Effect of Histone Deacetylase Inhibition on Ovarian Cancer Cell Survival

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:42004173">http://nrs.harvard.edu/urn-3:HUL.InstRepos:42004173</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Effect of Histone Deacetylase Inhibition on Ovarian Cancer Cell Survival

Ryan Sinapius

A Thesis in the Field of Biotechnology

For the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2019
Abstract

An estimated 15.5 million Americans have or are currently in remission from cancer and this number is likely to rise to 20.3 million by 2026 (Kimberly et al., 2017). Drug resistance is a major issue during cancer therapy. Therefore, there is a constant need for novel chemotherapy agents for all cancer types including ovarian cancer (Ma et al., 2014, Seguin et al., 2015). Ovarian cancer is the 5th leading cause of cancer-related death among women and, in 2018 alone; there were an estimated 14070 deaths (Torre et al., 2018). In the past several years, gene-regulatory chemotherapeutic agents that act on the epigenetic mechanisms have drawn considerable interest as a potentially important class of anti-cancer treatment. Due to the complexity of cell cycle, proliferation and apoptotic processes involved in cancer progression, the identification of specific gene controlling proteins is highly desirable for targeted therapy. In this study, I determined that histone deacetylase 3 (HDAC3) or histone deacetylase 8 (HDAC8) inhibition caused cell death in ovarian cancer cell lines with a similar effectiveness as generic pan-HDAC inhibition. Firstly, pan-HDAC inhibition was compared to HDAC class specific and HDAC protein specific inhibition with HDAC3 or HDAC8 inhibition causing cell death as indicated by elevated protein levels of the apoptosis indicator cleaved caspase 3. Next, the cellular pathways associated with HDAC3 or HDAC8 inhibition were interrogated. Both HDAC3 and HDAC8 inhibition caused upregulation of DNA damage markers, while HDAC3 inhibition also caused upregulation of unfolded protein response.
markers. Additionally, HDAC3 or HDAC8 inhibition combined with Bcl-xL inhibition had a synergistic effect on the percentage of dead cells post treatment. Finally, HDAC3 and HDAC8 inhibition were tested on multiple cell lines with HDAC3 inhibition causing cell death in all ovarian cancer cells line and a lung cancer cell line while and HDAC8 inhibition only causing cell death in ovarian cancer cell lines.
Acknowledgments

Reginaldo Prioli, Ph.D. – Reggie was my thesis adviser and I am very thankful for his time and support. He provided excellent, thorough, feedback on all my experiments. And really pushed me to make sure that my data was strong and defensible. His insight into how to design and follow through on experiments allowed this thesis to progress rapidly with very strong data.

Chris Fry, Ph.D. – Chris has previously mentored other Harvard Extension student. His advice and feedback throughout the proposal development and execution provided a greater understanding and confidence in my experimental design and data analysis.

Gary Kasof, Ph.D. – Gary provided excellent feedback on my cell death analysis and his expertise clarified some of the results I obtained.

Sarah L’Heureux – Sarah gave technical support when troubleshooting my cell cycle analysis.

Jeremy Fischer – Provided insight and was a valuable sounding board when developing experiments and thinking about follow up experiments.
Cell Signaling Technology, Inc. – This is my employer whose generous benefits have allowed me to pursue my master over the past 6 years. They allowed me to access our deep catalog of antibodies and this is where I performed all my research.
# Table of Content

Acknowledgments .................................................................................................................. v

List of Tables .......................................................................................................................... ix

List of Figures ......................................................................................................................... x

I. Introduction .......................................................................................................................... 1

  Epigenetic Regulation ............................................................................................................ 3

  HDAC Functional Classes ..................................................................................................... 4

  HDAC Upregulation in Cancer ............................................................................................. 7

  Identifying HDAC inhibition Targets in Cancer Cell Lines .............................................. 9

  Pan-HDAC Inhibition ........................................................................................................... 9

  Class Specific Inhibition ..................................................................................................... 10

  HDAC Protein Specific Inhibition ....................................................................................... 11

  Combination Therapy ......................................................................................................... 13

  Specific Aims ....................................................................................................................... 16

II. Material and Methods ...................................................................................................... 20

  Cell Culture ......................................................................................................................... 20

  Cell Treatments .................................................................................................................... 21

  Sample Preparations ........................................................................................................... 21

  Western Blot ........................................................................................................................ 22

  Cell Cycle Analysis ............................................................................................................. 24

  Cell Death Analysis ............................................................................................................. 25

III. Results .............................................................................................................................. 26
Specific Aim 1 – Comparison of the amount of cell death or cell arrest caused by vorinostat, and HDAC class or protein specific inhibitors ........................................ 26

Comparing Pan-HDAC Inhibition to HDAC Class I Protein Inhibition and HDAC Protein Specific Inhibition ................................................................. 29

Specific Aim 2 – To elucidate the possible anti-cancer effects that HDAC inhibition may have on OVCAR8 cells and to begin understanding the mechanism of action ........................................................................................................... 32

Analysis of Anti-Cancer Pathways Using Class Specific Inhibitor ...... 32

Analysis of anti-cancer pathways for protein specific inhibition ...... 35

Time Course Analysis of HDAC3 and HDAC8 Specific Inhibition ...... 38

Analysis of Pathways That Lead to Apoptosis .............................................. 42

Analysis of cell death in HDAC3, HDAC8 Bcl-xL inhibition combination treatment ........................................................................................................... 44

Specific Aim 3 – Comparing cell death induced by pan-HDAC inhibition to HDAC3 and HDAC8 inhibition across multiple cancer cells and PBMCs to determine if HDAC protein specific inhibitors can be used as a more target anti-cancer drug. .................................................................................................... 45

Comparison of Dead Cell Percentage in Various Cancer Cell Lines............................................................................................................................ 45

Discussion ........................................................................................................ 48

Bibliography .................................................................................................... 55
List of Tables

Table 1. Summary of HDAC inhibition studies ................................................................. 15

Table 2. List of chemical inhibitors used in thesis ............................................................ 17

Table 3. Protein markers for anti-cancer pathways ............................................................ 18

Table 4. List of cell lines used in thesis ............................................................................ 20

Table 5. List of antibodies used in thesis ........................................................................ 24
List of Figures

Figure 1. Diagram of lysine acetylation and deacetylation ........................................... 4

Figure 2. Diagram of HDAC classes .................................................................................. 5

Figure 3. Diagram of HDAC and HAT regulatory pathways ............................................... 7

Figure 4. HDAC expression in normal tissue and cancer tissue ........................................... 8

Figure 5. Cell death and cell cycle analysis of class specific inhibition ............................... 29

Figure 6. Cell death and cell cycle analysis of protein specific inhibition ............................ 31

Figure 7. Pathway analysis of class specific inhibition ....................................................... 34

Figure 8. Pathway analysis of protein specific inhibition .................................................. 37

Figure 9. Histone acetylation analysis of HDAC3 and HDAC8 inhibition ......................... 39

Figure 10. Time course analysis of HDAC3 and HDAC8 inhibition .................................... 41

Figure 11. Cell stress and damage pathway analysis .......................................................... 43

Figure 12. HDAC3 and HDAC8 combination treatment with Bcl-xL ................................. 45

Figure 13. HDAC4 and HDAC8 cell death analysis on various cell lines ............................ 47
Chapter I

Introduction

An estimated 15.5 million Americans have or are currently in remission from cancer as of 2016 and this number is likely to raise to 20.3 million by 2026 (Kimberly et al., 2017). Although cancer five-year survival increased from 49% in 1975 to 69% in 2013, long-term survival rates are still low and largely dependent upon the time of diagnosis (American Cancer Society, 2018). Highest survival rates are observed when cancer is detected within five years of onset (33%) but progressively worsen as time elapses falling to 4% after 25-20 years. (Kimberly et al., 2017). In addition to delayed diagnosis, drug resistance is a major obstacle for successful cancer therapy (Ma et al., 2014, Seguin et al., 2015). Chemotherapy can fail due to either tumor innate resistance or its ability to evade treatment. Molecular pathways that either upregulate cell survival and/or downregulate apoptotic pathways are the primary cause of resistance (Holohan et al, 2013). The elucidation of novel intracellular signaling pathways and the characterization of causal protein drivers are necessary for targeted therapy.

Dysregulation of histone deacetylase (HDAC) proteins has been reported in a variety of cancers (Weichert, 2009, De Ruijter et al., 2003). There are eleven known HDAC proteins that are divided into multiple classes, class I, class II which is further divided into class IIa and class IIb, class III, and class IV which regulate a wide variety of cellular pathways (Seto et al., 2014).
Currently there are four FDA approved pan-HDAC inhibition therapies used in cancer treatment namely: vorinostat for treating cutaneous T-cell lymphoma (CTCL), romidespin (FK228, depsipetide) for treating CTCL and peripheral T-cell lymphoma (PTCL), belinostat (PXD101) for treating PTCL, and panobinostat (LBH-589) for treating multiple myeloma (Zwerger et al., 2016). Clinical trials show response rates of 30% with vorinostat as determined by the severity weighted assessment tool (Mann et al., 2007), 34% with romidespin (Coiffier et al., 2012), 26% with belinostat (Poole, 2014), and 33% with panobinostat (Oki, 2013).

