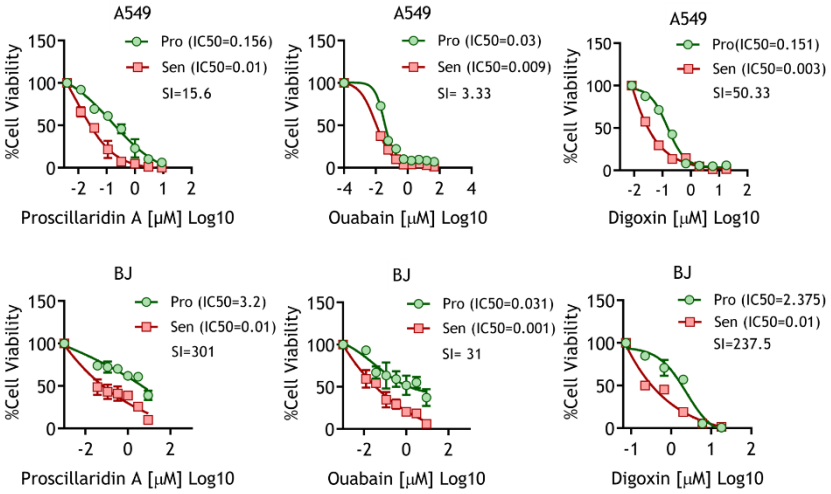

Figure 8. IC50 Validation of CGs in A549 and BJs. IC50 curves of A549 cells (upper part) and BJ primary cells (bottom part) treated with increased concentrations of Proscillaridine A, Ouabain, or Digoxin. Own authorship image.

Since Proscillaridin A is no longer used in the clinic, and neither is Ouabain, we decided to exploit and characterize Digoxin, widely used in the clinic, as a drug with senolytic potential.

To further evaluate the senolytic potential of Digoxin, we treated different human cancer cell lines (H1299: non-small cell lung cancer cells; H1755: non-small cell lung cancer; HaCat: spontaneously transformed keratinocytes; U373: astrocytoma; MCF-7: ER+ PR+ breast cancer) and human primary cells (HUVEC: normal primary human umbilical vein endothelial cells; ARPE-19: retinal pigment epithelial cells; T/C-28: human chondrocytes) with increasing concentrations of Digoxin after the induction of senescence or not. Again, we analyzed cell viability by measuring Hoechst fluorescence with Operetta® and confirmed that Digoxin has a senolytic effect in the

tested cell lines, regardless of their transformation status or tissue of origin (**Figure 9**).

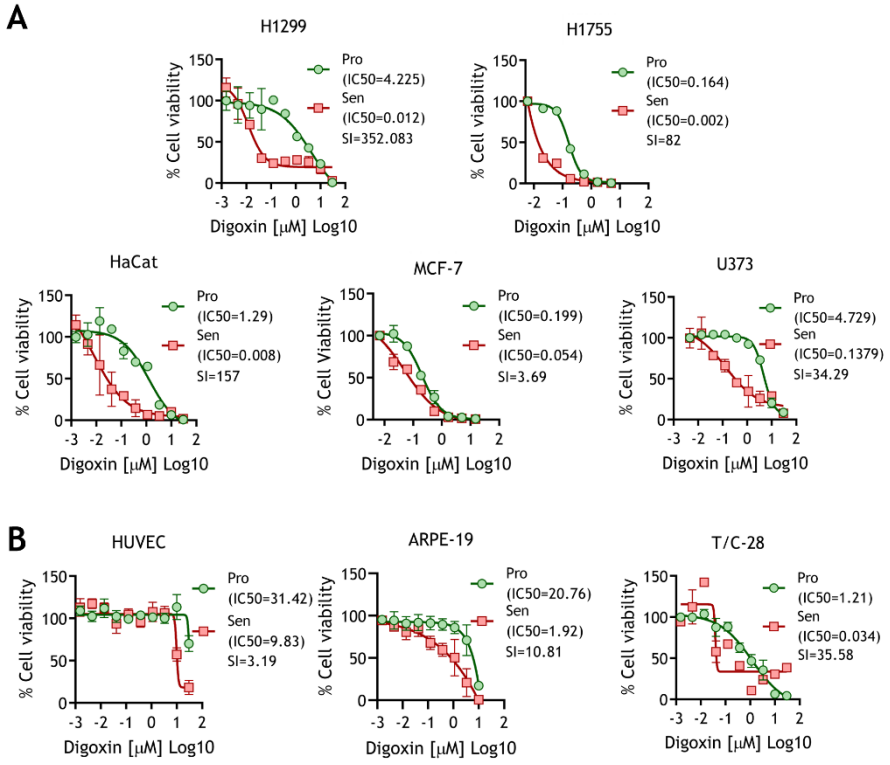

Figure 9. IC50 Validation in different cell lines. (A) IC50 curves of different cancer cell lines treated with increasing concentrations of Digoxin. (B) IC50 curves of different primary cells treated with increasing concentrations of Digoxin. Own authorship image.

1.2.2. Differential effect of Digoxin in senescent versus quiescent cells

We wanted to confirm this specificity in killing senescent cells by comparing them with non-proliferating cells. To do so, we cultured A549 tumor cells with low doses of FBS (0.05% instead of 10%) to induce in these cells a quiescent state. We analyzed the cell cycle of both A549-quiescent and A549-senescent cells to confirm that serum deprivation induces a similar, but reversible, cell cycle arrest. For this,

we stained the DNA of both quiescent and senescent cells using propidium iodide and measured the DNA content by flow cytometry. We distinguished between the G1, S, or G2/M phases, observing an accumulation of quiescent cells in the G1 phase (**Figure 10A**).

Then, we evaluated the senolytic potential of increasing concentrations of Digoxin in both conditions and assessed the IC50 value. The viability of the cells was measured by Hoechst quantification by using Operetta® and, again, we observed that Digoxin killed preferentially senescent cells above quiescent cells (**Figure 10B**).

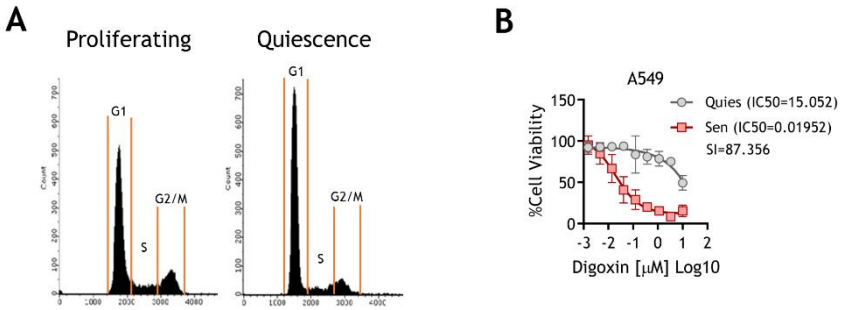

Figure 10. Effect of Digoxin in quiescent cells. (A) Flow-cytometry measurement of the cell cycle in both proliferative and quiescent A549 cells. (B) IC50 curve of quiescent vs senescent A549 cells treated with increasing concentrations of Digoxin. Own authorship image.

1.2.3. Effect of Digoxin in an in vitro model of osteoarthritis

Apart from the assays performed in established cell lines, we wanted to evaluate the senolytic potential of Digoxin in a cell system that more closely resembles a physiological context in which cellular senescence takes place. For this, we used chondrocytes derived from healthy individuals or patients with knee osteoarthritis (OA hereinafter referred to as).

First, we performed SABG staining to determine cellular senescence in our patient-derived cultures. We observed that, although our healthy chondrocytes have proliferated, some of these cells were positive for SABG while our OA chondrocytes have not proliferated and showed an enlarged morphology and positive staining (**Figure 11A**).

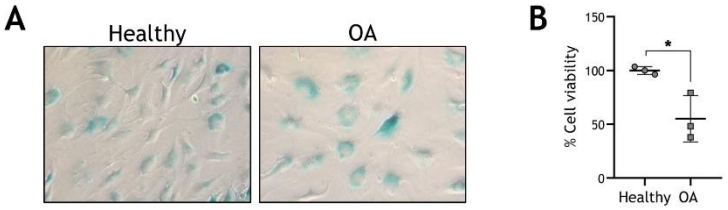

Figure 11. Evaluation of Digoxin effect over patient-derived chondrocytes. (A) SABG staining pictures of healthy (left panel) and osteoarthritic chondrocytes (right pannel). (B) Cell viability graph of healthy vs OA chondrocytes treated 24 hours with Digoxin. OA:osteoarthritis. Statistical significance assessed by the two-tailed Student's t-test: *p < 0.005. Own authorship image.

To analyze the potential senolytic effect of Digoxin on these patient-derive chondrocytes, we treated both healthy and osteoarthritic chondrocytes with Digoxin at 0.1 µM for 24 hours. At the end of the treatment, we tested cell viability by manual cell counting and observed a clear reduction in the viability of OA chondrocytes after Digoxin treatment compared to healthy ones **(Figure 11B**).

1.2.4. Cardiac Glycosides showed no effect on MEFs

It is well known that the main target of CGs is the Na^+/K^+-ATPase (hereinafter referred to as NKA) located at the surface of the cellular membrane. CGs, by binding the NKA, block the exchange of sodium and potassium leading to the accumulation of sodium within cells. This high concentration of intracellular sodium in turn leads to an accumulation of calcium in myocardial fibers increasing heart contraction. This effect provides the basis of its therapeutic benefit but at the same time represents the main cause of its toxicity.

To check if Digoxin has senolytic activity in senescent mouse embryo fibroblasts (MEFs), we first induced senescence in MEFs with Bleomycin at 20 µM for 5 days. Once these cells became senescent, we calculated the IC50 in both proliferative and senescent MEFs treated with increasing concentrations of Proscillaridine A or Digoxin for 24 hours. Cell viability was measured using Hoechst quantification by Operetta® (**Figure 12**).

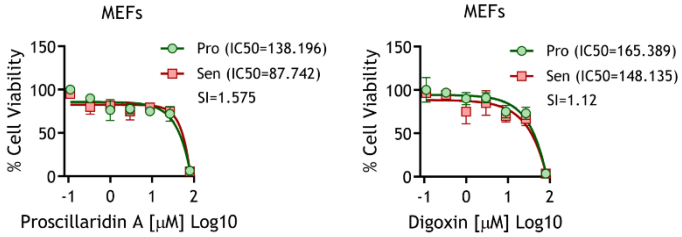

Figure 12. Senolytic validation of CGs in MEFs. IC50 curves of both Proscillaridine A and Digoxin treated MEFs. Own authorship image.

2. CHARACTERIZATION OF THE CELL DEATH MECHANISM TRIGGERED BY DIGOXIN[2]

2.1. ANALYSIS OF THE APOPTOTIC PATHWAY TRIGGERED BY DIGOXIN

Once confirmed that Digoxin killed specifically senescent cells, we wanted to evaluate the mechanism by which senescent cells are dying after Digoxin treatment.

First, we analyzed the activation of apoptosis since this is the most common pathway mediating cell death. For this, we measured two different apoptotic markers by flow cytometry in both A549 and BJ cells: Annexin-V and cleaved-caspase-3. The Annexin-V marker takes advantage of the exposure of phosphatidylserine residues out of the membrane when the cell is dying. The interaction of Annexin-V with these residues can be measured by fluorescence microscopy or flow cytometry as it is labeled with FITC. To do so, we treated proliferative and senescent A549 and BJ cells with Digoxin at 0.1 µM for 24 hours. At the end of the treatment, cells were collected and stained with

[2] The results from this chapter have already been published as Triana-Martínez F., Picallos-Rabina P., et al. Identification and characterization of Cardiac Glycosides as senolytic compounds. Nat Commun 10, 4731 (2019). https://doi.org/10.1038/s41467-019-12888-x

Annexin-V fluorescent probe, to be measured later by flow cytometry. We observed an increase in Annexin-V-positive cells after Digoxin treatment suggesting that the drug was triggering apoptosis (¡Error! No se encuentra el origen de la referencia.**A**).

In a complementary way, we measured cleaved-caspase-3, another apoptotic marker. During apoptosis, an enzymatic pathway is activated starting at the mitochondria. The mitochondrial outer membrane permeabilization (MOMP) caused in response to damage leads to the release of cytochrome c, the formation of the apoptosome, and the activation of a signaling cascade of caspases. The final effector of this cascade is caspase 3 which is cleaved to its active form by other caspases. We performed a similar protocol as we did to measure Annexin-V using this time a cleaved-caspase-3 fluorescent kit. Similar to Annexin-V, we observed an increase in the levels of cleaved-caspase-3 by flow cytometry after Digoxin treatment in both A549 and BJ-senescent cells (¡Error! No se encuentra el origen de la referencia.**B**).

To further validate the activation of the apoptotic pathway by Digoxin, we treated our A549 and BJ cells with a pan-caspase inhibitor (Z-VAD-FMK) with or without Digoxin to test if this treatment prevented cell death. Cell viability was measured by Hoechst quantification with Operetta®. The combination of Z-VAD-FMK with Digoxin showed a protective effect in senescent A549 and BJ cells from the senolytic effect of Digoxin (¡Error! No se encuentra el origen de la referencia.**C**). With all this evidence, we confirmed that the senolytic effect of Digoxin is mediated by the activation of the apoptotic signaling pathway.

2.2. OTHER CELL DEATH MECHANISMS THAT COULD BE IMPLICATED IN THE EFFECT OF DIGOXIN

Although we have observed that Digoxin triggers an apoptotic response in senescent cells, we wondered if the process of ferroptosis or necroptosis could be also mediating the senolytic effect of Digoxin.

We decided to evaluate the putative involvement of these two mechanisms of cell death on the senolytic potential of Digoxin. To do

so, we used specific inhibitors for each pathway: Ferrostatin-1 as an inhibitor of ferroptosis, and Necrostatin-1 as an inhibitor of the necroptosis pathway; in combination with Digoxin in A549-senescent cells. Cell viability was measured by Hoechst quantification with Operetta®. We did not observe any protection against the senolytic effect of Digoxin using the inhibitors suggesting that apoptosis was the main pathway triggered by Digoxin in A549-senescent cells (**Figure 13**).

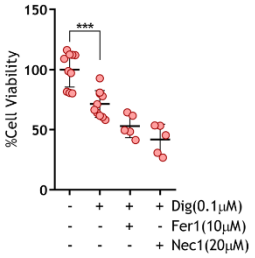

Figure 13. Inhibition of ferroptosis or necroptosis. Cell viability quantification of A549-sensecent cells treated with the combination of Digoxin plus Ferrostatin1, or Digoxin plus Necrostatin1. Dig: Digoxin. Fer1: Ferrostatin1. Nec1: Necrostatin1. Statistical significance assessed by the two-tailed Student's t-test:***p < 0.001. Own authorship image.

3. MECHANISM OF ACTION OF DIGOXIN AS A SENOLYTIC

Since the discovery of the medical use of extracts from *Digitalis* by William Withering in 1785 for the treatment of different heart conditions, research on the mechanism of action of digitalis-derived drugs did not take place until the 20th century after the Na^+-K^+-ATPase pump were discovered (Skou, 1957, 1960). Several investigators came to the idea that Cardiac Glycosides interact in some way with the Na^+/K^+ transport in cells inhibiting it (Schatzmann & Räss, 1965). These observations led to postulate that the inotropic mechanism of CGs in the myocardial contraction was mediated by the accumulation of sodium in the cells with the subsequent accumulation of calcium.

Although most of the research was made on the use of CGs for the treatment of heart disease, some data came from the mechanism of action of CGs in other cell types and diseases (Fozzard & Sheets, 1985; Katz, 1985).

Nowadays, the fact that CGs bind and inhibit the NKA is more than proven. However, recent research is focused on how this inhibition of the NKA plays different roles in different diseases besides cardiopathies, such as cancer.

With this information in mind, we wanted to decipher if Digoxin was targeting the NKA in our senescent cells and whether the inhibition of this pump was key in meditating the senolytic effect of Digoxin.

3.1. EVALUATION OF THE ROLE OF NKA IN THE SENOLYTIC POTENTIAL OF DIGOXIN[3]

The Na^+/K^+-ATPase pump (NKA) was discovered by Jens Christian Skou in 1957 who described its role in the exchange of sodium ions in nerve cells from crabs. Soon after, the mechanism by which CGs exert their ionotropic effect by targeting the NKA was further described, although it was not until the 90s were the full structure of the NKA was defined (Mijatovic et al., 2007). Thus, the NKA is a transmembrane active ion channel with an α catalytic subunit and a β regulatory subunit.

To evaluate if the α catalytic subunit of the NKA was the target of Digoxin-induced senolysis, we explored if the overexpression of the main isoform of the α subunit (the ATP1A1) could protect our cells from dying by Digoxin. We included in our analysis the overexpression of the mouse ortholog of this isoform since its lower sensitivity to CGs should lead to even stronger protection from senolysis.

To do so, we lentivirally transduced A549 cells to express the human *ATP1A1* or the mouse *Atp1a1* subunits. The lentiviral plasmid

[3] The results from this section have already been published as Triana-Martínez F., Picallos-Rabina P., et al. Identification and characterization of Cardiac Glycosides as senolytic compounds. Nat Commun 10, 4731 (2019). https://doi.org/10.1038/s41467-019-12888-x

FUGW, expressing GFP, was used as a control.We generated stable A549-*ATP1A1* and A549-*Atp1a1* by antibiotic selection with Puromycin. Once the stable cell lines were established, we induced senescence with Bleomycin at 20 µM for 5 days.

First, we validated our cellular model for the expression of both isoforms by measuring mRNA levels of human *ATP1A1* and mouse *Atp1a1* by qPCR (**Figure 14A**). Then, we evaluated our hypothesis by measuring cell viability after Digoxin treatment. The senolytic drug Navitoclax was used as a control. Cell viability was measured by Hoechst quantification with Operetta® (**Figure 14B**).

