THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON METASTATIC BREAST CANCER CELLS IN CONJUNCTION WITH CLINICALLY RELEVANT CHEMOTHERAPEUTIC AGENTS

by
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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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ABSTRACT

Mary Grace Stewart: The Effect of Histone Deacetylase Inhibitors on Metastatic Breast Cancer Cells in Conjunction with Clinically Relevant Chemotherapeutic Agents (Under the direction of Dr. Yu-Dong Zhou and Dr. Dale George Nagle)

Histone Deacetylases (HDACs) are enzymes charged with the job of loosening DNA packaged around histone proteins. This gives them the opportunity to affect the transcriptional regulation of certain cancer associated genes and proteins, but HDACs are not limited to only DNA modification. They also target non-chromatin proteins in the cytoplasm, and have been closely linked to the many pathways involved in metastatic breast cancer such as apoptosis evasion, cell migration, and angiogenesis. HDAC's effect on important cytoplasmic proteins could play a huge role in the largely unknown mechanisms of metastatic breast cancer. HDAC inhibitors are a rising class of chemotherapeutic agents. In this research, HDAC inhibitors selective for cytoplasmic target proteins are used in conjunction with nuclear based HDAC inhibitors and known microtubule stabilizers and destabilizers to test and explore the effects of HDAC inhibition on MDA-MB-231-derived metastatic breast cancer cell lines BoM-1833 (BoM) and LM-4175 (LM), and in MCF-7-derived metastatic breast cancer cell line MCF-7 BoM. Through viability, clonogenic, and combination assays, the HDAC inhibitors were not found to have complete inhibition of the tumor cells at any of the tested concentrations but show a trend suggesting potential effects on tumor inhibition at higher concentrations. There is also some evidence supporting a potential significance of HDAC inhibitors on ER+ breast cancer cells. Clonogenic assays upheld the inhibitory results of HDAC inhibitors on metastatic breast cancer cells, and emphasized the increased effectiveness of higher concentrations of HDAC inhibitors. Combination assays showed a trend towards antagonistic effects between HDAC inhibitors and microtubule stabilizes.

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LIST OF ABBREVIATIONS

BRCA1 - Breast Cancer gene 1

CYLD - Cylindromatosis

DMEM - Dulbecco's modified Eagle's media

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

ECM - Extracellular matrix

EMT - Epithelial–mesenchymal transition

EpoB - Epothilone B

ER+ - Estrogen receptor positive

FBS - Fetal Bovine Serum

GRK2 - G-Protein-Coupled Receptor Kinase 2

HAT - Histone Acetylase

HDAC - Histone deacetylase

HDACi - Histone Deacetylase inhibitor

HEF1 - Human enhancer filamentation 1

Her2+ - Human epidermal growth factor receptor 2-positive

HIF - Hypoxia-inducible factors

Hsp90 - Heat shock protein 90

IBC - Inflammatory Breast Cancer

IC50 – Half maximal inhibitory concentration

Lys - Lysine

MMP - Matrix metalloproteinase

NAD+ - Nicotinamide adenine dinucleotide coenzyme

NCI - National Cancer Institute

PBS - Phosphate-buffered saline

PR+ - Progesterone receptor positive

Prx - Peroxiredoxin

RunX2 - Runt Related Transcription Factor 2

SAHA - Vorinostat

SRB - Sulforhodamine B

Taxol - Paclitaxel

VEGF - Vascular endothelial growth factor

INTRODUCTION:

BREAST CANCER:

In 2019, the American Cancer Society predicts that there will be 1,762,450 new cases of breast cancer in the United States. Of these, 42,260 cases are predicted to end in death [1]. In 2018, The World Health Organization's International agency for Cancer Research documented an estimated 2,088,849 cases with 626,679 deaths worldwide. Breast cancer has the most incidences worldwide behind lung and skin cancers [2]. From 1989 to 2016, death associated with breast cancer in the U.S. was reduced by 40%. This is likely attributed to increased awareness, earlier diagnosis, and better available treatments, but since 2016, the death rates for women under 50 have plateaued [1]. Something new needs to happen. Early intervention can only go so far to help women, and with a 0.4% incident increase in the U.S. per year over recent years [1], one would expect the death rates to begin climbing again in the future if something new doesn't intervene.

Breast cancer, along with most all cancers, is caused by abnormal, out of control cell growth. In breast cancer, the growth and invasion of the cancerous cells can be denoted by staging. Small localized cancerous tumors in the breast duct or the mammary glands that have not spread or have spread minimally to the most commonly cancer associated lymph node, the sentinel lymph node, are classified as stage 1 breast cancer. Stage 2 tumors are larger and have generally spread to a few more nearby lymph nodes. Stage 3 tumors are even larger and have spread to most all nearby lymph nodes or have begun growing into the nearby skin and muscle tissues surrounding the breast. Stage 4 is metastatic breast cancer, which spreads to other parts of the body [4] (figure 1.1).

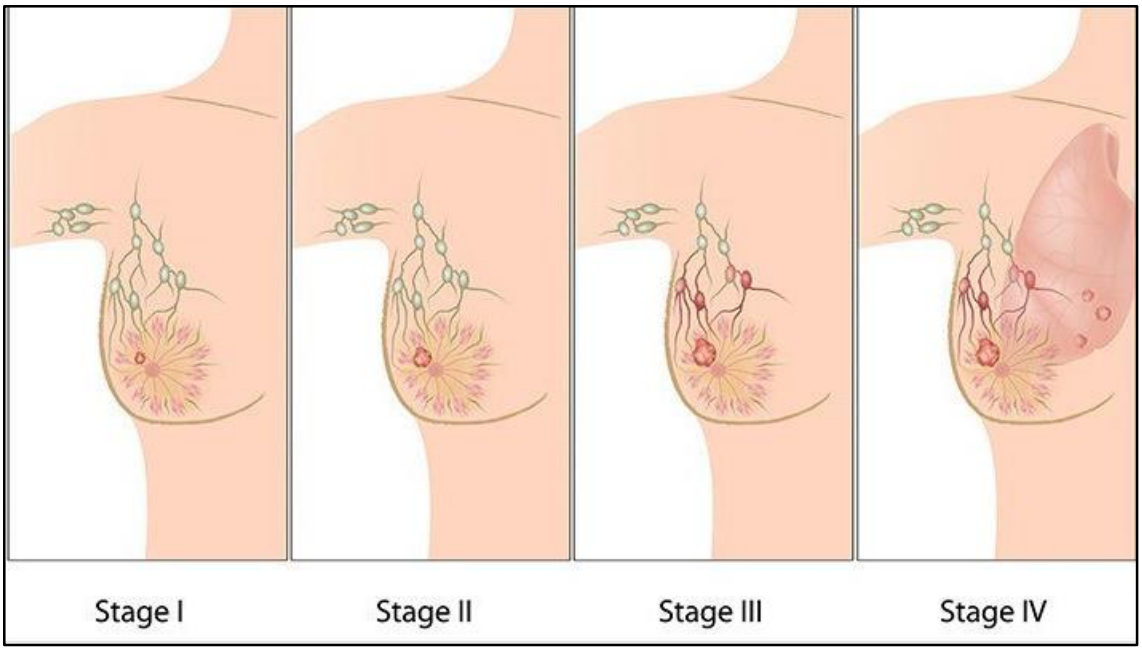

Figure 1 *Stages of Breast Cancer* (Siddique, M. (2017, April))

Breast tumors present as a mass or lump in the breast. The mass is usually hard and irregular and isn't associated with pain, but some can be soft, round, and painful. This is why diagnostic tools are so important. Most healthcare specialists use mammograms, specialized x-rays that can help visualize breast tissue and structures, and biopsies to identify the malignancy of tumors. These and more technological advances have helped to detect disease early on and reduce the incidence of metastasis. If caught early enough, these cancers can be very treatable. Some examples of treatments are chemotherapy, hormone therapy, targeted drug therapy, and oncoplastic surgery, in which the surgeon has to remove a tumor but reconstructs the breast afterward. The removal of a tumor from the breast is called a lumpectomy, which, along with partial mastectomies, can be done with early stage tumors. Later stage, larger tumors require full mastectomies and full reconstruction [4].