As with many chemotherapy agents these pan-HDAC inhibitors, which inhibit multiple HDAC proteins and classes, also have side effects. Thrombocytopenia, anemia and other hematologic side effects were observed in a clinical trial with vorinostat (Marks et al., 2009). A high number of cardiac adverse effects, including one sudden death, lead to the early termination of a clinical trial using romidespin (Shah et al., 2006). These studies demonstrated the possible risk of non-specific HDAC inhibition treatment since there are at least eleven distinct HDAC proteins functioning across multiple cellular pathways. Determining which HDAC to target for inhibition could prove useful in reducing deleterious effects and expanding the current understanding of the role HDAC proteins play in cancer causation. In addition, identification of which proteins and signaling pathways are affected by specific HDAC inhibition should aid to overcome resistance (Delcuve et al., 2012).
Epigenetic Regulation

Epigenetics is the alteration of the genomic structure and gene expression through transient modification of DNA and DNA associated proteins without the direct alteration of DNA sequences. Epigenetic modifications, such as methylation and acetylation, change the chromatin state leading to differential mRNA expression. DNA methylation enzymes like DNA (cytosine-5)-methyltransferase 1 cause the formation of silent chromatin; a structure consisting of 146 base pairs of DNA wrapped around four proteins Histone 2A, Histone 2B, Histone 3 and Histone 4, with histone 1 binding another 20bp. DNA methylation occurs on cytosines in CpG rich regions of DNA. HDAC induced deacetylation of histone proteins may also cause the formation of closed histone formation (heterochromatin) which causes the silencing of gene expression (Jaenish et al., 2003). Inversely, histone acetyltransferases (HATs) are gene activators and the balance between acetylated histones, CpG methylation and histone deacetylation controls gene expression (Figure 1) (Kuo et al. 1998, Jaenisch et al, 2003, De Ruitjer et al. 2003). Acetylation of histone proteins decreases the positive charge of the complex causing the negatively charged DNA molecule to become less tightly wound to the histone core (Rice et al., 2001). This structural relaxation allows transcription factors to bind the DNA and increase gene expression (De Ruitjer et al. 2003). Conversely, gene expression downregulation is achieved by increased binding tension following deacetylation. Similarly, histone methylation of the amino acid lysine on position 9 (lys9) on histone 3 (H3), performed by histone methyl transferases (HMTs), increases the affinity of histone proteins for DNA molecules. This suggests that
HDAC proteins, followed by HMTs, can lead to heterochromatin formation and silencing of transcription. (Rice et al., 2001). In opposing function, HATs catalyze the addition of an acetyl group by utilization cofactor acetyl-coenzyme A (Rice et al., 2001). Posttranslational modifications of histone occur preferentially on H3 or histone 4 (H4), although histone 1 (H1) and histone 2A (H2A) can also be modified. (Rice et al., 2001). The reversible nature of the histone acetylation/deacetylation states, dictates transcription of genes at specific locations on the genome.

Figure 1: Diagram illustrating the addition of an acetyl group (blue) to the lysine amino acid on histone by HAT and the removal of an acetyl group by HDAC proteins (Kuo et al., 1998).

**HDAC Functional Classes**

HDACs are a class of enzymes that function by removing acetyl groups from ε-amino groups on lysine of histones and other proteins. HDACs are grouped in two major families, namely the histone deacetylase family or Sir2 regulator family. The HDAC proteins are further divided into four classes based on their structural similarities to previously known yeast proteins with the Sir2 family being divided into its own class (Class III). Class I proteins HDAC1, HDAC2, HDAC3, and HDAC8 are homologous to Rpd3. Class II proteins HDAC4, HDAC5, HDAC6, HDAC7, HDAC9,
and HDAC10 have sequence similarity to Hda1 protein and are further divided into class IIa, HDAC4, HDAC5, HDAC7, HDAC9, and class IIb HDAC6, HDAC10. Class III are similar to Sir2 yeast protein and include SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7. Class IV is made of a single protein HDAC11. Class I and II proteins have a classical deacetylase domain while class III have a NAD+ dependent deacetylase domain or have a separate function like APD ribosyltransferase domain (Figure 2) (Seto et al., 2014).

Class I HDACs are expressed in every tissue type and are located usually in nucleus (Seto et al., 2014). Class I HDACs are responsible for deacetylating histone proteins, at the lysine residues on the N-terminus tail; although different HDAC proteins are slightly promiscuous and can overlap in function. HDAC1 has been shown to modify all histone proteins, while HDAC3 preferentially modifies H2A and

![Figure 2: Diagram showing HDAC class I, IIa, IIb, IV and Class III. Amino Acid number is designated on top right and catalytic domains are highlighted by color (Seto et al., 2014)](image-url)
H4. But during knockdown experiments if a specific HDAC is downregulated another HDAC will be able to function in its place. (Seto et al., 2014). HDAC class I proteins have a prominent role in cell proliferation and survival (Delcuve et al., 2012). There is also evidence that class I HDACs can function on non-histone proteins, including HDAC1 modification of α-tubulin, MyoD, tumor suppressor p53, E2F, and other transcription factors (Seto et al., 2014, De Ruitjer et al., 2003, Marks et al., 2003). Mass spectrometry studies have found that 3600 lysine acetylation sites occur in 1750 proteins with acetylation regulation being required for most cellular pathways suggesting HDAC function is widespread (Delcuve et al., 2012).

Class I HDAC proteins function within different protein complexes, such as HDCA1 and HDAC2 with Sin3 and NuRD (nucleosome remodeling and deacetylating). This protein complex is required for HDAC proteins 1, 2, and 3 binding to histones; although HDAC8 is an exception and can interact with histones solely (Delcuve et al., 2012; Marks et al., 2003). HDAC proteins require Zn²⁺ ions to catalyze the deacetylation of proteins. Some HDAC inhibitors, such as trichostatin A, chelate zinc at the active site of HDAC with its hydroxamic acid group interfering with the removal of acetyl groups from histones. (De Ruijter et al., 2003).

Class II HDAC proteins expression levels vary with different tissue types and are shuttled in and out of the nucleus. Class IIa HDAC 4, 5, and 7 can also deacetylate non-histone proteins (De Ruijter et al., 2003, Marks et al., 2003). Class II proteins may also function as chaperons by recognizing acetyl-lysine residues and not cleaving them. Instead, the binding of HDAC proteins recruits other protein complexes such as the complex of silencing mediator for retinoid-nuclear receptor
co-repressor 2 (SMRT-NCoR) (Fischle et al., 2002). Class IIb HDAC6 is specifically involved in alpha-tubulin deacetylation which controls microtubule and actin-dependent cell motility. (Yang et al., 2008). Taken together, class I and class II HDACs along with HATs play a role in regulation of the acetylation of lysine residues in a wide range of cellular functions (Figure 3).

**Figure 3**: Diagram showing HAT and HDAC regulation involved in multiple cellular pathways (Yang et al., 2007)

**HDAC Upregulation in Cancer**

It is known that improper regulation of epigenetic modifications and dysregulation of proteins responsible for altering epigenetic modifications play a role in the development of cancer (Zhang et al., 2017). These aberrant alterations in epigenetic modifications can cause upregulation of oncogenes or down regulation of apoptotic gene causing a cell to become cancerous. Multiple cancer cell types including cutaneous T-cell lymphoma, gastric, colorectal, hepatocellular, prostate,
ovarian, endometrial endometrioid, non-small cell lung, and breast carcinoma have upregulated HDAC proteins (Figure 4) (Weichert et al., 2009, De Ruijter et al., 2003). Metadata analysis shows that HDAC1 has a clinically significant hazard ratio (which is a ratio determined by the survival rates of two separate groups; in this case the survival was based on expression levels of HDAC1) in both ovarian and hepatocellular cancer suggesting that HDAC1 inhibition could be a viable chemotherapy target (Weichert et al., 2009). Inversely, this study did not show that specific HDAC protein inhibition had a clinically significant hazard ratio in cutaneous T-cell lymphoma (CTCL) although the FDA has already approved treatment of CTCL with pan-HDAC inhibition which suggested that HDAC inhibition could still work even without a significant hazard ratio for a specific HDAC protein (Weichert et al., 2009).

![Figure 4: HDAC expression levels in normal tissues and tumor tissue (De Ruijter et al., 2003)](image)
Identifying HDAC Inhibition Targets in Different Cancer Cell Lines

Effects of HDAC dysregulation varies by cancer cell line and tissue type and identifying which HDAC to potentially target as a chemotherapy agent is an ongoing activity in the scientific community.

Pan-HDAC Inhibition

Bladder cancer cell line BIU-87 was treated with TSA to determine if pan-HDAC inhibition would cause cell death. The results of this study showed that apoptosis occurred in 40% of the cells, while all cells underwent cell arrest with most arrested in G1. $p21^{WAF1}$ was upregulated in a TSA dosage dependent manner suggesting that $p21^{WAF1}$ regulated cell cycle arrest could be leading to apoptosis (Li et al., 2006).

Liver cell lines HepG2 and Huh7 were treated with pan-HDAC inhibitors TSA, vorinostat and sodium butyrate (NaBu) to determine if HDAC inhibition could be used to cause cell death. This study used qPCR and western blotting to determine that the tumor suppressing protein CLYD is upregulated in these cell lines upon treatment. Interestingly, the TSA treatment had different phenotypic outcomes in these liver cell lines with HepG2 been arrested in G1/S cell cycle whereas Huh7 underwent apoptosis (Kotantaki et al., 2016). This demonstrates that a pan-inhibitor, although effective in both liver cancer cell lines, maybe affecting different
cellular pathways. Little information regarding the inhibition of a specific HDAC protein is obtained when using pan-HDAC inhibitors due to the concomitant inhibition of multiple HDACs.

In a breast cancer cell line MDA-MB-231, HDAC5 may be the specific HDAC that can be inhibited for anti-cancer effects (Hsieh et al., 2015). This study treated MDA-MD-231 cells with pan-HDAC inhibitors TSA, calproic acid, sodium butyrate, splitomycin, apicidin, M3444 and demonstrated that pan-HDAC inhibition could cause cell death. Changes in microRNA and protein expression induced by pan-HDAC inhibition lead the authors to infer that HDAC5 was affected by the pan-HDAC inhibition. To corroborate this theory, cells were treated with shRNA specific to HDAC5 which caused the upregulation of the apoptotic marker protein cleaved caspase 3 indicating that HDAC5 inhibition is responsible for anti-cancer effects.

