AN INVESTIGATION OF CURCUMIN DERIVATIVES AND THEIR EFFECTS IN PROSTATE CANCER

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ABSTRACT OF THE THESIS

AN INVESTIGATION OF CURCUMIN DERIVATIVES AND THEIR EFFECTS IN PROSTATE CANCER

CANCER

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Prostate Cancer is one of the leading diagnosed cancers and sixth leading cause of cancer deaths worldwide. Prostate cancer has a variety of risk factors including age, diet susceptibility genes and somatic gene defects, all of which can drive prostate carcinogenesis. Phytochemicals have been shown to act as a chemoprevention agent in carcinogenesis, where the chemicals "delay" or inhibit cancer at any step of initiation, promotion, progression or metastasis. The phytochemical curcumin has been shown to possess chemopreventive anti-cancer and anti-inflammation properties, both of which have been indicated in prostate cancer models. The master regulator of anti-oxidative stress, nuclear-factor erythroid 2 related factor (Nrf2) is responsible for many detoxifying and anti-oxidation enzymes. In prostate carcinogenesis, oxidative stress and inflammation are major factors in the development of prostate cancer and nrf2 is necessary to maintain a stable, non-toxic environment.

In the present study, curcumin derivative FN1 was used to investigate its potential in restoring Nrf2 activity through modulation of epigenetic enzymes in mouse prostate cancer TRAMPC1 cells. Compounds were first tested on the HepG2-C8 stably transfected ARE-

luciferase cell line to determine its Nrf2/ARE induction potential. Real time PCR and western blot were performed to determine levels of expression of Nrf2 related enzymes at protein and mRNA level. Bisulfite genomic sequencing (BGS) and methylated DNA immunoprecipitation (MeDIP) were used to determine methylation status of Nrf2 CpG islands. Anchorage-independent colony-formation analysis was performed to examine the compounds potential as a tumor inhibitor. FN1 revealed to be a significant inhibitor of colony formation. FN1 was found to be a more potent inducer of the Nrf2/ARE pathway than curcumin. Expression of Nrf2 and its downstream targets were increased by FN1. Epigenetic modifying enzymes were also found to be downregulated. Methylation status of Nrf2 through BGS and MeDIP revealed reduced methylation of CpG sites. These results indicate that in TRAMPC1 cells, FN1 can increase level of Nrf2 and its downstream genes by activation of the Nrf2/ARE pathway.

In the next study, curcumin derivatives BDMC and DMC were used to investigate potential epigenetic activation of Nrf2 in human prostate cancer cells. LNCaP cells were treated with varying concentrations of the compound to determine cell viability. Real time PCR and western blot were performed to determine levels of expression of Nrf2 related enzymes as well as various epigenetic modifying enzymes, at protein and mRNA level. Bisulfite genomic sequencing (BGS) was used to determine methylation status of the human Nrf2 promoter region CpG islands. mRNA expression of Nrf2 and its downstream genes were induced by both curcumin derivatives. BDMC and DMC treatment resulted in inhibition of DNMT1 and DNMT3B while expression of DNMT3A was increased. Western blot analysis reflected mRNA results with increases of Nrf2 and its downstream targets at the protein level. HDAC proteins expression was inhibited by the curcumin derivatives.

Methylation of Nrf2 CpG sites were not reduced by BDMC and DMC treatment. The derivatives of curcumin have shown to activate Nrf2 and its phase II detoxifying genes, by which mechanism, remains unclear and requires further investigation. In summary, the potential of these curcumin derived chemicals to act as a chemoprevention agents in prostate cancer is high. FN1 displayed the ability to restore expression of Nrf2 and its downstream genes via hypomethylation of Nrf2's promoter CpG sites. BDMC and DMC displayed a potential to activate Nrf2, however, the two compounds were stronger inducers of Nrf2's downstream genes, HO-1 and NQO-1.

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TABLE OF CONTENTS

ABSTRACT OF THE THESIS ii

ACKNOWLEDGEMENT v

LIST OF FIGURES ix

Chapter 1 - Introduction and Background 1

1.1 Prostate Cancer 1

1.2 Prostate Cancer Risk Factors 2

1.2.1 Prostate Cancer as an Age-Related Disease 2

1.2.2 Diet Related Risk Factors 2

1.2.4 Somatic Gene Defects 4

Chapter 2 - Carcinogenesis and Chemoprevention 6

2.1 Carcinogenesis 6

2.2 Cancer Chemoprevention 8

2.2.1 The Master Regulator, Nrf2 9

2.2.2 Epigenetic Modifications in Prostate Cancer 10

2.3 Chemoprevention with Sulfphoraphane 12

2.4 Chemoprevention with PEITC 14

2.5 Chemoprevention with Curcumin 15

Chapter 3 - Epigenetic Reactivation of Nrf2 in Prostate TRAMPC1 cells by Curcumin Analogue FN1 18

3.1 Abstract 18

3.2 Introduction 19

3.2 Materials and Methods 21

3.2.1 Reagents and Antibodies 21

3.2.2 Materials and Chemicals 22

3.2.3 Cell Culture 23

3.2.4 Luciferase Reporter Activity Assay 23

3.2.5 MTS Assay 24

3.2.6 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR) 24

3.2.7 Preparation of Protein Lysates and Western Blotting 25

3.2.8 Bisulfite Genomic Sequencing (BGS) 26

3.2.9 Methylated DNA Immunoprecipitation (MeDIP) Analysis 27

3.2.10 Anchorage-Independent Colony-Formation Assay 27

3.3 Results 28

3.3.1 FN1 Induced ARE-Luciferase Reporter Activity 28

3.3.2 Cytotoxicity Measurement of FN1 by MTS 28

3.3.3 FN1 Enhanced Expression of Nrf2 and Its Downstream Antioxidant and Detoxifying Enzymes and Decreased Keap1 Expression 29

3.3.4 FN1 Reduced Colony Formation in TRAMP-C1 cells 30

3.3.5 FN1 Diminished the Methylation Ratio of the First Five CpG Regions in Nrf2 Promoter 30

3.3.6 FN1 Downregulated Epigenetic Modification Enzymes 31

3.4 Discussion 31

Chapter 4 - Epigenetic regulation of Nrf2 by curcumin derivatives –Demethoxycurcmin and Bisdemethoxycurcumin in Human Prostate Cancer Cells 36

4.1 Abstract 36

4.2 Introduction 37

4.3 Method 38

4.3.1 Cell Culture 38

4.3.2 MTS Assay 38

4.3.3 Western Blotting, mRNA expression and DNA Bisulfite Sequencing 39

4.4. Results 39

4.5 Conclusion 41

Chapter 5 Conclusion 43

References 62

LIST OF FIGURES

Figure 1. Three Stages of Carcinogenesis 46

Figure 2. Nrf2-ARE Transcription Pathway 47

Figure 3. Chemical structure of 48

Figure 4. ARE-Luciferase activity assay of effects of FN1, curcumin, and SFN 49

Figure 5. Cytotoxicity of FN1 against the TRAMP-C1 cell line. 50

Figure 6. Relative endogenous mRNA expression of Nrf2 target genes 51

Figure 7. Effect of FN1 on protein expression of Nrf2 target genes 52

Figure 8. Inhibitory effect of FN1 on the Anchorage-Independent Colony-Formation of TRAMP-C1 53

Figure 9. Methylation ratio of the first 5 CpGs of Nrf2 promoter region in TRAMP-C1 54

Figure 10. MeDIP (methylated DNA immunoprecipitation) assay for the effect of FN1 55

Figure 11. Effect of FN1 on DNMT protein expression 56

Figure 12. Percent Cell viability from the MTS assay in LNCaP cells treated with DMC and BDMC 57

Figure 13. Protein expression of Epigenetic Modifying Enzymes 58

Figure 14. mRNA expression levels of Nrf2 and its down-stream genes HO-1 and NQO1 59

Figure 15. The mRNA expression level of DNMTs 60

Figure 16. Total methylation levels of Nrf2 and its three major CpG sites 61

Chapter 1 - Introduction and Background

1.1 Prostate Cancer

Prostate cancer (PCa) is the most common non-skin cancer in the United States. It is estimated that in 2016, fourteen percent of men diagnosed with PCa will die from the disease, making it the second leading cause of cancer-related deaths in the US. Survival rates are relatively high. In the last 25 years, five-year survival rates for all stages of PCa have improved from 68% to 99% with ten and twelve year survival rates still over 95%.However, survival rates for distant (metastasis) remains low at 28% [1]. These favorable survival rates are in large part due to the introduction of the prostate specific antigen test (PSA). Although this test has diagnosed more than a million additional men over the last 20 years, there is still some debate surrounding the value of screening (via PSA or digital rectal exam) and whether the benefits outweigh the harms [2]. In many cases, men diagnosed with PCa will die as a result of causes unrelated to the disease. The controversy is that diagnosis leads to the treatment of cancer that is not necessarily an immediate danger. In addition, these tests can cause over diagnosis which ultimately always results in over-treatment. PCa treatment can have permanent side effects such as erectile dysfunction and urinary incontinence, all of which impair quality of life for men. In a meta-analysis of electronic databases (PubMed, Embase and Cochrane Registry of controlled trials) with eligible studies, it was found that overall mortality rates between PSA screened groups and non-PSA screened groups were insignificant [3, 4]. As a result, there has been growing consensus that men diagnosed with low-risk PCa should defer treatment and undergo active surveillance or watchful waiting management of their condition [5]. Watchful waiting is patient follow up with palliative care with no attempt at

curative therapy. In contrast, active surveillance involves regular follow ups and initiation of local curative therapy if there are signs of tumor progression [6, 7].

1.2 Prostate Cancer Risk Factors

PCa is an adenocarcinoma or a glandular cancer with relatively slow progression. It is currently known that age, dietary, lifestyle-related and environmental factors have an impact on its development.

1.2.1 Prostate Cancer as an Age-Related Disease

Prostate cancer is considered an age related disease because risk increases after the age of 50. Also, preneoplastic lesions or prostatic intraepithelial neoplasia (PIN) have been identified in men in their twenties but were found to be more common in men by their fifties. However, the progression of these lesions into clinically relevant PCa usually do not manifest until men reach their 60s or 70s [8].

1.2.2 Diet Related Risk Factors

Convincing epidemiological evidence has shown incidence of PCa is largely related to life style, with an emphasis on diet. The western diet or US diet is mainly comprised of animal fat and meat with very few fruit and vegetables. Incidence of PCa in the Asian counties have consistently reported lower rates, usually 50-60 fold less, than the US. However, there is still a large increase in incidence rates in these low-risk countries. These rates also reflect the increased incidence of diabetes and cancers of the colorectal and breast, which is attributed to the western diet of these low-risk populations [9]. In addition, a study showed that PCa rates for Japanese early-immigrants to the US were similar to American born

Japanese. When comparing homeland born Japanese to these rates, there is a significant difference between them which further implicates the role of westernization on Asian populations [10]. In a study of data collected from the Health Professionals Follow-up Study, a cohort of 51,529 men, aged 40-75, it was found that total fat, animal fat, and red meat consumption was directly related to risk of advanced prostate cancer. Although it is not known what components in red meat promote PCa, generation of heterocyclic aromatic amine and polycyclic aromatic hydrocarbon carcinogens through the cooking of meat is believed to play a role in PCa and animal testing of these carcinogens have demonstrated that these compounds can cause PCa [11].

1.2.3 Predisposition to Prostate Cancer through Susceptibility Genes

Inheritance of genes that may increase the risk of developing cancer is something that has been explored and observed for different cancers. One convincing piece of evidence of predisposition was the study of twins from Nordic countries, it was determined that the concordance for prostate cancer in male monozygotic twins was 0.21. This means that there is a twenty-one percent probability that the identical twin of a man with prostate cancer will have the same cancer. Overall, the proportion of susceptibility to cancer that was accounted for by genetic defects through inheritance was around 42%. It is believed that 5-10% of prostate cancer cases are primarily caused by high-risk inherited genetic factors or prostate cancer susceptibility genes [12]. There are two inherited susceptibility genes of note, RNASEL and MSR1. The latent ribonuclease (RNASEL) is involved in the interferon inducible RNA-degradation pathway and is activated to degrade cellular and viral RNA [13]. RNASEL is located on the HPC1 locus (implicated in PCa) and studies have shown that mutations to RNASEL have been found in familial prostate cancer. In one family, four

brothers with prostate cancer were found to be carriers of a mutated RNASEL gene. In another family, of six brothers, four had prostate cancer and carried a defective RNASEL gene. But the exact role of RNASEL and PCa is unknown. However it has been proposed that RNASEL is a tumor suppressor gene, based on its studied function [14]. Another possible PCa-susceptibility gene is the macrophage scavenger receptor 1 (MSR1) gene, which encodes for one subunit of the trimeric membrane glycoprotein macrophage scavenger receptor. These receptors are capable of recognizing a variety of ligands such as oxidized low density lipoprotein (LDL), oxidized high density lipoprotein (HDL), and lipopolysaccharide (LPS) [15]. In an analysis of MSR1 germ lines, one nonsense mutation and six rare nonsense mutations were found in families with hereditary PCa. In men of European descent, MSR1 mutations were found in 4.4% with non-hereditary PCa and 0.08% in unaffected [16].

1.2.4 Somatic Gene Defects

There are multiple genes that are responsible for prostate cancer which aren't necessarily inherited. Alterations to these somatic genes, whether by epigenetics or DNA modifications, can lead to greater risk of developing PCa. Prolonged accumulation of these defects have been identified in clinically significant prostate cancer cells [11].

Homeobox NKX3.1 is a prostate -specific human gene that is localized in the prostate epithelium. It is likely that NKX3.1 is essential for normal prostate development and is a potent growth suppressor. Mice with alterations in the expression of NKX3.1 have been shown to develop prostatic epithelial hyperplasia and dysplasia. The loss of one or both

NKX3.1 alleles resulted in accelerated and more aggressive prostate tumorigenesis. The absence of NKX3.1 was found in 20% of lesions of PIN, 22% of high stage PCa, 34% of androgen-independent PCa and 78% of PCa metastases. In other words, NKX3.1 expression decreases with disease severity, making this gene a potential biomarker for disease progression. Also it is believed that NKX3.1 works in a dose dependent manner in which gene dosage and subsequently, protein amount has an effect on tumor suppressor effects. NKX3.1 is mapped to chromosomal position 8p21 and has shown loss of heterozygosity (LOH) in studies of prostate carcinoma tissue. As a result of this, it is possible that LOH is responsible for the downregulation of NKX3.1 in PCa progression [17].

Another gene with strong association to PCa is glutathione S-transferase p1 (GSTP1). Normally, this gene is involved in DNA protection from electrophilic metabolites of carcinogens and reactive oxygen species by conjugating chemically reactive electrophiles to glutathione. Inactivation of GSTP1 leads to increased vulnerability to oxidant and heterocyclic amine carcinogens, which are both are implicated in prostate carcinogenesis. GSTP1 loss of expression has been heavily associated with PCa; it has been linked in more than 90% of PCa and in high grade PINs. The silencing of GSTP1 has been directly linked to hypermethylation of deoxycytidine residues of the entire gene. Methylation analysis of 131 CpG sites in prostate cancer cell lines and tumor samples determined that methylation of CpG islands correlated to downregulation of GSTP1 while methylation outside of the CpG islands had no effect on gene expression [18].

Chapter 2 - Carcinogenesis and Chemoprevention

2.1 Carcinogenesis

The pathway to carcinogenesis is driven by changes to DNA or to DNA repair mechanisms. Alterations to DNA can come from a variety of places, endogenous or exogenous. Exogenous sources can be environmental and/or diet related, while endogenous sources come from our own immune system which can generate DNA damaging entities. Continuous exposure to these entities can accumulate within the cell and ultimately result in carcinogenesis. There are various types of alterations to DNA that can drive carcinogenesis, this includes DNA oxidation, DNA methylation, mutations to DNA code and errors in repair mechanisms.

