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The Effects of Largazole, a Histone Deacetylase Inhibitor, on Breast Cancer Cell Viability and Metastasis

Hannah McCowan

THE EFFECTS OF LARGAZOLE, A HISTONE DEACETYLASE INHIBITOR, ON BREAST CANCER CELL VIABILITY AND METASTASIS

By
Hannah Kyle McCowan

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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Approved by

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Advisor: Dr. Yu-Dong Zhou

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Co-Advisor: Dr. Dale George Nagle

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Reader: Dr. Hoang V. Le

For everyone whose life has been touched by cancer

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The largazole graphs in the Results and Discussions section were created by Dr. Yu-Dong Zhou and were used for this thesis and the corresponding thesis by Hannah Carson.

ABSTRACT

Histone deacetylase enzymes modify epigenetic characteristics of a genome by removing acetyl groups from histone proteins in chromatin. Histone deacetylase inhibitors work by stopping this activity which can have various results in a cell including apoptosis, cell cycle arrest, differentiation, and migration. The purpose of these experiments was to see how largazole, a histone deacetylase inhibitor, affected cell viability for breast cancer and associated metastatic cell lines in both normoxic and hypoxic conditions. The experiment was completed by setting up two 96-well plates with varying concentrations of largazole and conducting a sulforhodamine viability assay. The specific cell lines used in the experiment were MCF-7, MDA-MB-231, MCF-7 BoM, and MDA-MB-231 BoM. Largazole showed promise in treating triple-negative breast cancer, triple-negative breast cancer metastasis, and estrogen receptor-positive breast cancers.

PREFACE

After my freshman year at the University of Mississippi, I had no doubt what the topic of my Honors College thesis would be: cancer research. Throughout my life, cancer had shaped and touched the lives of those around me until it eventually touched my life as well. Cancer is a non-discriminatory disease which affects the lives of millions of people around the world. This research project gave me insight on the complexity of cancer and the time required for proper and efficient research. I am incredibly thankful for the opportunity to study such an important disease which has affected so many people in my life.

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LIST OF ABBREVIATIONS

DNA – Deoxyribonucleic Acid

NCI – National Cancer Institute

HAT(s) – Histone Acetylase(s)

HDAC(s) – Histone Deacetylase(s)

HDACI(s) – Histone Deacetylase Inhibitor(s)

BRCA1 – Breast Cancer Gene 1

BRCA2 – Breast Cancer Gene 2

ER – Estrogen Receptor

PR – Progesterone Receptor

HER2 – Human Epidermal Growth Receptor 2

HIF – Hypoxia-Inducible Factor

ATCC – American Type Culture Collection

SRB – Sulforhodamine B

FCS – Fetal Calf Serum

PBS – Phosphate Buffer Saline

DMSO – Dimethyl Sulfoxide

TCA – Trichloroacetic Acid

1 Introduction

1.1 Cancer Introduction & Breast Cancer

Breast cancer is a disease which affects both women and men throughout the world. In 2020, the American Cancer Society estimates 276,480 women and 2,620 men will be diagnosed with invasive breast cancer while 42,690 people will die from breast cancer in the United States (American Cancer Society, 2020). Breast cancer is the second most common cancer in women in the United States and is the second most common cancer in both genders worldwide ("Breast Cancer Statistics and Resources"). Given these statistics, the development of treatments and potential cures for breast cancer should be a primary focus for cancer research.

Although the meaning of the word "cancer" might seem simple, it refers to over one hundred disease types which can affect nearly any tissue in the body. Cancer, in short, is a disease caused by an increase in cell proliferation and a decrease in cell death. The actions of a cell are guided by a specific sequence of DNA known as the cellular genome. When a cell divides and replicates, the genome template must be precisely copied and passed on to ensure proper cellular functioning. However, because the human body has an estimated 30 trillion cells, errors in DNA replication are bound to occur resulting in cancerous cells. Normally, the body is able to eliminate such dangerous cells by apoptosis (programmed cell death), immune destruction, and many other mechanisms. Cancer cells are now known to have certain hallmark abilities which allow them to evade normal destruction: resisting cell death, sustaining proliferative signals, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, and

inducing angiogenesis (Hanahan and Weinberg, 2011). Although anyone has a chance to develop cancer, some people are more prone than others.

Breast cancer has both modifiable and non-modifiable factors which can affect the potential for developing the disease (Kluttig and Schmidt-Pokrzywniak, 2009). Examples of modifiable risk factors include diet, alcohol consumption, smoking, physical activity level, and weight (Kluttig and Schmidt-Pokrzywniak, 2009). Currently, no consistent or strong associations have been made between breast cancer occurrence and diet, smoking, or physical activity level (Kluttig and Schmidt-Pokrzywniak, 2009). Despite some inconclusive results, smoking has been shown to increase the risk of breast cancer in subsets of women with specific genetic mutations (Kluttig and Schmidt-Pokrzywniak, 2009). Alcohol consumption has shown to be correlated with a greater risk of developing breast cancer; both pre- and post-menopausal women have shown a 7% increased risk for each additional 10 grams of alcohol consumed per day (Kluttig and Schmidt-Pokrzywniak, 2009). Weight had contrasting effects on women depending on if they were pre- or post-menopausal (Kluttig and Schmidt-Pokrzywniak, 2009). Pre-menopausal women with a larger BMI had decreased risks of breast cancer while post-menopausal women had increased risks of breast cancer (Kluttig and Schmidt-Pokrzywniak, 2009).

