5-2024

Simvastatin Induces Autophaghy-Mediated Cell Death In Metastatic Breast Cancer Cells

Jessica Allagoa
Texas Southern University

SIMVASTATIN INDUCES AUTOPHAGY-MEDIATED CELL DEATH IN METASTATIC BREAST CANCER CELLS

THESIS

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science in the Graduate School

of Texas Southern University

By

Jessica Allagoa B.S.,

Texas Southern University

2024

Approved by

Dr. Erica Cassimere
Chairperson, Thesis Committee

Dr. Mahesh Vanjani
Dean, The Graduate School

Approved by

Dr. Erica Cassimere	03/26/2024
Chairperson, Thesis Committee	Date
Dr. Mario Hollomon	03/26/2024
Committee Member	Date
Dr. Hector Miranda	03/26/2024
Committee Member	Date
Dr. Tuan Phan	03/26/2024
Committee Member	Date

SIMVASTATIN INDUCES AUTOPHAGY-MEDIATED CELL DEATH IN METASTATIC BREAST CANCER CELLS

By

Jessica Allagoa, M.S.

Texas Southern University, 2024

Associate Professor Erica Cassimere, Ph.D., Advisor

Breast cancer remains one of the leading causes of cancer deaths among women. Due to the limited effectiveness of current anticancer drugs, ongoing research has extended towards alternative drug categories for potential treatments. Recent findings indicate that statins possess the ability to suppress tumors across various cell types. Traditionally, statins are known as a class of cholesterol-lowering agents and function by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, a key enzyme in the mevalonate pathway. However, statins can also suppress cell proliferation and ultimately lead to cell death, which includes Type I apoptosis-induced cell death or Type II autophagy-induced cell death. Autophagy is a vital physiological cellular process that facilitates the intracellular degradation and removal of misfolded proteins and damaged organelles. Initially, autophagy was considered a pro-survival process, however, other reports have shown that improper balance of autophagic pathways can also exert pro-death pathways.

One type of statin, simvastatin, has been shown to induce autophagy in prostate cancer cells. However, its effects on other tumors remain poorly understood. In this study,

we hypothesize that simvastatin induces autophagy-mediated cell death in breast cancer cells. We used two metastatic breast cancer cell lines as a model for tumors which typically exert resistance to anticancer treatments. Cells were treated with simvastatin at various concentrations up to 48 hr. Cell morphology was examined microscopically, and induction of autophagy was measured using Western blotting. Following treatment with simvastatin, we observed increased rounding of cells in both MDA-MB-231 and MDA-MB-468 cells. Moreover, increase in protein expression of a classic autophagy marker, LC3-II, was markedly enhanced following a dose response treatment of simvastatin. To determine if the rounded cells were indicative of cell death, we performed a Trypan blue exclusion assay. Cell death was dramatically increased in a dose-dependent manner following simvastatin treatment, particularly at 48 hr. Moreover, we co-treated cells with simvastatin and chloroquine, an agent which blocks autophagy, and observed that cell death was reduced as compared to simvastatin alone, suggesting that autophagy contributed to cell death. These results demonstrate that simvastatin suppresses cell proliferation through induction of autophagy in breast cancer cells. Therefore, simvastatin may serve as an attractive anticancer agent to target advanced breast tumors.

TABLE OF CONTENTS

LIST OF TABLES

Tables Page

LIST OF FIGURES

Figures Page

LIST OF ABBREVIATIONS

AMPK: AMP-activated protein kinase

ASCO: American Society for Clinical Oncology

ATG: Autophagy-Related

BRCA1: Breast Cancer Gene 1

BRCA2: Breast Cancer Gene 2

BSA: Bovine Serum Albumin

CIPN: Chemotherapy-Induced Peripheral Neuropathy

CT: Computerized Tomography

CoA: Coenzyme A

CO2: Carbon dioxide

DCIS: Ductal carcinoma in situ

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

ECL: Enhanced Chemiluminescence

ER: Estrogen Receptor

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

HER2: Human epidermal receptor 2

HMG: 3-Hydroxy 3-methylglutaryl

H2O: Dihydrogen monoxide (Water)

HR: Hormone Receptor

HRP: Horse Radish Peroxidase

HT: Hyperthermia Therapy

LC3: Microtubule-associated Protein 1A/1B- Light Chain 3

MB: Metastatic Breast

MDA: Muscular Dystrophy Association

MRI: Magnetic Resonance Imaging

mTOR: Mechanistic Target of Rapamycin

PBS: Phosphate Buffered Saline

PE: Phosphatidylethanolamine

PET: Positron Emission Tomography

PI3KC3-C1: Class III Phosphatidylinositol 3-kinase complex

PMSF: Phenylmethylsulphonyl Fluoride

PR: Progesterone Receptor

P/S: Penicillin-Streptomycin

RIPA: Radio immunoprecipitation Assay

RPM: Revolutions per Minute

TBS-T: Tris-buffered Saline-Tween 20

TNBC: Triple Negative Breast Cancer

ULK1: Unc-51 Like Autophagy Activating Kinase 1

VITA

2022 …….……	B.S. Biology Texas Southern University Houston, Texas
2022-2024 …….……....	Graduate Teaching Assistant Department of Biology Texas Southern University Houston, Texas
Major Field…….	Biology

ACKNOWLEDGEMENTS

A sincere expression of gratitude is extended to my faith, family, and friends for their unwavering support and encouragement throughout my collegiate journey, enabling me to remain focused in the pursuit of my goals. A heartfelt thank you to my remarkable mother and father, Dr. Dennis, and Mrs. Peace Allagoa, whose love and myriad sacrifices have been indispensable in this journey. My deepest appreciation goes out to my friends Maxwell Orori, Assata Dockins, Favour Osawaru, Mesoma Mgbemeje, Sade Arnold, Devin Hurst, Sylvia Masango, Orlove Luvumbo and Joshua Avni who have all stood by me through thick and thin, ensuring I remained dedicated and focused on my goals. Their reassurance has been instrumental during moments of doubt and challenges.

A special acknowledgement is reserved for my mentor, thesis advisor and committee chair, Dr. Erica Cassimere, whose remarkable mentorship, and esteemed expectations have propelled me to strive for excellence and afforded me the platform to expand my research on a broader scale. This thesis and its extensive groundwork would not have materialized without the diverse resources she provided to enrich my understanding and approach to my area of focus. Her boundless enthusiasm, depth of knowledge, and meticulous attention to detail have served as a constant source of inspiration, steering my work toward completion with precision and purpose.

I also wish to acknowledge the invaluable contributions of Dr. Hector Miranda, Dr. Tuan Phan, and Dr. Mario Hollomon, outstanding members of my graduate thesis committee, for their steadfast support and expertise throughout this journey. I am

particularly thankful to Dr. Mario Hollomon for his commitment to my growth and development during my research. His insightful comments and constructive feedback on my lab techniques guided me towards necessary corrections and advancements. His mentorship was indeed the beacon of guidance I never anticipated. Finally, I am profoundly indebted to the Department of Biology, particularly to Dr. Alamelu Sundaresan, our department chair, and our administrative assistant, Mrs. Helen Pittman-Cockrell, for their collective support and generous expertise throughout this transformative journey.

CHAPTER 1

INTRODUCTION

On a global scale, breast cancer ranks as the most prevalent form of cancer among women. The disease is characterized by a complex interplay of genetic and clinical variations, resulting in multiple distinct subtypes. As a genetic disease, cancer originates from alterations in genes that affect cellular functions, particularly those related to growth and division mechanisms. This condition triggers uncontrolled cell proliferation and the spread of abnormal cells throughout the body. (National Cancer Institute, 2021). In contrast with normal cells, which strictly adhere to growth regulatory signals, cancer cells deviate significantly in behavior. They initiate growth even without external growth signals and ignore signals that would typically stop cell division or induce programmed cell death, known as "apoptosis."

Moreover, cancer cells promote the growth of blood vessels toward tumors, ensuring a continuous supply of oxygen and nutrients for their growth while eliminating waste products through these vessels. While normal cells remain mostly stationary within the body, cancer cells invade neighboring tissues and metastasize to distant body parts. These cells also have the capability to manipulate the immune system to support their growth and viability instead of attacking them. Cancer cells are prone to various chromosomal aberrations, including duplications and deletions. Due to their distinct nutrient requirements, they utilize specific energy-generating mechanisms that facilitate rapid growth, distinguishing them from normal cells (Farkas et al., 2021).

Autophagy is a physiological process known to serve diverse cellular functions, including adaptation to starvation, development, cell death, and tumor suppression (Mizushima et al., 2007). This mechanism initiates the formation of autophagosomes in response to various stressful conditions such as organelle damage, the presence of abnormal proteins, and nutrient deprivation (Russell et al., 2014). Autophagosomes capture degraded components and subsequently fuse with lysosomes for recycling. This autophagic process plays a critical role in safeguarding cellular organelles from toxins, regulating cell metabolism and energy balance, and promoting cell survival. Autophagy modulation assumes dual roles in cancer biology, contributing to both tumor promotion and suppression, thereby influencing cancer-cell development and proliferation (Lim et al., 2013).

Over the last decade, the landscape of cancer treatment has significantly expanded, offering patients a broad range of options. Evidence suggests that targeting epigenetic alterations responsible for drug resistance may potentially restore chemotherapy sensitivity in patients. However, a significant obstacle persists across various treatments: the emergence of drug resistance. Although researchers often study different resistance mechanisms independently, it is widely acknowledged that a single tumor likely harbors multiple resistance mechanisms. When cancer cells become resistant to treatment drugs, they can undergo regrowth and reform tumors, a process known as recurrence or relapse. Resistance can develop rapidly, sometimes within weeks of starting treatment, or it may evolve gradually over months or even years.

Considering the limited efficacy of current anticancer drugs due to the development of chemo resistance, ongoing research has expanded its exploration into alternative drug

categories for potential treatments. Recent findings suggest that statins have the capacity to induce autophagy across various cell types, including vascular endothelial cells, cardiac cells, respiratory tract mesenchymal cells, and transformed tumor cells (Araki et al., 2012). Statins, commonly used as cholesterol-lowering agents, work by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase, a key enzyme in the mevalonate pathway. Several signaling pathways, such as the AMPK/mTOR (AMP-activated protein kinase/mechanistic target of rapamycin) pathway and the AMPK/p21 pathway, are implicated in the regulation of statin-induced autophagy (Zhang et al., 2013).

These findings strongly suggest a delicate balance between apoptosis and autophagy regulation. This study illuminates a significant pharmaceutical property of simvastatin, demonstrating its ability to disrupt equilibrium, alter cell fate, and induce cell death through the activation of extracellular regulated proteins. Autophagy serves a pivotal role in limiting cancer necrosis and inflammation responses during the initial phases of cancer metastasis, thereby diminishing the invasion and migration of cancer cells from primary sites to other parts of the body. **The primary hypothesis proposes that simvastatin induces autophagy-mediated cell death in metastatic breast cancer cells.** Consequently, this approach may offer a promising treatment strategy for triple-negative breast cancer.

CHAPTER 2

LITERARY REVIEW

What is Cancer?

Cancer is a genetic disease originating from alterations in genes that regulate cellular functions, particularly in relation to growth and division mechanisms. This condition triggers unbridled cell proliferation and the subsequent dissemination of these abnormal cells throughout the body. Specific genetic mutations that contribute to cancer emergence can arise during cellular division or due to external risk factors in the environment. These factors encompass elements such as the chemicals found in tobacco smoke, the consumption of alcohol, dietary patterns, and the impact of ultraviolet rays emitted by the sun (National Cancer Institute, 2021).

The genetic modifications that play a role in cancer primarily impact three fundamental categories of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes. The first two categories are associated with cell growth and division, while the third category plays a role in repairing damaged DNA. All three categories are susceptible to undergoing modifications that can lead to the development of cancer. These alterations are commonly referred to as the "drivers" of cancer (Hausman et al., 2019).

Mechanism of Cancer

Ordinarily, the body efficiently removes cells bearing compromised DNA before they transition into a cancerous state. However, this natural safeguard diminishes with the passage of time as we age. This phenomenon contributes significantly to the heightened

susceptibility to cancer during the later stages of life. Every individual cancer case exhibits a distinctive array of genetic changes. As the cancer progresses, further modifications inevitably take place. Remarkably, even within a single tumor, divergent cells can harbor distinct genetic mutations as it can originate from almost anywhere in the human body which consists of trillions of cells. Typically, human cells grow and multiply through cell division to form new cells so when cells grow old or become damaged, they die, and are replaced by the newly formed cells. Occasionally, this orderly process falters, leading to the growth of abnormal or damaged cells when it should not occur. In such instances, the normal cell regulatory mechanisms that dictate cell behavior fail to suppress these processes as intended, resulting in a decline in the overall quality of life (Kroemer et al., 2008).

