

AN ABSTRACT OF THE DISSERTATION OF

John D. Clarke for the Doctor of Philosophy in Molecular and Cellular Biology
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Title: Prostate Cancer Prevention with Broccoli: From Cellular to Human Studies

Abstract approved:

Emily Ho

Prostate cancer is the second leading cause of cancer related death in American men. Epidemiologic studies suggest that cruciferous vegetable intake may lower the risk for many cancers, including prostate and colon. Isothiocyanates (ITC) are phytochemicals derived from cruciferous vegetables such as broccoli, Brussels sprouts, cauliflower, and cabbage that may have health promoting properties. Broccoli and broccoli sprouts are a good source of sulforaphane (SFN), a well studied chemopreventive ITC. SFN is known to inhibit histone deacetylase (HDAC) activity and alter epigenetic endpoints. A key factor in understanding the efficacy of SFN as a chemoprevention agent is to determine the metabolism, distribution and bioavailability of SFN, and the factors that alter these parameters. The present study was undertaken to provide further evidence that SFN can alter HDAC activity, alter prostate cancer cell proliferation, both *in vitro* and *in vivo* and expands our understanding of SFN metabolism and tissue distribution.

We characterized the effects of SFN in normal (PrEC), benign hyperplasia (BPH1) and cancerous (LnCap and PC3) prostate epithelial cells. We observed that 15 μ M SFN differentially induced cell cycle arrest and apoptosis in BPH1, LnCap and PC3 cells but not PrEC cells. SFN treatment also differentially decreased HDAC activity, and Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for *P21*, induced p21 expression and increased tubulin acetylation in prostate cancer cells. In PrEC cells, SFN caused only a transient reduction in HDAC activity

with no change in any other endpoints tested. Therefore, normal prostate cells were refractory to the cytotoxic and epigenetic effects of SFN.

In order for SFN to be an effective chemopreventive agent it must be metabolized and reach target tissues. Nrf2 wild-type and Nrf2 knockout (Nrf2^{-/-}) mice were treated with 5 or 20 μ moles of SFN, and SFN metabolites were detected in all tissues tested at 2 and 6 h in a dose dependent manner. Genotype only had marginal effects at 5 μ moles, whereas, at 20 μ moles the female Nrf2^{-/-} mice had dramatically higher levels. The relative abundance of each metabolite was not strikingly different between genders and genotypes, although different ratios between tissues were observed. In the transgenic adenoma of the mouse prostate model dietary SFN, fed as freeze-dried broccoli sprouts, increased SFN content in the prostate and decreased the severity of prostate cancer at 12 and 28 weeks of age.

In humans, the differences in metabolism of isothiocyanates between whole food and broccoli supplements have yet to be determined. Two separate human trials were conducted; the first was a randomized 7 day feeding study where subjects consumed either broccoli sprouts or a broccoli supplement, and the second study was a randomized single dose cross-over study with broccoli sprouts, followed by a washout period, then broccoli supplement. In plasma and urine, the total amounts of SFN and erucin (ERN) metabolites were greater and the peak concentration occurred sooner in subjects who consumed broccoli sprouts. Glutathione-S-transferase pi-1 polymorphisms did not affect ITC metabolism. Interconversion of SFN to ERN was observed. Histone deacetylase activity in peripheral blood mononuclear cells was inhibited only in subjects who consumed sprouts.

In conclusion, these data provide evidence that SFN alters HDAC activity and protein acetylation in cancerous prostate cells but not normal prostate cells. For the first time we show that SFN is bioavailable to many tissues types, including the prostate and are largely found as SFN metabolites not the parent SFN compound. We also show that the bioavailability of ITCs is markedly lower in human subjects who consume a broccoli supplement. Decreased HDAC activity in the peripheral blood of

subjects who consumed sprouts indicates that higher ITC plasma concentrations can alter HDAC activity *in vivo*. Taken together, these data show that SFN is an effective prostate cancer chemopreventive agent that can easily be utilized in the diet from whole food.

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Prostate Cancer Prevention with Broccoli: From Cellular to Human Studies

by

John D. Clarke

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APPROVED:

Major Professor, representing Molecular and Cellular Biology

Director of the Molecular and Cellular Biology Program

Dean of the Graduate School

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John D. Clarke, Author

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CONTRIBUTION OF AUTHORS

The author's responsibilities were as follows: EH-contributed to study concept and design, data interpretation and manuscript revisions; RHD-contributed to study design, data interpretation and manuscript revisions; AH-performed cell cycle and apoptosis analyses for cell culture, preparation and initiation of human studies, mouse tissue collection and processing; ZY-performed real-time PCR analysis from cell culture; DEW-data interpretation and manuscript revisions; JFS-mass spec method development and technical assistance; PT and JS-assistance with TRAMP study and prostate cancer pathology; KR-mass spec method development and execution, data interpretation and analysis, manuscript revision; DB-human study coordinator, registered dietitian for studies, and sample processing; SJS-data interpretation and manuscript revisions.

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Chapter 1 Introduction: Prostate Cancer Prevention with Broccoli:
From Cellular to Human Studies

John D. Clarke, Roderick H. Dashwood, Emily Ho

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1.1. Abstract

Isothiocyanates are found in cruciferous vegetables such as broccoli, Brussels sprouts, cauliflower, and cabbage. Epidemiologic studies suggest that cruciferous vegetable intake may lower overall cancer risk, including colon and prostate cancer. Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables and is especially high in broccoli and broccoli sprouts. SFN has proved to be an effective chemoprotective agent in cell culture, carcinogen-induced and genetic animal cancer models, as well as in xenograft models of cancer. Early research focused on the “blocking activity” of SFN via phase 2 enzyme induction, as well as inhibition of enzymes involved in carcinogen activation, but there has been growing interest in other mechanisms of chemoprotection by SFN. Recent studies suggest that SFN offers protection against tumor development during the “post-initiation” phase and mechanisms for suppression effects of SFN, including cell cycle arrest and apoptosis induction are of particular interest. In humans, a key factor in determining the efficacy of SFN as a chemoprevention agent is gaining an understanding of the metabolism, distribution and bioavailability of SFN and factors that alter these parameters. This review discusses the established anti-cancer properties of SFN, with an emphasis on the possible chemoprevention mechanisms. The current status of SFN in human clinical trials also is included, with consideration of the chemistry, metabolism, absorption and factors influencing SFN bioavailability.

1.2. Introduction

Cancer is the second leading cause of death in the United States. With over 1.5 million people estimated to have been diagnosed with cancer in 2010, preventive measures that target the various steps involved in cancer initiation and progression could significantly decrease the incidence and mortality of cancer. In particular, the use of dietary chemoprevention strategies has gained significant interest. Research investigating the use of diet-derived chemoprevention compounds may have significant impact on qualifying or changing recommendations for high-risk cancer

patients and thereby increase their survival through simple dietary choices with easily accessible foods. Epidemiologic studies suggest that cruciferous vegetable intake may lower overall cancer risk, including colon and prostate cancer, particularly during the early stages (1, 2). However, *in vitro* and *in vivo* data provide evidence that increasing cruciferous vegetable intake provides protection at every stage of cancer progression. Thus, there is growing interest in identifying the specific chemoprotective constituents in cruciferous vegetables and their mechanisms of action at all stages of cancer.

One such family of chemoprotective constituents are isothiocyanates (ITC) which are formed by hydrolysis of their parent compounds glucosinolates. Within the plant, glucosinolate content can vary greatly between and within members of the Cruciferae family depending on cultivation environment and genotype (3) and there are over 120 glucosinolates in the various varieties of cruciferous vegetables, each yielding different aglycone metabolic products (4). The general structure of a glucosinolate consists of a β -D-thioglucose group, a sulfonated oxime group, and a variable side chain. Many of the anticancer effects observed from cruciferous vegetables have been attributed to the ITCs rather than their parent glucosinolates. Two important and well studied isothiocyanates in cruciferous vegetables are sulforaphane (SFN) and indole-3-carbinol (I3C). The glucosinolate precursor to SFN, glucoraphanin, is abundant in broccoli, cauliflower, cabbage, and kale with the highest concentration found in broccoli and broccoli sprouts (5). Hydrolysis of glucoraphanin to its aglycone product SFN requires the activity of myrosinase enzymes released from the plant during consumption and other myrosinase enzymes present in our gut. The structures of glucoraphanin and SFN are shown in Figure 1.1. This review will focus on SFN in cancer development since a review of I3C was presented elsewhere (6).

The mechanisms of SFN chemoprevention have been well studied and reveal diverse responses depending upon the stage of carcinogenesis. SFN can function by blocking initiation via inhibiting phase 1 enzymes that convert procarcinogens to proximate or ultimate carcinogens, and by inducing phase 2 enzymes that detoxify carcinogens and facilitate their excretion from the body. Once cancer is initiated, SFN

can act via several mechanisms that modulate cell growth and cell death signals to suppress cancer progression. Prostate and colon cancer are the 1st and 4th most prevalent cancers in men in the United States, respectively. This review will discuss overall function and metabolism of SFN, with a focus on the mechanisms for chemoprevention of prostate and colorectal cancer development and discuss its capacity to act during both initiation and post-initiation stages. Due to space constraints it is not the intent of this review to cover all aspects of SFN chemoprevention but rather to thoroughly discuss several salient factors. For a comprehensive review refer to a recent review by Juge et al (7).

1.3.Molecular targets/Anticancer properties of sulforaphane

The molecular targets of SFN vary depending upon cancer stage and target tissue. Recent work has clearly implicated multiple targets of SFN action. Since SFN was first identified in 1992 as a potential chemopreventive agent (8), there has been a sharp increase in PubMed citations mentioning SFN. Early research focused on phase 2 enzyme induction by SFN, as well as inhibition of enzymes involved in carcinogen activation, but there has been growing interest in other mechanisms of chemoprotection by SFN. Recent studies also suggest that SFN offers protection against tumor development during the “post-initiation” phase and mechanisms for “suppression” effects of SFN are of particular interest. At the initiation stage of cancer, SFN blocks carcinogenesis through inhibition of phase 1 enzymes and induction of phase 2 enzymes. A summary of the “blocking” targets are shown in Table 1. These detoxification enzymes play an integral role in cellular resistance to carcinogenic insults. Post initiation, SFN can act to suppress cancer development through various molecular targets that are involved in controlling cell proliferation, differentiation, apoptosis, or cell cycle. A summary of “suppression” targets is presented diagrammatically in Figure 1.2. These targets may be aberrantly activated or silenced, depending on each specific case, and thus allow initiated cells to survive and proliferate. SFN can modulate these targets and redirect their activities towards

apoptosis or cell cycle arrest, thereby eliminating initiated cells from the general population.

1.3.1. Blocking mechanisms: phase 1 and phase 2 enzymes

SFN can modulate phase 1 metabolism through direct interactions with cytochrome P450 enzymes (CYP) or regulating their transcript levels within the cell. Phase 1 enzymes usually involve oxidation, reduction, or hydrolysis and generally lead to detoxification, but are also involved in converting procarcinogens to carcinogens. Inhibition of phase 1 enzymes is thought to be an important step in blocking chemically-induced carcinogenesis. A dose dependent inhibition of CYP1A1 and CYP2B1/2 by SFN was seen in rat hepatocytes. Similarly in human hepatocytes, SFN decreased the activity of CYP3A4 by decreasing its mRNA levels (9). Thus, SFN can modulate the levels and activities of various phase 1 enzymes leading to reduced activation of procarcinogens. There is additional indirect evidence that SFN can modulate the activity of different CYP enzymes. For a more thorough review, refer to Myzak et al (10).

The “blocking activity” of SFN has received significant attention and, although CYP inhibition is of interest, most work has focused on phase 2 enzyme induction via antioxidant response element (ARE)-driven gene expression. ARE-driven targets include NAD(P)H:quinone reductase (NQO1), heme oxygenase 1 (HO1) and gamma-glutamylcysteine synthetase (γ -GCS), a rate-limiting enzyme in glutathione (GSH) synthesis. Regulation of these genes, involved in detoxification of carcinogens and oxidants, is mediated by nuclear factor E2-factor related factor (Nrf2), a member of the basic leucine-zipper NF-E2 family, which binds to ARE sites as a cis-acting element in the 5'-flanking region of the genes for many of these phase 2 enzymes.

Kelch-like ECH-associated protein 1 (Keap1) is a cysteine rich protein which, in its dimeric form, interacts with Nrf2 sequestering it the cytosol, thereby inhibiting its transcriptional activity. Several models have been suggested to explain how Keap1 regulates Nrf2. In the most widely accepted model, two cysteine residues, C273 and

C288, are important for the dissociation of Keap1 from Nrf2. SFN is able to react with the thiol groups of Keap1 and form thionacyl adducts promoting Nrf2 dissociation from Keap1 and subsequent activation of ARE-driven gene expression.

The ability of SFN to activate these Nrf2 driven detoxifying genes is well documented both *in vitro* and *in vivo*. Potent induction of phase 2 enzymes has been found in prostate cell lines treated with 0.1-15 μ M SFN. Treatment with SFN or broccoli sprout extracts strongly induced NQO1 activity due to increased transcription of *NQO-1* (11). GSH is a crucial substrate in the metabolism of SFN. In human prostate cells, GSH synthesis was induced by SFN treatment due to increased levels of γ -GCS light chain mRNA but not γ -GCS heavy chain, although high levels of γ -GCS heavy chain message were present. This induction occurred concomitant with an increase in intracellular GSH levels and induction of glutathione S-transferase alpha (GST- α) and microsomal GST (11). More recent *in vivo* work in the F-344 rat model confirmed phase 2 enzyme induction in the prostate and colon after dietary supplementation of a broccoli or SFN diet. In one study the rats were fed a broccoli diet and a 4.5 fold induction of NQO1 activity was observed in the rat colon (12). In another study, rats gavaged with 50 mg/kg/day SFN for five days had increased NQO-1, total GST, and GST- μ activities in the prostate. In other tissues such as liver, kidney, and bladder, SFN induced NQO1 and total GST activity, with the highest induction in the bladder (13). Finally, the induction of phase 2 enzymes by SFN is also apparent in humans. *In vivo* jejunal perfusion of broccoli extracts (equivalent to ~1.2 g dry weight broccoli) resulted in an induction of GSTA1 and UGT1A1 in exfoliated enterocytes (14).

Importantly, the induction of these blocking genes after SFN treatment is dependent on Nrf2 as revealed from work done in Nrf2 knockout mice. Transcriptional profiling of the small intestine from wild-type Nrf2 mice (*nrf2*^{+/+}) and Nrf2 knockout mice (*nrf2*^{-/-}) fed 9 μ mol/day SFN revealed upregulation of the detoxification genes *NQO-1*, *GST*, γ -GCS, and *UDP-glucuronosyltransferases* (*UGT*). In addition, antioxidant genes *glutathione peroxidase*, *glutathione reductase*, *ferritin*, and

haptoglobin were also affected. The upregulation of these genes by SFN was significantly blunted in the Nrf2 knockout mice (15). Similar responses have been found in other tissues. In the liver, γ -GCS was induced as well as two GST- α subunits and three GST- μ subunits following 90 mg SFN/kg body weight. Many other gene groups were identified including antioxidants, ubiquitin/proteasome systems, cell cycle and cell growth, transcription factors, and several others (16). Another group reported an increase in NQO1 and GST activities in the stomach, small intestine, and liver of wild-type, but not Nrf2 knockout, mice after being fed a diet containing broccoli seed extracts. This was accompanied by increased protein levels of GSTA1/2, GSTA3, and GSTM1/2 in the wild-type mice (17). These studies defined the Nrf2 dependent SFN targets that are altered in the stomach, small intestine, and liver, tissues that play a critical role in absorption and detoxification, respectively. Collectively, these results highlight the importance of SFN in blocking the initiation stage of cancer by inhibiting phase 1 enzymes and stimulating phase 2 enzymes.

1.3.2. Suppression via anti-proliferative mechanisms

1.3.2.1. Cell cycle arrest

One hallmark of cancer is hyperproliferation due to loss of cell cycle regulatory mechanisms. The key regulators of cell cycle progression are the cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. The regulation of CDK complexes is dependent upon the phosphorylation status of the various components of the complex and whether or not CDK inhibitors are bound. The cyclin/CDK complexes promote cell cycle progression while the CDK inhibitors promote cell cycle arrest. Research suggests that the action of SFN on various CDKs, cyclins, and CDK inhibitors is complex and the regulation is likely affected by cell type, dose of treatment, and time of exposure.

In vitro experiments with SFN indicate a pronounced role for cell cycle arrest in its anticancer properties. In both prostate and colon cancer cells a predominant G₂/M cell cycle arrest is observed. The G₂/M transition in the cell cycle requires an active

cyclinB/CDK1 complex. Phosphorylation of CDK1 in its ATP binding loop by Wee1 and membrane associated tyrosine/threonine-1 (MYT1) kinases inactivates the complex while an opposing phosphatase, cell division cycle 25 (Cdc25) removes these inhibitory phosphorylations and promotes the progression into M-phase. Thus far, three mammalian Cdc25 isoforms have been identified; Cdc25A, Cdc25B, and Cdc25C. Chk1 and Chk2 kinases can phosphorylate and inactivate the various Cdc25 isoforms. Therefore Chk2 inactivates Cdc25 and the cyclinB/CDK complex arresting the cells in G₂/M phase. In PC3 cells, treated with 20 μ M SFN, a G₂/M cell cycle arrest occurred with concomitant decreases in levels of cyclin B1, Cdc25B, and Cdc25C. Cdc25C was phosphorylated at Ser-216 by Chk2 and sequestered to the cytoplasm by 14-3-3 β . This same Chk2-dependent G₂/M arrest was seen in the HCT116 human colon cancer cell line (18). Likewise, in LnCap prostate cancer cells, a dramatic increase in G₂/M phase arrest occurred in a concentration- and time-dependent manner concomitant with induction of cyclin B1, Chk2 kinase, and down-regulation of Cdk1 and Cdc25C protein levels (19). In addition, 10 μ M SFN treatment in DU145 prostate cancer cells reduced cell viability and induced G₂/M cell cycle arrest (20).

Although G₂/M arrest is the predominant stage of cell cycle arrest induced by SFN (21, 22), arrest at other phases of the cell cycle occurs in both prostate and colon cancer cells. In HT-29 cells, G₁ cell cycle arrest occurred concomitant with an increase in p21^{CIP1}, and a decrease in cyclin D1, cyclin A, and c-myc (23). A G₁/S block in LnCap and DU145 prostate cells has also been reported at concentrations at, or below, 10 μ M (24, 25).

Evidence indicates that dose and duration of SFN exposure may be responsible for these divergent cell fates. In human colon adenocarcinoma Caco-2 cells, a G₂/M arrest was observed at a dose of 20 μ M SFN, whereas concentrations >20 μ M induced accumulation of sub-G₁ cells and loss of mitochondrial membrane potential (26). Length of exposure to SFN also appears to play an important role. In p53 wild-type 40-16 colon cancer cells, transient SFN treatment for up to 6 h resulted in a reversible

G₂/M arrest and cytostatic growth effects. In contrast, exposures >12h resulted in irreversible G₂/M arrest and subsequent apoptosis. Interestingly, the cytostatic effects seen with 12 h exposure was sustained up to 72 h after SFN removal and IC⁵⁰s were indistinguishable from 72 h exposures (27).

The tumor suppressor and cell cycle inhibitor protein p21 appears to play an important role in SFN-induced cell cycle arrest. An induction of p21 is consistently observed regardless of cell type and p53 status. *In vitro*, SFN treatment induced *p21* expression in both p53 negative colon cell lines HT-29 and Caco-2 (21). *In vivo*, surgically resected colon tissue from three human volunteers treated with SFN for 2 h exhibited a strong induction of *p21* in cancer tissue, but not in normal tissue, in 2 out of 3 volunteers (28). In LnCap prostate cancer cells, p53 and p21 were induced by 20 μ M SFN. However, induction of p21 by SFN is also apparent in p53-null PC3 prostate cancer cells, suggesting p53-independent regulation of p21 (29). Supporting this p53-independent mechanism in LnCap cells, SFN-induced cell cycle arrest occurred after induction of p21 but not p53. Interestingly, in these experiments using LnCap cells, p21 siRNA knockdown potentiated cell cycle arrest with no affect on apoptosis as detected by histone fragmentation indicating a role for p21 in protecting against SFN-mediated arrest (19). This role of p21 merits further investigation in other cell lines. Taken together these data indicate a consistent induction of p21 following SFN administration in both p53 dependent and independent contexts.

1.3.2.2. Apoptosis

Apoptosis, or programmed cell death, can be accomplished either through the death-receptor caspase cascades or the mitochondria caspase cascades. Caspases are the effectors of apoptosis and some of the hallmarks of apoptosis are cytoplasmic histone associated DNA fragments, poly (ADP-ribose) polymerase (PARP) cleavage, changes in Bcl-2 protein family ratios (increased proapoptotic proteins and decreased antiapoptotic proteins), and cytochrome C release from the mitochondrial membrane.

There is substantial and compelling evidence for SFN mediated apoptosis in both prostate and colon cancer cells, but this may depend on SFN dose.

Administration of 10 μ M SFN reduced cell viability and induced apoptosis as indicated by PARP cleavage and increased release of histone associated DNA fragments in DU145 prostate cancer cells (20). In HCT116 colon cancer cell lines (both wild-type for p53 (40-16) or p53 knockout (379.2)), 15 μ M SFN induced activation of caspase 7 and caspase 9, and apoptosis independent of p53. This was accompanied by a consistent decrease in Bcl-x_L protein levels. Although the levels of Bax and Bak protein were not consistently decreased, a time-dependent change in Bax and Bak to Bcl-x_L protein ratios in favor of the pro-apoptotic factors Bax and Bak was observed concomitant with increased PARP cleavage (30). In HT-29 colon cancer cells, treatment with 15 μ M SFN increased Bax expression, release of cytochrome C from mitochondria, and PARP cleavage (22). In PC3 prostate cancer cell line apoptosis induction was apparent as indicated by an increase in sub-G₀/G₁ DNA content of treated cells, cytoplasmic histone associated DNA fragments, PARP cleavage, and an increased Bax:Bcl-2 ratio. Induction of apoptosis was associated with activation of caspases 3, 8, and 9 (31). This same group performed a PC3 xenograft experiment to determine the effects of SFN administration *in vivo*. In this experiment tumor growth was significantly inhibited as indicated by reduction in tumor volume and weight. Increased apoptosis and expression levels of Bax were also reported (31). From these data it is clear that apoptosis is induced in both prostate and colon cancer cells through the death receptor and mitochondrial pathways.

1.3.2.3. Histone deacetylase (HDAC) inhibition

HDAC inhibition is emerging as a fascinating and promising field in cancer chemoprevention and therapy. Increased HDAC activity and expression is common in many cancer malignancies, and can result in repression of transcription that results in a de-regulation of differentiation, cell cycle and apoptotic mechanisms. Moreover, tumor suppressor genes, such as *p21*, appear to be targets of HDACs and are “turned

off”, or transcriptional silenced, by deacetylation. HDAC1, a class I HDAC, is over-expressed and localized in the nucleus in hormone refractory prostate cancer (32). It has also been shown that Sirt1, the predominant class III NAD⁺ dependent HDAC, is over-expressed in both human and mouse prostate cancers (33). In human patient samples, global decreases in histone acetylation state corresponded with increased grade of cancer and risk of prostate cancer recurrence (34). In the Apc^{min} mouse model for colon cancer, the loss of *Apc* results in an over-expression of HDAC2. Moreover, HDAC2 knockdown promotes cell death in HT-29 colon cancer cells (35). Taken together these findings support the hypothesis that overactive HDAC activity and hypoacetylation may contribute to prostate and colon cancer progression.

Several clinical trials are currently ongoing aimed at establishing the chemotherapeutic efficacy of HDAC inhibitors, based on evidence that cancer cells undergo cell cycle arrest, differentiation and apoptosis *in vitro*, and that tumor volume and/or tumor number may be reduced in animal models. Strikingly, the effects of HDAC inhibition occur preferentially in cancer cells and not normal cells (36). Recent research has shown that SFN and its metabolites act as HDAC inhibitors.

SFN is metabolized via the mercapturic acid pathway, starting with GSH conjugation by GST and subsequent steps generate SFN-cysteine (SFN-Cys) followed by SFN-*N*-acetylcysteine (SFN-NAC) (Figure 1.3). Biochemical assays found that SFN metabolites did indeed inhibit HDAC activity *in vitro*, the greatest inhibition involving SFN-NAC and SFN-Cys. Molecular modeling in the active site of an HDAC enzyme provided evidence that SFN-Cys is acting as a competitive inhibitor (37). In BPH1, PC3, and LnCap prostate cancer cells, SFN inhibited HDAC activity with a concomitant increase in global histone acetylation, increased acetylated histone H4 interactions with the *P21* and *Bax* promoter, and induction of p21 and Bax mRNA and protein levels (29). The same effects were seen in HCT116 human colorectal cancer cells treated with SFN; namely HDAC inhibition, increase global histone acetylation, and selective increase in histone acetylation at the *p21* promoter (37). HDAC inhibition coincided with the induction of G₂/M phase cell cycle arrest and

apoptosis as indicated by multi-caspase activation (29). HT-29 colon cancer cells, which lack endogenous Nrf2 protein, and Nrf2^{-/-} mouse embryonic fibroblasts both exhibited an HDAC inhibitory response to SFN treatment. These results indicated the possibility of a separate SFN chemoprevention pathway distinct from the classic Nrf2 pathway (38).

In vivo, mice were given a single oral gavage dose of 10 μ M SFN or SFN-NAC and HDAC inhibition was observed with a concomitant increase in acetylated histones and induction of p21 in the colonic mucosa. In dietary studies, *Apc*^{min} mice were fed ~6 μ mol SFN/day for 10 weeks. In these experiments a significant decrease in intestinal polyps and an increase in global acetylated histones H3 and H4 were observed, with specific increases at the *Bax* and *p21* promoters (39). In PC3 xenograft studies, dietary SFN supplementation resulted in slower tumor growth and significant HDAC inhibition in the xenografts, as well as in the prostate and circulating peripheral blood mononuclear cells (38). From these studies it can be concluded that HDAC inhibition represents a novel chemoprevention mechanism by which SFN might promote cell cycle arrest and apoptosis.

1.3.2.4. Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) belong to the superfamily of serine/threonine kinases including the extracellular signal-regulated kinases (ERK), c-Jun NH₂-terminal kinases (JNK), and p38. Each is believed to play a role in carcinogenesis and cancer development. An important downstream effector protein of MAPKs is Activator protein-1 (AP-1), a dimeric basic region-leucine zipper (bZIP) protein that is activated by different MAPKs. The AP-1 family is comprised of heterogeneous and complex dimeric interacting partners, with divergent downstream targets depending on tissue context and cellular stimuli. Modulation of AP-1 members can have effects on both promoting and inhibiting carcinogenesis. These divergent responses observed are likely dependent on genetic background, cell type, tumor state, and signaling networks that are affected in response to specific agents.

In prostate and colon cancer, the activation of AP-1 by SFN appears to be a factor that plays an important role in the regulation of cell death. In HT-29 colon cancer cells activation of AP-1 luciferase activity occurred at low concentrations of SFN treatment ($\leq 35 \mu\text{M}$) while it was inhibited at higher concentration ($\geq 50 \mu\text{M}$). In corroboration with AP-1 activation, cyclin D1 levels increased at low concentrations of SFN and decreased at high concentrations. Interestingly, while AP-1 activity and cyclin D1 protein levels increased at lower concentrations and then decreased at higher concentrations, cell viability decreased in a direct dose dependent manner. Activation of the p46-JNK isoforms, and not the p54-JNK isoforms, was reported indicating that p54-JNK may be responsible for activation of AP-1 (40). Further work in HT-29 cells revealed that SFN was able to decrease cell viability and activate the MAPK pathways ERK, JNK, and p38. Furthermore, consistent with the previous report, activated JNK was able to decrease cyclin D1 levels at high concentrations of SFN (23). SFN treatment in human colon adenocarcinoma cells Caco-2 induced ERK activation but, in contrast to the above studies, not JNK or p38 (26). In prostate cancer cells it was reported that the ERK and JNK pathways are necessary to induce cell death in PC3 cells but only the JNK pathway in DU145 cells (20, 41). Another report by Kong et al., reveals that SFN can inhibit p38 activity. This is significant because active p38 can phosphorylate Nrf2 and promotes its association with Keap1 thereby suppressing Nrf2 translocation to the nucleus, thus inhibiting activation of phase 2 enzymes (42). Taken together these data indicate a diverse range of MAPK responses and possible cellular outcomes after SFN treatment in both prostate and colon cancer cells and highlights the importance of evaluating MAPK pathways in light of tissue context.

1.3.2.5. Nuclear factor kappa-B

The nuclear factor kappa-B (NF κ B) is a heterodimeric transcription factor that consists of a p50 and p65 subunit and, when active, promotes inflammatory gene expression, cell proliferation and cell survival. Inactive NF κ B is normally found in the cytosol, bound to its inhibitory subunits (I κ Bs). Upon activation, I κ Bs are rapidly phosphorylated by I κ B kinases (IKK), and ubiquitinated leading to I κ B degradation

and subsequent release of NF κ B and translocation to the nucleus. Constitutive activation of NF κ B is common in various human malignancies, including colon and prostate cancer, and leads to up-regulation of genes encoding adhesion molecules, inflammatory cytokines, growth factors, and anti-apoptotic genes (43, 44). Thus, inhibition of NF κ B activation has been postulated as a key target for cancer chemoprevention.

Several labs have shown down-regulation of NF κ B activity with SFN administration in prostate and colon cancer cells. This was observed in PC3 cells treated with 20 μ M SFN in which reduced nuclear localization of p65-NF κ B occurred after 1 h (45). Additionally, in PC3 cells inhibition of NF κ B activity coincided with expression of downstream targets, VEGF, cyclin D1, and Bcl-X_L. Decreased nuclear translocation and activation of p65-NF κ B was attributed to the inhibition of IKK phosphorylation, thereby attenuating I κ B α phosphorylation and degradation (46). Similar effects were seen in HT-29 colon cancer cells, depending on SFN dose. Specifically, SFN strongly inhibited LPS induced NF κ B activity at doses as low as 10 μ M, but a dose of 50 μ M SFN was required for significantly decreased LPS induced I κ B α phosphorylation. Decreased cell viability was observed at 25 μ M SFN which correlated to an induction of apoptosis via caspase 3 (47). Interestingly, a unique biphasic response was observed in LnCap cells, with an initial increase in NF κ B activity at 6-12 h, followed by inhibition 24 h after treatment. The inhibitor of apoptosis (IAP) family is one family of the downstream factors that are up-regulated by NF κ B activation. In LnCap and PC3 cells, the modulation of IAP levels was proportional to the level of NF κ B activity (45). Collectively, these results indicate that SFN can affect proliferation signals and apoptotic signals via modulation of NF κ B activity.

1.3.2.6. Reactive oxygen species

The production of reactive oxygen species (ROS) has been postulated to be a key mechanism by which SFN induces apoptosis. SFN administration to PC3 prostate

cancer cells resulted in ROS generation, which was accompanied by disruption of mitochondrial membrane potential, cytosolic release of cytochrome C, and apoptosis. All of these effects were reversed with administration of the antioxidant *N*-acetylcysteine and overexpression of catalase (48). The authors indicated that conjugation of SFN with GSH, a necessary step in SFN metabolism, depletes the intracellular concentration of GSH and potentially lowers the oxidative stress threshold of the cell. In their experiments SFN treatment increases mitochondrial ROS production and induces apoptosis as indicated by release of cytochrome C via both death-receptor and mitochondrial caspase cascades (48). In HT-29 colon cancer cells treated with 50 μ M SFN, the cell cycle arrest response were blocked by addition of antioxidants NAC or GSH, indicating that generation of ROS was indispensable for growth arrest under the assay conditions used (23). Generally, high doses of SFN are needed in order to induce ROS production, but one group reported a transient rise in ROS in DU145 after treatment with only 10 μ M SFN (20). Mitochondrial ROS generation and disruption of the mitochondrial membrane potential have both been shown to induce the formation of acidic vesicular organelles and autophagy in PC3 and LnCap human prostate cells at a dose of 40 μ M SFN. This response has unique morphological effects and, interestingly, has the ability to inhibit mitochondrial cytochrome C release and apoptosis (49). Therefore, ROS production after SFN exposure has the ability to influence cell death in prostate and colon cancer cells.

1.4. Metabolism and bioavailability

The metabolism of SFN is summarized in Figure 1.3. The initial reaction involves enzymatic hydrolysis of glucoraphanin, the glucosinolate precursor of SFN, found in the plant. This reaction is catalyzed by myrosinase, a β -thioglucosidase, which cleaves the glycone from the glucosinolate forming glucose, hydrogen sulfate and one of many different aglycones (e.g. thiocyanate, ITC, or a nitrile) depending on the glucosinolate, reaction pH, and availability of ions (50). At neutral pH, the major glucosinolate hydrolysis products are stable isothiocyanates. After absorption, SFN is predominantly metabolized via the mercapturic acid pathway. In these reactions, the

electrophilic central carbon of the $-N=C=S$ group in SFN reacts with the sulfhydryl group of GSH to form a dithiocarbamate GSH conjugate. The enzymes that catalyze GSH conjugation to SFN are the family of GST enzymes, and polymorphisms in these enzymes have a significant impact on overall ITC metabolism (discussed below). Interestingly, SFN is also able to induce its own metabolism via induction of GSTs. The final steps in SFN metabolism is formation of SFN-Cys and ultimately SFN-NAC (51).

The absorption and bioavailability of SFN is affected by several factors. The first factor involves the hydrolysis of SFN from glucoraphanin via myrosinase activity. This initial step is critical because only the ITC form is thought to be biologically active and exhibits the desired anticancer properties. Importantly, mammalian cells do not have endogenous myrosinase activity. Instead myrosinases are found in the plant or in the gut microbial flora. In the cruciferous plant, the myrosinase enzyme is physically separated from the glucosinolate, but upon physical disruption through chopping, cutting, and/or chewing, the enzyme is released and the ITC is formed. However, myrosinase is heat labile and thus cooking procedures can inactivate the enzyme and significantly reduce the bioavailability of SFN up to 3-fold (52). Another source of myrosinase activity is the intestinal microbial flora. Evidence from experiments done with isolated human fecal bacteria (53) and others using F344 rats dosed ip with glucoraphanin, indicates that glucoraphanin can be converted to SFN by colonic microbial flora and that enterohepatic circulation is requisite for efficient metabolism (54). However, the bioavailability of SFN is six times less when metabolism of the glucosinolate to the ITC had not occurred prior to ingestion (55), revealing a strong reliance on plant myrosinase activity, as opposed to the intestinal gut flora. Nonetheless, an important factor determining inter-individual SFN bioavailability is variability in the gut microbial flora when the non-hydrolyzed glucosinolate is consumed.

