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Anti-ovarian cancer effects of histone deacetylase inhibitors and calpain inhibitor

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Thesis

ANTI-OVARIAN CANCER EFFECTS OF
HISTONE DEACETYLASE INHIBITORS AND CALPAIN INHIBITOR

by

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ANTI-OVARIAN CANCER EFFECTS OF 
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KAROLINA EVA LAPINSKA

ABSTRACT

Ovarian cancer is the leading cause of death among gynecologic malignancies. The risk of developing ovarian cancer in a woman’s lifetime is 1 to 2 in 100. This high rate of development and death from the tumorigenesis is a result of its asymptomatic manifestation. Ovarian cancer is usually found in its advanced stage; therefore the survival rate is lower than for other types of cancers. The most common type of ovarian cancer, serous epithelial ovarian cancer, arises from the surface epithelium of the ovary and less frequent from the fallopian tubes or uterus. The treatment of surgery is limited by the fact that most ovarian cancers are detected after they have metastasized. Chemotherapy is often difficult because of the lack of sufficient target specific drugs. Typically, platinum in combination with other drugs is provided as the standard treatment. These combinations exhibit higher toxicity, are often not target specific, and frequently despite treatment, the tumor relapses. Current studies suggest epigenetics plays a significant role in carcinogenesis by the silencing of tumor suppressor genes (TSG). Histone modifications and the methylation of specific cytosine phosphate guanosine (CpG) residues in the upstream region of genes silence the TSG. Many clinical trials are in progress to develop combination therapies utilizing histone deacetylase inhibitors (HDACi), and DNA methyl transferase I (DNMTI) inhibitors, in combination with other cytotoxic agents. HDACi are known to be effective against different types of leukemia’s,
such as Cutaneous T-cell Lymphoma; however, they are not as effective against solid tumors when used as a single agent. Our laboratory was one of the first to demonstrate that HDACi, in addition to its known property to increase histone acetylation, additionally decrease CpG island methylation in the upstream region of TSG. This demethylation causes re-expression of TSG. Our laboratory hypothesizes that re-expression of TSG sensitize cancer cells to other cytotoxic drugs. In an effort to develop improved therapy for ovarian cancer, we employed a combination therapy, which includes epigenetic drugs, HDACi, in combination with calpain protease inhibitor, calpeptin. Calpain is a ubiquitous protease usually activated in cardiovascular diseases and cancer cells. The present study discerns that combination of HDACi and calpeptin produce more than additive growth inhibition of diverse ovarian cancer cells. HDACi re-expressed TSG. Additionally, the observed growth inhibition of ovarian cancer cells was caused by cell-cycle arrest, induction of apoptosis, followed by autophagy. The phosphorylation of growth promoting signaling protein, Mitogen Activated Protein Kinase 1 (ERK), was inhibited. In addition, the inhibitors also partially inhibited phosphorylation of anti-apoptotic protein V-ask Murine Thyomoma Viral Oncogene Homolog 1 (Akt). Collectively, the outcome of this study suggests that epigenetic drugs (HDACi) sensitize the diverse ovarian cancer cell lines by re-expression of TSG, followed by cell death, when treated in combination with calpain inhibitor, calpeptin.
# TABLE OF CONTENTS

- TITLE..................................................................................................................................... i
- COPYRIGHT............................................................................................................................... ii
- READER APPROVAL PAGE......................................................................................................... iii
- ACKNOWLEDGMENTS ................................................................................................................ iv
- ABSTRACT .................................................................................................................................. v
- TABLE OF CONTENTS ................................................................................................................ vii
- LIST OF TABLES ......................................................................................................................... viii
- LIST OF FIGURES ....................................................................................................................... ix
- LIST OF ABBREVIATIONS .......................................................................................................... x
- INTRODUCTION ......................................................................................................................... 1
- MATERIALS AND METHODS ................................................................................................... 8
- RESULTS ..................................................................................................................................... 13
- DISCUSSION ............................................................................................................................... 25
- REFERENCES .............................................................................................................................. 33
- CURRICULUM VITAE .................................................................................................................. 39
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>List of Primers Used for RT-PCR</td>
<td>10</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maintenance of Methylation by DNMTI</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>De Novo Methylation by DNMTI and Recruitment of HDAC and MDBP</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Cancer Progenitor Cells and Progression of Cancer</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Changes That Occur as a Tumor Cell Undergoes EMT and</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Subsequently Metastasizes at a Secondary Location</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Growth Inhibition and Morphological Changes of CAOV-3 and SKOV-3</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Ovarian Cancer Cells by HDACi and Calpeptin</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Transwell Inhibition of CAOV-3 and SKOV-3 Ovarian Cancer Cell Motility</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>Re-expression of Tumor Suppressor Genes: ARHI, p21, RARβ2</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>Cell Cycle Inhibition and Apoptosis</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Autophagy</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>Effects of HDACi and Calpeptin on Akt and ERK phosphorylation</td>
<td>24</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AKT…………………………………V-ask Murine Thyomoma Viral Oncogene Homolog 1
ARHI………………………………………………….Ras Homolog Member I
BRCA…………………………………………………Breast Cancer gene
BSA…………………………………………………..Bovine Serum Albumin
Calp……………………………………………………………..Calpeptin
CpG……………………………………………….Cytosine Phosphate Guanosine
DMEM……………………………………………Dulbecco’s Modified Eagle Medium
DMSO…………………………………………………….Dimethyl Sulfoxide
DNMTI……………………………………….DNA Methyl Transferase I
DNMTs…………………………………………DNA Methyl Transferases
DR4…………………………………………………..Death Receptor 4
ECM…………………………………………………..Extracellular Matrix
EMT……………………………………………… Epithelial-Mesenchymal Transition
ER……………………………………………………….Estrogen Receptor
ERK………………………………………………..Mitogen Activated Protein Kinase 1
FBS…………………………………………………..Fetal Bovine Serum
FITC…………………………………………….Fluorescein Isothiocynate
HAT………………………………………………Histone Acetyl Transferases
HDAC……………………………………………..Histone Deacetylases
HDACi……………………………………………Histone Deacetylase Inhibitors
HDAC1……………………………………………..Histone Deacetylase 1
HER2…………………………………………….Human Epidural Growth Factor Receptor 2
HRP………………………………………………..Horseradish Peroxide
LC3b………………………………………………….Cytoplasmic Protein
LOH…………………….................................................Loss of Heterozygosity
LDH…………………….................................................Lactate Dehydrogenase
LR……………………......................................................Lower Right
MMP……………………..................................................Matrix Metalloproteinase
MDBP…………………….............................................Methyl Domain Binding Protein
MDBP2…………………….............................................Methyl Domain Binding Protein 2
MET……………………..................................................Mesenchymal-Epithelial Transition
pAkt…………………….................................................Akt Phosphorylation
PBS……………………..................................................Phosphate Buffered Saline
pERK……………………..............................................ERK Phosphorylation
PL…………………….......................................................Propidium Iodide
PR…………………….....................................................Progesterone Receptor
qPCR……………………..............................................Real-time Quantitative PCR
RARβ2……………………............................................Retinoic Acid Receptor Beta 2
RNA Pol II……………………........................................RNA Polymerase II
RPMI…………………….............................................Roswell Park Memorial Institute
SB……………………......................................................Sodium Butyrate
SAHA……………………..........................................Suberoylanilide Hydroxamic Acid
TBST…………………….............................................Tris-Buffered Saline/Tween
TP53…………………….................................................Tumor Suppressor Protein p53
TSG…………………….................................................Tumor Suppressor Genes
UL……………………......................................................Upper Left
UR……………………......................................................Upper Right
INTRODUCTION

