Examining the effect of mitochondrial function manipulation in the development of cellular senescence

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A thesis submitted for the degree of Doctor of Philosophy

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November 2021

Abstract

Mitochondrial dysfunction and cellular senescence are key drivers of ageing and age-related disease, and are linked by several processes. The mitochondria are essential for the induction of senescence, and oxidative stress from mitochondrial respiratory dysfunction is one of the known triggers of senescence. Senescent cells accumulate with age and secrete pro-inflammatory cytokines and chemokines termed the Senescence-Associated Secretory Phenotype (SASP), which contributes to tissue dysfunction and disease. However, because cell cycle arrest of damaged cells is a beneficial tumour suppressor mechanism, this study aimed remove the harmful SASP while maintaining cell cycle arrest. To do this I screened inhibitors of the mitochondrial electron transport chain (ETC) to manipulate mitochondrial function at different respiratory complexes and examine the effects on SASP factor production.

I found that interrupting electron flow at multiple points in the ETC reduced SASP factor secretion *in vitro*. Data suggests that this reduction was a result of respiratory inhibition, as SASP factor reduction correlated with oxygen consumption rates. Additional markers of senescence including DNA damage foci, nuclear enlargement and SA-β-Gal activity were reduced following inhibition at respiratory complex I by the biguanide metformin, but cells remained arrested. Finally, I found that long-term treatment of an established senescent phenotype with senostatic drugs metformin and rapamycin, and CIII inhibitor myxothiazol, was sufficient to alleviate the SASP, but not other markers of senescence. Similarly, shorter treatments with myxothiazol or rapamycin after irradiation successfully inhibited secretion of some key SASP factors, but other markers remained unchanged.

Overall, this study shows that manipulation of mitochondrial electron transport rescues harmful aspects of the senescent phenotype without allowing cell-cycle re-entry. Moreover, I have provided evidence that treatment timing is key to effectiveness, and that the SASP appears more receptive to reversal than other senescent markers, offering a promising avenue of investigation in age-related disease treatment.

Acknowledgements

I would like to thank my supervisors Thomas von Zglinicki, Satomi Miwa, Joao Passos and Alberto Sanz for their guidance, advice and support during this project. Thomas and Satomi for accepting me into your lab for the last 2 years, and Alberto and Joao for supporting me during the first 2 years and since then advising me from Glasgow and the US. Your encouragement has been invaluable throughout. Thanks also to my assessment panel Laura and Viktor for your advice each year.

I have been lucky enough to work alongside colleagues from three different labs in my time at CAV, and would like to thank everyone for their help and support over the last few years. To my Sanz Lab friends Rhoda, Elise, Charlotte and Filippo, I couldn't have done it without you. I may not have been a 'fly person' but you made me feel welcome, talked me down from experimental despair, made me laugh when I wanted to cry, and have kept me going through lab moves and lockdowns. I will be forever grateful for your friendships and look forward to the next time we can meet in person. Until then I have the memories of beach trips, cocktails and escape rooms. Thanks for never letting me live down my spelling failures with 'Audi with an O'!

I would also like to thank my Passos lab colleagues James, Stella, Anthony, Leena, Mikolaj and Hanna for your help and guidance in my first year, though it may have been short it was most certainly sweet – if for no other reason than the sheer amount of cake! Thanks to Stella and Antho for your guidance in experiments and microscopy, and a special thank you to James who taught me everything I know about tissue culture and ELISAs.

To my newest lab colleagues Ed, Evon, Ghazal, Tengfei and Abbas I would like to thank you for welcoming me into your lab, and for your help over the last 2 years getting me to this finish line. Thank you for being my sounding boards during lab meetings and helping me to 'see the wood for the trees' in my data. Thanks also to my other office-mates over the years, Lucy, Yoana, George and Tetsushi. I would also like to give special thanks to Glyn, I could not have done any of my microscopy work without you, you truly are my bioimaging hero!

Thanks also to the wonderful DiMeN cohorts I've met and worked with, and in particular to Emily who has been such a help throughout and has gone above and beyond to support us all.

To Joe, my partner and best friend, I can't express how much your support has meant over the years. Not just these last four but the six before that too, never once doubting that I could achieve exactly what I wanted to. Your laughter, music (whether asked for or not!) and care has gotten me through the hardest of years. Thank you for always being there for me, I couldn't do any of this without you. Here's to the next adventure together!

Finally, to my family, especially my Mam, Dad and sister Rachel. Thank you for always believing in me and supporting me in everything I do. I wouldn't be where I am if not for your constant encouragement and continual dismissal of any self-doubt! I know you're always there when I need you, and can always come home for a cuppa and a hug. Thank you for being my constants over the years.

List of Abbreviations

4EBP	eIF4E-binding protein
53BP1	p53-binding protein 1
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATM	Ataxia-telangiectasis, mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasis and rad3-related
Aβ	Amyloid-beta
BCL-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CDC25	Cell-division cycle 25
CDK	Cyclin dependent kinase
cGAS	Cyclic DMP-AMP synthase
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CV	Complex V
DDF	DNA damage foci
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
DRP1	Dynamin-related protein 1
DSB	Double strand break
E2F1	E2F transcription factor 1
ECAR	Extracellular acidification rate
ELISA	Enzyme Linked Immunosorbent Assay
ETC	Electron transport chain

FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Fe-S	Iron-sulphur
FKBP12	FK506-binding protein 12
GPDH	Glycerol-3-phosphate deyhdrogenase
H2AX	Histone variant H2AX
H2O2	Hydrogen peroxide
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1-alpha
HuR	Human antigen R
IFNβ	Interferon beta
IR	Irradiation
KCN	Potassium cyanide
LDH	Lactate dehydrogenase
MDC1	Mediator of DNA Damage Checkpoint Protein 1
MDH1/2	Malate dehydrogenase 1/2
MiDAS	Mitochondrial dysfunction-associated senescence
MRN	MRE-11-RAD50-NBS1
mtDNA	mitochondrial DNA
mTOR	Mechanistic target of rapamycin
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
OCR	Oxygen consumption rate
OIS	Oncogene-induced senescence
OPA1	Optic atrophy 1
OXPHOS	Oxidative Phosphorylation
PARP	Poly(ADP-ribose) polymerase
PCNA	Proliferating Cell Nuclear Antigen
PDH	Pyruvate dehydrogenase
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PI3K	Phosphoinositide 3-kinase

PIKK	Phosphatidylinositol 3-kinase-like protein kinase
PINK1	PTEN-induced kinase 1
PTEN	Phosphatase and tensin homolog
RET	Reverse electron transport
ROS	Reactive Oxygen Species
S1QEL	Suppressors of Site I_Q Electron Leak
S3QEL	Suppressors of Site III_{Qo} Electron Leak
S6K1	S6 kinase beta-1
SAHF	Senescence Associated Heterochromatin Foci
SAMD	Senescence-associated mitochondrial dysfunction
SASP	Senescence Associated Secretory Phenotype
SA-β-Gal	Senescence-associated beta galactosidase
SIPS	Stress-induced premature senescence
SIRT	Sirtuin-1
SOD	Superoxide dismutase
SSB	Single strand break
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator or interferon genes
TFEB	Transcription factor EB
TGFβ	Transforming growth factor beta
TRF2	Telomeric repeat-binding factor 2
γH2AX	Phosphorylated histone variant H2AX

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Chapter 1: Introduction

1.1 Human ageing and disease

The ageing population in the UK is set to increase exponentially over the coming years, with current estimations predicting an additional 7.5 million people aged over 65 within the next 50 years (Office for National Statistics, 2021). With an ever-growing aged population comes increasing strain on health and social care systems to try to meet demand. Advanced age is associated with a plethora of chronic conditions, diseases and dysfunctions, which require medical care and social support, often long-term, in order to help manage symptoms and maintain quality of life. By attempting to understand why and how we age, and the drivers of age-related diseases, the ageing research field hopes to slow the ageing process and, more importantly, prevent the development of associated diseases. If successful, this would allow people to age healthily without the strain of disease – both on themselves, friends and family, and societal services.

Age is the greatest risk factor for conditions such as Alzheimer's disease (van der Flier and Scheltens, 2005), heart failure (North and Sinclair, 2012), diabetes (Chang and Halter, 2003), and arthritis (Loeser, 2017). As we age, our bodies are less able to combat damage and stress generated at the cellular level, and chronic accumulation of this damage can lead to disease. By targeting this damage and finding ways to prevent or repair it, we aim to alleviate some of the burden of age-related diseases. To date little progress has been made in slowing the ageing process, but a number of steps have been taken towards understanding the mechanisms behind it. Many age-related diseases, and ageing itself, have been linked to a state of irreversible cell cycle arrest in which cells accumulate damage over time, which eventually signals a permanent growth arrest. These cells continue to survive, but secrete a number of harmful products into the surrounding tissue microenvironment, producing a self-sustaining loop of damage and dysfunction culminating in disease. This state is known as cellular senescence (Hayflick and Moorhead, 1961).

1.2 Cellular senescence

Cellular senescence is a state of irreversible cell cycle arrest, first described by Hayflick and Moorhead in 1961, who identified a limit to the number of divisions proliferating cells could undergo in culture before halting cell cycle progression (Hayflick and Moorhead, 1961). This growth arrest was termed replicative senescence. Cells which undergo senescence are typically arrested in the G1 phase of the cell cycle (Stein and Dulic, 1995) and are resistant to cell death signalling due to upregulated pro-survival pathways such as BCL-2 family members (Yosef *et al.*, 2016). Though this resistance can differ depending on a range of factors that determine cell fate, including cell type, stimuli strength, and manipulation of pro- and anti-apoptotic proteins (Rebbaa *et al.*, 2003; Hampel *et al.*, 2004).

Senescent cells are known to accumulate with age in both animals (Wang *et al.*, 2009; Jurk *et al.*, 2014) and humans (Dimri *et al.*, 1995; Liu *et al.*, 2009a), and have been shown to impact lifespan (Baker *et al.*, 2016). Cellular senescence is largely thought to be specific to cells capable of proliferation; mitotic cells, which comprise tissues and organs that require continual renewal of the cell population, for example skin (Dimri *et al.*, 1995), liver (Wiemann *et al.*, 2002) and kidneys (Krishnamurthy *et al.*, 2004a). However, recent studies have also identified a senescent-like phenotype in post-mitotic cells (Jurk *et al.*, 2012; Oubaha *et al.*, 2016; Musi *et al.*, 2018; Anderson *et al.*, 2019), suggesting that stimuli such as oxidative or genotoxic stress are able to induce a persistent DDR and senescent-like growth arrest in postmitotic tissues.

Cellular senescence in the liver has been linked to cirrhosis (Wiemann *et al.*, 2002) and non-alcoholic fatty liver disease (NAFLD) (Ogrodnik *et al.*, 2017), while in the articular cartilage it has been shown to induce cartilage breakdown and onset of osteoarthritis (Loeser, 2009). This accumulation of senescent cells in tissue has also been well-established as a driver of ageing (Baker *et al.*, 2004; Baker;Jin and van Deursen, 2008; Wang *et al.*, 2009) and the specific removal of senescent cells in *in vivo* models has shown beneficial effects in alleviating disease and prolonging lifespan (Baker *et al.*, 2011; Baker *et al.*, 2016).

Senescent cells undergo a wide range of phenotypic changes which are discussed in detail later, and are responsible for both autocrine and paracrine effects which stabilise the existing phenotype (Takahashi *et al.*, 2006; Passos *et al.*, 2010) and promote senescence conversion in the wider tissue microenvironment (Nelson *et al.*, 2012). This accumulation of senescence is responsible for a multitude of tissue dysfunctions, but can also support beneficial wound healing processes (Jun and Lau, 2010) and provide protection against pre-malignant cells (Serrano *et al.*, 1997).

1.2.1. Senescence signalling pathways

Cellular senescence can be induced by a variety of stressors including oxidative stress (Passos *et al.*, 2007; Passos *et al.*, 2010), oncogene activation (Serrano *et al.*, 1997), and telomere attrition (van Steensel;Smogorzewska and de Lange, 1998). Though there are some variations in senescent phenotype, most stimuli converge on common signalling pathways. Which pathways are engaged, and to what extent, can have both cell- and species-specific differences (Smogorzewska and de Lange, 2002).

1.2.1.1 DNA damage response

In senescence the DNA damage response pathway (DDR) is triggered by DNA breaks, leading to cell-cycle arrest signalling at key checkpoints. DNA damage can be caused by oxidative stress (Chen *et al.*, 1995) oncogenic signalling (Serrano *et al.*, 1997), telomere attrition (d'Adda di Fagagna *et al.*, 2003), and genotoxic stress (Roberson *et al.*, 2005). Different DDR proteins are activated by different DNA breaks - double- or single-strand (DSBs or SSBs, respectively) – but converge on key points in the cascade (**Figure *1.1***).

DSBs are primarily detected by the DNA damage kinase ataxia-telangiectasia mutated (ATM) , or by DNA-dependent protein kinase (DNA-PK), both members of the phosphatidylinositol-3 kinase-like protein kinase (PIKK) family (Ciccia and Elledge, 2010). The MRE11-RAD50-NBS1 (MRN) complex, which is responsible for tethering DNA ends during repair, binds to DSBs and recruits ATM to the damage site (Mirzoeva and Petrini, 2001; Dupre;Boyer-Chatenet and Gautier, 2006). ATM then undergoes autophosphorylation (Bakkenist and Kastan, 2003), which activates ATM kinase activity and in turn phosphorylates the histone variant H2AX at serine 139, producing γH2AX (Burma *et al.*, 2001). γH2AX is recognised by mediator of DNA damage checkpoint 1

(MDC1) (Savic *et al.*, 2009), which triggers further activation of the MRN complex and ATM (Lieber, 2010; Lavin *et al.*, 2015) forming a positive feedback loop of ATM activity and γH2AX formation. γH2AX mediates the recruitment of chromatin remodelling proteins which help relax the chromatin to allow access for DDR proteins (Nakamura *et al.*, 2010). MDC1 also recruits additional DDR proteins such as p53 binding protein 1 (53BP1) to the damage site (Eliezer *et al.*, 2009). Sites of DNA damage can be identified by the accumulation of such DDR factors which localise at the point of damage, including γH2AX, 53BP1 or ATM (d'Adda di Fagagna *et al.*, 2003; Jackson and Bartek, 2009).

The checkpoint kinase Chk2 activates in response to ATM phosphorylation, stimulating its translocation to the nucleus (Buscemi *et al.*, 2004; Bekker-Jensen *et al.*, 2006) where along with ATM it promotes the dissociation of the transcriptional regulator p53 from its negative regulator HDM2 (Turenne *et al.*, 2001). p53 is also a major regulator in determining cell fate, and is thought to be an important communicator between senescent and apoptotic pathways (Seluanov *et al.*, 2001; Jackson and Pereira-Smith, 2006). This stabilisation of p53 allows it to promote transcription of the CDK inhibitor p21 (Jackson and Pereira-Smith, 2006), which blocks the apoptotic signalling cascade in the cytoplasm and is essential for cell-cycle arrest (Dutto *et al.*, 2015). p21 can induce cell cycle arrest by one of two pathways; i) inhibition of proliferating cell nuclear antigen (PCNA) (Cayrol;Knibiehler and Ducommun, 1998), or ii) inhibition of cyclin-dependent kinase CDK-1, -2, and -4/6 complexes. Inhibition of these CDK complexes results in hypo-phosphorylation of retinoblastoma protein (Rb) and reinforcement of its inhibitory binding to E2F1, culminating in cell cycle arrest (Brugarolas *et al.*, 1999; Georgakilas;Martin and Bonner, 2017). This p53-p21 signalling pathway is essential for senescent growth arrest (Di Leonardo *et al.*, 1994).

In addition to p53 stabilisation, Chk2 induces the degradation of cell-division cycle 25 (CDC25) phosphatase, which contributes to cell-cycle arrest by preventing G1-S phase transition (Mailand *et al.*, 2000).

Recognition of DSBs by DNA-PK activates non-homologous end joining (NHEJ) repair pathways. DNA-PK is recruited to DNA damage sites in combination with the endonuclease Artemis, and undergoes autophosphorylation upon binding with Artemis.

This activated Artemis-DNA-PK complex is able to remove DNA overhangs to create end structures which can be repaired by NHEJ ligase complexes (Chang *et al.*, 2017).

DSBs also stimulate the activity of the CDK inhibitor p16, which provides a second barrier to proliferation of damaged cells. p16 is a positive regulator of the pRb tumour suppressor pathway and can induce cell-cycle arrest in a similar manner to p21, by inhibiting CDK4/6 activity, preventing Rb phosphorylation and promoting its continued inhibition by the E2F1 transcription factor (Beausejour *et al.*, 2003). By preventing translocation of pRb to the nucleus, p16 activity prevents the transcription of key target genes involved in the transition from G1 to S phase (Rayess;Wang and Srivatsan, 2012). p16-pRb signalling is also essential for formation of senescence-associated heterochromatin foci (SAHF), which are known to silence proliferation genes, though once established these SAHFs do not require either p16 or pRb for maintenance (Narita *et al.*, 2003).

SSBs are recognised by the DNA damage kinase ATR (d'Adda di Fagagna, 2008) which recruits the Chk1 kinase (Wang *et al.*, 2006). Like Chk2, Chk1 also phosphorylates and causes degradation of CDC52, promoting cell cycle arrest (Furnari;Rhind and Russell, 1997; Mailand *et al.*, 2000). Both DSBs and SSBs also activate PARP1 and 2, which are essential for the recruitment of additional DNA repair proteins (Haince *et al.*, 2008).

DNA damage checkpoints, p53-p21, and p16-pRb signalling pathways maintain cell cycle arrest until the damage can be repaired. As senescent cells are often at a point of severe – but sub-apoptotic - accumulated damage, this arrest becomes permanent along with a persistent DDR as the damage cannot be repaired. This is especially the case in replicative senescence, which is caused by telomere attrition, as telomeres are inaccessible to DNA repair machinery and so the DDR stimulated by this form of ‘damage’ is persistent (Galbiati;Beausejour and d'Adda di Fagagna, 2017). Indeed, a persistent DDR is one of the core requirements of a senescent phenotype (Fumagalli *et al.*, 2014).

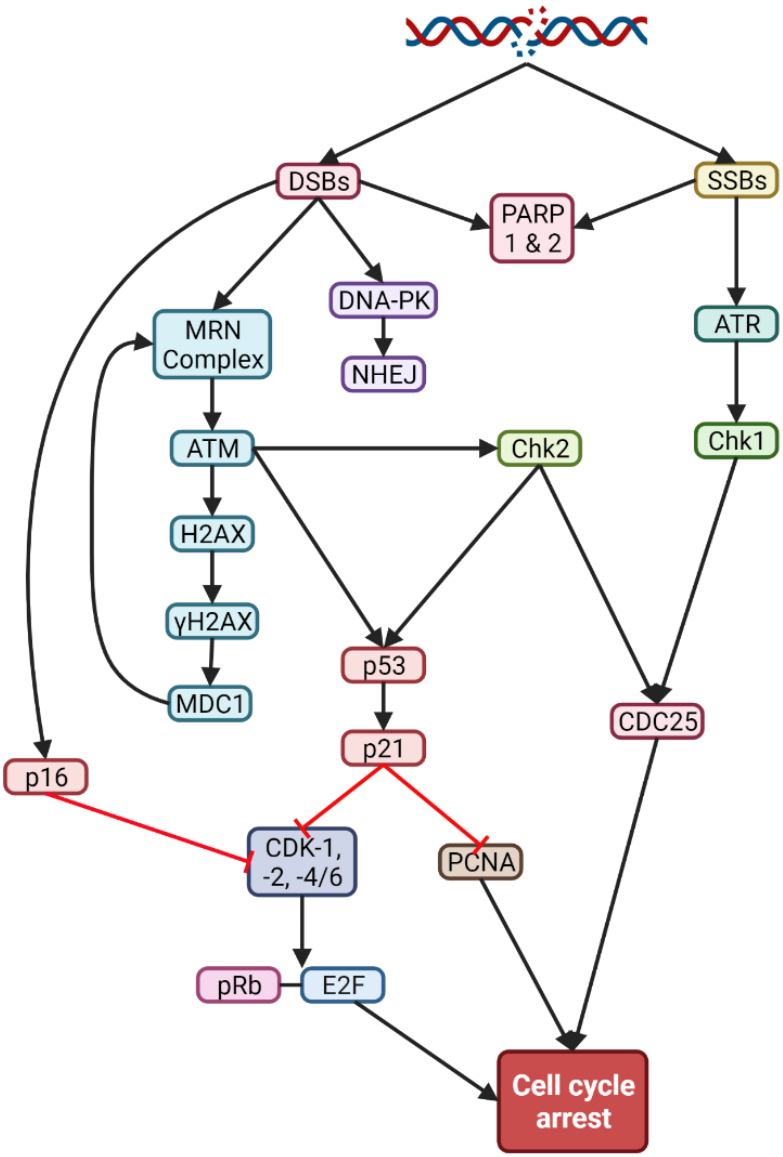


Figure 1.1: Overview of the DNA damage response. Many senescence-inducing stimuli such as telomere attrition, oxidative stress and genotoxic chemicals can cause DNA damage and trigger a DDR. Recruitment of the ATM and ATR kinases to sites of DNA damage leads to nuclear translocation of the Chk1 & 2 kinases, which phosphorylate and trigger degradation of CDC25 to promote cell-cycle arrest. ATM stimulates p53/p21 signalling and subsequent prevention of pRb phosphorylation though inhibition of key cyclin-dependent kinases, as well as formation of γH2AX in a self-sustaining loop with MDC1/MRN Complex activation which can be used to identify DNA damage foci. DSBs are also recognised by DNA-PK which promotes non-homologous end joining repair processes, and p16/pRb signalling pathways that further stimulate cell-cycle arrest through CDK inhibition and prevention of pRb phosphorylation.

1.2.1.2 mTOR

mTOR is a serine/threonine protein kinase and member of the PIKK family that functions as a sensor of nutrient and growth factor availability and regulates cellular responses such as proliferation, apoptosis and inflammation as required. Activation of mTOR can be triggered by changes in amino acid, oxygen, or lipid availability, or by an increase in the AMP (or ADP)/ATP ratio (Jewell;Russell and Guan, 2013). mTOR is formed of two primary complexes - mTORC1 and mTORC2. mTORC1 has been identified as a major regulator of mitochondrial functions including biogenesis (Cunningham *et al.*, 2007) and metabolism (Schieke *et al.*, 2006), and is formed of three core subunits: mTOR, regulatory protein associated with mTOR (Raptor), and mammalian lethal with Sec13 protein 8 (mLST8) (Kim *et al.*, 2002; Kim *et al.*, 2003). By comparison, mTORC2 is insensitive to rapamycin treatment and contains rapamycin insensitive companion of mTOR (Rictor) in place of Raptor (Jacinto *et al.*, 2004). mTORC1 plays a central role in regulating cell growth and division, including promoting protein synthesis, glucose metabolism, and protein turnover.

By phosphorylating S6K1 and 4EBP, mTORC1 both stimulates mRNA translation via S6K1 substrate activation (Holz *et al.*, 2005), and prevents 4EBP inhibitory effects by promoting its dissociation from eIF4E to allow translation to occur (Gingras *et al.*, 1999). mTORC1 has also been found to elevate glycolysis through HIF-1α stimulation (Duvel *et al.*, 2010) and downregulate OXPHOS, promote nucleotide synthesis required for DNA replication (Ben-Sahra *et al.*, 2013; Ben-Sahra *et al.*, 2016), and inhibit autophagy by both TFEB translocation inhibition (Martina *et al.*, 2012) and preventing early stage AMPK activation in the autophagic process (Kim *et al.*, 2011). It is clear from these observations that the mTOR pathway is a major regulator of cellular metabolism, and its manipulation has been a favoured target of the ageing field for many years.

mTORC1 is an important mediator of ageing (Johnson;Rabinovitch and Kaeberlein, 2013), and its dysregulation has been found in metabolic diseases such as diabetes and obesity (Khamzina *et al.*, 2005; Fraenkel *et al.*, 2008) which are common comorbidities for other age-related diseases. To date the only treatment identified which has been capable of extending lifespan in all tested models is rapamycin inhibition of mTORC1 (Harrison *et al.*, 2009; Lamming *et al.*, 2013). Rapamycin treatment has shown success as a longevity-

promoting drug in fruit flies (Kapahi *et al.*, 2004), worms (Vellai *et al.*, 2003), yeast (Kaeberlein *et al.*, 2005) and mice (Hurez *et al.*, 2015; Johnson *et al.*, 2015). Additionally, rapamycin has shown promise in improving immune response (Mannick *et al.*, 2014), alleviating frailty (Xue *et al.*, 2016) and age-related diseases such as diabetes (Zhou and Ye, 2018), osteoarthritis (Carames *et al.*, 2012), and Alzheimer's disease pathology (Siman;Cocca and Dong, 2015; Lin *et al.*, 2017). There are numerous pathways through which mTORC1 inhibition can produce these beneficial effects. Increased autophagy may promote clearance of dysfunctional mitochondria (Vellai *et al.*, 2003), and a reduction in mRNA translation may slow the accumulation of misfolded proteins and proteotoxic stress.

mTORC1 has also been implicated in the development of cellular senescence (Herranz *et al.*, 2015; Correia-Melo *et al.*, 2016). Indeed, many senescent cell models exhibit elevated mTORC1 activity, and mTORC1 is known to negatively regulate parts of the DNA damage repair pathway (Dominick *et al.*, 2017). Mice treated with rapamycin also develop age-related pathologies at a slower rate (Wilkinson *et al.*, 2012). mTORC1 is thought to be a major driver of the metabolic changes observed in senescent cells (Weichhart, 2018). Treatment with rapamycin has been shown to successfully inhibit SASP development both by activating the RNA-binding protein ZFP36L1 to promote its degradation of SASP components (Herranz *et al.*, 2015), and by suppressing translation of IL-1A and subsequently preventing transcription of pro-inflammatory NFκB-regulated genes (Laberge *et al.*, 2015).

1.2.1.3 AMPK

AMPK is another important mediator of senescent signalling, and its activation has been linked to lifespan extension in *c. elegans* (Mair *et al.*, 2011), and *Drosophila melanogaster* (Funakoshi *et al.*, 2011), but not in mice (Viollet *et al.*, 2003). AMPK is an important energy sensor that monitors AMP/ATP and ADP/ATP ratios within the cell, and is often referred to as a 'starvation pathway' that responds to conditions of energetic deficit (Greer and Brunet, 2009; Hardie and Ashford, 2014). Increases in these ratios as a result of cellular stresses activates AMPK, which in turn promotes ATP-producing pathways such as glycolysis, mitochondrial biogenesis and autophagy (Zong *et al.*, 2002; Kim *et al.*, 2011), and inhibits ATP-consuming processes such as fatty acid and protein synthesis, largely by

inhibiting mTORC1 signalling, in an attempt to restore energy homeostasis (Inoki;Zhu and Guan, 2003; Gwinn *et al.*, 2008).

The observed 'glycolytic state' of senescent cells is achieved by the diversion of pyruvate away from oxidative phosphorylation and towards aerobic glycolysis. This results from a promotion of lactate dehydrogenase (LDH) activity and inhibition of pyruvate dehydrogenase (PDH) and the malate-aspartate shuttle (MDH1 and MDH2) (Zwerschke *et al.*, 2003; Wiley and Campisi, 2016). The resulting decrease in cytosolic NAD+/NADH ratio and concomitant increase in the ADP/ATP ratio activates AMPK (Wiley *et al.*, 2016). Activation of PARP1 during the DDR exacerbates this decrease in NAD+/NADH ratio as PARP1 activity consumes NAD+ (Du *et al.*, 2003). AMPK then phosphorylates p53, promoting its stabilisation and triggering growth arrest, senescence induction and production of a SASP (Peyton *et al.*, 2012; Nacarelli *et al.*, 2019).

In addition to upregulation of p53 activity, AMPK also increases p16 expression by inhibiting the p16 destabilizer human antigen R (HuR), and inhibits cell proliferation by preventing phosphorylation of the Rb protein and downregulating the activity of genes involved in cell proliferation such as cyclin A, cyclin B1, and cyclin E (Peyton *et al.*, 2012). AMPK is also a key inhibitor of mTORC1 activity by inactivating the upstream activator of mTORC1, Rheb (Inoki;Zhu and Guan, 2003), and phosphorylating the Raptor subunit of mTORC1 (Gwinn *et al.*, 2008).

AMPK activation also has significant implications for mitochondrial function, and can promote mitochondrial fission by stimulating Drp recruitment to the mitochondria (Toyama *et al.*, 2016), as well as upregulating mitochondria biogenesis by direct phosphorylation of PGC-1α (Jager *et al.*, 2007). Interestingly, in mitochondrial dysfunction-associated senescence (MiDAS; discussed in the next section), AMPK activation has also been found to inhibit the IL-1 arm of the SASP through p53-mediated signalling (Wiley *et al.*, 2016).

1.2.2 Types of senescence

Senescence can be triggered by a range of cellular stresses, including telomere attrition, genotoxic stress, oncogene activation, and mitochondrial dysfunction. While these

different stimuli produce mostly overlapping phenotypes, there can be some stimuli-specific variations.

1.2.2.1 Replicative senescence

Replicative senescence was the first form of cellular senescence to be identified, when Hayflick and Moorhead demonstrated the limit of replicative capacity of cells in culture (Hayflick and Moorhead, 1961). This form of senescence results from the progressive shortening of telomere length with continued proliferation (Harley;Futcher and Greider, 1990), eventually reaching what is referred to as the 'Hayflick limit' at which point replicative senescence is initiated. Telomeres are unique chromatin structures formed of repetitive DNA (5'-TTAGGG-3' in vertebrates) and protective proteins (Blackburn, 1991) that prevent chromosome ends from being recognised as DNA strand breaks by DNA repair pathways, such as non-homologous end joining or recombination repair(d'Adda di Fagagna;Teo and Jackson, 2004). Telomeres are inaccessible to DNA repair machinery as components of the bound shelterin complex such as TRF2 inhibit the 'repair' of telomeres by inhibiting NHEJ (Bae and Baumann, 2007). However, during cell division DNA polymerase is unable to fully replicate these telomeric ends, and as such telomeres shorten slightly with each division until they reach a critical length (Harley;Futcher and Greider, 1990). At this point their protective capping structures are lost, triggering a DDR (d'Adda di Fagagna *et al.*, 2003). This means that the 'damage' cannot be repaired and results in a persistent DDR and induction of replicative senescence. Only a few short telomeres are sufficient to trigger a DDR and senescence (Hemann *et al.*, 2001).

The role of telomere shortening in senescence was confirmed by forced expression of telomerase, which prevented senescence and allowed cells to continually proliferate (Bodnar *et al.*, 1998). Telomere attrition has also been identified as an indicator of age-related disease risk, and a correlation discovered between telomere length and metabolic and cardiac disease outcome (Benetos *et al.*, 2013; D'Mello *et al.*, 2015). In addition to chronic telomere attrition during cell division, telomeric DNA is particularly susceptible to oxidative damage from ROS production (von Zglinicki, 2002; Reichert and Stier, 2017), and the formation of DNA damage foci (DDFs) at telomeres is a reliable marker of senescence (Takai;Smogorzewska and de Lange, 2003).

1.2.2.2 Oncogene-induced senescence

Cellular senescence was also found to be induced by aberrant oncogene signalling when an oncogenic form of RAS, a transducer of mitogenic signals, was expressed in normal human fibroblasts (Serrano et al 1997). This has since been corroborated by expression of other oncogenic proteins involved in cell growth and tumour suppression, such as BRAF (Michaloglou *et al.*, 2005), E2F (Lazzerini Denchi *et al.*, 2005), PTEN and p53 (Chen *et al.*, 2005). This oncogene-induced senescence (OIS) exhibits a similar phenotype to most other forms of senescence, demonstrating high p16 levels (Ohtani *et al.*, 2001), SAHF formation (Zhang *et al.*, 2005), and a persistent DDR (Di Micco *et al.*, 2006), but not all cells undergo OIS (Benanti and Galloway, 2004; Skinner *et al.*, 2004). In contrast to other forms of senescence, OIS is also often characterised by an initial hyperproliferative phase and associated DNA replication alterations. This results in recruitment of a DDR and eventual growth arrest (Di Micco *et al.*, 2006). In addition, oncogenic RAS has been shown to induce an amplified SASP in comparison to other forms of senescence (Coppe *et al.*, 2008). OIS is also thought to be triggered by excessive mitogenic stimulation (Lin *et al.*, 1998; Mathon *et al.*, 2001), supported by the observation that cells relieved of mitogenic pressure by culture in serum-free medium do not undergo oncogene-induced senescence (Woo and Poon, 2004).

Despite the tumour-suppressive role of cellular senescence, the telomerase-dependent extension of cell replication does not also promote malignant transformation, demonstrating a key difference between immortalised and tumorigenic cells (Morales *et al.*, 1999).

1.2.2.3 Stress-induced senescence

Some cells are also able to undergo senescence without telomere shortening, but instead as a result of stress or damage. This stress-induced senescence primarily involves elevated p16 expression and pRb signalling pathways in response to DNA damage (Parrinello *et al.*, 2003). In addition to replicative senescence as a result of telomere attrition, DNA damage can also induce senescence a) at sites other than telomeres, and b) at telomeres that are not critically shortened (Di Leonardo *et al.*, 1994). Such damage can be caused by ionising radiation, oxidative stress (Parrinello *et al.*, 2003), or genotoxic chemicals such as

chemotherapeutic drug treatments (Roninson, 2003). Such DNA-damaging agents cause accumulation of senescent cells in young animals (Le *et al.*, 2010; Shao *et al.*, 2014).

Additionally, studies have triggered stress-induced senescence by upregulation of key SASP factors IFNβ and TGFβ. While IGFβ signalling caused elevated ROS production and induction of a p53-dependent DDR (Moiseeva *et al.*, 2006), TGFβ signalling resulted in activation of the p16-pRb pathway and formation of heterochromatin foci (Vijayachandra;Lee and Glick, 2003; Zhang and Cohen, 2004), further demonstrating a link between pro-inflammatory cytokine secretion and senescence induction (Nelson *et al.*, 2012). IFNβ has also been shown to induce translocation of the transcription factor STAT3 to the mitochondria, downregulating the activities of mitochondrial respiratory complexes I and II (Cannino *et al.*, 2012), which are discussed in detail later. Similarly, TGFβ is able to induce mitochondrial ROS production, and its inhibition prevents both ROS production and DDR induction (Passos *et al.*, 2010).

1.2.2.4 Mitochondrial dysfunction-associated senescence (MiDAS)

Mitochondrial dysfunction and cellular senescence are strongly interlinked, and has each been shown to promote the other (Wiley *et al.*, 2016; Nelson *et al.*, 2018). Senescent cells exhibit changes in mitochondrial morphology and function, while mitochondrial dysfunction is a known cause of oxidative stress and senescence induction. Interestingly, however, senescence induced by mitochondrial dysfunction has been shown to exhibit a unique SASP, which lacks the pro-inflammatory IL-1 arm of the phenotype (Wiley *et al.*, 2016). This MiDAS phenotype comprised loss of LaminB1 protein, decreased NAD+/NADH ratio and elevated AMPK activation, along with increased p53 signalling, but reduced NFκB activity and lack of IL-1-dependent secretome.

Mitochondrial SIRTs are able to suppress senescence, and inhibition of SIRT3 specifically induced senescence with a distinct secretory phenotype lacking SASP factors including IL-1β, IL-6 and IL-8. From this, Wiley and colleagues suggested that mitochondrial function is required for expression of a specific subset of pro-inflammatory cytokines. This was supported by similar observations in cells subjected to mtDNA depletion, treatment with CI inhibitor rotenone, or depletion of the mitochondrial chaperone HSPA9. All treatments induced mitochondrial dysfunction, and triggered a senescent phenotype with a distinct secretory phenotype compared to other forms of senescence.

MiDAS also appears to be independent of DNA damage and ROS production as the phenotype is reversible in the presence of pyruvate, but not antioxidants. Mitochondrial dysfunction can reduce the NAD+/NADH ratio, and NAD+ levels are also known to decrease with age (Braidy *et al.*, 2011). Indeed, data subsequently demonstrated that this low NAD+/NADH ratio, primarily driven by accumulation of cytosolic NADH, can drive MiDAS through activation of AMPK and subsequent p53 activation. This increased p53 activity can suppress NFκB signalling and is thus the likely cause of the absent IL-1-dependent SASP arm.

1.2.3 Markers of cellular senescence

The senescent phenotype is characterised by increased levels of DNA damage (d'Adda di Fagagna *et al.*, 2003; Rodier *et al.*, 2009), reactive oxygen species production (Passos *et al.*, 2010), and secretion of pro-inflammatory cytokines and chemokines known as the senescence-associated secretory phenotype (SASP) (Acosta *et al.*, 2008). ROS production and SASP are discussed in detail later, but here I will cover the common biomarkers present in cellular senescence. Though it is important to note that none of these markers are exclusive to senescence, meaning that in practice multiple markers must be used to accurately identify the presence of senescent cells.

Double-strand DNA breaks occur more frequently with age and in cellular senescence. These breaks are detected by sensor kinases (ATM/ATR, DNA-PK) which trigger a DNA damage response, and lead to cell-cycle arrest through activation of p53 and p21 signalling (d'Adda di Fagagna *et al.*, 2003). DNA damage foci identified by expression of γH2A.X are known to increase with age, and foci which colocalize at the telomeres (telomere-associated foci, or TAFs) are persistent and difficult to repair as previously discussed (Hewitt *et al.*, 2012). As such, any double-strand breaks located within telomeres cannot be repaired and may continuously stimulate DNA damage response pathways leading to senescence induction (d'Adda di Fagagna *et al.*, 2003; Jurk *et al.*, 2014).

Increased activity of β-galacosidase (Dimri *et al.*, 1995) arises in response to elevated lysosomal activity in senescent cells (Kurz *et al.*, 2000). As such histochemical staining for

this senescence-associated β-galactosidase (SA-β-Gal) can be used as a robust markers of cellular senescence (Lee *et al.*, 2006).

The cyclin-dependent kinase inhibitors p21 (Fang *et al.*, 1999) and p16 (Takahashi *et al.*, 2006) are also common biomarkers of senescent cells, as major regulators of the DDR. Indeed, $p16^{INK4a}$-positive cells accumulate with age and negatively influence lifespan in mice and preferential elimination of such cells delays age-related pathologies in a progeroid mouse model with BubR1 insufficiency (Baker *et al.*, 2011). Mice without p16 are also prone to tumour formation, further supporting its role in tumour suppression (Serrano *et al.*, 1997).

Senescent cells also exhibit larger cell volume (Hayflick and Moorhead, 1961), nuclear size (Swanson *et al.*, 2013) and chromatin alterations (Adams, 2009) in the form of senescence associated heterochromatin foci (SAHFs) (Narita *et al.*, 2003). Changes in nuclear architecture can impact wide-ranging functions which rely on nuclear size, including cell cycle progression (Roca-Cusachs *et al.*, 2008) and transcription (Jorgensen *et al.*, 2007). Studies suggest that nuclear size is dependent on chromatin architecture (Funayama *et al.*, 2006), cytoplasmic volume (Jorgensen *et al.*, 2007), and factors which regulate nuclear structure such as lamin B protein (Liu *et al.*, 2000; Hoffmann *et al.*, 2002; Freund *et al.*, 2012). Many of these determining factors undergo changes during cellular senescence, culminating in nuclear enlargement.

Global loss of heterochromatin has been reported in worms (Haithcock *et al.*, 2005), flies (Brandt;Krohne and Grosshans, 2008), and humans with age (Scaffidi and Misteli, 2005), and studies have even suggested that alterations to heterochromatin structure could be a driver of ageing (Zhang *et al.*, 2015b). SAHFs can be identified by preferential staining of foci with DAPI, and have been found to both silence genes responsible for proliferation and restrain DDR signalling (Narita *et al.*, 2003). This is thought to be a pro-survival mechanism as chromatin relaxation by HDAC inhibitor treatment enhances the DDR, eventually resulting in cell death (Di Micco *et al.*, 2011). However, contradictory findings have also shown that histone deacetylase inhibition (HDAi) can induce senescence (Ogryzko *et al.*, 1996; Munro *et al.*, 2004), despite promoting the formation of active (euchromatin) over silent (heterochromatin) genes. This seemingly conflicting

relationship between chromatin alterations and senescence induction may be a result of differing gene expression or cell-type specific effects.

Cytosolic chromatin fragments (CCFs) can also be released into the cytoplasm of senescent cells as a result of nuclear lamina loss (Ivanov *et al.*, 2013), and these CCFs can trigger activation of the cGAS-STING pathway, along with further immune response and SASP induction (Gluck *et al.*, 2017). Absence of DNA replication in senescent cells can be determined by absence of the proliferation marker Ki67, though this can also be applied to quiescent or post-mitotic cells.

Senescent cells are arrested in the G1 phase of the cell cycle (Stein and Dulic, 1995), but nevertheless remain very metabolically active. Despite an increase in mitochondrial mass, senescent cells exhibit a shift towards glycolysis (Zwerschke *et al.*, 2003), which is thought to result from a failure of autophagy leading to accumulated dysfunctional mitochondria. In support of this, senescent cells exhibit mild uncoupling of oxidative phosphorylation and increased proton leak, which may be induced by oxidative stress (Stadlmann *et al.*, 2002). While senescent cells tend to be more glycolytic than proliferating cells, mitochondrial respiration is still enhanced due to the increase in mitochondrial content that accompanies expanding cell size, and exhibit higher ATP production (Dorr *et al.*, 2013). This increase in mitochondrial respiration allows cells to compensate for the greater proton leak caused by functional deterioration of mitochondria with advancing cell age (Hutter et al 2004).

1.2.4 Senescence Associated Secretory Phenotype (SASP)

Senescent cells develop a pro-inflammatory secretory phenotype with numerous roles in maintaining senescence, promoting immune signalling and targeted clearance of senescent cells, and eventually tumour progression. This SASP has been linked to the high metabolic rate observed in senescent cells, though whether it is a cause or consequence is still unclear (Wiley and Campisi, 2016). Nevertheless, studies have demonstrated a possible role for metabolic reprogramming of senescent cells in regulating pro-inflammatory immune responses (Van den Bossche;O'Neill and Menon, 2017). A persistent DDR is required to initiate pro-inflammatory cytokine secretion (Rodier *et al.*, 2009) and evidence suggests that components of the DDR influence SASP production

through both positive (Freund;Patil and Campisi, 2011; Kang *et al.*, 2015) and negative (Rodier *et al.*, 2009) regulatory roles. Interestingly, however, cells induced to senesce by overexpression of $p16^{INK4a}$ do not express a SASP, despite other hallmarks of senescence (Coppe *et al.*, 2010).

The SASP is primarily made up of cytokines, chemokines and growth factors that produce a pro-inflammatory environment and are involved in cell signalling and phenotype maintenance. Though SASP composition can vary between senescent stimuli and cell type, there are a number of common factors across most senescent phenotypes. IL-6 is the most common cytokine secreted in multiple SASP models, and has been linked to activation of a persistent DDR (Coppe *et al.*, 2008; Kojima *et al.*, 2013). IL-1α and IL-1β are both involved in paracrine signalling, triggering NFκB activation in neighbouring cells through interaction with corresponding cell surface receptors. IL-1α is also an upstream regulator of other SASP factors including IL-6 and IL-8 (Orjalo *et al.*, 2009), while IL-1β is known to induce p16 activity (Philipot *et al.*, 2014). Other common SASP factors include chemokines such as IL-8 and GROα/β, IGF-binding proteins, colony-stimulating factors, matrix metalloproteinases, and extracellular insoluble molecules such as fibronectin. These pro-inflammatory SASP factors have wide-ranging roles in regulating cell survival, immune cell recruitment, cell growth and differentiation. By comparison, anti-inflammatory cytokine expression remains relatively unaffected by a senescent phenotype (Coppe *et al.*, 2010).

The SASP has both autocrine and paracrine effects, and is known to maintain the senescent phenotype and stimulate senescence in neighbouring healthy cells. SASP factors including IL-6 and IL-8 reinforce the senescent phenotype in an autocrine manner (Acosta *et al.*, 2008), while the TGF-β family, VEGF and chemokines CCL2 and CCL20 are able to interact with cell-surface receptors of nearby healthy cells to trigger specific responses and senescence-associated pathways including the DDR (Acosta *et al.*, 2013). Exposure to senescent cells over time has been found to induce senescence in nearby intact cells (Nelson *et al.*, 2012). It is thought that this senescent bystander effect is mediated by ROS-dependent NFκB signalling, resulting from senescence-associated mitochondrial dysfunction (SAMD; discussed in further detail in section 1.5.2.1) (Nelson *et al.*, 2018).

A key role of the SASP with age lies in its proposed ability to signal the immune system and target senescent cells for clearance. Studies have demonstrated an upregulation in both chemoattractant SASP factors and innate immune cells following p53 activation in liver tumours. Their role in cell clearance was confirmed when treatments that interfered with immune cell function prevented tumour clearance (Xue *et al.*, 2007). In addition, liver fibrosis has been reduced by senescence-mediated immune clearance of activated hepatic stellate cells, which was impaired in *p53*$^{-/-}$ mice (Krizhanovsky *et al.*, 2008). Studies have suggested that this system may falter with age and allow chronic inflammation to develop (Ovadya *et al.*, 2018; Karin *et al.*, 2019). NFκB is a major transcriptional regulator of the SASP, and improved lifespan and healthspan observed following its inhibition show that the SASP may have an integral role in the ageing process and disease (Osorio *et al.*, 2012). This is supported by further studies which have shown improved lifespan through inhibition of other modulators of SASP, including mTOR and the JAK pathway (Laberge *et al.*, 2015) (Xu *et al.*, 2015).

Conversely, SASP is also implicated in chronic inflammatory diseases that occur with age. Elevated levels of pro-inflammatory SASP factors IL-6 and IL-8 have been observed in models of chronic obstructive pulmonary disease (COPD) (Birch *et al.*, 2015) and shortened telomere length has been linked to higher inflammatory cytokine expression (Amsellem *et al.*, 2011). Similarly, IL-6 secretion has been identified as a key predictor of osteoarthritis (Livshits *et al.*, 2009), and transplantation of senescent cells into the knee joints of mice caused an immune-mediated chronic inflammation and exacerbated an osteoarthritis-like phenotype (Xu *et al.*, 2017). Later studies found that this inflammatory disease burden could be alleviated by treatment with senolytic drugs (Xu *et al.*, 2018).

Alongside the SASP's numerous roles in ageing, immune signalling and cellular senescence, studies have also demonstrated a pro-tumorigenic capacity for the phenotype. The SASP can produce favourable conditions for malignant cell survival and proliferation (Coppe *et al.*, 2010; Zacarias-Fluck *et al.*, 2015; Ortiz-Montero;Londono-Vallejo and Vernot, 2017), as well as stimulating cancerous cell phenotypes through paracrine signalling pathways (Bavik *et al.*, 2006; Coppe *et al.*, 2008). Senescent cells have been shown to stimulate growth of malignant and premalignant cells irrespective of

senescent stimulus, and is at least partially caused by secretion of pro-inflammatory SASP factors (Krtolica *et al.*, 2001; Moiseeva *et al.*, 2013).

1.2.5 Senescence in wound healing

Despite the wealth of literature demonstrating the influence of senescent cells in ageing and disease, the phenotype also has important beneficial roles, particularly during development and wound healing. In fact, senescent cells have been linked to limb regeneration in both salamander and zebrafish models (Yun;Davaapil and Brockes, 2015; Da Silva-Alvarez *et al.*, 2020). Senescent cells are also present following cutaneous injury, and assist in the clearance of excess tissue fibrosis by targeted stimulation of enzyme production and immune clearance of affected cells (Jun and Lau, 2010). Similar findings were also observed in which Ccn1 expression both stimulated senescence and limited fibrosis in a model of cardiac injury (Meyer *et al.*, 2016). Wound healing is also accompanied by a dramatic increase in $p16^{INK4a}$ activation which resolves over 2-3 weeks, and is thought to represent immune clearance of the healing-associated senescent cells (Jun and Lau, 2010; Hoenicke and Zender, 2012; Demaria *et al.*, 2014).

The concept of immune clearance of senescent cells at sites of damage is supported by the presence of SASP factors and activation of NFκB following injury (Demaria *et al.*, 2014). Many SASP factors are able to recruit immune cells (Coppe *et al.*, 2010; Kang *et al.*, 2011b) to promote clearance of the affected cells, suggesting that this immunosurveillance is key to wound healing. Indeed clearance of $p16^{INK4a}$-positive cells delays the wound healing process (Baker *et al.*, 2016) supporting a role for senescent cells, but wound healing can still progress in animals deficient for $p16^{INK4a}$ suggesting that it is not p16 itself that is responsible for regulating this process (Demaria *et al.*, 2014). For example, the SASP factor PDGF-AA is known to accelerate wound closure (Demaria *et al.*, 2014). Noticeably, clearance of senescent cells at wound sites declines with age suggesting an age-dependent dysfunction of the immune response (Karin *et al.*, 2019).

Perhaps counterintuitively, senescent cells are also known to affect the proliferative potential of stem and progenitor cells which, in the case of muscle progenitor cells, can prohibit muscle regeneration after injury (Sousa-Victor *et al.*, 2015). Another contrasting observation of senescent cells in wound healing is demonstrated in chronic non-healing

wounds, in which the presence of senescent fibroblasts in ulcers has been associated with slower healing (Stanley and Osler, 2001), and the excessive immune response characteristic of chronic wound pathology may be a result of pro-inflammatory SASP secretion (Coppe *et al.*, 2008).

1.2.6 Senescence as a tumour suppressor mechanism

Before senescence was linked to ageing, its major role was thought to be in the prevention of tumour development (Chen *et al.*, 2005; Lazzerini Denchi *et al.*, 2005; Braig and Schmitt, 2006). Oncogene-induced senescence in particular provides much of the evidence for senescent growth arrest as a response to oncogenic mutations and pre-malignant cells. Growth arrest of such cells prevents the proliferation of damaged cells and division of daughter cells with accumulated dysfunction or mutation (Serrano *et al.*, 1997; Braig and Schmitt, 2006). Cancer incidence in mice is known to increase with reduced *p16INK4a* copy number (Donehower *et al.*, 1992; Sharpless *et al.*, 2001) and decrease with additional copies (Matheu *et al.*, 2004), demonstrating a significant influence of senescent cell signalling pathways on tumour formation. Moreover, loss of $p16^{INK4a}$ or p53 function are two of the most common mutation in many cancers, emphasising their beneficial roles as tumour-suppressors (Kandoth *et al.*, 2013). Interestingly, despite the role of p53 in pro-apoptotic signalling, it is its senescent signalling role that is most important in suppressing tumour formation (Brady *et al.*, 2011).

An increase in senescent markers was observed in studies of primary melanocytes subjected to overexpression of $BRAF^{V600E}$, demonstrating that in cells experiencing oncogenic activity a concurrent increase in senescent phenotype occurred (Peeper *et al.*, 2001). Furthermore, in biopsies of melanocytic nevi a similar increase in SA-β-Gal activity and negligible proliferation markers was found (Michaloglou *et al.*, 2005). In studies by Chen et al (2005), the role of senescence in preventing PTEN-impaired prostate hyperplastic lesions was confirmed in a conditional *PTEN*-deficient mouse model. Senescence development was induced by *PTEN* inactivation and eventually led to prostate cancer development over time, but on additional inactivation of *p53* prostate cancer development was both fast and aggressive, demonstrating the importance of p53-dependent senescence in preventing tumorigenesis (Chen *et al.*, 2005).

Tumour suppression is also supported by the interaction between senescent cells and the immune system. Immune surveillance and targeted clearance of senescent cells via SASP signalling promotes the removal of premalignant cells (Xue *et al.*, 2007; Kang *et al.*, 2011b), as well as limiting aspects of ageing such as accumulated fibrosis (Krizhanovsky *et al.*, 2008; Ovadya *et al.*, 2018). Interestingly, p53 actively restrains SASP production, suggesting that this prevention of an excessively pro-inflammatory and therefore pro-tumorigenic tissue microenvironment may be an additional mechanism by which p53 suppresses tumorigenesis (Coppe *et al.*, 2010). This could also represent another path through which p53 suppression results in malignant transformation and tumour progression.

Paradoxically, many SASP factors are linked to tumour growth and invasion (Di Mitri *et al.*, 2014; Eggert *et al.*, 2016; Lau *et al.*, 2019). This is thought to occur when immunosurveillance is unable to remove the senescent cells and their persistence leads to a chronic SASP with harmful results for the tissue microenvironment.

1.2.7 Senescence in age-related disease

The accumulation of senescent cells with age can contribute to overall tissue dysfunction (Janzen *et al.*, 2006; Molofsky *et al.*, 2006) and development of disease. The stresses associated with disease can in turn trigger greater conversion of cells to senescence, particularly as aged cells are less capable of combating stress and damage. Cellular senescence has been linked to numerous age-related diseases, including heart failure (Chimenti *et al.*, 2003; Schafer *et al.*, 2017), osteoarthritis (Loeser, 2009; Jeon *et al.*, 2017), and diabetes (Minamino *et al.*, 2009), and recent studies have even linked senescence within the central nervous system to Alzheimer's disease (Bhat *et al.*, 2012; Zhang *et al.*, 2019). These conditions have been found to exhibit higher levels of senescent markers, such as p16 expression or telomere shortening when compared to age matched controls. Moreover, clearance of senescent cells specifically has shown promise in prolonging lifespan and alleviating these conditions (Baker *et al.*, 2011; Zhu *et al.*, 2015; Roos *et al.*, 2016).

Cardiac ageing studies have found elevated cell death and hypertrophy of heart tissue with advanced age, along with accumulation of $p16^{INK4a}$-positive cells with substantial

telomere shortening (Chimenti *et al.*, 2003). The selective clearance of senescent cells has shown success in relieving SASP secretion and slowing progression of osteoarthritis in rat models (Peilin *et al.*, 2019). p53 expression and increased oxidative stress has also been linked to insulin resistance in diabetes, and adipose tissue samples from patients with type 2 diabetes exhibit prominent markers of senescence, including SA-β-Gal activity and pro-inflammatory cytokine expression (Minamino *et al.*, 2009).

Interestingly, senescent cell accumulation has been found to both promote and prevent aspects of the development of atherosclerosis, in that increased expression of $p16^{INK4a}$, ARF and $p15^{INK4b}$ has been associated with lower incidence of atherosclerotic vascular disease (Liu *et al.*, 2009b)liu. However, while the anti-proliferative role of senescent cells is favourable for preventing disease development, the pro-inflammatory aspect promotes atherogenesis which is relieved by targeted clearance of $p16^{INK4a}$-expressing cells (Childs *et al.*, 2016).

The SASP may also play a role in the development of age-related disease, as chronic inflammation is often observed in conditions such as atherosclerosis (Zhou *et al.*, 2006) and cancer (Thangavel *et al.*, 2011). Indeed, increased levels of SASP factors such as IL-6 and tumour necrosis factor (TNF) receptor in the blood have been identified as predictors of chronic disease with old age (Fabbri *et al.*, 2015). Moreover, work by Xu et al (2018) demonstrated that transplanting senescent cells into young mice resulted in chronic physical dysfunction that was relieved by senolytic treatment.

