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THE EFFECT OF THE SENOLYTIC ABT-263 ON ANDROGEN DEPRIVATION-INDUCED SENESCENT PROSTATE TUMOR CELLS

So Min Lee
Virginia Commonwealth University

THE EFFECT OF THE SENOLYTIC ABT-263 ON ANDROGEN DEPRIVATION-INDUCED SENESCENT PROSTATE TUMOR CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

So Min Lee
Bachelor of Science, Boise State University, 2018

Director: Dr. David A. Gewirtz
Professor, Pharmacology and Toxicology
Virginia Commonwealth University

Virginia Commonwealth University
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ABSTRACT

THE EFFECT OF THE SENOLYTIC ABT-263 ON ANDROGEN DEPRIVATION-INDUCED SENESCENT PROSTATE TUMOR CELLS

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Prostate cancer (PCa) is one of the leading causes of cancer-related deaths in men. Although standard treatments such as androgen deprivation therapies (ADT) and antiandrogens have increased survival for many patients, most men placed on these therapies will develop castration-resistant disease (CRPC). Previous studies have shown that these treatments have limited cytotoxicity and instead promote cell growth arrest. Our current work demonstrates that prostate tumor cells grown in the absence of androgens by using charcoal-stripped serum undergo senescence-mediated or senescent-like growth arrest, based on the cellular expression of senescence-associated-beta-galactosidase (SA-β-Gal). Our studies further suggest that this senescence is transient and "reversible," as cells are able to resume proliferation once androgen deprivation is terminated. Enrichment for senescent cells by SA-β-Gal-based FACS (fluorescence assisted cell sorting) confirmed that ADT-induced senescent cells can recover their proliferative capacity. While the transient nature of this senescence arguably creates the potential risk for disease recurrence, the senescent state induced by ADT may also provide a therapeutic strategy that can be exploited through the use of senolytics such as ABT-263 following ADT-induced senescence. ABT-263 selectively drives senescent, ADT-treated cells into apoptotic cell death while having minimal effect on non-senescent cells. Ongoing studies are designed to phenotypically characterize the recovered

senescent cells, to determine whether recovery from senescence promotes development of CRPC, and to what extent senolytics might delay the conversion from an ADT-sensitive to an ADT-resistant state.

Chapter 1: Introduction

PROSTATE CANCER

Prostate cancer (PCa) is one of the most common cause of cancer death in men. PCa is a disease that occurs in the prostate gland, and about one man in nine will be diagnosed during his lifetime [1]. It is estimated that 10.6% of all new cancer cases and 5.5% of all cancer deaths in men will be related to PCa in 2020 [2]. What causes prostate cells to become malignant is unknown, but there are several risk factors that may contribute to prostate carcinogenesis. A predominant factor is advanced age as the risk of developing PCa increases directly with age. It is reported that while 1 in 350 men under the age of 50 are diagnosed with PCa, 6 in 10 men over the age of 65 will be diagnosed with this disease [1,3]. Other strong risk factors are ethnicity, family history and genetic factors. African-American men have the highest risk among different racial groups and are frequently found with an aggressive form of the disease at a younger age [1,4]. Men with an immediate family member diagnosed with PCa will have up to a threefold higher risk of developing the cancer [5]. Furthermore, evidence shows that genetic predisposition to this disease can be inherited, contributing to 5% of all PCa cases [3].

While PCa is a serious disease, most prostate tumors are discovered in the early stages—localized or regional—where PCa cells are confined in the prostate or spread only to nearby locations. These tumors are relatively slow growing and are not life-threatening. In fact, the 5-year relative survival rate is nearly 98% [2], and the majority of these patients are more likely to die from other causes. Early stage treatments generally attempt to cure and remove all tumor cells; common treatment options include active surveillance, surgery, and radiation therapy. Despite a high survival rate, disease recurrence and advanced (or metastatic) PCa occur in 20% of men. The advanced disease is treated by hormonal therapy but will eventually become resistant to treatment within 2-3 years and progress into castration-resistant prostate cancer (CRPC) [6]. No optimal treatment is available at this time, and the 5-

year survival rate significantly decreases to about 30%, indicating the need for improved strategies for disease prevention and treatment [2].

ANDROGEN DEPRIVATION THERAPY

Androgen deprivation therapy (ADT) has been the standard of care for advanced PCa since the discovery of the treatment by Huggins and Hodges in 1941 [7]. Androgens are necessary for the growth of prostate cells, which is mediated by binding to androgen receptors in this tissue. Testosterone and its metabolite, dihydrotestosterone (DHT), are the primary androgens in the body. Testosterone is synthesized by the testes and adrenal gland, and is converted to DHT by the enzyme 5α-reductase primarily in prostate tissues. DHT has higher affinity for the androgen receptor than testosterone [9,12]. Likewise, the dependency of PCa cells on androgens for survival allows for androgen depletion to be an effective tumor-suppressing treatment.

The two main methods currently used to achieve reduced androgen production are medical castration and anti-androgens. Luteinizing hormone-releasing hormone (LHRH) agonists and antagonists are drugs that lower androgen levels from the testes and are also known as medical castration (Figure 1). When androgen levels are low, the hypothalamus produces LHRH, which stimulates the production and secretion of luteinizing hormone (LH) from the pituitary gland. Androgen is then produced by the testes in response to LH. Treatment with LHRH agonists, given by injection or implanted under the skin, will result in downregulation of pituitary LHRH receptors, which will inhibit LH secretion and ultimately androgen production. Furthermore, a phenomenon called “testosterone flare” causes a temporary increase in testosterone levels with the agonists, which led to the development of LHRH antagonists. This form of medical castration works similarly to the agonists but directly prevents LHRH from binding to its receptors, which in turn allows a rapid suppression of androgen levels and avoids the testosterone flare phenomenon [8-11].

Anti-androgen drugs are another effective and widely used method for ADT. These competitive inhibitors do not directly lower the androgen levels but are frequently used in combination with LHRH agonists to block androgen receptor binding in PCa cells to prevent cancer growth [8,11]. Also included with this class of drugs are androgen biosynthesis inhibitors. Unlike targeting the androgen receptor, these agents inhibit the enzyme CYP17, which is necessary for androgen production in the testes, adrenal gland, and prostate tumors [13]. While medical castration and anti-androgens lower androgen levels in the testes, the ability for androgen biosynthesis inhibitors to target other locations allow for a more comprehensive treatment. ADT alone cannot alleviate the cancer and combinational therapies are commonly used in recent treatment protocols to avoid tumor progression. The discovery of ADT has improved the survival of men with advanced PCa over the years. However, CRPC development is unavoidable and current treatment remains a challenge for overcoming resistance.

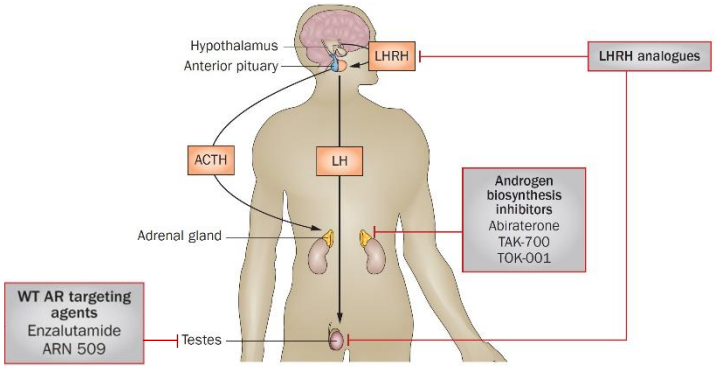


Figure 1
Adapted from Wong et al. 2014. Nature Reviews.

CELLULAR RESPONSE: SENESCENCE

Cells respond to ADT in various ways. A primary response is apoptosis, also known as programmed cell death, but studies have shown that apoptosis occurs in only a subpopulation of androgen sensitive PCa cells [14]. Cells that survive after ADT enter cell cycle arrest and exhibit characteristics of senescence [19,20]. Cellular senescence is part of a normal aging process and has

historically been defined as a stable, permanent cell cycle arrest initiated by a variety of stress mechanisms. Cells are induced into senescence by different stimuli. Replicative senescence is induced by telomere shortening in normal cells. Oncogene-induced senescence occurs from oncogene mutation (i.e. Ras) and stress-induced senescence is caused by DNA damage and oxidative stress (i.e. chemotherapy and radiation) [16]. These cells undergo growth arrest and express hallmarks that identify the cells as "senescent" (Figure 2). Some key features include enlarged and flattened morphology, altered gene expression, senescence-associated β-galactosidase (SA-β-gal) upregulation, and secretion of pro-inflammatory factors (such as IL-1β, IL-6, IL-8, and MMP3), termed the senescence-associated secretory phenotype (SASP). Other relevant biochemical markers are Lamin B1 and cathepsin D expression [16-18].

