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THE HISTONE DEACETYLASE INHIBITOR LARGAZOLE: A POTENTIAL CHEMOTHERAPEUTIC AGENT

By
Hannah Lynn Carson

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

Oxford

April 2020

Approved by:

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Advisor: Dr. Yu-Dong Zhou

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ABSTRACT
HANNAH LYNN CARSON: The Histone Deacetylase Inhibitor Largazole: A Potential Chemotherapeutic Agent
(Under the advisement of Drs. Yu-Dong Zhou and Dale Nagle)

Histone deacetylase enzymes are known for their inherent activity as epigenetic modifiers. Although, they have become recognized for their role in cancer progression and other diseases. But also, histone deacetylases have other non-histone targets, for example, microtubules, which play important roles in cancer metastasis, apoptosis, and replication. With histone deacetylase inhibitors (HDACi), our research explored HDACi effects on breast cancer cell lines. The overall goal was to understand the potential of largazole, a class one histone deacetylase inhibitor on breast cancer cell lines. The research consisted of two parts: sulforhodamine B (SRB) viability assays under hypoxic and normoxic conditions. The specific cell lines that were used were MCF-7, MCF-7 BoM, MDA-MB-231, and MDA-MB-231 BoM. The results of the experiments showed that histone deacetylase inhibitors, specifically Largazole is a useful chemotherapeutic agent against breast cancer. Also, Hypoxic conditions in combination with cycloheximide did lead to lower cell proliferation. The MCF-7 and MDA-231 had greater inhibition in normoxic condition. The BoM cell lines had greater inhibition in both conditions. And as expected the triple-negative did require a greater largazole concentration for effect to take place in the hypoxic conditions. The extremely aggressive cancer cell-line MDA-MB-231 gave the most conclusive results.

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Acknowledgements

I would like to thank Dr. Zhou and Dr. Nagle for all the time they have invested in walking me through this process. Dr. Zhou continually made herself readily available to guide and encourage me. Also, special thanks to Dr. Zhou for creating the graphs in the Results and Discussion section and my coresearcher partner Hannah McCowan as we processed the data together. Thank you to Dr. Nagle who taught me so much about cancer research. Thank you to the Sally McDonnell Barksdale Honors College for continuing to push me and make me better while providing me so much support through the ups and downs. And of course, my wonderful parents James and Rhonda Carson for always believing in me. Finally, I thank my husband Braxton Manning for pushing me and having just the right words to say to help me keep going.

List of Figures

List of Abbreviations

ATCC- American Type Culture Collection

BRCA1- Breast Cancer Gene 1

BRCA2- Breast Cancer Gene 2

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic Acid

ER- Estrogen Receptor

FCS- Fetal Calf Serum

HAT- Histone Acetylase

HDAC- Histone Deacetylase

HDACi- Histone Deacetylase inhibitor

HDACi(s)- Histone Deacetylase inhibitors

HER2- Human Epidermal Growth Receptors

HIF- Hypoxia-Inducible Factor

NCI- National Cancer Institute

PBS- Phosphate Buffer Saline

PR- Progesterone Receptors

SRB- Sulforhodamine

TCA-Trichloroacetic Acid

INTRODUCTION: BREAST CANCER

Through the years I have known many women who have triumphed through the diagnosis of breast cancer. This disease is the most common cancer and primary cause of cancer related death in females (Akram 1). Meaning that about 12% of women in the U.S. will develop breast cancer in their lifetime (breastcancer.org). This year alone, roughly 42,000 women are expected to die from breast cancer (breastcancer.org). These startling facts are what push researchers and medical professionals to work for a cure to ensure a healthier future for women.

When a student is learning about cancer the first piece of information that is taught is that there are eight remarkable Hallmarks of cancer, they are: "sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming energy metabolism, evading immune response, and activating invasion and metastasis."(Hanahan 1). Recognition of these cancer hallmarks are what lead researchers to understand more about cancer and potential ways to cure this disease. Although, research is fascinating and continues to make great discoveries every day. Women can take part in preventative medicine to reduce the risks of breast cancer. John Hopkins lists the following as preventative healthcare: "take care of yourself emotionally, physically, reduce stress, eat healthy, and see the doctor for regular health screenings" (John Hopkins, 1). Although these were listed for women to reduce the risk of breast cancer, this advice is useful for all cancer prevention.

The bad news is there is only so much that women can do, there are also non-modifiable factors, those cannot be prevented; such as age, race, mutated genes, family and personal history, late menopause, and even height are all associated with a predisposition to breast cancer. According to the Center of Disease Control and Prevention, 1 out of 10 women are diagnosed with some form of breast cancer under the age of 45 due to the BRCA1 and BRCA2 mutation, descent of the Ashkenazi Jewish heritage, exposure to radiation, or a close relative diagnosed with breast cancer or ovarian cancer before the age of 45 (CDC 1). Another non-modifiable factor is breast density, related to rather a woman's breast being more fibrous or glanderous than fat, leading to greater density in the breast causing it to be more difficult to locate tumors using a mammogram.

