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**ANTI-INFLAMMATORY, EPIGENETIC REGULATORY ROLE OF  
PHYTOCHEMICALS AND PKPD MODELING OF PHARMACOLOGICAL  
EFFECTS**

by

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Professor Ah-Ng Tony Kong

And approved by

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**ABSTRACT OF THE DISSERTATION**  
**ANTI-INFLAMMATORY, EPIGENETIC REGULATORY ROLE OF**  
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Chronic inflammation is considered to play an important factor in neoplastic progression via the induction of reactive oxygen, reactive nitrogen species and induction of growth promoting cytokines. Thus, it is very important to develop new drugs that can inhibit inflammation and prevent cancer formation or progression. Dietary phytochemicals are derived from natural sources and are found commonly in the fruits and vegetables consumed regularly as part of the diet. Understanding the mechanism by which the natural compounds inhibit inflammation and prevent cancers is very important and could pave way to developing new targets and drugs for chemoprevention. During my dissertation, I have studied the anti-inflammatory properties of phytochemicals like Curcumin, Phenethylisothiocyanate (PEITC) and Ursolic acid (UA). The importance of Nrf2, a transcription factor in attenuating inflammation was studied using macrophages from Nrf2 (+/+) and Nrf2 (-/-) mice. The results showed that Nrf2 plays a major role in the anti-inflammatory effects of Curcumin and PEITC as seen from the differences between Nrf2 (+/+) and Nrf2 (-/-) mice macrophages. The anti-inflammatory effect of Curcumin was further evaluated

in rats. The results showed that Curcumin suppressed lipopolysaccharide (LPS) induced inflammation as well as some epigenetic modifying genes like DNA methyltransferases and Histone deacetylases. The pharmacological response was modeled using an indirect response approach. The epigenetic modulatory role of PEITC was tested in LNCaP (androgen sensitive human prostate adenocarcinoma) cells, the results revealed that PEITC inhibits DNA methylation and increased transcription of RASSF1A, a tumor suppressor gene and in turn promotes apoptosis. Ursolic acid (UA), a pentacyclic triterpenoid was studied for its anti-inflammatory and epigenetic modulatory role in PTEN-CaP2 cells (Prostate specific PTEN null epithelial cell line). UA suppressed LPS induced inflammatory cytokines as well as inhibited HDAC protein expression along with increasing the expression of Nrf2 a master regulator of anti-oxidative stress response pathway and NQO1. Collectively, the results demonstrated the anti-inflammatory and epigenetic modulatory potential of phytochemicals *in vitro* and *in vivo*.

## PREFACE

This dissertation is submitted for the Degree of Doctor of Philosophy in Pharmaceutical Sciences at Rutgers, The State University of New Jersey. It serves as the documentation of my research carried out between September 2009 and March 2016 under the supervision of Dr. Ah-Ng Tony Kong at Department of Pharmaceutics. To the best of my knowledge, this work is original, except where suitable references are made to previous work.

This dissertation consists of six chapters. **Chapter 1** gives a general introduction to the studies done as part of the dissertation. **Chapter 2** gives an overview of the effects of curcumin on the epigenetic mechanisms involved in the inflammatory, cancer chemopreventive and neurological disorders and its role in attenuating them. This chapter has been published as a review in Current Pharmacology reports. **Chapter 3** investigate sthe role of Nrf2 in attenuating inflammation in mice macrophages on treatment with Curcumin and PEITC. The study used macrophages isolated from Nrf2 wild type and Nrf2 knockout mice. This study has been published in a peer-reviewed international journal. **Chapter 4** investigates the role of PEITC in epigenetic reactivation of tumor suppressor gene RASSF1A by regulating the epigenetic modifying enzymes. **Chapter 5** evaluated the Curcumin mediated suppression of LPS induced inflammation and described the pharmacological effects using Pharmacokinetic and Pharmacodynamic (PKPD) modeling based approach. **Chapter 6** elucidates the anti-inflammatory potential of Ursolic acid in prostate specific PTEN null epithelial cell line. Our studies collectively show the potential of phytochemicals in attenuating inflammation and in regulation of epigenetic modifying genes there by reactivating useful tumor suppressor genes.

Sarandeep Boyanapalli

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## **DEDICATION**

### **TO MY BELOVED FATHER**

Nothing of this would be possible without the constant encouragement that I used to get from my father ever since my undergraduate days and since I came to the US to pursue my graduate studies. He always encouraged me to pursue a doctoral degree and instilled the confidence in me. I am happy to fulfill his dream.



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## **ABBREVIATIONS**

PEITC: Phenethyl isothiocyanate

CUR: Curcumin

UA: Ursolic Acid

Nrf2: Nuclear factor-erythroid 2 (NF-E2)-related factor 2

COX-2: Cyclooxygenase-2

iNOS: inducible nitric oxide synthase

TNF- $\alpha$ : Tumor necrosis factor- $\alpha$

IL-1: Interleukin-1

IL-6: Interleukin-6

IL-8: Interleukin-8

HO-1: Hemeoxygenase-1

NQO1: NAD(P)H quinine oxidoreductase

LPS: Lipopolysaccharides

RIPA: Radio immune precipitation assay

BCA: bicinchoninic acid

ELISA: Enzyme linked immunosorbent assay

qPCR: quantitative Polymerase chain reaction

NO: Nitric Oxide

RASSF1A: RAS superfamily protein isoform 1A

DNMT: DNA methyl transferases

HDAC: Histone deacetylases

MSP: methylation specific PCR

BGS: bisulfite genomic sequencing

PCa: Prostate cancer

PK: Pharmacokinetics

PD: Pharmacodynamics

IDR: Indirect response

AUC: Area Under the Curve

MRT: Mean Residence Time

AUMC: Area under the moment curve

# 1 Introduction to studies

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## 1.1 Introduction

Identifying the anti-inflammatory properties of dietary compounds and phytochemicals isolated from plants, particularly tying their anti-inflammatory mechanisms to cancer chemopreventive role is an important area of research. Several phytochemicals like phenethyl isothiocyanate (PEITC) and SFN found abundantly in cruciferous vegetables, and other dietary compounds, such as curcumin, exhibit potential anti-cancer effects identifying the molecular mechanisms and establishing surrogate biomarkers for these compounds is of highest importance in translating these compounds to the clinic. The studies done as part of my dissertation are aimed to identify some of the aforementioned important aspects of drug discovery.

Curcumin (diferuloylmethane), a polyphenolic compound, is a component of *Curcuma longa*, commonly known as turmeric. It is a well-known anti-inflammatory, anti-oxidative, and anti-lipidemic agent and has recently been shown to modulate several diseases via epigenetic regulation. Many recent studies have demonstrated the role of epigenetic inactivation of pivotal genes that regulate human pathologies, such as neurocognitive disorders, inflammation, obesity and cancers. Epigenetic changes involve changes in DNA methylation, histone modifications or altered micro RNA expression patterns which are known to be interconnected and play a key role in tumor progression and failure of conventional chemotherapy. The majority of epigenetic changes are influenced by lifestyle and diets. Hence, dietary phytochemicals as dietary supplements have emerged as a promising source that are able to reverse these epigenetic alterations, to actively regulate gene expression and

molecular targets that are known to promote tumorigenesis, and also to prevent age-related diseases through epigenetic modifications. There have been several studies which reported the role of curcumin as an epigenetic regulator in neurological disorders, inflammation and in diabetes apart from cancers. The epigenetic regulatory roles of curcumin include: (i) it is an inhibitor of DNA methyltransferases (DNMTs), which has been well defined from the recent studies on its function as a DNA hypomethylating agent, and (ii) Regulation of histone modifications via regulation of histone acetyl transferases (HATs) and histone deacetylases (HDACs) and (iii) regulation of micro RNAs (miRNA). In chapter 2, the current knowledge on the effect of curcumin in the treatment and/or prevention of inflammation, neurodegenerative diseases and cancers by regulating histone deacetylases, histone acetyltransferases, and DNA methyltransferases has been extensively discussed.

Phenethyl isothiocyanate (PEITC) is a form of isothiocyanate found abundantly in cruciferous vegetables like broccoli, cabbage and water cress. Isothiocyanates like sulforaphane have been well studied for its chemopreventive activities. Anti-inflammatory and anti-oxidative properties of PEITC and the mechanism behind its chemopreventive role have been researched more recently. Nrf2 (nuclear factor E2-related factor 2) a transcription factor that plays a key role in anti-oxidative stress response as well as in chemoprevention. Our studies aimed to study the effect of Nrf2 in attenuating inflammatory response using Curcumin and PEITC. PEITC is also known for its epigenetic modulatory role. Studies have linked the transcriptional activation of silenced genes to PEITC mediated demethylation of the promoter region.

In chapter 3, we looked at the effect of Nrf2 on the anti-inflammatory effects of PEITC and Curcumin. The role of phytochemicals in preventive and therapeutic

medicine is a major area of scientific research. Several studies have illustrated the mechanistic roles of phytochemicals in Nrf2 transcriptional activation. The study aims to examine the importance of the transcription factor Nrf2 by treating peritoneal macrophages from Nrf2 (+/+) and Nrf2 (-/-) mice *ex vivo* with phenethyl isothiocyanate (PEITC) and curcumin (CUR). The peritoneal macrophages were pre-treated with the drugs and challenged with lipopolysaccharides (LPSs) alone and in combination with PEITC or CUR to assess their anti-inflammatory and anti-oxidative effects based on the gene and protein expression in the treated cells. LPS treatment resulted in an increase in the expression of inflammatory markers such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in both Nrf2 (+/+) and Nrf2 (-/-) macrophages, detected by quantitative polymerase chain reaction (qPCR). Nrf2 (+/+) macrophages treated with PEITC and CUR exhibited a significant decrease in the expression of these anti-inflammatory genes along with an increase in the expression of hemeoxygenase-1 (HO-1), which is an anti-oxidative stress gene downstream of the Nrf2 transcription factor battery. Although there was no significant decrease in the expression of the anti-inflammatory genes or an increase in HO-1 expression in Nrf2 (-/-) macrophages treated with either PEITC or CUR, there was a significant decrease in the protein expression of COX-2 and an increase in the expression of HO-1 in Nrf2 (+/+) macrophages treated with PEITC compared to CUR treatment. No significant changes were observed in the macrophages from knockout animals. Additionally, there was a significant decrease in LPS-induced IL-6 and TNF- $\alpha$  production following PEITC treatment compared with CUR in Nrf2 (+/+) macrophages, while no change was observed in the macrophages from knockout animals. The results from qPCR, western blot and ELISA analyses in macrophages from Nrf2 (+/+) and (-/-) mice

indicate that Nrf2 plays an important role in the anti-inflammatory and anti-oxidative effects of PEITC and CUR as observed by their decreased activities in the Nrf2 (-/-) macrophages.

In Chapter 4, the epigenetic reactivation of RASSF1A by PEITC was studied in prostate cancer cells *in vitro*. Epigenetic silencing of tumor suppressor genes is a phenomenon frequently observed in multiple cancers. Ras-association domain family 1 isoform A (*RASSF1A*) is a well-characterized tumor suppressor that belongs to the Ras-association domain family. Several studies have demonstrated that hypermethylation of the *RASSF1A* promoter is frequently observed in lung, prostate, and breast cancers. Phenethyl isothiocyanate (PEITC), a phytochemical abundant in cruciferous vegetables, possesses chemopreventive activities; however, its potential involvement in epigenetic mechanisms remains elusive. The study aimed to examine the role of PEITC in the epigenetic reactivation of *RASSF1A* and the induction of apoptosis in LNCaP cells. LNCaP cells were treated for 5 days with 0.01% DMSO, 2.5 or 5  $\mu$ M PEITC or 2.5  $\mu$ M azadeoxycytidine (5-Aza) with 0.5  $\mu$ M Trichostatin A (TSA). We evaluated the effects of these treatments on CpG demethylation using methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS). CpG demethylation was significantly enhanced in cells treated with 5  $\mu$ M PEITC and 5-Aza+TSA; therefore, the latter treatment was used as a positive control in subsequent experiments. The decrease in *RASSF1A* promoter methylation correlated with an increase in expression of the *RASSF1A* gene in a dose-dependent manner. To confirm that promoter demethylation was mediated by DNA methyltransferases (DNMTs), we analyzed the expression levels of DNMTs and histone deacetylases (HDACs) at the gene and protein levels. PEITC reduced DNMT1, 3A and 3B protein levels in a dose-dependent manner, and 5  $\mu$ M PEITC

significantly reduced DNMT3A and 3B protein levels. HDAC1, 2, 4 and 6 protein expression was also inhibited by 5  $\mu$ M PEITC. The combination of 5-Aza and TSA, a DNMT inhibitor and a HDAC inhibitor, respectively, was used as a positive control as this treatment significantly inhibited both HDACs and DNMTs. The function of *RASSF1A* reactivation in promoting apoptosis and inducing G2/M cell cycle arrest was analyzed using flow-cytometry analysis with Annexin V and propidium iodide (PI). In addition, we analyzed caspase-3 protein levels. Flow-cytometry analysis of cells stained with PI alone demonstrated that 5  $\mu$ M PEITC promotes early apoptosis and G2/M cell cycle arrest. Flow cytometry analysis of cells stained with Annexin V and PI also demonstrated an increased proportion of cells in early apoptosis in cells treated with 5  $\mu$ M PEITC or 5-Aza with TSA. In addition, 5  $\mu$ M PEITC significantly enhanced caspase-3 levels compared with the control treatment. Collectively, the results of our study suggest that PEITC induces apoptosis in LNCaP cells potentially by reactivating *RASSF1A* via epigenetic mechanisms.

In chapter 5, we evaluated the Pharmacokinetic and Pharmacodynamic effects of curcumin and how the PK drives the PD (inflammatory gene expression) using a semi-mechanistic indirect response modeling. Oxidative stress and inflammation are tightly coupled. Chronic inflammation appears to be a key driver of cancer development. Inflammation is directly related to the amount of nitrites which is regulated by inducible nitric oxide synthase (*iNOS*). Curcumin a compound present in the rhizomes of *curcuma longa* has long been studied for its anti-oxidant and anti-inflammatory properties. However, the acute pharmacokinetics (PK) and pharmacodynamics (PD) of curcumin in suppressing pro-inflammatory markers like *iNOS*, *TNF- $\alpha$* , *IL-6* as well as some epigenetic modulators like DNMT3A, HDAC2, 3



and 4 upon LPS stimulation is unclear. In this study, we evaluate the PK and PD of curcumin suppressing endotoxin lipopolysaccharide (LPS) mediated inflammation in rat lymphocytes. LPS alone or with Curcumin was administered intravenously to female Sprague-Dawley rats. Blood samples were drawn at 0, 10, 20, 30 min, 1, 2, 3, 4, 6, 8, 12 hr. Plasma were obtained from half of the blood samples and concentration of Curcumin was analyzed using a validated LC-MS/MS method. Lymphocytes were collected from the remaining blood samples using Ficoll-Paque™ Plus medium. RNA was extracted, converted to cDNA and quantitative real-time PCR analyses were performed. As part of the study the relative expression of several inflammatory genes like *iNOS*, *TNF- $\alpha$* , *IL-6* along with some epigenetic modulators like DNMT3A, HDAC2, 3 and 4 were analyzed. PK-PD modeling connecting the curcumin concentration to iNOS gene expression was conducted first using Jusko's indirect response model (IDR) and secondly utilizing our newly developed PKPD model integrating transition compartments describing the delayed response of LPS using ADAPT5. Plasma disposition kinetics of curcumin showed a bi-exponential decline and was described well by a two-compartment open model. Our results showed that LPS increased expression of *iNOS* mRNA in lymphocytes with peak expression around 3 h. Upon curcumin administration the *iNOS* gene expression was lowered. These effects were well captured using the two approaches involving the indirect response modeling, one without transit compartments and the other with the newly integrated transit compartments. This PK-PD modeling approach provides a framework for evaluating the acute effects of curcumin or other similar drugs in clinical trials.

In Chapter 6, we evaluated the anti-inflammatory and epigenetic modulatory role of Ursolic acid (UA), a pentacyclic triterpenoid commonly found in berries. Inflammation is a precursor for many disorders like cancer, neurological and

autoimmune diseases. Treatment and prevention of chronic inflammatory state therefore is of paramount importance to attenuate the chances of progression of cancers and other diseases. PTEN (phosphatase and tensin homolog deleted on chromosome 10) which is one of the most well-known tumor suppressors is known to be mutated in many cancers. Recent evidence has suggested that the silencing of PTEN is due to epigenetic modulation in melanomas and prostate cancers. Ursolic acid (UA), a natural triterpenoid present in fruits and plants is a well known anti-inflammatory agent which is recently being studied for cancer preventive properties. Studies have shown the prostate cancer preventive properties of UA in TRAMP mice. This study aimed to examine the anti-inflammatory role of UA *in vitro* in prostate specific PTEN-CaP2 cells which lack PTEN gene. PTEN-CaP2 cells were treated for 1 or 5 days with 0.01% DMSO or lipopolysaccharide LPS (1µg/mL) and/ or 3, 6, 6.25, 12 µM UA or 2.5 µM azadeoxycytidine (5-Aza) with 0.5 µMtrichostatin A (TSA). We evaluated the effects of these treatments on the nitrite production, regulation of pro-inflammatory and anti-oxidative gene expression by qPCR and regulation of protein expression of Nrf2, NQO1, HO-1 and other epigenetic modulators like HDAC1, 2, 3 and 4 using western blot analysis. Nitric oxide (NO) production was significantly reduced on 3 µM UA treatment compared with LPS treatment. qPCR analysis showed that 3 µM significantly lowered the gene expression of pro-inflammatory markers like iNOS, COX-2, IL-6, IL-1b and TNF-α compared to the LPS (1 µg/mL) treatment. We further evaluated the role of UA in activating Nrf2 and downstream targets by epigenetic modulation. The gene and protein expression analysis revealed a significant induction of Nrf2 and NQO1 gene and protein expression in a dose dependent manner on treatment with 3 and 6 µM UA in PTEN-CaP2 cells. This increase in gene and protein expression showed a correlation with a

decreased protein expression of HDAC 1, 2, 3 and 4 in the 5 day treatment group. There was a significant induction of Nrf2 and NQO1 in the PTEN-CaP2 cells treated with 3 and 6  $\mu$ M UA for 24 H. However, the fold induction was lower than the cells treated for 5 days. The PTEN-CaP2 cells treated with 2.5  $\mu$ M azadeoxycytidine (5-Aza) with 0.5  $\mu$ Mtrichostatin A (TSA) showed a significant inhibition of HDACs as these are known epigenetic modulators and hence are used as positive control. Collectively, our results revealed that Ursolic acid has potent anti-inflammatory effects and can be used as epigenetic modulator in reactivating the transcriptionally silenced genes.

## 2 "Curcumin, the king of spices"— Epigenetic regulatory mechanisms in the prevention of cancer, neurological and inflammatory diseases<sup>1, 2, 3</sup>

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### 2.1 Introduction:

Curcumin, commonly known as turmeric, is extracted from the rhizomes of the *Curcuma longa* plant. It has been the most commonly used spice in India for ages and is regularly used in ayurveda and traditional Chinese medicine. Curcumin has many medicinal properties that have been studied widely by researchers all over the world [1, 2]. Curcumin constitutes 80% of the curcuminoid complex, with the remainder constituted by demethoxycurcumin (17%) and bisdemethoxycurcumin (3%) [3]. Curcumin is a promising medicinal agent, as it regulates several key molecular signaling pathways that modulate survival, pathways governing anti-oxidative properties (e.g., nuclear factor E2-related factor 2 (Nrf2)), and inflammation pathways (e.g., nuclear factor kappa B (NF-κB)) [4]. Apart from its role in regulating Nrf2 in different types of cancers, curcumin modulates Nrf2 expression in many different types of human pathologies, including neurocognitive disorders, kidney disorders and diabetes.

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2 Key Words: Curcumin, Epigenetics, DNA methylation, Histone modification, Cancer, Nrf2.

3 Abbreviations: Nrf2- nuclear factor E2-related factor 2; NF-κB- nuclear factor kappa B; COX-2-cyclooxygenase; iNOS- inducible nitric oxide synthase; IL-6- Interleukin 6; miRNA- microRNA; DNMT- DNA methyltransferase; HDAC- Histonedecacetylase; HAT- Histone acetyl transferases

Curcumin is a well-known anti-inflammatory agent. It regulates the anti-inflammatory response by down regulating the enzymatic activities of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) by suppressing the transcription factor NF- $\kappa$ B, which in turn suppresses tumorigenesis [5, 6]. Curcumin also down regulates the expression levels of NF- $\kappa$ B-regulated gene products, such as tumor necrosis factor (TNF), 5-lipoxygenase (5-LOX), interleukins (IL-1, IL-6, IL-8), adhesion molecules, C-reactive protein (CRP) and chemokine receptor type 4 (CXCR-4) [7, 8].

Treating mouse liver and small intestine with curcumin, our group performed a global gene expression study and identified curcumin-regulated Nrf2-dependent genes. Our findings reveal the induction and suppression of several genes related to apoptosis and cell cycle control, cell adhesion, kinases and phosphatases, along with transcription factors. The results showed many phase II detoxification/antioxidant enzyme genes regulated by Nrf2 among these identified genes, demonstrating the potential roles of Nrf2 and curcumin in chemoprevention [9]. A recent study in rats identified curcumin as a neuroprotectant against hemin-induced damage in primary cultures of cerebellar granule neurons (CGNs), where in the protective effect of curcumin was attenuated by the inhibition of the heme oxygenase system or glutathione (GSH) synthesis by tin mesoporphyrin and buthioninesulfoximine, respectively in hemin induced toxicity. Furthermore, after 24 h incubation with curcumin, glutathione reductase, glutathione S-transferase and superoxide dismutase activities were increased by 1.4-, 2.3-, and 5.2-fold respectively, suggesting that Nrf2 and an antioxidant response may play important roles in the protective effect of this antioxidant against hemin-induced neuronal death [10]. Curcumin is also being researched for its beneficial effects in diabetes: curcumin attenuated glucose intolerance without affecting high-fat diet (HFD)-induced body weight gain. Curcumin treatment reversed the levels of total or

nuclear Nrf2 contents and its downstream target heme oxygenase-1 which were reduced by HFD-feeding, this was seen as a result of induction in the nuclear translocation of Nrf2 by curcumin [11]. A study conducted to determine the molecular mechanisms involved in the putative anti-oxidative effects of curcumin against experimental stroke showed that curcumin protects neurons against ischemic injury. This neuroprotective effect involves the Akt/Nrf2 pathway, which is consistent with the fact that Nrf2 participates in the neuroprotective effects of curcumin against oxidative damage [12]. The role of Nrf2 in reno-protection has been reported in several studies. In a study of ischemia-reperfusion injury, Nrf2<sup>-/-</sup> mice showed significantly worse renal function, vascular permeability and survival compared to wild-type mice. The Streptozotocin (STZ)-induced diabetic nephropathy model revealed that Nrf2<sup>-/-</sup> mice developed severe renal injury with greater oxidative DNA damage than wild-type mice. Nrf2<sup>-/-</sup> mice showed higher renal damage and interstitial fibrosis by cyclosporinA treatment [13-15]. It was also reported that curcumin (100 mg/kg) administration significantly decreased infiltration of renal macrophages and renal production of proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , along with NF $\kappa$ B inhibition in STZ diabetic animals [16]. A study showing attenuation of arsenic-induced hepatotoxicity and oxidative injuries upon curcumin treatment revealed that curcumin treatment relieved arsenic-induced elevation of serum alanine amino transferase (ALT) and aspartate aminotransferase (AST) activities, augmentation of hepatic malondialdehyde (MDA), and the reduction of blood and hepatic GSH levels via Nrf2 activation [17].

## **2.2 Epigenetic regulation by curcumin**

The studies discussed earlier have focused on the genetic modulation of various molecular aspects. However, epigenetic regulation which includes changes in DNA methylation, histone modifications, and alterations in microRNA (miRNA) expression levels without any changes in the DNA sequence constitutes an important mechanism by which dietary components, such as curcumin, can selectively activate or inactivate gene expression (Figure 2-1). DNA methylation and histone modifications are crucial epigenetic modifications of the genome that are involved in regulating many cellular processes, such as embryonic development, transcription, and chromatin structure and chromosome stability. DNA methylation represses transcription directly by inhibiting the binding of specific transcription factors and indirectly by recruiting methyl-CpG-binding proteins [18]. DNA methylation of several important genes has been reported in a wide variety of diseases, including several types of cancers, including those of the prostate, colon and lung, where one or more genes are repressed due to hypermethylation [19]. DNA methylation also plays important roles in several other disorders, such as diabetes, neurocognitive diseases, autoimmune disorders and inflammatory disorders [20]. While both DNA methylation and histone modification are involved in establishing patterns of gene repression during development, DNA methylation leads to stable long-term repression, whereas histone methylation causes local formation of heterochromatin that is readily reversible. Hence, understanding the cross-talk between DNA methylation and histone modification is paramount in understanding normal development, somatic cell reprogramming and tumorigenesis [21]. There are different types of histone modifications, including methylation, acetylation, phosphorylation, ubiquitylation and sumoylation, which regulate transcription, repair, replication or condensation [22]. The regulatory role of each histone modification is governed by the sites that are methylated or acetylated. The

histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two families of enzymes and have opposing actions that regulate the acetylation of lysines which is highly dynamic process. There are two major classes of HATs: type-A and type-B, and four classes of HDACs: HDACs 1, 2, 3 and 4 [23]. In this chapter, the role of curcumin in DNA methylation and different histone modifications in various diseases is summarized based on the current knowledge on the effects of curcumin in the treatment and/or prevention of inflammation, neurodegenerative diseases and cancers through its regulation on histone HDACs, HATs, and DNA methyltransferases.

### **2.3 DNA methylation and curcumin**

Methylation of cytosine residues in 5'-cytosine-guanosine (CpG) in the promoter regions of genes is an epigenetic mechanism that controls gene transcription, genome stability and genetic imprinting. Methylation is regulated by a group of enzymes called DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) in the presence of S-adenosyl-methionine (SAM); SAM serves as a methyl donor for the methylation of cytosine residues at the C-5 position to yield 5-methylcytosine. Several studies have demonstrated that the transcriptional silencing of several tumor suppressor genes (TSGs) is due to the aberrant hypermethylation of promoter CpG islands (>55% CG content), leading to a variety of solid and blood cancers [24]. Treatment with hypomethylating agents both *in vitro* and *in vivo* is an effective way of restoring gene expression and normal patterns of differentiation and apoptosis in malignant cells. Bioactive components from dietary supplements, such as the potent hypomethylating agent curcumin [25-27], may represent attractive agents for cancer prevention or treatment. The importance of methylation and its reversal by curcumin in different pathologies is reviewed.



## 2.4 Reversal of DNA methylation by curcumin in different disorders

As mentioned previously, DNA methylation of several important genes can be observed in a wide variety of diseases, including cancers of the prostate, colon and lung, where one or more genes are repressed due to hypermethylation. DNA methylation is also known to play an important role in several other disorders, such as diabetes, neurocognitive diseases, autoimmune disorders and inflammatory disorders.

Cancer epigenetics is characterized by heritable patterns of specific DNA methylation and disrupted cellular pathways mediated by DNA methyltransferases [28]. DNA methylation serves as an excellent target in the treatment of acute myeloid leukemia (AML) as it has been shown that inactivation of genes due to DNA methylation play a major role in AML development. However, the mechanism by which curcumin elicits its DNA hypomethylation to reactivate silenced tumor suppressor genes and to present a potential treatment option for AML remains unclear. A study reported that curcumin down regulated DNMT1 expression in AML cell lines, both *in vitro* and *in vivo*, and in primary AML cells *ex vivo*. In addition, curcumin reduced the expression of the positive regulators of DNMT1, p65 and Sp1, which correlates with reductions in the binding of these transcription factors to the DNMT1 promoter in AML cell lines, demonstrating the promise of curcumin as a potential treatment for AML [29]. More recently, dimethoxycurcumin, a synthetic structural analogue and curcumin, along with other epigenetic modifiers (e.g., 5-Azacytidine (5AZA) and decitabine) was shown to augment the effect of DNMT inhibitors on DNA methylation to re-activate the silenced genes in leukemia cells [30]. In addition to its regulatory role in blood cancers, curcumin is a potent epigenetic regulatory agent in many solid tumors. As a potent DNMT inhibitor, curcumin at a concentration of 5  $\mu$ M reverses CpG methylation at the promoter region

of Neurog1, a cancer methylation marker in LNCaP human prostate cancer cells; at 2.5  $\mu$ M, curcumin reverses the hypermethylation of Nrf2 in transgenic adenocarcinoma of mice prostate cells (TRAMP C1 cells) [26, 27]. A study conducted in three colorectal cancer cell lines, HCT116, HT29 and RKO, showed non-specific global hypomethylation with 5-aza-CdR treatment. In contrast, curcumin treatment resulted in methylation changes at selected, partially methylated loci instead of fully methylated CpG sites [31]. A recent study showed that curcumin induced a decrease in the AgNOR (argyrophilic nucleolar) protein, whose expression is elevated in malignant cells compared to normal cells and reflects the rapidity rate of cancer cell proliferation. This change in expression may be mediated by the global DNA hypermethylation that is observed following low-concentration curcumin treatment [32]. Curcumin treatment also significantly inhibits cell proliferation and increases the apoptosis rate by up-regulating phosphatase tensin homolog (PTEN), whose expression is associated with decreased DNA methylation. In addition, curcumin treatment causes the suppression of DNA methyltransferase 3b (DNMT3b) both *in vivo* and *in vitro* [33]. In addition to pure curcumin, the novel curcumin analogues EF31 and UBS109 also show demethylating effects. In one study, MiaPaCa-2 and PANC-1 cells were treated with a vehicle control, curcumin, EF31 or UBS109. The analogues resulted in significantly higher inhibition of proliferation and cytosine methylation compared to curcumin. Demethylation was associated with the re-activation of silenced p16, SPARC, E-cadherin, HSP-90 and NF- $\kappa$ B, leading to the down regulation of DNMT-1 expression [34]. A study using U251 and U81 glioblastoma cells also showed that treatment with 30  $\mu$ M curcumin for 4 days decreased the promoter hypermethylation of the receptor activator of NF- $\kappa$ B (RANK), resulting in RANK gene expression and activation [35]. Furthermore, in a different

study, curcumin treatment in MCF-7 cells enhanced the mRNA and protein levels of Ras-association domain family protein 1A (RASSF1A) and decreased its promoter methylation. This study also demonstrated that curcumin down regulated the DNA methylation activity of nuclear extracts and also down regulated the mRNA and protein levels of DNMT1 in MCF-7 cells. These effects may be associated with the disruption of binding between the NF- $\kappa$ B/Sp1 complex and the promoter region of DNMT1 induced by curcumin [36].

In addition to regulating cancer, curcumin also regulates inflammatory disorders through epigenetic modulation. Neprilysin (NEP, EP24.11), a zinc-dependent metallopeptidase, is expressed at relatively low levels in the brain due to its promoter hypermethylation [37]. NEP is emerging as a potent inhibitor of AKT/protein kinase B. One study showed that curcumin treatment induced the reactivation of the NEP gene via CpG demethylation. In curcumin-treated N2a/APP<sup>swe</sup> cells, the upregulation of NEP expression was also concomitant with AKT inhibition and the subsequent suppression of NF- $\kappa$ B and its downstream proinflammatory targets, including COX-2 and iNOS [38]. Furthermore, the Fanconianemia (FA) complementation group F (FANCF) gene plays an important role in the FA pathway and is frequently inactivated due to promoter hypermethylation in specific types of cancer [39]. Curcumin treatment produces a 5-fold increase in FANCF gene expression due to the demethylation of 12 of the 15 CpGs present in the promoter region [40]. In a study in non-small cell lung cancer cells comparing the effects of three different curcuminoids in demethylating the promoter region of Wnt inhibitory factor (WIF-1), bisdemethoxycurcumin possessed a strong demethylation function *in vitro* compared to the other two curcuminoids in restoring WIF-1 expression in A549 cells [41]. A recent study conducted in our

laboratory demonstrated that curcumin decreased the protein expression levels of DNA methyltransferases (DNMT1, 3A, and 3B) and induced the demethylation of Deleted in lung and esophageal cancer 1 (DLEC1) in human colorectal adenocarcinoma HT-29 cells. The results also showed that curcumin treatment decreased colony formation; however, the effect of curcumin was attenuated in DLEC1 knockdown cells. A study using MCF7 and MDA MB231 breast cancer cells showed that curcumin treatment decreased the expression levels of DNMT1, 3A and 3B and increased p21<sup>WAF1</sup> expression [42].

## **2.5 Histone modifications and curcumin**

Epigenetic alterations might occur as early events in carcinogenesis and might precede genetic alterations during oncogenic transformation [43]. The activation or repression of a gene is not determined by histone acetylation or methylation alone. Rather, it is determined by the modification of different residues. However, promoter DNA methylation generally suppresses gene expression. A histone octamer consisting of an H3/H4 tetramer and two H2A/H2B dimers wraps DNA, forming the nucleosome. Generally, histone modifications play a key role in maintaining the highly folded chromatin structure, which is closely linked to gene expression [44-46]. Histone acetylation in chromatin is a very widely studied histone modification. The different groups of histone-modifying enzymes that play crucial roles in histone acetylation are (i) HATs that add acetyl groups to lysine residues, (ii) HDACs or lysine deacetylases (KDACs) that remove acetyl groups from lysine residues, (iii) histone methyltransferases (HMTs) that add methyl groups to arginine or lysine residues, and (iv) histone demethylases (HDMs) that remove methyl groups from arginine or lysine residues, in turn regulating the conformation of chromatin structure

to facilitate or hinder the association of DNA repair proteins or transcription factors to chromatin [47, 48]. Collectively, the aberrant enrichment of HAT and HDAC activities may trigger carcinogenesis [49]. The different residues that are modified could play active roles in either activation or repression. For instance, trimethylation of lysine 4 on histone H3 (H3K4me3) results in transcriptional gene activation, whereas trimethylation of lysine 9 on histone H3 (H3K9me3) and trimethylation of lysine 27 on histone H3 (H3K27me3) at gene promoters repress transcription [47]. However, acetylation increases chromatin accessibility and is generally associated with transcriptional activation, as it neutralizes the DNA–histone interactions, resulting in a relaxed, open chromatin conformation that facilitates access of transcriptional activators to their cognate recognition elements and initiates/enhances transcription.