The last factor that affects SFN bioavailability is related to polymorphisms in phase 2 SFN metabolizing genes, such as GSTs, which play a significant role in

determining the detoxifying ability of an organism. In general, GST enzymes catalyze the conjugation of GSH to electrophiles such as SFN. There are six different classes of GST isoenzymes; α , μ , π , θ , σ , and κ , and each functional unit is composed of two subunits. Each is designated by the abbreviated Roman capital of each Greek letter followed by a number indicating subunit composition (ex. GST μ 1=GSTM1). In general, the substrate specificity for the different isoenzymes overlaps but, specifically, each class has varying degrees of reactivity for different substrates. GST null genotypes are quite prevalent in the population, with up to 50% of people being GSTM1 null and 47% GSTT1 null.

The effect that GST genotype has on cancer development and chemoprevention is complex. In the context of high cruciferous vegetable intake, evidence is mounting in favor of a GST null genotype providing a protective effect against lung, colon, and breast cancers (56, 57). Since GST activity is believed to play a critical role in SFN metabolism and subsequent excretion, lower GST activity in individuals with GST polymorphisms could result in slower elimination and longer exposure to isothiocyanates after cruciferous vegetable consumption. For example, one study investigating a population in Singapore reported higher ITC excretion among GSTT1 positive individuals in comparison to GSTT1 null (58), implying shorter exposure times to the potential beneficial metabolites of SFN for GSTT1 positive individuals. This finding corroborates with a later study in Singapore, indicating that the GST null genotype coupled with high cruciferous vegetable intake, reduced the risk of colorectal cancer (59). However, other studies have shown that the GSTM1 null genotype produced a slight increase in the area under time-concentration curve (AUC) for metabolite concentrations in plasma, a significantly higher rate of SFN metabolite excretion, and a higher percentage of SFN excreted 24 h after ingestion, indicating shorter retention times of SFN and its metabolites (60). In addition, in other studies, a GSTM1 null genotype had a non-significant increase in prostate cancer risk, while men in the high vegetable consuming group that have GSTM1 present exhibited the greatest reduction in cancer risk (1). These inconsistencies may be explained by

differences in the predominant ITC consumed (3-butenyl-ITC and 4-pentenyl-ITC versus SFN), the class of GST null genotype present (GSTT1 versus GSTM1), or the cancer of interest (colorectal versus prostate). Despite these conflicting results, the impact of polymorphism on nutrient bioavailability is an important area of research that will aid in our understanding of response variability to SFN and other phytochemicals in human populations.

1.5. Pharmacokinetics

The ability of SFN to be distributed throughout the body and reach target tissues has been investigated *in vitro*, in mouse models and in human subjects. In the human small intestine, SFN can be efficiently absorbed and conjugated to GSH. Human perfusion experiment showed that $74 \pm 29\%$ of SFN from broccoli extracts can be absorbed in the jejunum and that a portion of that returns to the lumen of the jejunum as SFN-GSH (14). Pharmacokinetic studies in both rats and humans also support that SFN can be distributed in the body and reach μM concentrations in the blood. In rats, following a $50 \mu\text{mol}$ gavage of SFN, detectable SFN was evident after 1 h and peaked at $\sim 20 \mu\text{M}$ at 4 h, with a half life of approximately 2.2 h (61). In human subjects given single doses of $200 \mu\text{mole}$ broccoli sprout isothiocyanate preparation, ITC plasma concentrations peaked between $0.943\text{--}2.27 \mu\text{M}$ 1 h after feeding, with half life times of $1.77 \pm 0.13 \text{ h}$ (62). Once SFN is distributed there is evidence that it can accumulate in tissues and produce anticancer blocking and suppressing effects. In a recent pilot study in human mammary tissue, an oral dose of a broccoli sprout preparation containing $200 \mu\text{mol}$ SFN 1 h prior to tissue removal showed mean accumulation of $1.45 \pm 1.12 \text{ pmol/mg}$ in the right breast and $2.00 \pm 1.95 \text{ pmol/mg}$ in the left breast. In these tissues the detoxification genes *NQO1* and *heme oxygenase-1 (HO-1)* were measured providing proof of principle that these measures can be made in a clinical trial (63). In another study mice supplemented with 300 or 600 ppm SFN, accumulated SFN and SFN-GSH plasma concentrations of $124\text{--}254 \text{ nM}$ and $579\text{--}770 \text{ nM}$, respectively. Also SFN and SFN-GSH concentrations in the small intestine were between 3 and 13 nmol/g of tissue and 14–32 nmol/g of tissue, respectively, which is equivalent to

roughly 3-30 μM of total SFN. Notably, the accumulation of SFN in colonic tissue corresponded with decreased adenoma formation in these animals (64). The last aspect of SFN pharmacokinetics concerns its excretion. When efficient metabolism of glucoraphanin occurs, SFN-NAC is the primary SFN metabolite excreted in the urine (12, 51, 52, 54). In humans, SFN and its metabolites were excreted with first-order kinetics (53, 62) and most data indicate that SFN and its metabolites are cleared from the body within 72 h of dosing. From these data it can be extrapolated that maintenance of SFN concentrations in the body can be achieved by consuming recommended servings of cruciferous vegetables once a day. Collectively, the published data indicate that SFN can be absorbed, reach μM concentrations in the blood, reach certain tissues and be maintained to achieve the anticancer effects.

1.6. Preclinical and clinical studies

In preclinical rodent models, there is significant data supporting the chemopreventive effects of SFN at several stages of carcinogenesis. SFN supplementation administered both pre- and post-initiation, decreased colonic aberrant crypt foci in azoxymethane (AOM)-induced rats. In contrast, supplementation with SFN-NAC was only effective post-initiation, suggesting different mechanisms of action between parent SFN and its metabolites (65). SFN supplementation also decreased polyp formation in Apc^{min} mice (39, 64). In other suppression studies, supplementation with SFN also decreased tumor growth in prostate xenograft studies (31, 66).

To date very few human clinical trials have evaluated the effects of SFN on cancer outcome, however, several pilot and phase 1 human SFN trials have been conducted utilizing different sources of SFN. The first study was a randomized, placebo-controlled, double-blind phase 1 clinical trial of healthy volunteers that used glucoraphanin or isothiocyanate as the SFN source (67). The phase 1 trial consisted of three study groups; 25 μmol of glucosinolate, 100 μmol of glucosinolate, or 25 μmol ITC for 7 days and examined parameters of safety, tolerance, and pharmacokinetics.

Importantly, there were no significant toxicities associated with taking the extracts at the doses employed. A second study was a randomized placebo-controlled chemoprevention trial performed in Qidong, People's Republic of China which used a hot drinking water infused with 3-day old broccoli sprouts (68). Residents of Qidong are at high risk for development of hepatocellular carcinoma, in part due to consumption of aflatoxin-contaminated foods, and are exposed to high levels of the airborne toxin phenanthrene. There was an inverse association for the excretion of dithiocarbamates and urinary aflatoxin-DNA adducts and *trans, anti*-phenanthrene tetraol, a metabolite of the combustion product phenanthrene in the intervention arm. Thus, an inverse correlation between SFN treatment and excretion of carcinogens was detected, suggesting induction of one or more phase 2 enzymes. Interestingly, in the Qidong trial, although there was consistency within an individual between doses, there was significant inter-individual variability in bioavailability of the dithiocarbamates. A third small preliminary human study interested in determining if the HDAC inhibition effects observed in cell culture and mice could be translated into humans was performed. After ingestion of 68g of broccoli sprouts, a significant decrease in HDAC activity was evident in peripheral blood mononuclear cells with a concomitant increase in acetylated histones H3 and H4 (66). The most recent pilot human study was performed in 8 healthy women who were undergoing elective reduction mammoplasty. Here the women were given an oral dose of broccoli sprout preparation containing 200 μ mol SFN 1 h prior to breast surgery. Mean epithelial-/stromal-enriched breast tissue dithiocarbamate concentration was 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue for the right and left breast, respectively. *NQO1* and *HO-1* transcript levels were measured in both breasts of all subjects (63). These clinical trials provide the important link to human relevance for SFN as a promising anticancer agent.

1.7. Conclusions

Cancer is a dynamic and multifaceted disease that impacts morbidity and mortality in every country in the world. Prostate and colon cancers are highly prevalent in the U.S., collectively representing 20% of all cancer deaths in men.

Lifestyle changes can potentially eliminate one third of these cancers, and epidemiological data indicates an inverse relationship between cruciferous vegetable intake and cancer risk. SFN has been postulated to be one of the principle ITCs found in cruciferous vegetables that possesses cancer chemopreventive properties. One of the challenges in cancer treatment is that each stage of cancer development presents unique obstacles to efficacious targeting and treatment of cancer. Since cancer is a multi-factorial process, with several molecular alterations, targeting more than one pathway by a chemopreventive agent is highly desirable. The ability of SFN to target both blocking mechanisms, via alteration in phase 1 and phase 2 enzymes, and suppressing mechanisms post-initiation via multiple cell proliferation targets (see Figure 1.2), make SFN a highly promising dietary chemoprevention and therapeutic agent.

An important key area of research that needs to be further addressed is the metabolism, bioavailability, and efficacy of SFN treatment in human populations. In clinical trials, SFN treatment appears to be safe and well tolerated. The blocking action of SFN is evident in the induction of phase 2 enzymes and inverse relationship between dithiocarbamate excretion and carcinogen excretion after ingestion of SFN. The suppression action of SFN is verified in humans by *ex vivo* induction of *p21* in resected colon and HDAC inhibition response in peripheral blood cells. However, other cancer suppressive mechanisms identified in cell culture and mouse models have not been studied in humans. Additionally, more extensive clinical studies are required to elucidate the changes in key cancer suppression biomarkers, such as apoptosis and cell cycle arrest, as well as tumor outcomes. Although preliminary clinical studies have shown promise, larger human studies are necessary in order to understand the full potential of SFN for cancer chemoprevention. In particular, a greater understanding of the mechanisms leading to inter-individual variation in responses to SFN supplementation is needed to delineate efficacious dose response, source of SFN (whole food or supplement), and effects of genotype in the population at large.

In closing, it is important to mention that there are other glucosinolates and metabolites found in cruciferous vegetables which possess similar chemistry, metabolism, and anticancer effects as SFN (eg. glucobrassicin- I3C, gluconasturtiin-phenethylisothiocyanate, glucoerucin-erucin (sulfide analog of SFN), glucoiberin-iberin). Also, there is a large body of research that has examined SFN effects on many other cancers such as breast, hepatic, bladder, osteosarcoma, glioblastoma, leukemia, pancreatic, and melanoma. Although larger scale clinical trials are necessary, dietary SFN shows promise as a safe and effective anti-cancer strategy that includes incorporating easily accessible foods into an individual's regular diet.

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Table 1.1 SFN responsive blocking and detoxifying genes.

Category	Gene	Change in activity
Phase 1 enzyme	CYP1A1	Decrease
	CYP2B1/2	Decrease
	CYP4A10	Increase
	CYP4A14	Increase
	CYP39A1	Increase
Phase 2 enzyme	NQO-1	Increase
	GST	Increase
	λ -GCS	Increase
	UGT	Increase
Antioxidants	Thioredoxin reductase 1 and 3	Increase
	Glutathione peroxidase 3	Increase
Proteasome subunits	Alpha 1 and 3, beta 5, and 26S	Increase
Cell cycle and cell growth	Cyclins D1, E2 and T2	Increase
	CDK 4, 7 and 9	Increase
Stress response	Heat shock proteins 1, 1 alpha, 1 beta, 8 and 105	Increase
Transcription factors	CREB binding protein	Increase
	Aryl hydrocarbon receptor nuclear translocator-like	Increase

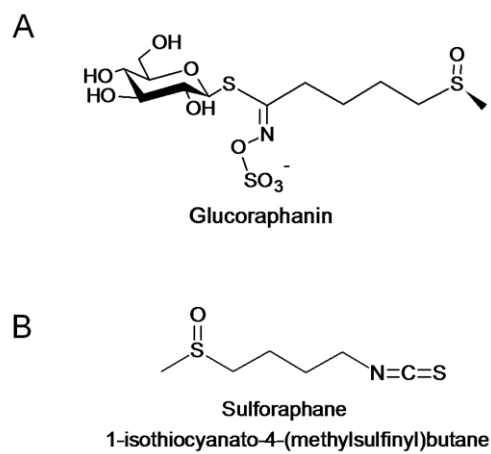


Figure 1.1 Chemical structures of glucoraphanin and sulforaphane.

Structures of glucosinolate precursor (A) glucoraphanin and its isothiocyanate hydrolysis product (B) sulforaphane.

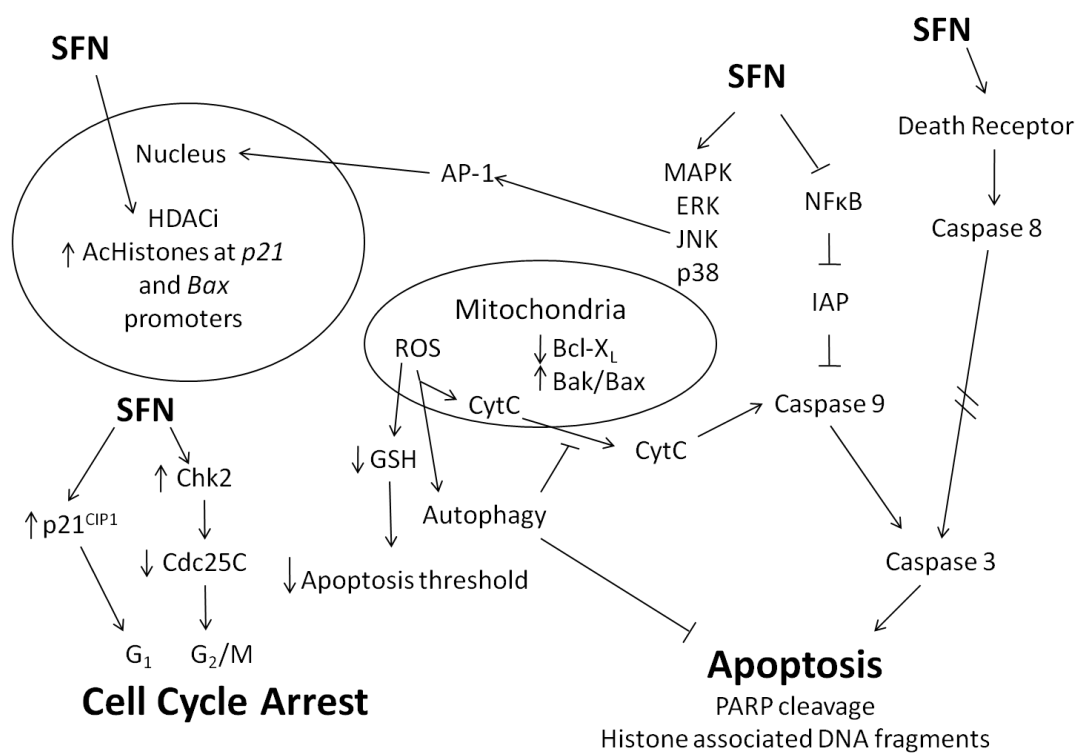


Figure 1.2 Proposed "suppression" chemoprevention mechanisms for sulforaphane.

Proposed "suppression" mechanisms of chemoprevention by SFN leading to alteration in cell cycle arrest, apoptosis and/or growth inhibition.

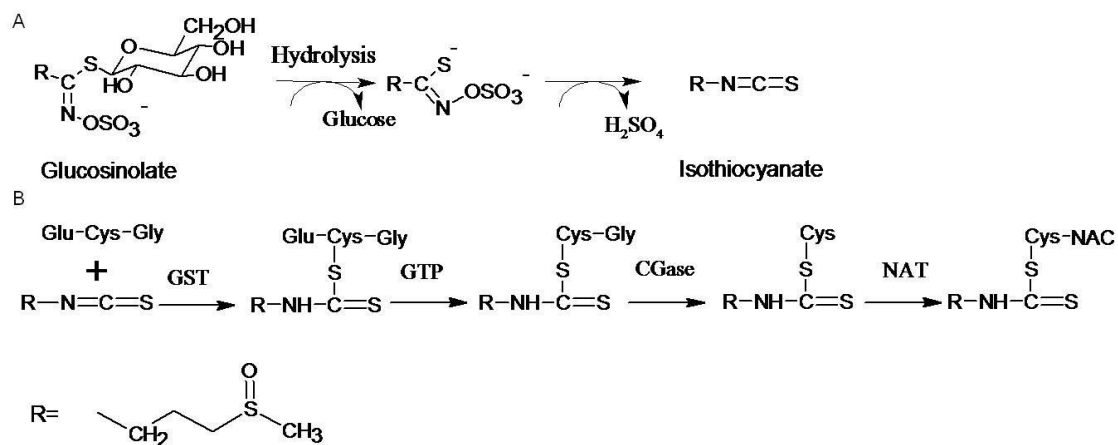


Figure 1.3 Glucoraphanin and sulforaphane metabolism.

Metabolism of sulforaphane involving (A) hydrolysis of glucosinolate to its isothiocyanate via myrosinase enzyme activity and (B) metabolism of SFN via the mercapturic acid pathway. GST=Glutathione-S-Transferase; GTP= γ -Glutamyltranspeptidase; CGase=Cysteinylglycinase; NAT=; N-acetyltransferase

Chapter 2 Differential effect of sulforaphane on histone deacetylases,
cell cycle arrest and apoptosis in normal prostate cells versus
hyperplastic and cancerous prostate cells

John D. Clarke, Anna Hsu, Zhen Yu, Roderick H. Dashwood, Emily Ho

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2.1. Abstract

Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables such as broccoli. The ability of SFN to inhibit histone deacetylase enzymes may be one mechanism by which it acts as a chemoprevention agent. The ability of a chemopreventive agent to specifically cause cytotoxicity in cancer, not normal cells is an important factor in determining its safety and clinical relevance. We characterized the effects of SFN in normal (PrEC), benign hyperplasia (BPH1) and cancerous (LnCap and PC3) prostate epithelial cells. We observed that 15 μ M SFN selectively induced cell cycle arrest and apoptosis in BPH1, LnCap and PC3 cells but not PrEC cells. SFN treatment also selectively decreased HDAC activity, and Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for *P21*, induced p21 expression and increased tubulin acetylation in prostate cancer cells. HDAC6 over-expression was able to reverse SFN-induced cytotoxicity. In PrEC cells, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. The differences in sensitivity to SFN in PrEC and PC3 are likely not due to differences in SFN metabolism or differences in phase 2 enzyme induction. From these data we conclude that SFN exerts differential effects on cell proliferation, HDAC activity and downstream targets in normal and cancer cells.

2.2. Introduction

Epidemiologic studies suggest that cruciferous vegetable intake may lower the overall risk of prostate cancer (6, 69). Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables such as broccoli and broccoli sprouts (70). The majority of chemoprevention studies have focused on the ability of SFN to act “pre-initiation” as a potent phase 2 enzyme inducer via Keap1-Nrf2 signaling and antioxidant response element (ARE)-driven gene expression. Additional evidence also suggests that SFN suppresses tumor development during the “post-initiation” phase of cancer via induction of cell cycle arrest and apoptosis (18, 25). Recently, a novel suppression mechanism involving the ability of SFN to inhibit histone deacetylase (HDAC) enzymes, alter histone acetylation and affect gene regulation has been reported (29, 37, 66).

Lysine acetylation and deacetylation is a dynamic process executed by histone acetyltransferases (HAT) and HDACs that can affect intermolecular interactions and/or the stability and activity of proteins. Currently there are 11 trichostatin A (TSA) sensitive HDACs which are divided into four classes (class I, IIa, IIb, and IV) according to structure, expression patterns, and subcellular localization. The class I HDACs consists of HDACs 1, 2, 3, and 8 and are expressed in all tissues. They are predominantly found in the nucleus and are mainly responsible for histone deacetylation. HDACs 4, 5, 7, and 9 belong to class IIa and have a more restricted expression pattern, being found predominantly in tissues such as muscle, brain, heart, endothelial cells, and thymocytes (71). The class IIb HDACs consists of HDAC 6 and 10, with HDAC6 being primarily localized in the cytoplasm and targeting non-histone substrates, while little is currently known about HDAC10.

In general, addition of acetyl groups to histones by HATs promotes gene expression by creating an “open” chromatin conformation and acts as a docking site for bromo domain containing transcription machinery, thereby facilitating access to DNA and organization of transcription. Removal of acetyl groups by HDACs results

in a “closed” conformation and expulsion of transcription activating components, ultimately repressing transcription. In addition to histone proteins, the acetylation of lysine residues on non-histone proteins by Class II HDACs plays a major role in many different cellular processes (72). A well studied example is HDAC6 which has multiple non-histone substrates including α -tubulin and HSP90 (73). The functional consequences of the acetylation and deacetylation of α -tubulin are alterations in microtubule dynamics and stability, cell migration, aggresome formation, and autophagy (74, 75). All of these processes are critically important in regulating cell cycle arrest, misfolded protein toxicity, and cell death. Currently little is known regarding the effects of dietary HDAC inhibitors on non-histone protein acetylation, and changes in Class I and Class II HDACs.

Pharmacological HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA), valproic acid, depsipeptide, and phenyl butyrate have been shown to be effective agents against prostate cancer in cancer cell lines and in xenograft models (76, 77). Similar to pharmacological HDAC inhibitors, SFN inhibits HDAC activity and suppresses prostate tumor growth both *in vitro* and *in vivo* (29, 66). Furthermore, HDAC inhibitors such as SAHA, TSA, and other novel hydroxamic acids are selectively growth inhibitory and cytotoxic to cancer cells rather than normal cells (78-83). For example, SAHA (Vorinostat) treatment in prostate cancer cells inhibited cell growth (LnCap, DU-145, and PC3) and induced cell death (LnCap and DU-145), whereas normal prostate cells (PrEC) were resistant to SAHA induced growth arrest and cell death (79). Thus, the goal of this study was to examine the cytotoxicity of SFN in normal and cancerous prostate epithelial cell lines by characterizing the effect of SFN on cell cycle arrest, apoptosis, HDAC activity, Class I and II HDAC expression, and histone/non-histone protein acetylation.

2.3. Materials and methods

Cell culture

Normal (PrEC) prostate epithelial cells were obtained from Lonza (Basel, Switzerland). Benign hyperplasia epithelial cells (BPH1) were a kind gift from Dr. Simon Hayward (Vanderbilt University Medical Center, Nashville, TN). Androgen dependent prostate cancer epithelial cells (LnCap) and androgen-independent prostate cancer epithelial cells (PC3) were obtained from American Type Tissue Collection (Manassas, VA). Cells were cultured at 5% CO₂ and 37°C. PrEC cells were cultured in PrEC basal media (PrEBM) with PrEC growth media (PrEGM) SingleQuots (Lonza) and BPH1, LnCap and PC3 cells in Roswell Park Memorial Institute (RPMI) 1640 supplemented with glutamine plus 10% fetal bovine serum (FBS) (Cellgro, Manassas, VA). The cells were treated with dimethylsulfoxide (DMSO) (vehicle control) or 15 μ M SFN (LKT laboratories, St. Paul, MN) for the time indicated and harvested for subsequent assays. This concentration of SFN was based on previous reports showing HDAC inhibition in prostate and colon cancer cells (29, 37). For HDAC6 over-expression 2.0 x 10⁴ cells per well were seeded in 96-well plates. For each well 20 μ L of Opti-MEM reduced serum media (Invitrogen) was mixed with 50ng of either empty vector (pcDNA 3.1, Invitrogen, Carlsbad, CA) or HDAC6 plasmid (cat # SC111132 Origene, Rockville, MD) and 0.1 μ L of PLUSTM reagent, and incubated for 10 min at room temperature. Lipofectamine LTXTM (0.3 μ L) was added and the solution was incubated for 30 minutes at room temperature. The DNA mix (20 μ L) was added to each well and cultured for 18-24 h. Cells were treated with DMSO or SFN as outlined above for 24 and 48 h. Transfection efficiency was ~50-65% using a GFP plasmid and measured by flow cytometry (Guava PCA). For protein over-expression the transfection procedure was scaled up to 12well format and western blots were performed as described below.

Western blot analysis

Proteins (10-20 μ g) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Novex, San Diego, CA) and transferred to nitrocellulose membrane (Invitrogen). Primary antibodies from Santa Cruz Biotechnologies, Santa Cruz, CA were as follows: HDAC2 (H-54) 1:200; HDAC3 1:200; HDAC4 1:200; HDAC6 1:200; HDAC8 1:200.

Other primary antibodies were: HDAC1, 1 µg/mL (Upstate, Billerica, MA); p21, 1:1000 (Cell Signaling, Danvers, MA); acetylated α -tubulin 1:5000 (Sigma, St. Louis, MO); poly (ADP-ribose) polymerase (PARP) (N-20) 1:2000 (BD); α -tubulin (Sigma); and β -actin, 1:5000 (Sigma). Detection was performed by using Supersignal West Femto Reagent (Thermo Scientific, Waltham MA) with image analysis on an AlphaInnotech photodocumentation system. Image quantification was determined by NIH ImageJ. Treatments were performed in triplicate.

Apoptosis/cell viability assays

Cell viability was determined using the trypan blue exclusion assay. Multicaspase assay was used as a marker of apoptosis using a flow-cytometry based assay kit (Guava Technologies, Hayward, CA). Briefly, 5×10^4 cells were stained in triplicate with a fluorochrome-conjugated caspase inhibitor, sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK) and 7-amino-actinomycin D (7-AAD) according to the manufacturer's instructions and analyzed on a Guava Personal Cell Analyzer (Guava PCA) (Guava Technologies).

Cell cycle analysis

A flow cytometric assay was performed to assess effects of SFN on cell cycle. One million cells were fixed in 70% ethanol. After fixation, cells were washed, pelleted, and resuspended in 0.04 mg/ml propidium iodide and 100 mg/ml RNase in PBS and analyzed on the Guava PCA. Multi-Cycle analysis software (Phoenix Flow Systems, San Diego, CA) was used to generate histograms and determine the number of cells in each phase of the cell cycle. Cells were treated in triplicate.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). Two micrograms of RNA were reverse-transcribed using the Invitrogen Super-Script® III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. About 50 ng cDNA was amplified using DyNAmo™ SYBR® Green qPCR Kit (New England Biolabs, Ipswich, MA) in a Chromo4 Real Time PCR

detections system (MJ Research, Waltham, MA) using primers for p21^{waf1/cip1} (F:CAGACCAGCATGACAGATTTC, R:GCGGATTAGGGCTTCCTCTT, annealing temp=56°C), GSTP1 (F: GCCCTACACCGTGGTCTATT, R: TGCTGGTCCTTCCCATAGAG annealing temp=58°C); HO1 (F: CTTCTTCACCTTCCCCAAC, R: GCTCTGGTCCTTGGTGTGATA, annealing temp=58°C); NQO1 (F: AAAGGACCCTTCCGGAGTAA, R: AGGCTGCTTGGAGCAAAATA, annealing temp=58°C); β -actin F: CTTCCAGCCTTCCTTCCTGGGCATG, R: GCTCAGGAGGAGCAATGATCTTGATC, annealing temp=56°C) PCR conditions were as follows: 95°C/10 min; 40 cycles: 94°C/10 sec, annealing temperature/20 sec and 72°C/20 sec; melting curve 60-95°C read every 0.2 sec; 72°C/10 min. Cells were treated in triplicate.

LC-MS/MS

The methods for LC-MS/MS analysis were adapted from Al Janobi et al (84). For cellular metabolite analysis, cells were treated in duplicate with SFN or vehicle for a time course up to 72 h and $\sim 5 \times 10^5$ cells were dedicated to MS analysis. Cell pellets were resuspended in 100 μ L of 0.1% formic acid in water, vortexed and frozen at -80°C. After being thawed the samples were vortexed again and centrifuged at 11,600 x g for 5 minutes at 4°C. The supernatant was collected and acidified with 10 μ L of trifluoroacetic acid. The samples were centrifuged as before and the supernatants were collected and filtered through a 0.2 μ m filter. For metabolite analyses in cell culture media, 500 μ L of spent media was saved and immediately acidified with pre-cooled (4°C) trifluoroacetic acid (10%). The samples were centrifuged at 11,600 x g for 5 minutes at 4°C and subsequently filtered through a 0.2 μ m filter.

Ten μ L of sample were separated on a Shimadzu Prominence HPLC (Shimadzu, Kyoto, Japan) using a reversed-phase Phenomenex synergi 4 μ m hydro RP 80Å 250 x 1.0 mm HPLC column. The flow rate was 0.1 mL/min using 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B). The gradient was as follows: 5% B increasing to 30% (7 min), held at 30% (6 min),

washed out with 90% B (10 min) and re-equilibrated to 5% B (5 min). The LC eluent was sprayed into an API triple quad mass spectrometer 3200 (Applied Biosystems, Foster City, CA) by electrospray ionization in positive mode. Tandem mass spectrometry using multiple reaction monitoring was used to detect the analytes with the following precursor and product ions: SFN (178>114), SFN-GSH (485>114), SFN-CG (356>114), SFN-Cys (299>114), SFN-NAC (341>114). Spike and recovery experiments confirmed that >95% of all compounds were recovered following the processing protocols outlined.

HDAC activity assays

HDAC activity was assayed using the 386 well format Fluor-de-Lys HDAC activity kit (Upstate) as described in (37). Five μ g of total cell lysate were added to each well and assay was performed according to the manufacturer's instructions. Fluorescence was measured and recorded as arbitrary fluorescence units (AFU) using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

Chromatin immunoprecipitation

ChIP assays were performed to examine the association of acetylated histone H3 with *P21* promoter as described previously (29). As a positive control PC3 cells were also treated with 0.3 μ M Trichostatin A (Enzo Life Sciences International, Inc, Plymouth Meeting, PA) for 8 h and harvested for the ChIP assay. An acetylated histone H3 ChIP kit (Upstate) was used according to the manufacturer's instructions. Quantification of ChIP DNA was performed by using DyNAmo™ SYBR® Green using primers for *P21*^{waf1/cip1} (F: GGTGTCTAGGTGCTCCAGGT, R: GCACTCTCCAGGAGGACACA). The immunoprecipitated DNA was normalized to input DNA and expressed as percentage of input DNA ($2^{CT_{input} - CT_{IP}} \times 10$) as described by Frank et. al. (85). PCR conditions were as follows: 95°C/10 min; 40 cycles: 94°C/10 sec, 63°C/20 sec and 72°C/20 sec; melting curve 60-95°C read every 0.2 sec; 72°C/10 min. Treatments were performed in triplicate.

Statistical analysis

All experiments except LC-MS/MS experiments were done in triplicate. The acetyl-histone ChIP and the HDAC6 over-expression experiments were analyzed by Student's t-test comparing the SFN treatment effect. The trypan blue exclusion time course was analyzed by two-way ANOVA. All other experiments were analyzed using one-way ANOVA with Dunnett's post-test comparing the mean SFN treatment effect for PrEC against each of the other cell lines.

2.4. Results

SFN preferentially induced apoptosis and cell cycle arrest in BPH1, LnCap and PC3 cells.

An induction of apoptosis was observed in BPH1 and PC3 cells following 48 h SFN treatment, as indicated by increases in multicaspase activity (Figure 2.1A). LnCap cell showed a trend towards increased caspase activity at 48 h but it was not significantly different than the SFN effect observed in PrEC cells. No effect on cell viability in PrEC cells with a dose range of 0-15 μ M for 24h was observed, as assessed by trypan blue exclusion assay ($p=0.63$ by one-way ANOVA). Two-way ANOVA analysis of 15 μ M SFN treatment in a time course up to 72 h in PrEC cells showed no significant treatment or interaction effect, although the time effect was significant ($p<0.0001$) (Figure 2.1B). A striking difference between PrEC cells and the other cell lines was seen in cell cycle kinetics at 24 and 48 h of SFN treatment. At 24 h SFN strongly induced a G₂/M cell cycle arrest in BPH1 and PC3 cells but had no effect on cell cycle in PrEC cells (Figure 2.2). Similar to previous published data (19), SFN caused significant changes in cell cycle distribution in LnCap cells at 48 h. Taken together these data provide evidence that SFN is preferentially cytotoxic to BPH1, LnCap and PC3 cells.

Induction of phase 2 enzymes in all cell lines and relationship to SFN metabolism.

The canonical chemoprevention pathway for SFN involves induction of phase 2 enzymes via activation of the Nrf2 transcription factor. We tested all four prostate cell

lines for the induction of several phase 2 enzymes known to be induced by SFN: glutathione-S-transferase- π 1 (GSTP1), Heme oxygenase (HO1) and NAD(P)H:quinone oxidoreductase (NQO1). No differences in the induction of HO1 and NQO1 were observed between the cell lines following 12 h SFN treatment. No induction of GSTP1 was observed at 12 h in any cell line (Figure 2.3). These data indicate that SFN treatment similarly induces the phase 2 enzymes HO1 and NQO1 in normal, hyperplastic and cancerous cell lines.

To confirm equivalent dosing and assess any possible differences in metabolism that may have contributed to the differences observed in cell fate, SFN and its metabolites were measured in culture media and the cell lysates for all four cell lines at selected time points using LC-MS/MS. All cell lines were similar regarding the metabolism of SFN at 24 and 48 h (data not shown) and therefore we chose to focus on normal PrEC and late-stage androgen independent PC3 and perform a rigorous SFN time course. The overall loss of SFN and its metabolites in both the media and in the lysate was similar in PrEC and PC3 cells, with a ~50% decrease detected after 24 h (Figure 2.4A). In the cell culture media the sum of all SFN compounds was similar between the cell lines (Figure 2.4A). For the individual SFN compounds a trend towards a 2-3 μ M shift in metabolite ratio from free SFN to the glutathione conjugate (SFN-GSH) was observed in the PrEC media at 24, 48, and 72 h, and the cysteinyl conjugate (SFN-Cys) was detected at similar levels in the media from both cell lines (Figure 2.4B). In the cell lysates, the sum of all SFN compounds was similar in both cell lines (2.8 and 2.7 nmol/mg in PrEC and PC3, respectively, at 0.5 hr) although a trend towards differences was observed at 2 and 5 h (Figure 2.4A). The cellular concentration of SFN-Cys, one of the putative HDAC inhibitor forms of SFN, was similar in both cell lines (Figure 2.4C). The other known metabolites of SFN, cysteinyl-glycine and N-acetylcysteine (SFN-NAC) conjugates, were not detected in either the cell lysate or the culture media. Overall these metabolite data show that the metabolite profiles and exposure times/concentrations are similar between PrEC and PC3 cell lines.

SFN inhibits HDAC activity in prostate cells.

At 24 h HDAC inhibition was observed in all cell lines and LnCap was the only line to have significantly greater HDAC inhibition compared to PrEC cells (Figure 2.5). By 48 h SFN induced HDAC inhibition was sustained in BPH1, LnCap and PC3 cells but not in PrEC, suggesting a transient inhibition of HDAC activity in normal prostate cells.