Approximately 20,000 women in the United States succumb to ovarian cancer each year [1]. These carcinomas are the eighth most common and the fifth leading cause of cancer deaths in women [1]. It is the source of more deaths in females as compared to any other gynecologic cancer. These ovarian carcinomas vary in their classification of cell types: serous, endometrioid, mucinous, and Brenner tumors. This heterogeneity is further categorized into benign, intermediate, and malignant clinical manifestations. These differences arise from the precise mechanisms of either inherited or sporadic ovarian tumorigenesis; however, they are not well understood [2]. Nevertheless, alterations in many gene expressions, which are observed to be involved in various cancers, such as shared genetic markers BRCA, HER2, ER, PR, and ARHI in breast cancers, are also implicated in ovarian cancer [3] [4]. Currently, most available chemotherapies for ovarian cancer are not target specific [5]. The chemotherapeutic agents, such as platinum-taxol combinations, are being used in treatment of ovarian cancer. These treatments are relatively ineffective and nonspecific, targeting both healthy and malignant tissues [6] [7]. Thus, overcoming the resistance and recurrence that develops in 30-40% of women by novel treatments and new therapeutic targets is necessary to successfully treat patients [8]. Recent research suggests that in addition to genetic alterations, epigenetic changes are associated with numerous pathway genes in ovarian cancer [9]. Our laboratory proposes that combination therapies involving epigenetic drugs are beneficial in combating drug resistance, and in decreasing toxicity.
The combination therapy will also exhibit more than an additive anti-cancer effect [10][11].

Altered epigenetic modulation of gene expression is a progression involved in various carcinomas. This modification manifests primarily through two mechanisms: histone modification and DNA methylation [12]. Increased regulation is involved around tightly bound basic proteins on DNA known as histones. The fundamental and structural arrangements of histones organize and condense the DNA molecules into chromosomes that are segregated during cell division. The amino acid residues on the histone tails are post-transcriptionally modified by acetylation, methylation, phosphorylation, and ubiquitination [13]. Histone acetylation and deacetylation is mediated by histone acetyl transferases (HATs) and histone deactylases (HDACs). Considerable data has shown abnormal histone modification as a cause of carcinogenesis [14]. With increasing therapeutic research, histone deacetylase inhibitors (HDACi) have shown promise [15]. HDACi result in increased acetylation activity of many intrinsic proapoptotic genes and are under clinical trials as anticancer agents. Recently, HDACi, such as suberoylanilide hydroxamic acid (SAHA, commercially known as Vorinostat), have been approved by the FDA in treatment of Cutaneous T-cell Lymphoma; however, HDACi are not useful in treating solid tumors [16]. Newly developed HDACi are under clinical trials in combination with other available drugs therapies against solid tumors, which include breast and ovarian cancers [17].
Another equally important epigenetic alteration observed during carcinogenesis is the silencing of tumor suppressor genes (TSG) by methylation of cytosine phosphate guanosine (CpG) islands around the promoter regions, as shown in Figure 1.

**Figure 1. Maintenance of Methylation by DNMT1.** After replication, methylation (shown in red) of the specific CpG residues (not shown) on the mother strand is copied onto the daughter strand by DNA methyltransferase I (DNMT1), with preservation of the methylation pattern on the mother strand [18].

DNA methyltransferase I (DNMT1) is the enzyme that maintains methylation in the newly replicated strand of DNA during cell division, as shown in Figure 1. Usually, the expression of DNMT1 varies during a cell cycle, being highest during the S phase [19]. In normal healthy cells, overall genome-wide methylation is higher, though specific regions near the promoters of TSG are hypomethylated. The reverse happens during carcinogenesis [20]. DNMT1 is highly expressed in all types of cancer, even during cell cycle arrest; however, the genome wide methylation of cancer cells are reduced compared to normal cells [19] [20] [21]. One possible reason for hypomethylation in cancer cells could be the higher expression of growth promoting genes. The hypermethylated CpG residues by DNMT1 around the promoter region of TSG in cancer cells, subsequently recruit histone deacetylase 1 (HDAC1) and methyl domain binding
protein 2 (MDBP2) [18]. These proteins hinder the binding of RNA polymerase II (RNA Pol II) to the promoter region, inhibiting transcription, as shown in Figure 2. Drug-induced demethylation of these CpG residues in the upstream regions of TSG, releases HDAC1 and MDBP2 from the CpG site and transcription resumes [21].

![Figure 2](image-url)

**Figure 2. De Novo Methylation by DNMT1 and Recruitment of HDAC and MDBP.** In de novo methylation, RNA polymerase II (RNA Pol II) and transcription factors bind to unmethylated upstream promoter region of a gene (shown in green), activating transcription. DNA methyl transferases (DNMTs) methylate specific CpG sites (not shown) within the promoter region. When a gene is methylated at CpG sites near the promoter region (shown in red), it allows binding of histone deacetylases (HDACs) and other methyl domain-binding proteins (MDBP) and prevents RNA Pol II from binding, thus inhibiting transcription. In addition, methylation in the intragenic region is seen, though the function is still not well understood [18].

Interestingly, genetic susceptibility to cancers has not been fully understood [22]. Genetic predisposition does not yet explain why people with the same types of genetic alterations or mutations develop cancer at different stages of their lives or why some develop and others do not [23]. Recent studies suggest that the environment plays an important role to induce carcinogenesis in these predisposed individuals [18] [21] [24]. One of the principal ways environmental factors, such as differential signaling
mechanisms and the differential effect of stromal cells on a tissue, induce carcinogenesis is most significantly revealed by the mechanisms of epigenetic alterations. This idea is supported by observations that conventional therapy is unable to kill cancer stem/progenitor cells and drug resistant cancer cells [25]. Our laboratory hypothesizes that epigenetic events initiate carcinogenesis and are also involved in cancer progression and metastasis, as shown in Figure 3 and 4 [4] [11] [21]. Recent studies have shown that treatment of platinum drug resistant ovarian cancer cells with epigenetic drugs made them susceptible to the platinum drugs once more [26]. Inhibiting the abnormal epigenetic progression may in fact kill drug resistant cancer cells, kill cancer stem/progenitor cells, inhibit epithelial-mesenchymal transition (EMT) of epithelial tumor cells, and perhaps mesenchymal-epithelial transition (MET) of circulating cancer cells [11].

