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HISTONE DEACETYLASE INHIBITORS AND BREAST CANCER METASTASIS: A REVIEW AND EXPLORATION OF HDAC(I)S AND OTHER 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

WILLIAM EVANS SISTRUNK: Histone Deacetylase Inhibitors and Breast Cancer Metastasis: A Review and Exploration of HDACi(s) and other Chemotherapeutic Agents
(Under the direction of Dr. Yu-Dong Zhou and Dr. Dale G. Nagle)

The traditional perspective of Histone Deacetylase enzymes is focused around their inherent epigenetic modification characteristics. While it is true that the histone modification these enzymes exhibit play a role in cancer and related diseases, Histone Deacetylase has a variety of non-histone targets. The non-histone targets include microtubules and are of specific interest because of the microtubules' role in cell line differentiation, replication, apoptosis, and cancer metastasis. Using a variety of Histone Deacetylase Inhibitors (HDACi) and other chemotherapeutic compounds, our research group explored the HDACi effect on breast cancer cell lines. Our goal was to indicate the presence of HDACi cell-line dependent cancer growth inhibition and to study the hypothesized non-histone mechanism of microtubule modification in HDACi(s). The experiment consisted of three parts: viability assay, clonogenic assay, and combination assay which analyzed HDACi(s) possible synergistic character with microtubule stabilizing compounds. The specific breast cancer cell lines used were MDA-MB-231 clones LM-4175 and BOM-1833, and MCF7-BOM. The results of our experiments indicated that there was cell line dependent growth inhibition with the treatment of HDACi(s). Specifically, MCF7-BOM showed to be more susceptible to treatment, and this could be due to it being an estrogen receptor positive ER+ cell line. However, the growth inhibition never reached complete inhibition and was most prominent at the highest concentrations of HDACi(s). Higher concentrations of HDACi(s) also had the most prominent effect on colony growth inhibition in the clonogenic assay. The

combination assay had an interesting result indicating an antagonistic trend between microtubule stabilizers and HDACi(s).

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LIST OF ABBREVIATIONS

BRCA1 - Breast Cancer gene 1

BRCA2- Breast Cancer gene 2

DMEM - Dulbecco’s modified Eagle’s media

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

EpoB - Epothilone B

ER+ - Estrogen receptor positive

FBS - Fetal Bovine Serum

HAT - Histone Acetylase

HDAC - Histone deacetylase

HDACi - Histone Deacetylase inhibitor

HDACi(s)- Histone Deacetylase Inhibitors

Her2+ - Human epidermal growth factor receptor 2-positive

Hsp90 - Heat shock protein 90

IC50 – Half maximal inhibitory concentration

NCI - National Cancer Institute

SAHA - Vorinostat

SRB - Sulforhodamine B

Taxol - Paclitaxel

Introduction: Breast Cancer

A profound statement that has resonated with me throughout my undergraduate research came from a newly diagnosed breast cancer patient named Carol. She described her first reaction like many of us would, “Why me?”; However, Carol’s next thought was, “Why not me?” (37). Unfortunately, Carol is correct in that the incidence of breast cancer does not discriminate among women. In other words, all have an absolute risk of developing the disease in their lifetime. Currently, one out of every eight women develop one of the many forms of breast cancer in their lifetime (15). That number is expected to increase as screening methods advance and progress throughout the world. Some experts predict that there will be 3.2 million new cases per year by 2050 compared to the 1,384,155 new cases registered in 2008 (29).

The emergence of cancer is simply a misprint of DNA. Imagine a cell as a factory containing thousands of printing presses. The assembly of pages (DNA) copied from the press (template strand DNA) is essentially the same process in which the body replicates DNA. However, that is just one factory; the body has an estimated 10^13 or thirty trillion cells in the body (17). Therefore, it is a question of when, not if, cancerous errors will occur. The human body has complex mechanisms known and unknown to correct these errors or apoptosis (self-terminate) corrupted cells. Despite this, an accumulation of errors ranging from single digits to thousands of misprints can develop into cancer. In the case of breast cancer, there are specific factors both non-

modifiable and modifiable that can increase the risk of developing the disease (15). Non-modifiable factors or factors that cannot be controlled include age, race, genes, age of menopause, breast density, body height, hormones, and prior history of breast biopsy or benign tumors. Before the age of 30, women have a very small chance of developing breast cancer. However, from the ages of 30 to 50 years of age, there is a dramatic increase of disease incidence that remains elevated after the age of 50 (15). Race and genetic factors are especially critical in addressing an individual's chances of breast cancer. Caucasian women over the age of 50 have an incidence rate of 351.9 per 100,000-compared to 292.2 per 100,000 African American women in the same age range (15). One of the most well-known breast cancer genetic mutations is BRCA 1 and BRCA 2. According to the Center for Disease Control, 50 out of 100 women with these mutations will develop breast cancer by the age of 70 (CDC). Thus, preemptive measures such as Mastectomy (removal of breast tissue) become a desirable choice in these hyper-predisposed women. Physical and chemical characteristics of an individual such as breast density, hormone levels, age of menopause, etc. can cause variability in breast cancer incidence. For instance, research suggests that the delay in menopause results in a 3% increase of breast cancer for every year of absence (15).

Unlike non-modifiable factors that are uncontrollable, modifiable factors such as smoking, diet and exercise, environment, and hormone therapies can be controlled to reduce the breast cancer risk. High alcohol consumption in an individual's diet is the most heavily correlated lifestyle factor associated with the risk of breast cancer (15). According to one study, alcohol consumption is the primary cause in 4% of new breast cancer diagnoses (27). Overall, modifiable risks other than alcohol consumption have a

marginal increase in breast cancer incidence. However, it is important to consider that avoiding known harmful modifiable factors can prevent a significant proportion of postmenopausal breast cancer cases (28).

Although breast cancer is unfortunately common among women, the prognosis is very heterogenous as each diagnosis depends on certain conditions. Tumor morphology, genetic typing, and histological grade coalesce in outcomes ranging from treatable to extremely malignant (29). Cancerous breast tumors are divided into several categories including infiltrating ductal carcinoma, infiltrating lobular carcinoma, tubular, mucinous, medullary, adenoid cystic carcinoma, and many other types (29). Each type of breast cancer has unique characteristics determining prognosis. For example, smaller tubular carcinomas are associated with a less advanced stage or progression at presentation of disease compared to infiltrating ductal carcinomas (29). The presence of estrogen receptors (ER positive) in a cancerous cell morphology indicates favorable outcomes (1). Also, the presence of genes that code for proteins such as HER2 (Human Epidermal Growth Factor Receptor 2) in tumor cells adds another trait in which physicians and scientists must consider for treatment. In contrast with ER positive breast cancer, Triple-Negative breast cancer (TNBC) is characterized with poor outcomes and high rates of relapse (8). The TNBC cells lack HER2 protein receptors, estrogen receptors, and progesterone receptors. In essence, the most effective way to destroy tumors is to consider them unique individuals through personalized medicine. This ideology centers around the mantra that there is both inter-heterogeneity among tumor types and intra-heterogeneity within the tumor itself (4). Ultimately, the

goal is to identify the breast cancer type and best course of treatment before the disease is in its final stage.

What is final stage breast cancer? What aspect of the disease ultimately causes death? These were two of my very first questions in the initial segment of research. The simple answer is that metastasis or M-stage is the final progression of breast cancer. Metastasis is the process in which cancerous cells spread to other parts of the body through the blood or lymph circulatory systems. Furthermore, the new tumor formed is of the same type as the primary tumor. For example, if cancerous cells spread from a breast tumor to the brain, these cancerous cells are still breast tissue (26). Distant metastases such as mentioned above (breast to brain) is the main cause of death in breast cancer patients (5).

