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Extraction, Purification and Evaluation of PRMT5-Inhibitory Phytochemical Compounds for the Treatment of Prostate Adenocarcinoma

Oliver H. Richmond III
Clark Atlanta University, oliver.richmond@students.cau.edu

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The development and advancement of prostate cancer is supported by a plethora of genetic and proteomic abnormalities, including events of post-translational modifications. The protein arginine methyltransferase 5 (PRMT5) enzyme regulates epigenetic events of histone modifications and protein post-translational modifications within protein signaling pathways. PRMT5 functions by catalyzing the symmetric dimethylation of terminal arginine residues on target protein substrates. Under abnormal conditions of overexpression and upregulation, PRMT5 methyltransferase activity constitutively drives the growth and proliferation of dysregulated cells. Overexpression or upregulation of PRMT5 correlates with disease progression as observed among numerous cancer types, including breast, colorectal, leukemia, lung, melanoma and prostate cancers. We demonstrated previously that PRMT5 knockdowns attenuated both
growth and proliferation of lung and prostatic tumors, *in vitro* and *in vivo*. Plants naturally produce chemical toxins as mechanisms of defense against microbial and other biological threats. Human exploitation, consumption and application of agents isolated from plants for therapeutic intervention dates back throughout the millennia. In this study, we extracted, purified and evaluated natural, small, chemical compounds from plant products that antagonize PRMT5 activity in prostate cancer cells. We found that crude and purified extracts of *Dendrobium aurantiacum var. denneanum* (*D. denneanum*) plants attenuated prostate tumor growth and proliferation by selective inhibition of PRMT5 methyltransferase activity. These findings establish the first set of natural PRMT5-specific inhibitors reported.
EXTRACTION, PURIFICATION AND EVALUATION OF PRMT5-INHIBITORY COMPOUNDS FOR THE TREATMENT OF PROSTATE ADENOCARCINOMA

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
OLIVER H. RICHMOND III

DEPARTMENT OF BIOLOGICAL SCIENCES

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LIST OF ABBREVIATIONS

µCi – microcurie

µg – microgram

µM – micromolar

AcN – acetonitrile

AdoMet – Adenosylmethionine

ATCC – American Type Culture Collection

BSA – bovine serum albumin

cDNA – copy DNA

CH₃ – methyl

CO₂ – carbon dioxide

Cₜ – cycle threshold

°C – degrees Celsius

°F – Fahrenheit

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid
DTT – Dithiothreitol

EDTA – ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptor

EGTA - ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EMT – epithelia-mesenchymal transition

ESI-MS – electrospray ion mass spectrometry

EtAc – ethyl acetate

EtOH – Ethanol

FBE – fetal bovine essence

GE – General Electric

H₂O – water

H³ – tritium

HCL – Hydrochloric Acid

HGF – hepatocyte growth factor

HOXA9 – homeobox protein A9

HPLC - high-pressure liquid chromatography

M – molar

Med – medical
MeOH – Methanol

mL – milliliter

mm – millimeter

mM – millimolar

MSKE – Muscadine Grape Skin Extract

NaHCO₃ – sodium bicarbonate

NC – nitrocellulose

OGT – N-acetylglucosamine transferase

p – peak

PAGE – Polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PET – positron emission tomography

pH – acidity

PI – Propidium iodide

PLB – passive lysis buffer

PRMT – Protein Arginine Methyltransferase

qRT-PCR – quantitative real-time polymerized chain reaction

rcf – relative centrifugal force
ROS – reactive oxygen species

rpm – repetitions per minute

RPMI – Roswell Park Memorial Institute

RT – room temperature

RT-PCR – real-time polymerized chain reaction

SAM – S-adenosylmethionine

SDS – sodium dodecyl sulfate

TBST – tris-buffered saline tween

TFA - trifluoroacetic acid

THF – Tetrahydrofuran

Tris - Trizma

UFLC – ultra-fast liquid chromatography

UPR – unfolded protein response

V – volt
CHAPTER I

INTRODUCTION

Metastatic Prostate adenocarcinoma is the leading cause of morbidity and the second-leading cause of cancer-associated mortality among American men.\textsuperscript{1,3} The National Cancer Institute’s (NCI) Physician Data Query (PDQ) of cancer information states that the propensity for disease and deaths related to prostate cancer is influenced by both genetic and environmental factors.\textsuperscript{4} Of all males autopsied age 80 or older; nearly 70\% displayed some grade of incidental prostate cancer.\textsuperscript{5-6} Prostate cancer is an age-related disease, however little is known regarding the specific biology underpinning the progression and recurrence of the disease.\textsuperscript{7} Data suggests that an accumulation of a variety of genomic and proteomic molecular events contributes to prostate tumor initiation and resiliency alike.\textsuperscript{8-9} According to the American Cancer Society (ACS), the five-year relative survival rate for American men diagnosed with localized or regionalized prostate cancer is 100\%.\textsuperscript{10-11} However, American men diagnosed with distant metastases of prostate cancer have an anticipated five-year survival rate of less than 30\%.\textsuperscript{10-11} In addition to other characteristics, unregulated cellular growth amid aberrant proliferation hallmarks the development and progression of malignant prostate cancer.\textsuperscript{12} A number of reports have implicated the family of protein arginine methyltransferases (PRMT) for their roles in the development and progression of
Protein arginine methyltransferases function by transferring methyl groups onto select arginine amino acid residues located on target substrates. Hyperactivity of PRMTs correlates with disease and is observable in multiple tissue types including bladder, blood, breast, colon, lung, ovarian and prostate cancers. Occurrence of nearly 80% of all mammalian asymmetric dimethylation activity is resultant of PRMT1. Whereas the majority of type II symmetric dimethylation events are resultant of the mammalian PRMT5. Previously, we published that localization of PRMT5 to the cytoplasm of lung cancer cells was essential for growth of lung cancer cells. Additionally, we found that overexpression of PRMT5 correlated with promotion of cellular growth and proliferation in prostate cancer cells.

1.1 Hypothesis

Whether PRMT5 hyperactivity promotes tumor resiliency by regulation of histone tails to effect gene expression, modification of protein signaling to activate or inactive signaling pathways and coax crosstalk of signaling pathways or another mechanism, is unknown. The upregulation or overexpression of PRMT5 in cancer presents a relevant opportunity for targeted therapeutic intervention. Chemical inhibition of PRMT5 potentiates opportunities to ameliorate its constitutive methyltransferase activity and mitigate the metastatic phenotype of prostate adenocarcinoma. Considering the results of our previously published findings and the chemotherapeutic data of medicinal molecules derived from plants, we investigated the hypothesis that, natural compounds are sufficient to antagonize growth and proliferation of prostate adenocarcinoma by way of inhibiting PRMT5 activity.
1.2 Purpose of the Study

We methodically and objectively evaluated the hypothesis and its alternatives according to the following three specific aims:

**Aim 1: To extract and purify natural chemical compounds from Dendrobium aurantiacum var. denneanum.** Natural chemical compounds obtained from plant, fruit and vegetable derivatives exhibited anti-tumorigenic effects against multiple cancer types.\textsuperscript{28-29} Our preliminary studies revealed successful extraction of a variety of natural crude products from another species of the orchid plant, *Dendrobium nobile* (*D. nobile*). We began optimizations for extraction of *D. denneanum*

**Aim 2: To test the purified chemical compounds for inhibition of PRMT5 activity.** Previously, we reported that PRMT5 and its enzymatic activity are required for cellular growth of prostate and lung cancer cells. Using an *in vitro* model, we evaluated crude and purified compounds for the ability to antagonize PRMT5 methyltransferase activity. We determined a minimum enzyme inhibitory concentration for the crude extract and each purified compound.

**Aim 3: To evaluate the effect of purified, natural compounds on growth and proliferation of cancer cells.** Previously, we observed and reported PRMT5 localized to the cytoplasm of prostate cancer cell drives growth and proliferation. We evaluated the crude and purified compounds in LNCaP and PC3 prostate cancer cell lines. Here, we
report the evaluation and characterization of the first known set of novel natural PRMT5-specific inhibitors with micromolar efficacy. This research extracts, purifies and evaluates novel, natural PRMT5-inhibitory small chemical compounds for the prevention and mitigation of advanced prostate cancer.

1.3 Statement of the Problem

The aberrant hyperactivity of PRMT5 underpins the age-related morbidity and metastatic-associated mortality of prostate cancer. Failure to address and abate perturbations of PRMT5 activity may contribute to greater rates of metastatic prostate cancer and augment cancer-related mortality rates. Currently, there exists limited availability of clinically relevant PRMT5-specific inhibitors. After using a combination of biochemical techniques to extract, purify and evaluate chemical compounds, we report here for the first time, natural, powerful probes that exhibit PRMT5-specific inhibitory activity.
CHAPTER II
LITERATURE REVIEW

2.1 Hallmarks of Cancer

Highlighting a conserved set of distinct characteristics observed to be indicative of cancers are six acquired biologic properties proposed by Hanahan and Weinberg reported in Figure 1.\textsuperscript{12,30} The organizing principle characterizes the initiation and development of neoplasia throughout the multistep succession to tumor pathogenesis. This comprehensive list of tumor qualities refers to phenotypic traits that enables unregulated growth and perpetuates proliferative states of cancers.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{The Hallmarks of Cancer.\textsuperscript{12} Six biological characteristics explain the acquired aberrant properties that cancer cells exhibit. Regulated function of each hallmark is innate to normal cellular biology.}
\end{figure}
2.1.1 Angiogenesis

Angiogenesis is a process of generating new blood vasculature for the maintenance of the cellular microenvironment.\textsuperscript{31} Blood vasculature innervates tissues of organ systems with an overarching duality of purpose: to transport nutrients and remove waste. In men, the generation of most blood vasculature occurs during periods of normal growth and development.\textsuperscript{31} Typically, abnormal physiologic conditions or rare circumstances, like the destruction or damage to preexisting blood vasculature, warrants activation of angiogenesis. More so than normal cells, cancer cells require nutrients in order to sustain the energetic demands associated with incessant development of dysregulated growth and divisions.\textsuperscript{10,32}

The major mechanism by which cancer cells acquire access to additional nutrients is achieved by manipulating protein signaling networks to coax the establishment of new blood vessels from preexisting blood vasculature.\textsuperscript{33-34} Tissue maintenance relies upon the balance of a number of inhibitory and stimulatory factors of angiogenesis. Cancer cells hijack and upregulate the secretion of molecules like vascular endothelial growth factor (VEGF) to chemically attract and direct the generation of new blood vasculature.\textsuperscript{35} Angiogenesis promotes the establishment and maintenance of the tumor microenvironment by directly innervating tumors with blood vasculature for nutrient delivery.\textsuperscript{31,34-37}

2.1.2 Invasion and Metastasis

Invasion and metastasis collectively refer to the migratory phenotypes exhibited by aberrant cells when exacerbating the motile propensity of normal cellular movement.
Cellular movement employed by normal cells typically occurs over short distances for processes of cellular turnover, during which nascent cells replenish preexisting cells. Cellular motility is essential for the normal growth and development of tissues, wound healing and tissue maintenance. More specifically, normal cells anchor to adjacent cells in order to maintain tissue-specific morphology and function and protect tissue architecture. Cells harboring anomalies with regard to anchorage-dependence, enables properties of cellular motility that disregard tissue integrity while promoting remote trafficking.

A compromise in tissue polarity due to abnormal tissue architecture with the loss of anchorage proteins like E-cadherin lays the foundation for both invasion and metastasis.\textsuperscript{38-39} The migration of cells from sites of primary tumors to distant organs of the body is an acquired trait that is indicative of an elite and resilient class of abnormal cells. The invasion and anchoring of tumorigenic cells into foreign tissues precipitates organ loss of function and subsequent organ failure. Consequentially, men who die of prostate cancer typically exhibit metastatic disease.

The acquired ability to engineer context-specific morphological plasticity, traverse cellular borders of nearby tissues, withstand detection by immune surveillance, withstand changes in pressure and pH within the blood vasculature and operate in environments of temporary nutrient deprivation for the formation of metastatic sites describes aspects of tumor growth and survival.\textsuperscript{37,40} Abandonment of tissue architecture and enablement of motile plasticity of aberrant cells promotes the tumor microenvironment.\textsuperscript{41}
2.1.3 Avoidance of Apoptosis

The acquired ability of cells to escape elaborate programs that instruct dysfunctional cells to commit programmed cell death is apoptosis. Programmed cell death functions as a major antagonist to cancer development by discouraging the formation of tumors. Tumor-suppressor and auxiliary proteins surveil the cellular microenvironment for signs of distress beyond repair. Upon experiencing extenuating genomic insults, individual cells typically sacrifice themselves by suicide in a tightly regulated mechanism to protect overall tissue integrity.\textsuperscript{42}

Under the auspice of the BH3 family of proteins, diverse intercellular damage signals regulate the fate of cells by promoting cell survival or apoptosis.\textsuperscript{43-44} Another means for regulation of cellular turnover, aberrant cells expressing high levels of c-Myc is sufficient to commit cells to apoptosis.\textsuperscript{45} Cancer cells develop mechanisms that enable evasion of apoptosis in order to prolong survival.\textsuperscript{43-45} During less severe events of cellular stress in which damage to DNA is repairable, cells invoke autophagy as a means of cellular recovery.\textsuperscript{46-47} Autophagy enables molecule-specific degradation as a mechanism that attempts to rescue damaged cells. Although there exists considerable and context-dependent overlap, apoptosis and autophagy function as opposite ends of a seesaw, regulating cellular fate.\textsuperscript{48} Under abnormal conditions, cells can rely more on autophagy as a mechanism for avoidance of apoptosis while contributing to the establishment and maintenance of the tumor microenvironment.\textsuperscript{48}
2.1.4 Replicative Immortality

In order to develop macroscopic tumors, cancer cells must sustain a significant level of division cycles that support size maintenance or augmentation. Programs that restrict replication to a limited number of division cycles or signal for cells to die after irreparable damage, govern cellular replication cycles under normal conditions. Strand length of deoxyribose nucleic acid (DNA) shortens with each cycle of replication until reaching a point at which normal cells can no longer divide. These cells typically do not die; however, they also do not replicate. They are senescent. Excessive or overwhelming damage to DNA that is beyond salvation in normal cells leads to cellular death for purposes of protecting the integrity of overall tissue.

