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The consumption of cruciferous vegetables is associated with several health benefits, including cancer prevention. Many of these benefits are attributed to the phytochemical, sulforaphane (SFN), which is derived from cruciferous vegetables such as broccoli and broccoli sprouts. These vegetables contain glucoraphanin (GFN), SFN's precursor, which is converted to SFN by the plant enzyme, myrosinase. Studies have shown that SFN influences a variety of biological pathways that are thought to be critical for maintaining health and preventing disease. For example, SFN has been shown to reduce inflammation and oxidative stress, and promote cancer cell-specific cell cycle arrest and apoptosis, leaving healthy cells intact. While several of the health-promoting effects of SFN may be mediated by the Keap1/nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/antioxidant response element (ARE) pathway, emerging evidence suggests that epigenetic mechanisms involving histone deacetylases (HDAC), DNA methyltransferases (DNMT) and microRNAs (miRNA) may also play a role (Chapter 1). Much of what is known about SFN comes from studies conducted in cell cultures and animal models using high doses of SFN and purified chemical forms. Observations from these studies may not reflect events that occur in humans who obtain SFN from dietary sources, such as broccoli

and broccoli sprouts. Only a few studies have been conducted to evaluate the effects of SFN in humans, and results sometimes vary from those seen in preclinical studies. Even fewer studies have evaluated effects of SFN in human tissue. Additionally, there is limited understanding of how dietary form (food form versus supplemental form), doses and dosing regimens may differ with respect to bioavailability and biochemical target. These differences could impact their ability to elicit specific health benefits in humans. Thus, the purpose of this dissertation is to translate mechanistic work with SFN into humans by evaluating responses of genetic and epigenetic molecular targets in human subjects following consumption of controlled doses of dietary SFN. We also evaluated the impacts of dietary form and dosing schedule on SFN absorption and metabolism in humans. The overarching hypothesis for this dissertation is that, in humans, SFN consumption decreases HDAC activity and increases histone acetylation, thereby promoting transcriptional activation of tumor suppressor genes and changes in prognostic biomarkers. We further hypothesize that, in humans, broccoli sprout consumption alters plasma metabolite profiles.

In a randomized, placebo-controlled, clinical trial, we evaluated the effects of consuming a broccoli sprout extract containing GFN, the precursor to SFN, on tumor biomarker responses in breast tissue collected from women (N=54) scheduled for breast biopsy. Following 4-8 weeks of supplementation with the sprout extract, we observed a significant reduction in expression of Ki-67 and HDAC 3 protein in benign breast tissue from pre- to post-supplementation, though responses were not significantly different from those in the placebo group. We also observed decreased HDAC activity in peripheral blood mononuclear cells (PBMC). Importantly, this study identified responses in tumor biomarkers in human breast tissue following consumption of a diet-relevant dose of SFN consumed as part of a broccoli sprout extract.

As the effects of SFN depend on its ability to be absorbed, metabolized and distributed to tissues, we evaluated SFN absorption and metabolism from a myrosinase-treated broccoli sprout extract containing SFN in its active form. Healthy adults (N=20) were randomized to consume a single dose of 200 μmol SFN equivalents from either fresh

broccoli sprouts or the SFN-rich broccoli sprout extract. Approximately 3 times more SFN was absorbed into the plasma and excreted in urine from sprout consumers compared to extract consumers. While this extract delivered higher amounts of SFN than the GFN extract used in the clinical trial, SFN was still relatively more bioavailable from fresh broccoli sprouts. Even though sprouts delivered higher amounts of SFN than the BSE, sprout and BSE consumers had similar changes in PBMC HDAC activity, and circulating levels of heme oxygenase-1 and p21. Furthermore, since SFN metabolites are rapidly metabolized and mostly excreted within 24 hours following a dose, we conducted a second study phase to evaluate the efficacy of a twice-daily dosing regimen on maintaining SFN metabolite levels in the plasma at the 24-hour time point. SFN metabolite levels were higher 24 hours following the divided dose compared to the same time point after subjects consumed a single dose.

To discover additional molecular targets of SFN, we used an untargeted metabolomics approach to screen for changes in the human plasma metabolome following consumption of fresh broccoli sprouts in healthy adults (N=10). This investigation revealed decreases in glutathione, glutamine, cysteine, dehydroepiandrosterone (DHEA), and several fatty acids (14:0, 14:1, 16:0, 16:1, 18:0, 18:1). Deoxyuridine monophosphate (dUMP) was increased. These metabolites are associated with antioxidant status and steroid, nucleotide and lipid metabolism and are possible molecular targets of SFN action. This information can aid in studying novel roles of SFN in human health and disease prevention.

In conclusion, these data provide the first evidence that SFN may alter cell proliferation and epigenetic mechanisms in human breast tissue. Additionally, we identified several metabolites detectable in human plasma that were altered with the consumption of fresh broccoli sprouts. Further study of pathways associated with these metabolites will improve understanding of existing and novel health benefits of SFN. We also demonstrated acceptable bioavailability of a SFN-containing broccoli sprout extract, providing critical information for future human studies with SFN. Taken together, this

work supports that consuming dietary sources of SFN may indeed contribute to improved health and prevention of disease in humans.

Characterizing the Roles of Dietary Sulforaphane in Human Health

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APPROVED:

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Lauren L. Atwell, Author

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CONTRIBUTION OF AUTHORS

Chapter 1: LLA researched and wrote the manuscript; LMB was involved with the design and writing of the manuscript; EH was involved with designing manuscript; and all authors reviewed/edited the manuscript.

Chapter 2: LLA and ZZ were involved with writing the manuscript and data analysis/interpretation; MM, JTV, AMN, KYO, and PT were involved with sample collection; PF assisted with recruitment and data collection; EH and JS assisted with study design and data interpretation; and all authors reviewed/edited the manuscript.

Chapter 3: LLA was involved with study design, recruitment, sample collection/processing, data analysis/interpretation, and wrote the manuscript; AH contributed to study design, recruitment and sample collection; CPW contributed to sample processing, and data interpretation; JFS assisted with sample processing and study design; DB assisted with study design, recruitment and data collection; TWY contributed to sample processing and data interpretation; CBP assisted with data analysis/interpretation and figure development; CVL assisted with data interpretation; JMC conducted pharmacokinetic analyses and assisted with data interpretation; RHD and DEW assisted with study design and data interpretation; EH contributed to study design, sample collection, and data interpretation; and all authors reviewed and edited the manuscript.

Chapter 4: LLA, AH, DB and EH assisted with study design and sample collection/processing; LMB assisted with sample collection/processing; LLA conducted data analysis/interpretation, and prepared the manuscript; LMB, JFS, JC and EH assisted with data interpretation; and all authors reviewed and edited the manuscript.

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EPIGENETIC REGULATION BY SULFORAPHANE: OPPORTUNITIES FOR BREAST AND PROSTATE CANCER CHEMOPREVENTION

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Abstract

Sulforaphane (SFN) is a phytochemical derived from cruciferous vegetables that has multiple molecular targets and anti-cancer properties. Researchers have demonstrated several chemopreventive benefits of SFN consumption, such as reductions in tumor growth, increases in cancer cell apoptosis, and disruption of signaling within tumor microenvironments both *in vitro* and *in vivo*. Emerging evidence indicates that SFN exerts several of its chemopreventive effects by altering epigenetic mechanisms. This review summarizes evidence of the impact of SFN on epigenetic events and how they relate to the chemopreventive effects of SFN observed in preclinical and clinical studies of breast and prostate cancers. Specific areas of focus include the role of SFN in the regulation of cell cycle, apoptosis, inflammation, antioxidant defense, and cancer cell signaling and their relationships to epigenetic mechanisms. Finally, remaining challenges and research needs for translating mechanistic work with SFN into human studies and clinical intervention trials are discussed.

1. Introduction

Epidemiological evidence suggests that consuming cruciferous vegetables (CV), such as broccoli and cauliflower, may lower risks of developing breast and prostate cancers [1]. Sulforaphane (SFN), a phytochemical derived from these vegetables, possesses many of the chemopreventive properties associated with consuming CV [2]. SFN is produced from glucoraphanin (GFN), a glucosinolate precursor found in CV. GFN is converted to SFN via the plant enzyme myrosinase. SFN is metabolized via the mercapturic acid pathway, generating several bioactive metabolites [3]. Early research has focused on the ability of SFN to activate nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and induce Phase 2 enzymes, as well as inhibit enzymes involved in activating carcinogens [4]. However, there has been growing interest in alternative mechanisms of chemoprevention by SFN that

include epigenetic targets [5]. Epigenetics refers to heritable changes in gene expression that are not caused by changes in DNA sequence. Epigenetic mechanisms work in concert to produce changes in chromatin structure and gene expression by modifying interactions among DNA, transcriptional machinery and regulatory protein complexes. Major epigenetic mechanisms include histone modifications, DNA methylation, non-coding RNAs (ncRNA), and chromatin remodeling. Unlike genetic mutations, epigenetic alterations are potentially reversible, making them attractive targets for cancer chemoprevention [6].

Dysregulation of epigenetic mechanisms is emerging as an important factor in cancer development and progression. Epigenetic alterations that improperly silence tumor suppressor genes and activate oncogenes allow cells to acquire cancer-promoting properties, such as uninhibited cell growth and proliferation [7]. In breast and prostate cancers, alterations in the expression of histone deacetylases (HDAC), histone methyltransferases (HMT) and miRNAs, as well as altered levels of histone modifications and DNA methylation, have been observed [8-11]. Reversing these aberrant epigenetic alterations is becoming a focus of many chemopreventive strategies. This review summarizes evidence from preclinical and clinical studies, with a focus on work conducted *in vivo*, that demonstrates the ability of SFN to attenuate breast and prostate carcinogenesis through epigenetic mechanisms (Fig. 1.1). Considerations for translating mechanistic work with SFN into human studies are also discussed.

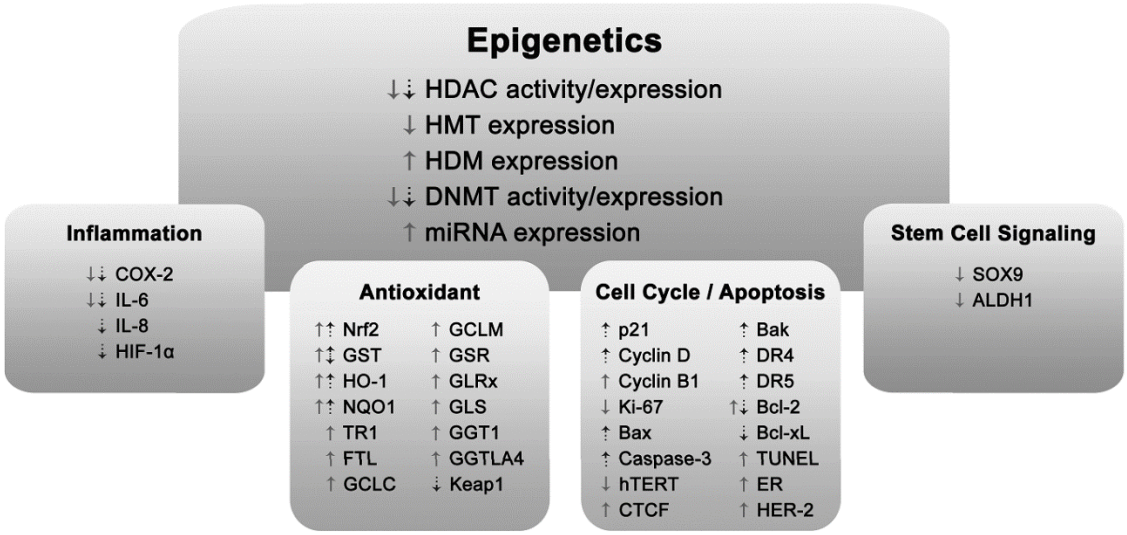

Figure 1.1. SFN-induced changes in epigenetic modifying enzymes and SFN targets that may be regulated in part through epigenetic mechanisms. Pictured are multiple targets relevant to breast (solid arrows) and prostate (dotted arrows) cancer prevention. CTCF, CCCTC-binding factor; COX-2, cyclooxygenase-2; DNMT, DNA methyltransferases; DR, death receptor; ER, estrogen receptor; FTL, ferritin, light polypeptide; GCLC, glutamate-cysteine ligase, catalytic unit; GCLM, glutamate-cysteine ligase, modifier unit; GGT1, gamma-glutamyltransferase 1; GGTLA4, gamma-glutamyltransferase-like activity 4; GLRx, glutaredoxin 1; GSL, glutaminase; GSR, glutathione reductase; GST, glutathione-*S*-transferase; HDAC, histone deacetylase; HDM, histone demethylase; HER-2, human epidermal growth factor receptor 2); HIF-1α, hypoxia-inducible factor 1α; HMT, histone methyltransferase; hTERT, human telomerase reverse transcriptase; IL, interleukin; Keap1, kelch-like ECH-associated protein 1; miRNA, microRNA; NQO1, NAD(P)H:Quinone Oxidoreductase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; TR1, thioredoxin reductase 1; TUNEL, terminal nucleotidyl transferase-mediated nick end labeling.