Some of the non-modifiable risk factors of breast cancer include age, race, history of breast cancer, breast density, and genetic factors. Generally, as people age, they become more prone to developing all types of cancer. For breast cancer, both incidence and mortality increase with increasing age (Kluttig and Schmidt-Pokrzywniak, 2009). The incidence of breast cancer also varies greatly by country; the United States and

Northern Europe had the highest standardized rates with 50-100 per 100,000, and Asia had the lowest incident rates with 10-30 per 100,000 (Kluttig and Schmidt-Pokrzywniak, 2009). For women under fifty, breast cancer incidence rates for Caucasians and African Americans were the same (Kluttig and Schmidt-Pokrzywniak, 2009). For women older than fifty, Caucasians had a higher incidence rate (351.9 per 100,000) compared to African Americans (292.2 per 100,000), but African Americans had greater breast cancer mortality rates (Kluttig and Schmidt-Pokrzywniak, 2009). Both high breast tissue density and a family history of breast cancer have been found to be associated with an increased risk for breast cancer (Kluttig and Schmidt-Pokrzywniak, 2009). Many genetic factors can also contribute to the likelihood of developing breast cancer. When mutated, BRCA1 and BRCA2–two well-known susceptibility-associated genes–can result in a 65% and 45% respective cumulative risk for a mutant carrier by age 70 (Kluttig and Schmidt-Pokrzywniak, 2009).

Breast cancer is considered a highly heterogenous disease, meaning cases can differ greatly from one another; some cancers will have a good prognosis while others can be aggressive and malignant (Tao, et al., 2015). The classification of breast cancer tumors is influenced by many factors including morphological characteristics, histological grade, and specific receptor expression (Tao, et al., 2015). The morphological types of breast cancer range from infiltrating ductal or lobular carcinoma to tubular carcinomas and more (Tao, et al., 2015). The histological grade of breast cancers can vary especially based on time of breast cancer discovery. Smaller, tubular carcinomas are associated with earlier stages while infiltrating ductal carcinomas have a higher histological grade and are often associated with a larger size and metastasis (Tao,

et al., 2015). Breast tumors have been classified into five subgroups depending on the expression of the HER2 (Human Epidermal Growth Factor Receptor 2) oncogene and two types of receptors: estrogen receptors (ER) and progesterone receptors (PR) (Tao, et al., 2015). Estrogen receptor-positive tumors are more common than ER-negative tumors, and ER-positive tumors are generally smaller and lower in grade (Tao, et al., 2015). In triple negative breast cancer (TNBC), cancer cells do not express ER, PR, or HER2 receptors. Triple-negative breast cancer makes up around 15% of breast cancer cases and is generally aggressive and associated with a poor prognosis (Tao, et al., 2015). An individual's specific breast cancer type should be identified to effectively treat the disease and prevent tumor progression.

Tumors can become significantly more difficult to treat due to metastasis. More than 90% of cancer-related deaths are associated with metastasis which significantly decreases the effectiveness of conventional cancer treatments such as surgery, chemotherapy, and radiation (Rankin and Giaccia, 2016). Metastasis occurs when cancerous cells break free from a primary tumor and travel through the circulatory and lymphatic systems to establish a secondary tumor at a different site. Metastatic tumors require different treatment as they behave more like the tumor of origin rather than a tumor of the tissue metastasized (Chiang and Massague, 2008). Many tumors preferentially metastasize to certain organs; for example, breast cancer often metastasizes to the bones, lungs, brain, and liver (Chiang and Massague, 2008). When studying breast cancer metastasis in vitro, various cell lines can be used to model different situations. For example, the MCF-7 cell line is ER-positive while the MDA-MB-231 cell line is representative of TNBC (Neve, et al., 2006). Metastatic lines can be derived by having

cells mimic transendothelial migration and grow in the presence of bone marrow stromal cells to produce BoM (bone metastatic) cell lines (Arrigoni, et al., 2016).

