THE LYSOSOMOTROPIC AGENT SIRAMESINE AND
THE TYROSINE KINASE INHIBITOR LAPATINIB
INDUCE CELL DEATH IN PROSTATE CANCER CELLS

by
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ABSTRACT

Prostate cancer is the most common cancer affecting men often resulting in aggressive tumors with poor prognosis. Novel therapeutic strategies to treat prostate cancer include combinational treatments aiming to reduce the negative effects of chemotherapy, such as drug resistance. The use of lysosomotropic agents offers a new treatment possibility since they disrupt lysosomal membranes and can trigger a series of events leading to cell death. In addition, combining lysosome disrupting agents with targeted inhibitors can induce synergistic cell death in different cancer types. In prostate cancer, these combination treatments have not been tested before. I found the lysosomotropic siramesine and the tyrosine kinase inhibitor lapatinib to be the most potent drug combination to induce cell death. I also investigated the mechanism by which cells were dying by siramesine and lapatinib treatment and found that increases in ROS caused significant cellular damage leading to mitochondrial dysfunction and high levels of lipid peroxidation. These effects were more prominent in PC3 cells, which are representative of the most aggressive type of the disease and therefore, this combination holds the potential to treat advanced prostate cancer.
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<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine mono phosphate kinase</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ASM</td>
<td>Acid sphingomyelinase</td>
</tr>
<tr>
<td>BAF1</td>
<td>Bafilomycin 1</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fer-1</td>
<td>Ferrostatin-1</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>HOXB13</td>
<td>Homeobox B13</td>
</tr>
<tr>
<td>HSP27</td>
<td>Heat shock protein 27</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MCL1</td>
<td>Induced myeloid leukemia cell differentiation</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>Nec-1</td>
<td>Necrostatin-1</td>
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</tbody>
</table>
LC50  Lethal concentration 50
LMP   Lysosome membrane permeabilization
PAGE  Polyacrylamide gel electrophoresis
PBST  1X PBS and 0.1% Tween20 Detergent
PBS   Phosphate buffered saline
PDGFR Platelet-derived growth factor receptor
PTEN  Phosphatase and tensin homolog
RIPK1/3 Receptor-interacting serine/threonine-protein kinase 1/3
Rb1   Retinoblastoma1
ROS   Reactive oxygen species
RAF   Rapid accelerated fibrosarcoma
RIPK1/3 Receptor-interacting serine/threonine-protein kinase 1/3
ROS   Reactive oxygen species
RPMI  Roswell Park Memorial Institute
RSL3  RAS Selective Lethal 3
SDS   Sodium dodecyl sulfate
SKII  Sphingosine kinase II
SOD   Superoxide dismutase
TMRM  Tetramethylrhodamine, methyl ester
TP53  Tumor protein 53
TNF-alpha Tumor necrosis factor alpha
TNFR  Tumor necrosis factor receptor
TRAIL TNF-related apoptosis inducing ligand
VEGFR Vascular endothelial growth factor receptor
CHAPTER 1: INTRODUCTION

1.1 PROSTATE CANCER

1.1.1 Prostate cancer

Prostate cancer is the most common cancer affecting men and it is estimated that 1 in 7 Canadian men will be diagnosed with the disease, according to Prostate Cancer Canada (1, 2, 3). Despite tremendous advancements made on improving early diagnosis and treatment in the last decades, resistance to current chemotherapy drugs still occurs (4, 5). If patients are diagnosed with prostate cancer during an early stage of the disease, the survival rate is close to 100%. However, close to 20% of men will be diagnosed with the more aggressive type, called advanced prostate cancer. This stage has a very poor prognosis where 3 out of 4 men diagnosed with it will die within 2 to 4 years (1). Currently, this is an incurable disease and the only strategy available is the use of chemotherapy and androgen deprivation therapy to prolong and improve the quality of life of patients (4, 6, 7).

Prostate cancer begins to develop when the cells of the prostate start to grow uncontrollably which leads to inflammation of the tissue and the increasing accumulation of mutations (4). These events trigger the transformation of cells into cancer cells and their metastasis to other organs (8). Advanced prostate cancer is the most aggressive type since the cancer cells have already spread to other organs and are more difficult to treat (9). This type of disease encompasses different stages and includes locally advanced prostate cancer where the cancer has started to migrate; or in the case of metastatic prostate cancer, it has already migrated to other parts of the body, more commonly to the bones and lymph nodes. It is estimated that up to 90% of patients at this stage show bone metastasis. Castration-resistant prostate cancer is another type that develops when the cancer has stopped responding to the first-line of androgen deprivation therapy and becomes resistant to treatment. Lastly, recurrent prostate cancer develops when the cancer has begun to grow again after a remission period (1).

To determine the severity and the type of prostate cancer clinicians use the Gleason scoring method. This technique studies tissue samples from two different locations under a microscope and classifies them based on how much they differ from non-malignant tissue samples. The score is assigned using numbers from 3-5 from each location and are then added together. A Gleason score of 6 or lower is a low-grade cancer and a score of 7 refers to an
intermediate type. Scores above 7 are samples that differ significantly from normal tissue and are referred to as poorly differentiated (10, 11, 12).

1.1.2 Current chemotherapy treatments and challenges

There are many approaches to treat prostate cancer and the most common one is active surveillance (5). This strategy is used when the cancer is small and slow growing, and it is most recommended for older people with other medical conditions and for which treatment is not expected to benefit them (13). Active surveillance involves following changes in the prostate specific antigen (PSA) levels and performing digital rectal exams, biopsies and CT scans to make sure the cancer has not progressed further (5). But when the cancer continues to grow, treatment such as surgery and radiation are considered, followed by androgen deprivation therapy (ADT) and chemotherapy (10). Surgery and radiation attempt to remove and/or decrease the number of cancerous cells but this technique can still lead to some cancer cells not being detected and removed (14, 15). This eventually triggers the return of the cancer. To prevent this, ADT drugs such as abiraterone and enzalutamide are administrated as the first line of treatment to reduce androgen levels since prostate cells require androgen hormones to grow, especially testosterone (7, 16, 17).

Chemotherapy is then included as a treatment strategy for recurrent or metastatic cancer in cases where the cancer does not respond to ADT and for this reason, a more aggressive approach is needed (1). Some of the most common chemotherapy drugs used alone or in combination with ADT are doxetacel, cabazitaxel, estramustine, mitoxantrone, olaparib and prednisone (16, 18, 19, 20, 21, 22). A more novel treatment approach is immunotherapy such as the sipuleucel-T and ipilimumab vaccines that specifically target prostate cancer cells. However, this approach has not shown significant tumor regression in patients with advanced prostate cancer compared to common chemotherapy drugs (7, 23, 24). Several new vaccines are under clinical trials now and their combination with other chemotherapy drugs is expected to show improved outcomes for patients in the future (10, 25).

When the cancer has spread to the bones, chemotherapy along with bone mineralization agents such as alendronate and denosumab are administered to improve the bone health of patients (26, 27). There is no effective treatment at this stage and the best approach is therefore, help to maintain bone health and reduce bone pain (28). Overall, there are several treatments
available for advanced prostate cancer but none of them can cure the disease. Drug resistance and cancer recurrence remain obstacles to finding a cure for advanced prostate cancer (29, 30).

1.1.3 Molecular pathogenesis

Advances in next generation genome sequencing have improved our understanding on the molecular patterns leading to the initiation and progression of cancer, including those that are only present in advanced prostate cancer patients (31). The goal of identifying molecular traits is to use them as indicators of disease progression to improve diagnosis, and for designing more effective drug treatments (32, 33). Several mutations accumulate early in disease development and one of the most common ones present in prostate cancer include the inhibition of cell cycle checkpoints, DNA repair pathways and expression of key receptors involved in initiating cell signalling cascades that promote cell proliferation, cell growth and survival (34, 35, 36, 37, 38, 39). These mutations are present in the majority of patients and although they drive cancer development and progression they represent a small portion of the vast heterogeneity of mutations. The most studied genes are TP53, PTEN, HOXB13, BRCA1 and BRCA2, c-MYC, RB1, EGFR and AR (10, 32, 40, 41, 42). These are mutations that are not used to determine the prognosis but are correlated with prostate cancer patients throughout their disease. HOXB13, BRCA1 and BRCA2 were shown to confer significant risk of prostate cancer but were only identified in early prostate diagnosis and not associated with disease progression.

TP53, RB1 and PTEN are tumor suppressor genes that allow cells to avoid cell cycle checkpoints and grow uncontrollably. MYC is an oncogene that is altered through gene amplification which provides cells with continuous signaling to proliferate and growth. Anti-apoptotic proteins such as HSP27 and Clusterin allow cells to escape cell death by blocking the apoptotic pathway (43, 44, 45, 46, 47, 48). AR is the genetic alteration most uniquely associated with prostate cancer (49, 50, 51). Cancer cells alter the androgen receptor to increase androgen signaling which is required for prostate cells to grow (50). Mutations in this receptor following androgen deprivation therapy are one of the main drivers of disease progression in advanced prostate cancer (52, 53). This alteration has been identified to be mutated by gene amplification or splice variants and point mutations leading to constitutive activation (54, 55).
1.2 HALLMARKS OF CANCER

Cancer develops from a variety of genetic modifications such as chromosome instability, somatic and germline mutations, also including post-translational modifications such as microRNAs and DNA methylation patterns. The main purpose of these alterations is to shift the normal balance between cell survival and cell death in all non-malignant cells to a state of continuous proliferation, growth and replicative immortality (56, 57, 58, 59). All cancer populations share common traits or adaptations that are acquired during the transformation process (56, 60, 61). The most up to date knowledge we have of these adaptations were published in The Hallmarks of Cancer paper by Hanahan and Weinberg in 2011 revolutionizing our view on how cancer develops. Based on our current knowledge, increased levels of cell proliferation, the ability to avoid cell growth suppressors while evading cell death signals constitute the first stages of cancer development (62, 63, 64). Cancer cells can also replicate indefinitely by behaving in similar patterns to those found in stem cells of non-malignant tissues (61, 65, 66). The development of new blood vessels connecting other tissues called angiogenesis, is essential to provide cancer cells with additional nutrients and routes to invade surrounding tissues. These events then contribute to metastasis, the establishment of cancer populations in other tissues (67, 68). In addition to these main alterations, increased genomic instability provides cells with a variety of novel genetic mutations to achieve all these developmental stages of cancer growth (69, 70). More recently, metabolic adaptations designed to supply cancer cells with additional nutrients and the ability to evade destruction from our immune system have been added as additional hallmarks of cancer (56, 65, 71, 72, 73). These hallmarks have all been identified in prostate cancer and include signals to increase cell proliferation and cell growth by use of selective mutations such as c-MYC, TP53 and PTEN (8, 30). Prostate cancer cells also acquire the ability to evade cell death signals through mutations in anti-apoptotic proteins such as HSP27 (74). The most characteristic hallmark of prostate cancer are the mutations found in the androgen receptor which confers cancer cells further proliferation capabilities (75, 76). This continuous growth is supported through alterations in metabolic pathways, one which occur through the AMP-activated protein kinase (AMPK) signalling pathway conferring cells the ability to increase ATP production (77, 78). Changes in de-novo cholesterol and fatty acid synthesis and steroid production further provide the tumor with additional nutrients (79, 80, 81). All these changes are recognized by the immune system and in order to evade destruction from
the immune system, prostate cancer cells alter their micro-environment to receive protection from the surrounding cells of the stroma (82, 83, 84). Once the tumor has been established within the prostate, the process of angiogenesis begins to develop through alterations in vascular endothelial growth factor (VEGF) signalling pathways allowing cells to acquire more nutrients to survive and establish in other tissues through an increase in blood vessel connections. Metastasis is then initiated through changes in cell migration and adhesion properties to invade the bones and lymph nodes (29, 85, 86). These selective mechanisms constitute the major hallmarks of prostate cancer and as we continue to analyze information from genomics and proteomics data, we will find more regulators of prostate cancer progression.

