PROSTATE CANCER CHEMOPREVENTION BY DIETARY PHYTOCHEMICALS

by

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ABSTRACT OF THE DISSERTATION

Prostate cancer chemoprevention by dietary phytochemicals

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Prostate cancer is one of the most frequent diagnosed forms of cancer among men in the United States. It displays considerable clinical, morphological and biological heterogeneity. Integrated information regarding candidate genes, transcription factors and pathways implicated in prostate cancer development is crucial to help stall prostate cancer. Currently the number of patients suffering from this invasive disease is on the rise and given the fact that when clinically significant this disorder is associated with a high mortality rate, prevention may prove to be the best approach. Chemoprevention entails the use of preferably dietary agents to block or suppress the various stages of prostate carcinogenesis. Flavonoids, the essential components of fruits and vegetables can modulate transcription factor AP-1 and its upstream signaling cascades mainly ERK-MAPK and JNK-MAPK in human androgen insensitive prostate cancer (PC3) cells. Soy isoflavone concentrate can upregulate the expression of several phase II detoxifying and antioxidant genes in an NF-E2-related factor 2 (Nrf2) dependent manner. In addition to detoxifying genes, soy isoflavone concentrate can also modulate the expression of genes such as LATS2, GREB1, calpain and many more in an Nrf2 dependent manner. The expression of most of these genes has been shown to be altered in prostate cancer progression. Animal

models that can mimick key events in prostate cancer progression provide a valuable tool in the development of anti cancer therapies. In one such transgenic model, curcumin or PEITC suppressed high grade PIN levels. However a combination of low doses of these agents worked remarkably well in suppressing tumors all together. A deeper insight at the process of cancer development in the same model revealed that indeed prostate carcinogenesis occurs by progressive suppression of Nrf2 and its related phase II detoxifying and antioxidant enzymes. Treatment with γ-tocopherol enriched mixed tocopherol diet, on the other hand, significantly increased expression of Nrf2 as well as phase II detoxifying and antioxidant enzymes. Likewise a mixed tocotrienol diet suppressed tumor incidence by modulating cell cycle control and pro-apoptotic proteins. Finally, the observed chemopreventive effects of the dietary agents were correlated with their and their metabolites' circulating plasma levels.

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Dedicated to

BABA

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE [1]

Cancer defines a disease that has the capacity to grow uncontrollably, invade surrounding normal tissues, and spread to distant sites ultimately killing the host. Despite the efforts of innumerable researchers worldwide to ameliorate the dismal outcomes of cancer, it stills continues to be a huge burden on mankind. In the United States alone, cancer accounts for one out of every four deaths. In 2007 it was estimated that approximately 559,650 Americans would die from cancer i.e. more than 1500 people a day [1].

1.1 Carcinogenesis and Chemoprevention:

The process of cancer development also known as carcinogenesis is thought to consist of three main phases: initiation, promotion and progression [2]. Initiation is often believed to be triggered by certain environmental carcinogens, radiations etc. This step is thought to be further modulated by metabolism, DNA repair processes and cell division. The initiated cell, the likely precursor of malignant cells, is thought to be more responsive, than normal cell, to subsequent stimuli that often cause further development to malignant state. Promotion and progression phases are relatively long-drawn processes. They often involve uncontrolled proliferation of the initiated cells often resulting in benign tumor formation which ultimately invade the surrounding tissues and spread to distant organs resulting in metastases [3, 4].

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1 Parts of this chapter have been published in ***Food and Chemical Tox 2007*** **as** Anticarcinogenesis by dietary phytochemicals: Cytoprotection by Nrf2 in normal cells and cytotoxicity by modulation of transcription factors NF-kappaB and AP-1 in abnormal cancer cells

The concept of chemoprevention was first introduced by Dr.Micheal Sporn, in 1976. Chemoprevention in simple terms means prevention or thwarting the process of cancer development by the use of relatively non-toxic chemicals. Since the inception of the concept of chemoprevention, researchers worldwide have focused on developing agents that can block each step of carcinogenesis. Most of these chemopreventive compounds originate from our normal diet or sources of diet [5].

Accumulated scientific evidence has suggested that atleast one –third of the estimated cancer cases in the United States is related to nutrition, physical activity and obesity. These data are important cues that link nutritional aspects with cancer. Increasing evidence substantiate the fact that a prudent diet has protective effects against various cancers versus a western diet which may be detrimental. The Western dietary pattern with its higher intake of red and processed meats, sweets and desserts and fried foods has been associated with higher risk of colon, breast, prostate and several other cancers. On the other hand, a prudent dietary pattern with its higher intake of fruits, vegetables, whole grains and cereals contributing to increased levels of antioxidants, essential minerals such as selenium, dietary fibers etc has been associated with a marked lower risk of many cancers. Thus it can be said that what really sets apart the oriental or prudent diet from the Western diet is the presence of a very vital component-dietary phytochemicals [6-8]. We and several researchers have shown that dietary phytochemicals can act as blocking agents by obstructing the initiation phase of carcinogenesis or suppressing agents retarding the promotion and progression phases of carcinogenesis. These dietary phytochemicals include isothiocyanates from cruciferous vegetables, catechins from green tea, resveratrol

from grape skin, flavonoids from fruits and vegetables, curcuminoids from turmeric and many more.

1.1.1 Dietary Phytochemicals and Cancer The primary research in our laboratory is focused towards understanding the beneficial health effects of these dietary phytochemicals. Recently, we showed that sulforaphane (SFN) a component of cruciferous vegetables inhibited adenoma formation in the gastro-intestinal tract of Apc 112 Min/+ mice[9]. In MCF-7, a breast cancer cell line and MCF-10F, a non-cancerous human breast cell-line, SFN has been shown to inhibit DNA adduct formation by polycyclic aromatic hydrocarbon – benzo[a]pyrene (BaP) and 1, 6-dinitropyrene [10]. Curcumin the beta diketone obtained from Indian spice turmeric has been long known as an anti-inflammatory and anti-cancer agent[11]. It has been shown to inhibit tumor initiation by BaP and DMBA (dimethylbenz[a]anthracene) and also inhibit tumor promotion induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) [12]. Phenylethylisothiocyanate (PEITC), another component of cruciferous vegetables has been shown to induce apoptosis in human colon cancer cell line HT-29 by activating the mitochondrial caspase cascade [13]. Interestingly a combination of curcumin and PEITC significantly inhibited tumor formation in athymic nude mice implanted with PC3 xenografts. Transgenic adenocarcinoma of the mouse prostate (TRAMP) is a transgenic mouse model that recapitulates all the salient features of human prostate cancer. Both curcumin and PEITC individually as well as in combination inhibited tumor development in these mice. Epigallocatechin gallate (EGCG) a popular component of green tea has been shown to promote apoptosis in T24 human bladder cancer cells by modulating PI3K/Akt signaling pathway and Bcl-

2 family proteins [14]. It is important to note that though each one of these phytochemicals is potent in inhibiting tumor/cancer development, they are also non-toxic to the normal cells. What really sets apart their differential effects in abnormal-cancer cells versus normal cells is their ability to induce apoptotic pathways to impede cancer in abnormal cancer cells and at the same time manipulate levels of metabolizing enzymes and induce detoxifying enzymes rendering them non-toxic to normal cells. Upregulating the expression of phase II detoxifying enzymes thus leading to enhanced excretion of the toxicants or carcinogens has been identified as one of the key steps in chemoprevention [6]. Accumulated evidence suggest that most dietary phytochemicals have the ability to modulate the expression of these detoxifying and antioxidant enzymes. SFN has been shown to increase mRNA and protein levels of quinone reductase (QR), UDP-glucuronyltransferase (UGT) and glutathione-s-transferase (GST) and heme oxygenase (HO-1) [15, 16]. It has also been shown to block benzo[a]pyrene induced forestomach cancers in ICR mice and this proctection resulted from the induction of GSTs and NQO-1[17]. Likewise PEITC, EGCG and curcumin have also been shown to induce phase II detoxifying enzymes – UGT, GST, HO-1, etc [9, 18-20]. Thus it can be inferred that careful manipulation of several physiological cascades by dietary phytochemicals within the cells could possibly contributing to the pleiotropic effects of these potential cancer chemopreventive agents.

1.2 Nrf2: Its role in chemoprevention

1.2.1 Nrf2 can modulate expression of detoxifying and antioxidant enzymes: While in search for binding proteins for the control region of b-globin locus, a subset

of leucine zipper proteins was isolated [21]. These proteins contain a CNC domain, named after the Drosophila cap ‘‘n’’ collar gene. This family includes p45-NFE2, Nrf 1, 2 and 3, Bach 1and 2 all of which serve as regulators in erythropoiesis to varying degrees. Nrf1 seems indispensable as demonstrated by embryonic lethality of Nrf1 (-/-) mouse while p45 and Nrf2 null mice demonstrate survivalability. It was later observed that the core binding region of Nrf1 and 2 highly resembles that of the ARE in the regulatory region of the human NQO-1 gene and Nrf2 was proposed to be a transcriptional activator for these phase 2 genes [22, 23]. More significantly we and others have shown using nrf2 knock-out mice that Nrf2 is essential for the induction of UGT, GST, NQO1 and HO-1 by butylhydroxyanisole, EGCG, curcumin, PEITC, SFN and soy derived phytochemicals. Besides enzymes such as aldehyde reductase (AR), glutathione reductase (GR) and epoxide hydrolase (EH) have also been shown to be regulated via Nrf2. These enzymes protect cells against toxic reactive species through a variety of reactions including conversion to a less reactive and toxic intermediate by conjugation with endogenous substrates such as glutathione, glucuronide or sulfate leading to an increase in their solubility and excretion as well as augmenting cellular antioxidant capacity by generation of endogenous antioxidant molecules such as GSH and bilirubin [24]. Nrf2 is often described as a multi-organ protector since it is widely expressed in numerous tissues and has been demonstrated to offer protection from toxic insults (Figure 1.1). The critical role portrayed by Nrf2 has been further confirmed from studies that compare differences in gene expression profiles between wild type and Nrf2 disrupted mice. Genome wide screening of wild type mice versus Nrf2 knock out mice has generated a battery of genes including

enzymes that provide co-factors, reducing equivalents, molecular chaperones/stress response genes, ubiquitin and proteosomal subunits etc that are mediated by Nrf2 thus laying emphasis on its elaborate cell defense activity.

1.2.2 Regulation of Nrf2: The cytoskeletal actin binding protein Keap1 (Kelch-like ECH-associated protein 1) was revealed to be one of the key regulators of Nrf2. Keap1 is thought to be responsible for the intracellular (cytoplasm to nucleus) shuttling of Nrf2 [23]. Keap1 is a cysteine-rich protein and its cysteine residues – C257, C273, C288 and C297 have been shown to interact with the Neh2 domain of Nrf2. A well-designed study using *keap1* disrupted mice by Wakabayashi and coworkers clearly demonstrated that Keap1 is responsible for sequestering Nrf2 in the cytoplasm thus shedding light on the crucial inhibitory role played by Keap1 in Nrf2 regulation [25]. Interestingly, in the livers of these mice the basal expression of most Nrf2 driven genes such as HO-1, γ-GCS remained unchanged. Moreover, recent work from our laboratory has demonstrated that indeed Nrf2 possesses multivalent NES/NLS motifs that have opposing driving forces. Under normal unstimulated the combined nuclear exporting forces of NES_{TA} and NES_{ZIP} counteract the nuclear importing forces of bNLS. As a result of this Nrf2 exhibits a whole cell distribution, predominantly. However upon stimulation, a condition wherein the net nuclear export driving force of NES_{ZIP} (NES_{TA} becomes disabled) being weaker than the net nuclear import force of bipartite bNLS is generated ultimately leading to enhanced nuclear translocation of Nrf2. Once in the nucleus, Nrf2 can bind to bZIP proteins such as small Maf proteins [26]. The Nrf2/Maf heterodimer formation further enhances the specificity and binding of Nrf2 to antioxidant response element (ARE) which is a cis-

acting regulatory element or enhancer sequence and is found in the promoter regions of most phase II detoxifying and antioxidant enzymes [27]. These discoveries suggest that Nrf2 may be regulated by both Keap-1 dependent and independent mechnasims.

Ubiquitination flags proteins so that they can be degraded by the proteosome. Such ubiquitination leading to proteosomal degradation appears to be one of the fates of Nrf2. Treatment with pharmacological inhibitors of proteosomal activity resulted in increased amounts of Nrf2 protein [28]. More conceptual evidence from various studies solidified the notion that Nrf2 is indeed a short-lived signaling molecule that is quickly degraded by 26S proteosome. This led researchers to hypothesize that liberation of Nrf2 from Keap1 is associated with the stabilization of Nrf2 [29, 30]. In a subsequent study, MacMahon demonstrated that the rapid degradation of Nrf2 requires the interaction of Nrf2 with Keap1 through the Neh2 domain. In addition, studies revealed that the mutations of the two residues C273 and C288 in Keap1 resulted in enhanced Nrf2 stability suggesting that these resides are vital in maintaining Nrf2 homeostasis [31, 32]. Thus it may be inferred that the existence of this protein within the cell is tightly controlled and regulated. Most putative chemopreventive agents can activate Nrf2 by both enhancing its release from Keap1 and suppressing its proteosomal degradation.

1.2.3 Upstream signaling cascades: Post-translational modification of the Nrf2 by various protein kinase signaling pathways can affect the process of liberation of Nrf2 from Keap1, its nuclear translocation and its inherent stability. Phosphorylation of Nrf2 at serine (S) and threonine (T) residues by kinases such as extracellular signal-

regulated kinase (ERK), c-Jun-NH2-terminal kinase (JNK), phosphatidyl-inositol 3-kinase (PI3K) and protein kinase C (PKC) are some of the signal transduction pathways identified.

MAPKs – a proline directed serine/threonine kinase family of proteins, is evolutionarily conserved among all eukaryotes. This family of proteins belongs to a group of important cell signaling components that relay, amplify and integrate signals from a variety of extracellular stimuli and convert them into intracellular responses through phosphorylation cascades. Several distinct MAPK cascades have been identified and studied. The c-Jun NH2 terminal kinase (JNK) represents one subgroup of MAPK that are primarily activated due to environmental stress. Its signaling module mainly consists of MEKK1/ASK/ TAK-MKK4/MKK7-JNK. ERK represents another important subgroup of MAPK. This well-established pathway involves recruitment of Raf1 by activated Ras, to the membrane resulting in subsequent activation of Raf1which further phosphorylates MEK ultimately resulting in the activation of ERK. Another well researched subgroup of MAPK is the p38 family of proteins which was first identified in 1994 as a target for endotoxins and hyperosmolarity in mammalian cells. Its signaling module mainly consists of TAK/ASK/MKK3-MKK6. Once activated p38 can phosphorylate a number of transcription factors such as ATF2, Elk1 and SAP1. While both ERK and JNK signaling cascades positively regulate ARE-driven gene expression, p38 is known to negatively regulate the same [33, 34].

PKC-like MAPK also belongs to a family of serine/threonine kinases. Abrogated nuclear translocation of Nrf2 led to loss of ARE-mediated gene expression by PKC

inhibitors. This suggested the putative role of PKC in Nrf2 transactivation. Pioneering works from the laboratories of Picket and Jaiswal established that activation of PKC leads to phosphorylation of Nrf2 at Ser40 [35-37]. This PKC phosphorylation site is conserved among many species. Nrf2 phosphorylation by upstream PKC led to its release from Keap1. However such phosphorylation does not warrant Nrf2 stabilization or nuclear translocation.

PI3K elicits a survival signal against various pro-apoptotic insults. Although the exact mechanism of PI3K induced activation of Nrf2 is unclear, the effective use of pharmacological inhibitors of PI3K helped reveal its involvement. Several studies indicate that activation of the PI3K/Akt signaling pathway leads to nuclear accumulation of Nrf2 and increased ARE-driven gene expression [38, 39].

PERK Cullinan and coworkers demonstrated that stresses on the endoplasmic reticulum can also activate the Nrf2-Keap1 system through the direct phosphorylation of Nrf2 by PKR-like endoplasmic reticulum-resident kinase (PERK). Upon exposure to ER stress inducing agents such as tunicamycin the ER localized PERK kinase senses the accumulation of unfolded proteins in the ER and phosphorylates Nrf2 thus promoting its release from Keap1 followed by nuclear translocation and inducing the expression of several pro-survival genes. Thus it can be speculated that the involvement and convergence of multiple signaling cascades could ultimately activate the Nrf2-ARE pathway [40, 41].

1.2.4 Dietary phytochemicals can activate Nrf2 and the upstream signaling cascades

Nrf2 and its upstream signaling cascades play a vital role in ARE-driven gene expression. Most potential chemopreventive agents can, hence, activate Nrf2 and its upstream signaling cascades. The different mechanisms by which these agents modulate the activity of Nrf2 are depicted in Figure 1.2.

SFN and PEITC, both are major components of cruciferous vegetables, have been shown to be potent inducers of ARE-driven phase II gene expression. Previous work from our laboratory have demonstrated that in human hepatoma (HepG2) and murine hepatoma (Hepa1c1c7) cells, SFN and tBHQ – a common food preservative, stimulated MEK-ERK2 pathway. Incubation with pharmacological inhibitor of ERK and co-transfection with dominant negative mutant ERK2 abrogated SFN and tBHQ induced quinone reductase activity [34]. The clear involvement of Nrf2 in SFN, PEITC and broccoli seed extract induced phase II gene expression was demonstrated using nrf2 disrupted mice. A host of phase II genes such as UGT, GST and γ-GCS were found to be highly induced in the wild type as compared to the nrf2 (-/-) mice [20, 42, 43]. SFN has been shown to bind covalently with the thiol groups of Keap1. The result of such interaction is liberation of Nrf2 from Keap1 with subsequent nuclear localization [44] . Likewise PEITC induced ARE-driven HO-1 expression in human prostate cancer (PC3). Dramatic increases in ARE-driven reporter gene expression on the one hand due to over-expression of Nrf2 and JNK while abrogated reporter gene expression on the other hand as a result of co-transfection with dominant mutants of the same implicated the involvement of Nrf2 and its upstream

signaling cascades in PEITC induced ARE activation [45]. SFN, in addition to activating Nrf2 and releasing it from Keap1 also suppresses its proteosomal degradation. Likewise another isothiocyanate – AITC was also found to induce activation of Nrf2 by enhanced phosphorylation, leading to increased nuclear accumulation but had little effect on delaying degradation [46].Green tea components such as Epigallocatechin gallate (EGCG), flavonoids such as kaempferol and genistein, t-BHQ and curcumin exemplify another major category of potential chemopreventive agents – polyphenols. By comparing the global gene expression profile between wild type and nrf2 disrupted mice, the involvement of Nrf2 in the activation of phase II detoxifying enzymes by these polyphenols became lucid [19, 47]. Modulation of MAPKs by flavonoid polyphenols such as kaempferol and genistein in PC3 cells has been shown [48]. Flavonoids have been shown to induce the expression of NQO1 and GST and while this induction may involve the release of Nrf2 from Keap1, the involvement of upstream PKC in such activation is debatable [49, 50]. EGCG has been shown to induce levels of HO-1 in endothelial cells. The upstream PI3K and ERK2 signaling cascades have been implicated in activating Nrf2 ultimately resulting in increased expression of HO-1 [51]. Curcumin (diferuloyl methane) is the principal active component of spice turmeric and is obtained from the rhizome of Curcuma longa. Curcumin has been reported to activate the expression of several intracellular defense systems *in vitro* and *in vivo*. Curcumin supplementation in mice results in increased expression of detoxification enzymes glutathione-s-transferases, glutathione reductase, epoxide hydrolase, HO-1, catalase, and NQO1 in the liver, small intestine and kidney tissues [18]. In vitro, curcumin has been shown to

activate NQO1 and HO-1 in numerous cells. Rushworth and coworkers have demonstrated that in human monocytes, curcumin can potently induce Nrf2/ARE pathway and this induction was abrogated by the use of PKC inhibitors and antisense oligonucleotides indicating that PKC activation is imperative for curcumin induced ARE-driven HO-1 and NQO1 expression [52]. Inactivation of the Nrf2-keap1 complex resulting in increased nuclear localization of Nrf2 has also been implicated in curcumin and caffeic acid phenylethyl ester (CAPE) induced HO-1 expression and the potential involvement of the MAPK pathway has been drawn in by the effective use of pharmacological inhibitors of the this pathway [46]. Diallyl sulfides such as diallyl sulfide (DAS), diallyldisulfide (DADS), and diallyl trisulfide (DATS) are the active principles in garlic and onions. They are lipophilic thioesters derived from oxidized allicin which is produced when garlic cloves are crushed. DAS has been shown to be protective against numerous chemically induced cancers and are potent inducers of phase II detoxifying enzymes. Extensive research from our laboratory and others has demonstrated that these diallyl sulfides can induce NQO1 and HO-1 in an Nrf2/ARE dependent fashion. These agents stimulate expression of Nrf2, its nuclear translocation and this effect can be abated by MAPK inhibitors [53]. In addition numerous other potential chemopreventive agents such as indole-3-carbinol (I3C), coffee diterpenes such as cafestol and sesquiterpenes such as parthenolide have been shown to induce Nrf2 mediated ARE-driven gene expression [46]. Taken together, it can be inferred that these naturally occurring dietary agents can activate ARE-driven phase II detoxifying enzymes and this activation clearly involves the transcription factor Nrf2. The upstream signaling cascades such as MAPK, PI3K and PKC may

participate either solely or in concert to bring about such activation of Nrf2. Suppressing the inhibitory action of Keap1 on Nrf2 and successfully averting the proteosomal degradation of Nrf2 are the other possible mechanisms by which these naturally occurring dietary agents can enhance the levels of Nrf2.

1.3 Role of transcription factor AP-1 in carcinogenesis and its modulation by dietary phytochemicals:

As a response to pro-inflammatory stimuli, various cytokines, chemokines, prostaglandins and leukotrienes are produced. These mediators play an active role in the promotion stage of carcinogenesis by altering myriad cellular cascades [54]. Prostaglandins are produced in abundance by the metabolic conversion of arachidonic acid by COX-2, which has been known to be upregulated in a number of malignancies. Four transcription factors including nuclear factor kappa B (NF-κB), CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1) and CRE-binding protein (CREB) have been identified to bind to the cis-acting elements in the promoter of COX-2 [55]. AP-1 was one among the first mammalian transcription factors to be identified and its activity is induced by numerous physiological stimuli and environmental insults. AP-1 is not a single protein but is a dimeric complex formed by basic leucine zipper proteins from Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1 and Fra-2) and Jun dimerization partners (JDP1 and JDP2) and closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) [56].

AP-1 activity is induced by growth factors, cytokines as well as several neurotransmitters. These stimuli activate the MAPK cascades that enhance AP-1 activity through the phosphorylation of distinct substrates [57]. Serum and growth

factors that induce AP-1 do so by activating the ERK family of MAPK whose members the translocate to the nucleus to phosphorylate and potentiate the transcriptional activity ternary complex factors (TCF) that bind to fos promoters. Furthermore, ERK can directly phosphorylate Fra-1 and 2 in response to serum stimulation possibly enhancing their DNA-binding in conjunction with c-Jun. On the other hand, the induction of AP-1 activity by pro-inflammatory cytokines and genotoxic stress is mostly mediated by JNK and p38 cascades. Once activated, the JNKs translocate to the nucleus where they phosphorylate c-Jun and thereby enhance its transcriptional activity. The JNKs also phosphorylate and potentiate the activity of ATF2 which heterodimerizes with c-Jun to bind divergent AP-1 sites in the c-jun promoter. The contribution of p38 to AP-1 activation is reported to be mediated by the direct phosphorylation and activation of ATF2, MEF2C and TCFs [58, 59].

AP-1 is very often portrayed as a general, nuclear decision maker that determines the final fate of the cell upon stimulation by extracellular signals. The role of AP-1 in apoptosis should be considered within the context of a wide array of complex network signaling as well as nuclear factors that often respond simultaneously. Cell death induced by Fas ligand (FasL) and its cell surface receptor (Fas) is a classic example of apoptosis induced by activation of JNK, Jun/AP-1 pathway. JNK activated by MAPK cascades phosphorylates Jun which results in enhanced transcription of target genes implicated in cellular stress induced apoptosis. On the other hand, some reports indicate that JNK activation can also signal cell survival. Thus it can be said that the ultimate fate of the cell relies on the relative abundance of

AP-1 complex, the composition of the AP-1 dimers, cell type and cellular environment [60, 61].

Given that AP-1 play a vital role in carcinogenesis, most dietary phytochemicals can modulate their activity via different mechanisms (Figure 1.3) and thereby amend the expression of its target genes. Recently Sakata and co-workers demonstrated that a green tea proanthocyanidin-PDGG caused a dose dependent inhibition of COX-2 at both mRNA and protein levels in RAW264 murine macrophage cells. Furthermore, they also demonstrated that PDGG inhibited the nuclear translocation of p65, the phosphorylation of c-Jun and activation of the three MAPKs – JNK, ERK and p38 kinase. Thus the conclusion drawn by the authors was that PDGG suppressed COX-2 expression by effectively suppressing MAPK-mediated NF-κB and AP-1 proteins [62]. Early work published from our laboratory clearly suggested that resveratrol – a stilbene compound present abundantly in grapeskin attenuated phorbol ester and UV induced AP-1 activity in human cervical cancer (HeLa) cells and did so by interfering with the MAPK cascade [63]. However in human colon cancer HT-29 cells resveratrol dose dependently increased LPS induced AP-1 activity. A coordinated increase in JNK activation was also observed. Likewise SFN and PEITC also increased AP-1 and its upstream JNK activation [64]. Flavonoids – kaempferol and genistein also induced AP-1 activity with coordinated increases in upstream MEK-ERK and JNK phosphorylation [48]. A more recent report by Mallikarjuna and coworkers established that silibinin treatment in UVB induced tumorigenesis resulted in strong phosphorylation of ERK1/2, JNK and p38. This suggests the possible involvement of the MAPKs in apoptotic effects of silibinin [65].

1.4 Role of reactive oxygen species in carcinogenesis and its modulation by dietary phytochemicals:

A substantial body of evidence links the production of reactive oxygen species (ROS) and subsequent oxidative stress and damage to the pathogenesis of cancer. During mitochondrial oxidative metabolism, the majority of oxygen consumed is reduced to water; however, an estimated 4-5 % of molecular oxygen is converted to ROS [66]. Neutrophils, eosinophils and macrophages are additional endogenous sources of cellular ROS. Activated macrophages often elicit a rapid but transient increase in oxygen uptake that gives rise to ROS such as superoxide anion, hydrogen peroxide and nitric oxide [67]. Other exogenous sources of ROS often result from xenobiotic metabolism of mainly toxicants and pro-carcinogens or radiation. The cell under normal conditions is well-equipped with antioxidant enzymes or species that help to keep ROS under check. The primary defense system includes superoxide dismutase, glutathione peroxidase, catalase and thioredoxin reductase. The secondary defense system mainly combats processes elicited by free radicals. Main compounds belonging to the secondary defense system include ascorbic acid, α-tocopherol, glutathione, β-carotene, vitamin A, and NADPH. However, when the antioxidant control mechanisms are exhausted or overrun the cellular redox potential shifts towards an oxidative stress. Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants in the favor of the former, resulting in an overall increase in cellular levels of ROS [68]. Several lines of evidence implicate oxidative stress as a putative mediator of apoptosis. This acts by decreasing intracellular glutathione, the major buffer of the cellular redox status and/or by increasing cellular reactive species.

On the contrary antioxidant enzymes are known to antagonize initiation and promotion phases of carcinogenesis and their levels are reduced in many malignancies [69]. The generation of large amounts of reactive oxygen intermediates may also contribute to the ability of some tumors to mutate, inhibit antiproteases and injure local tissues, thereby promoting tumor heterogeneity, invasion and ultimately metastases.

Dietary antioxidants are known to neutralize oxidative challenges either directly by intercepting free radicals or indirectly by modulating the expression of genes that detoxify these reactive intermediates or eliminate their damage products. Combined work from our laboratory and several other researchers have shown that SFN, PEITC, 3*H*-1,2-dithiole-3-thione (D3T), ethoxyquin, BHA can activate transcription of several protective genes through ARE. On the other hand, flavonoids from red wine and several other sources, owing to their structure, have been demonstrated to scavenge ROS and nitric oxide species both *in vitro* and *in vivo*[70-73].

Thus the exact mechanism through which ROS plays an important role in the initiation and progression of cancer and its ability to induce apoptosis is not fully understood. Therefore further efforts are necessary to fully elucidate the importance of free radical scavengers in the therapy of several diseases, especially cancer.

1.5 Animal models in carcinogenesis:

Given the fact that cancer development can be roughly divided into three stages of initiation, promotion and progression, control over this disease may only be achieved if molecular changes underlying each stage can be well-characterized and

recapitulated in animal models of carcinogenesis. Hence animal models are critically important and play a central role in expediting the development of new chemopreventive approaches and therapies for cancer.

1.5.1 Xenograft models of cancer: A very quick though rather non-mechanistic approach to evaluating the efficacy of chemopreventive agents in tumor suppression entails the use of immune suppressed athymic nude mice. Nude mice often present a routinely used valuable research tool since they can receive many types of tissue and tumor grafts and they offer no rejection response. The effects of the chemopreventive agents on the xenograft can be easily monitored by measuring the dimensions and the volume of the tumor graft. Innumerable researchers have used this model to evaluate the effectiveness of various anti-cancer agents. Research from our laboratory has clearly demonstrated that combination of dietary agents curcumin and phenylethylisothiocyanate can effectively regress tumor formation in nude mice bearing human prostate cancer xenografts and such regression was found be atleast in part due to suppression of the Akt and NF-κB signaling pathway ultimately resulting in enhanced expression of apoptosis biomarkers – caspase-3 and caspase-9 [74]. Apigenin a naturally occuring flavonoid compound has also been shown to suppress hypoxia-inducible factor 1 (HIF-1) and VEGF expression in tumor tissues of nude mice bearing A549 human lung cancer cell xenografts [75].

1.5.2 Genetically engineered mouse models of cancer: Once the efficacy of the chemopreventive agents has been established in nude mice the next logical step would be to obtain to good understanding of the molecular mechanisms underlying such effects and this is where authochthnous, germ-line transgenic and knockout

animals come into picture. These models are in sharp contrast to *in vitro* culture or orthotopic transplantation of cell lines or tumors from clinical specimens, as described above since these autochthnous or transgenic animals closely mimic the complex interactions that occur within the tumor microenvironment. By virtue of their design these animals provide a unique window of opportunity to investigate the molecular events related to various stages of cancer development. In other words, these animal models can very easily recapitulate most of the salient features of cancer progression. Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) is one such genetically modified mouse model that is commonly used to study prostate cancer chemoprevention and has received considerable attention over several past years. The TRAMP model was generated with a PB-Tag transgene containing the SV-40 early genes (T and t antigens, Tag) under the control of the minimal rat probasin gene. This strategy was designed to target the expression of large T antigen to abrogate the function of p53 and Rb tumor suppressor genes and to target small t antigens to inactivate protein phosphatase 2A [76]. The TRAMP transgenic mice develop high-grade prostatic intraepithelial neoplasia (PIN) within 12 weeks of birth and ultimately develop metastases by 30 weeks, primarily to the lungs, liver and lymph nodes. PIN is considered the precursor to invasive carcinoma since it is most often than not associated with malignant form of the disease and the primary architectural and cytological features of PIN resemble that of invasive carcinoma [77]. Hence, the ability to retard PIN formation is considered as an important merit in agents that are tested against prostate carcinoma. Genistein a very popular soy isoflavone in the diet has been shown to reduce the incidence of poorly differentiated

prostatic adenocarcinomas in a dose-dependent manner and down-regulate epidermal growth factor receptor, insulin-like growth factor-I, and extracellular signal-regulated kinase-1 mRNA expressions [78]. Likewise oral consumption of green tea polyphenols has also been shown to suppress the insulin- like growth factor 1 signaling in TRAMP mice [79]. Cancer phenotypes have also been described in various knockout mice. The Nkx 3.1 homeobox gene has been shown to be essential for prostate differentiation and function. Loss-of-function of Nkx 3.1 results in histopathological defects that resemble prostate cancer initiation in humans. These mutant mice represent an excellent model to study prostate cancer initiation.

1.5.3 Knockout rodent models of cancer: Most of the aforementioned knockout rodent models are tissue or site specific. However it is well understood that most cancers are multi-factorial and arise from multiple mutations. Hence knocking out a gene that exerts pleitropic effects or is pivotal to the development of several cancers presents a model that offers a more mechanistic approach to chemoprevention. Nrf2 is a master transcription factor that has been shown to regulate the expression of more than 200 genes, including those involved in Phase II detoxifying and antioxidant genes. One of the most prevalent mechanisms by which dietary phytochemicals effectively halt cancer formation/development is by manipulating the levels of detoxifying and antioxidant enzymes. This manipulation mainly involves regulation via Nrf2/ARE pathway. The use of Nrf2 disrupted mice, indeed, sheds more light into the mechanistic details of chemopreventive by these dietary agents. Since Nrf2 exerts pleiotropic effects, mice that lack this gene are spontaneously predisposed to a variety of cancers. Subjecting these mice to the putative chemopreventive agents aid in

elucidating the role of Nrf2 in chemoprevention. The Nrf2 knockout mouse model allows integration with other cancer models. Recently work from our laboratory established that Nrf2 deficient mice are more susceptible to DSS induced colitis and this was associated with increased levels of pro-inflammatory mediators such as COX-2, iNOS, IL-6 and TNF-α [80]. The Nrf2 knockout mouse model has been successfully integrated into skin cancer models that involve initiation with 7, 12-dimethylbenz[a]anthracene and promotion by 12-*O*-tetradecanoylphorbol-13-acetate. Work from our laboratory has demonstrated that Nrf2 deficient mice are greatly suspectible to development of skin cancer by DMBA/TPA applications as compared to the wild type mice and dietary agent sulforaphane could effectively suppress this effect in part by enhancing the expression of antioxidant enzyme HO-1 [81].

Each animal model discussed above has immense relevance and potential in the field of cancer chemoprevention and these animal models will undoubtedly facilitate the ability to discover the dietary factors effective at preventing cancer and the specific stage of carcinogenesis at which they render effective. Almost every model discussed may seem imperfect in one sense or another however each model has contributed to our current knowledge of chemoprevention. Further, cross-breeding to obtain double knockout mice may also greatly help in elucidating the mechanism of chemoprevention by dietary agents.

1.6 Prostate Cancer and Chemoprevention

Prostate cancer is the most frequently diagnosed forms of cancer among men in the United States. Most prostate cancer patients respond well to the first line of treatment – hormone ablation. However in most cases, the disease becomes hormone refractory

and unfortunately this form of the disease has no effective treatment options. Moreover, this disease typically has a long latency period. The progression from low grade prostatic lesions to high grade neoplastic lesions finally leading to aggressive adenocarcinoma may take several years. Hence this disease provides a unique window of opportunity for intervention by chemopreventive agents.

Given the important roles of transcription factors AP-1 and Nrf2 in cancer development – it is noteworthy that their roles in prostate cancer are less evolved. It has been shown that c-Jun a component of AP-1 is an androgen receptor co-activator. It increases the transcriptional activation by mediating receptor dimerization and DNA binding. Approximate 5 fold increase in AP-1 transcriptional activation was observed upon exposure to androgen. On the contrary, c-fos, another component of AP-1 behaves as a pro-apoptotic agent in TRAIL-induced apoptosis in the prostate. Though increased levels of oxidative stress have been noted in prostatic intraepithelial neoplasia, expression of Nrf2 in prostate cancer has not been studied thus far.

1.7 Summary

Both AP-1 and redox sensitive transcription factor Nrf2 have unique roles to play in the process of carcinogenesis and oxidative stress is one among their mechanisms of action. Dietary polyphenols can manipulate oxidative stress, thereby affecting AP-1 and Nrf2, by different mechanisms ultimately intercepting each stage of carcinogenesis, successfully. They can avert the initiation process by blocking carcinogen or ROS induced DNA damage. Loss of antioxidant defense mechanisms

and detoxifying systems has been shown in cancer. Dietary polyphenols can enhance the excretion of the toxicants or pro-carcinogens by inducing Phase II detoxifying and antioxidant enzymes may be through the Nrf2/ARE signaling pathway. By enhancing the activities of these detoxifying and antioxidant enzymes, the dietary polyphenols may avert promotion and progression stages of carcinogenesis.

Using both *in vitro* cell culture and *in vivo* animal models, we have examined the role of these key transcription factors in prostate carcinogenesis and the various mechanisms by which dietary polyphenols can manipulate them to effect chemoprevention. This dissertation compiles the results of the various investigations which are as follows: 1) Flavonoids can differentially modulate Activator-protein-1 and its upstream signaling cascades JNK-MAPK and ERK-MAPK. 2) Nrf2 regulates the expression of certain important prostate cancer related genes such as LATS2 and GREB1. Loss of Nrf2 may increase susceptibility to prostate cancer. Administration of soy isoflavone concentrate can regulate the expression of several prostate cancer specific genes in an Nrf2-dependent fashion. 3) TRAMP is a transgenic prostate cancer mouse model that closely mimics key events in human prostate cancer progression and thus provides a valuable tool in the development of anti-cancer therapies. We have shown that administration of curcumin or PEITC to these mice suppressed development of Prostatic Intraepithelial Neoplasia (PIN). However a combination of low doses of these agents worked remarkably well in suppressing tumors all together. Both curcumin and PEITC effectively modulate the Akt signaling pathway leading to enhanced apoptosis and suppressed proliferation. 4) A deeper insight at the process of cancer development in the same model revealed that indeed

prostate carcinogenesis occurs by progressive suppression of Nrf2 and its related phase II detoxifying and antioxidant enzymes. Treatment with γ-tocopherol enriched mixed tocopherol diet, on the other hand, significantly increased the expression of Nrf2 and its related genes and enzymes. 5) Several anti-cancer drugs under development are designed to block cell division. Herein, we show that mixed tocotrienols can effectively block cell cycle progression by suppressing the cyclins and increasing the expression of cyclin dependent kinase inhibitors p21 and p27 and driving the cell into apoptosis by increasing the levels of pro-apoptotic proteins. 6) Finally, in order to correlate the pharmacodynamic (chemopreventive) properties exerted by the dietary agents this chapter is dedicated to understanding the metabolism and pharmacokinetics/bioavailability of these agents. Since most of the dietary agents are extensively metabolized by phase I oxidative or phase II conjugation reactions, their circulating plasma levels are quite low. We have shown that Kaempferol (a putative chemopreventive flavonoid) undergoes extensive glucuronidation leading to enhanced excretion.

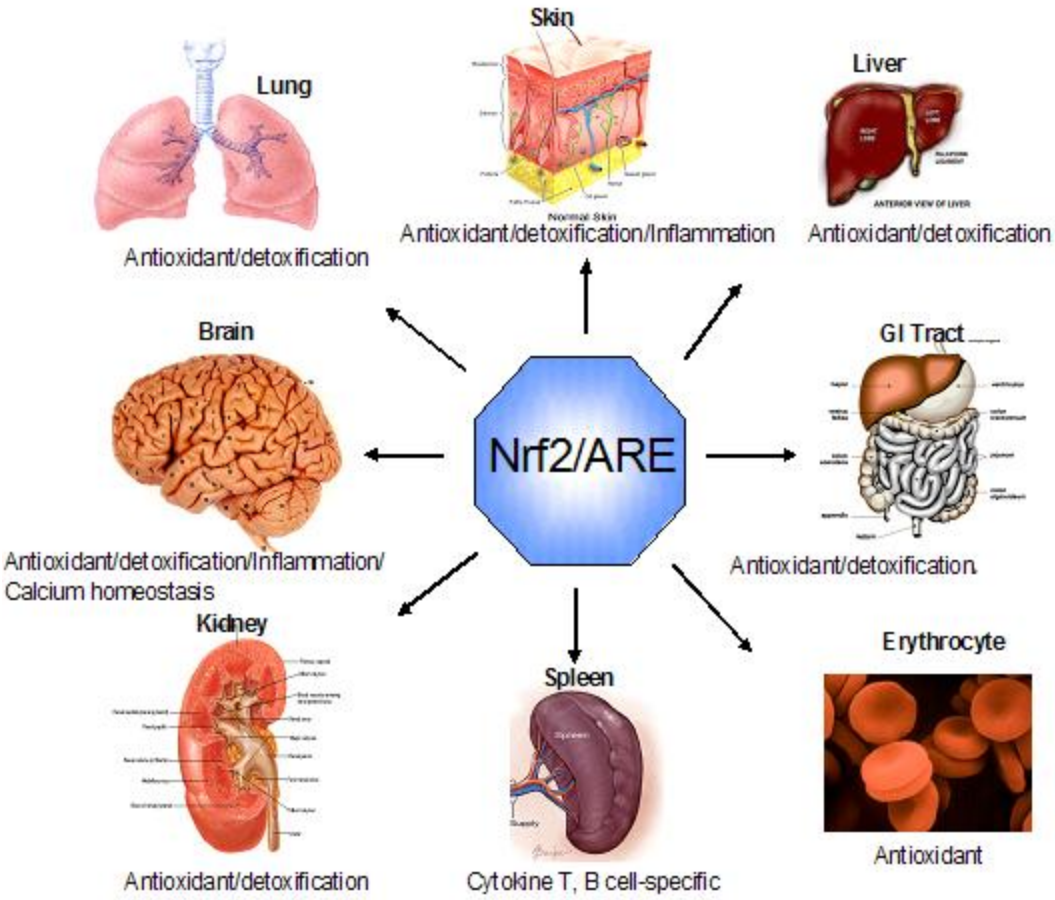

Figure 1.1 A multi-organ protector, Nrf2. Nrf2 protects various cell types by co-ordinately increasing ARE-driven detoxification and antioxidant genes. (Figure taken from Lee et al, *FASEB Journal*, 2005, **19** (9): 1061-6)

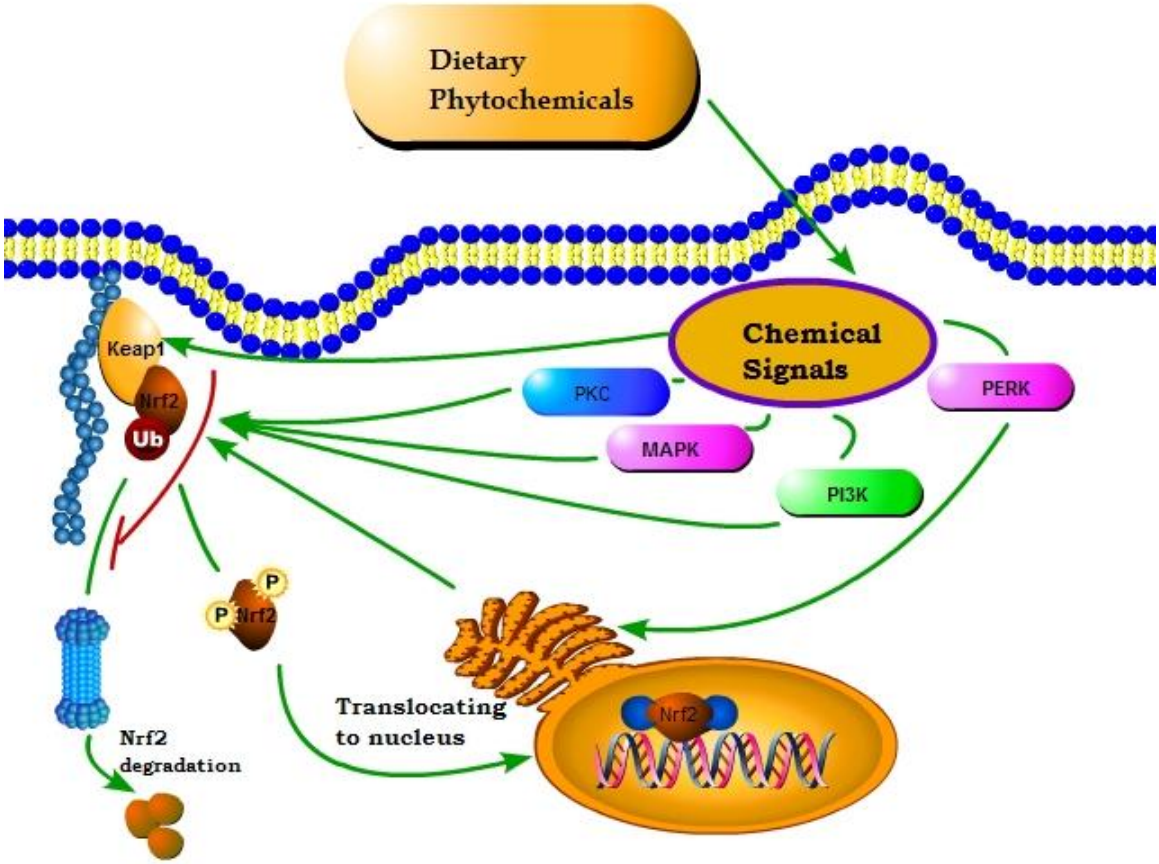

Figure 1.2: This figure depicts the regulation of detoxifying enzymes by dietary phytochemicals. These putative chemopreventive agents disrupt the association between Keap-1 and Nrf2, thereby releasing Nrf2 translocates into the nucleus, binds to antioxidant response elements present in the promoter region of phase II detoxifying genes, ultimately enhancing the gene expression of the detoxifying enzymes. Alternatively, these chemopreventive agents can inhibit proteosomal degradation of Nrf2, thereby increasing its half-life. Likewise, the activation of upstream signaling cascades such as MAPK, PI3K, PKC and PERK by these chemopreventive agents also results in increased nuclear translocation of Nrf2 and thereby enhanced gene transcription.