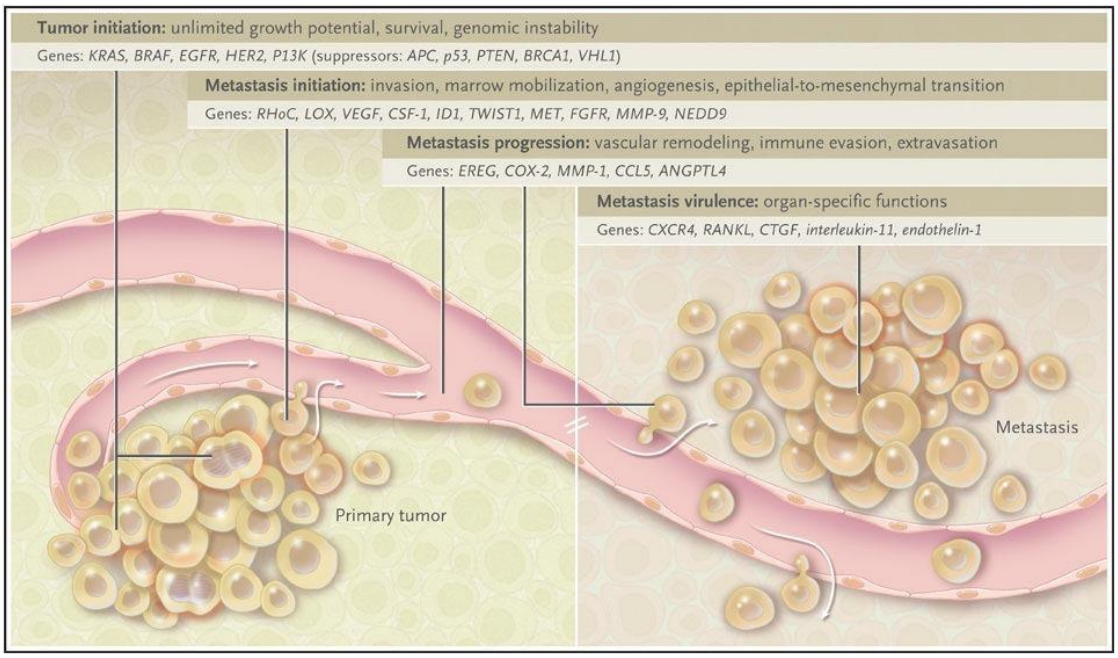

Figure 1: Tumor initiation and metastasis.

The microenvironment of a tumor also plays a role in breast tumor development and treatment. Tumors often have inadequate blood vessel vascularization which can result in hypoxia (lack of oxygen) in both primary and metastatic tumors (Knowles and Harris, 2001). Under normoxic (normal oxygen) conditions, oxygen prevents the accumulation and activation of hypoxia-inducible transcription factors HIF-1 and HIF-2 in the cell (Rankin and Giaccia, 2016). When a cell experiences hypoxia, however, HIF-1 and HIF-2 allow a cell to adapt to the low-oxygen environment by expressing genes which alter metabolism, apoptosis, cell proliferation, and more (Rankin and Giaccia, 2016). Hypoxia and HIF activation have been clinically associated with metastasis promotion, poor survival, and tumor resistance to chemotherapy and radiation (Rankin and Giaccia, 2016).

Currently, different treatments are available for breast cancer, and an individual's course of treatment depends on several factors. For nonmetastatic breast cancer, the goals of treatment are to prevent reoccurrence and eradicate the tumor (Waks and Winer, 2019). For people presenting with highly metastatic cancers, the goal is to prologue life while controlling symptoms (Waks and Winer, 2019). Breast cancer can be treated in many ways, including chemotherapy, radiation, surgery, and hormone therapy.

1.2 Histone Deacetylase Inhibitors (HDACIs)

Although a single cell holds an entire cell's genome, not every gene within the genome is expressed. The regularity of transcription for a gene varies greatly; some genes are transcribed constantly while others remain silenced. These differences in gene expression can result from epigenetic changes, or modifications to expressional patterns that do not alter the DNA sequence of a gene. Inside the nucleus of a cell, DNA is organized into chromatin which is made of repeating units called nucleosomes. A nucleosome contains 145-147 DNA base pairs wrapped around globular histone proteins made of H2A, H2B, H3, and H4 subunits connected by H1 linker histones (Biel, et al., 2005). The amino termini of histone proteins are flexible, conserved regions on the surface of the histone (Biel, et al., 2005). The availability of the amino termini of histones make post-translational modifications possible, such as acetylation by histone acetyltransferases (HATs) at particular lysine residues or deacetylation by histone deacetylases (HDACs) (Biel, et al., 2005). Histone acetylation is associated with transcriptional activation and is found to a higher degree in euchromatin, or actively transcribed chromatin, compared to heterochromatin, or condensed and transcription-inaccessible chromatin (Biel, et al., 2005).

Almost forty years ago, the addition of butyric acid to cells in culture resulted in increased histone acetylation via HDAC inhibition (Halsall and Turner, 2016). The ability to change the amount and frequency of gene acetylation could result in an alternation of cell phenotype without changing genotype. This concept sparked the interest of cancer drug researchers and eventually led to the use of histone deacetylase inhibitors (HDACIs) in cancer treatment. The HDACIs block the activity of HDACs in the cell which generally results in increased acetylation of histones and other protein targets (Ververis, et al., 2013). With HDACs inhibited, HATs are able to acetylate DNA segments allowing for transcriptional activation of many genes. The effects of HDACIs on a cell can vary widely due to targeting both histones and other intracellular proteins, and genes can actually be either up or down regulated in response (Ververis, et al., 2013). HDACIs can result in varied cell responses such as apoptosis, cell cycle arrest, differentiation, and migration (Ververis, et al., 2013).