In general, breast cancer is generally described at ductal or lobular, other forms exist as well but are less common such as tubular, mucinous, medullary, and papillary. Lobular carcinomas begin in the milk producing glands, whereas; ductal is in cells of the milk duct (MD Anderson 1). Each tumor is different and unique, and the prognosis is dependent on the type of tumor. For example, those diagnosed with mucinous (colloid) carcinoma tend to have a better prognosis than those diagnosed with an invasive ductal carcinoma (Susan G. Komen 1). There are also three types of molecular receptor-based classifications of breast cancers, these include HER2, ER, and triple-negative (MD Anderson 1). The HER2 (Human Epidermal Growth Factor receptor 2) is positive, then the cancer will be reluctant to respond to hormone therapies, because the receptor works specifically to cause growth of cancer cells. The ER-positive breast cancer (Estrogen Receptor) is another possible source of

promoting cancer cell growth. Triple-negative breast cancer is known to spread much faster because it lacks ER, PR (Progesterone Receptors), and does not overexpress HER2 growth receptors, anti-hormone therapies are not useful (cancer.org 1). As seen through all the breast cancer variations and the risk of women, the goal of researchers and doctors is to find the best course of action for each individual patient. Cancer results from a combination of mutations. The genes in our body are carried in the DNA molecules, and if a mutation occurs it can change the amount or activities carried out by particular proteins (Weinburg 1). Specifically, these cancer related genes are called proto-oncogenes and modifications of tumor suppressor genes, which lead to uncontrolled cell proliferation. Many proto-oncogenes relay growth signals from the outside of the cell to the deep interior, when the mutation occurs the growth signaling pathway remains continuously active when it should be silent (Weinberg 2). These growth factors promote cancer metastasis. For example, approximately one-third of women who are sentinel lymph node negative at the time of surgical resection of the primary breast tumor will subsequently develop clinically detectable secondary tumors (Hunter 1). Simply put with metastatic breast cancer there is risk that removal of the tumor could lead to an increase in the circulation of cancer cells in the body leading to cancer at a distal sight. Metastatic breast cancer is stage IV, although not always caused by surgery the cancer may just progress to the point of spreading to local or distal sights. With experiments and countless hours in the lab Drs. Zhou and Nagle allowed me and my lab partner Hannah McCowan to study possible ways to stop metastatic breast cancer. The use of histone deacetylase

inhibitors, specifically the marine natural product largazole, could be a potential answer to prevent malignant breast cancer.

Histone Deacetylase Inhibitors

The human genome contains approximately 3.2×10^9 nucleotides—is distributed over 24 different chromosomes (Johnson 1). The chromosome contains long, linear DNA associated with proteins and contains those that are used in gene expression and DNA replication and repair (Johnson 1). Histone modifications are what allow gene expression, for example histone acetylation allows for higher expression (Harb 2). “Histone acetylation status is regulated by two groups of enzymes exerting opposite effects, histone acetyltransferases (HATs) and histone deacetylases (HDACs).” (Harb 3).

The definition of an inhibitor is an agent that slows or interferes with a chemical action (Merriam webster). These inhibitors can interfere with epigenetic enzymes created through mutations caused by cancer in the body. These epigenetic enzymes cause modifications in the methylation and histone modifications and cause extensive changes without altering the DNA sequence (Han 1). Specifically, “Histone acetylation is an important epigenetic modification that mainly occurs in the N-terminal region of the histone tail.” (Han 5). The acetylation acts to weaken the binding between DNA and histones, ultimately leading to relaxed chromatin causing increased gene expression (Han 5).The acetylation occurs and cause neutralization of the positive charges by binding with lysine residues, this process can decrease the affinity for DNA. Because of this, researchers know that “histone acetylation alters

nucleosomal conformation" leading to increased availability of transcription regulatory proteins (Struhl 1). This could cause overexpression and lead to cancer. "Mutations in genes encoding HDACs are associated with the progression of tumors, owing to the abnormal transcription of key genes that regulate important cellular functions such as cell proliferation, cell cycle regulation and apoptosis," (Han 5) and certain histone deacetylase (HDAC) inhibitors can be used as anticancer agents. These are important agents because the histone acetylases are used in determination of gene expression (West 1). Whereas, the deacetylase is useful in the regulation process as it removes the acetyl group from the histone (West 1). The inhibition of HDAC1 and HDAC2 can cause hypersensitivity in cancer cells. But in the past, histone deacetylase inhibitors have only been useful on nontumor forming cancers. Specifically, blood cancers like leukemia and T-cell subcutaneous lymphoma.