In certain cases, abnormal or damaged cells exhibit an anomalous capacity to reproduce beyond normal bounds, leading to the potential formation of tumors—which are essentially lumps of tissue. Some tumors which are noncancerous are considered to be benign. Benign tumors lack the capacity to invade neighboring tissues and when surgically removed, they typically do not reappear. Conversely, cancerous tumors, which may also be called malignant tumors possess the ability to infiltrate adjacent tissues and migrate to other parts of the body, giving rise to new tumor growth in a process, termed metastasis. (Seyfried et al., 2013) (**Figure 1**).

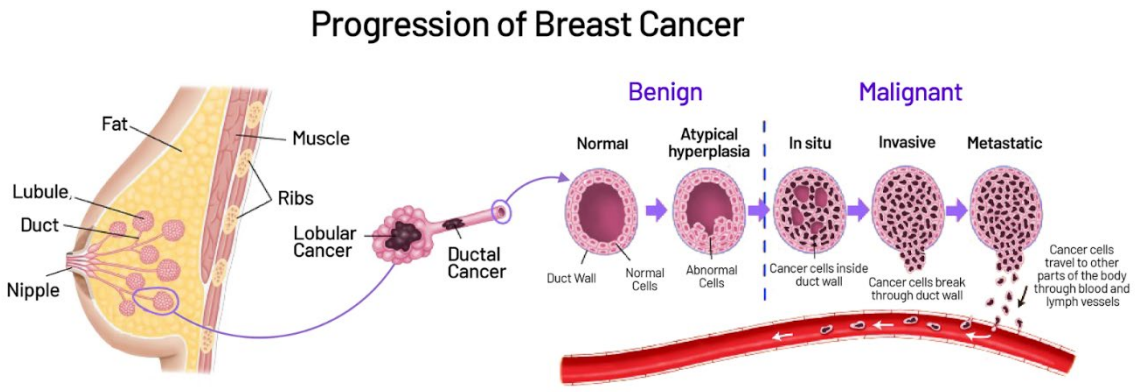

Figure 1: Stages of Breast Cancer (https://www.saintjohnscancer.org/breast/breast-cancer/types-of-breast-cancer/)

Characteristics of Cancer

Cancer cells exhibit several distinctions from normal cells. They initiate growth even in the absence of growth signals and disregard signals that would normally halt cell division or trigger programmed cell death, a process known as "apoptosis". In contrast, normal cells strictly adhere to these regulatory signals. While normal cells cease growth upon encountering other cells and remain mostly stationary within the body, cancer cells invade nearby tissues and disseminate to distant body parts (Kroemer et al., 2008). Furthermore, cancer cells can stimulate the growth of blood vessels towards tumors, ensuring a supply of oxygen and nutrients to sustain their growth, while concurrently expelling waste products through said vessels. These cells have the capability to manipulate the immune system, diverting it to support their growth and viability (Becker et al., 2016). Cancer cells are also prone to various chromosomal aberrations such as duplications and deletions (Farkas et al., 2021). Due to their distinct nutrient requirements, they generate energy in a specific manner that facilitates accelerated growth, setting them apart from normal cells (Kroemer et al., 2008).

Classification of Breast Cancer

There are more than 100 distinct types of cancer, often named after the organs or tissues from which they originate. These cancers can also be classified based on the specific cell type that initiated them, such as epithelial or squamous cells. Among these, carcinomas stand as the most prevalent category as they stem from epithelial cells, which cover the inside and outside surfaces of the body.

For this study, our focus will be directed towards the subtypes of breast cancer. On a global scale, breast cancer stands as the most prevalent form of cancer among women (Zhou et al., 2023). The most common breast cancers are ductal carcinoma in situ (DCIS) and invasive carcinoma. Both of which are adenocarcinomas originating from gland cells within the milk ducts or lobules—milk-producing glands. Breast cancer embodies a complex interplay of genetic and clinical variations, presenting multiple distinct subtypes. The classification of these subtypes has evolved over the years. A widely accepted classification system for breast cancer emerges from an immunohistochemical standpoint based on the expression of the following hormone receptors. These include the hormone receptors (HR); estrogen receptor (ER) and progesterone receptor (PR), and the human epidermal growth factor receptor (HER2), (Shaath et al., 2021).

The estrogen receptor (ER) plays a crucial role in diagnosis, as around 70-75% of invasive breast carcinomas express notably high proportions of ER (Zhang et al., 2013). In conjunction, progesterone receptor (PR) expression is found in over 50% of ER^+ patients and rarely in those with ER^- breast cancer (Hicks et al., 2016). ER influences PR expression, so PR levels provide insights into the functional ER pathway (Nicolini et al., 2018). Higher PR expression is linked to improved survival rates, delayed recurrence and

treatment failure or progression, while lower levels indicate a more aggressive disease course, high recurrence risk and poor prognosis (Purdie et al., 2014). Human epidermal growth factor receptor 2 (HER2) is a protein naturally synthesized by the body and genetically, it is essential for the growth and repair of healthy breast cells (Moasser et al., 2007). HER2 overexpression accounts for approximately 15-25% of breast cancers (Vaz-Luis et al., 2013). The amplification of this results in heightened activation of proto-oncogenic signaling pathways, driving uncontrolled cancer cell growth, and leading to worse clinical outcomes in $HER2^+$ cases. Furthermore, HER2 overexpression corresponds with a notably shorter disease-free interval (Krishnamurti et al., 2009).

Luminal A Subtype

Luminal A tumors represent the most prevalent molecular subtype, as it exhibits a comparatively slower growth rate compared to other cancer variants (Barnard et al., 2015). These tumors are characterized by their reliance on hormone receptors. The estrogen receptor (ER) and/or progesterone receptor (PR) are thus categorized as HR^+ (Reid et al., 2021). ER^+ and/or PR^+ cancers are fueled by estrogen and/or progesterone, so therapies that decrease the levels of these hormones have proven effective in managing this form of breast cancer (Higgins M.J., 2009). Furthermore, luminal A cancers are also known as $HER2^-$.

Luminal B subtype

Luminal B breast cancer cells are defined by a more rapid growth rate compared to the luminal A subtype, and they are recognized for their heightened aggressiveness. These cells display positivity for hormone receptors and an elevated level of HER2, leading to a

dual classification as ER^+ and $HER2^+$ (Li et al., 2015). Their advantages lie in the efficacy of hormone therapy and, to a greater extent, chemotherapy when contrasted with the preceding subtype (Lafci et al., 2022). Despite the prevalence of bone recurrence, they exhibit a heightened propensity for visceral recurrence, coupled with a diminished survival rate from initial diagnosis to relapse (Usman et al., 2022).

Triple-negative Subtype

Triple-negative breast cancer is a type of breast cancer where the cells lack receptors for estrogen, progesterone, and HER2. This form of breast cancer typically originates in the breast ducts with an estimate of two-thirds of women with breast cancer cells containing estrogen and progesterone receptors, and approximately 20 to 30 percent of breast cancers with an excess of HER2 receptors (Kumar et al., 2016). Healthy breast cells should possess receptors for said hormones along with the HER2 receptor but not in surplus amounts. Triple-negative breast cancer (TNBC) manifests aggressive characteristics, with 80% of breast cancer tumors attributed to the tumor suppressor genes BRCA1 and BRCA2 falling within this category (Loibi et al., 2017). The likelihood of TNBC development fluctuates based on factors such as genetics, ethnicity, age, body weight, breastfeeding practices, and parity (Collignon et al., 2016).

HER2-positive/HER2-enriched Subtype

$HER2^+$ breast cancer subtype is rendered among the more prevalent breast cancer variations in the United States. They are negative for estrogen receptor (ER) and progesterone receptor (PR) while exhibiting a positive status for HER2. The cancer cells linked to $HER2^+$ generate HER2 protein receptors due to an excessive number of copies of

the HER2 gene found on breast cells. In normal circumstances, these receptors govern the growth, division, and repair processes of healthy breast cells. Although, in excess, these receptors prompt cells to proliferate, leading to rapid and uncontrolled growth. Physicians frequently examine breast cancer tissue to detect surplus $HER2^+$ genes, aiding in the determination of whether the patient might benefit from targeted therapeutic approaches which can impede HER2's role in the sustenance of cancer cell growth (Li et al., 2015). The symptoms of $HER2^+$ breast cancer resemble those of other breast cancer types, including the presence of a breast lump, alterations in breast shape, discomfort, swelling, and abnormal discharge. Treatment approaches for $HER2^+$ breast cancer are based on the stage of the cancer and may incorporate an aggregation of various strategies (Figueroa-Magalhães et al., 2014).

Detection of Breast Cancer

In the initial stages of cancer, detection can significantly enhance the likelihood of a successful cure. Research indicates that screening tests can be lifesaving for certain types of cancer by detecting the disease at an early, treatable stage or elevated risk cases. The diagnostic process typically involves a combination of medical assessments as there isn't a singular test that can definitively diagnose cancer. This comprehensive approach helps healthcare professionals obtain a more accurate understanding of the underlying cause of the symptoms or abnormalities detected during screening (Song et al., 2016).

Physical Exam

A doctor may conduct a physical examination, feeling for lumps or abnormalities that could indicate the presence of cancer. Changes in skin color or organ enlargement may also be assessed during this examination (Abo Al-Sheikh et. al., 2021).

Laboratory Tests

These include urine and blood tests, helping your doctor identify cancer-related abnormalities. With reference to leukemia, which may be particularly detected through a complete blood count to analyze unusual white blood cell count (Park et al., 2022).

Imaging Tests

Noninvasive imaging tests allow your doctor to examine internal organs and bones. Examples include a computerized tomography (CT) scan, bone scan, magnetic resonance imaging (MRI) positron emission tomography (PET) scan, ultrasound, and X-ray (Wekking et al., 2023).

Biopsy

In a biopsy, cell samples are collected for laboratory testing. This option usually depends on the type and location of cancer. In many cases, a biopsy is the definitive method for diagnosing cancer. Cell samples scrutinized under a microscope to observe morphological differences. Normal cells exhibit uniformity in size and organized structure. Cancer cells appear less orderly, with variations in size and a lack of apparent organization (Matsutani et al., 2020).

Treatment of Breast Cancer

Cancer treatments are designed with diverse objectives, each serving a specific purpose tailored to encompass the primary goal of treatment, which is to achieve a cure for cancer, allowing the individual to lead a normal life span. The possibility of a cure varies depending on the specific characteristics of the cancer as well as the treatment administered.

Primary Treatment

Primary treatment is the complete removal of cancer from the body or eradication of cancer cells. Surgery is often the most frequent primary treatment for many common cancers. However, radiation therapy or chemotherapy may be employed if the cancer is particularly responsive to these modalities (Desai et al., 2021).

Adjuvant Treatment

This treatment eliminates any residual cancer cells post-primary treatment, reducing the risk of cancer recurrence. Any cancer treatment may serve as adjuvant therapy, with chemotherapy, radiation therapy, and hormone therapy being common choices (Desai et al., 2021).

Palliative Treatment

This form of treatment alleviates the side effects of treatment, or symptoms caused by cancer, particularly when a cure is not feasible. Surgery, radiation, chemotherapy, and hormone therapy are utilized to manage symptoms and control cancer spread. Palliative treatment can be administered concurrently with other interventions aimed at curing cancer. In certain cases, the treatment aims to achieve a complete cure, while in others, the

goal is to prevent the cancer from spreading further. (Van et al., 2022). Additionally, some treatments are administered to alleviate the side effects of other interventions and to relieve symptoms caused by either the cancer itself or its treatment. The treatment plan may evolve over time, adapting to the body's specific response and changing circumstances.

Surgery

This is the most common treatment approach to several cancer types. It involves the removal of cancerous mass (tumor) and adjacent tissue, sometimes to alleviate tumor-related side effects through operation. (Centers for Disease Control and Prevention, 2023). There are two primary types of surgeries for breast cancer treatment: A mastectomy involves the complete removal of breast tissue while a lumpectomy involves the removal of the tumor along with a small portion of healthy tissue surrounding it to ensure complete removal of cancer cells (Goethals et al., 2022).

Chemotherapy

The primary objective of chemotherapy is to suppress cell proliferation and hinder tumor multiplication, thereby preventing invasion and metastasis. This treatment is administered using drugs orally or through a blood vessel to eliminate cancer cells. Different drugs may be given concurrently or sequentially (American Cancer Society). However, a significant challenge associated with chemotherapy is its potential toxic effects on normal cells (Wu and Waxman, 2018). This is because chemotherapy drugs are designed to target rapidly dividing cells, which include both cancerous and healthy cells. As a result, normal cells can also be affected by chemotherapy, leading to various side effects and complications. There are several commonly used chemotherapy drugs for breast

cancer treatment, including Docetaxel, Paclitaxel, Doxorubicin, Epirubicin, Capecitabine, Carboplatin, Cyclophosphamide, Fluorouracil, Methotrexate, and Protein-bound Paclitaxel (Ge et al., 2022). Balancing the therapeutic benefits of chemotherapy with its potential toxicities remains a critical aspect of cancer treatment management.