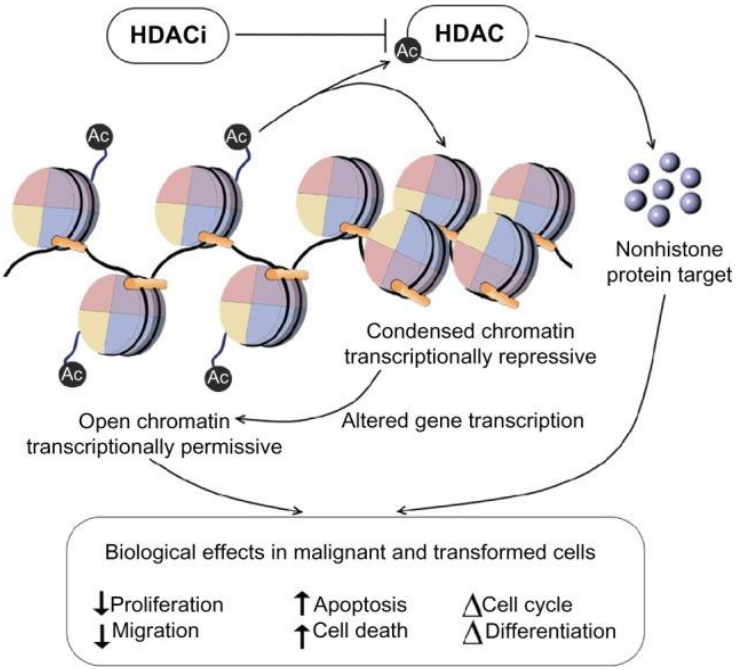

Figure 2: HDAC inhibitors promote the acetylation of histones and nonhistone proteins by inhibiting the activity of HDAC enzymes.

When HDACIs are introduced to a noncancerous cell, the cell needs to be able to adjust to the hyperacetylation without dying. Eukaryotic cells tolerate the hyperacetylation caused by HDACIs, but the exact tolerance mechanism is unknown (Halsall, et. al., 2015). In a study, cells showed an adaptive response to HDACIs by minimizing protein hyperacetylation, increasing methylation of histones at transcription start sites, and down regulating genes encoding lysine acetyltransferase (KAT) complexes to promote cell survival (Halsall, et. al., 2015). These three responses all contribute to alleviating the hyperacetylation caused by HDACIs. HDACIs are natural products which are often produced by bacteria. Since both bacteria and eukaryotic cells can live in the same environment, eukaryotic cells need to be able to mitigate the effects of HDACIs (Halsall, et. al., 2015).

Histone deacetylases (HDACs) are important biological enzymes which can influence development and disease via transcription (Haberland, et al., 2009). The HDAC superfamily contains many enzymes categorized into four classes: Class I HDACs including HDAC 1, 2, 3, and 8; Class IIa HDACs including HDAC 4, 5, 7, and 9; Class IIb HDACs including HDAC 6 and 10; and Class IV HDACs including HDAC 11 (Haberland, et al., 2009). The experiment focused on Class I HDACs which have relatively simple structures (Haberland, et al., 2009). Class I HDACs localize to the nucleus, show enzyme activity towards histone substrates, and are expressed ubiquitously (Haberland, et al., 2009). Highly involved with cell proliferation, cell cycle regulation, and apoptosis, the deregulation of Class I HDACs has been associated with many forms of cancer including renal, colorectal, lung, pancreatic, and breast cancers (Barneda-Zahonero and Parra, 2012).

Epigenetic changes are known to be associated with cancer, and HDACs play an important role in epigenetic modifications which can alter gene transcription of oncogenes and tumor suppressor genes (Li and Seto, 2016). Currently, there are at least twenty-two HDACIs under clinical investigation, and four HDACIs have been approved to treat cancer by the FDA (Li and Seto, 2016). In preclinical trials, HDACIs have been shown to re-sensitize tumor cells to treatments by other antitumor or chemotherapeutic agents to which cells had become resistant (Li and Seto, 2016). The HDACIs have shown synergistic relationships with some DNA methyltransferases (DNMTs) and have great potential for future cancer treatments (Li and Seto, 2016).

1.3 Largazole and Experiment Introduction

Largazole is a marine natural product produced by cyanobacteria of the genus *Symploca* (Hong and Luesch, 2012). Dolastin 10, a cancer clinical trial agent, is produced by cyanobacteria with the same genus (Hong and Luesch, 2012). Luesch and Paul were able to find another agent from the same genus of bacteria with antiproliferative activity in coastal Florida, and the agent was later identified and named largazole (Hong and Luesch, 2012). In preliminary tests, largazole showed differential cytotoxicity towards cancer cells, inhibiting them at nanomolar concentrations, while inhibiting other non-cancer cells to a considerably lesser extent (Hong and Luesch, 2012).