Metastatic or malignant tumors can cause death in a variety of ways depending on where the secondary tumors form. For example, malignant tumors are highly metabolic and can not only become strenuous to maintain but consume surrounding normal cells to strengthen their viability. Metastasis also causes terminal complications by interfering with bodily functions such as the immune system and circulatory system. Secondary infections, strokes, and other serious medical conditions resulting from the secondary tumor interference are the ultimate cause of death (30). Under these circumstances, a patient that has progressed to M-stage breast cancer is at a much higher risk than previous stages. Consequently, preventing the malignant transformation of a primary tumor is a promising target for therapy. Under the guidance of Dr. Yu-Dong Zhou and Dr. Dale G. Nagle, I along with my collaborators Mary

Grace Stewart and Henry Nguyen have pursued drug therapies using Histone Deacetylase Inhibitor (HDACi) class compounds to prevent malignant breast cancer.

Histone Deacetylase Inhibitors (HDACi)

The Human Genome Project represents a new era in the advancement of medicine. Using the combined resources of collaborators from around the world, in 2003 the entirety of the Human Genome was sequenced (25). In essence, the instruction manual of every cell in the human body is now available to be studied and perhaps manipulated. However, it is important to note that while some genes are constantly transcribed, many remain silent depending on the cellular environment and cell type. The gatekeepers of transcription are the histone proteins (3). Histones are proteins that have between 145-147 DNA base pairs wrapped around the core histone protein. Each core histone protein is globular in structure and consists of two histone protein subunits: H2A, H2B, H3, and H4 (3). The histone is designed to be post-translationally modified by the acetylation of lysine residues by histone acetylases (HATS). This function allows the histone to loosen the DNA tightly wrapped around the core protein and enable transcription to occur (3).

Imagine being able to control the acetylation of histones and eventually manipulate the phenotype of a cell without changing its inherent genotype. In 1977, scientists were able to complete such a feat by converting a cancerous erythroleukemia cell line into a non-dividing hemoglobin synthesizing cell using butyric acid, a histone deacetylase inhibitor (HDACi) (11). In order to change the cell in such a drastic way, the histone proteins must be denied the ability to be deacylated, which is the exact goal of

histone deacetylase inhibitors. The mechanism of HDACi consists of inhibiting the histone deacetylase enzyme's function of removing the acetyl group from lysine residues. With the HDAC inhibited by the HDACi, the histone acetylases can freely function to stimulate the transcription of DNA through the unwinding effect of the histone. Also, it is especially important to note that HAT and HDAC enzymes have non-histone targets in the cell. When HDACi(s) are introduced into the cell, the effects on both the histone and non-histone proteins coalesce into increased cell apoptosis, decreased migration, decreased proliferation, and cellular differentiation (32).

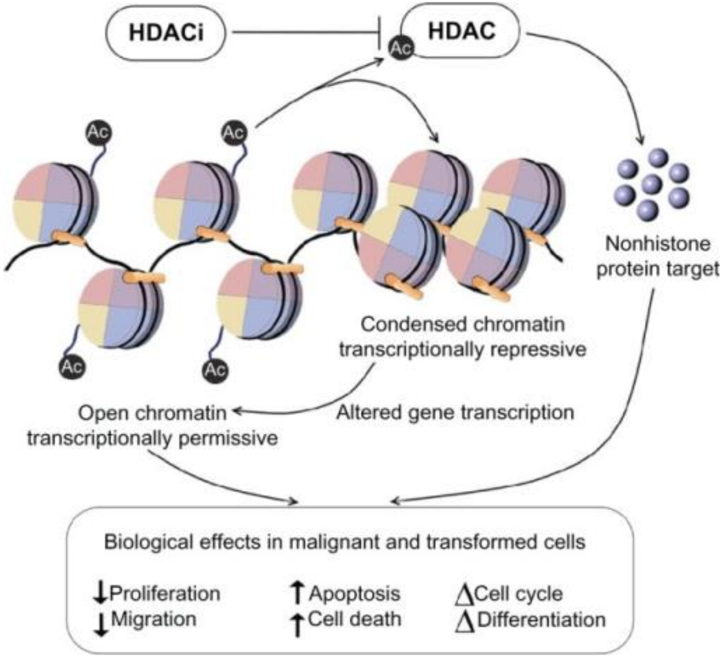


Figure 1 (20)

One pivotal question regarding the HDACi mechanism is: How do noncancerous cells (and some cancerous) survive the hyperacetylation of histones that cause the destruction/differentiation of many forms of cancer cells? While a concrete answer is unknown, one study suggests that body cells have an innate response to

downregulate histone acetylases during a period of hyperacetylation caused by HDACi (12). This theory is supported by histone acetylation falling dramatically after cells were removed from a HDACi rich environment. In other terms, the lack of HDACi induced hyperacetylation in the new environment post-HDACi caused an overcompensation of the innate cellular defense. How did this innate defense arise? Interestingly, HDACi activity is observed in many natural products produced by bacteria. Eukaryotic cells that were in the presence of these bacteria and thus the HDACi developed this innate response over thousands of generations in order to survive. The human body even has HDACi activity present in the large intestine where bacteria are believed to act as a weapon against eukaryotic organisms competing for the same resources (12).

Histone Deacetylase enzymes are a very diverse group of molecules affecting many components of the cell and body. In fact, there are four classes each representing numerous subtypes: HDAC class I, consisting of HDAC 1,2,3,8. HDAC class II(a), consisting of HDAC 4,5,6, and 7. HDAC class II(b), consisting of HDAC 6 and 10. HDAC class IV, contains HDAC 11 (10). While each HDAC subtype has many functions, the most crucial to my research are HDAC 1, 2, and 6. HDAC 1 and 2 are intricate in the processes of cell proliferation and apoptosis. The overexpression of HDAC 1 and 2 are associated with many forms of cancer including breast, lung, and classical Hodgkin’s lymphoma (2). HDAC 6 is mostly present in the cytoplasm and is a target of α-tubulin. The HDAC 6 mechanism with chaperon protein Hsp90 is known to increase the growth of some forms of prostate and breast cancer. HDAC 6 is also

associated with oral squamous cell carcinoma and expression increases in advanced stage cancers in comparison to early stage (2).

One of the non-histone targets of HDAC 6—as mentioned previously—is alpha-tubulin. Microtubules have a complex mechanism of elongation and degradation that enable cells to be motile in their external and internal environment. The acetylation of α-tubulin has a stabilizing effect that leads to long lived, less motile, and less dynamic microtubules (6). In contrast, HDAC 6 upregulation causes the deacetylation of microtubules that is associated with cell invasion and metastasis. Therefore, compounds inhibiting HDAC 6 are promising potential therapeutic agents for preventing metastasis in late stage cancers (6).

What is the current state of HDACi(s) in the clinical treatment of cancer? Four HDACi compounds have been FDA approved: Vorinostat (SAHA), Romidepsin, Panobinostat, and Belinostat. Vorinostat inhibits class I, II, and IV HDAC molecules and is approved for treatment of Cutaneous T-cell Lymphoma (CTCL). Romidepsin inhibits class I HDAC molecules and is approved for treatment of CTCL. Panobinostat inhibits class I, II, and IV HDAC molecules and is approved for treatment of CTCL and multiple Myeloma. Lastly, Belinostat inhibits class I, II, and IV HDAC molecules and is approved for treatment of Peripheral T-cell Lymphoma (PTCL) (17). The common denominator for each FDA approved HDACi(s) is that they are effective in the treatment of non-solid tumors (Myeloma, Lymphoma). While this clinical use is promising, one of the most exciting characteristics of HDACi(s) is its ability to work synergistically with other chemotherapeutic agents (7). HDACi(s) in combination with other epigenetic modifiers, reactive oxygen species, protease inhibitors, DNA damaging agents, and microtubule

stabilizers have increased chemotherapeutic effects. While numerous studies support the synergistic effect, the mechanism is generally unknown (7).

