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# Histone Deacetylase Inhibition Induces Apoptosis and Cell Cycle Dysregulation in Human and Murine Cancer Cell Lines

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HISTONE DEACETYLASE INHIBITION INDUCES APOPTOSIS AND CELL CYCLE DYSREGULATION IN  
HUMAN AND MURINE CANCER CELL LINES

by

Joseph Skurski

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

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August 2017

## ABSTRACT

### HISTONE DEACETYLASE INHIBITION INDUCES APOPTOSIS AND CELL CYCLE DYSREGULATION IN HUMAN AND MURINE CANCER CELL LINES

by

Joseph Skurski

The University of Wisconsin - Milwaukee, 2017  
Under the Supervision of Dr. Douglas Steeber, Ph.D.

Carcinogenesis is a complex multistep process that requires tumor cells to grow rapidly while overcoming growth inhibitory signals and sustained challenges from the host immune response. Mutations within promoter or enhancer regions, along with epigenetic changes, can induce aberrant expression of genes that regulate differentiation, cell cycle, and apoptosis, all of which enhance potential for cellular transformation. In recent years, our understanding of the biological processes that influence the activation and repression of transcription have evolved to highlight the role of chromatin architecture, and how chromatin remodeling may be utilized for the potential therapeutic benefit of genetic disease. Histone deacetylase inhibitors (HDACi) are small molecule drugs that affect the balance between acetylation and deacetylation of proteins, ultimately influencing cellular processes including gene transcription. There are currently three FDA-approved HDACi on the market (FK228, SAHA, Panobinostat) for cancer treatment, all of which have high systemic toxicity. Therefore, there is need for development of less toxic HDACi with improved tumor specificity. HDACi can exert anti-tumor effects by inducing transcriptional changes in tumor suppressors through modulating acetylation of histones and/or transcription factors. We hypothesize that the high systemic

toxicity of the current HDACi is due to their non-selective HDAC activity and that more class-specific HDACi would result in less potent effects in cellular proliferation, death, and off target activity. The present studies tested this hypothesis by comparing effects of multiple HDACi on cell cycle progression and cell death in the murine breast 4T1, human prostate DU145, and human myelomonocytic U937 cancer cell lines. Systemic toxicity was assessed *ex vivo* using primary murine leukocyte populations. Further, cytotoxic effects of HDACi were tested in myeloid derived suppressor cell populations harvested from 4T1 tumor – bearing mice. Using flow cytometry and fluorescence microscopy, we demonstrate that FK228 and Panobinostat induced apoptosis and cell cycle dysregulation of cancer cell populations *in vitro*. Ongoing studies are elucidating the mechanisms of cell death and determining the off target effects of these HDACi.

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## **Chapter 1 - Introduction**

### **1. Aims and Significance**

Cancer is a major global public health problem and is currently the second leading cause of death in the United States (Siegel et al., 2016). The number of estimated diagnoses of new cases of invasive cancer in the United States in 2016 was 1,685,210, or the equivalent of 4,600 new cancer diagnoses each day (Siegel et al., 2016). In 2016, these estimated cases resulted in roughly 595,690 deaths in the United States with lung, bronchus, prostate, and colorectal cancers being the deadliest cancers in men while lung, breast, and colorectal cancers are the deadliest among women (Siegel et al., 2016). Women in the United States face a 12.3% risk of lifetime diagnosis of breast cancer (Siegel et al., 2016). While there is currently an array of treatment options available to breast cancer patients in the United States and abroad, many approaches lack specificity and therefore contribute to systemic toxicity that may diminish quality of life. There is a distinct need for more targeted therapeutic options that utilize tumor-specific markers to selectively target malignant cells, leaving healthy tissues unaffected. Both human epidermal growth factor receptor (HER2) and estrogen receptors (ERs) are common markers that are overexpressed in a variety of breast cancers (Nahta et al., 2006). ER-positive breast cancers are susceptible to endocrine-based treatments targeting either estrogen or ER, and treatment options have demonstrated improved survival rates in both disease-free and overall survival (Nahta et al., 2006). Additionally, monoclonal antibodies targeted against HER2, such as trastuzumab, have been shown to improve patient outcomes (Burstein et al., 2003).

Despite these advancements there are still several challenges to be met. For example, multidrug resistance remains problematic, while HER2 is only overexpressed in approximately 25% of invasive breast cancers (Knuefermann et al., 2003). Of further concern to the development of new therapies is the genetic heterogeneity and biological complexity of cancerous cell populations.

Carcinogenesis is a complex multistep process that requires tumor cells to grow rapidly while overcoming growth inhibitory signals and sustained challenges from the host immune response. Tumor cells must also be able to replicate indefinitely and evade apoptosis, while achieving sustained growth and survival by maintaining a suitable intake of oxygen and nutrients from the host (Johnstone, 2002). Mutations that result in the constitutive activation of oncogenes and inactivation of tumor suppressor genes are critical events that can contribute to the continued growth of the tumor. Aberrant gene expression also plays an important role in the development and growth of cancerous cell populations. Mutations within promoter or enhancer regions, along with epigenetic changes, can induce aberrant expression of genes that regulate differentiation, cell cycle, and apoptosis, all of which enhance the potential for cellular transformation (Johnstone, 2002). In recent years, our understanding of the biological processes that influence the activation and repression of transcription have evolved to highlight the role of chromatin architecture, and how chromatin remodeling may be utilized for the potential therapeutic benefit of genetic disease. In cancer, many of the molecular events that cause irregular gene expression due to altered chromatin structure have been elucidated, and it has been shown that aberrant acetylation of histone tails is strongly linked to carcinogenesis (Johnstone, 2002).

Understanding how gene expression can be regulated allows for the development of novel molecular tools which may be used to reprogram transcription and inhibit tumor cell growth and progression. Histone deacetylase inhibitors (HDACi) are small molecule drugs that modulate the balance between histone acetylation and deacetylation, ultimately influencing gene transcription through altering chromatin conformation. HDACi can exert anti-tumor effects by inducing transcriptional changes in tumor suppressors through modulating acetylation of histones and/or transcription factors (Weichert et al., 2008). We hypothesized that more HDAC-selective inhibitors will result in less potent effects in cellular proliferation, death, and off-target activity than broad spectrum inhibitors. In contribution to the growing body of research regarding HDACi as a viable therapeutic strategy for a variety of cancers, the research proposed herein sought to:

1. Assess the efficacy of clinically approved HDACi FK228 and Panobinostat in the 4T1, DU145, and U937 tumor cell lines.
2. Characterize the potential pathways of cell death produced by these compounds.
3. Assess the off target effects of these compounds upon primary murine leukocytes *ex vivo*.
4. Determine the efficacy of these compounds in reducing immunosuppression in the tumor microenvironment by examining their potential cytotoxic effects upon myeloid derived suppressor cells (MDSCs) in the mouse 4T1 tumor model.

These aims were tested using qualitative techniques such as fluorescence microscopy and through a quantitative approach in flow cytometry. All *in vitro* analysis were performed using established tumor cell lines.

## 2. Rationale

Overexpression of HDACs in human tumors can serve as a biomarker for cancerous versus healthy tissue (Minamiya et al., 2011). In multiple cancers including prostate, colorectal, breast, lung, liver, and gastric, overexpression of individual HDACs correlates with decreases in both disease-free and overall survival, and can be used to predict poor patient prognosis independent of tumor type and disease progression (Rikimaru et al., 2007). The aberrant expression of HDACs has been linked to key oncogenic events, such as the epigenetic repression of the tumor suppressor gene *CDKN1A* (cyclin-dependent kinase inhibitor 1A), along with genes encoding DNA damage repair enzymes such as *BRCA1* and *ATR* (Liu et al., 2009). Genetic knockdown of individual HDACs including HDAC1, -2, -3, and -6 in multiple cancers such as colon, breast, and lung has resulted in apoptosis and cell cycle arrest (Duan et al., 2005). These events indicate that HDAC activity may be a key contributor to cell survival in multiple cancers. Further, the deacetylation of the tumor suppressor p53 by HDACs has been shown to decrease transcriptional activity, and the upregulation of oncogenes such as *BCL2* is induced by HDAC-mediated deacetylation of the transcription factors SP1 and C/EBP $\alpha$  (Bhaskara et al., 2008). The possibility of targeting the aberrant transcriptional processes that lead to neoplasia may provide an opportunity for therapeutic intervention at a crucial juncture of the transformation process. Since this approach may affect several molecular pathways, chromatin remodeling through HDACi may potentially be more powerful than a targeted disruption of any single pathway.

### **3. Literature Review**

#### **3 a. HDACs in Epigenetic Modification**

Epigenetics refers to heritable changes in gene expression that do not involve changes to the underlying DNA sequence. These changes can occur through a variety of mechanisms, including post-translational modifications of DNA-associated proteins. The fundamental structure of chromatin is the nucleosome, which is composed of a 146bp DNA strand wrapped around a core histone octamer (Deubzer et al., 2013). Histone proteins serve to compact lengthy strands of genomic DNA into structures that can be contained within the eukaryotic nucleus. These proteins can be post-translationally modified by a number of mechanisms including lysine acetylation and ubiquitination, serine phosphorylation, sumoylation, and lysine and arginine methylation (West and Johnstone, 2014). These structural changes to the histone surface influence their interactions with chromatin and chromatin-associated proteins, impacting transcriptional activity. The most relevant post-translational modifications to HDACi-based therapies are histone acetylation and deacetylation. These reversible processes are regulated by two classes of enzymes: histone acetyl transferases (HATs) and HDACs. HATs and HDACs perform opposite functions; HATs catalyze the transfer of an acetyl group from acetyl co-A to the  $\epsilon$ -amino site of lysine, neutralizing the positive charge of the histone resulting in an open chromatin conformation, facilitating increased DNA transcription (West and Johnstone, 2014). HDAC activity deacetylates lysine residues encouraging interactions between the negatively charged DNA and the positively charged histones. This results in a closed chromatin conformation that represses transcription.

There are 11 known HDACs with Zn<sup>2+</sup>-dependent (classes I, II, and IV) active sites. (Table 1). Class I HDACs associate with multiprotein repressor complexes, and include HDACs 1-3 and 8 (Leder and Leder, 1975). Class I HDACs are expressed ubiquitously in all cells, act almost exclusively in the nucleus, and have histone substrates (West and Johnstone, 2014). The deletion of HDACs 1 and 2 together, but not each separately, has been shown to cause cell death in tumor cells as well as produce neural precursor maturation defects (Lindemann et al., 2007). HDAC2 has been shown to suppress apoptosis in tumor cells, and there is increasing evidence for a role of HDAC1 and HDAC2 in the DNA damage response (Lindemann et al., 2007). Deletion of HDAC3 has been associated with interruption of cell cycle progression, DNA damage, and deficiencies in repair and apoptosis (Lindemann et al., 2007). Class II HDACs are comprised of HDACs 4-7, 9, and 10. These HDACs can be present in either the cytoplasm or the nucleus, and can often shuttle between these locations (Newbold et al., 2008). Class II HDACs are further subdivided into two subcategories: class IIa (HDACs 4, 5, 7, and 9), and class IIb (HDACs 6 and 10). Class II HDACs often regulate tissue specific processes, playing roles in the vascular and nervous systems, bone, and skeletal muscle (Newbold et al., 2008). The sole class IV HDAC is HDAC11. HDAC11 is found in the nucleus and cytoplasm, and is also overexpressed in several carcinomas and its depletion has been shown to cause apoptosis in cancer cells (Insinga et al., 2005).