Carcinogenesis is a multi-stage process in which multiple cellular events are affected by a carcinogenic agent that eventually transforms a normal healthy functioning cell into an irreversible malignant tumor. There are three major stages in the formation of tumors, initiation, promotion and progression, all of which can be seen in Figure 1. Initiation is a rapid and irreversible process where the uptake of a carcinogenic agent is distributed and transported to organs and tissue. Covalent interaction between reactive species and DNA forms a DNA adduct that can result in genomic damage [19]. Tumor promotion is the accumulation of a single clonal proliferating cell which has lost its ability to control proliferation and regulate apoptosis due to the carcinogenic agent. The dysregulation of these cellular functions ultimately results in the formation of preneoplastic cells [19]. Eventually, the preneoplastic cells irreversibly transform into malignant tumors during the tumor progression stage [20].

Chronic or acute inflammation are major driving forces of carcinogenesis and are responsible for the development of many different cancers, including PCa. Inflammation leads to the recruitment of mast cells and leukocytes. These immune cells, in response to cytokines release highly reactive chemical compounds (superoxide, hydrogen peroxide, reactive oxygen and reactive nitric oxide species) that can damage DNA in healthy tissues. Even though healthy tissue is being damaged, the conditions produced by inflammation creates a stressful environment for epithelial cells which are undergoing cell division to replace tissue that has been lost. Cells undergoing cell division in these conditions have an increased risk of mutations while synthesizing DNA, which can drive these tissues to carcinogenesis [21]. There is some consistent evidence demonstrating the link between inflammation and PCa, in which constant intake of anti-oxidants or nonsteroidal anti-inflammatory drugs decreased the risk of PCa [22].

Signs of chronic inflammation in prostate cancers have been described by De Marzo et al and have been labeled as proliferative inflammatory atrophy (PIA). These are lesions which possess abnormalities and are suspected to be a preliminary step in the development of PCa. They are believed to be linked to PIN since PIA lesions commonly appear in PCa and are observed to be directly adjacent to PIN lesions [23]. PIA lesions are characterized to be hyper-proliferative with reduced apoptotic rates, associated with inflammation and have the appearance of prostatic atrophy. Cells in the PIA region have demonstrated high expression of GSTP1, which is a response to a stressful oxidative environment, and elevated levels of Bcl-2 expression which contributes to reduced apoptosis [23]. There is a morphological transition from PIA to PCa in which PIA lesions which have somatic defects

in GSTP1 and are constantly bombarded by immune mediated stress signals, become vulnerable in progressing to PIN and eventually PCa [24].

Cancer cells and normal prostate cells rely on androgenic steroids and androgen receptors for proliferation. This dependence on androgen stimulation has been the backbone of androgen-deprivation therapy in treating the early stages of metastatic prostate cancer. However, these androgen dependent cells can progress to a point where androgen is no longer needed, leading to an increased potential for metastasis to other parts of the body [25, 26]. This newly formed independence from androgen or "castration resistance" is fueled by the secretion of growth factors allowing the cancer cells to form their own microenvironment and thrive. The progression to castration resistance prostate cancer (CRPC) is almost always associated with increases in levels of prostate-specific antigen (PSA), an organ-specific tumor marker [27]. Higher levels of PSA indicates a higher risk for metastatic disease or subsequent disease progression [28].

2.2 Cancer Chemoprevention

Chemoprevention is one approach, of many, when dealing with cancer control. This strategy uses natural or synthetic compounds to suppress, block or reverse initial carcinogenic process or progression to pre-malignant stages [20]. This term was coined by Dr. Michael Sporn in the 70s to describe the impediment of cancer progression by natural chemicals and possible synthetic analogues. Prevention of cancer relies on the notion of "delaying" cancer progression at a variety of steps through carcinogenesis. Cancer prevention through chemicals, or chemoprevention, is achieved by the classical notion of

using chemopreventive agents to block and suppress numerous cellular molecules and events. There have been many studies evaluating the effect of these agents and their preventive capabilities. Some notable chemicals are derived from plants, otherwise known as phytochemicals and this includes isothiocyanates from cruciferous vegetables, polyphenols from green tea and flavonoids from soybeans. Effects of chemopreventive agents can result in carcinogen activation/detoxification by xenobiotic metabolizing enzymes; DNA repair; cell-cycle progression; cell proliferation, differentiation and apoptosis; expression and functional activation of oncogenes or tumor-suppressor genes; angiogenesis and metastasis; and hormonal and growth-factor activity [29]. There are also many cellular signaling cascades that are targeted by chemoprevention agents, some of which are of particularly noteworthy. This includes the nuclear factor E2-related factor 2 (Nrf2), nuclear factor-kappa B (NF-kB) and cyclooxy-genases-2 (COX-2) with the focus of this paper being on Nrf2 [30].

2.2.1 The Master Regulator, Nrf2

The Nrf2-Keap1 signaling pathway provides cytoprotection from oxidative and electrophilic stress. The activation of this complex induces cellular defense mechanisms which is accomplished through phase II detoxifying enzymes, phase III transporters, anti-oxidative stress proteins and other anti-stress proteins that protect cells from ROS and/or RNS [31]. The Nrf2 protein is a helix-loop-helix basic leucine transcription factor that regulates antioxidant enzymes via binding to the antioxidant response element (ARE), located in the promoter region. Under stress-free conditions, Nrf2 is bound to and sequestered by Keap1 which prevents Nrf2 from translocating into the nucleus, thus

limiting Nrf2 to the cytoplasm, as show in Figure 2A. While in the cytoplasm, Nrf2 is under constant ubiquination by Cullin 3, which subsequently designates Nrf2 for proteosomal degradation. Upon oxidative stress, Nrf2 is phosphorylated by kinases releasing Nrf2 from Keap1, allowing for translocation to the nucleus where it dimerizes with small proteins, binds to ARE, ultimately resulting in the transcription of Nrf2-ARE target genes, shown in Figure 2B [32].

The phase II detoxifying enzymes reduce the toxicity of reactive intermediates produced by oxidative and electrophilic stresses. By reducing this toxicity, the chemical homeostasis within the cells are maintained. The phase II detoxifying enzymes encompass a variety of genes including, glutathione s-transferase (GST), UDP-glucuronosyltransferase (UGT), heme-oxygenase-1 (HO-1), NADP(H) quinoneoxidoreductase (NQO), glutamate cysteine ligase (GCL) and gamma glutamyl-cysteine-synthetase (γGCS). These genes are seen as strategic targets for chemoprevention due to their potential in blocking toxic and neoplastic processes in carcinogenesis [31].

2.2.2 Epigenetic Modifications in Prostate Cancer

Epigenetic changes effect the expression of genes but does not alter the genetic code of DNA. Epigenetic modifications can occur in many ways with covalent modifications being the most common. These covalent modifications include DNA methylation and histone modification, and can be affected by different factors such as environments, ageing, drugs or xenobiotics, and non-nutrient phytochemicals. Epigenetic regulation of genes involves modifications to the chromatin structure where certain genes are activated while

others are physically inaccessible for transcription. There are two families of enzymes which have opposing effects on chromatin structure. The first is Histone Acetyl Transferases (HAT) which catalyzes the transfer of an acetyl group to ε-amino group of lysine side chains causing relaxation of the chromatin structure. This allows for the binding of transcription factors and can significantly increase gene expression. The second is the histone diacetyl transferases (HDAC) class and work by opposing the effects of HAT by removing acetyl groups from lysine residues. This causes tighter wrapping of DNA around histones preventing transcription and thus decreasing gene expression [33]. In prostate cancer, Class 1 HDACs (HDAC 1, 2 and 3) have shown increased gene expression in majority of cancer cases and in PIN lesions. Upregulation of HDAC1 in PIN lesions indicates there is an early event in prostate carcinogenesis. HDAC2 expression is implicated as a prognostic marker as high expression levels have been linked to shortened PSA relapse-free survival times [34].

Another type of covalent epigenetic modification is DNA methylation of genes occurring on cytosine bases where DNA methyl transferase (DNMT) enzyme converts the base into 5-methylcytosine. Cytosine bases are most commonly located next to a guanine, giving rise to the name CpG island. There are three enzymes associated with methylation, DNMTs 1, 3A and 3B. DNMT1 is mainly responsible for maintaining methylation patterns following DNA replication and also shows a preference for methylating hemi-methylated DNA. DNMT 3A and 3B are responsible for de novo DNA methylation and cooperates with DNMT1 in DNA methylation [35]. There has been evidence linking methylation and gene expression where higher levels of methylation in promoter regions correlates to little or no transcription of the gene. In prostate cancer cells, there are many genes with

hypermethylated promoter regions that inactivate crucial tumor suppressor genes such as Adenomatous polyposis coli (APC), Retinoic acid receptor (RARβ) and Ras association domain family member 1 (RASSF1). Hypermethylation in PCa has also been linked to silencing of genes that are responsible for hormone signaling, DNA repair, cell adhesion, cell-cycle control, and apoptosis. The cellular damage defense gene and DNA repair gene, GSTP1, has highly methylated CpG island and is identified as an early event in PCa. Many PCa cases have reported methylation at both alleles, resulting in incomplete gene inactivation. GSTP1 is regularly expressed and hypomethylated in normal tissue [36]. Another type of histone modification comes in the form of histone methylation where methyl groups occur on basic residues including arginine, lysine and histidine. Lysines can be methylated in the form of monomethyalted, dimethylated or trimethylated. Depending on which residue is methylated and the degree of methylation, genes can be either activated or suppressed [37]. The enhancer of Zeste homolog 2 (EZH2) is a histone methyl transferase that catalyzes the trimethylation of histone H3 on lysine 27 (H3K27me3) which is thought to lead to transcription repression of tumor suppressor genes. EZH2 is amplified and overexpressed in PCa and has even higher expression in metastatic PCa. It has been demonstrated that EZH2 in PCa cell lines increase invasive characteristics while knockdown of EZH2 decreases proliferation potential [36].

2.3 Chemoprevention with Sulforaphane

Studies from multiple labs have described chemopreventive capabilities of various phytochemicals. They have been shown to act as blocking agents, impeding the initiation phase of carcinogenesis or as suppressing agents inhibiting the promotion and progression

phases of carcinogenesis. The phytochemical, sulforaphane (SFN), is an organosulfur compound found in cruciferous vegetables and has been shown to possess anti-cancer and anti-oxidative effects. In a study performed by Singh et al, they demonstrated SFN's ability to inhibit proliferation of cultured PC-3 human prostate cancer cells by inducing apoptosis through upregulation of the pro-apoptotic gene Bax, downregulation of anti-apoptotic gene Bcl-2, and activation of caspases 3, 8, and 9. In addition, oral administration of SFN significantly hindered growth of PC-3 xenografts in nude mice at concentrations generated by dietary intake of broccoli and other cruciferous vegetables [38]. Another possible mechanism by which SFN acts as a chemopreventive agent is through epigenetics. PCa is suggested to be associated with increased oxidative stress which is described by excess generation of ROS and/or reduction of antioxidant response. Studies have shown that Nrf2-deficient mice displayed increased susceptibility to prostate carcinogenesis, indicating Nrf2 as a key regulator in cellular defense of oxidative stress [39]. Zhang et al demonstrated that SFN can restore expression of Nrf2 and its downstream target gene, NQO1, by means of demethylation of the Nrf2 promoter region CpG islands in tumorigenic TRAMP C1 mouse prostate cells. Epigenetic demethylation of the CpG islands were achieved through suppression of methylation proteins DNMT1 and DNMT3a [40]. In PCa, HDAC activity is increased in varying degrees depending on cancer stage. A 15uM dose of SFN has also shown effective inhibition of HDAC while augmenting levels of acetylated histones in multiple human prostate cancer cell lines, BPH-1, LNCaP and PC-3 cells, with 40%, 30% and 40% inhibition, respectively. The downstream effects of HDAC inhibition by SFN are believed to affect chromatin remodeling, resulting in increased levels of acetylated histone H4 associated with the promoter region of pro-apoptosis gene Bax [41]. However further

studies need to be performed to rule out other mechanisms that may influence changes induced by SFN.

2.4 Chemoprevention with PEITC

Phenethylisothiocyanate (PEIT) is a naturally occurring isothiocyanate and like SFN, is found in cruciferous vegetables and has demonstrated anti-oxidative effects. As mentioned earlier, GSTP1 is a detoxifying phase 2 enzyme involved in the removal of toxins and oxidative chemicals. Loss of expression of GSTP1, due to aberrant methylation of the promoter region, has been identified in many clinical prostate tumors. In a study performed by Wang et al, PEITC was found to have dual epigenetic effects on GSTP1 expression and chromatin structure. Human PCa cell line, LNCaP, both androgen-dependent (AD) and androgen independent (AI), were treated with PEITC and a known DNA methylation inhibitor, 5-Aza-2'-deoxycytidine (5'-Aza). PEITC treatments demonstrated significant demethylation at GSTP1 CpG islands. CpG islands 1 and 3 in AD LNCaPs were heavily methylated at 89.5% and 61.8%, respectively. PEITC treated AD LNCaPs showed a significant reduction of methylation in the same CpG sites with 73.2% and 6.5%. PEITC also demonstrated demethylation activity when compared to 5-aza, suggesting PEITC can be a potent DNA methylation inhibitor. Consequently, GSTP1 was reactivated and protein expression was greatly enhanced. In addition to PEITC's demethylation effects on GSTP1, the compound was also found to inhibit the activity and level of HDACs and induce selective histone acetylation and histone methylation modifiers for chromatin folding. PEITC treated LNCaP-AD cells demonstrated more than a 2 fold increase of acetylated H3 and of mono/di/trimethylation of lysine 4 of histone H3. It has been suggested by the

authors that HDAC inhibition has a vital role in the reactivation of GSTP1 [42]. PEITC has also been found to suppress cancer cell motility through another type of epigenetic event, miRNAs. This type of epigenetic regulation involves short noncoding RNAs, about 20-22 nucleotides long, that post-transcriptionally repress the target gene by binding to the 3' untranslated region [43]. In PC-3 and LNCaP cells, miRNA-194 was found to be significantly induced by PEITC which subsequently downregulated the expression of oncogenic matrix metallopeptidases, MMP2 and MMP9. These oncogenes are heavily involved in the dissemination of tumors in various cancers and their downregulation resulted in inhibition of PCa cell invasion [44]. Based on various studies of PEITC's effect on PCa cell lines, it is safe to say that PEITC has a potent ability to modulate epigenetic events resulting in the impediment of PCa proliferation and metastasis.

2.5 Chemoprevention with Curcumin

Curcumin is a polyphenol component of turmeric (*Curcuma longa*) and is widely used in cultural dishes. Like the previous phytochemicals discussed, curcumin has anti-cancer and anti-inflammatory properties. Unfortunately, this compound has also demonstrated pro-tumorigenic activity in transgenic mouse models of lung cancer, suggesting that curcumin has organ-specific effects by enhancing ROS species formation in damaged lung epithelium of smokers and ex-smokers [45]. On the contrary, curcumin has been widely studied for its inhibitory effects on epigenetic regulators and several clinical trials have been performed based on the compound's safety and tolerability; two phase 1 clinical trials reported no-treatment related toxicity for oral doses up to 8,000 mg/day [46, 47]. Curcumin has been shown to modulate different cellular events such a inhibition of cell proliferation

and induction of apoptosis with alteration of AKT/mTor activity and downstream signaling [48, 49], suppressing inflammation by inhibition of NF-Kβ and cyclooxygenase-2 (COX-2) [50] and transcriptional activation of Nrf2 [51].

In a study using MV4-11 leukemia cells, curcumin was shown to exhibit epigenetic regulation affects where the DNMT1 analogue, M.SssI was inhibited, possibly through the blocking of the catalytic thiol of C1226 in DMT1 and inducing global DNA hypomethylation [52]. Also curcumin has demonstrated its potential as a DNA hypomethylation agent on the Neurog1 gene in a study performed by Shu et al. This gene is critical in neuronal differentiation, controls neurogenesis in the mouse pallium [53] and has been implicated to be highly methylated with perturbed expression in colorectal [54-56] and prostate cancer [57]. Hypermethylation of Neurog1 in colon cancers has been accepted as a sensitive cancer methylation marker [58]. Curcumin treated LNCaP cells displayed demethylation at the first 14 CpG islands of Neurog1 which resulted in the restoration of its expression. Levels of epigenetic modifying proteins (MBDD2, MeCP2, DNMT1, and DNMT3A) revealed that curcumin had limited effects on their expression. However, ChIP assays showed decreased binding of MeCP2 to the promoter of Neurog1. Methylated CpG binding protein 2 (MeCP2) is a DNA binding protein and is involved in histone modification related transcriptional repression of specific genes [59]. The decreased binding between MeCP2 and Neurog1 indicates that curcumin acts on Neurog1 as a demethylation agent [60].

