
Theses and Dissertations

Summer 2011

The effect of valproic acid on histone acetylation in FaDu-luc head and neck squamous cell carcinoma cells

Ali Pourian

University of Iowa

Copyright 2011 Ali Pourian

This thesis is available at Iowa Research Online: <http://ir.uiowa.edu/etd/1256>

Recommended Citation

Pourian, Ali. "The effect of valproic acid on histone acetylation in FaDu-luc head and neck squamous cell carcinoma cells." MS (Master of Science) thesis, University of Iowa, 2011.
<http://ir.uiowa.edu/etd/1256>.

Follow this and additional works at: <http://ir.uiowa.edu/etd>



Part of the [Other Dentistry Commons](#)

THE EFFECT OF VALPROIC ACID ON HISTONE ACETYLTATION IN FADU-LUC
HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS

by
Ali Pourian

A thesis submitted in partial fulfillment
of the requirements for the Master of
Science degree in Stomatology
in the Graduate College of
The University of Iowa

July 2011

Thesis Supervisor: Professor Frederick E. Domann

Copyright by
ALI POURIAN
2011
All Rights Reserved

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Ali Pourian

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Stomatology at the July 2011 graduation.

Thesis Committee:

Frederick E. Domann, Thesis Supervisor

Steven D. Vincent

Michael W. Finkelstein

To my wife Arshin,
who is always there for me whether she knows it or not.
To my father Habib,
who is always my inspiration for attaining higher education.
To my mother Fatimah,
who made me the person I am today.

ACKNOWLEDGMENTS

First I would like to thank Dr. Sherry Timmons, Dr. Christopher Squier, and Sara Anstoetter for bringing my attention to and helping me attain the NIH T32 grant that financially supported both me and the project. Without their help, none of this would be possible. Additionally, I would like to thank Dr. John Hellstein, and Dr. Timmons for their advice on my project and on the research process in general.

I would like to thank the members of my thesis committee for their intellectual support and direction in this project: Dr. Steven Vincent, Dr. Michael Finkelstein, and especially my thesis supervisor Dr. Frederick Domann. Dr. Domann went far beyond his responsibility as a mentor, supervisor, and advisor to accommodate my situation and help me finish this project. Beyond this, his excitement and passion for science was infectious and serves as inspiration in my future scientific endeavors.

I have sincere appreciation to everyone in the Domann lab that served as friends, mentors, and counselors. These include Dr. Joshua Madsen, Dr. Adam Case, Dr. Laura Wegman, Anthony Cyr, Trenton Place, and Matthew Fitzgerald. They guided me through obstacles that I did not even know existed. Special and heartfelt thanks are due to Dr. Madsen. For reasons still unbeknownst to me, Dr. Madsen helped me with unselfishness, kindness, patience, and genuineness in ways that are unparalleled in my life. This project would have been impossible without his help.

I would also like to thank my co-residents at the dental college for covering my duties and supporting me while I was in the lab. Dr. Nidhi Handoo, Dr. Rujuta Katkar, Dr. Gayle Reardon, Dr. Chelsia Sim, are thanked for this dubious task.

Finally, I would like to thank Dr. Andresa Borges Soares for support and advice during this process. Despite the distance, she was and always will be close.

TABLE OF CONTENTS

LIST OF TABLES	iv
LIST OF FIGURES	v
CHAPTER 1 INTRODUCTION	1
Cancer Overview	1
Epigenetics	2
DNA Methylation	3
Histone Acetylation	8
Valproic Acid	10
Aim	11
Hypothesis	11
CHAPTER 2 MATERIALS AND METHODS	12
Cell Culture and VPA Treatment	12
Histone Extraction	12
Histone Precipitation	13
Histone Quantification	13
Western Blot Analysis	14
Statistics	15
CHAPTER 3 RESULTS	16
CHAPTER 4 DISCUSSION	25
Significance	26
Future Directions	27
Conclusion	28
BIBLIOGRAPHY	29

LIST OF TABLES

Table 1: Chemical composition of running and stacking gels.....	14
Table 2: Protein ratio quantities of acetylated H3 to H3 levels. Samples were normalized to mock levels.	20
Table 3: Protein ratio quantities of acetylated H3 at lysine position 9 to H3 levels.	20
Table 4: ANOVA analysis of acetylated H3 protein showing a p value of 0.309.....	23
Table 5: ANOVA analysis of acetylated H3 lysine at position 9 protein showing a p value of 0.297.....	24

LIST OF FIGURES

Figure 1: DNA methylation effects on transcription..	5
Figure 2: Schematic representation of the nucleosome..	7
Figure 3: Schematic of histone acetylation and deacetylation.....	9
Figure 4: Chemical structure of 2-propylpentanoic acid (valproic acid).....	10
Figure 5: Western Blot Experiment 1.....	17
Figure 6: Western Blot Experiment 2.....	18
Figure 7: Western Blot Experiment 3.....	19
Figure 8: Plot of acetylated H3 levels to overall H3 levels with standard deviation bars.....	21
Figure 9: Plot of acetylated H3 levels to overall H3 levels with standard deviation bars.....	22

CHAPTER 1

INTRODUCTION

Cancer Overview

Cancer is a heterogeneous group of diseases characterized by uncontrolled cell growth, invasion with destruction of adjacent tissue, and sometimes metastasis, or spreading to other bodily locations via lymph or blood. If untreated, this uncontrolled growth and spread may result in death. It is estimated that over 1,529,000 non-dermal related cancers will be diagnosed in the United States.¹ Of these, it is estimated that approximately 570,000 will succumb to the disease, making cancer the second most common cause of death in the United States, second only to heart disease.²

Head and neck cancers are a related group of cancers that involve the oral cavity, pharynx and larynx. Globally, approximately 600,000 new cases of head and neck cancers are diagnosed yearly, resulting in approximately 300,000 deaths.¹ This ranks head and neck cancer as the sixth most common cancer worldwide, and third among developing countries.^{3,4} The majority of head and neck cancers are squamous cell carcinoma in histology, with approximately 94% of all oral malignancies being squamous cell carcinoma.⁵

Like most cancers, the cause of head and neck cancers are multifactorial. No single causative agent or factor (carcinogen) has been clearly defined or accepted. This has lead many investigators to propose the concept of cocarcinogenesis, in which extrinsic and intrinsic factors are needed to produce a malignancy.⁶ Tobacco smoking is the greatest extrinsic factor for head and neck cancer, with a higher risk for heavy and chronic smokers. Consumption of alcoholic beverages also increases the risk, with heavy drinkers having a tenfold risk. The combination of smoking and alcohol seems to have synergistic effect, further increasing the risk.⁶

Other extrinsic risk factors include betel quid and acra nut in India and Taiwan^{7,8}, inorganic acid mists containing sulfuric acid and mustard gas produced in manufacturing processes⁹, as well as poor oral health and frequent use of mouthwash.¹⁰ Large epidemiological studies have also shown human papilloma virus (HPV) as a causative agent of some head and neck cancers. HPV markers have been by identifying HPV DNA in biopsy tissues via polymerase chain reaction (PCR) methods, antibodies to HPV 16 capsids analyzed with enzyme linked immunosorbent assay (ELISA), and antibodies to HPV 16 E6 and E7 analyzed by ELISA.

Epigenetics

While the majority of extrinsic and intrinsic factors directly effect the genome, the study of cancer epigenetics has reached mainstream oncology.¹¹ The field of epigenetics is an expanding and promising field that has expanded our knowledge on the importance of epigenetic events in the control of both normal cellular processes and abnormal events associated with diseases. Conrad Waddington coined the term “epigenetics” in 1942 to describe the discipline in biology that studies “the interactions of genes with their environment that bring the phenotype into being.”¹² This definition initially referred to the role of epigenetics in reference to embryonic development only; however, since that time, it has evolved and implicated in a number of biological events that are not coded in the DNA sequence itself, namely imprinting, position-effect variegation, paramutations, and X-chromosome inactivation.¹³

The current definition of epigenetics is “the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence”. Thus, in the broadest sense, epigenetics represents an interface between genotype and phenotype in that it embodies the mechanism that modifies the final outcome of the genetic code without altering the code itself. The importance and validity of epigenetics is evident by

the fact that all cells in any given organism share an identical genome, yet have specific functional and morphological characteristics.