**Table 1.** Aberrant HDAC expression in multiple types of cancers

<b>HDAC</b>	<b>Cancer Expression</b>
HDAC 1 (Class I)	Gastric, breast, colorectal, lung, liver
HDAC 2 (Class I)	Gastric, prostate, colorectal, CTCL
HDAC 3 (Class I)	Gastric, breast, colorectal, decreased in liver
HDAC 4 (Class IIa)	Unknown
HDAC 5 (Class IIa)	Medullablastoma, decreased in lung
HDAC 6 (Class IIb)	Breast, CTCL, decreased in lung
HDAC 7 (Class IIa)	Acute lymphoblastic leukemia, decreased in lung
HDAC 8 (Class I)	Neuroblastoma
HDAC 9 (Class II)	Medullablastoma, acute lymphoblastic leukemia
HDAC 10 (Class IIb)	Unknown
HDAC 11 (Class IV)	Breast, renal, liver

### **3 b. HDACi in the Treatment of Cancers**

HDACi can induce tumor cell apoptosis, growth arrest, senescence, differentiation, increased immunogenicity, and inhibit angiogenesis (Nebbioso et al., 2005). Despite this broad range of effects, it has proven difficult to elucidate a specific mechanism of action responsible for all antitumor effects caused by HDACis due to the vast diversity among tumor types, as well as the diversity of specificity profiles among different classes of HDACi. Variability in biological effects and therapeutic outcomes can undoubtedly be attributed in part to the genetic heterogeneity characteristic of a wide variety of cancers. HDACi were initially described on their

ability to induce tumor cell differentiation; however, as the field expanded tumor cell apoptosis has come to be the most reported biological outcome of treatment (Duan et al., 2005). Preclinical models have established a direct link between HDACi-induced tumor cell apoptosis and therapeutic efficacy (Zhang et al., 2008); however, the role of the intrinsic apoptotic pathway mediated by inhibition of anti-apoptotic Bcl-2 family proteins vs the extrinsic pathway mediated by death receptors and their ligands remains contentious (Liu et al., 2009). Histone hyperacetylation observed at the promoters of apoptosis-inducing genes such as *TNFSF10* and *BMF* following HDACi treatment suggests that there is a link between altered gene expression and apoptosis. This hyperacetylation may influence changes in the activity of certain transcription factors, such as SP1 and C/EBP $\alpha$ , leading to the downregulation of anti-apoptotic protein Bcl-2 (Ungerstedt et al., 2005).

HDACi have also been shown to induce mitotic cell death of transformed tumor cells by causing mitotic defects. Through increasing histone acetylation, the structure and function of the centromere and pericentric heterochromatin are disrupted, causing a loss of binding to heterochromatin binding proteins (Weichert et al., 2008). Histone acetylation can also interfere with histone phosphorylation, disrupting the function of mitotic spindle proteins such as BubR1, hBUB1, CENP-F, and CENP-E (Weichert et al., 2008). HDACi treatment has also been shown to cause the degradation of the mitotic serine/threonine kinases aurora A and B, as well as survivin, which plays a paradoxical role in apoptosis and mitosis (Tan et al., 2006). Following treatment with HDACi, tumor cells show a transient arrest at prometaphase, followed by aberrant mitosis which results in death via apoptosis or mitotic cell death (Tan et al., 2006).



Therefore, it is reasonable to hypothesize that treatment of established cancer cell lines with HDACi will result in increased apoptosis and decreased proliferation.

HDACi can also induce cell death through autophagy. Previous studies have demonstrated that HeLa cells with Apaf-1 knockout or Bcl-X<sub>L</sub> overexpression cultured with the HDACi suberoylanilide hydroxamic acid (SAHA, a clinically approved HDACi) or butyrate (a short chain fatty acid HDACi, non-FDA-approved) succumbed to autophagic cell death (Bradner et al., 2010). More recently, combination treatments using OSU-HDAC42, a novel HDACi, along with SAHA have been shown to induce autophagy in hepatocellular carcinoma cells as evidenced by transmission electron microscopy, immunofluorescence and LC3-II recruitment (Krug et al., 2015). The authors concluded that this combination approach induced autophagy through the downregulation of Akt/mTOR signaling and induction of the ER stress response (Krug et al., 2015).

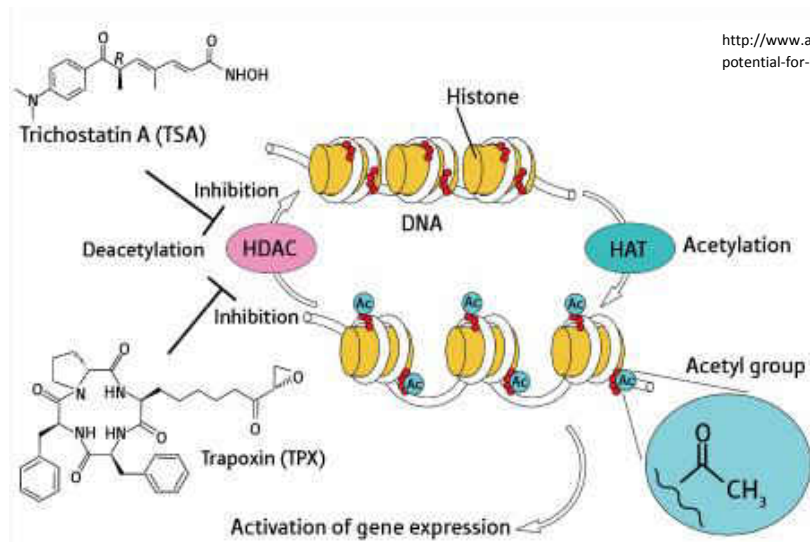
Accumulation of reactive oxygen species (ROS) has also been demonstrated in transformed cells cultured with HDACi including SAHA, FK228, Trichostatin A (TSA) (a class I and II HDACi), butyrate, and entinostat (an HDACi inhibiting HDAC1 and HDAC3 currently in clinical trials) (Furumai et al., 2002). This accumulation of ROS may be central to the selective induction of cell death in transformed cells but not in healthy tissue. HDACi up-regulates the expression of TBP-2, which binds to and inhibits thioredoxin activity, contributing to ROS accumulation in transformed but not in normal cells (Butler et al., 2002). Trx is an inhibitor of apoptosis-signal regulating kinase 1 (ASK1). ASK1 promotes apoptosis through the SET1-JNK and MKK3/MKK6p-38 signaling cascades, promoting the expression of the proapoptotic protein Bim by a positive

feedback of E2F1 activity (Ashkenazi and Dixit, 1998). Therefore, HDACi-induced Trx inhibition by TBP2 activates ASK1, which promotes cell death by apoptosis.

### **3 c. HDACi in the Clinic**

There has been much investigation regarding the clinical use and efficacy of HDACi as a cancer therapeutic option in recent years. There are currently four distinct classes of HDACi that are in clinical development: hydroxamic acids, cyclic peptides, short chain fatty acids, and benzamides (Krusche et al., 2005), each of which modulates histone acetylation levels in a reversible chemical process (Figure 1). SAHA (a hydroxamate-based inhibitor) was the first HDACi to be approved by the FDA (2006) for the treatment of refractory cutaneous T cell lymphoma. While initially believed to inhibit all class I, II, and IV HDACs in the low nanomolar range, more recent data suggests that SAHA may have only a weak inhibitory effect upon class IIa HDACs (Bossy-Wetzel and Green, 1999). SAHA has been shown to induce cellular differentiation in erythroleukemia cells and cause increased levels of p21, leading to G1 cell cycle arrest which inhibits cell growth in a variety of tumor cells, and animal models with limited toxicity (Aron et al., 2003). Being the first FDA-approved HDACi, SAHA has been studied in a number of clinical trials both in monotherapy and in combination with other compounds. FK228 was the second HDACi approved by the FDA (2009). Isolated from *Chromobacterium violaceum*, FK228 is a cyclic peptide with powerful activity, displaying inhibitory effects in the low nanomolar range. FK228 is a natural prodrug which is activated upon incorporation into the target cell through the reduction of an intramolecular disulfide bond by cellular reducing activity involving glutathione (Passmore et al., 2001). This compound has been shown to inhibit

human and mouse tumor growth in a variety of cancers through the inhibition of class I HDACs  
(Passmore et al., 2001).



**Figure 1.** Molecular structure of the HDACi TSA and Trapoxin illustrating their role in affecting histone acetylation levels.

## **Chapter 2 – Analysis of Cell Death and Cell Cycle Following HDAC Inhibition**

To investigate the cytotoxicity and mechanisms of cell death caused by treatment of human and murine cancer cell lines with HDACi, cell viability, apoptosis, and autophagy assays were performed. Additionally, cell cycle analyses were employed to elucidate the potential effects of HDACi treatment upon the cell cycle.

### **1. Materials and Methods**

#### **1 a. Cells**

The murine 4T1 mammary tumor cell line was originally isolated from a spontaneous breast tumor in BALB/c mice (Pulaski and Ostrand-Rosenberg, 2001). The 4T1 cell line has been chosen for its ability to human stage IV breast cancer, displaying highly metastatic behavior in mice. The 4T1 line has also been shown to induce the generation of myeloid-derived suppressor cells in mice. The cells utilized as described in this proposal have been obtained from ATCC (Manassas, VA, USA). The 4T1 cells were maintained in RPMI 1640 medium, supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 2mM L-glutamine, 100 units/mL penicillin, and 100 ug/mL streptomycin (all from Life Technologies, Grand Island, NY, USA). Cells were subcultured at approximately 70% confluency.

The human U937 cell line was used as a model for histiocytic lymphoma, as all currently approved HDACi have been approved for non-solid tumors. The U937 cell line was derived in 1974 from malignant cells recovered through pleural effusion of a patient with histiocytic lymphoma (Passmore et al., 2001). This line is one of only a few human cell lines that expresses monocytic characteristics exhibited by cells of histiocytic origin (Passmore et al., 2001). The

cells were subcultured at 400,000 cells/ml in the same media and conditions as the 4T1 cell line described above, with the addition of 55µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

In addition, the DU145 cell line was used to model highly metastatic prostate cancer. DU145 is a human cancer cell line that was originally isolated from a metastatic site in the brain (Wei et al., 2007). The DU145 cell line has previously been found to be sensitive to HDACi treatment and was cultured in the same media conditions as the 4T1 cell line described above (Pesce et al., 2015).