SFN selectively decreases HDAC protein expression in BPH1, LnCap and PC3 cells.

Class I HDACs (1,2,3,8) and Class II HDACs (4,6) were evaluated by immunoblotting (Figure 2.6). No change in Class I HDAC protein expression was apparent with SFN treatment in PrEC cells. In BPH1 cells the protein levels of HDAC2 and HDAC3 were decreased at 48 h after SFN treatment. In LnCap cells the only class I HDAC to decrease was HDAC3 at 48 h after SFN treatment. In PC3 cells the protein levels of HDAC3 was decreased at 48 h after SFN treatment (Figure 2.6A). Among the Class II HDACs, HDAC4 protein expression decreased in all cell lines at one or both time points depending on the cell line. The other class II HDAC tested, HDAC6, showed the most robust and consistent decrease in protein level in BPH1, LnCap and PC3 cells at both 24 and 48 h after SFN treatment. This decrease in HDAC6 was not observed in PrEC cells (Figure 2.6B). To assess the possibility that changes in HDAC6 protein expression contribute to the SFN mediated cell death, HDAC6 was overexpressed in PC3 cells followed by SFN treatment. Over-expression of HDAC6 protected PC3 cells from the SFN-induced decrease in cell viability (Figure 2.7).

SFN increases acetylated histone association on the P21 promoter and increases acetylation of cytosolic protein targets in BPH1, LnCap and PC3 cells.

P21 is commonly upregulated by HDAC inhibitors via increased acetylated histones associating with its promoter (86-88). We observed an increase in acetylated histone H3 at the *P21* promoter in PC3 cells (Figure 2.8A) at a level comparable to the positive control treatment (TSA) (data not shown). No increase in acetylation at the

P21 promoter was observed in PrEC cells after SFN treatment. An increase in histone acetylation at the *P21* promoter after SFN treatment in BPH1 cells has previously been reported (29). Concomitant with the increased association of acetylated histones to the *P21* promoter, SFN treatment preferentially increased *P21* mRNA (data not shown) and protein levels in BPH1, LnCap and PC3 but not PrEC cells (Figure 2.8B). The induction of p21 protein was so strong in LnCap cells that induction in BPH1 and PC3 did not reach significance in Dunnett's post-test but clearly demonstrated a trend for increased p21 in those cell lines.

Recent attention has focused on the non-histone deacetylation targets of HDACs. One example is α -tubulin acetylation which is regulated by HDAC6 and is linked to downstream apoptotic mechanisms (89, 90). An increase in acetylated α -tubulin was detected with SFN treatment in BPH1, LnCap and PC3 cells but not in PrEC cells (Figure 2.8C). These results indicate that SFN can increase both histone H3 acetylation and α -tubulin acetylation in BPH1, LnCap and PC3 cells while having no effect in normal PrEC prostate cells.

2.5. Discussion

It is well documented that SFN can target cancer cells through multiple chemopreventive mechanisms but here we show for the first time that SFN selectively targets benign hyperplasia cells and cancerous prostate cells while leaving the normal prostate cells relatively unaffected. Importantly, SFN selectively induced cell cycle arrest and apoptosis, specifically in hyperplastic and cancer cells. These findings regarding the relative safety of SFN to normal tissues has significant clinical relevance as the use of SFN moves towards use in human clinical trials. In addition, a reduction in HDAC activity and down-regulation of select class I and class II HDAC proteins, followed by an increase in acetylation of histone H3 at the *P21* promoter and increased acetylation of α -tubulin occurred specifically in the hyperplastic and cancer cells, not normal cells. Together these results highlight the use of dietary SFN as a

safe and relatively non-toxic chemopreventive agent that could be readily achieved by simple and affordable incorporation of SFN rich foods in the diet.

The level of HDAC activity within a cell can be altered via direct inhibition of the HDAC enzyme and changes in HDAC protein levels. Previous reports indicate that SFN and its metabolites SFN-Cys and SFN-NAC can directly inhibit HDACs (29, 37, 91), and in this report we show that SFN can also decrease protein levels of several different HDACs. We also observed that the HDAC inhibition in PrEC cells is more transient than in the other prostate cancer cell lines. The overall metabolite profiles for PrEC and PC3 cells were similar, particularly in the levels of SFN-Cys, the putative active HDAC inhibitor. Although small changes were observed between metabolite ratios in culture media and final metabolite concentrations in cell lysates, it is unlikely that a 2-3 μ M difference would exert a significant biological effect that would account for the marked increase in apoptosis and cell cycle arrest in PC3 cells. In contrast, a significant reduction in HDAC protein level was observed in BPH1, LnCap and PC3 cells which produced a greater and more persistent reduction in HDAC activity. It is possible that the differential responses in HDAC activity, HDAC protein levels and ultimately downstream acetylation of histone and non-histone targets may contribute to the differential cell fate responses observed after SFN treatment.

Over-expression of Class I HDACs has been reported in prostate cancer tumors (92, 93). Moreover, the effects of the inhibition of Class I HDACs on cycle arrest and apoptosis has been shown previously (93, 94). Colon cancer cells treated with 3,3'-diindolylmethane (DIM), an indole found in cruciferous vegetables, induced proteasome mediated degradation of HDACs 3 and 8, acetylation of histone H3 at the *P21* promoter and ultimately induced a G₂/M cell cycle arrest and apoptosis (94). Another report showed that siRNA knockdown of HDAC3 in SW480 colon cancer cells increased acetylated H4-K12 at the *P21* promoter, induced p21 expression and potentiated butyrate induced cell cycle arrest and growth inhibition (95). These reports are consistent with what we observed in our experiments, namely SFN

treatment caused a decrease in several class I HDAC proteins, induction of histone acetylation at the *P21* promoter and ultimately induction of G₂/M cell cycle arrest and apoptosis. This provides compelling evidence that the changes in class I HDAC proteins and histone acetylation may be responsible for the changes in cell fate.

Herein we also provide evidence that HDAC6 is potentially responsible for the selective effects of SFN in cancer cells. Tubulin is a well known deacetylation target for HDAC6 and this process has an impact on microtubule dynamics (73, 75). Importantly, tubulin deacetylation increases the turnover rate of microtubules, and over-expression of HDAC6 augments this process (74). Decreases in HDAC6 activity (91) and increases in tubulin acetylation (42) after SFN treatment has previously been reported by others (96). In Gibbs et. al., researchers found that HDAC6 over-expression in LnCap cells abrogated the effects of SFN on HSP90 acetylation and inhibited its association with the androgen receptor (AR) providing further evidence that inhibition of HDAC6 was a specific target for sulforaphane (91). Here we confirm the decrease in HDAC6 protein levels in LnCap after SFN treatment reported by Gibbs et. al., and report that HDAC6 protein levels decrease in BPH1 and PC3 cells after SFN treatment. Interestingly, Gibbs et. al. reported that changes in HDAC6 activity ultimately affected the activity of the AR but here we show that HDAC6 is also decreased in PC3 cells which are AR negative indicating that the effect of SFN on AR may not be the only target for HDAC6. Importantly, over-expression of HDAC6 rescues PC3 cells from SFN-induced decreases in cell viability suggesting that HDAC6 plays a critical role in mediating its cytotoxicity. Thus, the inhibition of HDAC6 could play a key role in the stabilization of microtubule networks, disruption of tubulin polymerization and ultimately contribute to the mitotic cell cycle arrest observed with SFN treatment (96, 97). The link between tubulin acetylation and selectively toxicity towards cancer cells has been reported after treatment with the HDAC6 specific inhibitor tubacin. Treatment with tubacin induced a dose and time dependent increase in α -tubulin acetylation and ultimately cytotoxicity in several multiple myeloma cell lines and bone marrow plasma cells but had no effect in normal

peripheral blood mononuclear cells (98). Autophagy is another cellular response that is partially mediated by HDAC6 because it functions to deliver polyubiquitinated proteins to aggresomes for degradation by binding both the polyubiquitinated proteins and the microtubule motor dynein (89). SFN treatment in PC3 and LNCaP prostate cells results in an induction of autophagy and partial inhibition of cytochrome C release and apoptosis (49). The decrease in HDAC6 protein we observed in BPH1, LNCaP and PC3 cells may divert the cell fate away from survival (autophagy) towards cell death (apoptosis) by decreasing the formation of autophagic aggresomes. Further investigation into the potential roles of α -tubulin acetylation and the reduction in HDAC6 levels in the selectivity of SFN will provide insights into the mechanisms for SFN mediated cancer cell death.

The use of HDAC inhibitors in cancer prevention and treatment has become an intense area of research. These data provide further support for the relevance of SFN as a dietary HDAC inhibitor and chemopreventive agent by showing that SFN can selectively target BPH1, LNCaP and PC3 prostate cells while leaving normal PrEC prostate cells virtually unaffected. This selectivity opens the door to a wide range of new scientific questions that will help in understanding the many mechanisms of action for SFN. The data presented here, taken with the previous reports of SFN action, show that SFN can target multiple steps in the carcinogenesis pathway and make it a promising cancer prevention agent.

Acknowledgements

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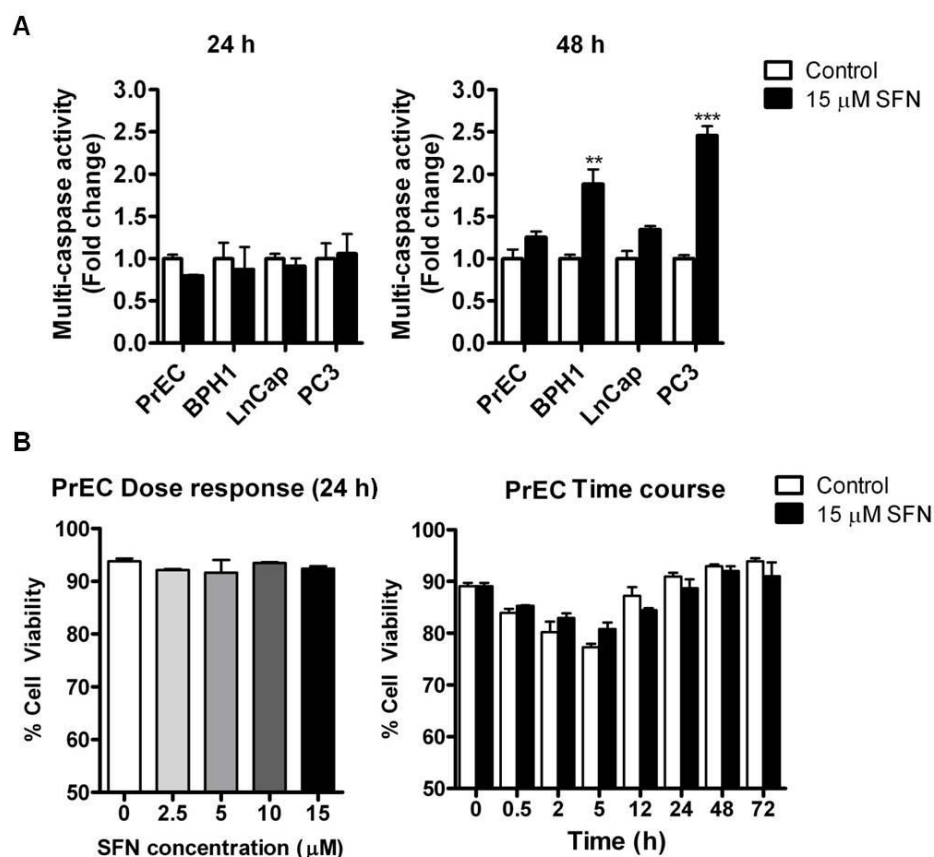


Figure 2.1 SFN preferentially induces apoptosis in BPH1 and PC3 cells.

Cells were treated with DMSO (control) (white bars) or 15 μ M SFN (black bars) for 24 and 48 h and harvested for apoptosis analysis. (A) Multi-caspase activity analyzed by one-way ANOVA. (B) Trypan blue exclusion assay in PrEC at increasing doses of SFN (left) and 15 μ M SFN 72 h time course (right), analyzed by one-way ANOVA and two-way ANOVA, respectively. Data in graphs represent mean \pm SEM (n=3). Statistical significance: **p<0.01, ***p<0.001 using Dunnett's post-test.

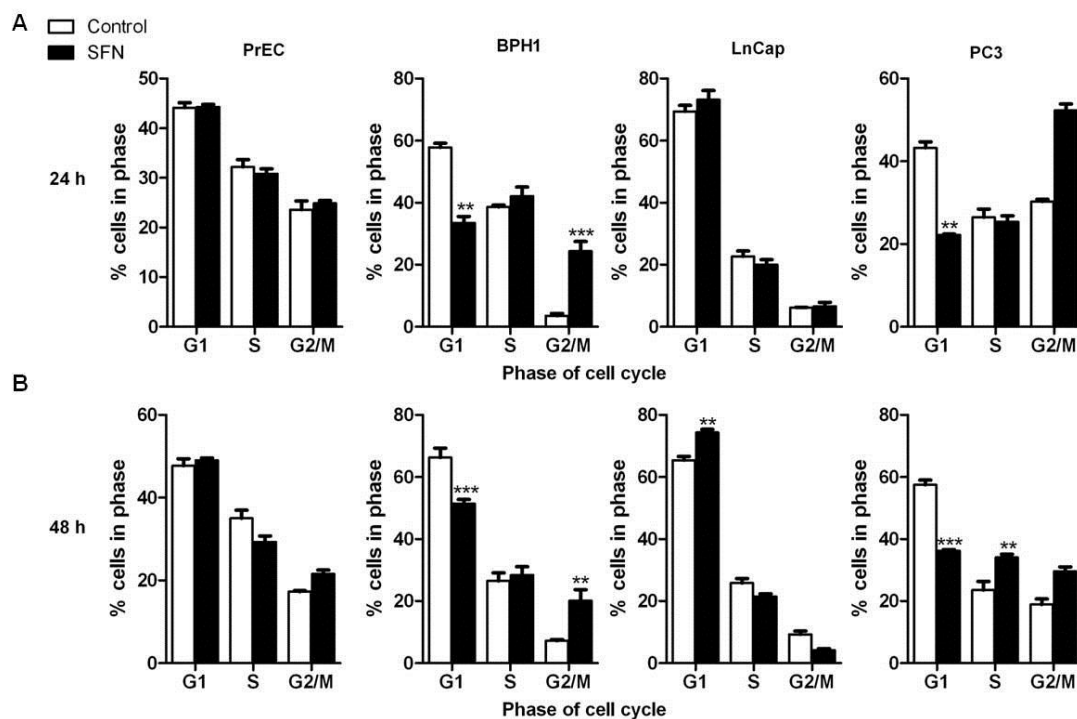


Figure 2.2 SFN preferentially induces cell cycle arrest in BPH1, LnCap and PC3 cells.

Cells were treated with DMSO (control) (white bars) or 15 μ M SFN (black bars) for 24 and 48 h and harvested for cell cycle analysis. Distribution of cells (in percentage) in the G₁, S, and G₂/M phases of the cell cycle. Data in bar graphs represent mean \pm SEM (n=3). Statistical significance: **p<0.01, ***p<0.001 using Dunnett's post-test.

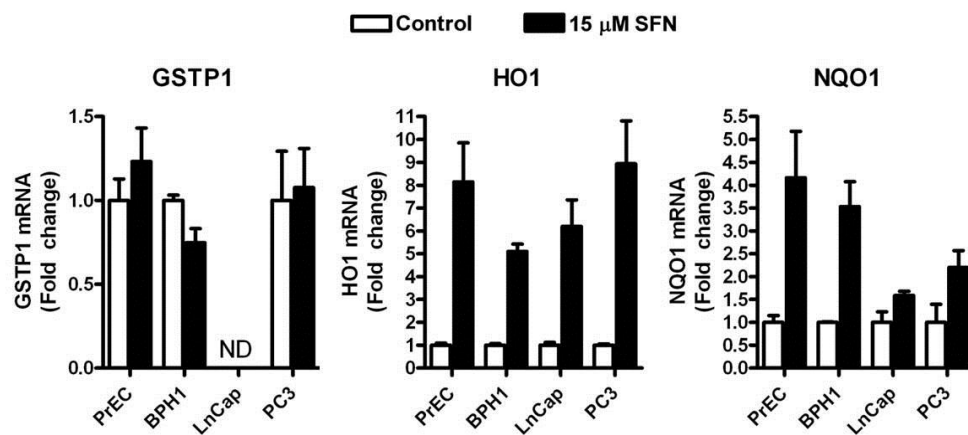


Figure 2.3 SFN increases the level of HO1 and NQO1 mRNA in PrEC, BPH1, LnCap and PC3 cells.

Cells were treated with DMSO (control) (white bars) or 15 μ M SFN (black bars) for 12 h and harvested for qRT-PCR. GSTP1 (left), HO1 (middle) and NQO1 (right). Data in bar graphs represent mean \pm SEM (n=3). ND=none detected. Data were analyzed by 1-way ANOVA and Dunnett's post-test.

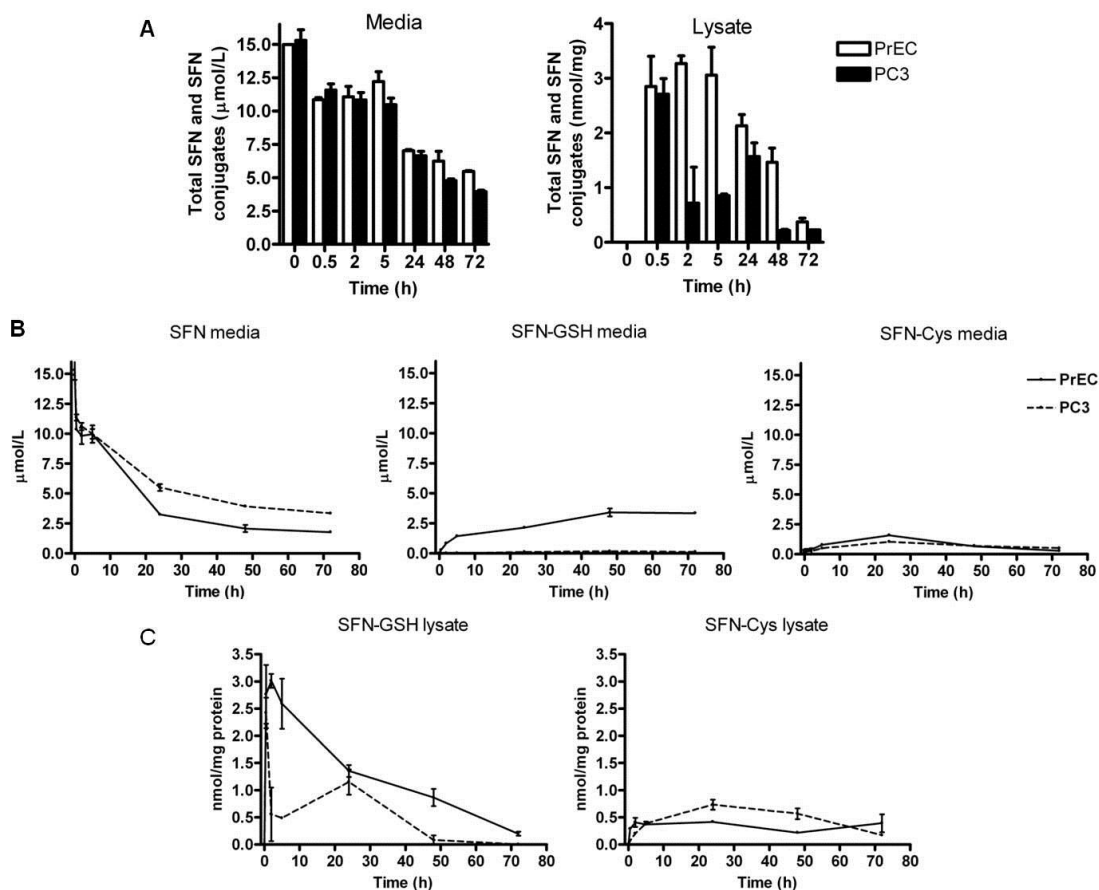


Figure 2.4 SFN is metabolized similarly in PrEC and PC3 cells.

Cells were treated and harvested as described in the methods. (A) The sum of all SFN compounds in culture media (left) and lysate (right) from PrEC (white bars) and PC3 (black bars). (B) Concentrations of free SFN (left), SFN-GSH (middle) and SFN-Cys (right) in the cell culture media from PrEC (dashed lines) and PC3 (solid lines). (C) Concentrations of SFN-GSH (left) and SFN-Cys (right) in the cell lysates. Data in bar graphs represent mean \pm SEM (n=2).

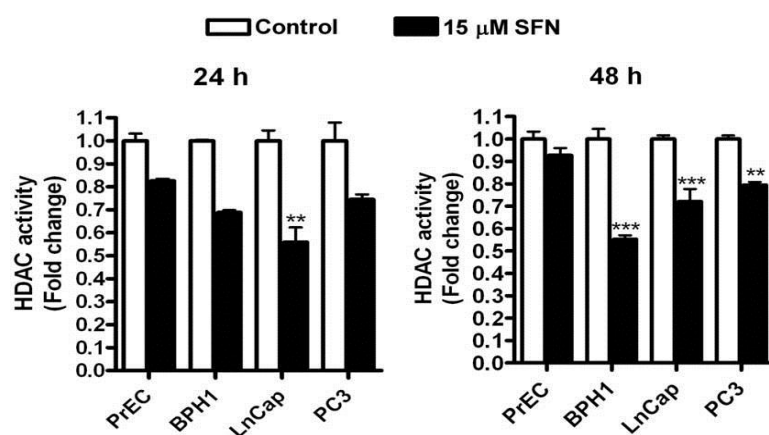


Figure 2.5 SFN selectively reduces HDAC activity in BPH1, LnCap and PC3 but not normal PrEC prostate cells.

Cells were treated with DMSO (control) (white bars) or 15 μM SFN (black bars) for 24 and 48 h and harvested for HDAC activity. Data in bar graphs represent mean \pm SEM (n=3). Statistical significance: **p<0.01, ***p<0.001 using Dunnett's post-test.

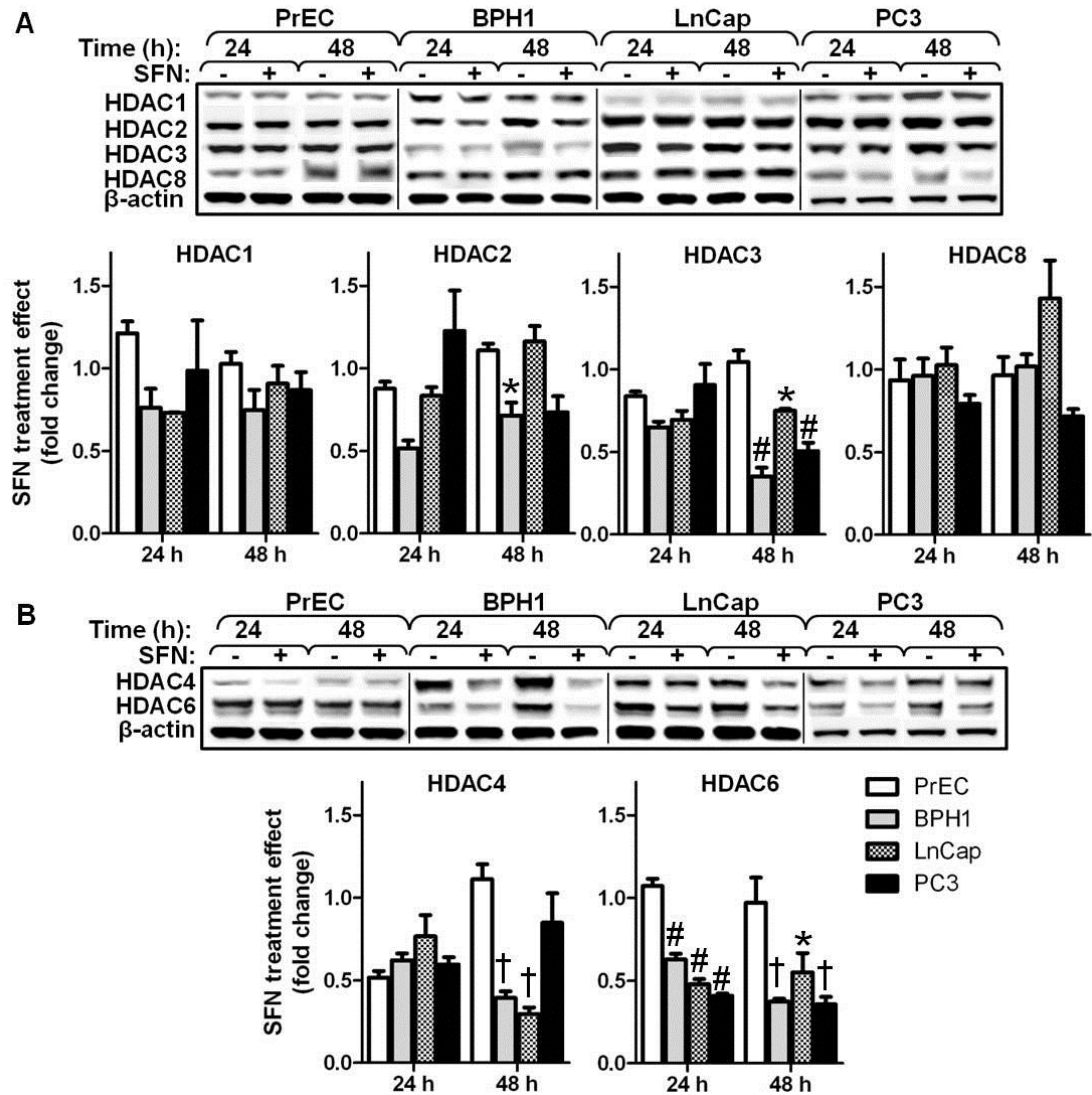


Figure 2.6 SFN selectively reduces several class I and class II HDAC proteins in BPH1, LnCap and PC3 but not normal PrEC prostate cells.

Western blot and densitometry of class I (A) and class II (B) HDACs at 24 and 48 h. Data in bar graphs represent mean \pm SEM (n=3). Statistical significance: *p<0.01, #p<0.01 †p<0.001 using Dunnett's post-test comparing PrEC to the other cell lines within each time point.

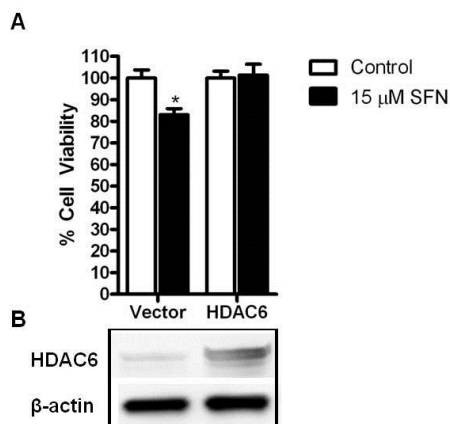


Figure 2.7 HDAC6 over-expression blocks SFN induced growth inhibition in PC3 cells.

A) Cells were transfected with empty vector or HDAC6 and treated with DMSO (control) (white bars) or 15 μ M SFN (black bars) for 48 hr and assayed for cell proliferation. Data in bar graphs represent mean \pm SEM (n=3). Statistical significance: * $p < 0.05$ comparing SFN effect in vector versus SFN effect in HDAC6 treated groups using Student's t-test. B) Western blots depicting relative expression levels of HDAC6 in empty vector and HDAC6 transfected cells.

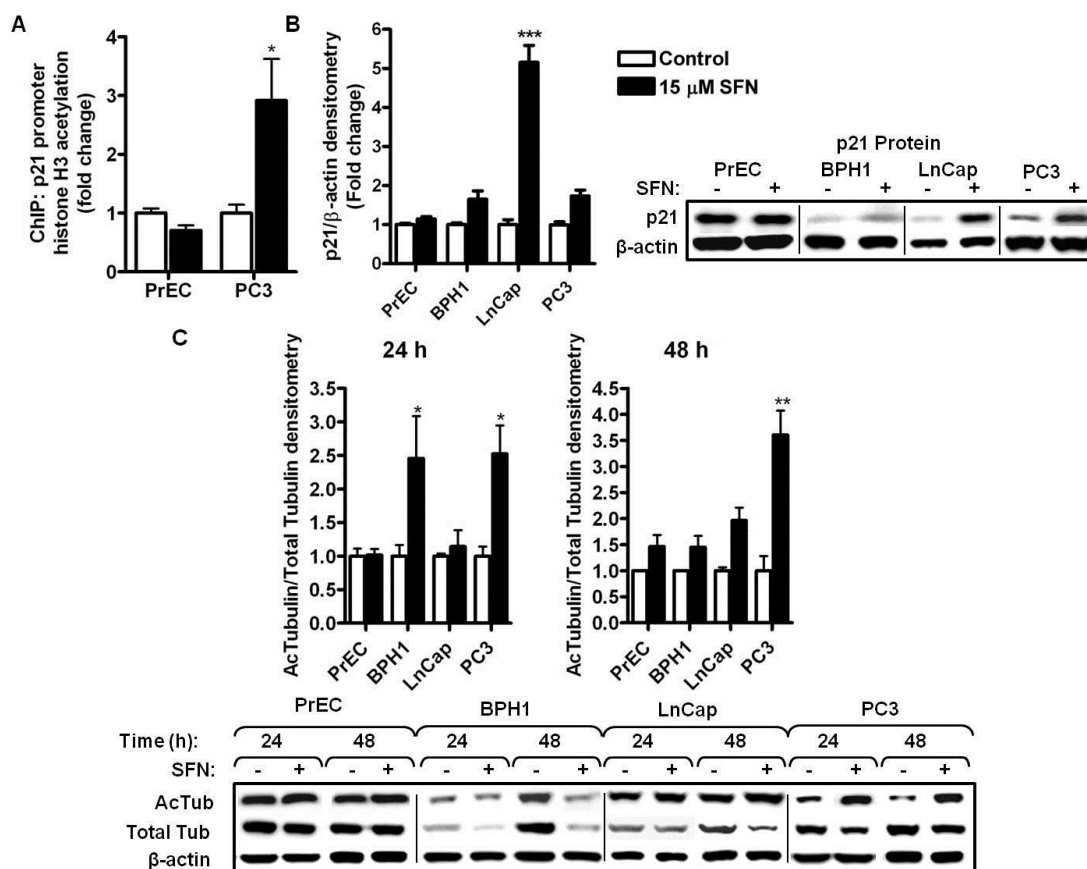


Figure 2.8 SFN treatment selectively induces histone H3 acetylation at the P21 promoter, p21 protein and tubulin acetylation in BPH1, LnCap and PC3 but not normal PrEC prostate cells.

Cells were treated with DMSO (control) (white bars) or 15 μ M SFN (black bars) for the time indicated and harvested for ChIP or western blots. (A) ChIP for acetylated histone H3 at the *P21* promoter at 48 h. For normalization between samples the immunoprecipitated DNA was expressed as percentage of input DNA ($2^{CT_{input} - CT_{IP}} \times 10$) and shown as fold change compared to control treatments. TSA was included as a positive control for histone acetylation (data not shown). Data were analyzed by Student's t-test; * $p < 0.05$. (B) Densitometry and western blots for p21 protein at 24 h. (C) Western blot and densitometry of acetylated tubulin and total tubulin at 24 and 48 h. Data in bar graphs represent mean \pm SEM ($n=3$). Data for B and C were analyzed by one-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Dunnett's post-test.

Chapter 3 Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice

John D. Clarke, Anna Hsu, David E. Williams, Roderick H. Dashwood, Jan F. Stevens, Philippe Thuillier, Jackilen Shannon, Emily Ho

3.1. Abstract

Purpose: To determine the metabolism and tissue distribution of the dietary chemoprotective agent sulforaphane following oral administration to wild-type and Nrf2 knockout (Nrf2^{-/-}) mice.

Methods: Male and female wild-type and Nrf2^{-/-} mice were given sulforaphane (5 or 20 μ moles) by oral gavage, and plasma, liver, kidney, small intestine, colon, lung, brain and prostate were collected at 2, 6 and 24 hours (h). The five major metabolites of sulforaphane were measured in tissues by high performance liquid chromatography coupled with tandem mass spectrometry. Transgenic adenoma of the mouse prostate mice were fed a broccoli-supplemented diet which is rich in glucoraphanin, the precursor to SFN, and tissue distribution of SFN and its metabolites, along with prostate cancer pathology, were examined.

Results: Sulforaphane metabolites were detected in all tissues at 2 and 6 h post gavage, with concentrations being the highest in the small intestine, prostate, kidney and lung. A dose- dependent increase in sulforaphane concentrations was observed in all tissues except prostate. At 5 μ mole, the Nrf2^{-/-} genotype had no effect on sulforaphane metabolism. Only Nrf2^{-/-} females given 20 μ moles sulforaphane for 6 h exhibited a marked increase in tissue sulforaphane metabolite concentrations. However, the relative abundance of each metabolite was not strikingly different between genders and genotypes. Long-term exposure to the broccoli sprout diet increased SFN metabolite levels in the prostate and slowed the progression of prostate cancer in the transgenic adenoma of the mouse prostate model.

Conclusions: Sulforaphane is metabolized and reaches target tissues in both wild-type and Nrf2^{-/-} mice. Together these data provide further evidence that sulforaphane is bioavailable and may be an effective dietary chemoprevention agent for several tissue sites.

3.2. Introduction

Epidemiological studies have shown an inverse association between cruciferous vegetable intake and cancer risk in many tissues including lung, bladder, colon and prostate (59, 99-101). Cruciferous vegetables contain high concentrations of glucosinolates, which are hydrolyzed to isothiocyanates (ITCs) by myrosinase, an enzyme endogenous in the plant and also present in colonic microflora (53). The ITCs have been extensively studied for their anti-cancer properties and shown promise in preclinical and clinical settings (6, 102, 103). Sulforaphane (SFN) is a well studied ITC derived from the glucosinolate glucoraphanin, which is abundant in broccoli and broccoli sprouts. The first identified and most studied mechanism for SFN-mediated chemoprevention is through the induction of phase II enzymes via Nrf2 (nuclear factor erythroid 2 (NF-E2) related factor 2) signaling. Nrf2 is well established as an important mediator of electrophile and reactive oxygen species toxicity (104). Upon electrophile- or reactive oxygen species-induced cellular stress, Nrf2 is released from cytoplasmic Keap1 and translocates to the nucleus where it binds antioxidant response element (ARE) sites on many phase I and II metabolism genes, and phase III transport genes. The upregulation of antioxidant and xenobiotic metabolism is believed to protect cells from carcinogen and/or oxidative stress exposure by expediting their inactivation and removal via metabolism and excretion. Studies utilizing the Nrf2^{-/-} mouse have highlighted the importance of Nrf2-mediated phase I, II and III enzyme induction in chemoprevention and its role in SFN mediated cytoprotection (104). Importantly, SFN is metabolized and removed from the body through Nrf2-mediated phase II and III proteins, such as glutathione-S-transferase (GST) and multidrug resistance associated protein-1 (105). Therefore, Nrf2 is believed to be an important player in SFN metabolism and export from the cell. However, it is currently not known what impact, if any, loss of Nrf2 has on SFN metabolism and tissue distribution *in vivo*. For example, one hypothesis is that Nrf2 target genes (such as GSTs) become induced by SFN and then convert the parent

compound to metabolites with important chemopreventive activity, which might be lost in the Nrf2 null background.

