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Cell Proliferation and Viability Inhibition by Resveratrol on Breast Cancer Cell Lines

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CELL PROLIFERATION AND VIABILITY INHIBITION BY RESVERATROL ON BREAST CANCER CELL LINES

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ABSTRACT
KYLE FORD GORDON: CELL PROLIFERATION AND VIABILITY INHIBITION BY RESVERATROL ON BREAST CANCER CELL LINES
(Under the direct of Drs. Yu-Dong Zhou and Dale G. Nagle)

Antioxidants are well-known for their various health benefits. They are able to protect cells from being damaged by free radicals that are produced by vital biochemical processes. It has long been known that antioxidants are important in our everyday health, but their potential as disease preventers and potential therapeutic agents is a relatively new field of study. Resveratrol, a natural polyphenol and well-known antioxidant, is found in plants, fruits, and products derived from them, like red wine. Resveratrol has been shown to have various properties, including antiaging, anti-aggregation of platelets, anti-inflammatory, and anticancer activities. Because of their many health benefits, antioxidants have become a hot topic in cancer research. Oxidative stress, which occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants, has been shown to be a potential cause of cancer development. Our research group tested the effects of resveratrol, vitamins C and E, and the green tea catechin, epigallocatechin gallate (EGCG), on various breast cancer cell lines, though this review will focus on the chemotherapeutic potential of resveratrol. In the experiments, breast cancer MCF-7, MDA-MB-231, BOM 231, and MCF-7-BOM cell lines were treated with various concentrations of resveratrol. A Sulforhodamine B viability assay was used to assess the percent inhibition of resveratrol on each cell line. The experiments showed that resveratrol is an inhibitor of breast cancer cells in a concentration and cell line dependent manner.

Table of Contents

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LIST OF FIGURES

LIST OF ABBREVIATIONS

BRCA1- Breast Cancer associated gene 1

BRCA2- Breast Cancer associated gene 2

CBR1- Carbonyl reductase 1

CI- Confidence interval

CSC- Cancer Stem Cell

CTC- Circulating tumor cell

ECM- Extracellular matrix

EGCG- Epigallocatechin gallate

EGFR- Epidermal growth factor receptor

EMT- Epithelial-mesenchymal transition

ER+- Estrogen receptor positive

ER/PR- Estrogen/progesterone receptor

FBS- Fetal bovine serum

GSH- Glutathione peroxidases

HER2- Human epidermal growth factor receptor 2

HIF- Hypoxia-inducible factor

HRT- Hormone replacement therapy

MET- Mesenchymal-epithelial transition

MMP-9- Matrix metallopeptidase 9

ROS- Reactive oxygen species

RR- Relative risk

SOD- Superoxide dismutase

SOD2- mitochondrial manganese SOD

SRB- Sulforhodamine B

TCA- Trichloroacetic acid

TNBC- Triple-negative breast cancer

TNM- Tumor, node and metastasis

uPA- Urokinase type plasminogen activator

VEGF- Vascular endothelial growth factor

VEGFR- Vascular endothelial growth factor receptor

Introduction: Breast Cancer

Cancer has become a major health problem across the globe. It is now the second leading cause of death in the United States. The American Cancer Society expects cancer to kill over 600,000 people in 2019 (27). Cancer is not only widespread but it is an extremely variable disease, as the National Cancer Institute recognizes over 100 different types of cancer. Cancer is a ruthless disease that takes advantage of a vital life process. The human body is made of trillions of cells. As old cells die, new ones divide and take their place. Cancer begins to take form when a buildup of cellular mutations cause some of these cells to become abnormal and divide uncontrollably. According to the World Health Organization, breast cancer is the most common form of cancer in the world (other than skin cancer), with over two million cases reported in 2018 (6). Most breast cancer occurs in women, with the number of cases in women occurring at a rate 100-times greater than in men (24). Like all cancers, breast cancer is an extremely complex and heterogeneous disease. Despite the variability of breast cancer, great strides have been made to identify risk factors that could lead to the development of breast cancer. Breast cancer associated gene 1 and 2 (BRCA1 and BRCA2) are two of the most well-known risk factors for breast cancer at the genomic level. These genes encode tumor suppressor proteins, which help to combat tumor growth when functioning properly. However, genetics are not solely responsible for the development of breast cancer. Aging, hormone levels, lifestyle, and gender can also greatly influence the development of breast cancer. Despite the high incidence of breast cancer, there have been new

developments in recent years to combat this disease. This is because of new therapies, early detection, and knowledge about the prevention of breast cancer.

The breasts are a very complex and important part of human anatomy. Breast tissue undergoes many changes during the course of a lifetime. From birth to the end of menopause they are constantly growing and their cells are dividing. After puberty, breast tissue undergoes even greater changes during breastfeeding and menopause. These changes are necessary to breastfeed children, but they also create an opportunity for cancer to manifest. Cancer results from the buildup of mutations over time, which are more common in tissues that have cells which divide often. Breast tissue contains approximately 20 lobes which contain smaller lobules that are responsible for producing milk. The lobules are able to move this milk to the nipple through tubes called ducts. Breast cancer typically begins in these ducts (ductal carcinoma) or in the lobules (lobular carcinoma) (2).

Breast cancer can form as a result of genetic pre-dispositions, environmental factors, or a combination of both. Every person has a unique set of DNA that codes for the human body and all of its processes. Genes are small sections of this DNA that code for individual traits and provide variability in a population. Every person has two copies of each gene: one from each parent. If one parent has a mutation in a certain gene, their child has a high chance of inheriting that gene. Recent advances in DNA technology have allowed researchers to gain a much better understanding of genetic involvement in cancer. Oncogenes and tumor suppressor genes are thought to be two of the biggest genetic players in the development of cancer. Oncogenes are mutated genes that have the potential to become cancerous, while tumor suppressor genes are responsible for tumor

repression. Just as the name suggests, a loss in function in a tumor suppressor gene can allow for tumor growth. Most inherited cases of breast cancer involve the genes BRCA1 or BRCA2. When these genes are not functioning properly, the risk for developing breast or ovarian cancer increases significantly. The BRCA1 and BRCA2 mutations account for approximately 10% of all breast cancer cases (29). Approximately 50% of women with either the BRCA1 or BRCA2 gene mutation will develop breast cancer at some point in their life (1). Various BRCA1 mutations can lead to dysregulation of the cell cycle, genetic instability, and eventually, apoptosis. The proteins encoded by BRCA2 regulate chromosomal breaks and repairs. If an individual inherits a deleterious BRCA1/2 gene, their risk for breast cancer increases dramatically. These genes are inherited in an autosomal dominant fashion, so only one mutated allele is necessary for deleterious effects to appear (24).