Clinically, breast cancer tumors can be classified by their genetic variation and receptor types. They can be broken down into four subgroups: luminal A, luminal B, Her2+

(human epidermal growth factor receptor 2-positive), and basal-like. Luminal A breast tumors have estrogen and progesterone receptors and can be treated with hormone therapy and chemotherapy. Luminal B tumors have estrogen receptors and are Her2 positive. These are generally treated with chemotherapy, hormone therapy, and targeted Her2 therapy. Her2+ tumors do not have hormone receptors but are positive for the Her2 oncogene which codes for a growth promoting Her2 protein. These tumors are approached with targeted therapy to shut down the Her2 protein in addition to chemotherapy [5]. Basal-like tumors are "triple-negative." They don't have estrogen receptors or progesterone receptors, and they do not overexpress Her2. Often, mutations in the BRCA1 gene, a tumor suppressor gene, are associated with triple-negative breast cancer. These are difficult to treat because there are no available target proteins or receptors for therapy. Chemotherapy is the treatment choice for basal like tumors. These are usually larger and more severe at presentation and are typically diagnosed with poorer overall outcomes [6]. Another, less common type of breast cancer is inflammatory breast cancer (IBC) which is associated with advanced stage presentation and axillary lymph node involvement which leads to a greater chance of metastasis. About 35% of IBC cases have distant metastasis which may be contributed to increased levels of Her2 protein and pro-angiogenic factors. There are so many types of breast cancer because the tumors have such heterogeneous pathology and genetic profiles [7].

METASTASIS:

When breast cancer cells break off the primary tumor, they can enter into the lymphatic or blood vasculature and disseminate to other organs. This is known as metastasis or stage 4 breast cancer. Breast cancer cells generally metastasize to the bones, lungs, liver, and brain. Some cases of metastasis present at initial diagnosis, which is known as de novo metastasis and generally results from a late diagnosis, but most occur after an initial diagnosis and treatment course. Some breast cancer cells can evade treatment and travel to other organs [8]. It is important to note that cancer that has metastasized to the bone, for example, is not bone cancer. It is still breast cancer and has the same genetic makeup as the primary breast tumor.

In order to travel over the body, epithelial breast cancer cells have to undergo a differentiation into mesenchymal stem cells. This differentiation is known as epithelial–mesenchymal transition (EMT.) The EMT process is reversible and it plays an important intrinsic role in organ development, wound healing, and fibrosis. The reversible nature of EMT makes it a source of interest when looking at possible drug targets. The shift from epithelial cells to mesenchymal stem cells results in a decrease in cell adhesions, and increase in cytoskeleton arrangement, matrix remodeling, and motility. Mesenchymal cell motility comes from specialized spindle morphology and cytoskeleton features [9]. There are two schools of thought on the factors that promote dissemination. Some believe in early dissemination and the importance of the microenvironment for selecting signaling pathways which lead to metastasis. Others believe in late dissemination in which the gradual mutation of the primary tumor results in the ability to metastasize [10]. Critical steps in the initial stages of EMT are the downregulation or silencing of E-cadherins, cell-

cell adhesion proteins, and the upregulation of N-cadherins and matrix metalloproteinases (MMPs), proteases that cleave endothelium proteins [11]. These steps work to break the cancer cells away from the surrounding epithelial cells and help them degrade and weaken the surrounding extracellular matrix (ECM). Once converted into mesenchymal stem cells, the MMP enzymes break down the extracellular matrix and deregulate ECM remodeling which allows the tumor to more easily invade local tissues [9]. The MMPs also help to break down the endothelial cells of lymphatic and blood vasculature. Due to tumor mediated angiogenesis, the blood vessels near the tumor are usually more permeable and leaky. Once inside the vasculature, the breast cancer cells are vulnerable to immune attack. They evade this by surrounding themselves with platelets and clotting factors. This most likely serves a dual purpose by also protecting the cells from anoikis, a form of apoptosis induced by the loss of cell-to-cell adhesions. Once safely in platelet clumps, the cancer cells travel within the vasculature until they come to a target organ with an ideal microvasculature, where the cancer cells attach to the wall of the blood or lymph vessels. Extravasation usually happens when a platelet clump associated with tumor cells is attracted to a weakened part of target organs microvasculature. The MMPs help to go through the endothelium of the vessels and into the parenchyma of the target organ [10]. The mesenchymal cells may then differentiate back into epithelial cancer cells in order to proliferate and form a tumor (figure 1.2). The target organ microenvironment must be conducive for proliferation, for example, the growth factor VEGF (vascular endothelial growth factor) is required to help ensure angiogenesis to support the newly growing secondary tumor [10]. This idea is known as the “seed and soil” theory. The microenvironment of the “soil” has to be specifically suited to the “seed” for it to grow

[11]. One major example of an extrinsic factor of microenvironment associated with breast cancer metastasis is hypoxia and hypoxia-inducible factors (HIFs.) These factors, specifically HIF-1 help promote motility, intravasation, platelet clumping in the vasculature, and formation of proper "soil" for metastatic invasion [11].

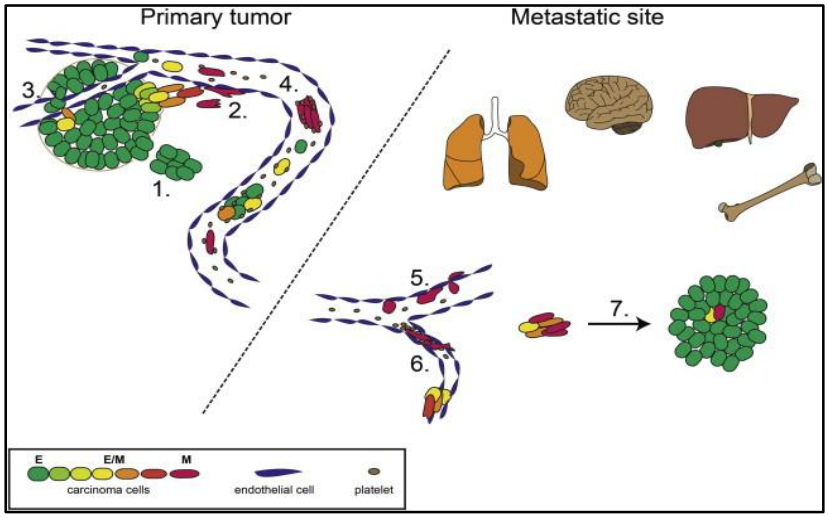

Figure 2 ***The significance of EMT and MET in breast cancer tumor metastasis***

Ruben, B. (2015, June 22)

Secondary symptoms can precede a diagnosis of metastatic breast cancer. Headaches, seizures, and drowsiness are associated with brain metastasis. Pain and frequent fracturing can be a symptom of bone metastasis. In the liver, metastasis presents with jaundice and a swelling of the abdomen. Lung metastasis is associated with chronic shortness of breath. Treatment plans to address metastasis are often primarily aimed at helping prolong and improve the quality of remaining life [12]. That means that all such treatments can do to address metastasis is treat symptoms and prevent further spreading of the cancer. Metastasis is sometimes approached surgically, but generally, based on the type of primary cancer, it is treated with hormone therapy or chemotherapy. There are some

newer targets that have been explored to help treat metastasis. Bevacizumab is a monoclonal antibody to VEGF. It is an antiangiogenic therapy used to prevent the formation of new blood vessels around tumors. It has shown an increase in progression-free survival time. Unfortunately, bevacizumab is a high-risk option specifically for breast cancer patients. It is associated with hemorrhaging, severely elevated blood pressure, and heart failure in breast cancer patients [10]. Another new drug therapy is called trastuzumab (Herceptin). It is a monoclonal antibody of the Her2+ antigen. This therapy has proven effective in metastasis of Her2+ cancers [10]. There remains considerable room for research and development in treating metastatic breast cancer.

HISTONE DEACETYLASES:

Eukaryotic DNA is packaged tightly together in the nucleus of every cell. Strands of DNA wrap around positively charged proteins rich in lysine and arginine called histones. The positive charge of histones helps to stabilize the negatively charged DNA. A histone octamer wrapped with 146 DNA base pairs, that make up a nucleosome. The octamer contains pairs of four core histones: H3, H4, H2A, and H2B. An H1 histone helps to ensure the DNA is wrapped effectively around the nucleosome [13]. A nucleosome is a subunit of chromatin, which coils tightly to form a eukaryotic chromosome.