1.2.8 Senolytics and senostatics

Many studies have investigated the use of senolytics and senostatics to alleviate the senescent burden *in vivo*. Senolytics are drugs which target senescent cells specifically for clearance and degradation (Zhu *et al.*, 2015), while senostatics are used to relieve harmful aspects of the senescent phenotype without removing the cells completely. Senolytics commonly target pathways which promote cell survival and protect against apoptosis. For example, the combination treatment of tyrosine kinase inhibitor dasatinib and PI3K inhibitor quercetin (D+Q) has been shown to reduce expression of BCL-xL; part of the anti-apoptotic BCL-2 family. D+Q treatment is able to clear senescent cells and improve health span in multiple mouse models of cellular senescence (Xu *et al.*, 2018), and has been

shown to improve vasomotor function and lung function in models of hypercholesterolaemia and pulmonary fibrosis, respectively (Roos *et al.*, 2016; Schafer *et al.*, 2017). Recently, D+Q treatment has also lowered SASP factor secretion and senescent cell burden in a mouse model of age-related intervertebral disc degeneration, improving cell viability and limiting degradation (Novais *et al.*, 2021). More importantly, the first evidence of beneficial D+Q use in humans has been reported in clinical trials in which patients with idiopathic pulmonary fibrosis exhibited improved physical function following a 3 week treatment course (Justice *et al.*, 2019), and a reduction in senescent cell burden and circulating pro-inflammatory cytokines was demonstrated in patients with diabetic kidney disease (Hickson *et al.*, 2019).

Navitoclax is another well-established senolytic which inhibits the BCL-2 family, and promotes release of pro-apoptotic factors (Zhu *et al.*, 2016). Treatment with navitoclax is able to selectively clear senescent cells *in vivo* and has been found to improve the ageing phenotype of haematopoietic and skeletal muscle stems cells in irradiated and aged mice (Chang *et al.*, 2016). Navitoclax-mediated senolysis has also been shown to improve atherosclerosis pathology by reducing plaque burden, number and size, along with a reduction in SA-β-Gal-positive fatty streak burden in the aortic arch (Childs *et al.*, 2016). However, its use in humans is limited by side effects such as thrombocytopenia, resulting from suppression of platelet activation and depletion of intracellular calcium stores (Vogler *et al.*, 2011).

Rapamycin has also been identified as an effective senostatic, and targets the mechanistic target of rapamycin complex 1 (mTORC1) after forming a complex with FKBP12 (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). This rapamycin-FKBP12 complex inhibits signalling pathways required for cell growth and proliferation (Chung *et al.*, 1992). Studies have demonstrated that treatment with rapamycin is able to extend health span, activate autophagy and improve CI activity (Carames *et al.*, 2012; Miwa *et al.*, 2014). It has also been shown to reduce elevated mitochondrial mass, levels of SASP factor expression, p21 expression, and DNA damage foci in models of senescence (Correia-Melo *et al.*, 2016). Furthermore, in studies of age-related diseases rapamycin treatment has been found to reduce IL-1β expression and alleviate cartilage degradation in experimental osteoarthritis models *in vivo* (Carames *et al.*, 2012), and relieve insulin resistance and inflammation in

rat models of type 2 diabetes (Zhou and Ye, 2018). Furthermore, rapamycin has shown success in preventing Alzheimer's disease pathology by protecting against tau-induced neurodegeneration and neuroinflammation (Siman;Cocca and Dong, 2015) and improved cognitive and cerebrovascular function in apolipoprotein E ε4 transgenic mice (Lin *et al.*, 2017).

However, dosage and treatment timing with rapamycin are important considerations, with acute rapamycin treatments able to improve insulin sensitivity (Tremblay and Marette, 2001; Krebs *et al.*, 2007), while longer treatment periods have been found to worsen hyperglycemia (Fraenkel *et al.*, 2008) and glucose intolerance (Chang *et al.*, 2009) in mouse models of diabetes. Disruption of mTORC2 has also been found to exacerbate insulin resistance (Lamming *et al.*, 2012). The use of mTOR inhibitors clinically is also limited by their associated side effects (Pallet and Legendre, 2013; Duran *et al.*, 2014), though some reports have found success in clinical trials at low doses (Mannick *et al.*, 2014).

1.3 Mitochondrial function

The mitochondria are essential organelles with roles in cell signalling, differentiation (Hamanaka and Chandel, 2010) and death (Frank *et al.*, 2001; Lee *et al.*, 2004). They are responsible for the majority of NADH and ATP production within the cell, and are involved in Ca^{2+} homeostasis (Baughman *et al.*, 2011; De Stefani *et al.*, 2011) and adaptive signalling responses to cell stress (Chandel *et al.*, 2000). They are also essential for synthesizing iron sulphur clusters needed to produce OXPHOS and DNA repair components and nucleotides (Lill and Muhlenhoff, 2008).

The mitochondrial genome held within the mitochondria encodes 13 proteins, which form the bulk of the electron transport chain (ETC) on the inner membrane of the organelle (Anderson *et al.*, 1981). The ETC oxidizes electron carriers produced by the tricarboxylic acid (TCA) cycle and drives ATP production through oxidative phosphorylation. Each of the mitochondria's roles within the cell depend on oxidative phosphorylation in some form, and can therefore be affected by changes in ETC function or impairment of the respiratory complexes.

1.3.1 Oxidative phosphorylation

The electron transport chain comprises five main respiratory complexes (I-V), the ubiquinone pool and cytochrome c, all involved in transporting electrons from donor sites to the final electron acceptor, oxygen, to form water (**Figure *1.2***). Specific metabolic substrates supply the ETC either via the NAD+/NADH pool to Complex I (NADH dehydrogenase), or directly through Complex II (succinate dehydrogenase). CI-linked substrates are often products of glycolysis (pyruvate) or the TCA cycle (malate/glutamate) and when the ETC is being driven by these CI-linked substrates electron transfer is in the forward direction through CI (Murphy, 2009). CII-linked substrates (succinate) supply electrons directly to the ubiquinone pool via CII, which is the only nuclear-encoded respiratory complex and is directly involved in the TCA cycle (Cecchini, 2003; Sun *et al.*, 2005). Other dehydrogenases can also introduce electrons to the ubiquinone pool, and are important contributors to electron flow, though are not addressed in this study. Such dehydrogenases include the electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) complex which forms a short electron transfer pathway with ETF to carry electrons from multiple mitochondrial dehydrogenases during fatty acid β-oxidation (Ruzicka and Beinert, 1977), and the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) which forms part of the glycerophosphate (GP) shuttle to deliver electrons from glucose and lipid metabolism to the ubiquinone pool (Mracek;Drahota and Houstek, 2013).

CI accepts electrons from the NAD+/NADH pool in the mitochondrial matrix. Electrons enter through the Flavin mononucleotide (Site I_F) and are transferred through the complex via iron-sulphur (Fe-S) clusters, to the Q-binding site (Site I_Q) where they enter the ubiquinone pool as ubiquinol. From here electrons are carried to complex III (ubiquinol-cytochrome c reductase), cytochrome c, and finally complex IV (cytochrome c oxidase), where oxygen acts as the final electron acceptor in the conversion to water by combining with 4 electrons and two protons.

Electron transfer is tightly coupled to proton pumping across the mitochondrial inner membrane into the intermembrane space by complexes I, III and IV. This action creates a proton gradient, producing both a pH and electrical difference across the membrane (mitochondrial membrane potential), which generates the protonmotive force necessary

to drive oxidative phosphorylation and ATP production at Complex V, otherwise known as ATP synthase (Hernansanz-Agustin *et al.*, 2017). Membrane potential is essential for other mitochondrial functions, including protein import and triggering responses to mitochondrial dysfunction (Mitchell, 1961; Neupert and Herrmann, 2007). At CV protons move through the complex, driven by the protonmotive force, and power the conversion of ADP + P_i to ATP.

1.3.1.1 ETC Inhibitors

Each stage of cellular respiration can be analysed by inhibition of individual respiratory complexes and supply of specific substrates. Rotenone is a CI-specific inhibitor that binds to the ubiquinone-binding I_Q site of the complex and supresses CI-linked respiration (**Figure *1.2***). This allows examination of cellular respiration driven by CII-linked substrates alone. The addition of a CIII inhibitor (antimycin A) reveals residual oxygen consumption observed when electron transfer from all major entry points is blocked (**Figure *1.2***). It should be noted that rotenone is a highly toxic compound known to induce a Parkinson-like phenotype in animal models (Cannon *et al.*, 2009), and so its use in researching potential therapeutic options is limited to a proof-of-concept model.

Metformin is an alternate CI inhibitor widely used in the treatment of diabetes (Hostalek;Gwilt and Hildemann, 2015). Though multiple mechanisms of action have been proposed, numerous studies have shown that metformin is able to block CI-linked respiration *in vitro* and *in vivo* (Bridges *et al.*, 2014; Janzer *et al.*, 2014; Wheaton *et al.*, 2014). Its specific binding site is debated in the literature, though has been shown to interact with both site I_Q and site I_F (Bridges *et al.*, 2014), providing a possible alternative mode of CI inhibition for examination. Its well-established safety profile makes it an interesting avenue of investigation for many diseases, particularly those with a causal link to mitochondrial or CI function specifically.

By inhibiting CII activity with malonate (Ralph *et al.*, 2011) it is possible to dissect ETC function when driven solely by CI-linked substrates. Malonate competes with succinate binding to the complex, preventing entry of electrons into the quinone pool at this point in the ETC (Dervartanian and Veeger, 1964).

Antimycin A and myxothiazol are both efficient inhibitors of CIII, though bind at different sites of the complex. Antimycin A occupies the inner ubiquinone-binding site (III_{Qi}) of CIII, while myxothiazol blocks the outer ubiquinone-binding site (III_{Qo}) (**Figure *1.2***). Inhibition of the III_{Qi} site prevents flow of electrons and halts them at the semiquinone occupying site III_{Qo} (Murphy, 2009). This can lead to a build up of electrons and subsequent overreduction of the ubiquinone pool, resulting in additional electron leak and formation of superoxide. Inhibition of the III_{Qo} site by myxothiazol can suppress this superoxide production (discussed in further detail in section 1.4).

Both potassium cyanide (Jensen *et al.*, 1984) and sodium azide (Bennett *et al.*, 1996; Leary *et al.*, 2002) are well-established CIV inhibitors, though literature has more recently suggested that azide may have additional inhibitory effects on CV (Hong and Pedersen, 2008). While cyanide inhibition is reversible in the presence of pyruvate (Nuskova *et al.*, 2010), chronic treatment with sodium azide has been shown to cause irreversible loss of CIV activity (Leary *et al.*, 2002). By providing cells with the artificial electron donors ascorbate and TMPD, which reduce cytochrome c, and inhibiting CIII activity, it is possibly to examine CIV activity specifically (Pesta and Gnaiger, 2012).

Inhibition of CV by oligomycin (Hong and Pedersen, 2008) reveals the non-phosphorylating portion of oxygen consumption, which represents intrinsic uncoupled respiration and compensation for proton leak across the membrane.

Uncoupling of respiration by FCCP titration allows us to assess the maximum capacity of the ETC for oxygen consumption. This collapses the electrochemical proton potential across the inner membrane and relieves pressure on the proton pumps CI, III and IV, allowing maximum electron flow in the non-coupled state (Pesta and Gnaiger, 2012). Maximum respiratory capacity per cell is known to increase with cell size and in senescent fibroblasts, though when corrected for citrate synthase activity or mitochondrial content no significant difference is apparent, suggesting increased respiratory capacity is a result of increased mitochondrial mass (Hutter *et al.*, 2004; Kitami *et al.*, 2012).

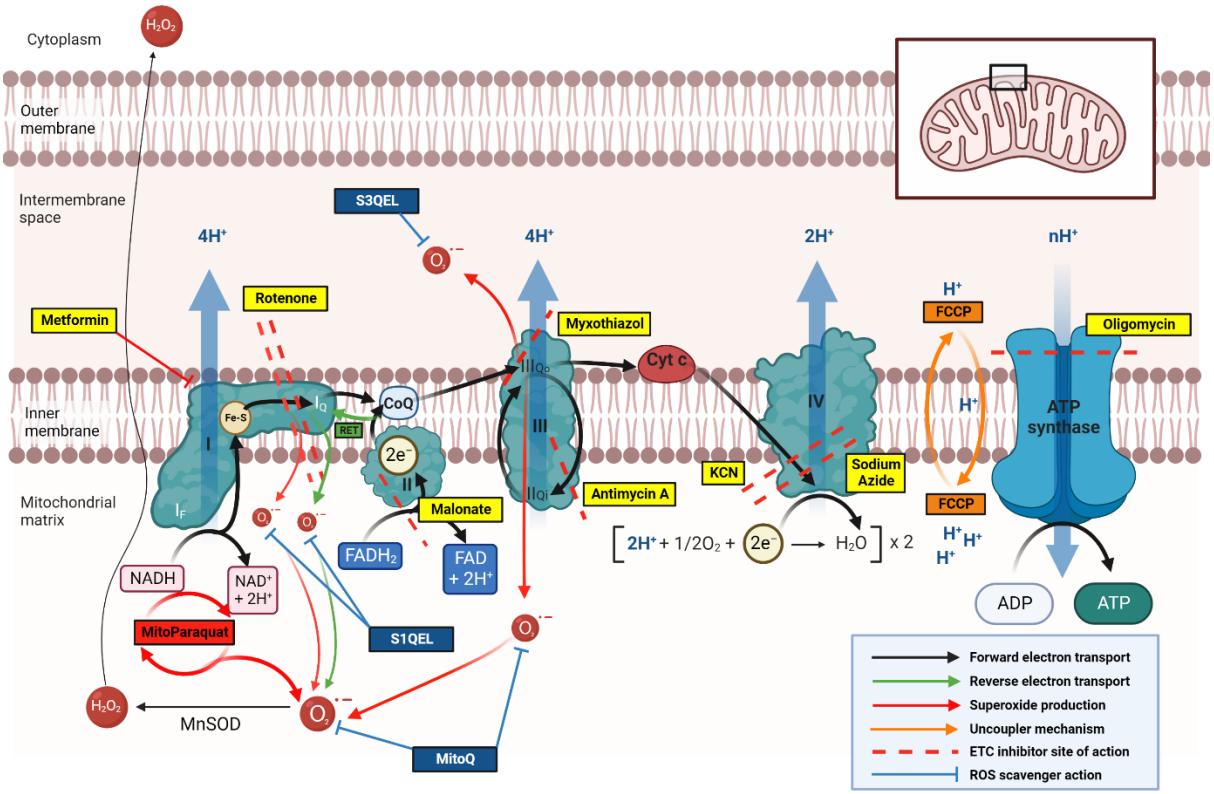


Figure 1.2: Mitochondrial electron transport chain and complex inhibitor effects. The ETC primarily consists of 5 respiratory complexes, the ubiquinone pool, and cytochrome c. Electrons enter the ETC either from CI-linked substrates which provide NADH for oxidation to NAD+ by CI, or from CII-linked substrates which provide $FADH_2$ for oxidation to FAD by CII. Electrons are passed from CI+II to the ubiquinone pool, and from there to site III_{Qo} of CIII, to cytochrome c, and finally to CIV and the final electron acceptor oxygen, to form water. Electron transfer at CI, III and IV is coupled to proton pumping across the inner membrane, which establishes the protonmotive force necessary to power ATP formation at CV (ATP synthase). When electron transfer is in the forward direction (black arrows), the CI inhibitor rotenone causes increased electron leak at the quinone-binding site (I_Q) by preventing exit of electrons into the ubiquinone pool, resulting in reduction of all available electron carriers within the complex (Fe-S clusters) and greater electron leak to form superoxide (red arrows). In conditions where the ubiquinone pool becomes over-reduced (such as increased contribution of CII-linked substrates) electrons can be forced back to CI in reverse electron transport (RET) (green arrows). This causes a significant increase in superoxide production from site I_Q of CI, which can be inhibited by rotenone

by preventing entry into the binding site. Metformin is also known to inhibit CI activity, but its specific binding site is less clear. Malonate is a well-known inhibitor of CII by competing with succinate for binding at the complex and preventing entry of electrons through CII into the ubiquinone pool. Antimycin A inhibits CIII activity by occupying the inner quinone-binding site (III_{Qi}), resulting in increased electron leak and superoxide production at the outer quinone-binding site (III_{Qo}). This superoxide production can be suppressed by myxothiazol, another CIII inhibitor that occupies site III_{Qo} and prevents electron leak at this site. KCN and sodium azide are both inhibitors of CIV activity. Oligomycin inhibits ATP synthase (CV) activity by blocking the proton channel (F0 subunit). The uncoupler FCCP (orange) interferes with mitochondrial membrane potential by shuttling protons across the inner mitochondrial membrane. Superoxide produced into the matrix is rapidly converted to hydrogen peroxide by MnSOD, and is then able to diffuse out of the mitochondria. Conversely, superoxide produced into the cytosolic side of the membrane largely requires the addition of exogenous SOD in order to be converted to hydrogen peroxide, as spontaneous dismutation is a poor competitor of other reactions that do not produce hydrogen peroxide. The redox cycler MitoParaquat (red) stimulates production of superoxide by reduction of oxygen. Comparatively, the ROS scavengers (blue) MitoQ, S1QEL and S3QEL detoxify superoxide produced in the mitochondria generally (MitoQ), or at specific sites (I_Q; S1QEL, III_{Qo}; S3QEL).

1.3.2 Mitochondrial quality control

Maintenance of the mitochondrial network is essential for cell function and survival. Numerous mitochondrial quality control pathways exist to ensure that dysfunctional mitochondria can be identified and removed from the network to preserve overall function. Such pathways include the mitochondrial unfolded protein response (mUPR), autophagy, mitophagy, and the balance of fission and fusion processes to regulate mitochondrial dynamics. Defects in mitochondrial quality, and in the quality control pathways themselves, have been linked to many age-related diseases and cellular senescence (Dalle Pezze *et al.*, 2014; Wong *et al.*, 2020). Mitochondrial dynamics have also demonstrated impairment with age, leading to reduced quality of the mitochondrial

network and accumulation of dysfunctional mitochondria (Reznick *et al.*, 2007; Crane *et al.*, 2010).

1.3.2.1 Autophagy

Autophagy is important for the maintenance of mitochondrial homeostasis and removal of harmful compounds within the cell. When material is targeted for autophagy it is engulfed by a phagophore, which expands to form an autophagosome vesicle, and subsequently fuses with the lysosome to form an autolysosome where the cargo is degraded. The autophagic process is also able to recycle these degraded materials to the cytosol when nutrients are scarce (Yang *et al.*, 2006). AMPK and mTOR are the key mediators of autophagy, and have important roles in mitochondrial function and longevity. High AMP/ATP ratios trigger AMPK activation and induction of autophagy, while mTORC1 activation suppresses autophagy. Impairment of these signalling pathways can lead to dysfunctional autophagy and contribute to the ageing process through accumulation of damaging compounds and organelles (Wong *et al.*, 2020).

Autophagy has been linked to cellular senescence, though its role in the process is still unclear and appears to be dependent on specific circumstances. For example, knockdown of the essential autophagy genes Atg5 and Atg7 have been shown to reduce pro-inflammatory factor secretion and prevent senescence development, and elevated p16 and p21 expression are able to induce autophagy (Young *et al.*, 2009; Capparelli *et al.*, 2012). Work by Young and colleagues demonstrated that knockdown of *ATG5* and *ATG7* lowered Ras-induced autophagy and delayed the senescence-associated increase in IL-6 and IL-8 secretion and SA-β-Gal activity. The report also provided evidence that suggests autophagy plays a role in the establishment, but perhaps not maintenance of senescence, as similar knockdown of these proteins in a model of established senescence was not sufficient to reverse senescent growth arrest. Similarly, Caparrelii et al (2012) found that in fibroblasts that overexpressed $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ markers of autophagy Lamp-1 and BNIP3 were increased, and cells were more susceptible to autophagy induction after a period of starvation, as evidenced by increased expression of LC3-I/II.

Conversely, other studies have shown that inhibition of autophagy in healthy cells can lead to induction of senescence via ROS- and p53-dependent pathways (Kang *et al.*, 2011a), and activation of autophagy by rapamycin treatment can suppress senescence

(Correia-Melo *et al.*, 2016). It seems likely that autophagy's role in senescence development may change depending on the specific type of senescence. For example, ULK3 overexpression enhances autophagy and stimulates oncogene-induced senescence *in vitro*, while mTORC1 activation and subsequent reduction in autophagic activity promotes SASP factor production (Young *et al.*, 2009). In comparison, in a model of MiDAS, elevated expression of *LCB3* and *ATG5* has been shown to delay senescence and extend replicative lifespan of cells via enhanced mitophagy, while oxidative stress promoted senescence induction due to compromised autophagy processes (Mai *et al.*, 2012).

1.3.2.2 Mitophagy

Mitochondria-specific autophagy – known as mitophagy – is an essential process in maintaining the quality and function of the mitochondrial network. The process is mediated by the Ser/Thr kinase PINK1 and the E3 ubiquitin ligase Parkin, which together target damaged or dysfunctional mitochondria for degradation by the autophagosome (Clark *et al.*, 2006; Park *et al.*, 2006; Narendra *et al.*, 2008). Initiation of mitophagy is often associated with a drop in mitochondrial membrane potential, which prevents degradation of PINK1 and stimulates accumulation of PINK1 at the outer membrane (Matsuda *et al.*, 2010; Narendra *et al.*, 2010) where it activates through autophosphorylation (Kondapalli *et al.*, 2012). Parkin is then recruited to translocate to the outer membrane (Kim *et al.*, 2008), where it 'tags' the dysfunctional mitochondria with polyubiquitin chains. These chains are recognised by receptor proteins to initiate recruitment of the autophagosome and clearance of the mitochondria (Lazarou *et al.*, 2015). Additionally, Parkin ubiquitinates key mediators of mitochondrial fusion – mitofusin 1 and 2 (MFN1 and MFN2) – on the outer membrane, targeting them for degradation, resulting in inhibition of fusion and promotion of fission processes (Ziviani;Tao and Whitworth, 2010).

Mitophagy, together with mitochondrial biogenesis, maintain mitochondrial content and may play an important role in cellular senescence. Though there is still much needed study in order to determine the nature of its involvement. Studies have shown that mitophagy activity is reduced in cellular senescence, which can contribute to accumulation of dysfunctional mitochondria (Dalle Pezze *et al.*, 2014). The tumour suppressor p53, which is upregulated in senescence, prevents translocation of Parkin to the outer membrane,

and subsequently suppresses mitophagy (Ahmad *et al.*, 2015). Moreover, activation of mitophagy by rapamycin is able to improve mitochondrial function and relieve the SASP (Correia-Melo *et al.*, 2016). However, some research has demonstrated that the activation of a DDR can cause a reduction in mitochondrial membrane potential, which would normally trigger a mitophagy response (Passos *et al.*, 2007). It could be suggested that the role of mitophagy in senescence may vary depending on circumstance; senescence model, cell type, nutritional availability. It has also been suggested that due to the increase in mitochondrial mass associated with senescence, such mitochondria are simply not able to be targeted for mitophagy as they are too large for degradation by the autophagosome (Gomes;Di Benedetto and Scorrano, 2011; Rambold *et al.*, 2011). Dysfunction in the lysosomal system is also a possibility in the context of senescence, as cells which lack regulatory components of mitochondrial dynamics, such as OPA1 or PINK1, have also exhibited impairment of the lysosome (Demers-Lamarche *et al.*, 2016). Moreover, cells with impaired mitochondrial respiratory chain machinery cannot stimulate lysosomal biogenesis (Fernandez-Mosquera *et al.*, 2019).

1.3.2.3 Fission/fusion

The balance of mitochondrial fission and fusion activity enables optimum function of the mitochondrial network. This balance is also intrinsically linked with mitophagy activity, and inhibition of PINK1 activity has been shown to suppress mitochondrial fission by preventing recruitment of Parkin to damaged mitochondria (Bueno *et al.*, 2015). Mitochondrial fusion allows the sharing of resources between mitochondria, including mtDNA, to improve survival (Tondera *et al.*, 2009; Rambold *et al.*, 2011). The dynamin-like GTPases Mitofusin 1, Mitofusin 2 and OPA1 are responsible for regulating fusion activity (Chen *et al.*, 2003; Ge *et al.*, 2020). Mitochondrial fission separates damaged or dysfunctional mitochondria from the network ready for degradation. DRP1 regulates fission processes by creating helical structures to surround and separate the targeted mitochondria (Labrousse *et al.*, 1999; Ingerman *et al.*, 2005). The mitochondrial network fragments significantly during apoptosis as a result of increased DRP1 recruitment, while increased fusion activity is able to prevent apoptosis (Lee *et al.*, 2004; Sugioka;Shimizu and Tsujimoto, 2004).

Disruption of either of these processes can lead to mitochondrial dysfunction. Impairments in both fusion and fission have been shown to cause deficiencies in oxidative phosphorylation, and while fusion defects can induce loss of mtDNA and disruption of mitochondrial motility, fission inhibition can result in elevated levels of ROS (Chen *et al.*, 2003; Parone *et al.*, 2008). Moreover, inhibition of fission has been shown to stimulate elongation of mitochondria and initiation of cellular senescence. This elongation is associated with a decline in membrane potential and increased ROS production, triggering a DDR (Yoon *et al.*, 2006). Senescent cells often demonstrate a hyper-fused mitochondrial network, which would be difficult to isolate impaired mitochondria for degradation (Dalle Pezze *et al.*, 2014). Studies have also demonstrated that stimulating mitochondrial fission is able to alleviate the senescent phenotype (Yoon *et al.*, 2006).

1.3.3 Cell death

Mitochondria also play an essential role in programmed cell death (Frank *et al.*, 2001; Lee *et al.*, 2004). When cells undergo significant stress or damage, pro-apoptotic signalling pathways can be activated. The intrinsic pathway of apoptosis involves mitochondrial outer membrane permeabilisation (MOMP) and release of cytochrome c. Pro-apoptotic factors activate the formation of what is known as the mitochondrial permeability transition pore (mPTP), causing the protonmotive force to collapse and oxidative phosphorylation to halt (Rottenberg and Hoek, 2017). The mPTP is formed as a result of activated Bax/Bak oligomerisation at the mitochondrial surface, creating a transitional pore through which apoptotic proteins can enter the cytosol. Cytochrome c is then released through the mPTP and triggers the formation of the apoptosome and recruitment of caspases (Nechushtan *et al.*, 2001; Karch *et al.*, 2013).

The balance of fission/fusion activities in mitochondria are also important for apoptotic cell death. The mitochondrial fission mediator DRP1 is increasingly recruited to mitochondria during apoptosis (Frank *et al.*, 2001), creating an extremely fragmented mitochondrial network able to be easily separated and degraded by autophagic vesicles. Conversely, increased mitochondrial fusion can protect cells from apoptotic cell death (Lee *et al.*, 2004; Sugioka;Shimizu and Tsujimoto, 2004) as OPA1 limits the release of proapoptotic components from the intermembrane space (Cipolat *et al.*, 2006).

Studies have demonstrated that cells are more predisposed to mPTP activation with age, with additional implications for more frequent activation of processes that rely on mPTP opening (Mather and Rottenberg, 2000). Increased ROS generation, which is observed with age and in many age-related diseases, can both inhibit cell growth and activate cell death processes (Lawless *et al.*, 2012). Dysregulation of these processes can therefore result in accumulation of damaged cells and cell components which contribute to disease.

1.4 Reactive Oxygen Species (ROS)

1.4.1 ROS production

During oxidative phosphorylation, electrons can leak from the respiratory complexes to form reactive oxygen species (ROS), formed by incomplete reduction of oxygen to water. A single electron reaction converts oxygen to superoxide, while transfer of two electrons forms hydrogen peroxide (Murphy, 2009). Furthermore, the highly reactive hydroxyl radical can be formed from Fenton reactions in which free iron reacts with superoxide or hydrogen peroxide (Lyngsie *et al.*, 2018), and by Haber-Weiss reactions where superoxide and hydrogen peroxide react together to form oxygen, hydroxyl radical and hydroxide (Kehrer, 2000). The hydroxyl radical is highly toxic and can damage all biological components of the cell, but is produced in much smaller quantities. Superoxide and hydrogen peroxide are the most common forms of ROS produced by the mitochondria, and have important roles in both redox signalling (Reczek and Chandel, 2015) and oxidative damage (Sena and Chandel, 2012). Superoxide produced into the mitochondrial matrix cannot cross the inner membrane (Muller;Liu and Van Remmen, 2004), so targets of superoxide interaction are limited to those nearby. It is also efficiently converted to hydrogen peroxide by mitochondrial superoxide dismutase (MnSOD) (Miwa *et al.*, 2003), meaning that under normal conditions superoxide does not exist in this form for long within the matrix.

As the primary source of ROS production, mitochondria are subject to much more oxidative damage compared to the rest of the cell, which can lead to dysfunction, disease, and contribute to the ageing process (Hollensworth *et al.*, 2000; Hoque *et al.*, 2021).

Oxidative stress has been identified as both a cause and consequence of mitochondrial dysfunction.

1.4.2 Sites of ROS production

The majority of ROS is produced in the mitochondria, and studies have identified respiratory complexes I and III as those with the highest capacity for ROS production (Miwa *et al.*, 2003; Brand, 2016). The flavin mononucleotide site of Complex I (site I_F) and the outer quinone-binding site of Complex III (site III_{Qo}) in particular are significant contributors to ROS production due to their close reliance on the NAD and ubiquinone pool redox statuses in determining electron availability. These two pools are common entry points to the ETC for many substrates, and as such these sites will often have access to electrons for ROS formation (Wong *et al.*, 2017). The relative production rates and overall contributions of individual sites can be assessed and altered by direct inhibition of electron transport, and by suppression or scavenging of ROS from specific points.

1.4.2.1 Mitochondrial electron transport chain

Complex I (CI) in particular has been implicated in age-related disease. Suppression of ROS production at CI has been shown to protect against ischemia-reperfusion injury (Brand *et al.*, 2016), while mild inhibition of the complex reduces levels of β-amyloid and phospho-tau in animal models of Alzheimer's disease (Zhang *et al.*, 2015a; Stojakovic *et al.*, 2021). Parkinson's disease pathology has also been linked to ROS production specifically from site I_F of CI (Sherer *et al.*, 2003).

When the ETC is driven by CI-linked substrates, electron transfer is in the forward direction and ROS production is rather low (Wong *et al.*, 2017). However, CI is known to leak electrons at both site I_Q and I_F in certain circumstances (Brand, 2016). Superoxide is produced exclusively into the mitochondrial matrix from CI due to its location on the inner surface of the mitochondrial inner membrane. Here, superoxide is efficiently converted to hydrogen peroxide by MnSOD. At site I_Q, if the ubiquinone pool becomes over-reduced (QH_2/Q ratio is high) then electrons can be forced back into CI, leading to a substantial increase in electron leak from this site. This is known as reverse electron transport (RET). The resulting ROS production from site I_Q can be inhibited by treatment with rotenone, which blocks the site and prevents exit of electrons from the quinone-binding site.

However, this is specific to ROS produced through reverse electron transport (RET). When electron transfer is in the forward direction, rotenone increases ROS produced at site I_Q by promoting reduction of all electron carriers within the complex and preventing exit into the ubiquinone pool, leading to increased electron leak (Miwa *et al.*, 2003).

Metformin is also able to inhibit ROS production from CI, though its binding site is less clear. The more recent discovery of S1QELs (Suppressors of Site I_Q Electron Leak) and S3QELs (Suppressors of Site III_{Qo} electron leak) allows site-specific suppression of ROS within the mitochondria without interference with OCR or electron transport elsewhere, rather than general reduction by other mitochondria-targeted antioxidants. S1QELs target site I_Q of CI and inhibit production of ROS from this site during reverse electron transport (RET) (Brand *et al.*, 2016), while S3QELs target ROS production from site III_{Qo} of CIII . The use of interventions such as rotenone, metformin and S1QELs allows us to examine the role of CI-linked ROS production in pathological conditions (Watson;Wong and Brand, 2019).

RET is often seen under conditions where CII is able to contribute a significant flow of electrons to the ubiquinone pool, e.g. high succinate concentrations (Kane *et al.*, 2010; Hernansanz-Agustin *et al.*, 2017; Scialo;Fernandez-Ayala and Sanz, 2017), or electron transfer upstream of the ubiquinone pool is blocked, for example by inhibition of CIII and CIV, though this is primarily an *in vitro* observation to date (Guaras *et al.*, 2016). Succinate has also been shown to accumulate during ischemia, which can cause long-lasting oxidative damage and RET-ROS production when the accumulated succinate is rapidly oxidized by CII upon reperfusion (Kohlhauer *et al.*, 2019). Studies have shown that when succinate is the driving substrate of the ETC, the majority of measurable ROS production can be attributed to site I_Q through RET (Quinlan *et al.*, 2013). Succinate can also increase mitochondrial membrane potential and reduction of the NADH pool (Robb *et al.*, 2018). This RET-ROS signalling can detect changes in membrane potential and electron flow, producing significant amounts of ROS exclusively at CI (Scialo;Fernandez-Ayala and Sanz, 2017).

ROS production at site I_F is generally low, but electron leak can occur when the NAD+/NADH ratio increases, or when site I_Q is blocked, for example by rotenone, preventing exit of electrons from the complex at the quinone-binding site. This leads to a

back-log of electrons within the complex and leak at site I_F. The importance of ROS production from any given site is also dependent on circumstances, for example, site I_F contributes the largest portion of overall ROS under conditions of intense exercise (Wong *et al.*, 2017). Under CI-linked substrate oxidation, the majority of ROS production is attributed to sites I_F, III_{Qo} and 2-oxoglutarate dehydrogenase equally (Quinlan *et al.*, 2013; Wong *et al.*, 2017).

ROS production from CI specifically has been shown to influence lifespan in multiple ageing models. Stimulating RET-ROS at CI improves health and delays ageing in flies (Scialo *et al.*, 2016), and inhibiting CI extends *C. elegans* lifespan in a ROS-dependent manner. The increase in ROS observed with age is also associated with a reduction in CI-linked respiration.

In contrast to CI, there is little evidence correlating CIII activity with age-related disease, and in fact differences in longevity related to ROS production are only seen when electron transfer through CI is promoted through supply of CI-linked substrates. When this electron transfer is inhibited, such as in the presence of succinate plus rotenone, longevity does not appear to be impacted by ROS production (Herrero and Barja, 1997).

The outer ubiquinone-binding site of CIII, Site III_{Qo}, possesses the highest maximum capacity for ROS production. Though this is only true under non-physiological conditions in the presence of antimycin A, which blocks centre *i* of CIII (site III_{Qi}). This causes electron transfer to halt at the semiquinone occupying site III_{Qo}, and prevents oxidation of the ubiquinone pool. As a result the QH_2/Q ratio rises rapidly, causing back-flow of electrons to downstream complexes and increased ROS production at site III_{Qo}. While CI can produce a mix of superoxide and hydrogen peroxide exclusively into the mitochondrial matrix (Pepelina *et al.*, 2009; Grivennikova and Vinogradov, 2013), site III_{Qo} produces superoxide only, but it is split between the matrix and the intermembrane space. This supports a role for ROS produced at CIII in wider cellular redox signalling.

Myxothiazol treatment can inhibit this ROS production by blocking site III_{Qo}, and S3QELs are able to suppress ROS production specifically from this site without interfering with electron flow or respiration (Wong *et al.*, 2017). In the absence of antimycin – when site

III_{Qi} is available for electron transfer – it is thought that site III_{Qo} is not capable of producing ROS in any great numbers (Miwa *et al.*, 2003; Brand, 2016).

The flavin mononucleotide site of CII (II_F) is thought to be capable of producing both superoxide and hydrogen peroxide, but under normal physiological conditions this production is negligible (Brand, 2016). In models of cancer involving CII subunit mutations, however, ROS production from this complex has been found to be important (Owens *et al.*, 2012; Li *et al.*, 2019). Nevertheless, it is an important consideration as the second primary site of electron entry to the ETC, and CII activity can be linked to overreduction of the ubiquinone pool and RET. In order for site II_F to produce ROS the flavin site must be open and able to interact with oxygen. Therefore only when substrates are provided which reduce the ubiquinone pool without occupying II_F, and the ubiquinone pool becomes sufficiently reduced (often through CIII inhibition), are electrons able to flow back into CII and produce ROS at II_F (Quinlan *et al.*, 2012). ROS production at site II_F is sensitive to inhibition by the CII inhibitor malonate (Ralph *et al.*, 2011). However, while the capacity of II_F for ROS production is high in isolated mitochondria, it has not been shown to be active in cells outside of certain cancer models.

1.4.2.2 Other sites of ROS production

There are a number of other sources of ROS production, but the levels of superoxide and hydrogen peroxide produced by these sites under physiological conditions are negligible. Mitochondrial glycerophosphate dehydrogenase (mGPDH) is located on the outer site of the inner mitochondrial membrane, and generates superoxide to both the mitochondrial matrix and intermembrane space. The quinone-binding site of mGPDH (G_Q) can produce large amounts of superoxide under CIII inhibition when the ubiquinone pool is reduced (Orr *et al.*, 2012). It forms part of the glycerophosphate shuttle (GPS) which transports NADH reducing equivalents directly to the ubiquinone pool, bypassing CI (Pecinova *et al.*, 2019).

Other quinone and flavin sites able to produce ROS include the O_F site of the 2-oxoglutarate dehydrogenase complex, the P_F site of the pyruvate dehydrogenase complex, the D_Q site of dihydroorotate dehydrogenase, and the A_F site of the aminoadipate dehydrogenase complex, but these are only under very specific conditions which do not often arise in vivo.

Fatty acid oxidation also supplies electrons to ubiquinone, and a very small amount of superoxide/hydrogen peroxide can result from enzymes involved in this process, such as acycl-CoA dehydrogenases (Brand, 2016).

1.4.3 Measuring mitochondrial ROS production

Experimental methods for the measurement of ROS production present a significant challenge. Most commonly, studies use redox-active fluorescent probes to visualise ROS production, though quantification of such visual measurements can be difficult, and small differences hard to distinguish. Many studies have inferred different species of ROS using different probes. For example, DCFH has been used to detect intracellular hydrogen peroxide, while MitoSOX reacts with mitochondrial superoxide. However, most of these probes are not as specific to ROS type as we would like, and can be activated by other oxidants to produce products with overlapping fluorescent spectra (Cheng *et al.*, 2018).

Fluorescent probes are also prone to artefact, largely as a result of their lack of specificity and ability to react with factors other than ROS. For example, a study by Cheng et al (2018) demonstrated that MitoSOX did not in fact measure mitochondrial superoxide exclusively, but indicated non-specific oxidation of the probe. Other probes, including DCFH, can produce intermediate radicals that undergo redox cycling, generating additional ROS alongside the fluorescent product. Some studies have used mass spectrometry to detect superoxide production using Mito-HE (MitoSOX), though as with other probes HE is not solely oxidized by superoxide (Michalski *et al.*, 2020). However, liquid chromatography can be applied to separate the specific oxidation product produced by reaction of Mito-HE with superoxide (Zhao *et al.*, 2003).

One probe which has shown better reliability as a measure of ROS production is Amplex Red. Amplex Red reacts with hydrogen peroxide, catalysed by HRP, to produce the fluorescent product resorufin. This fluorescent output can be quantified using a plate reader and used as a surrogate measurement for hydrogen peroxide production. Amplex Red has demonstrated far greater sensitivity than other commonly used probes, and a significant reduction in background noise (Mohanty *et al.*, 1997). Though it should be noted that the requirement of a peroxidase to catalyse the oxidation reaction may limit Amplex Red's use in *in vivo* studies. Moreover, as the peroxidase cannot enter the cells

itself, it secreted extracellular hydrogen peroxide that is measured by this technique, rather than site-specific ROS production.

1.4.4 Redox signalling

Under low physiological levels, ROS perform important redox signalling functions within the cell – hydrogen peroxide is particularly critical in this role as it is a more stable form of ROS and is able to translocate outside the mitochondria and interact with the wider microenvironment. It can then regulate the activity of target proteins in the mitochondria, the cytosol, or the nucleus (Sena and Chandel, 2012). Redox signalling pathways have also been suggested to have a key role in adapting to bioenergetic changes (Wong *et al.*, 2017), for example, responding to increased ATP demand or need for elevated Ca^{2+} signalling (Osellame;Blacker and Duchen, 2012). ROS are also involved in both cell differentiation (Tsatmali;Walcott and Crossin, 2005; Owusu-Ansah and Banerjee, 2009) and cell death (Kamata *et al.*, 2005).

In vivo¸ redox signalling by ROS has important roles in stress responses. For example, hydrogen peroxide is essential for stress responses in *Drosophila melanogaster*, and metabolism and brain function in mice. In order to respond to stresses such as hypoxia or thermal stress, fruit flies require redox signalling by hydrogen peroxide and do not survive for long under these conditions with depleted hydrogen peroxide levels (Scialo *et al.*, 2020). Reduction in hydrogen peroxide levels in mouse astrocytes results in altered glucose and glutathione metabolism and neuronal function (Vicente-Gutierrez *et al.*, 2019).

Superoxide produced via RET at CI has also been suggested as an important redox signalling pathway. RET is highly sensitive to changes in protonmotive force (Δp) and ubiquinone redox state, and has been linked to inflammation, lifespan extension in flies, and oxygen-sensing in the carotid body (Robb *et al.*, 2018).

1.4.5 Antioxidants

Cell antioxidant systems are generally able to detoxify ROS overproduction and prevent excessive oxidative stress. Imbalances in these antioxidant systems can result in a pro-oxidant shift and accumulating damage.

1.4.5.1 Enzymatic antioxidants

Superoxide dismutases SOD1 and 2 convert superoxide to hydrogen peroxide in the cytosol and mitochondrial matrix, respectively (Fridovich, 1997). SODs also play an important role in preventing the formation of $ONOO^-$ by removing superoxide (Brown and Borutaite, 2007), and in redox signalling by producing hydrogen peroxide required for oxidation of key cysteines of regulatory proteins, leading to changes in protein structure, function and localisation (Rhee, 2006). Catalase maintains the homeostasis of hydrogen peroxide as a signalling molecule, converting hydrogen peroxide to water and oxygen (Deisseroth and Dounce, 1970), which protects cells from oxidative damage induced by high levels of hydrogen peroxide (Tiedge *et al.*, 1998). Loss of catalase has been linked to numerous age-related diseases such as diabetes (Grankvist;Marklund and Taljedal, 1981), and its overexpression has shown promise in alleviating Alzheimer's disease pathology (Mao *et al.*, 2012). Glutathione peroxidases are present in the cytosol and mitochondria, and in some cases peroxisomal compartments, and are also responsible for reducing hydrogen peroxide to water and protecting against oxidative damage (Utsunomiya *et al.*, 1991; Singh *et al.*, 1994).

1.4.5.2 Non-enzymatic antioxidants

The major non-enzymatic antioxidants are NADPH and glutathione. Glutathione in the cytosol uses hydrogen peroxide to oxidise to glutathione disulphide, catalysed by glutathione peroxidases (Mailloux;McBride and Harper, 2013). NADPH is another small-molecule electron carrier produced largely as part of the pentose phosphate pathway, and which donates electrons to glutathione and thioredoxins, contributing indirectly to detoxification of ROS by not only these antioxidants but also glutaredoxins, peroxiredoxins, and glutathione peroxidases (Fernandez-Marcos and Nobrega-Pereira, 2016).

Cells with poor antioxidant defence tend to accumulate oxidative damage more quickly as a result of higher ROS levels, which has been implicated in brain ageing and age-related disease pathogenesis. Mitochondria-targeted antioxidants can also be administered as treatments, and are accumulated in the mitochondrial matrix by virtue of their attached positively charged lipophilic TPP cation, which relies on the mitochondrial membrane potential and highly negative-inside environment of mitochondria to transport it. MitoQ

is one such antioxidant; a ubiquinone derivative which forms a ubiquinol derivative when reduced by the mitochondrial respiratory chain, where it prevents lipid peroxidation. Its antioxidant activity is recycled after detoxifying a ROS (Kelso *et al.*, 2001).

Studies have also found that compounds with an attached TPP+ cation are capable of inhibiting oxidative phosphorylation. Fink et al (2012) found that MitoQ inhibited oxygen consumption directed at ATP turnover, but not basal respiration. Findings by Reiley et al (2013) also support this data, showing that MitoQ reduced oxygen consumption and prevented both oligomycin-induced decrease and FCCP-induced increase in OCR, and suggested that MitoQ may be interfering with electron transfer to oxygen.

The recently developed S1QELs (Suppressors of Site I_Q Electron Leak) and S3QELs (Suppressors of Site III_{Qo} Electron Leak) drugs hold a number of advantages over traditional mitochondria-targeted antioxidants. One of the problematic aspects of these traditional antioxidants is the non-specific suppression of ROS production, particularly when considering the beneficial signalling roles ROS are needed to perform. S1QELs and S3QELs are able to selectively suppress electron leak without interfering with other sites of electron flow or oxidative phosphorylation processes (Wong *et al.*, 2017; Watson;Wong and Brand, 2019). S1QELs target ROS production from site I_Q of CI via RET specifically, without impacting CI-linked respiration, though its mechanism of action is currently unknown (Brand *et al.*, 2016).

1.5 Mitochondrial role in ageing

During ageing and disease pathology mitochondrial homeostasis declines and ROS can increase dramatically, overwhelming the activities of local antioxidant systems and causing severe oxidative damage (Wallace, 2005). Genome-wide analysis has reported a reduction in gene expression of electron transport chain components and ATP synthase in flies, worms (McCarroll *et al.*, 2004), and rhesus monkeys, along with an upregulation of genes involved in inflammation and oxidative stress (Kayo *et al.*, 2001). At high levels ROS are capable of inducing lipid peroxidation, protein oxidation, and DNA damage (Cadet and Wagner, 2014; Su *et al.*, 2019), particularly in conditions of oxidative stress. This damage has been associated with many pathological conditions including cardiovascular

disease (Steven *et al.*, 2019), Parkinson's disease (Devi *et al.*, 2008), and Alzheimer's disease (Dragicevic *et al.*, 2010), where overproduction of ROS may contribute to disease progression. There is also an extensively debated relationship between ROS production and lifespan, primarily centred around the Mitochondrial Free Radical Theory of Ageing.

1.5.1 The Mitochondrial Free Radical Theory of Ageing

The Mitochondrial Free Radical Theory of Ageing was first stated by Denham Harman in 1956 (Harman, 1956), who proposed that mitochondria have an essential role in driving the ageing process. The basis of this theory was the observation that with age, dysfunctional mitochondria accumulate and produce high levels of ROS which cause oxidative damage within the cell. For a long time this theory went uncontested, but in more recent years contradictory evidence has arisen to bring it into question. It is true that an increase in dysfunctional mitochondria and ROS production can be seen with age (Choksi *et al.*, 2008; Gan *et al.*, 2012), and certain long-lived species produce less ROS (Barja *et al.*, 1994; Lambert *et al.*, 2007). However, studies have now shown that elevated ROS production does not always shorten lifespan (Van Raamsdonk and Hekimi, 2012), and directly manipulating ROS levels by treatment with antioxidants does not alter longevity (Sanz, 2016). This causes some controversy, as there is a clear disparity in the effects of ROS on lifespan between species. For example, modulation of antioxidant levels in mouse models of ageing shows no impact on lifespan (Perez *et al.*, 2009), whereas in *Drosophila melanogaster* and *C. elegans*, a mild increase in ROS production can extend lifespan by stimulating mitohormetic defence mechanisms and the mitochondrial unfolded protein response (mtUPR) (Yang and Hekimi, 2010; Owusu-Ansah;Song and Perrimon, 2013). Studies have also demonstrated that lifespan extension in worms with an inhibited mitochondrial electron transport chain is dependent on activation of HIF-1 by increased ROS levels (Lee;Hwang and Kenyon, 2010), and can promote longevity by stimulating pro-survival pathways in response to stress (Yee;Yang and Hekimi, 2014). While this information does raise questions regarding the original theory of ageing, it further highlights the idea that the mitochondria have essential roles in ageing.

Indeed, high levels of ROS production have been linked to both ageing and premature cellular senescence, and dysfunctional mitochondria are a common feature of aged cells

and major age-related diseases (ARDs) (Anderson *et al.*, 2009; Hoehn *et al.*, 2009; Ahmad *et al.*, 2015).

1.5.2 The role of mitochondria in cellular senescence

How cellular senescence develops and is regulated *in vivo* has been a subject of interest in the ageing field for many years, and particular attention has been paid to the role of the mitochondria. It has been well documented that dysfunctional mitochondria accumulate both with age (Bratic and Larsson, 2013; Chistiakov *et al.*, 2014), and in senescent cells (Passos *et al.*, 2007; Hara *et al.*, 2013; Correia-Melo *et al.*, 2016). This suggests a key role for mitochondria in ageing and senescence, but the specifics of that role are still unclear. A recent study by Correia-Melo and colleagues showed that mitochondria are indeed essential for the development of senescence, and that removing mitochondria from cells entirely via enforced mitophagy prevented development of the senescent phenotype. By targeting mitochondria for degradation via uncoupler-induced Parkin signalling, the report demonstrated that cells lacking mitochondria did not develop the classic markers of senescence following irradiation, including increased SA-β-Gal activity, ROS production, pro-inflammatory cytokine secretion and expression of CDK inhibitors p21 and p16.

A number of potential mechanisms for the role of mitochondrial dysfunction in senescence have been proposed. For example, studies have shown that senescent cells demonstrate a highly interconnected mitochondrial network, suggesting an impairment in the ability of the cells to remove and degrade old or dysfunctional mitochondria (Dalle Pezze *et al.*, 2014). Senescent cells display a distinct mitochondrial phenotype, involving increased mitochondrial mass, reduced antioxidant defence and increased ROS production, reduced membrane potential, and an imbalance of fission/fusion events favouring mitochondrial fusion. Data has also suggested that this rigid and elongated mitochondrial network can trigger onset of senescence, as such mitochondria produce higher levels of ROS and cannot be degraded by mitophagy processes. It is possible that this increased connectivity between mitochondria is a survival response, allowing the sharing of machinery and processes between dysfunctional mitochondria to regain function, and escape apoptosis by preventing membrane depolarisation and cytochrome

c release (Frank *et al.*, 2001; Gomes;Di Benedetto and Scorrano, 2011; Ziegler;Wiley and Velarde, 2015).

Increased antioxidant expression, or treatment with antioxidants, have both been shown to slow telomere shortening and delay onset of the senescent phenotype (Saretzki;Murphy and von Zglinicki, 2003), suggesting a role for ROS production in senescence development. In fact, a positive feedback loop has been identified linking mitochondrial ROS production and the persistent DDR required for senescence development. Moreover, mitochondrial ROS production is known to cause telomere dysfunction (Passos *et al.*, 2007). Excessive ROS production seen with age and in senescence can cause mitochondrial dysfunction resulting from oxidative damage, and subsequently increase electron leak and ROS production. High levels of ROS are able to continuously replenish short-lived DNA damage foci, maintaining a persistent DDR via p53/p21CIP1/WAF1 signalling. Although, studies have found that ROS production ceases to be essential for maintaining this loop and the senescent phenotype after approximately 9 days in the development of senescence, at which point the cell has been 'locked' into a senescent state (Passos *et al.*, 2010).

Defects in the electron transport chain are also known to induce premature senescence, including impairment of respiratory complexes, chronic uncoupling, and reduced ATP production (Zwerschke *et al.*, 2003; Stockl *et al.*, 2006; Moiseeva *et al.*, 2009). A number of these dysfunctions can lead to AMPK activation, which in turn triggers wide-ranging signalling pathways involved in senescent growth arrest, including p53 activation, downregulation of cyclin A, B1 and E, and p16/pRb signalling (Peyton *et al.*, 2012; Nacarelli *et al.*, 2019). Furthermore, mitochondrial CI is a major source of NAD+ within the cell, the depletion of which has been linked to senescence induction (Nacarelli *et al.*, 2019).

As discussed above, mitochondrial dysfunction also triggers a specific form of cellular senescence, termed mitochondria dysfunction-associated senescence, or MiDAS (Wiley *et al.*, 2016). A number of mitochondrial triggers of senescence were identified which tip the cell state in favour of senescence arrest and SASP production. Such triggers include a reduced NAD+/NADH ratio resulting in activation of AMPK and p53 signalling, mitochondrial depolarization, inhibition of ATP synthesis, NAD+ depletion, and elevated

ROS production (reviewed in (Ziegler;Wiley and Velarde, 2015). Of particular note, the SASP produced by MiDAS is distinct in that it lacks the pro-inflammatory IL-1 arm.

1.5.2.1 Senescence Associated Mitochondrial Dysfunction (SAMD)

A number of positive feedback loops have been proposed which link mitochondrial dysfunction and cellular senescence, with much emphasis on the role of mitochondrial dysfunction in triggering and maintaining the senescent phenotype. The other side of this observation is the role of senescence in causing further mitochondrial dysfunction, termed senescence-associated mitochondrial dysfunction, or SAMD. Studies have demonstrated a close relationship between mitophagy, SAMD, and the SASP. An impairment in mitophagy has been identified as a key mediator of dysfunctional mitochondria accumulation in senescent cells, and results in an overall reduction in mitochondrial quality and progressively more rigid networks (Dalle Pezze *et al.*, 2014). This failure of mitophagy also causes impaired mitochondrial membrane potential and respiratory coupling, and exacerbated oxidative stress, all resulting from a persistent DDR. Mitochondrial dysfunction is thought to both initiate and maintain the senescent phenotype, while the senescent DDR causes further mitochondrial dysfunction. Indeed, studies have now demonstrated that SAMD initiates the positive feedback loop between the DDR and ROS production, but also that SAMD is driven by p21 and p38MAPK signalling (Passos *et al.*, 2010).

Mutations in mitochondrial DNA (mtDNA) accumulate with age faster than mutations in nuclear DNA (Khrapko *et al.*, 1997; Wang;Wong and Cortopassi, 1997), and have been shown to induce senescent cell accumulation (Wiley *et al.*, 2016). In a study by Wiley and colleagues, they demonstrated that $POLG^{D257A}$ mice which have a mutation in mtDNA polymerase (PolG) and accumulate mtDNA mutations at a much faster rate than their wild-type counterparts, also exhibited a greater proportion of SA-β-Gal positive cells, lower nuclear HMGB1 levels, and increased $p16^{INK4a}$ expression than age-matched controls. Though this finding is not universal, and studies of fruit flies have shown that neither lifespan nor healthspan are impacted by the levels of mtDNA mutations that would reasonably occur in natural populations (Kauppila *et al.*, 2018). While the role of mtDNA mutations in ageing have been widely debated, studies have shown that an accumulation of mtDNA mutations does not necessarily lead to increased ROS production

and oxidative damage. In the same POLG^{D257A} mouse model it has been shown that markers of oxidative stress were not significantly difference to WT mice, and in fact direct measurement of ROS production from the mitochondria confirmed that ROS was not enhanced in these mice (Kujoth *et al.*, 2005; Trifunovic *et al.*, 2005). However, while mtDNA mutation may not be the cause of oxidative stress accumulation in ageing phenotypes, it has been demonstrated that the hydroxyl radical is capable of directly reacting with mtDNA and causing double-stranded DNA breaks (Chevion, 1988; Grishko *et al.*, 2001). Therefore, DDR-induced ROS production can lead to additional mutations in mtDNA and further accumulation of mitochondrial dysfunction (Passos *et al.*, 2010).

The observation of the MiDAS phenotype in which part of the SASP does not occur without mitochondrial dysfunction supports a role for SAMD in SASP development (Wiley *et al.*, 2016). Conversely, chronic NFκB-mediated inflammation enhances both senescent growth arrest and ROS production, demonstrating a positive feedback loop between SAMD and SASP (Nelson *et al.*, 2018). Furthermore, fragments of mtDNA have been proposed to escape into the cytosol during senescence, which are sensed by NFκB and cGAS-STING signalling pathways, triggering an innate immune response (West *et al.*, 2015). Finally, SAMD has been implicated in metabolic dysfunction with age, resulting from an inability to process fatty acids by dysfunctional mitochondria (Ogrodnik *et al.*, 2017).