Class Specific Inhibition

A study was performed in BRAF inhibitor resistance melanomas cell lines obtained from different patients. When cell line CM145 and CM150 were treated with BRAF inhibitor and the pan-HDAC inhibitor LBH589, cell death occurred through upregulation of pro-apoptotic proteins BIM or NOXA and down regulation of anti-apoptotic proteins Bcl-XL, MCL1 and x-linked inhibitor of apoptosis (XIAP). To determine which HDAC class was responsible for alteration of apoptotic pathway proteins, the experiment was repeated with class I specific inhibitor mocetinostat and class II specific inhibitor MC1568. The class I inhibitor had the most effect demonstrating that HDACs 1,2,3 and/or 8 were involved in cell death. Interestingly,
CM145 cells expressed high levels of NOXA and low levels of BIM whereas CM150 cells expressed reversed levels when treated with mocetinostat (Gallagher et al., 2018). This suggests that different cells lines from the same cancer type are affected differently even when treated with the same Class I inhibitor. Performing this experiment with more specific inhibitors could identify which HDAC is responsible.

Another study, using a class I inhibitor thailandepsin A (TDP-A) to treat breast cancer cell line MDA-MB-23, resulted in cell death with upregulation of Bax and downregulation of Bcl-2. This indicates that is it likely either HDAC1, 2, 3, and/or 8 that is upregulated in this breast cancer cell line (Xiao et al., 2015). These data contradict the shRNA data obtained in the study by Hsieh et al., 2015, which showed that HDAC5 should be targeted for inhibition.

**HDAC Protein Specific Inhibition**

A study done with pan-inhibitor vorinostat in prostate cancer cell lines DU145 and PC-3 cells, showed that vorinostat caused upregulation of BIM and BAX, two pro-apoptotic proteins, and down regulation of Bcl-2 and survivin, two anti-apoptotic proteins, along with G2/M arrest (Shi et al., 2017). A second study used TSA to inhibit HDACs in colorectal cancer cell line SW480 and prostate cancer cell PC3 caused reversal of EMT characteristics, including down regulation of vimentin and upregulation of e-cadherin. ChIP analysis with HDAC specific antibodies demonstrated that HDAC1 and HDAC2 were present at SLUG promotor regions suggesting that HDAC1 or HDAC2 were responsible for the reversal of EMT (Wang, et al., 2015). However a third study in castration-resistant prostate cancer
demonstrated that HDAC3 is a possible therapeutic target (Mcleod et al., 2018). This study compared vorinostat with HDAC3 specific inhibitor RGFP966 and demonstrated in 22Rv1 xenograft model that HDAC3 is the deacetylase responsible for anti-tumor effect. These contradictory data with pan-HDAC inhibition causing apoptosis of PC3 cells in one study and reversal of EMT in another and HDAC1 or 2 being implicated in the cell culture model of prostrate cancer and HDAC3 being implicated in the xenograft prostate model demonstrate the need for better understanding of the mechanism of HDAC inhibition and the need for more specific HDAC inhibition studies. The pan-inhibitors maybe causing too many off target effects to efficiently determine which HDAC is responsible for anti-cancer effects.

In colorectal cancer cell line HCT116, HDAC1 inhibition can lead to cell death by activating the autophagy pathway (Jo et al., 2018). This was determined by treating HCT116 cells with pan-inhibitors of HDACs (TSA, valproic acid, vorinostat) and comparing that to more specific inhibitors like CI-994. This work identified HDAC1 as a possible therapy target because the effects of CI-994 inhibiting, which according to the authors preferentially inhibits HDAC1 was similar to the pan inhibition. Although other data suggest that CI-994 inhibits HDAC1,2,3 and 8 (Zhou et al., 2018).

This approach seems ideal to identify specific HDACs to target in different cancer types. Because this approach is more clinically relevant than RNAi, due to the technical challenges RNAi has had as a therapeutic agent (Bobbin et al., 2016), it would be valuable to perform HDAC specific inhibition studies to help identify HDAC
targets in cancer cell lines. These HDAC chemical inhibitors could then be tested as possible chemotherapy reagents.

**Combination Therapy**

One study tested a set of 50 different cancer cell lines to determine how effective vorinostat treatment can be across a variety of tissue types. This study showed that treatment with vorinostat caused apoptosis in all cell lines but it can range from weak responders, 2.5% of cells being apoptotic, to strong responders, 97.4% of cells being apoptotic. Treatment with HDAC inhibitor caused upregulation of BIM and NOXA, and downregulation of Bcl-xL. Furthermore, this study combined HDAC inhibition with Bcl-xL inhibitor (A-1331852) with some of the weaker responders and found a greater cell death effect (Chüeh et al., 2017).

Combination therapy maybe appropriate due to the range of cell death responses in different cell lines and with evidence that HDAC inhibition alone will not suffice in treating breast cancer (Munster et al., 2011). A phase II clinical trial in hormone resistant breast cancer was done with HDAC inhibitor vorinostat combined with tamoxifen (an anti-estrogen drug). This trial showed 20% of patients responded to combination therapy. This is probably due to the HDAC inhibition reversal of estrogen receptor stabilization that can be caused when tamoxifen is administered alone (Munster et al., 2011).

A study done in patient derived pancreatic adenocarcinomas, which have high EMT markers ZEB1 and miRNA-203, showed these cells to be resistant to chemotherapy agent gemcitabine treatment alone. Use of pan-HDAC inhibitor TSA
or vorinostat had weak apoptotic effect and no apparent change on ZEB1 or miRNA-203 expression levels. Interestingly, treatment with a class I HDAC inhibitor, mocetinostat, did not enhance the apoptotic effects but down regulated ZEB1. When mocetinostat was combined with the nucleoside analog gemcitabine the apoptotic effect was greatly increased (Meidhof et al., 2015). This study demonstrates that HDAC combination therapy may be beneficial and identifying more specific HDAC inhibitors may actually be better than using a pan-inhibitor.

A phase I and subsequent phase II study was performed with a cumulative 45 patients with refractory advanced non–small cell lung cancer using combination therapy of the nucleoside analog azacitidine and HDAC class I inhibitor entinostat; although this inhibitor shows preference for HDAC1 and HDAC3. During this clinical trial all patients showed some side effects including vomiting, nausea and fatigue, but none of the side effects required medical treatment. One patient in this trial showed no signs of disease progression for 18 months, 10 had no progression for 12 weeks, but 22 had progression of the disease (Juergens et al., 2011). This clinical trial showed promise that a more specific HDAC inhibition can be used in combination therapy with other drugs. Because HDAC effects on cancers cells is fairly random more studies need to be done to determine which HDACs should be targets in combination therapies.

As outlined above, HDAC specific inhibition studies have been done in breast, prostate, colorectal, liver and bladder cancer cell lines to identify if HDAC inhibition could cause cell death. It is known that HDAC pan inhibition can cause cell death in some ovarian cancer cell lines (Chüeh et al., 2017). However, there has been no
study done using non pan-HDAC inhibition. My thesis aims to add to this knowledge by using HDAC specific inhibitors to determine if they can cause cell death and to identify which HDAC inhibition has the greatest effect. For this reason, I plan to perform HDAC specific inhibition screens to determine if a HDAC specific inhibitor can give similar results as a pan inhibitor.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cell Line</th>
<th>Treatment</th>
<th>Target</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>BIU-87</td>
<td>TSA</td>
<td>Pan-HDAC</td>
<td>p21, apoptosis</td>
</tr>
<tr>
<td>Liver</td>
<td>HepG2, huh7</td>
<td>TSA, Vorinostat, NaBu</td>
<td>Pan-HDAC</td>
<td>Tumor suppressor clyd upregulation</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CM145, CM150</td>
<td>LBH589</td>
<td>Pan-HDAC</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CM145, CM150</td>
<td>MC1568</td>
<td>Class II</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Breast</td>
<td>MDA-MB-231</td>
<td>TDP-A</td>
<td>Class I</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Breast</td>
<td>MDA-MB-231</td>
<td>shHDAC5</td>
<td>HDAC5</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>DU145, PC-3</td>
<td>Vorinostat</td>
<td>Pan-HDAC</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>Castration resistant prostate xenograph</td>
<td>RGFP966</td>
<td>HDAC3</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>SW480</td>
<td>TSA</td>
<td>Pan-HDAC</td>
<td>Reversal of EMT</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>HCT116</td>
<td>TSA, VPA, Vorinostat</td>
<td>Pan-HDAC</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>HCT116</td>
<td>CI-994</td>
<td>HDAC1</td>
<td>Autophagy</td>
</tr>
</tbody>
</table>

Table 1: Summary of HDAC inhibition experiments and the effects of inhibition
Specific Aims

The goal of this master's thesis project was to determine if the inhibition of specific HDAC proteins could replicate the apoptotic effects caused by treatment of ovarian cells with vorinostat, a pan-HDAC inhibitor and to identify activated anti-cancer cellular pathways. The project encompassed the following aims: 1) Comparison of the amount of cell death or cell arrest caused by pan-HDAC, class or HDAC protein specific inhibitors, 2) analysis of anti-cancer pathways altered with HDAC3 or HDAC8 inhibition, 3) analysis of the cell type specificity of HDAC3 or HDAC8 inhibition.