Inflammation is one of the major risks associated with carcinogenesis and regulation of cellular signals involving inflammation is important for chemoprevention. Curcumin has been described to have anti-inflammatory capabilities such as potent inhibition of the NF-

Kβ pathways through the inhibition of IκBα proteasomal degradation and thus nuclear translocation of the p65 subunit [61]. Additionally, curcumin is able to inhibit NF-Kβ through inhibition of IκBα and AKT activation which subsequently suppresses NF-Kβ-dependent gene products that suppress apoptosis and mediate proliferation, invasion, and angiogenesis [62].

[1] Chapter 3 - Epigenetic Reactivation of Nrf2 in Prostate TRAMPC1 cells by Curcumin Analogue FN1

3.1 Abstract

It has previously been shown that curcumin can effectively inhibit prostate cancer proliferation and progression in TRAMP mice, potentially acting through the hypomethylation of the Nrf2 gene promoter and hence activation of the Nrf2 pathway to enhance cell antioxidative defense. FN1 is a synthetic curcumin analogue that shows stronger anticancer activity than curcumin in other reports. We aimed to explore the epigenetic modification of FN1 that restores Nrf2 expression in TRAMP-C1 cells. Stably transfected HepG2-C8 cells were used to investigate the effect of FN1 on the Nrf2-antioxidant response element (ARE) pathway. Real-time quantitative PCR and Western blotting were applied to study the influence of FN1 on endogenous Nrf2 and its downstream genes. Bisulfite genomic sequencing (BGS) and methylated DNA immunoprecipitation (MeDIP) were then performed to examine the methylation profile of the Nrf2 promoter. An anchorage-independent colony-formation analysis was conducted to examine the tumor inhibition activity of FN1. Epigenetic modification enzymes, including DNMTs and HDACs, were investigated by Western blotting. The luciferase reporter assay indicated that FN1 was more potent than curcumin in activating the Nrf2-ARE pathway. FN1 increased the expression of Nrf2 and its downstream detoxifying enzymes. FN1 significantly inhibited the colony formation of TRAMP-C1 cells. BGS and

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MeDIP assays revealed that FN1 treatment (250 nM for 3 days) reduced the percentage of CpG methylation of the Nrf2 promoter. FN1 also downregulated epigenetic modification enzymes. In conclusion, our results suggest that FN1 is a novel anticancer agent for prostate cancer. In the TRAMP-C1 cell line, FN1 can increase the level of Nrf2 and downstream genes via activating the Nrf2-ARE pathway and inhibit the colony formation potentially through the decreased expression of keap1 coupled with CpG demethylation of the Nrf2 promoter. This CpG demethylation effect may come from decreased epigenetic modification enzymes, such as DNMT1, DNMT3a, DNMT3b, and HDAC4.

3.2 Introduction

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a key regulation factor of the phase II detoxifying enzymes that act as a defense system against oxidative stress. These enzymes include heme oxygenase-1 (HO-1), NAD[P]H/quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), glutathione *S*-transferase (GST), and γ-glutamyl cysteine ligase (γ-GCL) [63, 64]. These enzymes are mainly transcriptionally regulated by the antioxidant response element (ARE) and respond to the transcription factor Nrf2 [65]. Under normal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and is degraded by the ubiquitin-proteasome pathway through the Keap1- and Cullin 3-based-E3/Rbx1 ligase complex. Under stress conditions or when activated by enhancers, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds to AREs of target protective genes and activates transcription to protect impairment from oxidative stress, reactive carcinogenic metabolites, and carcinogenesis [66-68].

In the United States, prostate cancer (PCa) is the leading diagnosed non-cutaneous male cancer subtype [69]. Oxidative stress, which occurs when the reactive oxygen species (ROS) overwhelm the capacity of the antioxidant defense system, is one of the etiologic factors related to PCa. Epidemiological, experimental, and clinical studies have suggested an association between oxidative stress and risk of PCa development and progression [70-73]Excessive ROS induce DNA damage and mutation and cell and tissue damage, which could give rise to a variety of human pathogenesis, including cardiovascular, metabolic, inflammatory, and neurodegenerative diseases and cancer [74-76].

Epigenetic changes, mainly DNA methylation, histone modification, and microRNA regulation, are other hallmarks of PCa. DNA methylation is the most frequently studied of these changes [77]. Among all of the related genes, hypermethylated Nrf2 has shown a close relationship to PCa carcinogenesis. We have previously reported that Nrf2 transcription is suppressed in the prostate tumors of transgenic adenocarcinoma mouse prostate (TRAMP) mice and tumorigenic TRAMP-C1 cells due to the hypermethylation of the first five CpG islands in the Nrf2 promoter [32, 78].

Accumulating evidence has shown that Nrf2 activation can defend against and prevent PCa carcinogenesis [79-81]. Many dietary phytochemicals have beneficial effects and the ability to activate the Nrf2 signaling pathway. Isothiocyanates (cruciferous vegetables) [82], organosulfur compounds (garlic and onions) [83], polyphenols (green tea and spice turmeric) [84], and isoflavones (soy beans) [85] have been characterized as potent Nrf2 activators. These agents can stimulate various upstream kinases, interfere in the Keap1-Nrf2 interaction, and/or disturb cellular redox balance, all resulting in the activation of the Nrf2 pathway. Additionally, epigenetic modifications may contribute to the regulation of

the transcription activity of Nrf2. Since DNA methylation is reversible by intervention with DNMT inhibitors, combined treatment with 5-azadeoxycytidine (5-aza) and Trichostatin A (TSA) can restore Nrf2 expression in TRAMP-C1 cells [32]. In addition, a variety of bioactive nutrients (e.g., curcumin [86], tocopherols [78], sulforaphane [40, 87], and 3,3′-diindolylmethane (DIM) [88]) modulate DNA methylation and/or histone modification, thereby effectively restoring Nrf2 expression.

In our previous study, curcumin showed a PCa chemopreventive effect through the epigenetic modification of the Nrf2 gene and the restoration of the Nrf2-mediated antioxidative stress cellular defense capability [86]. (3*E*,5*E*)-3,5-Bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one (FN1) (Figure 3) is a newly synthesized curcumin analogue that is substantially more potent than curcumin in inhibiting PCa cell growth [89, 90]. However, it is not quite clear by what route FN1 exerts chemopreventive function for prostate cancer. Here, we will examine FN1 in inhibiting proliferation and colony formation of TRAMP C1 cells, its effects in activating the Nrf2 pathway, and the underlining mechanisms.

3.2 Materials and Methods

3.2.1 Reagents and Antibodies

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), penicillin–streptomycin (10,000 U/mL), versene, and trypsin-EDTA were supplied by Gibco (Grand Island, NY, USA). A Cell-Titer 96 Aqueous One Solution Cell Proliferation (MTS) Assay Kit was obtained from Promega (Madison, WI, USA).

Platinum Taq DNA polymerase was purchased from Invitrogen (Grand Island, NY, USA). Tris-HCl precast gels, turbo transfer buffer, and PVDF membranes were obtained from Bio-Rad (Hercules, CA, USA). Tris-glycine-SDS running buffer and Super Signal enhanced chemiluminescent substrate were purchased from Boston BioProducts (Ashland, MA, USA) and Thermo (Rockford, IL, USA), respectively. Antibodies against Nrf2 (C-20), HO-1 (C-20), NQO1 (H-90), UGT1A1 (V-19), and actin (I-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The protease inhibitor cocktail, radioimmunoprecipitation (RIPA) buffer, and antibodies against HDACs (HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6) were supplied by Cell Signaling Technology (Beverly, MA, USA). The anti-HDAC8 antibody was obtained from Proteintech Group (Chicago, IL, USA), and the anti-NQO1, -HDAC7, -DNMT3a, and -DNMT3b antibodies were from Abcam (Cambridge, MA, USA). Anti-DNMT1 was supplied by Novus Biologicals (Littleton, CO, USA). All other chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO, USA).

3.2.2 Materials and Chemicals

Synthesized FN1 (purity >95%) was obtained from Kun Zhang's laboratory (Laboratory of Natural Medicinal Chemistry & Green Chemistry, Guangdong University of Technology, Guangzhou, China). Dimethyl sulfoxide (DMSO) (purity ≥99.7%), 5-aza (purity ≥97%), and TSA (purity ≥98%) were from Sigma (St. Louis, MO, USA).

3.2.3 Cell Culture

The human hepatocellular HepG2-C8 cell line was previously established by stable transfection with an ARE luciferase construct [91]. The cells were cultured and maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. TRAMP-C1 cells were obtained from B. Foster (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY, USA). Cells were cultured in DMEM (pH 7.0) containing 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere, as described previously [32].

3.2.4 Luciferase Reporter Activity Assay

The stably transfected HepG2-C8 cells expressing the ARE-luciferase vector were used to study the effects of FN1, curcumin, and sulforaphane (SFN) on the Nrf2-ARE pathway. The ARE-luciferase activity in the HepG2-C8 cells was determined using a luciferase assay kit in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, HepG2-ARE-C8 cells (1.0×10^5 cells/well) were seeded in 12-well plates in 1 mL of medium containing 10% FBS, incubated for 24 h, and then treated with various concentrations of FN1, curcumin, and SFN samples. Afterward, the cells were lysed using the reporter lysis buffer, and 10 μL of the cell lysate supernatant was analyzed for luciferase activity using a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Normalization of the luciferase activity was performed based on protein concentrations, which were determined using a BCA protein assay (Pierce Biotech,

Rockford, IL, USA). The data were obtained from three independent experiments and are expressed as the inducible fold change compared with the vehicle control.

3.2.5 MTS Assay

TRAMP-C1 cells were seeded in 96-well plates at a density of 1×10^3 cells per 100 μL of DMEM per well, incubated for 24 h, and treated with either 0.1% DMSO (control) or various concentrations of FN1 in DMEM containing 1% FBS for 1, 3, or 5 days. Series diluted FN1 samples were dissolved in DMSO (final concentration in the medium of <0.1%), and the medium was changed every 2 days. Cell viability was estimated with a CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

3.2.6 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

TRAMP-C1 cells were seeded at a density of 1×10^5 cells in 10 cm dishes with 10% FBS/DMEM. After 24 h, the cells were treated with DMEM medium containing 1% FBS with FN1 (50 nM, 100 nM, and 250 nM) or with 0.1% DMSO as a control. The treatment medium was changed every 2 days. After the 3-day treatment, the total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. mRNA expression levels were determined using first-strand cDNA as a template by quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems,

Carlsbad, CA, USA) in an ABI7900HT system. The following primer sequences for Nrf2, HO-1, NQO1, and UGT1A1 were used: Nrf2, 5′-AGCAGGACTGGAGAAGTT-3′ (sense) and 5′-TTCTTTTTCCAGCGAGGAGA-3′ (antisense); HO-1, 5′-CCTCACTGGCAGGAAATCATC-3′ (sense) and 5′-CCTCGTGGAGACGCTTTACATA-3′(antisense); NQO1, 5′-AGCCCAGATATTGTGGCCG-3′ (sense) and 5′-CTTTCAGAATGGCTGGCAC-3′ (antisense); UGT1A1, 5′-GAAATTGCTGAGGCTTTGGGCAGA-3′ (sense) and 5′-ATGGGAGCCAGAGTGTGTGATGAA-3′ (antisense). β-Actin was used as an internal control with sense (5′-CGTTCAATACCCCAGCCATG-3′) and antisense (5′-ACCCCGTCACCAGAGTCC-3′) primers.

3.2.7 Preparation of Protein Lysates and Western Blotting

TRAMP-C1 cells were seeded at a density of 1×10^5 cells in 10 cm dishes with 10% FBS/DMEM. After incubation for 24 h, the cells were treated with 0.1% DMSO as a control and FN1 (50 nM, 100 nM, and 250 nM) in DMEM containing 1% FBS. Following treatment for 3 days, the cells were washed with ice-cold PBS and harvested in ice-cold 1× RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protein inhibitor cocktail (Sigma). The protein concentrations of the cell lysates were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Identical concentrations of protein (20 μg) were subjected to 4 to 15% SDS–polyacrylamide gel (Bio-Rad, Hercules, CA, USA) electrophoresis (SDS–PAGE) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA and sequentially incubated with specific primary antibodies and HRP-conjugated

secondary antibodies. The antibody-bound proteins were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and measured with a Gel Documentation 2000 system (Bio-Rad).

3.2.8 Bisulfite Genomic Sequencing (BGS)

TRAMP-C1 cells (1×10^5 cells per 10 cm dish) were treated with 0.1% DMSO as a control, FN1 (50 nM, 100 nM, and 250 nM), and a combination of 5-aza (500 nM) and TSA (100 nM) for 3 days. Genomic DNA was then extracted from the cells with a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Then, 500 ng of genomic DNA was subjected to bisulfite conversion with an EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA, USA) according to the manufacturer's instructions. The converted DNA was amplified with Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY, USA) and primers that amplify the first five CpGs of the murine Nrf2 gene. The primer sequences were 5′-AGTTATGAAGTAGTAGTAAAAA-3′ (sense) and 5′-AATATAATCTCATAAAACCCCAC-3′ (antisense). A TOPO TA Cloning kit (Invitrogen, Grand Island, NY, USA) was used to clone the PCR products into vector pCR4 TOPO. Plasmids containing PCR products from at least 10 colonies per treatment from three independent experiments were amplified and purified with a QIAprep Spin Miniprep kit (Qiagen), followed by sequencing (GeneWiz, South Plainfield, NJ, USA).

3.2.9 Methylated DNA Immunoprecipitation (MeDIP) Analysis

To verify the DNA methylation changes, methylated DNA was captured and quantified using methylated DNA immunoprecipitation coupled with quantitative real-time polymerase chain reaction analysis (MeDIP-qPCR), as described previously.(27) Briefly, the extracted DNA from treated cells was sonicated on ice using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ, USA) to a fragment size of approximately 200–1000 base pairs. The fragmented DNA was denatured at 95 °C for 2 min. Methylated DNA was isolated by immunoprecipitation with anti-5′-methylcytosine antibody using a Methylamp Methylated DNA capture kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's manual. After final purification and elution, the methylation status was quantified by qPCR amplification of MeDIP-enriched DNA using the primer set 5′-GAGGTCACCACAACACGAAC-3′ (forward) and 5′-ATCTCATAAGGCCCCACCTC-3′ (reverse) to cover the DNA sequence of the first five CpGs of murine Nrf2. The enrichment of methylated DNA in each treatment was calculated according to the standard curve of the serial dilution of input DNA. The relative methylated DNA ratios were then calculated with the basis of the control as 100% of DNA methylation.

3.2.10 Anchorage-Independent Colony-Formation Assay

TRAMP-C1 cells (2×10^4/ml) were suspended in 1 mL of basal medium Eagle (BME) containing 0.33% agar and plated over 3 mL of a solidified BME consisting of 0.5% agar and 10% FBS in 6-well plates in the presence of 50 nM, 100 nM, or 250 nM FN1. The cells were maintained at 37 °C in a 5% CO_2 incubator for 2 weeks. The cell colonies were

imaged using a Nikon ACT-1 microscope (version 2.20; LEAD Technologies) and counted using ImageJ (version 1.48d; NIH, Bethesda, MD, USA).

Statistical Analyses

The data are presented as the mean ± the standard deviation of three independent experiments. One-way analysis of variance (ANOVA) or unpaired Student's *t* test (SPSS 19.0, IBM, 2010, Chicago, IL, USA) were performed to identify significant differences between means ($p < 0.05$).