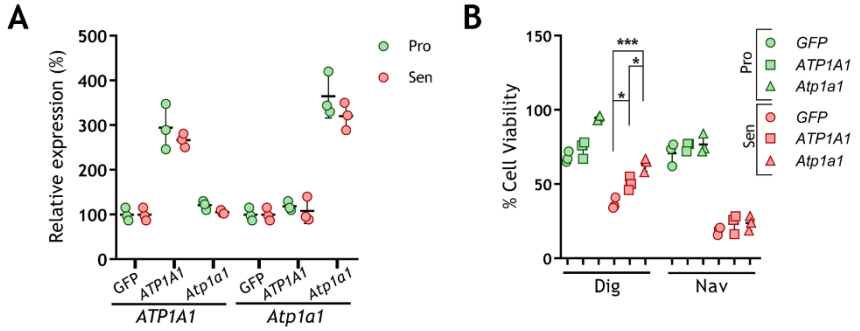

Figure 14. Overexpression of the alpha subunit of the Na^+/K^+-ATPase pump. (A) mRNA levels of the human ATP1A1 and the mouse atp1a1 in proliferative and senescent A549 cells. (B) Cell viability quantification of proliferative and senescent A549 cells expressing the human ATP1A1 or the mouse atp1a,1 treated with Digoxin or Navitoclax (as a control). Dig: Digoxin. Nav: Navitoclax. Statistical significance assessed by the two-tailed Student's t-test:***p < 0.001; *p < 0.05. Own authorship image.

In summary, what we observed was that the overexpression of the human *ATP1A1* isoform slightly prevented the senolytic effect of Digoxin, while the mouse *Atp1a1* showed a stronger protective activity against the senolytic effect of Digoxin.

3.2. EFFECT OF THE INHIBITION OF THE NKA ON ION DISBALANCE[4]

Following our results showing that Digoxin´s senolytic activity is exerted by targeting the NKA, we continued characterizing how this inhibition of the pump affected senescent cells.

3.2.1. Determination of ion concentrations after Digoxin

The inhibition of the NKA by CGs leads to the blockade of the active transport of sodium and potassium, causing the accumulation of sodium within the cell and a progressive reduction in potassium levels (Katz, 1985). To confirm that this blockade of the NKA was effective leading to the accumulation of sodium, we measured the amounts of sodium in A549 proliferating and senescent cells after Digoxin treatment. To do so, we used the fluorescence probe CoroNa Green, a sodium ion indicator that shows an increase in green fluorescence upon binding to sodium. We checked the fluorescence signal of CoroNa Green after overnight treatment with Digoxin in proliferative and senescent A549 cells and observed by microscopy that the green fluorescence signal increased after Digoxin treatment (**Figure 15A**). We further confirmed this increase in the fluorescence signal by measuring it using flow cytometry (**Figure 15B and C**).

This increase in sodium ions was common for both proliferating and senescent cells after Digoxin, but it was much higher in the senescent condition.

[4] The results from this section have already been published as Triana-Martínez F., Picallos-Rabina P., et al. Identification and characterization of Cardiac Glycosides as senolytic compounds. Nat Commun 10, 4731 (2019). https://doi.org/10.1038/s41467-019-12888-x

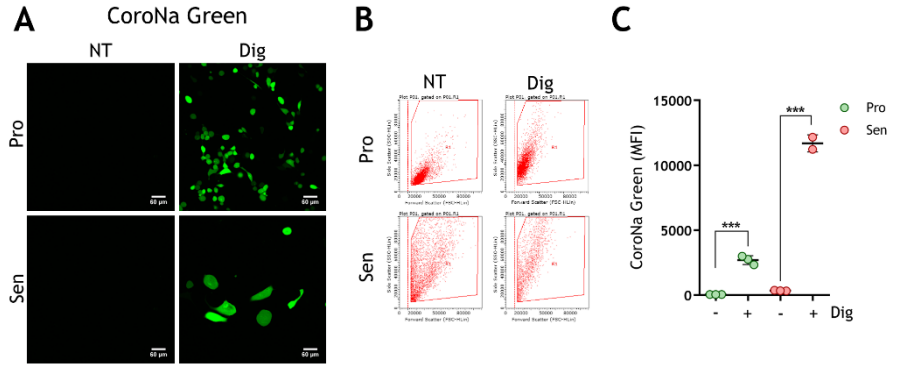

Figure 15. **Sodium ion determination.** (A) CoroNa Green signalling captured by fluorescence microscope pictures of proliferative and senescent A549 cells, treated or not with Digoxin. (B) Flow-cytomretry plots of the measure of CoroNa Green signal in proliferative and senescent A549 cells. (C) Quantification of the fluorescence signal by flow cytometry. Dig: Digoxin. MFI: mean fluorescence intensity. Statistical significance assessed by the two-tailed Student's t-test:***p < 0.001. Own authorship image.

To quantify potassium ions in a similar manner we used a potassium ion fluorescence indicator, PBFI. In this case, we did not observe a clear reduction in the concentration of potassium (**Figure 16**).

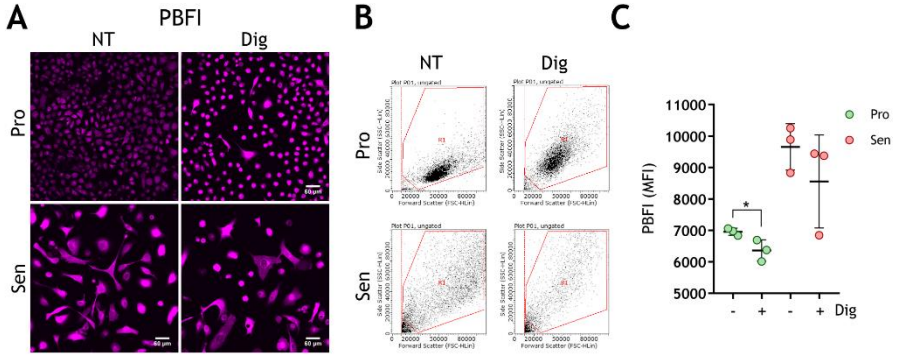

Figure 16. Potassium ion determination. (A) PBFI signalling captured by fluorescence microscope pictures of proliferative and senescent A549 cells, after 1 hour of treatment or not with Digoxin. (B) Flow-cytomretry plots of the measure of PBFI signal in proliferative and senescent A549 cells. (C) Quantification of the fluorescence signal by flow cytometry. Dig: Digoxin. MFI: mean fluorescence intensity. Statistical significance assessed by the two-tailed Student's t-test:*p < 0.05. Own authorship image.

The alteration of intracellular sodium induced by Digoxin could result in an accumulation of calcium. This increase in the levels of calcium is caused by the inhibitory effect of sodium ions over the Na^+/Ca^{2+}-Exchanger. The regulation of calcium levels is the main target in the treatment of heart diseases by CGs, since intracellular calcium levels regulate muscle contractility (Fozzard & Sheets, 1985; Katz, 1985).

We decided to measure calcium levels in our cells by using another fluorescent probe, Calcium-6. We first treated proliferative and senescent A549 cells with Digoxin for 6 hours and then incubated them with Calcium-6 and measured the signal with Hamamatsu FDSS® 7000EX. We observed that in basal conditions senescent cells showed higher levels of calcium than proliferating ones (**Figure 17A**).

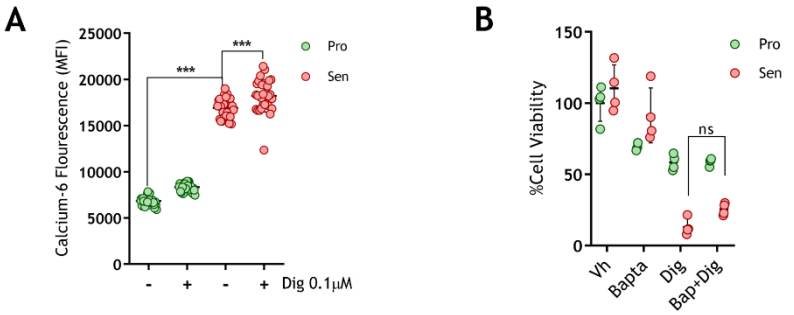

Figure 17. **Calcium determination.** (A) Calcium-6 fluorescence quantification after 6 hours of Digoxin treatment. (B) Cell viability quantification of proliferative and senescent A549 cells treated with BAPTA-AM, Digoxin or the combination. Dig: Digoxin. Bap: BAPTA-AM. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001; ns = non-significative. Own authorship image.

Interestingly, we observed an increase in calcium levels after Digoxin treatment, suggesting that maybe the uncontrolled accumulation of calcium could be mediating the death of senescent cells. To test this possibility, we used a calcium chelator, BAPTA-AM, to analyze if this could protect our cells from dying by Digoxin. For this, we treated proliferative and senescent A549 cells with BAPTA-AM, Digoxin, or the combination, and we analyzed cell viability by Hoechst quantification with Operetta®.

Co-treatment of BAPTA-AM with Digoxin did not produce any protective effect, suggesting that the accumulation of calcium within senescent cells did not seem to be responsible for the senolytic effect of Digoxin (**Figure 17B**).

3.2.2. Determination of membrane potential

The NKA, by allowing the active diffusion of sodium and potassium, causes an electrochemical gradient in the cell. This electrochemical gradient provides a membrane potential contributing to the osmotic regulation of the cell. The modulation of this membrane potential is one of the main characteristics of CGs that results in muscle contractility (Mijatovic et al., 2007).

As we have previously shown, the inhibition of the NKA by Digoxin caused the accumulation of sodium in the cell. Both the accumulation of sodium and the inhibition of the active diffusion of ions could be mediating different changes in the membrane potential. Due to the reported role of CGs in modulating the membrane potential, we wanted to evaluate whether Digoxin was affecting the membrane potential of senescent cells by targeting the NKA.

By using a fluorescent probe, DiBAC4(3), we measured the membrane potential in proliferating and senescent A549 cells. The probe DiBAC4(3) acts by binding to intracellular proteins or membranes in depolarized cells. As the depolarization increases, so does the fluorescence.

Similar to what we did for calcium determination, we treated our cells with Digoxin for 6 hours. At the end of the treatment, we incubated our cells with DiBAC4(3) and measured the fluorescence signal with Hamamatsu FDSS® 7000EX.

We noticed that in a basal state senescent cells showed higher fluorescence levels than proliferative ones, suggesting that senescent cells had a partially depolarized cellular membrane. This depolarization was even higher after Digoxin treatment (**Figure 18A**). We reasoned that this depolarization caused by Digoxin was being mediated by the alteration of the sodium/potassium balance within the cell, so we wondered whether reestablishing ion homeostasis could rescue the

membrane potential and the viability of our senescent cells. To test this hypothesis, we added KCl to the medium to replenish potassium ions in the cells during the treatment with Digoxin, and measured membrane potential and cell viability. Interestingly, the treatment of Digoxin in cells cultured with KCl added to the medium resulted in the restoration of the membrane potential (**Figure 18A**) and rescued the viability of our senescent cells (**Figure 18B**). This protective effect of KCl was reverted by using increasing concentrations of Digoxin, reinforcing the idea that KCl was protecting cells from the senolysis induced by Digoxin (**Figure 18B**).

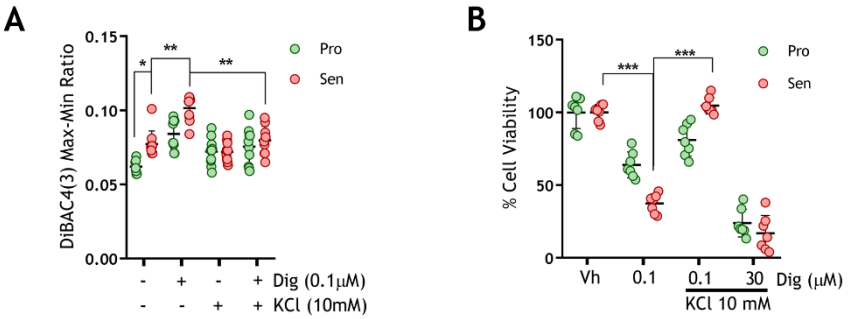

Figure 18. Effect of membrane potential impairment in the senolytic effect of Digoxin. (A) Membrane potential measurement by DiBAC4(3) quantification in proliferative and senescent A549 after treatment with Digoxin, KCl, or the combination. (B) Cell viability quantification of proliferative and senescent A549 cells treated with different concentrations of Digoxin, in combination or not with KCl. Own authorship image.

3.2.3. Determination of cytosolic pH

We had demonstrated how Digoxin, by targeting the NKA, caused an accumulation of sodium which led to an ion disbalance and depolarization of the cell membrane. It was also described in the literature how the accumulation of sodium by CGs can block the Na^+/H^+-Exchanger (NHE). By blocking the NHE, CGs cause the acidification of the cytosol, and this can trigger an apoptotic response in the cell (López-Lázaro, 2007).

To assess this possibility, we measured the intracellular concentration of protons with a fluorescence probe: pHrodo AM. This

probe emits fluorescence at neutral pH and the signal increases as pH decreases. We treated proliferative and senescent A549 cells with Digoxin for 6 hours and then incubated the cells with pHrodo AM and measured the fluorescence signal with Operetta®.

We noticed that senescent cells had higher amounts of protons than proliferative ones in the basal condition (**Figure 19A**). Even more, proton levels increased appreciably more after Digoxin treatment in senescent cells. Thus, although senescent cells have an already acidic cytosol, Digoxin seems to induce an even more acidic intracellular environment (**Figure 19A**).

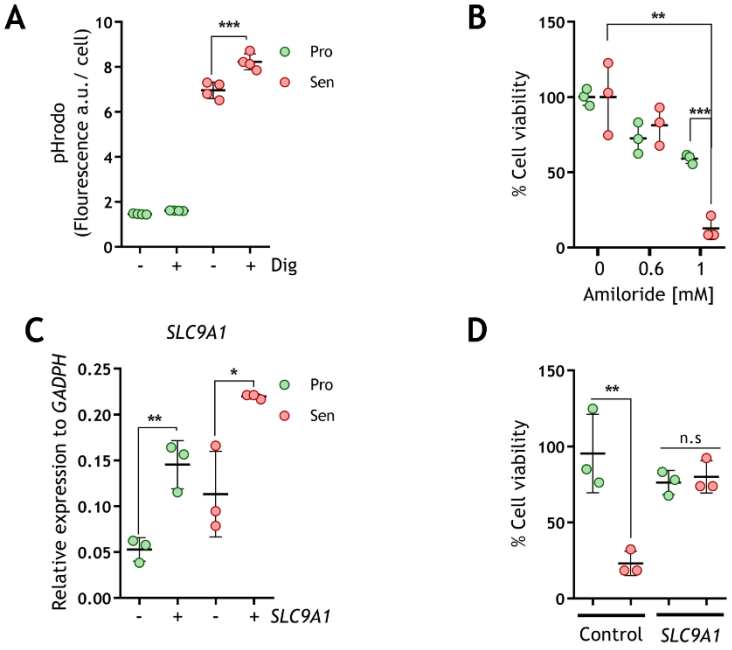

Figure 19. Effect of cytosolic acidification of the senolytic effect of Digoxin. (A) Fluorescence quantification of pHrodo in proliferative and senescent A549 cells after Digoxin treatment. (B) Cell viability quantification of proliferative and senescent A549 cells treated with different concentration of Amiloride. (C) mRNA levels of *SLC9A1* in proliferative and senescent cells lentivirally transduced to overexpress the NHE1. (D) Cell viability quantification of proliferative and senescent A549 cells, overexpressing the NHE1 or not, after Digoxin treatment. A.U.: arbitrary units. Dig: Digoxin. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001; **p < 0.01; *p < 0.05; ns = non-significative. Own authorship image.

To test if intracellular acidification could be promoting cell death in senescent cells, we pharmacologically inhibited the NHE with the idea that this inhibition should mimic the effect of Digoxin. To do so, we used Amiloride, a commonly used diuretic drug that inhibits the NHE. We treated proliferative and senescent A549 cells with different concentrations of Amiloride and observed a marked reduction in the cell viability of senescent cells (**Figure 19B**). This result suggests that intracellular acidification could have senolytic activity.

To further prove the relevance of the altered concentration of protons in Digoxin-induced senolysis, we reasoned that an increase in NHE activity through overexpression of a member of the NHE family of exchangers should have a protective effect. To this aim, we lentivirally transduced A549 cells to overexpress *SLC9A1*, encoding NHE1. We confirmed by qPCR the increase in mRNA levels of *SLC9A1* in both proliferative and senescent A549 cells (**Figure 19C**) and observed that the overexpression of *SLC9A1* rescued the viability of A549 senescent cells after Digoxin treatment (**Figure 19D**).

With all these findings, we concluded that Digoxin causes excessive intracellular acidification overwhelming the homeostasis of senescent cells, causing their death.

3.3. SIGNALING PATHWAYS REGULATED BY DIGOXIN

Although the mechanism of action of CGs through binding of the NKA and dysregulating ion homeostasis was described a long time ago, more recent research has led to the discovery of additional actions triggered by CGs that are relevant for their activities.

In the early 2000s, it was described how CGs, by binding the NKA could trigger the activation of different downstream intracellular signaling pathways. The binding of CGs changes the conformation of the NKA leading to the interaction of the pump with the SRC kinase, EGFR, and PI3K. These proteins, together with the NKA form a signalosome that activates different pathways that can regulate many cellular processes like cell proliferation, differentiation, cell cycle modulation, or apoptosis. Interestingly, the activation of this signaling cascade has been reported to occur at low concentrations CGs without

affecting the ion transport. Thus, CGs signaling activity was demonstrated to be independent of their regulation of ion homeostasis (Mijatovic et al., 2007; Prassas & Diamandis, 2008).

With all this in mind, even though we had obtained evidence of the involvement of an ion disbalance induced by CGs in senescent cells as responsible for the senolytic effect of Digoxin, we wanted to further explore whether Digoxin was activating these signaling transduction pathways in senescent cells. Deciphering the putative contribution of signaling pathways downstream of the NKA to Digoxin-induced senolysis could provide novel molecular targets to eliminate senescent cells, circumventing the toxic effects of Digoxin.

3.3.1. Evaluation of the signaling transduction caused by the binding of Digoxin to the NKA

Since there are descriptions in the literature of how CGs could modulate different signaling pathways downstream of the NKA, we wanted to explore whether this was also the case in our model. To do so, we analyzed different effectors of the three main pathways described as modulated by CGs: the PI3K-AKT, the RAS/RAF/MAPK, and the NF-κB pathways.