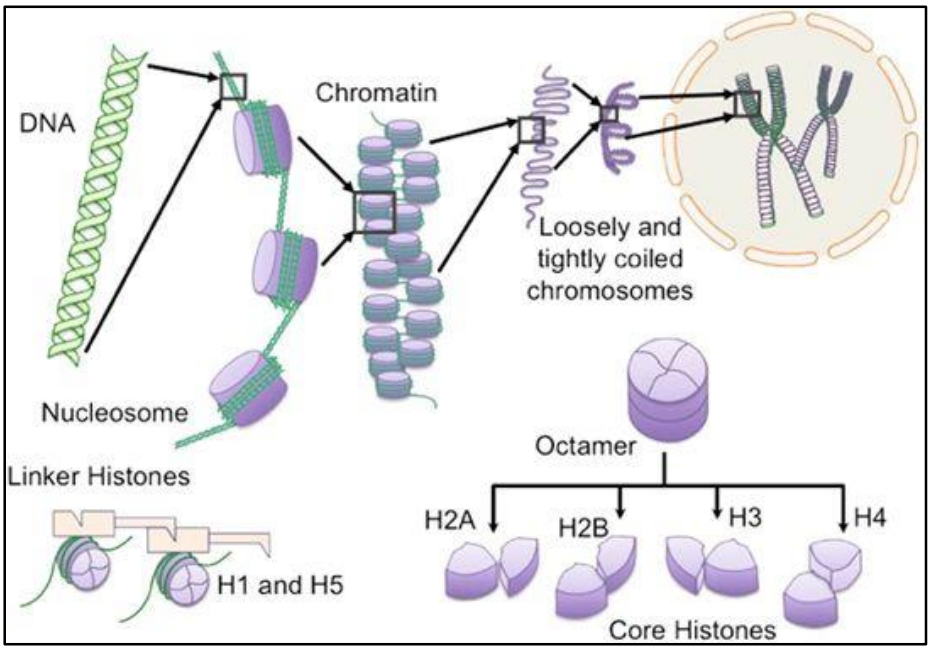

Figure 3 *Histone structure and organization (*Silk, E. (2017, May 25))

Transcriptional regulation is strongly influenced by the packaging of the DNA. Histone modifications can affect the accessibility of DNA and therefore the expression of genes. Acetylation is one of the ways that histones are modified. Histone acetyltransferase protein can acetylate the tail of histones in a nucleosome. The acetylation occurs at the ε-amino group of conserved lysine residues at the N-terminal of histone tails. In vivo, all core histones are acetylated, but H3, and H4 tails are more commonly acetylated than those of H2A/H2B dimers [14]. The added acetyl group acts to mask the positivity of the histones, loosening the tightly wrapped negatively charged DNA and exposing it for transcription. Therefore, generally, hyperacetylation results in an increase of gene expression, whereas, hypoacetylation results in the opposite effect [14].

Acetylation of histone tails is reversible and balanced by the activities of histone acetyltransferases (HATs), and histone deacetylases (HDACs), which deacetylate the histone tails [15]. In humans, there are 18 HDACs which are divided into four classes [13].

Class I is made up of HDAC 1, HDAC 2, HDAC 3, and HDAC 8. These are Rpd3-like proteins, meaning they have a similar mechanistic profile as that of the protein in Rpd3 yeast [15]. Class I HDACs are generally located in the nucleus and are ubiquitously distributed throughout the body [16]. Class II consists of HDAC 4, HDAC 5, HDAC 6, HDAC 7, HDAC 9, and HDAC 10. HDAC 10 and HDAC 6 are slightly different than the rest in this class and so are subdivided as Class IIb. All of these are Hda-1-like proteins, meaning they are similar to the Hda-1 yeast protein [15]. They can be moved between the nucleus into the cytoplasm, which suggests a potential for involvement with non-histone proteins. These HDACs are also expressed in a more tissue-specific fashion than Class I [16]. Class IV, as of now, is made up of only HDAC 11 and is homologous to Class I and II [15]. These three classes (I, II, IV) are known as the classical families (Table 1) [14]. Class III HDACs are Sir2-like proteins and have a mechanism involving NAD+, where the classical families have a zinc-dependent mechanism for deacetylation [15].

Table 1 *Classical Families of histone*

Class I	HDAC 1-3
	HDAC 8
Class II	HDAC 4-7
	HDAC 9,10
Class IV	HDAC 11

Not only can HDACs regulate gene expression through direct deacetylation of histones, they can also act in corepressor complexes with nuclear receptors, in direct

interaction with transcription factors, and in the deacetylation of non-histone proteins, making it possible for them to alter regulation of important cell functions and cellular homeostasis [16]. Specific HDACs have been linked to metabolic, inflammatory, immunological, cardiac, pulmonary, and neurodegenerative diseases, as well as cancer [15]. The loss of acetyl groups on Lys16 and the trimethylation of Lys20 of Histone 4 has been found to be a common event in human cancers. Methylation acts in opposition to acetylation. The reduction of acetylation of certain histones has also been more specifically linked to tumor invasion and metastasis [16]. Further exemplifying the relationship between HDACs and cancer, some gastric, prostate, colon, and breast cancer tumor samples have been found to have increased levels of HDAC 1. Similarly, HDAC 3 and HDAC 6 have been linked closely to colon and breast cancer. The increased levels of HDACs in tumor cells points to a potential involvement in the transcriptional repression of tumor suppressor genes [16]. They are also associated with cancer cell progression and metastasis through the modification of non-histone proteins. One example is the transcription factor SNAIL which recruits HDAC 1 and HDAC 2 along with their corepressor complex to repress the expression of E-cadherin, a crucial player in EMT [16]. Another non-histone target of HDACs closely linked to cancer is p53, an important tumor suppressor protein involved in the cell cycle. The deacetylation of p53 promotes its ubiquitination (degradation.) Without p53, cells can reenter the cell cycle with limited restriction. This contributes to cancer cell proliferation and tumor growth [15]. Tumor angiogenesis has also been associated with an increase in HDAC and their deacetylation and promotion of HIF-1〈, VEGF, and MMPs which are all important proteins associated

with tumor invasion [15]. All of this points to HDACs as prime drug targets for metastatic cancers.

HDAC 6 is unique in its tendencies to stay in the cytoplasm and primarily deacetylate non-histone proteins instead of chromatin proteins involved with gene expression [17]. Among many proteins that HDAC 6 is known for targeting are alpha-tubulin, cortactin, Hsp90 (heat shock protein 90), ®-caderin, and survivin. These proteins are associated with cell migration, F-actin binding, protein degradation, cell proliferation, and suppression of apoptosis respectively [17]. Hypoacetylation of alpha-tubulin promotes chemotactic cell movement. HDAC 6 appears to play a big role in tumor metastasis. One indication of this idea is the relationship between HDAC 6 and human enhancer filamentation 1(HEF1), a prometastatic scaffolding protein which phosphorylates HDAC 6, enhancing tubulin deacetylase activity and regulating cilia assembly by HDAC 6 [17]. Another role HDAC 6 plays in cells is colocalizing with G-Protein-Coupled Receptor Kinase 2 (GRK2) and interacting in the lamellipodia of migrating cells. The increased deacetylation of local tubulin proteins increases motility. HDAC 6 is required to modulate the invadopodia activity and invasion in many types of cancer. There is a significant link between higher levels of HDAC 6 in breast cancer cells and metastasis, which corresponds to the role it seems to play in the migration and movement of the cell. It also promotes angiogenesis by deacetylating cortactin in endothelial cells, helping to regulate migration and sprouting of cancer cells. Additionally, it has been found to upregulate HIF-1 and VEGF, both highly involved proteins in tumor angiogenesis [17]. HDAC 6 is also linked with cell proliferation. HDAC 6 associates with the transcription factor Runt Related Transcription Factor 2 (RunX2) which results in the repression of p21, a vital protein in

the cell cycle. The cylindromatosis (CYLD) gene, is a deubiquitinating enzyme and tumor suppressor gene. It controls cells growth, division, and cytokinesis through a-tubulin and microtubules. It also inhibits HDAC 6. Many types of cancers are found to have decreased levels of CYLD [17]. The regulation and repression of HDAC 6 may be a target to help increase expression of CYLD. In addition to proliferation and migration of the cell, HDAC 6 is involved in apoptosis. They regulate the Ku70 protein through deacetylation, preventing apoptosis. They also regulate the oncogenic protein survivin, which is expressed primarily in ER+ breast cancer. Survivin is acetylated in the nucleus, which masks its anti-apoptotic properties. HDAC 6 localizes to perinuclear regions and deacetylate the oncogenic protein, blocking apoptosis in cancer cells [17]. All of these facets, make HDAC 6 a prime target for anticancer drugs, especially in metastatic cancer cells, but a lot is still unknown about the mechanisms of its involvement in cancer.

The multifaceted roles of HDACs in cancer, give a wide range of anticancer drug targets. The mechanisms of anticancer effects are not uniform, but vary with the type of cancer being treated and with the individual inhibitor target HDAC class. There are five classes of HDAC inhibitors: hydroxamic acids, short- chain fatty acids, benzamides, cyclic tetrapeptides, and sirtuin inhibitors [18]. As of 2017, there were four approved HDAC inhibitor drugs to treat cancer. The first was a hydroxamic acid called Vorinostat, or SAHA. Vorinostat is a pan-HDAC inhibitor approved to treat cutaneous T-cell lymphoma. Belinostat is also a hydroxamic acid-based pan-inhibitor, and it is approved to treat peripheral T-cell lymphoma. Panobinostat is similar, but it is used in the treatment of multiple myelomas. Romidepsin is a cyclic tetrapeptide HDAC isoform-selective inhibitor which selects for class I HDACs. It is approved to treat cutaneous T-cell lymphoma [18].