3.3 Results

3.3.1 FN1 Induced ARE-Luciferase Reporter Activity

The relative fold changes of luciferase activity compared with HepG2-C8 cells which were stably transfected with the ARE-luciferase reporter vector are shown in Figure 4. FN1 induced a higher luciferase activity than the control in a dose-dependent manner from 50 to 250 nM which indicates that FN1 may possess the activation ability for those genes with an ARE sequence. When cells were treated with the same dosage (1000 nM), FN1 showed a greater effect than curcumin and SFN.

3.3.2 Cytotoxicity Measurement of FN1 by MTS

MTS measurement was performed to investigate the cytotoxicity of FN1 against TRAMP-C1 cells. Viability of TRAMP-C1 was found to be reduced by FN1 treatment in a time- and dose-dependent manner after 1, 3, and 5 days of treatment (Figure 5). The survival rate

of TRAMP-C1 treated with FN1 at concentrations less than 250 nM were higher than 80%, which indicates a lower toxicity than the high-concentration groups. Hence, doses of 0, 50, 100, and 250 nM of FN1 were chosen for further epigenetic study on Nrf2 reactivation.

3.3.3 FN1 Enhanced Expression of Nrf2 and Its Downstream Antioxidant and Detoxifying Enzymes and Decreased Keap1 Expression

TRAMP is a well-studied prostate carcinogenesis model. In our previous experiments with TRAMP-C1, C3 cells, TRAMP prostate tumor samples, and wild type prostate samples, we have demonstrated that the highly methylated CpG region in Nrf2 promoters often comes with a decreased expression of Nrf2 gene and its protein and vice versa [92]. Nrf2 is a vital regulating factor of antioxidant and detoxifying enzymes such as HO-1, NQO1, and UGT1A1 [93]. To test FN1 effects on Nrf2 and the downstream genes, we performed qPCR analysis to measure the mRNA level of Nrf2 and HO-1, NQO1, and UGT1A1 level in TRAMP-C1 cells after treating with FN1 for 3 days (Figure 6). As shown in Figure 6A, FN1 at 100 or 250 nM significantly upregulated the Nrf2 mRNA level ($p < 0.05$); FN1 at 100 or 250 nM significantly increased HO-1 mRNA expression (Figure 6B). FN1 at 50, 100, or 250 nM significantly increased NQO1 mRNA expression (Figure 6C) and FN1 at 250 nM significantly increased UGT1A1 mRNA compared with the controls (Figure 6D). We then carried out Western blotting experiments to analyze their protein expression after the same treatment. FN1 (50–250 nM) enhanced the protein expression of Nrf2, and that enhancement correlates positively with the dose of FN1 (Figure 7). Higher concentrations of FN1 also increased the protein expression of HO-1, NQO1, and UGT1A1. The above findings demonstrate that FN1 has the ability to enhance the expression of both mRNA and

protein level of Nrf2 and that it mediated antioxidant and detoxifying genes in TRAMP-C1 cells. Figure 7 also indicate that the FN1 concentration has an inverse relationship with the expression of Keap1, which also may explain the activation of Nrf2.

3.3.4 FN1 Reduced Colony Formation in TRAMP-C1 cells

The anchorage-independent growth capacity of cells indicates their tumorigenicity [94, 95]. FN1 at concentrations of 50, 100, and 250 nM significantly suppressed the colony formation of TRAMP-C1 cells by 80.48%, 83.36%, and 85.66%, respectively (Figure 8). These results suggest that FN1 plays a vital role in suppressing anchorage-independent growth of TRAMP-C1 cells and has the potential for decreasing tumorigenicity.

3.3.5 FN1 Diminished the Methylation Ratio of the First Five CpG Regions in Nrf2 Promoter

It has been demonstrated that the hypermethylation ratio of the first five CpGs in the Nrf2 promoter region correlated inversely with expression of Nrf2 [32]. BGS assay was then used to determine if FN1 can induce hypomethylation to the CpG region. The high methylation rate (88.13%) of the promoter CpGs was found in the control group (3 day-treatment with 0.1% DMSO) (Figure 9). Hypermethylation in the region was observed to reduce to 63.89%, 82.22%, and 73.55%, respectively, in 5-aza and the TSA group (3-day combination treatment of 5-aza (500 nM) and TSA (100 nM)), low dose FN1 group (100 nM for 3 days), and high dose FN1 group (3 days for 250 nM). We proceeded with the MeDIP-qPCR assay to verify the methylation profile from BGS. The first five CpGs of the

Nrf2 promoter region from sonicated fragmented DNA after being captured was expanded by qPCR. It demonstrates that combination treatment of 5-aza (500 nM)-TSA (100 nM) or FN1 (250 μM) induced the demethylation effect on the Nrf2 promoter region with statistical significance ($p < 0.01$) (Figure 10). These results suggest that FN1 can induce hypomethylation in the Nrf2 promoter region, which may restore Nrf2 expression.

3.3.6 FN1 Downregulated Epigenetic Modification Enzymes

The effects of FN1 on epigenetic modification enzymes, including DNMTs and HDACs, were further examined to explore the epigenetic mechanism of FN1 in promoter demethylation and the induction of Nrf2 gene transcription. We found that FN1 (50–250 nM) reduced the protein level of DNMT1, DNMT3a, and DNMT3b in TRAMP-C1 cells after 3 days of treatment (Figure 11A). In addition, FN1 has also reduced HDAC protein expression, especially HDAC4 ($p < 0.05$; Figure11B).

3.4 Discussion

PCa, a high-incidence and slow-progression disease, is typically diagnosed at the late stage of life. Hence, a modest delay in disease progression could have a significant impact on disease-related morbidity, mortality, and quality of life [96]. Natural phytochemicals, which have chemopreventive properties that delay the progress of carcinogenesis, have become as auspicious and practical approaches to deal with the increase of PCa [97-99]. Curcumin, for example, has demonstrated its chemopreventive activity in preventing PCa [98]. FN1, a newly synthesized curcumin analogue, has shown anti-carcinogenic

effects against PC-3, Panc-1, and HT-29 [89]. In our experiments, FN1 has shown to inhibit the proliferation of TRAMP-C1 cells, another tumorigenic prostate adenocarcinoma cell line. In addition, FN1 significantly inhibited the colony formation of TRAMP-C1 cells. Anchorage-independent colonies grown in soft agar indicate normal cell transformation or cancer cell tumorigenicity in vitro [95, 100]. In brief, FN1 has the ability to prevent prostate carcinogenesis in vitro.

Oxidative stress is believed to be mainly generated by the imbalance between ROS and cellular antioxidant defense capacity, which may be the cause of inflammation or PCa carcinogenesis [72, 77, 101]. It has long been known that Nrf2 plays a vital role in defending cells against oxidative damage by regulating antioxidant and detoxification enzymes, such as HO-1 (antioxidant), NQO1, and UGT1A1 (detoxification) [93, 102]. Because of its protective properties, Nrf2 expression will normally be reduced in the initiation of carcinogenesis. In human PCa, Nrf2 levels were found to be extensively decreased though the analysis of 10 human PCa microarray data sets [103]. Numerous dietary phytochemicals, such as curcumin [104], indole-3-carbinol (I3C) [105], tocopherols [78], and Z-ligustilide [106], can inhibit prostate tumorigenesis by enhancing the expression of Nrf2 and its downstream phase II antioxidant and detoxification enzymes, HO-1, NQO1, and UGT1A1. In our experiments, we found that FN1, an analogue of curcumin, could enhance the expression of those genes transcriptionally and post-transcriptionally, which suggests that FN1 may also exert its chemoprevention effects via enhancing the Nrf2 pathway.

It has been widely accepted that epigenetic modifications are closely related to PCa initiation and progression [70, 107, 108]. Hence, DNA methyltransferases (DNMTs) and

histone deacetylases (HDACs), which induce DNA methylation and histone acetylation modification and cause gene expression silencing are becoming new targets for prostate cancer prevention and therapy [32, 109-111]. We previously reported that Nrf2 is epigenetically silenced by a high percentage of CpG methylation in the promoter region during TRAMP prostate carcinogenesis in vivo and in vitro [32, 86, 101]. Many dietary compounds, such as apigenin, tanshinone IIA, and sulforaphane, were found to restore Nrf2 expression by epigenetic modification [40, 112, 113]. Curcumin can cause DNA demethylation and histone modification to TRAMP-C1 cells and thus restore the silenced Nrf2 expression arising from promoter region hypermethylation [86, 114]. We were therefore interested in exploring whether the curcumin analogue FN1 can activate Nrf2 expression though the same route. In the BGS test, we found that FN1 treatment (250 nM) exhibited a demethylation effect on the first 5 CpGs in the Nrf2 promoter in TRAMP-C1 cells (Figure 9). This demethylation effect was confirmed by the MeDIP assay, which indicated that the methylated DNA ratio of the same region was lower in TRAMP-C1 cells treated with FN1 (250 nM) than in control cells (Figure 10). The protein levels of DNMT1, DNMT3a, and DNMT3b and HDACs in TRAMP-C1 cells treated with FN1 were compared with controls to reveal the underlying mechanism of Nrf2 demethylation. After FN1 treatment, we found that the protein levels of DNMT1, DNMT3a, and DNMT3b decreased significantly in a dose-dependent manner (Figure 11A). The protein levels of HDAC2, HDAC4, HDAC7, and HDAC8 were all reduced after FN1 treatment, although only the decrease of HDAC4 was statistical significantly different from the control.

In the modulation of the Nrf2 pathway, Keap1 can inactivate the function of Nrf2 by sequestering it in the cytoplasm and preventing it from entering the nucleus [115]. Keap1

also serves as a bridge between Nrf2 and ubiquitination ligase Cullin-3 to help induce Nrf2 degradation [93]. ARE inducers can cause Nrf2 dissociation from Keap1, which helps Nrf2 to translocate into the nucleus and finally regulate the downstream antioxidant genes transcriptionally [116]. In our experiments, the protein expression of Keap1 was significantly reduced by treatment with 250 nM FN1 for 3 days (Figure 7) which suggests one of the other potential mechanisms of FN1 activation of the Nrf2 pathway.

Hyperactive proliferation and enhanced survival of cancer cells can be attributed to the elevated oxidative stress [117]. Oxidative stress and chronic inflammation, and chronic exposure to carcinogens and mutagens are crucial in the initiation of carcinogenesis [118]. Nrf2 pathway protects against oxidative stress and thus prevents carcinogenesis. In our previous in vivo study of treating TRAMP mice with broccoli sprouts, prostate tumorigenesis has been significantly inhibited via the activation of the Nrf2 pathway [119].

Anchorage-independent colony formation and growth are emblems of transformed cells [120]. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced JB6 cell transformation and the soft agar anchorage-independent colony formation model is a well-studied skin carcinogenesis model. By comparing with Nrf2 knock-down cells and control cells, it indicates that Nrf2 plays a vital role in the TPA-induced JB6 cell transformation and that the epigenetic reactivation of the Nrf2 pathway could potentially contribute to the attenuation of JB6 cellular anchorage-independent colony formation and inhibition of neoplastic transformation [113, 121].

In addition, as we also found previously that prostate carcinogenesis in the TRAMP model in vivo and in vitro are highly correlated with suppressed expression of Nrf2, which

mainly comes from epigenetically silence [32, 101, 104], it is very likely that hypermethylation in the first five CpG islands of Nrf2 is highly associated with the TRAMP cancer cell development and colony formation. Hence, the ability of FN1 in inhibiting the proliferation and colony formation of TRAMP C1 cells may probably be due to its capability in enhancing Nrf2 and its downstream antioxidant and detoxification enzymes by activation of the Nrf2-ARE pathway (Figures 4-8) through epigenetic reactivation of Nrf2, which includes DNA demethylation and histone modification effects (Figures 9-11) and inhibition of keap1 expression (Figure 7). We will further investigate the above hypothesis with Nrf2-knockdown TRAMP cells in our future study.

In conclusion, our findings reveal that FN1, an analogue of curcumin, can inhibit growth and colony formation in TRAMP-C1 cells. FN1 can increase mRNA and protein level of Nrf2 and its downstream detoxifying and antioxidant enzymes, including HO-1, NQO1, and UGT1A1. Our results also indicate that FN1 can restore the silenced Nrf2 gene in TRAMP-C1 cells probably through demethylation of the Nrf2 promoter region and histone modifications. In addition, the keap1 level was reduced by FN1 treatment. The epigenetic regulation and inhibition of keap1 therefore may be the mechanisms of restoring Nrf2 and its downstream target genes. FN1 thus demonstrates it effectiveness and potential in inhibiting the initiation, progression, and development of PCa. FN1 is a novel cancer chemopreventive agent for the management of PCa. However, its in vivo efficacy and pharmacokinetics profile need further investigation.

Chapter 4 - Epigenetic regulation of Nrf2 by curcumin derivatives – Desmethoxycurcmin and Bisdemethoxycurcumin in Human Prostate Cancer Cells

4.1 Abstract

Curcumin has been shown to be an effective chemopreventive agent in the TRAMPC1 prostate cancer cell model. It has also shown favorable effects as a hypomethylation agent in the Nuerog1 gene of human LNCaP cells. The poor systemic bioavailability of curcumin drives the need for an alternative agent that still retains the moiety of curcumin that makes it effective as an anti-cancer agent. BDMC and DMC are two curcumin derived compounds that have demonstrated anti-carcinogenic and ant-cancer effects. We look to investigate the epigenetic regulating effects that BDMC and DMC may have on Nrf2. Protein and mRNA expression of epigenetic modifying genes influenced by the two compounds were gathered through western blot and qPCR, respectively. Bisulfite sequencing of the Nrf2 gene was performed to examine the hypomethylation potential of BDMC and DMC. qPCR analysis revealed decreased mRNA levels of DNA methylating enzymes DNMT1 and DNMT3B, but increased levels of DNMT3A. mRNA expression of Nrf2 and its downstream genes, HO-1 and NQO-1, were found to be induced by the two chemicals. Protein levels of Nrf2, HO-1, and NQO-1 reflected the results of qPCR, where their expression was increased by treatment of BDMC and DMC. Histone modifying protein expression enzymes were decreased by the two treatment groups. Bisulfite sequencing analysis revealed little to no change in the methylation profile of Nrf2's three CpG sites. It was found that the two curcumin derivatives were able to induce Nrf2 and its downstream targets at high levels. However, it is not believed to be through the hypomethylation of Nrf2 but by way of histone modification as the results indicated HDACs 1, 3, and 4 were significantly

inhibited. Further experimentation of these two compounds are required to fully understand what mechanisms or what combination of mechanism are involved to induce expression of Nrf2.

4.2 Introduction

Previously, our lab has demonstrated that the diferuloylmethane compound, curcumin, was a potent demethylation agent and was able to restore the expression of Nrf2 in TRAMPC1 mice. Additionally, its demethylation properties has been shown to induce global DNA hypomethylation in the leukemia cell line, MV4-11 [52]. This suggests that curcumin can, at least in part, act as a chemopreventive agent in the carcinogenesis of prostate cancer. Curcumin's role in epigenetic regulation is largely attributed to its ability to modulate DNA methylation and/ or histone modification [86, 122]. As a result of curcumin's potential as a chemopreventive agent, many different analogues of curcumin have been explored to determine a more suitable compound with stronger anti-cancer activity. One drawback of curcumin is its poor bioavailability due to poor absorption and rapid metabolism [123]. FN1, which is a curcumin synthetic analogue, has demonstrated itself as a novel anticancer agent with stronger potential than curcumin. Although its bioavailability still needs to be studied, FN1 has displayed its epigenetic regulating effects by increasing Nrf2 levels and downstream genes via activation of Nrf2-ARE pathway in TRAMP-C1 mouse cells. All of which is achieved through reduction of epigenetic modifying enzymes involved in DNA methylation and histone modification [124]. Here we briefly, examine a set of curcuminoids or derivatives of curcumin, bisdemthoxycurcumin (BDMC) and

desmethoxycurcumin (DMC) and their epigenetic effects on Nrf2 in the human prostate cancer line, LNCaP.

4.3 Method

4.3.1 Cell Culture

The human hepatocellular HepG2C8 cell line was previously established by stable transfection with an ARE luciferase construct. Cells were cultured and maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. LNCaP cells were maintained in RPMI-1640 with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. All cells were maintained and grown at 37°C in a humidified 5% CO_2atmosphere.