To date, there is little precise information on the distribution of SFN and its metabolites in various tissues of the body following dietary administration. Pharmacokinetic (PK) studies in rodents have focused on either free SFN or its metabolite SFN-glutathione (SFN-GSH), and they have not attempted to measure other major SFN metabolites (106, 107). Only one human study included PK analysis of all five major SFN metabolites (60). In addition, a limited number of studies have examined tissues, such as small intestine and lung for SFN content (64, 108). One human study reported dithiocarbamate (a measure of total ITC content) concentrations in mammary tissue after consumption of a broccoli preparation (63). A comprehensive profiling of SFN bioavailability and tissue distribution *in vivo* is critical for understanding the potential efficacy of SFN as a dietary chemoprevention agent for various cancers.

Thus, we performed high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis of all five SFN metabolites in order to determine SFN metabolism and tissue distribution in both Nrf2^{-/-} and wild-type mice. SFN has been used in the Nrf2^{-/-} mouse model (109, 110) and the present study provides insight into how Nrf2^{-/-} impacts SFN metabolism. In addition, this is the first study to show tissue specific concentrations and metabolite profiles after oral SFN administration. Together, these data support the hypothesis that SFN is a promising chemopreventive agent that is bioavailable and can reach many different tissue sites.

3.3. Materials and methods

Materials

R,S-SFN, SFN-GSH, SFN-Cysteine (SFN-Cys) and SFN-*N*-acetylcysteine (SFN-NAC) were purchased from LKT laboratories (St Paul, MN). Deuterated *N*-acetylcysteine was synthesized according to Slatter *et al.* (111). For synthesis of the internal standard (deuterated SFN-NAC), 0.1 mM SFN, 10 mM deuterated NAC, and

0.04 M phosphate buffer at pH 7.8 were mixed together and stirred for 4 h at room temperature. The mixture was then acidified with 1 N HCl and applied to an equilibrated StrataX 33 μ m reverse phase cartridge (Phenomenex, Torrance, CA), washed, and then eluted in 50:50 acetonitrile and water. Trifluoroacetic acid (TFA) was purchased from EMD Chemicals (Darmstadt, Germany).

Treatment of animals

Wild-type (ICR) mice were purchased from Harlan Laboratories Inc. (Indianapolis, IN) and Nrf2 knockout mice were bred from a mouse colony originally obtained from RIKEN BioResource Center (Ibaraki, Japan). Mice were maintained at 22°C on a 12 hr light-dark cycle and given food and water ad libitum throughout the study. One week prior to SFN treatment the mice (~8 months of age) were switched from standard laboratory chow to a purified AIN93M diet that did not contain tert-butylhydroquinone (TBHQ). Mouse weights were recorded prior to treatment (males 48.1 ± 5.7 g and females 44.3 ± 8.2 g). Mice were treated with either 5 or 20 μ moles of SFN in corn oil, which is equivalent to 110 μ moles SFN/kg body weight or 440 μ moles SFN/kg body weight of SFN, respectively, or corn oil only (sham control) by gavage and sacrificed at 2, 6, and 24 h after treatment. At each time point the mice were euthanized with CO₂ and the following tissues were collected and flash frozen in liquid nitrogen: brain, liver, kidney, small intestine (SI) mucosa, colonic mucosa, lung, prostate, and plasma. For SI and colon, the intestine was removed, washed with PBS, and mucosal scrapings were collected. For plasma, whole blood was collected by cardiac puncture, transferred to a tube containing EDTA and centrifuged at high speed for 1 min to collect the plasma. The plasma was immediately acidified with 10% v/v ice cold TFA. Animal handling and procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Oregon State University.

Transgenic adenoma of the mouse prostate (TRAMP) mice were bred and treated at Oregon Health and Sciences University and maintained at 22°C on a 12 hr light-dark cycle and given food and water ad libitum throughout the study. Starting at 4

weeks of age, mice were fed either AIN93G diet (standard diet) or AIN93G diet supplemented with 15% broccoli sprouts (SFN diet) (n=20 in each group). The broccoli sprout powder was provided by Natural Sprout Company, LLC (Springfield, MO) and Research Diets, Inc (New Brunswick, NJ) formulated the diet. There was an equivalent of 400 mg SFN/kg diet in the broccoli sprout diet. Mice were euthanized by CO₂ asphyxiation at 12 and 28 weeks of age and the genito-urinary apparatus was removed, weighed and examined for gross abnormalities. The prostates were microdissected into the dorsolateral, ventral and anterior lobes. Portions of prostate, liver, kidney and colon from three mice were used for LC-MS/MS analysis of SFN compounds as described in the LC-MS/MS analysis section below. A portion of each lobe was fixed in multichamber cassettes and stained with hematoxylin and eosin for histology. Prostate pathology was scored from 1-6 according to the previously published methods (112, 113).

LC-MS/MS analysis

The methods for LC-MS/MS analysis were performed as described in Al Janobi *et al.* (84), with minor modifications. For most solid tissues ~50 mg of frozen tissue was homogenized using mortar and pestle and liquid nitrogen. After homogenization 50 µL of 10% TFA (v/v) in water was added to the sample along with 5 µL of 100 µM internal standard (deuterated SFN-NAC) and vortexed vigorously. The homogenate was frozen at -80°C then thawed, vortexed vigorously, centrifuged at 11,600 x g at 4°C for 5 min and the supernatant was subsequently filtered through a 0.2 µm pore size filter. For tissues such as brain and kidney, half of the brain and one entire kidney from each mouse was homogenized and used for metabolite analysis. For plasma the samples were acidified, centrifuged and filtered as described above.

Ten µL of filtered sample were separated on a Shimadzu Prominence HPLC (Shimadzu, Kyoto, Japan) using a reversed-phase Phenomenex Kinetex PFP 2.6 µm 100Å 100 x 2.6 mm HPLC column. The flow rate was 0.25 mL/min using 0.1% FA in water (solution A) and 0.1% FA in acetonitrile (solution B). The gradient was as follows: 5% B increasing to 30% over 1.5 min, held at 30% for 1.5 min, washed out

with 90% B for 3.5 min and re-equilibrated to 5% B for 3.5 min. The LC eluent was analyzed by an API triple quad mass spectrometer 3200 (Applied Biosystems, Foster City, CA) with electrospray ionization in positive mode. Tandem mass spectrometry using multiple reaction monitoring was used to detect the analytes with the following precursor and product ions: SFN (178>114), SFN-GSH (485>114), SFN-Cysteinylglycine (SFN-CG) (356>114), SFN-Cys (299>114), SFN-NAC (341>114). Spike and recovery experiments using the internal standard confirmed that >80% of all compounds were recovered following the processing protocols outlined. Quantification was performed by using a standard curve ranging from 0.156 to 25 μ M. Acquisition and quantification was performed using Analyst software (Applied Biosystems, Carlsbad, CA).

3.4. Results

In wild-type mice, individual SFN metabolites were quantified in tissues at 2, 6 and 24 h post gavage with SFN or sham control. Nrf2 wild-type mice had SFN metabolites present in all tissues tested after SFN treatment whereas no SFN metabolites were detected in any tissues from sham treated mice. Using the sum of all SFN metabolites, SFN treated mice exhibited a dose dependent increase in tissue concentrations (Figure 1A and B), with concentrations being the highest in the SI, prostate, kidney and lung. In plasma, SFN metabolite concentrations were highest at 2 h but were not detectable by 24 h. Most tissues followed similar kinetics as plasma, with highest concentrations at 2 h and completely cleared by 24 h. Colon, SI, and prostate had higher concentrations at 6 h, and only in the SI and prostate were low levels of metabolites still detectable at 24 h in mice given 20 μ mole SFN (Figure 2). Analysis of plasma concentrations with the different tissue concentrations revealed that only brain and lung correlated statistically with plasma concentrations (Table 1), despite the fact that most tissues followed similar kinetics as observed in plasma. No gender differences in SFN metabolism and tissue distribution were observed in the wild-type mice (data not shown).

To determine if the absence of Nrf2 has an impact, we compared the metabolism of SFN between Nrf2^{-/-} and Nrf2 wild-type mice. Initially it appeared that the Nrf2^{-/-} genotype resulted in markedly higher SFN metabolite concentrations; however, upon closer analysis a genotype effect was only apparent in female mice at the 20 μ mole dose and 6 h time point. Thus, a comparison between Nrf2^{-/-} and wild-type male mice at 6 h (Figure 3A and B) revealed a significant dose effect but no genotype effect on SFN tissue concentrations in nearly all tissues. Although not statistically significant, the exceptions were kidney and prostate at 5 μ mole SFN, which had lower levels in Nrf2 null versus wild type mice, and brain at the same SFN dose, which had higher levels in the knockout. Similar results were observed at 2 h when comparing male mice across genotypes and female mice across genotypes (data not shown). In female mice administered 5 and 20 μ mole SFN, a general trend was observed in which the tissue concentrations were higher in Nrf2^{-/-} compared with wild type mice, with certain exceptions (Figure 4A and B). Thus, Nrf2^{-/-} females in the 20 μ mole dose group and 6 h time point had drastically higher SFN metabolite concentrations compared to wild-type animals in all tissues, except the SI and Colon (Figure 4A and B). A similar trend was observed at the 5 μ mole SFN dose in most tissues, with higher metabolite concentrations in null versus wild type, although this was not always statistically significant. Data in the SI and colon provide indirect evidence that the differences noted in other tissues were unlikely to be due to an error in SFN dosing between the two genotypes.

Individual SFN metabolites were quantified, and the relative abundance of each was determined for each tissue. The relative abundance of each metabolite varied among the tissues, across gender and genotype, as well as across time and dose (Figure 5A and B). Surprisingly, there were no drastic differences across genotype, although at the 2 h time point there was a trend towards more SFN-GSH in the colon, liver, lung, brain and plasma from Nrf2^{-/-} mice compared to wild-type mice. For some tissues, such as lung and brain, the amount of SFN-NAC increased from the 2 h to 6 h time points. Other tissues such as SI, prostate, and plasma did not vary much across

genotype, gender, and time. SFN-GSH was the most abundant metabolite in liver, lung and brain, whereas SFN-NAC was the most abundant metabolite in prostate. Interestingly, metabolite ratios in the kidney at 2 h were somewhat variable across gender and genotype, but by 6 h SFN-Cys was the most abundant metabolite in both genders and genotypes. SI, colon, and plasma had a relatively even distribution of SFN-NAC, SFN-GSH and SFN-Cys. Only low concentrations of free SFN were detected, and only in a few tissues, with plasma having the highest percentage (<10%). Small quantities of SFN-CG were detected in a few plasma samples (<1%), but not in other tissues.

To determine if dietary SFN could alter the progression of prostate cancer, TRAMP mice were fed either a standard diet or a diet supplemented with 15% broccoli sprouts. SFN metabolites were detected in the prostates, as well as several other tissues, of mice fed the SFN diet (Figure 6A), although at much lower concentrations than observed in the mice from the SFN gavage study (Figure 1A). Most importantly, SFN reduced the severity of prostate cancer at both 12 and 28 weeks of age. At 12 weeks of age a majority of the mice fed the SFN diet were scored at grade 1 or 2 cancer severity, whereas a majority of the mice fed the standard diet were scored at stage 3 cancer severity. Similarly, at 28 weeks of age a majority of the mice fed the broccoli diet were grade 3 or below, whereas a majority of the mice fed the standard diet were considered grade 4 or above (Figure 6B).

3.5. Discussion

In the present study, we conducted experiments using *Nrf2*^{-/-} and *Nrf2* wild-type mice to determine the metabolism and tissue distribution of SFN following oral administration. Using LC-MS/MS analysis we sought to quantify SFN and four major metabolites (SFN-GSH, SFN-CG, SFN-Cys and SFN-NAC) in plasma, SI, colon, liver, kidney, lung, brain and prostate. Most studies in rodents and humans have reported peak plasma concentrations of SFN and its metabolites occurring between 1 and 3 h after SFN administration (60, 106, 107). Following SFN gavage, SFN

metabolites were detected in all tissues at 2 and 6 h, and a dose dependent increase in tissue concentrations was observed in all tissues except for the prostate. In liver, kidney, lung, brain and plasma the highest concentrations were at 2 h, but in SI, colon and prostate the highest concentrations were at 6 h. This indicated that SFN metabolites may be accumulating in certain tissues, and that peak plasma concentrations do not always align precisely with target tissues of major cancers, such as prostate and colon. Although no gender difference was observed in SFN metabolism in the wild-type mice, in *Nrf2*^{-/-} mice at the higher dose and 6 h time point there was a marked increase in tissue concentrations in the female mice compared to the male mice, and a similar trend also was seen at the lower SFN dose. Interestingly, the relative abundance of each metabolite was not strikingly different between genders and genotypes, despite some variability on a case-by-case basis. We also report that a 15% broccoli sprout diet, which is rich in glucoraphanin, the precursor to SFN, resulted in SFN metabolites in the livers, kidneys, colons and, importantly, prostates of TRAMP mice. This treatment resulted in reduced severity of prostate cancer in the TRAMP mice. This study is the first to show reduced severity of prostate cancer in TRAMP mice given a broccoli supplemented diet, detailed LC-MS/MS analysis of SFN metabolites in mouse tissues, and to compare SFN metabolite profiles between *Nrf2*^{-/-} and wild-type mice.

Although there have been several studies showing PK of SFN in rodents and humans, tissue distribution is still largely unknown. Herein we report that the tissue concentrations of SFN metabolites vary as much as 100 fold between different tissues. For example, SI had the highest concentration at 0.355 nmole SFN metabolites/mg of tissue whereas brain had 0.003 nmole SFN metabolites/mg of tissue (Figure 1). It has been reported that 74% of a SFN dose was absorbed in human jejunum (14), so high concentrations in the SI are likely not a result of poor bioavailability, although we did not directly test that here. Currently little is known regarding the ability of SFN metabolites to cross the blood-brain barrier, but here we report low concentration in the brain which likely indicates that SFN metabolites do not readily cross the blood-

brain barrier, Tissues such as liver, kidney, lung and prostate had comparable concentrations at 2h, ranging from 0.075 nmole SFN metabolites/mg of tissue in lung to 0.041 nmole SFN metabolites/mg of tissue in liver. The abundance of individual metabolites also varied between tissues. SFN-GSH, SFN-Cys and SFN-NAC represented the highest proportion of SFN metabolites in most tissues. For some tissues, such as prostate and lung, SFN-NAC and SFN-GSH were the most abundant metabolites, respectively. The *in vivo* bioactivity of each metabolite is still unclear, although it has been reported that the SFN-Cys and SFN-NAC metabolites are the bioactive intermediates targeting histone deacetylases (37).

Interestingly, because ITC thiol conjugates can dissociate into free ITCs under physiological conditions (114), it has been postulated that these thiol conjugates can be considered prodrugs of the parent compound (115). Studies have shown similar efficacy from either free ITC or the *N*-acetylcysteine (NAC) conjugated ITC *in vitro*, in cancer cells, or in rodent cancer models *in vivo* (24, 65, 115-118). The metabolism of the ITC and ITC-NAC compounds in the latter studies is unknown, but from the current investigation it can be expected that tissue concentrations in the mice that received the free ITCs were predominately in the form of thiol conjugates. The metabolism of orally administered ITC-NAC conjugates is unknown and would be an interesting area of future research. These differences in total and individual SFN metabolite tissue concentrations could impact the bioactivity of SFN in different tissues, and merits further investigation. From the current data it is clear that thorough accounting of SFN *in vivo* requires analysis of at least four of the five main metabolites. These data also support the hypothesis that repeated consumption of cruciferous vegetables is required to maintain SFN metabolite concentrations in tissues.

In our study we observed striking differences between male and female Nrf2^{-/-} mice at the 20 µmole dose and 6 h time point. It has been reported that female Nrf2^{-/-} mice have significantly higher morbidity and mortality, even in the absence of apparent exogenous stress (119). Interestingly, Ma *et al.* reported that 88% and 47%

of the death in Nrf2^{-/-} female and male mice, respectively, were caused by renal failure and severe glomerulonephritis. In the present study, the Nrf2^{-/-} female mice in the 20 μ mole SFN/6 h group had apparent toxicity after the SFN treatment manifested as lethargy and non-responsiveness, and had as much as 23-fold higher concentrations of SFN metabolites in all tissues (except for the SI and colon) compared to the corresponding wild-type females (Figure 4). The observation that the SI and colon were the only tissues that had similar concentrations as observed in the wild-type mice indicates that the differences in tissues concentrations occur post-absorption. We speculate that the female Nrf2^{-/-} mice in our study had some degree of glomerulonephritis as a result of lacking Nrf2, and upon receipt of the higher dose (20 μ mole SFN) underwent acute kidney failure and could not excrete the SFN metabolites in the urine, thus leading to an accumulation in tissues and ultimately toxicity. Furthermore, it is possible that this potential kidney damage is compounding the impact of reduced inducibility of phase III efflux transporters on clearance of SFN compounds from the tissues, thus contributing to the high tissue concentrations observed. Also, although differences between wild-type female and male mice in basal and inducible GST activity as well as hepatotoxicity have previously been reported (120-122), we did not observe a difference in SFN metabolism between wild-type males and wild-type females. Taken together, these data illuminate the need for caution when selecting either male or female Nrf2^{-/-} mice for xenobiotic studies.

The importance of Nrf2 in drug metabolism is well documented (123). As expected, it has been reported that Nrf2^{-/-} mice have much lower expression and lack the inducibility of phase I, II, and III proteins (119, 122, 124). Several groups have shown that Nrf2^{-/-} mice are more susceptible to experimentally induced colon cancer (125-127). In the context of SFN treatment, one study reported that the protective effects of SFN administration in a Parkinson's disease model was lost in Nrf2^{-/-} mice (110). Similarly, another group reported that topical SFN administration in a UVB induced skin inflammation model was only able to restore sunburn cells back to basal levels in mice that were wild-type for Nrf2 (109). The working hypothesis is that the

loss in SFN-mediated protection in these models is partially attributed to altered metabolism of SFN in Nrf2^{-/-} mice. In the current report we show that SFN metabolism and tissue distribution is nearly identical between wild-type and Nrf2^{-/-} mice (Figure 3 and 4), with the exception of female Nrf2^{-/-} mice given 20 μ mole for 6 h (see discussion above regarding these mice). Also, across genotypes there were no drastic differences in the relative abundance of each metabolite, even though the Nrf2^{-/-} females at the high dose and 6 h time point had dramatically higher tissue concentrations. Several aspects of drug metabolism could contribute to this apparent disconnect between SFN metabolism and Nrf2 status. For example, cross talk between the many different nuclear receptors involved in drug metabolism has been reported, and several members of the GST family are regulated independently of Nrf2 (128, 129). Indeed, it has been shown that dextran sulfate sodium treatment caused induction of GSTM1 protein in colonic tissues of Nrf2^{-/-} mice (125), indicating either a separate pathway for induction, or retention of inducibility, of GSTM1 in the colon of these mice. Also, it has been shown that SFN metabolites can undergo facile thiol exchange reactions (51), indicating that interconversion between SFN metabolites may occur, independent of enzymatic activity *in vivo*. From these data we conclude that Nrf2 status does not have a marked impact on SFN metabolism and tissue distribution in mice, and therefore the differences in SFN efficacy observed in other studies are likely not related to metabolism and biodistribution.

A large body of scientific evidence indicates that SFN is an effective anti-cancer agent. Despite extensive research in understanding the function and activity of SFN, little is known regarding the tissue distribution of SFN and its metabolites. The differences in bioavailability and distribution of specific metabolites to tissues could have a significant impact on efficacy and tissue-specific targets because SFN and its metabolites are known to work through multiple and potentially separate mechanisms of chemoprevention. These data are the first to show detailed SFN metabolism and tissue distribution profiles in mice. Herein we provide evidence that free SFN is not a major compound present in tissues of mice given SFN, but rather the glutathione,

cysteinyl, and *N*-acetylcysteine conjugates of SFN are the most abundant. The kinetics of SFN metabolism and tissue distribution appears to follow what is observed in the plasma for most tissues except SI, colon and prostate. We also show that Nrf2 is not required for efficient metabolism and tissue distribution of SFN, and provide evidence for a gender difference in the Nrf2^{-/-} mice, especially in response to higher doses of SFN. We report quantitative LC-MS/MS results in mice showing that the dietary anti-cancer agent SFN can be utilized in the diet and that its metabolites reach target tissues of carcinogenesis, such as colon and prostate.

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Table 3.1 Correlation between plasma and tissue concentrations in wild-type mice

Tissue	Correlation coefficient (p-value)			
	2h		6h	
	5 μ moles	20 μ moles	5 μ moles	20 μ moles
SI	0.21 (0.504)	0.57 (0.066)	0.42 (0.175)	-0.18 (0.572)
Colon	0.05 (0.868)	-0.26 (0.445)	0.03 (0.916)	0.03 (0.929)
Liver	0.06 (0.854)	0.18 (0.606)	0.22 (0.487)	0.38 (0.217)
Kidney	-0.01 (0.965)	0.30 (0.377)	0.07 (0.818)	0.37 (0.236)
Lung	0.56 (0.060)	0.83 (0.001)*	0.72 (0.008)*	-0.24 (0.453)
Brain	0.77 (0.003)*	0.76 (0.006)*	0.59 (0.043)*	0.72 (0.008)*
Prostate	0.29 (0.579)	-0.27 (0.601)	0.55 (0.254)	0.72 (0.103)

*p-value < 0.05 by correlation analysis

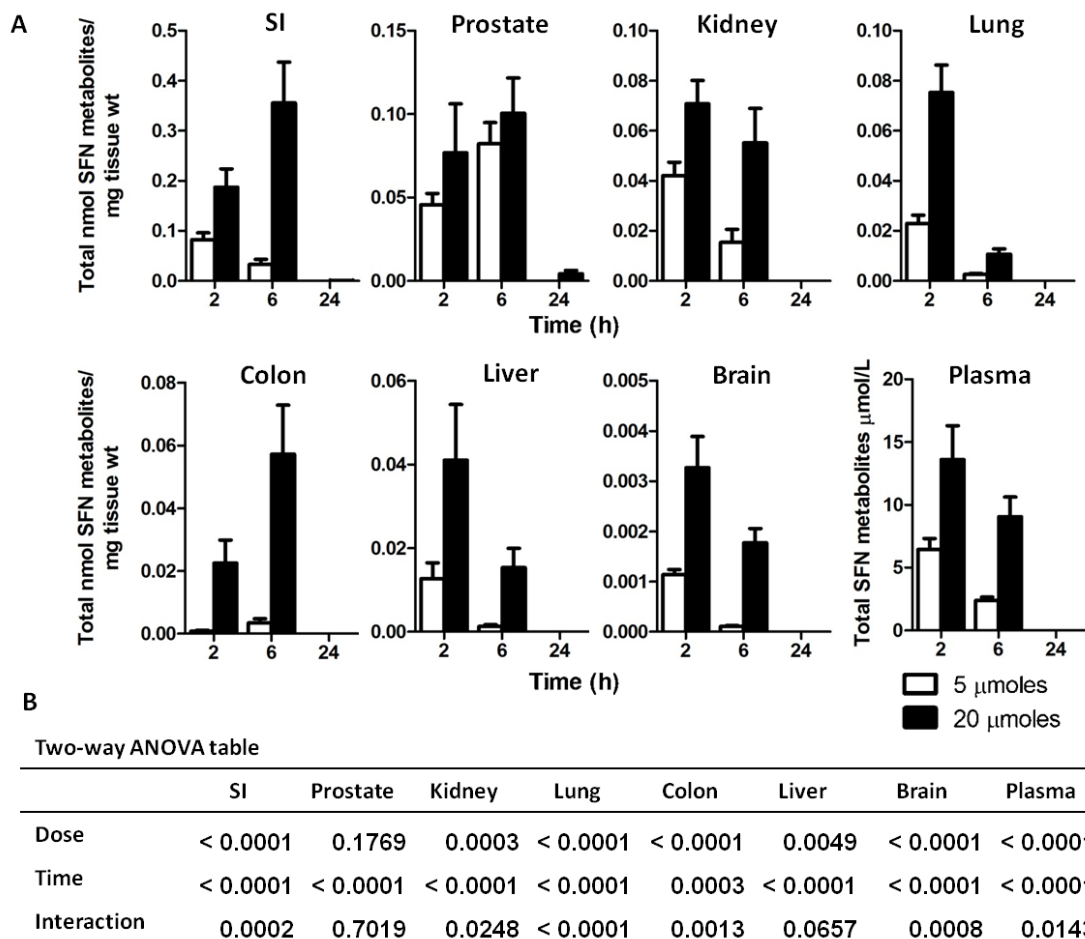


Figure 3.1 Dose dependent increase and rapid clearance of total SFN metabolites in most wild-type mouse tissues.

(A) Male and female wild-type mice received gavage of either 5 (open bars) or 20 (solid bars) μ moles of SFN and tissues were collected at 2, 6, and 24 h. Data in graphs represent the mean \pm SEM of the sum of all SFN metabolites normalized to tissue weight (n=12 for all tissues except prostate; prostate n=6). (B) Two-way ANOVA analysis of the data.

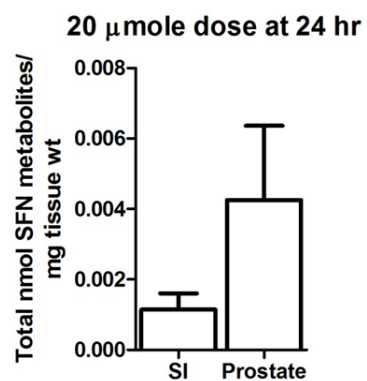


Figure 3.2 At 24 h low levels of SFN metabolites were detected in SI and prostate.

All tissues were tested for SFN metabolites but only SI and prostate had detectable levels. Data in graphs represent the mean \pm SEM of the sum of all SFN metabolites normalized to tissue weight (n=6).

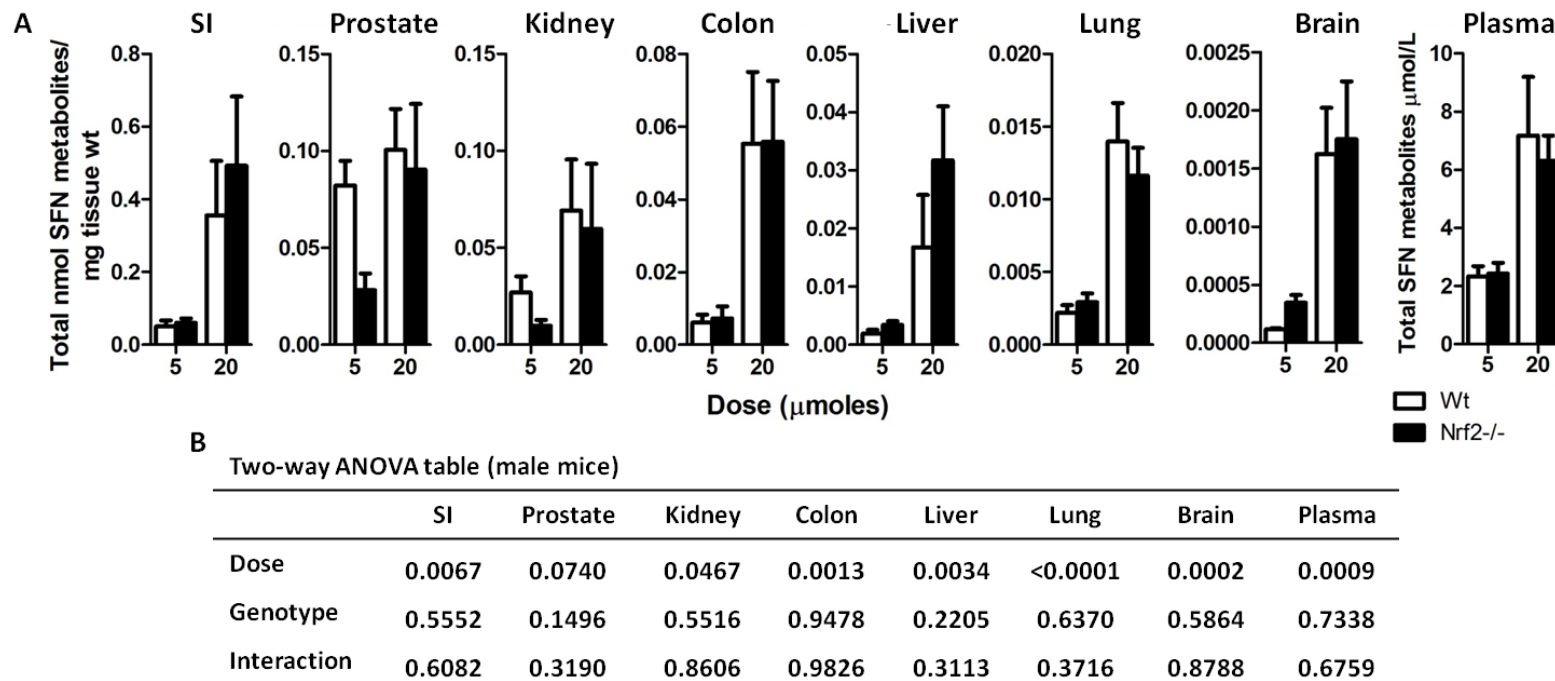
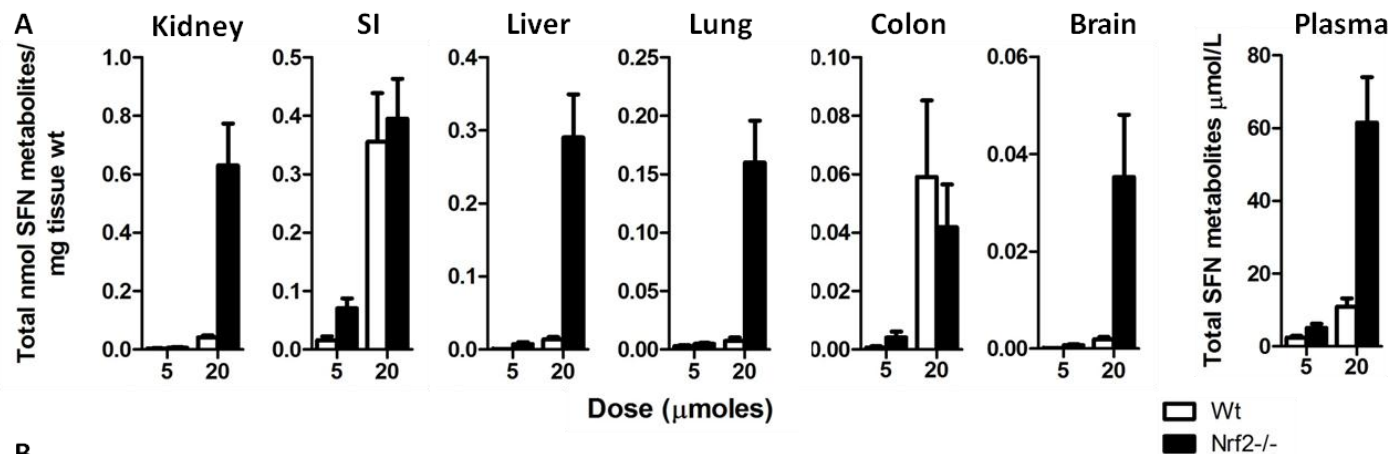


Figure 3.3 Nrf2 status has no effect on SFN metabolite concentrations in male mice.

(A) Data shown in graph are male wild-type (open bars) and male Nrf2^{-/-} (solid bars) mice treated with either 5 or 20 μ moles of SFN for 6 h. Data in graphs represent the mean \pm SEM of the sum of all SFN metabolites normalized to tissue weight (n=6).

(B) Two-way ANOVA analysis of the data.



B

Two-way ANOVA table (female mice)

	Kidney	SI	Liver	Lung	Colon	Brain	Plasma
Dose	0.0002	<0.0001	<0.0001	0.0003	0.0045	0.0102	<0.0001
Genotype	0.0005	0.4008	0.0001	0.0004	0.6489	0.0153	0.0005
Interaction	0.0006	0.8934	0.0002	0.0005	0.5011	0.0189	0.0013

Figure 3.4 Female Nrf2^{-/-} mice had dramatically higher SFN metabolite concentrations compared to female wild-type mice.

(A) Data shown in graph are female wild-type (open bars) and female Nrf2^{-/-} (solid bars) mice treated with either 5 or 20 μmoles of SFN for 6 h. Data in graphs represent the mean ± SEM of the sum of all SFN metabolites normalized to tissue weight (n=6). (B) Two-way ANOVA analysis of the data.

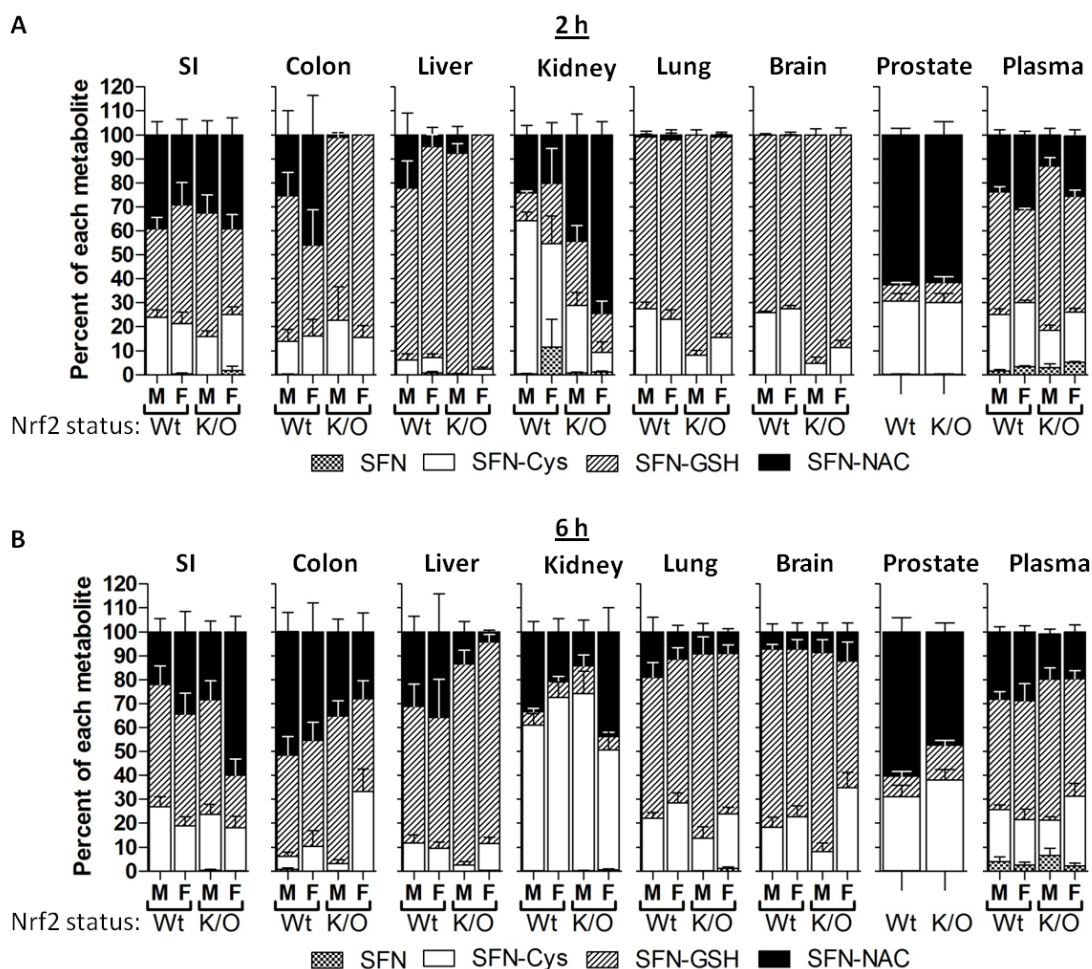


Figure 3.5 The relative abundance of each SFN metabolite is similar across genotype and gender but variable between tissues.