2. Sulforaphane and Epigenetics in Prostate and Breast Cancer

SFN has been shown to alter key epigenetic mechanisms *in vivo* and *in vitro* with corresponding impact on prostate and breast cancer development. Histone modifications, which occur on histone tails, alter interactions between histones and DNA and affect gene

transcription. Histone acetyltransferases (HAT) add acetyl groups to lysine residues within histone tails, thereby relaxing the chromatin structure and facilitating activation of gene transcription. Histone deacetylases (HDAC) suppress gene transcription by removing these acetyl groups [6]. Reduced HDAC activity in peripheral blood mononuclear cells (PBMC), prostates, and prostate cancer cell xenografts were reported in mice that consumed 443 mg/kg SFN in the diet for 3 weeks [12]. Prostates and xenografts exhibited corresponding increases in global acetylation of histones H3 and H4. Importantly, site-specific increases in histone acetyl marks were observed at gene promoters for p21 and Bax in tissues with corresponding increases in gene expression [13]. Additionally, SFN reduced protein levels of specific HDACs in prostate and breast cancer cell lines at concentrations ranging from 1-15 μM [14-16]. These concentrations have also been shown to inhibit HDAC activity and alter histone acetyl marks in breast cancer cell lines [16-18]. While SFN's effect on HAT expression and activity has received less attention, some studies have reported no change in HAT activity in breast cancer cell lines following SFN treatment [16, 18].

Emerging evidence suggests that SFN may alter additional epigenetic processes in the breast and prostate including DNA and histone methylation as well as ncRNAs. DNA methyltransferases (DNMT) add methyl groups to cytosine bases in DNA. High levels of DNA methylation are generally associated with gene silencing. DNMT1, often referred to as the "maintenance" DNMT, maintains methylation patterns through cell division. In contrast, DNMT3a and DNMT3b are responsible for *de novo* methylation and methylate DNA during development and according to environmental signals [9]. In human and mouse breast and prostate cancer cell lines, SFN treatment decreased DNMT activity and protein levels of DNMT1 and DNMT3a at SFN concentrations ranging from 1-30 μM. As a consequence, attenuated global and site-specific DNA methylation were linked to altered gene expression [15, 16, 18-20]. Histone methyltransferases (HMT) add methyl groups generally to lysine and arginine residues, and histone demethylases (HDM) remove them. Changes in chromatin structure resulting from histone methylation depend on the number of methyl groups and the residue modified [9]. ncRNAs are produced from non-coding regions of DNA and also play critical roles in modifying the epigenome. miRNAs are

ncRNAs that bind mRNAs with appropriate "seed sequences", which prevents the mRNA from being translated or enhances degradation of the mRNA template and have been implicated in cancer development [21, 22]. In human breast cancer cell lines, SFN treatment decreased protein levels of SUV39H1 (a HMT) and histone methyl marks (H3K27me3 and H3K9me3), increased protein levels of RBP2 (a HDM), and altered expression of the miRNA, miR-140, and its downstream targets [16, 18, 23]. To our knowledge, a direct interaction between SFN and chromatin remodeling complexes (*e.g.*, SWItch/Sucrose NonFermentable (SWI/SNF)) has not been established, but epigenetic events such as histone acetylation have been shown to influence nucleosome structure (reviewed by [24]).

3. Prostate Cancer

Chemopreventive properties of SFN have been demonstrated in the prostate *in vivo*. In mouse models of prostate cancer, SFN ingestion decreased tumor growth, increased cancer cell apoptosis, and prevented cancer progression [25, 26]. Several groups reported that SFN reached prostate tissue in rodents following oral consumption and had localized effects [12, 13, 27, 28]. Much of the epidemiological evidence suggests that individuals who consume greater amounts of cruciferous vegetables have a decreased risk of developing prostate cancer [29]. Additionally, a prospective study reported a decreased risk of prostate cancer progression with higher intakes of cruciferous vegetables [30]. There have also been a few human clinical prostate cancer trials conducted using SFN. In one such study involving men with recurrent prostate cancer, none of the patients experienced PSA doubling, a marker of disease severity, after consuming SFN daily for < 20 weeks [31]. In men diagnosed with high-grade prostatic intraepithelial neoplasia, a 12-month dietary broccoli intervention altered gene expression in prostate tissues relating to cell signaling pathways such as androgen, TGFβ1, insulin, and EGF signaling [32]. These findings suggest a potential role of SFN in preventing prostate cancer. Emerging evidence suggests that SFN's

chemopreventive activities in the prostate involve epigenetic regulation of the cell cycle, apoptosis, cellular antioxidant defenses, and chronic inflammation. Understanding the role of these epigenetic-related mechanisms in humans is an important future area of research.

3.1 Cell Cycle and Apoptosis

Uninhibited cell growth and evasion of apoptosis are classical hallmarks of cancer [33], and SFN has been shown to induce G1/S and G2/M cell cycle arrest and apoptosis specifically in human prostate cancer cells compared to non-cancerous cells [28, 34]. There is also *in vivo* evidence of SFN efficacy in prostate cancer prevention. SFN-fed mice showed a reduction in prostate tumor growth that was associated with increased apoptosis and decreased cell proliferation [25]. Furthermore, SFN consumption has been associated with increased expression of p21, cyclin D, Bax, caspase-3, Bak, and death receptors DR4 and DR5 and decreased expression of Bcl-2 and Bcl-X_L in prostate tissues [12, 13, 25, 26]. Similar observations were made when whole-food sources of SFN (broccoli and broccoli sprouts) were consumed [35, 36]. Some of these effects may be mediated by SFN's ability to inhibit HDACs. For example, mice that consumed 6 μmol SFN daily for 10 weeks had decreased levels of HDAC activity, increased acetylated histones H3 and H4, and increased expression of p21 in the prostate [13]. Like p21, cyclin D, Bak, Bax, Bcl-2, Bcl-X_L, caspase-3, DR4 and DR5, are often dysregulated in cancer cells through epigenetic modifications, thus SFN could be inducing changes in their expression through epigenetics mechanisms *in vivo* [37-40]. Recent research in cancer cells supports this idea. For example, in human prostate cancer cells treated with SFN, increased cyclin D2 expression was associated with decreased DNA methylation in its promoter. In these cells, SFN treatment also decreased mRNA and protein levels of DNMTs [19].

3.2 Antioxidant Defenses

Cellular antioxidant defenses protect cells against the damaging effects of oxidative stress and inflammation [41, 42]. SFN has been shown to stimulate these defense mechanisms *in vivo*, often through inducing Nrf2 [43, 44]. Keum *et al.* [36] reported that dried, ground broccoli sprouts administered in the diet increased Nrf2 and decreased Keap1 protein in mice. These changes were associated with induction of an Nrf2 target gene, heme oxygenase-1 (HO-1). Additionally, increases were observed in the activities of NAD(P)H:Quinone Oxidoreductase 1 (NQO1), the specific glutathione-*S*-transferase (GST) GSTM, and of total GSTs in the prostates of SFN-fed rats [45]. In rat prostates, Liu *et al.* [46] observed modest decreases in GSTP1 mRNA following consumption of a broccoli powder.

SFN is classically thought to induce Nrf2 by reacting with cysteine residues on Keap1, the cytosolic repressor/chaperone for Nrf2 [47]. Yet, recent evidence suggests that epigenetic mechanisms also contribute to SFN's regulation of Nrf2-mediated gene expression. Increased site-specific CpG methylation within the Nrf2 promoter was associated with reduced Nrf2 expression in mouse prostate tumors [48]. In prostate cancer cells, Zhang *et al.* [15] demonstrated that SFN could derepress Nrf2 by reducing CpG methylation. In these cells, they also observed dose-dependent decreases in expression of DNMT1, DNMT3a, HDACs 1, 4, 5 and 7, and increased binding of acetylated histone H3 (Ac-H3) to the Nrf2 promoter following SFN treatment. Epigenetic mechanisms have also been implicated in regulating expression of Keap1 and several GST enzymes [49-52]. There is likely interplay among the genetic and epigenetic mechanisms to elicit the chemopreventive effects of SFN, but more work is needed to understand the precise influence of SFN on its targets in the prostate.

3.3 Inflammation

Inflammation is a major driver of carcinogenesis [33], and epigenetic events play a role in inflammation-mediated cell transformation [53]. Consumption of *Brassica* vegetables has been shown to reduce markers of systemic oxidative stress and inflammation [54]. A major player that regulates inflammation is the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [53]. NF-κB has increased activity in prostate cancer and regulates the expression of many pro-inflammatory mediators through a well-described signaling pathway [55, 56]. In mice, SFN consumption was shown to reduce NF-κB activity and the expression of several NF-κB targets in prostate cancer cell xenografts. These targets included pro-inflammatory mediators interleukins IL-6 and IL-8, hypoxia-inducible factor 1α (HIF-1α), and cyclooxygenase-2 (COX-2) [25]. Several of these genes are known to be regulated by various epigenetic mechanisms, so it is possible that epigenetic alterations induced by SFN contributed to these changes in gene expression [39, 57]. For example, in prostate cancer cells, miR-101 inhibits COX-2 posttranscriptional expression [58], and IL-6 is regulated by the miRNA, let-7, in breast epithelial cells [53]. Additionally, stress-induced increases in IL-6 expression in mouse myoblasts were attenuated following treatment with the HDAC inhibitor, trichostatin A [59]. Importantly, Wong *et al.* [20] demonstrated that, in prostate cancer cells, 15 μM SFN significantly altered the DNA methylation status of the promoters of many genes that regulate inflammation and immune development, including the promoter of IL-6. These data support the possibility that SFN may work through epigenetic mechanisms in prostate tissue to reduce inflammation.

4. Breast Cancer

Several epidemiological studies have also indicated that consuming cruciferous vegetables may help prevent breast cancer [60-62]. Further evidence of SFN efficacy in breast cancer

prevention comes from dietary intervention studies conducted in rats, where consumption of SFN, broccoli, and broccoli sprout extracts was associated with reductions in multiplicity, size, and growth rate of mammary tumors and breast cancer cell xenografts [4, 23, 63-66]. Similar studies in humans are only starting to emerge, though there is evidence that dietary SFN can reach the breast tissue in humans. Cornblatt *et al.* [67] demonstrated in healthy, pre-menopausal women that consuming a broccoli sprout extract beverage containing SFN resulted in measureable levels of SFN metabolites in plasma, urine and breast tissue within 24 hours. This group also detected SFN metabolites in rat mammary tissues following SFN gavage and observed concurrent alterations in antioxidant gene expression. As discussed earlier (Chapter 1, Section 2), SFN decreased the activity and expression of HDACs, DNMTs and the HMT, SUV39H1, and altered histone marks, DNA methylation, and miRNA expression in human breast cancer cell lines [16-20, 23]. Below, we discuss the emerging evidence that these epigenetic mechanisms may mediate certain effects of SFN in the breast, including regulation of antioxidant defenses, cell cycling, apoptosis, and signaling within cancer stem cells (CSC) and tumor microenvironments. Additionally, several SFN targets that are epigenetically regulated in the prostate (*e.g.*, Bcl-2, COX-2) are also altered in breast cancer cells [68, 69]. Investigations to determine SFN's impact on these targets in breast tissue will provide further information on SFN's role in breast cancer prevention.

4.1 Antioxidant Defenses

SFN may be able to reduce oxidative stress in breast tissue by modulating the expression of antioxidant mediators. Several of these proteins are regulated by Nrf2 [47], and SFN has been shown to increase Nrf2 expression and activity in breast cell lines [70]. In rat mammary glands, SFN consumption increased the expression of NQO1 and HO-1, and activity of NQO1, with concurrent increases in the tissue levels of SFN and SFN metabolites [67]. Increased NQO1 and GST activities were also observed in mouse

mammary glands following SFN intake [71]. Importantly, Cornblatt *et al.* [67] demonstrated that NQO1 and HO-1 were detectable in human breast tissues, implicating that these genes could be useful for studying SFN mechanisms in human populations. Evidence from work in SFN-treated, human breast cancer cells suggests that SFN effects on NQO1, HO-1 and GSTs in the breast are mediated in part through epigenetic events [72, 73]. For example, in these cells, SFN exposure increased HO-1 and NQO1 expression, and these changes were associated with increased HAT (p300) recruitment to gene promoters and site-specific increases in H3K9Ac [72].

SFN also increases other antioxidant mediators in breast cells. In cancerous and non-tumorigenic, human breast epithelial cells, SFN exposure increased thioredoxin reductase 1 (TR1), ferritin, light polypeptide (FTL), and proteins involved in glutathione (GSH) metabolism, specifically catalytic and modifier subunits of glutamate-cysteine ligase (GCLC, GCLM), glutathione reductase (GSR), glutaredoxin 1 (GLRX), glutaminase (GLS), gamma-glutamyltransferase 1 (GGT1), and gamma-glutamyltransferase-like activity 4 (GGTLA4) [74, 75]. Epigenetic mechanisms have been implicated in regulating the expression of these genes [76-82]. For example, DNA methylation-dependent regulation of TR1 expression was demonstrated in human breast cancer cells, where a demethylating agent increased TR1 protein levels [78].

4.2. Cell Cycle and Apoptosis

Several studies have demonstrated SFN's ability to disrupt cell growth and proliferation and induce apoptosis in the breast. In mice, SFN injections decreased growth of breast cancer cell xenografts. These xenografts exhibited decreased cell proliferation and Ki-67 staining and increased apoptosis and staining of terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) in a dose-dependent manner with SFN treatment [65]. Stearns *et al.* [83] reported decreased Ki-67 expression in human breast cancer biopsies following treatment with the pharmacological HDAC inhibitor, Vorinostat, suggesting that

epigenetic events play a role in regulating Ki-67. Since SFN can inhibit HDAC activity in breast cancer cells [17], it may work through a similar mechanism to alter expression of Ki-67 in breast tissue.