1.5.2.2 Complex I dysfunction

Studies have demonstrated a decline in CI activity with age, accompanied by elevated ROS production (Navarro and Boveris, 2007). Such ROS production is linked to the induction of senescence (Passos *et al.*, 2010), and disrupted CI assembly has also been shown to induce premature senescence in primary cells (Miwa *et al.*, 2014). Partially assembled CI subcomplexes contribute to oxidative stress within the cell, as they possess the catalytic subunit capable of producing superoxide but do not form part of the electron transport chain and do not contribute to mitochondrial respiration. Importantly, work by Miwa et al (2014) provided a link between CI homeostasis and induction of senescence, as disruption of CI assembly by siRNA-mediated knockdown of the NDUFAF1 subunit triggered premature senescence. Conversely, low levels of partially assembled complexes corresponded to increased longevity. Efficient assembly of CI appears to be a key

determinant in ROS production – and subsequent senescence induction – as it is important to note that pharmacological inhibition of CI in senescent cells alleviates the pro-inflammatory SASP and lowers the incidence of DNA damage along with an associated reduction in ROS production (Moiseeva *et al.*, 2013; Le Pelletier *et al.*, 2021). Further work is needed to dissect the role of altered CI activity in cellular senescence.

CI dysfunction is also implicated in age-related diseases such as Parkinson's disease, Huntington's disease and Alzheimer's disease (Francis *et al.*, 2014). For example, PINK1 has been shown to regulate CI (Morais *et al.*, 2014), and greater CI deficiency in neurons is accompanied by elevated ROS production. A mutation of the PINK1 gene has also been linked to lower CI activity and elevated ROS levels in a genetic variant of Parkinson's disease (Morais *et al.*, 2014). In a study of Drosophila *pink1* mutants, overexpression of the CI subunit ND42 was sufficient to rescue a number of behavioural deficits and restore CI activity (Pogson *et al.*, 2014). Reduced levels of PINK1 and Parkin preceded a decline in CI-linked respiration in fruit flies, and PINK1 knockdown flies exhibited both lower CI-linked respiration and lifespan (Scialo *et al.*, 2016).

CI inhibition has also been shown to reduce both amyloid beta and phospho-tau levels in animal models of Alzheimer's disease. Zhang et al (2015) found that inhibiting CI increased neuronal response to stress, along with reduced ATP production and resultant increased AMP/ATP ratio – possibly implicating a role for AMPK activation.

1.5.2.3 The role of mitochondrial ROS in senescence

Senescent cells display an increased mitochondrial mass, reduced membrane potential and increased ROS production, thought to be a result of impaired antioxidant systems (Correia-Melo and Passos, 2015). High ROS levels have also been shown to accelerate telomere shortening and initiate DNA damage (Chen *et al.*, 1995; von Zglinicki, 2002), triggering the DNA damage response, which is essential for development of a senescent phenotype (d'Adda di Fagagna *et al.*, 2003; Rodier *et al.*, 2009). Despite a decreased membrane potential, mitochondria in senescent cells produce higher levels of ROS (Moiseeva *et al.*, 2009; Passos *et al.*, 2010) and are less able to regulate intracellular calcium homeostasis. During mitochondrial dysfunction, calcium is normally imported into the mitochondria to maintain electron transport chain function; however, this influx

leads to mitochondrial depolarization and decreased NAD+/NADH ratio, which can lead to senescence (Ziegler;Wiley and Velarde, 2015; Nacarelli *et al.*, 2019).

Increased ROS production is becoming a well-recognised aspect of cellular senescence, which can cause oxidative damage and result in mitochondrial dysfunction. Hydrogen peroxide has been identified as a potent inducer of senescence in numerous cell types (Sedelnikova *et al.*, 2004). ROS-mediated DNA damage and telomere shortening activates a persistent DDR, which is able to induce further ROS generation and mitochondrial dysfunction, demonstrating a positive feedback loop between mitochondrial dysfunction, ROS production and senescence (Passos *et al.*, 2010). Notably, studies have demonstrated that activation of p53, p21 and p16 – key DDR effectors – leads to elevated ROS production (Macip *et al.*, 2002; Macip *et al.*, 2003; Takahashi *et al.*, 2006), while treatment with antioxidants is able to prevent cell cycle arrest (Saretzki;Murphy and von Zglinicki, 2003).

1.5.2.4 SASP regulation

In addition to preventing the development of cellular senescence, Correia-Melo et al also demonstrated that mitochondria-depleted cells exhibited downregulation of many SASP factors, suggesting that mitochondria are required for the development of the SASP and may have a role in regulating its production.

A positive feedback loop has been proposed between mitochondrial ROS production and a persistent DDR which is required for establishment of the SASP. Mitochondrial ROS are able to replenish short-lived DNA damage foci and maintain the DDR, which in turn leads to senescence, SASP, and further damage to mitochondrial function (Passos *et al.*, 2010). The DDR has also been linked to increased mitochondrial biogenesis via PGC-1β activation, in which the initiator of the DDR, ATM, is activated in response to double-strand DNA breaks, in turn activates AKT and mTORC1, leading to PGC-1β-dependent mitochondrial biogenesis and ROS production, followed by DDR induction and SASP (Correia-Melo *et al.*, 2016). Studies have also shown that ATM can be activated by oxidative stress independently of DNA damage, and in fact has been implicated in regulating cell responses to oxidative stress (Guo *et al.*, 2010).

Changes in metabolite availability and redox status can also influence SASP production. An increased AMP/ATP ratio – often observed when mitochondrial ETC function is

impaired - activates AMPK, which in turn activates p53 signalling and recruitment of p21 and p16, resulting in development of senescence and SASP production (Peyton *et al.*, 2012; Nacarelli *et al.*, 2019).

1.6 Metformin

Metformin is a widely used anti-diabetic drug prescribed in the clinic for decades, but that has more recently shown benefits in alleviating a range of other age-related pathologies. Lifespan and healthspan extension has been demonstrated *in vivo* in both mice and worms with metformin treatment (Anisimov *et al.*, 2008; De Haes *et al.*, 2014). Moreover, in human studies metformin has been found to regulate expression of genes involved in DNA repair and mitochondrial fatty acid oxidation in muscle biopsies of older adults (Kulkarni *et al.*, 2018). Repurposing of metformin for use in other age-related conditions is now a vast area of research, covering cancer (Heckman-Stoddard *et al.*, 2017), cardiovascular disease (Han *et al.*, 2019), and neurodegenerative diseases (Kickstein *et al.*, 2010; Mor *et al.*, 2020).

1.6.1 Metformin and diabetes

Metformin is a well-established treatment for Type 2 diabetes that inhibits gluconeogenesis in the liver, increases glucose uptake by muscle, and increases fatty acid oxidation. These beneficial effects tend to require long-term treatment rather than single dosing (Wang *et al.*, 2019), but the liver receives relatively high concentrations of metformin through the portal circulation (Muller *et al.*, 2005).

Gluconeogenesis inhibition by metformin results from inhibition of key enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), energetic stress in the hepatocytes, and has been shown to be induced through both AMPK-dependent (Zhou *et al.*, 2001) and -independent (Foretz *et al.*, 2010) mechanisms *in vivo*. More recently it has been linked to inhibition of mitochondrial glycerophosphate dehydrogenase (mGPDH) (Madiraju *et al.*, 2014). An increased AMP/ATP ratio by metformin treatment also inhibits the activity of adenylate cyclase, responsible for converting ATP to cAMP. This reduction in cAMP eventually leads to suppression of gluconeogenesis (Miller *et al.*, 2013). Metformin also enhances insulin sensitivity by

reducing hepatic glucose production, which results from an AMP-induced inhibition of fructose-1,6-bisphosphatase-1; a rate-controlling enzyme in gluconeogenesis (Hunter *et al.*, 2018).

In skeletal muscle, activation of AMPK by metformin leads to increased glucose uptake, by increasing glucose transporter 4 (GLUT4) translocation to the cell membrane. Improved hyperglycemia is also achieved by inhibition of glucose production in the liver and improved insulin sensitivity (Hundal *et al.*, 2000; Takashima *et al.*, 2010).

1.6.2 Metformin and ageing

Metformin has been shown to extend lifespan in a ROS-dependent manner in *c. elegans* models, but this is not replicated in fruit flies (Sanz 2016). Lifespan and healthspan extension have also been observed in mice, though it is not known if this is mediated by ROS production (Martin-Montalvo *et al.*, 2013). In age-related disease, metformin has been tested in models of Alzheimer's disease and found to improve neuronal insulin signalling as well as neuropathological markers. Though this is contrasted with increasing Aβ levels, likely as a result of AMPK activation and upregulated BACE1 activity (Zhang *et al.*, 2015a). Chronic treatment with low dose metformin also inhibits age-associated atherosclerotic plaque formation in ApoE-/- mice (Karnewar *et al.*, 2018).

In a review of coronary artery disease (CAD) patient studies it was found that CAD patients exhibited reduced cardiovascular mortality, cardiovascular events and all-cause mortality when given metformin (Han *et al.*, 2019). Additionally, metformin treatment has shown benefits following myocardial infarction, and studies have demonstrated protection against hypertrophic and apoptotic remodelling *in vivo,* as well as a reduction of hypertrophic and apoptotic responses *in vitro* in response to stress (Loi *et al.*, 2019). Furthermore, metformin has been found to inhibit proliferation of cancer cells via the ATM-AMPK-p53/$p21^{CIP1}$ pathway and promote apoptotic responses to irradiation (Storozhuk *et al.*, 2013). Other reports have demonstrated a reduction in cell proliferation genes as an anti-tumour effect of metformin treatment (Ben Sahra *et al.*, 2008). Clinical trials have also reported the ability of metformin treatment to alleviate serum markers of breast cancer risk in women who had completed chemotherapy and radiotherapy treatment courses (Goodwin *et al.*, 2008; Campagnoli *et al.*, 2012).

More recently, metformin has shown great success as a senostatic treatment (Anisimov *et al.*, 2008; Algire *et al.*, 2012; Chen *et al.*, 2016; Hu *et al.*, 2020; Jiang *et al.*, 2020; Hansel *et al.*, 2021; Le Pelletier *et al.*, 2021). Notably, pro-inflammatory cytokine secretion in senescent cells is reduced by inhibition of the NFκB pathway by metformin independent of AMPK activation (Moiseeva *et al.*, 2013).

1.6.3 Mechanism of action

Metformin is part of the biguanide class of drugs, and exists as a positively charged hydrophilic cation, causing its accumulation within the mitochondria via high mitochondrial membrane potential and the strongly negative-inside environment of the mitochondrial matrix (Bridges *et al.*, 2014). Uptake of metformin into cells is primarily controlled by organic cation transporters (OCTs) (Moreno-Navarrete *et al.*, 2011), and accumulation in the mitochondria is slow, requiring hours before reaching effective concentrations. However it is important to note that concentrations within the mitochondria have been predicted to exist at up to 1000-times external concentrations as a result of its preferential accumulation (Owen;Doran and Halestrap, 2000; Bridges *et al.*, 2014). Phenformin and buformin are also anti-diabetic biguanides, but their clinical use has been suspended due to risk of lactic acidosis resulting from CI inhibition (Nattrass and Alberti, 1978; Kwong and Brubacher, 1998; Krishnamurthy *et al.*, 2004b).

Though metformin's mechanism of action is still debated within the literature, a number of pathways have been proposed for metformin, including inhibition of mitochondrial complex I, mTORC1 inhibition via activation of AMPK (Howell *et al.*, 2017; Wang *et al.*, 2019), and NFkB inhibition (Moiseeva *et al.*, 2013). It has also been shown to have antitumour effects due to inhibition of either gluconeogenesis or mTORC1, and antiproliferative effects which are not AMPK-dependent and are alleviated in the presence of antioxidants (Scotland *et al.*, 2013; Guo *et al.*, 2021). Metformin is also known to inhibit both ROS production and DNA damage following treatment with paraquat and induction of oncogenic Ras signalling, respectively (Algire *et al.*, 2012).

As a well-documented treatment with a good safety profile in humans, along with low cost and high efficacy, metformin represents an exciting potential therapy for a range of

conditions, but in particular there has been increasing interest in ageing and cellular senescence.

1.6.3.1 Complex I inhibition

The primary proposed mechanism of action of metformin is inhibition of mitochondrial respiratory complex I, by non-competitive inhibition of ubiquinone reduction (Bridges *et al.*, 2014; Wheaton *et al.*, 2014). This leads to decreased ATP production, elevated ADP/ATP ratio and subsequent activation of AMPK. Studies have shown that treatment with metformin reduces ADP-stimulated respiration with CI-linked substrates, but not CII-linked substrates, demonstrating CI-specific inhibition (Andrzejewski *et al.*, 2014). However, oral treatment of lean and obese Zucker rats has provided evidence that CI-linked respiration inhibition may not be replicated *in vivo* (Kane *et al.*, 2010), despite an observed reduction in ROS production and ADP-stimulated respiration inhibition in *ex vivo* models. One key observation in this study was that metformin doses capable of inhibiting mitochondrial respiration were two orders of magnitude greater than those required for inhibition of ROS production at CI, demonstrating the dose-dependent effects of metformin present across a number of its downstream targets.

The binding site at CI is thought to be formed by the 'core' subunits of CI that are conserved across all species (Bridges *et al.*, 2014)(and reviewed in (Hirst, 2013)), most likely an amphipathic region as the more hydrophobic biguanides produce greater CI inhibition. Metformin does not inhibit NADH oxidation, but in fact stimulates it, and does not interfere with intramolecular electron transfer between Fe-S clusters (Reda;Barker and Hirst, 2008). Studies suggest that biguanides may inhibit CI by trapping it in an open deactive-like conformation (Bridges *et al.*, 2014). However, as noted, significant inhibition of CI requires high concentrations of metformin, and the biguanide has been shown to have different dose-dependent effects.

At very high concentrations (>25mM) of metformin an increase in ROS production has been observed at the flavin site of CI (Bridges *et al.*, 2014), an effect which is increased when the NAD+/NADH pool is more reduced. ROS production at CI is likely stimulated by the reduced electron flow following inhibition and impaired ATP production, allowing more electrons to leak and form superoxide. Though these concentrations are extremely high and not achievable in a physiological setting, and many more reports have found

reductions in ROS production at lower concentrations of metformin, such as in superoxide production through RET at CI (Hou *et al.*, 2010; Marycz *et al.*, 2016; Robb *et al.*, 2018). However, this may be a result of changes in mitochondrial membrane potential or the ubiquinone redox status resulting from metformin treatment, rather than a direct inhibition of the ubiquinone-binding site (Robb *et al.*, 2018). The rate of ROS production at CI is largely dependent on the redox state of the NAD+/NADH pool (Murphy, 2009).

Metformin's dose-dependent inhibition of CI is reversible (Bridges *et al.*, 2014), in contrast to traditional CI inhibitors such as rotenone which bind competitively and irreversibly. This allows the inhibitory effect to respond to more favourable conditions and re-establish catalysis when possible.

Dose-dependent biphasic effects of metformin have been observed not only on ROS production rates, but also on NAD+/NADH ratio and CI-linked respiration. Low doses (<2nmol/mg) are able to promote a more oxidized NAD+/NADH pool, while high doses (>5nmol/mg) lead to a more reduced NAD+/NADH pool associated with CI inhibition. Similarly, low doses of metformin have been shown to promote CI activity, while supraphysiological doses are required to inhibit CI function (Wang *et al.*, 2019).

1.6.3.2 AMPK activation

Along with CI inhibition, studies have demonstrated that metformin activates AMPK both directly via phosphorylation of the catalytic α subunit at T172 (Hawley *et al.*, 2002), and indirectly by causing an increase in cellular AMP and ADP by inhibiting mitochondrial ATP synthesis (Hawley *et al.*, 2010). Importantly, metformin treatment does not improve hyperglycemia in a mouse model with specific knockout of the upstream AMPK regulator LKB1 (Shaw *et al.*, 2005), demonstrating AMPK dependence.

CI inhibition stimulates an increase in ADP/ATP ratio, and as such metformin is able to promote many downstream AMPK-dependent processes. This includes the inhibition of mTORC1 (Howell *et al.*, 2017), which is believed to contribute to insulin resistance in diabetes (Khamzina *et al.*, 2005). AMPK and mTORC1 have opposing influences on autophagy, with AMPK promoting phosphorylation of the mammalian autophagy-initiating kinase Ulk1, while mTORC1 prevents this activation (Kim *et al.*, 2011). This coordinated regulation of autophagy is important in the cellular response to nutrient

signalling. Activation of AMPK and inhibition of mTORC1 by metformin therefore promotes upregulation of autophagy, which can help to restore cellular function and aid in the clearance of dysfunctional organelles and misfolded proteins. Indeed, previous studies have demonstrated an increase in autophagosome and autophagolysosome presence in the cytoplasm of cells treated with low dose metformin, accompanied by AMPK activation, apoptosis suppression and a reduction in p16INK4a-positive cells (Chen *et al.*, 2016). Inhibition of the mTOR pathway, along with STAT3 signalling inhibition, by metformin is also able to prevent SASP production in models of senescence and enhance the efficacy of anticancer treatments (Hu *et al.*, 2020). A notable biphasic effect of metformin treatment has also been observed, in which low doses of metformin require AMPK activation in order to inhibit mTORC1, which higher doses demonstrate an AMPK-independent mechanism (Howell *et al.*, 2017).

By comparison, activation of AMPK by low dose treatment with metformin has also been found to induce senescence via p53-dependent signalling in proliferating hepatoma cells. In this context, metformin represents a potentially useful addition to cancer therapies (Yi *et al.*, 2013).

Metformin treatment also promotes mitochondrial biogenesis as a result of direct phosphorylation of PGC1α by AMPK, and has been shown to delay endothelial cell senescence (Karnewar *et al.*, 2018). Moreover, PGC1α activation is induced by elevated SIRT1 activity, which requires the increased NAD+ concentrations caused by AMPK's attempts to restore energetic balance (Canto *et al.*, 2009). SIRT1 is also involved in suppression of NFκB (Salminen *et al.*, 2008) and p53 signalling (Yi and Luo, 2010), demonstrating further beneficial effects downstream of metformin activity. Metformin is also able to inhibit mTORC1 independently of its action on AMPK, however, by inhibiting Rag GTPase-mediated mTORC1 signalling (Kalender *et al.*, 2010).

1.6.3.3 NFκB

Another known target of metformin action is the transcriptional regulator NFκB, which is a major regulator of pro-inflammatory cytokine secretion during senescence (Chien *et al.*, 2011; Ohanna *et al.*, 2011). Inhibition of NFκB by metformin therefore also causes a reduction in the expression of key pro-inflammatory cytokines, including MCP-1 and TGF-β (Gu *et al.*, 2014). Studies have demonstrated inhibition of NFκB nuclear translocation as

well as prevention of IκB and IKKα/β phosphorylation under metformin treatment. Metformin treatment also prevented induction of senescence in bystander cells when exposed to senescent conditioned media. Notably, however, metformin's effect on NFκB signalling is independent of its role in AMPK activation (Moiseeva *et al.*, 2013).

1.6.3.4 Other possible mechanisms

Low doses of metformin do not inhibit CI, but are still able to inhibit gluconeogenesis through a proposed redox-independent mechanism involving allosteric regulation of PFK1 and FBP1, and inhibition of the malate-aspartate shuttle (Alshawi and Agius, 2019). These low doses also produce a more oxidized mitochondrial NAD+/NADH pool, a more reduced cytoplasmic NAD+/NADH pool, and increased lactate/pyruvate ratio suggested to be a result of mitochondrial depolarisation from metformin accumulation.

An alternative proposed mechanism for gluconeogenesis inhibition by metformin is through mGPDH inhibition, which causes an elevated lactate/pyruvate ratio and inhibits gluconeogenesis specifically from reduced substrates (Madiraju *et al.*, 2014).

Metformin treatment has also been found to suppress glucagon signalling by blocking glucagon-induced cAMP production by adenylyl cyclase (Miller *et al.*, 2013). Furthermore, studies have demonstrated that metformin is able to inhibit mPTP opening in response to stressors. One such stressor is hyperglycemia, and studies have shown that treatment with antioxidants also prevents hyperglycemia-induced mPTP opening, providing a link between oxidative stress, high glucose production, PTP opening and metformin effects (Detaille *et al.*, 2005). Other noted effects of metformin treatment include inhibition of dipeptidylpeptidase 4, AMP deaminase, hexokinase II, and folate-related one-carbon metabolic pathways (Bridges *et al.*, 2014), as well as an elevated lactate/pyruvate ratio (Alshawi and Agius, 2019).

1.7 Aims and Objectives

Although the mitochondria are now known to be essential for the development of a senescent phenotype, less is known about the specifics of this role and how targeting mitochondrial function may benefit the pro-inflammatory senescent phenotype. We know that removing mitochondria alleviates the harmful SASP, but what is not known is the minimum effective intervention that may achieve a similar effect through less drastic means.

The aims of this study were:

a) To examine the role of mitochondrial respiratory function in senescence development
b) To identify whether manipulation of mitochondrial respiration could alleviate the SASP without restoring cell proliferation to damaged cells, and
c) To identify the minimum effective intervention that achieves this objective.

To do this, different experimental models and timepoints were examined in each chapter:

10 days treatment post-irradiation (Chapters 3 & 4):

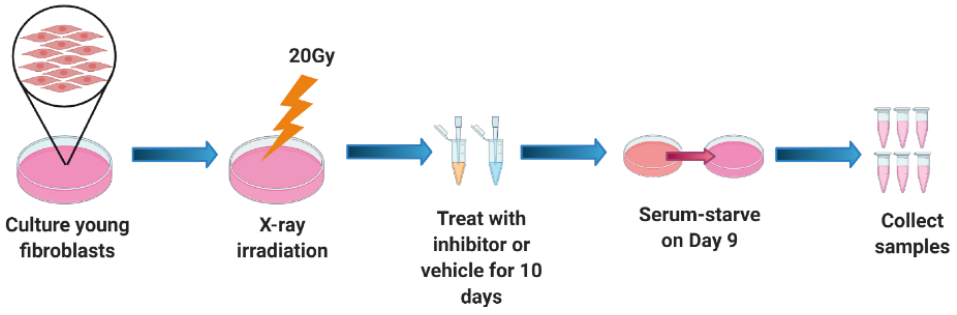


Figure 1.3: 10 day drug treatment experimental schematic immediately post-irradiation. Cells were seeded to plates and subjected to 20Gy irradiation the following day. Samples were then given drug-treated or control culture media for 10 days during senescence development. For ELISA analysis experiments, on Day 9 culture media was changed to serum-free for the final 24hrs to avoid serum interference with the assay. Samples were collected on day 10.

This experimental design allowed me to determine the effects of mitochondrial function manipulation on the senescent phenotype when cells were treated immediately following irradiation for the 10 days of senescence development.

Post-senescence treatment timepoints (Chapters 4 & 5):

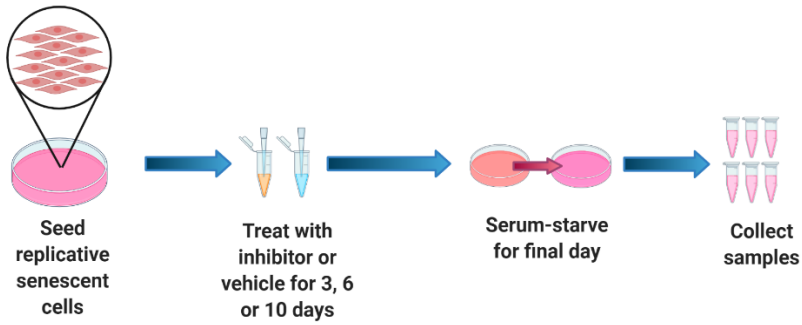


Figure 1.4: Replicative senescence experimental design schematic. In replicative senescence experiments, cells were cultured in flasks until proliferation slowed and eventually stopped, at which point samples were seeded to experimental plates. Treatments were initiated the following day, for either 3, 6 or 10 days. As before, in ELISA analysis experiments media was changed to serum-free for the final 24hrs before collection.

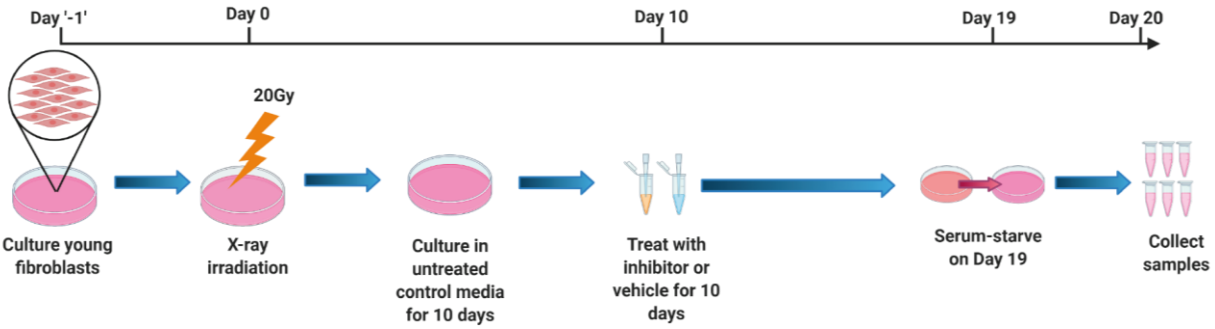


Figure 1.5: Post-senescence experimental design schematic for irradiation-induced senescence. In irradiation-induced senescence, cells were grown in untreated culture media for 10 days following irradiation, which was then switched to drug-treated media as appropriate at Day 10. Samples were then cultured for a further 10 days to match treatment times of earlier experiments. As before, in ELISA analysis experiments media was changed to serum-free for the final 24hrs before collection. In Chapter 5 an additional

3 day timepoint was examined in irradiation-induced senescence in which treatment timeline was shortened to 3 days.

These experimental designs allowed me to observe the difference in effect when senescent cells were treated after the phenotype had fully established, and determine if any differences existed between preventing a senescent phenotype and reversing one in two models of established senescence.

Shorter treatment times during senescence development (Chapter 5):

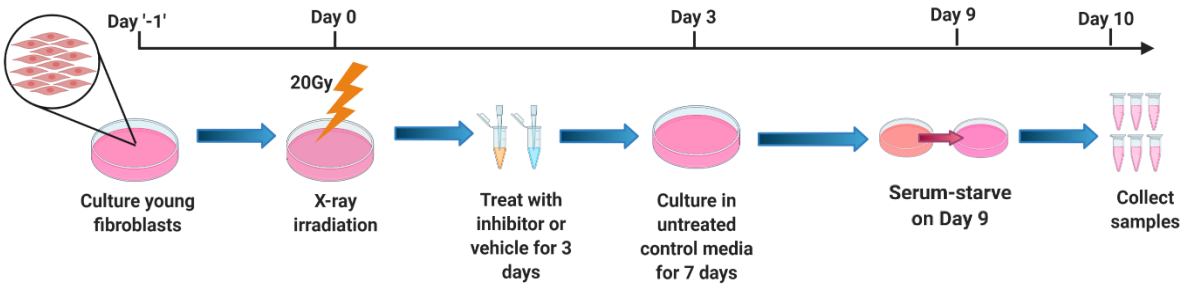


Figure 1.6: 3 day treatment during senescence development experimental design schematic. . Cells were seeded to culture plates and exposed to 20Gy irradiation the following day. Cells were then immediately given drug-treated or control media for the following 3 days. On Day 3 media was changed to untreated control for all samples for the remaining 7 days of senescence development. As before, in ELISA analysis experiments media was changed to serum-free for the final 24hrs before collection.

This experimental design allowed me to examine whether shorter treatment times of 3 days was sufficient to elicit a change in the resulting senescent phenotype, with the aim of identifying the minimum effective intervention to manipulate the phenotype

Chapter 2: Materials & Methods

2.1 Reagents

Reagent	Supplier	Catalogue number
(+)-Sodium L-ascorbate	Sigma	A4034-100G
2-deoxy-glucose	Merck	D8375-5G
Adenosine 5’-diphosphate sodium salt	Sigma	A2754-1G
Antimycin A	Santa Cruz Biotechnology	sc-202467A
Bovine Serum Albumin	Sigma	A7906-100G
Bovine Serum Albumin (fatty acid free)	Sigma	A7030-10G
Citric acid	Sigma	C0759-100G
cytochrome c from equine heart	Sigma	C2506-500mg
D-(+)-Glucose	Sigma	16325-1kg
D-(+)-Saccharose	VWR	27480.294
Digitonin	Santa Cruz Biotechnology	sc-280675
Dimethylformamide	Sigma	D4551-250ML
DMSO, sterile filtered	Santa Cruz Biotechnology	sc-359032
Dulbecco's Modified Eagle Medium - high glucose	Sigma	D5796
ELISA Substrate Reagent Pack	Biotechne	DY999
Ethyleneglycol-bis(2-amino-ethylether)-N,N,N',N'-tetracetic acid	Sigma	03777-10G
FCCP	abcam	ab120081
Fetal Bovine Serum	Sigma	
Fish skin gelatine	Sigma	G7765
Fluoroshield Mounting Medium With DAPI	abcam	ab104139
Galactose	Merck	G5388-100G

Reagent	Supplier	Catalogue number
Glutamate	Sigma	G1626
Glutaraldehye	Sigma	G5882
Goat anti-Mouse IgG (H+L), Alexa Fluor 488	Life technologies	A11001
Goat anti-Mouse IgG (H+L), Alexa Fluor 594	Life technologies	A11005
Goat anti-Rabbit IgG (H+L), Alexa Fluor 488	Life technologies	A11008
Goat anti-Rabbit IgG (H+L), Alexa Fluor 594	Life technologies	A11012
HEPES	Sigma	H3375-25G
Human CXCL10/IP-10 DuoSet ELISA	R&D Systems/biotechne	DY266-05
Human IL-6 DuoSet ELISA	R&D Systems/biotechne	DY206-05
Human IL-8/CXCL8 DuoSet ELISA	R&D Systems/biotechne	DY208-05
Hydrogen peroxide solution	Sigma	H1009-100ML
Ki67	Abcam	ab15580
L-(−)-Malic acid	Sigma	02288-10G
Lactobionic acid	Sigma	153516-100G
L-ascorbic acid	Sigma	A5960-25G
L-glutamine	Sigma	G7513-100ML
L-Glutamic acid monosodium salt hydrate	Sigma	G1626-100G
Magnesium Chloride, Hexahydrate	Santa Cruz Biotechnology	sc-203126A
Malonic acid	Sigma	M1296-500G
Metformin hydrochloride	Sigma	1115-70-4
MitoParaquat	Abcam	1821370-28-8
MitoQ	MedKoo Biosciences	317102
Monensin	Merck	M5273-500MG
Myxothiazol	Sigma	T5580-5MG

Reagent	Supplier	Catalogue number
N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride	Sigma	87890-25G
NADH disodium salt	Sigma	000000010128023001
Oligomycin A	Santa Cruz Biotechnology	sc-201551A
Penicillin-Streptomycin	Sigma	P4333-100ML
p21 Waf1 Cip1 (12D1)	Cell Signalling	2947S
PBS	Sigma	D5773
Peroxidase from horseradish	Sigma	P8250-50KU
Phospho-Histone H2AX (S139) clone JBW301	Merck millipore	05-636
Potassium cyanide	Sigma	60178-25G
Potassium ferricyanide	Sigma	702587-50G
Potassium ferro-cyanide	Sigma	P-9387
Potassium Phosphate, Dibasic, Anhydrous	Santa Cruz Biotechnology	sc-203210A
Potassium Phosphate, Monobasic	Santa Cruz Biotechnology	sc-203211A
ProLong® Gold Antifade Mountant with DAPI	Life technologies	P36935
Rapamycin	Merck	R8781-200UL
Rotenone	Santa Cruz Biotechnology	sc-203242
S1QEL1.1	Life Chemicals Europe	F2011-1277
S1QEL2.2	Life Chemicals Europe	F2068-0013
S3QEL2	Life Chemicals Europe	F6804-4818
Dulbecco's Modified Eagle Medium	Sigma	D5030
Seahorse XF24 FluxPak mini	Agilent	100867-100
Sodium azide 0.1M	Sigma	8591

Reagent	Supplier	Catalogue number
Sodium chloride	Sigma	S7653-1KG
Sodium phosphate dibasic	Sigma	S5136-100G
Sodium pyruvate	Sigma	P2256-25G
Sodium succinate dibasic hexahydrate	Sigma	S9637-500G
ELISA Stop solution	R&D Systems/biotechne	DY994
Taurine	Sigma	T0625-25G
Triton X-100	Promega	H5141
Trypsin-EDTA	Sigma	T4049
Tween 20	Sigma	P9416-100ML
X-gal	Sigma	3117073001

Table 2.1.1: Reagents used in this study

2.2 Cell culture

Cell culture work has been performed using human embryonic lung MRC5 fibroblasts acquired from ECACC, Salisbury, UK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, Dorset, UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100µg/ml penicillin, 100 ug/ml streptomycin and 2 mM glutamine at 37°C in a humidified atmosphere with 5% CO_2. Cell cultures were regularly maintained at 1 million cell density in 75cm^2 culture flasks, or 1.5 million in 150cm^2 flasks. Cells were trypsinized, collected and split every 2-3 days to maintain optimum confluency. Cells were counted at each passage and population doublings calculated prior to experiment setup, with no 'young' cell conditions exceeding PD32 by end of experiment timeline. Frozen cell stocks were stored in 95% FBS with 5% DMSO in aliquots of 1 million cells. Cryogenic vials were stored in a Nalgene Cryo freezing container at -80°C for minimum 24hrs before being transferred to -180°C storage in liquid nitrogen tanks. To defrost, cell vials were warmed to room temperature and immediately seeded to 75cm^2 culture flasks with warm DMEM media. Media was replaced the following day to remove any remaining DMSO.

2.3 Senescence induction

2.3.1 Irradiation-induced senescence

For stress-induced senescent cell cultures, cells were exposed to 20Gy X-ray irradiation using an X-Rad 225 irradiator (Precision X-Ray, N-Branford, CT USA) and media changed immediately after irradiation. For each condition; untreated senescent control, or senescent cells treated with specific ETC inhibitors, appropriate media was replaced every 2-3 days for 10 days until senescence development (Correia-Melo *et al.*, 2016). In immunostaining experiments senescence induction was considered successful by the presence of SA-β-Gal staining, DNA damage foci denoted by γH2AX-positive foci, and lack of proliferation marker Ki67.

2.3.2 Replicative senescence

For replicative senescent cell cultures, cells were maintained in culture until divisions ceased, usually beyond 40 population doublings, and considered senescent when cell size was expanded and appeared morphologically similar to irradiation-induced senescent cell cultures; increased cell size, increased nuclear size, and flatter cell body appearance. This was confirmed by SA-β-Gal test staining.

2.4 Cell treatments

For drug treatments, cells were grown in media containing the indicated drug concentration for the length of the experiment. Where cells were treated immediately post-irradiation, treated media was refreshed every 2-3 days for 10 days. Where cells were treated after establishment of a senescent phenotype, untreated media was use for the initial 10 days post-irradiation, changed to treated media on day 10, and refreshed every 2-3 days for 3 or 10 days respectively.

In experiments examining acute effects of treatments, drugs were administered directly during data collection for oxygen consumption rate and ROS production, to the

appropriate final concentration calculated for the experimental format (Seahorse XF Analyzer plates, or 96-well black plates).

Drug	Final concentration
Rotenone	0.1µM
Metformin	5mM
Malonate	5mM
Antimycin A	0.25µM
Myxothiazol	0.05µM
KCN	0.1mM
Sodium Azide	0.1mM
Oligomycin	0.25µM
FCCP	0.25µM
MitoQ	200nM
MitoParaquat	1µM
S1QEL1.1	100nM
S1QEL2.2	500nM
S3QEL2	10µM
Rapamycin	50nM

Table 2.4.1: Final drug treatment concentrations carried forward following dose range screening

2.5 High resolution respirometry

2.5.1 Oroboros Oxygraph-2k

High-resolution respirometry measurements of cellular oxygen consumption were carried out as described in (Pesta and Gnaiger, 2012), modified from a protocol for permeabilised muscle fibers, using OROBOROS Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) and using DatLab software for data acquisition and analysis.

Cells were cultured in 75cm^2 flasks for 10 days following irradiation of senescent samples. On Day 10 1 million cells were extracted per sample, centrifuged and resuspended in MiRO5 respiration buffer (110mM saccharose, 60mM lactobionic acid, 0.5mM EGTA, 3mM $MgCl_2 \cdot 6H_2O$, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES adjusted to pH7.1, and 1g/L BSA essentially fatty acid free) and added to the oxygraph chambers at 2ml chamber volumes ($0.5x10^6$ cells/ml). After 10 minutes to allow for stabilisation, OCR measurements for baseline respiration were taken (no additional substrates or inhibitors present). Oligomycin (2µg/ml) was added to inhibit ATP synthase activity and show proton leak-dependent OCR, and measurements taken after stabilisation. Maximum respiratory capacity was determined by titration of FCCP in 0.5µM steps until OCR no longer increased on addition of FCCP. Rotenone (0.5µM) and antimycin A (2.5µM) were then added to reveal residual oxygen consumption (non-mitochondrial OCR).

For permeabilised cell tests an optimum concentration of 32µM digitonin was administered in the chamber to permeabilise the plasma membrane. Complex-specific substrates were then provided; 5mM pyruvate and 2mM malate (CI), followed by 2mM ADP to stimulate oxidative phosphorylation, and 10mM succinate (CII) to activate the convergent electron flow from CI & II into the Q-cycle. 0.5µM rotenone was administered to show respiration when only CII was supplying electrons to the Q-cycle, followed by 2µM antimycin A to inhibit CIII function.

2.5.2 Seahorse AF Extracellular Flux Analyzers

Cellular oxygen consumption rates and extracellular acidification rates were measured in either a Seahorse XFe96 or XF24 Analyzer (Agilent Technologies). Cells were plated at seeding densities of 8,000 for XF96-well plates, and at 25,000 for young cell controls, and 6,000 for senescent conditions in XF24-well plates. Young cell samples were maintained in culture flasks until being seeded to plates the day before measurement, while senescent conditions were seeded directly to plates and maintained with media changes for the duration of senescence development (10 days). Cells were maintained in culture using media conditions as described above, before being changed to unbuffered media (DMEM (Sigma, D-5030) supplemented with 5mM D-glucose (Sigma), 2% L-Glutamate, 3%

calf serum) prior to OCR and ECAR measurement. Cell plates were then incubated at 37°C in a non-CO_2 incubator for 1 hour. Drug injection stocks (2.5µM oligomycin, 5µM FCCP, 5µM rotenone, and 25µM antimycin A) were loaded into injector ports of sensor cartridges, which were used to calibrate Analyzer machines prior to sample measurement. Cell plates were then loaded into the Analyzer and measurements taken for OCR and ECAR every 7 minutes for 90 minutes. Changes in oxygen consumption rates were measured after the addition of oligomycin (2.5µM), FCCP (0.5µM titration to 1uM final concentration), rotenone (0.5µM) and antimycin A (2.5µM).

Addition of oligomycin inhibited ATP synthase and revealed the level of proton leak present, and by subtraction from basal levels, the proportion of ATP-linked respiration. Titration of mitochondrial uncoupler FCCP allowed assessment of maximal respiratory capacity. Addition of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) showed the residual oxygen consumption – or non-mitochondrial oxygen consumption(Mavin *et al.*, 2020). OCR and ECAR measurements were used to calculate overall ATP production along with ATP generated specifically from oxidative phosphorylation or from glycolysis (Mookerjee and Brand, 2015).

Production of lactate from glycolysis, and CO_2 from the citric acid cycle, cause acidification of the unbuffered assay medium. The contribution of glycolysis to ATP production can be calculated from ECAR, as the 1:1 ratio of Lactate+H^+ to ATP production allows us to assume that the rate of proton production is equal to the rate of glycolytic ATP production. However, the acidification contribution from CO_2 produced by the citric acid cycle must be subtracted using the OCR. By multiplying OCR by 2.63 according to the 2.63:1 ratio of ATP to oxygen, we can calculate the ATP production rate per molecule of oxygen consumed. We can then estimate the amount of citric acid cycle activity needed in order to generate the amount of NADH required for a known OCR, and subtract that value from the total to give mitochondrial-derived ATP.

2.6 SA-β-Gal staining

Cells were seeded onto 19mm coverslips in 12-well plates and cultured as described above, young cells treated with control media for 2 days before fixation, and senescent cells with either control or drug-treated media for 10 days. Cells were then fixed with 2% PFA + 0.2% glutaraldehyde, and then washed with PBS. This was followed by incubation with 1ml/well of SA-β-Gal staining solution (containing 1M sodium chloride, 1M magnesium chloride, 0.5M citric acid, 0.1M sodium phosphate, potassium ferrocyanide, and X-gal at pH6) for 24hrs at 37°C. Coverslips were then washed with PBS and mounted onto microscope slides using ProLong Gold antifade reagent with DAPI labelling and detection media (Invitrogen by Thermo Fisher Scientific). Images were acquired using a Leica DM5500 microscope and LASX software.

2.7 Immunofluorescent staining

Following culture and treatment of cells (as detailed above), cells were fixed using 2% PFA and blocked using PBS. Cells were incubated with PBG-Triton (PBS, 0.5% BSA, 0.5% Triton X-100, 0.2% Fish skin gelatine) for 45 minutes, and then primary antibodies applied for incubation. Primary antibodies were diluted in PBG-Triton. Primary antibodies used were anti-p21 (Cell Signalling, Rabbit Mono, 2947S, 1:1000), anti-p16 (BD Biosciences, mouse, c 550834 / 4189810, 1:5), anti-Ki67 (Abcam, rabbit, ab15580, 1:250) and anti- yH2AX (Merck Millipore, mouse, 05-636, 1:2000). Secondary antibodies were diluted in PBG-Triton at a 1:4000 dilution for each, and included goat anti-mouse IgG (H+L), Alexa Fluor 594, goat anti-mouse IgG (H+L), Alexa Fluor 488, goat anti-rabbit IgG (H+L), Alexa Fluor 594, and goat anti-rabbit IgG (H+L), Alexa Fluor 488 (Life technologies, A11005, A11001, A11008, and A11012, respectively). Primary antibody incubations were carried out for 1hr in the dark at room temperature, and the process repeated for secondary antibody incubation. Coverslips were washed between incubations using PBG-Triton, and after secondary antibody incubation using PBS. Later experiments (Results Chapter 3) also used Sudan Black to reduce background interference in double-stained cells for p21 and yH2AX. This entailed a 30 second wash with 70% ethanol following the final PBS wash, 2 minute

block with 0.1% Sudan black dissolved in 70% ethanol, and a further wash with 70% ethanol to remove excess Sudan Black. Coverslips were washed again with PBS before mounting onto microscope slides using ProLong Gold antifade reagent with DAPI labeling and detection media (Invitrogen by Thermo Fisher Scientific).

2.8 Microscopy

2.8.1 Fluorescent microscopy

Immunofluorescent images were acquired using a Leica DMi8 widefield fluorescence inverted microscope (Leica Microsystems, Wetzlar, Germany) with a Hamamatsu C11440-22 camera through 40X oil immersion objective and assessed using LASX software.

2.8.2 Light microscopy

SA-β-Gal images were acquired using a Leica DM5500 widefield fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a DFC360FX camera through 20X dry objective using brightfield and DAPI channels and assessed using LASX software.

2.8.3 Analysis

Images were analysed using Leica Application Suite X software and ImageJ software. Analysis of cell count and percentage of positively stained cells for each fluorescent or histochemical staining was conducted manually by eye. p21 staining intensity, number of γH2AX-positive foci, and nuclear size were quantified using automated protocols in ImageJ software.

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Cells were cultured in 6 or 12-well plates at a seeding density of 150,000 or 75,000 cells per well, respectively. Non-irradiated controls were cultured separately until 2 days before media collection, when they were seeded to plates. Cells were incubated in half volume serum free DMEM for 24 hours at day 9 post-irradiation, after which the media was collected for ELISA analysis. Cells were trypsinized and counted to allow for data normalisation. ELISA analysis was carried out according to manufacturer's instructions

(R&D Systems; DY206/DY208/DY266) to measure concentrations of IL-6, IL-8 and IP-10 cytokines in cell culture media. 96-well half-area microplates with medium binding were coated with 240µg/ml capture antibody in PBS overnight on an orbital shaker. Wells were then washed twice in 150µl of 0.05% PBS-Tween20 buffer, followed by 90 minutes blocking with 150µl/well of 1% BSA in PBS solution. Wells were washed twice again with wash buffer, and standards and samples loaded to wells in 25µl volumes and incubated for 90 minutes on an orbital shaker. Following another two washes with buffer, detection antibody was added to wells in 25µl/well volumes of 1:100 dilution in either 1% (IL-6 and IP-10) or 0.1% (IL-8) BSA solution. Wells were washed twice and a 1:200 dilution of Streptavidin-HRP in 1% or 0.1% BSA solution as appropriate was added in 25µl/well volumes and incubated for 20 minutes. Wells were washed twice and 50µl/well of a 1:1 solution of Colour Reagents A and B was added. Plates were incubated in the dark for 20 minutes to allow for colour development, following which 50µl of $2NH_2SO_4$ was added to stop the reaction. Absorbance was measured at 450nm in a Promega GloMax Multi Detection System plate reader (Promega Corporation, Madison, WI, USA) and values converted to pg/ml of each cytokine using the standard curve measurements.

2.10 Cytokine Array

30µl aliquots of conditioned serum-free media, as used for ELISA analysis, were provided for Human Cytokine Array/Chemokine Array 42-Plex with IL-18 (HD42), carried out by Eve Technologies, Calgary, AB Canada.

2.11 Amplex Red Assay

Cells were seeded into 6-well plates and cultured in control or drug-treated DMEM media as described above. Young proliferating cells were cultured separately in $25cm^2$ flasks and seeded to plates 2 days before testing. On the day of testing cells were trypsinized and collected, cell counts taken, and samples centrifuged at 100*g* for 4 minutes. Samples were resuspended in 700µl PBS and added in 200µl triplicate samples to a black 96-well plate along with 1U/ml HRP and 50µM Amplex UltraRed Reagent per well. PBS with HRP and

AR was used as blank control. Fluorescent signal produced by the reaction between Amplex UltraRed and hydrogen peroxide was measured using a FLUOStar Omega Plate reader (BMG LABTECH, Offenburg, Germany) at Ex540/Em590 for 1 hour, taking measurements every 20 seconds. At 30 minutes, plate was extracted and hydrogen peroxide known concentration standard was added to a Blank well. Change in fluorescence upon standard addition was used to convert fluorescence values to pM hydrogen peroxide values during analysis, and change over time used to produce rates of production.

2.12 Statistical Analysis

Comparisons between two groups were analysed for statistical significance using 2-tailed unpaired t-tests. All staining, ELISA and ROS measurement data where more than two groups were compared, a one-way ANOVA analysis followed by Tukey tests was used, making multiple comparisons of each condition against young control and senescent control samples. All statistical analysis was conducted using GraphPad Prism 9.0, and statistical significance was determined at $p<0.05$. Where $n<3$ data is represented as mean±S.D., and where $n\geq3$ data is represented as mean±S.E.M.

2.13 Figure design

Graphical figures were produced using Adobe Illustrator 2021.

Illustrative figures were generated using BioRender.com

Chapter 3: Treatment with rotenone, antimycin A, myxothiazol, sodium azide and oligomycin alleviate SASP

3.1 Treatment with rotenone, antimycin A, myxothiazol, sodium azide and oligomycin all reduce pro-inflammatory cytokine secretion in senescent cells

In order to examine the influence of individual mitochondrial respiratory complexes in development of the SASP, I exposed MRC5 fibroblasts to 20Gy x-ray irradiation to trigger senescence, followed immediately by treatment with inhibitors of specific respiratory complexes for 10 days until development of a senescent phenotype (**Figure *3.1***). This timepoint was chosen as SASP development takes at least 7 days to develop (Coppe *et al.*, 2008). Culture media was exchanged for media without serum at day 9 to avoid interference with ELISA analysis, and at day 10 serum-free media was collected and secreted levels of key SASP factors IL-6, IL-8 and IP-10 were measured from proliferating, untreated and treated senescent cell samples by ELISA (Fig. 3.2-9).

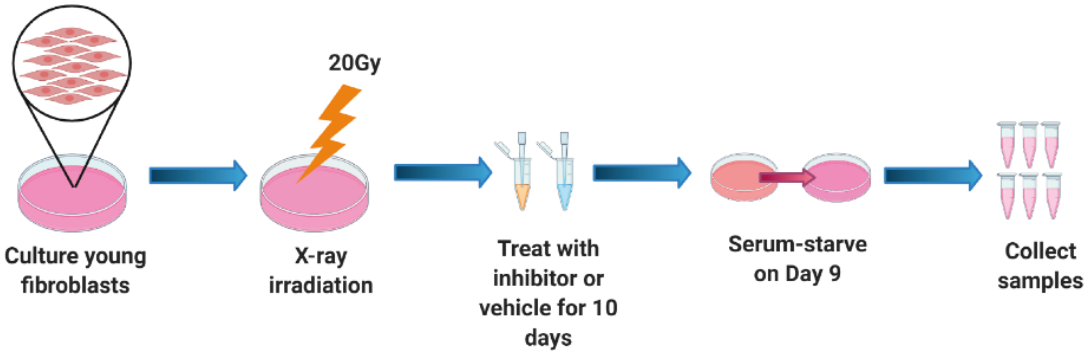


Figure 3.1: Schematic of senescent ELISA experimental design. Cells were seeded to 12-well plates and irradiated at 20Gy the following day, then given drug-treated or control media immediately post-IR and replenished every 2-3 days for 10 days. At Day 9 media was replaced with drug-treated or control serum-free media to avoid serum interference with ELISA. Conditioned medium was then collected on Day 10 and cell counts performed for data normalisation.

Dose ranges were tested to establish a concentration for each drug which was able to both inhibit OXPHOS activity and reduce secretion of pro-inflammatory SASP factors. This

was based on a 1:2 and 1:10 dilution from concentrations advised by Oroboros Oxygraph-2k respirometry protocols to fully inhibit the target complexes. Inhibitor efficacy was first confirmed using acute respiration analysis in young proliferating fibroblasts, in which increasing doses of drug were injected during Seahorse XF24 analysis and changes in oxygen consumption rate (OCR) measured. OCR was then measured in proliferating cells following 48hr treatment in culture with each of the inhibitors to account for factors such as slow drug accumulation or time-dependent increase or decrease in drug efficacy. Where possible, the lowest effective dose at inhibiting both OCR and SASP factor secretion was selected for all subsequent experiments for each inhibitor. I found that for most drugs the lowest doses tested were still able to inhibit the intended complex by at least 50% while also reducing SASP factor secretion. Exceptions to this were malonate, KCN, and FCCP. The inability of KCN to suppress OCR acutely is surprising, as it is a well-established CIV inhibitor and its acute effects on OCR are well-known (Jensen *et al.*, 1984; Leavesley *et al.*, 2008). As such, this may indicate a technical issue with these KCN treatment experiments as data following 48hrs in culture shows a significant decrease in OCR, confirming its effect.

3.1.1 Rotenone reduces OCR and inhibits SASP factor production

To examine the effect of CI inhibition on SASP production cells were treated with rotenone (**Figure *3.2***), a well-established CI inhibitor that binds the quinone-binding site of the complex, preventing exit of electrons into the intermembrane space and reduction of ubiquinone (Lambert and Brand, 2004). Acute treatment of proliferating cells with increasing doses of rotenone (0.1, 0.2, 0.4 and 0.5μM) demonstrated a significant and immediate dose-dependent reduction in OCR (Fig. 3.2A-B). This inhibition was still present when cultured for 48hrs in the presence of 0.1μM rotenone, confirming rotenone's ability to inhibit mitochondrial respiration over time (Fig. 3.2C). To determine the effect of CI inhibition in a senescent cell model, rotenone dose ranges were administered to cells following 20Gy irradiation, for 10 days until the senescent phenotype was fully established. Analysis of secreted levels of IL-6, IL-8 and IP-10 showed a significant reduction in all three markers at 10 days post-irradiation (Fig. 3.2D). This demonstrates that chronic treatment with low dose rotenone is able to alleviate SASP factor production in irradiation-induced senescent fibroblasts.

Based on these initial findings I determined that the lowest tested dose of 0.1µM rotenone was sufficient to inhibit SASP factor production while maintaining mild inhibition of CI activity, and minimising the risk of toxicity to cells. This dose was subsequently used for all future experiments with rotenone treatment. To expand on the picture of SASP suppression further, a set of serum-free culture media samples from proliferating, untreated control senescent cells and senescent cells treated with 0.1µM rotenone were subjected to cytokine array analysis (Eve Technologies, Calgary, AB Canada) (Fig. 3.2E). This analysis allowed me to examine a wider range of pro-inflammatory cytokines and chemokines known to make up the SASP (Coppe *et al.*, 2008; Coppe *et al.*, 2010). Results revealed an equivalent suppression of a number of major SASP factors, including GRO alpha, MIP-1a, IL-7 and IL-15 (Fig. 3.2F). Together these data demonstrate that treatment with rotenone over 10 days post-irradiation is sufficient to suppress production of a broad range of SASP factors; a promising indication for therapeutic targeting of mitochondrial function in senescence.

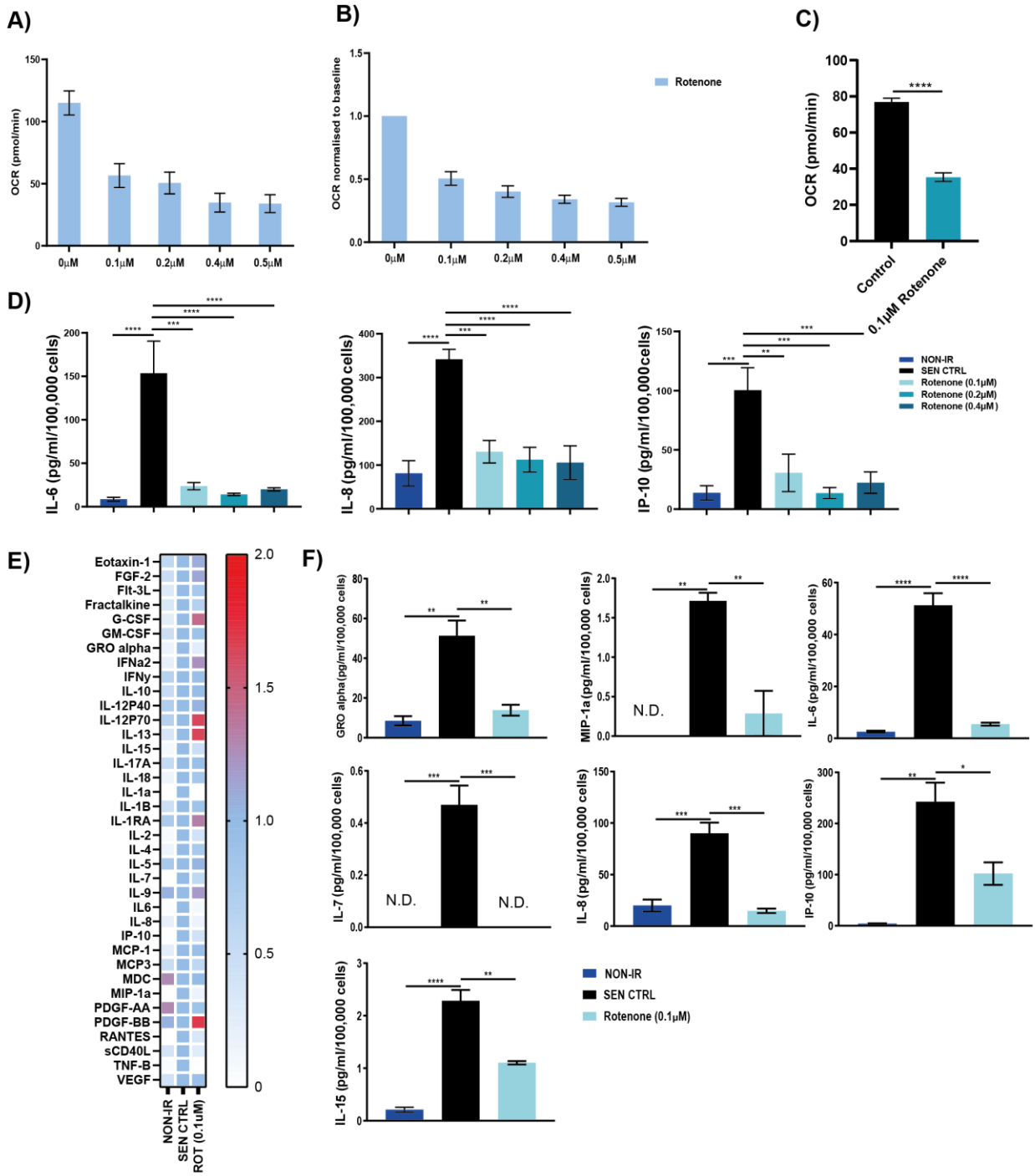


Figure 3.2: Rotenone reduces pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of rotenone on cellular respiration in young MRC5 fibroblasts analysed by Seahorse XF24 Analyzer, data taken from one experiment, n=5 technical replicates (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs in control or rotenone-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and rotenone-treated (0.1μM, 0.2μM, 0.4μM) senescent MRC5

fibroblasts measured by ELISA. Data taken from two independent experiments, n=6 technical replicates, (E) Cytokine array analysis of young non-irradiated, senescent, and rotenone treated (0.1μM) senescent fibroblasts represented as a heat map of fold-change in cytokine levels. Samples from one independent experiment as described in Fig. 3.1 used for analysis, n=3 technical replicates, (F) Levels of key cytokines analysed by cytokine array in (E) which showed significant increase in senescence and decrease with rotenone treatment. N.D. = Not detected; cytokine levels were below detectable limits. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.1.2 Malonate does not reduces OCR or inhibit SASP factor production

Acute treatment with increasing doses (0.5, 1, 2.5 and 5mM) of the CII inhibitor malonate (**Figure *3.3***) did not elicit any change in OCR of proliferating fibroblasts (Fig. 3.3A-B), nor after 48hrs in culture (Fig. 3.3C). This was unexpected, as malonate is widely known to inhibit CII activity, however I later became aware that malonate is unable to permeate intact cells (Prag *et al.*, 2020), and as such did not affect mitochondrial respiration in the models examined here. While malonate may be an effective inhibitor of CII in permeabilised cells or isolated mitochondria, for the purposes of this project it was not possible to design an experimental model which would allow effective treatment of senescent fibroblasts with malonate for 10 days. As such, it is unsurprising that chronic treatment with malonate in culture did not affect levels of secreted SASP factors in senescent cells (Fig. 3.3D). As a result, malonate treatment in intact cells provides a reasonable negative control with regards to both acute and long-term treatment in proliferating and senescent fibroblasts when measuring OCR and cytokine secretion.