The Hayflick limit refers to the maximum number of division cycles specific to that cell’s DNA strand length. Normal cell replication governs cellular division cycles by shortening the protective telomere cap on the end of DNA strands with each cycle of division.\textsuperscript{49-50} Telomeres shorten with each cycle of replication until reaching a critical point; beyond the critical number of divisions, cells enter into senescence and can no longer replicate.\textsuperscript{49-50} Dysfunctional cells achieve excessive rounds of division without consequences of cellular death or senescence. Tissue size augmentation potentiates disruption of tissue integrity.

In opposition to canonical cellular signaling, cancer cells undergo an undefined number of replicative events whereas normal cells are restricted to a limited number of division cycles.\textsuperscript{50} In a transgenic murine model, telomeres independently promoted proliferation of neuronal cells and suppressed signals for apoptosis.\textsuperscript{51} This finding
provides a novel implication that telomeres function to promote infinite division cycles independent of Hayflick’s limit. To combat restrictions imposed by Hayflick’s limit, cancer cells often upregulate activity of the enzyme telomerase in order to sustain an unspecified number of replicative events and promote the tumor microenvironment.52

2.1.5 Constitutive Proliferative Signaling

The prognostic evidence of proliferation is observable as augmentation in cell number resultant of a combination of growth and cellular divisions as illustrated in Figure 2.53 Under normal circumstances, checkpoints of proliferation and nutrient homeostasis regulate biologic demands by toggling growth and development with respect to energetic demands for tissue turnover, scaffolding, size and morphology.32,54 Chronic proliferation promotes dysfunction of tightly regulated checkpoints for the development and advancement of carcinogenesis.

Loss of function in the SNAIL1 transcription factor generates a loss in cellular polarity and tissue architecture leading to the upregulation of oncogenes, downregulation of epithelial cadherin (E-cadherin) and promotion of an epithelial-to-mesenchymal transition (EMT) phenotype, enabling constitutive proliferative signaling.39,55 Cancer stem cells (CSC) also contribute to proliferation by way of enabling accumulation of genetic mutations while exhibiting resistance to chemotherapy, resistance to apoptosis and properties of self-renewal.56 Dysregulation of one or a number of molecular events involving genetic mutations, EMT, or stemness potentiates conditions that promote sustained proliferative signaling for establishment and promotion of the tumor microenvironment, as demonstrated in Figure 2.
Figure 2. Loss of SNAIL promotes proliferation.57

2.1.6 Unregulated Growth

Tumor suppressor genes code for proteins that function to suppress the formation of tumors.58 Alternatively, oncogenes code for proteins that function to promote events that contribute to the formation of tumors.58 For purposes of sustaining signals that promote growth, cancer cells must circumvent the careful regulation of tumor suppressor
genes as reported in Table 1. Surveillance by an integrated network of internal and external signals regulates cellular entrance into Mitosis.

**Table 1.** Cancer Hallmarks Establish the Tumor Microenvironment.$^{57}$

<table>
<thead>
<tr>
<th>Factor</th>
<th>Contribution to Carcinogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMT</td>
<td>Promotes stem cell growth, metastasis</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>HIFs promote proliferation of CSCs and angiogenesis; alters metabolism</td>
</tr>
<tr>
<td>Autophagy</td>
<td>Promotes cell survival resultant of dysregulated signaling-mediated proliferation</td>
</tr>
<tr>
<td>CSCs</td>
<td>Dysregulation in “stemness”, quiescence, self-renewal, resistance to apoptosis</td>
</tr>
<tr>
<td>Cell Cycle Proteins</td>
<td>Dysregulated expression of cell cycle proteins (Rb, CDKs, cdk inhibitors)</td>
</tr>
<tr>
<td>Signal Transduction Pathways</td>
<td>Constitutive activation of multiple signaling pathways promotes uncontrolled proliferation</td>
</tr>
<tr>
<td>Altered Cell metabolism</td>
<td>Promotes altered survival and growth in adverse conditions</td>
</tr>
<tr>
<td>Hormone Signaling</td>
<td>Promotes the growth of hormone responsive cancers through constitutive activation of estrogen and androgen signaling pathways</td>
</tr>
<tr>
<td>Tumor Microenvironment</td>
<td>Stromal cell crosstalk promotes growth and metastasis of cancer stem cells</td>
</tr>
</tbody>
</table>

Intrinsic sensors or receptors mediate information received from transducers or signals that quickly respond to metabolic requirements for cellular growth. In order to maintain the delicate balance of tumor prevention, gene products that suppress tumors like p53 and RB, closely surveil DNA and intervene to arrest cell growth whenever genomic damage is beyond repair.$^{58,62-63}$ Additionally, loss of function in the tumor suppressor *Nf2* leads to restriction of EGFR in the cellular membrane attenuating contact-dependent inhibition of adjacent cells.$^{64}$ Similarly, loss of function of the tumor suppressor *LKB1* promotes multiple Hallmarks of Cancer including unregulated growth.$^{65}$ After experiencing a loss of function, a tumor suppressor gene fails to prevent the
formation of tumors, promoting unregulated cellular growth for establishment and promotion of the tumor microenvironment as reported in Table 1.

2.1.7 The Tumor Microenvironment

The Hallmarks of Cancer represent six biological characteristics essential to all cancer types. Each of the designated characteristics are not exclusively unique to cancer; rather the aberrant function of each characteristic beyond its canonical function is the basis for which cancer initiates and persists. One characteristic alone mentioned here is not sufficient to declare a tumor malignant. Rather, the authors stress that malignant tumor cells exhibit six phenotypes beyond the innate biological characteristics of normal cells that establish, maintain and promote aspects of the tumor microenvironment as depicted in Figure 3.

![Figure 3. Molecular mechanisms of the tumor microenvironment.](image-url)
To go on, cancer manifests as the culmination of a complex integration of canonical and awry signals and oncogenic events that vary across cell and organ type, as reported in Figure 3. A unique feature harbored within solid tumors are normal cells that maintain the ability to acquire oncogenic and tumorigenic hallmark traits while establishing and sustaining the tumor microenvironment. Given the consistency and prevalence of perturbations of each cancer characteristic, targeting each of the hallmarks for individual or concomitant inhibition potentiates promise for mitigating the onslaught of cancer disease incidence and progression.

2.2 The Human Prostate and Prostate Cancer

2.2.1 The Human Prostate

Similar in shape and size to that of a walnut, the prostate is a gland of the male reproductive organ situated around the urethra, inferior to the bladder and anterior to the rectum as depicted in Figure 4.

Figure 4. Anatomy and orientation of the male reproductive system. The prostate is located below the bladder and in front of the rectum. The prostate gland encircles the urethra.
The prostate assists with numerous physiological functions that promote optimal maintenance of male reproductive health. The smooth contractile muscles of the prostate influences management of urine flow rate as well as composition and secretion of seminal fluids. The prostate gland secretes anticlotting factors promoting the motility of semen to ensure a successful journey to the cervix. The prostate regulates the initiation for penile erections by controlling blood flow.

The prostate is an androgen-regulated exocrine gland. Androgens facilitate normal growth, development and maintenance of canonical prostatic physiology. Under aberrant conditions, androgen receptor (AR) signaling functions in overdrive greatly enhancing the growth and division of prostatic cells. Sustained aberrant signaling of the AR can lead to inflammation and enlargement of the prostate gland, generating a condition of benign prostate hyperplasia (BPH). The prostate expresses the enzyme, 5α-reductase, the major stimulator of the hormone that drives growth, development and maintenance of the prostate. Testosterone is the predominant circulating serum androgen in men. The enzyme 5α-reductase converts testosterone into dihydrotestosterone (DHT). While the AR binds both testosterone and DHT, the affinity for DHT to AR is two-folds greater than that of testosterone. Additionally, testosterone dissociates from the AR five times faster than DHT. Prostate cancer is an age-related disease, driven initially by constitutive signaling of the androgen receptor.

Factors like age, ethnicity, family history, accumulations of genetic abnormalities, caloric-rich and nutrient-deficient dietary practices, exposure to environmental carcinogens, and a sedentary lifestyle independently or concomitantly contributes to a
homeostatic imbalance of the cellular microenvironment.\textsuperscript{74-78} Dysregulation of the cellular microenvironment at the level of the prostate potentiates the development of prostatitis, subsequent enlargement of the prostate and ultimately, cancer of the prostate.\textsuperscript{70-71, 74}

2.2.2 Prostate Cancer

According to the NCI’s Surveillance, Epidemiology and End Results (SEER) program, there is an estimated 164,960 new cases of prostate cancer expected, accounting for nearly 10\% of all cancer types anticipated in 2018 nationwide (Figure 5).

![Figure 5](image)

\textbf{Figure 5.} Incidence and mortality trends in prostate cancer.\textsuperscript{1} United States prostate cancer incidence and mortality trends from 1992-2015 as reported by SEER. Total number of new cases of prostate cancer reported each year. Total number of deaths attributed to prostate cancer is reported.

The NCI estimates that 29,430 American men will die of prostate cancer-related deaths in 2018.\textsuperscript{1} Taken together, prostate cancer is currently the leading cause of morbidity and the second-leading cause of cancer-related mortality among American
According to the American Cancer Society, the five-year survival rate for American men diagnosed with regional prostate cancer is 99%, reported in Figure 6.

![5-Year Relative Survival](image)

**Figure 6.** Prostate cancer survival. SEER data reporting the stage-adjusted 5-year relative survival rate for prostate cancer after initial diagnosis. Data reported reflects statistics acquired from patients that followed recommended clinical treatments immediately following diagnosis.

However as reported in Figure 6, American men diagnosed with distant metastases of prostate cancer have an anticipated five-year survival rate of less than 30%.

Prostate cancer disproportionately affects men of African descent than Caucasian counterparts. The average time of incidence of prostate cancer is 66 years of age in males of Caucasian ancestry and approximately 55 years of age in males of African
ancestry. Like most other cancers, prostate cancer is an age-related disease. Of all men autopsied at least 70 years of age or older, 35% of Caucasian-American men and 50% of African-American men harbored undiagnosed tumors. While prostate cancer is chiefly a disease of accumulated genetic abnormalities, other variables such as environmental factors, lifestyle and diet serve as major possible influences to disease onset and progression. The aforementioned variables can also influence the patient’s prognosis in response to detection, treatment options and prognosis.

The current widely practiced treatments for advanced prostate cancer including hormone therapy, chemotherapy, radiotherapy and immunotherapy does little to ameliorate the prognosis for patients with metastatic prostate cancer. There exists a need for novel therapeutic practices that balance sensitivity and specificity when targeting biological perturbations for the treatment of late-stage prostate cancer.

2.2.3 Current Trends in Screening

Screening for prostate cancer involves the patient, physician, other medical practitioners or a combination of the aforementioned. Screening for prostate cancer begins at home with self-examination observing for complications with the male urinary system. However, many cases of prostate cancer are indolent and do not manifest with symptoms detectable by observation through self-examination alone. Methods that are more specialized including the assistance received from trained healthcare professionals, provides the greatest documented opportunity for detecting prostate cancer early. Practices that screen for prostate cancer are specialized in their ability to detect yet limited in their ability to diagnose. More specifically, mechanisms of screening defines
parameters that surveil for the detection of abnormal physiology as compared against known standards of normal physiology. Some parameters for comparison employed during screenings include age, organ type, physiological function, and previous medical history. Dedicated screening practices for prostate cancer lack diagnostic or even detection efficacy.

Although there currently exist controversy surrounding guidelines for recommended screening practices, there are two practices currently in-use. The accepted and widely practiced methods for the surveillance of prostate cancer include a recommended annual screening for all men older than 50 years of age utilizing the Digital Rectal Exam (DRE) and/or Prostate Specific Antigen (PSA) test.\textsuperscript{1, 10, 81-82} Current evaluations seek to determine if the recommendation of annual screening provides more benefit than harm.\textsuperscript{81, 83-85}

\textit{2.2.3.1 Digital Rectal Exam}

DRE is a moderately invasive, same-day manual examination of the prostate. While standing and bending at the waist or lying on one’s side, a trained urologist inserts two lubricated, gloved fingers into the rectum of the patient. While sliding fingers across the prostate, the physician observes for lumps, areas of rigid tissue or enlargement of the prostate among other possible anomalies. The patient is sometimes encouraged to take and calmly expel a deep-breath of air during the examination in order to assist with the procedure. Generally, a patient does not experience physical complications following a DRE screening, but may exhibit psychological unease.
2.2.3.2 Prostate Specific Antigen

The PSA test is a minimally invasive screening of blood for detection of the protein, prostate specific antigen secreted from cells of the prostate. Typically, a pathologist or other healthcare provider uses a syringe to draw a sample of blood from the arm of a male patient. Analysis of the blood sample occurs in a dedicated laboratory for quantification of PSA in the blood, reported in ng/mL. Possible complications that a patient may experience following a PSA test include but is not limited to acute pain due to the stretching of skin or rupture of hemorrhoids or fissures, if present.