SFN has been shown to alter the expression of additional cell cycle and apoptotic regulators in breast cancer cell lines. Meeran *et al.* [18] reported decreased hTERT expression in human breast cancer cells following SFN treatment. In these cells, decreased DNMT expression and CpG methylation within the binding region of hTERT's repressor, CTCF, occurred along with increased repressor binding. These events were accompanied by decreased HDAC activity, increased expression of RBP2, a HDM that regulates hTERT, and changes in histone acetyl and methyl marks within the *hTERT* promoter. In a separate study, SFN treatment increased expression of estrogen receptor (ER), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER-2), cyclin B1, Fas ligand, and Bcl-2 in human breast cancer cell lines [17]. Decreased HDAC activity was also observed in these cells following SFN treatment, but interestingly, without apparent changes in global histone acetylation. These changes were also associated with G2/M arrest and apoptosis.

SFN likely works through multiple epigenetic mechanisms to elicit changes in gene expression. In fact, both DNA methylation and histone acetylation were shown to be involved in regulating ER expression [84]. There is also evidence that epigenetic regulation contributes to changes in cyclin B1, Bcl-2 and HER-2 expression, but these mechanisms need to be validated in breast tissue [85-87]. Similar to prostate cancer, the chemopreventive effects of SFN may be attributed to a combination of genetic and epigenetic targets.

4.3 Cancer Stem Cell and Tumor Microenvironment Signaling

Dysregulation of cancer stem cell (CSC) signaling can increase risk of tumor development and progression [88]. SFN may be able to alter signaling in breast CSCs and the tumor

microenvironment through epigenetic modulation. Li *et al.* [23] reported that tumor-suppressive miR-140 was consistently decreased in early-stage and invasive subtypes of breast cancer compared to non-cancerous mammary cells and tissues. In breast CSCs derived from these subtypes, SFN restored expression of miR-140 by decreasing DNA methylation at a specific intronic gene locus. When breast CSCs were injected into mice, tumors resulting from CSCs that had been pre-treated with SFN had higher levels of miR-140, reduced expression of tumor-promoting CSC regulators (SOX9 and ALDH1), and decreased tumor size compared to tumors resulting from non-treated breast CSCs [23]. The ability of SFN to regulate CSC signaling in these tumors presents an opportunity for preventing the development of aggressive and therapy-resistant breast cancers.

There is also evidence that SFN interferes with the cross-talk that occurs between adipocytes and mammary stem cells that influences tumor promotion. When SFN was added to cultures containing breast CSCs and adipocytes, CSC migration was markedly reduced. When co-cultured CSCs were pre-treated with SFN and injected in to nude mice, resulting tumors were much smaller and stopped growing sooner than tumors arising from untreated, co-cultured CSCs. Similar results on tumor growth were found when mice were injected with SFN daily following xenograft implantation [89]. IL-6 mRNA was also lower than in untreated CSCs, suggesting that SFN's interference in the adipocyte-CSC cross-talk was mediated by altering cytokine expression [90]. This finding is highly relevant for cancer chemoprevention strategies, because adipocyte-secreted cytokines have been shown to promote tumor development and migration [91]. Furthermore, there may be an epigenetic interconnection with regards to IL-6 expression, as decreases in IL-6 promoter methylation have been observed in human breast cancer cells due to p53 deficiency [92]. This decrease in IL-6 methylation was associated with increases in IL-6 expression and an epigenetic reprogramming of the cells towards a basal-like/stem cell-like gene expression profile. This area of research warrants additional studies to investigate the mechanisms by which SFN may alter cross-talk between tumor cells and adipocytes *in vivo*.

5. Remaining Challenges and Research Needs

While there is evidence that SFN consumption may be beneficial for breast and prostate cancer chemoprevention, the optimal supplementation form and dosing regimens for SFN in humans still need to be established. This effort will involve clarifying the bioavailability and distribution of SFN and its specific metabolites to human tissues. To date, feeding studies and clinical trials investigating the effects of SFN use a variety of crucifers and extracts to deliver SFN or its precursors, but circumstances that dictate the use of specific sources, forms, or combinations are still emerging. In human feeding studies, consumption of raw or slightly cooked cruciferous vegetables resulted in higher levels of SFN in the body as compared to boiled or steamed vegetables [93, 94]. High cooking temperatures are thought to inactivate the myrosinase enzyme that is needed for deriving SFN from its glucosinolate precursor. It is possible that higher SFN bioavailability confers enhanced chemopreventive activity, substantiating the need for further work in this area as well as efficacy studies in humans. Fresh broccoli sprouts with active myrosinase have been identified as particularly rich dietary sources of SFN, but the high variability in SFN yield among sprout batches create logistical challenges for clinical researchers [95]. Supplemental forms of SFN or its precursor, GFN, have been used to circumvent issues of varying SFN yields from food sources; however, these forms can result in lower SFN absorption than from broccoli sprouts [96]. In an effort to improve SFN absorption from plant extracts, Cramer and Jeffery [97] demonstrated that SFN absorption from a GFN-rich broccoli powder devoid of myrosinase activity was enhanced when co-consumed with fresh broccoli sprouts. Results from another study raised the question of whether or not a combination of GFN and SFN sources can achieve additional benefits than consuming either alone [98]. More research is needed to evaluate and optimize specific formulations, combinations and dose schedules for SFN delivery *in vivo,* and especially in human subjects.

A better understanding of SFN distribution to target tissues will also help to establish the SFN doses and dosing schedules that achieve effective tissue concentrations.

Understanding the distribution of specific metabolites of SFN is important given that the parent compound and metabolites may have differing molecular targets and mechanisms of action. For example, SFN has been implicated as the compound responsible for releasing Nrf2 from Keap1, whereas SFN-Cysteine and/or SFN-N'acetylcysteine may be responsible for inhibiting HDACs [5, 99, 100]. SFN metabolites have been observed in multiple animal tissues (*i.e.*, adipose, bladder, brain, breast, colon, duodenum, heart, jejunum, kidney, liver, lung, pancreas, prostate, rectum, and skeletal muscle) [27, 28, 67], but SFN compounds have been detected in humans only in breast tissue [67]. Access to human tissues is limited, generally as clinical biopsies obtained from medical procedures not necessarily linked to predetermined research objectives. This highlights the importance of engaging in research collaborations and optimizing sample preparations to maximize the use of clinical biopsies and animal tissues for research purposes.

Another challenge for clinical researchers is the inter-individual variability in SFN metabolism, which has been observed in many controlled human feeding studies. The factors underlying variability in SFN metabolism remain poorly understood. Thus, it is important to identify and characterize key factors that may impact SFN metabolism and distribution to tissues. Such factors include the presence or severity of disease, SFN formulation or diet preparation, tissue type, gut microbiota composition, and genotype of GSTs [101]. Pharmacokinetic studies designed to systematically evaluate the impact of putative factors should consider stratifying subjects prior to randomization to increase statistical power and maximize the value of observations.

Ultimately, establishing key targets of SFN action in humans is critical to determine how SFN can be effectively utilized in chemoprevention strategies. It is worth noting that due to the differences in the genetic background between mice and humans, there may be additional challenges in translating SFN targets discovered in rodent models to the clinic. Nevertheless, once established, tissue-specific targets can be used as biomarkers to evaluate the chemopreventive efficacy of dietary SFN strategies in humans, as well as the impact of SFN metabolism and distribution on chemopreventive outcomes. The use of a single target is likely not sufficient for studying the totality of SFN's chemopreventive

effects, especially given that one specific target may not be altered significantly prior to or in the early stages of cancer development, which could limit its use as a biomarker at specific disease stages [14]. Additionally, the use of genetic (*e.g.*, Nrf2-regulated genes) and epigenetic mediators (*e.g.*, HDAC, DNMT, ncRNAs) either alone or in combination with proliferation and apoptosis markers could improve understanding of the timing and contributions of various epigenetic mechanisms to specific outcomes. Approaches such as metabolomics may also reveal new mechanisms and novel SFN targets within prostate, mammary, and other target tissues of interest. The outcomes of this research will rely on the ability to understand the physiological relevance of changes in SFN targets observed in human studies; thus, it is critical to quantify the effect sizes for SFN targets that are needed to elicit chemopreventive outcomes.

It is clear that SFN has multiple targets of action in the breast and prostate that may be coordinated by both genetic and epigenetic mechanisms. Future research will need to understand relationships among genetic and epigenetic targets as well as among the bioavailability of active compounds and defined molecular targets in tissues. Emerging bioinformatics technologies can evaluate information on a wide range of SFN targets simultaneously and integrate this information with the presence of SFN metabolites, which will help to clarify SFN mechanisms *in vivo.* In these evaluations, it will be important to consider the disease context and underlying cellular phenotype, as these factors are likely to impact relationships among SFN compounds, SFN targets, and resulting biological outcomes.

6. Conclusions

SFN is a promising dietary chemopreventive agent due to its ability to target multiple pathways involved in carcinogenesis. The ability to alter epigenetic events in the breast and prostate may underlie many of SFN’s chemopreventive effects in these tissues. More research is needed to determine the impact of SFN-induced changes in epigenetic

mechanisms and their cross-talk during cancer development. To inform chemoprevention strategies for breast and prostate cancers, investigations of SFN's chemopreventive efficacy should focus on tissue-specific effects and work to establish effective doses for different disease stages and human subpopulations.

CHAPTER 2

SULFORAPHANE BIOAVAILABILITY AND CHEMOPREVENTIVE ACTIVITY IN WOMEN SCHEDULED FOR BREAST BIOPSY

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Abstract

Epidemiological studies suggest a protective effect of cruciferous vegetables on breast cancer. Sulforaphane (SFN), an active food component derived from crucifers, has been shown to be effective in breast cancer chemoprevention. This study evaluated the chemopreventive effect of SFN on selective biomarkers from blood and breast tissues. In a 2-8-week double-blinded, randomized controlled trial, 54 women with abnormal mammograms and scheduled for breast biopsy were randomized into SFN (n = 27) or placebo (n = 27) intervention. Plasma and urinary SFN metabolites, peripheral blood mononuclear cell (PBMC) histone deacetylase (HDAC) activity, and tissue biomarkers (H3K18ac, H3K9ac, HDAC3, HDAC6, Ki-67, p21) were measured before and after the intervention in benign, ductal carcinoma *in situ* (DCIS), or invasive ductal carcinoma (IDC) breast tissues if available. Within the SFN group, Ki-67 (p = 0.004) and HDAC3 (p = 0.047) levels significantly decreased in benign tissue. Pre-to-post-intervention changes in these biomarkers were not significantly different between treatment groups after multiple comparison adjustment. SFN supplementation was associated with a marginally significant decrease in PBMC HDAC activity (p = 0.07). This association reached statistical significance among non-NSAIDs users (p = 0.03). No significant associations were observed between SFN and examined tissue biomarkers when comparing treatment groups. This study provides evidence that low-dose SFN supplementation for a few weeks is safe but may not be sufficient for producing changes in breast tissue tumor biomarkers. Future studies employing larger sample sizes should evaluate alternative dosing and duration regimens to inform dietary SFN strategies in breast cancer chemoprevention.

1. Introduction

Several lines of evidence indicate that increased consumption of cruciferous vegetables has a chemopreventive effect and may protect against several of the most common types of

cancer, including breast cancer [102]. Although the role of vegetable consumption in breast cancer risk remains controversial, several studies have demonstrated a decrease in breast cancer risk with increasing cruciferous vegetable intake [60, 62]. Cruciferous vegetables and their constituent biologically active food components, including indoles and isothiocyanates (ITC), such as sulforaphane (SFN), appear to modulate breast cancer risk at multiple stages of carcinogenesis through a variety of biological mechanisms.

Ductal carcinoma *in situ* (DCIS) is a non-invasive form of breast cancer that accounts for about 20% of newly diagnosed cases of breast cancer [103, 104]. DCIS lesions arise from terminal-duct-lobular units. Their presentation is considered a direct precursor, and thus a very high risk factor, for invasive cancer [104, 105]. While there have been recent improvements in the treatment of breast cancer, epidemiological studies have shown that women are more likely to change their lifestyle behaviors and medication use following diagnosis of DCIS [106]. Hence, there is a need for scientifically directed evaluation of the effect of alternative or supplemental therapies, such as dietary supplements that may effectively inhibit the progression of breast cancer in women. In this study, we evaluated the impact of SFN supplementation on molecular response biomarkers in blood and breast tissue (including tumor and non-tumor) from women that were scheduled for diagnostic biopsies following abnormal mammogram results. This is the first report of the effects of SFN on breast tissue physiology in women. Observations from this study will inform SFN supplementation strategies in women with DCIS with or without a component of invasion, as well as women that present with benign tissue.

SFN exists in particularly high amounts in broccoli and broccoli sprouts [64] as the glucosinolate precursor, glucoraphanin (GFN). When the plant is consumed, GFN is converted to SFN by myrosinases released from plant tissue and present in the human gut [107]. SFN has been shown to be an effective chemopreventive agent in both *in vitro* and *in vivo* models for breast cancer where SFN is able to selectively induce apoptosis and slow tumor growth [66, 108-110]. Mechanistic studies have identified several targets of SFN, including cell cycle proteins such as p21, which may be involved in its anti-cancer activities [65]. SFN has also been shown to decrease levels of Ki-67, a marker of cell proliferation,

in prostate tumor tissue [25]. Ki-67 is known as an important prognostic biomarker in women with breast cancer [111].

Recent work indicates that SFN targets epigenetic alterations and inhibits histone deacetylases (HDAC) [13, 112]. HDACs, along with histone acetyltransferases (HAT), facilitate an important mechanism of gene regulation which involves the removal and addition, respectively, of acetyl groups from histone proteins. Inhibiting HDACs can lead to increased histone acetylation and re-expression of tumor suppressor genes (*e.g.*, ERα, p21) that are often silenced in cancer cells [113, 114]. Pharmacological HDAC inhibitors have demonstrated anti-cancer effects in breast cancer cells both *in vitro* and *in vivo* [115, 116]. However, the adverse effects of these agents make them undesirable for long-term use in women with pre-invasive disease, such as DCIS [117]. Intake of cruciferous vegetables and dietary SFN are considered safe and have not been associated with any serious adverse side effects [118]. Therefore, we aimed to evaluate the efficacy of supplemental SFN intake in altering HDAC activity and improving biomarkers for prognosis in women with benign disease or DCIS with or without a component of invasion.