In the experiments the research group conducted, we examined the relationship of Histone Deacetylase Inhibitors and other chemotherapy agents on multiple breast cancer cell lines. The purpose of the viability, combination, and other cell assays was to replicate and further understand the therapeutic effect of HDACi(s) on breast cancer.

Histone Deacetylase Inhibitor Experiment

The experimental timeline and progression that has encompassed over two years began in the fall of 2016 under the direction of Dr. Nagle and Dr. Zhou. The first section was a seminar class that introduced new researchers to the many intricacies of breast cancer and beyond. The second section was devising a research plan in order to understand the effects of Histone Deacetylase Inhibitors on specific breast cancer cell lines. The HDACi research plan consisted of background research, viability assay, combination assay, and clonogenic assay. While it was a general goal to study all HDACi(s), HDAC 6 inhibitors were of specific interest because of the non-histone microtubule targets it influences. Therefore, microtubule stabilizers and destabilizers were used in viability studies as well as in combination with HDACi(s) to study this effect. Our general hypothesis stated HDACi(s) may exhibit cell-line dependent inhibitory activity against breast cancer cells.

MATERIALS AND METHODS

Background Research

In the spring of 2017, after the decision to pursue HDACi(s) with a concentration of the anticancer effects of HDAC 6 inhibitors, the initial plan of research was to conduct a background review on the topic. This process was completed in three steps: literature review, survey known HDACi/chemotherapy compound data, and selection of cell lines/compounds. Dr. Zhou emphasized the importance of literature review before conducting our experiment in order to maximize our chances of originality in the field. While it is difficult to produce new data because of the constant global competition, literature review was helpful because we could determine what had been researched and use that knowledge to focus on the selection of our topic. We learned that the role of HDAC 1, 2, and 3 and the inhibition of these HDACs in the treatment of breast cancer have been researched frequently in studies conducted over five years ago (22). However, we also learned more about HDAC 6 and the HDAC 6 inhibitor's role in microtubule stability (6). Since HDAC 6 interacts with microtubules, Dr. Zhou suggested using an HDAC 6 inhibitor in conjunction with paclitaxel and other microtubule stabilizers and destabilizers. In essence, there could be a synergistic property associated with the addition of HDAC 6 inhibitors with classic chemotherapy agents such as paclitaxel.

The next step of the background review was to survey known chemotherapy compounds and their inhibitory effects on each cell line. This procedure was completed using the National Cancer Institute Developmental Therapeutic Program named NCI-60

Human Tumor Cell Line Screen. The NCI-60 cell line assay results function as a database for the compound concentration needed to reach LC50 (LC50 is the lethal dose in which 50% of cells are destroyed in a given amount of time) in a particular cell line. In each compound our group was interested in, we would use the NCI-60 panel data to determine the general concentrations needed to reach LC50 and in turn determine a general guideline for the quantity of compound needed in our experiment.

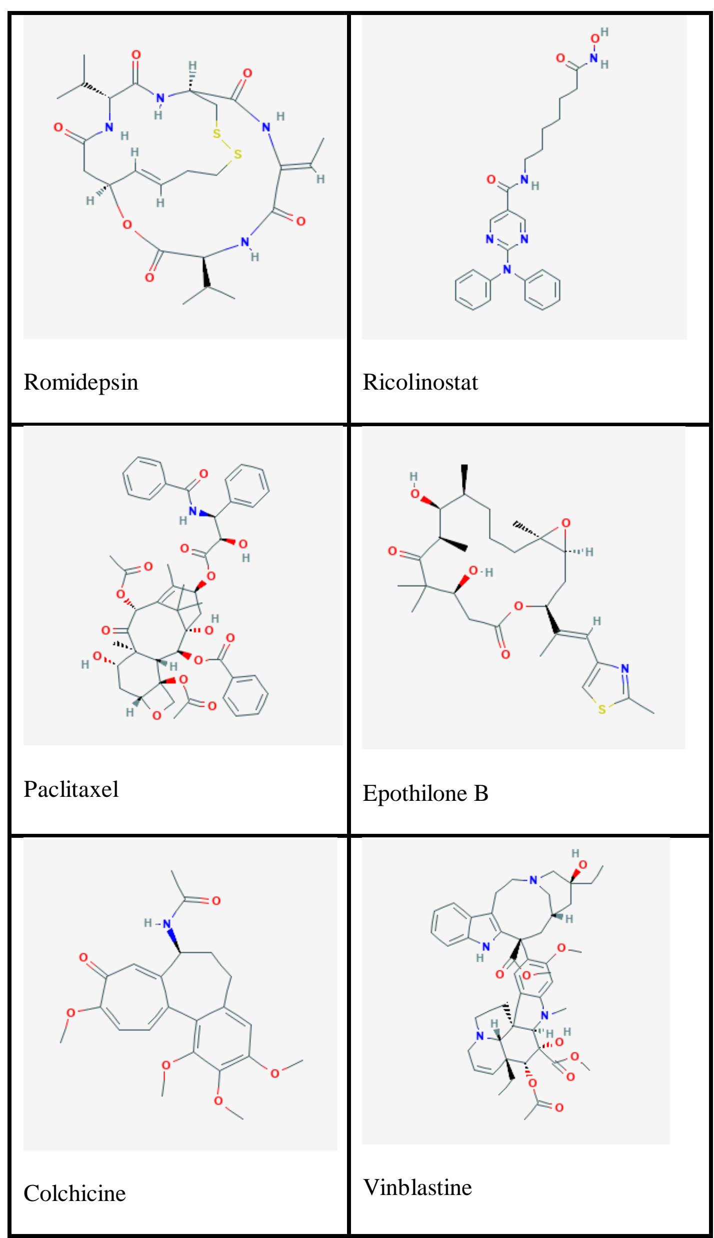
Compounds were selected to fill five classes of HDACi(s) and microtubule stabilizers/destabilizers: HDAC 1 and 2 inhibitors, HDAC 6 inhibitors, microtubule stabilizers, microtubule destabilizers, and pan-HDAC inhibitors. Among the compounds selected to fulfill these categories, further selections were based on logistics and availability of the compound.

Compound Data

Drug Classification	Compound Name	Purchased from	Solubility/stock solution
HDACi 1 and 2	Romidepsin	Cayman	100 μM
HDACi 6	Ricolinostat	Selleck	10 mM
MT Stabilizer	Paclitaxel	Sigma	10 mM
	Epothilone B	Selleck	10 mM
MT Destabilizer	Colchicine	Sigma	100 μM
	Vinblastine	Sigma	100 μM
Pan-HDACi	Vorinostat (SAHA)	Cayman	10 mM
	Panobinostat	Cayman	100 μM