## **2.6 Regulation of histone modifications by curcumin**

Curcumin, a HAT inhibitor, significantly reduces H3ac levels in the IL-6 promoter, along with IL-6 mRNA expression and IL-6 protein secretion by rheumatoid arthritis synovial fibroblasts RASFs. Thus, hyperacetylation of histone H3 in the IL-6 promoter induces the increase in IL-6 production by RASFs and thereby participates in rheumatoid arthritis (RA) pathogenesis [50]. One study showed that the anti-inflammatory activity of curcumin is regulated by epigenetically inhibiting the expression of Triggering receptor expressed on myeloid cells 1 (TREM-1), a potent amplifier of Toll-like receptor (TLR)-initiated inflammatory responses. The inhibition was observed both *in vitro* in primary bone marrow-derived macrophages and *in vivo* in the lungs of mice with sepsis. Curcumin inhibited p300 activity in the TREM-1 promoter region, leading to the hypoacetylation of lysine residues in histones 3 and 4

(H3Kac and H4Kac) [51]. In another study, the effect of curcumin on histone acetylation and proinflammatory cytokine secretion under high-glucose conditions in human monocytes was studied using human monocytic (THP-1) cells. The results showed that curcumin treatment not only significantly reduced HAT activity and the level of p300, a co-activator of NF- $\kappa$ B and acetylated CBP/p300 gene expression, but also induced HDAC2 expression. The results indicate that curcumin decreases high glucose-induced cytokine production in monocytes via epigenetic changes involving NF- $\kappa$ B [52]. Histone acetylation and HDAC regulation have been implicated as playing important roles in Alzheimer's disease (AD) [53-55]. In one study, HDAC alterations to histone acetylation at the promoter regions of AD-related genes, such as Presenilin 1 (PS1) and beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), were studied in neuroblastoma N2a cells. To establish the cellular models of AD, the cells were transfected with Swedish mutated human amyloid precursor protein (APP) (N2a/APPswe) and wild-type APP (N2a/APPwt). Curcumin, a natural selective inhibitor of p300 in HATs, significantly suppressed PS1 and BACE1 expression by inhibiting H3 acetylation at their promoter regions in N2a/APPswe cells [56]. When the combinatorial effects of SAHA and curcumin were investigated as a protective treatment against amyloid-beta neurotoxicity *in vitro*, the combination provided comprehensive protection against A $\beta_{25-35}$ -induced neuronal damage in PC12 cells via HDAC regulation [57]. The epigenetic regulatory role of curcumin was recently studied in the management of neuropathic pain in a chronic constriction injury model in rats. Chromatin immunoprecipitation (ChIP) analysis revealed the dose-dependent reduction in the recruitment of p300/CBP (CREB binding protein) and acetyl-histone H3/acetyl-histone H4 to the promoters of the BDNF and COX-2 genes upon curcumin administration, indicating the therapeutic role of curcumin in

neuropathic pain management [58]. In addition to this effect, it has previously been reported that curcumin plays a key role as a p300/CBP inhibitor of histone acetyltransferase [59, 60]. Lipopolysaccharide (LPS)-induced cardiac hypertrophy is known to accentuate p300 transcriptional activity, and LPS-mediated cardiac hypertrophy can be reversed by blocking p300-HAT activity upon treatment with curcumin at a dose of 100 µg/kg of body weight in mice [61]. A recent study revealed that diabetic rats treated with curcumin had significantly decreased blood urea nitrogen and creatinine and increased albumin; these variables are associated with the development of diabetic nephropathy. These results demonstrated that curcumin induced post-translational modifications of histone H3, expression of HSP-27 and MAP kinase p38 in diabetic kidneys [62]. Studies on the anti-cancer potential of curcumin in medulloblastoma reported that curcumin treatment of medulloblastoma cells induced apoptosis and cell cycle arrest at the G2/M phase. Curcumin also reduced histone deacetylase (HDAC) 4 expression and activity, while increasing tubulin acetylation, indicating its potential therapeutic role in medulloblastoma [63]. Neurog1, a gene that is epigenetically regulated and is considered an important biomarker in prostate cancer, is upregulated upon treatment with curcumin in LnCap cells by increasing HDAC1, 4, 5, and 8 expression, while decreasing HDAC3 expression. Curcumin also decreases the enrichment of H3K27me3 at both the Neurog1 promoter region and the global level [26]. In addition, DLEC1 expression is regulated by HDAC inhibition in human colorectal adenocarcinoma HT-29 cells upon curcumin treatment. Finally, curcumin inhibits subtypes of HDACs (i.e., HDACs 4, 5, 6, and 8).

## **2.7 Regulatory role of curcumin in miRNA expression**

MicroRNAs (miRNAs) are small non-coding RNA molecules ranging from 17 to 25 nucleotides in length that are endogenously expressed; they induce target mRNA degradation or repress mRNA translation by imperfect binding to their 3'-untranslated region. The RNA polymerase II, Drosha, transcribes miRNA gene into a primary transcript (pri-miRNA) in the nucleus, where the microprocessing complex, DGCR8 processes the hairpin stem-loop structure into a precursor miRNA (pre-miRNA). The 70-nucleotide-long pre-miRNA is then exported into the cytoplasm, where it undergoes a second processing by Dicer, where one strand of the hairpin is incorporated into the ribonucleoprotein complex called the miRNA-induced silencing complex (miRNA-RISC) [64, 65]. Studies have shown that miRNA control the self-renewal and differentiation of embryonic stem cells (ESCs), and the aberrant expression and/or functions of miRNAs have been implicated in tumorigenesis [66]. A significant proportion of miRNA genes (20–40%) are located close to CpG islands, and these miRNAs can be inactivated by epigenetic mechanisms [67]. Some of the signaling pathways that are regulated by miRNAs include the vascular endothelial growth factor (VEGF), NF- $\kappa$ B, protein kinase B and mitogen-activated protein kinase (MAPK) pathways, all of which are known to be targets of curcumin [68]. miRNAs represent very promising therapeutic targets for cancer treatment because their aberrant expression has been linked to cancer stem cell dysregulation and, thus, oncogenesis [69]. The function of histone lysine N methyl transferase (EZH2) in pancreatic cancer was studied in pancreatic cancer cells using difluorinated curcumin (CDF), a novel analogue of curcumin. The results revealed decreased EZH2 expression and increased expression of a panel of tumor-suppressor microRNAs (e.g., *let-7a, b, c, and d*; *miR-26a*; *miR-101*; *miR-146a*; and *miR-200b and c*), which are most commonly lost in pancreatic cancer [70]. In addition, studies have shown that



curcumin treatment alters miRNA expression in the PxBC-3 human pancreatic cancer cell line. For example, in one study, curcumin up regulated miRNA-22 and down regulated miRNA-199a expression. The up regulation of miRNA-22 expression by either curcumin or transfection with miRNA-22 mimetics in the PxBC-3 pancreatic cancer cell line suppressed the expression of its target genes, *SP1 transcription factor (SP1)* and *estrogen receptor 1 (ESR1)*. Conversely, inhibiting miRNA-22 with antisense enhanced SP1 and ESR1 expression [71]. Another study showed that treatment with curcumin and its analog, CDF, up regulated miR-200 expression and down regulated miR-21 expression, and the down regulation of miR-21 resulted in PTEN induction [72]. miR-203 is often hypermethylated in bladder cancers; curcumin induces the hypomethylation of the miR-203 promoter and subsequently up regulates miR-203 expression. As a result of increased miR-203 expression, the miR-203 target genes Akt2 and Src are down regulated, which lead to a decrease in proliferation and an increase in bladder cancer cell apoptosis [73]. A global miRNA expression profiling study performed in human ARPE-19 cells treated with curcumin showed that curcumin alters the expression of H<sub>2</sub>O<sub>2</sub>-modulated miRNAs that are known to be regulators of antioxidant defense and the renin-angiotensin system. These results were confirmed using RT-PCR and western blot. Compared with controls, cells treated with curcumin alone down regulated 20 miRNAs and up regulated 9 miRNAs. While exposure to H<sub>2</sub>O<sub>2</sub> down regulated 18 miRNAs and up regulated 29 miRNAs, cells that had been pre-treated with curcumin and were later exposed to H<sub>2</sub>O<sub>2</sub> showed a significant reduction in the H<sub>2</sub>O<sub>2</sub>-induced expression of 17 miRNAs [74]. In an engrafting melanoma model developed by injecting murine B78H1 cells in the flanks of C57BL/6 mice, oral curcumin treatment was administered through a 4% curcumin

diet. The results showed that curcumin intake altered miRNA expression, with mmu-miR-205-5p being expressed over 100-fold higher than controls[75].

## **2.8 Conclusion:**

The aberrant activation or inactivation of harmful and beneficial genes by epigenetic alterations has gained much focus in recent years. Epigenetic regulation, which includes changes in DNA methylation, and histone modifications and alterations in microRNA (miRNA) expression without any changes in the DNA sequence, constitutes an important mechanism by which dietary components, such as curcumin, can selectively activate or inactivate gene expression (Figure 2-2). The role of curcumin in regulating different disorders has been long studied. Contemporary research shows that curcumin can epigenetically regulate the expression of important genes by reversing DNA methylation and altering histone modifications and by targeting several miRNAs that play a key role in diseases (Table 2-1). Several studies have shown the potential of curcumin in regulating various intracellular pathways, thereby attenuating certain diseases, including cancers, diabetic nephropathy and neurocognitive disorders (e.g., Alzheimer's disease). Additionally, its potency can also be attributed to the regulation of epigenetic mechanisms, as has been shown recently. Furthermore, several studies have shown that curcumin is a potent DNMT and HDAC inhibitor and can regulate miRNA expression in several disorders. Therefore, it is imperative to identify more epigenetic targets of curcumin for the treatment of other disorders.

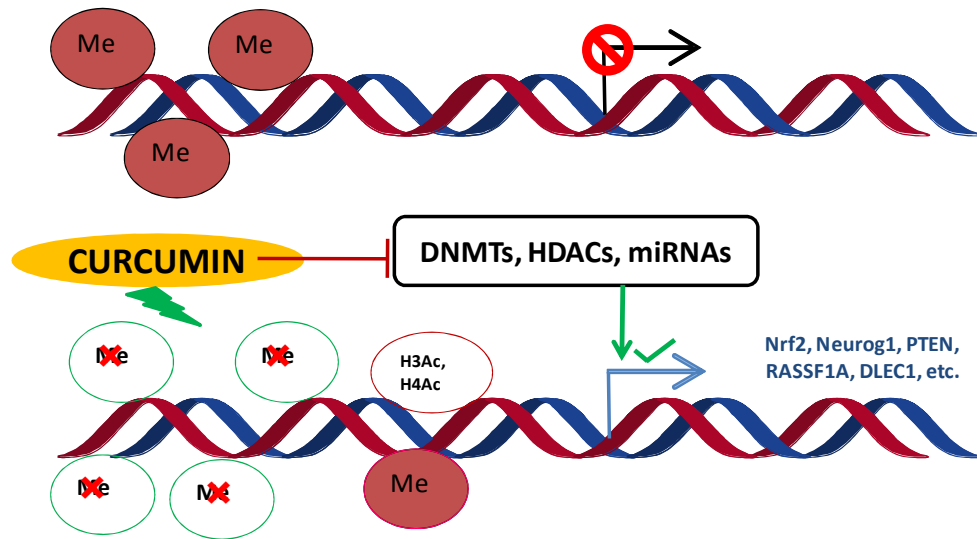
**Table 2-1: Examples of the effect of Curcumin on DNA methylation, histone modifications and miRNAs activity.**

	Dietary agent	Molecular mechanism	Validated target(s)	Experimental model	Concentration /Dose	Treatment duration	References
DNA methylation	Curcumin	↓ Methylation in promoter region	Nrf2	TRAMPC1 mouse prostate cells	2.5 µM	5 days	[27]
	Curcumin	↓Methylation in promoter region	Neurog1	LNCap human prostate cancer cells	5 µM	7 days	[26]
	Curcumin	↓DNMT3B	PTEN	HSC primary cells (RAT)	20µM	2 days	[33]
	EF31 (Curcumin analogs)	↓DNMT1	p16, SPARC, and E-cadherin	MiaPaCa-2 and PANC-1 pancreatic cancer cells	750nM and 1.25µM	2 days	[34]
	UBS109	↓DNMT1			250nM	2 days	[34]
	Curcumin	↓Methylation in promoter region	RANK	U87 and U251 glioblastoma cells	30µM	4 days	[35]
	Curcumin	↓DNMT1, 3A, 3B, ↓ MeCP2, ↓ MBD2	p21WAF1	MCF 7 and MDA MB 231 cells	10µM	4 days	[42]
	Curcumin	↓DNMT1	p15(INK4B)	K562, THP1, Kasumi and MV4-11 cells, Primary AML	10µM and 20µM	1 and 3 days	[29]

				cells			
	Curcumin	↓ Methylation in promoter region	FANCF	SiHa cell line	20μM	4 days	[40]
	Curcumin	↓ Methylation in promoter region	RASSF1A	MCF 7 cells	10μM and 20μM	3 days	[36]
Histonemodifications	Curcumin	↓Total activity, HDAC ↓ H3K27me3	Neurog1	LNCap human prostate cancer cells	5 μM	7 days	[26]
	Dimethoxycurcumin	↑ H3K36me3	p15 and CDH 1	CEM human leukemia cells	1μM	3 days	[30]
	Curcumin	↓ HDAC 1	p300	Raji cells (Human burkitts lymphoma)	12.5 μmol/l	6-48 hrs	[76]
	Curcumin	↓ H3ac and H4ac	PARP-Caspase 3	A172 (Human glioblastoma cell lines)	4μg/ml	4 days	[77]
	Curcumin	P300/CBP inhibition HAT	BDNF and COX-2	Chronic construction injury rat model (CCI)	40 and 60mg/kg	7 days	[58]
	Curcumin	↓H3K4me, ↓ H3ac and H4ac	TREM-1	BMDM (macrophages from WT mice)	10 μmol/L	1-4 hrs	[51]

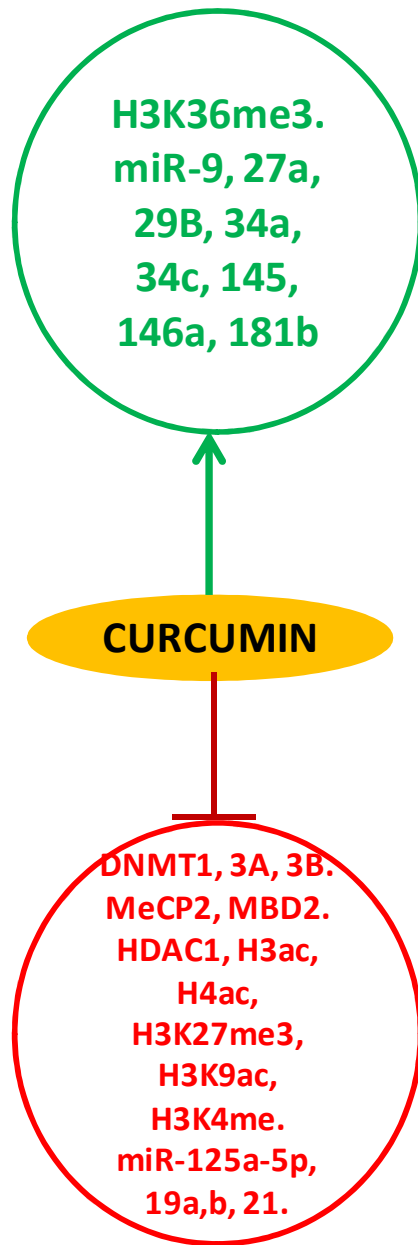
	Curcumin	↓ HDAC 1	p21WAF1	MCF 7 and MDA MB 231 cells	10μM	4 days	[42]
	Curcumin	↓ H3K9Ac	NKG2D	Jurkat and HUT78 cells	4μM	1 day	[78]
	Curcumin	↓ H3ac	IL-6	RA synovial fibroblasts	20μM	8 hrs	[50]
	Curcumin	↓ H3ac	PS1, BACE 1	N2a cells neuroblastoma cells	10μM	2 days	[56]
microRNAs	Curcumin	↑ miR-29B	PTEN (by decreasing DNMT3B)	HSC-T6 cells	20μM	2 days	[33]
	CDF(Difluorinatedcurcumin)	↑ miR-34a and miR-34c	Notch-1	SW620 colon cancer cell line; Archival formalin-fixed paraffin-embedded tissues from normal colonic mucosa and colon tumors	100 nM	3 days	[79]

	Curcumin	↓ miR-125a-5p	tumor protein 53 (TP53)	HONE 1 and HK1 Nasopharyngeal carcinoma cell lines	16 and 36μM	3 days	[80]
	Curcumin	↓ miR-19 a and ↓ miR-19B	PTEN, p-AKT, p-MDM2, p53	MCF7	1μM	4 days	[81]
	CDF( Difluorinatedcurcumin)	↑ miR-146a	EGFR	Human pancreatic cancer cell lines	200-500 nM	4 days	[82]
	Curcumin	↑ miR-181b	CXCL1 and CXCL2	MDA-MB-231	25μM	1 day	[83]
	Curcumin	↑ miR-9	Akt, FOXO1	SKOV3 ovarian cancer cell lines	10-60μM	3 days	[84]
	Curcuminoids	↑miR-27a	ZBTB10	SW-480 and HT-29	2.5-10 μg/mL	1 day	
	EF24 (Curcumin analog)	↓ miR-21	PTEN and PDCD4	DU145 and B16 murine melanoma cells	5μM	1 day	[85]
	Curcumin	↑ miR-145	Il-6 and sIL-6R	Head and neck cancer cells	10 or 20 μmol/L	1 day	[86]



**Figure 2-1 Schematic of epigenetic modulation by curcumin**

A) Represents the effect of epigenetic changes on transcription of genes upon curcumin treatment.



**Figure 2-2: Epigenetically modified genes by curcumin.**

Represents induced and suppressed Histone modifying enzymes, DNA methyl transferases and micro RNAs.





### 3 Nrf2 knockout attenuates the anti-inflammatory effects of phenethyl isothiocyanate and curcumin<sup>4, 5, 6</sup>

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#### 3.1 Introduction

Inflammation is a cellular defense mechanism that protects cells from pathogens. However, an increased period of inflammation leading to a chronic inflammatory state has been established as a major driving force behind carcinogenesis. Specifically, increased pro-inflammatory cytokine production from macrophages and lymphocytes is known to promote various stages of tumorigenesis [87, 88]. Chronic inflammatory damage has a negative impact on the regulation of signal transduction pathways by causing aberrant protein expression that could lead to inflammation-driven carcinogenesis [89]. Macrophages and monocytes act as a first line of defense against bacterial infections[90] as well as other disease states. Previous studies have established that activation of macrophages by lipopolysaccharides (LPSs) increases the levels of inflammatory mediators, such as cytokines, prostaglandins and nitric oxide, and provides a good

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<sup>4</sup> This work has been published as *Boyanapalli et. al.* in Chem. Res. Toxicol.; Nov. 2014.

<sup>5</sup> Key words: Nrf2, PEITC, Curcumin, Inflammation, COX-2, TNF- $\alpha$ , IL-6, NO, HO-1.

<sup>6</sup> Abbreviations: PEITC: Phenethyl isothiocyanate; CUR: Curcumin; Nrf2: Nuclear factor-erythroid 2 (NF-E2)-related factor 2; COX-2: Cyclooxygenase-2; iNOS: inducible nitric oxide synthase; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1: Interleukin-1; IL-6: Interleukin-6; IL-8: Interleukin-8; HO-1: Hemeoxygenase-1; NQO1: NAD(P)H quinine oxidoreductase; LPS: Lipopolysaccharides; RIPA: Radio immune precipitation assay; BCA: bicinchoninic acid; ELISA: Enzyme linked immunosorbent assay

model system to study inflammation [91, 92]. The nuclear factor-erythroid 2 (NF-E2)-related factor 2, commonly known as Nrf2, is a transcription factor that belongs to the cap 'n' collar subfamily containing a basic leucine zipper region. Nrf2 regulates downstream anti-oxidative stress genes, such as NAD(P)H quinine oxidoreductase (NQO1), hemeoxygenase-1 (HO-1) and phase II detoxifying enzymes upon being challenged by cellular stress, and it plays a key regulatory role in cellular stress defensive mechanisms[93, 94] by binding to the anti-oxidant redox element (ARE).

While the induction of anti-oxidant properties has been well attributed to Nrf2, we and others have demonstrated the role of Nrf2 in the regulation of inflammation. Transcriptional regulation of Nrf2 induces HO-1, which has well-known anti-oxidant properties as well as anti-inflammatory functions, as shown in studies conducted in colon, skin, neural and other tissues [95-98]. It has been shown that the Nrf2 pathway plays an important role in lowering both acute and chronic inflammation. Several studies in different animal models have illustrated the role of inflammation in cancer. In particular, studies conducted in Nrf2 knockout mice have demonstrated increased inflammation and tumorigenesis in the liver,[99] colon,[100, 101] brain[98] and lungs [102]. Nrf2 confers protection against inflammation as it has been shown that decreased Nrf2 expression leads to an increased inflammatory response in addition to increased electrophilic and oxidative stress[103].

Recently, our group conducted several studies to examine the anti-inflammatory effects of phytochemicals and their role in chemoprevention [104, 105]. Phytochemicals such as curcumin (CUR), which is an ingredient in turmeric from India, and isothiocyanates, specifically phenethyl isothiocyanate (PEITC), that have been found in broccoli sprouts, water cress and other vegetables have shown potent anti-inflammatory and anti-oxidant effects [8, 106, 107]. It has been well documented

that both these phytochemicals are potent Nrf2 inducers [107-109]. The anti-inflammatory effects of these compounds have been studied using the LPS-induced inflammation model, which is known to increase the expression of COX-2 in macrophages, endothelial cells and fibroblasts[110]. The anti-inflammatory role of phytochemicals, such as sulforaphane, which is an isothiocyanate present in broccoli, and eicosapentanoic acid and docosahexaenoic acid, which are constituents of fish oil and belong to polyunsaturated fatty acids, have been well documented in studies conducted in LPS-stimulated Nrf2 (-/-) macrophages, in which the absence of Nrf2 caused an increase in pro-inflammatory markers, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), interleukin-8 (IL-8), and a decrease in HO-1 expression, which is an anti-oxidative stress gene that also has anti-inflammatory effects [111-113]. Our current study builds on previous work and aims to examine the role of Nrf2 in regulating the anti-inflammatory properties in LPS-induced peritoneal macrophages isolated from Nrf2 (+/+) and Nrf2 (-/-) mice, as shown in the schematic. We hypothesize that although CUR and PEITC are not structurally similar and do not belong to the same class of phytochemicals; they act via the Nrf2 pathway. To evaluate our hypothesis, we compared the effects of CUR and PEITC in the presence and absence of Nrf2 in peritoneal macrophages. Furthermore, we examined the inhibitory effects of both these phytochemicals on TNF- $\alpha$ , NO and IL-6 production, while also examining their regulatory role in the expression of COX-2, iNOS, IL-6, IL-8 and HO-1.

### **3.2 Materials and Methods:**

### 3.2.1 Animals

The C57BL/6J mouse strain, which is homozygous WT for Nrf2, and Nrf2 (-/-) mice, was used as the control and the treatment groups for the current study. Specifically, 10-12-week-old male mice from the second filial generation of Nrf2 (-/-) mice and age-matched C57BL/6J male mice were used for the study. Nrf2 (-/-) mice were generated by backcrossing the F1 generation of Nrf2 (-/-) with C57BL/6J mice purchased from the Jackson laboratory (Bar Harbor, ME, USA) as described previously in studies by our group [111, 112]. The genotype of each animal was confirmed by performing a tail DNA extraction followed by polymerase chain reaction (PCR). Bands for Nrf2 (-/-) were visualized at 200 bp by agarose gel electrophoresis, while the WT mice displayed a band at 300 bp, as illustrated in our previous studies [111, 112]. All the animals used in the study were housed and maintained at Rutgers following the guidelines established by ICUAC and NIH guidelines for care and use of laboratory animals.

### 3.2.2 Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). Primary antibodies against HO-1, COX-2, iNOS and actin and secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). Curcumin (CUR) and phenethyl isothiocyanate (PEITC, 99%) were purchased from Sigma-Aldrich (St. Louis, MO). Thioglycollate broth was purchased from Edge Biological (Memphis, TN). Lipopolysaccharide (LPS from *E. coli* 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO). ELISA kits for the measurement of TNF- $\alpha$  (KMC 3012) and IL-6

(KMC 0062) was purchased from Invitrogen (Grand Island, NY). Taqman RT reagents were purchased from Life Technologies

### **3.2.3 Isolation of Peritoneal Macrophages**

The peritoneal macrophages were isolated using a previously established procedure in our laboratory [111, 112]. A volume of 800 µl of thioglycollate broth (TG broth) was injected intraperitoneally into the animals on Day 1. The animals were euthanized on Day 4, and the macrophages were harvested by flushing the peritoneal cavity with 1X ice cold PBS buffer (pH of 6.8 and 0.02% EDTA). The macrophages were then centrifuged at 1000 rpm for 10 min. Next, the cell pellet was re-suspended with 0.83% ammonium chloride to remove any residual red blood cells.

### **3.2.4 Cell Culture and Treatment**

The viability of the harvested macrophages was evaluated with the trypan blue exclusion method, and the cell viability was calculated from the cell population. Nrf2 (+/+) and Nrf2 (-/-) cells (macrophages) were cultured for 6 h in DMEM using 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. The macrophages were then pre-treated with the drugs CUR or PEITC alone at two non-toxic concentrations (5 and 10 µM, Figure 3-10) for 6 h followed by co-treatment with LPS (1 µg/ml) from *E. coli* to induce the pro-inflammatory cytokines for 8h before the RNA was extracted. For protein extraction the cells were pre-treated with CUR (5 and 10 µM) and PEITC (5 and 10 µM) alone for 6 h followed by co-treatment with LPS (1 µg/ml) for 18 h. LPS-only treatment served as a positive control. DMEM with 10% FBS served as negative control.

### **3.2.5 Biological Assays for Measurement of Nitrite Production (NO) and Cytokine Concentrations**

Nitrite production was assessed as described previously by measuring nitrite accumulation in the culture media fluorimetrically using an Infinite 200 PRO Tecanmicroplate reader [114]. The cells were pre-treated with drugs for 6 h and co-treated with (1 µg/ml) LPS for 8 h. A 50-µl solution of supernatant from the cultured media was added to 96-well black polystyrene flat bottom plates (Whatman Inc., Piscataway, NJ) to which 10 µl of freshly prepared 2,3 diamino naphthalene (0.05 mg/ml in 0.62 N HCl) was added and incubated for 10 min. This step serves as the start of the reaction. Next, 5 µl of 2.8 N sodium hydroxide was added to the solution to stop the reaction that results in the production of 2,3-diaminonaphthotriazole. The reactions were measured at an excitation of 360 nm and emission of 460 nm with a gain setting of 80% using an Infinite 200 PRO Tecanmicroplate reader. The levels of cytokines, TNF- $\alpha$  and IL-6, produced as a response to the inflammatory state, were measured in the supernatant of the culture medium by enzyme linked immunosorbent assay kits from Invitrogen (Grand Island, NY, USA), following the manufacturer's instructions.

### **3.2.6 Isolation of RNA and Reverse-Transcription and Quantitative Polymerase Chain Reaction**

Total RNA from the treatment and control groups was extracted using RNeasy Mini Kit (Carlsbad, CA), and the mRNA was quantified using a NanoDrop2000. Approximately 600 ng of mRNA was reverse transcribed into cDNA using Taqman RT reagents. This cDNA was used to perform the quantitative PCR using SYBR Green PCR master mix from ABI on the ABI7900HT system (Life Technologies,

Grand Island, NY). The primer sequences used for the different genes are listed in Table 1 and have been used in our previous studies [112].

### **3.2.7 Protein Extraction and Western Blotting**

Radio immune precipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO) was used to harvest the cells from the control and treatment groups. The concentrations of protein lysates were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Samples containing 20 µg of total protein were loaded onto a 4-12% electrophoresis gel. The proteins from the gel were then transferred onto a PVDF membrane at 1.3 A and 25 V for 5 minutes using the Transblot Turbo transfer system from Bio Rad. The PVDF membranes were then incubated with selected primary antibodies at 4° C overnight, followed by detection using HRP-conjugated secondary antibodies. The signals were enhanced using ECL reagents (Thermo Scientific) and were visualized using a Bio Rad Gel documentation system. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

### **3.2.8 Statistical Analysis**

All experiments were performed at least three times, and similar results were obtained. Statistical tests were performed using Student's *t* test. All p-values were two-sided, and a p-value < 0.05 was considered statistically significant.

## **3.3 Results**



### **3.3.1 Curcumin and PEITC inhibit nitrite Production in Nrf2 (+/+) Macrophages.**

Nitrite production as a result of CUR and PEITC treatment was assessed in LPS-stimulated Nrf2 (+/+) macrophages. The results showed that nitrite production was lowered by CUR at 10  $\mu$ M and PEITC in a dose-dependent manner at 5 and 10  $\mu$ M compared with the LPS-only treatment in Nrf2 (+/+) macrophages (

Figure 3-1). The nitrite production was also decreased by CUR and PEITC in Nrf2 (-/-) macrophages in comparison to the LPS treatment but the effect itself was not as pronounced as it was in Nrf2 (+/+) macrophages where the nitrite production was lower than 0.5 fold(

Figure 3-1). Overall, it can be observed that both CUR and PEITC lowered NO production in Nrf2 (+/+) macrophages in a dose-dependent manner in comparison to LPS treatment ( $p < 0.05$ ). While the lowering effect was also seen in Nrf2 (-/-) macrophages, the nitrite production itself was much higher than the WT cells, thus emphasizing the importance of Nrf2 (

Figure 3-1).

### **3.3.2 PEITC Decreases the TNF- $\alpha$ and IL-6 Levels in Nrf2 (+/+) Macrophages.**

To assess cytokine production in LPS-stimulated Nrf2 (+/+) macrophages treated with CUR and PEITC, we performed ELISA analysis of TNF- $\alpha$  and IL-6. We found that the level of TNF- $\alpha$  and IL-6 was significantly lower ( $p < 0.05$ ) after PEITC treatment (10  $\mu$ M) in Nrf2 (+/+) macrophages in comparison to LPS treatment only (Figure 3-2; Figure 3-3). Conversely, cytokine production in Nrf2 (-/-) macrophages treated with 10  $\mu$ M of PEITC was not lowered. By contrast, the effect of CUR on the level of cytokines was not nearly as significant compared with PEITC and the LPS-

only treatment in both the groups for IL-6 and TNF- $\alpha$ , as shown in (Figure 3-2 and Figure 3-3) respectively. PEITC and CUR show differential effects on cytokine production in Nrf2 (+/+) macrophages in terms of the respective gene expression. The differential effects of these compounds could be attributed to the post transcriptional regulation. It was previously published that CUR has differential expression of cytokines TNF-  $\alpha$  and IL-6 in adipocytes which was due to post transcriptional regulation [115].

### **3.3.3 Curcumin and PEITC Inhibit LPS Induced Gene Expression of COX-2, iNOS, TNF- $\alpha$ , IL-6 and Induced HO-1 Gene Expression in Nrf2 (+/+) Macrophages.**

It is known the LPS stimulation increases the expression of pro-inflammatory markers such as COX-2, TNF- $\alpha$  and IL-6 [91, 92]. The results of qPCR illustrate that CUR exerted an anti-inflammatory effect by lowering the LPS-induced expression of iNOS (Figure 3-5), TNF- $\alpha$  (Figure 3-6), IL-6 (Figure 3-7) and COX-2 (Figure 3-8). Similarly, treatment with PEITC showed a significant dose-dependent decrease at 5 and 10  $\mu$ M in the gene expression of LPS-induced pro-inflammatory markers, such as COX-2, iNOS, TNF- $\alpha$  and IL-6 in Nrf2 (+/+) macrophages, while no change was observed in Nrf2 (-/-) macrophages for TNF-  $\alpha$  and COX-2 (Figure 3-6 and Figure 3-8). iNOS gene expression was also decreased by PEITC in Nrf2 (-/-) macrophages in comparison to the LPS treatment but the effect itself was not as pronounced as it was in Nrf2 (+/+) macrophages. The fold change in Nrf2 (+/+) was 0.2 and 0.15 when treated with 5 and 10  $\mu$ M PEITC, whereas the same treatments in Nrf2 (-/-) macrophages showed fold change of 0.6 and 0.4 respectively as shown in Figure 3. B. Both CUR and PEITC also induced the expression of HO-1 (Figure: 3-4) at 5  $\mu$ M

and 10  $\mu$ M in Nrf2 (+/+) peritoneal macrophages in a dose-dependent manner. At the same time, there was no significant decrease in the pro-inflammatory markers or an increase in HO-1 induction in the Nrf2 (-/-) macrophages.

### **3.3.4 PEITC Induces HO-1 Protein Expression and Inhibits LPS-Induced COX-2 and iNOS Protein Expression**

The protein expression of COX-2, iNOS and HO-1 of macrophages upon pre-treatment with the drugs for 6 h and co-treatment with LPS for 18 h to induce inflammation were analyzed by western blotting. The results show that PEITC caused a dose-dependent decrease in COX-2 protein expression and a dose-dependent increase in HO-1 protein expression in Nrf2 (+/+) macrophages, while the effect was not similar in Nrf2 (-/-) macrophages, as shown in (Figure 3-9). The protein expression was consistent with the mRNA expression, as shown by qPCR analyses. Additionally, CUR caused a decrease in COX-2 expression; however, this decrease was not significant compared with that induced by PEITC. This result demonstrates that PEITC has a more potent anti-inflammatory effect, which is mediated by the Nrf2 pathway. Additionally, the presence of Nrf2 has more attenuating effects on COX-2 and iNOS.

## **3.4 Discussion**

The Nrf2 signaling pathway plays an important role in the regulation of oxidative stress and the inflammatory state both *in vitro* [116] and *in vivo* [103, 117-119]. It has been well documented that Nrf2 induction leads to increased expression of anti-oxidative stress genes, such as HO-1, that also confer anti-inflammatory effects by induction of IL-10 as an anti-inflammatory cytokine [120]. Several studies have demonstrated the anti-inflammatory effects of bioactive phytochemicals,

including flavonoids such as sappanchalcone,[121] chalcones such as 4,2',5'-trihydroxy-4'-methoxychalcone,[122] diterpenoids such as kaurenoic acid,[123] triterpenoids such as celastrol[124] and sesquiterpenoids such as costunolide[125] among others. These phytochemicals are regulated by the activation of the Nrf2 transcription factor and its target genes [126]. It has been shown that the lack of Nrf2 can lead to lethality by septic shock, as evidenced by comparative studies performed in Nrf2 (+/+) and Nrf2 (-/-) peritoneal neutrophils; these studies demonstrated that Nrf2 confers protection from LPS-induced inflammation [127]. The importance of Nrf2 as an anti-inflammatory target for phytochemicals has been illustrated in previous studies performed in Nrf2 (-/-) mice from our group [111, 112] as well as from others who showed that the lack of the Nrf2 gene makes these mice more susceptible to immune and inflammatory disorders[128]. Multiple studies have suggested that Nrf2-deficient mice are more prone to inflammatory, cytotoxic and genotoxic effects induced by oxidants as well as electrophiles [103, 129]. For instance, the lack of Nrf2 in the lungs led to reduced expression of Nrf2 target genes and an increase in pro-inflammatory cytokines such as IL-12 and IL-13[130].

