THE BIOAVAILABILITY OF SULFORAPHANE FROM BROCCOLI PRODUCTS IN MEN AND ITS EPIGENETIC ACTIVITY

BY

JENNA MARIE CRAMER

DISSERTATION

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Doctoral Committee:

Professor Emeritus John W. Erdman, Jr., Chair
Professor Emerita Elizabeth H. Jeffery, Director of Research
Assistant Professor Yuan-Xiang Pan
Assistant Professor Margarita De L. Teran-Garcia

ABSTRACT

Epidemiological data show a correlation between broccoli consumption and an anti-cancer benefit. This benefit is attributed to the isothiocyanate sulforaphane (SF). Sulforaphane is derived from the myrosinase-catalyzed hydrolysis of glucoraphanin. Both glucoraphanin and myrosinase are present in fresh or lightly cooked broccoli and broccoli sprouts. Like myrsoinase, gut microflora are capable of hydrolysis of glucoraphanin to SF, although to a much lesser extent than endogenous plant myrosinase (1, 2). It is well established that when glucoraphanin is consumed in the absence of myrosinase, as is the case for heavily cooked broccoli, much of the cancer preventative potential is not availed (1, 3). Similarly, many of the dietary glucoraphanin supplements on the market today lack myrosinase, and may not act as a source of SF. However, the efficacy of these supplements in delivering SF has not been previously evaluated, neither has the potential for restoring the availability of SF by ingesting an exogenous source of myrosinase concomitantly with sources of glucoraphanin that are devoid of endogenous myrosinase.

The mechanism of cancer protection by SF is multifaceted, but the best characterized involves the upregulation of detoxification enzymes through the nuclear factor (erythroid-derived 2)-like 2/antioxidant response element pathway (4). More recently, it was discovered that SF also inhibits cancer through epigenetic mechanisms, specifically by decreasing the activities of histone deacetylase and DNA methyltransferase enzymes (5, 6). These effects were most pronounced at 10-15 μM SF, concentrations that are not typically obtained through dietary means. Furthermore, it has not been previously determined whether the inhibition of DNA methyltransferase by SF correlates with increased expression of tumor suppressor genes.

The objective of this research was two-fold. First, to determine the plasma and urine levels of SF and metabolites following human ingestion of a glucoraphanin supplement alone or in combination with a myrosinase-rich food source. It was determined that the SF bioavailablity of a high-glucosinolate supplement that was devoid of myrosinase was enhanced by co-consumption with a food source that contained myrosinase. Specifically, plasma total isothiocyanate (ITC) concentration reached 2.86 ± 0.33 μM after the supplement was consumed with fresh broccoli sprouts whereas the peak plasma total ITC concentration after consumption of the glucoraphanin supplement did not reach significance over control meal or baseline values. The second objective was to evaluate the effects on DNA methylation and mRNA expression of cancer-related genes using physiologically attainable concentrations of SF similar to those identified in part one. While the promoters of *P16*, *MGMT* and *MLH1* were unaffected by SF, DNA methylation at the *P21* promoter was decreased by approximately 14% with a concurrent 1.92 ± 0.32 fold increase in mRNA. DNA methylation at the *BAX* promoter was decreased by a non-significant 11%, but was accompanied by a 1.64 ± 0.09 fold increase in mRNA, which did reach statistical significance. The activity of histone deacetylase and DNA methyltransferase enzymes was also assessed at this physiological concentration of SF. Histone deacetylase activity was unaffected, but DNA methyltransferase activity was decreased to 70.2 ± 9.8% of control after exposure to 5.0 μM SF.

These results indicate that the availability of dietary SF can be enhanced by co-consumption of glucoraphanin with an exogenous source of myrosinase. Separately, the results suggest that doses of SF attainable through diet may reduce the risk of cancer development partially by reducing the level of aberrant DNA methylation at the promoters of select tumor suppressor genes.

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CHAPTER 1

Introduction

1.1 Significance

Cancer is the second leading cause of death in the United States, accounting for over 23% of deaths (1). One in two men and one in three women will develop cancer in his or her lifetime (2). Lifestyle choices, especially smoking, patterns of dietary consumption and physical exercise, are estimated to account for 10-70% of all cancers (2, 3).

High rates of colon cancer are found in Westernized countries and evidence suggests that the causes of colon cancer are largely environmental, with the westernized diet being a key factor (4, 5). It is believed that colon cancer is mostly preventable by consuming an appropriate diet and other related factors (5). Epidemiological evidence suggests that consumption of cruciferous vegetables can reduce the risk of several types of cancer, including colorectal cancer (6).

Broccoli is a cruciferous vegetable and a rich source of many micronutrients in addition to the glucosinolate glucoraphanin (7-9). Unfortunately, most Americans consume less than one serving per week, possibly due to the perception of an unpleasant flavor (10, 11).

When the tissue of fresh broccoli is chewed, the enzyme myrosinase comes into contact with glucoraphanin and converts it to the cancer-preventative isothiocyanate sulforaphane (SF), which is partly responsible for the strong taste of fresh broccoli. If the broccoli has been thoroughly cooked, myrosinase is rendered inactive and the mild tasting glucoraphanin remains intact. As previously stated, some consumers may find the taste of fresh broccoli unappealing. In fact, the taste of glucoraphanin (found in well-cooked broccoli) was preferred 81:19 compared to SF in a study that evaluated the taste preference between a SF containing or a glucoraphanin containing

beverage (11). Dietary supplements are one way to overcome this obstacle. However, broccoli supplements typically incorporate glucoraphanin as the source of bioactive SF and lack myrosinase, thus putting their efficacy into question. It is a common public misconception that consumption of glucoraphanin, the inactive SF precursor from cooked broccoli or dietary supplements that have no myrosinase, provides health benefits comparable to those of SF.

The two studies completed in Aim 1 tested the hypothesis that combining a glucosinolate preparation devoid of myrosinase, typical of both well cooked broccoli and many dietary supplements on the market, with a second product rich in myrosinase would enhance glucosinolate conversion and ITC absorption. These are the first studies to examine a commercially available powdered broccoli preparation containing glucoraphanin, but lacking myrosinase and to examine effects of combining this glucoraphanin-rich broccoli preparation with an exogenous food source of myrosinase, broccoli sprouts. The results show that combination improved SF availability, likely because the endogenous myrosinase from broccoli sprouts allowed early hydrolysis of glucoraphanin not only from the broccoli sprouts, but also from broccoli powder, resulting in early and more complete absorption of SF. The effects of this combination open the door to development of products with enhanced chemo-protective potential. The importance of these studies was realized as they have been followed-up by two larger studies from an independent research group (12, 13).

In Aim 2, physiologically relevant concentrations of SF determined in Aim 1 and in other publications were used to evaluate the epigenetic activity of SF in colorectal cancer cells (14-16). Using physiologically relevant doses of SF, defined in this work as levels attainable in plasma through dietary consumption, is of interest as this approach mimics the situation that the majority of the population is exposed to this bioactive compound. Colorectal cancer frequently includes a

number of epigenetic changes that occur throughout the development of cancer. The gastrointestinal tract is constantly being exposed to food-derived compounds which may shift cellular balance towards harmful or beneficial outcomes depending on the foods consumed (5). It has been suggested that diet may be able to account for or prevent up to 80% of colon cancer incidence (17). For these reasons, it is reasonable to postulate that colorectal cancer may be particularity amenable to reversal of aberrant epigenetic changes through dietary means.

The cell line HCT 116 was selected for evaluation in Aim 2. HCT 116 cells are tumorigenic colorectal carcinoma cells with a mutation of the Ras proto-oncogene and have been utilized previously for work examining the connection between bioactive food components and epigenetic modifications. For the research conducted in Aim 2, the DNA methylation status of the O-6-methylguanine-DNA methyltransferase (*MGMT),* mutL homolog 1 (*MLH1),* cyclin-dependent kinase inhibitor 2A *(P16),* BCL2-associated X protein (*BAX)* and cyclin-dependent kinase inhibitor 1A *(P21)* gene promoters were investigated. Each of these genes has been reported to be upregulated by SF, with the exception of *MLH1* which was selected due to its predominance as a gene often hypermethylated in colorectal cancer (18, 19). Additionally, each gene selected for evaluation is involved in cancer, although they belong to different pathways (Figure 1.1). Of particular interest is *P21*. Stimulation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/antioxidant response element (ARE) pathway is most commonly attributed with the reduction of cancer observed after exposure to SF. However, *P21* lacks an ARE; therefore, it is likely that upregulation of P21 by SF acts through a NRF2-independent mechanism, such as DNA demethylation and/or histone acetylation (20).

In summary, combination of a glucoraphanin supplement with a source of myrosinase increases biomarkers of SF exposure in humans. Elevated exposure to SF may reduce the risk for and

progression of cancers, including colorectal cancer. Sulforaphane may contribute to the dietary protective effect of broccoli against colorectal cancer in part by decreasing DNA methylation and increasing expression of aberrantly silenced cancer-related genes at concentrations of SF that are attainable in humans through the diet.

1.2 Specific aims

Long Term Goal: To determine the bioavailability of SF from several broccoli products and the epigenetic activity of SF at physiological levels.

Central Hypotheses:

1) The bioavailiability of SF from a broccoli powder rich in glucoraphanin, but lacking myrosinase will be increased by simultaneously consuming broccoli sprouts containing myrosinase.
2) At physiologically relevant concentrations, SF will result in decreased DNA methylation of the promoters of several hypermethylated genes in HCT 116 human colorectal carcinoma cells. The demethylation will cause increased expression of these genes.

Aim 1: To evaluate SF absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men.

Hypothesis: The recovery of SF metabolites from urine and plasma will be greatest when additional myrosinase, in the form of broccoli sprouts, is provided concomitantly with the glucoraphanin-rich powder.

1.1: (Clinical study 1) To determine urinary sulforaphane-*N*-acetylcysteine (SF-NAC) and plasma total isothiocyanates following consumption of glucoraphanin powder and *air-dried*

broccoli sprouts alone and in combination.

1.2: (Clinical study 2) To determine urinary SF-NAC and a more kinetically complete data set of plasma total isothiocyanates following consumption of glucoraphanin powder and *fresh* broccoli sprouts alone and in combination.

Aim 2: To determine the effect of SF on the DNA methylation profile and mRNA expression level of selected genes.

Hypothesis: Sulforaphane will result in demethylation of the promoters of several genes selected based on their reported upregulation by SF. The mRNA level of genes found to be demethylated by SF will increase following SF treatment.

2.1: To determine the methylation status of the *MGMT*, *MLH1*, *P21*, *BAX* and *P16* gene promoters in control and SF treated HCT 116 cells.

2.2: To determine the associated changes in the mRNA expression levels of those genes from Aim 2.1 that responded robustly to SF exposure.

2.3: To determine the effect of SF on histone deacetylase and DNA methyltransferase activity.

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1.4 Figures

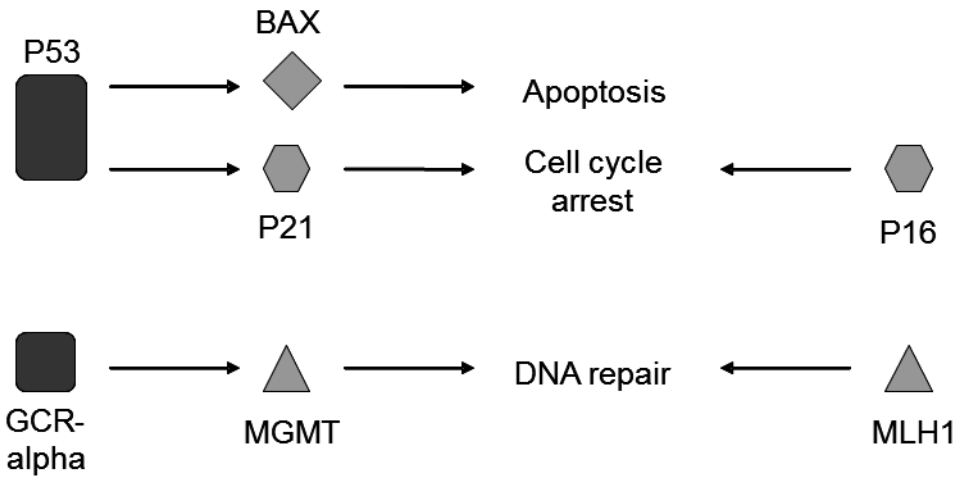

Figure 1.1. Tumor suppression pathways of genes selected for evaluation of DNA methylation (grey shapes).

CHAPTER 2

Literature Review

2.1 Cancer epidemiology and cruciferous vegetables

Consumption of fruits and vegetables is inversely correlated with the risk of developing cancer (1). In particular, epidemiological studies indicate a strong relationship between high intake of cruciferous vegetables and cancer prevention (2, 3). In fact, crucifers appear to offer substantially greater reduction of cancer risk compared to other groups of fruits and vegetables (2-6). Cruciferous vegetables are of the family Brassicaceae and are distinguished by the shape of their flowers in which the four petals resemble a cross. Broccoli, cauliflower, cabbage, kale, Brussels sprouts, arugula, horseradish and mustard are examples of cruciferous vegetables. High dietary intake of cruciferous vegetables is most consistently correlated with a decreased risk for cancers of the stomach, colon, rectum and lung, but evidence exists for a protective effect against other cancers including kidney, prostate, ovaries, bladder, pancreas and skin (2, 6-11). Cruciferous vegetables contain a unique class of sulfur containing organic compounds called glucosinolates. Most research on the reduction of cancer risk by cruciferous vegetables is focused on glucosinolates and isothiocynates, glucosinolate hydrolysis products. Isothiocyanates, also known as mustard oils, are responsible for the spicy taste associated with crucifers (12). Approximately 120 naturally occurring glucosinolates have been identified. Some of the most commonly studied include glucoraphanin, sinigrin, glucoerucin, glucoiberin, gluconasturtiin, glucobrassicin, neoglucobrassicin and progoitrin (13).

2.2 The bioactivity of broccoli

Mature broccoli and broccoli sprouts are reported to offer protection against cardiovascular disease, hypertension, Helicobacter pylori infection, rheumatoid arthritis, macular degeneration, neurological conditions and cancer (14). In particular, broccoli may be effective against cancers of the colon, rectum, bladder, breast and liver (15-18). Broccoli provides fiber, vitamins, minerals and antioxidants including tocopherols, carotenoids and flavonoids, which all may play a role in the chemoprotective activity of broccoli (19-21). Importantly, broccoli also contains glucosinolates with glucoraphanin (GRP) being the major glucosinolate present in broccoli. Glucoraphanin [4-(methylsulfinyl)butyl glucosinolate] likely plays a major role in the reduction of cancer risk that is attributed to broccoli consumption.

Glucoraphanin is a naturally occurring, biologically inactive compound that is abundant in broccoli and broccoli sprouts. Glucoraphanin gives rise to the biologically active isothiocyanate, sulforaphane (SF) [4-(methylsulfinyl)butyl glucosinolate], subsequent to hydrolysis by the endogenous thioglucoside glucohydrolase myrosinase (thioglucoside glucohydrolase; EC 3.2.1.147). It should be noted that SF is not the sole product that can form from the hydrolysis of GRP. Nitriles and thiocyanates can also be produced as a result of GRP hydrolysis depending on factors including pH and the presence of metal ions and/or other proteins (22-24). However, isothiocyanates and nitriles are the predominant hydrolysis products (Figure 2.1) (23). Sulforaphane is considered to be majorly responsible for the cancer preventative benefits of broccoli consumption (25). Broccoli seeds and young sprouts contain higher amounts of GRP and have higher myrosinase activity than mature plants, making them a better source of SF (13). Although GRP is the predominant glucosinolate in broccoli, it must be acknowledged that other glucosinolates such as glucobrassicin and sinigrin are also present in broccoli and can produce

isothiocyanates that may provide benefits in addition to those of SF. The research discussed in this literature review and presented in this dissertation is focused on SF, as SF is well known for its anti-cancer activity and is considered the primary anticarcinogen of broccoli (25).

A review of published literature suggests that SF prevents and controls cancer by several mechanisms. The induction of detoxification enzymes through the Kelch-like ECH-associated protein 1/nuclear factor (erythroid-derived 2)-like 2/antioxidant response element (Keap1/Nrf2/ARE) pathway is perhaps the best recognized (26). In the basal state of a cell, the negative repressor protein Keap1 sequesters Nrf2 in the cytoplasm directing Nrf2 toward ubiquitylation and subsequent degradation (27-29). When SF enters the cell, it reacts with cysteine residues of Keap1, preventing ubiquitylation of Nrf2. Nrf2 then translocates to the nucleus where it binds to the ARE sequence in DNA. The activation of gene transcription by Nrf2 upregulates expression of target genes including quinone reductase, glutathione transferases and antioxidant proteins (30). Upregulation of these genes increases cellular protection through detoxification of harmful, potentially carcinogenic compounds.

Other anti-carcinogenic mechanisms of SF include inhibition of phase 1 enzymes, inhibition of inflammation and induction of cell cycle arrest and apoptosis (26). More recently, epigenetic mechanisms of cancer prevention and control by SF, such as inhibition of histone deacetylase and decreased DNA methylation, have been identified and are being investigated (31, 32). These epigenetic mechanisms will be discussed in depth in section 2.5.

2.3 The importance of myrosinase

In mature broccoli and broccoli sprouts, myrosinase and the inactive SF precursor GRP are segregated in distinct cellular compartments (33). Myrosinase and GRP interact upon damage to

the plant tissue, such as during crushing or chewing. This interaction allows myrosinase to catalyze the hydrolysis of GRP to SF by cleavage of the β-thioglucosidic bond. It is therefore expected that SF metabolites will be detected in plasma soon after consumption of fresh broccoli or sprouts because the hydrolysis of GRP will occur in the upper gastrointestinal tract.

Plant myrosinase is not the only means of producing SF. Sulforaphane can also be formed by microflora of the mammalian colon that are capable of myrosinase-like activity (34, 35). Specifically, SF was detected following incubation of GRP in the cecum of rats, which is analogous to the human colon in terms of the location of gut microbes (35). Additionally, recovery of GRP hydrolysis products was completely abolished in humans by treatment with antibiotics and mechanical cleansing of the colon (34). These studies provide evidence that gut microbes are capable of GRP hydrolysis in the absence of myrosinase. However, SF recovery after ingestion of GRP without myrosinase is much lower than recovery when myrosinase is supplied concomitantly with GRP, indicating that the hydrolyzing efficacy of gut microflora is much less than that of plant myrosinase (34, 36). In the case where GRP products that lack myrosinase are the sole source of SF, such as well-cooked broccoli, broccoli supplements or broccoli powder lacking myrosinase, hydrolysis will be delayed until the GRP-containing meal reaches the colon and the microflora of the gut provide hydrolytic activity (34, 35, 37, 38). In these instances, the extent of hydrolysis will also be reduced. The consequence of late and incomplete hydrolysis by colonic microflora is a lower peak plasma level of cancer-preventative SF.

2.4 The bioavailability of sulforaphane from broccoli in humans

The most commonly used method to establish SF bioavailability in human subjects is the measurement of SF metabolites in plasma and urine after consumption of a SF-containing or producing meal. These methods are preferred over others due to the non-invasive methods of sample collection. The cyclocondensation assay, which utilizes a derivatization of isothiocyanates and their metabolites for detection, as well as direct detection of sulforaphane-*N*-acetylcysteine (SF-NAC) using HPLC are two common methods used in clinical studies to examine the bioavailability of SF in humans.