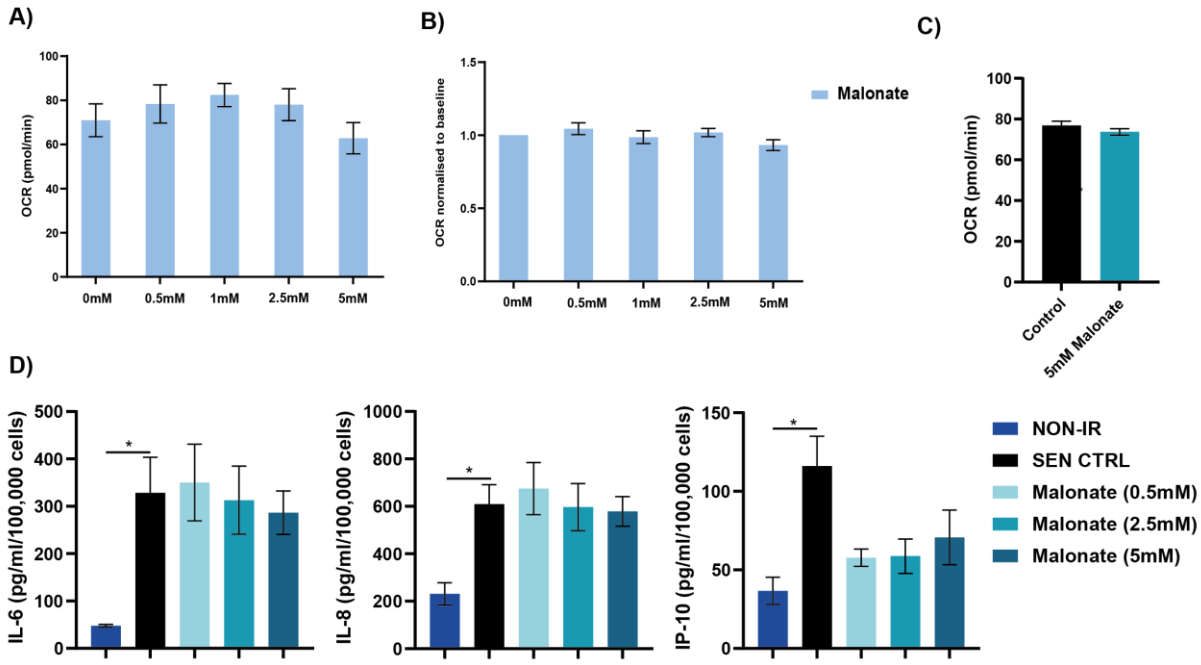


Figure 3.3: Treatment with malonate does not reduce pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of malonate on cellular respiration in young MRC5 fibroblasts measured by Seahorse XF24 Analyzer, data taken from one experiment, n=3 technical replicates, (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs in control or malonate-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and malonate-treated (0.5mM, 2.5mM, 5mM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05

3.1.3 Antimycin A and myxothiazol reduce OCR and inhibit SASP factor production

Acute treatment of proliferating cells with increasing doses (0.25, 0.5, 1.25 and 2.5µM) of the CIII inhibitor antimycin A (**Figure *3.4***) produced a significant (70%) and immediate reduction in OCR at all doses tested (Fig. 3.4A-B). After 48hrs in culture with antimycin-treated media this inhibition was reduced to 53% (Fig. 3.4C). Subsequent chronic treatment tests in senescent cells demonstrated a significant reduction in secreted IL-6

and IP-10 levels at all tested doses. However, IL-8 remained unaffected by long-term treatment at any tested concentration (Fig. 3.4D). As there appeared to be little difference in effect between doses on OCR and SASP factor measurements, the lowest dose of 0.25µM antimycin A was used for all subsequent experiments in order to minimise risk of cell toxicity and maintain the minimum effective intervention.

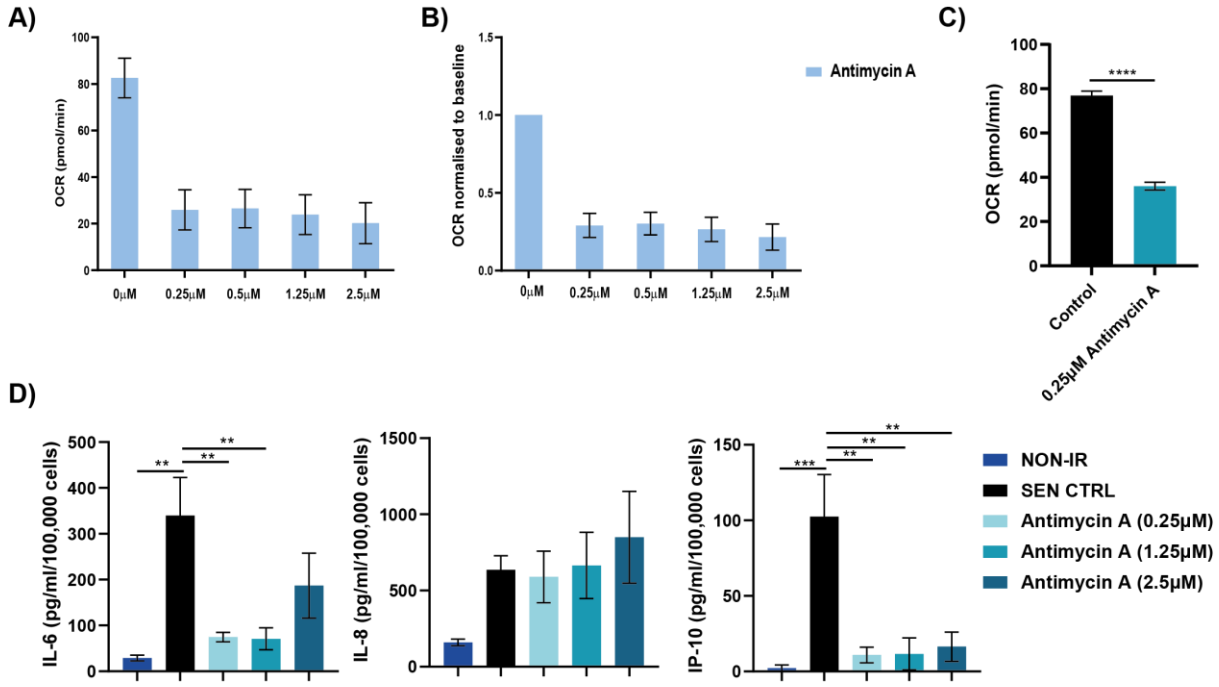


Figure 3.4: Antimycin A reduces some pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of antimycin A on cellular respiration in young MRC5 fibroblasts measured by Seahorse XF24 Analyzer, data taken from one experiment, n=5 technical replicates, (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1 (C) OCR measurements in young proliferating cells cultured for 48hrs in control or antimycin A-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and antimycin A-treated (0.25µM, 1.25µM, 2.5µM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates, Data was analysed using one-way ANOVA and Tukey's multiple comparisons test . Data is mean ±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Antimycin A blocks the inner quinone-binding site (site III_{Qi}) of CIII which causes backflow of electrons to downstream complexes and increased ROS production at site III_{Qo}, whereas myxothiazol blocks the outer quinone-binding site (III_{Qo}) and can reduce ROS production at CIII. Though both are inhibitors of CIII-linked respiration, in order to examine the influence of each site on SASP production, both inhibitors were tested in proliferating and senescent models. Acute treatment with increasing doses (0.05, 0.1, 0.25 and 0.5µM) of myxothiazol (**Figure *3.5***) elicited an almost complete inhibition of OCR, reaching 90% inhibition above 0.25µM (Fig. 3.5A-B), which was maintained when treated for 48hrs in culture (Fig. 3.5C). In senescent cells, all tested doses of myxothiazol successfully inhibited IL-6 and IP-10 levels, but as seen with antimycin A, IL-8 showed minimal change (Fig. 3.5D). Though there appeared to be a mild trend towards reduced IL-8 secretion, this difference was not statistically significant. However, analysis by cytokine array of the same myxothiazol-treated samples showed a comparable reduction in IL-8, suggesting a technical error may have occurred.

As the CIII inhibitor most effective at reducing SASP factor secretion, along with its established ability to reduce ROS production from CIII (Brand, 2016), I chose to examine the wider SASP profile of myxothiazol-treated senescent cells in more detail via cytokine array analysis (Fig. 3.5E-F). A number of pro-inflammatory cytokines and chemokines known to form part of the SASP were substantially reduced upon treatment with 0.05µM myxothiazol. This included G-CSF, CM-CSF, GROalpha, IFNa2 and MIP-1a, which have roles in macrophage maturation, cell proliferation and survival. Overall this data demonstrates that chronic treatment with inhibitors of CIII post-irradiation is sufficient to prevent the development of a SASP *in vitro* possibly with the exception of IL-8 as discussed.

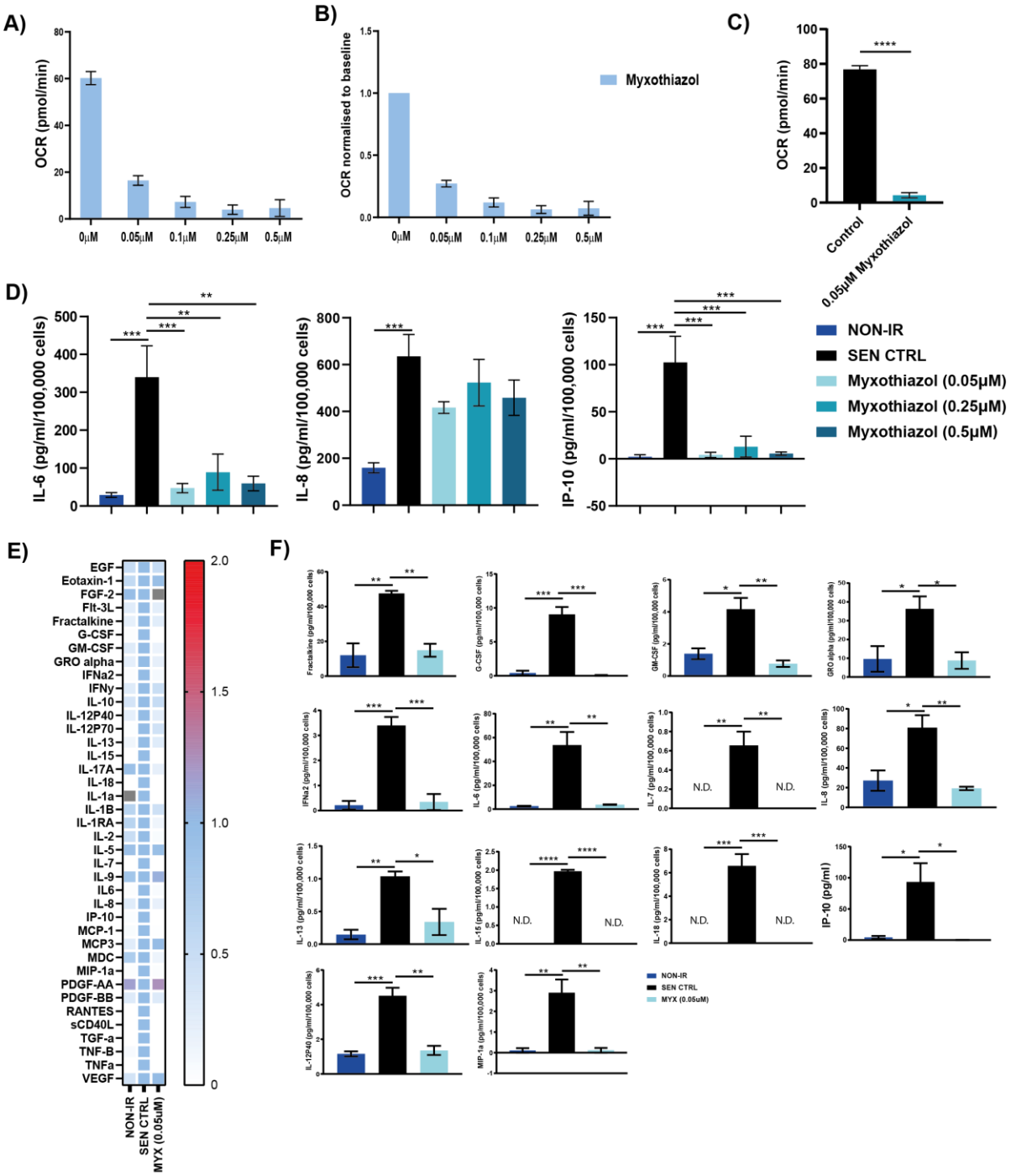


Figure 3.5: Myxothiazol reduces some pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of myxothiazol on cellular respiration in young MRC5 fibroblasts measured using Seahorse XF24 Analyzer, data taken from one experiment, n=3 technical replicates (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1 (C) OCR measurements in young proliferating cells cultured for 48hrs in control or myxothiazol-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and myxothiazol-treated (0.05μM, 0.25μM

and 0.5µM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates, (E) Cytokine array analysis of young non-irradiated, senescent, and myxothiazol-treated (0.05µM) senescent fibroblasts represented as a heat map of fold-change in cytokine levels. Grey colour indicates levels outside the detectable range. Samples from one independent experiment used for analysis, n=3 technical replicates, (F) Levels of key cytokines analysed by cytokine array in (E) which showed significant increase in senescence and decrease with myxothiazol treatment. N.D. = Not detected; cytokine levels were below detectable limits. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.1.4 KCN and sodium azide show differing effects on OCR and SASP factor production

Next, to examine the influence of CIV inhibition on SASP production I treated cells with increasing doses of CIV inhibitors KCN (0.1, 0.25, 0.5 and 1mM) (**Figure *3.6***) and sodium azide (**Figure *3.7***) (0.1, 0.5, 1 and 10mM). Acute treatment with KCN in proliferating cells showed minimal effect on OCR (Fig. 3.6A-B), however when measured after 48hrs in culture a 40% reduction in OCR was observed (Fig. 3.6C), suggesting a slower effect than the period of time observed during acute testing (roughly 10 minutes). Interestingly, in senescent cells treated with KCN the three major SASP factors all drastically increased (Fig. 3.6D). Based on findings with other electron transport inhibitors this result was unexpected, as OCR was unaffected suggesting an alternate influence on SASP factor secretion in this case.

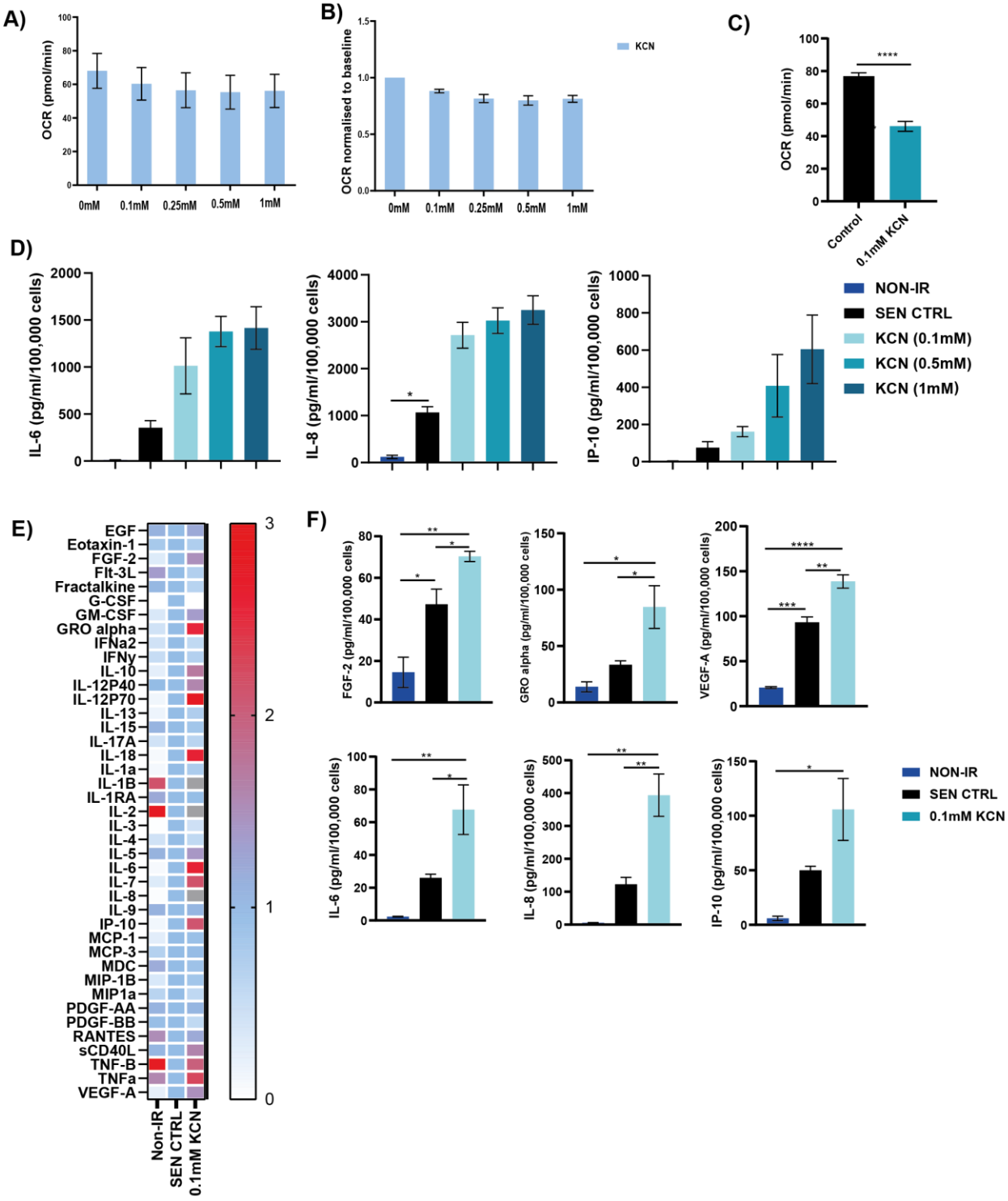


Figure 3.6: KCN does not reduce pro-inflammatory cytokine levels in senescent cells . (A) Effect of increasing doses of KCN on cellular respiration in young MRC5 fibroblasts measured using Seahorse XF24 Analyzer, data taken from one experiment, n=4 technical replicates (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs in control or KCN-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young

non-irradiated, senescent, and KCN-treated (0.1, 0.5 and 1mM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates, (E) Cytokine array analysis of young non-irradiated, senescent, and KCN-treated (0.1mM) senescent fibroblasts represented as a heat map of fold-change in cytokine levels. Grey colour indicates levels outside the detectable range. Samples from one independent experiment used for analysis, n=3 technical replicates, (F) Levels of key cytokines analysed by cytokine array in (E) which showed significant increase in senescence and further increase with KCN treatment. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Even more surprising was the comparison with the effects of sodium azide, which dramatically inhibited OCR both after acute treatment (Fig. 3.7A-B), and after 48hrs in culture (Fig. 3.7C). Correspondingly, IL-6, IL-8 and IP-10 all significantly decreased after 10 days treatment with sodium azide in senescent cells (Fig. 3.7D), demonstrating a dramatically different effect to KCN.

Cell culture media samples from both KCN- and sodium azide-treated senescent cells were analysed by cytokine array in an attempt to reveal a more detailed picture of their opposing effects on the SASP (Fig. 3.6E and 3.7E, respectively). As I observed in my own ELISA analysis, KCN further exacerbated a number of major pro-inflammatory cytokines, including FGF-2, GROalpha and VEGFA, compared to untreated senescent controls (Fig. 3.6F). Both VEGF and FGF-2 are key angiogenic growth factors, with FGF-2 also involved in promotion of cell proliferation and prevention of apoptosis (Sahni and Francis, 2004; Kim *et al.*, 2012). By comparison, sodium azide-treated cells exhibited significantly reduced levels of both VEGFA and GROalpha, along with other key SASP factors MCP-1, GM-CSF, G-CSF and IFNγ (Fig. 3.7F).

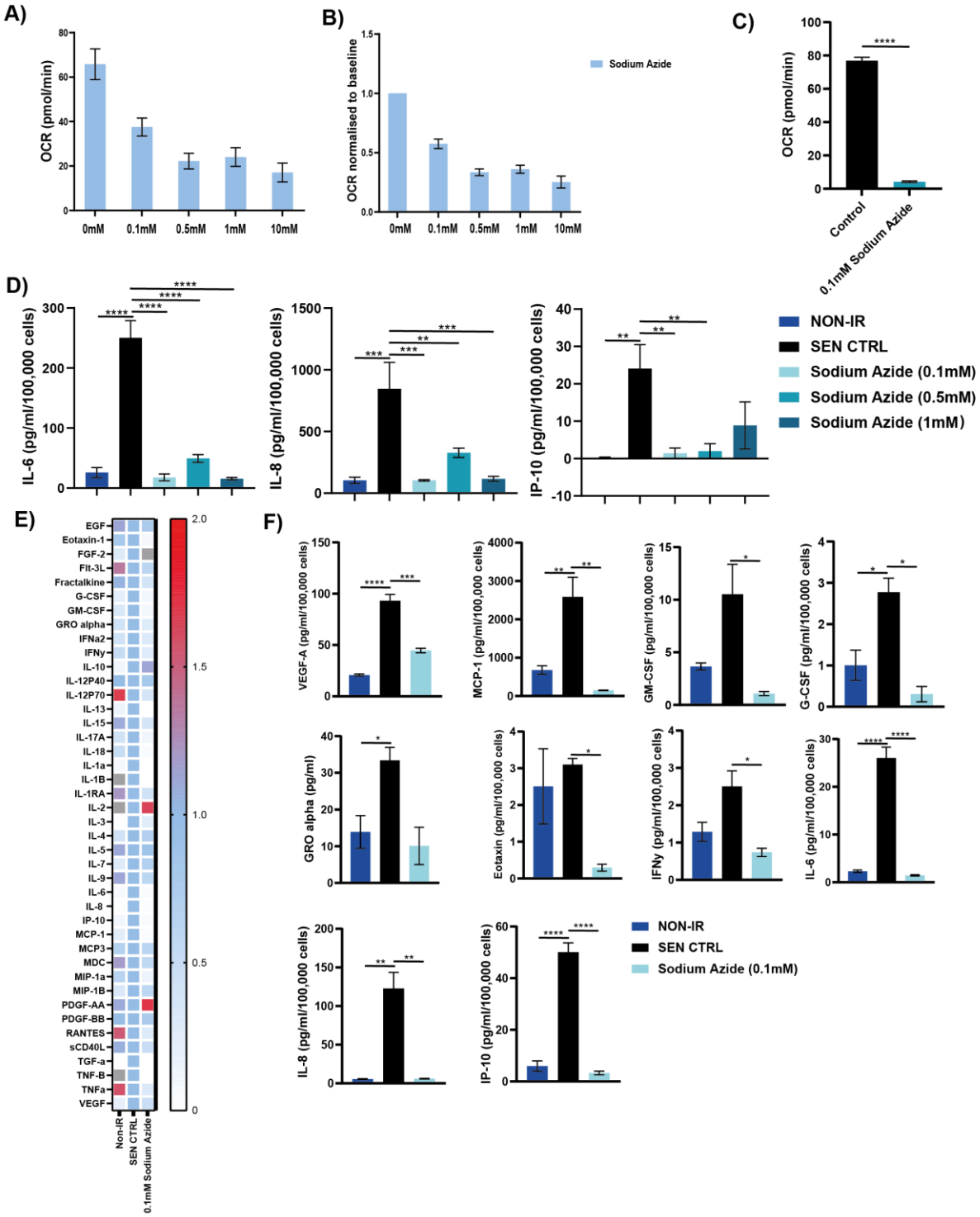


Figure 3.7: Sodium azide reduces pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of sodium azide on cellular respiration in young MRC5 fibroblasts measured by Seahorse XF24 Analyzer, data taken from one experiment, n=4

technical replicates (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs in control or sodium azide-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and sodium azide-treated (0.1mM, 0.5mM, 1mM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates, (E) Cytokine array analysis of young non-irradiated, senescent, and sodium azide-treated (0.1mM) senescent fibroblasts represented as a heat map of fold-change in cytokine levels. Grey colour indicates levels outside the detectable range. Samples from one independent experiment used for analysis, n=3 technical replicates, (F) Levels of key cytokines analysed by cytokine array in (E) which showed significant increase in senescence and decrease with sodium azide treatment. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

A possible explanation for this disparity is that KCN does not remain in the complex long-term, and its inhibition can be reversed by the presence of pyruvate (Nuskova *et al.*, 2010)which is more likely to occur over long-term cell culture. Additionally, the inhibitory effect of KCN on cellular respiration was much weaker than that observed by sodium azide, which would explain the contrasting effects if OCR is an important factor in SASP production. Studies have also shown that CIV must be inhibited by a minimum of 60% before a change in OCR can be observed (Leavesley *et al.*, 2008), so it is likely that the specific inhibition of CIV was not maintained at this level for long enough to produce a measurable change in OCR for long in culture. KCN can also act as an inhibitor of SOD1 (Malik *et al.*, 2020) and has been shown to increase ROS levels (Correia *et al.*, 2012) which could suggest a ROS-dependent increase in SASP if there is indeed a causal relationship between the two, particularly as OCR was unaffected while ROS (Section 3.7) and SASP both increased. Alternatively, or in addition to this explanation, studies suggest that sodium azide may also inhibit CV (Hong and Pedersen, 2008), and indeed I found that its effects on OCR and SASP closely matched that of oligomycin treatment, a known CV inhibitor. Further investigation into the mechanism of action of both drugs in the

senescent models tested is needed to determine if either of these proposed explanations are correct.

3.1.5 Oligomycin reduces OCR and inhibits SASP factor production

In order to assess the effect of CV (ATP synthase) inhibition on SASP production I treated cells with increasing doses of oligomycin (0.25, 0.5, 1.25, and 2.5µM) (**Figure *3.8***). Acute treatment in proliferating cells showed an immediate dose-dependent reduction in OCR which plateaued at 60% inhibition at 0.5µM and above (Fig. 3.8A-B). After 48hrs exposure to oligomycin in culture this inhibition increased to >80% (Fig. 3.8C). In senescent cells treated for 10 days post-irradiation, all three measured SASP factors were significantly reduced at the lowest tested dose (Fig. 3.8D). As the lowest effective dose, 0.25µM oligomycin was analysed by cytokine array (Fig. 3.8E), which showed a wider range of reduced SASP factor secretion following chronic treatment with oligomycin, including G-CSF, VEGFA, and IL-15 (Fig. 3.8F).

Together this data shows that chronic treatment with the CV inhibitor oligomycin at a dose that induces 50% inhibition of OCR is sufficient to significantly reduce secretion of pro-inflammatory SASP factors *in vitro*.

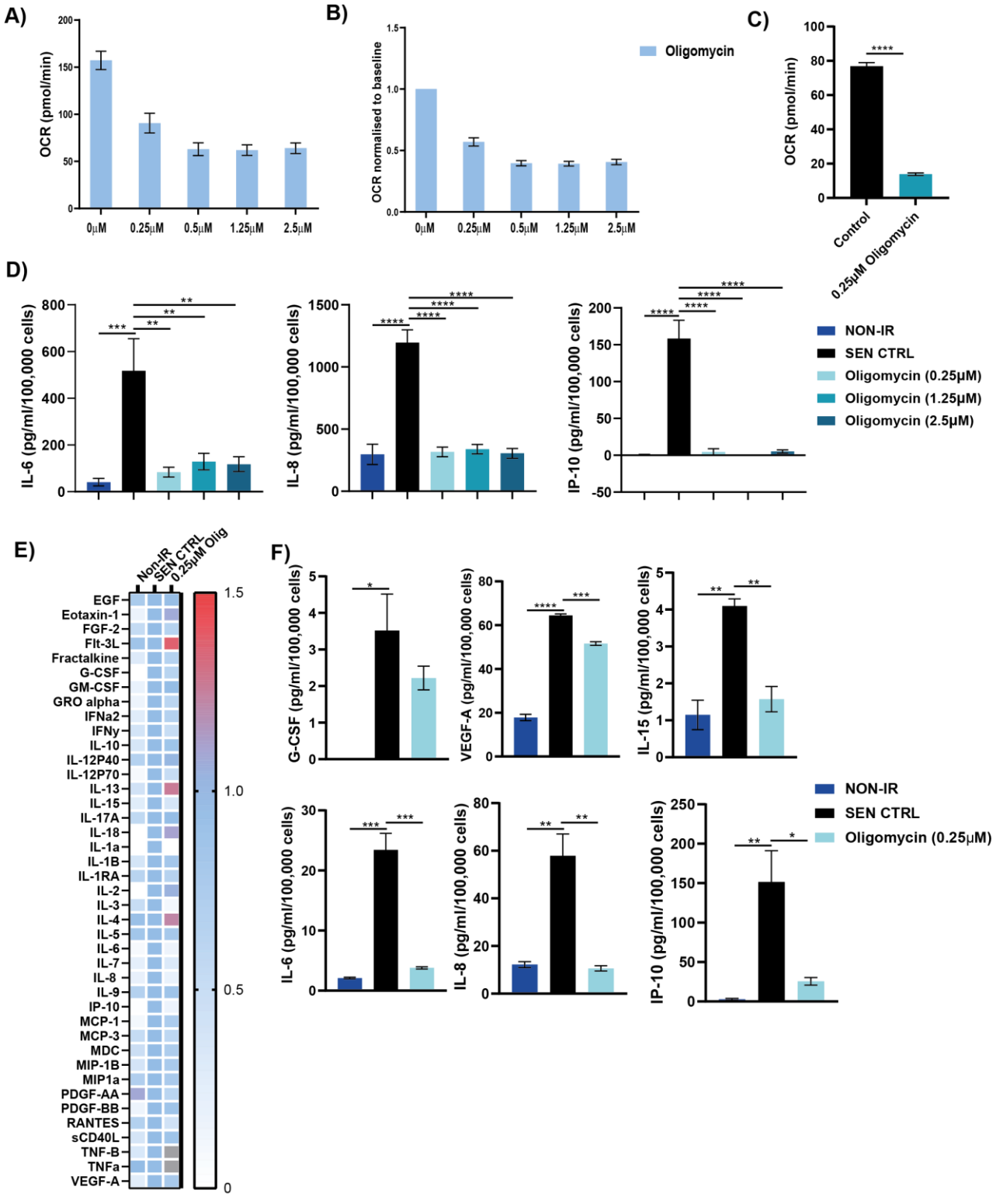


Figure 3.8: Oligomycin reduces pro-inflammatory cytokine levels in senescent cells. (A) Effect of increasing doses of oligomycin on cellular respiration in young MRC5 fibroblasts measured by Seahorse XF24 Analyzer, data taken from one experiment, n=5 technical replicates, (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs

in control or oligomycin-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and oligomycin-treated (0.25, 1.25 and 2.5µM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted as described in Fig.3.1, n=6 technical replicates, (E) Cytokine array analysis of young non-irradiated, senescent, and oligomycin-treated (0.25µM) senescent fibroblasts represented as a heat map of fold-change in cytokine levels. Grey colour indicates levels outside the detectable range. Samples from one independent experiment used for analysis, n=3 technical replicates, (F) Levels of key cytokines analysed by cytokine array in (E) which showed significant increase in senescence and decrease with oligomycin treatment. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. **p<0.05, *p<0.01, ***p<0.001, ****p<0.0001

3.1.6 FCCP elevates OCR and does not inhibit SASP factor production

Finally, in order to examine the influence of mitochondrial respiratory uncoupling on development of a SASP, I administered increasing doses of the mild uncoupler FCCP to proliferating and senescent cells (**Figure *3.9***). Acute treatment in proliferating cells produced a dose-dependent (0.25, 0.5, 1.25 and 2.5µM) increase in OCR (Fig. 3.9A-B), which was the expected response to respiratory uncoupling at the doses tested. This confirmed that FCCP functioned as expected. After 48hr treatment in culture OCR was only minimally decreased by around 15% compared to untreated controls (Fig. 3.9C), demonstrating minor influence on OCR as well as tolerability by the cells to prolonged exposure to the uncoupler.

A lower dose range was used in senescent cell experiments as 1.25 and 2.5µM FCCP showed large increases in OCR. In order to use doses capable of partially uncoupling respiration and potentially decreasing mitochondrial ROS production as noted in the literature (Miwa and Brand, 2003; Abramov;Scorziello and Duchen, 2007) without completely disrupting oxidative phosphorylation and ATP production, I tested chronic FCCP treatment in the range of 0.05-0.5µM in senescent cells. After treatment for 10 days at these doses, secretion of IL-6, IL-8 and IP-10 was only slightly reduced, with only IL-8 significantly reduced by 0.25µM FCCP (Fig. 3.9D). This data shows that treatment with low

dose FCCP does not have a strong impact on SASP development, and any change in cytokine secretion is unlikely to be mediated by changes in OCR here. While 0.25µM FCCP elicited a decrease in IL-8, and 0.5µM had no effect, this supports the idea that doses of ≥0.5µM would have been unlikely to change the SASP further. Of note, previous studies have shown that chronic FCCP treatment can inhibit cell proliferation and induce senescence in young healthy cells (Stockl *et al.*, 2007), so it may not be surprising that chronic treatment with FCCP in this project was unable to rescue SASP factor secretion. Stockl et al also showed that FCCP-induced cell cycle arrest could be rescued by antioxidant treatment, supporting a role for oxidative stress.

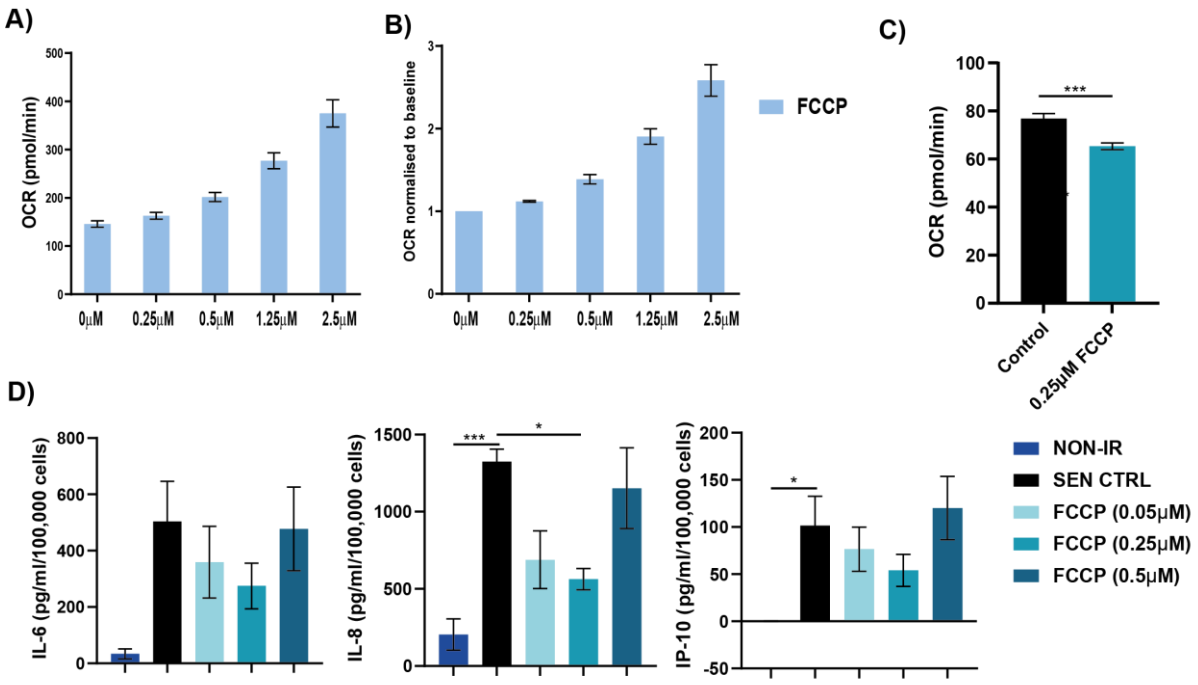


Figure 3.9: FCCP does not reduce pro-inflammatory cytokine levels in senescent cells. A) Effect of increasing doses of FCCPon cellular respiration in young MRC5 fibroblasts measured by Seahorse XF24 Analyzer, data taken from one experiment, n=4 technical replicates, (B) OCR data taken from (A) and represented as a proportion where untreated baseline control = 1, (C) OCR measurements in young proliferating cells cultured for 48hrs in control or FCCP-treated medium. (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and FCCP-treated (0.05µM, 0.25µM, 0.5µM) senescent MRC5 fibroblasts measured by ELISA. Data taken from two independent experiments conducted

as described in Fig.3.1, n=6 technical replicates. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, ***p<0.001

Overall, my data provides evidence that chronic treatment with inhibitors of respiratory complexes I, III, IV and V can prevent the development of a SASP *in vitro*. This finding suggests that no single respiratory complex is more important in regulating senescence, and that the role of mitochondria in senescence is more likely related to a function of mitochondrial respiration that is impacted by inhibition at multiple points in the ETC.

3.2 Inhibition of OCR correlates with SASP suppression

I next wanted to examine mitochondrial respiration in senescent cells treated for 10 days post-irradiation to determine a) whether there was a difference in acute and long-term effects, or between proliferating and senescent cells, on OCR by the tested inhibitors, and b) if the effects of chronic ETC inhibition on OCR correlated with those seen in SASP factor secretion. To do this I treated MRC5 fibroblasts with the lowest effective dose of each drug identified in Figures 3.2-3.9 for 10 days immediately after irradiation (**Figure *3.10***). OCR was analysed by Seahorse XF24 analysis and normalised to plated cell count. Plated cell count was used because the small size of XF24 plate wells - and therefore small sample sizes – made collecting and quantifying protein concentration at the end of experiments particularly difficult. Additionally, in preliminary tests in larger cell numbers I established that cell numbers in senescent conditions did not change dramatically following irradiation. Furthermore, at this point I also included the biguanide metformin as an alternate CI inhibitor to rotenone, as it is a well-established drug with a known safety profile in humans. Metformin is also thought to inhibit CI activity at the I_F site of CI as opposed to the I_Q site as with rotenone, offering a comparison of site-specific inhibition of CI. All tested drug doses were low enough to minimise cell toxicity, and all proliferating cell samples were plated the evening before analysis and measured the following morning, minimising the chance for further cell division.

Overall, I found that cells treated with metformin, myxothiazol and sodium azide retained a substantial inhibition of baseline OCR after 10 days, functioning at less than 20% that of untreated controls, lower even than young proliferating cells (Fig. 3.10A-B). Figure 3.10B demonstrates a more reproducible relationship change between drug treatments and untreated controls than shown by raw values. This finding corresponds with my previous data showing that myxothiazol and sodium azide were two of the most effective drugs at reducing SASP factor secretion and elicited the greatest acute effects on OCR, suggesting a strong influence of OCR in SASP development. Metformin was also found to have significant inhibitory effects on OCR acutely, and SASP factor production after chronic treatment for 10 days, and is discussed in greater detail in Chapter 4. Treatment with oligomycin also produced a significantly lower level of baseline OCR. Though this is not unexpected as inhibition of ATP synthase activity is predicted to have a considerable

impact on ETC function, this data shows that oligomycin retains its influence after long-term treatment at around 60% inhibition.

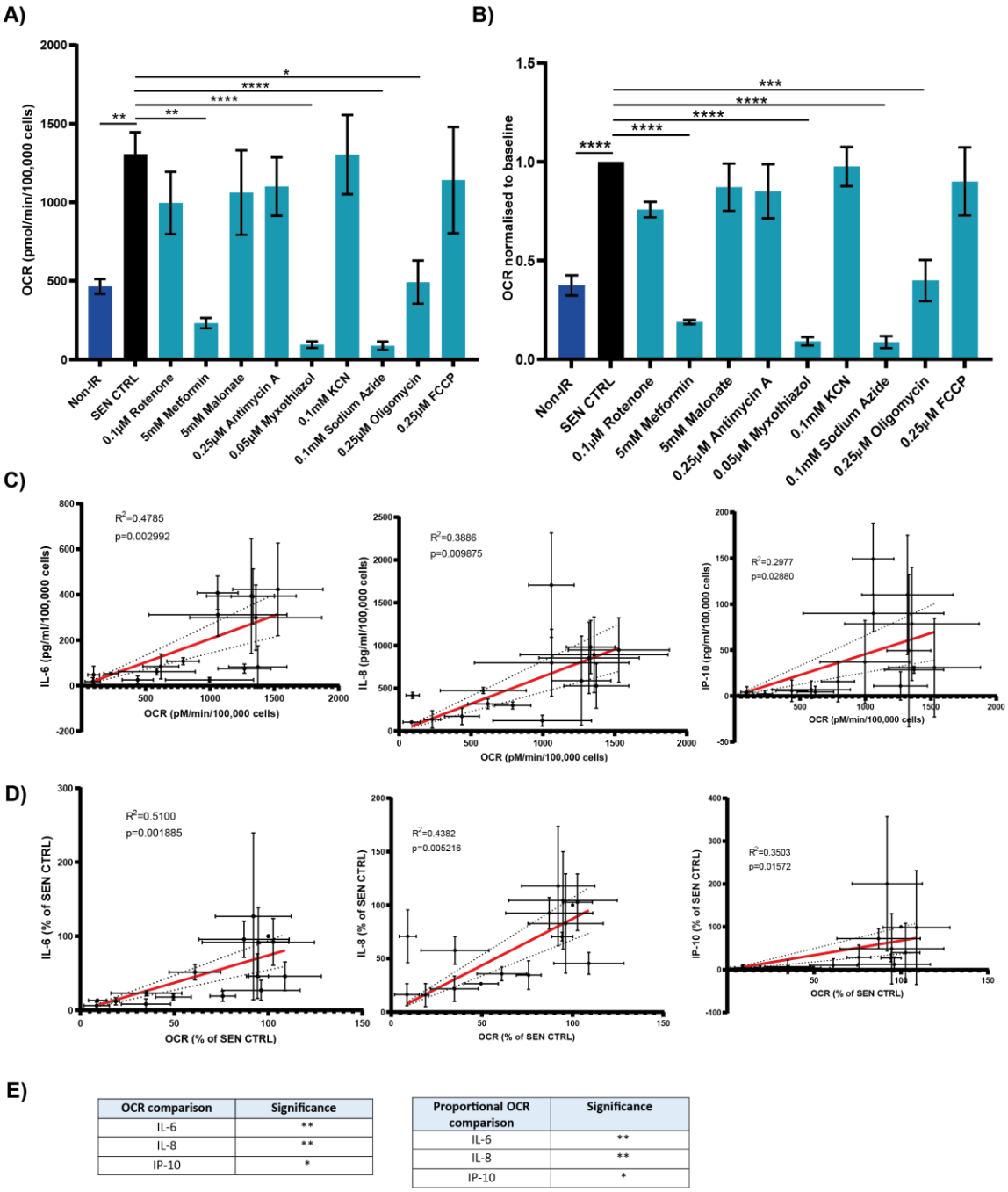
By comparison, treatment with rotenone and antimycin A did not retain a significant inhibition of OCR after 10 days treatment, demonstrating reduced effectiveness over time for these two inhibitors. Though a slight reduction in OCR is seen with rotenone, this was not statistically significant. This reduced effectiveness suggests an adaptive response by cells to long term treatment with certain inhibitors. Such adaptation could include degradation of the affected complexes or mitochondria, or turnover of the drug in culture over time. Indeed, some studies have shown increased mitophagy (Giordano *et al.*, 2014) and compensatory modifications to metabolic pathways (Worth *et al.*, 2014) in rotenone-treated primary neurons, indicating attempts by cells to adapt to rotenone treatment over time.

Malonate, KCN and FCCP again showed minimal effect on OCR following chronic treatment. It is clear from this data that malonate's lack of effect is not due to a slow accumulation in the cell or mitochondria, further confirming the likelihood that it cannot permeate the cell membranes. Minimal change following chronic treatment with FCCP was also expected based on the initial OCR measurements in proliferating cells. FCCP treatment again shows greater variability in OCR than most other inhibitors likely as a result of biological variation in the required dose for respiratory uncoupling. This data further confirms that FCCP does not have a significant effect on OCR after long-term treatment. It is also clear that long-term treatment with KCN does not retain the mild inhibitory effect seen after 48hrs in culture, at 10 days, supporting the idea that its effects are reversible.

These findings for each of the inhibitors were clarified further by examining the proportional change in OCR (Fig. 3.10B), by normalising OCR data to each corresponding untreated senescent control sample, which showed a reproducible relationship change in OCR when treated with each of these inhibitors. This analysis showed a much reduced variability for most of the inhibitors, proving that the proportion of OCR inhibited by each drug was consistent.

Overall, those inhibitors which demonstrated the greatest suppression of OCR were also most successful at inhibiting SASP factor production after 10 days. This supports a relationship between OCR and SASP production, and as such I conducted correlation analysis between baseline OCR measurements and each SASP factor (Fig. 3.10C). This analysis showed a significant positive correlation between OCR and all three measured SASP factors, IL-6, IL-8 and IP-10 (Fig. 3.10E). This analysis also included data points from ROS modifying drugs which are described later, in order to improve the robustness of the analysis. Overall, there appears to be a positive linear relationship between OCR and pro-inflammatory cytokine secretion in senescent cells, supporting a potential regulatory role for mitochondrial respiration in development of a SASP.

To further confirm this relationship I also conducted correlation analysis using proportional data for all 4 measurements, plotting values as a percentage of their corresponding untreated senescent control sample (Fig. 3.10D). This allowed me to determine whether there was a direct relationship between percentage inhibition of each measured product. This analysis again showed a positive linear correlation between inhibition of OCR and each SASP factor. The statistical significance of each correlation was mildly increased (Fig. 3.10E), likely associated with the reduced variability observed in proportional analysis compared to raw measurements.



E)

OCR comparison	Significance
IL-6	**
IL-8	**
IP-10	*

Proportional OCR comparison	Significance
IL-6	**
IL-8	**
IP-10	*

Figure 3.10: Correlation between OCR and SASP. (A) Oxygen consumption rates for young, untreated senescent, and senescent fibroblasts treated with inhibitors of the electron transport chain for 10 days post-irradiation measured by Seahorse XF24 Analyzer. Data represents averages taken from 3-5 independent experiments for each condition; n=3-5 (B) OCR taken from (A) and represented as a proportion where untreated senescent control = 1. n=3-5 (C) Correlation analysis of raw OCR data from (A) and levels of SASP factors IL-6, IL-8 and IP-10 measured by ELISA in the same conditions (Fig. 3.2-9). (D) Correlation analysis of OCR and SASP factor inhibition represented by correcting data

to senescent control = 100%. Note: for improved robustness correlation analysis also includes data points for ROS modifier drugs discussed later. For both measures an overall average value has been taken from all independent experiments completed; for OCR n=3-5, and for cytokine data n=6 technical replicates as before. (E) Significance of correlations between raw and proportional OCR and SASP factor measurements. Data is mean±S.E.M.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Taken together these findings provide evidence of a positive correlation between mitochondrial respiration and pro-inflammatory cytokine secretion in senescent cells. Treatment with inhibitors which substantially reduced OCR were most effective at preventing development of a SASP, suggesting mitochondrial respiration is key to SASP production and may be an important therapeutic target. Of note, despite the extremely low OCR of senescent cells treated with metformin, myxothiazol or sodium azide, there was minimal cell death observed and cells appeared to survive in culture long-term. Though there were perhaps some morphological changes in these cells, such as smoother cell bodies and smaller size identified by eye under microscopy, it seemed clear that such reduced OCR did not affect cell survival and so raised the question of the source of energy production in these cells.

3.3 Inhibition of electron transport at complexes I, III, IV and V force cells to rely on glycolysis for ATP production

Based on OCR data and the ability of senescent cells exhibiting almost no mitochondrial respiration to survive in culture I hypothesized that these cells were relying on glycolysis to provide the necessary ATP production. This would also allow me to investigate whether inhibitors that suppressed SASP did so as a result of metabolic reprogramming. To test this hypothesis I analysed ATP production rates obtained from Seahorse XF Analyzer analysis to determine how much ATP was derived from oxidative phosphorylation and glycolysis (**Figure *3.11***).

This analysis revealed that untreated senescent cells were producing significantly more overall ATP per cell than proliferating controls , but that this increase was reduced by treatment with metformin, myxothiazol, and sodium azide, corresponding with trends seen in OCR (Fig. 3.11A-B). This ATP production was not measured for mitochondrial mass or protein content normalisation, however, which may alter these trends due to increased cell size and mitochondrial mass of senescent cells. Of note, although senescent cells are generally considered to be more glycolytic than proliferating cells, this was not observed here. However, other studies have also demonstrated an increase in mitochondrial respiration along with glycolysis upregulation in senescence (Quijano *et al.*, 2012; Dorr *et al.*, 2013; Kaplon *et al.*, 2013). Importantly, all three of these inhibitors induced a complete shutdown of oxidative phosphorylation, meaning that cells treated with these inhibitors were relying entirely on glycolysis. This was also true of cells treated with oligomycin, confirming the accuracy of these measurements as oligomycin is known to decrease mitochondrial ATP production (Hong and Pedersen, 2008). However, in contrast to metformin, myxothiazol and sodium azide, overall ATP production was not substantially reduced following oligomycin treatment compared to untreated controls, and instead these cells compensated for reduced oxidative phosphorylation by upregulating glycolysis. In this case, while overall ATP production was not reduced, OXPHOS-derived ATP production was. This compensation was not present in metformin-, myxothiazol-, or sodium azide-treated cells.

Treatment with rotenone produced a mild reduction in overall ATP production which seemed to come solely from reduced OXPHOS-derived ATP, though it did not induce a

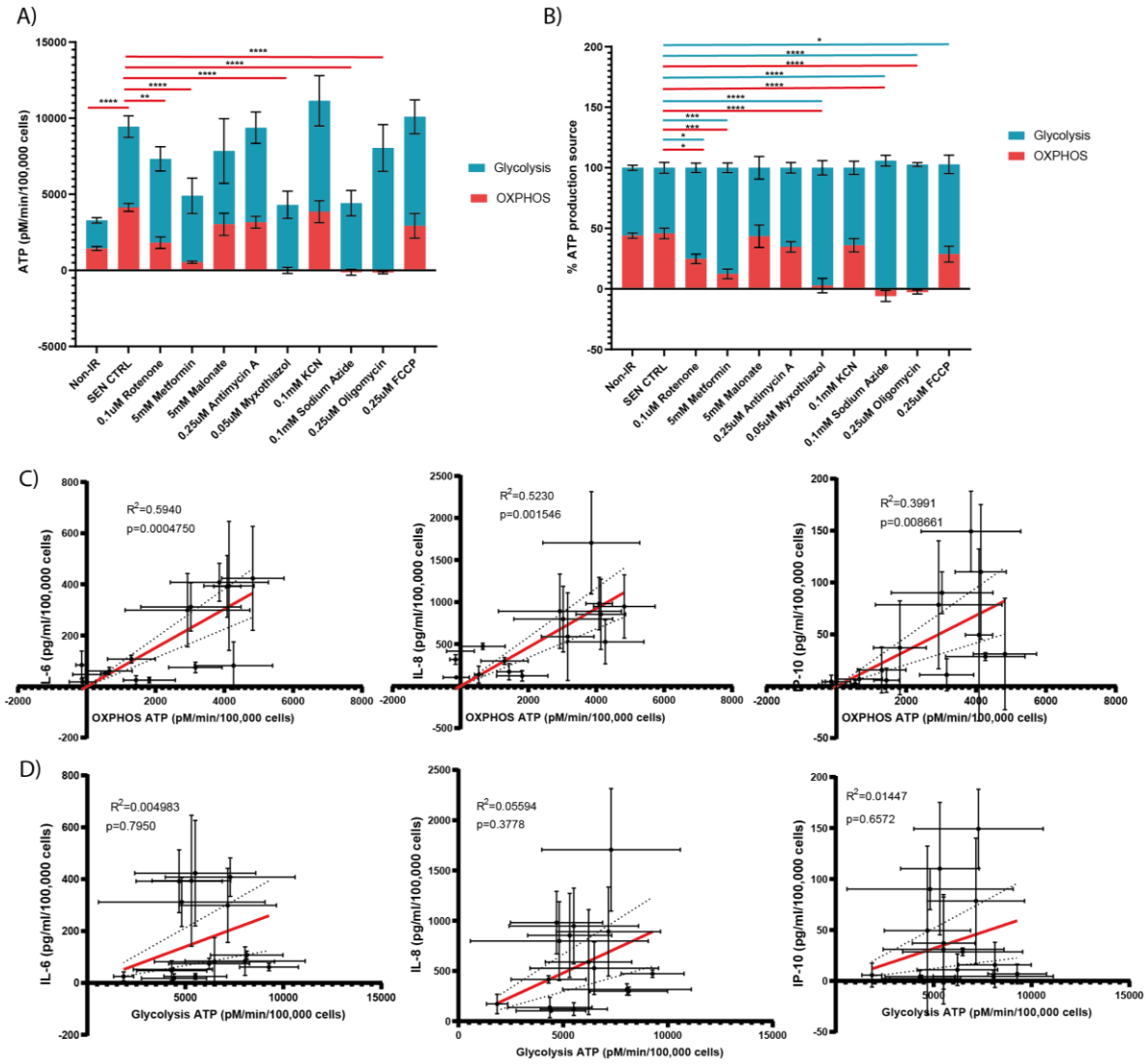
complete shutdown as observed with other inhibitors. This difference between rotenone and metformin – both CI inhibitors – in both OCR and ATP measurements could suggest an aspect of metformin's mechanism of action is more effective at suppressing CI activity over a longer period of time. Though metformin's mechanism of action is the subject of much debate, discussed later.