The first objective was to determine if HDAC class specific and HDAC protein specific inhibition caused a similar amount of cell death and/or cell cycle arrest as vorinostat. The ultimate goal is to cause cell death but cell cycle arrest is also desirable since it stops the spread of cancer cells and may indirectly lead to stimulation of the apoptosis pathway in a temporal fashion (Evans et al., 2001). Ovarian cancer cell OVCAR8 was chosen as a model system because it is a high-grade ovarian serous adenocarcinoma with a doubling time of 25 hours (Cowely et al., 2014; Mitra et al. 2015). OVCAR8 was treated with either vorinostat, HDAC class specific or HDAC protein specific inhibitors (Table 2). An initial experiment was performed with Class I and Class II HDAC inhibitors, due to lack of available drugs Class III and Class IV inhibition was not done. Based on these results more specific HDAC inhibition was performed. Effects of HDAC inhibition were measured by flow
cytometric analysis of cell cycle and by analysis of protein levels of cleaved caspase 3, an indicator of apoptosis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition Target</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorinostat</td>
<td>Pan-HDAC (Marks et al., 2007)</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>4SC-202</td>
<td>HDAC Class I (Hennings et al., 2010)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>MC1568</td>
<td>HDAC Class II (Mai et al., 2005; Duong et al., 2008)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>TMP195</td>
<td>HDAC Class IIa (Lobera et al., 2013)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>CI-994</td>
<td>Class I HDAC1, HDAC2, HDAC3, HDAC8 (Zhou et al., 2018)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>BRD6688</td>
<td>HDAC1, HDAC2, HDAC3 (Wagner et al., 2015)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>CAY10683</td>
<td>HDAC2 (Pavlik et al., 2013)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>RGFP966</td>
<td>HDAC3 (Malvaez et al., 2013)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>PCI-34051</td>
<td>HDAC8 (Balasubramanian et al., 2008)</td>
<td>Selleck Chemicals</td>
</tr>
<tr>
<td>ABT-263</td>
<td>Bcl-xL (Wong et al., 2012)</td>
<td>Selleck Chemicals</td>
</tr>
</tbody>
</table>

*Table 2:* List of chemical inhibitors with targets of inhibition.

For aim 2 treatments that induced apoptosis or cell cycle arrest from Aim 1 were analyzed by western blot with markers of different cellular pathways according to the current understanding of HDAC inhibition (Table 3). The most likely pathways affected were apoptosis, autophagy, DNA damage/cell cycle arrest, and epithelial to mesenchymal transition (EMT). Apoptosis is a cell death pathway
characterized by up regulation of caspase activity and down regulation of Bcl-xL (Gallagher et al., 2018). Autophagy is classically a pro-survival pathway but evidence suggests it can induce the apoptosis pathway or may directly lead to cell death in diseased cell or chemically treatment cells (Levine et al., 2005). Autophagy involves lysosomal degradation of cytoplasmic proteins and is characterized by an cleavage of Lc3b (Lin et al., 2018). HDAC inhibition can also lead to cell cycle arrest which can be determined by analysis of the DNA content with DNA stains such as DAPI (Gong-Cheng at al., 2016). EMT pathways can be affected by HDAC inhibition as marked by upregulation of e-cadherin and down regulation of n-cadherin (Wang et al. 2015). Although no previous studies have demonstrated that HDAC inhibition caused mitotic dysregulation or upregulated unfolded protein response, these pathways were investigated due to their known effects on cell death (Ricci et al., 2006; Hetz et al., 2012; Clarke et al., 2012). Possible mitotic arrest can be indicated by changes in Eg5 protein levels (Ricci et a, 2006), and ER Stress indicated by upregulation of eIF2a, phosphorylation of eLF2a at Serine 51, and upregulation of CHOP (Katsoulieris et al., 2010; Clarke et al., 2012; Zinszner et al., 1998).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved Caspase 3</td>
<td>Pro-apoptosis</td>
</tr>
<tr>
<td>Cleaved Caspase 7</td>
<td>Pro-apoptosis</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>e-cadherin</td>
<td>EMT</td>
</tr>
<tr>
<td>n-cadherin</td>
<td>EMT</td>
</tr>
<tr>
<td>Lc3b</td>
<td>Autophagy</td>
</tr>
<tr>
<td>p62</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Histone H2A.X</td>
<td>DNA Damage</td>
</tr>
<tr>
<td>Eg5</td>
<td>Mitotic Stress</td>
</tr>
<tr>
<td>eLF2a</td>
<td>ER Stress</td>
</tr>
<tr>
<td>CHOP</td>
<td>ER Stress</td>
</tr>
</tbody>
</table>

Table 3: Protein markers for various cellular pathways
For aim 3 optimized treatments from aim 1 and 2 were used on multiple cell lines to determine if the cell death caused by the HDAC specific inhibitions are specific to ovarian cancer cell lines. Cell death percentage was determined by staining cells with DNA dye DAPI.
Chapter II

Materials and Methods

The purpose of this study was to determine if HDAC protein specific inhibition could cause similar anti-cancer effects as pan-HDAC inhibition in ovarian cancer cells, could the mechanism of action be determined and if this effect was specific to ovarian cancer cells. The material and methods chapter outlines protocols used in this study.

Cell Culture

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tissue</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR8</td>
<td>Ovary</td>
<td>RPMI 1640, 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>Ovary</td>
<td>McCoy’s 5a Medium Modified 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>IGROV1</td>
<td>Ovary</td>
<td>RPMI 1640, 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>A459</td>
<td>Lung</td>
<td>F-12K Media, 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>PC3</td>
<td>Prostate</td>
<td>F-12K Media, 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>HepG2</td>
<td>Liver</td>
<td>TCC-formulated Eagle’s Minimum Essential Medium, 10% Fetal Bovine Serum</td>
</tr>
<tr>
<td>peripheral blood mononucleocytes (PBMCs)</td>
<td>Lymphocytes</td>
<td>RPMI 1640, 10% Fetal Bovine Serum</td>
</tr>
</tbody>
</table>

*Table 4: List of cells lines, tissue of origin and media used in this project.*
Multiple cell lines were used in this study (Table 4) cells were passaged every 2-3 days, incubated at 37°C in 5% carbon dioxide.

Cell Treatments

Cells were treated with HDAC inhibitors against class I, class II and class Ila and against specific HDAC proteins (Table 2)

All drugs were resuspended in DMSO and stored at -20°C until use. Treatments were performed at concentrations ranging from 2.5μM to 10μM with class specific inhibitors, 10μM to 100μM with HDAC protein specific inhibitors and 5μM with vorinostat. Treatment times varied from four to seventy-two hours as described in similar published experiments (Mai, Antonello, et al. 2005; Lobera, Mercedes, et al. 2013; Fu, Meili, et al, 2016). Cells were plated at 5x10⁴ cells/mL and allowed to rest overnight prior to the drug treatments.

Sample Preparations

Post treatment samples were analyzed by either western blot or flow cytometry. For western blot analysis, adherent cells were removed from the plate by physically scrapping the cells, transferred to a 50ml conical tube and pelleted by centrifugation at 1000 RPM for 5 minutes. Cells were washed with phosphate buffered saline pH 8.0 (PBS), pelleted and resuspended in 1x SDS loading buffer
with 41.6 μM DTT (#7722 Blue Loading Buffer Pack, Cell Signaling Technology). Samples were then heated at 98°C for 5 minutes and stored at -20°C until analysis.

For flow cytometric analysis, medium was first removed from cells and saved in a 50ml conical tube. Accutase (#At-104 Cell Detachment Solution, Innovative Cell Technologies) was added to adherent cells and incubated at 37°C until cells were detached from plate as determined by visual inspection. Accutase was used, instead of scraping, to keep the cells intact. Detached cells were added to previously collected medium to ensure collection of all treated cells both the attached cells and detached cells and pelleted by centrifugation. The cell pellet was washed with PBS and re-pelleted by centrifugation. The cells were fixed by resuspending the cell pellet in 4% paraformaldehyde and incubated for 10 minutes at room temperature. Fixed cells were washed once with PBS, pelleted by centrifugation and resuspended in 500μl of PBS. Permeabilization was achieved by drop wise addition of 4.5ml cold (-20°C) 100% methanol for a final concentration of 90% methanol. Samples were stored at -20°C until analysis.