There are many other HDAC inhibitors still undergoing various stages of trials and testing. One of these is Ricolinostat, a benzamide-based inhibitor, which selectively targets HDAC 6. HDAC inhibitors have been found to induce apoptosis, alter cell migration, activate cell differentiation, impair tumor angiogenesis, and upregulate the expression of tumor suppressor genes and proteins [18].

In this experiment we sought to look more closely at some HDAC inhibitors and their effects over multiple breast cancer cell lines. Specifically, we wanted to explore the behavior of HDAC inhibitors known for targeting non-histone microtubule proteins in comparison with nuclear HDAC inhibitors and known chemotherapeutic microtubule stabilizer and destabilizers. We tested multiple metastases lines in order to investigate the possibility of cell-line dependency on HDAC inhibition.

METHODS

BACKGROUND RESEARCH:

The undertaking of this thesis began over two years ago in the fall semester of 2016. We were involved in an introductory cancer research class in which we learned more about cancer and its mechanisms. In the spring of that year, we began to subdivide our class into specific interest groups. We began planning our approach to study the effects of HDAC inhibition in metastatic cancer cells with literary searches of existing research and scholarly sources. We began looking at different HDAC inhibitors and oncological compounds of interest. The layout of our experiment started by focusing on existing drug treatments and on the National Cancer Institute Developmental Therapeutics Program NCI-60 cell line database, a resource which makes relevant concentration responses known for different

compounds on specific cell lines. Under the guidance of Dr. Zhou, we were able to look at the available compounds and create an experimental layout. We wanted to make sure that each class of classical families of HDACs were represented through an inhibiting compound. We used two pan-HDAC inhibitors, an inhibitor selective to class 1 HDACs, and an inhibitor selective for the class II HDAC 6 enzyme specifically. The HDAC 6 inhibitors were of particular interest because of HDAC 6's known selectivity towards non-histone microtubule targets. We also included in our experiment known microtubule stabilizers and destabilizers in order to study them in conjunction with the activity of HDAC specific inhibition. The comparison might give a better understanding into the involvement of non-histone targets of HDACs such as the a-tubulin protein in microtubules.

DRUG COMPOUNDS:

Vorinostat (SAHA)

We purchased Vorinostat from Cayman Chemical Company. It was dissolved with DMSO to make a 10 mM stock solution. It is a pan-HDAC inhibitor

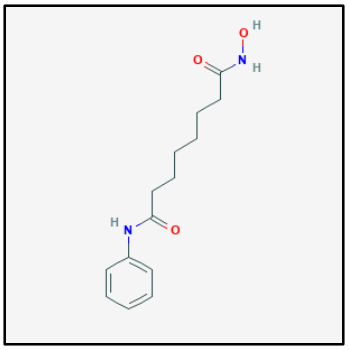

Figure 4 *Vorinostat*

Panobinostat

We purchased Panobinostat from Cayman Chemical Company. It was dissolved with DMSO to make 100 µM stock solution. It is a pan-HDAC inhibitor.

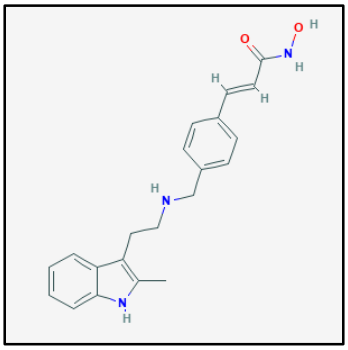

Figure 5 *Panobinostat*

Romidepsin

We purchased Romidepsin from Cayman Chemical Company. It was dissolved with DMSO to make a 100 µM stock solution. It is a selective inhibitor for class I HDACs.

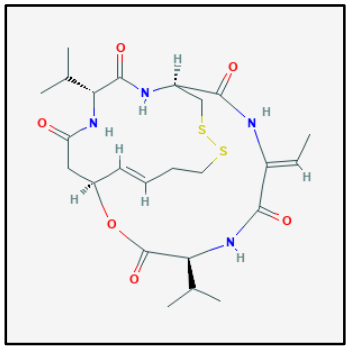

Figure 6 *Romidepsin*

Ricolinostat

We purchased Ricolinostat from Selleck Chemical Company. It was dissolved with DMSO to make a 10 mM stock solution. It is an inhibitor selective for HDAC 6.

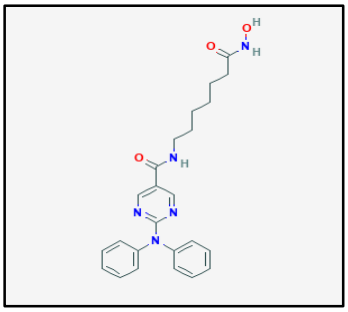

Figure 7 *Ricolinostat*

Paclitaxel (Taxol)

We purchased Paclitaxel from Sigma Aldrich. It was dissolved with DMSO to make a 10 mM stock solution. It is a microtubule stabilizer.

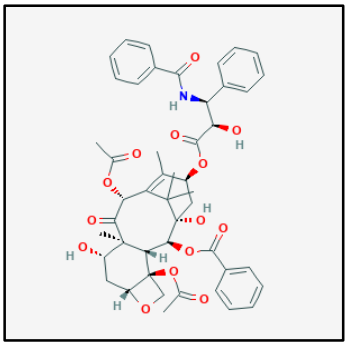

Figure 8 *Paclitaxel*

Epothilone B

We purchased Epothilone B from Selleck Chemical Company. It was dissolved with DMSO to make a 10 mM stock solution. It is a microtubule stabilizer.

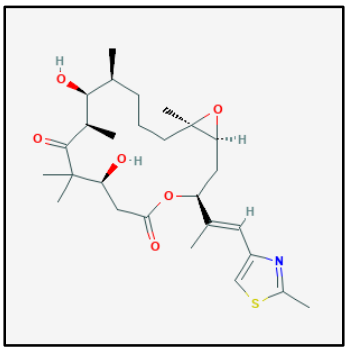

Figure 9 *Epothilone B*

Colchicine

We purchased Colchicine from Sigma Aldrich. It was dissolved with DMSO to make a 100 µM stock solution. It is a microtubule destabilizer.

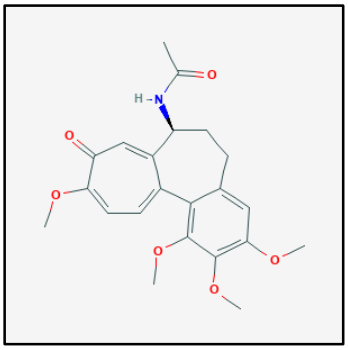

Figure 10 *Colchicine*

Vinblastine

We purchased Vinblastine from Sigma Aldrich. It was dissolved with DMSO to make a 100 µM stock solution. It is a microtubule destabilizer.

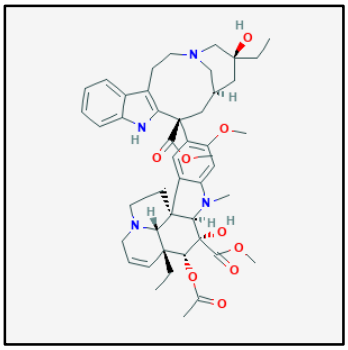

Figure 11 *Vinblastine*

CELL LINES:

We looked at the effects of HDAC inhibition over three cells lines: BOM, LM, and MCF-7-BOM. Both BOM and LM are derived from the triple-negative breast cancer cell line, MDA-MB-231. It was originally isolated over 40 years ago from a pleural effusion in a breast cancer patient with a metastatic relapse several years after her primary tumor removal [19]. MDA-MB-231 is highly selective for metastasis and has specific tissue dissemination selectivity to the bone and lung [20]. We are using a subpopulation derived from MDA-MB-231, BOM-1833, which is highly selective for metastasis to the bone when injected into the arterial circulation of immunodeficient mice [19]. Another derivative of MDA-MB-231 that we used is LM-4175, a derivative cell line, which is highly aggressive towards lung metastasis in mice [20]. MCF7-BOM is metastatic variant of derived from MCF7, a well characterized human breast cancer cell line. MCF7 is an ER positive breast cancer cell line that originated from a 69-year-old Caucasian female in the 1970's [24]. Both the MDA-MB-231 variant lines (BoM-1833, LM-4175) and the MCF-7 variant line (MCF7-BoM) were cultured in Dulbecco's modified Eagle's media (DMEM)/F12 that were supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics [19]. All of the metastases lines used in our research were first generated by Dr. J Massagué at the Sloan Kettering Cancer Center, New York City, New York [19]. Our research group obtained all of the metastatic clones from Dr. Kounosuke Watabe at Wake Forest University [24].

