Impact of Endosomal Recycling Inhibitors on Drug Resistant and Immune Tolerant Breast Cancers

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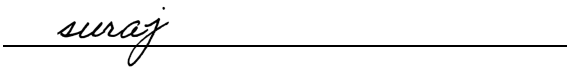
List of abbreviations:

BC	Breast Cancer
IHC	Immunohistochemistry
FISH	Fluorescent *in-situ* hybridization
TNBC	Triple-negative breast cancer
HER2	Human epidermal growth factor 2
TME	Tumour microenvironment
NSCLC	Non-small cell lung cancer
EGFR	Epidermal growth factor receptor
5-FU	5-fluorouracil
DDR	DNA damage repair
FEC	5-FU, epirubicin, cyclophosphamide
EMT	Epithelial mesenchymal transition
TGF-β	Transforming growth factor β
CSF	Colony simulating factor
SASP	Senescence associated secretory phenotype
SAHF	Senescence associated heterochromatin foci
SA-βGal	Senescence-associated β-galactosidase
TAF	Telomerase associated foci
IL	Interleukins
ROS	Reactive oxidative species
NF-κB	Nuclear-factor kappa-light chain-enhancer of activated B cells
OIC	Onco-induced senescence
TSLIS	Tumour-suppressor loss-induced senescence
PTEN	Phosphatase and tensin homolog deletion on chromosome 10
TIS	Therapy-induced senescence
IR	Ionizing radiation
SCAP	Senescent cell anti-apoptotic pathway
COP1	Coatomer complex-1
RNAi	RNA interference
NMTi	N-myristoylation inhibitors
FDA	Food and drug administration
DSB	Double-strand break

TfR	Transferrin receptor
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FTH1	Ferritin-heavy chain
NRF2, pNRF2	Nuclear factor–E2–related factor-2, phospho-NRF2
Keap1	Kelch-like ECH-associate protein 1
IKK	Inhibitor of NF-κB kinase
MAF	Musculo-aponeurotic fibrosarcoma
ERP	Endosomal recycling pathway
Cav-1	Caveolin - 1
CavME	Caveolin-mediated endocytosis
CME	Clathrin-mediated endocytosis
βPIX	Beta PAK-interacting nucleotide exchange factor
GIT	G-protein coupled receptor kinase interacting proteins
CD274	Cluster of differentiation 274
PD1	Programmed cell death protein 1
PDL1	Programmed cell death protein ligand 1
ICI	Immune checkpoint inhibitor
CMTM6	CKLF-like MARVEL transmembrane domain containing protein 6
DMEM	Dulbecco's Modified Eagle Media
RPMI 1640	Roswell Park Memorial Institute 1640
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

Declaration

“This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.”

Signed: 

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Abstract

Breast cancer (BC) is the most common and frequent of all the cancers, accounting for about 25% of all the cases of cancer detected in women annually. There are a number of approved drugs on the market that have been developed or repurposed for the treatment of BC. However, a recurrent problem faced in the clinic is the emergence of drug resistance, which can be intrinsic or acquired. Prolonged periods of chemotherapeutics and targeted therapy have resulted in the development of acquired resistance through a number of different adaptations.

This project focuses on resistance developed by various sub-types of breast cancer cells through drug-therapy-induced senescence. Previous studies have reported the cytoprotective nature of this cell cycle arrest phenomenon upon prolonged exposure to targeted- and chemotherapeutics. This study investigates specific molecular mechanisms governing therapy-induced senescence, the impact it has on the membrane trafficking of iron transporters, and the potential repurposing of endosomal recycling inhibitors (ERIs) to target these drug-tolerant cells.

Additionally, we examined the effects of ERIs on the total protein and surface levels of immune checkpoint proteins. This was done by targeting the recycling of the glycoprotein PDL1, which is expressed on the surface of some tumour cells and binds to its cognate receptors on the surface of T cells. PDL1-PD1 binding leads to T cell inactivation. A number of ERIs were used to determine their impact on the levels of PDL1.

Keywords:

Breast cancer, drug resistance, therapy-induced senescence, doxorubicin, lapatinib, primaquine, monensin, endosomal recycling pathway, iron transporters, transferrin receptor, ferritin, senolytics

1. Introduction

Breast cancer (BC) is the condition wherein some cells in the breast develop abnormally, leading to uncontrolled cell division, and formation of cancerous lumps. It is a malicious condition, which has proven to be a destructive force to the lives of millions of people around the world, and their families [1]. According to the World Cancer Research data, breast cancer is responsible for most cases of cancer detected annually, accounting for 12.5% of all recorded cases. It accounts for nearly 25% of all new cases of cancer detected annually in women in Ireland [2, 237]. The prognosis for BC is bleak in later stages of detection, and can financially and emotionally cripple patients and their families. While patients from higher income countries have a survival rate of around 90% for BC, middle and lower-income nations are plagued with lower survival rates, on account of lack of infrastructure, funding, coverage, and a general lack of awareness [3].

1.1. Risk Factor

There are several prevalent risk factors that contribute to the development of BC. The major contributing risk factor is sex, as women are nearly a hundred times more likely to develop BC. High concentrations of oestrogen and progesterone have been positively correlated to BC, and both pre- and post-menopausal women who exhibit high expression levels of sex hormones are more susceptible [4]. Additionally, a number of other factors also contribute to increased susceptibility to BC [5, 8].

a) Age: BC is commonly detected in women aged 50 and above, and the risk continues to rise with increasing age [Table 1]. According to the SEER database, the probability of developing BC is 2.4% for ages 50-59, 3.5% for ages 60-69, and 7.0% for ages 70 and older [6]. This reflects age-related changes in cellular

processes [6]. Additionally, younger women diagnosed with BC have lower survival rates, likely due to a higher prevalence of aggressive triple negative breast cancer in those aged 40 and younger [6].

b) Genetic component: Having a first-degree family history of BC, such as a mother, sister, or daughter with prior BC, significantly raises the risk of developing BC. Women with two first-degree relatives who had BC have triple the risk compared to those with no family history [6]. The association between positive family history and increased BC risk is often linked to gene abnormalities, particularly in the *BRCA1* and *BRCA2* genes. *BRCA1* mutation carriers have a cumulative BC risk of 72%, while *BRCA2* mutation carriers have a cumulative risk of 69% [6]. BC incidence tends to increase in early adulthood until around the ages of 35 for *BRCA1* carriers and 45 for *BRCA2* carriers [6]. Women with a family history of breast cancer have a slightly increased risk of developing BC in the opposite breast, with an estimated annual increase of 0.5% following the initial diagnosis [6]

c) Ethnicity: The prevalence of BC also varies depending on the ethnicity of the patient. There are genetic and environmental components to these differences, including geography, cultural factors, prevalent vegetation, access to nutrition, and other differences between ethnicities [6].

d) Physical attributes: Several physical attributes contribute towards increased susceptibility to BC. Height directly correlates with increased risk of developing BC. The relative risk is higher for patients taller than 1.75m [6]. The reason for this is unclear, but there are a number of studies that point to an association between nutritional uptake at childhood with chances of developing BC [8, 238]. Additionally, there is also an increased probability of occurrence in patients with

pre-existing conditions such as obesity, regular alcohol consumption and smoking, and history of hormonal therapy [6, 239]. Interestingly, there has been a negative corelation between multiparity (having 2 or more children), and elevated probabilities of developing BC [6].

	Incidence 2017-2019					Mortality 2017-2019				
	Annual case counts			ASR per 100,000*		Annual deaths			ASR per 100,000*	
	Male	Female	All	Male	Female	Male	Female	All	Male	Female
C50 Breast (all ages)	34	3507	3542	1.3	130.0	6	732	738	0.2	23.8
0-49 years	3	814	817	0.2	44.4	0	78	78	0	4.3
50-69 years	13	1713	1726	2.5	323.0	2	253	255	0.4	47.4
70+ years	18	980	998	8.5	391.3	4	400	404	1.8	147.8

Table 1: *Average annual number of total cases and deaths, and age-standardised rates (ASR) for breast cancer in Ireland 2017-2019 [9]*

1.2. Standard of Care for Breast Cancer

The current standards of care for the various stages of breast cancer involve chemotherapy, hormonal therapy, targeted radiotherapy and immunotherapy. Each stage has varying requirements, based on the differences in morphology, markers, and the degree of invasiveness [10, 11].

- Stage 1 Breast Cancer: Breast cancer that has not undergone much growth, and have barely reached the lymph nodes, are still in the primary stages. Treating stage 1 breast cancer usually involves surgery, which could either be a breast-conserving surgery (BCS), or mastectomy. In addition to this, radiation therapy is occasionally recommended. In such instances, patients are recommended to

undergo surgery after the radiation therapy is complete [10]. Occasionally, if tumour size is over 0.5cm across, chemotherapy might be recommended [10]. Fortunately, survival rate is very high in this stage of cancer, and there is very good prognosis [12, 17].

- Stage 2 Breast Cancer: The tumour is slightly larger than that of Stage 1 may have reached a few sentinel and axillary lymph nodes. This is usually tested through lymph node removal and analysis. Some additional standards of treatment include targeted therapy, immunotherapy, and hormonal therapy [10]. Prognosis at the second stage of breast cancer is also very good, with nearly a 90% 5-year survival rate [13, 17].
- Stage 3 Breast Cancer: The tumour is larger than in Stage 2, ranging from 5 to 8 cm across. It may have spread to an adjoining tissue at this stage, and would have reached several adjoining lymph nodes. There is also the possibility of inflammatory breast cancers, wherein the cancers have not spread beyond the lymph nodes. These require slightly different treatments to the regular stage 3 breast cancer. Any form of surgery is usually preceded by a line of chemotherapy, also known as neoadjuvant chemotherapy. This usually enables reduction of tumour size to allow surgical treatment. Post surgery treatment involves adjuvant, hormonal or targeted therapeutic approaches [10]. The prognosis during stage 3 breast cancer is not as favourable, with an approximately 70% 5-year survival rate [14, 17].
- Stage 4 Breast Cancer: Metastasis has occurred, and the cancer has spread to other parts of the body. Treatment can be hormone therapy, with drugs such as letrozole and anastrozole. If eventually the cells become unresponsive to the chemotherapeutic treatment, immunotherapy, or use of targeted therapies such as

PARP (Poly-ADP Ribose Polymerase) inhibitors (e.g., olaparib and talazoparib) can be recommended [11]. The prognosis in this stage is very bleak, with 5-year survival ranging between 20-25% [17]. Even with treatment, only a few years of survival can be observed [15].

Post-therapeutic prognosis of metastasised breast cancer is not promising either, on account of the possibility of a tumour recurrence. Metastasis is the spread of cancer from the primary or original tumour, to other parts of the body. The cancerous cells spread through the blood or lymph system, and continue to grow in the new location. Depending on the location of the tumour, the symptoms can vary. For instance, cancer that has spread to the brain can result in headaches, seizures and dizziness [240]. Breast cancer usually metastasizes into the brain, bones, liver and lungs. The five-year survival rate upon metastasis is between 25 to 85% based on how distant the spread is. Between the financial burdens that may be incurred on account of any treatment, and the uncertainty that follows treatment, there is an urgent need to tackle the problems associated with breast cancer on all fronts.

1.3. Drug Treatment by Sub-Type

Targeted therapy in breast cancer requires the selection of specific targets expressed by the tumour. The classification of breast cancer is performed on the basis of the presence of these unique markers, and their expression levels [Table 2].

1.3.1. HER2+ Breast Cancer Cells

HER2+ breast tumours over-express HER2 receptor. This is a receptor tyrosine kinase (RTK) that belong to the Epidermal Growth Factor Receptor (EGFR) family. About 15-20% of all breast cancers are HER2+ [16]. The HER2 receptor is unique in the EGFR family in that it does not bind with any ligand on its own. Its activation occurs through dimerization with other ligand activated EGFRs. This leads to phosphorylation of tyrosine residues, resulting in the activation of a number of downstream signalling cascades [19].

Quantification of HER2 expression level is usually performed through immunohistochemistry tests, which assign a score between 0 and 3 to the tumour. HER2-negative cells are assigned a score of 0 to 1, and HER2 overexpressed cells return a value of 3 [20]. One could also perform Fluorescent In-Situ Hybridisation (FISH) to determine the number of copies of the HER2 gene present in the samples. FISH distinguishes more comprehensively between HER2-positive and negative cells [20].

There are a wide range of treatments available for HER2-positive cancers. Surgery is usually the first line of treatment, mastectomy or lumpectomy, chosen based on the degree to which the tumour has grown [20]. In this classification of BC, targeted therapy is an effective treatment strategy, with many therapeutics available that target the HER2 protein specifically. These drugs can be classified into monoclonal

antibodies (trastuzumab, pertuzumab, margetumixmab), small molecule tyrosine kinase inhibitors (lapatinib, neratinib), or antibody-drug conjugates (Ado-trastuzumab emtansine, fam-trastuzumab deruxtecan) [18].

Tyrosine kinase inhibitors (TKIs) inhibit cellular signalling by inhibiting the intrinsic tyrosine kinase activity of HER2 [23].

The action of monoclonal antibodies such as trastuzumab has been well documented, in that they bind to the extracellular domain of the HER2-receptor and inhibit dimerization. This leads to inhibition of HER2-mediated signalling, which is crucial for cell proliferation. It has also been shown to facilitate antibody-dependent cellular cytotoxicity, causing cell death in HER2 positive cells [21].

ADC (Antibody Drug Conjugates) are monoclonal antibodies linked to chemotherapeutic drugs, wherein the antibody binds to the HER2 protein and acts as a homing signal for the chemotherapeutic drug to target the cancer cells specifically [22].

1.3.2. Hormone Receptor-Positive Breast Cancer

Hormone receptor-positive breast tumours express higher levels of hormone receptors. This could either be oestrogen receptor (ER+) and/or progesterone receptor (PR+) overexpressing [24]. The treatment for hormone receptor-positive breast cancer includes hormone therapies, also known as endocrine therapy. Tamoxifen is an example of an endocrine therapeutic drug. Aromatase inhibitors such as letrozole and selective estrogenic receptor modulators (SERMs) could also be used as hormone therapy [18, 25].

Much like in HER2-positive BC, the status of hormone positivity can be determined through immunohistochemistry (IHC), wherein the levels of oestrogen and

progesterone receptors expressed by the cancer cells can be determined [25]. There are a number of targeted therapy options as well, such as mTOR inhibitors (everolimus), PI3K inhibitors (alpelisib), AKT inhibitors (capivasertib), and CDK4/6 inhibitors like palbociclib and abemaciclib [18]. They target the cyclin dependant kinases, affecting the cell cycle machinery by interrupting intracellular and mitogenic hormone signals that stimulate proliferation of malignant cells [29]. Adjuvants and neo-adjuvants are often incorporated into treatment of hormone positive breast cancer (eg. Doxorubicin) [30].

Hormone receptor - positive cancers can further be classified into Luminal Type A and Type B cancers, which differ in a number of properties [16]. Luminal Type A cancers express lower levels of proliferation markers such as Ki-67, lower histological grade, and presents as the most common type of breast cancer, representing nearly 50% of all diagnosed. Luminal Type B is significantly more aggressive, has higher recurrence rate and higher levels of Ki-67, and is associated with much lower survival rates [16, 26, 27].

1.3.3. Triple Negative Breast Cancer

Triple Negative Breast Cancers (TNBC) under-express the three markers discussed so far. The absence of these cancer markers provides them with the appearance of epithelial cells. Hence, they are also called Basal-like Breast Cancer (BLBC) [28]. They present in 8 to 37% of all breast cancer cases, depending on the number of poorly differentiated grade 3 cells [16].

The standard of care for TNBCs ranges from neoadjuvant chemotherapy, to surgery. The initial three stages call for drugs like capecitabine, olaparib, and pembrolizumab [32]. Capecitabine can be taken orally, usually a course lasts 18-24 weeks and has

been reported to improve the prognosis of patients. Adjuvant capecitabine, when provided in six to eight cycles, showed significantly improved prognosis [33]. Studies have reported that low doses of capecitabine resulted in reduced recurrence of breast cancer in women presenting with TNBC through two pathways: angiogenesis and immune escape [243]. Olaparib is a targeted drug that act as a PARP inhibitor. Pembrolizumab is an adjuvant therapy, that is taken post-surgery (mastectomy)/radiotherapy [33]. Pembrolizumab is an immune checkpoint inhibitor that blocks the immunoprotective protein PD-1 on the surface of T cells, and preventing the cancer cell from suppressing the immune system [130]. Olaparib can also act as an adjuvant drug.

TNBC can be further classified on the basis of immune suppressive factors, into categories such as LAR (luminal androgen receptor), MES (mesenchymal), BLIA (basal-like immune-activated), and BLIS (basal-like immune-suppressed) [34].

Breast Cancer Sub-type		Prevalence	Diagnostic Markers	Prognosis	Standard of Care
HR-positive (Luminal)	Luminal A	~ 50 - 60% [16]	ER+, PR+	Good, with low relapse rate [16]	Hormone therapy (Tamoxifen, aromatase inhibitors), surgery, targeted therapy (Palbociclib, Abemaciclib) and broad range chemotherapy (Doxorubicin) [18]
	Luminal B	~ 15 - 20% [16]		Poor, with high relapse rate [16]	
HER2-positive (HER2+)		~ 15 - 20% [16]	HER2+	Poor, with high relapse rate [16]	Surgery (Mastectomy, lumpectomy) and targeted therapy (Trastuzumab, Lapatanib, Neratinib) [18, 19]
Basal-like breast cancer		~ 8 - 37% [16]	ER-, PR-, HER2-	Poor, with high relapse rate [16]	Neo-adjuvant Chemotherapy (Capecitabine, Olaparib, Pembrolizumab), Surgery [18]

Table 2: *Breast cancer subtypes, their diagnostic markers, prevalence, prognosis and standard of care.*

1.4. Drug Resistance in Breast Cancer

The current standards of care are very effective, but the occurrence of drug resistance can be a major setback for the overall prognosis of breast cancer. The development of drug resistance accounts for up to 90% of BC deaths [35]. There are two predominant forms of drug resistance – intrinsic and acquired resistance [36].

Intrinsic resistance refers to resistance that the patient demonstrates prior to exposure to drugs. This can be due to a pre-existing genetic mutation present in the tumour;

heterogeneity in tumours, resulting in selection of resistant clones of tumour cells; or activation of intrinsic pathways as defence mechanisms to certain drugs [36].