As with OCR, treatment with antimycin A produced little to no change in ATP production, either overall or specifically from OXPHOS or glycolysis sources. This supports the idea that antimycin A does not retain its inhibitory effect on mitochondrial respiration long term, and that its ability to inhibit SASP production may not rely on long-term suppression of OCR or ATP. Treatment with KCN also did not cause a significant change in ATP production, again demonstrating that its effects on mitochondrial respiration are not maintained in chronic treatment. This was also true of FCCP, which showed minimal difference in either total, OXPHOS- or glycolysis-derived ATP after 10 days, consistent with OCR data.

Overall, these findings show that in those inhibitors which triggered severe reduction in OCR, cells saw a complete shutdown of oxidative phosphorylation and relied solely on glycolysis for ATP production. Treatments which did not substantially inhibit OCR after 10 days showed little to no difference in ATP production. Most conditions which experienced OXPHOS shutdown exhibited significantly reduced ATP production overall, with the exception of those treated with oligomycin which compensated with increased glycolysis. This is also consistent with the observation that oligomycin induced a milder inhibition of OCR than metformin, myxothiazol or sodium azide.

In order to assess the relationship between OXPHOS- and glycolysis-derived ATP production and pro-inflammatory cytokine secretion in senescent cells, I conducted correlation analysis between each measure (Fig. 3.11C-D). This analysis revealed a significant positive correlation between OXPHOS-derived ATP and IL-6, IL-8, and IP-10. By comparison, there appeared to be no significant correlation between ATP derived from glycolysis and any of the measured SASP factors. This is an important finding as it provides further evidence that it is the activity of the mitochondrial electron transport chain that is key to SASP production. It is possible that this positive correlation between SASP and OXPHOS ATP is simply a result of the corresponding OCR effects, as reduction in

mitochondrial respiration would logically result in a reduction in ATP from oxidative phosphorylation. To examine this relationship further, experiments were conducted which would force reliance on either oxidative phosphorylation or glycolysis for ATP production without impacting OCR.



E)

OXPHOS ATP comparison	Significance
IL-6	***
IL-8	**
IP-10	**

F)

Glycolysis ATP comparison	Significance
IL-6	n.s.
IL-8	n.s.
IP-10	n.s.

Figure 3.11: Correlation between ATP production and SASP . (A) ATP production rates for young, untreated senescent, and senescent fibroblasts treated with inhibitors of the electron transport chain for 10 days post-irradiation measured by Seahorse XF24 Analyzer. Data represents averages taken from 3-5 independent experiments for each condition; n=3-5 (B) ATP measurements taken from (A) and represented as a proportion where total ATP = 100%. n=3-5 (C) Correlation analysis of raw OXPHOS ATP data from (A) and levels of SASP factors IL-6, IL-8 and IP-10 measured by ELISA in the same conditions (Fig.3.2-9). (D) Correlation analysis of raw glycolysis ATP data from (A) and levels of SASP

factors. Note: for improved data robustness correlation analysis also includes data points for ROS modifier drugs discussed later. For both measures an overall average value has been taken from all independent experiments completed; for OCR n=3-5, and for cytokine data n=2. (E) Significance of correlations between OXPHOS ATP production and SASP factor measurements. (F) Significance of correlations between glycolysis ATP production and SASP factor measurements. Data is mean±S.E.M. * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

3.4 Forced reliance on OXPHOS ATP production does not significantly affect pro-inflammatory cytokine secretion

By culturing proliferating and senescent cells in media supplemented with galactose instead of glucose for 10 days post-irradiation, I aimed to force cells to rely solely on oxidative phosphorylation for ATP production (**Figure *3.12***) (Aguer *et al.*, 2011). I hypothesized that this increased reliance on OXPHOS would result in elevated SASP factor secretion, based on my findings of reduced SASP factors in senescent cells with inhibited mitochondrial respiration.

However, no significant difference in IL-6 or IL-8 was observed between glucose- and galactose-cultured conditions, though a significant increase in IP-10 levels was found between senescent cells cultured in galactose compared to glucose (Fig. 3.12A). An increase in IL-8 levels was observed between proliferating controls cultured in galactose compared to glucose, but this was not statistically significant. I also examined OCR (Fig. 3.12C) and hydrogen peroxide production rates (Fig. 3.12B) under these same conditions and again found no significant difference between glucose- and galactose-cultured samples. ATP analysis confirmed that both proliferating and senescent cells cultured in galactose were relying heavily on OXPHOS for ATP production (>80%) (Fig. 3.12D-E). Therefore it seems that upregulating OXPHOS activity specifically, without affecting OCR, does not substantially affect pro-inflammatory cytokine secretion in senescent cells. As such, this would suggest that it is manipulation of OCR, and not oxidative phosphorylation-derived ATP production, that is key to preventing development of a SASP.

Based on my previous correlation analysis showing no significant relationship between glycolysis-derived ATP and SASP suppression, it would seem that this source of ATP production does not have a role here. In order to confirm this I conducted tests to promote reliance on glycolysis without impacting OCR.

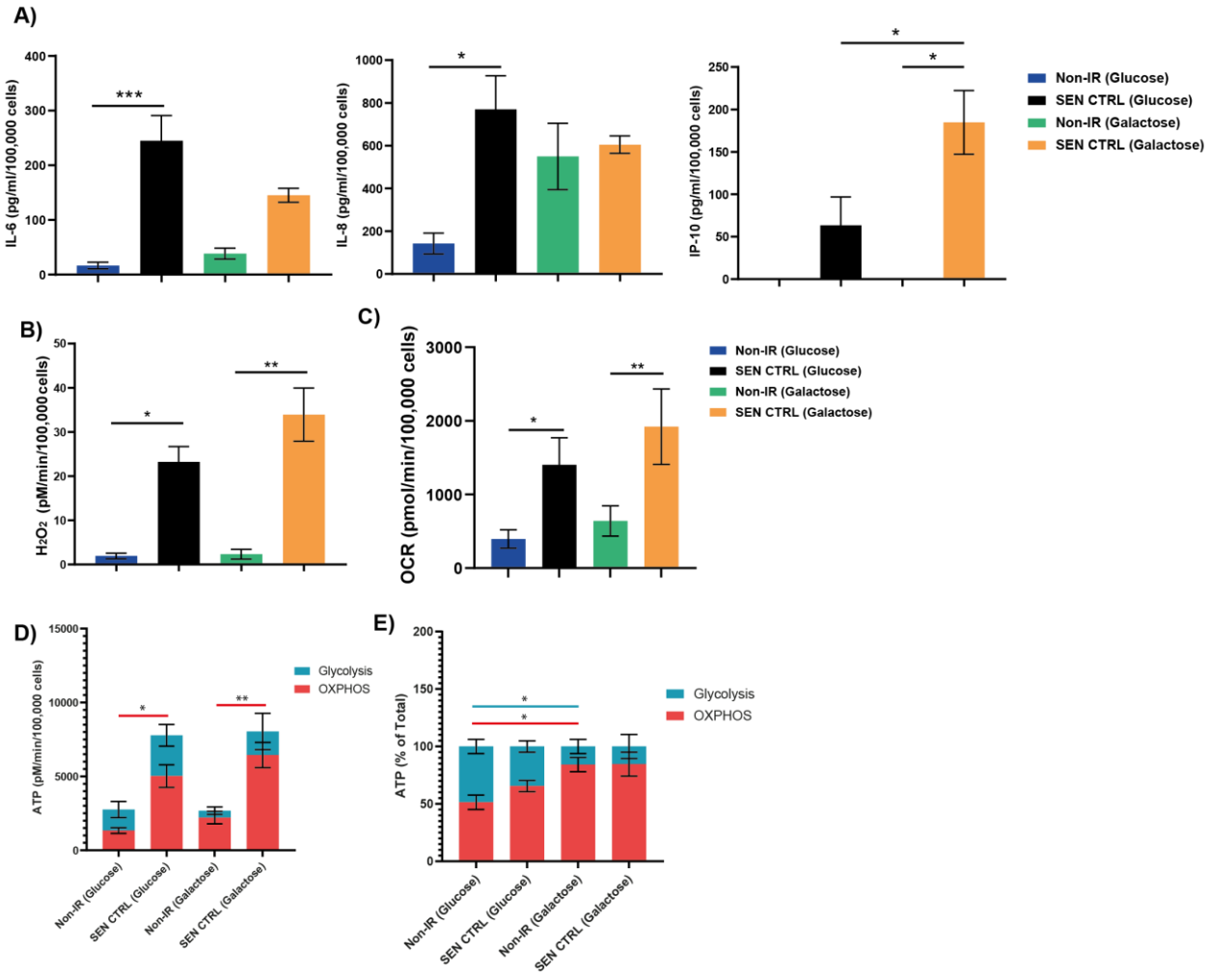


Figure 3.12: SASP factor secretion when cultured with galactose (A) Levels of IL-6, IL-8 and IP-10 secreted by young and senescent fibroblasts cultured in glucose (controls) and in galactose. n=3, (B) Hydrogen peroxide production rates measured by Amplex Red assay in young and senescent fibroblasts cultured in either glucose or galactose, n=3. (C) Oxygen consumption rates and (D) ATP production rates, measured by Seahorse XF24 Analyzer in young and senescent fibroblasts cultured in either glucose or galactose, n=3. (E) ATP measurements taken from (D) and represented as a proportion where total ATP = 100%, n=3. Data analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001

3.5 Treatment with monensin increases pro-inflammatory cytokine secretion

In order to evaluate the influence of glycolysis-derived ATP production in SASP production, I treated cells with the ionophore, monensin (**Figure *3.13***). Though there is some debate in the literature (Ibrahim *et al.*, 2020), the prevailing mechanism of action is generally thought to be promotion of ATP production from glycolysis (Mariani *et al.*, 1989; Erecinska *et al.*, 1991; Mookerjee;Nicholls and Brand, 2016) without interfering with OCR. Based on this I hypothesised that promotion of glycolysis reliance – and therefore reduction of OXPHOS influence - would reduce SASP factor secretion as suggested by the previous ETC inhibitor tests. However, during initial tests in young proliferating cells I instead found a dramatic increase in both IL-6 and IL-8 levels in cells treated with 0.5µM monensin for 3 days (Fig. 3.13A). Though this finding has caveats – monensin's cell toxicity was pronounced, and the dose tested could only be tolerated by cells for 3 days before widespread cell death was observed. Further tests are warranted to determine a more well-tolerated dose for long-term treatment.

In addition, acute respiratory analysis conducted by Dr Satomi Miwa using higher doses of monensin demonstrated no immediate effect following addition of 5µM monensin, while 10µM significantly increased both OCR (Fig. 3.13B) and ATP production (Fig. 3.13C-D). In contrast to much of the literature these high doses of monensin did not result in sole reliance on glycolysis for ATP production. 5µM monensin did not alter the balance of OXPHOS- and glycolysis-derived ATP production, but 10µM did cause a significant increase in the proportion of OXPHOS ATP and decrease in glycolysis ATP production, again an opposing effect to those noted in other studies. As such, it is possible that the observed effects on pro-inflammatory cytokine secretion by chronic monensin treatment is a result of factors other than OCR or ATP manipulation, though differences between acute and long-term effects of the drug are also possible.

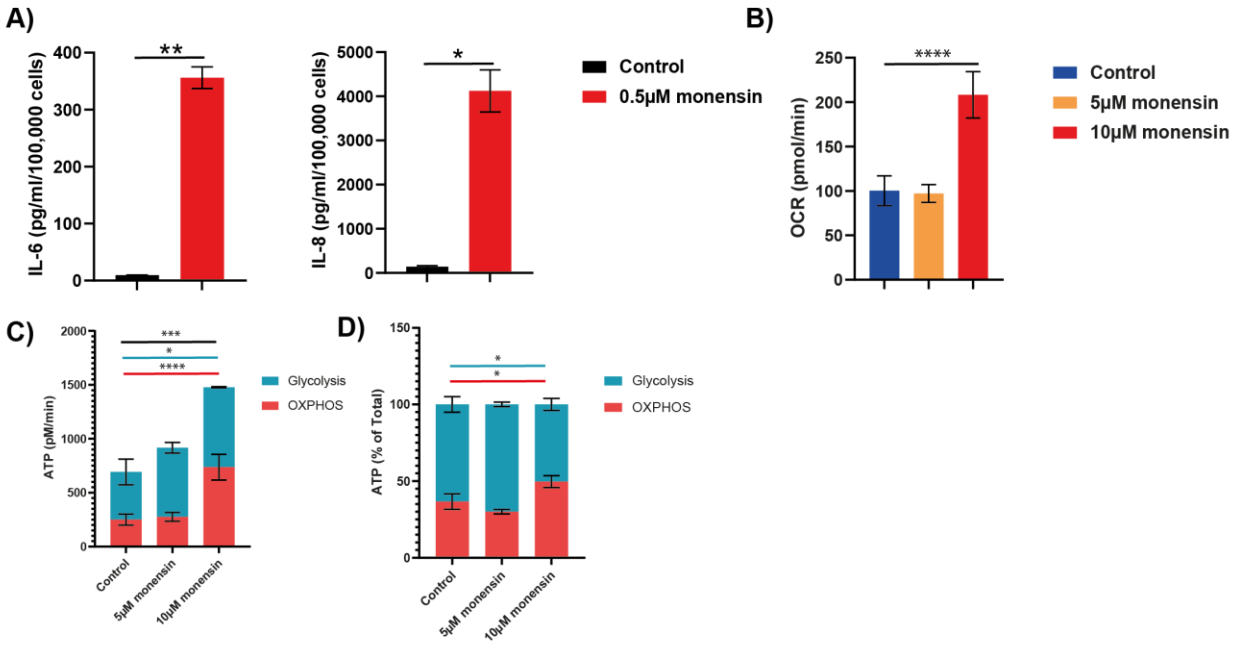


Figure 3.13: Young fibroblasts treated with monensin exhibit elevated SASP factor secretion, and acute treatment at high doses increases OCR and ATP production. (A) Levels of IL-6 and IL-8 in young untreated fibroblasts and young fibroblasts treated with 0.5µM monensin for 3 days, n=4 technical replicates, data analysed using 2-tailed unpaired t-test. Data is mean±S.E.M. (B) Oxygen consumption rates and (C) ATP production rates, measured by Seahorse XF24 Analyzer in young fibroblasts treated with 5 or 10µM monensin injected during measurement, n=2-7 technical replicates, Data is Mean±S.D. provided by Dr Satomi Miwa. (D) ATP measurements taken from (C) and represented as a proportion where total ATP = 100%, n=2-7 technical replicates. Data analysed using one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Based on my own observations and findings within the literature (Ketola *et al.*, 2010; Charvat and Arrizabalaga, 2016), I propose that the elevated cytokine secretion was in fact a result of highly elevated oxidative stress and mitochondrial damage caused by monensin treatment. A study by Charvat & Arrizabalaga (2016) found that treatment of the parasite *Toxoplama gondii* with monensin disrupted mitochondrial function and membrane potential, which was reversed by overexpression of antioxidant proteins, implicating a role for ROS production. They proposed that this ROS would likely be a result

of OXPHOS uncoupling, as monensin is capable of shuttling protons across mitochondrial membranes. Although this study used concentrations far below those required to affect mammalian cells, other studies have also found that monensin can cause mitochondrial damage in murine fibroblasts, along with proliferation inhibition, ionic imbalances and impaired ATP production (Souza *et al.*, 2005).

Though findings with monensin are not without flaws, data from galactose culture seems to confirm that OCR inhibition is key to SASP reduction, and not specifically OXPHOS activity or ATP production.

3.6 Does manipulation of ROS production specifically affect pro-inflammatory cytokine secretion?

I next wanted to examine the role of ROS production specifically in the development of senescence as it is possible that the observed effects of OCR inhibition could result from changes in ROS production. To do this I tested the effects of multiple ROS modifying drugs with the intention of manipulating ROS levels without impacting respiration. Here I measured the levels of total H_2O_2 secreted by cells in suspension using the established Amplex Red/resorufin fluorescent assay (Mohanty *et al.*, 1997).

3.6.1 Antioxidants

I first tested the effects of established and novel antioxidants on ROS production rates over 10 days of treatment post-IR (**Figure *3.14***). MitoQ is a well-known mitochondrially-targeted antioxidant which functions by forming a ubiquinol derivative in the mitochondria and preventing lipid peroxidation. A new class of ROS-targeting drugs have recently been developed, specific to individual ROS-producing sites of the ETC. S1QEL1.1 and S1QEL2.2 were identified in previous studies as effective suppressors of ROS from site I_{Qi} of complex I, while S3QEL2 targets site III_{Qi} of complex III (Brand *et al.*, 2016). While all four drugs were able to mildly reduce ROS production when given acutely in proliferating cells (**Figure *3.16***), after 10 days of treatment in senescent cells only MitoQ retained a mild inhibition of ROS, though even this was not statistically significant (Fig. 3.14A-B). This suggests that the effectiveness of these drugs is less potent over time.

To compare with previous data, I also tested the effects of MitoQ (Fig. 3.14C), S1QEL1.1 (Fig. 3.14D), S1QEL2.2 (Fig. 3.14E) and S3QEL2 (Fig. 3.14F) on SASP factor secretion along with OCR (Fig. 3.14G) and ATP levels (Fig. 3.14H-I). S1QEL1.1 did not show any influence on SASP factor secretion at the dose ranges tested, while S1QEL2.2 was able to reduce IL-6 only at 500nM concentrations. This is not surprising given that these drugs had no effect on either ROS production, OCR or ATP production in this model. S3QEL2 showed a mild reduction in all three measured cytokines at the highest dose tested of 10µM, but none were statistically significant. A higher dose may have been more effective, however I experienced issues with drug stability above certain doses in culture which made this problematic. S3QEL2 also showed no effect on ROS, OCR or ATP. Overall it appears that

the S1QEL and S3QEL drugs tested, while effective at reducing ROS production rates acutely in proliferating cells, do not have effects on any of the measured markers after 10 days in senescent cells, with the exception of S1QEL2.2 on IL-6.

Treatment with MitoQ showed a mild dose-dependent trend towards SASP reduction, though only IL-8 secretion after treatment with 200nM MitoQ showed a significant decrease. Based on MitoQ's mild impact on ROS production rates it seemed unlikely that this was a solely ROS-mediated effect, and indeed when assessing OCR measurements I found that MitoQ caused a significant reduction in mitochondrial respiration. This was accompanied by a substantial decrease in OXPHOS-derived ATP production and an upregulation of glycolysis, similar to the effects observed by previously tested ETC inhibitors. This lead me to believe that the observed effect on SASP factor secretion by MitoQ, though mild (with the exception of IL-8), was a result of MitoQ's effect on respiration, and not ROS.

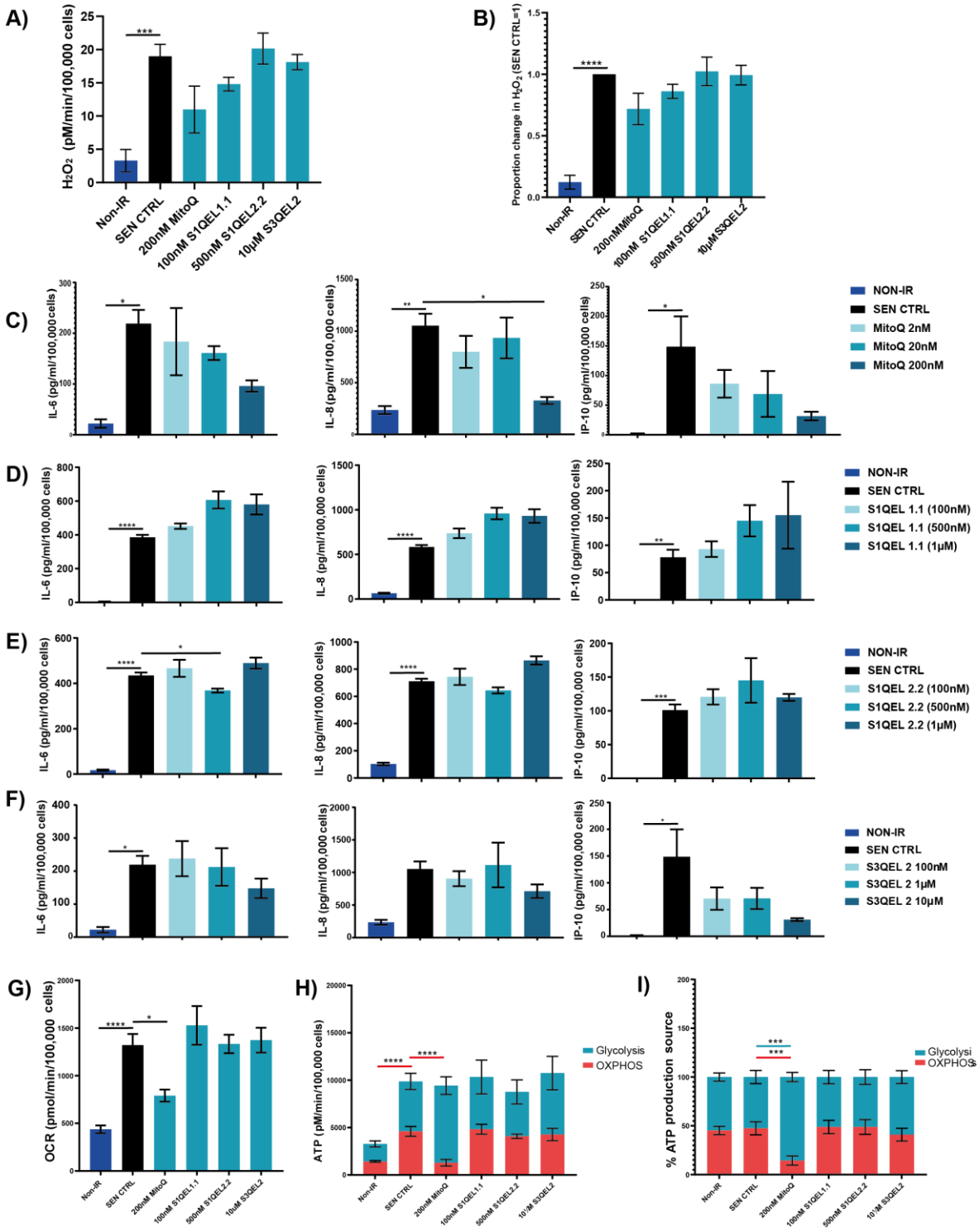


Figure 3.14: Treatment with site-specific mitochondrial ROS inhibitors does not reduce pro-inflammatory cytokine levels in senescent cells. (A) H_2O_2 production rates measured by Amplex Red assay in young, untreated senescent, and senescent fibroblasts treated with 200nM MitoQ, 100nM S1QEL1.1, 500nM S1QEL2.2 or 10µM S3QEL2, n=3-5 independent experiments (B) Effect on ROS production rates taken from (A) and represented as a proportion where senescent control = 1, (C-F) Levels of IL-6, IL-8, and IP-

10 secreted by senescent MRC5 fibroblasts treated with (C) MitoQ, CI-targeted antioxidants (D) S1QEL 1.1, or (E) S1QEL 2.2, and CIII-targeted antioxidant (F) S3QEL 2 . ELISA data taken from one (S1QEL1.1, S1QEL2.2) or two (MitoQ, S3QEL2) independent experiments, n=3 and 6 technical replicates respectively. (G) Oxygen consumption rates measured by Seahorse XF24 Analyzer in young, untreated senescent, and senescent fibroblasts treated with 200nM MitoQ, 100nM S1QEL1.1, 500nM S1QEL2.2, or 10µM S3QEL2, n=3 (H) ATP production rates in young, untreated senescent, and senescent fibroblasts treated with 200nM MitoQ,100nM S1QEL1.1, 500nM S1QEL2.2 or 10µM S3QEL2, n=3. (I) ATP production measurements from (H) represented as a proportion of total ATP. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

This was a particularly surprising result as MitoQ is generally accepted as a ROS scavenger with no effect on respiration (Kelso *et al.*, 2001), but further examination of the literature revealed that other studies have found the same inhibitory effect as seen here. Reilly et al (2012) found that multiple mitochondria-targeted compounds inhibited oxidative phosphorylation, irrespective of antioxidant activity. This was accompanied by an absence of OCR response to oligomycin or FCCP addition following MitoQ treatment, leading them to propose that MitoQ may interfere with the transfer of electrons from the ETC to oxygen. Other studies have also noted respiratory uncoupling in the presence of mitochondria-targeted antioxidants, as well as elevated basal acidification rate suggesting elevated glycolysis activity (Fink *et al.*, 2012). In support of these findings, a notable difference in this project between the inhibitory effects of MitoQ on ATP production and those of other tested ETC inhibitors is the compensatory increase in glycolysis-derived ATP. Where other ETC inhibitors produced an overall reduction in ATP levels, MitoQ appears to retain the same level of ATP production as untreated controls by increasing output from glycolysis.

Overall I was not able to scavenge ROS specifically without interference with OCR, and therefore the results given here are not able to answer the question of whether ROS

production specifically is influential on SASP factor secretion. However, data generated here is in accordance with an effect of OCR inhibition on SASP factor production.

3.6.2 MitoParaquat

To assess the effects of increased ROS production on development of a SASP I treated cells with increasing doses of the redox cycler mitoparaquat (Robb *et al.*, 2015) (**Figure *3.15***). Based on the well-documented link between oxidative stress and senescence I hypothesized that the burst of superoxide production resulting from mitoparaquat treatment would increase the production of SASP factors. Surprisingly, I found that all tested doses of mitoparaquat caused a significant reduction in IL-6, IL-8, and IP-10 (Fig. 3.15A). Of note, it was clear from observing cells in culture that the higher tested doses induced significant cell death (Fig. 3.15B), and although cytokine measurements were normalised to cell count, it is likely that the severe stress and reduced cell numbers caused by mitoparaquat may have influenced these results. Based on the cells apparent tolerance for the lowest dose tested, 1µM, I continued further experiments at this dose.

To confirm mitoparaquat's effects on ROS production I measured production rates of cellular hydrogen peroxide via Amplex Red assay, and surprisingly found that after 10 days treated with 1µM mitoparaquat ROS production rates showed a mild decrease, though this did not reach significance (Fig. 3.15C-D). Subsequent tests also revealed that OCR was significantly reduced by mitoparaquat treatment (Fig. 3.15E), which could explain the reduction in SASP factor secretion if there is indeed a causative link between the two. Interestingly, ATP analysis showed a similar picture to that of MitoQ, in which overall ATP levels were maintained, but OXPHOS-derived ATP production was reduced with compensatory upregulation of glycolysis (Fig. 3.15F-G). Further investigation into the mechanism of mitoparaquat is needed to confirm this data, but based on the information presented here it appears that mitoparaquat reduced SASP factor secretion in a manner similar to the previously tested ETC inhibitors, by inhibiting mitochondrial respiration. A number of possible factors could explain this unexpected result. For example, if the dose of mitoparaquat used causes substantial damage to the mitochondria, this would likely result in reduced respiration – though I would expect an increase in ROS production if this was the case, it is possible that mitoparaquat toxicity was pronounced enough that the resulting damage prevented even an initial burst of ROS.

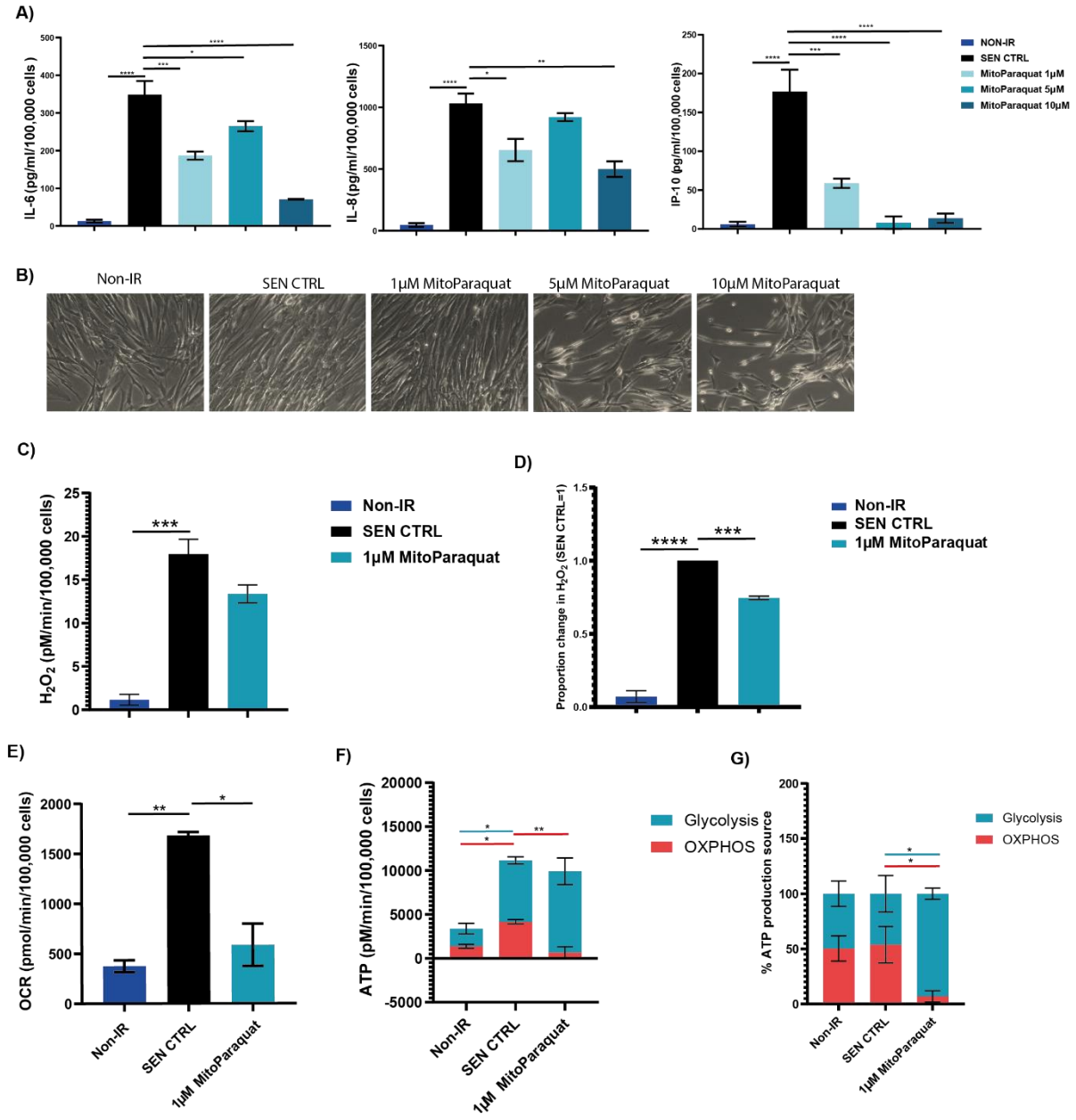


Figure 3.15: MitoParaquat reduces pro-inflammatory cytokine levels in senescent cells

(A) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, senescent, and MitoParaquat-treated (1μM, 5μM, 10μM) senescent MRC5 fibroblasts measured by ELISA. Data taken from one experiment conducted as described in Fig.3.2, n=3 technical replicates. (B) Representative brightfield images of cells in culture: young non-irradiated, untreated senescent, and senescent cells treated with increasing doses of MitoParaquat, showing increased cell death at higher concentrations. (C) Hydrogen peroxide production rates for young proliferating, untreated senescent, and senescent fibroblasts treated with 1μM MitoParaquat measured by Amplex Red assay, n=3 independent experiments, (D) ROS production rates taken from (C) and represented as a proportion where senescent

control = 1, Data is mean±S.E.M. (E) Oxygen consumption rates measured by Seahorse XF24 Analyzer, n=2. (F) ATP production rates, n=2 and (G) ATP measurements taken from (F) and represented as a proportion where total ATP = 100%. Data is mean±S.D. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.7 ROS production rates correlate with SASP suppression

To study the effects of ROS production in more detail, I measured levels of extracellular H_2O_2 in response to each of the previously tested drugs. To confirm the effectiveness of each drug I tested the acute effects on ROS production rates when administered directly during measurement (**Figure *3.16***). This demonstrated a downward trend in ROS production with most of the tested drugs. Exceptions to this include malonate, which showed minimal effect, and KCN which produced a mild increase. Surprisingly antimycin A elicited a reduction in ROS production in contrast to its established ability to increase ROS production from CIII. In this case I suggest that had measurements continued for a longer time period this initial decrease may have risen over time.

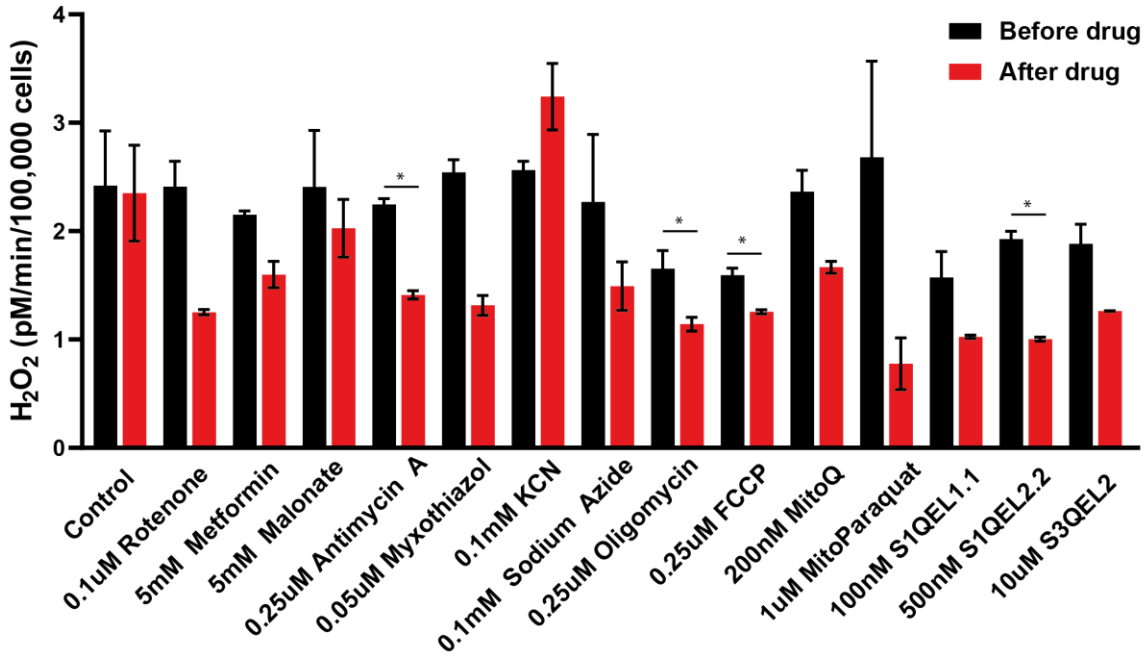
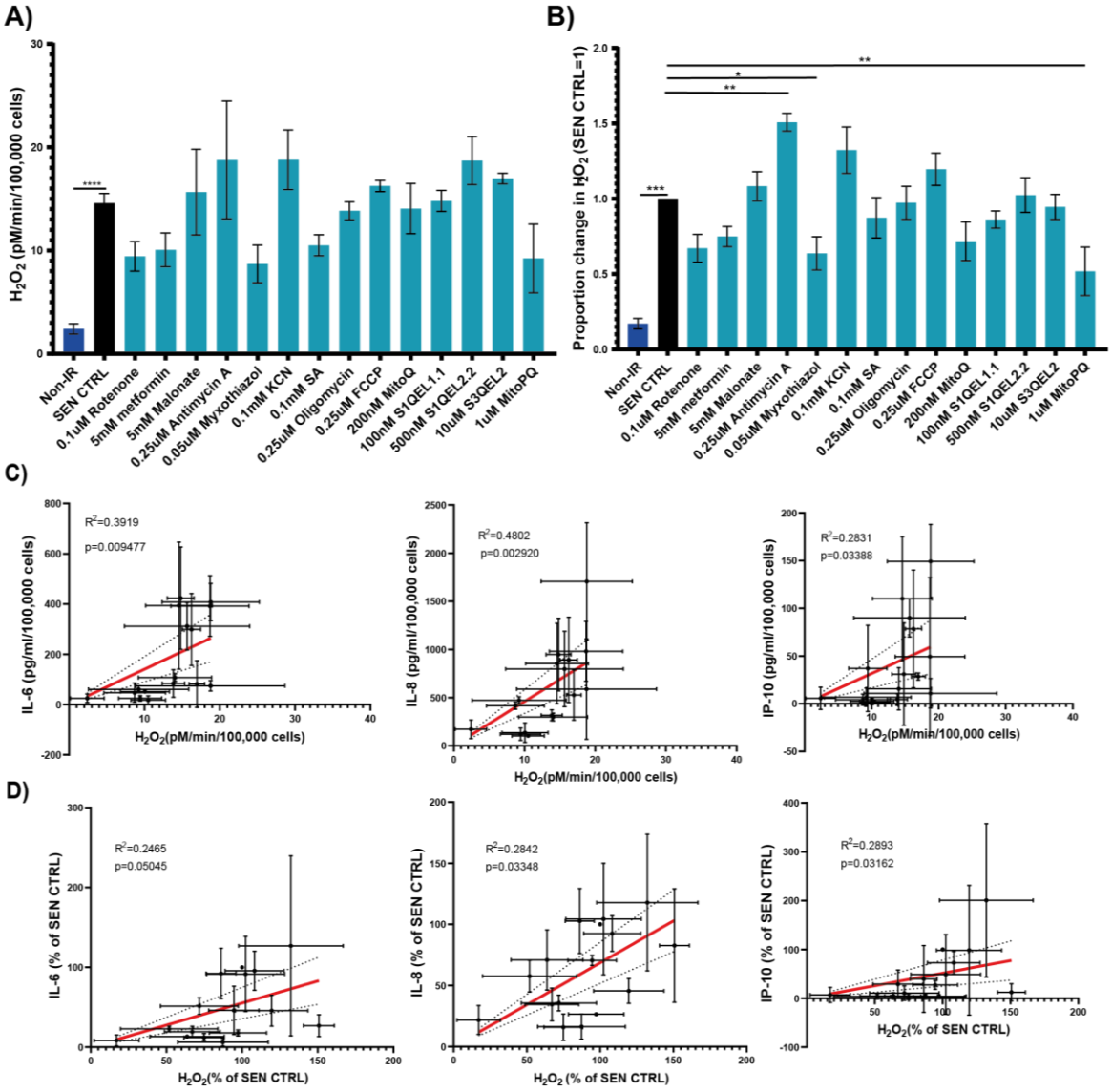


Figure 3.16: Acute ROS production rates in proliferating fibroblasts treated with ETC inhibitors and ROS modifiers. Hydrogen peroxide production rates measured by Amplex Red assay for young proliferating fibroblasts treated with inhibitors of the electron transport chain and ROS modifiers administered during ROS measurement, n=2-8 independent experiments. Data is mean±S.D. *p<0.05

I next treated senescent cells with the previously tested drugs (**Figure *3.17***). This analysis revealed that of the inhibitors tested, only myxothiazol produced a significant proportional reduction in hydrogen peroxide production rates compared to untreated controls (Fig. 3.17A-B). While a number of other inhibitors did appear to cause a reduction in ROS levels (rotenone, metformin, sodium azide), none of these were statistically significant. Antimycin also induced a significant proportional increase in ROS production rates, which was not an unexpected finding as the literature agrees that antimycin stimulates ROS production at CIII (Miwa *et al.*, 2003; Orr *et al.*, 2015). Though it should still be noted that this method of ROS measurement detects total secreted hydrogen peroxide rather than mitochondrial ROS specifically, and so further work is needed to confirm the source of this increase, despite the established mechanisms of this drug. These results show that after 10 days of treatment in senescent cells, the inhibitors tested – with the exception of myxothiazol and antimycin A - do not retain a significant effect on ROS production, despite strong acute effects.

However, despite not reaching statistical significance, there is a clear trend towards reduced ROS production with a number of drugs which also reduced both OCR and SASP production. To examine this observation in more detail I conducted correlation analysis between raw (Fig. 3.17C) and proportional (Fig. 3.17D) measurements of hydrogen peroxide production and secretion of IL-6, IL-8 and IP-10. This analysis showed a significant positive correlation between raw ROS production rates all three SASP factors (Fig. 3.17E). However, when I examined the relationship change between treated and untreated senescent conditions by normalising data to the corresponding untreated senescent condition, the significance of this correlation was noticeably reduced (Fig. 3.17F). The relationship between ROS and SASP appears to be weaker than that between OCR and SASP, and is likely an indirect effect of the latter connection given that ROS production is a product of mitochondrial respiration.



E)

H_2O_2 comparison	Significance
IL-6	**
IL-8	**
IP-10	*

F)

H_2O_2 proportional comparison	Significance
IL-6	n.s.
IL-8	*
IP-10	*

Figure 3.17: Correlation between ROS and SASP. (A) Hydrogen peroxide production rates measured by Amplex Red assay for young, untreated senescent, and senescent fibroblasts treated with inhibitors of the electron transport chain and ROS modifiers for 10 days post-irradiation, n=3-6 independent experiments. (B) Hydrogen peroxide production rates taken from (A) and represented as a proportion where untreated senescent control = 1, Data is mean±S.E.M. (C) Correlation analysis of ROS data from (A) and levels of SASP factors IL-6, IL-8 and IP-10 measured by ELISA in the same conditions (Fig. 3.2-9). (D)

Correlation analysis of percentage inhibition of ROS and SASP factors, represented by correcting to SEN CTRL = 100%. For both measures an overall average value has been taken from all independent experiments completed; for ROS n=3-6, and for cytokine data n=6 technical replicates as before, (E) Significance of correlations between ROS production and SASP factor measurements. (F) Significance of correlations between percentage inhibition of ROS and SASP factors. Data is mean±S.D. *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001

3.8 ROS production rates correlate with OCR

In order to further clarify the relationship between ROS, OCR and SASP, I compared the effects on ROS with those on OCR of all previously tested drugs (**Figure *3.18***). When looking at the proportional change of each measure compared to untreated senescent controls, a clear correlation was evident for a number of the drugs. Of those that elicited an effect on OCR and ROS production, rotenone and MitoQ exhibited comparable effects on both measures after 10 days of treatment post-IR. While they did not elicit an equivalent reduction in each measure, metformin, myxothiazol, sodium azide, oligomycin and mitoparaquat all demonstrated a significant reduction in OCR, and a milder, but still reduced, rate of ROS production. Antimycin and KCN both produced little to no effect on OCR compared to untreated controls, but increased ROS production. This finding of increased ROS is not unexpected based on previous literature on the mechanisms of both drugs (Gunasekar *et al.*, 1996; Correia *et al.*, 2012; Brand, 2016).

Correlation analysis based on both raw and proportional measurements of both factors (Fig. 3.18B) revealed a significant positive correlation between OCR and ROS production, which was largely expected as it is well known that increased mitochondrial respiration leads to increased ROS production (Murphy, 2009). Therefore, it is reasonable to suggest that the observed correlation between ROS production and SASP may simply be an indirect effect of the influence OCR has on SASP, and not a ROS-dependent effect at all.

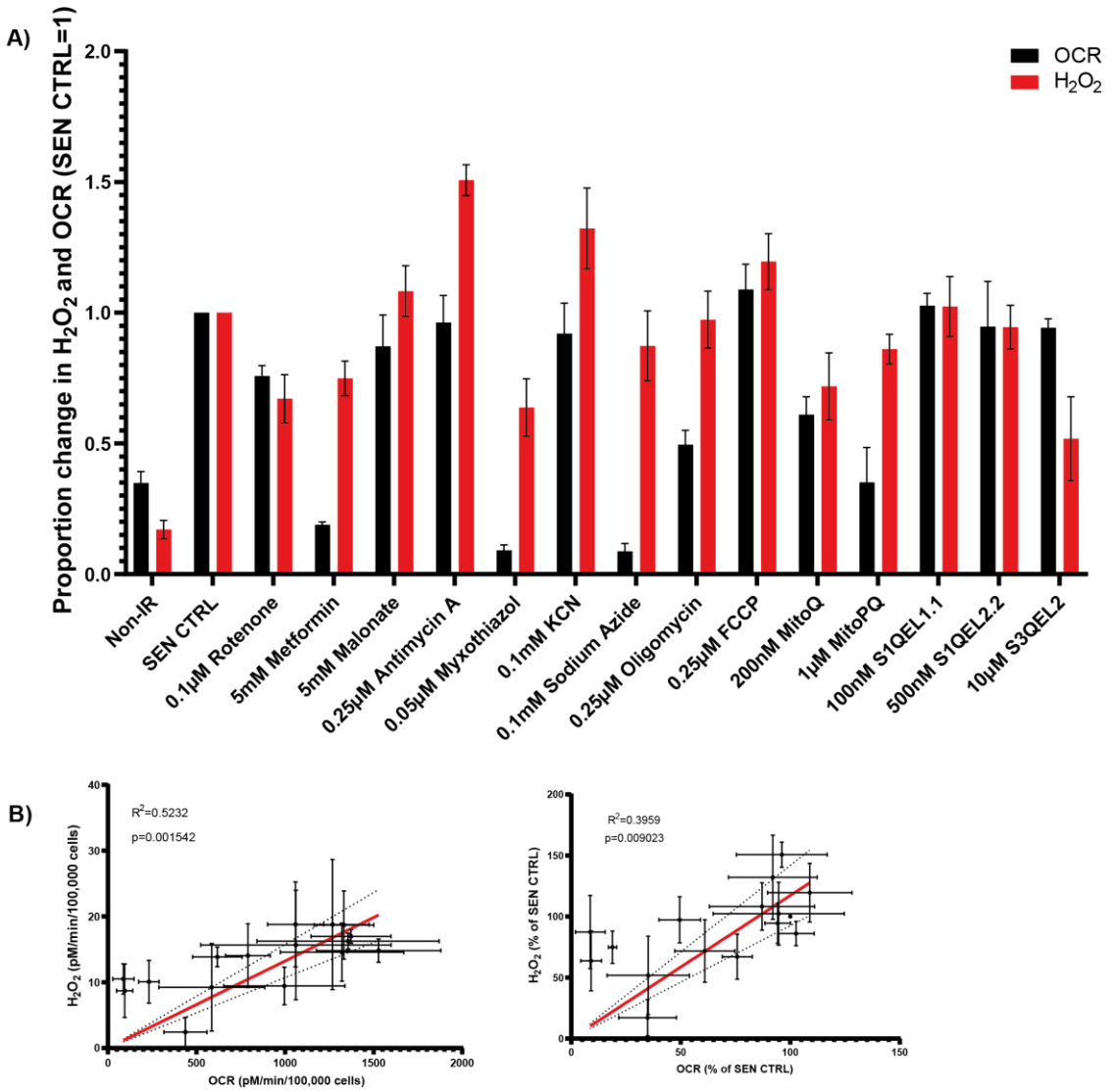


Figure 3.18: Correlation analysis between ROS production and OCR. (A) Proportion change in hydrogen peroxide production rates measured by Amplex Red assay for young, untreated senescent, and senescent fibroblasts treated with inhibitors of the electron transport chain or ROS modifiers for 10 days post-irradiation (n=3-6 independent experiments) plotted with OCR measurements from Seahorse XF24 Analyzer for corresponding conditions (n=3-5 independent experiments), represented by correcting data by SEN CTRL = 1. (B) Correlation analysis of raw ROS and OCR measurements in the same conditions. (C) Correlation analysis of percentage inhibition of OCR and ROS measurements by correcting for SEN CTRL = 100%. For both measures an overall average value has been taken from all independent experiments completed; for ROS n=3-6, and for OCR data n=3-5. Data is mean±S.D.

3.9 Discussion

3.9.1 Inhibition of electron transfer at multiple points in the ETC is sufficient to suppress the SASP

In this study, I provide evidence that interruption of electron transport at multiple points during oxidative phosphorylation is sufficient to prevent the production of a SASP *in vitro* in senescent human fibroblasts. Mitochondrial dysfunction is a common feature of senescent cells, and it has been shown that mitochondria are essential for the development of the senescent phenotype (Correia-Melo *et al.*, 2016). Previous reports have demonstrated that multiple manipulations of mitochondrial function can induce premature senescence, including partial respiratory uncoupling (Stockl *et al.*, 2007), disrupted respiratory complex assembly (Miwa *et al.*, 2014) and function (Yoon *et al.*, 2003; Stockl *et al.*, 2006). It is clear that mitochondrial function plays an important role in cellular senescence, but as yet the specifics of that role remain unclear. As such, I aimed to examine in finer detail how multiple aspects of mitochondrial function influence the senescent phenotype, through pharmacological inhibition of each mitochondrial respiratory complex, treatment with mild uncouplers of oxidative phosphorylation, and manipulation of mitochondrial ROS production.

As previously described, other studies have demonstrated a link between mitochondrial ROS production and induction of senescence and the SASP via a persistent DDR - and have even shown that suppression of ROS production can prevent onset of senescence (Passos *et al.*, 2010) - but few have taken a site-specific approach, opting instead to suppress ROS production generally (Saretzki;Murphy and von Zglinicki, 2003; Correia-Melo *et al.*, 2016). Though this relationship is not without debate (Stockl *et al.*, 2007), it seems clear that ROS production is an important factor in senescence, and identifying whether a specific site of ROS production holds more influence than others warrants investigation.

Screening of electron transport inhibitors and antioxidants allowed me to dissect the relationship between oxidative phosphorylation, mitochondrial ROS production, and induction of the senescent phenotype and SASP. I initially hypothesized that, as the primary source of ROS production within the mitochondria (Wong *et al.*, 2017) and the most commonly and severely affected respiratory complex with age and age-related

disease (Thomas;Keeney and Bennett, 2012; Pogson *et al.*, 2014; Hernansanz-Agustin *et al.*, 2017), inhibition of complex I function would be the most likely candidate for alleviating the senescent phenotype. Indeed, previous studies have shown that disrupted function (Moiseeva *et al.*, 2009) or assembly (Miwa *et al.*, 2014) of CI can induce senescence in healthy cells. These studies demonstrate that a dysfunctional CI is an integral part of senescence initiation. As such, by suppressing the activity of such dysfunctional CI in senescent cells, I aimed to remove the harmful impact of the impaired complex; namely, aberrant ROS production and oxidative stress, and subsequent SASP production. This could be achieved by use of CI inhibitors which suppress excessive ROS generation. However, I instead discovered that inhibition of electron transfer not only at complex I, but at complexes I, III, IV and V, was sufficient to prevent the induction of a SASP in irradiation-induced senescence (*Figure 3.19*).

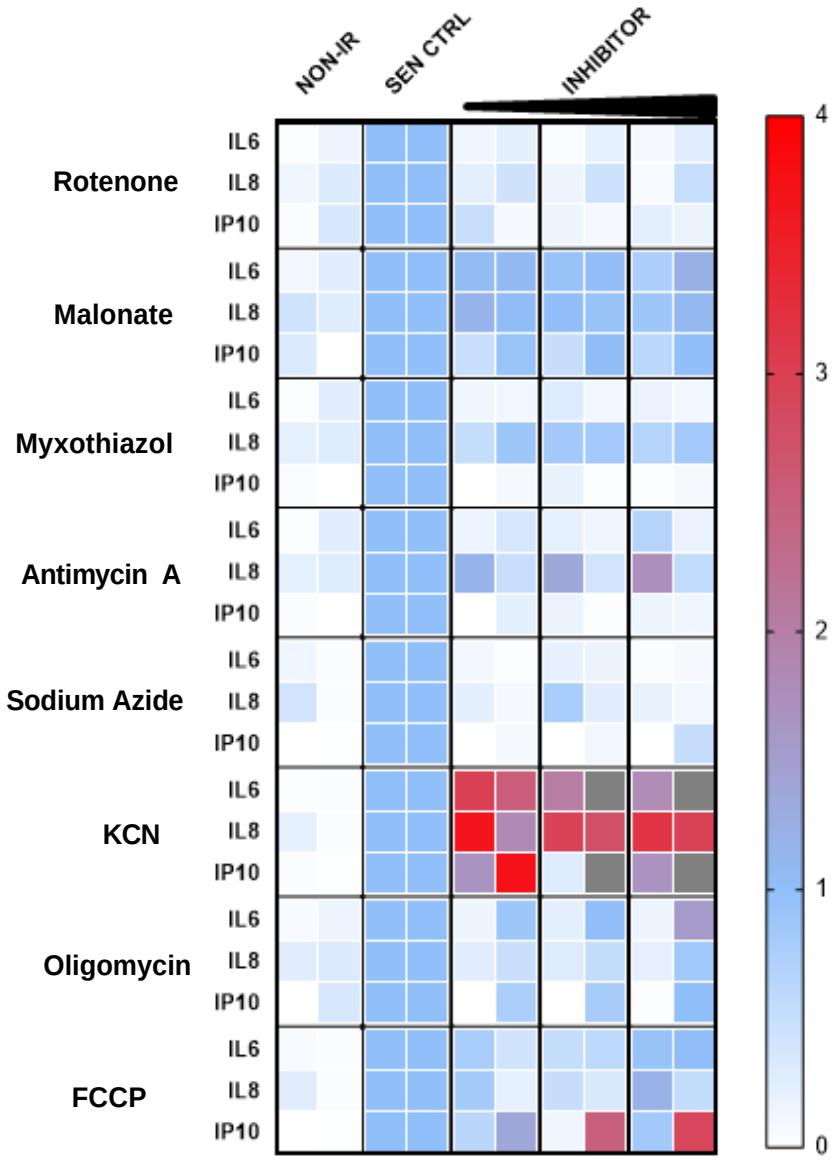


Figure 3.19: Heat map summary of the effect of electron transport chain inhibitor treatment on SASP factor production. Heat map represents the fold change in IL-6, IL-8 and IP-10 production in non-irradiated, untreated senescent, and senescent fibroblasts treated with increasing doses of inhibitors of mitochondrial CI (rotenone), CII (malonate), CIII (myxothiazol and antimycin), CIV (sodium azide and KCN), CV (oligomycin), and mitochondrial membrane potential (FCCP). Across all experiments data was normalised to the corresponding untreated Senescent Control condition. Overall, data demonstrates effective suppression of SASP factor production when senescent cells were treated with rotenone, myxothiazol, antimycin A, sodium azide, and oligomycin. n=6 technical replicates over 2 independent experiments.

Initial screening showed that treatment with inhibitors of complexes I (rotenone), IV (sodium azide) and V (oligomycin) was able to reduce secreted levels of IL-6, IL-8, and IP-10 in MRC5 fibroblasts when administered immediately after irradiation for 10 days. Inhibitors of CIII (antimycin and myxothiazol) were also able to significantly reduce levels of IL-6 and IP-10, though they showed minimal effect on IL-8 secretion outside of a slight downward trend with higher doses of myxothiazol (n.s.). These findings were true even if the target complexes were not fully inhibited (~50% inhibition), suggesting complete inhibition of mitochondrial complex activity was not necessary to alleviate the SASP. Inclusion of the anti-diabetic biguanide metformin - a widely accessible drug with a well-established safety profile in humans - represented a more physiologically practical CI inhibitor than rotenone, with therapeutic potential. In Chapter 4 I show that it too was able to successfully reduce SASP factor secretion after 10 days treatment post-irradiation. Inhibitors of CIV provided slightly more controversial results, with potassium cyanide (KCN) almost universally exacerbating secretion of multiple pro-inflammatory cytokines and chemokines, while sodium azide reduced production of these same factors. Possible explanations for this disparity are described above, but it appears that in my experimental conditions CIV inhibition by KCN did not work as expected. This limitation may be overcome by further review of the effects of KCN on respiration to clarify these findings.