Human epidermal growth factor receptor 2 (HER2) overexpression increases the number of cancer stem cells and is found in approximately 20% of primary breast cancers (24). Functioning as a tyrosine kinase, HER2 protein overexpression can lead to uncontrolled cell growth. Additionally, tumor cells that present high levels of HER2 have a poorer prognosis (15). Epidermal growth factor receptor (EGFR), also known as HER1, is a cell surface protein that promotes cell proliferation, invasion, angiogenesis, and apoptosis prevention. Patients with tumors that at are EGFR-positive have a much poorer prognosis than EGFR-negative patients (24). Furthermore, more than half of patients with triple-negative breast cancer (TNBC), the deadliest form of breast cancer, have EGFR overexpression. Estrogens (ERs) are sex hormones that promote the development and maintenance of the female sex characteristics. They are one of the most prominent

players in breast cancer because of their cell proliferating and growth stimulating capabilities. Estrogens are essential to the development of the lobes in the female breast, but they can promote breast cancer if they are present at high levels. In fact, in ER+ breast cancer patients, the concentration of estrogen in breast tissue has been shown to be 20-times higher than in normal breast tissue (18). Thus, a common treatment for breast cancer patients is to block estrogen from binding to its receptor through hormone therapy. There are two types of ERs that have different physiological roles; ERα and ERβ. Phytoestrogens, such as resveratrol, are found in plants and have been linked to a decrease in risk for developing breast cancer. Resveratrol is able to mitigate the cell proliferating capabilities of both subtypes of ERs but was shown to promote cell growth in in late stage cancer cells that lack ERβ (18).

Environmental risk factors for developing breast cancer range from lifestyle to aging. Aging, as it is in all cancers, is perhaps the biggest determinant in the development of breast cancer. Interestingly, the median age for the development of breast cancer varies with ethnicity. For example, according to the National Cancer Society, the median age of a breast cancer diagnosis for white women is 63, while that for African American women is 59 (3). Therefore, it is advised that women over the age of 40 have a mammography screening. Reproductive factors such as early menarche (first menstruation), late child bearing, low parity (number of child births), and late menopause can all increase the risk of developing breast cancer. Multiple successful pregnancies have been shown to decrease the risk of developing breast cancer after the age of 40 regardless of the age at first birth, but increases the risk for breast cancer diagnosis before 40 years of age (3). The relationship between reproductive factors and breast cancer development is very

complex, but a trend has emerged between certain factors and the development of estrogen/progesterone (ER/PR) positive or negative tumors. Both natural estrogen production and exogenous estrogen consumption are associated with breast cancer risk. Estrogen plays an important role in premenopausal women, but an ovariectomy can be performed to reduce the risk of breast cancer development in older women. Exogenous estrogen commonly comes in the form of oral contraceptives and hormone replacement therapy (HRT). Research suggests that oral contraceptives are more strongly associated with ER-negative tumors than ER-positive tumors, though the side effects of oral contraceptives has been greatly reduced since their original production. Women who stop using oral contraceptives for more than 10 years can completely eliminate their risk of developing breast cancer from exogenous estrogen. Additionally, studies have shown that the use of HRT can increase the risk of developing breast cancer. The Million Women Study in UK showed that women who currently take HRT have a relative risk (RR) of 1.66 for developing breast cancer when compared to those who have never used HRT (24). In 2003, the Women's Health Initiative published data reflecting the negative effects of HRT. Since then, the incidence rate of breast cancer caused by HRT has dropped 7% (24).

Unhealthy eating and drinking habits can also increase one's risk of developing breast cancer. It is recognized that early-life exposure to carcinogens can increase a women's lifetime risk of breast cancer. Breast cancer risk builds over the course of a lifetime, but research suggests that the majority of the buildup occurs between menarche and first pregnancy. There appears to be a critical period after the onset of menarche, when breast tissue is growing and dividing rapidly, all the way until the first pregnancy.

Thus alcohol, which is a breast carcinogen, if consumed early in life, could contribute to increased breast cancer risk. Specifically, daily excessive alcohol consumption can increase the risk of breast cancer by 32% (24). Case-control studies showed that alcohol consumption (1 drink/day) before the age of 30 increased the risk of premenopausal breast cancer by 34% (13). However, in the same study it was shown that alcohol consumption before the age of 30 did not affect postmenopausal women. This is further evidence that a critical period could exist in breast cancer development. Eating a healthy diet alone is not enough to prevent breast cancer, but it can contribute to lowering one's risk for developing it. Several vitamins and natural phenols have been identified as potential substances that can help to fight and or treat breast cancer. Resveratrol, a natural phenol, will be discussed later in this paper.

The development of breast cancer does not happen in one step, it is a multifaceted disease that takes time to develop. Some stages are much less dangerous than others, so doctors need a way to describe which stage a tumor is in at a certain time. In order to do this, doctors use a tumor, node, and metastasis (TNM) staging system (4). The following stages have been created to show tumor progression: normal epithelial cells, hyperplasia, hyperplasia with atypia, and carcinoma in situ invasive cancer. Typically, breast cancer begins in the milk-producing ducts of the breast. Often considered stage 0, ductal carcinoma in situ is a type of non-invasive breast cancer. It is characterized by the development of abnormal epithelial cells lining the milk ducts of the breast. While this stage is not considered life-threatening, treatment is still recommended to prevent further growth and spreading. In stage 1 breast cancer, the tumor is less than two cm in size, but the cancer is now considered invasive. The tumor has not spread to other parts of the

body at this point and successful treatment is still very likely. Even though stage 1 is not considered life threatening, treatment usually immediately follows a diagnosis. Stage 1 breast cancer is highly treatable, with radiation and/or surgery being the two most common treatment plans (20). In addition to radiation and surgery, a person with stage 1 breast cancer could also consider hormone therapy. Hormone therapy for breast cancer can be very effective if the particular type of breast cancer is sensitive to hormones. It works by blocking a hormone from attaching to its receptor or by blocking hormone production, thus blocking the function of the hormone (11). Stage 2 breast cancer is characterized by increased tumor growth, but the tumor is still contained in the breasts or nearby lymph nodes. Stage 2 is further divided in two groups: A and B. Stage 2A breast cancer can be applied to three different scenarios. The first scenario includes no tumor present but cancer cells have spread to the lymph nodes. The second scenario is that the tumor is less than two cm in size and has spread to the lymph nodes. Finally, the tumor has to be two to five cm in size and have not spread to the lymph nodes to be included in stage 2A. Stage 2B breast cancer is defined by 2 different situations. If the tumor is 2-5 cm in size and has spread to less than four nearby lymph nodes, then it is in stage 2B. Additionally, if the tumor grows to be larger than five cm, but has not spread to any lymph nodes, then it is in stage 2B (22)

Stage 3 breast cancer means the cancer has spread beyond the primary tumor site and may have invaded nearby tissues, but has not spread to distant organs. Stage 3 breast cancer is divided in to 3 groups: 3A, 3B, and 3C. The difference between stages is determined by the size of the tumor and whether the cancer has spread to the lymph nodes and surrounding tissue. Although stage 3 breast cancer is considered advanced,

there is a growing number of treatment options available. Stage 3 treatment options include mastectomy, radiation, hormone therapy, and chemotherapy. A combination of 2 or more treatments is typically the most effective for this stage (21). Stage 4 breast cancer means that the cancer has spread to other parts of the body, such as the brain, bones, lungs, and liver. Stage 4 breast cancer is considered incurable, but new treatments have prolonged the lives of stage 4 breast cancer patients (19). During this stage of cancer, patients can be exposed to experimental treatments if they are willing.