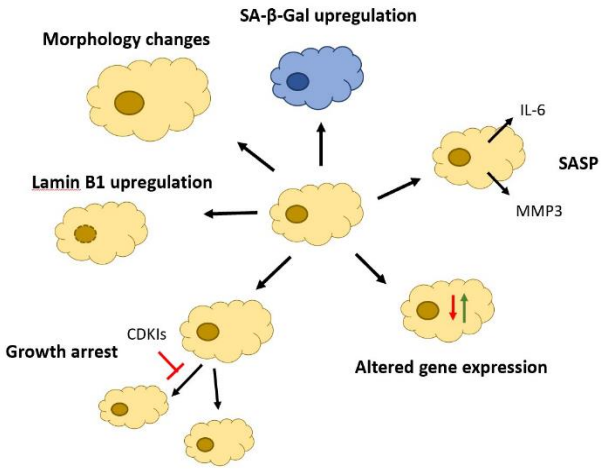


Figure 2
Figure adapted from Tareq Saleh

The ability of senescent cells to remain in a growth-arrested state is considered to contribute significantly to the suppression of tumorigenesis. While this is partially true, more recently there has been increasing evidence that senescence induced in tumor cells is a prolonged but *reversible* form of growth arrest [15,21]. Senescent cells can escape from growth arrest and potentially contribute to disease recurrence. For example, androgen deprivation (AD)-induced senescent cells showed

proliferative recovery when treated in charcoal-stripped serum (CS-FBS); repeated exposure to treatment promoted the outgrowth of castration resistant clones [20]. As this study was performed by following the bulk population of cells, our studies were designed to determine whether senescent cells specifically are able to escape growth arrest and if they can then be exploited for the prevention of CRPC.

SENOLYTICS AS A SENESCENCE INTERVENTION

One main attribute of senescence is its resistance to apoptosis, which provides an opportunity for therapeutic strategies. Apoptosis is characterized by distinct morphological changes, such as cell shrinkage, dense cytoplasm, and chromatin condensation via pyknosis [23]. Important factors in apoptosis involves the pro-apoptotic BH3-only proteins and pro-survival Bcl-2 family proteins (Figure 3). Apoptotic signals, such as DNA damage or oxidative stress, initiate BH3-only proteins to bind to core pro-apoptotic proteins BAX/BAK, which release cytochrome c and activate caspase-3 (executioner of apoptosis) into cell death. Conversely, the pro-survival proteins (i.e Bcl-2, Bcl-xl, and Mcl-1) prevent cell death by blocking BAX/BAK directly [22]. Overexpression of Bcl-2 and Bcl-xL are detected in cancer cells, including PCa, and contribute largely to tumor progression and therapy resistance [25-28]. Consequently, understanding the pro-survival pathways can provide strategies for direct elimination of senescent cells.

Senolytics are agents that selectively target senescent cells for apoptosis. Many senolytics have been identified, such as dasatinib and quercetin, HSP90 inhibitors, cardiac glycosides, and Bcl-2 family inhibitors. In particular, the BH3 mimetic ABT-263 (Navitoclax) is a well-studied senolytic drug that mimics the binding of BH3-only proteins and inhibits Bcl-2 and Bcl-xL (Figure 3) [24]. This small, oral bioavailable drug has been demonstrated to rapidly remove senescent cells and improve treatment outcomes *in vitro* and *in vivo* [24,30]. Moreover, ABT-263 proved to increase efficacy in combination with chemotherapeutic agents in lymphoma and multiple myeloma cells *in vivo* [24], dramatically

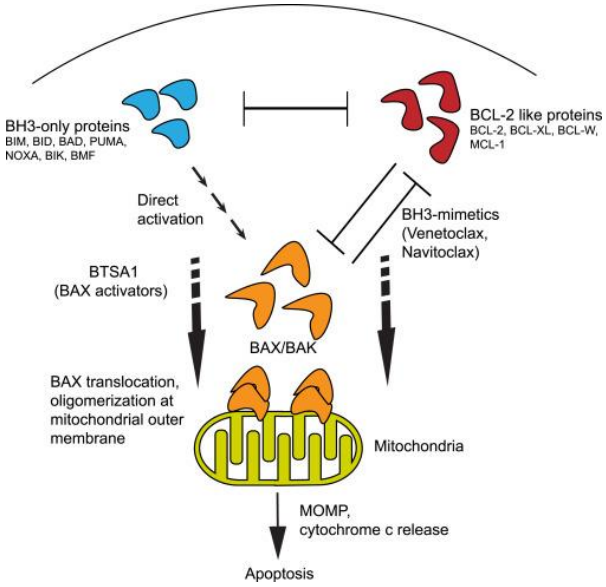


Figure 3.
Thandapani and Aifantis. 2017. Cancer Cell

increasing tumor growth inhibition. Our lab also demonstrated apoptosis in senescent lung and breast cancer cells by ABT-263 and reduced tumor volume with chemotherapy followed by ABT-263 [29]. This drug is also known to exhibit low cytotoxicity by targeting senescent cells only while having minimal effect on proliferating cells, which makes it of great interest. Like other senolytics, however, cytotoxic response from ABT-263 can vary from cell types and treatments. There have been relatively few studies on the PCa cell response to ABT-263.

In this project, we show that AD-induced senescent cells are able to escape and develop androgen independence. Treatment of ABT-263 following induction of senescence also demonstrates significant apoptotic cell death in senescent PCa cells and reduced outgrowth. Consequently, it is possible that ABT-263 could be used to delay CRPC by eliminating AD-induced senescent cells, thereby preventing proliferative recovery, and improving the overall survival in men with advanced prostate cancer.

Chapter 2: Methods

Cell culture

The murine prostate cancer Myc-CaP cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher, Waltham, MA USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gemini, West Sacramento, CA, USA) or 10% (v/v) charcoal-stripped serum (Life Technologies, Grand Island, NY, USA) in fetal bovine serum (CS-FBS) and 1% (v/v) 100 $U \cdot mL^{-1}$ penicillin G sodium/100$\mu g \cdot mL^{-1}$ streptomycin sulfate (Thermo Fisher) at 37°C in a humidified atmosphere of 5% CO_2. To assess the effects of androgen deprivation, cells were seeded in FBS for 24 hours prior to CS-FBS treatment for 7 days. Cells in DMEM were passaged at ~80% confluency and all media were changed every other day.

Cell line and drug treatment

The Myc-CaP cell line was purchased from ATCC (Manassas, VA, USA). ABT-263 (AbbVie, North Chicago, IL, USA) was prepared as a stock solution of 2 mM in dimethlysulfoxide (DMSO) and used in the dark at the indicated concentration. The drug was diluted to 2 µM in DMEM.

Cell viability assay

Cells were seeded at 1×10^4 cells per well in 6-well plates and allowed to adhere to the surface overnight. Cells were incubated in DMEM (10% FBS) for 24 h before treating them in CS-FBS for 7 days or ABT-263 for 48 h following CS-FBS or ABT-263 alone. On the indicated days, cells were washed with 1× phosphate buffered saline (PBS; Life Technologies), incubated in trypsin (0.25% trypsin-EDTA), and stained with trypan exclusion dye (0.4% trypan blue; Invitrogen, Carlsbad, CA, USA). The viable, unstained cells were then counted using a hemocytometer under a bright field microscope.

Clonogenic assay

Cells were seeded in 6-well plates at a low density (100 cells/well or 1×10^5 C12FDG high cells/well) and allowed to adhere to the plate for 24 h after which the media was replaced with CS-FBS. Cells were treated for 7 days or continuously, depending on the experiment. On the indicated days, colonies were washed in 1× PBS, fixed with 100% methanol, and stained with 0.1% (v/v) crystal violet dye (Sigma-Aldrich, St. Louis, MO, USA). Colonies with >50 cells were counted.

Senescence-associated β-galactosidase (SA-β-gal) assay

X-Gal staining

Cells were seeded at 2.5×10^4 cell per 60 mm dish and allowed to adhere to the surface overnight. Cells were treated with CS-FBS for 7 days. On the indicated days, cells were washed with 1× PBS, fixed with the fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS), and washed again with PBS. Cells were stained with a staining solution (20 mg/mL X-Gal in dimethylformamide, 0.2 M citric acid/sodium phosphate (NaPi, pH 6), 100 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), 100 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 5 M sodium chloride (NaCl), 1 M magnesium chloride ($MgCl_2$)) and incubated overnight in a CO_2-free incubator. Cells were then washed, and images were taken under 20× magnification using an inverted microscope.

C_{12}FDG staining

Cells were prepared as described above and seeded at 1.5×10^4 cells per well in 6-well plates. On the indicated days, cells were incubated with 100 nM bafilomycin A1 (BafA1; Sigma-Aldrich) for one hour followed by staining with 10 mM C_{12}FDG (Thermo Fisher) for two hours at 37°C. Cells were trypsinized, and the pellet was collected and washed with 1× PBS. Cell pellet was then resuspended in PBS and analyzed by flow cytometry (using BD FACSCanto II and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility).

Cell enrichment

Cells were seeded at a high density (~1×10^5 cells) in 20 cm plates and treated with CS-FBS after 24 hours of adhering to the surface. Media was changed every other day until the day of the sort (Day 6). Cells were treated with BafA1 (1:1000) for one hour and then stained with C_{12}FDG (1:1000) for two hours. Cells were trypsinized and centrifuged for five minutes at 1500×. Then the pellets were washed with sterile 1× PBS (Ca/Mg++ free) and centrifuged again. The pellet was resuspended in 4 mL of suspension media (1:1000 EDTA:PBS) and sorted by flow cytometry.