![Figure 3. Cancer Progenitor Cells and Progression of Cancer.](image)

Various epigenetic changes possibly initiate formation of cancer progenitor cells from predisposed cancer cells. The augmentation of cancer from the cancer progenitor cells is a consequence of the various environmental conditions and by certain intracellular alterations [20].
Figure 4. Changes That Occur as a Tumor Cell Undergoes EMT and Subsequently Metastasizes at a Secondary Location. As epithelial tumor cells (shown in blue) undergo epithelial-mesenchymal transition (EMT), they begin to lose their epithelial phenotype (shown after step 2). The decrease of cell-to-cell attachment receptors and integrins (shown in purple) continues to step 3 and persists after. Additionally, stromal cells near the cancer cell undergoing EMT are affected and start experiencing changes (shown as a transition from green to red cells). Once the cancer cells have completely undergone EMT, they travel to a new location, and multiple steps (not shown) involving mesenchymal-epithelial transition (MET) must occur for the metastatic cancer cell to infiltrate at a distant site to form a secondary tumor. Now, the stromal cells at the new tumor location will likewise undergo transformation [11]. (ECM: Extracellular Matrix; MMP: Matrix Metalloproteinase)

Our laboratory has been one of the first to discern that HDACi, in addition to their known ability to increase acetylation, produce demethylation of CpG residues on DNA [27]. The demethylation is a result of degradation of DNMTI, which leads to re-expression of TSG: p21 and p16, and RARβ2 [27]. Our laboratory presented outcomes in which treatment with two structurally different Class I and Class II HDACi, Sodium Butyrate (SB) and SAHA, re-express TSG, ARHI, in breast cancer cells [3]. Sarkar et al. proposed, that if HDACi re-express TSG, then consequently they should sensitize the cancer cells to other cytotoxic drugs [21]. Thus, this is one of the central hypotheses of our laboratory for developing a combination therapy. In support of this hypothesis, our
laboratory has shown that HDACi sensitize both breast and ovarian cancer cells to different cytotoxic drugs, including oligonucleotides designed against telomeres and inhibitors of calpain protease [3] [28]. Many studies are underway which involve the use of both HDACi and DNMTI inhibitors in combination with other drugs against solid tumors [4] [10].

In this study we investigated the effects of HDACi in combination with calpain protease inhibitor, calpeptin, on ovarian cancer cells. We employed two discrete ovarian cancer cells, namely CAOV-3 and SKOV-3. CAOV-3 is tumor suppressor protein p53 (TP53) mutated, while SKOV-3 is TP53 null. The distinction between the two is clearly noted in the differences of their response to our combination therapy. Recognizing the differences, CAOV-3 is understood to be less aggressive and less metastatic than SKOV-3. The combination of the two inhibitors, HDACi and calpeptin, produced enhanced growth inhibition in these ovarian cancer cells. Additionally, we identified the mechanisms of actions of the inhibitors. The inhibitors (i) inhibited cell cycle, (ii) decreased metastatic activity, (iii) induced apoptosis, and (iv) promoted autophagy. (v) TSG: ARHI, p21, and RARβ2 were re-expressed and (vi) we observed differential inhibition of two important signaling pathways, v-ask murine thyomoma viral oncogenes homolog 1 (Akt) and extracellular signal-regulated kinase (ERK). Thus, the comprehension and knowledge of the mechanisms involved in the growth inhibition and the death of ovarian cancer cells is essential to fully appreciate the usefulness of this combination therapy.
MATERIALS AND METHODS

1. Cell Culture

CAOV-3 and SKOV-3 ovarian cancer cells were grown in Roswell Park Memorial Institute (RPMI-1640) media containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 mL 10% heat-inactivated fetal bovine serum (FBS). Around 70-80% confluence, cells were exposed to dimethyl sulfoxide (DMSO) (control) or distinct concentrations of inhibitors. Cells were incubated for different times before being harvested.

2. Reagents

CAOV-3 and SKOV-3 ovarian cancer cells were obtained from American Type Culture Collection (Manassas, VA). SB and SAHA were purchased from Sigma (St. Louis, MO) and calpeptin was purchased from Nova Chemicals (Moon Township, PA). RPMI-1640 and Dulbecco’s Modified Eagle Medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). The annexin/PI apoptosis analysis kit was acquired from BD Biosciences (San Diego, CA). The cDNA preparation kit was obtained from Qiagen (Valencia, CA). The SYBR green RT-PCR mix was from Applied Biosciences (Foster City, CA). Diff-Quik Stain was obtained from IMEB Inc. (San Marcos, CA). All antibodies, including anti-phospho ERK, anti-ERK, anti-phospho-Akt, anti-Akt, and anti-L3Cb were obtained from Santa Cruz Biotech (Paso Robles, CA). The western blot developer was acquired from Thermo Scientific (Rockford, IL).
3. Cell Survival Assay

Equal number of ovarian cancer cells were grown in six-well plates and treated with desired concentrations of inhibitors, as described in the legend of Figures 5 (A and B). After 96 hours, viable cells were counted using a trypan blue exclusion assay. The media was aspirated and cells were washed with PBS. The cells were trypsinized and 20 μL cell suspensions were mixed with 20 μL trypan blue. The unstained (viable) cells were counted. The viable cell number in control (untreated) cells were plotted as 100%. Viable cell counts in inhibitor treated samples were normalized to the percentage compared to the control.

4. In-Vitro Cell Migration Assay

The effects of inhibitors on migration of the cells were tested by chemotaxis assay. This assay was performed in 24-well plates and contained transwell chambers with membranes, which included 8 μm pores. The cells were treated with specific concentrations, as described in the legend of Figure 6 (A and B), harvested two days later by trypsinization, and washed in serum-free RPMI growth medium. The upper chamber received 200,000 cells suspended in 500 μL serum-free RPMI growth medium and the lower chamber received 0.5 mL of conditioned media from the actively growing ovarian cancer cells. After 12 hours, the cells from the chambers were aspirated and wiped with cotton swabs. The membranes were stained with Diff-Quik Stain, obtained from IMEB Inc. (San Marcos, CA), and the migrated cells were viewed under a light microscope and counted.
5. Real-time Quantitative PCR (qPCR)

Ovarian cancer cells were grown in 10cm plates, treated with inhibitors as described in the legend of Figure 7 (A and B) and harvested. The total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturer’s protocol. The RNA was converted to cDNA after digesting any genomic DNA contamination. Real-time quantitative PCR was performed using prepared cDNA. Results were normalized to the expression level of β-actin transcripts within each sample with the same primers.