Tumor Formation:

Reactive oxygen species (ROS) consist of radical and non-radical oxygen species that have partially reduced oxygen molecules. These ROS are important regulators of homeostasis in moderate concentrations, but can become a major health problem if present in high concentration (7). Much of cellular ROS production is a by-product of oxidative phosphorylation occurring in the mitochondria but ROS can also be produced during an immune response to foreign substances. Oxidative stress can occur when there is an imbalance between the concentration of ROS and an antioxidant response. Oxidative stress caused by ROS has been shown to damage nucleic acids, proteins, and lipids (7). Additionally, ROS have the ability to promote disease formation (such as cancer) through signaling pathways. For example, ROS have been shown to promote cell proliferation, survival, and differentiation by activating the PI3K pathway. However, ROS have also been shown to induce apoptosis and cellular aging in certain cell lines. Therefore, how a cell reacts to ROS depends on its molecular background and surrounding environment. At high concentration, ROS can cause apoptosis in cancer cells

due to cellular damage. However, ROS plays a much larger role in signal transduction in tumor formation, progression, and survival.

Many cancers form solid masses of tissue called tumors after a period of time. The word tumor has a Latin origin that translates to “swelling” in English but not all swellings can be considered tumors. Wallace H. Clark, a celebrated pathologist, defined tumors as meeting the following criteria; they consist of cells experiencing temporally unrestricted growth, and the ability to grow in at least three different tissue compartments. Included in these compartments are the original site, the mesenchyme of the primary site, and the mesenchyme of a distant site. This definition of a tumor encompasses the benign beginning, invasive nature, and potential to spread that tumors have. Tumors consist of two separate but interdependent compartments. The parenchyma consists of neoplastic cells that exhibit uncontrolled growth. The other compartment, the stroma, consists of the connective tissue, blood vessels, and other components that keep the tumor alive and allow it to grow. All tumors that grow beyond one to two mm in size require stroma for survival. Tumor stroma is formed from circulating components of the blood. These elements are able to enter tissues in areas that consist of high levels of vascular endothelial growth factor (VEGF-A), which promotes high vascular permeability. The angiogenic growth factor, VEGF-A, is the most studied and well known in a family of proteins that also consists of vascular endothelial growth factors B, C, D, and E. Additionally, VEGF-A acts as a cytokine that serves many functions and is produced by both animals and humans. Among its many other activities, VEGF-A plays a large part in the immune response. Its vascular permeability inducing activity facilitates elements from the blood to enter wound sites and aid in the healing process. However,

VEGF-A plays a monumental role in the development of cancer. In addition to its role in stroma formation, VEGF-A contributes greatly to angiogenesis (the formation of new blood vessels) by binding to and activating vascular endothelial growth factor receptors (VEGFR). Upon VEGF binding to VEGFR, a tyrosine kinase cascade is activated, signaling new blood vessels to form. These new blood vessels are not the same as healthy, normal blood vessels. They are often leaky, disorganized, and have irregular blood flow. Further, VEGF-A plays a critical role in embryonic development and wound-healing in adults. However, VEGF-A is overexpressed in cancerous cells causing a large increase in angiogenesis, which is a hallmark of cancer.

The heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1), is the primary activator of VEGF-A (17). Under normoxic conditions, its hypoxia-regulated HIF-1α subunit is proteolytically degraded very quickly, with a physiological half-life of about five minutes. In the presence of oxygen, divalent iron ions bind to the proline or asparagine residues of HIF-1α and promote is rapid proteolysis. However, HIF-1α is stable and present in high levels in hypoxic environments, where it heterodimerizes with the constitutively expressed HIF-1β subunit, and activates the herterodimeric transcription factor complex known as HIF-1. Because of the irregularities of tumor vasculature, the tumor microenvironment is typically hypoxic, which leads to an increase in oncogene expression of HIF-1α. When activated by HIF-1, VEGF-A promotes tumor angiogenesis. Tumors are able to grow in size with the development of new blood vessels that feed the tumor. Thus, tumors are able to take advantage of the hypoxic environment that their vasculature creates in order to grow and survive.

The transcription factor HIF-1 also has a prominent role in the regulation of oxidative phosphorylation and ROS production. When ROS are present in high levels, HIF-1 can suppress ROS formation by inhibiting the mitochondrial tricarboxylic acid cycle. Low concentrations of ROS can trigger HIF-1 to increase ROS formation through NADPH oxidase (7). In healthy cells, ROS formation is a byproduct of oxidative phosphorylation and is present in minimal concentrations due to antioxidant systems. When these systems fail and ROS concentrations rise, oxidative damage can follow. Additionally, ROS have been shown to stabilize HIF-1α, which leads to HIF-1α accumulation and subsequent HIF-1 activation. Therefore, it is possible that ROS play a major role in tumor angiogenesis by stabilizing HIF-1α.

Metastasis:

One of the major issues with cancer is its ability to spread to other parts of the body. In a process called metastasis, cancer can spread locally into nearby tissues or to distant parts of the body through the circulatory or lymphatic systems. Nearly all types of cancer have the potential to become metastatic (6). Individual and environmental factors determine whether or not the cancer will become metastatic. There are three common ways metastasis can occur: by growing directly into tissue surrounding the tumor, traveling through the bloodstream, or by traveling through the lymphatic system. Metastasis is composed of several steps that leads to the spread of cancer throughout the body in a process known as the metastatic cascade. Metastasis begins when cancer cells detach from the primary tumor site in a process called invasion (25). The route of the detached cancer cell depends on the location of the primary tumor. For example, a breast

cancer tumor undergoing metastasis has the potential to invade the lungs, brain, lymph nodes, or liver. Many factors can contribute to cell detachment, including tumor growth, necrosis, and enzyme activity. Cancer cells can undergo an epithelial-mesenchymal transition (EMT) to gain migratory abilities. The process of EMT can be initiated by signaling from growth factors such as TGF-β or from cell proliferation proteins such as Wnt (25). Initiating EMT increases the expression of EMT transcription factors that are involved with the downregulation of epithelial cadherins (proteins that promote cell-cell binding). Many studies have shown that ROS are major activators of EMT by acting as secondary messengers. The growth factor, TGF-β, regulates urokinase type plasminogen activator (uPA) and matrix metallopeptidase 9 (MMP-9) through an ROS mechanism (12). This results in a weaker bond between cancer cells and the tumor, promoting detachment and leading to an increase in cell-stroma interactions (25). After a cancer cell detaches from the primary tumor, it begins a perilous journey around the body.