1.3 CELL DEATH PATHWAYS

All life forms are governed by the laws of cell survival and cell death signals. A cell dies when it reaches the end of its cell cycle as a natural process required during development (87, 88). Cellular death can also serve as a strategy to eliminate harmful or damaged cells that no longer support the growth and maintenance of the organism (89). This homeostatic process of life and death is often unbalanced in diseases such as heart disease and neurological damage where cells are selected to die more than to survive. On the contrary, some cells are capable of surviving and reproducing uncontrollably such as in cancer (62, 89). During the last decades, we have investigated several ways by which cells can selectively induce cell death through distinct mechanisms but there are also ways for cells to die uncontrollably in a non-regulated manner. These types of cell death have evolved throughout time based on environmental pressures and have also been adapted in a cell and context dependent manner (82, 91, 92). Since the discovery that cancer cells are capable of avoiding cell death by mutations in cell death pathways to achieve immortality and indefinite proliferating abilities, we have exploited these characteristics to find new therapeutic targets for cancer (62), including prostate cancer (94, 96, 97, 98, 99, 100).

1.3.1 Apoptosis

Apoptosis is the most evolutionary conserved model for cell death in eukaryotic organisms regulating embryogenesis, development, cell turnover and programmed cell death during stressful events (101, 102, 103, 104). In addition, this mechanism is the most manipulated
by cancer cells to avoid cell death, including in prostate cancer (102, 105). The most well-known types of apoptosis are the intrinsic and extrinsic apoptotic pathways. The equilibrium between cell survival and cell death is regulated by genes controlling B-Cell lymphoma/leukemia-2 (BCL2) family members’ expression and protein-protein interactions (106, 107, 108). The main event contributing to apoptotic cell death is the permeabilization of the outer mitochondrial membrane that results in the release of cytochrome c. This increase in cytosolic cytochrome c leads to the activation of caspases more specifically caspase-3, the final executor of the apoptotic pathway. Mitochondrial outer membrane permeabilization (MOMP) also leads to the translocation of the transcription factor AIF into the nucleus to initiate chromatic condensation, contributing to the induction of apoptotic cell death (109, 110).

The BCL2 family is composed of a large number of proteins sharing one or several domains (BH1-BH5). The different combination of domains among these proteins allows for specific binding between them. There are four main groups of BCL2 proteins involved in the apoptotic pathway: a) anti-apoptotic ‘mediators’ (BCL-2, BCL-XL, BCL-w, MCL-1, BFL-1/A1), b) pro-apoptotic ‘effectors’ (BAX and BAK), c) BH3-only ‘activators’ (BID, BIM, PUMA) and d) ‘sensitizers’ (BAD, BIK, NOXA, BMF, HRK). After stress signals such as DNA damage or growth factor withdrawal, expression of pro-apoptotic activators cause BAX and BAK to bind to each other as dimers and initiate MOMP. Under conditions that stimulate cell survival and expression of anti-apoptotic proteins, anti-apoptotic mediators function to indirectly inhibit the dimerization of BAX and BAK. By binding and ‘sequestering’ pro-apoptotic activators, anti-apoptotic mediators inhibit their ability to trigger BAX and BAK dimerization and by doing so, prevent apoptosis. Anti-apoptotic mediators can also directly inhibit BAX and BAK but this interaction is not commonly observed in cells. Lastly, the role of anti-apoptotic sensitizers is to free activators bound to BCL2 and by doing so, they can trigger BAX and BAK dimerization again. This mechanism neutralizes the function of anti-apoptotic BCL2 mediators (62, 103, 107, 111). In recent years, scientists have discovered that non-malignant cells and cancer cells can die by apoptosis through a mechanism independent of caspases, called caspase-independent cell death. This apoptotic pathway also includes MOMP and the release of the mitochondrial transcription factor AIF into the nucleus but it does not require the activation of the executioner caspases (110, 112, 113).
The other type of apoptosis called the extrinsic apoptotic pathway is an alternative mechanism that relies on the activation of receptors such as TNF, Fas and TRAIL. These receptors are activated after severe mechanical damage or significant cellular damage by environmental factors such as high pressure and high temperature, severe inflammation or high concentrations of drugs. This pathway also activates in particular the caspase executioner Caspase-8 which together with other signalling factors form the death-inducing signalling complex (DISC). DISC can initiate MOMP as observed in the intrinsic apoptotic pathway or it can activate Caspase-3 directly, leading to cell death (105, 106, 114, 115).

From all the cell death pathways, apoptosis is the mechanism most reported by which prostate cancer cells die despite efforts made by cancer cells to avoid cell death through genetic and epigenetic mutations (116, 117, 118). They also rely on these modifications to avoid apoptotic cell death during the transformation to androgen-independent cancer cells (119, 120, 121). Therapeutic efforts continue to focus on exploiting their unbalanced apoptotic signalling through the targeting of apoptotic proteins such as heat shock proteins (74, 122, 123). Other drug treatments inducing apoptosis in prostate cancer such as doxetacel and paclitaxel focus on disrupting cell division (124, 125).

1.3.2 Necrosis

There exists a less frequent type of cell death called necrosis which can be induced after excessive mechanical and/or chemical damage events. This type of cell death is known to be triggered and regulated in a more uncontrollable manner than apoptosis. An excessive level of mechanical damage as well as significant concentrations of certain drugs can lead to the rupture of the plasma membrane and cause DNA damage (126, 127). More recently, scientists have identified a type of programmed cell death named necroptosis (128, 129). This mechanism is independent of caspase activation and relies on the activation of the necroptosis death receptor TNF or Fas leading to the activation of the RIPK1 and RIPK3 kinases (130, 131). Several stimuli such as cell death ligands, DNA damage, viral sensors and hypoxia can trigger a necroptotic type of cell death (91, 134). In prostate cancer, necrotic events are usually observed within the core of the established prostate tumor where oxygen and nutrient availability are scarce leading to a necroptotic-type of cell death (135, 136). Necrosis at the core of the tumor has been reported to trigger the outgrowth of cancer cells and the initiation of angiogenesis and metastasis (136, 137).
Prostate cancer cells can undergo necroptotic cell death due to significant mechanical damage, following drug treatment, or when the tumor begins to lose blood vessel supply within the tumor core leading to decreased nutrient availability (105, 138, 139).

1.3.3 Autophagy- cell death

Autophagy is a conserved mechanism of cell survival present in eukaryotes. The purpose of autophagy is to recycle molecules and damaged organelles to build new cell structures and increase the flow of metabolic intermediates for energy production. Autophagy has been long characterized as a process that helps cells to support developmental, immunity and aging processes. Autophagy begins with the formation of autophagosome structures which carry the cargo destined for recycling. The fusion of autophagosomes with lysosomes, the organelles that contain proteolytic enzymes, lead to the degradation and recycling of these cellular compounds (140, 141, 142). Under severe and continuous stress, or under nutrient starvation, autophagy is activated as a way to protect the cell and sometimes this excessive state of autophagy can lead to a depletion of available nutrients and organelles; and ultimately, to the cell's own death. This process has been termed autophagic-cell death, and it has been implicated in several disease states such as cancer; therefore, targeting autophagy is a new therapeutic strategy to deplete cancer cells from a source of nutrients (142, 143).

Prostate cancer cells use autophagy as a protective mechanism to increase their nutrient availability in stressful events especially during tumor growth and tumor progression (144). Autophagy is actually a double-edge sword since increased autophagy can either sustain further tumor growth or lead to autophagic cell death. There are several drugs including natural compounds such as resveratrol that activate autophagic processes to levels that are unsustainable to cell survival (117, 142). Triggering autophagy to a critical homeostatic state is a new route for designing therapeutic agents. It is still not completely understood what susceptible pathways in prostate cancer cells can be exploited successfully in autophagy as the regulation of these events depend on the needs of cancer populations and that are also influenced by their environment (144, 145, 156).
1.3.4 Ferroptosis

The most newly discovered type of cell death, ferroptosis, is trigged by an increase in available labile iron within cells. Certain drugs such as sorafenib and erastin can lead to ferroptotic cell death by inhibiting enzymes responsible for maintaining iron homeostasis by decreasing the amount of chelatable and redox-active radicals (131, 147). The mechanism by which drugs can induce ferroptosis is by targeting the Xc-cysteine pathway, a system that exports glutamate and imports cysteine molecules. The Xc-cysteine pathway functions as an antioxidant designed to counteract free radicals binding with lipids, and to ensure a balance between labile iron pool levels (148, 149). Even though ferroptosis is a newly discovered type of cell death, it has already been linked with several diseases including neurological diseases, acute kidney injury, ischemia and tumorigenesis (149). The reason why some cancer cells undergo ferroptosis under selective circumstances is related to their altered iron and lipid metabolisms. In tissues where these pathways are upregulated or altered in unique ways, an increase in labile iron can potentially lead to an increase in reactive oxygen species which can target oxidation of lipids more specifically. An increase in lipid peroxidation rapidly leads to cell death since membranes become unstable and cannot longer sustain cell and organelle structure (147, 150).

To our knowledge, the role of ferroptosis in prostate cancer has not clearly been defined. There might not exist such relationship as prostate cancer does not rely as heavily on labile iron pools as other cancer types do; however, we need further investigation on how prostate cancer cells manage their iron levels. Evidence suggests prostate cancer cells are susceptible to ferroptotic cell death by specific drugs such as erastin but in general, we do not have much evidence on ferroptosis inducing drugs in advanced prostate cancer as compared to other cancer types (151). As we continue to investigate the effect of available natural and synthetic drugs on prostate cancer, we will be able to test for new inducers of ferroptosis in this type of cancer for therapeutic use (147, 151, 152).

1.4. REACTIVE OXYGEN SPECIES (ROS) AND LYSOSOMES IN CELL DEATH

1.4.1 ROS and cell death

ROS are produced from biochemical reactions and metabolic processes and include a number of oxygen-containing molecules such as hydroxyl radicals (·OH), superoxide anion (O₂⁻), and hydrogen peroxide (H₂O₂) (153). ROS are generated mostly during the production of
energy by the electron transport chain in the mitochondria (154). In addition, they are used by the cell in small quantities as signalling molecules during development, growth and differentiation, and most notably as a signal to induce cell death (155). Higher than normal ROS levels are often observed when a cell experiences damage due to mechanical stress, addition of drugs or DNA damage (156, 157).

The cell also has protective mechanisms against increased levels of toxic oxygen radicals by use of antioxidants such as vitamin A and E that are able to function as shields and convert them into more stable molecules. Enzyme antioxidants also perform these activities by incorporating ROS into functional macromolecules for other processes. Some of the most important ones include glutathione (GSH) and superoxide dismutase (SOD). In cases when severe damage by ROS levels cannot be neutralized by antioxidants and negatively impact the normal physiological functions of the cell, ROS can trigger the initiation of distinct cell death mechanisms (153).

In cancer, ROS levels tend to be higher than in non-malignant cells due to their increased metabolic needs as well as in other organelles that deal with increased levels of radicals such as in lysosomes. The unique adaptations of cancer cells also allow them to use ROS as signalling molecules to promote cell proliferation and invasion (158). Because of its importance in tumor development, cancer cells tightly control the regulation of ROS levels and even small changes in ROS by induction of drugs or mechanical damage can have a counteractive effect and switch the balance towards cell death. Prostate cancer has many mutations in ROS metabolism such as increased oxidative stress to induce inflammation of the prostate which promotes cancer migration (159, 160). For example, SOD3 and GSH levels are reduced while lipid peroxidation by-products and other reactive species are elevated throughout the course of the disease. Androgens also play a role by increasing autophagic flux through the induction of ROS (160, 161, 162).