Largazole

The natural product largazole is a cyclic depsipeptide, from marine *Symploca* sp. cyanobacteria with novel chemical scaffold that potently inhibits class I histone deacetylases. (Hong 1). The mechanism of action of largazole is similar to drugs like vorinostat (Zolinza®) and romidepsin (Istodax®), Vorinostat is a drug that inhibits class I, II, and IV HDAC molecules and romidepsin inhibits class I HDAC molecules (Hong1). The *Symploca* sp. extract was originally collected in Key Largo, Florida; where it was found to have remarkable antiproliferative activity (Hong 2).The name largazole was due to the occurrence in Key Largo and the "azole" because of the one thiozole linearly fused to a 4-methylthiazoline (Hong 2). Largazole contains only one

non modified standard amino acid and three stereogenic centers. Its 3-hydroxy-7-mercapto-hept-4-enoic acid is unique among marine natural products.

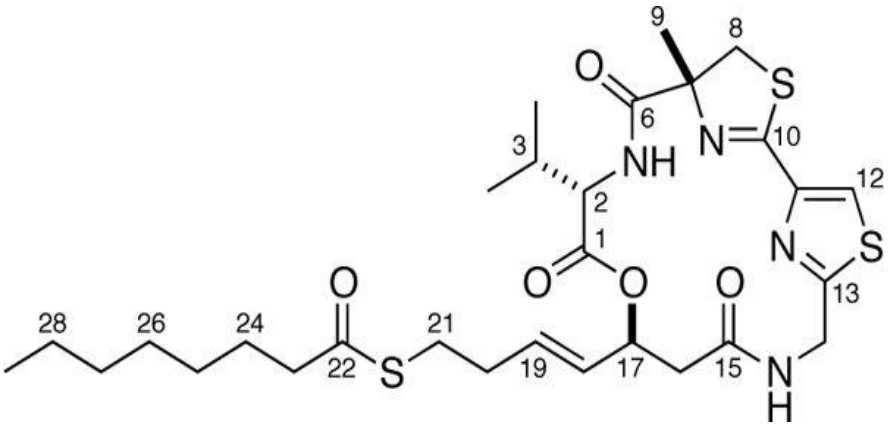

Figure 1. Largazole Structure

The cytotoxicity of the drug was tested on different tumor cell lines (i.e., colorectal carcinoma, breast cancer, neuroblastoma, and osteosarcoma) and was shown to inhibit their growth at nanomolar concentrations. Remarkably, non-transformed epithelial cells and fibroblasts were less sensitive to its effects (Hong2-3). Mechanism of action studies indicated that the antiproliferative activity of largazole results from the inhibition of HDAC enzymes (Hong 3). Because of the functionality of the thioester suggested that through hydrolysis largazole could be converted into a HDAC inhibitor (Hong 3). “Largazole activation appears to be induced through a general protein-assisted mechanism, which may explain why largazole itself displayed apparent activity in the in vitro enzymatic assay with recombinant HDAC1 enzyme, although with about 10-fold lower potency.” (Hong 3). The thiol in largazole presents itself to be stable in freeform and reversible adduct, could result in hijacking of proteins for delivery to the target site. But denatured proteins did not form adducts with largazole thiol. In addition, it has been found that largazole lacks acute toxicity, resulting in largazole being a potentially safe anticancer HDAC inhibitor (Hong 8).

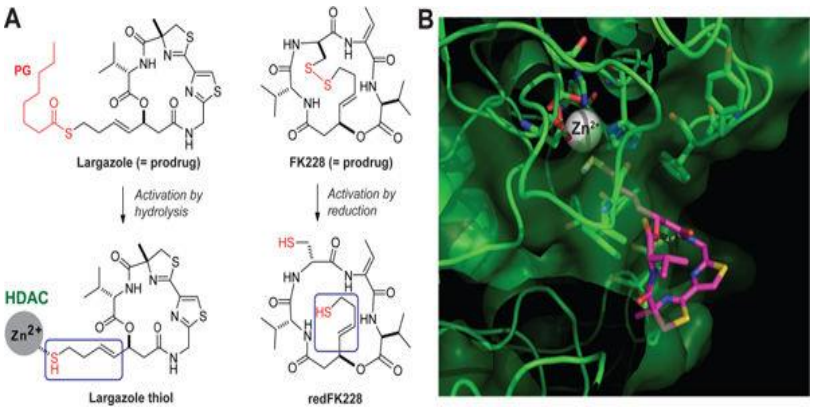

Figure 2: Mechanism of action. A) Modes of action of largazole and FK228 to liberate potent HDAC inhibitors. B) Molecular docking of largazole thiol into an HDAC1 homology model. PG = Protecting group.

Histone Deacetylase Inhibitor Experiment

The research for this project began in the Fall of 2018 under the guidance of Drs. Zhou and Nagle. The first part of the project began with a cancer research course to learn the background of cancer and specifics of breast cancer. The next semester the time was spent in the lab learning important laboratory procedures and techniques to sufficiently carry out the experiments. Beginning in Fall of 2019 the official research project began, the team developed a project and the actual experiments were put into place. The overall goal was to discover the effects of largazole in varying concentrations, on breast cancer cells. Therefore, the HDACi(s), specifically HDAC I inhibitors were studied. The general hypothesis of this experiment was to understand the effects of HDACi(s) on breast cancer cells.