### **1 b. MTT Assay**

The anti-proliferative effect of FK228 and Panobinostat was tested on the 4T1 and DU145 cell lines using the MTT assay. MTT, a yellow tetrazole, is reduced by mitochondrial succinate dehydrogenase to purple formazan in living cells. 200 µl of cell suspension (70,000 cells/ml) was plated into a 96 well plate and incubated for 24 hr at 37°C and 5% CO<sub>2</sub>. FK228 or Panobinostat (along with a 0.1% DMSO control) was added at various concentrations and incubated for another 48 hr. Cells were then washed twice with PBS followed by incubation with 200 µl of MTT solution (250 µg/ml) for 4 hr. The MTT solution was then aspirated, and the formazan crystals were solubilized by the addition of 200 µl of DMSO and by rotating the plate on a shaker for 10 min. Absorbance was determined at 570 nm with a reference wavelength of 690 nm using a microtiter plate reader (Infinite M200 Pro TECAN). All treatments were performed in triplicate. The anti-proliferative effect of FK228 or Panobinostat was expressed as the relative cell number (% control).

Relative cell number = (experimental absorbance – background absorbance)/(absorbance of DMSO control – background absorbance) x 100.

### **1 c. Cell Death**

To identify and quantify cell death taking place after HDACi treatments, live/dead assays using calcein AM and propidium iodide (PI) were performed. Non-fluorescent membrane permeable calcein AM dye is converted to fluorescent calcein through intracellular esterase activity in live cells. PI is membrane impermeant and will not enter live cells with intact membranes. PI enters through degraded plasma membrane in damaged cells and increases in fluorescence through the intercalation with nucleic acids. After staining cells, results can be obtained qualitatively through fluorescence microscopy and quantitatively through flow cytometry. 4T1 cells were harvested at approximately 70% confluency and plated at a density of 35,000 cells/well into a twenty-four well plate. Cells were then stained with calcein AM at 1 $\mu$ M for 30 min and incubated at 37°C, and with PI at 2 $\mu$ M for 15 min before being visualized by fluorescence microscopy as described above. In other experiments, cells were treated as above and then washed with and resuspended in PBS prior to analysis by flow cytometry. A minimum of 10,000 cells were collected for each sample on a BD FACSCalibur flow cytometer running BD CellQuest™ Pro software.

### **1 d. Apoptosis**

To characterize the mechanisms of death induced by HDAC inhibitors, calcein AM/Annexin V assays were performed to detect apoptosis. During apoptosis phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin

V binds specifically to PS in a calcium – dependent mechanism, allowing binding events to be visualized by fluorescence microscopy and quantified via flow cytometry. To quantify apoptotic activity of the 4T1 cells following treatment with FK228, PS expression was analyzed via flow cytometry through annexin V staining.

Similar to the calcein AM/PI assay, qualitative results were achieved through fluorescence microscopy while flow cytometry was used for quantitative data collection. Cells were washed with cold PBS and adjusted to a concentration of  $1 \times 10^6$  cells/ml. 100 $\mu$ l of cell suspension ( $1 \times 10^5$  cells) was stained with calcein AM (1 $\mu$ M) for 30 min, and 5 $\mu$ l of phycoerythrin (PE)-conjugated annexin V [in 1X binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl<sub>2</sub>)] for 15 min at 25°C in the dark with 5  $\mu$ l 7-Aminoactinomycin D (7AAD is a high affinity DNA intercalator). All reagents from BD Biosciences, San Jose, CA, USA. 400 $\mu$ l of binding buffer was added to the suspension and the samples were analyzed by flow cytometry within 1 hr.

### **1 e. Cell Cycle Analysis**

To further assess the cytotoxic effects of HDACi treatment on cell lines, cell cycle analyses were performed. With the data collected from these experiments, it may be possible to delineate cytotoxic effects of treatments from effects that may be merely anti-proliferative. Cells were washed with PBS and the concentration adjusted to  $0.5 \times 10^6$  cells/ml. Cells were then resuspended in 70% ice-cold ethanol that was added dropwise while vortexing. Cells were then incubated on ice for 30 min before being washed with PBS. Cells were then washed with PBS and stained with PI staining solution (500 $\mu$ l of 1mM PI stock, 150 $\mu$ l RNase 100 $\mu$ g/ml, 9.5 ml



0.1% glucose in PBS), and incubated for 30 min at 37°C in the dark. The cells were again washed with PBS and resuspended in 400µl PBS. Samples were then analyzed via flow cytometry.

#### **1 f. Autophagy**

To further interrogate the mechanism of cell death caused by HDACi treatment, autophagy assays were also performed. Cells were plated in a 24 well plate at a concentration of 35,000 cells/well and grown overnight. Cells were treated with HDACi or DMSO control for a 24 hr incubation. 30 µM chloroquine was used as a positive control. Cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min. Cells were washed with PBS and permeabilized with Tris buffered saline (TBS) containing 0.1% Tween 20 and 1% bovine serum albumin (BSA) for 1 hr at 4°C. Cells were washed with PBS, and stained with anti-LC3B polyclonal rabbit IgG (Thermo Fisher) at a dilution of 1:1000 and incubated at 4°C for 4 hr. Cells were washed with TBS and 1% BSA before incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson Labs) at a dilution of 1:500 for 1 hr at 4°C. Cells were washed with PBS and counter stained with DAPI nuclear stain at a concentration of 3µM for 15 min at room temperature in the dark, before a final wash with PBS. Cells were then imaged via fluorescence microscopy.

#### **1 g. Statistical Analysis**

Data are presented as mean ± SEM unless stated otherwise. Significant differences between sample means were calculated using a Mann – Whitney U test with p<0.05 being considered statistically significant.

## 2. Results

To obtain effective concentration (IC<sub>50</sub>) values for FK228 and Panobinostat in the DU145 cell line, the MTT assay was performed. FK228 produced a powerful anti-proliferative effect, suppressing cellular proliferation in the DU145 cell line even at 0.00001  $\mu$ M. The IC<sub>50</sub> of FK228 in the DU145 cell line was 0.006  $\mu$ M. Panobinostat displayed a dose-dependent effect upon cellular proliferation, as relative cell number increased from the 10  $\mu$ M dose to the 0.1% DMSO control, yielding an IC<sub>50</sub> of 1.875  $\mu$ M. The results from the MTT assay show that both FK228 and Panobinostat can have a strong antiproliferative effect on DU145 cells. To assess the effects of treatment with HDACi on cell viability in the 4T1 model, cells were treated with FK228 in multiple concentrations and stained with calcein-AM and PI. Figure 2 shows a dose-dependent effect of FK228 in 4T1 cells at a 24 hr time point, causing an increase in PI-stained populations (dead) and a decrease in calcein staining (live) as the concentration of FK228 increased. To quantify cell viability following treatment of 4T1 cells with FK228, calcein AM and PI staining was performed and analyzed via flow cytometry.

Following a 48 hr treatment with FK228, 4T1 cells showed increased cytotoxicity at the 10 nM concentration (Figure 3). At the 1nM concentration of FK228, the PI-positive population increased from 19% to 28%, relative to the DMSO control. This staining pattern suggests that treatment with FK228 causes plasma and nuclear membrane degradation increasing cell death.

To clarify the mechanism of cell death following exposure of 4T1, DU145, and U937 cells to FK228 and Panobinostat, PS expression was assayed as a marker for apoptosis. Further, by labelling with annexin V, cells were assayed for early, late, and total apoptosis. Cell populations

that stained positive for PS only were considered to be in early apoptosis, while cell populations that stained positive for both PS and 7AAD were considered to be in late apoptosis. The sum of both populations was taken as the measure of total apoptotic populations. After a 24 hr exposure to 10  $\mu$ M Panobinostat, PS expression increased in 4T1 cells compared to DMSO control (Figure 4A and B). After a 24 exposure, both FK228 and Panobinostat showed significant increases in early and total apoptosis compared to DMSO control (Figure 4C). Similar results were observed in the DU145 cell line; however, 0.1  $\mu$ M FK228 treatment did not result in a statistically significant difference from DMSO in late or total apoptotic populations (Figure 4D). Increases in early and total apoptosis were observed for both doses of FK228 and Panobinostat in U937 cells (Figure 4E).

To determine effects of HDACi treatment on cell cycle, 4T1, DU145, and U937 tumor cell lines were treated with FK228, Panobinostat, or DMSO, stained with PI and analyzed by flow cytometry. After a 24 hr incubation, 4T1 cells appeared to undergo cell cycle arrest in the G0/G1 phase (Figure 5C-D). Cell cycle analyses can also be used to approximate the portion of apoptotic cells in a population.

These results suggest that treatment with FK228 inhibits cell cycle progression in addition to producing cytotoxicity. Over a 24 hr time exposure, treatment of 4T1, DU145, and U937 cell lines with FK228 or Panobinostat all resulted in large increases in apoptosis compared to DMSO controls (Figure 5B-G). 4T1 cells treated with 1  $\mu$ M or 0.1  $\mu$ M FK228 resulted in a cell cycle arrest in the G0/G1 phase in addition to increased apoptosis (Figure 5B). 4T1 cells treated with 10  $\mu$ M Panobinostat resulted in increased apoptosis compared to DMSO control along

with a reduction in the G0/G1 phase and an increase in S phase, indicating a possible cell cycle arrest in S phase (Figure 5C). 1  $\mu$ M Panobinostat treatment of 4T1 cells caused an increase in apoptosis with no effects on the cell cycle (Figure 5C). DU145 cells treated with 1  $\mu$ M or 0.1  $\mu$ M FK228 also showed an increase in apoptosis compared to DMSO controls, with a large decrease in G0/G1 (Figure 5D). This decrease in G0/G1 phase was accompanied by a significant increase in cells in the S and G2/M phase (Figure 5D). DU145 cells treated with 10  $\mu$ M or 1  $\mu$ M Panobinostat showed similar results, leading to an increase in apoptosis accompanied by decreases in G0/G1 phase, and increases in S and G2/M (Figure 5E). U937 cells treated with 1  $\mu$ M or 0.1  $\mu$ M FK228 resulted in dramatically increased apoptosis compared to DMSO control, accompanied by very little to no change in cell cycle (Figure 5F). U937 cells treated with 10  $\mu$ M or 1  $\mu$ M Panobinostat also resulted in large increases in apoptosis compared to DMSO, with modest effects on the cell cycle (Figure 5G).