Western blotting

Cells were seeded at 1×10^4 cells per 10 cm dish and allowed to adhere to the plate for 24 h after which the media was replaced with the respective treatments. Cells were incubated in CS-FBS for 7 days or ABT-263 for 24 h. For CS-FBS + ABT-263 or + DMSO treatment, the drugs were incubated for 24 h after the end of CS-FBS treatment.

Protein extraction and quantification

To lysis proteins, cells were trypsinized, washed with 1× PBS, and resuspended in CHAPS lysis buffer containing protease inhibitors. The pellet was left on ice for 20 minutes and then centrifuged at 4°C at maximum speed for five minutes before transferring the supernatant to a clean tube. Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and each sample was adjusted to 25-30 µg.

SDS-PAGE

Protein samples were incubated in boiling water for 10 minutes and cooled at room temperature. Samples were loaded into 12% SDS-polyacrylamide gels and separated by electrophoresis at 80 V for 30 minutes (for stacking), followed by 120 V for 60 minutes in 1× running buffer (10× NuPAGE® MES SDS Running Buffer; Bio-Rad Laboratories).

Western blotting

SDS-polyacrylamide gels were transferred to PVDF membranes at 100 V for one hour in 1× transfer buffer (10× NuPAGE® MES SDS Transfer Buffer (Bio-Rad Laboratories) and 20% methanol). After transfer, membranes were blocked in 5% bovine serum albumin in TPBS (0.1% Tween® 20 in 1× PBS) for one hour on an orbital shaker at room temperature. Membranes were then incubated overnight at 4°C with the following primary antibodies: cleaved caspase 3 (1:1000; Cell Signaling, MA, USA), Bcl-xL (1:1000; Cell Signaling), Lamin B1 (1:1000; Cell Signaling), Cathepsin D (1:1000; arigo Biolaboratories, Taiwan) and GAPDH (1:1000; Cell Signaling). The following day, membranes were washed three times in TPBS for five minutes each. Membranes were then incubated with 1:2000 horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit; Cell Signaling) for two hours at room temperature. Protein signals were detected using a chemiluminescence kit (Pierce™ ECL Plus Western Blotting Substrate; Thermo Fisher), and western blots were analyzed using the Image J2 software.

Apoptosis Assay—Annexin V/Propidium Iodine

Cells were seeded at 1×10^4 cells per well in 6-well plates and allowed to adhere to the surface overnight. Cells were then incubated in CS-FBS for 7 days and then replaced back with DMEM. Cells treated with ABT-263 alone or ABT-263 after the end of CS-FBS treatment were removed after 18-24 h or 48 h exposure, respectively and replaced back with fresh media. Cells were collected, centrifuged at 10,000× for 3 minutes, and washed in 1× PBS on the indicated days. Samples were incubated in annexin V-FITC/propidium iodide (PI) staining as per manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences, NJ, USA). Samples were then transferred to flow tubes with additional 1× buffer to be read by flow cytometry.

Cell cycle analysis

Cells were seeded at 1×10^4 cells per well in 6-well plates and allowed to adhere to the surface overnight. Cells treated with CS-FBS were seeded in DMEM (10% FBS) for 24 hours followed by CS-FBS treatment for 7 days. On the indicated days, cells were collected, centrifuged, and washed in 1× PBS. Cells were fixed in 70% ethanol and stored in -20°C until ready to analyze by flow cytometry. Cells were resuspended in a propidium iodide staining solution (0.1 mg/ml PI, 1.2 mg/ml sodium citrate, 0.2 mg/ml DNase-free RNase A, and 0.1% Triton-X 100 in PBS) for one hour at room temperature in the dark before analysis.

QRT-PCR

Cells were seeded at 1×10^4 cells per well in 6-well plates and allowed to adhere to the surface overnight before treatment with CS-FBS for 6 days. Cells were pelleted, and RNA purification and real-time PCR were performed as described previously [29]. Primers were purchased from Qiagen (Germantown, MD, USA): IL-6: QT00083720; IL-1B: QT00021385; GAPDH: QT00079247. Relative mRNA expression was determined using the $\Delta\Delta C_t$ method.

Statistical analysis

All data were expressed as the means ± SEM of at least three independent experiments performed in duplicates or triplicates unless otherwise indicated. Statistical analysis was assessed using GraphPad Prism 8.0 software. One-way ANOVA Tukey post hoc was used for multiple conditions and unpaired *t*-test for comparing two conditions. A *P*-value of <0.05 was considered statistically significant.

Chapter 3: Results

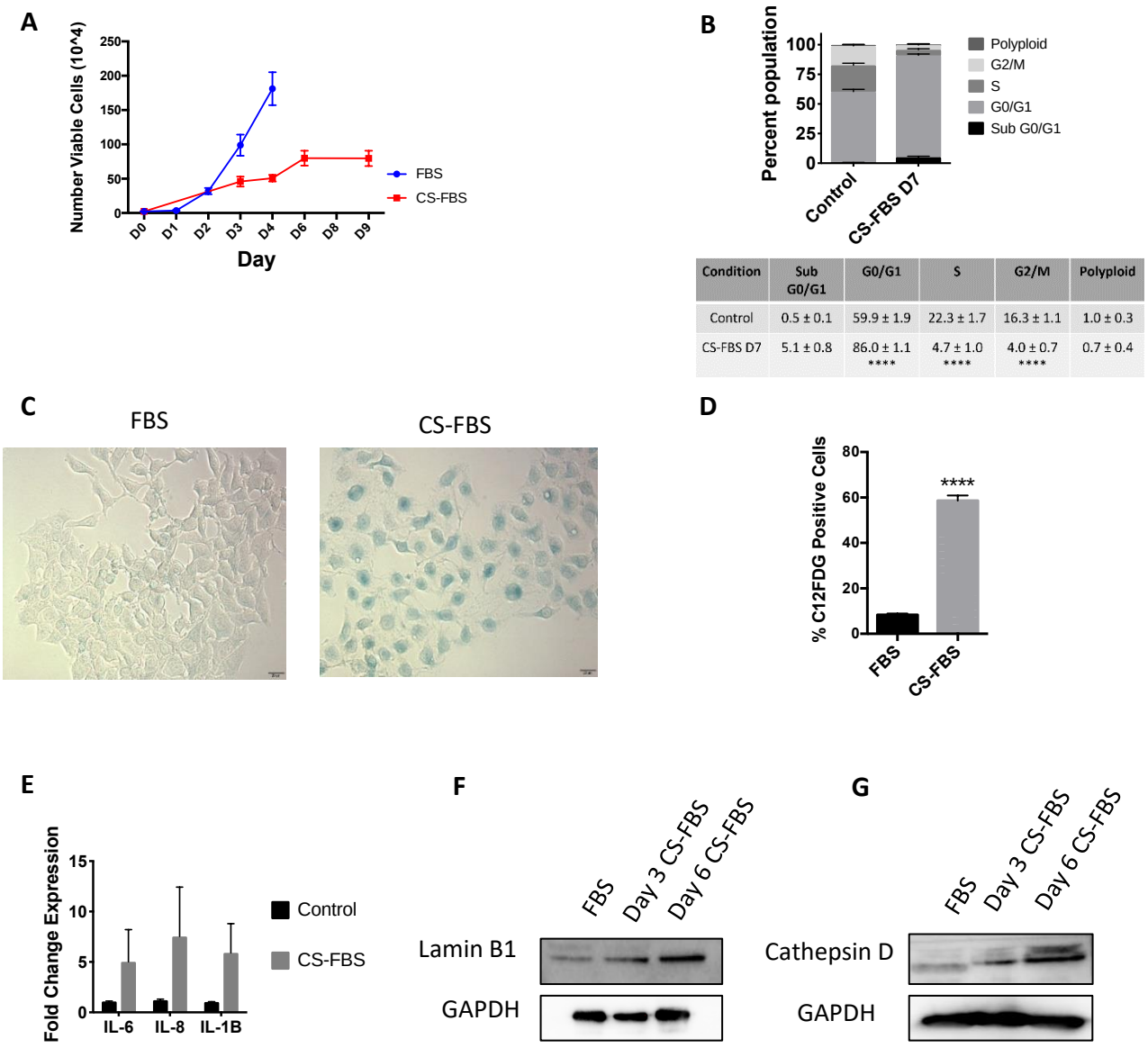
I. Androgen deprivation-induced growth arrested cells express senescent phenotypes

Cells deprived of androgen have been shown to enter into a prolonged growth arrest, which could indicate that the cells are in a state of senescence. To examine whether androgen deprivation (AD)-induced growth arrest is, in fact, due to senescence, biomarkers of senescence were assessed. Murine PCa Myc-CaP cells were treated with CS-FBS for 7 days and then replaced back with regular media. Cell viability was monitored on the indicated days using a Trypan blue exclusion assay. Figure 1A shows growth arrest based on the reduction in viable cell numbers in the androgen-deprived cells compared to the controls. To further assess that cells were growth arrested, a cell cycle analysis was performed at the end of the treatment (Day 7). Figure 1B shows a significant increase in the G0/G1 phase, indicating the reduced proliferation of the treated cells by the G0/G1 cell cycle arrest. There were subsequently reductions in the S and G2/M phase. The populations of sub-G0/G1 (total cell death) and polyploid cells were not significantly changed.