Figure 2

Molecular Structure



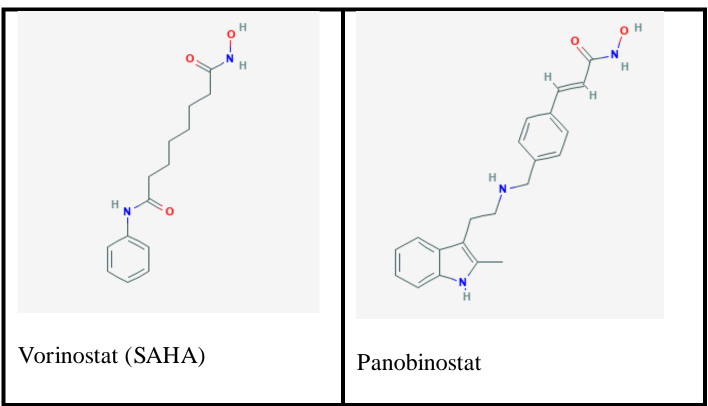
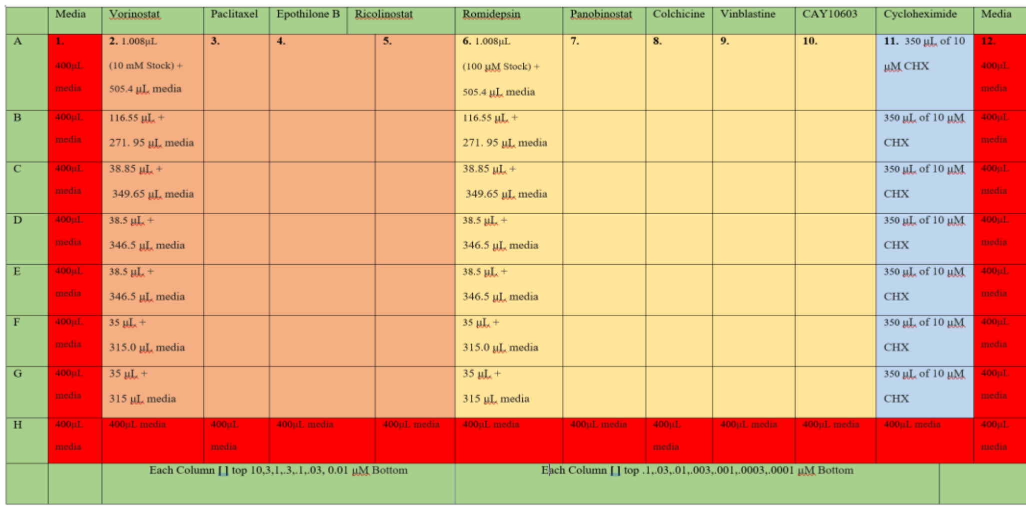


Figure 3

Preparation of HDACi and Chemotherapy Agent Dilution Plate

The chemotherapeutic agents were purchased from Cayman Chemical, Sigma-Aldrich, or Selleck Chemical. For our experiments, we set the stock solution concentration of 10 mM or 100 µM for each compound. Specific amounts of DMSO were added to each chemotherapeutic agent in order to configure the stock solution concentrations from the variable factory purchased compound concentrations. Serum free media was used to dilute the stock solutions to the highest tested concentration, and this was set at 2x the final concentration of the mother plate. The mother plate was prepared at 3.5x volume (of a single dose) because it was to be used on three SRB viability 96-well plates. A 1:10 serial dilution was performed creating a dilution pattern as seen in the table below (Top 10,3,1,.3,.1,.03,.01 µM Bottom). This process was completed in a stepwise pattern. For example, in one dilution we siphoned 35 µL of 0.1 µM solution into a well of 315 µL of serum free medium which created 350 µL of diluted solution at 0.01 µM. Each

dilution was calculated to have 350 μL of end product in each well.



	Media	Vorinostat	Paclitaxel	Epothilone B	Ricolinostat	Romidepsin	Panobinostat	Colchicine	Vinblastine	CAY10603	Cycloheximide	Media
A	**1.** 400μL media	**2.** 1.008μL (10 mM Stock) + 505.4 μL media	**3.**	**4.**	**5.**	**6.** 1.008μL (100 μM Stock) + 505.4 μL media	**7.**	**8.**	**9.**	**10.**	**11.** 350 μL of 10 μM CHX	**12.** 400μL media
B	400μL media	116.55 μL + 271. 95 μL media				116.55 μL + 271. 95 μL media					350 μL of 10 μM CHX	400μL media
C	400μL media	38.85 μL + 349.65 μL media				38.85 μL + 349.65 μL media					350 μL of 10 μM CHX	400μL media
D	400μL media	38.5 μL + 346.5 μL media				38.5 μL + 346.5 μL media					350 μL of 10 μM CHX	400μL media
E	400μL media	38.5 μL + 346.5 μL media				38.5 μL + 346.5 μL media					350 μL of 10 μM CHX	400μL media
F	400μL media	35 μL + 315.0 μL media				35 μL + 315.0 μL media					350 μL of 10 μM CHX	400μL media
G	400μL media	35 μL + 315 μL media				35 μL + 315 μL media					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
		Each Column [ ] top 10,3,1,.3,.1,.03, 0.01 μM Bottom				Each Column [ ] top .1,.03,.01,.003,.001,.0003,.0001 μM Bottom						

Figure 4: Serial Dilution Example 96-Well Plate

Breast Cancer Derived Cell Lines

The human breast cancer cell lines used in experimentation was MDA-MB-231, MDA-MB-231 subtypes BOM clone 1833 (bone metastasis) and LM clone 4173 (lung metastasis), and MCF7-BOM. MDA-MB-231 is a triple negative breast cancer that was derived from a pleural effusion in a 51-year-old Caucasian female in the 1970's (18). MDA-MB-231 is an aggressive tumor with poor prognosis which commonly metastasizes to the lung and bone to form secondary tumors. MDA-MB-231 BOM and LM are cell lines derived from these secondary tumors and were first generated by Dr. J Massagué at the Sloan Kettering Cancer Center (21). Our experiments acquired the specific MDA-MB-231 clones BOM 1833 and LM 4175 from Dr. Konosuke Watabe at Wake Forest University. MCF7 is an ER-positive breast cancer cell line that was originally derived from a 69-year-old Caucasian female in the 1970's (18). The MCF7-BOM cells that were used in our experimentation are another product of bone secondary metastasis generated in Dr. J Massagué's lab at the Sloan Kettering Cancer Center (23). Our lab acquired MCF7-BOM cells from Dr. Konosuke Watabe at Wake Forest University.

The MDA-MB-231 (BOM and LM) and MCF7-BOM cell lines were sustained in DMEM/F12 media containing L-glutamine (Mediatech, Manassas, VA), enriched with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT),50 units/mL of penicillin and 50 µg/mL streptomycin (Gibco, Grand Island, NY) were added, and the cells were temperature controlled at 37 °C in an environment of 95% air/5% CO_2 (34).

Sulforhodamine B (SRB) Viability Assay

The SRB viability assay procedure started after the 96-well master dilution plate was created and the specific cell lines developed to the desired density. The SRB viability assays are a rapid, relatively inexpensive, and reliable test to study the effects of particular compounds on cell line proliferation (24). The first step of the process was to trypsonize the cell cultures using 1 mL (Trypsin) and then wash the cells with 10 mL of 10% fetal bovine serum (FBS) media. The cells were then diluted with additional 10% FBS media until a density of 3 million cells per mL of 10% FBS was reached using a hemocytometer. Next, 100 µL of cell solution and 100 µL of media were seeded to each well in the desired number of 96-well plates (depending on the specific trial requirements) at a concentration of 30,000 cells per well. The plates were cultured at 37° C within a humid environment of 95% air and 5% $C0_2$. The 96-well cell containing plates were then infused with a specific amount of chemotherapy agent in the 96-well master dilution plate. The combination chemotherapy agent/cell plates were incubated for 48 hours. After the incubation process was complete, 100 µL of media was withdrawn from each well. The wells began the fixation process with the addition of 100 µL of 20% trichloroacetic acid (TCA) and 1% PBS solution to each well. The fixed plates were placed in the refrigerator for one hour at 4°C. After removal from the refrigerator, the plates were washed with tap water four times and set aside to dry. Each well was then stained using 100 µL of .4% SRB (w/v, 1% acetic acid) for 10 minutes at room temperature. The plates were further washed with 1% acetic acid four times and set aside to dry. Tris Base (100 µL of 10 mM) was added to each of the stained wells, and the plates were lightly shaken for 10 minutes using the microplate genie. The plates were

then read using the SpectaFlour plate and Magellen software at an absorbance range of 496-620 nm. The SRB dye binds to the basic amino acids present in the cell proteins allowing for Magellan software to calculate the inhibition value. This inhibition value was generated by the software using the total protein mass per well (flagged by the SRB dye) which is proportional to the cell density of that cell.