Table 1. List of Primers Used for RT-PCR.

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<thead>
<tr>
<th>Primer Name</th>
<th>Forward (5'→ 3')</th>
<th>Reverse (5'→ 3')</th>
</tr>
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<tbody>
<tr>
<td>ARHI</td>
<td>TGGGTAACGCCAGCTTTGGCT</td>
<td>TAACGTGGCGCTGAGCG</td>
</tr>
<tr>
<td>RARβ2</td>
<td>CAAACCGAATGCGACATCGG</td>
<td>GCGGAAAAAGCCCTTACATCC</td>
</tr>
<tr>
<td>p21</td>
<td>CTGGAGACTCTCAGGGTCGA</td>
<td>GGATTAGGCTTCCTCTTTGA</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTGGCACCAGCACAATG</td>
<td>GGACAGCGAGGCCAGGAA</td>
</tr>
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6. Cell Cycle and Apoptosis Assays

Ovarian cancer cells were treated with inhibitor concentrations, as described in the legend of Figure 8 (A, B, C, and D). Untreated control cells and treated cells were washed with phosphate buffered saline (PBS). For cell cycle analysis, cells were fixed with a medium containing 35% ethanol for 5 minutes at room temperature. The cells were stained for 30 minutes in the dark with 25 µg/mL propidium iodide (PI), containing 50 µg/mL RNAse in PBS, and then underwent FACS analysis. The apoptosis assay was performed with the annexin/PI assay. After treatment, untreated and treated cells were
harvested and suspended in 100 µL 1x binding buffer. Subsequently, 5 µL annexin
reagent conjugated to fluorescein isothiocynate (FITC) and 5 µL PI were added to each
sample as described in the kit protocol (BD Biosciences). The samples were kept in the
dark for 30 minutes at room temperature. 900 µL 1x binding buffer was added to each
sample follow by the measurement of the dual fluorescence of FITC and PI, in the FACS
machine.

7. Immuno Staining Procedure and Autophagy Assay

Ovarian cancer cells were grown in eight-well plates and treated with inhibitors,
as described in the legend of Figure 8 (G and H). Media was then aspirated from each
well. The cells were fixed for 15 minutes with 200 µL 4% paraformaldehyde at room
temperature and washed with PBS three times. Next, cells were treated for 30 minutes
with 200 µL solution, including 0.1% Triton X100 and 0.1% sodium citrate, at room
temperature. The solution was aspirated, the wells washed 3 times with PBS, and then
blocked for 1 hour with 300 µL bovine serum albumin (BSA) at room temperature. The
solution was aspirated and the wells washed three times with PBS. The primary anti-
LC3b was added in 3% BSA to the wells and incubated at 4°C overnight. The following
day, the solution was aspirated and the wells were washed three times with PBS. An
appropriate dilution of secondary antibody in 3% BSA (conjugated to FITC) was added
and incubated for one hour at room temperature. The solution was aspirated and the wells
washed three times with PBS. Then, 100 µL DAPI (Invitrogen) was added to each well
and incubated for 5-10 minutes. The solution was aspirated and the wells washed three
times with PBS. A drop of mounting solution (Prolong Gold, Invitrogen) was added to
each slide and a cover slip placed to hold in position. Each slide was sealed with nail oil, dried, and viewed and photographed under fluorescent microscope.

8. Protein Immunoblot Analysis

Ovarian cancer cells were grown in 10cm plates and treated with inhibitors, as described in the legend of Figure 9 (A and B). The cells were harvested with trypsin/EDTA, washed with PBS, and collected by centrifugation at 1,000 rpm. Subsequently, cells were lysed with ice cold 1% NP-40 lysis buffer, including vanadate and protease inhibitor cocktail tablet (1 tablet/10ml buffer) (Roche, Germany). For each sample, protein concentrations were calculated with BSA as the control, using a protein assay reagent (Bio-RAD). Equal amount of protein from each sample was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% non-fat milk powder or 5% BSA for phospho blots in Tris-Buffered Saline/Tween (TBST) and then incubated with the primary antibody at 4°C overnight. The membrane was washed with TBST three times for 10 minutes each. The secondary antibody was conjugated to horseradish peroxide (HRP), added, and then membrane was incubated for one hour at room temperature. The membrane was washed with TBST three times for 10 minutes, and was developed using a chemiluminescent reagent, and photographed using Image Quant (GE LAS4000).
RESULTS

1. Combination Treatment of HDACi and Calpeptin: Growth Inhibition and Morphological Changes in Ovarian Cancer Cells

To test the efficacy of the combination therapy, two characteristically different ovarian cancer cell lines, SKOV-3 and CAOV-3, were used in this study. These cells were treated with sub-optimal levels of chemically distinct HDACi (SB and SAHA) and calpain protease inhibitor, calpeptin. The combination treatment of HDACi and calpeptin resulted in a notable 80-90% inhibition of growth of the ovarian cancer cells after four days (Figure 5A and B). This decrease in growth reveals a significant effect of the combination therapy (SB and Calp: p value = 0.0002, and SAHA and Calp: p value = 0.0001, for CAOV-3 cells; and p value = 0.0001 for both combinations in SKOV-3 cells), since the effective dose of each HDACi and calpeptin was lower than the effective doses of HDACi and calpeptin when used as a single agent (not shown).

Distinct morphological changes were observed both in CAOV-3 and SKOV-3 ovarian cancer cells after treatment with the inhibitors. After four days, the cells decreased in number and assumed a more rounded shape with the addition of HDACi with calpeptin, which suggests cell death (Figure 5C and D). The distortion of cell structure in both ovarian cancer cell lines was more obvious when treated with calpeptin.
Figure 5. Growth Inhibition and Morphological Changes of CAOV-3 and SKOV-3 Ovarian Cancer Cells by HDACi and Calpeptin. A. CAOV-3 ovarian cancer cells were treated with 0.5 mM sodium butyrate (SB), 2 µM SAHA, 5 µg/mL calpeptin (Calp) or combination of SB and Calp (p value = 0.0002), and SAHA and Calp (p value = 0.0001), at the same concentrations. B. SKOV-3 ovarian cancer cells were treated with 1 mM SB, 10 µM SAHA, 10 µg/mL Calp or combination of SB and Calp (p value = 0.0001), and SAHA and Calp (p value = 0.0001), at the same concentrations. Viable cells were washed, trypsinized, and counted after four days. All treated cells are expressed as a percent relative to the arbitrarily set 100% of the dark shaded column of control cells. C. CAOV-3 ovarian cancer cells were treated with 1 mM SB, 10 µM SAHA, 20 µg/mL Calp, or a combination of SB and Calp, and SAHA and Calp, at the same concentrations. D. SKOV-3 ovarian cancer cells were treated with 1 mM SB, 10 µM SAHA, 20 µg/mL Calp, or a combination of SB and Calp, and SAHA and Calp, at the same concentrations. Morphology changes and number of cells are different in the cells with inhibitors when compared to the control cells. Photographs were taken after four days treatment.