We started by examining the modulation of some of the proteins from these pathways at short times after Digoxin treatment. We treated our proliferative and senescent A549 cells with Digoxin for 2, 4, 6, or 8 hours, and isolated protein extracts for Western blot analysis. We analyzed p-AKT/AKT, p-ERK/ERK and p-p65/p65 as readouts of the different signaling pathways.

We observed how in both, proliferative and senescent cells p-AKT and p-ERK were upregulated after just 2 hours of Digoxin treatment. The increase in the presence of these proteins was maintained throughout the following hours. However, at 8 hours post-treatment, we could observe how not only the levels of p-AKT and p-ERK dropped but also the total levels of AKT and ERK were reduced (**Figure 21**).

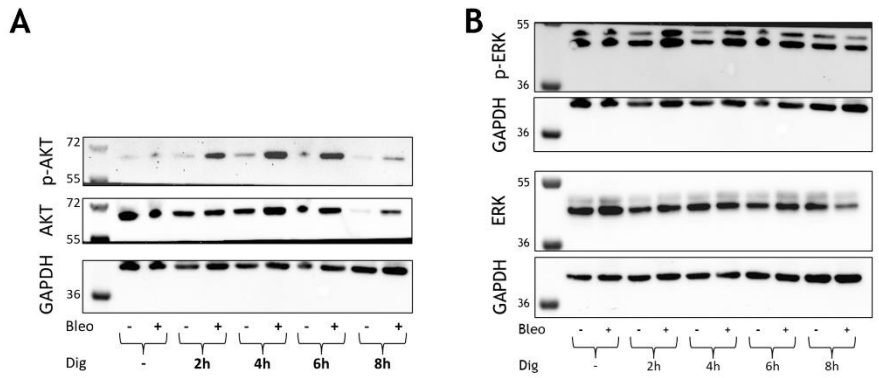

Figure 21. Modulation of the MAPK signaling pathway by Digoxin. (A) Western blotting of the time-course of Digoxin measuring p-AKT and AKT expression in proliferative and senescent A549. (B) Western blotting of the time-course of Digoxin measuring p-ERK and ERK expression in proliferative and senescent A549. Bleo: Bleomycin. Dig: Digoxin. Own authorship image.

Next we analyzed the expression of p65 (known as Rel-A), one of the most abundant isoforms of NF-κB. Contrary to what we observed with AKT and ERK, Digoxin reduced the levels of p-p65 while seemed to be increasing the total levels of p65 at 8 hours (**Figure 20**).

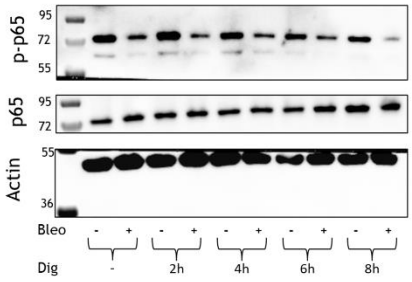

Figure 20. Modulation of the NF-κB signaling pathway by Digoxin. Western blotting of the time-course of Digoxin measuring p-p65 and p65 expression in proliferative and senescent A549. Bleo: Bleomycin. Dig: Digoxin. Own authorship image.

Given these results and since we observed that Digoxin modulated some key proteins in senescence induction and stability, we wanted to

examine what happens with other important proteins during senescence, like p53 and p21. Similar to the previous analysis, we analyzed p53 and p21 expression by Western blot after Digoxin treatment at different time points. We noticed that p53 and p21 levels decreased along Digoxin treatment in both proliferative and senescent A549 cells (**Figure 22**).

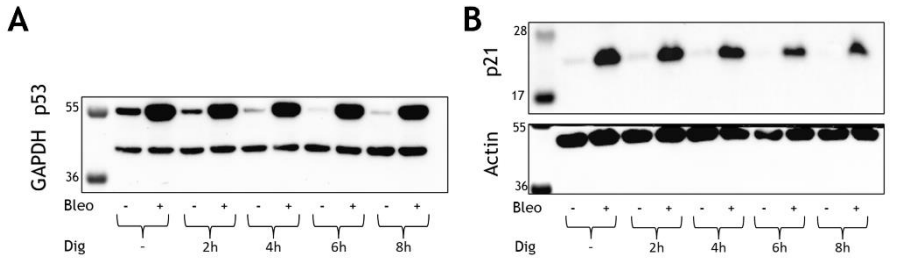

Figure 22. Modulation of p53 and p21 by Digoxin. (A) Western blotting of the time-course of Digoxin measuring p53 expression in proliferative and senescent A549 cells. (B) Western blotting of the time-course of Digoxin measuring p21 expression in proliferative and senescent A549 cells. Bleo: Bleomycin. Dig: Digoxin. Own authorship image.

Recently, it has been reported that the cytoplasmic fraction of p21 can protect senescent cells from senolysis and that reducing the cytoplasmic p21 levels promotes apoptosis in these senescent cells (Kartika et al., 2021; Koyanagi et al., 2022). This led us to consider the possibility of decreased levels of p21 induced by Digoxin as a putative senolytic mechanism triggered by CGs.

To test this possibility, we treated proliferating and senescent A549 cells with Digoxin for 8 hours. Then, we collected cell pellets from each condition and isolated the nuclear and the cytoplasmic fractions to measure p21 levels by Western blot.

We observed that cytoplasmic p21 levels increased in Bleomycin-induced senescent A549 cells, but these levels were reduced after 8 hours of Digoxin treatment (**Figure 23**).

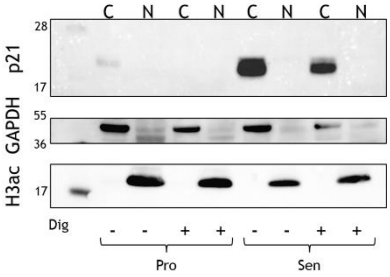

Figure 23. Subcellular localization of p21 during Digoxin treatment. Western blotting measuring cytoplasmic and nuclear p21 expression in proliferative and senescent A549 cells after Digoxin treatment. Bleo: Bleomycin. Dig: Digoxin. Own authorship image.

3.4. POTENTIAL MODULATION OF AUTOPHAGY BY DIGOXIN

The autophagy process has been described as a response to cellular stress with the aim of repairing and recycling cellular components in order to promote cell survival. However, in different contexts autophagy could also promote cell death to prevent the development of different alterations like cancer. In our case, CGs have been described as autophagy modulators capable of both, activating or inhibiting the autophagy process. The regulation of autophagy by CGs was described as a consequence of the inhibition of the NKA and subsequent activation of the SRC-MAPK pathway. Also, this modulation of autophagy could be calcium-dependent as CGs increased the levels of intracellular calcium. Therefore, different mechanisms have been implicated in autophagy modulation by CGs (Škubník et al., 2021).

In the context of cellular senescence, autophagy also acts as a double-edged sword, being pro or anti-senescence (Cassidy & Narita, 2022; Kwon et al., 2017). Thus, we wondered whether Digoxin modulates autophagy in our senescent cells and how this could be related to the senolytic effect of Digoxin.

3.4.1. Modulation of TFEB and its transcriptional functions by Digoxin

Previously, we observed that Digoxin caused an accumulation of intracellular calcium as a secondary effect of the inhibition of the NKA. Recently, it has been described that Digoxin, by modulating intracellular calcium storages, can activate TFEB, a master regulator of autophagy and lysosomal biogenesis (C. Wang et al., 2017). The inactive form of TFEB, phosphorylated by mTOR, is located at the cytoplasm. During starvation or under stress conditions that promote autophagy activation, TFEB is dephosphorylated and translocated into the nucleus where it activates the transcription of autophagy genes (Settembre et al., 2012).

With all of this in mind, we wanted to explore whether in our model Digoxin was also activating TFEB and its possible implications during senolysis. For this, we treated both proliferative and senescent A549 cells with Digoxin for 8 hours, collected cellular pellets and isolated the proteins from cytoplasmic and nuclear fractions for their analysis by Western blot.

We observed that after Digoxin treatment, TFEB tends to accumulate in the nucleus (**Figure 24A**). This nuclear translocation of TFEB was much more pronounced in senescent than in proliferative cells, suggesting that Digoxin seems to be activating TFEB.

To confirm this nuclear translocation of TFEB, we performed an immunofluorescence (IF) assay to visualize the location of TFEB after Digoxin treatment (**Figure 24B**). However, we have not been able to observe this nuclear translocation so clearly by IF.

The nuclear translocation of TFEB promotes the transcription of different pro-autophagy genes as well as lysosomal biogenesis genes. We wondered if Digoxin treatment, through the activation of TFEB, also activates these pathways at mRNA levels. We analyzed by qPCR key genes from the CLEAR network regulated by TFEB, some of which were genes involved in the autophagy process (*BENC1*, *UVRAG*, *LC3*, *SQSTM1*), and some others in lysosomal biogenesis (*LAMP1*, *MCOLN1*) (**Figure 24 C and D**).

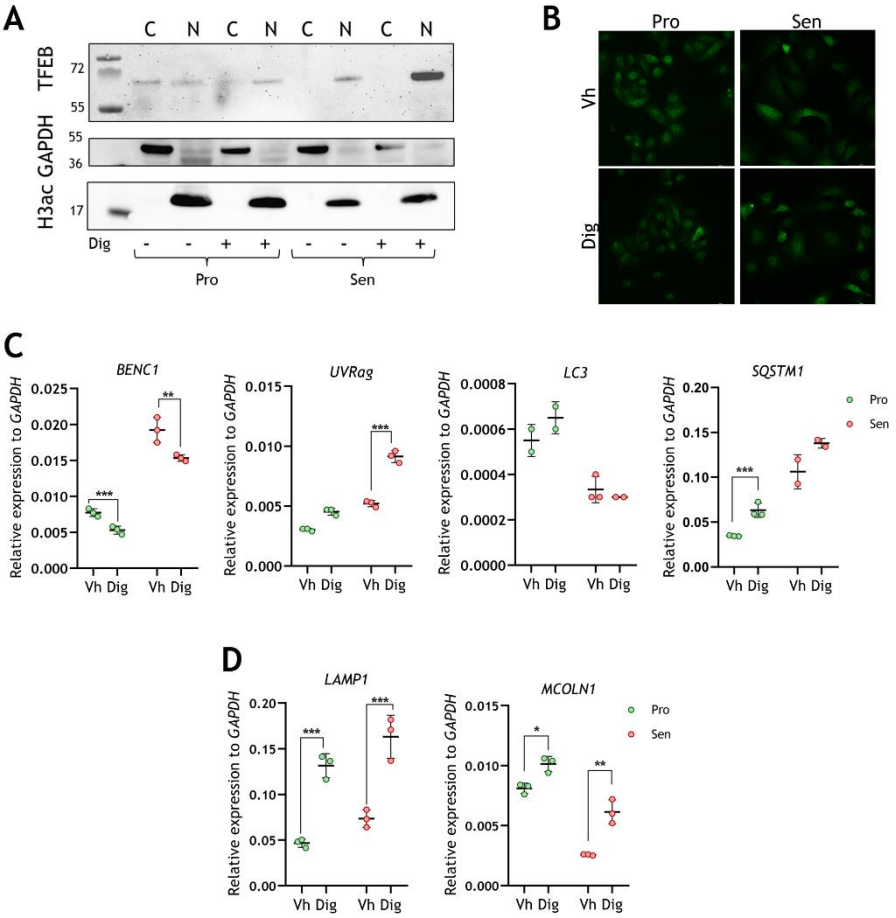

Figure 24. Modulation of TFEB by Digoxin. (A) Western blotting measuring cytoplasmic and nuclear TFEB expression in proliferative and senescent A549 cells after Digoxin treatment. (B) Fluorescence microscopy pictures of the IF of TFEB in proliferative and senescent A549 cells after 8 hours of Digoxin treatment. (C) mRNA levels of autophagy genes regulated by TFEB. (D) mRNA levels of lysosomal biogenesis genes regulated by TFEB. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001; **p < 0.01; *p < 0.05. Own authorship image.

We observed that, in general, these genes from the CLEAR network were overexpressed in senescent cells compared to proliferative ones in basal conditions. After 8 hours of Digoxin treatment, these CLEAR genes were upregulated in both conditions, which seems to indicate that the activation of TFEB by Digoxin is promoting its transcriptional functions.

3.4.2. Evaluation of the autophagy process after Digoxin treatment

The process of autophagy is carried out through different steps, starting with the nucleation of the membrane, the formation of the phagophore, which is subjected to an elongation and maturation process to form de autophagosome, which then will be fused with lysosomes to degrade its content (Aman et al., 2021). Due to the complexity of the autophagic process, we wanted to know not only if Digoxin activated autophagy through regulation of TFEB, but also if it could be acting over the autophagic flux *per se*.

For this, we analyzed key proteins from each step, from phagophore formation, elongation, and maturation to the fusion of the autophagosome with the lysosome. The aim of the study of every single step was to verify that there was a modulation of autophagy flux and whether the process was functional. We decided to assess BECLIN1 (as a regulator of the nucleation of the phagophore), LC3B (as a key marker for the elongation and maturation of the phagophore), p62 (as a marker of autophagosome-lysosome fusion and of completion of the process), and LAMP1 (as a marker of lysosomes). As controls of the different steps, we used Torin1 (an inhibitor of mTOR activating autophagy), Chloroquine (autophagy inhibitor by altering the acidic environment of the lysosomes preventing its fusion with the autophagosome), and Vacuolin1 (inhibitor of lysosomal maturation and exocytosis). We treated our proliferative and senescent A549 cells with vehicle (DMSO), Chloroquine (CQ, 1 µM), Vacuolin1 (Vac, 1 µM), Torin1 (0.25 µM), or Digoxin (Dig, 0.1 µM) for 8 hours. At the end of the treatments, cell pellets were collected, and protein extracts were used for Western blot analysis.

Starting with the first step of the process, we measured BECLIN1. As previously mentioned, BECLIN1 is responsible for the phagophore formation. We observed how in proliferating A549 cells after Digoxin BECLIN1 levels were reduced, in line with what we observed by qPCR. Interestingly, although we observed a similar reduction in mRNA levels of *BENC1* in A549-senescent cells, at the protein level we observed an accumulation of BECLIN1 after Digoxin treatment (**Figure 25A**).

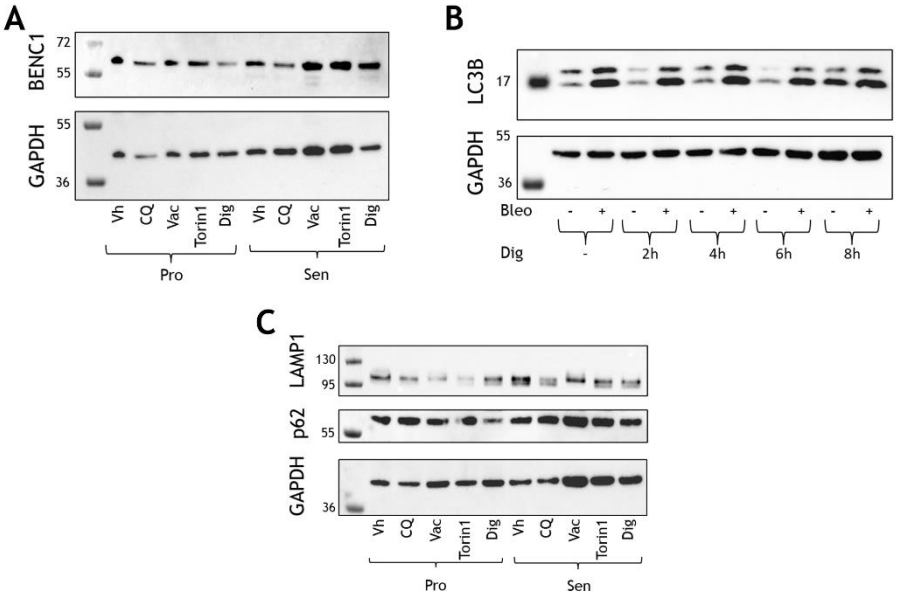

Figure 25. Modulation of key autophagy mediators by Digoxin. (A) Western blotting measurement of BENC1 in proliferative and senescent A549 cells after 8 hours of Digoxin treatment. (B) Western blotting measurement of LC3B in proliferative and senescent A549 cells at different time points of Digoxin treatment. (C) Western blotting measurement of p62 and LAMP1 in proliferative and senescent A549 cells after 8 hours of Digoxin treatment. Own authorship image.

The next step was the elongation of the phagophore to form the autophagosome. At this point, we focused on LC3B, a widely used marker for autophagic flux. During the elongation of the phagophore, LC3-I is conjugated by a complex of ATG proteins to a PE moiety to form LC3-II. Then, LC3-II binds to the membrane of the autophagosome and is degraded during the autophagosome-lysosome

fusion. Measuring LC3-II gives us a notion about autophagosome formation but cannot guarantee autophagosomal degradation by itself. We measured LC3B protein levels in both proliferating and senescent A549 cells at different time points after Digoxin trying to obtain a wide perspective of how Digoxin could be regulating the autophagy flux in a more dynamic way. We treated proliferative and senescent A549 cells for 2, 4, 6, and 8 hours with Digoxin. At the end of the treatments, cell pellets were collected and subjected to analysis by Western blot.

We observed how at short periods of time there seemed to be no changes in LC3B-II in proliferative A549, while after 8 hours post-treatment LC3B-II tended to accumulate. In contrast, we noticed that LC3B-II seemed to be unchanged in senescent A549 cells treated with Digoxin, suggesting that autophagosomes were being accumulated (**Figure 25B**).

Only by measuring the levels of LC3B, we could not confirm if the autophagic flux was being activated or not by Digoxin, so we decided to check SQSTM1/p62 to clarify the autophagosome-lysosome fusion status.

Similarly to what we did for BECLIN1, we measured p62 levels by Western blot after 8 hours of Digoxin treatment and with the different inhibitors as controls. We observed a reduction in the p62 levels in proliferative cells while in senescent A549 cells seems to be unchanged after Digoxin (**Figure 25C**).

Finally, we wanted to know if Digoxin was affecting lysosomes and impairing the autophagic flux by disrupting lysosomal integrity and preventing autophagosome-lysosome fusion. Although at mRNA levels we saw the upregulation of *LAMP1* after 8 hours of Digoxin in both proliferative and senescent cells, we were not able to observe the same behavior at protein levels. We observed a slight decrease in LAMP1 levels in A549-proliferating cells after 8 hours with Digoxin, which was more pronounced in senescent cells (**Figure 25C**).