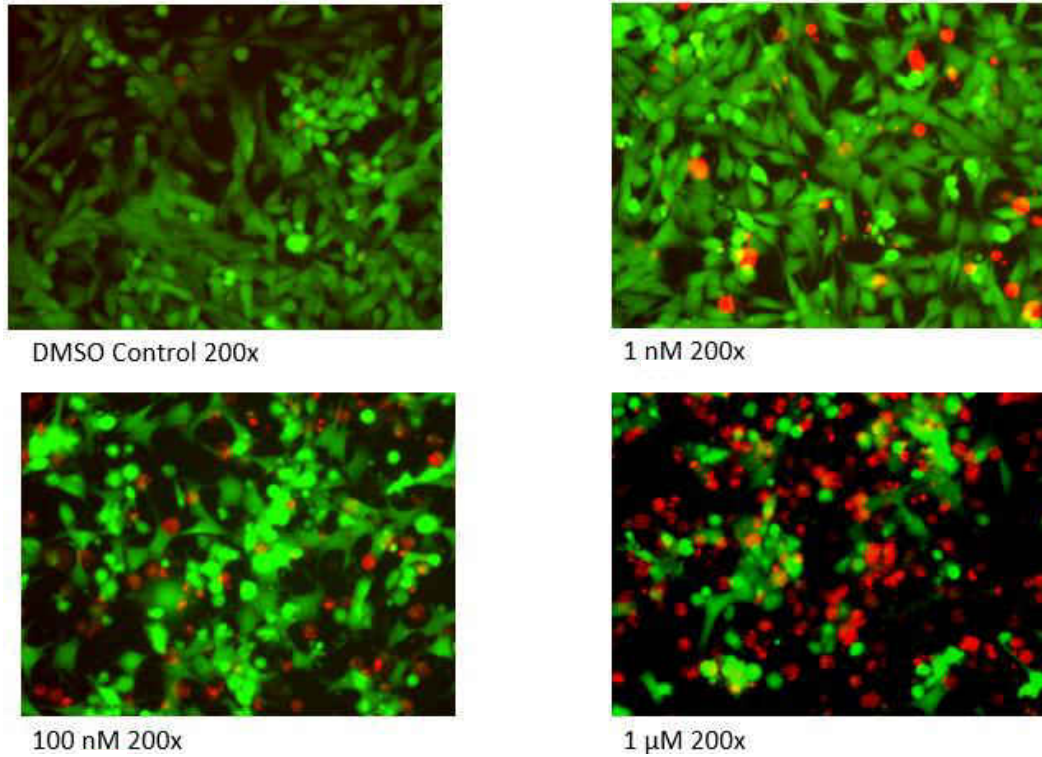
To further interrogate the mechanisms of cell death caused by treatments with HDACi, autophagy assays were performed by imaging LC3B recruitment to the autophagosome through immunofluorescence. 30  $\mu$ M Chloroquine was used as a positive control, while 0.1% DMSO served as the negative control. Both 4T1 and DU145 cells showed high levels of LC3B recruitment following a 24 hr treatment with 30  $\mu$ M chloroquine as expected (Figures 6 and 7). DMSO controls for both 4T1 and DU145 cells showed basal distribution of LC3B (Figures 6 and 7). 4T1 cells treated with FK228 and Panobinostat resulted in increased levels of LC3B recruitment compared to DMSO controls (Figure 6). DU145 cells also showed similar increases in LC3B recruitment compared to DMSO controls (Figure 7).

### 3. Discussion

The results of these studies show that both FK228 and Panobinostat are powerful HDAC inhibitors with strong antiproliferative effects. After assessing the impact of these inhibitors upon cellular proliferation, the potential for cytotoxicity and cell death was examined next. Through calcein/PI staining, 4T1 cells displayed high levels of cell death following exposure to FK228 at the 24 hr mark (Figure 2). The effect appeared to be dose dependent. At the 48 hr mark, cell death continued as the amounts of PI positive cell staining increased in the 4T1 cell line (Figure 3).

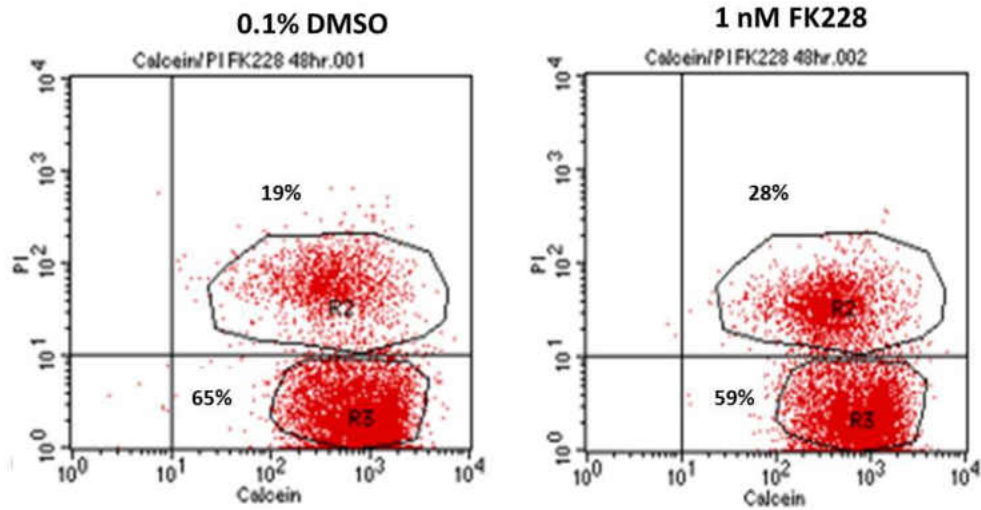
Following the examination of cell death through HDAC inhibition, the mechanism was then explored. PS exposure was assayed as a marker for apoptosis and detected by flow cytometry. In the 4T1, DU145, and U937 cell lines, 24 hr treatment with FK228 or Panobinostat resulted in large increases in PS expression compared to DMSO controls (Figure 4). This increase suggests that HDAC inhibition caused cell death *in vitro* through apoptosis. These results were confirmed through cell cycle analysis (Figure 5). All treatments showed increases in apoptosis compared to DMSO controls in the 4T1, DU145, and U937 cell lines (Figure 5). This cell death was also accompanied by cell cycle dysregulation and arrest in the 4T1 and DU145 cell lines (Figure 5). The U937 cell line did not show any cell cycle arrest following exposure to Panobinostat, only high levels of apoptosis (Figure 5). Treatment with FK228 yielded small decreases in G0/G1 phase in the U937 cell line, also accompanied by high levels of apoptosis. From these results, it is reasonable to conclude that the HDACi treatments were highly cytotoxic in the U937 cell line, with no substantial effects upon the cell cycle.

To further interrogate the cell death mechanism utilized following HDAC inhibition, 4T1 and DU145 cells were assayed for autophagy through LC3B recruitment (Figure 6). Both FK228 and Panobinostat treatments resulted in elevated LC3B recruitment in 4T1 and DU145 cell lines; however, it appeared that Panobinostat treatments caused the greatest recruitment (Figure 6). These results suggest that while apoptosis clearly plays a large role in cell death upon exposure to HDACi, autophagy seems to also take place, particularly following exposure to Panobinostat.



**Figure 2. 4T1 cells exhibited dose-dependent cytotoxicity following treatment with FK228 at 24 hr.**

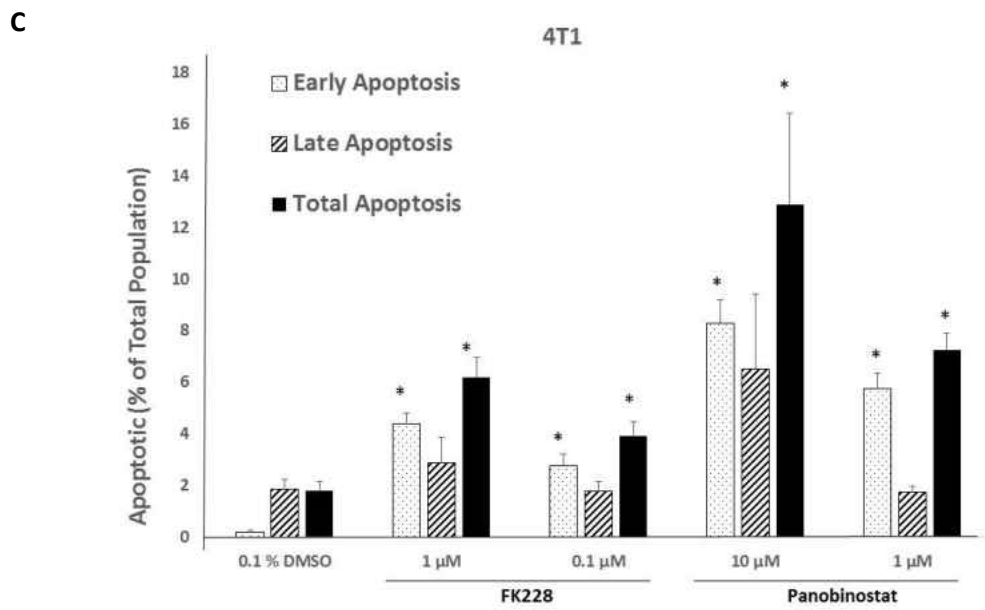
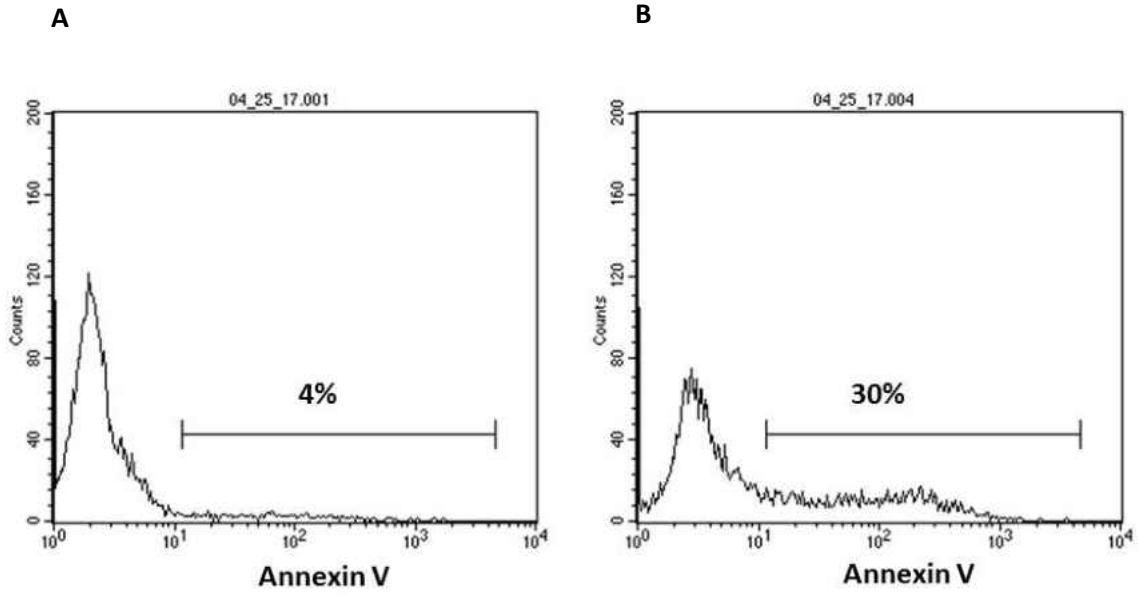
4T1 cells were treated with the indicated doses of FK228 or equivalent volume of DMSO as vehicle control. Calcein/PI staining was visualized by fluorescence microscopy and all images were taken at the same exposure setting. Green = calcein, red = PI.