PREPARING STOCK AND WORKING DILUTIONS:

To begin preparing the compounds for testing, we removed powdered compounds from 4ºC and let them warm up to room temperature for weighing on an analytic balance. Using DMSO, dimethyl sulfoxide, we prepared 10mM stock solutions for compounds with the higher range of concentration (vorinostat, paclitaxel, epothilone B, and ricolinostat) and 00 µM stock solutions for those needing a lower range (romidepsin, panobinostat, colchicine, and vinblastine.) Then using a serum free media, we diluted the stock solution to the highest tested concentration at 2x final concentration in the mother plate. We prepared the mother plate at 3.5x times of the required volume, since for the first round of viability we were working with three 96-well plates. We used a 1:10 serial dilution to prepare the working solutions at the lower concentrations. From the well on the mother plate with the highest concentration, we took same volume of solution and added it to a lower well in a stepwise fashion, and then we diluted it to the desired concentration using serum free media, achieving the desired 350 µL end volume for each well. For example, we took 35 µL of the 0.1 µM solution well and put in into a well with 315 µL of serum free media to create 350 µL of 0.1 µM solution as seen in **figure 12**.

	Media 1	Virinostat 2	Paclitaxel 3	Epothilone B 4	Ricolinostat 5	Romidepsin 6	Panobinostat 7	Colchicine 8	Vinblastine 9	CAY10603 10	Cycloheximide 11	Media 12
A	400µL media	1.008µL (10 mM Stock) + 505.4 µL media	10µM	10µM	10µM	1.008µL (100 µM Stock) + 505.4 µL media	0.1µM	0.1µM	0.1µM	0.1µM	350 µL of 10 µM CHX	400µL media
B	400µL media	116.55 µL (A) + 271. 95 µL media	3µM	3µM	3µM	116.55 µL (A) + 271. 95 µL media	0.03µM	0.03µM	0.03µM	0.03µM	350 µL of 10 µM CHX	400µL media
C	400µL media	38.85 µL(A) + 349.65 µL media	1µM	1µM	1µM	38.85 µL (A) + 349.65 µL media	0.01µM	0.01µM	0.01µM	0.01µM	350 µL of 10 µM CHX	400µL media
D	400µL media	38.5 µL (B) + 346.5 µL media	0.3µM	0.3µM	0.3µM	38.5 µL (B) + 346.5 µL media	0.003µM	0.003µM	0.003µM	0.003µM	350 µL of 10 µM CHX	400µL media
E	400µL media	38.5 µL (C) + 346.5 µL media	0.1µM	0.1µM	0.1µM	38.5 µL (C) + 346.5 µL media	0.001µM	0.001µM	0.001µM	0.001µM	350 µL of 10 µM CHX	400µL media
F	400µL media	35 µL (D) + 315.0 µL media	0.03µM	0.03µM	0.03µM	35 µL (D) + 315.0 µL media	0.0003µM	0.0003µM	0.0003µM	0.0003µM	350 µL of 10 µM CHX	400µL media
G	400µL media	35 µL (E) + 315 µL media	0.001µM	0.001µM	0.001µM	35 µL (E) + 315 µL media	0.0001µM	0.0001µM	0.0001µM	0.0001µM	350 µL of 10 µM CHX	400µL media
H	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media	400µL media

Figure 12 *Serial Dilution in 96-well plate*

SRB VIABILITY ASSAY:

Once we had our compounds and concentration ranges selected we ran several SRB viability assays. The SRB, or Sulforhodamine B Assay, is a rapid, sensitive, and relatively economical tool for looking at cell proliferation and chemosensitivity. To start the assay, the cells were taken out of incubator, the conditioned media removed, and the cells detached withtrypsin (1 ml per 10 cm plate), then the reaction stopped with 10 mL media that contains 10% FBS. The media used to grow the cells was RPMI-1640 with L-glutamine supplemented with 10% FBS and penicillin and streptomycin, known as "complete media." With the help of a hemocytometer to obtain average cell count, we were able to dilute the cells with additional 10% FBS media until we had a solution of three million cells per mL of media. We seeded the cells in a 96-well plate at a concentration of 30,000 cells per well. (100uL cell solution, 100uL media). The plates were cultured at 37ºC

in a humidified 5% CO_2/ 95% air environment for 24 hours. The cells were then treated with 10 µM-0.01 µM concentration scale of vorinostat, paclitaxel, epothilone B, and ricolinostat, and with a concentration scale of 0.1 µM - 0.0001 µM for romidepsin, panobinostat, colchicine, and vinblastine. The plates were incubated and removed after 48 hours. To fix the cells, 100 µL of conditioned media was removed from each well, and 100 µL of a 20% trichloroacetic acid (TCA) in 1x PBS solution added. These plates were set aside for an hour at 4ºC. The plates were washed with tap water four times and left to dry. To the dry wells, 75 µL of SRB (0.4% w/v in 1% acetic acid) was added and allowed to sit. After 10 minutes the plates were washed with 1% acetic acid three times then allowed to dry. To extract the dye, 100 µL of 10 mM Tris Base was added to each well, and the plates were gently shaken for 10 minutes on the microplate genie. Using a Spectrafluor plate reader and Magellan software, the plates were read at 490nm with background620 nm absorbance. The inhibition of each compound on each cell line can be found because the SRB dye binds to basic amino acids in cellular proteins of the cells [21]. The colorimetric valuation gives an estimate of the total protein mass per well which is proportional to cell number per well.

CLONOGENIC ASSAY:

Clonogenic assays are used to determine the effect of cytotoxic compounds on the reproductive and proliferative properties of cells. To determine the effectiveness of the chemotherapeutic agents used, we looked at the relative number of colonies proliferated over a period of ten days. We tested two cells lines, MCF7-BoM and BoM-1833. We looked at the effect of high and low concentrations of vinblastine, panobinostat,

romidepsin, SAHA, ricolinostat, paclitaxel, epothilone B, and colchicine over three the area six-well plates. That layout of these plates can be seen in **figure 16**. The cancer cells were seeded at 3,000 cells per well and incubated for 4 hours at 37ºC to allow the cells to adhere. The compounds were added with media at their respective concentrations to the wells for 24 hours. At that point, the compound filled media was swapped out for fresh (10%) FBS media with antibiotics. This was set aside for 5 days, at which time the media was switched out again. After 10 days, the cells were fixed with methanol and stained with crystal violet (1 mg/mL in 20% ethanol) in order to see the proliferation of the cells clearly. The concentrations of the compounds tested were chosen based on physiological relevance and cytotoxic potential (**Table 2**).

Table 2 *Clonogenic assay tested concentrations*

Compound	High Concentration	Low Concentration
Paclitaxel	1.0 µM	0.1 µM
Epothilone B	0.01 µM	0.001 µM
Colchicine	0.1 µM	0.01 µM
Vinblastine	0.1 µM	0.01 µM
SAHA	10 µM	1.0 µM
Ricolinostat	10 µM	1.0 µM
Romidepsin	0.01 µM	0.001 µM
Panobinostat	0.1 µM	0.01 µM

COMBINATION ASSAY:

We looked at histone deacetylase inhibitors in combination with other relevant chemotherapeutic agents. The procedure we followed was very similar to that of the viability assay. Paclitaxel and epothilone B are both microtubule stabilizer. Romidepsin,

ricolinostat, and panobinostat were the HDAC inhibitors that we used. We combined the stabilizers with the HDAC inhibitors as seen in **figure 13**. We chose to test these three HDAC inhibitors specifically because we wanted a range of HDAC class selectivity. Romidepsin is the most active and found to be selective for HDAC class 1. Panobinostat is a good control inhibitor, because it is a pan HDAC inhibitor. Ricolinostat is selective for HDAC 6. The stabilizers were selected based on their clinical effectiveness. Each compound was tested at a concentration range based on active concentrations found on the NCI database and taking into consideration the highest achievable concentration in patient plasma concentrations. We set up the experiment hoping to gauge the activity of the two compounds in combination whether they be synergistic, antagonistic, or additive.