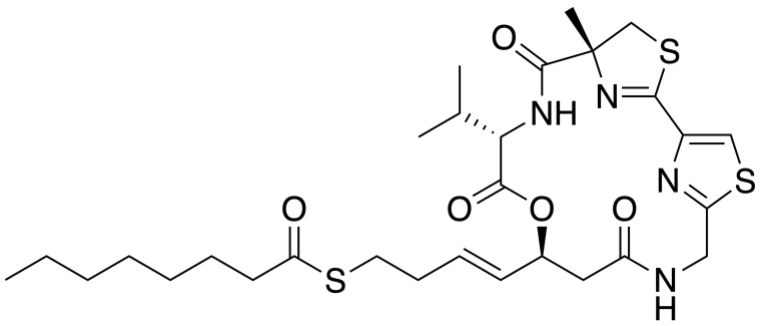

Figure 3: The chemical structure of largazole

Largazole is a cyclic molecule containing peptide bonds capable of inhibiting Class I HDACs (Hong and Luesch, 2012). Largazole shows great promise as an anticancer agent, and currently eleven different syntheses of the product have been recorded (Hong and Luesch, 2012). Largazole may interact with HDAC1, an enzyme which is overly expressed in some cancers (Hong and Luesch, 2012).

Under the guidance of Dr. Yu-Dong Zhou and with assistance from Hannah Carson, I tested the effectiveness of largazole on different breast cancer cell lines and associated BoM (bone metastatic) cell lines. The purpose of the experiment was to determine the effectiveness of largazole on breast cancer metastasis via a cell assay.

2 Methods

2.1 Background

The research project began with training in laboratory techniques in Spring 2019. After a change in topic, the final research project was designed and completed in Fall 2019. At the same time, Dr. Zhou taught a course on the basics of cancer and current treatment options. The final project design was to treat both breast cancer and derived metastatic breast cancer cell lines with largazole to determine cell viability.

2.2 Preparation of HDACi on Dilution Plate

Largazole was obtained from Professor Hendrik Luesch at the University of Florida College of Pharmacy. Largazole (a 100 µM stock solution) was serially diluted using a serum-free medium containing antibiotic. The compound was added at 2x the final concentration in volume to produce the correct concentration when added with the cells and their media. The mother plate was prepared at 2.5x volume to produce two SRB viability 96-well plates. The final concentrations of largazole used were 100, 30, 10, 3, 1, and 0.1 nM. Cycloheximide is a protein synthesis inhibitor which served as a positive control for the experiment as cycloheximide is known to reduce cell viability (PubChem Compound Database)

2.3 Cell Lines and Cell Culture

Human breast tumor MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC). The MCF-7-derived subclones MCF-7 BoM (BoM, bone metastatic) and MDA-MB-231-derived BoM were generated by J. Massagué at Memorial Sloan Kettering Cancer Center and were obtained from K. Watabe at Wake Forest. MDA-MB-231 is an aggressive, invasive, triple-negative breast cancer, and MCF-

7 is an ER-positive breast cancer cell line ("MDA-MB-231 Cell Line Profile"). The cells were maintained in RPMI 1640 media (Hyclone). Antibiotic was added to the cell media at a concentration of 1%, and the cells were grown at a temperature of 37ºC in an environment consisting of 95% air and 5% CO_2.

2.4 SRB Viability Assay under Normoxic and Hypoxic Conditions

The Sulforhodamine B (SRB) assay is a cost-effective way to test the cytotoxicity of a compound on various cell lines (Orellana and Kasinski, 2016). For the SRB viability assay, the different cell lines were seeded onto a 96-well plate in a volume of 100 µL 10% FCS complete culture media at the desired density. First, cells were trypsinized using 1 mL trypsin and 9 mL cell culture media. The cells were then washed with more culture media and diluted to the following densities using a hemocytometer: MCF7 at 30,000 cells/well, MDA-MB-231 at 20,000 cells/well, BoM lines to 15,000 cells/well. Next, 100 µL of the cell solution and 100 µL of the cell media were added to the respective positions on the 96-well plates to achieve the proper cell dilution and drug concentration. The cells were exposed to hypoxic (1% O_2/5% CO_2/94% N_2) or normoxia (5% CO_2/95% air) conditions for 48 hours. After incubation, 100 µL of media was removed from each well, and 20% trichloroacetic acid (TCA) and 1X PBS were added to each well. The plates were then put in the fridge at 4ºC for 1 hour, washed with water, and set in the hood to dry. Next, the wells were stained with 100 µL of 0.4% SRB for 10 minutes at room temperature. The plates were then washed with 3X acetic acid and were laid out to dry. Finally, 100 µL/well of 10 mM Tris base was added to the wells, and the plates were allowed to shake for 10 minutes. Absorbance was measured using Tecan

SpectraFluor Plus plate reader at a range of 496-620 nm, and the difference in absorbance was used to calculate cell viability.

3 Results and Discussion

The purpose of the SRB viability assay was to determine the percent inhibition of Largazole on MDA-MB-231, MDA-MB-231 BoM, MCF-7, and MCF-7 BoM cell lines. The goal was to find IC_{50} values, or the concentration of drug at which cell viability was inhibited by half. However, the percent inhibition never reached this value. Instead, the highest inhibitory concentration for largazole was found for each cell line in both normoxia and hypoxic conditions. Figures were made using Prism 8 and Microsoft Word.