In 1994, the United States Food & Drug Administration (USFDA) approved the use of the PSA test in conjunction with the DRE for surveillance and early detection of prostate cancer at clinic in asymptomatic men, with hopes of identifying cancer early enough to provide intervention and reduce morbidity and mortality. However, the United States Preventative Services Task Force (USPSTF) concluded from review of multiple screens of PSA clinical trials that a potential reduction in cancer death risk does not outweigh the benefits of screening and does not recommend the use of PSA as a prognostic marker for the detection and diagnosis of prostate cancer. Rather, the USPSTF recommends limiting PSA screenings to patients of African descent or those whom have a family history of the disease. Alternatively, the American Urological Association (AUA) maintains that screening for PSA after the diagnosis and treatment of prostate cancer can be a powerful tool for monitoring the status of the disease while in remission.
Results of the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial suggests minimum prognostic value for reliance on data obtained from using the DRE with regard to leveraging patient comfort and limitations due to a small sample size of patient participants of African descent while improving the accuracy and sensitivity for detecting early-stage prostate cancer. After evaluation of data obtained from a comprehensive study involving six medical university centers, investigators found that the DRE provided slightly less of a statistical value as a prognostic marker when compared to PSA analyses alone. The combination of screenings using the DRE combined with results from PSA analyses only slightly improved the prognostic value from either exam alone.

Although there exists a large body of conflicting scientific data available to the public regarding the best screening practices recommended for American men, the single, least-invasive, strongest trends in literature suggests that using PSA as a prognostic marker for screening of prostate cancer is more predictive than other currently available methods. More research is required to determine the methods that balance efficacy and safety for screening for prostate cancer without limitations resultant of results bias, poor sensitivity or poor target specificity. Leading data trends suggests that the safest practices encourage each patient to make informed decisions guided by support from his medical team given the potential advantages, risks and limitations of each screening or detection method. A major challenge facing patients and physicians alike is the avoidance of over-screening and over-diagnosis and subsequent over-treatment using the aforementioned mechanisms of surveillance and detection.
2.2.4 Current Trends in Diagnosis

Diagnosis of prostate cancer involves the patient, an urologist and a pathologist. Reliable detection of prostate cancer begins at clinic once the medical team acquires and analyzes a sample of the suspected tumor mass. The timing and type of test(s) used for the diagnosis of prostate cancer vary across medical practices and medical staff, alike. Additionally, the validity and sensitivity of each test possesses advantages and disadvantages, each specific to the patient’s status at presentation, timing and type of test administered, among other possible variables.

Evaluations employed for the diagnosis of prostate cancer are specific in their ability to diagnose cancers of the prostate but no other cancers. More specifically, diagnostic mechanisms utilize a small number of biological markers or molecular probes that are unique to prostatic tissues and typically aberrantly expressed in cells of the cancer but not normal cells. For its extreme level of sensitivity, reverse-transcription polymerized chain reaction (RT-PCR) analysis presents as a reliable tool for the detection of messenger ribonucleic acids (mRNA) as an indicator of biomarker presence in tissues sampled. Some prostate cancer diagnostic tools include tissue biopsy, magnetic resonance imaging (MRI) scan, computerized tomography (CT) scan, and a bone scan with or without x-rays, an ultrasound or a urine flow test. Dedicated diagnostic tools for prostate cancer lack efficacy for the detection or diagnosis of cancers not of the prostate.

2.2.4.1 Tissue Biopsy

A prostate tissue biopsy is an invasive procedure for the removal of a sample of cells from an aberrant prostatic mass. With the patient immobilized on his side, trained
medical personnel guided by ultrasound, insert a specialized needle for biopsy through the rectum into the prostate to collect a small sample of cells from the aberrant mass as well as a separate collection of normal cells adjacent to the aberrant mass. A trained pathologist evaluates the biopsied cells underneath a microscope to determine if the obtained sample reveals cancerous cells. Cellular morphology and molecular marker-specific colorimetric stains assist the pathologist in discerning cancer cells from non-cancer cells. Possible and sometimes common side effects following a needle biopsy include but are not limited to bleeding, blood in the urine, and blood in semen, difficulty with urination or infection of the sampled area.

2.2.4.2 Magnetic Resonance Imaging

An MRI scan is a non-invasive medical procedure that applies a considerably strong magnetic field across the area encompassing the patient’s prostate forcing the alignment of hydrogen molecules. Simultaneously, radio waves pass through the scan area generating high-resolution, cross-sectional images of solid tissues within organ systems. Performed by a staff of trained medical professionals, this procedure positions the patient anatomically inside of the MRI machine that scans the prostatic region in 360°. Upon conclusion of the scan, superimposed images generate a 3-dimensional (3D) composite of the gland and possible masses present. Alternatively, adjacently aligned images generate composites of the gland and possible masses for a topographical view. Sometimes, scanning for diagnosis using MRI relies on the assistance of injected dye to enable greater contrast of internal anatomical structures.
An MRI scan alone does not determine prostate cancer; rather, the scan merely identifies suspicious masses. Follow-up with a needle biopsy typically determines if the suspected abnormal mass is cancerous or not. The machine for MRI scans produces loud noises that could be damaging to patients. Additionally, the magnetic field generated is more than 10,000 times stronger than Earth’s core; it is imperative that patients harboring metal implants do not seek this method of scanning. Scanning by MRI is expensive and time-consuming in comparison to other methods available.

2.2.4.3 Computerized Tomography

A CT scan is a non-invasive medical procedure that utilizes x-rays for generation of internal gross anatomical structures. While a patient lies on his back inside of a CT machine, the patient is circles by a device that methodically passes x-rays through a patient’s diagnostic area of interest before collecting the x-rays as they exit the body. A collection plate mirrors and collects each x-ray passed through the patient, enabling measurement and interpretation scanned internal structures. Upon conclusion of the scan, superimposed images generate a 3-dimensional (3D) composite of the gland and possible masses present. Alternatively, adjacently aligned images generate composites of the gland and possible masses for a topographical view.

The detection of abnormal tissue architecture that differs from normal tissue architecture less than 10mm in size, evades the sensitivity of CT scans. When scanning lymph nodes by CT, physicians have reported success augmenting sensitivity to almost 70% and improving specificity to near 100% by employing size-detection limits coupled with fine-needle aspiration of lymph nodes. A CT scan demonstrates greatest
prognostic value when aberrations for detection exhibit higher density. Tissue masses of lower densities are not easily visible on a CT scan. A CT scan alone does not determine prostate cancer; rather the scan merely identifies suspicious masses. Follow-up with a needle biopsy typically determines if the suspected abnormal mass is cancerous or not. The use of positron emission tomography (PET) scans challenges the CT scan by enabling observation of metabolic activity of visualized masses. Both the CT scan and the PET scan rely on potentially damaging, ionizing radiation.\textsuperscript{94, 97}

2.2.4.4 \textit{Ultrasound}

During the medical procedure for an ultrasound, a transducer passes waves of sound through the diagnostic area of interest of a patient. Sound waves travel linearly through bodily cavities until striking the mass of an object. Sound waves reflected from the surface of objects with sufficient mass and return to the transducer. Emitting and receiving sound waves, a transducer relays information to a computer system that integrates, interprets and graphs perceived structures. Ultrasonic scans utilize high-frequency waves that are inaudible to the naked human ear. The leading ultrasonic diagnostic tool used today is the transrectal ultrasound (TRUS). During this procedure, a patient lies on his side with his knees bent and drawn toward his chest. A medical practitioner inserts a transducer probe into the rectum, scanning until reaching the site of the prostate. A tissue biopsy enables further analysis if a suspicious mass is evident. Video and images obtained during the scan enable diagnostic evaluation post-scanning. Contrast-enhanced ultrasonography (CEUS) as well as ultrasound electrography each demonstrates greater accuracy and reliability for sensitivity of detection when employing
ultrasonic technology for the diagnosis of prostate cancer compared to more popular techniques used recently.\textsuperscript{100} The low positive-predictive value of TRUS alludes to the scans inability to detect small tumors when present.\textsuperscript{101} However, TRUS is sufficient for the detection of tumors overlooked by digital screening methods.\textsuperscript{101-102} Prognostic value enhances when TRUS accompanies needle biopsies.\textsuperscript{103} Medical complications that follow a TRUS scan are uncommon.

2.2.4.5 Urine Flow Test

Measurement of the efficiency of the bladder, sphincters and urethra better enable medical personnel to assess and identify possible abnormal functions in the overall physiology of the male urinary system as it pertains to prostate cancer. The bladder is a membranous sac that collects and stores liquid waste as urine until clearance from the body.

2.2.5 Current Trends in Treatment

Current practices for the treatment of prostate adenocarcinoma vary greatly and factor into consideration the stage, aggressiveness or metastatic potential of the disease and patient medical history. Some of the more common treatments for prostate cancer include surveillance, androgen deprivation therapy, prostatectomy, cryotherapy, chemotherapy, radiation therapy, or combination therapy.

2.2.5.1 Watchful Waiting

During BPH or early-stage prostate cancer when the disease remains localized to a confined region, a medical staff may consult with the patient to advise a practice of watchful waiting. The surveillance of watchful waiting attempts to monitor the activity of
the tumor cells prior to determining if invasive, costly or potentially risky recommendations of medical action is necessary to improve disease prognosis. Medical personnel employ active surveillance by tracking tumor status comparing PSA levels or cellular morphology and localization over time.\textsuperscript{104-105}

\subsection*{2.2.5.2 Androgen Deprivation Therapy/Hormone Therapy}

In early-stage prostate cancer disease while cancerous cells are still confined to the primary tumor, a medical staff may recommend castration of the androgen-producing testes and chemical ablation of androgen receptor signaling using androgen deprivation therapy (ADT) and chemical inhibitors specific to the androgen receptor.\textsuperscript{106-107} This practice attempts to abate the molecular driving force of androgen-dependent disease.\textsuperscript{106-107}

\subsection*{2.2.5.3 Prostatectomy}

For men whose cancer remains localized and confined to the prostate but has spread throughout various regions of the organ, a medical staff may recommend surgery to remove the confined cancer cells during a radical prostatectomy.\textsuperscript{108-110} A prostatectomy attempts to remove the entire prostate gland. Men that undergo a radical prostatectomy may experience complications that lead to erectile dysfunction (ED), reduced sexual drive or incontinence among other symptoms.

\subsection*{2.2.5.4 Cryoablation Therapy}

Under conditions during which cancer manifests in isolated masses localized to the prostate, a patient may receive cryoablation therapy. Cryoablation therapy relies on the precision of medical probes that subjects cancer cells or diseased tissues to extreme
cold temperatures. Upon freezing, cancer cells die without harm to adjacent, normal cells.

2.2.5.5 *Radiation Therapy*

During localized, low-grade cancer, high-energy particles alone or in concert with hormone therapy function to kill cancer cells. Radiation therapy can capture lone cancer cells if surgical removal of the cancer fails to capture all of the cells.

2.2.5.6 *Chemotherapy*

For cancers that have metastasized or moved beyond the prostate, a medical staff typically employs intravenous or oral administration of anti-cancer pharmaceuticals that navigate the body and target remote cancer cells for death. Unique to this treatment course is the ability of target-specific chemical agents to locate and discriminate against aberrant cells. The chemical agents employed exploit one or more molecular target expressed on the surface of tumors.

Oftentimes in circumstances of metastatic disease, a medical staff may implement adjuvant care or treatments in tandem in order to maximize a patient’s prognosis and aggressively target remote and elusive cancer cells. There exists a variety of therapeutic treatments available designed to address each stage of prostate cancer, specific to time and status of disease upon diagnosis. Treatments for prostate cancer vary in levels of success, ranging from ineffective to total ablation of all clinically detectable cancer signs and symptoms. Whether applied in a hospital setting or at-home, each treatment for prostate cancer aims to reduce rates of cancer-related mortality.
It is important to narrow the symptoms at presentation to clinic as to avoid over-diagnosis of medical complications. Over-diagnosis can lead to unnecessary treatments involving costly, time-consuming, frightening, or even life altering medical procedures. Similarly, misdiagnosis as well as underdiagnoses can neglect the necessary medical required for timely intervention that could preserve the quality of life or life itself. Taken together, the patient and medical staff must work together in the decision making process to integrate information for the best prognosis possible for each patient. Each step of the screening, diagnostic, treatment and follow-up process requires the integration of accurate information for the most successful of patient outcomes.

2.2.6 Prostate Cancer Cell Lines

The prostate cancer cell lines used in this study represent the two overarching phenotypes of the disease, hormone-dependency and hormone-independency. The hormone-dependent LNCaP cell line established from cells isolated a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year old Caucasian male.\textsuperscript{118} The hormone-independent PC3 cell line established from highly metastatic cells isolated from the lower lumbar of a 62-year old Caucasian male.\textsuperscript{119}

2.3 Protein Arginine Methyltransferases

Following protein biosynthesis, biological systems rely on molecular mechanisms that execute events of posttranslational modification (PTM) for the generation of more specialized proteins that augment the functionality of the mammalian proteome. The complex regulation of PTM events occurs as an interconnected, multilayered process governing events of epigenetic regulation and signal transduction.\textsuperscript{120-121} Events of PTMs
range from cleavage or degradation of individual protein subunits or entire proteins to the transient addition of reversible covalent functional groups or proteins.\textsuperscript{121-122} Impacting a diverse range of cellular functions, PTMs regulate the cellular microenvironment throughout health and disease alike.\textsuperscript{123} Regulation of the cellular microenvironment by way of PTMs refers to one or more enzymatic or structural modifications by processes of phosphorylation, ubiquitination, acetylation, hydroxylation or methylation.\textsuperscript{120} While most events of PTMs manifests as events of phosphorylation, recent evidence suggests that protein methylation may be just as common.\textsuperscript{124}

2.3.1 Overview of Protein Methyltransferases

The protein arginine methyltransferase (PRMT) family of nine enzymes orchestrates a plethora of PTMs affecting protein signaling, transcriptional regulation, RNA metabolism and DNA repair among other cellular processes as illustrated in Figure 7.\textsuperscript{13, 125-131} Purified recombinant PRMTs evaluated \textit{in vitro} express relatively low constitutive activity under normal conditions, suggesting basal activity may be required for normal metabolic functions. Canonically, PRMT5 associates with MEP50 as a member of the methylosome. A single PRMT5 molecule situates inside of a pocket of proteins that interacts with motifs unique to PRMT5. Mutant Jak2 was reported to disrupt the association of MEP50 and PRMT5 at PRMT5’s TIM barrel.\textsuperscript{18, 132-133} Events of post-translational modification can function to alter the substrate specificity of PRMT enzymes. Multiple PRMTs have been characterized for their modification of histone tails.
Figure 7. PRMT5 regulates multiple biological mechanisms.\textsuperscript{131} Through events of symmetric dimethylation, PRMT5 participates in the regulation of RNA processing, signal transduction and suppression or promotion of gene transcription through modification of the tails of histone proteins.