An additional limitation to be addressed in future work is the inability of malonate to permeate intact cells and access the mitochondria. While we did not know at the beginning of this project that malonate was non-cell-permeable, attempts were made to address this issue once it came to light, by using proposed cell-permeable malonate pro-drugs (Ehinger *et al.*, 2016) (data not shown). Unfortunately, these drugs were also unsuccessful at modifying mitochondrial respiration. Further work is needed in order to truly examine the influence of CII activity in senescence, perhaps by testing alternative cell-permeable inhibitors such as diazoxide (Schafer *et al.*, 1969; Ralph *et al.*, 2011), thenoyltrifluoroacetone (TTFA) or atpenins (reviewed by (Kluckova *et al.*, 2013)).

SASP suppression following mild inhibition of multiple complexes points to a non-specific mechanism of action that does not rely on the function of a particular respiratory complex in order to prevent SASP development. It also suggests that no single complex is key to influencing SASP. Rather, interruption of electron flow – irrespective of location in the ETC

– is the important determining factor. More likely, these effects are mediated by an aspect of mitochondrial respiration which is impacted by inhibition of any respiratory complex. Prime candidates for this regulatory role that were subsequently investigated included OCR, ROS, and ATP production.

Further examination by cytokine array revealed that additional SASP factors with roles in cell survival (G-CSF, VEGF-A), differentiation (G-CSF, GM-CSF), and immune cell recruitment (GRO-alpha, MIP-1a, MCP-1, IFNγ) were also decreased when treated with these same drugs, demonstrating a broad suppression of pro-inflammatory SASP when electron transport is inhibited within the mitochondria. This could suggest that interfering with mitochondrial electron transport impacts an upstream regulator of the SASP, causing a global reduction in pro-inflammatory cytokines and chemokines, as opposed to affecting downstream targets or individual SASP factors. It is possible that inhibition of mitochondrial function reduces the levels of intermediates required for the production of these SASP factors at the transcriptional, translational or activation level. For example, a functioning ETC is required for pyrimidine synthesis (Khutornenko *et al.*, 2010), and pyrimidine deficiency has recently been linked to mtDNA release and innate immune signalling via the cGAS-STING pathway (Sprenger *et al.*, 2021). Studies have also shown that oncogene-induced senescence can be established and maintained by suppression of nucleotide metabolism (Aird *et al.*, 2013).

This data supports an important role for OCR, ROS, or overall mitochondrial function in regulating SASP. Targeting the electron transport chain specifically also offers a better approach than global removal of mitochondria, particularly as the observed SASP suppression can be achieved by pharmacological intervention targeted specifically to the mitochondria, such as with metformin which preferentially accumulates within the mitochondrial matrix (Wheaton *et al.*, 2014).

It is important to consider that there are other potential candidates in regulating SASP development, which could also be influenced by the inhibition of mitochondrial function demonstrated here. Manipulation of electron transport can have implications for the NAD/NADH ratio, and elevated NAD+ has been linked to SASP regulation (Nacarelli *et al.*, 2019). It is also possible that by modifying mitochondrial function in this way that these drugs also suppress leakage of mtDNA from the mitochondria – a known stimulator of

SASP (Pinti *et al.*, 2014) – by preventing oxidative stress (Cheng *et al.*, 2020) or even alter the balance of fission/fusion. These factors were not measured here, but may warrant further investigation in future.

3.9.2 Treatment with the uncoupler FCCP does not affect development of the SASP

In addition to respiratory complex inhibition, I also investigated the possibility of respiratory coupling efficiency as a mediator of SASP development (Hutter *et al.*, 2004). Treatment with the uncoupler FCCP immediately after irradiation did not alleviate SASP factor secretion and had variable effects on OCR and ROS measurements when given over 10 days, likely as a result of biological variation between samples and the dose of FCCP required to uncouple mitochondrial respiration. While previous studies demonstrate a reduced mitochondrial membrane potential in senescent cells (Hutter *et al.*, 2004), my data suggests that the development of the SASP is not dependent on mitochondrial membrane potential alone. Although mitochondrial membrane potential was not directly measured here, this can be done in future work to confirm these findings. Overall this data supports the idea that inhibition of respiration is key to suppressing SASP production rather than oxidative phosphorylation, as FCCP is known to prevent oxidative phosphorylation (Heytler, 1979; Park *et al.*, 2002; Grasmick *et al.*, 2018) but had no long-term impact on OCR and did not affect SASP factor secretion. A consideration for future examination of the effects of FCCP in senescence would be to evaluate higher doses of FCCP in order to truly assess the impact of mitochondrial uncoupling.

3.9.3 SASP suppression positively correlates with inhibition of OCR

Mitochondrial respiration data revealed a noticeable trend in which the drugs which induced the greatest inhibition of SASP factor secretion also produced the most significant decline in OCR. Each drugs inhibitory effect was also more pronounced when considering the proportional change compared to untreated senescent controls, as opposed to examining raw values.

Interestingly, following treatment with either rotenone or antimycin A for 10 days the respiratory inhibition was not as strong as seen in acute respiratory tests, suggesting an adaptive response by cells to long term treatment with certain respiratory complex inhibitors. Such adaptation could include degradation of the affected complexes or

mitochondria, or turnover of the drug in culture over time. Indeed, some studies have shown increased mitophagy (Giordano *et al.*, 2014) and compensatory modifications to metabolic pathways (Worth *et al.*, 2014) in rotenone-treated primary neurons, indicating attempts by cells to adapt to rotenone treatment over time. This difference in inhibitory effect between acute and long-term treatment provides convincing evidence that the efficiency of tested inhibitors must be confirmed in both circumstances, to compare the intended effect (OCR inhibition) with the hypothesized outcome (SASP factor secretion). Upon comparison, it seems clear that those drugs which did not elicit an immediate strong inhibition of OCR were not as effective at alleviating the SASP, and those which did induce a strong acute reduction in OCR successfully suppressed SASP even if OCR was recovered over time. It would also be of interest to test SASP factor levels following acute treatment with these inhibitors to compare.

The positive correlation identified between OCR and cytokine secretion levels at 10 days post-IR implies that in order for SASP production to occur in senescent cells, mitochondria must have functional oxidative phosphorylation machinery, though the possible reasons for this are numerous. This data also suggests that greater reliance on glycolysis should alleviate some of the burden on OXPHOS, though clearly not enough to fully rescue the senescent phenotype. My findings are in agreement with the knowledge that mitochondria are essential for senescence, and further, could suggest that development of the SASP requires a product of oxidative phosphorylation, though a functional ETC is needed for many mitochondrial processes, such as iron-sulphur cluster synthesis, intermediate metabolism and apoptotic signalling, and its impairment can have wide-ranging consequences. This study focused on production of ATP and ROS as major outputs of the ETC with links to the senescent phenotype (Zwerschke *et al.*, 2003; Passos *et al.*, 2010; Ogrodnik *et al.*, 2017). These products were examined in more detail by conducting correlation analysis between SASP factor secretion and ROS and ATP production in the same conditions, assessing OXPHOS- and glycolysis-derived ATP separately.

Closer comparison of the effect of the tested ETC inhibitors on OCR, ROS and SASP measurements when given acutely and over long-term treatment revealed a pattern which indicated the key determining factor in successful SASP suppression (Table 3.9.1). Drugs that caused a significant, immediate reduction in OCR – irrespective of whether this

inhibition was retained after 10 days – were able to effectively prevent induction of a SASP. This is clear when comparing the effects of rotenone and metformin. Both CI inhibitors caused a roughly 50% reduction in OCR at the concentrations tested when given acutely, but while metformin retained a strong inhibition after 10 days and rotenone did not, they both reduced SASP to comparable levels. In addition, antimycin A elicited a significant acute inhibition of OCR, which lessened over time, but long-term treatment actually exacerbated ROS production and yet SASP factor levels were still inhibited. Overall, data suggests that an immediate inhibition of OCR – not ROS – following significant genotoxic stress is sufficient to prevent development of a SASP. In section 5.4 I discuss in further detail the impact of shorter duration OCR inhibition immediately post-IR on the senescent phenotype.

Drug	OCR			SASP	ROS	
	Immediate	**48hr**	**10 days**	**10 days**	**Immediate**	**10 days**
Rotenone (0.1µM)	50% reduction	54% reduction	24% reduction	Inhibited	50% reduction	35% reduction (n.s.)
Metformin (5mM)	44% reduction	90% reduction	81% reduction	Inhibited	25% reduction	25% reduction (n.s.)
Malonate (5mM)	7% reduction	No change	13% reduction	No change	15% reduction	No change
Antimycin A (0.25µM)	71% reduction	53% reduction	15% reduction	Inhibited (excl. IL-8)	35% reduction	Exacerbated
Myxothiazol (0.05µM)	73% reduction	94% reduction	91% reduction	Inhibited (excl. IL-8)	50% reduction	35-40% reduction
KCN (0.1mM)	12% reduction	40% reduction	No change	Exacerbated	Exacerbated	Exacerbated
Sodium Azide (0.1mM)	42% reduction	95% reduction	91% reduction	Inhibited	35% reduction	10% reduction (n.s.)
Oligomycin (0.25µM)	43% reduction	82% reduction	60% reduction	Inhibited	30% reduction	No change
FCCP (0.25µM)	12% increase	15% reduction	10% reduction	Mild inhibition	20% reduction	Mild exacerbation

Table 3.9.1 Summary of ETC inhibitor effects on OCR, SASP and ROS production

3.9.4 SASP suppression positively correlates with OXPHOS-derived ATP production

Studies have demonstrated reduced ATP levels with increasing cell age (Zwerschke *et al.*, 2003), along with elevated glycolytic activity. It has been suggested that downregulation of mitochondrial activity may be an attempt to protect such cells from oxidative stress

(Brand, 1997). While this study observed the opposite with respect to ATP – a dramatic increase in overall ATP production in senescent cells – the idea that suppression of mitochondrial activity may benefit senescent cells appears to hold true in this case. Though it should be considered that measurements in this case were normalised to cell count, and the possibility of cell size- or mitochondrial mass-dependent differences in ATP production cannot be ruled out without further study. This is a wider limitation of this study, in that cell count is not an ideal method of correction, and in future data would be better served by normalisation to protein or mitochondrial content to remove any confounding influences of increased senescent cell size or mitochondrial mass.

Examination of ATP production rates from glycolysis and oxidative phosphorylation showed that the most effective suppressors of SASP also induced an almost complete shutdown of oxidative phosphorylation, forcing cells to rely on glycolysis as the primary source of ATP. This was true for senescent cells treated with metformin, myxothiazol, sodium azide, and oligomycin. While senescent cells are known to exist in a more glycolytic state than proliferating controls (Goldstein *et al.*, 1982; James *et al.*, 2015) (though this was not directly observed in this study), cells treated with these electron transport inhibitors exhibited almost no oxidative phosphorylation activity, resulting in a more complete swing towards glycolytic reliance. Despite this shutdown of OXPHOS systems, cells continued to survive in culture, with minimal loss of cell numbers compared to untreated controls. However, total ATP levels were closer to those of young proliferating controls than to senescent, suggesting these cells were likely existing in an energetically-stressed state as glycolytic compensation was not sufficient to maintain ATP levels equivalent to untreated controls.

The positive correlation identified between OXPHOS-derived ATP and SASP factor secretion supports a role for OXPHOS and electron transport chain activity in SASP development, as glycolytic ATP production appeared to have no influence on the measured SASP factors. This also provides evidence that it is specifically suppression of OXPHOS activity, and not upregulation of glycolysis, which is important in SASP development.

In contrast to this idea, however, young and senescent fibroblasts cultured in media supplemented with galactose instead of glucose to promote reliance on oxidative

phosphorylation (Aguer *et al.*, 2011) demonstrated no significant difference in SASP factor secretion. This indicates that increasing oxidative phosphorylation specifically does not promote the SASP – a perhaps contradictory finding to others demonstrated here, as all conditions that reduced OCR also reduced SASP. A possible explanation for this disparity may be that reduction of OXPHOS is sufficient to alleviate the SASP, but enhanced OXPHOS activity is not sufficient to stimulate it. An increase in IL-8 levels with young fibroblasts cultured in galactose compared to glucose may suggest induction of SASP factors when cells rely solely on OXPHOS, but this increase was not statistically significant and was not replicated in IL-6 or IP-10 measurements. OCR and hydrogen peroxide production rates under these same conditions again showed no significant difference between glucose- and galactose-cultured samples. Importantly, not all of the measured ROS production was derived from the mitochondria, and as such OCR provides a better determinant of OXPHOS-specific effects from galactose culture. Although culturing cells in galactose-supplemented media was sufficient to stimulate reliance on OXPHOS, based on the OCR, SASP and ROS data presented here I suggest that this was not sufficient to induce a level of mitochondrial dysfunction or ROS production needed to affect SASP factor secretion. Additionally, culture in galactose forced reliance on OXPHOS for ATP production but had no significant effect on either SASP production, OCR or ROS levels. This therefore suggests that it is OCR, and not OXPHOS ATP, that is important in SASP suppression.

By comparison, treatment with the ionophore monensin in proliferating cells resulted in substantial increases in both IL-6 and IL-8. Based on my own and others' observations (Ketola *et al.*, 2010; Charvat and Arrizabalaga, 2016), I suggested that increased cytokine secretion in response to chronic monensin treatment was a result of elevated oxidative stress and mitochondrial damage, and not the stimulation of glycolysis. This hypothesis was supported by additional respiratory analysis which showed that OXPHOS was not completely suppressed at 5µM, and even less so at 10µM acutely. 10µM monensin even increased OCR acutely, which could suggest that a subsequent burst in ROS production may also occur, and warrants further investigation. Though the SASP data in particular presented here should be interpreted with caution as this test was only conducted once, and there are clear toxicity and tolerability issues with monensin doses. Going forward, a

lower dose would be recommended in order to produce results based on better cell survival and higher final cell counts (current data based on counts of 5-15,000 fibroblasts per treated sample) and lower oxidative stress influence. Doses tested in acute OCR measurements are not feasible for long-term treatment, so acute OCR measurements following treatment with lower doses are necessary to compare. It would also be beneficial to measure oxygen consumption under long term treatment of monensin, if a tolerable dose can be maintained, to compare with SASP factor secretion changes. Furthermore, if monensin's toxic effects can be avoided with lower doses, additional tests in senescent cells would be of interest to examine its glycolysis-stimulating effects on SASP production.

Though findings with monensin are not without flaws, data from galactose culture seems to confirm that OCR inhibition is key to SASP reduction, and not specifically OXPHOS activity or ATP production.

3.9.5 Treatment with ROS modifiers shows minimal effect on SASP

My data strongly supports a role for mitochondrial respiratory function in the development of a SASP, though this was initially interpreted with caution until alternative influences of these treatments could be assessed, such as changes in ROS production resulting from altered OXPHOS activity. Examination of mitochondria-targeted antioxidants revealed a contrasting effect on SASP production between the traditional mitochondria-targeted MitoQ and the more specific S1QELs and S3QEL. Only MitoQ elicited any reduction in levels of measured pro-inflammatory cytokines after 10 days treatment post-IR, though the decrease was only statistically significant at the highest dose tested (200nM) in IL-8 secretion. By comparison, S1QEL1.1, S1QEL2.2 and S3QEL2 showed no influence on cytokine levels. This may suggest a mechanism of action specific to MitoQ which promotes the reduction of SASP, and may not be dependent on its antioxidant activity. This is particularly interesting as MitoQ can be given in humans and is non-toxic (Gane *et al.*, 2010; Snow *et al.*, 2010; Shill *et al.*, 2016), presenting a potentially clinically-relevant drug for future investigation.

Surprisingly, I found no significant reduction in ROS production when S1QELs or S3QELs were given for 10 days in senescent cells, despite a reduction being present when tested

acutely in proliferating cells. This data suggests that while these novel drugs do influence ROS production initially, the effect cannot be maintained long term. However, an important consideration to bear in mind is that while the method used here to measure ROS production rates offers the advantages of being extremely sensitive and specific as an enzymatic assay, it cannot detect mitochondrial ROS specifically in intact cells. As such, changes in ROS production measured here represent total secreted ROS which may mask source-specific changes. ROS reduction by MitoQ at 10 days was also mild, not reaching statistical significance, suggesting an alternative influence on SASP. Subsequent respiration analysis revealed that MitoQ in fact inhibited oxidative phosphorylation substantially, which has been observed in previous studies that suggested a disruption to electron transfer to oxygen (Fink *et al.*, 2012; Reily *et al.*, 2013), and supports my previous observations with ETC inhibitors. S1QELs and S3QEL showed no effect on OCR, further confirming the influence of OCR rather than ROS production over SASP development.

This finding, along with the minimal impact on ROS production over long term treatment, supports three key points: i) the mild reduction in SASP factor secretion by MitoQ was in fact mediated by its inhibition of oxidative phosphorylation, and not its role as an antioxidant; ii) a positive correlation between mitochondrial respiration inhibition and suppression of the SASP; and iii) it is not inhibition of ROS production specifically which is the determining factor in alleviating the SASP, but is instead another downstream result of OCR inhibition. It may also be important to examine any acute effects on OCR of these drugs, in the context of my previous hypothesis that acute OCR inhibition has a strong influence on subsequent SASP production. Comparison of the effects of these ROS-modifying drugs with the ETC inhibitors which suppress OCR acutely and SASP after long-term treatment, supports the idea that OCR, and not ROS, is the important factor in manipulating SASP production. Though we cannot yet discard the possibility that specific reduction of superoxide generation *per se* does not suppress the SASP also. Further work is needed to examine the specific sources of ROS production in these treatment conditions.

Surprisingly, cells treated with mitoparaquat exhibited reduced SASP factor levels, in contradiction with my initial hypothesis that the associated increase in superoxide production stimulated by mitoparaquat (Robb *et al.*, 2015) would increase pro-

inflammatory cytokine secretion. In fact, measurement of total cellular ROS levels found instead that ROS production was reduced. Identification of any source-specific changes, however, would require additional tests. This data is in contrast to much of the literature demonstrating a significant increase in superoxide production associated with mitoparaquat (Robb *et al.*, 2015). I propose that this may be due to a) compensatory mechanisms within the cell in an attempt to combat oxidative damage induced by mitoparaquat, or b) excessive mitochondrial dysfunction or cell damage, as evidenced by the increased cell death observed when trialling higher concentrations of mitoparaquat. Acute measurements of ROS production also showed an immediate reduction in ROS production when mitoparaquat was administered to young proliferating fibroblasts, suggesting that mitochondrial damage is the more likely explanation than adaptation. Additionally, I found that treatment with mitoparaquat significantly reduced basal OCR and triggered a similar switch to glycolytic reliance as seen in other treatments tested, but could also suggest damage-induced shutdown of OXPHOS. Though 1µM mitoparaquat and higher has been effective in other studies (Robb *et al.*, 2015; Antonucci *et al.*, 2019; Booty *et al.*, 2019), for long-term treatments a lower dose of mitoparaquat would be recommended in future experiments.

ROS analysis following treatment with the electron transport inhibitors produced a similar picture to that observed in OCR measurements. Though reductions in ROS production were milder than OCR inhibition, a similar pattern was observed showing the greatest decrease in ROS production by the same drugs that suppressed both OCR and SASP most strongly; namely, rotenone, metformin, myxothiazol, and sodium azide. I determined a positive correlation between H_2O_2 production rates and OCR measurements, which was not unexpected given that elevated oxidative phosphorylation activity is known to lead to greater electron leakage and formation of ROS (Murphy, 2009). Correlation analysis between ROS and SASP largely matched that seen for OCR and SASP, which may indicate that ROS trends are a result of OCR inhibition rather than a direct influence on ROS production.

A limitation to be considered in this analysis is that the methodology used here of examining intact cells using the Amplex Red/resorufin assay is that this does not allow for source-specific ROS analysis. As the peroxidase catalyst cannot access the mitochondria,

measurements obtained from this technique represent total secreted hydrogen peroxide, which consists of both mitochondrial superoxide converted to hydrogen peroxide by MnSOD and diffused out of the mitochondria, and extracellular hydrogen peroxide from other sources. This also means that not all mitochondrial superoxide is necessarily measured, though it is generally accepted that dismutation by MnSOD is an efficient process. In order to make interpretations based on site-specific ROS production, additional study would be required such as the use of isolated mitochondria, though this offers a less physiologically accurate model.

In summary, here I have demonstrated that inhibition of multiple mitochondrial respiratory complexes effectively suppresses multiple pro-inflammatory SASP factors. Data suggests that a general inhibition of mitochondrial electron transport successfully prevents the development of a SASP, suggesting that SASP production requires functioning OXPHOS machinery. Moreover, I have shown that the observed reduction in SASP factor production correlates strongly with mitochondrial respiration, but not with ROS production or glycolytic ATP levels. While the question of specific mitochondrial ROS production and its influence over senescence and the SASP could not be fully answered here, the ability of MitoQ to inhibit both OCR and SASP at the higher doses tested here lends support to the idea that it is OCR inhibition and not antioxidant activity that is the determining factor in SASP suppression. Finally, overall trends examined in this chapter suggest that a strong initial inhibition of OCR at the point of senescence induction may be key to preventing a SASP, while long-term inhibition is less important, though this requires further study. While CI was not the sole influencing factor in SASP production as predicted, it was nevertheless an effective target in SASP manipulation and the existence of drugs which target the complex and are safe for use in humans makes it an exciting avenue of investigation.

Chapter 4: Metformin treatment alleviates SASP and markers of senescence without restoring cell-cycle progression

The results of Chapter 3 provide strong evidence that interference with mitochondrial respiration at multiple points in the ETC is sufficient to prevent the production of a SASP *in vitro*. To develop a more detailed picture of the senescent phenotype under mitochondrial manipulation I next looked at the effects of mitochondrial respiration manipulation on a range of senescent markers and SASP factors. As a widely accessible drug with an established safety profile in humans and proven efficacy in the treatment of diabetes (Hostalek;Gwilt and Hildemann, 2015) as well as successful pre-clinical tests in other age-related diseases (Zhang *et al.*, 2015a), I chose to examine the effects of metformin specifically. While multiple respiratory complexes were effective targets for manipulation and SASP suppression, I chose to focus on CI as the primary mitochondrial source of ROS production (Brand, 2016) and the preferentially affected complex in ageing (Miwa *et al.*, 2014; Rygiel;Grady and Turnbull, 2014).

Although metformin's mechanism of action is still debated and potentially wide-ranging, it presents a more physiologically practical option for CI inhibition than other inhibitors such as rotenone. Metformin is also known to preferentially accumulate in the mitochondria over time, meaning that lower treatment doses can be used and still achieve concentrations over 100-fold higher within the mitochondria (Bridges *et al.*, 2014). Additionally, among the established mechanisms of metformin action is the inhibition of NFκB signalling (Saisho, 2015), which is a promising avenue for SASP modulation.

4.1 Metformin is a CI-specific inhibitor

Given the extensive debate around metformin's mechanism of action, in order to confirm its effects on mitochondrial respiration I conduced preliminary tests using Oxygraph 2k analysis. This allowed a more flexible methodology and ability to interrogate acute respiratory effects in individual complexes and at distinct respiratory states in greater detail. In samples of young proliferating cells I measured basal OCR before permeabilising cells by titration of digitonin. Once permeabilised, cells were provided with CI-linked substrates pyruvate and malate, which stimulate generation of NADH that is subsequently oxidized by respiratory complex I. This was followed by ADP to allow conversion to ATP (State 3 respiration). Cells were then given either metformin or water in 2.5mM steps – allowing OCR to stabilise between steps (roughly 5 minutes) - up to 10mM (Fig. 4.1A). Based on previous work within the lab of Joao Passos and the wider literature (Vazquez-Martin *et al.*, 2011; Moiseeva *et al.*, 2013), 5mM metformin was known to be an effective dose in senescent cells studies, so in order to confirm its effects on respiration a range between 50% (2.5mM) and 200% (10mM) of this dose was tested.

A clear and immediate reduction in OCR was evident in cells given metformin, and at 10mM OCR was fully inhibited. To determine whether metformin inhibited CI-linked respiration specifically, I administered saturating concentrations of the CII-linked substrate succinate, which restored electron transfer downstream of CI. This resulted in a complete restoration of OCR in metformin-treated cells to levels almost equivalent to untreated controls. This strongly suggests that metformin inhibited CI-linked respiration specifically, which was rescued by stimulating the ETC downstream of the affected complex.

To corroborate this finding, I conducted a second test in permeabilised proliferating cells, first administering the CI inhibitor rotenone at a dose known to fully suppress activity of CI (Fig. 4.1B). This was followed by succinate to allow electron transfer downstream of CI, and ADP to promote conversion to ATP. Subsequent additions of metformin in 2.5mM steps produced no effect on OCR, suggesting that metformin cannot elicit any effect from its target – CI – because rotenone treatment has already fully inhibited its activity. Taken together these results provide evidence that metformin is a CI-specific inhibitor.

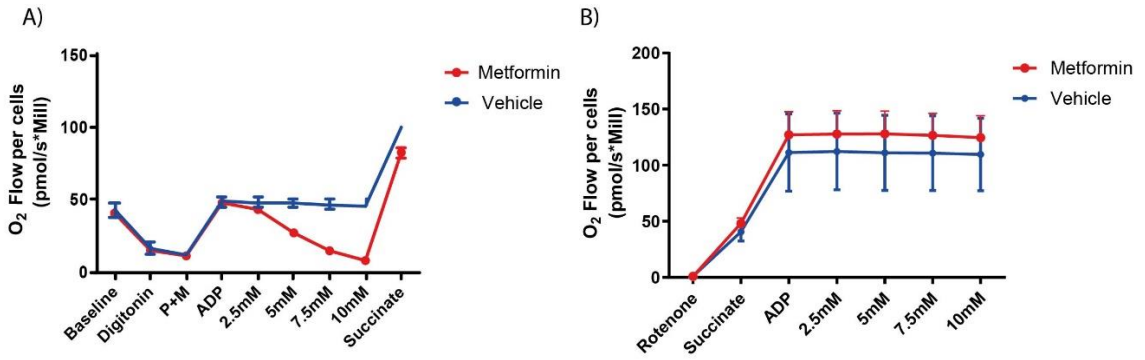


Figure 4.1 High dose metformin inhibits complex I-linked respiration (A) Effect of increasing doses of metformin on oxygen consumption of young MRC5 fibroblasts in Oxygraph-2k analysis, given either metformin or water to observe CI-linked respiration inhibition. Cells were permeabilised with digitonin and provided with CI-linked substrates (5mM pyruvate, 2mM malonate) followed by 2mM ADP to stimulate oxidative phosphorylation. Metformin or water was given in 2.5mM steps to full OCR inhibition. Addition of 10mM succinate (CII-linked substrate) restored electron transport at CII n=3 (B) Young permeabilised MRC5 fibroblasts given rotenone to inhibit CI, followed by succinate and ADP to allow electron transport from CII onwards. Subsequent metformin addition gave no further inhibition of respiration. n=3. Data is mean±S.E.M.

4.2 Metformin inhibits CI-linked respiration and triggers a glycolytic switch in senescent cells

Once metformin's target complex was confirmed, I next examined cellular respiration in young and senescent fibroblasts (**Figure *4.2***). I first confirmed that senescent cells respire at higher rates than young controls (Fig. 4.2A) by measuring the basal OCR in the absence of complex-specific substrates or inhibitors. This was in agreement with previous reports which showed a significant increase in mitochondrial respiration as a result of increased mitochondrial content in senescent cells (Hutter *et al.*, 2004). This was followed by addition of oligomycin to inhibit ATP synthase (CV) activity and prevent conversion of ADP to ATP and reveal the oligomycin-insensitive proportion of OCR derived from proton leak (LEAK). LEAK respiration was significantly higher in senescent cells than in young proliferating cells, suggesting a greater amount of proton leak and possibly greater respiratory uncoupling, again in line with previous studies (Hutter *et al.*, 2004; Passos *et al.*, 2007). The uncoupler FCCP was then titrated to determine the maximum electron transport system capacity (ETS) by uncoupling electron transfer from proton pumping, and revealed a higher maximum capacity in senescent cells compared to young controls. This again may be a result of increased mitochondrial content in senescent cells. Mitochondrial content was not measured in this study, but warrants confirmation in future work. The addition of inhibitors rotenone (CI) and antimycin A (CIII) inhibit electron transfer from the major entry points of the ETC, and reveal the residual oxygen consumption rate (ROX), or non-mitochondrial respiration. No significant difference in ROX was observed between young and senescent cells, confirming that the prior differences in OCR observed were purely from mitochondrial respiration.

In a further examination of ATP production gathered by Seahorse XF24 Analyzer assessment, I identified a significant increase in total ATP production in senescent cells compared to young controls (Fig. 4.2B). This appears to be in contrast to some of the literature which has demonstrated a reduced capacity for ATP production in senescence (Ogrodnik *et al.*, 2017), but obviously this increase could be due to the increased mitochondrial content that comes with enlarged cell size in senescence. The proportional contributions of oxidative phosphorylation and glycolysis to total ATP production were

only mildly altered in senescent compared to young cells, with a slight upregulation in glycolysis compared to OXPHOS (Fig. 4.2C). Previous studies have shown a more significant reliance on glycolysis by senescent cells (Zwerschke *et al.*, 2003), but this observation of only a mild difference was reproducible across all experiments conducted in this project.

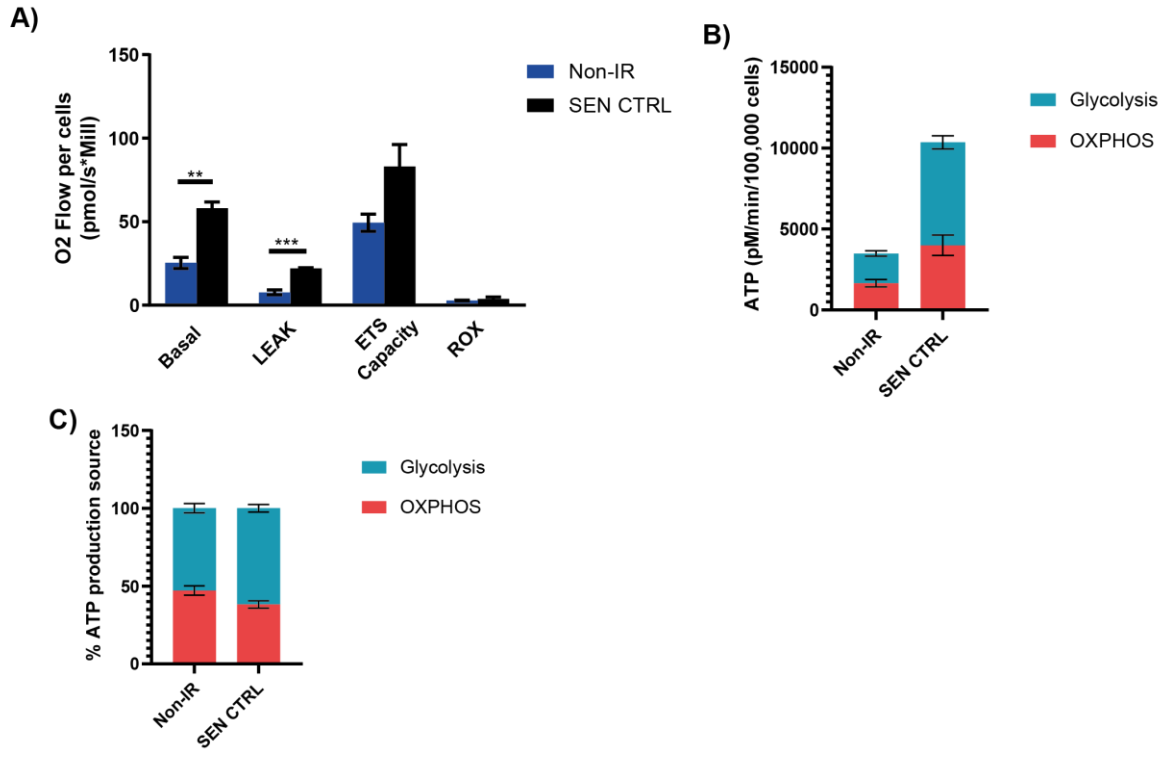


Figure 4.2 Senescent fibroblasts respire more than young controls and produce higher levels of ATP. (A) Oxygen consumption rate (OCR) analysis using Oroboros Oxygraph-2k of intact young and senescent MRC5 fibroblasts. Basal respiration represents baseline OCR without additional substrates or inhibitors. Addition of oligomycin to inhibit ATP synthase activity provides the LEAK respiration (electron flow coupled to proton pumping to compensate for proton leak). Titration of FCCP to collapse the proton gradient across the mitochondrial inner membrane measures the maximum electron transfer system capacity (ETS). Finally, addition of rotenone and antimycin A inhibits electron entry at complexes I and III, revealing the residual oxygen consumption (ROX) or non-mitochondrial respiration (B) ATP production rates from oxidative phosphorylation and glycolysis sources in young and senescent MRC5 fibroblasts obtained from Seahorse XF24

Analyzer analysis. (C) Data from (B) represented where total ATP = 100% to show the proportional contribution of OXPHOS and glycolysis to ATP production. n=3 Data is mean±S.E.M. **p<0.01, ***p<0.001

I next examined the effect of metformin treatment on cellular respiration in senescent cells. When compared to untreated controls, metformin again elicited a significant inhibition of OCR at basal, LEAK, and maximum capacity stages (Fig. 4.3A). This demonstrates a significant global suppression of mitochondrial respiration by metformin treatment that is unchanged in a senescent model. To study this in more detail I conducted analysis in permeabilised cells to interrogate the activity of individual complexes (Fig. 4.3B). Cells were permeabilised by titration of digitonin, and provided with CI-linked substrates pyruvate and malate followed by ADP to promote NADH production and activation of CI, and conversion of ADP to ATP. Again, metformin-treated cells exhibited significantly lower OCR than untreated controls. However, on addition of the CII-linked substrate succinate this inhibition was fully rescued as seen in proliferating cells, and metformin-treated cells returned to an OCR equivalent to – and even mildly higher than – untreated senescent controls. This again demonstrates that metformin's mechanism of action in mitochondrial respiration inhibition is targeted specifically to CI activity, as restoration of electron transfer downstream of CI restored OCR completely.

Given the significant inhibition of mitochondrial respiration in senescent cells treated with metformin, it was surprising that these cells survived so well in culture on perceivably no active respiration. This led me to examine the ATP production rates of these cells, based on the hypothesis that they were compensating with energy production from elsewhere. Indeed, ATP measurements from Seahorse analysis revealed that in senescent cells treated with metformin, while overall ATP production was significantly decreased (Fig. 4.3C), the remaining ATP production was almost exclusively from glycolysis (Fig. 4.3D). This corroborated an observation in culture that cells treated with metformin over long periods of time produced a change in culture media colour from pink to orange, indicating acidification. Furthermore, cells have also been reported to survive in culture following selective removal of all mitochondria, again demonstrating the ability of cells to

compensate for this respiratory loss (Correia-Melo *et al.*, 2016). My findings here were also in agreement with previous studies that have demonstrated increased glycolysis after treatment with metformin (Andrzejewski *et al.*, 2014; Yang *et al.*, 2021), although this is not without dispute (Hu *et al.*, 2019). While overall ATP production was decreased, this conversion to glycolysis reliance was clearly sufficient to maintain cell survival over long-term culture, though likely in a stressed state.

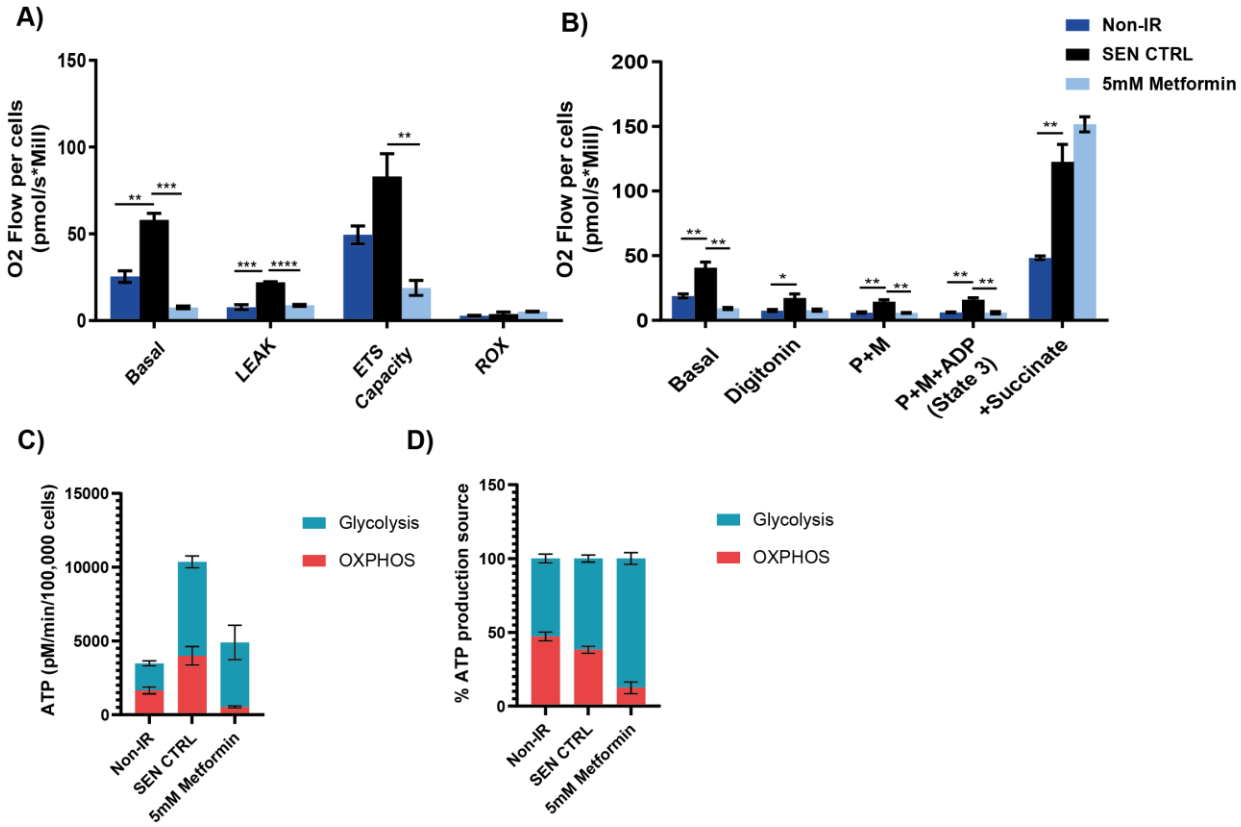


Figure 4.3 Metformin treatment inhibits OXPHOS respiration and triggers a glycolytic switch in senescent cells. (A) Oxygen consumption rate (OCR) analysis using Oroboros Oxygraph-2k of intact young, untreated senescent, and senescent MRC5 fibroblasts treated with 5mM metformin for 10 days post-irradiation. (B) OCR analysis in permeabilised young, untreated senescent, and senescent MRC5 fibroblasts treated with 5mM metformin for 10 days post-irradiation. Cells were permeabilised with digitonin and supplied with CI-linked substrates (5mM pyruvate and 2mM malate) plus 2mM ADP to stimulate oxidative phosphorylation (State 3 respiration). Addition of 10mM succinate to promote electron transfer through CII recovered respiratory inhibition in metformin-

treated senescent fibroblasts. (C) ATP production rates from oxidative phosphorylation and glycolysis sources in young, untreated senescent, and metformin-treated senescent MRC5 fibroblasts obtained from Seahorse XF24 Analyzer analysis. (D) Data from (C) represented where total ATP = 100% to show the proportional contribution of OXPHOS and glycolysis to ATP production. n=3 Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.3 Metformin treatment reduces markers of senescence and the SASP without restoring cellular proliferation in irradiation-induced senescence

To build a more detailed picture of the effects of metformin treatment on the senescent phenotype, it was important to look not only at metformin's direct effects on CI and mitochondrial respiration, but on the downstream effects of this inhibition on the SASP and senescent markers. Here I cultured senescent cells in metformin-treated media for 10 days following irradiation, and collected serum-free media samples for cytokine analysis on day 10 (**Figure *4.4***). By measuring the secreted levels of three major SASP factors IL-6, IL-8 and IP-10 by ELISA analysis I was able to assess the impact of metformin treatment of development of a SASP. I observed a significant reduction in the levels of all three cytokines in metformin-treated senescent cells compared to untreated controls, to levels equivalent to proliferating controls (Fig. 4.4A). This finding correlates with previous work that has shown inhibition of multiple other SASP factors by metformin at the mRNA level (Hu *et al.*, 2020; Hansel *et al.*, 2021), and demonstrated a reduction of SASP production by metformin through modulation of IKK/NFκB signalling (Moiseeva *et al.*, 2013).

To expand this picture of SASP suppression by metformin treatment, samples underwent cytokine array analysis (Fig. 4.4B), which revealed that metformin also rescued elevated production of a number of other key SASP factors in senescent cells, namely, IL-15, RANTES, IFNa2, GM-CSF, MCP-3, and MCP-1 (Fig. 4.4C). These cytokines have roles in immune response, cell proliferation and survival, and have also been implicated in tumour growth and progression. This broad suppression of SASP factors suggests that metformin treatment may have important anti-inflammatory and anti-tumour effects that are applicable to numerous age-related diseases.

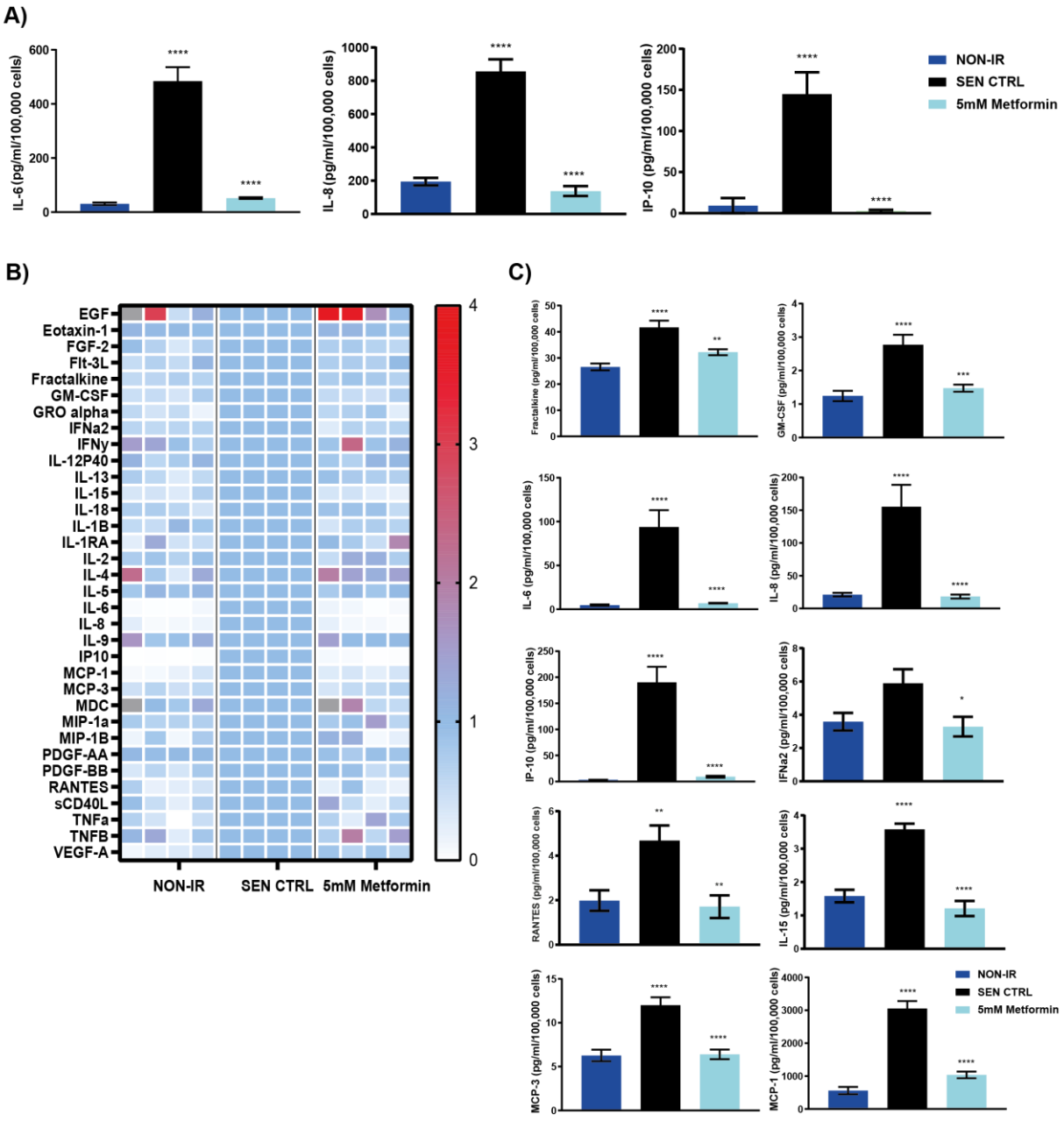


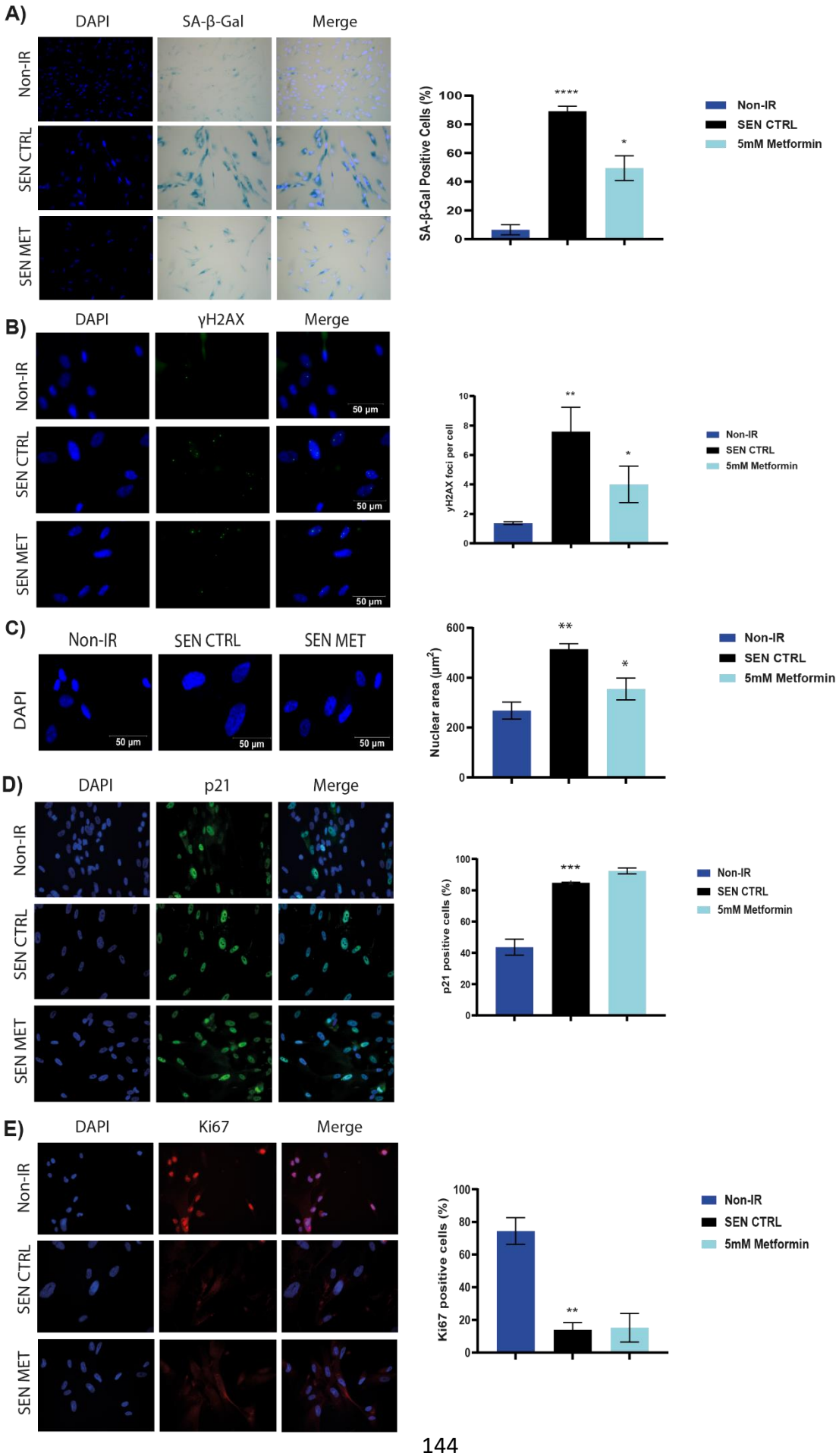
Figure 4.4 Treatment with metformin reduces pro-inflammatory cytokine secretion in senescent cells. (A) Levels of SASP factors IL-6, IL-8 and IP-10 measured by ELISA in young, untreated senescent, and senescent fibroblasts treated with 5mM metformin for 10 days post-irradiation. n=4 (B) Cytokine array analysis of young, untreated senescent, and senescent fibroblasts treated with 5mM metformin for 10 days post-irradiation, represented as a heat map of fold-change in cytokine levels. n=4 (C) Key cytokines identified during cytokine array in (B) which which showed significant increase in senescence and decrease with metformin treatment in senescent cells. Grey colour indicates levels outside the detectable range. Cytokine data was analysed using one-way

ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Data shown here suggests that treatment with supraphysiological doses of metformin for 10 days post-irradiation is sufficient to alleviate the pro-inflammatory SASP *in vitro*. This is particularly promising as metformin is a safe, affordable, widely-used drug in humans, which presents exciting possibilities for its use in ageing research and treatment of age-related disease. Indeed, its preferential accumulation within the mitochondria has also been observed in *in vivo* models (Kane *et al.*, 2010), further supporting its use clinically and suggesting a similar anti-inflammatory effect may be expected in animals and humans.

In order to provide more context to this finding and the wider senescent phenotype, I next measured a number of senescent markers by histochemical and immunofluorescent staining (**Figure *4.5***). As an increase in beta-galactosidase activity is a common marker of the senescent phenotype, I first examined the presence of senescence-associated beta galactosidase (SA-β-Gal) in metformin-treated senescent cells. Here I found that as expected, senescent fibroblasts exhibited much higher levels of SA-β-Gal than proliferating cells, and importantly that metformin was able to significantly reduce SA-β-Gal activity (Fig. 4.5A) when compared to untreated senescent controls. I next examined the prevalence of DNA damage by immunofluorescent staining for γH2AX, as a marker of DNA damage foci. The number of DDFs per nuclei in senescent cells treated with metformin were significantly reduced compared to untreated controls (Fig. 4.5B). This suggests that metformin was able to prevent the development of some long-term foci. As senescent cells are commonly associated with enlarged nuclei, I also assessed nuclei size to determine whether metformin was able to prevent changes to the nuclear architecture (Fig. 4.5C). Here I found that treatment with metformin also resulted in reduced nuclear size compared to untreated controls. Nuclear size is a key hallmark of senescence (Mehta *et al.*, 2007), and so this finding further supports the idea that metformin is able to alleviate numerous aspects of the senescent phenotype.

Notably, the number of cells expressing p21 were not changed by metformin treatment (Fig. 4.5D). This may not be surprising given the concurrent finding that the marker of cellular proliferation, Ki67, was also unchanged by metformin (Fig. 4.5E). This finding also demonstrated that metformin treatment did not allow senescent cells to re-enter the cell cycle; an important indication that metformin does not remove the key tumour-suppressor capabilities of the senescent phenotype. As an inhibitor of cell-cycle progression, it would be reasonable then to see no change in the level of p21-positive cells if no change in proliferation is also evident. Though some studies have also demonstrated minimal change in p21 levels following metformin treatment (Kim *et al.*, 2021), many more show reduced p21 expression (Karnewar *et al.*, 2018; Jiang *et al.*, 2020; Hansel *et al.*, 2021).


A)
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SEN MET
SA-β-Gal Positive Cells (%)
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*
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5mM Metformin
B)
DAPI
γH2AX
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50 μm
γH2AX foci per cell
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C)
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SEN CTRL
SEN MET
DAPI
Nuclear area (μm²)
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*
D)
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Merge
p21 positive cells (%)
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E)
DAPI
Ki67
Merge
Ki67 positive cells (%)
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Figure 4.5 Metformin treatment rescues markers of senescence in irradiation-induced senescence. (A) Representative images and quantification of SA-β-Gal staining in young, untreated senescent, and senescent fibroblasts treated with metformin for 10 days post-irradiation. n=3, (B) yH2AX staining and quantification. (C) Nuclear size (μm^2) DAPI staining and quantification, (D) p21 staining and quantification. (E) Ki67 staining and quantification, n=3 Data is mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

These findings provide evidence that treatment with metformin can prevent a number of aspects of the senescent phenotype. Of particular interest is metformin's ability to lower the development of DNA damage foci, which may indicate a reduced DDR. In addition to the previously observed SASP inhibition, this suggests that metformin is able to alleviate parts of the senescent phenotype involved in harmful positive feedback loops and induction of additional damage in existing senescent cells and nearby healthy cells.

4.4 Metformin treatment rescues the SASP in models of established senescence

Once I established that metformin treatment is able to successfully suppress the SASP and markers of senescence when given immediately after the senescent stimulus (20Gy irradiation), I next wanted to know if this was also possible in a model of established senescence. This would provide a more physiologically relevant model as any practical applications in humans would involve treatment of cells which were already senescent, with no guarantee of targeting cells at the point of senescence induction. For example, chronic treatment with metformin in middle-aged individuals would represent a similar setting. The next important question was to identify whether metformin treatment was able not only to prevent parts of the senescent phenotype, but also reverse them (**Figure *4.6***). Here, cells were exposed to 20Gy x-ray irradiation and cultured in untreated control media for 10 days until the senescent phenotype was fully established. On Day 10, media was either maintained untreated (senescent control) or changed to metformin-treated media. Cells treated for 3 days post senescence development were collected on day 13, 6 day treatment at day 16, and 10 day treatment and untreated controls at day 20 (Fig. 4.6A).

I first tested metformin treatment in MRC5 fibroblasts that had reached senescence through prolonged culture – replicative senescence (Fig. 4.6B-D). As previous experiments involved treatment for the 10 days required for senescence development, this was used as a starting point. I also wanted to know if shorter treatment timepoints would be sufficient to alleviate the SASP, and thus identify the minimum effective intervention. Measurements of IL-6, IL-8 and IP-10 were taken for replicatively senescent cells treated for 3, 6 and 10 days with 5mM metformin. This assessment revealed that in replicative senescence, only treatment for 10 days was able to successfully inhibit SASP factor production. All three measured cytokines were significantly reduced after 10 days of metformin treatment. This is an important finding, as it demonstrates that the SASP can be alleviated in cells that are ‘locked in’ to senescence long after the initial 10 day development period.

Next, I tested these same treatment timepoints in irradiation-induced senescence (Fig. 4.6E-F). This allowed me to construct a timeline of SASP factor levels after 3, 6, and 10

days of metformin treatment following the development of the senescent phenotype. Data showed a time-dependent decrease in all three cytokines. In this case, 6 days of metformin treatment was sufficient to significantly reduce levels of IL-6, IL-8, and IP-10. This may be because this model of irradiation-induced senescence was not as 'locked in' as in replicative senescence, or had not reached the point of 'deep senescence'. Alternatively, this could result from physiological differences between irradiation-induced and replicative senescence that influence SASP severity or receptiveness to treatment. For example, replicative senescence is telomere-dependent, while irradiation-induced senescence is not. As such, influencing the activity of a persistent DDR in each of these models may require different approaches. These phenotypes also differ at the protein expression level (Dierick *et al.*, 2002), and studies have found that certain genes are uniquely involved in specific senescent phenotypes. For example, fatty acid anabolism mediators are downregulated in replicative senescence, but not SIPS, while parts of cholesterol metabolism are downregulated in SIPS, but not in replicative senescence (Kural *et al.*, 2016). There are numerous possible differences between these senescent phenotypes that could influence their differing responsiveness to metformin treatment, but further work would be required in order to dissect this further.