2. Materials and Methods

2.1 Participants

This double-blind, randomized, placebo-controlled clinical trial was conducted in collaboration with clinicians and researchers at Oregon Health and Science University's (OHSU) Center for Women's Health Breast Center in Portland, OR. English-speaking women were recruited to participate in the study from OHSU, Kaiser Permanente Northwest and Epic Imaging Clinics. Inclusion criteria included: ≥ 21 years of age, diagnostic mammogram with results that required biopsy. Exclusion criteria included: invasive breast cancer without DCIS or atypical ductal hyperplasia (ADH), pregnancy (determined by clinically administered urine pregnancy test), patient reported

breastfeeding, significant active medical illness, history of or active liver disease or baseline total bilirubin greater than institutional upper limit of normal, allergy to cruciferous vegetables, use of oral antibiotics (except doxycycline) within three months prior to randomization, oral steroid therapy at enrollment, current therapy with valproate acid or suberoyl + anilide + hydroxamic acid (SAHA), current and planned continuous use of SFN-containing supplements, herbal remedies or pharmaceutical HDAC inhibitors, additional surgical operations scheduled within 30 days of study start date, neoadjuvant radiation or chemotherapy for currently-diagnosed disease prior to or during study supplementation, or any condition possibly exacerbated by participating. Eligible women met with study coordinators at the OHSU Clinical and Translational Research Center (CTRC) to review the study's purpose and exclusion criteria. All participants provided informed consent. Study protocols were approved by OHSU and Kaiser Permanente Northwest committees for the protection of human subjects (ClinicalTrials.gov Identifier: NCT00843167).

2.2 Study Design

The study sample size flowchart is depicted in Figure 2.1 following CONSORT guidelines [119]. Consented subjects (N = 54) were randomized to consume a minimum two-week supply of either ~250 mg of broccoli seed extract (30 mg sulforaphane glucosinolate) from BroccoMax™ supplements (2 pills, 3 times daily) (n = 27), which are commercially available, or matching placebos containing ingredients of microcrystalline cellulose (n = 27).

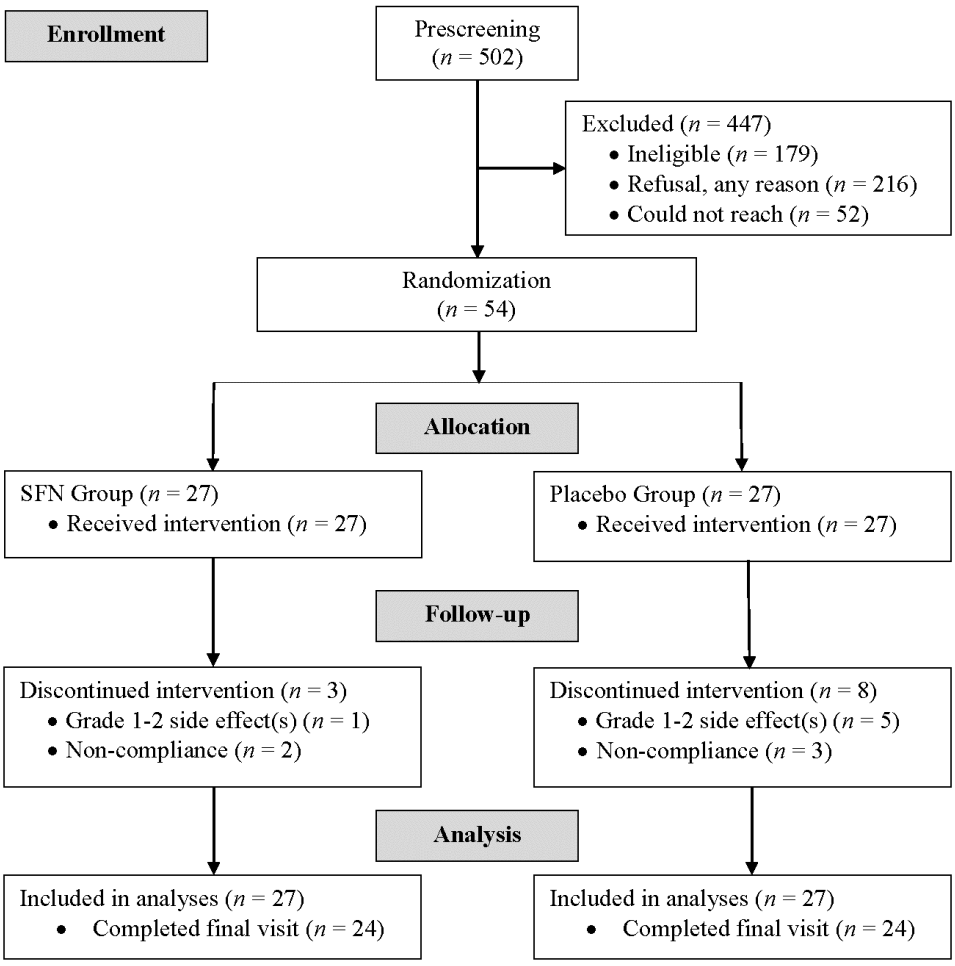

Figure 2.1. Randomized controlled trial sample size flow chart.

Subjects always began supplementation following diagnostic biopsy and informed consent. For those who were diagnosed with DCIS or ADH with or without a component of invasive cancer, supplementation ended the day before operation. The maximum supplement intervention period was 8 weeks (56 days). Women with surgery scheduled earlier than 2 weeks post-biopsy were not eligible for enrollment, such that participation in the study would not delay surgery. Subjects assigned to the placebo received capsules identical to the BroccoMax™ capsules three times a day. The dose of BroccoMax™ supplements used

in this study yielded 210 mg GFN per day, which is similar to the amount of broccoli sprout extract administered in our pilot study and other trials that achieved a significant increase in blood and urine isothiocyanate levels and reduced histone activity within one month with no reported adverse effects [12, 118, 120]. All pills and containers were provided by Jarrow Formulas® (Los Angeles, CA) and were dispensed by the OHSU Research Pharmacy. Administration of the intervention was extended up to an eight-week supply for women experiencing surgical operation delays not related to the dietary intervention or for whom operation was not indicated or chosen post-biopsy. After consenting to the research, subjects also completed family history and risk factor questionnaires, as well as two dietary history questionnaires: a modified National Cancer Institute (NCI) diet history questionnaire and the Arizona cruciferous vegetable food frequency questionnaire (CVFFQ) [121]. Additional questionnaires were administered throughout the duration of each woman's participation on and 30 days after the intervention to monitor cruciferous vegetable intake, safety, and any changes in medications, supplement use or dietary intake. For any reported adverse event characterized as grade 3 or higher, according to the NCI Common Terminology Criteria for Adverse Events Version 3.0, the responsible clinician was notified, the event was determined related/not-related to the intervention, and the event was recorded. Adherence to study protocol ($\geq 80\%$) was determined by Research Pharmacy count of returned pills.

2.3 Sample Collection

Two, 30-ml whole blood specimens and a spot urine sample were collected from each participant at the baseline visit prior to starting the intervention and at the final visit. Plasma was isolated by centrifugation, immediately acidified with 10% (v/v) pre-cooled trifluoroacetic acid (TFA), and stored at -80°C. PBMCs were isolated using Histopaque (Sigma, St. Louis) separation, suspended in DMSO, and stored in liquid nitrogen until further analysis (HDAC activity, acetylated histone status). Urine samples were acidified

immediately with 10% (v/v) pre-cooled TFA and stored at -80°C. Diagnostic tissue specimens were formalin fixed, subjected to routine processing and paraffin embedding, and maintained in the pathology archive according to standard clinical protocols. All breast biopsy or surgical tissues were evaluated for the presence of DCIS and/or ADH or invasive cancer immediately after these procedures by board certified pathologists.

2.4 Preparation of Mass Spectrometry Standards

Chemical standards for (R,S-) SFN and its metabolites (SFN-GSH, SFN-Cys, SFN-NAC) were purchased from LKT Laboratories, Inc. (St. Paul, MN) and Toronto Research Chemicals (Canada), respectively. Deuterated SFN-NAC (SFN-NAC-D3) and SFN-cysteinylglycine (SFN-CG) were prepared in-house as previously described [96]. GFN and glucotropaeolin (GTP) were purchased from The Royal Veterinary School of Denmark and AppliChem (Darmstadt, Germany), respectively. All final standard dilutions were prepared in 0.1% (v/v) formic acid (FA) in H2O. Consistent and high (>80%) recoveries of isothiocyanate and glucosinolate standards from both biological matrices and 0.1% (v/v) FA in water were confirmed through a series of spike and recovery experiments using appropriate internal standards.

2.5 Dietary SFN Supplementation Analysis

Each batch of BroccoMax™ supplements administered to subjects during the trial was analyzed in duplicate for GFN content. Our method was adapted from Tian, *et al*. [122]. BroccoMax™ powder (~450-480 mg) was dissolved into 100% methanol and homogenized 5 min with an Omni homogenizer (Omni International, Kennesaw, GA). Mixtures were centrifuged (5 min, 25°C, 10,000 x g). Supernatants were transferred to fresh tubes and set on ice. Methanol extractions were performed a total of 3 times on the

same pill matter. Preliminary experiments revealed > 95% GFN recovery within first 3 extractions (data not shown). All extracts were combined and filtered through Spin-X® centrifuge tube filters (VWR, Radnor, PA) by centrifugation (5 min, 25°C, 10,000 x g). Filtrates were diluted with 0.1% (v/v) FA in H2O to final concentrations of 250 µM GFN and stored at -20°C until analysis by HPLC/MS-MS. GTP was used as an internal standard. Pill extracts (10 µl) were injected in duplicate. HPLC-MS/MS conditions were similar to those in our previous study [28], except that analysis was performed in negative ion mode using a 4-µm Synergi Hydro-RP, 80 Å, 150 x 2.0 mm reversed phase column (Phenomenex, Torrance, CA) with a 0.2 µm guard column (Optimize Technologies, Inc., Oregon City, OR). The following precursor and product ions were used for detection: GFN (436 > 96/97) and GTP (408 > 166). Quantitation was based on a 6-point standard curve prepared in 0.1% (v/v) FA in H2O with concentrations spanning extract concentrations.

2.6 Isothiocyanate Analysis in Urine and Plasma

Following protein precipitation by centrifugation (3184 x g, 4 min, 2°C), plasma and urine supernatants were filtered twice through Spin-X® centrifuge tube filters (12,000 x g, 3.5 min, 2°C). Plasma filtrates were stored at -80°C until further analysis by HPLC-MS/MS. Urine filtrates were diluted 1:2 with 0.1% (v/v) FA in H2O prior to storage. Matched pre and post samples were analyzed for SFN, SFN-Cys (299 > 114), SFN-GSH (485 > 179), SFN-CG (356 > 114), and SFN-NAC (341.1 > 114) in duplicate following a 10-µl injection. Instrumentation and HPLC-MS/MS conditions were the same as used previously [28], except SFN metabolites were detected with an Applied Biosystems MDS Sciex 4000 Q TRAP HPLC-MS/MS system. Metabolite levels were quantified using standard curves prepared in 0.1% (v/v) FA in H2O with concentrations spanning metabolite concentrations.

2.7 PBMC HDAC Activity Analysis

Analyses were performed by the Cancer Chemoprevention Program's Core Laboratory at the Linus Pauling Institute. PBMC HDAC activity was evaluated using the positive control, sodium butyrate, as previously described [112]. Substrates and standards for the assay were custom synthesized by AAPPTec, LLC (Louisville, KY). HDAC activity is expressed relative to PBMC protein content and negative control (DMSO).

2.8 Immunohistochemistry (IHC) Staining

IHC was performed on paraffin-embedded, breast biopsy samples as described by Elsheikh, *et al*. [123]. Briefly, slides of paraffin-embedded breast tissue specimens were deparaffinized in xylenes (3 x 3 min), rehydrated with graded alcohols, washed 10 min in Tris-buffered saline (pH 7.2-7.6), heated 10 min in a Russell-Hobbs programmable pressure cooker in 0.01 M citrate buffer (pH 6.0), treated 5 min with 3% aqueous H2O2 solution, blocked 1 h at 25°C in 3% goat serum, incubated 1 h at 25°C with primary antibodies for acetylated histone H3 at lysine 9 (H3K9) (1:1000) and 18 (H3K18) (1:2000), p21 and Ki-67 (Abcam, Cambridge, MA), and HDAC 6 and HDAC 3 (Santa Cruz Biotechnology, Inc., Dallas, TX) followed by mouse Envision (Dako, Glostrup, Denmark), counterstained 1 min with Gill's hematoxylin, rinsed, dehydrated, and coverslipped using Permount. Positive and negative control slides were assessed for technical adequacy of staining. Stained slides were blindly evaluated by a collaborating pathologist on a Leica DMLS microscope. Benign breast tissues were scored separately from *in situ* disease or invasive carcinoma, where available. A modified Histo-score (H-score) was recorded, which involved semi-quantitative assessment of both staining intensity (graded as 1-3 with 1 representing weak staining, 2 moderate staining, and 3 strong staining) and percentage of positive cells. The range of the H-score was 0-300.

2.9 Statistical Methods

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The intent-to-treat analysis was performed for the primary outcomes and included all randomized participants. Baseline characteristics were expressed as means and standard errors (SEs) for continuous variables, and counts (n) and percentages (%) for categorical variables, stratified by treatment group. The comparability of the two treatment groups for baseline characteristics were tested using independent two-sample *t*-tests for continuous variables and Chi-square tests for categorical variables. Any of the baseline characteristics found to be significantly different between groups, and also associated with the primary endpoints, were considered as possible adjustment variables in the final models.