The cyclocondensation reaction and measurement of SF metabolites in plasma

Isothiocyanates and their dithiocarbamate metabolites (hereafter referred to as total ITC) react stoichiometrically with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione, which can be detected at 365 nm (Figure 2.2) (12, 39). This derivatization is referred to as the cyclocondensation reaction and has successfully been used to measure the very low concentrations of total ITC found in human plasma following a brassica meal (36, 40). This assay is advantageous as it provides an overall measurement of total ITC exposure. The limitation is that it does not allow for the differentiation between the isothiocyanate, isothiocyante –glutathione, -cysteine-glycine, -cysteine, or –*N*-acetylcysteine conjugates. LC-MS/MS techniques that are capable of detecting each isothiocyante and isothiocyanate-conjugate individually are available for studies that require this type of differentiation (41). The disadvantage of these techniques is that the total ITC measurement is not provided. Therefore, these techniques are advantageous only when a particular isothiocyanate or isothiocyanate

metabolite is being investigated. These techniques are also associated with higher cost and a greater time requirement as a standard for each compound of interest must be prepared.

Direct detection of SF-NAC in urine by HPLC

Metabolites of SF accumulate in urine before being excreted from the body. Therefore, the amount of SF metabolites present in urine following a brassica meal are much greater compared to the levels present in plasma. For this reason, detection of individual metabolites in urine samples is possible using HPLC methods. Sulforaphane-*N*-acetylcysteine is the predominant SF metabolite present in urine after exposure to SF and is often used as a marker of bioavailability (36, 41).

Approximately 74% of dietary SF is absorbed in the jejunum (42). After absorption, SF is conjugated to glutathione by glutathione transferases in the intestinal epithelium and liver (42). SF-glutathione is then sequentially metabolized to SF-cysteinyl-glycine and SF-cysteine (43). Finally, SF-cysteine is conjugated to SF-NAC by the activity of *N*-acetyltransferase in the kidney and excreted in urine (43). Small amounts of SF and SF-cysteine are also excreted, but SF-NAC is the major SF metabolite appearing in urine (41). Therefore, SF-NAC is often used as a marker of bioavailability.

Clinical studies of SF bioavailability

The metabolic fate of glucosinolates has been investigated following human ingestion of steamed broccoli, fresh broccoli, broccoli sprouts, preformed isothiocyanates from sprout homogenate, and GRP from broccoli sprout homogenate without myrosinase. The inclusion of sufficient washout periods and glucosinolate-free diets during broccoli feeding studies ensures that the

major isothiocyanate measured in plasma or urine is SF and SF metabolites. The half-life of SF is reported to be approximately 2 hours (40, 44).

Plasma metabolites

Because plasma concentrations of compounds relate to effects at the site of action, peak plasma levels of total ITC are measured as an indication of cancer-preventative potential from SF. A higher peak plasma level of total ITC indicates an increased potential for cancer prevention. Peak plasma total ITC levels range between 0.07-7.3 μM after consumption of various broccoli products and preparations (36, 40, 41, 45, 46). Most studies report a C_{max} between 2-3 μM. The C_{max} of plasma total ITC is largely dependent on the dose of SF or GRP consumed, which varies with whether myrosinase is present. For example, the peak plasma total ITC concentration following consumption of fresh broccoli was approximately three times higher than the concentration reached after an equimolar dose of GRP from well-steamed broccoli that lacked myrosinase (36). Other factors that may influence the C_{max} of plasma total ITC include variations of subject genotype, especially of the glutathione-S-transferase (GST) gene, and differences in gut microflora composition. However, two recent studies from independent research groups indicated that polymorphisms in the *GSTP1*, *GSTM1* and *GSTT1* genes did not affect SF metabolism or excretion (47, 48). Repeated dosing of broccoli does not lead to accumulation or higher plasma levels of SF over time (45).

The time of peak plasma concentration is also dependent on whether the source of SF is preformed isothiocyanate or GRP. In the case of GRP ingestion, the time of peak plasma concentration also depends on whether or not myrosinase is present. Preformed isothiocyanates or GRP in the presence of myrosinase result in earlier peak plasma total ITC than GRP without

myrosinase (36, 40, 44). Peak plasma total ITC concentration occurs approximately 1-2 h after consumption of fresh broccoli or preformed isothiocyanate (40, 41, 44, 45). The peak plasma total ITC concentration is delayed to approximately 6 h post-consumption following ingestion of GRP without a source of myrosinase (36, 44). Plasma total ITC return to baseline values by 24 h post-consumption regardless of the source of ITC, GRP or presence of myrosinase (36, 41, 44).

Urinary metabolites

The appearance and elimination profile of SF metabolites in urine closely mimics the pattern in plasma. Glucoraphanin without myrosinase results in lower and delayed appearance of SF metabolites compared to preformed isothiocyanates or GRP with myrosinase present. Conaway et al. found that excretion of urinary isothiocyanate metabolites was approximately three times greater from fresh broccoli than from well-steamed broccoli, where myrosinase had been heat-inactivated leaving GRP to be hydrolyzed to SF by gut microflora (36). A similar study found that the bioavailability of SF from fresh broccoli was 11 times greater than that from cooked broccoli (44). Another study evaluated the appearance of total ITC in urine following ingestion of broccoli sprout homogenates that contained either 111 µmol preformed isothiocyanates or 111µmol unhydrolyzed glucosinolates (49). The authors found that excretion of urinary isothiocyanate metabolites was approximately 7 times greater after consumption of the preformed isothiocyanates than the unhydrolyzed glucosinolates (49). Recovery from uncooked, intact fresh sprouts without added myrosinase was reported to be ~50% (49). As seen in plasma, repeated dosing with either SF or GRP did not lead to an increase in daily urinary SF recovery (48).

Chewing of broccoli was shown to increase the bioavailability of SF from a plant matrix (49). Glucosinolate conversion to isothiocyanate was increased by 68% with thorough chewing of fresh, uncooked broccoli sprouts (49). Additionally, it seems that the meal matrix that broccoli is served with does not affect the conversion of GRP to SF, but may have an impact if SF is ingested as a preformed isothiocyanate (50). In this study, the absorption of preformed allyl isothiocyanate was 1.3 fold higher when consumed as part of a meat-containing meal than with a meal devoid of meat, but no difference in SF absorption was detected when broccoli was served with or without meat (50). The body of work encompassing the effect of meal matrix on GRP to SF conversion and subsequent uptake in humans is comprised only of this single piece of work. Therefore, more research is needed before definitive statements can be made.

As is the case with the time required to reach peak plasma total ITC concentration, the time required to reach peak urinary excretion of SF metabolites is also dependent on whether the source of SF is preformed isothiocyanate or GRP and if myrosinase is present. Urinary excretion of SF metabolites reaches peak concentration 3-6 h after consumption of fresh broccoli, but is delayed until 6-12 h after consumption of GRP (44). SF metabolites are almost completely eliminated from urine by 24 h post-consumption regardless of whether GRP was consumed in the absence or presence of myrosinase (36, 44).

Overall, glucosinolate availability and excretion is dictated by conversion to isothiocyanate. Thus, myrosinase is a key factor to isothiocyanate bioavailability. The result of GRP consumption without myrosinase is lower and delayed exposure to bioactive SF. Exogenous myrosinase from white mustard or daikon radish has been used to hydrolyze GRP to SF *ex vivo* as a way to prepare preformed isothiocyanates or to quantify GRP (40, 51). Our studies detailed in chapters 3 and 4 are the first to evaluate the possibility that myrosinase from a second food

source could provide hydrolytic activity to a food containing GRP, but lacking myrosinase *in vivo*.

2.5 Epigenetic basis of cancer

Epigenetics is defined as "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence" (52). Epigenetic mechanisms include DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs (52). Collectively, these elements are referred to as the epigenome. The epigenome is responsible for regulating access to genetic information. All cells in the body have identical DNA, but differ in their epigenetic landscape. The epigenome is thus responsible for cellular diversity in an organism (52). Improper epigenetic alterations, or epimutations, can significantly impact cancer development through activation or inhibition of various biological pathways, primarily by regulating access to genes. Interestingly, it has been suggested that epigenetic changes, rather than genetic mutations, may be responsible for the silencing of as many as half of the tumor suppressor genes that are aberrantly silenced in cancer (53). Thus, the packaging of the genome seems to be of equal importance to the genome itself in regulating cellular processes and contributing to the development and outcome of several chronic diseases.

DNA methylation

DNA methylation is catalyzed by a group of enzymes called DNA methyltransferases (DNMT). DNA methylatransferases act by transferring a methyl group from S-adenosylmethionine to the 5-position of the pyrimidine ring of cytosine (54). There are currently three known DNMT enzymes: two *de novo* methyltransferases (DNMT3A and 3B) and one maintenance

methyltransferase (DNMT1) (52, 55). The *de novo* DNMTs are independent of DNA replication (52). The maintenance DNMT has a preference for methylating hemimethylated DNA and acts during DNA replication, reproducing the methylation pattern of the parent strand (52). However, this model may be oversimplified as both groups of DNMTs have been shown to be capable of some level of both maintenance and *de novo* methylation (56).

Many cancers have an epigenetic basis whereby certain genes show an aberrantly high degree of promoter DNA methylation and an associated transcriptional silencing of expression (57). In mammals, DNA methylation occurs most typically on the cytosine residues within CpG islands, which are areas of DNA with a high concentration of CpG dinucleotides and often located in the promoter region of genes. In normal cells, most CpG sites throughout the genome are methylated, but islands of CpG sites in the promoter regions of genes remain unmethylated (52). In cancer cells, the DNA methylation pattern shifts to one characterized by global hypomethylation with site-specific hypermethylation of promoter CpG islands (52). These changes lead to genome instability and silencing of tumor suppressor genes, which results in a condition of uncontrolled growth, accumulated DNA damage and resistance to cell death (Figure 2.3) (52).

The mechanisms by which changes in the DNA methylation pattern occur and specific genes are targeted for hypermethylation are not well understood (52). One suggested mechanism involves the targeting of DNMT to specific genes by oncogenic transcription factors (58). Another proposed mechanism is that a reversible DNA binding polycomb protein is replaced by more stable DNA methylation through recruitment of DNMT by the polycomb protein, leading to permanent silencing of tumor suppressor genes (59). Yet another hypothesis is that a repressive epigenetic landscape can spread along DNA resulting in *de novo* DNA methylation and silencing

of tumor suppressor genes (60). Additionally, a general elevation in the expression level of DNMT enzymes likely plays a role in the observed changes of DNA methylation in cancer (61). In fact, DNMTs have been reported to be overexpressed in a variety of cancers including those of the breast, colon, lung and prostate (62-65). This may be due to loss or inhibition of the retinoblastoma protein, a tumor suppressor protein that is dysfunctional in nearly all cancers (66). Retinoblastoma is a repressor of the transcription factor E2F, which upregulates transcription of genes including DNMT1 (66). Therefore, when retinoblastoma is inactivated in cancer, the activity of E2F increases, resulting in elevated transcription of DNMT. Development of tools to decrease the expression and/or activity of DNMT enzymes has been a promising method for reducing the aberrant DNA methylation and thus permitting re-expression of tumor suppressor genes that are associated with cancer prevention. Currently, two DNA demethylating drugs have been approved by the Food and Drug Administration for use in certain cancers. These demethylating agents are 5-azacytidine and 5-aza-2'-deoxycytidine, which are detailed later in this section. Other demethylating agents are under development. The potential impact that dietary agents have on DNA methylation in cancer prevention is becoming of increasing interest. This is discussed in section 2.6.

Histone modifications

Post-translational modification of histone tails also plays a regulatory role within a cell. Histone tails can be methylated, acetylated, ubiquitylated, sumoylated, and phosphorylated (52). Together these modifications form a dynamic "histone code" that can regulate cellular activities by controlling the structure and activity of different regions of the chromosome (52). Histone modifications can lead to activation or repression of gene expression depending on the type of

histone modification and where the modification is located (52). Acetylation of histones is generally associated with transcriptional activation and is accomplished by histone acetyltransferase (HAT) enzymes (52). Histone deacetylase (HDAC) enzymes remove acetyl groups from histone tails and are involved in gene silencing. The opposing actions of HAT and HDAC determine the acetylation status of histones. Histone deacetylases are often found to be overexpressed in cancer, resulting in a global loss of histone acetylation (67, 68). By altering the acetylation state of histones, HDAC limits the access of transcriptional machinery to DNA, effectively silencing tumor suppressor genes (57, 69). Therefore, in the presence of an HDAC inhibitor, histones are more acetylated and the gene of interest is activated.

Epigenetic mechanisms act in concert

In addition to exerting individual mechanisms of control, DNA methylation and histone modifications can act cooperatively. Importantly, methylated DNA can recruit HDAC through methyl-CpG-binding proteins (70). Conversely, histone modifying enzymes can recruit the DNA methylation machinery (71). Therefore, HDAC and DNA methylation can work together to effectively silence genes.

Colon cancer

The global burden of cancer is increasing, largely because of population growth and increased life span, but also because of adopted habits including smoking, physical inactivity and consumption of a "westernized" diet (72). Fortunately, risk reduction of some cancers is possible by educating the public to avoid smoking, exercise regularly and consume a nutritionally balanced diet rich in vegetables.

Worldwide, an estimated 1.2 million new cases of colorectal cancer were diagnosed and over 600,000 deaths resulted from colorectal cancer in 2008 (72). This puts colorectal cancer as the third and second most commonly diagnosed cancer and the fourth and third cause of death worldwide for males and females, respectively (72). The incidence of colorectal cancer is rapidly increasing in geographical areas of historical low risk (72). Undesirable changes in dietary patterns as well as increases in obesity and smoking are believed to contribute to this concerning trend (72).

In the United States, the average overall cancer incidence increased between the years 1975 and 2000 (73). After 2000, the incidence rate began to decrease (73). However, partly because the mortality rate from heart disease has steadily decreased since 1975, cancer is now the leading cause of death for individuals under 85 years of age (73). According to the National Cancer Institute, colorectal cancer is the third most common cancer and third leading cause of cancer-related deaths in the United States. This is true for both men and women (73). Ironically, the United States is currently the only country with a decreasing trend of colorectal cancer incidence and mortality in both men and women (72, 73). This decrease is reflective of improvements in methods to detect and treat precancerous lesions (72, 73).

Epigenetic changes often occur during the earliest stages of cancer development, including colon cancer, providing an opportunity for epigenetically targeted dietary compounds to impact cancer prevention (53, 74). Evidence is accumulating supporting the hypothesis that epigenetic alterations play a major role in the initiation and progression of colorectal cancer (75, 76). As is the case in many cancers, the earliest stages of colorectal cancer are characterized by a depletion of global DNA methylation, leading to increased genomic instability (77). Hypermethylation of tumor suppressor and DNA repair gene promoter regions is also a predominant occurrence and

the most extensively characterized epigenetic abnormality of colorectal cancer (78). In colorectal cancer, many genes showing hypermethylated promoter regions are involved in signaling events that regulate cell cycle, DNA repair/stability, apoptosis, adhesion, angiogenesis, invasion and metastasis, among others (78). Dysregulation of histone acetylation and histone methylation also plays a well-established role in the progression of colorectal cancer (78). Additionally, both DNMT and HDAC enzymes are typically overexpressed in colorectal cancer, contributing to the state of gene silencing (64, 79, 80).

Two drugs currently approved by the Food and Drug Administration for used in certain malignancies are 5-azacytidine and 5-aza-2'-deoxycytidine (81). These drugs are nucleoside analogs that induce DNA hypomethylation by being incorporated into the growing DNA strand while it is synthesized during replication. Once incorporated, the nucleoside analogs trap DNMT enzymes onto the DNA, rendering them unable to methylate DNA at other sites (52). The trapping of DNMTs by these drugs leads to depletion of DNMT within the cells and results in successful inhibition of DNMT (52). Therefore, 5-azacytidine and 5-aza-2'-deoxycytidine are effective therapeutic agents. Unfortunately, these drugs commonly induce nausea, vomiting, diarrhea and myelosuppression (82). They also have the potential to form mutagenic lesions, which if not repaired, can lead to additional cancers (82). Thus, undesired side effects limit the treatment dose and duration of these drug therapies. Preventative treatment with these drugs may be an option for high-risk patients, but in most cases, these hypomethylating drugs are too toxic to use for preventative measures (53). Other nuceoside analogs as well as non-nucleoside, small molecule drugs are currently being investigated for effective DNMT inhibition with lower toxicity.

Because of direct contact, the digestive tract may be exposed to higher levels of dietary bioactive compounds compared to other organs. Therefore, dietary factors with epigenetic targets may offer a unique opportunity for cancer prevention and/or reduced tumor progression through a role in maintaining normal epigenetic regulation of gene expression. Due to lower toxicity, use of dietary factors rather than pharmaceutical agents may also help to minimize or eliminate the deleterious toxic side effects of present-day epigenetic therapies including hypomethylating drugs (83).

2.6 Dietary anticarcinogens have epigenetic effects

Several dietary bioactive components have been found to modulate the epigenome by altering DNA methylation and/or histone acetylation levels. This is often accompanied by an increase in expression of tumor suppress or genes (54, 84-86). Included among this group of bioactive dietary constituents are genistein, tea polyphenols/epigallacatechin 3-gallate, selenium and SF. Several differences have been observed concerning effective doses, responsive genes and responsive cell lines in regard to the epigenetic activity of the dietary bioactive agents that are discussed in the following subsections. These differences demonstrate potential for substantial specificity of dietary components to target genes and cell lines, which is likely to be of importance for future applications. These differences also highlight the need for properly designed animal and small clinical studies to confirm the observed *in vitro* effects and proposed mechanisms of action as well as to establish optimal doses and dosing frequencies before large scale clinical trials are commenced (87). Finally, it should be understood that using concentrations relevant to dietary consumption is critical when attempting to evaluate possible dietary means to reduce the risk of cancer. By studying concentrations that are attainable

through diet, results can be more easily translated to human use and can be used to establish non-toxic treatment levels not only demonstrated through a history of safe use, but also through simple toxicity studies.

Genistein

DNA demethylation has been observed in a variety of cancer cell types following exposure to genistein, an isoflavone from soy. A physiologically relevant, non-toxic dose of genistein was found to demethylate the promoter region of *GSTP1* in the MDA-MB-468 human breast cancer cell line (85). The demethylation was associated with restoration of expression of this previously silenced gene (85). Interestingly, this dose of genistein did not affect DNA methylation of the retinoic acid receptor, beta (*RAR-beta)* gene promoter, suggesting specificity (85). Genistein was also found to decrease DNA methylation and induce mRNA expression of *WNT5a* in SW1116 human colorectal adenocarcinoma cells (86). Additionally, genistein effectively reversed DNA hypermethylation and restored expression of *RAR-beta*, *P16* and *MGMT* in KSYE 510 human esophageal squamous cell carcinoma cells (88). Promoter demethylation and increased gene expression of *RAR-beta* was observed in LNCaP and PC3 human prostate cancer cells following genistein exposure, but the effective dose was much greater than that required to reverse methylation of the esophageal cancer cells, again suggesting specificity (88). Genistein treatment was also associated with repressed transcription of the human telomerase reverse transcriptase (*hTERT*) gene (89). Mechanistically, genistein was found to inhibit DNMT activity and protein expression (88-90). Histone acetylation was also increased following exposure to genistein (90, 91). The increase in acetylated histones occurred concurrently with both a

decrease in HDAC activity and an increase in histone acetyltransferase activity and expression (88, 90).