Expanding on this principle that epigenetics is a conduit between genotype and phenotype, epigenetics can be viewed as the changes that are stably transmitted over many rounds of cell division, but that do not alter the nucleotide sequence. Major epigenetic mechanisms can be divided into four main categories: DNA methylation, covalent histone modifications, non-covalent mechanisms such as incorporation of histone variants and nucleosome remodeling, and non-coding RNAs including microRNAs (miRNAs).¹³ It is these mechanisms that allow such a stable propagation of gene activity states from one generation of cells to the next. This epigenetic activity is essential for embryonic development, cell differentiation, and could be the mechanism of integration of endogenous and environmental signals during the life of an organism.¹⁴⁻¹⁶ Analogously, it is important to note that the deregulation of epigenetic states is intimately linked to human diseases, most notably cancer.¹⁵⁻¹⁷

DNA Methylation

DNA methylation and histone acetylation and methylation are the major epigenetic modifications that are most intensively studied in the context of gene transcription and abnormal events that lead to oncogenesis.^{15,17} Once the epigenetic modifications have been established, they are autonomously propagated through cell lines. It has been shown that disruption of one of these two epigenetic mechanisms inevitably affects the other.¹⁸⁻²¹ For example, hypermethylation of CpG islands in gene promoters triggers deacetylation of local histones, whereas lower levels of histone acetylation (hypoacetylation) seem to predispose to target DNA methylation. Therefore, there appears to be an intimate relationship between the mechanisms. However, the overlying hierarchical order of epigenetic events during gene silencing in tumor cells is yet to be determined.

DNA methylation is perhaps the most extensively studied epigenetic mechanism.¹³ The methylation of DNA refers to the covalent addition of a methyl group to the 5-carbon (C⁵) position of cytosine bases, yielding 5-methylcytosine (5-mC). While this does not alter the DNA sequence directly, it may have major consequences on gene regulation by acting as a stable gene silencing mechanism. In mammals, DNA methylation occurs in regions where a cytosine nucleotide occurs next to a guanine nucleotide in a linear sequence. These “CpG” (-C-phosphate-G-) dinucleotides are not randomly distributed within the genome, but rather tend to occur in CpG-rich stretches (~300-3000 base pairs in length) representing “CpG islands”. These regions of repetitive sequences are found in centromeres, retrotransposon elements, and rDNA.^{22,23} Notably, these islands are preferentially located at the 5’ end of genes and occupy ~60% of human gene promoters.²⁴

From a regulatory standpoint, DNA methylation serves as a repressive mechanism inhibiting gene transcription. This primarily occurs through two mechanisms. The first involves direct interference with transcription factor binding. Methylation of CpG nucleotides prevents normal binding of these proteins to DNA, and thus inhibits gene transcription. (Figure 1-1) The second mechanism involves the recruitment and binding of specialized methyl-DNA binding proteins. These proteins bind to 5-mC bases and serve as a protein scaffold that recruits transcription repression factors, as well as families of histone modifying enzymes.

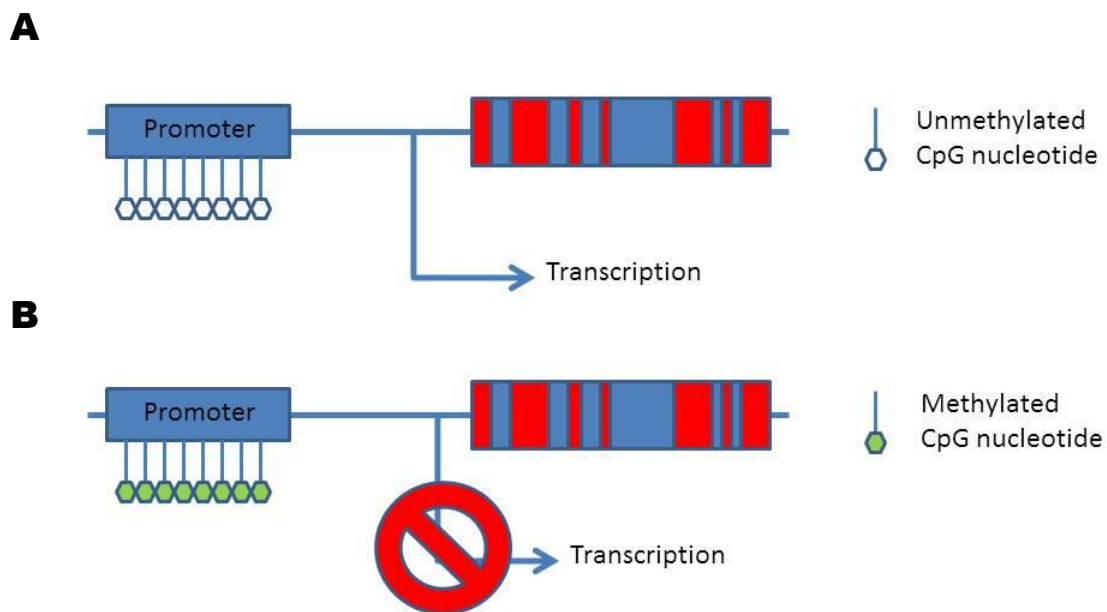


Figure 1: DNA methylation effects on transcription. A) The CpG islands in the promoter region are unmethylated allowing for the binding of transcription factors and permitting transcription of the gene to occur. B) The CpG islands are methylated and thus block the binding of transcription factors in effect silencing gene expression.

Aberrant DNA methylation is closely connected to a wide variety of human malignancies.²⁵⁻²⁹ Two forms of aberrant DNA methylation are found in human cancer: the overall loss of 5-methyl-cytosine (global hypomethylation and gene promoter-associated (CpG island-specific) hypermethylation).^{15,17} When hypermethylated, gene promoters become unable to bind the factors that are responsible for gene expression. The gene thus becomes inactivated. A large number of studies indicate that the silencing of tumor suppressor genes and other cancer-related genes may occur though

hypermethylation of their promoters. Unscheduled hypermethylation of gene promoters represents an attractive target for early diagnosis, risk assessment, and cancer prevention.³⁰⁻³³

Genomic DNA is packaged into a highly compacted DNA-protein complex, called chromatin. It functions to pack DNA into a smaller volume within the cell structure, to strengthen the DNA, coordinate mitosis, and to serve as a control of gene expression and DNA replication. This DNA-protein complex is often divided into two distinct forms: heterochromatin, a condensed and highly repressive form, and euchromatin, a more “relaxed” form that can be generally permissive for transcription. Chromatin is made of repeating units of nucleosomes, which consists of ~146 base pairs of DNA wrapped 1.6 times around an octamer of four core histone proteins. This octamer is composed of two H3-H4 histone dimers bridged together as a stable tetramer that is flanked by two separate H2A-H2B dimers. (Figure 1-2) The addition of other factors, such as linker histone H1 and nonhistone chromatin proteins, results in higher order chromatin organization and compaction³⁴⁻³⁶ Histone proteins are composed of a structured globular central domain by which they interact together and that is close contact to DNA and a less structured amino-terminal domain^{35,36} Each of the four core histones contains 20-30 amino acids in their N-terminal domain, referred to as the “tail”. Histone tails extend from the surface of the nucleosome and are sites for posttranslational modifications. Various histone modifications have been identified including acetylation, phosphorylation, methylations, ubiquitination, sumoylation, ADP-ribosylation, demination, proline isomerization, and propionylation.^{37,38} Combination of different histone modifications can dictate the status of a gene by changing the chromatin structure, thus regulating transcription, DNA replication, and DNA repair.³⁹ Site-specific combinations and interdependence of different histone modifications form the so-called “histone code”.

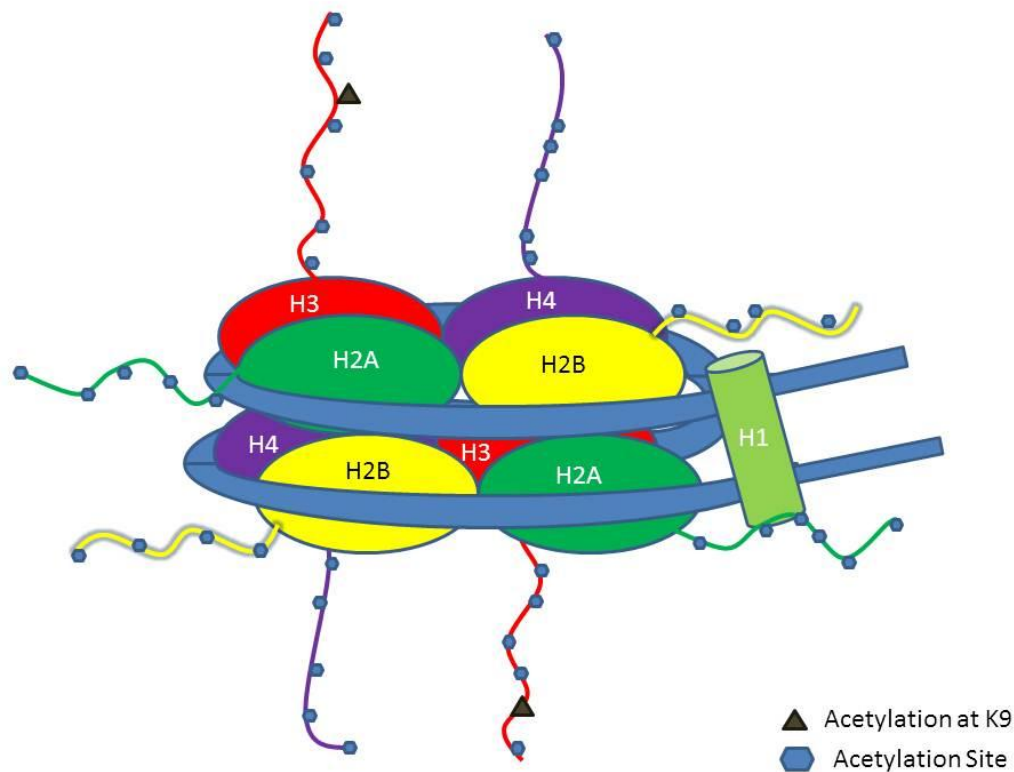


Figure 2: Schematic representation of the nucleosome. DNA is wrapped around histone octamer of the four core histones H2A, H2B, H3, and H4. Histone H1, the linker protein, is bound to DNA between nucleosomes. Different amino acids constituting histone tails are represented along with acetylation sites (hexagon). At position 9 of the H3 tail, acetylation of lysine may also occur.