	Romidepsin V V	V V	V V	**Ricolinostat** V V	V V	V V	**Panobinostat** V V	V V	V V	V V
Taxol 0nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
Taxol 10nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
Taxol 100 nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
Taxol 1µM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
EpoB 0nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
EpoB 10nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
EpoB 100nM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM
EpoB 1µM ===>	0.001 µM	0.01 µM	0.1 µM	0.1 µM	1 µM	10 µM	0.0001 µM	0.001 µM	0.01 µM	0.1 µM

Figure 13 *Combination assay plate layout*

RESULTS AND DISCUSSION

VIABILITY ASSAY RESULTS:

The percent inhibition of the HDAC inhibitors, microtubule stabilizers, and destabilizers at different concentrations in different cell lines was graphed to display any relationship between concentration and inhibition or cell line selectivity. The graphs were put together using the software Prism 8 (GraphPad).

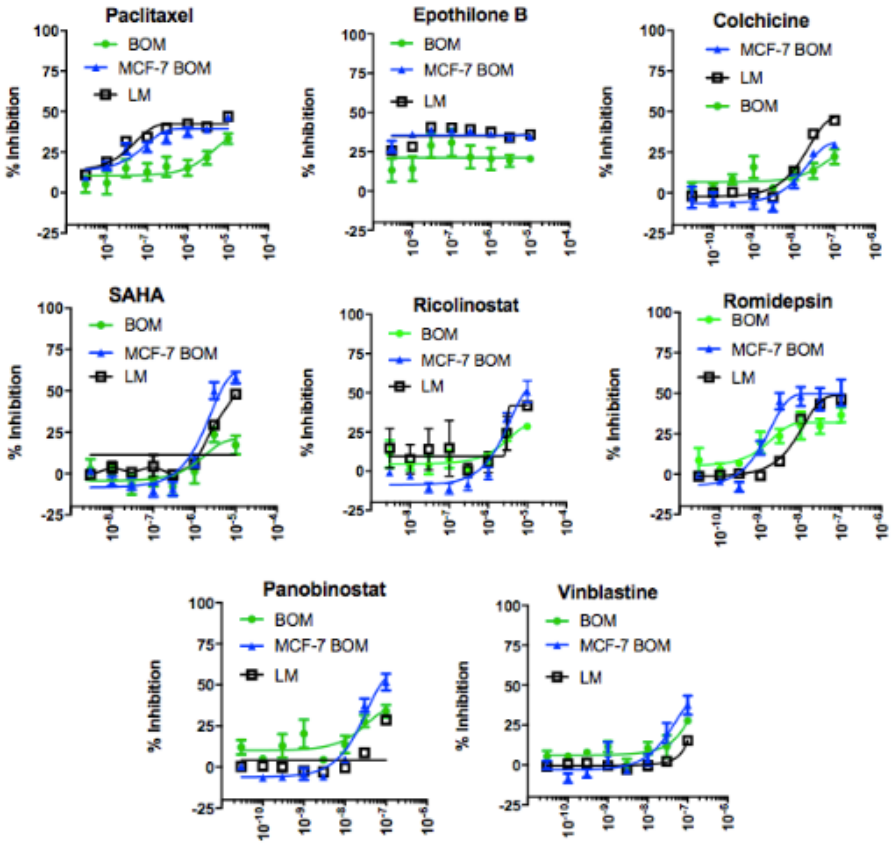

Figure 14 *Inhibition of BOM, LM, and MCF7-BOM over different drug concentrations.*

None of the compounds were able to inhibit cell proliferation enough to establish an IC_{50}, a measure used to indicate the concentration at which a compound can inhibit cell growth at one-half of the maximum inhibition. The $IC5_0$ is a commonly used tool which helps to measure drug activity [22]. The concentration ranges used for the compounds may

have been too low. In the future, more testing may help to see the effectiveness of these compounds at higher concentrations. While IC_{50} values were not achieved, we did note the concentration with the highest inhibitory effect for each compound over the three cell lines. This can be seen in **Table 3**.

Table 3 *Highest average Inhibitory Concentrations over three cell lines*

Compound	Highest Inhibitory Concentration					
	BOM		MCF7-BOM		LM	
	% Inhibition	Concentration	% Inhibition	Concentration	% Inhibition	Concentration
Paclitaxel	33%	10μM	47%	10μM	47%	10μM
Epothilone B	31%	0.1μM	41%	0.03μM	39%	0.1μM
Colchicine	22%	0.1μM	44%	0.1μM	30%	0.1μM
Vinblastine	28%	0.1μM	16%	0.1μM	38%	0.1μM
Vorinostat (SAHA)	24%	3μM	48%	10μM	58%	10μM
Romidepsin	36%	0.1μM	46%	0.1μM	50%	0.1μM
Ricolinostat	29%	10μM	42%	10μM	51%	10μM
Panobinostat	34%	0.1μM	29%	0.1μM	52%	0.1μM

The highlighted cells in **Table 3** indicate that the highest inhibitory concentration is the highest concentration of the compound that was tested. This is the case for almost all of the compounds, suggesting that, if tested again at higher concentrations, the percent Inhibition may increase with concentration. The outlier of this group of compounds was Epothilone B, a microtubule stabilizer known for the its potency in the treatment of paclitaxel-resistant cells [23]. Epothilone showed inhibition even at the lowest concentration tested and had consistent inhibition over its tested concentration range. There

was not a trend to suggest a sensitivity to increasing concentration on inhibition though. Further testing may help to shed light onto the effect of epothilone B on these three breast cancer cell lines.

While complete inhibition was not achieved by any of the compounds, some of the highest inhibition percentages (74% with romidepsin) were found in the plates containing the MCF-7 BOM cells. The MCF-7 BoM is an ER+ cell line unlike BoM and LM which are both triple-negative breast cancers [24]. This may suggest that HDACs are more involved in the mechanisms surrounding the ER+ breast cancer than in triple negative breast cancer. HDAC inhibition has been connected to the inhibition of MCF-7 breast cancer cells in previous studies. Specifically, their relationship involving the Akt serine-threonine family of kinase, which signal regulation of proliferation, protein translation, and apoptosis [25]. The down regulation of Akt proteins is just one potential mechanism among many that may be associated with HDAC inhibition in ER+ breast cancer. Many mechanistic properties involved with HDACs and HDAC inhibition are not well known, but may play a role in cancer cell proliferation.

Another round of viability assays was performed specifically with the BoM-1833 cell line. The results of which can be seen in Figure 16.

While the mechanisms behind the HDAC inhibitors are complex and largely still under investigation, these viability assays show a clear effect on the tumor cells. HDAC inhibitors, whether it be epigenetically or through non-histone target, seem to inhibit tumor cell growth in most cases.

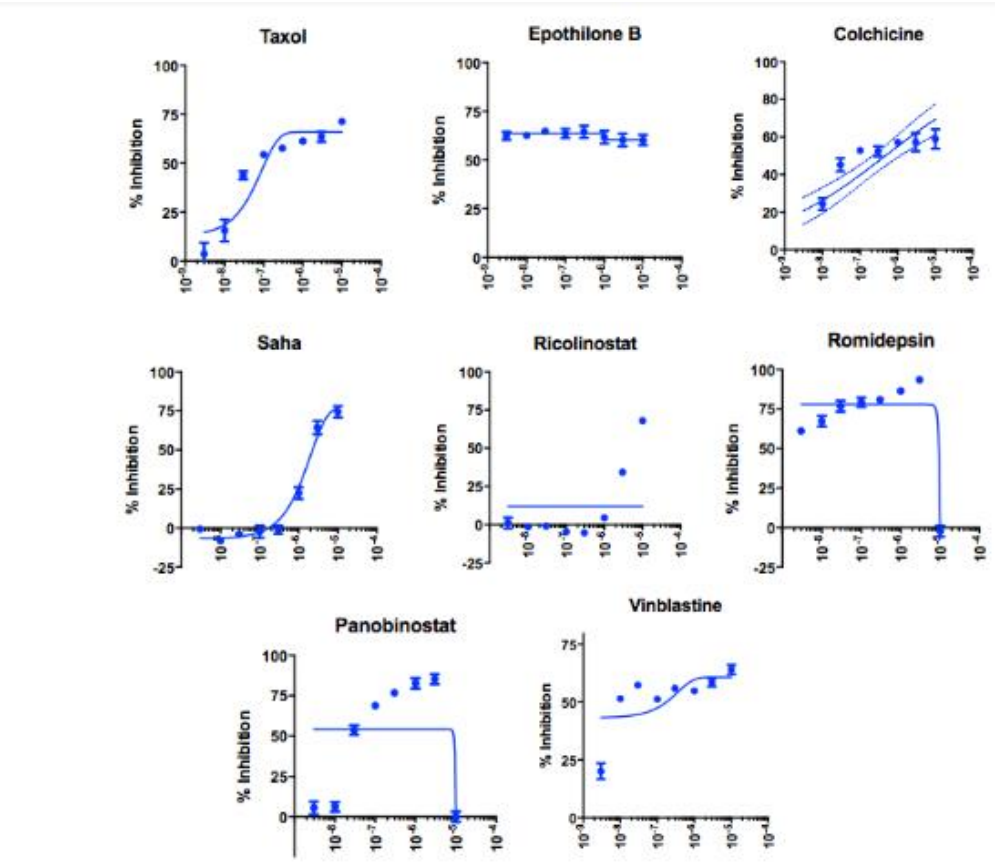

Figure 15 *Inhibition over different drug concentrations in BOM cells.*

These viability assays came out a little more strangely. Like the earlier, multi-cell line assay, none of these were able to completely inhibit the breast cancer cells and, therefore, IC_{50} values were not established. Again, these compounds should be subsequently evaluated again at higher concentrations. These BoM assays held a general pattern of increased inhibition with increased concentration, suggested by the prior results. The percent inhibition in general was larger in this experiment compared to the previous viability assay's inhibition. This could be because of an initial decline in the health of the BoM cells at the start of the second round of assays or a difference in lab technique proficiency between the time of the two assays. There were also a couple of compounds that produced a dramatic drop in inhibition at higher concentrations: panobinostat and romidepsin. These exhibited no inhibition at their highest tested concentration. This could