Three structurally distinct sub-classes simplify classification of the methyltransferases according to the type of methylation pattern each carries out: Type I PRMTs are responsible for the generation of asymmetric dimethylarginine (aDMA); type II PRMTs are responsible for the generation of symmetric dimethylarginine (sDMA); type III PRMTs are responsible for the generation of asymmetric monomethylarginine (aMMA).\textsuperscript{134-135} The enzymology for PRMT-generated transfers of methyl groups is
similar across all subclasses with respect to the plane of symmetry about the length of recipient arginine residues.

Protein arginine methylation is carried out by the transfer of one or more methyl groups from the methyl donor of $S$-adenosylmethionine (AdoMet) onto terminal (ω-omega) guanidino nitrogen atoms of arginine residues on target substrates. Favorably positioned for interaction with hydrogen bond acceptors, the sidechain of the terminal arginine amino acid contains five unique protonated hydrogen atoms.

In addition to arginine methylation, the other two major classes of protein methyltransferases are lysine and membrane-bound methyltransferases. The functional significance of methyltransferases is grouped and distinguishable according to their structural composition. The AdoMet-dependent methyltransferases are divisible by three classes. Class I methyltransferases harbor a seven-stranded β-sheet motif. Class II methyltransferases contain a SET lysine methyltransferase domain. Class three methyltransferases are the only known membrane-associated methyltransferases. Characterized protein substrates methylated by PRMTs typically harbor motifs that are rich in glycine and arginine (GAR). Figure 8 provides a list of common substrates of PRMT methylation, by enzyme. Target substrates reported in literature are unique to each enzyme for proteins listed in Figure 8 and does not represent a comprehensive listing.
The binding pocket for enzymatic activity is formed of AdoMet in concert with I, post-I and THW, depicted in Figure 9. \textsuperscript{136-137}

### Figure 8. List of PRMT substrates.
A comprehensive list of known PRMT targets. Targets of PRMT methylation typically harbor a GAR motif.

<table>
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<th>PRMT6</th>
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Figure 9. Domain homology of human PRMT enzymes. Comparison of PRMT family structural homology depicting conserved motifs and domains for functional diversity.

The methylosome is a 20s cytosolic complex containing pICln (regulates the assembly of U1, U2, U4 and U5)\textsuperscript{139}, spliceosomal snRNP, Sm core proteins, the WD repeat protein, WDR77/MEP50/WD45/p44 and PRMT5.\textsuperscript{140-142} In the cytosol, this complex executes the symmetric dimethylation of arginine residues on target substrates. The methylosome enhances the PRMT5-mediated transfer of methyl groups.
2.3.1.1 Type I: Asymmetric Dimethylation

Asymmetric dimethylation is the predominant type I PRMT of all methylated arginine residues to occur in mammalian cells.\textsuperscript{134,143} As depicted in Figure 10, this class of enzymes asymmetrically deposits two methyl groups onto target substrates.\textsuperscript{144} Current literature suggests that all PRMT enzymes are first monomethylated prior to further modification and subsequent functional designations.\textsuperscript{145} Enzymes 1, 2, 3, 4, 6, and 8 are all type I PRMTs according to their enzymatic activity.\textsuperscript{15}

2.3.1.2 Type II: Symmetric Dimethylation

Symmetric dimethylation is the predominant type II PRMT of all methylated arginine residues to occur in mammalian cells.\textsuperscript{146}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Generation of type methylation by protein arginines.\textsuperscript{2} Generation of type I asymmetric dimethylation, type II symmetric dimethylation or type III monomethylation by classes of PRMT enzymes.}
\end{figure}
Type II PRMTs symmetrically deposit methyl marks onto target substrates as shown in Figure 10.\textsuperscript{2,146}

Enzymes 5 and 9 comprise the only type II PRMTs given the pattern of methyl marks that they execute.\textsuperscript{15}

2.3.1.3 Type III: Monomethylation

Each arginine methylation event may illicit different post-translational mechanisms to regulate biological events\textsuperscript{7}. Type I PRMTs comprises PRMTs 1, 3, 4, 6 and 8. Type II PRMTs comprises both PRMTs 5 and 7. PRMT 7 is also currently the only Type III PRMT discovered to date as depicted in Figure 10.\textsuperscript{2}

2.3.1.4 Protein Arginine Methyltransferase 5

Originally discovered as the Janus Kinase-Binding Protein 1 (JBP1) or Jak2, PRMT5 is the predominant effector of symmetric deposition of most methylation marks in mammals.\textsuperscript{146} The functional product of \textit{PRMT5} is a 72kDa protein. Each molecule of PRMT5 adopts the formation of a TIM barrel at its amino terminal, shown in Figure 11.\textsuperscript{18,132}
A molecule of MEP50 interacts with a molecule of PRMT5 through its TIM barrel shown in Figure 11.\textsuperscript{18,132} Structurally, four PRMT5 molecules arrange adjacent to one another forming a tetramer; four MEP50 molecules externally flank the PRMT5 tetramer. Evolutionarily conserved in humans and mice, \textit{PRMT5} and its ortholog are located on chromosome 14 according to the NCBI human genome sequence. Alternative splicing of \textit{PRMT5} mRNA generates six isoforms as reported in the NCBI human genome sequence.
Nine residues have been identified as necessary for maintaining the interaction between PRMT5 and MEP50. The hetero-octameric union of PRMT5 and MEP50 is approximately 453kDa. The catalytic site of PRMT5 adopts the canonical type I PRMT AdoMet binding domain. The catalytic site of PRMT5 contains a SAM domain, a Rossman fold and a β–sandwich. The SAM domain enables binding of both substrates and cofactors while the β–sandwich assists with substrate binding. The Rossman fold enables binding of nucleotides. Protein backbone interactions largely facilitate substrate interaction with PRMT5. The binding pocket of the PRMT5 active site contains a double-E loop that harbors two conserved residues, Glu435 and Glu444. The glutamate residues 435 and 444 establishes salt-bridges with the guanidino side chains of substrate-arginines in preparation for the transfer of methyl group.

Protein arginine methyltransferase 5 functions by catalyzing the monomethylation and symmetric dimethylation of target substrates. Symmetric dimethylation by PRMT5 occurs in a mechanism of successive distribution, validated by the observation that events of dimethylated product occur at rates slower than events of monomethylated product. A monomethyl peptide released from PRMT5 is recycled for carrying out the second methylation event generating a dimethylated product only after the concentration of monomethylated product exceeds the concentration of dimethylated products. Mammalian PRMT5 requires tight association with MEP50 for catalytic activity. Regulation of the PRMT5-MEP50 complex was demonstrated by observation of phosphorylation at T5 on MEP50, by cyclin D1 – CDK4. The co-substrate, SAM serves as a methyl donor to PRMT5 for methylation of a variety of substrates.
PRMT5, the predominant type II protein arginine methyltransferase, was originally identified as a transcriptional repressor.\textsuperscript{146, 153} The regulation of gene expression is possible through PRMT5 methylation of histone tails. Histone substrates reported to be targets of PRMT5 methylation include H2AR3, H2R3, H3R8, and H4R3.\textsuperscript{144, 154-156} Chiefly, PRMT5 generates two patterns of methyl marks on target substrates: H3R8me2 and H4R3me2.\textsuperscript{157-158}

The majority of symmetric dimethylated protein substrates is resultant of regulation by PRMT5. Binding partners of PRMT5, including those appearing in Table 2, can alter the enzyme’s substrate specificity to effect methylation targets and biological signaling. Known PRMT5 binding partners include the chromatin remodeling complex SWI/SNF, RNA-binding protein Y14, methylosome adaptors RIOK1 and ICLN, histone-binding protein cooperator CORP5 and HSP90.\textsuperscript{154-155, 159-161}

**Table 2.** Major PRMT5 interacting proteins.\textsuperscript{132} List of proteins that associate with PRMT5 and alter its biological activity.

<table>
<thead>
<tr>
<th>PRMT5 Binding Partner</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP50 (Wdr77/P44)</td>
<td>Essential for PRMT5 histone methylation</td>
</tr>
<tr>
<td>pICln</td>
<td>Contributes to spliceosome assembly and directs PRMT5 methylation to Sm proteins</td>
</tr>
<tr>
<td>RioK1</td>
<td>Competes with pICln for PRMT5 binding and recruits nucleolin for methylation</td>
</tr>
<tr>
<td>Menin/MEN1</td>
<td>Adapter protein for MML methyltransferase that targets PRMT5 to chromatin</td>
</tr>
<tr>
<td>CoPR5</td>
<td>Mammalian nuclear protein that targets PRMT5 to chromatin</td>
</tr>
<tr>
<td>hSWI/SNF</td>
<td>Targets PRMT5 to chromatin and methylation of histone H3</td>
</tr>
<tr>
<td>JAK kinases</td>
<td>Mutant Jak2 found in leukemia phosphorylates PRMT5 and reduces its activity</td>
</tr>
<tr>
<td>Blimp1</td>
<td>Localization of PRMT5 in primordial germ cells</td>
</tr>
<tr>
<td>AJUBA</td>
<td>Coordinates PRMT5 interaction with SNAIL</td>
</tr>
<tr>
<td>Piwi</td>
<td>Recruitment via Tudor domain proteins to piRNA pathways</td>
</tr>
</tbody>
</table>
2.3.2 PRMTs in Cancer

Overexpression and hyperactivity of each of the PRMTs has been reported in multiple cancer types. Dysregulation of canonical PRMT activity is achieved through a variety of mechanisms. Research suggests that hyperactivity of PRMT functions promotes the tumor microenvironment and perpetuates cancer progression.

2.3.2.1 Type I Protein Arginine Methyltransferases

The protein arginine methyltransferase PRMT1 has been observed to be hyperactive in multiple cancer types, correlating with disease. Asymmetric dimethylation of arginine 3 on histone 4 (H4R3me2a) results in transcriptional activation. In fact in prostate cancer, protein expression of PRMT1 correlates positively with tumor grade and accurately predicts recurrence. DNA repair proteins MRE11 and p53 binding protein 1 (p53BP1) appear to promote cancer phenotypes through dysregulated PRMT1 methylation. Inhibition of methyltransferases perturbs the p53BP1 response to damaged DNA.

During events of PTMs, PRMT4 is phosphorylated in a process that eliminates its methyltransferase activity. However, overexpression of N-acetylglucosamine transferase (OGT) was reported to inhibit phosphorylation of PRMT4 at S217, subsequently enabling unregulated mitotic events. Acetylation of lysine 18 on histone 3 (H3K18ac) enables asymmetric dimethylation of at arginine 17 (H3R17me2a) of PRMT4.

Methylation of the inner centromere protein INCEP by PRMT1 enhances the mitotic activity of cancer cells. In estrogen-receptor positive breast cancer cells,
PRMT2 knockdown resulted in attenuation of cellular growth and migration.\textsuperscript{175} Silencing of PRMT6 in PC3 cells upregulated p21, p27 and CD44.\textsuperscript{176} PRMT6 overexpression impairs Histone 3 arginine 2 asymmetric dimethylation contributing to a global pattern of hypomethylation across DNA and correlates with cancer.\textsuperscript{177} Overexpression of PRMT8 has been demonstrated to be correlated with breast, ovarian and gastric cancers.\textsuperscript{178}

2.3.2.2 Type II Protein Arginine Methyltransferases

The dissociation of PRMT5 from MEP50 inhibits the methyltransferase potential of PRMT5.\textsuperscript{133} More specifically, regulation of the PRMT5-MEP50 complex was demonstrated by observation of phosphorylation at T5 on MEP50, by cyclin D1 – CDK4.\textsuperscript{151} Hyperphosphorylation of T5 on PRMT5 augments the methyltransferase activity of PRMT5, driving growth and proliferation of cells.\textsuperscript{179}

Cytoplasmic localization of PRMT5 is correlated with growth and proliferation of LNCaP cells.\textsuperscript{25} Overexpression of PRMT5 in LNCaP cells generated greater rates of growth and proliferation.\textsuperscript{25} Inhibition of PRMT5 enzymatic activity alleviated growth and proliferation of LNCaP cells.\textsuperscript{25} PRMT5 protein expression was observed to elevated in a panel of lung cancer cells, compared to normal lung cells. Knockdown of PRMT5 resulted in attenuation of A549 cells growth and proliferation.\textsuperscript{21} Inhibition of PRMT5 \textit{in vivo} resulted in reduced tumor volume and mass.\textsuperscript{21}

Elevated levels of PRMT5 protein is apparent in a variety of transformed cells. Knockdown of PRMT5 in transformed cells retards cellular growth while overexpression of PRMT5 correlates with high rates of proliferation.\textsuperscript{157, 180} The transition of cells from an epithelial phenotype to a mesenchymal phenotype is facilitated by aberrant PRMT5
expression. By way of an interaction with SNAIL the adaptor protein AJUBA, PRMT5 enables downregulation of \textit{E-Cadherin}.\textsuperscript{181} Loss of E-cadherin compromises the architectural integrity of polarized cells, enabling motile conditions. Silencing of PRMT5 using siRNA also suppresses \textit{E-Cadherin}. Cullin proteins (CUL4A and CUL4B) are adaptors for ubiquitination that enable the modification of protein function or signal for degradation. Hyperactivity of PRMT5 suppresses \textit{CUL4A} and \textit{CUL4B}, enabling neoplastic potential of cells.