Acquired resistance refers to the gradually declining efficacy of drug treatment. There are several mechanisms by which cancer cells acquire resistance; activation of a second proto-oncogene that becomes the new driver gene; mutation or altered expression levels of the drug target; or changes in tumour microenvironment (TME) [36]. Table 3 provides a more comprehensive view of the various factors that could be responsible for drug resistance in breast cancer [Table 3].

1.4.1. Drug Efflux

Chemotherapy resistance can be caused by a decrease in intracellular drug accumulation, which is caused by a dysfunction in the ABC transporter genes that control the transmembrane transporters. The ABC superfamily consists of 48 genes within 7 subfamilies (ABCA to ABCG), among which ABCB1, ABCC2 and ABCG2 are reported to be highly involved in multi-drug resistance [36, 241].

ABCB1 transporter contains two transmembrane domains that bind and hydrolyse ATP, following which it binds and releases substrates such as etoposide, doxorubicin, paclitaxel and vinblastine [56, 132 - 136]. Though most tumours express high levels of ABCB1 prior to therapy, haematological malignancies demonstrated lower levels prior to chemotherapeutic treatment [138-140]. Unlike ABCB1, which pumps out amphipathic and lipid-soluble compounds, ABCC1 transporters exclusively pump organic anionic anti-cancer agents, such as epipodophyllotoxins, camptothecins, and methotrexate [141-144]. ABCC1 overexpression has been observed in breast cancer, as well as prostate and lung cancer [145-147].

ABCG2 is largely expressed in breast cancer, but also present in lung cancer and leukemia [148, 149]. It is reported to transport positively- and negatively-charged substrates. This includes chemotherapeutic drugs such as mitoxantrone, bisantrene, and flavopiridols, as well as TKIs such as gefitinib and imatinib [56, 137, 148].

1.4.2. Alternation of Drug Targets

Targeted therapy (e.g., lapatinib, gefitinib, erlotinib) relies on the inhibitions of specific target proteins to prevent or delay tumour development. Cells can develop resistance to these drugs through the alteration of drug targets; either through a secondary mutation, or by varying expression levels of the target protein [36]. This has been observed in non-small cell lung cancer (NSCLC) for epidermal growth factor receptor (EGFR) targeting drugs, where 50% of the patients treated with TKIs such as erlotinib developed a threonine-to-methionine mutation (T790M) within one year. This varied the EGFR protein structure, enhancing binding affinity to ATP, while concomitantly impairing drug binding [57, 150, 151]. Extended exposure to tamoxifen, a drug that targets hormone-positive cancer, rendered breast cancer cells resistant to the drug. This was caused by the downregulation of oestrogen receptor [152, 153].

1.4.3. Enhanced DNA Damage Repair

Drugs like cisplatin and 5-fluorouracil (5-FU) cause cell death through the induction of DNA damage. This has been shown to upregulate DNA damage repair (DDR) genes, such as FEN1, FANCG, and RAD23B, leading to insensitivities to chemotherapeutics [60]. A suggested strategy to overcome this form of resistance is through the deregulation of DDR, though it is suspected that this might cause genomic instability and result in the initiation of another round of carcinogenesis [36].

1.4.4. Epigenetic Alterations

Epigenetic modifications such as DNA methylation and lncRNA modification have been implicated in the induction of drug resistance. For instance, demethylation of DNA and active modification of histone H3 can lead to the enrichment of G-actin monomer binding protein thymosin β4, which induced a stem-like property in hepatocellular carcinoma, and induced resistance to VEGFR inhibitor sorafenib [61].

In another instance, lncRNA urothelial cancer-associated 1 was recorded to be upregulated in cisplatin resistant bladder cancer cells. This led to increased expression of wingless-type MMTV integration type family member 6, promoting cell survival [154].

1.4.5. Tumour Heterogeneity and Microenvironment

Heterogeneity causes varying sensitivities to drug treatment, causing the selection of cells that have high tolerance to the drug to proliferate [156-159]. There are four levels of heterogeneity: genetic, cell-type, metabolic and temporal heterogeneity [155]. The presence of any form of heterogeneity made it nearly impossible to kill all tumour cells with a single line of therapeutics, and this led to the development of combinatorial therapies and cocktail therapies, such as FEC (5-FU, epirubicin and cyclophosphamide) for breast cancer [36].

Another factor that could influence variations of drug-tolerance in cells is the tumour microenvironment (TME). This includes extracellular ECM, immune cells, blood vessels, fibroblasts etc., and it is reliant on the right TME for the tumour cells to survive and proliferate. Each TME factor could induce drug-resistance via a variety of strategies (Refer Table 3). A notable strategy is through the manipulation of pH levels. Tumour cells are shown to establish a “reverse pH gradient”, with increased

intracellular pH and a reduced extracellular pH level [65, 161]. Acidic intracellular pH has been reported to induce resistance to chemotherapeutics, through the impairment of distribution of weak base anticancer drugs. This is known as “ion trapping”, and enables cancer cells to evade apoptosis [64, 160].

1.4.6. Epithelial-Mesenchymal Transition

The process of epithelial cells detaching from each other and acquiring mesenchymal properties is known as epithelial-mesenchymal transition (EMT). It is essential for the initiation of metastasis [36]. EMT has been shown to implement a number of strategies to resist apoptosis induction, contributing to drug resistance [68]. One such strategy is based on transforming growth factor β (TGF-β), which is an integral cytokine in EMT. Inhibition of TGF-β pathway has been shown to reverse EMT, and increase sensitivity to drugs. Wnt, Notch and Hedgehog signalling pathways are also reported to contribute to drug resistance. For instance, Wnt activates the Wnt/ β-catenin pathway to promote an EMT-like phenotype upon treatment with trastuzumab [162, 163].

1.4.7. Senescence Escape

The focus of this thesis is to study acquired resistance, and understand its impact on cellular phenotype with a view to identifying vulnerabilities that can be targeted to overcome drug resistance. Prolonged periods of targeted drug treatment and chemotherapeutic treatment has been found to induce phenotypes resembling cellular senescence [37]. Cellular senescence is defined as a permanent or temporary cell-cycle arrest, characterised by a number of qualities. These include, but are not restricted to beta-galactosidase activity, senescence associated secretory phenotype (SASP), cellular enlargement, and senescence associated heterochromatin foci (SAHF) [38 - 40]. SASP is characterized by widespread changes in protein expression

and secretion that leads to the arrest of the cell cycle in senescent cells while maintaining metabolic activity. Coppe *et al.* have extensively characterized SASP in human epithelial cells, and human and mouse fibroblasts [81].

The development of SASP results in inhibition of cell division via genetic instability, oncogene inactivation, and promotion of immune clearance in cells [50].

Senescent cells also differ in cell shape and size to a normal cell. They show increased protein content, an elevation in the amount of anti-apoptotic proteins, increased lysosomal hydrolase activity (presented through senescence-associated β-galactosidase (SA-βGal)), DNA damage, and telomere associated foci (TAFs) [52]. Some notable SASPs include pro-inflammatory interleukins (IL-6, IL-7, IL-1α, IL-1β, IL-13, IL-15), chemokines (GRO-α, GRO-β, MCP-1, MCP-2, eotaxin-3), growth factors (EGF, bFGF, HGF, VEGF), proteases (MMP-1, MMP-3, MMP-10, MMP-12, MMP-13, MMP-14, capthesin B), and extracellular matrix elements like fibronectin and altered collagen [52, 81] [Figure 1].

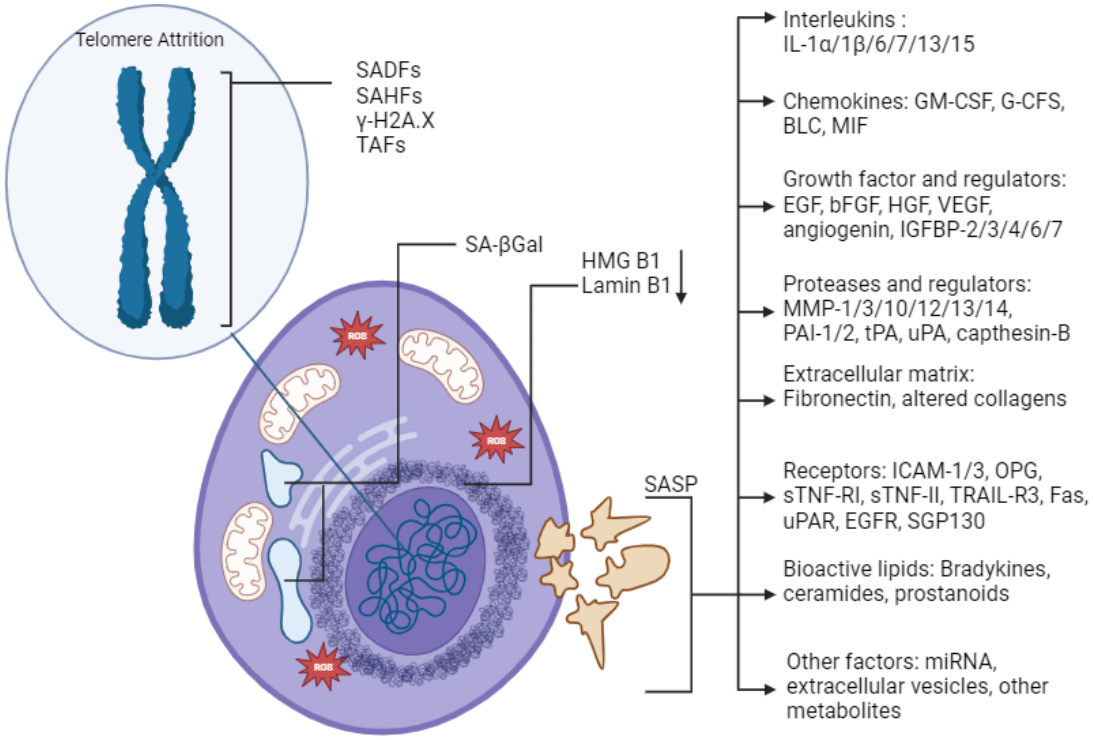

Figure 1: ***Characterization of senescence, senescence markers and SASPs:*** *Various features of senescent cells that have been described in prior literature. These are not conserved in different types of senescence, but are mostly consistent [52]*

1.4.7.1. Key Senescence Markers

Oxidative stress markers are potential markers of senescence, on account of the multiple avenues through which oxidative damage is incurred by senescent cells [54]. A upregulation in reactive oxidative species (ROS) acts as an inflammatory mediator for Nuclear-factor kappa-light chain-enhancer of activated B cells (NF-κB) by upregulating the levels of inflammatory factor IL-1α, thus inducing senescence [112].

There are contrasting reports on lysosomal activity. On the one hand, there have been studies stating that senescence causes an increase in dysfunctional mitochondria, and a corresponding decrease in lysosomal activity [127]. In contrast, increased pro-autophagic activity has also been reported to elevate lysosomal activity [128]. These

contradictory findings suggest a cell specific corelation between lysosomal activity and induction of senescence, with an increase in some cases and a decrease in others.

1.4.7.2. Induction of Senescence

Cells can undergo senescence under a variety of circumstances. Cells naturally attains senescence after completing a number of divisions, at which point they reach the end of their replicative lifespan, called the Hayflick limit [83]. This is called replicative senescence and occurs due to the telomerase-induced reduction of telomere length after each replication cycle. Due to its intrinsic nature, it is sometimes also referred to as intrinsic senescence [63]. In contrast, extrinsic senescence is independent of telomerase activity, and can be categorized as onco-induced, tumour-suppressor loss-induced, or therapy-induced [63]. Onco-induced senescence (OIS) occurs upon onco-gene activation, while tumour-suppressor loss-induced senescence (TSLIS) results from the inactivation of tumour suppressors, such as phosphatase and tensin homolog deletion on chromosome 10 (PTEN). Both OIS and TSLIS are reported to protect cells from neoplastic growth, and further transformation into malignancy [63].

Therapy-induced senescence (TIS) occurs as a result of drug-treatment or radiation therapy [83]. Ionizing radiation (IR) is commonly used in cancer therapy, and has been shown to induce cellular senescence in doses ranging from 2Gy to 10Gy. [84, 85, 86]. $p21^{Cip1}$ plays a key role in cell-cycle inhibition, and p53 status was shown to demonstrate a crucial role in induction of ionizing radiation-induced senescence [86, 87]. This type of senescence was also reported to be telomere shortening independent, and ATM/Chk2, p38 MAPK, AMPK/NF-κB dependent [88, 90, 91]. Radiation activates the DNA damage response through the ATM/Chk2 immediately, followed by a series of slower events, including p38 MAPK activation, that contributes to SASP

production [90]. 5-adenosine monophosphate-activated protein kinase (AMPK) - NF-κB pathways become activated upon exposure to radiation, and contribute to the induction of senescence [91]. There have also been reports of the presence of the multi-functional protein securin pushing cells towards apoptosis when irradiated with IR [89], and the inhibition of glycolysis, or deficiency of PTEN attenuating IR induced senescence [91, 92].

A wide range of drugs (chemotherapy or targeted-therapy) have been reported to induce senescence in a variety of tumour cells. While there is no robust senescence-inducing agent that universally targets all tumour types, there have been studies into different types of tumours, and the agents that could induce senescence in them. *In vitro* and *in vivo* studies have reported that TIS can be induced through a number of different pathways, including DNA damage, oxidative stress, post-translational modifications in proteolytic processing and kinase signalling [42]. To identify senescence inducing drugs, Ewald *et al.* (2009) screened a 4160-compound library of known bioactive compounds and natural products. This study discovered over 225 cytotoxic compounds, out of which 51 were identified as potentially senescence inducing [93].

Unlike in IR-induced senescence, drug therapy induced senescence is reported to be independent of p53 status [94]. However, it was determined that each drug had their own unique mode of induction of senescence. The most potent pre-senescence response was from DNA-damaging agents, like doxorubicin and cisplatin, while the weakest was from drugs targeting microtubules, like taxol and vincristine [95].

Dosage is an important aspect of drug-induced senescence; lower concentrations would not produce any effect while higher concentrations may induce apoptosis-

mediated cell death [93, 96]. It was also noted that senescent markers were usually observed after a period of treatment ranging from 3 to 7 days, and sometimes even be longer [42].

Several pro-senescence drugs are reported to induce DNA damage via double- or single-stranded breaks, suggesting a commonality between drug-induced senescent cancer cells, stress-induced senescent cells, and replicative senescent cells [84, 97].

Although therapy induced senescence does not demonstrate any telomere-dependency, or telomeric shortening [102, 103], DNA damage in therapy induced senescence usually occurs through means of alteration of DNA structure by indirect means; for instance, the inhibition of DNA methyltransferase using 5-azacytidine, or modifying chromatin structure using histone DNA acetyl inhibitors like Sirtinol [42].

The literature on cell cycle effectors in senescent cancer cells is contradictory, with many studies reporting varied relationships between drug-induced senescence and p53, $p16^{INK4a}$ and p21[46, 98, 99, 100, 101]. For instance, one study suggests a central role for p53 and p21, while $p16^{INK4a}$ maintains senescence [46]. Another suggests that though p53 and p21 does positively regulate senescence, it is not absolutely required for its induction [98]. These inconsistencies could be attributed to the varying choice of cell lines and drugs used in these studies, providing an incomplete picture of the pathways involved.

Induction of TIS is frequently associated with double-stranded breaks in genomic DNA [53]. However, it could also be induced upon oxidative stress caused by chemotherapeutics such as pyrithione, which are detrimental to mitochondrial function [42]. This dysfunction can affect glycolysis, glutaminolysis and other

metabolic pathways that regulate double-strand break (DSB) repair and DNA damage checkpoints [55].

1.5. A Link Between Therapy-Induced Senescence and Iron Metabolism

Mitochondrial dysfunction has been previously reported to correlate strongly with cellular senescence, accompanied with elevated intracellular iron accumulation (by nearly 30-fold) [69, 70]. This section discusses the contradictory evidence that exists in the literature on the impact of drug resistance and senescence on the expression levels of iron regulatory proteins [69 – 72].

Recently, Li *et al.* (2023) reported evidence of a direct correlation between elevation of intracellular iron levels, mitochondrial dysfunction and senescence [69]. Intracellular iron assists in the generation of hydroxyl radicals through Fenton's reaction. Fenton's reaction refers to the oxidation of ferrous iron to ferric iron in the presence of peroxide. It generates hydroxyl and hydroperoxyl radicals, which are unstable/reactive oxygen pollutants. With the critical role that iron plays in the generation of hydroxyl radicals, it is unsurprising that the induction of senescence modifies the expression levels of iron regulatory proteins such as transferrin, transferrin receptor (TfR), and ferritin, and thus oxidative stress [Figure 2].

