MEDICINAL PLANTS WITH ANTIOXIDANT AND ANTIINFLAMMATORY

PROPERTIES IN CANCER PREVENTION

by

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ABSTRACT OF THE DISSERTATION

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Plants extracts have been used in the folk medicine for hundreds of years because it is believed that they constitute a natural approach in dealing with many diseases including cancer. To detect, prevent and treat cancer before it arises is a great promise in personalized medicine and chemopreventive compounds that have the ability to suppress or block tumor progression are an important approach that has gained increasing attention due their low side effects. Many of these plants and their bioactive compounds have the ability to modulate reactive oxidative species, inflammation, apoptosis mediators and Nrf2/ARE signaling pathway. In this dissertation, we have explored the effect of glucosinolates and the main bioactive compounds of *Radix Angelicae Sinensis* and *Matricaria recutita* L. in order to have a better understating on how they are able to activate Nrf2 signaling and the

role that they play in Nrf2 interaction with anti-inflammatory, pro-apoptosis and epigenetic signals. We have also discussed the emerging field of cancer and obesity on that crosstalking of these pathways play a pivotal role worsening cancer outcomes representing an emergent and challenging field for prevention and treatment that needs to be further addressed in the future.

Preface

This dissertation is submitted for the degree of Doctor of Philosophy in Pharmaceutical Science at Rutgers, The State University of New Jersey. It serves as documentation of my research work carried out between September 2010 and December 2014 under the supervision of Dr. Ah-Ng Tony Kong in the Department of Pharmaceutics. To the best of my knowledge, this work is original, except where suitable references are made to previous work.

The dissertation consists of seven chapters. Chapter one introduced the importance of plant extracts in cancer field with focus in chemoprevention and the significance of the research work. The following six chapters contain papers that are published or intended to be submitted to a journal indexed by PubMed Central or that have been included in books. Chapter two illustrated the state of the art of natural glucosinolates biological activities that includes sulforaphane, phenethyl isothiocianate, indole-3-carbinol, 3,3'-diindolylmethane in oxidative stress/inflammation, Nrf2-ARE signaling pathway modulation, epigenomics and *in vivo* cancer chemopreventive efficacy. Chapter three investigated the chemopreventive effect of *Radix Angelicae Sinensis* (RAS) extract and its main bioactive phtalide Z-ligustilide (Ligu) in prostate cancer by using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model focusing in their effects in cell cycle and apoptosis regulation *in vitro* and *in vivo* and epigenetic restoration of silenced Nrf2 *in vivo*.

Chapter four demonstrated the role of apigenin and luteolin from *Matricaria recutita* L. (chamomile) in Nrf2 activation coupled with anti-inflammatory effects in the suppression of LPS-induced inflammation in HepG2 cells. Chapter five investigated the epigenetic modulation exerted by apigenin in 15 critical CpG sites in the promoter region

of Nrf2 in JB6 P+ cells leading to re-expression of Nrf2 and its downstream genes by targeting DNA methyltransferases and histone deacetylases. Chapter six explored the emergent field of obesity, inflammation and cancer providing an overview from epidemiological to molecular events and chapter seven focuses in the relationship between obesity and non-melanoma skin cancer (NMSC) providing insights for future studies in the field of chemoprevention. These chapters explored various aspects of the biological effects of plant extracts and their main active compounds with focus in RAS and chamomile in cancer prevention, Nrf2 activation, anti-inflammatory and pro-apoptotic effects, epigenetic modulation and future directions in cancer research.

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I acknowledge also the encouragement and moral support that I received from my colleagues and friends in the clinical setting to pursue my career further. Finally, I owe my

deepest gratitude to my family and friends who offered me their unconditional love and understanding throughout all this time.

Dedication

I wish to dedicate this thesis to my mom, my grandparents, my husband, my daughter, my parents in law and my friends. Their unconditional love, support, patient and guidance inspired me to be the person I am today and to continue my travel in this life. This work is especially dedicated to the memory of all my patients who lost the battle against cancer and the healthcare professionals who believe that finding a cure and provide better quality of life for cancer patients are possible. Very especially, I wish to dedicate this work to all minority students in STEM from underrepresented countries and specially women, who struggle hard to persevere with their goals and balance personal life and work. Hopefully, this dissertation serves as an inspiration that everything is possible.

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5-azadeoxycytidine	5-aza
3.3'-diindolylmethane	DIM
7,12-dimethylbenz[a]anthracene	DMBA
12-O-tetradecanoylphorbol 13-acetate	TPA
Antioxidant response element	ARE
Apigenin	API
Aryl-hydrocarbon receptor	AhR
Azoxymethane	AOM
Body mass index	BMI
Cytosolic phospholipase A2	cPLA2
Dextran sodium sulfate	DSS
Dimethyl sulfoxide	DMSO
DNA methyltransferase	DNMT
Erythroid 2p45 (NF-E2)-related factor 2	Nrf2
Fatty acid	FA
Genitourinary apparatus	GU
Heme oxygenase-1	HO-1

High-fat diet	HFD
Histone deacetylase	HDAC
Indole-3-carbinol	I3C
Inducible nitric oxide synthase	iNOS
Insulin grow factor 1	IGF-1
Interleukin	IL
Isothiocyanates	ITCs
Lipopolysaccharide	LPS
Luteolin	LUT
Macrophage chemo-attractant protein 1	MCP1
MicroRNA	miRNA
Mitogen-activated protein kinases	MAPK
Monocyte chemoattractant protein-1	MCP-1
NAD (P)H:quinone oxidoreductase 1	NQO-1
Nitric oxide	NO
Non-melanoma skin cancer	NMSC
Nuclear factor kappa B	NF-κB
Phenethyl isothiocyanate	PEITC

Phosphatidylinositol 3-kinase	PI3K
Plasminogen activator inhibitor type 1	SERPINE1
Prostate Cancer	PCa
Prostatic intraepithelial neoplasia	PIN
Radix Angelicae Sinensis	RAS
Reactive oxygen species	ROS
Sulforaphane	SFN
Tissue inhibitor of metalloproteinases-1	TIMP-1
Toll-like receptor	TLR
Transgenic adenocarcinoma of mouse prostate	TRAMP
Trichostatin A	TSA
Tumor-associated macrophage	TAM
Twist-related protein	TWIST
White adipose tissue	WAT
Z-Ligustilide	Lig

Chapter 1.   Introduction to the studies

Over many centuries plant extracts have been used to treat a broad variety of diseases and they have played important roles in the development of many traditional anticancer agents. It is estimated that almost 50% of the 175 new worldwide approved small molecule with antitumor activities (from 1940s to 2010) are either natural products or directly derived from them [1]. Drugs commonly used in cancer treatment that have been obtained from plants are for example: etoposide and tenoposide from *Podophyllum peltatum*, vincristine and vinblastine from *Catharanthus roseus*, irinotecan from *Camptotheca acuminata* and paclitaxel from *Taxus brevifolia* [2]. Plant extracts can target several pathways against carcinogenesis including inhibition of cell division, induction of apoptosis, targeting oxidative stress and blocking inflammation and it is believed that their main bioactive compounds may act alone or in concert to reduce cancer risk.

Growing evidence supports that these anticancer effects are exerted in part by their ability to modulate biological activities related with balance of reactive oxygen species (ROS). ROS are highly reactive molecules or molecular fragments which are generated through various processes such as ultraviolet irradiation, inflammatory processes, lipid peroxidation, mitochondria-catalyzed electron transport reactions and environmental pollutants [3]. They are important secondary messengers in cell signaling that are necessary for maintaining various biological processes in normal cells and their balance is tightly controlled to maintain cellular homeostasis. In this context, NF-E2-related factor 2 (Nrf2) that is a transcription factor from the basic leucine zipper family plays an important role regulating the antioxidant defense system. Nrf2 activity is regulated in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) that is homolog of the Drosophila actin-

binding protein Kelch, a cytoskeleton protein capable of binding actin filaments preventing Nrf2 translocation into the nucleus acting as transcriptional repressor during basal conditions. Nrf2 is rapidly targeted towards ubiquitination and 26S proteasomal degradation dependent of Keap-1 in an extremely efficient system [3].

Nrf2 release from Keap-1 can be triggered by several effectors including modification of cysteine residues in Keap1, Nrf2 phosphorylation by Protein Kinase C (PKC) at Ser40, direct phosphorylation of Nrf2 by the MAPK cascade including extracellular signal-regulated kinases (ERKs), JNK, and protein 38 (p38) or phosphorylation mediated by phosphoinositide-3-kinase (PI3K) [4, 5]. Indeed, Nrf2 possess various threonine, tyrosine and serine residues that can be targeted by phosphorylation (Appendix A). After release, Nrf2 translocates into the nucleus and heterodimerizes with small Maf (musculoaponeurotic fibrosarcoma) proteins, activates transcription factor (ATF) and/or members of the AP-1 family of leucine zipper proteins to trigger the transcription of its target genes. Most of the antioxidant genes (phase II genes) contain cis-acting antioxidant response elements (AREs) with a functional consensus sequence of 5′-RTGAYnnnGCR-3′ on that R = A or G and Y = C or T. Nrf2 recognizes and binds this cis-acting ARE region and it may recruits the whole transcription machinery including coactivators and/or corepressor such as p160 family coactivator, CREB-binding protein (CBP)/p300, RNA polymerase II, silencing mediator of retinoid and thyroid receptors (SMRT), histone deacetylase (HDAC) and others to produce activation/inactivation of Nrf2-targeted ARE gene expression [4, 5]. ARE genes include over 100 cytoprotective and detoxification genes, including ferritin, the conjugating enzymes glutathione-S-reductase (GSR), reduction enzymes NAD(P)H:quinone

oxidoreductase 1 (NQO1), and stress response enzymes such as heme oxygenase-1 (HO-1) [4, 5].

Increasing evidence strongly suggest an important cross talking between Nrf2 signaling pathway and Nuclear factor-kappa B (NF-κB). NF-κB is important because it is involved in the expression of over 500 genes involved in human diseases [6]. Potent NF-κB signaling pathway activators are the pro-inflammatory cytokines such as (IL-1, IL-17, IL-18, TNF family), epidermal growth factor (EGF), lipopolysaccharides (LPS) and ROS among many others. NF-κB is a central coordinator of immune responses and inflammation that is highly controlled under normal conditions however, constitutive activation and overexpression are often observed in many cancers [6]. NF-κB family consist of five members: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA or p65, RelB, and c-Rel with a highly conserved N-terminal region of 300 amino acid called the Rel Homology Domain. NF-κB/ Rel proteins are able to target several factors due their ability to combine and form homodimers or heterodimers (with the exception of RelB) such as p50/RelA, RelA/RelA, p50/p50, p50/cRel, p52/c-Rel that are able to process differential binding specificities and target gene activation [2, 5, 7]. After activation, nuclear translocation and post-transcriptional modification of NF-κB/ Rel-family transcription factors trigger the expression of a variety of proteins involved in different aspects of carcinogenesis including cell proliferation, survival, inflammation, angiogenesis, epithelial–mesenchymal transition, invasion and metastasis [2, 5, 7]. On the other hand, the transcriptional activity NF-κB /Rel proteins is silenced by interactions with inhibitory IkB family of proteins present in the cytoplasm (IkBa, IkBb, IkBe, IkBg or NF-κB essential modulator, Bcl-3, and the precursor proteins p100 and p105) [2, 5, 7].

The best characterized NF-κB pathway is the classical or canonical, that is activated by TNF, Toll like, or T cell by a variety of stimuli including cytokines, LPS and ROS [2, 5, 7]. The classical pathway is important because is related to the transcriptional activity of the p50/p65 heterodimer which it is believed being intimately involved in inflammatory response [2, 7, 8]. After binding to the receptor, a serial recruitment of adaptors to the cytoplasmic domain of the receptor is produced. The adaptors in this pathway include: Myeloid differentiation primary response gene (MyD88), Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF), transforming growth factor beta activated kinase-1 (TAK1) and they produce the recruitment into the cytoplasm of an IKK complex formed by the catalytic kinase subunits IKKβ and IKKα, and the regulatory non-enzymatic scaffold protein IKKγ (that is also known as NF-κB essential modulator or NEMO) [2, 7, 8]. The inactivation of NF-κB is mediated in the cytoplasm by regulatory proteins called inhibitors of κB (IκB) and the most important are IκBα, IκBβ, and IκBε [2, 7, 8]. When the IKK complex is activated it mediates the phosphorylation of IκB proteins that leads to ubiquitination and proteasomal degradation of the IkB inhibitor by the 26S subunit freeing the NF-κB/Rel complexes in the cytoplasm [2, 7, 8]. After phosphorylation-mediated activation the NF-κB/Rel complexes translocate into the nucleus and induce target gene expression by binding to κB enhancer elements of target genes [2, 7, 8]. NF-κB activation produce by itself the activation of the IkBa gene that silence NF-κB subunits and terminates the transcriptional activity [2, 7, 8].

Interestingly, it has been observed that Nrf2-deficient mice under conditions of head injury show a greater cerebral NF-κB activation compared with their Nrf2 wild-type

mice [9]. Similarly, enhanced inflammation has been reported in Nr2 knockout mice challenged with dextran sulfate sodium (DSS)-induced inflammation and in Nrf2 knockout mice exposed to UVB irradiation [10-13]. Conversely, Nrf2 overexpression has been reported strongly decrease NF-κB activity [14]. Although, the regulatory loop between Nrf2 and NF-κB signaling pathways is not totally elucidated it seems that the ability of Nrf2-ARE-mediated gene activation can induce NF-κB inactivation by different mechanisms. For example, S-glutathionylation of p65 NF-κB can produce NF-κB inactivation [15]. Also, it has been observed that inhibition of NF-κB activation by phospholipid hydroperoxide glutathione peroxidase and 15-lipoxygenase is concomitant with up-regulation of HO-1 one of the key targets genes of Nrf2 signaling pathway. HO-1 exhibits important immunomodulatory and anti-inflammatory functions and it is able to attenuate the expression of cyclooxygenase-2, iNOS, TNF-α and IL-6 after LPS challenge.

Likewise, NF-κB p65 subunit is able to repress the transcriptional activity of Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating the recruitment of the corepressor HDAC3 with either CBP or MafK [5, 16]. Indeed, multiple alignments of Nrf2 and NF-κB 1 genes in five mammalian species – human, chimpanzee, dog, mouse and rat-showed a canonical first-generation biological network for Nrf2–NF-κB1 involved in inflammation/carcinogenesis and an important amount of mediators are MAPKs family members [17]. It is believed that redox-based regulation of Nrf2 and NF-κB cell signaling plays a pivotal role in this relationship and although the relationship between Nrf2, NF-κB and cell survival pathways at the transcription and transduction level are not fully understood; growing evidence indicates that they are closely related impacting tumorigenesis [18, 19]. Furthermore, it has been observed that many natural compounds

and plant extracts that have the ability to trigger Nrf2-ARE system, inflammatory responses are also able to target cancer cell survival pathways continuing an interesting approach for cancer chemoprevention. In this dissertation, we will discuss molecular and epigenetic targets regulated by plant phytochemicals and their main bioactive compounds with focus in Nrf2 signaling, NF-κB pathway, apoptosis and epigenetic modulation.

Chapter 2.   Dietary Glucosinolates Sulforaphane, Phenethyl Isothiocyanate, Indole-3-Carbinol/3,3'-Diindolylmethane: Antioxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and *in vivo* cancer chemopreventive efficacy [1,2,3]

2.1.   Introduction

Cancer chemoprevention is a major cancer preventive strategy that utilizes naturally occurring dietary phytochemicals or therapeutic drugs with relatively low toxicity to inhibit the malignant transformation of initiated cells at the promotion or progression stages [20, 21]. Thus, chemoprevention can involve preventing carcinogens from reaching target sites, undergoing metabolic activation, or subsequently interacting with crucial cellular macromolecules (e.g., DNA, RNA, and proteins) at the initiation stage [4, 22]. Interestingly, prevention and/or protection from chemical carcinogens by phytochemicals present in glucosinolate-containing cruciferous vegetables is of great interest because they

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[1] This chapter has been submitted for publication as *review article* to Current Pharmacology Reports as "Dietary Glucosinolates Sulphoraphane, PEITC, DIM, I3C/DIM: Antioxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and *in vivo* cancer chemopreventive efficacy" by Fuentes F*, Paredes-Gonzalez X * and Kong AN. **Equal contribution*

[2] Key Words: cancer, chemoprevention, DNA methylation, epigenetics, histone modifications, isothiocyanates, indoles, microRNA

[3] Abbreviations: SFN, sulforaphane; PEITC, phenethyl isothiocyanate; I3C, indole-3-carbinol; DIM, 3.3'-diindolylmethane; DMBA, 7,12-dimethylbenz[a]anthracene; AOM, azoxymethane; DSS, dextran sodium sulfate; TPA, 12-O-tetradecanoylphorbol 13-acetate.

may provide a safe and cost-effective strategy for combating cancer [23, 24]. In this context, numerous epidemiological and pharmacological studies have revealed that the consumption of cruciferous vegetables has substantial potential for human cancer chemoprevention [25].

Isothiocyanates (ITCs) and indoles are biologically active molecules formed from glucosinolate precursors present in a large number of edible species existing in sixteen families of dicotyledonous angiosperms [26]. It has been described more than 200 different naturally-occurring glucosinolates isolated from plants, with a relatively high content in cruciferous vegetables such as broccoli, cabbage, cauliflower, turnip, horseradish, watercress, and brussels sprouts [27, 28]. Some naturally occurring glucosinolates and their breakdown products have received considerable attention as chemopreventive agents, including the ITCs 4-methylsulphinylbutyl isothiocyanate (sulforaphane, SFN) and phenethyl isothiocyanate (PEITC); and the indoles indole-3-carbinol (I3C) and 3.3'-diindolylmethane (DIM) [25, 29, 30]. The glucosinolates have undergone several human clinical trials for treatment evaluation for various diseases, including cancer (www.clinicaltrials.gov). Thus, the protective role of dietary glucosinolates has been extensively studied using *in vitro* and *in vivo* approaches in cancer and cardiovascular and neurological diseases using rodent and human models [31, 32]. These studies have shown that glucosinolates and their derivatives may modulate many relevant processes, such as the induction of cytoprotective enzymes, inhibition of inflammatory processes, modulation of cancer signaling pathways including cellular proliferation, angiogenesis, the epithelial–mesenchymal transition, cancer stem cell self-renewal and suppressing diverse oncogenic signaling pathways, including nuclear factor-κB, hormone receptor, and signal transducer

and activator of transcription [33-35]. More recently, increasing evidence has also shown that glucosinolate derivatives have the potential to modulate epigenetic alterations, such as DNA methylation, histone modifications, non-coding microRNAs (miRNAs), regulation of polycomb group proteins and epigenetic cofactor modifiers, which all may contribute to carcinogenesis [33, 36]. Here, we review the cancer chemopreventive role of naturally occurring glucosinolate derivatives as inhibitors of carcinogenesis, particularly emphasizing specific molecular and epigenetic alterations in *in vitro* and *in vivo* animal models of human cancers.

2.2.   Biosynthesis and metabolism of glucosinolates

Glucosinolates are a group of sulfur-containing glycosides found in the plant order Brassicales, which includes the Brassica or Cruciferous vegetables such as broccoli, cabbage, brussels, and cauliflower [37]. These plants have been used for food or medicinal purposes, with the latter partially due to their relatively high content of glucosinolates, which distinguish them from other plant species [38]. Thus far, nearly 200 different glucosinolates with different substituents have been reported, which can be classified into three groups based on the structure of different amino acid precursors: aliphatic glucosinolates, indole glucosinolates, and aromatic glucosinolates [28] (Figure 2.1.A). The content of glucosinolate in plants depends on many factors, such as plant variety, growing conditions, climate and the tissue-specific distribution in a plant [39]. For example, in Brassica vegetables, 0.5-28 μmol aliphatic/aromatic glucosinolates per gram of dry weight and 0.7–8 μmol indole glucosinolates per gram of dry weight have been reported [38]. Glucosinolates are relatively biologically inert glucosides; however, their hydrolysis by myrosinase (b-thioglucosidase) enzymes after chopping vegetables, chewing of raw

vegetables or insect attack leads to the conversion of biologically active compounds, such as ITCs, thiocyanates, nitriles and epithionitriles, depending on glucosinolate substrate, pH, temperature, presence of ferrous ions, and level and activity of specific protein factors, including thiocyanate-forming protein (TFP) and epithiospecifier protein (ESP) [37, 39] (Figure 1.B). Nevertheless, when the plant myrosinase enzyme is inactivated by heat during the cooking process, the action of myrosinase originated from gastrointestinal tract bacteria allows the formation and absorption of dietary ITCs and indoles in mammals [40].

Several epidemiological and pharmacological studies have demonstrated that dietary glucosinolates and their breakdown products, isothiocyanates, may reduce the risk of carcinogenesis and particular human diseases [32]. Isothiocyanates from dietary vegetables currently investigated for use as chemopreventive agents include SFN from broccoli, cauliflower, and kale, PEITC from watercress, radish and turnip, allyl isothiocyanate (AITC) from cabbage, mustard, and horseradish, benzyl isothiocyanate (BITC) from lepidium cress, 3-methylsulfinylpropyl (iberin) from broccoli, brussels sprouts and cabbage, 4-methylthiobutyl from arugula, and 3-methylthiopropyl from cabbage [31]. Similarly, the indole I3C, which upon exposure to gastric acid undergoes self-condensation to form DIM, is also present in cruciferous vegetables, including broccoli, cabbage, cauliflower, brussels sprouts, collard greens and kale and is used as a chemopreventive agent [41].

After ingestion, isothiocyanates are absorbed from the gastrointestinal tract by passive diffusion into the capillary blood network, reversibly binding to free plasma protein thiols (protein thiocarbamoylation) and crossing the plasma membrane into the cells of tissues [38]. Thus, isothiocyanates are metabolized by the mercapturic acid pathway and

initially conjugated to glutathione by glutathione S-transferases (GSTs) and successively cleaved by γ-glutamyltranspeptidase (γ-GT), cysteinylglycinase (CGase), and N-acetyltransferase (AT), creating N-acetylcysteine conjugates (mercapturic acids), which are transported into the kidney and actively secreted in urine for elimination from the body (Figure 2.1.C) [32].

2.3. Dietary glucosinolate derivatives and modulation of phase I and phase II biotransformation enzymes

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is an essential metabolic process for maintaining cellular chemical homeostasis; however, their production at low to moderate concentrations is essential for normal physiological processes [42]. Consequently, the oxidative stress produced by high levels of ROS/RNS during normal cell metabolism leads to potential damage, causing oxidative damage to large biomolecules, such as lipids, proteins, and DNA, which may eventually lead to mutations and ultimately, cancer development [43]. Similarly, oxidative stress also has a significant association with many other chronic diseases, such as neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis), cardiovascular disease, diabetes, and inflammatory diseases [44-46]. In this context, the major chemoprevention mechanisms mediated by dietary glucosinolate derivatives include modulation of phase I drug metabolic enzymes (e.g., cytochrome P450 family, CYP), which prevent procarcinogenic molecule formation and the induction of phase II/detoxifying enzymes (e.g., GST; UDP-glucuronosyl transferases, and UGT), which catalyze conjugation reactions to inactivate or detoxify exogenous (e.g., carcinogens and

other xenobiotics) and endogenous compounds (e.g., sex steroid hormones) related to cancer development [47-49].

Most evidence suggests that dietary glucosinolate derivatives upregulate phase II/detoxifying enzymes through interaction with the cytoplasmic-anchoring protein Kelch-like ECH-associated protein 1 (Keap 1), which represses the transcription factor NF-E2-related factor 2 (Nrf2), a basic-region leucine zipper (bZIP) transcription factor that binds in combination with small Maf proteins to antioxidant response elements (AREs) in the promoter regions of many antioxidant and phase II biotransformation enzymes, including GST, UGT, heme oxygenase-1 (HO-1), NADP(H):quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCL) and gamma glutamylcysteine synthetase (γGCS) (Figure 2.2) [50, 51]. Thus, the effects of dietary glucosinolate derivatives upregulating phase II enzymes have been extensively reported using different *in vivo* and *in vitro* approaches [31, 52]. For example, sulfur-containing dietary glucosinolate derivatives, such as SFN and PEITC, are potent phase II gene inducers, and these inductions are Nrf2-dependent [31, 53]. SFN attenuates Nrf2 degradation by modifying the Keap1-Nrf2 interaction, which results in the translocation of Nrf2. SFN can react with thiols within Keap1 by forming thionoacyl adducts, thereby releasing Nrf2 from Keap1 binding [54]. Similarly, PEITC can induce the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) and subsequently, phosphorylate Nrf2 and induce its nuclear translocation [55, 56]. Indole glucosinolate hydrolysis products, such as I3C and DIM, also induce both phase I drug metabolic and phase II/detoxifying enzymes by direct interaction with aryl-hydrocarbon receptor (AhR) or increasing the binding affinity of AhR to xenobiotic response elements (XREs) in target genes [49]. Upon binding chemical ligands,

cytosolic AhR translocates into the nucleus and dimerizes with its nuclear protein partner Ah receptor nuclear translocator (ArnT), and then, the AhR complex binds to specific DNA sequences and activates transcription [50] (Figure 2.2).

The activation of phase II gene expression and enzyme activity by dietary glucosinolate derivatives has been well documented in *in vitro* and *in vivo* studies. For example, different studies have reported that SFN significantly induces phase II enzyme expression and activity in human and mouse cells lines, including LNCaP, PC-3, TSU-Pr1, MDA PCa 2a, MDA PCa 2b, MDA-MB-231, TRAMP-C1, HeLa, HT-29, Caco2, HepG2, Hepa1c1c7 and MCF-7 [57-61]. In contrast, we found that SFN is capable of inhibiting 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumorigenesis in C57BL/6 mice mediated by Nrf2 [62]. More recently, we also demonstrated that re-expression of Nrf2 and the subsequent induction of Nrf2 downstream target genes are involved in the cellular protection mediated by SFN during TPA-induced tumor transformation in mouse skin epidermal JB6 (JB6 P+) cells, suggesting the anti-cancer effects of SFN against the TPA-induced neoplastic transformation of mouse skin [63]. Similarly, we also demonstrated that PEITC enhances the expression of various genes, including drug detoxifying enzymes, through the Nrf2 signaling pathway in *in vivo* and *ex vivo* studies [64, 65]. Furthermore, PEITC has been demonstrated to stimulate tissue differences in the modulation of rat cytochrome P450 and phase II conjugation systems, showing increased hepatic GST activity, although not in the lung or kidney [66]. Similarly, the expression of the antioxidant enzyme HO-1 has also been shown to be strongly increased by PEITC treatment in PC-3 cells [56]. Interestingly, differences in the basal expression level of Nrf2 and resultant changes in GSH levels in

human breast cancer cell lines may be an important determinant of sensitivity to PEITC-induced apoptosis [67]. Indole-containing compounds, such as I3C and DIM, have also been described to possess potent cancer chemopreventive effects, potentially through multi-targets [68], including the induction of endogenous Nrf2, phase II genes (e.g., GSTm2, UGT1A1 and NQO1) and antioxidant genes (e.g., HO-1 and SOD1), as reported in a human liver hepatoma cell line (HepG2-C8) [69].

2.4. Dietary glucosinolate derivatives and inflammation modulation

Sustained generation of ROS/RNS has been shown to contribute to the pathological consequences of chronic inflammation, which is believed to be the cause of many human diseases, including cancer [70]. If this crosstalk between inflammation and oxidative stress is prolonged, excessive cellular ROS/RNS will be produced, resulting in genetic changes and/or epigenetic alterations, which lead to the deregulation of oncogenes and tumor suppressor genes [24, 71]. Cytokines, chemokines, nuclear factor (NF)-κB, nitric oxide synthase-2 (NOS2), cyclooxygenase-2 (COX2), hypoxia inducible factor-1α (HIF1-α), signal transducer and activator of transcription 3 (STAT3), Nrf2 and nuclear factor of activated T cells (NFAT) are key molecular players linking inflammation to cancer [72]. In this context, Nrf2 is a crucial regulator that has been shown to modulate the innate immune response and survival during experimental sepsis using Nrf2-deficient mice and Nrf2-deficient mouse embryonic fibroblasts [73]. Some findings have suggested that there is crosstalk between Nrf2 and inflammation [74]. Interestingly, the Nrf2 pathway has been connected to the inflammatory response in studies using the TRAMP mouse model of prostate carcinogenesis [75]. Similarly, lower induction of phase II antioxidant and detoxification enzymes, such as HO-1, NQO1, UGT1A1, and GSTM1, and higher

induction of proinflammatory biomarkers, such as interleukin IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), and COX2, were observed in Nrf2-KO mice [76].

NF-κB is a transcription factor and a key molecular link between inflammation and cancer that regulates several genes whose products inhibit apoptosis and enhance cell cycle progression, angiogenesis and metastasis [70, 77]. Additionally, a considerable number of NF-κB target genes encode mediators of the innate immune response and inflammation, which include cytokines, chemokines, proteases, NOS2 and COX2 [70, 78]. In this context, dietary glucosinolate derivatives have been shown to inhibit NF-κB-mediated processes *in vitro* and *in vivo*, playing an important role because NF-κB is involved in the expression of over 500 genes involved in human diseases, including cancer [33, 79]. Thus, glucosinolate derivatives are capable of inhibiting NF-κB regulated pathways triggered by these activators by blocking pro-inflammatory signals at various levels; however, the molecular mechanisms by which these interactions are exerted are complex and poorly understood [33, 79].

Several cellular targets of glucosinolate derivatives have been investigated for modulating the NF-κB signaling pathway. For example, SFN is capable of suppressing the TLR4 signaling cascade by affecting the downstream effectors MyD88, p38 mitogen-activated protein kinase (MAPK) and JNK by interacting with glutathione or other redox regulators, such as thioredoxin or Ref-1, which are indirectly capable of impairing NF-κB DNA binding ability and directly binding the essential thiol groups of p50, affecting NF-κB DNA binding with the potential involvement of Akt regulation [33, 80, 81]. In addition, DIM, PEITC and SFN have been described to repress IKK/IkB phosphorylation and p65

NF-κB nuclear translocation, inhibiting the transcriptional activity of NF-κB and affecting important mediators, such as IL-6, iNOS, TNF-α and COX-2 [33, 82, 83]. Similarly, PEITC is also capable of decreasing the iNOS and COX-2 protein expression levels, leading to reduced expression of both pro-inflammatory mediators, and has also been reported to suppress the phosphorylation of interferon regulatory factor 3 (IRF3) induced by stimulation of the Toll-like receptor that decreases the activation of type I interferons (IFNs) and IFN-inducible genes [31, 84].

Recent evidence suggests an important crosstalk between NF-κB and Nrf2 signaling, and the strong mechanism by which glucosinolate derivatives affect NF-κB may be partially mediated by their ability to activate the Nrf2-ARE signaling cascade (Figure 2.2). For example, Nrf2 knockout mice subjected to pro-inflammatory stimuli simultaneously demonstrated decreased levels of anti-oxidant/phase 2 enzymes and upregulation of NF-κB pro-inflammatory mediators, such as COX-2, iNOS, IL-1, IL-6, cPLA2 and TNF-α [76, 85-87]. Modulation of Nrf2 and NF-κB crosstalk is not well characterized, but it appears to occur through a common MAPK network because common regulatory sequences in the transactivation domains of Nrf2 and NF-κB have been described [33]. In addition, NF-κB can antagonize Nrf2 activity at the transcriptional level by interacting with the co-activator CREB-binding protein (CBP), which is required for translocation, and concomitant recruitment of histone deacetylase (HDAC). In contrast, ARE-mediated gene activation by Nrf2 can inactivate NF-κB by different mechanisms. For example, upregulation of HO-1, one of the key target genes of the Nrf2 signaling pathway, has been suggested to inhibit NF-κB nuclear translocation [88]. Additionally, GSH/Grx-1-dependent *S*-glutathionylation of p65 NF-κB produces NF-κB inactivation [89].

Accordingly, C57BL/6 mice pre-treated with SFN in the presence of dextran sodium sulfate (DSS) demonstrated significantly reduced expression of inflammatory markers, such as IL-6 and interferon γ, with increased expression of Nrf2-dependent genes [90]. Similarly, SFN treatment of WT but not Nrf2 KO mice restored the number of sunburn cells to their basal level post-UVB irradiation, demonstrating decreased inflammatory biomarker activity in SFN-treated WT compared with Nrf2 KO mice, revealing a protective role for Nrf2 when activated by SFN against UVB-induced skin inflammation [91]. Moreover, SFN has also been reported to induce significant downregulation of pro-inflammatory microRNA-155 by epigenetic mechanisms that together with the regulation of other target NF-κB coactivators, such as CCAAT-enhancer binding proteins, cAMP response element binding protein, and activator protein-1 (AP-1), open new frontiers in the complex activities exerted by glucosinolate derivatives [31, 92].

2.5. Dietary glucosinolate derivatives and epigenetic mechanisms modulating carcinogenesis, inflammation and reactive oxygen species.

Epigenetic regulation comprises DNA modifications without changes in sequence that result in changes in gene expression or phenotype [49]. Recently, a large amount of evidence has demonstrated that epigenetic alterations, such as DNA methylation, histone modifications and non-coding miRNAs, consistently contribute to carcinogenesis, and constituents in the diet, including dietary glucosinolate derivatives, have the potential to alter a number of these epigenetic events [33, 36, 44]. Although most research on the cellular effects of dietary glucosinolate derivatives has primarily focused on detoxifying enzyme effects, increasing evidence has demonstrated the chemopreventive effects of dietary glucosinolate derivatives on the regulation of silenced genes in cancer.

DNA methylation was the first epigenetic alteration to be observed in cancer cells, and it represents the most common molecular alteration in the origin of many cancers [93, 94]. DNA methylation occurs at the 5' position of cytosine residues within CpG dinucleotides through addition of a methyl group by DNA methyltransferases (DNMTs), which include DNMT1, DNMT3A and DNMT3B, leading to transcriptional silencing of tumor suppressors and other genes with important biological functions. Conversely, global hypomethylation causes genome instability and inappropriate activation of oncogenes and transposable elements [44, 95, 96]. In this context, dietary glucosinolate derivatives, such as SFN, PEITC and DIM, have been shown to inhibit the carcinogenic process, enhance xenobiotic metabolism, induce cell cycle arrest and apoptosis and affect the cancer epigenome in various human cancers and cancer mouse models, demonstrating relevance as chemopreventive agents [31, 41, 97]. In different studies, the treatment of human and mouse cells with different dietary glucosinolate derivatives has resulted in the downregulation of DNMT activity, with concomitant promoter demethylation and re-expression of genes such as GSTP1 (glutathione S-transferase pi 1), Nrf2, hTERT (telomerase reverse transcriptase), TGFBR1 (transforming growth factor, beta receptor I) and CYR61 (Cysteine-rich angiogenic inducer 61) (Table 2.1) [29, 41, 58, 63, 98-101].

Interestingly, Wong et al. described the genome-wide effects of SFN and DIM on promoter methylation in normal prostate epithelial cells and prostate cancer cells [29]. Accordingly, both SFN and DIM treatment decreased the expression of DNMTs in normal prostate epithelial cells (PrEC) and androgen-dependent (LNCaP) and androgen-independent (PC3) prostate cancer cells. Specifically, SFN and DIM altered promoter methylation in different sets of genes in normal prostate epithelial cells and prostate cancer

cells; however, they shared similar gene targets in a single cell line, reversing many of the cancer-associated methylation alterations, including aberrantly methylated genes that are dysregulated during cancer progression (e.g., cell migration, cell adhesion, cell-cell signaling, and transcriptional regulation).