Tea polyphenols/epigallocatechin-3-gallate

Green tea, a popular beverage consumed worldwide, contains polyphenols that possess anti-carcinogenic activities. (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol found in green tea. Exposure of LNCaP cells to EGCG or a combination of green tea polyphenols resulted in re-expression of silenced *GSTP1* which correlated with demethylation of the *GSTP1* promoter and inhibition of DNMT protein expression and activity (92). The green tea polyphenol mix inhibited DNMT activity and increased *GSTP1* expression more effectively than EGCG alone (92). (-)-Eigallocatechin-3-gallate also increased mRNA and protein levels of silenced tumor suppressor genes *P16* and *P21* and decreased global DNA methylation as well as DNMT mRNA, protein and activity in A431 human epidermoid carcinoma cells (84). Another study confirmed the reversal of hypermethylation at *P16*, *RAR-beta*, *MGMT* and *hMLH1* by EGCG in KYSE 510 esophageal squamous cell carcinoma cells (93). The loss of methylation correlated with increased mRNA and protein expression of these genes (93). This study determined that EGCG inhibited DNMT activity through competitive inhibition by forming hydrogen bonds with proteins in the catalytic site of the DNMT enzyme (93). Computational modeling of EGCG and DNMT confirmed the likelihood of a direct inhibitory interaction (94). In contrast to demethylation causing increased gene expression, demethylation of the promoter region of *hTERT* by EGCG resulted in decreased mRNA expression in MCF-7 breast cancer cells (95). Interestingly, histone acetylation was decreased by EGCG treatment in this case (95). This paper proposed that DNA demethylation allowed the repressor complex including HDAC to

bind to the promoter of *hTERT* and resulted in decreased mRNA transcription and histone acetylation (95).

Green tea polyphenols were also found to increase histone acetylation and decrease HDAC activity, mRNA and protein level (84, 92). The changes in HDAC were accompanied by the changes in DNA methylation that were detailed in the previous paragraph (84).

Selenium

Selenium is a trace element and anti-oxidant that possesses anti-carcinogenic activity which may be partly due to alteration of the epigenome (54). LnCaP cells treated every 48 h for 7 days with 1.5 μM selenium in the form of sodium selenite resulted in DNA demethylation of the promoters and re-expression of the tumor suppressor genes *GSTP1*, adenomatous polyposis coli and cellular stress response 1 (96). The effect was both dose and time dependent for *GSTP1* (96). Conversely, a dose-dependent increase in DNA methylation of the *P53* promoter was reported in Caco-2 cells after exposure to 0, 1, or 2 μM sodium selenite (97). Sodium selenite did not affect promoter DNA methylation of *P16* (97). Sodium selenite effectively decreased the mRNA for DNMT1 and 3A with a reduction in protein level observed only for DNMT1 (96). Other studies confirm the decreased levels of DNMT1 protein and activity (98, 99). Additionally, HDAC activity was decreased with a concomitant increase in histone acetylation at H3-Lys 9 (96). Decreased histone methylation at H3-Lys 9 was also observed (96). Increased acetylation and decreased methylation at H3-Lys 9 are both associated with activation of gene expression. Selenium also appears to protect cultured cancer cell lines as well as rodents from DNA hypomethylation (97, 99). As discussed in the *DNA methylation* subsection of section 2.5, genomic DNA hypomethylation is a phenomenon characteristic of virtually all types of

neoplastic cells (100). Collectively, these observations suggest that regulation of DNMT and alteration of the histone code may be important epigenetic mechanisms of chemoprevention by sodium selenite and other selenium compounds. Interestingly, broccoli has the capacity to accumulate selenium when grown in an environment enriched with selenium (101, 102). The potential health benefits of this interaction will be discussed later in the subsection titled *Combination diets and therapies*.

Sulforaphane

Sulforaphane has repeatedly been demonstrated to inhibit HDAC activity in a variety of cell types including colorectal, breast and prostate cancer cells (31, 32, 80). In all cases, inhibition of HDAC activity was associated with an increase in histone acetylation levels. It is interesting to evaluate the research discussed in this section bearing in mind that SF concentrations over ~7 μM do not carry relevance to dietary SF (46).

A 15 μM dose of SF inhibited HDAC activity in prostate cancer cells and increased histone acetylation at the *P21* and *BAX* gene promoters, which was accompanied by increased protein expression of these genes (80). Sulforaphane also dose dependently inhibited HDAC activity in a colorectal carcinoma cell line (31). In this study, activity of HDAC was significantly decreased by 3 μM SF, although the effect was most pronounced with 15 μM SF treatment (31). In addition to inhibition of HDAC activity, an increase in histone acetylation and P21 protein expression was also observed at a concentration of 15 μM SF (31). Using molecular modeling, the mechanism of HDAC inhibition by SF was hypothesized to be direct inhibition by the SF metabolite SF-cysteine at the active site of the HDAC enzyme (31). In support of this hypothesis, inhibition of HDAC activity was fully reversed 18 h after SF was removed from the

culture medium of cells that had been exposed to SF for 6 h, demonstrating that SF or a metabolite of SF must be present to maintain HDAC inhibition (79). While some researchers report that HDAC protein levels remain unaltered with only the activity of HDAC being influenced by SF (31), other reports have claimed that HDAC protein levels decrease in response SF (79). The discrepancy between these observations was not due to the dose used as both studies utilized 15 μM SF. However, the duration of exposure may be of importance. Myzak et al. assayed HDAC protein expression 47 h after exposure to SF and saw no change in protein level (31) whereas Rajendran et al. assayed 24-36 h post-exposure and saw decreased HDAC protein expression (79). The cell type used may have also been a factor. Myzak et al. used human embryonic kidney 293 cells whereas Rajendran et al. used HCT 116 colon cancer cells (31, 79). Interestingly, the observed decrease in HDAC protein expression may have actually been the result of an increase in HDAC protein of the vehicle-treated control cells. In fact, the authors reported that HDAC activity in vehicle-treated control cells increased while activity in SF treated cells remained unchanged over time (79). Another group, using breast cancer cells, reported that SF had no effect on histone acetyltransferase activity (32). Therefore, it appears that SF may increase the acetylation level of histones through an inhibition of HDAC activity, independent of any change in histone acetyltransferase. Whether alteration of HDAC protein expression plays a role in the observed decrease in HDAC activity is not established. Overall, the mechanism by which SF results in decreased HDAC activity is not yet fully understood. One animal and one human study have examined the inhibition of HDAC by SF (80, 103). Mice treated orally with a single 10 μmol dose of SF showed significant inhibition of HDAC activity and increased histone acetylation in the colonic mucosa 6 h after dosing (80). Protein expression of p21 was unchanged at 6 h, but was doubled at the 24 and 48 h measurements (80). Daily

consumption of 6 μmol SF/day for 10 weeks resulted in a trend for increased histone acetylation and p21 expression in the ileum, colon, prostate and peripheral blood mononuclear cells (80). However, the only significant changes were for histone acetylation and p21 expression in mononuclear cells and for p21 expression in the ileum (80). Inhibition of HDAC activity in the prostate was also significant (80). Tumor development was suppressed in this mouse model and histone acetylation was increased in colonic polyps (80). The promoters for *p21* and *bax* showed a trend toward enrichment with acetylated histones in the ileum and colon of SF treated mice, but increases were only significant in the ileum (80). Protein expression of bax was increased in ileal, but not colonic polyps of SF treated mice (80). It was not reported whether the protein expression of p21 was measured in polyps. In another study, daily dietary treatment with 7.5 μmol SF suppressed the growth of prostate cancer xenographs in nude mice (103). Histone deacetylase activity was also decreased in the xenographs, prostates and mononuclear blood cells with a trend towards increased global histone acetylation and expression of bax protein (103). Finally, a single 600 μmol dose of SF given to human subjects through the consumption of broccoli sprouts inhibited HDAC activity in peripheral blood mononuclear cells 3 h post-consumption (103). The inhibition of HDAC activity was no longer significant 6 h post-consumption (103). Histone deacetylase activity returned to baseline level by 24 h (103). Overall, these results demonstrate that dietary SF inhibits HDAC activity *in vivo*.

A previous investigation of gene expression changes in Caco-2 cells after exposure to SF revealed an approximately 2-fold decrease of *DNMT1* mRNA (104). However, these cells were treated with 50 μM SF, a very high dose, which may have had unintended and/or non-specific effects on cellular activities. It was recently reported that 5 and10 μM SF downregulated DNMT1 and DNMT3a protein expression and resulted in decreased DNA methylation of the

hTERT gene in human breast cancer cells (32). The effects were more robust with 10 μM SF treatment compared to 5 μM SF (32). Contrary to the typical association of DNA methylation and increased gene expression, the observed DNA demethylation in this study correlated with *repression* of the gene of interest (32).

Combination diets and therapies

Whereas consuming a single bioactive component may prevent or reverse epimutations, there is potential to synergistically or additively enhance the effect by combining dietary bioactive components together or with drugs targeted to the epigenome. Combinations of dietary agents may provide enhanced health benefit by affecting different epigenetic mechanisms of cancer protection. Combining dietary agents with pharmaceuticals may allow for lower doses of potentially toxic drugs to be used, or for enhanced efficacy through targeting a variety of mechanisms.

It is well established that combining drugs targeted to different epigenetic modifications has beneficial outcomes in cancer studies. For example, the combination of the DNMT inhibitor 5-aza-2'-deoxycytidine with HDAC inhibitors such as valproic acid or trichostatin A has shown promising synergistic effects in clinical and non-clinical trials (105-108). Combining 5-aza-2'-deoxycytidine with conventional chemotherapeutic agents has also shown encouraging results in clinical trials, but further research is needed (109-111).

The potential for enhanced chemoprevention through the combination of bioactive dietary factors has already been realized. It was found that combining tomato and broccoli reduced the development of prostate tumors to a greater extent than either tomato or broccoli alone (112). The combination of SF with selenium synergistically increased the activity of thioredoxin

reductase in a cell culture model using human hepatoma cells (113). Additionally, broccoli enriched with selenium induced glutathione peroxidase activity while broccoli without selenium enrichment did not affect glutathione peroxidase activity (114). The synergistic effect between broccoli and selenium was also evident in rat models where selenium enriched broccoli or broccoli sprouts resulted in fewer mammary tumors and aberrant colon crypts compared to selenium with or without low-selenium broccoli (101). Whereas these studies demonstrate that foods and/or food components can be combined for enhanced efficacy in cancer risk reduction, few data exist that describe an additive or synergistic effect on cancer-related epigenetic endpoints in response to food-food or food-drug combinations. In a cell culture model, SF plus trichostatin A increased acetylated histones H3 and H4 to a greater extent than either treatment alone (31). Additionally, SF plus trichostatin A inhibited HDAC activity without affecting HDAC protein expression to any greater extent than SF or trichostatin A alone (31). A synergistic effect on the suppression of *hTERT* was observed after co-treatment with genistein and 5-aza-2'-deoxycytidine (89). In a separate study, combination of genistein with either trichostatin A or 5-aza-2'-deoxycytidine resulted in increased mRNA expression of *RAR-beta* and *P16* to a greater extent than individual treatments (88). Additionally, combination of genistein with SF enhanced the expression of *RAR-beta*, *P16* and *MGMT* in a synergistic or additive manner (88). Epigenetic endpoints such as DNA methylation and histone acetylation were not assessed in this study, but this is an important next step for establishing the mechanism(s) by which the expression of these genes were increased in response to the combination treatments utilized in this study. Despite the limited size of the body of information pertaining to epigenetic effects on cancer through the combination of dietary bioactive

components, either in a food-food or food-drug combination, the need to conduct these types studies has been recognized (54, 55).

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2.8 Figures

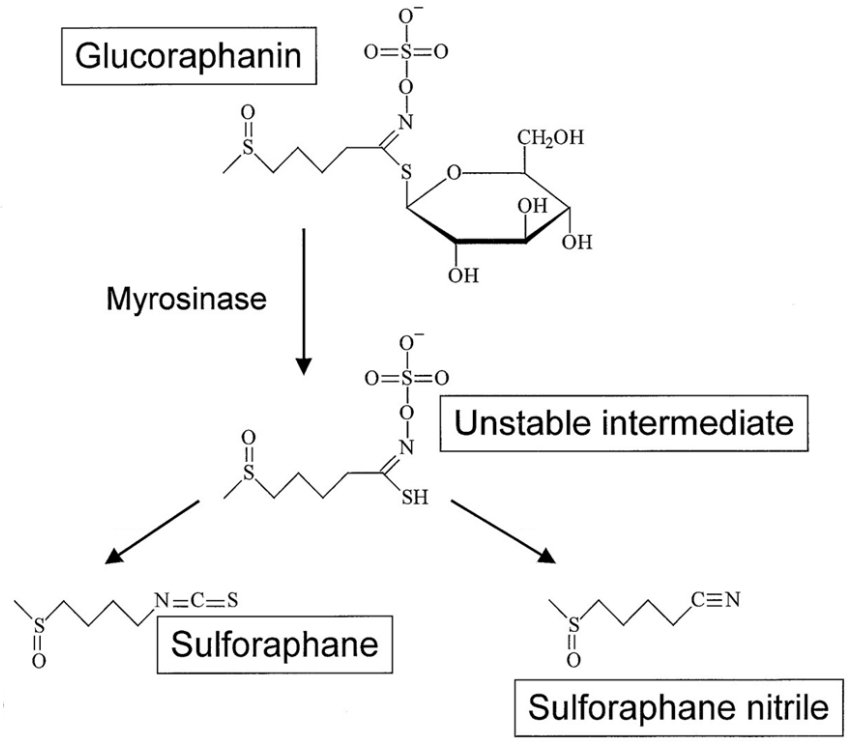

Figure 2.1. The myrosinase catalyzed hydrolysis of glucoraphanin leads to the production of sulforaphane and sulforaphane nitrile. Adapted from (115).

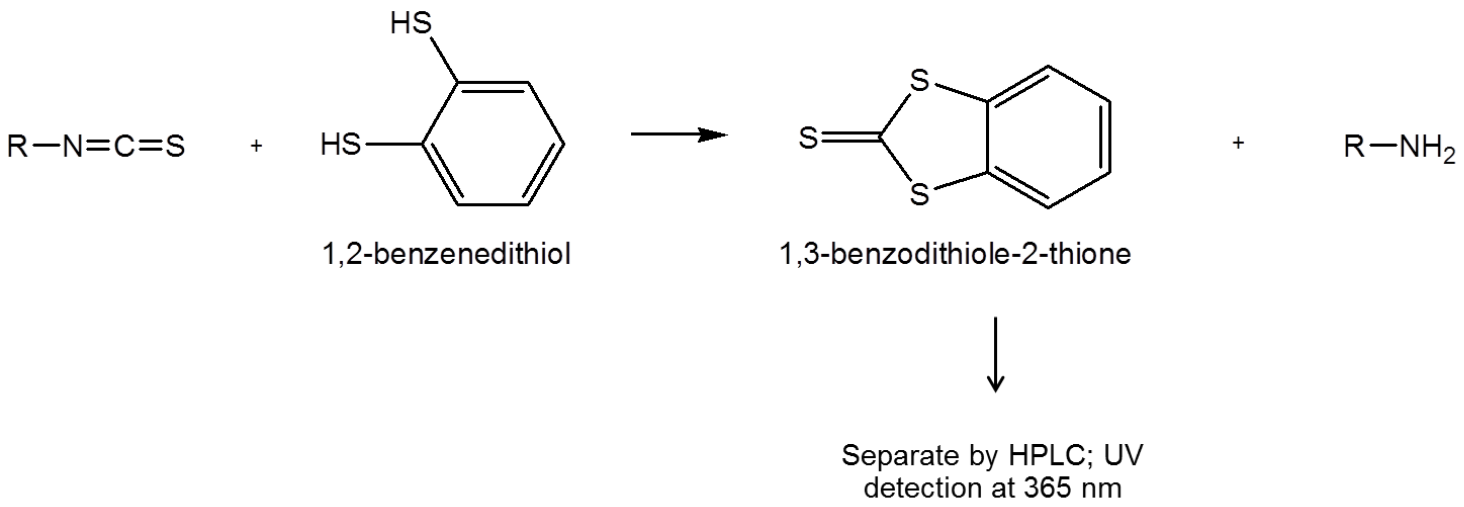

Figure 2.2. Isothiocyanates and isothiocyanate metabolites react with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione, which is detected for quantification of total ITC in the cyclocondensation reaction.

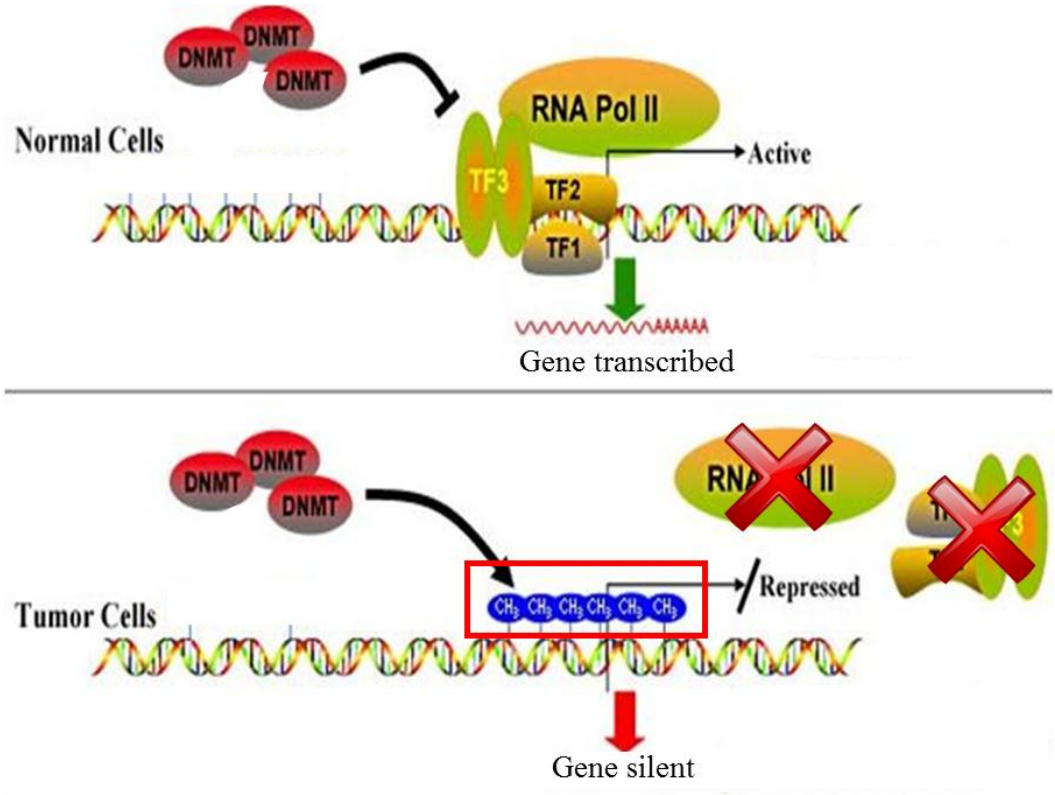

Figure 2.3. DNA methylation of the promoter region of tumor suppressor genes in normal cells and tumor cells. In normal cells, the promoter region of tumor suppressor genes is not methylated, allowing for gene transcription (upper panel). In tumor cells, the promoter region of tumor suppressor genes can become aberrantly hypermethylated through the activity of DNMT enzymes, blocking gene transcription (lower panel). Adapted from (116).