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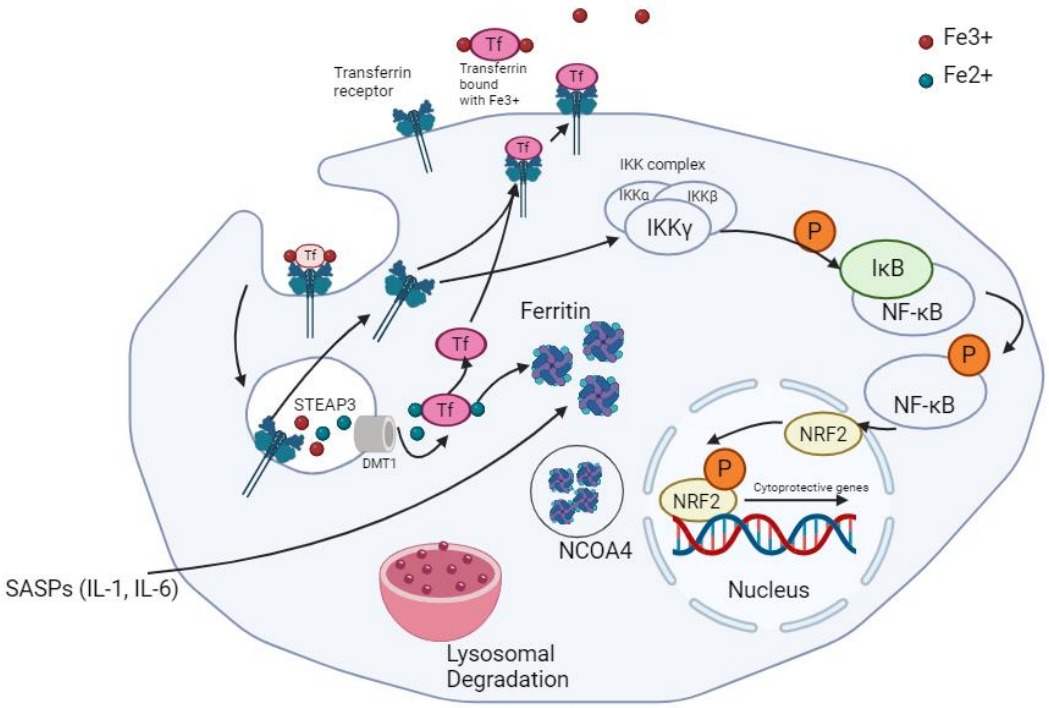


Figure 2: ***Iron metabolism in senescent cancer cells***. *Ferric iron is bound to transferrin and endocytosed with the help of TfR. Conversion of ferric to ferrous iron is followed by the uptake of this ferrous iron by ferritin. NCOA4 supports the degradation of these molecules via ferritinophagy. Simultaneously, free TfR could either get recycled, or utilized in the activation of stress response via NRF2 activation.*

The transferrin receptor (TfR) is a membrane protein responsible for the uptake of iron. It transports iron-bound transferrin into the cell. TfR has been reported to be over-expressed in breast cancer cells [73]. The protein levels of TfR in each of the sub-types of breast cancer has been studied, and was found to be elevated in luminal subtype, albeit negatively correlated with estrogen receptor (ER). [74].

Ferritin is a protein complex that captures intracellular ferrous iron (Fe^{2+}) and converts it into ferric iron (Fe^{3+}) through intrinsic ferroxidase activity in order to reduce intracellular damage caused by reactive oxidative species (ROS) [75]. This

ROS is generated as a product of Fenton's reaction from the less oxidatively stable Fe^{2+} [131].

FTH1 (ferritin-heavy chain) is the active component of this protein complex and directly correlates to Ferritin activity [76]. It has been reported that FTH1 levels are significantly higher in TNBC than in other breast cancer cell subtypes [76].

Ferritin plays a protective role against an iron-dependent cell death pathway called ferroptosis, and pushes cells towards senescence [77]. Ferroptosis is different to apoptosis, autophagy and necrosis. Unique properties include activation by the small molecule xCT inhibitor erastin and a redox state imbalance [117, 118]. Studies have since associated ferroptosis with ischemic and degenerative disorders, as well as carcinogenesis [119, 120].

Another protein involved in the regulation of oxidative stress and ferroptosis is the nuclear factor–E2–related factor – 2 (NRF2). NRF2 is a key transcription factor that maintains intracellular oxidative stress damage, and in turn, ferroptosis. It is a basic Leucine Zipper (bZIP) transcription factor that regulates the expression of proteins such as glutathione S-transferase, heme oxygenase 1, and NADPH quinone oxidoreductase [122]. NRF2 activation is regulated by Kelch-like ECH-associate protein 1 (Keap1) based on the redox state of the cell. Unstimulated, Keap1 binds to NRF2 in the cytoplasm, actively contributing to ubiquitination and proteasomal degradation of the transcription factor [123]. As per Yuan *et al.,* when stimulated by inhibitor of NF-κB kinase (IKK), NF-κB activates NRF2 via phosphorylation [78]. Interestingly, IKK has been reported to be activated by TfR [71]. Upon NRF2 phosphorylation, Keap1 is modified, and phospho-NRF2 (pNRF2) is translocated into the nucleus, where it heterodimerizes with Musculo-aponeurotic fibrosarcoma (Maf)

protein. This protein complex then binds to apoptotic/electrophilic response elements, promoting transcription of cytoprotective genes [79, 123 - 126].

With senescent cells demonstrating a strong anti-apoptotic tendency despite the apoptotic expression, it stands to reason that the levels of pNRF2 is a prominent indicator for senescent activity. Interestingly, silencing of the NRF2 genes has been shown to induce premature senescence in human embryonic fibroblasts [79, 121].

1.6. Membrane Trafficking Pathway and Senescence

A crucial element of membrane trafficking is the endosomal recycling pathway (ERP), which plays an important role in regulating the composition of the plasma membrane. This involves the internalization of cargo from the cell surface by a process called endocytosis. Endocytosed cargo is delivered to an intracellular organelle called early endosome, where its fate is decided. It can be either returned to the surface via the ERP, or sent to lysosome for degradation [182]. There are two recycling pathways, based on the speed at which the process is carried out – in the fast-recycling pathway, cargo is directly transported to the surface from the early endosome; and the slow recycling pathway, where the cargo is transported back to the plasma membrane via a perinuclear localized endosomal recycling compartment [Figure 3] [183, 184].

A number of studies report that disruption of endocytosis results in the induction of cellular senescence [177-179]. For instance, the disruption of clathrin-mediated endocytosis results in DNA damage and changes in mitogenic signaling, leading to centrosome overduplication, which is accompanied by induction of senescence [178]. Clathrin mediated endocytosis, and caveolin mediated endocytosis are the different types of endocytosis. Clathrin is the molecular scaffold that forms vesicles to uptake

cargo [209], and caveolin is a special cholesterol-binding protein that assists in the formation of cave-like structures in the membrane, called caveolae [210].

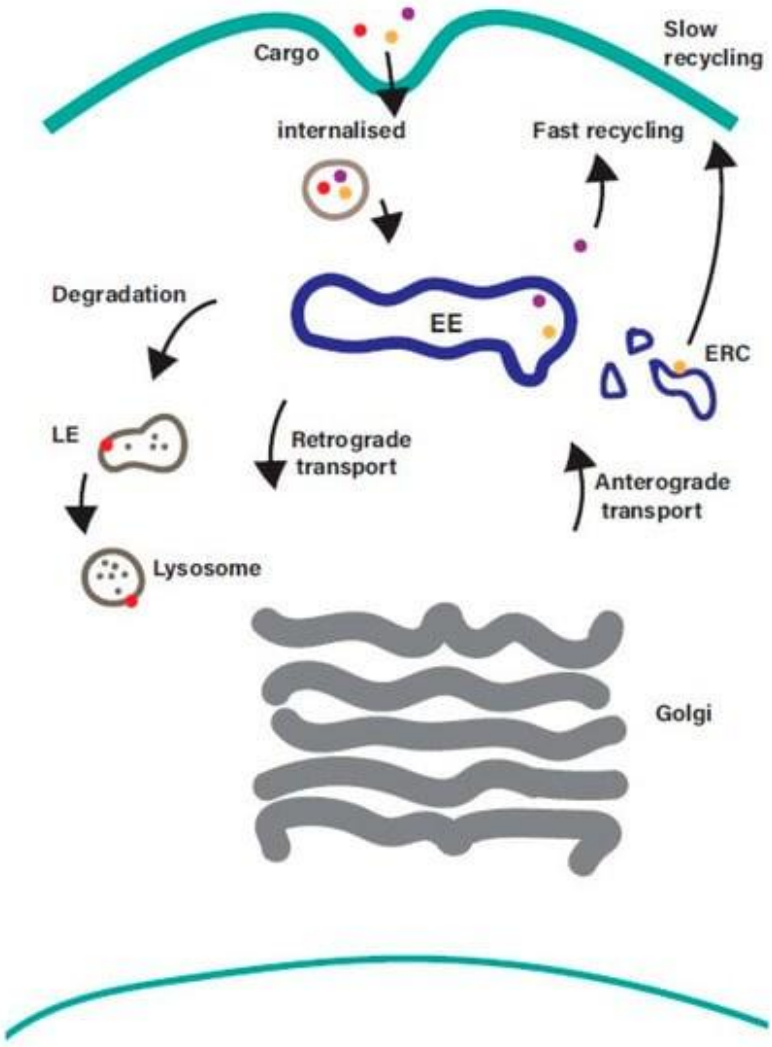


Figure 3: ***Endosomal recycling pathway.*** *Simplified flow diagram representing the endosomal recycling pathway. Internalization or endocytosis occurs through either clathrin- or caveolin-mediated pathway, leading to the early endosome. Here sorting happens, where the proteins to be degraded gets sent to the late endosomes, where it is transported to the lysosomes. It could also undergo recycling, wherein proteins are returned to the plasma membrane with the help of recycling endosomes [198]*

1.6.1. Caveolin Mediated Endocytosis

Senescent cells have been reported to express increased levels of Caveolin-1 (Cav1), a major structural and functional unit of Caveolae-mediated endocytosis (CavME) [167]. CavME involves the pinching off of vesicles by dynamin, followed by transportation to endosomal compartments [169]. It has been shown to be associated with lipid metabolism. A caveolin mutant was shown to result in decreased levels of free cholesterol and increased neutral lipid storage in lipid droplets [168]. Cav1, which is the most common isoform of caveolin, has been reported to be associated with a large number of signaling proteins. This includes RTKs, Src family kinase, and nitric oxide synthase 3 [170-172]. This was further confirmed when deletion or overexpression of Cav1 resulted in altered signaling activities [173]. It has also been reported that Cav1 inhibits NRF2 and other oxidative stress regulators, actively contributing to cellular ROS [174, 175].

It must be noted that only caveolar caveolin is involved in membrane trafficking, and that non-caveolar caveolin is more involved with signaling molecules. Furthermore, it was established that non-caveolar caveolin contributes towards hypo-responsiveness, generation of ROS or senescence phenotypes [166]. The exact role of caveolar Cav1 in senescence is largely unknown, as it is difficult to distinguish between caveolar and non-caveolar Cav1.

1.6.2. Clathrin Mediated Endocytosis

Parallelly, another mechanism of endocytosis is clathrin-mediated endocytosis (CME), where a group of adapter proteins are recruited to assemble and mediate invagination and incision of coated vesicles [166]. It was noted that amphiphysin-1, a key regulator of CME was reported to be downregulated in senescent cells [176]. Notably, this downregulation coincided with reduced endocytosis of TfR, and

restoration of amphyphysin-1 returned the endocytic capacity of the cell [176]. It was suggested that the reduced functionality of CME played an important role in the induction of senescence [166].

Beta PAK-interacting nucleotide exchange factor (βPIX), which is a GTPase activator, binds to G-protein coupled receptor kinase interacting proteins (GIT) and act as adaptor proteins with ArfGAP activity (a regulator of Arf family of GTPases), which as noted in the following section, is a part of the coatomer complex-1 (COPI) pathway [180]. Studies have shown that βPIX-GIT complex plays a critical role in amphihysin cleavage [166]. Both βPIX silencing and GIT knockdown have been shown to induce senescence, just as βPIX levels are shown to reduce in senescent cells [181]. Reduced βPIX levels resulted in destabilization of the paxillin-calpain complex, which cleaves amphiphysin and deactivates it [176]. Thus, senescence is linked to reduced CME through reduced amphiphysin activity.

1.7. Targeting Therapy-Induced Senescence as a Treatment Strategy

The differences in the morphology and functional properties of senescent cancer cells can render them a potent target and has led to many efforts to identify vulnerabilities that might be exploited to treat drug resistant cancers. One of the reported therapeutic advantages of senescence is the restriction in tumour progression. It has also been reported that senescent properties could spread from one cancer cell to the other, restricting the proliferation of neighbouring cancer cells as well [84, 104].

There are, however, detrimental effects to the induction of senescence via therapy. It has been shown to develop EMT and increase invasiveness of pre-malignant epithelial cells through the IL6- and CXCL8-dependent pathways [105].

The SASP provides an opportunity to develop a “one-two punch” strategy in treatment of cancers, with the help of senotherapeutics (senomorphics and senolytics, are small molecules that suppress some senescent properties or kill senescent cells respectively) [51, 52] [Figure 4]. Senotherapeutics is a growing area of drug discovery that has seen the usage of targeted approaches, reverse-pharmacology, drug-repurposing and other traditional methodologies [51]. A strategy that has been implemented to target senescence is the use of senomorphics or senostatics. These drugs suppress SASP, and in turn, decrease inflammation. The drawback of using senomorphics is that SASP is critical for wound healing [52].

It was first hypothesised that senescent cells upregulate anti-apoptotic and pro-survival pathways. This was confirmed by the continued survival of these cells even upon upregulation of pro-apoptotic phenotypes, indicating the presence of factors that can counter-act this cell death pathway. This led to the development of senolytics, which specifically target these anti-apoptotic pathways in senescent cells [106, 113]. These senescent cell anti-apoptotic pathways (SCAP) demonstrate similar properties to the anti-apoptotic pathways in B cell lymphoma and leukocytic leukaemia cells [114, 115]. Senolytic compounds were considered more potent if they could target more than a single SCAP node at a time, like BCL2 pathway inhibitors (e.g., N(ABT-263), A1331852, A1155463). This would allow them to attack a wider range of senescent cell types [107, 110].

Recent studies have identified COPI vesicles as a senolytic target in drug therapy-induced senescent cells [164]. The COPI vesicle system transports cargo to and from the Golgi apparatus. The COPI transporters (encoded for by *COPB2, COPG1* and *ARF*1) regulates a number of membrane-trafficking events by mediating transport in the early secretory pathway, via the Golgi apparatus [165]. Disrupting *COPB2* leads

to Golgi dispersal and induce apoptosis in senescent cells [164]. COPI vesicle formation is regulated by the ARF family of GTPases. GBF1, guanine nucleotide exchange factor is required for the activation of ARF GTPases. Conventionally, GBF1 inhibitors, like brefeldin A are used to disrupt COPI vesicle formation, but they are not clinically approved. However, N-myristoylation inhibitors (NMTi), a clinically approved treatment for parasitic protozoan infections, was shown to phenocopy COP1 inhibitors and act as a potent senolytic [164, 211].

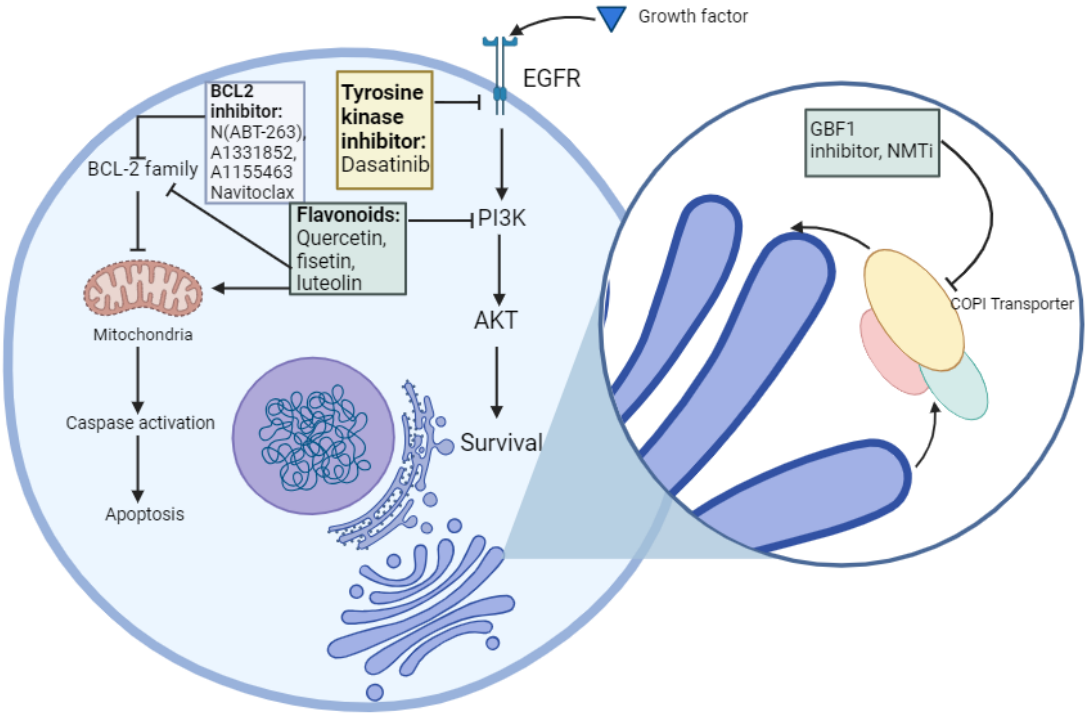


Figure 4: ***First generation senolytics and their corresponding target SCAPs.*** *Currently approved senolytics, their molecular targets and their mode of action. The senolytes have been classified into their respective categories [116, 164]*

Ideal senolytics should target several SCAPs, be able to be administered orally, and be already approved by the United States Food and Drug Administration (FDA) [116]. A wide-range of senotherapeutics have been identified so far, such as dasatinib,

quercetin, fisetin, luteolin, curcumin and navitoclax, which ticks all these boxes [106 - 111].

1.8. Immune-Tolerance and Membrane Trafficking

This thesis also looks into the innate defence mechanisms that tackle tumors, and the strategies implemented by breast cancers to overcome it; specifically, the capacity of tumor cells to develop immune-tolerance and survive attacks from T-lymphocytes. Immune-checkpoint proteins are those present on the surface of cells like macrophages, that bind to complementary sites on the surface of immune cell and trigger an "off" switch in order to prevent it from destroying the cell [182].

One such immune checkpoint protein is the programmed-death protein ligand 1 (PDL1), also known as cluster of differentiation 274 (CD274). PDL1 is a transmembrane protein usually located on the surface of macrophages, with N-terminal binding sites that bind to programmed cell death protein 1 (PD1) receptor on the surface of T cells. When bound, it restricts T cell activity, and evades destruction [183]. A dysfunction in this protein has been linked to the occurrence of auto-immune disorders, such as systemic lupus erythematosus (SLE) [184].

About 5-40% of tumor cells express PDL1, which can provide them immune-tolerance, and inhibit cytokine production and cytolytic activity [185, 186]. A number of studies have delved into the reduction of PDL1 expression levels [187-190]. There are currently nine approved immune checkpoint inhibitors on the market, seven of which block PDL1-PD1 binding to some capacity [191].