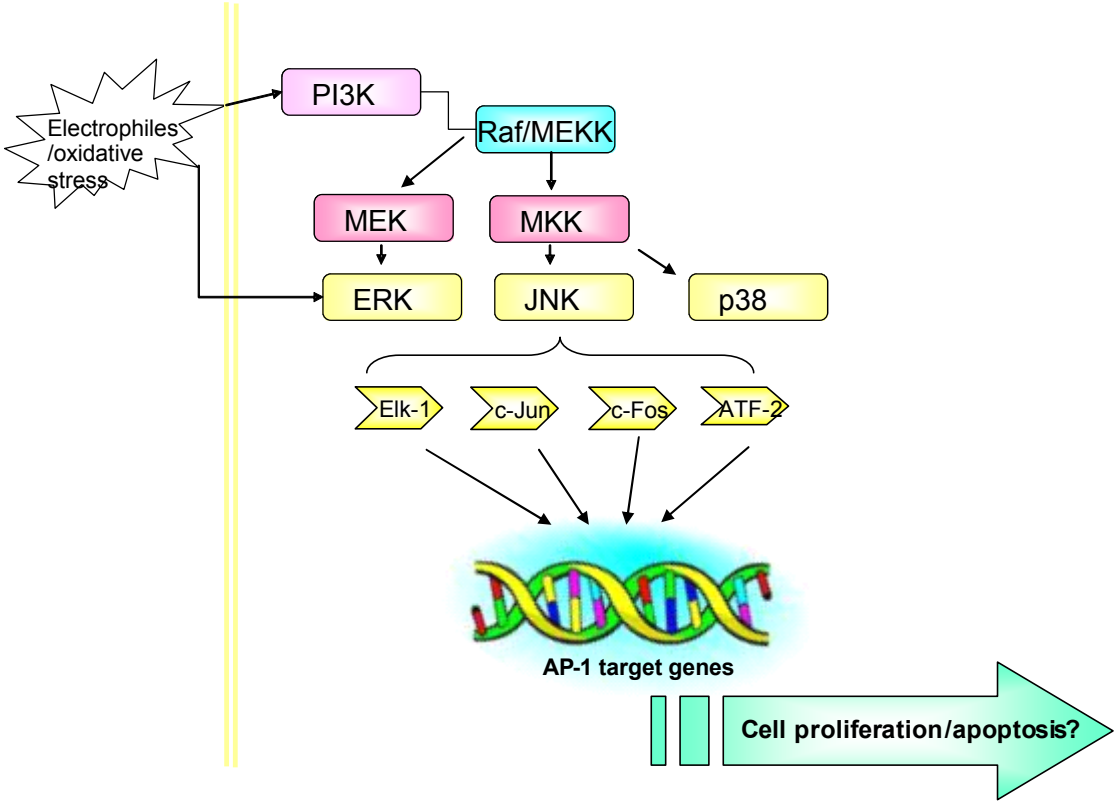

Figure 1.3: Regulation of AP-1 and MAPK signaling by chemopreventive compounds. Activation of AP-1 is primarily mediated by MAPK, especially through the phosphorylation of JNK which leads to translocation of AP-1 and subsequent transcriptional activation of target genes.

CHAPTER 2: MODULATION OF ACTIVATOR PROTEIN-1 (AP-1) AND MAPK PATHWAY BY FLAVONOIDS IN HUMAN PROSTATE CANCER PC-3 CELLS [2].

2.1 Abstract

Flavonoids have been demonstrated to exhibit a wide range of biological activities including chemopreventive properties. In an attempt to study the chemopreventive action of these flavonoids on human prostate cancer cells, we constructed a stable cell line expressing activator protein (AP-1) luciferase and screened several flavonoids for their ability to modulate AP-1 luciferase activity. The most induction was observed with 20 μM quercetin, chrysin, genistein and kaempferol. To elucidate the mechanism by which these flavonoids modulate AP-1, western blots of phosphorylated MAPK proteins were performed. Most of the flavonoids activated both phospho-JNK and phospho-ERK. Furthermore, results from western blot of phosphorylated MEK and pretreatment with inhibitors of JNK and ERK revealed that only kaempferol potently activated the MEK-MAPK pathway, while genistein induced AP-1 luciferase activity was abolished by the JNK inhibitor. Neither inhibitors abolished apigenin nor naringenin induced AP-1 luciferase activity. Furthermore, we observed the effects of these flavonoids on the mRNA levels of transcription factors ATF-2, Elk-1 and cyclin D1 that are downstream to the MAPK family of proteins and are known to be under the control of AP-1 promoter.

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2 This chapter has been published in *Archives of Pharmacal Research* as Modulation of AP-1 and MAPK by flavonoids.

The transcriptional activation of Elk-1 and ATF-2 by kaempferol and genistein indeed correlated well with the translational activation of MEK-ERK and JNK by these agents.Taken together, the MAPK pathway may play important roles in kaempferol and genistein induced AP-1 activity, respectively. Thus, flavonoids as chemopreventive agents might have different physiological and biological actions on signaling cascades such as MAPKs which, in part, may play a role in the intervention of prostate cancer progression.

2.2 Introduction

Activator protein-1 (AP-1) transcription factors comprise a family of ubiquitously expressed proteins that include the Jun (e.g., cJun, JunB, JunD) and Fos (e.g., cFos, FosB, Fra-1, Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K) and ATF (ATF2, LRF1/ ATF3) proto-oncoproteins. The resulting homodimeric (Jun-Jun) or heterodimeric (Jun-Fra or Fos-Jun) complex binds a palindromic DNA sequence, known as 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element. This element is known to be present within the regulatory region of several genes including c-jun [82-84]. The AP-1 complex mediates responses to cellular signals by binding to DNA and producing changes in gene transcription that ultimately leads to physiological functions in the cell. Several lines of evidence indicate that AP-1 activity is involved in many different cellular processes including cell proliferation, differentiation, apoptosis, and stress responses. Depending upon the composition of the AP-1 complex, different genes could be activated as a result of AP-1 binding. Although the relevance of AP-1 in human diseases is not completely understood, it is thought that AP-1 proteins may play a significant role in the pathogenesis of human cancers [85].

Dong and co-workers demonstrated that blocking AP-1 activity by pharmacological or biological inhibitors impaired neoplastic transformation by tumor promoters such as UV light and PMA [86]. On the other hand a report by Zerbini *et al*. showed that the aberrant activation of AP-1 transcription factors in human prostate cancer cells results in deregulation of interleukin-6 (IL-6), which is associated with androgen-independent human prostate cancer. This suggests that regulation of AP-1 activity may be important in prostate cancer development/progression [87].

During prostate carcinogenesis the normal paracrine function of the androgen receptor signaling is converted into an autocrine function whereby the binding of androgen receptor within the cancer cells directly stimulates their survival and proliferation. Thus, androgen withdrawal produces an initial response of apoptosis in these androgen-dependent cells. Unfortunately, this androgen-dependent cell survival is nearly universally followed by a relapse to a refractory state in which the cancer cells continue to survive and proliferate despite a low, circulating androgen environment. Several lines of evidence suggest multiple molecular changes that result in these cancer cells acquiring alternative ways of activating survival and proliferative pathways without requiring physiological levels of circulating androgens[88] . Thus, identifying the signal transduction pathways responsible for the survival and proliferation of androgen-independent prostate cancer cells is critical for future targeted drug development. Race, age and diet are identifiable risk factors associated with prostate cancer occurrence. An increasing body of evidence suggests that several essential nutrients present ubiquitously in fruits, vegetables and other beverages such as tea and wine can suppress prostate cancer development [89]. Identifying these

factors and understanding their chemopreventive mechanisms presents a noninvasive strategy for decreasing the incidence and severity of this disease.

Strong evidence from experimental and epidemiological studies have established a positive link between consumption of several yellow-green vegetables that are rich in flavonoids and fresh fruits, especially grapes, and the reduced incidence of cancer. Procyanidins, a diverse group of flavonoids, are the principle constituents of grape seed extract and are also prevalent in a number of fruits and vegetables. A study performed by Agarwal and coworkers demonstrated that grape seed extract induced apoptotic death in DU145 human prostate cancer cells [90]. A more recent study by Rana *et al*. demonstrated that grape seed extract inhibited advanced human prostate tumor growth and angiogenesis and also inhibited insulin-like growth factor binding protein-3, high levels of which is known to be positively correlated with increased risk of prostate cancer [91]. Another study performed by Huang *et al*. demonstrated that baicalin (baicalein 7-D-β-glucuronate) could inhibit the proliferation of several human prostate cancer cells including PC3, DU145, LNCaP [92].

Taken together, there is an imminent need for chemopreventive strategies for prostate cancer and it is almost certain that dietary constituents play a significant role in trying to fulfill this need. In line with this, we screened several flavonoids for their potential chemopreventive properties on human prostate cancer cells. Thus, the goal of this study was to investigate the chemopreventive mechanisms and the signaling pathways induced by these chemopreventive agents within the cell. In order to achieve the specific aims of this study, we stably transfected androgen-independent human prostate cancer cell line (PC3) with the AP-1 luciferase reporter gene. This cell line

was then used to screen eight flavonoids belonging to four different classes namely flavonols, flavones, flavonones and isoflavones. Since AP-1 activity can be induced by several growth factors, cytokines, bacterial and viral infection and a variety of physical and chemical stresses, and since these stimuli can activate MAPK cascades that enhance AP-1 activity through phosphorylation of a variety of substrates, we studied the modulation of the MAPK pathway by these flavonoids as well.

2.3 Materials and Methods

Materials Quercetin, kaempferol, apigenin, chyrsin, naringenin, hesperitin, genistein, biochanin, SP600125 (specific JNK inhibitor) and U0126 (MEK inhibitor) were purchased from Sigma chemicals Co. (St. Louis, MO). All other chemicals were purchased from commercial sources and were of analytical grade. Human prostate cancer cell line PC3 was obtained from American Type Culture Collections (Manassas, VA). AP-1 luciferase reporter plasmid construct containing AP-1 consensus binding site was a kind gift provided by Dr. Anning Lin (University of Chicago, Chicago, IL).

Cell Culture and Stable Transfection PC3 cell line was maintained in Minimal Essential Medium supplemented with 10% bovine serum albumin and antibiotics at 37 °C with 5% CO_2. AP-1 luciferase construct containing the AP-1 consensus binding site (-TGACTCA-) and pcDNA3.1 neomycin plasmid was co-transfected into PC3 cells by Lipofectamine TM 2000 (LF2000, Invitrogen life technology, Carlsbad, CA) following the manufacturer's instruction, and stable clones were selected with 0.5 mg/ml of G418 sulfate (Invitrogen Life Technology, Carlsbad, CA). One of the stable clones was subcultured and used for further studies.

AP-1 Luciferase Assay The cells were subcultured in 6-well plates at a density of 1 × 10^5 cells/well. 12-16 hours prior to treatment, the cells were starved with a medium containing 0.5% FBS. The cells were treated with the various flavonoids and incubated for 24 hours else they were pre-treated with the JNK or MEK inhibitors for 30 minutes followed by treatment with the respective flavonoids and then incubated for 24 hours. Luciferase activity was assayed with a luciferase kit from Promega (Madison, WI) by using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein concentration and expressed as fold induction over control values.

MTS Assay for cell viability PC3 AP1 cells were seeded in 24 well plates at a density of 10^5cells/well. 12-16 hours prior to treatment, the cells were starved with a medium containing 0.5% FBS. The cells were incubated with different doses of the flavonoids for a period of 24 hours. The MTS [3,4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with Cell Titer 96 Aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI) by manufacturer's instruction. After 2 hours, the absorbance was measured at 490 nm with μ Quant ELISA reader (BIO-TEK Instruments, Inc., Madison, WI). The cell viability was determined by the percentage of treated over the control that was treated with DMSO (0.1%).

Western Blot Analysis The cells were washed with ice-cold PBS after treatment and harvested with 200 μl of a lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonylfluoride and 0.5% Triton-X 100. The supernatant was collected

and twenty micrograms of total protein, as determined by Bio-Rad protein assay, was mixed with 4x loading buffer, and pre-heated at 95°C for 3 min. The samples were then loaded on a 10% SDS–polyacrylamide gel, and run at 200 V. The proteins were transferred onto PVDF membrane for 1.5 h using semi-dry transfer system (Fisher, Pittsburg, PA). The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature, and then incubated overnight at 4°C with indicated primary antibody (1:1000 dilution). After hybridization with primary antibody, the membrane was washed with TBST (Tris buffered-saline Tween-20) three times, and incubated with HRP-labeled secondary antibody for 45 min at room temperature. Final detection was performed with ECL™ (Enhanced Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech).

RNA Extraction and Quantitative real-time PCR PC3-AP1 cells were treated with the respective flavonoids for 5 or 10 hours. RNA was isolated using RNEasy Mini Kit (Qiagen) according to manufacturer's instructions. ATF-2, cyclin D1 and Elk-1 genes are known to be under the control of AP-1 promoter and are downstream to the MAPK pathway and hence were selected. Beta-actin was selected as the housekeeping gene. The primers for these genes are listed in Table 2.1. First-strand cDNA was synthesized using 4 μg total RNA following the protocol of SuperScript III First-Strand cDNA Synthesis System (Invitrogen). The PCR was performed using 100 times diluted cDNA, 60nM of each forward and reverse primer followed by SYBR Green mix. The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 40 cycles and 72°C for 10

minutes. The gene expression was normalized with the house-keeping gene – beta actin.

Statistical Analysis Statistical significance of differences in all other measurements was determined by one-way analysis of variance followed by Tukey's procedure for all pairwise multiple comparisons, excluding the control group. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 Effects of chemopreventive agents on the induction of AP-1 luciferase in PC3 cells

To investigate and compare the effects of various natural chemopreventive agents on the transcriptional activation of AP-1, we constructed a PC3 cell line that was stably transfected with the AP-1 luciferase reporter gene. TPA (12-O-tetradecanoylphorbol-13-acetate), LPS (lipopolysacchrides) and H_2O_2 (hydrogen peroxide) are known to activate AP-1 transcriptional activation in several cell models. However, according to a study performed in our laboratory, none of these agents increased the transcriptional activity of AP-1 dramatically in the PC3 cell line (unpublished results). Hence these agents could not be used as positive controls in any of our experiments.

Four different groups of flavonoids, namely flavonols, flavones, flavanones and isoflavones, were tested for their effects on AP-1 luciferase activity. Figure 2.1 shows the structures of all the flavonoids assessed for their chemopreventive properties in this study. Figure 2.2A demonstrates the effects of flavonols on AP-1 luciferase activity. Quercetin and Kaempferol are both flavonols. Quercetin and Kaempferol both induced maximal AP-1 luciferase activity ~3 fold at 20 μM and 50 μM

concentrations respectively. At higher concentrations, both compounds caused a slight inhibition of AP-1 luciferase activity. Figure 2.2B demonstrates the effects of flavones on AP-1 luciferase activity. Apigenin and Chrysin are both flavones. They differ from quercetin and kaempferol in that they lack the hydroxyl group on ring C. Chrysin induced AP-1 activity ~3 fold over control at 20 μM concentration. Apigenin on the other hand did not demonstrate much dose-dependency in AP-1 transcriptional activation. Figure 2.2C demonstrates the effects of isoflavones on AP-1 luciferase activity. Isoflavones differ from flavones in that the phenyl ring is attached to position 3 on ring C instead of position 2. Genistein induced AP-1 activity to almost equal levels (~3 fold) at both 20 μM and 50 μM concentrations. Biochanin on the other hand did not induce any AP-1 activity. Figure 2.2D demonstrates the effects of flavonones on AP-1 luciferase activity. Naringenin and Hesperitin are both flavonones. The structural difference between flavonones and flavones is that in flavonones the double bond in ring C is reduced. Neither naringenin nor hesperitin demonstrated a pronounced effect on the transcriptional activation of AP-1.

2.4.2 Effects of flavonoids on cell viability

To investigate whether the slight inhibition of AP-1 luciferase activity modulated by the flavonoids at higher concentrations is due to cytotoxicity, we measured the cell-viability of PC3-AP1 cells using the MTS assay. The results are as depicted in Figure 2.3. The data is expressed as percent cell viability as compared to the control cells which were treated with 0.1% DMSO. The concentrations of the flavonoids ranged from 10 μM to 500 μM. Treatment with 500 μM chrysin resulted in ~70% cell viability as opposed to kaempferol and apigenin that resulted in ~78% and ~82% cell

viability. All the other flavonoids even at the highest concentrations produced negligible cell death. Interestingly, at 100 μM concentration, the highest concentration used for luciferase assay, chrysin and kaempferol demonstrated ~80% cell viability while all the other flavonoids exhibited ~90% or >90% cell viability.

2.4.3 Activation of MAPK

To investigate whether the increased AP-1 activity by these flavonoids can be correlated to changes in the expression of MAPKs, we examined the phosphorylation of JNK, ERK, p38 and MEK using western blot analyses. We tested and compared the effects of four of the eight flavonoids namely kaempferol, apigenin, genistein and naringenin. Apigenin was chosen over chrysin, although chrysin clearly demonstrated better AP-1 transcriptional activation, to purpotedly include atleast one flavonoid that did not follow the general pattern of AP-1 activation among its class. In the control cells, although only small amounts of both p-ERK and p-JNK were detected, all flavonoid treatments induced both p-ERK and p-JNK. p-ERK was induced by kaempferol, apigenin and genistein. Kaempferol induced p-ERK at 20 μM which corresponds well with the luciferase activity results. Naringenin did not cause any appreciable change in the levels of p-ERK.On the other hand, treatment with kaempferol and genistein resulted in maximum JNK activation though higher concentrations of both appeared to suppress p-JNK. On the contrary, JNK activation by apigenin and naringenin was not very pronounced and lacked any dose-dependency. None of the flavonoids induced phosphorylation of p38, another member of the MAPK family (results not shown). In order to confirm the involvement of ERK pathway in the AP-1 luciferase activity induced by flavonoids, we examined the

phosphorylation of MEK. As expected treatment with kaempferol, apigenin and genistein resulted in activated MEK. However, 50 μM apigenin induced p-MEK as well as lack of MEK activation by higher concentrations of genistein did not corroborate well with p-ERK activation demonstrated by these flavonoids. Again, naringenin treatment did not affect p-MEK levels. From this data, it may be inferred that the MEK-MAPK pathway may play a role in the transcriptional activation of AP-1 by kaempferol, apigenin and genistein.

2.4.4 Effects of specific JNK and MEK inhibitors on the AP-1 luciferase activity induced by flavonoids

In order to corroborate the results from the Western blot analyses and the luciferase activity and to discern whether or not the MAPK pathway plays an important role in the transcriptional activation of AP-1 by the flavonoids, we treated the cells with specific JNK and MEK inhibitors and observed their effects on the AP-1 luciferase activity. The cells were pretreated with 10 μM SP600125 (specific JNK inhibitor) or 10 μM U0126 (specific MEK inhibitor) and then incubated with the respective flavonoids for a period of 24 hours. The results are as depicted in Figure 2.5. Figure 2.5A demonstrates the effects of these inhibitors on kaempferol induced AP-1 transcriptional activity. Treatment with the MEK inhibitor markedly abolished kaempferol induced luciferase activity while the JNK inhibitor did not show any pronounced effect. Interestingly, neither the JNK inhibitor nor the MEK inhibitor reduced apigenin induced AP-1 transcriptional activity (Figure 2.6B). Treatment with the JNK inhibitor, on the other hand, effectively abolished AP-1 activity induced by gensitein even at 10 μM concentration (Figure 2.6C). The MEK inhibitor did not

show much effect on genistein induced AP-1 activity. Hence though apigenin and genistein did slightly induce MEK-ERK, this pathway may not result in the activation of AP-1. On the contrary, the JNK-MAPK pathway seems to be predominant in activating AP-1 luciferase activity induced by genistein. Also, it may be deduced that the JNK and ERK-MAPK pathways may not play a pivotal role in the transcriptional activation of AP-1 by apigenin and naringenin.

2.4.5 Effect of flavonoids on gene expression profiles of ATF-2, Elk-1 and cyclin D1:

Furthermore, in order to discern whether the above demonstrated translational data corroborates well with possible changes in the transcriptional machinery, we performed q-RT PCR. We observed the effects of flavonoids on the gene expression profiles of the transcription factors - ATF-2, Elk-1 and cyclin-D1 that are downstream to the MAPK pathway and known to be controlled by the AP-1 promoter. Temporal gene expression profile of these genes elicited by only those concentrations of flavonoids that demonstrated maximal AP-1 luciferase activity was observed. Results are as depicted in table 2.2. No appreciable change was observed on the positive cell cycle regulator cyclin-D1. Furthermore, we observed 1.8 fold induction in the mRNA levels of Elk-1 by 20 μM Kaempferol while 20 μM Apigenin and Genistein induced it by 1.2 and 1.35 fold, respectively. On the other hand, we observed a 1.65 fold induction of ATF-2 by 20 μM Genistein while 20 μM Kaempferol and Apigenin induced it by 1.35 fold. Naringenin treatment did not cause any appreciable change in the mRNA levels of ATF-2 and Elk-1. In general, the transcriptional changes

observed after five hour treatment with flavonoids were more pronounced than those observed after a ten hour treatment.

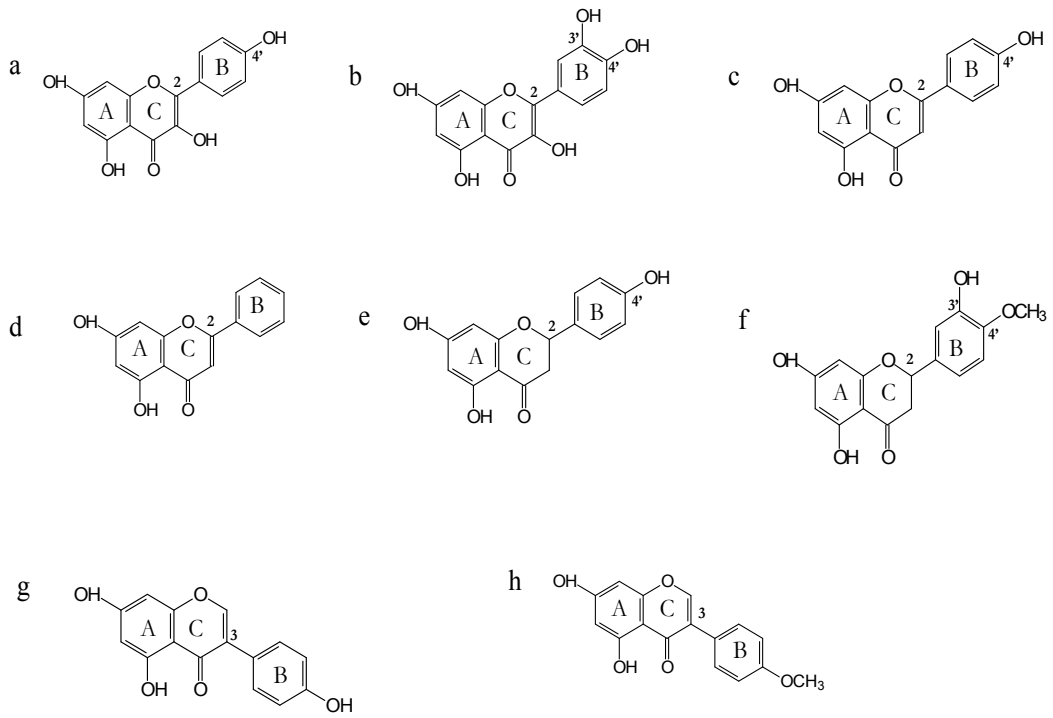

Figure 2.1: Structures of the different flavonoids. a) Kaempferol, b) Quercetin, c) Chrysin, d) Apigenin, e) Naringenin, f) Hesperitin, g) Genistein, h) Biochanin

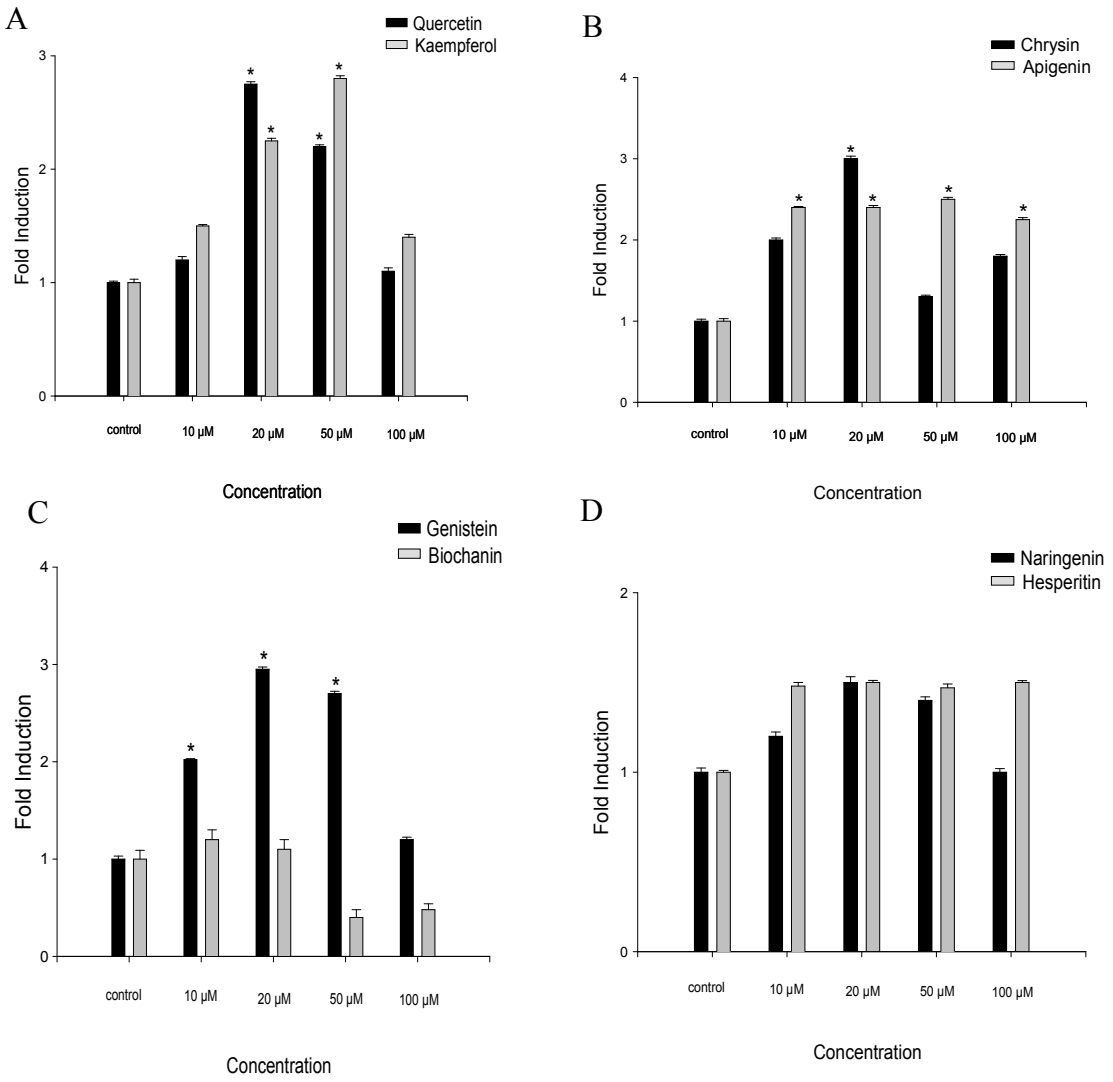

Figure 2.2: Transactivation of AP-1 luciferase reporter by various flavonoids. PC3-AP1 were seeded in six well plates and treated with the flavonoids. The AP-1 luciferase activity was measured relative to vehicle control (DMSO) after 24 hours of incubation and normalized against protein concentration. Values represent mean ± S.E. Significance of difference was analyzed by ANOVA followed by Tukey's posthoc analysis. * P value <0.05 was considered significant.

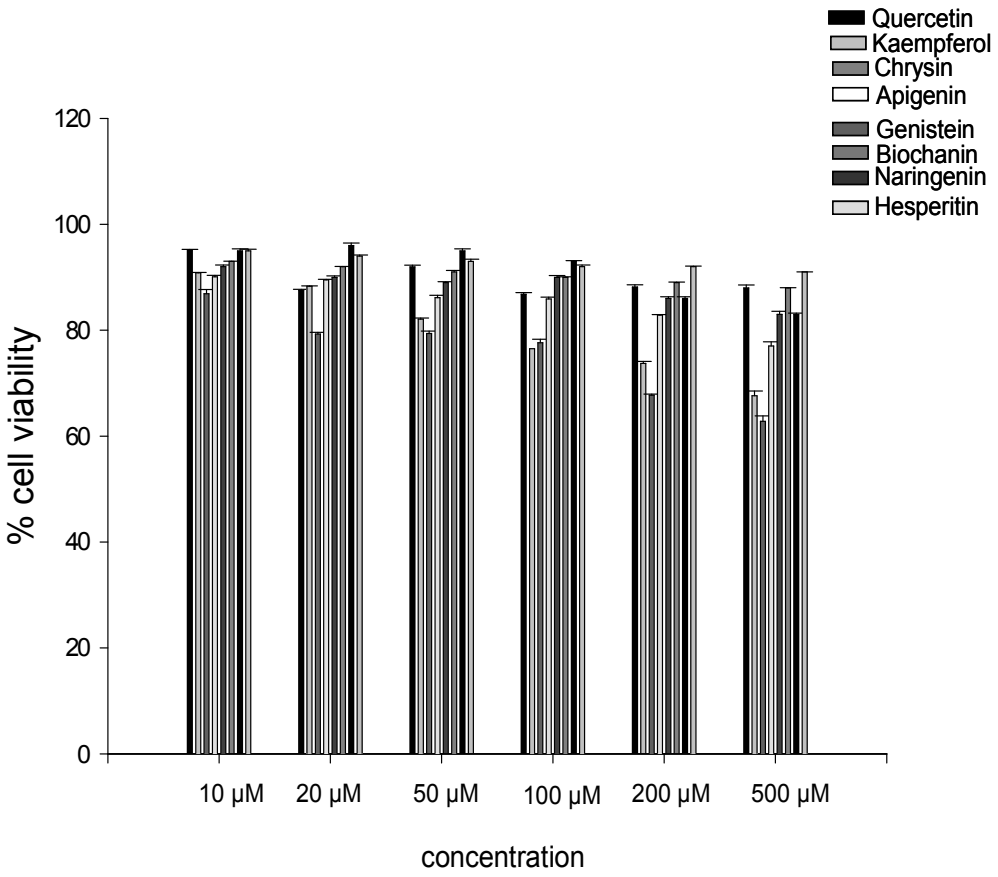

Figure 2.3: Viablity of PC3-AP1 cells with different concentrations of flavonoids. PC3-AP1 cells were treated with the flavonoids for twenty four hours followed by treatment with the MTS assay reagent to test cell viability. Values represent mean ± S.E.

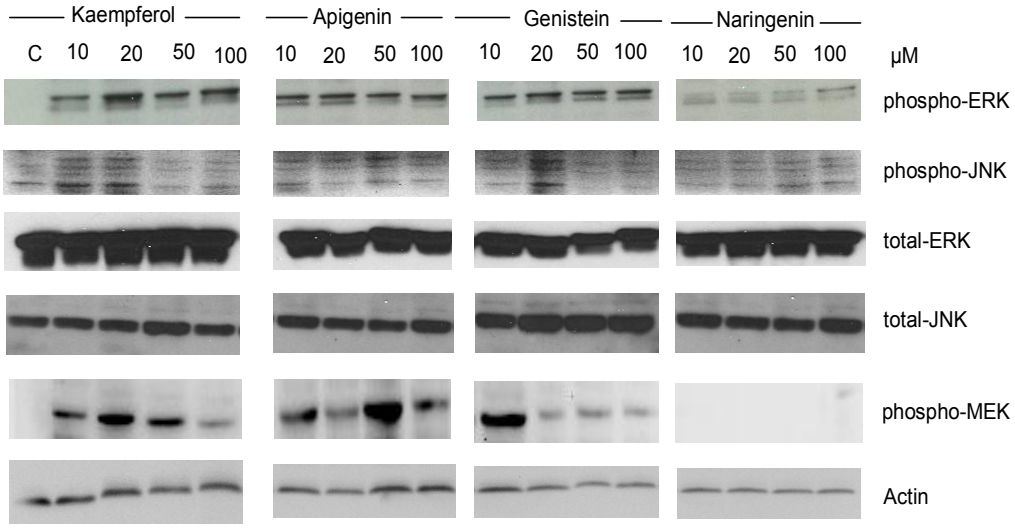

Figure 2.4: Expression of p-ERK, p-JNK, total ERK, total JNK and p-MEK following treatment with flavonoids. PC3- AP1 cells were treated with different concentrations of flavonoids for two hours. Cells were harvested and proteins were immunoblotted using specific antibodies using actin as a loading control.

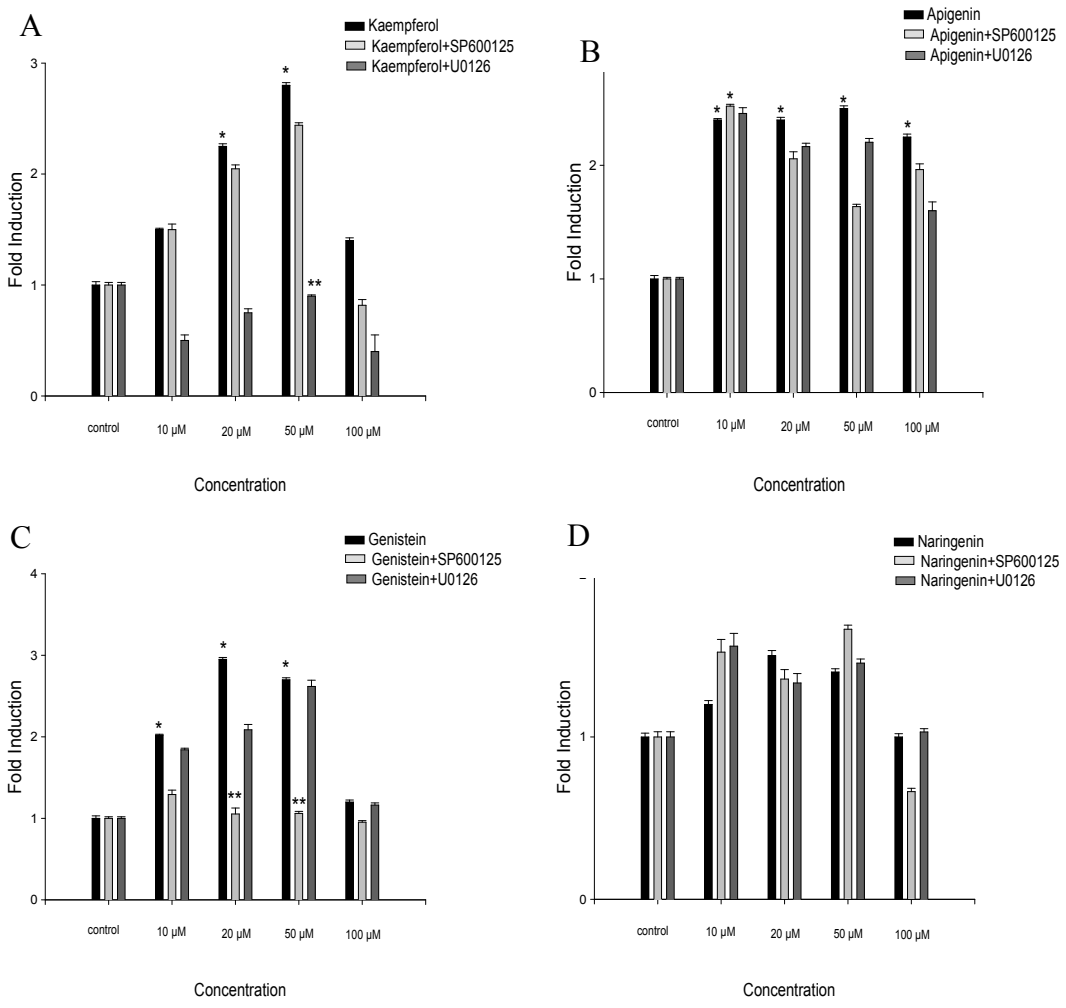

Figure 2.5: Effect of pharmacological inhibitors on the transactivation of AP-1 luciferase reporter by various flavonoids. PC3-AP1 were seeded in six well plates and pretreated with indicated inhibitor for 30 minutes followed by treatment with the flavonoids. The AP-1 luciferase activity was measured relative to vehicle control (DMSO) after 24 hours of incubation and normalized against protein concentration. Values represent mean ± S.E. Significance of difference was analyzed byANOVA followed by Tukey's posthoc analysis. * P value <0.05 was considered significant.

Table 2.1: Primers of genes used for quantitative real-time PCR.

Gene Name	Genebank Accession number	Forward primer	Reverse primer
Homo sapiens Elk-1	NM_002259	5'-TGGACCCATCTGTGACGCT-3'	5'-GAATTCACCACCATCCCGTG-3'
Homo sapiens ATF-2	NM_001880	5'-GGTCATGGTAGCGGATTGGT-3'	5'-GCTGGAGAAGCCGGAGTTTC-3'
Homo sapiens cyclin-D1	NM_053056	5'-CCCGCACGATTTCATTGAAC-3'	5'-CACAGAGGGCAACGAAGGTC-3'
Homo sapiens beta-actin	M10277	5'-CCCAGCCATGTACGTTGCTA-3'	5'-CAGTGTGGGTGACCCCGT-3'

Table 2.2: Temporal gene expression profiles elicited by flavonoids – kaempferol, apigenin, gensitein and naringenin.

	Elk-1		**ATF-2**		**Cyclin D1**	
	5 hours	10 hours	5 hours	10 hours	5 hours	10 hours
Kaempferol	1.81± 0.17*	1.25 ± 0.1	1.35 ± 0.23	0.86 ± 0.11	0.65 ± 0.26	0.87 ± 0. 18
Apigenin	1.2 ± 0.23	0.9 ± 0.12	0.9 ± 0.16	0.91 ± 0.32	0.89 ± 0.12	0.97 ± 0.14
Genistein	1.35 ± 0.3	1.1 ± 0.16	1.65 ± 0.35	1.1 ± 0.15	0.57 ± 0.31	0.36 ± 0.11
Naringenin	0.65 ± 0.11	0.81 ± 0.21	0.87 ± 0.18	0.98 ± 0.28	0.78 ± 0.19	0.45 ± 0.19

2.5 Discussion

Flavonoids are a class of natural compounds that occur ubiquitously in food, plants and vegetables. Chemically, they have a phenylchromanone structure (C_6-C_3-C_6) and usually have at least one hydroxyl group substituent or a hydroxyl group derivative such as a methoxy group. They have been found to possess various clinically relevant properties such as anti-tumor, anti-platelet, anti-ischemic, and anti-inflammatory activities and these effects are believed to be an outcome of their antioxidant properties [93]. Compelling data from *in vitro* and *in vivo* laboratory studies and epidemiological investigations indicate that flavonoids exert important effects on cancer chemoprevention and therapy.

Silibinin, a component of silymarin, is a classic flavonoid exhibiting potential anticancer efficacy against prostate cancer and various other cancers too [94]. It is also sold as a dietary supplement in the United States and Europe. Both silymarin and silibinin are exceptionally well tolerated and are reported to be non-toxic in acute, chronic, sub-chronic tests in various animals with no known LD_{50}. It has been shown that silibinin/silymarin feeding to mice upto 2 gm/kg did not show any signs of toxicity and also that it is physiologically available in different organs of the mice [95]. Likewise, several epidemiological studies suggest that consumption of green tea lowers the risk of cancer. Soy isoflavones including genistein, daidezin and glycitein, mainly derived from soybean have been found to inhibit cancer growth *in vivo* and *in vitro*[89]. Geller and co workers reported that genistein inhibits the growth of malignant and benign prostatic hyperplasia [96].

It is increasingly becoming clear that these dietary agents exert their pleiotropic effects on cancer cells, affecting cell survival and physiological behavior. However, the precise molecular mechanisms of action of these compounds have not been elucidated, although the data from published literature indicate that these compounds regulate cellular signal transduction pathways such as NF-κB, MAPK, Akt etc. In this study, we demonstrate the effects of various flavonoids on the transcriptional activation of AP-1 and their modulation of the MAPK pathway.

Ishikawa and Kitamura reported that, in mesangial cells, quercetin abolished H_2O_2 induced AP-1 activity [97]. Herein, we report that, at lower concentrations, quercetin dose-dependently increased AP-1 activity while at higher concentrations slight inhibition was observed. Similarly, kaempferol also induced AP-1 activity at lower concentrations and exhibited some inhibition at higher concentrations. Apigenin increased the transcriptional activity of AP-1 ~ 2.5 fold over control at 20 μM concentration while chrysin increased the transcriptional activity of AP-1 ~3 fold over control at the same concentration. Similarly, both apigenin and chrysin showed some inhibition of AP-1 activity at higher concentrations. Frigo and coworkers demonstrated that in endometrial Ishikawa and HEK 293 cells, apigenin markedly increased AP-1 activity and it was further enhanced by cotreatment with phorbol-12-myristate-13-acetate (PMA), thus confirming our results [98]. The isoflavones - genistein and biochanin and flavonones - naringenin and hesperitin induced AP-1 activity at lower doses while at higher concentrations all three flavonoids except hesperitin slightly inhibited AP-1 activity. Taken together, most of the flavonoids tested in this report increased AP-1 activity at low doses. This observation is similar

to a previous study performed in our laboratory where we demonstrated that in HT-29 colon cancer cells stably transfected with AP-1, EGCG a flavan-3-ol induced AP-1 luciferase activity about 12 fold over control at 100 μM concentration. Co-treating HT-29 cells with EGCG (100 μM) and TPA produced 14 fold increase in AP-1 activity. Likewise sulforafane an isothiocyanate compound also increased AP-1 activity ~3 fold over control and cotreating it with TPA increased AP-1 activity ~10 fold over control [99].