CHAPTER 3

Sulforaphane Absorption and Excretion Following Ingestion of a Semi-Purified Broccoli Powder Rich in Glucoraphanin and Broccoli Sprouts in Healthy Men

(Cramer and Jeffery. Nutr Cancer. 2011;63(2):196-201.)

3.1 Introduction

Sulforaphane (SF), an isothiocyanate derived from the hydrolysis of glucoraphanin found naturally in broccoli, is considered responsible for the cancer preventative benefits of broccoli consumption. The inactive precursor glucoraphanin is hydrolyzed to SF by the thiohydrolase myrosinase, found endogenously in broccoli and broccoli sprouts, or by microflora present in the colon (1).

A few small clinical studies have examined the bioavailability of SF in humans. The major metabolite of SF appearing in urine, SF-*N*-acetylcysteine (SF-NAC), is often used as a marker of bioavailability, although it is not the only metabolite present in urine (2, 3). Isothiocyanates and their dithiocarbamate metabolites (hereafter referred to as total ITC) react stoichiometrically with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione, which can be detected at 365 nm (4, 5). This cyclocondensation reaction has successfully been used to measure the very low concentrations of total ITC found in human plasma following a brassica meal (6, 7).

The metabolic fate of glucosinolates has been investigated following human ingestion of steamed or fresh broccoli (6). Excretion of urinary ITC metabolites was approximately three times greater from fresh broccoli than from steamed, where myrosinase had been heat-inactivated leaving glucoraphanin to be hydrolyzed to SF by gut microflora (6). A similar study evaluated appearance of total ITC in urine following ingestion of broccoli sprouts that had been either

completely hydrolyzed to ITC using exogenous myrosinase or contained only glucosinolates (myrosinase destroyed by boiling) (8). They found that 90% of the preformed ITC and only 12% of the glucosinolate dose was recovered as total ITC in urine (8). The fate of the remaining glucosinolates was not determined. Recovery from uncooked sprouts without added myrosinase was 50% (8). The study concluded that glucosinolate availability and excretion is dictated by conversion to ITC, and that myrosinase contributed to ITC bioavailability in sprouts (8).

It is a common public misconception that consumption of glucoraphanin, the inactive SF precursor, from well-cooked broccoli or dietary supplements that have no myrosinase provides health benefits comparable to those of SF. Here we tested the hypothesis that combining a glucosinolate preparation devoid of myrosinase, typical of many dietary supplements on the market, with a second product rich in myrosinase would enhance glucosinolate conversion and ITC absorption. Four healthy human males were provided two broccoli products, alone or in combination; dried broccoli sprouts containing myrosinase and a broccoli powder rich in glucoraphanin, but lacking myrosinase. This is the first study to examine a commercially available powdered broccoli preparation containing only glucoraphanin and to examine effects of combining this glucoraphanin-rich broccoli preparation with an exogenous food source of myrosinase, broccoli sprouts. The results show that combination improved availability, opening the door to development of products with enhanced chemo-protective potential.

3.2 Methods

All chemicals were purchased from Sigma Chemical, St. Louis, MO unless stated otherwise. Air-dried broccoli sprouts and broccoli powder were a gift from Caudill Seed, Inc., Louisville, KY.

Human subjects study population. Four healthy men of normal body weight, ages 18-30 years, were recruited by fliers at the University of Illinois at Urbana-Champaign. All subjects completed baseline questionnaires regarding dietary, tobacco and other drug or supplement use. All subjects signed informed consent documents before participating in the study. The study was approved by the University of Illinois Institutional Review Board in October 2007 and took place November 27-December 8, 2007.

Study design, meal administration and sample collection. Subjects were randomly assigned to a four by four crossover design. Meals were given on Tuesdays and Fridays resulting in a two or three day washout between meals; the half life for SF is reported as approximately 2 h (7, 9). Subjects were given a list of foods known to contain glucosinolates and asked to avoid these foods for three days prior to and during the entire duration of the study. Subjects were also requested to avoid use of dietary supplements and more than two alcoholic beverages per day during the study.

The morning of each trial, subjects were instructed to ingest a meal according to the crossover design. Meals included two grams of ground, air-dried broccoli sprouts or broccoli powder alone or in combination (2 g of sprouts plus 2 g of powder) added to one cup dry cereal (Go Lean Crunch, Kashi Company, La Jolla, CA) and ½ cup plain fat free yogurt (Stonyfield Farm, Londonderry, NH). The control meal included cereal and yogurt only. Blood (20 mL) was drawn into EDTA (for cyclocondensation) and/or Lithium Heparin (for liver panel) vacutainer tubes (BD, Franklin Lakes, NJ) immediately prior to ingestion of each meal (0 h), and at three and 24 h following the meal. Plasma was immediately prepared by centrifugation and stored at -80°C until analyzed. Urine samples were collected at baseline (0 h), 0-6, 6-12 and 12-24 h after

meal consumption. Baseline urine samples were kept on ice and ascorbic acid was added to the urine samples at 1 g/L urine no later than one hour following collection. All other urine samples were collected into 500 mL bottles containing 0.5 g ascorbic acid. Subjects were instructed to store samples in a cooler provided and return them the following morning at the 24 h blood draw. Samples were then stored at -80°C until analyzed.

Sulforaphane analysis. Air-dried broccoli sprouts were ground using a coffee grinder. In triplicate, ground broccoli sprouts (50 mg) were then added to 1.6 mL distilled H_2O, vortexed, and left to hydrolyze in the dark for 24 hours. The mixture was then centrifuged for 5 minutes at 14,000 x g and filtered through a 0.45 µm nylon filter. The supernatant was diluted 5-fold with distilled H_2O and an internal standard of benzyl isothiocyanate was added. In triplicate, broccoli powder was hydrolyzed in the same manner as broccoli sprouts described above except that 0.8 U white mustard myrosinase was added. The analysis of the broccoli powder was also conducted in the absence of added myrosinase to confirm the necessity of myrosinase in the conversion of glucoraphanin to SF. Isothiocyanates were extracted into dichloromethane for analysis by gas chromatography. Briefly, 1 µL extract was injected onto an Agilent 6890N gas chromatography system equipped with single flame ionization detection (Agilent Technologies, Santa Clara, CA). Samples were separated using a 30 m x 0.32 mm J&W HP-5 capillary column (Agilent Technologies). After an initial hold at 40°C for 2 min, the oven temperature was increased by 10°C/min to 260°C and held for 10 min. Injector temperature was 200°C; detector temperature was 280°C. Helium carrier gas flow rate was 25 mL/min. Data output was analyzed by Agilent ChemStation software and compared to SF (LKT Laboratories, St. Paul, MN) and benzyl isothiocyanate standard curves.

Hepatic function panel. Blood samples were collected in Lithium Heparin tubes and centrifuged at 1000 x g for 30 minutes. Plasma was collected and stored frozen at -80°C in the dark until routine hepatic function panel analysis by Provena Covenant Medical Center (Urbana, IL).

Plasma isothiocyanate analysis. Blood samples collected in EDTA tubes were centrifuged at 1000 x g for 30 minutes. Plasma was collected and analyzed using a slight modification of the cyclocondensation method previously described (7). Briefly, 200 μL of 100 mmol/L potassium phosphate buffer and 400 μL of 20 mmol/L 1,2-benzenedithiol were combined with 200 μL plasma in a 4.0 mL sealable glass vial. The mixture was incubated for two hours at 65°C, cooled to room temperature and centrifuged at 10,000 x g for two minutes. The supernatant (100 μL) was injected onto an analytical C_{18} reverse-phase column (ES Industries Marvel ODS-3, 5 μm, 250 x 4.6 mm, West Berlin, NJ) attached to a Waters HPLC system (Waters, Milford, MA). The solvent system was operated isocratically with 80% methanol/20% water at a rate of 1.0 mL/min. A five-minute time interval was assigned for column washing between sample injections. The cyclocondensation product, 1,3-benzodithiole-2-thione, was analyzed by absorption at 365 nm. For quantification, the peak area of 1,3-benzodithiole-2-thione (eluting between 10-11 minutes) was integrated using Empower Pro software (Waters) and compared to a standard developed by reacting known concentrations of pure allyl isothiocyanate with 1,2-benzenedithiol.

Urinary N-acetylcysteine conjugate analysis. Urine samples were analyzed as previously described (6, 10). The filtered urine (50 μL) was analyzed by HPLC using a Hypersil C18 ODS column (10 μm, 250 x 4.6 mm, Phenomenex, Torrance, CA) and detected at 254 nm using the

Waters HPLC system described above. A gradient solvent system with a flow rate of 1 mL/min consisted of a starting solvent system of 5% aqueous acetonitrile and 95% water. The acetonitrile was linearly increased to 20% over three minutes, held four minutes, then increased to 100% over two minutes and held thirteen minutes to rinse the column. Both solvents contained 1.0% acetic acid. A standard was generated in control urine using SF-NAC synthesized as previously described (10).

Statistical analysis. Data were evaluated using the GLIMMIX procedure of SAS Statistical software (version 9.1; SAS Institute, Cary, NC). Levels of SF metabolites in blood and urine were tested for interactions of treatment and time. Differences were separated using the SLICEDIFF option. Values were considered different from controls at $p<0.05$.

3.3 Results

Sulforaphane content of hydrolyzed broccoli sprouts and powder. Upon incubation in water at room temperature for 24 h, air-dried broccoli sprouts produced 36.9 ± 1.8 µmol SF per gram (approximately 70 µmol / 2 g dose), whereas broccoli powder produced no SF. Upon addition of myrosinase, broccoli powder produced 61.7 ± 2.1 µmol SF per gram (approximately 120 µmol / 2 g dose).

Hepatic function panel. The mean measurements for each meal at 24 h post-consumption are given in Table 3.1a. Among meals, no differences were detected for any parameter measured. Most readings were within the normal range; individual panel results outside the normal range are shown in Table 3.1b. All measurements of direct and total bilirubin, alkaline

phosphatase and total protein fell within reference ranges.

Plasma total isothiocyanates. Three hours post-ingestion, broccoli sprout and combination meals showed elevated total ITC levels in plasma with mean concentrations of 0.9 ± 0.0 and 2.1 ± 0.5 µmol/L, respectively (Figure 3.1). These levels returned to baseline values by 24 h. Values for total ITC in plasma following the broccoli powder meal were not different from control at any of the time points measured. When plasma values following the broccoli powder meal were compared longitudinally, despite an observed increase between 0 h (0.2 ± 0.0 µmol/L) and 24 h (0.5 ± 0.1 µmol/L) post-consumption, the difference was not significant (Figure 3.1).

Sulforaphane-N-acetylcysteine in urine. The amounts of SF-NAC excreted in the urine collected over different time periods within the first 24 hours after ingestion of control and treatment meals are shown in Figure 3.2 and Table 3.2. When individuals ingested broccoli sprouts in combination with broccoli powder, they excreted a mean of 93.8 ± 11.8 µmol SF-NAC over 24 hours post-ingestion; 49% of the dose ingested (Figure 3.2). After ingestion of broccoli sprouts alone, a mean of 52.0 ± 1.4 µmol SF-NAC was excreted; 74% of the ingested dose. However, after ingesting broccoli powder alone, a mean of only 22.6 ± 2.1 µmol SF-NAC was excreted, 19% of the dose ingested.

Urine collection was separated into discrete intervals for evaluation of SF-NAC excretion: urine was collected for the first 6 h after meal ingestion (T1), from 6-12 h post ingestion (T2) and from 12-24 h post ingestion (T3). Significant differences were seen among dietary groups, with substantial levels of SF-NAC excretion detected during T1 for both the combination meal and the broccoli sprouts meal (44% and 37% of total SF-NAC excreted within 24 h, respectively),

but less than 10% during this first 6 h period from those receiving the broccoli powder meal (Table 3.2). In contrast, only 22% of total from the combination or sprouts meals was excreted in the second 12 h period (T3), whereas 39% of SF-NAC excreted following the broccoli powder meal was excreted during this later time period.

The *N*-acetylcysteine conjugate of erucin, the reduced form of SF, was detected in trace amounts in urine from those receiving both the combination and the sprouts meals (data not shown). No erucin metabolite was detected following the powder meal alone. Treatment of urine samples with excess myrosinase did not yield free ITC, indicating that glucoraphanin was not present in urine (data not shown).

3.4 Discussion

In the present study, we measured metabolites in plasma and urine to compare the absorption of SF among two broccoli products, alone and in combination; dried broccoli sprouts rich in myrosinase and a broccoli powder rich in glucoraphanin, but lacking myrosinase. Each of four subjects ate each meal separated by two or three days washout. Four subjects is a small study population and unfortunately, most clinical studies focusing on glucoraphanin and SF have similar small numbers (6-8, 11). This study was intended to provide data as a pilot study and future large scale work is needed to provide greater statistical power and precision.

The hypothesis, that myrosinase from the sprouts would enhance SF absorption from the broccoli powder, was confirmed. Within whole sprouts, myrosinase is located separately from the glucosinolates and hydrolysis of glucoraphanin to SF is therefore not initiated until the onset of chewing. The trend for greater levels of SF-NAC to be excreted early (during T1 and T2) from the combination and sprouts meals is consistent with metabolism occurring in the upper

gastrointestinal tract in the presence of dietary myrosinase (Table 2). For the broccoli powder meal, hydrolysis may not occur until the meal reaches the colon, the microflora of the gut being the source of myrosinase-like thiohydrolase activity (1, 12, 13). The consequence of late and incomplete hydrolysis by colonic microflora is a lower peak plasma level of cancer-preventative SF.

Previous reports providing an ITC extract from hydrolyzed broccoli sprouts (7, 8, 12) showed that SF was highly bioavailable, with approximately 80% of the SF dose being recovered as metabolites in the urine. Although we found a very similar recovery from the dried, ground sprouts, fresh unhydrolyzed sprouts have been reported to differ in bioavailability depending upon the extent of chewing before swallowing (8). It remains to be determined if fresh sprouts can enhance bioavailability of SF from a broccoli preparation lacking myrosinase. Here we found that plasma levels of total ITC were elevated 3 h after consumption of dried, ground sprouts. Broccoli sprouts are an excellent source of SF, as they contain 10-100 fold higher concentrations of glucoraphanin compared to mature broccoli. However, they have a spicy "radish" taste which may be un-appetizing to some individuals. To these individuals, broccoli powder may be a tempting alternative, as it lacks flavor and has a high concentration of glucoraphanin compared to commercial mature broccoli or sprouts. The glucoraphanin-rich broccoli powder is also heat stable with a prolonged shelf life. Therefore it can be used in other products, such as dietary supplements, many of which are on the market today. However, since it lacks the myrosinase enzyme, SF bioavailability and hence potential for cancer prevention by this product is in question. This study is the first to show the poor yield of SF from such a product and the potential for a separate dietary source of myrosinase to improve the SF yield.

The broccoli powder, lacking myrosinase, was a poor dietary source of SF compared to broccoli sprouts, with only 20% of the dose being recovered as SF-NAC in urine. This value is comparable to reported recovery when cooked sprouts or mature broccoli plants are ingested (6, 8, 12). Plasma total ITC was not altered at 3 h in response to the powder meal, but there was a trend toward an increase in plasma total ITC at 24 h, suggestive of delayed absorption (9). This delayed hydrolysis is consistent with a lack of myrosinase in the powder resulting in late conversion of glucoraphanin to SF by gut microflora. The possibility was considered that the maximum plasma ITC level was delayed rather than absent following ingestion of the powder, and thus was not measured in our study. However, urinary recovery of SF-NAC was low not only for the first 6 h, but for the entire 24 h period post consumption, suggesting that any possible spike in plasma ITC levels would not be very great relative to that observed for sprouts or the combination meal.

Data from the combination meal were interesting as they identified possible synergy among the sprouts and powder at early time point measurements of plasma and urine recovered metabolites. This indicates that endogenous myrosinase from the broccoli sprouts had the opportunity to hydrolyze glucoraphanin not only from the broccoli sprouts but also from the broccoli powder. Whereas our results show that the full 24 h urinary excretion of SF-NAC was additive in the combined meal relative to the individual sprouts and broccoli powder meals, plasma total ITC for the combined meal at 3 h suggested synergism, in that the 3 h plasma total ITC value for the combined meal was significantly greater than the sum of the values obtained for the two individual meals at 3 h. This is further supported by the finding that excretion of SF-NAC following the combination meal was earlier than from the broccoli powder alone. The early elevated plasma ITC would translate into an increased potential for cancer-preventative activity.

With only 50% of the dose from the combination meal recovered as SF-NAC, it is possible that the large amount of unhydrolyzed glucoraphanin from the combination meal may have exceeded the capacity of the myrosinase enzyme in the sprouts, leaving myrosinase as the limiting factor in this meal. If this was the case, then increasing the proportion of sprouts to broccoli powder may have given even higher SF absorption from the broccoli powder. Detection of low amounts of SF-NAC for a few baseline readings is unaccounted for, but may be due to accidental ingestion of glucoraphanin-containing products during the washout period. No significant carry-over effect was detected.

With regard to safety, the doses provided in this study were similar to or less than that provided as whole broccoli in an earlier study (6). Values of alanine and aspartate aminotransferase below normal range (Table 3.1b) do not indicate liver damage or toxicity. The high value for alanine aminotransferase of subject 101 following the broccoli powder meal was also observed following the control meal for that individual, suggesting that the elevation was not caused by the broccoli meal and that slight elevation of this value may be normal for this individual. No other listed abnormal values were significant. We conclude that none of the broccoli meals caused any hepatotoxicity.

Taken together, myrosinase from broccoli sprouts allowed early hydrolysis of glucoraphanin from broccoli powder, resulting in early and more complete absorption.

3.5 References

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3.6 Tables and figures

Table 3.1a. Mean Values of Liver Function Test

	Normal Range	Control	Powder	Sprout	Combination
ALT (U/L)	21-72	38.8 ± 16.2	34.3 ± 14.9	34.3 ± 12.7	34.0 ± 12.6
AST (U/L)	20-57	28.3 ± 8.7	27.5 ± 9.5	27.8 ± 8.2	28.0 ± 9.0
Albumin (g/dl)	3.4-4.8	4.5 ± 0.1	4.4 ± 0.1	4.5 ± 0.0	4.5 ± 0.0
Alk Phos (U/L)	38-126	67.0 ± 6.5	66.8 ± 7.0	67.3 ± 8.5	66.8 ± 6.8
Bilirubin, D (mg/dl)	0.0-0.3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Bilirubin, T (mg/dl)	0.0-1.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.1
Bilirubin, I (mg/dl)	0.2-1.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
T Protein (g/dl)	6.4-8.3	7.4 ± 0.2	7.4 ± 0.2	7.5 ± 0.2	7.5 ± 0.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Alk Phos, alkaline phosphatase; D, direct; T, total; I, indirect. Mean ± SEM (n=4) of liver function test values at 24 h following test meal consumption.