Histone Acetylation

Acetylation is a reaction that introduces an acetyl functional group into a chemical compound. Specifically, it involves the substitution of an acetyl group for an active hydrogen atom. Acetylation of lysine residues on histone tails is controlled via two types of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs use acetyl-CoA to transfer an acetyl group on the N-terminal tails of histones⁴⁰, while HDACs reverse this modification.^{41,42} Most HATs come in the form of large multiprotein complexes with various complexes ensuring locus targeting and chromosomal-domain and substrate-specificity.⁴³ Similarly, HDACs are also composed of large multiprotein complexes. The dynamic equilibrium, and ultimately gene availability and expressivity, is governed by the opposing actions of HATs and HDACs.

Within the histone octamer, positively charged histone tails protrude out from the central domain of the nucleosome (Figure 1-2) and are believed to bind the negatively charged DNA through charge interactions or mediate interactions between nucleosomes contributing in chromatin compaction.^{36,44} Lysine acetylation is believed to neutralize the positive charge of histone tails, weakening histone-DNA⁴⁵ or nucleosome-nucleosome interactions.^{44,46} This results in destabilizing nucleosome and chromatin structure, thus facilitating access to the DNA for different nuclear factors, such as the transcription complex. Thus, hyperacetylation of histones is considered a hallmark of transcriptionally active chromatin. Deacetylation of histones by HDACs results in a decrease in the space between the nucleosome and the DNA, leading to a closed (heterochromatin-like) chromatin conformation that diminishes accessibility for transcription factors. (Figure 1-3) In this way, HDACs can influence the epigenetic status of a cell, thereby changing gene expression patterns in the absence of mutations to the genome itself.

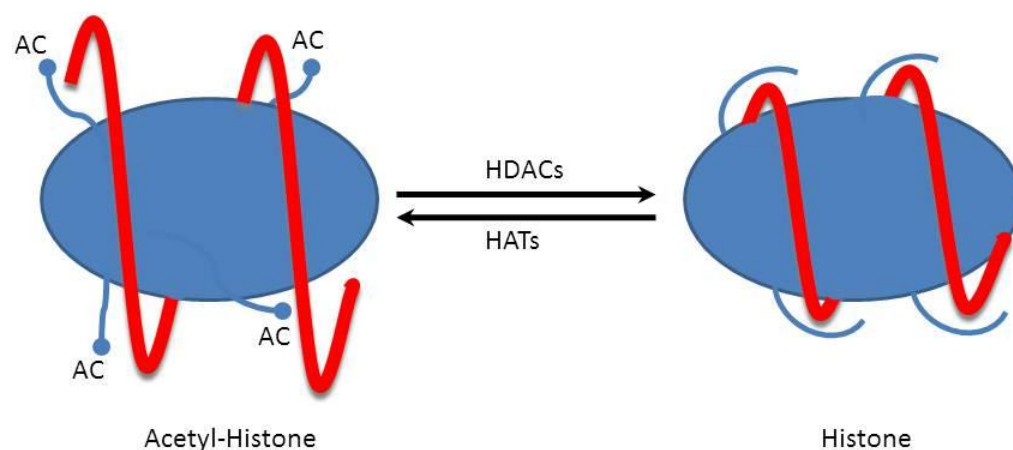


Figure 3: Schematic of histone acetylation and deacetylation. These reactions are catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. Acetylation brings in a negative charge, acting as a repelling force to the negatively charged DNA. As a consequence, the condensed chromatin is transformed into a more relaxed structure which allows for gene transcription. HDAC activity reverses this relaxation, and in effect blocks transcription.

It has long been known and well documented that aberrant gene regulation and ultimately, aberrant gene expression, are the hallmark features of neoplastic cells. Therefore, pharmacological manipulation methods of the epigenome have been investigated, with considerable effort to develop HDAC inhibitors (HDACi). A number of preclinical studies have demonstrated that HDAC inhibitors have been found to have potent anticancer activities, with remarkable tumor specificity, and some have demonstrated promising therapeutic potential.^{47,48} HDAC inhibitors under current development include benzamides, hydroxamic acids, epoxyketones, as well as small

molecular weight carboxilates such as sodium butyrate, sodium penylbutyrate, and pivaloyloxymethyl butyrate, and valproic acid.

Valproic Acid

Valproic acid (VPA) is a branching chain alkyl carboxylic acid with the chemical formula $C_8H_{16}O_2$ (Figure 1-4). It is an analogue of the straight chain valeric acid ($C_5H_{10}O_2$), which is found naturally in the perennial flowering plant valerian (*Valeriana officinalis*). Valproic acid was thought to be physiologically inert until 1963 when it was used as a molecular carrier that prevented pentylenetetrazol-induced convulsions in rodents.⁴⁹ In the human brain, VPA increases the function of the neurotransmitter gamma-Aminobutyric acid (GABA) by increasing synthesis of GABA, inhibiting GABA degradation and decreasing GABA turnover.⁵⁰ Moreover, VPA blocks Na^+ channels, Ca^{2+} channels, and voltage-gated K^+ channels.⁵¹ Clinically, VPA can be used as an anticonvulsant and moodstabilizing drug, but also to control absence seizures, tonic-clonic seizure, complex partial seizures, seizures associated with Lennox-Gastaut syndrome, and schizophrenia.

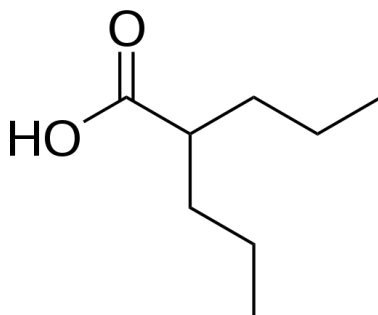


Figure 4: Chemical structure of 2-propylpentanoic acid (valproic acid).

Additionally, VPA has been noted for its HDAC inhibitory abilities, which allows it to alter expression of genes. It has also been shown that VPA has the ability to induce

methylation of histone 3, with this occurring primarily at lysine 9.⁵²⁻⁵⁴ Methylation at this histone site is associated with increased transcriptional activity. Thus, the use of VPA as an epigenetic drug is encouraging because it targets chromatin through associated proteins and may be applicable to a wide range of pathologic processes, especially cancer. Specifically epigenetic drugs could be used to target repressed tumor suppressor genes. To date, VPA's antitumor effects have been used in pre-clinical studies to treat glioma,⁵⁵⁻⁵⁷ neuroblastoma,^{57,58} breast cancer,^{59,60} colon cancer,⁶¹⁻⁶³ prostate cancer,^{64,65} thyroid cancer,⁶⁶⁻⁶⁸ cervical cancer,⁶⁹ melanoma,⁷⁰ ovarian cancer,⁷¹ as well as numerous other malignancies. Further, HDAC inhibitors have been introduced in both phase I and phase II clinical trials promising results.⁷²⁻⁷⁶ At this time, valproic acid is used solely or in conjunction with other drugs in over 200 clinical trials.

Aim

For all the signaling pathways modulated and clinical trials conducted, the effects of VPA on head and neck squamous cell carcinoma lines have not been investigated. The aim of this study is to determine the effects of VPA on histone acetylation on FaDu-luc head and neck squamous cell carcinoma cell line.

Hypothesis

There is a direct relationship between VPA administration and histone acetylation. The null hypothesis (H_0) being that VPA will not have an affect on histone acetylation, and the alternative hypothesis (H_a) is that there is a difference in the means.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture and VPA Treatment

Human squamous cell carcinoma FaDu-luc cells were grown in suspension in Dulbecco's modified Eagle's medium (DMEM) low-glucose medium with 2mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin-streptomycin antibiotic in a fully humidified atmosphere of 95% air and 5% CO₂ (v/v) at 37°C.

VPA was prepared as a 100mM stock solution in water and stored at -20°C. Cells were maintained in exponential growth phase until experiments were performed with cells that were plated at a cell density of 5×10^5 cells per 100mm dish. Cells were then allowed to grow for 24 hours before treatments. In complete DMEM media, VPA was then added in concentrations of 0.02mM, 0.1mM, 0.5mM, 2.5mM, and 12.5mM and incubated at 37° for 48 hours with no additional drug given.

Histone Extraction

Following incubation, cells were harvested and histones were extracted using the following protocol.