Percent each SFN metabolite represents within the SFN metabolites in different tissues in male (M) or female (F) and wild-type (Wt) or Nrf2^{-/-} (K/O) mice at 2 (A) and 6 (B) h after 20 μ mole dose of SFN. Data in graphs represent mean \pm SEM (n=6).

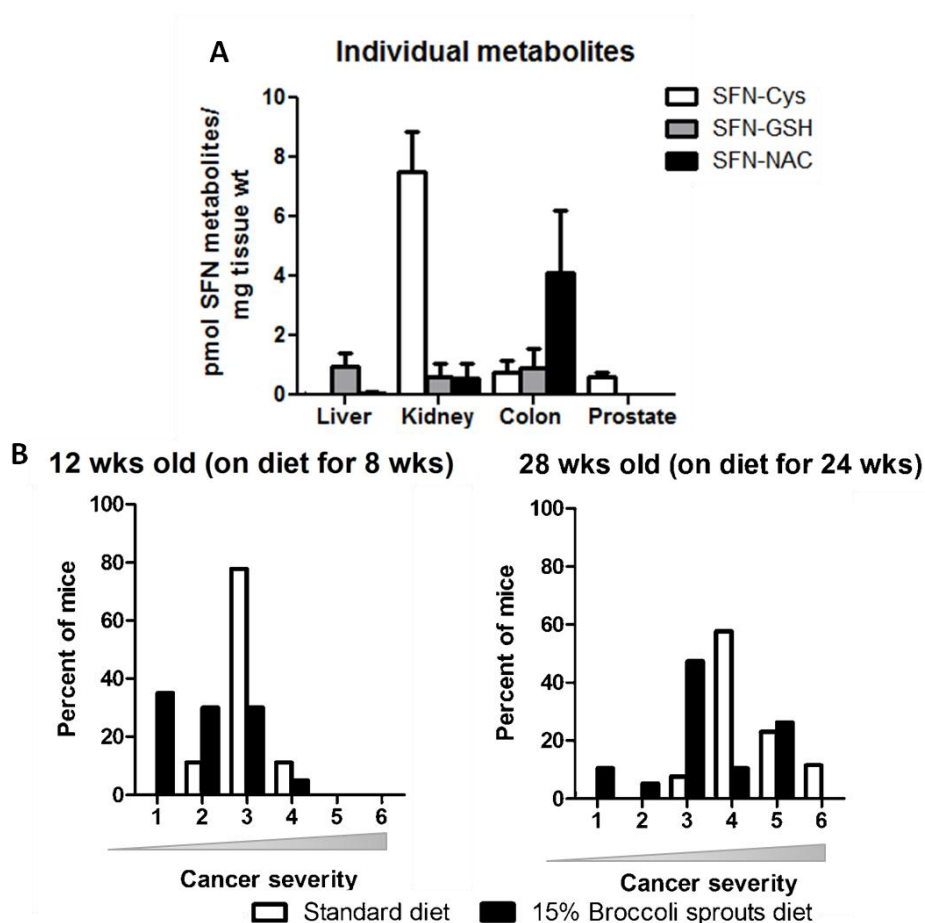


Figure 3.6 Dietary SFN results in SFN metabolites in prostate and slows the progression of prostate cancer in TRAMP mice.

(A) Liver, kidney, colon and prostates were collected from mice fed standard diet and mice fed a 15% broccoli diet and processed according to the procedure in the Materials and Methods section (n=3). No SFN compounds were detected in the control mice. (B) Prostate cancer severity in mice fed standard diet and 15% broccoli diet (n=20 in each group). Range of cancer severity: 1) normal, 2) prostatic intraepithelial neoplasia, 3) cribriform, 4) early adenoma 5) moderate adenoma, 6) poorly differentiated adenoma.

Chapter 4 Bioavailability and inter-conversion of sulforaphane and erucin in human subjects after consumption of broccoli sprouts or a broccoli supplement in a cross-over study design

John D. Clarke, Anna Hsu¹, Ken Riedl, Deborah Bella, Steven J. Schwartz, Jan F. Stevens, and Emily Ho

4.1. Abstract

Background: Broccoli consumption may reduce the risk of various cancers and many broccoli supplements are now available. The bioavailability and excretion of the bioactive isothiocyanates after human consumption of broccoli supplements has not been tested. Two important isothiocyanates from broccoli are sulforaphane and erucin.

Methods: We employed a cross-over study design in which 12 subjects consumed 40 grams of fresh broccoli sprouts followed by a 1 month washout period and then the same 12 subjects consumed 6 pills of a broccoli supplement. As negative controls for isothiocyanate consumption four additional subjects consumed alfalfa sprouts during the first phase and placebo pills during the second. Blood and urine samples were collected for 48 hours during each phase and analyzed for sulforaphane and erucin metabolites using LC-MS/MS.

Results: The bioavailability of sulforaphane and erucin is dramatically lower when subjects consume broccoli supplements compared to fresh broccoli sprouts. The peaks in plasma concentrations and urinary excretion were also delayed when subjects consumed the broccoli supplement. *GSTP1* polymorphisms did not affect the metabolism or excretion of sulforaphane or erucin. Sulforaphane and erucin are able to interconvert *in vivo* and this interconversion is consistent within each subject but variable between subjects.

Conclusions: This study confirms that consumption of broccoli supplements does not produce equivalent plasma concentrations of the bioactive isothiocyanates compared to broccoli sprouts. This has implications for people who consume the recommended serving size (1 pill) of a broccoli supplement and believe they are getting equivalent amounts of isothiocyanates.

4.2. Introduction

Epidemiological studies have shown an inverse association between cruciferous vegetable intake and cancer risk in many tissues including lung, bladder and prostate (99-101). A recent analysis of the EPIC-Heidelberg cohort study showed that the risk of prostate cancer decreased significantly over the quartiles of total glucosinolate intake (99). Importantly, glucosinolates are not the putative bioactive compounds in cruciferous vegetables, rather the hydrolysis products of glucosinolates, the isothiocyanates (ITCs), are the putative bioactive compounds. This is important because hydrolysis of the glucosinolate to the ITC is dependent upon a β -thioglucoside glucohydrolase enzyme called myrosinase. When humans consume cruciferous vegetables the only sources of myrosinase activity are from plant endogenous enzymes and from intestinal microflora; there is no evidence that mammalian cells are capable of metabolizing glucosinolates. Within cruciferous vegetables there are many different glucosinolates, each yielding a different ITC. In broccoli and broccoli sprouts two of the most abundant glucosinolates are glucoraphanin and glucoerucin (130) and myrosinase hydrolysis of these glucosinolates form sulforaphane (SFN) and erucin (ERN), respectively. Some of the ITCs have been investigated for their anti-cancer properties and there is a preponderance of evidence from *in vitro* studies in cell culture and *in vivo* studies in rodent models of cancer that SFN is an effective anti-cancer agent with the ability to both prevent and fight many types of cancer (7, 102, 103). Thus far ERN has not been widely studied but similar results are reported for bioactivity of ERN, although the potency and specific targets are variable between the two ITCs (26, 131-134). An important area of research about cruciferous vegetables and cancer prevention is a better understanding of the bioavailability of bioactive ITCs after human consumption of glucosinolates.

There are many factors that can affect the bioavailability of bioactive dietary constituents including food matrix, cooking, co-ingestion of other factors or the presence of proper enzymes for metabolism. Lycopene is a well known example where heat processing the tomato juice yields a 2-3 fold increase in serum

concentrations whereas the unprocessed tomato juice produces no change in serum concentrations (135). Also, tomato paste was shown to yield 2.5 fold higher lycopene plasma concentrations than fresh tomatoes (136). In the case of lycopene, cooking and coingestion of fats are both believed to increase its bioavailability. For glucosinolates it has been shown that cooking of cruciferous vegetables inactivates the myrosinase and decreases the bioavailability of ITCs. In contrast, coingestion of a myrosinase source with glucosinolates has been reported to increase ITC bioavailability (137). For consumers who do not enjoy eating broccoli but still want the benefits of ITCs in their diet, many different broccoli supplements have become available on the market. Importantly these supplements do not contain active myrosinase and therefore will likely not produce an equivalent amount of bioavailable ITCs.

A human feeding trial was performed to determine if there is a difference in the bioavailability of ITCs between a whole food source and a supplement source. To date a few studies have examined differences between ITC levels in humans that have consumed either fresh or cooked broccoli (52, 55, 138) and another study directly tested a powder similar to broccoli supplements in that there is no active myrosinase (137). The limitation of these studies was the use of the cyclocondensation assay or other means of surrogate ITC measurements, which do not directly measure and distinguish between different ITCs and their metabolites of which there are five main compounds of interest for each ITC. Herein, we used the specific and quantitative method of LC-MS/MS to measure SFN and ERN metabolite concentrations in both plasma and urine in a cross-over study design. It is hypothesized that polymorphisms in GST enzymes may impact ITC metabolism and excretion (139). To date, mixed results have been reported depending on the GST analyzed, glucosinolate source and study methodologies. As a component of this study we compared the metabolism of ITCs in relation to the *glutathione-S-transferase-P1* (*GSTP1*) polymorphism, since this is hypothesized to affect ITC metabolism.

4.3. Materials and methods

Participants

Sixteen subjects, aged 19-50 years were recruited in and around Corvallis, Oregon. The study was conducted in the Nutrition and Exercise Sciences department's metabolic kitchen and clinical collection lab at Oregon State University. Exclusion criteria included: smokers, vegetarians, anemic, engaged in vigorous activity for more than 6 hours (h) per week, or history of viral diseases, high blood pressure, high blood cholesterol, abnormal blood chemistries or urinary tract problems. All subjects gave written, informed consent to participate in the study. The study protocol was reviewed and approved by the Institutional Review Boards at Oregon State University and the Ohio State University.

Interventions

Subjects were randomized into two groups and processed through a blinded cross-over study design. One group (n=12) received broccoli sprouts in the first phase followed by BroccoMax®, a commercially available broccoli supplement, in the second phase. The other group (n=4) receiving alfalfa sprouts in the first phase followed by placebo pills in the second phase. Subjects were not told which type of sprouts or pills they received. A single lot of broccoli sprouts were obtained from Sprouters Northwest, Inc (Kent, WA). A single lot of alfalfa sprouts were obtained from a local grocery store. BroccoMax® pills and placebo pills was obtained from Jarrow Formulas (Los Angeles, CA) and were designed to be indistinguishable from each other. Sprouts and supplements were quality controlled for glucosinolate content both before and after trial by methods described below. Subjects avoided eating foods, such as other cruciferous vegetables, that contain glucosinolates or isothiocyanates for 24 h prior to the beginning of the study and throughout the duration of the study. Subjects participated in a pre-study meeting in which the protocol was explained and subjects were taught how to accurately keep dietary records by a registered dietitian (RD). The RD was available throughout the study to assist with diet records. Subjects kept 3-day dietary records during the study period. Subjects fasted every morning prior to sprouts or supplement and blood draws. On the

first day of the first phase subjects consumed 40 g of broccoli sprouts (150 and 71 μ moles glucoraphanin and glucoerucin, respectively) or alfalfa sprouts. On the first day of the second phase subjects consumed 6 BroccoMax® pills (~3 g of freeze dried broccoli sprouts) (121 and 40 μ moles glucoraphanin and glucoerucin, respectively) or 6 placebo pills during the second phase with a breakfast consisting of bagels with cream cheese and orange juice every morning. Days 2 and 3 the same breakfast was served except without sprouts or pills.

Study protocol

Complete urine and whole blood were collected at different times in the study. On day 1, urine was collected prior to consumption of sprouts or pills and total urine was collected during the following time blocks: 0-3, 3-6, 6-12, 12-24, and 24-48 h after sprouts or pills. Each urine bottle contained granulated boric acid to acidify the urine immediately upon collection to stabilize the isothiocyanate compounds. Urine volume was recorded and aliquots were frozen at -80 °C until analysis. Ten mL of whole blood were collected by venipuncture into a vacutainer containing EDTA at the same time points urine samples were collected. An additional tube of whole blood was collected at time zero during the first phase of the study for isolation of genomic DNA and subsequent analysis of *GSTP1* polymorphisms. Immediately following each blood draw, 1 mL of whole blood was removed from the vacutainer and centrifuged at high speed for ~1 min. The plasma was removed and acidified with trifluoroacetic acid (TFA) to a final concentration of 10% (v/v). The plasma was then centrifuged at 11,600 x g for 5 min at 4°C and the resulting supernatant was used for analysis on the UPLC-MS/MS system described below. Phlebotomy was performed in the Nutrition and Exercise Sciences Department Clinical Collection Lab by a trained phlebotomist.

DNA isolation and GSTP1 PCR-restriction fragment length polymorphism genotyping

Genomic DNA from whole blood was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and used to determine the genotypes of the *GSTP1*

gene using the PCR-RFLP method (140). The GSTP1 polymorphism screened was a guanine to adenine transition at nucleotide 313 (A³¹³G) that results in an isoleucine to valine substitution at codon 105 (I¹⁰⁵V), which is located in the substrate binding site of GSTP1. The primers for the PCR reactions were *GSTP1* (sense) 5'-CCAGTGACTGTGTGTTGATC-3' and (antisense) 5'-CAACCCTGGTGCAGATGCTC-3' (189-bp fragment). The PCR reactions were carried out in a 20 µL mixture containing 50 ng sample DNA, 2X Taq polymerase master mix (New England Biolabs, Ipswich, MA), and 0.5 M of each oligonucleotide primer. Amplification was achieved by 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, followed by a final extension step for 7 min at 72°C. PCR product was subjected to BsmAI enzyme (New England Biolabs) digestion and analyzed by gel electrophoresis on 3% low-melt agarose gel. The presence of the polymorphic BsmAI restriction site yields 148- and 41-bp fragments, indicating the presence of at least one G allele and *GSTP1105Val* genotypes.

Glucosinolate analysis

Glucoraphanin and glucoerucin were purchased from The Royal Veterinary School of Denmark. Extraction: Fresh broccoli sprouts were freeze-dried, ground to a powder with mortar and pestle and stored at -80°C until analysis. A portion of the powder (0.25 g) was dispersed in 10 mL of boiling water and boiled for five minutes by immersing the loosely capped vials containing the sprout suspension in a boiling water bath. Samples were cooled to room temperature and centrifuged for 5 min at 20,000 x g. The pellet was resuspended in 10 mL of room temperature water and extracted a second time for 10 min at room temperature. Samples were centrifuged again and extracted once more for 10 min before pooling the three extracts. An aliquot of the pooled extract was diluted 100-fold with 0.1 % (v/v) formic acid in water for HPLC-MS/MS analysis. A portion of the Broccomax supplement (0.25g) was extracted three times with 10 mL of water in the same way as described above for the freeze-dried broccoli sprout powder.

HPLC-MS/MS: Quantitative HPLC-MS/MS analysis for glucosinolates was conducted with an HPLC (Agilent 1200 SL+, Agilent, XXX) coupled to a quadrupole/ion trap hybrid mass spectrometer (QTrap 5500, AB Sciex, Concord, Canada) operated as a triple quadrupole instrument in electrospray negative mode. Reversed phase chromatography employed a cyanopropyl column (4.6 x 250mm, 5 μ m; Zorbax Stable Bond CN, Agilent, USA) with a gradient of 0.1 % (v/v) formic acid in water (A) versus 0.1 % (v/v) formic acid in acetonitrile (B) at 1.5 mL/min and 30°C. The initial condition was 0% B held isocratically for 3 min then increased linearly to 10% B at 4 min, 50% B at 8 min and then re-equilibrated by 12 min. Mass spectrometer source settings and MS/MS transitions were optimized for selected reaction monitoring of each glucosinolate based on common liberation of the HSO_4^- anion (m/z 97) from each glucosinolate (glucoraphanin and glucoerucin) with dwell times of 140 ms. Instrumental parameters included turbospray desolvation at 550°C, declustering potential 70 V, entrance potential 10 V, exit potential 11 V, collision energy 30, ion spray 4.5 kV, gas 1 60 psi, gas 2 55 psi, curtain gas 30 psi. Extinction coefficients used for external calibration of the three glucosinolates were as described in Tian et al. (130).

Preparation of isothiocyanate standards

Sulforaphane (SFN) and erucin (ERN) were purchased from LKT laboratories Inc (St. Paul, MN). SFN-cysteinylglycine (SFN-CG), ERN-glutathione (ERN-GSH), ERN-cysteinylglycine (ERN-CG), ERN-cysteine (ERN-Cys) and ERN-N-acetylcysteine (ERN-NAC) were prepared following methods described by Vermeulen et al. (141) in which isothiocyanates and their respective conjugate groups were reacted, to generate the various metabolites, e.g. SFN + CG for SFN-CG. The reaction mixtures were purified by semi-preparative reversed phase chromatography (10 x 250mm, 5 μ m C18 Bondapak, Waters Corp, Milford, MA) with a water/acetonitrile mobile phase. ACN in metabolite fractions was removed by Rotovap and the remaining aqueous phase freeze-dried to achieve a powder. Powders were weighed and extinction coefficients at appropriate wavelengths determined as the

average of triplicate determinations. Each metabolite displayed spectra with two characteristic UV features at 250 and 270 nm. HPLC was performed and compounds were shown to be spectrally pure (all >95%). The extinction coefficients at 270 nm were 7551 SFN-Cys, 4796 SFN-CG, 7334 SFN-GSH, 6832 SFN-NAC, 2302 ERN-CG, 1609 ERN-Cys, 1653 ERN-GSH, 3391 ERN-NAC each in methanol.

Analysis of isothiocyanates

For sample preparation, urine was diluted 1:10 with 0.1 % (v/v) formic acid in water.

Plasma processing was slightly modified from that of Janobi et al. (84). Cold TFA (0°C) was added at 10 % (v/v) to plasma that had been pre-chilled on ice. After five minutes on ice the cloudy suspension was centrifuged at 16,000 x g for 5 min to pellet the proteins and recover metabolites in the supernatant. Supernatant was injected directly (10 µL) for quantitative analysis.

UPLC chromatography was as follows: Acquity BEH C18 (2.1 x 100mm, 1.7 µm) with a mobile phase of 0.1 % (v/v) formic acid in water versus 0.1 % (v/v) formic acid in acetonitrile at 0.45 mL/min at 40°C. Five uL was injected onto the column. Initially, the mobile phase was 0% B increased linearly to 10% B at 1min, 33.3% B at 2.5 min, 72% B at 4 min (curve 7) and returned to 0% B by 6 min. HPLC eluent was interfaced without flow splitting to a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, UK) via an electrospray probe operated in positive mode. Selected reaction monitoring (SRM) MS/MS transitions were developed for each of 9 analytes using collision induced dissociation (CID) – sulforaphane (178>114), SFN-GSH (485>136), SFN-CG (356>136), SFN-Cys (299>136), SFN-NAC (341>114), ERN-ERN (469>179), ERN-CG (340>103), ERN-Cys (283>103), and ERN-NAC (325>164) with dwell times of 80-150 ms. Source parameters included capillary 3.2 kV, desolvation temperature 450 C, cone voltage 35 V, RF1 12.5 V, collision energy (10-18 eV), and CID argon pressure (3×10^{-3} mBar). Reproducible chromatography

and MS response could not be achieved with free ERN and thus it was omitted from the analysis.

Statistical analysis

Repeated measures two-way ANOVA and linear regression were performed as indicated in the applicable tables and graphs.

4.4. Results

Table 4.1 shows the subjects demographics and percent of required dietary intake for calories, protein, carbohydrates and fat. There were no statistical differences between the two treatment groups in these measures. We also tested the percent of required dietary intakes across phases of the study and did not see any statistical differences.

Following consumption of 40 g of alfalfa sprouts or 6 placebo pills no SFN or ERN metabolites were detected in plasma or urine from the four subjects in the control group (Figure 4.1). In contrast subjects who consumed 40 g of broccoli sprouts (150 and 71 μ moles glucoraphanin and glucoerucin, respectively) or 6 supplement pills (121 and 40 μ moles glucoraphanin and glucoerucin, respectively) had considerable amounts of SFN and ERN metabolites in both plasma and urine. During the first phase of the study (broccoli sprouts), total SFN metabolite and total ERN metabolite concentrations in plasma were highest at 3 h post consumption and were almost completely cleared from the plasma by 24 h (Figure 4.1A). Urinary excretion during this phase peaked between 3 and 6 h (Figure 4.1B). In contrast, during the second phase of the study total SFN metabolite and total ERN metabolites in the plasma were at the highest concentrations 6 h post consumption (Figure 4.1A). The peak in urinary excretion was delayed in the second phase such that the peak did not occur until between the 6 and 12 h time points (Figure 4.1B). Although similar doses of glucoraphanin and glucoerucin were given in the sprouts and supplement, the total amounts of SFN and ERN metabolites in the plasma and urine were much lower in the supplement group compared to the sprout group. Two-way ANOVA analysis of the

total 24 h urinary excretion for the total SFN metabolites showed that *GSTP1* genotype had no effect on excretion but the source of glucosinolates had a significant effect (Table 4.2). The same results were observed for the total 24 h urinary excretion for ERN metabolites (Table 4.2).

We were able to quantify all of the major metabolites for both SFN and ERN in plasma and urine. The relative abundance of each metabolite within SFN and ERN in the plasma (Table 4.3) and urine (Table 4.4) was analyzed by 2-way ANOVA. *GSTP1* genotype did not have an effect on the relative abundance of each metabolite but glucosinolate source had a significant effect for all metabolites except SFN glutathione (GSH). In the plasma the most abundant metabolite for SFN and ERN was the cysteine-glycine (CG) conjugate, representing ~85 and ~70% of the total, respectively, and the percentage of the CG conjugates increased when subjects consumed supplement compared to sprouts (Table 4.3). Similar to what was observed in the plasma, within the urine *GSTP1* genotype did not have an effect on the relative abundance of each metabolite but glucosinolate source had a significant effect in several of the metabolites. Interestingly, the most abundant metabolites for SFN and ERN, which were the N-acetylcysteine (NAC) conjugates, were not significantly different between the treatment groups. The free SFN and the SFN-Cysteine (Cys) had the largest shift in percentages; free SFN increased and SFN-Cys decreased when subjects consumed supplement compared to sprouts (Table 4.4).

To assess the possibility of SFN and ERN interconversion *in vivo* we calculated the ratio of ERN metabolites to SFN metabolites in the plasma and the urine. To obtain this ratio we divided the sum of ERN metabolites by the sum of SFN metabolites for each subject at each time point. In the broccoli sprouts the ratio of glucoerucin to glucoraphanin was 0.47 and in the broccoli supplement it was 0.32, indicating that more glucoraphanin was present than glucoerucin in both sprouts and supplement. Interestingly, the ratio in the plasma and urine increased compared to the ratio of glucoerucin and glucoraphanin present in the broccoli sprouts or broccoli supplement in nearly all subjects indicating that some SFN was converted to ERN

(Figure 4.2). The plasma ratio in a particular subject during the sprout phase correlated to the plasma ratio in the same subject during the supplement phase indicating that within each subject there was a consistent interconversion across treatments and a one month period of time (Figure 4.2A). A similar correlation between phases was also found in urine samples (Figure 4.2B). Although the interconversion ratios within each subject were consistent, there was higher inter-subject variation in conversion suggesting individual differences in the ability to interconvert SFN and ERN.

4.5. Discussion

This cross-over study compared SFN and ERN bioavailability from a whole food source to a dietary supplement. We demonstrate that the whole food had both higher bioavailability and altered kinetics compared to a myrosinase-inactivated supplement. The whole food, which contains myrosinase, produced peak plasma concentrations that were 7 and 12 fold higher for SFN and ERN, respectively, compared to peak plasma concentrations after consumption of the broccoli supplement, which does not contain myrosinase. Similarly in the urine total 24 h excretion was 5 fold and 8 fold higher for SFN and ERN, respectively, when subjects consumed the whole food versus the supplement. These data are the first to show detailed LC-MS/MS analysis of both SFN and ERN in human subjects after consuming broccoli sprouts and a broccoli supplement.

The necessity for myrosinase has been considered in many different studies involving glucosinolate and ITC metabolism (142) and in this report we provide further evidence that myrosinase activity is necessary for maximal bioavailability of ITCs. Lack of myrosinase can affect two main aspects of metabolism; bioavailability and kinetics. For bioavailability several studies in humans have examined the difference between cooked and raw broccoli (52, 138, 143) and another even compared excretion when broccoli sprouts were chewed versus swallowed intact (55). In all cases it has been concluded that inactivation or inaccessibility of myrosinase led

to lower plasma and urine concentrations of the ITCs. Recently a paper was published testing the differences in absorption and excretion between a broccoli powder and broccoli sprouts (137). In this study the authors reported that only 19% of the SFN was recovered after consumption of the broccoli powder compared to 74% recovery after consumption of the broccoli sprouts. From these data we can conclude that the presence of myrosinase is important for maximal bioavailability of ITCs.

Not only were the ITCs more bioavailable from the whole food source but the peak plasma and urine concentrations occurred sooner when subjects consumed the whole food. Cramer et al concluded that there was a delay in the appearance of SFN in subjects who consumed the broccoli powder compared to those who consumed the broccoli sprouts (137). In congruence with that report, herein we observed that the peaks in plasma concentrations and urinary excretion were delayed when subjects consumed the broccoli supplement.

The lower bioavailability and this delayed appearance of ITC metabolites in the plasma and urine when subjects consumed the supplement likely reflects the reliance on microflora in the colon for glucosinolate hydrolysis. When subjects are given fresh sprouts the ITCs are formed and released when consumed and can readily be absorbed in the jejunum (14). In contrast, unhydrolyzed glucosinolates need to be metabolized by gut microflora before the ITCs can be absorbed. In fact, a recent report demonstrated that cecal microbiota can hydrolyze glucosinolates and that SFN can be absorbed through the cecal enterocytes in rats (144). These factors relating to glucosinolate metabolism and ITC absorption play important roles in the bioavailability and kinetics of ITCs in humans.

In this report we provide further evidence that SFN and ERN interconvert in humans. Only one other study in humans has reported interconversion between SFN and ERN (145). We show for the first time that conversion is variable between subjects, but consistent within subjects across time and glucosinolate source. In our study the ratio of glucoerucin to glucoraphanin in the broccoli sprouts and broccoli supplement was 0.47 and 0.32, respectively. This is significant because the ratio in

the plasma of most subjects ≥ 0.4 , indicating that some SFN had been converted to ERN. The variability between subjects in the plasma ratio ranged from ~ 0.2 to ~ 1.2 . The variability in the urine ratio between individual subjects was even wider ranging from ~ 0.1 to ~ 2.3 . The conversion of SFN to ERN appears to occur after absorption because in the plasma the ratio started ~ 0.4 and did not reach ~ 0.8 until 12 to 24 h post consumption. In contrast, regardless of time, the average ratio in the urine was ~ 0.8 . This implies that when the ITCs are absorbed they are closer to the starting ratio of glucoerucin to glucoraphanin but as they are metabolized and excreted in the urine some SFN is converted into ERN. It is not clear what drives the conversion of the sulfoxide in SFN to the sulfide in ERN but is an important area for future research.

In conclusion, our data provide further evidence that bioavailability of SFN and ERN is dramatically lower when subjects consume broccoli supplements compared to fresh broccoli sprouts. Furthermore, we provide strong evidence that the interconversion between SFN and ERN is consistent within each subject but variable between subjects. Together these data further characterize the bioavailability and kinetics of ITCs from a whole food source versus a dietary supplement, and have implications regarding consumer choices of how to best incorporate the chemopreventive effects of cruciferous vegetables into their diets.

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Table 4.1 Subject demographics and caloric and macronutrient intake (%)

	Demographics ^{1,2}			% intake ^{1,2}			
	Gender ³	Age	BMI ⁴	Caloric	Protein	Carbs.	Fat
Broccoli group	4(M) 8(F)	31.8 ± 2.6	25.0 ± 0.9	87.1 ± 4.4	142.4 ± 9.9	87.4 ± 4.9	94.8 ± 7.8
Alfalfa group	2(M) 2(F)	24.5 ± 1.7	23.8 ± 2.1	83.2 ± 10.3	151.6 ± 10.3	81.8 ± 16.0	77.6 ± 9.1

¹Mean ± SEM²No significant difference between treatment groups by Student's t-test³M=male, F=female⁴BMI=body mass index

Table 4.2 Total μmol of SFN compounds and ERN compounds excreted in urine during 24 h after consumption of broccoli sprouts or broccoli supplement

μmol	Broccoli sprouts ¹		Broccoli supplement ¹		p-values ²		
	A/A ³	A/G ⁴	A/A	A/G	Genotype	Treatment	Interaction
SFN	171.6 \pm 23.4	135.1 \pm 14.8	35.6 \pm 11.8	30.1 \pm 7.0	0.227	<0.0001	0.408
ERN	114.1 \pm 17.4	98.8 \pm 19.7	15.3 \pm 4.1	14.4 \pm 3.5	0.550	<0.0001	0.620

¹Mean \pm SEM.

²Repeated measures 2-way ANOVA

³Positive GSTP1 genotype (n=7)

⁴Heterozygous A³¹³G GSTP1 genotype (n=5)

Table 4.3 Percent of each SFN compound and each ERN compound within each group of compounds in the plasma during the first 12 hrs after consumption of broccoli sprouts or broccoli supplement

	Broccoli sprouts ¹		Broccoli supplement ¹		p-values ²		
	A/A ³	A/G ⁴	A/A	A/G	Genotype	Treatment	Interaction
ITC compounds ⁵	%	%	%	%			
SFN	0.30 ± 0.04	0.27 ± 0.06	0.04 ± 0.04	0.05 ± 0.05	0.797	0.0004	0.647
SFN-GSH	2.64 ± 0.08	2.87 ± 0.14	2.43 ± 0.19	2.58 ± 0.11	0.194	0.125	0.815
SFN-CG	82.0 ± 1.02	81.0 ± 1.20	89.0 ± 1.19	88.2 ± 0.90	0.574	<0.0001	0.761
SFN-Cys	3.44 ± 0.12	3.67 ± 0.31	3.06 ± 0.18	3.00 ± 0.18	0.740	0.003	0.336
SFN-NAC	11.6 ± 0.96	12.2 ± 0.94	5.51 ± 0.99	6.17 ± 0.87	0.646	<0.0001	0.944
ERN-GSH	8.39 ± 0.29	9.98 ± 1.09	7.11 ± 0.95	7.76 ± 1.04	0.271	0.043	0.548
ERN-CG	69.4 ± 1.38	67.6 ± 1.19	72.9 ± 1.78	75.1 ± 1.44	0.916	<0.0001	0.022
ERN-Cys	13.6 ± 0.59	14.6 ± 1.20	12.09 ± 0.42	12.1 ± 1.59	0.686	0.002	0.360
ERN-NAC	8.56 ± 1.15	7.77 ± 0.75	7.92 ± 1.67	5.01 ± 1.13	0.311	0.048	0.189

¹Mean ± SEM. ²Repeated measures 2-way ANOVA, ³Positive GSTP1 genotype (n=7), ⁴Heterozygous A³¹³G GSTP1 genotype (n=5), ⁵For both SFN and ERN compounds abbreviations are: GSH=glutathione, CG=cysteine-glycine, Cys=cysteine, NAC=N-acetylcysteine

Table 4.4 Percent of each SFN compound and each ERN compound within each group of compounds in the urine during the first 12 hrs after consumption of broccoli sprouts or broccoli supplement

	Broccoli sprouts ¹		Broccoli supplement ¹		p-values ²		
	A/A ³	A/G ⁴	A/A	A/G	Genotype	Treatment	Interaction
ITC compounds ⁵	%	%	%	%			
SFN	9.72 ± 1.68	7.99 ± 2.71	16.8 ± 4.08	16.3 ± 5.30	0.784	0.034	0.853
SFN-GSH	0.02 ± 0.006	0.02 ± 0.004	0.09 ± 0.03	0.09 ± 0.03	0.885	0.009	0.921
SFN-CG	0.63 ± 0.21	0.42 ± 0.17	0.86 ± 0.27	1.59 ± 0.56	0.428	0.053	0.171
SFN-Cys	17.9 ± 1.37	20.2 ± 2.69	14.1 ± 1.06	16.5 ± 0.50	0.197	0.024	0.957
SFN-NAC	71.7 ± 1.33	71.4 ± 1.03	68.1 ± 4.25	65.6 ± 5.72	0.717	0.205	0.747
ERN-GSH	0.02 ± 0.005	0.01 ± 0.005	0.21 ± 0.12	0.35 ± 0.14	0.466	0.015	0.443
ERN-CG	0.11 ± 0.01	0.12 ± 0.04	0.89 ± 0.31	2.00 ± 0.77	0.160	0.005	0.167
ERN-Cys	16.4 ± 1.38	18.4 ± 2.42	15.4 ± 2.51	16.9 ± 2.68	0.566	0.378	0.849
ERN-NAC	83.4 ± 1.37	81.4 ± 2.38	83.5 ± 2.25	80.8 ± 2.62	0.415	0.810	0.789

¹Mean ± SEM, ²Repeated measures 2-way ANOVA, ³Positive GSTP1 genotype (n=7), ⁴Heterozygous A³¹³G GSTP1 genotype (n=5), ⁵For both SFN and ERN compounds abbreviations are: GSH=glutathione, CG=cysteine-glycine, Cys=cysteine, NAC=N-acetylcysteine

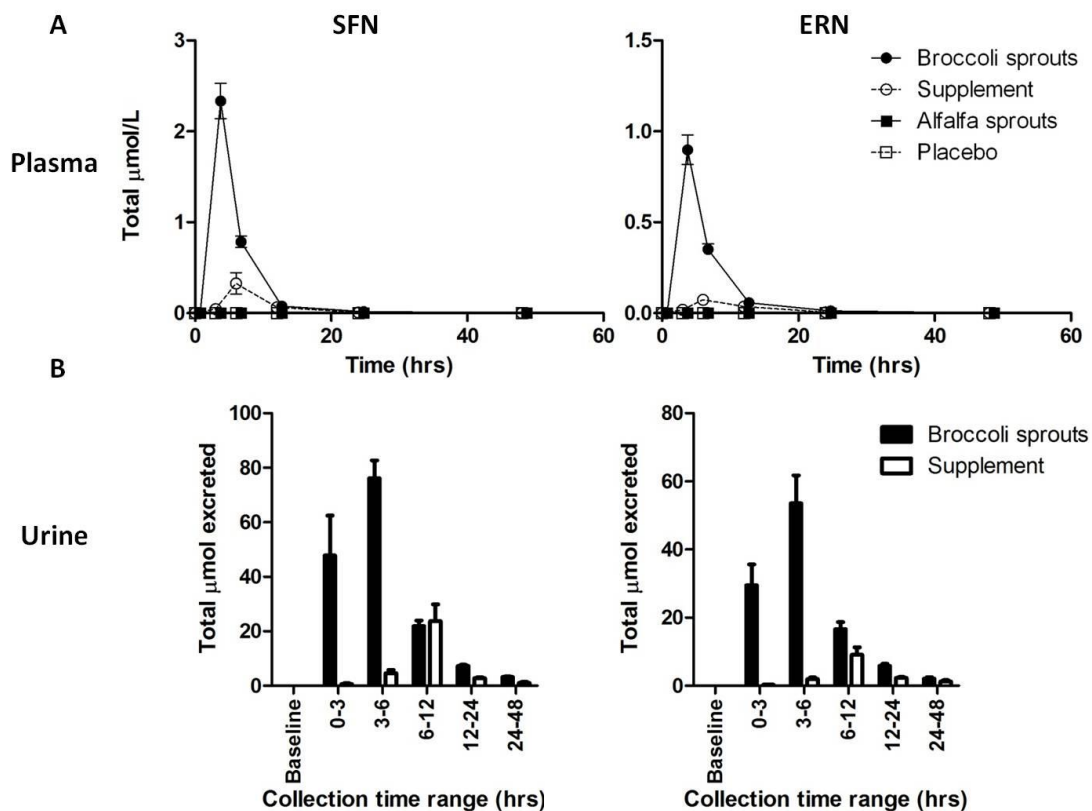


Figure 4.1 Higher amounts of total SFN and ERN compounds in plasma and urine after consumption of broccoli sprouts.