Table 3.1b. Abnormal Liver Function Test Values

Test	Reference Range	Subject	Treatment	Time	Observed Value
ALT	21-72 U/L	101	Control	24 h	84
		101	Powder	24 h	77
		102	Control	24 h	12
		102	Powder	0 h	12
		102	Powder	24 h	14
		102	Sprout	24 h	12
		102	Combination	24 h	12
		104	Control	24 h	19
		104	Powder	0 h	13
		104	Powder	24 h	14
		104	Sprout	24 h	18
		104	Combination	24 h	18
AST	20-57 U/L	102	Control	24 h	18
		102	Powder	0 h	17
		102	Powder	24 h	20
		102	Sprout	24 h	18
		102	Combination	24 h	18
		103	Powder	24 h	18
		104	Control	24 h	19
		104	Powder	0 h	15
		104	Powder	24 h	16
		104	Sprout	24 h	17
		104	Combination	24 h	18
Bilirubin, indirect	0.2-1.0 mg/dl	104	Powder	0 h	0.2
Albumin	3.40-4.80 g/dl	101	Control	24 h	4.87
		102	Powder	24 h	4.82

ALT, alanine aminotransferase; AST, aspartate aminotransferase. No abnormal values were found at any time point for the following determinations: alkaline phosphatase (38-126 U/L); bilirubin, direct (0.00-0.30 mg/dl); bilirubin, total (0.00-1.00 mg/dl); total protein (6.4-8.3 g/dl).

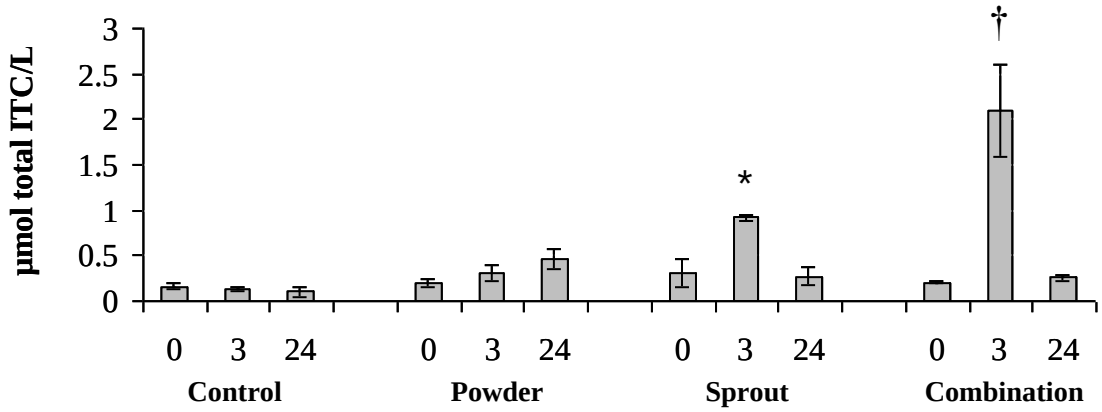

Figure 3.1. Mean values of total ITC in plasma at 0, 3, and 24 h after consumption of test meals. Data are presented as means of four subjects per group ± SEM. Values indicated by an asterisk (*) are significantly different from the control, powder and combination groups, $p<0.05$. Values indicated by a dagger (†) are significantly different from the control, powder and sprout groups, $p<0.05$.

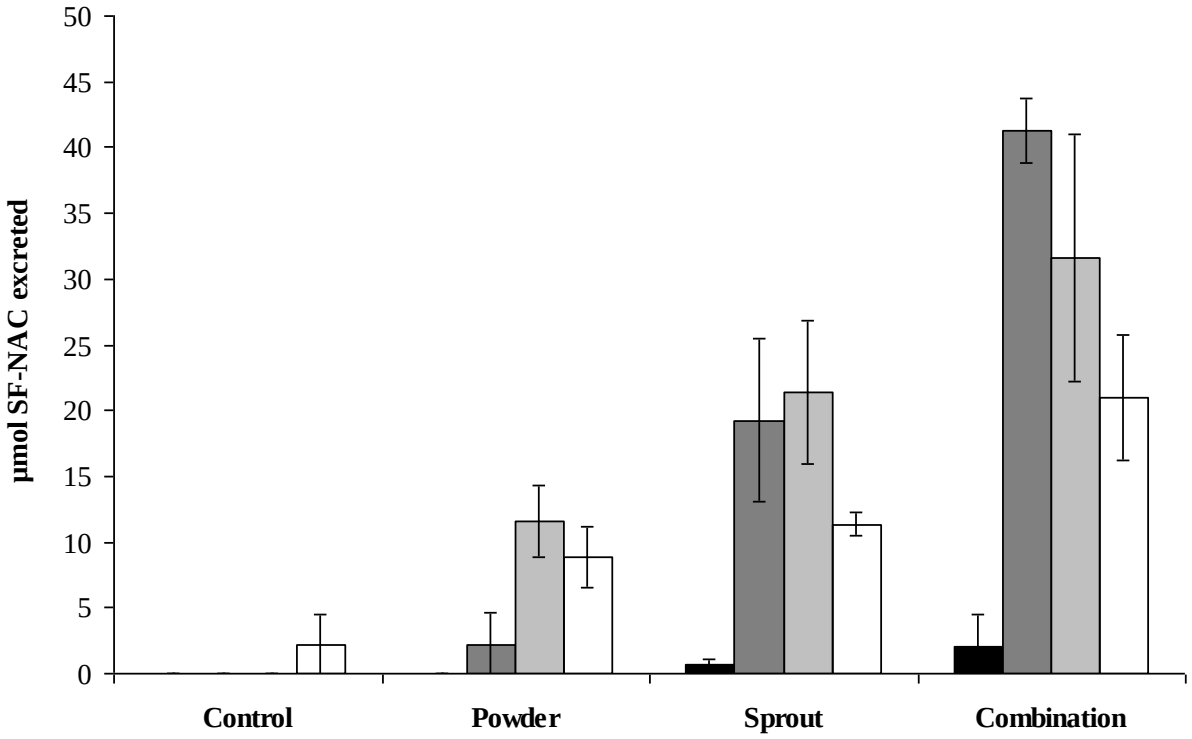

Figure 3.2. Urinary SF-NAC excretion after consumption of control, powder, sprout and combination meals. Baseline (black bars), T1 = 0-6 h (dark grey bars), T2 = 6–12 h (light grey bars), T3 = 12-24 h (open bars) urine collection post consumption. Data are presented as means of four subjects per group ± SEM. Statistical significance is detailed in Table 2.

Table 3.2. Twenty-Four Hour SF-NAC Excretion

	Baseline (0 h)	T1 (0-6 h)	T2 (6-12 h)	T3 (12-24 h)
Control	nd^{a,1}	nd^{a,1}	nd^{a,1}	2.1 ± 2.4^{a,1}
Powder	nd^{a,1}	2.1 ± 2.5^{a,1,2}	11.6 ± 2.7^{b,3}	8.9 ± 2.4^{a,2,3}
Sprout	0.7 ± 0.5^{a,1}	19.3 ± 6.2^{b,2,3}	21.4 ± 5.5^{b,2}	11.4 ± 1.0^{a,3}
Combination	2.1 ± 2.4^{a,1}	41.3 ± 2.4^{c,2}	31.6 ± 9.4^{c,3}	21.0 ± 4.8^{b,4}

nd, below the limit of detection. Data are presented as means (μmol) of four subjects per group ± SEM. Mean excretion was compared for each time period between control, powder, sprout, and combination meals. Within **each column** (between-meal values) or **each row** (within-meal values) means that do not share a superscript letter or number, respectively, are significantly different, $p<0.05$.

CHAPTER 4

Enhancing Sulforaphane Absorption and Excretion in Healthy Men through the Combined Consumption of Fresh Broccoli Sprouts and a Glucoraphanin-Rich Powder

(Cramer et al. Br J Nutr. 2011;DOI: 10.1017/S0007114511004429.)

4.1 Introduction

Sulforaphane (SF), found in broccoli as its inactive precursor glucoraphanin (GRP), is considered to be responsible for the reduction of cancer risk that is associated with broccoli consumption. Upon crushing or chewing of fresh broccoli or broccoli sprouts, GRP is hydrolyzed to SF by the plant thiohydrolase myrosinase. In instances of myrosinase inactivation, such as over-cooking of broccoli, GRP can be hydrolyzed to SF by microflora present in the lower gut (1, 2). However, GRP hydrolysis by microflora of the lower gut is far less efficient than hydrolysis by endogenous broccoli myrosinase (3-6).

Sulforaphane protects against the incidence and progression of cancer via several mechanisms including inhibiting phase I cytochrome P450 enzymes, inducing cell cycle arrest and apoptosis, reducing inflammation, and perhaps most well-characterized, modulating the nuclear factor-erythroid-2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway (7). In the body, SF is metabolized by the mercapturic acid pathway and excreted in urine, mostly as sulforaphane-*N*-acetylcysteine (SF-NAC) (8, 9). The fate of non-hydrolyzed GRP is less well understood. A recent study reported that low amounts of intact GRP were recovered in the urine of human subjects after consumption of a GRP-rich beverage, but not after consumption of SF-

rich beverage (9). Another study reported that low amounts of intact GRP were recovered in the urine, but not in the feces of rats that were fed purified GRP (10).

Several small clinical studies have examined the absorption and excretion of SF in humans. When urinary excretion of isothiocyanates (ITC) was measured following ingestion of fully cooked or fresh/lightly cooked broccoli, urinary ITC metabolites were approximately three times greater from fresh/lightly cooked broccoli than from fully cooked broccoli where myrosinase had been heat-inactivated (3, 4). Similar studies evaluated the appearance of total ITC in urine following ingestion of broccoli sprouts that had been either completely hydrolyzed to ITC using exogenous myrosinase or contained only GRP where the myrosinase had been destroyed by boiling (5, 9). It was found that ITC excretion was much greater after consumption of the preformed ITC compared to the GRP preparations (5, 9). The results of these studies suggest that the conversion of GRP to SF and subsequent ITC bioavailability is dependent on active myrosinase.

In a previous study, a commercially available GRP-powder devoid of myrosinase, typical of many dietary GRP supplements on the market, was examined for its potential to deliver bioactive SF to human subjects alone or in combination with air-dried broccoli sprout powder, which served as an exogenous food source of myrosinase (6). The results showed that the combination improved the absorption of SF, and thus opened the door to the potential for enhanced cancer risk reduction not only from GRP supplements, but also from specifically designed foods or food combinations (6).

Due to commercial availability and consumer preferences, intact fresh broccoli sprouts are more likely to be ingested by humans than the air-dried broccoli sprout powder used in our previous study. However, fresh broccoli sprouts may present additional variables such as matrix effects or

product variability that were not present when examining the air-dried broccoli sprout powder. Therefore, the current study examined the same commercially available GRP-powder used in our previous study, but here, intact fresh broccoli sprouts were used as the exogenous food source of myrosinase. The present study also expanded the number of blood samples collected to better capture the differences in SF appearance in plasma from the test meals. The study sought to determine if the fresh broccoli sprouts would enhance GRP conversion and ITC absorption from the GRP-powder.

4.2 Methods

Fresh broccoli sprouts were donated by Tiny Greens Organic Farm, Urbana, IL, USA. Broccoli powder was a gift from Caudill Seed, Inc., Louisville, KY, USA.

Human subjects study population. Four healthy men, ages 18-30, were recruited by fliers at the University of Illinois at Urbana-Champaign. Before participating in the study, each subject completed baseline questionnaires regarding dietary supplement, tobacco and other drug use. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Illinois Institutional Review Board. Written informed consent was obtained from all subjects. The study took place January 31-February 25, 2009.

Study design, meal administration and sample collection. Subjects were randomly assigned to a four by four crossover design. Subjects were given a list of foods known to contain glucosinolates and asked to avoid these foods for three days prior to and throughout the entire

duration of the study. Subjects were also requested to avoid the use of dietary supplements and to limit alcohol consumption to no more than two alcoholic beverages per day during the study. Experimental meals were given each Tuesday for four weeks resulting in a six day washout period between meals; the half life for SF is reported as approximately 2 h (11, 12). The morning of each trial, subjects were instructed to ingest a meal according to the crossover design. In the case of sprout-containing meals, subjects were also instructed to chew the sprouts thoroughly. Meals included 5 day old intact fresh broccoli sprouts of the calabrese variety (approximately 42 g) or GRP-powder (2 g) in an amount that produced 70 or 120 μmol SF, respectively, determined by bench hydrolysis. The combination meal contained both intact fresh broccoli sprouts (approximately 42 g) and GRP-powder (2 g). The GRP-powder was a proprietary dry, defatted broccoli seed powder preparation that did not contain myrosinase. Experimental meals were accompanied by one cup (53 g) of dry cereal (Go Lean Crunch, Kashi Company, La Jolla, CA, USA) and ½ cup (113.5 g) french vanilla fat free yogurt (Stonyfield Farm, Londonderry, NH, USA) to serve as a vehicle and control meal. Thus, the control meal included cereal and yogurt only. Blood (8 ml) was drawn into EDTA vacutainer tubes (BD, Franklin Lakes, NJ, USA) immediately before ingestion of each meal (0 h), and at 0.5, 1.0, 1.5, 3.0 and 24 h following the meal. Plasma was immediately prepared by centrifugation and stored at -80°C until analyzed. Urine samples were collected at baseline (0 h), 0-6, 6-12 and 12-24 h after meal consumption. All urine voided during these time intervals was collected. The volumes were recorded and used to calculate total μmol of SF-NAC excreted. Baseline urine samples were kept on ice and ascorbic acid (Fisher Scientific, Pittsburgh, PA, USA) was added to the urine samples at 1 g/l urine no later than one hour following collection. All other urine samples were collected into 500 ml bottles containing 0.5 g ascorbic acid. Subjects were

instructed to store urine samples in a provided cooler and return them the following morning when the 24 h blood samples were collected. Urine samples were then stored at -80°C until analyzed.

Sulforaphane analysis. In triplicate, GRP-powder (50 mg) was added to 1.6 ml distilled H_2O containing 0.8 U white mustard myrosinase (Sigma Chemical, St. Louis, MO, USA), vortexed, and left to hydrolyze in the dark for 24 hours. The mixture was then centrifuged for 5 minutes at 14,000 x g and filtered through a 0.45 µm nylon filter. The supernatant was diluted 5-fold with distilled H_2O and an internal standard of benzyl isothiocyanate (Sigma Chemical, St. Louis, MO, USA) was added. The analysis of the GRP-powder was also conducted in the absence of added myrosinase to confirm the necessity of myrosinase in the conversion of GRP to SF. Fresh broccoli sprouts were obtained the day before each trial meal and analyzed for SF production upon hydrolysis using a modification of a previously reported method (13). In triplicate, 0.25 g fresh broccoli sprouts were heated at 90°C for 5 min in a glass vial containing 2 ml dH_2O. Following heating, samples were cooled on ice, homogenized and 0.5 U white mustard myrosinase was added. Samples were vortexed and left to hydrolyze at room temperature for 2 h. The samples were then centrifuged for 5 min at 14,000 x g. The supernatant filtered through a 0.45 µm nylon filter and diluted 4-fold with distilled H_2O. An internal standard of benzyl isothiocyanate was added. Isothiocyanates were extracted into dichloromethane for analysis by gas chromatography, as previously described (6).

Plasma total isothiocyanate analysis. Blood samples were collected in EDTA-coated tubes and centrifuged at 1000 x g for 30 minutes. Plasma was collected and analyzed as

previously described (6). This method provides a single total measurement for SF, other ITCs and metabolites (14, 15).

Urinary sulforaphane-N-acetylcysteine analysis. Urine samples were analyzed as previously described (6). Briefly, the filtered urine (50 µL) was analyzed by HPLC using a Hypersil C18 ODS column (10 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA, USA) and detected at 254 nm using a Waters HPLC system. A gradient solvent system with a flow rate of 1 ml/min consisted of a starting solvent system of 5% aqueous acetonitrile (Fisher Scientific, Pittsburgh, PA, USA) and 95% water. The acetonitrile was increased linearly to 20% over three minutes, held four minutes, then increased to 100% over two minutes and held thirteen minutes to rinse the column. Both solvents contained 1.0% acetic acid (Fisher Scientific, Pittsburgh, PA, USA). A standard was generated in control urine using SF-NAC synthesized as previously described (16).

Statistical analysis. Data were evaluated using the GLIMMIX procedure of SAS Statistical software (version 9.1; SAS Institute, Cary, NC). Levels of SF metabolites in urine and blood were tested for interactions of treatment and time. Differences were separated using the SLICEDIFF option. Values were considered different at $p<0.05$.

4.3 Results

Sulforaphane content of hydrolyzed broccoli sprouts and GRP-powder. Upon incubation in water at room temperature for 24 h with the addition of 0.8 U myrosinase, GRP-powder produced 61.7 (SE 2.1) µmol SF/g powder. No SF was produced in the absence of added

myrosinase. Fresh broccoli sprouts produced 1.69 (SE 0.12) µmol SF/g fresh weight.

Plasma total isothiocyanates. Plasma ITC were elevated compared to baseline at 0.5 h in both the sprout and combination meals (Table 4.1). The combination meal reached peak plasma concentration (2.86 (SE 0.33) µmol/l) 1.5 h after ingestion. The sprout meal peaked at 3 h (1.53 (SE 0.22) µmol/l), but this value was not different than the value at 1.5 h (1.43 (SE 0.21) µmol/l). The GRP-powder meal showed slightly elevated plasma concentration levels 3 h post consumption (0.37 (SE 0.25) µmol/l). However, values following the GRP-powder meal were not different from the control meal or baseline measurements at any of the time points measured. All values returned to baseline values by 24 h.

Sulforaphane-N-acetylcysteine in urine. The amount of SF-NAC excreted in the urine over 24 h following ingestion of each meal is shown in Figure 4.1. After ingestion of fresh broccoli sprouts in combination with GRP-powder, individuals excreted a mean of 123.8 (SE 8.8) µmol SF-NAC over 24 hours post-ingestion, 65% of the dose ingested. After ingestion of broccoli sprouts alone, a mean of 42.0 (SE 11.8) µmol SF-NAC was excreted, 60% of the ingested dose. However, after ingesting GRP-powder alone, a mean of only 29.2 (SE 5.0) µmol SF-NAC was excreted, 24% of the dose ingested.

Urine collection was separated into discrete intervals for evaluation of SF-NAC excretion: urine was collected for the first 6 h after meal ingestion, from 6-12 h and from 12-24 h post ingestion. Significant differences were observed between dietary groups. Considerable levels of SF-NAC were excreted during the first six hours from individuals who received the combination meal or the broccoli sprouts meal (61% and 62% of the total SF-NAC that was excreted during the entire

24 h urine collection, respectively), but less than 22% of total 24 h SF-NAC was excreted during this first 6 h period from those receiving the GRP-powder meal alone. In contrast, less than 10% of total 24 h SF-NAC recovered from the combination or sprouts meals was excreted during the 12-24 h period, whereas 42% of total 24 h SF-NAC excreted following the GRP-powder meal was excreted during this later time period.

4.4 Discussion

The main findings of this study were that combining fresh broccoli sprouts with the GRP-powder *(a)* increased the appearance of SF metabolites in plasma and urine and *(b)* removed the delay of metabolite appearance observed after the GRP-powder, shifting the absorption/elimination pattern to one similar to that seen after the consumption of fresh broccoli sprouts alone. This is the first study to determine if combining two commercially available broccoli products, one containing and the other lacking myrosinase, would enhance SF availability from GRP. This research could be extrapolated to hypothesize that combining fresh broccoli sprouts with well-cooked broccoli, where myrosinase is inactive, would also enhance SF availability. Additionally, it could be hypothesized that other sources of myrosinase, such as mustard, horseradish, cabbage, Brussels sprouts and watercress, would also enhance the conversion of GRP to SF.