Cryotherapy

This is also known as cryosurgery, it employs very cold gas to freeze and eliminate cancer cells (Doroshow, 2020). This treatment approach is gaining international traction with reference to its recent inclusion in the 2020 guidelines by the American Society for Clinical Oncology (ASCO) to prevent Chemotherapy-Induced Peripheral Neuropathy (CIPN). Although, it's important to note that while this practice is considered "promising," it is not yet firmly established (Loprinzi, 2020). It is occasionally utilized to treat cells with the potential to develop into cancer, termed pre-cancerous cells, on areas like the skin or cervix. Moreover, doctors can utilize a specialized instrument to apply cryotherapy to tumors located internally, such as those in the liver or prostate.

Immunotherapy (Biological Therapy)

Immunotherapy harnesses the body's inherent infection-fighting capability, namely the immune system (National Cancer Institute, 2022). It employs substances produced naturally by the body or synthesized in a laboratory to enhance the immune system's efficacy, fostering a more targeted approach to combat cancer cells or to manage side effects from other cancer treatments. (Centers for Disease Control and Prevention, 2023). Pembrolizumab is an FDA-approved therapy, in combination with chemotherapy both before and after surgery, is utilized for treating high-risk, early-stage, and triple-negative

breast cancers. On the other hand, Trastuzumab and Pertuzumab are agents that target ERBB2 monoclonal antibodies thereby halting signaling pathways associated with cell proliferation (Trayes et al., 2021). Immunotherapy functions through several key mechanisms to combat cancer effectively. Firstly, it can halt or slow down the growth of cancer cells, impeding the spread to other areas of the body, preventing metastasis. Additionally, this treatment enhances the proficiency of the immune system, empowering it to identify and eliminate cancer cells more effectively, thereby bolstering the body's natural defenses against the disease.

Hyperthermia

Hyperthermia applies heat to damage and eliminate cancer cells and can be targeted to a small area (tumor), parts of the body (organ or limb), or the entire body. Clinical trials investigating the effects of Hyperthermia Therapy (HT) have shown a noteworthy reduction in tumor size in combination with other treatments. Interestingly, HT at temperatures ranging from 41 to 44ºC did not demonstrate toxicity to normal cells, yet it induced toxicity specifically in cancerous cells (Ahmed et al., 2013). It is typically delivered externally with a machine or internally using a needle/probe placed inside the tumor (National Cancer Institute, 2022).

Radiation Therapy

During radiation therapy, high-powered energy beams like x-rays particles, or radioactive seeds and protons destroy cancer cells (National Cancer Institute, 2022). Cancer cells exhibit a faster rate of growth and division compared to normal cells in the body. Since radiation is particularly detrimental to rapidly dividing cells, radiation therapy

inflicts more damage on cancer cells than on normal cells. This hinders the growth and division of cancer cells, ultimately resulting in cell death (American Cancer Society, 2022). An estimate of about 50% of all cancer patients will undergo radiation in their treatment cycle (Delaney et al., 2005) as it yields approximately 40% of curative treatments (Barnett et al. 2009). There are two main types of radiation therapy used in cancer treatment. The first is external beam radiation therapy, which is the most common approach. It involves directing x-rays or particles at the tumor from outside the body. The second type is internal beam radiation therapy, also known as brachytherapy, which involves placing radiation sources inside the body. This method can include using radioactive seeds near the tumor, taking a liquid or pill orally, or administering radiation through a vein intravenously (IV).

Hormonal Therapy

This treatment is targeted for cancer growth fueled by hormones, such as breast, ovarian and prostate cancer as it involves the use of surgery or drugs to remove natural hormones from the body or block their effects to impede cancer cell growth. (Drăgănescu et al., 2017). It encompasses the use of various medications that target estrogen receptors (ER) in breast cancer especially. These drugs include ER modulators like Tamoxifen, which is taken orally and prevents estrogen from binding to breast cancer cells. Another type is selective ER down regulators such as Fulvestrant, which disrupts estrogen receptors. Aromatase inhibitors like Letrozole, Anastrozole, and Exemestane are also used; they reduce estrogen production in the body. Typically prescribed after surgery, hormone therapy helps reduce the risk of tumor recurrence and regression (Saeaib et al., 2020).

Laser Therapy

Laser therapy employs an exceptionally narrow and focused beam of light to eradicate cancer cells. It is commonly administered using a thin, illuminated tube inserted into the body. Delicate fibers at the tube's end guide the light toward cancer cells (Doroshow, 2020). This treatment has a wide range of applications in oncology, including reducing or eliminating tumors size, assisting in the management of cancer symptoms like bleeding, and sealing nerve endings and lymph vessels post-surgery to alleviate pain, reduce swelling, and prevent the spread of tumor cells.

Photodynamic Therapy

Photodynamic therapy involves administering a person a drug sensitive to a specific type of light through an injection (National Cancer Institute, 2022). This drug lingers in cancer cells for a more extended period than in healthy cells. The use of a laser triggers a transformation in the drug, turning it into a substance that effectively kills the cancer cells (Doroshow, 2020).

Targeted Therapies

This treatment targets specific abnormalities within cancer cells crucial for their survival by using drugs to impede cancer growth with minimal harm to normal cells (American Cancer Society, 2022). Conventional chemotherapy functions by eliminating both cancer cells and some normal cells. In contrast, targeted treatment precisely focuses on specific targets (molecules) within cancer cells that influence proliferation. The monoclonal antibodies trastuzumab (Herceptin) and pertuzumab (Perjeta), which target the

HER2, have significantly enhanced the prognosis for HER2-positive breast cancer patients (Slamon et al., 1989).

Stem Cell Transplant (Bone Marrow Transplant)

A bone marrow transplant can involve the utilization of either the patients' cells or cells obtained from a donor (American Cancer Society, 2022). It is commonly used to treat blood cancers and cancers originating in the lymph nodes as it involves replacing bone marrow cells lost due to intensive chemotherapy or radiation therapy. Administering this treatment enables higher doses of chemotherapy and can replace diseased bone marrow (Centers for Disease Control and Prevention, 2023).

Clinical Trials

The first clinical trial was initially employed for assessing cancer treatments in the mid-1950s (Frei et al., 1958). It has emerged as a valuable method for evaluating the comparative efficacy of treatments. In a matter of two decades, we have gathered a significant body of knowledge that offers objective data regarding cancer treatments. Today, thousands of ongoing cancer clinical trials contribute to advancing treatment methodologies. Other treatments may also be available, contingent on the specific type of cancer. The diverse array of treatment options underscores the multidimensional approach taken by medical professionals in addressing the complexities of cancer and tailoring interventions based on factors such as cancer type, stage, and the individual needs of patients (Desai et al., 2021).

Cancer Treatment Resistance and Reoccurrence

Over the past decade, the landscape of cancer treatment has expanded significantly, providing patients with a broader array of options. Some of these treatments yield remarkable responses, leading to the complete elimination of tumors, even in cases where cancer has spread extensively. However, a major obstacle persists across various treatments: the emergence of drug resistance. This phenomenon poses a formidable challenge for both cancer researchers and patients. When cancer cells develop resistance to the effects of treatment drugs, they can undergo regrowth and reform tumors, a process known as recurrence or relapse. Resistance can manifest rapidly, sometimes within weeks of initiating treatment, or it may evolve over months or even years.

Combining cancer drugs is a potential strategy to overcome or slow down the development of resistance by treating patients with combinations of various drugs. A strategy for combination treatment is to "simultaneously administer drugs that function through distinct molecular mechanisms," (Al-Lazikani et al., 2016). This method aims to enhance the killing of tumor cells, reduce the risk of drug resistance, and mitigate overlapping toxicity. An alternative strategy involves administering drugs that target the specific resistance mechanism developed by tumors. Subsequently, patients are treated once more with the drug to which they developed resistance. The rationale is that this combined approach may potentially "re-sensitize" patients to the initial treatment.

Immunotherapies, which aim to enhance the immune system's ability to combat cancer, have shown robust and enduring responses in patients with diverse cancer types. However, like other treatments, immunotherapies exhibit ineffectiveness in certain patients, and for some, their efficacy wanes after an initial positive response. Researchers

are optimistic that combining immunotherapies with either other immunotherapies or various types of cancer drugs could counteract the development of resistance (Merghoub et al., 2016). While different resistance mechanisms are typically studied independently, there is a consensus that a single tumor likely harbors multiple resistance mechanisms. Nevertheless, the considerable challenge lies in the extensive number of drug combinations requiring testing to identify the most suitable match for each patient. This presents a significant obstacle to the precision approach.

Autophagy

Autophagy, a vital physiological cellular process that facilitates the intracellular degradation and removal of misfolded proteins and damaged organelles. It is known to serve various cellular functions such as adaptation to starvation, development, cell death, and tumor suppression (Mizushima et. al., 2007). This mechanism triggers the formation of autophagosomes under various stressful conditions like organelle damage, the presence of abnormal proteins, and nutrient deprivation (Russell et al., 2014). This autophagic process plays a critical role in safeguarding cells organelles from toxins, regulating cell metabolism and energy balance, and fostering cell survival. The modulation of autophagy assumes dual roles in cancer biology, contributing both to tumor promotion and suppression, thus influencing cancer-cell development and proliferation (Lim et al., 2013). Moreover, autophagy regulates cancer stem-cell properties by maintaining stemness, inducing recurrence, and fostering resistance to anticancer agents. Certain anticancer medications have the capacity to modulate autophagy. Consequently, chemotherapy strategies that regulate autophagy may impact cancer-cell survival or death (Rosenfeldt et al., 2011).

Metastasis, the process by which cancer cells infiltrate and colonize new tissues and organs via the vascular and lymphatic systems, is a defining feature of cancer progression. During metastasis, cancer cells from the primary site exhibit heightened motility, migrating towards secondary locations. In primary cancer cells, autophagy is induced by hypoxia and nutrient deprivation, serving as a protective mechanism against cell necrosis and inflammation (Sosa et al., 2014). Autophagy exhibits both pro-metastatic and anti-metastatic effects (Kenific et al., 2010). In its anti-metastatic role, autophagy limits cancer necrosis and inflammation responses during the initial stages of cancer metastasis, also reducing invasion and migration of cancer cells from primary sites. However, in advanced stages of metastasis, autophagy switches to a pro-metastatic role by promoting cancer cell survival and colonization at secondary sites. (Hamurcu et al., 2018).

The autophagic process can be broken down into distinct steps (**Figure 2**). During initiation, ULK1 protein complex activates the PI3KC3-C1 protein complex. These complexes are transported to the site where the phagophore assembles, aiding in the formation of autophagosomes. Next, in the extension step, ATG12 is activated by ATG7, which creates an ATG12-ATG5 complex and a subsequent interaction with either ATG16 or ATG16L1 forms an ATG12-ATG5-ATG16L complex. Concurrently, ATG7 helps in attaching phosphatidylethanolamine (PE) to LC3-I to form LC3-II. The maturation step involves the formation of closed autophagosomes which finally fuse with the lysosomes. (Zhao et al., 2023)

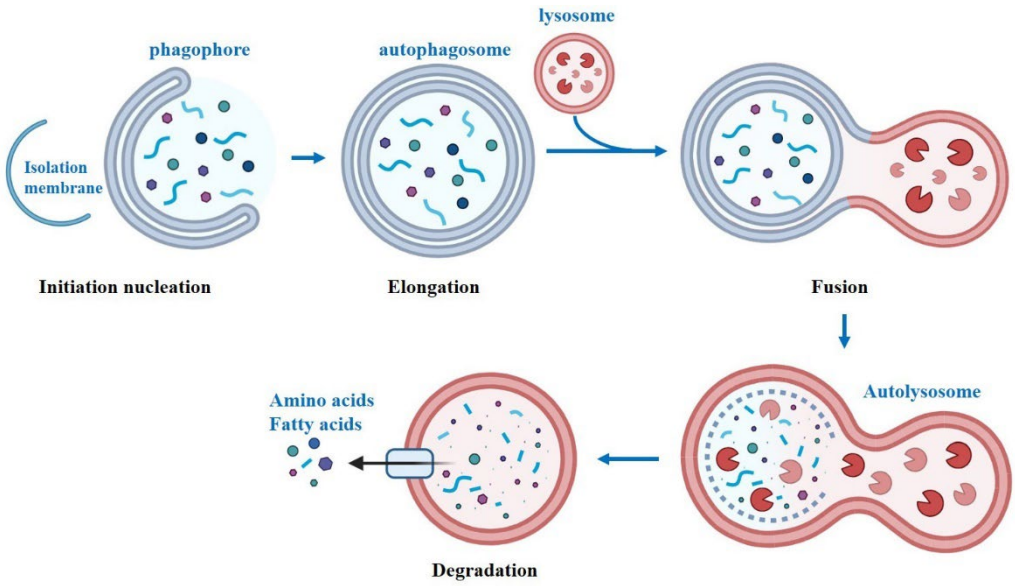

Figure 2: The Classical Process of Autophagy (https://www.frontiersin.org/files/Articles/1228045/fendo-14-1228045-HTML/image_m/fendo-14-1228045-g001.jpg)

Simvastatin

In response to the limited effectiveness of current anticancer drugs resulting from the emergence of chemoresistance, ongoing research has extended its exploration into alternative drug categories for potential treatments. Research findings indicate that statins possess the ability to induce autophagy across various cell types, including vascular endothelial cells, cardiac cells, respiratory tract mesenchymal cells, and transformed tumor cells (Araki et al., 2012). Statins, a class of drugs long used as cholesterol-lowering agents, function by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), a key enzyme in the mevalonate pathway. Several signaling pathways are involved in the regulation of statin-induced autophagy, including the AMPK/mTOR (AMP-activated protein kinase/mechanistic target of rapamycin) pathway and the AMPK/p21 pathway.