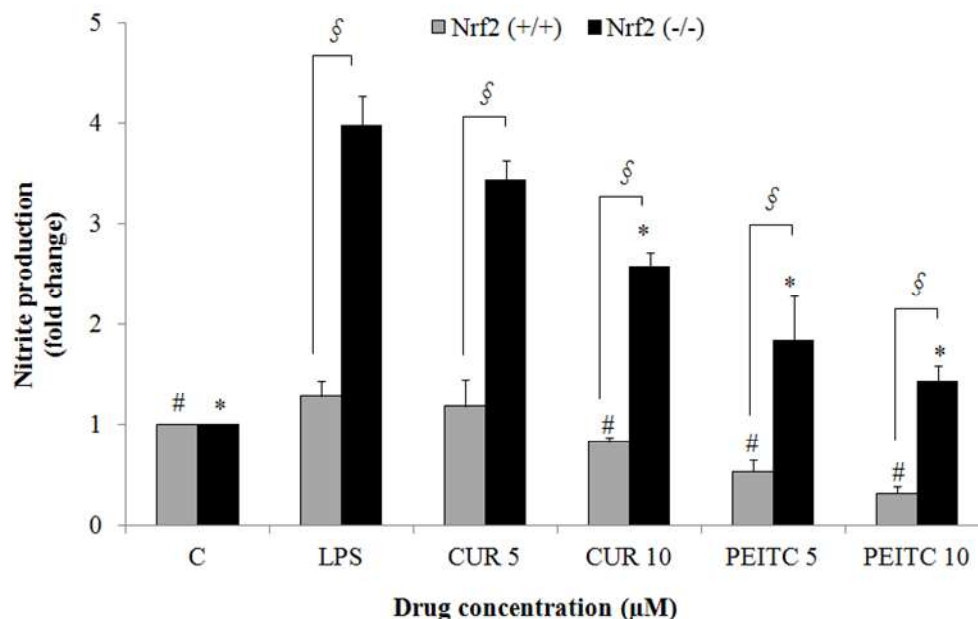
We have previously reported that curcumin and PEITC in combination with sulforaphane have a strong inhibitory effect on inflammation in RAW 264.7 cells by reducing iNOS and COX-2 expression [131]. We also showed that the combination of curcumin and PEITC reduces the growth of PC3 cells in a xenograft model *in vivo* [132, 133]. In this study, we provide mechanistic insight into the role of the Nrf2 pathway in ameliorating inflammation by using these phytochemicals to explore the anti-inflammatory and the anti-oxidant effects of PEITC and curcumin mediated by Nrf2. Our results indicate that PEITC acts via the Nrf2 pathway to reduce inflammation, as demonstrated by its regulation of pro-inflammatory cytokines,

decrease IL-6 and TNF- $\alpha$ , as revealed by ELISA in Nrf2 (+/+) mice compared with the Nrf2 (-/-) mice (Figs. 2A and 2B). However, the same phenomenon was not as clear in CUR-treated macrophages, indicating an alternative pathway for attenuating inflammation, for instance by inhibiting TLR4 mediated NF- $\kappa$ B signaling pathways [134]. Though there was an increase in the expression of HO-1 in the Nrf2 (+/+) macrophages by both CUR and PEITC, differential expression in the cytokine production was observed suggesting there is a lack of direct correlation between Nrf2 expression and these cytokines. This was also observed in our previous work where EPA and DHA showed changes in cytokine production which were not of the same magnitude of HO-1 expression [112], others reported tBHQ which induced the expression of Nrf2 and HO-1 in a dose-dependent manner while not having similar effects on the cytokine expression like TNF- $\alpha$  [135]. Although low levels of NO are required to maintain normal homeostasis in the body, one of the direct consequences of an inflammatory state is the increased expression of iNOS in macrophages and other immune cells, which exacerbates chronic inflammation by changing the normal tissue environment; [136] curcumin and PEITC were shown to have anti-inflammatory effects due to the suppression of iNOS and consequent reduction nitrite production [136-139]. In this context, our results show that PEITC treatment exhibits a dose-dependent suppression of nitrite production, as demonstrated in the NO assay of Nrf2 (+/+) macrophages. By contrast, the lack of Nrf2 reduces the impact of the drug on the reduction of NO production (Figure 1). Hemeoxygenase breaks down heme as the rate-limiting step and produces several active biological molecules that can serve as secondary messengers for cellular processes, such as inflammation [140]. It has also been observed that lack of Nrf2 in macrophages decreased the expression of HO-1, which confers protection against inflammation. Additionally, LPS-induced iNOS

expression in the macrophages was also lowered by an increase in HO-1 expression in the Nrf2 (+/+) mice [141, 142]. The anti-oxidant gene HO-1 was clearly regulated by PEITC and CUR in Nrf2 (+/+) macrophages as demonstrated by quantitative PCR (Figure 3A) and western blotting analysis (Figure 4A, 4B). Previous studies from our group as well as others showed that increased expression of HO-1 has anti-inflammatory effects [111, 112, 143]. Accordingly, our results in the present study clearly indicate that PEITC and CUR increase the gene (Figure 3.A) and protein expression (Figure 4A and 4B) of HO-1 in LPS-induced Nrf2 (+/+) macrophages in a dose-dependent manner, while the effect was not observed in the Nrf2 (-/-) macrophages. There have been several reports on the anti-inflammatory effects of CUR and PEITC acting via induction of HO-1[131] and suppression of NF-kB [144-146]. It is logical to conclude that the induction of HO-1 and suppression of inflammatory cytokines as well as prostaglandins such as COX-2 is mediated via the Nrf2 pathway, which is further supported by the induction of HO-1 gene and protein expression. Furthermore, it was clearly observed that PEITC inhibited IL-6 and TNF- $\alpha$  production, as measured by ELISA (Figs 2A, 2B). Additionally, the mRNA expression was clearly attenuated by CUR and PEITC, as illustrated in (Figure 3D and 3C). Nevertheless, the effect was not prominent in the CUR-treated macrophages, as shown in (Figure 2A and 2B), which depict cytokine measurements by ELISA. In addition to the cytokines, the mRNA and protein expression of COX-2, which is a classic inflammatory mediator, was lowered upon treatment by CUR and PEITC in a dose-dependent manner, as shown in (Figure 3 E and Figure 4.A and B).

In conclusion, our study indicates that Nrf2 plays a critical role in mediating the anti-inflammatory properties of PEITC and CUR, as demonstrated by their effects in Nrf2 (+/+) and Nrf2 (-/-) macrophages upon induction with LPS. This result further

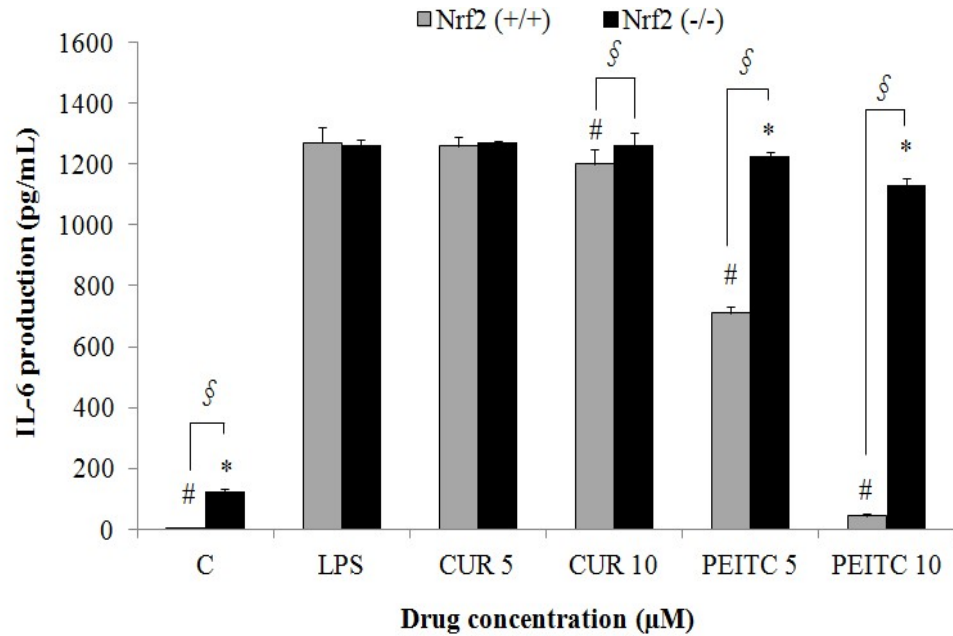
strengthens the hypothesis that Nrf2, apart from regulating the phase II and III drug metabolizing enzymes and transporters, also plays a crucial role in attenuating inflammation, which contributes to many acute and chronic diseases, including autoimmune disease, neurological, cardiovascular disease and cancer.



**Figure 3-1: Inhibitory effect of Curcumin and PETIC on LPS induced NO (Nitric Oxide) production**

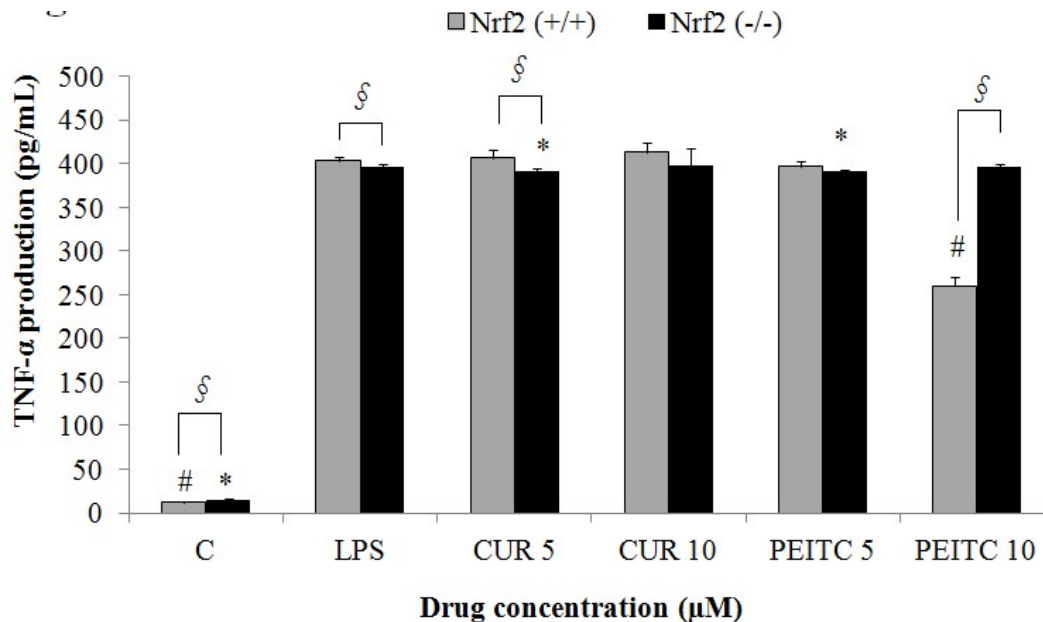
LPS induced Nitrite production is decreased by the treatment of peritoneal macrophages Nrf2 (+/+) with CUR and PEITC but no significant decrease was seen in Nrf2 (-/-) macrophages. The peritoneal macrophages were pretreated with the drugs for 6 h followed by co-treatment with the drug and LPS for 8h. The results are shown as mean  $\pm$  S.E of three independent experiments (n=3). (§) represents statistical significance between Nrf2 (+/+) and Nrf2 (-/-) macrophages; (#) represents statistical significance between LPS and treatment groups in (+/+) macrophages; (\*) represents statistical significance between LPS and treatment groups in (-/-) macrophages.





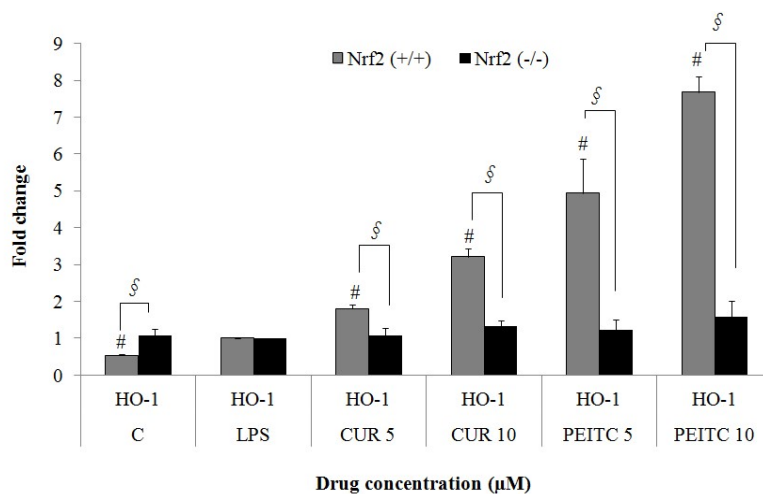
**Figure 3-2: Effect of Curcumin and PEITC on IL-6 cytokine production**

Inhibition of IL-6 secretion in Nrf2 (+/+) macrophages upon treatment with PEITC were significantly higher than CUR. Both the treatment groups compared with the LPS only treatment which served as positive control. The cells were pre-treated with CUR or PEITC alone for 6 h and co-treated with LPS and CUR or PEITC for 8 h and the supernatant was used for ELISA. (n=3) (§) represents statistical significance between Nrf2 (+/+) and Nrf2 (-/-) macrophages; (#) represents statistical significance between LPS and treatment groups in (+/+) macrophages; (\*) represents statistical significance between LPS and treatment groups in (-/-) macrophages.

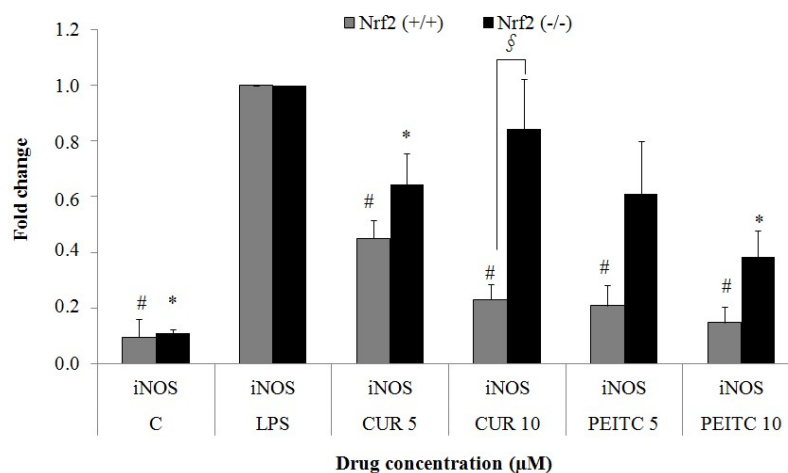


**Figure 3-3: Effect of Curcumin and PEITC on TNF- $\alpha$  production**

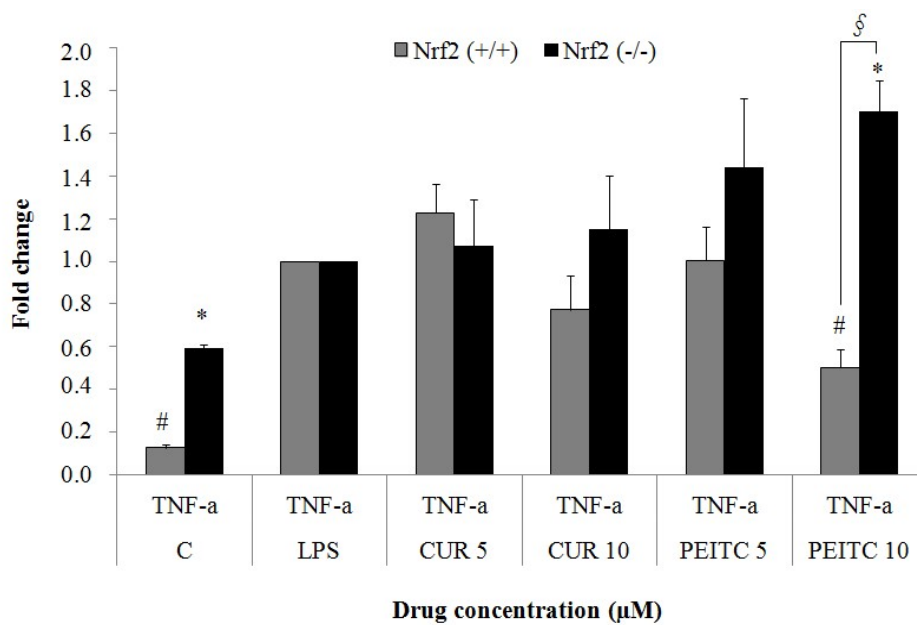
Inhibition of TNF- $\alpha$  secretion in Nrf2 (+/+) macrophages upon treatment with PEITC were significantly higher than CUR. Both the treatment groups compared with the LPS only treatment which served as positive control. The cells were pre-treated with CUR or PEITC alone for 6 h and co-treated with LPS and CUR or PEITC for 8 h and the supernatant was used for ELISA. (n=3) (§) represents statistical significance between Nrf2 (+/+) and Nrf2 (-/-) macrophages; (#) represents statistical significance between LPS and treatment groups in (+/+) macrophages; (\*) represents statistical significance between LPS and treatment groups in (-/-) macrophages.



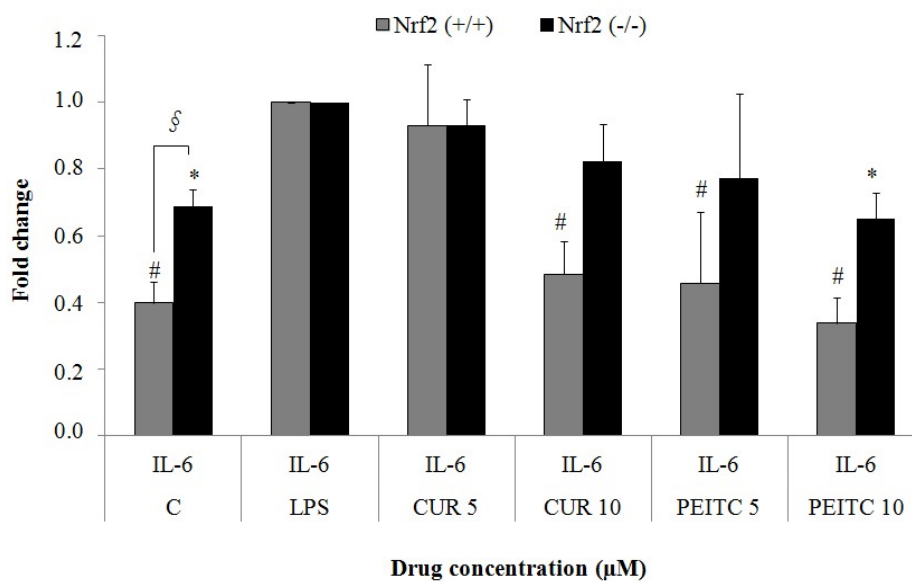
**Figure: 3-4: Effect of Curcumin and PEITC on Hemeoxygenase-1 gene expression**



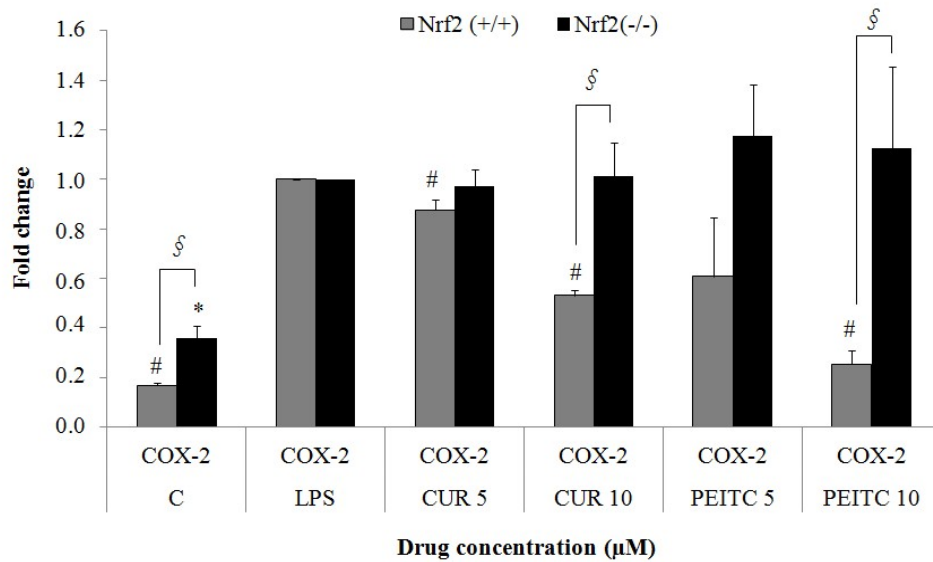
**Figure 3-5: Effect of Curcumin and PEITC on inducible nitric oxide synthase (iNOS) gene expression**



**Figure 3-6: Effect of Curcumin and PEITC on TNF- $\alpha$  gene expression**

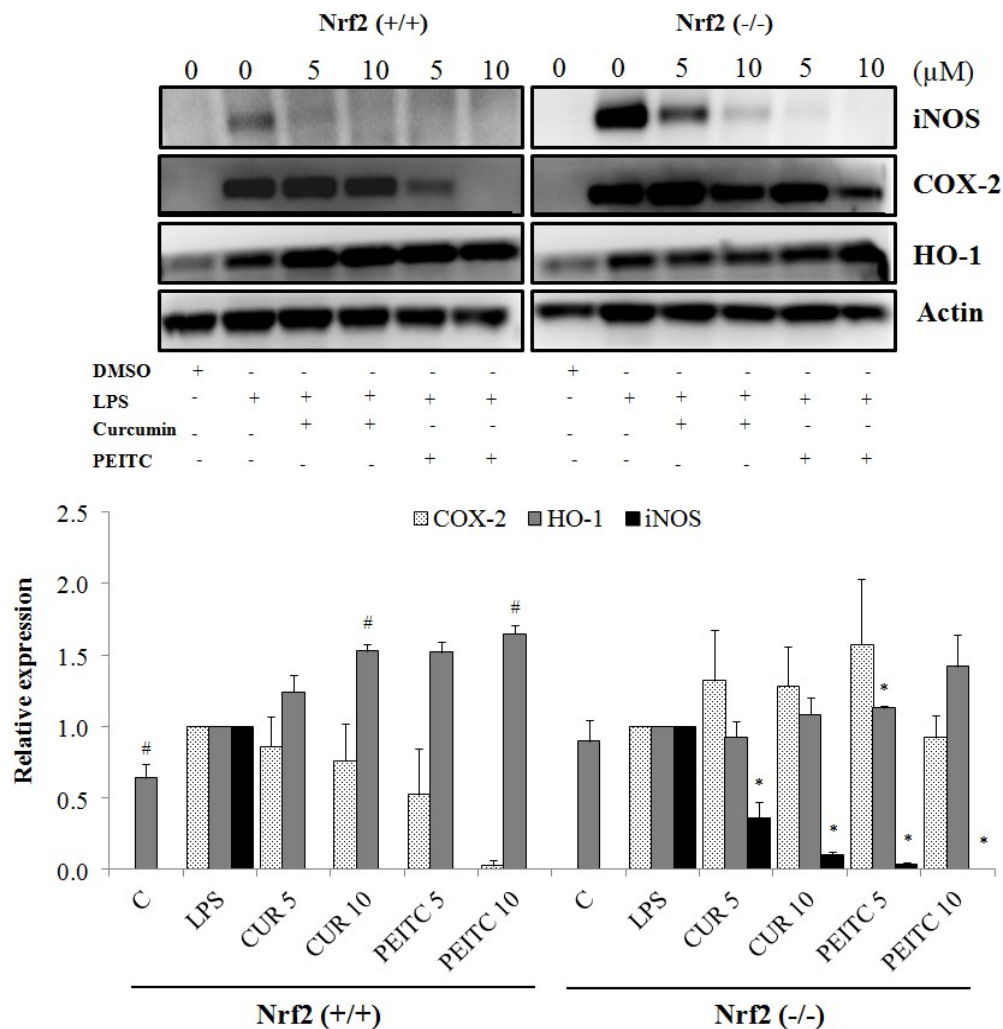


**Figure 3-7: Effect of Curcumin and PEITC on IL-6 gene expression**



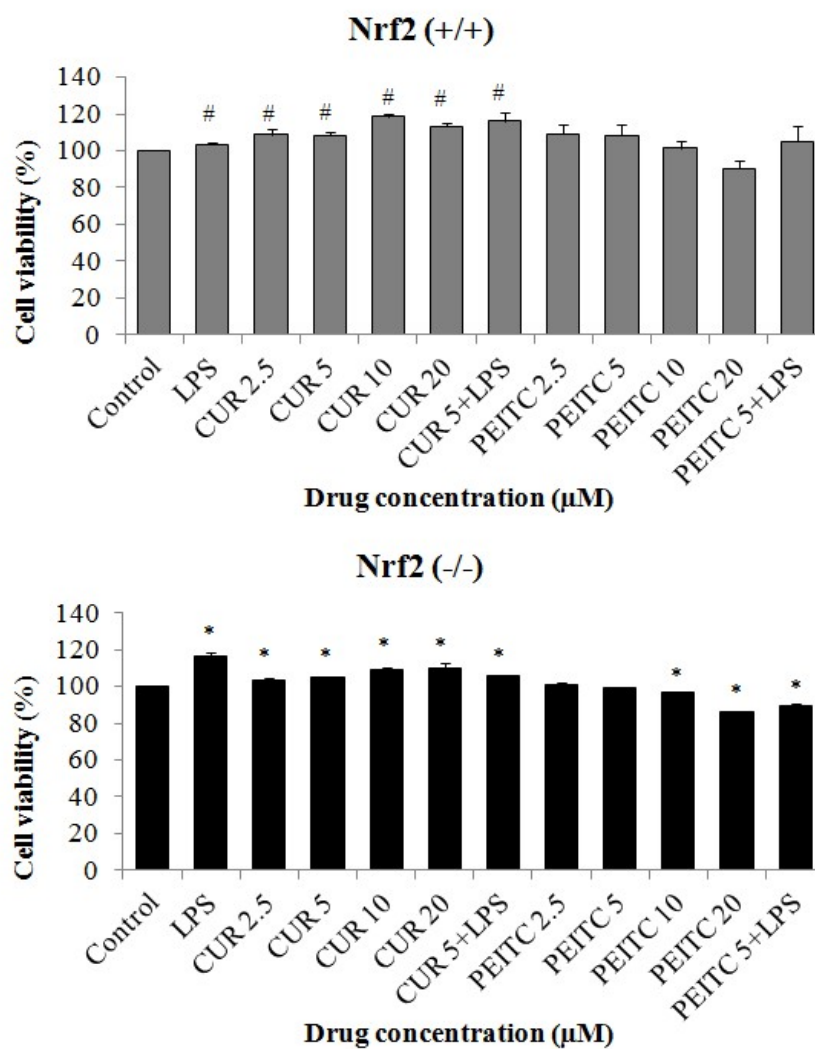
**Figure 3-8: Effect of Curcumin and PEITC on COX-2 gene expression**

qPCR analyses showed that LPS induced gene expression of pro-inflammatory markers such as iNOS, COX-2, IL-6, TNF- $\alpha$  was significantly reduced by PEITC in Nrf2 (+/+) macrophages in a dose dependent manner. While the effect was comparably less in CUR treatment it still lowered LPS induced gene expression of those genes at 10  $\mu$ M concentrations. However, the same effect of PEITC and Curcumin was not seen in the Nrf2 (-/-) macrophages. PEITC and CUR also increased the expression of HO-1 in Nrf2 (+/+) macrophages significantly while the effect was not seen in the Nrf2 (-/-) macrophages. The results are shown as mean  $\pm$  S.E of three independent experiments. (n=3)(§) represents statistical significance between Nrf2 (+/+) and Nrf2 (-/-) macrophages; (#) represents statistical significance between LPS and treatment groups in (+/+) macrophages; (\*) represents statistical significance between LPS and treatment groups in (-/-) macrophages.



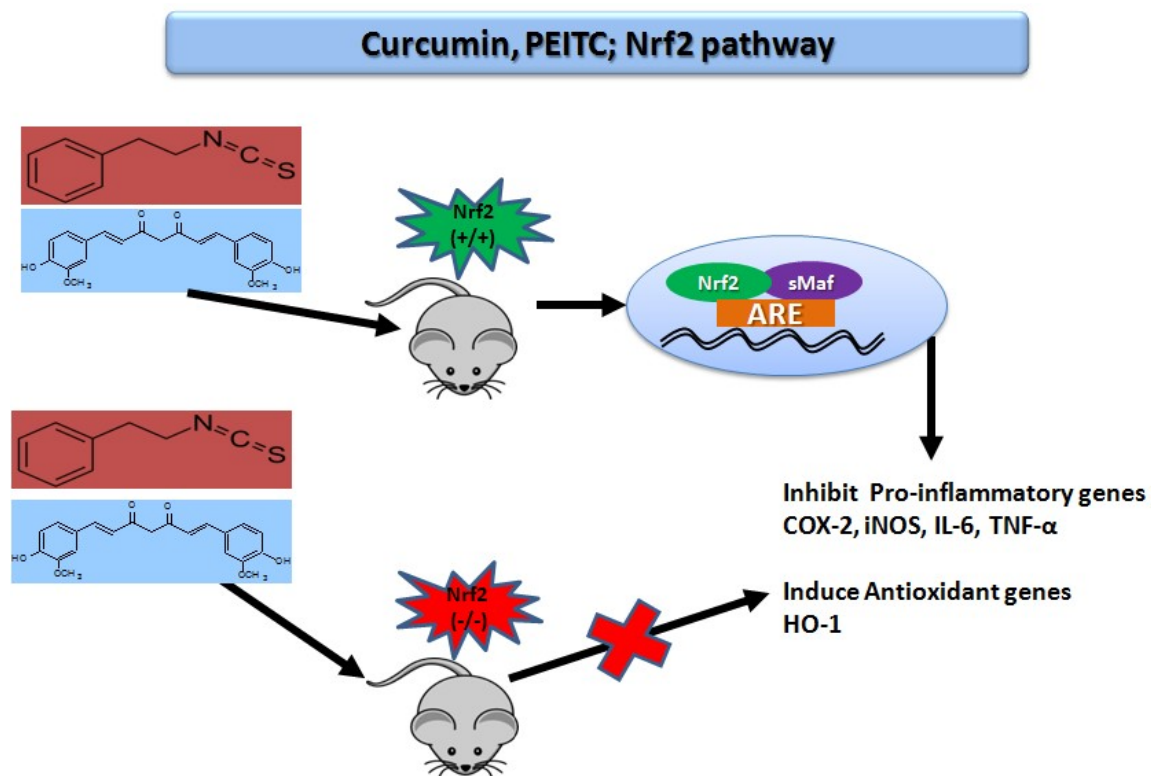
**Figure 3-9: Effect of Curcumin and PEITC on COX-2, iNOS and HO-1 protein expression**

Western blot analyses showed that PEITC significantly reduced LPS induced protein expression of COX-2 in a dose dependent manner while the effect was lower in CUR in Nrf2 (+/+) macrophages. There was no significant lowering of COX-2 protein expression in Nrf2 (-/-) macrophages upon treatment with either PEITC or CUR. HO-1 protein expression was significantly increased in Nrf2 (+/+) macrophages in dose dependent manner upon treatment with PEITC and CUR while the induction was not as pronounced in Nrf2 (-/-) macrophages. The macrophages were pre-treated with the drugs for 6 h and co-treated with drugs and LPS for 18h. The results are shown as mean  $\pm$  S.E of three independent experiments. (n=3). (#) represents statistical significance between LPS and treatment groups in Nrf2 (+/+) macrophages; (\*) represents statistical significance between LPS and treatment groups in Nrf2 (-/-) macrophages.



**Figure 3-10: Cell viability of macrophages upon treatment with Curcumin, PEITC or LPS**

. Effect of Curcumin, PEITC, or LPS on cell viability in Macrophages. Cells were treated with Curcumin, PEITC or LPS (1  $\mu\text{g/mL}$ ) during 24 hours, as described in the “Materials and Methods” section. The data are expressed as the mean  $\pm$  SD of three independent experiments. \* indicate significant differences ( $p < 0.05$ ) in cell viability compared to LPS-induced DMSO treatment, which was used as a negative control.



**Figure 3-11: Summary of studies showing the impact of Nrf2 on pro-inflammatory and anti-oxidant genes**



**Table 3-1: qPCR primers used for GAPDH, HO-1, iNOS, TNF- $\alpha$ , IL-6 and COX-2 genes.**

<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5'-TGA AGC AGG CAT CTG AGG G-3'	5'-CGA AGG TGG AAG AGT GGG AG-3'
Hemeoxygenase-1 (HO-1)	5'-CCT CAC TGG CAG GAA ATC ATC-3'	5'-CCT CGT GGA GAC GCT TTA CAT A-3'
Inducible nitric oxide synthase 2 (iNOS)	5'-CCT GGT ACG GGC ATT GCT-3'	5'-GCT CAT GCG GCC TCC TTT-3'
Tumor necrosis factor-alpha (TNF- $\alpha$ )	5'-TCT CAT GCA CCA CCA TCA AGG ACT-3'	5'-ACC ACT CTC CCT TTG CAG AAC TCA-3'
Interleukin-6 (IL-6)	5'-ATC CAG TTG CCT TCT TGG GAC TGA-3'	5'-TAA GCC TCC GAC TTG TGA AGT GGT-3'
Cyclooxygenase-2 (COX-2)	5'-TGC CTG GTC TGA TGA TGT ATG CCA-3'	5'-AGT AGT CGC ACA CTC TGT TGT GCT-3'

## 4 Epigenetic Reactivation of RASSF1A by Phenethyl Isothiocyanate (PEITC) and promotion of apoptosis in LNCaP cells<sup>7, 8, 9</sup>

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### 4.1 Introduction:

Prostate cancer (PCa) is one of the most frequent causes of cancer-related deaths in men in the United States. The most recent data indicate that greater than 233,000 new cases of PCa are diagnosed and an estimated 29,480 cases of prostate cancer-related deaths occur in men in the US each year[147]. Lifestyle factors, such as diet and nutrition, play a significant role in the pathogenesis of PCa, and multiple studies have revealed that intensive nutrition and lifestyle changes might modulate gene expression in the prostate. These findings highlight the potential role of dietary phytochemicals in the treatment of PCa[148-150]. Studies evaluating epigenetic modifications in cancer, specifically those that lead to the inactivation or silencing of key regulatory genes, such as tumor suppressor genes, has led to the realization that genetic and epigenetic changes play a critical role in tumorigenesis[151]. Recent advances in the field of epigenetics, which are partly due to next generation sequencing techniques, have redirected the focus of cancer researchers.

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<sup>7</sup> The work done in this chapter is submitted for peer-review in an international journal.

<sup>8</sup> Keywords: RASSF1A, Prostate cancer, epigenetics, DNA methylation, DNMTs, HDACs, apoptosis.

<sup>9</sup> Abbreviations: RASSF1A- RASsuperfamily isoform1A; MSP- Methylation specific PCR; BGS- Bisulfite genomic sequencing; DNMT- DNA methyl transferase; HDAC- Histone deacetylase; PI- Propidium iodide; Prostate cancer (PCa); PCR- polymerase chain reaction; Aza- Azadeoxycytidine; TSA-Trichostatin A

These advances have led to the realization that the development of this deadly disease involves interplay between genetic alterations and epigenetic aberrations. DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, such as microRNAs, are specific epigenetic aberrations that induce the expression of oncogenes or silence the expression of tumor suppressor genes[152].

In recent years, epigenetic silencing of tumor suppressor genes has gained much attention in cancer research, and the significance of this phenomenon in driving tumorigenesis is now well established. Recent developments in this field have led to the hypothesis that reactivating tumor suppressor genes that have been silenced by promoter methylation might be an effective targeted therapy for treating cancer[73]. Several studies have demonstrated that the epigenetic silencing of several genes, including *RAR $\beta$ 2*, *RAR $\beta$ 4*, *RASSF1A*, *CDH13*, *APC*, *CDH1*, *FHIT*, *CDKN2A* and *Nrf2*, is due to increased promoter methylation exclusively in prostate tumor tissues[134, 153-156]. Various changes in DNA methylation patterns can be detected before PCa becomes invasive, suggesting that epigenetic changes are pivotal events in tumor progression and that epigenomic changes play a critical role in PCa[157].