1.8.1. Endosomal Recycling of PDL1

The proteasomal-dependent degradation and autophagy-mediated degradation of PDL1 has been well characterized and summarized by Gou *et al.* [192] [Figure 5]. HIP1R, an autophagy induced protein, binds to PDL1, inducing lysosomal degradation [193]. However, cancer cells protect PDL1 from degradation using

palmitoylation modification via DHHC3, which is a palmitoyltransferase, or through the binding of CKLF-like MARVEL transmembrane domain containing protein 6 (CMTM6) [194, 195]. DHHC3 palmitoylates PDL1 at Cystine-272, which inhibits the ubiquitination and subsequent autophagy-mediated degradation of PDL1[195]. CMTM6 assists in the protection of PDL1 through promotion of recycling, whereupon it increases in stability [194]. It has also been noted that H1A reduces the affinity of CMTM6 to PDL1, promoting degradation [196].

Glycosylation of PDL1 has also been reported to inhibit the degradation of PDL1. EGFR, when activated, induces B3GNT3, an *N*-glycosyltransferase, which glycosylates PDL1 and in turn, inhibits tumor suppression [197].

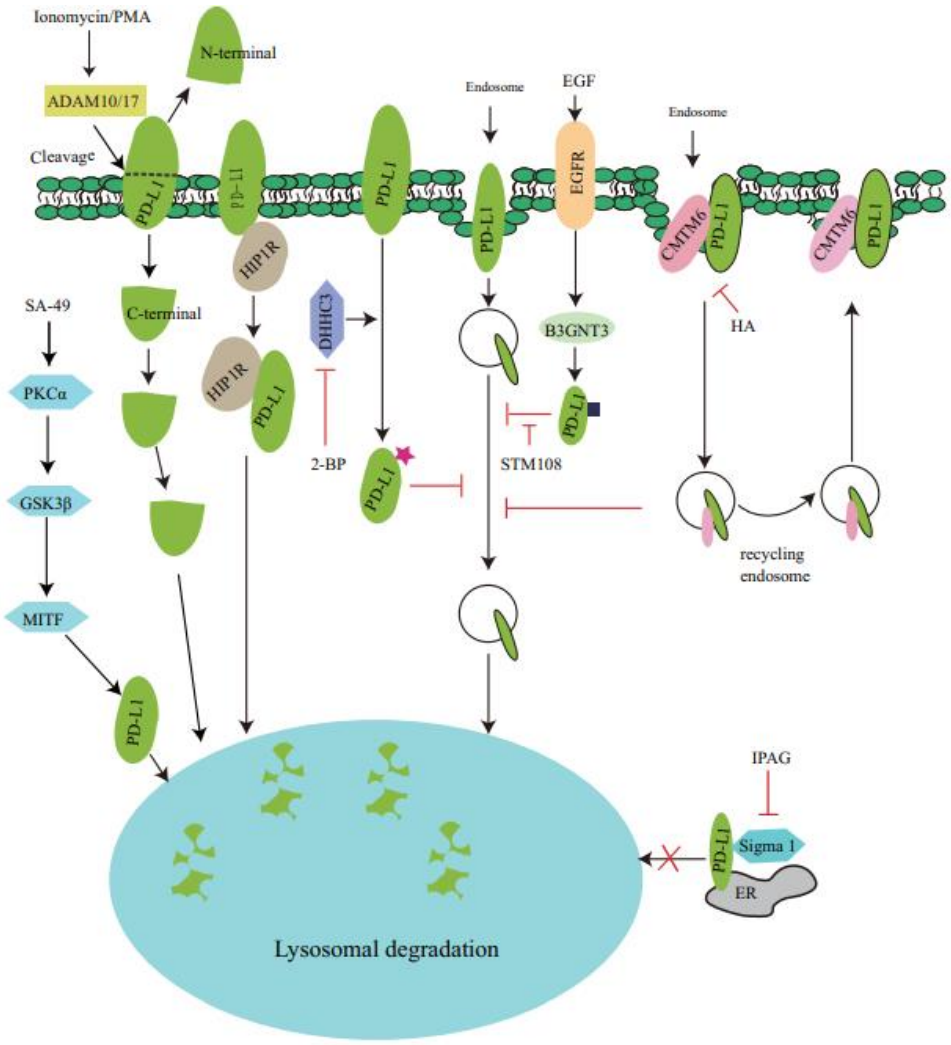


Figure 5: ***PDL1 autophagic degradation pathway.*** *Flowchart detailing the recycling and degradation of PDL1, along with the various regulating surface and messenger proteins involved in these processes[192]*

To better characterize PDL1 recycling, it is crucial to explore each element of the endosomal recycling pathway. The impact of surface PDL1 levels upon inhibition of various aspects of the recycling pathway could provide a comprehensive picture of the PDL1 recycling process.

One strategy that has been suggested for the inhibition of recycling is through the use of small molecule inhibitors [198]. These are organic compounds that weigh less than 900Da, and are capable of easy permeation of cell membranes on account of their small size. For instance, Plinabulin (BPI-2358) and 5-Benzylglycinyl-amiloride (UCD38B) are two such small molecule inhibitors that are currently being clinically tested for the treatment of NSCLC and high-grade glioma [199, 200] [Table 4]. The following is a list of small molecule inhibitors:

Drug:	Effect:
Primaquine	Interferes with recycling from endosomes to plasma membrane by inhibiting calmodulin function at endosome [203]
Monensin	Disrupts the recycling of cargo from the surface of the perinuclear recycling compartment and the endosome to the cell surface [204, 205]
Amlodipine	Inactivates Calpain through the reduction of intracellular calcium, leading to blockade of recycling endosomes [206]
Simvastatin	Impairs Rab5 driven endocytosis [207]
Resveratrol	Inhibits Rab coupling protein (RCP)-induced Zeb1 expression through blocking β1 integrin recycling and EGFR activation [208]
Plinabulin	Inhibits the polymerization of microtubules, leading to accumulation of KRAS in endosomal vesicles and an inhibitory effect on Akt phosphorylation and an increase on ERK activation. [200]
5-Benzylglycinyl-amiloride	Causes mitochondrial depolarization, leading to release and nuclear translocation of apoptosis inducing factors. This causes nuclear condensation and caspase-independent necrosis [199].

Table 3: *Small molecule endosomal recycling inhibitors and their effects*

Primaquine (PQ) is an endosomal recycling inhibitor that is currently approved on the market for malarial treatment. Derived from plasmoquine, it is the first available anti-malarial agent. PQ has been reported to inhibit endosomal recycling via the crucial calmodulin-dependent processes. Calmodulin is an intracellular calcium ion-bound protein that is important for transportation of messenger signals within the cells [228]. Several studies have suggested the repurposing of PQ for the treatment of tumours [229, 230]. Recent work from our group has also confirmed the synergistic benefits of PQ in the treatment of HER2+ cells [212].

Monensin is another endosomal recycling inhibitor which is currently approved in the market as an antibiotic. It has been reported that monensin blocks the recycling from early endosomes and perinuclear recycling compartment [204]. The drug was shown

to inhibit cell-cycle progression, proliferation and in some cases, induce apoptosis. Prior work from our group explores the anti-tumour properties of monensin in hormone-receptor rich cancer cells [231].

The aim of this study is to investigate the feasibility of using ERIs to treat drug resistant breast cancer. During the course of this study, we will attempt to discern the impact of PQ and monensin on the viability of drug therapy-induced resistant breast cancer cells, and the specific impact ERIs have on the iron metabolism in these cells. We will also explore the efficacy of a wide range of ERIs in the reduction of immune-suppression in HER2+ cells.

2. Materials and methods

2.1. Materials

All tissue culture media, materials and supplements were purchased from Sigma Aldrich (London, UK). The plasticware used was from Sarstedt. Doxorubicin was from Selleckchem (Cologne, Germany), monensin was from Sigma Aldrich, and the primaquine, lapatinib, simvastatin, resveratrol, amlodipine and MTT were from Carbosynth (Dublin, Ireland), UK. The drugs were reconstituted in DMSO (doxorubicin, lapatinib, simvastatin, resveratrol, and amlodipine), methanol (monensin), and saline (primaquine), and stocks were prepared for future treatments as follows:

Drug	Stock concentration and vehicle	Supplier
Lapatinib	50 mM in DMSO	Carbosynth
Doxorubicin	10 mM in DMSO	Selleckchem
Simvastatin	100 mM in DMSO	Carbosynth
Primaquine	50 mM in Saline	Carbosynth
Monensin	25 mM in Methanol	Sigma Aldrich
Resveratrol	100 mM in DMSO	Carbosynth
Amlodipine	40 mM in DMSO	Carbosynth

Table 4: *Stock concentrations, vehicle and supplier for each of the drugs ordered*

LysoTracker Red DND-99 was obtained from ThermoFisher, and made into stocks of 150 μM.

Antibodies specific for Transferrin Receptor (TfR) (Mouse) was sourced from Invitrogen, Ferritin Heavy Chain (FTH1) (Mouse) was sourced from Santa Cruz (Heidelberg, Germany), LAMP1 (Mouse) and pNRF2 (Rabbit) were sourced from AbCam (Cambridge, UK), PDL1 (Rabbit) was sourced from Cell Signalling Technologies, and α-Tubulin (Mouse, #T5168) was sourced from Sigma-Aldrich. 680RD-conjugated goat anti-Mouse (#C80619-5) and 800CW conjugated goat anti-Rabbit (#C80426-08) secondary antibodies were from LI-COR (Cambridge, UK). Plasticware to store the diluted antibodies was from Sarstedt (Wexford, Ireland).

2.2. Cell Culture

This study utilized HER2-positive, lapatinib sensitive cells (BT474 and SKBR3), HER2-positive JIMT1 and MDA-MB453 cells, which have innate resistance to lapatinib, hormone receptor positive (ER/HR) cells (MCF7 and ZR75-1), and Triple Negative Breast Cancer Cells (MDA-MB231 and BT549). MCF7 and SKBR3 were grown and cultured in Dulbecco's Modified Eagle Media (DMEM), JIMT1 and MDA-MB453 (Kind gift from Dr Alex Eustace, DCU, Ireland) were grown and cultured in Roswell Park Memorial Institute (RPMI 1640) media, BT474 was obtained from CalTag Medsystems (Buckingham, UK) and cultured in DMEM, ZR75-1 and MDA-MB231(kind gift from Prof. Rosemary O'Connor)(ATCC/LGC Standard, Middlesex, UK) were cultured in RPMI1640 and DMEM respectively. BT549 cells (ATCC/LGC Standard) were cultured in RPMI1640.

The culture media for each of these cell lines were supplemented with 10% foetal bovine serum(FBS), 1% L-Glutamine and 1% Penicillin-Streptomycin. All cell lines were cultured at 37^{o}C with 5% CO_2, verified to be mycoplasma free and authenticated occasionally by STR analysis (Eurofins, Ebersberg, Germany).

2.3. MTT Cell Viability Assay

The MTT assay, a method assessing mitochondrial reducing power, was employed to determine cell quantity and health. These assays, utilizing colorimetric techniques, gauge cellular metabolic activity and offer insights into cell viability, cytotoxicity, and proliferation. In the course of the assay, diphenyltetrazolium bromide undergoes reduction by metabolically active cells, forming formazan crystals, resulting in a colour change from yellow to purple/blue. Viable cells employ NAD(P)H-dependent oxidoreductase enzymes to reduce MTT to formazan. The crystals are dissolved by a solubilization solvent, and quantification was achieved by measuring absorbance at 570 nm using a multi-well spectrophotometer. A darker solution indicates a higher number of viable cells [232].

To assess the cytotoxic impact of various drug treatments, MTT assays were conducted. Cells were seeded in a 96-well plate at 5,000 cells/well with 100 µl of growth medium. Following the required duration and concentrations of drug exposure, 20 µl of 5mg/ml MTT solution was added to each well for 2-3 hours. The solution in each well was then removed and replaced with 100 µl of MTT solvent (4 mM HCl, 0.1% NP-40 in isopropanol) to solubilize purple crystals. A MultiSkanGo plate reader (Thermo Scientific) measured OD_{570} and OD_{630}. Wells were treated in triplicate, and each experiment included three or more biological replicates.

2.4. Fluorescence Microscopy

Cells were seeded onto 10 mm glass coverslips in a 24-well plate, at 50,000 cells/well in 500 µL of media. Once the cells had adhered, the required drug treatment was carried out. The plates were incubated for the required period of drug treatment, and then the media was removed. The wells were washed twice with pre-warmed PBS buffer, and fixed in warm 4% paraformaldehyde for 15 minutes.

After fixing, the wells were washed with PBS once and treated with quenching buffer (50 mM ammonium chloride/PBS) for 20 minutes. The quenching buffer was then removed, and the wells were washed with PBS. Permeabilization buffer (0.05% saponin, 0.2% BSA in PBS) was added to each well, and left on for 15 minutes in order to permeabilize and block the cells. Once the permeabilization buffer was removed, the wells were washed once more with PBS. The slides were taken out of the 24-well plate, and transferred into humid chambers.

About 50 µL of permeabilization buffer containing primary antibody was used to cover the cover slips. After an hour, the slides were washed twice with PBS. A solution of secondary antibody/permeabilization buffer with DAPI was prepared and added to the cover slips for an hour.

The cover slips were mounted on glass slides in 8 µL of MOWIOL solution. It was left in the dark overnight to dry and viewed with Nikon E600 fluorescence microscope the following day.

2.5. Senescence Assays

Cells were seeded into two 100 mm dishes, with one seeded with a twice the number of cells as the other. They were left to adhere overnight, and the dish with the higher cell count was treated with senescence-inducing agent. The dish with the lower cell count was treated with equal volume of vehicle (DMSO) for control.

The treatment was performed for the required duration, depending on cell lines. The concentration of therapy was determined by plating onto 6-well plate with varying concentrations. The cells were routinely checked for senescence under the microscope for change in morphology, and further verified using SA-βGal assay.

After the treatment, the cells were trypsinized and were plated into separate experiments, as mentioned below:

2.5.1. Determination of Viability to ERIs

The cells were plated onto 96 well plated and left overnight to recuperate and adhere after trypsinization. They were then treated with different concentrations of PQ and Monansin. The concentration ranged from 0.125x to 4x of IC50 for each drug, prepared through serial dilution. IC50 was assumed via literature or, in the absence of which, data on similar cell lines. The control was left untreated (in the case of PQ) or treated with methanol, which is the vehicle for Monansin. The treatment was carried out for a period of three days, following which MTT cell viability assay was performed.

GraphPad Prism was used to determine the IC50 value for each treatment in each cell line, and dose response data was generated. The difference between the senescent and control cell viability was noted.

2.5.2. SA-βGal Assay

The cells were plated in two separate 35 mm dishes after senescence treatment. They were left overnight to adhere to the plate wall. The plates were washed twice with pre-warmed PBS, and fixed with Fixation Buffer (0.2% Gluteraldehyde + 2% formaldehyde) for 20 minutes. During the fixation step, fresh SA-βGal staining solution was prepared (20 mM X-Gal + 500 mM Phosphate Buffer + 100 mM Citric Acid + 200 mM Sodium Dibasic Phosphate solution). Once the fixation buffer was removed, the plates were washed once with PBS, and then treated with 2 ml of staining solution each. It was left in 37^{o}C in a non-CO_2 incubator for 12-16 hours.

The staining solution was removed, and the plates were washed with PBS. The plates were quickly washed with 1 ml of 100% Methanol in the fume hood. After removing the methanol, the plates were left to dry for half an hour.

The cells were counted using a brightfield microscope, and the number of blue cells and colourless cells were counted. The ratio of blue cells to total cells was counted, and compared between drug treated and control cells. This protocol was carried out as suggested in Debacq-Chainiaux *et al.* [233].

2.5.3. Lysotracker Assay

After the required treatment to induce senescence, the cells were trypsinized and plated onto separate wells in a 24-well plate, with coverslips at the bottom of these wells. They were left overnight to adhere to the coverslips, and were then treated with 75 nM concentration of LysoTracker Red DND-99, and incubated at 37ºC in a humidified atmosphere with 5% CO_2 for an hour.

The wells were washed with pre-warmed PBS twice, and then fixed with pre-warmed 4% paraformaldehyde for 15 minutes. After discarding the paraformaldehyde, the wells were washed again with PBS, and quenched using 50 mM Ammonium Chloride/PBS for 20 minutes. After one more wash, the wells were treated with 1:10,000 ratio of DAPI/PBS solutions in order to label the nuclei of the cells. This provides an unbiased frame for imaging, and helps check the samples for the presence of mycoplasma.

After two more washes with PBS, the coverslips were mounted onto the slides with the help of 8 µL of MOWIOL. The slides were left to dry overnight, and viewed under confocal microscope for lysotracker intensity.

2.6. Immunoblot Analysis

Cell protein lysates were prepared using RIPA lysis buffer, supplemented with protease inhibitors (1% TX-100; 50 mM Hepes, pH7.4; 150 mM NaCl; 1.5 mM MgCl2, 1 mM EGTA; 10% glycerol + 10 mM NaF + 1 mM NaOV + 1X PIC + 10 mM Na. pyrophosphate + AEBSF + 10 mM b-glycerophosphate). Plates were washed with ice-cold PBS twice, then incubated on ice with RIPA buffer on, and shaken every 5 minutes. It was then centrifuged at 14000 rpm for 10 minutes at 4ºC. The supernatant was transferred into a fresh tube and the pellet (cellular debris) was discarded. Bradford assay was performed to determine protein concentration of supernatant. 4x loading buffer was added in 1:3 ratio and the samples were heated at 95ºC for 5 minutes to denature the proteins.

The proteins samples were first resolved using SDS-PAGE, where the protein sizes were tracked using Chameleon Duo Pre-Stained Protein Ladder. This was then transferred onto a nitro-cellulose blot through Western Blotting. The total protein level of each sample was measured by reversible staining using Revert 700 Total Protein stain (LiCor). The blots were scanned using the Odyssey Infrared Imaging system. The revert staining was washed off, and blocking was carried out using a solution of 1% Fish Gelatin/TBS for an hour. The blocking agent was discarded, and the primary antibody was added and left overnight.

The primary antibody was removed the following day, and the blot was washed twice with TBST, and once with TBS. It was then treated with IRDye conjugated secondary antibody for an hour. Once scanned on the Odyssey, the LiCor ImageStudio software was used to quantify the intensities of the bands, and the protein levels in the individual samples were calculated.

This was performed by using the protein image from the revert for normalisation, and comparing the relative intensity of the bands (against the control) to the revert values for that sample. The normalisation was done to account for errors in loading and transfer.