One type of statin, simvastatin, has been demonstrated to trigger autophagy in prostate cancer cells. This characteristic makes simvastatin a promising candidate for potential use in treating various other types of cancer as well. Simvastatin was the first statin approved by the FDA is recommended to be administered orally alongside dietary adjustments. It is categorized as a hexahydro naphthalene, belonging to the same class as lovastatin from which is derived from through semi-synthetic processes but differing in the ester group substitution; with a 2, 2-dimethylbutyrate ester group in place of the 2-methylbutyrate ester moiety found in lovastatin (**Figure 3**). Simvastatin aids in decreasing cholesterol synthesis, mitigating complications related to dyslipidemia, and treating cardiovascular diseases (Talreja et al., 2024)

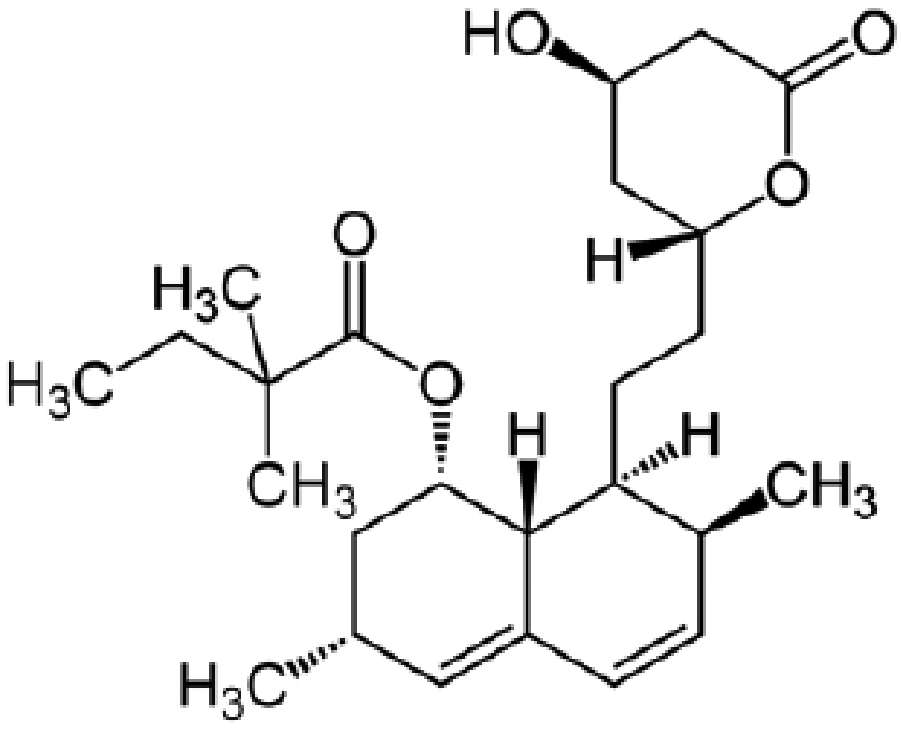

Figure 3: Chemical Structure of Simvastatin (https://www.researchgate.net/figure/Chemical-Structure-of-Simvastatin_fig1_262462596)

Rationale for This Study

Autophagy plays a pivotal role in intracellular degradation and the removal of misfolded proteins and damaged organelles. Initially perceived as a pro-survival mechanism, recent studies have highlighted the potential of autophagy to trigger pro-death pathways when its balance is disrupted. Recent research suggests that statins, traditionally recognized as cholesterol-lowering agents, possess the capability to suppress tumors across various cell types. In addition to their cholesterol-lowering effects, statins exhibit the ability to suppress cell proliferation, ultimately leading to cell death through Type I apoptosis-induced cell death or Type II autophagy-induced cell death. Simvastatin, a specific type of statin, has been demonstrated to induce autophagy in prostate cancer cells. However, its impact on other tumor types remains inadequately understood. **We hypothesize that simvastatin induces autophagy-mediated cell death in metastatic breast cancer cells.** The foundation of this hypothesis lies in the anti-metastatic and autophagic properties exhibited by simvastatin within the prostate cancer cells. In this study, we aim to investigate the hypothesis by elucidating the effects of simvastatin on breast cancer cells, as we seek to enhance our understanding of the potential therapeutic applications of statins in cancer treatment.

CHAPTER 3

DESIGN OF THE STUDY

Cell Culture

The metastatic breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were obtained and cultured in growth media containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin (P/S) (VWR). Cells were incubated at 37℃ in a water-jacketed incubator containing 5% CO2 and split routinely every 2-3 days depending on the confluency. For routine maintenance, growth media was aspirated from a T75cm2 flask and cells were washed with about 4 mL of Phosphate Buffered Saline (PBS). Next, 1 mL of 0.25% trypsin was then added to each flask before incubating for 2-3 minutes prior to trypsinization check under microscope. The flask was taken back under the hood and 9 mL of growth media was added, mixed and drawn up in a total volume of 10 mL, which was transferred into a 15 mL centrifuge tube.

Drug Treatment

Cells were seeded at a density of 3 x 105 cells in a 6–well plate in replicates. Both 6-well plates were removed from the incubator and viewed for confluency under the microscope. Old media was aspirated and each well in the plate was washed with 2 mL of PBS. New growth media was then added in 2 mL quantities after the PBS was aspirated. Cells were treated with varying concentrations of vehicle control, dimethyl sulfoxide

(DMSO), simvastatin (0 μM to 2 μM) or chloroquine (0 μM to 10 μM) for 24 and 48 hours, respectively. Following treatment, morphology was visualized using a Nikon inverted microscope. Alternatively, cells were harvested for protein expression for Western blotting or Trypan Blue Exclusion Assay.

Trypan Blue Exclusion Assay

Treated cells were harvested and separated into 15 mL centrifuge tubes and centrifuged for 3 minutes at 1500 RPM. The media was removed, and cells were resuspended in 1 mL of growth media. From this, 300 μL of cell suspension was added to 300 μL of 0.4% trypan blue solution. Following gentle mixing, cell viability was measured using a Bio-Rad automated cell counter.

Harvesting of Cells

Cells were to be collected at the 24 hour and 48-hour timepoint respectively. A total of six 15 mL centrifuge tubes were labelled to the predetermined drug concentrations. The 6-well plate was extracted from the incubator and cell death was viewed in each well to confirm accuracy based on timepoint and drug concentration under the microscope. The growth media was then transferred from each well to the respective labeled tubes according to the corresponding concentration. Each well was washed with 1mL of PBS which was also transferred into the respective labeled tubes. Thereafter, each well was trypsinized with 500 μL of 0.25% trypsin and incubated for 2-3 minutes. The plate was viewed under the microscope to confirm trypsinization and neutralized with 500 μL of growth media. The total contents of each well were transferred into the corresponding 15 mL centrifuge tube to bring up to a total of 4 mL in each tube. The empty dish was viewed under the

microscope to ensure most cells were collected. The 15 mL centrifuge tubes were then centrifuged at 4℃, 1500 rpm for 5 minutes. Next, the media was aspirated leaving the pellet behind and 1 mL of PBS was added to each tube with mixing to break the pellet before transferring to the appropriately labeled mini centrifuge tube, which were then centrifuged in the mini refrigerated centrifuge at 4℃, 5000 rpm for 5 minutes. The PBS was then extracted from each mini centrifuge tube and the remaining suspension was spun again on the tabletop centrifuge for 5 seconds. The remaining suspension was carefully collected leaving behind the pellet. Each tube was labeled with the timepoint (24/48 hours) and stored at -80℃.

Protein Lysate Preparation

The amount of lysis buffer needed to be transferred to the pellet was predetermined based on the pellet size and that information was then used to confirm the amount of lysate that needs to be transferred to the new labeled tube. To prepare protein lysates, 3 mL of Radio Immunoprecipitation Assay (RIPA) lysis buffer was placed in a 15 mL centrifuge tube. Next, 30 µL of each of the protease inhibitors were added: 100 mM sodium orthovanadate, 200 mM phenylmethylsulphonyl fluoride (PMSF), and 1X protease inhibitor cocktail were added to the 15 mL centrifuge tube and mixed. Then, the predetermined volume of lysis buffer- protease inhibitor mix ranging between 30 µL -100 µL was added to the mini centrifuge tube containing the cell pellets from each timepoint which was then incubated on ice for 15 minutes. Following incubation, the mini centrifuge tubes were centrifuged in the refrigerated micro centrifuge at 12,000 RPM for 10 minutes at 4℃. The supernatant was transferred to a new pre-labeled tube and stored.

Protein Estimation

The protein lysate samples were extracted from -80℃ freezer and thawed on ice alongside 5 mg/ml Bovine Serum Albumin (BSA). 1 mL of Bio-Rad Protein Assay Dye Reagent Concentrate (Bradford Reagent) was diluted with 4 mL dH_2O in a 15 mL centrifuge tube and vortexed to mix. Microcentrifuge tubes were labeled and set up from row A1-A4 and B1-B4. 200 µL of diluted reagent was added to each tube in row "A". 10 µL of H_2O was added to tubes B2, B3, B4 and 20 µL of 5 mg/ml BSA to B1. Then, 10 µL was transferred from B1 to B2, mixed and another 10 µL from B2, repeated to B3 but not B4. 1 µL was then added from B1 to A1 and the same pattern was followed for B2, B3 and B4. In the sample preparation step, 200 µL of diluted reagent was added to separate tubes for protein estimation of the actual samples. 200 µL of diluted reagent was transferred to the sample tube before 1 µL of sample was added per tube with respect to sample number and drug concentration for each timepoint. 85 µL of prepared sample was then transferred to each well in duplicates into a 96-well plate. The plate was then read using a microplate reader (Molecular Devices) and the data was extracted and saved under a template. The required microgram (µg) for the western blot step was documented according to the sample number that correlates the dosage concentration from both timepoints.

Western Blot

The samples were heated at a 100℃ for 5 minutes, centrifuged and loaded according to protein estimated concentration alongside the 4 µL protein marker on both ends and in-between to demarcate the 24 hr. from the 48-hr. lysate. The proteins were resolved on a 12% gel at 100V for 2 hours and transferred onto a nitrocellulose membrane at 85 V for 1 hour. The membrane was then blocked in 5% (w/v) nonfat milk containing

1X Tris-buffered Saline-Tween 20 (1X TBS-T) for 1 hour at room temperature. Next, the membrane was rinsed with 1X TBS-T and probed overnight with an LC3 antibody (1:5000). The following day, the membrane was washed three times with 1X TBS-T for 10 minutes each then incubated with Goat-anti-rabbit horse-radish peroxidase (HRP) conjugated secondary antibody (1:5000) for 1 hr. The membrane was washed again three times in 1X TBS-T for 10 minutes each, followed by enhanced chemiluminescent (ECL) detection using Santa Cruz Biotechnology Luminol Reagent or Thermo Scientific Super Signal West Pico PLUS Chemiluminescent Substrate. The membrane was then placed in between the autoradiography cassette film and developed in the dark room. Exposures were repeated for minute to second intervals and fed through the developer. The x-ray film was marked according to the protein ladder and labeled with the conditions used to detect the protein of interest. The membrane was then stored in 1X TBS-T at 4°C till the next day for the reprobing step following the same procedure with a GAPDH-specific antibody (1:5000) to confirm equal loading of protein across gel.

CHAPTER 4
RESULTS AND DISCUSSION

To test the effects of Simvastatin on breast cancer cells, we first employed the MDA-MB-231 metastatic breast cancer cells. Cells were routinely cultured using standard protocols in media containing specific agents, as outlined in the materials and methods section. Regular microscopic inspections were conducted before the cells reached peak confluency to confirm their viability and overall health in the growth media. Cells were seeded in replicates into 100 mm cell culture dishes.