Sum of all SFN compounds (left) and sum of all ERN compounds (right) in plasma (A) and urine (B) after consumption of broccoli sprouts (closed circle), broccoli supplement (open circle), alfalfa sprouts (closed square) or placebo pills (open square) throughout the course of the study. (B) Sum of all SFN compounds (left) and sum of all ERN compounds (right) in urine after consumption of broccoli sprouts (closed bars) and broccoli supplement (open bars). No ITCs were detected in either plasma or urine from subjects who consumed alfalfa sprouts or placebo pills. Sprouts and supplement $n=12$; alfalfa and placebo $n=4$.

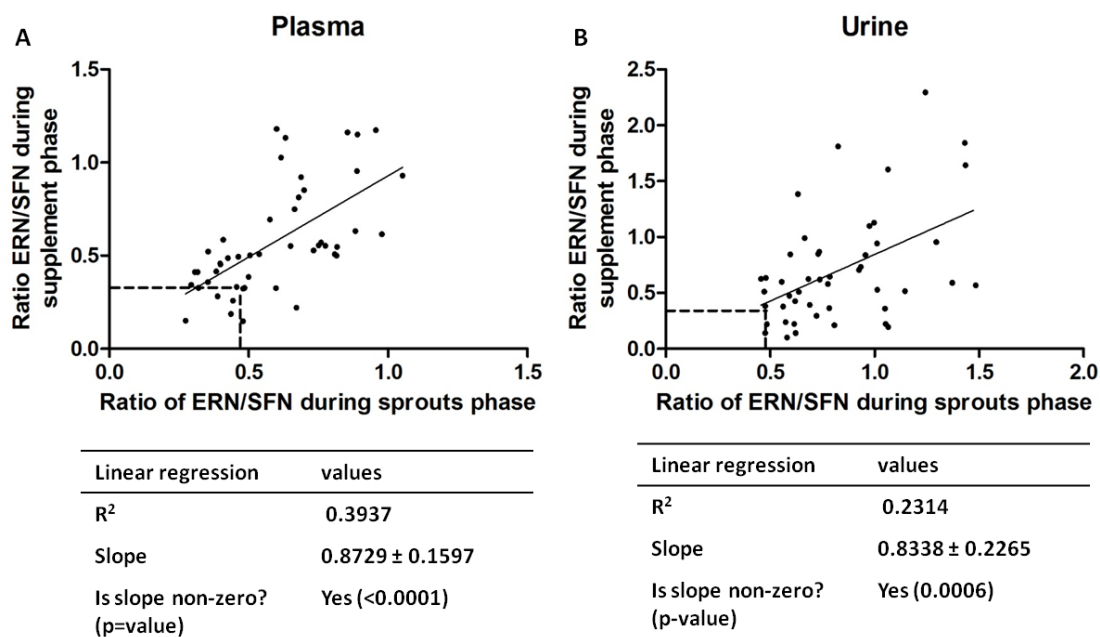


Figure 4.2 The ratio of ERN compounds to SFN compounds within the plasma and urine of a subject during the sprout phase of the study correlates with the ratio within the plasma and urine of the same subject in the supplement phase of the study.

The ratio observed in both the plasma (A) and urine (B) during the sprout phase correlates with the ratio observed in the plasma during the supplement phase. Each data point represents the ratio of total ERN metabolites divided by the total SFN metabolites (without free SFN) for one subject at the same time point in the sprouts phase (x-axis) and the supplement phase (y-axis). The dashed lines indicate the ratios of glucorucin to glucoraphanin in the broccoli sprouts and broccoli supplement. The values for R^2 , slope and test for linearity are shown in the table below the graphs.

Chapter 5 Comparison of isothiocyanate metabolite levels and histone deacetylase activity in human subjects after consumption of broccoli sprouts or a broccoli sprout supplement

John D. Clarke, Ken Riedl, Deborah Bella, Steven J. Schwartz, Jan F. Stevens, and
Emily Ho

5.1. Abstract

Background: Broccoli consumption may reduce the risk of various cancers and many broccoli supplements have become available. The possible differences in metabolism of active isothiocyanates between the whole food and these supplements have yet to be determined.

Objective: We evaluated isothiocyanate excretion profiles in healthy subjects who consumed broccoli sprouts or broccoli supplement with equivalent glucosinolate content.

Design: Twenty-four subjects (12 male and 12 female) were recruited into a randomized study in which 12 subjects consumed fresh broccoli sprouts and 12 subjects consumed a broccoli supplement. Urine metabolites of two major isothiocyanates, sulforaphane and erucin, were measured by liquid chromatography coupled with tandem mass spectrometry.

Results: Subjects that consumed broccoli sprouts excreted 5-8 times more sulforaphane and erucin metabolites during a 24 h period compared to subjects who consumed the supplement. The peak excretion of sulforaphane and erucin metabolites was also delayed by 6 h in the subjects that consumed the supplement. A subject dependent shift in the ratio of urinary sulforaphane to erucin metabolites was observed in subjects in both treatment groups indicating interconversion of sulforaphane to erucin. Lower histone deacetylase activity was observed in the peripheral blood mononuclear cells only in subjects consuming sprouts.

Conclusions: Consumption of a broccoli supplement results in lower amounts of ITC metabolites (sulforaphane & erucin) that are excreted in the urine which is likely due to the lack of myrosinase enzymes in the supplement. We also show that the interconversion between sulforaphane and erucin occurs in humans following the consumption of broccoli products.

5.2. Introduction

Epidemiological data indicates that dietary consumption of cruciferous vegetables may reduce the risk of prostate, breast, lung and colorectal cancers (6). Cruciferous vegetables contain high levels of glucosinolates, a class of phytochemicals not found in any other vegetable. Glucosinolates are stable compounds that require enzymatic hydrolysis by myrosinase, a β -thioglucosidase present only in the plant and in gut microbial flora, to form many different metabolites. Myrosinase cleaves the glucosinolate forming glucose, hydrogen sulfate and either a thiocyanate, nitrile, or an isothiocyanate (ITC) depending on the starting glucosinolate, reaction pH, and availability of ions (50). ITCs are the putative bioactive constituents of cruciferous vegetables. Once consumed, ITCs are metabolized through the mercapturic acid pathway and ultimately excreted in the urine predominately as the N-acetylcysteine conjugates. Intense interest surrounds the use of ITCs to inhibit cancer development and growth at many stages in cancer progression. Sulforaphane (SFN) is an aliphatic ITC that has shown promise as a chemopreventive agent both *in vitro* and *in vivo* (102). The closely related aliphatic ITC erucin (ERN) has also received attention due to its similar structure and activity to SFN. The classic chemoprevention mechanism for SFN involves induction of phase 2 enzymes thereby facilitating detoxification of carcinogens and other genotoxic stresses. Another chemopreventive mechanism by which SFN is reported to work is through inhibition of histone deacetylases (HDACs) which causes an increase in histone acetylation at the promoters of aberrantly silenced tumor suppressor genes, reactivating the tumor suppressor and ultimately inducing cell cycle arrest and/or apoptosis (29, 37, 39, 66). Broccoli contains high amounts of glucoraphanin and glucoerucin, the glucosinolate precursor to SFN and ERN, respectively (130). Although glucoraphanin content increases as the plant matures, the concentration of glucoraphanin is highest in the broccoli sprouts (146). The amount of pre-clinical and clinical data for the chemopreventive effects of broccoli and ITCs is strong and growing (147).

Many cancer patients in the United States use dietary supplements which represents a large proportion of complementary and alternative medicine (148). A meta-analysis of vitamin and mineral supplement use in US adults after cancer diagnosis reported that between 64% and 81 % of cancer survivors (combined cancer site data) reported using a supplement (148). Although a multitude of *in vitro* evidence for the chemopreventive properties of many individual dietary constituents is evident there are limited data regarding *in vivo* efficacy of whole foods versus supplements. In order for a dietary compound to be efficacious it must be metabolized and reach target tissues. Currently little is known regarding the metabolism of glucosinolates in supplement form. One of the main issues regarding the utility of broccoli supplements is the efficacious conversion of the glucosinolates to ITCs because the supplements do not contain active myrosinase enzymes. A small number of studies in animals and humans have found that inactivation of myrosinase decreases availability of ITCs (52, 54, 55, 138, 149). Broccoli supplements contain glucosinolates rather than their cognate ITCs because glucosinolates are more stable than ITCs. It has been speculated that consuming broccoli supplements will not produce high plasma and urinary concentrations of the bioactive ITCs (149). To date, a majority of the research has focused on the pharmacokinetics and bioactivity of SFN while only one human study has looked into pharmacokinetics of ERN. The current study was undertaken to assess differences in the metabolism and excretion of glucoraphanin and glucoerucin between broccoli sprouts and a broccoli supplement and test if consumption of an ITC rich food will alter HDAC activity in healthy human subjects.

5.3. Subject and methods

Participants

Twelve men and 12 women aged 19-50 y were recruited in and around Corvallis, Oregon. The study was conducted in the Nutrition and Exercise Sciences department's metabolic kitchen and clinical collection lab at Oregon State University.

Exclusion criteria included: smokers, vegetarians, anemic, engaged in vigorous activity for more than 6 h per week, or history of viral diseases, high blood pressure, high blood cholesterol, abnormal blood chemistries or urinary tract problems. All subjects gave written, informed consent to participate in the study. The study protocol was reviewed and approved by the Institutional Review Boards at Oregon State University and the Ohio State University.

Interventions

Subjects were randomized into two groups; either broccoli sprouts or BroccoMax® a commercially available broccoli supplement. A single lot of broccoli sprouts were obtained from Sprouters Northwest, Inc (Kent, WA) and BroccoMax® was obtained from Jarrow Formulas (Los Angeles, CA). Subjects avoided eating foods that contain glucosinolates or isothiocyanates for 24 h prior to the beginning of the study and throughout the duration of the study. Subjects participated in a pre-study meeting in which the protocol was explained and subjects were taught how to accurately keep dietary records by a registered dietitian (RD). The RD was available throughout the study to assist with diet records. Subjects kept dietary records for the first 7 days of the study. Subjects fasted every morning prior to sprouts or supplement and blood draws. Subjects consumed either 68 g of broccoli sprouts or 6 BroccoMax® pills (~3 g of freeze dried broccoli sprouts) with a breakfast consisting of bagels with cream cheese and orange juice every morning for 7 days (Figure 5.1). On days 8-10 the same breakfast was served except without sprouts or supplement.

Study protocol

Complete urine and whole blood were collected at different times in the study (Figure 5.1). On day 1 urine was collected prior to consumption of sprouts or supplement and total urine was collected during the following time blocks: 0-6, 6-12, and 12-24 h after sprout or supplement. On day 7, urine was collected prior to consumption of sprouts or supplement and total urine was collected during the following time blocks: 0-6, 6-12, 12-24, and 24-48 h post sprout or supplement. Urine

volume was recorded and aliquots were frozen at -80 °C until analysis. Ten mL of whole blood were collected by ventipuncture into a vacutainer containing EDTA at the same time points urine samples were collected. In addition whole blood was also collected on the mornings of days 3, 4, 5, 6 and 10 (Figure 5.1). Phlebotomy was performed in the Nutrition and Exercise Sciences Department Clinical Collection Lab by a trained phlebotomist.

Peripheral blood mononuclear cell (PBMC) isolation

Approximately 10 mL of whole blood were carefully layered on 10 mL of room temperature Histopaque® 1077 (Sigma-Aldrich, St. Louis, MO). The blood was then centrifuged at 400 RCF for 30 minutes at room temperature. The PBMCs form a cloudy band at the interface between the Histopaque and the plasma. The PBMCs were collected and washed with PBS. Following the wash the PBMCs were centrifuged again at 300 RCF for 10 minutes at room temperature. To ensure no red cell contamination in the PBMCs, the cell pellet was resuspended in 1 mL of red cell lysis buffer (0.15 M NH_4Cl , 1.0 mM KHCO_3 and 0.1 mM EDTA) and allowed to sit on ice for 5 minutes. The cells were washed 2 more times with PBS. Cells were then lysed in IP lysis buffer with protease inhibitors and proteins were quantified using RC DC™ protein assay (Bio-Rad, Hercules, CA).

Glucobriferin, glucoraphanin and glucorucin were purchased from The Royal Veterinary School of Denmark. The glucosinolate quantitative method as described by Tian et al. (130) was used with some modification. Broccoli sprouts were frozen fresh in liquid nitrogen and stored at -80°C until analysis. Prior to extraction, frozen sprouts were steamed in a strainer above a pot of boiling water with a lid for five minutes to destroy endogenous myrosinase activity. Steamed sprouts were chopped finely and 5 g was extracted three times with 20 mL of 70 % (v/v) methanol/water, each time for 10 min at room temperature in a bath sonicator. A portion of the pooled extract was centrifuged, filtered through a 0.22 μm nylon syringe filter and diluted twenty fold with 0.1 % (v/v) formic acid in water prior to HPLC-MS/MS analysis as described by Tian et al. (130). Broccomax supplement capsules (0.7 g) were extracted

with 5 mL of 70% aqueous methanol three times. HPLC was conducted on an Acquity system (Waters Corp., Milford, MA) and eluent interfaced with a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, UK) for MS/MS analysis.

Preparation of isothiocyanate standards

Sulforaphane (SFN) and erucin (ERN) were purchased from LKT laboratories Inc (St. Paul, MN). SFN-cysteinylglycine (SFN-CG), ERN-glutathione (ERN-GSH), ERN-cysteinylglycine (ERN-CG), ERN-cysteine (ERN-Cys) and ERN-*N*-acetylcysteine (ERN-NAC) were prepared following methods described by Vermeulen et al. (141) in which isothiocyanates and their respective conjugate groups were reacted, to generate the various metabolites, e.g. SFN + CG for SFN-CG. The reaction mixtures were purified by semi-preparative reversed phase chromatography (10 x 250mm, 5 μ m C18 Bondapak, Waters Corp, Milford, MA) with a water/acetonitrile mobile phase. ACN in metabolite fractions was removed by Rotovap and the remaining aqueous phase freeze-dried to achieve a powder. Powders were weighed and extinction coefficients at appropriate wavelengths determined as the average of triplicate determinations. Each metabolite displayed spectra with two characteristic UV features at 250 and 270 nm. HPLC was performed and compounds were shown to be spectrally pure (all >95%). The extinction coefficients at 270 nm were 7551 SFN-Cys, 4796 SFN-CG, 7334 SFN-GSH, 6832 SFN-NAC, 2302 ERN-CG, 1609 ERN-Cys, 1653 ERN-GSH, 3391 ERN-NAC each in methanol.

Analysis of isothiocyanates

For sample preparation, urine was diluted 1:10 with 0.1 % (v/v) formic acid in water.

Plasma processing was slightly modified from that of Janobi et al. (84). Cold TFA (0°C) was added at 10 % (v/v) to plasma that had been pre-chilled on ice. After 5 minutes on ice the cloudy suspension was centrifuged at 16,000 x *g* for five min to pellet the proteins and recover metabolites in the supernatant. Supernatant was injected directly (10 μ L) for quantitative analysis.

UPLC chromatography was as follows: Acquity BEH C18 (2.1 x 100mm, 1.7 μ m) with a mobile phase of 0.1 % (v/v) formic acid in water versus 0.1 % (v/v) formic acid in acetonitrile at 0.45 mL/min at 40°C. Five μ L was injected onto the column. Initially the mobile phase was 0% B increased linearly to 10% B at 1 min, 33.3% B at 2.5 min, 72% B at 4 min (curve 7) and returned to 0% B by 6 min. HPLC eluent was interfaced without flow splitting to a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, UK) via an electrospray probe operated in positive mode. Selected reaction monitoring (SRM) MS/MS transitions were developed for each of nine analytes using collision induced dissociation (CID) – sulforaphane (178>114), SFN-GSH (485>136), SFN-CG (356>136), SFN-Cys (299>136), SFN-NAC (341>114), ERN-ERN (469>179), ERN-CG (340>103), ERN-Cys (283>103), and ERN-NAC (325>164) with dwell times of 80-150 ms. Source parameters included capillary 3.2 kV, desolvation temperature 450°C, cone voltage 35 V, RF1 12.5 V, collision energy (10-18 eV), and CID argon pressure (3×10^{-3} mBar). Reproducible chromatography and MS response could not be achieved with free ERN and thus it was omitted from the analysis.

HDAC activity assay

HDAC activity was assayed using the 386 well format Fluor-de-Lys HDAC activity kit (Upstate) as described in (37). Approximately 5 μ g of total PBMC lysate were added to each well and assay was performed according to the manufacturer's instructions. Fluorescence was measured and recorded as arbitrary fluorescence units (AFU) using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Repeated measures two-way ANOVA and Student's t-test were performed as indicated in the applicable tables and graphs.

5.4. Results

Twenty four subjects, 12 males and 12 females, were enrolled in the study. Subjects' gender, age and body mass index (BMI) are shown in Table 5.1. No significant differences were found in these subject characteristics between dietary groups. One male and one female subject were not included in further biochemical analyses due to issues with protocol compliance. Diet records indicate that the total intake and percent of required intake for calories, protein, carbohydrates and fats were similar between the broccoli sprout and broccoli supplement groups during the study period, although the percent of intake for protein was significantly higher in the sprout group (Table 5.2).

Three glucosinolates, glucoraphanin, glucoerucin and glucoiberin were quantified in the sprouts and supplement. Table 5.3 shows the total μmol of each glucosinolate that was received per consumption of 68 g of sprouts or 6 pills of supplement. Total glucosinolate content was equal between the broccoli sprout and Broccomax supplement intervention groups. Interestingly, even though the amounts of glucosinolates administered to each group was the same, there was a 5-fold lower amount of SFN metabolites and a 8-fold lower amount of ERN metabolites excreted in the urine during 24 hours after consumption in the supplement consumers compared to the sprout consumers (Table 5.4). There was no change or accumulation in the amount of metabolites excreted after repeated 7 –day consumption of sprouts or supplement (Table 5.4), which is consistent with previous reports (150). Although plasma samples were collected during this study we were unaware of the need to acidify the plasma in order to stabilize the metabolites as per Al Janobi et al (84). However, despite possible degradation of metabolites, we observed that the relative quantity of the metabolites in the plasma was also much lower in the supplement group as compared to the sprout group (data not shown), suggesting that differences in excretion concentrations between sprout and supplement groups were due to differences in uptake rather than retention.

A closer look at the time course within the 24 h following consumption of the intervention materials revealed that the timing of metabolite excretion was also

different between sprout and supplement groups. The peak SFN and ERN metabolite excretion rate was at 0-6 h post consumption for the sprout group, but at 6-12 h post consumption in the supplement group (Figure 5.2). This peak in excretion can also be observed when looking at the total μ moles of each metabolite over all time points collected (Figure 5.3). As expected SFN-NAC was the major metabolite followed by SFN-Cys, free SFN, SFN-CG and then SFN-GSH (Table 5.5 and Figure 5.2). Similarly, ERN-NAC was the most abundant of the ERN compounds followed by ERN-Cys, ERN-CG and then ERN-GSH (Table 5.5 and Figure 5.4). Although striking differences were observed in the total amounts and timing of SFN and ERN metabolites, the relative percent of each ITC metabolite within its respective group of metabolites was not drastically different between the two groups. For the ERN metabolites there was no difference in the percent of each metabolite between the two treatment groups (Table 5.5). Similar results were observed among the SFN metabolites although a small but significant shift in metabolite ratios was observed between the treatment groups, with a higher percent of SFN-Cys and lower percent of SFN-NAC observed in the sprout group compared to the supplement group (Table 5.5).

We also looked at the ratio of ERN-NAC to SFN-NAC that was excreted in the urine and found that, although the starting ratio of glucoerucin to glucoraphanin was approximately 1:3, all but two subjects had an increase in this ratio indicating that some SFN may have converted to ERN (Figure 5.5A). Within each subject the ratio was consistent at all time points of the study (see error bars in Figure 5.5A) and each subject had a unique ratio ranging from a dominance of SFN-NAC in one subject to a dominance of ERN-NAC in another subject, with a majority of subjects ($n=18$) having more SFN-NAC than ERN-NAC. There was no difference in the ratios between the treatment groups indicating that the source of the glucosinolates did not affect the conversion of SFN to ERN (Figure 5.5B). Several groups have reported this interconversion between SFN and ERN (51, 54, 145) and here we provide further evidence for this possibility.

HDAC activity in PBMCs was measured throughout the course of the study. Using repeated measures two-way ANOVA both treatment group and time have significant effects on HDAC activity (Figure 5.6). The level of HDAC activity in the supplement group was at or above baseline activity indicating that the lower *in vivo* concentrations had no inhibitory effect. In contrast the HDAC activity in the sprout group was below the baseline at most time points. At every time point tested the supplement group had higher HDAC activity than the sprout group although the difference was only statistically significant 12 and 48 h after the final dose of sprouts or supplement. These data provide further evidence that SFN can alter HDAC activity *in vivo*.

5.5. Discussion

The current study shows that subjects who consumed a broccoli supplement, which does not contain active myrosinase, have 5 times lower excretion amounts of SFN metabolites, 8 fold lower excretion amounts of ERN metabolites and a delayed peak excretion compared to subjects that consumed fresh broccoli sprouts (Table 5.4 and Figure 5.2). Although several studies have looked at the effect of inactive myrosinase on ITC bioavailability and excretion (52, 54, 55, 138, 149), this is the first study to directly compare the excretion of ITCs and bioactivity in subjects who consumed fresh broccoli sprouts to subjects who consumed a broccoli supplement.

Broccoli supplements do not have active myrosinase and therefore conversion of the glucosinolates to the isothiocyanates in the subjects from that group was primarily dependent on the myrosinase activity from the gut microbial flora. This conversion was not as effective as the conversion from the plant endogenous myrosinase but was sufficient to produce as much as 25 μ moles of SFN metabolites at the excretion peak (Figure 5.3A). A recent study showed that rats given glucoraphanin either by gavage or in the diet only recovered 27.6% and 23.8% of the dose, respectively, in the urine after 24 h. In contrast, recovery in rats fed a broccoli floret diet, which contained myrosinase was 62.5%. Interestingly, hepatic NQO1 activity was only induced in rats

fed the floret diet, not those fed glucosinolates without myrosinase, indicating that the induction of phase 2 enzymes, one bioactivity attributed to the ITCs, was lost when myrosinase was not present (149). Cooking broccoli also inactivates the myrosinase enzymes and it has been observed that the bioavailability of SFN is three to ten times greater from fresh broccoli compared to cooked broccoli (52, 138). Similar to our data, there is a delay in peak SFN plasma concentration with heat-inactivation of myrosinase in cooked broccoli (138). Another study showed that ITCs are six times more bioavailable than glucosinolates and chewing sprouts thoroughly to release the plant myrosinases rather than swallowing them intact increased the ITC excretion approximately two fold (55). Together these data confirm the importance of active myrosinases in increasing bioavailability of ITCs *in vivo*.

This is the first report to show detailed ERN metabolite data in human subjects using LC-MS/MS analysis. The metabolism and excretion of ERN metabolites were similar to SFN metabolites with respect to peak excretion times and a predominant N-acetylcysteine metabolite. One difference between ERN and SFN was in the percentage of each metabolite that was excreted. There was no change in the percentage of the various ERN metabolites between the treatment groups whereas subjects who consumed broccoli sprouts had an increase in the percent of SFN-Cys and a decrease in the percent of SFN-NAC metabolites excreted in the urine during 24 h post consumption as compared to the subjects who consumed the supplement (Table 5.5). This is consistent with the report by Gasper et al in which the treatment group that had the higher amount of SFN metabolites excreted in the urine had a slight and significant increase in the percentage of SFN-Cys and a slight but non-significant decrease in the percentage of SFN-NAC (60). In that study, the authors found that subjects who consumed three time more SFN metabolites (50 μ moles compared to 16 μ moles) had approximately three times higher peak plasma concentrations (7.3 μ mol/L compared to 2.3 μ mol/L). Although we could not accurately test the plasma concentration directly in our study, it is conceivable that the subjects who consumed broccoli sprouts could have 2-4 times higher plasma concentrations of SFN

metabolites than subjects who consumed 50 μ moles from Gasper et al since the subjects in our study consumed >300 μ moles of glucosinolates every day for 7 days, with approximately 220 μ moles of those glucosinolates being glucoraphanin, the precursor to SFN. Importantly, a phase I placebo controlled, double-blind, randomized clinical trial to assess toxicities of either glucosinolate (300 μ moles/day) or isothiocyanate (75 μ moles/day) preparations showed no toxicities or abnormal events occurred with any of the test extracts (67). Consistent with this, no adverse events were reported in our study indicating that the high dose of glucosinolates was well tolerated in our subjects.

Herein we provide further evidence for the possible interconversion between SFN and ERN and for the first time report that this interconversion is consistent within a single subject through a period of 10 days. This unique balance was variable between subjects and ranged from 43% to 80% SFN metabolites. This is significant because the starting ratio of glucoraphanin to erucin was 3:1, or 75% glucoraphanin and 25% glucoerucin, indicating that some of the SFN was converted ERN *in vivo*. Currently it is not known what drives the balance between oxidation of the sulfide in ERN and reduction of the sulfoxide in SFN, although one report indicates that ERN can be converted to SFN after reaction with hydroperoxides (151). Currently much less is known about the mechanism and bioactivity of ERN compared to SFN but due to their structural similarities it is expected that they act in a similar manner. There have been several reports that have looked into the bioactivity of ERN and found that indeed the bioactivity of these two compounds is similar but not identical. Multiple reports have indicated similar induction of phase II enzymes by ERN and SFN in rat lung (131), duodenum and urinary bladder (132), although some enzyme and tissue specificity for each compound was noted. Another study showed that both ERN and SFN induced phase III detoxification via induction of multidrug resistance pump 1 and 2 (MRP1 and MRP2, respectively), although SFN was substantially more potent than ERN (133). Another report indicated that ERN was able to inhibit proliferation and modulate p53 and p21 protein expression in human lung cancer A549 cells, but again

to a lesser degree than observed after SFN treatment (134). In contrast to these results, a study performed in Caco-2 colon cancer cells showed that ERN was substantially more effective at inducing G2/M cell cycle arrest, cell death, phase II enzymes and MRP2 (26). This may indicate that the potency of each compound may be dependent on the endpoint and/or cell line of interest. It has also been suggested that ERN can directly scavenge certain ROS and be converted to SFN (151). This possibility is intriguing because it would indicate that a higher amount of ERN would favor a modest induction of phase 2 enzymes by ERN itself as well as direct scavenging of ROS thereby forming SFN which is a more potent inducer phase 2 enzymes. More research is required to determine what drives the consistent interconversion of SFN and ERN within each individual and whether this interconversion is important for the chemopreventive effects of cruciferous vegetables.

Previously our group reported a preliminary human trial showing that consumption of broccoli sprouts can inhibit HDAC activity in PBMCs (66). Here we provide further evidence for this by showing that subjects consuming sprouts had higher urinary ITC levels and evidence of HDAC inhibition whereas subjects consuming the supplement had lower urinary ITC levels and did not demonstrate HDAC inhibition (Figure 5.6). Concerns have been raised regarding the impact of consuming dietary HDAC inhibitors on healthy individuals and a discussion regarding dietary HDAC inhibitors and their relative potencies compared to pharmacological HDAC inhibitors has been published (152). Importantly, here we show that the difference in HDAC activity between the groups was never greater than 30% and the decrease from baseline was never greater than 20%. This likely reflects the fact that the dietary HDAC inhibitors are weak ligands for the HDAC enzymes relative to pharmacological HDAC inhibitors. It still remains to be determined whether this change in human PBMC HDAC activity translates to changes in HDAC activity in specific tissues and whether it can influence chemoprevention.

In conclusion, we show that consumption of a broccoli supplement results in significantly lower amounts of ITC metabolites that are excreted in the urine which is

likely due to the lack of myrosinase enzymes in the supplement. This finding has significant implications for people who consume broccoli supplements and believe they are getting equivalent amounts of the bioactive ITCs as if they were consuming fresh broccoli sprouts. We also conclude that the interconversion between SFN and ERN is a consistent occurrence. Overall, these data provide further information regarding the metabolism and bioactivity of SFN and ERN in human subjects.

Acknowledgements:

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Table 5.1 Demographics of subjects

	Sprouts ¹		Supplement ¹	
	Age	BMI	Age	BMI
Male	27.8 ± 3.1	25.4 ± 1.6	28.8 ± 2.1	29.5 ± 1.5
Female	31.5 ± 5.7	21.7 ± 1.3	29.8 ± 3.9	22.5 ± 1.0

¹Mean ± SEM.

Table 5.2 Total caloric intake and macronutrient intake from 7 days of food records

	Sprouts ¹		Supplement ¹	
	Total intake	% required	Total intake	% required
Caloric (kcal)	2552 ± 172	102.1 ± 6.8	2484 ± 174	91.6 ± 5.3
Protein (g)	96.2 ± 7.1	171.9 ± 15.1	86.0 ± 7.7	131.3 ± 9.2 ²
Carbohydrate (g)	365.1 ± 21.3	106.3 ± 6.2	344.9 ± 23.1	93.0 ± 5.8
Fat (g)	77.3 ± 7.6	99.0 ± 9.0	85.2 ± 7.9	95.2 ± 11.8

¹Mean ± SEM²p<0.05, Student's t-test between percent required for sprouts and supplement groups

Table 5.3 Total μmol of glucosinolates per consumption of broccoli sprouts or broccoli supplement

Glucosinolate ¹	Sprouts ($\mu\text{mol}/68\text{g FW}^2$)	Supplement ($\mu\text{mol}/6$ pills)	t-test ³ p-value
Glucoraphanin	218.4 ± 24.4	220.3 ± 3.3	0.964
Glucoerucin	67.1 ± 8.5	75.9 ± 0.8	0.553
Glucoiberin	33.8 ± 4.0	31.7 ± 0.4	0.764

¹Mean \pm SEM

²Fresh weight

³Student's t-test between sprouts and supplement groups for each glucosinolate

Table 5.4 Total μmol of SFN compounds and ERN compounds excreted in urine during 24 h after consumption of broccoli sprouts or broccoli supplement

	Sprouts ¹		Supplement ¹	
Total μmol	Day 1:	Day 7:	Day 1:	Day 7:
	single dose	repeated dose	single dose	repeated dose
	(n=10 ²)	(n=10)	(n=11)	(n=11)
SFN ³	192 \pm 23	217 \pm 27	41.3 \pm 14.8	40.8 \pm 10.6
ERN ⁴	125 \pm 10	134 \pm 19	13.1 \pm 1.4	18.5 \pm 3.2

¹Mean \pm SEM. No significant dose number x treatment group interactions (repeated measures two-way ANOVA).

²n=10 because one subject did not have an entire urine collection at one time point and therefore could not be included in the two-way ANOVA.

³Significant treatment group, $p < 0.0001$ (repeated measures two-way ANOVA). Subject matching, $p < 0.0001$

⁴Significant treatment group, $p < 0.0001$ (repeated measures two-way ANOVA). Subject matching, $p = 0.0015$

Table 5.5 Percent each SFN compound and each ERN compound represent within each group of compounds excreted during 24 h (Day1 and Day7) after consumption of broccoli sprouts or broccoli supplement¹

	Sprouts	Supplement	
	% ³	%	P-value
SFN compounds			
SFN	7.8 ± 3.2	9.4 ± 4.3	0.3339
SFN-GSH	0.09 ± 0.23	0.01 ± 0.01	0.2914
SFN-CG	0.25 ±0.20	0.13 ± 0.09	0.0768
SFN-Cys	23.2 ± 7.2	16.1 ± 6.4	0.0231
SFN-NAC	68.7 ±6.8	74.4 ±5.7	0.0437
ERN compounds			
ERN-GSH	0.15 ± 0.47	0.02 ± 0.04	0.3913
ERN-CG	0.19 ± 0.42	0.001 ± 0.006	0.1641
ERN-Cys	18.4 ± 4.7	17.1 ± 6.7	0.6235
ERN-NAC	81.3 ± 4.6	82.8 ± 6.7	0.5363

¹Mean ± SEM.