MATERIALS AND METHODS

Background Research

With the decision to pursue the use of HDAC(i)'s as a form of cancer treatment, the real research began. This process began with mini lectures each time learning more and more of what each experiment would entail. Afterwards we began to gather what was needed to conduct the experiments. Dr. Zhou specifically mentioned studying largazole and its effects on breast cancer. The overall research design was to discover the effects of differing concentrations of largazole on four different cancer cell lines. Largazole was obtained from Professor Luesch at the University of Florida College of Pharmacy. The human cancer cell lines were the MCF-7 (ductal carcinoma), MDA-MB-231 (breast adenocarcinoma), and subtypes MDA-MB-231 BoM and MCF-7 BoM, these were all from ATCC. The MDA-MB-231 was from a 51-year-old Caucasian woman with triple negative breast cancer from a pleural effusion. Sadly, this is an aggressive tumor with a poor prognosis. The MCF-7 was from a 69-year-old Caucasian woman with triple-negative breast cancer, the unique aspect about this tumor is its ER-positive, meaning it grows with response to the hormone estrogen. The MCF-7 BoM cell line was from a bone secondary metastasis acquired from Dr. J Massagué's lab at the Sloan Kettering Cancer Center. And our research group specifically received the cells from Dr. Konosuke Watabe at Wake Forest University.

Sulforhodamine B (SRB) Viability Assay

The SRB Viability Assay was developed to measure the drug-induced cytotoxicity (voigt 1). The method is useful in discovering the cell density determination depending on the amount of cellular protein content (Viachi 1). This is a useful method because it can test large samples in a matter of days and the materials used are inexpensive. Therefore, SRB is a useful and cost-effective method of screening. For this experiment to take place it is broken up into four main steps: preparation of treatment, incubation of the cells with a treatment, fix the cells and add the SRB staining, and measure the absorbance (Orellana 1). Beginning the experiment, the cell cultures were trypsinized with 1 mL (Trypsin). The cells were then washed with 10 mL of 10% fetal calf serum (FCS) media. Then the 100 µL of solution and 10% FCS were seeded to each well on a 96-well plate, at varying concentrations ranging from 30,000 cells/mL, 20,000 cells/mL, and 15,000 cells/mL. The cells were then cultured at 37℃ with 95% air and 5% CO_2 creating a humid environment. The 96-well plates were then infused with the chemotherapeutic drug, largazole which was prepared by serial dilutions. The cells were then incubated for 48 hours and afterwards a 100 µL of media was removed for the cell wells. A cell count was then performed to ensure that cells were seeded. And the process of cell fixation began by adding 100 µL of 20% trichloroacetic acid (TCA) and 1% phosphate-buffered saline (PBS) solution into each of the wells. The PBS that was available was 10% so it was diluted with autoclaved water. The plates were then refrigerated at 4℃ for an hour. After the removal of the plates from the refrigerator the plates were then washed down with tap water from the sink four separate times. After the wells in the plates were dry, the wells were then stained with 100 µL of 4% SRB containing 1% Acetic acid and the plates sat at room temperature with the stain for a total of 10 minutes. Afterwards each well was washed three times with 1% acetic acid and then left to dry. A 100 µL of 10%

Tris buffer was added to the stained wells and were lightly shaken for 10 minutes on the microplate genie. The plates were then read by the SpectraFlour plus machine and Magellen software at the absorbance range of 490 nm to 620 nm. Overall, the sulforhodamine B dye is what binds to the proteins of the cell specifically the basic amino acids, which allows for the calculation of the inhibition value using the Magellen software. This inhibition value can be calculated by using the total protein mass per wall (indicated by the SRB dye) which is causally related to the cell density of that specific cell.

Hypoxic and Normoxic Experiments

Hypoxic conditions are defined as cells, tissues, or organs that lack sufficient oxygen. Hypoxia can be caused by the presence of tumors in the body and the cells can learn to adapt to that environment. During hypoxia, hypoxia-inducible factor-1 (HIF-1) is activated to regulate various genes such as those involved in angiogenesis or oxygen transport. The stabilization of this transcription factor is a hallmark of hypoxia, therefore detecting elevated levels of HIF-1 is used to screen for tumors hypoxia. (Wu 1). Part of the experiment after the SRB Assay was performed, the research team decided to see the effects of hypoxia on the cells after treatment with largazole. This was accomplished by putting one set of plates in the Hypoxia chamber with a petri dish containing sterilized water, this allows for adequate humidification of the cells. The other set of cells were placed in normoxic conditions as a control. In the hypoxia chamber the conditions were set at 1% O_2 gas mixture. In order to remove most of the oxygen from the chamber it was flushed by opening the gas tank. And

the gas flow was turned off and the chamber was closed completely. Following the de-gasing process the chamber was then returned to normal incubation.