The primary outcomes examined include isothiocyanate levels, HDAC activity, Ki-67, p21, and levels of acetylation of H3K9 and H3K18. Our primary interest was to determine whether changes from pre- to post-treatment were significantly different between placebo and supplement groups. The analysis was conducted separately for each primary endpoint as well as for each specimen type (*e.g.*, blood, urine, normal tissue, cancer tissue). Shapiro-Wilks Normality tests were conducted for all continuous variables. Comparison between pre- and post-intervention levels of SFN metabolites and PBMC HDAC activity within each treatment group was conducted using either a paired *t*-test or Wilcoxon signed rank sum test. For urinary and blood SFN metabolites, Wilcoxon signed rank tests were conducted for the pre- to post-treatment changes between treatment groups. Tissue biomarkers were log2 transformed in order to obtain approximate normality.

To assess treatment group differences in the changes in primary outcome biomarkers, linear mixed effects models were conducted separately for each outcome to calculate adjusted least square means (LSMEANS) and 95% confidence intervals (95% CI), and to test the statistical significance of the difference between pre- and post-treatments within each group, as well as between treatment groups. The mixed effects model has the advantage of accommodating incomplete data as well as within-subject correlation due to repeated measurements (*i.e.*, pre- and post-intervention). NSAIDs use

was included as a covariate in all models due to the baseline difference between treatment groups. To adjust for multiple comparisons of the primary endpoints, we applied the method of False Discovery Rate (FDR) [124]. The FDR *p* values were provided in addition to the standard *p* values for the overall treatment comparisons.

Adverse events and compliance between the treatment groups were analyzed using Chi-square tests or Fisher's exact test, as appropriate. Tests of statistical significance were conducted using two-sided tests, and a p-value ≤ 0.05 was considered statistically significant unless otherwise noted.

The sample size needed for the study was initially determined to be 60 (30 in each group) for analyses of isothiocyanate levels and HDAC activity in PBMCs, and 40 (20 in each group) for IHC analyses of Ki-67 and HDAC change. Sample sizes were determined to detect a biologically meaningful difference between SFN and placebo groups with at least 80% power using a two-sample t-test or binomial test. All power analyses were performed assuming two-sided 5% overall significance level accounting for seven primary endpoints.

3. Results

3.1 Patient Characteristics and Adverse Events

From December 23rd, 2008 to March 27th, 2013, a total of 54 participants aged 25-83 years (54 ± 12) were randomized into this trial at OHSU. Table 2.1 describes the baseline characteristics of the 54 patients stratified by treatment group. The supplement group reported significantly higher proportion of NSAIDs use than the placebo group ($p = 0.002$). There was no statistically significant difference in age, BMI, height, cruciferous vegetable intake, race, family history of breast cancer, smoking, alcohol, income, education, marital status or menopausal status. There was no difference between treatment groups for each specific type of adverse event and total number of adverse events (Table 2.2). In addition,

no statistically significant difference was observed in terms of compliance to the treatment plan between the two treatment groups ($p = 0.88$).

Table 2.1. Basic characteristics of women in the study[a,b,c]

[a] *t*-tests were conducted for continuous variables (age, BMI, height and cruciferous vegetable intake); chi-square tests were conducted for categorical variables with expected cell frequencies ≥ 5; and Fisher's exact tests were conducted for categorical variables with expected cell frequencies < 5.

[b] *p*-value for *t*-tests or chi-square tests between supplement and placebo groups, $^{*}p < 0.05$.

[c] For the categorical variables with missing values, chi-square tests or Fisher's exact tests were conducted without including missing group.

[d] Percentages may not add up to 100 due to rounding values.

[e] Cruciferous vegetable intake includes sources from food lines, mixed dishes and condiments.

[f] For the 13 women that did not report their menopausal status, those aged < 45 years were grouped into Pre-menopausal status, those aged between 45 and 55 years were grouped into Peri-menopausal status, and those aged > 55 years were grouped into Post-menopausal status. The chi-square test *p*-value between supplemental and placebo groups among the 41 women who initially reported menopausal status was 0.86.

[g] In supplement group, malignant category includes 7 subjects with DCIS or DCIS + ADH, 5 subjects with invasive cancer with DCIS component; in placebo group, malignant includes 10 subjects having DCIS/DCIS+ADH and 4 subjects having invasive cancer with DCIS component.

Table 2.1. Basic characteristics of women in the study[a,b,c]

Participant Characteristics	Sulforaphane (n = 27)	Placebo (n = 27)	p
	Mean (SD)	Mean (SD)	
Age, yr	53.52 (9.54)	55.30 (14.27)	0.59
BMI (kg/m^2) at baseline	26.69 (5.38)	27.95 (5.80)	0.42
Height (cm) at baseline	165.8 (5.78)	164.5 (5.88)	0.42
Cruciferous vegetable intake (g/day) [e]	83.0 (64.5)	80.3 (50.3)	0.86
	n (%[d])	n (%[d])	
Race			0.30
White	26 (96.3)	24 (88.9)	
Non-White	1 (3.7)	3 (11.1)	
Family history of Breast Cancer			0.26
Yes	12 (44.4)	8 (29.6)	
No	15 (55.6)	19 (70.4)	
Smoking			0.51
Current	0 (0.0)	2 (7.4)	
Former	7 (25.9)	5 (18.5)	
Never	17 (63.0)	15 (55.5)	
Missing	3 (11.1)	5 (18.5)	
Alcohol			0.55
Current	13 (48.1)	8 (29.6)	
Former	3 (11.1)	3 (11.1)	
Never	8 (29.6)	10 (37.0)	
Missing	3 (11.1)	6 (22.2)	
Income			0.20
≤ $25,000	3 (11.1)	9 (33.3)	
$25,000 - $50,000	2 (7.4)	2 (7.4)	
> $50,000	14 (51.9)	11 (40.7)	
Refuse/Don’t know/missing	8 (29.6)	5 (18.5)	

Table 2.1. Basic characteristics of women in the study[a,b,c]

(Continued)

Education			1.00
≤ 12 years	3 (11.1)	3 (11.1)	
Some college/technical	6 (22.2)	5 (18.5)	
≥ College graduate	13 (48.1)	13 (48.1)	
Missing	5 (18.5)	6 (22.2)	
(Continued)			0.81
Marital status	10 (37.0)	8 (29.6)	
Single, Divorced, Widowed	14 (51.9)	13 (48.1)	
Married/partner	3 (11.1)	6 (22.2)	
Missing			
Use of NSAIDS			0.002
Yes	20 (74.1)	8 (29.6)	
No	7 (25.9)	18 (66.7)	
Missing	0 (0.0)	1 (3.7)	
Menopausal Status[f]			0.40
Pre	8 (29.6)	11 (40.7)	
Peri	8 (29.6)	4 (14.8)	
Post	11 (40.7)	12 (44.4)	
Pathology Biopsy Diagnosis			0.21
Benign	15 (58.3)	13 (50.0)	
Malignant[g]	12 (41.7)	14 (50.0)	

Table 2.2. Incidence of reported grade 2 adverse events in the BroccoMax™ trial (treatment-related)

Adverse Events (AE)	Sulforaphane (*n* = 27)		Placebo (*n* = 27)		
	Number	%[b]	Number	%[b]	*p*[a]
Bloating	5	18.5	5	18.5	1.00
Gas/Flatulence	1	3.7	4	14.8	0.35
Burping	0	0	0	0	1.00
Diarrhea	1	3.7	2	7.4	1.00
Nausea/vomiting	0	0	1	3.7	1.00
Headache	1	3.7	3	11.1	0.61
Taste Alteration	0	0	2	7.4	0.49
Bruising	1	3.7	1	3.7	1.00
Other[c]	5	18.5	7	25.9	0.74
All[d]	8	29.6	9	33.3	1.00

[a] Fisher's exact tests.
[b] Number of subjects reporting event/total number of study subjects (%).
[c] Other changes to health included tingling tongue sensation, tiredness, change of sleep pattern, arthritic pain, allergy, heartburn, bulbitis, cramping.
[d] Count of subjects who experienced at least one of the grade 2 adverse events.

3.2 Urinary and Blood Biomarkers

Table 2.3 presents all continuous outcomes of isothiocyanates from urine and plasma and HDAC activity in PBMCs. Pre- to post-intervention changes in total urinary SFN isothiocyanates and in individual SFN metabolites (SFN-NAC, SFN-Cys, SFN-GSH, and SFN) were statistically higher in the SFN group compared to the placebo group. In plasma, pre- to post-intervention changes in total SFN isothiocyanates and individual SFN metabolites (SFN-NAC, SFN-GSH and SFN-CG) were statistically significant in the SFN group only. No SFN metabolites were detected in plasma from the placebo group. Changes in SFN-Cys levels in plasma were not significantly different between treatment groups. We also compared the means of pre- and post-intervention PBMC HDAC activity levels within each treatment group. For the SFN group, the average change in HDAC activity from pre-

to post-intervention was a decrease of 80.39 pmol/min/mg protein (p = 0.11); for the placebo group, the average change from pre- to post-intervention was an increase of 27.52 pmol/min/mg protein (p = 0.40). Comparing the two groups, changes in HDAC activity were marginally different (p = 0.07). In a sub-analysis stratified by NSAIDs use, we observed a statistically significant difference in HDAC activity among non-NSAID users (p = 0.03), and no significant difference among NSAID users (Figure 2.2).

Table 2.3. SFN and SFN metabolite levels in urine and plasma as well as HDAC activity changes from pre- to post-treatment by treatment group.

[a] Urinary metabolite concentrations normalized to creatinine in millimolar concentration. All SFN metabolite concentrations are shown in micromolar concentrations.

[b] Change = post-intervention level minus pre-intervention level. The mean changes value may not be equal to mean in the post-intervention group minus mean in the pre-intervention group due to different sample sizes in these two groups.

[c] *p*-values were calculated for difference of change in means across treatment groups. *t*-test was used for change of HDAC activity, which had normal distribution; Mann-Whitney U test was used for all other variables due to non-normal distribution.

[d] HDAC activity values represent pmol/min/mg protein.

	Sulforaphane Mean (SE)			Placebo Mean (SE)			*p*[c]
	Urinary Metabolites[a]						
	Pre-intervention	Post-intervention	Changes[b]	Pre-intervention	Post-intervention	Changes[b]	
	n = 21	*n* = 22	*n* = 21	*n* = 22	*n* = 22	*n* = 22	
Urinary Creatinine[a]	13.02 (1.80)	10.83 (1.61)	-1.75 (2.52)	11.07 (1.46)	9.56 (1.53)	-1.50 (1.33)	0.63
Total SFN Metabolites[a]	0.07 (0.01)	1.06 (0.32)	1.00 (0.33)	0.10 (0.02)	0.05 (0.007)	-0.05 (0.02)	**<0.0001**
SFN-NAC[a]	0.02 (0.005)	0.77 (0.24)	0.75 (0.25)	0.04 (0.01)	0.02 (0.002)	-0.02 (0.01)	**<0.0001**
SFN-Cys[a]	0.02 (0.005)	0.22 (0.06)	0.20 (0.06)	0.04 (0.006)	0.02 (0.003)	-0.02 (0.004)	**<0.0001**
SFN-GSH[a]	0.004 (0.001)	0.007 (0.002)	0.002 (0.009)	0.005 (0.001)	0 (0)	-0.005 (0.001)	**0.0004**
SFN-CG[a]	0.01 (0.004)	0.02 (0.006)	0.008 (0.007)	0.02 (0.004)	0.01 (0.002)	-0.006 (0.002)	**0.03**
SFN[a]	0.001 (0.001)	0.05 (0.02)	0.05 (0.02)	0.004 (0.002)	0.002 (0.001)	-0.002 (0.002)	**<0.0001**
	Plasma Metabolites[a]						
	Pre-intervention	Post-intervention	Changes[b]	Pre-intervention	Post-intervention	Changes[b]	
	n = 16	*n* = 17	*n* = 16	*n* = 18	*n* = 21	*n* = 18	
Total SFN Metabolites[a]	0 (0)	0.25 (0.07)	0.27 (0.07)	0 (0)	0 (0)	0 (0)	**<0.0001**
SFN-NAC[a]	0 (0)	0.07 (0.01)	0.08 (0.01)	0 (0)	0 (0)	0 (0)	**<0.0001**
SFN-Cys[a]	0 (0)	0.02 (0.02)	0.03 (0.03)	0 (0)	0 (0)	0 (0)	0.32
SFN-GSH[a]	0 (0)	0.06 (0.01)	0.07 (0.01)	0 (0)	0 (0)	0 (0)	**<0.0001**
SFN-CG[a]	0 (0)	0.10 (0.04)	0.10 (0.05)	0 (0)	0 (0)	0 (0)	**0.03**
	HDAC Activity in PBMCs						
	Pre-intervention	Post-intervention	Changes[b]	Pre-intervention	Post-intervention	Changes[b]	
	n = 23	*n* = 24	*n* = 23	*n* = 25	*n* = 24	*n* = 24	
HDAC Activity[d]	447.51 (48.80)	371.14 (44.78)	-80.39 (48.53)	432.76 (42.00)	476.57 (43.83)	27.52 (32.58)	0.07

Table 2.3. SFN and SFN metabolite levels in urine and plasma as well as HDAC activity changes from pre- to post-treatment by treatment group.