The *RASSF1A* gene, a well-characterized tumor suppressor, belongs to the Ras-association domain family of genes and is located on chromosome at 3p21.3. The *RASSF1* gene encodes several isoforms, including *RASSF1A*, *RASSF1B*, and *RASSF1C*, all of which are generated from alternative mRNA splicing and promoter usage[158]. *RASSF1A* transcriptional silencing is frequently observed feature in multiple types of cancer as a result of promoter hypermethylation; however, few reports are available describing the mechanisms underlying this phenomenon [159-162]. *RASSF1A* transcription is suppressed by hypermethylation in breast, ovarian, gastric, nasopharyngeal, and bladder cancers as well as neuroblastoma and renal cell

lines [163-168]. Hypermethylation of the *RASSF1A* gene at CpG islands has been observed in 70% of prostate cancer cases, and the restoration of *RASSF1A* expression inhibited tumorigenesis in prostate and renal tumor cell lines[169, 170]. The tumor suppressor function of *RASSF1A* has been confirmed by studies demonstrating that the exogenous expression *RASSF1A* decreased colony formation, suppressed anchorage-independent growth and reduced tumor formation in lung, kidney and breast cancers *in vivo*. In light of the tumor suppression functions of *RASSF1A* and the observation that restoring *RASSF1A* expression in tumor cell lines decreases tumorigenicity, restoring *RASSF1A* expression and elucidating the mechanism by which hypomethylation occurs have significant implications in the context of preventing tumor growth [165, 169, 171]. Recent meta-analyses have revealed that *RASSF1A* methylation in tumor tissues was directly correlated with the Gleason Score (GS), serum prostate-specific antigen (PSA) level and tumor stage in PCa, indicating that *RASSF1A* promoter methylation might be a potential biomarker for prostate cancer prognosis and therapy[172, 173]. Several studies have described the effect of phytochemicals, such as curcumin and mahanine, in reversing *RASSF1A* promoter hypermethylation and restoring *RASSF1A* expression in breast cancer and prostate cancer cells[36, 174].

Phenethyl isothiocyanate (PEITC), an abundant component of cruciferous vegetables, such as broccoli and watercress, exerts chemoprevention activity by modulating epigenetic modifications [175, 176]. PEITC is a novel epigenetic regulator of both histone deacetylases (HDACs) and DNA methyltransferases (DNMTs), factors that govern the expression and suppression of several genes, including GSTP1 and p21[177-179]. Recent studies demonstrated that PEITC is an effective HDAC inhibitor and reduces HDAC enzyme activity. These events induce growth arrest and

apoptosis in cancer cells both *in vitro* and *in vivo* [177, 180]. A recent study revealed that PEITC regulated H3-acetylation (H3-Ac) and site-specific H3 lysine methylations (H3K27me3 and H3K9me2), which are epigenetic modifications associated with attenuating cell proliferation in a concentration- and time-dependent manner in human colon cancer cells [181].

We hypothesized that PEITC induces *RASSF1A* promoter demethylation in LNCaP cells and that epigenetic reactivation of *RASSF1A* induces apoptosis in LNCaP cells. To test this hypothesis, we treated LNCaP cells with 0.01% DMSO, 2.5 and 5  $\mu$ M PEITC or 2.5  $\mu$ M azadeoxycytidine (5-Aza) with 0.5  $\mu$ M trichostatin A (TSA). The demethylating effect of the test compounds was assessed using the methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS). We also evaluated the reactivation of *RASSF1A* expression and the role of *RASSF1A* activation in promoting apoptosis. The results of our BGS and protein expression analyses indicate that PEITC reactivates *RASSF1A* expression by promoting demethylation and inhibiting HDAC activation.

## 4.2 Materials and methods

### 4.2.1 Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). The cells were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. PEITC, 5-Aza, TSA, bacteriological agar, puromycin, and ethidium bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Annexin V and propidium iodide (PI) apoptosis kit was purchased from Life

Technologies. Then,  $3.5 \times 10^4$  LNCaP cells/ml were seeded in 10-cm tissue culture plates for 24 h and treated with DMSO 0.01% (negative control), PEITC (2.5  $\mu$ M or 5  $\mu$ M) or 5-Aza with TSA (2.5  $\mu$ M and 0.5  $\mu$ M, respectively) (positive control) for 5 days. The drugs and media were replenished every 2 days. TSA (0.5  $\mu$ M) was added to the 5-Aza treatment group 20 h prior to harvesting the cells.

#### **4.2.2 DNA methylation analysis**

LNCaP cells were plated in 10-cm plates for 24 h and subsequently treated with 0.1% DMSO (control), 2.5  $\mu$ M 5-Aza and 500 nM TSA, or 2.5 or 5  $\mu$ M PEITC for 5 days. The medium was changed every other day. For cells treated with 5-Aza and TSA, 500 nM TSA was added 20 h before the cells were harvested. On day 5, the cells were harvested for further analyses. Genomic DNA was isolated from the treated cells using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Next, 600 ng of genomic DNA was used as a template for bisulfite conversion using the EZ DNA Methylation Gold Kit (Zymo Research Corp., Orange, CA, USA) following the manufacturer's instructions. To obtain products for sequencing, the converted DNA was amplified using semi-nested PCR as previously described by Dammann et al. [161]. Briefly, the DNA sequences were amplified by mixing bisulfite-converted DNA (500 ng) with primer MU379 (50 pmoles) and primer ML730 (50 pmoles) in reaction buffer (20  $\mu$ l) containing dNTPs (200  $\mu$ M each dNTP) using Platinum PCR Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The reaction conditions consisted of 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 74 °C for 2 min. A semi-nested PCR using the amplified products at a 1:50 dilution, the internal primer ML561 and primer MU379 was performed using similar PCR conditions as described above. The PCR products containing bisulfite-resistant cytosines were ligated into the

pCR2.1 vector (Invitrogen), and several clones were sequenced to confirm the presence of the amplified DNA. A minimum of 10 clones per biological replicate from each treatment group were randomly selected and sequenced (Genewiz, Piscataway, NJ, USA). The percentage of methylated CpG sites was calculated by dividing the number of methylated CpG sites by the total number of CpG sites in the DNA sequence. The primer sequences used for BGS are listed in Table 1.

Methylation-specific PCR (MSP) was performed using the bisulfite-converted genomic DNA as template. The primer sequences used for the PCR amplification of the methylated (M) and unmethylated regions of the *RASSF1A* gene are listed in Table 4-1. The amplification products were separated on a 2% agarose gel by electrophoresis and visualized using ethidium bromide staining and a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA). The densities of the bands were quantified using ImageJ software (Version 1.48d; NIH, Bethesda, Maryland, USA).

#### **4.2.3 RNA isolation and qPCR**

Total RNA from the treatment and control groups was extracted using the RNeasy Mini Kit (Carlsbad, CA), and the mRNA was quantified using a NanoDrop 2000. Approximately 600 ng of mRNA was reverse-transcribed into cDNA using Taqman RT reagents. The relative expression levels of *RASSF1A*, *DNMT1*, *DNMT3A* and *DNMT3B* mRNA were determined using qPCR with the cDNA template and the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in an ABI7900HT system (Applied Biosystems). The sequences of the forward and reverse primers used to analyze these genes are listed in Table 4-1.

#### **4.2.4 Protein lysate preparation and Western blot assays**

Protein lysates were prepared using radio immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, St. Louis, MO, USA) supplemented with a protein inhibitor cocktail (Sigma–Aldrich). Total protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Briefly, 20 µg of total protein were separated using 4 to 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electro-transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% BSA (bovine serum albumin; Fisher Scientific, Pittsburgh, PA, USA) in Tris-buffered saline with 0.1% Tween 20 (TBST) (Boston Bioproducts, Ashland, MA, USA), the membranes were sequentially incubated with specific primary antibodies and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using the Supersignal West FemtoChemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA). Densitometry of the bands was analyzed using ImageJ (Version 1.48d; NIH). The primary antibodies were obtained from the following sources: anti-β-ACTIN from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-DNMT 3A and 3B from IMGENEX (San Diego, CA, USA); and anti-HDAC1, 2, 4 and 6 from Cell Signaling Technology (Boston, MA, USA). The secondary antibodies were purchased from Santa Cruz Biotechnology.

#### **4.2.5 Flow cytometry analysis of cell cycle distribution and apoptosis**

PEITC- or 5-Aza-treated LNCaP cells were stained with PI (Sigma-Aldrich Co., St. Louis, MO) or PI and Annexin V-FITC (Life Technologies) and analyzed by flow cytometry using a Beckman Coulter Gallios Flow Cytometer (Brea, CA) in The Flow



Cytometry/Cell Sorting & Confocal Microscopy Core Facility at Rutgers University. Briefly, LNCaP cells ( $3.5 \times 10^4$  cells/ml) were seeded in 10-cm plates, allowed to attach overnight and treated (as previously described) with the indicated drugs. Then, the cells were trypsinized, and the pellet was collected by centrifugation at  $700 \times g$  for 5 min. The cells were fixed with 3 ml of ice-cold 70% ethanol for 48 h at 4 °C prior to the cell cycle analysis. The cells were rinsed twice with phosphate-buffered saline (PBS), treated with RNaseA (1 mg/ml) for 30 min at 37 °C, stained with 5 µl of 1 mg/ml PI in the dark at room temperature for 30 min and analyzed according to the manufacturer's instructions. Following the incubation, the samples, including floating and adherent cells, were collected and prepared for the analysis of apoptosis using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions.

#### 4.2.6 Statistical analysis

The data are presented as the mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant and are indicated with \*. *P*-values less than 0.01 are indicated with #.

### 4.3 Results

#### 4.3.1 PEITC induced *RASSF1A* expression in LNCaP cells by inducing promoter demethylation

Hypermethylation of the 16 CpG sites in the *RASSF1A* promoter region has previously been observed in multiple prostate cancer cell lines, including LNCaP cells[182]. The reversal of *RASSF1A* promoter hypermethylation in LNCaP cells

treated with PEITC was analyzed using MSP and BGS via the semi-nested PCR method. The extent of methylation in MSP was quantified by analyzing the density of the gel bands. The results of the MSP analysis revealed high levels methylated DNA in untreated LNCaP cells, suggesting that the *RASSF1A* promoter was hypermethylated in untreated cells. However, in the cells treated with PEITC (2.5 or 5  $\mu$ M) or 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M), an increase in unmethylated DNA regions was noted compared with the control (Figure 4-2). To further analyze the extent of promoter methylation, we used BGS to analyze the 16 CpG sites in fragment containing the 3Sp1 consensus binding sites and the putative transcription and translation initiation sites present in Exon 1 $\alpha$  (-197 and +438).(Figure 4-3). The Genbank accession number of the cDNA sequence is AF132675 [161]. In the control group, an average 98.1% of the 16 CpG sites were methylated. However, in cells treated with 2.5  $\mu$ M or 5  $\mu$ M PEITC, the percentage of methylated CpG sites decreased to 93.3% and 90.7%, respectively, indicating that CpG demethylation was induced in cells treated with PEITC compared with the control group. The percent of methylated CpG sites in the positive control group treated with 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) was 83.6% (Figure 4-4). The data obtained from MSP and BGS demonstrated that 5  $\mu$ M PEITC significantly reduced CpG methylation in the *RASSF1A* gene. Previous studies have reported that reactivation of *RASSF1A* expression is mediated by promoter demethylation in various types of cancers [36, 183, 184]. We hypothesized that PEITC-induced *RASSF1A* promoter demethylation increases *RASSF1A* expression in LNCaP cells. Quantitative polymerase chain reaction (qPCR) analysis revealed that *RASSF1A* expression was significantly enhanced following treatment with 2.5  $\mu$ M and 5  $\mu$ M PEITC in a dose-dependent manner, suggesting that the reactivation of *RASSF1A* resulted from promoter

demethylation (Figure 4-5). Treatment with 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) significantly enhanced *RASSF1A* expression, consistent with the observation that the *RASSF1A* promoter was demethylated in these cells (Figure 4-5).

#### 4.3.2 Regulation of DNA methyltransferases by PEITC

To further investigate PEITC-induced demethylation of the *RASSF1A* promoter, we evaluated the effect of PEITC on DNMTs, which are enzymes that catalyze the addition of methyl group. Given that we observed a decrease in *RASSF1A* promoter methylation in cells treated with PEITC, we hypothesized that PEITC and 5-Aza might repress the transcription of DNMTs. The results from the qPCR analysis demonstrated that the expression of DNMT1 (Figure 4-6) and DNMT3A mRNA was significantly reduced in cells treated with PEITC (5  $\mu$ M) or 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) (Figure 4-7). In addition, the expression of DNMT 3B mRNA transcripts was significantly reduced in cells treated with 5  $\mu$ M PEITC (Figure 4-8). However, the levels of all the DNMTs probed were not significantly affected in cells treated with 2.5  $\mu$ M PEITC.

To determine whether the reduction in gene expression translated to a reduction in protein expression, we evaluated DNMT protein levels using Western blot analysis. Consistent with the reduction in gene expression, the protein levels of the all DNMTs evaluated were significantly reduced in cells treated with 2.5  $\mu$ M or 5  $\mu$ M PEITC compared with the control cells. In addition, analysis of densitometry demonstrated that DNMT protein levels were reduced in the positive control group of cells treated with 5-Aza as expected (Figure 4-9).

### 4.3.3 Regulation of HDAC protein expression by PEITC

HDACs are enzymes that catalyze the removal of acetyl groups, an epigenetic modification associated with the induction of gene expression. PEITC is an HDAC inhibitor[179] that promotes apoptosis. We hypothesized that PEITC treatment would alter HDAC expression, and we used western blot analysis to investigate this hypothesis. Treatment with 5  $\mu$ M PEITC or 2.5  $\mu$ M 5-Aza and 0.5  $\mu$ M TSA significantly reduced HDAC1, HDAC2, HDAC4 and HDAC6 protein levels (Figure 4-10). PEITC at a concentration of 2.5  $\mu$ M also significantly reduced HDAC 4 and HDAC 6 protein levels (Figure 4-10); however no significant differences in the other HDACs evaluated were noted between the control and the treatment groups (data not shown). These data indicated that the repression of DNMTs and HDAC1, 2, 4 and 6 induced by treatment with PEITC in LNCaP cells led to *RASSF1A* promoter demethylation and subsequent activation of *RASSF1A*.

### 4.3.4 PEITC-induced *RASSF1A* expression induces p21 and caspase-3 expression and promotes apoptosis

Based on the results of the western blot analysis, we further hypothesized that the increase in *RASSF1A* expression induced by promoter demethylation promotes cell cycle arrest by increasing expression of the p21 gene (Figure 4-11) and promotes apoptosis by increasing caspase-3 expression (Figure 4-12). To test this hypothesis, we performed flow cytometry analysis to determine the cell cycle distribution of PI-stained cells. PEITC significantly enhanced the proportion of cells in the sub-G0 phase, indicating that these cells were undergoing early apoptosis. In addition, a significant increase in the proportion of cells in the G2/M phase was noted in the PEITC (5  $\mu$ M) and 5-AZA (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) treatment groups, indicating

that these cells were arrested in the G2/M phase of the cell cycle (Figure 4-13). To confirm whether the growth inhibition induced by PEITC resulted from apoptosis, we performed flow-cytometry analysis of LNCaP cells stained with Annexin V and PI that were treated with PEITC (2.5  $\mu$ M and 5  $\mu$ M) and 5-AZA (2.5  $\mu$ M) for 5 days. The results of this analysis demonstrated that treatment with PEITC (5  $\mu$ M), 5-AZA (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) increased the number of cells in early apoptosis (Annexin V-positive cells) from 4.6% in control cells to 11.1% in cells treated with PEITC (5  $\mu$ M) and 53.7% in cells treated with 5AZA (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) (Figure 4-14). No difference was observed in the cells treated with 2.5  $\mu$ M PEITC compared with the control group (data not shown). With respect to late apoptosis (PI-positive cells), a similar trend was observed in cells treated with 5  $\mu$ M PEITC, which exhibited a 2-fold increase in the proportion of cells in late apoptosis compared with the control group (Figure 4-14). Together, these results demonstrate that the reactivation of *RASSF1A* gene expression by PEITC-induced promoter demethylation promoted cell cycle arrest and apoptosis.

#### 4.4 Discussion

*RASSF1A* is one of the most frequently hypermethylated genes in human cancers, and *RASSF1A* hypermethylation is associated with the loss of *RASSF1A* expression in several types of cancer, including lung, breast, bladder and gastric cancer [164, 165, 167, 185, 186]. In contrast to the *RASSF1C* isoform, *RASSF1A* is expressed at higher levels in normal tissues than in lung and breast cancer tumor tissues, suggesting the *RASSF1A* acts as a tumor suppressor[187]. Previous studies reported that the silencing of *RASSF1A* expression was a frequently observed phenomenon in advanced stages of

prostate cancer[170, 173] and that reversing *RASSF1A* methylation using phytochemicals, such as mahanine and soy phytoestrogens, reactivated *RASSF1A* transcription[188, 189]. Several studies that evaluated the mechanism underlying *RASSF1A* inactivation by analyzing CpG methylation and histone acetylation revealed that reduced histone acetylation or H3K4me2 methylation and increased dimethyl-H3-K9 methylation played a crucial role in *RASSF1A* promoter methylation and the corresponding inactivation of *RASSF1A* in prostate cancer [190]. Other studies that investigated the tumor suppressor function of *RASSF1A* by analyzing microtubule dynamics and apoptosis induction revealed that *RASSF1A* exerted its tumor suppressor effects primarily by restricting cell cycle progression [191].

The use of PEITC as a chemopreventive agent has been well studied in prostate cancer and colon cancer by our group and several others[132, 192, 193]. Recent investigations into the use of PEITC as a demethylating agent and HDAC inhibitor have provided new insights into its role in regulating epigenetic modifications. We hypothesized that PEITC induces demethylation of the *RASSF1A* promoter in LNCaP cells and that reactivating *RASSF1A* by reversing this epigenetic modification induces apoptosis in LNCaP cells *in vitro*. Using MSP and BGS analysis, we demonstrated that PEITC promotes the demethylation of the *RASSF1A* promoter (Figure 2A and 2C). *RASSF1A* promoter demethylation was also observed in cells treated with 5-Aza and TSA. As treatment with 5-Aza and TSA is known to reverse the methylation of several genes, these agents were used as a positive control [194]. The demethylation of the *RASSF1A* promoter was partly due to the inhibition of DNMT1, 3A and 3B gene expression that resulted from treatment with PEITC at both concentrations evaluated (2.5 and 5  $\mu$ M) (Figure 4-6, Figure 4-6 and Figure 4-8). However, the protein levels of DNMT3A and 3B but not DNMT1 were significantly inhibited by 5

$\mu\text{M}$  PEITC (Figure 4-9). The tumor suppressor function of *RASSF1A* is attributed to its ability to modulate cell cycle progression and mitotic spindle dynamics [195, 196]. In this study, we observed that PEITC (5  $\mu\text{M}$ ) significantly up-regulated *RASSF1A* gene expression and p21 expression (Figure 4-5 and Figure 4-11), thus resulting in the induction of G2/M cell cycle arrest (Figure 4-13). The ability of *RASSF1A* to induce p21 expression and promote cell cycle arrest at the G1 and G2/M cell cycle phases was previously demonstrated in tumors from immunodeficient mice [196, 197].

Several studies demonstrated that the *RASSF1* gene exhibits pro-apoptotic activity and that the *RASSF1A* isoform exhibits stronger pro-apoptotic activity than the *RASSF1C* isoform. *RASSF1A* increases caspase-3/7 activation to promote apoptosis in breast, lung and ovarian tumors and simultaneously down-regulates the expression of cyclinD1 [187, 198]. Our study revealed that promoter demethylation activated *RASSF1A* gene expression in LNCaP cells treated with 5  $\mu\text{M}$  PEITC. This effect might account for the increase in apoptosis we observed as caspase-3 protein expression was significantly enhanced in these cells (Figure 4-12). We further analyzed the pro-apoptotic role of PEITC in LNCaP cells using flow cytometry analysis of Annexin V- and PI-stained normal, necrotic and apoptotic cells and observed a 2-fold increase in apoptotic cells following PEITC treatment (5  $\mu\text{M}$ ). This effect was stronger than that observed in the positive control group of cells treated with 5-Aza and TSA, which exhibited a 1-fold increase in the number of apoptotic cells (Figure 4-14). This study is one of the first to demonstrate the role of PEITC in activating *RASSF1A* and promoting apoptosis *in vitro*.

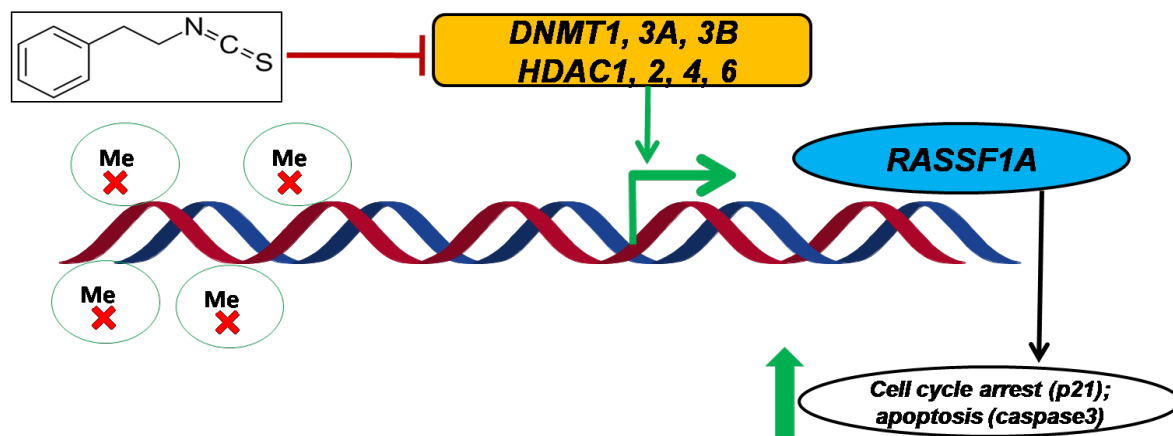
In conclusion, our findings provide new insight into the role of phytochemicals as epigenetic modulators. Here, 5  $\mu\text{M}$  PEITC significantly induced promoter demethylation and consequently increased *RASSF1A* gene expression. In addition,

PEITC-induced activation of *RASSF1A* promoted apoptosis and cell cycle arrest. These results further emphasize the DNMT and HDAC inhibitory function of PEITC and its role in promoting apoptosis.

#### **4.5 Conclusion**

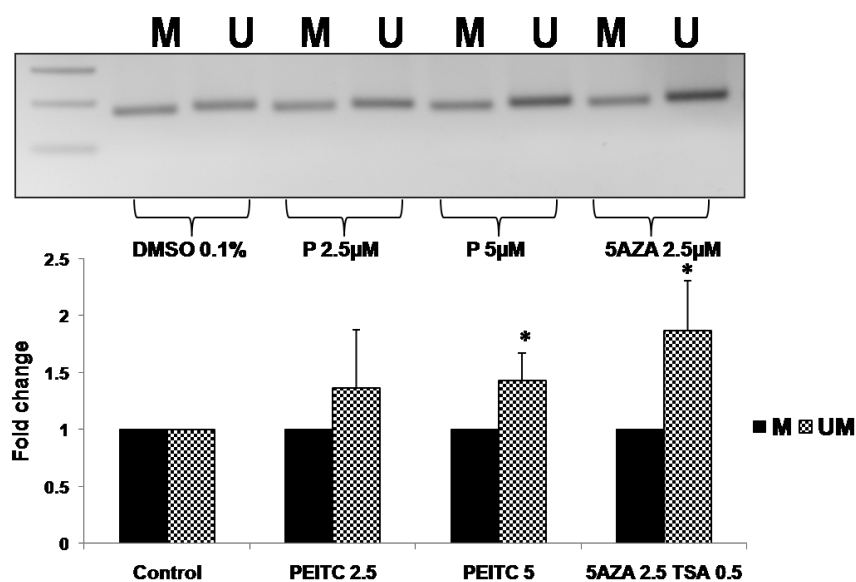
Our study revealed that PEITC is a potent regulator of DNA methylation that reverses transcriptionally silenced *RASSF1A* by inducing promoter demethylation by inhibiting DNMTs and HDACs. *RASSF1A* activation by PEITC in LNCaP cells induced G2/M cell cycle arrest and apoptosis, indicating that *RASSF1A* is a potential molecular marker that can be targeted by phytochemicals to prevent prostate cancer.





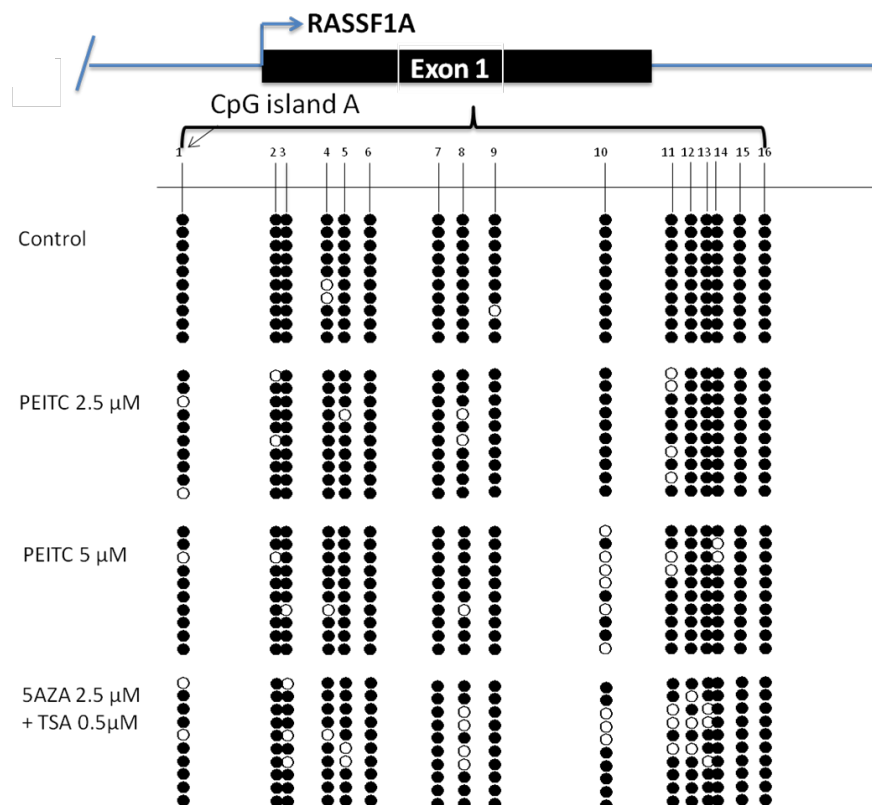
**Figure 4-1: Schematic of epigenetic regulation of RASSF1A by PEITC**

Schematic illustrating the role of PEITC in epigenetically regulating the transcription of *RASSF1A* and its role in cell cycle and apoptosis.



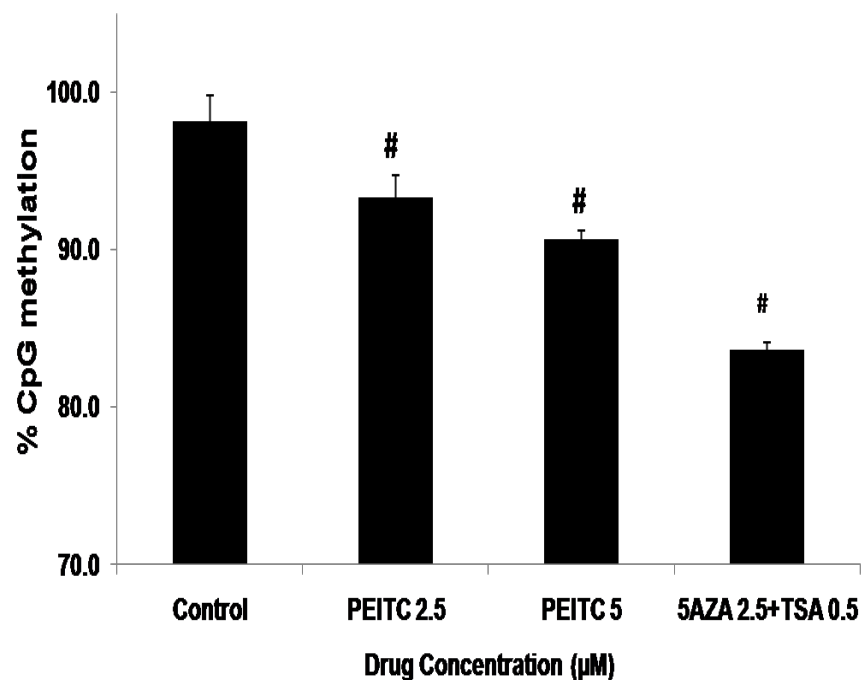
**Figure 4-2: Effect of PEITC on promoter demethylation analyzed by Methylation specific PCR (MSP)**

LNCaP cells ( $3.5 \times 10^4$  cells/ml) were seeded in 10-cm tissue culture plates. LNCaP cells were treated with 0.01% DMSO, PEITC (2.5 or 5  $\mu$ M), or 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) for 5 days. The media containing the drugs was changed on day 3 and day 5. Cells were harvested on day 6 for all the experiments. Representative images from the methylation-specific PCR experiments. Genomic DNA was extracted from cells harvested on day 6, and the bisulfite conversion was subsequently performed. M: methylated, U: unmethylated. The relative intensity of the methylated and unmethylated bands was measured using ImageJ software, and the values are presented in the lower panel. \*  $P < 0.05$  versus the control group. # $P < 0.01$  versus the control group.



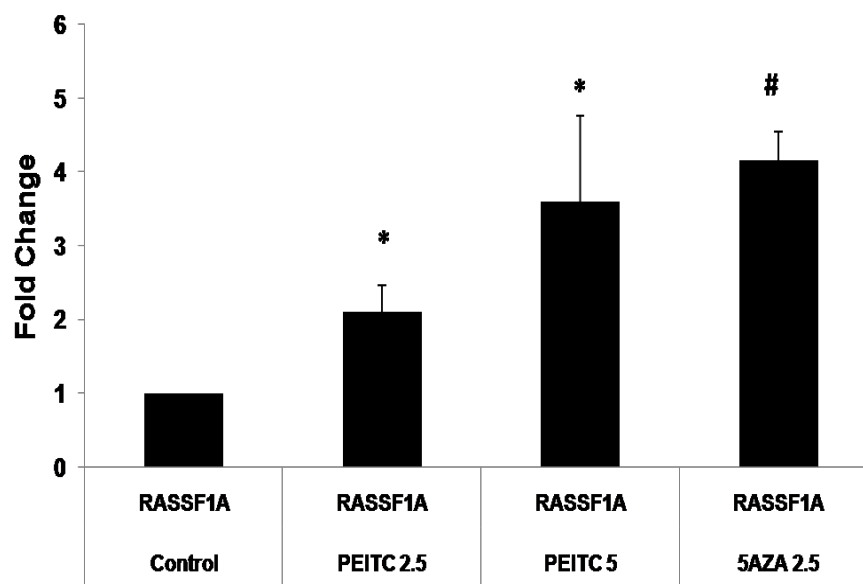
**Figure 4-3: Effect of PEITC on promoter demethylation as analyzed by bisulfite genomic sequencing (BGS) analysis**

LNCaP cells ( $3.5 \times 10^4$  cells/ml) were seeded in 10-cm tissue culture plates. LNCaP cells were treated with 0.01% DMSO, PEITC (2.5 or 5  $\mu$ M), or 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) for 5 days. The media containing the drugs was changed on day 3 and day 5. Cells were harvested on day 6 for all the experiments. The methylation pattern of the 16 CpG sites in the *RASSF1A* promoter was analyzed using bisulfite genomic sequencing. Solid circles indicate methylated CpG sites, and empty circles indicate unmethylated CpG sites. Ten representative clones from each of the 3 independent experiments were selected for analysis.



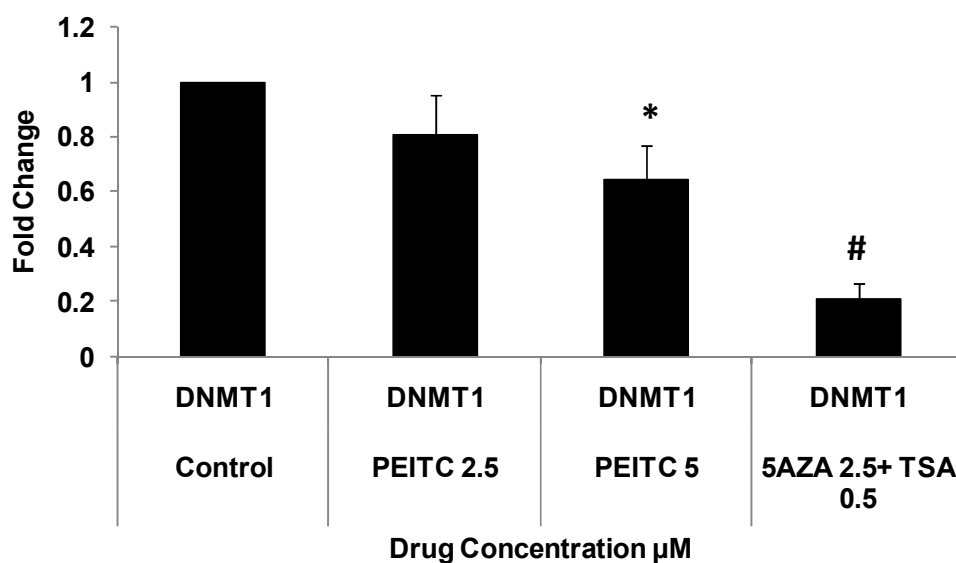
**Figure 4-4: Effect of PEITC on promoter demethylation on CpG demethylation**

LNCaP cells ( $3.5 \times 10^4$  cells/ml) were seeded in 10-cm tissue culture plates. LNCaP cells were treated with 0.01% DMSO, PEITC (2.5 or 5 μM), or 5-Aza (2.5 μM) and TSA (0.5 μM) for 5 days. The media containing the drugs was changed on day 3 and day 5. Cells were harvested on day 6 for all the experiments. The percentage of methylated CpG sites is presented. The methylation percentage was calculated as the mean of the number of methylated CpG sites over the total number of CpG sites from 3 independent experiments. \*  $P < 0.05$  versus the control group. <sup>#</sup> $P < 0.01$  versus the control group.

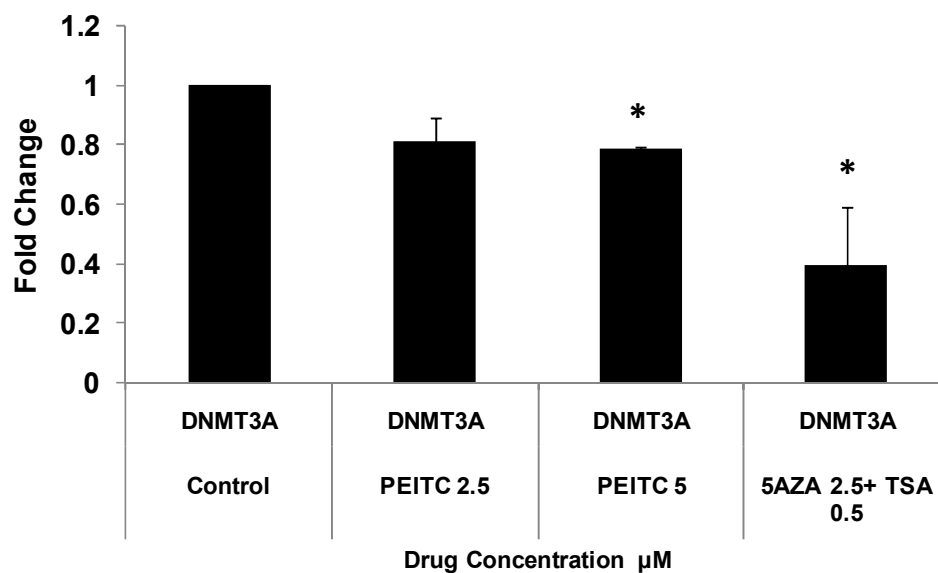


**Figure 4-5: Effect of PEITC on RASSF1A gene expression**

LNCaP cells ( $3.5 \times 10^4$  cells/ml) were seeded in 10-cm tissue culture plates. LNCaP cells were treated with 0.01% DMSO, PEITC (2.5 or 5  $\mu$ M), or 5-Aza (2.5  $\mu$ M) and TSA (0.5  $\mu$ M) for 5 days. The media containing the drugs was changed on day 3 and day 5. Cells were harvested on day 6 for all the experiments. The effect of PEITC on *RASSF1A* mRNA expression. Total mRNA was isolated and analyzed using quantitative real-time PCR. The data are presented as the mean  $\pm$  SEM of 3 independent experiments. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.