For instance, PRMT5 catalyzes the symmetric dimethylation of p53, E2F1, RB, homeobox A9 (HOXA9) and RAD9.\textsuperscript{182-185} PRMT5 exhibits regulatory effects on opposite ends of the axis for epidermal growth factor receptor (EGFR)-RAS-ERK signaling. At arginine 1175, PRMT5 methylates EGFR, enabling phosphorylation of tyrosine 1173 to facilitate docking of SHP1.\textsuperscript{186} Inhibition of arginine 1175 methylation promotes growth, proliferation invasion and migration of mammary gland epithelial cells through hyperactive EGFR signaling.\textsuperscript{186} Alternatively, Melanoma cells treated with hepatocyte growth factor (HGF) augmented phosphorylation of MEK1, MEK2, ERK1 and ERK2.\textsuperscript{187} Transcriptional suppression of tumor suppressor genes by PRMT5 potentiates oncogenic perturbations and contributes to carcinogenic phenotypes. Hyperactivity of PRMT5 downregulates the expression of ST7 (correlated with autism) and \textit{NM23} (correlated with neuroblastomas) by symmetric dimethylation of arginine 8 on histone 3 (H3R33me2s).\textsuperscript{154} In myoproliferative neoplasms, phosphorylation of Jak2 lead to downregulation of PRMT5 methyltransferase activity, promoting new neoplasms.\textsuperscript{133}
PRMT5 effects biological functions by executing methylation of chromatin-associated proteins and transcription factors, alike. Acetylation of lysine 9 of histone 3 (H3K9ac) blocks PRMT5-mediated symmetric dimethylation (H3R8me2s).\(^{154}\) Interestingly, acetylation of lysine 5 on histone 4 (H4K5ac) alters the methyl marks of motif H4R3 such that PRMT5 methylation suppresses H4R3 rather than PRMT1 activating H4R3.\(^{158}\) Events of PTMs can function to influence crosstalk between protein arginine modification and histone proteins.

In cancers of the bladder, blood, breast, colon, lung, ovaries and prostate, PRMTs were reported upregulated or overexpressed.\(^{20-25}\) More research is required to develop novel mechanisms of therapeutic intervention that can function to induce remission and reduce disease progression. The evidence that links PRMTs to cancer is growing and strengthening as investigators continue to uncover the biological consequences of PRMT hyperactivity. The overwhelming observations of PRMT hyperactivity in multiple cancer types suggest that targeting one or more PRMTs may prove relevant for therapeutic intervention.

2.3.3 PRMT Inhibitors

Arginine methylation has been evaluated under the auspice of small-molecule inhibitors. The only natural and global methyltransferase inhibitor identified to date, Sinefungin disrupts the methyltransferase activity of both lysine and arginine methyltransferases.\(^{188-189}\) Sinefungin is an analog to the AdoMet and restricts methylation by inhibiting the transfer of methyl groups dependent upon the binding of AdoMet,
including PRMT-mediated methylation. Hosts of other methyltransferase inhibitors have been discovered through screening techniques.

Eight compounds obtained from ChemBridge were identified by Wang et al. to interact with the substrate-binding site within PRMT5 to inhibit its methyltransferase activity. One (C9a) of these compounds demonstrated efficacy in vitro and in vivo to inhibit cellular proliferation, halt cellular growth and generated reductions in tumor volume and size.\textsuperscript{21, 25, 190}

The small chemical inhibitor designed by Smil et al., DS-437 competes for binding to the AdoMet to inhibit PRMT5 methyltransferase activity.\textsuperscript{191} This dual inhibitor inhibits the methyltransferase activity of both PRMT5 and PRMT7, but not 29 other targets. DS-437 demonstrated its PRMT5-specific inhibition in the low micromolar range (6µM).\textsuperscript{190}

PRMTs have been shown to be upregulated in a number of different cancers and plays an essential role in growth of various cancer cells.\textsuperscript{132, 192} Consistent with these observations, PRMT5-inhibitors suppressed growth of lymphoma, AML, and MCL cells in tissue culture.\textsuperscript{193-195} More recently, EPZ015666 demonstrated antitumor activity in multiple MCL xenograft models.\textsuperscript{195}

\textbf{2.4 Plant-Based Medicine}

Developed largely as mechanisms of defense against microbial threats, plants continue to evolve producing protective and adaptive strategies to promote self-survival.\textsuperscript{196} People indigenous to an area often referred to nature to remedy their symptoms and escape the grasp of disease. Human exploitation, consumption and
application of agents isolated from plants dates back throughout the millennia. Worldwide, more than 28,000 species of plants account for medicinal usage, while fewer than 16% of these plant species receive accurate references in publication. Despite the lack of documented pre-clinical or clinical research, the use of plants for therapeutic intervention and prevention of disease is gaining in popularity. The therapeutic value of responsibly administered plant-based medicine provides and potentiates efficacious relief from a variety of conditions including but not limited to disease of infection, deficiency, heredity, and physiology as well as the Human Immunodeficiency Virus (HIV).

2.4.1 Natural Alternatives to Chemotherapy

Exposure to non-dietary environmental factors provide evidence of positive outcomes influencing the onset, management and prognosis of prostate cancer. Oral administration of the chemical inhibitor, Finasteride in human clinical trials demonstrated a reduction in the prevalence-risk of prostate cancer by way of inhibiting the conversion of testosterone to dihydrotestosterone. Administration of the chemical, Dutasteride in human clinical trials reduced the risk of early-stage prostate cancer in comparison to control groups by way of inhibiting the conversion of testosterone to dihydrotestosterone. The naturally occurring inhibitors Finasteride and Dutasteride competitively inhibit 5\(\alpha\)-reductase preventing the conversion of testosterone to dihydrotestosterone in a mechanism that reduces incident prostate cancer observed in short-term clinical trials.

Treatment of prostate cancer cells using Muscadine Grape Skin Extract (MSKE) averted autophagy-mediated cell survival mechanisms suppressing Bcl2 expression and
sensitized cells to apoptosis via activation of PARP, caspases and the Unfolded Protein Response (UPR) in comparison to controls. Treatment of breast and prostate cancer cells using MSKE suppressed SNAIL1 expression and phosphorylation of STAT3, preventing Cathepsin L (CatL) expression and abrogated invasion and metastasis in advanced breast and prostate cancer cells. Chemical intervention using the antioxidant, MSKE administered to prostate cancer cells assisted Superoxide Dismutase (SOD) in reverting the aberrantly expressed profiles of E-cadherin and vimentin to near normal expression and reverted the pro-cancer effects of EMT and Reactive Oxygen Species (ROS) by targeting overexpressed SNAIL1. Evaluated in randomized human clinical trials, MSKE did not demonstrate significant effects for lowering values of classical prognostic markers used in prostate cancer detection.

Advanced prostate cancer cells treated with the phytoalexin, camalexin, experienced a reduction in viability and an induction in apoptosis, mediated through ROS generation. In comparison to primary epithelial and hormone-dependent prostate cells, camalexin did not exhibit similar results. Although the exact mechanism has to be determined, camalexin selectively antagonized metastatic prostate cancer cells through a ROS-dependent mechanism.

2.4.2 Dendrobium aurantiacum ver. denneanum

The flowering Orchidaceae (orchid) plant, D. denneanum, is native to the southern provinces of China and the western Himalayas. This orchid congeneric grows robustly in the Sichuan province and does not require protection from cultivation. The plant germinates in the shallow soils, along the base of mountainsides.
More than 1000 species of plants comprise the genus *Orchidaceae*. Southern China was once home to more than 74 species of wild *Dendrobium*.\textsuperscript{213} Resultant of overharvesting to meet the growing interest of clinical trials, many species of *Dendrobium* appear on national or international lists of endangered plants. The five endangered species most notable for owning the medicinal benefits ascribed to *Dendrobium* known colloquially as “Shi Hu,” and their congeneric relatives appear in the 2010 Chinese Pharmacopoeia.\textsuperscript{214} Traditional Chinese medicinal practices relied on Shi Hu for ailments of imbalance rumored to replenish yin. Contrary to popular trends in the use of botanicals for medicinal application, Shi Hu relies on the stems of orchid plants rather than the flower petals or leaves.\textsuperscript{215}

Previous experimental evaluations of the plant source *D. denneanum* yielded more than 20 secondary metabolites possessing some medicinal value for the prevention of inflammation, fever, aging and macular degeneration.\textsuperscript{214, 216} The compound, Gigantol isolated from *D. denneanum* suppressed gene expression to inhibit the formation of galactose-induced cataracts in rats.\textsuperscript{217} A phenolic glycoside from *D. denneanum* selected against acute myeloid leukemia cells but not control cells and antagonized glutamate to induce apoptosis in PC12 cells.\textsuperscript{218}
Figure 12. Dendrobium denneanum. (A) Fully grown and labeled *D. denneanum* (B) Traditional methods of consumption of *D. denneanum* including tea, broth and stew, from top to bottom, respectively. (C) Image of *D. denneanum* stems used for extraction.
CHAPTER III
EXPERIMENTAL METHODS AND TECHNIQUES

3.1 Cell Culture

Adherent monolayer cultures of androgen-dependent and androgen-independent human LNCaP and PC3 prostate cancer cell lines obtained from the American Type Culture Collection (ATCC) from were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium from Gibco by Invitrogen (31800-089) completed by supplementation with 10% FB Essence (FBE) from Seradigm (3100-500), 1% L-Glutamine from Gibco by Invitrogen (25030-081) and 100mM Penicillin/Streptomycin from Corning (30-002Cl) in Corning T-75 flasks (430641). Cell lines were grown at 37°C in humidified atmospheric conditions of 5% CO2. Cell culture media was replenished every third day after washing using 1x phosphate buffered saline (PBS). Once LNCaP and PC3 populations reached near-confluence, cells were split 1:3 and 1:6, respectively.

3.2 Growth Assay

LNCaP and PC3 cells were seeded into Nunclon ∆ Surface 24 well-plates (142485) at 1.0x10^4 and 6.0x10^3 cells per well respectively and permitted to attach overnight. Cells were washed using 1xPBS. On the following day, culture medium was
replaced using RPMI 1640 supplemented with 2% FBE. Experimental cells received titrations of crude or purified plant extracts. Control cells received DMSO, determined by and equivalent to the largest volume of plant extract utilized for treatments. Cell lines were grown for 72 hours at 37°C in humidified atmospheric conditions of 5% CO₂. Cells were harvested using Trypsin-EDTA from Sigma (T4174). In a microcentrifuge tube, a subpopulation of cells was reconstituted into an equivalent volume of RPMI1640 and Trypan Blue Solution (Sigma T8154) stain and counted manually using a hemocytometer.

3.2.1 Cell Counting Using a Hemocytometer

Hemocytometer chambers were stored while submerged in at least 70% ethanol prior to use. Air-dried chambers were positioned on a flat surface prior to loading and covered using a hemocytometer glass coverslip carefully positioned atop of the chamber for counting. A 20µL aliquot of treated or untreated cell suspension was introduced into the loading chamber of a hemocytometer using a suitable pipette. Cells appearing on or beyond the boundary lines of any quadrant were excluded from observed cell counts. These cells were not counted. Similarly, cells appearing within the boundary lines that denote the cross-structure were omitted from observed counts. These cells were not counted. Only cells appearing definitively within quadrants 1, 2, 3, or 4 were observed for counts. After counting, each hemocytometer chamber and accompanying coverslip was cleared of cells by manual wiping with a ChemWipe containing 70% ethanol and permitted to air-dry prior to successive counts.
3.3 Cell Cycle Analysis

LNCaP and PC3 cells were seeded into 100mm VWR Tissue Culture Dishes (10062-880) at 4.8x10^5 and 2.4x10^5 cells per dish, respectively. Cells were permitted to attach overnight. On the following day, cells were washed using 1xPBS and culture medium was replaced using RPMI 1640 supplemented with 2% FBE. Experimental cells received titrations of crude or purified plant extracts. Control cells were treated with DMSO determined by and equivalent to the largest volume of plant extracts utilized for treatment. Cell lines were grown for 72 hours at 37°C in humidified atmospheric conditions of 5% CO₂. Media containing concentrations of treatments were removed using needle-aspiration. Cells were washed using cold 1xPBS. Dishes containing cells and PBS were placed on ice to limit protein degradation due to ambient temperatures. While in 1xPBS, cells were harvested manually by gently detaching the monolayer of cells and transferred to pre-chilled 15-mL conical tubes. Cells were pelleted using a desktop centrifuge at 4°C, 0.6xRCF, for 2 minutes. Supernatants were removed by pipetting. Cells were resuspended into 100µL of cold 1xPBS. Under gentle vortexing exercising caution not to lyse cells, each sample of cells received 3mL of cold 70% ethanol. Cells were incubated overnight in 70% ethanol and 1xPBS at 4°C.

On the following day at room-temperature (RT), cells were pelleted using the conditions previously describe with a desktop centrifuge. Supernatants were removed by pipetting. Cells were resuspended into Propidium Iodide (PI) solution (10µg/mL final concentration) containing RnaseA (0.2mg/mL final concentration), transferred to
microcentrifuge tubes and incubated for 20 minutes at RT. After 20 minutes, samples were transferred to ice and protected from white light until same-day analysis using the Accuri C6 Flow Cytometer.