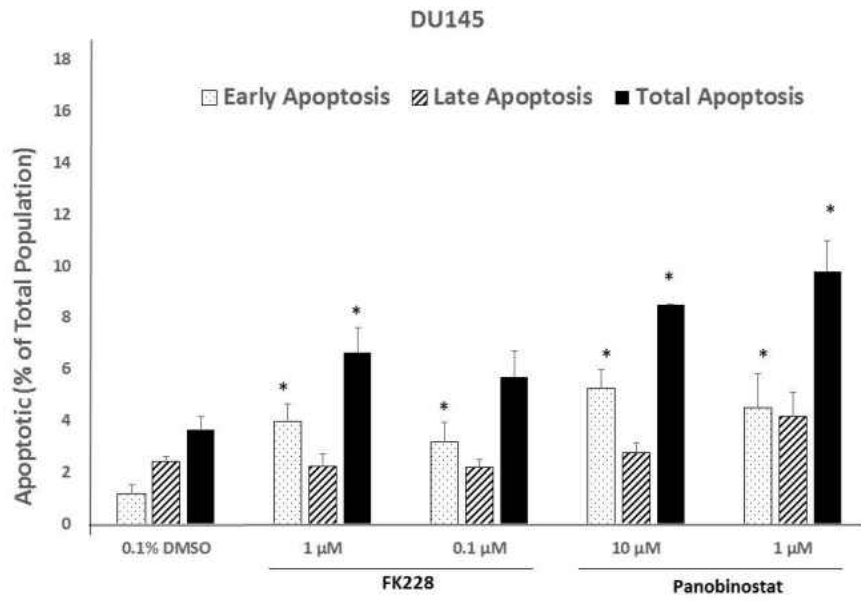
**Figure 3. FK228 treatment induces cytotoxicity in 4T1 cells.**

4T1 cells were treated with FK228 or DMSO control for 48 hr, stained with calcein AM and PI and analyzed via flow cytometry. Numbers indicate the percent of cells found within the indicated gate.

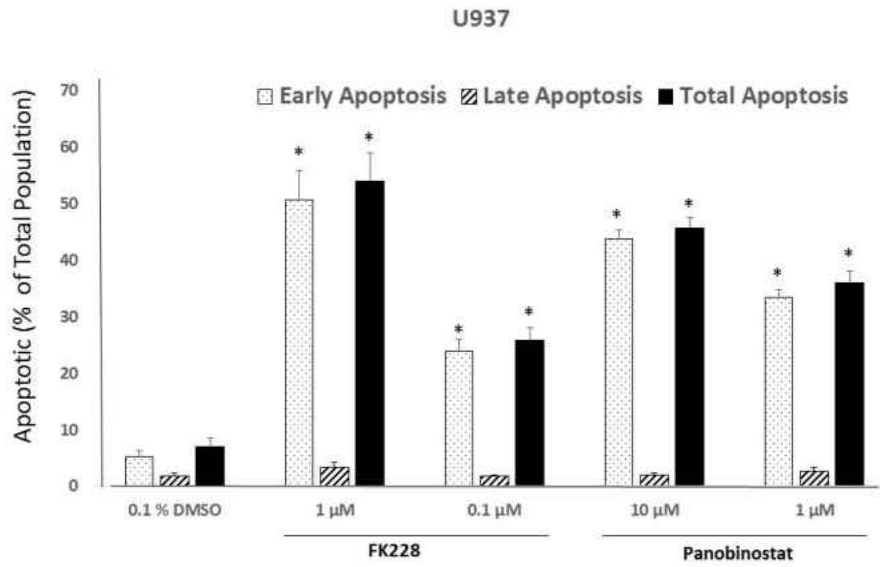




D



E



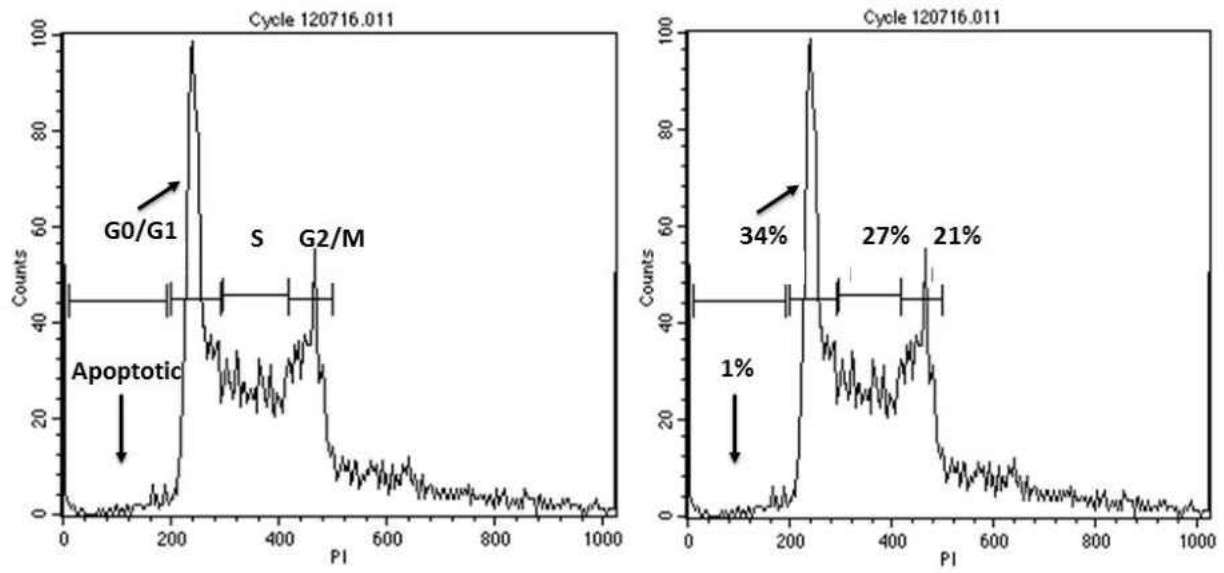
**Figure 4. Treatment of 4T1, DU145, and U937 cells with FK228 or Panobinostat increased apoptotic populations.**

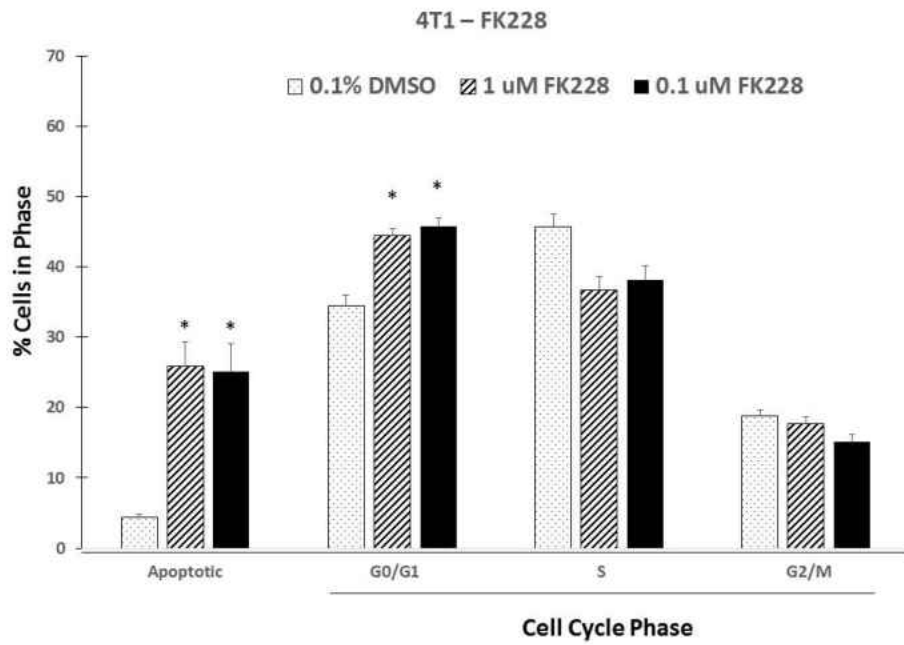
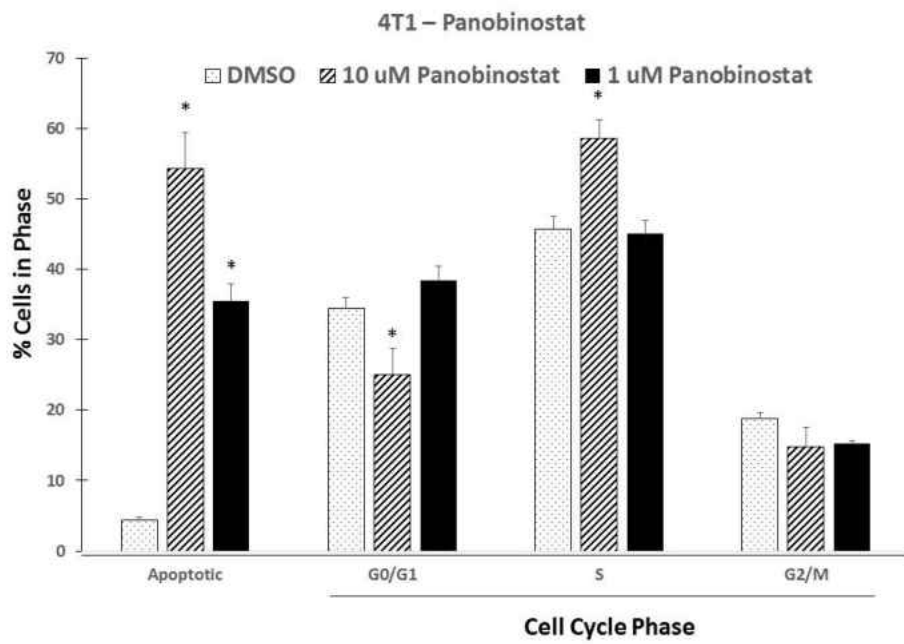
Cells treated with HDACi or DMSO control for 24 hr, stained with annexin V – PE and 7AAD for apoptosis analysis and analyzed via flow cytometry.