AP-1 activation is often considered as an indicator of external stimuli and several signal transduction pathways converge at the level of this transcription factor. It is, therefore, a potential target for multiple signaling cascades [100]. Thus, it has been shown to be an excellent marker to demonstrate whether various chemopreventive compounds can modulate signal transduction pathways. The ability of the lower doses of flavonoids to activate AP-1 transcriptional activity and higher doses to inhibit AP-1 transcriptional activity without causing cell death as evident from the cell viability assays, suggests the presence of multiple signaling mechanisms.

Expression of AP-1 mediated genes is regulated in two ways: a) phosphorylation and activation of individual AP-1 components and b) expression of AP-1 components jun and fos. Both jun and fos are heavily regulated by MAPK pathways. MAPKs are important in controlling the cellular events such as proliferation, differentiation, and apoptosis. ERK activation usually leads to elevated AP-1 activity via c-fos induction. This results in increased synthesis of c-fos, which upon translocation to the nucleus dimerizes with the pre-existing Jun proteins to form AP-1 dimers. The JNKs were characterized as stress-activated protein kinases on the basis of their activation in

response to stress activators as well as inhibition of protein synthesis. JNK is known to bind and phosphorylate the DNA binding protein c-jun and increase its transcriptional activity [84, 101]. In human A549 lung cancer cells, kaempferol induced growth inhibition and apoptosis and this was mediated by the activation by MEK-MAPK pathway [102]. Paweletz *et al*. reported by protein microarray analysis that prostate cancer progression was associated with increased Akt and decreased ERK phosphorylation. Further solidifying this relationship, it has been shown in poorly differentiated prostate cancer the serine/threonine kinase Akt is highly phosphorylated as compared to ERK [103] . Boldt and coworkers demonstrated that taxol, an agent that targets microtubules, etoposide, an agent that targets topoisomerase II, and ceramide, a synthetic lipid that is a second messenger to TNF, all induce MAPK signaling cascades that results in an apoptotic response in different cancer cells [104]. A more recent report by Mallikarjuna and coworkers established that silibinin treatments in UVB induced tumorigenesis resulted in strong phosphorylation of ERK1/2, JNK and p38. This suggests the possible involvement of the induced MAPKs in apoptotic effects of silibinin [105]. Consistent with these reports, the activation of the MAPK cascade is suggested as a possible mechanism by which these flavonoid compounds exert their chemopreventive action. ATF-2 can form a heterodimer with c-*Jun* and control the induction of c-*Jun* in an AP-1 dependent manner, however, both ATF-2 and c-*Jun* can be activated by JNK. Genistein induced p-JNK correlated well with the ATF-2 activation observed. Biochemical studies have indicated that Elk-1 is a good substrate for ERK1/2 in vitro, and the kinetics of its modification correlated with MAPK activation in vivo. Thus

the transcriptional activation of Elk-1 translated well with the increased phosphorylation of ERK1/2 by kaempferol.

In summary, the PC3 cells stably transfected with AP-1 luciferase reporter gene can be used as a potential tool to screen a variety of chemopreventive agents. It is known that most of these agents modulate intracellular signal transduction pathways potentially leading to chemoprevention. However, our study clearly demonstrates that the activation of these pathways could be dependent on several parameters such as the chemical structures of the compounds, concentration and the incubation period. Also, since almost every pathway has cross-talk with other signal transduction pathways, the activation or inhibition of one pathway may not completely account for the chemopreventive action of these natural compounds. In the current study, we have demonstrated that while kaempferol and genistein clearly activate the MEK-ERK and JNK MAPK cascades, apigenin and naringenin did not activate either pathway. Whether or not other signal transduction pathways are involved and if they are involved, which pathway is more significant towards eliciting the chemopreventive action of these compounds needs further studying. Thus, from our current study we can infer that each flavonoid compound exhibited a different level of potency in modulating intracellular signaling cascades. The possibility that combination of these compounds may be more effective in slowing prostate cancer progression than single agents cannot be ruled out. More studies in this direction are currently ongoing in our laboratory.

Acknowledgements

We thank Dr. Michael Karin (University of California, San Diego) and Dr. Anning Lin (University of Chicago) for providing the AP-1 reporter gene construct.

CHAPTER 3: PHARMACOGENOMIC PROFILE OF SOY ISOFLAVONE CONCENTRATE IN THE PROSTATE OF Nrf2 DEFICIENT AND WILD TYPE MICE [3].

3.1 Abstract

The putative involvement of Nrf2- a bZip transcription factor in soy isoflavones induced protection against oxidative stress and cancer has been reported. To gain a deeper insight into the role of Nrf2 in prostate cancer chemoprevention by soy isoflavones, we examined the pharmacogenomics and gene expression profiles elicited by soy isoflavones in the prostates of C57BL/6J/Nrf2 (-/-) and C57BL6J (+/+) wildtype. The profiles were analyzed using 45,000 Affymetrix mouse genome 430 2.0 array and Genespring 7.2 software. The results obtained from microarray were further validated by real-time reverse transcription-PCR. Clusters of genes that were induced or suppressed more than 2 fold were identified as Nrf2 regulated soy isoflavone induced or suppressed genes. Classification based on their biological function revealed that genes mainly belonging to the categories of electron transport, phase II metabolizing enzymes, cell growth and differentiation, apoptosis, cell cycle, transcription factors, transport, mRNA processing, carbohydrate homeostasis etc were either induced or suppressed by soy isoflavone and regulated by Nrf2. In addition, modulation of novel target genes such as LATS2 and GREB1 were identified to be mediated by Nrf2.

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3 This chapter been published in *Journal of Pharmaceutical Sciences* as Pharmacogenomic profile of soy isoflavones in the prostates.

Thus by way of such research, we provide a link between cancer chemopreventive properties of soy derived phytochemicals and the transcription factor Nrf2.

3.2 Introduction

Nuclear factor 2 related factor 2 (Nrf2) is a basic leucine zipper, redox-sensitive transcription factor that has been widely studied to gain an insight into its role in chemoprevention. It is known to be involved in the regulation of antioxidant response element mediated gene transcription. Under normal conditions, Nrf2 remains sequestered in the cytoplasm by Kelch-like ECH associated protein (Keap1). However under conditions of oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, and subsequently dimerizes with small Maf proteins and other coactivators ultimately leading to the transcription of genes encoding several antioxidant enzymes such as heme oxygenase -1, glutathione-S-tranferase, glutamylcysteine synthetase as well as and detoxifying enzymes such as phase II metabolizing enzymes. Several potentially chemopreventive, dietary phytochemicals activate this Nrf2/ARE axis leading us to believe that by activating the Nrf2/ARE pathway, these agents ultimately induce antioxidant and detoxifying enzymes and this is an important weapon against cancer [106-108]

Prostate cancer is one of the leading causes of cancer related deaths among men in the United States . Since the refractory form of this disease does not respond to hormone ablation, chemoprevention appears to be the best choice in order to ameliorate this disease . Epidemiological studies have continually documented the healthful effects of soy products and have established an inverse relationship between the consumption

of soy intake and the low incidence of prostate cancer in Asian versus Caucasian populations . Soy foods such as soybeans, soy milk products and soy flour have the highest recorded levels of isoflavones and represent one of the largest sources of dietary isoflavones. Other sources of dietary isoflavones include legumes, lentils and chickpeas. Di and trihydroxy isoflavones –daidzein and genistein are the two primary constituents of soy products and have been the focus of several anti prostatic cancer research programs [109, 110]. These have been shown to inhibit prostate cancer both *in vitro* and *in vivo*. They have been shown to reverse DNA hypermethylation in PC3 and LNCaP cells and also cause apoptosis in these cells by modulating the cell-cycle genes [111] . In vivo these agents have been shown to be effective against prostatic lesions developed in a transgenic mouse model of prostate cancer (TRAMP model) . Immunodeficient nude mice transplanted with PC3 xenografts and subject to soy isoflavones in the diet demonstrated significantly reduced tumor cell proliferation and increased apoptosis [112]. Additional pieces of evidences to further solidify this inverse relationship between soy isoflavones and prostate cancer are the results from clinical trials that have established that 100mg/kg of soy isoflavone (Novasoy) reduced prostate specific antigen (PSA) levels in human volunteers diagnosed with prostate cancer and that the isoflavones that were found to be highly effective in stalling prostate cancer exhibited minimal genotoxicity [113] .

There are several reports indicating that isoflavones can induce the expression of antioxidant and detoxifying genes such as heme-oxygenase 1, glutathione reducatase and γ glutamyl cysteine synthetase in various cell lines . Genistein and daidizein have been shown to offer protection against oxidative stress induced endothelial injury by

evoking increased expression of γ glutamyl cysteine synthetase and nuclear expression of Nrf2 [114] . Hence to gain a deeper insight into identifying the various other genes, in addition to phase II detoxifying and antioxidant genes, that may be modulated by soy isoflavones and involve signaling through transcription factor Nrf2, this research study is aimed at looking at the global gene expression profile of Nrf2(-/-) knockout mice treated with a commercially available over the counter isoflavone mixture – Novasoy. Novasoy was a kind gift to us by Archer Daniel Midland Company, Decatur, IL.

3.3 Materials and Methods

Animal care and treatments

Nrf2(–/–) mice (C57BL/SV129) have been described previously. Nrf2(–/–) mice were backcrossed with C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME). To confirm the genotype from each animal, DNA was extracted from the tail and analyzed by PCR using the following primers: 3'-primer, 5'-GGAATGGAAAATAGCTCCTGCC-3'; 5'-primer, 5'-GCCTGAGAGCTGTAGGCCC-3'; lacZ primer, 5'-GGGTTTTCCCAGTCACGAC-3'. Nrf2 (–/–) and Nrf2 (+/+) mice exhibited one band at 200 and 300 bp, respectively. The second generation (F2) of male Nrf2 knockout mice were used in this study. Age-matched male C57BL/6J mice were purchased from The Jackson Laboratory. Nine- to 12-week-old mice were used and housed at Rutgers Animal Facility and maintained under 12-hour light/dark cycles. All animals were allowed water and food *ad libitum*. After 1 week of acclimatization, mice were put on AIN-76 A diet. Prior to treatment they were starved for ~ 8 hours and then divided into the following treatment groups

Group I: Nrf2 (-/-) mice orally dosed once with 20 mg/kg of Novasoy

Group II: Nrf2(-/-) mice orally dosed once with vehicle (50:50 PEG, saline).

Group III: wild type mice orally dosed once with 20 mg/kg of Novasoy

Group IV: wild type mice orally dosed once with vehicle ((50:50 PEG, saline).

Each treatment group consisted of four animals. Mice were given AIN-76A diet 3 hours after dosing. Twelve hours after dosing, the animals were sacrificed. A modest vegetarian diet yields ~ 74.5 mg/kg of isoflavones [115]. Inorder to obtain this concentration of isoflavones, a 20 mg/kg dose of Novasoy was selected. Table 3.1 indicates the precise quantities of the different isoflavones in Novasoy. All animal use procedures were in accordance with the NIH Guide for the Care and Use for Laboratory Animals, and were approved by the Rutgers Institutional Animal Care and Use Committee.

RNA Extraction, Microarray Hybridization, and Data Analysis

A scheme of the experimental design is depicted in Figure 3.1. Total RNA from the prostate tissues were isolated by using a method of Trizol (Invitrogen, Carlsbad, CA) extraction coupled with the RNeasy Midi kit from Qiagen (Valencia, CA) according to the manufacturer's protocol. After RNA isolation, all the subsequent technical procedures, including quality control, concentration measurement of RNA, cDNA synthesis, and biotin labeling of cRNA, hybridization, and scanning of the arrays, were done at CINJ Core Expression Array Facility of Robert Wood Johnson Medical School (New Brunswick, NJ). Affymetrix mouse genome 430 2.0 array containing > 45,101 probe sets was used to probe the global gene expression profile in mice following soy isoflavone treatment. Each array was hybridized with cRNA derived

from a pooled total RNA sample from four mice per treatment group. A total of four chips were used in this study. After hybridization and washing, the intensity of the fluorescence of the array chips was measured by the Affymetrix GeneChip Scanner. The expression analysis file created from each sample (chip) scanning was imported into GeneSpring 6.1 software (Silicon Genetics, Redwood City, CA) for further data characterization. A new experiment was generated after importing data from the organ in which data were normalized to the 50th percentile of all measurements on that array. Data filtration based on flags present in at least one of the samples was generated. Lists of genes that were either induced or suppressed >2-fold between treated and vehicle group of same genotype were created by filtration-on-fold function within the presented flag list. By using color-by-Venn-diagram function, lists of genes that were regulated >2-fold only in C57BL/6J mice in the prostate tissue were created.

Quantitative Real-time PCR for Microarray Data Validation

To verify the microarray data, several genes (including the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase) from different categories were chosen for quantitative real-time PCR analyses. The primers for these genes are listed in Table 3.2 . Instead of using pooled RNA from each group, RNA samples isolated from individual mice as described above were used in real-time PCR analyses. First-strand cDNA was synthesized using 4 μg total RNA following the protocol of SuperScript III First-Strand cDNA Synthesis System (Invitrogen). Real-time PCR was done as described previously [17]. The gene expression was determined by normalization with control gene glyceraldehyde-3-phosphate dehydrogenase. The correlation between corresponding microarray data and real-time PCR data was

validated by Spearman rank correlation method. The results of such correlation are depicted in Figure 3.3.

3.4 Results

3.4.1 Soy isoflavone altered gene expression pattern in the prostate.

Oligonucleotide microarray was used to analyze the gene expression profiles in the prostate of mice treated with soy isoflavones. Genes that were regulated by soy isoflavones in C57BL/6J mice but not in C57BL/6J Nrf2 (-/-) were considered to be Nrf2 dependent genes. Among these Nrf2-dependent genes the expression levels of 1428 genes were induced > 2 fold while the expression levels of 1338 genes were suppressed < 2 fold at 12 hours.Results are as depicted in figure 3.2.

3.4.2 Soy isoflavone induced Nrf2 dependent genes in the prostate.

Genes that were induced only in the wild type mice but not in the Nrf2 (-/-) mice were considered to be soy isoflavones induced Nrf2-dependent genes. Based on their biological functions these genes were classified into categories such as cell adhesion, apoptosis and autophagy, cell cycle, cell differentiation, DNA associated proteins (transcription factors, replication and repair enzymes), mRNA processing and splicing, transport, electron transport, lipid metabolism, signaling molecules, carbohydrate homeostasis, inflammatory response, ubiquitination, phase II metabolizing enzymes, G-protein coupled receptors, cytoskeleton, extracellular matrix and smooth muscle associated proteins, kinases and phosphatases and protein folding and proteolysis.

Cyp2b10, Cyp2c and Cyp3a13 genes that are involved in xenobiotic metabolism were found to upregulated by 2.441, 8.018 and 2.336 fold, respectively. Since the major

constituent of Novasoy is isoflavones, it is not surprising that a robust induction of cytochrome P450 family 2c and 3a was observed. Although the involvement of Nrf2 in the regulation of Cyp2b10 is known, the ability of soy isoflavones to induce this family of P450 has not been shown earlier and thus proves to be novel. As expected from earlier studies, we observed quite a robust induction of phase II metabolizing enzymes belonging mainly to the UDP-glucuronyltransferase, glutathione-s-transferase, catalytic subunits of glutamate-cysteine ligase and sulfotransferases. Upon human consumption, majority of isoflavones are either glucuronidated or sulfated and since we observed the induction of both the enzymes involved in such transformation, these results are in agreement with other numerous studies suggesting the same. Hence these results clearly demonstrate that xenobiotic detoxifying enzymes are indeed regulated by the transcription factor Nrf2 and Novasoy – a mixture of isoflavones is a potent inducer of these detoxifying enzymes.

Genes regulating cell cycle and cell growth were also identified as Nrf2-dependent and soy isoflavone inducible genes. These mainly include epiregulin (23.25 fold), cyclin dependent kinase inhibitor 1A (4.224 fold), transforming growth factor β (2.144 fold), signal transducer and activator of transcription (2.094 fold), insulin like growth factor (2.316 fold) and hepatocyte growth factor (7.131 fold), to name a few. Genes involved in transport was the other major category that was found to be upregulated. These include gamma aminobutyric acid receptor (25.49 fold), fatty acid binding protein, liver (5.981 fold), aquaporin (2.025 fold), cytoglobin (2.417 fold), oxysterol binding protein-like 9 (3.187 fold), ATP-binding cassette family (2.354

fold) and many more. A huge battery of genes belonging to the solute carrier family was also upregulated.

Several kinases and phosphatases were also found to be upregulated. To name a few – MAPKKK5 or Ask-1 (2.358 fold), MAPKKK4 or MEK4 (2.253 fold), Protein tyrosine phosphatase, diacylglycerol kinase (2.229 fold). A variety of transcription factors were found to be induced by Novasoy too. These include nuclear factor erythroid derived 2 like 2 (2.479 fold), transcription factor 23 (10.29 fold), glucocorticoid receptor DNA binding factor (2.013 fold), CREB regulated transcription coactivator 3 (3.035 fold) etc.

Besides these categories a number of genes belonging to cell adhesion such as protocadherin 1 (2.821 fold), chondroitin sulfate proteoglycan (3.837 fold), cadherin 20 (9.589 fold), apoptosis such as Bcl2 associated anthanogene (2.31 fold), angiopoietin like 4 (15.03 fold), mRNA processing and splicing such as cleavage stimulation factor (22.96 fold), ubiquitination genes such as proteosome subunit (6.365 fold), ubiquitin specific peptidase 27, X chromosome (4.016 fold) and many more were found to be Nrf2-dependent and soy isoflavone induced genes.

3.4.3 Soy isoflavone suppressed Nrf2 dependent genes in the prostate.

Table 3.4 lists the genes that were found to be inhibited by soy isoflavones in an Nrf2 dependent fashion. Based on their biological functions these genes were classified into the following categories: carbohydrate homeostasis and glucose metabolism, DNA associated proteins including transcription factors, DNA repair enzymes and DNA replication enzymes, proteolysis, phase II detoxifying enzymes, apoptosis, transport, cell cycle, signaling proteins, ubiquitination, cell adhesion, cell growth and

differentiation, mRNA processing, electron transport, inflammatory and immune response proteins, cytoskeleton associated proteins, G-protein coupled receptors, kinases and phosphatases, lipid metabolism, nucleotide biosynthesis and metabolism and spermatogenesis.

A number of genes involved in electron transport such as cyp4f, cyp2j and cyp24a were found to be downregulated. These results are in conformance with previous studies that demonstrate similar effects post treatment with isoflavones. Interestingly the phase II detoxifying genes glutathione- s- transferase mu 7 and sulfotransferase 1E were found to be inhibited by soy isoflavones.

In the DNA associated transcription factor category E2F8 (0.205 fold), E2F2 (0.132 fold), activating transcription factor Atf6 (0.489 fold) and many more were found to be downregulated. The DNA repair enzyme - DNA cross link repair enzyme 1B, PSO2 homolog was found to be significantly inhibited (0.485 fold) by soy isoflavone in an Nrf2 dependent manner.

A variety of genes involved in transport were also found to be inhibited by soy isoflavones. These include major urinary protein 5 (0.348 fold), orosomucoid 2 (0.28 fold), orosomucoid 3 (0.29 fold) and a number of solute carrier family proteins. A number of cell cycle and cell differentiation genes such as cyclin B3 (0.141 fold), G1 to S phase transition 2 (0.399 fold) MAD2-like 1 (0.22 fold) and Microtubule associated protein 1b (0.433 fold) were found to be downregulated. Several kinases and phosphatases including MAPKKK3 and phosphoserine phosphatase were found to be downregulated (0.355, 0.381 fold, respectively).

Table 3.1: Percentage of Isoflavone aglycone quantities in Novasoy.

	Isoflavone (aglycone)	
Genistein	Genistein glucopyranoside	13.671 %
	Mal-genistein	0.003050 %
	Ac-genistein	0.466 %
	Genistein	0.2307 %
Daidzein	Daidzein glucopyranoside	11.044 %
	Mal-daidzein	0.001 %
	Ac-daidzein	0.394 %
	Daidzein	0.3364 %
Glycetin	Glycetin glucopyranoside	2.193 %
	Mal-glycetin	0.01 %
	Ac-glycetin	0.07 %
	Glycetin	0.1958 %
Total isoflavone aglycone content		28.647 %

Note: Novasoy contains ~30% type A and type B saponins. The remaining mass is mainly made up of proteins, ash and moisture.

Table 3.2: Oligonucleotide primers used for quantitative real-time PCR.

Gene name	Accession number	Forward primer	Reverse primer
CREB regulated transcription coactivator 3 (Crtc3)	NM_173863	5'-CCTCTGGTCTCCAAAGTTCTCG-3'	5'-TGAGTGTGGCTTGGATGGAAG-3'
Cytochrome P450, family 2, subfamily c, protein 37 (Cyp2c37)	NM_010001	5'-CTGCATGACAGCACGGAGTT-3'	5'-GTGGCCAGGGTCAAACACTT-3'
Cyclin dependent kinase inhibitor 1A (p21)	NM_007669	5'-AATCCTGGTGATGTCCGACC-3'	5'-ACAACGGCACACTTTGCTCC-3'
Glutathione-s-transferase, mu 3 (GSTmu3)	NM_010359	5'-ATGGACACCCGCATACAGCT-3'	5'-CTCAAAATCGGGACTGCAGC-3'
Large tumor suppressor 2 (LATS2)	NM_015771	5'-AAAAGCTCTCAGGGAAATCCG-3'	5'-GACTCGTTGGCAAAAGGCAG-3'
Rho-related BTB domain containing 3 (Rhobtb3)	NM_028493	5'-CCTCCTCAGCTTGAACAGCC-3'	5'-TGCCTCAGCTTTCAAGACAGG-3'
Gene regulated in breast cancer 1 (GREB1)	NM_015764	5'-CGGGCTCTTTAATTTGTACCATG-3'	5'-ACGTGTAGGTGGTTGGCTCC -3'
Fork-head binding protein 4 (Fkbp4)	NM_010219	5'-AGGTCCATGCACTCCGACTG-3'	5'-GGTGACACATGGCCAGATTG-3'
G1 to S phase transition 2 (Gstp2)	NM_008179	5'-GTGGTCTTCATTGGGCATGTG-3'	5'-TTGTCCTCCAATGGTTGACTTG-3'
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_001001303	5'-CAGGAGCGAGACCCCACTAA-3'	5'-ATACTCAGCACCGGCCTCAC-3'

Table 3.3: Novasoy-induced Nrf2-dependent genes in mouse prostate.

GenBank accession number	Gene symbol	Gene title	Fold induction [a]
		Cell adhesion	
NM_029357	Pcdh1	Protocadherin1	2.8
NM_011580	Thbs1	Thrombospondin 1	4.8
NM_008608	Mmp14	Matrix metallopeptidase 14	3.6
NM_011985	Mmp23	Matrix metallopeptidase 23	2.2
NM_011800	Cdh20	Cadherin 20	9.6
NM_130448	Pcdh18	Protocadherin18	2.9
NM_023160	Cml1	Camello-like 1	5.9
	Cspg4	Chrondoitin sulfate proteoglycan	3.9
		Apoptosis and Autophagy	
NM_020581	Angptl3	Angiopoietin like 4	15.0
NM_145392	Bag2	Bcl2 associated anthanogene	2.3
NM_028133	Egln3	EGL nine homolog 3	2.2
NM_007609	Casp4	Caspase 4	2.22
NM_152823	Unc5c	Unc-5 homolog C (C.elegans)	17.62
NM_011125	Pltp	Phospholipid transfer protein	2.0
		Cell cycle	
NM_007950	Ereg	Epiregulin	23.2
NM_175155	Sash1	SAM and SH3 containing domain	2.0
NM_011035	Cdkn1a	Cyclin dependent kinase inhibitor 1A (p21)	4.2
NM_009772	Bub1	Budding uninhibited by benzimidazoles 1	2.1
BC04787	Nusap1	Nucleolar and spindle associated protein 1	5.7
NM_198604	Plekhg6	Pleckstrin homology domain containing	5.0
NM_028760	Cep55	Centrosomal protein 55	2.4
NM_153103	Kif1c	Kinesin family member 1C	2.4
NM_009794	Capn2	Calpain 2	2.0
		Cell differentiation	
NM_009365	Tgfb1i1	Transforming growth factor beta 1 induced	2.1
U06922	Stat3	Signal transducer and activator of transcription	2.0
NM_008716	Notch3	Notch gene homolog 3	3.5
NM_007463	Speg	SPEG complex locus	3.0

NM_008048	Igfbp7	Insulin-like growth factor binding protein 7	2.6
NM_010514	Igf2	Insulin-like growth factor 2	2.3
D10213	Hgf	Hepatocyte growth factor	7.1
NM_011352	Sema7a	Sema domain	2.5
DNA associated proteins (replication, transcription and repair enzymes)			
NM_020296	Rbms1	RNA binding motif, single stranded interaction	2.3
NM_011243	Rarb	Retinoic acid receptor beta	2.1
NM_009054	Trim27	Tripartite motif protein 27	2.0
NM_139294	Braf	Braf transforming gene	2.0
NM_145624	Zfp709	Zinc finger protein 709	2.0
NM_011385	Ski	Sloan Kettering viral oncogene homolog	2.8
NM_013468	Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	2.7
NM_007770	Crx	Cone-rod homeobox containing gene	2.6
NM_010296	Gli1	GLI-Kruppel family member GLI1	2.5
NM_172392	Zfp759	Zinc finger protein 759	8.0
NM_026058	Lass4	Longevity assurance homolog 4 (S.crevisiae)	2.9
NM_010902	Nfe2l2	Nuclear factor,erythroid derived 2 like 2	2.4
NM_009238	Sox4	SRY-box containing gene 4	2.4
NM_009010	Rad23a	RAD23 homolog (S.crevisiae)	2.4
NM_010419	Hes5	Hairy and enhancer of split 5 (Drosophila)	2.4
NM_033563	Klf7	Kruppel-like factor 7 (ubiquitous)	2.3
NM_053085	Tcf23	Transcription factor 23	10.2
AF098967	Ilf3	Interleukin enhancer binding factor 3	2.3
NM_008719	Npas2	Neuronal PAS domain protein 2	2.3
NM_178646	Tigd5	Tigger transposable element driven 5	4.4
NM_172739	Grlf1	Glucocorticoid receptor DNA binding factor	2.0
NM_008416	Junb	Jun-B oncogene	2.2
NM_173863	Crtc3	CREB regulated transcription coactivator 3	3.0
	Cbx5	Chromobox homolog 5	2.0
NM_007624	Cbx3	Chromobox homolog 3	9.6
AY158954	Hist2h3c1	Histone2, H3c1	7.5
NM_133702	Nol11	Nucleolar protein 11	2.2
NM_017462	Polg	Polymerase (DNA directed), gamma	2.7
AB208640	Brdt	Bromodomain, testis-specific	2.4

		mRNA processing, splicing	
NM_024199	Cstf1	Cleavage stimulation factor	22.9
NM_026554	Ncbp2	Nuclear cap binding protein subunit 2	2.1
		Transport	
NM_010250	Gabra1	Gamma-aminobutyric acid receptor	25.4
NM_017474	Clca3	Chloride channel calcium activated 3	2.1
NM_017399	Fabp1	Fatty acid binding protein 1, liver	5.9
NM_011125	Pltp	Phospholipid transfer protein	2.0
NM_007472	Aqp1	Aquaporin1	2.0
NM_008428	Kcnj8	Potassium inwardly rectifying channel	2.0
NM_011887	Scn11a	Sodium channel, voltage gated type	2.0
NM_031169	Kcnmb1	Potassium large conductance calcium-active channel.	4.2
NM_030888	C1qtnf3	C1q and tumor necrosis factor protein 3	7.8
NM_172675	Stx16	Syntaxin 16	3.4
NM_008766	Slc22a6	Solute carrier family 22	
NM_009973	Csn1s2b	Casein alpha s2-like B	2.9
BC026927	Osbpl9	Oxysterol binding protein-like 9	3.1
NM_013927	Cgnb3	Cyclic nucleotide gated channel beta 3	3.2
NM_133254	Slc5a2	Solute carrier family 5	2.6
NM_133891	Slc44a1	Solute carrier family 44, member 1	2.6
NM_011390	Slc12a7	Solute carier family 12, member 7	2.6
NM_021411	Rab37	RAB37, member of Ras family	2.5
NM_001003950	Rab3ip	RAB3A interacting protein	2.0
NM_133685	Rab31	RAB31, member RAS oncogene	2.2
NM_030206	Cygb	cytoglobin	2.4
NM_053195	Slc24a3	Solute carrier family 24	2.6
NM_011955	Nubp1	Nucleotide binding protein 1	2.3
BC090629	Abcc5	ATP-binding cassette, sub-family C	2.3
NM_133930	Creld1	Cysteine-rich with EGF-like domains 1	2.1
NM_008489	Lbp	Lipopolysacchride binding protein	2.2
NM_025618	Sri	Sorcin	2.1
		Electron transport	
NM_011723	Xdh	Xanthene dehydrogenase	2.2

BC060973	Cyp2b10	Cytochrome P450, family 2, subfamily b, protein 10	2.4
NM_010001	Cyp2c37	Cytochrome P450, family 2, subfamily c, protein 37	8.0
NM_177282	Mical2	Microtubule associated monoxygenase	2.4
NM_007819	Cyp3a13	Cytochrome P450, family 3, subfamily a, protein 13	2.3
		Lipid metabolism	
NM_147779	Sftpb	Surfactant associated protein B	2.0
NM_008875	Pld1	Phospholipase D1	2.0
NM_013829	Plcb4	Phospholipase C, beta 4	34.6
NM_009695	Apoc2	Apolipoprotein C-II	3.0
NM_009692	Apoa1	Apolipoprotein A I	36.3
		Signaling proteins	
AF067816	Vav3	Vav3 oncogene	2.1
NM_010728	Lox	Lysyl oxidase	2.1
NM_028186	Nkd2	Naked cuticle 2 homolog (Drosophila)	2.0
NM_026835	Ms4a6d	Membrane-spanning 4-domains	5.0
NM_029035	Spsb1	SpIA/ryanodine receptor domain	7.3
NM_010815	Grap2	GRB2-related adaptor protein 2	5.5
NM_011682	Utrn	utrophin	3.3
NM_011505	Stxbp4	Syntaxin binding protein 4	3.4
NM_009528	Wntb	Wingless-related MMTV integration site 7B	2.1
NM_145852	Ropn1l	Ropporin 1-like	2.1
AF153350	Adam28	A disintegrin and metallopeptidase domain	10.0
NM_019549	Plek	Pleckstrin	2.8
NM_019840	Pde4b	Phosphodiesterase 4B, cAMP specific	2.7
NM_008560	Mc2r	Melanocortin 2 receptor	2.5
NM_011019	Osmr	Oncostatin M receptor	2.4
NM_008965	Ptger4	Prostaglandin E receptor 4 (subtype EP4)	2.0
NM_009779	C3ar1	Complement component 3a receptor 1	2.3
NM_008719	Npas2	Neuronal PAS domain protein 2	2.3
NM_019662	Rrad	Ras-related associated with diabetes	7.4
NM_001025612	Snx22	Sorting nexin 22	2.2

		Ubiquitination	
NM_011967	Psma5	Proteosome subunit	6.3
NM_031881	Nedd4l	Neural precursor cell expressed	4.4
NM_019461	Usp27x	Ubiquitin specific peptidase 27, X chromsome	4.0
NM_026346	Fbxo32	F-box only protein 32	2.3
		Phase II metabolizing enzymes	
NM_152811	Ugt2b1	UDP glucuronyltransferase family	2.2
NM_016771	Sult1d1	Sulfotransferase family 1D	4.4
NM_010359	Gstm3	Glutathione-s-transferase, mu 3	2.9
NM_010295	Gclc	Catalytic subunit of glutamate-cystein ligase	2.0
		G-protein coupled receptors	
NM_001081342	Gpr133	G-protein couple receptor 133	2.1
NM_029522	Gpsm2	G-protein signaling modulator 2	2.6
NM_022881	Rgs18	Regulator of G-protein signaling 18	2.5
		Cytoskeleton, extracellular matrix and smooth muscle associated	
NM_009922	Cnn1	Calpolin	2.1
NM_010858	Myl4	Myosin light polypeptide 4	2.1
NM_011890	Sgcb	Sarcoglycan beta	2.0
NM_001039392	Tmsb10	Thymosin beta	5.6
NM_011902	Tekt2	Tektin 2	2.0
NM_139300	Mylk	Myosin light polypeptide kinase	3.2
NM_026131	Pdlim7	PDZ and LIM domain 7	2.9
NM_001081390	Palld	Palladin, cytoskeleton associated protein	2.6
NM_011891	Sgcd	Sarcoglycan delta	2.5
NM_145575	Cald1	Caldesmon 1	2.4
NM_008216	Has2	Hyalouranan synthase 2	2.4
		Kinases and phosphatases	
NM_009647	Ak3l1	Adenylate kinase 3 alpha-like 1	2.1
NM_022563	Ddr2	Discoidin domain receptor family	2.0
NM_011218	Ptprs	Protein tyrosine phosphatase	2.0
NM_010831	Snf1lk	SNF1-like kinase	4.0
NM_145475	Cerk	Ceramide kinase	2.2
NM_138306	Dgkz	Diacylglycerol kinase zeta	2.2
NM_178630	Agbl3	ATP/GTP binding protein-like 3	14.6
NM_010633	Uhmk1	U2AF homology motif kinase 1	2.6

NM_011985	Mmp23	Matrix metallopeptidase 23	2.3
NM_001033257	Phactr2	Phosphatase and actin regulator 2	2.0
NM_152804	Plk2	Polo-like kinase 2	3.1
NM_008580	Map3k5	MAPKKK5 (ASK-1)	2.3
NM_011948	Map3k4	MAPKKK4 (MEK 4)	2.2
NM_028444	Prkcdbp	Protein kinase C, delta binding protein	2.1
		Protein folding and proteolysis	
NM_007798	Ctsb	Cathepsin B	2.0
NM_001038619	Dnm3	Dynamin 3	2.3
NM_026419	Ela3	Elastase 3, pancreatic	6.4
NM_145427	Atpaf2	ATP synthase mitochondrial F1 complex	4.2
NM_172845	Adamts4	A disintegrin-like and metallopeptidase	4.8
NM_008608	Mmp14	Matrix metallopeptidase 14	3.3
NM_007801	Ctsh	Cathepsin H	2.2
NM_028066	F11	Coagulation factor XI	9.0
NM_001024698	Cpa2	Carboxypeptidase A2, pancreatic	8.4
NM_011645	Prss3, 1	Protease, serine, 3/1	2.4
NM_008609	Mmp15	Matrix metallopeptidase 15	2.3
NM_008872	Plat	Plasminogen activator, tissue	2.2
NM_010221	Fkbp10	FK506 binding protein	2.1
NM_013868	Hspb7	Heat shock protein 7	4.4
NM_030704	Hspb8	Heat shock protein 8	2.1
NM_013560	Hspb1	Heat shock protein 1	2.8

[a] Genes that were induced >2-fold by Novasoy only in prostate of Nrf2 wild-type mice but not in that of Nrf2 knockout mice compared with vehicle treatment at 12 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

Table 3.4: Novasoy- suppressed Nrf2-dependent genes in mouse prostate.

GenBank accession number	Gene symbol	Gene title	Fold induction [a]
		DNA associated proteins (transcription factors)	
NM_011547	Tcfap2a	Transcription factor, AP-2 alpha	0.4
NM_007624	Cbx3	Chromobox homolog 3	0.4
NM_023258	Pycard	PYD and CARD domain containing	0.2
NM_009566	Zfp92	Zinc finger protein 92	0.1
NM_025594	Zmat2	Zinc finger, matrin type 2	0.1
NM_001013368	E2f8	E2F transcription factor 8	0.2
NM_177733	E2f2	E2F transcription factor 2	0.1
NM_031404	Actl6b	Actin-like 6B	0.1
NM_009343	Phf1	PHD finger protein 1	0.2
NM_033476	Tcfcp2	Transcription factor CP2	0.4
NM_007623	Cbx2	Chromobox homolog 2 (Drosophila Pc class)	0.4
AF194420	Tef	Thyrotroph embryonic factor	0.4
NM_028716	Phf19	PHD finger protein 19	0.4
NM_172856	Lass6	Longevity assurance homolog 6 (S. cerevisiae)	0.4
NM_207677	Dedd2	Death effector domain-containing DNA binding protein	0.4
NM_010906	Nfix	Nuclear factor I/X	0.4
NM_010916	Nhlh1	Nescient helix loop helix 1	0.04
NM_019957	Dnase 2b	Deoxyribonuclease II beta	0.01
NM_080847	Asb15	Ankyrin repeat and SOCS box-containing protein 15	0.05
NM_080847	Rbl1	Retinoblastoma-like 1 (p107)	0.4
AB043687	Solt	SoxLZ/Sox6 leucine zipper binding protein in testis	0.4
NM_054056	Pawr	PRKC, WT1 regulator	0.4
NM_013685	Tcf4	Transcription factor 4	0.4
NM_033476	Tcfcp21	Transcription factor CP2-like 1	0.4
NM_134111	Eaf2	ELL associated factor 2	0.4

XM_992568	Atf6	Activating transcription factor 6	0.4
BC_011094	Dcler1b	DNA cross-link repair 1B, PSO2 homolog (S. cerevisiae)	0.4
NM_011265	Rfx3	Regulatory factor X, 3	0.3
NM_008452	Klf2	Kruppel-like factor 2 (lung)	0.4
NM_011751	Zfp207	Zinc finger protein 207	0.4
NM_010800	Bhlhb8	Basic helix-loop –helix domain containing, class B, 8	0.3
NM_007959	Etv2	ets variant gene 2	0.3
NM_008089	Gata	GATA binding protein 1	0.3
	Trim 17	Tripartite motif protein 17	0.2
NM_023739	Nfx1	Nuclear transcription factor, X-box binding 1	0.3
NM_011139	Pou2f3	POU domain, class 2, transcription factor 3	0.2
NM_023472	Rfxank	Regulatory factor X-associated ankyrin-containing protein	0.3
NM_009545	Pcgf2	Polycomb group ring finger 2	0.3
NM_080793	Setd7	SET domain containing (lysine methyltransferase) 7	0.2
NM_011565	Tead2	TEA domain family member 2	0.2
		Proteolysis	
NM_031156	Ide	Insulin degrading enzyme	0.4
NM_013903	Mmp20	Matrix metallopeptidase 20	0.1
NM_024434	Lap3	Leucine aminopeptidase 3	0.4
NM_010643	Klk1b24	Kallikrein 1-related peptidase b24	0.4
NM_026111	Qpctl	Glutaminyl-peptide cyclotransferase –like	0.4
NM_009390	Tll1	Tolloid-like	0.3
NM_010808	Mmp24	Matrix metallopeptidase 24	0.2
		Phase II metabolizing enzymes	
NM_026672	Gstm7	Glutathione S-transferase , mu 7	0.4
NM_023135	Sult1E	Sulfotransferase 1E	0.3
		Apoptosis	
NM_009402	Pglyrp1	Peptidoglycan recognition protein 1	0.5
NM_019516	Lgals12	Lectin, galactose binding, soluble 12	0.4
		Transport	
NM_020049	Slc6a14	Solute carrier family 6 (neurotransmitter	0.4

		transport)	
NM_008649	Mup5	Major urinary protein 5	0.3
NM_023852	Rab3c	RAB3C, member RAS oncogene	0.2
NM_018768	Stx8	Syntaxin 8	0.1
NM_019741	Slc2a5	Solute carrier family 2 (facilitated glucose transporter)	0.1
NM_028052	Synpr	synaptoporin	0.1
NM_011397	Slc23a1	Solute carrier family 23 (nucleobase transporters)	0.01
NM_053248	Slc5a5	Solute carrier family 5 (sodium iodide symporter)	0.014
NM_009209	Slc6a2	Solute carrier family 6 (neurotransmitter transporter)	0.044
NM_020332	Ank	Progressive ankylosis	0.4
NM_013623	Orm3	Orosomucoid 3	0.2
NM_011016	Orm2	Orosomucoid 2	0.2
NM_026232	Slc25a30	Solute carrier family 25, member 30	0.2
NM_009001	Rab3a	RAB3A, member RAS oncogene family	0.4
NM_139295	Mcfd2	Multiple coagulation factor deficiency 2	0.4
NM_175328	Slc6a15	Solute carrier family 6 (neurotransmitter transport)	0.2
NM_001004148	Slc13a5	Solute carrier family 13 (sodium dependent citrate transport)	0.2
NM_025841	Kdelr2	KDEL endoplasmic reticulum protein 2	0.4
NM_021350	Chml	Choroidermia-like	0.4
NM_031874	Rab3d	RAB3D, member RAS oncogene family	0.2
NM_133352	Tm9sf3	Transmembrane 9 superfamily member 3	0.4
NM_028493	Rhobtb3	Rho-related BTB domain containing 3	0.3
NM_019810	Slc5a1	Solute carrier family 5 (sodium/glucose cotransporter)	0.3
NM_023179	Atp6v1g2	ATPase, H+ transporting, lysosomal V1 subunit G2	0.4
NM_027491	Rragd	RAS-related GTP binding D	0.4
NM_019632	Napb	N-ethylmaleimide sensitive fusion protein attachment	0.3
NM_018803	Syt10	Synaptotagmin X	0.3
NM_016773	Nucb2	Nucleobindin 2	0.07

NM_032541	Hamp1	Hepcidin antimicrobial peptide 1	0.2
NM_019697	Kcnd2	Potassium voltage gated channel	0.4
NM_194341	Ap1gbp1	AP1 gamma subunit binding protein 1	0.3
NM_144552	Stxbp6	Syntaxin binding protein 6	0.2
		Cell cycle	
NM_019499	Mad2l1	MAD2-like 1	0.2
NM_029633	Clasp2	CLIP associating protein 2	0.2
NM_183015	Ccnb3	Cyclin-B3	0.1
	Arhgef16	Rho guanine nucleotide exchange factor (GEF)	0.4
NM_010615	Kif11	Kinesin family member 11	0.4
NM_009282	Stag1	Stromal antigen 1	0.2
NM_008634	Mtap1b	Microtubule associated protein 1b	0.4
NM_029550	Keg1	Kidney expressed gene 1	0.3
NM_008179	Gspt2	G1 to S phase transition 2	0.3
NM_133350	Mapre3	Microtubule-associated protein, RP/EB family, member 3	0.3
		Signaling proteins	
NM_009920	Cnih2	Cornichon homolog 2 (Drosophila)	0.1
NM_011067	Per3	Period homolog 3 (Drosophila)	0.4
NM_007785	Csn1s2a	Casein alpha s2-like A	0.1
NM_007412	Admr	Adrenomedullin receptor	0.4
NM_008306	Ndst1	N-deacetylase/N-sulfotransferase	0.4
NM_007641	Ms4a1	Membrane spanning 4-domains, subfamily A, member 1	0.4
NM_009500	Vav2	Vav2 oncogene	0.4
NM_016893	Fut8	Fucosyltransferase 8	0.4
NM_009850	Cd3g	CD3 antigen, gamma polypeptide	0.4
NM_010570	Irs1	Insulin receptor substrate 1	0.04
NM_011268	Rgs9	Regulator of G-protein signaling 9	0.4
NM_172964	Arhgap28	Rho GTPase activating protein 28	0.4
AY_294283	Grip1	Glutamate receptor interacting protein 1	0.1
NM_133248	Glmn	Glomulin, FKBP associated protein	0.4
NM_023543	Chn2	Chimerin 2	0.3
NM_008731	Npy2r	Neuropeptide Y receptor Y2	0.3
AH007445	Gng2	Guanine nucleotide binding protein (G protein)	0.1

		gamma 2	
NM_030614	Fgf16	Fibroblast growth factor 16	0.3
AH005928	Sim1	Single-minded homolog 1 (Drosophila)	0.2
NM_009953	Crhr2	Corticotrophin releasing hormone receptor 2	0.3
		Ubiquitination	
NM_148943	Usp9y	Ubiquitin specific peptidase 9, Y chromosome	0.1
NM_020575	March7	Membrane-associated ring finger (C3HC4)	0.4
NM_010241	Fts	Fused toes	0.4
NM_007659	Cdc20	Cell division cycle 20 homolog	0.3
NM_025692	Ube1dc1	Ubiquitin-activating enzyme E1-domain containing 1	0.2
		Cell adhesion	
NM_053147	Pcdhb22	Protocadherin beta 22	0.1
NM_011836	Lamc3	Laminin gamma 3	0.4
XM_907532	Cdh9	Cadherin 9	0.04
NM_008463	Klra5	Killer cell lectin-like receptor, subfamily A, member 5	0.4
NM_016898	CD164	CD164 antigen	0.4
		Electron transport	
NM_007717	Cmah	Cytidine monophospho-N-acetylneuraminic acid hydroxylase	0.1
NM_023140	Txnl2	Thioredoxin-like 2	0.4
NM_015764	Greb1	Gene regulated by estrogen in breast cancer protein	0.1
NM_007805	Cyb561	Cytochrome b-561	0.4
NM_018881	Fmo2	Flavin containing monoxygenase 2	0.4
NM_025862	Acad8	Acyl-coenzyme A dehydrogenase family, member 8	0.4
NM_009996	Cyp24a1	Cytochrome P450, family 24, subfamily a, polypeptide 1	0.2
NM_024444	Cyp4f18	Cytochrome P450, family 4, subfamily f, polypeptide 18	0.4
NM_145548	Cyp2j13	Cytochrome P450, family 2, subfamily j, polypeptide 13.	0.4

		Cytoskeleton associated proteins	
NM_028513	Actrt2	Actin-related protein T2	0.1
NM_144800	Mtss1	Metastasis suppressor 1	0.4
NM_010665	Krt32	Keratin 32	0.4
NM_013512	Epb4.1l1	Erythrocyte protein band 4.1-like 1	0.3
		G-protein coupled receptor	
NM_173365	Gpr20	G-protein coupled receptor 20	0.2
NM_026081	Gprasp1	G-protein couple receptor associated sorting protein 1	0.4
		Kinases and phosphatases	
NM_009871	Cdk5r1	Cyclin dependent kinase 5, regulatory subunit (p35)	0.1
NM_146151	Tesk2	Testis-specific kinase 2	0.4
NM_011947	Map3k3	Mitogen activated protein kinase kinase kinase 3	0.3
NM_133900	Psph	Phosphoserine phosphatase	0.3
NM_011361	Sgk	Serum/glucocorticoid regulated kinase	0.3
NM_008978	Ptpn20	Protein tyrosine phosphatase, non receptor type 20	0.3
		Protein folding, chaperones	
NM_009839	Cct6b	Chaperonin subunit 6b (zeta)	0.4
NM_029669	Dnajc18	DnaJ (HSP40) homolog, subfamily C, member 18	0.4
NM_010219	Fkbp4	FK506 binding protein 4	0.4
		Lipid metabolism	
NM_010726	Phyh	Fatty acid alpha oxidation	0.4
NM_023530	Pla2g12b	Phospholipase A2, group XIIB	0.4

[a] Genes that were suppressed < 2-fold by Novasoy only in prostate of Nrf2 wild-type mice but not in that of Nrf2 knockout mice compared with vehicle treatment at 12 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

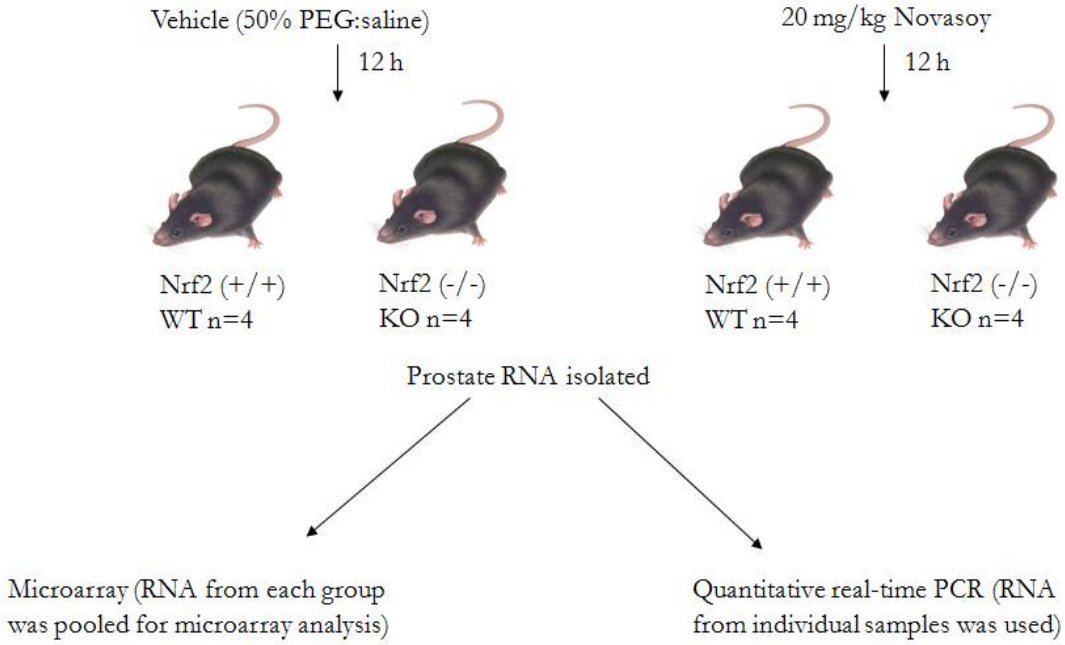

Figure 3.1: Schematic representation of the experimental design.