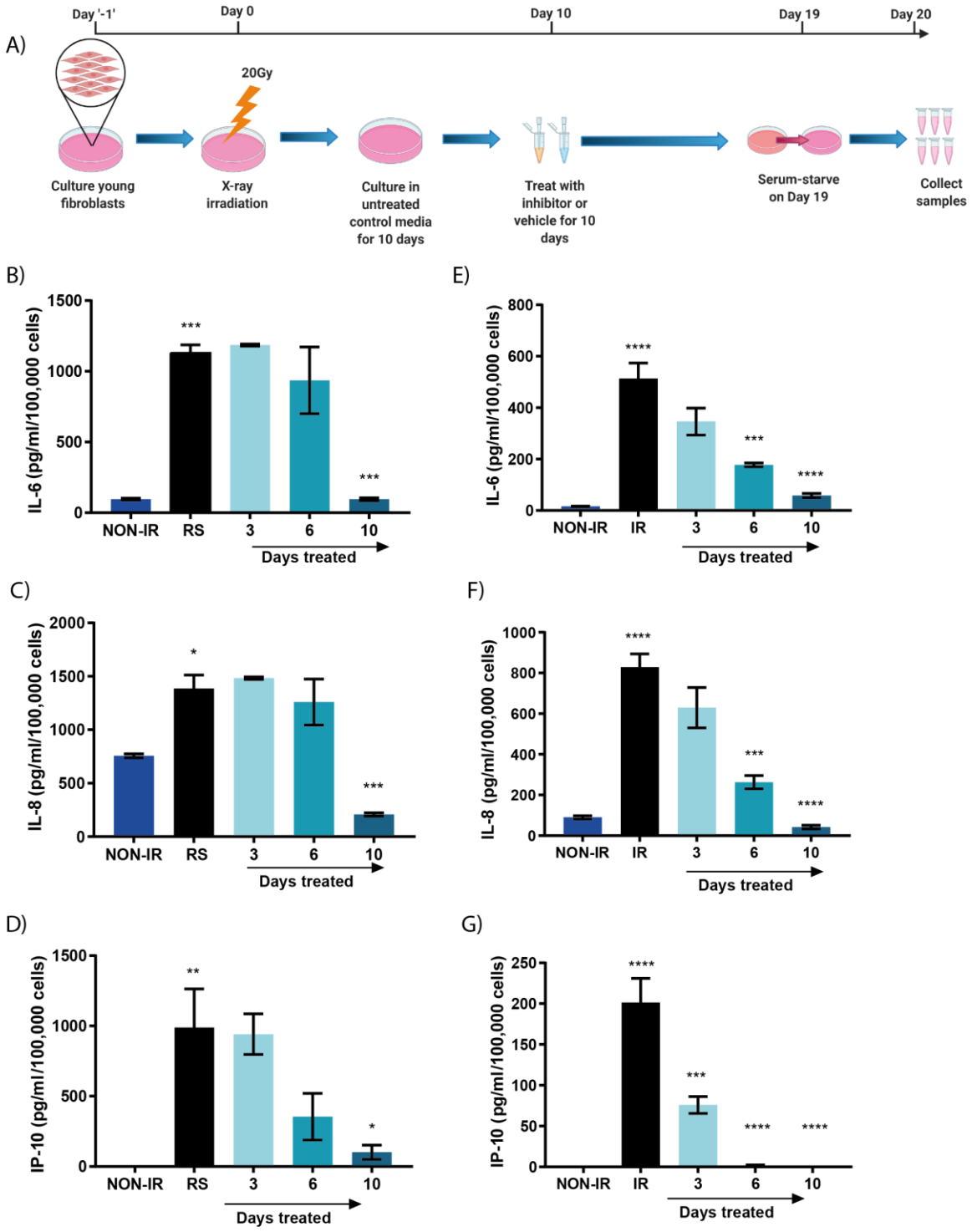


Figure 4.6 Metformin reduces pro-inflammatory cytokine secretion in established senescence. (A) Schematic of experimental design; cells were seeded to plates and irradiated the next day, then cultured in untreated control media immediately post-IR and replenished every 2-3 days for 10 days. At Day 10 culture media was replaced with drug-treated or control media as appropriate, and replenished every 2-3 days for a further 3, 6

or 10 days. (B-D) Levels of IL-6, IL-8, and IP-10 (pg/ml/100,000 cells) measured by ELISA in replicative senescent MRC5 fibroblasts treated for 3, 6, or 10 days with 5mM metformin. (E-G) Levels of IL-6, IL-8, and IP-10 (pg/ml/100,000 cells) in irradiation-induced senescent MRC5 fibroblasts treated for 3, 6, or 10 days with 5mM metformin following initial development of senescence for 10 days. All data taken from one independent experiment, n=3-4 technical replicates. Data is mean±S.E.M., analysed using one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Overall, these findings show that treatment with metformin is able to successfully reduce SASP factor secretion in an established senescent phenotype. This is a very promising indication for potential uses in humans, where a more heterogeneous cell population of senescence progression would be present. This also confirms that the SASP is reversible, which offers an interesting option for 'breaking' the damaging positive feedback loops involving SASP, ROS and a persistent DDR. The difference in effectiveness between the examined replicative and IR-induced senescent models may also suggest that timing of treatment is important. The model in which cells were senescent for a shorter period of time demonstrated greater receptiveness to treatment, while replicative senescence – or 'deep' senescence – was less affected. However it should also be considered that SASP composition between senescent models does vary, and so this should be explored as another explanation for the difference.

4.5 Metformin treatment does not rescue other senescent markers in models of established senescence

4.5.1 Replicative senescence

To examine the wider senescent phenotype in these same models, I measured key senescent markers SA-β-Gal, γH2AX, p16, p21, and the proliferation marker Ki67 (**Figure *4.7***). p16 staining was only possible in replicative senescence tests due to issues with antibody efficacy. Here I found that metformin treatment was not able to affect any of the tested markers after 10 days of treatment in replicative senescence. The proportion of cells positive for SA-β-Gal remained at 100% after 3, 6 and 10 days of treatment (Fig. 4.7A), showing that metformin does not influence SA-β-Gal activity when senescence is fully established. This was also true of γH2AX foci, and numbers of foci per cell remained unchanged by metformin treatment at any of the timepoints tested (Fig. 4.7B). It is therefore clear that metformin treatment does not impact the formation of DNA damage foci in established senescence after 10 days, and as such is unlikely to have any influence on DDR signalling as observed in cells treated immediately after senescence induction. Treatment times of >10 days should not be ruled out however. These findings demonstrate a difference in effect between treatment immediately post-IR and treatment once the senescent phenotype is 'locked in'. It is clear that by the time senescence is fully established, both SA-β-Gal activity and DNA damage foci formation can no longer be modified by metformin. This also aligns with the model of senescence becoming 'locked in' with time (d'Adda di Fagagna *et al.*, 2003; Coppe *et al.*, 2008).

Cells positive for p21 staining remained high at all timepoints tested (Fig. 4.7C), though as before this may not be surprising given that this marker was also unaffected when treatment started immediately post-IR, so it would be unexpected to find greater influence of metformin in 'deep senescence'. A similar finding was present for Ki67 staining (Fig. 4.7D), which again demonstrated no significant increase at 3, 6, or 10 days of treatment. In fact, after 10 days the proportion of cells positive for Ki67 were significantly decreased further when compared to untreated controls.

Immunofluorescent staining for the senescent marker p16 also showed no significant change in the proportion of cells positively stained (Fig. 4.7E), though a mild decreasing trend does seem evident this was not statistically significant and was highly variable. Additional tests to confirm this finding, and possibly the use of longer treatment times, may provide more detail on this mild trend. However, issues with immunofluorescent antibody efficacy mean this data is interpreted with caution.

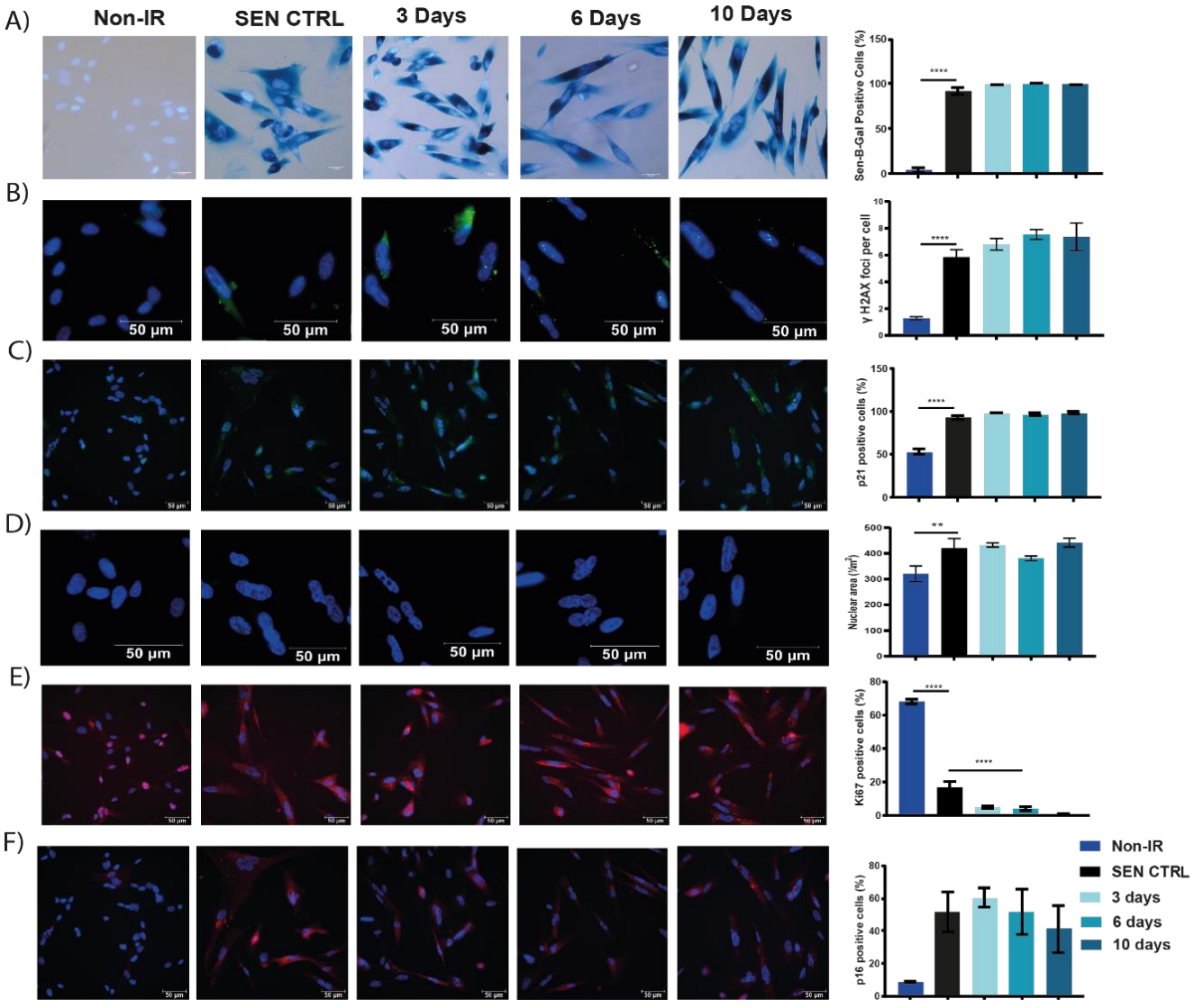


Figure 4.7: High dose metformin does not rescue markers of senescence in established replicative senescence. (A) Representative images of SA-β-Gal staining and bar chart quantification in young, untreated replicative senescent, and replicative senescent fibroblasts treated with 5mM metformin for 3, 6 and 10 days. (B) γH2AX staining and

quantification, (C) p21 staining and quantification, (D) Nuclear size DAPI staining and quantification, (E) Ki67 staining and quantification. (F) p16 staining and quantification. All data taken from one independent experiment, n=3-4 technical replicates. Data is mean±S.E.M., analysed using one-way ANOVA and Tukey's multiple comparisons test. ****p<0.0001

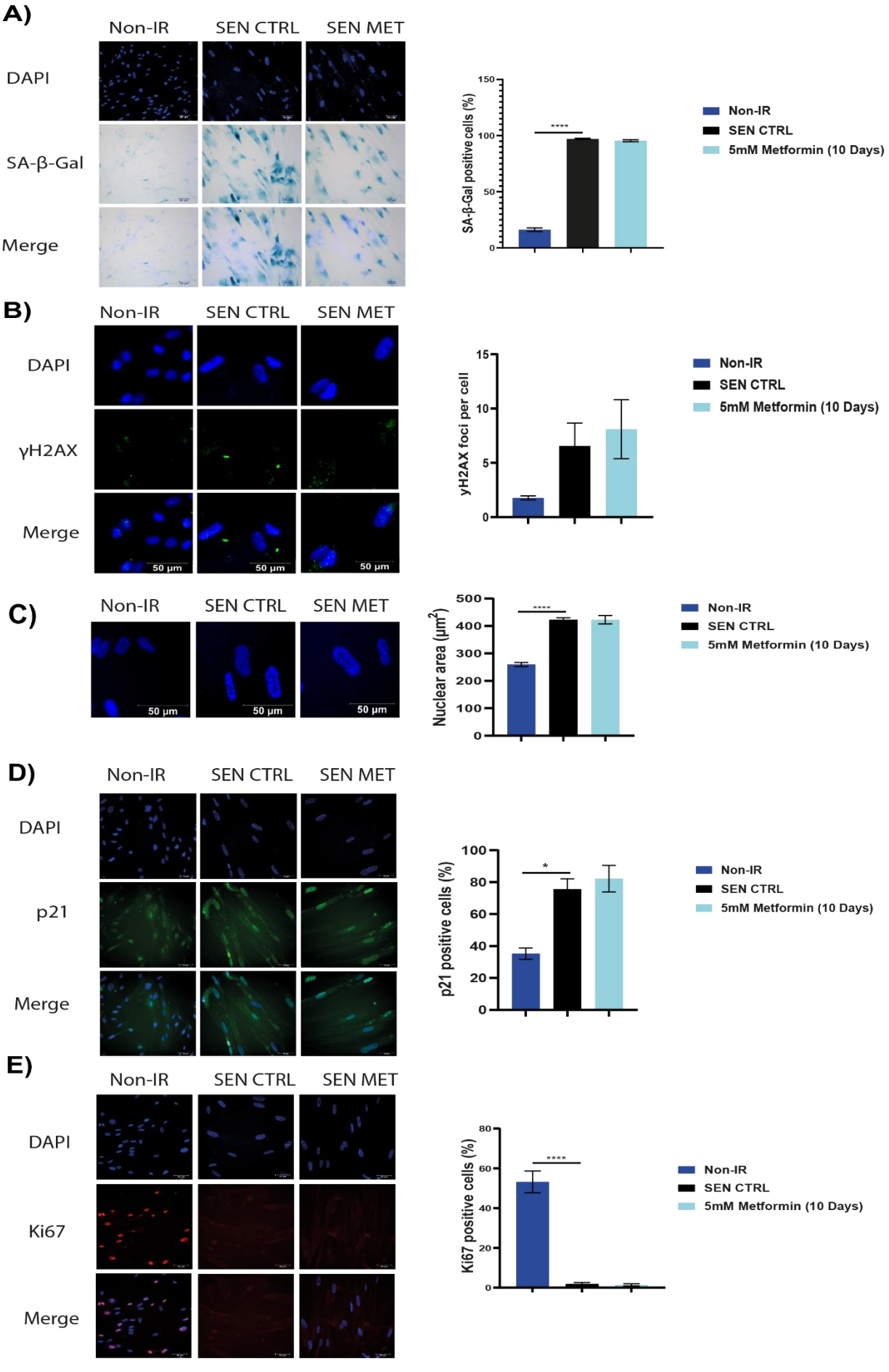
4.5.2 IR-induced senescence

Based on the previous findings demonstrating a greater effectiveness of metformin treatment in suppressing SASP in a model of established irradiation-induced senescence compared to replicative senescence, I next wanted to determine if this trend could also be true for other senescent markers (**Figure *4.8***). Based on my hypothesis that treatment of senescent cells from day 10 after irradiation was more effective because these cells were not as deep into senescent progression as replicatively senescent cells, it seemed reasonable that this may also transfer to other markers of senescence.

Here I measured treatment for only 10 days initially based on the minimal effect metformin treatment had in replicative senescence. I again discovered that treatment with metformin in established irradiation-induced senescence did not significantly alter any of the measured markers. SA-β-gal-positive cells (Fig. 4.8A) remained unchanged after 10 days. A noticeable decrease in the intensity of SA-β-Gal staining in metformin-treated samples was noted by eye, though not quantified here. It could suggest that perhaps longer treatment times may eventually lead to a reduction in SA-β-Gal activity in this model.

DDF numbers (Fig. 4.8B) were also unaffected by metformin treatment, again supporting the idea that DDR signalling cannot be influenced by metformin in established senescence.

The proportion of cells positive for p21 (Fig. 4.8C) and Ki67 (Fig. 4.8D) staining was not affected by metformin, though again unsurprising given their resistance to influence by metformin when treated immediately post-IR.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5mM Metformin (10 Days)
B)
Non-IR
SEN CTRL
SEN MET
DAPI
γH2AX
Merge
50 µm
50 µm
50 µm
γH2AX foci per cell
15
10
5
0
Non-IR
SEN CTRL
5mM Metformin (10 Days)
C)
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SEN CTRL
SEN MET
50 µm
50 µm
50 µm
Nuclear area (µm²)
500
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300
200
100
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5mM Metformin (10 Days)
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p21 positive cells (%)
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DAPI
Ki67
Merge
Ki67 positive cells (%)
80
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Non-IR
SEN CTRL
5mM Metformin (10 Days)

Figure 4.8: Metformin does not rescue markers of senescence in established irradiation-induced senescence. (A) Representative images and quantification of SA-β-Gal staining and quantification in young, untreated irradiation-induced senescent, and senescent fibroblasts treated with 5mM metformin. Senescent cells were irradiated and given control media for 10 days post-irradiation, followed my metformin-treated media for an additional 10 days. (B) γH2AX staining and quantification, (C) Nuclear size (μm^2) DAPI staining and quantification, (D) p21 staining and quantification, (E) Ki67 staining and quantification. n=1. Data is mean±S.E.M., analysed using one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001

Overall these findings demonstrate a key difference between the effectiveness of metformin when treatment is given at the point of senescence induction, and after the senescent phenotype is established. When treatment is initiated before the senescent phenotype has progressed, metformin is able to successfully prevent the development of a SASP, and reduce SA-β-Gal activity, nuclear enlargement, and formation of persistent DDFs, without restoring cell proliferation. By comparison, if treatment with metformin is given only after senescence is established, the SASP can be reduced while other senescent markers remain unchanged, though examination of ROS levels would provide further useful context to this interpretation. This also provides an important insight into the SASP and senescence, as it is clear that cell cycle arrest and DNA damage are uncoupled from the production of a pro-inflammatory phenotype. While the production of SASP factors can be alleviated in deep senescence, other markers of the senescent phenotype are resistant to manipulation if not treated at the point of induction.

It should be noted that at this stage of the project, all experiments conducted in models of established senescence were carried out once as preliminary tests. These findings led to further study in Chapter 5, and the timeline of treatment necessary to elicit beneficial effects in senescence.

4.6 Discussion

Following the findings in Chapter 3 that interruption of electron transfer at multiple points in the ETC is sufficient to prevent the development of a SASP, likely through the inhibition of OCR, I wanted to examine the effects of altered mitochondrial function on the wider senescent phenotype. Studies have repeatedly linked mitochondrial respiratory complex dysfunction to senescence, with evidence for declining function of each complex with age. However, while CII (Yoon *et al.*, 2003; Bowman and Birch-Machin, 2016; Lafargue *et al.*, 2017), CIII (Stockl *et al.*, 2006; Moiseeva *et al.*, 2009), and CIV (Xin *et al.*, 2003) all demonstrate reduced function in senescence, it is CI that is most commonly linked to ageing, senescence and lifespan (Sanz *et al.*, 2010; Stefanatos and Sanz, 2011; Hur;Stork and Walker, 2014; Miwa *et al.*, 2014; Pogson *et al.*, 2014).

As previously discussed, mitochondrial CI is the primary source of elevated ROS production with age and is frequently impacted in diseases such as Parkinson's disease (Mortiboys *et al.*, 2008; Flinn *et al.*, 2009), Alzheimer's disease (Terada *et al.*, 2021), and heart failure (Karamanlidis *et al.*, 2013). As such, I chose to focus my next investigation on the manipulation of CI activity by treatment with metformin. This allowed me to study changes in senescent cells and markers of senescence following treatment with a well-established drug already used in clinics (Hostalek;Gwilt and Hildemann, 2015), and with the potential for wider applications in numerous other age-related diseases (Zhang *et al.*, 2015a; Han *et al.*, 2019; Mor *et al.*, 2020). Of note, among the age-related diseases senescence is associated with is diabetes (Kuki *et al.*, 2006; Thompson *et al.*, 2019) for which metformin is an effective treatment. Though metformin's benefits in diabetes are thought to be a result of gluconeogenesis inhibition rather than CI inhibition (Alshawi and Agius, 2019; Pecinova *et al.*, 2019), it does provide an interesting precedent for treatment of senescent cells in age-related diseases, and metformin's use specifically.

Upon further examination of the effect of metformin treatment on the senescent phenotype, I have demonstrated 4 key findings. First, that metformin does indeed inhibit CI-linked respiration as shown by the restoration of OCR upon succinate addition in metformin-treated permeabilised fibroblasts, and a negligible effect on OCR in rotenone-treated fibroblasts. Second, that treatment with metformin induces an almost total shutdown of oxidative phosphorylation, which promotes reliance on glycolysis for ATP

production. Third, that chronic treatment with metformin immediately post-irradiation is able to prevent the onset of both the SASP and multiple senescent markers (increased SA-β-Gal activity, nuclear size, and γH2AX foci denoting DNA damage), but does not rescue senescent cell-cycle arrest. Finally, preliminary experiments suggest that treatment with metformin in models of established replicative and irradiation-induced senescence is not sufficient to rescue these same markers of senescence, but is able to reduce secretion of pro-inflammatory SASP factors after 10 days.

4.6.1 Metformin inhibits CI-linked respiration

Studies have previously shown that metformin is a mild CI inhibitor (Bridges *et al.*, 2014; Wheaton *et al.*, 2014), but there is still debate within the literature as to metformin's mechanism(s) of action, particularly with regards to high and low dose effects (Madiraju *et al.*, 2014; Wang *et al.*, 2019). Here I have shown that 5mM metformin does indeed inhibit CI-linked respiration using permeabilised MRC5 fibroblasts. My data demonstrated that mitochondrial respiration was suppressed in a dose-dependent manner by metformin, and subsequently restored when CII-linked substrates were provided, reinstating electron transfer downstream of CI. This was corroborated by analysing cells treated with rotenone, and supply of CII-linked substrates to restore respiration. Subsequent metformin addition had no effect on OCR, confirming that metformin's inhibitory effect was CI-specific. It is also worth noting that metformin is known to accumulate slowly within the mitochondria as a result of its positive charge and the negative-inside state of the mitochondrial matrix. As such, while these high concentrations effectively inhibited CI activity immediately, it would be reasonable to assume that lower doses could be tested over a longer period of time to achieve the same effect.

4.6.2 Metformin triggers a reliance on glycolysis for ATP production

In senescent cells treated for 10 days immediately post-irradiation with metformin, I found that oxidative phosphorylation was almost entirely shut down, despite both cell morphology and number appearing similar to untreated senescent controls when examined in culture. From this I hypothesized that metformin-treated cells must be using alternative ATP sources to maintain cell survival, and indeed found that they were relying almost solely on glycolysis. Though this did not compensate for the reduction in oxidative

phosphorylation-derived ATP, and overall ATP production was lower than untreated senescent control samples suggesting cells were likely surviving in an energetically stressed state. Senescent cells are generally considered to be more glycolytic than healthy controls (Bittles and Harper, 1984; Zwerschke *et al.*, 2003). However, here I observed only a mild increase in glycolytic reliance for ATP production in senescent compared to proliferating controls, which was reproducible across all bioenergetics experiments conducted in this and the previous chapter. Nevertheless, metformin treatment induced a significant swing towards glycolysis reliance as a result of almost total oxidative phosphorylation shutdown. Though it does not appear that metformin induced an upregulation of glycolysis, the loss of OXPHOS activity results in a significant difference in the proportional contribution of glycolysis to total ATP production.

Mitochondrial metabolism in senescence is a widely-covered area of research, and there are numerous ways in which ATP production and the balance between oxidative phosphorylation and glycolysis activity can influence senescence and cell health. Previous reports have shown that a reduction in ATP production from the ETC can induce cellular senescence (Stockl *et al.*, 2006), likely as a result of elevated AMP (or ADP)/ATP ratios which stimulate growth arrest (Zwerschke *et al.*, 2003) via AMPK activation and p53-dependent signalling. (Peyton *et al.*, 2012). Though findings here demonstrated an overall increase in ATP production in senescent cells, this does not necessarily rule out an increase in the ADP/ATP ratio. If ADP levels were to increase proportionally then the ratio would be maintained, and any additional increase in ADP would result in a higher ADP/ATP ratio. Moreover, the response of AMPK to a reduction in ATP is to upregulate ATP-producing processes and inhibit ATP-consuming processes, in an attempt to restore homeostasis. One suggestion is that this may be what is represented by the data shown here; a compensatory increase in ATP levels 10 days after senescent stimulus.

Nevertheless, the known ability of AMPK activation to induce growth arrest would suggest that reduction of ATP production in senescent cells treated with metformin should re-inforce growth arrest through the same signalling pathway. However, studies of metformin have demonstrated that treatment can in fact delay endothelial senescence via AMPK activation, by enhancing SIRT3 and PGC-1α activity stimulating mitochondrial biogenesis, and leading eventually to an increase in hTERT and prolonging replicative

lifespan (Karnewar *et al.*, 2018). It is possible that in this case metformin is not acting through AMPK, but instead production of the SASP is incredibly energetically demanding, and the observed reduction in ATP levels following metformin treatment prevents its production. In particular, as metformin is able to alleviate other aspects of the senescent phenotype that would be reinforced by an AMPK-stimulated p53-dependent growth arrest, it seems unlikely that metformin is acting through AMPK under these conditions. Further study is needed to determine metformin's specific mechanism of action in these circumstances.

A more in depth analysis of SASP suppression following metformin treatment demonstrated a broad inhibition of pro-inflammatory SASP factors. Although this suppression is not a CI-specific effect as seen in Chapter 3, it does provide a promising indication that metformin is able to alleviate numerous cytokines and chemokines with potentially harmful roles in inflammation, cell survival and tumour progression. These findings are aligned with previous reports that show that metformin suppresses the SASP through inhibition of NFκB signalling, which may be the case here (Moiseeva *et al.*, 2013). Although my previous data seems to suggest it is an OCR-dependent effect, there is much to still be understood about metformin's mechanism of action specifically.

4.6.3 Metformin alleviates markers of senescence without restoring cell proliferation

Increases in nuclear size (Mehta *et al.*, 2007) and DNA damage (d'Adda di Fagagna *et al.*, 2003; Hewitt *et al.*, 2012) are well-established markers of senescence, and the CDK inhibitor p21 (Fang *et al.*, 1999) responsible for triggering cell-cycle arrest is known to mediate induction of senescence. SA-β-Gal activity is also a sensitive and reliable marker of cellular senescence (Dimri *et al.*, 1995). Here I showed that treatment with metformin for 10 days post-irradiation prevented increases in multiple senescent markers but did not restore proliferation.

Treatment with 5mM metformin at the point of senescence induction prevented much of the increase in SA-β-Gal activity associated with senescence. Though SA-β-Gal activity was not reduced to the level of healthy proliferating cells, the decrease was distinct when compared to untreated controls. Activation of AMPK by metformin is also known to result in mTORC1 inhibition (Howell *et al.*, 2017). If this is the case here, the data shown here is

in agreement with other studies that have found that inhibition of mTORC1 leads to both reduced SASP and SA-β-Gal activity (Wang *et al.*, 2017; Park *et al.*, 2020). While SA-β-Gal is a common feature of multiple forms of senescence, there are circumstances under which it is not expressed in senescent cells, and although it is frequently apparent in most models it is not a requirement of the senescent phenotype (Lee *et al.*, 2006). As such, multiple markers are needed to corroborate the presence of senescent cells.

A persistent DDR is the most common requirement for senescence induction, and monitoring the formation of DNA damage foci (DDFs) has been established as an effective and reliable marker of senescent cells. The cellular response to DNA double-strand breaks involves phosphorylation of H2AX, making γH2AX a reliable indicator of DNA damage in senescence (Hovest *et al.*, 2006).

Metformin treatment during the development of the senescent phenotype reduced the formation of DNA damage foci identified by γH2AX immunostaining. By Day 10 it is expected that the DDFs present in senescent cells would mostly be persistent, long-lived DDFs (Passos *et al.*, 2010; Hewitt *et al.*, 2012). As such, the reduction in number of these foci following treatment with metformin could suggest a) that metformin prevented the induction of a proportion of DNA damage required to stimulate DDF formation, or b) that metformin prevented some of the smaller short-term foci from developing into persistent DDFs. Previous work has shown that metformin is in fact able to stimulate a DDR including increased levels of γH2AX (Vazquez-Martin *et al.*, 2011), in contrast to my findings here. However, the report by Vazquez-Martin et al was conducted in cancer cells, while other studies in senescent cells have produced similar findings of reduced DDR signalling and γH2AX foci as shown here in this project (Kim *et al.*, 2021), suggesting possible differences between cellular models.

Nuclear size was significantly reduced in metformin-treated cells compared to untreated controls. This could suggest a number of possible influences of metformin, including prevention of chromatin remodelling, laminB1 or histone H1 reduction, or simply an intervention upstream of all of these observed senescence-associated changes in nuclear architecture (Pathak;Soujanya and Mishra, 2021). Further work would be required to truly delineate metformin's role in preventing nuclear enlargement.

As cell cycle arrest is an established marker of senescence it was important to monitor cellular proliferation via the marker Ki67, which revealed no change in the proportion of proliferating cells when treated with metformin. This indicates that while metformin can alleviate a number of harmful aspects of the senescent phenotype, it does not restore cell-cycle progression. While this may be expected given metformin's documented ability to induce cell-cycle arrest via AMPK and p53 signalling, it is particularly important as it confirms that metformin does not remove the essential tumour suppressor function of senescent growth arrest; a promising finding for therapeutic applications.

Surprisingly, metformin treatment showed no effect on p21-positive cell proportions. Other studies have shown that metformin can reduce p21 expression levels in a number of experimental models, including epithelial lung cell cultures induced to senesce by radiotherapy (Hansel *et al.*, 2021), and in HK-2 cells after 2hrs of treatment (Jiang *et al.*, 2020). Though my findings contradict these reports, it could be argued that as p21 is essential in triggering senescent growth arrest, and based on the observation that Ki67-positive cells remained low it seems clear that senescent cells treated with metformin did not continue to progress through the cell cycle.

4.6.4 Metformin reduces pro-inflammatory cytokine secretion in both replicative and established irradiation-induced senescence

Evaluation of key SASP factors in both models of established senescence revealed two key findings. First, that the SASP is reversible in established senescence after 10 days of 5mM metformin treatment. Second, that a shorter treatment timeline is successful in established irradiation-induced senescence compared to replicative senescence. The first is exciting as it offers promising indications that the harmful pro-inflammatory phenotype that accompanies senescent growth arrest can be targeted by treatments even after senescence is fully developed. This means that it may be effective *in vivo* and in human studies at alleviating chronic inflammation and the damage it causes. The second observation – while additional experiments are needed to corroborate it – suggests that time is an important factor in targeting the SASP for treatment. While the irradiation-induced senescent cells had senesced for 10 days post-stimulus, the replicatively senescent cells had been established for longer. This is largely due to the nature of replicative senescence in prolonged cell culture. Noticeably, the cells which had been

senescent for longer were more resistant to treatment, suggesting that earlier treatment has a greater effect, and the noted 'locking in' of the senescent phenotype may still be progressing to some degree even after the accepted 10 day timeline. Studies have shown that most markers of senescence, such as DDF frequencies, ROS production, p53 activation and p21, stabilise around 2 days after induction of SIPS without intervention (Passos *et al.*, 2010). The full senescent phenotype with pro-inflammatory SASP requires 7-10 days (Coppe *et al.*, 2008; Rodier *et al.*, 2009). While interventions capable of rescuing senescent growth arrest have been shown to be effective if initiated within 9 days of senescence induction, beginning treatments after this point becomes less effective (Passos *et al.*, 2010). One proposed explanation for this is the influence of changes to chromatin organisation, which increase in parallel with irreversible growth arrest. It is therefore unsurprising that most senescent markers were not responsive to treatment in both models of established senescence, though it does not provide an explanation for the disparity between SASP alleviation. It should also be considered, however, that this difference in treatment efficacy could also be a result of difference in SASP makeup between senescent models. Further experiments would be required to examine this discrepancy in more detail.

4.6.5 Metformin does not reduce other markers of senescence when the phenotype is already established

Treatment with metformin in models of established replicative and irradiation-induced senescence showed no effect on any of the measured senescent markers. Studies have previously noted that the senescent phenotype becomes 'locked in' after 9 days, no longer requiring constant stimulation by factors such as ROS-mediated oxidative damage. This oxidative damage replenishes DNA damage markers and promotes the presence of larger, more persistent foci which are more difficult to repair (Passos *et al.*, 2010). This supports our findings that DNA damage foci are unaffected by treatment in models of established senescence, as the majority of foci remaining at this point in senescence are persistent and long-lived. Additionally, this is in agreement with other reports that have shown that manipulation of the DDR is unable to rescue senescent growth arrest once senescence is established (d'Adda di Fagagna *et al.*, 2003; Sang;Coller and Roberts, 2008).

SA-β-Gal activity also remained unchanged after metformin treatment in established senescence, which could suggest that lysosomal activity remains permanently elevated, triggering SA-β-Gal enhancement. Nuclear size was also unresponsive to metformin treatment in both replicative and irradiation-induced senescence, suggesting that nuclear enlargement falls within the 'locked in' senescent phenotype.

Levels of p21- and Ki67-positive cells were also unaffected following metformin treatment, though this is not unexpected following similar findings when metformin is given immediately post-irradiation. This corroborates our findings that metformin treatment does not allow senescent cells to re-enter the cell cycle and continue to proliferate; an important finding when considering clinical applications.

These findings suggest that timing of treatment is an important determinant in alleviating the senescent phenotype. Further work is warranted to discover where the critical change occurs that leaves these specific markers unresponsive to treatment, while SASP remains receptive to modification. Surprisingly, these findings are in contrast to other papers which have shown an almost opposite effect, in which the 'locking in' of senescence is mediated via the SASP, which remains refractory to modification while other markers can be alleviated.

In summary, here I have shown that metformin can inhibit major markers of the senescent phenotype without restoring cell proliferation. This is a key requirement for any senescence-targeting treatment, allowing harmful aspects of the phenotype to be suppressed while preventing damaged cells from re-entering the cell cycle. Data here also demonstrates inhibition of CI-linked respiration and shutdown of oxidative phosphorylation activity by metformin, resulting in reliance on glycolysis for ATP production that does not reach the levels of untreated cells. This may point to energetic deficit as a determining factor in metformin's suppression of the senescent phenotype. Preliminary data in this chapter also suggests that the SASP is particularly receptive to modification by metformin, as demonstrated by reduced SASP production when treated both acutely and chronically with metformin, and both from the point of senescence induction and in a model of established senescence. Further work is required to examine in further detail the treatment dose-, length-, and initiation point-dependent effects of metformin on the senescent phenotype, and identify a minimum effective intervention.

Chapter 5: Treatment with metformin, myxothiazol and rapamycin in a model of established senescence produce differing effects on SASP production

To build on the preliminary findings in models of an established senescent phenotype, I decided to expand on this by also examining the effects of the CIII inhibitor myxothiazol, and the senostatic rapamycin. CIII is another significant contributor to cellular ROS production, and treatment with myxothiazol from the point of senescence induction was able to dramatically decrease SASP factor secretion. As such, it was of particular interest to compare the effects in other senescent markers based on manipulation of a different ROS source and respiratory complex activity. Comparison of the effects of metformin and myxothiazol allowed me to demonstrate whether metformin was likely mediating its effects on the SASP through inhibition of the ETC. This also allowed me to investigate whether the observed effects in metformin were site- or mechanism-specific.

Addition of rapamycin provided a well-established positive control with proven success in alleviating the senescent phenotype, as numerous previous works have shown that rapamycin can reduce both the SASP and a number of other senescent markers (Pospelova *et al.*, 2012; Correia-Melo *et al.*, 2016). Furthermore, as an inhibitor of mTOR, rapamycin provided an interesting comparison with metformin, which also inhibits mTOR indirectly as a result of CI inhibition and AMPK activation. Should the resulting phenotypes of cells treated with these two drugs be comparable, this could provide preliminary evidence that metformin's beneficial effect in senescence is linked to mTOR inhibition.

5.1 Alleviating the SASP requires 10 days of treatment with electron transport inhibitors in established senescence

To evaluate the effect of metformin, myxothiazol and rapamycin treatment in established IR-induced senescence over time, cells were irradiated at 20Gy and fresh culture media given immediately afterwards (**Figure *5.1***). Culture media was untreated for the first 10 days following irradiation, replenished every 2-3 days until Day 10, at which point media was switched to drug-treated or untreated as appropriate. Cells were then treated for either 3 (Fig. 5.1A) or 10 (Fig. 5.1B) days. For SASP factor analysis by ELISA culture media was changed to serum-free for the last 24hrs to avoid serum interference with ELISA.

IL-6 (Fig. 5.1A) levels were significantly reduced after 10 days of treatment with metformin, myxothiazol, or rapamycin. By comparison, after 3 days of treatment none of the tested drugs were able to significantly reduce IL-6 levels. Although there is a definite downward trend for all three drugs, none reached statistical significance.

IL-8 (Fig. 5.1D) levels demonstrated a slightly different picture, with only metformin eliciting a significant reduction after 10 days of treatment. Myxothiazol, while it did demonstrate a clear reduction compared to untreated controls, was not statistically significant. This corresponds to my earlier findings that myxothiazol's effects on IL-8 secretion were not as strong as IL-6 or IP-10. However, the observation of any decrease in IL-8 with myxothiazol is in contrast to my earlier findings which showed no effect of myxothiazol on IL-8 levels, and so may suggest a technical error in my earlier ELISAs when also considering the cytokine array analysis of the same samples did demonstrate a reduction in IL-8. Interestingly, after 10 days of treatment with rapamycin IL-8 levels were significantly increased. This is in contrast to much of the literature which identifies IL-8 as rapamycin-sensitive (Lin *et al.*, 2014; Park *et al.*, 2020; Sasaki;Itakura and Toyoda, 2020), however other studies have also found a stimulatory effect of rapamycin on certain cytokines and chemokines (Lee *et al.*, 2013; Gillespie *et al.*, 2015), suggesting a more complicated relationship that first thought. In this study, elevated IL-8 secretion was reproducible across all independent experiments and appears to increase over time. None of the tested drugs were able to reduce IL-8 levels significantly after 3 days of treatment.

IP-10 (Fig. 5.1E) levels were significantly reduced after 10 days of treatment with metformin, myxothiazol or rapamycin. Additionally, 3 days of treatment with either metformin or rapamycin was also sufficient to reduce IP-10 levels. Myxothiazol treatment demonstrated a clear reduction after 3 days but this was not statistically significant. These findings suggest that IP-10 levels can be reduced by shorter treatment timepoints, however IP-10 measurements were more variable and generally harder to detect by ELISA due to low baseline levels. As such IP-10 data should be used to support inferences from SASP analysis based on the wider picture including IL-6 and IL-8, and not IP-10 alone.

Overall, here I found that in a model of established IR-induced senescence 10 days of treatment with metformin is required to significantly reduce secreted levels of IL-6, IL-8 and IP-10. Treatment with myxothiazol or rapamycin for 10 days was also sufficient to reduce levels of both IL-6 and IP-10, but rapamycin exacerbated IL-8 production by an unknown mechanism. Based on these findings it appears that 10 days of treatment would be an optimal starting point in cells which are already senescent. Further examination of longer treatment times may reveal a further time-dependent decrease in SASP.

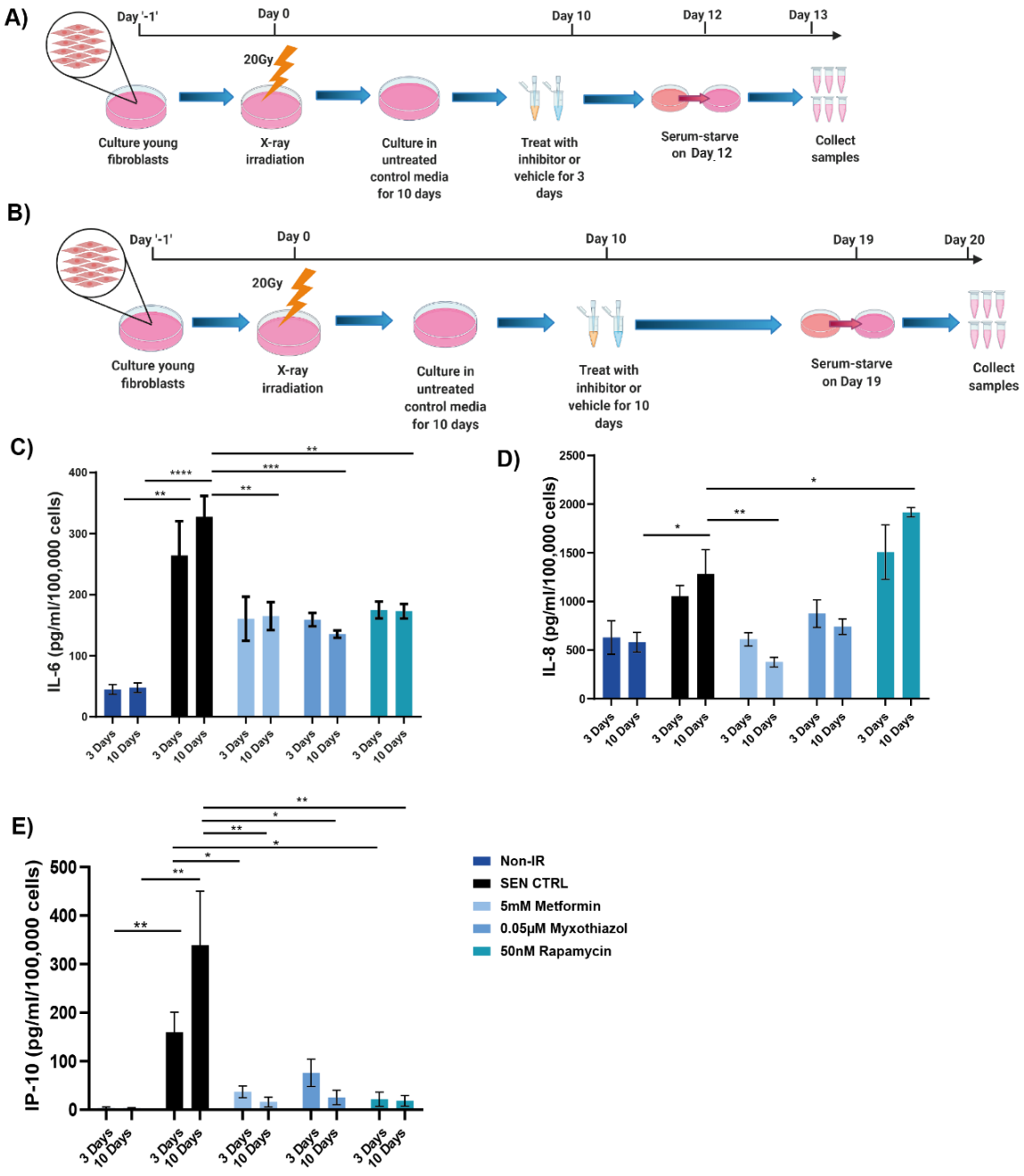

Figure 5.1: Treatment with metformin, myxothiazol, and rapamycin for 10 days in a model of established senescence reduces pro-inflammatory cytokine secretion in senescent cells. Levels of IL-6 (C), IL-8 (D) and IP-10 (E) secreted by young non-irradiated, untreated senescent, and senescent fibroblasts treated with metformin (5mM), myxothiazol (0.05µM), or rapamycin (50nM) for 3 (A) or 10 (B) days after the development of senescence (total 13 or 20 days post-irradiation, respectively), measured by ELISA. n=3. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.2 Treatment with metformin, myxothiazol or rapamycin for 10 days is not sufficient to rescue senescent markers in established senescence

I next wanted to examine the wider senescent phenotype in established senescence to determine whether treatments which successfully rescued SASP production could also alleviate additional markers of senescence. Here I found that 10 days of treatment with metformin, myxothiazol or rapamycin was not sufficient to reduce any of the tested senescent markers, confirming earlier preliminary findings that the SASP is more receptive to modification.

SA-β-Gal activity (**Figure *5.2***) remained high after both 3 and 10 days of treatment with all tested drugs, with the proportion of positively stained cells in all experiments above 80%.

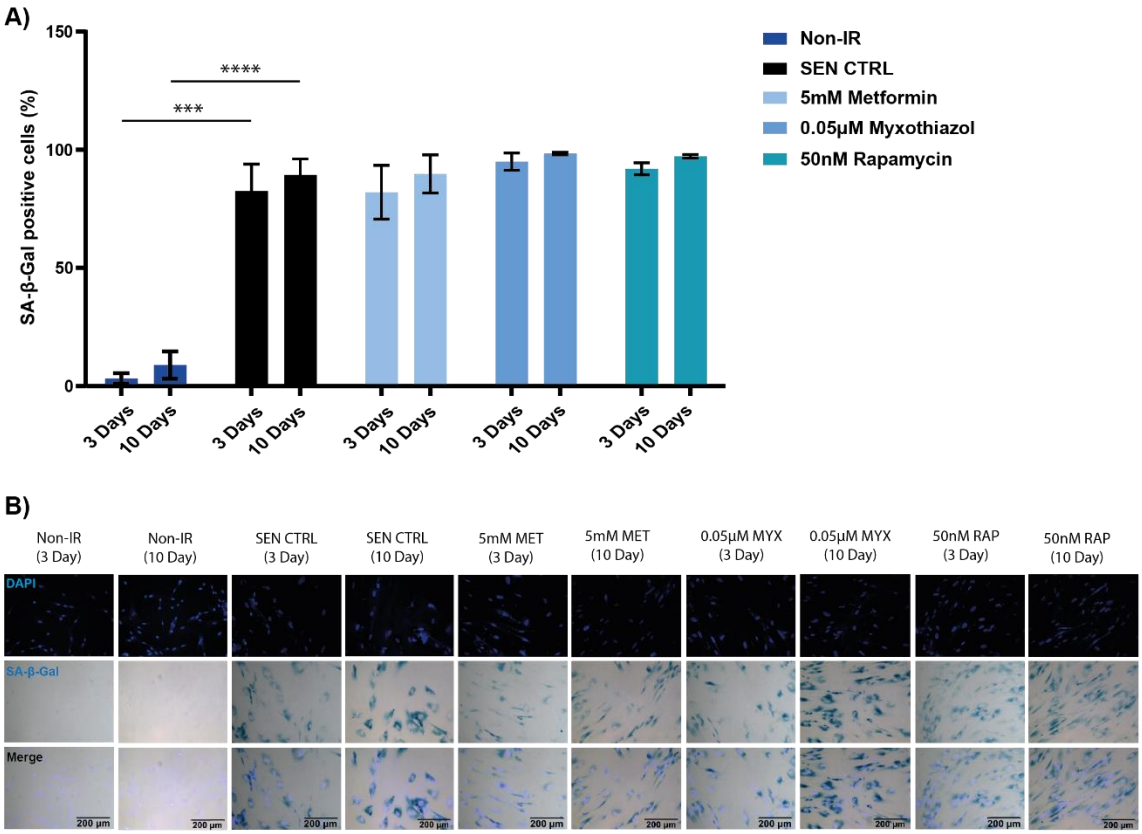

Figure 5.2: Treatment with metformin, myxothiazol, and rapamycin for 3 and 10 days in a model of established senescence does not affect SA-β-Gal activity. (A) Percentage of cells positive for SA-β-Gal in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM

rapamycin for 3 or 10 days after the development of senescence (total 13 or 20 days post-irradiation, respectively) identifiedby histochemical staining of SA-β-Gal, n=3. (B) Representative images of SA-β-Gal staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ***p<0.001, ****p<0.0001

DDF foci measured by immunostaining of γH2AX demonstrated no change after either 3 or 10 days of treatment with metformin, myxothiazol or rapamycin (**Figure *5.3***). As a result of one independent experiment with particularly high staining intensity across the board, the raw measurements of γH2AX foci per cell appear highly variable (Fig. 5.3A). However, when using a threshold of ≥3 foci to identify a cell as positive for γH2AX foci (Meier *et al.*, 2007), this variability was dramatically reduced in all but rapamycin conditions (Fig. 5.3B). This data shows that none of the tested drugs were able to influence DDF formation in established senescence.

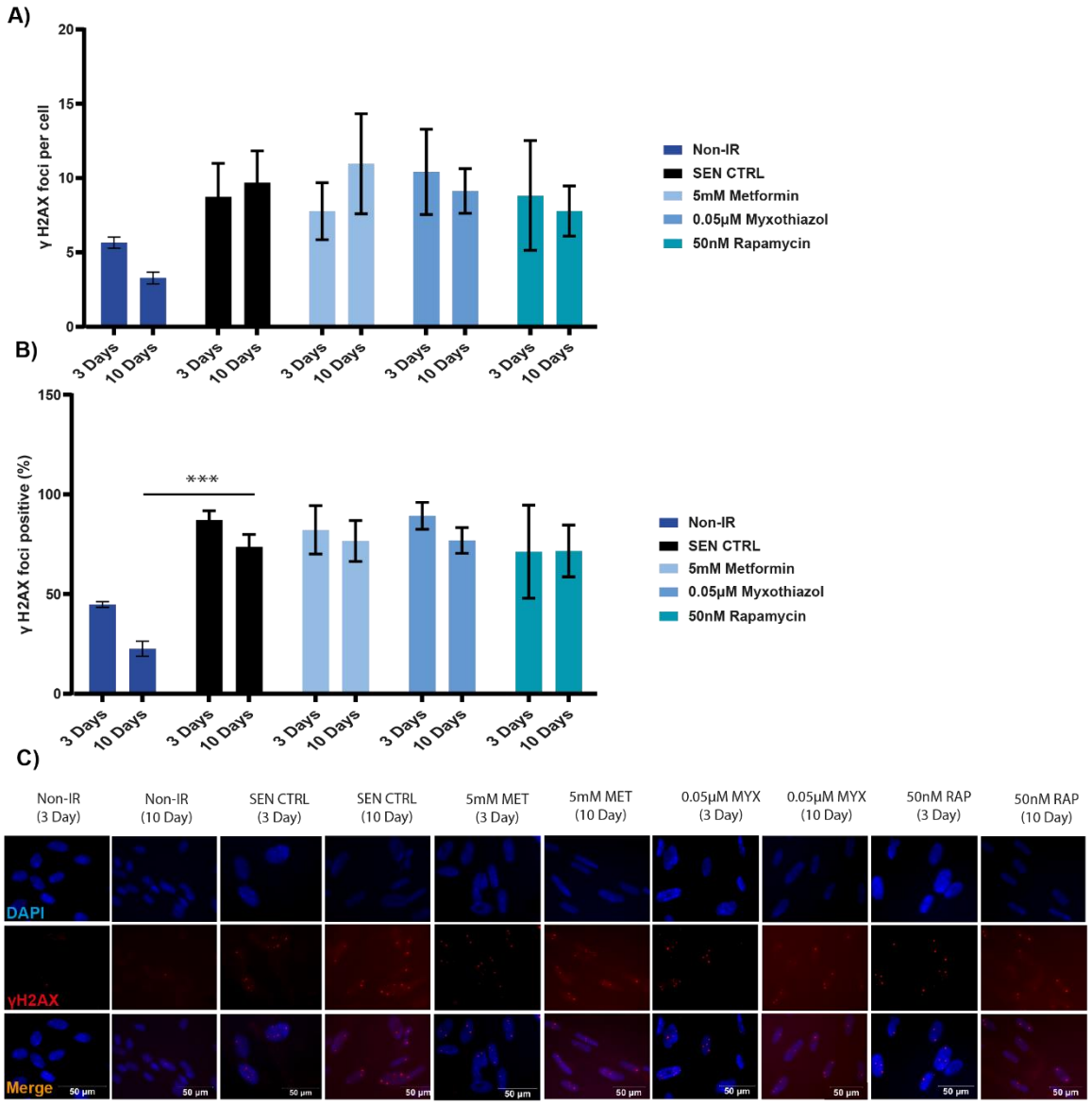

Figure 5.3: Treatment with metformin, myxothiazol, and rapamycin for 3 and 10 days in a model of established senescence does not affect formation of DNA damage foci. (A) Number of γH2AX foci per cell of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05μM myxothiazol, or 50nM rapamycin for 3or 10 days after the development of senescence (total 13 or 20 days post-irradiation, respectively) measured by immunofluorescent staining and automated ImageJ analysis, n=3. (B) Percentage of nuclei with 3 or more foci, considered 'positive' for γH2AX. (C) Representative images of immunofluorescent staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ***p<0.001

Nuclear size also remained unaffected by all three treatments after both 3 and 10 days (**Figure *5.4***). Despite 10 days of metformin treatment effectively preventing nuclear enlargement when given immediately post-IR (Chapter 4), it was unsuccessful in established senescence, suggesting that nuclear enlargement is irreversible after 10 days post-irradiation and unresponsive to treatment with metformin, myxothiazol or rapamycin.