### 1.4.2 Lysosomes and cell death

Lysosomes are small acidic organelles that function as the recycling centers of the cell by providing additional nutrients and building blocks for routine biological processes, but they are also an important source of energy and macromolecules that are needed during starvation or under cellular damage. Lysosomes contain numerous acidic hydrolytic proteins called cathepsins
that perform these recycling activities but the role of lysosomes is not solely confined to recycling (163). Lysosomes contain important membrane lipids and enzymes that serve as signalling molecules to regulate levels of cell survival and cell death signals as well as regulating lipid metabolism (164, 165). In some situations, lysosomes can lead to cell death through a process that involves the permeabilization and destabilization of lysosome membranes. If lysosomes are significantly damaged, it can lead to the release of acidic hydrolases and toxic compounds into the cytoplasm (166, 167). Release of lysosome cathepsins can act as mediators of different types of cell death, and in the case of apoptosis, by activation and cleavage of key pro-apoptotic factors such as Bid (167).

### 1.4.3 Lysosomotropic agents and their use in cancer therapy

Lysosomotropic agents are molecules able to penetrate lysosome membranes and induce lysosome membrane permeabilization (LMP). Many of these molecules were designed for clinical use as antihistamines or antidepressants and are used by people every day (168). A study conducted in 2016 found several compounds with weak amino bases with a pKa~ 8-10 that were capable of penetrating and accumulating within lysosomes and inducing cell death (169). As the interest on lysosomotropic agents for cancer therapy increased, several studies were conducted to test compounds sharing these same characteristics in *in-vitro* models of cancer such as breast, lung, chronic lymphocytic leukemia (CLL) and glioblastoma; all which were effective at inducing cell death at relatively low doses (170, 171, 172).

The mode of action of these lysosomotropic agents involves their accumulation within lysosomes causing damage to lysosome membranes and often resulting in leakage of their contents into the cytosol triggering a series of events leading to cell death (168). In general, lysosomotropic agents are more effective in cancer cells than in non-malignant cells. This is due to the higher metabolic demands needed for a cancer cell to survive. Cancer cells have a higher number of lysosomes, are larger in size, and have acquired favorable genetic lysosomal alterations in order to support these increased metabolic demands (173). These adaptations can contribute to the development of tumor metastasis since lysosomes contain more hydrolytic enzymes to help them break down non-malignant cellular membranes to facilitate tissue invasion (174, 175).
1.4.4 Susceptible pathways targeted by lysosomotropic agents

Lysosomotropic agents accumulate within lysosomes ultimately causing cell death but the severity and type of damage depends on the unique genetic adaptations of cancer cells. These adaptations make them more susceptible to cell death by lysosomotropic agents than non-malignant cells (168). Disruption of autophagic processes is often observed after treatment due to an increase in lysosomal pH that block autophagosome and lysosome fusion resulting in significant autophagy stress leading to cell death (163, 176, 177, 178).

In-vitro models of lung cancer and glioblastoma were reported to be sensitive to ferroptotic cell death by the anti-depressant and lysosomotropic agent siramesine due to their increased iron metabolism. Siramesine triggered the release and accumulation of toxic labile iron pools from lysosomes causing cell death (170). Another consequence of lysosomotropic includes the intercalation of these compounds within organelle membranes such as lysosomes and mitochondria and within the plasma membrane (179). Enzymatic function is also inhibited by disrupting protein-protein interactions and through binding affinity to some enzymes. For example, siramesine is known to inhibit the lysosomal enzyme acid sphingomyelinase (ASM) responsible for converting sphingomyelin into ceramide and vice-versa (180). Enzyme inhibition can shift the sphingolipid balance of cell survival and cell death in addition to disrupting cellular membranes by intercalation (179). Lysosomotropic agents are also known to induce generation of soluble ROS, lipid ROS and mitochondrial dysfunction all which have important down-stream effects leading to cell death (157, 181).

1.4.5 Lipid peroxidation and lysosomotropic agents

Lipid peroxidation is one of the most common side-effects of lysosomotropic agents in cancer cells due to increased levels of reactive oxygen species and intercalation of these compounds within membranes (182). Cancer cells usually experience higher levels of reactive oxygen species that allows them to surpass certain biological thresholds to increase cell proliferation, growth, and metabolism processes (158) while at the same time tightly regulating the levels of antioxidants such as alpha-Tocopherol (171, 182, 183). The lysosomotropic siramesine is one of the most potent inducers of lipid ROS in cancer cells (180). In CLL, acute myeloid leukemia (AML) and breast cancer cells treatment with low doses of siramesine was
shown to induce massive lipid peroxidation and mitochondria dysfunction since lipid peroxidation can decrease the mitochondrial membrane potential leading to cell death (157).

The exact cross-talk between lysosomes, lipid peroxidation and mitochondrial dysfunction is still being investigated. What we know so far is that certain cancer cells alter the levels of sphingosine molecules, as well as enzymes such as sphingosine kinase and acid ceramidase (157, 184). These alterations ensure that the balance between cell death and cell survival is tipped towards cell survival. Other examples of genetic alterations used by cancer cells to promote cell survival involves decreasing the levels of sphingolipid enzymes such as acid sphingomyelinase. Siramesine and desipramine are well-known ASM inhibitors and have been the focus of several recent papers investigating cancer therapeutic options through inhibition of key sphingolipid enzymes (180, 185, 186).

1.5 TYROSINE KINASE INHIBITORS

Amongst the genetic alterations adopted by cancer cells the ability to overexpress many receptor kinases driving the growth, motility, differentiation, cell survival and metabolic processes of the cell are one of their most effective strategies (187). There are several receptors modified in cancer cells and among those, the epidermal growth factor receptor (EGFR) and the growth factor receptor-2 (HER2) are important drivers of cancer metastasis, especially EFGR in prostate cancer (188, 189, 190). Therefore, targeted inhibition of these receptors is a promising strategy to suppress tumor growth. Lapatinib is a small molecule targeting these two receptors simultaneously and is currently approved by the Food and Drug Administration (FDA) for the treatment of breast cancer. Given its effectiveness shown in clinical trials to treat breast cancer, and its mechanism of action targeting HER2 receptors altered in prostate cancer, lapatinib was considered as a new strategy for the treatment of advanced prostate cancer (202, 203). However, treatment with lapatinib as a single agent was not effective in clinical trials. In one phase II clinical trial, treatment with lapatinib in patients with hormone-sensitive prostate cancer showed no significant antitumor activity although the drug was well tolerated by patients. In a separate phase II trial, a small population of patients with castration-resistant prostate cancer showed some reduction in bone metastasis (7 out of 29) and PSA levels (1 out of 21). Since lapatinib showed a minor positive result in some patients, combination treatments are now being investigated to improve these outcomes (226). It is still unknown whether combining tyrosine
kinase inhibitor lapatinib with lysosomotropic agents may lead to triggering events of cell death in advanced prostate cancer cells.

The use of combination treatments in cancer therapy is a strategy designed to lower drug concentrations since high doses may promote drug resistance and cancer reoccurrence (193, 194, 195). Lysosome disrupting agents in combination with tyrosine kinase inhibitors were shown to cause synergistic cell death in cancer types such as breast, lung, CLL and glioblastoma cell lines (170, 172, 196). Therefore, this strategy shows potential therapeutic uses to treat advanced prostate cancer since one of the main challenges in treating this disease involves the development of drug resistance.
CHAPTER 2: RATIONALE, HYPOTHESIS AND RESEARCH AIMS

2.1 RATIONALE

Prostate cancer is the most common cancer affecting men and despite tremendous advancements made on improving early diagnosis and treatment, resistance to current chemotherapy drugs still occurs (197, 198). There are no effective treatments to eradicate the advanced form of this disease (124, 198) and novel strategies include the use of combinational treatments aiming to reduce the negative effects of chemotherapy by using lower drug concentrations (3,4). Another approach for treatment is targeting lysosomes since damaged lysosomes can leak their contents into the cytosol triggering a series of events leading to cell death (163, 200).

Combining lysosomotropic agents with targeted inhibitors, such as tyrosine kinase inhibitors, has been recently shown by our lab to induce cell death in glioblastoma, lung, CLL and breast cancer at relatively low doses (170, 196, 201). Lapatinib is a small inhibitor molecule targeting EGFR and HER2 (202). These receptors are often mutated in many cancers and contribute to cancer cell survival and proliferation (203, 204). Currently, lapatinib is approved for the treatment of metastatic breast cancer in patients, administered alone or in combination with other chemotherapy agents, with close to 30% of patients showing a complete or partial treatment response. In prostate cancer, combinational therapies using the tyrosine kinase inhibitors sorafenib, lapatinib and erlotinib are being explored (230, 231).

LMP generally results in increased reactive oxygen species and redox-active iron leading to major lipid peroxidation and mitochondrial dysfunction, among other events less reported (200, 206). These lysosomotropic agents are more effective in cancer than in non-malignant cells due to higher metabolic demands in cancer cells that require increased levels of cellular breakdown by lysosomes (168, 173). Cancer cells have higher lysosome numbers and size and carry genetic lysosomal alterations to drive these metabolic changes (174, 207). Lysosomes also contribute to metastasis since they contain hydrolytic enzymes that can break down cell membranes and facilitate tissue invasion (208, 209).
2.2 HYPOTHESIS

We hypothesized that lysosomotropic agents in combination with tyrosine kinase inhibitors will induce lysosome membrane permeabilization in advanced prostate cancer cells resulting in significant cell death.

2.3 RESEARCH AIMS

The aims of this study were to:

1. Determine whether lysosomotropic agents induce cell death in prostate cancer cells and to determine the extent and type of cell death.
2. Determine whether lysosomotropic agents in combination with tyrosine kinase inhibitors result in significant cell death and to determine the extent and type of cell death.
CHAPTER 3: MATERIALS AND METHODS

3.1 CELL CULTURE

3.1.1 Cell culture

Adherent human cancer cell lines were chosen for the experiments described in this thesis. The three cell lines used were PC3, DU145 and LnCaP and their characteristics are listed in Table 3.1. Cell lines were maintained in a humidified 5% CO\textsubscript{2} environment (normoxia = 21% O\textsubscript{2}) at 37°C. PC3 cells were cultured in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; Gibco, Life Technologies), DU145 and LnCaP cells were cultured in RPMI 1640 Medium (Gibco, Life Technologies) and all cell lines were supplemented with 5% (v/v) fetal bovine serum (FBS; Life Technologies) and 1% Penicillin /Streptomycin (Pen/Strep) (Gibco, Life Technologies). Cells were grown in 100 x 20 mm tissue culture plates (Sarstedt). Cells were passaged upon reaching ~80% confluency (see below).

<table>
<thead>
<tr>
<th>Table 3.1 Characteristics of advanced prostate cancer cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Human</td>
</tr>
<tr>
<td><strong>Tissue/Cell Type</strong></td>
</tr>
<tr>
<td><strong>Disease</strong></td>
</tr>
<tr>
<td><strong>Androgen and PSA status</strong></td>
</tr>
<tr>
<td>Genetic characteristics</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td><strong>Culture Properties</strong></td>
</tr>
<tr>
<td><strong>Gender/age/ethnicity</strong></td>
</tr>
<tr>
<td><strong>Culture medium</strong></td>
</tr>
<tr>
<td><strong>Doubling time</strong></td>
</tr>
<tr>
<td><strong>Karyotype</strong></td>
</tr>
<tr>
<td><strong>Source</strong></td>
</tr>
</tbody>
</table>

### 3.1.2 Passaging of cells

All three cell lines were passaged at a 1:4 ratio after reaching ~80% confluency. To detach strongly adherent PC3 cells from culture plates, 3 mL of Trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.05%) (Gibco) was added and incubated at 37°C for 5-6 minutes. For DU145 cells, 2 mL of trypsin-EDTA was added and incubated for 5 minutes. Trypsin-sensitive LnCaP cells were incubated with 2 mL of trypsin for a maximum of 2 minutes. To stop the proteolytic reaction, cells were neutralized with media up to 10 mL in total. Cells were centrifuged at 1200 rpm for 5 minutes and resuspended in 10 mL of fresh growth media and dispensed into new 100 x 20 mm tissue culture plates. Fresh growth media was added every 2-3 days.