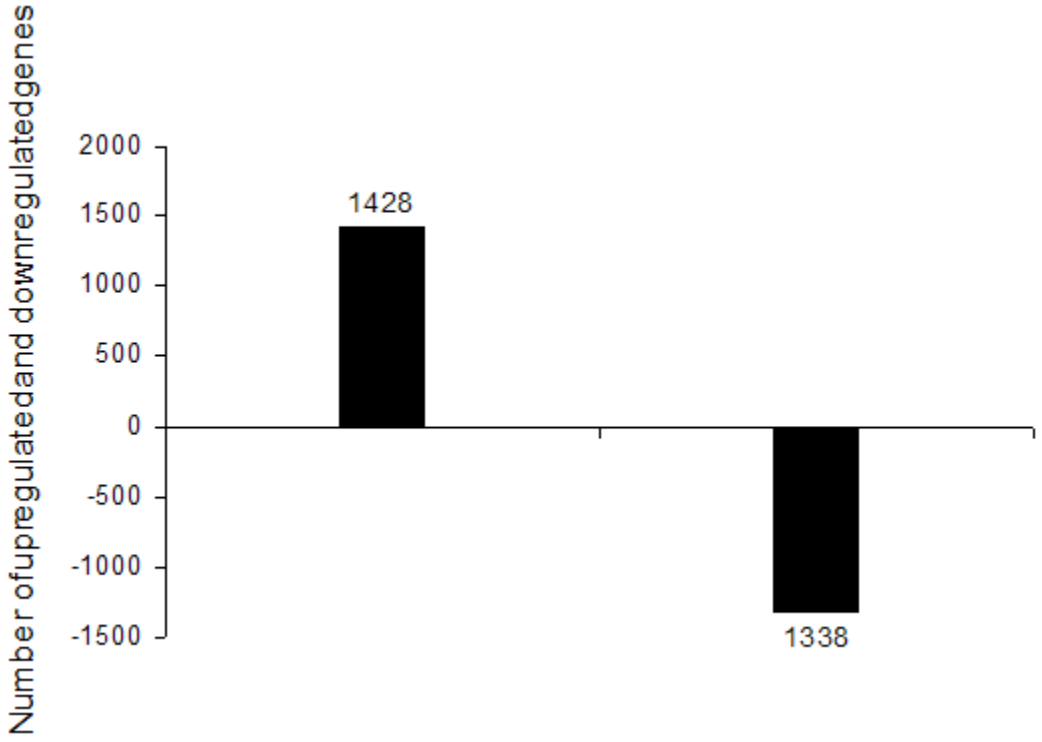

Figure 3.2: Regulation of Nrf2 dependent gene expression by soy isoflavones in Novasoy in the mouse prostate. Gene expression patterns were analyzed 12 hours after oral administration of 20 mg/kg of Novasoy. The positive numbers on the Y-axis refer to number of genes being induced; the negative numbers on the Y-axis refer to the number of genes being suppressed.

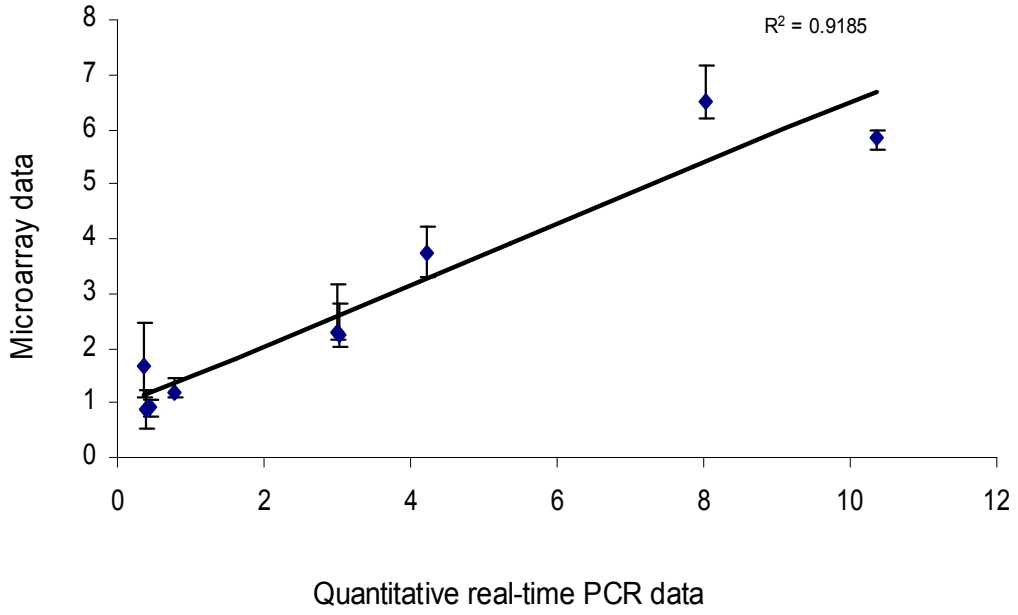

Figure 3.3: Correlation of the microarray data with quantitative real-time PCR data. Fold changes in gene expression obtained by oligonucleotide microarray were plotted against corresponding gene expression fold changes measured by quantitative real-time PCR. The data obtained from the two methods were found to be highly correlated (r^2=0.9185)

3.5 Discussion

Despite immense efforts in improving treatment modalities the overall mortality rates for most cancers have not declined significantly in the past several years and prostate cancer is no exception to this. Prostate cancer is the leading causes of cancer related death among men in the United States. Preventive measures of chemoprevention, thereby, are an integral part of this ongoing war against prostate cancer. The term chemoprevention was first coined by Dr. Michael J Sporn in 1976 and he advocated the use of chemopreventive agents to combat cancer. Since then thousands of naturally occurring dietary compounds have been screened for their potential cancer chemopreventive properties.

One of the successful mechanism by which these chemopreventive agents attain pharmacological effect is by modulating the levels of biotranformation enzymes that facilitate the elimination of endogenous and exogenous carcinogens. Results obtained from this oligonucleotide microarray analysis clearly demonstrate that soy isoflavones can induce the levels of several cytochrome P450 enzymes mainly cyp2b10, cyp2c and cyp3a13. Klaassen et al have shown that trans stilbene oxide can activate cyp2b10 via the transcriptional regulation of Nrf2 [116]. Hence the finding that Cyp2b10 induction involves Nrf2 is not novel however this is the first record indicating that soy derived isoflavones can also induce cyp2b10 via Nrf2. Delclos and coworkers demonstrated that genistein can induce cyp2c which conforms with the results we obtained [117]. Cyp3a is known to be involved in estrogen metabolism and hence it is no wonder that the isoflavones in Novasoy could induce cyp3a13. Another strategy adopted by most of the chemopreventive agents is upregulation of Phase II

detoxifying enzymes. EGCG – from green tea, curcumin – from turmeric, PEITC and sulforaphane from cruciferous vegetables, BHA – a popular food preservative and several other naturally chemopreventive agents have demonstrated a robust induction of phase II enzymes such as UDP-glucuronyltransferase, catalytic subunit of glutamate cysteine ligase, sulfotransferases and glutathione-s-transferases. Adding to this pool, we now demonstrate that soy isoflavones also induce phase II detoxifying enzymes. The role of Nrf2 in the transcriptional activation of these detoxifying enzymes is well-described and hence the results from this study reiterate the pivotal role of Nrf2 is chemoprevention.

The efficacy of genistein against prostate cancer has been established by several researchers. One of the major mechanisms by which genistein exerts its chemopreventive action is by causing a G2/M cell cycle arrest in prostate cancer cells thereby resulting in decreased proliferation [118]. Inhibiting protein tyrosine kinase, DNA topoisomerase II, activating apoptosis related proteins, modulating the transcriptional machinery and regulating some of the key signal transduction pathways are some of the possible mechanisms that have been postulated by several researchers to be the answer to genistein's ability to stall various cancers including that of the prostate. Likewise daidzein, another prevalently studied isoflavone has been shown to induce apoptosis and inhibit several growth factors induced cell growth in prostate cancer cells[119, 120] . Thus, needless to say, we observed significant alterations in the levels of several cell cycle associated genes, cell growth and apoptosis related genes after treatment with Novasoy. Cyclin B3 was significantly suppressed while p21 was induced 4.4 fold over control. Novasoy- the

agent of interest in this research study is a mixture of isoflavones both aglycones and glycosides. As mentioned above, numerous reports linking individual isoflavones and cancer chemoprevention have been described. However the effects of a complex mixture of isoflavones have not been studied in the same light. Choi and coworkers compared the pro-apoptotic and cytotoxic effects of genistein (aglycone) with genistin (glycoside) in human ovarian cancer cells and concluded that both forms were equipotent in inducing cell cycle arrest and apoptosis [121]. Kwon and coworkers compared the pharmacokinetics and disposition of genistein versus genistin and concluded that bioavailability of the glycoside is better than aglycone [122]. Based on this it is tempting to speculate that since the bioavailability of glycoside form is better, it has better access to the target tissue leading to enhanced effects. However detailed studies comparing the two forms will need to be performed to support such a contention.

We report that soy isoflavones can inhibit cell invasion and metastasis by regulating several cell adhesion molecules such as laminA, integrin and E-cadherin family of proteins. Though it has been shown earlier that genistein is capable of causing a sustained increase in intracellular Ca^{++} levels leading to activation of the negative regulator of cell cycle – calpain ultimately resulting in activated caspase 12 in human breast cancer cells [123], the involvement of Nrf2 in such activation has not been reported. Our results for the first time indicate that soy derived isoflavones indeed activate calpain in an Nrf2-dependent fashion. A disintegrin and metallopeptidase (ADAM) family of membrane-anchored metalloproteases is responsible for the cleavage of membrane-anchored proteins and releasing them into the cell. High

expression levels of ADAM relate well with the progression of cancer. Here we report that soy isoflavone treatment resulted in the inhibition of ADAM2 (0.485 fold) and since the involvement of transcription factor Nrf2 in such regulation of ADAM2 has not been shown earlier, this finding is truly novel and opens up another avenue to study chemopreventive actions of soy derived phytochemicals.

LATS is a recently identified Drosophila tumor suppressor belonging to a subfamily of protein kinases involved in the regulation of cell cycle progression and cell morphogenesis. The function of LATS 2 is not very clear. Localized within the nucleus, LATS2 possesses kinase activity and phosphorylates itself. Recently it has been demonstrated that LATS2 is involved in cell cycle regulation and cell growth by controlling G2/M transition and downregulating cyclin E. While one report suggests the involvement of LATS2 in androgen-mediated transcription, its role in cancer is largely unknown [124, 125]. In this study we report a robust induction of LATS2 (10.2 fold) by soy isoflavones and this induction involves the transcription factor Nrf2. This finding is strickingly original and indeed lays a renewed emphasis on the immense chemopreventive attributes of isoflavones and transcription factor Nrf2.

The advancement of prostate cancer progression from benign prostatic hypertrophy to localized prostate cancer and finally hormone refractory prostate cancer involves the amplification and suppression of numerous genes. Basic calponin 1 is one such an actin-binding protein that is involved in the regulation of smooth muscle contractile activity, the expression of which is drastically suppressed during the development of prostate carcinogenesis [126]. On the other hand the expression levels of gene regulated in breast cancer 1 (GREB1) have been reported to dramatically increased

[127]. We now report that soy isoflavones can activate calponin and at the same time suppress GREB1 in an Nrf2 dependent manner further substantiating the cancer chemopreventive potential of soy isoflavones and the role of Nrf2 in such activity.

Plasma levels of apolipoprotein A1 - the major components of high density lipoproteins (HDL) and HDL have been inversely correlated with the risk of prostate cancer. This inverse association is due to the important role played by HDL and apolipoprotein A1 in the reverse cholesterol pathway. Elevated cholesterol levels in prostate cancer cells have been found to result from aberrant regulation of cholesterol metabolism. Prostate cancer cells are known to rely on cholesterol for transducing several cell survival signals. Hence a scenario wherein abnormal cholesterol metabolism influences signal transduction events that promote cell survival, tumor growth and suppress apoptotic signals is generated and thereby agents that can inhibit or reverse cholesterol biosynthesis have been widely researched as anti-prostate cancer agents[128]. The data from this oligonucleotide microarray clearly demonstrates that soy isoflavones can induce apolipoprotein A1 roughly 36 fold and high density lipoprotein ~ 4.4 fold thus confirming other reports that show increased HDL and Apo A1 are effects of suppressed cholesterol metabolism and androgen suppression thereby suggesting that soy isoflavones can effectively suppress the effects of amplified cholesterol signaling in the prostate.

The transcriptional activation of Nrf2 dependent genes is heavily regulated by the interacting partners - co-activators and co-repressors of the gene transcription machinery. Some of identified and well characterized co-activators of Nrf2 include SRC1, RAC3, CBP while the co-repressors include Ncor1 and Nrip1. Yamamoto and

coworkers demonstrated that CBP can bind directly to the Nrf2 transactivation domains Neh4 and Neh5 [129]. Likewise using a Gal4-Nrf2 construct which contains the transactivation domain, we showed that RAC3/SRC3 can enhance the transactivation of the chimera. Combined results from previous studies and the current research indicate that these nuclear co-activators- Ncoa1 and Mafg induced by BHA and tunicamycin, CBP induced by EGCG and soy isoflavones and co-repressors Ncor1 and Nrip1 suppressed by BHA and tunicamycin are regulated by Nrf2 hence it may be speculated that soy isoflavone induced chemopreventive effects may involve a complex interactive association between transcription factor Nrf2 and its binding partners though only detailed functional and biochemical assays can support this hypothesis.

In summary, we have identified genes regulated by Nrf2, in addition to phase II metabolism and detoxification associated genes and this highlights the protective role portrayed by Nrf2 in cancer chemoprevention. By comparing the gene expression profiles elicited by soy isoflavones in Nrf2 deficient and wild type mice we have identified numerous genes performing a variety of biological and physiological functions and exhibit Nrf2 dependency. The aberrant behavior of cancer cells reflects the upregulation of several oncogenic signaling pathways that promote proliferation, inhibit apoptosis and aid in spreading cancer by evoking angiogenesis. This clearly suggests the involvement of numerous signaling pathways within the cell. The data from our oligonucleotide microarray lucidly demonstrate that soy isoflavones upregulate pro-apoptotic genes, downregulate cell growth and metastasis related genes and also modulate several other genes involved in one way or another in

prostate carcinogenesis. Multifocal signal modulation therapy (MSMT) has been ongoing to combat prostate cancer [130] and from the results we obtained, it is apparent that soy isoflavones appears to be an ideal candidate to consider for such therapy. Thus this research not only portrays the chemopreventive attributes of soy isoflavones but also describes the significant role played by the redox transcription factor Nrf2 in modulating various signaling pathways ultimately leading to their putative chemopreventive effects.

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CHAPTER 4: MURINE PROSTATE CANCER INHIBITION BY DIETARY PHYTOCHEMICALS - CURCUMIN AND PHENYLETHYLISOTHIOCYANATE[4]

4.1 Abstract

Prior studies from our laboratory have demonstrated the efficacy of a combined treatment of low doses of dietary agents curcumin and phenylethylisothiocyanate in effectively suppressing prostate cancer *in vitro* in human prostate cancer PC3 cells as well as *in vivo* in immunodeficient mice implanted with PC3 cells. Hence, this study was undertaken to examine the potential chemopreventive properties of the two agents against transgenic adenocarcinoma of the mouse prostate. The efficacy of AIN-76A diet supplemented with 2% curcumin or 0.05% PEITC or a combination of 1% curcumin and 0.025% PEITC for periods of 10 weeks and 16 weeks were tested against adenocarcinoma of the mouse prostate. Immunohistochemistry and Western blot analysis were used to examine the expression of proliferation and apoptotic biomarkers. All statistical tests were two-sided. Supplementing AIN-76A diet with dietary phytochemicals curcumin or PEITC either alone or in combination, significantly decreased incidence of prostate tumor formation (P=0.0064). Immunohistochemistry revealed a significant inhibition of high-grade PIN (P= 0.0006, 0.000069, 0.00029 for a treatment period of 10 weeks and P=0.02582, 0.022179, P= 0.0317 for a treatment period of 16 weeks) along with decreased proliferation and increased apoptotic index in the curcumin, PEITC or curcumin and PEITC treated animals, respectively.

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4 This chapter been published in *Pharmaceutical Research* as Murine prostate cancer inhibition by curcumin and phenylethylisothiocyanate.

Furthermore, Western blot analysis revealed that downregulation of the Akt signaling pathway may in part play a role in decreasing cell proliferation ultimately retarding prostate tumor formation. Our data lucidly evidence the chemopreventive merits of dietary phytochemicals curcumin and PEITC in suppressing prostate adenocarcinoma.

4.2 Introduction

Prostate cancer (CaP) is one of the most frequently diagnosed form of cancer and the second leading cause of cancer related death among men in the United States. CaP when truly confined only to the prostate can be cured by radiation therapy or androgen ablation. Such treatment causes apoptosis in androgen-dependent cells and substantially reduces tumor growth in ~ 80% of patients. However, in all too many cases, androgen-independent tumor cells, that are refractory to hormone therapy, ultimately develop and eventually result in a relapse [88]. Currently, the number of patients suffering from this invasive disease is on the rise and given the fact that when clinically significant this disorder is associated with a very high mortality rate, prevention may prove to be the best approach to combat it. The use of recently developed, genetically manipulated animals provides a good strategy to study CaP chemoprevention [131]. The transgenic adenocarcinoma of the mouse prostate (TRAMP) is one such genetically manipulated model that recapitulates many salient aspects of the progressive forms of human prostatic cancer. In this model, expression of the SV40 early genes (T and t antigens, Tag) are driven by the prostate-specific promoter probasin that leads to cell transformation within the prostate. The TRAMP transgenic mice develop high-grade prostatic intraepithelial neoplasia (PIN) within 12

weeks of birth and ultimately develop metastases by 30 weeks, primarily to the lungs, liver and lymph nodes [132] . PIN is considered the precursor to invasive carcinoma since it is most often than not associated with malignant form of the disease and the primary architectural and cytological features of PIN resemble that of invasive carcinoma. Hence the ability to retard PIN formation is considered as an important merit in agents that are tested against prostate carcinoma.

Epidemiological studies have continually supported the contention that naturally occurring dietary agents demonstrate chemopreventive properties and can suppress several malignancies including that of the prostate [89]. Antineoplastic effect of cruciferous vegetables is mainly attributed to isothiocyanates which occur as thioglucoside conjugates (glucosinolates) in a variety of edible plants including broccoli, watercress, cabbage etc. Organic isothiocyanates are generated due to hydrolysis of corresponding glucosinolates through catalytic activity of enzyme myrosinase which is released on damage of plant cells during processing (cutting or chewing) of cruciferous vegetables. Phenylethylisoathiocyanate (PEITC) is one such naturally occuring isothiocyanate compound that has attracted a great deal of attention due to its remarkable cancer chemopreventive properties [13,20,45]. Data from literature clearly demonstrates that PEITC can effectively suppress proliferation of human prostate cancer cells by inducing apoptosis and cell cycle arrest [133]. Curcumin (diferuloyl-methane), the yellow pigment found in spice turmeric extracted from the rhizome of the plant *Curcuma longa* has been shown to possess strong anti-oxidant and anti-inflammatory effects [11]. Due to these properties it has been very widely investigated for its potential chemopreventive activity. Curcumin has been

shown to inhibit the induction of cancers of the skin, forestomach, duodenum and colon in models of chemical carcinogenesis in mice and rats [12[134, 135]. It has also shown therapeutic efficacy against human prostate cancer xenografts in nude mice [136].

Recent studies from our laboratory have established that combined treatment of low doses of PEITC and curcumin can suppress human prostate cancer cell growth *in vitro* as well as in immunodeficient (Nu/Nu) mice bearing xenografts of androgen-independent human prostate cancer cells (PC-3) [137]. The following research study was undertaken to extend the promising chemopreventive effects of a combined treatment of PEITC and curcumin to the TRAMP mouse model. Our results clearly demonstrate that a combination of PEITC and curcumin can effectively inhibit the development and progression of CaP in TRAMP mice.

4.3 Materials and Methods

Animals: Female hemizygous C57BL/TGN TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were bred on same genetic background and maintained in the Animal Care Facility of Rutgers University. Housing and care of the animals was in accordance with the guidelines established by the University's Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP x C57BL/6] F1 or as [TRAMP x C57BL/6] F2 offspring. Identity of transgenic mice was established by the PCR-based DNA screening. Throughout the experiment the animals were housed in cages with wood chip bedding in a temperature-controlled

room (68˚F -72˚F) with a 12 hour light dark cycle, at a relative humidity of 45% to 55%. The animals were fed with irradiated AIN-76A diet (Research Diets Inc, New Brunswick, NJ).

Study Design: Curcumin and phenylethylisothiocyanate (PEITC) were obtained from Sigma chemicals Inc (St.Louis, MO). Two studies were initiated using 8-week old TRAMP males. One was a short term study involving treatment for 10 weeks (i.e. till the mice were 18 weeks old) while the second was a long term study involving treatment for 16 weeks (i.e. till the mice were 24 weeks old). In addition, 4 TRAMP males of age 8 weeks and 12 weeks were used as untreated controls. A summary of study design is as shown in Figure 4.1A.

All the animals were put on AIN-76A diet one week prior to the study. The control animals received AIN-76A diet throughout the experiment while the treatment groups received the corresponding chemopreventive agent supplemented AIN-76A diet. The animals were weighed weekly and monitored on a regular basis for their general health. At each time point the mice were killed by cervical dislocation and the genitourinary apparatus (GUT) consisting of the seminal vesicles, prostate and the bladder were isolated for further analyses.

Histopathology: The dorso-lateral prostate was excised and fixed overnight in 10% formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin embedded tissue and mounted on slides. The sections were stained with Haematoxylin and Eosin to observe any neoplastic changes. Sections were blindly evaluated by histopathologist Dr. Xingpei Hao to classify PIN lesions. Lesions were classified as PIN I, PIN II, PIN III and PIN IV as described by Park and co-workers.

Briefly prostatic tissue displaying columnar epithelial cells with surrounding stroma consisiting of two to three layers of smooth muscle with loose connective tissue was classified as normal. Tissue displaying fairly small foci with one or two layers of atypical cells with or without papillary structures and none of them being secondary branched was classified as PIN I. In PIN I the cells appear slightly taller than normal cells, though the fibromuscular stromal sheath appears intact. PIN II displays larger foci with multiple layers of atypical cells that do not, however, fill up the lumen space. The underlying fibromuscular stromal sheath appears intact, though the epithelial cells display papillary, cribiform or tufting patterns. The atypical cells appear the same as in PIN I though there is increased nuclear pleomorphism and hyperchromasia. PIN III displays foci with multiple layers of atypical cells that fill up roughly 2/3 rd of the lumen. The epithelial cells exhibit either papillary with multiple branches or cribiform or tufting patterns. The atypical cells have inverted nuclear to cytoplasmic ratio. They appear poorly oriented with increasingly severe nuclear pleomorphism and hyperchromasia. Mitotic figures are present. PIN IV displays foci with multiple layers of atypical cells that fill up the lumen of the ducts. The ducts appear distorted with irregular or absent fibromuscular sheath. The epithelial cells form solid, cribiform or tufting growth patterns. The nuclear to cytoplasmic ratio is inverted. The atypical cells display increasingly severe nuclear pleopmorphism and hyperchromasia. Mitotic figures are present.

For the purpose of ease, PIN I and PIN II have been grouped as low grade PIN while PIN III and PIN IV have been grouped as high grade PIN.

Apoptosis and proliferation. The apoptotic cells were detected using an ApopTag *In Situ* Apoptosis Detection Kit (Chemicon). The assay was performed according to the manufacturer's manual. After deparaffinization, the tissues sections were incubated in proteinase K for 15 minutes in room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for one hour, washed in three changes of PBS and incubated with anti-digoxigenin conjugate in a humidified chamber at room temperature for 30 minutes. The color was developed by incubating the sections with peroxidase substrate and then counterstained with haematoxylin for 30 seconds. For detection of proliferative cells, PCNA antibody (1: 50; Dakocytomaton, CA) was used. The assay was performed following the manufacturer's protocols. A positive control slide of rat mammary glands provided by the manufacturer was used as positive control for the *In Situ* apoptosis detection assay. For the PCNA staining, mouse intestinal crypt cells were used as a positive control. The apoptotic or proliferative index was calculated as number of positive cells/total cells counted x 200.

Immunoblot analysis: The dorso-lateral prostate tissues removed from both treated and control groups were pooled and homogenized with RIPA buffer (50mM NaCl, 0.5% Triton-X 100, 50mM Tris-HCl, pH 7.4, 25mM NaF, 20mM EGTA, 1mM DTT, 1mM Na_3VO_4, protease inhibitor cocktail tablet (Roche, Manheim, Germany) at a concentration of 10µg/ml and placed on ice for 40 minutes, followed by centrifugation at 14,800 x g for 15 minutes. The protein concentrations were measured by Bicinchonic Acid (BCA) solution (Pierce, Rockford,IL). 20 µg of protein was loaded onto Biorad pre-cast gels (4-12%) and after electrophoresis,

transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA in 0.1% Tween-20 in PBS (TBST) for 1 hour followed by incubation with primary antibody in 3% BSA overnight at 4˚C. After three 15-minute washes with TBST, the membrane was incubated with horseradish peroxidase conjugated secondary antibody in 3% BSA for 1 hour at room temperature followed by washing with TBST. The proteins were then visualized using Supersignal West Femto (Pierce, Rockford, IL).

Statistics: The results for tumor incidence and poorly differentiated carcinoma between 18 and 24 week old control males were analyzed by Student's test. Statistical significance of differences in all other measurements was determined by one-way analysis of variance followed by Tukey's procedure for all pairwise multiple comparisons, excluding the control group. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Effects of curcumin and/or PEITC supplemented diet and general health observations:

The overall health of all the mice was observed on a daily basis and was found to be good throughout the study period. No significant change in the body weights of the animals was found throughout the experimental period. Results are as depicted in figures 4.1B and C.

4.4.2 Effect of 10 weeks of curcumin and/or PEITC supplemented diet on prostate tumorigenesis:

The mice were put on respective diets for a period of 10 weeks and sacrificed when they were 18 weeks old. The effect of such treatment for a period of 10 weeks on the wet weight of the GUT apparatus is shown in Figure 4.1D. Curcumin and/or PEITC supplemented diet significantly decreased the GUT weight with P values of 0.000322, 0.000295, 0.000357 respectively. Such treatment resulted in decreased hyperplasia in the GUT apparatus, especially in the seminal vesicles as evidenced by their reduced size. Hematoxylin and Eosin staining of the dorso-lateral prostates of these animals revealed that the curcumin or PEITC or a combination of curcumin and PEITC treated animals demonstrated the presence of significantly higher proportion of normal prostatic tissue as compared to the control animals that demonstrated almost no normal prostatic tissue (P=0.0069, 0.0019, 0.0019, respectively). In addition the treated animals demonstrated low levels of high grade PIN as compared to the control animals (P =0.007, 0.00533 and 0.00883 , respectively). Although PEITC treated animals demonstrated higher levels of normal tissue, the degree of inhibition of high-grade PIN was the same as the curcumin treated animals. A combination diet, surprisingly, did not inhibit high grade PIN as much as individual agents curcumin or PEITC did, although this difference was not found to be statistically significant. Results are as depicted in figure 4.3B. Thus, it may be inferred that a combination of low doses of potential chemopreventive agents curcumin and PEITC for a treatment period of 10 weeks, lacked any synergistic effect.

4.4.3 Effects of 16 weeks of curcumin and/or PEITC supplemented diet on prostate tumorigenesis:

The mice were put on the respective diets for a period of 16 weeks and sacrificed when they were 24 weeks old. The effect of such treatment on the wet weight of the GUT is summarized in figure 4.1E. There was a statistically significant reduction in GUT weight followed by curcumin and/or PEITC supplemented diet (P= 0.025, 0.04 and 0.01345, respectively). A significant reduction in the incidence of palpable tumor was observed in the treated animals (P= 0.0064). 6 out of 9 control animals fed AIN-76A diet developed palpable tumors. In contrast, only two curcumin fed and one PEITC fed animal demonstrated the presence of palpable tumors. None in the curcumin and PEITC fed groups of TRAMP males developed palpable tumors. Results are as depicted in figure 4.2. These findings were further confirmed by histological observations that showed that 8 out of the 9 control animals developed poorly differentiated carcinoma with one of them demonstrating necrosis. In contrast only one 18 week old control animal developed poorly differentiated carcinoma (P=0.00022). This confirms previous findings that as age of the TRAMP mouse advances, the incidence of carcinoma increases. Results are as depicted in figure 4.3D. Among the treated groups, 1 out of 9 combination diet fed animal demonstrated poorly differentiated carcinoma although there was no evidence of any palpable tumor. Of most importance is the observation that the invasiveness of CaP was suppressed in the treated groups as compared to the control. This is evidenced by effective inhibition of high grade PIN in the curcumin, PEITC and combination diet fed animals (P= 0.0023, 0.014, 0.0036 respectively). Thus it can be inferred that a combination of low doses of chemopreventive agents curcumin and PEITC can

effectively suppress prostate tumor growth and this effect is moderately synergistic when treated for longer periods.

4.4.4 Effect of curcumin and/or PEITC supplemented diet on Proliferation Marker (PCNA) and Apoptosis:

PCNA serves as an auxiliary protein for DNA polymerase δ-driven DNA synthesis and is known to be cell-cycle regulated. Treatment with either curcumin or PEITC or the two in combination for periods of 10 weeks resulted in significantly low levels of PCNA (P=0.001818, 0.0005657, 0.01288, respectively) as observed by immunohistochemical analysis. A similar statistically significant decrease in PCNA levels was observed in TRAMP males fed curcumin and/or PEITC diet for a period of 16 weeks (P=0.00240, 0.00075, 0.002509, respectively). Results are as shown in figures 4.4A and B.

The percentage of apoptotic cells in the dorso-lateral prostates of the animals fed curcumin or curcumin and PEITC supplemented diet for 10 weeks was significantly higher than control animals (P=0.0072 and 0.00523 respectively.) Although the number of apoptotic cells in the PEITC fed animals was higher than the control, this difference was not found to be statistically significant. In contrast, all the long term treatment groups - curcumin and/or PEITC demonstrated significantly higher percentage of apoptotic cells as compared to the control (P=0.001656, 0.000159, 0.0163 respectively). Results are as shown in figures 4.5A and B.

4.4.5 Effect of curcumin and/or PEITC supplemented diet on the expression levels of p-PDK1, p-Akt, p-FKHR , Bad and caspase 3 proteins.

In order to examine the molecular targets involved in curcumin and/or PEITC mediated CaP regression, we investigated the expression levels of both proliferative and pro-apoptotic biomarkers of tumor progression - PDK1, Akt, FKHR, Bad and caspase 3 proteins. Results are as depicted in Figure 4.6A. Western blot analysis demonstrate reduced expression levels of phospho-PDK1, phospho-Akt (Ser 473) and phospho-FKHR proteins in the prostatic tissues of the treated animals as compared to the control. The bar graphs represent the levels of expression as determined by densitometric analysis. From the figure, it is clear that as age of the TRAMP mouse advances the levels of the proliferative biomarkers p-PDK-1, p-Akt and p-FKHR progressively increase while that of the pro-apoptotic biomarkers either progressively decrease as in the case of Bad, or are undetectable in the case of cleaved caspase-3. Interestingly, curcumin and PEITC treatment alone for a period of 10 weeks suppressed the levels of PDK-1, Akt and FKHR however, a combination diet did not suppress these levels as much on the other hand did increase levels of pro-apoptotic biomarkers Bad and cleaved caspase 3. The most interesting observation is that curcumin when fed for a period of 16 weeks, did not effectively suppress the proliferative biomarkers though it did significantly increase the levels of both Bad and cleaved caspase 3 proteins. Thus it may be inferred that a diet supplemented with curcumin and/or PEITC can impede the progression of PIN at least in part by downregulating the Akt pro-survival pathway.

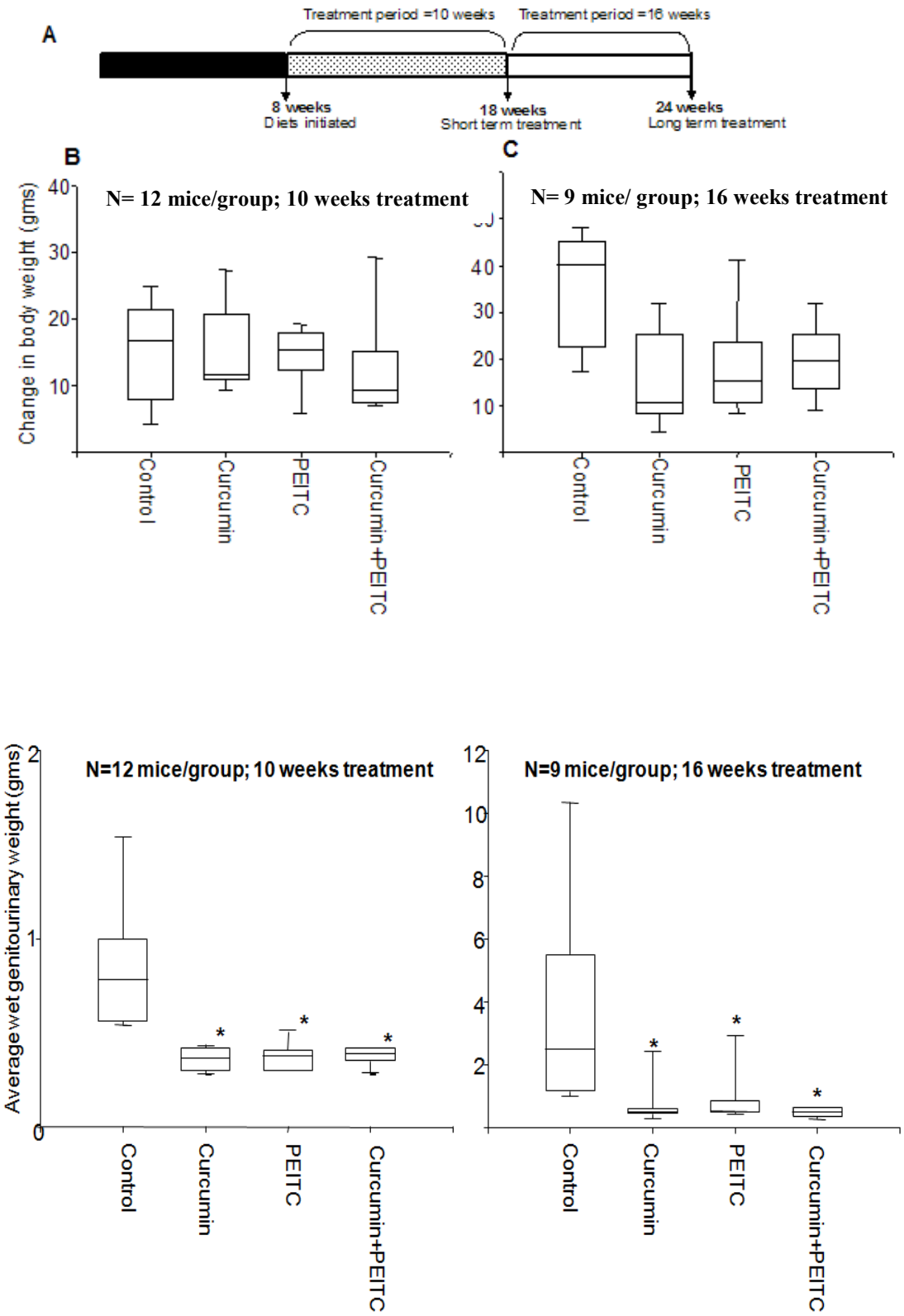

A
Treatment period =10 weeks
Treatment period =16 weeks
8 weeks
Diets initiated
18 weeks
Short term treatment
24 weeks
Long term treatment
B
C
N= 12 mice/group; 10 weeks treatment
N= 9 mice/ group; 16 weeks treatment
Change in body weight (gms)
Control
Curcumin
PEITC
Curcumin +PEITC
N=12 mice/group; 10 weeks treatment
N=9 mice/group; 16 weeks treatment
Average wet genitourinary weight (gms)
Control
Curcumin
PEITC
Curcumin+PEITC

Figure 4.1: The effects of curcumin and/or PEITC supplemented diet on the genitourinary weight. **A)** Time line. Eight week old TRAMP males were put on an AIN-76A diet supplemented with either (2%) curcumin or (0.05 %) PEITC alone or a combination of the two chemopreventive agents (1% and 0.025%). The diets were maintained for two treatment periods of 10 weeks and 16 weeks till the age of the mice were 18 weeks and 24 weeks, respectively at which point they were sacrificed and the genitourinary apparatus consisting of the prostate, seminal vesicles and bladder, the lungs, livers and periaortic renal lymph nodes were harvested. **B, C)** Effects of curcumin and/or PEITC on the body weights of animals treated for periods of 10 weeks and 16 weeks, respectively. The upper boundary of the box represents the 75th percentile while the lower boundary of the box represents the 25th percentile of the data distribution, the horizontal line within each box represents the median value and the error bars represent the 95% confidence intervals. No statistically significant change was observed **D, E)** Effects of curcumin and/or PEITC on the genitourinary weights of animals treated for periods of 10 weeks and 16 weeks, respectively. *Significantly different from the control.