Combination Assay

One of the main objectives of these series of experiments was to explore the synergistic or possibly antagonistic effects of HDACi(s) and other chemotherapeutic compounds. Therefore, a combination assay was conducted using a cross of HDACi compounds and microtubule stabilizing chemotherapeutic agents' paclitaxel and epothilone B. The procedure of the combination assay was very similar to the viability assays performed. The difference was the bidirectional compound addition where the microtubule stabilizers (Taxol and epothilone) were added from left to right and the HDACi(s) (romidepsin, ricolinostat, panobinostat) were added from top to bottom. The specific HDACi(s) tested were chosen based on the range of HDACi activity they exhibit. Romidepsin is a class 1 HDACi that showed strong and consistent activity in our viability screening. Ricolinostat is a class 6 HDAC inhibitor and was selected because of our interest in its specific effect on microtubule stabilization and destabilization. Panobinostat was selected because it is a pan-HDACi, meaning it inhibits all classes of HDAC. The concentration ranges for both HDACi(s) and microtubule stabilizers were determined based on concentrations used in the National Cancer Institute Database of both experimental and physiological relevance.

Combination Assay	**Romidepsin**			**Ricolinostat**			**Panobinostat**			
	V V	V V	V V	V V	V V	V V	V V	V V	V V	V V
Taxol 0 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 10 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 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 1 µM ===>	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 0 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
EpoB 10 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
EpoB 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
EpoB 1 µM ===>	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 5: Combination Dilution 96-Well Plate

Clonogenic Assay

The Clonogenic assay is used in assessing the survivability of a cell line in the presence of a cytotoxic agent. Essentially, the ability for a single cell to grow into a colony under cytotoxic conditions (9). The number of six-well plates used was a total of six: three for the BOM and three for MCF7-BOM cell lines. The first plate in each cell line had two media control wells and four chemotherapeutic compound wells. The second and third plates of each cell line had all six wells containing chemotherapeutic compounds. The concentrations of the chemotherapeutic agents were based on physiological relevance (low concentration) and cytotoxic potential (high concentration).

Chemotherapeutic Compound	Cytotoxic Potential	Physiological Relevance
Vorinostat (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
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

Figure 6

The cells were seeded in a density of 3,000 cells per well, and six-well plates were incubated for four hours at 37°C allowing the cells to adhere. After a period of 24 hours, the media solution was replenished with fresh FBS (10%) with antibiotics. This incubation process was then conducted over ten days with fresh medium replenished every five days. After the incubation period, the cells were fixed with methanol and stained using crystal violet solution (1 mg/mL in 20% ethanol). Pictures of the final Clonogenic assay product were taken on an iPhone XR.

Results and Discussion

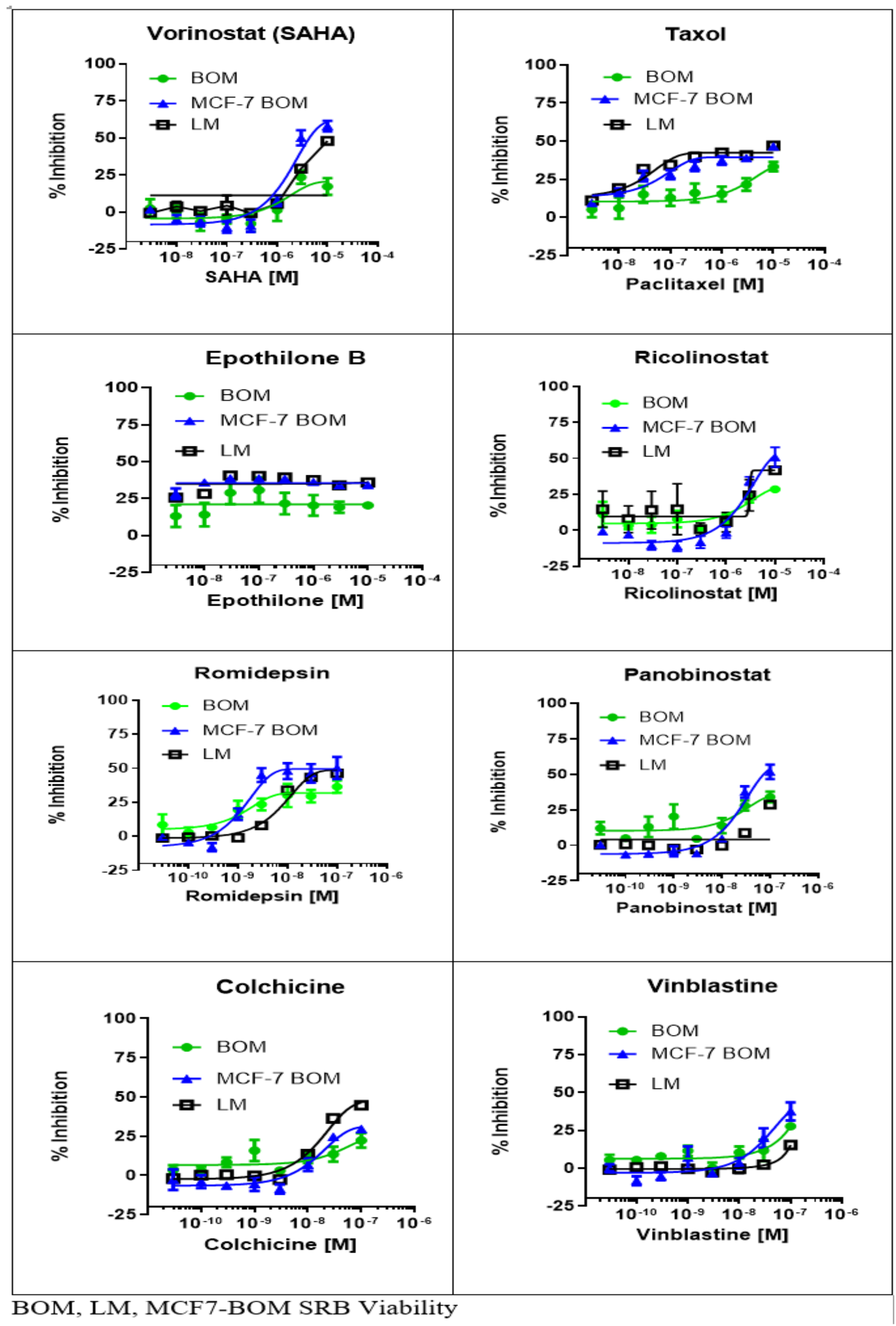
SRB Viability Assay

The results of the Sulforhodamine B viability assay reflect the percent inhibition values of the chemotherapeutic agents tested on MDA-MB-231 BOM, MDA-MB-231 LM, and MCF7-BOM cell lines. The initial goal of viability testing was to acquire an IC50 value (drug concentration where cell growth is inhibited by half of total inhibition). However, while our results had significant inhibition, they never reached this value. Instead, our group categorized each chemotherapeutic drug into a highest inhibitory concentration category under the parameters of cell line, percent inhibition, and drug concentration of highest inhibition. The highest inhibitory concentration results and trends of the viability tests are located in the figures below. The graphs were configured using prism GraphPad 8.