Cell Line	MCF7		MCF7-BoM		MDA-MB-231		MDA-MB-231 BoM	
Condition	Con [ ]	% Inhib	Con [ ]	% Inhib	Con [ ]	% Inhib	Con [ ]	% Inhib
Normoxia	1 nM	25.33%	100 nM	22%	1 nM	32.67%	1 nM	40.67%
Hypoxia	.1 nM	4.33%	100 nM	25.33%	30 nM	1%	1 nM	18%

Figure 4: Percent inhibition of cell viability by largazole

Both MCF-7 and MDA-MB-231 show greater inhibition in normoxic conditions. The triple-negative breast cancer cell line MDA-MB-231 required a much greater concentration of largazole in the hypoxic condition to achieve only 1% inhibition. The BoM cell lines show a greater percent inhibition in both normoxia and hypoxic conditions compared to their corresponding non-metastatic lines. The MCF-7 BoM cell line showed relatively the same percent inhibition in both normoxia and hypoxic conditions when treated with largazole (100 nM). The MDA-MB-231 BoM line showed greater inhibition in the normoxia condition, but was still inhibited in the hypoxia condition, with a treatment of only 1 nM of largazole.

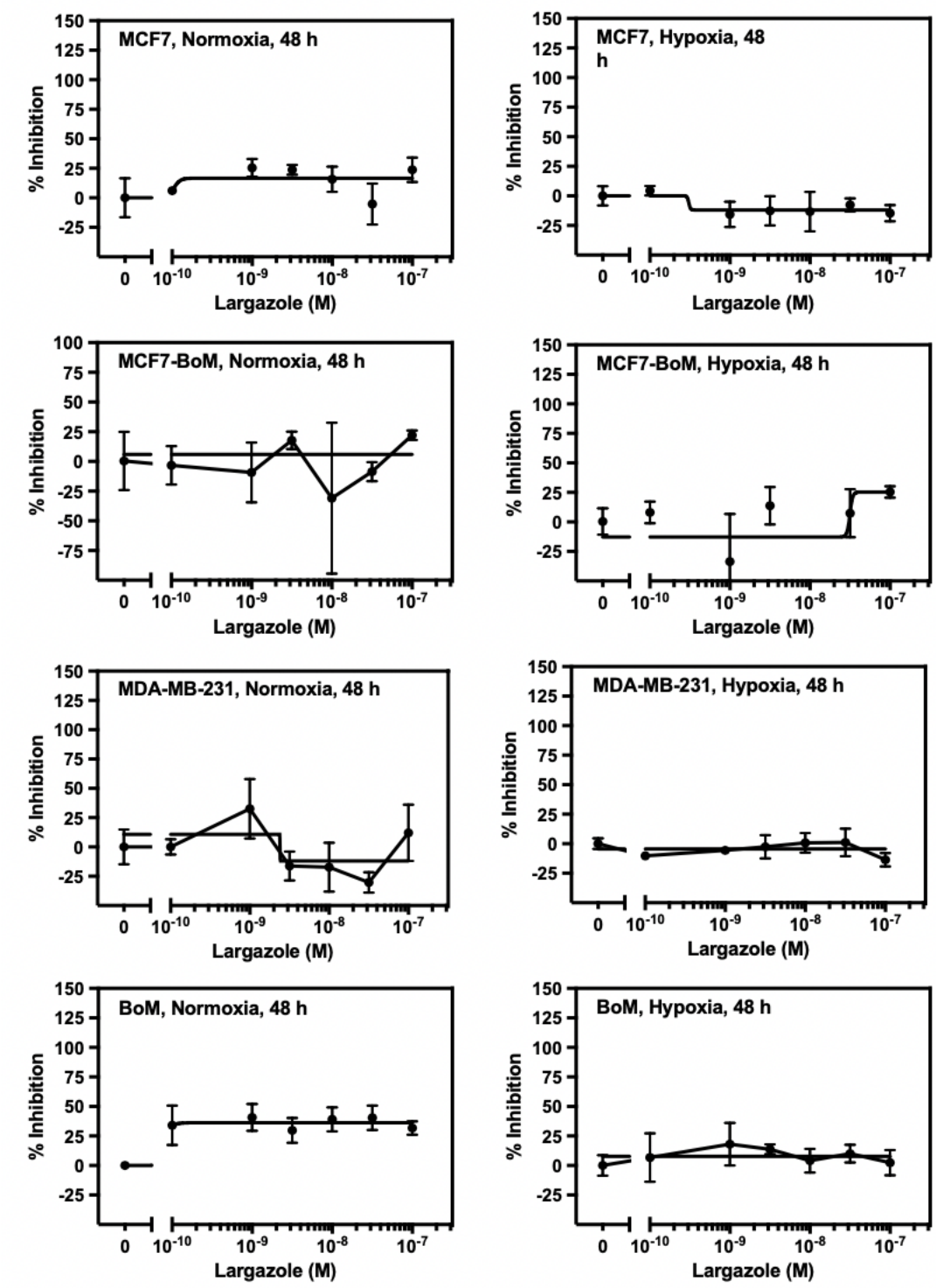

Figure 5: Largazole SRB results

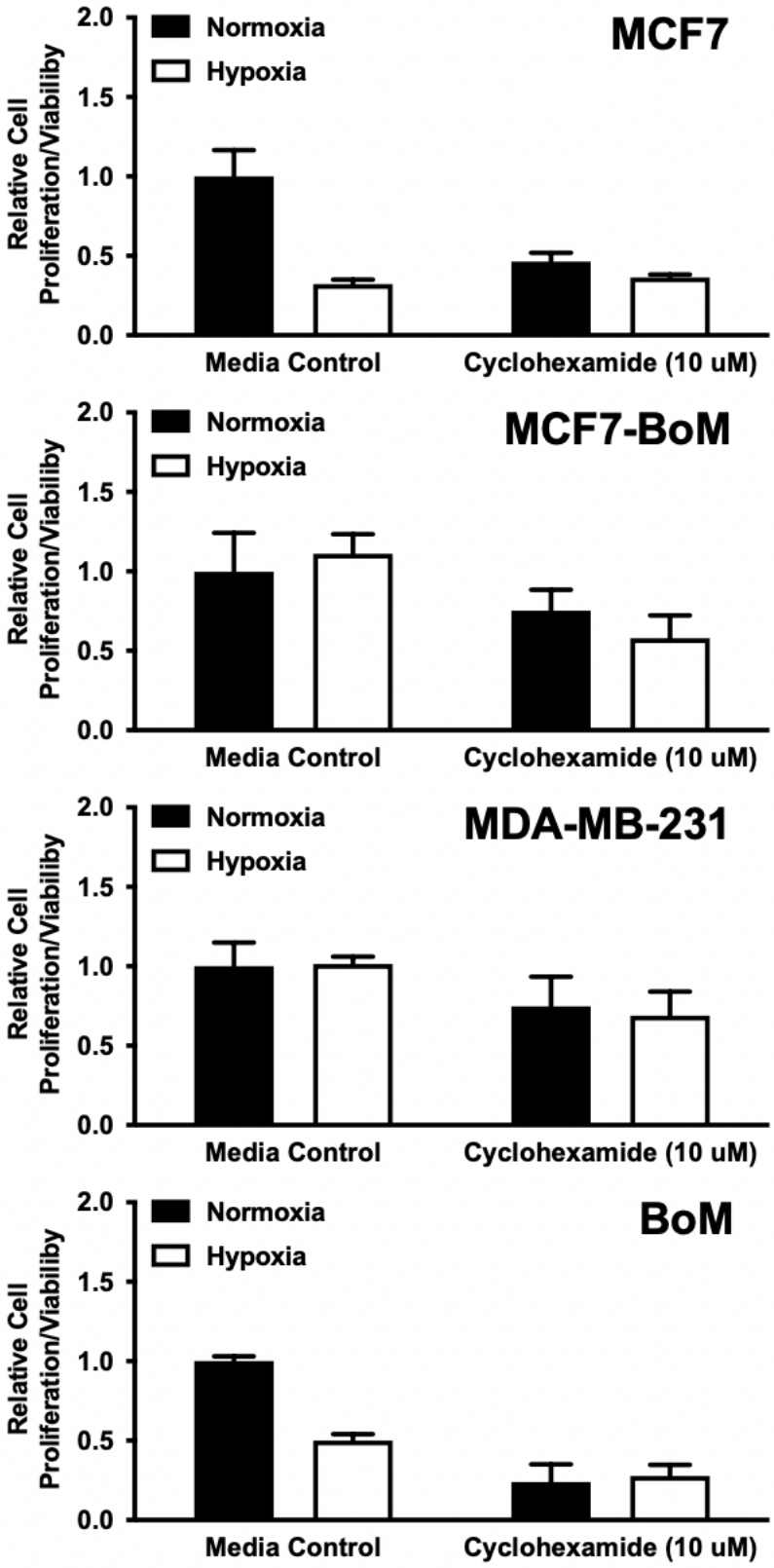

Figure 6: Effect of cycloheximide on cell viability under normoxic and hypoxic conditions

Finally, Figure 6 shows the effects of cycloheximide compared to the media control on the cell lines in normoxia and hypoxic conditions. Overall cycloheximide reduced cell viability compared to the media control. However, in the MCF-7 and BoM lines, the hypoxia condition also significantly reduced cell viability in the media control. These results would suggest those two cell lines are very sensitive to oxygen concentrations.

In the figures above, both overlapping and large error bars can be seen. Since this was the experimenters' first-time practicing cell culture, laboratory technique error was introduced into the experiment. Errors were also introducing via pipetting techniques which were constantly being improved throughout the entire process.

4 Conclusion

The purpose of the experiment was to test how largazole affected both breast cancer cell viability and metastasis in both normoxic and hypoxic conditions. The two metastatic breast cancer lines, MDA-MB-231 BoM and MCF-7 BoM, were generally found to have the overall highest inhibition in normoxic and hypoxic conditions. However, the MCF-7 BoM line required a much higher concentration of largazole in both conditions. Largazole was also found to be efficient in treating the non-metastatic lines, MDA-MB-231 and MCF-7, under normoxic conditions. Largazole, and potentially other histone deacetylase inhibitors, shows promise in treating triple-negative breast cancer, triple negative breast cancer metastasis, and ER-positive breast cancers. For future research, this experiment could be repeated with better laboratory technique to gain better insight. Additionally, this experiment could be repeated with different cell lines to see if largazole affects all ER-positive and TNBC cell lines in the same fashion.