Intravasation is the process by which cancer cells enter the circulatory system through blood or lymphatic vessels. These circulating tumor cells (CTCs) are often killed by stress or the immune system, or they can become lodged in a capillary bed where they form a new tumor. This circulation process is very inefficient, with only 0.01% of CTCs actually forming a distant tumor (8). However, it is very important to study and understand intravasation because it is a major part of the deadly metastatic cascade. The content of the tumor microenvironment, proteases, and signaling molecules all play a part in the process of intravasation. Angiogenesis is the process by which new blood vessels form, so it follows that tumors with a higher degree of angiogenesis could also experience higher levels of intravasation. As mentioned earlier, VEGF-A plays a large

part in increasing vascular permeability and blood vessel formation. Additionally, VEGF-A leads to an increase in microvessel density, which has been linked to an increase in intravasation (8). The primary activator of VEGF-A, HIF-1, is also an activator of proteases known as matrix metalloproteinases (MMPs). These zinc-dependent endopeptidases are responsible for the breakdown of the extracellular matrix (ECM), which serves as the physical barrier between tumor cells and blood and lymphatic vessels. Once cancer cells are able to enter into the circulatory system, they have the potential to invade other parts of the body. If these cells are able to establish themselves in other tissues, the result is often lethal.

Extravasation is the process by which CTCs exit the circulatory system to form secondary tumors. Once CTCs are in their new location, they go through a mesenchymal-epithelial transition (MET) to form a new tumor. Extravasation is a very dynamic process and is not as well understood as the previous steps of the metastatic cascade. However, further research should be conducted on this process because of its potential as a target for anti-metastatic drugs.

Treatment:

Breast cancer is the deadliest form of cancer among women in the world (18). It is more prevalent in developed countries, but the mortality rates are much higher in poorer countries because of fewer and inferior treatment options. The high incidence of breast cancer has made it become one of the most widely studied forms of cancer. Early detection in the form of biomarkers has served as a huge step towards curing breast cancer. Estrogen (ER) and progesterone (PR) receptors have been the most widely

studied biomarkers for early breast cancer detection and prevention. Additionally, advances in DNA technology have allowed researchers to target early breast cancer detection at the level of DNA processing. However, thousands of people still die from breast cancer every year and countless more suffer from a lower quality of life from the side effects of the toxic drugs that they have to take to survive. The most common form of treatment for metastatic breast cancer is surgery, but almost all treatment plans use a combination of therapies. Radiotherapy, chemotherapy, and immune therapy are also common treatment options. Resistance to therapies and high toxicity of primary treatment options have led researchers to look in to more natural options for breast cancer treatment. Phytochemicals, a large family of bioactive compounds found in plants, have emerged as leading candidates for the management and prevention of breast cancer (18). Several phytochemicals have been shown to induce apoptosis in breast cancer cells and prevent the growth of tumors by inhibiting angiogenesis, causing DNA damage through oxidative stress, controlling proliferation, and overtaking cell signaling pathways. In this review, breast cancer cell lines were treated with resveratrol, a non-flavonoid polyphenol.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene, $C_{14}H_{12}O_3$) is a naturally occurring polyphenol that is present in grapes, berries, peanuts, soy beans, and certain fungi (18). Resveratrol is thought to have beneficial effects on the human heart. The French consume a high amount of red wine, which is rich in saturated fat, yet they have few heart related problems. This “French Paradox” is thought to be related to the beneficial effects of resveratrol. Additionally, resveratrol has been shown to have various benefits in coronary, neurological, hepatic, and cardiovascular systems (18). It has been shown to fight inflammation, reduce reactive oxygen species, freeze the cell cycle, restore

apoptosis, and slow cancer cell growth in various cell lines. Mitochondrial Complex I releases superoxide radicals into the mitochondrial matrix, where mitochondrial manganese SOD (SOD2) converts the superoxide radicals to hydrogen peroxide. Next, hydrogen peroxide is converted to water by glutathione peroxidases (GSH). Resveratrol has been shown to be a scavenger of certain oxidants including superoxide radicals and hydrogen peroxide. However, resveratrol is only effective at scavenging low levels of ROS. Instead, resveratrol's best antioxidant properties work at the level of DNA transcription (28).

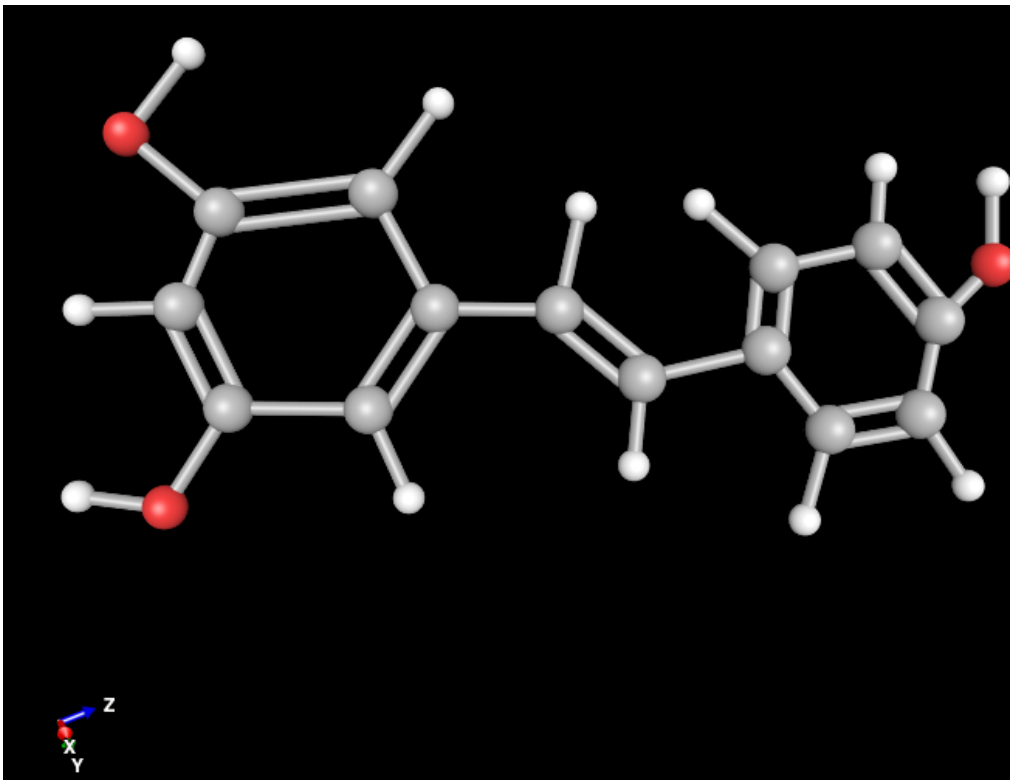


Figure 1. Resveratrol Structure. This image was created using ChemDoodle3D.