**Western Blot**

Varying amounts of SDS cell lysates were loaded into a 4-20% Mini-PROTEAN TGX gel (#456-1096, Bio-Rad). Proteins were separated by electrophoresis at 230 volts for 20 minutes using Mini-PROTEAN Tetra System (#1658004EDU, Bio-Rad). Samples were then transferred to a nitrocellulose membrane at 2.5 amps, 25 volts for 7 minutes using the Trans-Blot Turbo
The nitrocellulose membrane was incubated at room temperature for 1 hour with gentle shaking in Tris Buffer Saline, 0.1% Tween-20 (TBST) and 5% milk. The nitrocellulose membrane was washed 3 times with TBST before addition of the primary antibody at a 1:1000 dilution in TBST with 5% milk or 5% bovine serum albumin (BSA) depending on antibody used (Table 5). Primary antibody was incubated at 4°C overnight with gentle shaking. The following day the primary antibody was removed, and the membrane washed 3 times with TBST with a 5 minute incubation for each wash. Anti-rabbit HRP conjugated antibody (#7074, Cell Signaling Technology) diluted 1:1000 in TBST with 5% milk was added to the membrane and incubated for 1 hour at room temperature with gentle shaking. After 1 hour the membrane was washed 3 times with TBST for 5 minutes each wash. Antibody signal was visualized using a 1:1 mixture of LumiGLO® and Peroxide (#7004, Cell Signaling Technology) and imaged on the ChemiDoc Imaging System (#12003153, BioRad).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Buffer containing</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb</td>
<td>Milk</td>
<td>9664</td>
</tr>
<tr>
<td>Cleaved Caspase-7 (Asp198) (D6H1) Rabbit mAb</td>
<td>BSA</td>
<td>8438</td>
</tr>
<tr>
<td>β-Actin (D6A8) Rabbit mAb</td>
<td>BSA</td>
<td>8457</td>
</tr>
<tr>
<td>E-Cadherin (24E10) Rabbit mAb</td>
<td>BSA</td>
<td>3195</td>
</tr>
<tr>
<td>Bcl-xL (54H6) Rabbit mAb</td>
<td>Milk</td>
<td>2764</td>
</tr>
<tr>
<td>p21 Waf1/Cip1 (12D1) Rabbit mAb</td>
<td>BSA</td>
<td>2947</td>
</tr>
<tr>
<td>N-Cadherin (D4R1H) XP® Rabbit mAb</td>
<td>BSA</td>
<td>13116</td>
</tr>
<tr>
<td>LC3B (D11) XP® Rabbit mAb</td>
<td>BSA</td>
<td>3868</td>
</tr>
<tr>
<td>Antibody Name</td>
<td>Buffer</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>Acetylated-Lysine (Ac-K-100) MultiMab™ Rabbit mAb mix (HRP Conjugate)</td>
<td>BSA</td>
<td>6952</td>
</tr>
<tr>
<td>SQSTM1/p62 (D5L7G) Mouse mAb</td>
<td>BSA</td>
<td>88588</td>
</tr>
<tr>
<td>Caspase-3 (D3R6Y) Rabbit mAb</td>
<td>BSA</td>
<td>14220</td>
</tr>
<tr>
<td>Histone H2A.X (D17A3) XP® Rabbit mAb</td>
<td>BSA</td>
<td>7631</td>
</tr>
<tr>
<td>Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb</td>
<td>BSA</td>
<td>9718</td>
</tr>
<tr>
<td>eIF2α (D7D3) XP® Rabbit mAb</td>
<td>BSA</td>
<td>5324</td>
</tr>
<tr>
<td>Phospho-eIF2α (Ser51) (D9G8) XP® Rabbit mAb</td>
<td>BSA</td>
<td>3398</td>
</tr>
<tr>
<td>Eg5 (E1L3W) Rabbit mAb</td>
<td>Milk</td>
<td>14404</td>
</tr>
<tr>
<td>CHOP (L63F7) Mouse mAb</td>
<td>BSA</td>
<td>2895</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys23) (D6Y7M) Rabbit mAb</td>
<td>BSA</td>
<td>14932</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys14) (D4B9) Rabbit mAb</td>
<td>BSA</td>
<td>7627</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys36) (D9T5Q) Rabbit mAb</td>
<td>BSA</td>
<td>27683</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys27) (D5E4) XP® Rabbit mAb</td>
<td>BSA</td>
<td>8173</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb</td>
<td>BSA</td>
<td>9649</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys18) (D8Z5H) Rabbit mAb</td>
<td>BSA</td>
<td>13998</td>
</tr>
<tr>
<td>Acetyl-Histone H2B (Lys12) Antibody</td>
<td>BSA</td>
<td>5410</td>
</tr>
<tr>
<td>Acetyl-Histone H2B (Lys5) (D5H1S) XP® Rabbit mAb</td>
<td>BSA</td>
<td>12799</td>
</tr>
<tr>
<td>Acetyl-Histone H2B (Lys15) (D8H1) XP® Rabbit mAb</td>
<td>BSA</td>
<td>9083</td>
</tr>
<tr>
<td>Acetyl-Histone H2B (Lys20) (D7O9W) Rabbit mAb</td>
<td>BSA</td>
<td>34156</td>
</tr>
<tr>
<td>Acetyl-Histone H2A (Lys5) Antibody</td>
<td>BSA</td>
<td>2576</td>
</tr>
<tr>
<td>Acetyl-Histone H4 (Lys12) (D2W60) Rabbit mAb</td>
<td>BSA</td>
<td>13944</td>
</tr>
<tr>
<td>Acetyl-Histone H4 (Lys5) (D12B3) Rabbit mAb</td>
<td>BSA</td>
<td>8647</td>
</tr>
<tr>
<td>Acetyl-Histone H4 (Lys5) (D12B3) Rabbit mAb</td>
<td>BSA</td>
<td>13534</td>
</tr>
</tbody>
</table>

*Table 5*: List of antibody used in this study, buffer conditions and Cell Signaling Technology’s catalog number for that product.

**Cell Cycle Analysis**

24
Cells prepared for flow cytometric analysis were counted using a Countess II FL (Thermo Fisher Scientific) cell counting device; 100,000 cells were washed with PBS and then resuspended in PBS, 0.5% BSA and DAPI (#4083, Cell Signaling Technology) diluted 1:10000. Cells were incubated for 10 minutes at room temperature in the dark and then analyzed on a flow cytometer (iCyt Eclipse, Sony Biotechnology).

Cell Death Analysis

Live cells were harvested as previously described but without fixation and permeabilization. Following treatment, cells were washed 2 times with PBS, stored in PBS on ice until stained with DAPI and analyzed as described above.
CHAPTER III

Results

The results from this work showed that HDAC3 or HDAC8 inhibition caused cell death by upregulation of apoptosis in a similar fashion to class I HDAC inhibitors (Aim 1 and 2). Aim 3 demonstrated that HDAC8 inhibition caused cell death in a more targeted manner than pan-HDAC inhibitor vorinostat.

Specific Aim 1 – Comparison of the amount of cell death or cell arrest caused by vorinostat, and HDAC class or protein specific inhibitors.

To determine if inhibition of different classes of HDAC proteins (Class I, II or IIa) could cause cell death similarly to the pan-HDAC inhibitor vorinostat; ovarian cancer cell line OVCAR8 was treated in a dose dependent manner (10μM, 5μM, and 2.5μM) for 72 hours and compared to pan-HDAC inhibition using vorinostat at 5μM for the same time period. The effects of each treatment were evaluated in three separate assays; the first analysis was to observe and estimate the percent cell confluency at 24, 48 and 72 hour time points visually. Both pan-HDAC inhibition (vorinostat) and class I inhibition (42C-202) prevented cells from reaching 30% confluency for at least three days. HDAC class II and HDAC class IIa inhibition had the same growth rate as the untreated control cells (Figure 5a). These data suggest
that the pan-HDAC inhibition and class I inhibition caused either a cell cycle arrest leading to no growth or caused enough cell death to decrease the amount of cells at the end of the treatment when compared to the untreated healthy control cells.

The cells were collected at 72 hours, lysed for western blot analysis and analyzed for the presence of cleaved caspase 3 and cleaved caspase 7 which are indicators of cell death (Nicholson, et al.; 1995; Lippke, et al.; 1995). Pan-HDAC inhibition (vorinostat) and class I inhibition (4SC-202) caused a significant induction of cleaved caspase 3 and cleaved caspase 7 over the untreated healthy control (Figure 5e,f). The HDAC class II and IIa inhibition using MC1568 and TMP195 respectively does appear to induce some apoptosis as indicated by the signal of cleaved caspase 3 and caspase 7 but this induction is minimal when compared to pan inhibition or class I (Figure 5e).

The treated cells also underwent cell cycle analysis. This provided information about both the cell cycle state of the treated cells and the percentage of cells that were apoptotic as indicated by cells with sub-G1 DNA content (Zhou et al.; 2018). Pan inhibition with vorinostat and HDAC class I inhibition with 4SC-202 showed a similar increase of apoptotic cells with the percentage of cells in sub-G1 cell cycle phase between 35% and 39% (Figure 5b). While HDAC class II inhibition with MC1568 and HDAC class IIa inhibition with TMP195 have similar percentage of sub-G1 cells as the untreated control with around 15% of events being sub-G1 (Figure 5b). Due to a relatively high amount of apoptotic cells in the untreated cells this experiment was repeated with a similar pattern of results. In the 2\textsuperscript{nd}
experiment pan-HDAC inhibition had 34.6% sub-G1 events, HDAC class I inhibition had 25.8%, 14.3%, 9.9% apoptotic cells with treatment concentrations of 10μM, 5μM and 2.5μM respectively. The untreated controls cells, HDAC class II, and class IIa inhibition had apoptotic cell percentage around 5% (Figure 5C).

Cell cycle analysis also showed that HDAC class I inhibition caused a G2 arrest at higher concentrations as noted in the increase percentage of G2 cells compared to G1 cells as determined by a ratio of G2 to G1 (Figure 5d). Together these data clearly demonstrated that HDAC class I inhibition with 4SC-202 is able to cause similar cell death as the pan-HDAC inhibition with vorinostat. Since class I is made up of four HDAC proteins, it merited further investigation on whether more specific inhibition of HDAC 1, 2, 3, or 8 could cause cell death.
Comparison of Pan, Class I and HDAC Protein Specific Inhibitions

To determine if HDAC protein specific inhibition could cause cell death similar to pan-HDAC inhibition five different HDAC inhibitors were used (CI-994 which inhibits HDAC1,2,3, and 8, BRD6688 which inhibits HDAC1,2, and 3, CAY10683 which inhibits HDAC2, RGFP966 which inhibits HDAC3, PCI-34051 which inhibits HDAC8). Ovarian cancer cell line OVCAR8 was treated with different doses (10µM, 5µM, and 2.5µM) for 72 hours and compared to pan-HDAC inhibition with vorinostat at 5µM for the same time period. These dosages (besides pan-HDAC inhibition) did not appear to reduce cell growth and all treatments, appear the same as untreated (data not shown). Due to this, the dosage for the HDAC protein specific inhibitors was increased to 100µM, 50µM, 25µM, and 10µM. This dosage allowed for differential responses and testing proceeded with these conditions. First cellular confluence was monitored visually at 48 and 72 hours post treatment.
All HDAC inhibitions, with the exception of HDAC2 inhibition, caused a decrease in the cell growth when compared to untreated cells. On day 3, cells treated with CI-994 (HDAC1,2,3,8 inhibitor) were at 1%, 10%, 30%, 100% confluency at 100μM, 50μM, 25μM, 10μM respectively; cells treated with BRD6688 (HDAC1,2,3 inhibitor) or with CAY10683 (HDAC2 inhibitor) were at 80%, 100%, 100%, 100% confluency respectively; cells treated with RGFP966 (HDAC3 inhibitor) cells were at 10%, 50%, 100%, 100% confluency respectively; the cells treated with PCI-34051 (HDAC8 inhibitor) were at 5%, 30%, 80%, 100% confluency respectively (Figure 6a,b). Western blot analysis was performed to determine the level of the apoptosis marker cleaved caspase 3. All inhibitions, with the exception of HDAC2 inhibition, showed induction of cleaved caspase 3 in a dose dependent manner (Figure 6c). This clear dose dependent response validates the notion that the effects are due to the treatment and not due to another independent variable. Cell cycle analysis of cells treated with 50μM for 72 hours was performed but no meaningful conclusion could be drawn since the treatments caused such cellular disruption and death. It should be noted that the cell cycle analysis for the untreated control sample and HDAC2 inhibited samples showed normal cell cycle profiles. This demonstrates that technically the experiment worked correctly, which suggests that the cell cycle disruption is a true valid data point and is a result of the treatments and not a technical artifact.