Cells were treated with simvastatin (0 μM to 2 μM) or vehicle control (DMSO) for 24 hr. and 48 hr., respectively. After the incubation process, morphological alterations we observed and recorded using a Nikon inverted microscope at the same intervals. A comparative analysis of these changes was conducted between the simvastatin-treated samples and the untreated control group. During this analysis, it was observed that the number of rounded cells visibly increased with increasing drug concentration from the 24-hour to the 48-hour period, with the most rounded cells being observed with 2 μM treatment at 48 hr. **(Figure 4).**

The autophagic flux in metastatic breast cancer cells was investigated using simvastatin at concentrations ranging from 0 μM to 2 μM. The first set of cells incubated with the drug were harvested after 24 hr., followed by another batch after 48 hours. Following treatment, the cells were collected into pellets and lysed to estimate their protein content using a microplate reader. After protein estimation, Western blot

analysis was performed using predetermined protein concentration levels. The expression levels of cleaved LC3-II, an autophagic marker were examined. Results from this experiment depicted a resultant up-regulation of LC3-II with increased simvastatin concentrations in MDA-MB-231 cell lines. To ensure for equal protein sample loading, a GAPDH-specific antibody was probed on the same membrane **(Figure 5)**.

To rule out the possibility that the increased expression was a cell-line specific phenomena, a similar experiment was conducted using the MDA-MB-468 metastatic breast cancer cells. Cells were treated as previously described. Microscopic images revealed decreased viability with the appearance of more rounded cells with increased dosage over time, albeit to a lesser extent than MDA-MB-231 cells (**Figure 6)**. Western blot analysis was similarly performed in MDA-MB-468 cells treated with varying concentrations of simvastatin. LC3-II protein expression levels revealed an increase in elevated autophagy levels in response to higher drug concentrations in the MDA-MB-468 cell line, particularly after 24 hr. However, an increase in LC3-II expression was also observed in the samples treated for 48 hr. A GAPDH-specific antibody was conducted on the same membrane to confirm equal loading of protein samples **(Figure 7)**. These results indicate that simvastatin induces autophagy in these metastatic breast cancer cells.

To determine whether simvastatin induces autophagy-mediated cell death rather than apoptosis in metastatic breast cancer cells, we treated MDA-MB-231 cells with chloroquine, an established chemical inhibitor of autophagy which blocks autophagosome-lysosome fusion. MDA-MB-231 cells were treated with concentrations ranging from 0 μM to 10 μM for 24 hr. and 48 hr. intervals. Cells were harvested for Western blot analysis.

Results indicated a dose-dependent accumulation of LC3-II protein expression with increasing concentrations of chloroquine, indicating a lack of LC3-II turnover **(Figure 8)**. This was supported by the observation that there was little cell death microscopically viewed (data not shown). We chose to use 5 μM chloroquine as our optimized concentration for subsequent experiments.

To test whether simvastatin induced rounded morphology was exerted through autophagic pathways, MDA-MB-231 cells were co-treated with simvastatin and chloroquine. For controls, cells were treated with vehicle control (DMSO), chloroquine alone. Alongside these treatments, cells were co-treated with simvastatin +/- chloroquine. The phenotypic comparison of chloroquine treatment alongside simvastatin yields apparent differences against control sample. Of note, the treatment with vehicle control or 5 μM of chloroquine resulted in little to no rounded cells. On the other hand, treatment with 1 μM and to a greater extent, 2 μM simvastatin resulted in more rounded cells (as previously observed), particularly at the 48-hr period. However, the co-treatment of 1 μM simvastatin with chloroquine showed microscopically more attached cells when compared to the treatment with 1μM of simvastatin alone from 24 hr. to 48 hr. **(Figure 9)**. Interestingly, enhanced magnification of the data in Figure 9 revealed a noticeable contrast when examining the 48 hr. timepoint of the 2 μM treatment of simvastatin, wherein more rounded cells were observed with simvastatin along when compared to co-treatment with chloroquine **(Figure 10)**. These data reveal that rounded morphology is rescued as a consequence of chloroquine treatment.

To determine if the rounded cells visualized thus far were indicative of cell death, a Trypan blue exclusion assay was performed. Following treatment of simvastatin on MDA-MB-231 cells, cell suspensions were collected at each concentration, mixed with Trypan blue dye, and then loaded onto cell-count slides. The cell viability was assessed using a Bio-Rad automated cell counter, which excluded non-viable (dead) cells stained with Trypan blue dye. The results indicated a decrease in cell viability in MDA-MB-231 cells following dose-dependent treatment with simvastatin across three trials **(Figure 11, 12 & 13)**. Of note, the highest dose of simvastatin treatment (2 µM) resulted in the lowest cell viability (12%, 12% and 29%, respectively). Collectively, these data suggest that simvastatin induces autophagy-induced cell death.

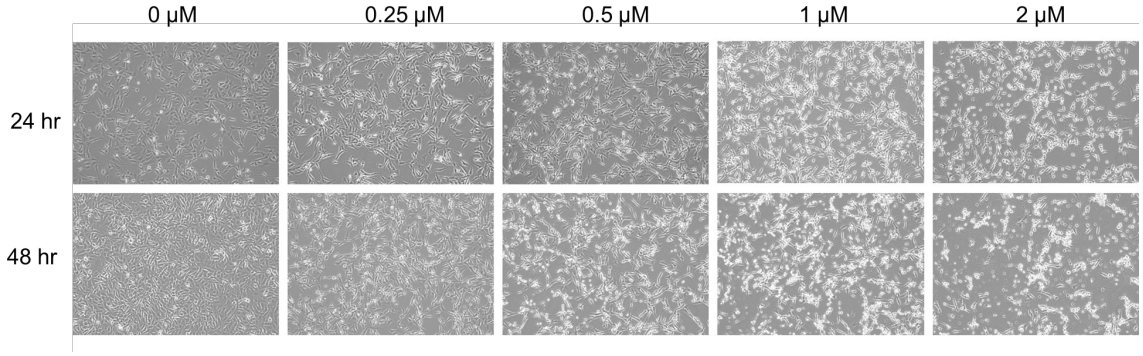

Figure 4: Morphology of MDA-MB-231 Metastatic Breast Cancer Cells Treated with Simvastatin

MDA-MB-231 cells were treated with simvastatin and incubated for the appropriate time interval. Following incubation, morphological changes were observed and captured with a Nikon inverted microscope between the 24- and 48-hour timepoints. Morphological changes and apparent cell number were compared between treated and non-treated (control) samples.

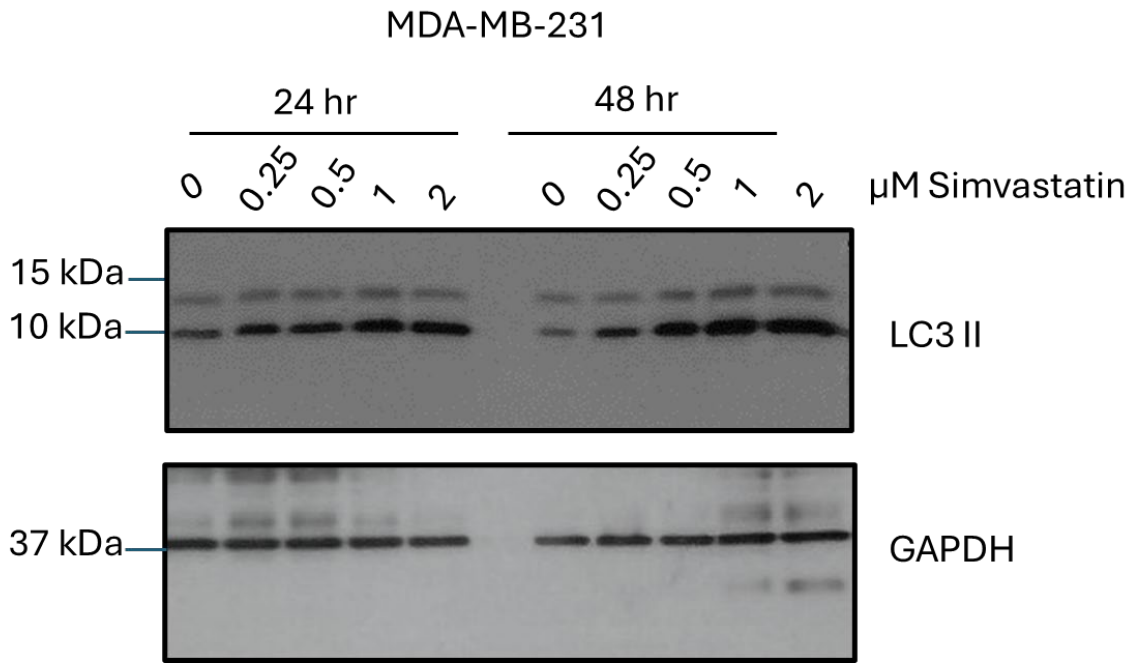

Figure 5: Western blot Analysis for LC3-II Protein Expression in MDA-MB-231 Metastatic Breast Cancer Cells treated with Simvastatin

Autophagic flux in MDA-MB-231 cells was examined via use of Simvastatin, at a concentration range from 0 μM-2 μM. Cells were incubated under standard culture conditions within a 24-hour and 48-hour timepoint. Cells were harvested and LC3-II expression was verified via Western blot analysis employing an LC3-II-specific antibody. To ensure equal protein loading, the same membrane was probed using a GAPDH-specific antibody.

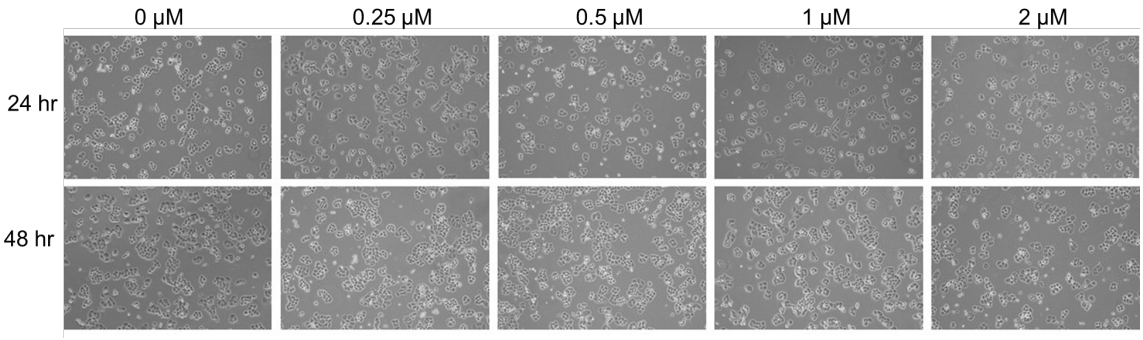

Figure 6: Morphology of MDA-MB-468 Metastatic Breast Cancer Cells Treated with Simvastatin.

MDA-MB-468 cells were treated with simvastatin and incubated for the appropriate time interval. Following incubation, morphological changes were observed and captured with a Nikon inverted microscope between the 24- and 48-hour timepoints. Morphological changes and apparent cell number were compared between treated and non-treated (control) samples.

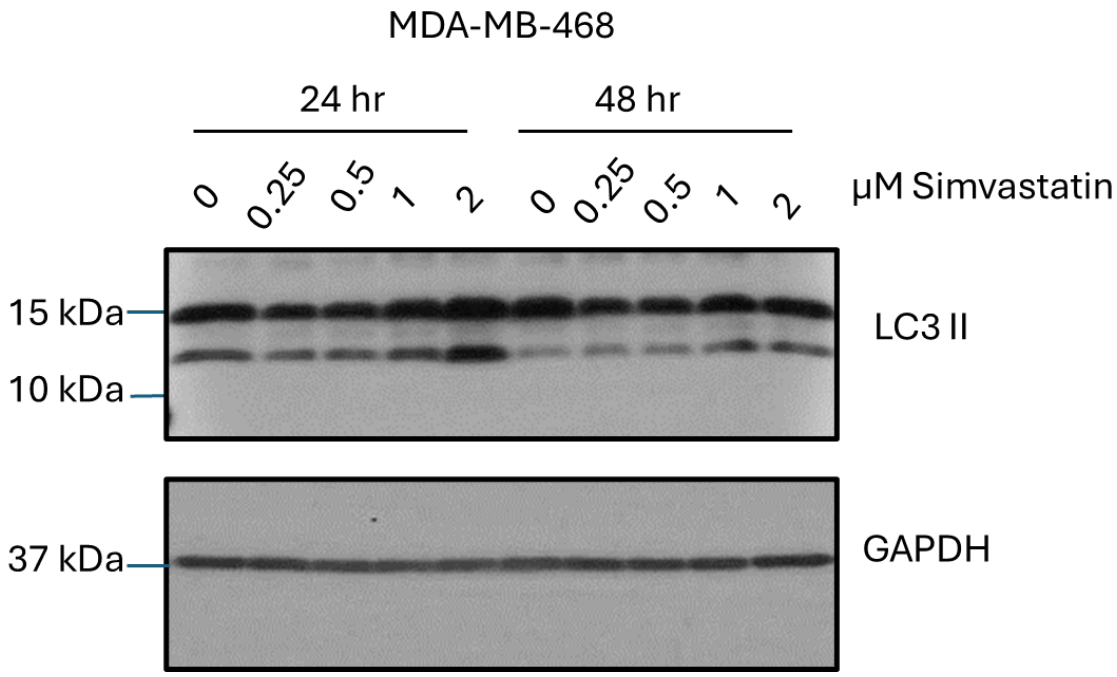

Figure 7: Western blot Analysis for LC3-II Protein Expression in MDA-MB-468 Metastatic Breast Cancer Cells Treated with Simvastatin

Autophagic flux in MDA-MB-468 cells was examined via use of Simvastatin, at a concentration range from 0 μM-2 μM. Cells were incubated under standard culture conditions within a 24-hour and 48-hour timepoint. Cells were harvested and LC3-II expression was verified via Western blot analysis employing an LC3-II-specific antibody. To ensure equal protein loading, the same membrane was probed using a GAPDH-specific antibody.