Results and Discussion

SRB Viability Assay

The results of the sulforhodamine B viability assay show the percent inhibition of values of largazole tested on MCF-7, MCF-7 BoM, MDA-MB-231, and MDA-MB-231 BoM cell lines. One goal of the viability test was to determine the IC_{50} values (the half-maximal inhibitory concentration when measuring the potency of a substance in inhibition). Although the results provided inhibition, the IC_{50} value was not attained because more than 50% was inhibited. The figures below show the results of viability due to varying largazole concentrations depending on hypoxic and normoxic conditions. Also, bar graphs in comparing media control versus cycloheximide. My lab partner and I configured the data together and processed the graphs through graph pad.

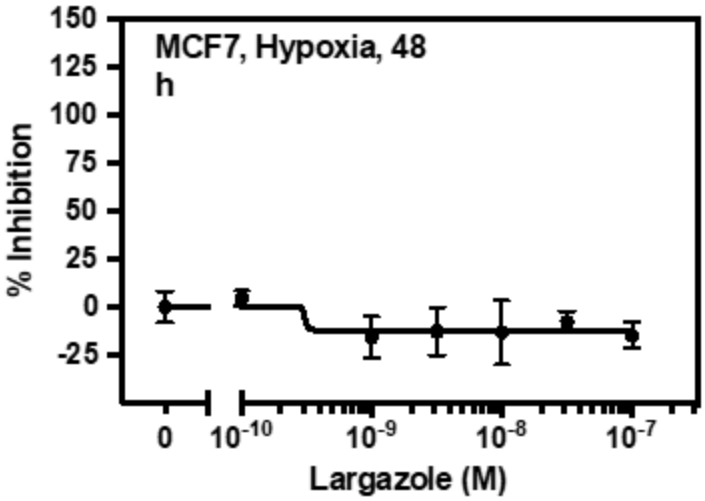

Figure 3. The effect of largazole on MCF-7 cell proliferation/viability under hypoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate.

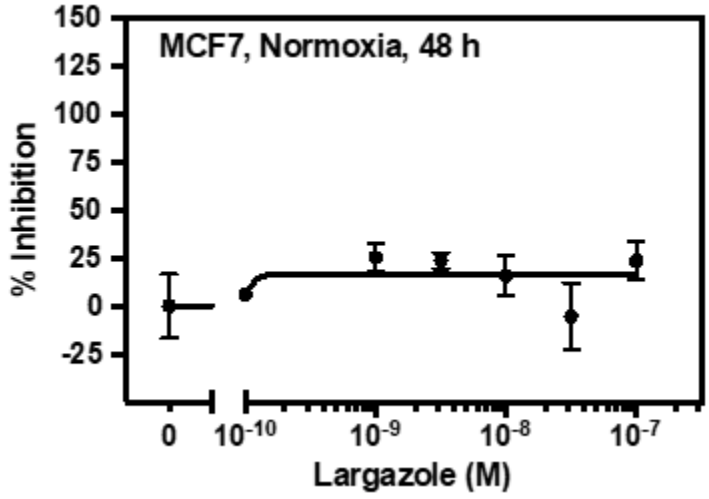

Figure 4. The effect of largazole on MCF-7 cell proliferation/viability under normoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

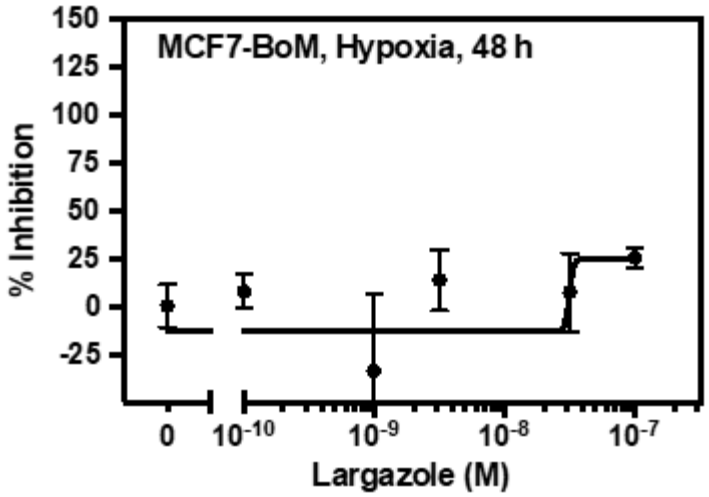

Figure 5. The effect of largazole on MCF7-BoM cell proliferation/viability under hypoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