### 3.1.3 Cryopreservation of cells

For long term preservation, cells were frozen in liquid nitrogen tanks and for short-term
use, stored in -80°C. When cells reached 80% confluency, they were trypsinized and pelleted as described above. Cell pellets were resuspended in 1:1 of growth media to freezing media and stored in cryovials (Sarstedt). Immediately after adding freezing media, cryovials were placed in Mr. Frosty Freezing Container (Nalgene). The freezing media consisted of a 5:3:2 ratio of corresponding growth media, 10% FBS and 20% dimethyl sulfoxide (DMSO, Sigma), respectively.

3.1.4 Thawing of cells

Frozen cryovials containing cells were immediately placed in a water bath at 37°C and gently shaken side to side until no ice crystals were visible, approximately 1-2 minutes. Cells were resuspended in a total of 10 mL of corresponding fresh growth media and centrifuged at 1200 rpm for 5 minutes. Cell pellets were resuspended in 10 mL of fresh growth media and dispensed into 100 x 20 mm tissue culture plates.

3.1.5 Cell counting and cell seeding

After reaching 80% confluency, cells were trypsinized and resuspended in 10 mL of fresh media as described above. 10 uL of media containing cells were dispensed into both sides of a dual chamber counter slide (Bio-Rad) and counted using the TC20 Automated Cell Counter (Bio-Rad) which calculates the number of cells per mL. Cell debris was excluded based on the cell size being used. For flow cytometry experiments, cells were seeded at a concentration of 1.0x10^5 cells/mL in 12-well plates and 3.0x10^5 cells/mL for western blot experiments. To achieve the necessary cell density for experiments, cells were diluted in fresh growth media.

3.2 DRUGS AND STIMULI

3.2.1 Drugs

The drugs used in this study are summarized in Table 3.2. Drugs were stored in single use aliquots and were used fresh for each experiment. A 24h dose-response curve was generated for PC3 cells using siramesine, desipramine, clemastine, loratadine, desloratadine, lapatinib, gefitinib, sorafenib, paclitaxel and etoposide. A dose-response curve for siramesine was tested in all three cell lines. In addition, the LC_{50} for siramesine, desipramine and clemastine was determined for assays to study their effects on cell lines. For drug combination experiments, the concentrations used were based on the lowest concentration of each drug that induced between
10-15% cell death at 24h. The specific concentrations of each drug employed for experiments are summarized in Table 3.3.

**Table 3.2 Drugs used in this study**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Class</th>
<th>Concentrations tested</th>
<th>Solvent/ Storage temperature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siramesine</td>
<td>Antidepressant (Lysosomatropic agent)</td>
<td>10-25 uM</td>
<td>DMSO/Room temperature</td>
<td>Sigma</td>
</tr>
<tr>
<td>Desipramine</td>
<td>Antidepressant</td>
<td>10-100 uM</td>
<td>Water/-20°C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clemastine</td>
<td>Antihistamine</td>
<td>10-80 uM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Loratadine</td>
<td>Antihistamine</td>
<td>10-50 uM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Desloratadine</td>
<td>Antihistamine</td>
<td>10-100 uM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>TKI</td>
<td>0.5-10 uM</td>
<td>DMSO/-20°C</td>
<td>LC labs</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>TKI</td>
<td>10-60 uM</td>
<td>DMSO/-20°C</td>
<td>Cedarlane labs</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>TKI</td>
<td>10-60 uM</td>
<td>DMSO/-20°C</td>
<td>LC labs</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase inhibitor</td>
<td>25-200 uM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

- **TKI= Tyrosine kinase inhibitor**

**Table 3.3 Specific drug concentrations used in this study**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Class</th>
<th>Concentrations Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siramesine</td>
<td>Antidepressant (Lysosomatropic agent)</td>
<td>10, 15, 20, 25, 35, 40, 45 uM</td>
</tr>
</tbody>
</table>
### Desipramine
Antidepressant (Lysosomotropic agent) | 10, 100, 150 uM

### Clemastine
Antihistamine (Lysosomotropic agent) | 10, 60, 80 uM

### Loratadine
Antihistamine (Lysosomotropic agent) | 10, 25, 50 uM

### Desloratadine
Antihistamine (Lysosomotropic agent) | 10, 50, 100 uM

### Lapatinib
Tyrosine kinase inhibitor | 0.5 uM

### Gefitinib
Tyrosine kinase inhibitor | 10 uM

### Sorafenib
Tyrosine kinase inhibitor | 10 uM

### Etoposide
Topoisomerase inhibitor | 100 uM

#### 3.2.2 Stimuli and Inhibitors
All stimuli and inhibitors used in this study are summarized in Table 3.4. Compounds were stored in single use aliquots and were used fresh for each experiment. N-acetyl cysteine (NAC) and alpha-Tocopherol solutions were prepared fresh from stock before experiments. One hour before treatment, stimuli or inhibitors were added to treatment wells. For each stimuli or inhibitor, a dose response curve was performed based on literature values and the specific concentrations used in experiments were based on the lowest concentration that provided the best results without compromising cell viability.

**Table 3.4 Stimuli and inhibitors**

<table>
<thead>
<tr>
<th>Stimuli/Inhibitor</th>
<th>Class</th>
<th>Concentrations tested</th>
<th>Solvent/Storage temperature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-MA</td>
<td>Autophagy inhibitor</td>
<td>2 mM</td>
<td>DMSO/Room temperature</td>
<td>Sigma</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Autophagy</td>
<td>30 uM</td>
<td>Water/-20°C</td>
<td>Sigma</td>
</tr>
<tr>
<td>inhibitor</td>
<td>concentration</td>
<td>storage temperature</td>
<td>supplier</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------</td>
<td>----------------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>BafA1 Autophagy inhibitor</td>
<td>50 nM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>Spautin-1 Autophagy inhibitor</td>
<td>5 µM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>z-VAD-fmk Apoptosis inhibitor</td>
<td>10 µM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>Necrostatin-1 Necrosis inhibitor</td>
<td>10 µM</td>
<td>DMSO/-20°C</td>
<td>LC labs</td>
<td></td>
</tr>
<tr>
<td>Ferrostatin-1 Ferroptosis inhibitor</td>
<td>5 µM</td>
<td>DMSO/-20°C</td>
<td>Cedarlane labs</td>
<td></td>
</tr>
<tr>
<td>alpha-Tocopherol Lipid ROS scavenger</td>
<td>200 µg/mL</td>
<td>ethanol/4°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>N-acetylcysteine Antioxidant/ FSH precursor</td>
<td>5 mM</td>
<td>Water/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>SKII Sphingosine kinase inhibitor</td>
<td>5, 10, 20 µM</td>
<td>DMSO/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>D- Sphingosine Chemical compound</td>
<td>2.5, 5, 10 µM</td>
<td>Chloroform/-20°C</td>
<td>Sigma</td>
<td></td>
</tr>
</tbody>
</table>
3.3 FLOW CYTOMETRY

3.3.1 Cell death assays

PC3, DU145, and LnCaP cells were added to 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42hs before treatment. On the day of treatment, fresh media was added before addition of drugs and incubated at 37°C for 24h, unless otherwise indicated. For experiments where inhibitors were used, cells were incubated with inhibitors for 1 hour before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After treatment, cells were collected, together with the media, and resuspended in phosphate saline buffer (PBS) and stained with 10 μL of 0.04% Trypan blue (Sigma). Trypan blue can enter cells through membrane pores of dying cells while being excluded from live ones. Cells were analyzed within 5 minutes of trypan blue addition using the Novocye flow cytometer (Acea Biosciences). 20,000 events were collected from each sample and gated using the PER-CP channel and data was analyzed using CellQuest software. To detect early and late apoptotic events, cells were resuspended in 1X Binding Buffer with AnnexinV (BD) and 7AAD (BD) dyes diluted in 1X Binding Buffer. Cells were incubated at 37°C for 15 minutes and immediately analyzed by flow cytometry. 20,000 events were collected and gated using FITC and PER-CP channels. Events positive for either FITC and PER-CP were considered apoptotic cells.

3.3.2 Lysosome membrane permeability assay

PC3, DU145, and LnCaP cells were seeded in 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After 4h treatment the media was removed from cells and Lysotracker Deep Red dye (50 nM, Invitrogen) was diluted in fresh media and added to cells for 15 minutes at 37°C in the dark. Cells were collected, together with the media, and resuspended in PBS and analyzed using the Novocye flow cytometer (Acea Biosciences). 20,000 events were collected from each sample and gated using the PER-CP channel. Data was analyzed using CellQuest software.
### 3.3.3 ROS detection assay

PC3, DU145, and LnCaP cells were seeded in 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After 4h or 24h treatment, cells were collected, together with the media, and resuspended in PBS and stained with 3.2 μM dihydroethidium (DHE, Invitrogen) for 30 minutes at 37°C in the dark. Cells were analyzed using the Novocyt flow cytometer (Acea Biosciences) and Cellquest software. 20,000 events were collected from each sample and gated using the PER-CP channel.

### 3.3.4 Mitochondria membrane potential assay

PC3 cells were seeded in 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After 24h treatment, cells were collected together with the media and resuspended in PBS and stained with 25 nM tetramethylrhodamine (TMRM, Invitrogen) for 30 minutes at 37°C. Cells were analyzed using the Novocyt flow cytometer (Acea Biosciences) and Cellquest software. 20,000 events were collected from each sample and gated using the PE channel.

### 3.3.5 Mitochondria superoxide detection assay

PC3 cells were seeded in 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After 24h treatment, cells were collected together with the media and resuspended in PBS and stained with 5 μM Mitosox Red (Invitrogen) for 10 minutes at 37°C in the dark. Cells were analyzed using the Novocyt flow cytometer (Acea Biosciences) and Cellquest software. 20,000 events were collected from each sample and gated using the PE channel.

### 3.3.6 Lipid peroxidation assay

PC3 cells were seeded in 12-well plates at a concentration of $1.0 \times 10^5$ cells/mL and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After 24h treatment, cells were collected together with the media and resuspended in PBS and stained with 1 μM C11-BODIPY (Invitrogen) for 30 minutes at
37°C in the dark. Cells were analyzed using the Novocyt flow cytometer (Acea Biosciences) and Cellquest software. 20,000 events were collected from each sample and gated using the PE channel.

3.4 WESTERN BLOT

3.4.1 Cell harvesting and sample preparation

PC3 cells were cultured as described above and seeded in 6-well plates at a concentration of 3.0x10^5 cells/mL, and allowed to grow for 42h before treatment. Depending on the solvent used to dilute drugs, DMSO or water was added as vehicle control. After treatment, cells were collected and resuspended in 100-150 μL 1% NP40 Lysis buffer containing Complete Mini Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 2 and 3 (Sigma). Resuspended cells were vortexed and placed in ice for 10 minutes 3 times. After lysis, cells were centrifuged 16,000 x g for 10 minutes at 4°C. Protein supernatant was collected and placed on ice. Protein was quantified using the Denovix DS-11 UV-Vis spectrophotometer using 1.5 μL of sample, and blanked with NP40 Lysis buffer without phosphatases and proteases.