3.4 Proliferation Assay

3.4.1 Preparation of Coverslips

Glass coverslips were prepared inside of a class II sterilized biological safety cabinet. One coverslip for each well of Nunclon Δ Surface 6 well-plates (140675) was incubated at RT for 15 minutes in 100% EtOH then permitted to air-dry from a vertical position. Coverslips were then subjected to one round of ultraviolet light for 15 minutes. Coverslips were placed squarely into individual wells of the 6 well-plates. Coverslips were carefully handled using forceps for the duration of the entire time for preparation.

3.4.2 Bromodeoxyuridine (BrdU) Incorporation Assay

LNCaP and PC3 cells were seeded onto coverslips in 6 well-plates at densities of 1.5x10^6 and 2.5x10^6 cells per well, respectively, in RPMI 1640 complete medium as described in the Cell section. After 24 hours, medium was removed and cells were washed twice using 1 x phosphate buffered saline (PBS), before replacing media with fresh RPMI (+2%FBE) with dissolved Dimethyl Sulfoxide (DMSO) or plant extracts. Control cells received DMSO, determined by and equivalent to the largest volume of plant extract utilized for treatments. Experimental cells received a titrated range of crude or purified plant extracts. Cells were permitted to grow under these conditions unaltered for 72
hours. Cells were then washed using 1x PBS and replenished with fresh RPMI media containing BrdU (Beckton Dickinson Biosciences 247580). Cells were incubated for 2 hours with BrdU (10µM) and immunostained using anti-BrdU (1:400) antibody (Beckton Dickinson Biosciences) with strict adherence to a protocol published previously. Coverslips were then relocated onto appropriately charged, labeled, clean slides in final preparation for microscopy. Cells were imaged using a Carl Zeiss Confocal-LSM 700 Microscope.

3.5 Minimum Inhibitory Concentration (IC₅₀)

LNCaP and PC3 cells were seeded into Nunclon ∆ Surface 24 well-plates (142485) at 1.0x10⁴ and 6.0x10³ cells per well respectively and permitted to attach overnight. Cells were washed using 1xPBS. On the following day, culture medium was replaced using RPMI 1640 supplemented with 2% FBE. Control cells received DMSO, determined by and equivalent to the largest volume of plant extract utilized for treatments. Experimental cells received a titrated range of crude or purified plant extracts. Triplicates of experimental cells received titrations of crude or purified plant extracts. Cell lines were incubated for 72 hours at 37°C in humidified atmospheric conditions of 5% CO₂. Medium was removed using needle-aspiration. Cells were washed once using 1x PBS. Cells were harvested using Trypsin-EDTA. In a microcentrifuge tube, a subpopulation of cells was reconstituted into an equivalent volume of RPMI1640 and Trypan Blue Solution stain and counted manually using a hemocytometer. Minimum enzyme inhibitory concentrations were determined by identifying the treatment
concentration at which half of the seeded population of cells remained viable at calculated between fold-change ranges of crude or purified compounds. Data sets were analyzed and graphed using Microsoft Excel.

3.6 Protein Isolation and Quantification

Protein samples were isolated using cell scrapers and 1x protein Passive Lysis Buffer (PLB) (Promega E194A), supplemented with Phosphatase Inhibitor Cocktail (Santa Cruz 45044) and Protease Inhibitor Cocktail (Active Motif 100510) for total protein. Protein concentrations were ascertained using the Protein Assay Dye Reagent Concentrate (Bio-Rad 500-0006), Bovine Serum Albumin (BSA) (New England BioLabs B9001S) and the Bradford Assay of the DU800 Spectrophotometer (Becton Coulter). Standard concentrations used were 1, 2, 3, 4 and 5µg/mL. Standards and samples were measured in triplicate with each coefficient R value ≥ 0.998.

3.7 Western Blot Analysis

Protein samples were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were transferred overnight at low voltage (~6000mV) to a nitrocellulose (NC) membrane. In order to reduce the potential for non-specific antibody interaction, NC blots were blocked for 30 minutes at room temperature using 3% non-fat milk (Santa Cruz 2324) in 1 x Tris-Buffered Saline Tween20 (TBST) while shaking at low speeds. Primary antibodies were reconstituted into 2% BSA in 1x T-BST. Blots were incubated in a primary antibody for 2 hours at room temperature while shaking at low speeds. Blots were washed 3 times using 1x TBST while shaking at
moderate speeds over 5 minutes with each wash. Secondary antibodies were reconstituted into 2% BSA in 1x TBST. Blots were incubated in a secondary antibody conjugated with HRP at room temperature for 1.5 hours while shaking at low speeds. Blots were then washed 4 times, each in 5 minute increments using 1x TBST. Protein bands were visualized using an enhanced chemiluminescence substrate (PerkinElmer NEL103001EA) according to specifications recommended by the manufacturer. Densitometry was used to determine the relative concentrations of target protein as normalized according to β-actin for use as an internal standard.

3.8 Antibodies

Anti-dimethyl-arginine, symmetric (SYM10) (07-421) polyclonal rabbit antibody was obtained from EMD Millipore. BrdU (247580) antibody was obtained from Beckton Dickinson Biosciences. Anti-β-Actin (A2103) polyclonal rabbit antibody was obtained from SigmaAldrich. Anti-BrdU (347580) was obtained from Beckton Dickinson Biosciences.

3.9 Radio-Labeled Protein Methylation Assay

Experimental samples were prepared using various concentrations of plant extract, 1µg of SmD3, PRMT enzyme (0.2µg of PRMT1, 0.2µg of PRMT3 or 0.8 µg of PRMT5), BSA, and H2O to volume. Positive control samples were prepared using DMSO, SmD3, PRMT enzyme (0.2µg of PRMT1, 0.2µg of PRMT3 or 0.8 µg of PRMT5), BSA, and H2O to volume. Positive control samples lack an inhibitor of methyltransferase activity. Negative control samples were prepared using DMSO, SmD3,
BSA, and H₂O. Negative control samples lack a PRMT enzyme to execute methyltransferase activity.

Reactions (40µL total volume) were initiated with the addition of a reaction mixture containing reaction buffer (0.5M Tris-HCL, pH 7.5, 10mM EDTA, 10mM EGTA, 0.1M DTT, 10mM SAM, 1µCi of \( S\)-[methyl-H] adenosylmethionine (PerkinElmer) and H₂O) and were incubated at 30°C for 2 hours. Reaction samples were analyzed utilizing one of the two following methods:

3.9.1 Analysis by SDS-PAGE

Reaction samples for analysis using SDS-PAGE were terminated with the addition of 10µL of 5x SDS sample reducing buffer. The final concentration of SDS in 50µL of total sample (40µL of sample + 10µL of 5x SDS) is 1x. Samples were incubated at 95°C for five minutes using a heat-block. Samples were separated using 15% gels over 100V of electricity. Gels were fixed for 30 minutes in 50% water, 40% methanol and 10% acetic acid (v/v/v), incubated in Amplify (Amersham by GE Healthcare) for 10 minutes. Fixed gels were immobilized onto filter paper, positioned on the surface of dried a gel dryer (BioRad model 583) and covered with generic plastic wrap. Gels were dried and affixed to filter paper for 2 hours at 80°C. Using a dark room, dried gels were exposed to X-ray film (Phenix F-BX57) and stored inside of a radiographic cassette. Cassettes were stored at -80°C for ≥15 hours. After the incubation period for film exposure, cassettes were removed from the -80°C and allowed to come to room temperature. Exposed film were developed using an AFP MiniMed 90 x-ray processor.
Films were scanned using a ChemiDoc Imager XRS+ system. Values of IC$_{50}$ were calculated by densitometry. Results were analyzed and graphed using Microsoft Excel.

3.9.2 Analysis by Liquid Scintillation

Reaction samples to be analyzed using scintillation were spotted onto DE81 Whatman Paper Discs. Spotted discs were washed 3x using NaHCO$_3$ at 5 minutes each, decanting solutions after each wash. Next, spotted discs were washed 1x using H$_2$O at 5 minutes and then spotted discs were washed 1x using 100% ethanol. Discs were permitted to air-dry overnight. The following day, dried discs were transferred into separate scintillation vials using forceps. Each scintillation vial received an aliquot of 5mL of ScintiVerse BD Cocktail (SX18-4) scintillation solution. Samples were analyzed by scintillation counts for H$^3$ over 1 minute in a Beckman Coulter LS 5000 TD Scintillator Counter. Results were analyzed and graphed using Microsoft Excel.

3.10 Real Time – qPCR

Total RNA was isolated from cells using the TRIzol reagent (ThermoFisher). Strands of cDNA were generated using the Superscript III First-Strand Synthesis System (ThermoFisher). Real-time PCR was performed with Go-Taq qPCR master mix (Promega) and specific primers (40 cycles of 15 seconds at 95°C and 20 seconds at 60°C). Gene expression was defined as the cycle threshold (C$T$) number. Fold changes in
mean expression levels of the target genes were calculated using the comparative \( C_T \) method (RUN; 2-\( \Delta C_T \)).

### 3.11 Protein Expression and Purification

According to previously used methods, PRMT 1, PRMT3, PRMT5 and SmD3 cDNAs were subcloned into pET15b vectors purchased from Novagen and transfected into BL21 (ED3) competent *E. coli* cells. Amino-terminal His (6x) tagged proteins were expressed and purified using Ni-NTA Agarose (Qiagen) as reported previously. Proteins were separated across 10% SDS-PAGE gels. Proteins were detected using Coomassie Blue R-250 Stain for 2 hours. Gels were destained while washing in water, methanol and acetic acid 50/40/10 (v/v/v) for 15 hours. Recombinant human histone H4 and H2A proteins were obtained from SigmaAldrich.

### 3.12 Organic Extraction

Sun-dried and packaged spikes (stems) of *Dendrobium aurantiacum var. denneanum* were purchased and shipped from the Taipei Orchid Farm in China. Products were shipped in sealed plastic packages or airtight glass containers. Magnolia leaves and pods were pruned from live trees growing on the campus of Clark Atlanta University. Collected leaves and pods were air-dried in the laboratory on the lab bench at room temperature over 48 hours. Pre-packaged, whole Fennel Seeds were purchased from the Great Wall Supermarket located at 2300 Pleasant Hill Road, Duluth GA, 30096, USA. Plant materials (100g) were pulse-grounded into medium parts using a commercial Lab Waring Blender (7011BU). Grounding by way of pulsing was employed to avoid
excessive generation of heat as to avoid damage to plant materials. Medium parts were
generated in order to maximize the surface-to-volume ratio of plant material to extraction
solvent.

Extractions were initiated by incubating 1L of H$_2$O, ethyl acetate (EtAc) or
Tetrahydrofuran (THF) alone with slow or gentle agitation to suspend solid plant
materials. Crude compounds were extracted from plant materials at 4, 26 and 50°C.
Crude compounds were extracted from plant materials over 24, 48 and 72-hour time-
courses. Supernatants containing crude extracts of plant materials were separated from
whole plant materials using a separatory funnel. Solid-plant materials were discarded;
liquid crude extracts were collected and retained.

3.12.1 Water Extraction

Crude extracts were extracted against H$_2$O using vigorous shaking for
approximately 5 minutes. Separation of the aqueous phase (H$_2$O) from the organic phase
(EtAc) was facilitated using a Beckman Coulter J-E floor centrifuge at 5000rpm over 5
minutes at room temperature with rotor F-10. Extraction by water was repeated twice
more for samples of EtAc containing crude plant materials. Both aqueous and organic
liquid samples were immobilized by submerging glass bottles for lyophilization into
liquid nitrogen. Samples were dried using a FreeZone 4.5 Liter Benchtop Freeze Dry
System by Labconco.
### 3.12.2 Hexane Extraction

Dried samples of crude extracted materials were reconstituted into 15-25mL of Methanol (MeOH). Reconstituted samples were extracted against equal volumes of hexane or cyclohexane using vigorous shaking for approximately 5 minutes. Separation of the aqueous phase (MeOH) from the organic phase (hexane or cyclohexane) was facilitated using a Beckman Coulter J-E floor centrifuge at 5000rpm over 5 minutes at room temperature with rotor F-10. Extraction by hexanes was repeated twice more for samples of MeOH containing crude plant materials.

Both aqueous and organic liquid samples were immobilized by submerging glass bottles for lyophilization into liquid nitrogen. Samples were dried using a FreeZone 4.5 Liter Benchtop Freeze Dry System by Labconco. Methyltransferase inhibitory potential was evaluated using radiolabeled protein methylation assays as mentioned previously, at each step along the extraction process.

### 3.13 Chromatography

Both the Xterra 125Å, 3.5µm, 3.9mm x 100mm and the Xterra 125Å, 10µm, 7.8mm x 150mm C-18 preparatory columns for reverse-phase chromatography were purchased from Waters. Dried samples from extraction were reconstituted into 5mL of EtAc. Samples were aliquotted evenly among 5x microcentrifuge tubes that were pre-massed. Aliquotted samples were dried using a FreeZone 4.5 Liter Benchtop Freeze Dry System by Labconco. *Dendrobium* samples were separated over 80 minutes, incrementally increasing the buffer acetonitrile (AcN) from 10% to 100%. Samples of
Fennel or Magnolia was separated over 80 minutes, incrementally increasing the buffer MeOH from 10% to 100%. Both Fennel and Magnolia samples were separated using 0.1% trifluoroacetic acid (TFA), total volume.