2. Inhibition of Motility by HDACi and Calpeptin.

Growth inhibition does not necessarily imply that the inhibitors would be effective against a cancer cell’s metastatic property. Therefore, we determined the effects of HDACi and calpeptin on the invasiveness property of ovarian cancer cells. As a marker of invasive potential, the degree of motility of ovarian cancer cells was assessed by an in-vitro cell migration (transwell) assay [11]. Conditioned media from the untreated control cells was used as a chemo-attractant. The inhibitor-treated cells and untreated
control cells were placed in the upper chambers and the conditioned media was added to the lower chamber. The movement of the cells across the membrane separating the two chambers was measured by staining the cells that crossed the membrane. The number of control, untreated cells, which moved across the membrane, was assigned as 100% of cells being motile. The percent of treated cells, which moved across the membrane, were plotted (Figure 6). Approximately 70-90% reduction in motility of both CAOV-3 and SKOV-3 ovarian cancer cells was observed in HDACi and calpeptin treated cells (Figure 6A CAOV-3 and 6B SKOV-3, respectively). The combination, at the concentrations used, did not produce any additional inhibition.

Figure 6. Transwell Inhibition of CAOV-3 and SKOV-3 Ovarian Cancer Cell Motility. A. CAOV-3 ovarian cancer cells were treated with 0.5 mM sodium butyrate (SB), 2 µM SAHA, 5 µg/mL calpeptin (Calp) or combination of SB and Calp, and SAHA and Calp, at the same concentrations. B. SKOV-3 ovarian cancer cells were treated with 1 mM SB, 10 µM SAHA, 10 µg/mL Calp or combination of SB and Calp, and SAHA and Calp, at the same concentrations. After 48 hours, ovarian cancer cells were harvested, suspended in serum-free media and added to the upper chamber. The lower chamber contained conditioned media from control cells. After 12 hours, cells were removed from the upper chamber and the membrane was stained and viewed under the microscope. Cell counts were taken. Untreated control cell counts were normalized to 100% motility. HDACi, Calp, and combination treatment results are shown in percentage motility compared to the control.
3. Re-expression of Tumor Suppressor Genes by HDACi

Our laboratory has previously demonstrated that treatment with HDACi caused demethylation of methylated CpG islands in the upstream regions of tumor suppressor genes (TSG). Demethylation re-expressed these silenced TSG [3,28]. Three TSG: ARHI, p21, and RARβ2 are epigenetically silenced during ovarian carcinogenesis. ARHI is a maternally imprinted, pro-apoptotic TSG. Normally, in healthy tissue, ARHI is expressed only from the paternal allele. In 40% of breast and ovarian cancer, the paternal allele is silenced causing a loss of heterozygosity [4,21]. p21 is a cell cycle inhibitor and RARβ2 is a marker of differentiation. To determine the status of these three TSG after HDACi and calpeptin treatment, we performed qPCR assays with treated and untreated ovarian cancer cells. HDACi, 10 µM SAHA, resulted in a 6-fold re-expression of ARHI in CAOV-3 ovarian cancer cells and a 2.2-fold re-expression of ARHI in SKOV-3 ovarian cancer cells, with respect to each untreated control cells (Figure 7A and B, ARHI). p21 re-expression with 10 µM SAHA produced a 5-fold increase in CAOV-3 ovarian cancer cells and 8-fold increase in SKOV-3 ovarian cancer cells (Figure 7A and B, p21). RARβ2 re-expression was enhanced 85-fold in CAOV-3 ovarian cancer cells and 6-fold in SKOV-3 ovarian cancer cells after treating with 10µM SAHA. (Figure 7A and B, RARβ2).
Figure 7. Re-expression of Tumor Suppressor Genes: ARHI, p21, RARβ2. A. CAOV-3 ovarian cancer cells were treated with 10 µM SAHA for 48 hours. B. SKOV-3 ovarian cancer cells were treated with 10 µM SAHA for 48 hours. Total RNA was isolated, then cDNA was prepared. qPCR for ARHI, p21, and RARβ2 transcripts were performed. The results were normalized for each sample to the level of β-actin transcripts for each gene in each sample. Data was expressed as a fold re-expression compared to the control cells.

4. Inhibition of Cell Cycle, Induction of Apoptosis, and Autophagy

The results from the inhibition of growth and re-expression of TSG, including cell cycle inhibitor p21 and pro-apoptotic gene ARHI, suggested our laboratory to test both cell cycle inhibition and apoptosis by HDACi and calpeptin. CAOV-3 and SKOV-3 ovarian cancer cell lines displayed a relatively similar type of cell cycle phase inhibition with the addition of SB, SAHA, and calpeptin (Figure 8A and B). All treated CAOV-3 ovarian cancer cells were arrested in the S-phase of the cell cycle. The CAOV-3 cancer cells treated with combination of both SB and SAHA with calpeptin exhibited a significant 16-18% S-phase arrest, with the amount of cells in the G1 phase being relatively lower (all compared to its respective untreated control cells) (Figure 8A). Similar to the CAOV-3 cancer cells, the SKOV-3 ovarian cancer cells exhibited S-phase arrest; however, certain drug-treatments portrayed considerably increased inhibition than
the CAOV-3 cancer cells (Figure 8B). SKOV-3 cells treated with SB, calpeptin, and combination of SB and calpeptin presented a substantial cell cycle arrest in the S-phase. SAHA and calpeptin combination treated SKOV-3 cancer cells were significantly arrested in the G2/M phase of the cell cycle (13-14%), as compared to their respective untreated control cells.