These data demonstrates that a more specific HDAC inhibition can cause cell death in a similar manner as pan-HDAC inhibition using vorinostat. Combined inhibition of HDAC1, 2, 3, and 8 using CI-944, combined inhibition of HDAC1,2, and 3
using BRD6688, inhibition of HDAC3 only using RGFP966, and inhibition of HDAC8 only using PCI-34051 caused significant upregulation of apoptosis as indicated by elevated levels of cleaved caspase 3. Although CI-944 gave the strongest results it also inhibited multiple HDAC proteins but because HDAC3 or HDAC8 inhibition alone can cause apoptosis it may suggest that the effects of CI-944 could mainly be attributed to the inhibition of HDAC3 or HDAC8; similarly the inhibitory effects seen on cells treated with BRD6688, which inhibits HDAC1, 2, and 3, could be attributed mainly to the inhibition of HDAC3. Due to this and the desire to have a more targeted inhibition, the analysis of cell death and anti-cancer pathways proceeded with inhibition of HDAC3 (RGFP966) and HDAC8 (PCI-34051).

Figure 6: a) Cellular confluence data for protein specific inhibition. b) Western blot analysis of apoptosis indicator cleaved caspase 3 and loading control marker β-actin. Pan-HDAC inhibition controls cells are labeled with (+) and untreated controls cells are labeled with (-).
Specific Aim 2 – To elucidate the possible anti-cancer effects that HDAC inhibition may have on OVCAR8 cells and to begin understanding the mechanism of action

Analysis of Anti-Cancer Pathways Using Class Specific Inhibitors

To begin understanding the possible effects that class specific and protein specific HDAC inhibition have on treated cells, the class specific inhibition dose response samples were analyzed by western blot for markers of pathways that had previously been identified as affected by HDAC inhibition; autophagy, apoptosis, epithelial to mesenchymal transition (EMT switch), p21 driven apoptosis and total acetylation level that maybe affected with HDAC inhibition (Zhou et al., 2018). Figure 7 shows that pan-HDAC inhibition and class I inhibition using 4SC-202 caused the greatest increase in acetylation levels at the molecular weight for histone H3 (17 kDa) and histone H4 (11kDa), while class II and class IIa inhibition only caused a weaker increase at histone H4 and very minimal increase at histone H3 molecular weight when compared to the untreated sample. This is to be expected because class I HDAC are localized in the nucleus while class II HDACs are shuttled in and out of the nucleus and may not have as much effect on histone proteins (De Ruijter et al., 2003)

When observing the EMT switch both, pan-HDAC inhibition with vorinostat and class I HDAC inhibition with 4SC-202, caused an increase in e-cadherin protein levels although only HDAC class I inhibition causes clear down regulation of tumor
invasion associated n-cadherin (Figure 7b,c). This suggested that HDAC class I inhibition may also be able to downregulate metastasis of the cancer. Interestingly HDAC class IIa inhibition appears to cause an upregulation of n-cadherin which is considered a detrimental effect because upregulation of n-cadherin is associated with metastasis (Hazen et al.; 2004). Analysis of Lc3b upregulation and cleavage was performed to determine the effects on the autophagy pathway, which under normal conditions is considered a pro-survival pathway but may affect cell death pathways during drug treatment (Yuan et. al, 2005; Stacey et. al, 2006). Neither pan-HDAC inhibition with vorinostat, class I inhibition with 4SC-202, or class II inhibition with MC1568 caused strong alterations in Lc3b expression levels indicating that these inhibitors may not have an effect autophagy marker Lc3b (Figure 7d). The class IIa inhibitor does cause upregulation of Lc3b suggesting that it maybe increasing autophagy within the cell.

Apoptosis was analyzed by western blot for the expression of cleaved caspase 3 as previously shown in Figure 6 and by downregulation of the anti-apoptotic protein Bcl-xL; only class I inhibition caused a downregulation of Bcl-xL, pan-HDAC inhibition and class II inhibitors had no effect while class IIa caused an upregulation of Bcl-xL (Figure 7f). Previous studies have shown that apoptosis in HDAC inhibited cells maybe caused by upregulation of transcription factor p21 (Zhou et al.; 2018). In all treatment conditions used in this experiment, p21 is upregulated when compared to untreated control cells (Figure 7g). Pan-HDAC and Class I inhibition showed the greatest increase in p21 with milder increases in the
class II and class IIa inhibition. This increase in p21 may begin to explain the increased apoptosis in pan-HDAC and class I inhibition.

Figure 7: Pathway analysis of class specific inhibition. a) Levels of acetylated lysines at molecular weights 17kDa and 13kDa. b,c) Protein expression levels of EMT switch proteins e-cadherin and n-cadherin d) protein levels of autophagy marker lc3b e,f) protein levels of apoptosis protein cleaved caspase 3 and anti-apoptosis protein Bcl-xL, g) protein levels of p21 h) protein levels of loading control β-actin. Pan-HDAC inhibition controls cells are labeled with (+) and untreated controls cells are labeled with (-).
Analysis of anti-cancer pathways upon HDAC protein specific inhibition.

Similarly to class specific inhibition, HDAC protein specific inhibition was performed and analyzed on previously identified pathways that maybe affected by HDAC inhibition. Cells treated with different amount of BRD6688 (HDAC 1,2,3 inhibitor), RGFP966 (HDAC3 inhibitor), PCI-34051 (HDAC8 inhibitor), CAY10683 (HDAC2 inhibitor) and CI-994 (HDAC 1,2,3,8 inhibitor) were analyzed by western blot to monitor total acetylation levels, EMT phenotype, autophagy, apoptosis and p21 expression.

Interestingly not all class I protein specific inhibitors caused an increase in acetylation levels at the molecular weight for histone H3 or H4 (Figure 8a). Both HDAC8 inhibition with PCI-34051 and HDAC2 inhibition with CAY10683 caused no increase in acetylation levels while HDAC8 inhibition might have caused a decrease in of acetylation levels at higher dosages. Although HDAC8 inhibition did not cause increase in acetylation signal it does still have significant effects on other pathways as shown in figure 8.

All class I protein inhibitions beside HDAC2 inhibition caused an upregulation of e-cadherin and down regulation of n-cadherin (Figure 8b,c) suggesting that this treatment could reverse the cadherin switch and cause a decrease in metastatic phenotype. The effects on the autophagy pathway appears to be more pronounced in protein specific inhibition than was previously shown in class specific inhibition (Figure 7). Inhibition of HDAC8 and HDAC3 caused a clear
decrease in Lc3b with a transition to the lower molecular weight Lc3b in a dosage dependent manner which is an indicator of autophagy activity (Kabeya et. al, 2004). P62 levels also go down with HDAC8 inhibition but go up with HDAC3 inhibition (Figure 8d,e). This increase in p62 and decrease in Lc3b protein levels with HDAC3 inhibition is a contradictory result and may suggest that autophagy regulation is not being directly affected by the HDAC inhibition.

Apoptosis was upregulated in a dosage dependent manner with combined inhibition of HDAC1,2,3, and 8 with CI-944, combined HDAC1,2, and 3 inhibition with BRD6688, HDAC3 inhibition with RGFP966, and HDAC8 inhibition with PCI-34051 as indicated by the strongest presence of cleaved caspase 3 at 100μM treatment with decreasing signal in the 50μM, 25μM and minimal to no signal in the 10μM treatment; although HDAC2 inhibition did not cause an increase in cleaved caspase 3 (Figure 8g). The pro-apoptotic results agree with the expected anti-apoptotic protein Bcl-xL levels, with caspase 3 going up in higher doses and Bcl-xL going down in higher dosage (Figure 8f).

P21 protein levels were also upregulated in all HDAC protein specific inhibitions including HDAC2 inhibition that previously had shown no effect on acetylation levels or any other pathway (Figure 8h). Interestingly, in the HDAC8 inhibition p21 is completely down regulated at higher dosage while unregulated in lower dosage suggesting that p21 may not play a regulatory role in apoptosis of HDAC8 inhibited cells or it may play a role in earlier stages of the treatment. The p21 results for HDAC8 inhibition differs from other treatments suggesting that class
I inhibition results may differ from inhibition of individual class I proteins when compared to simultaneous inhibition of all HDAC class I proteins.

**Figure 8:** Western blot analysis of protein specific inhibition a) acetylated lysine levels at molecular weights 17kDa and 13kDa, b,c) EMT switch indicators e-cadherin and n-cadherin, d,e) autophagy indicators p62 and lc3b, f,g) apoptosis indicator cleaved caspase 3 and anti-apoptosis protein Bcl-xL, h) p21 protein levels, i) expression levels of loading control protein β-actin. Untreated controls cells are labeled with (-).