2.7. Flow Cytometry

JIMT-1 cells were seeded onto 6-well (100,000 cells/well) or 12-well plates (75,000 cells/well) depending on the experiment and allowed to grow till 65-70% confluency was achieved. The wells were treated with the required drugs for the planned interval, and incubated in the CO_2 incubator during this period.

After the required interval was complete, the media was removed, and the wells were washed with PBS twice. Each well was treated with 200 µL Accutase and left in CO_2 incubator till the cells were detached. Note that depending on the treatment, the cells may take different intervals to detach, so they were treated with Accutase until enough cells were detached in the slowest well. 0.8 ml of c-RPMI media was added to each well, and the net content was transferred into a 1.5 ml Eppendorf tubes.

The tubes were centrifuged at 500 rad/s for 5 minutes at room temperature, and the supernatant was discarded. The pellet was washed twice with Hank's Balanced Salt Solution (HBSS), and centrifuged at 500 rad/s for 5 minutes both times. The pellet was resuspended in incubation buffer (0.5% Bovine Serum Albumin (BSA) in 100 ml of HBSS), and each sample was split into two 1.5 ml dark Eppendorf tubes. One of each sample was transferred without staining into ice, and the other was treated with 1:10,000 ratio of PE Anti-Human PDL1 Antibody, vortexed and transferred to ice.

After an hour, the stained and unstained samples are centrifuged at 500 rad/s for 5 minutes. The pellet was resuspended in 500 µL of HBSS, and all the samples were

analysed on the BD Accuri C6 Flow Cytometer in the PE channel. The shift was recorded for all the samples, and the readings in unstained samples is subtracted from the stained samples in order to account for auto-fluorescence.

2.8. Statistical Analysis

Most experiments were performed in biological triplicates. All exceptions were specified. Student's T Test was used to determine statistical significance. 1-way analysis of variance (ANOVA) was used in specified cases using GraphPad Prism. Data was represented as a combination of the mean of all results, and the standard deviation between them. Significance was classified by p-value: *<0.05, **<0.01, ***<0.001, and ****<0.0001.

3. Results

3.1. Senescent Breast Cancer Cells Are More Sensitive to Endosomal Recycling Inhibitors

Several studies have reported that prolonged treatment with chemotherapeutics and targeted therapy have been reported to induce senescence in cancer cells, as a means of tolerating the stress caused by said therapy. This induction of cell-cycle arrest could open up channels for novel strategies to target drug tolerant persistor cells, based on the altered phenotype of the senescent cells.

Prior work from our lab has determined that BT474, a HER2-positive cell line becomes more sensitive to small molecule endosomal recycling inhibitor primaquine upon the induction of senescence [212]. This increased sensitivity was attributed to primaquine being an autophagy inhibitor. BT474, which demonstrated increased reliance on autophagic pathways upon induction of senescence, developed increased sensitivity to autophagy inhibitors.

Simultaneously, other studies have shown that monensin also demonstrates anti-tumour activity on drug-resistant cells [213]. However, monensin does not demonstrate autophagy inhibition, but rather affects the early endosomes and endocytosis, leading to an elevation in autophagic activity.

This section examines the effects of primaquine and monensin on senescent cells generated from cell lines representing different BC sub-types in order to develop a more detailed picture of the action of these ERIs on senescent breast cancer cells. ERIs have the potential to be re-purposed to treat resistant cancers [212].

3.1.1. Determination of pro-senescence agents for each cell line

This project focuses on drug-therapy induced senescence. To avoid variability, it would be best to use a common pro-senescence agent. However, there is no universal pro-senescence agent and we had to test a number of chemotherapies and therapies to induce senescence in the BC cell lines. The chemotherapeutic doxorubicin induced senescence in the TNBC and HR-positive cell lines. Lapatinib, a HER2- and EGFR-targeting TKI, induced senescence in the HER2-positive cell lines [63]. The dosage of treatment were determined experimentally or from concentrations reported in literature [Table 5].

Cell line	Sub-type	Drug	Dosage	References	Treatment duration	Primaquine	Monensin
JIMT1	HER2+	Lapatinib	10 µM	[212]	10 days	20 µM	1 µM
SKBR3	HER2+	Lapatinib	1 µM	-	5 days	10 µM	3.125 µM
BT474	HER2+	Lapatinib	100 nM	[212]	10 days	1 µM	-
MCF7	HR+	Doxorubicin	100 nM	[63]	4 days	20 µM	0.5 µM
ZR75-1	HR+	Doxorubicin	500 nM	-	5 days	20 µM	2 µM
MDA-MB-231	Basal-like	Doxorubicin	250 nM	[63]	8 days	12.5 µM	0.5 µM
BT549	Basal-like	Doxorubicin	100 nM	-	4 days	10 µM	1 µM

Table 5: *Pro-senescence treatment per cell line, and IC50 value of each ERI*

Two methods were used to confirm the induction of senescence; SA-βGal staining and labelling of lysosomes with LysoTracker Red. It was important to use two methods for verification of senescence, as not all cells will display increased SA-βGal staining upon induction of senescence. For instance, BT549 showed virtually no SA-βGal staining upon pro-senescence treatment but did not display increased LysoTracker Red fluorescence intensity [Figure 6 (*f*)]. In contrast, senescent and non-senescent MCF7 cells had similar LysoTracker Red intensities, but senescent MCF7 cells displayed much greater SA-βGal activity [Figure 6 (*c*)]. As reported by Dimri *et al.,* SA-βGal is not a universal indicator of senescence [214]. However, the cells demonstrated other senescent characteristics, such as elongation, and other changes in morphology. Furthermore, the lysotracker assay indicated the upregulation of autophagy, which is also characteristic for senescent cells. Ivanov *et al* has previously demonstrated the use of LysoTracker Red in the determination of senescence [242].

Conversely, MCF7 did not show much variation in lysotracker activity, but it demonstrated an elevation in SA-βGal activity, with a large number of cells being stained blue [Figure 6 (*c*)]. This does not indicate an absence of autophagic activity, as was confirmed via degradative autophagy-lysosomal marker LAMP1 [Figure 7] [223].

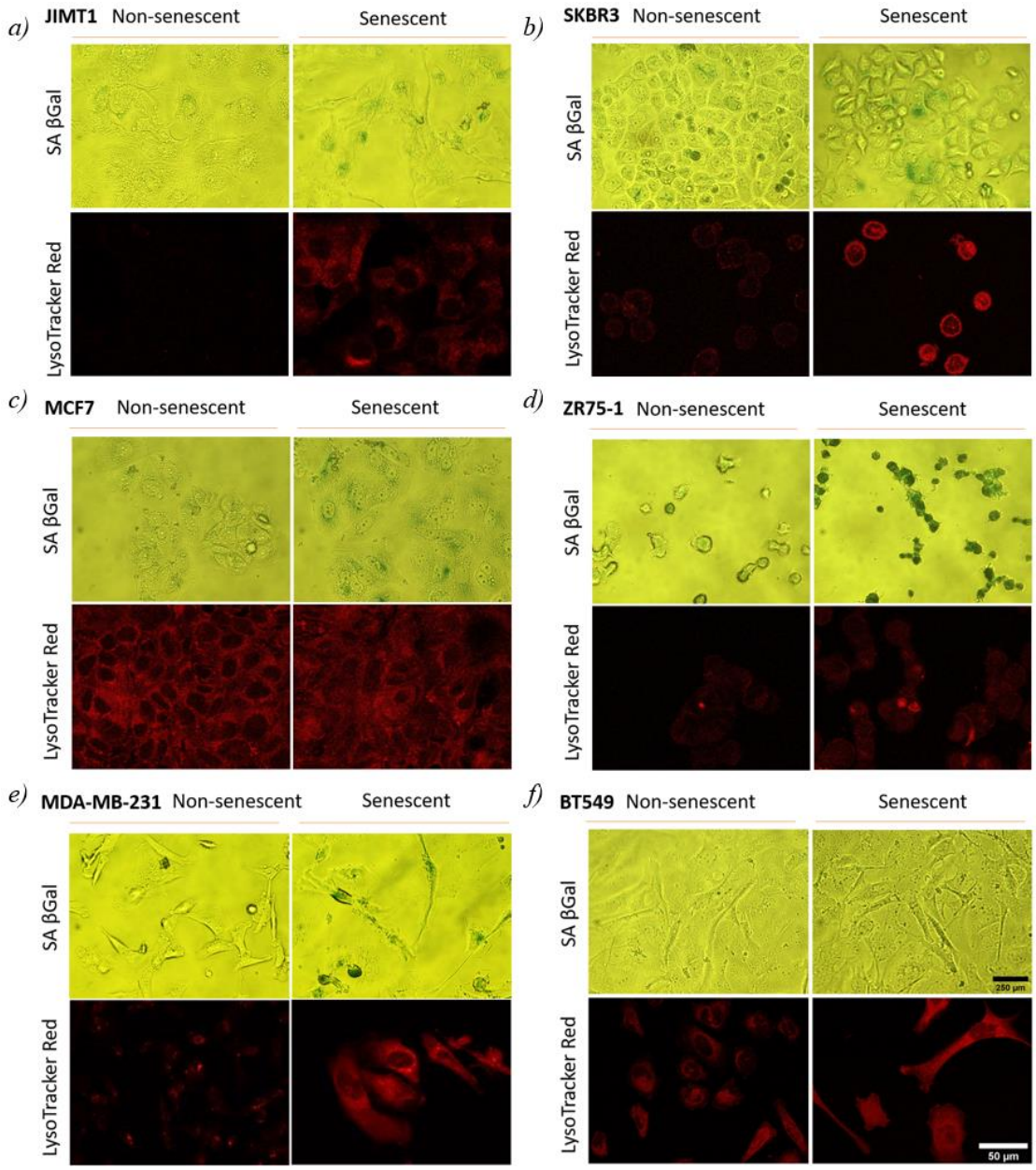


Figure 6: ***Induction of senescence in breast cancer cell lines***. *JIMT1 (a) SKBR (b) MCF7 (c), ZR75-1 (d), MDA-MB-231 (e), and BT549 (f) cells were treated with the indicated drug, or vehicle control for 4-10 days [Table 5] and tested for SA-βGal activity and observed with a brightfield microscope, or labelled with LysoTracker Red and imaged in confocal microscope. Scale bar, 250µm, 35µm.*

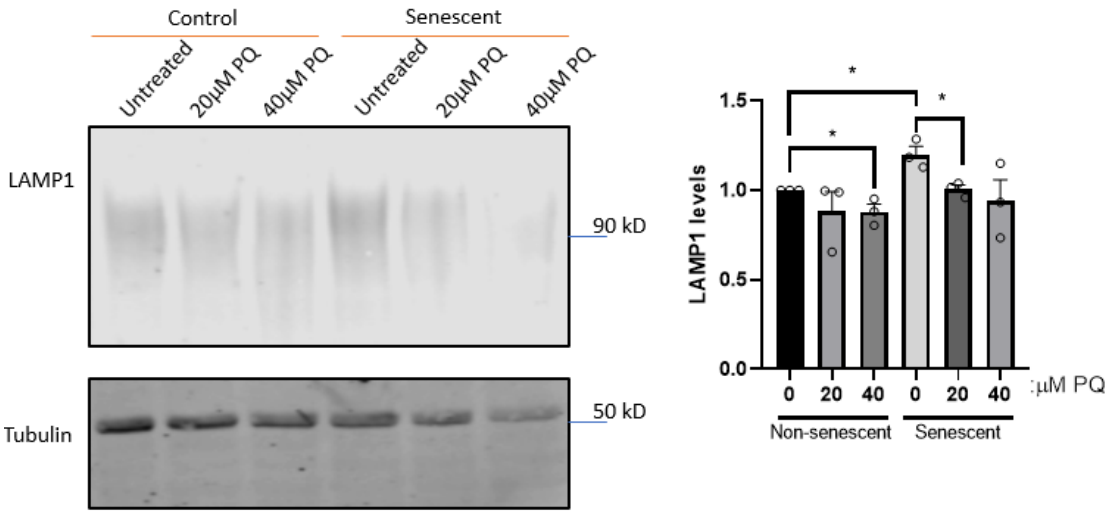


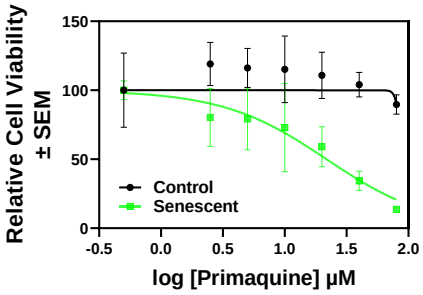
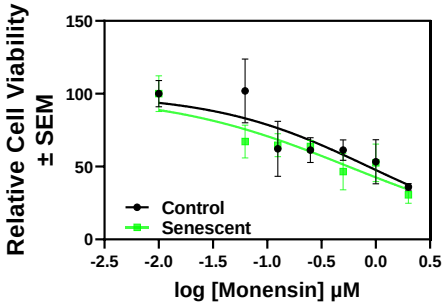
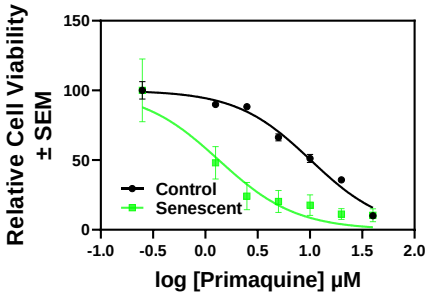
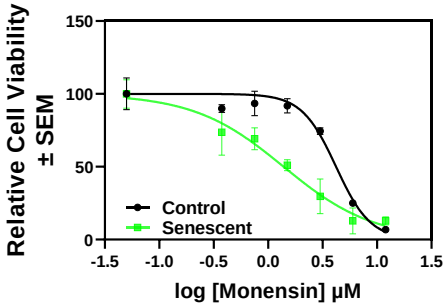
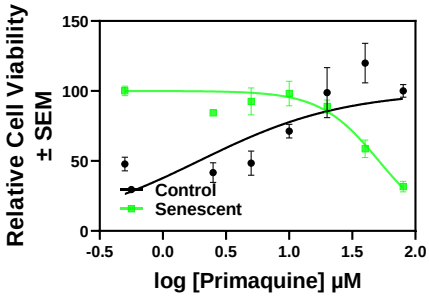
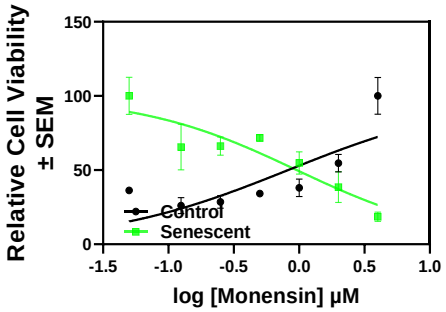
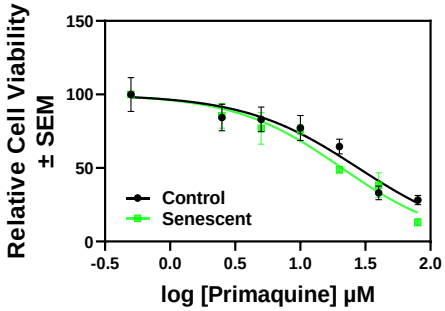
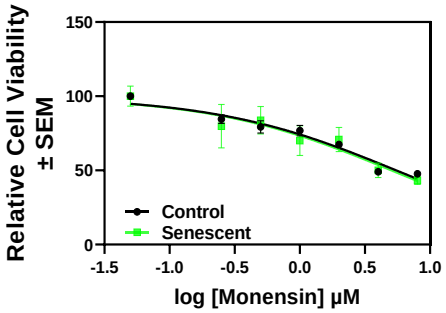
Figure 7: ***Confirmation of degradative lysosomal markers in MCF7****. Cells were treated with pro-senescence agents [Table 5] for the required durations, and then treated with IC50 and 2xIC50 values of PQ and lysates were prepared. Western blots were run and imaged on Odyssey Infrared Imaging System.*

3.1.2. Comparing viability of pro-senescence agents with endosomal recycling inhibitors

The IC50 value for primaquine and monensin was determined for each cell line by treating non senescent cells with increasing concentrations of each ERI for 72 hours and calculating the remaining viable cells using colorimetric MTT assay [Table 6].

To determine if senescent cells are more sensitive to the ERIs, the BC cell lines were seeded into two 100 mm dishes and treated with the senescence inducer, or vehicle control, for the required number of days [Table 5]. This produces pools of senescent and non-senescent cells, which were then seeded into 96-well and 24-well plates for further analysis. The cells in the 96-well plate were treated with increasing concentrations of both ERIs, followed by an MTT assay to construct a dose-response curve [Figure 8]. Induction of senescence was confirmed by performing SA-b-Gal

assays and LysoTracker Red staining on the cells seeded into the 24-well plates. The dose-response curves of the senescent and non-senescent cells were compared to determine if there is a difference in sensitivity to the ERIs depending on the cell state.