Although growth arrested cells represent one of the characteristics of senescence, it is not enough to distinguish senescence from other forms of growth arrest, such as quiescence, which is caused by growth factor and nutrient deprivation [32]. The expression of the lysosomal enzyme β-galactosidase (β-gal) is an indicator of senescent cells and can be detected by chromogenic or fluorescent assays. This pH-sensitive enzyme will show an increase in activity for senescent cells only at the suboptimal pH of 6.0. Figure 1C shows the results of an X-gal assay where X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), a conjugated substrate, stains cells blue when cleaved by the enzyme. Cells treated with CS-FBS produced dark blue staining compared to the controls, supporting the conclusion that the cells had become senescent. In addition, a common marker of senescence includes morphological changes such as flattening and enlargement of cytoplasm as shown in Figure 1C. A

fluorescent assay C_{12}FDG was performed via flow cytometry for quantification of the expression of senescence-associated (SA) β-gal; the substrate C_{12}FDG fluoresces when cleaved by β-gal. Figure 1D shows a significant increase in the level of the substrate, again supporting the conclusion that androgen-deprived cells undergo senescence. It is also to be observed that while our data shows evidence of senescence, we cannot confirm that all growth arrested cells in the mixed population of cells are senescent, as some may potentially be in a state of quiescence as part of a normal cell cycle arrest.

To establish that growth arrested cells are truly induced into senescence, we examined other important hallmarks of senescence: senescence-associated secretory phenotype (SASP), Lamin B1 and cathepsin D expression. We quantified the expression of SASP factors and saw an upregulation of IL-1β, IL-6, and IL-8 (Figure 1E). Western blot analysis provided evidence for the accumulation of Lamin B1 (Figure 1F) and cathepsin D (Figure 1G). Altogether, these results lead to the conclusion that growth arrested cells induced by ADT are senescent.

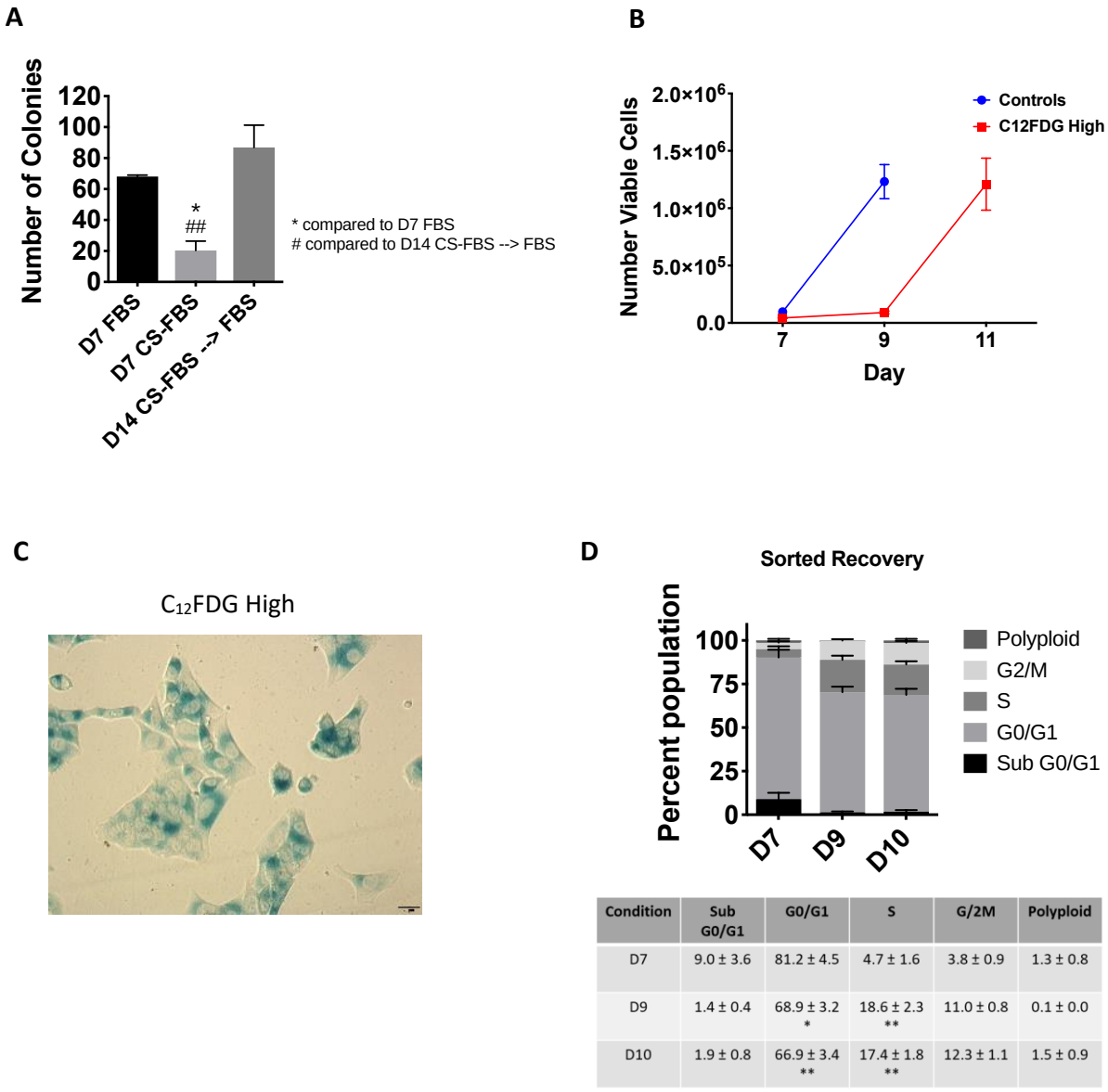


Condition	Sub G0/G1	G0/G1	S	G2/M	Polyploid
Control	0.5 ± 0.1	59.9 ± 1.9	22.3 ± 1.7	16.3 ± 1.1	1.0 ± 0.3
CS-FBS D7	5.1 ± 0.8	86.0 ± 1.1 ****	4.7 ± 1.0 ****	4.0 ± 0.7 ****	0.7 ± 0.4

Figure 1. Androgen deprivation-induced markers of senescence in Myc-CaP cells. Myc-CaP cells were treated with 10% CS-FBS for 7 days and replaced back with regular media. **A. Cell viability.** Cells were trypsinized and stained with trypan blue and unstained cells were then counted with a hemocytometer on the indicated days. N=3. **B. Cell cycle analysis.** Cell cycle analysis was performed by flow cytometry. N=3. $^{****}P < 0.0001$. **C. SA-β-gal staining.** Cells were treated with CS-FBS for 6 days (Day 6) and then fixed and stained with X-gal staining solution for 24 h. Images were taken at 200×. **D. Quantification of SA-β-gal.** Cells were treated with bafilomycin A1 (100 μM) for 1 h and stained with $CD_{12}FDG$ for 2 h before quantifying the expression of SA-β-gal by flow cytometry on Day 6. N=3. $^{****}P < 0.0001$. **E. Expression of SASP.** SASP factors were assessed by qRT-PCR in cells treated with CS-FBS for 6 days. N=3. **F, G. Western blot.** Cells were treated with CS-FBS and collected on the indicated days. N=3.

II. Androgen deprivation-induced senescent cells show reversibility

Myc-CaP cells deprived of androgen were shown to become senescent and have a prolonged growth arrest. However, previous studies from our lab and others have shown that senescent cells can escape growth arrest and resume proliferation [15,21]. We first observed the formation of colonies from a bulk population of cells that were treated in CS-FBS followed by regular media (Figure 2A), and colonies that were growth arrested in CS-FBS were able to resume proliferation after switching back to regular media. In order to determine if AD-induced senescent cells specifically can recover, we then sorted the cells via flow cytometry to ensure the separation of non-senescent and senescent cells. The top 25% of cells were sorted on Day 6, re-cultured with CS-FBS to finish treatment (Day 7), and replenished with regular media the following day. We assessed the increased SA-β-gal activity of the sorted cells by X-gal staining (Figure 2C) to confirm that the sorted cells were senescent, and cell viability was again monitored using the Trypan blue exclusion assay. In Figure 2B, the senescent cells (C_{12}FDG High) show proliferation after the end of treatment, which indicates that senescent cells can recover and proliferate again. To further analyze the reversibility, we performed a cell cycle analysis via flow cytometry, and Figure 2D confirms that the recovered cells are in its proliferative state based on the profile analogous to the untreated/controls (Figure 1B).