Interestingly, resveratrol has been shown to have prooxidative and antioxidative properties. Resveratrol is well-known as an antioxidant, but it can act as a prooxidant in

the presence of certain transition metals such as copper (18). As described earlier, ROS are able to damage macromolecules such as DNA when present in high concentrations. If ROS could be contained to a tumor site, then ROS generation could potentially be an avenue for cancer treatment by damaging DNA and preventing cell growth and proliferation. On the other hand, resveratrol is known to be a scavenger of ROS. It is able to stabilize free radicals by forming a resonance-stabilized peroxyl radical, thus diminishing the effects of ROS. In this way, resveratrol is preventing the accumulation of ROS, which can lead to cancer by damaging healthy DNA. Resveratrol is also involved with an increase in antioxidant enzymes, such as superoxide dismutase (SOD). The antioxidant properties of resveratrol have also been shown to have a synergistic effect when combined with certain chemotherapeutic agents. Many chemotherapeutic drugs work through a pro-oxidant mechanism, leading to a buildup of ROS that can cause toxicity in the body. In one study, Resveratrol protected against cardiotoxicity in H9c2 cardiomyocytes that were treated with doxorubicin by blocking ROS production through the upregulation of manganese superoxide dismutase (18). This review focuses on the antioxidant properties of resveratrol.

Cancer stem cells (CSCs) are cancer cells that have similar properties to normal stem cells, specifically the ability to become any cell type found in the body. Cancer stem cell theory has gained much traction over recent years. This theory suggests that there is a small subpopulation of cells within the tumor that are responsible for the persistence of the disease. Other cells can still cause problems, but the CSCs are the root of the issue according to this theory (23). Cancer stem cell theory suggests that if the CSCs are not destroyed but the signs of cancer have been eliminated, then the cancer will grow back.

Most chemotherapies and radiotherapies do not target CSCs. Thus, many new anticancer drugs are now focusing on this small subpopulation of cells to combat tumor growth. These drugs could work by re-sensitizing CSCs to chemotherapy drugs or blocking self-renewal pathways. In a number of studies, resveratrol has been shown to slow or halt the differentiation of the CSCs directly and indirectly (18).

Adjuvant therapy is treatment that is given in addition to the primary treatment (surgery, chemotherapy, etc.) Phytochemicals, such as resveratrol, have proven to be one of the most effective adjuvant treatment drugs. These compounds are able to reverse drug resistance in some tumors and increase sensitivity to drugs in others. Resveratrol has been shown to increase the antitumor activities of drugs such as doxorubicin, which belongs to the class of chemotherapy drugs known as anthracyclines. Anthracyclines inhibit topoisomerase 2, which cells need to divide and grow. Resveratrol, when combined with doxorubicin, has been able to increase the power of doxorubicin without affecting normal cells. Carbonyl reductase 1 (CBR1) catalyzes the reaction of doxorubicin to doxorubicinol, which causes cardiotoxicity and diminishes the antitumor potential of doxorubicin. If CBR1 is present in high levels, chemoresistance of tumor cells is enhanced. Resveratrol has the ability to bind to CBR1, thus re-sensitizing cancer cells to doxorubicin. A case control study performed from 1993-2003 examined the effect of resveratrol intake in breast cancer patients. There were 369 patients that were given resveratrol, and 602 patients that were not. The study showed that breast cancer risk was decreased in the patients taking resveratrol (18).

In the experiments performed by the research group, we studied the effects of resveratrol, vitamins C and E, and epigallocatechin gallate (EGCG) on several breast

cancer cell lines. This review will focus on the antioxidant therapeutic capabilities of resveratrol.

Natural Product Studies:

The research team began working in the spring of 2019 and conducted research under the direction of Dr. Zhou and Dr. Nagle until spring 2020. We began with literature research into breast cancer to gain a better understanding of the mechanisms underlying this complicated disease. After deciding to pursue research regarding the effects of certain compounds on breast cancer cell lines, Dr. Zhou advised us to find a less studied compound to increase the originality. While it is difficult to be completely original in this field, background research increased our knowledge of the field and helped us select a topic. Literature review showed us that resveratrol has been studied in various ways over the past decade. For example, resveratrol has been shown to have positive effects on the heart, kidneys, and cancer patients (18). After selecting a compound to study, a plan was created to study the effects of resveratrol, vitamins C and E, and EGCG on various breast cancer cell lines. The chemotherapeutic experiment consisted of literature research, cell culture procedures, and a viability assay. Experiments were concluded in November 2019 and the data analysis and writing process began soon after. Our general hypothesis stated that these compounds (resveratrol, vitamins C and E, EGCG) would cause damage to certain breast cancer cell lines or inhibit their growth.

Materials and Methods:

Sulforhodamine B (SRB) Viability Assay:

The Sulforhodamine B (SRB) assay is a widely used method to determine cytotoxicity in cell-based studies in a relatively inexpensive and efficient manner (16). Sulforhodamine B is a fluorescent dye that binds to proteins under acidic conditions and can be used to determine cell inhibition/apoptosis. This assay was used after the cells were seeded in a 96-well plate with a cell density of 30,000 cells/well and 100 mL/well of media. First, cells were trypsonized with 1.0 mL Trypsin. This step was performed to prevent cell-cell attachment. Next, the cells were washed with 10 mL of 10% fetal bovine serum (FBS) media. Then, the plates were cultured at 37° C for 24 hours. After 24 hours, test compounds diluted in serum-free RPMI 1640 media with L-glutamine (2 mM), penicillin, and streptomycin were added (100 mL/well.) The compounds were prepared as stock solutions in DMSO with a final concentration of 0.25% (v/v). After 48 hours, 100 μL of media was removed from each well. 100 μL of 20 % trichloroacetic acid (TCA) in 1x PBS solution were then added to each well to begin the staining process. The wells were then incubated at 4° C for 1 hour. Next, the wells were washed with tap water and then stained with 100 μL of 0.4% SRB (w/v, 1% acetic acid). The plates were then washed 4 times with 1% acetic acid and allowed to dry. Then, Tris Base (100 μL of 10 mM) was added to each well at a volume of 200 mL/well. The wells were shaken for 10 minutes and were then ready to be analyzed using the SpectaFluor plate and Magellan software. The absorbance range was set to 496-620 nm. The Magellan software was able to calculate the inhibition value by analyzing the total protein mass density in each well.