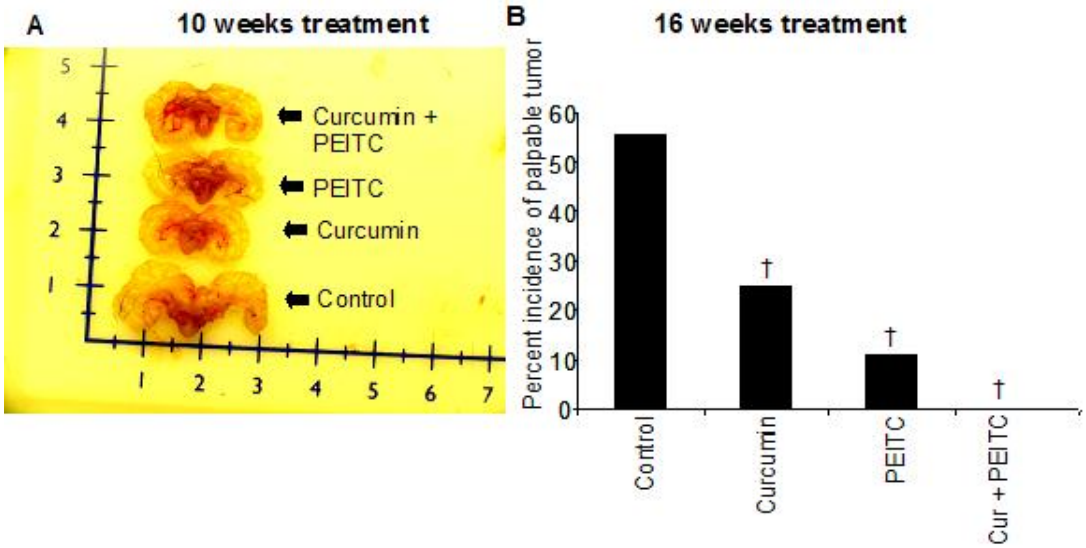
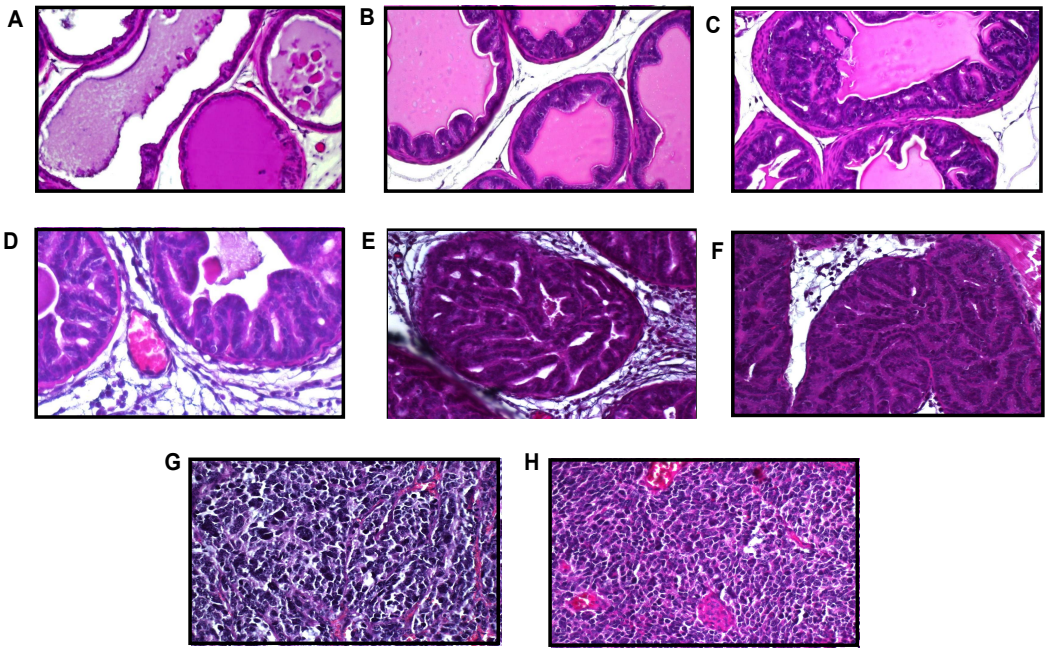


Figure 4.2: Incidence of palpable tumor in the 24 week old control versus treated animals. † Significantly different based on Student's t-test. Data represented is the mean value and the error bars represent ± SE.


A
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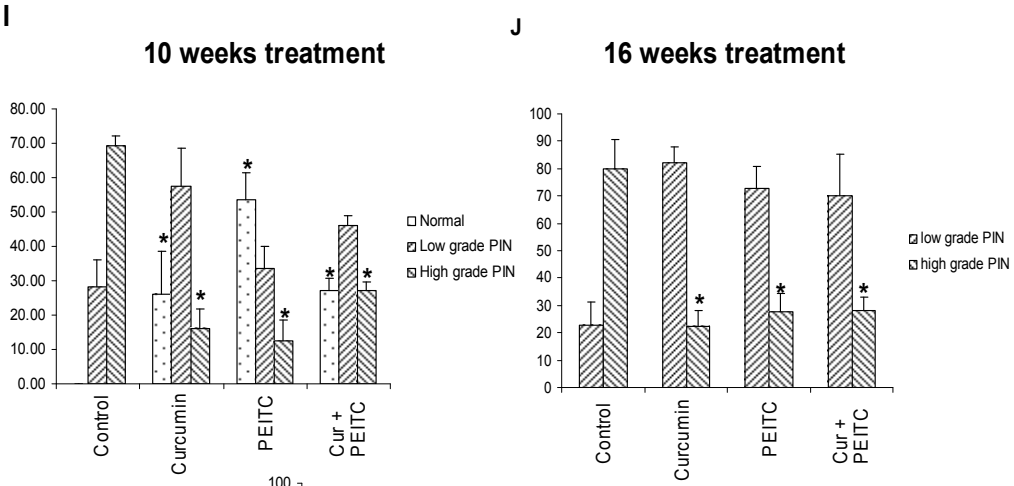
I
10 weeks treatment
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Control
Curcumin
PEITC
Cur + PEITC
Normal
Low grade PIN
High grade PIN
J
16 weeks treatment
100
90
80
70
60
50
40
30
20
10
0
Control
Curcumin
PEITC
Cur + PEITC
low grade PIN
high grade PIN

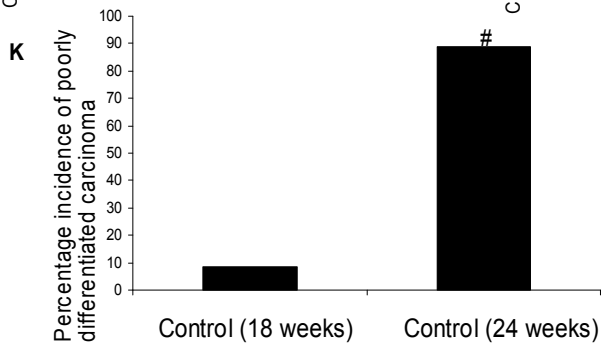
K
Percentage incidence of poorly differentiated carcinoma
100
90
80
70
60
50
40
30
20
10
0
#
Control (18 weeks)
Control (24 weeks)

Figure 4.3: Histological evaluation of prostatic intra-epithelial lesions and adenocarcinoma and their inhibition by curcumin and/or PEITC supplemented diets. Representative sections depicting **A)** normal tissue, **B)** PIN I, **C)** PIN II, **D)** PIN III, **E)** PIN IV, **F)** PIN IV with metastasis, **G)** anaplastic carcinoma and **H)** metastasis to lymph nodes. **I)** Levels of low and high grade PIN in animals treated for a period of 10 weeks. *Significantly different from the control. **J)** Levels of low and high grade PIN in animals treated for a period of 16 weeks. *Significantly different from the control. **K)** Bar graph representing number of 18 and 24 week old control animals that demonstrated poorly differentiated carcinoma, histologically. # Significantly different based on Student's t test. Data represented is the mean value and the error bars represent ± SE.

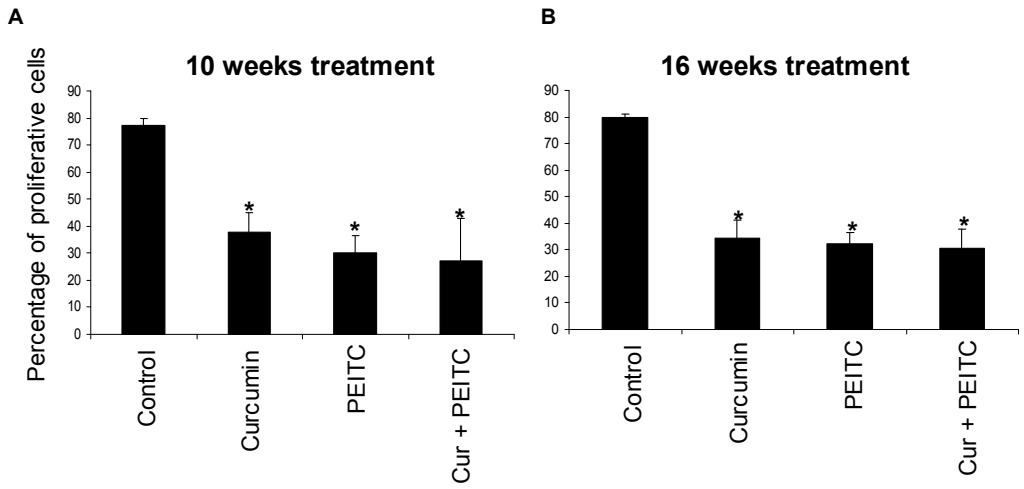

Figure 4.4: Immunohistochemical analysis of the effect of curcumin and/or PEITC supplemented diet on the proliferation marker PCNA. **A,B)** PCNA expression followed by treatment with curcumin and/or PEITC for a period of 10 weeks and 16 weeks, respectively. *Significantly different from the control. Data represented is the mean value and the error bars represent ± SE.

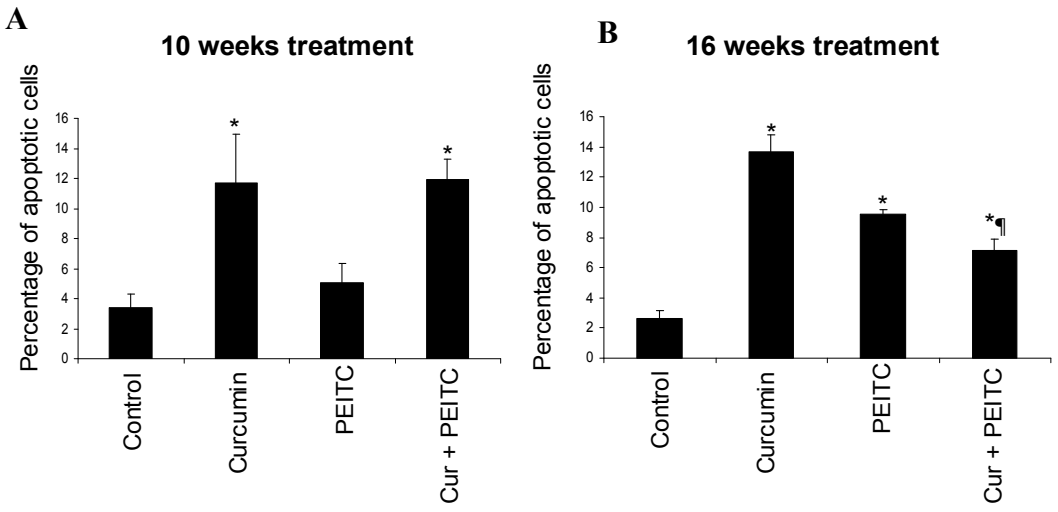

Figure 4.5: Immunohistochemical analysis of the effect of curcumin and/or PEITC supplemented diet on apoptosis. **A, B)** Apoptotic cells counted under 40X were quantified and presented as a percentage of apoptotic cells followed by curcumin and/or PEITC supplemented diet for periods of 10 weeks and 16 weeks, respectively. *Significantly different from the control. ¶ significantly different from curcumin treatment, Tukey's test. Data represented is the mean value and the error bars represent ± SE.

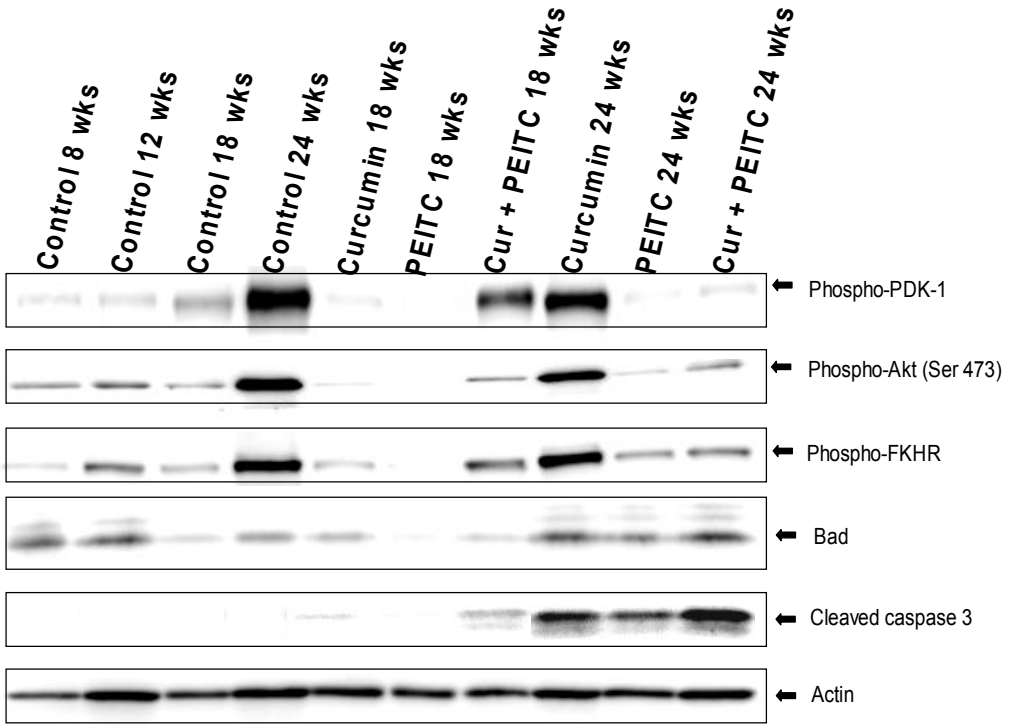

Figure 4.6: Western blot analysis of proliferative and apoptotic biomarkers – PDK-1, Akt (Ser 473), FKHR, Bad and cleaved caspase 3 in the dorsolateral prostates of mice treated with curcumin and/or PEITC. A 20 µg protein sample was loaded onto each lane.

4.5 Discussion

Earlier studies from our laboratory have demonstrated that intraperitoneal injections of curcumin and/or PEITC retarded the growth of PC3 xenografts in immunodeficient mice [74]. This study clearly demonstrated the efficacy of the two agents as chemopreventive rather than cancer therapeutic. Hence, as an extension to the above study, we evaluated the chemopreventive properties of curcumin and PEITC in a genetically engineered mouse model which has revolutionized the process of investigating molecular mechanisms of prostate carcinogenesis, the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model. The major strength of this model is that cancer arises from normal prostate epithelial cells in their natural tissue microenvironment and progresses through multiple stages thus exhibiting both histological and molecular features similar to human prostate cancer.

It is known that by approximately 6 weeks of age, TRAMP mice exhibit low-grade PIN which progresses to high grade PIN by 12-16 weeks. Focal adenocarcinoma develops between 12-18 weeks and progresses to poorly differentiated carcinoma within 24 weeks. By 28 weeks of age, 100% of these transgenic mice harbor metastatic prostate cancer in liver, lymph nodes and lungs [132]. In the current study we demonstrate that by 24 weeks of age, 6 out of 9 animals had developed palpable tumors and demonstrated metastasis mainly to the lymph nodes. Curcumin or PEITC either alone or in combination were found to be highly effective in decreasing the incidence of tumor formation in these mice.

Histologic analysis of the prostate of control animals clearly displayed major epithelial proliferation, hyperchromatic nuclei and mitotic figures. In contrast the

prostate sections of mice fed curcumin and/or PEITC diets displayed no indication of neoplasia. Analysis of other tissues such as lungs, liver and lymph nodes showed normal morphology thus indicating that curcumin and/or PEITC did not have any deleterious effects on these organs (data not shown).

For cancer progression it is vital that cell survival signals are amplified and pro-apoptotic signals are minimalized. The Akt is one such pathway that is often involved in amplifying cell-survival signals. Besides, there is also a rich body of evidence suggesting that prostate cancer progression may be mediated by the Akt signaling pathway[138, 139]. The PTEN gene encodes a dual specificity phosphatase active against protein and lipid substrates and is known to be a direct antagonist to the PI3K signaling pathway. The most important downstream target of the PI3K is serine-threonine Akt kinase family. Activation of PI3K caused by suppression of PTEN leads to an increase in its lipid products that facilitate the recruitment of Akt and PDK-1 to the plasma membrane. Once recruited, to the plasma membrane, Akt is phosphorylated by PDK-1 [140, 141]. From the data we provide, it is clear that the progression from prostatic lesions to highly invasive carcinoma in the control animals relates well with the gradual increase in levels of activated Akt, thus reinforcing the fact that cell-survival signals are amplified in cancer progression. Treatment with curcumin or PEITC lowers the levels of activated Akt and its upstream kinase PDK-1. Slightly aberrant from the trend is the observation that curcumin treatment for a period of 16 weeks did not significantly alter levels of Akt and PDK-1. This observation is truly intriguing, although it is also suggestive that *in vivo* long term treatment with PEITC may prove better than curcumin, in suppressing the Akt

signaling pathway. Curcumin and PEITC put together demonstrated significant inhibition of the Akt signaling pathway again when treated for longer periods of time. A short term treatment with the combination did not alter the Akt pathway which is consistent with the observation that such treatment did not affect levels of high grade PIN as much as individual agents did. Since immunohistochemical analysis demonstrates that such treatment did decrease the levels of proliferation marker – PCNA and induce apoptosis in the cells, it is alluring to make a conjecture that other cell-survival amplifying pathways may play a role.

Akt is known to possess two phosphorylation sites at Thr 308 and Ser 473/474. PDK-1 is known to phosphorylate Akt at the Thr 308 position [142]. Although our results demonstrate modulation of Akt activity by curcumin and/or PEITC at Ser473/474 position, we did not observe a similar modulation at the Thr 308 position. Prior studies in our lab have demonstrated that in human PC3 cells a combination of curcumin and PEITC can modulate PDK-1 and Akt Ser 473/474 as well as Thr 308 activity. However lack of phosphorylation of Akt at the Thr 308 position *in vivo* suggests that studies aimed to correlate observations made *in vitro* with those made *in vivo* are imminent. The ability of curcumin and PEITC to modulate Akt activity at Ser 473/474 site directly without the involvement of its upstream kinase PDK-1 might also in part explain such observation. Activated Akt is known to promote cell survival by inactivating its downstream targets including Bad and members of the forkhead family [140]. From figures it is clear that activated Akt in the untreated controls led to suppression of Bad and activation of phosphorylated forkhead proteins while curcumin and/or PEITC treatment could effectively activate pro-apoptotic protein

Bad and suppress phosphorylated forkhead proteins. The finding that curcumin when treated for a period of 16 weeks does induce pro-apoptotic Bad without suppressing the Akt signaling pathway is indicative of the intervention of certain other signal transduction pathways. Another possible explaination for this finding could be that as the levels of Akt increase with invasiveness of the carcinoma, curcumin alone may not be able to suppress Akt and thus proliferation. It would do so in a much better fashion when combined with other chemopreventive agents mainly PEITC. This also explains the findings that a combination of curcumin and PEITC was far more effective in suppressing tumor incidence as well as activating cleaved caspase 3 – a hallmark feature of apotosis. Hence, though we did not observe any synergistic effects of combined therapy with curcumin and PEITC when administered for a shorter period of time (10 weeks), a long term treatment definitely could prove beneficial.

In summary, we show that dietary agents - curcumin and PEITC can suppress levels of high grade PIN in the TRAMP mouse model by downregulating the Akt signaling pathway and upregulating members of the pro-apoptotic family ultimately resulting in inhibited prostate tumor formation. To the best of our knowledge, this study is the first to demonstrate the efficacy of these agents both alone and in combination in the TRAMP mouse model.

CHAPTER 5: γ-TOCOPHEROL ENRICHED MIXED TOCOPHEROL DIET INHIBITS PROSTATE CARCINOGENESIS IN TRAMP MICE [5]

5.1 Abstract:

γ-tocopherol (γ-T) alone or in combination with α-tocopherol has been shown to suppress biomarkers of oxidative stress in asthamatics and human subjects with metabolic syndrome. Oxidative stress has been implicated as a key event in prostate carcinogenesis. Hence the purpose of this study was to examine the effects of γ-tocopherol enriched mixed tocopherol diet on prostate carcinogenesis in a murine prostate cancer model (TRAMP). 8 week old TRAMP males were fed 0.1 % γ-T enriched mixed tocopherol diet that contained 20 fold higher levels of γ-tocopherol, and roughly 3 fold higher levels of α-tocopherol. The effect of such diet on tumor and PIN development was observed. The expression of phase II detoxifying, antioxidant enzymes and Nrf2 mRNA and protein were determined by RT-PCR, immunohistochemistry and western blotting techniques. Treatment with γ-T enriched mixed tocopherols significantly suppressed the incidence of palpable tumor and Prostate Intraepithelial Neoplasia (PIN) development without affecting the expression of the transgene (SV-40). Tumor progression occurred with a significant suppression of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, heme-oxygenase-1 and phase II detoxifying enzymes.

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5 This chapter has been accepted for publication in *International Journal of Cancer* as γ-Tocopherol enriched mixed tocopherols inhibit prostate carcinogenesis in TRAMP mice.

Treatment with γ-T enriched mixed tocopherol diet upregulated the expression of most detoxifying and antioxidant enzymes. Nrf2 – a redox sensitive transcription factor known to mediate the expression of phase II detoxifying enzymes, was also significantly upregulated following treatment with γ-T enriched mixed tocopherol diet. γ-T enriched mixed tocopherols significantly up-regulated the expression of Nrf2 and its related detoxifying and antioxidant enzymes thereby suppressing PIN and tumor development.

5.2 Introduction

Reactive oxygen species (ROS) such as superoxide radical anion, hydroxyl radical and hydrogen peroxide are generated as by-products of aerobic mitochondrial respiration. The human body has robust defense mechanisms comprising of detoxifying enzymes such as glutathione-s-transferase, UDP glucuronyl transferases and antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase as well as non-enzyme antioxidant species that, under physiological conditions, are known to quench these reactive moieties. However, a scenario where excessive reactive oxygen species are generated and the body's antioxidant defenses are overwhelmed leads to a state of oxidative stress. Oxidative stress can cause adducts and permanent damage to cellular macromolecules such as DNA/RNA, proteins, lipids, etc. A rich body of evidence substantiates the role of such oxidative stress in carcinogenesis. Needless to say, increased levels of ROS coupled with suppressed antioxidant defense have been reported in human prostate intraepithelial neoplasia and prostate cancer [143-146].

The antioxidant activity of vitamin E has persuaded many researchers to study its ability to ameliorate chronic diseases, especially those in which oxidative stress plays a key role such as arthrosclerosis, cardiovascular diseases and cancer [147]. Vitamin E is a generic name for structurally related tocopherols and tocotrienols. A standard American diet contains large amounts of γ-T compared to European diet due to high intake of soybeans and corn oil. Despite high intake of γ-T, plasma concentrations of γ-T are about 10 times lower than that of α-T. The reason for the plasma preference for α-T is in its specific selection by α-T transfer protein (α-TTP). α-TTP not only specifically selects the α form of all tocopherols but also has a preference for 2R-stereoisomers [148, 149]. As a result, the cancer chemopreventive activities of Vitamin E have been extensively studied with α-T [150, 151]. However the protective effects of γ-T having only recently been recognized are now the subject of active investigation. A γ-T enriched mixed tocopherol diet (containing more than 50% γ-T) has been shown to suppress azoxymethane induced crypt foci – a precursor of colon cancer, in rats [152]. A similar diet has also shown to suppress N-methyl-N-nitrosourea induced mammary tumors in rats [153]. A recent study by Devaraj et al reports that in human subjects with metabolic syndrome, supplementation with γ-T alone or in combination with α-T significantly decreased TNF levels. However nitrotyrosine levels (a biomarker for oxidative stress) were decreased only by γ-T and not by α-T [154]. A nested case-control study in Washington County, MD that examined the effects of α-T, γ-T and selenium on the incidence of prostate cancer in human subjects found that the inverse relationship between high levels of selenium and α-T held true when the levels of γ-T were high. Interestingly, a 5-fold reduction

in prostate cancer was observed in men who also had the highest plasma γ-T concentrations [155].

γ-T possesses certain unique properties that are relevant to cancer chemoprevention. Jiang and coworkers demonstrated that both γ-T and its catabolic metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) possess anti-inflammatory attributes. They significantly suppress cyclooxygenases and other inflammatory proteins such as interleukins and cytokines [156, 157]. Owing to its strong nucleophilic properties, γ-T exhibits more efficiency than α-T in trapping ROS and reactive nitrogen species (RNS) [158]. Cooney et al reported that γ-T was superior to α-T in suppressing the transformation of murine fibroblasts incubated with chemical carcinogen 3-methylcholanthrene [159]. A study by Gysin et al reported that γ-T inhibited cell proliferation, cell cycle progression and DNA synthesis in prostate cancer cells better than α-T [160].

To our knowledge the chemopreventive effects of a γ- T enriched mixed tocopherol diet that provides more than 60 % γ-T, have not been explored in genetically modified murine model for prostate cancer (TRAMP). This model recapitulates many salient features of the progressive forms of human prostate cancer. This study was aimed at observing the effects of a γ-T rich mixed tocopherol diet on prostate carcinogenesis in TRAMP mice. The effects of such treatment on the levels of PIN (a recognized precursor for prostate cancer) and the expression of phase II detoxifying and antioxidant enzymes were determined.

5.3 Materials and Methods

Animals: Female hemizygous C57BL/TGN TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were bred on same genetic background and maintained in the Animal Care Facility of Rutgers University. Housing and care of the animals was in accordance with the guidelines established by the University's Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP x C57BL/6] F1 or as [TRAMP x C57BL/6] F2 offspring. Identity of transgenic mice was established by the PCR-based DNA screening. Throughout the experiment the animals were housed in cages with wood chip bedding in a temperature-controlled room (68˚F -72˚F) with a 12 hour light dark cycle, at a relative humidity of 45% to 55%.

Diets and study design: All animals were fed AIN-76A diet obtained from Research Dyets (Easton, PA). Treated animals were fed 0.1% mixed tocopherols incorporated in AIN-76A diet. The mixed tocopherols were obtained from Cognis Corporation (Kankakee, IL) and contained <60% γ-tocopherol, 12% α-tocopherol, 21% δ-tocopherol and also insignificant levels of β-tocopherol. This diet contained about 20 fold higher levels of γ-tocopherol, and roughly 3 fold higher levels of α-tocopherol. All the animals were put on AIN-76A diet one week prior to the study. The control animals (n=17) received AIN-76A diet throughout the experiment while the treatment group (n=11) received 0.1% γ-T enriched mixed tocopherol diet for a period of 24 weeks. Fresh diets were added to the cages twice a week. The animals were weighed

weekly and monitored on a regular basis for their general health. At each time point mice were killed by cervical dislocation and the genitourinary apparatus (GUT) consisting of the seminal vesicles, prostate and the bladder were isolated for further analyses.

Histopathology: The dorso-lateral prostate was excised and fixed overnight in 10% formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin embedded tissue and mounted on slides. The sections were stained with hematoxylin and eosin to observe any neoplastic changes. Sections were blindly evaluated by a histopathologist to classify PIN lesions. Lesions were classified as PIN I, PIN II, PIN III and PIN IV as described by Park and co-workers. For the purpose of ease, PIN I and PIN II have been grouped as low grade PIN while PIN III and PIN IV have been grouped as high grade PIN.

Immunohistochemistry staining: Sections of 4μm thickness were cut from formalin fixed, paraffin-embedded blocks and mounted on glass slides. The slides were deparafinized in xylene and antigen retrieval enhanced by boiling in 10mM sodium citrate buffer (pH 6.0) for 10 minutes in a microwave oven. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 30 minutes. Non-specific binding was blocked by incubating slides with 5% BSA for 1 hour. Incubation with primary antibody (Nrf2 – 1:100, GSTM1 1:50) was carried out overnight at 4°C. For the subsequent reaction, a streptavidin-biotin complex peroxidase kit was used according to manufacturer's instructions and the slides were counterstained with hematoxylin for 5 minutes. For both Nrf2 and GSTM1 staining, a modified semi-quantitative scoring method was used. The degree of positive staining for all

antibodies was evaluated on a scale from 0-4 for percentage of positive cells and on a scale from 0-3 for strength of staining intensity. The percentage of positive cells was evaluated using the following scale: 0, no staining of the epithelial cells in any field; 1+, <25% of the epithelium stain positive; 2+, 25–50% stain positive; 3+, 50–75% stain positive; and 4+, >75% stain positive. The strength of intensity of staining was estimated using the following scale: 0, no staining of epithelial cells; 1+, mild staining; 2+, moderate staining; and 3+, intense staining. The final total score was generated by adding the score for percentage of positive cells and the strength of stain intensity. Hence, the minimum and maximum score for an area were 0 and 7, respectively.

Immunoblot analysis: The dorso-lateral prostate tissues removed from each treated and control groups were pooled accordingly and homogenized with RIPA buffer (Roche, Manheim, Germany). The protein concentrations were measured by Bicinchonic Acid solution (Pierce, Rockford, IL). 20 μg of protein was loaded onto Biorad pre-cast gels (4-12%) and transferred to polyvinyl difluoride membranes. The membranes were blocked with 5% BSA in TBST for 1 hour followed by overnight incubation with primary antibodies and horse-radish peroxidase conjugated secondary antibody for 1 hour. Protein bands were visualized using Supersignal West Femto (Pierce, Rockford, IL).

RNA extraction and semi-quantitative RT-PCR: Total RNA was isolated with a combined method using Trizol (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA samples were converted to single-stranded cDNA by the Superscript First-Strand Synthesis System III (Invitrogen) and the

resulting cDNA was amplified by the PCR supermix kit (Invitrogen). PCR conditions are as follows: 94°C for 5 min followed by cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The forward and reverse primers used for amplifying UGT1A1 were 5'-GTGGCCCAGTACCTGACTGT-3' and 5'-CGATGGTCTAGTTCCGGTGT-3'. The primers used for amplifying NQO1 were 5'-CAGATCCTGGAAGGATGGAA-3' (forward) and 5'-AAGTTAGTCCCTCGGCCATT-3' (reverse). The forward and reverse primers used for amplifying Nrf2 were 5'-TGAAGCTCAGCTCGCATTGATCC-3' and 5'AAGATACAAGGTGCTGAGCCGCC-3'. Actin was used as an internal control and was amplified with the primers: 5'-TGTTACCAACTGGGACGACA-3' and 5'-TCTCAGCTGTGGTGGTGAAG-3'. PCR products were resolved on 1.5% agarose gels and visualized under UV lamps.

GST assay: Prostate tissue homogenates were centrifuged and the supernatants were analyzed for total GST activity. GST activity was determined by the change in absorbance at 340 nm of chlorodinitrobenzene incubated in the presence of GSH.

Statistics: For a dichotomous measure like the presence or absence of a palpable tumor prior to necropsy Chi square test was used. For all other determinations statistical significance was determined using Students' t test at significance ($p < 0.05$).

5.4 Results

5.4.1 General health observations: The overall health of the mice throughout the study period was found to be good. No significant changes in the body weights were observed in animals given control or tocopherol mixture supplemented diet.

5.4.2 γ-T enriched mixed tocopherol diet reduces tumor incidence in TRAMP mice. A significant reduction in the tumor incidence was observed as recorded by palpation prior to necropsy (Table 5.1). 13 out of 17 animals in the control group versus 2 out of 11 in the treated group developed palpable tumors. Autopsy revealed significant difference between wet weight of the genitourinary apparatus of all animals in control and γ-T enriched mixed tocopherol treated groups. The γ-T enriched mixed tocopherol treated group demonstrated about 60% reduced weight as compared to the control group.

Although primary prostate tumor was not grossly palpable in four control animals at the time of sacrifice, histological analysis revealed a relatively well differentiated carcinoma, marked stromal thickening and hypercellularity. Hematoxylin and eosin staining further revealed multiple layers of atypical cells with significantly reduced luminal space. The epithelial cells demonstrated a characteristic cribiform or tufting growth pattern. High-grade PIN was also characterized by nuclear atypia and an increased mitotic index. Control animals demonstrated ~ 60% incidence of high grade PIN and ~40% incidence of low-grade PIN (Figure 5.1B). Four control animals demonstrated distinct lymph node metastasis. No pulmonary or liver metastases were observed. In contrast, histological analysis of age-matched non-transgenic animals demonstrated the presence of ~100% normal prostatic tissue (Figure 5.1B). Normal

prostatic acinus was characterized by the presence of single-layered columnar epithelial cells and a distinct lumen. Surrounding stroma consisted of layers of loose connective tissue. Histological evaluation revealed that γ-T enriched mixed tocopherol treatment resulted in ~ 50% low grade PIN, ~30% high-grade PIN and 20% normal tissue. Low grade PIN was characterized by a couple of layers of atypical cells that did not fill up the luminal space. The cells displayed tufting or cribiform growth patterns though the underlying fibromuscular sheath in most cases was almost intact. Tumor incidence and decrease in PIN levels by γ-T enriched mixed tocopherol treatment were not a result of suppression of SV-40 transgene expression (results not shown).

5.4.3 Suppression of Phase II detoxifying enzymes during tumor progression: The three enzymes UGT1A1, NQO-1 and GST-M1 were consistently suppressed at 24 weeks compared to 8 week old prostate tissue. Both UGT1A1 and GSTM1 showed a slight peak in the expression levels at 12 weeks followed by reduced expression at 16 and 24 weeks. Conversely, the expression of NQO-1 was relatively high even at 16 weeks but eventually was reduced at 24 weeks (Figure 5.2A). Similarly, the mRNA levels of both UGT1A1 and GSTM1 were reduced at 24 weeks compared to 8 week old samples (Figure 5.2B). However the mRNA levels of NQO-1 were not significantly reduced.

5.4.4 γ-T enriched mixed tocopherol diet increases the expression of phase II detoxifying enzymes: The γ-T enriched mixed tocopherol treatment significantly increased the expression levels of UGT1A1 and GSTM1. Results are as shown in Figure 3A. No effect was observed on the expression levels of NQO-1 (results not

shown). Furthermore, immunohistochemical staining with GSTM1 antibody confirmed the results from western blotting. The results of the immunohistochemistry staining are as depicted in Figure 5.3B. Tocopherol treatment increased the expression of GSTM1 as compared to the control animals. The mean total score for control and tocopherol mixture treated slides was 4.9 ± 1.1 and 6 ± 0.6. This difference was not found to be statistically significant.

Furthermore, an *in vitro* assay to determine the catalytic activity of GST was also performed to further corroborate the results from expression data. The results are as depicted in Figure 5.3C. A comparison with age-matched non-transgenic animals revealed the catalytic activity of GST was significantly suppressed in control animals. Treatment with γ-T enriched mixed tocopherols restored the activity significantly.

5.4.5 γ-T enriched mixed tocopherols induce the expression of antioxidant enzymes: The expression of glutathione peroxidase, heme-oxygenase 1, superoxide dismutase 1 and catalase were significantly suppressed in 24 weeks compared to the age-matched non transgenic prostate tissue samples. The expression of most of these enzymes with the exception of glutathione peroxidase showed a slight increase in expression at 8 weeks followed by complete suppression at 24 weeks. Expression of glutathione peroxidase was completely abolished even in the 8 week old TRAMP prostate samples. However treatment with γ-T enriched mixed tocopherol diet significantly increased the expression levels of all antioxidant enzymes (Figure 5.4).

5.4.6 γ-T enriched mixed tocopherols induce the expression of Nrf2: Tumorigenesis in TRAMP mice progresses with significant suppression of Nrf2 protein and mRNA as depicted in Figure 5.5 A and B. Of note, the enhanced

expression level of Nrf2 at 12 weeks of age corresponds well with the increased expression of UGT1A1 and GSTM1 at 12 weeks as described above. Age advancement in control animals clearly demonstrates low expression of Nrf2 compared to age-matched non transgenic animals. γ-T enriched mixed tocopherols significantly upregulated the expression of Nrf2 as demonstrated by western blotting and immunohistochemistry staining (Figure 5.5C and D). The treated mice clearly demonstrated a stronger staining for Nrf2, indicative of its increased expression levels of the same. The mean scores for control and treated slides were 3.1± 0.8 and 5.2 ± 0.5. This difference was found to be statistically significant ($p<0.05$).

Table 5.1: γ-T enriched mixed tocopherols inhibit prostate carcinogenesis.

	Number of animals	Incidence of palpable tumor [1]	Average wet weight of genitourinary apparatus (gm)
Control	17	13/17 [a]	3.9 ± 1.2 [b]
γ-T enriched mixed tocopherol treated	11	2/11 [a]	1.6 ± 0.71 [b]

[1] Tumors detected by palpation prior to necropsy.
[a] Represents the number of animals that showed presence of tumor upon palpation. Chi's square test was used to compare incidence of tumor between control and treated mice at 24 weeks of age. P values <0.05 were considered significant.
[b] Numbers represent mean ± S.E. Student's t test was used to evaluate significance. P values <0.05 were considered significant.

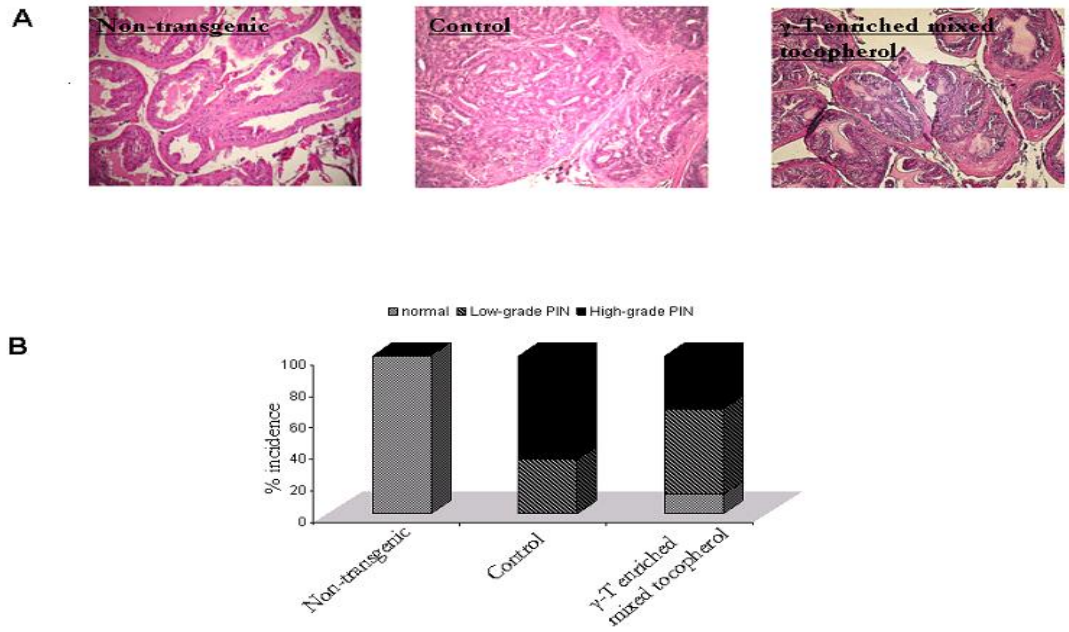


Figure 5.1: γ-T enriched mixed tocopherol diet suppresses tumorigenesis in TRAMP mice. **A)** Representative photomicrographs (100X magnification) of H&E stained dorso-lateral section of non-transgenic, control and treated mice at 24 weeks of age. The genitourinary apparatus was removed *en bloc.* The dorsolateral prostate was microdissected and analyzed for PIN evaluation. **B)** Percentage incidence of normal tissue, low grade and high grade PIN displayed by non-transgenic, control and γ-T enriched mixed tocopherol treated 24 week old mice. The bars represent mean values ± S.E. Statistical significance of the difference was analyzed by Chi's square test. P values < 0.05 were considered significant.

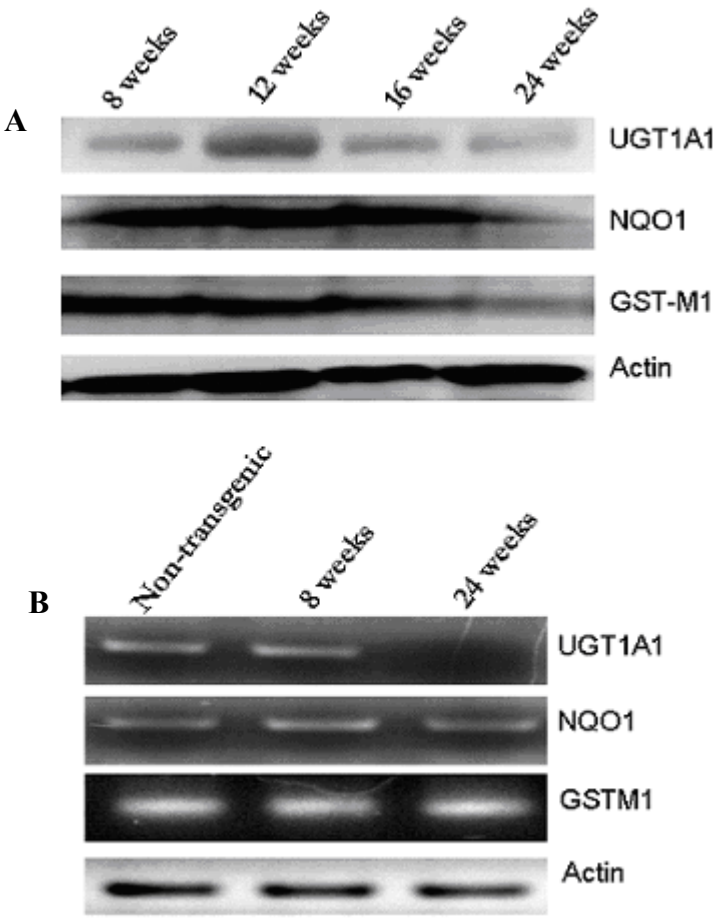
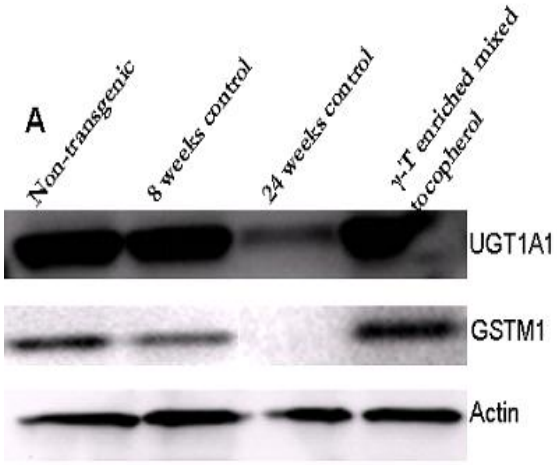
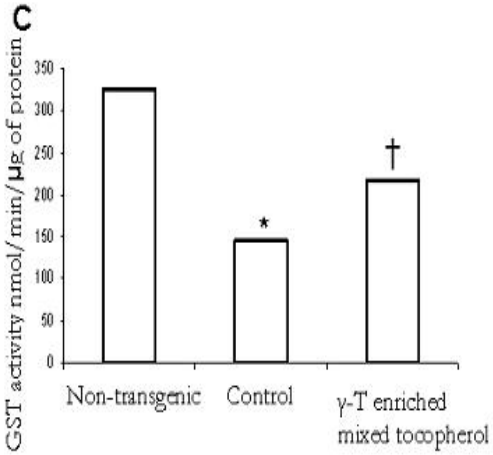
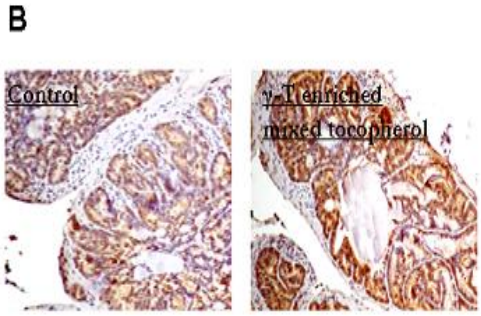


Figure 5.2: Phase II detoxifying enzymes are suppressed during prostate tumorigenesis in TRAMP mice. **A)** Western blot analysis of UGT1A1, NQO-1 and GSTM1 in 8, 12, 16 and 24 week old TRAMP mice. **B)** RT PCR analysis of UGT1A1 (30 cycles), NQO-1 (30 cycles) and GSTM1 (30 cycles) in 24 week old non-transgenic, 8 week and 24 week old TRAMP mice.