Cell line	Primaquine	Monensin
JIMT1		
SKBR 3		
MCF7		
ZR75-1		

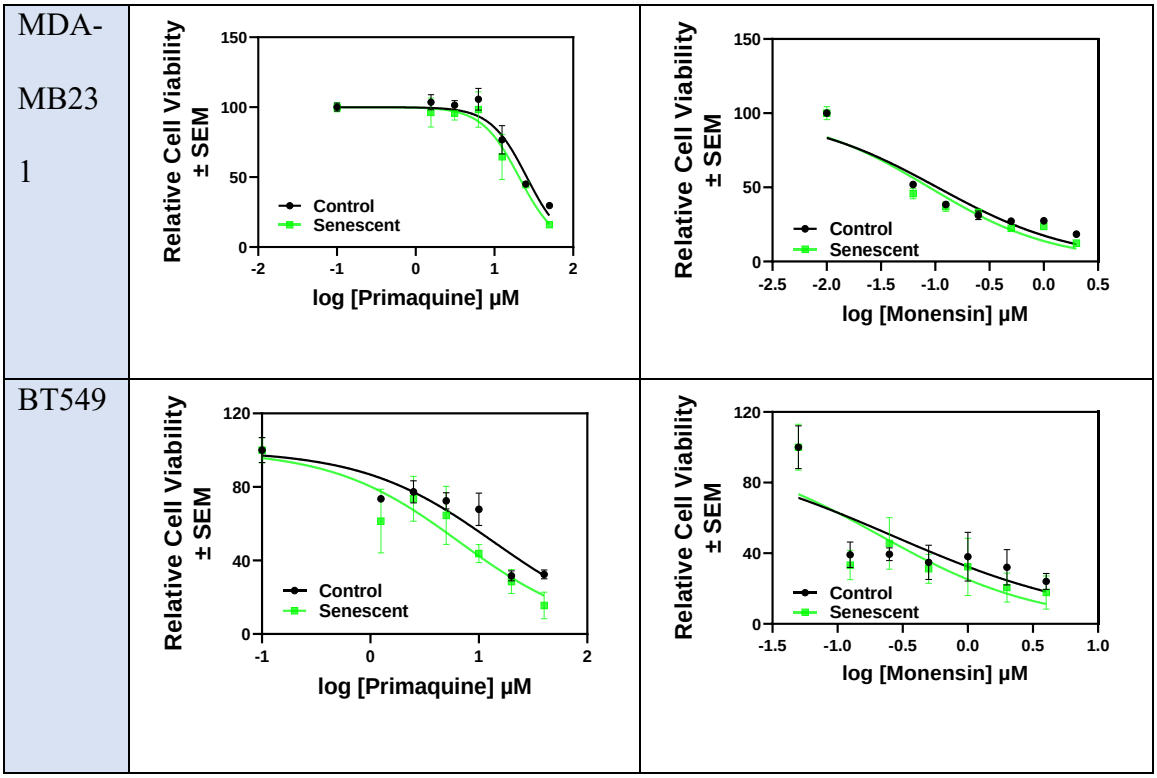


Figure 8: ***Senescent HER2-positive BC cells are more sensitive to ERIs.***

Representative dose response curves displaying the sensitivity of non-senescent and senescent cells to PQ and monensin. See Table 5 for quantification. Each experiment was carried out in triplicates.

Cell line	IC50 calculated for ERI (μM)							
	PQ				Monensin			
	Non-senescent		Senescent		Non-senescent		Senescent	
	Mean	Variation	Mean	Variation	Mean	Variation	Mean	Variation
JIMT1	39.93	10.07	27.65	8.51	0.176	0.081	0.060	0.022
SKBR3	10.16	2.19	1.345	0.94	4.205	0.88	1.386	0.645
MCF7	-	-	51.49	18.06	-	-	0.959	0.85
ZR75-1	27.92	13.15	20.86	6.96	5.506	2.62	5.054	5.198
MDA-MB-231	25.79	7.25	20.60	7.04	0.104	0.071	0.088	0.057
BT549	13.79	9.7	6.650	5.504	0.262	0.406	0.212	0.281

Table 6: *Calculated IC50 values for ERIs on senescent and non-senescent cells*

It was observed that each cell line behaved differently. JIMT1 and SKBR3 cells demonstrated heightened sensitivity to primaquine treatment, consistent with the observations made from previous work in our lab [212]. This indicates that HER2-positive senescent cells are collectively reliant on the autophagy pathway, and inhibition of this can result in apoptosis. The senescent SKBR3 cells were also more sensitive to monensin, whereas there was no difference in monensin sensitivity in the JIMT1 cells. SKBR3, like BT474 and unlike JIMT1, is highly responsive to lapatinib treatment. Monensin has been previously shown to affect the endocytosis of surface EGFR [224-226]. So, it is likely that TKI sensitivity might play a crucial factor in the endocytosis of surface EGFR.

MCF7 displayed an interesting trend as treatment with the ERIs induced proliferation of the non-senescent cells. This was consistent with prior observations [215], which

stated that these cells do not display any reduction in proliferative factors upon treatment with PQ. However, the reason for this phenomenon is unexplained. It is possible that upon induction of senescence, the cells lose capacity for proliferation, but generate substantially high number of interleukins via SASP and inflammatory response, leading to loss of cellular integrity, and eventually, cell death. No significant difference in sensitivity to ERIs was observed between senescent and non-senescent ZR75-1, MDA-MB-231 and BT549 cells.

Collectively, these results suggest that luminal B and triple negative sub-types of breast cancers are less sensitive, while HER2+ and luminal A sub-type of breast cancers are sensitive to the action of endosomal recycling inhibitors.

3.2. Therapy Induced Senescence Impact Iron Metabolism Pathways Varyingly

Prior studies have reported the impact of senescence on various iron transporters [69-79, 117-126]. It has been observed that there is approximately a thirty-fold increase in intracellular iron levels. While the collective consensus is that senescence can affect the expression levels of iron transporters, there have not been any clearly defined pathways for the same. Some studies have reported that the altered SASP can affect the iron metabolism, while others point at the oxidative and oncological stress generated by senescence. We will attempt to develop a comprehensive picture of the impact of senescence on iron metabolism.

Furthermore, we will also attempt to deduce the impact of endosomal recycling inhibitor PQ on this altered iron metabolism. The endosomal recycling of the transferrin receptor, an iron transporter that is responsible for the entry of ferrous iron into the cell, has been reported in prior studies [198]. It therefore stands to reason that

inhibition of this recycling could significantly impact the iron metabolism in senescent breast cancer cells.

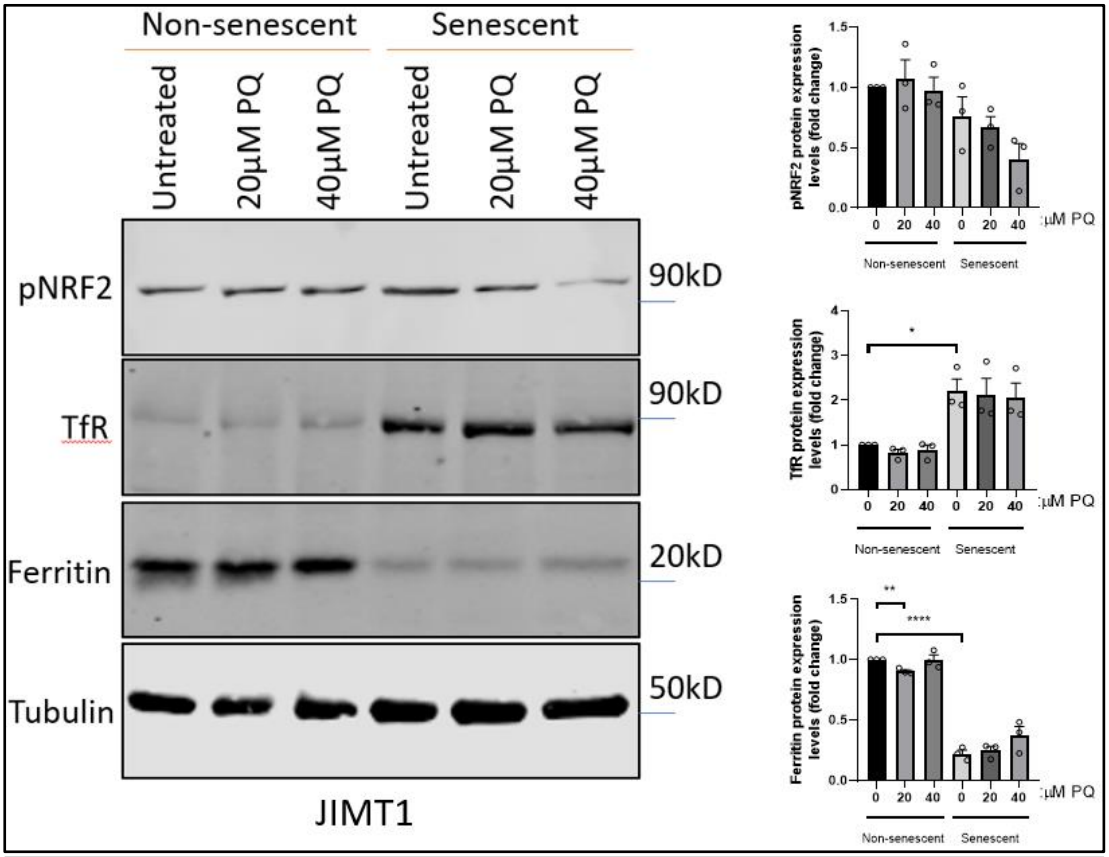
In order to develop a comprehensive picture of the impact of ERIs on the altered iron metabolism in senescent cells, lysates of senescent and non-senescent cells treated with two concentrations of PQ for 72 hours were prepared and the levels of proteins involved in iron transport were determined by Western blot. The control and senescent cells were treated with primaquine of IC50 and 2xIC50 in order to determine the sensitivity of iron metabolites to primaquine treatment. Lysates were prepared and run on Western blots.

It was observed that each cell line displayed varying characteristics, with some cells showing significant difference upon treatment with ERI, while others demonstrated no significant difference, even upon induction of senescence.

3.2.1. HER2-positive cells

JIMT1 cells did not demonstrate any significant changes in pNRF2 levels upon treatment with the ERIs, or upon induction of senescence, suggesting an inaction in the stress-response of the cell. Given the crucial role of recycled TfR in the activation of the NRF2 cytoprotection, their levels were also checked. Interestingly, there was a significant upregulation in the TfR levels in senescent cells, with a little over two-fold increase. This is consistent with observations made in prior studies, which is further substanciated by the elevation in intracellular iron content upon induction of senescence [71]. These results were determined by preparing lysates and running them on Western blots [Figure 9].

a)


Non-senescent
Senescent
Untreated
20μM PQ
40μM PQ
Untreated
20μM PQ
40μM PQ
pNRF2
TfR
Ferritin
Tubulin
90kD
90kD
20kD
50kD
JIMT1
pNRF2 protein expression levels (fold change)
TfR protein expression levels (fold change)
Ferritin protein expression levels (fold change)
0 20 40 0 20 40 μM PQ
Non-senescent
Senescent

b)

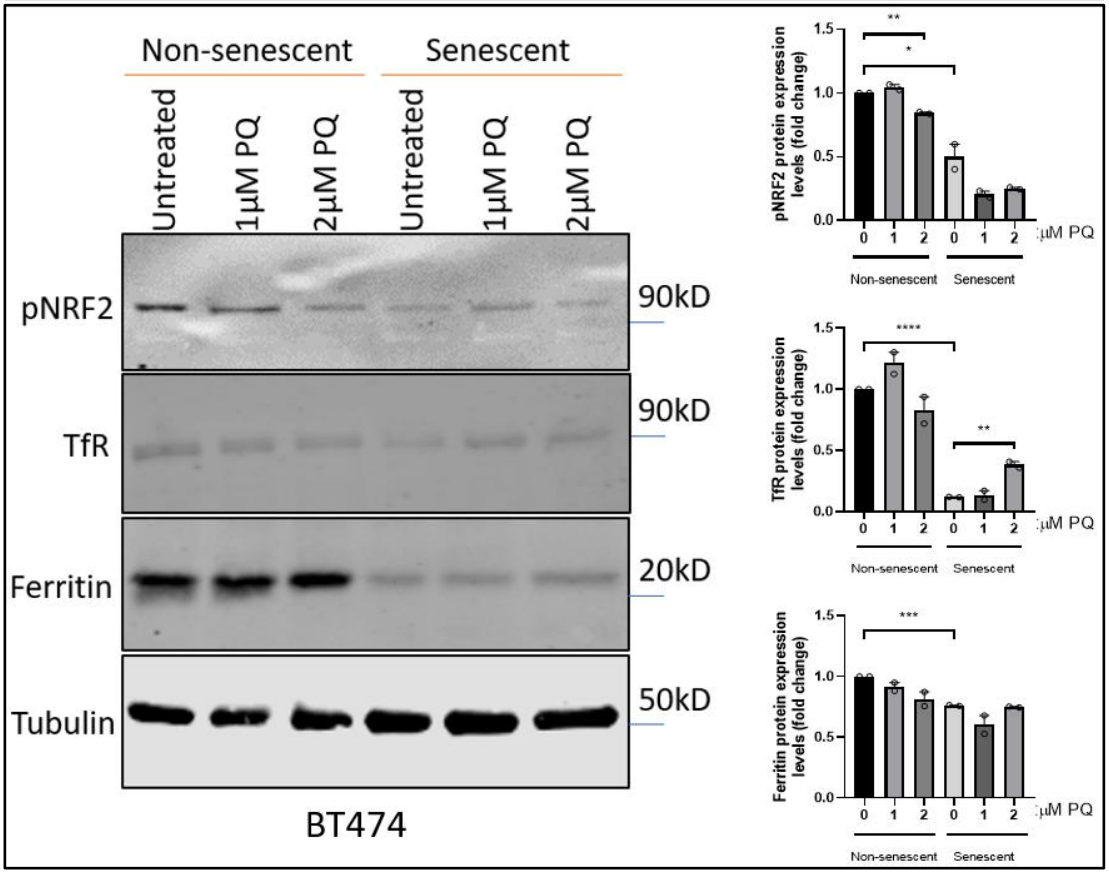

Non-senescent
Senescent
Untreated
1μM PQ
2μM PQ
Untreated
1μM PQ
2μM PQ
pNRF2
TfR
Ferritin
Tubulin
90kD
90kD
20kD
50kD
BT474
pNRF2 protein expression levels (fold change)
TfR protein expression levels (fold change)
Ferritin protein expression levels (fold change)
0 1 2 0 1 2 μM PQ
Non-senescent
Senescent

Figure 9: ***Impact of therapy-induced senescence and ERIs on iron metabolism in HER2-positive BC cells.*** *Representative blots depicting varying trends of the impact of therapy-induced senescence and ERI on iron transporters TfR and ferritin, and stress response transcription factor pNRF2 on (a) JIMT1 and (b) BT474.*

The upregulation in free iron should also be accompanied by a drastic decrease in the levels of intracellular iron-carriers such as ferritin. Ferritin levels were tested and determined to be drastically decreased, as expected [Figure 9 (*a*)]. It might be worth exploring the impact of senescence on transporter protein NCOA4, which is responsible for carrying ferritin molecules to lysosome for degradation, to better understand this reduction.

BT474 cells, which are more sensitive to TKI treatment demonstrated a very different behavior to JIMT1. There was a significant downregulation of pNRF2 levels in senescent cells, suggesting an inhibition to stress response [Figure 9 (*b*)]. This was suspected to be on account of reduced TfR which, as previously discussed, is crucial for the activation of the NRF2 pathway. This is consistent with the observed levels of TfR in senescent BT474.

This is an interesting observation, as it implicates primaquine in downregulating the stress response in control cells without affecting transferrin recycling. It is possible that primaquine is directly acting on the inhibitor of NF-κB kinase (IKK), which is crucial for the phosphorylation and activation of NRF2. There have been reports that suggest that primaquine could inhibit the NF-κB pathway [216].

The upregulation of TfR in senescent cells is consistent with an elevation in labile iron pool in the cell upon induction of senescence [218].

3.2.2. Hormone receptor-positive cells

It was observed that pNRF2 levels decreased in MCF7 cells. Much like in the previous instances, a corresponding decline in TfR was observed [Figure 10].

High doses of ERI treatment also resulted in reduced pNRF2. However, no such corresponding feedback was observed in TfR levels. This indicates that the activation of stress response pathway upon treatment with ERI does not affect the TfR recycling pathway. This could be the inhibitor of NF-κB kinase (IKK), which is crucial for the phosphorylation and activation of NRF2. There have been reports that suggest that primaquine could inhibit the NF-κB pathway [216].