Condition	Sub G0/G1	G0/G1	S	G/2M	Polyploid
D7	9.0 ± 3.6	81.2 ± 4.5	4.7 ± 1.6	3.8 ± 0.9	1.3 ± 0.8
D9	1.4 ± 0.4	68.9 ± 3.2 *	18.6 ± 2.3 **	11.0 ± 0.8	0.1 ± 0.0
D10	1.9 ± 0.8	66.9 ± 3.4 **	17.4 ± 1.8 **	12.3 ± 1.1	1.5 ± 0.9

Figure 2. The C_{12}FDG High Myc-CaP cells show proliferative recovery indicative of escape in growth arrest. A. Clonogenic assay. Cells were plated at low density and then were treated for either 7 days of regular FBS medium, 7 days of CS-FBS medium, or 7 days of CS-FBS followed by 7 days of regular FBS medium. Cell colonies were fixed with methanol and stained with crystal violet on the indicated days, and colonies (>50 cells) were counted. N=3. $^{*}P < 0.05$ vs D7 FBS; $^{**}P < 0.01$ vs D14 CS-FBS→FBS. **B. Cell viability.** Senescent cells (C_{12}FDG High) were sorted using flow cytometry, and the number of viable cells were counted on the indicated days. Control cells were not sorted. N=3. **C. SA-β-gal staining.** Sorted cells were stained with X-gal for 24 h after end of CS-FBS treatment (Day 7). Images were taken at 200×. **D. Cell cycle analysis.** Cell cycle analysis was performed by flow cytometry. $^{*}P < 0.05$ vs D7; $^{**}P < 0.01$ vs D7. N=3.

III. Androgen deprivation-induced senescent cells can escape during continual androgen deprivation which contribute to castration-resistance

We have demonstrated that PCa Myc-CaP cells with ADT treatment undergo senescence, which allows the cells to escape their prolonged dormant state by recovering their proliferative activity. While this escape may promote recurrence, we needed to resolve its contribution to castration-resistance. We assessed whether senescent cells could escape and recover in a continuous state of androgen deprivation. Sorted senescent cells were plated at a low density, treated with CS-FBS, and stained with crystal violet on the indicated days. Recovered senescent cells in continual ADT treatment showed persistent clonal growth (Figure 3), signifying that escaped senescent cells may encourage resistance.

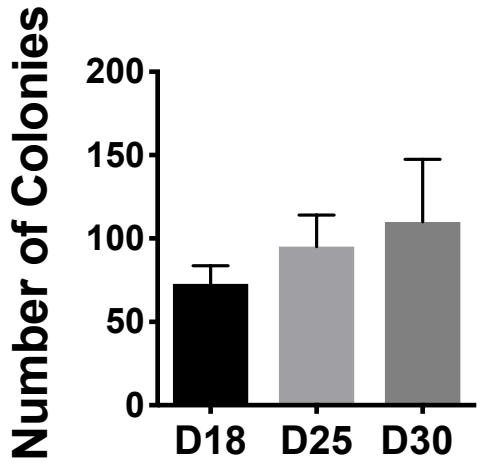


Figure 3. AD-induced senescent cells contribute to resistance outgrowth. Sorted cells (C_{12}FDG High) were treated with CS-FBS until the end of the experiment. Cell colonies were fixed with methanol and stained with crystal violet on the indicated days, and colonies (>50 cells) were counted. N=2.

IV. ABT-263 induces apoptosis in senescent cells

The proliferative recovery of AD-induced senescent cells from both after and continual treatment indicate that these cells can develop androgen independence and castration-resistant like features. We then decided to treat a bulk population of escaped senescent cells maintained in continuous CS-FBS with ABT-263, a senolytic agent that selectively eliminates senescent cells, to assess whether the agent will interfere with the recovered senescent cells or already progressed tumor (i.e. castration-resistance). Colony formation of AD-treated cells was observed over a prolonged period with or without ABT-263; Figure 4A demonstrates that while colonies without ABT-263 treatment continued to grow, exposure to ABT-263 significantly reduced colony formation.

Senolytics are a class of drugs that could potentially play a role in treating diseases, and studies suggest that anti-apoptotic Bcl-2 family proteins may represent a target for therapy; more specifically, the Bcl-2/Bcl-xL inhibitor ABT-263 (navitoclax), a BH3 mimetic, could behave as a potential senolytic. We demonstrate that ABT-263 may essentially delay or even prevent prostate tumor cell progression by using a sequential strategy of allowing Myc-CaP cells to enter AD-induced senescence followed by the removal of cells via ABT-263 treatment.

To determine the effect of ABT-263 on senescent cells, it was necessary to measure the cell viability under each experimental condition. Figure 4B shows the comparison between 0 μM of ABT-263 and 2 μM of ABT-263 in untreated, control cells and/or CS-FBS treated cells. Cells were counted using the Trypan blue exclusion assay. The cell viability of the untreated cells was determined on Day 2 after a 48-hour treatment of ABT-263; there was no significant difference between 0 μM and 2 μM of ABT-263. The CS-FBS treated cells were treated with ABT-263 for 48 hours at the end of CS-FBS treatment, and the cell viability was determined on Day 9, which is the reason for the high cell number compared to the untreated cells. There was a significant increase in cell death with ABT-263 treatment, indicating that

the senolytic is not effective against the untreated, non-senescent cells but is markedly effective against ADT-induced senescent cells.

We continued the study to determine if apoptosis was playing a role in the sensitization by ABT-263 by performing an Annexin V/Propidium Iodine assay. Figure 4C shows the percent of apoptotic cells for the controls (FBS; 0 μM of ABT-263), controls + ABT-263, CS-FBS, and CS-FBS + ABT-263. The extent of cell death significantly increased in CS-FBS + ABT-263 cells compared to the CS-FBS only cells, reflecting what was presented in Figure 4B. We then ran a western blot (Figure 4D) to further assess that apoptosis is induced by ABT-263 in senescent cells. There was an upregulation of cleaved caspase-3 (c-Casp3), an executioner caspase in apoptosis, in the CS-FBS + ABT-263 cells and no (or minimal) increase of proteins in the other conditions, validating that senescent cells are sensitive to ABT-263 as it induces apoptosis.