These data demonstrate that HDAC3 and HDAC8 inhibition can cause cell death in ovarian cancer cell line OVCAR8 and that they may be causing cell death in different manners compared to each other. To try to get a more detailed order of events a time-course study with HDAC3 inhibition and HDAC8 inhibition was done to see if causation can begin to be understood and analysis of histone specific levels was done.
Time Course Analysis of HDAC3 and HDAC8 Specific Inhibition

As shown in previous western results total levels of acetylated lysine were increased with HDAC3 inhibition at the molecular weights of histone H3 and H4 but showed no apparent increase in acetylation levels when HDAC8 was inhibited. To confirm that the increase acetylation are associated with histone proteins OVCAR8 cells were treated with HDAC3 or HDAC8 inhibitors at 50μM and collected 4, 8, 24, 48 and 72 hours post treatment. These samples were analyzed with antibodies against specific acetylated lysine histone proteins as outline in Table 5.

Inhibition of HDAC3 with RGFP966 caused increased acetylation on all analyzed histone proteins and at all amino acids analyzed. While HDAC8 inhibition with PCI-34051 caused no increase in acetylation levels at any sites on any histone proteins when compared to the untreated control (Figure 9). This would suggest that the HDAC8 inhibition maybe be causing changes in non-classic HDAC8 targets.
**Figure 9:** Western blot analysis of acetylated histones western or HDAC3 and HDAC8 time course experiment.

To determine what maybe direct effects of the HDAC3 and HDAC8 inhibition and to see what might be downstream effects a time course was done with sample taken at 4 hours, 8 hours, 24 hours, 48 hours and 72 hours. This allowed the observation of early changes and not just the end result at 72 hours. Firstly total acetylation levels were analyzed. For HDAC3 inhibition with RGFP966 acetylation levels increased as early as 4 hours and stay increased throughout the time course
while HDAC8 inhibition with PCI-34051 caused no acetylation increase compared to the untreated sample but does show an increase at the 24 hour time point when compared to 4 and 8 hours (Figure 10a).

EMT protein markers were analyzed throughout the time course with e-cadherin protein levels increasing at 24 hours in OVCAR8 cells treated with both HDAC3 and HDAC 8 inhibitors. Unlike in the dosage study the levels of n-cadherin do not come down (Figure 10b,c).

Autophagy markers p62 and Lc3b were analyzed and the p62 levels show no decrease over the time course while Lc3b shows mild down regulation at 48h and 72hours. For these protein markers the untreated healthy control cells have a more dramatic decrease in autophagy markers (Figure 10d,e). This suggested that autophagy is not strongly influenced by HDAC inhibition and autophagy activity may actually be reduced compared to untreated. Because of this it seems unlikely that HDAC3 or HDAC8 inhibition altered autophagy has a major impact on cell death for these treatments.

The apoptosis and p21 levels show a more direct effect with both HDAC3 and HDAC8 inhibition. When OVCAR8 cells are inhibited with an HDAC3 inhibitor, p21 levels are increased at 4 hours time point along with total caspase 3 being upregulated at 4 hours with cleavage of caspase 3 starting to show up at 24 hours in this time course (Figure 10f,h). For HDAC8 inhibition p21 is upregulated at 4 hours with very mild up regulation of total caspase 3. At the 24 hour time point caspase 3 shows a strong induction followed by cleavage of caspase 3 observed at 48hours
Interestingly for both HDAC3 and HDAC8 inhibited cells, levels of the anti-apoptotic protein Bcl-xL do not go down suggesting that Bcl-xL levels are not directly affected by the inhibition of HDAC3 or HDAC8 (Figure 10g). This time course analysis along with dosage response experiments demonstrated that pro-apoptosis indicators are reproducibly affected by HDAC3 or HDAC8 inhibition, while autophagy is not affected as indicated by a lack of down regulation of p62. EMT proteins are altered but at later time points. This suggests that apoptosis related proteins and pathways maybe directly affect by HDAC3 or HDAC8 inhibition. Because of this pathways related to cell death or pathways leading to apoptosis were further examined.

![Figure 10: Western blot analysis of time course experiment a) acetylated lysine levels at 17kDa and 13kDa b,c) EMT indicators e-cadherin and n-cadherin d,e) autophagy indicators p62 and lc3b f,g) apoptosis indicator caspase 3, anti-apoptosis protein Bcl-xL, h) p21 expression levels, i) protein levels of loading control β-actin.](image-url)
Analysis of Pathways That Lead to Apoptosis

To determine if other cells death pathways are upregulated, western blot analysis was performed with antibodies against markers for DNA damage, histone H2A.X and for phosphorylation of Histone H2A.X at Serine 139 (Yuan et al., 2004; Burma et al., 2001), possible mitotic arrest indicated by changes in Eg5 protein levels (Ricci et a, 2006), and ER Stress makers eIF2a, phosphorylation of eIF2a at Serine 51, and CHOP (Katsoulieris et al., 2010; Clarke et al., 2012; Zinszner et al., 1998).

HDAC3 inhibition caused an upregulation of Eg5 at early time points with degradation of Eg5 being at 42 hours. This could suggest mitotic disruption but further experiments would be needed to confirm this result. HDAC8 inhibition had no early effects on Eg5 protein levels with some upregulation happening post 24 hours but with no indication of degradation of Eg5 (Figure 11a). Protein eIF2a which is involved early in the unfolded protein response was upregulated and more importantly had increased phosphorylation at early time points when compared to untreated sample, followed by minimal expression of CHOP detected at 24, 48 and 72 hours when HDAC3 was inhibited suggesting that HDAC3 maybe directly causing ER stress when inhibited. HDAC8 inhibition showed no effect on any ER stress protein levels when compared to untreated control. (Figure 11b,c,d).
One common effect of HDAC3 and HDAC8 inhibition was an increase in the DNA damage markers. In both inhibitions, total and phosphorylated levels of Histone H2A.X were upregulated at the 4 hour time point and became dramatically upregulated at 24 hours when compared to the untreated control. This is suggesting that both HDAC3 and HDAC8 inhibition are causing DNA damage to occur early in the drug treatment (Figure 11e,f). Because neither drug causes direct damage to DNA it is possible that these treatments are causing dis-regulation of cell cycle control and DNA repair proteins leading to DNA damage.

**Figure 11:** Cell stress and damage pathway analysis of time course experiment a) mitotic stress protein Eg5 b,c,d) proteins expression of ER stress pathways eIF2a, phosphorylated eIF2a and CHOP e,f) DNA double strand break markers Histone H2AX and phosphorylated Histone H2AX, g) protein levels of loading control β-actin.
Analysis of Cell Death in HDAC3, HDAC8 Bcl-xL Inhibition Combination Treatment

These experiments suggested that the end point caused by HDAC3 or HDAC8 inhibition treatments was upregulation of the apoptotic pathway possibly through multiple different cell damage responses. But this testing has also shown that the anti-apoptotic protein Bcl-xL does not always respond to this treatment directly. Because of this fact, combination treatment was attempted with HDAC3 and HDAC8 inhibition combined with Bcl-xL inhibition. For this experiment cells were treated with 50\(\mu\)M RGFP966 (HDAC3 inhibition) alone, 50\(\mu\)M PCI-34051 (HDAC8 inhibition) alone, 1\(\mu\)M ATB-262 (Bcl-xL inhibition) and in combination for 48 hours. To determine percentage of dead cells, treated cells harvested at 48 hours were stained with a DNA dye with positively stained cells indicating cell death. HDAC3 and HDAC8 inhibition had 15.1% and 11.8% cell death percentage respectively with Bcl-xL inhibition having 4.3% percent dead cells. When combined together the treatments had a synergistic effect with both HDAC3/Bcl-xL treatment and HDAC8/Bcl-xL treatment more than doubling the percentage of dead cells 35.2% and 23.0% respectively. The untreated cells had 1.26% dead cells (Figure 12).
Specific Aim 3 – Comparing cell death induced by pan-HDAC inhibition to HDAC3 and HDAC8 inhibition across multiple cancer cells and PBMCs to determine if HDAC protein specific inhibitors can be used as a more target anti-cancer drug.

Comparison of Dead Cell Percentage in Various Cancer Cell Lines

Now that it is clear that inhibition of HDAC3 or HDAC8 has apoptotic effects in ovarian cancer cell line OVCAR8 it is important to determine if this inhibition is effective in multiple ovarian cancer cell lines and to see if the treatment is more targeted than pan-HDAC inhibition with vorinostat by testing it on non-ovarian
cancer cell lines. For this experiment ovarian cancer cell lines OVCAR8, SKOV3, IGROV1 were treated along side lung cancer cell line A459, prostate cancer cell line PC3, liver cancer cell line HepG2 and human peripheral blood mononucleocytes (PBMCs). All cells lines were treated with either 50µM RGFP966 (HDAC3 inhibition), 50µM PCI-34051 (HDAC8 inhibition), 5µM vorinostat (pan-HDAC inhibition), or untreated and harvested 48 hours post treatment. None of the treatments caused increase in cell death in the HepG2 sample due to high amount of death in the untreated. Vorinostat caused an increase in percent dead cells when compared to untreated control in all samples with very mild effect in ovarian cancer cell line SKOV3 and PBMCs. HDAC3 inhibition and HDAC8 inhibition do show a differential effect with HDAC3 inhibition causing death to increase in ovarian cancer cell lines OVCAR8, ovarian cancer cell line IGROV1, lung cancer cell line A459 and with a very mild effect in ovarian cancer cell SKOV3, although this is the same effect as vorinostat. HDAC8 inhibition caused an increase in cell death in ovarian cancer cell line OVCAR8, ovarian cancer cell line IGROV1 but appears to cause minimum cell death in all other cell types (Figure 13).
Figure 13: Cell death analysis for lung cancer cell line A459, prostate cancer cell line PC_3, liver cancer cell line HepG2, human peripheral blood mononucleocytes and ovarian cancer cell lines SKOV3, OVCAR8 and IGROV1.
CHAPTER IV

Discussion

This study had three aims: 1) Comparison of the amount of cell death or cell arrest caused by pan-HDAC inhibition with vorinostat, and HDAC class or protein specific inhibitors, 2) analysis of anti-cancer pathways altered by HDAC3 or HDAC8 inhibition, 3) analysis of the cell type specificity of HDAC3 or HDAC8 inhibition.