Dried *Dendrobium* samples were reconstituted into 40% acetonitrile (AcN) by volume. Dried Magnolia or Fennel Seeds were reconstituted into MeOH with 0.1% trifluoroacetic acid total volume. For analytical determination of peak-fraction resolution, 50-200µL volumes of sample were loaded onto a Shimadzu Ultra-Fast Liquid Chromatography apparatus. For mass preparation of peak-fraction collection, 2mL of sample were loaded onto the high-pressure liquid chromatography (HPLC) machine. Peak-fractions eluted from the HPLC were collected manually using microcentrifuge tubes or graduated sample collection tubes. Samples for empirical analysis were transferred to pre-massed microcentrifuge tubes and dried using a desktop SpeedVac to immobilize liquid samples, connected to the FreeZone 4.5 Liter Benchtop Freeze Dry System by Labconco.

Each sample collected for mass-preparation was transferred to individual 50mL conical tubes designated by peak-fraction and dried using the FreeZone 4.5 Liter Benchtop Freeze Dry System by Labconco. Each 50mL conical tube was snap-frozen by submerging capped tubes into liquid nitrogen to immobilize contents. After contents were frozen, caps were replaced with plastic wrap anchored by rubber bands around the sides, approximately ½ inch below the brim. Small holes to enable gas exchange were
introduced into the plastic covering of each sample using a sterile syringe, changed with each utilization.

3.14 Mass Spectrometry

Electrospray Ion-Mass Spectrometry was facilitated by Simian Wang and her Department of Chemistry staff in the Natural Science Center at Georgia State University.

3.15 Chemicals and Reagents

HPLC-grade of the following reagents: Ethyl Acetate (E195-4), Methanol (A412-4), Water (W5-4) and Acetonitrile (BDH 83639.400) were acquired from FisherScientific, Inc. FB Essence by Seradigm was purchased from VWR.

3.16 Statistical Analysis

All experiments were validated by obtaining similar results from a minimum of three independent, empirical evaluations. Data reported are represented by the mean of three independent experiments ± the standard deviation. A 2-tailed, unpaired, student t-test was used to determine whether differences between control and experimental samples were statistically significant. For these studies, statistical significance was attributed for any P-value less than 0.05.
4.1 Overview of Extraction Protocol

It has been reported that a variety of plants and plant derivatives harbor agents that possess chemotherapeutic and anti-tumorigenic properties. We began our investigation by determining whether PRMT5-inhibitory compounds could be extracted from Dendrobium plants. Several species of orchids have been documented for having demonstrated antimicrobial and anti-tumorigenic activity. We extracted compounds from the stems of dried orchid plant, Dendrobium aurantiacum var. denneanum as shown in Figure 13.

![Diagram of extraction process]

**Figure 13.** Organic extraction of D. denneanum. Crude plant materials were extracted using organic reagents. The initial extraction used EtAc to liberate crude extracts. Polar compounds were excluded by extracting EtAc against H2O, prior to extracting MeOH against Hexane. The final product of extract was largely non-polar.
Using *D. denneanum*, we attempted to extract a source of chemical constituents possessing methyltransferase inhibitory potential for use against PRMT5. Dried stems of *D. denneanum* were extracted with water, ethanol, methanol or ethyl acetate. Extractions of *D. denneanum* were carried out at temperatures of up to 50°C for periods of up to 72 hours. The solvents ethanol, methanol and ethyl acetate extracted chemical constituents that exhibited inhibitory activity against PRMT5. Water as a solvent did not extract PRMT5-inhibitory chemical constituents from *D. denneanum*.

We also attempted to obtain PRMT5-inhibitory chemical constituents from a relative of *D. denneanum*, *Dendrobium nobile* (*D. nobile*) by extraction. Attempts to extract chemical compounds from *D. nobile* using organic reagents of ethanol, methanol or ethyl acetate, but not water yielded PRMT5-inhibitory chemical constituents. Extractions of *D. nobile* were conducted under the same time and temperature parameters as those used for *D. denneanum*. The organic extraction of each species of *Dendrobium*, yielded chemical constituents that exhibited inhibition against PRMT5 methyltransferase activity. We selected ethyl acetate as the extraction solvent of choice for further analysis of *D. denneanum* (Figure 13).

After separation of supernatant from the plant pellet, the liquid extract product suspended in ethyl acetate was separated by water partition. Water partition of the plant extract in ethyl acetate was carried out three times in effort to remove water-soluble components from the extract. The dried extract was dissolved into methanol and partitioned three times using equal volumes of hexane to remove lipid-soluble components from the extract.
4.2 Optimization of Extraction Conditions for *D. denneanum* Extract.

Next, we wanted to determine the best conditions necessary for the extraction of *D. denneanum* compounds. Using ethyl acetate, ground *D. denneanum* was extracted while varying time and temperature conditions. *D. denneanum* was extracted at temperatures of 4, 25 and 50ºC. *Dendrobium* was extracted over periods of 24, 48 and 72 hours. As shown in Figure 14, the extract of *Dendrobium* at 25º over 72 hours possessed the greatest fraction of phytochemical compounds as measured assessing methyltransferase activity.

![Figure 14](image)

**Figure 14.** Time and temperature-dependent extraction of *Dendrobium d*. Crude compounds from *D. denneanum* were extracted while varying time and temperature in order to optimize extraction conditions for methyltransferase-inhibitory compounds. Methylation or inhibition of methylation for the substrate, SmD3 was assessed by protein methylation assay and radiolabeled isotope, H3.

The yield of PRMT5-inhibitory product obtained from the crude extract was calculated to be approximately 0.1% (Figure 14). This was designated the crude extract.

4.3 Determination of PRMT-enzyme selectivity.

A radiolabeled methyltransferase assay confirmed enzyme-specific inhibitory potential of extracted compounds against methylation of the SmD3 substrate (Figure 15). In order to determine if the crude compounds exhibit preferential inhibition against
PRMT activity, we assessed the methyltransferase activity *in vitro*, in the presence and absence of *D. denneanum* titrations.

![Figure 15](image.png)

*Figure 15.* *D. denneanum* selectively inhibits PRMT activity. (A) Assessment of methylated SmD3 in an in vitro assay using titrations of *D. denneanum* crude extract against type I, II and III PRMTs. (B) Determination of minimum *D. denneanum* required for PRMT5 inhibition, in vitro.

In a concentration-dependent manner, *Dendrobium* exhibited varied inhibition of the SmD3 product of all types of PRMTs (Figure 15A). More interesting, crude *Dendrobium* selectively inhibited the type II methyltransferase activity of PRMT5 the as compared to type I and type II PRMTs, depicted in Figure 15. Although levels of methyltransferase inhibition were detected, *D. denneanum* inhibited type II methyltransferase activity (Figure 15A, PRMT5) more thoroughly than type I methyltransferase activity (Figure 15A, PRMT1 and PRMT3).

Further analysis of *D. denneanum* extract against the type II methyltransferase PRMT5, revealed potential for inhibition in the microgram-range of compound, as shown in Figure 15B. The crude extract strongly inhibited the arginine methylation of SmD3 by
PRMT1, PRMT3 and PRMT5 with the IC<sub>50</sub> values of 70, 90 and 15 mg/mL, respectively (Figure 15).

4.4 Isolation and purification of <i>D. denneanum</i> crude extract

4.4.1 Separation and Purification of Individual Compounds.

In order to isolate individual compounds from the crude extract of <i>D. denneanum</i>, samples were further separated using chromatography (Figure 16A).

![Figure 16](image)

**Figure 16.** Separation of compounds from crude <i>Dendrobium denneanum</i>. (A) Crude materials extracted from <i>D. denneanum</i> were separated using reverse-phase chromatography using a gradient of AcN over 80 minutes. (B) In vitro analysis of SmD3 methylation by PRMT5 after exposure to peak-fractions purified from <i>D. denneanum</i>.
With HPLC, the crude extract was further separated using a linear gradient of acetonitrile from 10-100% in water. Peaks eluted from the column were collected and dried under vacuum (Figure 16A). An *in vitro* methylation assay indicated that 4 peaks (P2, P3, P4 and P5) depicted in figure 16 with retention times ranging from 50 minutes to 57 minutes, contained PRMT5-inhibitory chemical constituents. The four peak-fractions obtained and analyzed differentially inhibited SmD3 methylation by PRMT5.

Using the same buffer conditions for with HPLC, *D. nobile* was separated. As shown in Figure 17, two PRMT5-inhibitory chemical constituents were collected from the eluate.

![Figure 17](image)

*Figure 17. Separation of compounds from Dendrobium nobile. Crude materials obtained from the orchid, D. nobile were separated using a revere-phase column. Compounds were separated over 80 minutes of an incrementally increasing gradient of AcN.*

Peak-fractions 35, 54 and 56 of *D. nobile* demonstrated significant inhibitory potential against PRMT5-methyltranferase activity (Figure 18). Peak-fractions eluted from
the column during times ranging from 51 to 57 minutes. Peak fractions 35, 54 and 56 demonstrated selective inhibitory activity against type II methyltransferase activity but not type I methyltransferase activity as shown in Figure 18 A and B. Peak-fraction 3 exhibited the greatest level of PRMT5 inhibition as compared to other peak-fractions separated from \textit{D. denneanum} (Figure 16B). Peak-fraction 3 was selected for further analysis.

![Figure 18](image)

\textbf{Figure 18.} \textit{Dendrobium nobile} inhibits PRMT5 activity. (A) Analysis of SmD3 methylation by PRMT5 after exposure to peak-fractions purified using reverse-phase chromatography. (B) Analysis of SmD3 methylation by PRMT1 after exposure to peak-fractions 54 and 56 purified using reverse-phase chromatography.

4.4.2 Purity of Compounds Isolated from \textit{D. denneanum}

After verifying PRMT5-inhibitory potential of multiple compounds isolated from crude \textit{D. denneanum}, we sought to measure the level of purity of each compound. Four
peak-fractions obtained from *D. denneanum* demonstrated potential for inhibition of PRMT5 methyltransferase activity. Peak-fraction 2 (lane 5) exhibited poor resolution as depicted in Figure 16A. We rationalized that the inhibitory results observed for this peak-fraction 2 was likely due to a combination of multiple compounds present in one peak-fraction. Additionally, the elution volume of peak-fraction 2 was nearly negligible in comparison to other peak-fraction volumes.

**Figure 19.** Determination of purity of peak-fraction 3. Assessment of purification of compound 3 separated from *D. denneanum* using ESI-MS analysis.

We did not seek to measure the purity of this peak-fraction. Peak-fractions 3, 4, and 5 were outsourced for electrospray ion mass spectrometry analysis (ESI-MS) at the Georgia State University. As shown in Figure 19, peak-fraction 3 was purified to 100%, with a molecular weight of 592.2766 Daltons (Figure 19).

Mass-spectral analysis resolved a single compound present in peak-fraction 3. Two isoforms of peak-fraction 3 were also identified at 593.2797 and 594.200 Daltons each (Figure 19). The ration between the three isotopic peaks is 48:20:5.
The two compounds from *D. nobile* exhibiting the strongest inhibition of PRMT5 activity were subjected to mass spectrometry analysis. Peak-fraction 54 was purified to 100% with a molecular mass of 428.210 Daltons (Figure 20). Peak-fraction 56 was purified to 100% with a molecular weight of 536.16 Daltons (Figure 20). Multiple compounds were obtained from two different species of *Dendrobium* demonstrating inhibitory potential against PRMT5 activity.

![Figure 20. Determination of purity of peak-fractions p54 and p56. Mass determination of compounds p54 and p56 separated from *D. nobile*. using ESI-MS analysis.](image)

### 4.5 Purified Compounds’ IC\textsubscript{50} Values

#### 4.5.1 *D. denneanum* compounds *in vitro* IC\textsubscript{50}

To assess the functional concentration of compound required to inhibit protein arginine methyltransferase enzymatic activity without competition, we conducted *in vitro* analyses, verified using protein methylation assays. As shown in Figure 21, each compound (P2, P3 and P4) demonstrated concentration-dependent inhibition of SmD3 methylation by types I and II protein arginine methyltransferases, as compared to controls. Compound 5 least-inhibited PRMTs as compared to other compounds.Compound 4 demonstrated
slightly less effectiveness against PRMTs than compound 3. The compound in peak-fraction 3 demonstrated the greatest level of inhibition for methylation of SmD3 by all protein arginine methyltransferases as shown in Figure 21 with an IC$_{50}$ of 0.2µg/mL (0.3µM). Concentrations of compounds required for inhibition of methyltransferase activity were calculated by measuring SmD3 methylation using densitometry.

**Figure 21.** Determination of in vitro minimum enzyme inhibitory values. Determination of the required minimum concentration of compounds purified from *D. denneanum* against type I, II and III PRMTs for inhibition of SmD3. Analyses were carried out using a cell-free assay.

4.5.2 Determination of purified *D. denneanum in vivo* IC$_{50}$ values.

To test whether the purified compounds could inhibit protein methyltransferase activity *in vivo* and assess the minimum required concentration of compounds required for
such, we subjected prostate cancer LNCaP and PC3 cells to titrations of *D. denneanum* compounds. Figure 22A displays the concentration-dependent inhibition of prostate cancer cell growth achieved independently using compounds P2, P3, or P4. Values of concentration required for enzyme inhibition were determined by cell counting.

![Graph showing concentration-dependent inhibition of prostate cancer cell growth](image)

**Figure 22.** *Dendrobium denneanum* diminished cell growth but not PRMT5 protein. (A) Assessment of cell growth after treatment with crude *D. denneanum*. (B) Relative protein expression levels of PRMT5 and Wdr77 after treatment with crude *D. denneanum*.

During determination of the IC₅₀ values, we noticed that the compounds strongly reduced cell growth of both LNCaP and PC3 prostate cancer cells, but did not alter expression levels of PRMT5 or Wdr77 protein as shown in Figure 22B.
4.6 *D. denneanum* induces growth arrest in PC3 prostate cancer cells.