5 List of References

1. American Cancer Society. *Cancer Facts and Figures 2020*. Atlanta, Ga: American Cancer Society; 2020. https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2020/cancer-facts-and-figures-2020.pdf
2. Arrigoni, C., Bersini, S., Gilardi, M., & Moretti, M. (2016). In vitro co-culture models of breast cancer metastatic progression towards bone. *International Journal of Molecular Sciences*, *17*(9), 1405. https://doi.org/10.3390/ijms17091405
3. Barneda-Zahonero, B., & Parra, M. (2012). Histone deacetylases and cancer. *Molecular Oncology*, *6*(6), 579-589. https://doi.org/10.1016/j.molonc.2012.07.003
4. Biel, M., Wascholowski, V., & Giannis, A. (2005). Epigenetics—an epicenter of gene regulation: histones and histone-modifying enzymes. *Angewandte Chemie International Edition*, *44*(21), 3186–3216. https://doi.org/10.1002/anie.200461346
5. "Breast Cancer Statistics and Resources." *Breast Cancer Research Foundation*, Breast Cancer Research Foundation, www.bcrf.org/breast-cancer-statistics-and-resources.
6. Chiang AC, Massague J. Molecular basis of metastasis. *New England Journal of Medicine*, 2008;359:2814–23.
7. PubChem Compound Database, Cycloheximide, *PubChem Compound Database*, U.S. National Library of Medicine, pubchem.ncbi.nlm.nih.gov/compound/Cycloheximide.

8. Haberland, M., Montgomery, R. L., & Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nature Reviews. Genetics*, *10*(1), 32-42. https://doi.org/10.1038/nrg2485
9. D. Hanahan, R.A. Weinberg. Hallmarks of cancer: the next generation. *Cell*, 144 (2011), pp. 646-674. https://doi.org/10.1016/j.cell.2011.02.013
10. Halsall, J. A., & Turner, B. M. (2016). Histone deacetylase inhibitors for cancer therapy: An evolutionarily ancient resistance response may explain their limited success. *BioEssays*, *38*(11), 1102–1110. https://doi.org/10.1002/bies.201600070
11. Halsall, J. A., Turan, N., Wiersma, M., & Turner, B. M. (2015). Cells adapt to the epigenomic disruption caused by histone deacetylase inhibitors through a coordinated, chromatin-mediated transcriptional response. *Epigenetics & Chromatin*, *8*(1), 29. https://doi.org/10.1186/s13072-015-0021-9
12. Hong, J., & Luesch H. (2012). Largazole: From discovery to broad-spectrum therapy. *Natural Product Reports*, 29(4), 449-456. doi: 10.1039/c2np00066k
13. Kluttig, A., & Schmidt-Pokrzywniak, A. (2009). Established and suspected risk factors in breast cancer aetiology. *Breast Care (Basel, Switzerland)*, *4*(2), 82–87. https://doi.org/10.1159/000211368
14. Knowles, H. J., & Harris, A. L. (2001). Hypoxia and oxidative stress in breast cancer. Hypoxia and tumourigenesis. *Breast Cancer Research*, *3*(5), 318–322. https://doi.org/10.1186/bcr314
15. Li, Y., & Seto, E. (2016). HDACs and HDAC inhibitors in cancer development and therapy. *Cold Spring Harbor Perspectives in Medicine*, *6*(10), a026831. https://doi.org/10.1101/cshperspect.a026831

16. *mda-mb-231-cell-line-profile.pdf*. (n.d.-a). Retrieved from https://www.phe-culturecollections.org.uk/media/133182/mda-mb-231-cell-line-profile.pdf
17. Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayani, N., Coppe, J. P., Tong, F., Speed, T., Spellman, P. T., DeVries, S., Lapuk, A., Wang, N. J., Kuo, W. L., Stilwell, J. L., Pinkel, D., Albertson, D. G., Waldman, F. M., … Gray, J. W. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, *10*(6), 515–527. https://doi.org/10.1016/j.ccr.2006.10.008
18. Orellana, E. A., & Kasinski, A. L. (2016). Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. *Bio-Protocol*, *6*(21), e1984. https://doi.org/10.21769/BioProtoc.1984
19. Rankin, E. B. & Giaccia, A. J. Hypoxic control of metastasis. Science (2016) 352(6282):175-180. doi:10.1126/science.aaf4405
20. Tao, Z., Shi, A., Lu, C., Song, T., Zhang, Z., & Zhao, J. (2015). Breast Cancer: Epidemiology and Etiology. *Cell Biochemistry and Biophysics*, *72*(2), 333–338. https://doi.org/10.1007/s12013-014-0459-6
21. Ververis, K., Hiong, A., Karagiannis, T. C., & Licciardi, P. V. (2013). Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. *Biologics: Targets & Therapy*, *7*, 47–60. https://doi.org/10.2147/BTT.S29965
22. Waks AG, Winer EP. Breast Cancer Treatment: A Review. *Journal of the American Medical Association.* 2019;321(3):288–300. doi:10.1001/jama.2018.19323

https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2020/cancer-facts-and-figures-2020.pdf