Histone modifications have been broadly recognized as critically important triggers of gene silencing via post-translational modifications of histones at amino-terminal tails [44]. For example, the open chromatin state and gene activation is mediated by histone acetyltransferases (HATs), which transfer acetyl groups to the ε-amino group of lysine residues in histone tails, whereas the condensed chromatin state and the respective gene silencing is commonly regulated by HDAC enzymes, which remove histone acetyl groups by catalyzing their transfer to coenzyme A (CoA) [44, 102]. Similarly, the histone methylation of lysine and arginine residues mediated by histone methyltransferases (HMTs) and demethylases (HDMs) has also been described as a mechanism activating or repressing the gene expression in various forms of cancer [33, 103]. For example, methylation of H3K4, H3K36 and H3K79 has been associated with transcriptionally active chromatin, whereas methylation of H3K9, H3K27 and H4K20 has been associated with transcriptionally repressed chromatin, constituting two of the important silencing mechanisms in mammalian cells [44, 104].

Thus far, several studies have demonstrated the effects of dietary glucosinolate derivatives on histone modification mechanisms in *in vitro* and *in vivo* animal cancer models [24, 36]. Thus, the HDAC inhibitory activity of different dietary glucosinolate derivatives has been widely reported to alter the tumorigenesis processes, with a concomitant increase in the expression of tumor suppressor, pro-apoptotic, anti-oxidant

and anti-inflammatory genes [63, 105-107]. Dietary glucosinolate derivatives, such as SFN, PEITC and DIM, have been specifically associated with HDAC inhibitory activity in the peripheral blood mononuclear cells of human patients who consumed broccoli sprouts, different cancer cell lines (kidney, colon, prostate, leukemia and breast) and *in vivo* and *in vitro* cancer mouse models, such as APCmin/+ and TRAMP-C1 mice and the JB6P+ skin cell line (Table 2.1) [58, 63, 105, 108]. Specifically, SFN and PEITC treatments in human breast and prostate cancer cell lines have been shown to increase H3Ac, H3K9Ac and H4Ac acetylation and H3K4 methylation and decrease the methylation of H3K9 and H3K27 [41, 100, 106]. Similarly, DIM treatments in the androgen-dependent LNCaP prostate cancer cell line have also been demonstrated to increase H3K4me3 in the promoter regions of the TGFBR1 and CYR61 genes, as revealed by ChIP assays [29]. Interestingly, analysis of the impact of SFN on the level and function of polycomb group (PcG) proteins in SCC-13 skin cancer cells revealed that SFN treatment causes a concentration-dependent reduction in PcG protein (Bmi-1, Ezh2) expression and reduced histone H3 lysine 27 trimethylation, which is correlated with the accumulation of cells in G2/M phase, reduced levels of cyclin B1, cyclin A, cyclin dependent kinases 1 and 2, and increased p21Cip1 expression [109].

These results were also observed in other skin-derived immortalized cells and transformed cell lines. In contrast, DIM has been reported to significantly decrease HDAC2 protein expression but not HDAC1, HDAC3, HDAC4, HDAC6 or HDAC8 protein expression in androgen-insensitive PC-3 and androgen-sensitive LNCaP prostate cancer cell lines [110]. Interestingly, the same study was observed that I3C treatment slightly inhibits HDAC activity in LNCaP cells with no HDAC inhibition in PC-3 cells. Similarly,

DIM has been shown to suppress the expression of the HDAC2 and HDAC3 proteins in TRAMP-C1 cells, with a concomitant increase in apoptosis, decrease in cell proliferation and enhanced Nrf2 and Nrf2-target gene NQO1 expression in prostate tissues [101]. DIM can selectively induce the proteasome-mediated degradation of class I histone deacetylases (HDAC1, HDAC2, HDAC3, and HDAC8) without affecting class II HDAC proteins in human colon cancer cells *in vitro* and *in vivo* in tumor xenografts [111]. Thus, the HDAC depletion was associated with DNA damage induction, which triggered apoptosis.

miRNAs have become an important component of epigenetic gene regulation in mammals [24]. Typically, miRNAs are a class of endogenous small non-coding RNA molecules 20-25 nucleotides in length cleaved from approximately 70-100 nucleotide hairpin pre-miRNA precursors that regulate gene expression by inhibiting translation and/or triggering the degradation of target messenger RNAs (mRNAs) [112, 113]. Different studies in cancer have shown that miRNAs interact with genes in many different cellular pathways, displaying a differential gene expression profile between normal and tumor tissues and between tumor types [96, 114]. For example, the overexpressed miR-17-92 oncogenic cluster may function as an oncogene and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control differentiation or apoptosis, such as E2F1 (a cell cycle and apoptosis regulator), BIM (a pro-apoptotic gene that counteracts BCL2) and PTEN (a negative regulator of the oncogenic pro-survival PI3K/AKT signaling pathway) [114]. In contrast, downregulation of the let-7 and miR-15/miR-16 miRNAs, which target the RAS and BCL2 oncogenes, respectively, has been previously described [96]. For example, altered expression of a number of miRNA molecules in the lung following the exposure of rats to environmental cigarette smoke

(ECS) can be attenuated by dietary agents, such as PEITC and I3C [30]. Thus, the ECS-downregulated miRNAs affected by PEITC have a variety of functions, such as the stress response, TGF-β expression, NF-κB activation, Ras activation, cell proliferation, apoptosis, and angiogenesis. In addition, I3C-regulated miRNAs are involved in p53 function, TGF-β expression, Erbb2 activation, and angiogenesis [30, 115](Table 2.1). Similarly, DIM treatment has been reported to cause alterations in the expression of several miRNAs, including miR200 and the let-7 family, which were increased in gemcitabine-resistant pancreatic cancer cells with a concomitant reversal of the mesenchymal phenotype to an epithelial phenotype [116].

Interestingly, I3C is also capable of reducing the effects of vinyl carbamate (a potent carcinogen causing lung tumors) in the lung by modulating the expression of several oncomiRs [117]. Other studies have shown that treatment of breast cancer cell lines with DIM increases the expression of miR-21, exhibiting dose-dependent inhibition of cell proliferation and the development of breast tumors in an *in vivo* MCF-7 xenograft model [118]. Similarly, in human prostate cancer, interventions including high bioavailability formulations of DIM for 2 to 4 weeks in patients prior to radical prostatectomy demonstrated an association between the re-expression of miR-34a and decreased androgen receptor (AR) signaling, prostate specific antigen (PSA) and Notch-1 [119]. Furthermore, in the same patient group, DIM supplementation increased the expression of *let* family miRNAs and decreased the expression of the histone methyltransferase EZH2 [120]. More recently, SFN has been shown to mediate the induction of miR-let-7a expression, which in turn inhibits K-ras expression and cancer stem cell (CSC) characteristics during pancreatic ductal adenocarcinoma (PDA) progression [121].

Moreover, SFN can also modulate the expression of several miRNAs, including miR-140, miR-29a, and miR-21, in basal-like ductal carcinoma *in situ* (DCIS) stem-like cells, inducing significant changes in the exosomal secretion of miRNAs more closely resembling that of non-stem cancer cells, representing a promising chemopreventive strategy in the early stages of non-invasive breast cancer [122]. Taken together, the current studies using both *in vitro* and *in vivo* approaches suggest that dietary glucosinolate derivatives may function as miRNA regulators in a number of cancer types and target systems.

2.6.   Dietary glucosinolate derivatives: *in vivo* studies

Naturally occurring glucosinolates and their breakdown products have been extensively used as chemopreventive agents in *in vivo* studies, including chemically induced rodent cancer models and oncogene-driven cancer development in transgenic mice [123]. Key studies documenting cancer chemoprevention by glucosinolates in chemically induced rodent cancer and transgenic mouse models are summarized in Table 2.2. For example, we have shown that topical application of SFN decreases the incidence of DMBA/TPA-induced skin tumors in Nrf2 (+/+) wild type (Nrf2 WT) mice but not in sulforaphane-treated Nrf2 KO mice, demonstrating that the chemopreventive effects of SFN in DMBA/TPA-induced skin tumors is mediated by Nrf2 [62]. Similarly, inhibition of skin tumorigenesis was also observed using SFN in chemically induced skin cancer in CD-1 mice during the promotion stage [124]. In contrast, we have also reported that SFN treatments in ApcMin/+ mice lead the regulation of different sets of genes involved in apoptosis, cell growth/maintenance and inflammation in small intestinal polyps, as revealed by gene expression profile analysis using Affymetrix microarrays [125].

These results are in agreement with other studies from our laboratory in which SFN treatments reduced the number of polyps by inhibiting phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated-Akt (p-Akt), COX-2, and cyclin D1 protein expression in ApcMin/+ mice [126, 127]. In transgenic adenocarcinoma of mouse prostate (TRAMP), an oral gavage of 6 μmol SFN three times per week for 17 to 19 weeks inhibited prostate intraepithelial neoplasia and pulmonary metastasis by reducing cell proliferation and augmenting NK cell lytic activity [128]. In this context, we have reported that TRAMP mice fed with 240 mg of broccoli sprouts/mouse/day for 16 weeks exhibit significant retardation of prostate tumor growth, with a concomitant increase in the expression level of the Nrf2, HO-1, cleaved-caspase-3, cleaved-PARP and Bax proteins and a decrease in the Keap1 and Bcl-XL proteins. Furthermore, the phosphorylation and/or expression level of Akt and its downstream kinase and target proteins (e.g., mTOR, 4E-BP1 and cyclin D1) were also reduced [129]. These data correlate with our previous findings, in which oral administration of SFN was capable of inducing Nrf2-dependent detoxification phase I and II drug metabolizing enzymes and phase III transporters in livers of C57BL/6J and C57BL/6J/Nrf2(-/-) mice using the Affymetrix 39K oligonucleotide microarray [130].

In contrast, ApcMin/+ mice fed with a diet supplemented with 0.05% PEITC for 3 weeks developed significantly less and smaller polyps than those fed with a basal diet (47). We have also reported that PEITC in an azoxymethane (AOM)-initiated and DSS-promoted colon cancer mouse model is capable of lowering tumor incidence and colon tumor multiplicities with smaller polyps compared with mice fed on a basal diet [131]. Thus, in this study, PEITC was associated with an increase in apoptosis (increased cleaved

caspase-3 and caspase-7) and cell cycle arrest (increased p21). In the polyoma middle-T antigen transgenic breast cancer mouse model, dietary feeding with an 8 mmol PEITC/kg diet resulted in smaller mammary cancer lesions with a progressive loss of ERα and FOXA1 but persistence of GATA-3 expression (48). In contrast, in TRAMP mice, a diet supplemented with 0.05% PEITC for periods of 10 and 16 weeks decreased the incidence of prostate tumors and was associated with downregulation of the Akt signaling pathway, ultimately decreasing cell proliferation and retarding prostate tumor formation [132]. Similarly, in TRAMP mice, the administration of a 3 mmol PEITC/kg diet suppressed prostate cancer progression by inducing autophagic cell death and overexpressing E-cadherin. Interestingly, PEITC treatment was not associated with a decrease in cellular proliferation, apoptosis induction, or neoangiogenesis inhibition [133]. In studying the chemopreventive efficacy of I3C in TRAMP mice, we observed that I3C suppressed the incidence of palpable tumors and reduced the genitourinary weight [134]. In addition, in this study, I3C induced the expression of Nrf2 and NQO-1 and cell cycle- and apoptosis-related biomarkers in prostate tissue. More recently, the expression of Nrf2 was found to be controlled by epigenetic alterations, such as DNA methylation and histone modifications, and dietary phytochemicals, such as DIM, could decrease the incidence of tumorigenesis and metastasis and increase apoptosis, decrease cell proliferation and enhance the expression of Nrf2 and the Nrf2-target gene NQO1 in prostate tissues [101].

Although there has been extensive research on dietary phytochemicals contributing to the overall understanding of glucosinolate derivatives in terms of their chemical and biological functions and beneficial effects in human health, clinical studies of human participants on the biological effects of dietary glucosinolate are lacking and limited to

determining the effects of raw cruciferous vegetables or their extracts under some biological parameters [123, 135]. For example, the inhibitory effects of watercress on the oxidative metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the peripheral blood cells of participants have been described [136]. Similarly, a randomized and placebo-controlled trial utilizing a beverage infused with broccoli sprouts exhibited an inverse association between the excretion of dithiocarbamates and urinary aflatoxin-DNA adducts [137]. Similarly, the consumption of broccoli sprouts decreased histone deacetylase activity in peripheral blood mononuclear cells in humans subjects [108].

Thus far, thirty-one clinical studies have been registered using SFN (www.clinicaltrials.gov; accessed Oct. 22, 2014). Of these studies, ten have been completed and reported data from patients treated with prostate and breast cancer, cardiovascular disease, immune diseases and autism. Similarly, PEITC, which has had fewer registered studies, comprises one of four studies completed for preventing lung cancer in individuals who smoke. Four studies of seven registered for I3C treatment have been completed for patients with prostate and breast cancer and a specific study on the prevention of cancer in healthy participants. Finally, four studies of ten registered for DIM for patients treated with prostate and cervical cancer as well as specific studies of preventing cancer in healthy participants have been completed. In summary, these findings suggest that dietary glucosinolate derivatives could be extensively utilized in further prospective epidemiological and chemopreventive studies.

2.7. Concluding remarks

Naturally occurring glucosinolates have been extensively used in *in vitro*, *in vivo*, preclinical and clinical studies, supporting the idea that dietary glucosinolates and their

derivatives have potential beneficial effects for cancer prevention. In extensive mechanistic studies, robust chemopreventive effects have been observed by glucosinolate derivatives, such as SFN, PEITC, 3IC and DIM, demonstrating that they can modulate oxidative stress and inflammatory damage caused by exposure to various toxicants, such as environmental pollutants, carcinogens, dietary mutagens, and solar radiation, which can result in genetic mutations and molecular alterations that cause the initiation of carcinogenesis in normal cells. In contrast, accumulating evidence has shown that cancer initiation and progression are driven not only by acquired genetic alterations or mutations but also epigenetic disruption of gene expression. Epigenetic alterations and modifications through dietary glucosinolate derivatives can largely restore the expression of many tumor suppressor genes. Although *in vitro* approaches have greatly contributed to understanding the regulation of the molecular pathways involved in different cancers, including the epigenetic network exerted by glucosinolate derivatives, *in vivo* data are lacking for most of these dietary compounds. Still, the health effects of dietary glucosinolates in humans are considered promising; however, there are several challenges and limitations to better understanding the molecular mechanisms underlying the chemopreventive effects of these dietary compounds, such as the safety profile of dosage regimens and potential interactions between different glucosinolates and other constituents in the diet. Notably, emerging technologies and research tools, such as RNA interference, microarrays, proteomics and genome-wide DNA methylation/histone modifications/miRNA profiling, have been addressing novel mechanisms through which glucosinolate derivatives may prevent cancer.

Table 2.1. Examples of the effect of dietary glucosinolate derivatives on DNA methylation, histone modifications and miRNAs mechanisms

Epigenetic mechanism	Dietary agent	Molecular mechanism	Validated target(s)	*In vitro* model	*In vivo* model	Concentration	Treatment exposure	Ref
DNA methylation	SFN	↓methylation in promoter region, ↓ DNMT1, ↓ DNMT3a	Nrf2	TRAMP-C1 mouse prostate cells		2.5 µM	5 days	[58]
	SFN	↓methylation in promoter region, ↓ DNMT1, ↓ DNMT3a, ↓ DNMT3b	Nrf2	JB6 P+ mouse skin cancer cells		2.5 µM	5 days	[63]
	SFN	↓DNMT1 expression		Caco-2 human colon cancer cells		50 µM	5 days	[98]
	SFN	↓methylation in promoter region, ↓ DNMT1 and ↓DNMT3a	hTERT	MCF-7 and MDA-MB-231 human breast cancer cells		10 µM	6 days	[41, 99]
	PEITC	↓methylation in promoter region	GSTP1	LNCaP (androgen-dependent/independent) human prostate cancer cells		2.0 µM	5 days	[100]
	DIM	↓methylation in promoter region, ↓ DNMT1,	Nrf2	TRAMP-C1 mouse prostate cells	TRAMP mice prostate tumors	5 µM / 1% DIM diet	5 days / 24 weeks	[101]

		↓ DNMT3a, ↓ DNMT3b					
	DIM (formulation with higher bioavailability)		miR-34a	LNCaP and C4-2B human prostate cancer cells	6 μM	5 days	[119]
	DIM	↓methylation in promoter region, ↓ DNMT1, ↓ DNMT3b	TGFBR1, CYR61	LNCaP human prostate cancer cells	15 μM	48 hours	[29]
Histone modifications	SFN	↓HDAC1, ↓HDAC4, ↓HDAC5, ↓HDAC7, ↑H3Ac	Nrf2	Mouse prostate TRAMP-C1 cells	2.5 μM	5 days	[58]
	SFN	↓HDAC1, ↓HDAC2, ↓HDAC3, ↓HDAC4	Nrf2	Mouse skin JB6 P+ cells	2.5 μM	5 days	[63]
	SFN	↓HDAC activity, ↑H3Ac, ↑H4Ac	p21, bax	Apc^{min} mice colon tumors	Single oral dose of 10 μM / ~6 μM	6 hours / 10 weeks	[105]
	SFN	↓HDAC activity, ↑H3Ac, ↑H4Ac	p21	Human colorectal HCT116 cells	15 μM	47 hours	[138]
	SFN	↓HDAC activity, ↑H3Ac, ↑H4Ac	p21, bax	Human prostate BPH-1, LNCaP and PC-3 cells	15 μM	48 hours	[139]
	SFN	↓HDAC activity, ↓HDAC1		Human embryonic kidney 293 cells	15 μM	47 hours	[138]
	SFN	↓HDAC activity, ↑global histone acetylation		Human PC-3 prostate cancer xenografts in nude mice	7.5 mumol per animal	21 days	[108]

Compound	Effect	Target	Model	Concentration	Time	Ref
SFN	↓HDAC activity, ↑H3Ac, ↑H4Ac		Human peripheral blood mononuclear cells (PBMC)	68 g Broccoli Sprouts	3 and 6 hours following consumption	[108]
SFN	↓HDAC activity		Human breast MDA-MB-231, MDA-MB-468, MCF-7, and T47D cell lines	25 μM	48 hours	[140]
SFN	↓HDAC activity, ↑H3Ac, ↑H4Ac, ↑H3K9Ac, ↓H3K9, ↓H3K27, ↑RBP2	hTERT	MCF-7 and MDA-MB-231 human breast cancer cells	10 μM	6 days	[41, 99]
SFN	↓H3K27		Human SCC-13 skin cancer cells	20 μM	48 hours	[109]
PEITC	↓HDAC expression, ↑H3Ac, ↑H3K4, ↓H3K9, ↓ HDAC1		Human prostate LNCaP cells	0.1-20 μM	36 hours	[100]
PEITC	↑H3Ac, ↑H3K4, ↓H3K9	p21	Human prostate LNCaP cells	10 μM	30 hours	[106]
Phenylhexyl isothiocyanate	↓HDAC activity, ↑H3Ac, ↑H4Ac, ↑H3K14, ↑H3K4, ↓H3K9	p21	Human leukemia HL-60 cells	40 μM	7 hours	[141]
DIM	↓HDAC1, ↓HDAC2, ↓HDAC3, ↓HDAC4		Human colon HT-29 cells	60 μM	24 hours	[142]
DIM	↓HDAC1, ↓HDAC2, ↓HDAC3, ↓HDAC4	p21, p27	Human colon HT-29 and SW620 cells Human colon cancer xenografts in nude mice	60 μM / 300 mg/kg/day	24 hours / 2 days	[111]

	DIM	↑histone acetyl transferase p300, ↑H4Ac	COX-2	Human breast MCF-7 cells	10 µM	15 minutes	[107]
	DIM	↓HDAC1, ↓HDAC2, ↓HDAC3, ↓HDAC4, ↓HDAC8		Mouse prostate TRAMP-C1 cells	5 µM	5 days	[101]
	DIM	↑H3K	TGFBR1, CYR61	LNCap human prostate cancer cells	15 µM	48 hours	[29]
microRNAs	SFN	miR-140, miR-29a, and miR-21		MCF10DCIS,MCF-7, MDA-MB-231 breast cancer cells	10 µM	7 days	[122]
	SFN	miR-let7-a	K-ras	BxPc-3, MIA-PaCa2 pancreatic cancer cells	10 µM	72 hours	[121]
	DIM	miR-200b, miR-200c, let-7b, let-7c, let-7d, and let-7e	ZEB1	MiaPaCa-2, Panc-1, and Aspc-1 pancreatic cancer cells	25 µM	48 hours	[116]
	DIM	miR-146a	EGFR, IRAK-1, and MTA-2	Colo357 and Panc-1 pancreatic cancer cells	25 µM	48 hours	[143]
	DIM	miR-21	Cdc25A	MCF-7 and MDA-MB-468 breast cancer cells Human MCF-7 breast cancer xenografts in nude mice	30-60 µM / 5 mg/kg	24-96 hours / 7 weeks after cell injection	[118]
	DIM (formulation with higher bioavailability)	let-7a, let-7b, let-7c, and let-7d	EZH2	LNCaP, C4-2B and PC3 human prostate cancer cells Human prostate samples from patients with stage I or stage II PC undergoing radical prostatectomy	25 µM / 225 mg orally twice daily x 14-72 days (based on scheduling of surgery)	24 hours / 2–4 weeks prior to surgery	[120]

I3C	miR-21, miR-31, miR-130a, and miR-146	Human lung carcinoma A549 cells	Vinyl carbamate-induced female A/J mice lung tumors	100 - 150 µM	24 hours / 15 weeks	[117]
I3C	miR-10a, miR-26a, miR-34b, miR-125a-prec		Sprague-Dawley rats lung cancer samples induced by environmental cigarette smoke (ECS)	2,500 mg/kg diet	28 days	[30]
PEITC	let-7a, let-7c, miR-26b, miR-99b, miR-123-prec, miR-125b, miR-146-prec, miR-192, miR-222-prec		Sprague-Dawley rats lung cancer samples induced by environmental cigarette smoke (ECS)	500 mg/kg diet	28 days	[30]

Table 2.2.   Chemopreventive effect of dietary glucosinolate derivatives in rodents *in vivo* models

Compound	Animal model	Experimental protocol	Effect	Ref
SFN	DMBA/TPA - CD-1 mice	Topical application of 1, 5 or 10 micromol/mouse in anti-promotion protocol (SFN from 1 week after carcinogen until the end of the study) or a combined anti-initiation, anti-promotion protocol (SFN 7 days prior to carcinogen until the end of the study)	Inhibition of skin tumorigenesis	[124]
	DMBA/TPA-induced skin tumorigenesis in C57BL/6 mice	Topical application of 100 nmol of SFN once a day for 14 days prior to DMBA/TPA applications	Decreasing the incidence of skin tumor	[62]
	C57BL/6J wild type and C57BL/6J/Nrf2(-/-) knock-out mice	Topical application of 100 nmol of SFN in 100 µL acetone for 4 and 5 d and irradiated with a single dose of UVB (300 mJ/cm^2) during 10 min	Decreasing of inflammation and restored sunburn	[91]
	Apc$^{min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of 600 ppm SFN/day during 1 to 5 days	Regulation of different set of genes involving apoptosis, cell growth/maintenance and inflammation in the small intestinal polyps	[125]
	Apc$^{min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of 300 and 600 ppm of SFN for 3 weeks	Suppression of polyps in the small intestine with higher apoptotic and lower proliferative indices	[127]
	Apc$^{min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of 300 ppm SFN during 10 weeks	Reduction of colon tumor numbers, decreasing levels of prostaglandin E2 or leukotriene B4 in intestinal polyps and inhibition of cell survival and growth-related signaling pathways	[126]
	Apc$^{min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of ~6 µmol SFN/day for 10 weeks	Suppression of polyps formation	[105]
	Apc$^{min/+}$ mouse model of gastrointestinal cancer	Dietary feeding of 300 or 600 ppm of SFN for 3 weeks	Suppression of polyps in the small intestine	[127]

	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Oral gavage of 6 µmol SFN thrice a week for 17 to 19 weeks	Inhibition of prostate intraepithelial neoplasia and pulmonary metastasis	[128]
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Feeding with 240 mg broccoli sprouts/mouse/day for 16 weeks	Inhibition of prostate tumor growth	[129]
	C57BL/6J and C57BL/6J/Nrf2(-/-) knock-out mice	Oral gavage of 90 mg/kg SFN (0.2 ml) for 3 and 12 hours	Increasing the expression of Nrf2-dependent detoxification phase I, II drug metabolizing enzymes and phase III transporters genes	[130]
PEITC	AOM/DSS - C57BL/6 mice colon cancer model	Dietary feeding of 0.05% PEITC and 1% DBM during 20 weeks	Inhibition of colon tumor multiplicity	[131]
	Apc[min/+] mouse model of gastrointestinal cancer	Dietary administration of 0.05% PEITC for 3 weeks	Inhibition of intestinal polyp development and reduced intestinal tumor size	[144]
	Polyoma middle-T antigen (PyMT) transgenic mouse model of breast cancer	Dietary feeding of 8 mmol PEITC/kg for 4 to 16 weeks	Reducing size of mammary cancer lesions	[145]
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary feeding of 0.05% PEITC for 10 and 16 weeks	Inhibition of prostate tumor incidence	[132]
	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary administration of 3 mmol PEITC/kg for 19 weeks	Inhibition of incidence and burden of poorly-differentiated prostate tumor	[133]
I3C	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary administration of 1% I3C for 8 and 12 weeks	Inhibition of incidence of palpable tumor and increased expression of Nrf2, NQO-1, as well as cell cycle and apoptosis related biomarkers	[134]

DIM	Transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer	Dietary administration of 1% I3C for 12 and 16 weeks	Decreasing of incidence of tumorigenesis and metastasis; increasing of apoptosis, decreasing of cell proliferation and enhanced Nrf2 and Nrf2-target gene NQO1 expression in prostate tissues	[101]

Figure 2.1. Representative figures of Glucosinolates **(A)**, their hydrolysis **(B)** and metabolism **(C)**

Examples of aliphatic, indole and aromatic glucosinolates found in Brassicaceae vegetables **(A).** General model of glucosinolate hydrolysis by myrosinase and specifier proteins indicated as TFP (thiocyanate-forming protein) and ESP (epithiospecifier protein) **(B).** Metabolism of isothiocyanates by the mercapturic acid pathway. Isothiocyanates are conjugated to glutathione by glutathione S-transferases (GSTs) and successively cleaved by γ-glutamyltranspeptidase (γ-GT), cysteinylglycinase (CGase), and N-acetyltransferase (AT) to create N-acetylcysteine conjugates (mercapturic acids) **(C).**

A

B

$$R-C(=N-OSO_3^-)(S-C_6H_{11}O_6) \xrightarrow[\text{(+H}_2\text{O)}]{\text{Myrosinase}} \left[R-C(=N-OSO_3^-)(S-) \right] + C_6H_{12}O_6$$

Glucosinolate   Unstable Intermediate   Glucose
(Aglycone)

$-SO_4^{2-}$

Lossen-Type
Rearrangement

pH 7   **TFF**   **ESP**

$+Fe^{2+}$
pH 4 $-S$

Isothiocyanate   Thiocyanate   Oxazolidine-thione   Nitrile   Epithionitrile

R−N=C=S   **R−S−C≡N**   **CH₂=CH−CH−CH₂**   **R−C≡N**   **CH₂−CH−CH₂−C≡N**

O   NH   **S**

C
‖
S

C

γ-Glu-Cys-Gly   γ-Glu-Cys-Gly   Cys-Gly   Cys   Cys-NAc

SH   **S**   **S**   **S**   **S**

+   **GST**   **γ-GT**   **CGase**   **AT**

R−N=C=S   **R−NH−C=S**   **R−NH−C=S**   **R−NH−C=S**   **R−NH−C=S**

Mercapturic acid

Figure 2.2. Chemopreventive effects of natural dietary glucosinolate derivatives

Nrf2-mediated antioxidative stress and anti-inflammatory signaling pathways
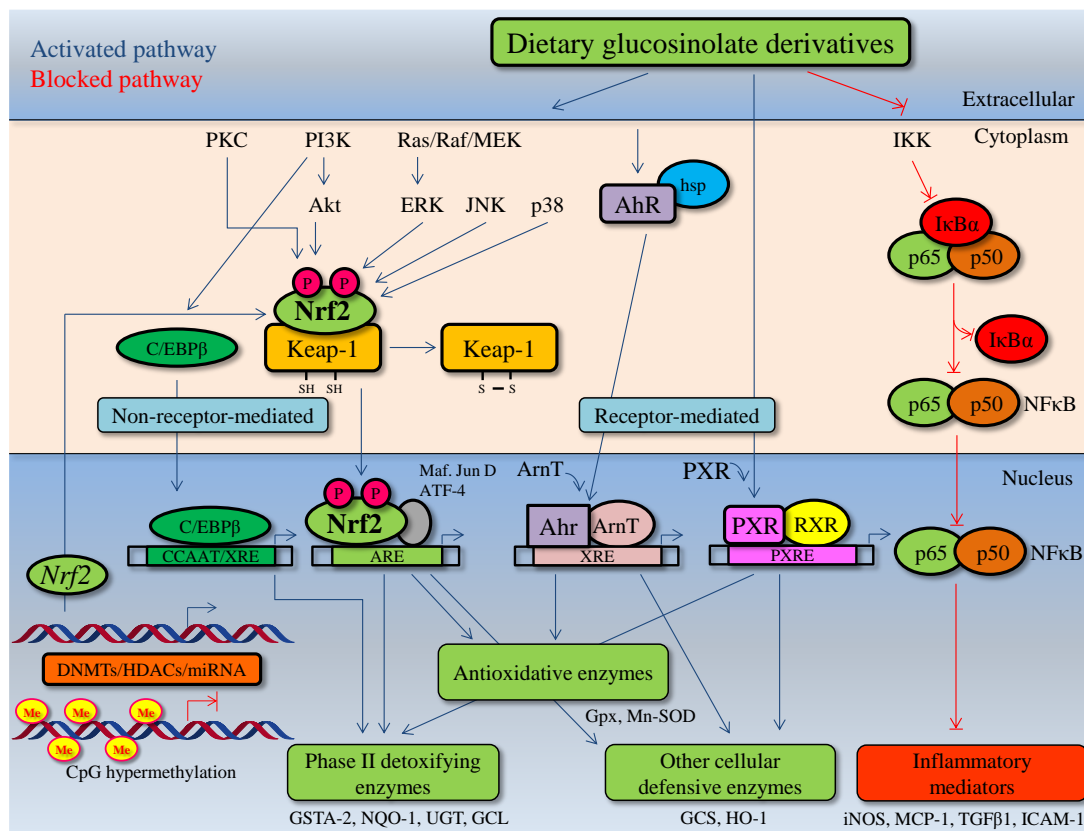
Chapter 3.   Chemopreventive effects of *Radix Angelicae Sinensis* (Danggui) in TRAMP model [4,5,6]

3.1.   Introduction

Prostate cancer (PCa) is a slow-growing tumor that is generally diagnosed in men above 65 years of age and constitutes one of the leading causes of cancer-related death among men in the USA [146]. Estimates indicate that 233,000 new cases will be diagnosed and that 29,480 males will die of PCa in the USA in 2014 (NCI; http://www.cancer.gov/cancertopics/types/prostate). PCa is characterized by progressive genetic and molecular alterations at the cellular and tissue levels that produce a gradual and slow transition from a normal phenotype to increasing grades of dysplasia and, ultimately, to an invasive and metastatic phenotype [147]. Although its pathogenesis is not clear, epidemiological evidence suggests that relationships exist between PCa and the serum levels of testosterone, advanced age, family history, ethnicity, cadmium exposure and poor diet [148]. Epidemiological studies indicate that a high intake of vegetables and fruits combined with lifestyle changes can significantly reduce the risk of PCa development [149, 150]. In the last few years, strategies for prevention of PCa using dietary

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[4] This chapter is intended to be submitted for publication as *original research paper* as "Effects of *Radix Angelicae Sinensis* (Danggui) in TRAMP model" by Paredes-Gonzalez X, Saw CL, Fuentes F, Shu L, Liu Y, Guo Y, Boyanapalli SS, Suh N and Kong AN.

[5] Key Words: *Radix Angelicae Sinensis*, Z-Ligustilide, apoptosis, Nrf2, TRAMP

[6] Abbreviations: RAS, *Radix Angelicae Sinensis*; Lig, Z-Ligustilide; TRAMP, transgenic adenocarcinoma of mouse prostate; PCa, Prostate Cancer.

phytochemicals targeting multiple changes related to tumorigenesis have gained increasing attention due their low side effects and promising activity [21].

Radix Angelicae Sinensis (RAS), which is commonly known as 'Danggui,' has a long history in traditional Chinese medicine as a treatment for various diseases and as a healthful food tonic [151]. Recent studies indicate that RAS exerts various biological activities, such as anti-inflammatory, immunomodulatory, antioxidant, antiangiogenic and anticancer effects [151-155]. Although, the exact mechanism through which RAS produces these effects remains unclear, the modulation of reactive oxygen species (ROS) appears to be an important event related to the biological activities of this herb [156]. Oxidative stress is a major risk factor for various diseases, including PCa; therefore, one effective defense mechanism may be the induction of phase II detoxifying and antioxidant enzymes that are mediated by the nuclear erythroid-related factor 2 (Nrf2) pathway [151]. Nrf2 is an important factor that mediates the transcriptional regulation of the antioxidant response element (ARE) that is present in the promoter region of many phase II drug metabolizing/detoxifying/antioxidant enzymes [151, 157]. Recently, we have demonstrated that RAS extract and the main bioactive phthalide components present in the lipophilic fraction, including Z-ligustilide (Lig), can induce the expression of Nrf2 and the downstream genes NAD(P)H:quinone oxidoreductase 1 and superoxide dismutase 1 in HepG2-C8 cells, which is in agreement with the results previously reported by Dietz et al. [151, 158]. Recent evidence suggests that the progressive loss of expression of Nrf2 and its downstream target genes occurs in PCa [159].

Accordingly, in the transgenic adenocarcinoma of mouse prostate (TRAMP) model and in human prostate cancer tissues, we have observed that Nrf2-ARE gene expression is

significantly diminished, and this loss of expression appears to be mediated through the epigenetic methylation of CpG sites in the Nrf2 promoter [160, 161]. This event positively correlates with the hypermethylation of Nrf2 observed in TRAMP-C1 and LNCaP cell lines [160, 161]. We have also observed that this hypermethylated status of Nrf2 in mice can be reversed by dietary phytochemicals such as 3,3′-diindolylmethane and a γ-tocopherol-rich mixture of tocopherols, as well as by sulforaphane, apigenin, Lig and RAS *in vitro* [157, 162-165]. Interestingly, TRAMP-C1 cells treated with Lig and RAS displayed a significant dose- and time-dependent inhibition of cell viability [157]. However, the effect of RAS and Lig on cell proliferation and cell cycle progression in the TRAMP model has not been explored thus far. This current study seeks to examine the activities of RAS and its main bioactive compound Lig in regard to cell cycle arrest and apoptosis in TRAMP-C1 cells and to determine the effects of RAS-supplemented diet on the TRAMP mouse model.

3.2. Materials and Methods

3.2.1. Reagents and cell culture

TRAMP-C1 cells were obtained from B. Foster (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, NY, USA). The cells were cultured in DMEM (pH 7.0) containing 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere, as described previously [157].

Lig was obtained from ChromaDex, Inc. (Irvine, CA, USA) and reconstituted as a 100 mM stock solution in DMSO. The RAS extract was prepared by supercritical fluid carbon dioxide extraction of the milled decoction pieces (40 mesh) (HA220-50-01) in the laboratory of Dr. Q. Wu (Beijing University of Chinese Medicine, BJ, China). The

separation process was executed in two phases, as previously described [151, 157]. The Lig content of the extract was 62.3%, as measured by high performance liquid chromatography (HPLC). Throughout the entire study, the extract was stored at -20 °C until use.