A
Non-transgenic
8 weeks control
24 weeks control
γ-T enriched mixed tocopherol
UGT1A1
GSTM1
Actin


C
GST activity nmol/ min/ μg of protein
350
300
250
200
150
100
50
0
*
†
Non-transgenic
Control
γ-T enriched mixed tocopherol


B
Control
γ-T enriched mixed tocopherol

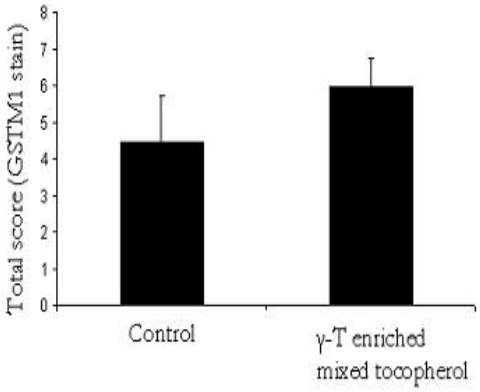

Total score (GSTM1 stain)
8
7
6
5
4
3
2
1
0
Control
γ-T enriched mixed tocopherol

Figure 5.3: γ-T enriched mixed tocopherol diet increases expression of phase II detoxifying enzymes. **A)** Western blot analysis of UGT1A1 and GSTM1 in 24 week old non-transgenic, 8 week and 24 week old TRAMP control and γ-T enriched mixed tocopherol diet treated samples. **B)** Immunohistochemical analysis of GSTM1 in control and γ-T enriched mixed tocopherol diet treated 24 week old mice. Bars represent mean ± S.E. of relative total score of GSTM1 positively stained cells in control and treated 24 week old mice. No statistically significant difference was observed between control and treated samples. **C)** Catalytic activity of GST in non-transgenic, control and treated 24 week old mice. GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate. The reaction product, GSH-DNB conjugate absorbs at 340nm. The rate of increase in absorption is directly proportional to the GST activity in the sample. Bars represent mean values. Students t test was used to evaluate significance. * Significantly different compared to non-transgenic. ‡ Significantly different compared to control.

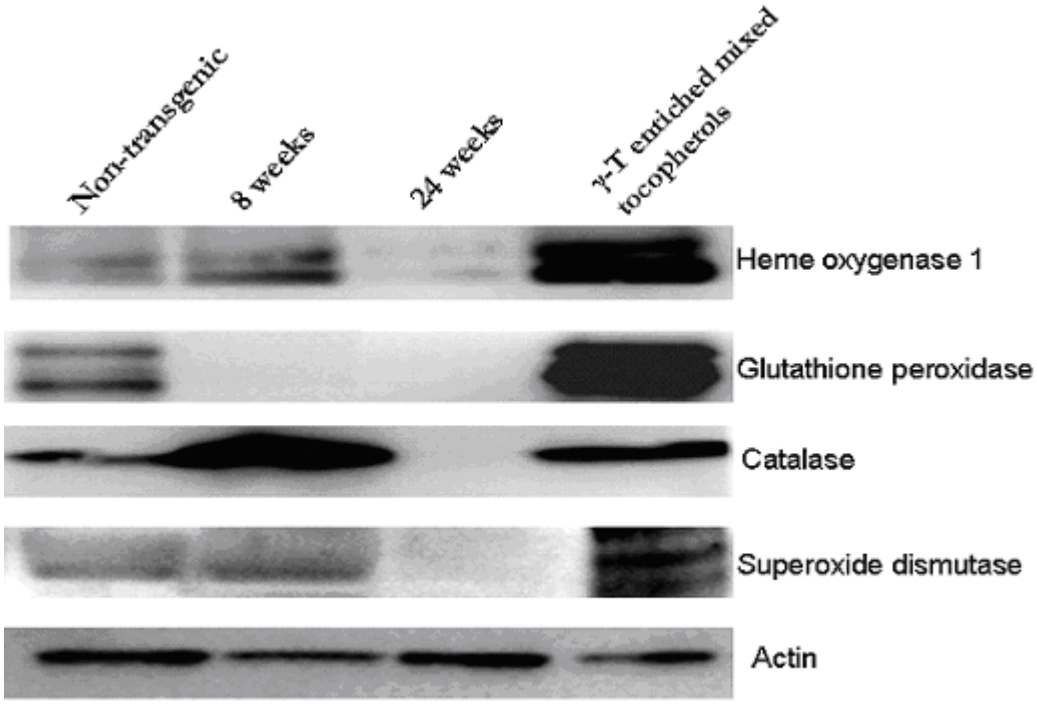


Figure 5.4: γ-T enriched mixed tocopherol diet increases expression of antioxidant enzymes – hemeoxygenase -1, glutathione peroxidase, catalase, superoxide dismutase. The expression of the enzymes was compared between non-transgenic 24 week old, 8 week old TRAMP, 24 week old TRAMP and treated samples. Actin was used as a loading control.

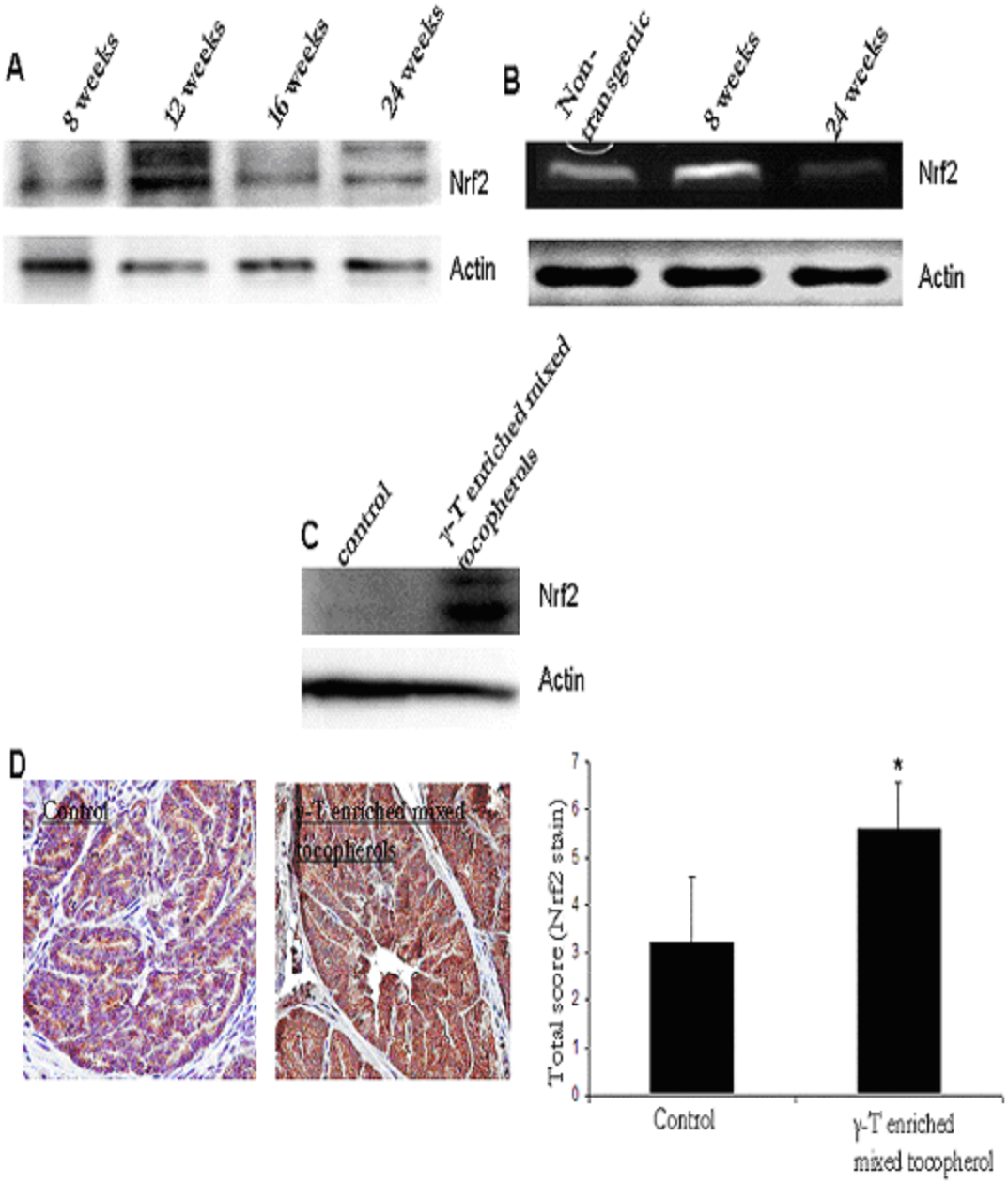

A
8 weeks
12 weeks
16 weeks
24 weeks
Nrf2
Actin
B
Non-transgenic
8 weeks
24 weeks
Nrf2
Actin
C
control
γ-T enriched mixed tocopherols
Nrf2
Actin
D
Control
γ-T enriched mixed tocopherols
Total score (Nrf2 stain)
0
1
2
3
4
5
6
7
*
Control
γ-T enriched mixed tocopherol

Figure 5.5: γ-T enriched mixed tocopherol diet upregulates the expression redox-sensitive transcription factor Nrf2 that is known to mediate the expression pf phase II detoxifying and antioxidant enzymes. **A)** Western blot analysis of Nrf2 in 8, 12, 16 and 24 week old TRAMP mice. **B)** RT PCR analysis of Nrf2 (32 cycles) in 24 week old non-transgenic, 8 week and 24 week old TRAMP mice. The forward and reverse primers are described in *Materials and Methods*. **C)** Western blot analysis of Nrf2 in control and γ-T enriched mixed tocopherol diet treated 24 week old TRAMP mice. **D)** Immunohistochemical analysis of Nrf2 in control and γ-T enriched mixed tocopherol diet treated 24 week old TRAMP mice. Bars represent mean ± S.D. of the relative total score of Nrf2 postively stained cells in control and treated sections. * Significantly different from control using Students' t test. P value <0.05 was considered significant.

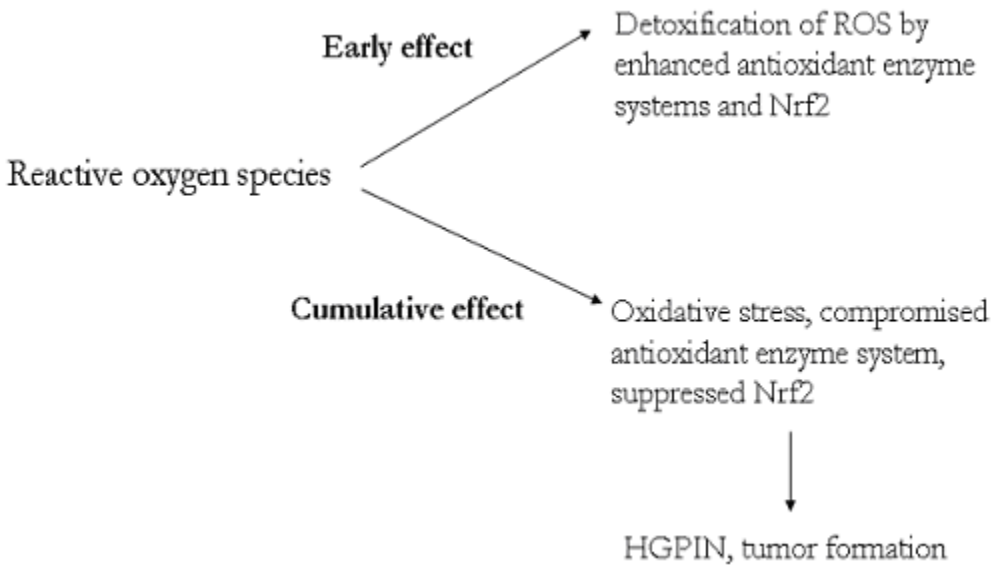


Figure 5.6: A schematic representation of the putative role of reactive oxygen species/oxidative stress and its modulation of Nrf2 and related phase II antioxidant and detoxifying enzymes in prostate carcinogenesis in TRAMP mice.

5.5 Discussion

The efficacy of a 0.1% γ-T enriched mixed tocopherol diet against azoxymethane-induced aberrant crypt foci and N-methyl-N-nitrosourea induced mammary tumors has been recently reported [152, 153]. The present study was undertaken to examine the chemopreventive properties of a similar γ-T enriched mixed tocopherol diet on prostate tumor progression in TRAMP mice – a mouse model that closely mimics and captures most pathological and biochemical events leading to human prostate cancer. γ-T rich diet significantly attenuated PIN levels and the development of tumors. We report that antitumor efficacy of γ-T rich diet is in part due to its antioxidant properties. Nrf2 is a redox-sensitive transcription factor that is involved in the transcriptional regulation of many detoxifying and antioxidant genes. Under normal unstimulated conditions, Nrf2 remains sequestered in the cytoplasm due to actin-bound Keap1. It has been shown that treatment with oxidants such as H_2O_2, oxidative stress or electrophiles results in conformational changes due to oxidation of thiol groups present on Nrf2/Keap1 and/or activate upstream signaling cascades causing dissociation of Nrf2 from Keap1 and nuclear translocation of Nrf2 molecule leading to enhanced expression of phase II detoxifying and antioxidant enzyme expression [161-163]. In a recent study, Kode and coworkers demonstrated that short term treatment with oxidants from cigarette smoke caused dissociation of Nrf2 from Keap1 and nuclear translocation of Nrf2. Long term treatment with oxidants, on the other hand, formed protein carbonyl adducts with the sulfhydryl groups of Nrf2/Keap1 thereby modulating them in a manner such that Nrf2 failed to translocate into the

nucleus and thus did not enhance the transcription of detoxifying and antioxidant enzymes [164].

Since oxidative stress has been shown to be implicated in prostate carcinogenesis, we believe that sustained oxidative stress for long periods may suppress instead of enhance the expression of Nrf2 as evident from our study. Indeed, we observed that although the expression of Nrf2 and certain phase II detoxifying and antioxidant enzymes were enhanced in the prostates of TRAMP mice upto 12 weeks of age, the neoplasms in 24 week old mice expressed low to almost nil levels of Nrf2 and its regulated enzymes. Thus sustained oxidative stress may lead to concerted suppression of Nrf2 and detoxifying enzymes, leading to high-grade PIN and tumor progression in TRAMP mice. Figure 5.6 portrays this dual role played by ROS in modulating Nrf2 protein and thereby carcinogenesis in TRAMP mice. The exact mechanism by which enhanced oxidative stress suppresses Nrf2 is currently being investigated.

Tissues expressing high levels of GST, UGT1A1 and NQO-1 are often protected from cytotoxic damage by electrophiles [165, 166]. The expression and activities of these enzymes and their modulation in tissues that are subjected to physiological or environmental stimuli offers an important tool for monitoring the detoxification potential of cellular systems. The expression of these detoxifying enzymes was significantly suppressed during PIN development and tumor formation in TRAMP mice. On the other hand, treatment with γ-T enriched mixed tocopherol diet restored the activity and expression levels of these enzymes, perhaps, enhancing the detoxification of ROS and effectively retarding PIN and tumor formation. The exact mechanism by which γ-tocopherol exerts these effects is not known. However, in

light of the knowledge that γ-tocopherol possesses chain breaking properties, it may be speculated that it can break oxidative stress induced protein carbonyl adducts thereby breaking the Nrf2/Keap1 dissociation and increase translocation of Nrf2 and thereby transcription/translation of its related genes. However detailed biochemical assays are necessary to prove such interaction [167, 168].

Superoxide dismutases are a family of enzymes responsible for the detoxification of superoxide free radicals. SODs are expressed in many human tissues including the prostate. It has been shown that individuals with homozygous Ala genotype are at a greater risk for prostate cancer. Further, this elevation of prostate cancer risk associated with Ala allele was particularly observed in smokers with low Vitamin E intake indicating that individuals with low antioxidant status are at higher risk for developing PCa [169]. It has been shown that malignant epithelial cells in prostatic adenocarcinoma express no SOD, GPx and catalase. Similarly, Aydin and coworkers have also shown that levels of Cu-Zn SOD and GPx in the erythrocytes of prostate cancer subjects were lower than in benign prostate hyperplasia and control subjects [170]. We have now shown that as age advances in TRAMP mice, SOD, catalase and GPx expression is suppressed. With low GPx activity, catalase alone may be incapable of detoxifying hydrogen peroxide resulting in an accumulation of the same leading to higher production of hydroxy radical. Additionally suppressed SOD causes accumulation of superoxide radicals that are notorious in causing deleterious effects at sites distant from the tumor. Thus, suppression of SOD, catalase and GPx causes the prostate to be predisposed to oxidative insults leading to PIN and tumor

formation. Administering γ-T enriched mixed tocopherol diet perhaps shifts the redox balance within the prostatic mileu ultimately resulting in tumor inhibition.

From the current study, we can infer that tumorigenesis in TRAMP mice occurs with progressive suppression of Nrf2 and related phase II detoxifying enzymes UGT1A1 and GSTM1 and the antioxidant enzymes heme oxygenase 1, catalase, superoxide dismutase 1 and glutathione peroxidase. By exerting an effect on Nrf2 and the antioxidant and detoxifying enzymes, γ-T enriched mixed tocopherol diet perhaps enhanced the detoxification of reactive moieties leading ultimately to suppression of PIN and tumor development. Based on these findings, we believe that mixed tocopherols, and especially γ-tocopherol, may have potential as a chemopreventive agent against the development of prostate cancer.

CHAPTER 6: MIXED TOCOTRIENOLS INHIBIT MURINE PROSTATE CARCINOGENESIS [6].

6.1 Abstract:

The biological activities of the tocotrienols are just being unraveled. Their efficacy against various cancers including breast and prostate cancer has recently been demonstrated. Their *in vivo* efficacy in cancer prevention, though, needs to be established. Herein, we show the efficacy of a mixed tocotrienol diet against prostate tumor progression in a transgenic mouse model that closely mimics human prostate tumors. Male TRAMP mice at 8 weeks of age were fed 0.1 % (n=8), 0.3 % (n=9) or 1 % (n=9) mixed tocotrienols in AIN-76 A diet upto 24 weeks of age. Likewise, a positive control group consisting of male TRAMP mice (n=11) fed regular AIN -76 A diet and a negative control group consisting of mice (n =4) that were non transgenic wild types fed regular AIN-76 A diet upto 24 weeks of age were also included. Our results showed that mixed tocotrienol fed groups had a lower incidence of tumor formation along with a significant reduction in the average wet weight of genitourinary apparatus ($p<0.05$). Mixed tocotrienols significantly reduced the levels of high grade neoplastic lesions as compared to the positive controls. This decrease in levels of high grade neoplastic lesions was found to be the result of increased expression of pro-apoptotic proteins BAD and cleaved caspase-3 and cyclin dependent kinase inhibitors p21 and p27 ($p<0.01$). Expression of cyclins A and E were found to be substantially decreased following mixed tocotrienol administration ($p<0.01$).

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6 This chapter is submitted to '*Nutrition and Cancer*' for review.

Together, our results show that by modulating cell cycle regulatory proteins and increasing expression of pro-apoptotic proteins, mixed tocotrienols effect prostate tumor suppression in TRAMP mice.

6.2 Introduction:

Interest in the use of alternative and complementary therapies, mainly nutraceuticals as chemopreventives is high in prostate cancer. The reason being that prostate cancer is the second leading cause of cancer related death among men in the United States [171] and this disease which initially responds well to hormone ablation, most often than not becomes hormone refractory ultimately resulting in death [172]. This disease has a long latency period and hence offers a unique window for chemoprevention [173]. Chemoprevention may not necessarily inhibit tumor formation completely but may help in delaying the progression from neoplasms to a more advanced state of the disease. This delay in progression is thought to be the result of modulation of complex signal transduction pathways that lead up to tumor formation [174].

The natural vitamin E family includes eight chemically distinct molecules: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol. Structurally, vitamin E consists of a chroman ring with either a saturated side chain (tocopherols) or an unsaturated side chain (tocotrienols). Isoforms of tocotrienol differ from each other based on the methyl groups on the chroman ring (Figure 6.1). For chemoprevention research, α – tocopherol is among the most studied component of Vitamin E. The biological activities of the other isoforms are largely unknown and being unraveled. This represents a major void in Vitamin E research. Significance of the void is enhanced

by the observation that the biological functions of the different homologues of natural vitamin E are not identical. We and other researchers have shown that γ – tocopherols are potent against various cancers. Likewise, during the last couple years, tocotrienol research has also gained substantial momentum. Palm oil is one of the richest sources of tocotrienols. Tocotrienols extracted from crude palm oil mainly consist of a mixture of α-, γ-, and δ-tocotrienols and some α-tocopherols, referred to as tocotrienol-rich fraction (TRF). Tocotrienol-rich fraction from palm oil has been shown to inhibit proliferation and growth of human breast cancer cells [175-177]. Recent studies have shown that γ-tocotrienols inhibit cell proliferation by decreasing Akt and activation of NF-κB, implicated in the regulation of cell growth, cell cycle, and apoptosis [178]. More recently, researchers have demonstrated that TRF imparts differential anti-proliferative and apoptotic effects in human prostate cancer cells versus normal cells and preferentially sensitizes xenograft bearing nude mice to radiation therapy [179, 180]. Based on the documented anticancer efficacy of TRF, we evaluated the chemopreventive efficacy of a mixed tocotrienol diet against prostate cancer growth and progression in the TRAMP mouse model.

The TRAMP mouse model was developed in C57BL/6J mice using minimal rat probasin promoter to drive the expression of SV40 early genes specifically in prostatic epithelium. These mice demonstrate the progression of the disease from early PIN (prostatic intraepithelial neoplasia) lesions to a more aggressive metastatic adenocarcinoma which closely mimics the various stages in human prostate cancer. Since this model bears close relevance to the human form of the disease we evaluated the efficacy of mixed tocotrienols in this mouse model. We present data to

demonstrate a dose dependent increase in chemopreventive efficacy of mixed tocotrienols against prostate cancer in the TRAMP mice.

6.3 Materials and Methods:

Animals and study design: Female hemizygous C57BL/TGN TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were bred on same genetic background and maintained in the Animal Care Facility of Rutgers University. Housing and care of the animals was in accordance with the guidelines established by the University's Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP x C57BL/6] F1 or as [TRAMP x C57BL/6] F2 offspring. Identity of transgenic mice was established by the PCR-based DNA screening. Throughout the experiment the animals were housed in cages with wood chip bedding in a temperature-controlled room (68˚F -72˚F) with a 12 hour light dark cycle, at a relative humidity of 45% to 55%.

Diets and study design: All animals were fed AIN-76A diet obtained from Research Dyets (Easton, PA). Treated animals were fed 0.1, 0.3 or 1 % mixed tocotrienols incorporated in AIN-76A diet. The mixed tocotrienols (Tocomin®) were a kind gift from Carotech Bhd (Perak, Malaysia) and contained D-α tocotrienol (12-14%), D-β tocotrienol (1%), D-γ tocotrienol (18-20%), D-δ tocotrienol (4-6%) and D- α-tocopherol (12-14%). All the animals were put on AIN-76A diet one week prior to the study. The control animals (n=11) received AIN-76A diet throughout the

experiment while the treatment group received 0.1 % (n=8), 0.3 % (n=9) or 1 % (n=9) mixed tocotrienol diet for a period of 24 weeks. Fresh diets were added to the cages twice a week. The animals were weighed weekly and monitored on a regular basis for their general health. At each time point mice were killed by cervical dislocation and the genitourinary apparatus (GUT) consisting of the seminal vesicles, prostate and the bladder were isolated for further analyses.

Histopathology: The dorso-lateral prostate was excised and fixed overnight in 10% formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin embedded tissue and mounted on slides. The sections were stained with hematoxylin and eosin to observe any neoplastic changes. Sections were blindly evaluated by a histopathologist to classify PIN lesions. Lesions were classified as PIN I, PIN II, PIN III and PIN IV as described by Park and co-workers. For the purpose of ease, PIN I and PIN II have been grouped as low grade PIN while PIN III and PIN IV have been grouped as high grade PIN.

Immunoblot analysis: The dorso-lateral prostate tissues removed from each treated and control groups were pooled accordingly and homogenized with RIPA buffer (Roche, Manheim, Germany). The protein concentrations were measured by Bicinchonic Acid solution (Pierce, Rockford, IL). 20 μg of protein was loaded onto Biorad pre-cast gels (4-12%) and transferred to polyvinyl difluoride membranes. The membranes were blocked with 5% BSA in TBST for 1 hour followed by overnight incubation with primary antibodies and horse-radish peroxidase conjugated secondary antibody for 1 hour. Protein bands were visualized using Supersignal West Femto (Pierce, Rockford, IL).

Statistics: Chi square test was used to assess the significance of incidence of palpable tumor. For all other determinations statistical significance was determined using Students' t test at significance ($p< 0.05$ or $p<0.01$).

6.4 Results:

6.4.1 General health observations: The overall health of the mice throughout the study period was found to be good. No significant changes in the body weights were observed in animals given control or mixed tocotrienol supplemented diet.

6.4.2 Mixed tocotrienols reduce tumor incidence in TRAMP mice. A significant reduction in the tumor incidence was observed as recorded by palpation prior to necropsy (Figure 6.2A). 8 out of 11 animals in the control group versus 3 out of 8, 3 out of 9 and 2 out of 9 animals in the 0.1, 0.3 and 1 % mixed tocotrienols treated groups developed palpable tumors. Autopsy revealed significant difference between wet weight of the genitourinary apparatus (GUT) of all animals in control and mixed tocotrienol treated groups (Figure 6.2B). The 0.3 and 1 % mixed tocotrienols significantly decreased the weight as compared to the control group ($p<0.05$). The decrease in GUT weight by 0.1 % mixed tocotrienols was not statistically significant.

Histological analysis was performed to determine percentage levels of PIN in the animals (Figure 6.3). A brief description of the histology displayed by normal prostatic tissue, low grade and high grade PIN is as follows: Normal prostatic acinus was characterized by the presence of single-layered columnar epithelial cells and a distinct lumen. Surrounding stroma consisted of layers of loose connective tissue. Low grade PIN was characterized by a couple of layers of atypical cells that did not

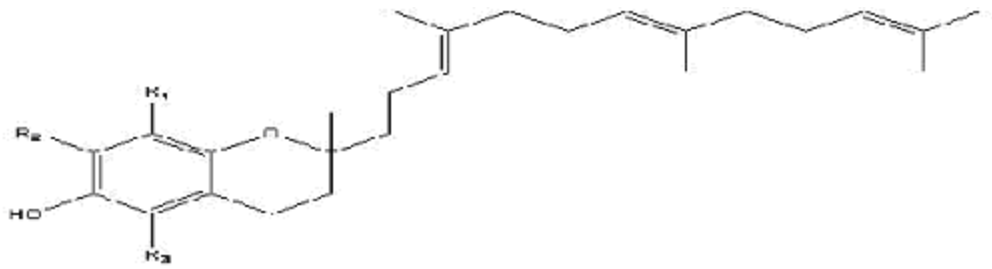
fill up the luminal space. The cells displayed tufting or cribiform growth patterns though the underlying fibromuscular sheath in most cases was almost intact. High-grade PIN was characterized by multiple layers of atypical cells with significantly reduced luminal space. The epithelial cells demonstrated a characteristic cribiform or tufting growth pattern.

All non transgenic animals displayed approximately > 95 % normal prostatic tissue and < 5 % low grade PIN. Histological analysis of the all control animals including the ones without a palpable tumor revealed well differentiated carcinoma, marked stromal thickening and hypercellularity. Control animals demonstrated ~ 75-80 % incidence of high grade PIN and ~25-30 % incidence of low-grade PIN (Figure 6.3B). Lymph node metastasis was evident in control mice bearing tumors. No pulmonary or liver metastases were observed. 0.1 % mixed tocotrienol treatment decreased the percentage of high grade PIN by 40 %. On the other hand, 0.3 and 1 % mixed tocotrienol treatment decreased the percentage of high grade PIN resulting in higher percentages of low grade PIN, significantly ($p<0.05$). Immunoblot analysis for SV-40 transgene expression revealed that tumor incidence and decrease in PIN levels by mixed tocotrienols were not a result of suppression of transgene expression (results not shown).

6.4.3 Mixed tocotrienols effect apoptosis and cell-cycle control proteins. The suppression of tumor formation by mixed tocotrienols led us to investigate their effects on pro-apoptotic and cell cycle regulatory proteins. Figure 6.4 demonstrates the immunoblot analysis of pro-apoptotic proteins BAD and cleaved caspase 3 as well

as cyclins and cyclin dependent kinase inhibitors with the densitometric data after adjusting with beta-actin as a loading control (Note beta-actin blot not shown).

Results showed that the mixed tocotrienols diet enhanced expression of pro-apoptotic proteins BAD and cleaved caspase 3 (~ 1.5 to 3 fold and 2 to 3 fold over control, respectively). The prostatic samples from control animals demonstrated significantly increased expression of cyclin E and cyclin A ~3.5 and 2.2 fold, respectively over the non transgenic controls. Likewise the suppression of cyclin-dependent kinase inhibitor p21 and p27 were significantly suppressed ~ 0.2 fold below the non transgenic control animals. Interestingly, the non transgenic prostatic samples demonstrated low expression of p27 as compared to p21. Mixed tocotrienol diet significantly increased the expression of p21 and p27 several folds over the control and suppressed the expression of both cyclin A and cyclin E. Together, these results demonstrate that tocotrienols decrease the expression of cyclins and increase the expression of Cdk inhibitors thus regulating cell cycle progression ultimately leading to increased apoptosis and tumor suppression.



R1= R2=R3=CH_3	α-tocotrienol
R1= R3=CH3, R2=H	β-tocotrienol
R1=H, R2=R3=CH3	γ-tocotrienol
R1=R2=H, R3=CH3	δ -tocotrienol

Figure 6.1: Structures of the different isomers of tocotrienols.

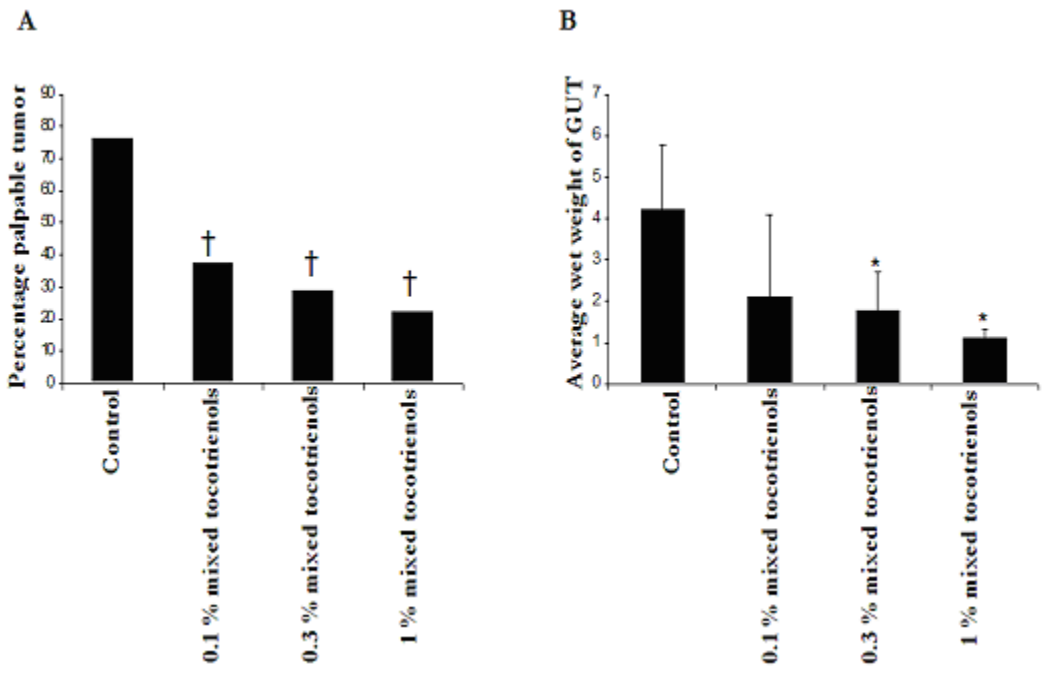


Figure 6.2: Mixed tocotrienols suppress tumor incidence in TRAMP mice. **A)** Percentage incidence of palpable tumor in the control and mixed tocotrienol treated groups. The tumor was detected by palpation prior to necropsy. **B)** Average wet of the genitourinary apparatus of control and tocotrienol treated groups. At the time of necropsy, the genitourinary apparatus consisting of seminal vesicles, prostate and bladder was removed *en bloc* and weighed. The bars represent mean values ± S.E. Statistical significance of the difference between control and treated groups was analyzed by Student's t test. P values < 0.05 were considered significant.

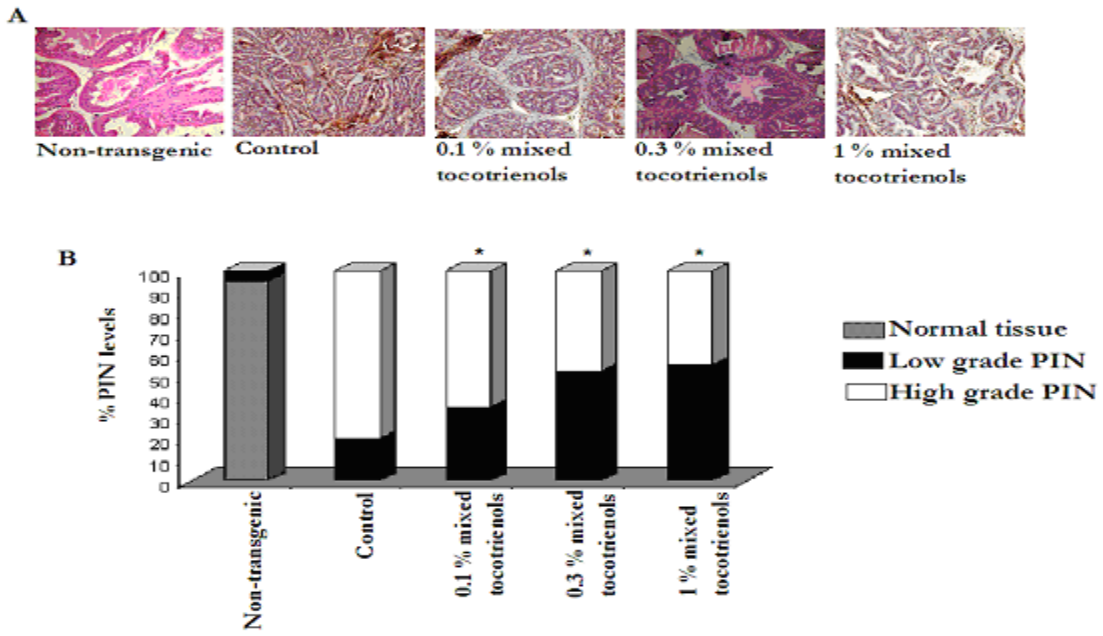


Figure 6.3: Mixed tocotrienols inhibit tumorigenesis in TRAMP mice. **A)** Representative photomicrographs (100X magnification) of H&E stained dorso-lateral section of non-transgenic, control and mixed tocotrienol fed mice at 24 weeks of age. B) Percentage levels of normal tissue, low grade and high grade PIN displayed by non-transgenic, control and mixed tocotrienol fed 24 week old mice. At the time of necropsy, the genitourinary apparatus was removed *en bloc.* The dorsolateral prostate was microdissected and analyzed for PIN evaluation. Normal tissue was characterized by single-layered epithelial cells and a distinct lumen, low grade PIN typically displayed a couple of layers of atypical cells that did not, however, fill up the luminal space and high grade PIN characterized by marked stromal thickening and hypercellularity and significantly reduced luminal space. The bars represent mean values ± S.E. Statistical significance of the difference between control and treated groups was analyzed by Student's t test. P values < 0.05 were considered significant.

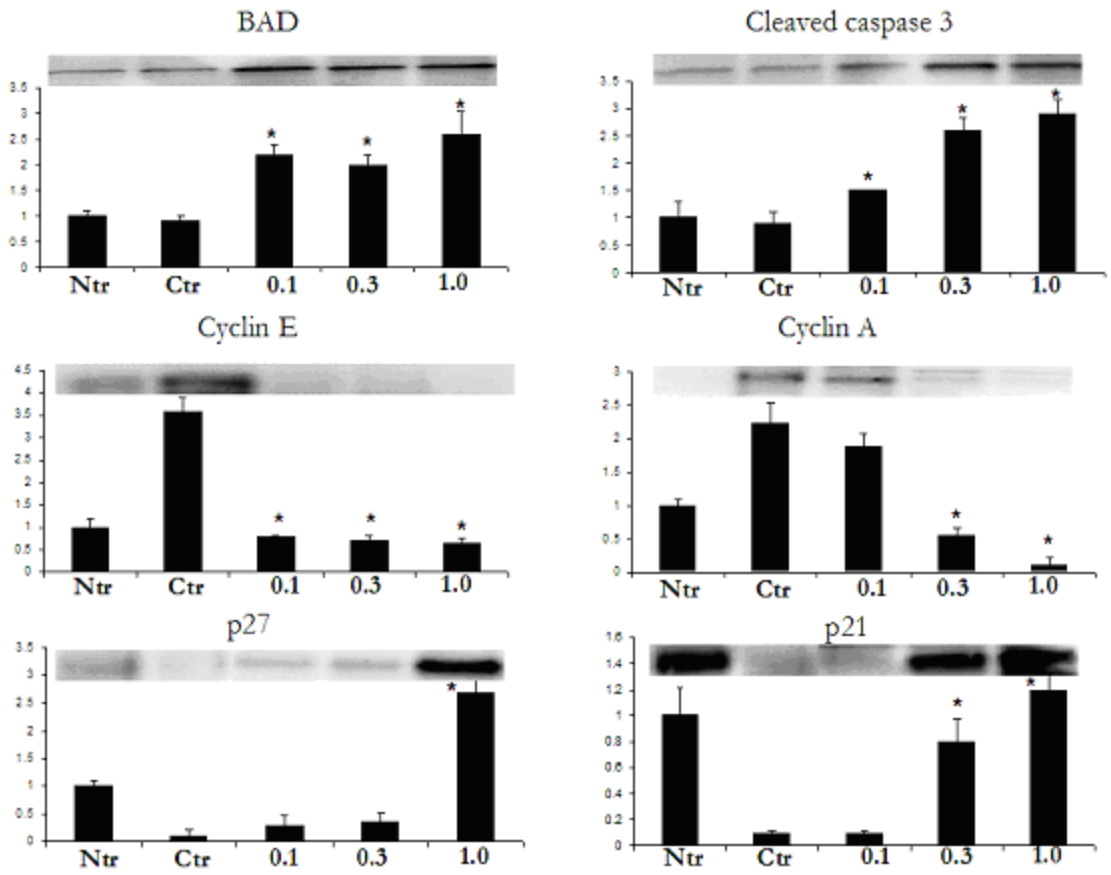


Figure 6.4: Mixed tocotrienols alter the expression levels of apoptotic proteins BAD and cleaved caspase 3 and cell cycle regulatory proteins cyclin E, cyclin A, p21 and p27. Densitometric analysis of the band intensity of each protein was adjusted with β-actin (loading control) and is shown as histograms. Note β-actin band is not shown. Ntr: Non-transgenic control, ctr: control, 0.1: 0.1 % mixed tocotrienol fed, 0.3: 0.3 % mixed tocotrienol fed, 1: 1% mixed tocotrienol fed. The bars represent mean values ± S.E. Statistical significance of the difference between control and treated groups was analyzed by Student's t test. P values < 0.01 were considered significant.

6.5 Discussion:

A direct link between the incidence of cancer and nutrition has been long established. Cancer chemoprevention entails the use of select agents preferably of a dietary source to prevent cancer. Chemoprevention may not essentially inhibit tumor formation all together but may help in preventing the progression of neoplastic lesions to fully developed tumor [174]. In our view, chemoprevention may be most useful to prevent cancers that do not have a remedial effective chemotherapeutic agent and those that have long latency periods like prostate cancer. Prostate cancer is the second leading cause of cancer related deaths among men in the United States. Prostate cancer patients usually respond well to hormone ablation but in the later stages, this disease becomes hormone-refractory. Unfortunately, this hormone refractory stage of the disease has no effective treatment options. In prostate cancer patients, the progression from benign to neoplastic lesions to fully developed tumors may take several years and hence this disease provides a window for intervention by chemopreventive compounds.

Vitamin E, an important micronutrient in our diets, exists as eight isoforms. Owing to its unique structure (a chroman head with a phytyl side chain) it can behave both as a pro-oxidant and an anti-oxidant. Emerging evidence in the literature suggests Vitamin E as a potential chemopreventive agent [181, 182]. Support for the possible use of Vitamin E as a chemopreventive came from a study that demonstrated high incidence of prostate cancer in subjects with low nutritional intake of Vitamin E [183]. However, most of the Vitamin E chemoprevention research involves efficacy of α-tocopherol. The biological activities of the other isoforms of Vitamin E were largely

unknown till recent. We and others have shown that γ-tocopherols are effective against prostate, breast as well as colon cancer [180, 184, 185]. Likewise, only recently, the potency of tocotrienols against cancer has been established.

In this report, we show that mixed tocotrienols are effective in preventing prostate cancer growth in a transgenic mouse model (TRAMP). Prostate cancer progression in this mouse model closely resembles that in humans and therefore, provides a useful tool to test efficacy of chemopreventive compounds.

Mice that were fed mixed tocotrienols demonstrated low incidence of tumor detected by palpation. Histopathology revealed that mixed tocotrienol treatment significantly decreased the levels of high grade neoplastic lesions while increasing the levels of low grade PIN. No characteristics of normal tissue were detected in the tocotrienol treated groups. The anti-prostate cancer effect of tocotrienols was accompanied by significant increase in expression of pro-apoptotic proteins BAD and cleaved caspase-3 as well as cyclin dependent kinase inhibitors p21 and p27. Tocotrienol treatment significantly suppressed the expression of cyclins E and A. The expression of cyclin D1 did not vary much with tocotrienol treatment.