- 1) Cells were harvested via scraping from plates with phosphate buffered saline (PBS) and centrifuged at 500 x g at 4°C, obtaining a pellet.
- 2) The pellet was washed again with 10mL PBS and spun again. The pellet was re-suspended in 1 mL hypotonic lysis buffer and transferred to 1.5mL tubes.
- 3) The tubes were then placed on a rotator at 4°C for 30 minutes to promote hypotonic swelling of cells and lysis by mechanical shearing during rotation.
- 4) A pellet was then obtained by spinning on a tabletop centrifuge at 10,000x g for 10 minutes at 4°C.

- 5) The supernatant was discarded, and this process was repeated with another 1 mL of lysis buffer and rotated for an additional 30 minutes.
- 6) The supernatant was again discarded, and the remaining pellet was re-suspended in 800 μ L 0.4 N H₂SO₄.
- 7) The suspension is vortexed and placed on rotator overnight.
- 8) Nuclear debris was removed by spinning samples in tabletop centrifuge at for 10 minutes at 16,000 x g at 4°C.

Histone Precipitation

- 1) The supernatant was transferred to 1.5mL tubes and 132 μ L of 100% (trichloroacetic acid) (TCA) was added drop by drop.
- 2) The solution was cooled on ice for 30 minutes.
- 3) The suspension was then centrifuged for 10 minutes at 16,000 x g at 4°C.
- 4) The supernatant was removed and the remaining pellet was washed with 1mL 100% ice-cold acetone.
- 5) The pellet was again centrifuged for 10 minutes at 16,000 x g at 4°C.
- 6) The pellet was again washed and centrifuged (repeating steps 4 and 5).
- 7) The histone pellet was air dried for 20 minutes at room temperature and then dissolved with tris buffer 10mM, pH 8.

Histone Quantification

The concentration of each sample was obtained using a Bradford protein assay.

The procedure is as follows:

- 1) Bovine serum albumin (BSA) at a concentration of 10mg/mL was diluted 1:10 to obtain a final concentration of 1mg/mL.
- 2) A standard curve was developed using BSA with concentrations of 0, 1, 2, 4, 8, and 16 μ L in water with a total volume of 800 μ L for each sample.

- 3) Duplicate histone samples were mixed with 200 μ L of Bradford reagent (BioRad Protein Assay Dye Reagent).
- 4) Spectrophotometer readings were taken at 595 nm.
- 5) Standard curves were constructed plotting the 595 nm values (y-axis) versus their concentration in μ g/mL.

Western Blot Analysis

Proteins quantified on the Bradford assay were run on a SDS-PAGE gel. H3, acetylated H3 (acH3), and acetylated H3 only lysine position 9 (acH3-K9) were run for separation of different-sized proteins. The procedure is as follows:

- 1) 12% gels were constructed with the following recipe (Table 1):

Running Gel	Stacking Gel
10.35ml ddH ₂ O	3.1ml ddH ₂ O
7.5ml 4X running buffer (80% H ₂ O, 10% 10X buffer, 10% SDS)	1.25ml 4X stacking buffer (70% H ₂ O, 10% 10X buffer, 20% methanol)
12ml acrylamide	0.67ml acrylamide
0.15ml ammonium persulfate*	0.025ml ammonium persulfate*
0.02ml TEMED* (tetramethylethylenediamine)	0.005ml TEMED*
*APS and TEMED added just before pouring of the gels	

Table 1: Chemical composition of running and stacking gels.

- 2) Proteins were run for separation for 1 hour at 120V.

- 3) The proteins were transferred from the gel to a nitrocellulose membrane for 1 hour at 100V.
- 4) After transfer, the H3 and acH3 nitrocellulose membranes blocked with Tri Buffered Saline (TBS – 1.8L H₂O, 100mL Tris pH 8.0, 100 mL 3M NaCl) and 5% milk. The acH3-K9 nitrocellulose membrane was blocked with TBS and 5% BSA. All membranes were blocked for 1 hour.
- 5) Primary antibodies in concentrations of 1:2000 were applied to membranes overnight. Antibodies used were:
 - Rb pAb α H3 total, ab 1791, Lot 668712, (Abcam)
 - Rb α acH3 ab06599, Lot DAM1422332, (Upstate)
 - Rb pAb α H3K9, ab 4481, Lot 677405, (Abcam)
- 6) Membranes were washed, and goat versus rabbit IgG horseradish- peroxidase-tagged secondary antibodies in concentrations of 1:50,000 were applied to membranes for 1 hour.
- 7) Chemiluminescent substrate was added to the blot for exposure of products, and film was exposed and qualitatively quantified for protein amount.

Statistics

Data are expressed as means and standard deviation. All experiments were performed at least three different cell cultures. Significance between drug levels was analyzed by analysis of variance (one way). A *p*-value of less than 0.05 was considered significant.

CHAPTER 3

RESULTS

A total of three experiments examining the effect of VPA at various concentration levels (0.0 mM (mock), 0.02 mM, 0.1 mM, 0.5 mM, 2.5 mM, 12.5 mM) on histone acetylation. Specifically, acetylated H3 (acH3) levels and acetylated H3 levels at lysine position 9 (acH3K9) were examined and compared with the overall histone levels. The findings demonstrated the following:

1. Treatment with VPA shows a qualitative trend in accumulation of acetylated histones.
2. This positive relationship is dose dependent.
3. A one-way ANOVA model was created for each drug level. No statistically significant difference on histone acetylation with VPA treatment was found (p=0.309 acH3, p=0.296)

Western blot analysis was used to detect specific proteins by using gel electrophoresis to separate acetylated H3 and acetylated H3 K9 proteins. After transfer to a nitrocellulose membrane, specific secondary antibodies marked these proteins. Chemiluminescent detection was used to capture a digital image of the Western blot. (Figures 5, 6, and 7) This image was analyzed by densitometry, yielding quantities based on optical density. Quantities were normalized in relation to mock levels (Table 2 and Table 3), and there was found to be a direct relationship with VPA levels and histone acetylation (Figure 3-4). Levels of acetylated H3 and acetylated H3 K9 were compared to total H3 (figure 3-5)

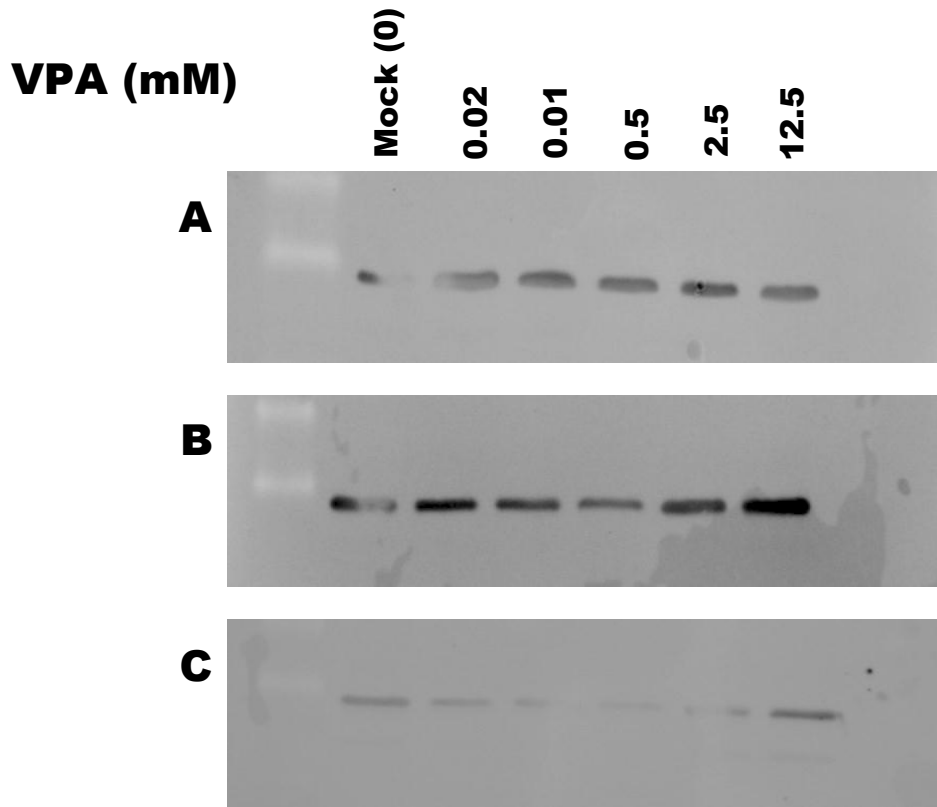


Figure 5: Western Blot Experiment 1. A) H3 protein levels: Consistent (same intensity bands) at various levels of VPA treatment. B) Acetylated H3 protein levels: Protein increases as VPA levels increase, with the highest protein levels (darkest band) at 12.5 mM of VPA. C) Acetylated H3 at lysine position 9: Protein levels increase as VPA level increase, with the highest protein levels (darkest intensity bands) at 12.5 mM of VPA.