3.2.2. MTS and trypan blue exclusion assays

For the MTS assay, TRAMP-C1 cells were plated in 6-well plates in medium containing 1% FBS at an initial density of 1×10^4 cells/mL. After overnight incubation, the medium was replaced with fresh DMEM/1% FBS and Lig (25 μM or 50 μM), RAS (4.25 μg/mL or 8.5 μg/mL) or 0.1% DMSO (control), and the cells were incubated for additional 24 or 48 h in a humidified atmosphere of 95% air and 5% CO_2. The cytotoxicity of the drugs was tested using the CellTiter 96® aqueous nonradioactive cell proliferation MTS assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] from Promega (Madison, WI, USA) and a μQuant Biomolecular Spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT, USA). The absorbance of the formazan product was determined at 490 nm. The cell viability was calculated by comparing the optical density of the treated samples with the optical density of the negative control (DMSO).

In the case of the trypan blue dye exclusion assay, TRAMP-C1 cells were plated in 6-well plates at a density of 1×10^4 cells/mL and allowed to attach overnight. Then, the cells were treated with the desired concentrations of the indicated compounds for 24 or 48 h, similar to the assay described above. Both floating and adherent cells were collected and pelleted by centrifugation at 700 x g for 5 min. The cells were resuspended in 1 mL of phosphate buffered saline (PBS). Twenty microliters of the cell suspension was mixed with

20 µL of 0.4% trypan blue solution, and the cells were counted using a TC20™ Automated Cell Counter from Bio-Rad (Hercules, CA, USA) according to the manufacturer's protocol.

3.2.3. Cell cycle distribution and apoptosis analysis by flow cytometry

Lig- and RAS-treated TRAMP-C1 cells were stained with propidium iodide (PI) from Sigma-Aldrich Co. (St. Louis, MO, USA) or PI/Annexin V-FITC from BD Bioscience (Franklin Lakes, NJ, USA) and were analyzed by flow cytometry using a Beckman Coulter Gallios Flow Cytometer (Brea, CA, USA) in the Flow Cytometry/Cell Sorting & Confocal Microscopy Core Facility at Rutgers University. Briefly, 1×10^4 cells/mL of TRAMP-C1 cells were seeded in 10-cm plates, allowed to attach overnight and treated as described above for 48 h. Next, the cells were trypsinized, collected and pelleted by centrifugation at 700 x *g* for 5 min. For cell cycle analysis, the cells were fixed with 3 mL of ice-cold 70% ethanol for 48 h at 4°C. After being rinsed twice with PBS, the cells were treated with RNase A at 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 an internal protocol. For apoptosis analysis, after incubation, the treated samples, including the floating and adherent cells, were collected and were prepared for analysis using an Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions.

3.2.4. Protein lysate preparation and western blotting

TRAMP-C1 cells were treated with the test compounds for 48 h, as described above. The protein lysate was prepared using RIPA buffer from Cell Signaling (Danvers, MA, USA); the protein concentration determination was performed using the bicinchoninic acid (BCA) method; and western blotting was executed using a standard protocol from our laboratory, as described previously [157, 165]. Twenty micrograms of total proteins from

each sample were mixed with 5 µL of Laemmli's SDS sample buffer and denatured for 5 min at 95 °C. The proteins were separated using a Bio-Rad 4-15% SDS-polyacrylamide gel, and then they were transferred to a Millipore polyvinylidene difluoride (PVDF) membrane (Bedford, MA, USA), followed by blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. Then, the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were visualized by a SuperSignal enhanced chemiluminescence (ECL) detection system and recorded using a Bio-Rad Gel Documentation 2000 system (Hercules, CA). Anti-caspase-3 was purchased from Cell Signaling (Boston, MA, USA) and anti-β-actin, anti-PARP, anti-p21, anti-p27, anti-cyclin A, and anti-cyclin D1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The images were analyzed using ImageJ software, as described previously [165].

3.2.5. Animals

Female C57BL/TGN TRAMP mice (hemizygous line PB Tag 8247NG) and male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were bred on the same genetic background, and transgenic males were obtained for the studies as [TRAMP × C57BL/6] F1 or [TRAMP × C57BL/6] F2 offspring. The genotypes of the transgenic mice were established by DNA genotyping using PCR with the following primers, as suggested by The Jackson Laboratory: Tcrd Forward 5′-CAA ATG TTG CTT GTC TGG TG-3′; Tcrd Reverse 5′-GTC AGT CGA GTG CAC AGT TT-3′; SV1 5′-GGA CAA ACC ACA ACT ATG CAG TG-3′; SV5 5′-CAG AGC AGA ATT GTG GAG TGG-3′. All mice were maintained in the Laboratory Animal Service facility at Rutgers University in accordance with the guidelines established by the

university's Animal Research Committee according to the NIH Guidelines for the Care and Use of Laboratory Animals, as we have described previously [162, 163]. The study was performed using a protocol approved by the Institutional Animal Care and Use Committee at Rutgers University.

3.2.6. Diet and study design

Before entry into the study, the mice were maintained on irradiated PicoLab Rodent Diet 20 from WF Fisher & Son Inc. (Somerville, NJ, USA). At 8 weeks of age, the mice were assigned randomly to the experimental diets prepared by Research Diets Inc. (New Brunswick, NJ, USA). The dose was selected based on previous reports [155, 166]. In the present study, the AIN-93M diet was supplemented with 0.2% of RAS extract (RAS-LD) or 0.5% of RAS (RAS-HD). The control TRAMP males (n = 14) received an AIN-93M diet without RAS extract starting from 8 weeks of age. Starting from 8 weeks of age, the treated TRAMP males received RAS-LD in AIN-93M chow (n = 12) or RAS-HD in AIN-93M chow (n = 13) (Fig. 5.A). Fresh control and experimental feed was administered twice weekly.

The mice were weighed every week throughout the experiment, and the overall healthcondition of the animals was monitored on a regular basis. Prostatic intraepithelial neoplasia (PIN) lesions presumably started to form at a time corresponding to sexual maturity (8 weeks old), as reported by Greenberg et al. [167]. At the age of 24 weeks, the mice were sacrificed by carbon dioxide euthanasia, and the genitourinary apparatus (GU), consisting of the bladder, prostate, and seminal vesicles, was collected for further analyses.

3.2.7. Histopathology

The dorsolateral prostates of the animals in the control, RAS-LD and RAS-HD groups (n=7) were excised and fixed using 10% formalin for 24 h, and then the samples were transferred into 70% ethanol for an additional 24 h. Tissue processing, sectioning and evaluation were performed as previously described in our laboratory [168, 169]. The sections were stained with hematoxylin and eosin, as described previously, to observe the neoplastic changes [162, 168]. The sections were evaluated by an independent pathologist in a blinded manner to classify the prostatic PIN lesions using the classification described by Park et al. as PIN I, PIN II, PIN III, or PIN IV. PIN I and PIN II were grouped as low-grade (LG) PIN, whereas PIN III and PIN IV were grouped as high-grade PIN, as we have reported previously [162, 168].

3.2.8. Immunohistochemistry

Immunohistochemistry (IHC) to determine the levels of proliferating cell nuclear antigen (PCNA), Nrf2 and 5-mC was performed on formalin-fixed, paraffin-embedded prostate tissue sections of the control, RAS-LD and RAS-HD groups (n=3) using a standard protocol with 3,3′-diaminobenzidine and counterstaining with Mayer's hematoxylin, as previously described [162, 168]. A 1:100 dilution was used for the antibodies. The sections were examined with an inverted Olympus BX51 microscope, and images were acquired with Olympus MicroSuite™ Five Software (Soft Imaging System, Lakewood, CO, USA). The quantitative assessment the IHC staining was performed using the Aperio ScanScope® GL system according to the manufacturer's protocol (Aperio Technologies Inc., Vista, CA, USA). Briefly, the slides were quantified using an area quantification algorithm to detect

both cytoplasmic and nuclear staining. The images were scored according to the percentage of positive cells, as described previously [161].

3.2.9. Statistical analysis

All values are reported as the mean ± standard deviation of the mean (SD). All experiments were performed at least three times with similar results (unless indicated). The statistical tests were performed using Student's t-tests for independent samples, unless specified otherwise. Distribution of the GU weight data of mice was presented by box plot on which the upper edge indicates the 75th percentile of the dataset whereas the lower edge indicates the 25th percentile. The line in the box show the media value of the data and the error bars represents the 95% confidence intervals. Differences in mean body weight of mice (evaluated weekly) were assessed with Tukey's multiple-comparison test. All p-values were two-sided, and a p-value of < 0.05 was considered significant.

3.3. Results

3.3.1. Cytotoxicity of Lig and RAS extract in TRAMP-C1 cells

In our previous reports, we observed a significant time- and dose-dependent decrease in the cell viability of treated TRAMP-C1 cells by the MTS assay [157]. MTS, an indicator of metabolically active mitochondria, may overestimate the number of viable cells compared to the ATP-, DNA-, or trypan blue-based determinations used in chemopreventive studies [170]. Therefore, in this study, we tested the cell viability using MTS and trypan blue dye exclusion assays, which are broadly used in proliferation and cell death studies. As shown in Figure 3.1.A and 3.1.B, we observed a significant decrease in the viability of treated cells compared with the controls (p<0.05) at 24 h and 48 h using the trypan blue assay, which provided more evident results compared to the MTS assay. These

results implied that Lig and RAS are able to control the growth of murine prostate cancer cells.

3.3.2. Lig and RAS induce cell cycle arrest and apoptosis in TRAMP-C1 cells

To elucidate whether Lig (25 µM and 50 µM) and RAS (4.25 µg/mL and 8.5 µg/mL) cause cell growth inhibition and/or cell death by apoptosis, the effect of the compounds on cell cycle progression and apoptosis were determined by flow cytometry over a 48-h period of exposure. Cell cycle analysis revealed that TRAMP-C1 cells treated with Lig and RAS for 48 h demonstrated significant cell cycle arrest at the G1 phase in a dose-dependent fashion (Figure 3.2). Lig and RAS dose-dependently induced a significant proportion of cells to arrest at the G0-G1 phase, accompanied by a simultaneous decrease in the number of cells in the S phase, after 48 h of treatment ($p < 0.05$). No statistically significant differences were observed with either compound for the G2/M phase. To confirm whether the growth inhibition induced by Lig and RAS extract was caused by apoptosis, we analyzed the Annexin V/propidium iodide (PI)-stained TRAMP-C1 cells after treatment with the compounds for 48 h by flow cytometry. We observed that the highest dose of Lig and both doses of RAS increased the number of cells in early apoptosis (Annexin V‑positive cells) from 7.38% (control) to 13.02% (Lig 50 µM), 18.63% (RAS 4.25 µg/µL) and 28.41% (RAS 8.5 µg/µL), whereas no statistically significant difference was observed with Lig at the lowest dose. For late apoptosis, a similar trend was observed, with significant differences between the control sample (18.57%) and cells treated with Lig or RAS at the highest dose (over 30% PI-positive cells) (Figure 3.3).

3.3.3. Lig and RAS induce protein expression of cell cycle regulators and proapoptotic mediators in TRAMP-C1 cells

We investigated the effect of Lig and RAS on the protein expression of regulators of apoptosis and the cell cycle. Western blot analysis was performed following the treatment of the TRAMP-C1 cells for 48 h with Lig or RAS. We observed significant poly(ADP-ribose) polymerase (PARP) cleavage of the signature 86-kDa fragment in cells treated with Lig ($p<0.05$; Figure 4.A) and a moderate effect of RAS at 4.25 μg/mL and 8.5 μg/mL, suggesting that Lig induces an apoptotic response, as PARP is a downstream target of activated caspase-3/7. Accordingly, a significant increase in caspase-3 cleavage was observed with Lig treatment, and a moderate effect on caspase-3 expression was observed with RAS treatment at both doses (Figure 3.4.A). In the case of cell cycle-related proteins, we observed increased expression of cyclin A, an indicator for cell entry into the S phase with both Lig and RAS, along with a significant effect on cyclin D1, which also affects the cells progression from G1 to S phase, by both doses of RAS [171, 172]. p21 and p27 are able to arrest cells at both the G1/S and G2/M checkpoints [173]. We observed that TRAMP-C1 cells treated with Lig and RAS displayed a moderate increase in the protein levels of p21 for the highest dose of Lig, whereas p27 was affected by RAS at the highest dose (Figure 3.4.B). These results suggest that the main mechanism through which Lig and RAS reduce the proliferation of TRAMP-C1 cells is by inducing G1 arrest and apoptosis.

3.3.4. RAS-supplemented diet reduces GU weight and the incidence and proliferation index of poorly differentiated adenocarcinoma in TRAMP mice

During the study, all of the mice were weighed and monitored every week, and no significant changes in the body weights or overall health were found in any group.

However, the weight gain of the RAS-HD group was lower than that observed for the control and RAS-LD groups (Table 3.1). Nevertheless, the gross autopsy of these animals failed to reveal signs of important toxicity. A statistically significant decrease was observed in the wet weight of the genitourinary apparatus in both the RAS-LD- and RAS-HD-treated groups ($p < 0.05$) compared with the control AIN-93M-treated group (Figure 5.B). Upon necropsy, we observed a dose-dependent decrease in the number of mice with palpable tumors for the RAS-treated animals. The effects of RAS on the incidence of palpable tumors and metastasis are summarized in Table 3.2. Fifty-seven percent of the control mice developed primary palpable prostate tumors, and one of these tumors was associated with lymph node metastases, although no lung or liver metastases were observed. Histological analyses of seven mice per group revealed that only 21% of the control mice possessed normal glands; 52% of the animals exhibited LG-PIN and 27% HG-PIN (Figure 3.6.B), one of which was classified as a carcinoma.

For the RAS-LD and RAS-HD groups, 24% and 34% decreases in the number of animals with palpable tumors were observed compared with the control group, respectively. No metastases were observed in mice fed with RAS at low or high doses. Histological analysis revealed that the RAS-LD group exhibited a significant increase (24%) in the percentage of normal glands and a 25% decrease in the number of HG-PIN animals, compared with the control mice, whereas no differences were found for LG-PIN compared with the controls (Figure 3.6.B). No evidence of carcinoma was reported for the RAS-LD group. For the RAS-HD group, histological analysis revealed a significant increase of 50% in the number of animals with normal glands, compared with the control group, and a 23% decrease in the number of LG-PIN animals, with no HG-PIN cases

reported. Two mice were reported to contain carcinomas upon the histological examination of the RAS-HD animals. The RAS treatments did not affect the expression of the SV-40 transgene (data not shown).

To assess the *in vivo* effect of dietary administration of RAS extract on the proliferation index of the dorsolateral prostate, tissue samples from the control and RAS-LD- and RAS-HD-treated groups were analyzed for PCNA expression through immunostaining (n=3). The qualitative microscopic examination of PCNA-stained sections indicated a substantial decrease in the percentage of PCNA-positive cells in the RAS-fed groups, compared with the positive control (Figure 4.A), which significantly differed from the RAS-HD group ($p < 0.01$). The quantification of the PCNA staining confirmed that $50 \pm 5\%$ and $20 \pm 3\%$ of the cells were positive for PCNA in the RAS-LD- and RAS-HD-fed groups, respectively, compared with $70 \pm 8\%$ for the positive control (Fig. 3.4.A), accounting for a decrease in the proliferation indices of both groups; this difference was statistically significant for the RAS-HD group compared with the control group ($p < 0.05$). Collectively, our data suggest that RAS exerts dose-dependent inhibitory effects on PCa tumor formation and progression.

3.3.5. RAS extract feeding restores Nrf2 expression in TRAMP mice and suppresses global CpG methylation staining by 5-MC

We have previously demonstrated that the expression of Nrf2 is epigenetically suppressed in TRAMP-C1 cells and in the prostate tumors of TRAMP mice by promoter methylation related to MBD2 and histone modifications [160]. Lig and RAS are potent Nrf2 activators, and they are able to epigenetically restore the methylation status of five

key CpGs in the Nrf2 gene promoter region in TRAMP-C1 cells [151, 157]. Therefore, in the present study, we decided to test whether Nrf2 expression is restored in the prostate tissue of TRAMP mice after long-term exposure to RAS. Figure 3.7.A shows that treatment of TRAMP mice with RAS significantly increased Nrf2 staining by IHC in the prostate tissue for both the RAS-LD and the RAS-HD group in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively). Next, we tested whether the level of 5-methylcytosine (5-MC), which is generated when a methyl group is added to the C5 position of DNA, is affected by RAS treatment. Figure 3.7.B shows that RAS significantly and dose-dependently decreased the level of 5-MC IHC staining in the prostate tissue of both RAS-LD- and RAS-HD-treated animals ($p < 0.05$ and $p < 0.01$, respectively). These data suggest that RAS is able to epigenetically modulate the progression of tumorigenesis in the TRAMP model.

3.4. Discussion

Prostate cancer progression is a multistage process involving the initial development of a small carcinoma of low histologic grade that progresses slowly to aggressive lesions with a higher grade [174]. The TRAMP model is considered to closely mimic the human development and progression of prostate cancer in a stochastic fashion and to be a suitable model to study the chemopreventive efficacy of agents against prostate cancer [175]. In this study, we used TRAMP-C1 cells, an androgen receptor-positive epithelial prostate cell line derived from a prostate tumor of a 32-week-old TRAMP mouse, and TRAMP mice to assess the effect of RAS and its main bioactive compound Lig on PCa progression [176]. We have previously demonstrated that the viability/proliferation of the TRAMP-C1 cell line is reduced significantly in the presence of Lig and RAS extract in a dose- and time-dependent manner using the MTS assay [157]. Moreover, RAS has been

reported to be cytotoxic to various tumor cell lines [155]. Therefore, in the present study, we determined the effect of Lig and RAS on the cell survival and apoptosis of TRAMP-C1 cells and the effect of the long-term feeding of RAS on TRAMP mice. We observed that RAS and its main bioactive compound Lig are able to decrease the proliferation of TRAMP-C1 cells by targeting G1/S arrest (Figure 3.2) and inducing apoptosis (Figure 3.3), which is in agreement with other reports [155, 177]. We observed that Lig and RAS induce cleavage of PARP and caspase-3, an effect that was more pronounced with Lig at the highest dose (Figure 3.4.A). Lig and RAS have been reported to modulate key apoptosis players, such as p53, Bcl-2 and Bax and caspase-3, in different cell models, and RAS is suggested to induce apoptosis by both p53-dependent (through the phosphorylation of p53) and p53-independent mechanisms involving the cyclin/CDK/CKI system [155, 178, 179]. In addition, Lig has also been reported to attenuate H_2O_2-induced cell death, reduce increases in intracellular reactive oxygen species (ROS) levels, decrease Bax expression and cleave caspase-3 and cytochrome C [156].

In our study, we observed increased levels of cyclin A resulting from RAS and Lig treatment, as well as decreased levels of cyclin D1. Interestingly, differential effects were observed with Lig and RAS in regards to the levels of p21 and p27 expression. Although we focused our attention on Lig in this study, we cannot discard the possibility that the differential effect on p21 and p27 protein expression observed with TRAMP-C1 cells may have been induced by other lipophilic bioactive compound(s) with similar structure(s) present in RAS, namely n-butylidenephthalide (Buty), which has also been reported to cause a G1 cell cycle arrest and apoptosis in various malignant cell lines [180, 181]. In our supercritical extract, the phthalide contents measured by high performance liquid

chromatography were 62.3% for Lig and 1.75% for Buty [151]. Although the amount of Buty in the extract was low, the potent antiproliferative effects of RAS on colon cancer HT-29 cells have been suggested to be potentially generated by the synergistic effects of its main phthalides, and the ability to induce apoptosis and cell cycle arrest and decrease proliferation appears to be related to the chemical structure of the phthalides themselves [180, 182]. For example, 4-(3′,3′-dimethylallyloxy)-5-methyl-6-methoxyphthalide has been reported to induce G1 cell cycle arrest and apoptosis in human cervical adenocarcinoma HeLa cells through the upregulation of p21, p16, p73, JunB, FKHR, PUMA, NOXA, Bax, Bad, Bid and Bim at the mRNA level and the upregulation of p53, p73, p27, caspase-3, caspase-8 at the protein level; additionally, the compound is able to decrease the proliferation of the MDA-MB-231 and MCF7 breast cancer cell lines [183, 184]. Moreover, Buty has been reported to induce apoptosis in LNCaP human prostate cancer cells in a concentration- and time-dependent manner by affecting the protein expression of several proteins related to metabolic processes, cell signaling, apoptosis and cell cycle progression, including L-lactate dehydrogenase A chain, apoptosis-inducing factor 1, cytochrome c, cyclin-dependent kinase inhibitor 2A and PCNA, and many others [181]. Accordingly, our *in vivo* study demonstrated that the long-term RAS treatment of TRAMP mice reduced TRAMP mouse tumor growth (Table 3.2) and the occurrence of HG-PIN lesions in the animals in a dose-dependent manner (Figure 3.6.B), which positively correlates with the decrease in PCNA-positive cells in the prostate tissues of RAS-LD- and RAS-HD-fed mice (Figure 3.7.B). Notably, our results regarding cell cycle arrest, apoptosis and inhibition of tumor progression in TRAMP mice are close to the findings reported by Tsai et al. using a chloroform extract of RAS on glioblastoma

multiforme *in vitro* and *in vivo* [155]. In addition, similar trend was observed in a breast cancer model (Appendix B).

We previously reported that RAS and its main phthalides Lig and Buty can induce the Nrf2-ARE signaling pathway in HepG2-C8 cells [151]. In addition, the strong protective effect of Lig against brain damage caused by ischemia-reperfusion *in vitro* and *in vivo* is reported to be in part mediated by its ability to activate the Nrf2-ARE signaling pathway [185]. Moreover, Lig and RAS modulate the DNA methylation of the Nrf2 promoter in 5 key CpG sites in TRAMP-C1 cells compared with 5-aza-2′-deoxycytidine and trichostatin A, which were used as positive controls, highlighting the potential function of RAS and Lig as epigenetic modifiers [151, 157]. In our studies, we have consistently reported that Nrf2 expression in TRAMP-C1 cells and the prostate tumors of TRAMP mice is abolished in part by CpG hypermethylation in the promoter region, which positively correlates with the CpG methylation of the human NRF2 promoter in clinical PCa samples and in LNCaP cells [160, 161, 163, 164]. Therefore, we tested whether the expression of Nrf2 could be restored in prostate tissue of TRAMP mice after long-term treatment with RAS. Using immunohistochemistry, we observed that Nrf2 was significantly induced in prostate tissue from TRAMP mice treated with RAS (Figure 3.7.A), which was positively correlated with 5-methylcytosine-positive cells (Figure 3.7.B), in agreement with our previous findings in TRAMP-C1 cells [157]. Hypermethylation of Nrf2 leading to gene silencing is important because Nrf2-disrupted mice are more susceptible to chemically induced DNA damage and oxidative stress-induced diseases, such as cancer, compared with wild-type mice [160]. In addition, the hypermethylation of various genes, including RARβ, TNFRSF10C, RASSF1A, Neurog1 and GSTP1, has been reported as a common

feature of human prostate cell lines and/or prostate cancer samples [161, 186, 187]. Therefore, the ability of RAS to restore silenced Nrf2 expression and decrease global hypermethylation in a dose-dependent manner supports the suggestion that RAS extract may be a promising agent in prostate cancer prevention.

An important concern in chemoprevention is the potential toxicity associated with the treatments. RAS has been reported to be a nontoxic agent *in vivo*, even at high doses [155]. In our present study, RAS feeding for 24 weeks, starting at eight weeks of age. did not generate any evident toxic effects, and weight gain was observed in all groups of mice during the study. However, we cannot discard the possibility of some degree of toxicity in the RAS-HD group because the weight gain of these mice was significantly lower than that observed for the RAS-LD and control groups from the 4th week of treatment (Table 3.1) until sacrifice. Similarly, a decrease in the overall weight of the GU apparatus in the RAS-HD group was observed, compared with the control (Figure 3.5.B). Nevertheless, no abnormalities were found during the histological analysis of the prostate in this group.

3.5. Conclusion

Taken together, our findings suggest that RAS and its main bioactive phthalide Lig may be useful agents for prostate cancer prevention. Additional studies are needed to assess whether the anti-prostate cancer efficacy of RAS involves other molecular changes or perhaps synergistic effects exerted by its other main phthalides. To our knowledge, this is the first study to demonstrate that RAS exerts important antiproliferative and proapoptotic effects in the TRAMP model and that it restores the expression of epigenetically silenced Nrf2 *in vivo*.

Table 3.1.   Body weights of TRAMP mice measured weekly

Week	Control			RAS-LD				RAS-HD			
							Weight (g) ±SD				
8	22.5	±	1.0	22.5	±	1.4	ns	21.8	±	1.3	ns
9	21.9	±	1.0	22.2	±	1.4	ns	21.6	±	1.2	ns
10	22.5	±	1.2	21.9	±	1.5	ns	21.7	±	1.2	ns
11	22.7	±	1.1	23.1	±	1.5	ns	22.1	±	1.3	ns
12	23.4	±	1.0	23.5	±	1.3	ns	22.0	±	1.2	*
13	23.5	±	1.2	23.8	±	1.6	ns	22.4	±	1.4	*
14	23.5	±	1.0	23.8	±	1.6	ns	22.5	±	1.1	*
15	24.1	±	1.2	24.2	±	1.4	ns	22.6	±	1.1	*
16	24.7	±	1.2	24.4	±	1.4	ns	23.0	±	1.1	*
17	24.6	±	0.8	24.7	±	1.5	ns	23.0	±	0.9	*
18	24.8	±	0.8	25.1	±	1.6	ns	23.3	±	0.9	*
19	24.8	±	1.0	25.3	±	1.4	ns	23.5	±	0.9	*
20	25.1	±	1.0	25.5	±	1.5	ns	22.9	±	1.0	*
21	25.2	±	1.2	25.6	±	1.6	ns	22.9	±	1.1	*
22	25.6	±	1.2	25.8	±	1.5	ns	23.0	±	1.3	*
23	26.0	±	1.4	26.1	±	1.9	ns	23.1	±	1.3	*
24	26.1	±	1.2	25.9	±	1.9	ns	23.3	±	1.9	*

* p values < 0.05 were considered as significant. ns= no significant.

Table 3.2. RAS feeding inhibits the growth of palpable tumors and metastasis in TRAMP males at 24 weeks of age

Treatments	Number of animals	Incidence of palpable tumor	Lymph nodes
Control	14	8/14	1/14
RAS-LD	12	4/12	0/12
RAS-HD	13	3/13	0/13

Figure 3.1. Effect of RAS and Lig on TRAMP-C1 cell survival based on MTS **(A)** and trypan blue dye exclusion **(B)** assays

For the MTS assay **(A)**, TRAMP-C1 cells were treated in 96-well plates with RAS (4.25 µg/mL or 8.5 µg/mL) or Lig (25 µM or 50 µM) for 24 or 48 h. MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium] was introduced into 96-well plates containing cells after the incubation period, according to the manufacturer's instructions. For the trypan blue assay **(B)**, cells were treated in 6-well plates with RAS (4.25 µg/mL or 8.5 µg/mL) or Lig (25 µM or 50 µM) for 24 or 48 h. At the end of the treatment, trypan blue stain was added to an aliquot of cells to assess the live/dead cell ratio, and the results were normalized against control-treated cells.* and # indicate a significant difference of $p < 0.05$ and $p < 0.01$ vs. control, respectively.
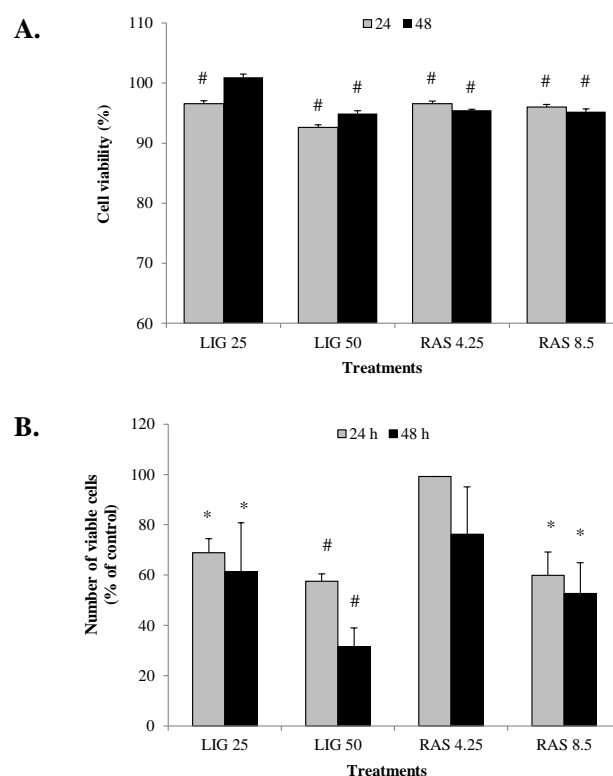
Figure 3.2.   Effect of RAS and Lig on the cell cycle in TRAMP-C1 cells

Cells were treated in 10-cm plates with RAS, Lig or DMSO as a control for 48 h. The cells were fixed with ethanol and stained with propidium iodide (PI), and a cell cycle analysis was performed by flow cytometry, as described in the Materials and Methods section. Three separate experiments were conducted, and representative results are shown. * and # indicate a significant difference from the control, $p<0.01$ and $p<0.05$, respectively.
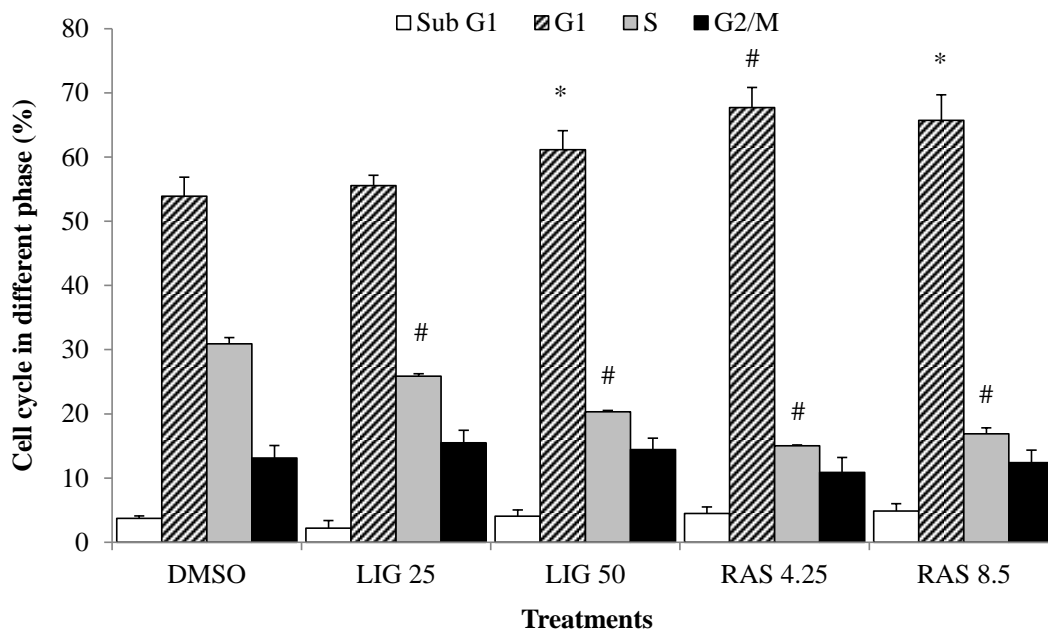
Figure 3.3. Induction of TRAMP-C1 cell apoptosis by RAS and Lig

Cells were treated in 6-cm plates with RAS, Lig or DMSO as a control for 48 h. Then, the cells were stained with Annexin V/ PI, and apoptotic cell death was determined by flow cytometry. Two separate experiments were conducted with similar results. Representative pictures are shown.
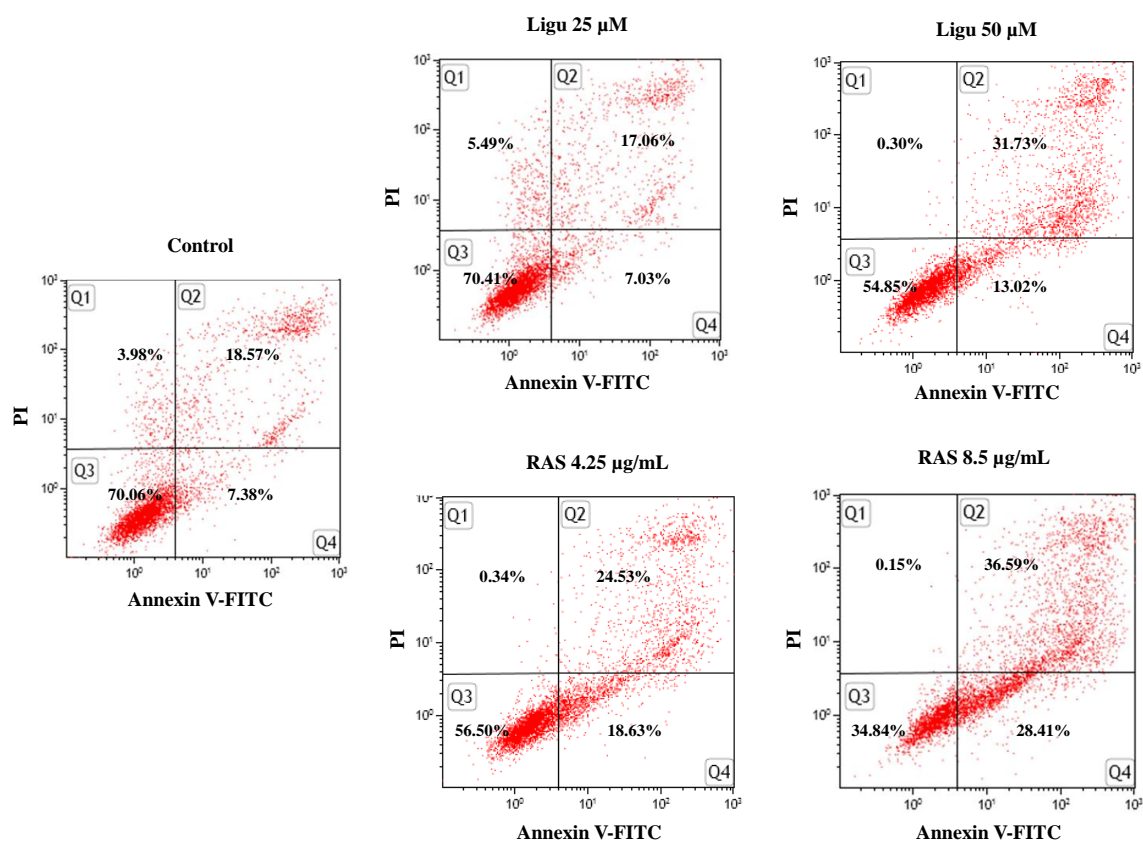
Figure 3.4. Western blot of biomarkers for apoptosis **(A)** and cell cycle regulation **(B)** affected by treatment of TRAMP-C1 cells with Lig and RAS

TRAMP-C1 cells were treated with various doses of Lig or RAS extract for 48 h, and the total cell extracts were collected and subjected to western blotting, with β-actin used as an internal control. **(A)** Lig and RAS induced caspase-3 and PARP cleavage. **(B)** Effects of Lig and RAS on cyclin A, cyclin D1, p21 and p27 expression. The images were analyzed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in relative protein expression compared with control DMSO.
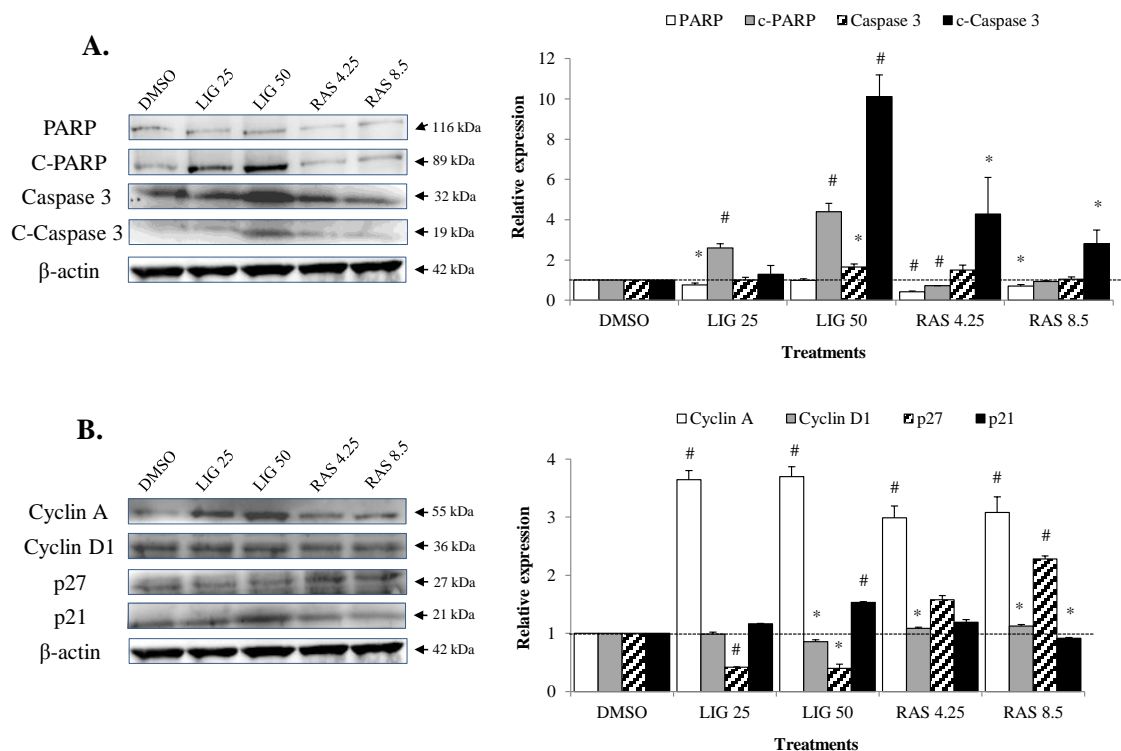
Figure 3.5. Effect of a RAS-supplemented diet on TRAMP mice at 24 weeks of age; design of the study **(A)** and GU weight of the mice **(B)**

(A) TRAMP mice at eight weeks of age were randomly divided into three groups: control diet AIN-93M (n=14), RAS-LD (n=12) and RAS-HD (n=13). The mice were treated with the diets until 24 weeks of age. **(B)** The average wet weights of the genitourinary apparatuses of the TRAMP mice are shown. The boxplot shows the average weight of the wet genitourinary apparatus of the mice at the moment of sacrifice (24 weeks old). * $p < 0.05$, significantly different from the control (ANOVA).