potentially be attributed to experimental error since it directly opposes the results of the first viability assay and is such a dramatic change in effect. More testing would help to clarify these results.

CLONOGENIC ASSAY RESULTS:

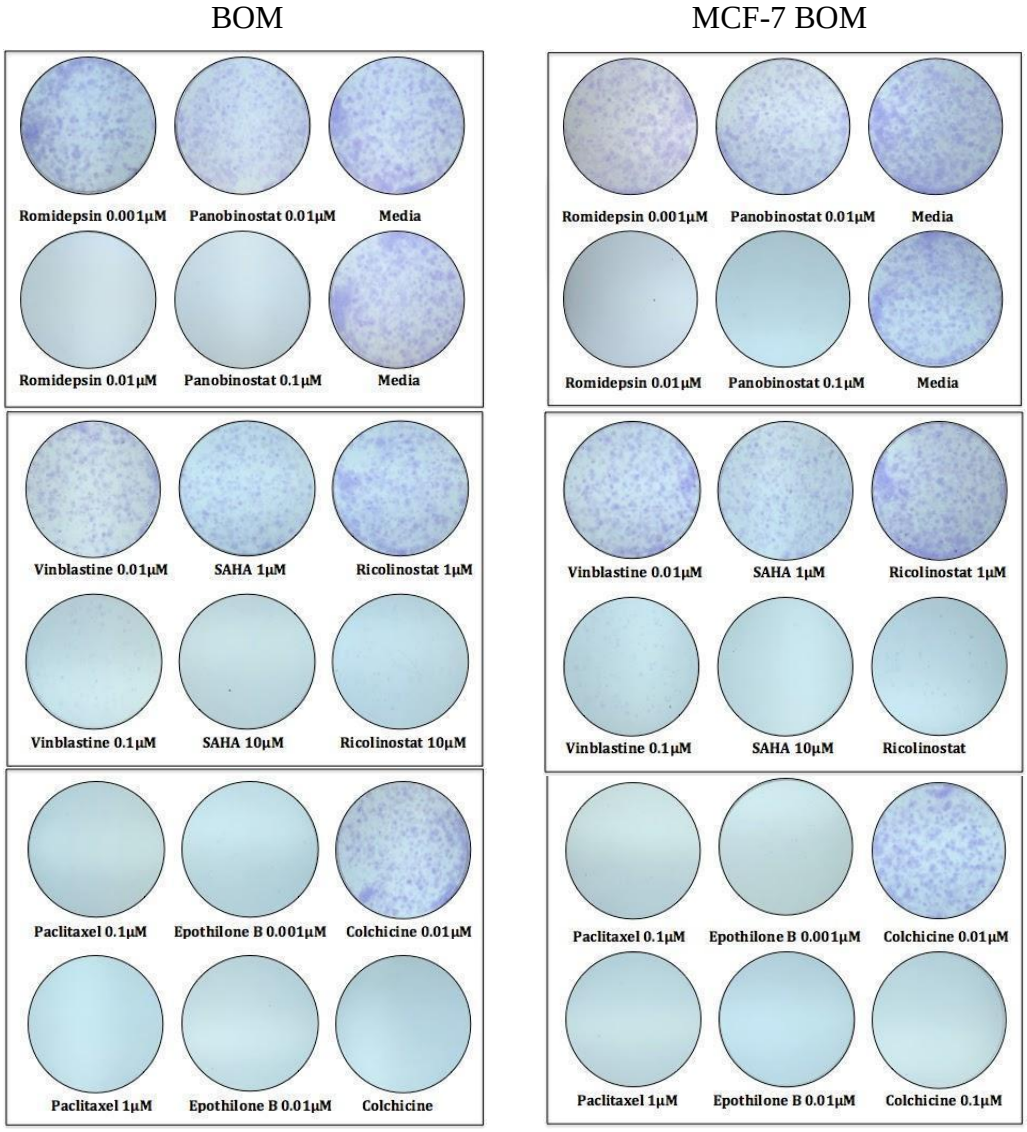

Figure 16 *Clonogenic Assay Plates*

These clonogenic plates show that the BoM and MCF-7 BoM cell lines seem to react similarly to each compound concentration. These results do not support cell-line-dependent inhibition. None of the HDAC inhibitors are effective at their lower tested concentrations. All of the HDAC inhibitors showed inhibition at their higher tested

concentration, though ricolinostat appeared not as effective as the others at its higher concentration. It is interesting that ricolinostat shows slightly different results than the other HDAC inhibitors. This could be accounted for by a change in tested concentration, but it is important to note that ricolinostat is the only HDAC class 2 inhibitor, being highly selective for HDAC 6 inhibition. These HDACs are more cytoplasmic and have more non-histone targets [16]. The slight difference could possibly be accounted for because of mechanistic differences between the class 2 histones and the others. The microtubule stabilizers tested (paclitaxel and epothilone B) showed apparent complete inhibition at both high and low tested concentrations. Neither of the destabilizers tested (colchicine and vinblastine) completely inhibited the cells at their lower concentrations. Colchicine seemed to inhibit the cells more fully than vinblastine at their higher tested concentration. Overall, this assay helps to assert that there is indeed a correlation between increased HDAC inhibitor concentration and the survival of the tumor cells.

COMBINATION ASSAY RESULTS:

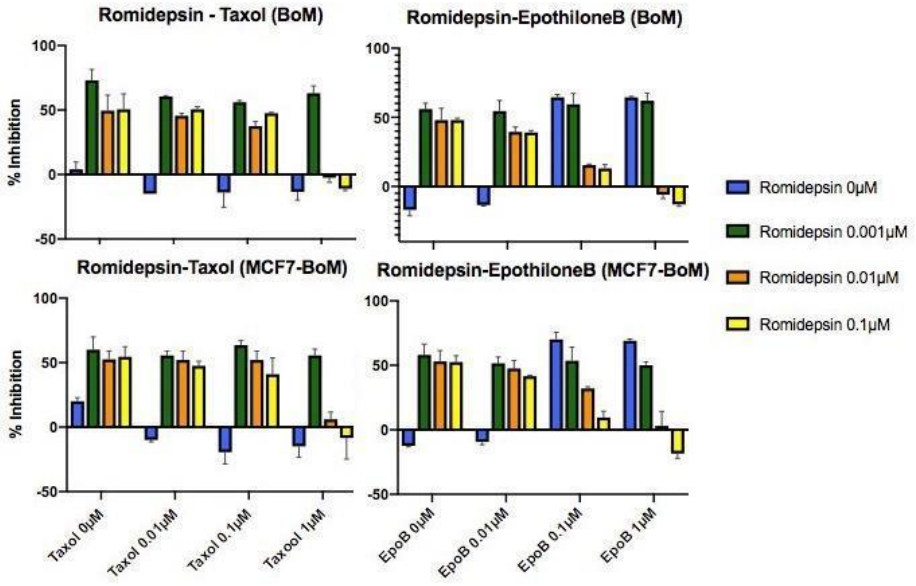

Figure 17 *Combination results between Romidepsin and microtubule stabilizers*

This figure shows the combination between Romidepsin, a class I selective HDAC inhibitor and the microtubule stabilizers Paclitaxel and Epothilone B in both BOM-1833 and MCF-7 BOM metastatic breast cancer cells. Taxol by itself in this test seems to have no inhibitory effect on the tumor cells. Epothilone B doesn't appear to add to the percent inhibition until it reaches higher concentrations (0.1 μM.) This is a different result than can be seen in the viability and clonogenic assays, in which epothilone B is inhibitory at very small concentrations. Once romidepsin and epothilone B are combined, there seems to be a downward trend in cell inhibition. They appear to be slightly antagonistic towards each other. As higher concentration of the HDAC inhibitor are added, the inhibition in reduced. Taxol and romidepsin show similar antagonistic results. Both of these combination results are supported by the National Cancer Institute (NCI) Combination Almanac database [27].