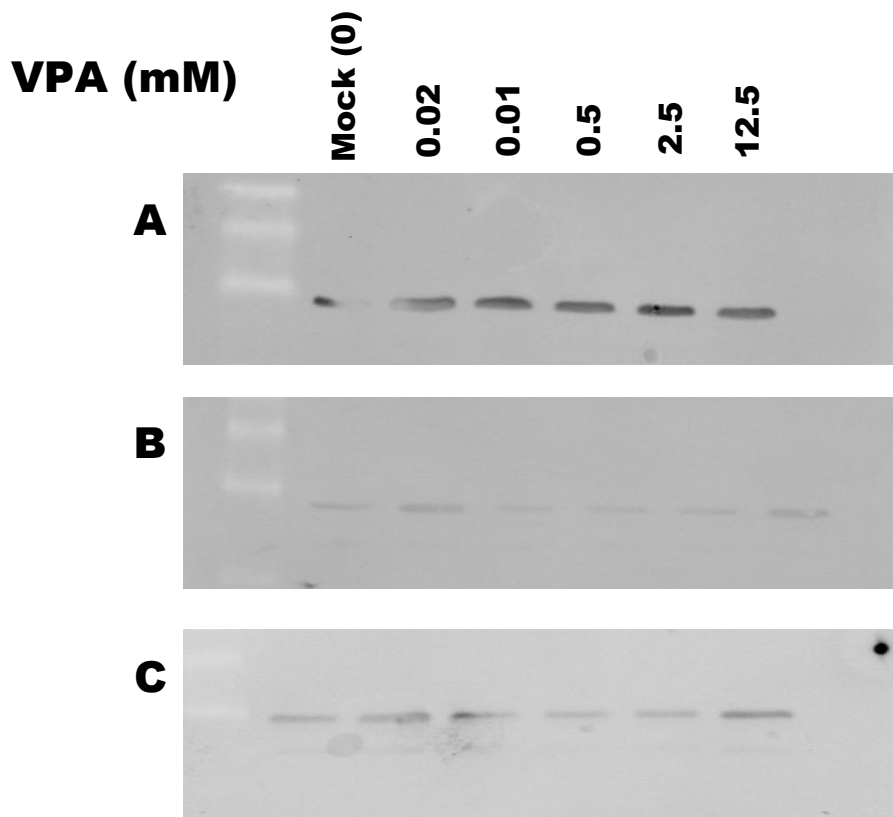


Figure 6: Western Blot Experiment 2. A) H3 protein levels: Consistent (same intensity bands) at various levels of VPA treatment. B) Acetylated H3 protein levels: Protein increases as VPA levels increase, with the highest protein levels (darkest band) at 12.5 mM of VPA. C) Acetylated H3 at lysine position 9: Protein levels increase as VPA level increase, with the highest protein levels (darkest intensity bands) at 12.5 mM of VPA.

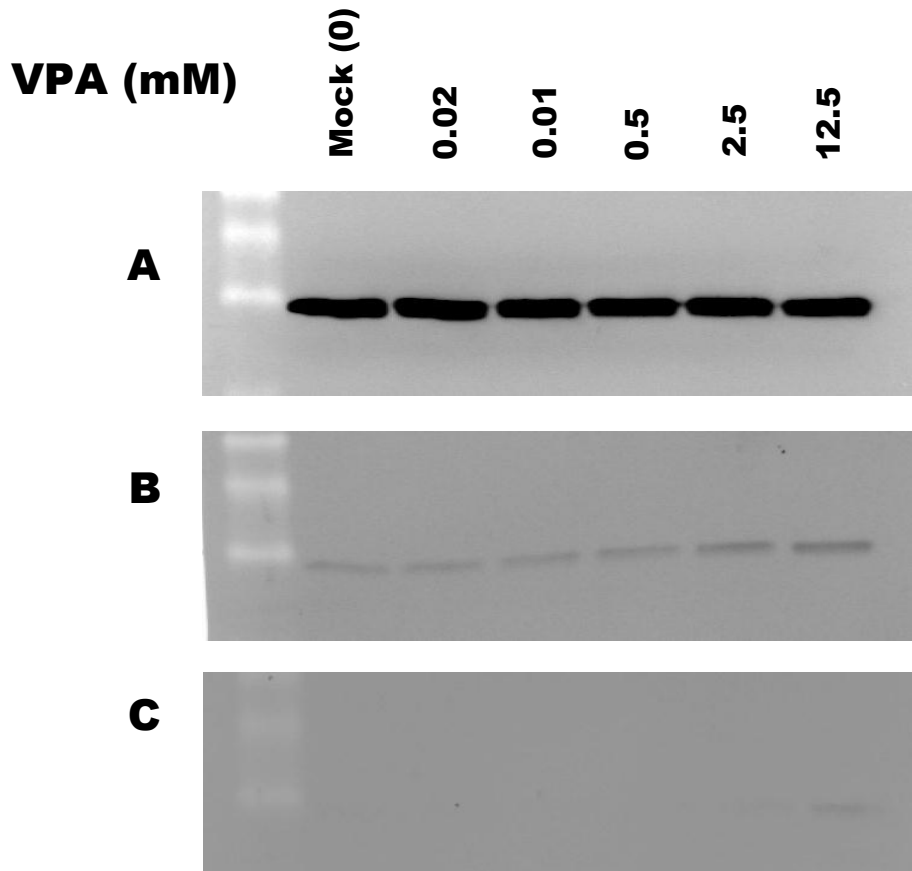


Figure 7: Western Blot Experiment 3. A) H3 protein levels: Consistent (same intensity bands) at various levels of VPA treatment. B) Acetylated H3 protein levels: Protein increases as VPA levels increase, with the highest protein levels (darkest band) at 12.5 mM of VPA. C) Acetylated H3 at lysine position 9: Protein levels increase as VPA level increase, with the highest protein levels (darkest intensity bands) at 12.5 mM of VPA.

AcH3/H3						
Experiment	Mock (0mM)	0.02 mM	0.1 mM	0.5 mM	2.5 mM	12.5 mM
1	1	0.801	1.212	1.113	1.492	1.627
2	1	1.001	0.697	0.902	0.847	1.083
3	1	1.199	1.191	1.059	1.064	1.491
Average	1	1.000	1.033	1.025	1.134	1.400
SD	0	0.199	0.291	0.110	0.328	0.283

Table 2: Protein ratio quantities of acetylated H3 to H3 levels. Samples were normalized to mock levels.

AcH3K9/AcH3						
Experiment	Mock (0mM)	0.02 mM	0.1 mM	0.5 mM	2.5 mM	12.5 mM
1	1	0.943	0.998	1.233	1.302	0.943
2	1	0.609	0.683	0.950	1.014	1.076
3	1	0.882	0.901	0.833	0.960	0.946
Average	1	0.811	0.860	1.005	1.092	0.988
SD	0	0.178	0.161	0.206	0.184	0.076

Table 3: Protein ratio quantities of acetylated H3 at lysine position 9 to H3 levels. Samples were normalized to mock levels.

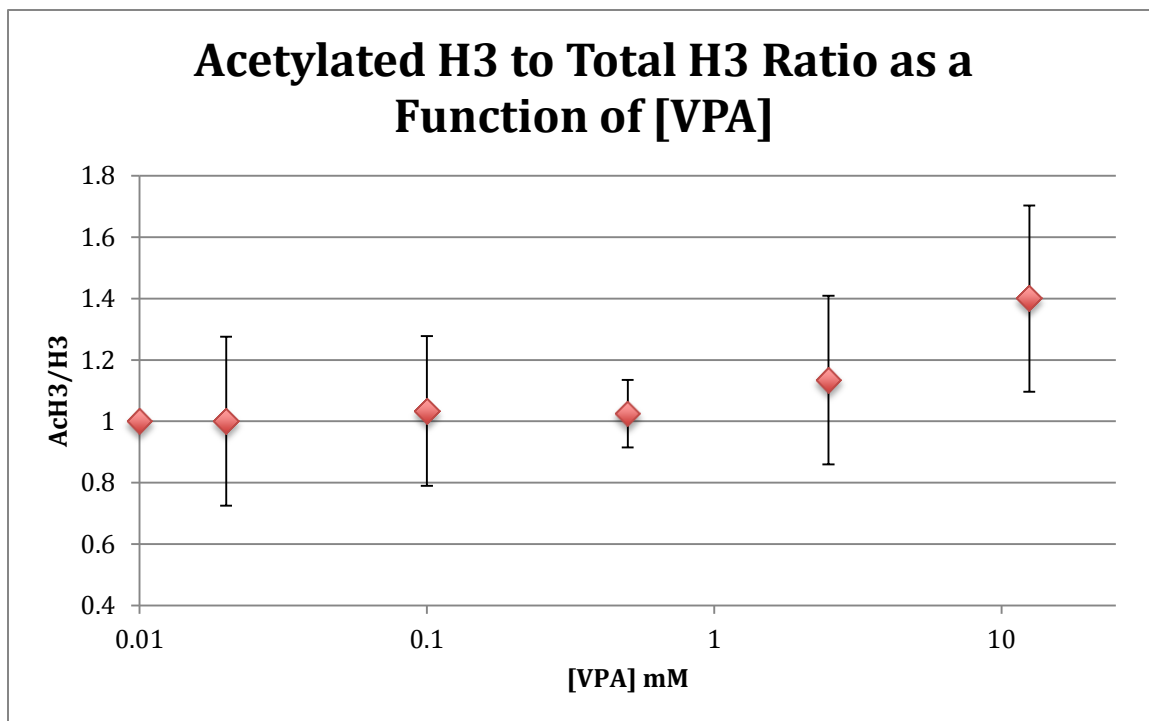


Figure 8: Plot of acetylated H3 levels to overall H3 levels with standard deviation bars. There is a trend between acetylation and higher levels of VPA concentration.