Cell viability data is presented as percent control using the formula: % Control = $(OD_{compound}/OD_{control})$ x 100.

	MCF-7			BOM			MD-MBA-231			MCF-7-BOM		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Media Control 100 µL (negative control)											
B	Media Control 100 µL (negative control)											
C	1 µM Resveratrol											
D	10 µM Resveratrol											
E	30 µM Resveratrol											
F	100 µM Resveratrol											
G	300 µM Resveratrol											
H	10 µM Cycloheximide (positive control)											

Figure 2. Resveratrol Concentrations in *96-Well Plate Layout*

Natural Product Dilution Plate

The chemotherapeutic agents were purchased from Sigma Aldrich or The Association of Clinical Research Organizations (ACRO). For this experiment, we set the stock solution concentration at a range from 1 µM to 300 µM for each compound. DMSO was added to each chemotherapeutic agent to acquire the desired stock concentration for the experiment. Fetal bovine serum (FBS) was used as media to dilute the stock solutions to the highest tested concentration. A 1:10 serial dilution was performed in a stepwise manner as can be seen in the table below.

Resveratrol solution	Stock Dilution	Media Volume
300 μM	50.4 μL of 50 μM	4.15 mL
100 μM	1.1 mL of 300 μM	2.2 mL
30 μM	330 μL of 300 μM	2.97 mL
10 μM	330 μL of 100 μM	2970 mL
1 μM	300 μL of 10 μM	2700 mL

Figure 3. Serial Dilution of Resveratrol

Cell Lines:

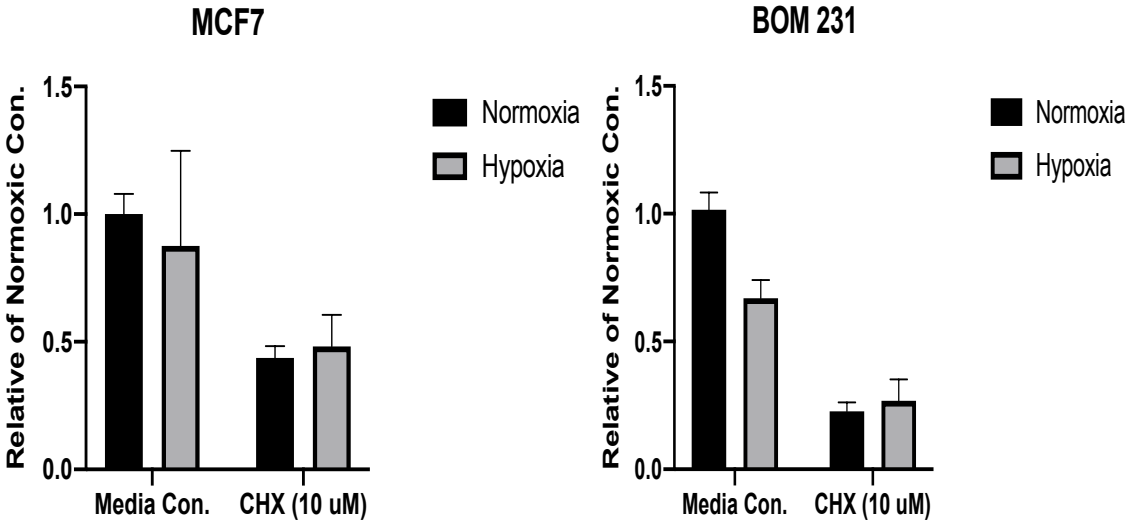
Four cancer cell lines were used in this experiment: MCF-7, BOM 231, MDA-MB-231, and MCF-7-BOM. The MCF-7 is a human breast cancer cell line with estrogen, progesterone, and glucocorticoid receptors (5). It was derived from a 69-year old Caucasian woman with adenocarcinoma in 1970 by Dr. Herbert Soule. MCF-7 is very useful for in vitro studies because of its ability to process estrogen as an in vivo breast cancer cell would. The MDA-MB-231 cell line is a triple-negative breast cancer cell line that was derived from a 51-year-old Caucasian female with invasive ductal carcinoma in 1970 (26). This cell line is highly aggressive, invasive and lacks estrogen and progesterone receptors, and does not overexpress HER2. Additionally, this cell line has a mutated p53 (tumor suppressor) gene. MDA-MB-231 cells are invasive when implanted and metastasize to lymph nodes in vivo. This cell line also commonly metastasizes to the lung and bone to form secondary tumors. The BOM 231 is a cell line from metastatic bone cancer that was derived from the secondary tumors of MDA-MB-231. The BOM 231 cell line was generated in Dr. J. Massagúe's lab at Memorial Sloan Kettering Cancer Center, New York City, New York. The specific BOM 231 cell line that we used was acquired from Dr. Konosuke Watabe at Wake Forest University. The MCF-7 BOM is also a metastatic bone cancer cell line that was derived from secondary tumors formed by

the breast cancer cell line MCF-7. The MCF-7-BOM was also generated in Dr. J. Massagúe's lab at Memorial Sloan Kettering Cancer Center, New York City, New York. We acquired MCF-7-BOM cells from Dr. Konosuke Watabe at Wake Forest University.

All cell lines were maintained in RPMI 1640 media with L-glutamine (2 mM) (Mediatech), supplemented with fetal bovine serum [FBS, 10% (v/v), Hyclone], penicillin (50 units/mL), and streptomycin (50 μg/mL) (Lonza) in an atmosphere containing 5% CO_2 and 95% air at 37° C.

Results:

Figure 4 was created to compare results under normoxic and hypoxic conditions, as well as treatment with cycloheximide, which was the positive control in this experiment. In all cell lines except for MCF-7-BOM, cell viability and proliferation from the media control was reduced under hypoxic conditions compared to normoxic conditions. Hypoxic conditions result when there is an absence of oxygen in the physiological system, which can lead to stress on a cell because they have to switch to anaerobic respiration to produce energy. Anaerobic respiration is not as efficient as aerobic respiration, which could lead to cell stress and eventually apoptosis. The graphs from Figure 4 were created relative to the normoxic control, which was set at 100% cell survival.