Highest Inhibitory Concentration

Chemotherapeutic Agent	LM		BOM		MCF7-BOM	
	Con [ ]	% Inhibit	Con [ ]	% Inhibit	Con [ ]	% Inhibit
Vorinostat (SAHA)	10 µM	58	3 µM	24	10 µM	48
Ricolinostat	10 µM	51	10 µM	29	10 µM	42
Panobinostat	0.1 µM	52	0.1 µM	34	0.1 µM	29
Romidepsin	0.1 µM	50	0.1 µM	36	0.1 µM	46
Vinblastine	0.1 µM	38	0.1 µM	28	0.1 µM	16
Colchicine	0.1 µM	30	0.1 µM	22	0.1 µM	44
Epothilone B	0.1 µM	39	0.1 µM	31	0.1 µM	41
Paclitaxel	10 µM	47	10 µM	33	10 µM	47

Figure 7

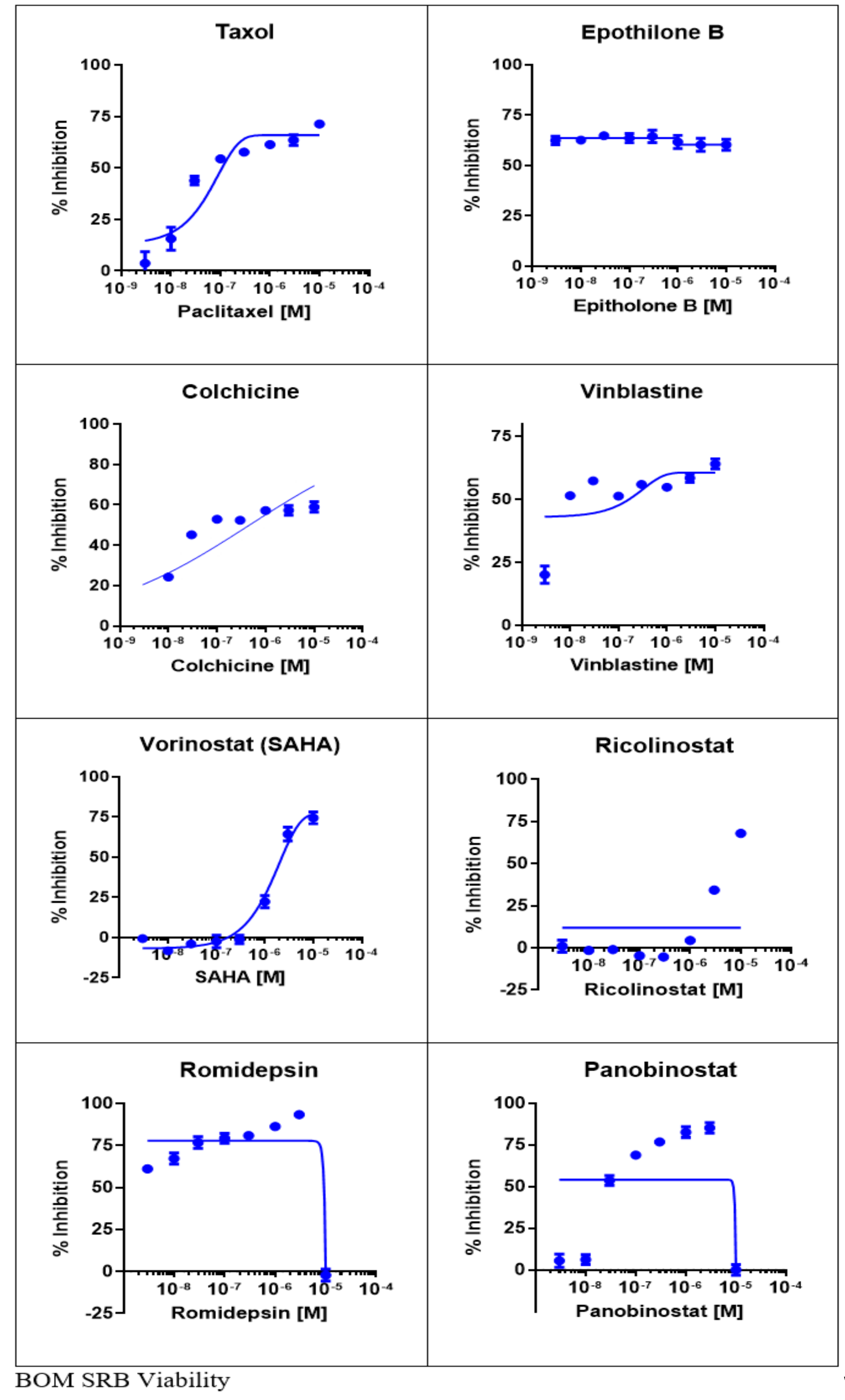


BOM, LM, MCF7-BOM SRB Viability

Figure 8

In the Highest Inhibitory Concentration table, the cells that were highlighted represent values in which the greatest growth inhibition was not at the highest concentration of chemotherapeutic agent tested. However, in the case of epothilone B, inhibition was relatively similar across all cell lines from the lowest to highest concentration. The results were conflicting in the MDA-MB-231 BOM vorinostat (SAHA) data. The first and second highest concentrations tested were relatively similar, possibly indicating a plateau in inhibitory effect at these concentrations. Further testing for both MDA-MB-231 BOM vorinostat (SAHA) and epothilone B SRB viability would be ideal to further specify the data.

An interesting aspect of our results was the sensitivity of the MCF7-BOM cell line compared to the MDA-MB-231 BOM and LM cell lines. There was a consistent trend of MCF7-BOM being the most susceptible or a close second in every trial. This could be due to the MCF7-BOM cell lines being estrogen receptor positive (ER+) compared to the triple-negative cell lines of MDA-MB-231 BOM and LM. One previous study found that HDACi enhances ER(+)-stress mediated cell death in some cancers (14). That being said, one of the highest percent inhibition values was romidepsin, a HDAC 1 and 2 inhibitor, at 74% inhibition in the MCF-7 BOM cell line. Interestingly, the HDAC 6 inhibitor ricolinostat had the most effect on the MCF-7 BOM cell line with a sharp slope. This finding could be promising as a higher drug concentration could lead to increased cell growth inhibition. Overall, there is a clear (though somewhat small) inhibitory effect of HDACi(s) on these specific breast cancer cell lines.



BOM SRB Viability

Figure 9

There was a second round of SRB viability testing conducted on the MDA-MB-231 BOM cell line in order to replicate our findings in the first viability experiment and to fine tune our laboratory skills in the viability procedure. In general, our results were very similar to the original MDA-MG-231 BOM viability assay. The epothilone B assay exhibited a high inhibition at both low and high concentrations, and the other drug assays exhibited similar inhibitory effects. However, there were a few differences that are noteworthy. First, the percent inhibition values were larger in general. These numbers could be caused from a variety of reasons including the cell line being weakened (stressed) to begin with and/or our lab technique was slightly superior in delivering the chemotherapeutic agents. Second, romidepsin and panobinostat exhibited around 0% inhibition at their highest concentration. This finding directly contradicts the first viability experiment and is most likely an error in the lab. More testing is required to clarify the contradiction of results.