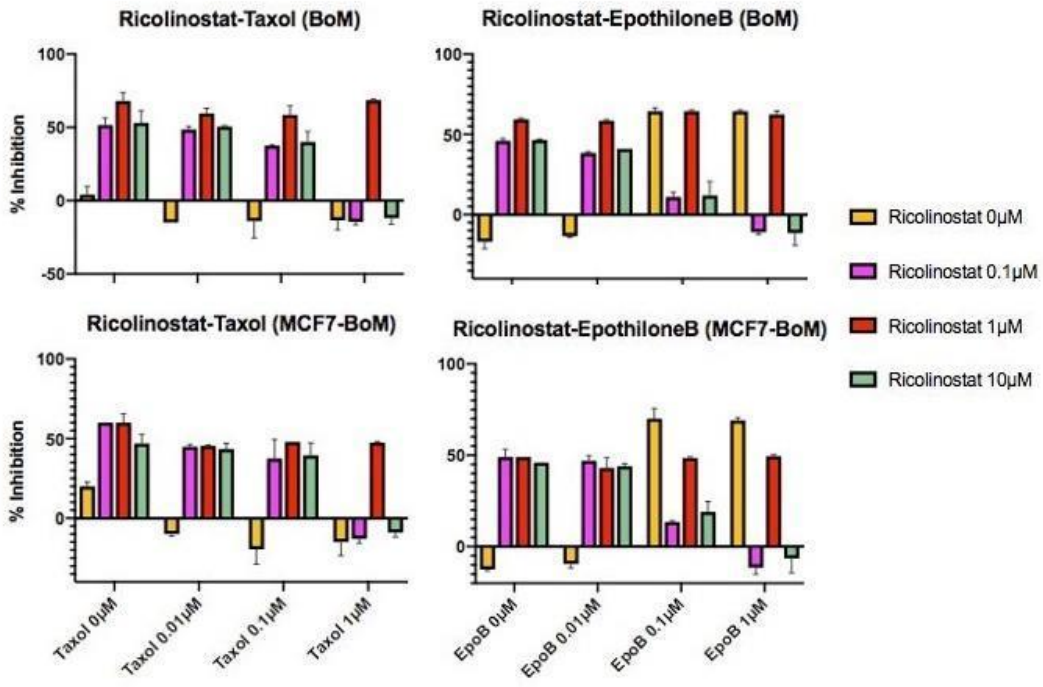

Figure 18 *Combination results between ricolinostat and microtubule stabilizers*

This figure shows the combination between ricolinostat, a class II HDAC 6 selective HDAC inhibitor and the microtubule stabilizers paclitaxel and epothilone B in both BOM and MCF-7 BOM metastatic breast cancer cells. Again, here Taxol seems to not be inhibitory towards the cells by itself and epothilone only seems to be effective at higher concentrations. The combination bars of these graphs are abnormal, especially surrounding the last two concentrations groupings of both microtubule stabilizer graphs. The results are too sporadic and opposing to make sense. There must have been some lab error involving those combinations. Because of the lack of information surrounding higher concentrations of epothilone B and Taxol, it is hard to say what the relationship there appears to be between the stabilizers and HDAC 6 inhibitor. This combination should be rerun to find more accurate and replicable results. Although the results from this assay set

are hard to interpret the general trend seems to be more antagonistic. However, previous research has shown that the combination treatment of paclitaxel and ricolinostat will be synergistic in the suppression of tumor growth. In cell lines from multiple solid tumor lineages, combination treatment with an HDAC-6 inhibitor and paclitaxel enhanced inhibition of proliferation and increased cell death compared to either single compound alone [26.]. Along with other effects, the inhibition of HDAC 6 results in the hyperacetylation of α-tubulin, which increases microtubule stability. This suggests that inhibition of HDAC 6 by selective or pan-HDAC inhibitors could increase in efficacy when in combination with microtubule stabilizers [26.]

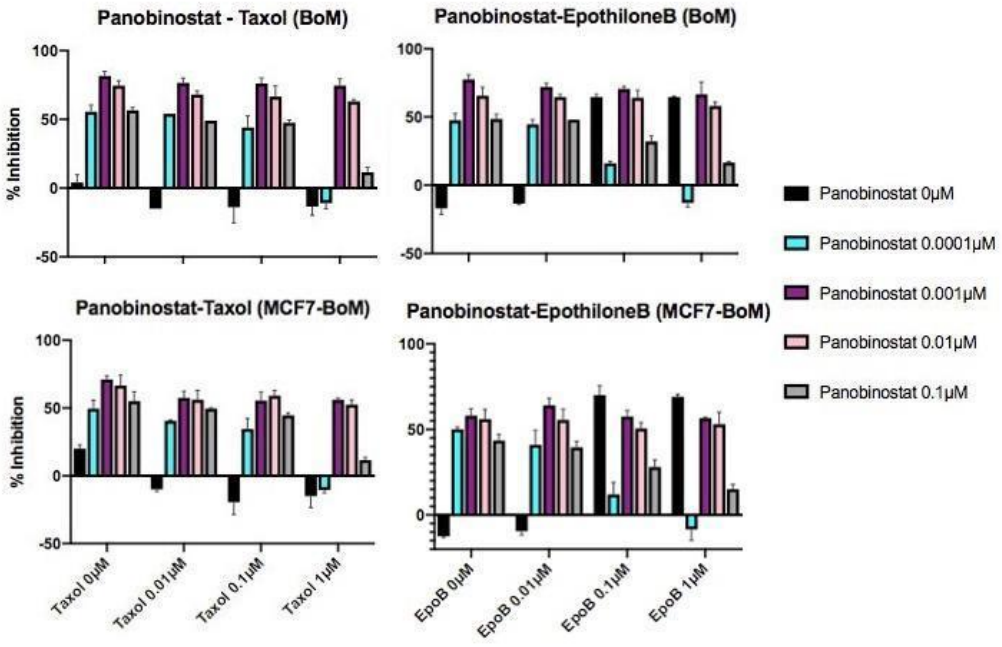

Figure 19 *Combination results between panobinostat and microtubule stabilizers*

Figure 19 shows the combination between Panobinostat, a pan selective HDAC inhibitor and the microtubule stabilizers paclitaxel and epothilone B in both BOM and

MCF-7 BOM metastatic breast cancer cells. Taxol and epothilone B show the same trends of inhibition as singular compounds as in the other two combination assays. The results suggest an antagonistic relationship in both the panobinostat/paclitaxel and panobinostat/epothilone B combinations. The inhibition appears to be strongest with 0.001 μM of panobinostat in all applications. The inhibition decreased as the concentration of the HDAC inhibitor increases.

These combination assays were done in replicate with $n = 2$.
In future rounds of these combinations, they would be repeated with at least $n = 3$ in order to ensure more accurate results.

The lack of independent inhibition from paclitaxel in all of these combinations was initially surprising because Taxol is an established chemotherapeutic drug. But recent studies have shown that while high doses of paclitaxel result in mitotic arrest, clinically relevant low concentrations of paclitaxel can increase multipolar spindle formation and subsequently increased the rate of aberrant mitosis and cell death [26].

CONCLUSIONS:

Using multiple experimental assays, the effects of HDAC inhibitors were tested on MDA-DB-231 derivatives, BOM-1833 and LM-4175 as well as on ER+ MCF-7 BOM metastatic breast cancer cells. Through viability assays, curves for percent inhibition were produced which did not have enough inhibition to find IC50 values, but did have a strong trend towards a potential for increased percent inhibition with further testing at higher concentration ranges. The viability assay also suggested a slight selectivity of inhibition between HDAC inhibitors and ER+ MCF-7 BOM cells over the two triple negative MDA-

MB-231 derivative cell lines. Clonogenic assays supported the effectiveness of higher concentrations of HDAC inhibitors on the inhibition of tumor cell growth, but presented evidence against cell-line-dependent inhibition. When combined with microtubule stabilizers, paclitaxel and epothilone B, the HDAC inhibitors showed an overall antagonistic effect. These results indicate that HDACs may be valuable targets for anti-cancer drugs over multiple cell lines. In future research, fluorescent protein labeling could be done to determine the molecular interactions involved in the inhibition of breast cancer cells by the HDAC inhibitors. This might better demonstrate the potential role of non-histone microtubule targets in tumor inhibition.

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