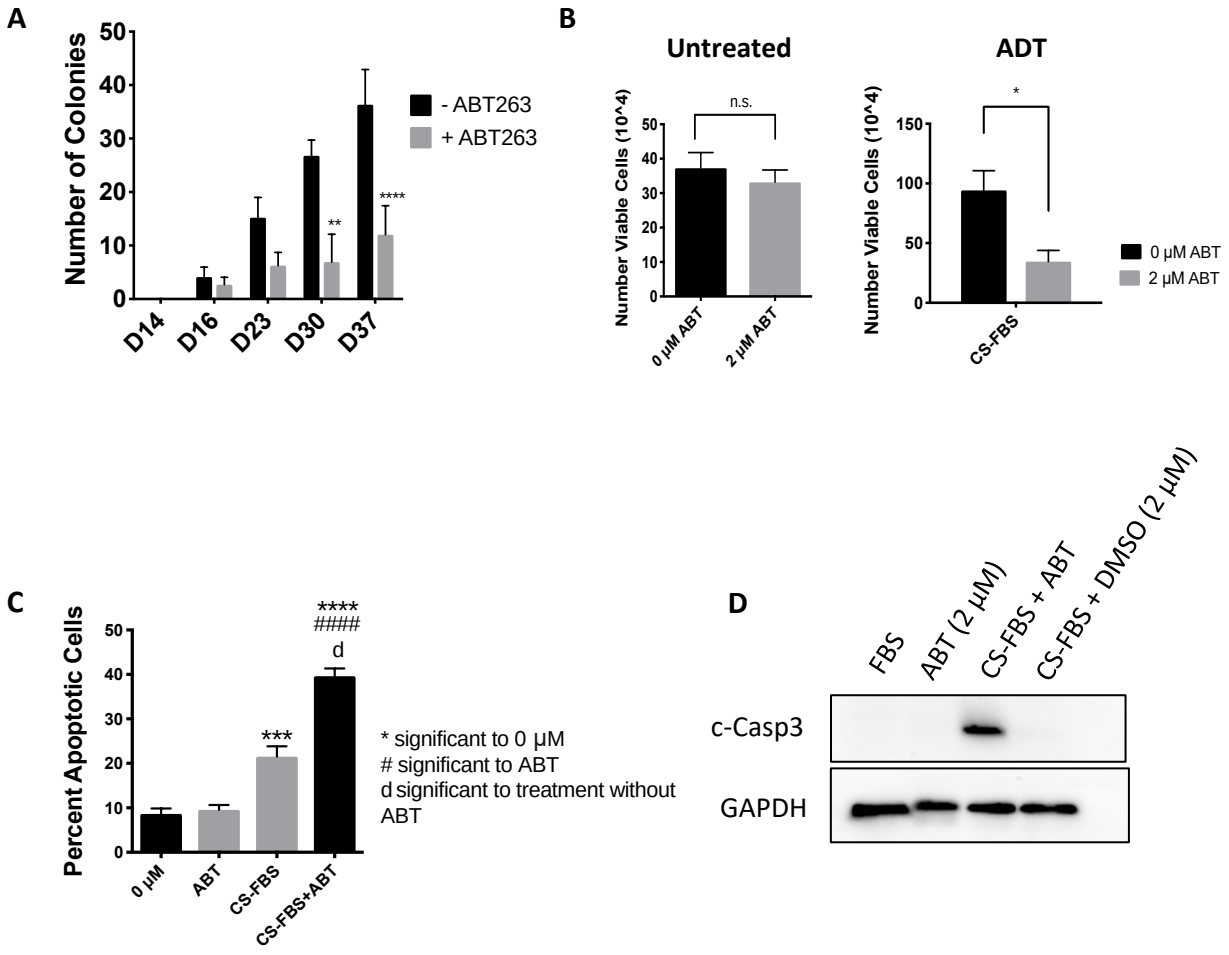


Figure 4. The senolytic agent, ABT-263, induces apoptosis in senescent cells. A. Clonogenic assay. Cells were continuously treated in CS-FBS and exposed to ABT-263 (2 µM) for 48 h prior to the indicated days after Day 14. Cell colonies were fixed with methanol and stained with crystal violet on the indicated days, and colonies (>50 cells) were counted. N=3. $^{***}P < 0.001$; $^{****}P < 0.0001$. Data generated by Valerie Carpenter. **B. Cell viability.** Cells were treated with ABT-263 for 48 h following the end of treatment with CS-FBS (Day 7). The cells were stained with trypan blue and the number of viable cells were counted using a hemocytometer. There was no significance between controls (0 µM ABT-263) and ABT-263 treated cells, but a significant difference was evident when the cells were androgen deprived. N=3; $^{*}P < 0.05$. **C. Annexin V/PI staining.** Cells were treated with ABT-263 for 18-24 hours on Day 7 CS-FBS. Apoptosis was analyzed by flow cytometry. N=3; $^{***}P < 0.001$ vs control; $^{****}P < 0.0001$ vs control; $^{\#\#\#\#}P < 0.0001$ vs ABT-263; $^{\delta}P < 0.05$ vs CS-FBS. **D. Western blot.** Cells were treated with ABT-263 or DMSO for 24 h on Day 8 CS-FBS. The expressed level of cleaved caspase-3 (c-Casp3) was determined at the indicated conditions. N=3.

V. The survival of androgen deprivation-induced senescent cells is dependent on Bcl-xL

As we have demonstrated that ABT-263 can induce apoptosis in senescent cells, we wanted to demonstrate the pathway that the senolytic takes in the Myc-CaP cells. The senolytic ABT-263 is known to target Bcl-2 and Bcl-xL; the specific inhibitors A-1155463 (Bcl-xL) and ABT-199 (Bcl-2) were used to determine the importance of each potential cell target in senescent PCa cells in inducing apoptosis. Figure 5A shows a significant promotion of apoptosis with the inhibition of Bcl-xL by A-1155463 while inhibition of Bcl-2 induced minimal cell death in cells treated with bicalutamide, an anti-androgen, suggesting that inhibition of Bcl-xL is essential to target cell death. We ran a western blot to further determine the specific target for ABT-263 in cells treated with CS-FBS; Figure 5B shows an increase in upregulation of Bcl-xL throughout the treatment, which strongly suggests that killing of AD-induced senescent cells is mediated by inhibition of Bcl-xL.

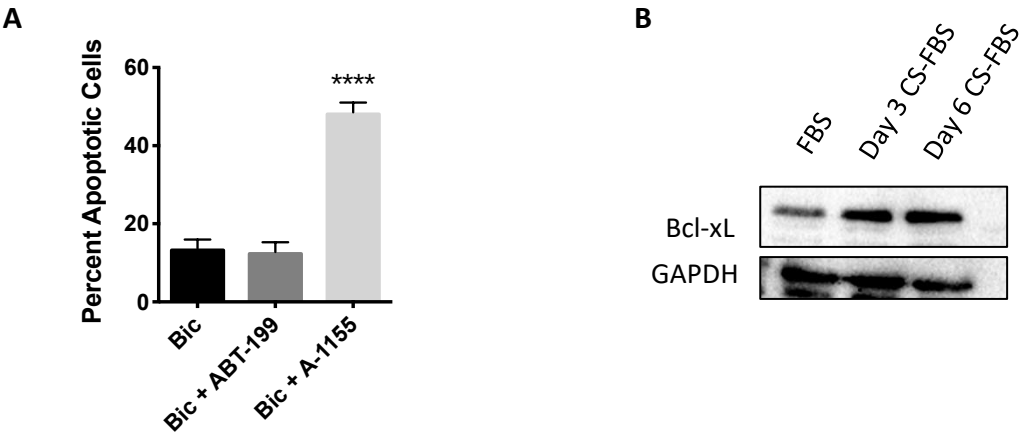


Figure 5. Cell killing by ABT-263 appears to be mediated by Bcl-xL. A. Annexin V/PI staining. Cells were treated with 35 µM bicalutamide (Bic) followed by 24 h treatment with ABT-199 (2 µM) or ABT-1155 (2 µM). N=3. $^{****}P < 0.0001$ vs Bic. Data generated by Valerie Carpenter. **B. Western blot analysis showing increased expression of Bcl-xL.** Cells were treated with CS-FBS and collected on the indicated days (Days 3 and 6). N=3.

Chapter 4: Discussion

Cellular senescence is a unique form of cell cycle arrest that is initiated as a response to various stressors. Senescence may be protective during tumorigenesis by inhibiting (pre-)malignant cell division, while senescence as a response to chemotherapy has often been deemed a desirable response due to the prolonged growth arrest. However, senescence plays a dual role in cancer progression, as it can also have deleterious effects when senescent cells accumulate and promote the growth of neighboring cells via factors such as senescence-associated secretory phenotypes (SASP) [21]. In addition to the negative consequences of the SASP, recent studies have demonstrated that senescent cells can escape their dormant-like state and exhibit aggressive growth, which may contribute to drug resistance [15,21,32]. These observations have led to increased interest in the use of senolytics as potential adjuvant therapies [33], and recent pre-clinical literature has shown the successful elimination of senescent tumor cells by senolytic therapies both *in vitro* and *in vivo* [29].

PCa patients usually present with androgen-dependent disease and often have a long latency period before the disease progresses, at which point patients inevitably become unresponsive to ADT and develop castration-resistant prostate cancer (CRPC). While it has been shown in prior literature that ADT can induce senescence in PCa cells and in PCa patient tumors [19,34], it was previously unknown whether AD-induced senescent cells can recover from the senescent growth arrest and potentially serve as a therapeutic target for prevention of the development of CRPC.

In this study, we determined that AD induces a form of senescence in Myc-CaP cells that is associated with only a transient growth arrest, and that the senescent cells are capable of escaping the arrest and developing androgen independence. Senescent cells are heterogeneous, and the senescent phenotype varies within cells. Further, senescent-like phenotypes, such as cell cycle arrest and morphology changes can be shown in other cellular states, thus limiting how senescent cells are

identified since there is no single or distinct marker of senescence. We therefore assessed multiple markers of senescence for confirmation that the cell population was recovering from a senescent-like arrest, rather than another type of cell cycle arrest, and found that AD resulted in multiple senescence-associated phenotypes, including the SASP (Figure 1).

SASP factors are reported to promote senescence escape and tumor cell proliferation [21]. With this, the increase in the secretion of SASP factors in AD-induced senescent cells may be associated with the facilitation of cellular escape, although this was not investigated during this study. Our results showing recovery in the bulk population confirms previous reports, such as Burton *et al*. and Malaquin *et al*, which demonstrated the recovery of PCa cells from a senescence-associated growth arrest following CS-FBS or enzalutamide, respectively [20,35]. However, because AD results in an impure population that consists of both senescent and non-senescent cells, it could not be confirmed from these studies if the outgrowths from a subpopulation of cells were directly from senescent cells or rather partially due to a bystander effect via SASP factors that enhance growth of non-senescent cells [20]. Our cell sorting method provided a homogenous population of senescent cells to eliminate the possible bystander effects, and our results confirm our previous findings in models of breast and lung cancer that senescent cells can indeed escape growth arrest and resume proliferation.

Although the capacity of senescent PCa cells to reenter the cell cycle following androgen restoration is an important observation in itself (as it defies the traditional definition of senescence as an irreversible arrest that cells cannot escape even after the senescence-inducing stress is removed), PCa patients undergo a long-term ADT and will often progress during the course of treatment [6]. We therefore examined the outgrowth of senescent cells in continuous CS-FBS for clinical relevance. Substantially, our sorted population of senescent cells exhibited outgrowths in colonies that were continuously exposed to ADT (Figure 3). We counted significant number of colonies by Day 18 of CS-FBS treatment, and these colonies sustained their growth the following days. The re-emergence of cells from

a sorted population in continuous CS-FBS exposure strongly suggest that senescent cells are capable of developing androgen independence and may potentially contribute to CRPC via direct escape during prolonged ADT.

Our results, however, fail to define *how* senescent cells develop androgen independence. Burton *et al*. also characterized the outgrowth of castration-resistant clones from repeated exposure to androgen deprived conditions [20]. In addition to the high proliferative activity, they observed morphology changes, reduced SA-β-gal activity, and increased androgen receptor expression compared to the parental cells. These cells present phenotypes of "castration-resistance" and have no effect of either androgenic stimulation or treatment with antiandrogens on growth rate. Although the resistant clones from our study have not yet been characterized, we expect similar castration-resistant phenotypes. Additionally, Burton *et al*. selected their castration-resistant clones from the bulk population after multiple cycles of androgen deprivation and repletion [20], which contrasts to our sorted, SA-β-gal positive cells maintained under androgen deprived conditions. This raises interesting observations for future studies, such as the response to AD in senescent cells that have escaped senescence under androgen replete (FBS switch-back) conditions. Molecular changes may occur during escape and whether senescent cells that escape following reversion back to FBS have developed resistance to treatment is unknown. While our studies in continuous CS-FBS likely mimic the clinical state in which patients are on continuous ADT for 28-36 months, these studies may help identify what occurs in patients that are taken off therapy following complete remission, only to develop recurrence years later.