To prepare samples for gel electrophoresis, 30 μL of protein were diluted in 1 x Laemmli loading buffer (Bio-rad) and beta-mercapto. Water was added when necessary to reach 20 μL. Samples were centrifuged for 30 seconds and incubated at 98°C in a hot plate for 10 minutes. Before loading samples into the gel, samples were centrifuged again for 30 seconds and placed on ice for 5 minutes. For short-term storage, samples were stored at -20°C.

3.4.2 SDS-PAGE gel electrophoresis and membrane transfer

Samples were loaded into pre-made 4-20% acrylamide Mini-PROTEAN TGX Stain-Free Protein gels (Bio-rad). PageRuler Plus Prestained Protein ladder (Thermo Fisher) was loaded in one well to determine the molecular weight of proteins. Samples were electrophoresed at 200V for 30 minutes, or until the stained samples reached the bottom of the gel. After gel electrophoresis, samples were transferred into a 0.22 μm nitrocellulose membrane at 24V for 15 minutes using the Trans-Blot Turbo transfer system (Bio-rad) following manufacturer’s instructions.
3.4.3 Western blot

After transfer, membranes were blocked for 1h in 5% bovine serum albumin (BSA) in 1X PBS and 0.1% Tween20 Detergent (PBST). Primary antibodies used are listed in Table 3.5 were added to membranes and incubated overnight at 4°C. Membranes were washed 3 times in PBST for 10 minutes and incubated with secondary antibodies for 1h, which are listed in Table 3.6. After incubation, membranes were washed again 3 times in PBST for 10 minutes. Membranes were developed by enhanced chemiluminescence using the Pierce ECL Western Blotting substrate (Thermo Fisher) according to manufacturer’s instructions. The membranes were imaged using the ImageQuant LAS 500 gel imager (GE Healthcare Life Sciences). For all western blot membranes, the reference gene Actin was used as a loading control. Protein images obtained from the gel imager were quantified using ImageJ software (v2.0). The band intensity of samples was normalized to their corresponding Actin loading control.

Table 3.5 Primary antibodies

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<th>Antigen</th>
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<th>Dilution</th>
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<td>Sigma</td>
<td>A2066</td>
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<tr>
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<td>1:1000 in 1XPBST</td>
<td>Cell signaling</td>
<td>#9662</td>
</tr>
<tr>
<td>Cleaved Caspase-3</td>
<td>Rabbit</td>
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<td>#9664</td>
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Table 3.6 Secondary antibodies

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<th>Conjugate</th>
<th>Dilution</th>
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<td>1:10,000 in 1XPBST</td>
<td>Bio-rad</td>
<td>170-6516</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>Horse radish peroxidase</td>
<td>1:5000 in 1XPBST</td>
<td>Bio-rad</td>
<td>170-6515</td>
</tr>
</tbody>
</table>
3.5 STATISTICAL ANALYSIS

All graphs were generated using GraphPad Prism 7. Statistical analysis for flow cytometry experiments were conducted using GraphPad Prism 7. Statistical significance was determined using a two-tailed unpaired t-test for all treatments and control samples. A p-value <0.05 was considered statistically significant (represented by *) in addition to a p-value <0.01 (represented by **), and a p-value <0.001 (represented by ***)). Error bars represent standard error of the mean for each treatment, and a minimum of three independent replicates were included for each experiment. For normalization of protein expression levels, Microsoft Excel 2017 was used.
CHAPTER 4: RESULTS

4.1 Lysosomotropic agents induce lysosome membrane permeabilization in advanced prostate cancer cells

Lysosomotropic agents are weak bases that accumulate within lysosomes and can generate large enough pores within the membranes allowing toxic lysosome contents to be released. Two types of lysosomotropic agents were tested: H1 antihistamines clemastine, loratadine and desloratadine, and the ASM inhibitors desipramine and siramesine. To determine whether these compounds were able to induce LMP in prostate cancer cell lines, PC3 cells were treated with increasing doses of these lysosomotropic agents for 4 hours and analyzed by flow cytometry using the Lysotracker fluorescent dye. A decrease in fluorescence indicates an increase in LMP.

The ASM inhibitor siramesine was used to treat PC3 cells at concentrations of 10, 15 and 20 μM. At all of these concentrations, siramesine induced statistically significant levels of LMP in a dose-dependent manner up to 12% (Figure 4.1.1a). The second most potent LMP inducer was loratadine. Prostate cancer cells were treated with loratadine at concentration of 10, 25 and 50 μM and showed a significant 11% increase in LMP but only at the highest concentration (Figure 4.1.1b). LMP induced by desipramine reached 8% at the highest concentration of 150 μM (Figure 4.1.1c). The H1 antihistamine desloratadine failed to significantly induce LMP even at concentrations of 100 μM (Figure 4.1.1d). Lastly, treatment of prostate cancer cells with 10, 60 and 80 μM of clemastine did not induce a statistically significant increase in LMP (Figure 4.1.1e). From these results, the most potent lysosome disrupting agent was siramesine followed by loratadine, desipramine, desloratadine and clemastine (Figure 4.1.1).

Since siramesine was the most potent lysosome disrupting agent LMP was tested in the other two advanced prostate cancer cell lines. DU145 showed the most significant percentage of LMP when treated with 10, 35 and 45 μM siramesine reaching up to 75% at the highest dose (Figure 4.1.2a.). On the other hand, when LnCaP cells were treated with 10, 25 and 40 μM siramesine, LMP significantly increased up to 33% (Figure 4.1.2b.). DU145 and LnCaP cells showed higher levels of LMP than PC3 cells, and this effect was most pronounced in DU145 cells (Figure 4.1.2).
Figure 4.1.1: Lysosomotropic agents induce lysosome membrane permeabilization at 4h in PC3 cells. PC3 cells were treated with siramesine (a), loratadine (b), desipramine (c), clemastine (d) and desloratadine (e) for 4h and stained with the fluorescent dye Lysotracker (50 nM) and analyzed by flow cytometry. A decrease in fluorescence indicates an increase in LMP. Results are representative of at least three independent replicates (N=3).

Figure 4.1.2: Lysosomotropic agents induce lysosome membrane permeabilization in DU145 and LnCaP cells. DU145 (a) and LnCaP (b) cells were treated with increasing doses of siramesine for 4h and stained with the fluorescent dye Lysotracker (50 nM) and analyzed by flow cytometry. A
decrease in fluorescence indicates an increase in LMP. Results are representative of at least three independent replicates (N=3).

4.2 Lysosomotropic agents induce ROS

Lysosome membrane permeabilization can lead to significant increases in reactive oxygen species resulting in cell death. To determine if the most potent LMP inducers could increase significant ROS levels in advanced prostate cancer cells, PC3 were treated for 4 hours and stained with DHE, a dye that changes fluorescence when it reacts with superoxide. After treating cells with 10, 15 and 20 μM siramesine, ROS levels significantly increased up to 40% and this effect was similar at 15 and 20 μM (Figure 4.2.1a). On the other hand, desipramine increased significant levels of ROS only at 150 μM, and while it increased ROS levels in a dose-dependent manner, it induced 20% less ROS than siramesine even at the highest concentration tested (Figure 4.2.1b). When cells were treated with clemastine at 10, 60 and 80 μM concentrations, a significant increased in ROS levels were observed only at 80 μM (Figure 4.2.1c). Clemastine showed the lowest levels of ROS compared to the other two drugs, with increases up to 8% (Figure 4.2.1). From these results, we concluded siramesine was the most potent ROS inducer even at the lowest concentration tested.

To test the ability of siramesine to induce similar ROS levels in the other prostate cancer cell lines, DU145 and LnCaP cells were treated with increasing concentrations of siramesine for 4 hours and stained with DHE, followed by flow cytometry analysis. Siramesine induced the least amount of ROS in DU145 cells compared to PC3 cells with only a 20% significant increase in ROS when treated with 35 and 45 μM concentrations (4.2.2a). In LnCaP cells, siramesine significantly induced the highest increase in ROS when treated with 10, 25 and 40 μM concentrations and this effect was observed in a dose dependent manner, reaching a maximum of 55% increase in fluorescence (4.2.2b). In conclusion, all prostate cancer cell lines tested showed significant increases in ROS levels when treated with siramesine, and LnCaP cells showed the highest increase followed by PC3 and DU145 cells (Figure 4.2.2).
Figure 4.2.1: Siramesine, desipramine and clemastine increase ROS levels at 4h in PC3 cells. PC3 cells were treated with siramesine (a), desipramine (b) and clemastine (c) for 4h, stained with the fluorescent dye dihydroethidium (DHE, 50 nM), and analyzed by flow cytometry. An increase in fluorescence indicates an increase in ROS levels. Results are representative of at least three independent replicates (N=3).

Figure 4.2.2: Siramesine increases ROS levels in DU145 and LnCaP cells. DU145 (a) and LnCaP (b) cells were treated with increasing doses of siramesine for 4h, stained with the fluorescent dye dihydroethidium (DHE, 50 nM), and analyzed by flow cytometry. An increase in fluorescence indicates an increase in ROS levels. Results are representative of at least three independent replicates (N=3).
4.3 The ASM inhibitor siramesine is the most potent lysosomotropic agent to induce cell death

To determine which lysosomotropic agent induced the highest percentage of cell death in prostate cancer cell lines, a death curve was generated in PC3 cells using the most potent LMP and ROS inducers previously tested. To determine the amount of cell death, cells were treated for 24 hours and stained with Trypan blue, a dye that stains cells with damaged plasma membranes, which is an indicator of cell death. Siramesine (10-50 μM) and desipramine (10-100 μM) induced cell death in a dose-dependent manner with an LC₅₀ of 25 μM and 80 μM respectively (Figure. 4.3.1a, b). The ASMase inhibitor siramesine was the most potent drug to induce cell death in PC3 cells therefore, a dose response for siramesine was generated using the other two prostate cancer cell lines DU145 and LnCaP. In these cell lines, siramesine induced less cell death than in PC3 cells (LC₅₀= 35 μM and 40 μM respectively) (Figure. 4.3.2a,b). From these results, we concluded siramesine was the most appropriate lysosomotropic agent to test for combinational treatments.

Figure 4.3.1: Siramesine and desipramine induce cell death at 24h in PC3 cells. PC3 cells were treated with siramesine (a) and desipramine (b) for 24h and stained with the fluorescent dye Trypan blue (0.4%), and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).
Figure 4.3.2: Siramesine induces cell death in DU45 and LnCaP cells. DU145 (a) and LnCaP (b) cells were treated with increasing doses of siramesine for 24h and stained with the fluorescent dye Trypan blue (0.4%), and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).

4.4 Siramesine induces significant cell death with the tyrosine kinase inhibitors lapatinib and sorafenib, and the chemotherapy agent etoposide.

Combining lysosome disrupting agents with tyrosine kinase inhibitors has previously been reported to induce synergistic cell death in several cancer types such as breast, lung and glioblastoma. From the initial results, the ASM inhibitors siramesine and desipramine were selected for combination experiments with tyrosine kinase inhibitors since these two drugs were the most potent at inducing LMP, ROS and cell death in PC3 cells. The concentration used for combination treatments was selected based on the lowest concentration of each drug that induced between 5-10% cell death. After treating PC3 cells with 10 uM of siramesine and 0.5 uM lapatinib, cell death increased approximately 70% (Figure 4.4.1.a). When 10 uM desipramine was combined with 0.5 uM lapatinib, the addition of the two drugs combined increased cell death by only 15%. (Figure 4.4.1.b). Therefore, since siramesine and lapatinib induced the highest percentage of cell death in PC3 cells, this combination was tested in the other two cell lines at the same concentration. Significant cell death was also observed in DU145 and LnCaP but the amount of cell death was lower than PC3 cells (Figure 4.4.2.a, b).
increased up to 32% (Figure 4.4.2a) and in LnCaP cells increased to 40% (Figure 4.4.2b). Based on these results, siramesine but not desipramine in combination with lapatinib induced a significant amount of cell death in all prostate cancer cell lines and this effect was more prominent in the advanced prostate cancer cell line PC3, followed by DU145 and LnCaP cells.