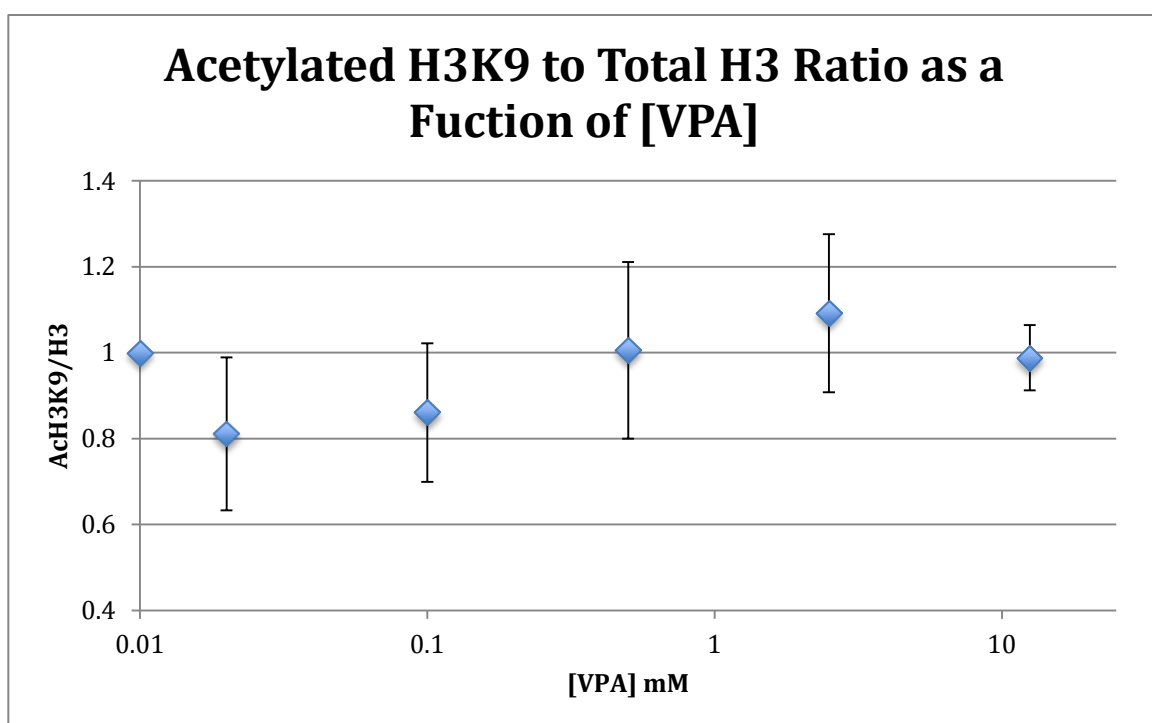


Figure 9: Plot of acetylated H3 levels to overall H3 levels with standard deviation bars. There is a trend between acetylation and higher levels of VPA concentration.

Analysis of Variance (One-Way) AcH3/H3						
<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
<i>Mock</i>	3	3.	1.	0.E+0		
<i>0.02</i>	3	3.001	1.000	0.039		
<i>0.1</i>	3	3.100	1.033	0.084		
<i>0.5</i>	3	3.074	1.024	0.012		
<i>2.5</i>	3	3.402	1.134	0.107		
<i>12.5</i>	3	4.201	1.400	0.080		
ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
Between Groups	0.36408	5	0.07282	1.34796	0.30976	4.16175
Within Groups	0.64823	12	0.05402			
<i>Total</i>	1.01231	17				

Table 4: ANOVA analysis of acetylated H3 protein showing a p value of 0.309.

Analysis of Variance (One-Way) acH3K9/H3							
Summary							
	<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
	0.001	3	3.	1.	0.E+0		
	0.02	3	2.433	0.811	0.031		
	0.1	3	2.581	0.860	0.026		
	0.5	3	3.016	1.005	0.042		
	2.5	3	3.276	1.092	0.033		
	12.5	3	2.964	0.988	0.005		
ANOVA							
	<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
	Between Groups	0.161	5	0.032	1.389	0.297	4.167
	Within Groups	0.279	12	0.023			
	<i>Total</i>	0.441	17				

Table 5: ANOVA analysis of acetylated H3 lysine at position 9 protein showing a p value of 0.297.

CHAPTER 4

DISCUSSION

Epigenetics describes heritable changes in gene expression that are not coded in the DNA sequence. To clarify, these modifications are to the DNA and its associated proteins with affected the underlying base-pair sequence. Epigenetic events are important in the physiology of normal cells as well as aberrant conditions such as cancer. During embryonic development, hypermethylation silences specific sequences of genes that dictate the path of differentiation. Likewise, silencing of tumor suppressor genes is central to the development of cancer.

Physiological hypermethylation in promoter regions in areas of CpG islands cause interfering with the binding of transcriptional proteins to gene promoters, thus halting the expression of that gene and leading to long-term gene silencing. In a similar way, aberrant DNA methylation of the promoter region of certain genes is now thought to be a key mechanism for carcinogenesis.

DNA does not exist as a bare molecule; rather it is associated with proteins forming a complex structure known as chromatin. Chromatin is composed of nucleosomes of 146 base pairs of DNA surrounding a histone octamer. Conformational changes to this structure can have profound effects on gene expression. De-acetylation of histones gives them a positive charge that interacts with the negative charge of DNA producing a closed structure. When in a condensed, tight-knit configuration, gene expression factors cannot bind to promoter regions, thus effectively turning the gene off. Histone de-acetylase (HDAC) thus mediates gene silencing. Conversely, HDAC inhibitors change the chromatin structure to a loose, more open structure, making promoter regions accessible, and thus genes are in effect, turned on. Thus, HDAC inhibitors can reverse gene silencing in certain instances.

VPA has been used in the clinical treatment for epilepsy and other seizure disorders.^{77,78} VPA has been used in this capacity for over 30 years because it is generally well tolerated, has good oral bioavailability and a favorable toxicity profile.⁷⁹ More recently, VPA has been identified as an effective HDAC inhibitor and used as a new class of targeted cancer therapeutics. Several HDAC inhibitors, including VPA, are currently in clinical trials and promising anti-cancer effects at well-tolerated doses have been observed for both hematological and solid cancers.^{80,81} The anti-cancer effects of HDAC inhibitors such as VPA are caused in part by the accumulation of acetylated nuclear histones with altered transcription of tumor suppressor genes.

Significance

The experiments described herein link VPA administration of head and neck squamous cell carcinoma FaDu-luc cell line to increased histone acetylation. Specifically, histone acetylation of H3 and H3 at the lysine 9 position. While overall experiments do not show statistical significance, a trend is seen with histone H3 acetylation and VPA concentrations between 0.5mM and 12.5mM. Similarly, a trend is seen with histone H3 acetylation at lysine position 9 and VPA concentrations between 0.1mM and 2.5mM.

The trends found in the study are consistent with finding of Coombes et al⁸², who showed the HDAC inhibitor 5-aza-2'dexocytidine increases histone acetylation and reactivates p16 in Tu159 in head and neck squamous cell carcinoma cells. Similar trends are compatible with the interaction of trichostatin A (TSA) with the active site of mammalian HDAC homolog histone-deacetylase-like protein⁸³, as well as the interaction with TSA and other active inhibitors with the catalytic site of HDAC. Specifically regarding VPA, similar trends have also been linked to histone acetylation in bladder^{84,85}, prostate⁸⁶, colon⁸⁷, pancreas⁸⁸, lung⁸⁹, cervical⁹⁰, gastric⁹¹, and hepatocellular⁹² cancer

cell lines. This is, to our knowledge, the first such trend investigated and demonstrated in head and neck cell lines using VPA.

Although the ANOVA failed to show a difference in any of the means, the sample size and the effect size was small to modest. Also of note, the variance was high within the samples. With an increased number of experiments, and thus higher power, it would be expected that the trend seen would manifest as significance. Reducing the variance with improved laboratory technique and a compressed time schedule would also allow for significant results. This would allow for more consistent laboratory techniques as well as allowing for usage of materials from the same sample stock, reducing external variability. Reducing variance could also be obtained by utilizing more sensitive detection techniques such as individual protein immunoprecipitation (IP) or chromatin immunoprecipitation methods (ChIP).