Our studies suggest that senescent cells may directly contribute to CRPC by escaping the growth arrest and developing androgen independence, and thus we hypothesized that their elimination may provide a therapeutic benefit. We therefore investigated whether these cells were susceptible to the senolytic agent ABT-263, which targets senescent cells and promotes apoptosis. Previous studies have

demonstrated the ability of ABT-263 to selectively remove senescent cells induced by therapy, such as PARP inhibitors, doxorubicin, etoposide, and radiation [29,36,37]. We show here for the first time that AD-induced senescent cells are also sensitive to senolysis by ABT-263, entering rapid apoptosis following exposure. Additionally, ABT-263 reduced the total number of clones capable of developing androgen independence (Figure 4A). Because we have shown that a subpopulation of senescent cells can escape to form androgen-independent clones, we speculate that the reduction in clonal outgrowth from ABT-263 is primarily due to the direct elimination of cells that escape during CS-FBS to form the castration-resistant like colonies. However, it is possible, and perhaps even likely, that the removal of senescent cells from the population also reduces clonal outgrowths by reducing paracrine signaling via the SASP. Therefore, the relative contribution of direct escape from senescence versus paracrine signaling from senescent cells should be pursued in future studies.

Moreover, we examined the mechanism by which ABT-263 induces senolysis, which has previously been identified to rely on either Bcl-w or Bcl-xL inhibition, but not Bcl-2 inhibition [24,29,38]. Our data shows that Bcl-xL inhibition induces substantial cell death in senescent cells, with no effect of Bcl-2 inhibition. Although our current studies do not examine the role of Bcl-w in promoting senescent cell viability or in ABT-263's mechanism, it appears that Bcl-xL inhibition is sufficient to eliminate ADT-induced senescent cells. Besides, it has been established that upregulation of BH3-only proteins plays an important part in regulating the sensitivity of ABT-263. Cancer cells are constantly in a stressed state (i.e. androgen deprivation) and activate BH3-only proteins, like Bim, to induce apoptosis. These cells, however, overcome the predisposition by overproducing anti-apoptotic proteins while removing active Bim into a complex with the Bcl-2 family proteins. ABT-263 disrupts the Bim-Bcl-xL complex and releases Bim towards cell death [24]. Cells in a normal state have inactive pro-apoptotic proteins, and consequently ABT-263 does not promote apoptosis, presumably because the Bim-Bcl-xL complex is not formed; however, this needs to be confirmed by additional experiments.

Our findings complement those of other recent studies that identified Bcl-xL as a major mediator in apoptotic resistance to senescent cells [29,37]. In contrast to our results, Malaquin *et al*. reported that enzalutamide-induced senescent LNCaP cells are resistant to ABT-263, suggesting DNA damage-induced senescence as a requirement for sensitization [35]. Cell death induced by Bcl-2 family inhibitors were shown to previously require that senescence was induced by DNA damage, but enzalutamide-induced senescent phenotypes lacked DNA damage and were insensitive to the senolytic agent. Their conclusion directly opposes Shahbandi *et al*. who showed that DNA damage is not required via Nutlin-3a induced senescence model [37]. We have also independently validated that LNCaP cells are insensitive to senolysis by ABT-263, but this may be due to the cell line, as our work has demonstrated that ADT-induced senescent cells are amendable to clearance by ABT-263. Nonetheless, ABT-263 sensitivity in AD-induced senescent cells is important to determine appropriate treatment options, and contradicting observations to the sensitivity of the senolytic require further investigation.

Alternatively, we need to take into account the heterogeneity of the cancer cells. Prostate tumors are highly heterogenous, and various types of cells are found within the same tumor. Some are susceptible to ADT or senescence, while others are initially resistant to treatment. Our data show that only a subpopulation of the cells is induced into senescence and/or become androgen independent from ADT. This implies that not all arrested cells are senescent, nor all proliferative recovery is from senescent cells. In addition to the androgen-independence from senescence, other types of cells are likely to resist treatment and contribute to CRPC. Targeted therapy has enhanced treatment outcomes by exploiting survival pathways but remains limited in completely eliminating progressed cancer cells due to further mutation and formation of a dynamic population that is difficult to sustain. Heterogeneity is yet poorly understood and will require in-depth assessments to fully optimize treatment outcomes.

We address several limitations in this project. CS-FBS is commonly used to cultivate cells under androgen-free conditions by removing steroid hormones via charcoal. There have been recent growing

concerns about using this serum due to the risk of removing essential molecules for cell growth or lot-to-lot variability [39,40]. However, we validate the use of CS-FBS as a relevant method for mimicking ADT by having performed a simultaneous study with bicalutamide. The results (not shown) were analogous to CS-FBS treatment. We have also extended our work to the human prostate cell line, LAPC-4 cells, and are currently investigating the determinants of sensitivity to ABT-263 in order to better explain the contradictory results with the LNCaP cell line.

A related limitation is that the recovery of the AD-induced growth arrested cells followed by FBS medium (Figure 2) does not validate that it is the stimulation of the androgen receptor that is responsible for the recovery. This may be addressed by adding testosterone instead of FBS back to the CS-FBS treated cells and adding a combination of testosterone + bicalutamide (or testosterone + enzalutamide), which would demonstrate direct regulation of androgen receptors. The addition of testosterone is likely to reverse the arrest of the tumor cells. Bicalutamide (or enzalutamide) directly inhibit the androgen receptor activity. While adding testosterone back to the androgen depleted condition would activate androgen receptors and promote tumor cell growth, exposure to bicalutamide (or enzalutamide) would inhibit the receptors and the cancer cells would likely remain static. These additional studies would provide more rigorous evidence that androgen receptor is important for the recovery of the cells.

Additionally, a considerable limitation for taking this work into the clinic is the toxicity of ABT-263 through the production of thrombocytopenia. Platelets express Bcl-xL for survival but ABT-263's selective inhibition causes them to undergo rapid death [41]. Although the toxicity can be dose-limiting and creates a challenge for clinical translation, trials have shown encouraging results with an effort to reduce platelet toxicity while targeting tumor cells. For example, a phase I trial in patients with relapsed lymphoid malignancies showed that administering a lead-in dose reduced acute platelet nadirs and

grade 4 thrombocytopenia [42]. Ongoing studies are being evaluated to identify the safest way to deliver the drug while enhancing its efficacy.

In conclusion, ADT induces cellular senescence in PCa cells and senescent cells can trigger deleterious effects by recovering from their dormant-like state and becoming resistant to treatment. The senolytic agent ABT-263 is an effective drug for eliminating these senescent cells, and its ability to delay proliferative recovery shows promising results. Therefore, the use of senolytic agents following ADT has potential as a novel therapeutic approach that could improve the survival of men with the advanced disease. Our future work will involve screening for less toxic agents and *in vivo* studies to examine the response of AD-induced senescent cells to ABT-263 as well as other senolytics.

REFERENCES

[1] American Cancer Society. (2019). Prostate Cancer. Available online: https://www.cancer.org/cancer/prostate-cancer.html

[2] SEER*Explorer: An interactive website for SEER cancer statistics [Internet]. Surveillance Research Program, National Cancer Institute. [Cited 2020 Sep 14]. Available online: https://seer.cancer.gov/explorer/.

[3] Rawla, P. Epidemiology of Prostate Cancer. *World J Oncol* **2019**, *10*, 63–89.

[4] Centers for Disease Control and Prevention. Prostate Cancer. Available online: https://www.cdc.gov/cancer/prostate/index.htm

[5] Pernar, C.H.; Ebot, E.M.; Wilson, K.M.; Mucci, L.A. The Epidemiology of Prostate Cancer. *Cold Spring Harb Perspect Med* **2018**, *8*, a030361.

[6] Karantanos, T.; Corn, P.G.; Thompson, T.C. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene* **2013**, *32*, 5501–5511.

[7] Huggins, C.; Hodges, C.V. Studies on prostatic cancer. I. The effects of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* **1941**, 1, 293–297.

[8] Wong, Y.N.S.; Ferraldeschi, R.; Attard, G.; de Bono, J. Evolution of androgen receptor targeted therapy for advanced prostate cancer. *Nat Rev Clin Oncol* **2014**, *11*, 365–376.

[9] Harris, W.P.; Mostaghel, E.A.; Nelson, P.S.; Montgomery, B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. *Nat Rev Urol* **2009**, *6*, 76–85.

[10] Miyamoto, H.; Messing, E.M.; Chang, C. Androgen deprivation therapy for prostate cancer: Current status and future prospects. *Prostate* **2004**, *61*, 332–353.

[11] National Cancer Institute. Hormone Therapy for Prostate Cancer. Available online: https://www.cancer.gov/types/prostate/prostate-hormone-therapy-fact-sheet

[12] Chandrasekar, T.; Yang, J.C.; Gao, A.C.; Evans, C.P. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). *Transl Androl Urol* **2015**, *4*, 364-380.

[13] Rice, M.A.; Malhotra, S.V.; Stoyanova, T. Second-Generation Antiandrogens: From Discovery to Standard of Care in Castration Resistant Prostate Cancer. *Front. Oncol.* **2019**, *9*, 801.