A.
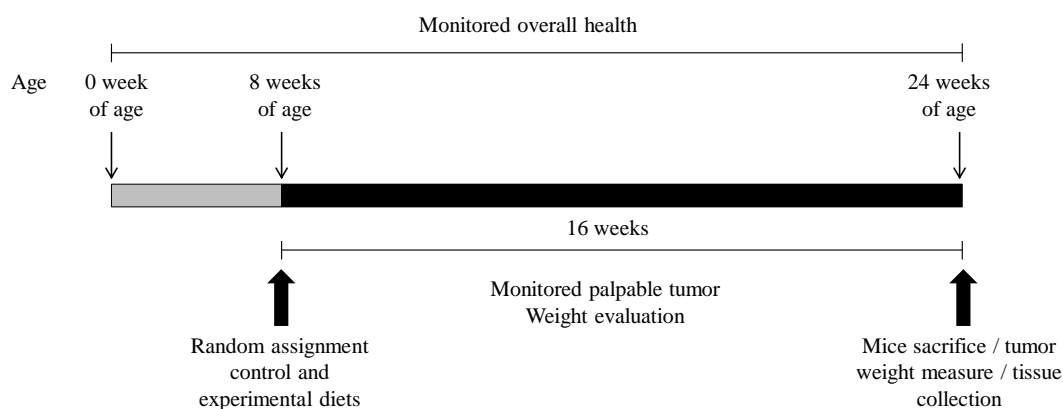
B.
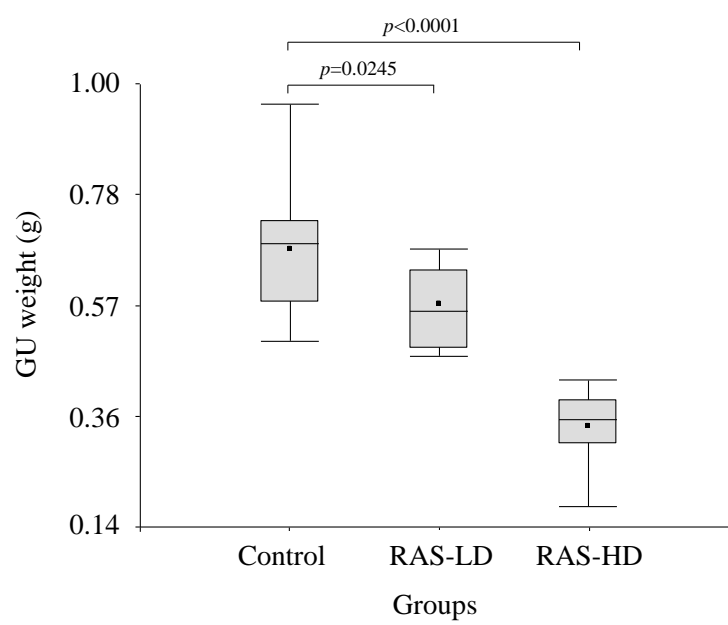
Figure 3.6. Effect of the RAS-supplemented diet on TRAMP mice at 24 weeks of age. Representative micrographs of prostate sample tissue **(A)**, effect of RAS on PIN lesions **(B)** and PCNA immunostaining **(C)**

(A) Representative photomicrographs (×400 magnification) of H&E staining of the dorsolateral section of control and treated mice at 24 weeks of age. Representative figures of normal, LG-PIN and HG-PIN samples. **(B)** The dorsolateral prostate was microdissected, H&E stained and blinded for PIN evaluation (n=7). p-values < 0.05 were considered significant. **(C)** Immunohistochemical analysis of PCNA. Representative photomicrographs (×40 magnification) of PCNA in TRAMP prostate tissue (n=3). The percentage of positive cells (%) with IHC staining of PCNA was evaluated in control, RAS-LD and RAS-HD samples using an Aperio ScanScope® GL system, as described in the Materials and Methods section. The bars represent the mean values ± S.D. p-values < 0.05 were considered significant.

A.

| Normal | LG PIN | HG PIN |

B.

C.

| Control | RAS-LD | RAS-HD |
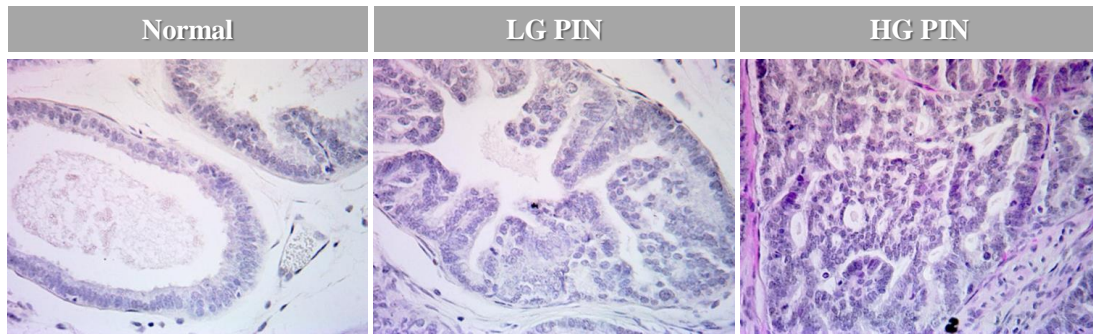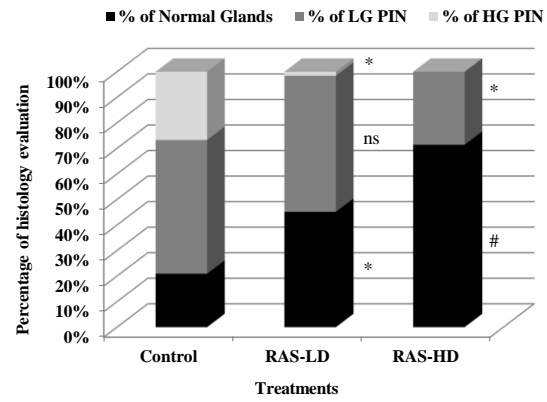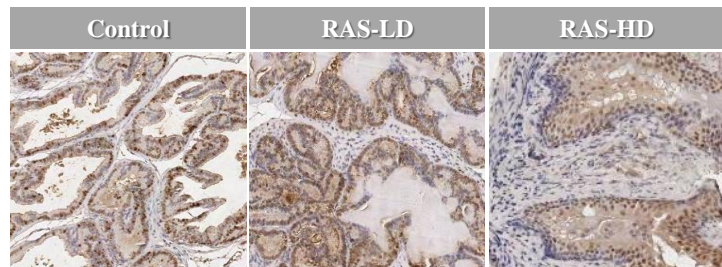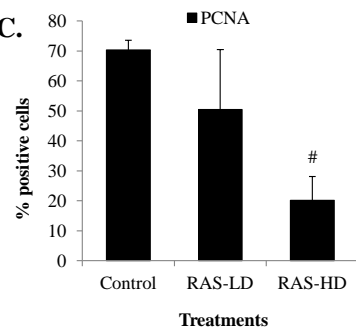
Figure 3.7. RAS-supplemented diet induces Nrf2 expression **(A)** and decreases 5-mC **(B)** in prostate samples from TRAMP mice at 24 weeks of age

Immunohistochemical analysis of Nrf2 and 5-methylcytosine. Representative photomicrographs ($\times$40 magnification) of stained TRAMP prostate tissue and the percentage of positive cells are shown (n=3). The percentage of positive cells (%) with IHC staining of Nrf2 or 5-methylcytosine was evaluated in control, RAS-LD and RAS-HD samples using an Aperio ScanScope® GL system, as described in the Materials and Methods section. The bars represent the mean values ± S.D. p-values < 0.05 were considered significant.
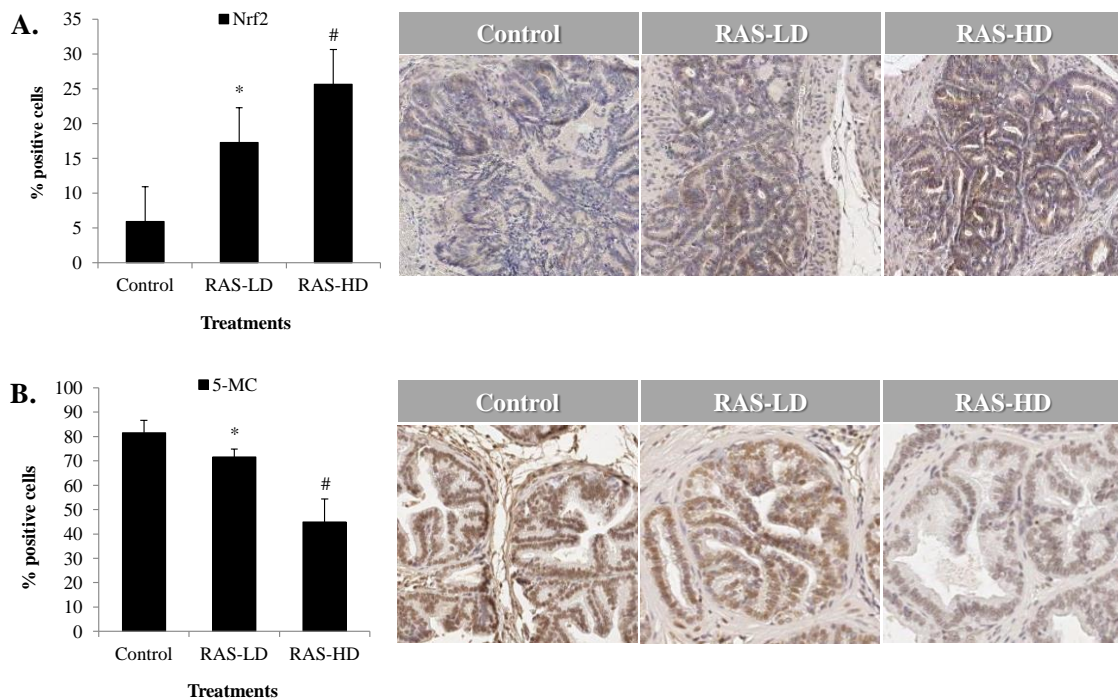
Chapter 4.　Induction of Nrf2-mediated gene expression by Dietary Phytochemical Flavones Apigenin and Luteolin. [7,8,9]

4.1.　Introduction

Increased consumption of fruits and vegetables is associated with a decreased risk of cancer, and this correlation is thought to be driven by the high levels of phytochemicals with anticancer properties that are contained in fruits and vegetables [3]. Apigenin (4′,5,7,-trihydroxyflavone; API) and luteolin (3′,4,5,7-tetrahydroxyflavone; LUT) are low molecular weight polyphenolic compounds and two of the most common dietary flavonoids in the human diet (Figure 4.1.A and 4.1.B, respectively) [188]. These compounds are abundant in a wide variety of common herbs and vegetables, such as parsley, chamomile, celery, and citrus fruits [188, 189]. Although API and LUT are usually found in small quantities, their intake is associated with a decreased risk of several types of cancer [190, 191]. In this context, various studies suggest that these flavonoids inhibit critical events associated with carcinogenesis, including cell transformation, invasion, metastasis, and angiogenesis, by modulating kinases, inhibiting transcription factors, regulating the cell cycle, and inducing apoptosis [192, 193]. Subsequently, several molecular targets have been identified to contribute to these effects, including the activation of phosphatidylinositol 3'-kinase (PI3K)/Akt, nuclear factor kappa B (NF-kB),

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mitogen-activated protein kinase (MAPK), p53, and many others; however, the mechanisms underlying their chemopreventive effects are not completely understood [193, 194].

It has been increasingly reported that the biological activities of LUT and API are related to their ability to modulate reactive oxygen species (ROS) [195, 196]. ROS are highly reactive chemical species that play important roles in cell signaling and homeostasis when they are present at low levels but can lead to carcinogenesis at high levels by promoting DNA damage, genomic instability, and neoplastic transformation [69, 197]. The induction of cytoprotective mechanisms, such as the expression of phase II detoxification/antioxidant enzyme orchestrated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is believed to have an important role in preventing carcinogenesis [4, 198]. After translocation into the nucleus, Nrf2 induces the transcriptional activity of a *cis*-acting DNA element known as the antioxidant response element (ARE)/electrophile response element (EpRE), which leads to the expression of protective genes, such as phase II drug metabolizing enzymes UDP-glucuronosyltransferases (UGT), glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO-1), and anti-oxidative stress enzymes heme oxygenase-1 (HO-1) [198].

API and LUT have been reported to protect against oxidative stress through the upregulation of glutamate cysteine ligase, the glutamate cysteine ligase catalytic subunit, and HO-1 in primary rat hepatocytes via the Nrf2 signaling pathway [199]. In addition, we have previously demonstrated that API targets key CpG sites in the Nrf2 promoter region of epithelial JB6 P+ cells through epigenetic mechanisms, and this leads to the reactivation of Nrf2-dependent antioxidant/detoxifying enzymes [165]. Interestingly, increasing

evidence has indicated that important cross talk occurs between Nrf2 and NF-κB under conditions of oxidative stress, suggesting that Nrf2 plays an important role in the regulation of inflammation [200-203]. For example, HepG2 cells challenged with lipopolysaccharide (LPS) show significantly enhanced NF-κB transcriptional activity when Nrf2 is silenced [204]. Additionally, we reported that sulforaphane (SFN) treatment of Nrf2 (+/+) but not Nrf2 (-/-) mice restored the number of healthy cells back to basal levels by 8 days after UVB irradiation, demonstrating a decreased activity of inflammatory biomarkers in SFN-treated Nrf2 (+/+) mice compared to KO mice and revealing a protective role of Nrf2 against UVB-induced skin inflammation [91]. Subsequent studies in our lab demonstrated that the protective effects of Nrf2 in response to UVB irradiation are mediated in part by increased HO-1 protein expression [10].

API and LUT have been extensively reported to possess strong anti-inflammatory activities by repressing NF-kB and inhibiting proinflammatory mediators *in vitro* and *in vivo* [193, 194, 205, 206]. Therefore, we examined the Nrf2-ARE activation mediated by API and LUT and the anti-inflammatory activity of both compounds in LPS-stimulated HepG2 cells. Our research suggests that API and LUT activate the anti-oxidative Nrf2-ARE pathway and that this activation may be involved in their attenuation of LPS-induced inflammation.

4.2.   Materials and Methods

4.2.1. Reagents and cell culture

Dimethyl sulfoxide (DMSO), API (Fig. 4.1A), and LUT (Fig. 4.1 B) were purchased from Sigma (St. Louis, MO). Sulforaphane (SFN) was purchased from LKT laboratories (St. Paul, MN). The inhibitors of phosphatidylinositol 3-kinase (PI3K)

(LY294002), p38 MAPK (SB203580), JNK-1/2 (SP600125), and ERK-1/2 (PD98059) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Human hepatoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The HepG2-C8 cell line was established in Dr. Ah-Ng Tony Kong's laboratory by transfecting HepG2 cells with a pARE-T1-luciferase construct (kindly provided by Dr. William Fahl, University of Wisconsin) using the FuGENE 6 method as previously described [12]. The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.17 g/l sodium bicarbonate, 100 units/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown to 80% confluence, split, and then sub-cultured in fresh medium three times per week after washing with Versene and detaching with trypsin (Gibco, Carlsbad, CA, USA).

4.2.2. Cell viability assay

HepG2 cells were seeded in 96-well plates in medium containing 1% FBS per well (1×10^4 cells/well) at an initial density of 1×10^5 cells/ml in a volume of 100 µl. After overnight incubation, the cells were treated with DMEM/1% FBS and various concentrations of API and LUT (1.56 µM–100 µM) using 0.1% DMSO as a control for 24 h. The cytotoxicity of the API and LUT was tested by using the CellTiter 96® aqueous non-radioactive cell proliferation MTS assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). Absorbance of the formazan product was measured at 490 nm using a µQuant Biomolecular spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT).

The cell viability was calculated by comparing the optical density of the treated samples to the optical density of DMSO, which was used as the negative control.

4.2.2. Evaluation of ARE reporter gene activity by luciferase assay

HepG2-C8 cells were seeded in 12-well tissue culture plates at a density of 1×10^5 cells/ml in 1 ml of DMEM/1% FBS per well. The cells were treated with various concentrations of API and LUT, and the negative control group was treated with 0.1% DMSO. The positive control group was treated with 6.25 µM SFN, a well-known inducer of ARE activity [12, 14]. After 6 h and 12 h of incubation, the luciferase activities in the cell extracts were measured with a Promega luciferase kit (Madison, USA) according to the manufacturer's protocol. The inhibitors LY294002 (50 µM), SB203580 (10 µM), SP600125 (50 µM), or PD98059 (50 µM) were added to the cultures one hour prior to treatment and incubated with the cells for 12 h during stimulation according to the manufacturers' instructions. Briefly, after the treatment, the cells were washed with ice-cold phosphate buffered-saline (1x PBS, pH 7.4) and immediately harvested in 1x Promega Luciferase Cell Culture Lysis Buffer (Madison, USA). The homogenates were centrifuged at 12,000 rpm at 4°C for 5 min. An aliquot of 10 µl of supernatant was assayed for luciferase activity using a SIRIUS luminometer from Berthold Detection Systems GmbH (Pforzheim, Germany). The luciferase activity was normalized relative to the protein concentration, which was determined using the BCA protein assay from Pierce (Rockford, USA). The results are expressed as the fold induction over the luciferase activity of the control DMSO-treated cells.

4.2.3. Evaluation of of nitric oxide (NO) increase

The concentrations of NO in the supernatants of HepG2 cells were detected using a fluorometric assay, as reported previously [151]. Briefly, HepG2 cells were seeded at a density 1.0×10^5 cells/ml in 96-well plates overnight. Then, the medium was discarded, and the cells were pretreated with API or LUT for 12 h. Lipopolysaccharide (LPS) from Sigma, Inc. (St. Louis, MO, USA) was then added to a final concentration of 1 μg/ml and incubated for 24 h as described by Kang et al. [207]. The MTS assay was used to evaluate any potential toxicity as described above. The controls used were 0.1% DMSO with and without LPS. After 24 h of treatment, 50 μl of supernatant from each well was mixed with 10 μl of 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) from Sigma, Inc. (St. Louis, MO, USA), and the NO concentration was determined by comparison to a calibration curve generated using NO standards in deionized water. After 10 min of incubation at room temperature in the dark, the reaction was terminated with 5 μl of 2.8 M NaOH. The formation of 2,3-diaminonaphthotriazole in black opaque 96-well plates was measured with an FLx-800 microplate fluorescence reader from Bio-Tek Instruments Inc. (Winooski, VT) at 360 nm excitation and 460 nm emission using a gain setting of 75%. The results are expressed as the fold of NO increase as follows: NO increase (fold) = [(nitric oxide content in LPS or sample treatments (μM)] / (nitric oxide content in DMSO control (μM)].

4.2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted from treated HepG2 cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. The cDNA was used as the

template for quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an ABI7900HT system. The following primers were used: Nrf2, 5'-GGCAGAGACATTCCCATTTGTAG-3' (sense) and 5'-TCGCCAAAATCTGTGTTTAAGGT-3' (antisense); NQO1, 5'-CAGAAATGACATCACAGGTGAGC-3' (sense) and 5'-CTAAGACCTGGAAGCCACAGAAA-3' (antisense); HO-1, 5'-GCTCGAATGAACACTCTGGAGAT-3' (sense) and 5'-TCCAGAGAGAAAGGAAACACAGG-3' (antisense). β-actin was used as an internal control with 5'-CGTTCAATACCCCAGCCATG-3' (sense) and 5'-GACCCCGTCACCAGAGTCC-3' (antisense) primers.

4.2.5. Protein lysate preparation and western blotting

The HepG2 cells were treated with API and LUT according to the procedures described previously. DMSO and SFN were used as negative and positive controls, respectively. After 12 h of treatment, the cells were harvested using RIPA buffer supplemented with a protein inhibitor cocktail from Sigma (St. Louis, MO). The protein concentrations of the cleared lysates were determined using the bicinchoninic acid (BCA) method according to the protocol provided by Pierce (Rockford, IL). Then, 20 μg of total protein from each sample was mixed with 5 μl Laemmli SDS sample buffer from Boston Bioproducts (Ashland, MA) and denatured for 5 min at 95°C. The proteins were separated on a Bio-Rad 4-15% SDS-polyacrylamide gel (Hercules, CA) and transferred to a Millipore polyvinylidene difluoride (PVDF) membrane (Bedford, MA), and the membranes were blocked with 5% BSA in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. Then, the membrane was sequentially incubated with specific primary antibodies and HRP-

conjugated secondary antibodies. The bands were visualized using the SuperSignal enhanced chemiluminescence (ECL) detection system and recorded using a Bio-Rad Gel Documentation 2000 system (Hercules, CA). The primary antibodies were purchased from the following sources: anti-Nrf2 from Abcam (Cambridge, MA), anti-HO-1 from Cell Signaling (Boston, MA), and anti-cPLA2, anti-iNOS, anti-NQO-1, and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA).

4.2.6. Statistical analysis

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

4.3. Results

4.3.1. API and LUT display differential profiles in inducing ARE-luciferase reporter activity

The MTS assay was used to determine the cytotoxicity of API and LUT in HepG2 cells after 24 h of treatment. Neither compound presented significant toxicity at concentrations up to 12.5 µM (Figure 4.2.A and 4.2.B); therefore, these concentrations were used for the subsequent studies. To evaluate the transcriptional activation of ARE-luciferase by API and LUT, HepG2-C8 cells were incubated with different concentrations of these compounds (1.56 µM-6.25 µM) for 6 and 12 h. The results demonstrated that API and LUT both induced ARE-luciferase after as little as 6 h of exposure at all the concentrations evaluated, exhibiting significant differences at 6 h for LUT and 12 h for API that were comparable with the effects of the positive control, SFN (6.25 µM) ($P< 0.05$; Figure 4.3.A and 4.3.B).

4.3.2. API and LUT increase ARE-luciferase reporter activity in part through the PI3K and MAPK signaling pathways

We next decided to examine the potential pathways involved in the underlying effect of the ARE activation by API and LUT. It has been suggested that these compounds activate Nrf2 and ARE-related genes through the extracellular signal-regulated protein kinase 2 (ERK2) [199] and PI3K/Akt pathways [208]. Therefore, we treated HepG2-C8 cells with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K; an upstream kinase of Akt), or with PD98059, an inhibitor of the ERK-1/2 mitogen-activated protein kinase (MAPK) pathway, prior to exposure to API or LUT. The luciferase activity of HepG2-C8 cells with the inhibitors alone were examined; however, no luciferase activity was registered for these treatments (data not shown), whereas, the luciferase activity induced by both compounds was nearly abolished in the presence of the PI3K inhibitor (Figure 4.4.A and 4.4.B). Interestingly, the luciferase activity was significantly decreased ($P < 0.05$) in the presence of the ERK-1/2 inhibitor PD98059 in cells treated with API (Figure 4.4.A) but not in cells treated with LUT (Figure 4.4.B). In addition, we did not observe any changes in the ARE induction in cells treated with the inhibitors of p38 MAPK (SB203580) or JNK-1/2 (SP600125) (data not shown).

4.3.3. API and LUT increase the mRNA and protein levels of Nrf2 and Nrf2-regulated genes

Because API and LUT showed the strongest induction of ARE-luciferase activity at 12 h (Figures 4.3.A and 4.3.B), we decided to evaluate the expression of Nrf2 and its target genes NQO-1 and HO-1 at the mRNA and protein levels in HepG2 cells by quantitative real-time polymerase chain reaction and western blotting, respectively. The

results revealed a moderate increase in NQO-1 expression and a significant increase in the mRNA levels of Nrf2 and HO-1 ($P< 0.05$) after treatment with API and LUT compared with the control; this induction was comparable to that observed in the positive control, SFN (6.25 µM) (Figure 4.5.A). At the protein level, we observed a moderate elevation of NQO-1 with all compounds after 12 h of treatment, whereas an increase of Nrf2 and HO-1 expression was observed with API and LUT for all doses evaluated ($P< 0.05$; Figure 4.5.B). Interestingly, when cells were treated with LUT, the Nrf2 protein level was increased by approximately 5-fold, which is greater than that induced by SFN (6.25 µM).

4.3.4. API and LUT inhibit NO production and reduce iNOS and cPLA2 protein expression in LPS-induced HepG2 cells

Nrf2 and its downstream gene HO-1 have been reported to be key anti-inflammatory mediators that suppress the nuclear factor (NF) kappa B (NF-κB) signaling pathway in LPS induction experiments [209, 210]. Furthermore, API and LUT have been reported to block this pathway in both *in vitro* and *in vivo* studies [205, 206, 211]. Therefore, we decided to investigate whether the activation of the Nrf2 signaling pathway by API and LUT is related to their potential anti-inflammatory effects by evaluating NO production in LPS-induced HepG2 cells. HepG2 cells were pre-treated with API or LUT for 12 h before being challenged with LPS for 24 h. No toxicity was observed in the cells treated with LPS in combination with API or LUT, as demonstrated by the MTS assay (Figure 4.6.A). Next, we evaluated the nitric oxide level in LPS-treated cells with and without pretreatment with API and LUT. We observed that API and LUT significantly reduced LPS-stimulated NO production in a dose-dependent manner ($P< 0.05$; Figure 4.6.B). LUT exhibited a stronger inhibitory activity than API at all concentrations. Because

cPLA2 and iNOS are important inflammatory mediators [212], we further examined whether their protein levels were affected by API or LUT relative to control LPS-treated cells. Thus, we found that LPS stimulated the expression of iNOS and cPLA2 (Figure 4.6.C) and that API and LUT markedly blocked the induction of these proteins in a dose-dependent manner ($P < 0.05$).

4.4.   Discussion

Extensive reports have shown that the plant flavones API and LUT exert a variety of biological activities, including the modulation of inflammation and carcinogenesis, and thus constitute promising molecules for cancer prevention [190, 213]. Recent data indicate that API and LUT protect against oxidative stress through the upregulation of HO-1 mediated via Nrf2 [165, 199]. In agreement with the above findings, our data show that API and LUT significantly induced ARE-luciferase activity in HepG2-C8 cells in a dose-dependent manner at 6 h (Figure 4.3.A) and that this activity was maintained or enhanced after 12 h of treatment (Figure 4.3.B). We also observed that API and LUT significantly induced the mRNA and protein levels of Nrf2 and its target gene HO-1 after 12 h of treatment (Figure 4.5.A and 4.5.B). The Nrf2-ARE system is regulated by several kinases, such as extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal protein kinase (JNK), p38, and phosphoinositide 3-kinase (PI3K), and API and LUT exert important biological activities through these pathways [193, 194, 198]. Therefore, we evaluated the role of MAPKs in the ARE activation mediated by the flavones by using the inhibitors PD98059, SB203580, and SP600125 to perturb signaling through ERK-1/2, p38 MAPK, and JNK, respectively. In the presence of the ERK-1/2 inhibitor, the induction of ARE activity by API was significantly decreased, but the other MAPK inhibitors had no effect,

indicating a potential contribution of ERK-1/2 to ARE modulation by API (Figure 4.4.A). In contrast, the ARE activity induced by LUT was not affected by the three MAPK inhibitors evaluated (Figure 4.4.B).

These contrasting results may be explained by the nature of the stimuli, the cell type, the sequence of the ARE, or the chemical characteristics of these flavones [214]. Although the Nrf2-ARE system has been reported to be modulated by different MAPKs, this topic is poorly studied and highly controversial because modulation may occur through indirect mechanisms with limited effects [215]. Accordingly, after 12 h of treatment, we did not observe substantial differences in the activation of the Nrf2 signaling pathway based on the ARE-luciferase activity or in the mRNA levels of the flavones. However, at the protein level, LUT had a moderately stronger effect on Nrf2 expression, but no significant differences between the flavones were observed with regard to HO-1 expression (Figure 4.5.B). In contrast, we observed that the PI3K inhibitor strongly abrogated ARE activation by API and LUT (Figure 4.4.A and 4.4.B), which is consistent with a previous report by Lim et al. regarding the stimulation of the Nrf2 signaling pathway through PI3K/Akt by the related compound luteolin 6-C-beta-D-glucoside in HepG2 cells [208].

Increasing evidence suggests that Nrf2 is involved in the activation of antioxidant/phase 2 gene transcription machinery and in the regulation of inflammation by modulating the NF-κB pathway through a regulatory feedback loop [203, 216, 217]. For example, Nrf2-deficient mice challenged with LPS or dextran sulfate sodium (DSS) show a dramatic increase in the activity of the NF-κB inflammatory signaling cascade, and reports indicate that Nrf2 affects the redox status of the cells and modulates NF-κB activation [144, 210]. Similarly, Nrf2 knockdown by siRNA in LPS-stimulated HepG2

cells significantly increased NF-κB transcriptional activity and the levels of NF-κB-dependent transcription factors, such as interleukin 6, B-cell lymphoma 2, tumor necrosis factor alpha, and cellular inhibitor of apoptosis 2, whereas Nrf2 overexpression reduced NF-κB transcriptional activity, perhaps by regulating the activity MafK-dependent NF-κB [204]. We have previously observed that LPS-induced NF-κB activation can be attenuated by Nrf2 inducers such as phenethyl isothiocyanate, sulforaphane, and curcumin [218]. Although the mechanism by which Nrf2 modulates inflammatory signals is not fully understood, some studies suggest that the Nrf2 target gene HO-1 may be involved in this activity because its upregulation has been correlated with cytoprotective effects in various inflammatory diseases [219].

API and LUT both significantly increased HO-1 expression at the mRNA and protein levels (Figures 4.5.A and 4.5.B); therefore, we hypothesized that API and LUT would demonstrate anti-inflammatory activity in HepG2 cells challenged with LPS because NF-κB plays a pivotal role in the response to LPS via Toll-like receptor 4 (TLR4) [220, 221]. Activated TLR4 induces the expression of a broad spectrum of mediators, including inducible nitric oxide synthase (iNOS), which has been shown to depend of NF-κB activation [222]. iNOS catalyzes the oxidative deamination of l-arginine to produce NO, which is a potent pro-inflammatory and tumorigenesis mediator [222-224]. In our study, we observed that the treatment of LPS-induced HepG2 cells with API or LUT reduced the production of NO in a dose-dependent manner (Figure 4.6.B); this result correlates with the suppression of iNOS protein expression by these flavones (Figure 4.6.C) and is consistent with the results of other studies regarding their anti-inflammatory activities [225, 226].

In addition, we have reported that Nrf2 plays an important role in the regulation of proinflammatory mediators, such as cyclooxygenase (COX2), arachidonate 5-lipoxygenase (5-LOX), prostaglandin E2, leukotriene B4, iNOS, and cytosolic phospholipase A2 (cPLA2), by using Nrf2-knockout mice [12, 144, 203]. cPLA2 catalyzes the release of arachidonic acid, which is the limiting substrate for enzymes such as 5-LOX and COX, and we have observed that cPLA2 is a consistent pro-inflammatory pathway affected by Nrf2 deletion [12, 144, 203]. cPLA2 is of interest because its overexpression has been associated with the pathogenesis of several types of cancer by stimulating proliferation and angiogenesis, which affect tumor progression and resistance to therapies [227, 228]. In our study, we observed that API and LUT significantly suppressed the induction of cPLA2 protein in LPS-treated HepG2 cells in a dose-dependent manner (Figure 4.6.C).

4.5. Conclusion

The results of this study demonstrate that the natural flavones API and LUT both activate Nrf2 and downstream genes, particularly HO-1, in HepG2 cells at low doses, and this activation of Nrf2 seems to be related to their potent anti-inflammatory activities seen in LPS-stimulated HepG2 cells. Therefore, API and LUT hold great promise as chemopreventive agents that promote anti-oxidative/anti-inflammatory activities.

Figure 4.1.   Chemical structures of API **(A)** and LUT **(B)**.

A.