The present study measured urinary SF-NAC excretion and plasma total ITC levels. The measurement of SF metabolites after consumption of broccoli, broccoli sprouts, and other broccoli related preparations has been a useful tool for assessing human exposure to SF, a compound associated with reduced risk for cancer (1, 3-6, 9, 17, 18). Sulforaphane metabolites in plasma reflect the amount of SF that tissues are being exposed to and are therefore important

biomarkers of exposure to this cancer preventative agent. Sulforaphane metabolites in urine reflect the absorption, metabolism and excretion of an ingested dose (5). The major metabolite of SF appearing in urine, SF-NAC, is often used as a marker of bioavailability, although it is not the only metabolite present in urine (8, 9).

Only 24% of the GRP dose from the GRP-powder was recovered as SF-NAC in the urine, making it a poor source of dietary SF compared to fresh broccoli sprouts. This value is comparable to the reported recovery following ingestion of well-cooked sprouts or well-cooked mature broccoli that both also lacked myrosinase (3, 5, 18). Urinary values of SF-NAC after the GRP-powder meal displayed a non-significant trend of increasing excretion over 24 hours, suggestive of delayed absorption. The delayed absorption and low SF recovery was likely due to the lack of myrosinase in the powder and the resulting hydrolysis of GRP occurring by microflora after transit of GRP to the lower gut (1, 2, 18, 19). Plasma total ITC was not altered in response to the GRP-powder, but there was a slight non-significant elevation at 3 h, also suggestive of delayed ITC absorption with low availability. It could be questioned if the maximum plasma ITC level was further delayed rather than absent following ingestion of the GRP-powder, and thus was not measured in this study. Indeed, a slight, but non-significant peak 6 h post-consumption was observed in a study of well-cooked broccoli (3). However, urinary recovery of SF-NAC was low not only for the first 6 h of the present study, but for the entire 24 h period following consumption of the GRP-powder meal, confirming the absence of any significant elevation in plasma ITC levels. The low levels of SF metabolites detected in plasma and urine after consumption of the GRP-powder may indicate lower anti-cancer potential for this product and other similar dietary supplements. For instance, it was reported that similar GRP products lacking myrosinase induced detoxification enzymes in the colon, but not liver of rats,

whereas unheated broccoli florets with functional myrosinase induced activity in both colon and liver (20).

Interestingly, data from the combination meal identified possible synergy among the fresh sprouts and GRP-powder at early time points for SF and metabolites appearance in plasma and urine. This indicates that GRP, not only from the broccoli sprouts, but also from the GRP-powder, was hydrolyzed by endogenous myrosinase from the broccoli sprouts. Additionally, excretion of SF-NAC following the combination meal was earlier than from the GRP-powder alone and more similar to the excretion pattern following consumption of broccoli sprouts alone, indicating that the fresh sprouts not only supported hydrolysis of the GRP-powder, but also caused it to occur earlier, resulting in earlier and more complete SF absorption. The trend for greater levels of SF-NAC to be excreted early (during the first 12 h following meal consumption) from the combination and sprouts meals is consistent with metabolism occurring in the upper gastrointestinal tract in the presence of dietary myrosinase. A similar trend was observed in plasma where in both sprout and combination meals, plasma ITC levels were elevated by 0.5 h, and to a much higher level in the combination meal. Higher levels of SF metabolites in plasma and urine may indicate greater reduction of cancer risk from consumption of this food combination.

It has been reported that approximately 74% of dietary SF is absorbed in the jejunum (21). Elevation of plasma ITC levels at 0.5 h post-consumption of the sprout and combination meals may indicate that a portion of SF was absorbed at a point in the digestive tract as early as the stomach and/or duodenum. Considering the small volume of food and low amount of fat consumed in these trial meals, the observation that the highest levels of plasma ITC occurred

between 1.5-3 h post-consumption was consistent with the expected majority of SF absorption occurring in the jejunum/upper intestine.

Recovery of preformed ITC or ground, air-dried broccoli sprouts was reported to be between 75-90% of ingested doses (5, 6, 22). This recovery decreased when a plant matrix was introduced, as is evidenced by several published papers, as well as the present paper where intact, but thoroughly chewed fresh sprouts resulted in a 60% recovery of the dose (3, 5, 22). Interestingly though, comparing an equimolar dose of SF from fresh sprouts (used here) to air-dried sprouts (6) when combined with the GRP-powder, an improved 24 h urinary recovery (65% versus 50% of the ingested dose, respectively), and an elevated peak plasma ITC level (C_{max} 2.9 versus 2.1µmol total ITC/l, respectively) was observed in the combination using fresh intact broccoli sprouts. Based on this evidence, we conclude that fresh broccoli sprouts aided the conversion of GRP to SF from GRP-powder to a greater extent than air-dried broccoli sprouts. More research with larger study populations is needed.

One limitation of the present study is its small sample size. However, most human studies focusing on the bioavailability of SF use similar small population sizes (3, 5, 6, 11). The intent of this study was to provide direction as a pilot study. Future large scale work is needed.

In conclusion, the GRP-powder that lacked myrosinase was a poor dietary source of SF compared to broccoli sprouts. Fresh intact broccoli sprouts were able to synergistically enhance the hydrolysis of GRP from the GRP-powder, perhaps more efficiently than ground, air-dried broccoli sprouts. Because efficacy is related to plasma levels, the elevation seen in plasma levels likely translates to greater potential for cancer risk reduction. These findings provide important insights into the protective health benefit of broccoli products and preparations and can be used to develop foods with enhanced anti-cancer properties.

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4.6 Tables and figures

Table 4.1. Total ITC in Plasma Following Test Meal Consumption

	0 h		0.5 h		1 h		1.5 h		3 h		24 h	
(μmol total ITC/l)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
control	$0.22^{a,1}$	0.09	$0.18^{a,b,1}$	0.11	$0.24^{a,1}$	0.06	$0.16^{a,1}$	0.13	$0.05^{a,1}$	0.03	$0.05^{a,1}$	0.04
GRP-powder	$0.09^{a,1}$	0.07	$0.11^{a,1}$	0.07	$0.07^{a,1}$	0.03	$0.12^{a,1}$	0.11	$0.37^{a,1}$	0.25	$0.16^{a,1}$	0.10
fresh sprout	$0.13^{a,1}$	0.12	$0.46^{b,1}$	0.11	$0.97^{b,2}$	0.15	$1.43^{b,3}$	0.21	$1.53^{b,3}$	0.22	$0.19^{a,1}$	0.07
combination	$0.10^{a,1}$	0.07	$1.26^{c,2}$	0.22	$2.14^{c,3}$	0.15	$2.86^{c,4}$	0.33	$2.57^{c,4}$	0.38	$0.30^{a,1}$	0.07

ITC, isothiocyanates.

Data values are μmol total ITC/l, n=4 subjects per group. Within each column (between-meal values) or each row (within-meal values) means that do not share a superscript letter or number, respectively, are significantly different, $p<0.05$.

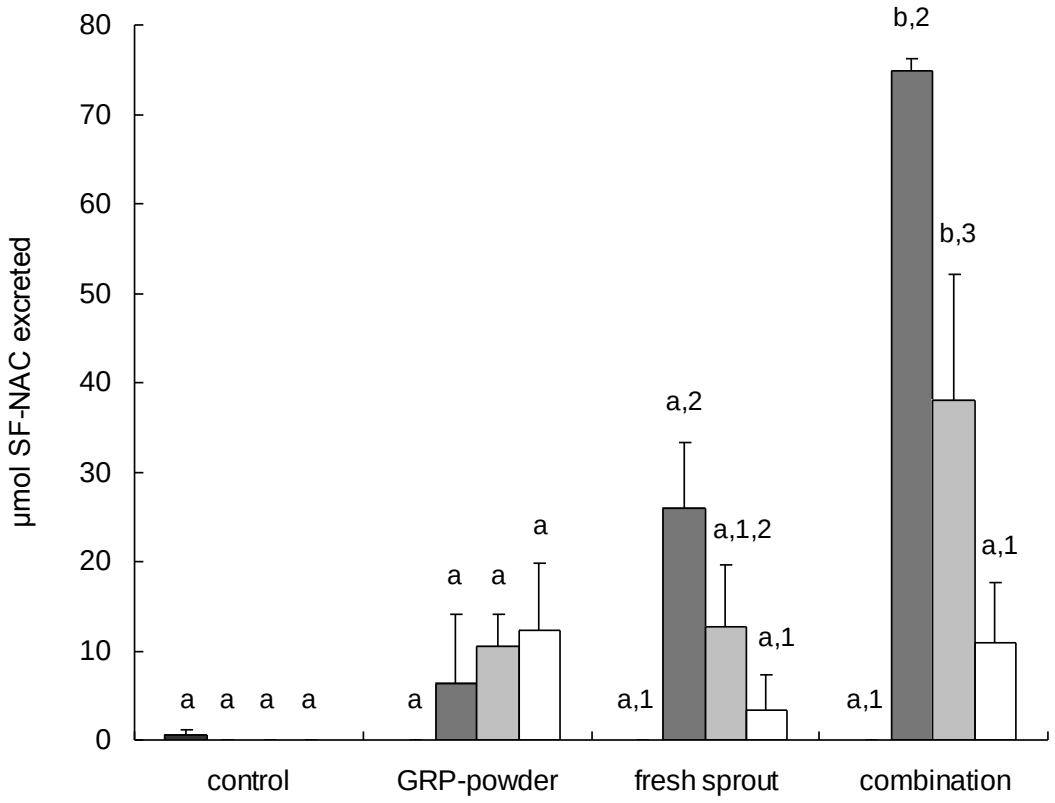

Figure 4.1. Urinary SF-NAC (sulforaphane-*N*-acetylcysteine) excretion after consumption of four different meals: control, GRP-powder, fresh broccoli sprout and combination. Baseline (black bars), 0-6 h (dark grey bars), 6–12 h (light grey bars), 12-24 h (open bars) urine collection post consumption. Data are presented as means of four subjects per group with standard errors represented by vertical bars. Between-meal values that do not share a letter or within-meal values that do not share a number, are significantly different, $p<0.05$.

Chapter 5

Sulforaphane Decreases DNA Methylation and Increases Gene Expression of *P21* in Human Colorectal Carcinoma Cells

5.1 Introduction

Sulforaphane (SF) is an isothiocyanate derived from the hydrolysis of glucoraphanin, a natural constituent of broccoli, and is considered to be responsible for the chemoprotective benefit associated with broccoli consumption. Sulforphane is known to activate the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/Antioxidant Response Element (ARE) pathway causing upregulation of detoxification enzymes that protect against the incidence and progression of cancer (1-3). The Nrf2/ARE pathway is the best-characterized mechanism by which SF exerts its anti-cancer effect (4). However, additional anti-carcinogenic pathways involving epigenetic gene regulation are being explored.

Histone acetyltransferases are enzymes that transfer acetyl groups to histones, whereas histone deacetylases (HDAC) remove acetyl groups from histones (5). The balance between histone acetyltransferase and HDAC enzymes determines the acetylation level of histones. Greater acetylation of histones is generally associated with transcriptional activation of a gene (5). By decreasing the acetylation status of nucleosomes, HDAC can limit the access of transcriptional machinery to DNA (6). Thus, inhibition of HDAC activity is associated with gene activation (6, 7). Trichostatin A (TSA) is a classic HDAC inhibitor and is often used as a positive control in studies evaluating inhibition of HDAC activity (7-9).

DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMT). Hypermethylation at the promoter regions of several tumor suppressor genes is associated with gene silencing in

cancer (6). Thus, inhibition of DNMT activity is a promising method for reducing promoter hypermethylation and restoring expression of tumor suppressor genes in cancer. The nucleoside analog 5-azacytidine (AZA) been shown to reduce DNA methylation by inhibiting DNMT and is used clinically for this purpose (5, 10, 11). 5-azacytidine is also frequently used as a positive control in studies evaluating DNA demethylation.

Histone post-translational modifications, including histone acetylation level, are linked to DNA methylation (12-14). Histone modifying enzymes can recruit the DNA methylation machinery and conversely, methylated DNA can recruit histone-modifying complexes, including HDAC (12, 13). Interestingly, the HDAC inhibitor valproic acid stimulates gene expression by both preventing histone deacetylation and concurrently stimulating DNA demethylation (15, 16). Therefore, HDACs and DNA methylation can work together to effectively regulate gene expression.

Many cancers, including colorectal cancer (CRC), have an epigenetic basis involving dysregulation of histone modifications and DNA methylation with an associated aberrant transcriptional silencing of gene expression (6, 17-19). Furthermore, both HDAC and DNMT enzymes are typically overexpressed in CRC, contributing to the state of gene silencing (20-22). Epigenetic changes, rather than genetic mutations, are responsible for as many as 50% of the tumor suppressor genes silenced in cancer, and aberrant DNA methylation is a hallmark of numerous cancers (19, 23, 24). Sulforaphane is an inhibitor of class I HDAC, ubiquitously expressed nuclear HDAC enzymes involved in gene silencing, suggesting it may affect epigenetic gene regulation (8, 22). Sulforaphane treatment was also recently reported to down regulate DNMT enzymes (25). Thus, the chemoprotective effect of dietary SF may be due to a role in normalizing epigenetic regulation of gene expression.

A few dietary anticarcinogens, including epigallocatechin-3-gallate, lycopene and genistein, have been shown to increase gene expression by inducing DNA demethylation of aberrantly hypermethylated gene promoters (26-28). Sulforaphane may similarly decrease DNA methylation levels. As stated previously, SF treatment was recently reported to down regulate DNMT1 and DNMT3a resulting in decreased DNA methylation in breast cancer cells (25). However, the gene evaluated in this study was telomerase reverse transcriptase, a gene that is activated by methylation. Therefore, demethylation correlated with repression of gene expression (25). In the present study, SF was tested for the ability to stimulate DNA demethylation and upregulate gene expression of cyclin-dependent kinase inhibitor 1A *(P21),* BCL2-associated X protein (*BAX),* cyclin-dependent kinase inhibitor 2A *(P16),* O-6-methylguanine-DNA methyltransferase (*MGMT)* and mutL homolog 1 (*MLH1)* using human CRC cells. *P21, BAX, P16* and *MGMT* were selected based on their reported upreguation by SF as well as their status as tumor suppressor genes (22, 29, 30). *MLH1* was selected because this tumor suppressor gene is commonly hypermethylated in CRC (31, 32).

5.2 Methods

Cell culture. HCT 116 human colorectal carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained with McCoy's 5A medium supplemented with 10% fetal bovine serum. Cells were cultured at 37ºC in a humidified atmosphere of 95% air and 5% carbon dioxide. A preliminary study was carried out in duplicate to determine if SF caused loss of cell growth and/or cell death at 5.0 µM SF. Cells were plated at 5×10^4 cells/well in 6 well plates and allowed to attach for 24 h. On days 1, 3, 5, and 7 medium was removed and replaced with fresh medium containing 5 µM SF (LKT Laboratories, St. Paul, MN) or no treatment (control).

The treatment and control contained 0.1% DMSO. Trypsinization followed by trypan blue staining and cell counting was used on days 1, 3, 5, 7 and 9 to carry out a growth curve assay and evaluate cytotoxicity. Adherent cells that did not accumulate trypan blue were counted as live. Adherent cells that accumulated trypan blue and all floating cells were counted as dead. Next, HCT 116 cells were again plated at 5 x 10^4 cells/well in 6 well plates and allowed to attach for 24 h. Medium was removed and replaced with fresh medium containing 5 μM SF or no treatment (control) on days 1, 3 and 5. The treatment and control contained 0.1% DMSO. On day 7, cells were harvested by trypsinization. DNA or RNA was isolated for use in methylation-specific PCR and quantitative real-time RT-PCR as described below. Trypan blue staining and cell counting were used to measure cell growth and cytotoxicity in triplicate on day 7. Adherent cells that did not accumulate trypan blue were counted as live. Floating cells were not assessed.

Methylation-specific PCR. DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA was treated with sodium bisulfite using the Zymo Research EZ DNA Methylation-Gold Kit. Gene promoter sequences for primer design were obtained from ensembl.org. Primers, listed in Table 5.1, were designed using MethPrimer software (33). PCR was performed using the following program: 15 min at 95˚ C, followed by 35 cycles of 30 sec at 95˚ C, 30 sec at the annealing temperature listed in Table 5.1, 30 sec at 72˚ C, and final extension at 72˚ C for 5 min. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide and quantified by densitometry (ChemiImager 4400, Alpha Innotech, San Leandro, CA).

Quantitative real-time RT-PCR. RNA was isolated using the Qiagen RNeasy Mini Kit with RNase-free DNase; a denaturing agarose gel confirmed RNA integrity. cDNA was generated using the Invitrogen SuperScript VILO cDNA synthesis kit. Primers were designed using Primer Express software (version 2; Applied Biosystems, Carlsbad, CA) according to the manufacturer and are shown in Table 5.2. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with a TaqMan ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer protocol. Data were analyzed using Sequence Detection System software version 2.4 (Applied Biosystems) and normalized to beta-actin. Results are expressed as fold change over control using the comparative C_T method.

HDAC activity assay. HCT 116 human colorectal carcinoma cells plated at a density of 2×10^5 cells/100 mm tissue culture dish. Cells were maintained and treated as described above except that treatments included 1.0, 2.5 or 5 μM SF or no treatment (control). The treatments and control contained 0.1% DMSO. On day 7, cells were harvested by trypsinization and nuclear extracts were prepared using the EpiQuick Nuclear Extraction Kit (Epigentek, Brooklyn, NY). Nuclear extracts (20 μg) from cells were assayed for HDAC activity using a colorimetric HDAC activity assay kit (Actif Motif, Carlsbad, CA). Trichostatin A was added directly to nuclear extract from control cells to serve as a positive control.

DNMT activity assay. Cells were maintained and treated as described for the HDAC activity assay. Nuclear extracts (5 μg) were assayed for DNMT activity using the colorimetric EpiQuick DNMT Activity/Inhibition Assay Ultra Kit (Epigentek, Brooklyn, NY). Cells treated with 1.0 or 5.0 μM AZA served as a positive control. Additionally, nuclear extract from control cells was

directly treated with a final concentration of 5.0 μM SF to determine whether SF was a direct inhibitor of DNMT.

Statistical analysis. Data were evaluated by one-way analysis of variance (ANOVA) using SAS Statistical software (version 9.1; SAS Institute, Cary, NC). Where a significant effect was found (*F*-ratio, $P<0.05$), Fisher's LSD post-hoc test was used to determine significant differences between means. Data are presented as mean ± SD.

5.3 Results

Cell growth and cytotoxicity assays. HCT 116 cells were grown with and without 5.0 μM SF and harvested on day 7. As predicted from preliminary studies (Figures 5.1a and 5.1b), triplicate studies of adherent cells showed that growth was inhibited by approximately 53% in the SF treated cells compared to untreated control cells (Figure 5.1c). Cell survival was 99 ± 0.01% in SF treated cells which was indistinguishable from the untreated controls (99 ± 0.01% survival) indicating that 5.0 μM SF treatment for 7 days was not cytotoxic (data not shown).

Methylation-specific PCR. Quantitative methylation-specific PCR (MSP) analysis of DNA from cells treated with 5.0 μM SF for 7 days showed a 14% reduction of promoter DNA methylation for the *P21* tumor suppressor gene and a non-significant 11% reduction of DNA methylation at the promoter of *BAX* (Figure 5.2). No significant change in DNA methylation status was observed at the promoters of *MGMT*, which remained 100% methylated for control and SF treatments (data not shown), or *P16*, which showed 52.3 ± 4.9 and 49.7 ± 3.1%

methylation for control and SF treatments, respectively (Figure 5.2). The promoter of *MLH1* was 0% methylated in control cells leaving no opportunity for SF to result in demethylation of the promoter of this gene (data not shown). As expected, cells treated with 5.0 μM SF also revealed 0% methylation at the *MLH1* promoter (data not shown).