To test whether the significant increase in cell death from combining siramesine and lapatinib is specific to lapatinib or, if other tyrosine kinase inhibitors could induce the same effect, PC3 cells were treated with 10 uM sorafenib and 10 uM gefitinib. The results showed a significant increase in cell death when siramesine was combined with sorafenib but not with gefitinib. Combining 10 uM siramesine with 10 uM sorafenib resulted in a 78% cell death (Figure. 4.4.3a). But when 10 uM siramesine was combined with 10 uM gefitinib, the percentage of cell death was much lower than when combined with sorafenib since cell death increased by only 14%. (Figure 4.4.3b). Lastly, to investigate whether this combination strategy was also capable of inducing a significant amount of cell death with common chemotherapy drugs used in prostate cancer, etoposide and paclitaxel were tested. 10 uM siramesine in combination with 200 uM etoposide induced 38% cell death (Figure 4.4.4a). Paclitaxel was not able to induce significant cell death when combined with siramesine since 1 uM paclitaxel alone and 10 uM siramesine induced only 9% cell death (Figure 4.4.4b) In conclusion, lapatinib was the best candidate for combination treatments with tyrosine kinase inhibitors, followed by sorafenib and etoposide.
Figure 4.4.1: Siramesine and lapatinib induce cell death at 24h in PC3 cells. PC3 cells were treated with siramesine (S, 10 uM) and lapatinib (L, 0.5 uM) (a) or desipramine (D, 10 uM) and Lapatinib (L, 0.5 uM) (b) for 24h and stained with the fluorescent dye Trypan blue (0.4%) and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).

Figure 4.4.2: Siramesine and lapatinib induce cell death in DU145 and LnCaP cells. DU145 (a) and LnCaP (b) cells were treated with siramesine (S, 10 uM) and lapatinib (L, 0.5 uM) for 24h and stained with the fluorescent dye Trypan blue (0.4%) and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).

Figure 4.4.3: Siramesine and sorafenib induce cell death in PC3 cells. PC3 cells were treated with siramesine (Sira, 10 uM) and sorafenib (Sora 10 uM) (a) or siramesine (S, 10 uM) and gefitinib (G, 10 uM) (b) for 24h and stained with the fluorescent dye Trypan blue (0.4%) and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).
analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).

Figure 4.4.4: Siramesine and etoposide induce cell death in PC3 cells. PC3 cells were treated with siramesine (Sira, 10 uM) and etoposide (E, 200 uM) (a) or siramesine (S, 10 uM) and paclitaxel (P, 1 uM) (b) for 24h and stained with the fluorescent dye Trypan blue (0.4%) and analyzed by flow cytometry. An increase in fluorescence indicates an increase in cell death. Results are representative of at least three independent replicates (N=3).

4.5 Siramesine and lapatinib induce apoptosis after 24h treatment

To determine what type of cell death mechanism is involved after treatment with siramesine and lapatinib, inhibitors for autophagy (3-MA, Spautin-1, BafA1, NH₄Cl), ferroptosis (Fer-1), necroptosis (Nec-1), and apoptosis (z-VAD) were added 1h before treatment and cell death was measured at 24h by Trypan blue and analyzed by flow cytometry. When siramesine and lapatinib were combined, several replicates induced nearly 100% cell death and for this reason increases or decreases in cell death after addition of inhibitors was difficult to assess (data not shown).

When cells were treated with the autophagy inhibitor 3-MA (2 mM) cell death increased by 10% at 24h and decreased 6% after 48h (Figure 4.5.1a). The necroptosis inhibitor Nec-1 also increased cell death at 24h by 8% and the amount of cell death did not change at 48h (Figure 4.5.1b). A similar pattern was observed when the ferroptosis inhibitor Fer-1 was added since cell death increased by 10% at 24h and did not reduce nor increase cell death at 48h (Figure 4.5.1c). Lastly, the apoptosis inhibitor z-VAD slightly increased cell death at 24h by 5% and did not
reduce the amount of cell death by siramesine after 48h (Figure 4.5.1d). From these results, there was no statistically significant decrease in cell death with any of the inhibitors tested since the use of inhibitors only resulted in an increase in cell death at 24h and did not have an effect in cell death after 48h.

There are other more specific ways by which cells can die by apoptosis through a mechanism independent of caspases, which could provide an explanation for the failure of the caspase-dependent inhibitor z-VAD to reduce cell death (Figure 4.5.1d). To test whether PC3 cells were dying by apoptosis regardless of the role of caspases, cells were treated with 10 uM siramesine and 0.5 uM lapatinib and cell death was analyzed using the apoptotic assay AnnexinV/7AAD, and analyzed by flow cytometry. This assay can identify early and late apoptotic events by detecting the apoptotic marker phosphatidyl serine in the outer leaflet of the plasma membrane. After 24h treatment, there was a significant increase in apoptotic events up to 70% (Figure 4.5.2a). To further investigate if these apoptotic events were caspase dependent or independent, a western blot analysis was performed to measure protein expression levels of caspase-3 and cleaved caspase-3. The western blot results showed a 0.9 decrease in cleaved caspases expression after the combination treatment in comparison to untreated lysates suggesting a caspase-independent cell death (Figure 4.5.2b). Further western blot experiments need to be conducted to include another positive for apoptotic cell death since these samples did not show large qualitative detectable traces of cleaved caspase expression compared to control samples (Figure 4.5.2b).
Figure 4.5.1: Inhibitors of autophagy, necroptosis, ferroptosis and apoptosis fail to block cell death after treatment with siramesine. PC3 cells were treated with siramesine (20 uM) for 24h and 48h. Before treatment, cells were incubated with either 3-MA (2 mM) (a), Nec-1 (10 uM) (b), Fer-1 (5 uM) (c) and z-VAD (10 uM) (d) for 1h at 37C. Cell death was quantified by Trypan blue (0.4%) and analyzed by flow cytometry. Results are representative of three independent replicates (N=3).

Figure 4.5.2: Siramesine and lapatinib induce apoptotic cell death. PC3 cells were treated with siramesine alone (S,10 uM), lapatinib alone (L, 0.5 uM) or in combination for 24h. (a) Cells were incubated with AnnexinV/7AAD dyes for 15 minutes and analyzed by flow cytometry. (b) Cells were lysed after 12h treatment and protein expression for cas-3 and cleaved cas-3 were determined by western blot. Actin was used as a loading control and lapatinib (10 uM) was added as a positive control. Results are representative of three independent replicates (N=3)
4.6 Siramesine in combination with lapatinib induce lysosome membrane permeabilization in PC3 cells

Lysosome membrane permeabilization increased in all advanced prostate cancer cell lines tested when treated with siramesine alone. To further investigate whether LMP is a siramesine-induced event or if this LMP effect increases in combination with lapatinib, LMP was measured at 15 min, 1h and 4h. PC3 cells were treated with siramesine alone, lapatinib alone or in combination and incubated with the Lysotracker dye (50 nM) for 30 min and analyzed by flow cytometry. Increases in LMP were time-dependent but these effects showed only a 1% increase after treatment with siramesine alone (Figure 4.6.1). After 15 min treatment, combination with lapatinib increased only 1% than siramesine alone (Figure 4.6.1). And at 1h, the combination of siramesine and lapatinib induced higher LMP levels than at 1h (Figure 4.6.1). The highest increase in LMP was observed at 4h where both siramesine and lapatinib induced a 2% LMP and when combined, LMP increased to 4% (Figure 4.6.1). In conclusion, even though treatment with siramesine and lapatinib showed increases in LMP, these results were not much significantly higher than using siramesine alone, and were much lower than what it was observed in other cancer types.

![Figure 4.6.1: Siramesine and lapatinib induce lysosomal membrane permeabilization in PC3 cells.](image)

PC3 cells were treated with siramesine alone (10 uM), lapatinib alone (0.5 uM) or in combination for 15 min, 1h and 4h. Cells were stained with Lysotracker red (50 nM) for 30 minutes at 37C after treatment. LMP was quantified by flow cytometry where loss of
fluorescence indicates lysosome membrane disruption. Results are representative of at least three
independent replicates (N=3).

4.7 Siramesine and lapatinib increase ROS levels, lipid peroxidation while decreasing
mitochondrial membrane potential and generating mitochondrial super oxide in PC3 cells

Since the combination of siramesine and lapatinib induced minor lysosome membrane
permeabilization compared to what has been observed in other cancer types, the mechanism of
cell death induced by siramesine and lapatinib still needs to be elucidated in prostate cancer cells.
It has been reported that siramesine increases the levels of ROS and this can be accompanied by
increases in lipid peroxidation and mitochondrial damage. For this reason, levels of ROS and
lipid peroxidation were measured in addition to changes in mitochondrial membrane potential in
prostate cancer cell line PC3 treated with siramesine and lapatinib. Furthermore, levels of
mitochondrial superoxide were measured to determine if this combination treatment increased
reactive oxygen species within mitochondria in order to identify more specific causes for
mitochondrial damage.

After treatment with siramesine and lapatinib for 24 hours, there was a significant
increase in ROS levels in PC3 cells up to 70% (Figure 4.7.1a). To investigate whether this
increase in ROS included lipid ROS causing lipid peroxidation, cells were treated with
siramesine and lapatinib and stained for lipid radicals with the dye C11 BODIPY. After 24h, this
combination induced approximately 60% lipid peroxidation (Figure 4.7.1b). Siramesine alone
induced this 60% increase in lipid peroxidation but not with lapatinib which suggests siramesine
alone induces lipid peroxidation (Figure 4.7.1b.). In addition, a significant increase of 60% in
superoxide levels was observed with siramesine and lapatinib, whereas siramesine alone
increased mitochondrial oxide up to 25% and lapatinib showed no increase (Figure 4.7.1c).

Since the combination treatment with siramesine and lapatinib increased mitochondrial
superoxide levels, mitochondrial damage was tested by measuring the mitochondrial membrane.
A decrease in fluorescence indicates levels of mitochondrial dysfunction. Similarly to what was
observed for superoxide levels, the mitochondrial membrane potential decreased up to 70%
when siramesine and lapatinib were combined. However, the mitochondrial membrane potential
did not significantly increase when siramesine or lapatinib were added alone (Figure 4.7.d). In
conclusion, treatment with siramesine and lapatinib caused significant increases in the levels of
reactive oxygen species which include generation of mitochondrial super oxide while decreasing mitochondrial membrane potential. Lipid peroxidation was also observed but only by siramesine alone.

**Figure 4.7.1:** Siramesine and lapatinib increase reactive oxygen species, lipid peroxidation while decreasing mitochondrial membrane potential and generating mitochondrial super oxide. PC3 cells were treated with 10 uM siramesine and 0.5 uM lapatinib for 24h. (a) To detect reactive oxygen species, cells were stained with 3.2 uM DHE for 30 minutes at 37C. An increase in fluorescence indicates an increase in reactive oxygen species. (b) Lipid peroxidation was detected by staining cells with 1 uM C11-BODIPY for 30 minutes at 37C. An increase in fluorescence indicates increased lipid peroxidation. for 30 minutes at 37C. An increase in fluorescence indicates an increase in reactive oxygen species. (c) Changes in mitochondrial membrane potential were measured by staining cells with 25 nM TMRM for 30 minutes at 37C.
Increases in fluorescence indicate a decrease in mitochondrial membrane potential. (d) Mitochondrial superoxide levels were measured by staining cells with 5 uM Mitosox red for 10 minutes at 37°C. An increase in fluorescence indicates an increase in mitochondrial superoxide. All these experiments were analyzed by flow cytometry. Results are representative of three independent replicates (N=3).