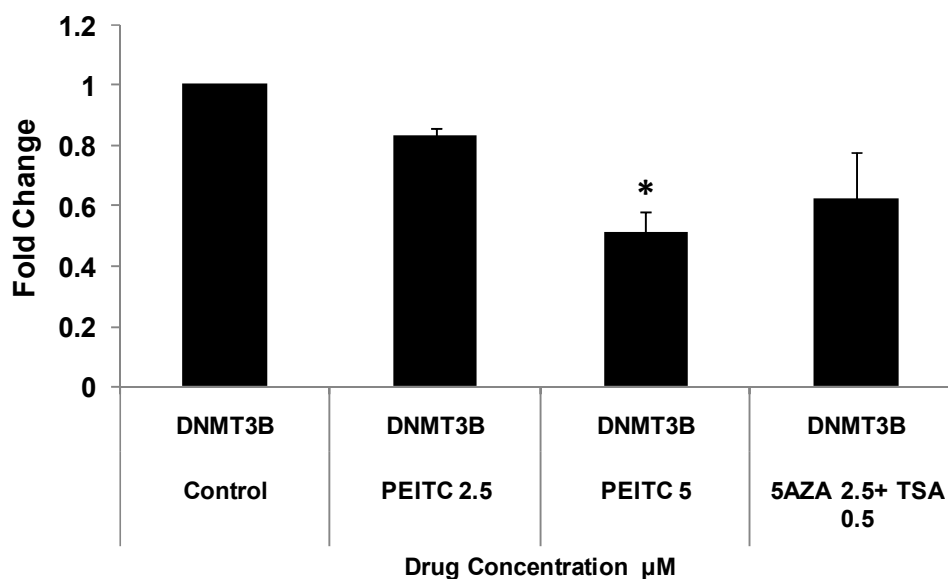


**Figure 4-6: Effect of PEITC on DNMT1 gene expression**



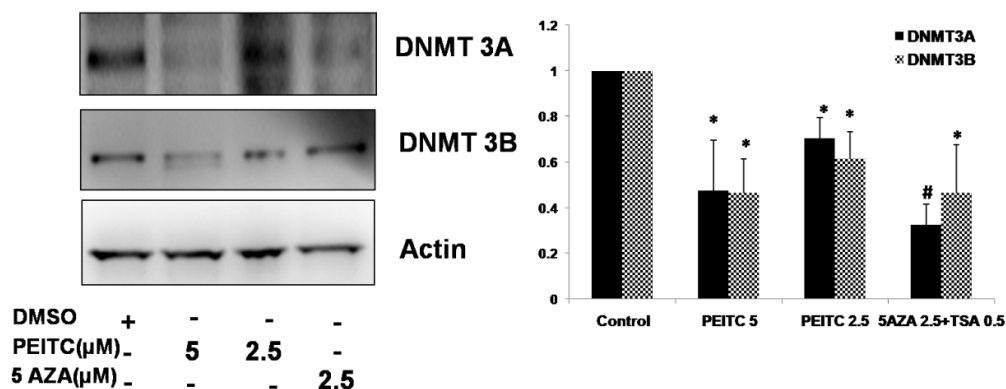
**Figure 4-7: Effect of PEITC on DNMT 3A gene expression**

Effect of PEITC on DNMT 1 and DNMT 3A gene expression in LNCaP cells. Total mRNA was isolated and analyzed using quantitative real-time PCR. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



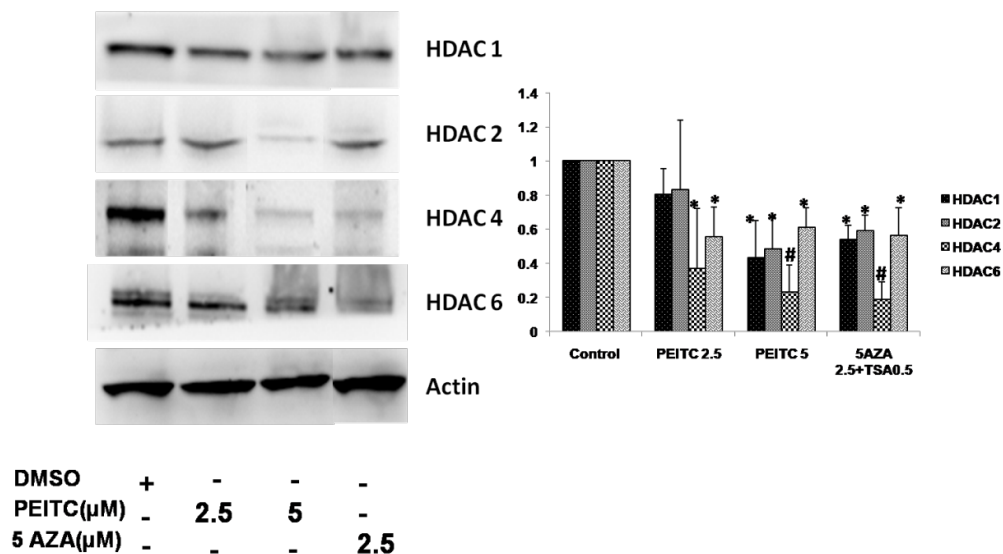
**Figure 4-8: Effect of PEITC on DNMT 3B gene expression**

Effect of PEITC on DNMT 3B gene expression in LNCaP cells. Total mRNA was isolated and analyzed using quantitative real-time PCR. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



**Figure 4-9: Effect of PEITC on protein expression of DNMT 3A and DNMT 3B s Western Blot analysis**

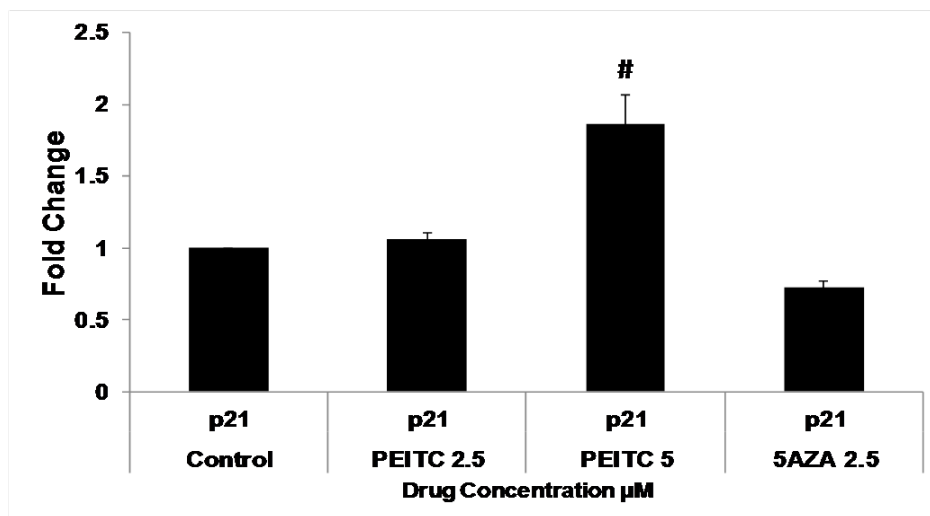
Effect of PEITC on DNMT protein expression in LNCaP cells. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of the protein band by the intensity of the control protein band, and the values were normalized to  $\beta$ -actin levels using ImageJ Representative Western blot images of DNMT3A and 3B protein expression. Data are presented as the mean  $\pm$  SEM protein expression levels of 3 independent experiments. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



**Figure 4-10: Effect of PEITC on HDAC protein expression**

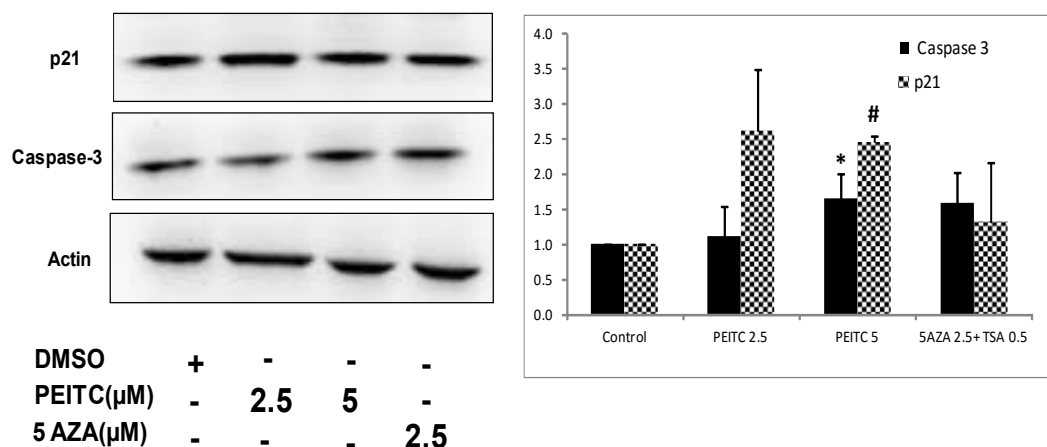
Effect of PEITC on HDAC protein expression in LNCaP cells. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of the protein band by the intensity of the control protein band, and the values were normalized to  $\beta$ -actin levels using ImageJ. A) Representative western blot images of HDAC1, 2, 4 and 6 protein expression. B) Data are presented as the mean  $\pm$  SEM protein expression levels of 3 independent experiments. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.





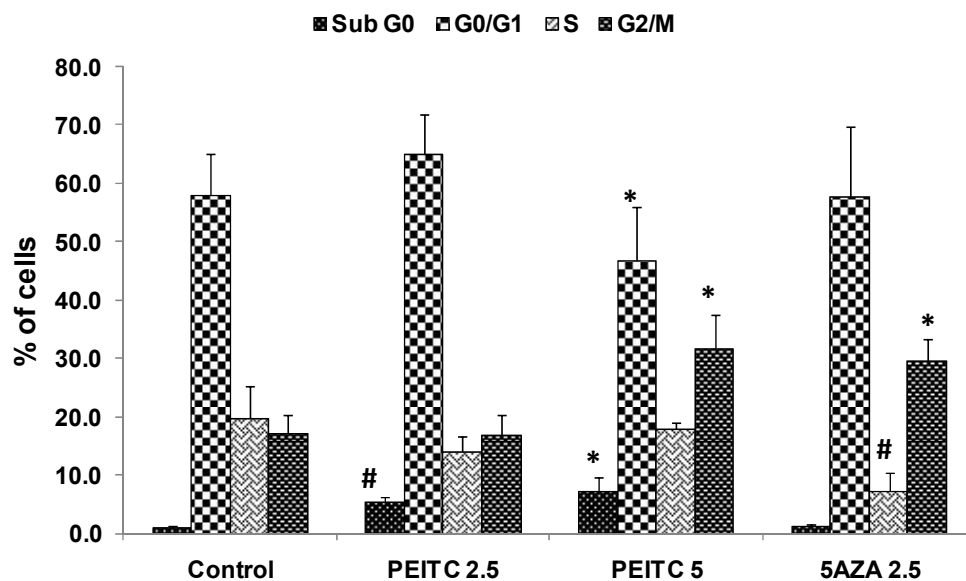
**Figure 4-11: Effect of PEITC on p21 gene expression**

Effect of PEITC on A) p21 gene expression. Total mRNA was isolated and analyzed using quantitative real-time PCR. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



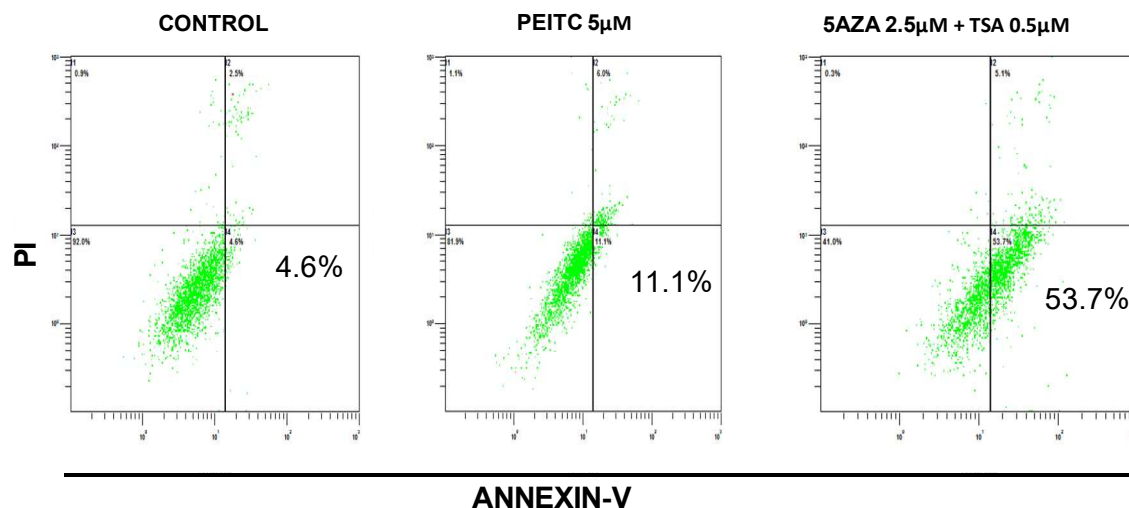
**Figure 4-12: Effect of PEITC on p21 and caspase 3 protein expression**

Representative western blot images of p21 and caspase-3 protein expression. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of each protein band by the intensity of the control protein band, and the values were normalized to  $\beta$ -actin levels using ImageJ. C) Data are presented as the mean  $\pm$  SEM protein expression of 3 independent experiments. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



**Figure 4-13: Effect of PEITC on Cell cycle progression**

Cell cycle progression in LNCaP cells treated with PEITC or 5-Aza and stained with propidium iodide (PI) was analyzed using flow cytometry with a Beckman Coulter Gallios Flow Cytometer. The legend presents the distribution of cells in the sub G<sub>0</sub>, G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle. \*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.



**Figure 4-14: Effect of PEITC on apoptosis in LNCaP cells**

Percentage of normal, necrotic and apoptotic cells as determined by flow cytometry of cells stained with PI/Annexin V-FITC. In all panels, the bottom left quadrant represents normal cells, the top right quadrant represents necrotic cells and the bottom right quadrant represents apoptotic cells. The control group is presented in the panel on the left, cells treated with 5  $\mu$ M PEITC are presented in the center panel, and cells treated with 5-Aza and TSA are presented in the panel on the right.

\*  $P < 0.05$  versus the control group. #  $P < 0.01$  versus the control group.

**Table 4-1: Primer sequences for qPCR, MSP and BGS**

<b>qPCR primers</b>		
	<b>Forward</b>	<b>Reverse</b>
DNMT1	5'-CCGAGTTGGTGATGGTGTGTAC-3'	5'-AGGTTGATGTCTGCGTGGTAGC-3'
DNMT3A	5'-TATTGATGAGCGCACAAAGAGAGC-3'	5'-GGGTGTTCCAGGGTAACATTGAG-3'
DNMT3B	5'-GACTTGGTGATTGGCGGAA-3'	5'-GGCCCTGTGAGCAGCAGA-3'
RASSF1A	5'-CCTCTGTGGCGACTTCATCTG-3'	5'-CAACAGTCCAGGCAGACGAG-3'
<b>MSP primers</b>		
RASSF1A (M)	5'-GTGTTAACGCGTTGCGTATC-3'	5'-AACCCCGCGAACTAAAAACGA-3'
RASSF1A (UM)	5'-TTTGGTTGGAGTGTGTTAATGTG-3'	5'-CAAACCCACAAACTAAAAACAA-3'
<b>BGS primers</b>		
RASSF1A MU730	5'-ACCCTCTTCTCTAACACAATAAACTAACC-3'	
RASSF1A MU561	5'-CCCCACAATCCCTACACCCAAAT-3'	
RASSF1A MU379	5'-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT-3'	



## 5 Pharmacokinetics, Pharmacodynamics and PKPD modeling of curcumin in regulating anti-inflammatory and epigenetic gene expression<sup>10, 11, 12</sup>

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### 5.1 Introduction

Chronic inflammation is the pathophysiological condition which may overlap with the onset of cancer and also drive the development of cancers as seen in several cancers like colorectal, esophageal and hepatocellular carcinoma [199]. Nitric oxide synthase (NOS) is an enzyme which is responsible for production of nitric oxide. It is categorized into subfamilies like neuronal tissues (*nNOS*) and vascular endothelial cells (*eNOS*), and inducible NOS (*iNOS*) based on location of expression in the body. *iNOS* is expressed in a variety of cell types under both normal and pathological conditions, including macrophages, microglial cells, keratinocytes, hepatocytes, astrocytes and vascular endothelial and epithelial cells. Pro-inflammatory stimuli and other infectious conditions induce *iNOS* protein to produce NO in a micro molar range [200]. A recent study also showed that *iNOS* plays a crucial role in angiogenesis, inflammation, and collagen deposition in *iNOS* gene (*iNOS*<sup>-/-</sup>) and wild-type (WT) mice where the hemoglobin content, VEGF

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<sup>10</sup> The work done in this chapter is being submitted to an international journal for peer- review.

<sup>11</sup> Keywords: Curcumin, inflammation, Pharmacokinetics, Pharmacodynamics, Indirect response modeling

<sup>12</sup> Abbreviations: PK- Pharmacokinetics; PD- Pharmacodynamics; NO- Nitric oxide; iNOS- inducible nitric oxide synthase; DNMT- DNA methyl transferase; HDAC- Histone deacetylase; TNF- $\alpha$ - Tumor necrosis factor  $\alpha$ ; IL-6- Interleukin 6; IDR- Indirect response model; AUC- Area under the curve; MRT- Mean residence time.

levels and number of vessels were decreased in the implants of *iNOS*<sup>-/-</sup> mice compared to WT mice [201]. The role of iNOS expression in tumors and adjacent inflammatory microenvironment has been documented. Strong iNOS expression was seen in prostate tumor cells whereas the normal and hyperplastic prostate tissue showed weak or no iNOS expression [202]. A study examined the effects of methylprednisolone (MPL) which is well known anti-inflammatory agent and the dynamics of lung *iNOS* mRNA expression and plasma NO after LPS-induced acute inflammation in rats along with the plasma disposition kinetics. Their results showed a very good concentration dependent decrease in the *iNOS* expression upon MPL administration which was modeled using Jusko's indirect modeling approach [203].

Lipopolysaccharide (LPS), a gram negative bacterial toxin is a ligand for Toll like receptor 4 (TLR4) and LPS induced systemic inflammation remains a classic animal model that has been studied for several years [204, 205]. Research in this area has thus focused on the role of macrophages and mediators, e.g., cytokines, released from those cells during the host response. Studies *in vivo* and *in vitro* have demonstrated that LPS exerts adjuvant effects on macrophages, resulting in an inflammatory cascade defined by early production of pro-inflammatory cytokines [206]. *iNOS* is known to be induced by LPS and several other pro-inflammatory cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  [207]. Studies have shown the effects of LPS on inducing pro-inflammatory markers using an indirect response approach and receptor mediated modeling based approach [208, 209].

Curcumin, a component of the rhizomes of *Curcuma longa* has long been used in traditional medicine in India for its anti-inflammatory and anti-oxidant properties [2]. Recent studies on Curcumin from our lab as well as others have shown that it is a potent regulator of epigenetic modifying genes that play a crucial role in prevention of

several cancers [26, 27, 31]. The low oral bioavailability of curcumin is one of the challenges in the drug reaching adequate circulating levels to cause the Pharmacodynamic response [210]. There have been several studies which have looked at improving the bioavailability of curcumin by adding compounds like piperine and bioperine to the formulation [211-213]. The role of Curcumin as an anti-inflammatory agent and the inhibition of pro-inflammatory markers like *COX-2*, *iNOS*, *TNF- $\alpha$*  and *IL-6* has been well studied [8, 214, 215]. A study showed the effect of Curcumin, diacetylCurcumin and diglutarylCurcumin to have potent anti-inflammatory properties when studied using a carrageenan induced paw edema model *in vivo* compared to aspirin [216]. Curcumin also showed to have decreased the *iNOS* tyrosine phosphorylation in RAW 264.7 murine macrophages via the inhibition of ERK 1/2 activation resulting in a lowered *iNOS* enzyme activity, indicating that Curcumin promotes the ubiquitination and degradation of *iNOS* after LPS stimulation [217]. The role of Curcumin as an epigenetic modulator is being established recently, with studies showing that Curcumin can inhibit several DNMTs and HDACs and thereby reverse the silencing of some key genes like Neurog1, DLEC1, Nrf2 [26, 27, 218]. A recent study showed that anti-inflammatory role of Curcumin in Alzheimer's disease is by restoration of epigenetically silenced gene called NEP which inhibits Akt signaling [38]. The present study aims to quantitatively describe the effect of Curcumin on LPS induced inflammation using a PKPD modeling approach.

## 5.2 Materials and Methods

### 5.2.1 Chemicals and reagents

Curcumin (98%), Isorhamnetin (>95%, used as internal standard), Lipopolysaccharide (LPS), ethanol (99%), diisopropyl ether (98%), ammonium



acetate (99%), and formic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) and pure water used for the LC mobile phase were purchased from Honeywell Burdick & Jackson (Muskegon, MI). De-ionized water was obtained from a MiliQ system (Milipore, Bedford, MA). Heparin sodium injection (1000U/mL) and sodium chloride injection (0.9%) were purchased from Baxter Healthcare Corporation (Deerfield, IL) and Hospira Inc. (Lake Forest, IL), respectively. Ficoll-Paque<sup>TM</sup> PREMIUM 1.084 was purchased from GE Healthcare (Piscataway, NJ).

### **5.2.2 Animals**

Female Sprague-Dawley rats weighing 250-300 g with cannulated jugular vein were purchased from Hilltop Laboratories (Scottsdale, PA). The rats were housed in the Laboratory Animal Service facility at Rutgers University with free access to food and water. The rats were acclimatized for three days, during which they were fed the AIN-76A diet free of antioxidant (Research Diets Inc., New Brunswick, NJ). The experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Rutgers University.

### **5.2.3 Dosing of animals, collection of plasma and isolation of lymphocytes**

On the day of the study, the cannula was taken out and connected to polyethylene tubes using adapters (Instech Laboratories, PA) for dosing and blood collection. Heparinized saline (50U/mL) was used to flush the cannulae and tubes. Curcumin and LPS were suspended in a vehicle consisting of Cremophor, Tween 80, ethanol and water at the ratio of 1:1:1:7. Curcumin 40 mg/kg (n=6), Lipopolysaccharide 50µg/kg (n=6) or vehicle control (n=4) were administered intravenously through the jugular

vein. Blood samples (0.3 mL) were withdrawn at 0, 10, 20, 30 min, 1, 2, 3, 4, 6, 8, 12 and 24 hours after LPS alone or with Curcumin and LPS administration, followed by injection of an equal volume of saline to rats. All blood samples were immediately centrifuged at 2500 rpm at 4°C to obtain plasma samples, which were stored at -80°C until analysis. Due to the limited amount of blood withdrawn at each time point from rats, there were small amounts of mononuclear cells in the blood. Hence, the blood cells obtained at the same time point were pooled and re-suspended in 4X volume of Hank's balanced salt solution (HBSS, Invitrogen, Grand Island, NY). Lymphocytes were isolated using Ficoll-Paque<sup>TM</sup> PREMIUM 1.084, following the manufacturer's instruction, and the cell pellets were stored at -80°C until further analysis.

#### **5.2.4 Sample Preparation and Liquid Chromatography–Mass Spectrometry**

##### **Analysis of Curcumin in plasma**

Aliquots of original or diluted rat plasma samples (100 µL) spiked with isorhamnetin as an internal standard were extracted twice with 200 µL of extraction solvent (diisopropyl ether: ethanol=90:10, v/v), and the layers were separated by centrifugation at 10,000 rpm for 2 min at room temperature. The upper organic layer was transferred to a clean microcentrifuge tube. The combined organic extracts were dried using a vacuum pump, and the dried sample was reconstituted in 200 µL of ACN and water (50:50, v/v). The reconstituted samples were centrifuged at 17,500 rpm for 5 min at 18-20°C, and the supernatant was collected for analysis.

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed using a Finnigan LTQ (Thermo Fisher Scientific Inc., San Jose, CA) consisting of a Surveyor quaternary pump with a build-in degasser, a Surveyor auto-sampler, and a Surveyor PDA detector. Chromatographic separation was achieved using a Zorbax

Eclipse XDB C18 column (3.5  $\mu\text{m}$ , 4.6 $\times$ 50 mm, Agilent Technologies, Santa Clara, CA). The aqueous mobile phase A was composed of 0.01% formic acid in water, and the organic mobile phase B was acetonitrile (ACN). The column oven and auto-sampler temperatures were 40°C and 4°C, respectively. The mobile phase was 50:50 (A: B) for the first 1 min, followed by 15:85 (A: B) for 5 min. The mobile phase was maintained at 5:95 (A: B) for an additional 2 min and then returned to 50:50 (A: B) for 3 min. The flow rate was 200  $\mu\text{L}/\text{min}$ . The volume of the injected sample was 100  $\mu\text{L}$ . The total run time for the present analysis was 11 min. MS/MS system was operated in positive ESI. The analytes were detected in selected ion reaction monitoring (SRM) mode. The ion transition was  $m/z$  369-175 for curcumin and  $m/z$  317-285 for isorhamnetin with normalized collision energy of 30 and 45 respectively. The lower and upper limits of quantification were 2 ng/mL and 20  $\mu\text{g}/\text{mL}$ , respectively. The plasma calibration curve was linear, with  $r^2 > 0.99$ .

#### **5.2.5 Measurement of mRNA expression in mononuclear cells**

Total RNA was extracted from mononuclear cells using Picopure RNA isolation kits from Life technologies and was reversed transcribed to cDNA. The quantitative PCR reactions were performed using Power SYBR Green mix on an ABI Prism 7900HT system (Applied Biosystems, Foster City, CA). Relative gene expression was calculated using a  $\Delta\Delta\text{Ct}$  method.

#### **5.2.6 Pharmacokinetic and Pharmacodynamic Modeling**

The secondary PK parameter like the area under the concentration-time curve (AUC) was determined using the linear trapezoidal rule. The terminal half-life ( $t_{1/2}$ ) was estimated from the equation  $t_{1/2} = \ln 2 / K_{(z)}$ , where  $K_{(z)}$  was determined from the

terminal slope of the concentration-time curve. Clearance (CL) was calculated by dividing the dose by AUC. The mean residence time (MRT) was obtained by dividing AUMC by AUC, where AUMC is the area under the first moment curve. The volume of distribution (Vss) was calculated by  $V_{ss} = CL \times AUMC / AUC$ .

The plasma concentration data were analyzed by using the compartmental method in ADAPT 5 (Biomedical Simulations Resource, University of Southern California, Los Angeles, CA)[219]. A two-compartmental model was used to describe the PK profile of CURCUMIN. The differential equations are shown in Eq.1 and Eq.2.

$$\frac{dX_1}{dt} = -\frac{CL + CL_d}{V_c} \times X(1) + \frac{CL_d}{V_p} \times X(2) + R(1); \quad X(1)(t=0) = Dose \dots \dots Eq. 1$$

$$\frac{dX_2}{dt} = \frac{CL_d}{V_c} \times X(1) - \frac{CL_d}{V_p} \times X(2); \quad X(2)(t=0) = 0 \dots \dots \dots Eq. 2$$

$CL_d$  Represents the inter-compartmental clearance between the central (C) and peripheral (P) compartments, respectively;  $CL$  is the elimination from the central compartment;  $X_1$  and  $X_2$  represent the amount of CURCUMIN in the central and peripheral compartments, respectively.

Relative gene expression of *iNOS* was measured as PD response (R) and modeled using an indirect response model with and without transit compartments (Figure 2 and Figure 5) differential equations shown Eq. 3 and indirect response model with transit compartments with differential equations shown Eq. 4 to 8.

$$\frac{diNOS_{mRNA}}{dt} = K_{in} \times (1 + K_{lps}) \times \left(1 - \frac{I_{max} \times Cur_c}{IC_{50} + Cur_c}\right) - K_{out} \times iNOS_{mRNA};$$

$$iNOS_{mRNA}(t = 0) = 1 \dots \dots Eq. 3$$

$K_{in}$  represents the zero-order rate constant for production of the iNOS mRNA,  $K_{out}$  denotes the first-order rate constant for degradation of the iNOS mRNA, and  $R$  is assumed to be stationary and set as 1 arbitrarily in the initial condition. The stimulation of iNOSmRNA is triggered by  $(1 + K_{lps})$  and the duration (T) of which is fixed for 3H as observed from the LPS stimulation in all the animal groups. The inhibition of iNOS mRNA by Curcumin is represented by

$$\left(1 - \frac{I_{max} \times Cur_c}{IC_{50} + Cur_c}\right). \text{ The process is described in Eq.3.}$$

$$\frac{dLPS}{dt} = -K_1 \times LPS; \quad LPS(t = 0) = 1 \dots \dots \dots Eq. 4$$

$$\frac{dM_1}{dt} = \frac{1}{\tau} (LPS - M_1); \quad M_1(t = 0) = 0 \dots \dots \dots Eq. 5$$

$$\frac{dM_2}{dt} = \frac{1}{\tau} (M_1 - M_2); \quad M_2(t = 0) = 0 \dots \dots \dots Eq. 6$$

$$\frac{dM_3}{dt} = \frac{1}{\tau} (M_2 - M_3); \quad M_3(t = 0) = 0 \dots \dots \dots Eq. 7$$

$$\frac{diNOS_{mRNA}}{dt} = K_2 (1 + K_{lps}) \times \left(1 - \frac{I_{max} \times Cur_c}{IC_{50} + Cur_c}\right) - K_{out} \times iNOS_{mRNA};$$

$$iNOS_{mRNA}(t = 0) = 1 \dots \dots Eq. 8$$

The indirect response model with transit compartments is described by equations 4-8. Where Eq. 3 represents LPS kinetics and  $K_1$  represents the rate of change of LPS,  $\tau$  represents the mean transit time between compartments,  $M_n$  represents the response in the transit compartments and  $K_2$  represents the zero order rate of formation of iNOS mRNA.  $I_{\max}$  is the maximum ability of CURCUMIN to inhibit  $K_{in}$ , and  $IC_{50}$  represents the CURCUMIN concentration producing 50% of the maximum inhibition of iNOS. The value of  $I_{\max}$  was fixed to 1. PK parameters in Eq.1 and Eq.2 were estimated using the generalized least squares method in ADAPT5; then PK and PD data was fitted simultaneously to estimate PD parameters, when the PK parameters were fixed using the values from the PK model only.

### 5.3 Results

#### 5.3.1 Gene expression by qRT-PCR

The gene expression data from the LPS dosed group showed an increase over time reaching maximum expression around 3 or 4 h for the pro-inflammatory genes like iNOS, TNF- $\alpha$  and IL-6 (Figure 5-1); epigenetic modulatory genes like DNMT3A, HDAC2, HDAC3 and HDAC 4 (Figure 5-1) also showed increased expression reaching maximum expression around 4h. However, upon dosing with Curcumin and LPS, the expression of the pro-inflammatory genes and epigenetic modulatory genes was inhibited over the time (Figure 5-1).

### 5.3.2 Method validation of pharmacokinetic analysis

**Specificity and Selectivity:** Retention times for Isorhamnetin and Curcumin were approximately 3.4 min and 5.5 min respectively. Figure 5-8 shows representative chromatogram of extracted plasma spiked with Isorhamnetin and Curcumin. Neither endogenous matrix plasma peaks nor any in vivo metabolites of Curcumin were found to interfere with any of the compounds evaluated.

**Sensitivity:** 100  $\mu$ L of plasma was used for sample extraction using liquid-liquid extraction method. The extracted samples were reconstituted 200  $\mu$ L of ACN and water (50:50, v/v), and 100  $\mu$ L was used as the LC injection volume. The upper limit of quantification was 20 $\mu$ g/mL and the lower limit of quantification was 2ng/mL for Curcumin.

**Linearity** Plasma calibration curve for CURCUMIN was linear over the concentration range of 2ng/mL- 20 $\mu$ g/mL. The calibration curve was  $y=0.001x+0.184$  and correlation coefficients were 0.997.

### 5.3.3 Pharmacokinetics of Curcumin

Curcumin plasma concentration-time profiles are displayed in Figure 5-3. The two-compartment PK estimated parameters are listed in Table 5-1. The pharmacokinetics parameters appear that the two compartmental PK model fitted well for the plasma concentration versus time for 40mg/kg dose administered. The software generated two-compartment PK parameters of Curcumin were then used to fit the PD data. Elimination clearance (CL) was 119.5 L/h/Kg; distribution clearance was 54.7 L/h/Kg; Volume of central and peripheral compartments was 32.5 and 89 L/kg. Secondary PK parameters like AUC (0-6) was 406.93 h\*ng/mL and AUC (0- $\infty$ ) was 444.08 h\*ng/mL; terminal half-life 0.11 h.

#### 5.3.4 Pharmacokinetic-Pharmacodynamic correlation

The *iNOS* and *TNF- $\alpha$*  mRNA expression was measured by qRT-PCR (Figure 5-1). Indirect response model with and without transit compartments was used to describe the quantitative aspects of the gene expression. The Pharmacokinetic-Pharmacodynamic analysis results were fitted using ADAPT5. The fold changes of the mRNA expression were calculated for *iNOS* and *TNF- $\alpha$*  mRNA upon dosing with LPS alone or with Curcumin and LPS. The profiles show that *iNOS* (Figure 5-4 and Figure 5-6) and *TNF- $\alpha$*  (Figure 5-7) was induced in the lymphocytes by LPS and then suppressed with the Curcumin administration described by the concentration of Curcumin. The estimated Pharmacodynamic parameters for *iNOS* and *TNF- $\alpha$*  are shown in Table 5-2 and Table 5-3.

#### 5.4 Discussion

Curcumin is a well-known anti-inflammatory agent which is being studied more for preventive properties due to the fact that inflammation serves as a precursor for several diseases like cancers and neurological disorders [220, 221]. The role of curcumin as an epigenetic modulator and its role in prevention of cancers and neurological disorders were shown by its ability to reactivate key genes like DLEC1, NEP responsible for preventing cancers and neurological disorders [38, 218, 222]. In this study, the concentration of Curcumin was measured in rat plasma and the gene expression of pro-inflammatory and epigenetic regulatory markers was measured in the lymphocytes isolated from the blood samples. The concentration and the gene expression data were then fit using a 2 compartment PK model; indirect response model and indirect response model with transit compartments.



Curcumin disposition showed a bi-exponential decline well describe a by a two compartment model with linear clearance (Figure 5-3). The results from the PK showed high clearance and volume of distribution which suggest extensive tissue distribution of Curcumin. The time course of iNOS and TNF- $\alpha$ mRNA showed an increase upon LPS dosing after an initial delay of 1 h; followed by stimulation reaching maximum at 3h. Curcumin dosing along with LPS reduced the expression of iNOS mRNA with the maximum expression at 3h which was considerably lower than LPS alone administration. Curcumin dosing along with LPS also reduced TNF- $\alpha$ mRNA production. The induction of iNOS mRNA expression was modeled using a simple indirect response model as well as indirect response model with transit compartments. The transit compartments were used to serve as downstream genes that were delaying the production of mRNA expression (Figure 5-5 and Figure 5-6). The indirect response model alone was able to describe the gene expression data of iNOS (Figure 5-2 and Figure 5-4). However, the duration of the effects of LPS were fixed in this model based on the observed gene expression data in all the animals on LPS dosing.