B.
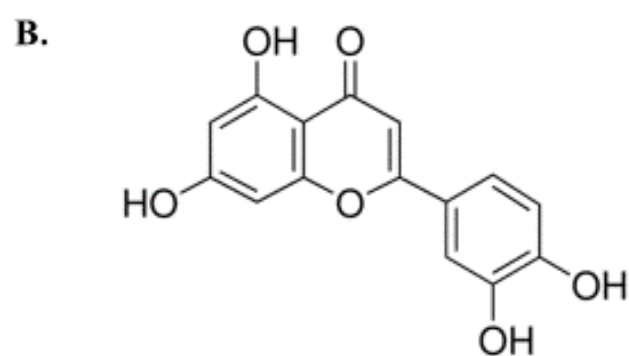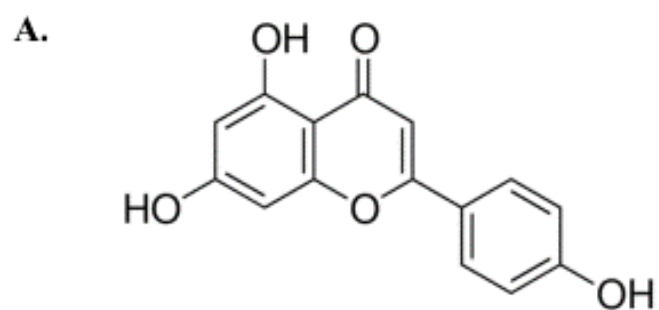
Figure 4.2.   Viability of HepG2 cells after treatment with API **(A)** or LUT **(B)** for 24 h

Cells were seeded in 96-well plates in 10% DMSO for 24 h. The cells were then incubated

in fresh medium with different concentrations (1.56 to 100 μM) of API or LUT for 24 h,

as described in the Materials and Methods section. Cell viability was determined and

calculated using the MTS assay. The data are expressed as the mean ± SD (n=3). Asterisks

indicate significant differences (p<0.05) in cell viability compared to the control

A.

B.
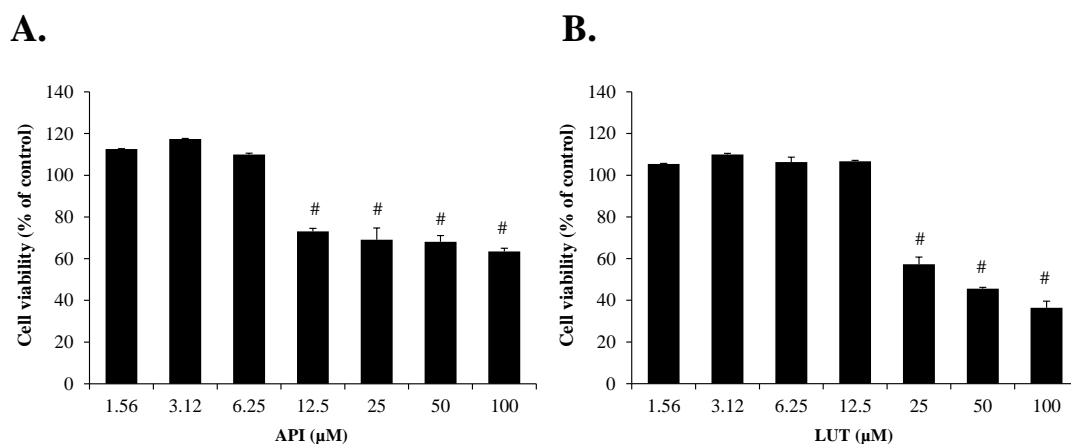
Figure 4.3. Induction of ARE-luciferase activity by API and LUT at concentrations from 1.56 to 6.25 μM in HepG2-C8 cells after 6 h **(A)** and 12 h **(B)**

The normalization of the luciferase activity was performed based on the protein concentrations, which were determined using a BCA protein assay, as described in the Materials and Methods section. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the vehicle control. Asterisks and number signs indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between the treatment and the control group
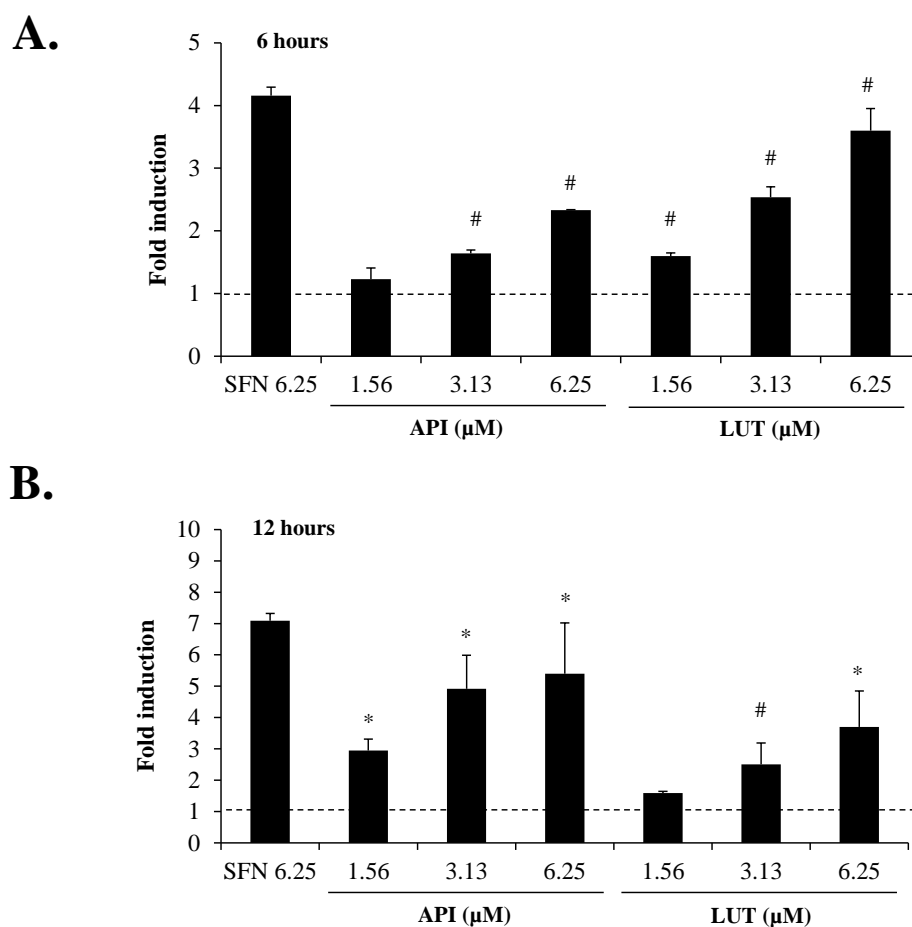
Figure 4.4. Induction of ARE-luciferase activity by the treatment of HepG2-C8 cells with API **(A)** and LUT **(B)** in combination with the phosphoinositide 3-kinase inhibitor, LY294002, or the ERK-1/2 inhibitor, PD98059, during a 12-h treatment

HepG2-C8 cells were treated as described in the Materials and Methods section. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the vehicle control. Asterisks and number signs indicate significant differences (p<0.05 and p<0.01, respectively) between the treatment and control groups
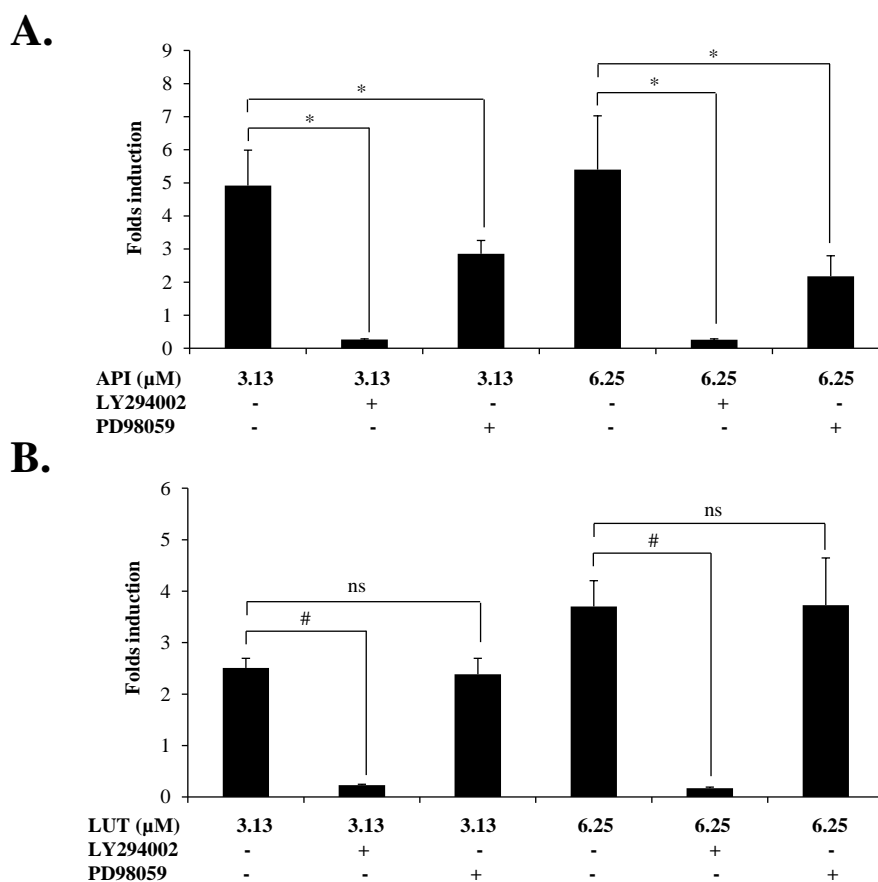
Figure 4.5. Effect of API and LUT on the relative fold changes of the mRNA **(A)** and protein **(B)** levels of Nrf2 and the Nrf2 target genes NQO-1 and HO-1 in HepG2 cells

Cells were incubated with different concentrations of API and LUT (1.56–6.25 μM) for 12 h, as described in the Materials and Methods section. Normalization of the mRNA expression data was performed using β-actin as an internal control. The protein expression level was normalized to β-actin (a complete description of the procedure and antibodies used is provided in the Materials and Methods section). The images were analyzed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The data are expressed as the mean ± SD of three independent experiments. Asterisks and number signs indicate significant differences (p<0.05 and p<0.01, respectively) in the relative mRNA and protein expression levels compared to DMSO, which was used as a negative control.
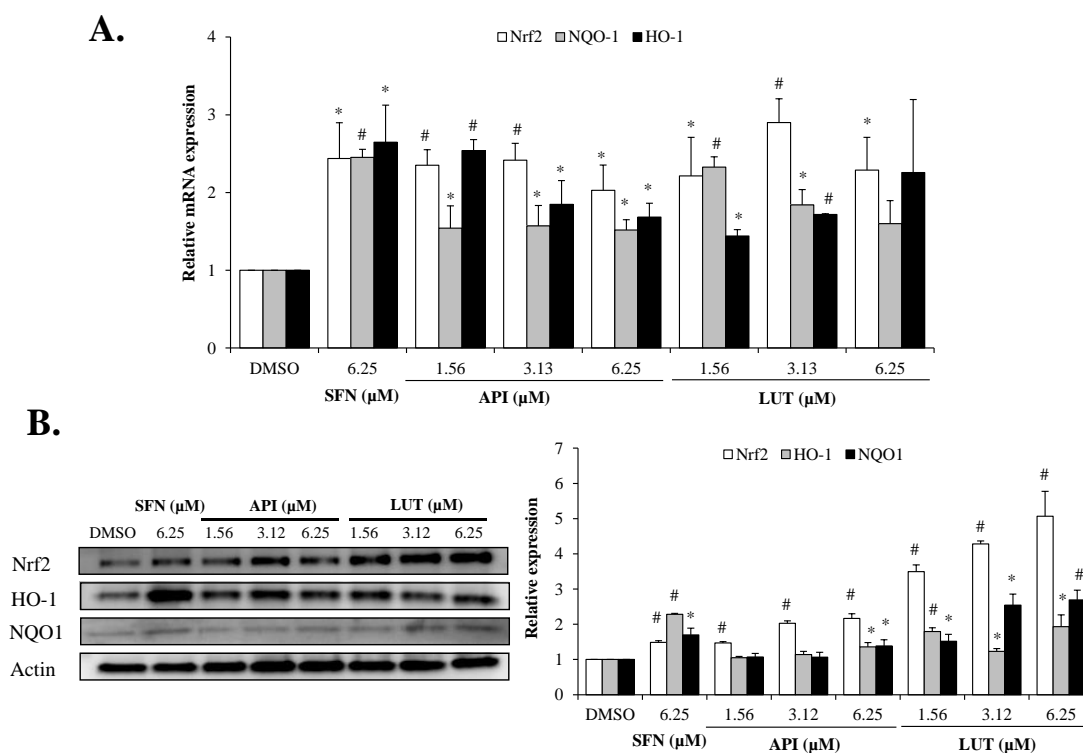
Figure 4.6.   Effect of API and LUT on cell viability **(A)**, NO increase **(B)** and iNOS and cPLA2 protein expression **(C)** in LPS-stimulated HepG2 cells

HepG2 cells were pre-treated for 12 h before being challenged with 1 µg/ml LPS for 24 h, as described in the Materials and Methods section. The protein expression level was normalized to β-actin, and the images were analyzed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The data are expressed as the mean ± SD of three independent experiments. Asterisks in **(A)** indicate significant differences ($p < 0.05$) in cell viability compared to DMSO, which was used as a negative control. Asterisks and number signs in **(B)** and **(C)** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in NO production and relative protein levels compared to LPS treatment
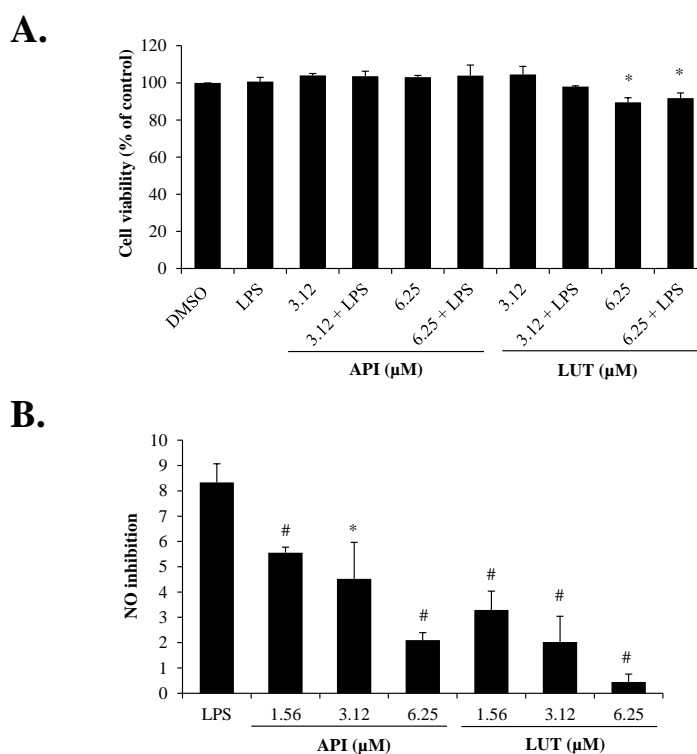
C.
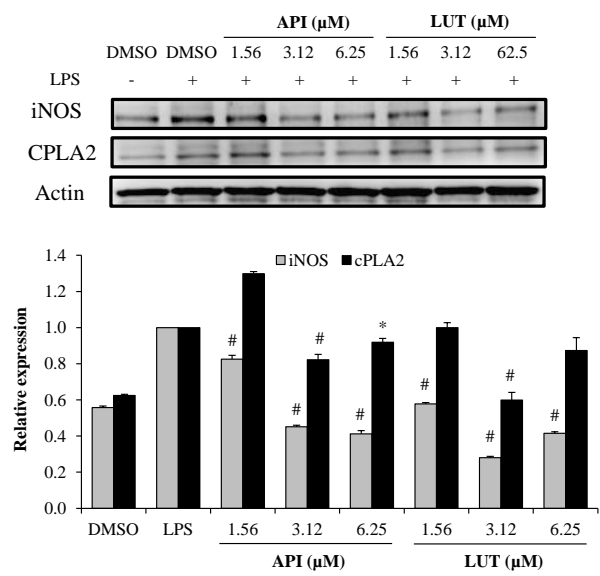
Chapter 5.   Apigenin reactivates Nrf2 anti-oxidative Stress signaling in mouse epidermal JB6P+ cells through epigenetics modifications [10],[11],[12]

5.1.   Introduction

Skin cancers are the most common types of cancer in the US and worldwide [229, 230]. It is estimated that 81,220 men and women will be diagnosed with the disease in 2014 and that 12,980 will die [231]. Ultraviolet radiation (UVR) between 200 and 400 nm is the most important risk factor leading to DNA damage, inflammation, immune impairment and epigenetic modifications [232-234]. Accumulating evidence shows that epigenetic silencing of critical tumor suppressor genes plays a role in cancer development and that enhanced DNA methylation at the C5 cytosine position of the CpG dinucleotides in the CpG island is associated with a transformed phenotype [235]. In epidermis that is chronically exposed to UVR, there is a strong correlation among DNA methylation, augmented activity of DNA methyltransferases (DNMTs) and histone modifications [235]. Thus, DNA methylation represents an early molecular event that precedes the observation of actual neoplastic lesions on the epidermis [236]. In this context, diverse studies have revealed aberrant promoter methylation of many genes involved in critical cellular

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[10] This chapter has been published in the AAPS journal as *original research paper* as "Apigenin reactivates Nrf2 anti-oxidative stress signaling in mouse epidermal JB6P+ cells trough epigenetics modifications" by Paredes-Gonzalez X, Fuentes F, Su ZY and Kong AN. July 2014, Volume 16, Issue 4, pp 727-735, DOI:10.1208/s12248-014-9613-8

[11] Key Words: Apigenin, Nrf2, skin cancer, epigenetics, DNMTs, HDACs, JB6 P+.

[12] Abbreviations: API, apigenin; Nrf2, erythroid 2p45 (NF-E2)-related factor 2; ROS, reactive oxygen species; TSA, trichostatin A; 5-aza, 5-azadeoxycytidine; HDAC, Histone deacetylase; DNMT, DNA methyltransferase

processes, including CDH1, CDH3, LAMA3, LAMC2, RASSF1A, BCL7a, PTPRG, thrombospondin 4, p73, p16, CHFR, p15 and TMS1 [234, 236-238].

The deregulation of the antioxidant defense system has been gaining increased attention because it promotes toxicity and the neoplastic progression of cancer [3, 4, 239]. In this context, we have reported that erythroid 2p45 (NF-E2)-related factor 2 (Nrf2), a basic region leucine zipper (bZIP) transcription factor that regulates the expression of many phase II detoxifying/antioxidant enzymes, is suppressed epigenetically by promoter CpG methylation/histone modifications in association with MBD2 in prostate tumors from TRAMP mice and tumorigenic TRAMP-C1 cells [160]. In addition, we have recently demonstrated that Nrf2 is downregulated in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of mouse skin epidermal JB6 P+ cells [239]. Thus, the reversible nature of epigenetic modifications is an attractive strategy to correct abnormal skin DNA methylation patterns using DNMT inhibitors, such as 5-azadeoxycytidine (5-aza) and 5-aza′-2-deoxycytidine (decitabine), or histone deacetylase (HDAC) inhibitors, such as romidepsin, trichostatin A (TSA) or vorinostat. However, the therapeutic use of these agents has been limited because of unacceptable toxic effects and the lack of gene modulation specificity [240]. Thus, phytochemicals with minor side effects that have DNA methylation modulating properties are a promising alternative for skin cancer chemoprevention [241].

Apigenin (API) is the most important and active flavonoid present in chamomile flowers (*Matricaria recutita* L.), a common component of many skin formulations used in folk medicine since the time of Hippocrates in 500 BC [242]. API has long been recognized to be active against skin carcinogenesis, exerting a broad spectrum of activities, including

DNA damage prevention, cell cycle arrest and apoptosis induction, as well as immunomodulatory and anti-inflammatory effects with high tumor specificity activity, low toxicity and the ability to penetrate deep skin layers in mice and humans [194, 243-245].

Thus, topical application of API potently suppresses epidermal ornithine decarboxylase (ODC) activity, which directly promotes skin carcinogenesis by enhancing cell proliferation, angiogenesis and metastasis in susceptible mouse strains after administration of TPA or UV irradiation [246, 247]. Moreover, API treatment of SENCAR mice reduces papilloma incidence induced by TPA/7,12-dimethylbenz (a)anthracene (DMBA) by more than 50%, thereby reducing the conversion from papilloma to carcinoma and increasing the latency period for tumor conversion [246]. Similarly, a reduction in tumorigenesis has been observed after topical application of API in SKH-1 mice exposed to chronic UV irradiation [247] and in intraperitoneal administration in syngeneic C57BL6 mice injected with B16-BL6 cells [248]. Interestingly, API induces epigenetic modifications due to its capacity to produce 5-methylcytosine inhibition in the human KYSE-510 esophageal squamous cell carcinoma cell line [249] and histone deacetylase (HDAC) inhibition in PC3 and 22Rv1 prostate cancer cell lines [250]. In this study, we investigated the potential of API to restore the expression of Nrf2 through DNA methylation in the preneoplastic epidermal JB6 P+ cell line.

5.2. Materials and Methods

5.2.1. Reagents and cell culture

All chemicals, including apigenin (API), dimethyl sulfoxide (DMSO), 5-aza-2′-deoxycytidine (5-aza) and trichostatin A (TSA), were purchased from Sigma (St. Louis, MO). The murine skin epidermal JB6 P+ cell line, Cl 41-5a, was purchased from American

Type Culture Collection (ATCC, Rockville, MD). JB6 P+ cells were maintained in Minimum Essential Media (MEM) supplemented with 5% (V/V) fetal bovine serum (FBS; GiBco, Invitrogen Corp., USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

5.2.2. MTS assay

JB6 P+ cells were cultured in 96-well plates using MEM containing 5% FBS at densities of 2 x 10^5 cells/ml for the 1 and 3 day treatments and 5 x 10^4 cells/ml for the 5 day treatment. After 24 h, the cells were treated with MEM/1% FBS and various concentrations of API using 0.1% DMSO as a control. For the 3- and 5-day treatments, the medium containing drugs was changed every 2 days. The cytotoxicity of API was tested using the CellTiter 96 aqueous non-radioactive cell proliferation MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). After the 1, 3 and 5 day treatments, the JB6 P+ cells were treated with the MTS solution for 1 h at 37°C. Absorbance of the formazan product was read at 490 nm using a µQuant Biomolecular Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), and cell viability was calculated in comparison with the control DMSO.

5.2.3. RNA extraction and quantitative real-time PCR

JB6 P+ cells were cultured in 10-cm plates using MEM containing 1% FBS at a density of 5 x 10^3 cells/ml. After 24 h, the cells were treated with 1.56 or 6.25 µM API for 5 days using 0.1% DMSO and 100 nM 5-aza as negative and positive controls, respectively. The medium containing drugs was changed every 2 days. For the 5-aza/TSA treatment, 100 nM TSA was added to the 5-aza-containing medium on day 4, and the cells were

cultured for an additional 24 h. The cells were then harvested for RNA isolation. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was used as the template for quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an ABI7900HT system. The following sequences for the Nrf2 and NQO1 primers were used: Nrf2, 5'-GGCAGAGACATTCCCATTTGTAG-3' (sense) and 5'-TCGCCAAAATCTGTGTTTAAGGT-3' (antisense); and NQO1, 5'-CAGAAATGACATCACAGGTGAGC-3' (sense) and 5'-CTAAGACCTGGAAGCCACAGAAA-3' (antisense). *β*-Actin was used as an internal control with sense (5'-CGTTCAATACCCCAGCCATG-3') and antisense (5'-GACCCCGTCACCAGAGTCC-3') primers.

5.2.4. Protein lysate preparation and western blotting

JB6 P+ cells were treated using the procedures described above. After the 5 day of treatment, cells were harvested using RIPA buffer supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain whole protein lysate. In the case of nuclear protein extraction, the NEPER Nuclear and Cytoplasmic Protein Extraction Kit (Pierce, Rockford, IL) was used according to the manufacturer's instructions. The protein concentrations of the cleared lysates were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL), and 20 μg of total protein from each sample was mixed with 5 μl of Laemmli's SDS-sample buffer (Boston Bioproducts, Ashland, MA, USA) and denatured at 95°C for 5 min. The proteins were separated using 4-15% SDS-

polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) followed by blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. The membrane was then sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were visualized by the SuperSignal enhanced chemiluminescence (ECL) detection system and recorded using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). The primary antibodies were purchased from different sources as follows: anti-Nrf2, anti-NQO1, anti-β-actin and anti-Lamin A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-DNMT1, anti-DNMT3a and anti-DNMT3b antibodies were purchased from IMGENEX (San Diego, CA); anti-HDAC1 to anti-HDAC6 antibodies were purchased from Cell Signaling (Boston, MA); anti-HDAC7 antibody was purchased from Millipore (Bedford, MA); and anti-HDAC8 antibody was purchased from Proteintech (Chicago, IL).

5.2.5. Bisulfite genomic sequencing (BGS)

Genomic DNA was isolated from DMSO-, API- or 5-aza/TSA-treated JB6 P+ cells using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). The bisulfite conversion was performed using 500 ng of genomic DNA with the EZ DNA Methylation Gold Kit (Zymo Research Corp., Orange, CA) following the manufacturer's instructions. The converted DNA was amplified by PCR using Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) and primers that amplify the 15 CpG sites located between -1226 and -863 of the murine Nrf2 gene with the translational start site defined as +1. The sequences of the primers were as follows: 5'- AGTTATGAAGTAGTAGTAAAAA-3' (sense) and 5'-AATATAATCTCATAAAACCCCAC-3' (antisense) for the first five CpG sites; and 5'-

TTAAAAATAGTTTGAGAGGTG-3' (sense) and 5'-ACCCCAAAAAAATAAATAAATC-3' (antisense) for the next 10 CpG sites. The PCR products were cloned into the pCR4 TOPO vector using a TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids from at least 10 colonies of each treatment group were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and were sequenced (Genwiz, Piscataway, NJ).

5.2.6. Methylated DNA immunoprecipitation (MeDIP) analysis

MeDIP analysis was performed with a MagMeDIP Kit (Diagenode, Denville, NJ) according to the manufacturer's instructions as described previously [160, 251]. Briefly, the extracted DNA from the treated JB6 P+ cells was sonicated on ice to approximately 100-500 bp. The fragmented DNA was denatured at 95°C for 3 min and subjected to immunoprecipitation overnight at 4°C. After incubation, the DNA contained on the magnetic beads with an anti-methylcytosine antibody (Anaspec, Fremont, CA) and an anti-c-Myc antibody (as a negative control; Santa Cruz, Santa Cruz, CA) was isolated for use in PCR and qPCR assays. The primers, 5′-TTTCTAGTTGGAGGTCACCACA-3' (sense) and 5′-CCCAGGGAGATGGATGAGT-3′ (antisense), were used to cover the DNA sequence of the 15 CpG sites of murine Nrf2 from position −1226 to −863 with the translational start site defined as +1. The enriched MeDIP DNA content was calculated on the basis of the calibration of the serial dilution of input DNA by qPCR, and the relative methylated DNA ratios were calculated on the basis of the control, which was defined as 100% methylated DNA [21].

5.2.7. Statistical analysis

All experiments were performed at least three times with similar results. 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.

5.3. Results

5.3.1. API exhibits cytotoxicity against JB6 P+ cells

The cell viability of JB6 P+ cells was analyzed to determine the cytotoxic effect of API using a MTS assay. The results showed that API treatments decreased cell viability in a dose-dependent manner at 1, 3 and 5 days after treatment (Figure 5.1). Low concentrations of API (< 6.25 µM) were much less toxic than higher concentrations (50 µM). Because the viability of cells treated with 6.25 µM API was greater than 70%, API concentrations of 1.56 and 6.25 µM were selected for subsequent studies of epigenetic modifications of the Nrf2 gene promoter.

5.3.2. API decreases the methylation level of 15 CpG sites in the Nrf2 promoter in JB6 P+ cells

We previously demonstrated that the transcriptional activity of Nrf2 is significantly downregulated when the first 15 CpG sites of the Nrf2 promoter are hypermethylated in JB6 P+ cells [239]. Therefore, we performed bisulfite genomic sequencing (BGS) to determine whether API treatment demethylated these CpG sites during a 5-day treatment with 0.1% DMSO and the combination of 5-aza/TSA (100 nM/100 nM) as controls. We found that JB6 P+ cells treated with DMSO were highly methylated (85%; Figure 5.2), which was in agreement with our previous studies [239]. Treatments with API significantly decreased (p < 0.05) the methylation status of these CpG sites in a dose-dependent manner

(Figure 5.2). Similarly, the combination of the DNMT/HDAC inhibitors (5-aza/TSA) showed significant demethylation ($p < 0.05$) compared to DMSO-treated cells (63%; Figure 5.2). These results strongly suggested that API demethylated the Nrf2 promoter in a dose-dependent manner and acted preferentially on the last 10 CpG sites analyzed (Figure 5.2).

5.3.3. API significantly decreases the binding of anti-methyl cytosine antibody to the 15 CpG sites in the Nrf2 promoter in JB6 P+ cells

To confirm our findings, we performed a methylated DNA immunoprecipitation (MeDIP) assay in which methylated DNA fragments are enriched via immunoprecipitation with an anti-methylcytosine (anti-MeCyt) antibody that binds specifically to methylated cytosine. The enriched methylated DNA was used as the template for qPCR analysis to amplify the Nrf2 promoter region containing the CpG sites of interest (Figure 5.3). The qPCR results showed that API and the 5-aza/TSA combination significantly reduced the total amount of MeCyt enrichment of the 15 CpG sites in the Nrf2 promoter compared to the control ($p < 0.05$). Thus, these results strongly suggested that API can reverse the methylation level of these specific CpG sites in the Nrf2 promoter in JB6 P+ cells.

5.3.4. API increases mRNA and protein expression levels of Nrf2 and Nrf2 downstream genes

We have previously demonstrated that Nrf2 and Nrf2 downstream genes are decreased in JB6 P+ cells [239]. To evaluate whether the demethylation induced by API in the promoter region of Nrf2 contributed to the transcriptional activation of Nrf2 and Nrf2 downstream genes, we first examined the expression of Nrf2 and NQO1 by qPCR (Figure 5.4.A). The results revealed that the gene expression levels of Nrf2 and the ARE-mediated

gene NQO1 were significantly ($p < 0.05$) increased in API-treated JB6 P+ cells in a dose-dependent manner compared with the control. Similarly, the gene expression levels of Nrf2 and NQO1 were also significantly ($p < 0.05$) increased in the 5-aza/TSA treatment. We next assessed the protein levels of Nrf2 and NQO1 by western blot analysis. Figure 5.4.B shows that the protein levels of the Nrf2 and the Nrf2 downstream gene NQO1 were in agreement with the previous findings, especially at the higher dose of API for Nrf2. Interestingly, NQO1 protein expression was high in the 5-aza/TSA treatment. In order to confirm the effect of API in JB6 P+ cells, we assessed the nuclear translocation of Nrf2 induced by API. After 5 days of treatment, API at 1.56 and 6.25 μM significantly increased the nuclear translocation of the Nrf2 protein in JB6 P+ cells compared with the control group (Figure 5.5). These results suggest that API has the potential to increase the Nrf2-mediated mRNA and protein expression of Nrf2 downstream genes, which might be correlated with the increased cellular expression and nuclear translocation of Nrf2 in JB6 P+ cells.

5.3.5. API induces modifications in the expression of DNMT/ HDAC proteins

Because DNA methylation occurs at the 5′ position of the cytosine residue within CpG dinucleotides through the addition of a methyl group by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B [3], we next evaluated the effect of API on DNMT protein expression. Figure 6.A shows that API decreased the expression of all DNMTs, especially DNMT1 and DNMT3b, at the higher dose. Furthermore, the expression of all DNMTs was also decreased by the combination treatment of 5-aza/TSA as expected.

Previous studies have reported that API decreases HDAC class I activity, specifically HDAC1 and HDAC3 proteins, in prostate cancer cells with a concomitant global acetylation of histones H3 and H4 [250]. To assess the effect of API on HDAC proteins in JB6 P+ cells, we evaluated HDAC class I and class II proteins. We observed that API decreased the expression of HDACs (1-8) in a dose-dependent manner (Figure 5.6.B). Nevertheless, HDAC1 and HDAC5 proteins were slightly decreased at the higher dose of API, but the expression of HDAC3, HDAC4, HDAC6 and HDAC8 was affected by both doses. Interestingly, the combination of 5-aza/TSA dramatically decreased the protein expression of all HDAC proteins assessed, except for HDAC7, which was not affected compared to the control. Taken together, API decreased DNMT and HDAC expression in a dose-dependent manner, which was in general comparable with the 5-aza/TSA combination treatment at the higher dose.

5.4. Discussion

The generation of reactive oxygen species (ROS) is an essential metabolic process for maintaining homeostasis in the human body, and it serves as an important sensor in skin [3, 252]. Thus, ROS at low levels initiate cell cycle arrest and promote DNA repair mechanisms, but ROS at high levels initiate apoptosis though activation of death receptor signaling [252]. The imbalance between the generation of ROS and the anti-oxidative stress defense systems in the body can be highly deleterious to the cells because they need effective mechanisms for detoxification, such as the ARE gene machinery orchestrated by Nrf2, which contributes to the preservation of skin homeostasis [3, 253]. Previously, we have reported the critical role of Nrf2, a key transcription factor that regulates the expression of many phase II detoxifying/antioxidant enzymes, in the classical 2 stage

carcinogenesis model with the DMBA chemical carcinogen and the tumor promoter TPA-induced skin cancer in Nrf2 KO mice [254]. Thus, the transcriptional repression of Nrf2 and ARE target genes contributes to cell transformation allowing enhanced effects of carcinogens due to impaired functional protection [255, 256]. We have also demonstrated that Nrf2 expression is regulated by epigenetic mechanisms during tumor promoter TPA-induced mouse skin cell transformation, thereby suggesting the epigenetic reactivation of Nrf2 and the subsequent induction of Nrf2 downstream target genes involved in cellular protection [239].

In contrast, *in vitro* and *in vivo* studies have shown that API is an active compound against skin carcinogenesis [194] and targets many pathways as follows: G1 cell cycle arrest with the induction of p21/WAF1 [257] and G2/M cell cycle arrest inducing the inhibition of the p34 (cdk2) kinase independent activation of p21/WAF1 [258], p53 stabilization and p53 expression through the modulation of p53-HuR protein interaction [259], blockade of protein kinase C activity [260], inhibition of inducible cyclo-oxygenase-2 (COX-2) [261, 262] and inhibition of Src kinase [198]. Thus, more than 150 human cellular targets associated with API have been identified revealing the complex biological network targeted by this flavone [263]. In addition, API induces epigenetic modifications that inhibit 5-cytosine DNA methyltransferase activity in human KYSE-510 esophageal squamous cell carcinoma cells and histone deacetylase (HDAC) inhibition in PC3 and 22Rv1 prostate cancer cells [249, 250, 264]. However, there are no data revealing the molecular mechanisms of API behind the epigenetic regulation of Nrf2 in mouse skin cells. In this study, we presented data demonstrating that API reversed the methylation status of 15 specific CpG sites in the promoter region of the Nrf2 gene in JB6 P+ cells by BGS and

MeDIP analyses (Figure 5.2 and 5.3). Moreover, the results from this study also revealed that the expression of Nrf2 and Nrf2 downstream targets, namely NQO1, in JB6 P+ cells was restored after treatment with API and the DNMT/HDAC inhibitors (5-aza/TSA) (Figure 5.4), and that API was able to induce the nuclear translocation of Nrf2 in a dose-dependent manner (Figure 5.5).

To elucidate the specific demethylation effects of API on the CpG sites in the Nrf2 promoter, we evaluated the protein expression of DNMT1, DNMT3a and DNMT3b. The decreased DNMT expression, especially DNMT1 and DNMT3b (Figure 5.6.A), observed in the present study was in agreement with previous studies indicating that API has high affinity for GC-rich DNA in the GSTP-1 promoter sequence of prostate cancer cells, thus preventing the methylation of this promoter sequence when incubated with the MssI enzyme [265]. Similarly, API has been reported to inhibit the 5-cytosine DNA methyltransferase activity in nuclear extracts of KYSE 510 cells [249]. Nevertheless, the specific effect on the maintenance of DNMT1 and the *de novo* methyltransferases, namely DNMT3a and DNMT3b, have not been fully elucidated.