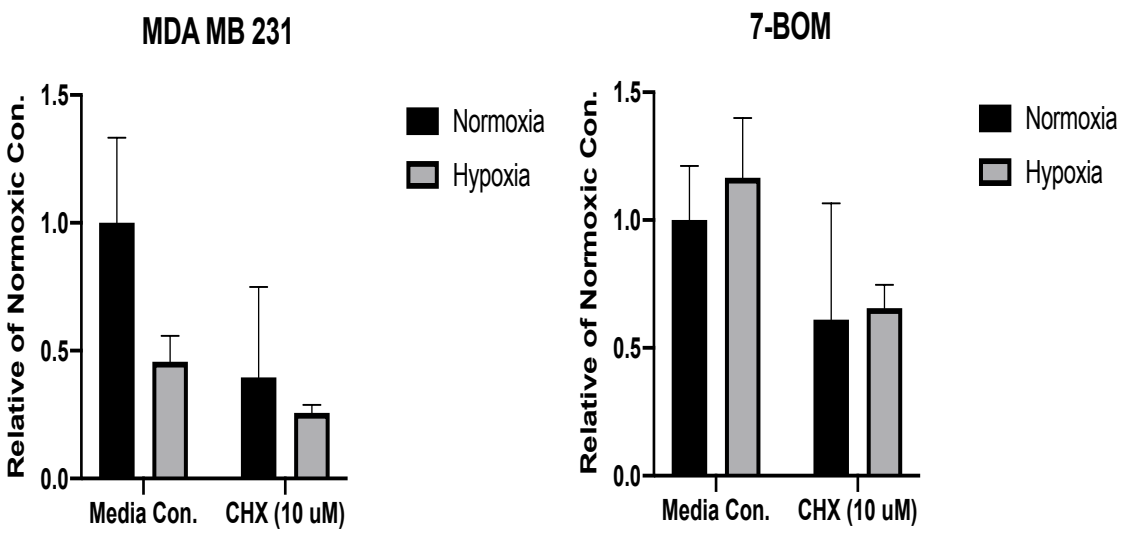
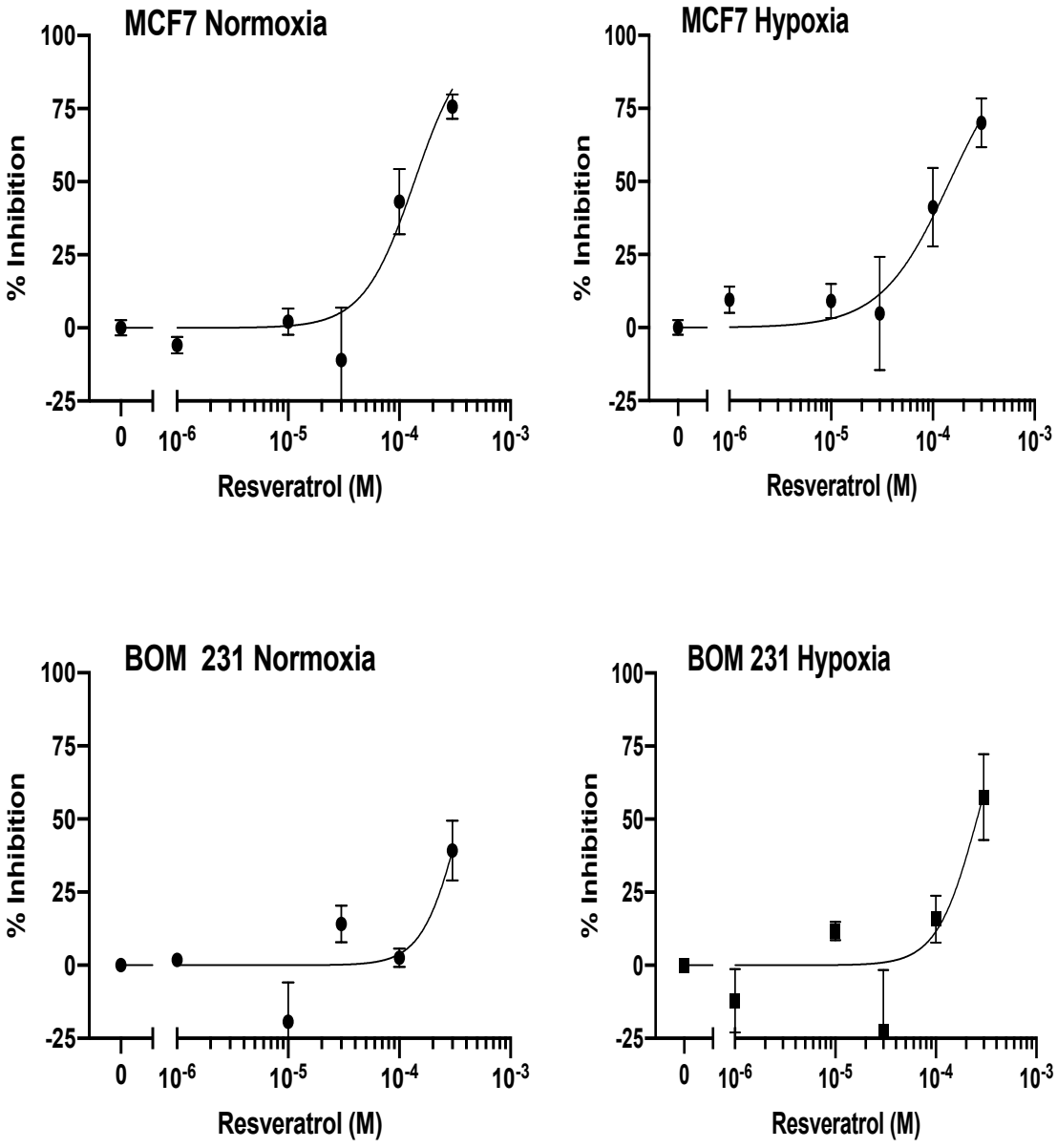


Figure 4. Normoxia, hypoxia, and cycloheximide (CHX) compared to normoxic media control.

SRB Viability Assay

The results of the Sulforhodamine B viability assay reflect the percent inhibition values of resveratrol tested on the cell lines MCF-7, BOM-231, MDA-MB-231, and MCF-7-BOM. The goal of this assay was to acquire an IC_{50} value, which is the drug concentration where cell growth is inhibited by 50%. This means that a lower IC_{50} value indicates a more effective drug. However, while each cell line and condition showed inhibition, only MCF-7 normoxia and hypoxia, and BOM 231 hypoxia were inhibited enough for an IC_{50} value to be calculated within the highest concentration of resveratrol tested. Prism Graphpad 8 was able to calculate an IC_{50} value for all cell lines, but only three of the cell lines fell within the range of concentrations tested. The other cell lines had an IC_{50} value over 300 μM. The following charts reflect percent inhibition of

resveratrol on each cell line that were incubated under either of two conditions: normoxia or hypoxia. These graphs were created using Prism GraphPad 8.