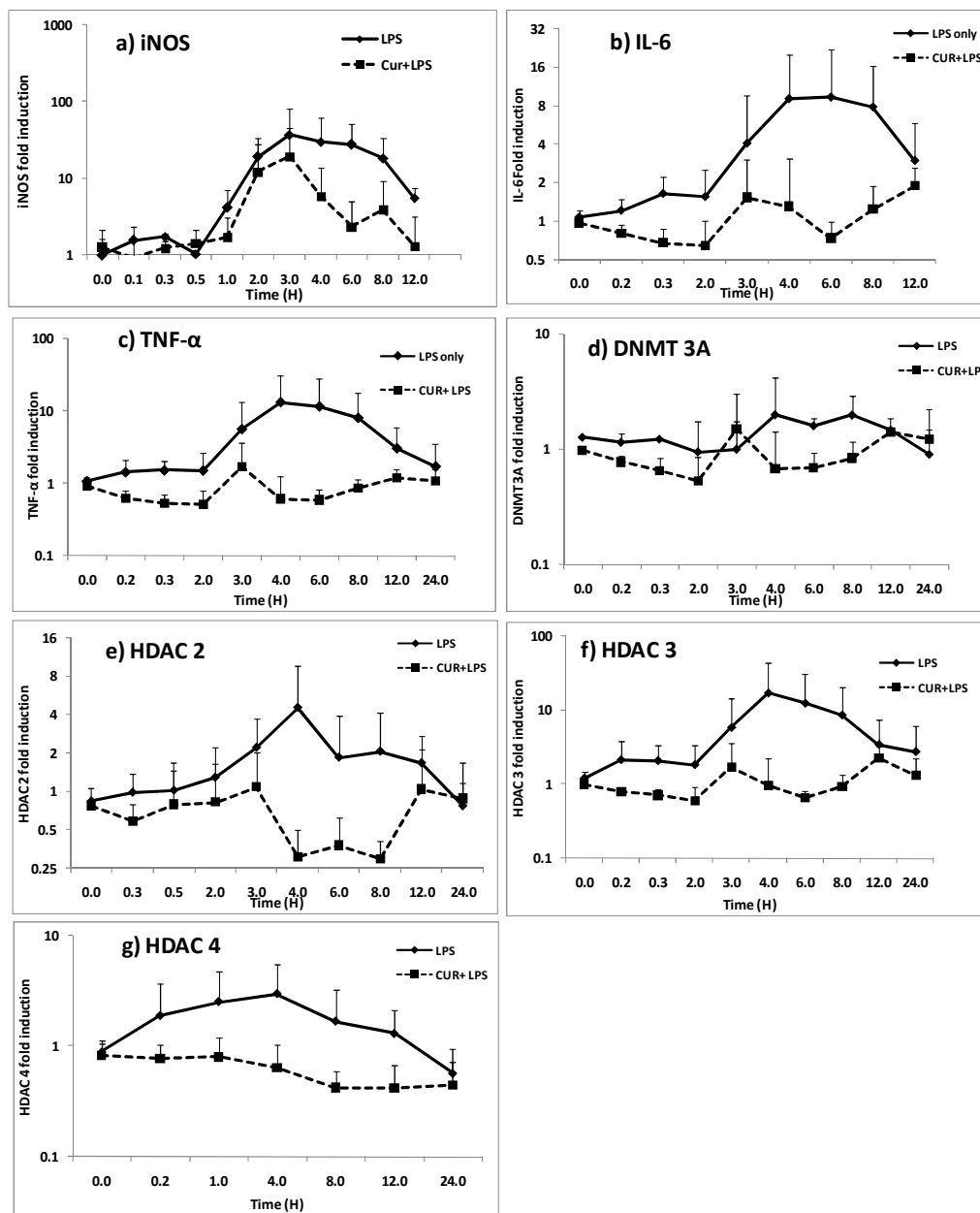
In order to further understand the delay, an IDR model with transit compartment model was used so that it could describe the delay in the iNOS mRNA expression which could be attributable to the signal transduction process that is involved in the activation of inflammatory molecules. The stimulation of iNOS and TNF- $\alpha$  by LPS was estimated in LPS alone dosing group as ( $K_{lps}$ ) and the value was used to estimate the other model parameters in the Curcumin and LPS dosed group. This approach can be explained by the fact that the presence of Curcumin can alter the mRNA dynamics and hence the only parameter that could be constant between both the groups is the

stimulation of gene expression by LPS ( $K_{LPS}$ ). This model under predicted the iNOS mRNA expression in both LPS alone and LPS along with Curcumin treated groups. The maximum effects of LPS and Curcumin on iNOS expression were not captured, likely due to the simplified model used in the transit compartments. The delay in the response was successfully captured by both modeling approaches; the transit compartments with the indirect response model could capture the delay which largely arises from the signal transduction process that drives the mRNA synthesis. Similar results were seen when TNF- $\alpha$  gene expression was modelled using the indirect response model with the transit compartments. The model described the effect of LPS on TNF- $\alpha$  induction. However, the effect of Curcumin and LPS was not very described in the later time points. This lack of flexibility in effectively modelling both the genes was improved by estimation of the initial conditions of the transit compartments which were close to zero (data not shown). This approach however was not considered as it is not ideal to estimate the initial conditions which have to be normalized across the transduction process to a fixed value.

Our current model could be used as an initial model that could describe the effects of LPS and Curcumin in the transcriptional regulation of iNOS and TNF- $\alpha$ . This model was able to describe the observed data but did not provide flexibility required to capture the dynamics of iNOS and TNF- $\alpha$  in the curcumin and LPS treatment. This kind of approach did not completely capture all the observed data points. However, this model could be further enhanced by measuring the downstream gene expression of TLR4 signaling pathway at multiple time points and thereby building a more robust model that could explain the intermediary steps involved in the

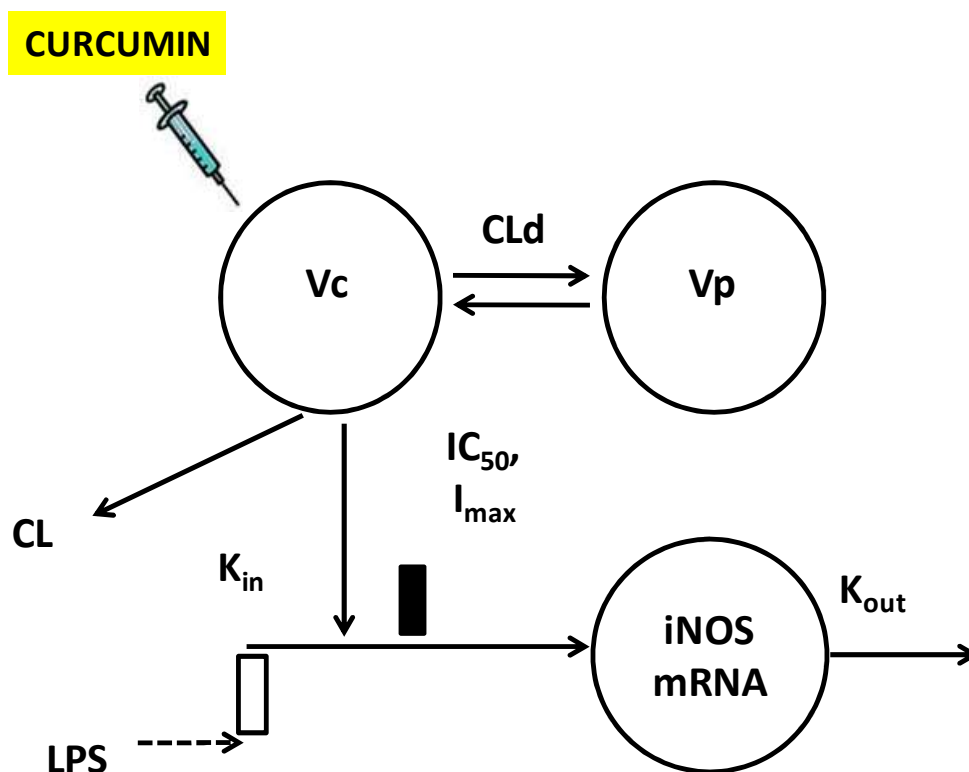
transcription of down stream genes in the LPS and TLR4 signaling cascade as previously described by Foteinou et. al. [209].

Collectively, we demonstrate that Curcumin at 40mg/kg inhibits the expression of pro-inflammatory genes, including iNOS, TNF- $\alpha$  and IL-6 along with epigenetic modulatory genes like DNMT 3A, HDAC 2, 3 and 4 in rat lymphocytes compared to LPS alone treatment. This dose of 40 mg/kg corresponds to a dose of approximately 440mg for a 70 kg human calculated based on US FDA published “Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy” in 2005. This corresponding dose of 440mg is well below the maximum tolerated dose of 12000 mg in humans as evidenced from the clinical trials and the dose escalation studies [3]. Furthermore, the description of iNOS and TNF- $\alpha$  by PD model suggests that these two may be good surrogate PD markers for future anti-inflammatory trials using curcumin.



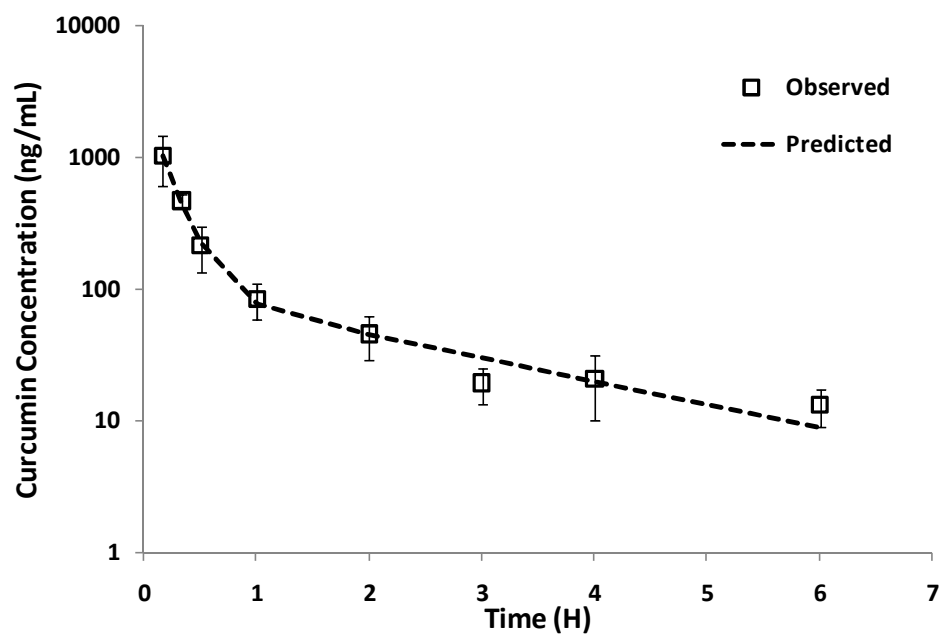
**Figure 5-1: Gene expression changes upon LPS and Curcumin dosing in rats**

Gene expression changes of pro-inflammatory markers a) iNOS b) IL-6 c) TNF- $\alpha$  d) DNMT3A e) HDAC2 f) HDAC3 g) HDAC4 fold induction in rat lymphocytes upon dosing with LPS alone (50  $\mu$ g/kg) or with Curcumin (40mg/kg) and LPS (50  $\mu$ g/kg).



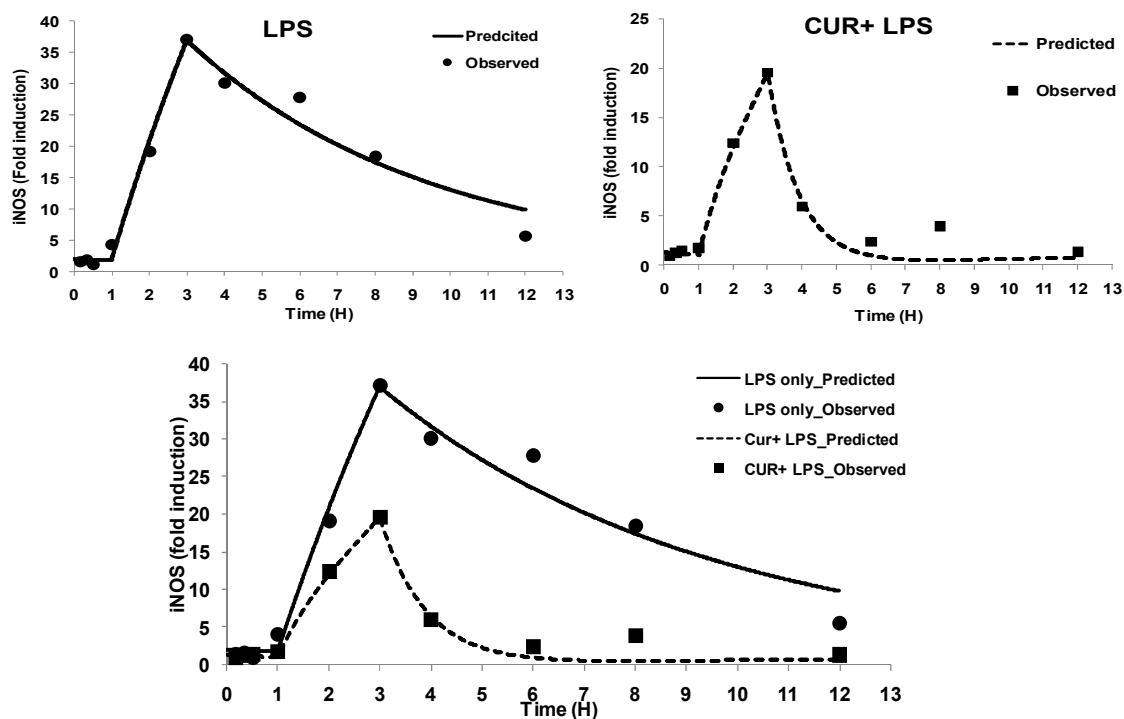
**Figure 5-2: Schematic of integrated PKPD model for simple indirect response model**

Schematic showing integrated PKPD model for LPS induced iNOS suppression by Curcumin. The model is defined by equations 1-3 in the text.  $K_{in}$  represent rate of formation of iNOS mRNA,  $K_{out}$  represent rate of elimination of iNOS mRNA



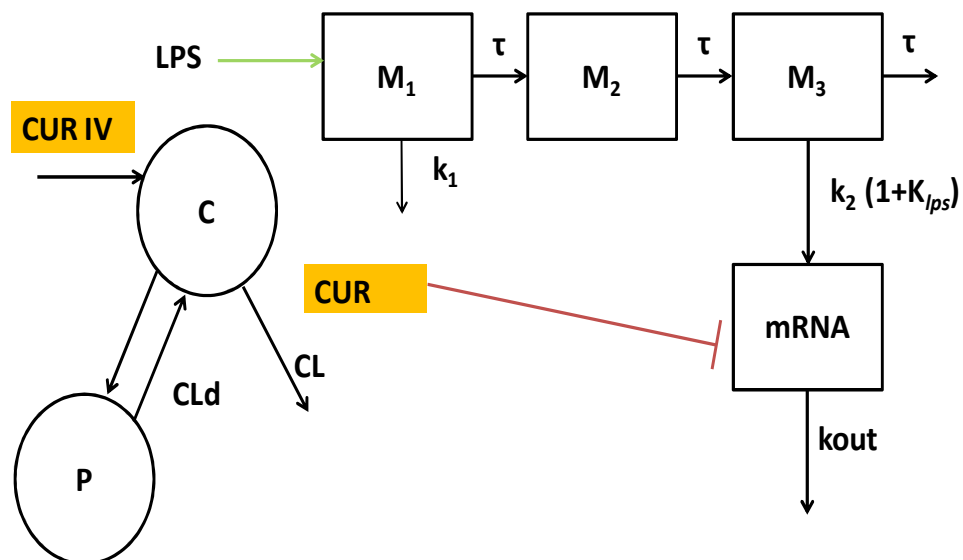
**Figure 5-3: Pharmacokinetics of Curcumin**

Curcumin Pharmacokinetics described by a 2 compartment model; dashed line Represents model fitting for 40 mg/kg dose of Curcumin; diamonds represent the observed data in rat plasma



**Figure 5-4: Effect of Curcumin on iNOS gene expression described by simple IDR model**

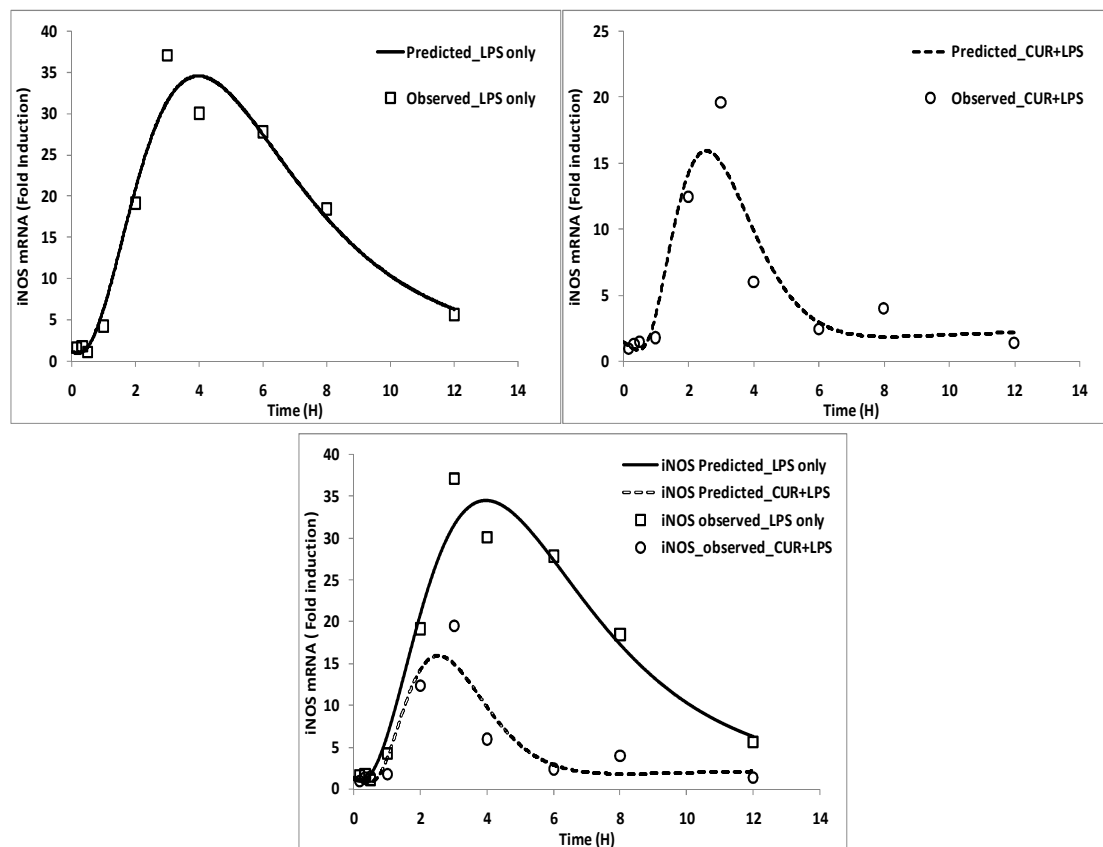
iNOS gene expression described by indirect response model in LPS only group (top left) Curcumin and LPS group (top right); circles and squares represent observed data in rat lymphocytes and solid line and dashed lines represent predicted data using the model in LPS alone and Curcumin with LPS group respectively. Mean of observed data is presented.



**Figure 5-5: Schematic showing IDR model with transit compartments**

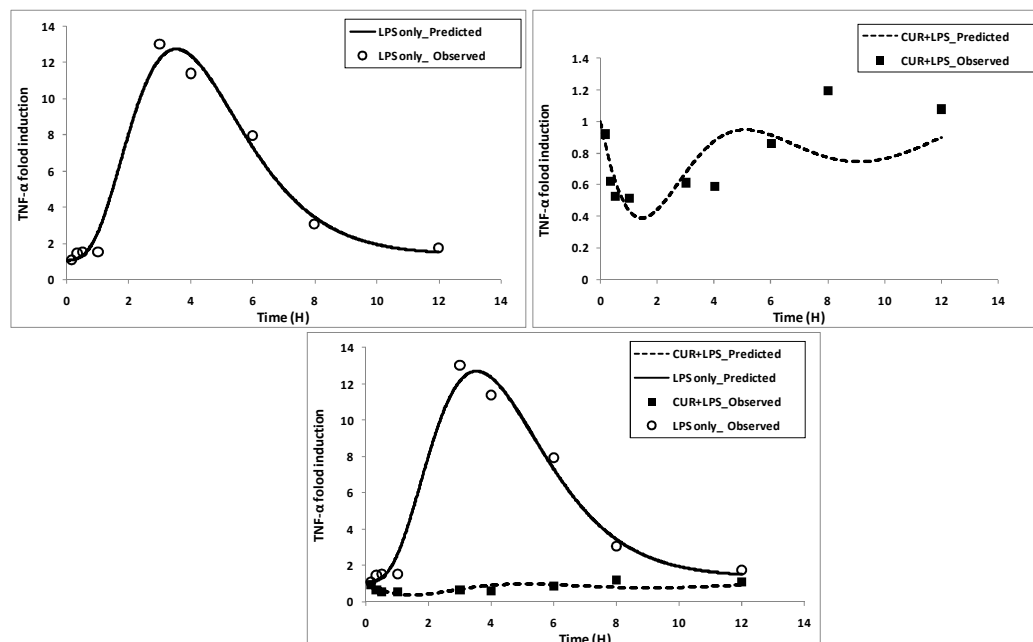
Schematic showing integrated PKPD model with transit compartments for LPS induced iNOS suppression by Curcumin. The model is defined by equations 1-8 in the text





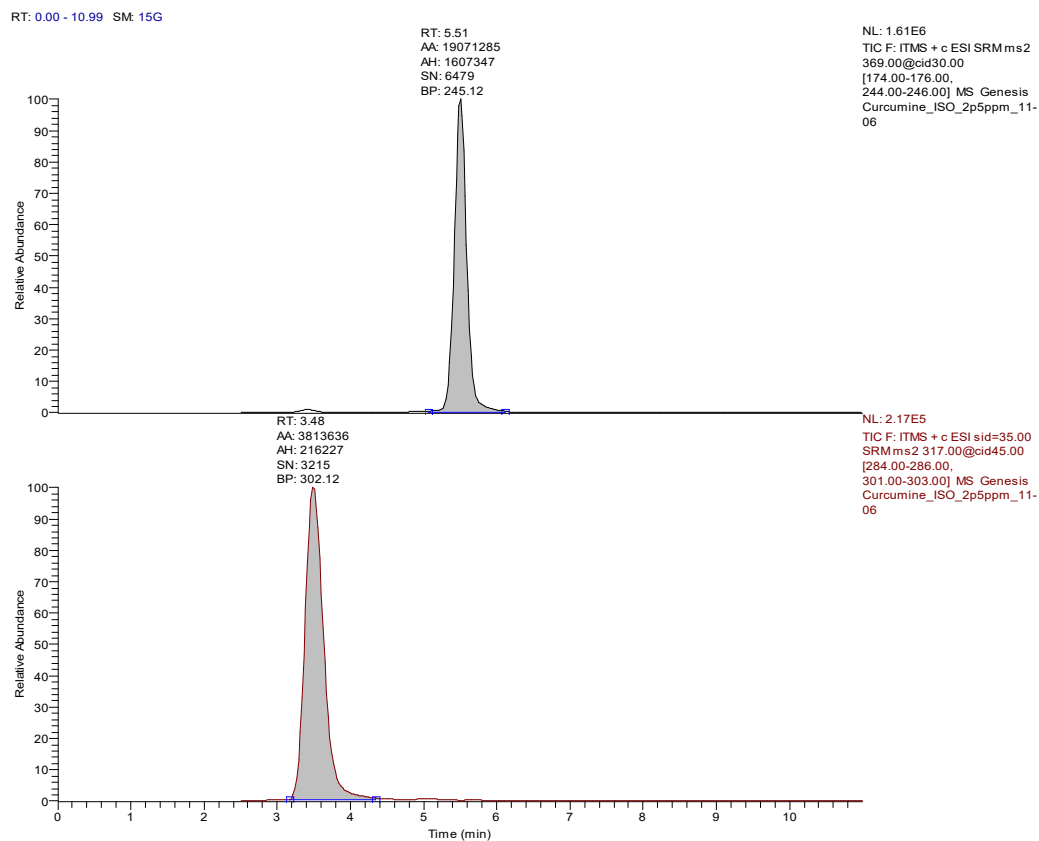
**Figure 5-6: Effect of Curcumin on iNOS gene expression modeled by IDR model with transit compartments**

iNOS gene expression described by indirect response model with transit compartments in LPS only group (top left); Curcumin and LPS group (top right); circles and squares represent observed data in rat lymphocytes and solid line and dashed lines represent predicted data using the model in LPS alone and Curcumin with LPS group respectively. Mean of observed data is presented.



**Figure 5-7: Effect of Curcumin on TNF- $\alpha$  gene expression modeled by IDR model with transit compartments**

TNF- $\alpha$  gene expression described by indirect response model with transit compartments in LPS only group (top left); Curcumin and LPS group (top right); circles and squares represent observed data in rat lymphocytes and solid line and dashed lines represent predicted data using the model in LPS alone and Curcumin with LPS group respectively. Mean of observed data is presented.



**Figure 5-8: Representative chromatogram from LC-MS analysis**

Representative chromatogram of spiked rat plasma with Isorhamnetin (internal standard in the bottom panel) and Curcumin (top panel) with retention times of approximately 3.4 and 5.5 minutes respectively.

<b>Pharmacokinetic Parameters</b>		
<b>Parameter</b>	<b>Estimate (CV%)</b>	<b>Description</b>
<b>CL</b> (L/h/kg)	119.5 (3.92)	Elimination clearance
<b>V<sub>c</sub></b> (L/kg)	32.5 (2.44)	Volume of central compartment
<b>V<sub>p</sub></b> (L/kg)	89.0 (23.8)	Volume of Peripheral compartments
<b>CL<sub>D</sub></b> (L/h/kg)	54.7 (8.37)	Distribution Clearance
<b>Secondary parameters</b>		
<b>AUC<sub>(0-∞)</sub></b> (h*ng/mL)	444.08	Area under the curve
<b>MRT</b> (h)	1.37	Mean residence time
<b>T<sub>1/2</sub></b> (h)	1.95	Terminal Half-life
<b>Vss</b> (L/kg)	132.7	Volume of distribution at steady state

**Table 5-1: Curcumin Pharmacokinetic parameters**

Parameter	Description	Estimates by model	
		IDR only	TC+ IDR
$K_1$ (1/h)	Zero-order rate of formation of iNOS mRNA	1.115	--
$K_2$ (1/h)	Zero-order rate of formation of iNOS mRNA	–	3.698
T (h)	Duration of LPS effect	3 (Fixed)	–
$K_{out}$ (1/h)	Elimination constant of iNOS mRNA	1.496	1.639
$K_{lps}$	Stimulation of iNOS mRNA by LPS	131.8	65.03
$IC_{50}$ (ng/mL)	50% inhibition of iNOS by Curcumin	5.82	12.47
$\tau$ (h)	Transit time	–	0.52
$K_1$ (1/h)	Rate constant for LPS elimination	–	1.06

**Table 5-2: iNOS Pharmacodynamic Parameters**

Pharmacodynamic parameters of iNOS expression estimated from indirect response model with transit compartments.

Parameter	Description	Estimates by model
		TC + IDR
$K_1$ (1/h)	Zero-order rate of formation of TNF- $\alpha$ mRNA	--
$K_2$ (1/h)	Zero-order rate of formation of TNF- $\alpha$ mRNA	1.235
T (h)	Duration of LPS effect	–
$K_{out}$ (1/h)	Elimination constant of TNF- $\alpha$ mRNA	1.090
$K_{lps}$	Stimulation of TNF- $\alpha$ mRNA by LPS	72.04
IC <sub>50</sub> (ng/mL)	50% inhibition of TNF- $\alpha$ by Curcumin	2.056
$\tau$ (h)	Transit time	0.9482
$K_1$ (1/h)	Rate constant for LPS elimination	1.829

**Table 5-3: TNF- $\alpha$  pharmacodynamic parameters**

Pharmacodynamic parameters of TNF- $\alpha$  expression estimated from indirect response model with transit compartments.



## 6 Anti-inflammatory effects of Ursolic Acid in *in vitro* PTEN-CaP2 cells<sup>13, 14, 15</sup>

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### 6.1 Introduction

The idea that inflammation may be a precursor to cancer has been proposed for many types of cancer. Epidemiological evidence supports this theory in the respiratory, genitourinary, reproductive and gastrointestinal systems. Chronic inflammation occurs due to macrophages, lymphocytes and plasma cells infiltrating damaged tissue. All of these leukocytes can release potentially harmful products such as reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), cytokines, and other mediators, which may contribute to the onset of cancer [223, 224]. Many inflammatory cytokines and chemokines promote tumor progression by converging on and stimulating the IKK2/NF- $\kappa$ B signaling axis [225]. Prostatic inflammation has been linked to prostate cancer through epidemiological, histopathological and molecular pathological studies [226].

The *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene is one of the most frequently mutated/deleted genes in various human cancers. Germ line mutations in the *PTEN* gene have been associated with Cowden syndrome and related diseases in which patients develop hyperplastic lesions (hamartomas) in multiple organs with increased risks of malignant

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<sup>13</sup>The work done in this chapter is being submitted to an international journal for peer-review.

<sup>14</sup> Keywords: Ursolic Acid, inflammation, PTEN, Prostate cancer

<sup>15</sup> Abbreviations: UA- Ursolic acid; NO- Nitric oxide; iNOS- inducible nitric oxide synthase; DNMT- DNA methyl transferase; HDAC- Histone deacetylase; TNF- $\alpha$ - Tumor necrosis factor  $\alpha$ ; IL-6- Interleukin 6.



transformation [227]. *PTEN* alteration is strongly implicated in prostate cancer development. *PTEN* deletions and/or mutations are found in 30% of primary prostate cancers and 63% of metastatic prostate tissue samples, placing *PTEN* mutation among the most common genetic alterations reported in human prostate cancers. It was observed in an animal model with constitutive active expression of *IKK2* alone is insufficient in promoting prostate tumorigenesis, but when combined with heterozygous loss of *Pten*, *IKK2* activation leads to an increase in tumor size, accompanied by increased inflammatory cytokines secreted from the stromal microenvironment of the prostate as a result of *PTEN* loss to drive epithelial prostate tumor cells toward metastasis [228]. It has also been shown that in Prostate specific *Pten* null mice; there is increase expression of *CXCL8/IL-8* which is a pro-inflammatory chemokine promoting tumorigenesis [229]. Studies have connected transcriptional silencing of *PTEN* to epigenetic modulation as seen in prostate cancers and melanomas [230, 231].

In that perspective, it is imperative to look at the effect of the phytochemicals in the prostate specific *PTEN* KO mice model to establish a link between inflammation and prostate cancer in the KO mice model that can be translated to clinical settings using the phytochemicals. To understand the role of *PTEN* in prostate cancer an androgenic AR positive prostate epithelial cell line was developed from *PTEN* knockout mice with homozygous (*PTEN*-CaP2 and CaP-8) and heterozygous deletion (*PTEN*-P2 and P8) of *PTEN* by Wu et.al. [232]. Studies have shown that dietary intervention with the phytochemicals like isothiocyanates led to modulation of gene expression led by *PTEN* deletion in a mice model [233].

Ursolic acid (UA), a pentacyclic triterpenoid present naturally in fruits like cranberries, apples and plants like basil and peppermint. A study done in TRAMP

mice showed that UA down-regulated activation of various pro-inflammatory mediators like AKT, STAT3, NF- $\kappa$ B, IKK $\alpha/\beta$  phosphorylation resulting in the reduced serum levels of TNF- $\alpha$  and IL-6. UA also significantly down-regulated the expression levels of cyclin D1 and COX-2[234]. Triterpenoids like UA inhibited lipopolysaccharide-induced cytokines and inducible enzyme production via the nuclear factor-kappaB signaling pathway in lung epithelial cells [235]. UA inhibited the carbon tetrachloride induced inflammatory response in mouse kidney by modulating the STAT3 and NF- $\kappa$ B signaling pathways [236]. Recent studies revealed inhibition of miRNA-21 expression and activation of PI3K/Akt/mTOR signaling pathway along with induction of PTEN gene expression by UA in rat glomerular mesangial cells [237].

We hypothesized that UA inhibits LPS induced pro-inflammatory markers along with induction of Nrf2 and downstream target genes in PTEN-CaP2 cells. Our results showed that 3  $\mu$ M treatment of UA significantly inhibited iNOS, COX-2, IL-6, IL-1b and TNF- $\alpha$  gene expression. The decrease in iNOS gene expression was correlated with a decrease in NO production. PTEN-CaP2 cells treated with UA at 3 and 6  $\mu$ M showed increased Nrf2 and NQO1 gene and protein expression along with decreased HDAC protein levels indicating a possible epigenetic role in their Nrf2 regulation.

## **6.2 Materials and methods**

### **6.2.1 Cell culture and reagents**

The mouse prostate cancer cell line with homozygous PTEN deletion, PTEN-CaP2 obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). PTEN-CaP2 cells were maintained in DMEM supplemented with 10% fetal

bovine serum (FBS; Gibco, Grand Island, NY), 25 µg/mL bovine pituitary extract, 5 µg/mL bovine insulin, and 6 ng/mL recombinant human epidermal growth factor (Sigma-Aldrich). The cells were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Ursolic Acid (UA), azadeoxycytidine (5AZA), trichostatin A (TSA) and ethidium bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA).  $3 \times 10^5$  cells/ml PTEN-CaP2 cells were seeded in 10 cm tissue culture plate and 24 h after the seeding, the cells were treated with DMSO 0.01% (negative control), UA 3µM, UA 6 µM, UA 6.25 µM, 5Aza+TSA (2.5 µM+ 0.5µM) (positive control) for 5 days with the drugs and media replenished every 2 days. TSA 0.5µM is added to the 5Aza treatment group 20h prior to harvesting the cells. For evaluating the inflammatory gene expression, the cells were pre-treated with the drugs for 6 H and then co-treated with LPS for 18 H.

### **6.2.2 Biological Assays for Measurement of Nitrite Production (NO)**

Nitrite production was assessed as described previously by measuring nitrite accumulation in the culture media fluorimetrically using an Infinite 200 PRO Tecanmicroplate reader.[114]the cells were pre-treated with drugs for 6 h and co-treated with (1 µg/ml) LPS for 18 h. A 50-µl solution of supernatant from the cultured media was added to 96-well black polystyrene flat bottom plates (Whatman Inc., Piscataway, NJ) to which 10 µl of freshly prepared 2,3 diamino naphthalene (0.05 mg/ml in 0.62 N HCl) was added and incubated for 10 min. This step serves as the start of the reaction. Next, 5 µl of 2.8 N sodium hydroxide was added to the solution to stop the reaction that results in the production of 2, 3-diaminonaphthotriazole. The reactions were measured at an excitation of 360 nm and emission of 460 nm with a gain setting of 80% using an Infinite 200 PRO Tecanmicroplate reader.