[14] Etheridge, T.; Damodaran, S.; Schultz, A.; Richards, K.A.; Gawdzik, J.; Yang, B.; Cryns, V.; Jarrard, D.F. Combination therapy with androgen deprivation for hormone sensitive prostate cancer: A new frontier. *Asian J Urol* **2019**, *6*, 57–64.

[15] Chakradeo, S.; W. Elmore, L.; A. Gewirtz, D. Is Senescence Reversible? *Current Drug Targets* **2016**, *17*, 460–466.

[16] Hernandez-Segura, A.; Nehme, J.; Demaria, M. Hallmarks of Cellular Senescence. *Trends in Cell Biology* **2018**, *28*, 436–453.

[17] Barascu, A.; Le Chalony, C.; Pennarun, G.; Genet, D.; Imam, N.; Lopez, B.; Bertrand, P. Oxidative stress induces an ATM-independent senescence pathway through p38 MAPK-mediated lamin B1 accumulation: Senescence through lamin B1 accumulation. *The EMBO Journal* **2012**, *31*, 1080–1094.

[18] Byun, H.O.; Han, N.K.; Lee, H.J.; Kim, K.B.; Ko, Y.G.; Yoon, G.; Lee, Y.S.; Hong, S.I.; Lee, J.S. Cathepsin D and Eukaryotic Translation Elongation Factor 1 as Promising Markers of Cellular Senescence. *Cancer Research* **2009**, *69*, 4638–4647.

[19] Ewald, J.A.; Desotelle, J.A.; Church, D.R.; Yang, B.; Huang, W.; Laurila, T.A.; Jarrard, D.F. Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo. *Prostate* **2013**, *73*, 337-345.

[20] Burton, D.G.A.; Giribaldi, M.G.; Munoz, A.; Halvorsen, K.; Patel, A.; Jorda, M.; Perez-Stable, C.; Rai, P. Androgen Deprivation-Induced Senescence Promotes Outgrowth of Androgen-Refractory Prostate Cancer Cells. *PLoS ONE* **2013**, *8*, e68003.

[21] Saleh, T.; Tyutynuk-Massey, L.; Cudjoe, E.K.; Idowu, M.O.; Landry, J.W.; Gewirtz, D.A. Non-Cell Autonomous Effects of the Senescence-Associated Secretory Phenotype in Cancer Therapy. *Front. Oncol.* **2018**, *8*, 164.

[22] Kale, J.; Osterlund, E.J.; Andrews, D.W. BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ* **2018**, *25*, 65–80.

[23] Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol* **2007**, *35*, 495–516.

[24] Tse, C.; Shoemaker, A.R.; Adickes, J.; Anderson, M.G.; Chen, J.; Jin, S.; Johnson, E.F.; Marsh, K.C.; Mitten, M.J.; Nimmer, P.; et al. ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. *Cancer Research* **2008**, *68*, 3421–3428.

[25] Lebedeva, I.; Rando, R.; Ojwang, J.; Cossum, P.; Stein, C.A. Bcl-xL in Prostate Cancer Cells: Effects of Overexpression and Down-Regulation on Chemosensitivity. *Cancer Research* **2000**, *60*, 6052-6060.

[26] Castilla, C.; Congregado, B.; Chinchón, D.; Torrubia, F.J.; Japón, M.A.; Sáez, C. Bcl-xL Is Overexpressed in Hormone-Resistant Prostate Cancer and Promotes Survival of LNCaP Cells via Interaction with Proapoptotic Bak. *Endocrinology* **2006**, *147*, 4960–4967.

[27] Bah, N.; Maillet, L.; Ryan, J.; Dubreil, S.; Gautier, F.; Letai, A.; Juin, P.; Barillé-Nion, S. Bcl-xL controls a switch between cell death modes during mitotic arrest. *Cell Death Dis* **2014**, *5*, e1291–e1291.

[28] Zhu, Y.; Tchkonia, T.; Pirtskhalava, T.; Gower, A.C.; Ding, H.; Giorgadze, N.; Palmer, A.K.; Ikeno, Y.; Hubbard, G.B.; Lenburg, M.; et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell* **2015**, *14*, 644–658.

[29] Saleh, T.; Carpenter, V.J.; Tyutyunyk-Massey, L.; Murray, G.; Leverson, J.D.; Souers, A.J.; Alotaibi, M.R.; Faber, A.C.; Reed, J.; Harada, H.; et al. Clearance of therapy-induced senescent tumor cells by the senolytic ABT-263 via interference with BCL-X_L –BAX interaction. *Mol Oncol* **2020**, *14*, 2504-2519.

[30] Chang, J.; Wang, Y.; Shao, L.; Laberge, R.-M.; Demaria, M.; Campisi, J.; Janakiraman, K.; Sharpless, N.E.; Ding, S.; Feng, W.; et al. Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med* **2016**, *22*, 78–83.

[31] Blagosklonny, M.V. Cell cycle arrest is not senescence. *Aging* **2011**, *3*, 94–101.

[32] Milanovic, M.; Fan, D.N.Y.; Belenki, D.; Däbritz, J.H.M.; Zhao, Z.; Yu, Y.; Dörr, J.R.; Dimitrova, L.; Lenze, D.; Monteiro Barbosa, I.A.; et al. Senescence-associated reprogramming promotes cancer stemness. *Nature* **2018**, *553*, 96–100.

[33] Short, S.; Fielder, E.; Miwa, S.; von Zglinicki, T. Senolytics and senostatics as adjuvant tumour therapy. *EBioMedicine* **2019**, *41*, 683–692.

[34] Blute, M.L.; Damaschke, N.; Wagner, J.; Yang, B.; Gleave, M.; Fazli, L.; Shi, F.; Abel, E.J.; Downs, T.M.; Huang, W.; et al. Persistence of senescent prostate cancer cells following prolonged neoadjuvant androgen deprivation therapy. *PLoS ONE* **2017**, *12*, e0172048.

[35] Malaquin, N.; Vancayseele, A.; Gilbert, S.; Antenor-Habazac, L.; Olivier, M.-A.; Ait Ali Brahem, Z.; Saad, F.; Delouya, G.; Rodier, F. DNA Damage- But Not Enzalutamide-Induced Senescence in Prostate Cancer Promotes Senolytic Bcl-xL Inhibitor Sensitivity. *Cells* **2020**, *9*, 1593.

[36] Fleury, H.; Malaquin, N.; Tu, V.; Gilbert, S.; Martinez, A.; Olivier, M.-A.; Sauriol, A.; Communal, L.; Leclerc-Desaulniers, K.; Carmona, E.; et al. Exploiting interconnected synthetic lethal interactions between PARP inhibition and cancer cell reversible senescence. *Nat Commun* **2019**, *10*, 2556.

[37] Shahbandi, A.; Rao, S.G.; Anderson, A.Y.; Frey, W.D.; Olayiwola, J.O.; Ungerleider, N.A.; Jackson, J.G. BH3 mimetics selectively eliminate chemotherapy-induced senescent cells and improve response in TP53 wild-type breast cancer. *Cell Death Differ* **2020**, *27*, 3097–3116.

[38] Leverson, J.D.; Phillips, D.C.; Mitten, M.J.; Boghaert, E.R.; Diaz, D.; Tahir, S.K.; Belmont, L.D.; Nimmer, P.; Xiao, Y.; Ma, X.M.; et al. Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci. Transl. Med.* **2015**, *7*, 279ra40-279ra40.

[39] Cao, Z.; West, C.; Norton-Wenzel, C.S.; Rej, R.; Davis, F.B.; Davis, P.J.; Rej, R. Effects of resin or charcoal treatment on fetal bovine serum and bovine calf serum. *Endocr Res* **2009**, *34*, 101-108.

[40] Sikora, M. J.; Johnson, M. D.; Lee, A. V.; Oesterreich, S. Endocrine Response Phenotypes Are Altered by Charcoal-Stripped Serum Variability. *Endocrinology* **2016**, *157*, 3760–3766.

[41] Zhang, H.; Nimmer, P.M.; Tahir, S.K.; Chen, J.; Fryer, R.M.; Hahn, K.R.; Iciek, L.A.; Morgan, S.J.; Nasarre, M.C.; Nelson, R.; et al. Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ* **2007**, *14*, 943–951.

[42] Wilson, W.H.; O'Connor, O.A.; Czuczman, M.S.; LaCasce, A.S.; Gerecitano, J.F.; Leonard, J.P.; Tulpule, A.; Dunleavy, K.; Xiong, H.; Chiu, Y.-L.; et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. The Lancet Oncology 2010, 11, 1149–1159.

[43] Frame, F.M.; Noble, A.R.; Klein, S.; Walker, H.F.; Suman, R.; Kasprowicz, R.; Mann, V.M.; Simms, M.S.; Maitland, N.J. Tumor heterogeneity and therapy resistance - implications for future treatments of prostate cancer. *JCMT* **2017**, *3*, 302.

VITA

So Min Lee was born on March 12, 1995, in Daegu, South Korea. She graduated from Meridian Medical Arts Charter High School, Meridian, Idaho in 2014. She received her Bachelor of Science in Chemistry from Boise State University, Boise, Idaho in 2018.