These results, together with the observation of LC3B-II and p62 accumulation suggest that Digoxin impaired lysosomal integrity preventing the autophagosome-lysosome fusion.

3.4.3. Effect of Digoxin on lysosomal integrity

We previously showed the modulation of *MCOLN1* and *LAMP1* at mRNA, and also LAMP1 at the protein level, after Digoxin treatment. These results together with the literature reporting that Digoxin can modulate lysosomal-calcium depletion, led us to consider that maybe Digoxin was somehow acting over lysosomes.

To evaluate the integrity of the lysosomes, we decided to perform an Acridine Orange staining (hereinafter referred to as AO). AO is a fluorescent dye commonly used to detect lysosomal viability due to its ability to change the spectrum of fluorescence emission depending on the pH. If the vacuole has acidic pH the fluorescence will be red, and it will turn to green as the pH becomes neutral. Functional lysosomes have acidic pH so they will emit red fluorescence, while the cytosol or the nucleoli emit green fluorescence since they have a more neutral pH.

We treated proliferative and senescent A549 cells with Digoxin for 8 hours. As controls of lysosomal pH impairment, we used Chloroquine (CQ, 1 µM) which causes a moderate basification of the lysosomes, and Bafilomycin A1 (Baf, 0.1 µM) which causes a more intense basification of the lysosomes. At the end of the treatments, we incubated our cells with AO and measured the fluorescence by confocal microscopy and flow cytometry. For image acquisition, we used confocal microscopy at two different emission ranges for detecting both red and green signals.

We observed functional lysosomes in both proliferating and senescent cells marked in red, with a higher amount of lysosomal content in senescent cells than proliferative ones. However, after Digoxin treatment we noticed that the red fluorescence decreased and turned more yellow/green, which means a change in the lysosomal pH (**Figure 26 A and B**).

In order to have a more robust measurement of AO signal we repeated the experiment for flow-cytometry quantification, obtaining similar results (**Figure 26 C and D**).

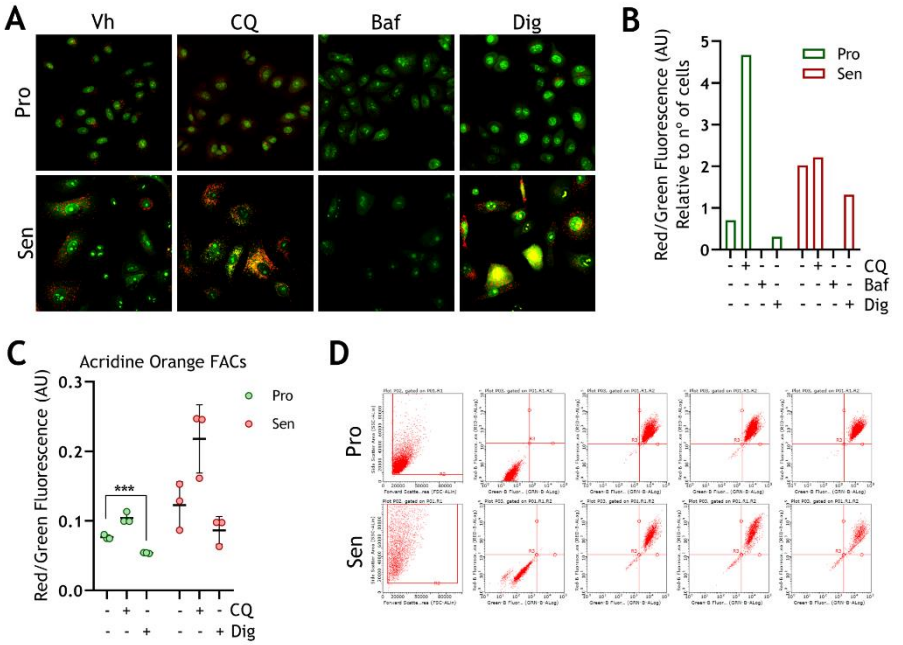

Figure 26. Determination of the lysosomal integrity by AO after Digoxin. (A) Confocal microscope pictures of AO staining proliferative and senescent A549 cells treated with Digoxin. (B) Quantification of the AO fluorescence. (C) AO quantification by flow cytometry after Digoxin. (D) Flow cytometry plots of the measurement of AO. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001. Own authorship image. Own authorship image.

In view of the potential effect of Digoxin over lysosomes changing their pH, we thought that maybe basification of the lysosomes could be responsible for the senolytic effect of Digoxin. To test this possibility, we tried to counteract the basification of the lysosomes caused by Digoxin combining it with increasing concentrations of NH_4Cl in A549-senescent cells. We analyzed cellular viability after 24 hours of treatment by MTT and we observed a mild protective effect when combining Digoxin with 25 mM of NH_4Cl (**Figure 27A**).

Although we did not observe a marked protective effect by blocking the impairment of the lysosomal homeostasis, we considered that maybe lysosomal disruption could be a potential senolytic target. To prove this hypothesis, we decided to test Leu-Leu-OMe (LLOMe),

a lysosomotropic agent that disrupts the lysosomal membrane causing lysosomal-membrane permeabilization (hereinafter referred to as LMP). We analyzed the effect of LLOMe on cell viability after the treatment of proliferative and senescent A549 cells with increasing concentrations of LLOMe. We measured cell viability after 24 hours of treatment by counting Hoechst-positive cells with Operetta® and calculated the IC50. We observed a greater predisposition to eliminate A549 senescent cells than proliferative ones with LLOMe (**Figure 27B**).

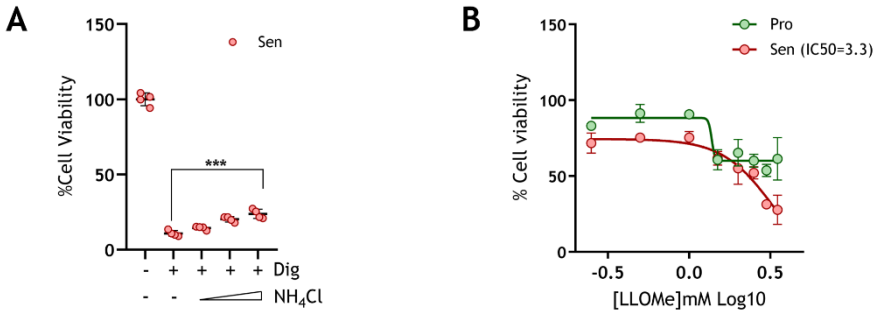

Figure 27. Lysosomal membrane permeabilization as potential senolytic target. (A) Cell viability measured by MTT of senescent-A549 cells treated with Digoxin in combination with increasing concentrations of NH_4Cl. (B) IC50 curve of proliferative and senescent A549 cells treated with increasing concentrations of LLOMe. Dig: Digoxin. LLOMe: Leu-Leu-OMe. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001.

3.5. EVALUATION OF THE EFFECT OF DIGOXIN ON MITOCHONDRIAL ACTIVITY

Different CGs can affect mitochondrial respiration and homeostasis either by signaling through the RAS-RAF-MAPK pathway increasing ROS (reactive oxygen species) production or by increasing intracellular sodium leading to the accumulation of mitochondrial calcium storage (T. Liu et al., 2010; Škubník et al., 2021). For this reason, we wanted to evaluate whether Digoxin was affecting the mitochondria in our cellular model.

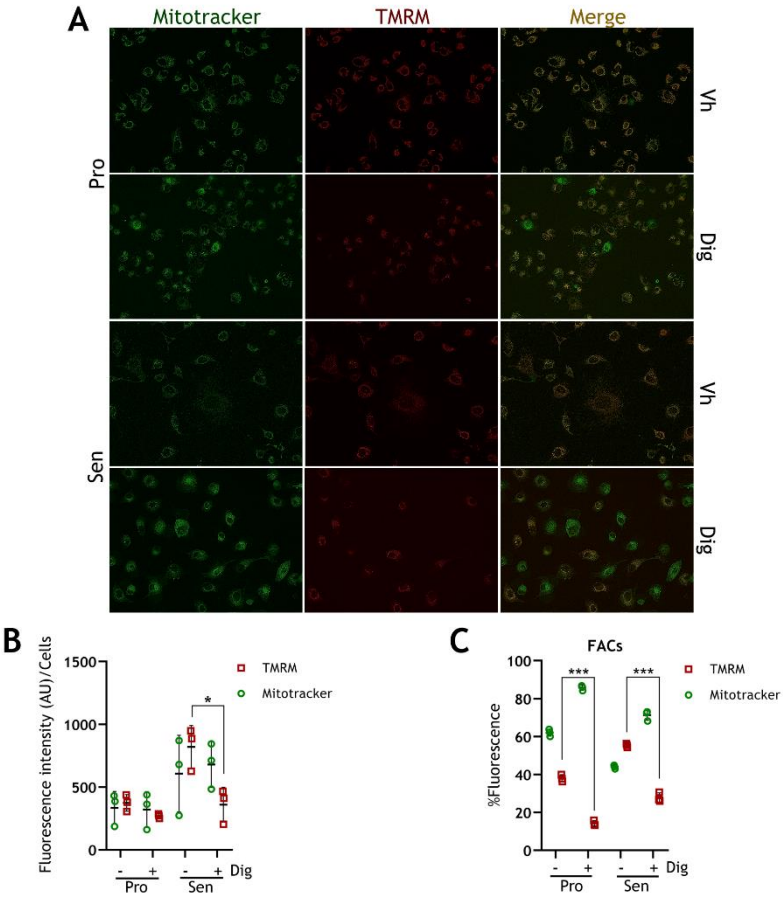

Figure 28. Mitochondrial impairment by Digoxin. (A) Fluorescence microscopy pictures showing Mitotracker (green), TMRM (red) fluorescence, double-fluorescence merge, and merge with brightfield, of proliferative and senescent A549 cells treated with Digoxin. (B) Quantification of the fluorescence from microscopy. (C) Flow cytometry quantification of fluorescence. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001 *p < 0.05. Own authorship image.

To do so, we treated proliferative and senescent A549 cells with Digoxin for 8 hours. At the end of the treatment, we cultured the cells with Mitotracker Green, a fluorescent probe that marks mitochondria, together with TMRM, another fluorescent probe that labels in red those

mitochondria with functional membrane potential. We obtained images by using fluorescence microscopy and quantified the green and red fluorescence relative to the number of cells with Image J software. We observed that the majority of cells were double-positive while after Digoxin treatment some lack the TMRM signal, meaning that they lost their mitochondrial membrane potential (**Figure 28A, B**).

To get a more robust quantification of the fluorescence signals, we repeated the experiment to measure Mitotracker and TMRM levels by flow cytometry obtaining similar results (**Figure 28C**).

4. *IN VIVO* DETERMINATION OF THE SENOLYTIC POTENTIAL OF DIGOXIN

The main goal of discovering new senolytic drugs is to improve the response of cancer patients to therapy, apart from ameliorating aged conditions. We know that different cancer treatments can induce senescence in tumors, limiting tumor growth. However, accumulation of senescent tumor cells, because of the SASP, impair therapy response of cancer patients. Thus, the "one-two punch" strategy combining TIS with senolytics can provide a more efficient therapeutical approach.

To bring the senolytics identified at *in vitro* screenings closer to the reality of cancer patients, we must test their effect in *in vivo* models.

4.1. EVALUATION OF THE *IN VIVO* SENOLYTIC POTENTIAL OF DIGOXIN IN COMBINATION WITH CHEMOTHERAPY-INDUCED SENESCENCE[5]

Although we confirmed that Digoxin had a quite broad senolytic effect in different cellular models, we had to validate its senolytic potential *in vivo*.

[5] The results showed in this section have already been published as Triana-Martínez F., Picallos-Rabina P., et al. Identification and characterization of Cardiac Glycosides as senolytic compounds. Nat Commun 10, 4731 (2019). https://doi.org/10.1038/s41467-019-12888-x

For this, we established a cancer model using A549 tumor cells. These A549 cells were previously lentivirally transduced to express Luciferase which allowed us to follow the tumor progression *in vivo*. We subcutaneously injected these A549-Luciferase cells into immunodeficient nude mice and let them grow until the tumors were palpable. At this point, we started therapy regimens with Gemcitabine, as a chemotherapy-inducing senescence agent, Digoxin, or the (Triana-Martínez et al., 2019) combination of both intraperitoneally.

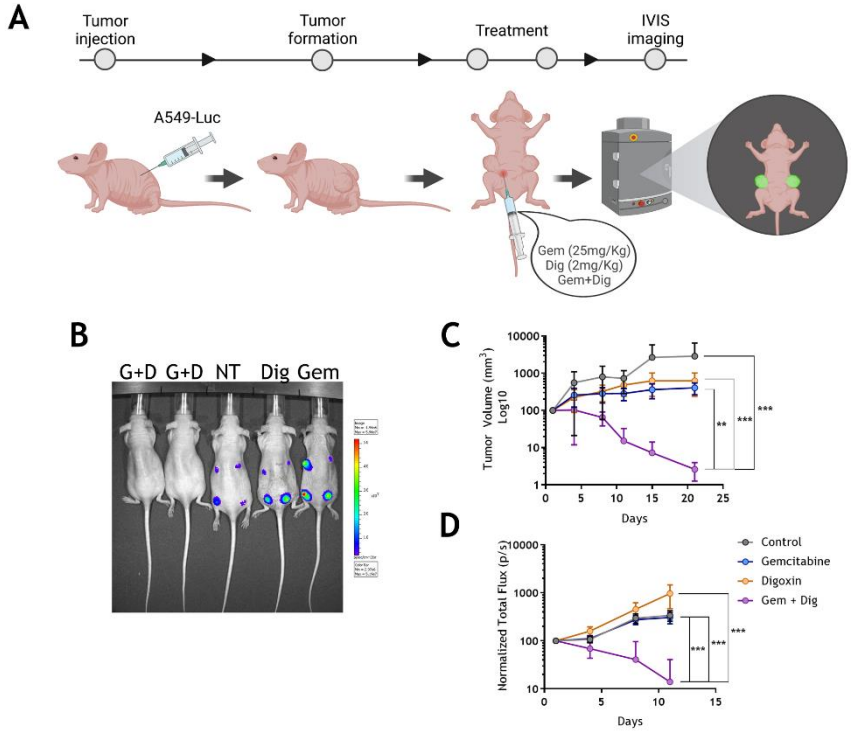

Figure 29. Evaluation of the senolytic effect of Digoxin in an *in vivo* of chemotherapy-induced senescence. (A) Schematic diagram of the experimental plan in which A549 tumor cells expressing luciferase were subcutaneously injected in immunodeficient nude mice. Gemcitabine (Gem), Digoxin (Dig), or the combination were administered IP. Created with BioRender.com. (B) IVIS imaging of the tumors after the different regimens (Triana-Martínez et al., 2019). (C) Tumor volume quantification measured with caliper. (D) Tumor volume quantification measured by luminescence using IVIS. G+D: Gemcitabine+Digoxin. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001; **p < 0.01; *p < 0.05. Own authorship image.

The therapy regime was twice a week for two weeks, and at the same time we measured tumor volume by using caliper and by luminescence imaging using IVIS (**Figure 29A and B**).We observed how treatments with Gemcitabine or Digoxin alone delayed tumor growth by tumor volume and luminescence measurements. However, only the combination of Gemcitabine with Digoxin achieved the total elimination of the tumors (**Figure 29C and D**).

At the end of the treatments, mice were euthanized, and tumors were collected to perform immunohistochemical analysis.

First, we performed whole-mount SABG staining of the tumors to evaluate senescence induction after the different treatments. We observed SABG-positive cells after Gemcitabine treatment. Intense blue staining was detected also in the condition of the combination treatment (**Figure 30A and B**). These strongly blue giant cells were multinucleated immune cells.

To further confirm senescence induction by Gemcitabine treatment we measured p21 as one of the key markers of senescence and Ki67 as a marker of proliferation. We noticed double-positive cells for SABG and p21 after Gemcitabine treatment, whereas these double-positive populations of cells were reduced after the combination therapy. The use of the Ki67 marker could also confirm the induction of growth arrest after Gemcitabine treatment, being positive for the proliferating control tumor (**Figure 30A and B**).

Moreover, only by detecting a reduction in senescent markers in the combination therapy of Gemcitabine plus Digoxin was not enough to demonstrate the senolytic effect of Digoxin. For this reason, we also analyzed the Cleaved-Caspase 3 (CCasp3) apoptotic marker. We observed some positive CCasp3 cells, suggesting that Digoxin was inducing cell death in the tumor after senescence induction (**Figure 30A and B**).

Finally, we analyzed two cytokeratin (CK) clones AE1 and AE3 for identifying epithelial cancer cells. We identified tumoral CK AE1/AE3 positive cells surrounded by SABG positive cells after combination therapy of Gemcitabine plus Digoxin (**Figure 30C**).