### **6.2.3 RNA isolation and qPCR**

Total RNA from the treatment and control groups was extracted using RNeasy Mini Kit (Carlsbad, CA), and the mRNA was quantified using a NanoDrop 2000. Approximately 600 ng of mRNA was reverse-transcribed into cDNA using Taqman RT reagents. Relative iNOS, TNF- $\alpha$ , COX-2, IL-6, IL-1 $\beta$ , Nrf2 and NQO1 expression was determined by qPCR using cDNA as the template and the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in an ABI7900HT system (Applied Biosystems).

### **6.2.4 Protein lysate preparation and western blotting**

Protein lysates were prepared using radio immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, St. Louis, MO, USA) supplemented with protein inhibitor cocktail (Sigma–Aldrich). Briefly, 20  $\mu$ g of total protein as determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was separated by 4–15% SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electro-transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% BSA (Fisher Scientific, Pittsburgh, PA, USA) in Tris-buffered saline–0.1% Tween 20 (TBST) buffer (Boston Bioproducts, Ashland, MA, USA), the membranes were sequentially incubated with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using Supersignal West Femtochemiluminescent substrate (Pierce, Rockford, IL, USA) and documented using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA). Densitometry of the bands was analyzed using ImageJ (Version 1.48d; NIH). The primary antibodies were obtained from different sources: anti- $\beta$ -ACTIN, Nrf2 and NQO1 from Santa Cruz Biotechnology

(Santa Cruz, CA, USA); anti-HDAC1, 2, 3 and 4 from Cell Signaling Technology (Boston, MA, USA). The secondary antibodies were purchased from Santa Cruz Biotechnology.

### **6.2.5 Statistical analysis**

The data are presented as the mean  $\pm$  SEM (standard error of the mean). The statistical analyses were performed using Student's *t*-test. *P* values less than 0.05 were considered statistically significant and are indicated with \*; *P* values less than 0.01 are indicated with #.

## **6.3 Results**

### **6.3.1 Effect of UA and LPS on PTEN-CaP2 cell viability**

The percentage cell viability of PTEN-CaP2 cells on treatment with LPS alone or with UA was evaluated. The results revealed 3 and 6  $\mu$ M of UA along with 1  $\mu$ g/mL of LPS showed no noticeable cell death. However, PTEN-CaP 2 cells on treatment with 12  $\mu$ M of UA and 1  $\mu$ g/mL LPS decreased the cell viability to approximately 50 % (Figure 6-2).

### **6.3.2 Ursolic acid inhibits LPS Induced Gene Expression of COX-2, iNOS, TNF- $\alpha$ , IL-6 and IL1-b in PTEN-CaP2 cells**

Several studies have reported the induction of pro-inflammatory gene expression on treatment with LPS. PTEN-CaP2 cells on pre-treatment with 3  $\mu$ M UA for 6 H followed by co-treatment with LPS 1  $\mu$ g/mL for 18 H inhibited the transcription of pro-inflammatory gene expression as seen by qPCR analyses. Our results revealed

significantly lower gene expression of TNF- $\alpha$ , COX-2, IL-6 IL-1b in the 3  $\mu$ M UA treated group compared with LPS only treatment (Figure 6-3). 3  $\mu$ M UA also lowered iNOS gene expression compared to LPS only treated group, however, the effect was not significant (Figure 6-3).

### **6.3.3 Effect of Ursolic acid on inhibition of LPS induced nitrite production in PTEN-CaP2 cells**

Nitrite production is a downstream effect of iNOS induction. Effect of UA treatment was assessed in LPS-stimulated PTEN-CaP2 cells. The results showed that LPS induced nitrite production was lowered significantly by UA in a dose dependent manner at 3, 6, and 12  $\mu$ M compared with the LPS-only treatment (Figure 6-4). The nitrite production was decreased significantly by 12  $\mu$ M UA compared to the 3 and 6  $\mu$ M treatment groups.

### **6.3.4 UA Induces Nrf2 and NQO1 Gene and Protein Expression while inhibiting HDAC Protein expression in PTEN-CaP2 cells**

The gene and protein expression of Nrf2 and NQO1 on treatment with 3, 6  $\mu$ M UA for 24 H was analyzed using qPCR and western blot. The results revealed significant induction of Nrf2 and NQO1 gene expression in a dose dependent manner (Figure 6-5A). The protein levels of NQO1 showed a significant induction in a dose dependent manner on treatment with 3 and 6 UA for 24 H. However, there was no significant induction of Nrf2 protein expression in the same treatment group (Figure 6-5B). We hypothesized that UA modulates the transcription of these genes by epigenetic modulation. To test this PTEN-CaP2 cells were treated with 3, 6  $\mu$ M UA and 2.5  $\mu$ M 5Aza + 0.5  $\mu$ M TSA for 5 days. In the 5Aza treatment group, TSA was

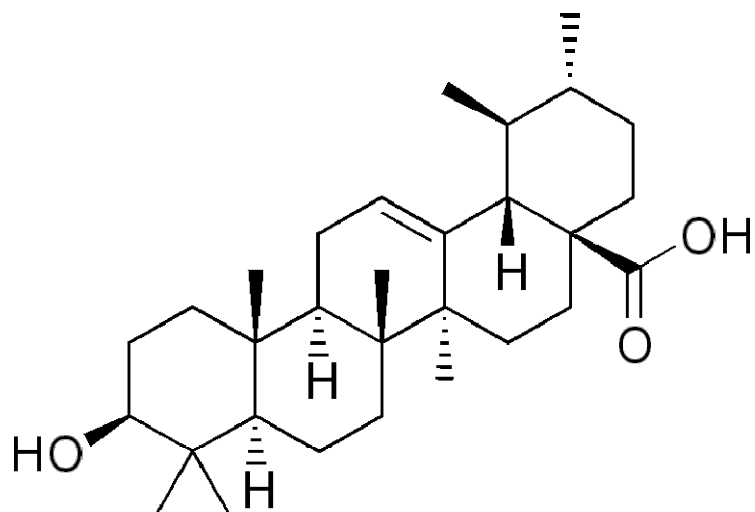
added 20 H prior to harvesting the cells on the sixth day. The results revealed that UA induced the protein expression of Nrf2 and NQO1 in a dose dependent manner (Figure 6-6A). There was a significant induction in the protein levels of Nrf2 and NQO1 in 5Aza treated cells. To analyze the epigenetic role, several histone deacetylases were probed for their protein expression. HDAC1, 2 and 3 protein levels were significantly inhibited by 3 and 6  $\mu$ M UA treatment. The inhibition was also seen in the 5Aza treatment group which is used as a positive control. However, 3 $\mu$ M UA showed no inhibition of HDAC4, while 6  $\mu$ M UA significantly inhibited HDAC4 protein expression (Figure 6-6B).

#### **6.4 Discussion and future directions**

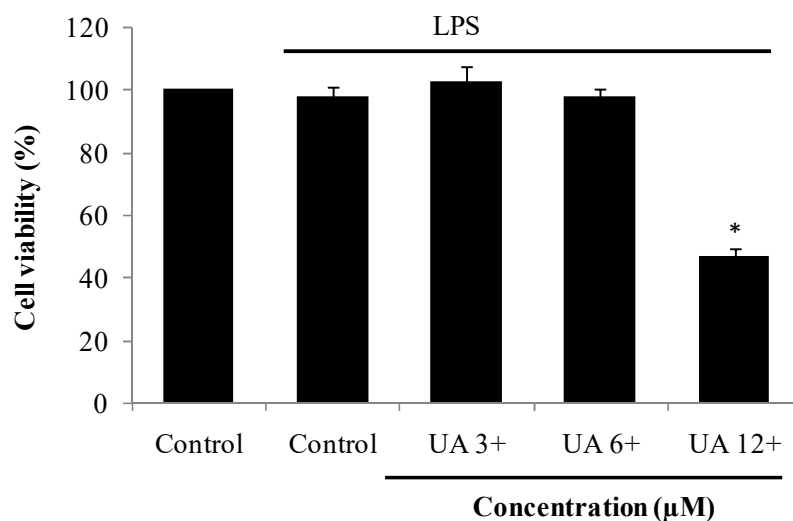
Prostate cancer development is often driven by chronic inflammation, the mechanisms underlying inflammatory processes to cancer progression have become a very important target as part of prostate cancer chemoprevention. Studies have shown the role of PTEN in prostate cancer where it is most frequently mutated or hypermethylated. Loss of PTEN resulted in an up regulation of inflammatory and cytokine receptor signalling pathways [238]. Research has reported that bacterial-induced inflammation promotes basal-to-luminal differentiation and accelerates prostate cancer initiation in Pten-null basal cells [239, 240]. Hence, inhibition of inflammation by xenobiotics and finding the mechanisms by which they act could be considered as one of the important part of cancer chemoprevention. Increased levels of DNMTs and HDACs have been reported in prostate cancers [241]. HDAC inhibitors have been found to trigger both pro- and anti-inflammatory effects in cell types with relevant inflammatory pathophysiology. Studies have reported that HDAC inhibitors showed efficacy in animal models of several inflammatory diseases [242,

243]. Taken together, induction of Nrf2 by epigenetic mechanisms could be one of the ways through which the phytochemicals inhibit inflammation thereby enhancing their chemo preventive properties. Our study hypothesized that UA, a triterpenoid decreased LPS induced inflammation in PTEN-CaP2 cells. Our results revealed that UA at 3  $\mu$ M significantly inhibited the transcription of pro-inflammatory molecules and cytokines like COX-2, TNF- $\alpha$ , IL-6 and IL1- $\beta$  compared to LPS only treatment (Figure 3). Our results also showed that 3 $\mu$ M UA treatment also lowered iNOS gene expression (Figure 3) which resulted in the lowered NO production (Figure 2). Studies done in the past have also revealed the importance of Nrf2 and the downstream genes in the anti-inflammatory effects of phytochemicals [103, 214, 244]. Epigenetic regulation of Nrf2 is mediated by histone deacetylases and DNA methyltransferases as previously indicated in several studies [134, 155, 245]. PTEN-CaP2 cells on treatment with UA showed a dose dependent induction of Nrf2 and NQO1 protein levels. The UA treatment also showed a dose dependent inhibition of HDAC 1, 2, 3 and 4 showing that the increased expression of Nrf2 could be linked to the epigenetic mechanisms. Collectively, our results show that UA is potent anti-inflammatory agent, whose epigenetic modulatory role and the mechanism is not yet fully understood. Further studies that are being carried out as part of this project will help bridge the epigenetic regulatory role of UA and connecting it to its anti-inflammatory and cancer preventive properties.



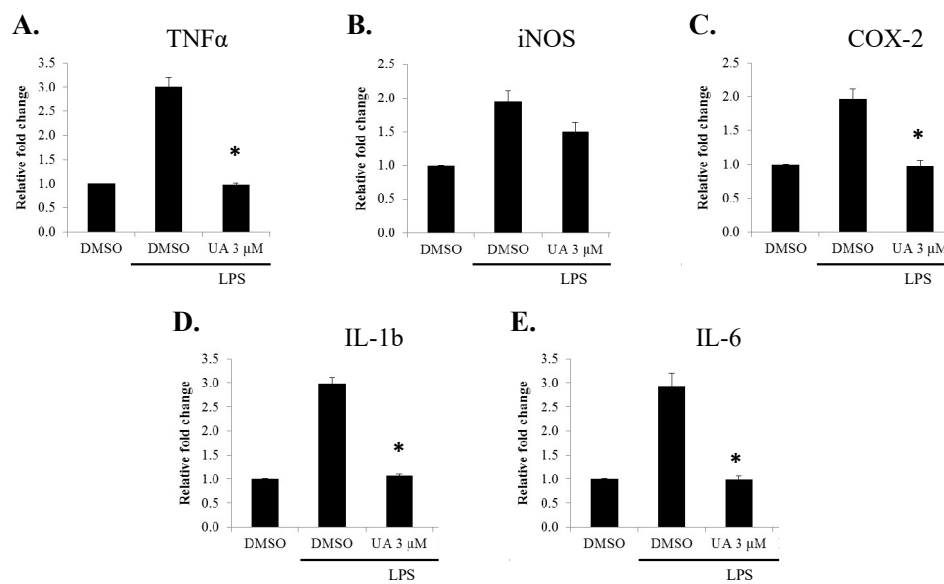


**Figure 6-1: Chemical structure of Ursolic acid**



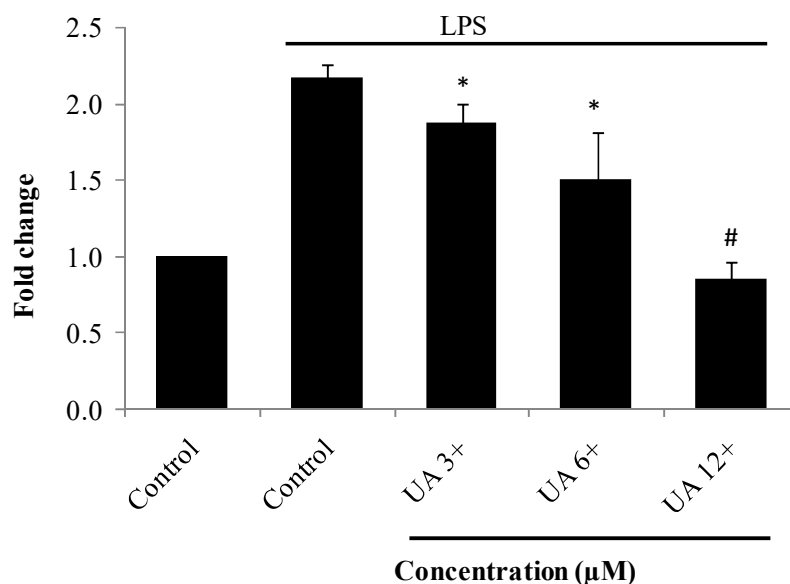
**Figure 6-2: MTS assay showing cell viability upon UA and LPS treatment in PTEN-CaP2 cells**

Effect of Ursolic acid (UA) on cell viability in LPS-stimulated PTEN-CaP2 cells. PtenCap2 cells were pre-treated with UA during 6 hours and co-treated with LPS (1  $\mu\text{g/mL}$ ) during 24 hours, as described in the “Materials and Methods” section. The data are expressed as the mean  $\pm$  SD of three independent experiments. \* indicate significant differences ( $p < 0.05$ ) in cell viability compared to LPS-induced DMSO treatment, which was used as a negative control.



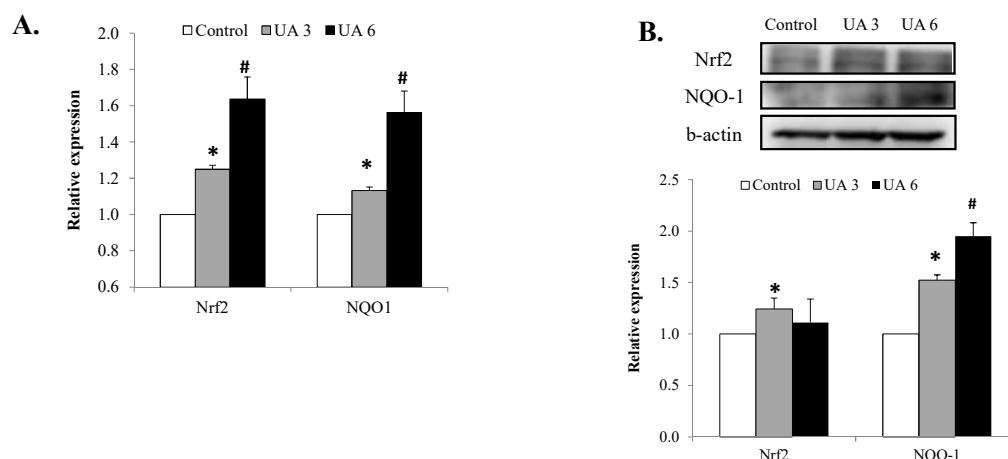
**Figure 6-3: Effect of UA on Pro-inflammatory gene expression**

Effect of Ursolic acid (UA) on the relative mRNA expressions of TNF $\alpha$ (**A**), iNOS (**B**), COX-2 (**C**), IL-1b (**D**) and IL-6 (**E**) genes in LPS-stimulated PtenCaP2 cells using quantitative real-time PCR (qPCR), with  $\beta$ -actin serving as endogenous housekeeping gene. PtenCaP2 cells were treated with UA during 6 hours and co-treated with LPS (1  $\mu$ g/mL) during 24 hours. RNAs were extracted from three independent experiments. Data are expressed as mean  $\pm$  SD. \* indicates significant differences ( $p < 0.05$ ) in the relative mRNA expression levels compared to DMSO, which was used as a negative control.



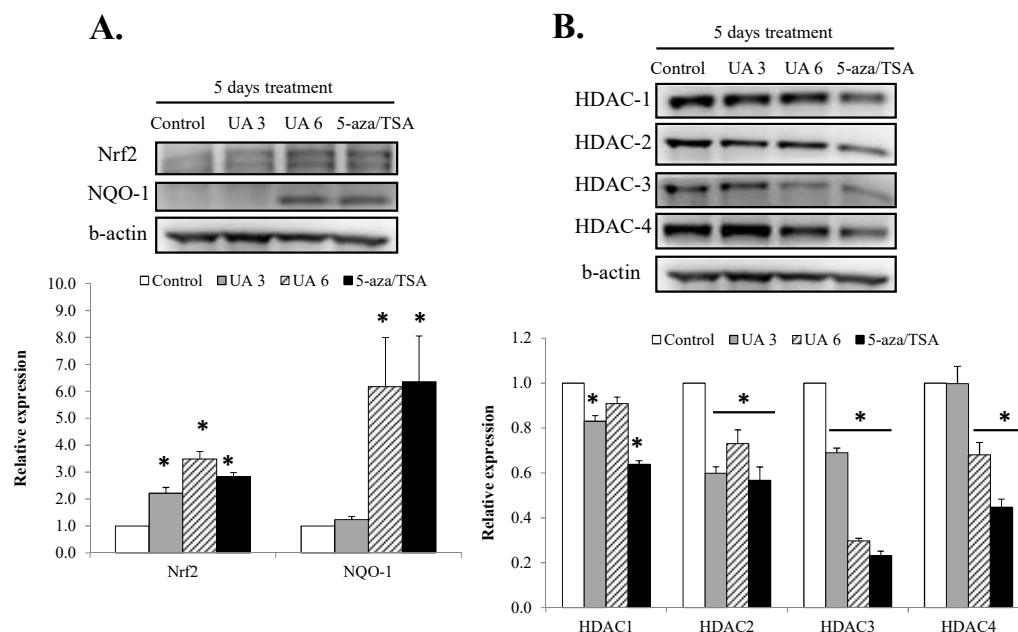
**Figure 6-4: Effect of UA on NO production in PTEN-CaP2 cells**

Effect of Ursolic acid (UA) on Nitrite production in LPS-stimulated PtenCap2 cells. PtenCap2 cells were pre-treated with UA during 6 hours and co-treated with LPS (1  $\mu\text{g/mL}$ ) during 24 hours, as described in the Materials and Methods section. The data are expressed as the mean  $\pm$  SD of three independent experiments. \* indicate significant differences ( $p < 0.05$ ) and # indicate significant differences ( $p < 0.01$ ) in NO production compared to LPS-induced DMSO treatment, which was used as a negative control.



**Figure 6-5: Effect of UA on Nrf2 and NQO1 gene expression in PTEN-CaP2 cells**

Effect of Ursolic acid (UA) on the relative mRNA expressions (**A**) and protein levels (**B**) of Nrf2 and NQO1 in PtenCaP2 cells using quantitative real-time PCR (qPCR) and western blot, respectively. Cells were treated with UA using different concentrations during 24 hours. RNAs and proteins were extracted from three independent experiments. Data are expressed as mean  $\pm$  SD. \* indicates significant differences ( $p < 0.05$ ) and # indicate significant differences ( $p < 0.01$ ) in the relative mRNA and protein levels compared to DMSO, which was used as a negative control. Images were analyzed by using ImageJ software (NIH, <http://rsbweb.nih.gov/ij/>).



**Figure 6-6: Effect of UA on Nrf2, NQO1 and HDAC protein expression on treatment with UA for 5 days**

Effect of Ursolic acid (UA) on the protein level of Nrf2 and NQO1 (A), and HDACs (1-4) in PtenCaP2 cells. Cells were treated with UA during 5 days. Data are expressed as mean  $\pm$  SD. \* indicates significant differences ( $p < 0.05$ ) in the relative protein levels compared to DMSO, which was used as a negative control. Protein expression level was normalized with  $\beta$ -actin. Images were analyzed by using ImageJ software (NIH, <http://rsbweb.nih.gov/ij/>). The different sources of the antibodies are described in “Material and Methods Section”.



## 7 Summary and Future Perspectives

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Inflammation is a key event in the progression of several disorders. Discovering drugs and identifying the targets by which they elicit the anti-inflammatory effects is of importance in ameliorating these diseases. Nrf2, a transcription factor plays a key role in anti-oxidative stress response and cancer prevention. It is suggested that several phytochemicals reactivate epigenetically silenced Nrf2 gene expression. Epigenetic silencing of genes especially tumor suppressors is a key phenomenon in cancer formation and progression. In this dissertation, we have shown the importance of Nrf2 a transcription factor known for its anti-oxidative properties in the anti-inflammatory properties of phytochemicals like Curcumin, PEITC. In agreement with our studies on the role of Nrf2 in anti-inflammatory effects of Curcumin, a recent study has shown the effect of Curcumin in activating airway inflammation [246].

In chapter 3, we provide mechanistic insight into the role of the Nrf2 pathway in ameliorating inflammation by using these phytochemicals to explore the anti-inflammatory and the anti-oxidant effects of PEITC and curcumin mediated by Nrf2. Our results indicate that PEITC acts via the Nrf2 pathway to reduce inflammation, as demonstrated by its regulation of pro-inflammatory cytokines, decrease IL-6 and TNF- $\alpha$ , as revealed by ELISA in Nrf2 (+/+) mice compared with the Nrf2 (-/-) mice. Our results showed that PEITC treatment exhibits a dose-dependent suppression of nitrite production, as demonstrated in the NO assay of Nrf2 (+/+) macrophages. Contrary to the results in the wildtype group, the lack of Nrf2 reduced the impact of the drug on the reduction of NO production.



Chapter 4 evaluated the promoter demethylation of RASSF1A in androgen sensitive prostate cancer cells (LNCaP). RASSF1A is a tumor suppressor gene. It is known to mediate important cellular processes like cell cycle progression, microtubule stability, and the induction of apoptosis. Loss of function of RASSF1A leads to accelerated cell cycle progression and resistance to apoptotic signals, resulting in increased cell proliferation[247]. Results from various studies showed that RASSF1A is one of the most frequently hypermethylated genes in prostate cancer[173]. Our studies showed that PEITC caused promoter demethylation of RASSF1A, led to a G2/M cell cycle arrest and increased apoptosis in LNCaP cells. Our results showed that promoter demethylation activated *RASSF1A* gene expression in LNCaP cells treated with 5  $\mu$ M PEITC. This affect might account for the increase in apoptosis we observed as caspase-3 protein expression was significantly enhanced in these cells and a 2-fold increase in apoptotic cells following PEITC treatment (5  $\mu$ M) was seen. This study is one of the first to demonstrate the role of PEITC in activating *RASSF1A* and promoting apoptosis *in vitro*. The interaction of RASSF1A and PTEN has been well documented and both of these genes are known to be hypermethylated in several cancers. As a perspective for future studies, further studying the role of RASSF1A and linking its pro-apoptotic role to PTEN using phytochemicals could provide further insights to the underlying mechanisms through which the chemopreventive agents elicit their action. This work can be further enhanced by performing *in vivo* studies linking the expression of RASSF1A and its role in inhibiting inflammation by suppression of NF-kB.

In chapter 5, we built on the anti-inflammatory properties of Curcumin from chapter 3 and analyzed the effect of Curcumin in LPS induced inflammatory and epigenetic gene expression in rats. In general, inflammatory stimulus caused due to

either natural or experimental conditions results in an increase of pro-inflammatory biomarkers. The phytochemicals acting as anti-inflammatory agents act typically either by inhibiting the pro-inflammatory molecules or by inducing anti-inflammatory genes. The pharmacological response of the drugs are quantified using an array of simple or complex indirect response, signal transduction models [248]. Our results demonstrated that the indirect response model with transit compartments could describe the anti-inflammatory effects of Curcumin. The disposition kinetics of Curcumin showed a bi-exponential decline well described by a two compartment model with linear clearance. The time course of iNOS and TNF- $\alpha$  mRNA showed an increase upon LPS dosing after an initial delay of 1 h; followed by stimulation reaching maximum at 3h. Curcumin dosing along with LPS reduced the expression of iNOS mRNA with the maximum expression at 3h which was considerably lower than LPS alone administration. Curcumin dosing along with LPS also reduced TNF- $\alpha$  mRNA production. The induction of iNOS mRNA expression was modeled using a simple indirect response model as well as indirect response model with transit compartments. The transit compartments were used to serve as downstream genes that were delaying the production of mRNA expression. The indirect response model alone was able to describe the gene expression data of iNOS and the delay was better described without fixing any parameters by the transit compartment model.

However, to increase the flexibility and describe the TLR4 signaling cascade through which LPS mediated inflammation is driven, there is a need to optimize the model with the ligand receptor dynamics as well as modeling the downstream genes as in the signal transduction model.

Chapter 6 evaluated the anti-inflammatory and the epigenetic modulatory role of Ursolic acid (UA) in prostate specific PTEN null cells (PTEN-CaP2). Our results

revealed that UA significantly inhibited the transcription of pro-inflammatory molecules and cytokines like COX-2, TNF- $\alpha$ , IL-6 and IL1- $\beta$  compared to LPS only treatment. PTEN-CaP2 cells on treatment with UA showed a dose dependent induction of Nrf2 and NQO1 protein levels. The UA treatment also showed a dose dependent inhibition of HDAC 1, 2, 3 and 4 showing that the increased expression of Nrf2 could be linked to the epigenetic mechanisms. The role of UA on DNA methylation has been studied extensively. However, studies done on promoter methylation of Nrf2 in PTEN-CaP2 cells showed very little basal methylation suggesting that the expression of Nrf2 in PTEN null models may be mediated by histone modifications. Further studies are to be carried out to efficiently link the epigenetic modulatory role of UA in increasing the expression of Nrf2 and inhibition of pro-inflammatory genes.

Collectively, this dissertation shows an exciting new prospect of regulation of epigenetic modifying genes as well as inflammatory genes by phytochemicals like Curcumin, Phenethylisothiocyanate and Ursolic acid. The molecular markers that are identified may be further developed as robust biomarkers for future clinical studies that are in the stage of conception using these phytochemicals.

## Bibliography

1. Aggarwal, B.B. and B. Sung, *Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets*. Trends Pharmacol Sci, 2009. **30**(2): p. 85-94.
2. Ammon, H.P. and M.A. Wahl, *Pharmacology of Curcuma longa*. Planta Med, 1991. **57**(1): p. 1-7.
3. Lao, C.D., et al., *Dose escalation of a curcuminoid formulation*. BMC Complement Altern Med, 2006. **6**: p. 10.
4. Hatcher, H., et al., *Curcumin: from ancient medicine to current clinical trials*. Cell Mol Life Sci, 2008. **65**(11): p. 1631-52.
5. Surh, Y.J., et al., *Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation*. Mutat Res, 2001. **480-481**: p. 243-68.
6. Jobin, C., et al., *Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity*. J Immunol, 1999. **163**(6): p. 3474-83.
7. Skommer, J., D. Wlodkowic, and J. Pelkonen, *Gene-expression profiling during curcumin-induced apoptosis reveals downregulation of CXCR4*. Exp Hematol, 2007. **35**(1): p. 84-95.
8. Rao, C.V., *Regulation of COX and LOX by curcumin*. Adv Exp Med Biol, 2007. **595**: p. 213-26.
9. Shen, G., et al., *Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin*. Mol Cancer Ther, 2006. **5**(1): p. 39-51.
10. Gonzalez-Reyes, S., et al., *Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats*. Oxid Med Cell Longev, 2013. **2013**: p. 801418.
11. He, H.J., et al., *Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice*. World J Diabetes, 2012. **3**(5): p. 94-104.
12. Wu, J., et al., *Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway*. PLoS One, 2013. **8**(3): p. e59843.
13. Liu, M., et al., *Transcription factor Nrf2 is protective during ischemic and nephrotoxic acute kidney injury in mice*. Kidney Int, 2009. **76**(3): p. 277-85.
14. Shin, D.H., et al., *The NRF2-heme oxygenase-1 system modulates cyclosporin A-induced epithelial-mesenchymal transition and renal fibrosis*. Free Radic Biol Med, 2010. **48**(8): p. 1051-63.
15. Jiang, T., et al., *The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy*. Diabetes, 2010. **59**(4): p. 850-60.
16. Soetikno, V., et al., *Curcumin ameliorates macrophage infiltration by inhibiting NF-kappaB activation and proinflammatory cytokines in streptozotocin induced-diabetic nephropathy*. Nutr Metab (Lond), 2011. **8**(1): p. 35.

17. Gao, S., et al., *Curcumin attenuates arsenic-induced hepatic injuries and oxidative stress in experimental mice through activation of Nrf2 pathway, promotion of arsenic methylation and urinary excretion*. Food Chem Toxicol, 2013. **59**: p. 739-47.
18. Robertson, K.D., *DNA methylation and chromatin - unraveling the tangled web*. Oncogene, 2002. **21**(35): p. 5361-79.
19. De Smet, C., et al., *DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter*. Mol Cell Biol, 1999. **19**(11): p. 7327-35.
20. Robertson, K.D., *DNA methylation and human disease*. Nat Rev Genet, 2005. **6**(8): p. 597-610.
21. Cedar, H. and Y. Bergman, *Linking DNA methylation and histone modification: patterns and paradigms*. Nat Rev Genet, 2009. **10**(5): p. 295-304.
22. Kouzarides, T., *Chromatin modifications and their function*. Cell, 2007. **128**(4): p. 693-705.
23. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications*. Cell Res, 2011. **21**(3): p. 381-95.
24. Herman, J.G. and S.B. Baylin, *Gene silencing in cancer in association with promoter hypermethylation*. N Engl J Med, 2003. **349**(21): p. 2042-54.
25. Liu, Z., et al., *Curcumin is a potent DNA hypomethylation agent*. Bioorg Med Chem Lett, 2009. **19**(3): p. 706-9.
26. Shu, L., et al., *Epigenetic CpG demethylation of the promoter and reactivation of the expression of Neurog1 by curcumin in prostate LNCaP cells*. AAPS J, 2011. **13**(4): p. 606-14.
27. Khor, T.O., et al., *Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation*. Biochem Pharmacol, 2011. **82**(9): p. 1073-8.
28. Yoo, C.B. and P.A. Jones, *Epigenetic therapy of cancer: past, present and future*. Nat Rev Drug Discov, 2006. **5**(1): p. 37-50.
29. Yu, J., et al., *Curcumin down-regulates DNA methyltransferase 1 and plays an anti-leukemic role in acute myeloid leukemia*. PLoS One, 2013. **8**(2): p. e55934.
30. Hassan, H.E., et al., *Curcumin and Dimethoxycurcumin Induced Epigenetic Changes in Leukemia Cells*. Pharm Res, 2014.
31. Link, A., et al., *Curcumin modulates DNA methylation in colorectal cancer cells*. PLoS One, 2013. **8**(2): p. e57709.
32. Lewinska, A., et al., *Curcumin-mediated decrease in the expression of nucleolar organizer regions in cervical cancer (HeLa) cells*. Mutat Res Genet Toxicol Environ Mutagen, 2014. **771**: p. 43-52.
33. Zheng, J., et al., *Curcumin up-regulates phosphatase and tensin homologue deleted on chromosome 10 through microRNA-mediated control of DNA methylation--a novel mechanism suppressing liver fibrosis*. FEBS J, 2014. **281**(1): p. 88-103.
34. Nagaraju, G.P., et al., *Novel synthetic curcumin analogues EF31 and UBS109 are potent DNA hypomethylating agents in pancreatic cancer*. Cancer Lett, 2013. **341**(2): p. 195-203.

35. Wu, B., et al., *Epigenetic reactivation of RANK in glioblastoma cells by curcumin: involvement of STAT3 inhibition*. DNA Cell Biol, 2013. **32**(6): p. 292-7.
36. Du, L., et al., *Reactivation of RASSF1A in breast cancer cells by curcumin*. Nutr Cancer, 2012. **64**(8): p. 1228-35.
37. Chen, K.L., et al., *The epigenetic effects of amyloid-beta(1-40) on global DNA and neprilysin genes in murine cerebral endothelial cells*. Biochem Biophys Res Commun, 2009. **378**(1): p. 57-61.
38. Deng, Y., et al., *Curcumin inhibits the AKT/NF-kappaB signaling via CpG demethylation of the promoter and restoration of NEP in the N2a cell line*. AAPS J, 2014. **16**(4): p. 649-57.
39. Taniguchi, T., et al., *Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors*. Nat Med, 2003. **9**(5): p. 568-74.
40. Parashar, G., N.C. Parashar, and N. Capalash, *Curcumin causes promoter hypomethylation and increased expression of FANCF gene in SiHa cell line*. Mol Cell Biochem, 2012. **365**(1-2): p. 29-35.
41. Liu, Y.L., et al., *Hypomethylation effects of curcumin, demethoxycurcumin and bisdemethoxycurcumin on WIF-1 promoter in non-small cell lung cancer cell lines*. Mol Med Rep, 2011. **4**(4): p. 675-9.
42. Mirza, S., et al., *Expression of DNA methyltransferases in breast cancer patients and to analyze the effect of natural compounds on DNA methyltransferases and associated proteins*. J Breast Cancer, 2013. **16**(1): p. 23-31.
43. Nephew, K.P. and T.H. Huang, *Epigenetic gene silencing in cancer initiation and progression*. Cancer Lett, 2003. **190**(2): p. 125-33.
44. Berlowitz, L. and D. Pallotta, *Acetylation of nuclear protein in the heterochromatin and euchromatin of mealy bugs*. Exp Cell Res, 1972. **71**(1): p. 45-8.
45. Luger, K., et al., *Crystal structure of the nucleosome core particle at 2.8 Å resolution*. Nature, 1997. **389**(6648): p. 251-60.
46. Tremethick, D.J., *Higher-order structures of chromatin: the elusive 30 nm fiber*. Cell, 2007. **128**(4): p. 651-4.
47. Choudhuri, S., Y. Cui, and C.D. Klaassen, *Molecular targets of epigenetic regulation and effectors of environmental influences*. Toxicol Appl Pharmacol, 2010. **245**(3): p. 378-93.
48. Zhang, K. and S.Y. Dent, *Histone modifying enzymes and cancer: going beyond histones*. J Cell Biochem, 2005. **96**(6): p. 1137-48.
49. Mottet, D. and V. Castronovo, *Histone deacetylases: target enzymes for cancer therapy*. Clin Exp Metastasis, 2008. **25**(2): p. 183-9.
50. Wada, T.T., et al., *Aberrant histone acetylation contributes to elevated interleukin-6 production in rheumatoid arthritis synovial fibroblasts*. Biochem Biophys Res Commun, 2014. **444**(4): p. 682-6.
51. Yuan, Z., et al., *Curcumin mediated epigenetic modulation inhibits TREM-1 expression in response to lipopolysaccharide*. Int J Biochem Cell Biol, 2012. **44**(11): p. 2032-43.
52. Yun, J.M., I. Jialal, and S. Devaraj, *Epigenetic regulation of high glucose-induced proinflammatory cytokine production in monocytes by curcumin*. J Nutr Biochem, 2011. **22**(5): p. 450-8.