²Student's t-test between sprouts and supplement groups for percent of each metabolite

³The percentage each compound represents within the its respective group of compounds (i.e. SFN compounds and ERN compounds)

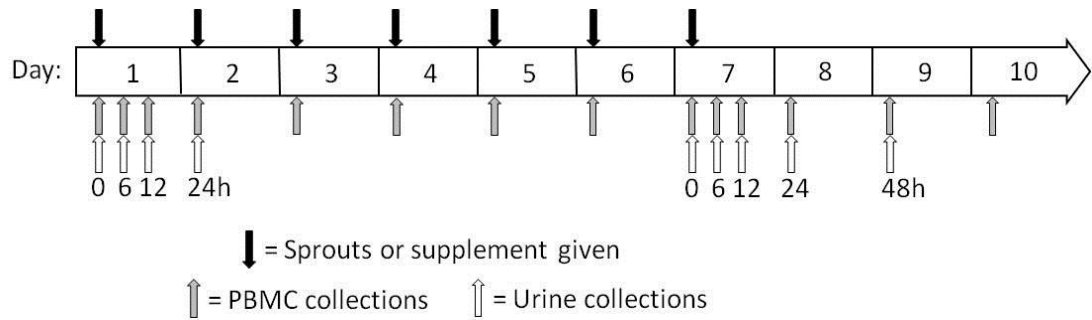


Figure 5.1 Study timeline showing when subjects received sprouts or supplement and when blood or urine were collected.

Black arrows indicate when subjects received sprouts or supplement, grey arrows indicate when blood was drawn and PBMCs were isolated and white arrows indicate when a complete urine collection was sampled.

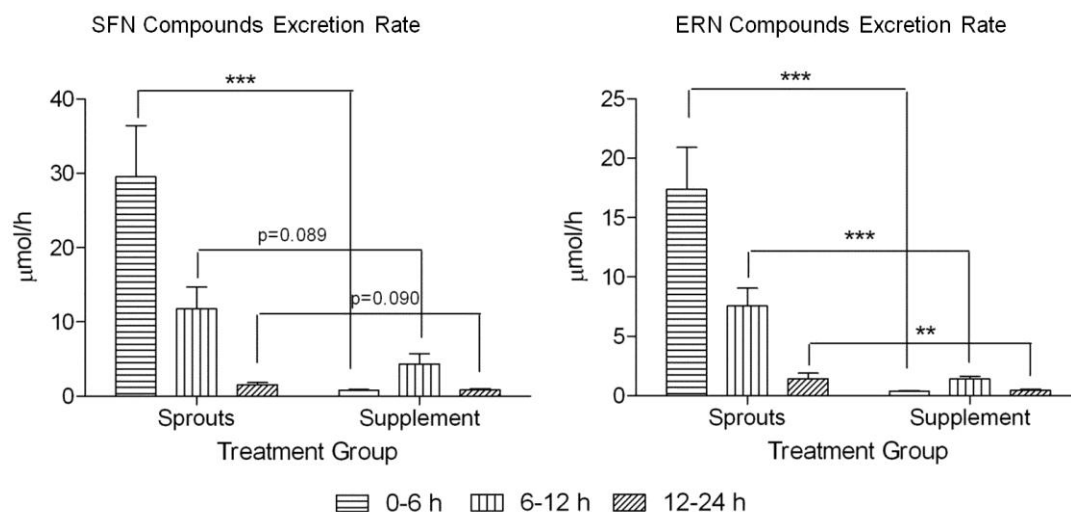


Figure 5.2 The peak in SFN and ERN metabolite excretion occurs earlier in subjects consuming sprouts compared to supplements.

The peak in ERN and SFN metabolite excretion occurred during the 0-6 h time block in subjects consuming sprouts and during the 6-12 h time block in subjects consuming supplement. Data in bar graphs represent mean \pm SEM (n=11). Statistical significance: **p<0.01, ***p<0.001 or p-value indicated using Student's t-test.

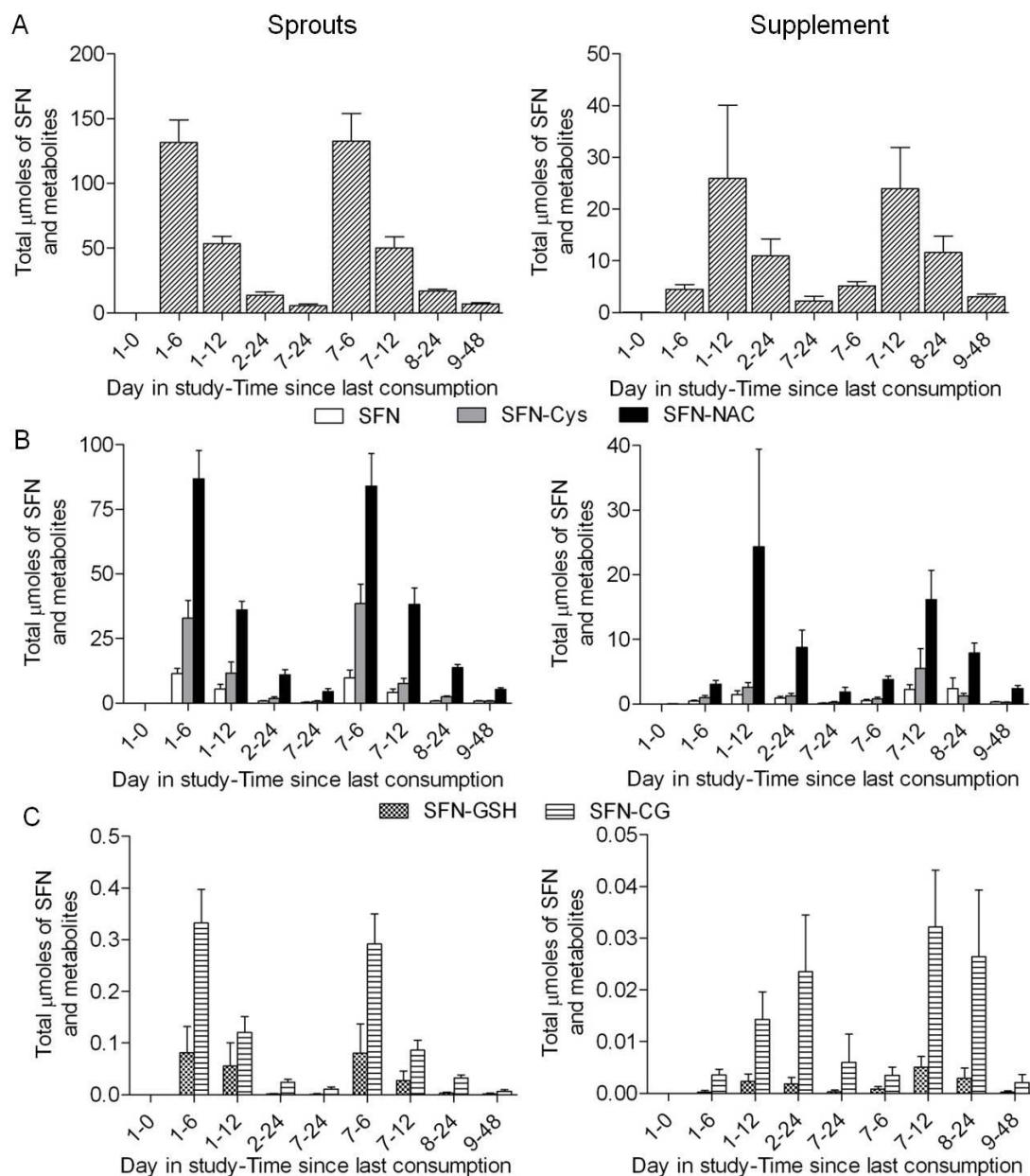


Figure 5.3 The μ moles of SFN and SFN metabolites excreted in the urine over the course of the study.

The time indicated is the time since last consumption of sprouts or supplement and complete urine was collected from the previous time point to the time point indicated. A) All SFN compounds summed for each time block. B) The major metabolites in the urine, SFN-NAC and SFN-Cys. C) The minor metabolites in the urine, SFN-CG and SFN-GSH. Data in bar graphs represent mean \pm SEM (n=11).

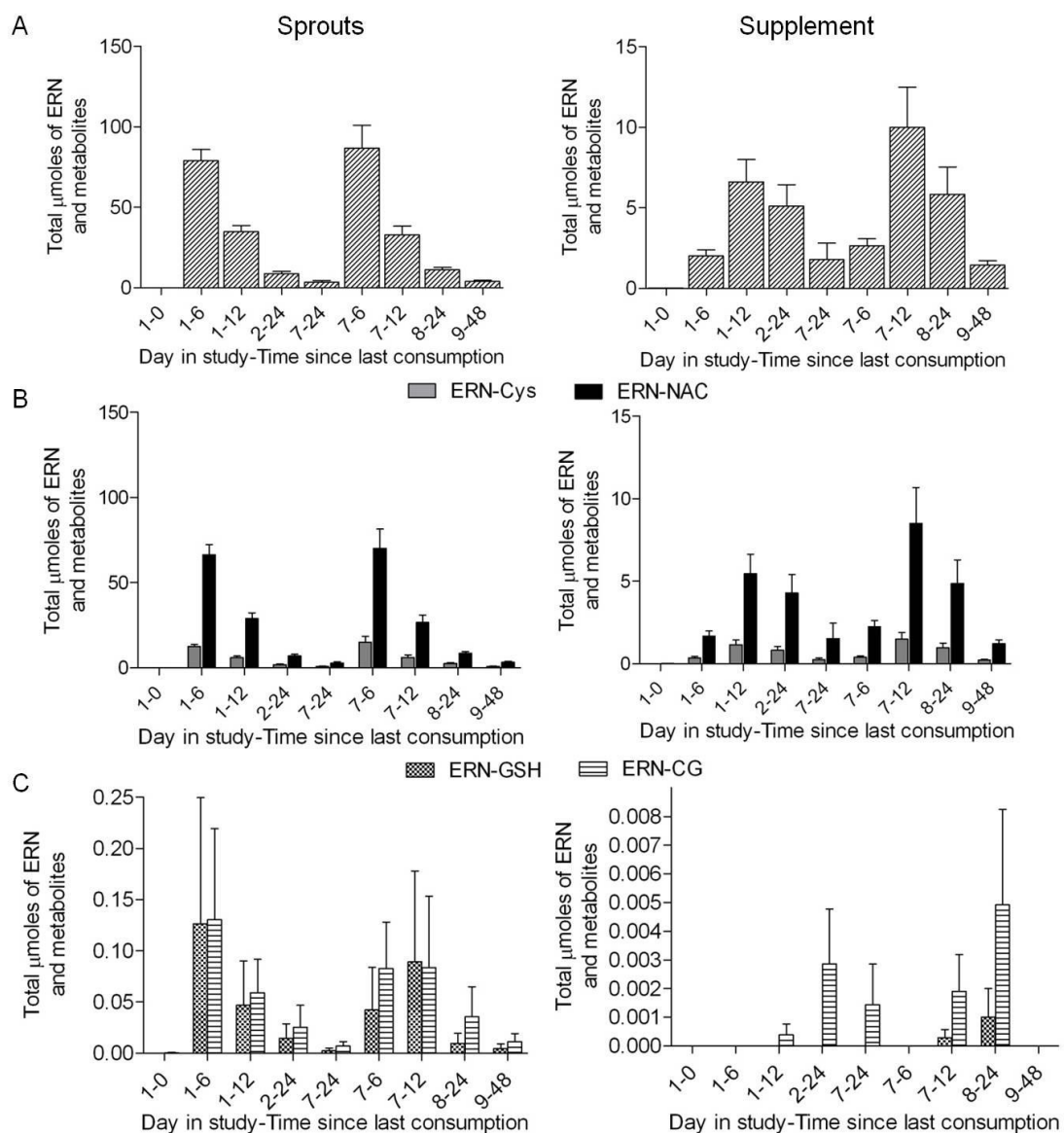


Figure 5.4 The μ moles of ERN metabolites excreted in the urine over the course of the study.

The time indicated is the time since last consumption of sprouts or supplement and complete urine was collected from the previous time point to the time point indicated. A) All ERN compounds summed for each time block. B) The major metabolites in the urine, ERN-NAC and ERN-Cys. C) The minor metabolites in the urine, ERN-CG and ERN-GSH. Data in bar graphs represent mean \pm SEM (n=11).

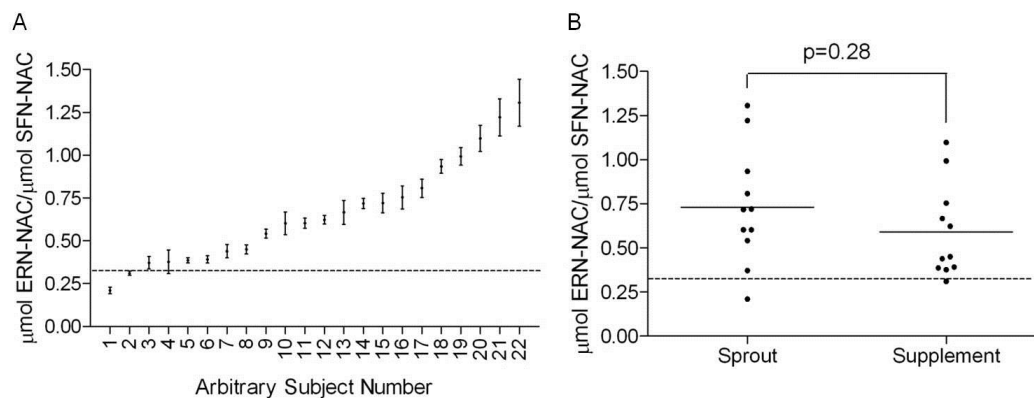


Figure 5.5 Shift in the ratio of ERN-NAC to SFN-NAC is subject dependent not treatment group dependent.

The dotted line in each graph represents the ratio of glucoerucin to glucoraphanin (in μmoles) each subject received in the sprouts or supplement (0.324 ± 0.009). A) The ratio of μmoles of ERN-NAC over μmoles of SFN-NAC for every subject. Each data point represents one subject and the subjects were ordered from lowest to highest ratio. Error bars represent SEM of all nine time points for that subject. B) Each data point represents all nine time points for one subject. No significant difference between treatment groups as determined by Student's t-test.

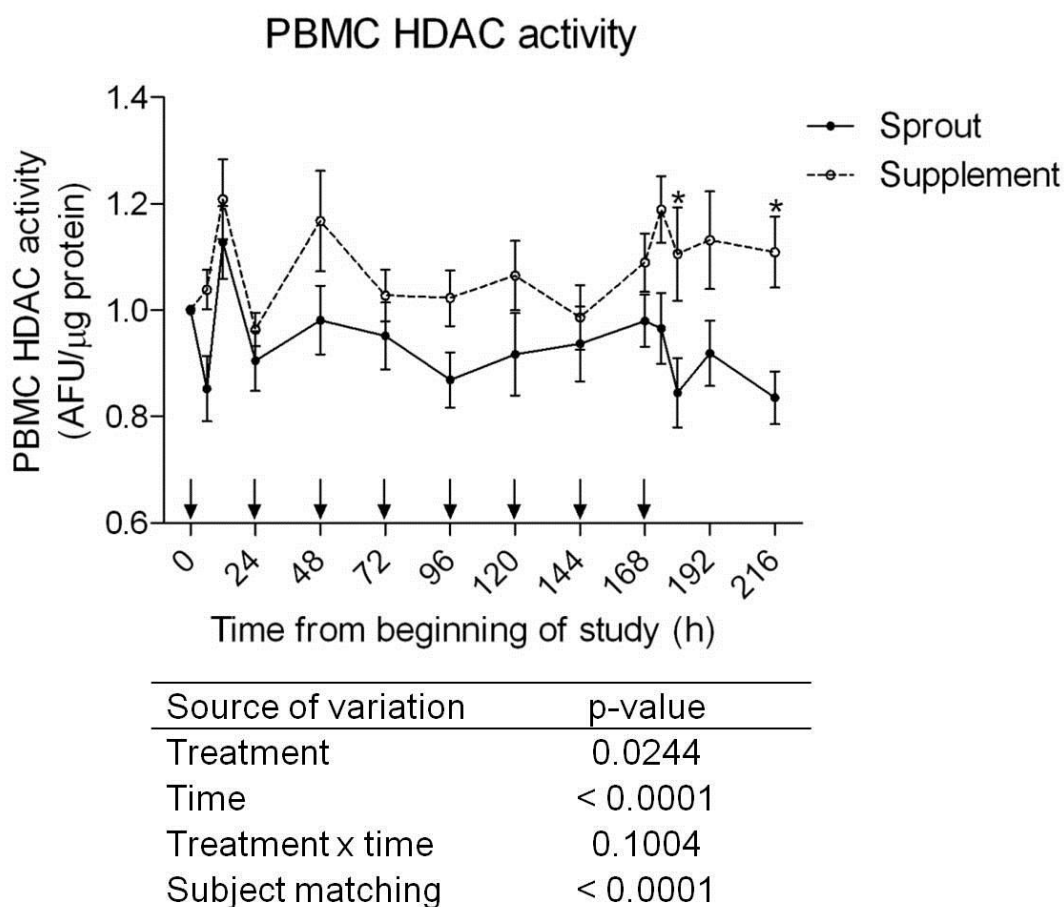


Figure 5.6 Subjects who consumed sprouts had lower HDAC activity in their PBMCs compared to subjects who consumed supplement.

Each subject was normalized to their baseline HDAC activity (time zero). Solid circles and lines represent the sprout group and open circle and dotted lines represent the supplement group. Arrows along the x-axis indicate when sprouts or supplement were consumed. Each data point represent mean \pm SEM (n=11). The data were analyzed by two-way ANOVA and p-values are shown below the graph.

Chapter 6 General conclusions

Prostate cancer is the second leading cause of cancer related death in American men, and it is estimated that one in six men will be diagnosed with prostate cancer in his lifetime. It has been estimated that one third of cancers could be prevented by changes in lifestyle, such as diet and physical activity. Indeed, the evidence to support cruciferous vegetables consumption to help prevent carcinogenesis in many tissues is growing and undeniable. Studies involving isothiocyanates (ITCs), the bioactive constituents derived from cruciferous vegetables, have shown promise in cell culture and rodent models of cancer. Sulforaphane (SFN) is one of the most studied ITCs and much is known regarding its “blocking” and “suppressing” chemoprevention mechanisms of action (See chapter 1). The transition of several nutritional compounds from experimental models to human clinical trials has been somewhat of a disappointment due to poor outcomes in many of the clinical trials. For example, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was stopped early because data indicated that selenium and vitamin E in combination or alone did not prevent prostate cancer. In fact, a slight, non-significant increase in prostate cancer risk was observed in men taking vitamin E alone (153). In many cases involving nutritional compounds, such as the SELECT study noted here, data on specific dose, supplement forms and compound toxicity had not sufficiently been studied, and may have contributed to the failure of these studies. Although there is strong evidence for chemoprevention of prostate cancer by SFN, a greater understanding of its effects on normal prostate cells and its metabolism, bioavailability and biodistribution within the body are critical to transition SFN from preclinical to clinical research. Thus, the focus of this dissertation was to characterize the effects of SFN on normal prostate cells and further characterize the metabolism and distribution of SFN *in vivo*.

An ideal anti-cancer agent is one that will target cancer cells while having minimal effects in normal cells and tissues. Pharmacological HDAC inhibitors appear to be selectively cytotoxic to cancer cells and data from the studies presented here provide evidence that SFN, a dietary HDAC inhibitor, has this same characteristic. In

human clinical trials, administration of SFN-rich products has no adverse effects. In a phase 1 clinical trial that utilized either ITCs or glucosinolates, the authors observed no adverse effects as indicated by numerous hematology and chemical tests commonly used to assess safety in humans (67). Likewise, in our studies with human subjects, no adverse side effects were reported from our subjects. Although reports from human studies are important for the whole organism aspect of toxicity, an analysis of events occurring at the molecular and cellular level are also required. Herein we report that a normal prostate epithelial cell line, PrEC, is refractory to the cytotoxic and epigenetic effects of SFN. Similarly, it recently was reported that normal colon cells were resistant to the cytotoxic effects of SFN (154). From these initial *in vitro* studies it is becoming clear that normal cells are more resistant than cancer cells to the cytotoxic effects of SFN. These data imply that consuming the recommended intake, or even a greater amount of cruciferous vegetables, similar to what was consumed in the phase 1 trial and our human studies, will have no detrimental effects in healthy populations.

The ability of SFN to modulate epigenetics, particularly histone acetylation that impacts tumor suppressor gene expression, has been postulated to be one of the chemopreventive mechanisms for SFN (66, 155). In this dissertation we further characterized the role of SFN in modulating histone deacetylase (HDAC) activity and impacting protein acetylation. Herein we report that SFN can decrease the expression of several specific class I and II HDACs, with subsequent changes in histone and non-histone protein acetylation. The family of trichostatin A sensitive (Class I and II) HDACs includes eleven different HDACs, and there is growing interest in developing HDAC inhibitors specific for particular subtypes, potentially producing more targeted and efficacious treatments. Herein we report that the expression of HDACs 3 and 6 were consistently reduced in cancer cells, indicating that SFN treatment may be affecting specific cellular processes either through changes in corepressor complexes and subsequently their activity on histones at gene promoters, or through non-histone related processes via altered protein stability and function. Although the mechanism that accounts for specific decreases in HDAC expression is unclear, this potential for

specificity in targeting the epigenome is intriguing because changes in the activity of these HDACs can potentially be linked to the cell death and growth arrest effects of SFN (See section 2.5). It still remains to be determined if SFN-Cys or SFN-NAC, the HDAC inhibitor forms of SFN, have HDAC subtype specificity. As mentioned above, we also show that the cytotoxic and epigenetic effects of SFN were only apparent in the cancer cells, not the normal cells. Furthermore, we show that consumption of broccoli sprouts, a food rich in glucoraphanin the precursor to SFN, results in high concentrations of SFN metabolites in the plasma of healthy human subjects and inhibition of HDAC activity in their peripheral blood mononuclear cells (PBMCs). We also show in this dissertation that a broccoli sprout powder given in the diet of mice reduced the severity of prostate cancer compared to controls in the transgenic adenoma of the mouse prostate (TRAMP) model for prostate cancer. Future analysis of epigenetic marks in prostates from these mice will be performed. Importantly, histone acetylation is only one of many different epigenetic marks that can occur on chromatin and influence gene transcription. Indeed, it has been shown that DNA methylation is associated with gene silencing because corepressor complexes that contain HDAC proteins can bind to methylated DNA and contribute to gene silencing (156). Currently, several labs at Oregon State University, including our lab, are exploring how SFN treatment affects DNA methylation and other epigenetic pathways. These data support the role of SFN in counteracting aberrant epigenetic changes that occur in cancer cells, inducing cancer cell death and slowing the progression of prostate cancer.

Bioavailability is one of the critical factors that can influence drug/nutrient targeting and bioactivity in the body. The bioavailability of ITCs can be affected by factors involving the food source, such as plant variety, cultivation and processing, and it can potentially be affected by heterogeneity in metabolism enzymes in the human population. In order for SFN to be efficacious as a chemopreventive agent *in vivo*, first it must be formed by myrosinase mediated hydrolysis of glucoraphanin. Myrosinase is not present in mammalian cells and therefore hydrolysis of

glucoraphanin is dependent on endogenous myrosinase activity in cruciferous vegetables and/or myrosinase activity from the gut microbial flora. It has been reported that myrosinase is heat labile and inactivated by cooking cruciferous vegetables, resulting in lower bioavailability of ITCs (52, 138, 149). Interestingly, commercially available broccoli supplements do not contain myrosinase and therefore it is hypothesized that ITCs will be less bioavailable from supplements. Herein we report that ITCs are far less bioavailable from broccoli supplement, with up to 12 fold lower plasma concentrations compared to subjects who consume an equivalent dose of glucosinolates from fresh broccoli sprouts. Importantly, because there is no myrosinase activity in the supplements, the hydrolysis of glucosinolates to ITCs was likely carried out by the myrosinase activity from the gut microbial flora resulting in lower bioavailability and delayed peak concentrations. This is significant for consumers who believe taking the recommended daily dose of supplement will deliver equal or higher concentrations of ITCs compared to the whole food. Also, variability in glucosinolate metabolism by intestinal bacteria could potentially impact the bioavailability of the ITCs in situations where myrosinase is inactive or not present, such as in cooked cruciferous vegetables or supplements. From these data it is clear that the presence of myrosinase in the whole food affords greater bioavailability of ITCs compared to the supplement which does not contain myrosinase.

Once SFN has been formed by myrosinase mediated hydrolysis it is metabolized through the mercapturic acid pathway to form, in sequence: SFN-glutathione (SFN-GSH), SFN-cysteinyl-glycine (SFN-CG), SFN-cysteine (SFN-Cys) and SFN-*N*-acetylcysteine (SFN-NAC). As mentioned above, the bioavailability and bioactivity of ITCs can potentially be affected by polymorphisms in xenobiotic metabolism enzymes, such as the glutathione-*S*-transferase enzymes (GSTs). Mixed results have been reported regarding the influence of GST polymorphism on ITC metabolism (see section 1.4), and herein we show that GSTP1 polymorphism does not affect SFN or ERN metabolism and excretion in human subjects. Importantly, there are multiple polymorphisms that can occur in the various GSTs that can potentially

influence bioavailability; therefore larger studies considering multiple GSTs are merited. Due to the conflicting data on GST polymorphisms and ITC bioavailability, it is likely that factors such as presence/absence of myrosinase and differences in intestinal bacteria will have a greater impact on the bioavailability of ITCs. Again, further studies regarding these factors are an important area for future research.

Differences in bioactivity between free ITCs and their metabolites have been considered by some researchers, and therefore it is possible that the metabolism of these bioactive compounds is a major factor in determining specific biological effects. In support of that idea, it has been reported that SFN-Cys and SFN-NAC are competitive inhibitors of the HDAC enzymes (37). This contributes to the epigenetic effects reported previously (29, 39, 66) and reported in this dissertation. In contrast, it has been postulated that the ITC thiol conjugates can be considered prodrugs of the parent compound (115), because ITC thiol conjugates can dissociate into free ITCs under physiological conditions (114). Several studies have shown similar efficacy from either free ITC or the *N*-acetylcysteine (NAC) conjugated ITC in cancer cells and in rodent cancer models (24, 65, 115-118), although it is unknown which ITC compounds were most abundant in the different treatment groups and therefore unclear which form is truly exerting the bioactive effects. In this dissertation we show that mice given an oral dose of SFN have predominately SFN-GSH, SFN-Cys, and SFN-NAC, present in plasma and many other tissues, with some free SFN present in some tissues. Furthermore, from previous reports and from work presented here it has been shown that SFN-GSH and SFN-Cys are the predominant intracellular metabolites *in vitro* (105, 157, 158). In our human studies we report that the cysteinyl-glycine conjugate was the most abundant form of SFN and erucin (ERN) in the plasma and the NAC conjugates were the most abundant forms in the urine. We also show that in mice SFN and its metabolites are distributed throughout the body and, importantly, reach the prostate. In fact, concentrations in the prostate were second highest of all tissues measured in our experiments, indicating that SFN may be particularly active in the prostate. We also show that SFN metabolites are present in SI, colon, lung, liver,

kidney and brain. These data provide insight into the most abundant intracellular and *in vivo* forms of SFN and potentially have implications for future research directions and formulation of more bioavailable and bioactive chemical forms of ITCs.

In conclusion, this dissertation supports the role of SFN in prostate cancer prevention. Through its “blocking” and “suppressing” mechanisms, SFN has potential to reduce the morbidity and mortality associated with many cancers, including prostate cancer. The ability of SFN to target the aberrant epigenetic modifications that occur in cancer cells along with the relative resistance of normal prostate epithelial cells to the cytotoxic and epigenetic effects of SFN, make SFN an ideal means to prevent prostate cancer. By identifying the most abundant chemical forms of SFN within the cell and *in vivo* we provide necessary information to aid in bringing SFN from the lab into the clinic. SFN is an ideal chemopreventive agent because it can be easily utilized in the diet via consumption of a whole food that contains many other health beneficial constituents. It is possible that synergism among bioactive constituent in the whole food will potentiate the chemopreventive effects of SFN while reducing the toxicity often associated with high doses of isolated compounds. Furthermore, these data strongly support advancing SFN, not only as a chemopreventive agent in the diet, but also as a potential therapeutic agent in cancer clinical trials. Taken together, these data provide strong contributions to the scientific literature regarding cancer prevention with SFN and open many new research opportunities for the future.

Bibliography

1. Joseph MA, Moysich KB, Freudenheim JL, Shields PG, Bowman ED, Zhang Y, et al. Cruciferous Vegetables, Genetic Polymorphisms in Glutathione S-Transferases M1 and T1, and Prostate Cancer Risk. *Nutr Cancer*. 2004; 50(2):206-213.
2. Lin HJ, Probst-Hensch NM, Louie AD, Kau IH, Witte JS, Ingles SA, et al. Glutathione Transferase Null Genotype, Broccoli, and Lower Prevalence of Colorectal Adenomas. *Cancer Epidemiol Biomarkers Prev*. 1998; 7(8):647-652.
3. Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA, et al. Variation of Glucosinolates in Vegetable Crops of Brassica Oleracea. *J Agric Food Chem*. 1999; 47(4):1541-1548.
4. Mithen R. Glucosinolates – Biochemistry, Genetics and Biological Activity. *Plant Growth Regul*. 2001; 34(1):91-103.
5. Zhang Y, Talalay P, Cho CG, and Posner GH. A Major Inducer of Anticarcinogenic Protective Enzymes from Broccoli: Isolation and Elucidation of Structure. *Proc Natl Acad Sci U S A*. 1992; 89(6):2399-2403.
6. Higdon JV, Delage B, Williams DE, and Dashwood RH. Cruciferous Vegetables and Human Cancer Risk: Epidemiologic Evidence and Mechanistic Basis. *Pharmacol Res*. 2007; 55(3):224-236.
7. Juge N, Mithen RF, and Traka M. Molecular Basis for Chemoprevention by Sulforaphane: A Comprehensive Review. *Cell Mol Life Sci*. 2007; 64(9):1105-1127.
8. Zhang Y, Talalay P, Cho CG, and Posner GH. A Major Inducer of Anticarcinogenic Protective Enzymes from Broccoli: Isolation and Elucidation of Structure. *Proc Natl Acad Sci U S A*. 1992; 89(6):2399-2403.
9. Maheo K, Morel F, Langouet S, Kramer H, Le Ferrec E, Ketterer B, 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):3649-3652.
10. Myzak MC and Dashwood RH. Chemoprotection by Sulforaphane: Keep One Eye Beyond Keap1. *Cancer Lett*. 2006; 233(2):208-218.
11. Brooks JD, Paton VG, and Vidanes G. Potent Induction of Phase 2 Enzymes in Human Prostate Cells by Sulforaphane. *Cancer Epidemiol Biomarkers Prev*. 2001; 10(9):949-954.
12. Keck AS, Qiao Q, and Jeffery EH. Food Matrix Effects on Bioactivity of Broccoli-Derived Sulforaphane in Liver and Colon of F344 Rats. *J Agric Food Chem*. 2003; 51(11):3320-3327.
13. Jones SB and Brooks JD. Modest Induction of Phase 2 Enzyme Activity in the F-344 Rat Prostate. *BMC cancer*. 2006; 6:62.
14. Petri N, Tannergren C, Holst B, Mellon FA, Bao Y, Plumb GW, et al. Absorption/Metabolism of Sulforaphane and Quercetin, and Regulation of Phase II Enzymes, in Human Jejunum *in Vivo*. *Drug Metab Dispos*. 2003; 31(6):805-813.
15. Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, and Biswal S. Identification of Nrf2-Regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray. *Cancer Res*. 2002; 62(18):5196-5203.
16. Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, 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):170-192.
17. McWalter GK, Higgins LG, McLellan LI, Henderson CJ, Song L, Thornalley PJ, 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):3499S-3506S.
 18. Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, Kamath R, et al. Sulforaphane-Induced G2/M Phase Cell Cycle Arrest Involves Checkpoint Kinase 2-Mediated Phosphorylation of Cell Division Cycle 25c. *J Biol Chem.* 2004; 279(24):25813-25822.
 19. Herman-Antosiewicz A, Xiao H, Lew KL, and Singh SV. Induction of P21 Protein Protects against Sulforaphane-Induced Mitotic Arrest in Lncap Human Prostate Cancer Cell Line. *Mol Cancer Ther.* 2007; 6(5):1673-1681.
 20. Cho SD, Li G, Hu H, Jiang C, Kang KS, Lee YS, et al. Involvement of C-Jun N-Terminal Kinase in G2/M Arrest and Caspase-Mediated Apoptosis Induced by Sulforaphane in Du145 Prostate Cancer Cells. *Nutr Cancer.* 2005; 52(2):213-224.
 21. Parnaud G, Li P, Cassar G, Rouimi P, Tulliez J, Combaret L, et al. Mechanism of Sulforaphane-Induced Cell Cycle Arrest and Apoptosis in Human Colon Cancer Cells. *Nutr Cancer.* 2004; 48(2):198-206.
 22. Gamet-Payraastre L, Li P, Lumeau S, Cassar G, Dupont M-A, Chevolleau S, et al. Sulforaphane, a Naturally Occurring Isothiocyanate, Induces Cell Cycle Arrest and Apoptosis in Ht29 Human Colon Cancer Cells. *Cancer Res.* 2000; 60(5):1426-1433.
 23. Shen G, Xu C, Chen C, Hebbar V, and Kong A-N. P53-Independent G1 Cell Cycle Arrest of Human Colon Carcinoma Cells Ht-29 by Sulforaphane Is Associated with Induction of P21cip1 and Inhibition of Expression of Cyclin D1. *Cancer Chemother Pharmacol.* 2006; 57(3):317-327.
 24. Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A, and Conaway CC. Sulforaphane and Its Metabolite Mediate Growth Arrest and Apoptosis in Human Prostate Cancer Cells. *Int J Oncol.* 2002; 20(3):631-636.
 25. Wang L, Liu D, Ahmed T, Chung FL, Conaway C, and Chiao JW. Targeting Cell Cycle Machinery as a Molecular Mechanism of Sulforaphane in Prostate Cancer Prevention. *Int J Oncol.* 2004; 24(1):187-192.
 26. Jakubikova J, Sedlak J, Mithen R, and Bao Y. Role of Pi3k/Akt and Mek/Erk Signaling Pathways in Sulforaphane- and Erucin-Induced Phase II Enzymes and Mrp2 Transcription, G2/M Arrest and Cell Death in Caco-2 Cells. *Biochem Pharmacol.* 2005; 69(11):1543-1552.
 27. Pappa G, Bartsch H, and Gerhäuser C. Biphasic Modulation of Cell Proliferation by Sulforaphane at Physiologically Relevant Exposure Times in a Human Colon Cancer Cell Line. *Mol Nutr Food Res.* 2007; 51(8):977-984.
 28. Traka M, Gasper AV, Smith JA, Hawkey CJ, Bao Y, and Mithen RF. Transcriptome Analysis of Human Colon Caco-2 Cells Exposed to Sulforaphane. *J Nutr.* 2005; 135(8):1865-1872.
 29. Myzak MC, Hardin K, Wang R, Dashwood RH, and Ho E. Sulforaphane Inhibits Histone Deacetylase Activity in Bph-1, Lncap and Pc-3 Prostate Epithelial Cells. *Carcinogenesis.* 2006; 27(4):811-819.