Quantitative real-time RT-PCR. The *P21* and *BAX* mRNA showed a 1.92 ± 0.32 and 1.64 ± 0.09 fold increase over mRNA from untreated control HCT 116 cells in response to 5.0 μM SF treatment, indicating an increase in gene expression concordant with DNA demethylation (Figure 5.3).

HDAC activity assay. HDAC activity was not changed in response to any concentration of SF tested (Figure 5.4). However, HDAC activity was decreased in the TSA treated nuclear extracts from control cells that were included as a positive control, indicating that the assay was functioning correctly.

DNMT activity assay. DNMT activity following 7 day treatment with 1.0, 2.5 or 5.0 μM SF was 111.9 ± 7.3, 81.5 ± 11.4 and 70.2 ± 9.8% respectively, of untreated controls (Figure 5.5). However, only the 5.0 μM SF treatment caused a significant decrease in DNMT activity. Both 2.5 and 5.0 μM SF resulted in significantly lower DNMT activity than 1.0 μM SF, but were not different from each other (Figure 5.5). Although 1.0 μM AZA had no effect on DNMT activity, 5.0 μM AZA decreased DNMT activity to 58.0 ± 10.0% of untreated control (Figure 5.5). A final concentration of 5.0 μM SF added directly to nuclear extract of untreated control cells did

not alter DNMT activity, indicating that SF does not inhibit DNMT through direct inhibition (Figure 5.5).

5.4 Discussion

According to the National Cancer Institute, CRC is the third most common cancer and third leading cause of cancer-related deaths in the United States with 142,570 new cases and 51,370 deaths estimated for 2010. If cancer prevention is to be an effective strategy for decreasing cancer deaths, then it is necessary to identify and inhibit the early changes of the cancer process. Epigenetic changes often occur during the earliest stages of CRC development providing an opportunity for epigenetically targeted compounds to impact cancer prevention (23, 34). Because of direct contact, the digestive tract may be exposed to higher levels of dietary bioactive compounds compared to other organs and epidemiological data indicate that cancers of the digestive tract are particularly responsive to dietary components (35). Additionally, higher consumption of broccoli and other related vegetables correlate with reduced risk of CRC (36). In one study, the diets of 586 adult male patients with colon or rectal cancer were compared to the diets of 1411 control adult males who did not have these cancers (36). An increase in risk of colon and rectal cancer was found with lower frequency of vegetable intake; specifically cabbage, Brussels sprouts and broccoli (36). Therefore, it is interesting to determine if dietary factors, such as SF, impact epigenetic targets to provide a unique opportunity for CRC prevention and/or reduced tumor progression.

Sulforaphane is a natural agent present in broccoli known to inhibit HDAC activity and is a prime candidate for mediating the chemoprotective effects of broccoli consumption towards CRC. Levels of SF metabolites in plasma can reach 7 μM, but more commonly reach C_{max} at

2-3 μM (37-41). To test the potential efficacy of dietary SF as an anti-cancer treatment, concentrations in the range of 1.0-5.0 μM SF were chosen for this study. Treatment of HCT 116 cells with 5.0 μM SF over 7 days resulted in cell survival indistinguishable from the untreated controls (99 ± 0.01% survival in both groups) indicating no cytotoxic effects of the SF. However, 7 days of SF treatment inhibited cell growth by approximately 53% compared to control untreated cells. Thus, the 5 μM SF obtainable in circulating plasma through broccoli consumption is both non-toxic and growth-suppressive on CRC cells.

Many CRCs have epigenetic alterations, including aberrant DNA methylation patterns, associated with their transformation. To determine if SF may be exerting its anti-cancer effects through targeting epigenetic mechanisms, DNA methylation analysis was performed on several candidate tumor suppressor genes (*P21, BAX*, *P16*, *MGMT*) known to be upregulated by SF (22, 29, 30). DNA methylation was also assessed at the promoter of *MLH1* because this tumor suppressor gene is commonly hypermethylated in CRC (31, 32). Quantitative methylation-specific PCR analysis showed a gene-specific effect in which SF treatment resulted in a 14% reduction of promoter DNA methylation for the *P21* tumor suppressor gene and a non-significant effect at the promoter of *BAX*. No change in DNA methylation status was observed at the promoters of *MGMT, P16* or *MLH1*. The DNA demethylation of *P21* is particularly interesting because the *P21* gene encodes a cyclin-depedent kinase inhibitor that is involved in regulation of the cell cycle, specifically blocking the progression from G1 to S phase. Sulforaphane has been reported to inhibit G1 to S phase transition, although the mechanism has not been established (42-44). Therefore, *P21* demethylation may be involved in the observed growth inhibition by SF.

If it has biological significance, the DNA methylation status of promoter CpG islands can be expected to correlate with gene expression. Therefore, quantitative real-time RT-PCR (qRT-PCR) was used to assay the *P21* and *BAX* mRNA expression levels in response to SF treatment. The *P21* and *BAX* mRNA both showed an increase over untreated control in response to SF treatment, indicating an increase in gene expression concordant with DNA demethylation. Previously, treatment of MCF-7, MDA-MB-231 and MCF10A breast cancer cells with 5 μM SF was shown to decrease promoter DNA methylation of telomerase reverse transcriptase, although the effect was more robust at 10 μM SF (25). Therefore, the results reported here might be more robust if examined at higher SF concentrations, but it must be kept in mind that such levels would no longer carry relevance to dietary SF from broccoli. At a physiologically relevant level for dietary protection, we demonstrated that SF was an activator of *P21* and *BAX* mRNA expression in addition to being efficient at stimulating DNA demethylation.

Histone modifying machinery as well as DNA methylation machinery are involved in regulating the epigenetic landscape of cells. The epigenetic machinery responsible for histone deacetylation and DNA methylation can interact to effectively silence genes (12, 13). HDAC inhibition by SF was previously reported over a range of 3.0-15.0 μM SF (8). Therefore, HDAC and DNMT activity were assayed in order to elucidate a potential mechanism for the observed decrease in promoter DNA demethylation of SF treated cells. Specifically, we sought to determine whether the decrease in DNA methylation was due to the coupled action of HDAC and DNMT inhibition, or if the observed promoter DNA demethylation was independent of HDAC inhibition. In our study, no effect on HDAC was observed following any concentration of SF treatment, despite SF being a known competitive inhibitor of HDAC. However, it has been shown that SF must be present to maintain HDAC inhibition (21). In the present study, the

final SF treatment was administered to confluent cells 48 h before harvest. Unpublished data from our lab indicate that the concentration of SF in medium devoid of cells after 48 h incubation at 37ºC is negligible. Therefore, it is not surprising that a decrease in HDAC activity was not observed, as it is likely that SF had been depleted. Cells treated with increasing concentrations of SF showed a pattern of decreased DNMT activity and reached significance at 5.0 μM SF. However, nuclear extract from untreated control cells treated acutely with 5.0 μM SF did not show decreased DNMT activity, suggesting that the inhibition of DNMT activity by SF is not acting directly at the enzyme. Therefore, the underlying mechanism for the decrease in promoter DNA demethylation at 5.0 μM SF appears to involve indirect inhibition of DNMT activity. In support of this hypothesis, Meeran et al. observed decreased DNMT protein expression following SF treatment in breast cancer cells (25).

DNA methylation is not the only SF-affected pathway relating to cancer. Triggering the Nrf2/ARE pathway is the mechanism most commonly attributed with the reduction of cancer observed after exposure to SF. However, none of the genes evaluated in the present study were found to be regulated by Nrf2/ARE and *P21* has specifically been shown to lack an ARE (8, 45). Therefore, it is likely that upregulation of *P21* by SF occurs through a Nrf2-independent mechanism, consistent with a biological role for SF affecting DNA demethylation. In addition, only *P21* (and *BAX* to a lesser extent) was associated with loss of methylation by SF, indicating selectivity of the impact of SF on DNA demethylation. Demethylation of specific gene targets is important since global genome demethylation has been associated with undesirable effects (46). These data suggest that administration of SF, either through dietary broccoli or through other means, can affect the epigenome beneficially, with the potential to slow or prevent cancers, particularly those of the colon.

5.5 References

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5.6 Tables and figures

Table 5.1. Primer Sequences and Annealing Temperatures for Methylation-Specific PCR

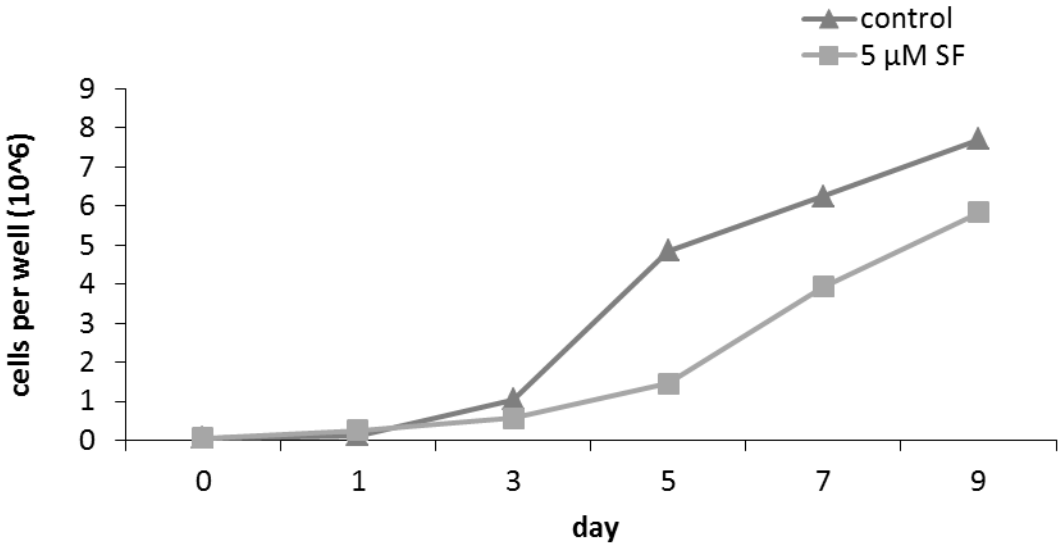
Gene	MSP reaction	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (˚C)
P21	M	ATGTGTTTAGCGTATTAACGTAGGC	GACTCCACAAAAAACTAACTTCGAC	50
	U	TGTGTTTAGTGTATTAATGTAGGTGA	AACTCCACAAAAAACTAACTTCAAC	50
BAX	M	GTTGGGGAGAGTTTAAATTTTGTTC	GCTAAACGTACGTCCTCCACGTA	50
	U	GTTGGGGAGAGTTTAAATTTTGTTT	CCCACTAAACATACATCCTTCACATA	50
P16	M	AGTAGTTGGGATTATAGGTATGCGT	ATTCTAAAAAACCGAAACAAACG	48
	U	GAGTAGTTGGGATTATAGGTATGTGT	ATTCTAAAAAACCAAAACAAACAAA	48
MGMT	M	GAGAGATTCGCGTTTCGGGTTTAG	AACGACGCCTTCCCAACTTC	53
	U	TTGGGTATGTGGTAGGTTGTTTGT	ACACCAACACACCAACCCTA	53
MLH1	M	AGTGAAGGAGGTTACGGGTAAGTC	TATCGCCGCCTCATCGTAACT	53
	U	AGAGGTGGTAGAGTTTGAGGTTTG	CACCACCTCATCATAACTACCCA	53

Abbreviations: MSP, methylation-specific PCR; M, methylated; U, unmethylated

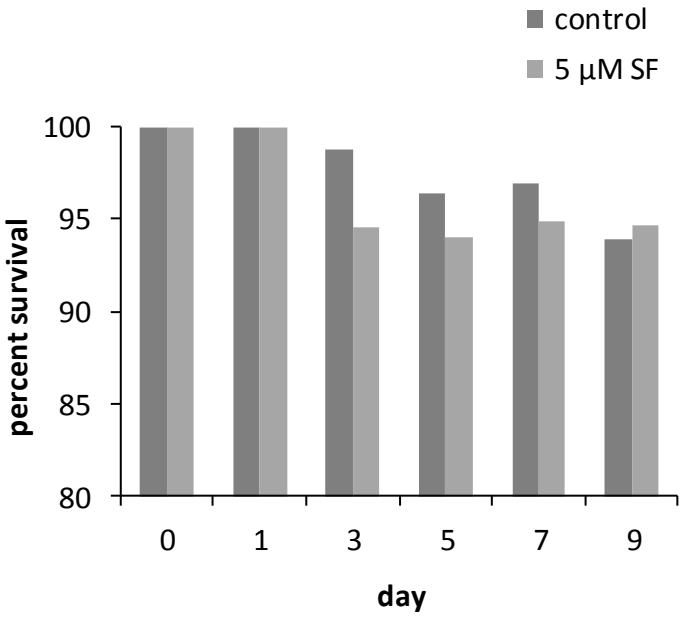
Table 5.2. Primer Sequences for Quantitative Real-Time RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
P21	GAGACTCTCAGGGTCGAAAACG	TGGTAGAAATCTGTCATGCTGGTC
BAX	TGTCGCCCTTTTCTACTTTGC	GTGCACAGGGCCTTGAGC
ß-actin	CTGGCACCCAGCACAATG	CTTGCGCTCAGGAGGAGC

5.1a.


control
5 µM SF
cells per well (10^6)
0
1
2
3
4
5
6
7
8
9
0
1
3
5
7
9
day

5.1b.


control
5 µM SF
percent survival
100
95
90
85
80
0
1
3
5
7
9
day

5.1c.

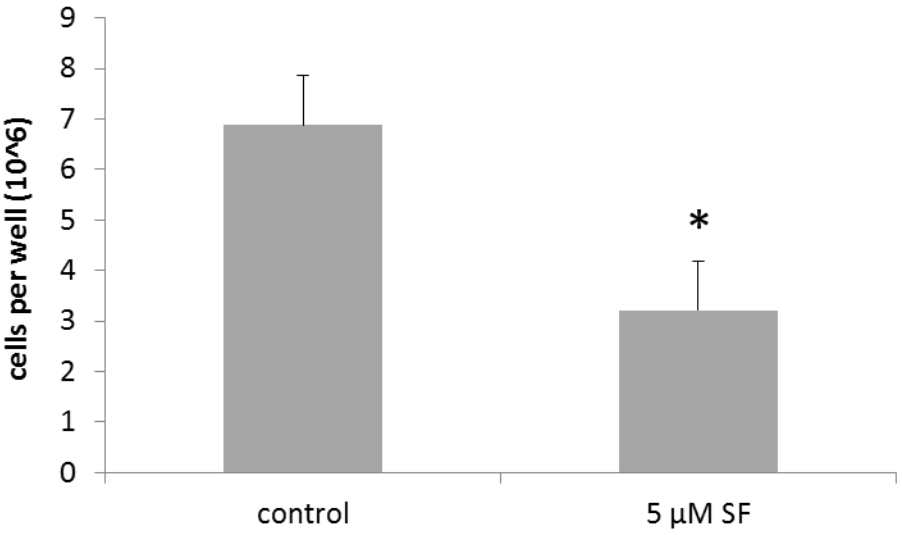


Figure 5.1. The effect of SF treatment on cell growth and cytotoxicity. a) Growth curve and b) cytotoxicity was determined after treatment of HCT 116 cells with 5.0 µM SF on experimental days 1, 3, 5, 7 and 9. At each time point, cells were harvested, stained with trypan blue and counted. Adherent cells that did not accumulate trypan blue were counted as live. Adherent cells that accumulated trypan blue and all floating cells were counted as dead. Data represent the mean of duplicate samples. c) In a separate experiment, HCT 116 cells were treated with 5.0 µM SF for 6 days; on experimental day 7, cells were harvested, stained with trypan blue and counted. Adherent cells that did not accumulate trypan blue were counted as live. Floating cells were not assessed. Data represent mean ± SD of three samples. *$P<0.05$.

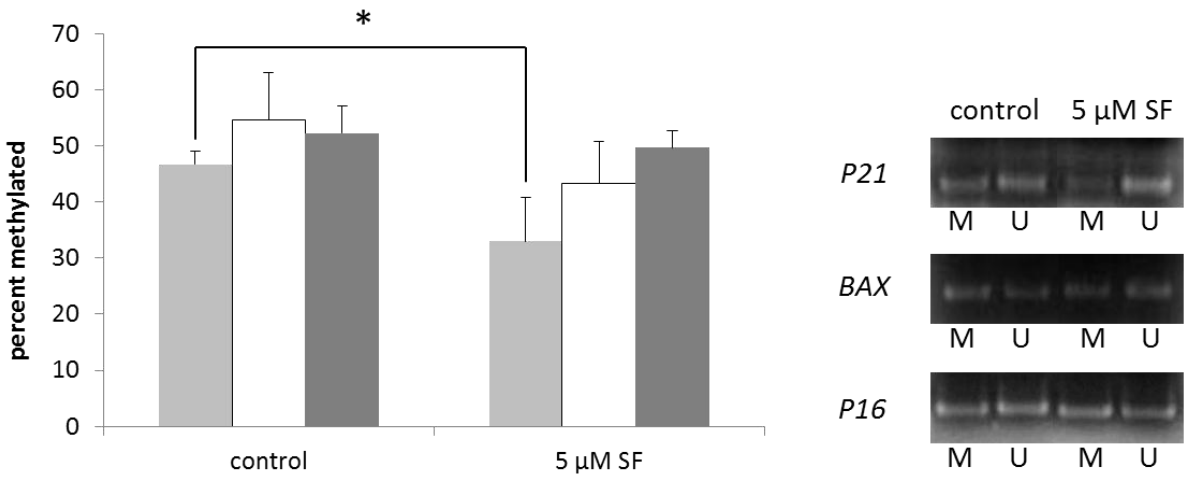


Figure 5.2. The effect of SF treatment on promoter DNA methylation. HCT 116 cells were treated with 5.0 μM SF or no treatment (control) every 48 h for 6 days and harvested on day 7. The effect of 5.0 μM SF on the DNA methylation status of *P21* (light grey bars), *BAX* (open bars), and *P16* (dark grey bars) was assayed by MSP and quantified by densitometry, as described in methods. Data represent mean ± SD of three independent experiments. One representative gel image of PCR band intensities is shown. *P<0.05. M, methylated; U, unmethylated.

5.3a. 5.3b.