53. Miller, G., *Epigenetics. A role for epigenetics in cognition*. Science, 2010. **329**(5987): p. 27.
54. Xu, K., et al., *Targeting HDACs: a promising therapy for Alzheimer's disease*. Oxid Med Cell Longev, 2011. **2011**: p. 143269.
55. Kilgore, M., et al., *Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease*. Neuropsychopharmacology, 2010. **35**(4): p. 870-80.
56. Lu, X., et al., *Histone acetyltransferase p300 mediates histone acetylation of PS1 and BACE1 in a cellular model of Alzheimer's disease*. PLoS One, 2014. **9**(7): p. e103067.
57. Meng, J., et al., *The anti-tumor histone deacetylase inhibitor SAHA and the natural flavonoid curcumin exhibit synergistic neuroprotection against amyloid-beta toxicity*. PLoS One, 2014. **9**(1): p. e85570.
58. Zhu, X., et al., *Curcumin alleviates neuropathic pain by inhibiting p300/CBP histone acetyltransferase activity-regulated expression of BDNF and cox-2 in a rat model*. PLoS One, 2014. **9**(3): p. e91303.
59. Morimoto, T., et al., *The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats*. J Clin Invest, 2008. **118**(3): p. 868-78.
60. Puglia, C., et al., *Curcumin loaded NLC induces histone hypoacetylation in the CNS after intraperitoneal administration in mice*. Eur J Pharm Biopharm, 2012. **81**(2): p. 288-93.
61. Chowdhury, R., et al., *Curcumin attenuation of lipopolysaccharide induced cardiac hypertrophy in rodents*. ISRN Inflamm, 2013. **2013**: p. 539305.
62. Tikoo, K., et al., *Change in post-translational modifications of histone H3, heat-shock protein-27 and MAP kinase p38 expression by curcumin in streptozotocin-induced type I diabetic nephropathy*. Br J Pharmacol, 2008. **153**(6): p. 1225-31.
63. Lee, S.J., et al., *Curcumin-induced HDAC inhibition and attenuation of medulloblastoma growth in vitro and in vivo*. BMC Cancer, 2011. **11**: p. 144.
64. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. Cell, 2004. **116**(2): p. 281-97.
65. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs*. Cell, 2009. **136**(4): p. 642-55.
66. Calin, G.A. and C.M. Croce, *MicroRNA signatures in human cancers*. Nat Rev Cancer, 2006. **6**(11): p. 857-66.
67. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers*. Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2999-3004.
68. Sarkar, F.H. and Y. Li, *Cell signaling pathways altered by natural chemopreventive agents*. Mutat Res, 2004. **555**(1-2): p. 53-64.
69. Papagiannakopoulos, T. and K.S. Kosik, *MicroRNAs: regulators of oncogenesis and stemness*. BMC Med, 2008. **6**: p. 15.
70. Bao, B., et al., *Curcumin analogue CDF inhibits pancreatic tumor growth by switching on suppressor microRNAs and attenuating EZH2 expression*. Cancer Res, 2012. **72**(1): p. 335-45.
71. Sun, M., et al., *Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells*. Mol Cancer Ther, 2008. **7**(3): p. 464-73.

72. Ali, S., et al., *Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF*. *Cancer Res*, 2010. **70**(9): p. 3606-17.
73. Saini, S., et al., *Curcumin modulates microRNA-203-mediated regulation of the Src-Akt axis in bladder cancer*. *Cancer Prev Res (Phila)*, 2011. **4**(10): p. 1698-709.
74. Howell, J.C., et al., *Global microRNA expression profiling: curcumin (diferuloylmethane) alters oxidative stress-responsive microRNAs in human ARPE-19 cells*. *Mol Vis*, 2013. **19**: p. 544-60.
75. Dahmke, I.N., et al., *Curcumin intake affects miRNA signature in murine melanoma with mmu-miR-205-5p most significantly altered*. *PLoS One*, 2013. **8**(12): p. e81122.
76. Chen, Y., et al., *Curcumin, both histone deacetylase and p300/CBP-specific inhibitor, represses the activity of nuclear factor kappa B and Notch 1 in Raji cells*. *Basic Clin Pharmacol Toxicol*, 2007. **101**(6): p. 427-33.
77. Kang, S.K., S.H. Cha, and H.G. Jeon, *Curcumin-induced histone hypoacetylation enhances caspase-3-dependent glioma cell death and neurogenesis of neural progenitor cells*. *Stem Cells Dev*, 2006. **15**(2): p. 165-74.
78. Fernandez-Sanchez, A., et al., *DNA demethylation and histone H3K9 acetylation determine the active transcription of the NKG2D gene in human CD8+ T and NK cells*. *Epigenetics*, 2013. **8**(1): p. 66-78.
79. Roy, S., et al., *Expression of miR-34 is lost in colon cancer which can be re-expressed by a novel agent CDF*. *J Hematol Oncol*, 2012. **5**: p. 58.
80. Gao, W., J.Y. Chan, and T.S. Wong, *Curcumin exerts inhibitory effects on undifferentiated nasopharyngeal carcinoma by inhibiting the expression of miR-125a-5p*. *Clin Sci (Lond)*, 2014. **127**(9): p. 571-9.
81. Li, X., et al., *Curcumin Modulates miR-19/PTEN/AKT/p53 Axis to Suppress Bisphenol A-induced MCF-7 Breast Cancer Cell Proliferation*. *Phytother Res*, 2014. **28**(10): p. 1553-60.
82. Ali, S., et al., *Deregulation of miR-146a expression in a mouse model of pancreatic cancer affecting EGFR signaling*. *Cancer Lett*, 2014. **351**(1): p. 134-42.
83. Kronska, E., et al., *miR181b is induced by the chemopreventive polyphenol curcumin and inhibits breast cancer metastasis via down-regulation of the inflammatory cytokines CXCL1 and -2*. *Mol Oncol*, 2014. **8**(3): p. 581-95.
84. Zhao, S.F., et al., *Induction of microRNA-9 mediates cytotoxicity of curcumin against SKOV3 ovarian cancer cells*. *Asian Pac J Cancer Prev*, 2014. **15**(8): p. 3363-8.
85. Yang, C.H., et al., *The curcumin analog EF24 targets NF-kappaB and miRNA-21, and has potent anticancer activity in vitro and in vivo*. *PLoS One*, 2013. **8**(8): p. e71130.
86. Yu, C.C., et al., *miR145 targets the SOX9/ADAM17 axis to inhibit tumor-initiating cells and IL-6-mediated paracrine effects in head and neck cancer*. *Cancer Res*, 2013. **73**(11): p. 3425-40.
87. Aggarwal, B.B., et al., *Inflammation and cancer: how hot is the link?* *Biochem Pharmacol*, 2006. **72**(11): p. 1605-21.
88. Condeelis, J. and J.W. Pollard, *Macrophages: obligate partners for tumor cell migration, invasion, and metastasis*. *Cell*, 2006. **124**(2): p. 263-6.



89. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability*. Carcinogenesis, 2009. **30**(7): p. 1073-81.
90. Rietschel, E.T. and H. Brade, *Bacterial endotoxins*. Sci Am, 1992. **267**(2): p. 54-61.
91. Fujihara, M., et al., *Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex*. Pharmacol Ther, 2003. **100**(2): p. 171-94.
92. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nat Rev Immunol, 2008. **8**(12): p. 958-69.
93. Lee, J.M. and J.A. Johnson, *An important role of Nrf2-ARE pathway in the cellular defense mechanism*. J Biochem Mol Biol, 2004. **37**(2): p. 139-43.
94. Nguyen, T., P. Nioi, and C.B. Pickett, *The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress*. J Biol Chem, 2009. **284**(20): p. 13291-5.
95. Horvath, K., et al., *The involvement of heme oxygenase-1 activity in the therapeutic actions of 5-aminosalicylic acid in rat colitis*. Eur J Pharmacol, 2008. **581**(3): p. 315-23.
96. Pae, H.O., et al., *Heme oxygenase-1 attenuates contact hypersensitivity induced by 2,4-dinitrofluorobenzene in mice*. Immunopharmacol Immunotoxicol, 2008. **30**(2): p. 207-16.
97. Syapin, P.J., *Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders*. Br J Pharmacol, 2008. **155**(5): p. 623-40.
98. Foresti, R., et al., *Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells*. Pharmacol Res, 2013. **76**: p. 132-48.
99. Osburn, W.O., et al., *Genetic or pharmacologic amplification of nrf2 signaling inhibits acute inflammatory liver injury in mice*. Toxicol Sci, 2008. **104**(1): p. 218-27.
100. Osburn, W.O., et al., *Increased colonic inflammatory injury and formation of aberrant crypt foci in Nrf2-deficient mice upon dextran sulfate treatment*. Int J Cancer, 2007. **121**(9): p. 1883-91.
101. Khor, T.O., et al., *Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer*. Cancer Prev Res (Phila), 2008. **1**(3): p. 187-91.
102. Ishii, Y., et al., *Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema*. J Immunol, 2005. **175**(10): p. 6968-75.
103. Kim, J., Y.N. Cha, and Y.J. Surh, *A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders*. Mutat Res, 2010. **690**(1-2): p. 12-23.
104. Su, Z.Y., et al., *Epigenetic reactivation of Nrf2 in murine prostate cancer TRAMP C1 cells by natural phytochemicals Z-ligustilide and Radix angelica sinensis via promoter CpG demethylation*. Chem Res Toxicol, 2013. **26**(3): p. 477-85.
105. Saw, C.L., et al., *Effects of natural phytochemicals in Angelica sinensis (Danggui) on Nrf2-mediated gene expression of phase II drug metabolizing enzymes and anti-inflammation*. Biopharm Drug Dispos, 2013. **34**(6): p. 303-11.

106. Rose, P., et al., *Beta-phenylethyl and 8-methylsulphinyloctyl isothiocyanates, constituents of watercress, suppress LPS induced production of nitric oxide and prostaglandin E2 in RAW 264.7 macrophages*. Nitric Oxide, 2005. **12**(4): p. 237-43.
107. Balogun, E., et al., *Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element*. Biochem J, 2003. **371**(Pt 3): p. 887-95.
108. Saw, C.L., et al., *Impact of Nrf2 on UVB-induced skin inflammation/photoprotection and photoprotective effect of sulforaphane*. Mol Carcinog, 2011. **50**(6): p. 479-86.
109. Huang, C.S., et al., *Isothiocyanates protect against oxidized LDL-induced endothelial dysfunction by upregulating Nrf2-dependent antioxidant and suppressing NFkappaB activation*. Mol Nutr Food Res, 2013. **57**(11): p. 1918-30.
110. Hempel, S.L., M.M. Monick, and G.W. Hunninghake, *Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes*. J Clin Invest, 1994. **93**(1): p. 391-6.
111. Lin, W., et al., *Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway*. Biochem Pharmacol, 2008. **76**(8): p. 967-73.
112. Wang, H., et al., *Role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages by polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid*. Mol Pharm, 2010. **7**(6): p. 2185-93.
113. Malhotra, D., et al., *Denitrosylation of HDAC2 by targeting Nrf2 restores glucocorticosteroid sensitivity in macrophages from COPD patients*. J Clin Invest, 2011. **121**(11): p. 4289-302.
114. Misko, T.P., et al., *A fluorometric assay for the measurement of nitrite in biological samples*. Anal Biochem, 1993. **214**(1): p. 11-6.
115. Gonzales, A.M. and R.A. Orlando, *Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes*. Nutr Metab (Lond), 2008. **5**: p. 17.
116. Wu, T.Y., et al., *Anti-inflammatory/Anti-oxidative stress activities and differential regulation of Nrf2-mediated genes by non-polar fractions of tea Chrysanthemum zawadskii and licorice Glycyrrhiza uralensis*. AAPS J, 2011. **13**(1): p. 1-13.
117. Levonen, A.L., et al., *Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo*. Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 741-7.
118. Itoh, K., et al., *Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin j(2)*. Mol Cell Biol, 2004. **24**(1): p. 36-45.
119. Rangasamy, T., et al., *Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice*. J Clin Invest, 2004. **114**(9): p. 1248-59.
120. Piantadosi, C.A., et al., *Heme oxygenase-1 couples activation of mitochondrial biogenesis to anti-inflammatory cytokine expression*. J Biol Chem, 2011. **286**(18): p. 16374-85.

121. Jeong, G.S., et al., *Effects of sappanchalcone on the cytoprotection and anti-inflammation via heme oxygenase-1 in human pulp and periodontal ligament cells*. Eur J Pharmacol, 2010. **644**(1-3): p. 230-7.
122. Lee, D.S., et al., *4,2',5'-trihydroxy-4'-methoxychalcone from Dalbergia odorifera exhibits anti-inflammatory properties by inducing heme oxygenase-1 in murine macrophages*. Int Immunopharmacol, 2013. **16**(1): p. 114-21.
123. Lyu, J.H., et al., *ent-kaur-16-en-19-oic Acid, isolated from the roots of Aralia continentalis, induces activation of Nrf2*. J Ethnopharmacol, 2011. **137**(3): p. 1442-9.
124. Seo, W.Y., et al., *Celastrrol induces expression of heme oxygenase-1 through ROS/Nrf2/ARE signaling in the HaCaT cells*. Biochem Biophys Res Commun, 2011. **407**(3): p. 535-40.
125. Pae, H.O., et al., *Costunolide inhibits production of tumor necrosis factor-alpha and interleukin-6 by inducing heme oxygenase-1 in RAW264.7 macrophages*. Inflamm Res, 2007. **56**(12): p. 520-6.
126. Kumar, H., et al., *Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases*. Nat Prod Rep, 2013. **31**(1): p. 109-39.
127. Thimmulappa, R.K., et al., *Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolidine*. Biochem Biophys Res Commun, 2006. **351**(4): p. 883-9.
128. Ma, Q., L. Battelli, and A.F. Hubbs, *Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2*. Am J Pathol, 2006. **168**(6): p. 1960-74.
129. Chan, K., et al., *NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development*. Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13943-8.
130. Li, Y.J., et al., *Disruption of Nrf2 enhances susceptibility to airway inflammatory responses induced by low-dose diesel exhaust particles in mice*. Clin Immunol, 2008. **128**(3): p. 366-73.
131. Cheung, K.L., T.O. Khor, and A.N. Kong, *Synergistic effect of combination of phenethyl isothiocyanate and sulforaphane or curcumin and sulforaphane in the inhibition of inflammation*. Pharm Res, 2009. **26**(1): p. 224-31.
132. Cheung, K.L. and A.N. Kong, *Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention*. AAPS J, 2010. **12**(1): p. 87-97.
133. Khor, T.O., et al., *Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice*. Cancer Res, 2006. **66**(2): p. 613-21.
134. Khor, T.O., et al., *Epigenetic DNA methylation of antioxidative stress regulator NRF2 in human prostate cancer*. Cancer Prev Res (Phila), 2014. **7**(12): p. 1186-97.
135. Rockwell, C.E., et al., *Th2 skewing by activation of Nrf2 in CD4(+) T cells*. J Immunol, 2012. **188**(4): p. 1630-7.
136. Suschek, C.V., O. Schnorr, and V. Kolb-Bachofen, *The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all?* Curr Mol Med, 2004. **4**(7): p. 763-75.
137. Menon, V.P. and A.R. Sudheer, *Antioxidant and anti-inflammatory properties of curcumin*. Adv Exp Med Biol, 2007. **595**: p. 105-25.

138. Chen, Y.H., H.J. Dai, and H.P. Chang, *Suppression of inducible nitric oxide production by indole and isothiocyanate derivatives from Brassica plants in stimulated macrophages*. *Planta Med*, 2003. **69**(8): p. 696-700.
139. Murakami, A., *Chemoprevention with phytochemicals targeting inducible nitric oxide synthase*. *Forum Nutr*, 2009. **61**: p. 193-203.
140. Ryter, S.W., J. Alam, and A.M. Choi, *Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications*. *Physiol Rev*, 2006. **86**(2): p. 583-650.
141. Surh, Y.J., et al., *Role of Nrf2-mediated heme oxygenase-1 upregulation in adaptive survival response to nitrosative stress*. *Arch Pharm Res*, 2009. **32**(8): p. 1163-76.
142. Ashino, T., et al., *Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages*. *Mol Immunol*, 2008. **45**(7): p. 2106-15.
143. Otterbein, L.E., et al., *Heme oxygenase-1: unleashing the protective properties of heme*. *Trends Immunol*, 2003. **24**(8): p. 449-55.
144. Biswas, S.K., et al., *Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity*. *Antioxid Redox Signal*, 2005. **7**(1-2): p. 32-41.
145. Murakami, A., M. Song, and H. Ohigashi, *Phenethyl isothiocyanate suppresses receptor activator of NF-kappaB ligand (RANKL)-induced osteoclastogenesis by blocking activation of ERK1/2 and p38 MAPK in RAW264.7 macrophages*. *Biofactors*, 2007. **30**(1): p. 1-11.
146. Xu, C., et al., *Suppression of NF-kappaB and NF-kappaB-regulated gene expression by sulforaphane and PEITC through IkappaBalpha, IKK pathway in human prostate cancer PC-3 cells*. *Oncogene*, 2005. **24**(28): p. 4486-95.
147. Siegel, R., et al., *Cancer statistics, 2014*. *CA Cancer J Clin*, 2014. **64**(1): p. 9-29.
148. Abbas, A., W. Patterson, 3rd, and P.T. Georgel, *The epigenetic potentials of dietary polyphenols in prostate cancer management*. *Biochem Cell Biol*, 2013. **91**(6): p. 361-8.
149. Ornish, D., et al., *Changes in prostate gene expression in men undergoing an intensive nutrition and lifestyle intervention*. *Proc Natl Acad Sci U S A*, 2008. **105**(24): p. 8369-74.
150. Guo, Y., Z.-Y. Su, and A.-N.T. Kong, *Current Perspectives on Epigenetic Modifications by Dietary Chemopreventive and Herbal Phytochemicals*. *Current Pharmacology Reports*, 2015.
151. Jones, P.A. and S.B. Baylin, *The epigenomics of cancer*. *Cell*, 2007. **128**(4): p. 683-92.
152. Sharma, S., T.K. Kelly, and P.A. Jones, *Epigenetics in cancer*. *Carcinogenesis*, 2010. **31**(1): p. 27-36.
153. Maruyama, R., et al., *Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features*. *Clin Cancer Res*, 2002. **8**(2): p. 514-9.
154. Woodson, K., et al., *Heterogeneous gene methylation patterns among pre-invasive and cancerous lesions of the prostate: a histopathologic study of whole mount prostate specimens*. *Prostate*, 2004. **60**(1): p. 25-31.

155. Yu, S., et al., *Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice*. PLoS One, 2010. **5**(1): p. e8579.
156. Majumdar, S., et al., *Aberrant DNA methylation and prostate cancer*. Curr Genomics, 2011. **12**(7): p. 486-505.
157. Perry, A.S., et al., *The epigenome as a therapeutic target in prostate cancer*. Nat Rev Urol, 2010. **7**(12): p. 668-80.
158. Dammann, R., et al., *The tumor suppressor RASSF1A in human carcinogenesis: an update*. Histol Histopathol, 2005. **20**(2): p. 645-63.
159. Pfeifer, G.P., et al., *Methylation of the RASSF1A gene in human cancers*. Biol Chem, 2002. **383**(6): p. 907-14.
160. Richter, A.M., G.P. Pfeifer, and R.H. Dammann, *The RASSF proteins in cancer; from epigenetic silencing to functional characterization*. Biochim Biophys Acta, 2009. **1796**(2): p. 114-28.
161. Dammann, R., et al., *Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3*. Nat Genet, 2000. **25**(3): p. 315-9.
162. Zhang, H., et al., *Methylation of RASSF1A gene promoter is regulated by p53 and DAXX*. FASEB J, 2013. **27**(1): p. 232-42.
163. Yoon, J.H., R. Dammann, and G.P. Pfeifer, *Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas*. Int J Cancer, 2001. **94**(2): p. 212-7.
164. Byun, D.S., et al., *Frequent epigenetic inactivation of RASSF1A by aberrant promoter hypermethylation in human gastric adenocarcinoma*. Cancer Res, 2001. **61**(19): p. 7034-8.
165. Burbee, D.G., et al., *Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression*. J Natl Cancer Inst, 2001. **93**(9): p. 691-9.
166. Lo, K.W., et al., *High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma*. Cancer Res, 2001. **61**(10): p. 3877-81.
167. Lee, M.G., et al., *Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma*. Cancer Res, 2001. **61**(18): p. 6688-92.
168. Yang, Q., et al., *Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma*. Clin Cancer Res, 2004. **10**(24): p. 8493-500.
169. Dreijerink, K., et al., *The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis*. Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7504-9.
170. Kuzmin, I., et al., *The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells*. Cancer Res, 2002. **62**(12): p. 3498-502.
171. van der Weyden, L., et al., *The RASSF1A isoform of RASSF1 promotes microtubule stability and suppresses tumorigenesis*. Mol Cell Biol, 2005. **25**(18): p. 8356-67.
172. Ge, Y.Z., et al., *The association between RASSF1A promoter methylation and prostate cancer: evidence from 19 published studies*. Tumour Biol, 2014. **35**(4): p. 3881-90.
173. Pan, J., et al., *Association between RASSF1A promoter methylation and prostate cancer: a systematic review and meta-analysis*. PLoS One, 2013. **8**(9): p. e75283.

174. Agarwal, S., et al., *Mahanine restores RASSF1A expression by down-regulating DNMT1 and DNMT3B in prostate cancer cells*. Mol Cancer, 2013. **12**(1): p. 99.
175. Veeranki, O.L., et al., *Cruciferous Vegetables, Isothiocyanates, and Prevention of Bladder Cancer*. Current Pharmacology Reports, 2015.
176. Royston, K.J. and T.O. Tollefsbol, *The Epigenetic Impact of Cruciferous Vegetables on Cancer Prevention*. Curr Pharmacol Rep, 2015. **1**(1): p. 46-51.
177. Beklemisheva, A.A., et al., *Epigenetic mechanism of growth inhibition induced by phenylhexyl isothiocyanate in prostate cancer cells*. Anticancer Res, 2006. **26**(2A): p. 1225-30.
178. Wang, L.G., et al., *Dual action on promoter demethylation and chromatin by an isothiocyanate restored GSTP1 silenced in prostate cancer*. Mol Carcinog, 2007. **46**(1): p. 24-31.
179. Wang, L.G., et al., *De-repression of the p21 promoter in prostate cancer cells by an isothiocyanate via inhibition of HDACs and c-Myc*. Int J Oncol, 2008. **33**(2): p. 375-80.
180. Liu, K., et al., *Synergistic effect of paclitaxel and epigenetic agent phenethyl isothiocyanate on growth inhibition, cell cycle arrest and apoptosis in breast cancer cells*. Cancer Cell Int, 2013. **13**(1): p. 10.
181. Liu, Y., S. Chakravarty, and M. Dey, *Phenethylisothiocyanate alters site- and promoter-specific histone tail modifications in cancer cells*. PLoS One, 2013. **8**(5): p. e64535.
182. Liu, L., et al., *Frequent hypermethylation of the RASSF1A gene in prostate cancer*. Oncogene, 2002. **21**(44): p. 6835-40.
183. Montenegro, M.F., et al., *Reactivation of the tumour suppressor RASSF1A in breast cancer by simultaneous targeting of DNA and E2F1 methylation*. PLoS One, 2012. **7**(12): p. e52231.
184. Gray, S.G., et al., *Gemcitabine reactivates epigenetically silenced genes and functions as a DNA methyltransferase inhibitor*. Int J Mol Med, 2012. **30**(6): p. 1505-11.
185. Hesson, L.B., W.N. Cooper, and F. Latif, *The role of RASSF1A methylation in cancer*. Dis Markers, 2007. **23**(1-2): p. 73-87.
186. Mengxi, D., et al., *Effect of DNA methylation inhibitor on RASSF1A genes expression in non-small cell lung cancer cell line A549 and A549DDP*. Cancer Cell Int, 2013. **13**(1): p. 91.
187. Reeves, M.E., et al., *The RASSF1 Gene and the Opposing Effects of the RASSF1A and RASSF1C Isoforms on Cell Proliferation and Apoptosis*. Mol Biol Int, 2013. **2013**: p. 145096.
188. Jagadeesh, S., et al., *Mahanine reverses an epigenetically silenced tumor suppressor gene RASSF1A in human prostate cancer cells*. Biochem Biophys Res Commun, 2007. **362**(1): p. 212-7.
189. Vardi, A., et al., *Soy phytoestrogens modify DNA methylation of GSTP1, RASSF1A, EPH2 and BRCA1 promoter in prostate cancer cells*. In Vivo, 2010. **24**(4): p. 393-400.
190. Kawamoto, K., et al., *Epigenetic modifications of RASSF1A gene through chromatin remodeling in prostate cancer*. Clin Cancer Res, 2007. **13**(9): p. 2541-8.

191. Donninger, H., et al., *Cell cycle restriction is more important than apoptosis induction for RASSF1A protein tumor suppression*. J Biol Chem, 2014. **289**(45): p. 31287-95.
192. Barve, A., et al., *Murine prostate cancer inhibition by dietary phytochemicals-curcumin and phenethylisothiocyanate*. Pharm Res, 2008. **25**(9): p. 2181-9.
193. Khor, T.O., et al., *Chemoprevention of familial adenomatous polyposis in Apc(Min/+) mice by phenethyl isothiocyanate (PEITC)*. Mol Carcinog, 2008. **47**(5): p. 321-5.
194. Shen, W.J., et al., *Regulation of demethylation and re-expression of RASSF1A gene in gastric cancer cell lines by combined treatment of 5-Aza-CdR and NaB*. World J Gastroenterol, 2008. **14**(4): p. 595-600.
195. Vos, M.D., et al., *A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability*. Cancer Res, 2004. **64**(12): p. 4244-50.
196. Rong, R., et al., *Tumor suppressor RASSF1A is a microtubule-binding protein that stabilizes microtubules and induces G2/M arrest*. Oncogene, 2004. **23**(50): p. 8216-30.
197. Thaler, S., et al., *RASSF1A mediates p21Cip1/Waf1-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt*. Cancer Res, 2009. **69**(5): p. 1748-57.
198. Fu, L. and S. Zhang, *RASSF1A promotes apoptosis and suppresses the proliferation of ovarian cancer cells*. Int J Mol Med, 2014. **33**(5): p. 1153-60.
199. Coussens, L.M. and Z. Werb, *Inflammation and cancer*. Nature, 2002. **420**(6917): p. 860-7.
200. Griffith, O.W. and D.J. Stuehr, *Nitric oxide synthases: properties and catalytic mechanism*. Annu Rev Physiol, 1995. **57**: p. 707-36.
201. Cassini-Vieira, P., et al., *iNOS Activity Modulates Inflammation, Angiogenesis, and Tissue Fibrosis in Polyether-Polyurethane Synthetic Implants*. Mediators Inflamm, 2015. **2015**: p. 138461.
202. Aaltoma, S.H., P.K. Lipponen, and V.M. Kosma, *Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer*. Anticancer Res, 2001. **21**(4B): p. 3101-6.
203. Sukumaran, S., et al., *Pharmacokinetic/pharmacodynamic modeling of methylprednisolone effects on iNOS mRNA expression and nitric oxide during LPS-induced inflammation in rats*. Pharm Res, 2012. **29**(8): p. 2060-9.
204. Thomas, L., *The physiological disturbances produced by endotoxins*. Annu Rev Physiol, 1954. **16**: p. 467-90.
205. Juskewitch, J.E., et al., *LPS-induced murine systemic inflammation is driven by parenchymal cell activation and exclusively predicted by early MCP-1 plasma levels*. Am J Pathol, 2012. **180**(1): p. 32-40.
206. Terenzi, F., et al., *Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages*. J Biol Chem, 1995. **270**(11): p. 6017-21.
207. Melillo, G., et al., *Regulation of nitric-oxide synthase mRNA expression by interferon-gamma and picolinic acid*. J Biol Chem, 1994. **269**(11): p. 8128-33.
208. Yang, Q., et al., *A dual negative regulation model of Toll-like receptor 4 signaling for endotoxin preconditioning in human endotoxemia*. Math Biosci, 2011. **232**(2): p. 151-63.

209. Foteinou, P.T., et al., *Modeling endotoxin-induced systemic inflammation using an indirect response approach*. Math Biosci, 2009. **217**(1): p. 27-42.
210. Gutierrez, V.O., et al., *Curcumin Pharmacokinetic and Pharmacodynamic Evidences in Streptozotocin-Diabetic Rats Support the Antidiabetic Activity to Be via Metabolite(s)*. Evid Based Complement Alternat Med, 2015. **2015**: p. 678218.
211. Shoba, G., et al., *Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers*. Planta Med, 1998. **64**(4): p. 353-6.
212. Jager, R., et al., *Comparative absorption of curcumin formulations*. Nutr J, 2014. **13**: p. 11.
213. Klickovic, U., et al., *Human pharmacokinetics of high dose oral curcumin and its effect on heme oxygenase-1 expression in healthy male subjects*. Biomed Res Int, 2014. **2014**: p. 458592.
214. Boyanapalli, S.S., et al., *Nrf2 knockout attenuates the anti-inflammatory effects of phenethyl isothiocyanate and curcumin*. Chem Res Toxicol, 2014. **27**(12): p. 2036-43.
215. Brouet, I. and H. Ohshima, *Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages*. Biochem Biophys Res Commun, 1995. **206**(2): p. 533-40.
216. Jacob, J.N., et al., *Evaluation of the in vivo anti-inflammatory and analgesic and in vitro anti-cancer activities of curcumin and its derivatives*. Nat Prod Commun, 2013. **8**(3): p. 359-62.
217. Ben, P., et al., *Curcumin promotes degradation of inducible nitric oxide synthase and suppresses its enzyme activity in RAW 264.7 cells*. Int Immunopharmacol, 2011. **11**(2): p. 179-86.
218. Guo, Y., et al., *Curcumin inhibits anchorage-independent growth of HT29 human colon cancer cells by targeting epigenetic restoration of the tumor suppressor gene DLEC1*. Biochem Pharmacol, 2015. **94**(2): p. 69-78.
219. D'Argenio D, S.A., Wang X, *ADAPT 5 user's guide: pharmacokinetic/pharmacodynamic systems analysis software*. 2009.
220. Deguchi, A., *Curcumin targets in inflammation and cancer*. Endocr Metab Immune Disord Drug Targets, 2015. **15**(2): p. 88-96.
221. Boyanapalli, S.S. and A.N. Tony Kong, *"Curcumin, the King of Spices": Epigenetic Regulatory Mechanisms in the Prevention of Cancer, Neurological, and Inflammatory Diseases*. Curr Pharmacol Rep, 2015. **1**(2): p. 129-139.
222. Rodriguez-Paredes, M. and M. Esteller, *Cancer epigenetics reaches mainstream oncology*. Nat Med, 2011. **17**(3): p. 330-9.
223. Demaria, S., et al., *Cancer and inflammation: promise for biologic therapy*. J Immunother, 2010. **33**(4): p. 335-51.
224. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer*. Cell, 2010. **140**(6): p. 883-99.
225. Schmid, J.A. and A. Birbach, *IkkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB*. Cytokine Growth Factor Rev, 2008. **19**(2): p. 157-65.
226. De Marzo, A.M., et al., *Inflammation in prostate carcinogenesis*. Nat Rev Cancer, 2007. **7**(4): p. 256-69.
227. Wang, S.I., R. Parsons, and M. Ittmann, *Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas*. Clin Cancer Res, 1998. **4**(3): p. 811-5.



228. Birbach, A., et al., *Persistent inflammation leads to proliferative neoplasia and loss of smooth muscle cells in a prostate tumor model*. Neoplasia, 2011. **13**(8): p. 692-703.
229. Maxwell, P.J., et al., *Potentiation of inflammatory CXCL8 signalling sustains cell survival in PTEN-deficient prostate carcinoma*. Eur Urol, 2013. **64**(2): p. 177-88.
230. Mirmohammadsadegh, A., et al., *Epigenetic silencing of the PTEN gene in melanoma*. Cancer Res, 2006. **66**(13): p. 6546-52.
231. Phin, S., M.W. Moore, and P.D. Cotter, *Genomic Rearrangements of PTEN in Prostate Cancer*. Front Oncol, 2013. **3**: p. 240.
232. Jiao, J., et al., *Murine cell lines derived from Pten null prostate cancer show the critical role of PTEN in hormone refractory prostate cancer development*. Cancer Res, 2007. **67**(13): p. 6083-91.
233. Traka, M.H., et al., *The dietary isothiocyanate sulforaphane modulates gene expression and alternative gene splicing in a PTEN null preclinical murine model of prostate cancer*. Mol Cancer, 2010. **9**: p. 189.
234. Shanmugam, M.K., et al., *Ursolic acid inhibits the initiation, progression of prostate cancer and prolongs the survival of TRAMP mice by modulating pro-inflammatory pathways*. PLoS One, 2012. **7**(3): p. e32476.
235. Lee, C.H., et al., *Eriobotrya japonica leaf and its triterpenes inhibited lipopolysaccharide-induced cytokines and inducible enzyme production via the nuclear factor-kappaB signaling pathway in lung epithelial cells*. Am J Chin Med, 2008. **36**(6): p. 1185-98.
236. Ma, J.Q., et al., *Ursolic acid ameliorates carbon tetrachloride-induced oxidative DNA damage and inflammation in mouse kidney by inhibiting the STAT3 and NF-kappaB activities*. Int Immunopharmacol, 2014. **21**(2): p. 389-95.
237. Lu, X., et al., *Ursolic acid attenuates diabetic mesangial cell injury through the up-regulation of autophagy via miRNA-21/PTEN/Akt/mTOR suppression*. PLoS One, 2015. **10**(2): p. e0117400.
238. Garcia, A.J., et al., *Pten null prostate epithelium promotes localized myeloid-derived suppressor cell expansion and immune suppression during tumor initiation and progression*. Mol Cell Biol, 2014. **34**(11): p. 2017-28.
239. Liu, X. and A.S. Goldstein, *Inflammation promotes prostate differentiation*. Proc Natl Acad Sci U S A, 2014. **111**(5): p. 1666-7.
240. Kwon, O.J., et al., *Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin*. Proc Natl Acad Sci U S A, 2014. **111**(5): p. E592-600.
241. Patra, S.K., A. Patra, and R. Dahiya, *Histone deacetylase and DNA methyltransferase in human prostate cancer*. Biochem Biophys Res Commun, 2001. **287**(3): p. 705-13.
242. Grabiec, A.M., P.P. Tak, and K.A. Reedquist, *Function of histone deacetylase inhibitors in inflammation*. Crit Rev Immunol, 2011. **31**(3): p. 233-63.
243. Halili, M.A., et al., *Histone deacetylase inhibitors in inflammatory disease*. Curr Top Med Chem, 2009. **9**(3): p. 309-19.
244. Qin, T., et al., *Sinomenine activation of Nrf2 signaling prevents hyperactive inflammation and kidney injury in a mouse model of obstructive nephropathy*. Free Radic Biol Med, 2016. **92**: p. 90-9.

- 245. Zhang, C., et al., *Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation*. *Biochem Pharmacol*, 2013. **85**(9): p. 1398-404.
- 246. Liu, L., et al., *Curcumin ameliorates asthmatic airway inflammation by activating nuclear factor-E2-related factor 2/haem oxygenase (HO)-1 signalling pathway*. *Clin Exp Pharmacol Physiol*, 2015. **42**(5): p. 520-9.
- 247. Amin, K.S. and P.P. Banerjee, *The cellular functions of RASSF1A and its inactivation in prostate cancer*. *J Carcinog*, 2012. **11**: p. 3.
- 248. Mager, D.E., E. Wyka, and W.J. Jusko, *Diversity of mechanism-based pharmacodynamic models*. *Drug Metab Dispos*, 2003. **31**(5): p. 510-8.