30. Pappa G, Lichtenberg M, Iori R, Barillari J, Bartsch H, and Gerhauser C. Comparison of Growth Inhibition Profiles and Mechanisms of Apoptosis Induction in Human Colon Cancer Cell Lines by Isothiocyanates and Indoles from Brassicaceae. *Mutat Res.* 2006; 599(1-2):76-87.
31. Singh AV, Xiao D, Lew KL, Dhir R, and Singh SV. Sulforaphane Induces Caspase-Mediated Apoptosis in Cultured Pc-3 Human Prostate Cancer Cells and Retards Growth of Pc-3 Xenografts in Vivo. *Carcinogenesis.* 2004; 25(1):83-90.
32. Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, and Robson CN. Upregulation and Nuclear Recruitment of Hdac1 in Hormone Refractory Prostate Cancer. *Prostate.* 2004; 59(2):177-189.
33. Huffman DM, Grizzle WE, Bamman MM, Kim J-s, Eltoum IA, Elgavish A, et al. Sirt1 Is Significantly Elevated in Mouse and Human Prostate Cancer. *Cancer Res.* 2007; 67(14):6612-6618.
34. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, et al. Global Histone Modification Patterns Predict Risk of Prostate Cancer Recurrence. *Nature.* 2005; 435(7046):1262-1266.
35. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen K-P, and Gottlicher M. Induction of Hdac2 Expression Upon Loss of Apc in Colorectal Tumorigenesis. *Cancer Cell.* 2004; 5(5):455-463.
36. Johnstone RW. Histone-Deacetylase Inhibitors: Novel Drugs for the Treatment of Cancer. *Nat Rev Drug Discov.* 2002; 1(4):287-299.
37. Myzak MC, Karplus PA, Chung FL, and Dashwood RH. A Novel Mechanism of Chemoprotection by Sulforaphane: Inhibition of Histone Deacetylase. *Cancer Res.* 2004; 64(16):5767-5774.
38. Dashwood RH and Ho E. Dietary Histone Deacetylase Inhibitors: From Cells to Mice to Man. *Semin Cancer Biol.* 2007; 17(5):363-369.
39. Myzak MC, Dashwood WM, Orner GA, Ho E, and Dashwood RH. Sulforaphane Inhibits Histone Deacetylase in Vivo and Suppresses Tumorigenesis in Apc-Minus Mice. *Faseb J.* 2006; 20(3):506-508.
40. Jeong WS, Kim IW, Hu R, and Kong AN. Modulation of Ap-1 by Natural Chemopreventive Compounds in Human Colon Ht-29 Cancer Cell Line. *Pharm Res.* 2004; 21(4):649-660.
41. Xu C, Shen G, Yuan X, Kim JH, Gopalkrishnan A, Keum YS, et al. Erk and Jnk Signaling Pathways Are Involved in the Regulation of Activator Protein 1 and Cell Death Elicited by Three Isothiocyanates in Human Prostate Cancer Pc-3 Cells. *Carcinogenesis.* 2006; 27(3):437-445.
42. Keum Y-S, Yu S, Chang PP-J, Yuan X, Kim J-H, Xu C, 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):8804-8813.
43. Rayet B and Gelinas C. Aberrant Rel/Nfkb Genes and Activity in Human Cancer. *Oncogene.* 1999; 18(49):6938-6947.
44. Baldwin AS. Control of Oncogenesis and Cancer Therapy Resistance by the Transcription Factor Nf-Kappa-B. *J Clin Invest.* 2001; 107(3):241-246.

45. Choi S, Lew KL, Xiao H, Herman-Antosiewicz A, Xiao D, Brown CK, et al. D,L-Sulforaphane-Induced Cell Death in Human Prostate Cancer Cells Is Regulated by Inhibitor of Apoptosis Family Proteins and Apaf-1. *Carcinogenesis*. 2007; 28(1):151-162.
46. Xu C, Shen G, Chen C, Gelinas C, and Kong AN. Suppression of Nf-Kappa-B and Nf-Kappa-B-Regulated Gene Expression by Sulforaphane and Peitc through Ikkappa, Ikk Pathway in Human Prostate Cancer Pc-3 Cells. *Oncogene*. 2005; 24(28):4486-4495.
47. Jeong WS, Kim IW, Hu R, and Kong AN. Modulatory Properties of Various Natural Chemopreventive Agents on the Activation of Nf-Kappa-B Signaling Pathway. *Pharm Res*. 2004; 21(4):661-670.
48. Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, et al. Sulforaphane-Induced Cell Death in Human Prostate Cancer Cells Is Initiated by Reactive Oxygen Species. *J Biol Chem*. 2005; 280(20):19911-19924.
49. Herman-Antosiewicz A, Johnson DE, and Singh SV. Sulforaphane Causes Autophagy to Inhibit Release of Cytochrome C and Apoptosis in Human Prostate Cancer Cells. *Cancer Res*. 2006; 66(11):5828-5835.
50. Bones AM and Rossiter JT. The Myrosinase-Glucosinolate System, Its Organisation and Biochemistry. *Physiol Plant*. 1996; 97(194-208).
51. Kassahun K, Davis M, Hu P, Martin B, and Baillie T. Biotransformation of the Naturally Occurring Isothiocyanate Sulforaphane in the Rat: Identification of Phase I Metabolites and Glutathione Conjugates. *Chem Res Toxicol*. 1997; 10(11):1228-1233.
52. Conaway CC, Getahun SM, Liebes LL, Pusateri DJ, Topham DKW, Botero-Omary M, et al. Disposition of Glucosinolates and Sulforaphane in Humans after Ingestion of Steamed and Fresh Broccoli. *Nutr Cancer*. 2000; 38(2):168-178.
53. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, and Talalay P. Human Metabolism and Excretion of Cancer Chemoprotective Glucosinolates and Isothiocyanates of Cruciferous Vegetables. *Cancer Epidemiol Biomarkers Prev*. 1998; 7(12):1091-1100.
54. Bheemreddy RM and Jeffery EH. The Metabolic Fate of Purified Glucoraphanin in F344 Rats. *J Agric Food Chem*. 2007; 55(8):2861-2866.
55. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, and Talalay P. Chemoprotective Glucosinolates and Isothiocyanates of Broccoli Sprouts: Metabolism and Excretion in Humans. *Cancer Epidemiol Biomarkers Prev*. 2001; 10(5):501-508.
56. Seow A, Vainio H, and Yu MC. Effect of Glutathione-S-Transferase Polymorphisms on the Cancer Preventive Potential of Isothiocyanates: An Epidemiological Perspective. *Mutat Res*. 2005; 592(1-2):58-67.
57. Lampe JW and Peterson S. Brassica, Biotransformation and Cancer Risk: Genetic Polymorphisms Alter the Preventive Effects of Cruciferous Vegetables. *J Nutr*. 2002; 132(10):2991-2994.
58. Seow A, Shi CY, Chung FL, Jiao D, Hankin JH, Lee HP, et al. Urinary Total Isothiocyanate (Itc) in a Population-Based Sample of Middle-Aged and Older Chinese in Singapore: Relationship with Dietary Total Itc and Glutathione S-Transferase M1/T1/P1 Genotypes. *Cancer Epidemiol Biomarkers Prev*. 1998; 7(9):775-781.

59. Seow A, Yuan JM, Sun CL, Van Den Berg D, Lee HP, and Yu MC. Dietary Isothiocyanates, Glutathione S-Transferase Polymorphisms and Colorectal Cancer Risk in the Singapore Chinese Health Study. *Carcinogenesis*. 2002; 23(12):2055-2061.
60. Gasper AV, Al-Janobi A, Smith JA, Bacon JR, Fortun P, Atherton C, et al. Glutathione S-Transferase M1 Polymorphism and Metabolism of Sulforaphane from Standard and High-Glucosinolate Broccoli. *Am J Clin Nutr*. 2005; 82(6):1283-1291.
61. Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, et al. In Vivo Pharmacokinetics and Regulation of Gene Expression Profiles by Isothiocyanate Sulforaphane in the Rat. *J Pharmacol Exp Ther*. 2004; 310(1):263-271.
62. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, and Talalay P. Quantitative Determination of Dithiocarbamates in Human Plasma, Serum, Erythrocytes and Urine: Pharmacokinetics of Broccoli Sprout Isothiocyanates in Humans. *Clin Chim Acta*. 2002; 316(1-2):43-53.
63. Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, et al. Preclinical and Clinical Evaluation of Sulforaphane for Chemoprevention in the Breast. *Carcinogenesis*. 2007; 28(7):1485-1490.
64. Hu R, Khor TO, Shen G, Jeong WS, Hebbar V, Chen C, et al. Cancer Chemoprevention of Intestinal Polyposis in Apcmin/+ Mice by Sulforaphane, a Natural Product Derived from Cruciferous Vegetable. *Carcinogenesis*. 2006; 27(10):2038-2046.
65. Chung FL, Conaway CC, Rao CV, and Reddy BS. Chemoprevention of Colonic Aberrant Crypt Foci in Fischer Rats by Sulforaphane and Phenethyl Isothiocyanate. *Carcinogenesis*. 2000; 21(12):2287-2291.
66. Myzak MC, Tong P, Dashwood WM, Dashwood RH, and Ho E. Sulforaphane Retards the Growth of Human Pc-3 Xenografts and Inhibits Hdac Activity in Human Subjects. *Exp Biol Med*. 2007; 232(2):227-234.
67. Shapiro TA, Fahey JW, Dinkova-Kostova AT, Holtzclaw WD, Stephenson KK, Wade KL, et al. Safety, Tolerance, and Metabolism of Broccoli Sprout Glucosinolates and Isothiocyanates: A Clinical Phase I Study. *Nutr Cancer*. 2006; 55(1):53-62.
68. Kensler TW, Chen J-G, Egnor PA, Fahey JW, Jacobson LP, Stephenson KK, et al. Effects of Glucosinolate-Rich Broccoli Sprouts on Urinary Levels of Aflatoxin-DNA Adducts and Phenanthrene Tetraols in a Randomized Clinical Trial in He Zuo Township, Qidong, People's Republic of China. *Cancer Epidemiol Biomarkers Prev*. 2005; 14(11):2605-2613.
69. Kristal AR and Lampe JW. Brassica Vegetables and Prostate Cancer Risk: A Review of the Epidemiological Evidence. *Nutr Cancer*. 2002; 42(1):1-9.
70. Fahey JW, Zhang Y, and Talalay P. Broccoli Sprouts: An Exceptionally Rich Source of Inducers of Enzymes That Protect against Chemical Carcinogens. *Proc Natl Acad Sci USA*. 1997; 94(19):10367-10372.
71. Haberland M, Montgomery RL, and Olson EN. The Many Roles of Histone Deacetylases in Development and Physiology: Implications for Disease and Therapy. *Nat Rev Genet*. 2009; 10(1):32-42.
72. Yang XJ and Seto E. Hats and Hdacs: From Structure, Function and Regulation to Novel Strategies for Therapy and Prevention. *Oncogene*. 2007; 26(37):5310-5318.
73. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, et al. Hdac6 Is a Microtubule-Associated Deacetylase. *Nature*. 2002; 417(6887):455-458.

74. Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, et al. In Vivo Destabilization of Dynamic Microtubules by Hdac6-Mediated Deacetylation. *Embo J*. 2002; 21(24):6820-6831.
75. Boyault C, Sadoul K, Pabion M, and Khochbin S. Hdac6, at the Crossroads between Cytoskeleton and Cell Signaling by Acetylation and Ubiquitination. *Oncogene*. 2007; 26(37):5468-5476.
76. Korkmaz CG, Fronsdal K, Zhang Y, Lorenzo PI, and Saatcioglu F. Potentiation of Androgen Receptor Transcriptional Activity by Inhibition of Histone Deacetylation--Rescue of Transcriptionally Compromised Mutants. *J Endocrinol*. 2004; 182(3):377-389.
77. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, et al. Suberoylanilide Hydroxamic Acid, an Inhibitor of Histone Deacetylase, Suppresses the Growth of Prostate Cancer Cells in Vitro and in Vivo. *Cancer Res*. 2000; 60(18):5165-5170.
78. Xu WS, Parmigiani RB, and Marks PA. Histone Deacetylase Inhibitors: Molecular Mechanisms of Action. *Oncogene*. 2007; 26(37):5541-5552.
79. Dokmanovic M, Perez G, Xu W, Ngo L, Clarke C, Parmigiani RB, et al. Histone Deacetylase Inhibitors Selectively Suppress Expression of Hdac7. *Mol Cancer Ther*. 2007; 6(9):2525-2534.
80. Ungerstedt JS, Sowa Y, Xu WS, Shao Y, Dokmanovic M, Perez G, et al. Role of Thioredoxin in the Response of Normal and Transformed Cells to Histone Deacetylase Inhibitors. *Proc Natl Acad Sci USA*. 2005; 102(3):673-678.
81. Atadja P, Gao L, Kwon P, Trogani N, Walker H, Hsu M, et al. Selective Growth Inhibition of Tumor Cells by a Novel Histone Deacetylase Inhibitor, Nvp-Laq824. *Cancer Res*. 2004; 64(2):689-695.
82. Piekarczyk R and Bates S. A Review of Depsipeptide and Other Histone Deacetylase Inhibitors in Clinical Trials. *Curr Pharm Des*. 2004; 10(19):2289-2298.
83. Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W, et al. Phase I Clinical Trial of Histone Deacetylase Inhibitor: Suberoylanilide Hydroxamic Acid Administered Intravenously. *Clin Cancer Res*. 2003; 9(10 Pt 1):3578-3588.
84. Al Janobi AA, Mithen RF, Gasper AV, Shaw PN, Middleton RJ, Ortori CA, et al. Quantitative Measurement of Sulforaphane, Iberin and Their Mercapturic Acid Pathway Metabolites in Human Plasma and Urine Using Liquid Chromatography-Tandem Electrospray Ionisation Mass Spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006; 844(2):223-234.
85. Frank SR, Schroeder M, Fernandez P, Taubert S, and Amati B. Binding of C-Myc to Chromatin Mediates Mitogen-Induced Acetylation of Histone H4 and Gene Activation. *Genes Dev*. 2001; 15(16):2069-2082.
86. Richon VM, Sandhoff TW, Rifkind RA, and Marks PA. Histone Deacetylase Inhibitor Selectively Induces P21waf1 Expression and Gene-Associated Histone Acetylation. *Proc Natl Acad Sci U S A*. 2000; 97(18):10014-10019.
87. Ocker M and Schneider-Stock R. Histone Deacetylase Inhibitors: Signalling Towards P21cip1/Waf1. *Int J Biochem Cell Biol*. 2007; 39(7-8):1367-1374.

88. Bruserud O, Stapnes C, Ersvaer E, Gjertsen BT, and Ryningen A. Histone Deacetylase Inhibitors in Cancer Treatment: A Review of the Clinical Toxicity and the Modulation of Gene Expression in Cancer Cell. *Curr Pharm Biotechnol*. 2007; 8(6):388-400.
89. Rodriguez-Gonzalez A, Lin T, Ikeda AK, Simms-Waldrip T, Fu C, and Sakamoto KM. Role of the Aggresome Pathway in Cancer: Targeting Histone Deacetylase 6-Dependent Protein Degradation. *Cancer Res*. 2008; 68(8):2557-2560.
90. Simms-Waldrip T, Rodriguez-Gonzalez A, Lin T, Ikeda AK, Fu C, and Sakamoto KM. The Aggresome Pathway as a Target for Therapy in Hematologic Malignancies. *Mol Genet Metab*. 2008; 94(3):283-286.
91. Gibbs A, Schwartzman J, Deng V, and Alumkal J. Sulforaphane Destabilizes the Androgen Receptor in Prostate Cancer Cells by Inactivating Histone Deacetylase 6, *Proc Natl Acad Sci U S A*, Vol. 106, 2009, pp. 16663-16668.
92. Nakagawa M, Oda Y, Eguchi T, Aishima S, Yao T, Hosoi F, et al. Expression Profile of Class I Histone Deacetylases in Human Cancer Tissues. *Oncol Rep*. 2007; 18(4):769-774.
93. Weichert W, Roske A, Gekeler V, Beckers T, Stephan C, Jung K, et al. Histone Deacetylases 1, 2 and 3 Are Highly Expressed in Prostate Cancer and Hdac2 Expression Is Associated with Shorter Psa Relapse Time after Radical Prostatectomy. *Br J Cancer*. 2008; 98(3):604-610.
94. Li Y, Li X, and Guo B. Chemopreventive Agent 3,3'-Diindolylmethane Selectively Induces Proteasomal Degradation of Class I Histone Deacetylases. *Cancer Res*. 2010; 70(2):646-654.
95. Spurling CC, Godman CA, Noonan EJ, Rasmussen TP, Rosenberg DW, and Giardina C. Hdac3 Overexpression and Colon Cancer Cell Proliferation and Differentiation. *Mol Carcinog*. 2008; 47(2):137-147.
96. Azarenko O, Okouneva T, Singletary KW, Jordan MA, and Wilson L. Suppression of Microtubule Dynamic Instability and Turnover in MCF7 Breast Cancer Cells by Sulforaphane. *Carcinogenesis*. 2008; 29(12):2360-2368.
97. Jackson SJ and Singletary KW. Sulforaphane: A Naturally Occurring Mammary Carcinoma Mitotic Inhibitor, Which Disrupts Tubulin Polymerization. *Carcinogenesis*. 2004; 25(2):219-227.
98. Hideshima T, Bradner JE, Wong J, Chauhan D, Richardson P, Schreiber SL, et al. Small-Molecule Inhibition of Proteasome and Aggresome Function Induces Synergistic Antitumor Activity in Multiple Myeloma. *Proc Natl Acad Sci U S A*. 2005; 102(24):8567-8572.
99. Steinbrecher A, Nimptsch K, Husing A, Rohrmann S, and Linseisen J. Dietary Glucosinolate Intake and Risk of Prostate Cancer in the Epic-Heidelberg Cohort Study. *Int J Cancer*. 2009; 125(9):2179-2186.
100. Tang L, Zirpoli GR, Jayaprakash V, Reid ME, McCann SE, Nwogu CE, et al. Cruciferous Vegetable Intake Is Inversely Associated with Lung Cancer Risk among Smokers: A Case-Control Study. *BMC cancer*. 2010; 10(162):1-9.
101. Tang L, Zirpoli GR, Guru K, Moysich KB, Zhang Y, Ambrosone CB, et al. Intake of Cruciferous Vegetables Modifies Bladder Cancer Survival. *Cancer Epidemiol Biomarkers Prev*. 2010; 19(7):1806-1811.

102. Clarke JD, Dashwood RH, and Ho E. Multi-Targeted Prevention of Cancer by Sulforaphane. *Cancer Lett.* 2008; 269(2):291-304.
103. Cheung KL and Kong AN. Molecular Targets of Dietary Phenethyl Isothiocyanate and Sulforaphane for Cancer Chemoprevention. *The AAPS journal.* 2010; 12(1):87-97.
104. Kwak M-K and Kensler TW. Targeting Nrf2 Signaling for Cancer Chemoprevention. *Toxicol Appl Pharmacol.* 2010; 244(1):66-76.
105. Zhang Y and Callaway EC. High Cellular Accumulation of Sulphoraphane, a Dietary Anticarcinogen, Is Followed by Rapid Transporter-Mediated Export as a Glutathione Conjugate. *Biochem J.* 2002; 364(Pt 1):301-307.
106. Hanlon N, Coldham N, Gielbert A, Kuhnert N, Sauer MJ, King LJ, et al. Absolute Bioavailability and Dose-Dependent Pharmacokinetic Behaviour of Dietary Doses of the Chemopreventive Isothiocyanate Sulforaphane in Rat. *Br J Nutr.* 2008; 99(3):559-564.
107. Keum YS, Khor TO, Lin W, Shen G, Kwon KH, Barve A, et al. Pharmacokinetics and Pharmacodynamics of Broccoli Sprouts on the Suppression of Prostate Cancer in Transgenic Adenocarcinoma of Mouse Prostate (Tramp) Mice: Implication of Induction of Nrf2, Ho-1 and Apoptosis and the Suppression of Akt-Dependent Kinase Pathway. *Pharm Res.* 2009; 26(10):2324-2331.
108. Singh SV, Warin R, Xiao D, Powolny AA, Stan SD, Arlotti JA, et al. Sulforaphane Inhibits Prostate Carcinogenesis and Pulmonary Metastasis in Tramp Mice in Association with Increased Cytotoxicity of Natural Killer Cells. *Cancer Res.* 2009; 69(5):2117-2125.
109. Saw CL, Huang MT, Liu Y, Khor TO, Conney AH, and Kong AN. Impact of Nrf2 on Uvb-Induced Skin Inflammation/Photoprotection and Photoprotective Effect of Sulforaphane. *Mol Carcinog.* 2010; Published online ahead of print Dec 28 2010(DOI: 10.1002/mc.20725).
110. Jazwa A, Rojo AI, Innamorato NG, Hesse M, Fernandez-Ruiz J, and Cuadrado A. Pharmacological Targeting of the Transcription Factor Nrf2 at the Basal Ganglia Provides Disease Modifying Therapy for Experimental Parkinsonism. *Antioxidants & redox signaling.* 2011; Published online ahead of print March 28 2011(DOI 10.1089/ars.2010.3731).
111. Slatter JG, Rashed MS, Pearson PG, Han DH, and Baillie TA. Biotransformation of Methyl Isocyanate in the Rat. Evidence for Glutathione Conjugation as a Major Pathway of Metabolism and Implications for Isocyanate-Mediated Toxicities. *Chem Res Toxicol.* 1991; 4(2):157-161.
112. Kaplan-Lefko PJ, Chen TM, Ittmann MM, Barrios RJ, Ayala GE, Huss WJ, et al. Pathobiology of Autochthonous Prostate Cancer in a Pre-Clinical Transgenic Mouse Model. *Prostate.* 2003; 55(3):219-237.
113. Suttie A, Nyska A, Haseman JK, Moser GJ, Hackett TR, and Goldsworthy TL. A Grading Scheme for the Assessment of Proliferative Lesions of the Mouse Prostate in the Tramp Model. *Toxicol Pathol.* 2003; 31(1):31-38.
114. Conaway CC, Krzeminski J, Amin S, and Chung FL. Decomposition Rates of Isothiocyanate Conjugates Determine Their Activity as Inhibitors of Cytochrome P450 Enzymes. *Chem Res Toxicol.* 2001; 14(9):1170-1176.

115. Conaway CC, Wang CX, Pittman B, Yang YM, Schwartz JE, Tian D, et al. Phenethyl Isothiocyanate and Sulforaphane and Their N-Acetylcysteine Conjugates Inhibit Malignant Progression of Lung Adenomas Induced by Tobacco Carcinogens in a/J Mice. *Cancer Res.* 2005; 65(18):8548-8557.
116. Yang YM, Jhanwar-Uniyal M, Schwartz J, Conaway CC, Halicka HD, Traganos F, et al. N-Acetylcysteine Conjugate of Phenethyl Isothiocyanate Enhances Apoptosis in Growth-Stimulated Human Lung Cells. *Cancer Res.* 2005; 65(18):8538-8547.
117. Tang L, Li G, Song L, and Zhang Y. The Principal Urinary Metabolites of Dietary Isothiocyanates, N-Acetylcysteine Conjugates, Elicit the Same Anti-Proliferative Response as Their Parent Compounds in Human Bladder Cancer Cells. *Anti-cancer drugs.* 2006; 17(3):297-305.
118. Chiao JW, Wu H, Ramaswamy G, Conaway CC, Chung FL, Wang L, et al. Ingestion of an Isothiocyanate Metabolite from Cruciferous Vegetables Inhibits Growth of Human Prostate Cancer Cell Xenografts by Apoptosis and Cell Cycle Arrest. *Carcinogenesis.* 2004; 25(8):1403-1408.
119. Ma Q, Battelli L, and Hubbs AF. Multiorgan Autoimmune Inflammation, Enhanced Lymphoproliferation, and Impaired Homeostasis of Reactive Oxygen Species in Mice Lacking the Antioxidant-Activated Transcription Factor Nrf2. *Am J Pathol.* 2006; 168(6):1960-1974.
120. Blackburn AC, Matthaei KI, Lim C, Taylor MC, Cappello JY, Hayes JD, et al. Deficiency of Glutathione Transferase Zeta Causes Oxidative Stress and Activation of Antioxidant Response Pathways. *Mol Pharmacol.* 2006; 69(2):650-657.
121. Dai G, He L, Chou N, and Wan YJ. Acetaminophen Metabolism Does Not Contribute to Gender Difference in Its Hepatotoxicity in Mouse. *Toxicol Sci.* 2006; 92(1):33-41.
122. Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, et al. Loss of the Nrf2 Transcription Factor Causes a Marked Reduction in Constitutive and Inducible Expression of the Glutathione S-Transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 Genes in the Livers of Male and Female Mice. *Biochem J.* 2002; 365(Pt 2):405-416.
123. Shen G and Kong AN. Nrf2 Plays an Important Role in Coordinated Regulation of Phase II Drug Metabolism Enzymes and Phase III Drug Transporters. *Biopharm Drug Dispos.* 2009; 30(7):345-355.
124. Anwar-Mohamed A, Degenhardt OS, El Gendy MA, Seubert JM, Kleeberger SR, and El-Kadi AO. The Effect of Nrf2 Knockout on the Constitutive Expression of Drug Metabolizing Enzymes and Transporters in C57bl/6 Mice Livers. *Toxicol In Vitro.* 2011; Published online ahead of print Jan 31 2011(doi:10.1016/j.tiv.2011.01.014).
125. Khor TO, Huang MT, Kwon KH, Chan JY, Reddy BS, and Kong AN. Nrf2-Deficient Mice Have an Increased Susceptibility to Dextran Sulfate Sodium-Induced Colitis. *Cancer Res.* 2006; 66(24):11580-11584.
126. Osburn WO, Karim B, Dolan PM, Liu G, Yamamoto M, Huso DL, et al. Increased Colonic Inflammatory Injury and Formation of Aberrant Crypt Foci in Nrf2-Deficient Mice Upon Dextran Sulfate Treatment. *Int J Cancer.* 2007; 121(9):1883-1891.
127. Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, et al. Increased Susceptibility of Nrf2 Knockout Mice to Colitis-Associated Colorectal Cancer. *Cancer Prev Res (Phila).* 2008; 1(3):187-191.

128. Falkner KC, Pinaire JA, Xiao GH, Geoghegan TE, and Prough RA. Regulation of the Rat Glutathione S-Transferase A2 Gene by Glucocorticoids: Involvement of Both the Glucocorticoid and Pregnane X Receptors. *Mol Pharmacol.* 2001; 60(3):611-619.
129. Urquhart BL, Tirona RG, and Kim RB. Nuclear Receptors and the Regulation of Drug-Metabolizing Enzymes and Drug Transporters: Implications for Interindividual Variability in Response to Drugs. *J Clin Pharmacol.* 2007; 47(5):566-578.
130. Tian Q, Rosselot RA, and Schwartz SJ. Quantitative Determination of Intact Glucosinolates in Broccoli, Broccoli Sprouts, Brussels Sprouts, and Cauliflower by High-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry. *Anal Biochem.* 2005; 343(1):93-99.
131. Hanlon N, Coldham N, Sauer MJ, and Ioannides C. Modulation of Rat Pulmonary Carcinogen-Metabolising Enzyme Systems by the Isothiocyanates Erucin and Sulforaphane. *Chem Biol Interact.* 2009; 177(2):115-120.
132. Munday R and Munday CM. Induction of Phase II Detoxification Enzymes in Rats by Plant-Derived Isothiocyanates: Comparison of Allyl Isothiocyanate with Sulforaphane and Related Compounds. *J Agric Food Chem.* 2004; 52(7):1867-1871.
133. Harris KE and Jeffery EH. Sulforaphane and Erucin Increase Mrp1 and Mrp2 in Human Carcinoma Cell Lines. *J Nut Biochem.* 2008; 19(4):246-254.
134. Melchini A, Costa C, Traka M, Miceli N, Mithen R, De Pasquale R, et al. Erucin, a New Promising Cancer Chemopreventive Agent from Rocket Salads, Shows Anti-Proliferative Activity on Human Lung Carcinoma A549 Cells. *Food Chem Toxicol.* 2009; 47(7):1430-1436.
135. Stahl W and Sies H. Uptake of Lycopene and Its Geometrical Isomers Is Greater from Heat-Processed Than from Unprocessed Tomato Juice in Humans. *J Nutr.* 1992; 122(11):2161-2166.
136. Gartner C, Stahl W, and Sies H. Lycopene Is More Bioavailable from Tomato Paste Than from Fresh Tomatoes. *Am J Clin Nutr.* 1997; 66(1):116-122.
137. Cramer JM and Jeffery EH. Sulforaphane Absorption and Excretion Following Ingestion of a Semi-Purified Broccoli Powder Rich in Glucoraphanin and Broccoli Sprouts in Healthy Men. *Nutr Cancer.* 2011; 63(2):196-201.
138. Vermeulen M, Klopping-Ketelaars IW, van den Berg R, and Vaes WH. Bioavailability and Kinetics of Sulforaphane in Humans after Consumption of Cooked Versus Raw Broccoli. *J Agric Food Chem.* 2008; 56(22):10505-10509.
139. Lampe JW. Interindividual Differences in Response to Plant-Based Diets: Implications for Cancer Risk. *Am J Clin Nutr.* 2009; 89(5):1553S-1557S.
140. Zhao M, Lewis R, Gustafson DR, Wen WQ, Cerhan JR, and Zheng W. No Apparent Association of Gstp1 a(313)G Polymorphism with Breast Cancer Risk among Postmenopausal Iowa Women. *Cancer Epidemiol Biomarkers Prev.* 2001; 10(12):1301-1302.
141. Vermeulen M, Zwanenburg B, Chittenden GJ, and Verhagen H. Synthesis of Isothiocyanate-Derived Mercapturic Acids. *Eur J Med Chem.* 2003; 38(7-8):729-737.
142. Rungapamestry V, Duncan AJ, Fuller Z, and Ratcliffe B. Effect of Cooking Brassica Vegetables on the Subsequent Hydrolysis and Metabolic Fate of Glucosinolates. *Proc Nutr Soc.* 2007; 66(1):69-81.

143. Rungapamestry V, Duncan AJ, Fuller Z, and Ratcliffe B. Effect of Meal Composition and Cooking Duration on the Fate of Sulforaphane Following Consumption of Broccoli by Healthy Human Subjects. *Br J Nutr.* 2007; 97(4):644-652.
144. Lai R-H, Miller MJ, and Jeffery EH. Glucoraphanin Hydrolysis by Microbiota in the Rat Cecum Results in Sulforaphane Absorption. *Food and Function.* 2010; 1:161-166.
145. Vermeulen M, van den Berg R, Freidig AP, van Bladeren PJ, and Vaes WH. Association between Consumption of Cruciferous Vegetables and Condiments and Excretion in Urine of Isothiocyanate Mercapturic Acids. *J Agric Food Chem.* 2006; 54(15):5350-5358.
146. Rangkadilok N, Nicolas ME, Bennett RN, Premier RR, Eagling DR, and Taylor PWJ. Developmental Changes of Sinigrin and Glucoraphanin in Three Brassica Species (*Brassica Nigra*, *Brassica Juncea* and *Brassica Oleracea* Var. *Italica*). *Sci Hortic (Amsterdam).* 2002; 96(1-4):11-26.
147. Jeffery EH and Keck AS. Translating Knowledge Generated by Epidemiological and in Vitro Studies into Dietary Cancer Prevention. *Mol Nutr Food Res.* 2008; 52 Suppl 1(S7-17).
148. Velicer CM and Ulrich CM. Vitamin and Mineral Supplement Use among Us Adults after Cancer Diagnosis: A Systematic Review. *J Clin Oncol.* 2008; 26(4):665-673.
149. Zhu N, Soendergaard M, Jeffery EH, and Lai RH. The Impact of Loss of Myrosinase on the Bioactivity of Broccoli Products in F344 Rats. *J Agric Food Chem.* 2010; 58(3):1558-1563.
150. Hanlon N, Coldham N, Gielbert A, Sauer MJ, and Ioannides C. Repeated Intake of Broccoli Does Not Lead to Higher Plasma Levels of Sulforaphane in Human Volunteers. *Cancer Lett.* 2009; 284(1):15-20.
151. Barillari J, Canistro D, Paolini M, Ferroni F, Pedulli GF, Iori R, et al. Direct Antioxidant Activity of Purified Glucoerucin, the Dietary Secondary Metabolite Contained in Rocket (*Eruca Sativa* Mill.) Seeds and Sprouts. *J Agric Food Chem.* 2005; 53(7):2475-2482.
152. Dashwood RH, Myzak MC, and Ho E. Dietary Hdac Inhibitors: Time to Rethink Weak Ligands in Cancer Chemoprevention? *Carcinogenesis.* 2006; 27(2):344-349.
153. Dunn BK, Richmond ES, Minasian LM, Ryan AM, and Ford LG. A Nutrient Approach to Prostate Cancer Prevention: The Selenium and Vitamin E Cancer Prevention Trial (Select). *Nutrition and cancer.* 2010; 62(7):896-918.
154. Zeng H, Trujillo ON, Moyer MP, and Botnen JH. Prolonged Sulforaphane Treatment Activates Survival Signaling in Nontumorigenic Ncm460 Colon Cells but Apoptotic Signaling in Tumorigenic Hct116 Colon Cells. *Nutr Cancer.* 2011; 63(2):248-255.
155. Myzak MC and Dashwood RH. Histone Deacetylases as Targets for Dietary Cancer Preventive Agents: Lessons Learned with Butyrate, Diallyl Disulfide, and Sulforaphane. *Curr Drug Targets.* 2006; 7(4):443-452.
156. Dobosy JR and Selker EU. Emerging Connections between DNA Methylation and Histone Acetylation. *Cell Mol Life Sci.* 2001; 58(5-6):721-727.
157. Callaway EC, Zhang Y, Chew W, and Chow HHS. Cellular Accumulation of Dietary Anticarcinogenic Isothiocyanates Is Followed by Transporter-Mediated Export as Dithiocarbamates. *Cancer Lett.* 2004; 204(1):23-31.

158. Clarke JD, Hsu A, Yu Z, Dashwood RH, and Ho E. Differential Effects of Sulforaphane on Histone Deacetylases, Cell Cycle Arrest and Apoptosis in Normal Prostate Cells Versus Hyperplastic and Cancerous Prostate Cells. *Mol Nutr Food Res.*, Published online ahead of print Mar 4 2011 (DOI: 10.1002/mnfr.201000547).