4.6.1 Growth-Inhibition in LNCaP and PC3 cells by *D. denneanum*

Treatment of LNCaP and PC3 cells with purified *D. denneanum* generated a concentration-dependent ablation of cellular growth as shown in Figure 23.

![Figure 23](image)

**Figure 23.** Purified *Dendrobium denneanum* thwarts growth of prostate cancer cells. (A) Assessment of PC3 cells treated with varying concentrations of compounds purified from *Dendrobium d.* (B) Assessment of PC3 cells treated with varying concentrations of compounds purified from *Dendrobium d.*

Each of the compounds purified from *D. denneanum* significantly halted growth of both LNCaP and PC3 prostate cancer cells at micromolar concentrations. Evaluation of compounds p54 and p56, from *D. nobile* also abolished cell growth of LNCaP cells shown...
in Figure 24. Other compounds purified from *D. nobile* did not alter growth of LNCaP cells, significantly.

![Figure 24. Assessment of Purified *Dendrobium nobile* on LNCaP cell growth. LNCaP cells treated with peak-fractions purified *D. nobile* alters cell growth.](image)

Evaluation of compounds p54 and p56, from *D. nobile* also abolished cell growth of PC3 cells shown in Figure 25. Other compounds purified from *D. nobile* did not alter growth of PC3 cells, significantly.
Figure 25. Assessment of Purified *Dendrobium nobile* on PC3 cell growth. PC3 cells treated with peak-fractions purified from *D. nobile* alters cell growth.
4.6.2 Growth-arrest of prostate cancer cells by *D. denneanum*

To examine more precisely how this growth ablation was achieved, we analyzed cell cycle progression of PC3 cells treated with the crude compounds. Illustrated in Figure 26, *D. denneanum* significantly elicited an arrest of PC3 cells in the G1-phase of the cell cycle as shown in Figure 26.

![Figure 26](image).

*Figure 26. Dendrobium denneanum arrests cell growth. Growth arrest of PC3 cells treated with *D. denneanum*. Cell cycle analysis of PC3 cells treated with crude *D. denneanum*.***
Furthermore, there was not a significant change in the treated population of cells in sub-G1-phase as compared against control cells as shown in Figure 26. Together, this data suggests that crude *D. denneanum* arrests cell growth without induction of apoptosis. Given the results that *D. denneanum* halts cell growth by forcing cells to arrest at G1, we next wanted to investigate the potential for proliferating cells to respond to treatment as well.

### 4.7 Suppression of proliferation by *D. denneanum*

To assess the effect of compounds on prostate cancer cells, we treated PC3 cells with crude and purified *D. denneanum*. Cells were incubated with Bromodeoxyuridine (BrdU) and counter stained using anti-BrdU. Depicted in Figure 27, crude *D. denneanum* abated proliferation in both PC3 cells. In a concentration-dependent manner, crude *D. denneanum* arrested proliferation of aggressive prostate cancer cells as compared to cells that did not receive compound treatments. Inhibition of proliferation is reported in Figure 27B and 27C as compared to 27A. Cells positive for BrdU are quantitated in Figure 27F.

Similarly, PC3 cells were incubated for 2 hours using BrdU and counterstained as mentioned previously. Compound 3 purified from *D. denneanum* also thwarted proliferation of PC3 prostate cancer cells, shown in Figure 27. In a concentration-dependent manner, *D. denneanum* suppressed proliferation in prostate cancer cells depicted in Figure 27D and 27E, as compared to control cells in Figure 27A. Cells positive for BrdU are quantitated in Figure 27F.
Figure 27. Inhibition of PC3 cell proliferation by D. denneanum. (A) Control cells treated with DMSO. (B) Cells treated with crude extract. (C) Cells treated with crude extract. (D) Cells treated with purified extract. (E) Cells treated with purified extract. (F) Quantitation of BrdU-positive cells.
4.8 Regulation of cellular targets by PRMT5-inhibition.

Inhibition of PRMT5 methyltransferase activity results in differential expression at the genomic and proteomic levels as shown in Figure 28. Depicted in Figure 28A, PRMT5-inhibition generated a concentration dependent reduction in histone H2A protein levels.

Figure 28. Inhibition of PRMT5 alters gene and protein levels. (A) Assessment of histone modification post-exposure of cells to D. denneanum. (B) Analysis of PRMT5 gene targets post-exposure of cells to D. denneanum.
Reduction in histone H2A protein levels was also achieved using other known PRMT5 inhibitors, EPZ0015666 and C9a, but not with compound 4 purified from D. denneanum as shown in Figure 28.

Inhibition of PRMT5 by crude and purified D. denneanum generated differential alterations in the expression of multiple genes of PC3 cells. The BTG family member 2 anti-apoptosis gene, BTG2 was overexpressed following PRMT5 inhibition (Figure 28). The receptor tyrosine kinase family member, ErbB3 was suppressed upon inhibition of PRMT5 (Figure 28). The fibroblast growth factor receptor 3 FGFR3 was also suppressed following PRMT5 inhibition (Figure 28). The kinetochore associated gene, ZWINT was downregulated upon inhibition of PRMT5 (Figure 28). The differential gene expression observed is attributed the D. denneanum mediated inhibition of PRMT5 methyltransferase activity.
CHAPTER V
DISCUSSION

While studies have reported diverse groups of compounds in *Dendrobium* plants which possess various biological activities\(^1\), there have been no reports of PRMT-inhibitory constituents in these plants. In the present study, we provide evidence, for the first time, that two *Dendrobium* species contain multiple constituents that inhibited the activity of both type I and II protein arginine methyltransferases. Three such constituents have been purified to homogeneity with molecular weights of 592.2766, 427.21, 535.15 Da. The purified constituents inhibited growth of prostate cancer cells, which correlated well with their inhibitory activity on symmetric protein arginine dimethylation.

**5.1 *Dendrobium* Plants Contain PRMT-Inhibitory Compounds**

By conventional extraction and chromatographic methods, we isolated 4 and 2 PRMT-inhibitory constituents from *D. nobile* and *D. denneanum*, respectively. These constituents inhibited the activity of both type I (PRMT1 and PRMT3) and type II (PRMT5) protein arginine methyltransferases with IC\(_{50}\) values from 0.2 to 32 \(\mu\)g/ml. Some constituents demonstrated the moderate (about 4-fold) selectivity for PRMT5 than PRMT 1 and PRMT3. One constituent from *D. nobile* contained a compound with the molecular weight of 592.2766 Da and demonstrated the highest inhibitory activity towards PRMT5 with the
IC50 at 0.2 g/ml (0.3 M), which is comparable with PRMT5-specific inhibitors recently identified by screening chemical libraries. These results suggested that *Dendrobium* plants contain multiple potent PRMT inhibitors. The *Dendrobium* PRMT-inhibitors also inhibited the protein arginine methylation in prostate cancer cells and affected expression of some PRMT5-target genes. The future study would use a genome-wide approach to determine genes whose expression is affected by *Dendrobium* inhibitors of PRMTs.

### 5.2 Inhibition of PRMT5 Methyltransferase

In a cell-free assay using purified recombinant proteins, we observed alterations in methylation patterns of known PRMT5 histone substrates. Consistent with published observations, the arginine methylation at histone H2A was detected (Figure 28A, lane 1), which was significantly inhibited by the crude extract (lanes 2 and 3) as well as the compound 3 peak-fraction (lane 4). Two identified PRMT5 inhibitors (C9a and EPZ) were included in the assay as positive controls (Figure 28A, lanes 5 and 6). Thus, PRMT-inhibitory compounds from *D. denneanum* inhibited the symmetrical arginine dimethylation of histones in cells. These results suggested that *Dendrobium* plants contain multiple potent PRMT inhibitors.

A set of genes regulated by PRMT5 and mediated its functions was identified. To investigate whether purified compounds affected expression of these genes, RT-PCR was performed with total RNAs isolated from PC3 cells treated with the crude extract (100 µg/ml) or the compound 3 peak-fraction (1.2 or 2.4 g/ml). We found that...
expression of a PRMT5 down-regulated gene (BTG2) was up-regulated when PC3 cells were treated with the crude extract or the A set of genes regulated by PRMT5 and mediated its functions was identified. To investigate whether purified compounds affected expression of these genes, RT-PCR was performed with total RNAs isolated from PC3 cells treated with the crude extract (100 µg/ml) or compound 3 peak-fraction (1.2 or 2.4 µg/ml). We found that expression of a PRMT5 down-regulated gene (BTG2) was up-regulated when PC3 cells were treated with the crude extract or compound 3 peak-fraction (Figure 28B). On the other hand, expression of PRMT5-upregulated genes (ErbB3, FGFR3, and ZWINT) was down-regulated by the crude extract or the purified compound 3 peak-fraction. These results are consistent with the results obtained with PRMT5 silencing experiments. Therefore, the isolated compound from D. denneanum inhibited PRMT5 activity and regulated its target gene expression in cells. These data indicate that PRMT5-inhibitory compounds regulate PRMT5 functions in vivo. A future study would use a genome-wide approach to determine genes whose expression is affected by PRMT inhibitors of Dendrobium.

5.3 PRMT5-Mediated Growth Suppression

Given the fact that PRMT5 plays critical roles in the growth of cancer cells, we tested whether the purified PRMT-inhibitory compounds could inhibit growth of prostate cancer PC3 and LNCaP cells. The crude extract inhibited growth of both PC3 and LNCaP cells in a dosage-dependent manner with IC50 values of 33 mg/mL and 85 mg/mL, respectively (Figure 22A). Similarly, all three HPLC peak fractions also
inhibited growth of PC3 and LNCaP cells in a dosage-dependent manner (Figure 23) and their IC\textsubscript{50} values are in the ranges from 4.6 to 12.65 µg/mL. The protein levels of PRMT5 and WDR77 were not affected by the treatment of PC3 cells with the crude extract, suggesting that cell growth inhibition induced by the crude extract is not through down-regulation of PRMT5 or WDR77 expression rather than inhibition of PRMT5 activity.

Silencing PRMT5 expression resulted in cell cycle arrest in the G1 phase.\textsuperscript{21} Both crude extract and the purified compound 3 peak-fraction induced G1 cell cycle arrest and decreased cell populations in the S phase (Figure 26), consistent with the observation that crude extract and the purified compound 3 inhibited PRMT5 activity. It was observed that the purified constituent compound 3 also significantly decreased the cell population in the G2 phase. The crude extract and purified compound did not induce apoptosis because they did not affect subG1 cell populations (Figure 26).

We previously showed that the enzymatic activity of PRMT5 is required for cellular proliferation.\textsuperscript{25} The BrdU incorporation assay was used to measure proliferative cells. Thirty-one percent of PC3 cells were BrdU-positive when cells were cultured in the presence of DMSO (Figure 27F). In contrast, the treatment of PC3 cells with the crude extract (Figure 27 A, B and C) or the purified compound 3 peak-fraction (Figure 27 A, D and E) significantly decreased cell populations of BrdU-positive cells in a dosage-dependent manner, suggesting that the crude extract and the purified compound inhibited cellular proliferation. Similar results were observed with LNCaP cells.
CHAPTER VI
CONCLUSION

We found in literature other studies providing evidence of extraction of phytochemical compounds from the orchid species *Dendrobium d*.\textsuperscript{215-216} A number of studies have evaluated the species of orchid reported here, as well as other species of orchid plants, for use against a number of maladies, including cancer.\textsuperscript{136, 223-227} We did not find published studies utilizing our methods of extraction conditions or inhibition of protein arginine methyltransferase activity.

PRMT5 is frequently overexpressed or upregulated in multiple cancer types. Hyperactivity of PRMT5 correlates with cancer progression. PRMT5 localized to the cytoplasm in LNCaP cells promoted growth of prostate cancer cells. Knockdown of PRMT5 ablated cellular growth in A549 cells. Furthermore, mutation of PRMT5’s catalytic domain rendering the enzyme catalytically inactive thwarted growth of A549 and LNCaP cells.\textsuperscript{21, 25, 228} Chemically targeting PRMT5 for inhibition of its methyltransferase activity generated reduced tumor volume and size \textit{in vivo}. Many studies have reported a number of compounds designed to inhibit PRMT5 methyltransferase activity.\textsuperscript{21, 125, 132, 144, 190, 228-230} There is also a number of current studies seeking to discover new PRMT5 inhibitors. Scientific interest for the development of chemical probes that inhibit PRMT5 activity is growing. Less attention has been allotted
to the discovery of natural probes that target PRMT5 for inhibition of its methyltransferase activity.

PRMTs have been shown to be upregulated in a number of different cancers and plays an essential role in growth of various cancer cells. Consistent with these observations, PRMT5-inhibitors suppressed growth of lymphoma, AML, and MCL cells in tissue culture. More recently, EPZ015666 demonstrated antitumor activity in multiple MCL xenograft models. Previous studies used RNA interference technologies revealed an essential role PRMT5 in growth of lung cancer cells and lung tumor xenografts. We have shown in this report that identified *Dendrobium* PRMT-inhibitors inhibited growth of prostate cancer cells. This inhibitory effect on cell growth is through the arrest of cells in G1 phase of the cell cycle, not through induction of apoptosis. These observations are consistent with the documented role of PRMT5 in the control of cellular proliferation. These data imply that the observed inhibitory effect of *Dendrobium* isolates on growth of prostate cancer cells may be due to the suppression of the PRMT5 activity. In the future, we plan to evaluate the observed effects in a murine model. In addition to resolving the structure of compound 3 from *D. denneanum*, we plan to evaluate the enzyme kinetics for association, stability, and mechanism of inhibition. We intend to determine the effects of *D. denneanum*, if any, on protein lysine methyltransferases and the modification of histone protein tails. Lastly, we plan to conduct screens of other plant sources for compounds that demonstrate PRMT5-inhibitory potential.
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