Clonogenic Assay

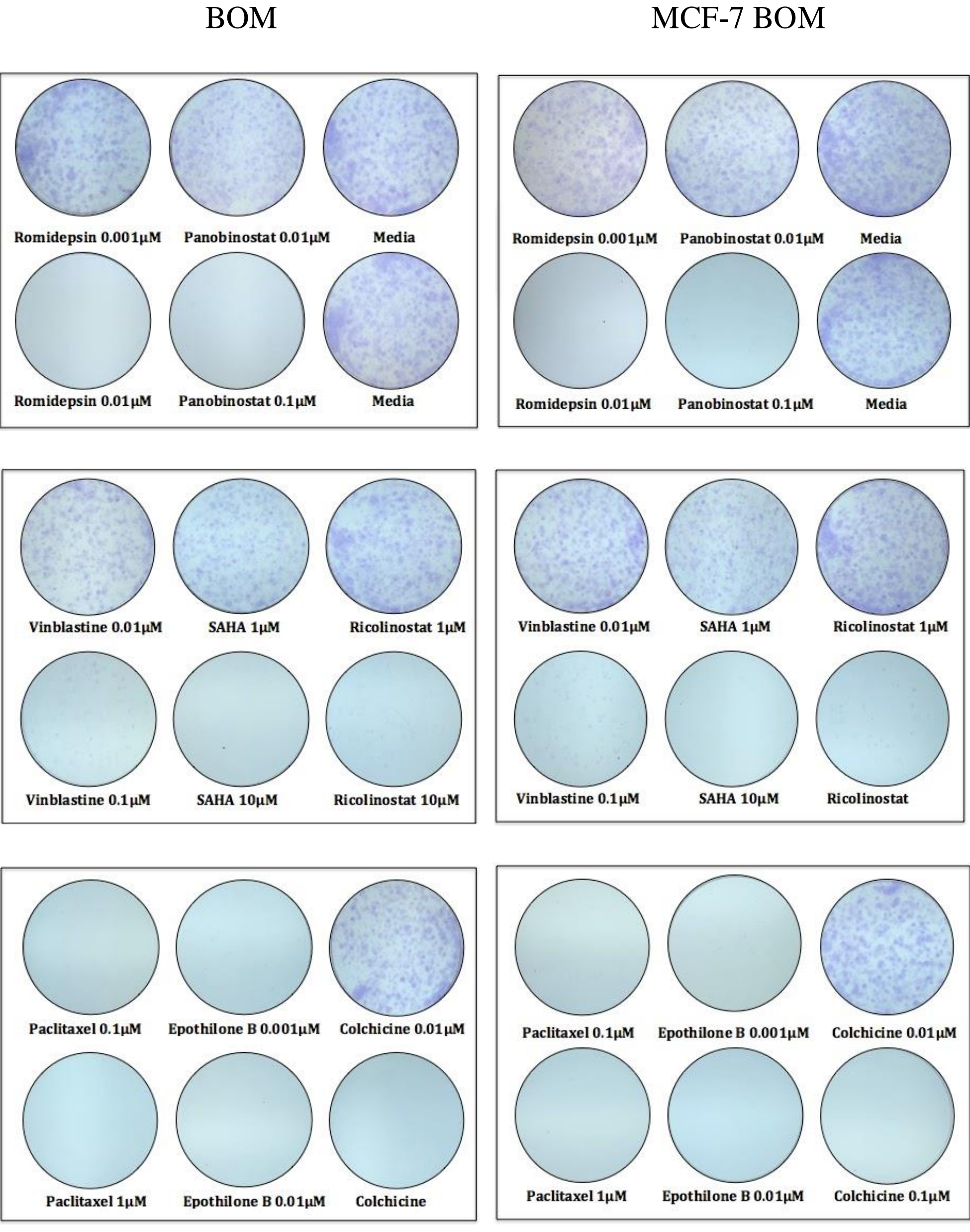
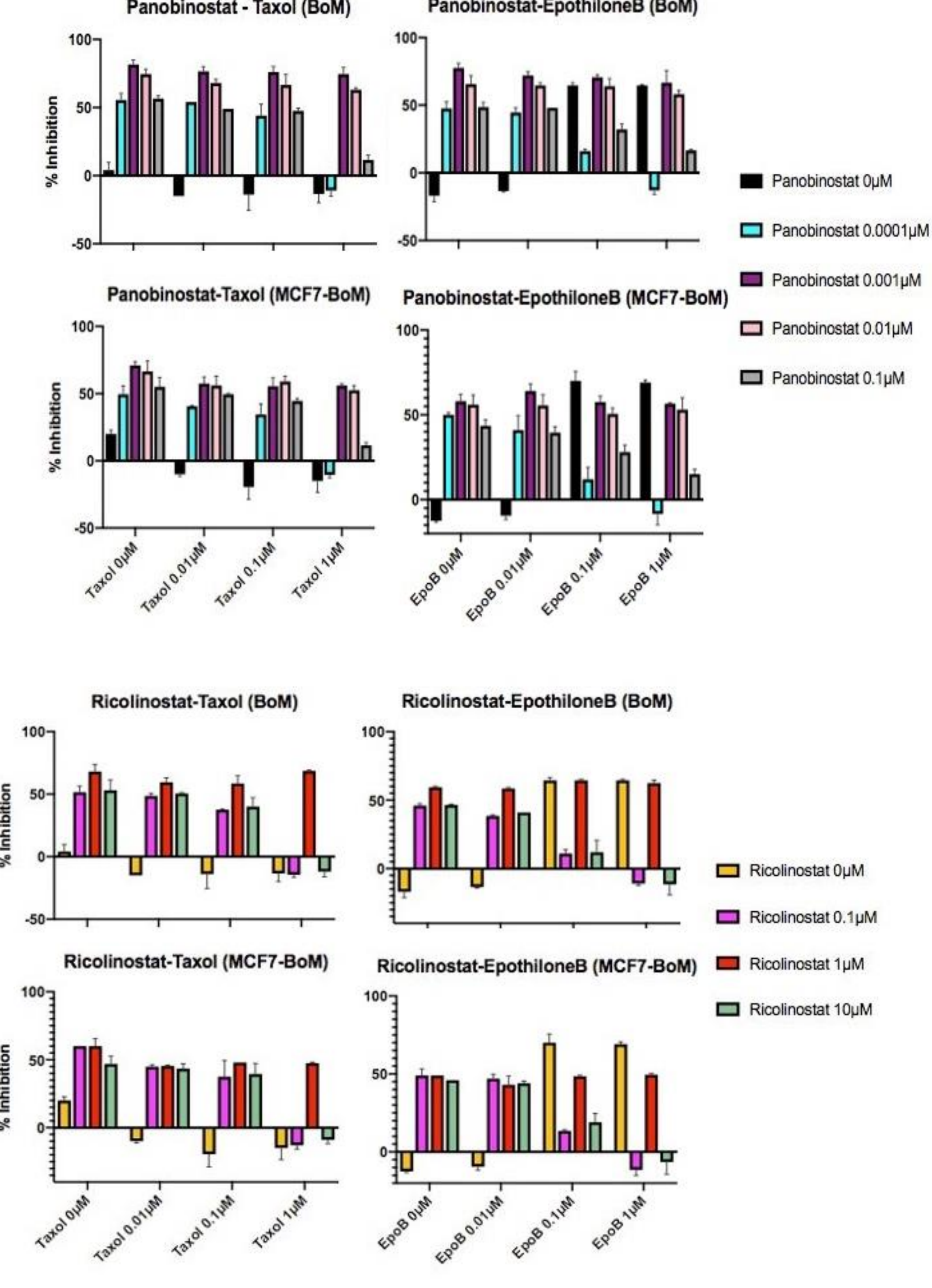


Figure 10

The figures above represent two three-plate clonogenic assays on the MDA-MB-231 BOM cell line and MCF-7 BOM. In a general visual comparision of the two plates, relativly similar clonogenic inhibition occurred between the two cell lines. This tends to suggest that there is relativly little cell line dependence in clonogenic inhibition between the two cell lines. What is interesting is the difference between the physiologically relevent concentration inhibition of HDACi(s) and the cytotoxic potential. In every HDACi physiological (lower) concentration well, there was some form of colony growth. On the other hand, cytotoxic potential concentrations of HDACi exhibited virtually complete colony growth inhibition. The only other chemotherapuetic compounds that had such inhibition were the microtubule stabilizers paclitaxel and epothilone B at both physiological and cytotoxic relevence. This HDACi concentration dependent colony growth inhibition could be due to the microtubule stabilizing or destabilizing effect taking place at the cytotoxic potential concentration. In essence, the microtubule effect of HDACi(s) (as suggested by previous studies) could be exhibiting the same properties as epothilone B and paclitaxel (6). Overall, the clonogenic assay provided further evidence of the inhibitory effects that HDACi(s) have on breast cancer cell lines. It also suggests further avenues of research regarding HDACi(s) effect on microtubule destabilization or stabilization.