In order to determine whether the cell death observed from the morphological alterations (Figure 5C and D) was caused by induction of apoptosis and autophagy, we investigated both possibilities to discern the methodologies. Firstly, to measure apoptosis, the annexin/propidium iodide (PI) assay was utilized [27]. Annexin is bound to the inner surface of the plasma membrane. When the cell undergoes the early phase of apoptosis, the membrane flips, resulting in exposure of annexin to the external environment. PI binds the released inactive and nuclear DNA during apoptosis. The combination of annexin and PI attachment provides a comprehensive measurement of all the phases of apoptosis. As shown in Figure 8C and D, CAOV-3 and SKOV-3 ovarian cancer cells exposed to HDACi and calpeptin exhibited an increase in apoptosis at the given concentrations. The lower right (LR) quadrant depicts cells in the early stage of apoptosis. This is the point when the cell membrane reverses and only allows annexin to bind. The increase in apoptosis presented in the results however, was more pronounced in the later phases of apoptosis, as observed from the upper right (UR) and upper left (UL) quadrants values (Figure 8C and D). UR is the intermediate stage of apoptosis when the membrane is flipped and the DNA in nucleus is becoming increasingly accessible to PI. UL is the
late stage of apoptosis, when the cell membrane has completely fallen apart and the entire DNA is exposed. Essentially at this stage, all the cells are dead.

SKOV-3 ovarian cancer cells portrayed a much higher apoptosis percentage when treated with HDACi, calpeptin, and combination treatments; however, analogously increased results were observed when both ovarian cancer cells lines were treated with the combination of SAHA and calpeptin. Figure 8E and F demonstrated the total apoptosis of CAOV-3 and SKOV-3 ovarian cancer cells after the treatments.

The induction of autophagy similarly plays a role in cancer cell death. The morphological changes observed in Figure 5C and D indicate that treated cancer cells possibly underwent autophagy, particularly calpeptin-treated ovarian cancer cells. This likelihood is supported by the observation that ARHI is re-expressed by HDACi (Figure 7A and B, left panel). ARHI is also known to induce autophagy in cancer cells [21] [29]. With these reasons we determined the status of autophagy in HDACi and calpeptin treated CAOV-3 and SKOV-3 ovarian cancer cells. During autophagy, lysosomes fuse with vesicles containing the isolated constituents of the cell to produce phagolysosomes, in which the cargo materials are degraded. Cytoplasmic protein, LC3b, translocates to the phagolysosomal membrane during this process. Detection of LC3b on the phagolysosome membrane by the immuno staining procedure is a measure of autophagy. The translocation of LC3b was portrayed in different patterns after treatment with certain drugs in CAOV-3 and SKOV-3 ovarian cancer cells (Figure 8G and H). In CAOV-3 ovarian cancer cells, the LC3b protein was primarily associated with the phagolysosomes in increased amount after calpeptin treatment and SAHA and calpeptin combination
treatment (Figure 8G). In contrast, in SKOV-3 ovarian cancer cells, both HDACi and calpeptin treated cells portrayed the LC3b protein around phagolysosomes (Figure 8H). Furthermore, the shape of the SKOV-3 ovarian cancer cells were more contorted in an elongated shape as compared to the CAOV-3 ovarian cancer cells. However, in both cell lines, LC3b association was more pronounced in SAHA and calpeptin combination treatments. Untreated controls cells showed a diffused staining of lower intensity in the cytoplasm.

Taken together, the results from cell cycle arrest, apoptosis induction, and induction of autophagy, provided mechanisms of how the number of live ovarian cancer cells decreased with HDACi and calpeptin combination therapy. The data observed on CAOV-3 and SKOV-3 ovarian cancer cells, at given concentrations, suggest overlapping of all these processes in the inhibition of cell growth and in cell death.
Figure 8 A-F. Cell Cycle Inhibition and Apoptosis. A. CAOV-3 ovarian cancer cells were treated for 48 hours with 1 mM sodium butyrate (SB), 10 uM SAHA, and 10 ug/mL calpeptin (Calp) or combination of SB and Calp, and SAHA and Calp, at the same concentrations. B. SKOV-3 ovarian cancer cells were treated for 48 hours with 1 mM SB, 10 uM SAHA, and 10 ug/mL Calp or combination of SB and Calp, and SAHA and Calp, at the same concentrations. The cells were stained with PI and FACS analysis was performed to determine the stages of cell cycle inhibition in each sample. C. CAOV-3 ovarian cancer cells were treated with 1 mM SB, 10 uM SAHA, and 10 ug/mL Calp or combination of SB and Calp, and SAHA and Calp, at the same concentrations. D. SKOV-3 ovarian cancer cells were treated with 1 mM SB, 10 uM SAHA, and 10 ug/mL Calp or combination of SB and Calp, and SAHA and Calp, at the same concentrations. After a 48-hour treatment, the ovarian cancer cells were stained with annexin conjugated to FITC and PI. Apoptosis quantities were measured with flow cytometry and plotted. E. Graph representation of CAOV-3 cancer cell apoptotic percentages from data of C. F. Graph representation of SKOV-3 cancer cell apoptotic percentages from data of D
Figure 8 G-H. Autophagy. G. CAOV-3 ovarian cancer cells were treated for 96 hours with 0.5 mM SB, 2 µM SAHA, 5 µg/mL Calp or combination of SB and Calp, and SAHA and Calp at the same concentrations. H. SKOV-3 ovarian cancer cells were treated for 96 hours with 1 mM SB, 10 µM SAHA, 10 µg/mL Calp or combination of SB and Calp, and SAHA and Calp at the same concentrations. Next, cells were treated with antibodies followed by DAPI addition and incubated. Cells were viewed and photographed with a fluorescent microscope.

5. Effects of HDACi and Calpeptin on the phosphorylation of ERK and Akt

Recent work from our laboratory has revealed that HDACi inhibit ERK phosphorylation in prostate and breast cancer cell lines [27]. ERK activation is known to activate many pro-growth pathways by inducing the expression of genes involved. Research from other laboratories has shown that calpeptin inhibits Akt phosphorylation [30]. Akt activity is important for cell survival and often enhanced in cancer cells. Interestingly, in addition to their role in major signaling pathways, both ERK and Akt have been shown to regulate methylation of genes silenced in cancer cells [10] [21]. To determine whether HDACi and calpeptin inhibit phosphorylation of ERK and Akt in ovarian cancer cells, a western analysis of HDACi and calpeptin treated and untreated ovarian cancer cells was performed. Figure 9A (upper left) showed reduced phosphorylation of Akt in CAOV-3 ovarian cancer cells when the cells were treated in
combination with either HDACi SB or SAHA with calpeptin. In contrast, SKOV-3 ovarian cancer cells did not show much inhibition of phosphorylation of Akt (Figure 9A, upper right).