**A)** 4T1 cells treated with 0.1% DMSO control, **B)** 4T1 cells treated with 10  $\mu$ M Panobinostat, **C)** 4T1 cells, **D)** DU145 cells, and **E)** U937 cells.

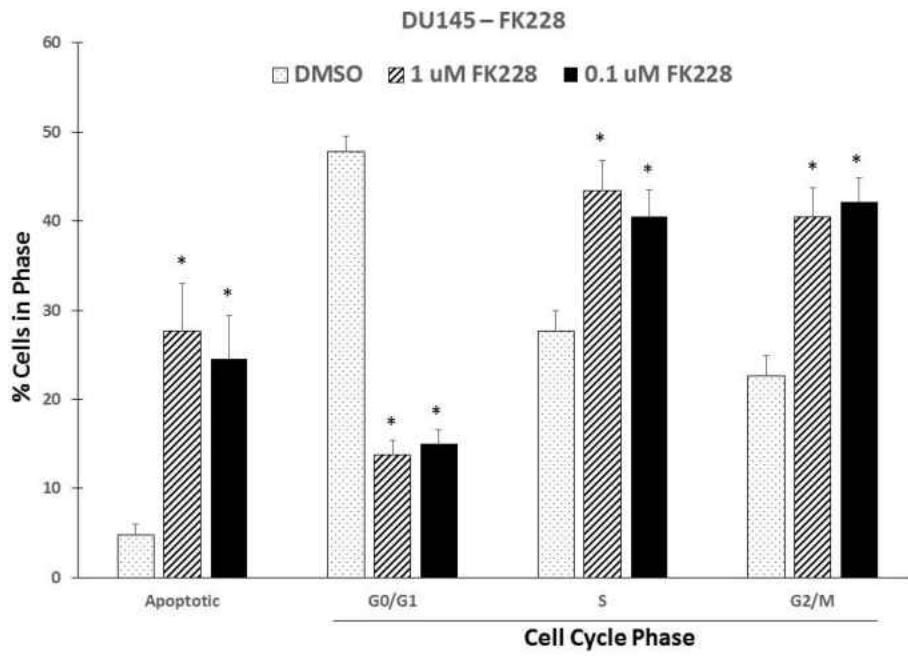
Results represent means  $\pm$  SEM for three to four independent experiments. \* indicates differences between values from treatment groups compared to the corresponding DMSO control were significantly different;  $P < 0.05$ .

A

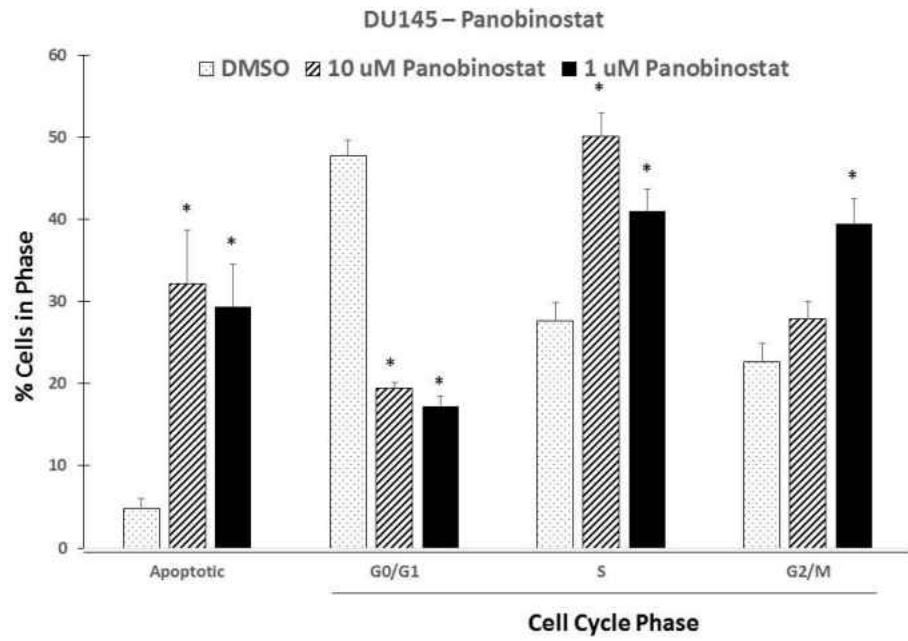


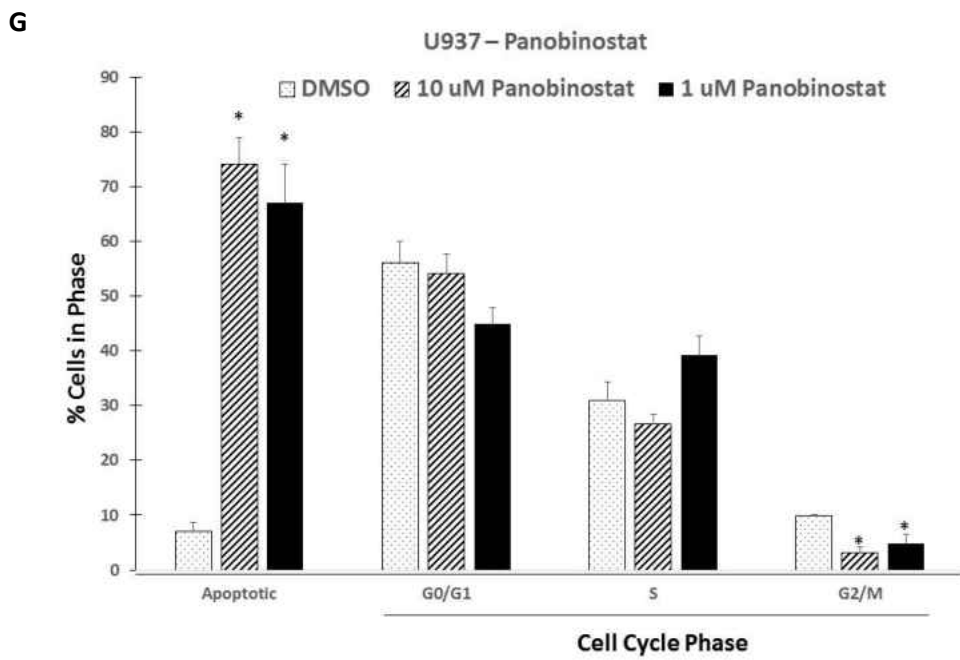
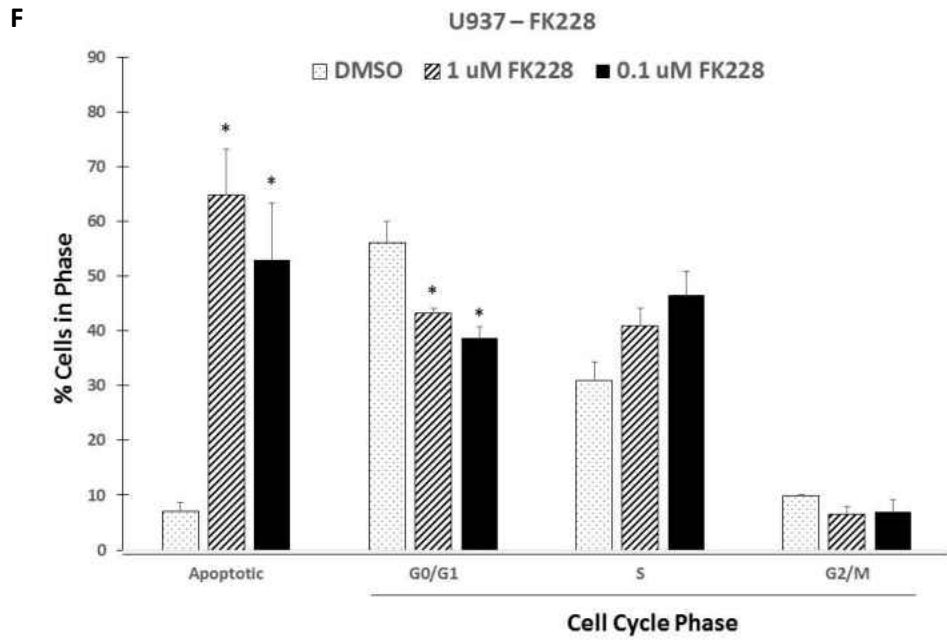
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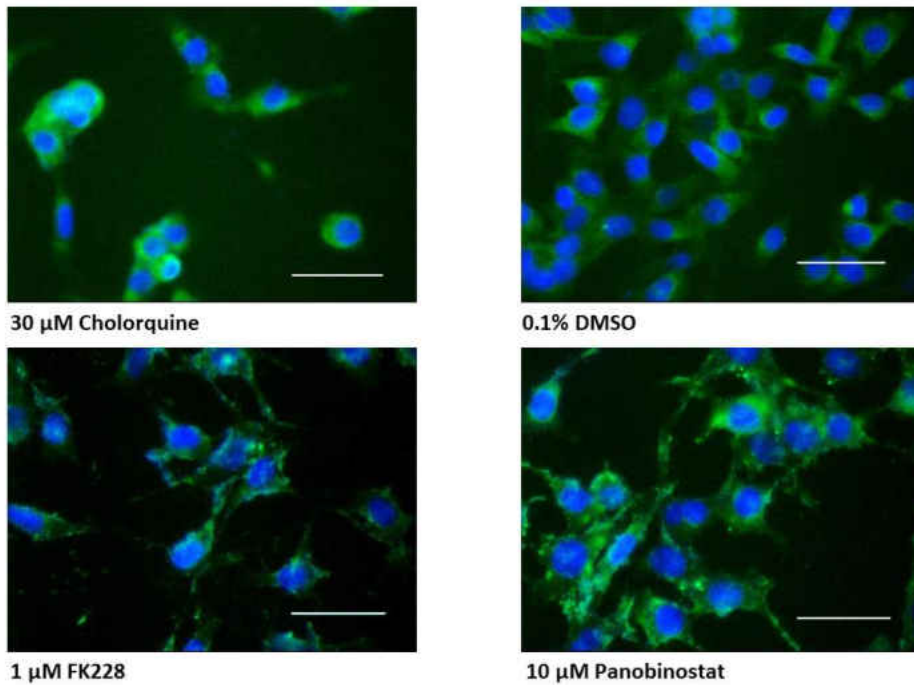
**Figure 5. Treatment of 4T1, DU145, and U937 cells with FK228 or Panobinostat resulted in apoptosis and cell cycle dysregulation.**

Cells treated with HDACi or DMSO control for 24 hr, stained with PI for cell cycle analysis and analyzed via flow cytometry.

**A)** 4T1 cells treated with 0.1% DMSO, **B)** 4T1 treated with FK228, **C)** 4T1 treated with Panobinostat, **D)** DU145 treated with FK228, **E)** DU145 treated with Panobinostat, **F)** U937 treated with FK228, and **G)** U937 treated with Panobinostat.