It has been observed that UVB-induced skin tumors in mice show elevated expression and activity of the DNMT1, DNMT3a and DNMT3b with potential role in the suppression of the immune system and inflammatory response [234]. Similarly, DNMT3a and DNMT3b have been reported highly expressed in human metastatic melanoma and they are associated with poor prognosis [266]. This suggests that API could potentially may play an important role in controlling the expression of DNMTs during UVB-induced skin cancer.

We also evaluated the effect of API on the expression of HDAC proteins in JB6 P+ cells because API has been reported to inhibit HDAC1 and HDAC3 proteins in human prostate cancer cells, resulting in global histone H3 and H4 acetylation as well as localized hyperacetylation of histone H3 on the p21/waf1 promoter, leading to induction of growth arrest and apoptosis [250]. Although studies of histone modifications are scarce in non-melanoma skin cancer, it has been reported that elevated expression of HDACs is associated with closed chromatin and transcriptional repression which may play an important role in DNA repair mechanisms after environmental insults such as Ultraviolet radiation [234, 267]. Our results revealed that API is not only able to target HDAC class I proteins (HDAC1, HDAC2, HDAC3, HDAC8) but also class IIA (HDAC4, HDAC7) and class IIB (HDAC6) proteins, thereby inhibiting almost all HDACs evaluated (1-8), except for HDAC5, in a dose-dependent manner (Figure 5.6.B).

5.5. Conclusion

In summary, the results of this study demonstrated that API demethylates the Nrf2 gene promoter at 15 specific CpG sites in JB6 P+ cells *in vitro* and that its activity is mediated though the inhibition of DNMT and HDAC proteins, which restore the cellular expression and nuclear translocation of Nrf2 and the Nrf2 downstream target, NQO1. These findings provide new insights into earlier observations that have shown differential Nrf2 expression during tumor promoter TPA-induced mouse skin cell transformation [239]. All in all, the present study supports the conclusion that API may play an important role in the prevention and treatment of skin cancer by epigenetic modifications leading to therapeutic development as a primary chemopreventive agent and/or as an adjuvant therapy.

Figure 5.1.   API exhibits cytotoxicity against JB6 P+ cells

Cells were seeded in 96-well plates in 5% MEM for 24 h. The cells were then incubated in fresh medium with API for 1, 3 or 5 days at different concentrations (1.56 to 50 μM), as described in the "Materials and Methods". Cell viability was determined and calculated using the MTS assay. The data are expressed as the mean ± SD (n=3). * indicates significant differences ($p < 0.05$) in cell viability in comparison with the control.
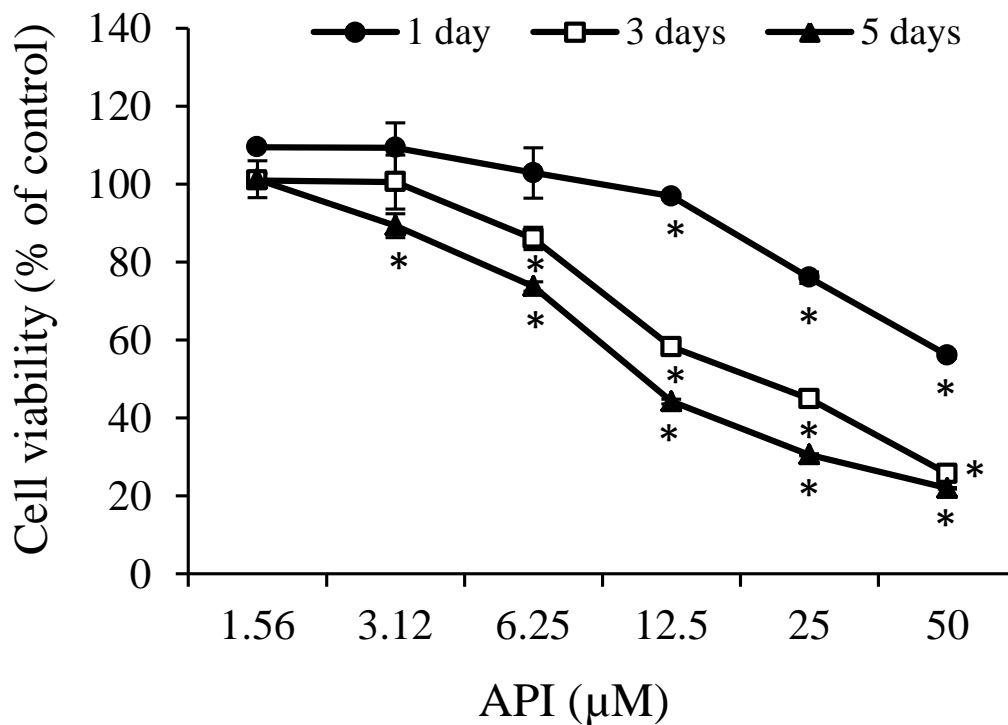
Figure 5.2. API decreases the methylation level of 15 CpG sites in the Nrf2 promoter in JB6 P+ cells

The methylation level was determined by using bisulfite genomic sequencing (BGS), as described in the "Materials and Methods". The 15 CpGs sites were located between -1226 and -863 of the murine Nrf2 gene with the translational start site defined as +1. The cells were seeded and treated for 5 days with 1.56 and 6.25 μM API as well as the combination of 5-aza/TSA. Black dots indicate methylated CpGs, and open circles indicate non-methylated CpGs. The values are the mean ± SD of at least 10 clones from three independent experiments. * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in methylation level in comparison with control DMSO.
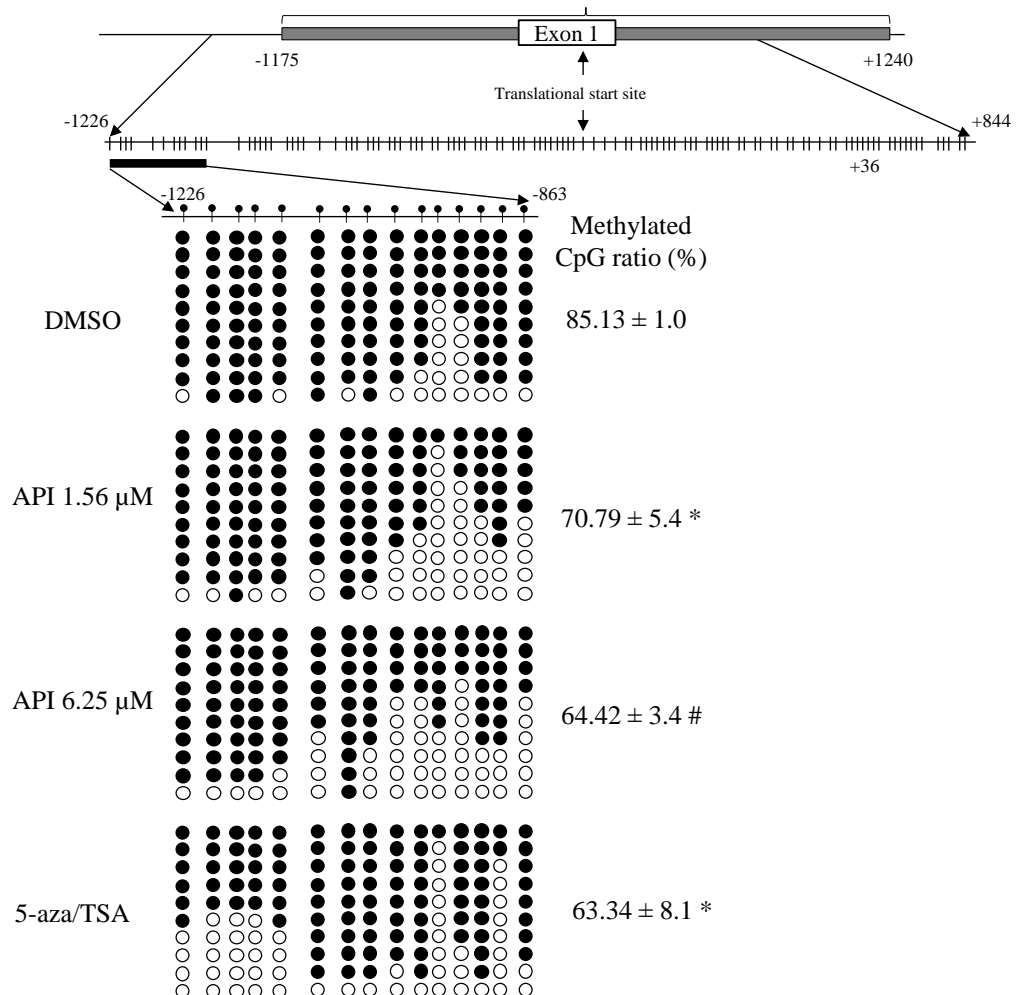
Figure 5.3. API significantly decreases the binding of anti-methyl cytosine antibody to the 15 CpGs sites in the Nrf2 promoter in JB6 P+ cells

Methylated DNA immunoprecipitation (MeDIP) was performed as described in the "Materials and Methods" using the MagMEDIP kit. The immunoprecipitated DNA and inputs were analyzed by qPCR using primers covering the 15 CpG sites in the Nrf2 promoter. The relative amount of MeDIP DNA was calculated using a standard curve of delta CT values obtained by serial dilution of the inputs. The relative values were then compared with the DMSO control, which was defined as 100% of methylated DNA. The values are expressed as the mean $\pm$ SD of three separate samples. * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in the relative methylated DNA ratio in comparison with control DMSO.
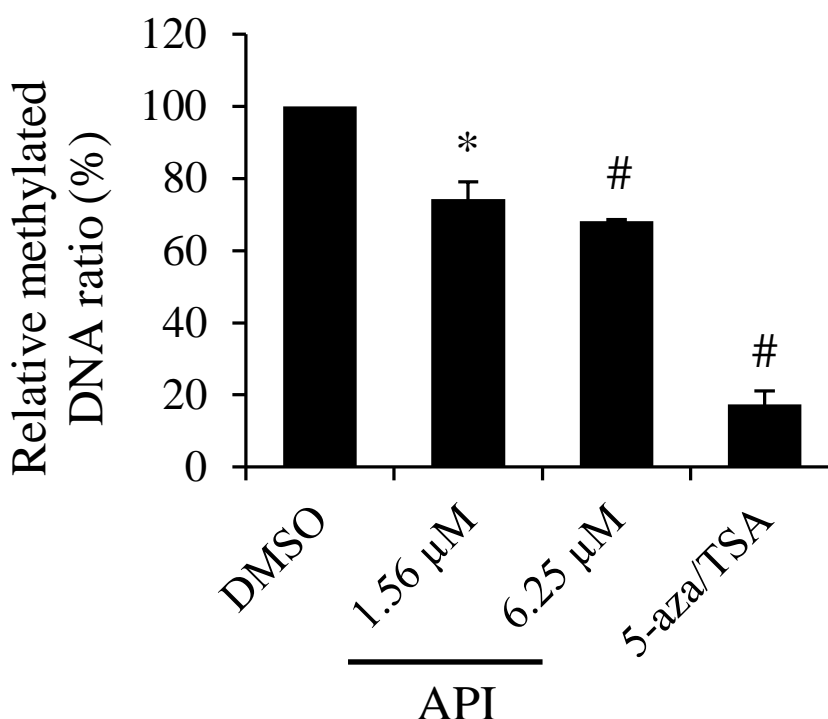
Figure 5.4. API increases the level of mRNA (A) and protein expression (B) of Nrf2

and the Nrf2 downstream gene, NQO1

(A) After 5 days of treatment, mRNA expression was determined using the ABI7900HT

qPCR system. Normalization of gene expression data was performed using β-actin as an

internal control. Relative fold changes of mRNA expression of Nrf2 and NQO1 in JB6 P+

cells treated with API, DMSO and the DNMT/HDAC inhibitor (5-aza/TSA) combination.

(B) Protein expression level of Nrf2 and the downstream gen NQO1 in JB6 P+ cells treated

with DMSO, API and the DNMT/HDAC inhibitor (5-aza/TSA) combination for 5 days.

The protein expression level was normalized with β-actin (a complete description of the

procedure and antibodies used is presented in the "Materials and Methods"). The images

were analyzed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/).* and # indicate

significant differences ($p < 0.05$ and $p < 0.01$, respectively) in relative protein expression

in comparison with control DMSO in the case of mRNA and protein expression.
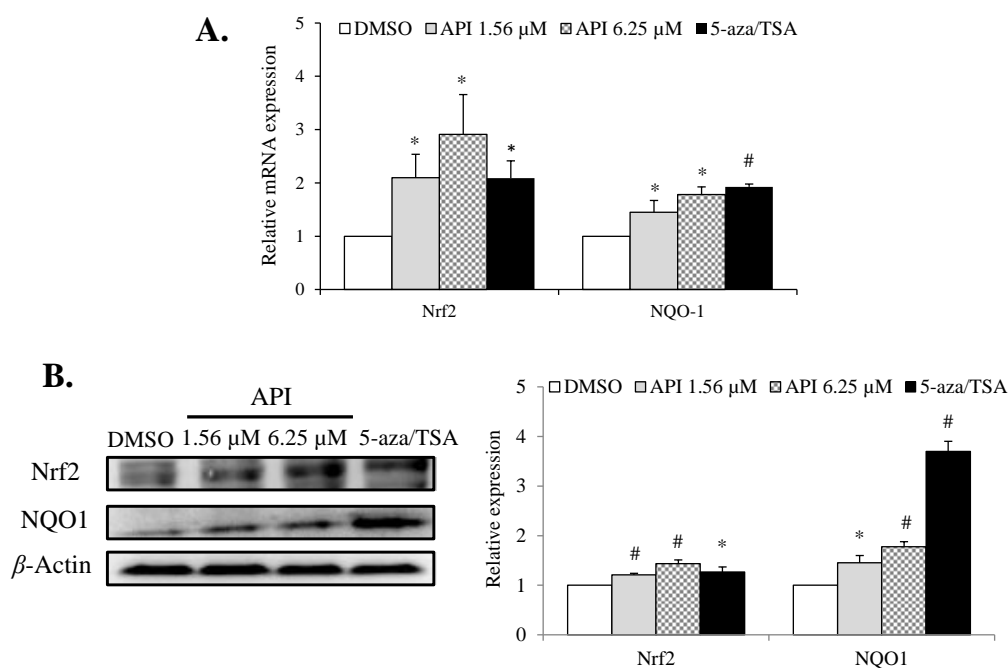
Figure 5.5.   API increases the level of the nuclear translocation of the Nrf2 protein

JB6 P+ cells were treated with DMSO and API for 5 days. * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in relative protein expression in comparison with control DMSO. The images were analyzed by ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The protein expression level was normalized with Lamin A (a complete description of the procedure and antibodies used is presented in the "Materials and Method").
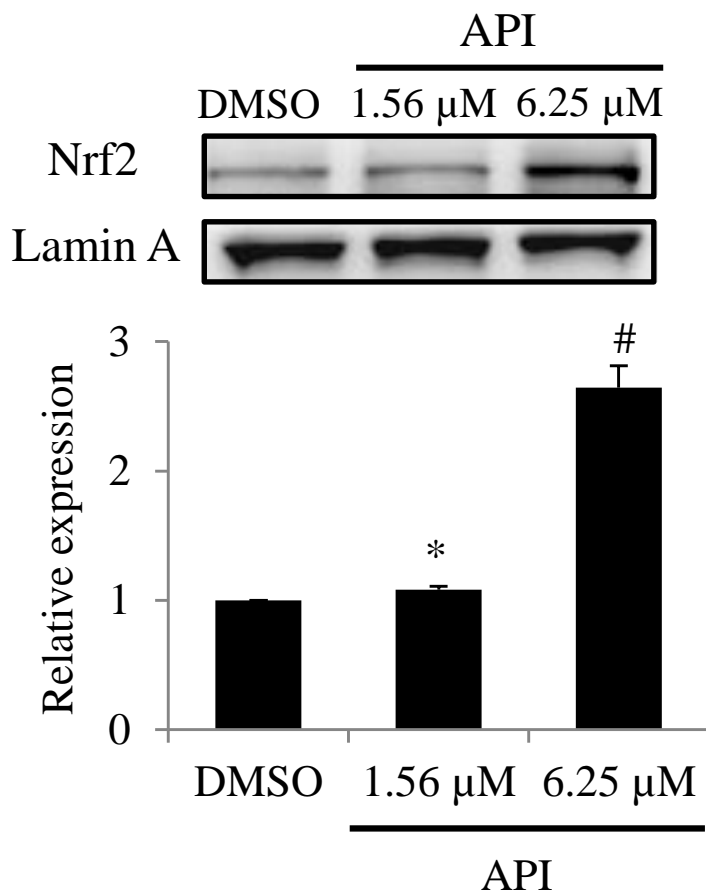
Figure 5.6.   API changes the expression of DNMT **(A)** and HDAC **(B)** proteins

(A) Protein expression level of DNMT1, DNMT3a and DNMT3b. **(B)** Protein levels of the HDAC (1–8) enzymes. JB6 P+ cells were treated with DMSO, API or the DNMT/HDAC inhibitor (5-aza/TSA) combination for 5 days. * and # indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in relative protein expression in comparison with control DMSO. The images were analyzed by ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The protein expression level was normalized with β-actin (a complete description of the procedure and antibodies used is presented in the "Materials and Method").
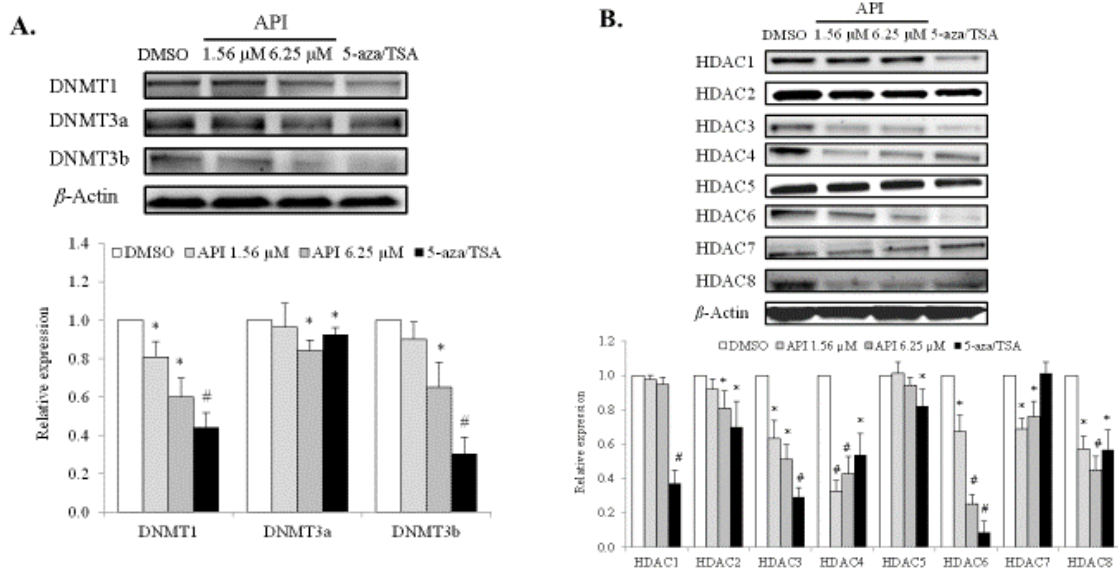
Chapter 6.   Overview of Obesity, Inflammation and

Cancer [13,14,15]

6.1.   Introduction

Obesity has reached epidemic proportions and is recognized as a major cause of cancer worldwide [268, 269]. Obesity can be defined as an excess of body adiposity, which is evaluated according to the body weight. The body mass index (BMI) correlates weight and height (BMI= weight in kilograms divided by the square of the height in meters (kg/m2), and since 1980, BMI has been considered the standard measure for evaluating whether a person is obese. In general, an individual with a BMI between 25 and 30 is classified as overweight or pre-obese, while an individual with a BMI over 30 is classified as obese; different intervals are established according to the mortality risk (Table 6.1) [269, 270].

Although data from the US indicate that the incidence of obesity is increasing slowly or leveling off when compared to the past decade [271, 272], more than 1 billion people worldwide are overweight or obese [273]. It has been estimated that 14% and 20% of all cancer deaths in men and women, respectively, can be attributed to excess body weight [274-276]. Obesity is associated with some types of cancer [277], such as colon,

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[13] This chapter has been published as *book chapter* in book: Inflammation, oxidative stress, and cancer: Dietary approaches for cancer prevention; as "Overview of obesity, inflammation and cancer" by Paredes-Gonzalez X, Khor TO, Shu L, Saw CL, Kong AN. Publisher: CRC Press, Editor: Ah-Ng Tony Kong, 2013.

[14] Key Words: Obesity, inflammation, cancer, chemoprevention, dietary phytochemicals.

[15] Abbreviations: IGF-1; insulin growth factor 1; TAM, tumor-associated macrophage; MCP1, macrophage chemo-attractant protein 1; PI3K, phosphatidylinositol 3-kinase; MAPK, Mitogen-activated protein kinases; IL, interleukin; BMI, body mass index; FA, fatty acid.

breast (postmenopausal), endometrial, kidney (renal cell), esophageal (adenocarcinoma), pancreatic, colorectal, and, potentially, gall bladder carcinoma [278, 279]. In addition, a prospective study published in 2003 that considered more than 900,000 U.S. adults found that non-Hodgkin's lymphoma, prostate cancer and multiple myeloma are also associated with obesity [274].

If obesity can be controlled, the incidence and mortality of obesity-associated cancers may be reduced significantly with weight loss [280]. The American Cancer Society (ACS) Board of Directors has proposed the ambitious and challenging goal of reducing cancer mortality by 50% and cancer incidence by 25% by the year 2015 [281-283]. Moreover, a better understanding of the relationship between cancer and obesity, beyond epidemiology and basic research, may lead to the development of new targeted therapies to prevent obesity-related cancers.

6.2. Obesity and cancer risk

An association between obesity and cancer was clearly observed in an experimental setting as early as the 1940-1950s, when overfed animals exhibited a higher incidence of neoplasia [284]. In 1949, Waxler and Brecher developed a novel obese murine model by injecting mice with a single dose of gold thioglucose, and in 1953, Waxler reported that these obese mice developed spontaneous mammary tumors earlier than non-obese controls [285, 286]. However, Lew and Garfinkel (1979) published the first significant epidemiological evidence that excessive weight or obesity increases the risk of mortality from cancer [287]. Since then, experimental and clinical studies have yielded a vast amount of information.

A meta-analysis of data from prospective studies published between 1966 and 1997 for six cancer types (breast, colon, endometrial, prostate, kidney and gallbladder) reported that 3% and 6% of the attributable risks for men and women, respectively, were caused by excessive body mass [288]. Other studies that included more types of cancers have confirmed these findings and reported a higher population-attributable risk in women [289, 290]. In addition, for all cancers combined, the male and female populations with the highest BMI values (Class III) have death rates that are 52% and 62% higher, respectively, than those of populations with normal weight [274]. Therefore, it is now well-established that being overweight or obese increases the risk of developing and dying from cancer [274, 291-295]. A 5 kg/m² increase in BMI is significantly associated with esophageal adenocarcinoma in both sexes, thyroid, colon, and renal cancers in men, and endometrial, gallbladder, and renal cancers in women [289].

Furthermore, it is not surprising that losing weight can reduce the probability of developing cancer; thus, gastric bypass has been found to decrease cancer risk by 24% and the risk of death from cancer by 46% [296]. Interestingly, this impact is especially significant in women, for whom surgery decreases the overall risk of cancer by 42% [297], with a large impact on breast and endometrial cancers [298]. As mentioned previously, weight gain has a larger effect on female cancer development than male cancer development; moreover, The Million Women study developed in the UK, which included a 5.4-year follow-up for cancer incidence and a 7-year follow-up for cancer mortality, revealed a significant increase in cancer incidence and mortality risks with increasing BMI for 10 of 17 cancers that were examined in women 50 years of age or older [299].

6.2.1. Breast cancer

Although breast cancer incidence rates in the US were stable in 2004-2008 [300], breast cancer still represents the most common cause of death among Hispanic women and the second most common cause of death among white, black, Asian/Pacific Islander, and American Indian/Alaska Native women [301], with different epidemiological traits in pre- and postmenopausal women [300, 302]. Obese women have an increased risk of cancer [303-305] and a poorer survival prognosis than lean women [306-309]. Obesity has been associated with an increased risk of postmenopausal breast cancer [289, 310, 311], but the effect of obesity on premenopausal women is controversial because some studies have demonstrated a negative relationship [289, 312-315].

Women older than 50 years of age, who are usually post-menopausal, constitute the majority of breast cancer diagnoses; until recently, the incidence of breast cancer has followed an increasing trend in this group. Among post-menopausal women, nulliparity and obesity are associated with higher risk of breast cancer incidence and mortality, particularly in non hormone therapy users [316]. Obesity has been associated with the production of increased levels of estrogen in adipose tissue [317], which is positively correlated with tumors that express estrogen hormone receptors (ER) and progesterone (PG) hormone receptors [318, 319].

In premenopausal women, who constitute approximately 35% of breast cancer diagnoses [320], the risk of breast cancer is more likely to be associated with genetic predisposition [321], and the effects of obesity are unclear. Nevertheless, epidemiological data from the Iowa Women's Health Study have suggested that weight gain between 18 years of age and menopause is consistently associated with a higher risk of developing

breast cancer after menopause [322], particularly hormone receptor-sensitive breast cancer [323]. In addition, breast cancer survival may be reduced in premenopausal women [324].

The relationship between obesity and breast cancer risk varies among different races and ethnicities. Some studies have shown that obesity plays an important role in breast cancer in Caucasian women but may not have an influence in Hispanic women [325, 326]. In Hispanic women, breast cancer is increasingly diagnosed at a younger age, with a more advanced stage at diagnosis and worse prognosis than for non-Hispanic whites. In addition, this group is more likely to have estrogen receptor-negative tumors [326]. However, it remains unclear whether obesity plays a role in this trend. In African-American women, the link between obesity and cancer risk is controversial; despite higher rates of obesity, BMI differences do not explain the reduced breast cancer survival in this group [327-329]. A recent case-control study found an important role for obesity in influencing adverse outcomes (development of distant metastases and death from breast cancer) in white but not black women with breast cancer [330]. In Asian women, a stronger association between obesity and breast cancer has been observed for both post- and premenopausal women [289, 294].

6.2.2. Endometrial and colorectal cancers

Excess weight and obesity increase the risk of endometrial cancer by more than 40% [289, 290, 331] and are associated with a poor prognosis [274, 332]. There appears to be a linear weight–risk relationship between increased BMI and endometrial cancer risk [333]. This association appears to be independent of menopausal status but has a stronger effect in postmenopausal woman, particularly those who have never used hormone replacement therapy [334, 335]. Through the aromatization of estrogen precursors [336] as

well as inflammation and insulin resistance [337], excessive estrogen production by adipose tissue in obese women has been suggested to play a complex role in the relationship between obesity and endometrial cancer. Furthermore, obesity in combination with diabetes [338-342] and a lack of exercise [343, 344] have been associated with a poor predictive outcome [345-347].

Obesity is associated with an increased risk of colorectal cancer in both men and women [348-353], with a stronger association in men. Interestingly, compared to cancer in the proximal or distal colon, the distribution of fat may play a greater role in the increased risk of colorectal cancer, particularly in men, who more often develop abdominal obesity [351, 354, 355]. The endocrine and paracrine effects of adipose tissue may lead to systemic alterations such as chronic inflammation and alterations in the levels of adipokines, sex steroids, insulin, insulin-related growth factor (including proinsulin and insulin-like growth factors I and II) and vascular endothelial growth factor (VEGF), which may play an important effect in promoting colon cancer development [356, 357].

6.2.3. Esophageal and pancreatic cancers

Like colorectal cancer, esophageal adenocarcinoma has been strongly associated with obesity (particularly abdominal obesity), through similar mechanisms; the incidence of esophageal adenocarcinoma is four times higher in obese patients [289, 357-362], and men exhibit the strongest association between obesity and esophageal adenocarcinoma [363, 364]. Obesity may exacerbate esophageal inflammation, particularly in the presence of esophageal reflux [365, 366]; however, the effect of obesity on Barrett's esophagus remains unclear [367-369], as does the association between obesity and squamous carcinoma [370, 371]

There is a slightly increased risk of pancreatic cancer in both men and women who are overweight or obese [372-374], particularly for individuals with abdominal obesity [375, 376]. It has been suggested that obesity is correlated with an earlier age of disease in a dose-response manner [377], with lower overall survival in older patients [378].

6.2.4. Kidney and prostate cancers

In 2008, Renehan et al. reported [289] a strong association between obesity and renal cancer in both men and women, which has been confirmed by several studies [290, 379-390]. In renal cell carcinoma, which represents the majority of kidney cancers, this relationship appears to be stronger in obese women [391-394]. The mechanisms by which obesity may increase renal cell cancer risk and progression are poorly understood [395, 396], but hormonal changes, such as increased levels of leptin and decreased levels of adiponectin, hyperinsulinemia and increases in related insulin growth factors, and increased levels of estrogen, lipid peroxidation and defects in the immune response may be responsible [374, 385, 397-401]. Interestingly, in patients with renal cell carcinoma and melanoma, a mutation in SUMO (small ubiquitin-like modifier) on microphthalmia-associated transcription factor was identified, which could impair the adaptation of cells to stress and initiate tumor formation, thus increasing the risk of developing renal cell carcinoma, melanoma or both five-fold [402]. However, the mechanism (if any) by which this genetic predisposition may be correlated with obesity remains to be elucidated.

The relationship between obesity and prostate cancer has been extensively studied but is not completely conclusive [403]. Obesity may cause stromal changes in sex steroid production and signaling pathways, which may affect prostate cancer growth via intracrine/paracrine mechanisms involving certain hormones and growth factors such as

IGF-1 [404, 405]. Three large, prospective cohort studies performed in the US found that obesity is not associated with prostate cancer and may even reduce the risk of less aggressive tumors while increasing the risk of more aggressive tumors [406-408], with a positive mortality association [294, 409, 410]. A recent meta-analysis suggests that obesity may have the dual effect of decreasing the risk for localized prostate cancer and increasing the risk for advanced prostate cancer [411].

This apparent paradox may be explained by the reduced detectability of prostate cancer among obese men with asymptomatic, clinically localized disease [412]. Parekh et al. [413] analyzed three representative cross-sectional surveys in the US (NHANES III, NHANES 2001-2004 and NHIS 2000) and concluded that there prostate-specific antigen (PSA)-driven biopsy rates are lower in men with BMIs above 30. In fact, overweight men may have more frequent PSA screening [414], but due to a possible hemodilution from their large plasma volume [415], their PSA levels may appear normal, leading to lower biopsy rates in this group. Moreover, obesity is associated with a larger prostate size, which may also affect the early detection of prostate cancer [416]. Therefore, to clarify the relationship between obesity and prostate cancer, further studies considering all of these factors are needed.

6.2.5. Others types of cancer

Diverse meta-analyses reporting a positive correlation between excessive body weight and the risk of cancer have been published for gallbladder cancer [417], non-Hodgkin's lymphoma, Hodgkin's lymphoma [418, 419], leukemia [420], liver cancer [421] and, possibly, myeloma [422, 423]. In addition, obesity may be involved in the development of non-alcoholic, fatty liver disease contributing to the development of

hepatocellular carcinoma [424-426]. The mechanisms involved are not well understood but may involve the same mechanisms previously discussed, such as increased levels of hormones, inflammation, and immunity impairment.

6.3. Molecular pathways

The mechanism(s) by which obesity contributes to some or all of the hallmarks of cancer is an open question. Although the link between cancer and obesity has been clearly demonstrated by many epidemiological studies and several molecular pathways have been proposed [427], the interaction between obesity and cancer development and progression remains poorly understood. A cooperative mechanism involving increased insulin and IGF-1 axis signaling, chronic inflammation, changes in adipokine levels, increased availability of lipids and hormones, microenvironment changes and immunity impairment as well as genetic factors may play a role in the conversion of normal epithelial cells to an invasive tumor.

6.3.1. Insulin and IGF-1

Weight gain induces progressive metabolic dysfunction, leading to insulin resistance, in which metabolic tissues (adipose tissue, liver and muscle) are unable to respond to the anabolic actions of insulin. Thus, impaired glucose disposal in muscle and enhanced triglyceride lipolysis in adipose tissue results in hyperinsulinemia, hyperglycemia and hyperlipidemia. As a compensatory mechanism, β-pancreatic cells subsequently increase the production of insulin, and the liver, which exhibits partial insulin resistance, performs lipogenesis but not gluconeogenesis. As a result, the β-cells become exhausted and contribute to sustained hyperglycemia and type-2 diabetes.

High levels of insulin and IGF-1 may promote tumor growth in a cooperative manner through a variety of pathways such as the phosphatidylinositol-3-kinase (PI3K) and serine/threonine protein kinase AKT (or PKB—protein kinase B), mTOR, S6 kinase and mitogen activated protein kinase pathways [428]. Insulin and insulin-related growth factors exert their effects after interacting with the tyrosine kinase receptors on the cell surface, which are homologous to the tyrosine kinase class oncogenes. The insulin receptor (INSR) exists in two variant isoforms, A and B. INSR isoform A interact with insulin and IGF whereas isoform B which is more commonly expressed on tumors .interact just with insulin. The insulin-like growth factor-1 receptor (IGF1R) is a hybrid receptor that is able to interact with insulin as well as IGF-1 [429].

The downstream signaling pathways of insulin and IGF-1 are similar but not identical; insulin and IGF-1 can concurrently activate both the Phosphatidylinositol 3-kinase (PI3K) and MAPK pathways. After binding to its receptor, insulin induces ERK activation through GRB2/SHC–SOS–Ras–Raf and may also activate p38 and JNK with a regulatory role exerted by p85 (PI3K regulatory subunit), inducing cell-cycle arrest and apoptosis [430-432]. Insulin and IGF-1 can also recruit the PI3K to the plasma membrane, triggering its activation by increasing the production of PIP3 from PIP2 (conversely, PTEN converts PIP3 to PIP2) and promoting the phosphorylation of AKT on Thr308 by PDK1 and on Ser473 by the mammalian target of rapamycin complex 2 (mTORC2), also called PDK-2, through acetylation of Rictor [433-435]. These events produce the full activation of AKT, leading to cell growth and proliferation by a variety of downstream pathways that are involved in promoting cell survival and division. These pathways involve not only the activation of vascular endothelial growth factor (VEGF) expression, which may be

regulated by hypoxia-inducible factor (HIF), hypoxia-inducible factor-1α (HIF-1α), signal transducer and activator of transcription 3 (STAT3) and peroxisome proliferator-activated receptor gamma (PPAR-γ), [436, 437] but also include the activation of nuclear factor-kappaB (NF-κB) [438]; the activation of mTORC1 signaling pathway [439, 440] the inhibition of p53 [437, 441]; the inhibition of BAD and BAX [442, 443] as well as the activation of cyclin D1 [444] and the inhibition of chK1 [445]. AKT also phosphorylates forkhead box O (FOXO) transcription factors, resulting in nuclear exclusion and enhanced oxidative phosphorylation [446].

The increase in insulin levels inhibits the production of insulin growth factor binding protein 1 (IGFBP1) and insulin growth factor binding protein 2 (IGFBP2), leading to a decrease in the circulating levels of IGFBP-1 and IGFBP-2 as well as an increase in hepatic sensitivity (IGF-1 is mainly produced in the liver [447]. These changes result in increased circulating levels of IGF-I [448]. Due to their sequence homology, IGF-1 is able to interact with the same receptors as insulin; however, insulin growth factor 2 (IGF-2), which is required for early development, can interact with its own receptor (IGF-2R) and with IGF-1R [449]. Cancer cells express IGF receptors on their surface and have increased expression of the insulin receptor [429], producing enhanced sensitivity to the circulating levels of IGF-1 as well as insulin levels, which may increase the effect of IGFR expression on the downstream signaling pathways that lead to carcinogenesis.