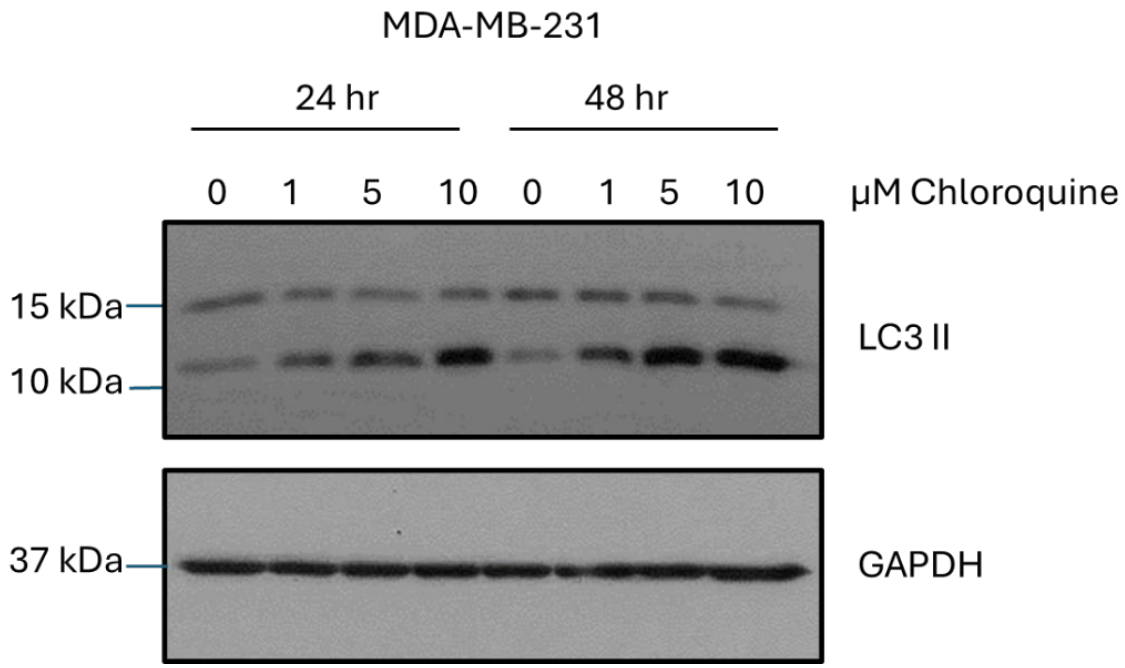

Figure 8: Western Blot Analysis for LC3-II Protein Expression in MDA-MB-231 Metastatic Breast Cancer Cells Treated with Chloroquine

Autophagic flux in MDA-MB-231 cells was examined via use of Chloroquine, an autophagy inhibitor, at a concentration ranging from 0 μM-10 μM. Cells were incubated under standard culture conditions within a 24-hour and 48-hour timepoint. Cells were harvested and LC3-II expression was verified via Western blot analysis employing an LC3-II-specific antibody. To ensure equal protein loading, the same membrane was probed using a GAPDH-specific antibody.

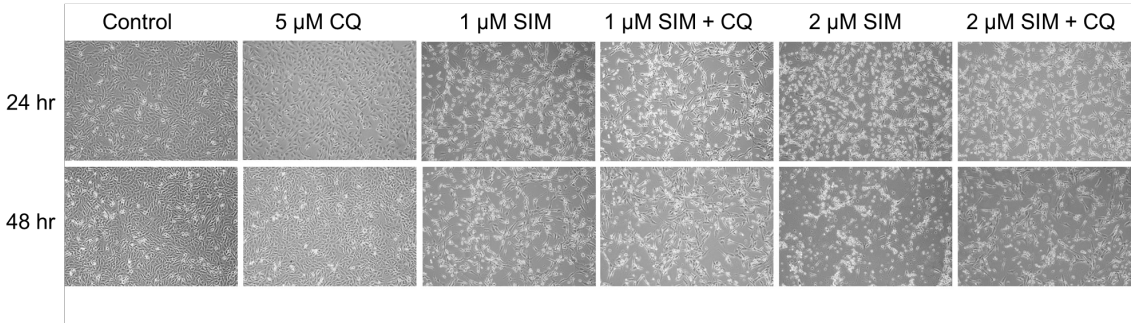

Figure 9: Morphology of Simvastatin Co-treatment with Chloroquine of MDA-MB-231 Metastatic Breast Cancer Cells

MDA-MB-231 cells were treated with both simvastatin (SIM) and chloroquine (CQ) and incubated for the appropriate time interval. Following incubation, morphological changes were observed and captured with a Nikon inverted microscope between the 24- and 48-hour timepoints. Phenotypic differences of chloroquine treatment alongside simvastatin treatment were compared against control sample.

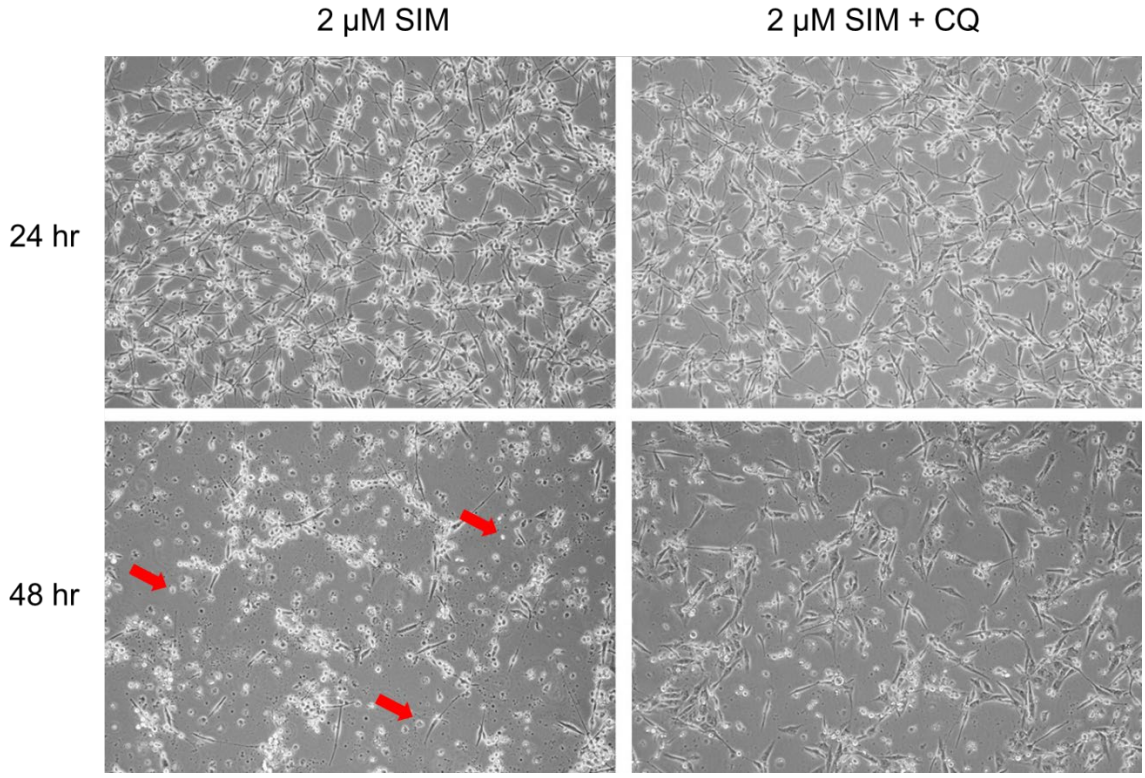

Figure 10: Enhanced Visualization of Morphology of Simvastatin Co-treatment with Chloroquine in MDA-MB-231 Metastatic Breast Cancer Cells

MDA-MB-231 cells were treated with simvastatin and chloroquine and incubated for the appropriate time interwall. Following incubation, morphological changes were observed and captured with a Nikon inverted microscope between the 24- and 48-hour timepoints. Phenotypic differences of chloroquine treatment alongside simvastatin treatment were compared against control sample.

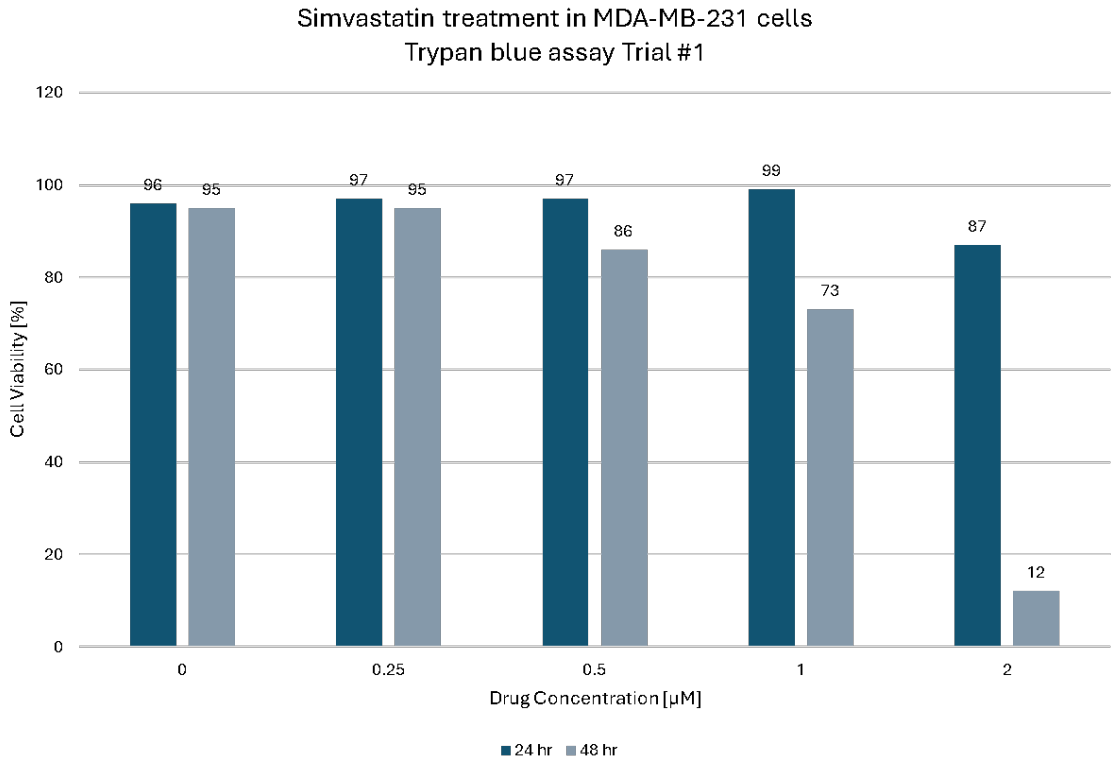

Figure 11: Cell Viability Assay Trial #1 of MDA-MB-231 Cells Treated with Simvastatin

A trypan blue exclusion assay was performed to determine the effect of various concentrations of simvastatin on viability of MDA-MB-231 metastatic breast cancer cells. Cells were treated, harvested, and resuspended in growth media. Trypan blue was added to cell suspension to measure cell viability using an automated cell counter.