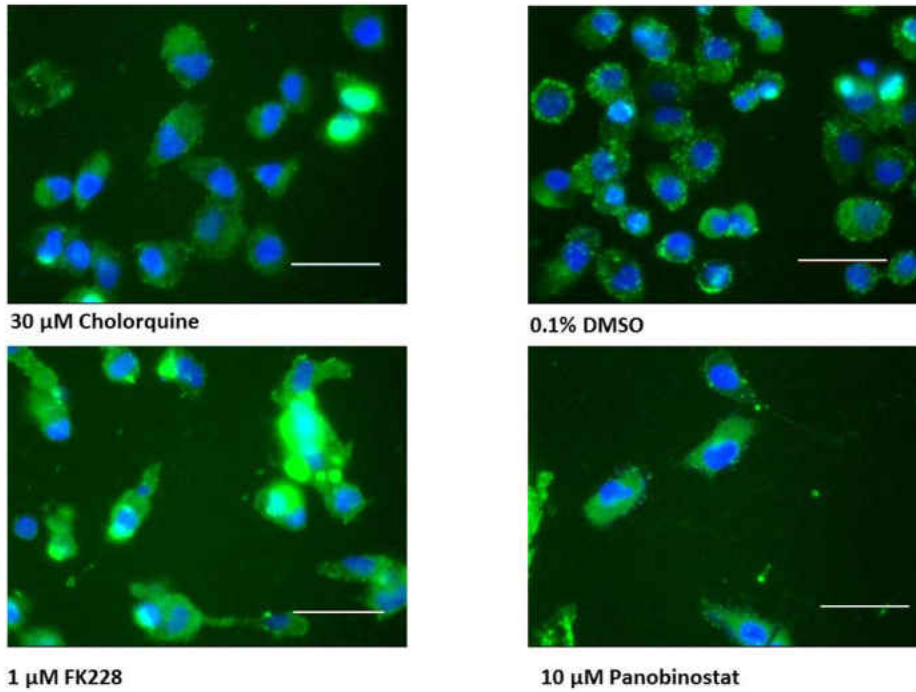
Results represent means  $\pm$  SEM for three to four independent experiments. \* indicates differences between values from treatment groups compared to DMSO control were significantly different;  $P < 0.05$ . Apoptotic populations were gated separately from that of the cell cycle analysis.





**Figure 6. Treatment of 4T1 cells with FK228 or Panobinostat resulted in increased autophagy.**

4T1 cells were treated with the chloroquine or indicated doses of FK228 or equivalent volume of DMSO as vehicle control and stained with anti-LC3B antibody to detect autophagy. Staining was visualized by fluorescence microscopy and all images were taken at the same exposure setting. Size bar = 50 microns. Green = LC3B localization, blue = DAPI counterstain (nuclear).



**Figure 7. Treatment of DU145 cells with FK228 or Panobinostat results in autophagy.**

DU145 cells were treated with chloroquine or the indicated doses of FK228 or equivalent volume of DMSO as vehicle control and stained with anti-LC3B antibody to detect autophagy. Staining was visualized by fluorescence microscopy and all images were taken at the same exposure setting. Size bar = 50 microns. Green = LC3B localization, blue = DAPI counterstain (nuclear).

## **Chapter 3 – The Effects of HDAC Inhibition on Myeloid Derived Suppressor Cells (MDSC)**

A major barrier to cancer immunotherapeutic treatments in general has come from the complex, immunosuppressive microenvironment that envelops solid tumors. MDSC populations play an instrumental role in this immunosuppression. This chapter seeks to assess the potential cytotoxic effects of HDACi on MDSC populations taken from 4T1 tumor – bearing BALB/c mice, to examine the feasibility for HDACi treatment in solid tumors. To assess cytotoxicity, cell viability and cell count assays were performed. In addition, HDACi treatments were performed upon conventional murine leukocyte populations as a measure of off target activity *ex vivo*.

### **1. Materials and Methods**

#### **1 a. Myeloid Derived Suppressor Cell Viability**

To examine the effects of FK228 and Panobinostat upon MDSC populations, cell viability assays were performed. To generate MDSCs, female BALB/c mice were injected with 4T1 cells ( $1 \times 10^4$  cells in 50  $\mu$ l) in supplement free RPMI medium subcutaneously into the mammary fat pad. After four weeks, spleen tissue was harvested from the tumor-bearing mice. To achieve a single – cell suspension, spleen tissue was mechanically disrupted in PBS, filtered through a 70  $\mu$ m filter mesh, and resuspended in red blood cell lysis buffer (Thermo Fisher) 10x stock diluted to 1x in PBS. Cells were then washed twice more with PBS before being seeded into a 24 well plate ( $0.5 \times 10^6$  cells/well) containing 500  $\mu$ l RPMI 1640 medium, supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 2mM L-glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin (all from Life Technologies, Grand Island, NY, USA) with 55 $\mu$ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). FK228 and Panobinostat concentrations were

then made 2x and 500  $\mu$ l of each concentration was added to each well (or a 0.1% DMSO control). After a 24 hr incubation, cells were then washed with PBS and stained with rat anti-mouse Ly6G and Ly6C (myeloid monocytic markers) IgG (BD Pharmingen) at a 1:100 dilution for 30 min on ice. Cells were then washed with PBS before the addition of 5  $\mu$ l 7AAD before analysis by flow cytometry.

### **1 b. Primary Leukocyte Cell Viability**

Single – cell suspension was achieved from the spleen of a non-tumor-bearing BALB/c mouse as described above. Cells were then treated with FK228, Panobinostat, or 0.1% DMSO and cultured for 24 hr at 37°C and 5% CO<sub>2</sub> in RPMI 1640 medium, supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 2mM L-glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin (all from Life Technologies, Grand Island, NY, USA) with 55 $\mu$ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). To assess viability after HDACi treatments, live/dead assays using calcein AM and propidium iodide (PI) were performed. Cells were stained with 0.5  $\mu$ M calcein AM and 2  $\mu$ M PI for 15 min, washed with PBS and resuspended in 400  $\mu$ l PBS for analysis via flow cytometry. Cells were then stained with PE-conjugated rat anti-mouse B220, FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8, and FITC conjugated rat anti-mouse CD11b (all from BD Pharmingen). All samples were analyzed via flow cytometry on “Hi” setting (a rate of 60  $\mu$ l/min) for 3:00 min. Cell number/sample was calculated by:  $(400 \mu\text{l}) \times (\text{event count})/180 \mu\text{l} = \text{cell number}$ .

## 2. Results

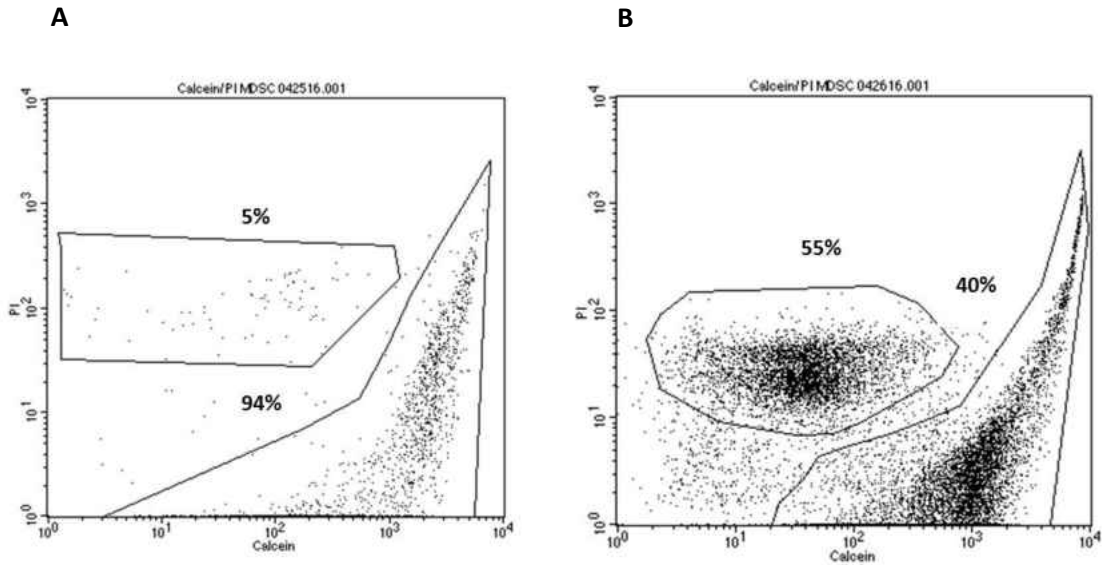
To determine the sensitivity of MDSC to HDACi treatment, MDSC harvested from 4T1 tumor-bearing mice were used. Figure 10A depicts a high proportion of live calcein-positive cells (greater than 90%) with a relatively small population of PI-positive dead cells prior to treatment with FK228. In contrast, roughly 55% of the cells were dead (staining positive for PI) following 24 hr of 10 nM FK228 treatment (Figure 8B). These results suggest that treatment of MDSC with FK228 dramatically reduces viability. Following a 24 hr incubation with FK228 or Panobinostat, both cell number and cell viability decreased sharply compared to DMSO controls (Figure 9A-B).

We next sought to determine the effects of FK228 and Panobinostat on primary murine lymphocyte populations as a measure of off target activity. After a 24 hr incubation with FK228 or Panobinostat, conventional primary murine leukocyte populations only experienced a modest if any reduction in relative cell number as a percentage of DMSO control (Figure 10A). After a 48 hr incubation with FK228 or Panobinostat cell numbers decreased (Figure 10B); however, none of these decreases were statistically significant. Therefore, neither FK228 or Panobinostat had significant off target effects upon primary murine leukocyte populations *ex vivo*.

### **3. Discussion**

Following treatment with FK228 or Panobinostat, MDSC populations showed marked decreases in both absolute cell number and cell viability compared to DMSO controls (Figure 9). These results are consistent with the high levels of apoptosis that followed treatment of U937 cells with FK228 or Panobinostat (Figure 5), suggesting a susceptibility of cell populations derived of myeloid lineage to cytotoxicity due to HDAC inhibition. The strong effect that both FK228 and Panobinostat had on cell number and viability of MDSC further suggests that HDAC expression may change along the myeloid developmental pathway, and that HDAC expression in MDSC populations may be similar to that found in tumor cell populations.

It is also worth noting that while HDAC inhibition is not a tumor-selective mechanism, conventional primary murine leukocyte populations exposed to FK228 or Panobinostat did appear to be much less sensitive following exposure in the same concentrations at the same time points. These results suggest that aberrant HDAC expression in the 4T1 and DU145 cell lines causes these cells to be more susceptible to HDAC inhibition.