The cyclins are an integral part of the cell cycle process. Cell cycle progression is regulated by a series of cyclin dependent kinases (Cdks). The Cdks are regulated by activating (CDK-activating kinases) and inhibitory (Wee) phosphorylations as well as Cdk inhibitors. The Cdk inhibitors are known to bind to the cyclin-Cdk complexes and inhibit their activity. The Cip/kip family of Cdk inhibitors includes p21, $p27^{Kip1}$ and p57 [186]. We observed enhanced expression of the cyclins and suppression of p21 and p27 in the prostatic lysates of control animals and this demonstrates

unstoppable cell cycle leading to vast increase in cell numbers ultimately leading to tumor formation. The expression of p21 and p27 has been shown to be significantly downregulated in various cancers including prostate cancer [187]. A recent study demonstrated that human subjects maintaining a high expression of p27 had significantly longer disease-free intervals and longer survival as compared to subjects with low p27 expression [188-190]. In the present study, we observed that tocotrienol treatment reversed this effect by significantly enhancing the expression of p21 and p27. Increased levels of p21 and p27 inhibit the kinases thereby in capacitating the cyclins. This cell cycle arrest may drive the cell into apoptosis by recruiting caspases and BAD. Thus, the anti-cancer effects of tocotrienols, at least in part, could be the result of mediation of the CDK inhibitors/ cyclin axis.

In summary, the novel findings, we report here are that mixed tocotrienols inhibited prostate tumor growth in TRAMP mice without any signs of toxicity or affecting the expression of the transgene SV-40. Mixed tocotrienols significantly suppressed the progression of high grade prostatic neoplastic lesions to fully developed tumor by modulating the cell cycle and affecting the expression of pro-apoptotic proteins. These findings further support the potential use of tocotrienols as prostate cancer chemopreventive agents in humans.

CHAPTER 7: METABOLISM & PHARMACOKINETICS OF KAEMPFEROL IN THE RAT [7].

7.1 Abstract:

The purpose of this study was to examine the metabolism and pharmacokinetics of Kaempferol in the rat. The current report presents evidence for phase I NADPH dependent oxidative metabolism of Kaempferol to Quercetin and phase II UDPGA dependent conjugation using both liver and small intestinal microsomes. Rats bearing indwelling jugular vein cannulae were administered Kaempferol intravenously (10 and 25 mg/kg) or orally (100 and 250 mg/kg). The concentration of Kaempferol was assayed using HPLC and metabolites were identified using authentic standards by cochromatography. Non compartmental analysis was performed to calculate the pharmacokinetic parameters. The Cl_{total} values after IV and oral dose were high with large volumes of distribution. The Cl_{renal} values did not significantly differ between IV and oral doses. The dose normalized AUC values after oral administration did not superimpose each other. The bioavailability and rate of absorption constant ka decreased with higher doses. The low oral bioavailability of Kaempferol was due to moderate absorption as well as significant first pass effects. Most of the aglycone existed in its conjugated glucuronide forms or as metabolites identified to be Quercetin and Isorhamnetin which were also further glucuronidated. From this data it is evident that Kaempferol is moderately absorbed, extensively metabolized and is rapidly excreted.

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7 This chapter is being considered for submission to '*Nutrition Research*' for review.

7.2 Introduction

Kaempferol (3, 4', 5, 7-tetrahydroxyflavone) is a flavonoid that is widely distributed in onion, kale, endive and tea. It has been shown to exhibit chemopreventive efficacy against a wide variety of cancers including hepatocellular carcinoma, breast, lung and prostate. Its chemopreventive attributes are a combined result of its ability to inhibit the phase I carcinogen activating genes and enzyme levels while induce the phase II detoxifying genes and enzyme levels [191]. It has been reported that on account of its behavior as a pro-oxidant it induces oxidative stress resulting in apoptosis in cancer cells. There is plenty of evidence relating its chemopreventive efficacy to its antioxidant properties as well [192-194].

To better understand the *in vivo* pharmacological properties of any chemopreventive agent, it is important to have a thorough understanding of its metabolism and pharmacokinetics. The metabolism of Kaempferol has been studied to some extent. Quercetin has been identified as the product of oxidative metabolism while the 7-O-glucuronide has been identified as the major product of conjugative metabolism using rat liver microsomes [195-197] . In this report we compare the rates of oxidative and conjugative metabolism between rat liver and small intestinal microsomes. In addition by performing detailed pharmacokinetic studies we have examined the absolute bioavailability of Kaempferol in the rat. In our view, such a study examining both metabolism and pharmacokinetics of Kaempferol in the rat has not performed and was deemed important.

7.3 Materials and Methods

Materials: Kaempferol was purchased from Indofine Chemicals (Hillsborough, New Jersey). All solvents were of HPLC grade (Fisher Scientific)

Incubations with NADPH or UDPGA fortified microsomes: NADPH-Dependent Phase I Metabolism: Liver and small intestinal microsomes were prepared as described elsewhere. Phase I metabolism of Kaempferol was studied using Sprague-Dawley rat liver and small intestinal microsomes. Incubations in a final volume of 200µL consisted of microsomes (0.1 mg protein/ml) suspended in 100 mM potassium phosphate buffer (pH 7.4) and varying concentrations of Kaempferol. Reaction was initiated by the addition of 5mM NADPH. Reactions were carried out for various time points upto 120 minutes at 37°C and stopped by equal volume addition of cold methanol containing the IS. After vortex-mixing and spinning in a centrifuge (14,000 rpm for 10 min), supernatants were analyzed by HPLC. Turnover was calculated using a standard curve derived by incubating Quercetin with cold microsomes. K_m and V_{max} values were obtained using WinNonlin (Pharsight, Mountainview, CA).

UDPGA-dependent Phase II metabolism: Phase II metabolism of Kaempferol was studied using Sprague-Dawley rat liver and small intestinal microsomes. Incubations in a final volume of 200µL consisted of microsomes (0.1mg protein /ml) suspended in 100 mM potassium phosphate buffer (pH 7.4) with alamethicin (1 µg/10 µg protein) and varying concentrations of Kaempferol. Reaction was initiated by the addition of 2 mM UDPGA. Reactions were carried out for various time points upto 120 minutes at 37°C and stopped by addition of equal volume of cold methanol containing the IS. The samples were vortex mixed followed by centrifugation at

14,000 rpm for 10 minutes and the supernatents were analyzed by HPLC. Turnover was calculated as the individual glucuronide peak area divided by the sum of glucuronide and parent peak areas. Both K_m and V_{max} parameters were calculated using WinNonlin (Pharsight, Mountainview, CA).

Animal Treatment: Male Sprague Dawley rats (250-300 gms) bearing indwelling jugular vein or portal vein cannulae were obtained from Hilltop Labs (Scottsdale, PA). The animals were housed at the Animal Care facility at Rutgers University under 12 hour light day cycles with free access to food and water. The animals were allowed to acclimatize for 3 days before commencement of the study during which time they were put on an antioxidant free AIN-76A diet (Dyets Inc, PA). On the day of the study, the cannulae were exteriorized on the dorsal side of the neck and connected to a long polyethylene tube wrapped in a wire coil (Instech, Plymouth, PA) for blood collection. Heparinized saline (50 U/ml) was used to flush the cannula. Rats (n=4) were also administered kaempferol (10 mg/kg and 25 mg/kg) in a vehicle composition of cremaphor/ tween-80/ PEG/ ethanol and water (2:1:1:1:5) intravenously in a final injection volume of 0.15ml. Rats (n=5) were fasted overnight and administered kaempferol (100 mg/kg) in a similar vehicle composition by oral gavage in a final volume of 0.7 ml. Likewise an oral dose of 250 mg/kg was also administered to the rats (n=4), however, due to solubility issues Kaempferol was suspended in corn oil. Blood samples (0.2 ml) were withdrawn at regular time intervals for upto 24 hours following which 0.2 ml of heparinized saline was flushed into the cannula. Blood samples were centrifuged to obtain plasma. The plasma

samples were acidified with 50 µL of acetic acid (0.5 mol/L) and then stored at -20 °C until further analysis.

To assess gastro-intestinal first pass effects male rats bearing indwelling portal vein cannulae were used. Rats (n=3) were administered kaempferol (100mg/kg) in a similar vehicle composition by oral gavage in a final volume of 0.7 ml. Portal blood was withdrawn at regular intervals for upto 24 hours and were immediately centrifuged to obtain plasma. The portal plasma samples were acidified with 50 µL of acetic acid (0.5 mol/L) and then stored at -20 °C until further analysis.

In addition urine from each animal was also collected over a period of 96 hours post IV and oral dose. The time intervals for urine collection were as follows 0-4, 4-8, 8-24, 24-48, 48-72 and 72-96 hours. The volume of urine collected at each time interval was recorded. The urine samples were likewise stored at -20 °C until further analysis.

Sample preparation: One aliquot of plasma (50µl) spiked with 0.1 µg/ µl Biochanin A as IS was extracted twice with a mixture of ethylacetate: methanol (95:5 v/v). The layers were separated by centrifugation 4000g for 10 minutes. The combined organic extracts were dried under nitrogen followed by reconstitution in 100 µl of mobile phase B. Another aliquot of plasma (50 µl) was incubated with 1 X 10^5 U/l β-glucuronidase/ sulfatase (crude extract from Helix pomatia, Sigma- Aldrich, MO) at 37°C overnight followed by addition of IS and subsequent extraction as described earlier. Similar extraction procedure using a 50µl urine aliquot was followed for extraction of K and its metabolites.

HPLC conditions: A Shimadzu HPLC system (SCL-10A VP) consisting of a binary pump (FCV-10AL VP), an autosampler (SIL-10AD VP) that was maintained at 4 °C,

and a UV-Vis detector (SPD-10AV VP). Reverse phase chromatography was performed with an analytical Shimadzu C18 column (240 mm × 2.0 mm, 5-µm, Shimadzu, MD) protected with a SecurityGuard™ cartridge system (Phenomenex) and a 0.45-µm in-line filter.

The aqueous phase (Mobile phase A) and organic phase (Mobile phase B) used were water: acetonitrile: trifluoroacetic acid (90:10:0.1 v/v/v) and acetonitrile: water: trifluoroacetic acid (95:5:0.1 v/v/v), respectively. The flow rate was set at 0.7 ml/min. Chromatographic separation was achieved using a linear gradient elution from 0- 25 minutes from 25 % Mobile phase B to 100 % Mobile phase B. Mobile phase B was maintained at 100 % for an additional 2.5 minutes followed by return to initial mobile phase conditions in 2.5 minutes. Peaks of the HPLC chromatograms were evaluated using Class-VP program (7.1.1, Shimadzu, MD).

The various flavonols extracted from the plasma samples were identified by comparing the retention times with those of the authentic standards. The retention times for M1 (Quercetin), Kaempferol, M2 (Isorhamnetin) was found to be 8.8 ± 0.2, 10.3 ± 0.3, and 11.4 ± 0.2 minutes respectively. The absolute recovery of Kaempferol was 89 %. The absolute recoveries for Quercetin, Isorhamnetin and Biochanin A were found to be 92 %, 88 % and 71 %, respectively. Peaks were considered if the peak height was atleast five times the baseline.

Pharmacokinetic analysis: Pharmacokinetic analysis was carried out using WinNonlin software package (Pharsight, Mountain View, CA). Noncompartmental analysis was used to calculate the other parameters. Based on the cumulative urinary excretion, fraction excreted in the urine (fe is calculated by dividing total cumulative

amount of flavonoid excreted in urine by the dose), renal clearance was calculated by multiplying fe by Cl_{plasma}.

Statistics: Experimental values are expressed as mean ± SE. Statistical analysis was performed using Student's t test. A p value of < 0.05 was considered significant.

7.4 Results:

7.4.1 In vitro metabolism of Kaempferol: Table 7.1 summarizes the percentage of parent remaining after a 120 minutes incubation of Kaempferol with rat liver and small intestinal microsomes fortified with NADPH or UDPGA. The glucuronidation rate for Kaempferol as a function of concentration was measured in both small intestinal and liver microsomes. The corresponding kinetic parameters are listed in Table 7.2. V_{max} values for liver microsomes were consistently high in the hepatic microsomes as compared to the small intestinal microsomes. Likewise the Michelis Menten constant K_m was generally smaller for the hepatic microsomes as compared to the small intestinal microsomes. The rate of formation of metabolite M1 as a function of concentration of Kaempferol was also measured in small intestinal and liver microsomes. Similar to the glucuronidation profiles, the liver microsomes demonstrated lower K_m and higher V_{max} values as compared to the small intestine, thus, signifying the role of hepatic metabolism over small intestinal metabolism. Meanwhile, the higher propensity for phase II UDPGA mediated conjugation reactions as compared to phase I NADPH mediated oxidative reactions is also evident. Thus metabolic clearance due to conjugation appears to be more efficient than phase I oxidation.

The metabolic profile between rat liver and small intestinal microsomes was identical except that the rate for formation of M1 was slower in the small intestinal microsomes. The phase I NADPH mediated oxidative metabolism resulted in the formation of a single metabolite M1 (rt: 8.8 ± 0.2 minutes). Based on retention time match M1 was identified to be Quercetin. Phase II UDPGA mediated conjugation reaction led to the formation of 4 glucuronide peaks (G1 –G4). These peaks were identified to be glucuronide peaks following glucuronidase incubation resulting in their disappearance (results not shown). G1 and G4 were smaller peaks and their peak areas did not change significantly with time. G2 (rt: 5.1 ± 0.1 min) and G3 (rt: 5.7 ± 0.1 min) on the other hand were more predominant peaks. Differences in the rate of formation for G2 and G3 were observed between rat liver and small intestinal microsomes. G3 (rt: 5.7 ± 0.1 min) appeared to be the more predominant peak in the liver microsomes as compared to G2 (rt: 5.1 ± 0.1 min) that was the predominant peak in the small intestinal microsomes. The chromatograms depicting the in vitro metabolic profiles are as shown in Figure 7.1.

7.4.2 Pharmacokinetics and oral bioavailability of Kaempferol in rats: The pharmacokinetic parameters following IV and oral doses of K are listed in Table 7.3. Figure 7.2A shows the time-course changes in the plasma concentration of K following intravenous administration (10 mg/kg and 25 mg/kg). The plasma profile is best described by a two-compartment pharmacokinetic model. The plasma concentrations for both the doses drop to 50 % in approximately 3-4 hours followed by a steady decline upto ~ 12 hours. No free Kaempferol even for the higher dose was detected at 24 hours. The dose normalized AUC values were independent of the

doses and were not significantly different from each other. The V_d was significantly large (12 ± 0.4 l/kg). Figure 7.2B shows the time-course changes in the plasma concentration of K following oral doses of 100 mg/kg and 250 mg/kg. The half life following both oral doses ranged between 2-3 hours and followed by a steady decline upto 6 hours. No free K was detected at time points beyond six hours. The AUC values in orally treated rats did not increase proportionally with doses. A comparison of the dose normalized AUC_{po} and AUC_{iv} values reveals very low bioavailability (~ 2%). After accounting for the conjugated Kaempferol the bioavailability increased to approximately 5-6 fold for the two oral doses. In addition to the K-glucuronides, two major metabolites M1 (Quercetin) and M3 and their glucuronides were also found to be circulating in the plasma (Figure 7.3A). Based on retention time match, M3 was identified to be the 3'-O-methylated metabolite of M1 – isorhamnetin. After oral administration of 100 mg/kg Kaempferol and portal vein sampling, the AUC_{portal} value was ~ 10 times that after $AUC_{systemic}$. The major metabolites identified from portal plasma were K-glucuronide, Isorhamentin, Isorhamnetin glucuronide and Quercetin glucuronide (Figure 7.3B).

Urine collected from rats administered K intravenously demonstrated significant amounts of aglycone K, K-glucuronide and insignificant amounts of other metabolites (M1 and M3). On the other hand, in addition to aglycone Kaempferol and its glucuronide, peaks corresponding to M1 and M3 were also observed in urine collected from rats administered Kaempferol orally (Figure 7.3C). Approximately 16-18 % and 3-4 % of the dose was excreted as parent K after IV and oral administration, respectively.

Table 7.1: Percentage of the parent peak remaining after 120 minutes incubation with NADPH or UDPGA.

		Phase I NADPH dependent metabolism		Phase II UDPGA dependent conjugation	
		0'	**120'**	**0'**	**120'**
RLM	**1µM**	103 ± 1.23	37 ± 4.56	103 ± 1.23	17 ± 4.56
	5 µM	109 ± 1.3	44.5 ± 6.98	109 ± 1.3	19.5 ± 6.98
	10 µM	110 ± 0.98	49.61 ± 5.31	110 ± 0.98	29.61 ± 5.31
	25 µM	106 ± 0.67	47.34 ± 3.69	106 ± 0.67	37.34 ± 10.98
	100 µM	111 ± 1.46	60.97 ± 4.4	111 ± 0.92	50.94 ± 4.21
RSiM	**1 µM**	102 ± 0.98	65.31 ± 9.31	102 ± 0.98	25.31 ± 12.31
	5 µM	106 ± 0.67	69.03 ± 8.64	106 ± 0.67	39.03 ± 18.64
	10 µM	109 ± 0.99	72.11 ± 10.23	109 ± 0.99	42.11 ± 11.23
	25 µM	107 ± 1.32	74.11 ± 11.81	111 ± 1.32	54.31 ± 18.81
	100 µM	111 ± 2.66	81.11 ± 6.8	107 ± 1.68	60.18 ± 4.56

Table 7.2: Kinetic constants obtained from Phase I oxidative and Phase II conjugation reactions

		Km (μM)	Vmax (nmol of product/min/mg protein)
Phase I oxidative metabolism			
RLM		14.25 ± 1.19	95.69 ± 9.12
RSiM		28.11 ± 1.6	58.54 ± 2.38
Phase II conjugation			
RLM	**G2**	20 ± 1.1	112.19 ± 17.2
	G3	8.47 ± 0.845	1736 ± 53.6
RSiM	**G2**	14.55 ± 3.6	229.4 ± 26.2
	G3	25.29 ± 2.4	151.91 ± 16

Table 7.3: Pharmacokinetic parameters following IV and oral administration of Kaempferol.

	IV (10 mg/kg)	IV (25 mg/kg)	PO (100 mg/kg)	PO (250 mg/kg)
AUC (min*µg/ml)	190.2 ± 17.24	402.8 ± 12.6	45.59 ± 5.7	77.20 ± 2.4
Vd (ml/kg)	12714 ± 459	8229.2 ± 209		
t1/2 (min)	245.3 ± 9.7	207.60 ± 12.6		
Cmax (µg/ml)			253.9 ± 48	190.01 ± 29.35
Ka (min^{-1})			0.0128	0.0031
Cl_{total}(ml/min*kg)	52.58 ± 1.89	62.1 ± 4.1	2212 ± 306	3228 ± 268
Cl_{renal} (ml/min*kg)	39.31 ± 2.66	42.1 ± 11.6	66.9 ± 10.84	59.5 ± 12.63
$Cl_{nonrenal}$ (ml/min*kg)	13.27 ± 1.51	21 ± 6.36	2141 ± 187	3160 ± 158
% F			2.7 ± 0.2	1.9 ± 0.11

Table 7.4: Comparison of total plasma and portal plasma AUC values for free and total (free + conjugated) Kaempferol 100 mg/kg.

	Total plasma	Portal plasma
AUC free(min* µg/ml)	45.6 ± 5.7	419.13 ± 29.67
AUC total (min * µg/ml)	205.19 ± 4.7	654.21 ± 19.63

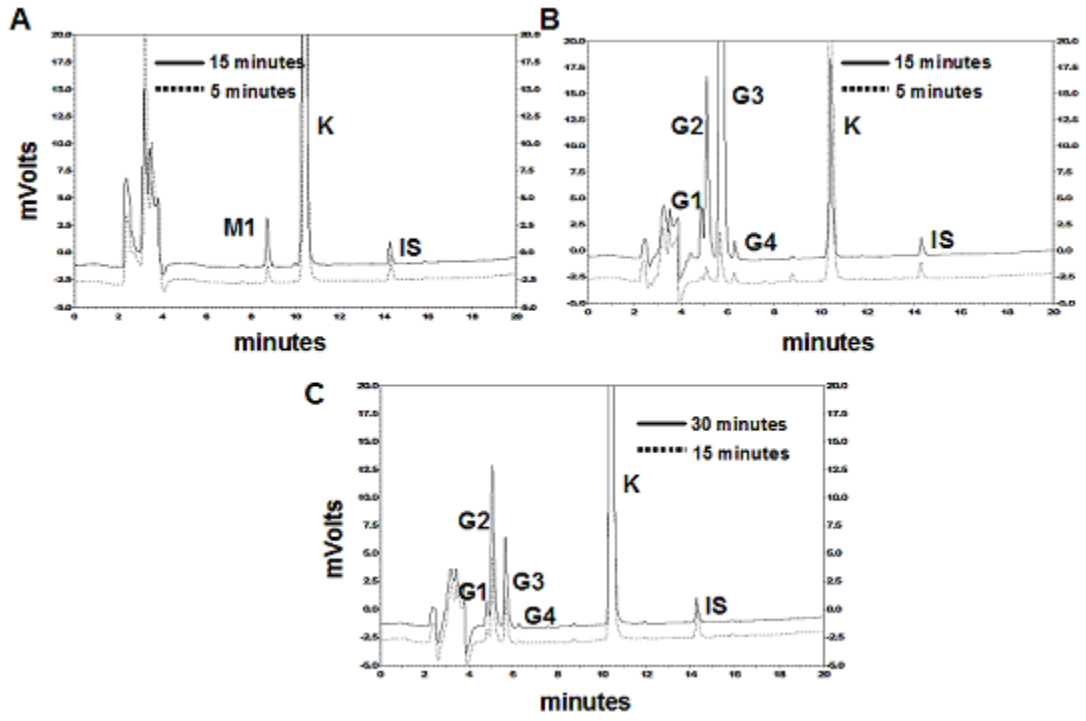

Figure 7.1: HPLC elution profiles following NADPH or UDPGA dependent metabolism **(A)** 0.1 mg/ml rat liver microsomes fortified with 5mM NADPH at 37° C for 5 or 15 minutes. **(B)** 0.1 mg/ml rat liver microsomes fortified with 2mM UDPGA at 37° C for 5 or 15 minutes **(C)** 0.7 mg/ml rat small intestinal microsomes fortified with 2mM UDPGA at 37° C for 15 or 30 minutes. For details of procedure, see Materials and Methods section.

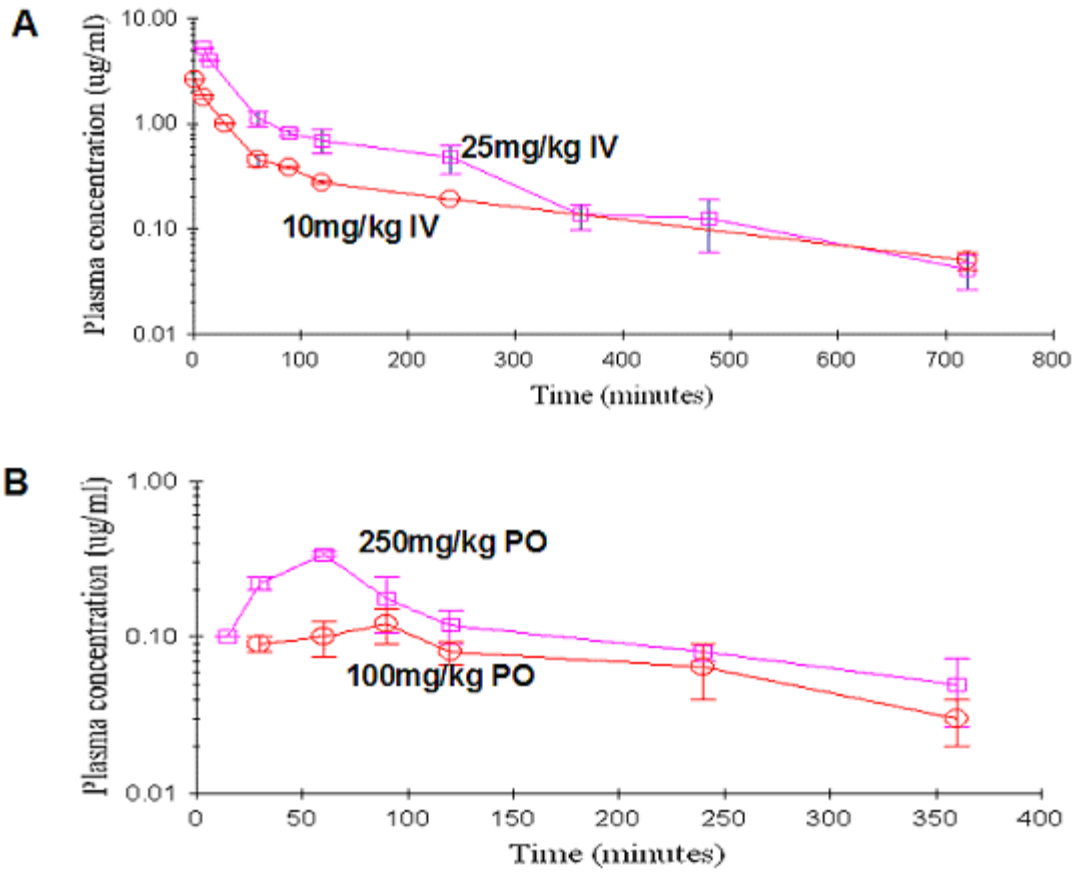

Figure 7.2: Plasma concentration versus time curves in rats following **(A)** intravenous (10 and 25 mg/kg) and **(B)** oral (100 and 250 mg/kg) exposure to Kaempferol. Blood samples were withdrawn upto 24 h post. Bars represent mean ± SE. For details of procedures, see Materials and Methods section.

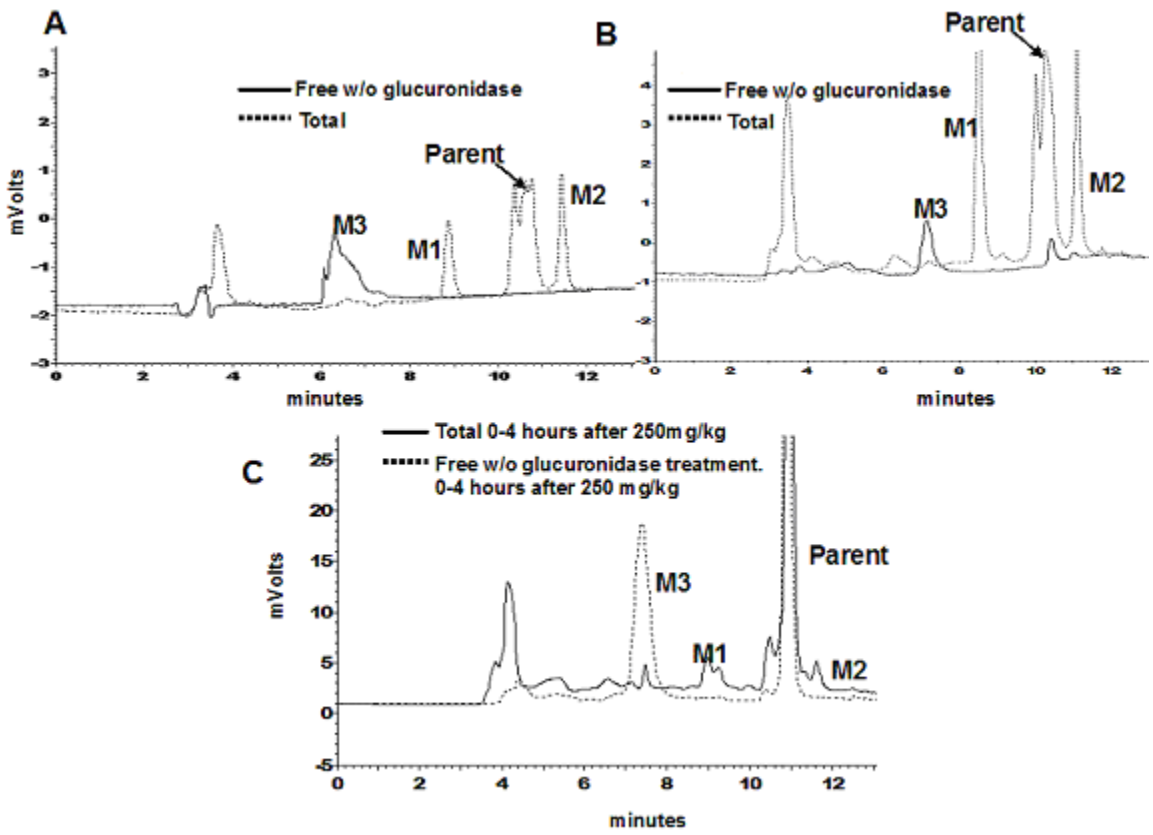

Figure 7.3: Determination of Kaempferol in rat plasma and urine. **(A)** Representative chromatogram of plasma sample obtained six hour post oral dose (250 mg/kg). Free (without glucuronidase treated) and Total (glucuronidase treated) sample chromatograms are indicated. **(B)** Representative chromatogram of portal plasma sample obtained 90 minutes post oral dose (100 mg/kg). Free (without glucuronidase treated) and Total (glucuronidase treated) sample chromatograms are indicated. **(C)** Representative chromatogram of cumulative urine sample obtained 4 hours post oral dose (250 mg/kg). Free (without glucuronidase treated) and Total (glucuronidase treated) sample chromatograms are indicated. For details of procedures, see Materials and Methods section.

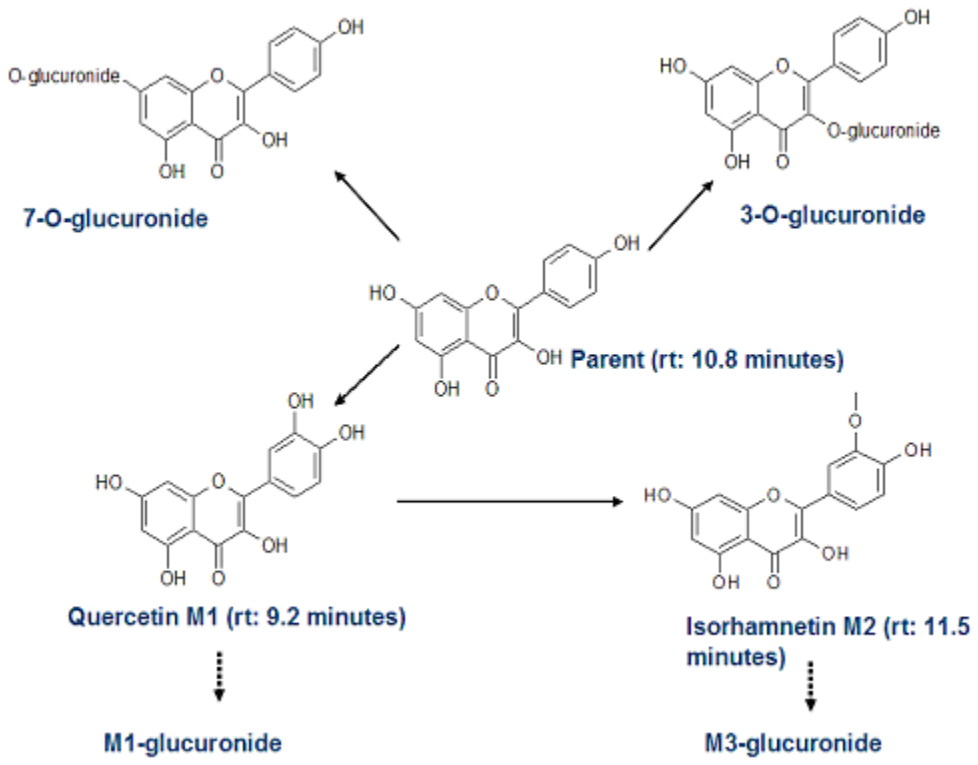

Figure 7.4: Potential NADPH and UDPGA metabolism of Kaempferol in the rat.

7.5 Discussion:

Hepatic and intestinal conjugation with subsequent excretion of the phase II conjugates has been shown to be an important component of first-pass metabolism for many flavonoids [198, 199]. Several studies including those using $CaCO_2$ cells showed that intestinal conjugation followed by excretion of the conjugates depends of the native structure of the flavonoid [200, 201]. Thus significant differences in the conjugation and excretion rates between the liver and small intestine as well as flavonoids bearing close structural similarity exist. Quercetin bearing close structural similarity to Kaempferol has been extensively studied. The role of the liver and small intestine in metabolizing Quercetin is well appreciated and most of its metabolites are fully characterized too [202]. The *in vitro* metabolism of Kaempferol to Quercetin was reported earlier [195]. However differences between hepatic and intestinal disposition of Kaempferol are largely unknown. This study was therefore aimed to obtain an appreciation for the role of small intestine in metabolizing Kaempferol both *in vitro* and *in vivo*.

Liver and small intestinal microsomes fortified with UDPGA revealed a four peak pattern with two major predominant peaks G2 and G3. Yodogawa et al reported a similar four peak pattern for glucuronides formation in the liver and the major glucuronide being the 7-O-glucuronide. Based on this data G3 most likely is the 7-O-glucuronide [197]. UGT catalyzed glucuronidation is known to occur via an S_N2 mechanism characterized by a nucleophilic attack by the hydroxyl group on the pyranose acid ring of UDPGA. Resonance structures are easily formed for hydroxyl groups on positions other than 3 position on ring C. Resonance structures may

weaken the nucleophilicity thus making the hydroxyl on position 3 a good candidate for glucuronidation [203]. Hence we believe G2 most likely is the 3-O-glucuronide. Chen et al reported that in human liver microsomes the two predominant glucuronide peaks formed by Kaempferol were catalyzed by different isoforms of UGT namely UGT1A3 and UGT1A9 [196]. Hence the possibility that G2 and G3 are the result of different isoforms of UGT catalyzed glucuronidation exists. Besides tissue specific differences in expression of UGTs between liver and small intestine have also been reported earlier [204, 205]. Thus differences in the expression patterns of UGT isoforms between liver and small intestine could offer an explanation for the different rates of formation of G2 and G3 between liver and small intestine.

The kinetic constants obtained from the *in vitro* metabolism reveal that rates of glucuronidation are much higher than phase I oxidative metabolism. The *in vivo* data parallel the *in vitro* findings since extensive glucuronidation of the parent occurred after both intravenous and oral administration. DuPont et al studied the pharmacokinetics and excretion kinetics of Kaempferol in humans after administration of endive soup. They reported a second reentry peak in the plasma concentration time curve indicating enterohepatic recirculation. Kaempferol mainly existed in the conjugated form both in plasma as well as urine [206]. This parallels the observations we made after an IV and oral dose of Kaempferol. Reentry peaks were not very evident from the plasma concentration time curves however Kaempferol mainly existed as the glucuronide. However in addition to the conjugated forms, Kaempferol also underwent oxidative conversion to Quercetin which was further subject to methylation yielding Isorhamnetin. Both Quercetin and

Isorhamnetin were also glucuronidated. This metabolic route however is uncommon to humans since no such phase I oxidative metabolism is reported to occur in humans [207-209]. Preliminary data from our laboratory (unpublished results) recorded using human liver microsomes fortified with NADPH also demonstrated no peak corresponding to Quercetin. Since there is no evidence for the formation of hydroxylated metabolite Quercetin, the likelihood of formation of Isorhamnetin as metabolite M2 in humans is minimal.

Bioavailability of any drug depends on the extent of absorption and the rate of clearance (including all possible routes of clearance). The amount of the drug that can be taken up by the intestinal cells into mesenteric architecture and into the liver depends on various factors including lipophilicity, uptake by transporters etc. Crespy et al studied the splanchic metabolism of several flavonoids including Kaempferol. They reported that Kaempferol demonstrated a relatively high (66 to 86%) net transfer directed toward the serosal side. The net absorption of Kaempferol (8.50 ± 0.40 nmol/min) was high owing to the less abundant secretion of its conjugates into the lumen. On the other hand, total Kaempferol conjugates excreted in the bile were quite high [210]. This parallels our study since we observed measurable amounts of the free aglycone in the portal blood that was subject to further hepatic metabolism. The AUC_{portal} for free Kaempferol was approximately 10 times $AUC_{systemic}$ (419.13 ± 29.67 versus 45.6 ± 5.7) while the AUC_{portal} for total Kaempferol including glucuronides and other metabolites was approximately 3 times $AUC_{systemic}$ (654.21 ± 19.63 versus 205.19 ± 4.7).

The Cl_{renal} values did not change significantly between IV and oral doses although the Cl_{total} values (using non compartmental analysis) were significantly apart. Since Cl_{total} is a reflection of all routes of clearance renal and non renal (including metabolic), it is evident that after an oral dose Kaempferol undergoes significant first pass metabolic clearance as evident from the *in vitro* and *in vivo* metabolism studies, leading to enhanced excretion. Although bile and fecal samples were not analyzed, it is reasonable to expect the presence of Kaempferol and its metabolites and conjugates in these samples. Thus the low oral bioavailability of Kaempferol is most likely due to moderate absorption rate, extensive metabolic clearance in the liver > small intestine, potential fecal excretion. In summary, the present paper is the first study describing both the *in vitro* and *in vivo* metabolism of Kaempferol followed by its pharmacokinetics in the rat.

CHAPTER 8: SUMMARY

We have in this dissertation shown that Nrf2 can indeed regulate a plethora of genes that are vital to cancer development. The ability to induce the expression of defense and antioxidant enzymes by activating the Nrf2-ARE and also to modulate transcription factors such as AP-1 to effect apoptosis are two effective strategies adopted by chemopreventive agents. Compared to normal cells, abnormal cancer cells constitutively express several proteins that either amplify or are integral molecules of tumor progression and promotion signaling pathways. We have shown that prostate tumor progression in TRAMP mice occurs by significant suppression of Nrf2 and its related phase II detoxifying and antioxidant enzymes as well as cell cycle inhibitory proteins such as p21 and p27. While the expression of proliferative biomarkers such as Akt, PDK-1, FKHR and cell cycle regulatory proteins such as cyclins A and E are significantly amplified during tumor progression, the expression of pro-apoptotic biomarkers such as BAD and cleaved caspase 3 are downregulated (Figure 8.1). Dietary phytochemicals suppressed prostate tumorigenesis by modulating the expression of Nrf2, its related phase II detoxifying and antioxidant enzymes as well as other cell cycle regulatory and proapoptotic proteins. Soy isoflavones modulated the expression of several genes such as GREB1, LATS2 and calponin in an Nrf2 dependent manner. Thus we have provided convincing data to support the chemopreventive potential of dietary phytochemicals in rodent models of carcinogenesis.

The ultimate proof of efficacy of any putative chemopreventive compound can only be provided by long term phase III clinical intervention studies. Agent classes under

active clinical investigation with potential for prostate cancer chemoprevention include antiandrogens (finasteride, bicalutamide), phytoestrogens (soy isoflavones), antioxidants (Vitamin E, selenium, lycopene) and non steroidal anti-inflammatory agents (sulindac). Prostate Cancer Prevention Trial (PCPT) an NCI sponsored Phase 3 primary prevention trial using finasteride was the first large scale chemoprevention trial with prostate cancer as the primary outcome. The prevalence of prostate cancer was reduced by 24.8 % in subjects randomized to finasteride versus placebo- control [211]. A double-blind randomized study designed to test whether selenized yeast could prevent recurrence of nonmelanoma skin cancer in 1312 patients showed a statistically significant increase in nonmelanoma skin cancer although a secondary end-point analysis revealed a striking reduction in prostate cancer incidence [212]. Selenomethionine has been selected for the SELECT trial which is an ongoing phase III prevention trial using selenium and vitamin E supplementation to prevent prostate cancer. This prospective randomized double-blind placebo-controlled prevention trial involves healthy men with a normal digital rectal examination and a serum PSA level below 4ng/ml. The participants are required to take 200 µg/day L-selenomethionine and/or vitamin E (400 IU/day of α-tocopheryl acetate) supplementation for a minimum of 7 years. In addition there are other ongoing trials using higher concentrations of selenium too. Unlike these trials in which prostate cancer incidence is a secondary end point, clinical diagnosis of prostate cancer is the primary end point of the SELECT trial [213-215]. The α-tocopherol ß-carotene cancer prevention trial (ATBC) in 29,133 male smokers found a 32% reduction in CaP incidence and a 41% lower mortality in those receiving 50 mg α-tocopherol daily for 5 to 8 years [216].

Clinical responses have been seen with the combination of high-dose calcitriol, a vitamin D analog and dexamethasone, in a large randomized trial in men with an androgen-independent CaP. In this study, calcitriol potentiated the antitumor effects of docetaxel [217]. A case control study, conducted in southeast China during 2001 to 2002, reported a reduced CaP risk with increasing frequency, duration, and quantity of green tea consumption [218]. In another study, patients with asymptomatic androgen-independent metastatic prostate carcinoma and progressive PSA elevation were evaluated after ingestion of 6 g of green tea per day. Only one patient manifested a decline in serum PSA, and no patient manifested a tumor response on radiographic assessment or physical examination. Thus, a limited antineoplastic effect with a maximum response rate of 2% was seen with green tea [219]. Similar results were seen in another clinical trial involving patients with hormone refractory CaP. Green tea extract capsules, prescribed at a dose level of 250 mg twice daily, showed minimal clinical activity against the disease. Both these studies were conducted in end-stage disease, signifying that green tea may be more effective if used in the early stages of the disease or in patient at high risk [220].

Thus, the in vivo efficacy of chemopreventive compounds in animal models being established, yet not every phase III clinical trial in human subjects has turned into a success story. The road is littered with failures. Some of these failures could be attributed to inappropriate study design, sub optimal dose, unvalidated end-point etc [221.

Prostate cancer is heterogenous disease that occurs with substantial comorbid conditions. There are a number of points of intervention to inhibit, reverse or

modulate the natural history of prostate cancer development to its progression to clinically active disease. Thus, a strategy that incorporates well characterized natural and synthetic agents with a clinically relevant and validated end point/ biomarker in high risk cohorts provides an efficient pathway for evaluating and approving new chemopreventive agents for prostate cancer [222-223]. In that regard identification and targeting valid biomarkers is critical. Reduction in cancer incidence as an end point is FDA accepted measure of clinical benefit. The suppression of high grade PIN can be a surrogate for clinical benefit by reducing and delaying clinical development of cancer, the need for therapy and hopefully reducing mortality [225]. Target populations of interest arise from the biologic continuum that underlies prostatic neoplastic progression. These populations include: (1) the general population of men over 50 to 55 years of age with PSA less than 4 ng/mL; (2) subjects with hereditary/family history of PCa; (3) subjects with biochemical risk factors such as elevated PSA and/or serum IGF-1; (4) subjects with HGPIN; (5) subjects with low volume/low Gleason grade cancer; and (6) subjects with unfavorable prognostic factors before and after radical prostatectomy, for example, rising PSA[226-229]. Combining these at-risk populations into a multivariare risk model for prostate cancer similar to the Gail model for risk of breast cancer could prove beneficial [230].