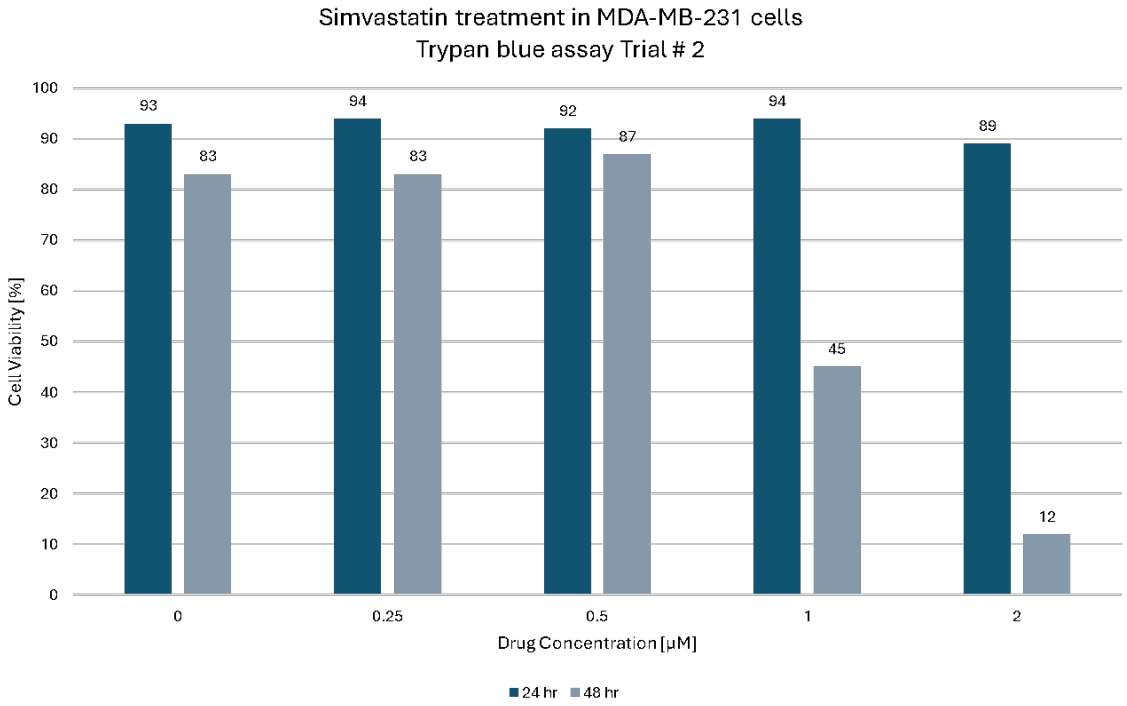

Figure 12: Cell Viability Assay Trial #2 of MDA-MB-231 Cells Treated with Simvastatin

A trypan blue exclusion assay was performed to determine the effect of various concentrations of simvastatin on viability of MDA-MB-231 metastatic breast cancer cells. Cells were treated, harvested, and resuspended in growth media. Trypan blue was added to cell suspension to measure cell viability using an automated cell counter.

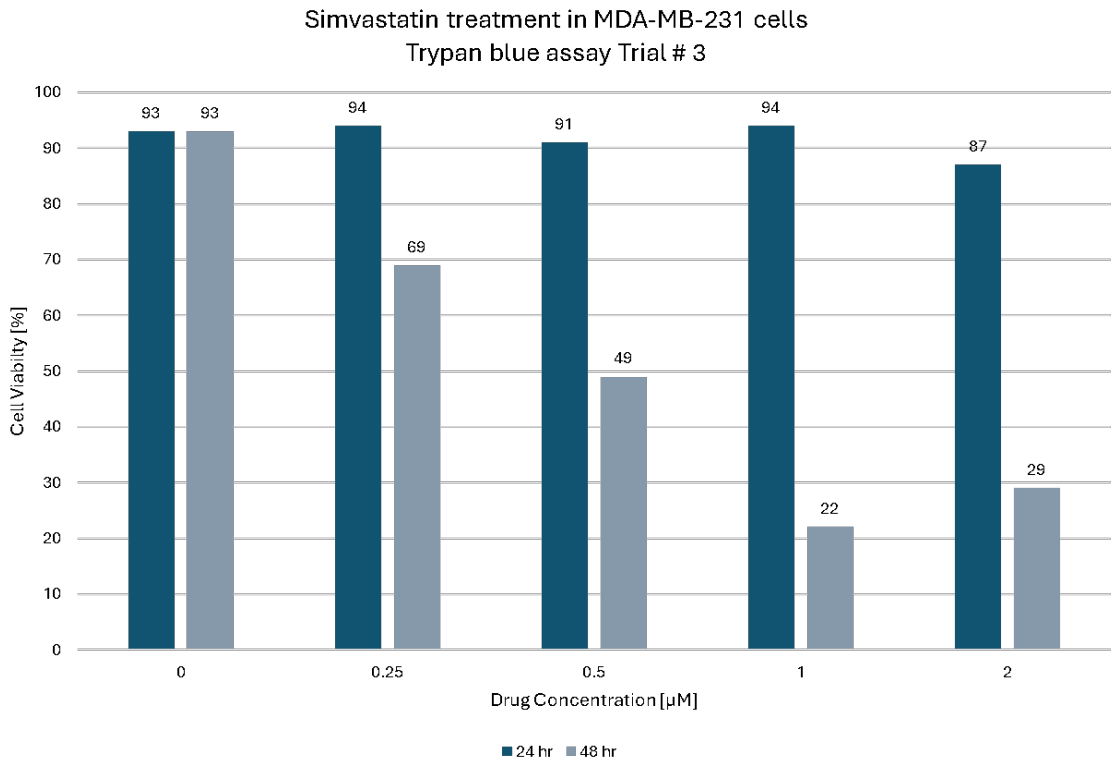

Figure 13: Cell Viability Assay Trial #3 of MDA-MB-231 Cells Treated with Simvastatin

A trypan blue exclusion assay was performed to determine the effect of various concentrations of simvastatin on viability of MDA-MB-231 metastatic breast cancer cells. Cells were treated, harvested, and resuspended in growth media. Trypan blue was added to cell suspension to measure cell viability using an automated cell counter.

Discussion

To adequately access simvastatin's role in promoting autophagy within metastatic breast cancer cells, we utilized the MDA-MB-231 and MDA-MB-468 cell lines as investigative models. These cells were cultured according to standard protocols or in media supplemented with specific agents (refer to materials and methods for details). Prior to reaching peak confluency, cells underwent periodic splitting and were inspected microscopically using a Nikon inverted microscope and digitally using an automated cell counter to conduct the trypan blue exclusion assay. This additional step was performed pre-treatment to confirm their viability (live cell count) and health status in the growth media before the administration of simvastatin. Upon achieving the targeted confluency, we treated the cells with varying simvastatin concentrations, from 0 μM to 2 μM. Observations at 24- and 48-hour post-treatment involved detailed microscopic analysis of cell morphology, where we noted an increase in rounded and/or detached cells (**Figure 4**). To determine if these morphological changes were indicative of cell death, another trypan blue exclusion assay was performed post-treatment, which demonstrated a concentration-dependent increase in cell mortality. Following this, the cells were collected, lysed, and subjected to protein concentration measurement via a microplate reader.

For the analysis of protein expression changes induced by simvastatin in metastatic breast cancer cells, Western blotting was meticulously performed using proteins with concentrations determined prior. In each experimental trial, membranes were incubated with primary antibodies targeting the specific protein of interest, LC3-II, an autophagy marker. The primary antibody LC3 (rabbit), was used to evaluate the expression levels of this key marker. Following the development of the blots, particular attention was paid to

the expression levels of LC3-II which showed an upregulation, correlating with increased concentrations of simvastatin in both MDA-MB-231 and MDA-MB-468 cell lines (**Figures 5 and 7**), suggesting a dose-dependent induction of autophagy by the drug. To confirm the accuracy of protein loading across the samples, a control Western blot analysis was concurrently performed using an antibody specific to GAPDH. Probing the same membrane with a GAPDH-specific antibody ensured that any observed changes in target protein expression were not due to variations in the amount of protein loaded onto the gel. This step is essential for validating the reliability of the observed upregulation in autophagy markers, providing a framework for the interpretation of the results.

To ascertain whether simvastatin induces autophagy-mediated cell death rather than apoptosis in metastatic breast cancer cells, we first conducted an optimization treatment with chloroquine, an established autophagy inhibitor in the MDA-MB-231 cell line. The range of concentrations tested was from 0 μM to 10 μM, using identical cell culture and protein estimation methodologies as described previously, prior to Western blot analysis, with a dose of 5 μM being used at the optimal dose (**Figure 8**). The morphological evaluation of chloroquine treatment against simvastatin reveals significant differences when compared to the control group. Specifically, the presence of rounded cells appears to decrease notably in the chloroquine treatment group compared to simvastatin treatment alone. Furthermore, when comparing the co-administration of simvastatin and chloroquine, there is a clear distinction as the number of visible live cells is significantly higher than in the simvastatin treatment alone at the same concentration. This observation supports the notion that the autophagic pathway has indeed been blocked. Since this approach aimed to

dissect the specific pathway through which simvastatin exerts its cytotoxic effects on cancer cells, the inclusion of chloroquine treatment led to a noticeable decrease in cell death compared to the administration of simvastatin alone. This outcome underscores the pivotal role of autophagy in the mechanism of action of simvastatin in inducing cell death in these cancer cells. Our results, therefore, highlight the therapeutic potential of leveraging autophagy as a mechanism to target and eliminate cancer cells, with simvastatin being a promising agent in this regard.

CHAPTER 5
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Breast cancer is recognized as the most common type of cancer affecting women worldwide as it represents a specialized cellular subset known for their pronounced resistance to traditional cancer treatments. These cancer cells are integral to the process of metastasis and relapse due to their ability to self-renew and differentiate into the diverse cell types that comprise tumors. The challenge posed by the resilience of breast cancer cells against conventional treatments underscores the urgent need for the development of targeted therapies capable of effectively neutralizing these cells, thereby hindering the progression and recurrence of breast cancer. Delving into the molecular mechanisms behind the drug resistance is essential for the enhancement of breast cancer therapies and the improvement of patient survival rates. This goal of this study focuses primarily on the effects of simvastatin, a commonly prescribed cholesterol-lowering medication, on metastatic breast cancer cells, specifically examining its role in inducing autophagy-mediated cell death.

Autophagy, a cellular process involved in the degradation and recycling of cellular components, has been identified as a potential therapeutic target in cancer treatment. Simvastatin's repurposing for cancer treatment is based on its ability to induce cell death in cancer cells without significantly affecting healthy cells. The impact of simvastatin on the autophagic pathway in breast cancer cells was evaluated by treating MDA-MB-231 and

MDA-MB-468 cell lines with varying concentrations of simvastatin for durations spanning 24 to 48 hours. To assess cell viability, the trypan blue assay was utilized. A notable decrease in the number of viable cells was observed as the concentration of simvastatin increased, indicating that simvastatin curtailed cell proliferation in a dose-responsive manner. The subsequent analysis of protein expression through western blotting techniques highlighted an escalation in LC3-II levels correlating with the increased dosage, signaling the activation of autophagy that led to cell death in these specific cell lines.

Considering the promising outcomes, we aim to conduct future experiments using a variety of other breast cancer cell lines (**Table 1**). This will strengthen our assertion that autophagy is not limited to the specific cells studied in this research. Incorporating additional cell lines will help dispel any misconceptions regarding apoptosis as a potential factor in cell death resulting from simvastatin treatment.

Moreover, identifying the molecular mechanism governing autophagy-induced cell death in these breast cancer cells is of great interest. Considering that statins have been previously reported to be involved in mTOR/ AMPK signaling, it is plausible that simvastatin exerts its effects through these pathways. Thus, chemical inhibition in the presence of simvastatin may shed light into the factors contributing to the observations gained in this study.

One limitation of our study was the lack of backdating for the samples used in protein estimation. This will impede our ability to revisit and compare older samples using western blot analysis. However, we are taking proactive steps to address this limitation. We plan to generate new samples with dating in mind, ensuring that we can conduct multiple trials and generate more comprehensive data on the morphological changes

observed post-treatment. This broader approach will not only enhance the reliability of our results but also facilitate a deeper understanding of the mechanisms of action and possible applications in cancer treatment.

Conclusively, this research highlights the effectiveness of simvastatin in targeting metastatic breast cancer cells, which are known for their aggressive nature and resistance to conventional treatments. By inducing autophagy-mediated cell death, simvastatin presents a novel approach to combat metastasis in breast cancer. The findings suggest potential clinical applications of simvastatin as a complementary therapy in the treatment of metastatic breast cancer. Further investigation and clinical trials are essential to fully understand its efficacy and safety in cancer treatment, potentially integrating it into existing treatment strategies. Exploring dosages and treatment regimens will maximize therapeutic outcomes while minimizing side effects. This also opens avenues for future studies to explore the combination of simvastatin with other therapeutic agents, aiming to enhance its anti-cancer effects. Investigating the role of genetic and molecular factors in modulating the response to simvastatin could lead to personalized treatment strategies for metastatic breast cancer.

Table 1: Cell Lines for Future Studies

Triple Negative Cell Lines for Future Study
MDA-MB-468
HCC1599
HCC1937
HCC1143
DU4475
HCC38
HCC70

Panel of Triple Negative Breast Cancer Cell Lines to be used to test the effects of Simvastatin on autophagy-induced cell death.