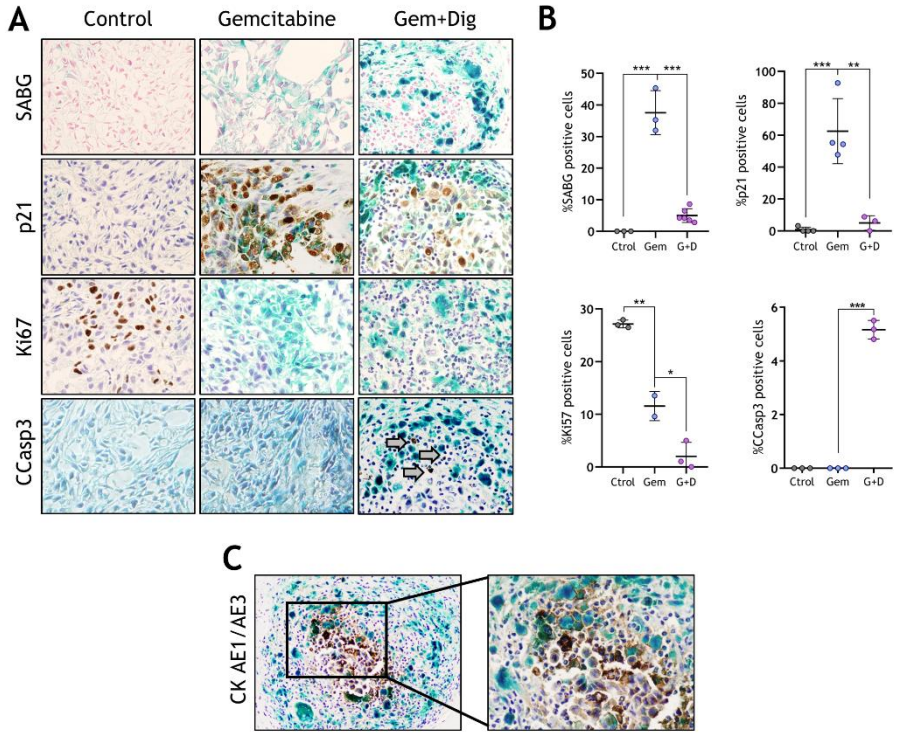

Figure 30. Immunohistochemical determination of the senolytic effect of Digoxin *in vivo*. (A) IHC sections of the tumors from the A549-Luc *in vivo* model after the different regimens. Tumors were whole mount stained for SABG, and then marked for p21, Ki67, and CCasp3 (Triana-Martínez et al., 2019). (B) Quantification of the IHC stainings from figure A. (C) IHC sections of tumors marked with CK AE1/AE3 (Triana-Martínez et al., 2019). Gem: Gemcitabine. Dig: Digoxin. SABG: senescence-associated beta-galactosidase. CCasp3: cleaved caspase 3. CK: cytokeratin. Statistical significance assessed by the two-tailed Student's t-test: ***p < 0.001; **p < 0.01; *p < 0.05. Own authorship image.

4.2. EVALUATION OF THE SENOLYTIC POTENTIAL OF DIGOXIN ON TUMOR-DERIVED EXPLANTS

Nowadays we have different preclinical models to mimic the tumor context observed in patients, such as tumor organoids or patient-derived xenografts (PDXs). Although the use of these models helps advance cancer research, these approaches have some drawbacks such as the reproducibility of tumor architecture or the tumor microenvironment.

The use of tumor-derived explants, not only from the patients but also from *in vivo* experiments, is a tool that allows us to perform high-throughput assays for drug discovery by studying the effect of different therapies in the real context of the tumor, in a non-invasive manner. For this reason, we wanted to find out if we could evaluate the senolytic potential of Digoxin on tumor-derived explants.

As a proof of concept, we used an *in vivo* model of melanoma by subcutaneously injecting SK-Mel-103 melanoma cells in nude mice. Once tumors were palpable, we started a chemotherapeutic regimen by using the CDK4/6 inhibitor Palbociclib, a well-known drug inducing cell-cycle arrest by preventing the phosphorylation of Rb protein. Palbociclib was administered by oral gavage every day for a week. At the end of the treatment, mice were euthanized, and tumors were resected.. These explants were cultured in p96 well plates and treated with Navitoclax (ABT-263), as a positive control of senolysis, or with Digoxin, for 24 hours. At the end of the treatment, we performed whole-mount staining of the explants (**Figure 31A**).

We observed SABG-positive staining on those explants derived from mice treated with Palbociclib, while the population of SABG positive cells was clearly reduced after Navitoclax or Digoxin treatment (**Figure 31B**).

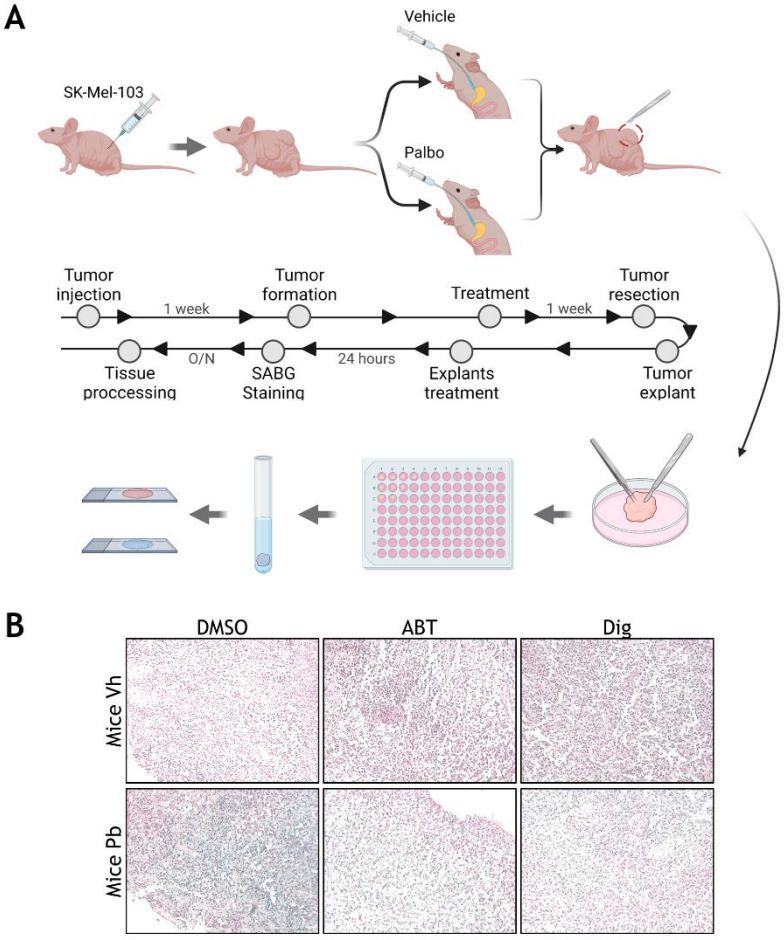

Figure 31. Determination of the senolytic effect of Digoxin on tumor-derived explants. (A) Schematic diagram of the in vivo procedure and the tumor explant generation. Created with BioRender.com. (B) SABG staining of the explants after the treatments. Palbo/Pb: Palbociclib. SABG: senescence-associated beta-galactosidase. Vh: vehicle. ABT: ABT-263/Navitoclax. Dig: Digoxin. Own authorship image.

With these results we can prove that we were able to stablish tumor-derived explants for the evaluation of the senolytic potential of Digoxin representing a novel tool for the characterization of novel senolytic drugs.

DISCUSSION

1. THE IDENTIFICATION OF NEW SENOLYTICS

Cellular senescence was originally identified as a proliferative arrest in response to damage caused by consecutive cell divisions reflecting the progressive loss of function of the organism observed during aging (Campisi, 2013; Hayflick & Moorhead, 1961). Afterwards, it was described as a crucial cellular response during embryo development and regeneration, and it has also been pointed as an essential antitumoral mechanism preventing the progression of premalignant cells (Collado et al., 2005; Muñoz-Espín & Serrano, 2014; Serrano et al., 1997).

Despite the beneficial effects provided by cellular senescence during physiological development and regeneration, uncontrolled accumulation or inefficient elimination of aberrant senescent cells is related with the appearance or aggravation of different pathologies. Focusing on the context of cancer, the induction of senescence by OIS or TIS represents a therapeutic benefit by preventing tumor growth. However, the accumulation of these senescent tumor cells impairs the total recovery of the patients as they can promote therapeutic resistance or tumor relapses (Demaria et al., 2017).

Therapeutic targeting of cellular senescence emerged after observing the beneficial effects of eliminating senescent cells ameliorating different age-related diseases (Baker et al., 2011, 2016; Chang et al., 2016; Y. Zhu et al., 2015). Apart from aging, and despite the anticancer properties of senescence induction, the elimination of senescent cancer cells with senolytics demonstrated a promising approach for improving the therapeutic response of cancer patients (L. Wang et al., 2022). Even though different genetic engineering tools allow us to identify senolytic targets, pharmacological approaches appeared as a new therapeutic opportunity to eliminate senescent cells with a clinical outcome. These new drugs were coined as senolytics, and the firsts described were the combination therapy of Dasatinib plus Quercetin (DQ) and Navitoclax (Chang et al., 2016; Y. Zhu et al., 2015). However, the lack of a specific target for DQ, or the thrombocytopenia caused by Navitoclax, limits their clinical use. For

this reason, it is still necessary to discover new senolytic compounds. In line with this, the most efficient and easiest way to find new senolytics with a clinical application is by drug discovery and repositioning. Using high-throughput screenings of chemical libraries containing regulatory agencies-approved drugs offers the opportunity to identify new senolytic compounds that can be repositioned for their faster clinical application. Also, these drug screenings could provide clues about new vulnerabilities of senescent cells to exploit them therapeutically.

For this reason, we decided to perform a high-throughput screening in order to discover new compounds with senolytic potential. First, we originally developed a cellular system that allowed us to identify potential senolytics by using co-cultures of fluorescently labeled proliferative and senescent cells. To do so, we used human lung adenocarcinoma A549 cells lentivirally transduced to express GFP or RFP. Then, A549-RFP cells were induced to senescence by using Bleomycin. At the end of the treatment, we performed co-cultures of A549-GFP proliferative cells together with A549-RFP senescent cells. We screened the Prestwick chemical library® containing 1,280 drugs and small molecules already approved for their clinical use. Among the potential hits obtained in the primary screening, we could identify and validate Proscillaridin A, a Cardiac Glycoside (CG).

CGs are a family of naturally derived molecules produced by different species of plants and as part of the venoms from toads. All the CGs share a common structure composed of a steroid group bound to an unsaturated lactone ring on one side and a sugar moiety on the other side. Thus, the CGs family is divided into two sub-groups depending on structural variations giving rise to the group of Cardenolides, derived from plants, and Bufadienolides, derived from venoms. These structural differences are in the lactone ring, which provides the specific activity of the molecule, and the sugar residue, which is a determinant of their pharmacodynamics and toxicity (Mijatovic et al., 2007) (**Figure 32**).

The best known CGs derived from plants are Digoxin and Digitoxin (from the *Digitalis*), Ouabain and Oleandrin (from *Nerium*

oleander). On the other hand, the most commonly known CGs derived from toads are Bufalin and Marinobufagenin.

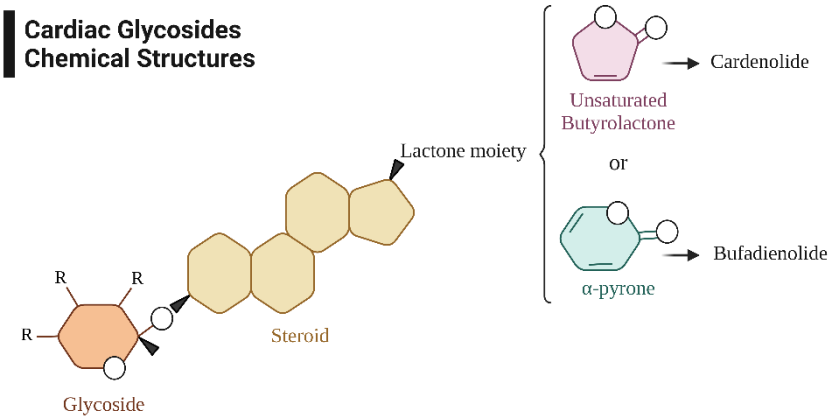

Figure 32. Cardiac Glycosides chemical structure. All the CGs share a common structure formed by sugar moiety or glycoside part bound to a steroid. Depending on the lactone moiety, these CGs will be classified into a Cardenolide or a Bufadienolide. Own authorship created with BioRender.com

The relevance of this family of molecules was originally described in 1785 when William Withering reported the therapeutic benefits of *Digitalis* derivatives in several diseases highlighting the alleviation of heart conditions as a consequence of the treatment of "dropsy" (or edema) (Withering, 1785). Since then, the therapeutic use of digitalis drugs, such as Digoxin or Digitoxin, has been spread all over the world to this day. Later, since many people were under digitalis treatment, new properties of these drugs were reported. Cancer patients on digitalis therapy showed low recurrences and less aggressiveness of tumors, and also a reduction on the risk of developing some types of cancers (Haux, 1999; Stenkvist, 1999). In line with this, different *in vitro* and *in vivo* reports suggested the anticancer properties of CGs (Mijatovic et al., 2007; Prassas & Diamandis, 2008).

All this evidence led us to further explore Proscillaridin A as a promising drug with senolytic potential. For this reason, in order to determine whether the senolytic property of Proscillaridin A was exclusive or a common feature of this family of compounds we

characterized the senolytic potential of other well-known CGs such as Digoxin and Ouabain. We could confirm the senolytic effect from the three compounds, Proscillaridin A, Ouabain, and Digoxin, not only in tumor A549 cells but also in primary BJ cells after senescence induction with Bleomycin. This observation gave us the opportunity to further exploit Digoxin as a potential senolytic since Proscillaridin A is no longer used in the clinic whereas Digoxin is.

It is worth mentioning that data from our collaborators proved the senolytic effect of other members of the CGs family derived from toad venoms (Triana-Martínez et al., 2019). Also, similar results were demonstrated by the group of Jesus Gil reporting Ouabain as a senolytic (Guerrero et al., 2019).

To evaluate the broad-spectrum senolytic effect of Digoxin we treated different senescent tumor cell lines and normal senescent primary cells confirming its senolytic potential. Also, data from our laboratory demonstrated the senolytic effect of Digoxin not only in different cell lines but also after different TIS stimuli or after OIS (Triana-Martínez et al., 2019). However, Digoxin cannot eliminate efficiently A549-quiescent cells suggesting that the effect of Digoxin did not depend on the growth arrest of the cells but on the senescence program.

Apart from the *in vitro* cancer models, we wanted to explore whether Digoxin could have senolytic effect in another pathological context different from cancer. To this aim, we tested the effect of Digoxin on osteoarthritic patient-derived chondrocytes versus healthy chondrocytes.

Osteoarthritic (OA) disease is one of the most common conditions observed during aging. OA is characterized by the loss of the ability of the chondrocytes to maintain the homeostasis of the cartilage. However, during aging, these chondrocytes lose their phenotype from tissue remodeling to a more inflammatory and profibrotic one. Senescent chondrocytes were found both during the aging process and in patients with post-traumatic OA, and selective elimination of senescent chondrocytes improves the regeneration of the joint, suggesting a

detrimental role of cellular senescence during OA development (Jeon et al., 2017, 2018). For this reason, first, we tried to determine cellular senescence in these OA chondrocytes by SABG staining. However, in this case, SABG staining was not the best marker to determine cellular senescence since healthy chondrocytes were also positive despite their active proliferation. This SABG staining background could be due to the heterogeneity of chondrocytes isolated from the patients, and maybe other senescence markers should be used for a more specific determination of cellular senescence in this model. Nevertheless, Digoxin showed specific elimination of the OA chondrocytes without affecting the healthy ones. Thus, Digoxin has senolytic effect in an *in vitro* model of an age-related disease demonstrating its efficacy beyond cancer.

Even though Digoxin demonstrated a broad-spectrum senolytic effect in different human normal and tumor cell lines, and also in an *in vitro* model of OA disease, we could not observe a senolytic effect in MEFs. Since CGs can be found in different plants and herbs, mice and rats that feed on them evolved with mutant forms of the alpha subunit of the Na^+-K^+-ATPase pump (NKA), the main target of CGs, making them more resistant to its toxicity (Mijatovic et al., 2007). This seems to be the reason why Digoxin has no senolytic activity in senescent MEFs.

With these data, we can conclude that the family of CGs have senolytic potential, and particularly Digoxin, showing the specific elimination of senescent cells in different *in vitro* models with the exception of MEFs.

2. CHARACTERIZATION OF THE CELL DEATH MECHANISM TRIGGERED BY DIGOXIN

After confirming Digoxin as a senolytic drug *in vitro*, we wanted to decipher the mechanism by which Digoxin was killing senescent cells. First, we analyzed apoptosis activation by flow cytometry using

two different apoptosis markers: Annexin V and cleaved-caspase-3. With these methods, we could observe that after Digoxin the apoptotic response was higher in senescent cells.

To confirm that apoptosis was the main death response triggered by Digoxin, we combined Digoxin with a pan-caspase inhibitor, Z-VAD-FMK (ZVF), with the purpose of protecting cell killing. Consequently, we observed that the combination of Digoxin plus ZVF has a protective effect against senolysis.

However, apart from apoptosis, other different mechanisms could be contributing to cell death, such as ferroptosis or necroptosis. We analyzed whether these pathways could be involved, at least in part, in the senolytic effect of Digoxin. To do so, we used ferroptosis and necroptosis inhibitors, Ferrostatin-1 and Necrostatin-1, in combination with Digoxin. Contrary to the effects observed with ZVF, neither Ferrostatin-1 nor Necrostatin-1 protected against the senolytic effect of Digoxin.

With all this evidence, we can confirm that the senolytic effect of Digoxin is mediated by the activation of the apoptotic caspase signaling pathway.

3. MECHANISM OF ACTION OF DIGOXIN AS A SENOLYTIC

3.1. EVALUATION OF THE ROLE OF NKA IN THE SENOLYTIC POTENTIAL OF DIGOXIN

Despite CGs being identified more than two centuries ago, their mechanism of action was not described until the mid-20th century. In 1957 the Na^+-K^+-ATPase (NKA) was first isolated from crabs and its role was described in nerve stimulation by a sodium-derived electrochemical gradient (Skou, 1957). Three years later, the enzymatic activity of the NKA was further explored and, serendipitously, strophanthin (a CG) was found to have the capacity to inhibit the ATPase exchange activity (Skou, 1960). These findings by JC Skou earned him the Nobel Prize in Chemistry in 1997. Soon after the

observations of JC Skou, HJ Schatzmann reported that CGs inhibited the NKA in erythrocytes, triggering research on the foundations of the mechanism of action of CGs in the clinic (Fozzard & Sheets, 1985; Katz, 1985; Schatzmann & Räss, 1965).

In terms of structure, the NKA is an ATPase located at the cell plasma membrane and it is responsible for the active transport of sodium and potassium with the expenditure of ATP. This ATPase is composed of an α catalytic subunit and a β regulatory subunit (**Figure 33**). At the same time, each subunit is constituted by different isoforms: four isoforms for the α subunit and three isoforms for the β subunit. The combination of subunits and isoforms is responsible for the different enzymatic reactions of the NKA among tissues (Mijatovic et al., 2007).