4.3.2 MTS Assay

LNCaP cells were seeded in 96-well plates at a density of $3x10^3$ cells per 100 uL of 1% FBS RMPI-1640 per well, incubated for 20 h, and treated with either 0.1% DMSO (control) or various concentrations 6.25uM and 12.5uM BDMC, 6.25 and 12.5uM DMC, 5uM CURC and 5-Aza/TSA (2.5uM/500nM) for 5 days. Medium was changed every five days. Cell viability was estimated with CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

4.3.3 Western Blotting, mRNA expression and DNA Bisulfite Sequencing

Extracted genomic DNA was bisulfite converted and used as template for converted fragment amplification, the PCR products were then run on agarose gel. PCR product was then extracted from gel and subjected to TA cloning to determine methylation profiles of the Nrf2 sequence target.

Total RNA was extracted from 5 day treated cells and reverse transcribed to cDNA for real time PCR analysis. Relative expression was determined using DMSO control and GAPDH endogenous control.

Total protein lysate was extracted from cells treated for 5 days. 20ug of protein was run through gel electrophoresis and transferred to a PVDF membrane. Membrane was stained with antibodies targeting various proteins with β-Actin as control. Stained membrane was visualized using a chemiluminescent imager.

4.4. Results

Cytotoxicity of BDMC and DMC in LNCaP cells were determined by MTS measurement. As shown in Figure 12A and 12B, viability of the cells were found to be reduced by BDMC and DMC, through separate treatments, in a time- and dose-dependent matter starting with the lowest concentration of 1.5625 uM. Survival rates of LNCaPs by BDMC at concentrations less than 6.25 uM higher than 80% indicating lower toxicity. Thus, concentrations of 6.25 and 12.5 uM of both compounds chosen to test.

Western Blot analysis showed 1.3 fold induction of Nrf2 when treated with DMC 6.25 uM and 1.2 fold induction with DMC 12.5 uM. Downstream targets of Nrf2, NQO-1 and HO-1 displayed greatest protein levels for treatments of DMC 12.5 uM with 1.7 and 4.4 fold induction, respectively (Figure 13A). Epigenetic modulator (Figure 13B) protein expression of HDACs 1, 3, and 6 had significant reduction in both DMC treatments (6.25 and 12.5uM), with DMC 12.5uM having the greatest fold change of 0.7 for HDAC1, 0.3 for HDAC3 and 0.5 for HDAC6. At concentrations of 12.5 uM, BDMC had similar expression levels as 12.5uM of DMC, with HDAC1 having a 0.8 fold change.

To test the effects of these curcumin derivatives, qPCR analysis was carried out to measure mRNA expression of Nrf2, HO-1 and NQO-1. Results (Figure 14) indicated that there was some correlation between expression of proteins and mRNA expression. Concentrations of 12.5uM of BDMC and DMC treated cells displayed at least 2 fold induction of HO-1, while NQO-1 induction was significantly induced for treatments of 12.5 uM BDMC and all treatments of DMC. Additionally, mRNA expression of DNA methylation genes were measured to determine the influence of these derivatives (Figure 15). All concentrations of BDMC and DMC showed significant induction of DNMT3A, whereas DNMT1 and DNMT3B has at least a 0.6 fold change when treated with 12.5 uM BDMC, 6.25 uM and 12.5 uM DMC, revealing a possible inhibitory effect.

Relative total methylation profiles were used to determine methylation status of the three major CpG sites in the Nrf2 promoter (Figure 16). 5-aza control showed a decrease in methylation, confirming its status as a DNMT inhibitor and consequently, lower relative total methylation. All concentrations of the curcumin derivatives showed no change in total

methylation, with some treatments even showing an increased as with the case of 12.5 uM DMC.

4.5 Conclusion

Delaying the progression of prostate carcinogenesis with phytochemicals is one of many approaches in cancer therapeutics. Curcumin and its synthetic analogue have both shown the capacity to reduce the methylation status of the first 5 CpG sites in the Nrf2 promoter in TRAMPC1, resulting in reactivation of the oxidative stress master regulator and its downstream enzymes [86, 124]. In this study, we discovered that the higher concentrations of BDMC and DMC induced higher levels of Nrf2 mRNA when compared to control. However, total relative methylation showed no change, whereas curcumin and known DNMT inhibitor had reduced methylation at the three major CpG sites. Although the methylation profile revealed no change, there was a slight increase in expression of Nrf2 at both protein and mRNA levels, across most of the treatments, which may be connected to the higher levels of DNMT3A mRNA as this gene is involved in de novo methylation. The same treatments showed an inhibition of the DNMT1 and DNMT3B genes. An in vitro study of cells lacking DNMT1 exhibited decreased cellular DNA methyltrasnferase activity but only had a 20% decrease in overall genomic methylation. This suggests that there are other factors involved in methylation of genes besides DNMT1 [125]. It is suspected that DNMT3A plays a larger role in methylation and would explain the lack of change in Nrf2's methylation status.

Levels of HDACs 1, 2 and 3 have been shown to be highly expressed in prostate cancer with HDAC2 being a possible biomarker [34]. Our results show that HDAC levels of 1, 3

and 6 are significantly reduced for the curcumin derivatives, with DMC being the most promising. Additionally, many studies have shown that HDAC inhibition can go one to stimulate Nrf2 signaling and subsequently inducing its target genes NQO-1 and HO-1.

With the limited data that is presented, there is potential epigenetic regulation of Nrf2 by these two curcumin derivatives. The exact mechanism by which it is regulated is still unclear and must be further investigated. What is known, is that BDMC and DMC have the capability to inhibit two major DNA methylating enzymes and inhibit a selection of histone deacetylation enzymes in human prostate cancer cell.

Chapter 5 Conclusion

Prostate cancer is one of the most common diseases in men and is responsible for the second most cancer related deaths in the US. Although survival rates have improved with the last two decades, the disease remains complex in diagnosis, treatment and in prevention. There are various factors that can cause PCa, some which are more impactful than others. Diet has been shown to be impactful where studies involving transplantation of low-risk PCa individuals to high-risk PCa populations have shown these individuals to develop similar PCa rates as the high-risk populations. These high-risk populations had diets that consisted of increased levels of fat and red meat intake which has been directly linked to PCa. These types of diets have the potential to develop carcinogenic compounds which can lead the production of two major factors that drive carcinogenesis, inflammation and oxidative stress. Cellular systems have defenses in place to prevent the accumulation of reactive oxidants caused by inflammation. Central to these defenses is master regulator of the anti-oxidative pathway, Nrf2, which is responsible for the induction of phase II detoxifying/antioxidant genes and reduction of stressful conditions generated by inflammation and oxidation. The Nrf2 pathway can be activated by oxidative stress, carcinogens, and phytochemicals. Use of phytochemicals in particular, leads to the idea of chemoprevention where phytochemicals, synthetic or natural, are used to impede the development of cancer at different stages. The mechanism of activation of Nrf2 varies with each phytochemical but the end results are the same, induction of its downstream targets necessary for detoxification. The isothiocyanate, PEITC, induced phosphorylation of ERK and JNK and subsequent phosphorylation of Nrf2 to induce its translocation to the nucleus.

SFN, another isothiocyanate, interacts with thiols of Keap1 resulting in the release of Nrf2 and its translocation into the nucleus.

In cancer cells, genes can be activated, suppressed or silenced through epigenetic mechanisms. DNA methylation of tumor suppressor genes are associated with cancer where hypermethylation of the promoter regions with DNA methylation enzymes results in the silencing of these crucial genes. Changes in chromatin structure initiated by histone modifying enzymes can impact gene expression by activating or inactivation gene transcription. Various phytochemicals have been shown to influence epigenetic mechanisms, by way of DNMT and/or HDAC inhibition. Curcumin is a potent epigenetic modulator in TRAMPC1 cells where it has effectively reduced methylation levels of the first 5 CpG sites in the Nrf2 promoter, ultimately resulting in the reactivation of Nrf2 and its downstream genes. Demethylation potential of curcumin was also found in human LNCaP cells, in which the first fourteen CpG sites of Neurog1 were found to be highly demethylated. Curcumin has diminished clinical usefulness due to poor absorption and low oral bioavailability, hence, alternatives need to be identified. The synthetic derivative of curcumin, FN1, has revealed itself as a possible candidate based on preliminary anti-cancer studies. In TRAMPC1 cells, FN1 was able to reactivate Nrf2 by way of CpG demethylation and induce downstream genes. Additionally, FN1 was able to inhibit tumor colony formation suggesting FN1s potential for decreasing tumorigenicity. Two other curcumin derivatives are also being investigated for their potential effects in human Nrf2. BDMC and DMC are curcuminoids that have shown to reactivate the expression of Nrf2 and its downstream genes, HO-1 and NQO-1. However, the methylation profiles of the major CpG sites of human Nrf2 promoter regions were remained unchanged relative to controls

suggesting that the Nrf2 expression is controlled by other mechanisms. Results from the study suggest that inhibition of certain HDACs by BDMC and DMC play a larger role in regulation of Nrf2. In conclusion, this thesis examines curcumin derivatives in activating Nrf2 and epigenetics effects that could potentially contribute to prostate cancer chemoprevention and treatment. Further studies encompassing in vivo animal models undergoing treatment with FN1 are needed to confirm the results that were obtained from in vitro studies. Additional experiments concerning BDMC and DMC are also required to fully understand what combination of mechanisms are responsible for Nrf2 activation in prostate cancer.

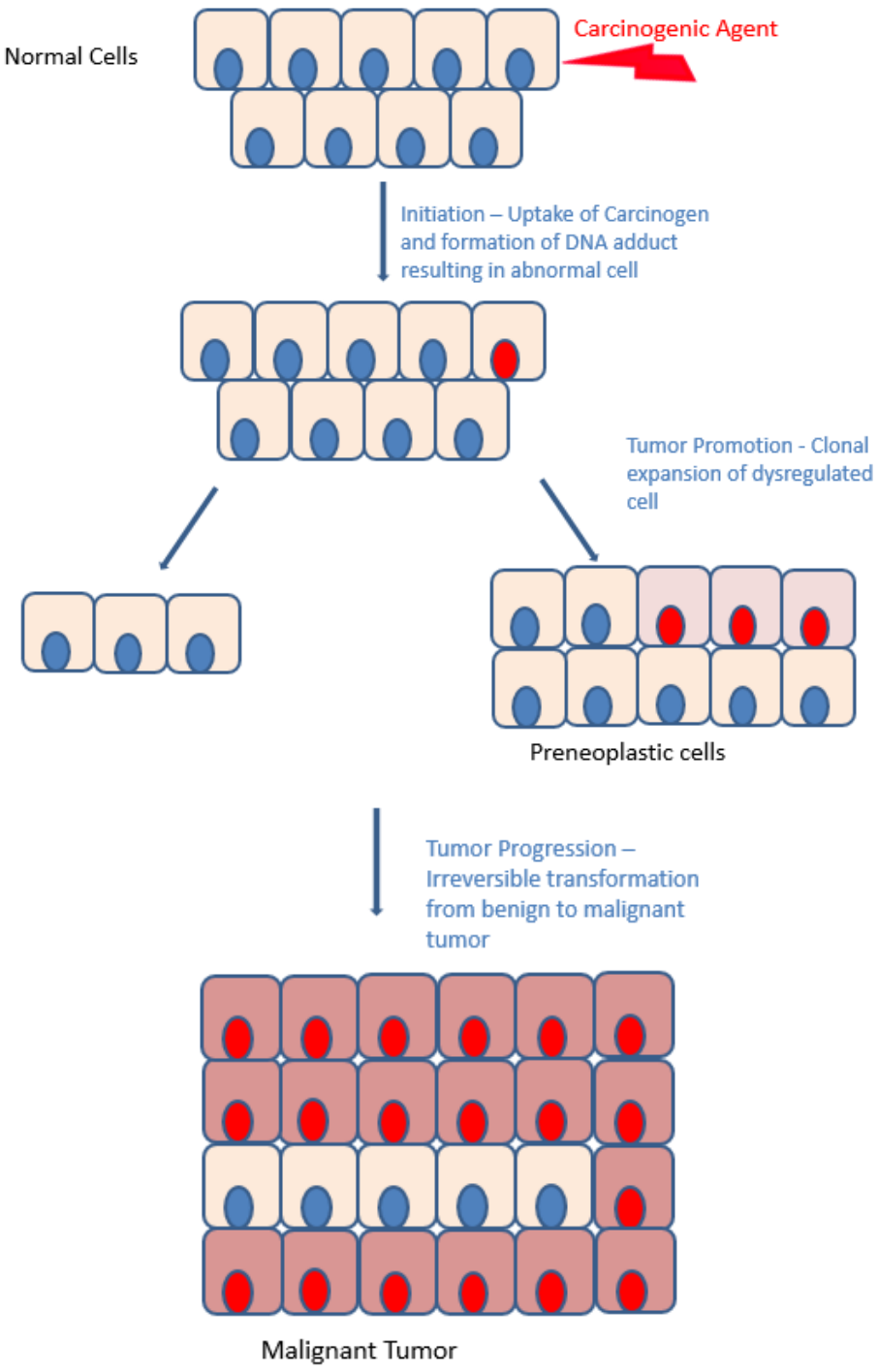


Figure 1. Three Stages of Carcinogenesis

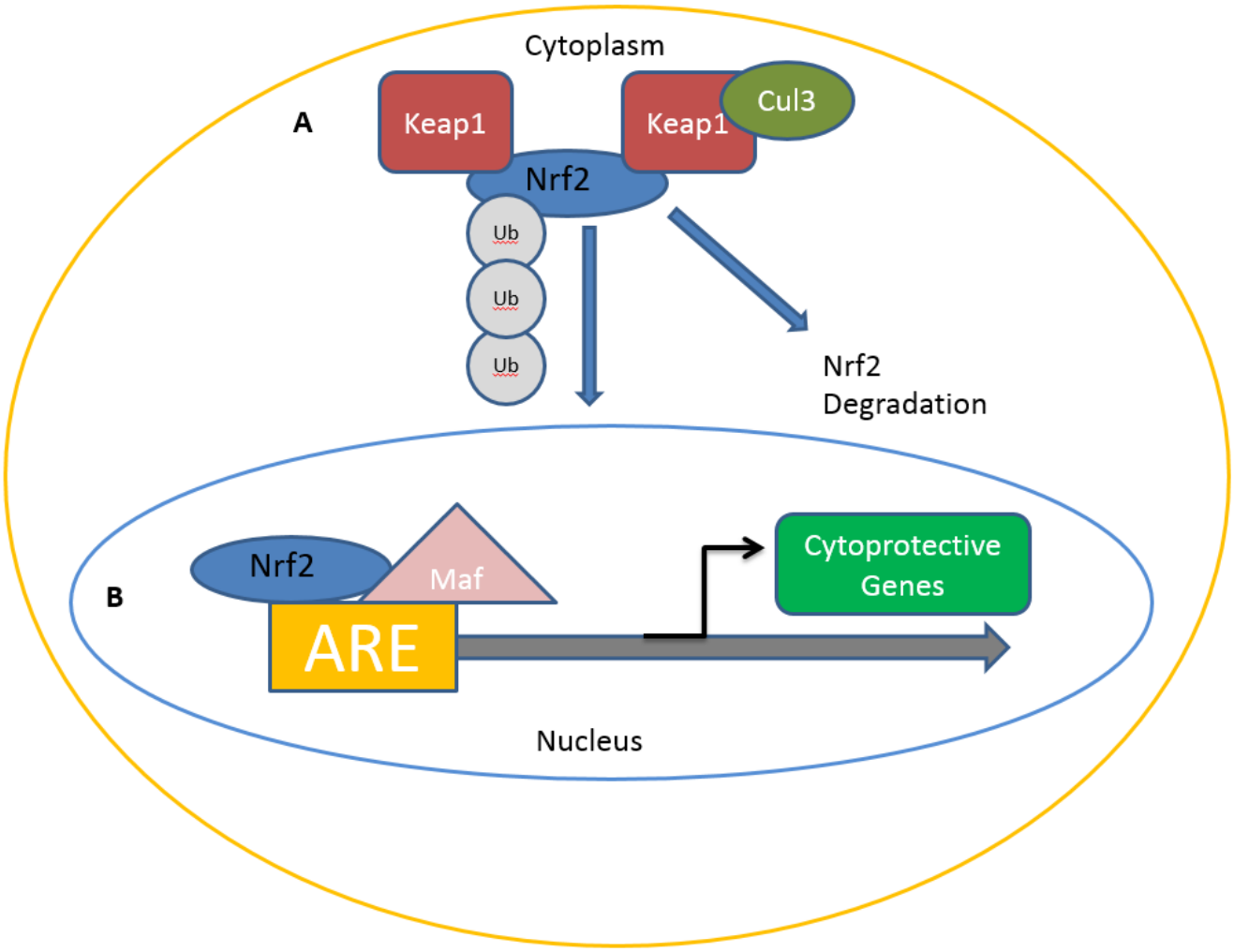
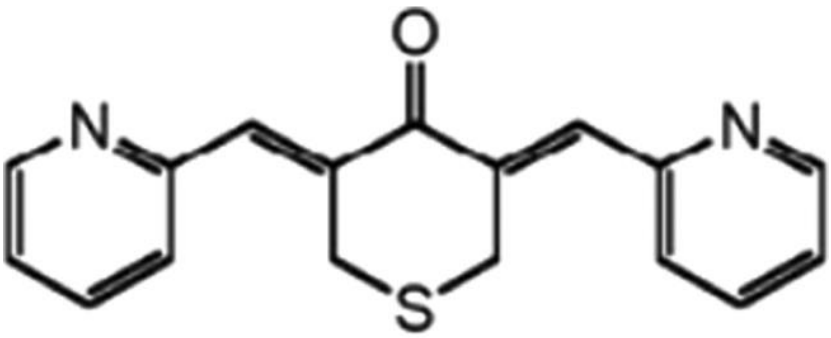


Figure 2. Nrf2-ARE Transcription Pathway. A) Basal conditions of Nrf2 in the cytoplasm where it is sequestered by Keap1 and degraded by the ubiquitin-proteasome pathway. B) In response to stress conditions, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to ARE with Maf proteins and incudes expression of target cytoprotective genes.