4.8 Lipid ROS scavenger alpha- Tocopherol decreases siramesine-induced cell death

To identify whether treatment with siramesine exclusively induces lipid ROS or whether it also increases soluble ROS, 200 ug/mL of the lipid ROS scavenger alpha-Tocopherol and 5 mM of the hydrophilic ROS scavenger NAC were added 1h prior to treatment with 20 uM siramesine. As a positive control, 175 uM H2O2 was used as it is known to induce the generation of reactive oxygen species. After treatment, only alpha-Tocopherol was able to decrease cell death from 18% to 9% (Figure 4.8.1a.) compared to cell death after NAC treatment which only decreased 1% (Figure 4.8.1b.) When the amount of ROS was measured after alpha-Tocopherol treatment, ROS levels decreased from 41% to 17% (Figure 4.8.1c); however, these results only could only show a pattern since they were not statistically significant. Addition of NAC did not decrease ROS levels since cell death increased from 29% to 33%, instead of decreasing ROS (Figure 4.8.1d). In conclusion, only the lipid ROS scavenger alpha-Tocopherol reduced cell death. Lastly, the lipid antioxidant was able decreased ROS levels but more replicates are needed to validate these results.
Figure 4.8.1: alpha-Tocopherol decreases cell death after treatment with siramesine. PC3 cells were treated with 20 uM siramesine for 24h. Before treatment, cells were incubated with 200 ug/mL alpha-tocopherol (200 ug/mL) (a,c) or 2 mM N-acetyl cysteine (NAC) (b,d) for 1h at 37C. 175 uM Hydrogen peroxide (H202) was used as a positive control. Cells were stained with 0.4% Trypan blue for cell death assays (a,b) or 3.2 uM DHE (c,d) and incubated fo 30 minutes at 37C for ROS detection and analyzed by flow cytometry. Results are representative of three independent experiments (N=3).

4.9 Sphingosine failed to sensitize cells to siramesine and lapatinib-induced cell death

Cancer cells usually have altered lipid composition and metabolism, such as increased levels of sphingosine kinase that converts sphingosine to sphingosine-1-phosphate, resulting in a balance towards cell survival. Therefore, to investigate if disruption of sphingosine levels can sensitize cells to siramesine and lapatinib induced- cell death, PC3 cells were pre-treated with different concentrations of sphingosine or sphingosine kinase inhibitor II and cell death was measured after 24 hours. Results indicated that addition of sphingosine to the combination treatment failed to significantly increase cell death since in all concentrations tested, we observed no statistically significant difference (Figure 4.9.1a). The sphingosine kinase inhibitor II showed a statistically significant increase in cell death however, the percentage of cell death was less than 10% (Figure 4.9.1b). In addition, sphingosine in combination with lapatinib failed to increased cell death (data not shown). Based on these results, we concluded altering the levels of sphingosine does not play a major role in siramesine and lapatinib induced cell death.
Figure. 4.9.1: The sphingosine kinase inhibitor II sensitizes PC3 cells to siramesine and lapatinib-induced cell death. PC3 cells were treated with different concentrations of sphingosine and sphingosine kinase inhibitor (SKII) for 24h. Before treatment, cells were incubated with these inhibitors for 1h at 37°C. Cells were stained with 0.4% Trypan blue and analyzed by flow cytometry. Results are representative of three independent experiments (N=3).
CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1. DISCUSSION

Drug resistance is the main obstacle for effectively treating advanced prostate cancer. When prostate cancer cells have metastasized to other organs, or have become resistant to current therapy drugs, there are no options to eradicate the disease. Therefore, a new therapeutic strategy to prolong the life of patients has become the ultimate goal in advanced prostate cancer research (198, 210). Many approaches have been developed in an attempt to reduce tumor growth and to increase the lifespan of patients, such as vaccines or the use of combination treatments of chemotherapy drugs, but we need more efficient treatments to significantly improve these outcomes (211).

Targeting lysosomes in combination with tyrosine kinase inhibitors to treat aggressive cancer stages had significant positive results in several in-vitro models of breast, lung, glioblastoma and CLL (170, 172, 196). However, it has not been tested yet whether combining these two types of targeted drugs have similar outcomes for advanced prostate cancer. For this reason, we aimed to test this approach as a potential new therapy in advanced prostate cancer cell lines PC3, DU145 and LnCaP. We found the combination of the lysosomotropic agent siramesine and the tyrosine kinase inhibitor lapatinib to induce significant cell death after 24h in all cell lines tested. Treatment with siramesine and lapatinib resulted in a small increase in lysosome membrane permeabilization, significant increases in lipid ROS and mitochondrial damage in PC3 cells, as the mitochondrial membrane potential decreased while the levels of superoxide radicals increased. These events were evident as early as four hours after treatment. We also found cell death decreased when the lipid ROS scavenger alpha-Tocopherol was added before treatment suggesting siramesine damages cells by generating lipid ROS and disrupting organelle membranes. In addition, we found this combination to be more effective in PC3 than in DU145 and LnCaP cells, the former which is representative of the most advanced type of prostate cancer (212, 213) and therefore, holds the potential to become a new therapeutic strategy.

The idea of repurposing lysosomotropic agents originally designed to treat health conditions such as depression, or the use of antihistamines with lysosomotropic properties, had successful therapeutic results in a wide range of cancer types (169). Since then, studies have
found several more compounds that accumulate within lysosomes that induce cell death in a relatively short time (206). In CLL cells, treatment with antihistamines (loratadine, desloratadine, clemastine) and antidepressants (siramesine and desipramine) significantly increased cell death compared to non-malignant cells (157,196). We found clemastine, desipramine and siramesine to induce to cell death in PC3 cells, and siramesine showed to be the most potent drug even at low concentrations. These results based on siramesine as the most potent lysosomotropic agent agree with another study using similar concentrations and time points (180). H1 antihistamines desloratadine and loratadine did not induce cell death at any of the concentrations tested despite its effectiveness in other cancer types. These differences between results could be due to prostate cancer cells being less dependent on lysosome function supported by having less lysosome numbers or size, and the type and level of toxic content inside lysosomes generally used to provide cancer cells with additional building blocks.

It has also been reported that some cancer cell lines possess increased lysosomal genetic alterations in key enzymes such as Hsp70 which provides lysosome membranes with extra protection from lysosome-induced cell death (214). The lower number of lysosomotropic agents reported to kill prostate cancer cells compared to other cell lines could suggest that either prostate cancer is less susceptible to lysosome damage as antihistamines loratadine and desloratadine were much more effective at killing CLL cells (168). Perhaps these drugs do not readily diffuse through lysosome membranes, and are more readily tolerated and more efficiently metabolised by lysosomes. The question still remains as to why siramesine is so effective as a lysosomotropic agent in all cancer cell lines tested regardless of the amount of lysosome damage it can cause to cancer cells.

Siramesine is not FDA approved for cancer therapy since it was developed as an antidepressant drug and did not achieve significant clinical results however, several researchers and our group have found their potential as a lysosomotropic agent and an inducer of cell death in many cancer cells (157, 215 216). Breast, CLL, lung and glioblastoma cell lines showed significant cell death after 24h when treated with the lysosomotropic agent siramesine. In these cases, cell death was associated by an increased in lysosome membrane disruption, lipid ROS and mitochondrial damage (170, 172, 196). Despite the number of reports conducted on the effects of siramesine on cancer cell lines, its mechanism of action is not fully understood yet as it
displays a large variation on its effects on cellular processes; which seem to be induced in a cell and context dependent manner (206, 216).

If we think about inducers of LMP, several drugs come to mind. Compounds that possess weak bases are known for their ability to diffuse into lysosomes with an acidic pH (217). Once inside, they accumulate without possibility of escaping due to their now acquired positively charged nature. As these drugs continue to accumulate, they are able to disrupt lysosomal membrane protein function and they can also intercalate within the lysosomal membrane creating a chaotic and unorganized membrane that eventually leads to disruption of cellular homeostasis (179, 218). It is important to remember that these events in combination can result in cell death which are based on drug-to-protein interactions, the concentrations used, and the unique genetic alterations that rend them susceptible to lysosomal damage. LMP is therefore a context and cell line dependent event.

We found that in prostate cancer cells these effects on lysosome membrane permeabilization were lower than what was reported in other cancer cell lines previously tested by our group (170, 172, 196). We suspect these differences in results are due to prostate cancer cells being more adapted to their environment in order to protect lysosomes from increased drug turnover; or on the other hand, they have acquired genetic adaptions that lower the need of the recycling function provided by lysosomes, which is higher during other stages of cancer growth such as early metastatic events. Since advanced prostate cancer is an already established cancer that has metastasized to other organs, there is a possibility they do not readily rely on increased lysosome function to provide them with additional nutrients during these periods of growth, angiogenesis and metastasis.

In this study, we also tested levels of LMP after siramesine treatment in the other two cell lines DU145 and LnCaP. Unexpected results showed DU145 having significant higher LMP levels compared to PC3 cells, followed by LnCaP cells. There are many possible explanations for these results that could be addressed in future studies, such as the possibility DU145 cells have increased lysosomal alterations needed for angiogenesis and metastasis since they are representative of a middle metastatic stage between the two other cell lines. A more in-depth study is required to determine whether key lysosomal enzymes regulating events required to support cell survival are altered in DU145 cells compared to PC3 and DU145 cells. Regardless of these differences in LMP levels after treatment, we concluded siramesine is very effective at
inducing cell death mostly in PC3 cells and more interestingly, LMP was not the major cellular event leading to cell death by siramesine.

Lysosomotropic agents can also induce other cellular events leading to cell death. One of the most widely characterized consequences of lysosome membrane permeabilization is the release of lysosomal proteases into the cytosol, as the membrane become porous for lysosome contents to diffuse into the cytosol. Among these, cathepsin proteases are one of the most toxic enzymes (219). Their function is to degrade molecules destined to be recycled by lysosomes but when they are released into the cytosol they are capable of activating proteins associated with cell death signals, such as Bid and Bak (167). Treatment with siramesine triggers the release of cathepsins into the cytosol followed by a decrease in mitochondrial membrane potential and ultimately, cell death (180). Cathepsin release is triggered after a massive induction of LMP and since we observed a minor increased in lysosome damage we did not test for cathepsin release in prostate cancer cells. In a study conducted using immortalized keratinocyte HaCaT cells and glioblastoma U-87MG cells, cathepsin release into the cytosol was not observed after treatment and using higher concentrations of siramesine than the one used in this study (220). These results support our results that siramesine does not trigger massive LMP in prostate cancer cells however, other studies have found cathepsin release in the same cell lines (221).

As the accumulation of lysosomotropic agents within lysosomes occurs, this often leads to an increase in reactive oxygen species as these drugs destabilize lysosome membranes by intercalating within membranes, inhibiting or activating key enzymes, and in some cases working as a detergent (218, 222). Production of ROS is not only tied to lysosomes as it can interfere with cytosolic or lipids components in other organelles such as mitochondria, destabilizing the mitochondria membrane potential (164, 223). We found this to be the case when we treated advanced prostate cancer cell lines with siramesine and observed significant increases in lipid ROS accompanied by a decreased in mitochondrial membrane potential and increased levels of mitochondria superoxide. In the same study conducted with HaCaT and U87 cells, siramesine induced mitochondrial damage and induction of lipid peroxidation (220).

Treatment with the lipophilic antioxidant alpha-Tocopherol was able to decrease cell death and restore mitochondrial function as reported in a previously mentioned study (220). We tested the role of lipid ROS as a trigger for siramesine-induced cell death by treating with alpha-Tocopherol and measuring cell death after 24h. Our results agree with this study as the same
concentration of tocopherol (200 ug/mL) decreased cell death after 24h treatment with siramesine. This was not the case when 2 mM of the cytosolic antioxidant NAC was added since cell death and ROS levels remained the same as control samples treated with siramesine, suggesting siramesine-induced cell death is triggered by lipid peroxidation and not soluble ROS. Another protective mechanism used by cells to neutralize mitochondrial ROS is the expression of the enzyme Heme oxygenase-1 (HO-1) (150). Whether prostate cancer displays altered levels of tocopherol and HO-1 is a question waiting to be answered in future studies.