Aim one began by analyzing the effects of pan-HDAC inhibition and class specific inhibition in OVCAR8 cells by analyzing cell growth, cell cycle and upregulation of cleaved caspase protein levels. As shown in figure 5, class I HDAC inhibition caused a similar decrease in cell growth as compared to pan-HDAC inhibition. The cell cycle analysis showed that class I inhibition caused similar amount of apoptotic cells as the pan-inhibitor. Although the cell cycle analysis does show that class I inhibition caused a G2 arrest in dosage dependent manner whereas the pan-HDAC inhibition caused more cells to be in G1 than the untreated. These data showed that class specific inhibition causes similar amounts of cell death when compared to pan-HDAC inhibition but it maybe occurring through different mechanisms.

When class I individual proteins were inhibited all HDAC proteins, with the exception of HDAC2, caused an increase in cleaved caspase 3 signal (Figure 8). These treatments caused so much cell cycle disruption that it was not possible to
analysis the cell cycle so it is not known if these caused a G2 arrest as seen in the class I HDAC inhibition.

The goal was to determine if single HDAC protein inhibition could cause similar cell death as pan-inhibition and this data showed it to be the case. This could indicate that more specific HDAC protein inhibition could be used as a chemotherapy agent. This more targeted treatment may lead to fewer side effects due to less cellular pathways being interrupted.

The goal of aim two was two fold. First to determine if the mechanism of cell death could be determined and if HDAC protein specific inhibition caused changes in other anti-cancer pathways. Both HDAC3 and HDAC8 inhibition caused an increased in apoptosis marker cleaved caspase 3 (Figure 8) but the cell death mechanism appears to be differ. HDAC8 inhibition caused upregulation of p21, increased in DNA damage markers, followed by caspase 3 upregulation and cleavage. This suggests that the cell death mechanism maybe under p21 control as noted in other papers or is being caused by DNA damage. While HDAC3 inhibition upregulated more cell death pathways. HDAC3 inhibition has the same effect as HDAC8 in regards to DNA damage and p21 upregulation but also cause upregulation of the unfolded protein responses and caused degradation of mitotic protein Eg5. This difference between HDAC3 and HDAC8 can probably be explained by the difference in histone acetylation levels. HDAC3 inhibition caused clear increase in acetylation levels on all histone proteins analyzed which suggest that the acetylation increase is very wide spread and could be causing upregulation of proteins from a
variety of different cellular pathways. This is somewhat unexpected because previous studies suggest that HDAC3 prefers to modify Histone 2A and Histone 4 so it would have been expected to see an increase of acetylation on H4 and H2A but not H3 and H2B. While HDAC8 inhibition caused no detectable increase in acetylation of histones when compared to untreated samples. This could suggest that HDAC8 inhibition is more targeted and therefore causes less histone acetylation or it could be that the acetylation is being increased on non-classic HDAC targets. Further experiments, using techniques such as mass spectrometry would be needed to determine the proteins with increased acetylation levels caused by HDAC8 inhibition. Although this lack of wide spread acetylation could be useful if HDAC8 inhibition is used a chemotherapy agent. These results suggest that HDAC8 inhibition is more targeted than HDAC3 inhibition.

These data also show some inconsistency regarding the effects of HDAC protein inhibition. In the dosage response experiment, HDAC3 and HDAC8 inhibition caused upregulation of e-cadherin with down regulation of n-cadherin and caused upregulation of cleaved caspase with down regulation of Bcl-xL. This is an ideal response because it affects complementary sides of the pathways. E-cadherin levels should go up while n-cadherin levels should do down; the same with upregulation of pro-apoptotic protein caspase 3 and down regulation of anti-apoptotic protein Bcl-xL. If the detrimental proteins Bcl-xL and n-cadherin don't go down it can cause less effective treatments. Unexpectedly in the time course experiment HDAC3 and HDAC8 inhibition did not cause the down regulation of Bcl-xL or n-cadherin as they did in the dosage response experiment.
These data demonstrate that the downregulation of proteins is not as reproducible with HDAC inhibition. This is a somewhat expected result because HDAC inhibition causes an increase of acetylation levels on histone proteins, which leads to greater binding of transcription factors and subsequent increase in mRNA expression levels leading to increased protein expression. Because histone acetylation does not directly down regulate genes, in order for HDAC inhibition to cause down regulation of a protein it would either need to be through the upregulation of other proteins that subsequently cause protein degradation or alteration in the chromatin state surrounding causing down regulation proteins levels or mRNA levels. The lack of repetition of the down regulation is unfortunate but the upregulation of proteins in this study appears to be robust and reproducible.

Because of this lack of consistent down regulations of the anti-apoptotic protein Bcl-xL, I choose to perform a combination treatment with HDAC and Bcl-xL inhibition. This experiment showed a clear synergist effect suggesting that combination of HDAC3 or HDAC8 inhibitions with Bcl-xL maybe a possible chemotherapy treatment.

These data also show some possible negative effects of HDAC inhibition. When HDAC class IIa proteins were inhibited, they had multiple negative effects with an increase in anti-apoptotic protein Bcl-xL and in metastasis marker n-cadherin (Figure 7). Further studies would be needed to determine if HDAC3 or HDAC8 inhibition causes detrimental effects in other cell lines. Also HDAC3 or
HDAC8 inhibition would have to be tested on healthy tissue compared to cancerous cell models.

Other studies have shown that HDAC inhibition caused an upregulation of p21 levels which leads to cell death. Data from aim 2 showed an increase in p21 levels and subsequent upregulation of apoptosis from both HDAC3 and HDAC8 inhibition. This result is in agreement with other reported HDAC inhibition studies done in other cancer types. Inversely data from aim 2 for HDAC class IIa and HDAC2 inhibition caused a clear upregulation of p21 levels when compared to untreated control cells but neither treatment lead to cell death. This suggests that in ovarian cancer cell line OVCAR8 that p21 upregulation may not be a strong driver for apoptosis due to the fact the p21 upregulation alone was not an indicator of apoptosis.

Aim 2 provided conclusive data showing that HDAC3 or HDAC8 inhibition caused cell death through the apoptosis pathway.

Another interesting result of HDA3 and HDAC8 inhibition is the upregulation of DNA double strain break markers and an alteration in mitotic protein Eg5 with HDAC3 inhibition. Because the two inhibitors do not directly cause DNA damage it suggests that the damage is indirect. This could be caused by the misregulation of cell cycle and DNA repair proteins. Performing mRNA-seq would allow direct quantification of mRNA transcript levels that could indicate which proteins are being upregulated or down regulated. Doing this experiment along with microscopy experiments that visualize double strand breaks like tunel or by analysis of mitotic
cells could provide supporting data that the DNA is physically breaking. The western blot results are indirect indicators because it is based of protein levels associated with DNA double strand breaks.

Although there are still more experiments that could be done these data show that HDAC3 or HDAC8 inhibition caused the desired effect of inducing cell death in OVCAR8 cells.

Because aim 1 and aim 2 showed that HDAC3 or HDAC8 can cause cell death in ovarian cancer cell line it was important to determine if this more specific treatment could possibly be used as a more specific chemotherapy agent and if it would work in other ovarian cell lines. HDAC3 inhibition caused similar cell death in all ovarian cancer cells line as compared to pan-HDAC inhibition showing that it is possible to do a more target treatment, but HDAC3 inhibition also caused cell death in lung cancer cell line A459. While HDAC8 inhibition caused cell death in two ovarian cancer cell lines but not in IGROV1. HDAC8 inhibition also caused no cell death in non-ovarian cancer cell lines.

While these data need to be expanded upon it does prove that more targeted HDAC inhibition can cause similar cell death as pan-HDAC inhibition. HDAC8 inhibition could be promising for a targeted therapy with further investigation. It would be important to expand the number of cell lines treated with HDAC8 inhibitors to confirm that the pattern seen in this small study holds true. Repeating this experiment with the combination therapy could also boost the HDAC8
inhibition cell death while possibly keeping the more targets effects of HDAC8 inhibition when compared to HDAC3 or pan-HDAC inhibition.

In conclusion this study was performed because HDAC inhibition is a novel and promising chemotherapy agent. Most research has been performed using pan-HDAC inhibition but now that new and more targeted HDAC inhibition drugs are becoming available, more studies using targeted HDAC inhibition are needed. This thesis is the first study to demonstrate that HDAC3 or HDAC8 inhibition alone could cause cell death in ovarian cancer cell lines. Both treatments caused cell death through the apoptosis pathways. HDAC3 and HDAC8 caused the upregulation of DNA double strand break proteins while HDAC3 inhibition also caused degradation of Eg5 and upregulation of ER stress proteins with which had not been previously reported.
Bibliography


Coiffier, Bertrand, et al. "Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy." *Journal of Clinical Oncology* 30.6 (2012): 631-636


Poole, Raewyn M. "Belinostat: first global approval." *Drugs* 74.13 (2014): 1543-1554.


Yuan, Jingsong, Rachel Adamski, and Junjie Chen. "Focus on histone variant H2AX: to be or not to be." *FEBS letters* 584.17 (2010): 3717-3724.