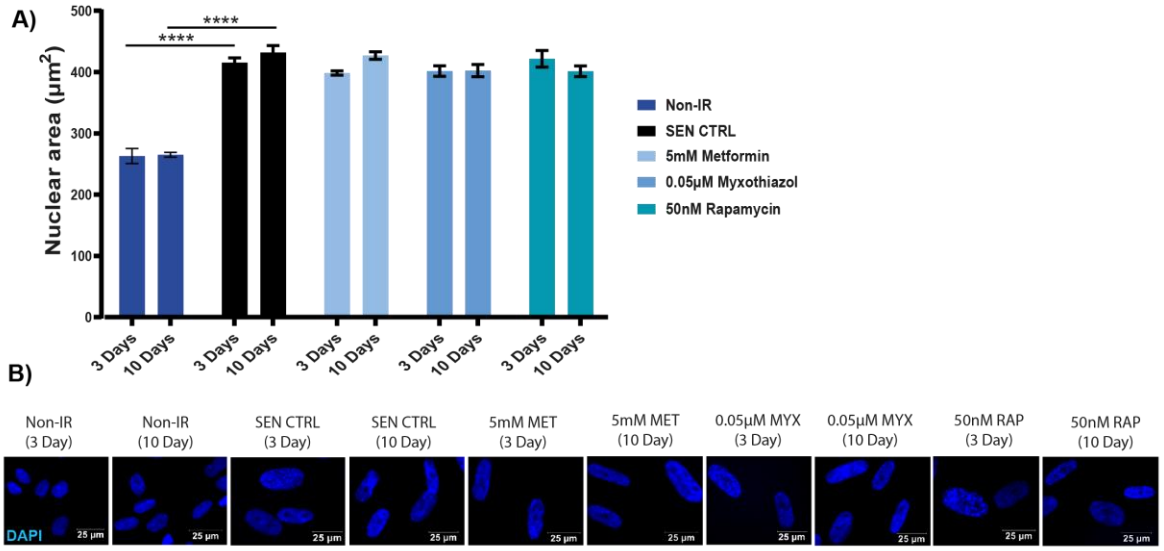

Figure 5.4: Treatment with metformin, myxothiazol, and rapamycin for 3 and 10 days in a model of established senescence does not affect nuclear size. (A) Nuclear area (μm^2) of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3 or 10 days after the development of senescence (total 13 and 20 days post-irradiation, respectively) measured by ImageJ analysis of DAPI immunostaining, n=3. (B) Representative images of DAPI staining in the above conditions showing difference in nuclear size. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ****p<0.0001

The proportion of cells expressing p21 (**Figure *5.5***) was not impacted by either 3 or 10 days of treatment with any of the three drugs (Fig. 5.5A), remaining above 80% in all senescent conditions. This trend was also replicated when examining immunofluorescent staining intensity for p21 (Fig. 5.5B). While a mild trend towards reduced staining intensity is apparent after 10 days of treatment with myxothiazol or rapamycin, this is not statistically significant. Nevertheless, it may warrant further study with longer treatment times to determine whether this trend continues. The finding that p21 expression cannot be rescued by treatment with these three drugs in established senescence is unsurprising, as earlier findings in a developing senescent phenotype showed no change when metformin was administered from the point of senescence induction.

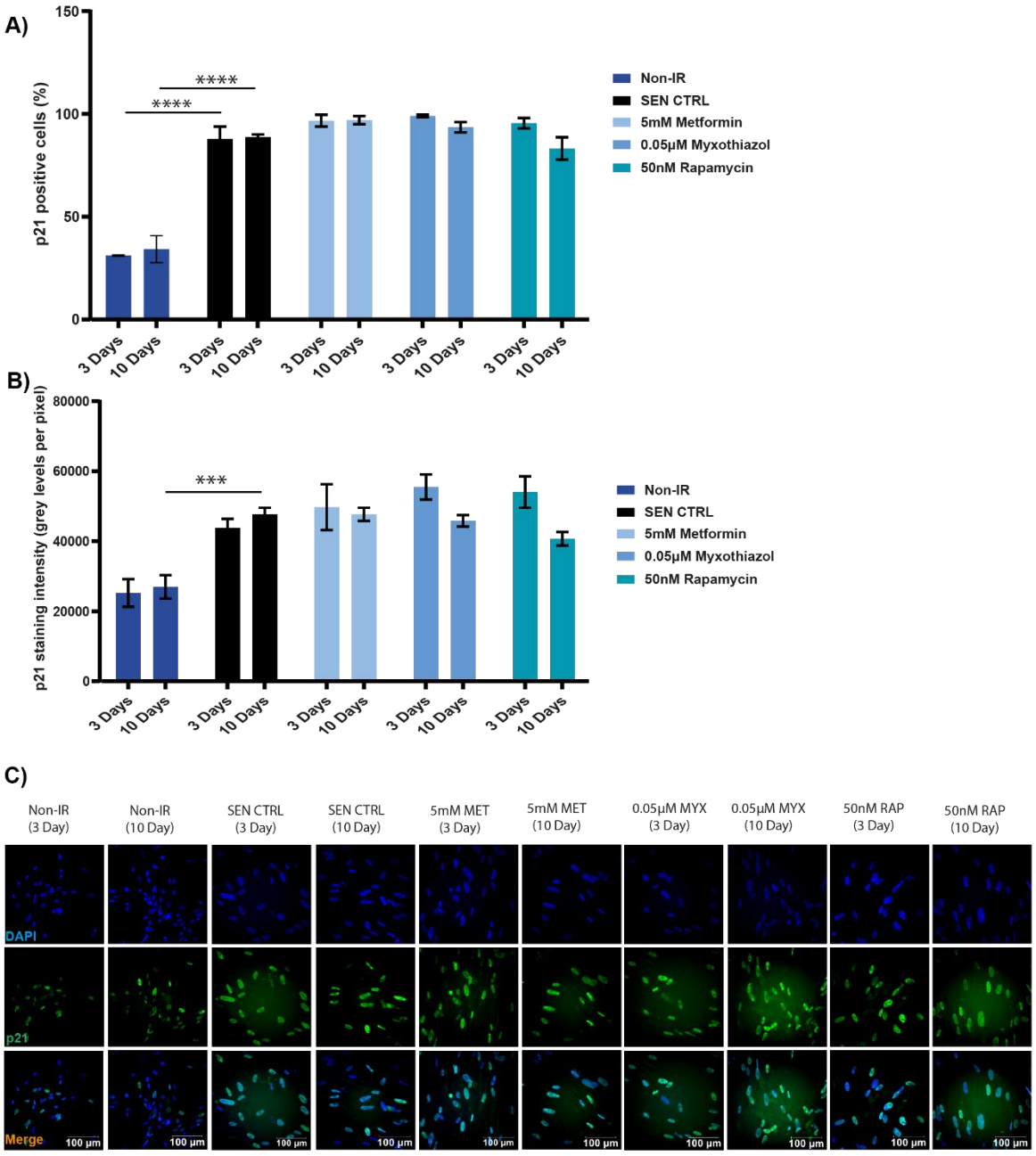

Figure 5.5: Treatment with metformin, myxothiazol, and rapamycin for 3 and 10 days in a model of established senescence does not affect p21 expression. (A) Percentage of cells positive for p21 in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3or 10 days after the development of senescence (total 13 or 20 days post-irradiation, respectively) measured by immunofluorescent staining and automated ImageJ analysis, n=3. (B) p21 staining intensity measured by automated ImageJ quantification. (C) Representative images of immunofluorescent staining in the above

conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ***p<0.001, ****p<0.0001

Senescent growth arrest was also maintained after treatment with metformin, myxothiazol and rapamycin for 3 and 10 days (**Figure *5.6***). Ki67-expressing cells remained low in all senescent cultures, significantly reduced compared to young control cells. This was expected given the lack of effect of metformin when treatment was initiated at the point of senescence induction.

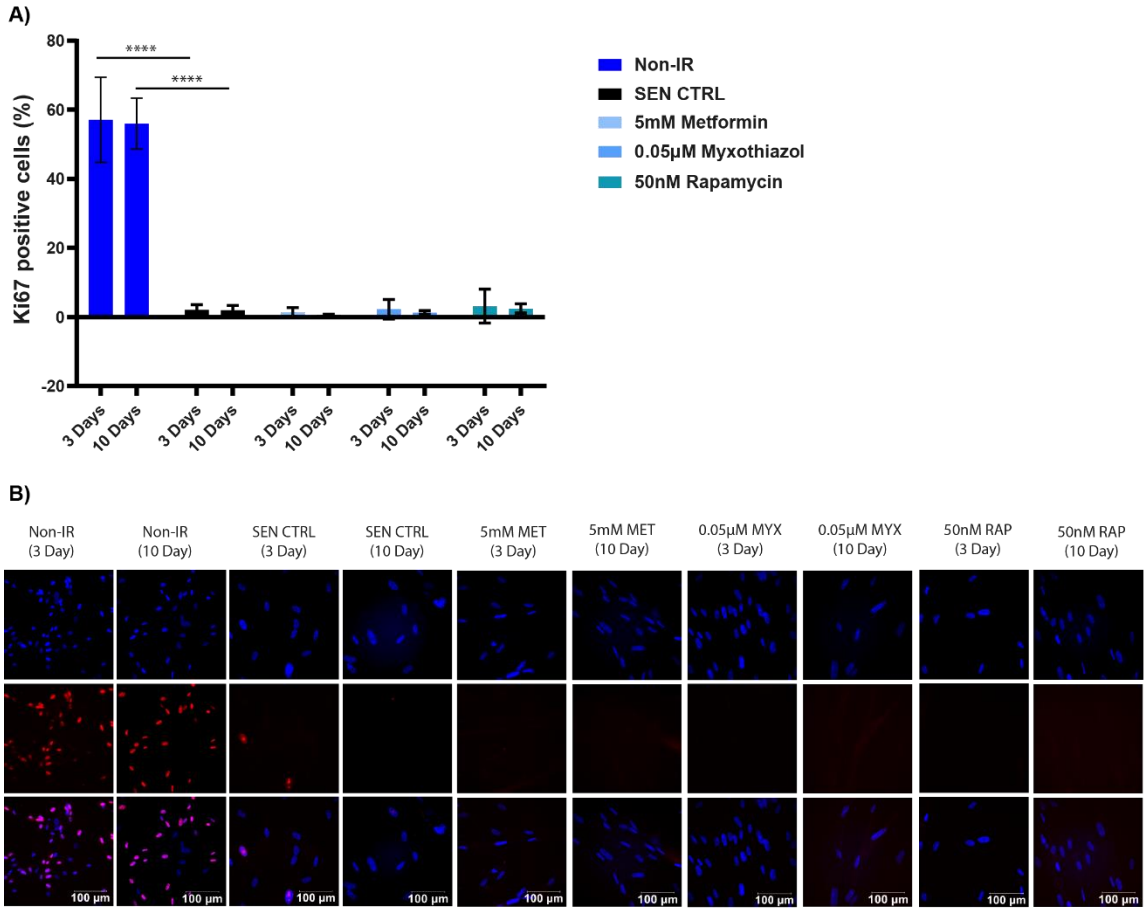

Figure 5.6: Treatment with metformin, myxothiazol, and rapamycin for 3 and 10 days in a model of established senescence does not affect cell proliferation. (A) Percentage of cells positive for Ki67 in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3 or 10 days after the development of senescence (total 13 or 20 days post-irradiation, respectively) identified by immunofluorescent staining for Ki67, n=3. (B)

Representative images of Ki67 staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ****p<0.0001

Based on the data presented here these senescent markers appear to be refractory to treatment in established senescence, though other treatments, concentrations or durations cannot be ruled out. While I have shown here that none of the tested treatments were able to substantially rescue markers of senescence at the doses and treatment lengths tested, this does provide a promising indication that SASP reduction can be achieved without restoring cell-cycle progression to senescent cells.

5.3 Treatment with myxothiazol for 3 days post-irradiation is sufficient to reduce the SASP

My next question was whether a shorter treatment duration of 3 days could also be effective in alleviating harmful aspects of senescence if given at the point of senescence induction (**Figure *5.7***). Cells were treated for 3 days post-IR, followed by untreated control media for the remaining 7 days of senescence development (Fig. 5.7A). This allowed me to determine whether 3 days of treatment with either metformin, myxothiazol or rapamycin was sufficient to prevent the development of a SASP (Fig. 5.7B).

After 3 days of treatment with metformin, none of the measured SASP factors (IL-6, IL-8 and IP-10) were significantly reduced in senescent cells. There was great variability in IL-8 and IP-10 secreted levels, and IP-10 even appeared to increase after 3 days of metformin treatment, though as mentioned previously IP-10 measurements were more difficult to obtain and were generally more variable. By comparison, treatment with myxothiazol for 3 days reduced the secreted levels of all three cytokines, though only IL-6 significantly. Rapamycin treatment also significantly reduced IL-6 levels after 3 days of treatment, and showed a mild reduction in IP-10 though this was not statistically significant.

Due to the variability between experiments I examined this data by normalizing each dataset to the corresponding untreated senescent control to try to remove any inter-experiment variation (Fig. 5.7C). This also allowed me to look at the relationship change between each drug and untreated senescent cells. This revealed a much clearer picture of SASP suppression. Here I was able to see that myxothiazol treatment significantly reduced all three SASP factors, rapamycin inhibited IL-6 and IP-10, and metformin did not affect IL-6 or IL-8 but increased IP-10 secretion. Rapamycin again caused an increase in IL-8 secretion. Overall this is a promising indication that shorter treatment times – particularly with myxothiazol - may be successful at reducing the SASP, and suggests that prevention of the initial 'burst' of activity following a severe senescent trigger may be sufficient to prevent the development of a SASP.

That myxothiazol was able to reduce secretion of all three cytokines significantly after 3 days of treatment while metformin did not offer an interesting point of comparison. While both drugs were able to suppress the SASP after 10 days of treatment post-IR (Fig. 5.7C),

myxothiazol appears to have the stronger – or faster – effect. To dissect the possible reason for this, I tested the effects of rotenone treatment for the same length of time on SASP factor secretion (Fig. 5.7B) to compare the effects of metformin with another CI inhibitor. This analysis revealed that 3 days of rotenone treatment actually increased IL-6 secretion (though proportional change was not significant), and had no effect on either IL-8 or IP-10 levels. This suggests that the difference in effectiveness between metformin and myxothiazol may be a site-specific effect, as both CI inhibitors were unable to impact SASP production after 3 days. Further research into this difference is needed, and is discussed later.

Comparison of the effects of these drugs after 3 (Fig. 5.7B-C) and 10 days (Fig. 5.7D) of treatment (with the exception of rapamycin which was not included in earlier stages of this project and so does not have 10 day data) demonstrates a clear time-dependent suppression of SASP with rotenone, metformin and myxothiazol. Further study is warranted to determine the minimum required intervention for SASP suppression. 3 days may not be sufficient to fully suppress the SASP with these drugs, but it is reasonable to suggest that 4, 5, or 6 days may have greater effect and allow shorter exposure time of cells to drugs such as rotenone or myxothiazol and minimize potential side effects.

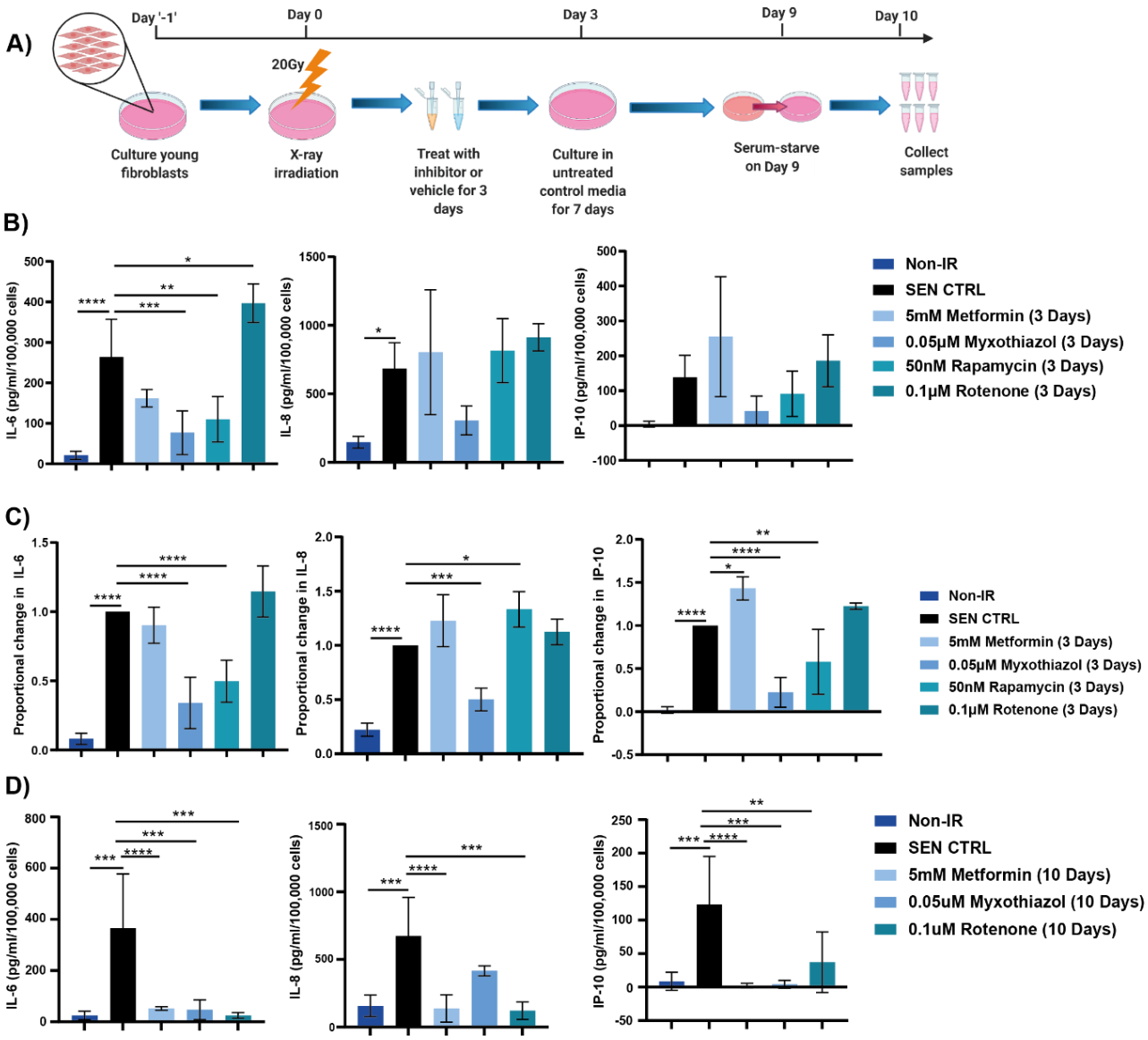

Figure 5.7: Treatment with myxothiazol for 3 days post-irradiation is sufficient to reduce pro-inflammatory cytokine secretion in senescent fibroblasts. (A) Schematic of experimental design; cells were seeded to plates and x-ray irradiated at 20Gy the following day, then given drug-treated or control media immediately post-IR, before reverting to untreated media for all conditions on day 3 and for the remaining 7 days of senescence development. (B) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, 50nM rapamycin or 0.1µM rotenone for 3 days immediately post-IR, measured by ELISA, n=3.Data are mean±S.E.M. (C) Cytokine data taken from (B) and normalised to independent senescent control values for each experiment, (D) Levels of IL-6, IL-8 and IP-10 secreted by young non-irradiated, untreated senescent, and senescent

fibroblasts treated with 5mM metformin, 0.05µM myxothiazol or 0.1µM rotenone for 10 days post-IR as shown in Fig.4.4, 3.5 and 3.2, respectively, conducted as described in Fig.3.1, measured by ELISA, n=2-4. Data are mean±S.D. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.001, ***p<0.001, ****p<0.0001

5.4 Treatment with electron transport inhibitors for 3 days post-irradiation is not sufficient to rescue senescent marker expression

Based on these findings of successful SASP suppression, I next wanted to determine whether 3 days of treatment with metformin, myxothiazol or rapamycin was sufficient to alleviate any of the previously tested senescent markers, to help identify the minimum effective intervention.

The proportion of cells positively stained for SA-β-Gal was unaffected by 3 days of treatment with any of the tested drugs (**Figure *5.8***). However, a noticeable decrease in the intensity of SA-β-Gal staining was evident with rapamycin treatment, though not enough to be considered negative for SA-β-Gal activity. A ~25% reduction in SA-β-Gal-positive cells was seen with rapamycin, but this was not statistically significant. Previous studies have found a complete reduction in SA-β-Gal activity after 10 days with rapamycin treatment (Correia-Melo *et al.*, 2016; Park *et al.*, 2020), and so this could suggest an optimum minimum treatment time somewhere between 3 and 10 days. The difference observed by eye seems to suggest that rapamycin caused a faster decline in SA-β-Gal activity than metformin or myxothiazol, and so further study is warranted into additional treatment timepoints.

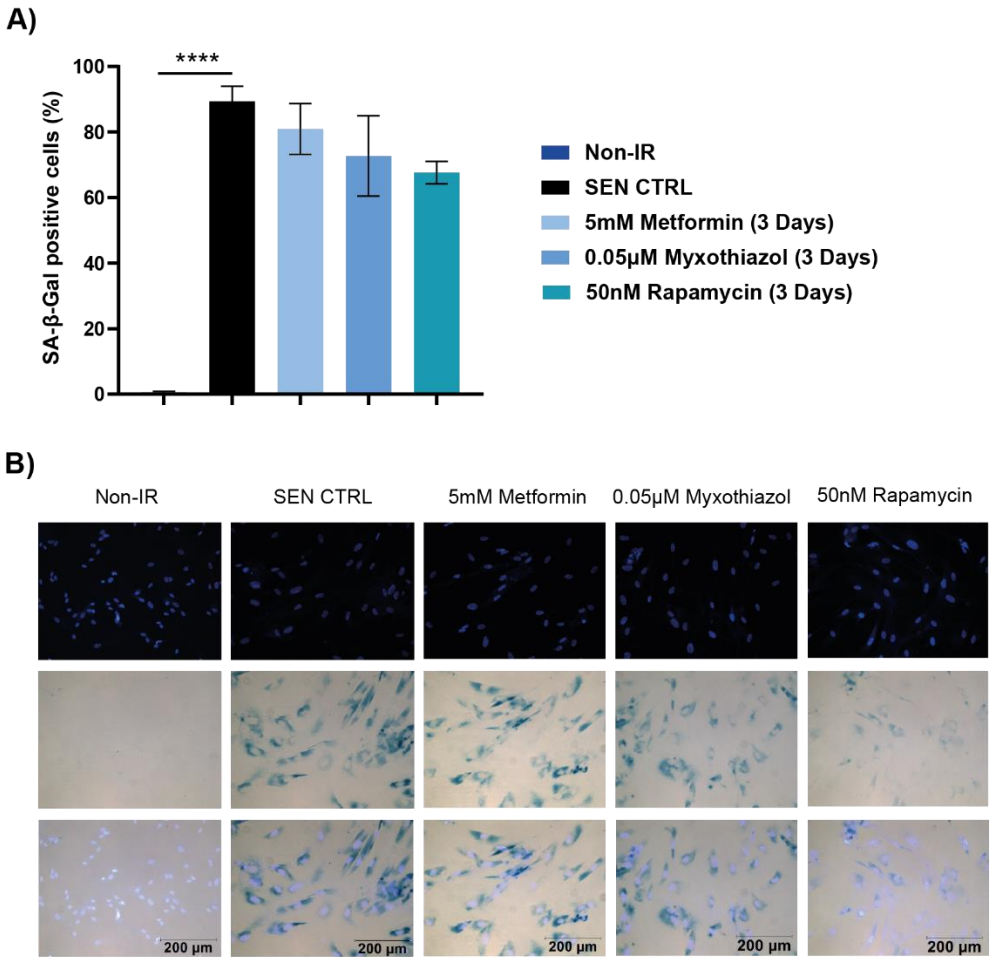

Figure 5.8: Treatment with metformin, myxothiazol, and rapamycin for 3 days post-irradiation does not affect SA-β-Gal activity. (A) Percentage of cells positive for SA-β-Gal in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05μM myxothiazol, or 50nM rapamycin for 3 days after irradiation, identified by histochemical staining of SA-β-Gal, n=3. (B) Representative images of SA-β-Gal staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ***p<0.001

The number of γH2AX foci present in senescent cells treated with metformin, myxothiazol, or rapamycin for 3 days did not change dramatically (**Figure *5.9***), although raw foci numbers were variable between experiments (Fig. 5.9A). This was largely a result of one independent experiment producing particularly high staining intensities across the board. Additional experiments may help to reduce this variability. To help improve the variability of the current data I again used a threshold of 3 or more foci to determine cells 'positive' for γH2AX, which revealed a much more reproducible picture of DDF distribution (Fig. 5.9B). Overall the proportion of cells which were positive for γH2AX foci remained high in cells treated with metformin, myxothiazol, or rapamycin for 3 days post-IR. This suggests that 3 days is not sufficient to prevent the formation of DDFs.

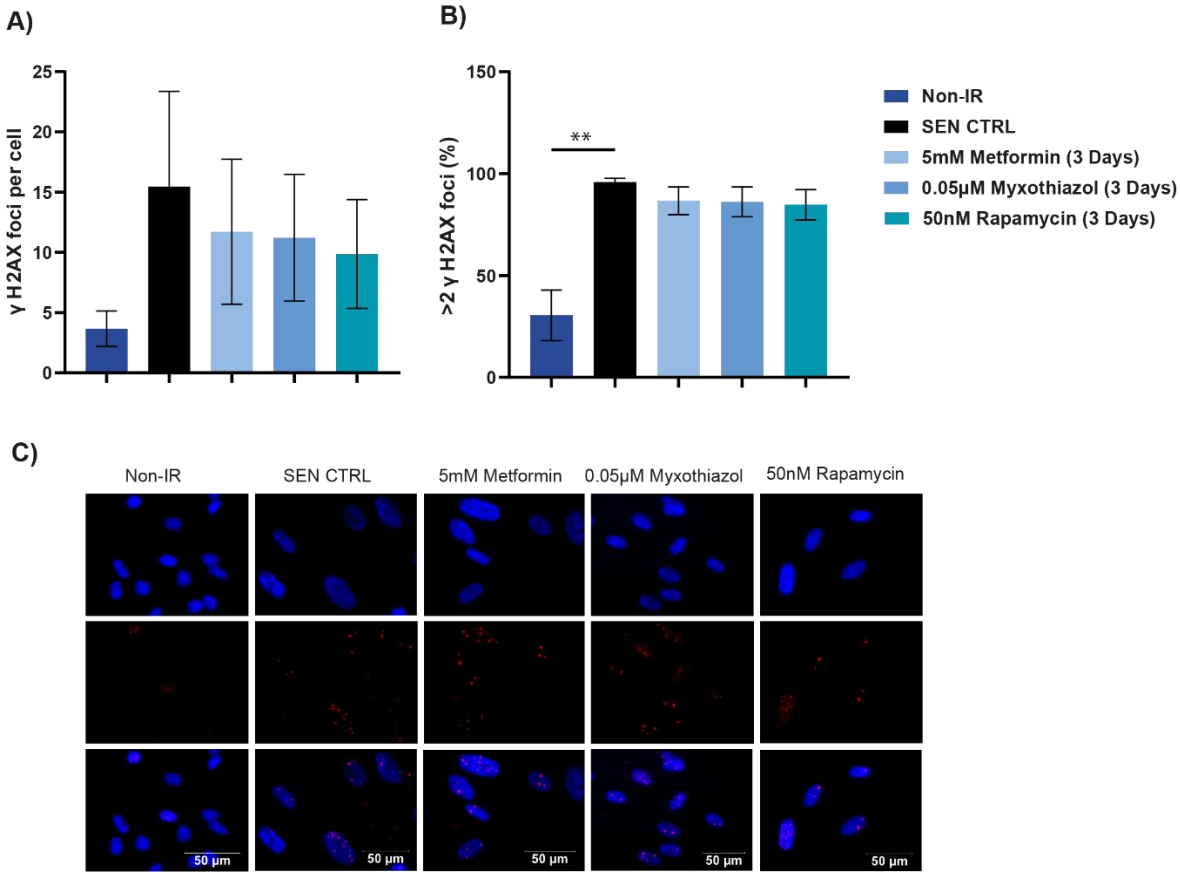

Figure 5.9: Treatment with metformin, myxothiazol, and rapamycin for 3 days post-irradiation does not affect DNA damage foci formation. (A) Number of γH2AX foci per cell in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05μM myxothiazol, or 50nM rapamycin for 3 days after irradiation, identified by immunofluorescent staining, n=3. (B) Percentage of cells with 3

or more γH2AX foci and therefore considered 'positive' for γH2AX foci, n=3. (C) Representative images of γH2AX staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. **p<0.01

Nuclear size also remained unchanged after 3 days of treatment with metformin, myxothiazol and rapamycin (**Figure *5.10***). This again suggests that prevention of nuclear enlargement requires a longer treatment time than 3 days. Additional work exploring later timepoints of treatment may reveal the minimum effective treatment time required to reduce nuclear size in senescence.

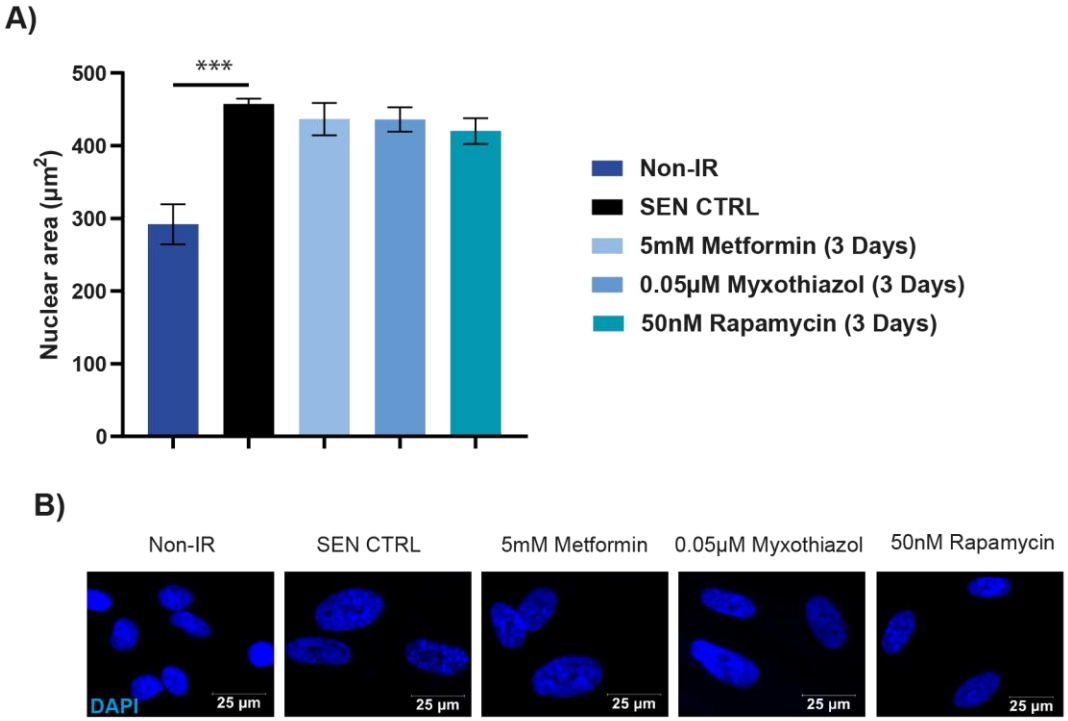

Figure 5.10: Treatment with metformin, myxothiazol, and rapamycin for 3 days post-irradiation does not affect nuclear size.(A) Nuclear area (µm^2) of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3 days after irradiation, measured by ImageJ analysis of DAPI immunostaining, n=3.(C) Representative images of DAPI staining in the above conditions showing the difference in nuclear size. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ***p<0.001

Cells positive for p21 expression did not respond to 3 days of treatment with either metformin, myxothiazol or rapamycin (**Figure *5.11***). Staining intensity demonstrated wide variability (Fig. 5.11A), largely a result of the same independent experiment as described above exhibiting higher staining intensities across the board. By using staining intensity thresholds for each experiment to identify cells 'positive' for p21 staining I was able to determine a more reproducible picture of drug effects on p21 expression (Fig. 5.11B). Importantly, while staining intensity of some NI cells was particularly high, the number of cells which exhibited this intensity was much lower compared to senescent cells. Here I found that 3 days of treatment with any of the drugs tested was not sufficient to influence p21 expression. For metformin this is unsurprising as 10 days of treatment was also ineffective as detailed in Chapter 4.

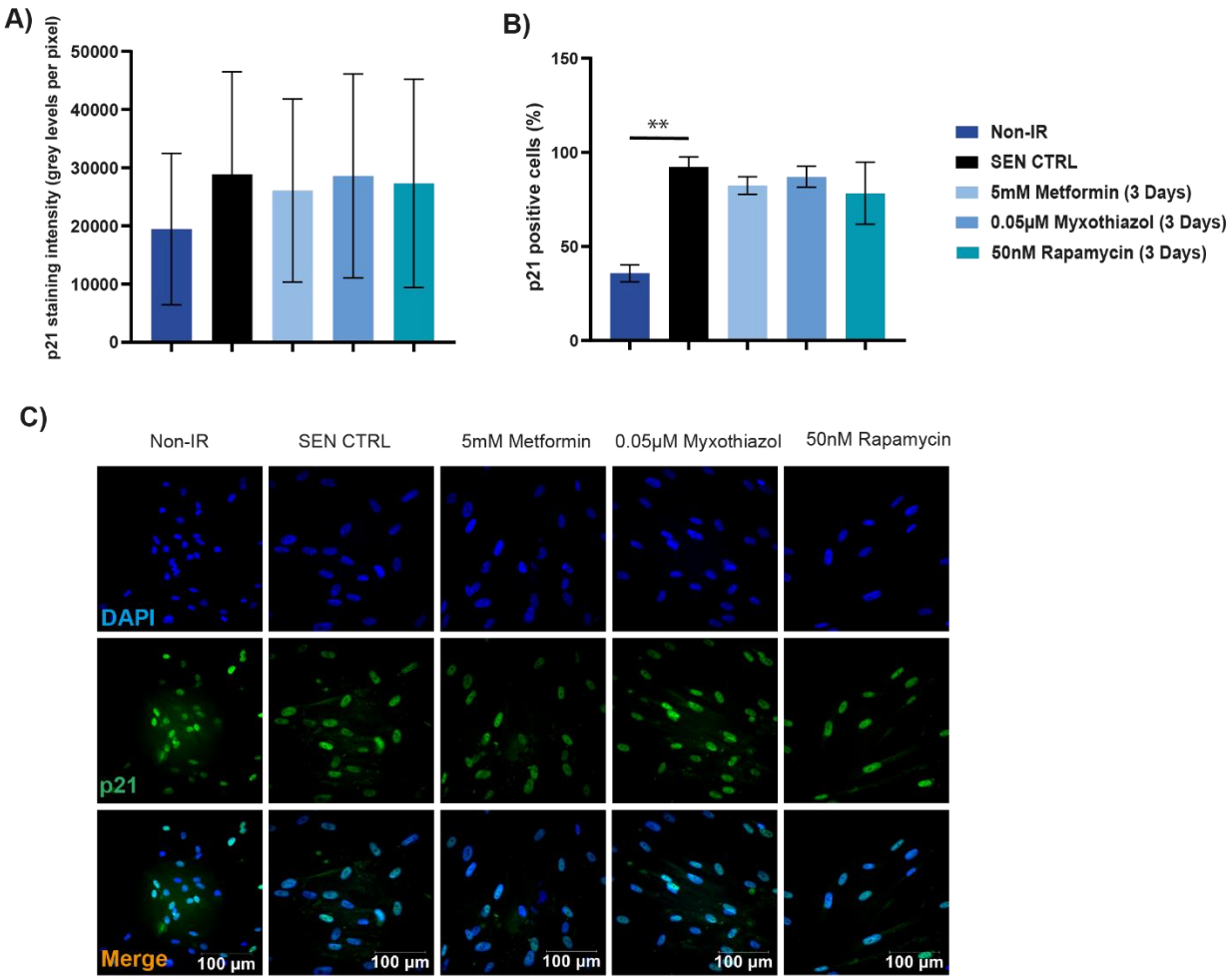

Figure 5.11: Treatment with metformin, myxothiazol, and rapamycin for 3 days post-irradiation does not affect p21 expression. (A) p21 staining intensity in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3 days after irradiation, identified by immunofluorescent staining, n=3. (B) Percentage of cells positive for p21 staining, n=3. (C) Representative images of p21 staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. **p<0.01

Finally, Ki67-positive cells were also unaffected by treatment with any of the three drugs (**Figure *5.12***). As before this was expected for metformin as 10 days of treatment was also unable to change Ki67-positive cell proportions. Here I show that neither metformin, myxothiazol nor rapamycin are able to influence cell proliferation in senescent cells treated for 3 days post-IR.

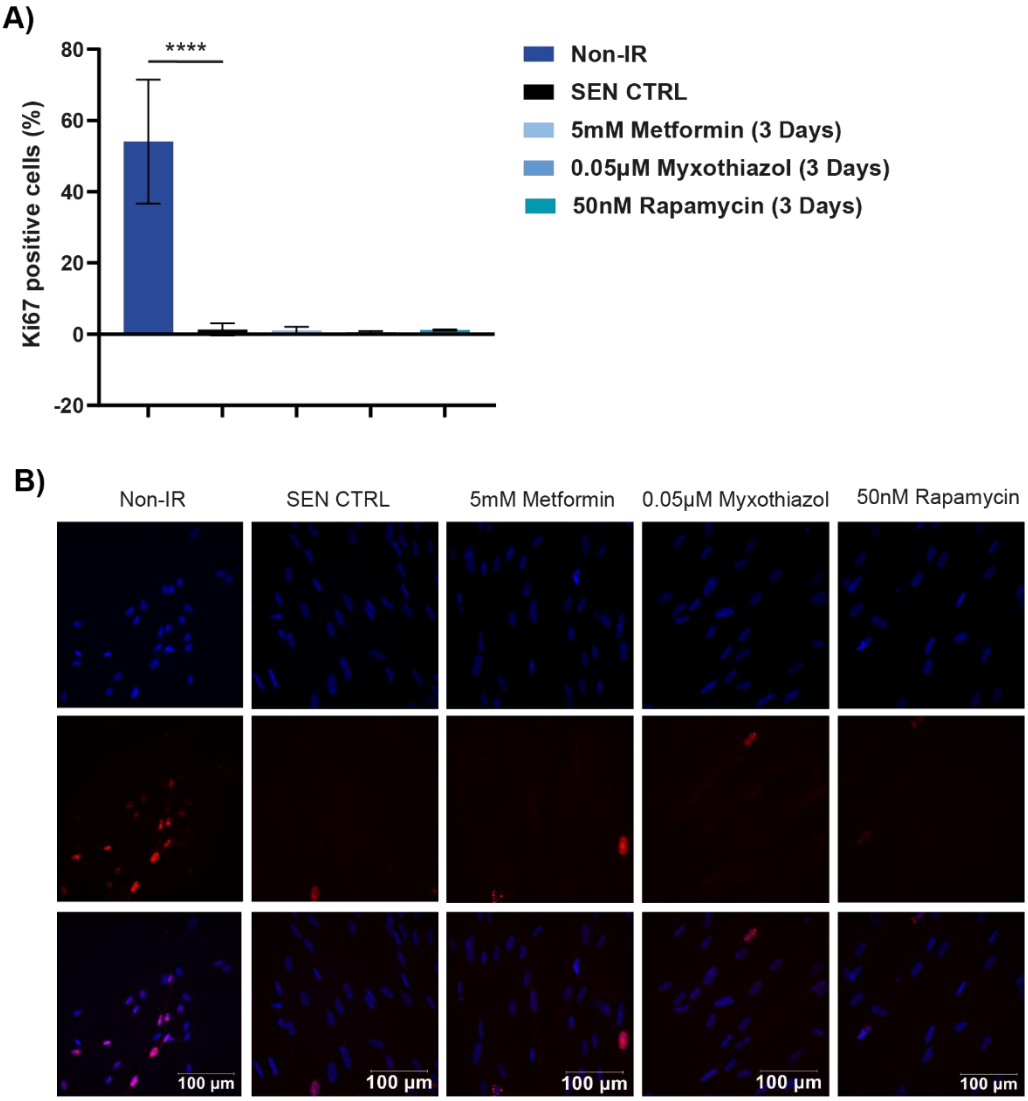

Figure 5.12: Treatment with metformin, myxothiazol, and rapamycin for 3 days post-irradiation does not affect cell proliferation. (A) Percentage of cells positive for Ki67 in cultures of young non-irradiated, untreated senescent, and senescent fibroblasts treated with 5mM metformin, 0.05µM myxothiazol, or 50nM rapamycin for 3 days after irradiation, identified by immunofluorescent staining for Ki67, n=3. (B) Representative images of Ki67 staining in the above conditions. Data was analysed using one-way ANOVA and Tukey's multiple comparisons test. Data are mean±S.E.M. ****p<0.0001

Overall I have shown that SASP factor secretion can be reduced by 3 days of treatment post-IR with myxothiazol and rapamycin to some degree. This is a promising indication that shorter treatment timelines with these drugs may be sufficient to alleviate the SASP. By comparison metformin and rotenone were both unable to reduce SASP after 3 days. Furthermore, SASP factor secretion can also be successfully reduced in an established senescent phenotype by 10 days of treatment with metformin, myxothiazol and rapamycin, with the exception of IL-8 after rapamycin treatment.

However, while 3 days of treatment appears to be the starting point for effective SASP suppression, it is clear that other senescent markers either cannot be reverted by these drugs or require longer treatment times. Findings in Chapter 4 for metformin treatment would suggest longer treatment times are sufficient in this case, but longer durations require testing for myxothiazol and rapamycin. p21 and Ki67 were unaffected by treatment with any of the tested inhibitors for 3 days post-IR (and 10 days in metformin treatment), as well as 3 and 10 days of treatment after the development of a senescent phenotype. By comparison, SA-β-Gal activity, DDF formation, and nuclear enlargement were all significantly reduced by 10 days of metformin treatment post-IR (Chapter 4), but not 3 days. 3 days was also insufficient treatment with myxothiazol and rapamycin to impact these markers, and no measured marker responded to treatment in an established senescent phenotype.

Therefore, the SASP appears to be an aspect of the senescent phenotype that is more receptive to manipulation, even when the phenotype is perceivably ‘locked in’ and other features are irreversible.

5.5 Discussion

In this chapter I have provided evidence that the timing of senescent cell treatment is an important determining factor in alleviating both the SASP and essential senescent markers. Perhaps unsurprisingly, treatment is more effective when administered at the point of senescence induction compared to a model of established senescence. This is particularly true for the reduction of senescent markers such as SA-β-Gal activity, DDF formation and nuclear enlargement. These markers were unaffected by both shorter treatment times during senescence development, and treatment in established senescence. Only after 10 days of metformin treatment given at the point of senescence induction were any markers other than SASP significantly affected, as shown in Chapter 4. Additionally, while 3 days of treatment with myxothiazol and rapamycin was sufficient to reduce SASP factor secretion when given immediately post-IR, this shorter timeline was not effective in an established senescent phenotype, suggesting that the SASP is more receptive to treatment earlier in the senescent timeline. This may indicate that it is easier to prevent the development of a senescent phenotype than reverse it, which would not be unexpected given previous reports of reduced treatment efficacy at points later than 9 days after senescence induction (Passos *et al.*, 2010), though other studies have demonstrated the reversibility of some senescent phenotypes in replicative senescence (Matsushita *et al.*, 2001; Chen *et al.*, 2002).

In a model of established IR-induced senescence, all three tested drugs alleviated at least two of the three measured SASP factors, showing that the SASP can be reduced even after the phenotype is 'locked in'. This correlates with previous work which has shown that the SASP can be modulated *in vivo* in aged mice (De Cecco *et al.*, 2019), and *in vitro* through a number of mechanisms in established senescence (Xu *et al.*, 2015; Dou *et al.*, 2017). Additionally, work by both Dou et al (2017) and Xu et al (2015) found that interventions that were able to suppress SASP did not necessarily have an effect on other senescent markers, again demonstrating a separation of these two aspects of the senescent phenotype. This is nevertheless a promising indication for future work in both animal and human studies, as it demonstrates that the SASP specifically can be reduced in a model closer to normal physiological circumstances, in which cells exist as a mosaic across a spectrum of senescence progression.

These findings suggest that the tested drugs are unable to reduce lysosomal activity, based on consistently high SA-β-Gal activity observed in all conditions. As SA-β-Gal activity was significantly reduced by metformin treatment when administered at the point of senescence induction, this also suggests that SA-β-Gal activity may form part of the 'locked in' senescent phenotype and become resistant to manipulation at some point in the first 10 days of senescence progression. This is also true of DDFs, though this was expected as continual stimulation of DDFs is no longer required to maintain senescence at timepoints later than 9 days, as the phenotype stabilises and DDFs become persistent. At this point interruption of DDR signalling is less efficient in alleviating the phenotype (Passos *et al.*, 2010).

Although the treatments tested here were unable to alleviate senescent markers such as DNA damage foci or nuclear enlargement in established senescence, the reversibility of these markers has been shown elsewhere (Matsushita *et al.*, 2001; Chen *et al.*, 2002). Rapamycin in particular is known to be an effective senostatic when treated over longer durations than tested here (Correia-Melo *et al.*, 2016). mTORC1 inhibition is known to stimulate autophagy resulting in increased mitochondrial clearance and reduced OXPHOS activity. This may therefore suggest a similar effect to the previously tested ETC inhibitors, with greater reliance on glycolysis. Further study would be needed to examine this.

Furthermore, while 3 days of treatment in established senescence was not sufficient to elicit a significant decrease in SASP factors with any of the tested drugs, a downward trend is clear, suggesting that treatment between 3 and 10 days may provide the minimum effective intervention for SASP suppression in this model.

As stated, while SASP factor secretion was receptive to treatment in established senescence, this was not the case for any of the measured senescent markers. No significant differences were seen in SA-β-Gal activity, DDF formation, nuclear size, p21 expression, or cell proliferation with either metformin, myxothiazol or rapamycin treatment. This specific inhibition of SASP production alone points to a mechanism that either impacts cells ability to produce pro-inflammatory cytokines, or promotes their clearance, rather than a manipulation of one of the core senescent signalling pathways which would allow re-entry into the cell cycle and alleviation of DDR signalling and DDF formation. Though this would require further study to confirm. Additionally, it seems clear

that alleviation of the SASP alone is not sufficient to 'break' the positive feedback loops between SASP production, DDR signalling and ROS production in maintenance of the senescent phenotype.

In this study the findings of time-dependent SASP and senescent marker suppression are primarily demonstrated by metformin treatment immediately post-IR, in which 3 days is insufficient to modify SASP and senescent markers, but 10 days is effective. Reduction in IL-6 and IP-10 levels with rapamycin after 3 days may suggest a faster efficacy, and 10 days of treatment warrants investigation. This is in agreement with previous reports which have shown that rapamycin promotes SASP factor degradation by activating the RNA-binding protein ZFP36L1 (Herranz *et al.*, 2015). Studies have demonstrated its effectiveness at reducing markers of senescence in a similar model when treated for 10 days (Correia-Melo *et al.*, 2016).

Furthermore, based on successful SASP suppression by myxothiazol after 3 and 10 days post-IR, further work may determine whether myxothiazol treatment could also alleviate other markers of senescence if treated for longer than 3 days. To my knowledge CIII inhibition, either by pharmacological (Stockl *et al.*, 2006) or genetic manipulation (Moiseeva *et al.*, 2009), has to date only been studied as an inducer of senescence, and not as a potential therapeutic target, likely due to the lack of available CIII inhibitors with an acceptable safety profile in humans.

I have also shown here that the time-dependent decrease in SASP suppression with rotenone, metformin and myxothiazol varies between treatments. When drugs were administered at the point of senescence induction, all three were able to significantly suppress SASP factor secretion after 10 days of treatment as seen in Chapter 3. By comparison, only myxothiazol reduced the SASP after 3 days. I have not included rapamycin in this assessment as it was not measured at 10 days. This suggests that myxothiazol requires a shorter period of time to elicit a change in the senescent phenotype. The reason for this is unclear, and could stem from myxothiazol's site of inhibition, effect on ROS production, or another unknown mechanism. While the findings of Chapter 3 provided implications for OCR inhibition in SASP suppression, the specific timeline of these effects are yet to be elucidated. Despite comparable effects on OCR, ROS, and SASP after 10 days post-IR by metformin and myxothiazol, here we see that

myxothiazol's impact on SASP is much faster than metformin. One possible explanation is that CI inhibition by metformin still allows electron transfer to occur downstream with electrons supplied by CII-linked substrates or fatty acid oxidation, while inhibition of CIII interrupts electron flow from all sources as well as functions such as iron-sulphur cluster or pyrimidine synthesis (Khutornenko *et al.*, 2010).

As CIII inhibition has not been explored as a potential therapeutic target in senescence there is little literature to base conclusions on, but it is possible that this difference between CI and CIII inhibitors relates to a site-specific mechanism, perhaps ROS-dependent. However, confirming site-specific ROS production as a determining factor is difficult in whole cell studies, as the activities and ROS production rates of respiratory complexes can vary dramatically based on substrate availability, cell stress responses, and culture conditions. Data presented here by S1QEL and S3QEL treatments aimed at suppressing site-specific ROS production does not support a ROS-dependent difference between rotenone and myxothiazol effects, though these experiments require further confirmation with direct ROS measurements in isolated mitochondria to confirm effects on mitochondrial ROS at the concentrations used. Additionally, intact cells are able to swiftly switch to glycolysis reliance in response to stressful conditions, whether this be respiratory complex inhibition or downregulation, or simply a lack of available substrates (Vander Heiden;Cantley and Thompson, 2009; Heiss *et al.*, 2014; Rafikov *et al.*, 2015). This in turn induces an overall decline in ROS production resulting from downregulated ETC activity, confounding attempts to accurately measure the contribution of individual ROS-producing sites.(Lee and Yoon, 2015)

Further work is needed to examine this difference in effect between CI and CIII inhibitors in SASP suppression. This could include the use of isolated mitochondria to confirm inhibitor effects on respiratory complex activity and ROS production without the confounding influences of non-mitochondrial dehydrogenases or endogenous substrates, and measurement of NAD+ and NADH levels in senescence and in response to specific complex inhibition. It could also be important to evaluate the specific enzymatic inhibition of these drugs, as it is not known to what degree each respiratory complex is inhibited by the tested drugs in order to produce the observed reductions in OCR, ROS and SASP production. The inhibitory thresholds for producing these effects for each complex may

vary. Finally, investigation of additional drug concentrations as conducted in Chapter 3 drug screening may reveal any concentration-dependent effects on ROS production and other markers of senescence.

However, the influence of myxothiazol treatment on senescence after 3 days appears to be limited to the SASP. Based on previous findings of successful reduction in senescent markers after 10 days of metformin treatment, it would interesting to examine these markers after longer treatment with myxothiazol to compare any time-dependent effects.

Importantly, in all of the models examined in this chapter the SASP demonstrated greater potential for manipulation than any of the other senescent markers. While 3 days of myxothiazol or rapamycin treatment reduced SASP factor secretion when given immediately post-IR, and 10 days with all three tested inhibitors reduced SASP in established senescence, no other tested marker showed any change. The reason for this likely lies in the transient nature of the pro-inflammatory cytokines and chemokines that form the SASP. As a secreted factor that is perpetually produced, it is more practical to prevent cytokine secretion or upregulate their clearance than to reverse markers such as DNA damage foci or nuclear enlargement which are far more permanent in nature. The processes required to repair DNA damage foci or reverse nuclear enlargement are likely far more complicated than suppressing the active secretion of pro-inflammatory products.

Overall, here I have shown that when targeting the SASP for treatment, both the point of initiation and duration of treatment are key in determining their effectiveness. The success of short-term treatment post-IR in alleviating the SASP also suggests that interruption of the initial burst of activity following irradiation may be sufficient to prevent SASP development, but not other markers of senescence.

Chapter 6: Conclusions

The interplay between mitochondrial dysfunction and cellular senescence has been the subject of investigation for many years, stemming from their shared accumulation with age and links to age-related disease. More recently, their influence on each other has been given greater context, both by the confirmation of mitochondria's essential role in senescence development (Correia-Melo *et al.*, 2016) and identification of a mitochondrial dysfunction-induced senescent phenotype (Wiley *et al.*, 2016), and the clarified role of senescence in promoting mitochondrial dysfunction (Nelson *et al.*, 2018). Work presented in this thesis provides further detail on the role of mitochondria in senescence, how manipulating mitochondrial function can modify the senescent phenotype, and provides evidence for possible determining factors in alleviating the SASP by targeting specific mitochondrial processes such as mitochondrial respiration. These observations offer important context in the pursuit of age-related disease treatments and improved healthspan by targeting senescent cells.

Firstly, while great focus has been given to Complex I dysfunction and ROS production in ageing and age-related disease, here I have shown that it is possible to alleviate pro-inflammatory SASP secretion by interrupting electron flow at CI, III, IV and V. This shows that SASP suppression following mitochondrial respiration inhibition is not necessarily a site- or complex-specific effect, and can be achieved by general interruption of electron transport. Secondly, data here also presents evidence that suppression of overall mitochondrial respiration is the key determining factor in preventing SASP factor secretion, and not specific oxidative phosphorylation activity or OXPHOS-derived ATP production or ROS production. While both of these products may have some influence on senescence, based on findings presented here they are not as important as the overall respiratory inhibition, which is in line with knowledge that many aspects of senescence, SASP production and synthesis of necessary intermediate products all require a functional ETC. It is also possible that other interventions may have similar effects, such as preventing electron provision from the TCA cycle or entry of pyruvate into the mitochondria, though at this point these are purely specutalive.

Thirdly, metformin is an effective senostatic treatment capable of alleviating the SASP, DNA damage, nuclear enlargement, and SA-β-Gal activity when given chronically from the point of senescence induction. While it retains its ability to modify SASP production in models of established senescence, other markers of senescence become refractory to this intervention once the phenotype is 'locked in'. A noticeable difference is presented by preliminary experiments between IR-induced and replicative senescence, which require further study to determine whether greater SASP suppression by metformin in IR-induced compared to replicative senescence is a time- or phenotype-specific effect.

The precise mechanism of metformin action is the subject of great debate, and has yet to be fully elucidated. While a number of key targets and pathways have been identified, including CI inhibition, AMPK activation, gluconeogenesis inhibition, mTORC1 and NFκB inhibition, even these appear to be context-dependent. Studies have demonstrated biphasic effects of metformin between high and low doses, acute and chronic treatment, as well as AMPK-dependent and –independent roles. Here I have shown that high dose metformin inhibits mitochondrial CI specifically, both acutely and over long-term treatment, in agreement with previous reports, and that while it is an effective modulator of senescent markers when given from the point of senescence induction, it is unable to alleviate cell-cycle arrest. Whether this is an AMPK-dependent or –independent effect is unclear. Maintenance of cell-cycle arrest could suggest reinforcement of p53-dependent signalling via AMPK activation, while the lack of enhancement of other senescent markers that occur downstream of p53 signalling implies this is not the case, though metformin may simply be preventing ATP production required for these processes. Further work is required to dissect the mechanism of metformin's effects in these conditions, but these findings are promising as metformin can be used as a therapy and has been prescribed in humans for decades, making it a prime candidate for repurposing.

Finally, duration and point of treatment initiation is also important in determining effective senescence modification. Here I have shown that treatment with multiple senostatic drugs while the senescent phenotype is developing is sufficient to prevent SASP production, in some cases after only 3 days of treatment, while treatment in established senescence requires longer times to be effective. Moreover, I have provided evidence that the SASP is more receptive to treatment in all models examined here than any other

tested senescent marker. This provides encouraging evidence that the harmful pro-inflammatory aspect of the senescent phenotype can be modified even in 'deep senescence' without allowing damaged cells to re-enter the cell cycle and proliferate, and represents a much more physiologically relevant model of senescence applicable to further *in vivo* study. Moreover, the interventions tested here may not be able to relieve proliferation arrest, but can be beneficial in preventing and alleviating the harmful 'bystander effect' caused by SASP. ROS and SASP are the major inducers of cell damage resulting from senescence; both as part of a positive feedback loop of increasing production, and through the bystander effect. Removing one or both of these harmful aspects should be beneficial in ageing and ARDs, while maintaining the tumour-suppressor aspect of the senescent phenotype by maintaining cell cycle arrest.

Importantly, I have shown that it is essential to consider both acute and chronic effects of mitochondrial manipulations and their consequences, as cell adaptations over time are taken into account. Furthermore, shorter treatment times may be sufficient to prevent the SASP, but not reverse it, demonstrating that the minimum effective intervention varies between conditions.

Taken together, my data provides support for functional mitochondrial respiration as a determining factor in development of a SASP, and highlights the importance of treatment initiation and duration in defining treatment efficacy. Finally, I have provided evidence that manipulation of mitochondrial electron transport at multiple points successfully prevents the SASP, opening up a wider avenue of investigation for additional senostatic drugs and treatments for age-related diseases based on a general interruption of mitochondrial respiration.

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