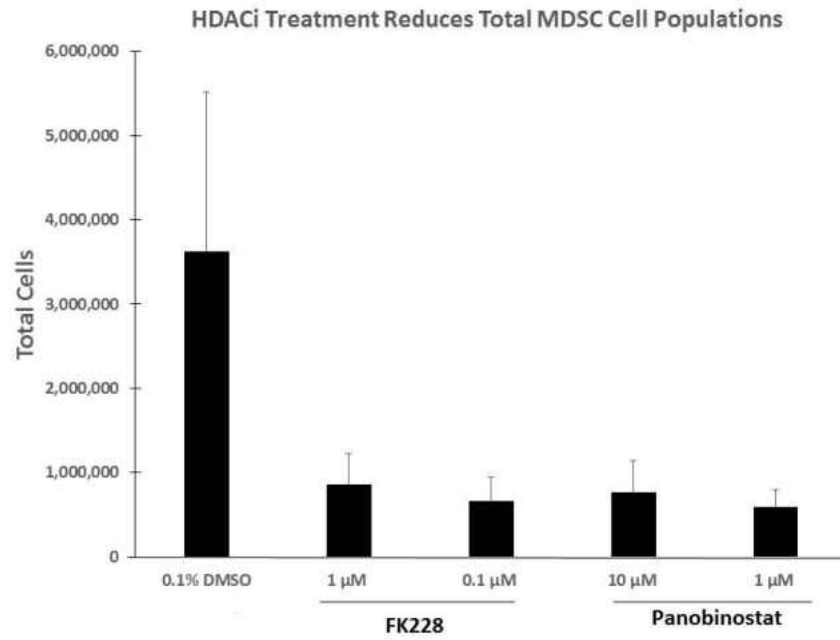


**Figure 8. FK228 treatment produced cytotoxicity in mononuclear cells harvested from 4T1 tumor – bearing mice.**

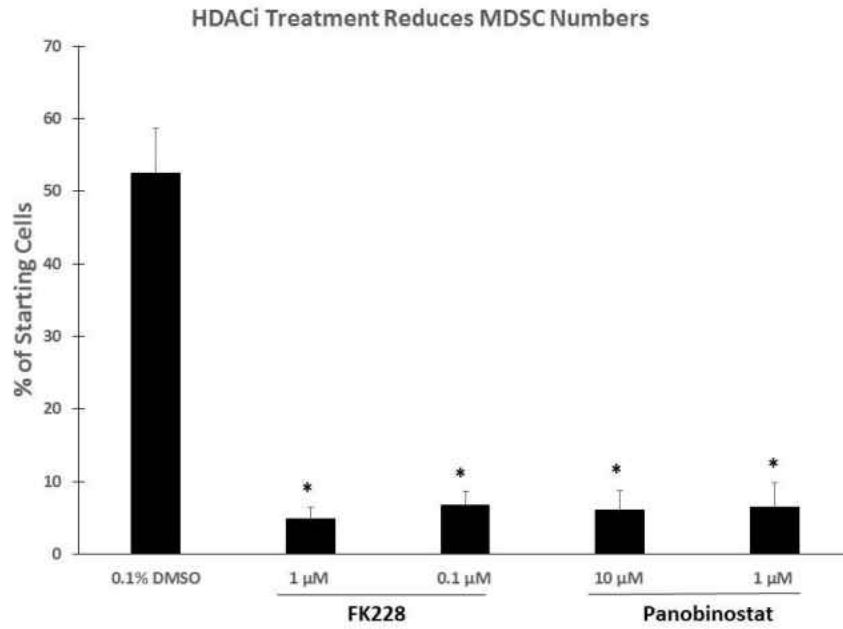
Splenocytes were harvested from 4T1 tumor – bearing mice and treated with FK228 or 0.1% DMSO control, stained with calcein AM and PI in a viability assay and analyzed via flow cytometry.

**A)** Cells treated with 0.1% DMSO control at 0 hr **B)** Cells following incubation with 10 nM FK228 for 24 hr. Numbers indicate the percentage of cells located within the indicated gate.

**A**



**B**





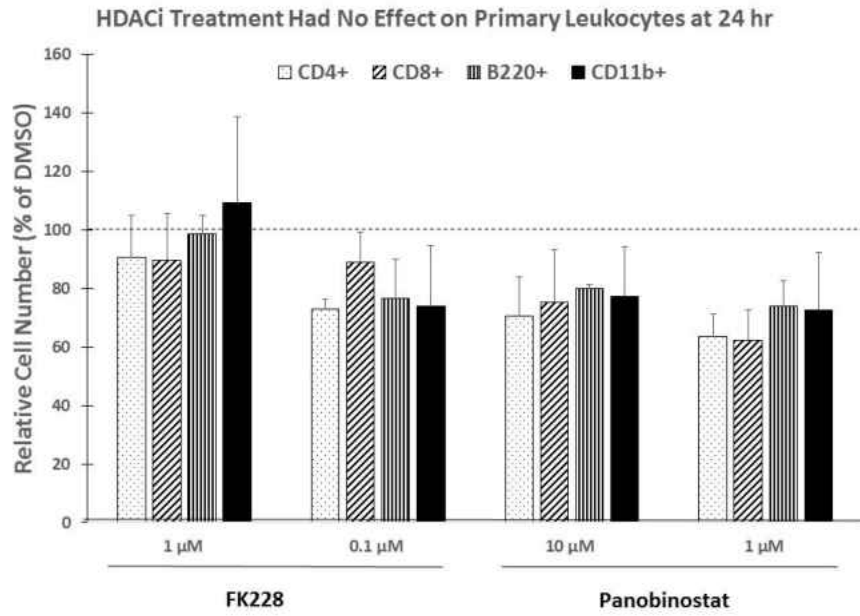
**Figure 9. HDACi treatment for 24 hr produced cytotoxicity in myeloid derived suppressor cells.**

Myeloid derived suppressor cells were harvested from the spleen of 4T1 tumor-bearing mice, treated with HDACi or equivalent volume DMSO control for 24 hr, stained with anti-Ly6G/Ly6C antibodies, and analyzed via flow cytometry.

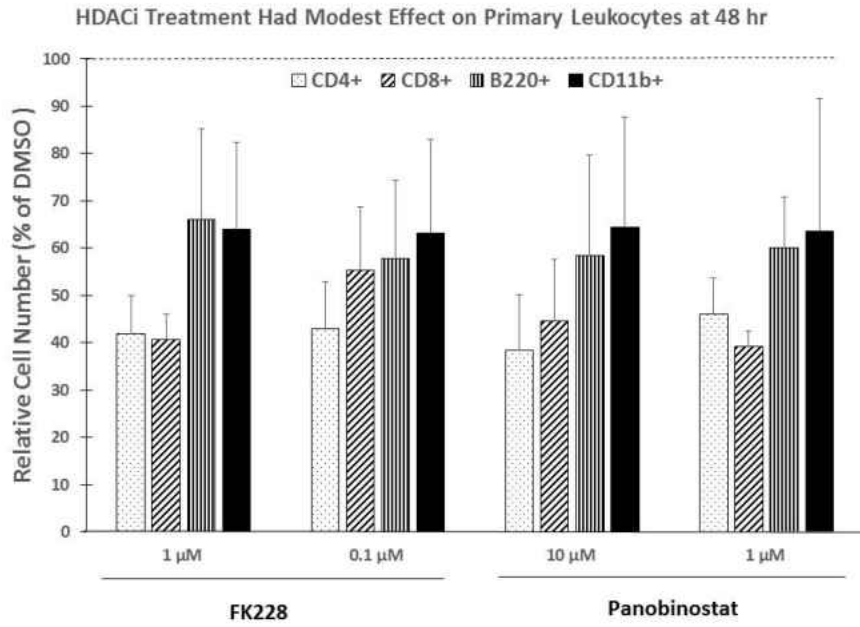
**A)** Total cell number of MDSC, **B)** Percentage of MDSC remaining at the end of culture.

Results represent means  $\pm$  SEM for three to four independent experiments. \* indicates differences between values from treatment groups compared to DMSO control were significantly different;  $P < 0.05$ .

**A**



**B**



**Figure 10. Treatment of primary murine leukocytes with FK228 or Panobinostat had only a modest effect on cell viability.**

Primary murine leukocytes were harvested from BALB/c mice and treated with HDACi or equivalent volume DMSO control for 24 or 48 hr. Cells were then stained for CD4, CD8, B220, or CD11b and analyzed via flow cytometry.

**A) 24 hr, B) 48 hr.**

Results represent means  $\pm$  SEM for three to four independent experiments. \* indicates differences between values from treatment groups compared to DMSO control were significantly different;  $P < 0.05$ . Horizontal dashed line indicates 100% control value.

## Chapter 4 – Conclusions

The studies herein show that both FK228 and Panobinostat show powerful anti-proliferative and cytotoxic effects in 4T1, DU145, and U937 cell lines. 24 hr treatments of both FK228 and Panobinostat caused high levels of cell death in all three cell lines, mostly through apoptosis. Although apoptosis did appear to be the main cell death mechanism through which most cellular damage occurred following treatments with FK228 and Panobinostat, autophagy appeared to increase as a result of HDACi treatment, and therefore may contribute to the increased cell death. Autophagy was most common in DU145 cells treated with a high dose (10  $\mu$ M) of Panobinostat, indicating that the different mechanisms of the two drugs has an effect on cell death. Both FK228 and Panobinostat also showed strong effects on the cell cycle of all three cell lines, resulting in increased apoptosis and cell cycle arrest. Interestingly, MDSC populations also had a strong response to FK228 and Panobinostat treatment compared to the DMSO controls. MDSC populations experienced a sharp decrease in cell number and viability following 24 hr drug treatments indicating MDSC populations may also express HDACs aberrantly. From these results it is reasonable to postulate that HDACi treatment may result in less immunosuppression in the solid tumor microenvironment, a yet untested idea as there are currently no inhibitors approved for the treatment of solid tumors.

In contrast to the results obtained from treatments of MDSC populations, neither FK228 nor Panobinostat appeared to have a significant effect on primary murine leukocytes (Figure 10). When compared to the viability of 4T1 and DU145 cell lines following exposure to FK228 or Panobinostat, primary murine leukocytes showed a modest response. These results suggest that off target activity was limited *in vitro*. This may be due to normal HDAC expression in non-

cancerous cells, along with the lowered metabolic activity of primary leukocytes *ex vivo*. However *in vivo* treatment with HDACi could have a more significant effect on immature populations of these cells. Taken together, the results of these studies show that HDAC inhibitors induce a powerfully cytotoxic response in the 4T1, DU145, and U937 cell lines, while also drastically reducing the viability of MDSC populations that are found in the complex microenvironment of solid tumors. These results suggest that HDAC inhibitors may warrant further investigation into the treatment of solid tumors.

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