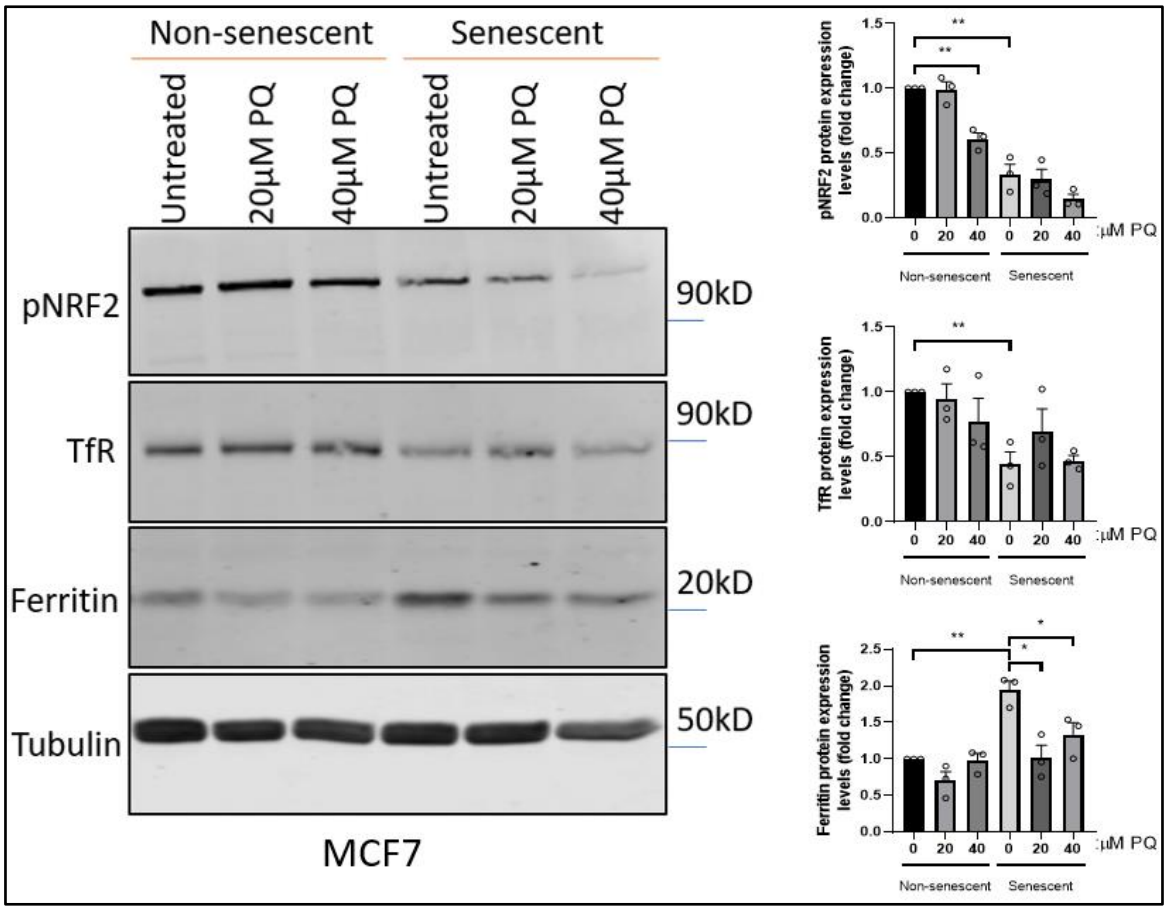


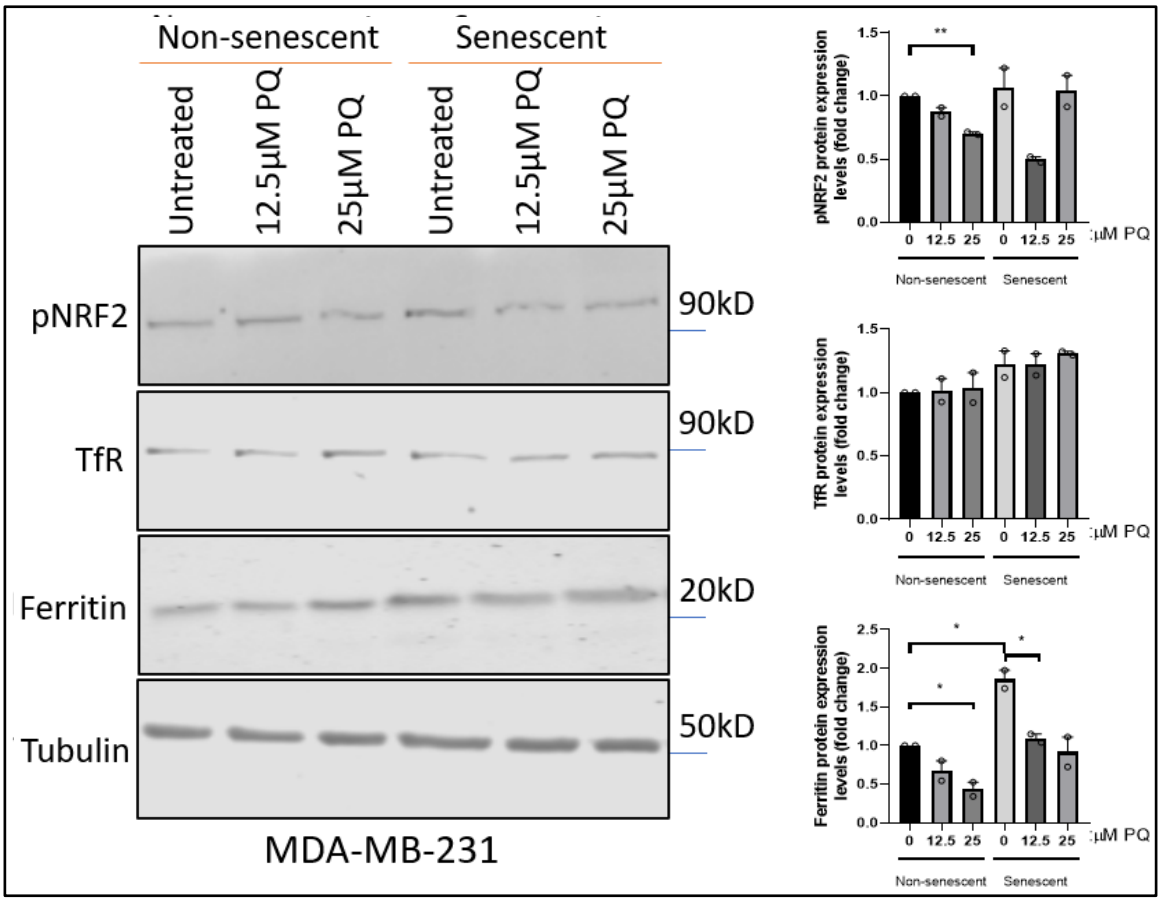
Figure 10: ***Varying impact of therapy-induced senescence and ERIs on iron metabolism in hormone-positive BC cells.*** *Representative blots depicting varying trends of the impact of therapy-induced senescence and ERI on iron transporters TfR and ferritin, and stress response transcription factor pNRF2 on MCF7 cells*

Contrastingly, ferritin levels increased dramatically in senescent MCF7 cells [Figure 10(*a*)], despite the decline in TfR. This is interesting, as prior studies have shown that ferritin drives proliferative activity in MCF7 [217]. However, senescence is characterised by the absence of proliferation, suggesting an altogether new model of drug-therapy induced senescence. Once again, NCOA4 levels might provide a better insight into the specifics of this occurrence.

3.2.3. Triple negative cells

MDA-MB231 cells did not display any drastic changes pNRF2 or TfR levels that could be characterised as a result of senescence or ERI treatment [Figure 11]. It did, however, demonstrate interesting trends in ferritin levels. It was observed that induction of senescence significantly upregulated the levels of ferritin, while treatment with ERI downregulated it. This suggests that chemotherapeutic induction of senescence in MDA-MB231might be an inhibitor to the ferritinophagic pathway.

a)


Non-senescent
Senescent
Untreated
12.5μM PQ
25μM PQ
Untreated
12.5μM PQ
25μM PQ
pNRF2
TfR
Ferritin
Tubulin
90kD
90kD
20kD
50kD
MDA-MB-231
pNRF2 protein expression levels (fold change)
TfR protein expression levels (fold change)
Ferritin protein expression levels (fold change)
0 12.5 25 0 12.5 25 μM PQ
Non-senescent
Senescent

b)

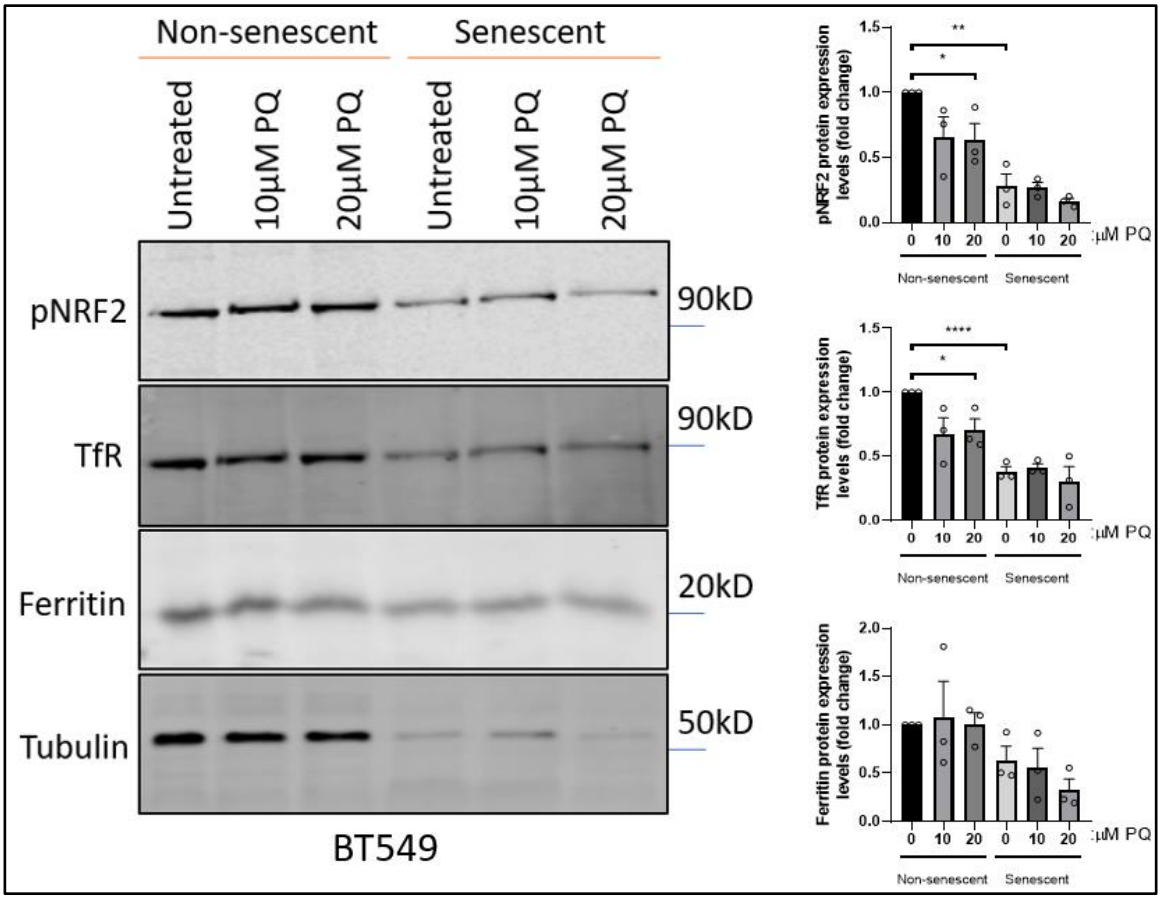

Non-senescent
Senescent
Untreated
10μM PQ
20μM PQ
Untreated
10μM PQ
20μM PQ
pNRF2
TfR
Ferritin
Tubulin
90kD
90kD
20kD
50kD
BT549
pNRF2 protein expression levels (fold change)
TfR protein expression levels (fold change)
Ferritin protein expression levels (fold change)
0 10 20 0 10 20 μM PQ
Non-senescent
Senescent

Figure 11: ***Varying impact of therapy-induced senescence and ERIs on iron metabolism in triple-negative BC cells.*** *Representative blots depicting varying trends of the impact of therapy-induced senescence and ERI on iron transporters TfR and ferritin, and stress response transcription factor pNRF2 on (a) MDA-MB-231, and (b) BT549.*

A decrease in ferritinophagy is associated with elevation in ferritin, but also a decrease in lysosomal activity. As observed from Figure 6(*e*), the LysoTracker Red result suggests a very autophagy-like elevation in lysosomal activity. It is unclear as to what these results collectively suggest.

BT549 cells showed no difference in ferritin levels, but demonstrated significant downregulation in pNRF2 and TfR levels, both upon induction of senescence, as well as upon treatment with high doses of ERI [Figure 11(*b*)]. However, upon induction of senescence, ERI did not contribute to any significant changes.

These findings collectively suggest that primaquine could affect the interplay between TfR and NRF2. Simultaneously, induction of senescence reduced internal TfR levels, but inexplicably, the iron metabolism of these cells seems unaffected by ERI treatment. It might be worth looking into the labile iron levels to confirm this, and run a proteomic analysis in the future to determine the variations in the action of ERI in senescent BT549s.

It was also observed that in BT549, there was downregulation in tubulin, which was being used as a housekeeping protein. This has been reported in other papers, so to indicate uniformity, images of the REVERT Total Protein Stain are represented in Supplementary Image (1).

3.3. Effect of endosomal recycling inhibitors on PDL1 in JIMT1 cells

PD-L1 has previously been reported to undergo endosomal recycling [198]. We hypothesised that chemical inhibition of endosomal recycling could lead to a reduction of PD-L1 protein levels, and thus be an alternative means of downregulating PD-L1 activity. To investigate this, we first determined the IC50 concentrations of five ERIs using MTT assays in JIMT1 cells [Table 7]. The high levels of PDL1 in JIMT1 provide clearer insight into the changes in PDL1 levels upon treatment with said ERI.

Endosomal recycling inhibitor	IC50
Amlodipine	40μM
Resveratrol	100μM
Simvastatin	70μM
Monensin [previously established]	1μM
Primaquine [previously established]	20μM

Table 7: *IC50 values determined for each ERI*

Next, the JIMT1 cells were treated with the ERIs at IC50 concentration for 24 and 48 hours. Lysates were prepared, and the level of PDL1 was determined by Western blots [Figure 12].

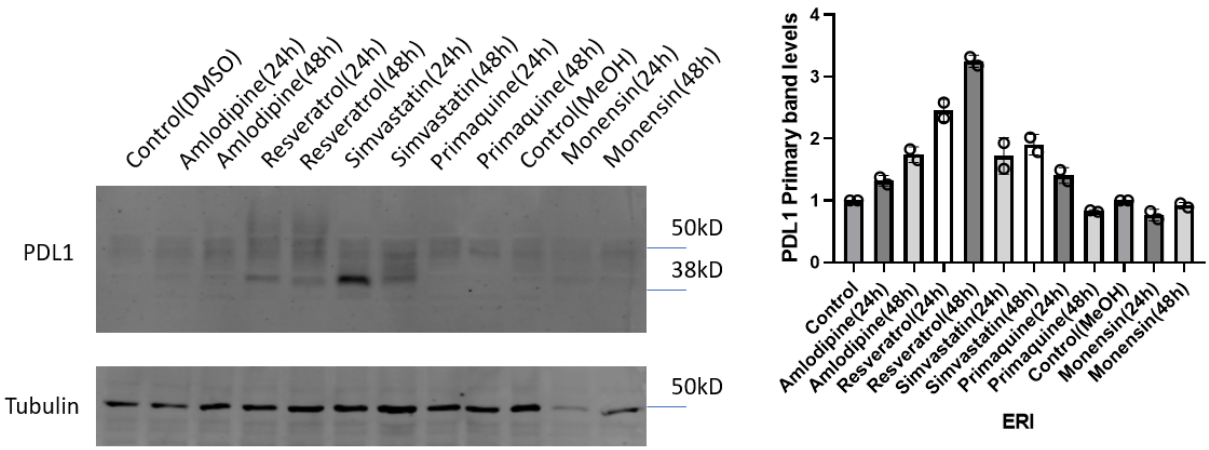


Figure 12: ***Western blot for PDL1 levels in JIMT1 upon treatment with ERIs for 24 and 48 hours****. DMSO was used as control vehicle for amlodipine, resveratrol, simvastatin and primaquine, while methanol is used as control for monensin. Histogram represents levels of PDL1 bands at ~50kD size, known as primary band.*

It was observed that resveratrol, simvastatin, primaquine and monensin had varying effects on PDL1 levels. Most of the treatments generated a lower (~37kD mark) band, which, as per literature, could be an unstable isoform of PDL1 [222]. It was observed that resveratrol induced a time-dependent elevation of PDL1 levels [Figure 12]. This phenomenon has been observed previously and been attributed to resveratrol targeting the Wnt pathway [219]. Dysfunction of Wnt has been reported to result in alteration of PDL1 levels, on account of a cross-talk between the Wnt activity and PDL1 expression [227]. Surprisingly, the levels of PDL1 were also elevated upon treatment with simvastatin. Simvastatin has been reported to reduce PDL1 levels in previous studies by antagonizing tumour necrosis factor-α, which when downregulated, leads to PDL1 depletion [220, 221].

Primaquine was observed to elevate PDL1 levels in 24 hours, but demonstrated a steep decline upon 48 hours of treatment. This suggested the elevated expression of an

unstable PDL1, that could have undergone degradation over time. This instability could be attributed to the dysfunction of post-translational modifications (e.g., glycosylation, ubiquitination), which are crucial for the stability of PDL1 [192].

Contrarily, monensin and amlodipine do not show any significant changes on the PDL1 levels. However, the band developed at the ~37kD mark appears to be upregulated at 24 hours, and reduced at 48. Literature suggests that this lower band could have formed on account of post-translational modification of PDL1 [222].

The above was repeated with the help of de-glycosylation agent tunicamycin in order to verify the de-glycosylation of the bands [Figure 13].

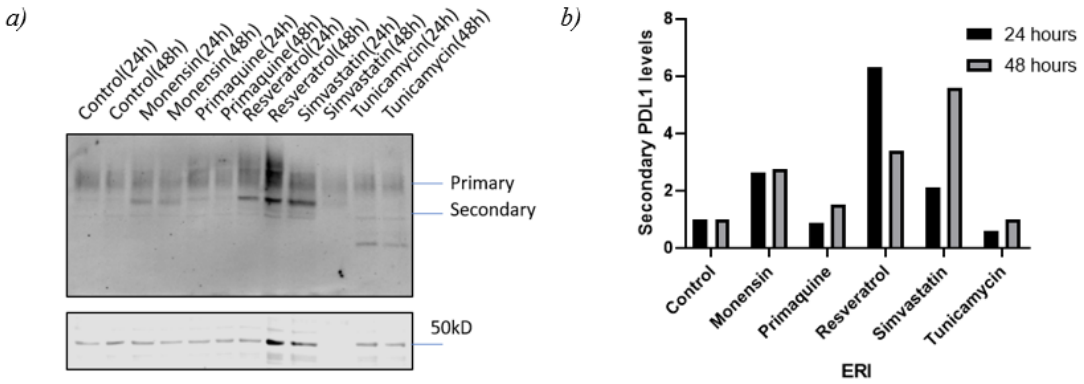


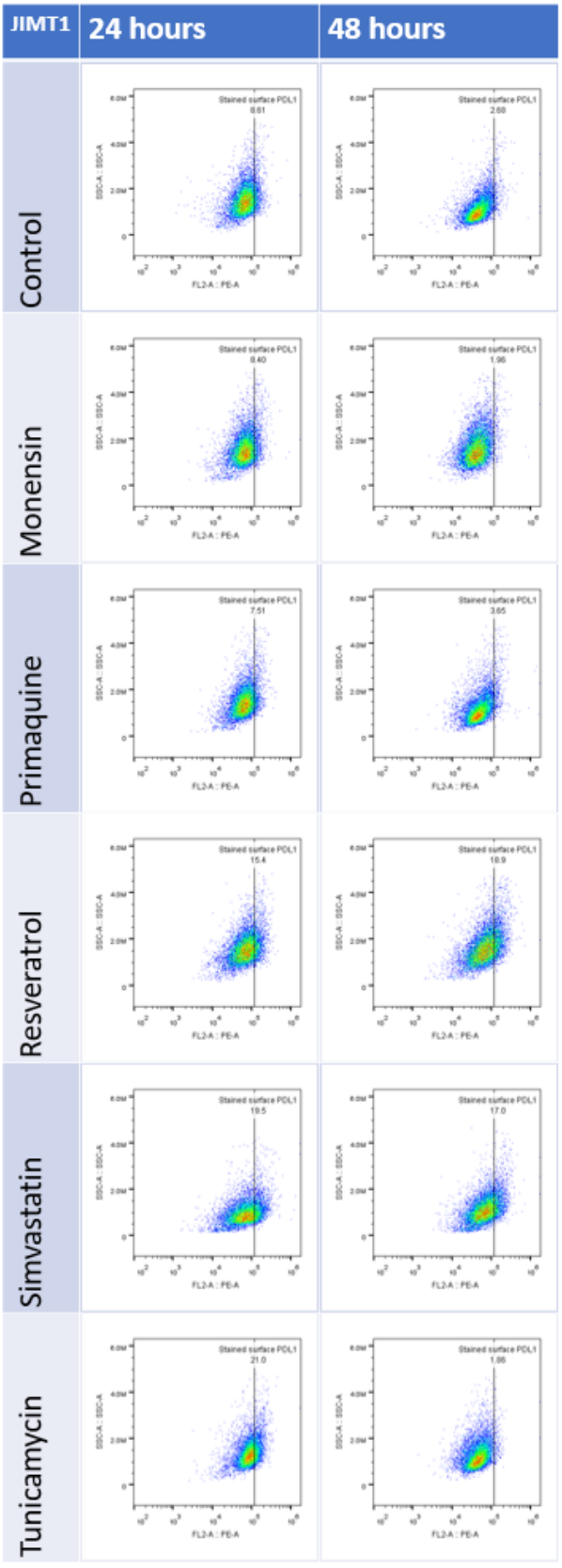
Figure 13: ***Verification of de-glycosylation of PDL1****. The lysates were prepared by treating the cells with ERIs as well as tunicamycin for 48 and 24 hours. (a) The Western blots were generated, and (b) the secondary bands (~37kD) were quantified and tabulated.*

The bands generated do not coincide with the de-glycosylated bands generated upon tunicamycin treatment. It is possible that other post-translational modifications might

be responsible for the secondary bands. This will have to be confirmed in future studies via mass-spectrophotometry.