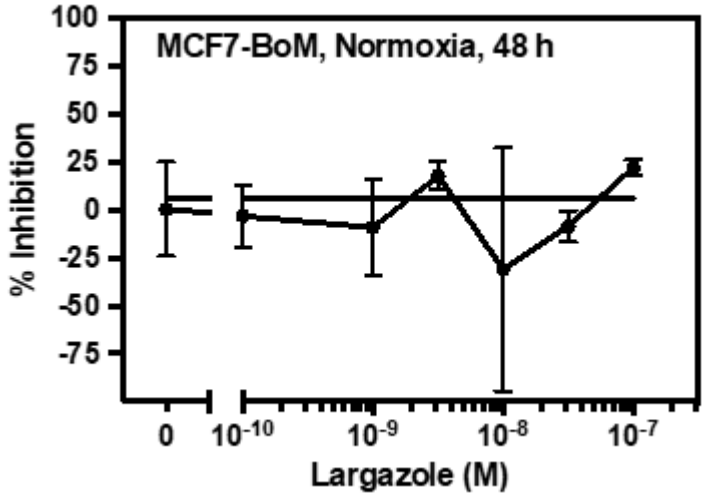

Figure 6. The effect of largazole on MCF7-BoM cell proliferation/viability under normoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

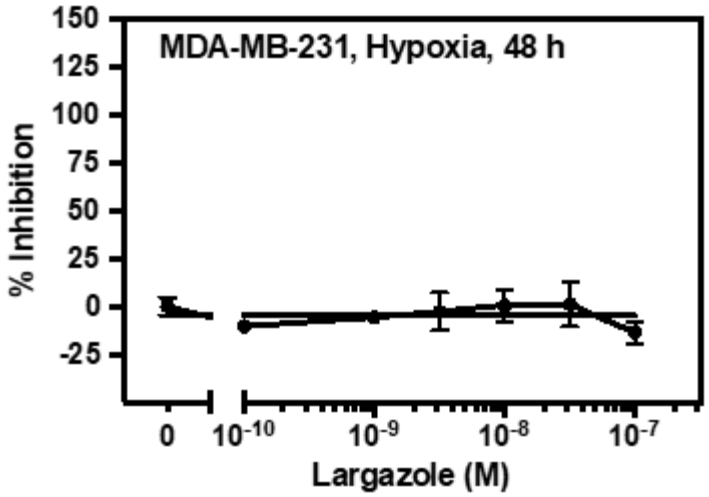

Figure 7. The effect of largazole on MDA-MB-231 cell proliferation/viability under hypoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

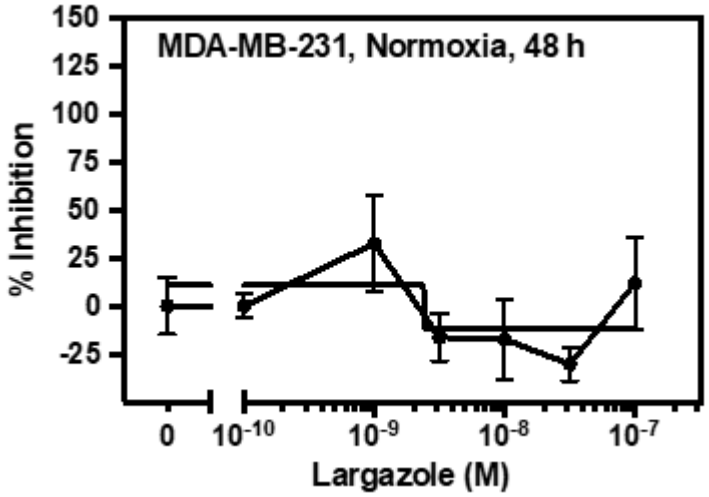

Figure 3. The effect of largazole on MDA-MB-231 cell proliferation/viability under normoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

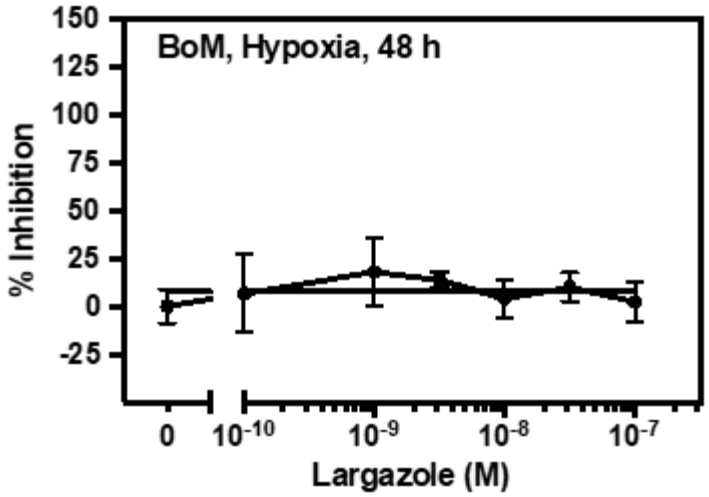

Figure 9. The effect of largazole on BoM cell proliferation/viability under normoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