Future Directions

Radiation is a well-established and widely used therapy modality to treat cancer worldwide. Data indicates that approximately 50% of cancer patients are treated with radiation for either therapeutic or palliative purposes.⁹³ It is clear that the therapeutic index of radiotherapy can be improved with the use of chemical agents that sensitize cancer cells to the toxic effects of ionizing radiation. Therefore, the development of such compounds with the purpose of cell sensitization to radiation has been pursued. Recently, it has been reported that VPA possesses radiation sensitizing properties both in vitro and in vivo.⁹⁴ It was believed that histone hyperacetylation and the radiation sensitizing mechanism suppressed DNA repair leading to cell death. Given these findings, further studies can be done on head and neck squamous cell carcinoma FaDu-luc cells to investigate the correlation of enhancement of radiation-induced cell-death by VPA. This

is not only to elucidate applications for head and neck cancers, but also to confirm treatment modalities for other applications as well.

The radiosensitizing effects of VPA could be investigated with a clonogenic assay. Gerster et al found radiosensitivity on head and neck squamous cell carcinoma cells at 6 Gy when targeting polo-like kinase 1.⁹⁵ Chen et al utilized DNA methyltransferase 1 on FaDU-C225-R cells and also found radiosensitivity at 6 Gy.⁹⁶ Given these findings, treatment with VPA on FaDu-luc cells treated with VPA and radiation doses of 0 – 12 Gy could be of interest. A sample clonogenic assay could entail administering ionizing radiation (0, 6, 12 Gy) delivered 24 hours after treatment treated with VPA (0 – 12.5 mM).

Conclusion

VPA has been shown to increase histone acetylation in various cancer cell lines and is actively pursued in clinical trials. To date, VPA has not been utilized for head and neck squamous cell carcinoma therapy. The findings show that VPA has the propensity to act as a HDAC inhibitor and may be used to radiosensitize these cells for higher efficiency of treatment.

BIBLIOGRAPHY

1. Boyle P, Levin B, International Agency for Research on Cancer, ebrary I. *World cancer report*. IARC Press; 2008.
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA: a cancer journal for clinicians*. 2010;caac. 20073v1.
3. Parkin DM, Bray F, Devesa S. Cancer burden in the year 2000. The global picture. *Eur J Cancer*. 2001;37:4-66.
4. Muir C, Nectoux J. International patterns of cancer. *Cancer epidemiology and prevention*. 1996.
5. Davies L, Welch HG. Epidemiology of head and neck cancer in the United States. *Otolaryngology--Head and Neck Surgery*. 2006;135(3):451.
6. Zygogianni A, Kyrgias G, Mystakidou K, et al. Potential role of the alcohol and smoking in the squamous cell carcinoma of the head and neck: review of the current literature and new perspectives. *Asian Pac J Cancer Prev*. 2011;12(2):339-344.
7. Warnakulasuriya S, Trivedy C, Peters TJ. Areca nut use: an independent risk factor for oral cancer. *BMJ*. 2002;324(7341):799.
8. Ko Y, Huatig Y, Lee C, Chen M, Litt L, Tsai C. Betel quid chewing, cigarette smoking and aicohoi consumption related to orai cancer in Taiwan. *J Oral Pathol Med*1995. ;24:450-453.
9. Siemiatycki J, Richardson L, Straif K, et al. Listing occupational carcinogens. *Environ Health Perspect*. 2004;112(15):1447.
10. Guha N, Boffetta P, Wünsch Filho V, et al. Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *Am J Epidemiol*. 2007;166(10):1159.
11. Rodríguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. 2011:330-339.
12. Waddington C. The epigenotype. *Endeavour*. 1942;1(1):18-20.
13. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010;31(1):27.
14. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003;33:245-254.

15. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nature Reviews Cancer*. 2004;4(2):143-153.
16. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis*. 2007;22(2):91.
17. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128(4):683-692.
18. Wade PA, Geggion A, Jones PL, Ballestar E, Aubry F, Wolffe AP. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet*. 1999;23(1):62-66.
19. Pikaart MJ, Recillas-Targa F, Felsenfeld G. Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes Dev*. 1998;12(18):2852.
20. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem*. 2003;278(6):4035.
21. Hellebrekers D, van Engeland M. DNA Methylation and Histone Deacetylation. .
22. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev*. 2002;16(1):6.
23. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A*. 2002;99(6):3740.
24. Wang Y, Leung FCC. An evaluation of new criteria for CpG islands in the human genome as gene markers. *Bioinformatics*. 2004;20(7):1170.
25. Estecio MR, Issa JP. Dissecting DNA hypermethylation in cancer. *FEBS Lett*. 2010.
26. Huang L, Frampton G, Liang LJ, Demorrow S. Aberrant DNA methylation profile in cholangiocarcinoma. *World J Gastrointest Pathophysiol*. 2010;1(2):23-29.
27. Lewandowska J, Bartoszek A. DNA methylation in cancer development, diagnosis and therapy--multiple opportunities for genotoxic agents to act as methylome disruptors or remediators. *Mutagenesis*. 2011.
28. Sceusi EL, Loose DS, Wray CJ. Clinical implications of DNA methylation in hepatocellular carcinoma. *HPB (Oxford)*. 2011;13(6):369-376.

29. Watanabe Y, Maekawa M. Methylation of DNA in cancer. *Adv Clin Chem.* 2010;52:145-167.
30. Wang J, Chen H, Fu S, Xu ZM, Sun KL, Fu WN. The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. *Oral Oncol.* 2011.
31. Syeed N, Husain SA, Sameer AS, Chowdhri NA, Siddiqi MA. Mutational and promoter hypermethylation status of FHIT gene in breast cancer patients of Kashmir. *Mutat Res.* 2011;707(1-2):1-8.
32. Kikuchi R, Tsuda H, Kanai Y, et al. Promoter hypermethylation contributes to frequent inactivation of a putative conditional tumor suppressor gene connective tissue growth factor in ovarian cancer. *Cancer Res.* 2007;67(15):7095-7105.
33. Janson PC, Marits P, Thorn M, Ohlsson R, Winqvist O. CpG methylation of the IFNG gene as a mechanism to induce immunosuppression [correction of immunosuppression] in tumor-infiltrating lymphocytes. *J Immunol.* 2008;181(4):2878-2886.
34. Clausell J, Happel N, Hale TK, Doenecke D, Beato M, Aramayo R. Histone H1 subtypes differentially modulate chromatin condensation without preventing ATP-dependent remodeling by SWI/SNF or NURF. *PLoS One.* 2009;4(10):e0007243.
35. Eickbush TH, Moudrianakis EN. The histone core complex: an octamer assembled by two sets of protein-protein interactions. *Biochemistry (N Y).* 1978;17(23):4955-4964.
36. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature.* 1997;389(6648):251.
37. Kouzarides T. Chromatin modifications and their function. *Cell.* 2007;128(4):693-705.
38. Liu B, Lin Y, Darwanto A, Song X, Xu G, Zhang K. Identification and characterization of propionylation at histone H3 lysine 23 in mammalian cells. *J Biol Chem.* 2009;284(47):32288.
39. Grant PA. A tale of histone modifications. *Genome Biol.* 2001;2(4):0003-0001.
40. Loidl P. Histone acetylation: facts and questions. *Chromosoma.* 1994;103(7):441-449.

41. Brownell JE, Allis CD. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in *Tetrahymena* macronuclei. *Proceedings of the National Academy of Sciences*. 1995;92(14):6364.
42. Walkinshaw DR, Tahmasebi S, Bertos NR, Yang XJ. Histone deacetylases as transducers and targets of nuclear signaling. *J Cell Biochem*. 2008;104(5):1541-1552.
43. Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. *Nature Reviews Molecular Cell Biology*. 2007;8(4):284-295.
44. Fletcher TM, Hansen JC. Core histone tail domains mediate oligonucleosome folding and nucleosomal DNA organization through distinct molecular mechanisms. *J Biol Chem*. 1995;270(43):25359.
45. Steger DJ, Workman JL. Remodeling chromatin structures for transcription: What happens to the histones? *Bioessays*. 1996;18(11):875-884.
46. Luger K, Richmond TJ. The histone tails of the nucleosome. *Curr Opin Genet Dev*. 1998;8(2):140-146.
47. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nature Reviews Drug Discovery*. 2006;5(9):769-784.
48. Carey N, La Thangue NB. Histone deacetylase inhibitors: gathering pace. *Current Opinion in Pharmacology*. 2006;6(4):369-375.
49. MEUNIER H, CARRAZ G, NEUNIER Y, EYMARD P, AIMARD M. Pharmacodynamic properties of N-dipropylacetic acid. *Therapie*. 1963;18:435-438.
50. Mesdjian E, Ciesielski L, Valli M, et al. Sodium valproate: kinetic profile and effects on GABA levels in various brain areas of the rat. *Prog Neuro-Psychopharmacol Biol Psychiatry*. 1982;6(3):223-233.
51. VanDongen A, VanErp M, Voskuyl R. Valproate reduces excitability by blockage of sodium and potassium conductance. *Epilepsia*. 1986;27(3):177-182.
52. Nightingale KP, Gendreizig S, White DA, Bradbury C, Hollfelder F, Turner BM. Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. *The Journal of biological chemistry*. 2007;282(7):4408.
53. Harikrishnan K, Karagiannis TC, Chow MZ, El-Osta A. Effect of valproic acid on radiation-induced DNA damage in euchromatic and heterochromatic compartments. *Cell cycle*. 2008;7(4):468-476.