PDL1 functions by binding to its complementary PD1 on the surface of T-cells. It is therefore the surface-PDL1 that contributes to immune-tolerance in cancer. Fluorescence associated cell sorting was performed to determine the surface levels of PDL1 upon treatment with ERIs, in order to see if they correspond to the data obtained from total PDL1 [Figure 14].

a)


JIMT1
24 hours
48 hours
Control
Monensin
Primaquine
Resveratrol
Simvastatin
Tunicamycin

b)

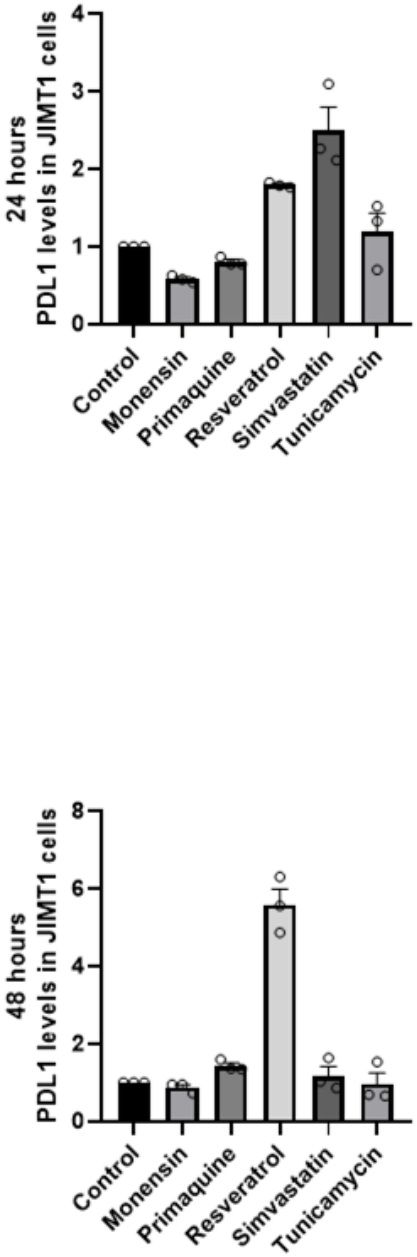

24 hours
PDL1 levels in JIMT1 cells
0
1
2
3
4
Control
Monensin
Primaquine
Resveratrol
Simvastatin
Tunicamycin
48 hours
PDL1 levels in JIMT1 cells
0
2
4
6
8
Control
Monensin
Primaquine
Resveratrol
Simvastatin
Tunicamycin

Figure 14: ***FACS frameshift to determine surface-PDL1 levels upon 24 and 48 hours of treatment with ERI****. a) FACS frameshift recorded in JIMT1 upon various ERI treatments (Control, Monensin, Primaquine, Resveratrol, Simvastatin, Tunicamycin), b) Graphical representation of PDL1 levels. All experiments were carried out in triplicates.*

It was observed that surface levels of PDL1 reduced upon 24 hours of treatment in both primaquine and monensin treated samples. However, while PDL1 levels remained low in the monensin treated samples, the primaquine treated samples demonstrated upregulation upon 48 hours of treatment [Figure 14(*a*)(*b*) and (*g*)].

If surface PDL1 levels are reduced due to the downregulation of its endosomal recycling, this would imply that both primaquine and monensin inhibit the return of PDL1 to the surface. However, it appears as though monensin has a long term affect on PDL1. It is also possible that monensin affects the stability of endocytosed PDL1, which is likely, considering Figure 13.

Both resveratrol and simvastatin demonstrate a significant upregulation in surface PDL1 at 24 hours, possibly on account of inhibited endocytosis. However, simvastatin treated samples show a steep decline in surface PDL1. This could be on account of the pleitropic action of statins which could affect the post-translational modification in surface proteins, as has been reported in prior studies [221].

Taken together, it appears as though primaquine enables a short term reduction of surface PDL1 levels, but does not reduce total PDL1; monensin inhibits recycling and also possibly affects the stability of PDL1; Resveratrol inhibits PDL1 uptake, and contributes to the upregulation of PDL1, and therefore likely to be detrimental for the

reduction of immune-tolerance; Simvastatin inhibits PDL1 uptake initially as well, but affects the stability of PDL1, resulting in deterioration of the protein.

4. Discussion

Breast cancer is the most prevalent form of cancer. It accounts for annual mortality rate of over 600,000 people globally. Conventional treatments have contributed towards the reduction of mortality and improved standards of living in patients. Among these are the usage of chemotherapies and targeted therapeutics. Annually, millions of euros are spent towards these drugs, for both pre- and post-surgery applications.

However, prolonged periods of treatment with these drugs have resulted in the development of resistance to them. This can increase the mortality of patients and worsen standards of living, so it is crucial to understand this development of drug resistance.

One of the avenues through which drug resistance is developed is therapy-induced senescence. This is the quiescent state that cells attain in order to develop resilience to therapy. This can be attained through prolonged treatment with radiation or drugs. In the present study, senescence was induced through treatment with Lapatinib (HER2+ cells) or doxorubicin (non-HER2+ cells) for specific durations, at fixed concentrations per cell line. The induction was verified by staining for SA-βGalactosidase, which is a characteristic of senescent cells, and cross-verified with LysoTracker Red assay to check for lysosomal level, which is also elevated in senescent cells.

Prior work from our lab has established the heightened sensitivity of therapy-induced senescent BT474 HER2+ cells to an endosomal recycling inhibitor called primaquine [212]. Here, we have determined that other HER2+ cells also demonstrate the same elevated sensitivity to PQ, but not to other ERIs. Interestingly, senescent JIMT1 and MDA-MB-453 [Refer to Supplementary Images (2)], both of which have innate

resistance to lapatinib, showed elevated sensitivity to primaquine, but not to monensin. Meanwhile SKBR3, which is highly lapatinib sensitive, displayed heightened sensitivity to both. We concluded that lapatinib-resistant HER2+ cells demonstrate increased sensitivity to primaquine, but do not demonstrate any difference in sensitivity to other ERIs tested.

We observed that exposure to ERIs led to increased proliferation of the hormone receptor positive MCF7 BC cell line. This contradicts a previous study, that did not find any effect of ERIs on the proliferating capacity of MCF7 [215], and present results indicate that ERIs may actively contribute to the proliferation of these cells. However, ERIs have a highly cytotoxic effect on senescent MCF7 cells.

This was specific to MCF7 cells, as there was no difference in ZR75-1 cells, which are also HR positive. This difference in behaviour is quite interesting, as these cells have several characteristics that distinguish them from each other. The main difference is ZR75-1 cells are a Luminal B sub-type of HR+ BC, while MCF7s are Luminal A. It is possible that the high content of proliferative marker Ki-67 in the Luminal B sub-type could be responsible for the difference in sensitivities between the two cell lines. Literature has characterised the recycling and regulation of Ki-67; it has been suggested that Ki-67 is transferred to the endoplasmic reticulum, from where it can either be degraded or recycled, depending on the requirement, thereby regulating it [234]. It is likely that the ERIs are affecting the Ki-67 levels in non-senescent cells, upregulating it. This might also suggest that the induction of senescence can alter the recycling pathway, since in this proliferative-arrest, it is likely to promote degradation of Ki-67.

Furthermore, this would also explain the lack of difference in ERI-sensitivity between senescent and non-senescent triple-negative MDA-MB-231 and BT549 cell lines. TNBC, like Luminal B sub-type, have significantly higher Ki-67 content than HER2+ and Luminal sub-type A cells [235]. It is possible that the ample expression of Ki-67 in TNBCs is compensating for any degradation that occurs.

A characteristic feature of senescence is the presence of oxidative stress. This is usually on account of the development of SASP, the expression of anti-apoptotic proteins, or simply stress induced by change in the tumour microenvironment. In any case, the presence of stress can usually be tested through the detection of the stress response transcription factor NRF2, or more specifically, the activated form, phospho-NRF2, where NRF2 is phosphorylated at Serine-40. Theoretically, induction of senescence, which causes oxidative stress through SASPs, should elevate pNRF2 levels, which should further be amplified by the stress caused by treatment with ERIs. However, as observed in BT474, MCF7 and BT549, this was not the case. The induction of senescence significantly reduced the levels of pNRF2. Furthermore, non-senescent BT474, MCF7, MDA-MB-231 and BT549 demonstrated some degree of reduction in pNRF2 upon treatment with ERI, while none of the senescent cells demonstrated any significant changes. This could be on account of a breakdown in the stress response pathway.

The activation of NRF2 occurs when NF-κB phosphorylates it, leading to the detachment of Keap1 protein from it. NF-κB is further activated by the IKK complex, which phosphorylates an inhibitory protein bound to NF-κB. There is evidence in literature to support the role of TfR in the activation of the IKK complex [71]. The altered endocytosis of TfR in senescent cells has been documented [166], and it is possible that the effect that ERIs have on the uptake of TfR could contribute to the

dysfunction of the oxidative stress response system. This could, in-turn, suggest impacts of senescence on the other iron transporters, and iron metabolism in general.

Hence, we examined the effect that senescence induction has on iron transporter levels. It was observed that MCF7, BT474 and BT549 cells downregulated their levels of TfR. This could either be due to increased lysosomal degradation of TfR (lysosomal activity is typically increased in senescent cells), or a reduction in expression of the protein. If the former was true, that would indicate the passage of TfR to the late endosome, where it would be transported to lysosomes for degradation. Upon treatment with primaquine, which affects the early and recycling endosomes, it was observed that TfR levels had been elevated in senescent BT474 cells, but not in the non-senescent cells. This might suggest that the primaquine affects the action of late endosomes, restricting the degradation of TfR in senescent cells. No such changes were observed in MCF7 and BT549 cells upon treatment with primaquine, suggesting that it was more likely a reduction in the expression of TfR that was responsible in these cell lines. This can be confirmed going forward by analysing TfR mRNA levels in these cells.

Interestingly, JIMT1 cells demonstrated an upregulation of TfR in senescent cells, which is consistent with prior literature indicating an increase in intracellular iron levels. TfR transports ferrous iron into the cell bound to transferrin. The elevation in iron levels in senescent cells correlates with an elevation in TfR [73]. This altered iron homeostasis is characterised by impaired ferritinophagy, as a means to escape iron-dependent cell death ferroptosis [70]. However, it is observed that ferritin levels, which are expected to increase when ferritinophagy is downregulated instead decreases. It is possible that JIMT1 cells are undergoing ferritinphagy while simultaneously endocytosing large amounts of iron.

This downregulation in ferritin was also observed in senescent BT474 cells. It is possible that impaired ferritinophagy is not characteristic of HER2+ senescent cells. This, in accompaniment with the downregulation of TfR in BT474 might indicate a depletion in intracellular iron, contrary to literature, which reports an upregulation in mouse embryonic fibroblasts [70]. Both of these inferences can be confirmed by verifying the iron levels between senescent and non-senescent cells in future work.

In summation, there a number of different factors that could be explored to develop a more comprehensive picture of therapy induced senescence. Quantification of Ki-67 levels in senescent cells and upon treatment with ERIs could provide more conclusive evidence to our hypothesis with regards to the sensitivities of the various cell lines to ERIs. A holistic picture of iron metabolism in senescent cancer cells could be developed by examining the intracellular iron levels, as well as look at additional iron transporters such as ferritin-transporter NCOA4 and metalloreductase Steap1. The verification of these findings in additional cell lines would also be important.

Besides the development of acquired resistance, as seen in the case of therapy-induced senescence, breast cancer cells also present with some intrinsic defence mechanisms to protect itself from the immune system. One of these is the presentation of immune-suppressive surface glycol-protein programmed death ligand-1 (PDL1). This immune checkpoint protein binds to the complementary programmed death receptor-1 (PD1) present on the surface of T cells, inactivating it and providing immune-tolerance to the cancer cells.

Therapeutics targeting surface PDL1 called immune-checkpoint inhibitors (ICI) are consequential in reducing immune-tolerance. Currently, there are a number of approved drugs on the market that are designed to target these proteins, such as

durvalumab and pembrolizumab. It must be noted that ICIs are not very effective at eliminating cancers altogether, and work only on specific tumour cells [236]. They are also reported to be deleterious to non-tumorous cells. It is therefore prudent to look for alternative strategies to target these immune checkpoint proteins and reduce immune tolerance of breast cancer cells.

Considerable research has looked into the regulation of PDL1 levels in cancer cells, and several studies have reported that it undergoes endosomal recycling [192, 194, 195, 198]. We believe that repurposing endosomal recycling inhibitors could prove to be an effective therapeutic strategy towards lowering immune-tolerance. By preventing the return of internalised PDL1 to the plasma membrane and diverting it into lysosomes for degradation. To that end, we looked into the effects of several endosomal recycling inhibitors on the total and surface PDL1 levels.

Upon treatment with the various ERIs, it was determined that PQ enables short term reduction in surface PDL1, but does not affect the total PDL1 levels. Monensin inhibits recycling and may affect PDL1 stability, and resveratrol upregulated surface PDL1 levels, and could possibly be inhibiting PDL1 endocytosis. Simvastatin also provides evidence suggesting inhibition of uptake, but it also appears to reduce the stability of surface PDL1, leading to an eventual degradation of the protein.

These results could be enhanced by looking into the PDL1-PD1 interaction, and determining if the impact of these ERIs would weaken this interaction. If so, this could further be verified in other cell lines to test for efficacy across multiple sub-types of breast cancer.

The two strategies of treatment discussed in this project aims to answer a singular question: can ERIs be effectively repurposed for the treatment of breast cancer?

First, we explored the possibility of using ERIs as means to overcome drug resistance developed as a result of drug-therapy-induced senescence. It proved effective against senescent HER2+ BC. However, this strategy was ineffectual against TNBC and Luminal B sub-types and even promoted proliferation in Luminal A sub-type MCF7 cells. Treatment with ERIs could, in fact, prove to be detrimental to therapy.

This result showed us that the effect of ERIs could be dependant on proliferation marker Ki-67, which has been associated with the iron metabolism in cancer cells. Looking into the effect of ERIs on various iron transporters could help us paint a clearer picture of the action ERIs in senescent breast cancer. While we determined several sections of the story, further look into the effects of other ERIs and on other cell lines would help us conclusively decide on whether we need to continue to explore or dismiss ERIs as a therapeutic strategy for overcoming drug resistance.

Second, we explored the role of ERI as a potential alternative therapeutic strategy to immune checkpoint inhibitors. While none of the ERIs tested immediately increased the lysosomal degradation of PDL1, monensin and simvastatin demonstrated some promising results by seemingly reducing the stability of the protein. Further exploration into the effects of ERIs on other cell lines, and on the dynamics of PDL1-PD1 interaction could prove to be promising avenue towards a clearer strategy for reducing immune-tolerance in breast cancer cells.

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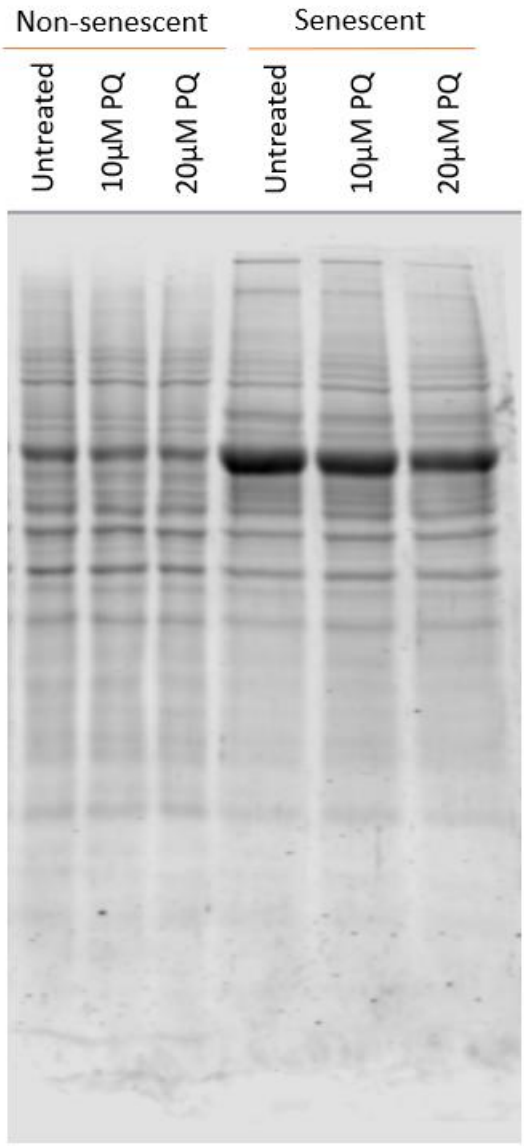
Supplementary Images

1. REVERT Total protein stain for BT549 blots



2. Dose-response curves for MDA-MB-453 senescent and non-senescent cells upon treatment with primaquine and monensin