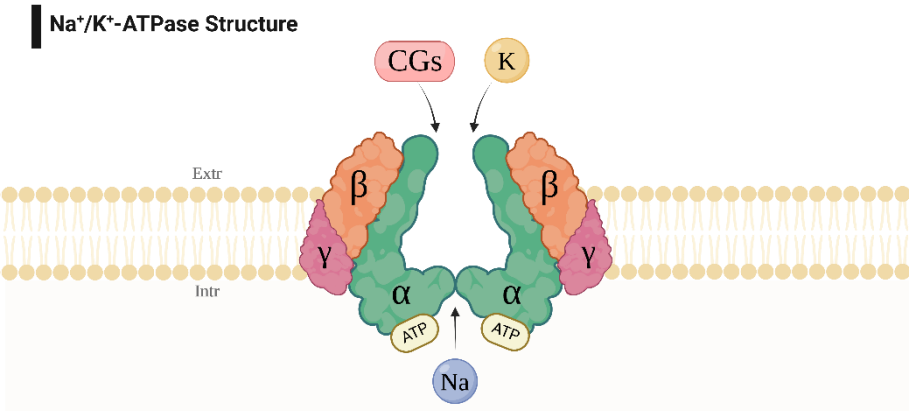

Figure 33. NKA Structure. The NKA is an ATPase formed by the α catalytic subunit and the β regulatory subunit. The third subunit is a complementary γ regulatory subunit present in different tissues. This ATPase actively transports sodium and potassium across the cellular membrane creating a membrane potential. However, CGs can compete with potassium for binding the NKA and inhibiting its transport. Own authorship created with BioRender.com

The catalytic α subunit contains the binding sites for sodium, potassium, ATP, and CGs, representing the main part of the protein. However, and despite around 90% of homology between species, different mutations were described in this α subunit from humans to rodents (Mijatovic et al., 2007). These mutations could be the cause of the previously mentioned senolytic resistance of MEFs to Digoxin.

With all this evidence, we wanted to evaluate whether the senolytic activity of Digoxin depends on its binding to the α catalytic subunit of the NKA. To do so, we overexpressed human *ATP1A1*, the main isoform of the α subunit of the NKA with the aim to protect our cells from dying by Digoxin. In parallel, we also overexpressed the mouse ortholog of this isoform, the *Atp1a1*, as a resistant form against Digoxin, demonstrating a better protective response. We observed that the overexpression of the human *ATP1A1* slightly reduced the senolytic effect of Digoxin in our *in vitro* model, with this protection being more evident after the overexpression of the mouse ortholog, *Atp1a1*. Similar results were reported by A. Guerrero and colleagues by overexpressing the rat ortholog of the α_1 isoform of the NKA protecting IMR90 cells against the senolytic effect of Ouabain after oncogenic RAS-induced senescence (Guerrero et al., 2019).

Even though the protective effect of overexpressing *ATP1A1* did not reach 100% of cell viability, we consider that Digoxin is senolytic mainly by targeting the NKA, although further validations were performed and will be discussed later. This partial protective effect of the overexpression of the α_1 subunit could be due to the role of the other α isoforms on the binding of Digoxin to the NKA. The conserved expression of the α_1 subunit across different tissues could provide the basis for the potential broad-spectrum senolytic effect of Digoxin.

3.2. EFFECT OF THE INHIBITION OF THE NKA ON ION DISBALANCE

The NKA is a protein complex belonging to the P-type family of ATPases which depends on the hydrolysis of ATP to actively transport three ions of Na^+ outside the cell in exchange for two ions of K^+. For this process, the NKA has two conformational states, E1 and E2. In E1, Na^+ and ATP bind to the NKA phosphorylating it, leading to the capture of three Na^+ ions and releasing them outside the cell. In this state, known as E2-P, the NKA is still phosphorylated. Once the Na^+ is released, K^+ binds to the NKA for its dephosphorylation, changing its conformation to the E2 state and capturing two K^+ ions that will be released to the cytosol (**Figure 34**) (Scheiner-Bobis, 2002). With this ion exchange, the NKA plays a critical role in maintaining an electrochemical gradient across the plasma membrane for the osmotic

regulation of the cell, which is crucial for physiological processes such as neuronal communication and muscle contractility (Mijatovic et al., 2007). The clinical relevance of using CGs relies on the modulation of this electrochemical gradient in heart muscle stimulating its contractility. Also, the observations of W. Withering, when he first identified CGs for the treatment of edema, could be related to this osmotic regulation in cells.

Therefore, and after previously observing that Digoxin is targeting the NKA for its senolytic effect, we wanted to further characterize all the disbalance triggered by the inhibition of the NKA by Digoxin and how this can be an Achilles heel of senescent cells.

3.2.1. Determination of ion concentrations after Digoxin

We first measured Na^+ and K^+ ion levels in proliferative and senescent A549 cells treated or not with Digoxin. Using a fluorescent probe, CoroNa Green, as a Na^+ ion indicator we measured sodium levels and observed an accumulation of sodium both in proliferative and senescent A549 cells after Digoxin treatment. This accumulation is markedly higher in the senescent condition compared to the proliferative one. On the other hand, we also measured K^+ ions with another fluorescent probe, the PBFI, but in this case, we cannot see a clear reduction in potassium levels after Digoxin treatment.

The observation of the accumulation of sodium after Digoxin is in line with the inhibitory effect of the NKA by CGs. However, we cannot see the reduction in potassium levels that we expected. This result could be due, on the one hand, to improper experimental timing since depletion of potassium levels could take longer than the increase in sodium levels. On the other hand, we could speculate that it could be due to an overall disbalance in the cells making them inefficient to release the amounts of potassium. This hypothesis will be in line with the observations of PI. Deryabin and colleagues. They reported that senescent A549 and IMR90 cells are more sensitive to potassium disbalance as they lack compensatory mechanisms to overcome this issue, while senescent endometrial mesenchymal stem cells are resistant to CGs by overexpressing compensatory channels replenishing potassium requirements of the cell (Deryabin et al., 2022).

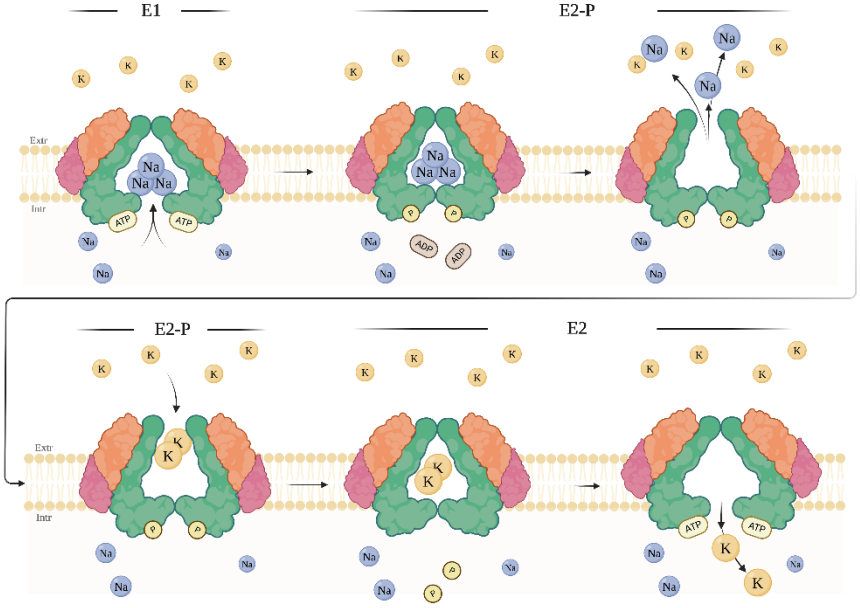

Figure 34. Schematic representation of the transport of sodium and potassium through the NKA. Own authorship created with BioRender.com

Apart from the alterations in Na^+/K^+ balance, we analyzed the potential modulation in Ca^{2+} levels since the main therapeutic effect of CGs on heart conditions is based on the increase in intracellular calcium to potentiate the force of myocardial contraction. The Na^+-Ca^{2+}-Exchanger allows the active transport of Na^+ inside and Ca^{2+} outside the cell generating an electrochemical gradient essential for muscle contractility. Thus, CGs base their positive ionotropic effect on the rise of intracellular calcium levels by the inhibition of the NKA, but also by the release of calcium from the intracellular storages, such as the endoplasmic reticulum (Fontana et al., 2013). We used a fluorescent probe, Calcium-6, to measure the levels of intracellular calcium in our cells after Digoxin treatment. We observed that in a basal condition, A549 senescent cells have higher amounts of Ca^{2+} than proliferative ones. It was described that senescent cells showed an elevation of calcium levels as a response to senescence-inducing stress. Also, these

high levels of calcium were required as regulators of the SASP in two different ways. On the one hand, the activation of IL1α (the initiator for the production of pro-inflammatory cytokines) by calpains (calcium-regulated proteases). On the other hand, the transcriptional regulation of the SASP by NF-κB, which is activated by intracellular free calcium (Martin & Bernard, 2018).

However, after Digoxin treatment the levels of calcium increased even more, which seems to be another consequence of the NKA inhibition by Digoxin. To prove if the rise of calcium levels has a role in the senolytic effect of Digoxin, we treated our cells with the combination of Digoxin with a calcium chelator, BAPTA-AM. In this case, we could not observe any protective effect of sequestering free calcium suggesting that the accumulation of intracellular calcium might not be the main player in Digoxin-induced senolysis. Even though we could not determine the role of the rise in calcium levels by Digoxin, we can speculate that Digoxin could still be acting over the different reservoirs of calcium, such as the endoplasmic reticulum, mitochondria, or lysosomes, promoting calcium signaling, and maybe leading to apoptosis (Orrenius et al., 2003). Whether the modulation of calcium levels by Digoxin in senescent cells has any senolytic potential must be further explored.

3.2.2. Determination of membrane potential

As previously described, the NKA allows the active transport of Na^+ and K^+ ions across the plasma membrane generating an electrochemical gradient. Thus, the NKA contributes to the maintenance of the membrane potential for the secondary transport of other substances required for the homeostasis of the cell (Mijatovic et al., 2007). Therefore, inhibition of the NKA by CGs alters the membrane potential of the cell.

After observing that Digoxin is targeting the NKA as part of its senolytic effect, we wanted to evaluate whether Digoxin was affecting the membrane potential and how this could be mediating the senolytic effect of Digoxin. We used a fluorescent probe, DiBAC4(3), to measure the membrane potential of proliferative and senescent A549 cells treated or not with Digoxin. We observed that in a basal state, senescent

cells have a slightly depolarized plasma membrane compared to proliferative cells, and this depolarization was even higher after Digoxin treatment. This observation confirms again the targeting of the NKA by Digoxin being the depolarization of the plasma membrane a consequence of the accumulation of intracellular sodium (Katz, 1985). Taking this into account, we wanted to check whether the depolarization of the plasma membrane was mediating the senolytic effect of Digoxin. For this, we reasoned that reestablishing the balance between Na^+ and K^+ could rescue the cell viability of senescent cells after Digoxin treatment by restoring the membrane potential. Increasing serum potassium levels in patients under digitalis prevents the toxicity of these drugs (Fozzard & Sheets, 1985; Katz, 1985). In line with this, we supplemented the culture medium with KCl to replenish potassium ions in the cells during the treatment with Digoxin. Interestingly, we could restore the membrane potential to a basal state in A549 senescent cells when we treated them with Digoxin in combination with KCl. Consequently, the addition of KCl also protected against the senolytic effect of Digoxin. However, at higher concentrations of Digoxin, KCl could not rescue the cell viability, suggesting that CGs compete with K^+ to bind the NKA. Similar observations were reported by the groups of Jesús Gil and Jean-Yves Thuret (Guerrero et al., 2019; L'Hôte et al., 2021).

3.2.3. Determination of cytosolic pH

After observing how Digoxin caused an ion disbalance and the depolarization of the plasma membrane by targeting the NKA leading to senolysis, we wanted to further analyze the effects of NKA inhibition on intracellular pH. The accumulation of Na^+ within the cell has been reported to reduce the activity of the Na^+-H^+-Exchanger (NHE), which allows the counter-transport of H^+ ions in exchange for Na^+. This exchange can occur in both directions depending on the sodium gradient maintaining intracellular pH.

We used a fluorescent probe, pHrodo AM, to measure intracellular pH. We observed that A549 senescent cells had higher levels of H^+ than proliferative cells in a basal state. These findings suggest that senescent cells have a more acidic cytosol compared to proliferative tumor cells.

After Digoxin treatment, these levels of H^+ increased even more in senescent cells. Then, to evaluate whether this intracellular acidification could be mediating the senolytic effect of Digoxin, we used a well-known inhibitor of the NHE, Amiloride. We observed that Amiloride phenocopied the senolytic effect of Digoxin. On the contrary, the overexpression of *SLC9A1*, encoding for NHE1, a member of the NHE family, protected against the senolytic effect of Digoxin.

These results demonstrate that since senescent cells have a lower cytosolic pH than their proliferative counterparts, forcing a reduction in pH levels in senescent cells promotes their elimination. The fact that senescent cells have an acidic cytosol could be due to the accumulation of acidic vacuoles such as lysosomes. We can speculate that Digoxin has senolytic effect at least in part by targeting lysosomes since senescent cells have a characteristic accumulation of these organelles. This hypothesis would be based on the fact that the main proton channels in lysosomes are V-ATPases which share structural and functional similarities with the NKA, making them more prone to be targeted by Digoxin (Morth et al., 2011; S. Zhu et al., 2020).

Altogether, the results presented in this part of the thesis demonstrate that Digoxin, by targeting the NKA, exerts its senolytic effect by causing an ion disbalance and a membrane depolarization in senescent cells exacerbating an already altered homeostatic state. These multiple homeostatic disturbances keep senescent cells at the edge between life and death representing a great vulnerability that could be exploited for senolytic interventions. Hence, recently the use of Nigericin was reported to target senescent cells in the same way that CGs do (Deryabin et al., 2022).

3.3. SIGNALING PATHWAYS REGULATED BY DIGOXIN

Around the ’60s, the mechanism of action of CGs was mainly described as the modulation of the ion transport in cells by targeting the NKA. However, it was not until the early 2000s that novel functions of the NKA under the action of CGs were described. Apart from the ion transport, the NKA participates in the activation of different signaling cascades by acting as a scaffold for the assembly of different protein complexes and forming a signalosome. This CGs-mediated signaling is

independent of its modulation of ion trafficking and includes the activation of SRC kinase, the transactivation of EGFR, the activation of the RAS-MAPK pathway, modulation of NF-κB, or the generation of ROS by altering mitochondria (**Figure 35**) (Mijatovic et al., 2007; Prassas & Diamandis, 2008).

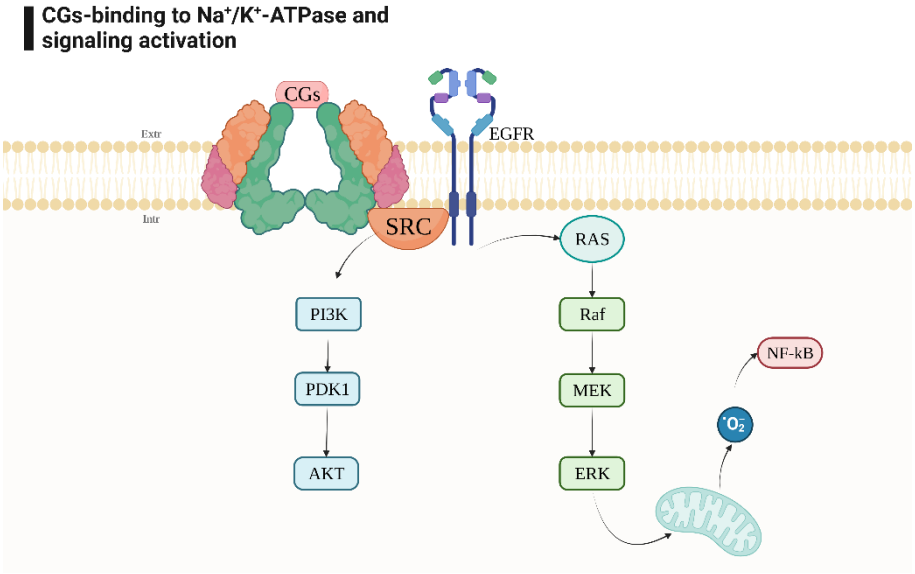

Figure 35. CGs, by binding the NKA, activates different signaling cascades through its interaction with SRC and EGFR. Own authorship created with BioRender.com

Despite proving that Digoxin is exerting its senolytic effect through the inhibition of the NKA causing a homeostatic disbalance in senescent cells, we wanted to further characterize whether Digoxin was signaling through the NKA and its potential implications during senolysis. For this, we analyzed different effectors of the main pathways described as modulated by CGs, such as AKT, ERK, and p65 expression at different time points after Digoxin treatment.

First, we measured p-AKT/AKT and p-ERK/ERK protein levels in proliferative and senescent A549 cells every two hours up to 8 hours post-treatment with Digoxin. After just 2 hours of Digoxin, we could observe an accumulation of p-AKT and p-ERK in both proliferative and

senescent cells, levels that were maintained throughout the following hours. However, both phosphorylated forms and total protein levels of AKT and ERK decreased after 8 hours of treatment.

Both AKT and ERK are proteins well characterized due to their important roles mainly in proliferation, differentiation, and cell survival (H. Lavoie et al., 2020; Vivanco & Sawyers, 2002). Thus, AKT and ERK have been found upregulated in different human cancers, playing important roles during tumorigenesis (H. Lavoie et al., 2020; Nitulescu et al., 2018; Vivanco & Sawyers, 2002). However, AKT and ERK are also involved during senescence as well. The PI3K-AKT pathway hyperactivation leads to senescence induction, and this pathway is related to SASP production through its downstream effector mTOR (Soto-Gamez et al., 2019; S. Zhu et al., 2020). Similarly, ERK, one of the best-known MAPK kinases, can activate the senescence program in response to aberrant RAS activation, DNA damage, or oxidative stress, upregulating p21 expression and leading to cell-cycle arrest. Also, ERK can regulate SASP production through the signaling of NF-κB (Anerillas et al., 2020).