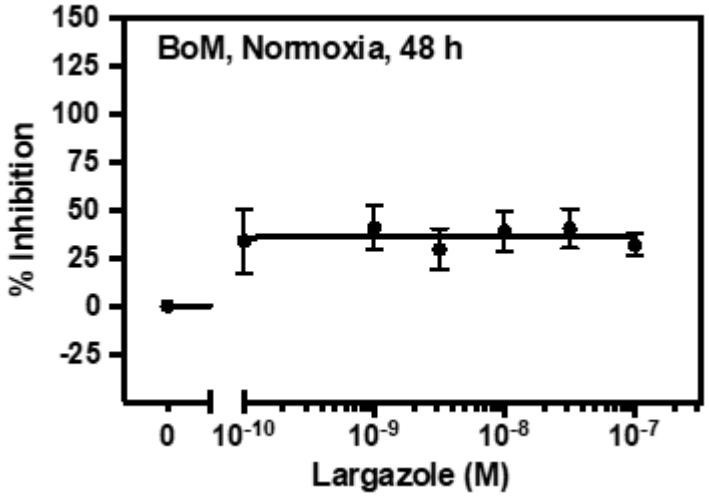

Figure 10. The effect of largazole on BoM cell proliferation/viability under normoxic conditions. Data shown are average +/- standard deviation of the mean from one experiment performed in triplicate

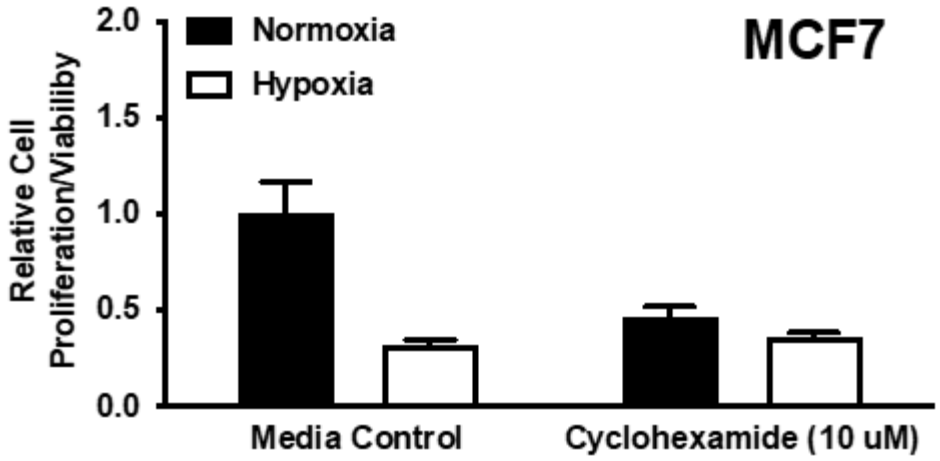

Figure 11. MCF-7 cell line effects on cell growth/viability under hypoxic/normoxic and the effects of cycloheximide.

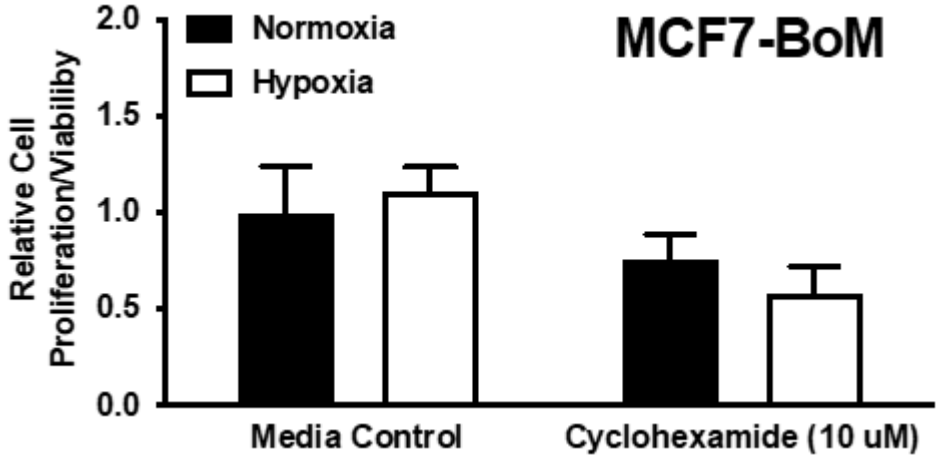

Figure 12. MCF7- BoM cell line effects on cell growth/viability under hypoxic/normoxic and the effects of cycloheximide