FN1

Figure 3. Chemical structure of (3*E*,5*E*)-3,5-bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one (FN1).

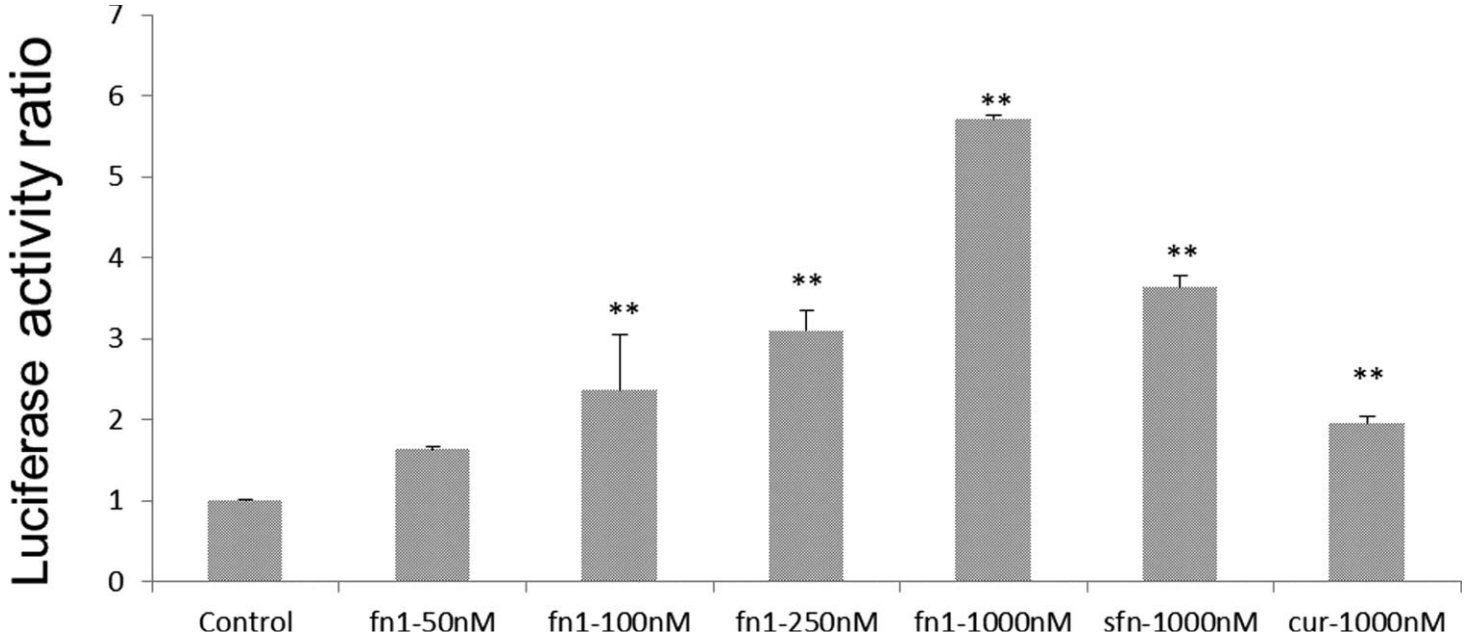


Figure 4. ARE-Luciferase activity assay of effects of FN1, curcumin, and SFN on the Hep-G2 C8 cell line. The BCA protein assay was determined to normalize the luciferase activity. The data obtained from three independent experiments expressed the inducible fold change compared with the vehicle control. *, $p < 0.05$; **, $p < 0.01$ comparing with control group.

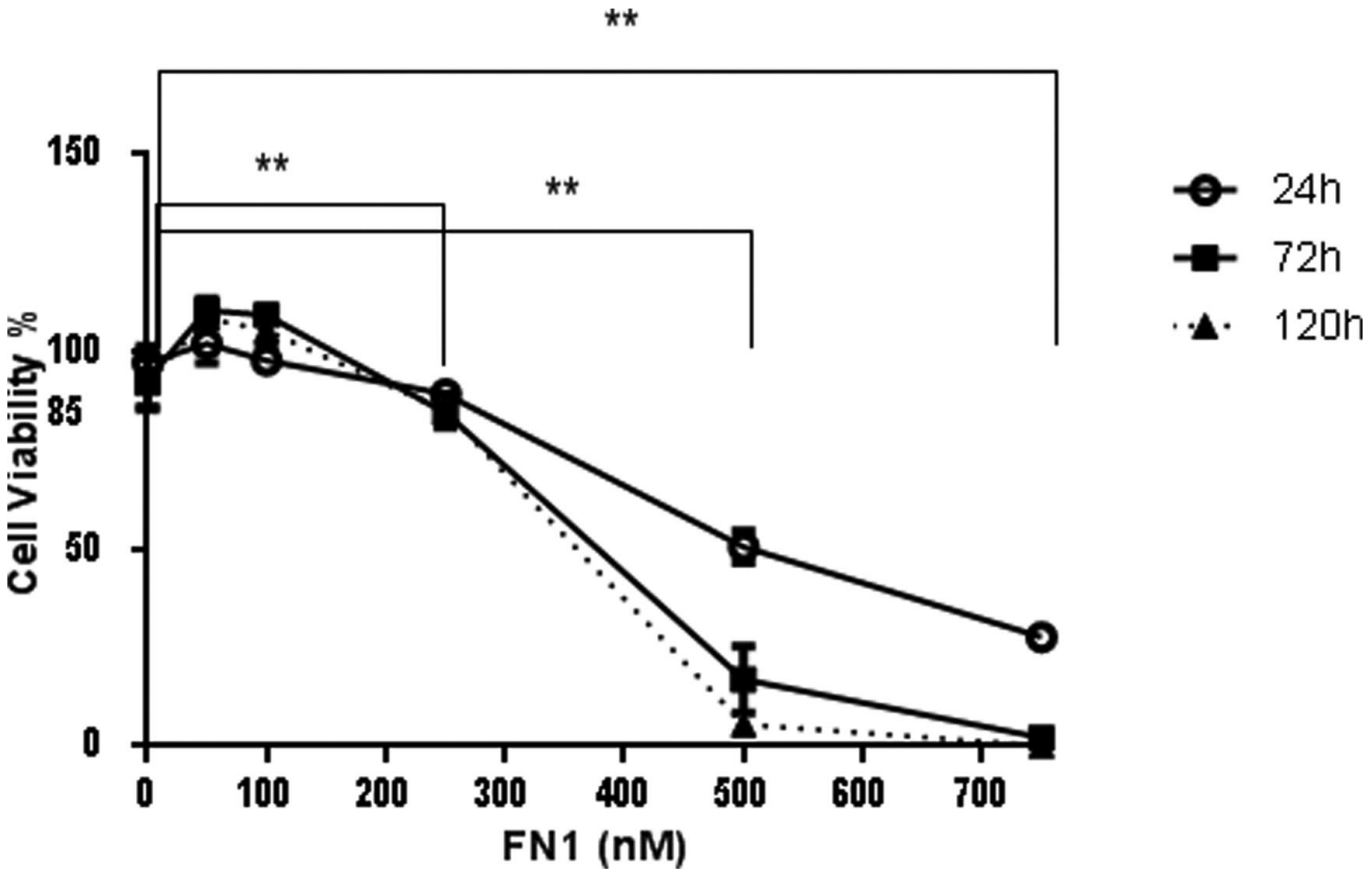


Figure 5. Cytotoxicity of FN1 against the TRAMP-C1 cell line. Cells were seeded in a 96-well plate for 24 h and then treated with various concentrations of FN1 for 1, 3, or 5 days. Cytotoxicity was determined by a MTS assay. The data are expressed as the means ± SD (n = 3). **, $p < 0.01$ compared with the control group.

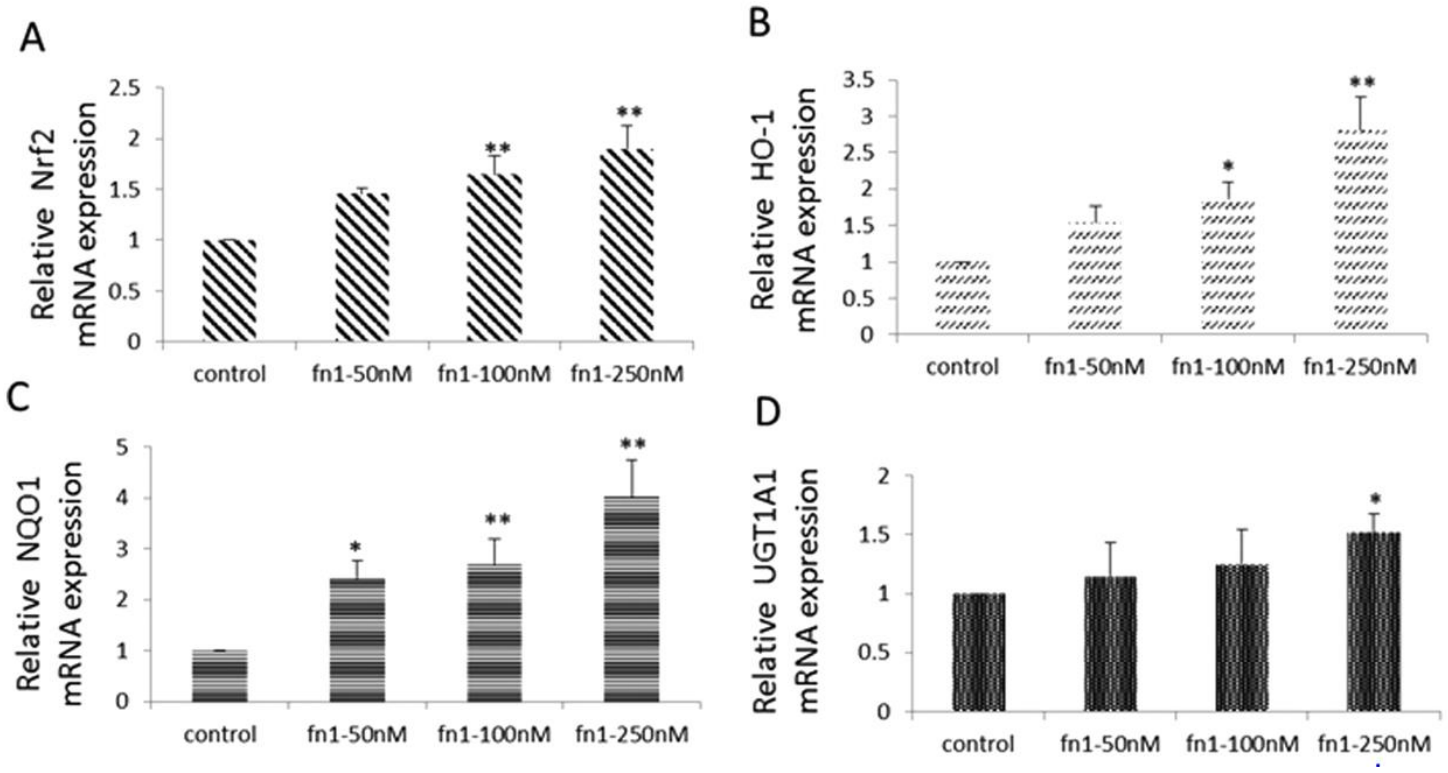


Figure 6. Relative endogenous mRNA expression of Nrf2 target genes; (A), HO-1 (B), NQO1 (C), and UGT1A1 (D) in TRAMP-C1 cells from three independent experiments after treatment with FN1 (50, 100, and 250 nM) for 3 days. The data are expressed as the mean ± SD. β-Actin was used as an internal control. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group.

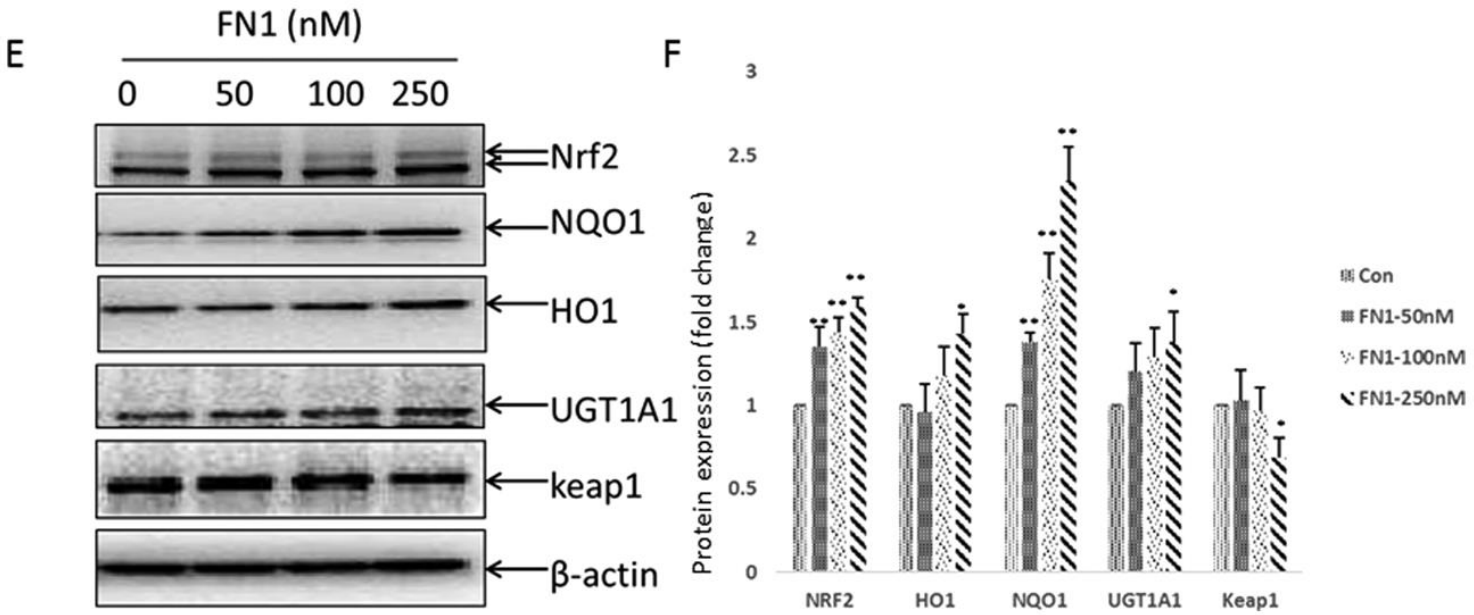


Figure 7. Effect of FN1 on protein expression of Nrf2 target genes (HO-1, NQO1, and UGT1A1) and keap1 in TRAMP-C1 cells. FN1 was applied (50, 100, and 250 nM) for 3 days. The relative protein expression levels were quantified based on the signal intensity of the corresponding bands from 3 independent experiments and normalized using β-actin for the total cellular protein level and are presented as the mean ± SD. *, $p < 0.05$ and *, $p < 0.01$ compared with the control group.