The consequences of activating these signaling pathways by CGs were reported as cell-type dependent since most research has been made on cardiomyocytes. On the one hand, the activation of the PI3K-AKT pathway by CGs in normal cells can promote both growth stimulation and inhibition, being reported as a cause of hypertrophic growth in terminally differentiated cardiomyocytes (L. Liu et al., 2007). On the other hand, CGs-induced activation of RAS through SRC activates ERK expression which mediates mitochondrial ROS generation, and subsequently promotes aberrant cell proliferation. Similar to AKT, activation of RAS-MAPK by CGs also causes cardiac hypertrophy (Xie & Askari, 2002). During senescence, targeting the PI3K-AKT pathway with Dasatinib or Fisetin has been suggested to be the basis of their senolytic activity since they are involved in the anti-apoptotic features of senescence (L. Zhang et al., 2022). Also, targeting the MAPK pathway by Dasatinib and Quercetin induces apoptosis in senescent cells (Anerillas et al., 2020; L. Wang et al., 2022).

Our results suggest that Digoxin, by targeting the NKA, not only impairs ion exchange but also seems to activate the NKA-dependent signalosome as observed by the phosphorylation of AKT and ERK. It has been reported that the key mediator in the activation of the RAS-MAPK pathway is SRC phosphorylation, while the activation of AKT signaling is through the binding of PI3K to the NKA. The results from A. Guerrero and V. L'Hôte also demonstrate an increase in p-AKT and p-ERK after 6 hours of Ouabain treatment, confirming our experimental observations (Guerrero et al., 2019; L'Hôte et al., 2021). Additionally, we showed that after 8 hours of Digoxin treatment the levels of these proteins start to decrease, suggesting a potential inhibition of the pro-survival effect of AKT and ERK leading to senolysis.

Furthermore, we decided to analyze p-p65/p65 expression as the main isoform of NF-κB. During cellular senescence, NF-κB is the master regulator of SASP production, which accounts for the beneficial and detrimental effects of senescence induction. Thus, SASP modulation by targeting NF-κB could provide promising interventions to improve aging conditions and cancer therapy (Chien et al., 2011; L. Wang et al., 2022). In addition, CGs have been implicated in the regulation of NF-κB by two different mechanisms: the modulation of intracellular calcium levels and NKA-dependent signaling (Prassas & Diamandis, 2008). The inhibition of NF-κB seems to mediate the anticancer properties of CGs since NF-κB expression promotes proliferation in cancer cells (Mijatovic et al., 2007).

Our results showed a reduction in the levels of p-p65, while the total levels of p65 seemed to be increased at 8 hours post-treatment. In view of this result, the modulation of NF-κB in senescent cells by Digoxin requires a more detailed characterization. An assumption could be that Digoxin is inhibiting the activation of p65 preventing SASP production. In line with this, A. Guerrero observed that Ouabain decreases different SASP markers (Guerrero et al., 2019). On the other hand, we previously showed that Digoxin increased intracellular free calcium in senescent cells which could be caused not only by the inhibition of the Na^{+}-Ca^{2+}-Exchanger but also by targeting calcium storages, such as lysosomes, and in consequence modulating NF-κB.

Thus, CGs, such as UNBS1450, can induce lysosomal-membrane permeabilization (LMP) leading to the release of calcium content (Mijatovic et al., 2006). With all this evidence, we speculate that Digoxin may have a dual role in regulating NF-κB during cellular senescence: first, by directly repressing the phosphorylation of p65 and second, by promoting the accumulation of p65 due to the increase in intracellular calcium levels.

All these data suggest that Digoxin might be activating signaling cascades through NKA. However, the relevance of the modulation of these pathways in our model must be further investigated during senolysis. Interestingly, recent data demonstrated that the senolytic effect of CGs, more specifically Ouabain, is mainly through the activation of the NKA-dependent signaling instead of the homeostatic disbalance, suggesting that the mechanism of action of CGs as senolytics could vary according to the cell type (L'Hôte et al., 2021).

Apart from the potential implications of the signaling cascade triggered by Digoxin during senolysis, we wondered whether Digoxin could be acting over other senescence-specific markers. We measured the expression of p53 and p21 after Digoxin at different time points and observed a gradual reduction in the expression of both p53 and p21. During the senescence program, p53 activation is crucial for the arrest of the cell cycle by transcriptionally activating the cyclin-dependent kinase inhibitor p21. Moreover, the expression of both p53 and p21 also contribute to the pro-survival features of senescent cells, and targeting p53 regulatory networks has senolytic properties (Sturmlechner et al., 2022; L. Wang et al., 2022; L. Zhang et al., 2022). CGs were already described to reduce the levels of p53 in different cancer cell lines in an NKA-dependent manner through the signaling of the SRC-MAPK pathway since SRC or ERK inhibitors rescued p53 levels (Z. Wang et al., 2009). However, the potential implications of the reduction in p53 and p21 levels by Digoxin during senolysis need to be further explored.

Even though a similar reduction in p21 levels after CGs was reported by A. Guerrero and colleagues, they suggest that this observation represents a reduction in the levels of senescence (Guerrero et al., 2019). In contrast, we hypothesize that this modulation of p53

and p21 could be linked to their pro-survival mechanism during senescence, providing specificity to CGs targeting senescent cells. To further explore this possibility, we measured the differential expression between cytosolic and nuclear p21, since recently it has been reported that cytoplasmic p21 protects senescent cells from senolysis (Kartika et al., 2021; Koyanagi et al., 2022). We clearly showed that during Bleomycin-induced senescence, p21 accumulates in the cytoplasm and its levels were reduced after Digoxin treatment.

Even though we cannot confirm that the reduction in p53 and p21 levels are linked to the senolytic properties of Digoxin, we could speculate that in senescent cells Digoxin could be acting beyond the NKA-dependent ion disbalance by targeting different essential pathways for the senescence program.

3.4. POTENTIAL MODULATION OF AUTOPHAGY BY DIGOXIN

Although basal autophagy is a crucial mechanism for the maintenance of cellular homeostasis implicated during development, stem cell renewal, cell differentiation, and plasticity, the autophagic program is also relevant during stress response (Klionsky et al., 2021). The autophagy process is a complex set of molecular pathways to repair and recycle cellular components to maintain energy homeostasis and to promote cell survival. Even though the term "autophagy" is always used in a broad sense, this term usually refers to the process of macroautophagy. During this process, cellular components are isolated in vacuoles, called autophagosomes, where they fuse with the lysosome for the degradation of their contents (Cassidy & Narita, 2022).

Interestingly, autophagy and senescence share common characteristics since both are stress responses that can act during physiological and pathological settings. However, the implications of autophagy during cellular senescence are poorly understood. There is some evidence demonstrating that autophagy inhibition can lead to senescence both *in vitro* and *in vivo*, and restoring a normal autophagic program can improve the healthspan. Also, the autophagy program has a role in SASP production during senescence since the inhibition of autophagy can change SASP secretion (Cassidy & Narita, 2022). Contrary to the pro-senescence effects of autophagy, it has also been

proposed that targeting autophagy in senescent cells, either activating or inhibiting it, can induce senolysis (Kucheryavenko et al., 2019; Wakita et al., 2020).

In line with these dual effects of autophagy, CGs have been identified as autophagy modulators that can act both activating or inhibiting the autophagic process in a cell type-dependent manner. The effects of CGs in modulating autophagy can be NKA-dependent or calcium-dependent (Škubník et al., 2021). We wondered whether Digoxin could be modulating the autophagy process in our *in vitro* model of senescence. This would provide us with some clues on its possible implication in Digoxin-induced senolysis.

3.4.1. Modulation of TFEB and its transcriptional functions by Digoxin

TFEB is a transcription factor from the MiT family, crucial for autophagy and lysosomal biogenesis and function, through the regulation of the CLEAR network. The CLEAR network was described by the group of Andrea Balabio after observing that several lysosomal and autophagic genes were expressed under an E-box promoter whose master regulator is TFEB (Palmieri et al., 2011; Sardiello et al., 2009; Settembre et al., 2012). Later, they described that TFEB activation was dependent of mTORC1 regulation since mTORC1 keeps TFEB phosphorylated and inactive in the cytoplasm (Martina et al., 2012; Settembre et al., 2012). Under stress conditions, such as nutrient deprivation, mTORC1 becomes inactive and lysosomes release Ca^{2+} through mucolipin1 (MCOLN1), activating calcineurin and dephosphorylating TFEB which then translocates into the nucleus (Medina et al., 2015).

Although the expression of TFEB during senescence was not fully investigated, there are some reports describing the role of TFEB improving healthspan and lifespan (Cassidy & Narita, 2022). In line with this, Digoxin has been reported recently to induce lysosomal-calcium release. This in turn activates TFEB, causing its nuclear translocation, and the transcriptional activation of autophagic genes. The activation of this signaling cascade results in an increase in the

lifespan of *C. elegans* (C. Wang et al., 2017). Therefore, we wondered whether Digoxin was regulating TFEB in our cellular model.

We measured cytoplasmic and nuclear fractions of TFEB in proliferative and senescent A549 cells after Digoxin. A clear nuclear translocation of TFEB was evident in senescent cells after Digoxin. To confirm this nuclear translocation of TFEB, we performed an immunofluorescence (IF) assay to visualize the location of TFEB after Digoxin treatment. However, we failed to observe a clear translocation to the nucleus by IF, perhaps due to technical problems. In order to monitor the translocation of TFEB, it would be more accurate to use a cell line expressing a fluorescently-tagged version of TFEB.

In addition, to prove that this nuclear translocation of TFEB was taking place and it is promoting the transcription of CLEAR components, we analyzed the expression of some autophagy (*BECN1, UVRAG, LC3,* and *SQSTM1*) and lysosomal (*LAMP1* and *MCOLN1*) genes known to be regulated by TFEB.

First, we observed that *BECN1* expression levels were higher in senescent A549 cells than in proliferative ones in a basal state, with Digoxin causing a reduction in its levels. BECLIN1 is required for the initiation of the autophagic process by its interaction with PI3KC3. Also, BECLIN1 is considered a tumor suppressor gene being deleted in many breast, ovarian, and prostate cancers (Wijshake et al., 2021). Interestingly, BCL-2 and BCL-xL interact with BECLIN1 resulting in a block of its function during autophagy. Although TFEB positively regulates the transcription of *BENC1*, the BECLIN1 protein inhibits TFEB via mTOR regulation (Ma et al., 2015). We speculate that Digoxin is downregulating *BECN1* resulting in the activation of TFEB.

Then, we observed that *UVRAG* and *SQSTM1* were upregulated in senescent cells compared to proliferative ones, and that their expression increased after Digoxin treatment. First, UVRAG forms a PI3KC3 complex with BECLIN1 regulating autophagosome formation and fusion with lysosomes. This complex interacts with Rubicon to inhibit the autophagosome-lysosome fusion stopping the autophagic flux at this point (Nah et al., 2021). Also, UVRAG was described as an anti-

inflammatory mediator and its loss of function caused age-related cancer development (Song et al., 2020). Second, *SQSTM1*, which encodes for p62 protein, is implicated during selective autophagy by targeting ubiquitylated proteins forming aggregates for their degradation by the lysosome. Thus, p62 together with LC3B are common markers for autophagic flux. Unfortunately, we have not been able to properly measure mRNA levels of LC3 in our cells.

Focusing on lysosomal biogenesis genes regulated by TFEB, we measured *LAMP1* and *MCOLN1*. LAMP1, together with LAMP2, are major components of the lysosomal membrane and are essential for the maintenance of lysosomal integrity. *MCOLN1* encodes for a lysosomal-membrane channel allowing ion trafficking, especially Ca^{2+}.

We observed a small increase in *LAMP1* mRNA levels in senescent cells compared to proliferative ones, which could be partially reflecting the dysfunctional lysosomal biogenesis characteristic of senescent cells. However, the levels of *LAMP1* were greatly increased after Digoxin treatment in both proliferative and senescent cells. Similar to previous reports showing that CGs improve lysosomal turnover (Hundeshagen et al., 2011), perhaps Digoxin could be promoting lysosomal biogenesis through TFEB activation. In line with this, we saw that basal levels of *MCOLN1* were lower in senescent cells than in proliferative ones. The increase in *LAMP1* together with the low levels of *MCOLN1* could reflect the accumulation of defective lysosomes in senescent cells, since they actively promote lysosomal biogenesis in response to an impairment in lysosomal functions in a feedback loop (Robbins et al., 1970). However, Digoxin upregulates *MCOLN1* expression in both proliferative and senescent A549 cells. Recently, it has been described how TFEB could be activated by the calcium released from lysosomes through the MCOLN1 channel. We can speculate that maybe Digoxin could be enhancing the activation of TFEB by the lysosomal-calcium release in a feedback loop. This would support the above-mentioned observations demonstrating that Digoxin increased the levels of cytosolic free calcium (Medina et al., 2015).

Altogether, these results seem to prove that TFEB transcriptional regulation is activated by Digoxin as shown by the upregulation of

several CLEAR genes. However, the implications of these transcriptional regulations in Digoxin-induced senolysis need to be further investigated. Even so, this knowledge could give us some notions about novel potential targets to eliminate senescent cells.

3.4.2. Evaluation of the autophagy process after Digoxin treatment

Autophagy is a highly selective program for the maintenance of cellular homeostasis through the degradation and recycling of cellular components. Although autophagy is regulated in normal conditions, its modulation could be triggered as a stress response, and impairment in the autophagic program is related to the development of different age-related diseases such as neurodegenerative disorders, lung fibrosis, or cancer. Interestingly, there is a close relationship between the alterations in the autophagic program and cellular senescence in these aging conditions (Aman et al., 2021; Klionsky et al., 2021).

As previously mentioned, CGs have been proven to modulate autophagy, either by their interaction with the NKA or by calcium release. However, the modulation of the autophagy flux varies among the different CGs and in a cell-type-dependent manner. Thus, despite proving that Digoxin is activating TFEB and hence the transcription of different autophagic and lysosomal genes, we wanted to evaluate the whole autophagy flux, since Digoxin could be acting over the autophagosome formation or lysosomal viability.

The autophagy flux is carried out through different steps: the nucleation of the membrane to form a phagophore and encapsulate protein aggregates; its maturation, leading to the autophagosome formation; and finally, its fusion with the lysosome for the degradation of the contents (**Figure 36**). We measured protein levels of some key components of each step of the process that we previously showed to be transcriptionally modulated by Digoxin, such as BECLIN1, LC3B, LAMP1, and p62.

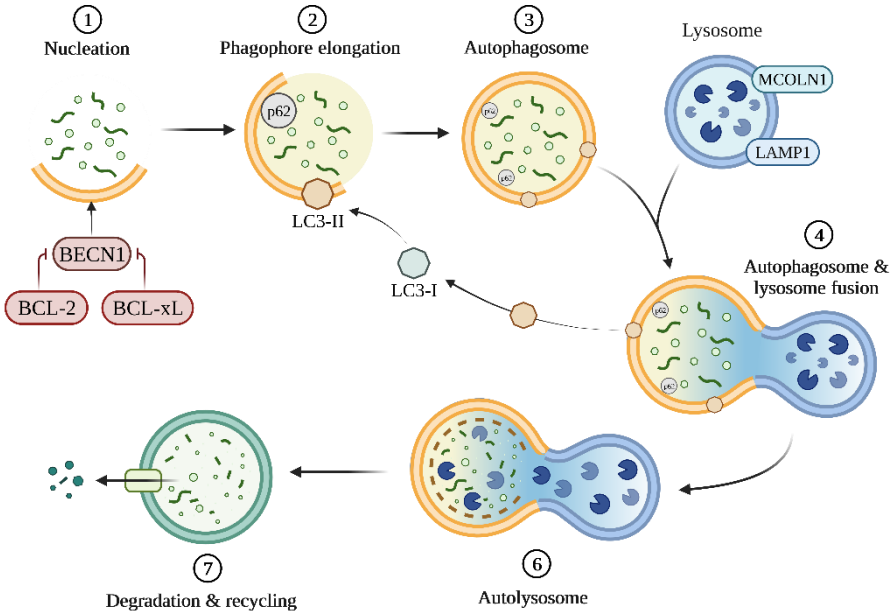

Figure 36. Autophagy flux. The autophagy process begins with the formation of the phagophore in which Beclin1 plays an important role during the nucleation of the membrane (1). During the elongation of the phagophore, LC3-I is transformed into LC3-II which binds the phagophore membrane, and p62 is captured inside the phagophore with the cellular debris (2). Once the autophagosome is formed (3), it can be fused with lysosomes for the degradation of its content. During the autophagosome and lysosome fusion, LC3-II is released and recycled for a new autophagic process (4). The fusion of the autophagosome and the lysosome gives rise to the autolysosome in which the hydrolytic enzymes degrade the content (6). The result of the degradation of the autolysosome content is then recycled for the cell for the generation of new proteins and molecules (7). Own authorship created with BioRender.com

First, we measured the levels of BECLIN1 as a first step in the autophagic flux for its role in regulating the nucleation of the phagophore. Despite observing that A549 senescent cells upregulate *BECN1* compared to proliferative cells in a basal state, we observed the opposite at protein levels with a slight reduction in BECLIN1 expression in senescent cells. This tendency of senescent cells to have lower levels of BECLIN1 could be due to the previously mentioned interaction of BCL-2 and BCL-xL with BECLIN1 inhibiting its autophagic role and preventing autophagic cell death (Soto-Gamez et

al., 2019). However, after Digoxin, BECLIN1 levels were reduced in proliferative cells while they seem to be increased in senescent cells. Even though the regulation and functions of BECLIN1 during cancer and senescence are far from defined (Salminen et al., 2013), a hypothesis could be that Digoxin is modulating BCL-2/xL expression releasing BECLIN1 and promoting phagophore nucleation in senescent cells among other functions, such as promoting cell death.