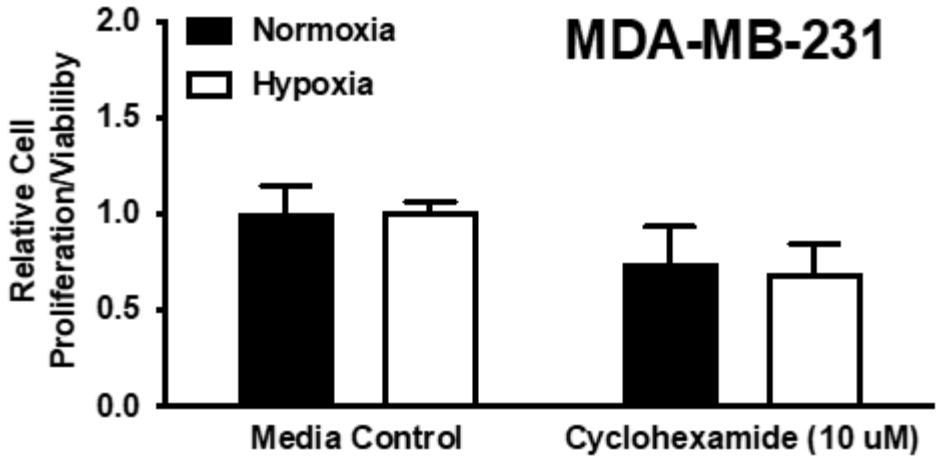

Figure 13. MDA-MB-231 cell line effects on cell growth/viability under hypoxic/normoxic and the effects of cycloheximide

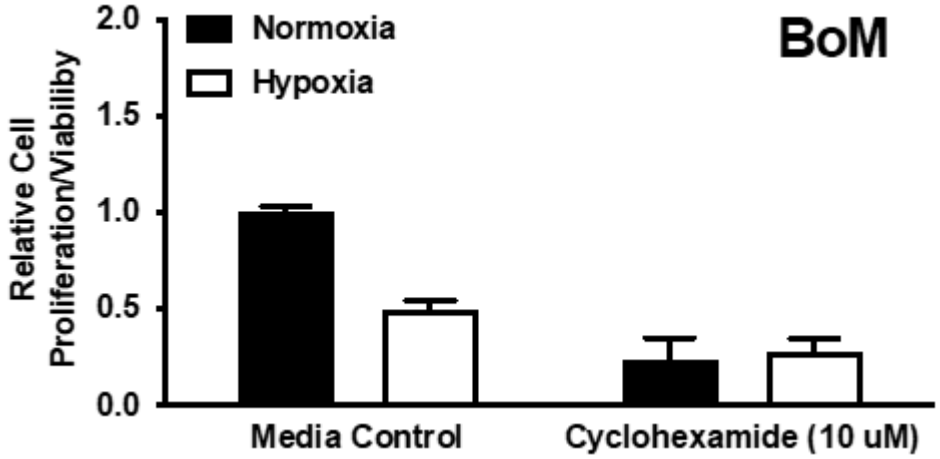

Figure 14. MDA-MB-231 BoM cell line effects on cell growth/viability under hypoxic/normoxic and the effects of cycloheximide.

The first eight graphs in the figures above represent tumor cell viability in relation to hypoxic and normoxic controls and the effects of cycloheximide in comparison to media controls. In each graph it is evident that the hypoxic control leads to a smaller percent inhibition in comparison to normoxic conditions. Therefore, it can be said that hypoxia

conditions cause the cell lines to be less susceptible to cytotoxic effects of the drug largazole. As expected, the triple negative required a higher concentration of largazole in hypoxic conditions. Triple negative breast cancer is one of the most difficult to treat, therefore requiring a higher drug concentration. The hypoxic and normoxic conditions had the least effects on the MCF-7 and MDA-MB-231 BoM cell lines. It can be stated that the normoxic control does produce a higher percent inhibition overall. The MCF-7 BoM cell line provided drastic results between the hypoxic and normoxic conditions, further proving that normoxic conditions produce a much larger percent inhibition. The negative values are evidence of error for example improper serial dilutions. For more reliable data all four cell lines should be further tested to attain IC_{50} values.

An interesting data trend was provided by the last four figures. The media control was overall less effective than cycloheximide, a highly toxic fungicide. In each of the finding less cell proliferation was found in each cell line due to the use of 10 μM cycloheximide. Also, in the data shown above, the majority of the time the hypoxia condition leads to equal or lower cell proliferation in regard to media control and cycloheximide; with exception to the MCF-7 BoM, the media control hypoxic condition was higher than under normoxic conditions. Overall, the growth inhibition never reached complete inhibition and was most prominent at the highest concentrations of largazole.

Conclusion

An overview of the analysis of the SRB viability assay and the hypoxia versus normoxia conditions of multiple different cell lines, our hypothesis largazole does have an impact on cell lines, as does hypoxic and normoxic conditions. The MCF-7 and MDA-231 had greater inhibition in normoxic condition. The BoM cell lines had greater inhibition in both conditions. And as expected the triple-negative did require a greater largazole concentration for effect to take place in the hypoxic conditions. The four cell lines, MCF-7, MCF 7 BoM, MDA-MB-231, and MDA-MB-231 BoM had variation in inhibitor effects in SRB viability assays. This is most likely due to human error, possibly not accurate dilutions. Therefore, further testing should be done. Also, more research should be conducted at higher concentrations to attain IC_{50} values of the marine natural product largazole. The use of cycloheximide did prove to be effective and the hypoxic conditions did lead to lower cell proliferation. In order, to improve the results more testing should be done at a higher cycloheximide concentration direct comparison of largazole and cycloheximide.

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