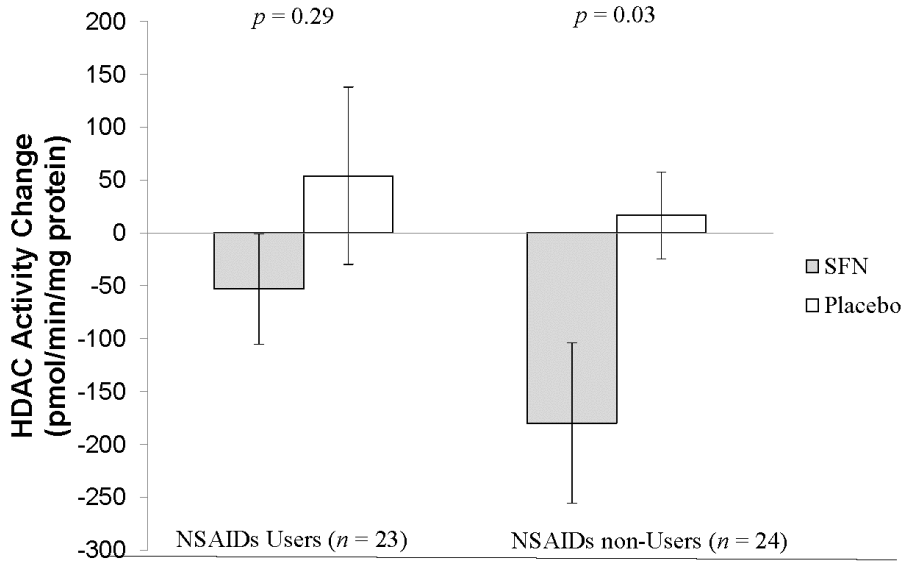

Figure 2.2. Comparison of PBMC HDAC activity change between SFN and placebo groups stratified by NSAIDs use. Changes in HDAC activity from pre- to post-intervention between treatment groups were compared and stratified by NSAIDs use using a mixed effect model. Values shown indicate least-squares means (lsmean ± SE) of pre-to-post change of HDAC activity by treatment group and NSAIDs use group.

3.3 IHC Biomarkers

Fifty (92%) women (24 in SFN group and 26 in placebo group) consented to analysis of breast biopsy tissue and were included in IHC analysis. Levels of H3K18ac, H3K9ac, HDAC3, HDAC6, Ki-67 and p21 were evaluated by IHC from pre-treatment biopsies followed by post-treatment biopsies lumpectomy or mastectomy specimens (when available). Interaction tests between NSAIDs use and treatment group did not show any statistical significance; therefore, NSAIDs use was adjusted in all models as a single variable, not as an interaction term. Table 2.4 shows the log2-transformed LSMEANS of the tissue biomarkers by treatment groups and the *p*-values comparing pre-to-post changes of biomarkers between and within treatment groups after adjusting NSAIDs use. Through

multiple comparison adjusted *p*-value using the Benjamini-Hochberg False Discovery rate, there was no statistical significance between treatment groups for pre-to-post changes of all the examined tissue biomarkers including H3K18ac, H3K9ac, HDAC3, HDAC6, Ki-67 and p21 levels in all the three tissue types. Before adjusting for false discovery rate, a significant difference in pre-to-post changes of Ki-67 was present between the two treatment groups among benign tissues, but not among DCIS or IDC tissues. Comparing pre- and post-treatment levels within each treatment group, there was a significant decrease in Ki-67 and HDAC3 in benign tissues in the SFN group and a significant decrease in H3K9ac in DCIS tissue in the placebo group.

Table 2.4. Log2-transformed LSMEANS of immunohistochemistry H-score of selected breast tissue biomarkers in women scheduled for breast biopsy

	n of Subjects / *n* of Observations Used[b]	SFN Group	Placebo Group	*p* comparing treatment groups[a]	False Discovery Rate *q* Values[e]
		Pre-to-Post Change LSMEANS (95% CI)[c]	Pre-to-Post Change LSMEANS (95% CI)[c]		
H3K18ac					
Benign	29/32	-1.17 (-3.62, 1.28)[f]	-0.62 (-3.91, 2.67)	0.34	0.58
DCIS	16/23	-1.40 (-2.95, 0.14)[f]	-0.55 (-1.60, 0.50)	0.29	0.58
IDC	13/20	-0.36 (-2.92, 2.20)	-0.36 (-3.19, 2.46)	1.00	1.00
H3K9ac					
Benign	41/51	-0.95 (-2.21, 0.31)	-1.12 (-2.26, 0.02)[f]	0.82	0.88
DCIS	18/29	-0.49 (-2.18, 1.20)	**-1.39 (-2.48, -0.30)[g]**	0.34	0.58
IDC	15/18	0.93 (-4.99, 6.86)	-1.62 (-6.22, 2.98)	0.28	0.58
HDAC3[4]					
Benign	33/37	**-1.32 (-2.60, -0.04)[g]**	-0.38 (-1.73, 0.97)	0.16	0.58
DCIS	10/12	n/a	n/a	n/a	n/a
IDC	4/5	n/a	n/a	n/a	n/a
HDAC6[d]					
Benign	33/40	-0.27 (-1.51, 0.97)	-0.76 (-2.01, 0.50)	0.51	0.71
DCIS	13/14	n/a	n/a	n/a	n/a
IDC	7/7	-0.71 (-4.42, 3.00)	n/a	n/a	n/a
Ki-67					
Benign	48/62	**-1.39 (-2.23, -0.55)[h]**	0.23 (-0.61, 1.07)	**0.01**	0.14
DCIS	19/28	0.42 (-1.18, 2.02)	-0.48 (-1.69, 0.72)	0.32	0.58
IDC	13/16	0.98 (-3.89, 5.85)	0.28 (-3.22, 3.78)	0.37	0.58
p21					
Benign	47/63	-0.60 (-1.62, 0.43)	-0.27 (-1.16, 0.61)	0.62	0.79
DCIS	20/29	-0.64 (-2.40, 1.11)	-0.90 (-2.39, 0.59)	0.80	0.88
IDC	13/18	0.44 (-1.99, 2.87)	-1.36 (-4.42, 1.71)	0.23	0.58

[a] Mixed effect model adjusting NSAIDs use.
[b] Each subject has maximum of 2 observations.
[c] The unit of the LSMEANS is log2(H-score).
[d] HDAC3 and HDAC6 were added late in the study. Differing numbers of participant specimens available reflect reasons such as: depleted blocks, tissue falling off stained slides and non-existent epithelial tissue to stain/review.
[e] Multiple comparison adjusted *p*-values using the Benjamini–Hochberg False Discovery Rate. [f] $p \leq 0.1$ [g] $p \leq 0.05$ [h] $p \leq 0.01$.

4. Discussion

In this analysis of 54 women who participated in this randomized, placebo-controlled trial, we found that SFN supplementation was associated with reduced PBMC HDAC activity. Specifically, the SFN supplementation was more significantly associated with reduced PBMC HDAC activity levels among non-NSAIDs users. In addition, we observed significant pre-to-post changes in Ki-67 and HDAC3 within the SFN supplementation group. However, we did not observe significant differences between SFN and placebo groups for any of the tissue biomarkers examined including H3K9ac, H3K18ac, HDAC3, HDAC6, Ki-67 and p21.

As a study quality control, plasma and urinary levels of SFN and SFN metabolites were used to indicate SFN exposure. We observed SFN and SFN metabolites in the urine and the plasma of subjects that consumed the BroccoMax™ supplements, but SFN isothiocyanates were not detected in placebo subjects, as expected. This supports previous reports that this supplement can reach detectable levels of bioactive SFN in humans. Supplement subjects consumed ~210 mg GFN daily for approximately 4 weeks. Isothiocyanate concentrations in plasma and urine were lower than observed in a previous study [96], but biological fluids were collected at time of surgical operation rather than at a designated time point following supplement intake. SFN is metabolized rapidly in the body. Plasma levels peak between 1 and 3 hours, and the majority of SFN metabolites are excreted 12 hours following consumption [96]. It is likely that the samples in this study were collected after the peak in plasma levels but within the initial 12 hours following intake.

Ki-67 is a marker of cell proliferation. We observed significant decreases in Ki-67 levels via IHC following SFN supplementation in benign tissue. The difference between treatment groups was not significant after adjusting for multiple comparisons; however, the change in the SFN group was significant and quite different than that of the placebo group, which had a non-significant increase in Ki-67 levels. There is evidence that Ki-67 gene expression is regulated in part through epigenetic mechanisms involving HDACs. For

example, Stearns, *et al*. [83] reported significant decreases in Ki-67 gene expression, but not protein levels, in invasive breast tissue obtained from women treated with the pharmacological HDAC inhibitor, Vorinostat, compared to untreated subjects. Similar to this report, we also did not observe changes in Ki-67 protein levels following SFN supplementation in cancer tissue. It may be that cancer cells have regulatory events that prevent changes in Ki-67 protein levels when gene transcription increases. Cell proliferation is a hallmark of cancer, but it is possible that increased proliferation may support viability of transformed cells at specific stages and may be subject to distinct regulation within cancer cells. In addition, cancer cells may have become independent of normal proliferation regulatory mechanisms that involve HDACs. Specific situations when cells experience decreased cell proliferation in response to SFN are not clearly defined. Our observations, along with previous reports, support that timing (pre-disease vs. disease) and disease stage may influence a cell's response to SFN.

There are several reports that SFN inhibits HDAC activity in cultured cells and animal models, but only a few human studies report decreases in HDAC activity with SFN consumption [12, 13, 17, 125, 126]. One study in healthy individuals reported that PBMC HDAC activity was lower after consuming BroccoMax™ supplements compared to a placebo, though changes from pre-intervention levels were not statistically significant [126]. Another human study reported larger decreases in PBMC HDAC activity in healthy adults following consumption of broccoli sprouts [12]. That study used a small sample size (n = 3), so it is unclear whether or not the magnitudes of changes they observed are widely achievable in the population at similar SFN doses. Furthermore, Pledgie-Tracy, *et al*. [17] reported decreased HDAC activity in multiple breast cancer cell lines, including the DCIS-like cell line, T-47D. In the current study, we observed a marginally statistically significant difference of the changes in PBMC HDAC activity between the two treatment groups. This observation suggests that the decreases in HDAC activity observed may have been related to higher SFN exposure in the supplementation group. Because NSAIDs use was different between supplement and placebo groups, we compared changes in HDAC activity between groups among NSAIDs users and separately among NSAIDs non-users. Among NSAIDs

non-users, mean fold change in HDAC activity was significantly different in supplement consumers compared to placebo consumers (Fig. 2.2). NSAIDs inhibits the synthesis of prostaglandins, which could suppress regulatory protein expression via recruitment of HDAC [127]. Increased recruitment of HDACs to chromatin may have prevented inhibition associated with SFN consumption, which is one potential explanation for our observations. Future studies on SFN with larger sample size are needed to confirm our findings.

Specific HDAC proteins have been reported to be inhibited by SFN. In colon cancer cell lines, HDAC3 and HDAC6 were among HDACs that showed the largest decrease in protein expression following SFN exposure [128]. Clarke *et al*. [14] further demonstrated decreases in HDAC3 and HDAC6 in prostate cancer cells treated with SFN. We evaluated changes in HDAC3 and HDAC6 protein expression in breast biopsy tissue as targets of SFN. HDAC3 was significantly decreased in the supplement group, which may have contributed to the non-significant decreases in total HDAC activity we observed. HDAC6, however, was not decreased with SFN supplementation. Rajendran *et al.* [128] demonstrated that changes in HDAC protein expression following SFN treatment are time-dependent, where decreases in HDAC6 occurred after decreases in HDAC3.

Decreases in histone acetylation have been reported to occur with progression of normal breast epithelium to DCIS [129]. Studies in prostate and breast cancer cells, as well as in an *in vivo* rat model of breast cancer, have shown that decreasing HDAC activity can result in increased histone and protein acetylation [130, 131]. However, another study in breast cancer cells, reported that decreases in HDAC activity were not associated with increased histone H3 or H4 acetylation [17]. In this study, we did not observe increased histone acetylation at H3K18 and H3K9, despite decreases in HDAC3 expression and total HDAC activity comparing supplement and placebo groups. We did observe a significant decrease in H3K9 acetylation in DCIS tissue among placebo consumers, which could be related to cancer progression, as put forth by Suzuki *et al.* [129]. Though it cannot be determined from the present data, SFN supplementation may have mitigated decreases in histone acetylation. Another possible reason for observing decreased H3K9 acetylation in

the placebo group relates to the observation that pre-intervention levels were higher in the placebo group compared to the SFN group.

p21 is a major cell cycle regulator. Increased expression of p21 in breast cancer cells leads to cell cycle arrest [132]. SFN has been shown to increase p21 expression in breast cancer cell lines [65], and the regulation of p21 expression has been shown to involve HDACs [133, 134]. In this study, statistically significant changes in p21 protein expression were not observed in breast tissue with or without SFN supplementation. The intake threshold for increasing expression of p21 in human breast tissue is unclear and may not have been achieved by the doses consumed in this study. There could have also been other reasons why p21 may not have changed, including post-transcriptional and post-translational modifications reviewed recently by Jung *et al.* [135]. Additionally, it is possible that the regulation of p21 expression was altered in the tumors we analyzed and may be less responsive to changes in HDAC activity. Of course, this hypothesis is less consistent with the fact that no changes in p21 levels were observed in benign tissue either. Overall, our observations suggest that SFN does not alter cell proliferation during all stages of breast tumorigenesis.