6.3.2. Chronic inflammation

Obese people often have chronic low-level, or "sub-acute," inflammation, which has been associated with an increased cancer risk due to the presence of pro-inflammatory cytokines that are released from phagocytes and other immune cells; these cytokines

infiltrate adipose tissues and contribute to insulin resistance. Circulating blood monocytes migrate from the vasculature to the extravascular compartments, where they mature into tissue macrophages that infiltrate adipose tissue. Inflammatory factors such as interleukin-6 (IL-6), interleukin-17 (IL-17), interleukin 1 beta (IL-1β), plasminogen activator inhibitor 1 (PAI1), and tumor necrosis factor-alpha (TNF-α) are then released. These macrophages may possess a pro-inflammatory phenotype [450]; infiltration by classically activated macrophages may coincide with the onset of insulin resistance, which may be produced through the inhibitor of nuclear factor κB-kinase β (IKK). Additionally, alternatively activated macrophages present in lean adipose tissue may play a crucial role in maintaining the insulin sensitivity of adipocytes via the secretion of interleukin-10 (IL-10) [451].

These cytokines activate multiple signal pathways linked with carcinogenesis, such as PI3K/Akt, MAPK, and signal transducer and activator of transcription 3 (STAT3). In hepatocellular carcinoma, high levels of TNF and cytokine IL-6, which are associated with obesity, can transform healthy cells into malignant cells through chronic low-grade inflammation [452]. TNF and cytokine IL-6 are considered master regulators of tumor-associated inflammation and tumorigenesis [453] an insulin receptor (INSR). They can activate the Janus-family tyrosine kinases (JAK)-STAT3 and NF-κB pathway as well as the MAPK and PI3K/Akt pathways. TNF and cytokine IL-6 are also able to activate proliferation via cyclin D1 and cyclin-dependent kinase 2 (CDK2), which are known cell cycle regulators, thus inhibiting apoptosis and promoting proliferation and metastasis [451, 454]. TNF-α, which is the best-known member of the TNF family, may contribute to insulin resistance by intervening in the intracellular insulin signaling cascade [455, 456].

Inflammatory cytokines can prevent apoptosis by activating inhibitors of apoptosis (IAPs) as well as BCL-2 family factors. The increased expression of growth factors and of cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM1) and E-selectin (ELAM1) can stimulate metastasis. Furthermore, the expression of matrix metalloproteinases (MMPs), which can increase microenvironment remodeling phenomena [427, 457], is increased. Plasminogen activator inhibitor-1 (PAI1) may also play a role through the increased production of adipose tissue [458]. As an inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), PAI1 may act to increase vascularization in a cooperative synergism with VEGF, which is induced by insulin and IGF [459].

6.3.3. Leptin, adiponectin and estrogen

Adipocytes secrete the hormones leptin and adiponectin, which are common homeostatic components that act as feedback peripheral sensors for the hypothalamus in the control of appetite and energy expenditure. In the presence of weight gain, increased levels of the hormone leptin released from adipocytes affect the capacity to conduct negative feedback to the hypothalamus through their action on central leptin receptor (OBR). Leptin can also interact with peripheral receptors that are present at lower levels under normal conditions but are increased in breast, prostate and colon cancer. Leptin can stimulate the IL-6 receptor, activating JAK/STAT signaling through STAT3 (an oncogenic transcription factor) and concomitantly activating the PI3K/Akt/ mTOR axis, which results in the promotion of invasion and migration [460-462]. Leptin can mediate tumorigenesis in endometrial cancer cells through the JAK2/STAT3, MAPK/ERK, and PI3K/AKT pathways, leading to the functional activation of COX-2 [463]. In addition, leptin may act

on the fat tissue, which produces excess amounts of estrogen. Leptin stimulates the expression and activity of aromatase and the transactivation of estrogen receptor-α in breast cancer cells, which has mitogenic and antiapoptotic effects. High levels of estrogen have been associated with an increased risk of post-menopausal breast and endometrial cancer; the main source of this estrogen is adipose tissue, through the aromatization of adrenal androgens (and decreased production of sex hormone binding globulin) [464, 465].

The adipokine adiponectin appears to have an opposing role to leptin in cancer development; adipokine levels are decreased in obese individuals. Adiponectin may have a role in cancer due to its possible antiproliferative effects in prostate [466] and colon cancer cells [467]. Adiponectin acts on a variety of tissues to regulate glucose and lipid metabolism by binding its receptors ADIPOR1 and ADIPOR2. Many of the effects of adiponectin are mediated by increasing the conversion of ceramide to sphingosine-1-phosphate (S1P); alterations in sphingolipid metabolism may modulate tumorigenesis through caspase 8 in a manner that is apparently independent of AMP-activated kinase (AMPK) signaling, which is a key regulator of proliferation in response to nutrient status [468]; however, only a few studies have focused on the effects of adiponectin on cancer development.

6.3.4. Microenvironment changes

As the mass of adipose tissue is increased by the proliferation of adipocyte progenitors in the stromal-vascular fraction, angiogenesis increases to provide oxygen and nutrients. TNF-α contributes to this process by increasing the levels of endothelial-immune cell adhesion molecules and immune trafficking. Leptin secreted by adipocytes plays an important role through the over-production and sensitivity of nitric oxide, which in turn

leads to angiogenesis. Additionally, the chemokines released from macrophages, stromal cells, and adipocytes as well as changes in endothelial permeability or endothelial dysfunction and increased levels of hormones enhance the overall process. The infiltration of activated macrophages may contribute to tissue invasion, angiogenesis and metastasis. Tumor-associated macrophages (TAMs) release macrophage chemo-attractant protein 1 (MCP1), which enhances cell infiltration into the tissue; the presence of these factors has been associated with a poor outcome in some types of cancer [451, 469, 470].

The tumor microenvironment includes a variety of innate and adaptive immune cells that can communicate with each other in autocrine and paracrine manners to control tumor growth. Additionally, these immune cells can communicate with the surrounding stroma, which contains fibroblasts, endothelial cells, pericytes, and mesenchymal cells, which also appear to have an important role in tumorigenesis. The increased hypoxia induces fibrosis mediated by hypoxia-inducible factor α (HIF1-α), leading to hypertrophy with abnormal deposition and accumulation of excess lipids. Hypertrophic adipocytes release free fatty acids (FFAs), which directly impair endothelial function by activating Jun kinases (JNK), which, in combination with the cytokines, enhance the production of reactive oxygen species (ROS) and activate stress signaling pathways, resulting in cell death. Cancer-associated fibroblasts (CAFs) and pre-adipocytes are important that also may enhance tumor growth and metastasis [451, 471]. Accordingly, Barone has proposed a bi-directional crosstalk between cancer cells and cancer-associated fibroblasts (CAFs) in breast cancer through a positive feedback between leptin/EGF that supports tumor progression, proliferation, migration and invasiveness [472].

6.3.5. Changes in lipid availability

Decreased high-density lipoprotein levels, increased triglyceride levels and other lipid abnormalities are common in obesity and may be key factors for tumor development and progression due their functions in the production of new membrane structures and the regulation of growth factor receptors. In tumors and their precursor lesions, glycolysis and the de novo biogenesis of fatty acids (FAs) such as phosphatidylinositol, phosphatidyl serine and phosphatidyl choline are increased independent of their circulating levels. These signaling lipids are known to be important factors that activate and mediate pathways such as PI3K/AKT, Ras or Wnt [427, 473].

Adipose tissue is an important site of endogenous fatty acid (FA) synthesis beyond the liver. By shifting from oxidative to glycolytic metabolism, tumor cells are able to catabolize glucose at a rate that exceeds their bioenergetic requirement, producing energy for tumors and the precursors for FA synthesis through the pentose phosphate pathway. In obesity, the upregulation of fatty acid synthase (FASN) in adipocytes has been observed; the enhanced activity of FASN may provide the necessary support for the development of new membranes to support tumor expansion. In hormonally responsive tumors, the overproduction of steroid hormones may have a role in regulating FASN gene expression and FASN biosynthesis, leading to proliferation [474]. The monoacylglycerol lipase (MAGL) pathway, which can control the production of free fatty acids (FFAs), is also upregulated in some aggressive cancers that are associated with obesity. However, whether those pathways are important in tumor initiation and metastasis remains controversial [474, 475]. Peroxisome proliferator-activated receptor-α (PPARα) may play a role in tumor

development via its ability to increase peroxisome-mediated fatty acid oxidation, which increases the levels of ROS, which can drive mutagenesis and carcinogenesis [451, 476].

6.3.6. Other pathways

Novel genetic variants that are associated with obesity and/or BMI have been reported in recent years, such as single nucleotide polymorphisms (SNPs) that are associated with the fat mass and obesity (FTO) associated gene, melanocortin 4 receptor (MC4R) and other variants [477-479]. Evidence of a correlation between these SNPs and obesity-related cancers is inconsistent for breast [480, 481], colorectal [482, 483] and endometrial cancers [484, 485].

SNPs in obesity genes such as adiponectin (ADIPOQ), leptin (LEP), adiponectin receptor 1 (ADIPOR1), adiponectin receptor 2 (ADIPOR2), paraoxonase 1 (PON1), resistin (RETN) and leptin receptor (LEPR) have also been reported. SNPs in ADIPOQ gene may play a role in endometrial cancer [486], and SNPs in LEP, LEPR and PON1 genes may have roles in breast cancer [487-496]. LEP and LEPR SNPs have been linked with non-Hodgkin's lymphoma [497, 498], while SNPs in LEPR and ADIPOQ have been linked with colon and rectal cancers [499-501]. LEP and LEPR are associated with oral squamous cell carcinoma [502] as well as prostate cancer [503, 504], whereas LEP has been linked with increased susceptibility to non-small cell lung cancer [505]. Finally, correlations between microsatellite instability, obesity and cancers have gained attention [506]

6.4.   Concluding remarks

Although obesity may be influenced by genetic, biological, behavioral and cultural factors, dietary and physical activity habits have been identified as the major risk factors for obesity [507]. Therefore, the consumption of dietary compounds that have anti-obesity properties, along with caloric restriction and physical activity, may be an effective strategy for the prevention of obesity-related cancers. A growing body of evidence indicates that natural dietary compounds may possess anti-obesity/anti-cancer effects with minimal or no side effects [508]. Among the dietary compounds that have been reported to potentially possess anti-obesity effects are curcumin [509], EGCG [510], soy isoflavone [511-513], bitter melon extracts [514-516], resveratrol [517], pterostilbene [518], eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) [519], dibenzoylmethane (DBM) [520], oleanolic acid [521], capsiate [522], and many traditional herbs (reviewed in [523]). Severe side effects have hindered the development of anti-obesity drugs, and currently, only two FDA-approved drugs, phentermine and orlistat, are available for the treatment of obesity [524]. Therefore, the clinical development of natural dietary compounds for use as anti-obesity drugs is an ideal strategy for the treatment of obesity and obesity-associated diseases.

Table 6.1.   The International classification of underweight, overweight and obese adults according to BMI*

Classification	BMI
Underweight	< 18.50
Normal Range	18.50-24.99
Overweight	≥ 25.00
Pre-obese	25.00-29.99
Obese	≥ 30.00
Class I	30.00-34.99
Class II	35.00-39.99
Class III	≥ 40.00

*Source: Adapted from WHO 1995, WHO 2000 and WHO 2004.

Chapter 7. Targeting Obesity-Related Inflammation in Skin Cancer: Molecular and Epigenetics insights for Cancer Chemoprevention by Dietary Phytochemicals [16,17,18]

7.1. Introduction

Non-melanoma skin cancers (NMSCs), including basal and squamous cell carcinoma, are the most common cancers in the U.S., and chronic sun exposure is a recognized risk factor for its development [525]. Obesity, which may be defined as an excess of body adiposity has been considered an emerging risk factor for carcinogenesis [526]. Although data from the U.S. show that the obesity trend has been increasing slowly over the last decade, it has been estimated that 14% and 20% of all cancer deaths in men and women, respectively, may be attributed to an excess of body weight [527]. Among skin cancers, the role of obesity has been well established in epidemiological studies for malignant melanoma [528]. However, obesity's role in NMSC remains poorly studied due to technical limitations and controversies, the latter relating to the negative association that has been observed between obesity and carcinogenesis, which is partly explained by lower sun exposure among people with a higher body mass index (BMI) [529].

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[17] Key Words: Non-melanoma skin cancer, obesity, oxidative stress, epigenetics, dietary phytochemicals.

[18] Abbreviations: NMSC, Non-melanoma skin cancer; HFD, high-fat diet; BMI, body mass index; IGF-1, insulin grow factor 1; TIMP-1, tissue inhibitor of metalloproteinases-1; SERPINE1, plasminogen activator inhibitor type 1; MCP-1, monocyte chemoattractant protein-1; TWIST, Twist-related protein; miRNA, microRNA.

Early studies conducted by Black et al [530] have shown the stimulatory effect of a high-fat diet (HFD) on the development of skin tumors induced by UV exposure in mice with a positive association between HFD, actinic keratosis and skin cancer in humans. Similarly, *in vivo* studies using SKH-1 and C5BL6 mice irradiated with UVB and ICR mice challenged with DMBA/TPA support the hypothesis that obesity is a major risk factor in enhanced skin carcinogenesis and that the surgical removal of fat (the parametrial fat pad) provides a protective effect against UVB-induced skin cancer [531-534]. Studies performed in the early 1940s by Tannenbaum [535] demonstrated that mice fed by calorie restriction instead of *ad libitum* showed a dramatic decrease in the incidence of chemically induced skin tumorigenesis (as well as spontaneous tumors) with a direct relationship between caloric intake and tumor incidence. Interestingly, a protective effect has also been observed related to the reduction of adipose tissue that occurs in obese patients following gastric bypass, wherein the risk of developing cancer is decreased by 24% and the risk of dying of cancer is decreased by 46% after surgery [296].

Because the interaction between molecular and epigenetic events related to obesity and cancer development and progression remain poorly understood, certain cooperative mechanisms involving several factors such as increased insulin and insulin-like growth factor 1 (IGF-1) axis signaling, chronic inflammation, the deregulation of adipokines, microenvironmental changes and immunity impairment with the participation of molecular/epigenetic modifications and genetic factors have been described that favor the conversion of normal epithelial cells to an invasive tumor phenotype following environmental insults such as ultraviolet radiation exposure [428, 536, 537].

7.2. Molecular link between obesity and NMSC

7.2.1. Insulin and the IGF-1 axis

It is recognized that weight gain induces progressive metabolic dysfunction that leads to insulin resistance in tissues such as liver, muscle and adipose tissue, which become unable to respond to the anabolic actions of insulin. Furthermore, impaired glucose disposal in muscle (which is worsened by pancreatic β-cell dysfunction) and enhanced triglyceride lipolysis in adipose tissue produce hyperlipidemia, hyperinsulinemia and hyperglycemia, creating a suitable environment for tumor development and/or progression when the conditions are sustained. It has been proposed that high levels of insulin and IGF-1 may promote tumor growth in a cooperative manner through a variety of pathways such as serine/threonine protein kinase Akt, phosphatidylinositol-3-kinase (PI3K), mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) [428] (Figure 7.1). For example, it has been observed *in vitro* that insulin protects keratinocytes from UVB-induced apoptosis and that the activation of the IGF-1 receptor (IGF-1R) is required for the survival signal involving the PI-3 kinase and the MAP kinase pathways [538]. Moreover, a decrease in the development of NMSC has been reported in patients with type 2 diabetes who are receiving insulin and metformin therapies compared with patients receiving other oral glucose-lowering drugs [539, 540]. Similarly, metformin has been recently reported to inhibit skin tumor promotion induced by TPA in FVB/N mice fed with either 10 Kcal% fat or 60 Kcal% fat in a dose-dependent manner [541]. This activity is linked to the activation of AMPK mediated by metformin and through decreased TPA-mediated activation of the mTORC1 signaling pathway in the epidermis [541].

7.2.2. Inflammation and oxidative stress

Weight gain is accompanied by an increase in the white adipose tissue (WAT), which comprises mainly adipocytes (lipid storage cells) contained in a framework of collagen fibers and other components such as fibroblastic connective tissue cells, leukocytes, macrophages and pre-adipocytes [537]. Saturated free fatty acids (FFAs) from the diet as well as cytokines and/or lipopolysaccharide (LPS) can induce WAT inflammation through the activation of pathways modulated by the Toll-like receptor (TLR) family and the tumor necrosis factor receptor (TNF-R), producing a pro-inflammatory cascade including several cytokines and transcription factors with cooperative activity between the adipocytes and the immune cells recruited to the area [542] (Figure 7.1). The up-regulation of tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) in obesity is well documented, and their expression in fat and their plasma concentrations correlate with the development of glucose intolerance, with macrophages being an important source of their production. Additionally, adipocytokines such as plasminogen activator inhibitor type 1 (PAI-1 or SERPINE1), resistin, leptin and adiponectin (which is often decreased in obesity) are released locally into the systemic circulation, modulating a broad spectrum of biological functions leading to the maintenance of a chronic inflammatory state that is considered one of the hallmarks of obesity [543]. In this environment enriched with T lymphocytes that are producing increased levels of interferon γ (IFNγ) and a high level of infiltration by activated macrophages with the M1 phenotype (classically pro-inflammatory), a dramatic increase in reactive oxygen species (ROS) has been observed. [544].

ROS are a group of highly reactive free radicals that can cause cellular damage, produce mutagenic changes and are able to activate molecular pathways such as PI3K/Akt. ROS may lead to progressive genetic instability that along with the decreased clearance of damaged cells, appears to favor tumor progression and metastasis by augmenting the deleterious effects of carcinogens such as UVB [545]. A scenario that is commonly observed in obesity involves increased levels of phosphorylation of ERK1/2, JNK, and p38 proteins with greater activation of PI3K and NF-κB signaling pathways, cyclooxygenase-2 expression and prostaglandin-E2 production, as well as higher levels of proinflammatory cytokines in skin and serum, including TNF-α, IL-1β and IL-6, whose levels have been augmented already upon UVB radiation exposure [531, 546, 547]. For example, enhanced skin carcinogenesis has been observed in both genetically obese Lep^{ob}/Lep^{ob} (leptin-deficient) C57BL/6 mice and wild-type C57BL/6 mice fed with an HFD after chronic UVB exposure (120 mJ/cm^2) in which greater oxidative stress, oxidative damage and susceptibility to a UVB-induced inflammatory response were observed in comparison with leaner control mice [547]. Interestingly, skin and adipose samples from non-irradiated obese mice demonstrated lower basal levels of detoxification enzymes in comparison to lean controls, which were modulated via the transcription factor nuclear factor E2–related factor 2 (Nrf2), leading to a significant reduction in the levels of glutathione (GSH), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) in both obese and control leaner mice [546, 548]. Thus, the Nrf2 pathway plays an essential role in maintaining skin homeostasis by regulating the expression of several antioxidant and phase II detoxifying enzymes and is considered a critical factor for UVB cytoprotection through the establishment of a paracrine glutathione/cysteine-mediated gradient in the epidermis

[254, 256, 549]. In this context, the impaired anti-oxidative response and the increased UVB-induced inflammation in skin has been reported in Nrf2 knockout mice [549], along with an increased incidence of papillomas and carcinomas with DMBA/TPA treatment in comparison to Nrf2 wild-type mice [254].

Down-regulation and even the loss of Nrf2 expression and the downstream ARE-gene HO-1 have been implicated in the development of skin carcinogenesis induced by DMBA/TPA in mice [254], and it appears that Nrf2 silencing may favor the progression of skin carcinogenesis [239]. Interestingly, independent microarray profile studies performed in adipose tissue from genetically obese and diet-induced obesity mouse models have shown significant down-regulation of glutathione S-transferases (GSTs), contributing to enhanced oxidative stress and metabolic dysfunction [550, 551]. Nrf2 is able to regulate lipid synthesis and adipocyte differentiation, although its specific function and mechanisms are not well understood. It is likely an important player because it can interact with a variety of metabolic pathways, including peroxisome proliferator-activated receptor gamma (PPARγ), retinoid X receptor alpha (RXRα), the transcription of CCAAT/enhancer-binding protein β (C/EBPβ), the aryl-hydrocarbon receptor (AHR), and fibroblast growth factor 21 (FGF21) [552, 553]. Indeed, the transcriptional activity of Nrf2 can be affected by increased levels of insulin leading to phosphorylation of Nrf2, nuclear exclusion and degradation [554]. Thus, it is reasonable to expect that down-regulation of Nrf2 and ARE-genes may play an important role in the enhanced effect of obesity in skin cancer (Figure 7.1).

7.2.3. Role of TIMP-1, MCP-1, SERPINE1 and TWIST1 genes in obesity

Mice fed with an HFD particularly rich in omega-6 have shown an increased rate of skin tumorigenesis upon UVB exposure, exhibiting in the epidermis a high differential expression of chemokines such as tissue inhibitor of metalloproteinases-1 (TIMP-1), lipopolysaccharide-induced CXC chemokine, soluble TNF-receptor Type I and macrophage inflammatory protein-1γ in comparison with controls in keratoacanthomas and invasive carcinomas [555]. Accordingly, these chemokines appear to be implicated in tumor formation and/or progression in the epidermis [533, 534]. Notably, the partial removal of the parametrial fat pad exerts a protective effect against UVB-induced skin tumorigenesis because the dermal fat appears to constitute a type of energy source for tumor development [533, 534]. For example, the average thickness of the dermal layer directly below tumors is much thinner than the fat layer away from tumors, with a significant positive linear correlation between the number of tumors per mouse and the thickness of the dermal fat layer away from the tumors [556]. Increasing evidence shows that TWIST1, which is highly expressed in adipose tissue, is capable of controlling apoptosis, and its overexpression is related to insulin and insulin-like growth factor 1 signaling [557, 558]. Additionally, SKH-1 mice fed with an HFD and irradiated with UVB demonstrated elevated mRNA levels of monocyte chemoattractant protein-1 (MCP-1), SERPINE1, and TIMP-1 in the parametrial fat pad and plasma, which are significantly decreased upon lipectomy of the compensatory fat pad [555]. These events strongly correlate with the increase of caspase 3 (active form) in the epidermis and the decreased rate of tumors [555]. Altogether, it appears that the adipose tissue is secreting a variety of

substances that enhance carcinogenesis and inhibit the apoptosis of DNA-damaged cells, facilitating the pernicious effect of UVB radiation in the epidermis [534].

7.2.3.1.   TIMP-1

HFD increases the levels of several adipokines, particularly TIMP-1, a glycoprotein that is an inhibitor of the matrix metalloproteinases (a group of peptidases involved in the degradation of the extracellular matrix). TIMP-1 inhibits apoptosis and protects human epithelial cells against intrinsic and extrinsic apoptotic pathways and it is proposed as an oncogenic factor [559]. The overexpression of TIMP-1 in HPV16 transgenic mice increases epithelial carcinogenesis through different mechanisms such as by enhancing keratinocyte hyperproliferation, favoring the occurrence of chromosomal aberrations in premalignant cells and by affecting the stabilization of collagen fibrils [560]. TIMP-1 levels are increased in visceral fat in humans and obese mouse models, and the adipokine may be released by both mature adipocytes and by components of the stromal vascular fraction (SVF) [561]. Although the role of TIMP-1 in obesity is unclear, its overexpression has been linked to adipocyte hypertrophy, impaired metabolic profiles with high levels of blood glucose and insulin, accelerated insulin resistance, increased levels of circulating non-esterified free fatty acids, and hepatic triacylglycerol accumulation *in vivo* [561]. Interestingly, in 3T3-L1 adipocytes cultured in the presence of TNFα, IL-6 and IL-1 β, the levels of TIMP-1 are up-regulated, highlighting the importance of circulating cytokines in TIMP-1 regulation [562] (Figure 7.1).

7.2.3.2.   SERPINE1

SERPINE1, also known as PAI-1, is a serine protease inhibitor that is considered to be the major physiological inhibitor of the urokinase plasminogen activator and is

involved in matrix proteolysis, cellular adhesion and migration, inflammation and angiogenesis [563]. The overexpression of SERPINE1 has been described as an essential factor in promoting the early steps of skin carcinoma progression and other malignancies [564]. Overexpression of SERPINE1 is often observed in obese conditions with a positive correlation with BMI and is associated with an increased risk of cardiovascular disease and thromboembolic incidents in obese patients due to its angiogenic properties [565].

SERPINE1 has also been suggested to play an important role in the metabolic syndrome in obesity because it participates in insulin signaling and adipogenesis, as well as being a predictive marker of type 2 diabetes [566]. The overexpression of SERPINE1 in adipocytes and macrophages from the SVF is a complex process that has not been fully elucidated. Its synthesis can be induced by IL-6 but it has been suggested that leptin, insulin, transforming growth factor β (TGF-β) and TNF-α are primarily responsible for the expression of SERPINE1 with a cooperative mechanism between TGF-β and TNF-α [566, 567] (Figure 7.1).

7.2.3.3. MCP-1

Monocyte chemoattractant protein-1 is an important chemokine that can be produced by many cell types and is able to regulate the migration and infiltration of several immune cells, including monocytes/macrophages, memory T lymphocytes, and natural killer cells, with monocytes/macrophages generally representing the major source of MCP-1 production [568]. HaCaT keratinocytes exposed to UVB radiation exhibit increased levels of MCP-1, TNF-α and cytokines such as IL-1β, IL-6, IL-8 and IL-10, whereas in adipose tissue, MCP-1 constitutes one of the classic overexpressed adipocytokines related to obesity-mediated inflammation [569, 570] (Figure 7.1). It has been considered that

dietary fatty acids present in HFD can stimulate MCP-1 expression in immune cells through interaction with the Toll-like receptor pathway in favoring the migration of monocytes from the bloodstream across the vascular endothelium and into the adipose tissue, enhancing the inflammation process [532]. *In vitro* studies performed using 3T3-L1 adipocytes and THP-1 human macrophages have shown that after being challenged with saturated free fatty acids (oleic, elaidic and palmitic), the MCP-1 expression is virtually unaffected in adipocytes, whereas the MCP-1 expression in macrophages is increased upon challenge with palmitic acid [570]. Interestingly, when the exposure to free fatty acids occurs in the presence of glucose-enriched medium, a significant increase in the adhesion and chemotaxis of monocytes and the up-regulation of NF-κB and MCP-1 in adipocytes have been observed [571]. These effects appear to be mediated by diet-induced ROS generation and can be reversed by SOD, catalase or N-acetylcysteine [571]. It has also been demonstrated that MCP-1 derived from adipocytes strongly contributes to macrophage infiltration into adipose tissue, thus increasing the inflammatory loop between adipocytes and the macrophages through the interplay of MCP-1, IL-6, and TNF-α [572, 573]. Notably, TNF-α is able to control the secretion and the expression of SERPINE1, MCP-1 and adiponectin in 3T3-L1 differentiated adipocytes, suggesting a feedback loop between all these factors in which ROS generation induced by components of the diet and high levels of glucose appear to be crucial elements [566, 567].

7.2.3.4.   TWIST1

TWIST1 (also called twist related protein 1) is a highly conserved transcription factor that is involved in cell differentiation and programmed cell death processes and whose overexpression has been reported in several types of cancers [574]. In epithelial

cancers, TWIST1 can halt terminal differentiation, regulate apoptosis through both p53-dependent and p53-independent pathways in response to DNA damage, leading to cell survival and invasiveness and which therefore has long been proposed as a candidate oncogene [574, 575]. TWIST1 may be activated by different signaling transduction pathways, including signal transducer and activator of transcription 3, Ras, Wnt, Akt, SRC-1, MSX2, HIF-1α, integrin-linked kinase, and MAPK [576]. Moreover, cross-talk between TWIST1and the NF-κB signaling pathway appears to be important in the carcinogenesis process [575]. It has been reported that IL-6 may increase TWIST1expression via transcription-independent mechanisms in head and neck squamous cell carcinoma cell lines [577]. Importantly, studies using highly metastatic B16BL6 melanoma cells cultured in serum-containing conditioned medium from *ob/ob* mice (with high expression of resistin, insulin, SERPINE1, IL-6, TNF-α, and MCP-1) demonstrated an increased expression of Snail and Twist-1, promoting epithelial-mesenchymal transition (EMT) [578]. Accordingly, similar results have been obtained in melanoma cells cultured with conditioned medium obtained from 3T3-L1 adipocytes [579].

TWIST1 is abundantly expressed in adipose tissue in mice and humans. Although it does not appear to participate in adipogenesis processes, it appears to be a key player in adipose tissue remodeling and inflammation [557, 580] (Figure 7.1). Studies performed in human white adipocytes isolated from the abdominal tissue of subjects undergoing surgery and liposuction have suggested that TWIST1is a key regulator of fatty acid oxidation and the expression of IL-6, TNF-α, and MCP-1 by direct interaction with specific promoter regions of all these factors; a cross-talking and regulatory loop appears to exist between them [581, 582]. Recent studies also suggest that TWIST1is able to modulate oxidative

stress directly by lowering the level of intracellular ROS by interacting with Cdo1, Mgst3, ApoD, Dhcr24, Aox1 and Osgin1 genes, which appears to be related to the mechanisms by which TWIST1could protect cells against c-Myc-induced apoptosis with a potential involvement in cancer initiation and progression [583].

7.3.   Epigenetic modifications in obesity and NMSC

Epigenetic mechanisms are reversible heritable changes in gene expression that cannot be explained merely by changes in the DNA sequence that include DNA methylation, histone modifications and non-coding RNAs including microRNAs (miRNAs) that are able to regulate chromatin architecture and gene expression [584]. Increasing evidence suggests that inflammation induces aberrant epigenetic modifications in the early stages of carcinogenesis [585]. Likewise, a strong correlation has been demonstrated between chronic exposure of the epidermis to UVR and DNA methylation, increased DNA methyltransferase (DNMT) activity and histone modifications in which hypermethylation in the promoter regions of key genes with progressive global hypomethylation and genetic instability appears to represent an early event that precedes the observation of neoplastic lesions in the epidermis [236, 536] (Figure 7.1).

Specific DNA methylation patterns in skin tumorigenesis are not totally elucidated; however, abnormal promoter methylation in mice and/or humans genes that are involved in cell cycling, cell adhesion and migration, such as p*16*, p*14*, CDH1, CDH3, RASSF1A, LAMC2 and LAMA3, have been reported [234, 236, 237]. In addition, DNA repair and cellular adhesion-related genes such as O(6)-methylguanine-DNA methyltransferase (MGMT), Snail, E-cadherin, and methylated in liver tumor-1 (MLT1) have been observed to be hypermethylated in the mouse skin carcinogenesis model with a loss of both

acetylated histone H4 and dimethylated lysine 4 histone H3 [536]. These particular changes in the CpG methylation of MGMT, MLT1, and Snail promoter appear to occur at the beginning of the carcinogenic process along with the loss of acetylated Lys16 and trimethylated Lys20 residues of histone H4 in the DNA repetitive elements [586]. Similarly, promoter CpG methylation of chemokine receptor CMKAR4, IGF binding protein-3, antioxidant enzyme peroxiredoxin-1 and Nrf2 have been related to skin tumorigenesis [239, 254, 536].

Alternatively, CpG methylation and histone modifications play essential roles in the regulation of glucose homeostasis and adipogenesis [587]. Genome-wide analysis of peripheral blood from obese subjects has shown significant enrichment of several genes for obesity-related diseases such as hypertension, dyslipidemia, type 2 diabetes, and colon and breast cancer [588]. Studies using transgenic mice overexpressing DNMT3a have revealed high gene expression levels of inflammatory cytokines in obese adipose tissue such as TNF-α and MCP-1 compared with wild-type mice fed with HFD [589]. Similarly, the hypomethylation of CpG sites in the MCP-1 promoter region has been described in obese subjects with type 2 diabetes and is correlated with the increased serum MCP-1 levels that are observed as a characteristic feature [590]. Interestingly, the methylation of leptin, TNF-α and SERPINE1 appears to be involved in the chronic inflammatory status of adipose tissue, and therefore, their methylation status has been proposed as a parameter to evaluate the effect of therapeutic strategies in obesity and related diseases [591, 592].

It is recognized that TWIST1is able to produce transcriptional regulation in mesenchymal cell lineages mediated in part by epigenetic mechanisms [580]. For example, TWIST1can inhibit the activity of histone-remodeling enzymes such as cAMP-response

element binding protein (CREB), CREB-binding protein (CBP), p300 and p300/CBP-associated factor, thereby affecting the activity of transcription factors that recruit these histone acetyltransferases, and TWIST1may act through direct interactions with HDACs [576, 580]. Additionally, TWIST1may be regulated by histone methyltransferases, considering that the multiple myeloma SET domain has been described to directly target its locus, leading to an increase in H3K36me2 expression [593]. TWIST1has also been significantly implicated in the regulation of cytokine expression in epithelial and adipose tissues [582]. *In vitro* silencing of TWIST1in human adipocytes obtained from white adipose tissue (WAT) of patients undergoing elective surgery revealed significant decreases in IL-6, TNF-α and MCP-1 levels [581]. Intriguingly, ChIP analysis has suggested that TWIST1can directly regulate the transcriptional expression of MCP-1, IL-6, and TNF-α in adipocytes by binding directly on E-box sites in the promoter regions of these genes, which may have important implications in the enhanced expression of these factors under obesity conditions [79].

Although miRNAs have been studied more extensively in obesity than in NMSC, recent evidence suggests that they are important factors in the relationship between inflammation, the immune response and cancer [594]. These endogenous non-coding RNA transcripts of ∼22 nucleotides in length are able to control diverse biological processes at a post-transcriptional level, and their deregulation may be crucial given that they are able to target TLR signaling pathways [595]. miRNAs have been involved in the up-regulation of pro-inflammatory cytokines and conversely in the binding of cytokines to death receptors, affecting target genes involved in apoptotic and survival pathways [595]. In this regard, differentially expressed miRNAs are found upon UV radiation exposure in skin

squamous cell carcinoma and melanoma with potential effects on cell growth and proliferation [596]. Similarly, miRNAs have also been involved in several processes in adipose tissue, such as adipogenesis, apoptosis, angiogenesis, insulin signaling, glucose homeostasis, oxidative stress and the immune response, with an increasing number of studies showing alterations in the expression levels of several miRNAs in obesity [597]. Some examples are the up-regulation of miR-21, miR-27a, miR-27b, miR-34a, miR-100, miR-125b, miR 146b, miR-221, and miR-222 and the down-regulation of miR-103, miR-107, miR-143, miR-185 and miR-200 among the many others that have been described in obesity [598, 599].

miR-146b is an intergenic miRNA that is able to regulate the inflammatory process in skin. The up-regulated condition of this miRNA in patients with malignant melanoma has been recognized to play an important role in cancer development and progression because miR-146b is able to modulate the TLR4 signaling pathway and is conversely regulated by cytokines [600, 601]. miR-146b is highly expressed in mature human adipocytes and both IL-6 and TNF-α are able to up-regulate its transcriptional activity by interacting with its promoter region [601, 602]. In fact, TNF-α is able to regulate the expression of various miRNAs deregulated in obesity, such as miR-21, miR-103, miR-143, miR-221, and miR-222, which have also been implicated in tumorigenesis [599, 603-605]. Similarly, miR-155, miR-183, and miR-872 contribute to the production of proinflammatory adipocytokines, oxidative damage, and apoptosis in 3T3-L1 adipocytes by repressing the anti-inflammatory, antioxidative, and anti-apoptotic effects of HO-1 [606]. Down-regulation of miR-200 is also likely to be critical because the miR-200 family is well known to inhibit EMT by targeting ZEB1 and ZEB2, which are transcriptional

repressors of E-cadherin [598, 607]. Importantly, TWIST1is known to repress several miRNAs, including the miR-200 family and miR-205, as well as to activate oncogenic miRNAs such as miR-10b and miR-223 [608-610]. Thus, TWIST1itself appears to be controlled by several miRNAs that are able to interact with the 3'UTR coding region [611]. Based on these facts, it is reasonable to expect that miRNAs play a key role in the enhanced oxidative stress/inflammation and pro-survival signals, leading to the enhanced tumorigenesis observed in UVB-irradiated mice under HFD conditions.