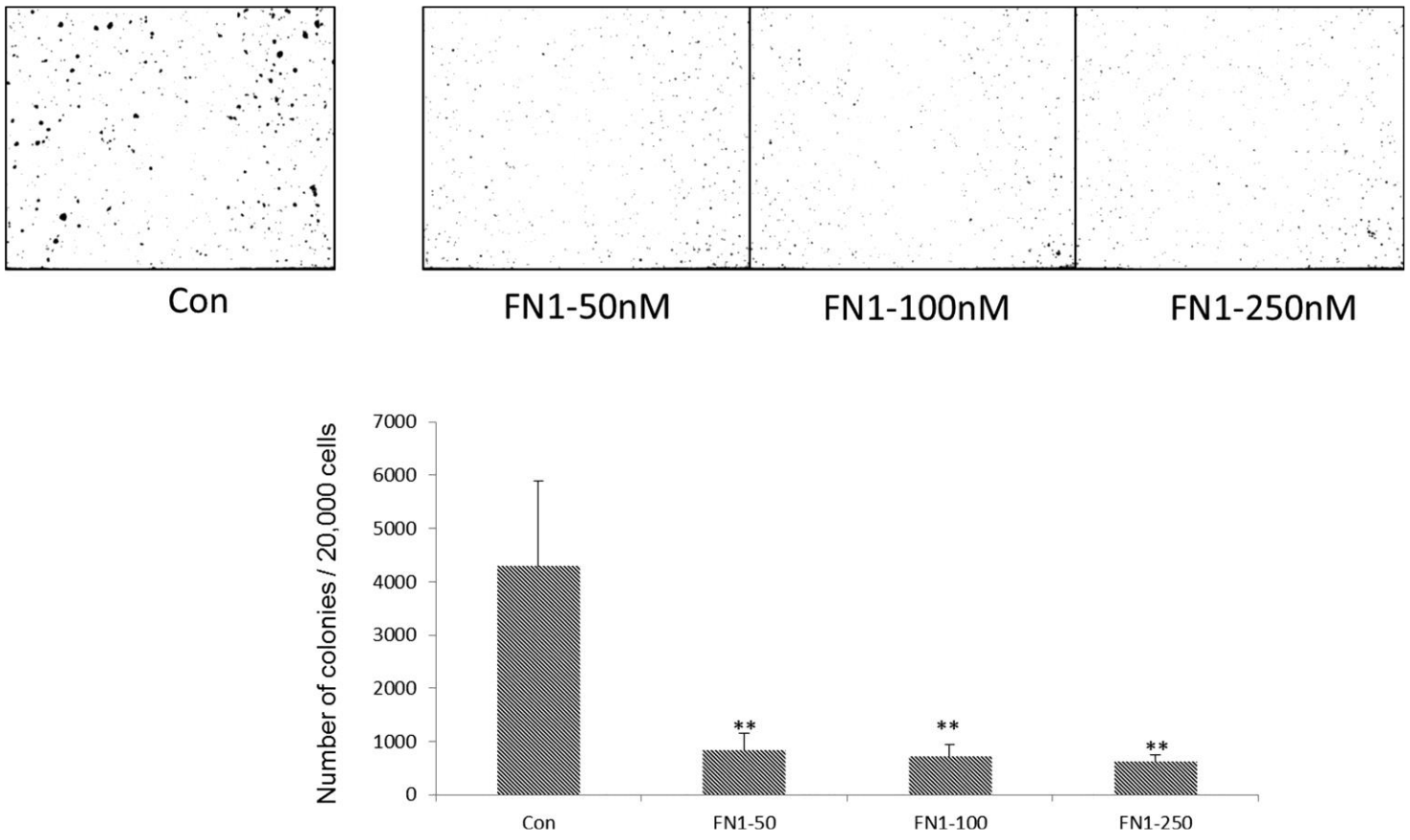


Figure 8. Inhibitory effect of FN1 on the Anchorage-Independent Colony-Formation of TRAMP-C1 cells. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The graphical data are presented as the average of triplicate results from 3 independent experiments. **, $p < 0.01$ compared with the control group.

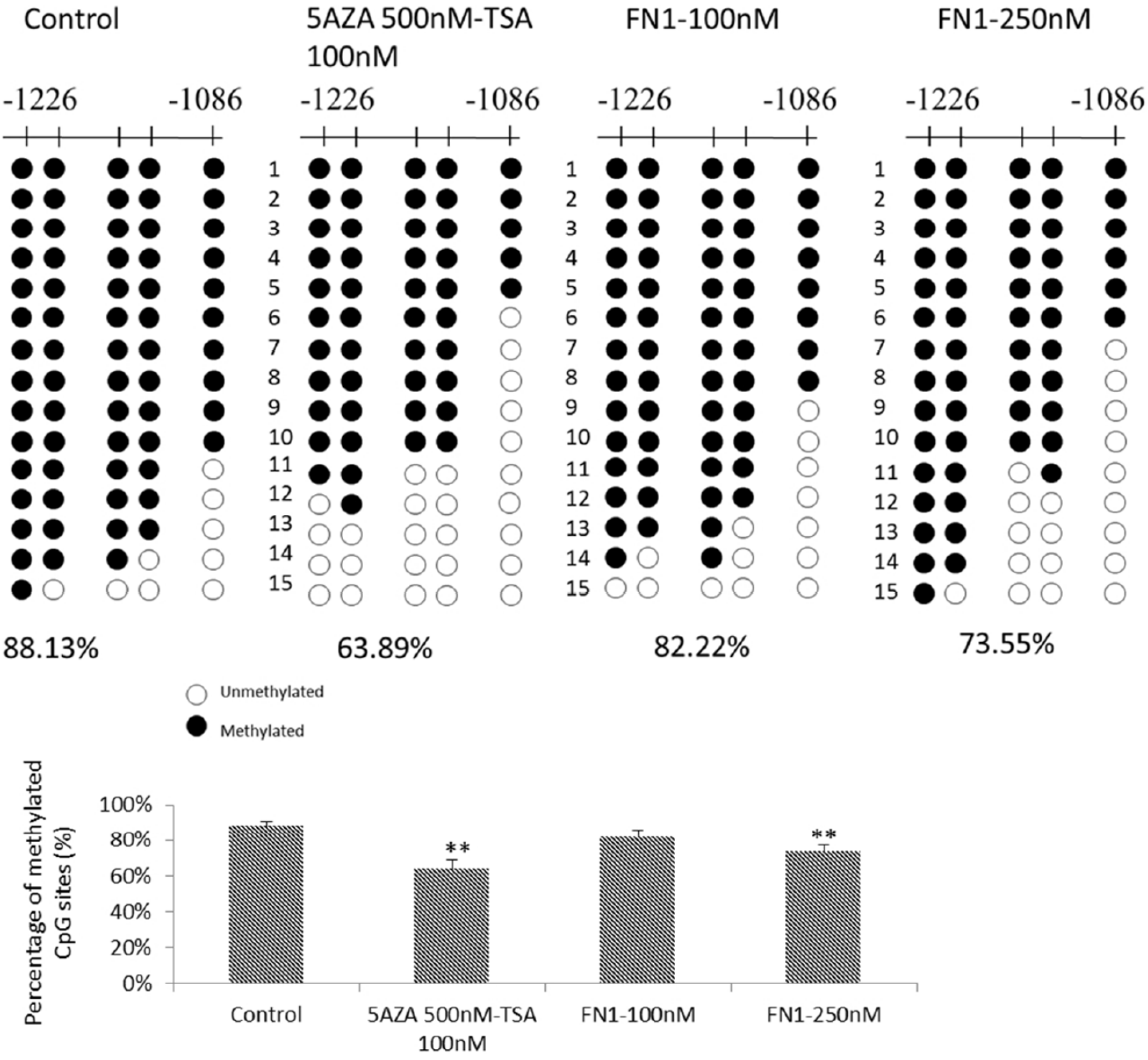


Figure 9. Methylation ratio of the first 5 CpGs of Nrf2 promoter region in TRAMP-C1. Cells were treated for 3 days. * $p< 0.05$ and ** $p < 0.01$ compared with the control group.

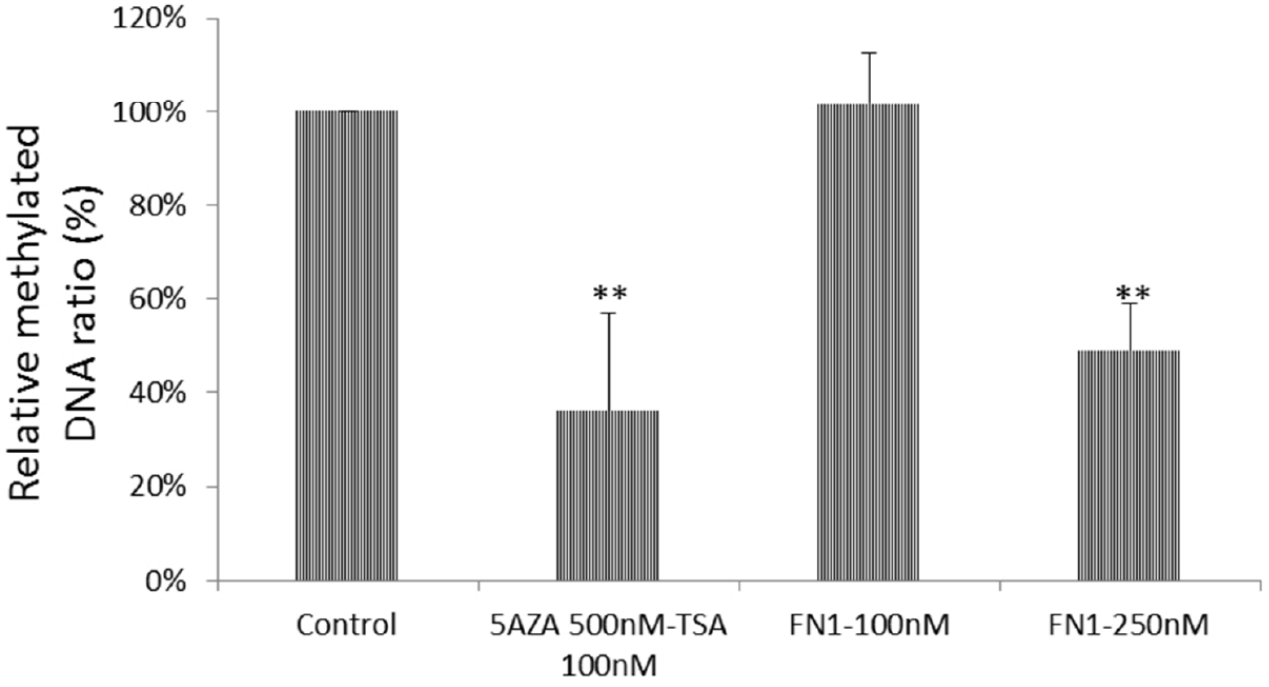


Figure 10. MeDIP (methylated DNA immunoprecipitation) assay for the effect of FN1 on the methylation of theNrf2 promoter region in TRAMP-C1 cells.

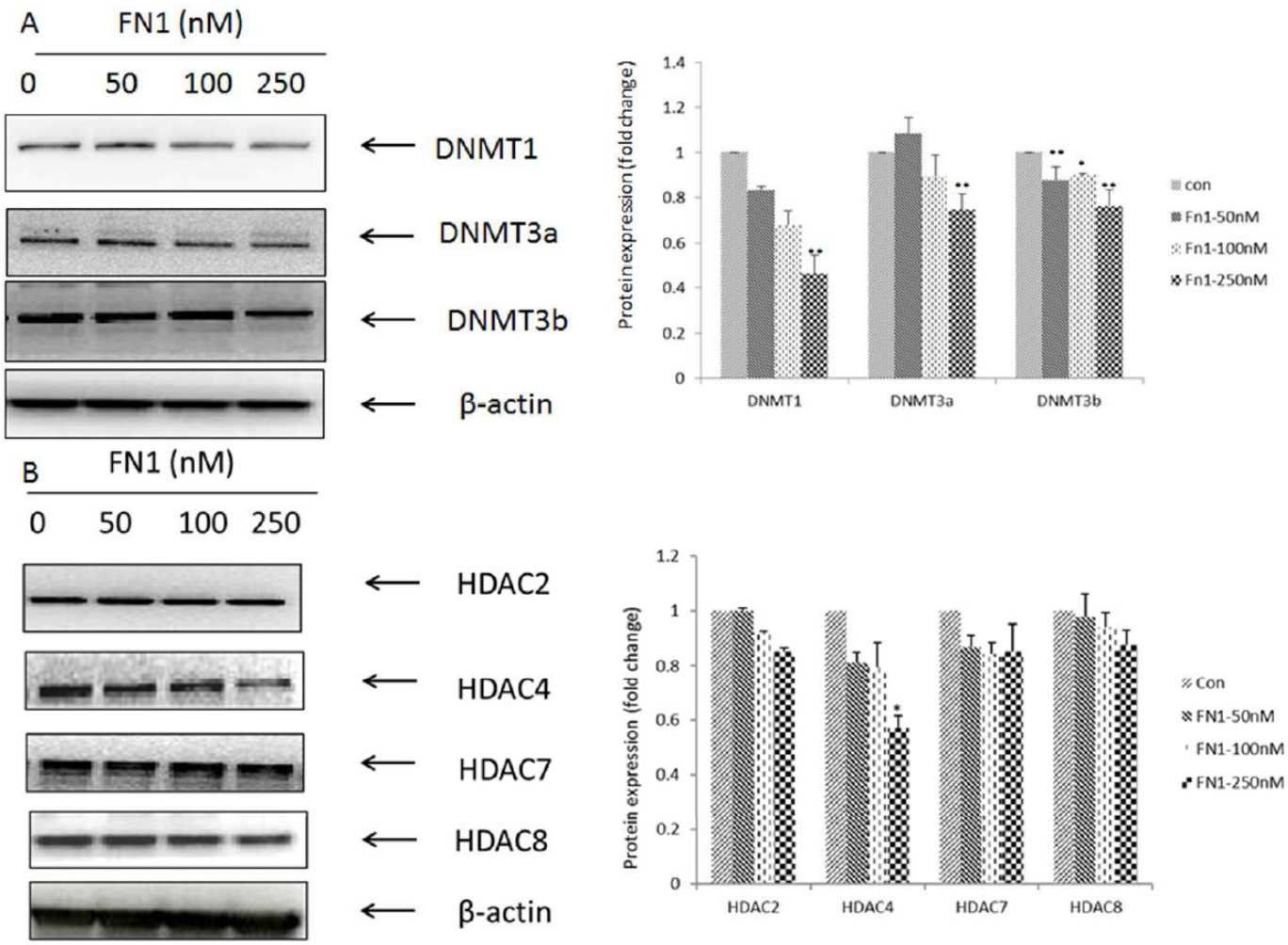
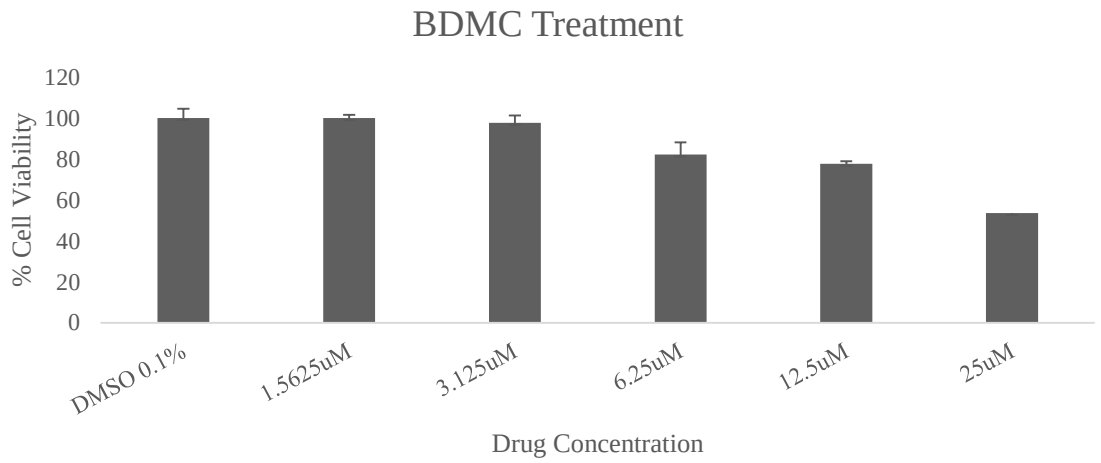


Figure 11. Effect of FN1 on DNMT protein expression (A) and protein expression of HDACs (B) in TRAMP-C1 cells. Cells (1 × 105/10-cm dish) were incubated with FN1 (50, 100, and 250 nM) for 3 days. The relative expression levels were quantified based on the signal intensity of the corresponding bands and normalized using β-actin. The graphical data are represented as the mean ± SD from 3 independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group.