Our study has several strengths. First, we were able to analyze biomarkers from both breast tissues and PBMC among pre- and post-intervention samples. Second, we were able to collect various types of breast tissues, including benign, DCIS and IDC, to examine potential effects of SFN on biomarkers from different lesional and non-lesional breast tissue. Third, information on adverse events and changes to diet and medication use was collected at each visit among maximum 9 visits during the up to 8 weeks supplement intervention period and approximately 30 days after participants' surgical or post-intervention appointment. Study coordinators and subjects were both blinded in terms of treatment assignment; therefore, information bias was minimized. Study limitations include small sample size, limited tissue availability in some cases, and hospital-based study design. However, most of our comparisons met the minimum sample size required to detect a biologically meaningful difference between SFN and placebo groups with at

least 80% power. Importantly, evaluation of SFN effect on various biomarkers from population-based participants may increase generalizability of our results.

In conclusion, this study provides evidence for chemopreventive activity of SFN in human breast tissue. We demonstrated effects of SFN on known cancer biomarkers as well as epigenetic targets *in vivo*. Additional studies are needed to evaluate dose-responses and responses of other relevant molecular targets to consuming SFN. While the supplements used in this study were well-tolerated, recent work has indicated that other SFN supplements may be more bioavailable and should be considered for use in future studies to provide higher SFN doses [136]. Overall, this work provides important information for future larger population-based clinical trials with SFN.

CHAPTER 3

ABSORPTION AND CHEMOPREVENTIVE TARGETS OF SULFORAPHANE IN HUMANS FOLLOWING CONSUMPTION OF BROCCOLI SPROUTS OR A MYROSINASE-TREATED BROCCOLI SPROUT EXTRACT

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Abstract

Sulforaphane (SFN), an isothiocyanate derived from crucifers, has numerous health benefits. SFN bioavailability from dietary sources is a critical determinant of its efficacy in humans. A key factor in SFN absorption is the release of SFN from its glucosinolate precursor, glucoraphanin (GFN), by myrosinase. Dietary supplements are used in clinical trials to deliver consistent SFN doses, but myrosinase is often inactivated in available supplements. We evaluated SFN absorption from a myrosinase-treated broccoli sprout extract (BSE) and are the first to report effects of twice-daily, oral dosing on SFN exposure in healthy adults. Subjects consumed fresh broccoli sprouts or the BSE, each providing 200 μmol SFN daily, as a single dose and as two 100-μmol doses taken 12 hours apart. Using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), we detected ~3x higher SFN metabolite levels in plasma and urine of sprout consumers, indicating enhanced SFN absorption from sprouts. Twelve-hour dosing retained higher plasma SFN metabolite levels at later time points than 24-hour dosing. No dose responses were observed for molecular targets of SFN (*i.e.*, heme oxygenase-1, histone deacetylase activity, p21). We conclude that the dietary form and dosing schedule of SFN may impact SFN absorption and efficacy in human trials.

1. Introduction

Consuming cruciferous vegetables is associated with many health benefits, including cancer chemoprevention. Sulforaphane (SFN), a major isothiocyanate (ITC) derived from these vegetables, is widely studied for its abilities to prevent cancer onset and progression. SFN facilitates carcinogen metabolism by inducing Phase 2 enzymes and promotes carcinogen excretion by inhibiting Phase 1 enzymes [137-139]. More recently, SFN metabolites were reported to inhibit histone deacetylases (HDAC). HDACs remove acetyl groups from proteins, including histones, which alters gene expression and protein

function [12, 112, 126]. SFN's ability to alter epigenetic marks may be important for helping re-express tumor suppressor genes that are often silenced during cancer development. Given these properties, SFN is a promising dietary chemopreventive agent. Here, we focus on how the dietary form and frequency of SFN intake impact SFN absorption. The influence of these factors on specific, putative chemopreventive targets of SFN is also evaluated.

Evidence indicates that SFN absorption is affected by the form consumed. One major determining factor of SFN absorption is the formation of SFN from its glucosinolate precursor, glucoraphanin (GFN). GFN is present in cruciferous vegetables and dietary supplements. The majority of SFN is formed when GFN is hydrolyzed by the plant enzyme, myrosinase, upon plant tissue damage (*e.g.*, chopping, chewing). When plant myrosinase is inactive or absent, a small amount of SFN may still be formed by gut bacteria-derived myrosinase activity [107]. Clarke *et al.* observed limited SFN absorption in healthy adults after consuming GFN supplements with inactivated myrosinase, which was sevenfold lower than when subjects consumed equivalent levels of GFN from fresh broccoli sprouts containing the active enzyme. These supplements were also less effective than sprouts at lowering HDAC activity [96, 126]. Cramer and Jeffery [97] demonstrated that SFN absorption from a GFN powder devoid of myrosinase activity improved when consumed along with a source of active myrosinase (air-dried broccoli sprouts). These studies clearly demonstrate differences in SFN bioavailability from whole foods and dietary supplements. Since substantial variation in plant glucosinolate content limits the use of whole foods in controlled clinical trials, researchers often employ dietary supplements to deliver consistent SFN doses [140]. Thus, there is a need to identify a suitable supplemental form of SFN. Broccoli sprout extracts (BSE), containing SFN rather than GFN, have been developed and may have enhanced bioavailability compared to GFN supplements lacking active myrosinase. We conducted a human feeding study to evaluate SFN absorption and excretion in individuals consuming fresh broccoli sprouts or a myrosinase-treated BSE containing SFN. Since SFN is mostly excreted within 24 hours following consumption, we tested if consuming two doses of SFN 12 hours apart (divided dose) could prolong SFN

exposure compared to consuming the total amount at a single time point [96, 98, 141]. Finally, to identify chemopreventive pathways influenced by SFN in humans, we evaluated cancer-relevant, putative molecular targets of SFN in subjects before and after SFN consumption. Our observations provide fundamental information for the design of SFN supplementation trials to study the efficacy of dietary SFN in cancer chemoprevention.

2. Materials and Methods

2.1 Participants

Twenty healthy adults, 19-50 years, were recruited in Corvallis, Oregon. This study was conducted in the Moore Family Center metabolic kitchen and clinical collection laboratory at Oregon State University (OSU). Exclusion criteria included: smoking, body-mass-index (BMI) < 18.5 and > 30 kg/m^2, vegetarianism, and use of drugs altering lipid metabolism. All participants provided informed consent. Study protocols were approved by the Institutional Review Board at OSU (OSU IRB #4995).

2.2 Dietary Interventions

Study design and interventions are depicted in Figure 3.1. Subjects were randomized to consume fresh broccoli sprouts or a myrosinase-treated BSE (n = 10), on day 1 of two separate study phases (single- and divided-dose). In the single-dose phase, subjects (fasting) consumed 200 µmol SFN equivalents from fresh broccoli sprouts or the BSE at 8 AM.

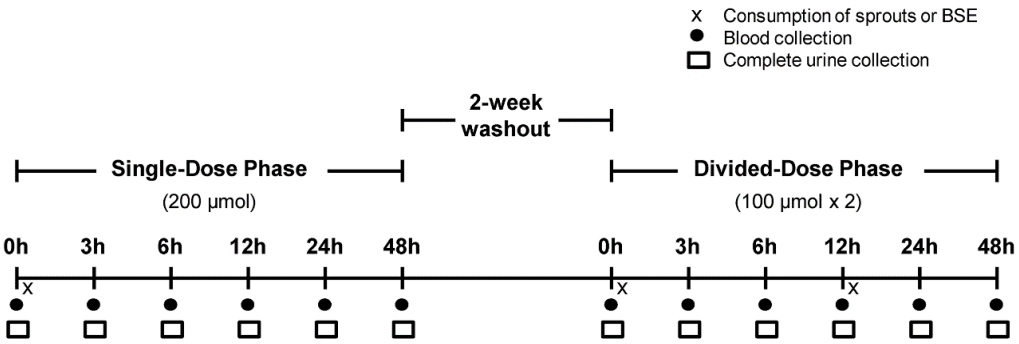

Figure 3.1. Human feeding study design. Subjects consumed single and divided doses of 200 µmol SFN equivalents from either fresh broccoli sprouts or myrosinase-treated broccoli sprout extract (BSE) supplements (n = 10). Divided doses were consumed 12 hours apart within a single day.

In the divided-dose phase (two weeks later), subjects (fasting) consumed half the original dose (100 µmol SFN equivalents) at 8 AM from sprouts or the BSE and the other half (not fasting) 12 hours later. Sprouts and the BSE were served with bagels, cream cheese, orange juice, milk or coffee. The same breakfast was provided on days 2 and 3 without sprouts or the BSE. Sprouts were obtained from Sprouters Northwest, Inc. (Kent, WA), and the BSE from Johns Hopkins University (Baltimore, MD). Details of the BSE preparation prior to encapsulation are reported by Egner, *et al.*, except supplements used in this study provided 100 µmol SFN per capsule [98]. SFN was determined stable within the BSE stored ≤ 900 days at ≤ -20°C by manufacturers. The BSE was stored in our lab at -20°C ≤ 200 days before consumption and were removed from freezers only moments before being consumed. SFN levels and stability within the BSE were verified in-house, and the "SFN potential" of sprouts was determined to match BSE doses (Chapter 3, Section 2.5). Subjects were instructed to avoid consuming foods containing glucosinolates and/or ITCs for 1 week before and throughout each study phase. Subjects attended a pre-study meeting with staff and a registered dietitian regarding study protocol and training for documenting food

intake. Self-reported, 3-day diet records collected during each study phase were analyzed using Food Processer® SQL (ESHA, Salem, OR).

2.3 Sample Collection

Complete urine collections were obtained following a 12-hour overnight fast before SFN consumption and at 3, 6, 12, 24 and 48 hours post-consumption. Processing protocols were modified slightly from Janobi *et al.* [142]. While in subjects' possession, urine was refrigerated or kept on ice in opaque jugs containing granulated boric acid (~20 mg/ml) to stabilize SFN metabolites. Urine was acidified with trifluoroacetic acid (TFA) to a final concentration of 10% (v/v) in urine storage tubes (VWR, Radnor, PA) before storing at -80°C. Whole blood (20 ml) was collected by venipuncture into EDTA vacutainers (VWR, Radnor, PA) before SFN consumption and at 3, 6, 12, 24, and 48 hours post-consumption. Whole blood was immediately centrifuged 1 min at high speed. Plasma was removed and acidified with TFA to a final concentration of 10% (v/v), vortexed, snap-frozen in liquid N_2, and stored at -80°C. Remaining whole blood was processed to isolate peripheral blood mononuclear cells (PBMC) as described previously [126]. PBMC protein concentrations were determined using the RC/DC protein assay (Bio-Rad, Hercules, CA). PBMC lysates were frozen at -80°C until analysis of HDAC activity. Phlebotomy was performed in the Moore Family Center clinical collection laboratory by a certified phlebotomist.

2.4 Preparation of Standards

SFN was purchased from LKT Laboratories, Inc. (St. Paul, MN). SFN-glutathione (SFN-GSH), SFN-cysteine (SFN-Cys), and SFN N-acetyl-cysteine (SFN-NAC) were purchased from Toronto Research Chemicals (Canada). Deuterated SFN-NAC (SFN-NAC-D_3) and SFN-cysteinylglycine (SFN-CG) were prepared in-house as described previously [96].

2.5 Sulforaphane Content of Sprouts and the Broccoli Sprout Extract

Sprouts (215 mg) were analyzed for "SFN potential" upon receipt and on nights before consumption. Fresh sprouts were homogenized in 1 ml deionized water and incubated 2 hours in the dark at 60°C with 2 mg *Sinapis alba* thioglucosidase (Sigma-Aldrich, St. Louis, MO). Following incubation, sprouts were filtered using 0.22-µm nylon Spin-X® centrifuge tube filters (VWR, Radnor, PA) (16,000 x *g*, 5 min, 25°C). The supernatant was diluted 20-fold in 0.1% (v/v) formic acid in water. To confirm SFN content of the BSE, 300-400 mg BSE powder was dissolved into 1.5 ml DMSO and diluted to 2 µM in 0.1% (v/v) formic acid in water. Sprout and BSE extracts were immediately stored at -80°C until HPLC-MS/MS analysis. We performed quality control experiments to confirm repeatability of extract and analysis procedures, and that SFN within the BSE remained stable over time (data not shown). For HPLC-MS/MS analysis, ten microliters of extracts were injected in duplicate, along with the internal standard, SFN-NAC-D_3. HPLC-MS/MS conditions were identical to those in our previous study [28]. The following precursor and product ions were used for detection: SFN (178 > 114), SFN-NAC-D_3 (344.1 > 114). Quantitation was based on an 8-point standard curve prepared in 0.1% (v/v) formic acid in water. Sprout and BSE doses given to participants were matched to provide equivalent SFN content (Table 3.1).

Table 3.1. Sulforaphane (SFN) content of broccoli sprouts and broccoli sprout extracts (BSE) consumed by participants during the study.

	Single-Dose Phase		Multiple-Dose Phase	
	Amount consumed	SFN content (μmols)[a]	Amount consumed	SFN content (μmols)[a]
Sprout	127.6 grams	205 ± 19.9	46.8 grams	102 ± 9.24
BSE	2 pills	203 ± 8.09	1 pill	101 ± 4.05
p-value		0.835		0.879

[a]SFN content (mean ± SEM). [b]Subjects consumed the doses shown at a single time point (sprouts, $n = 4$; BSE values were generated by doubling SFN measurements from individual BSE supplements ($n = 2$)). [c]Subjects consumed the doses shown 12 hours apart within a single day (sprouts, $n = 3$; BSE, $n = 2$). The same total daily dose of 200 μmols SFN was consumed during each study phase.