54. Lunke S, El - Osta A. The emerging role of epigenetic modifications and chromatin remodeling in spinal muscular atrophy. *J Neurochem*. 2009;109(6):1557-1569.

55. Bojic U, Ehlers K, Ellerbeck U, et al. Studies on the teratogen pharmacophore of valproic acid analogues: evidence of interactions at a hydrophobic centre. *Eur J Pharmacol*. 1998;354(2-3):289-299.

56. Das CM, Aguilera D, Vasquez H, et al. Valproic acid induces p21 and topoisomerase-II (α/β) expression and synergistically enhances etoposide cytotoxicity in human glioblastoma cell lines. *J Neurooncol*. 2007;85(2):159-170.

57. Knüpfer M, Hernaiz-Driever P, Poppenborg H, Wolff J, Cinatl J. Valproic acid inhibits proliferation and changes expression of CD44 and CD56 of malignant glioma cells in vitro. *Anticancer Res*. 1998;18(5A):3585-3589.

58. Michaelis M, Michaelis UR, Fleming I, et al. Valproic acid inhibits angiogenesis in vitro and in vivo. *Mol Pharmacol*. 2004;65(3):520.

59. Hodges-Gallagher L, Valentine CD, Bader SE, Kushner PJ. Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells. *Breast Cancer Res Treat*. 2007;105(3):297-309.

60. Mongan NP, Gudas LJ. Valproic acid, in combination with all-trans retinoic acid and 5-aza-2'-deoxycytidine, restores expression of silenced RAR β 2 in breast cancer cells. *Molecular cancer therapeutics*. 2005;4(3):477.

61. Wilson AJ, Byun DS, Popova N, et al. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem*. 2006;281(19):13548.

62. Pan L, Lu J, Huang B. HDAC inhibitors: a potential new category of anti-tumor agents. *Cell Mol Immunol*. 2007;4(5):337-343.

63. Huang X, Guo B. Adenomatous Polyposis Coli Determines Sensitivity to Histone Deacetylase Inhibitor-Induced Apoptosis in Colon Cancer Cells. *Cancer Res*. 2006;66(18):9245.

64. Angelucci A, Valentini A, Millimaggi D, et al. Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathways. *Anticancer Drugs*. 2006;17(10):1141.

65. Thelen P, Schweyer S, Hemmerlein B, Wuttke W, Seseke F, Ringert RH. Expressional changes after histone deacetylase inhibition by valproic acid in LNCaP human prostate cancer cells. *Int J Oncol*. 2004;24(1):25-31.

66. Shen WT, Wong TS, Chung WY, et al. Valproic acid inhibits growth, induces apoptosis, and modulates apoptosis-regulatory and differentiation gene expression in human thyroid cancer cells. *Surgery*. 2005;138(6):979-985.
67. Fortunati N, Catalano MG, Arena K, Brignardello E, Piovesan A, Boccuzzi G. Valproic acid induces the expression of the Na⁺/I⁻-symporter and iodine uptake in poorly differentiated thyroid cancer cells. *Journal of Clinical Endocrinology & Metabolism*. 2004;89(2):1006.
68. Catalano MG, Fortunati N, Pugliese M, et al. Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. *Journal of Clinical Endocrinology & Metabolism*. 2005;90(3):1383.
69. Chen J, Ghazawi FM, Bakkar W, Li Q. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. *Mol Cancer*. 2006;5:71.
70. Valentini A, Biancolella M, Amati F, et al. Valproic acid induces neuroendocrine differentiation and UGT2B7 up-regulation in human prostate carcinoma cell line. *Drug Metab Disposition*. 2007;35(6):968.
71. Takai N, Kawamata N, Gui D, Said JW, Miyakawa I, Koeffler HP. Human ovarian carcinoma cells: histone deacetylase inhibitors exhibit antiproliferative activity and potently induce apoptosis. *Cancer*. 2004;101(12):2760-2770.
72. Atmaca A, Al-Batran S, Maurer A, et al. Valproic acid (VPA) in patients with refractory advanced cancer: a dose escalating phase I clinical trial. *Br J Cancer*. 2007;97(2):177-182.
73. Chavez-Blanco A, Segura-Pacheco B, Perez-Cardenas E, et al. Histone acetylation and histone deacetylase activity of magnesium valproate in tumor and peripheral blood of patients with cervical cancer. A phase I study. *Mol Cancer*. 2005;4(1):22.
74. Korn EL. Nontoxicity endpoints in phase I trial designs for targeted, non-cytotoxic agents. *J Natl Cancer Inst*. 2004;96(13):977.
75. Munster P, Marchion D, Bicaku E, et al. Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clinical Cancer Research*. 2009;15(7):2488.
76. Münster P, Marchion D, Bicaku E, et al. Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. *Journal of clinical oncology*. 2007;25(15):1979.

77. Pinder RM, Brogden RN, Speight TM, Avery GS. Sodium valproate: a review of its pharmacological properties and therapeutic efficacy in epilepsy. *Drugs*. 1977;13(2):81-123.
78. Perucca E. Pharmacological and therapeutic properties of valproate: a summary after 35 years of clinical experience. *CNS drugs*. 2002;16(10):695-714.
79. Brown R. US experience with valproate in manic depressive illness: a multicenter trial. *J Clin Psychiatry*. 1989.
80. Kelly WK, Marks PA. Drug insight: Histone deacetylase inhibitors--development of the new targeted anticancer agent suberoylanilide hydroxamic acid. *Nature clinical practice.Oncology*. 2005;2(3):150.
81. Piekarz R, Bates S. A review of depsipeptide and other histone deacetylase inhibitors in clinical trials. *Curr Pharm Des*. 2004;10(19):2289-2298.
82. Coombes MM, Briggs KL, Bone JR, Clayman GL, El-Naggar AK, Dent SYR. Resetting the histone code at CDKN2A in HNSCC by inhibition of DNA methylation. *Oncogene*. 2003;22(55):8902-8911.
83. Finnin MS, Donigian JR, Cohen A, et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature*. 1999;401(6749):188-193.
84. Ozawa A, Tanji N, Kikugawa T, et al. Inhibition of bladder tumour growth by histone deacetylase inhibitor. *BJU Int*. 2009.
85. Xu XS, Wang L, Abrams J, Wang G. Histone deacetylases (HDACs) in XPC gene silencing and bladder cancer. *J Hematol Oncol*. 2011;4:17.
86. Fortson WS, Kayarthodi S, Fujimura Y, et al. Histone deacetylase inhibitors, valproic acid and trichostatin-A induce apoptosis and affect acetylation status of p53 in ERG-positive prostate cancer cells. *Int J Oncol*. 2011;39(1):111-119.
87. Chen X, Wong P, Radany E, Wong JY. HDAC inhibitor, valproic acid, induces p53-dependent radiosensitization of colon cancer cells. *Cancer Biother Radiopharm*. 2009;24(6):689-699.
88. Iwahashi S, Shimada M, Utsunomiya T, et al. Histone deacetylase inhibitor augments anti-tumor effect of gemcitabine and pegylated interferon-alpha on pancreatic cancer cells. *Int J Clin Oncol*. 2011.
89. Platta CS, Greenblatt DY, Kunnimalaiyaan M, Chen H. Valproic acid induces Notch1 signaling in small cell lung cancer cells. *J Surg Res*. 2008;148(1):31-37.

90. Sami S, Hoti N, Xu HM, Shen Z, Huang X. Valproic acid inhibits the growth of cervical cancer both in vitro and in vivo. *J Biochem*. 2008;144(3):357-362.
91. Yagi Y, Fushida S, Harada S, et al. Effects of valproic acid on the cell cycle and apoptosis through acetylation of histone and tubulin in a scirrhous gastric cancer cell line. *J Exp Clin Cancer Res*. 2010;29:149.
92. Tatebe H, Shimizu M, Shirakami Y, et al. Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells. *Cancer Lett*. 2009;285(2):210-217.
93. Gunderson LL, Tepper JE. *Clinical radiation oncology*. Churchill Livingstone; 2007.
94. Camphausen K, Cerna D, Scott T, et al. Enhancement of in vitro and in vivo tumor cell radiosensitivity by valproic acid. *International journal of cancer*. 2005;114(3):380-386.
95. Gerster K, Shi W, Ng B, et al. Targeting polo-like kinase 1 enhances radiation efficacy for head-and-neck squamous cell carcinoma. *International Journal of Radiation Oncology* Biology* Physics*. 2010;77(1):253-260.
96. Chen CC, Chen WC, Wang WH, et al. Role of DNA methyltransferase 1 in pharyngeal cancer related to treatment resistance. *Head Neck*.