A



B

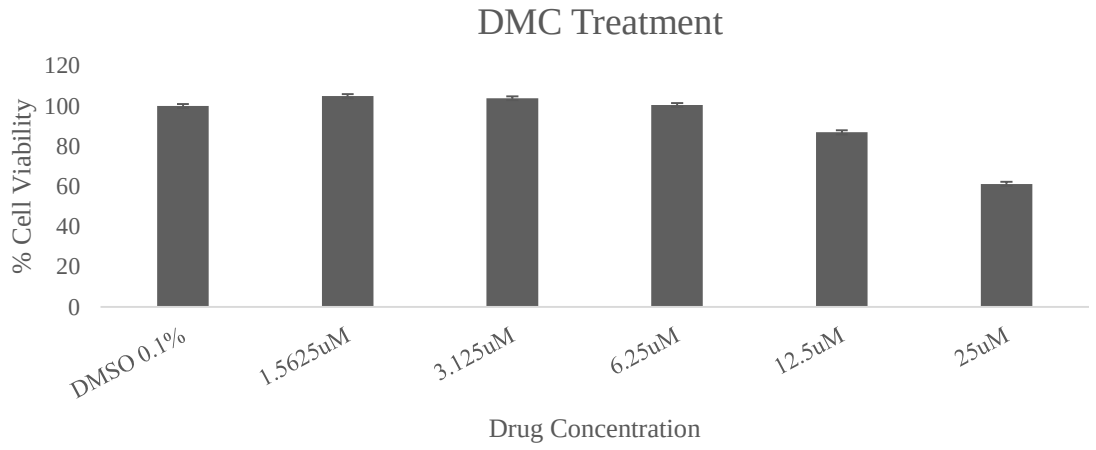


Figure 12. Percent Cell viability from the MTS assay in LNCaP cells treated with DMC and BDMC

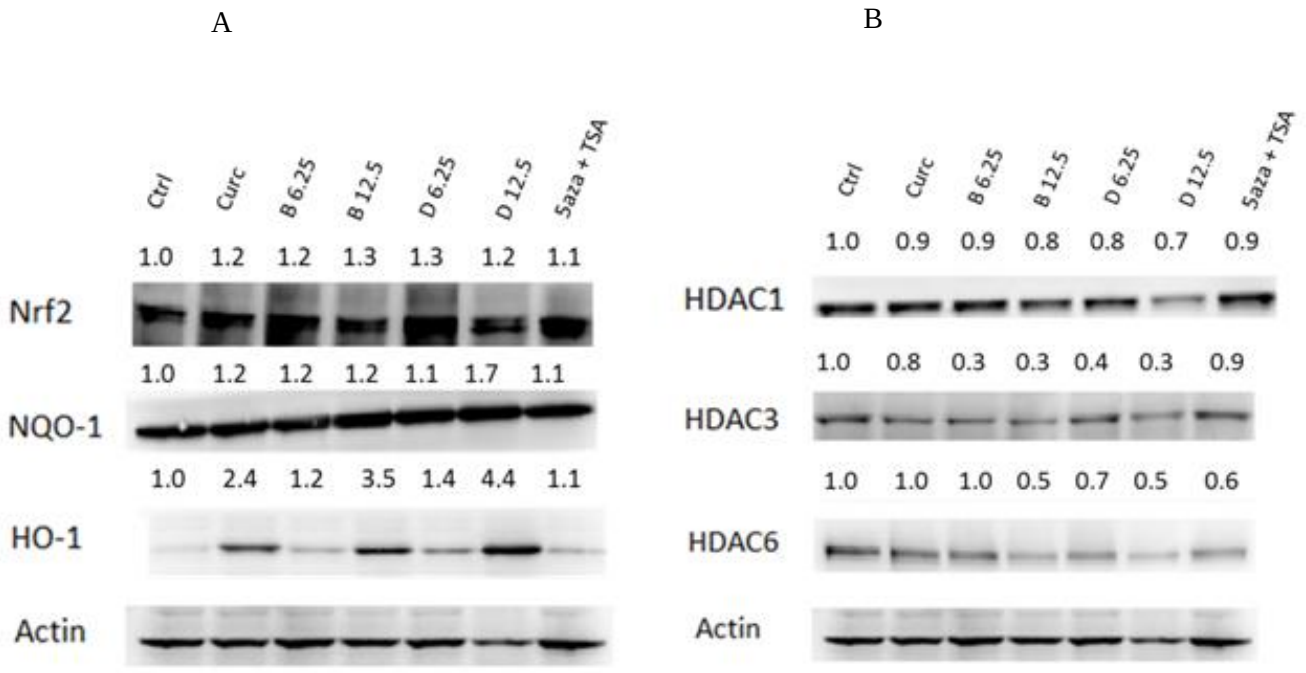


Figure 13. Protein expression of Epigenetic Modifying Enzymes

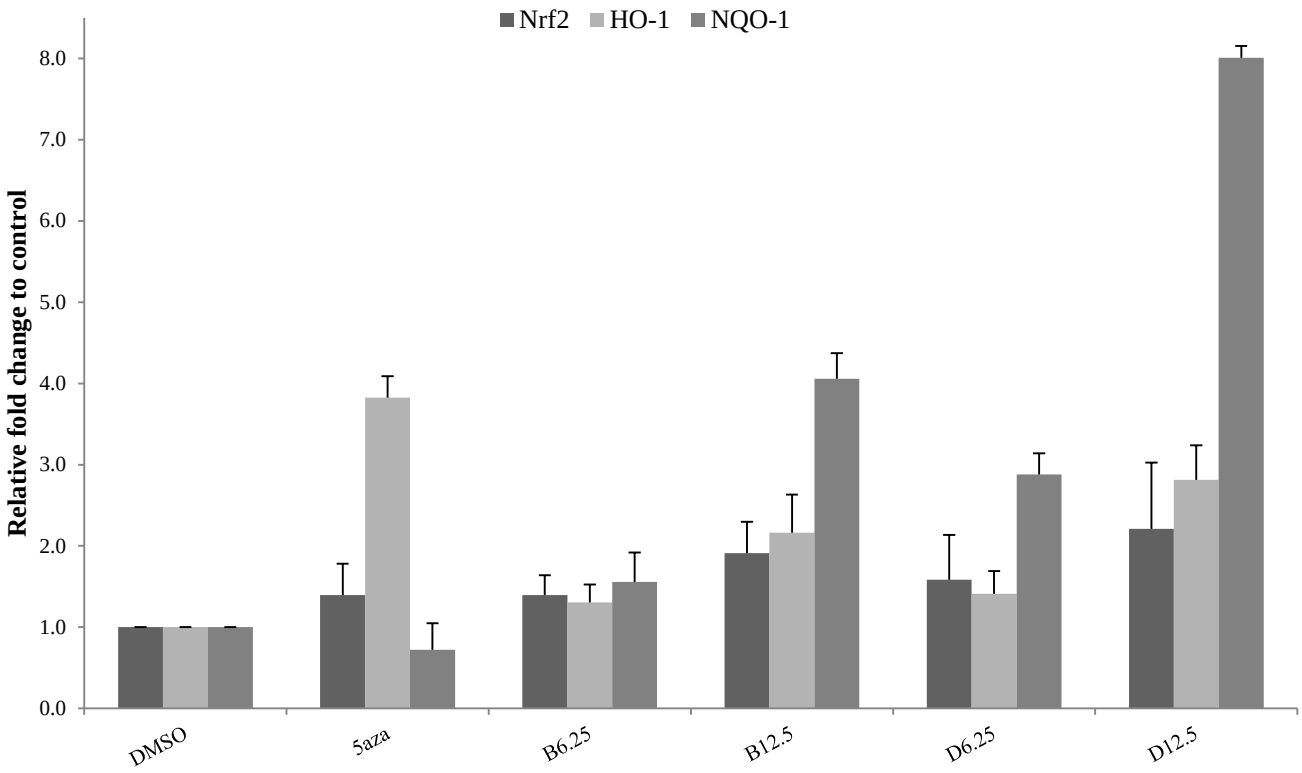


Figure 14. mRNA expression levels of Nrf2 and its down-stream genes HO-1 and NQO1

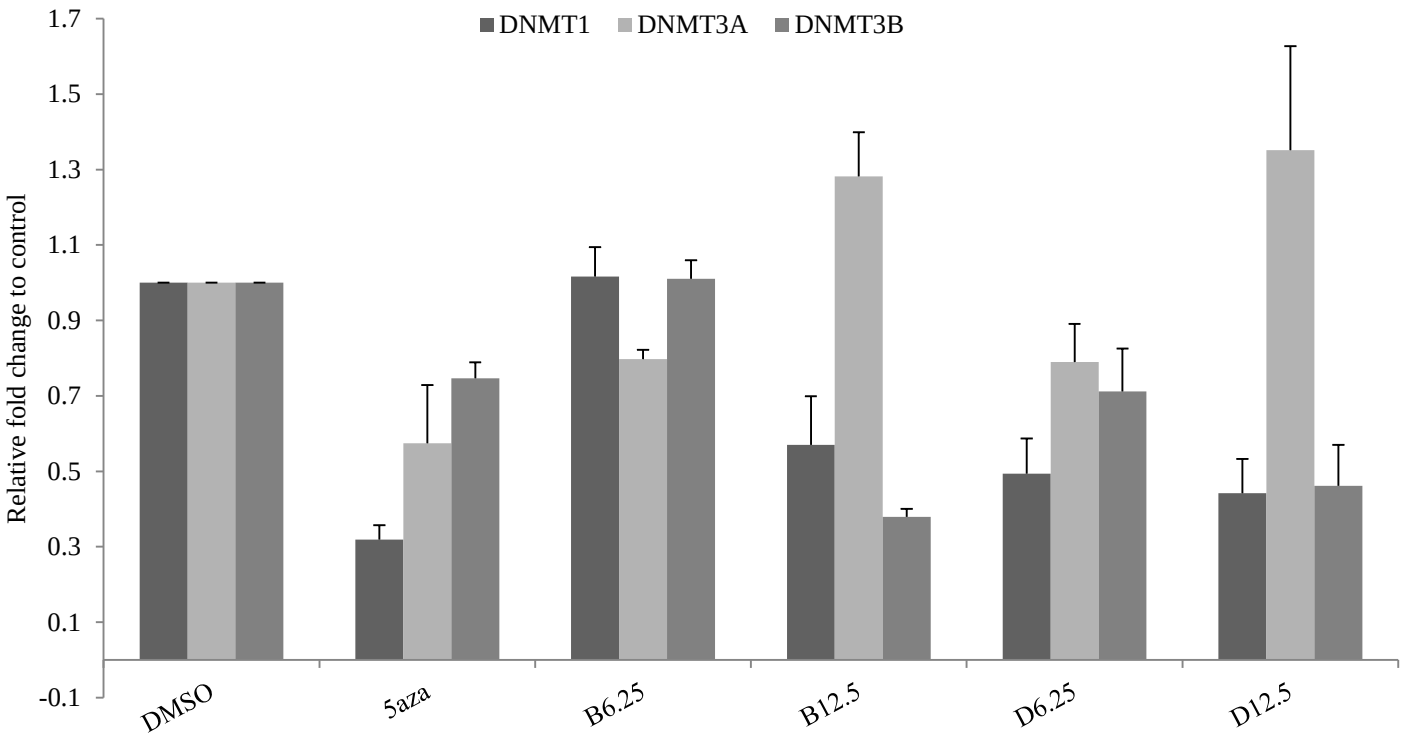


Figure 15. The mRNA expression level of DNMTs

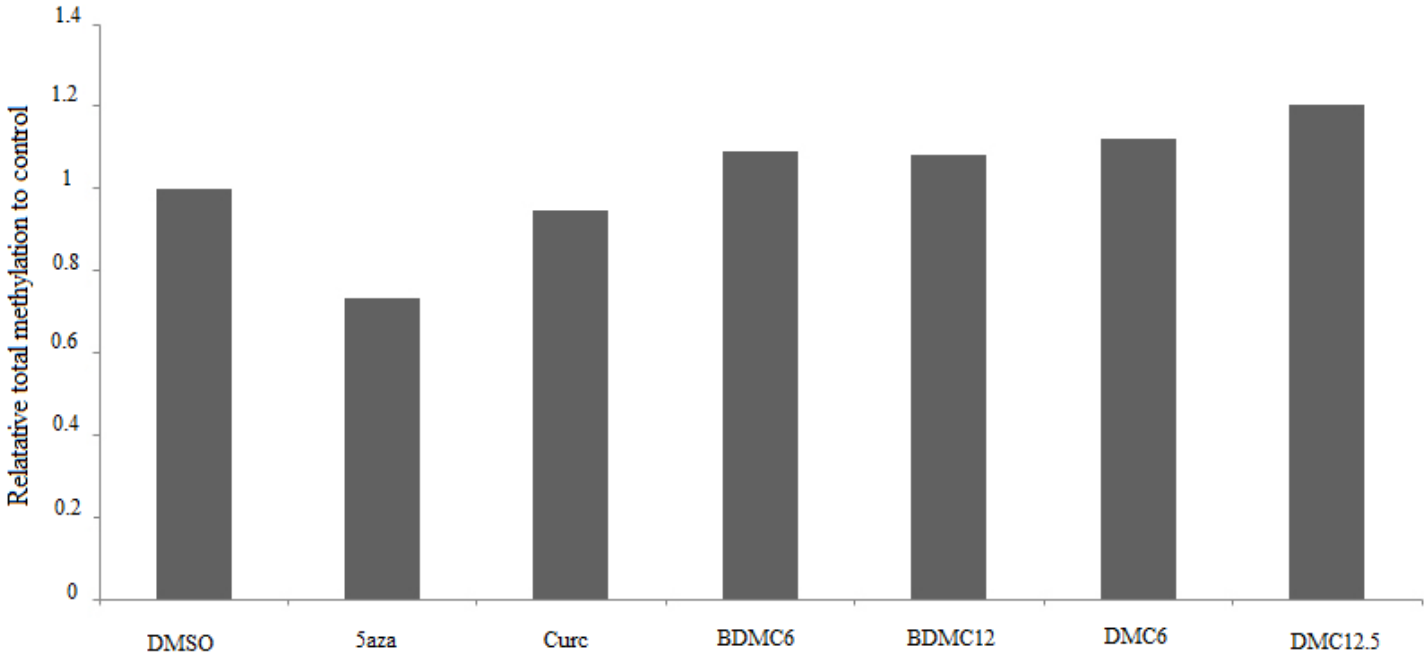


Figure 16. Total methylation levels of Nrf2 and its three major CpG sites relative to the control

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