Analysis of ERK phosphorylation provided different results in CAOV-3 and SKOV-3 ovarian cancer cells. Results from these experiments demonstrated that CAOV-3 cancer cells had decreased phosphorylation of ERK when exposed to SAHA, as compared to the untreated control cells (Figure 9B, lower left). Calpeptin did not produce much decrease in ERK phosphorylation in CAOV-3 cancer cells by itself; however, the combination treatments demonstrated significant inhibition. It is important to note that SB, at the concentration used (1mM) was not as effective as SAHA (10 µM), which is a much better inhibitor. SKOV-3 ovarian cancer cells showed a similar inhibition pattern of ERK phosphorylation as in CAOV-3 cancer cells (Figure 9B, lower right). The lower panel of each figure demonstrate similar loading of Akt and ERK, respectively.
Figure 9. Effects of HDACi and Calpeptin on Akt and ERK phosphorylation. A. CAOV-3 ovarian cancer cells (upper left panel) and SKOV-3 ovarian cancer cells (upper right panel) were treated with 1 mM sodium butyrate (SB), 1 uM SAHA, 20 ug/mL calpeptin (Calp), or combination of SB and Calp, and SAHA and Calp, at the same concentrations. Western analysis was performed for Akt phosphorylation (pAkt) in CAOV-3 and SKOV-3 ovarian cancer cells. B. CAOV-3 ovarian cancer cells (lower left panel) and SKOV-3 ovarian cancer cells (lower right panel) were treated with 1 mM SB, 10 uM SAHA, 20 ug/mL calpeptin (Calp), or combination of SB and Calp, and SAHA and Calp, at the same concentrations. Western analysis was performed for ERK phosphorylation (pERK) in CAOV-3 and SKOV-3 ovarian cancer cells. Blots were stripped and a western blot followed for Akt and ERK, respectively.
DISCUSSION

Epigenetics is a relatively new field in the realm of scientific discoveries. However, since its recognition in the 1980’s, epigenetic drugs have already been approved for certain types of leukemia and T-cell lymphoma’s [16]. Recent clinical trials though, have shown that epigenetic drugs (i.e. DNMTI inhibitors and HDACi) do not work well in solid tumors when used as a single agent [4] [10]. Even the combination of two types of epigenetic drugs is not as effective against solid tumors. Our laboratory hypothesized that the combination of epigenetic drugs with other cytotoxic drugs, such as chemotherapeutics, should be more effective against solid tumors [4] [11] [18] [21]. Our premise was based on the observation that epigenetic drugs, such as HDACi alone or in combination with DNMTI inhibitors, re-express TSG, which should sensitize cancer cells towards other chemotherapeutic agents [21] [31]. In fact, our laboratory and other investigators have determined that HDACi in combination with additional inhibitors are very successful against breast and ovarian cancer cells. One study described HDACi in combination with inhibitory antibody to death receptor 4 (DR4) reduced breast cancer in a xenographed tumor model [31]. Additionally, we previously demonstrated that HDACi combined with telomere analogues GT oligonucleotides were effective against ovarian cancer cells of diverse characteristics [28] [33]. Our laboratory has also established that HDACi in combination with calpeptin induced growth inhibition of both estrogen responsive and triple negative breast cancer cell lines [3].

In this study, we presented results that validated that the combination of HDACi with calpain protease inhibitor, calpeptin, produced enhanced growth inhibition of two
ovarian cancer cell lines of different characteristics, (i) CAOV-3 (TP53 mutated) and (ii) SKOV-3 (TP53 null and highly metastatic). We employed two structurally different Class I and Class II HDACi, sodium butyrate (SB) and SAHA, respectively, to determine that the effects we observed were certainly due to the inhibition of HDAC activity. We chose to utilize calpeptin as the other drug in our combination treatment since calpain has been shown to regulate many signaling pathways, including Akt, which are involved in cellular growth and apoptosis [34]. Additionally, our laboratory and others have previously shown that the inhibition of calpain has anti-thrombotic properties [35] [36]. Calpain plays a significant role in platelet activation and is highly activated during thrombosis [35]. Treatment of ovarian cancer cells with high concentrations of HDACi or calpeptin was capable of inhibiting growth (not shown). We however performed a titration of concentrations for all inhibitors and chose sub-optimal concentrations of each, which do not inhibit the growth significantly when used as a single agent (Figure 5). The reasoning behind these sub-optimal concentrations was to demonstrate that the combination of HDACi and calpeptin produce a greater inhibition of ovarian cancer cell growth than the inhibitors alone, when used at the same sub-optimal doses (Figure 5A and B). Interestingly, the combination doses of both HDACi and calpeptin were lower for CAOV-3 than for SKOV-3 ovarian cancer cells. This is consistent with the fact that SKOV-3 cancer cells are a more aggressive and resistant ovarian cancer cell line, which is difficult to treat. These results additionally suggested that these types of combinations can be used against diverse types of ovarian cancer cell lines and that the effective doses will vary as well. Interestingly, the addition of calpeptin further drastically altered the
morphology of both types of ovarian cancer cell lines (Figure 5C and D). These results suggest the inclusion of calpeptin-induced cell death.

In addition to uncontrollable cell growth, one of the characteristics of malignant tumors is metastasis. The aggressiveness of a tumor is dependent upon how motile the individual cancer cells are. Theoretically, the most effective drugs, in addition to inhibiting growth and promoting cell death, should inhibit metastasis as well. Many available chemotherapeutic agents are not effective against these two characteristics. Some drugs that inhibit metastasis are not effective in inhibiting cancer cell growth, and the opposite is true as well. Figure 6A and B demonstrated that both HDACi and calpeptin inhibited the motility of both CAOV-3 and SKOV-3 ovarian cancer cell lines. Though the individual inhibitors and combinations showed similar effects, we believe that HDACi and calpeptin concentrations used for this experiment were substantial enough to inhibit cell motility even when used as a single agent. To determine whether the combination would produce a more significant outcome, a separate experiment would need to be generated to titrate HDACi and calpeptin and discover the sub-optimal doses. Nonetheless, collectively, the results (Figures 5 and 6) showed that the combination of HDACi and calpeptin inhibited growth and reduced tumor cell motility.

Successively, we further investigated the mechanisms of growth inhibition of these ovarian cancer cells after drug treatments. As described in the introduction, our laboratory has demonstrated that HDACi degrade DNMTI and thus demethylate CpG residues in the upstream region of TSG [21] [28]. Figure 7A and B showed that three TSG: ARHI, p21, and RARβ2 were re-expressed in both CAOV-3 and SKOV-3 ovarian
cancer cell lines after treatment with HDACi SAHA. These three TSG have distinctly different functions. ARHI is an imprinted pro-apoptotic gene, which is similar to RAS, a proto-oncogene, but opposite in function [32]. The maternal allele of ARHI is silenced by methylation in normal breast and ovarian tissue and the gene is expressed from the paternal allele. In 40% of breast and ovarian cancer cells; however, the paternal allele is also silenced by methylation, causing loss of heterozygosity (LOH) [37] [38]. It has been shown that re-expression of ARHI can induce apoptosis and autophagy [32] [37]. p21 is a cell cycle inhibitor that is usually silenced in many types of cancer cells, including ovarian cancers, which facilitates uncontrolled cell-cycle progression. RARβ2 is a marker of differentiation and is silenced by methylation, since rapid growth of tumor cells is antagonistic to differentiation. Theoretically, re-expression of RARβ2 can limit uncontrolled cell growth, the re-expression of p21 can cause cell cycle arrest, and re-expression of ARHI may result in induction of apoptosis and autophagy. As we observed the re-expression of these three genes in ovarian cancer cells after HDACi treatment, we further investigated the effects of these inhibitors on cell cycle progression, apoptosis, and autophagy.