We then hypothesized that lysosomotropic agents would work to significantly increase cell death with lapatinib, and whether siramesine could induce these effects on cell death when combined with other tyrosine kinase inhibitors. We obtained some unexpected results since siramesine only induced significant cell death when combined with lapatinib. When other tyrosine kinase inhibitors were tested, sorafenib but not gefitinib induced significant cell death. Since one of the tyrosine kinase inhibitors tested was able to significantly increase the amount of cell death when combined with siramesine, we further investigated the effect of siramesine in combination with common chemotherapy agents used in prostate cancer. Etoposide but not paclitaxel worked in combination with siramesine. During the duration of this study, we were not able to identify why siramesine worked in combination with etoposide and not with paclitaxel due to time constraints however, we have shown siramesine as a potential combination agent to be explored with etoposide.

A plausible explanation for the results obtained with tyrosine kinase inhibitors suggests lysosomotropic agents are not as potent in prostate cancer cells with only siramesine showing a inducing effect on cell death with lapatinib. The tyrosine kinase inhibitors lapatinib and sorafenib worked in combination with siramesine but not gefitinib probably due to sorafenib targeting several tyrosine kinase receptors (VEGR, PDGFR, RAF) (224) in comparison to gefitinib. Gefitinib only targets the EGFR receptor whereas lapatinib targets EGFR and HER2 receptors (225). Lapatinib could have an off-target effect in addition to EGFR however, we do not have sufficient data on additional mechanisms in prostate cancer that could differentiate lapatinib from those of other tyrosine kinase inhibitors already investigated.

The potent effect of lapatinib when combined with siramesine but not gefitinib might suggest targeting the HER2 receptor is a key regulator in this scenario. There is no sufficient evidence to support this hypothesis since there are contradictory results about the status of the
HER2 receptor and protein levels from prostate cancer studies (226, 227, 228). In a phase II clinical trial using lapatinib, elevated levels of HER2 protein were found in serum of castration-resistant prostate cancer patients suggesting inhibition of this receptor might actually lead to increased activation and further supporting tumor growth (226). Lastly, results on the combination with chemotherapy drugs etoposide and paclitaxel added new questions as to why only some of these drugs work to synergy with siramesine. The explanation for these results is out of the scope for this study but provide new opportunities to repurpose commonly used chemotherapy drugs in combination with lysosomotropic agents in the treatment of advanced prostate cancer.

Treatment with lysosomotropic agents and tyrosine kinase inhibitors can lead to different types of cell death that are cell and context specific. The type of cell death induced by these drugs is an important piece of information on how these cancer types operate and what makes them susceptible to cell death after drug treatment. For example, breast, lung and glioblastoma cells treated with siramesine and lapatinib died by ferroptosis after 24h treatment whereas CLL cells died by apoptosis (172, 196, 215). In the former, cells showed an increase in LMP, cytosolic labile iron and decreased HO-oxygenase levels, an enzyme required to neutralize toxic iron. These alterations in iron metabolism forced these cells to die by a mechanism dependent on iron which can serve as a key evidence to develop future therapeutic treatments.

Apoptosis by lysosomotropic agents and lapatinib is usually been reported as a consequence of mitochondrial damage and increased lipid peroxidation which ultimately leads to the collapse of organelles and the plasma membrane (215). Lapatinib inhibits the EGFR/HER2 receptor dimerization which is involved in signalling pathways that support cell proliferation and cell survival therefore, lapatinib disrupts the signals destined to avoid programmed cell death (229). Recently, it was reported that tyrosine kinase inhibitors increase the degradation of the anti-apoptotic MCL1 protein in prostate cancer cell lines through a novel mechanism that require further investigation (230). Degradation of MCL1 by lapatinib would therefore support death by apoptosis in prostate cancer. Other tyrosine kinase inhibitors in combination also resulted in apoptotic cell death in these prostate cancer cell lines (231, 232, 233). We also found evidence that PC3 cells might die by apoptosis through a mechanism independent of caspases, as the caspase-dependent z-VAD inhibitor did not decrease cell death and we did not observe higher caspase cleavage levels compared to control samples. None of the other cell death inhibitors
were able to decrease cell death after siramesine treatment suggesting apoptosis is the major cell death mechanism induced by siramesine and lapatinib.

Previous studies conducted by Jaattela et al. in 2013 found lysosomotropic agents siramesine and desipramine inhibited ASM in a wide range of cancer cell lines, including in PC3 cells (180). ASM is an enzyme located within the lysosomal membrane that converts sphingomyelin into ceramide and phosphatidyl choline by detachment from lysosome membranes (234). Cancer cells tend to have lower levels of ASM leading to an increase in sphingomyelin levels which are bulky sphingolipids and are crucial for maintaining membrane integrity and fluidity (168, 235). Further increases in sphingomyelin have been reported to induce cell death however its role as a pro-apoptotic sphingolipid is not well understood compared to ceramide (236). It is possible that increased sphingomyelin levels trigger cell death signals produced within lysosome membranes. Therefore, inhibiting ASM function could lead to increased sphingomyelin levels by lysosomotropic agents and could shift the sphingolipid balance so tightly regulated by cancer cells that could ultimately lead to cell death, especially if other cellular organelles result damaged at the same time. Understanding how sphingolipid metabolism is altered in advanced prostate cancer would provide additional evidence on why combining lapatinib with siramesine is so effective at inducing cell death.

The ultimate goal of these studies on lysosomotropic agents and tyrosine kinase inhibitors is to translate them into a clinical setting for the treatment of advanced prostate cancer. We first need to investigate how these combination treatments will affect non-malignant prostate cells in-vitro and to assess its safety if delivered to patients with advanced prostate cancer. We suspect non-malignant cells will be less affected since these lysosomotropic agents were previously tested in clinical trials for treatment of other diseases at doses much higher than the ones used in this study. These new hypotheses will be the focus of future investigations.

5.2 CONCLUSIONS

Combining lysosomotropic agents with tyrosine kinase inhibitors were reported to induce significant cell death in a number of cancer cell lines but it has not been yet tested in prostate cancer cells. The results of this study found combining siramesine and lapatinib to yield positive results on cell death much higher than what was reported in other cell lines therefore, this drug combination holds the potential for therapy to treat patients diagnosed with advanced prostate
cancer. We identified lipid ROS to be the major inducer of cell death when treated with siramesine, and this was accompanied with mitochondria destabilization and an increase in superoxide levels within the mitochondria when cells were treated with siramesine alone or in combination with lapatinib. These cells were observed to die by apoptosis and potentially through a mechanism independent of cathepsins. The lipid antioxidant alpha-Tocopherol significantly decreased siramesine-induced cell death further supporting the role of lipid ROS as the main mechanism of action for this drug. We did not find this combination to increase high levels of lysosomal membrane permeabilization as it was previously shown in other types of cancer but the evidence of why it did not occur in prostate cancer cell lines remains unknown.

In conclusion, the results of this study found siramesine and lapatinib as a potential strategy for treating advanced prostate cancer and provided new evidence on how the combination of these two drugs can induce cell death through a mechanism dependent on lipid ROS and mitochondrial dysfunction. To understand the mechanism of action of this combination treatment, I have developed a model highlighting lipid ROS as the major inducer of cell death (Figure 5.1). In addition, ASMase inhibition by siramesine, although not tested in this study, was included as it has been reported in other studies. The results of this study could guide us in determining whether exploring this combination is a feasible option to translate these results into a clinical setting for treatment of patients with advanced prostate cancer in order to the extend their quality of life and hopefully, to reduce the prognosis of this disease.
Figure 5.1: Siramesine and lapatinib mechanism of action in advanced prostate cancer cells. Siramesine alone and in combination with lapatinib triggers minor lysosomal membrane permeabilization while increasing lipid peroxidation. In the mitochondria, this combination treatment increases superoxide levels and decreases mitochondria membrane potential. Lapatinib is a HER2/EGFR inhibitor, which is involved in promoting cell survival and cell proliferation. The blockage of this pathways primes the mitochondria for apoptosis through the activation of pro-apoptotic proteins Bak and Bax. Together, siramesine and lapatinib induce significant cell death in advanced prostate cancer cells through the apoptotic pathway.
CHAPTER 6: FUTURE DIRECTIONS

6.1 Future directions

The combination treatment using siramesine and lapatinib is a potential therapeutic strategy to explore for the treatment of advanced prostate cancer. However, additional research is required to investigate potential off-targets of siramesine and lapatinib as well as understanding why this combination is so effective in prostate cancer cells compared to other cancer cell lines. In order to bring this therapy to the clinical setting, studies conducted in-vivo models are needed to determine whether this combination can reduce cell death in cancers cells but not in non-malignant cells.

In this study, we found lipid ROS to be the major inducer of cell death after siramesine and lapatinib treatment. However, we do not have supporting evidence to conclude what is the main target of these drugs alone and in combination. Since siramesine has been reported to be an ASM inhibitor, an enzymatic assay measuring levels of ASM and sphingomyelin before and after treatment is recommended. Prostate cancer cells have altered sphingolipid metabolism and we did not analyze in this study the ratio of sphingolipids before and after treatment using mass spectroscopy to quantify other sphingolipids levels to understand other potential targets for siramesine. Lastly, since siramesine induced significant LMP levels in other cancer cell lines it is important for the validity of this study to further investigate why these prostate cancer cells were not susceptible to LMP. Several techniques could be applied to answer this question. Fluorescent microscopy using the dye Acridine Orange or Lysotracker Red to quantify lysosome numbers might be a good starting point to determine any differences in lysosome numbers that might render prostate cancer cells less susceptible to lysosome damage. A transcriptome analysis selecting for lysosome genes would be an appropriate source of supporting evidence to understand these differences.

Before translating these results into in-vivo models to validate its efficacy in a 3D environment that is more representative of the spatial arrangement that human cancer cells experience, the same in-vitro experiments conducted in this experiment in addition to those suggested in this section need to be replicated in non-malignant cells. RPW-1 is a non-malignant prostate epithelial cell line commonly used as a control to prostate cancer cells. These results would provide additional information on any differences that are unique to prostate cancer cells and also whether non-malignant cells are less susceptible to this combination treatment.
The following step into this investigation would be to test this combination treatment in 3D models such as spheroids and organoids since they are more rapidly reproducing than animal models and are a more ethically conscious choice for research. Organoids can be formed from tissues of advanced prostate cancer patients and spheroids can utilize the cell lines employed in this study for prostate cancer investigations. A step further to validate the efficacy of siramesine and lapatinib in *in-vivo* models would be to include components of the microenvironment into these 3D models such as stroma cells which are shown to play an important role in supporting cancer cells with additional nutrients in addition to providing enhanced protection from cells of the immune system.

Application of siramesine and lapatinib in a clinical setting for treatment of advanced prostate cancer would be feasible only if these experimental questions are answered and whether they are able to yield significant positive results as a potential therapy that induces significant cell death at low doses and that targets cancer cells more significantly than non-malignant cells. Drug resistance still is a major concern for treatment of advanced prostate cancer and we are in need of new therapeutic strategies that decrease the concentrations of the drugs being delivered while killing a significant number of cancer cells. Since lysosomotropic agents and tyrosine kinase inhibitors are well-tolerated drugs when delivered as a monotherapy, we hope repurposing these drugs and combining them will improve our strategy plans in treating advanced prostate cancer to prolong the life of patients.
CHAPTER 7: REFERENCES


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