7.4. Perspectives in the targeting of NMSC and obesity with natural dietary compounds

Diet-derived chemopreventive agents with low side effects may prevent NMSC via molecular/epigenetic modifications, and some of these modifications have been recently described to inhibit HFD-induced obesity, constituting an interesting approach in NMSC chemoprevention related to obesity [526, 587]. Among the most extensively studied agents in NMSC with potential activities in obesity are sulforaphane (SFN) from cruciferous vegetables and (−)-epigallocatechin-3-gallate (EGCG) from green tea [526].

SFN is an isothiocyanate from cruciferous vegetables with proven effects against carcinogenesis in various types of cancers using *in vitro* and *in vivo* approaches [612]. SFN is able to modulate the initiation, promotion and progression stages of carcinogenesis by a variety of mechanisms, such as the activation of c-Jun NH_2-terminal kinase; activation of extracellular signal-regulated kinase-1/2; interaction with redox-sensitive proteins; induction of cell cycle arrest; transcriptional regulation of AP-1, MAPK and death receptors; and the active suppression of prosurvival signals and Nrf2, a master regulator of many critical anti-oxidative stress genes [612, 613]. Nrf2 plays an important role in the

SFN-mediated suppression of induced skin inflammation and tumorigenesis by either DMBA/TPA or UVB [254, 549]. Furthermore, it has been reported that SFN can also target adipose tissue, preventing HFD-induced obesity in *in vivo* studies by down-regulation of PPARγ and C/EBPα and by suppressing lipogenesis through activation of the AMPK pathway [614]. In *in vitro* studies, SFN has been shown to induce cell cycle arrest in adipocytes at the G(0)/G(1) phases by inhibiting the early stages of adipogenesis and by promoting lipolysis via hormone-sensitive lipase activation mediated by the AMPK signaling pathway [615]. Additionally, SFN inhibits the expression of MCP-1, TIMP-1, TNF-α, COX-2, iNOS, IL-6, and TWIST1and induces apoptosis, suggesting that SFN may be useful to modulate the expression of these deregulated factors [616-619].

Recent evidence has demonstrated the potential effect of SFN as a potent epigenetic modifier [620]. Accordingly, the epigenetic mechanisms involved include the inhibition of DNMT and HDAC activity, alteration of histone acetylation, modulation of polycomb group proteins, and regulation of miRNA [621]. For example, in our previous studies, we reported that SFN is effective in blocking TPA-induced anchorage-independent growth in JB6 P+ cells in part by acting on DNMT and HDAC proteins, leading to restored Nrf2 expression [239]. It has also been observed that SFN reduces polycomb-group (PcG) protein levels via a proteasome-dependent mechanism, inhibiting PcG-dependent pro-survival epigenetic events by suppressing Bmi-1 and Ezh2 levels, leading to reduced H3K27me3 formation in skin cancer cells [622].

Epigallocatechin gallate (EGCG) constitutes the major green tea catechin, and its antitumor and chemopreventive properties have been reported in various cancer cell lines, animal models and clinical studies targeting several pathways related to cell cycle arrest,

proliferation, apoptosis, oxidative stress, inflammation, angiogenesis and metastasis, among many others [623]. Oral and topical administration of green tea polyphenols including EGCG significantly reduces UVB-induced tumor incidence, tumor multiplicity and tumor growth in *in vivo* models, in part mediated by diminishing the pernicious effects of UVB by enhancing the activity of nuclear excision repair genes, inhibiting the activation of activator protein-1 and p38/MAPK pathways, protecting against oxidative cellular damage and producing immune-modulatory and anti-inflammatory effects, which are mediated by stimulating the release of interleukin 12 [624, 625]. Similar to SFN, EGCG is recognized as an epigenetic modifier that is able to restore aberrant DNA methylation of tumor suppressor genes p16INK4a and Cip1/p21 in skin cancer cells by decreasing the activity of DNMT1, DNMT3a and DNMT3b proteins in the promoter regions and by affecting the levels of global DNA methylation through the increase of acetylated H3-Lys 14 and acetylated H4 at Lys 5, Lys 12 and Lys 16 [626]. EGCG can also alter specific miRNA expression in human dermal fibroblasts, which are implicated in UVB-mediated cellular damage and are involved in cell proliferation [627]. We have previously observed that SKH-1 mice treated with green tea and challenged with UVB demonstrated not only a decreased number of tumors/mouse but also a decreased size of the parametrial fat pads and a decreased thickness of the dermal fat layer away from tumors and under tumors [556]. Cumulative evidence suggests that EGCG can prevent obesity through a variety of mechanisms, including the modulation of pathways such as IGF-1, insulin, PPARγ and TLR4, exhibiting a strong anti-adipogenic effect via the activation of AMP-activated protein kinase [628]. Accordingly, mice chronically supplemented in the diet with EGCG have shown a reduction in overall body fat and a reduction in epididymal fat pads

accompanied with a significant reduction in the levels of leptin and stearoyl-CoA desaturase-1, which is implicated in the synthesis of monounsaturated fatty acids and skin integrity [629]. A summary figure of the relationship between obesity and NMSC and potential targets for chemoprevention with dietar phytochemicals is presented in Figure 7.2.

7.5. Concluding remarks

Substantial biological evidence appears to exist to support the role of obesity as an important risk factor in the pathogenesis of non-melanoma skin cancer. Evidence also indicates that enhanced inflammation, oxidative stress and apoptosis are significantly involved in the molecular/epigenetic events related to enhanced tumorigenesis. Many issues remain that will require further investigative efforts on this topic such as the elucidation of the actual impact of obesity in human NMSC through carefully designed epidemiological studies, achieving a better understanding of the molecular/epigenetics events leading to enhanced rates of NMSC in obesity conditions and identifying achievable therapeutic strategies that can be used to target deregulated pathways with high efficacy and low toxicity. In this regard, SFN and EGCG constitute promising molecules for further clinical development in the area of obesity-related diseases such as cancer. Therefore, more in-depth *in vitro* and *in vivo* experiments are needed in this area in the future.

Figure 7.1.   Molecular/epigenetics events involved in the relationship between obesity and NMSC
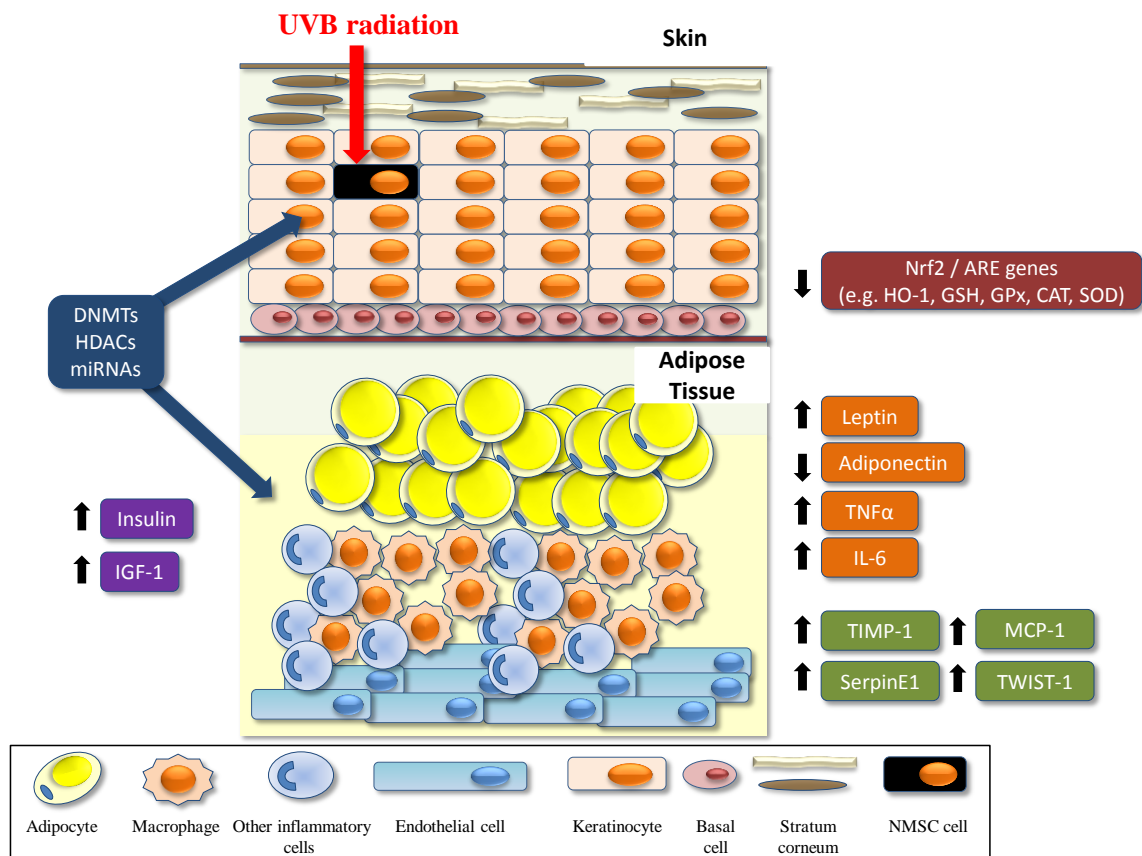
Figure 7.2. Graphical abstract of the relationship between obesity and NMSC and potential targets for chemoprevention with dietary phytochemicals
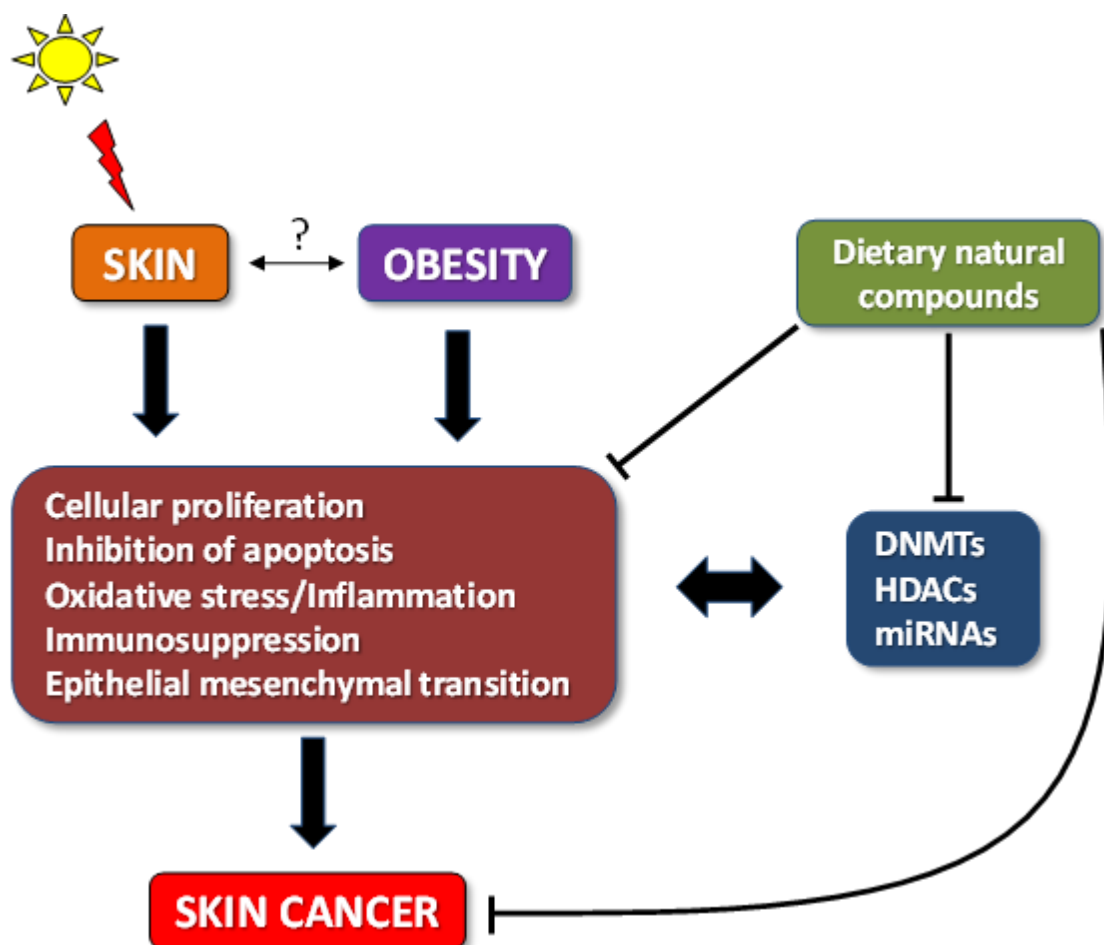
Chapter 8.   Summary

In summary, we reviewed the effects of plant extracts and their main bioactive compounds in cancer chemoprevention through Nrf2-ARE pathway, NF-κB modulation, cell cycle modulation, apoptotic effects and epigenetic regulation with focus in glucosinolates from cruciferous vegetables, apigenin and luteolin from chamomille and Ligu and RAS extract.

In chapter two, we discussed the role of glucosinolates in the prevention of the initiation of carcinogenesis via the induction of cellular defense detoxifying/antioxidant enzymes and their epigenetic mechanisms, including modification of the CpG methylation of cancer-related genes, histone modification regulation and changes in the expression of miRNAs. In this chapter, we further explored the cancer chemopreventive role of naturally occurring glucosinolate derivatives as inhibitors of carcinogenesis, with particular emphasis on molecular targets such as Nrf2, NF-κB, apoptotic pathways and epigenetic alterations in *in vitro* and *in vivo* human cancer animal models.

In chapter three, we focused in the effects of RAS extract that has been long time used as a healthful tonic in the traditional Chinese medicine. Increasing body of publications that are highlighting its potential against carcinogenesis. In this study, we investigated the effect of Lig (25 and 50 µM) and RAS (4.25 and 8.5 µg/mL) on TRAMP-C1 cells and the effect of RAS (0.2% or 0.5% RAS-supplemented diet) on TRAMP mice from 8 to 24 weeks of age. RAS and Lig demonstrated dose- and time-dependent cytotoxicity against TRAMP-C1 cells, inducing G1 arrest and apoptosis after 48 h of exposure, with significant cleavage of caspase-3 and PARP in the case of Lig. TRAMP mice fed with RAS exhibited decreased tumorigenesis and a significant increase (60%) in

the percentage of normal glands in mice treated with the highest dose of RAS. Similarly, a significant decrease was observed in the number of high-grade prostatic intraepithelial neoplasia lesions and proliferating cell nuclear antigen-positive cells, from 70% to 20%, in mice treated with the highest dose of RAS, compared with the control. In addition, TRAMP mice fed with RAS demonstrated a significant increase in the percentage of Nrf2-positive cells and a decrease in the percentage of 5-methylcytosine-positive cells compared with the control group, as evaluated by immunostaining. Our results indicate that RAS and Lig induce G1 phase arrest and apoptosis in TRAMP-C1 cells, which positively correlates with their ability to decrease tumorigenesis in TRAMP mice. This observed activity could be related to RAS's effects on global methylation levels and the restoration of Nrf2 expression *in vivo*.

In chapter four, we explore the effect of the main bioactive flavonoids of chamomile apigenin and luteolin. Apigenin and luteolin have been used as therapeutic agents in folk medicine for thousands of years. These compounds exert a variety of biological activities, including anti-cancer, anti-oxidant, and anti-inflammatory activities. In this study, we investigated whether apigenin and luteolin could activate Nrf2-antioxidant response element (ARE)-mediated gene expression and induce anti-inflammatory activities in human HepG2 cells. The compounds did not exhibit any substantial toxicity at low doses (1.56-6.25 μM). We assessed the induction of ARE activity in HepG2-C8 cells, which are stably transfected with a plasmid containing an ARE luciferase reporter gene, after treatment with low doses of apigenin and luteolin for 6 and 12 h. We found that the induction of ARE activity by these compounds at the higher doses was comparable to the effects of the positive control, SFN at a dose of 6.25 μM. Exposure to the PI3K inhibitor

LY294002 abolished ARE activation by both apigenin and luteolin, whereas the ERK-1/2 inhibitor PD98059 only decreased ARE activity induced by apigenin. Both compounds significantly increased the endogenous mRNA and protein levels of Nrf2 and Nrf2 target genes with important effects on heme oxygenase-1 (HO-1) expression. Apigenin and luteolin significantly and dose-dependently decreased the production of nitric oxide (NO), nitric oxide synthase (iNOS), and cytosolic phospholipase A2 (cPLA2), which were induced by the treatment of HepG2 cells with 1 µg/ml of lipopolysaccharide (LPS) for 24 h. Our results indicate that apigenin and luteolin significantly activate the PI3K/Nrf2/ARE system at low doses, and this activation may be responsible for their anti-inflammatory effects, as demonstrated by the suppression of LPS-induced NO, iNOS, and cPLA2.

Nrf2 is a crucial transcription factor that controls a critical anti-oxidative stress defense system and is implicated in skin homeostasis. Apigenin is a potent cancer chemopreventive agent that protects against skin carcinogenesis and elicits multiple molecular signaling pathways. However, the potential epigenetic effect of apigenin in skin cancer chemoprotection is not known. In chapter five, we performed bisulfite genomic DNA sequencing and methylated DNA immunoprecipitation in order to investigate the demethylation effect of apigenin at 15 CpG sites in the Nrf2 promoter in mouse skin epidermal JB6 P + cells. In addition, qPCR and Western blot analyses were performed to evaluate the mRNA and protein expression of Nrf2 and the Nrf2 ARE downstream gene, NQO1. Finally, the protein expression levels of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) were evaluated using apigenin and the DNMT/HDAC inhibitor 5-aza/trichostatin A. Our results showed that apigenin effectively reversed the hypermethylated status of the 15 CpG sites in the Nrf2 promoter in a dose-dependent

manner. Apigenin enhanced the nuclear translocation of Nrf2 and increased the mRNA and protein expression of Nrf2 and the Nrf2 downstream target gene, NQO1. Furthermore, apigenin reduced the expression of the DNMT1, DNMT3a, and DNMT3b epigenetic proteins as well as the expression of some HDACs (1-8). Taken together, our results showed that apigenin can restore the silenced status of Nrf2 in skin epidermal JB6 P + cells by CpG demethylation coupled with attenuated DNMT and HDAC activity. These results may provide new therapeutic insights into the prevention of skin cancer by dietary phytochemicals.

Obesity that is a chronic inflammatory disease has gained increasing attention due the body of evidence indicating that is importantly correlated with tumor progression and survival in different types of cancers. Thus, natural compounds derived from plants that are able to target important deregulated pathways in obesity-cancer relationship with low side effects and high efficacy are an excellent approach to be further explored. In chapter six, we provided an overview in obesity, inflammation and cancer focusing in the state of art in this subject from epidemiological studies in different cancers and molecular events involved in cancer-obesity relationship, whereas in chapter seven, we focused in the relationship between obesity and non-melanoma skin cancer (NMSC) that is one of the most common cancers in the US. *In vivo* studies have consistently demonstrated that obese mice challenged with UVB radiation show increased skin tumorigenesis in comparison with leaner control mice. Cumulative evidence suggests that enhanced inflammation, oxidative stress and impaired apoptosis may play important roles in the development of skin cancer. Interventions such as voluntary exercise and the surgical removal of parametrial fat have been demonstrated to be effective in reducing adipose tissue that may

influence the development of skin cancer; however, these interventions are not achievable in all obese patients. Therefore, the use of dietary natural phytochemicals that may modify and reverse the deregulated molecular and epigenetic events related to obesity and cancer development might represent a potential therapeutic modality due to their potential efficacy and low toxicity. In this review, we aim to provide the molecular and epigenetic basis of the NMSC-obesity relationship and to highlight the potential anti-cancer chemopreventive benefits of phytochemicals.

In sum, we investigated the role of RAS extract and Lig in cancer progression in the TRAMP mice model; Nrf2 activation and suppression of LPS-induced inflammation in HepG2 cells by apigenin and luteolin and we demonstrated the effects of apigenin targeting key CpG sites in the murine Nrf2 promoter of JB6 P+ cells reactivating epigenetically silenced Nrf2 expression. In addition, we explored new frontiers of cancer chemoprevention in the field of obesity-cancer relationship with focus in NMSC. We demonstrated that plant phytochemicals could act as cancer blocking agents, suppressing agents of relevant cancer molecular pathways and act as epigenetic modulators through the Nrf2/ARE axis and its anti-inflammatory crosstalk. These studies were conducted to contribute to our understanding of the chemopreventive effects and molecular/epigenetic targets of plant phytochemicals in order to design better chemopreventive compounds that can be used as a primary therapeutic agents or adjuvant therapies in cancer treatment.

Appendices

Appendix A.   Prediction of phosphorylation sites of Nrf2

NetPhos 2.0 and NetPhosK 2.0 softwares (http://www.cbs.dtu.dk/services/) were used to identify potential serine (S), threonine (T) and tyrosine (Y) phosphorylation sites in human **(A)** and mouse **(B)** Nrf2 proteins. In human, 33 S, 6 T, and 2 Y phosphorylation sites were identified whereas, in mouse 41 S, 10 T and 2 Y were observed. Distribution of the sites in the sequence is presented. In addition, kinase specific prediction of phosphorylation sites in human **(C)** and mouse **(D)** Nrf2 proteins were determined. Kinase specific prediction of phosphorylation sites of human **(C)** and mouse **(D)** by using Nrf2 NetPhosK software revealed several phosphorylation sites targeted by kinases. Scores with potential phosphorylation sites are presented (the higher the score the greatest is the possibility of being phosphorylated by kinases). The highest score was observed in both human and mouse Nrf2 by PKC at serine 371 and serine 378, respectively. For both analysis, the input FASTA sequences of human and mouse Nrf2 proteins were obtained from NCBI or GenBank (AAB32188.1 and NP_035032.1, respectively).

(A)
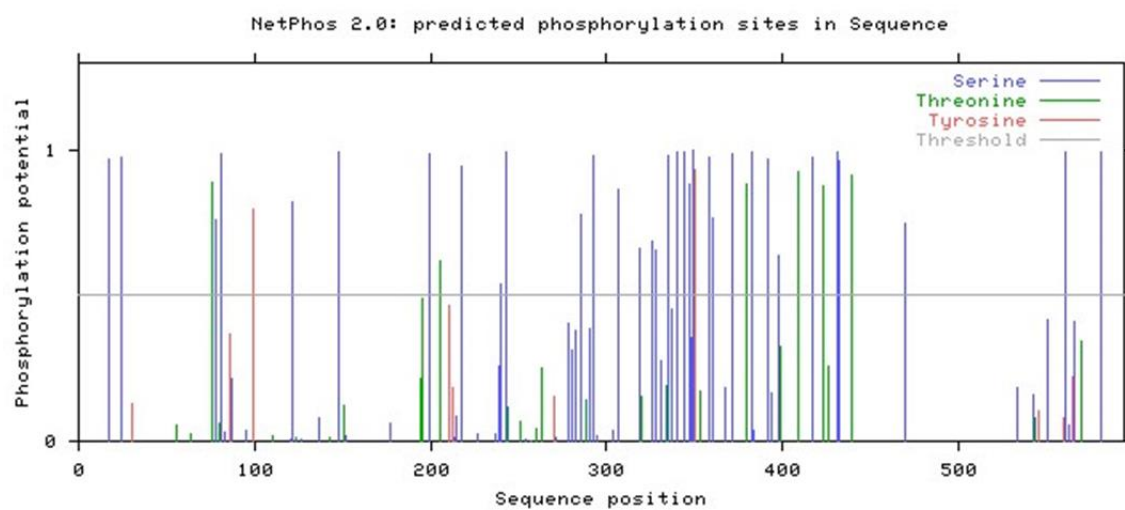
(B)
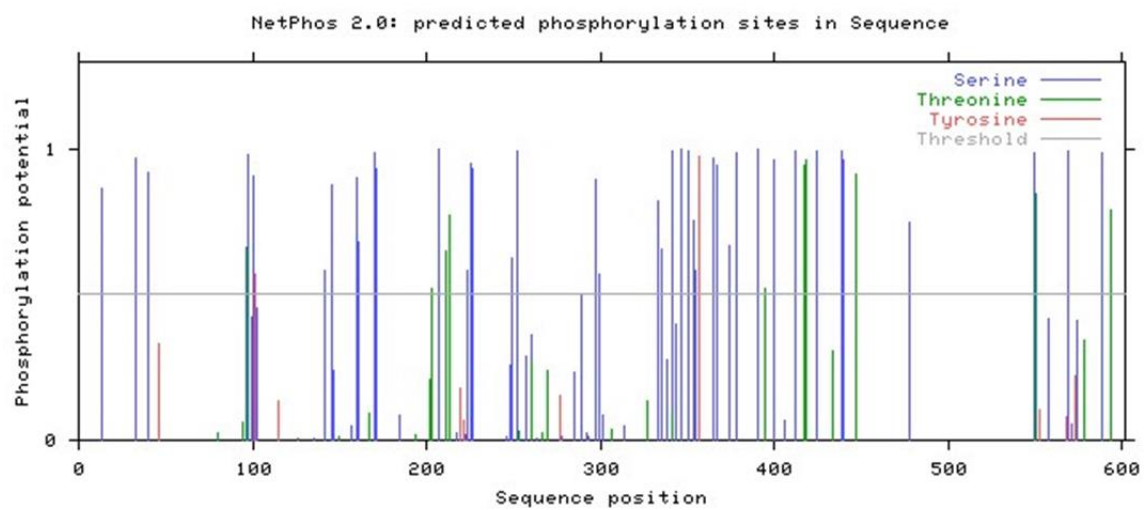
(C)

Site	Kinase	Score	Site	Kinase	Score	Site	Kinase	Score
S-17	cdc2	0.55	S-254	PKA	0.59	S-392	p38MAPK	0.61
S-24	DNAPK	0.61	T-263	CKII	0.59	S-392	GSK3	0.51
S-24	ATM	0.56	S-271	CKII	0.56	S-394	DNAPK	0.60
S-24	PKC	0.79	S-271	cdc2	0.55	S-394	ATM	0.59
T-64	CKII	0.54	S-280	cdc2	0.52	S-394	GSK3	0.50
T-76	DNAPK	0.60	S-282	DNAPK	0.52	S-417	GSK3	0.52
T-76	cdc2	0.50	S-282	cdc2	0.53	S-417	cdk5	0.54
S-78	cdc2	0.50	S-285	cdk5	0.59	T-423	cdk5	0.66
S-87	DNAPK	0.56	T-288	PKC	0.51	T-426	PKC	0.89
S-137	GSK3	0.51	S-292	DNAPK	0.51	S-431	CKII	0.50
S-137	cdk5	0.52	S-319	CKII	0.62	S-432	CKII	0.53
S-148	p38MAPK	0.58	S-326	cdc2	0.53	T-439	CKII	0.55
S-148	cdk5	0.51	S-331	CKI	0.52	S-470	CKII	0.57
S-177	CKII	0.55	S-335	GSK3	0.51	S-542	PKA	0.60
S-199	GSK3	0.50	S-335	cdk5	0.58	T-543	PKC	0.58
S-199	cdk5	0.51	S-337	cdc2	0.50	T-543	PKA	0.61
T-205	PKG	0.50	S-340	p38MAPK	0.61	T-543	cdc2	0.54
S-215	PKC	0.53	S-340	cdk5	0.67	S-550	CKII	0.55
S-218	DNAPK	0.53	S-347	CKI	0.59	S-550	cdc2	0.51
S-227	p38MAPK	0.57	S-348	CKII	0.51	Y-560	INSR	0.54
S-239	CKII	0.56	S-358	CKII	0.72	S-561	CKII	0.50
S-239	CKI	0.53	S-358	cdc2	0.52	S-561	GSK3	0.50
T-244	CKII	0.53	S-360	CKII	0.68	S-581	PKC	0.57
T-244	PKC	0.64	S-360	cdc2	0.58	T-379	p38MAPK	0.52
S-254	DNAPK	0.52	**S-371**	**PKC**	**0.93**	T-379	GSK3	0.53
						T-379	cdk5	0.59

(D)

Site	Kinase	Score	Site	Kinase	Score	Site	Kinase	Score
S-13	DNAPK	0.55	S-249	CKI	0.51	S-367	CKII	0.70
S-13	ATM	0.58	S-252	CKII	0.54	S-367	cdc2	0.52
S-33	cdc2	0.55	S-252	PKC	0.53	**S-378**	**PKC**	**0.93**
S-40	DNAPK	0.61	T-253	CKII	0.56	S-391	GSK3	0.52
S-40	ATM	0.57	T-253	PKC	0.79	S-400	p38MAPK	0.61
S-40	PKC	0.70	S-257	DNAPK	0.61	S-400	GSK3	0.51
Y-46	INSR	0.51	S-257	ATM	0.62	S-412	DNAPK	0.62
T-80	CKII	0.56	S-257	cdc2	0.53	S-412	ATM	0.54
T-94	cdc2	0.53	S-261	CKI	0.50	T-417	PKC	0.63
T-96	PKC	0.64	S-261	PKC	0.78	T-417	cdc2	0.51
S-103	ATM	0.58	S-278	CKII	0.56	T-418	PKC	0.58
S-135	CKII	0.56	S-278	cdc2	0.53	S-425	p38MAPK	0.53
S-142	CKII	0.52	S-285	CKI	0.55	S-425	GSK3	0.52
S-147	PKC	0.56	S-292	cdc2	0.51	S-425	cdk5	0.52
S-157	PKC	0.72	S-297	DNAPK	0.55	T-434	PKC	0.86
S-160	CKII	0.53	S-299	PKA	0.53	S-439	CKII	0.50
S-160	cdc2	0.51	S-301	CKII	0.54	S-439	PKA	0.51
S-161	DNAPK	0.58	T-307	cdc2	0.51	S-440	CKII	0.53
T-167	CKI	0.50	S-333	CKII	0.52	T-447	CKII	0.55
S-170	CKII	0.55	S-338	CKI	0.52	S-478	CKII	0.57
S-170	PKG	0.51	S-342	GSK3	0.51	S-550	RSK	0.70
S-171	CKII	0.52	S-342	cdk5	0.64	S-550	PKA	0.88
S-171	CKI	0.55	S-344	cdc2	0.50	T-551	PKA	0.84
T-202	PKC	0.50	S-347	p38MAPK	0.57	S-558	CKII	0.55
T-203	PKG	0.56	S-347	cdk5	0.65	S-558	cdc2	0.51
S-207	p38MAPK	0.51	S-354	CKI	0.55	Y-568	INSR	0.54
S-207	cdk5	0.52	S-355	CKI	0.54	S-569	CKII	0.50
T-213	CKII	0.50	Y-357	SRC	0.55	S-569	GSK3	0.50
S-226	CKII	0.60	Y-357	EGFR	0.60	S-589	PKC	0.71
S-227	DNAPK	0.59	S-365	CKII	0.69	T-594	PKC	0.71

Appendix B. Effect of *Radix Angelicae Sinensis* (RAS) extract in breast cancer model

Female nude mice (5–6 weeks old) were purchased from Charles River Laboratories and they were allowed to acclimate to the facilities for 2 weeks, at which time they were injected (7–8 weeks old) with human MDA-MB 231 cells ($2*10^6$ cells; 10 mice per group) into the mammary fat pad and treatment began the following day. They were treated for 4 weeks with control diet AIN-93M (Group 1) or AIN-93 M diet supplemented with RAS at 0.2% (Group 2) or at 0.5% (Group 3). Total body weights were evaluated weekly and no significant changes were observed between the groups during the 4 weeks study (data not shown). All animal studies were approved by the Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University.

At the end of the study, four mice in the control group developed mammary tumors whereas 7 and 9 mice in group 2 (RAS-LD) and group 3 (RAS-HD), respectively. Tumors were palpated twice per week and measured by using a vernier caliper. Animals were sacrificed 4 weeks after injection and tumors were excised for H&E analysis. Tumor volume (V) in cm3 was calculated by considering the perpendicular diameters as follows: V = Dd2/2, where D (cm) and d (cm) are perpendicular diameters largest and smallest, respectively. Distribution of the mammary tumor volume was presented by box plot on which the upper edge indicates the 75th percentile of the dataset whereas the lower edge indicates the 25th percentile. The line in the box show the media value of the data. The error bars represents the 95% confidence intervals. **(A)** Representative boxplot of the tumor size of mice at moment of sacrifice and **(B)** the H&E staining of the tumor samples (10X magnification) showing decrease in nuclear staining of mice tumors treated with RAS.

(A)
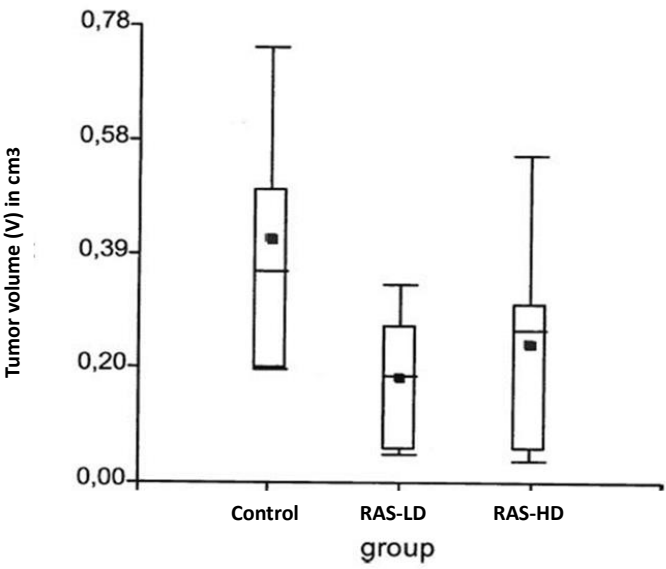
(B)
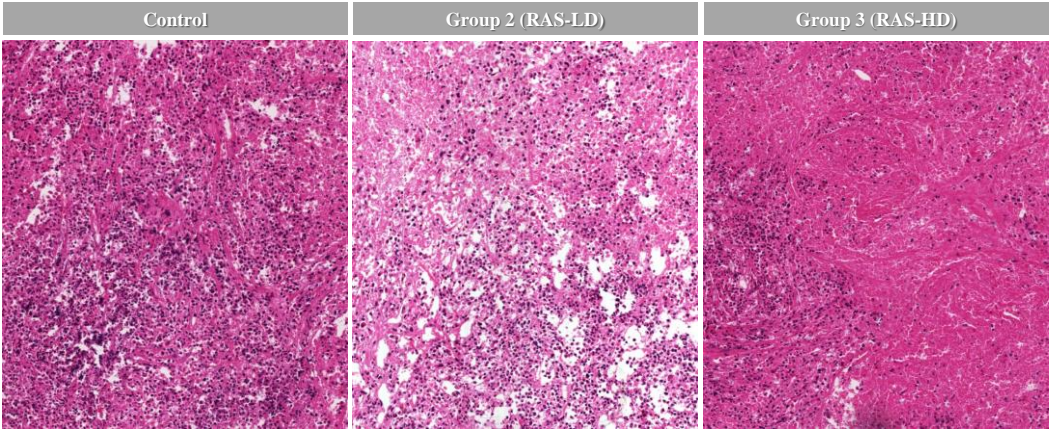
10X

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