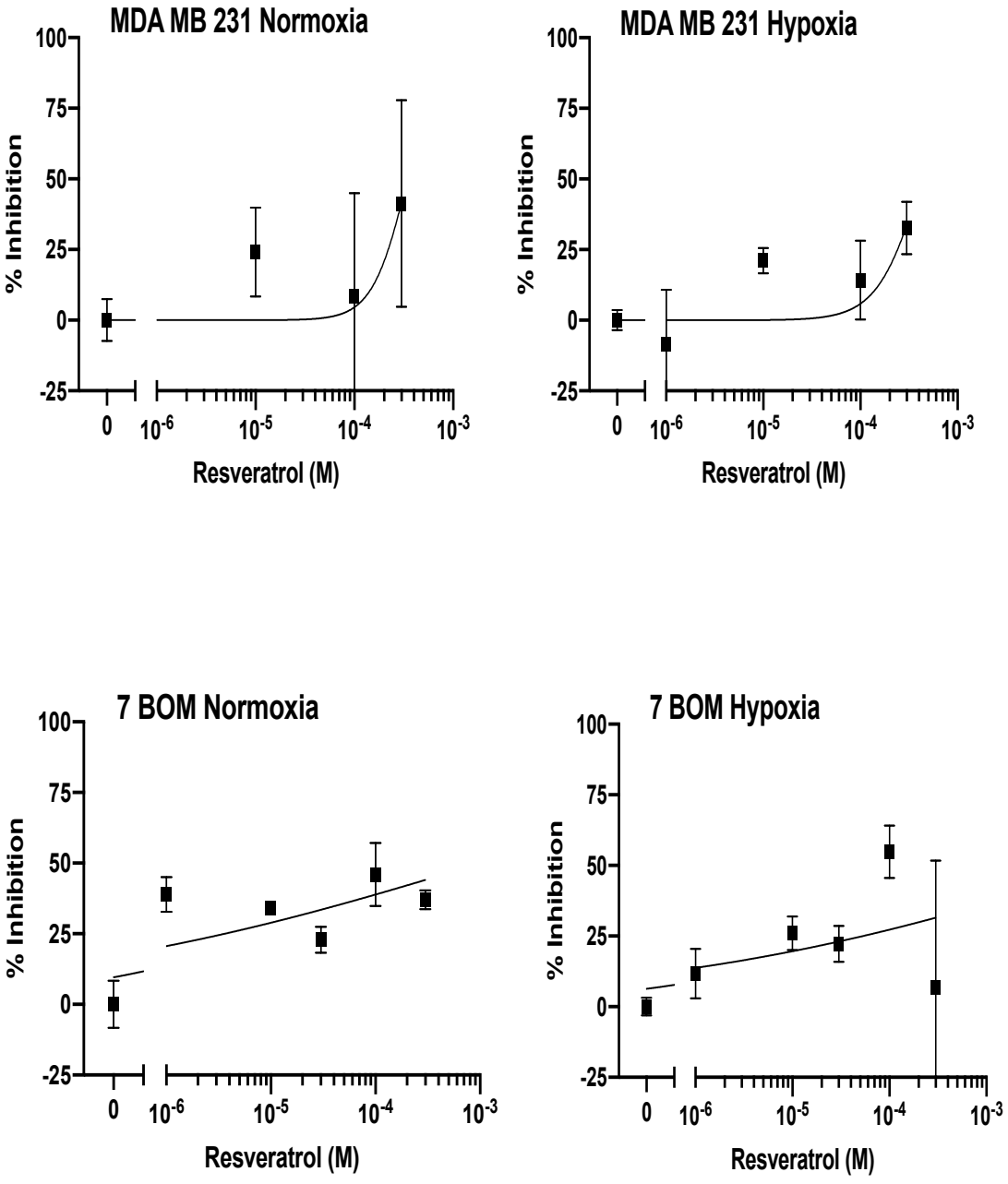


Figure 5. Percent Tumor Cell Inhibition Data

Discussion

From the SRB assay, resveratrol was shown to be capable of inhibiting cell proliferation in a concentration and cell line dependent manner. The MCF-7 cell line proved to be the most susceptible to resveratrol treatment in both normoxic and hypoxic conditions at maximum resveratrol concentration. This cell line also showed an increase in % inhibition when the concentration of resveratrol was increased in both conditions. The MCF-7 cell line could be the most susceptible because it is an ER+ cell line and resveratrol has shown to have phytoestrogen properties. The resveratrol IC_{50} value was the smallest in the MCF-7 normoxia cell line, indicating that this cell line was the most susceptible to resveratrol treatment. Resveratrol was also effective at inhibiting the MCF-7 hypoxia cell line, which produced the second smallest IC_{50} value. The BOM 231 hypoxia was the only other cell line that produced an IC_{50} value that was lower than the maximum resveratrol concentration tested. All IC_{50} values were calculated and fall into a 95% confidence interval (CI) as shown in Figure 6. These values were calculated using prism GraphPad 8. The cell lines that produced IC_{50} values less than the maximum concentration of resveratrol (300 μM) tested are highlighted in green, while those that had an IC_{50} value greater than 300 μM are highlighted in red.

Cell line	IC_{50} (M)	95% CI (M)
MCF-7 Normoxia	0.0001364	0.000095995 to 0.001
MCF-7 Hypoxia	0.0001467	.00009323 to 0.001
BOM 231 Normoxia	0.0003572	0.0002736 to 0.001304
BOM 231 Hypoxia	0.0002563	0.0001634 to 0.0005

MDA-MB-231 Normoxia	0.0003442	0.0001206 to 0.0009
MDA-MB-231 Hypoxia	0.0004277	0.0002177 to 0.001
MCF-7 BOM Normoxia	0.001024	0.00009873 to 2.652
MCF-7 BOM Hypoxia	0.01951	0.0001263 to 3.12

Figure 6. IC_{50} Values for all Cell Lines

This study was successful in showing that resveratrol is capable of slowing and/or inhibiting cell growth in certain breast cancer cell lines. However, the exact mechanism of how this occurs is an area that requires further study. One potential area of study could be examing resveratrol antioxidant effects in certain cancer cell processes. For example, as mentioned earlier, the PI3K pathway relies on ROS to promote cellular proliferation. Resveratrol is a known scavenger of ROS and could potentially inhibit this pathway. Additonally, the role of resveratrol as a pro-oxidant in breast cancer treatment could be an interesting avenue for further research. A major hurdle to this research would be to find a way to limit ROS generation to the tumor site, which could lead to cancer cell apoptosis.

Conclusions

In conclusion, resveratrol was tested on several different cancer cell lines to determine its efficacy as a chemotherapeutic compound. Resveratrol proved to be capable of inhibiting cell growth and proliferation in cell lines and the results were much more profound in specific cell lines. While this study produced results that could lead to resveratrol gaining a bigger role as a chemotherapeutic compound, much more research needs to be conducted to understand the exact mechanism by which this compound works. Additionally, these experiments should be repeated in order to ensure that the data provided from this report is accurate. The hypothesis that resveratrol would show cell line-dependent inhibition is supported by the percent inhibition data produced from the SRB viability assay. The MCF-7 cell line was the most sensitive to resveratrol, possibly because of the presence of estrogen receptors in that cell line. However, more experiments should be conducted using higher concentrations of resveratrol to acquire precise IC_{50} values for all cell lines.

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