The next step in the autophagic flux that we analyzed was the elongation of the phagophore and the formation of the autophagosome. For this, we measured LC3B and p62 protein levels. LC3 is transformed from its unprocessed form, proLC3, to LC3-I. Then, LC3-I is conjugated by different ATG proteins into a phosphatidylethanolamine-conjugated form, LC3-II, which binds to autophagosomes. During the autophagy process, LC3-II bound to the autophagosome membrane is released once the autophagosome is fused with the lysosome and recycled for a new autophagic process. In contrast, p62 is captured in the autophagosome with the protein aggregates and degraded during the autophagosome-lysosome fusion. Thus, LC3-II levels together with p62 degradation, reflects the degradative process during autophagy. We measured LC3B-II at different time points after Digoxin to visualize the dynamic process of formation and degradation of autophagosomes. We observed that at basal levels, senescent cells have higher levels of LC3B-II compared to proliferative cells. This increase in LC3B-II could reflect an impairment in LC3B turnover, perhaps due to an inefficient autophagic flux during senescence. However, after Digoxin, the levels of LC3B-II vary over time in proliferative cells while in senescent cells remain unchanged. The increase in LC3B-II levels at 8 hours post-treatment in proliferative cells could be caused by the overstimulation of autophagosome formation (active autophagic flux) or the inhibition of autophagosome-lysosome fusion (inactive autophagic flux) triggered by Digoxin. In contrast, the maintenance of the same levels of LC3B-II in senescent cells after Digoxin could reflect an impairment in the formation or degradation of the autophagosomes (inactive flux).

To further explore what is happening at this point we measured p62 at 8 hours post-Digoxin treatment to decipher whether autophagosomes were accumulated by an impairment in autophagosome-lysosome fusion. We could observe that p62 levels were reduced after Digoxin treatment in proliferative cells, which suggests that autophagosomal p62 is being degraded during the autophagosome-lysosome fusion and that the autophagic flux seems to be activated. In this case, autophagy activation in proliferative A549 cells could be a survival mechanism in response to Digoxin treatment. However, p62 levels in senescent cells after Digoxin seems to remain unchanged, which suggests, together with the accumulation of LC3B-II, that Digoxin is impairing the autophagic flux and autophagosomes are accumulated. Similar observations were reported by Valentin L'Hôte demonstrating that Ouabain impairs the autophagic flux in senescent cells in an NKA-signaling-dependent manner (L'Hôte et al., 2021).

After observing that there seems to be a detention in the autophagic flux in senescent cells due to the accumulation of autophagosomes caused by Digoxin, we decided to measure the levels of LAMP1, a key marker of lysosomes, to decipher whether the fusion between lysosomes and autophagosomes was affected. In a basal state, senescent cells showed higher levels of LAMP1 compared to their proliferative counterparts, which could be reflecting the characteristic accumulation of lysosomes during senescence. After Digoxin, we observed that there was a slight decrease in LAMP1 levels in proliferative cells which was more pronounced in senescent cells.

With these data, we observed that A549 senescent cells seem to have a dysfunctional autophagy program. We speculate that in our model, Digoxin is impairing autophagy in senescent cells by preventing the autophagosome-lysosome fusion by affecting lysosomal integrity. In addition, we suggest that Digoxin could be stimulating autophagy as a survival mechanism in proliferative A549 cells.

3.4.3. Effect of Digoxin on lysosomal integrity

Lysosomes are vesicles full of hydrolytic enzymes crucial for degradative processes, such as autophagy or phagocytosis, but also as signaling mediators for maintaining energy homeostasis and metabolic

regulation. These lysosomal functions are based not only on their enzymatic content but also on several V-ATPase ion channels and transporters. Due to these transport and communication mechanisms, lysosomes also store various ions such as Ca2+, Na+, or K+ (S. Zhu et al., 2020).

Lysosomal damage is associated with the development of different pathologies such as cancer, neurodegenerative disorders, or cardiovascular diseases. The disruption of the lysosomal integrity leads to the release of their acidic content and lytic enzymes causing cellular damage and cell death (S. Zhu et al., 2020).

Even though there is evidence reporting that CGs could promote lysosomal biogenesis it can also lead to inhibition of V-ATPases. This causes the release of lysosomal calcium and with that triggers their disruption (Hundeshagen et al., 2011; Mijatovic et al., 2006; C. Wang et al., 2017).

Our previous results showed that Digoxin upregulates the expression of *LAMP1* and *MCOLN1* under the regulation of TFEB, which means that Digoxin could be favoring lysosomal turnover. However, at the protein level, we observed that Digoxin reduced LAMP1 preventing autophagosome-lysosome fusion in senescent cells. We wondered whether Digoxin could be also affecting lysosomal integrity. Using Acridine Orange, a marker of pH as a reporter of lysosomal viability, we observed that senescent cells seem to have more lysosomal content than proliferative cells, which is a hallmark of senescence. Interestingly, these lysosomes showed a reduction in their pH after Digoxin treatment which could reflect an impairment in their function and viability. To prove whether this lysosomal impairment is related to the senolytic effect of Digoxin, we co-treated senescent A549 cells with the combination of Digoxin with increasing concentrations of NH_4Cl to protect from lysosomal damage. We observed a mild protection in the senolytic effect of Digoxin, suggesting that Digoxin can be causing cell death by lysosomal disruption although it might not be its main mechanism of senolysis.

These data, together with the increase in the levels of free intracellular calcium, could suggest that Digoxin is affecting lysosomal integrity promoting calcium release. This lysosomal calcium release could be involved in Digoxin-induced cell death. However, this might now be the main mechanism mediating senolysis since we could not rescue the viability of senescent cells by using BAPTA, a calcium chelator. Still, targeting lysosomal integrity could be a potential senolytic target. We tested this idea by causing lysosomal-membrane permeabilization (LMP) with LLOMe. LLOMe showed senolytic effect in A549 senescent cells, suggesting that disrupting the lysosomal compartment of senescent cells is a potential senolytic target.

Altogether, these data indicate that Digoxin could be affecting lysosomal integrity in senescent cells by targeting the V-ATPases in the lysosome and thus causing LMP. These observations could also be related with the previously mentioned acidification of the cytosol and the increase in the levels of intracellular calcium. This would suggest that the Digoxin is acting beyond NKA inhibition and that senescent cells are more susceptible to the lysosomal-disrupting action of CGs due to the increased lysosomal biomass.

3.5. Evaluation of the effect of Digoxin on mitochondrial activity

After observing that Digoxin is acting at different cellular compartments and not only by targeting the NKA, we wondered whether Digoxin could be acting on mitochondrial function. The effects of CGs on the mitochondria were described as a consequence of the activation of RAS-MAPK pathway which caused mitochondrial-ROS production and ion depletion. Consequently, this alteration in mitochondrial homeostasis by CGs is reflected as a depolarization of the mitochondrial membrane leading to cell death (T. Liu et al., 2010; Škubník et al., 2021). We quantified mitochondrial content and viability with two different fluorescent probes, and observed that Digoxin was reducing the mitochondrial membrane potential both in proliferative and in senescent cells. We speculate that senescent cells, due to their characteristic mitochondrial dysfunction, could be prone to the

alteration of the mitochondrial homeostasis caused by Digoxin contributing to its senolytic effect.

It is worth mentioning that similar observations were reported after Ouabain treatment. Ouabain can cause mitochondrial membrane depolarization due to cellular ion disbalance and ROS production (Deryabin et al., 2021; López-Lázaro, 2007).

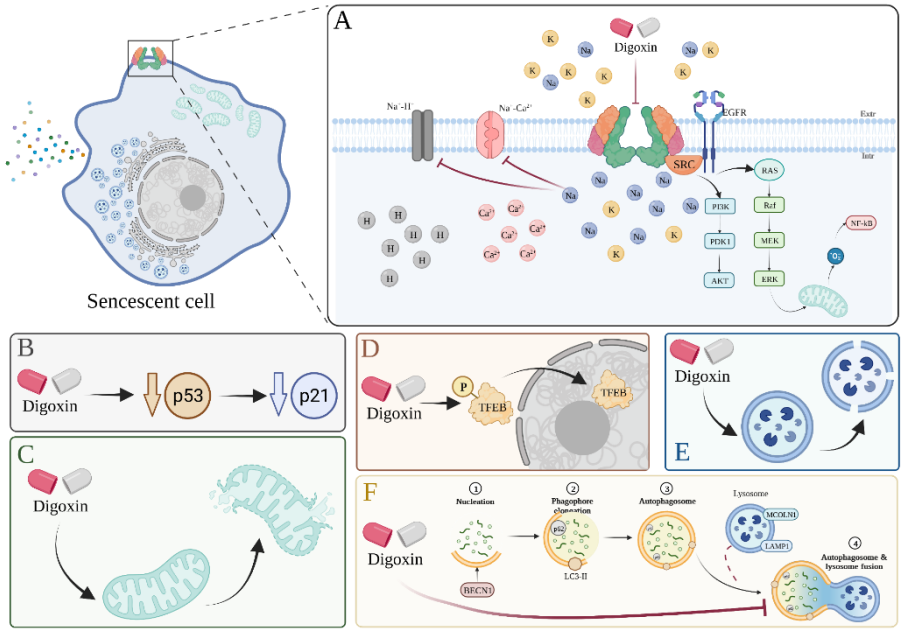

Figure 37. Effects of Digoxin in senescent cells. A) Digoxin, by targeting the NKA, causes an ion disbalance due to the accumulation of sodium, and triggers the NKA-dependent signaling. B) Digoxin reduces the levels of p53 and p21. C) Digoxin disrupts the mitochondrial membrane potential. D) Digoxin promotes the nuclear translocation of TFEB with the subsequent transcription of CLEAR components. E) Digoxin impairs lysosomal integrity. F) Digoxin disrupts autophagy flux by impairing the autophagosome-lysosome fusion. Own authorship created with BioRender.com

Taken together, our results suggest that, in senescent cells, Digoxin targets the NKA causing both ion disbalance and the activation of the NKA-dependent signaling cascade, the activation of TFEB and its transcriptional targets, the impairment of the autophagic flux due to lysosome disruption, and an impairment of the mitochondrial membrane potential (**Figure 37**). We could only confirm the

homeostatic disbalance resulting from the inhibition of the NKA by Digoxin as its main mechanism mediating senolysis. The other cellular alterations could be contributing to the senolytic effect of Digoxin. However, these other potential targets of Digoxin need to be further explored as they could provide us the opportunity to improve the effect of Digoxin as a senolytic drug. Also, these observations could give us some clues about novel vulnerabilities of senescent cells for developing new senolytic strategies.

4. *IN VIVO* DETERMINATION OF THE SENOLYTIC POTENTIAL OF DIGOXIN

4.1. EVALUATION OF THE *IN VIVO* SENOLYTIC POTENTIAL OF DIGOXIN IN COMBINATION WITH CHEMOTHERAPY-INDUCED SENESCENCE

After demonstrating that Digoxin has senolytic effect in different *in vitro* cancer models, it was essential to characterize this senolytic property *in vivo.* We tested the senolytic potential of Digoxin using a cancer model by subcutaneously injecting A549 cancer cells expressing a luciferase reporter in nude mice. When tumors were formed we established four groups of mice: control, Gemcitabine treatment, Digoxin treatment, or a combination of both treatments. Gemcitabine is an antineoplastic drug from the group of "antimetabolites" commonly used in the treatment of pancreatic cancer that causes genotoxic stress and, in turn, induces senescence (Song et al., 2016). We followed tumor progression by measuring tumor volume with a caliper and luciferase emission by IVIS. Single treatments with both Gemcitabine or Digoxin had mild antitumor effect. However, we observed that the combination of Gemcitabine plus Digoxin reduced almost completely the tumor volume.

To validate that Gemcitabine was inducing senescence and that Digoxin was inducing senolysis, we performed SABG staining together with other markers of senescence on the tumors post-mortem. We showed that Gemcitabine-treated tumors presented an increase in both SABG and p21 senescence markers together with the lack of the

proliferative marker Ki67, implying that Gemcitabine was inducing senescence in our *in vivo* cancer model. In this condition, we could not observe any cleaved-caspase-3 positive cell, suggesting that Gemcitabine alone was not inducing apoptosis. We measured the same markers in tumors treated with the combination of Gemcitabine and Digoxin. Despite observing intense blue staining after SABG, we concluded that the combination of Gencitabine and Digoxin was not inducing senescence. These intensily blue stained cells were not of tumor origin and were identified as multinucleated immune cells surrounding some remaining p21 cells and a few caspase-3 positive apoptotic cells. Additionally, we measured a cytokeratin marker for the identification of epithelial cancer cells which were surrounded also by these giant SABG-positive cells.

These data demonstrate that Digoxin, in combination with chemotherapy-induced senescence, has a senolytic effect *in vivo* favoring the almost complete elimination of the tumor.

It is worth mentioning that Digoxin also showed senolytic activity in another model using breast cancer PDXs treated with Doxorubicin. In addition, we observed senolytic effect of Digoxin using an *in vivo* model of lung fibrosis (Triana-Martínez et al., 2019). Altogether, our results provide robust evidence for the senolytic property of Digoxin after different senescence-induced stimuli and for different pathologies *in vivo*.

4.2. EVALUATION OF THE SENOLYTIC POTENTIAL OF DIGOXIN ON TUMOR-DERIVED EXPLANTS

We have mainly demonstrated that Digoxin has senolytic activity in cancer but it also works in other age-related diseases, such as osteoarthritis or lung fibrosis, both *in vitro* and *in vivo*.

Ex vivo models using tumor-derived explants are currently exploited for drug screening purposes providing the opportunity to test a wide range of different drugs in the same tumor preserving its microenvironment and architecture (Powley et al., 2020). Despite having used *in vivo* models to test Digoxin´s senolytic activity, we

wanted to develop an *ex vivo* system that would allow us to test in the future tissue directly derived from cancer patients.

As a proof of concept, we tried to test the senolytic activity of Digoxin on tumor-derived explants obtained from an *in vivo* model of melanoma by subcutaneously injecting SK-Mel-103 cells in nude mice. In this model, we used the CDK4/6 inhibitor Palbociclib as a senescence-inducing agent once the tumors were palpable, and at the end of the treatment, the tumors were resected. Then, these explants were treated *in vitro* with Navitoclax, as control of senolysis, or with Digoxin, and we measured SABG staining. We showed that explants derived from Palbociclib-treated mice and treated with vehicle showed SABG-positive staining, while treatemnt with Navitoclax or Digoxin were negative. More specific senescence markers should be implemented to further confirm the complete elimination of senescent cells in the explants.

After these observations, our aim is to exploit the use of tumor-derived explants to further validate the mechanisms of action of Digoxin observed *in vitro* and also for high-throughput screenings in the future.

CONCLUSIONS

1. We have developed an *in vitro* system to perform high-throughput screenings for the discovery of senolytics.
2. We performed a high-throughput screening of the Prestwick chemical library and we have identified the family of Cardiac Glycosides (CG) as potential senolytics in an in vitro model of lung adenocarcinoma and chemotherapy-induced senescence.
3. We validated the CG, and more specifically Digoxin, as senolytic drugs in:
    - 3.1 Different cancer and normal cell lines in vitro after Bleomycin-induced senescence.
    - 3.2 Patient-derived chondrocytes with osteoarthritic disease.
    - 3.3 An in vivo model of cancer after chemotherapy-induced senescence.
    - 3.4 In tumor-derived explants from mice treated with Palbociclib.
4. We characterized the mechanism of action of Digoxin as a senolytic, and found that:
    - 4.1 The main mechanism by which Digoxin induces cell death is apoptosis.
    - 4.2 Digoxin acts by inhibiting NKA in senescent cells and the overexpression of the pump almost completely restores cell viability.
    - 4.3 Digoxin-mediated NKA inhibition causes intracellular sodium accumulation, which consequently causes an ionic imbalance in senescent cells.
    - 4.4 The replacement of potassium ions in senescent cells has a protective effect against Digoxin.
    - 4.5 Digoxin, by targeting the NKA, activates the NKA-dependent signaling cascade.
    - 4.6 Digoxin reduces the levels of p53 and p21 in senescent cells.
    - 4.7 Digoxin promotes the nuclear translocation of TFEB, which transcriptionally regulates pro-autophagy genes.
    - 4.8 Digoxin affects the autophagosome-lysosome fusion by impairing the lysosomal integrity. This observation

suggests that lysosomal membrane permeabilization is a senolytic target.

4.9 Digoxin reduces the mitochondrial membrane potential.

COMPETING INTERESTS

Mª del Pilar Picallos Rabina, the autor of this thesis, has no competing interests.

PUBLISHED DATA INFORMATION

Mª del Pilar Picallos Rabina

The results shown in the chapters and sections indicated have already been published in the paper shown below.

Identification and characterization of Cardiac Glycosides as senolytic compounds

Francisco Triana-Martínez [1,2,15], Pilar Picallos-Rabina [1,15], Sabela Da Silva-Álvarez [1], Federico Pietrocola [3], Susana Llanos [4], Verónica Rodilla [5], Enrica Soprano [6], Pablo Pedrosa [1], Alba Ferreirós [1], Marta Barradas [7], Fernanda Hernández-González [3,8], Marta Lalinde [5], Neus Prats [3], Cristina Bernadó [5], Patricia González [9], María Gómez [9], Maria P. Ikonomopoulou [10], Pablo J. Fernández-Marcos [7], Tomás García-Caballero [11], Pablo del Pino [6], Joaquín Arribas [5,12], Anxo Vida [13], Miguel González-Barcia [14], Manuel Serrano [3,12], María I. Loza [2], Eduardo Domínguez [2*] & Manuel Collado [1*]

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Mª del Pilar Picallos Rabina and Francisco Triana Martinez are co-first authors of the article.

Performance and design of most of the experiments together with Francisco Triana-Martinez, and writing the manuscript with Manuel Collado.

Quality index: Nature Communications Journal has actually an impact factor of 17.690 (2021 Journal Citation Reports) and an impact factor of 12.121f the publication of the paper (2019 Journal Citation Reports).

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Cellular senescence is an anticancer mechanism preventing the proliferation of damaged cells. However, the uncontrolled accumulation or the inappropriate elimination of senescent cells has been related to the development of different age-related diseases and cancer. For this reason, and in order to prevent the detrimental effects of senescence, in recent years new approaches have emerged to specifically eliminate these senescent cells by using senolytics. In this thesis, we performed a high-throughput screening of a chemical library to identify novel senolytic compounds, and we found Digoxin as a promising senolytic drug. We characterized the senolytic potential of Digoxin in different models of cancer in vivo and in vitro, and described the mechanisms of action behind the senolytic activity of Digoxin.