REFERENCES

1. Abo Al-Shiekh SS, Ibrahim MA, Alajerami YS. Breast Cancer Knowledge and Practice of Breast Self-Examination among Female University Students, Gaza. Scientific World Journal. 2021 Apr 27;2021:6640324. doi: 10.1155/2021/6640324. PMID: 34007246; PMCID: PMC8100409.
2. Ahmed K., Zaidi S.F. Treating cancer with heat: Hyperthermia as promising strategy to enhance apoptosis. *J. Pak. Med. Assoc.* 2013;63:504–508.
3. American Cancer Society website. Treatment types. www.cancer.org/treatment/treatments-and-side-effects/treatment-types.html.
4. Araki M., Maeda M., Motojima K. Hydrophobic statins induce autophagy and cell death in human rhabdomyosarcoma cells by depleting geranylgeranyl diphosphate. Eur. J. Pharmacol. 2012;674:95–103. doi: 10.1016/j.ejphar.2011.10.044.
5. Barnard ME, Boeke CE, Tamimi RM. Established breast cancer risk factors and risk of intrinsic tumor subtypes. Biochim Biophys Acta. 2015 Aug;1856(1):73-85. doi: 10.1016/j.bbcan.2015.06.002. Epub 2015 Jun 10. PMID: 26071880.
6. Barnett GC, West CM, Dunning AM, Elliott RM, Coles CE, Pharoah PD, Burnet NG. Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. Nat Rev Cancer. 2009; 9:134–142.

7. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell. 2016 Dec 12;30(6):836-848. doi: 10.1016/j.ccell.2016.10.009. PMID: 27960084; PMCID: PMC5157696.

8. "Cancer Treatments." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 15 May 2023, www.cdc.gov/cancer/survivors/patients/treatments.htm

9. Charles L. Loprinzi et al., Prevention and Management of Chemotherapy-Induced Peripheral Neuropathy in Survivors of Adult Cancers: ASCO Guideline Update. *JCO* 38, 3325-3348(2020). DOI:10.1200/JCO.20.01399

10. Collignon J, Lousberg L, Schroeder H, Jerusalem G. Triple-negative breast cancer: treatment challenges and solutions. Breast Cancer (Dove Med Press). 2016;8:93. https://doi.org/10.2147/BCTT.S69488 .

11. Delaney G, Jacob S, Featherstone C, Barton M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer. 2005;104:1129–1137.

12. Desai P, Aggarwal A. Breast Cancer in Women Over 65 years- a Review of Screening and Treatment Options. Clin Geriatr Med. 2021 Nov;37(4):611-623. doi: 10.1016/j.cger.2021.05.007. Epub 2021 Jul 30. PMID: 34600726.

13. Doroshow JH. Approach to the patient with cancer. In: Goldman L, Schafer AI, eds. Goldman-Cecil Medicine. 26th ed. Philadelphia, PA: Elsevier; 2020:chap 169.

14. Drăgănescu M, Carmocan C. Hormone Therapy in Breast Cancer. Chirurgia (Bucur). 2017 Jul-Aug;112(4):413-417. doi: 10.21614/chirurgia.112.4.413. PMID: 28862117.

15. Farkas G, Kocsis ZS, Székely G, Dobozi M, Kenessey I, Polgár C, Jurányi Z. Smoking, chromosomal aberrations, and cancer incidence in healthy subjects. Mutat Res Genet Toxicol Environ Mutagen. 2021 Jul;867:503373. doi: 10.1016/j.mrgentox.2021.503373. Epub 2021 Jun 18. PMID: 34266629.

16. Frei, E., III, Holland, J. F., Schneiderman, M. A., Pinkel, D., Selkirk, G., Freireich, E. J., Silver, R. T., Gold, G. L., and Regelson, W. A comparative study oftwo regimens of combination chemotherapy in acute leukemia. Blood 13: 1126 (1958).

17. Goethals A, Rose J. Mastectomy. [Updated 2022 Oct 6]. In: Stat Pearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538212/

18. Hausman DM. What Is Cancer? Perspect Biol Med. 2019;62(4):778-784. doi: 10.1353/pbm.2019.0046. PMID: 31761807.

19. Hicks DG, Lester SC. Hormone Receptors (ER/PR). Diagn Pathol. 2016:430–439. https://doi.org/10.1016/B978-0-323-37712-6.50067-3 .

20. Higgins MJ, Stearns V. Understanding Resistance to Tamoxifen in Hormone Receptor-Positive Breast Cancer. Clini Chem. 2009;55(8):1453–1455. https://doi .org/10.1373/clinchem.2009.125377 .

21. Kenific C.M., Thorburn A., Debnath J. Autophagy and metastasis: Another double-edged sword. Curr. Opin. Cell Biol. 2010;22:241–245. doi: 10.1016/j.ceb.2009.10.008.

22. Krishnamurti U, Hammers J, Atem F, Storto P, Silverman J. Poor prognostic significance of unamplified chromosome 17 polysomy in invasive breast carcinoma. Mod Pathol. 2009:1044–1048. https://doi.org/10.1038/modpathol.2009.61 .

23. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008 Jun;13(6):472-82. doi: 10.1016/j.ccr.2008.05.005. PMID: 18538731.

24. Kumar P, Aggarwal R. An overview of triple-negative breast cancer. Arch Obstet Gynaecol. 2016;293(2):247–269. https://doi.org/10.1007/s00404-015-3859-y .

25. Lafci O, Celepli P, Oztekin PS, Kosar PN. DCE-MRI Radiomics Analysis in Differentiating Luminal A and Luminal B Breast Cancer Molecular Subtypes. Acad Radiol. 2022. https://doi.org/10.1016/j.acra.2022.04.004 .

26. Li J, Chen Z, Su K, Zeng J. Clinicopathological classification and traditional prognostic indicators of breast cancer. Int J Clin Exp Pathol. 2015 Jul 1;8(7):8500-5. PMID: 26339424; PMCID: PMC4555752.

27. Lim K.H., Staudt L.M. Toll-like receptor signaling. Cold Spring Harbor Perspect. Biol. 2013;5:a011247. doi: 10.1101/cshperspect.a011247.

28. Loibl S, Gianni L. HER2-positive breast cancer. Lancet. 2017;389(10087):2415–2429. https://doi.org/10.1016/S0140-6736(16)32417-5

29. Matsutani A, Udagawa C, Matsunaga Y, Nakamura S, Zembutsu H. Liquid biopsy for the detection of clinical biomarkers in early breast cancer: new insights and challenges. Pharmacogenomics. 2020 Apr;21(5):359-367. doi: 10.2217/pgs-2019-0130. Epub 2020 Apr 14. PMID: 32284011.

30. Mizushima N. Autophagy: Process and function. Genes Dev. 2007;21:2861–2873. doi: 10.1101/gad.1599207.

31. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007 Oct 4;26(45):6469-87. doi:

10.1038/sj.onc.1210477. Epub 2007 Apr 30. PMID: 17471238; PMCID: PMC3021475.

32. National Cancer Institute website. Types of cancer treatment. www.cancer.gov/about-cancer/treatment/types.

33. Nicolini A, Ferrari P, Duffy MJ. Prognostic and predictive biomarkers in breast cancer: Past, present and future. Semin Cancer Biol. 2018;52(1):56–73. https://doi .org/10.1016/j.semcancer.2017.08.010,

34. Park S, Ahn S, Kim JY, Kim J, Han HJ, Hwang D, Park J, Park HS, Park S, Kim GM, Sohn J, Jeong J, Song YU, Lee H, Kim SI. Blood Test for Breast Cancer Screening through the Detection of Tumor-Associated Circulating Transcripts. Int J Mol Sci. 2022 Aug 15;23(16):9140. doi: 10.3390/ijms23169140. PMID: 36012405; PMCID: PMC9409068.

35. Purdie CA, Quinlan P, Jordan LB, Ashfield A, Ogston S, Dewar JA, et al. Progesterone receptor expression is an independent prognostic variable in early breast cancer: a population-based study. Br J Cancer. 2014;110(3):565–572. https://doi.org/10.1038/bjc.2013.756.

36. Reid S, Haddad D, Tezak A, Weidner A, Wang X, Mautz B, Moore J, Cadiz S, Zhu Y, Zheng W, Mayer IA, Shu XO, Pal T. Impact of molecular subtype and race on HR+, HER2- breast cancer survival. Breast Cancer Res Treat. 2021 Oct;189(3):845-852. doi: 10.1007/s10549-021-06342-0. Epub 2021 Jul 31. PMID: 34331630; PMCID: PMC8511072.

37. Russell R.C., Yuan H.X., Guan K.L. Autophagy regulation by nutrient signaling. Cell Res. 2014;24:42–57. doi: 10.1038/cr.2013.166.

38. Saeaib N, Peeyananjarassri K, Liabsuetrakul T, Buhachat R, Myriokefalitaki E. Hormone replacement therapy after surgery for epithelial ovarian cancer. Cochrane Database Syst Rev. 2020 Jan 28;1(1):CD012559. doi: 10.1002/14651858.CD012559.pub2. PMID: 31989588; PMCID: PMC7027384.

39. Seyfried TN, Huysentruyt LC. On the origin of cancer metastasis. Crit Rev Oncog. 2013;18(1-2):43-73. doi: 10.1615/critrevoncog.v18.i1-2.40. PMID: 23237552; PMCID: PMC3597235.

40. Shaath H, Elango R, Alajez NM. Molecular Classification of Breast Cancer Utilizing Long Non-Coding RNA (lncRNA) Transcriptomes Identifies Novel Diagnostic lncRNA Panel for Triple-Negative Breast Cancer. Cancers. 2021;13(21):5350. https://doi.org/10.3390/cancers13215350.

41. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science.* 1989;244(4905):707-712.

42. Song JL, Chen C, Yuan JP, Sun SR. Progress in the clinical detection of heterogeneity in breast cancer. Cancer Med. 2016 Dec;5(12):3475-3488. doi: 10.1002/cam4.943. Epub 2016 Oct 24. PMID: 27774765; PMCID: PMC5224851.

43. Sosa M.S., Bragado P., Aguirre-Ghiso J.A. Mechanisms of disseminated cancer cell dormancy: An awakening field. Nat. Rev. Cancer. 2014;14:611–622. doi: 10.1038/nrc3793.

44. Talreja O, Kerndt CC, Cassagnol M. Simvastatin. 2023 Jun 5. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 30422514.

45. Trayes KP, Cokenakes SEH. Breast Cancer Treatment. Am Fam Physician. 2021 Aug 1;104(2):171-178. PMID: 34383430.

46. “Tests and Procedures Used to Diagnose Cancer.” *National Cancer Institute*, www.cancer.gov/about-cancer/diagnosis-staging/diagnosis.

47. Usman A. The Survival of Patients with Triple Negative Breast Cancer with Chemotherapy Along with Lifestyle Change Interventions in Survivors. Arch Cancer Res. 2022;10(1)

48. Van Christ Manirakiza A, Pfaendler KS. Breast, Ovarian, Uterine, Vaginal, and Vulvar Cancer Care in Low- and Middle-Income Countries: Prevalence, Screening, Treatment, Palliative Care, and Human Resources Training. Obstet Gynecol Clin North Am. 2022 Dec;49(4):783-793. doi: 10.1016/j.ogc.2022.08.004. PMID: 36328680.

49. Vaz-Luis I, Winer EP, Lin NU. Human epidermal growth factor receptor-2-positive breast cancer: does estrogen receptor status define two distinct subtypes? Ann Oncol. 2013;24(2):283–291. https://doi.org/10.1093/annonc/mds286 .

50. Wekking D, Porcu M, De Silva P, Saba L, Scartozzi M, Solinas C. Breast MRI: Clinical Indications, Recommendations, and Future Applications in Breast Cancer Diagnosis. Curr Oncol Rep. 2023 Apr;25(4):257-267. doi: 10.1007/s11912-023-01372-x. Epub 2023 Feb 7. PMID: 36749493.

51. “What Is Cancer?” *National Cancer Institute*, 11 Oct. 2021 www.cancer.gov/about-cancer/understanding/what-is-cancer.

52. Zhang J, Yang Z, Xie L, Xu L, Xu D, Liu X. Statins, autophagy and cancer metastasis. Int J Biochem Cell Biol. 2013 Mar;45(3):745-52. doi: 10.1016/j.biocel.2012.11.001. Epub 2012 Nov 10. PMID: 23147595.

53. Zhang MH, Man HT, Zhao XD, Dong N, Ma SL. Estrogen receptor-positive breast cancer molecular signatures and therapeutic potentials (Review). Biomed Rep. 2013;2:41–52. https://doi.org/10.3892/br.2013.187 .

54. Zhao X, Bie LY, Pang DR, Li X, Yang LF, Chen DD, Wang YR, Gao Y. The role of autophagy in the treatment of type II diabetes and its complications: a review. Front Endocrinol (Lausanne). 2023 Sep 21;14:1228045. doi: 10.3389/fendo.2023.1228045. PMID: 37810881; PMCID: PMC10551182.

55. Zhou JS, Liu ZN, Chen YY, Liu YX, Shen H, Hou LJ, Ding Y. New advances in circulating tumor cell-mediated metastasis of breast cancer (Review). Mol Clin Oncol. 2023 Jul 24;19(3):71. doi: 10.3892/mco.2023.2667. PMID: 37614367; PMCID: PMC10442766.