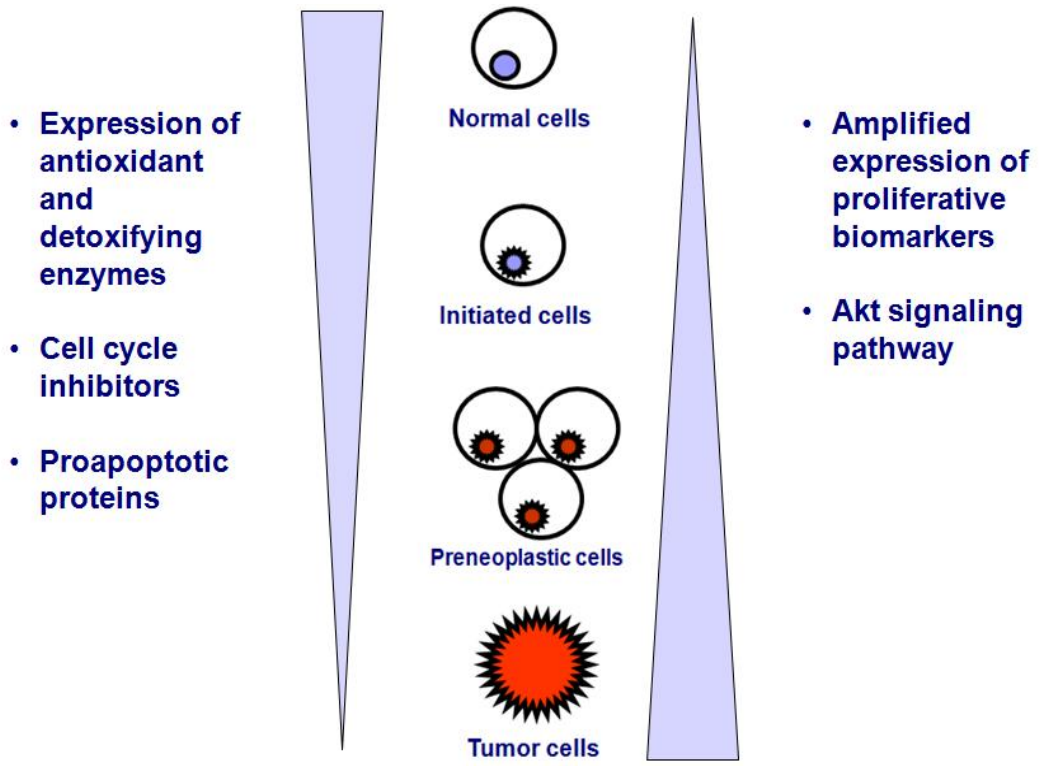

Figure 8.1: Prostate tumor progression in TRAMP mice follows significant suppression of Nrf2, its related phase II detoxifying enzymes, cell cycle inhibitory proteins and proapoptotic proteins. The expression of proliferative biomarkers such as the Akt family of proteins as well as the cyclins are significantly upregulated.

REFERENCES:

1. Jemal, A., et al., *Cancer statistics, 2007.* CA Cancer J Clin, 2007. 57(1): p. 43-66.

2. Sporn, M.B., *Carcinogenesis and cancer: different perspectives on the same disease.* Cancer Res, 1991. 51(23 Pt 1): p. 6215-8.

3. Sporn, M.B., *Approaches to prevention of epithelial cancer during the preneoplastic period.* Cancer Res, 1976. 36(7 PT 2): p. 2699-702.

4. Moolgavkar, S.H., *The multistage theory of carcinogenesis and the age distribution of cancer in man.* J Natl Cancer Inst, 1978. 61(1): p. 49-52.

5. Surh, Y.J., *Cancer chemoprevention with dietary phytochemicals.* Nat Rev Cancer, 2003. 3(10): p. 768-80.

6. Chen, C. and A.N. Kong, *Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects.* Trends Pharmacol Sci, 2005. 26(6): p. 318-26.

7. Chen, C. and A.N. Kong, *Dietary chemopreventive compounds and ARE/EpRE signaling.* Free Radic Biol Med, 2004. 36(12): p. 1505-16.

8. Thomasset, S.C., et al., *Dietary polyphenolic phytochemicals--promising cancer chemopreventive agents in humans? A review of their clinical properties.* Int J Cancer, 2007. 120(3): p. 451-8.

9. Hu, R., et al., *Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable.* Carcinogenesis, 2006. 27(10): p. 2038-46.

10. Singletary, K. and C. MacDonald, *Inhibition of benzo[a]pyrene- and 1,6-dinitropyrene-DNA adduct formation in human mammary epithelial cells bydibenzoylmethane and sulforaphane.* Cancer Lett, 2000. 155(1): p. 47-54.

11. Sharma, O.P., *Antioxidant activity of curcumin and related compounds.* Biochem Pharmacol, 1976. 25(15): p. 1811-2.

12. Huang, M.T., et al., *Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice.* Cancer Res, 1994. 54(22): p. 5841-7.

13. Hu, R., et al., *The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells.* Carcinogenesis, 2003. 24(8): p. 1361-7.

14. Qin, J., et al., *A component of green tea, (-)-epigallocatechin-3-gallate, promotes apoptosis in T24 human bladder cancer cells via modulation of the PI3K/Akt pathway and Bcl-2 family proteins.* Biochem Biophys Res Commun, 2007. 354(4): p. 852-7.

15. Keum, Y.S., W.S. Jeong, and A.N. Kong, *Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms.* Mutat Res, 2004. 555(1-2): p. 191-202.

16. Keum, Y.S., W.S. Jeong, and A.N. Kong, *Chemopreventive functions of isothiocyanates.* Drug News Perspect, 2005. 18(7): p. 445-51.

17. Maheo, K., et al., *Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes.* Cancer Res, 1997. 57(17): p. 3649-52.

18. Shen, G., et al., *Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin.* Mol Cancer Ther, 2006. 5(1): p. 39-51.

19. Shen, G., et al., *Comparison of (-)-epigallocatechin-3-gallate elicited liver and small intestine gene expression profiles between C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice.* Pharm Res, 2005. 22(11): p. 1805-20.

20. Hu, R., et al., *Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray.* Life Sci, 2006. 79(20): p. 1944-55.

21. Yu, X. and T. Kensler, *Nrf2 as a target for cancer chemoprevention.* Mutat Res, 2005. 591(1-2): p. 93-102.

22. Jaiswal, A.K., *Regulation of genes encoding NAD(P)H:quinone oxidoreductases.* Free Radic Biol Med, 2000. 29(3-4): p. 254-62.

23. Venugopal, R. and A.K. Jaiswal, *Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene.* Proc Natl Acad Sci U S A, 1996. 93(25): p. 14960-5.

24. Jeong, W.S., M. Jun, and A.N. Kong, *Nrf2: a potential molecular target for cancer chemoprevention by natural compounds.* Antioxid Redox Signal, 2006. 8(1-2): p. 99-106.

25. Wakabayashi, N., et al., *Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation.* Nat Genet, 2003. 35(3): p. 238-45.

26. Li, W., S.W. Yu, and A.N. Kong, *Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain.* J Biol Chem, 2006. 281(37): p. 27251-63.

27. Shen, G., et al., *Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein.* J Biol Chem, 2004. 279(22): p. 23052-60.

28. Sekhar, K.R., X.X. Yan, and M.L. Freeman, *Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2.* Oncogene, 2002. 21(44): p. 6829-34.

29. Nguyen, T., et al., *Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome.* J Biol Chem, 2003. 278(7): p. 4536-41.

30. Stewart, D., et al., *Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium.* J Biol Chem, 2003. 278(4): p. 2396-402.

31. McMahon, M., et al., *Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression.* J Biol Chem, 2003. 278(24): p. 21592-600.

32. McMahon, M., et al., *Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron.* J Biol Chem, 2004. 279(30): p. 31556-67.

33. Keum, Y.S., et al., *Mechanism of Action of Sulforaphane: Inhibition of p38 Mitogen-Activated Protein Kinase Isoforms Contributing to the Induction of Antioxidant Response Element-Mediated Heme Oxygenase-1 in Human Hepatoma HepG2 Cells.* Cancer Res, 2006. 66(17): p. 8804-13.

34. Yu, R., et al., *Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals.* J Biol Chem, 1999. 274(39): p. 27545-52.

35. Bloom, D.A. and A.K. Jaiswal, *Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression.* J Biol Chem, 2003. 278(45): p. 44675-82.

36. Huang, H.C., T. Nguyen, and C.B. Pickett, *Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2.* Proc Natl Acad Sci U S A, 2000. 97(23): p. 12475-80.

37. Huang, H.C., T. Nguyen, and C.B. Pickett, *Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription.* J Biol Chem, 2002. 277(45): p. 42769-74.

38. Kang, K.W., et al., *Activation of phosphatidylinositol 3-kinase and Akt by tert-butylhydroquinone is responsible for antioxidant response element-mediated rGSTA2 induction in H4IIE cells.* Mol Pharmacol, 2001. 59(5): p. 1147-56.

39. Nakaso, K., et al., *PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells.* FEBS Lett, 2003. 546(2-3): p. 181-4.

40. Cullinan, S.B. and J.A. Diehl, *PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress.* J Biol Chem, 2004. 279(19): p. 20108-17.

41. Nair, S., et al., *Toxicogenomics of endoplasmic reticulum stress inducer tunicamycin in the small intestine and liver of Nrf2 knockout and C57BL/6J mice.* Toxicol Lett, 2007. 168(1): p. 21-39.

42. Hu, R., et al., *Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice.* Cancer Lett, 2006. 243(2): p. 170-92.

43. McWalter, G.K., et al., *Transcription factor Nrf2 is essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates.* J Nutr, 2004. 134(12 Suppl): p. 3499S-3506S.

44. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proc Natl Acad Sci U S A, 2002. 99(18): p. 11908-13.

45. Keum, Y.S., et al., *Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC).* Pharm Res, 2003. 20(9): p. 1351-6.

46. Jeong, W.S., et al., *Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells.* J Biochem Mol Biol, 2005. 38(2): p. 167-76.

47. Nair, S., et al., *Pharmacogenomics of phenolic antioxidant butylated hydroxyanisole (BHA) in the small intestine and liver of Nrf2 knockout and C57BL/6J mice.* Pharm Res, 2006. 23(11): p. 2621-37.

48. Gopalakrishnan, A., et al., *Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells.* Arch Pharm Res, 2006. 29(8): p. 633-44.

49. Lee-Hilz, Y.Y., et al., *Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression.* Chem Res Toxicol, 2006. 19(11): p. 1499-505.

50. Leung, H.W., et al., *Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes.* Food Chem Toxicol, 2007.

51. Wu, C.C., et al., *Upregulation of heme oxygenase-1 by Epigallocatechin-3-gallate via the phosphatidylinositol 3-kinase/Akt and ERK pathways.* Life Sci, 2006. 78(25): p. 2889-97.

52. Rushworth, S.A., et al., *Role of protein kinase C delta in curcumin-induced antioxidant response element-mediated gene expression in human monocytes.* Biochem Biophys Res Commun, 2006. 341(4): p. 1007-16.

53. Chen, C., et al., *Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals.* Free Radic Biol Med, 2004. 37(10): p. 1578-90.

54. Perkins, N.D., *Integrating cell-signalling pathways with NF-kappaB and IKK function.* Nat Rev Mol Cell Biol, 2007. 8(1): p. 49-62.

55. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. 357(9255): p. 539-45.

56. Karin, M., Z. Liu, and E. Zandi, *AP-1 function and regulation.* Curr Opin Cell Biol, 1997. 9(2): p. 240-6.

57. Karin, M., *The regulation of AP-1 activity by mitogen-activated protein kinases.* Philos Trans R Soc Lond B Biol Sci, 1996. 351(1336): p. 127-34.

58. Foletta, V.C., *Transcription factor AP-1, and the role of Fra-2.* Immunol Cell Biol, 1996. 74(2): p. 121-33.

59. Kaminska, B., et al., *Modulation of the composition of AP-1 complex and its impact on transcriptional activity.* Acta Neurobiol Exp (Wars), 2000. 60(3): p. 395-402.

60. Hess, J., P. Angel, and M. Schorpp-Kistner, *AP-1 subunits: quarrel and harmony among siblings.* J Cell Sci, 2004. 117(Pt 25): p. 5965-73.

61. Matsui, K., et al., *Role of activator protein-1 in TCR-mediated regulation of the murine fasl promoter.* J Immunol, 2000. 164(6): p. 3002-8.

62. Hou, D.X., et al., *Green tea proanthocyanidins inhibit cyclooxygenase-2 expression in LPS-activated mouse macrophages: Molecular mechanisms and structure-activity relationship.* Arch Biochem Biophys, 2007.

63. Yu, R., et al., *Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by interfering with mitogen-activated protein kinase pathways.* Mol Pharmacol, 2001. 60(1): p. 217-24.

64. Jeong, W.S., et al., *Modulation of AP-1 by natural chemopreventive compounds in human colon HT-29 cancer cell line.* Pharm Res, 2004. 21(4): p. 649-60.

65. Mallikarjuna, G., et al., *Silibinin protects against photocarcinogenesis via modulation of cell cycle regulators, mitogen-activated protein kinases, and Akt signaling.* Cancer Res, 2004. 64(17): p. 6349-56.

66. Ozben, T., *Oxidative stress and apoptosis: impact on cancer therapy.* J Pharm Sci, 2007. 96(9): p. 2181-96.

67. Laviano, A., et al., *Oxidative stress and wasting in cancer.* Curr Opin Clin Nutr Metab Care, 2007. 10(4): p. 449-56.

68. Aydin, A., et al., *Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia.* Clin Biochem, 2006. 39(2): p. 176-9.

69. Zhao, W., D.I. Diz, and M.E. Robbins, *Oxidative damage pathways in relation to normal tissue injury.* Br J Radiol, 2007. 80 Spec No 1: p. S23-31.

70. Hassig, A., et al., *Flavonoids and tannins: plant-based antioxidants with vitamin character.* Med Hypotheses, 1999. 52(5): p. 479-81.

71. Lairon, D. and M.J. Amiot, *Flavonoids in food and natural antioxidants in wine.* Curr Opin Lipidol, 1999. 10(1): p. 23-8.

72. Vinson, J.A., *Flavonoids in foods as in vitro and in vivo antioxidants.* Adv Exp Med Biol, 1998. 439: p. 151-64.

73. Wickramasinghe, S.N., R. Hasan, and Z. Khalpey, *Differences in the serum levels of acetaldehyde and cytotoxic acetaldehyde-albumin complexes after the*

consumption of red and white wine: in vitro effects of flavonoids, vitamin E, and other dietary antioxidants on cytotoxic complexes. Alcohol Clin Exp Res, 1996. 20(5): p. 799-803.

74. Khor, T.O., et al., *Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice.* Cancer Res, 2006. 66(2): p. 613-21.

75. Liu, L.Z., et al., *Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer.* Mol Pharmacol, 2005. 68(3): p. 635-43.

76. Greenberg, N.M., et al., *The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice.* Mol Endocrinol, 1994. 8(2): p. 230-9.

77. Greenberg, N.M., et al., *Prostate cancer in a transgenic mouse.* Proc Natl Acad Sci U S A, 1995. 92(8): p. 3439-43.

78. Mentor-Marcel, R., et al., *Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP).* Cancer Res, 2001. 61(18): p. 6777-82.

79. Gupta, S., et al., *Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols.* Proc Natl Acad Sci U S A, 2001. 98(18): p. 10350-5.

80. Khor, T.O., et al., *Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis.* Cancer Res, 2006. 66(24): p. 11580-4.

81. Xu, C., et al., *Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2.* Cancer Res, 2006. 66(16): p. 8293-6.

82. Karin, M., Z. Liu, and E. Zandi, *AP-1 function and regulation.* Curr Opin Cell Biol, 1997. 9(2): p. 240-6.

83. Vogt, P.K. and T.J. Bos, *jun: oncogene and transcription factor.* Adv Cancer Res, 1990. 55: p. 1-35.

84. Adcock, I.M., *Transcription factors as activators of gene transcription: AP-1 and NF-kappa B.* Monaldi Arch Chest Dis, 1997. 52(2): p. 178-86.

85. Karin, M. and T. Smeal, *Control of transcription factors by signal transduction pathways: the beginning of the end.* Trends Biochem Sci, 1992. 17(10): p. 418-22.

86. Dong, Z., V. Lavrovsky, and N.H. Colburn, *Transformation reversion induced in JB6 RT101 cells by AP-1 inhibitors.* Carcinogenesis, 1995. 16(4): p. 749-56.

87. Zerbini, L.F., et al., *Constitutive activation of nuclear factor kappaB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer.* Cancer Res, 2003. 63(9): p. 2206-15.

88. DeMarzo, A.M., et al., *Pathological and molecular aspects of prostate cancer.* Lancet, 2003. 361(9361): p. 955-64.

89. Denis, L., M.S. Morton, and K. Griffiths, *Diet and its preventive role in prostatic disease.* Eur Urol, 1999. 35(5-6): p. 377-87.

90. Agarwal, C., R.P. Singh, and R. Agarwal, *Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release.* Carcinogenesis, 2002. 23(11): p. 1869-76.

91. Singh, R.P., et al., *Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3.* Int J Cancer, 2004. 108(5): p. 733-40.

92. Chan, F.L., et al., *Induction of apoptosis in prostate cancer cell lines by a flavonoid, baicalin.* Cancer Lett, 2000. 160(2): p. 219-28.

93. Bors, W., et al., *Flavonoids as antioxidants: determination of radical-scavenging efficiencies.* Methods Enzymol, 1990. 186: p. 343-55.

94. Singh, R.P. and R. Agarwal, *A cancer chemopreventive agent silibinin, targets mitogenic and survival signaling in prostate cancer.* Mutat Res, 2004. 555(1-2): p. 21-32.

95. Singh, R.P. and R. Agarwal, *Prostate cancer chemoprevention by silibinin: bench to bedside.* Mol Carcinog, 2006. 45(6): p. 436-42.

96. Geller, J., et al., *Genistein inhibits the growth of human-patient BPH and prostate cancer in histoculture.* Prostate, 1998. 34(2): p. 75-9.

97. Ishikawa, Y. and M. Kitamura, *Anti-apoptotic effect of quercetin: intervention in the JNK- and ERK-mediated apoptotic pathways.* Kidney Int, 2000. 58(3): p. 1078-87.

98. Frigo, D.E., et al., *Flavonoid phytochemicals regulate activator protein-1 signal transduction pathways in endometrial and kidney stable cell lines.* J Nutr, 2002. 132(7): p. 1848-53.

99. Jeong, W.S., et al., *Modulation of AP-1 by natural chemopreventive compounds in human colon HT-29 cancer cell line.* Pharm Res, 2004. 21(4): p. 649-60.

100. Gonzalgo, M.L. and W.B. Isaacs, *Molecular pathways to prostate cancer.* J Urol, 2003. 170(6 Pt 1): p. 2444-52.

101. Angel, P. and M. Karin, *The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.* Biochim Biophys Acta, 1991. 1072(2-3): p. 129-57.

102. Nguyen, T.T., et al., *Kaempferol-induced growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK.* J Cell Physiol, 2003. 197(1): p. 110-21.

103. Grubb, R.L., et al., *Signal pathway profiling of prostate cancer using reverse phase protein arrays.* Proteomics, 2003. 3(11): p. 2142-6.

104. Boldt, S., U.H. Weidle, and W. Kolch, *The role of MAPK pathways in the action of chemotherapeutic drugs.* Carcinogenesis, 2002. 23(11): p. 1831-8.

105. Mallikarjuna, G., et al., *Silibinin protects against photocarcinogenesis via modulation of cell cycle regulators, mitogen-activated protein kinases, and Akt signaling.* Cancer Res, 2004. 64(17): p. 6349-56.

106. Itoh, K., et al., *Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles.* Genes Cells, 2003. 8(4): p. 379-91.

107. Itoh K, et al., *An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying genes through antioxidant response elements.* Biochem Biophys Res Commun, 1997. 236: p. 313-22.

108. Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes Dev, 1999. 13(1): p. 76-86.

109. Perabo, F.G., et al., *Soy isoflavone genistein in prevention and treatment of prostate cancer.* Prostate Cancer Prostatic Dis, 2007.

110. deVere White, R.W., et al., *Effects of a genistein-rich extract on PSA levels in men with a history of prostate cancer.* Urology, 2004. 63(2): p. 259-63.

111. Onozawa, M., et al., *Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP.* Jpn J Clin Oncol, 1998. 28(6): p. 360-3.

112. Wang, J., I.E. Eltoum, and C.A. Lamartiniere, *Genistein chemoprevention of prostate cancer in TRAMP mice.* J Carcinog, 2007. 6: p. 3.

113. Hussain, M., et al., *Soy isoflavones in the treatment of prostate cancer.* Nutr Cancer, 2003. 47(2): p. 111-7.

114. Mann, G.E., et al., *Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression.* Cardiovasc Res, 2007. 75(2): p. 261-74.

115. McVey, M.J., G.M. Cooke, and I.H. Curran, *Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones.* J Steroid Biochem Mol Biol, 2004. 92(5): p. 435-46.

116. Slitt, A.L., et al., *trans-Stilbene oxide induces expression of genes involved in metabolism and transport in mouse liver via CAR and Nrf2 transcription factors.* Mol Pharmacol, 2006. 69(5): p. 1554-63.

117. Laurenzana, E.M., et al., *Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P-450 enzymes, and estrogen receptor alpha expression.* Food Chem Toxicol, 2002. 40(1): p. 53-63.

118. Sarkar, F.H. and Y. Li, *Mechanisms of cancer chemoprevention by soy isoflavone genistein.* Cancer Metastasis Rev, 2002. 21(3-4): p. 265-80.

119. Yu, L., G.L. Blackburn, and J.R. Zhou, *Genistein and daidzein downregulate prostate androgen-regulated transcript-1 (PART-1) gene expression induced by dihydrotestosterone in human prostate LNCaP cancer cells.* J Nutr, 2003. 133(2): p. 389-92.

120. Steele, V.E., et al., *Cancer chemoprevention agent development strategies for genistein.* J Nutr, 1995. 125(3 Suppl): p. 713S-716S.

121. Choi, Y.H., et al., *Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells.* Int J Oncol, 1998. 13(2): p. 391-6.

122. Kwon, S.H., et al., *Comparison of oral bioavailability of genistein and genistin in rats.* Int J Pharm, 2007. 337(1-2): p. 148-54.

123. Sergeev, I.N., *Genistein induces Ca2+ -mediated, calpain/caspase-12-dependent apoptosis in breast cancer cells.* Biochem Biophys Res Commun, 2004. 321(2): p. 462-7.

124. Jimenez-Velasco, A., et al., *Downregulation of the large tumor suppressor 2 (LATS2/KPM) gene is associated with poor prognosis in acute lymphoblastic leukemia.* Leukemia, 2005. 19(12): p. 2347-50.

125. Powzaniuk, M., et al., *The LATS2/KPM tumor suppressor is a negative regulator of the androgen receptor.* Mol Endocrinol, 2004. 18(8): p. 2011-23.

126. Meehan, K.L., J.W. Holland, and H.J. Dawkins, *Proteomic analysis of normal and malignant prostate tissue to identify novel proteins lost in cancer.* Prostate, 2002. 50(1): p. 54-63.

127. Rae, J.M., et al., *GREB1 is a novel androgen-regulated gene required for prostate cancer growth.* Prostate, 2006. 66(8): p. 886-94.

128. Platz, E.A., et al., *Statin drugs and risk of advanced prostate cancer.* J Natl Cancer Inst, 2006. 98(24): p. 1819-25.

129. Katoh, Y., et al., *Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription.* Genes Cells, 2001. 6(10): p. 857-68.

130. McCarty, M.F., *Targeting multiple signaling pathways as a strategy for managing prostate cancer: multifocal signal modulation therapy.* Integr Cancer Ther, 2004. 3(4): p. 349-80.

131. Abate-Shen, C. and M.M. Shen, *Mouse models of prostate carcinogenesis.* Trends Genet, 2002. 18(5): p. S1-5.

132. Gingrich, J.R., et al., *Metastatic prostate cancer in a transgenic mouse.* Cancer Res, 1996. 56(18): p. 4096-102.

133. Xiao, D. and S.V. Singh, *Phenethyl isothiocyanate-induced apoptosis in p53-deficient PC-3 human prostate cancer cell line is mediated by extracellular signal-regulated kinases.* Cancer Res, 2002. 62(13): p. 3615-9.

134. Shankar, S., et al., *Curcumin enhances the apoptosis-inducing potential of TRAIL in prostate cancer cells: molecular mechanisms of apoptosis, migration and angiogenesis.* J Mol Signal, 2007. 2: p. 10.

135. Shankar, S., X. Chen, and R.K. Srivastava, *Effects of sequential treatments with chemotherapeutic drugs followed by TRAIL on prostate cancer in vitro and in vivo.* Prostate, 2005. 62(2): p. 165-86.

136. Li, M., et al., *Curcumin, a dietary component, has anticancer, chemosensitization, and radiosensitization effects by down-regulating the MDM2 oncogene through the PI3K/mTOR/ETS2 pathway.* Cancer Res, 2007. 67(5): p. 1988-96.

137. Kim, J.H., et al., *Inhibition of EGFR signaling in human prostate cancer PC-3 cells by combination treatment with beta-phenylethyl isothiocyanate and curcumin.* Carcinogenesis, 2006. 27(3): p. 475-82.

138. Pommery, N. and J.P. Henichart, *Involvement of PI3K/Akt pathway in prostate cancer--potential strategies for developing targeted therapies.* Mini Rev Med Chem, 2005. 5(12): p. 1125-32.

139. Fresno Vara, J.A., et al., *PI3K/Akt signalling pathway and cancer.* Cancer Treat Rev, 2004. 30(2): p. 193-204.

140. Nakamura, N., et al., *Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN.* Mol Cell Biol, 2000. 20(23): p. 8969-82.

141. Vazquez, F. and W.R. Sellers, *The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling.* Biochim Biophys Acta, 2000. 1470(1): p. M21-35.

142. Shukla, S., et al., *Constitutive activation of P I3 K-Akt and NF-kappaB during prostate cancer progression in autochthonous transgenic mouse model.* Prostate, 2005. 64(3): p. 224-39.

143. Bostwick, D.G., et al., *Human prostate cancer risk factors.* Cancer, 2004. 101(10 Suppl): p. 2371-490.

144. Chen, J.Z. and F.F. Kadlubar, *Mitochondrial mutagenesis and oxidative stress in human prostate cancer.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2004. 22(1): p. 1-12.

145. De Marzo, A.M., et al., *Human prostate cancer precursors and pathobiology.* Urology, 2003. 62(5 Suppl 1): p. 55-62.

146. Pathak, S.K., et al., *Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies.* Eur J Cancer, 2005. 41(1): p. 61-70.

147. Thompson, I.M., *Chemoprevention of prostate cancer: agents and study designs.* J Urol, 2007. 178(3 Pt 2): p. S9-S13.

148. Wen, X.Q., et al., *Reduced expression of alpha-tocopherol-associated protein is associated with tumor cell proliferation and the increased risk of prostate cancer recurrence.* Asian J Androl, 2007. 9(2): p. 206-12.

149. Liebler, D.C., *The role of metabolism in the antioxidant function of vitamin E.* Crit Rev Toxicol, 1993. 23(2): p. 147-69.

150. Ni, J., et al., *Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway.* Cancer Res, 2005. 65(21): p. 9807-16.

151. Kim, J., et al., *Changes in serum proteomic patterns by presurgical alpha-tocopherol and L-selenomethionine supplementation in prostate cancer.* Cancer Epidemiol Biomarkers Prev, 2005. 14(7): p. 1697-702.

152. Newmark, H.L., M.T. Huang, and B.S. Reddy, *Mixed tocopherols inhibit azoxymethane-induced aberrant crypt foci in rats.* Nutr Cancer, 2006. 56(1): p. 82-5.

153. Suh, N., et al., *Mixed tocopherols inhibit N-methyl-N-nitrosourea-induced mammary tumor growth in rats.* Nutr Cancer, 2007. 59(1): p. 76-81.

154. Devaraj, S., et al., *Gamma-tocopherol supplementation alone and in combination with alpha-tocopherol alters biomarkers of oxidative stress and inflammation in subjects with metabolic syndrome.* Free Radic Biol Med, 2008. 44(6): p. 1203-8.

155. Helzlsouer, K.J., et al., *Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer.* J Natl Cancer Inst, 2000. 92(24): p. 2018-23.

156. Jiang, Q., et al., *gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells.* Proc Natl Acad Sci U S A, 2000. 97(21): p. 11494-9.

157. Jiang, Q. and B.N. Ames, *Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats.* Faseb J, 2003. 17(8): p. 816-22.

158. Campbell, S., et al., *Development of gamma (gamma)-tocopherol as a colorectal cancer chemopreventive agent.* Crit Rev Oncol Hematol, 2003. 47(3): p. 249-59.

159. Cooney, R.V., et al., *Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol.* Proc Natl Acad Sci U S A, 1993. 90(5): p. 1771-5.

160. Gysin, R., A. Azzi, and T. Visarius, *Gamma-tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulation of cyclins.* Faseb J, 2002. 16(14): p. 1952-4.

161. Kwak, M.K., N. Wakabayashi, and T.W. Kensler, *Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers.* Mutat Res, 2004. 555(1-2): p. 133-48.

162. Yu, X. and T. Kensler, *Nrf2 as a target for cancer chemoprevention.* Mutat Res, 2005. 591(1-2): p. 93-102.

163. Jaiswal, A.K., *Nrf2 signaling in coordinated activation of antioxidant gene expression.* Free Radic Biol Med, 2004. 36(10): p. 1199-207.

164. Kode, A., et al., *Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells.* Am J Physiol Lung Cell Mol Physiol, 2008. 294(3): p. L478-88.

165. Bostwick, D.G., I. Meiers, and J.H. Shanks, *Glutathione S-transferase: differential expression of alpha, mu, and pi isoenzymes in benign prostate, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma.* Hum Pathol, 2007. 38(9): p. 1394-401.

166. Canada, A.T., et al., *Glutathione and glutathione S-transferase in benign and malignant prostate cell lines and prostate tissues.* Biochem Pharmacol, 1996. 51(1): p. 87-90.

167. Gao, L., et al., *Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3.* J Biol Chem, 2007. 282(4): p. 2529-37.

168. Bruno, R.S. and M.G. Traber, *Vitamin E biokinetics, oxidative stress and cigarette smoking.* Pathophysiology, 2006. 13(3): p. 143-9.

169. Kang, D., et al., *Functional variant of manganese superoxide dismutase (SOD2 V16A) polymorphism is associated with prostate cancer risk in the prostate, lung, colorectal, and ovarian cancer study.* Cancer Epidemiol Biomarkers Prev, 2007. 16(8): p. 1581-6.

170. Aydin, A., et al., *Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia.* Clin Biochem, 2006. 39(2): p. 176-9.

171. Jemal, A., et al., *Cancer statistics, 2008.* CA Cancer J Clin, 2008. 58(2): p. 71-96.

172. Petrylak, D.P., *The current role of chemotherapy in metastatic hormone-refractory prostate cancer.* Urology, 2005. 65(5 Suppl): p. 3-7; discussion 7-8.

173. Klein, E.A., *Can prostate cancer be prevented?* Nat Clin Pract Urol, 2005. 2(1): p. 24-31.

174. Singh, R.P. and R. Agarwal, *Mechanisms of action of novel agents for prostate cancer chemoprevention.* Endocr Relat Cancer, 2006. 13(3): p. 751-78.

175. Nesaretnam, K., et al., *Tocotrienol-rich fraction from palm oil and gene expression in human breast cancer cells.* Ann N Y Acad Sci, 2004. 1031: p. 143-57.

176. Shun, M.C., et al., *Pro-apoptotic mechanisms of action of a novel vitamin E analog (alpha-TEA) and a naturally occurring form of vitamin E (delta-tocotrienol) in MDA-MB-435 human breast cancer cells.* Nutr Cancer, 2004. 48(1): p. 95-105.

177. Takahashi, K. and G. Loo, *Disruption of mitochondria during tocotrienol-induced apoptosis in MDA-MB-231 human breast cancer cells.* Biochem Pharmacol, 2004. 67(2): p. 315-24.

178. Shah, S.J. and P.W. Sylvester, *Gamma-tocotrienol inhibits neoplastic mammary epithelial cell proliferation by decreasing Akt and nuclear factor kappaB activity.* Exp Biol Med (Maywood), 2005. 230(4): p. 235-41.

179. Srivastava, J.K. and S. Gupta, *Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells.* Biochem Biophys Res Commun, 2006. 346(2): p. 447-53.

180. Kumar, K.S., et al., *Preferential radiation sensitization of prostate cancer in nude mice by nutraceutical antioxidant gamma-tocotrienol.* Life Sci, 2006. 78(18): p. 2099-104.

181. Lee, I.M., J.M. Gaziano, and J.E. Buring, *Vitamin E in the prevention of prostate cancer: where are we today?* J Natl Cancer Inst, 2006. 98(4): p. 225-7.

182. Heinonen, O.P., et al., *Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial.* J Natl Cancer Inst, 1998. 90(6): p. 440-6.

183. Rodriguez, C., et al., *Vitamin E supplements and risk of prostate cancer in U.S. men.* Cancer Epidemiol Biomarkers Prev, 2004. 13(3): p. 378-82.

184. Newmark, H.L., M.T. Huang, and B.S. Reddy, *Mixed tocopherols inhibit azoxymethane-induced aberrant crypt foci in rats.* Nutr Cancer, 2006. 56(1): p. 82-5.

185. Suh, N., et al., *Mixed tocopherols inhibit N-methyl-N-nitrosourea-induced mammary tumor growth in rats.* Nutr Cancer, 2007. 59(1): p. 76-81.

186. DelSal, G., M. Loda, and M. Pagano, *Cell cycle and cancer: critical events at the G1 restriction point.* Crit Rev Oncog, 1996. 7(1-2): p. 127-42.

187. Claudio, P.P., et al., *Expression of cell-cycle-regulated proteins pRb2/p130, p107, p27(kip1), p53, mdm-2, and Ki-67 (MIB-1) in prostatic gland adenocarcinoma.* Clin Cancer Res, 2002. 8(6): p. 1808-15.

188. Cote, R.J., et al., *Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma.* J Natl Cancer Inst, 1998. 90(12): p. 916-20.

189. Cordon-Cardo, C., et al., *Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic carcinoma.* J Natl Cancer Inst, 1998. 90(17): p. 1284-91.

190. Yang, R.M., et al., *Low p27 expression predicts poor disease-free survival in patients with prostate cancer.* J Urol, 1998. 159(3): p. 941-5.

191. Moon, Y.J., X. Wang, and M.E. Morris, *Dietary flavonoids: effects on xenobiotic and carcinogen metabolism.* Toxicol In Vitro, 2006. 20(2): p. 187-210.

192. Ramos, S., *Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention.* J Nutr Biochem, 2007. 18(7): p. 427-42.

193. Ramos, S., *Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways.* Mol Nutr Food Res, 2008. 52(5): p. 507-26.

194. Abdulla, M. and P. Gruber, *Role of diet modification in cancer prevention.* Biofactors, 2000. 12(1-4): p. 45-51.

195. Nielsen, S.E., et al., *In vitro biotransformation of flavonoids by rat liver microsomes.* Xenobiotica, 1998. 28(4): p. 389-401.

196. Chen, Y., et al., *Glucuronidation of flavonoids by recombinant UGT1A3 and UGT1A9.* Biochem Pharmacol, 2008. 76(3): p. 416-25.

197. Yodogawa, S., et al., *Glucurono- and sulfo-conjugation of kaempferol in rat liver subcellular preparations and cultured hepatocytes.* Biol Pharm Bull, 2003. 26(8): p. 1120-4.

198. Chen, J., H. Lin, and M. Hu, *Metabolism of flavonoids via enteric recycling: role of intestinal disposition.* J Pharmacol Exp Ther, 2003. 304(3): p. 1228-35.

199. Zhang, L., Z. Zuo, and G. Lin, *Intestinal and hepatic glucuronidation of flavonoids.* Mol Pharm, 2007. 4(6): p. 833-45.

200. Zhang, L., G. Lin, and Z. Zuo, *Position preference on glucuronidation of mono-hydroxylflavones in human intestine.* Life Sci, 2006. 78(24): p. 2772-80.

201. Chen, J., H. Lin, and M. Hu, *Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model.* Cancer Chemother Pharmacol, 2005. 55(2): p. 159-69.

202. van der Woude, H., et al., *Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems.* Chem Res Toxicol, 2004. 17(11): p. 1520-30.

203. Yin, H., G. Bennett, and J.P. Jones, *Mechanistic studies of uridine diphosphate glucuronosyltransferase.* Chem Biol Interact, 1994. 90(1): p. 47-58.

204. Shelby, M.K., et al., *Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family.* Drug Metab Dispos, 2003. 31(3): p. 326-33.

205. Radominska-Pandya, A., et al., *UDP-glucuronosyltransferases in human intestinal mucosa.* Biochim Biophys Acta, 1998. 1394(2-3): p. 199-208.

206. DuPont, M.S., et al., *Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans.* Eur J Clin Nutr, 2004. 58(6): p. 947-54.

207. de Vries, J.H., et al., *Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake.* Am J Clin Nutr, 1998. 68(1): p. 60-5.

208. Nielsen, S.E., et al., *Human absorption and excretion of flavonoids after broccoli consumption.* Cancer Lett, 1997. 114(1-2): p. 173-4.

209. Breinholt, V.M., et al., *In vitro investigation of cytochrome P450-mediated metabolism of dietary flavonoids.* Food Chem Toxicol, 2002. 40(5): p. 609-16.

210. Crespy, V., et al., *The splanchnic metabolism of flavonoids highly differed according to the nature of the compound.* Am J Physiol Gastrointest Liver Physiol, 2003. 284(6): p. G980-8.

211. Feigel, P., et al., *Design of the prostate cancer prevention trial (PCPT).* Control Clinical Trials, 1995. 16: p. 150-163.

212. Clark, L.C., et al., *Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized control trial.* JAMA, 1996. 276: p. 1957-63.

213 Lippman, S.M., et al., *Designing the selenium and vitamin E cancer prevention trial (SELECT).* J Natl Cancer Inst, 2005. 97: p. 94-102.

214 Dufflied-Lillico, A.J., et al., *Selenium supplementation, baseline plasma selenium status and incidence of CaP: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial.* BJU Int, 2003. 12: p. 608-12.

215 Sabichi, A.L., et al., *Selenium accumulation in prostate tissue during a randomized, control short-term trial of 1-selenomethionine - a Southwest Oncology Group Study.* Clin Cancer Res, 2006. 12: p. 2178-84.

216 Heinonen, O.P., et al., *Prostate cancer and supplementation with alpha-tocopherol and beta-carotene. Incidence and mortality in a controlled trial.* J Natl Cancer Inst, 1998. 90: 440-446.

217 Beer, T.M., et al., *Calcitrol in cancer treatment: from lab to clinic.* Mol Cancer Ther, 2004. 3: 373-81.

218 Jian, L., et al., *Protective effects of green tea against CaP: a case control study in Southeast China.* Int Journal Cancer, 2004. 108: p. 130-5

219 Jatoi, A., et al., *A phase II trial of green tea in the treatment of patients with androgen-independent metastatic prostate carcinoma.* Cancer, 2003. 97: p. 1442-6.

220 Choan, E., et al., *A prospective clinical trial of green tea for hormone refractory CaP: an evaluation of the complementary and alternative therapy approach.* Urol Onco, 2005. 23: p. 108-13.

221 Greenwald, P., et al., *Chemoprevention trials for prostate cancer.* Prostate Canceer in 21st century, Totowa, NJ, Humana Press Inc, 2001. p: 499-518

222 Kelloff, G.J., et al., *Chemoprevention of prostate cancer: concepts and strategies.* Euro Urol, 1999. 35: p. 342-350.

223 Kelloff, G.J., et al., *Strategies for chemoprevention of prostate cancer.* Pros Cancer and Pros Dis, 1999. 2: p. 420-7

224 Strausberg, R.L., et al., *New opportunites for uncovering the molecular basis for cancer*. Nat Genetics, 1997. 15: 415-6

225 Lieberman, R., et al., *Development of new cancer chemoprevention agents: the role of pharmacokinetic-pharmadynamic and intermediate endpoint biomarker monitoring*. Clin Chem, 1998. 44: p. 420-7.

226 Bostwick, D.G., et al., *Target populations and strategies, for chemoprevention trials of prostate cancer*. J Cell Biochem Suppl, 1994. 19: p. 191-6

227 Partin, A.W., et al., *Selection of men at high risk for disease recurrence for experimental adjuvant therapy following radical prostatectomy.* Urology, 1995. 45: p. 831-8.

228 Blute, M.L., et al., *Use of Gleason score, prostate specific antigen, seminal vesicle and margin status to predict biochemical failure after radical prostatectomy.* J Urol, 2001. 165: p. 119-125.

229 Keetch, D.W., et al., *Serial prostate biopsies in men with persistently elevated serum prostate-specific antigen values.* J Urol, 1994. 151: p. 1571-4

230 Pound, C.R., et al., *Natural history of progression after PSA elevation following radical prostatectomy.* JAMA, 1999. 281: p. 1591-1617.

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Mixed tocotrienols inhibit prostate carcinogenesis. *(Submitted to Nutrition Cancer)*

Barve A, Khor TO, Nair S, Suh N, Reuhl K, Reddy B, Newmark H, Kong AN.
γ-tocopherol enriched mixed tocopherols inhibit prostate carcinogenesis in TRAMP mice *(International Journal of Cancer- Article in press)*

Barve A, Khor T, Nair S, Lin W, Yu S, Jain MR, Chan JY, Kong AN.
Pharmacogenomic profile of soy isoflavone phytochemicals (Novasoy) in the prostate of Nrf2 deficient and wild type mice. Journal of Pharmaceutical Sciences 97(10), 4528-45, 2008.

Barve A, Khor T, Keum YS, Xingpei Hao, Chung S Yang, Bandaru Reddy, Kong AN.
Murine prostate cancer inhibition by dietary phytochemicals- curcumin and phenyethylisothiocyanate. Pharmaceutical Research – special issue on chemoprevention 25 (9), 2181-9, 2008.

Gopalakrishnan A, Kong AN.
Anticarcinogenesis by dietary phytochemicals – cytoprotection by Nrf2 in normal cells and cytotoxicity in cancer cells. Food and Chemical Toxicology 46(4), 1257-70, 2007.

Kwon KH, **Barve A**, Yu S, Huang MT, Kong AN
Cancer chemoprevention by phytochemicals: potential molecular targets, biomarkers and animal models. Acta Pharmacologica Sinica 28(9), 1409-21, 2007.

Gopalakrishnan A, Xu CJ, Nair SS, Chen C, Hebbar V, Kong AN.
Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. Archives of Pharmacal Research 29(8), 633-644, 2006.

Nair SS, Hebbar V, Shen G, **Gopalakrishnan A**, Khor TO, Yu S, Xu C, Kong AN.
Synergestic effects of a combination of dietary factors sulforaphane and epigallocatechin gallate in HT-29 AP1 human colon carcinoma cells. Pharm Res 25(2), 387-99, 2008.

Nair S, Xu C, Shen G, Hebbar V, **Gopalakrishnan A**, Hu R, Jain MR, Liew C, Chan JY, Kong AN.
Toxicogenomics of endoplasmic reticulum stress inducer tunicamycin in the small intestine and liver of Nrf2 knockout and C57BL/6J mice. Toxicol Lett. 168(1), 21-39, 2007.

Nair S, Xu C, Shen G, Hebbar V, **Gopalakrishnan A**, Hu R, Jain MR, Lin W, Keum YS, Liew C, Chan JY, Kong AN.
Pharmacogenomics of phenolic antioxidant butylated hydroxyanisole (BHA) in the small intestine and liver of Nrf2 knockout and C57BL/6J mice. Pharm Res. 23(11), 2621-37, 2006.

Hu R, Xu C, Shen G, Jain MR, Khor TO, **Gopalkrishnan A**, Lin W, Reddy B, Chan JY, Kong AN.
Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray. Life Sci. 79(20), 1944-55, 2006.

Hu R, Xu C, Shen G, Jain MR, Khor TO, **Gopalkrishnan A**, Lin W, Reddy B, Chan JY, Kong AN.
Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice.
Cancer Lett. 243(2), 170-92, 2006.

Shen G, Xu C, Hu R, Jain MR, **Gopalkrishnan A**, Nair S, Huang MT, Chan JY, Kong AN.
Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin.
Mol Cancer Ther. 5(1), 39-51, 2006.

Khor TO, Keum YS, Lin W, Kim JH, Hu R, Shen G, Xu C, **Gopalakrishnan A**, Reddy B, Zheng X, Conney AH, Kong AN.
Combined inhibitory effects of curcumin and phenethyl isothiocyanate on the growth of human PC-3 prostate xenografts in immunodeficient mice.
Cancer Res. 66(2), 613-21, 2006.