The cell cycle profiles have revealed that HDACi in combination with calpeptin arrested the cell-division cycle at the S-phase in both CAOV-3 and SKOV-3 ovarian cancer cells, whereas calpeptin alone did not produce much inhibition (Figure 8A and B). Since HDACi re-expressed p21 in both CAOV-3 and SKOV-3 ovarian cancer cell lines, these results were expected. We are further not surprised that calpeptin was not as
effective in inhibiting the cell cycle progression. Inhibition of calpain is customarily linked to induction of apoptosis and autophagy [34].

To measure apoptosis in the two ovarian cancer cell lines, we utilized an annexin/PI fluorescent apoptosis assay. The assay essentially examined the binding of annexin to the flipped cellular membrane during the early stage of apoptosis and made the membrane permeable for propidium iodide to enter the cell and bind the DNA of dying cells. The analysis with the assay is more precise when applied in the early phase of apoptosis, when most cells possess the reversed cellular membrane. In the late phase of apoptosis, most dying cells lose their membranes and minimum annexin binding is observed. Therefore, to optimize the time of initial induction of apoptosis is extremely important to examine a “perfect window” of cells to perform this type of assay. Figure 5A and B indicated that HDACi and calpeptin were successful at much lower concentrations to inhibit the growth of CAOV-3 ovarian cancer cells as compared to SKOV3 ovarian cancer cells. Therefore, theoretically, we should have observed increased levels of apoptosis in CAOV-3 ovarian cancer cells. Induction of apoptosis however was more apparent in SKOV-3 ovarian cancer cells as compared to the CAOV-3 ovarian cancer cells (Figure 8C and D). The observed decreased amount of apoptosis is perhaps a reflection of the majority of cells having been already dead when the cells were harvested and the assay was performed. In support of this conclusion, we discovered that the number of live CAOV-3 ovarian cancer cells was much lower when compared to SKOV-3 ovarian cancer cells, under the conditions of the experiment (not shown). Most of the
cells were smaller and rounded in shape, suggesting that they were already progressing through cell death.

Autophagy is a process of degradation of cellular components through phagosomal and lysosomal fusion. During the formation of this phagolysosomal complex, cytoplasmic LC3b protein translocates to the membrane of the phagolysosome complex. Inhibition of calpain is known to cause autophagy [34]. In addition, the drastic change in morphology (Figure 5C and D) after calpain treatment (as a single agent or in combination) and the re-expression of ARHI (Figure 7A and B, left panel) suggested a possible induction of autophagy. To analyze for autophagy, we used an immunofluorescent assay to detect the presence of LC3b on phagolysosomes after HDACi and calpeptin treatment. CAOV-3 ovarian cancer cells illustrated distinct formation of phagolysosomes, which contained fluorescently stained LC3b when calpeptin was used (Figure 8G). In SKOV-3 cancer cells, HDACi also detected LC3b on phagolysosomes, though in a lower amount as compared to the combination treatment. The combination of HDACi and calpeptin revealed the formation of phagolysosomal complexes with fluorescent LC3b protein in both CAOV-3 and SKOV-3 ovarian cancer cells. As discussed in the apoptosis assay, though we expected a larger signal of LC3b protein fluorescence in CAOV-3 cancer cells, the results demonstrated an apparent lower amount translocated, as compared to SKOV-3 cancer cells. We attribute this fact to the same reason that most of the CAOV-3 ovarian cancer cells were already dead and degraded when this assay was performed.
Our laboratory has previously demonstrated HDACi inhibition of DNMTI through the inhibition of ERK kinase in prostate cancer cells [21] [28]. In this study, we investigated whether HDACi and calpeptin have any effect on the ERK kinase phosphorylation in ovarian cancer cells (Figure 9B). HDACi SAHA inhibited ERK phosphorylation in both CAOV-3 and SKOV-3 ovarian cancer cells. SB and calpeptin alone, at the concentrations used, were not as effective. However, the combination treatment indicated the inhibition of ERK phosphorylation in both ovarian cancer cell lines. The inhibition of ERK phosphorylation suggests that in addition to the degradation of DNMTI to demethylate TSG for their re-expression, these drug combinations inhibit an imperative pro-growth signaling pathway mediated by ERK kinase.

Interestingly, inhibition of calpain is known to negatively regulate Akt mediated signaling [30]. Akt is a principal kinase, anti-apoptotic in nature and often constitutively phosphorylated in various types of cancer cells. Inhibition of Akt phosphorylation is associated with induction of apoptosis and initiation of autophagy. Figure 9A illustrated the status of phosphorylated Akt in CAOV-3 and SKOV-3 ovarian cancer cells after HDACi and calpeptin treatments. The inhibition of Akt phosphorylation by calpeptin however, was not as prominent as hypothesized. The concentrations of calpeptin in these experiments were employed at 20 µg/mL. This is a sub-optimal dose for inhibition of calpain. The complete inhibition of calpain activity is usually achieved at 40 µg/mL. Though a much lower dose of calpeptin is sufficient for our combination studies, a much higher dose might be required in a biochemical assay to show complete inhibition of Akt phosphorylation.
In conclusion, this study has demonstrated that combination of HDACi and calpeptin is effective against the growth of ovarian cancer cells, though the inhibitory concentrations vary for CAOV-3 and SKOV-3 ovarian cancer cell lines. The apparent reduction of viable cells was a result of cell cycle arrest, induction of apoptosis and autophagy. We observed the re-expression of three epigenetically silenced TSG: ARHI, p21, and RARβ2 designating their roles in the above processes. In addition to regulating methylation process, the inhibition of ERK and Akt kinases presumably played a role in growth inhibition and induction of apoptosis and autophagy. Regulation of epigenetic silencing of TSG by ERK and Akt is a current focus of our laboratory, however it is beyond the scope of this thesis proposal. These findings provide an understanding of how these inhibitors are involved in upregulating different pathways, which block cancer cell growth and induce apoptosis and autophagy. Our results suggest that the combination of epigenetic drugs with other cytotoxic agents can generate improved therapies and treatment for ovarian cancer.
REFERENCES


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