Combination Assay



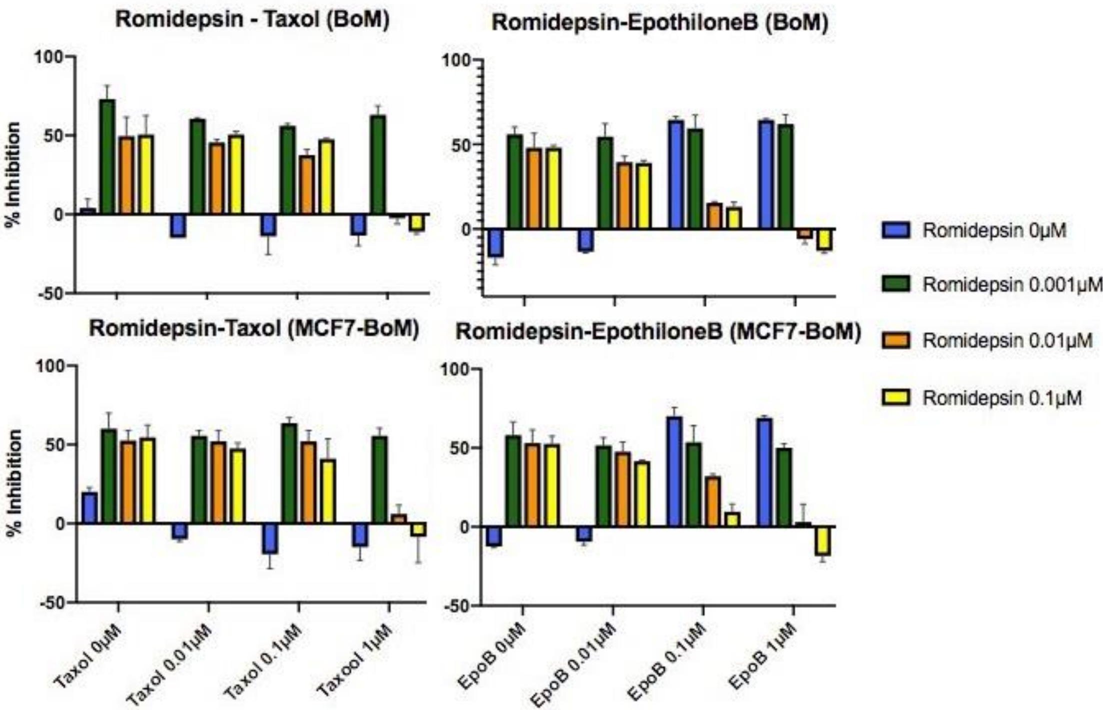


Figure 11, 12, 13

The goal of the combination assays conducted was to determine the possible synergistic or antagonistic effects associated with the combination of these chemotherapeutic compounds. The results obtained were then compared to the US National Cancer Institute's Combination Almanac. This reference system provides previous crosses of the compounds of interest in a variety of cell lines. However, the crosses in the almanac are not necessarily the same as our experiment. Therefore, some direct comparisons are not possible.

The combination assay represented in figure 11 is a cross between panobinostat (pan-HDAC inhibitor), epothilone B, and paclitaxel in two different cell lines: MDA-MB-231 BOM and MCF7-BOM. In comparing the two cell lines, there also was

relatively little cell line dependent inhibition. There was only a slight variation in magnitude where every trend is the same.

In both cell lines, the panobinostat-paclitaxel cross and the panobinostat-epothilone B inhibition cross percent inhibition values were highest when panobinostat was at 0.001 μM across all ranges of microtubule stabilizer concentrations. In regard to the synergistic or antagonistic characters of these compounds in combination, there is an antagonistic relationship between panobinostat-paclitaxel and panobinostat-epothilone B. This is evident because of the decrease in percent inhibition as the microtubule stabilizer concentration increased along with the increase in concentration of HDACi.

The combination Assay represented in figure 12 is a cross between ricolinostat (HDAC 6 Inhibitor), epothilone B, and paclitaxel. The same cell lines were used in figure 12 as figure 11. In comparison between MDA-MB-231 BOM and MCF7-BOM, there is no evidence of cell line dependent inhibition. After discussion within the research group, we concluded that the graphs represented are abnormal. In both paclitaxel-ricolinostat and epothilone B-ricolinostat crosses, the results at high combination are sporadic and vary. This could be due to cell line stress, human lab error, or an unknown factor. However, a general antagonistic trend can be seen in both crosses as the microtubule stabilizer concentration increases and HDACi concentration increases.

The combination assay represented in figure 13 is a cross between romidepsin (HDAC 1 and 2 Inhibitor), epothilone B, and paclitaxel. The same cell lines were used in figure 13 as figures 11 and 12. There is also a similar effect as seen in figures 11 and 12 in that there is no cell line dependent inhibition between MDA-MB-231 BOM

and MCF7-BOM. In the paclitaxel-romidepsin cross, the highest inhibiting combination of compounds were present at 0.001 μM across all ranges of paclitaxel. There was also a strong antagonistic trend that increased when paclitaxel concentrations were elevated and romidepsin concentrations were elevated. In this particular cross, there had been previous data on the NCI Almanac reference tool. According to the reference, both MCF7 and MDA-MB-231 cell lines showed an antagonistic relationship (NCI). This provided some validation to our group as we were able to compare similar research to our own data. The epothilone B-romidepsin cross had the highest inhibition values at 0 μM romidepsin in high concentrations of epothilone. It appeared that romidepsin and epothilone B had an antagonistic relationship similar to previous trends of the other compound combinations. However, these were some of the most antagonistic as there was a sharp decline in inhibition at the highest concentrations of epothilone B and romidepsin.

Overall, there was an antagonistic trend with the increased concentrations of microtubule stabilizers and HDACi(s). This was also supported by relevant data at the NCI Combination Almanac. However, our data should be supplemented in the future by a replicate number of n=3 compared to our n=2. There could be many possible answers to why this antagonistic trend occurs. One answer could be a microtubule destabilizing effect that is present in HDACi competing with the microtubule stabilizing compounds. In order to answer this question, further research must be conducted on HDACi(s) effect on microtubules.

Conclusion

In reflecting on the analysis of the SRB viability assays, clonogenic assays, and combination assays across a variety of cell lines, our hypothesis that histone deacetylase inhibitors have cell line-dependent inhibition is supported. In the MDA-MB-231 clones BOM-1833 and LM-4175 as well as MCF7-BOM breast cancer cell lines, there was a variation in inhibitory effects in SRB viability testing. This could be due to the presence of ER receptors. However, more research must be conducted and higher concentrations should be tested in order to find the IC50 values of the HDACi(s) tested. The clonogenic assay also provided evidence of HDACi tumor growth inhibition. At the cytotoxic potential or highest concentration, HDACi(s) had a great degree of colony growth inhibition. However, at physiologically relevant concentrations, there was only mild inhibition. When HDACi(s) were combined with the microtubule stabilizers epothilone B and paclitaxel, an antagonistic trend was observed. Therefore, this combination may not be desired for chemotherapeutic therapy. However, this evidence provides another step in understanding the non-histone targets of HDACi(s) and their role in microtubule stabilization and destabilization. In order to further this experiment, fluorescent labeling of microtubules and other structure proteins would benefit the understanding of this potential mechanism.

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