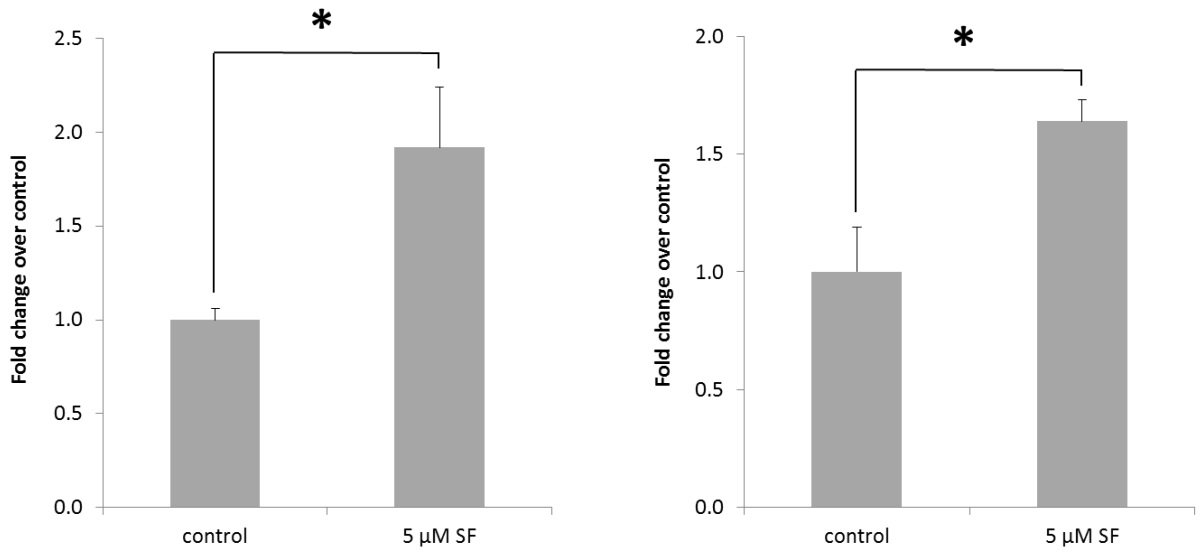


Figure 5.3. The effect of SF treatment on *P21* (a) and *BAX* (b) mRNA expression. HCT 116 cells were treated with 5.0 μM SF or no treatment (control) every 48 h for 6 days and harvested on day 7. The effect of 5.0 μM SF treatment on *P21* and *BAX* mRNA expression level was assayed by qRT-PCR, normalized to β-actin, and expressed as fold change over untreated control using the comparative C_T method. Data are presented as mean ± SD of three independent experiments. *P<0.05.

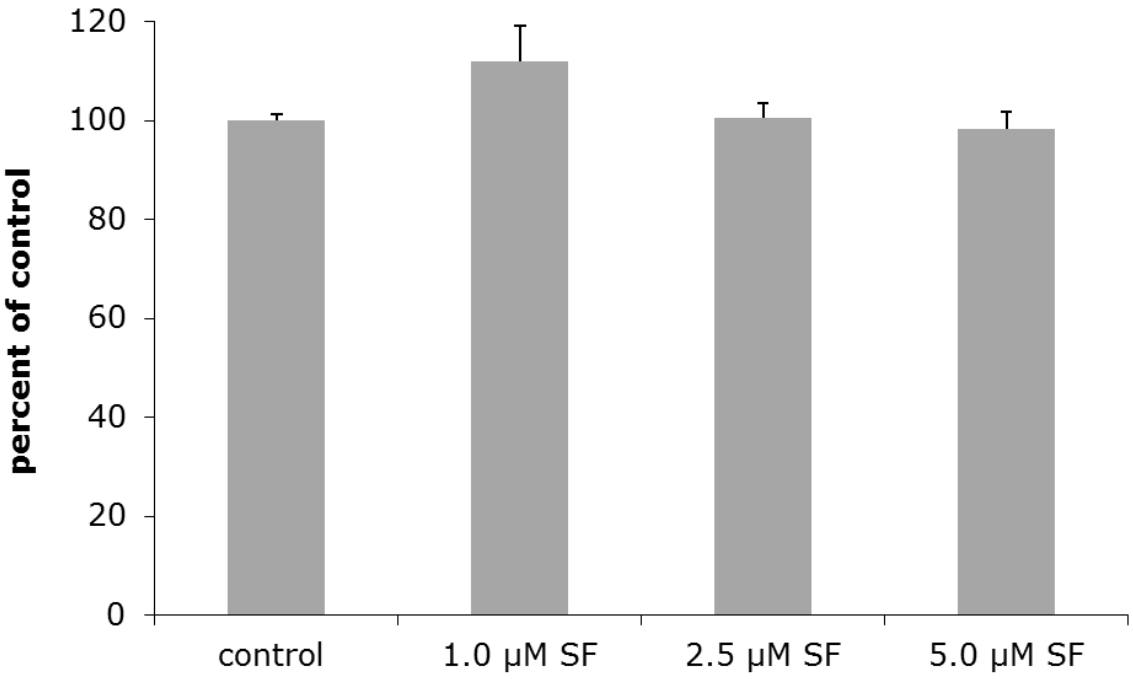


Figure 5.4. The effect of SF treatment on HDAC activity. HCT 116 cells were treated with 1.0, 2.5 or 5.0 μM SF or no treatment (control) every 48 h for 6 days and harvested on day 7. The effect of SF treatment on HDAC activity was assessed. A final concentration of 1.0 μM Trichostatin A added directly to nuclear extract of control cells during the HDAC activity assay served as a positive control for the assay and inhibited HDAC activity to 74.2 ±10.2 percent of untreated control. Data are expressed as percent activity of untreated control and presented as mean ± SD of three independent cultures, each measured in duplicate. At $p<0.05$ compared to untreated control, only the Trichostatin A positive control significantly inhibited HDAC activity.

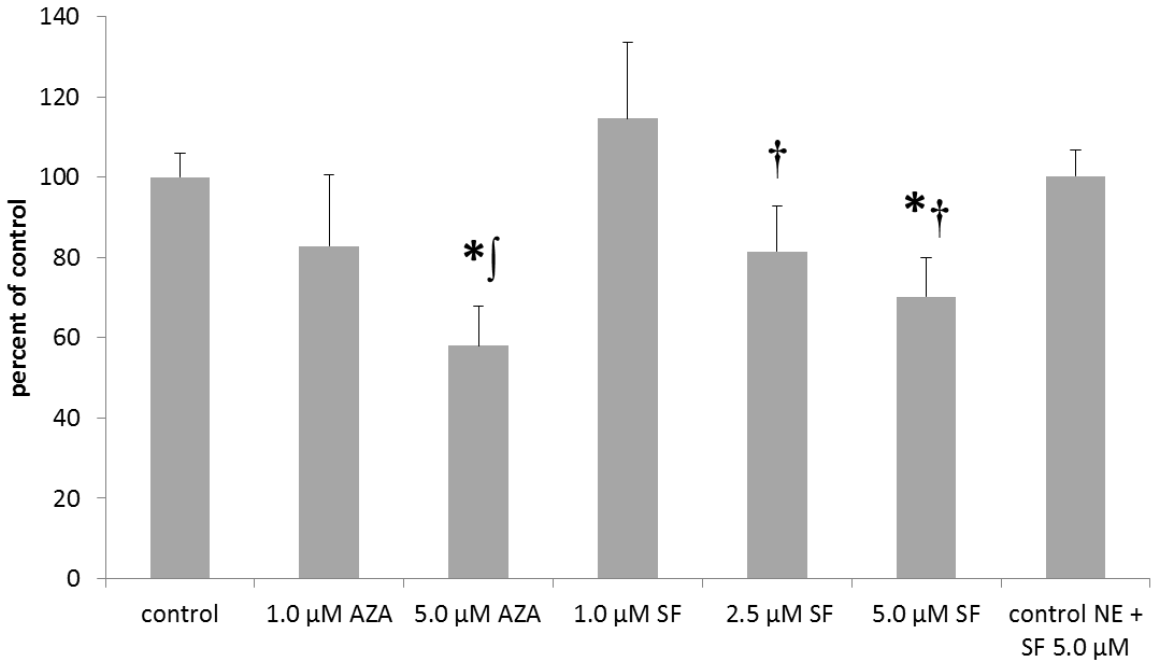


Figure 5.5. The effect of SF on DNMT activity. HCT 116 cells were treated with 1.0, 2.5 or 5.0 μM SF; 1.0 or 5.0 μM 5-azacytidine (AZA) (positive controls); or no treatment (control) every 48 h for 6 days and harvested on day 7. The effect of these treatments on DNMT activity was assessed. Additionally, a final concentration of 5.0 μM SF was introduced directly to nuclear extract of control cells during the DNMT activity assay (control NE + SF 5.0 μM). Data are expressed as percent activity of untreated control and presented as mean ± SD of three independent cultures each measured in duplicate. $P<0.05$ compared to untreated control (*), 1.0 μM SF (†) or 1.0 μM AZA (∫).

CHAPTER 6

Conclusion and Future Directions

6.1 Conclusion

The overall conclusion of this body of work is that combined consumption of glucoraphanin and a source of myrosinase could be an effective way of attaining plasma concentrations of SF through the diet that are reflective of concentrations correlated with an epigenetic impact in a cell culture model. Specifically, consumption of glucoraphanin with broccoli sprouts as the source of myrosinase resulted in enhanced exposure to SF indicated by elevated concentrations of total ITC in plasma and greater recovery of SF-NAC in urine. Plasma total ITC levels reached 2.86 ± 0.33 μM at 1.5 h post-ingestion of the fresh broccoli sprout plus glucoraphanin powder combination meal. A concentration of SF similar to that attained in human plasma after consumption of the combination meal was associated with markers of epigenetic activation of tumor suppressor genes in a cell culture model of CRC. In particular, 5 μM SF for 7 days decreased DNA methylation at the promoter of *P21*. The demethylation correlated with an increase in *P21* mRNA expression and an inhibition of DNMT activity. The activity of HDAC was not affected by this study. Therefore, a physiologically relevant concentration of SF resulted in decreased promoter DNA methylation through a mechanism that likely involved decreased DNMT activity.

A few studies have recently been published that extend our findings concerning the bioavailability of glucoraphanin supplements in humans. In one such study, ingestion of 221 μmol glucosinolates from fresh broccoli sprouts with active myrosinase resulted in an approximately eight fold greater peak plasma total ITC concentration and more than a four fold

greater recovery of urinary SF and metabolites compared to ingestion of 161 μmol glucosinolates from a myrosinase-lacking broccoli supplement (1). This study, like ours, also demonstrated a delay in peak plasma total ITC and urinary recovery of SF and metabolites following the supplement alone in comparison to the fresh broccoli sprouts alone (1). Additionally, they showed that SF and erucin, the reduced form of SF, were able to be interconverted in humans (1), confirming studies from rats (2). Finally, when fed equivalent doses of glucosinolates from fresh broccoli sprouts or a broccoli supplement, these authors showed that excretion of urinary SF and erucin metabolites occurred earlier and to a greater extent after consumption of the broccoli sprouts (3). Inhibition of HDAC activity in mononuclear cells from blood samples of these subjects was decreased only by the broccoli sprout meal (3). The peak plasma concentration of total ITC that correlated with inhibition of HDAC activity was not reported (3). HDAC inhibition following human consumption of broccoli sprouts has been reported previously (4). However, the concentration of SF metabolites in plasma that correlated with HDAC inhibition was not measured (4). Therefore, the concentration of SF metabolites in plasma that correlates with a decrease in HDAC activity in humans remains to be established. A second group also extended our studies, reporting that ingestion of 800 μmol glucoraphanin resulted in urinary recovery of only approximately 22 μmol SF and metabolites, whereas 150 μmol preformed SF resulted in approximately 84 μmol recovered (5). This translates to an almost four fold higher recovery of urinary SF metabolites from a 5.3 fold lower dose. Together, these studies confirm the low bioavailability from glucoraphanin supplements reported in our clinical studies which further demonstrates the importance of improving the bioavailability of these products. As we have shown in chapters 3 and 4, one way to accomplish this is to include a source of myrosinase with the supplement in order to increase the hydrolysis and

subsequent biological exposure to the anti-cancer agent SF. It is known that SF is an inhibitor of HDAC activity in human mononuclear cells from blood (3, 4). While this provides evidence that dietary SF has epigenetic bioactivity in humans, the effective plasma concentration to obtain this result is not established. Future work is needed to determine whether dietary SF can also affect the activity of DNMT and DNA promoter methylation in animal and human models.

6.2 Future directions

Future work for clinical studies

Most importantly, I think a clinical study with a larger subject population should be conducted in order to confirm the possible synergy that was observed in plasma markers of SF exposure following the combination meal. This study would ideally include multiple plasma sampling time-points throughout the first 24 h following consumption of the test meals. To clarify, our current work identifies the plasma kinetics of total ITC appearance following consumption of broccoli products from 0-3 h with an additional reading at 24 h post-consumption. Extending the time period to include the plasma profile of total ITC between 3 and 24 h would capture any further rise in plasma total ITC resulting from the myrosinase-lacking glucoraphanin powder meal as well as facilitate the calculation of an area under the curve for each meal.

Next, it would be interesting to conduct an experiment at the bench to determine if glucoraphanin from cooked broccoli could be hydrolyzed by co-incubation with other myrosinase-containing vegetables such as radish, kale, horseradish or mustard. If the results of these bench experiments indicate that hydrolysis of broccoli gluocraphanin does occur through the activity of other plant myrosinases, then conducting a feeding study in humans would be necessary to ensure the applicability of this finding to humans. The novel aspect of this work would be that broccoli

glucoraphanin can be hydrolyzed by other plant myrosinases rather than only myrosinase from broccoli. There are data supporting this hypothesis. For example, glucoraphanin can be hydrolyzed by incubation with myrosinase isolated from white mustard or daikon radish (6-9). The application and benefit to humans would be that cooked broccoli could be combined with a variety of foods, rather than broccoli sprouts as the only option, for an appealing dish with added health benefits.

It may also be of interest to conduct a long-term randomized controlled trial designed to determine whether increased dosing of SF from broccoli sprouts, glucoraphanin supplements and the combination of the two would result in superior protection from cancer over time. A study population could be asked to consume these products 3-5 times per week and incidence of colon, rectum, bladder, liver, prostate, gastric, and other cancers could be followed for decades to definitively determine if these products reduce the incidence of cancer or impact other biological factors. I think it would be fascinating to include an arm in this study where broccoli feeding begins at an early age, perhaps at the point when an infant progresses beyond a liquid-only diet, by incorporating a broccoli-based baby food into the diet. This eating pattern would then continue throughout the lifetime by consuming 3-5 servings of the designated broccoli product weekly. Cancer incidence and other endpoints of interest could be assessed throughout the lifetime. This study design would also allow for a statement regarding the effects of consuming broccoli products throughout the whole lifetime rather than only later in life. Animal studied could precede the clinical studies in order to save costs, generate hypotheses and assess safety. Finally, because supplements for glucoraphanin are available, but are essentially ineffective compared to SF, I think the development of a commercialized product that utilizes preformed isothiocyanate, freeze-dried broccoli sprouts or glucoraphanin with a source of myrosinase for

use in blended beverages, cooking, etc is warranted. However, the consumer acceptance of the taste and smell of such a product would need to be considered.

Future work in epigenetics and broccoli

I think the most important first step to carrying this research further is to confirm the demethylating effect of SF on the *P21* gene using bisulfite sequencing, a state of the art technology that is capable of quantifying the degree and specific CpG location of changes in DNA methylation. Next, this technique should be applied to explore the effects of SF on other genes of interest including NAD(P)H dehydrogenase, quinone 1 (*NQO1),* nuclear factor (erythroid-derived)-like 2 (*NRF2),* π-class glutathione S-transferase (*GSTP1)* and tumor protein p53 *(P53)*. The pathways of these genes are detailed in Figure 6.1. As previously discussed, the Nrf2/ARE pathway is most commonly associated with the cancer risk reducing potential of broccoli and SF. Of the genes listed here and examined in the research presented in this dissertation, only *Nqo1* was reported to be affected in a *Nrf2* knockout model (10). Indeed, *NQO1* contains an ARE sequence and is regulated by NRF2 (10). *GSTP1* also contains an ARE sequence and is regulated by NRF2 (11). Therefore, the question arises as to whether the increases in NQO1 and GSTP1 protein caused by SF are due to traditional ARE-directed upregulation or if epigenetic regulation is also having an effect. Interestingly, it was recently reported that *Nrf2* itself was hypermethylated in a prostate cancer model and that this hypermethylation inhibited *Nrf2* transcription (12). Induction of Nqo1 was also inhibited in this hypermethylated state (12). Restoration of N*rf2* transcription by the DNMT inhibitor 5-aza-2'-deoxycytidine resulted in increased expression of Nrf2 and induction of Nqo1 (12). Thus, epigenetic regulation of *NRF2* may be of significance since the nuclear level of NRF2 protein is

ultimately responsible for regulation of many genes that are upregulated by SF through interaction with the ARE (13). Although SF has been shown to upregulate NRF2, there are no studies evaluating the epigenetic effect of SF on *NRF2*.

BAX, as well as *P21,* are controlled by the "master regulator" P53 (14). *P53* is a tumor suppressor gene and restoration of P53 is a promising anti-cancer strategy as it leads to apoptosis and cell cycle arrest (15). P53 was reported to be upregulated by SF (16), but any involvement of epigenetic mechanisms was not evaluated. Thus, it would be interesting to observe any effect of SF on DNA promoter methylation of the *P53* gene.

After the effects of SF on DNA methylation in HCT 116 cells are established, it would be extremely interesting to pursue the same study using LS123 cells. LS123 cells are human colonic epithelial cells that serve as a model of pre-cancerous cells. To elaborate, these cells are immortalized and abnormal, but well differentiated and non-invasive with no tumorigenic potential. They have little mitotic activity and grow in confluent monolayers. These cells are aneuploid and release small amounts of carcinoembryonic antigen. Preliminary data suggest that several of the aforementioned genes are hypermethylated in this cell line (data not shown). DNA methylation occurs early in cancer development, often preceding neoplastic transformation and detection of malignancy. At early stages of cancer, when epigenetic changes are only beginning to be established, these genes may not be in as heavily of a repressed state as during later stages of cancer. Dietary intervention may be most effective at early stages of cancer due to the fact that diet is a chronic and mild effector, compared to conventional chemotherapeutic methods, to prevent and/or slow tumor progression. Therefore, I hypothesize that SF treatment will result in decreased DNA methylation and increased mRNA expression in the LS123 cell model of pre-cancer, perhaps more robustly than in the more invasive HCT 116 cells. This experiment would

provide interesting and novel information pertaining to the plasticity of DNA methylation in early colorectal cancer in response to SF.

Finally, the epigenetic effects of treatment combinations with SF, particularly with lycopene or selenium, should be investigated. The importance of this type of study and reasons for specific interest in SF combination with lycopene or selenium was discussed in section 2.6. Unless being evaluated for use as a drug, all potential future research examining the epigenetic effects of broccoli, alone or in combination with other bioactive dietary agents, should consider including physiologically relevant concentrations of dietary components for the purpose of maintaining a study that is meaningful to the human diet.

6.3 References

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6.4 Figures

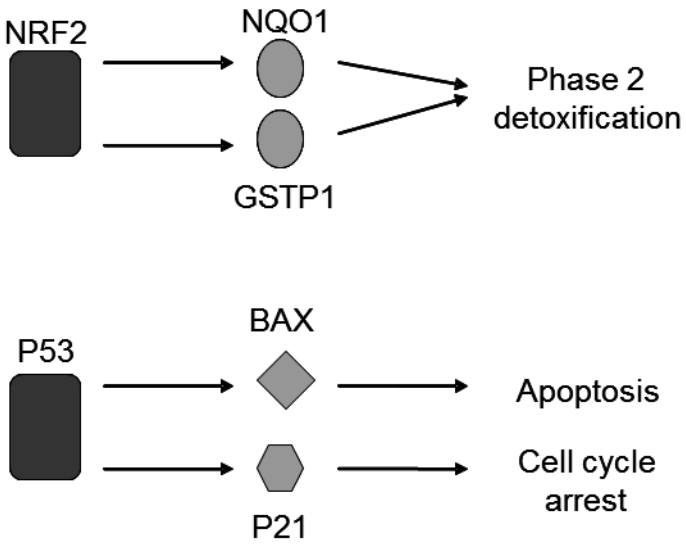

Figure 6.1. Pathways of additional tumor suppressor genes that could be evaluated in an effort to enhance the understanding of the effect of SF on DNA methylation.