2.6 Sulforaphane Metabolites in Plasma and Urine

Plasma was thawed and centrifuged (12,000 x *g*, 5 min, 4°C) to precipitate proteins. Supernatants were filtered twice through Spin-X® centrifuge tube filters (VWR, Radnor, PA) by centrifugation (12,000 x *g*, 3.5 min, 4°C). Final filtrates were snap-frozen in liquid N_2 and stored at -80°C until HPLC-MS/MS analysis. Urine samples were thawed and centrifuged (400 x *g*, 5 min, 4°C) to precipitate proteins. Supernatants were filtered twice, as with plasma samples. Final filtrates were diluted 1:2 with 0.1% (v/v) formic acid in water, snap-frozen in liquid N_2, and stored at -80°C until analysis. Both plasma and urine were analyzed for SFN, SFN-Cys (299 > 114), SFN-GSH (485 > 179), SFN-CG (356 > 114), and SFN-NAC (341.1 > 114). Ten microliters of plasma or urine were injected in duplicate. The same instrumentation and HPLC-MS/MS conditions were used as for sprout and BSE analyses. Quantitation was based on standard curves prepared in 0.1% (v/v) formic acid in water. Spike and recovery experiments using an internal standard confirmed

consistent and high (> 80%) recovery using the documented processing protocols and comparable quantitation of standards prepared in biological matrices versus 0.1% (v/v) formic acid in water.

2.7 Urine Creatinine

Creatinine was determined using the standard Jaffe reaction method with alkaline picrate [143] and measuring absorbance at 490 nm using a Spectra Max M2 fluorescent plate reader (Molecular Devices, Sunnyvale, CA). Urinary SFN metabolite levels were normalized to urinary creatinine levels where indicated.

2.8 Gene Expression

Whole blood was preserved using PAXgene Blood RNA Tubes. Total RNA was isolated using the PAXgene Blood miRNA kit (PreAnalytiX, Hombrechtikon, Switzerland). RNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for quantitative real-time PCR (qPCR) (Life Technologies, Grand Island, NY). Gene expression was quantified by qPCR using primers specific for p21, heme oxygenase-1 (HO-1), and the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalizing gene copy number (Table 3.2). All qPCR reactions were done using Fast SYBR Green Mastermix on 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY). Gene copies were determined based on standard curves generated from serial dilutions of purified plasmid DNA encoding for genes of interest.

Table 3.2. Primer sequences used for qPCR analysis of gene expression.

	qPCR Primer Sequences	
	Forward	Reverse
GADPH	CGAGATCCCTCCAAAATCAA	TTCACACCCATGACGAACAT
HO-1	CTTCTTCACCTTCCCCAACA	GCTCTGGTCCTTGGTGTCAT
p21	CAGACCAGCATGACAGATTTC	GCGGATTAGGGCTTCCTCTT

Levels of GADPH, HO-1, and p21 mRNA were determined using qPCR analysis and the forward and reverse primer sequences shown. GADPH served as the housekeeping control. GADPH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1.

2.9 Protein Expression

Plasma HO-1 protein was evaluated in neat, non-acidified plasma obtained during the single-dose phase using an HO-1 ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. Optical density was read at 450 nm using a Spectra Max M2 fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

2.10 HDAC Activity

HDAC activity was measured in PBMC lysates obtained during the single-dose phase in the Cancer Chemoprevention Program's Core Laboratory at the Linus Pauling Institute as previously described, with sodium butyrate as the positive control [112]. HDAC substrate and deacetylated standard were custom synthesized (AAPPTec, LLC, Louisville, KY). HDAC activity is expressed relative to lysate protein content.

2.11 Statistical Analyses

Initial analyses for SFN metabolite levels included time points where multivariate normality was reasonable (no or few zero responses). Multivariate, repeated measures analyses assessed evidence regarding treatment (sprout or BSE) effect, time effect and treatment-by-time interaction. When necessary, the repeated measures variance-covariance structure was allowed to vary between treatments (likelihood ratio tests). With strong evidence of time effects or time-by-treatment interactions, treatment effects were evaluated at each time point (unpaired *t*-tests) and compared between pairs of time points (paired *t*-tests). With little to no evidence of treatment effects (main or interaction), treatment groups were combined to evaluate changes in responses over time. Use of exact two-sided Wilcoxon rank-sum and exact signed rank tests accommodated time points with non-normal data (zeroes or non-detectable levels). For molecular responses (HDAC, HO-1 and p21), we analyzed fold change relative to baseline using multivariate, repeated measures analyses including all time points. Two responses (HDAC and HO-1) required additional covariate adjustment for baseline due to significant downward linear trends in fold change with increasing baseline. Whenever residuals exhibited right skew, responses were also analyzed on the log transformed scale with no changes in conclusions. An extreme outlier in the sprouts group for HO-1 protein level led to two different analyses giving similar conclusions: non-parametric analysis (Wilcoxon at each time point) with outlier included and multivariate, repeated measures analysis with outlier excluded. Student's *t*-tests were used to compare subject characteristics, reported dietary intakes, and SFN doses between treatment groups (unpaired) and study phases (paired). These analyses were performed using SAS 9.3 (Cary, NC). Pharmacokinetic parameters were analyzed using WinNonLin, version 5.3 (Pharsight Corporation, St. Louis, MO) and compared using paired and unpaired Student's *t*-tests. Both compartmental and non-compartmental analyses were performed and returned similar results. Maximum concentration (C_{max}) and time at C_{max} (T_{max}) were determined graphically. Significance for all analyses was determined at $\alpha < 0.05$.

3. Results

3.1 Subject Characteristics

Age, gender, and BMI were similar between sprout and BSE consumers (Table 3.3). Total intakes of calories and macronutrients were similar between treatment groups and study phases (Table 3.4). Records indicated compliance with avoiding confounding food items.

Table 3.3. Characteristics of study participants.

	Sprout		BSE		*p*-value[b]	
	Age (years)	BMI (kg/m^2)	Age (years)	BMI (kg/m^2)	Age (years)	BMI (kg/m^2)
Male (*n=8)*	36 ± 5.3	24 ± 1.7	28 ± 2.6	26 ± 1.4	0.19	0.39
Female (*n*=12)	27 ± 2.6	22 ± 0.9	31 ± 1.8	23 ± 1.5	0.23	0.46
p-value[c]	0.11	0.22	0.33	0.21		

Each treatment group (n = 10) consisted of 6 females and 4 males. Age (years) and BMI (kg/m^2) were compared between sprout and BSE groups[b] and between males and females[c]. Values represent mean ± SEM.

Table 3.4. Total intakes of calories and macronutrients consumed by participants during the study.

	Single-Dose Phase	Multiple-Dose Phase	Between Phases
	Total intake[a]	Total intake[a]	*p*-value[c]
Calories (kcal)			
Sprout	2630 ± 220	2410 ± 191	0.199
BSE	2410 ± 301	2290 ± 239	0.594
p-value[b]	0.313	0.502	
Protein (g)			
Sprout	95.2 ± 15.2	84.9 ± 7.08	0.296
BSE	89.3 ± 15.0	93.7 ± 13.7	0.704
p-value[b]	0.633	0.327	
Carbohydrate (g)			
Sprout	352 ± 31.5	306 ± 26.3	0.055
BSE	347 ± 53.2	310 ± 37.3	0.329
p-value[b]	0.881	0.874	
Fat (g)			
Sprout	81.5 ± 10.3	80.5 ± 9.41	0.904
BSE	70.5 ± 9.19	75.0 ± 9.58	0.560
p-value[b]	0.174	0.481	

[a]Total intakes (mean ± SEM). Self-recorded dietary intake began on the day prior to each study phase and continued through the second day of each study phase. Intakes of calories and macronutrients were compared between sprout and BSE groups[b] (n = 10) and between single- and divided-dose phases[c]. kcal, kilocalories; g, grams.

3.2 Effects of Sulforaphane Form and Dose

Plasma and urinary levels of total SFN metabolites were ~3-5 times higher in sprout consumers compared to BSE consumers (Fig. 3.2, Table 3.5). During both study phases, total SFN metabolites in plasma were significantly higher in sprout consumers at 3, 6, 12 and 24 hours after consuming SFN (Figs. 3.2A and 3.2C).

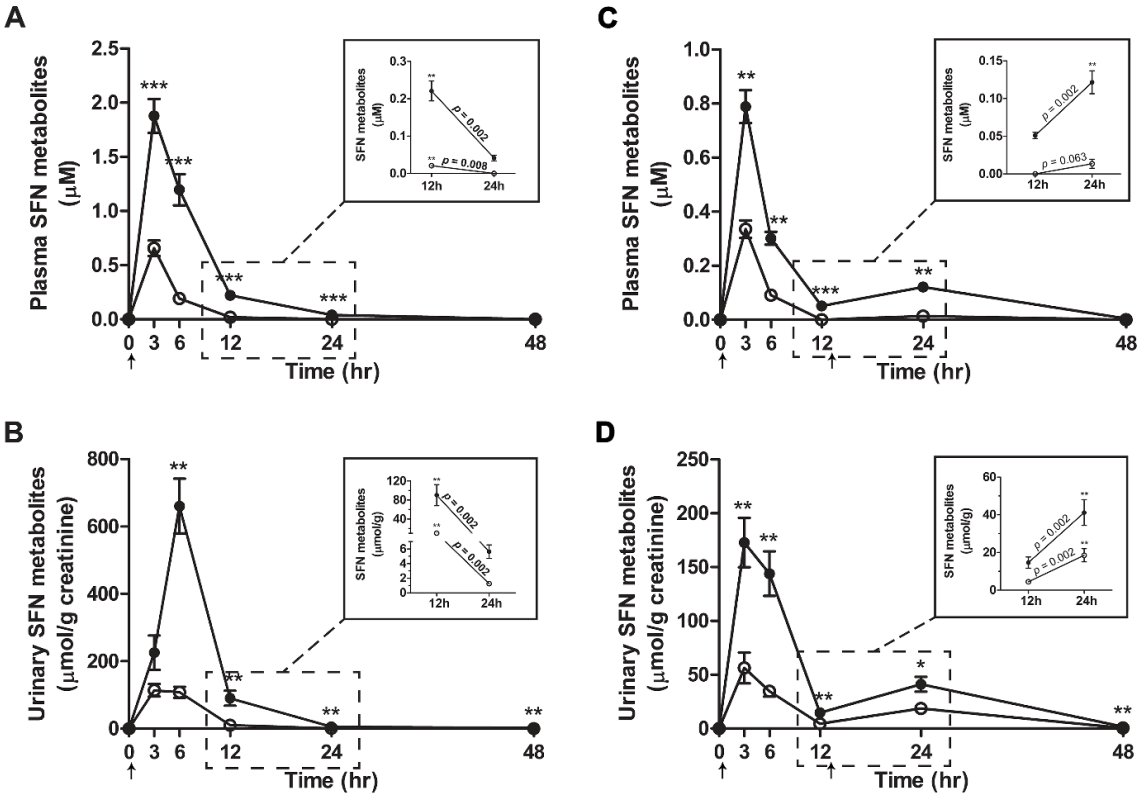

Figure 3.2. Sulforaphane (SFN) metabolite levels in plasma and urine following consumption of fresh broccoli sprouts and the broccoli sprout extract (BSE). Total levels (mean ± SEM) of SFN metabolites in plasma (A and C) and urine (B and D) of subjects at time 0 and at 3, 6, 12, 24 and 48 hours after consuming broccoli sprouts (closed) or the BSE (open). A and B) SFN metabolite levels detected following the single dose of 200 μmol SFN equivalents. C and D) SFN metabolite levels following two 100-μmol SFN doses consumed within a single day. Insets: Close-up of SFN metabolite levels at 12 and 24 hours in A and C) plasma and B and D) urine. Arrows indicate times of SFN consumption. Free SFN was only detected in 4 subjects at 3 hours following the single dose and comprised < 2% of total SFN metabolites (data not included). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 3.5. Pharmacokinetic parameters of sulforaphane (SFN) and SFN metabolites following consumption of broccoli sprouts and broccoli sprout extract (BSE) supplements.

	Sprout	BSE	*p*-value
Plasma SFN Metabolites[a]			
C_{max} (μM)	1.9 ± 0.5	0.7 ± 0.2	< 0.001
T_{max} (h)	3	3	n/a
$AUC_{(0\text{-}48h)}$ (μmol × h/L)	13.8 ± 4.1	3.0 ± 0.9	< 0.001
$t_{1/2}$ (h)	3.7 ± 0.8	1.9 ± 0.4	< 0.001
Urinary SFN Metabolites			
Total Excretion (μmols)[b]			
Single-Dose	345.7 ± 52.5	109.7 ± 28.1	< 0.001
Divided-Dose	200.1 ± 71.9	67.6 ± 18.4	< 0.001
Fraction Absorbed[c]			
Single-Dose	1.7 ± 0.3	0.5 ± 0.1	< 0.001
Divided-Dose	1.0 ± 0.4	0.3 ± 0.1	< 0.001

[a]Following consumption of a single 200-μmol SFN dose. [b]Cumulative excretion of SFN metabolites from baseline collection through the 48-hr study period. [c]Bioavailability F calculated based on total micromoles excreted in urine. C_{max}, maximum concentration observed; AUC, area under the curve; h, hour; L, liter; $t_{1/2}$, half-life; T_{max}, time at C_{max}. Values represent mean ± SD, $n = 10$.

In the single-dose phase, urinary levels peaked between 3 and 6 hours in sprout consumers and between 0 and 6 hours in BSE consumers. In the divided-dose phase, mean urinary levels peaked in both groups between 0 and 3 hours following the first 100-μmol SFN dose (Fig. 3.2D). While SFN absorption from sprouts and the BSE was different, the distribution of SFN metabolites in plasma and urine at T_{max} was similar, with the exception that plasma SFN-Cys ($p < 0.05$) was slightly lower in sprout consumers (Figs. 3.3A and 3.3B, Table 3.6). SFN-Cys and SFN-CG were the major SFN metabolites detected in plasma at T_{max}.

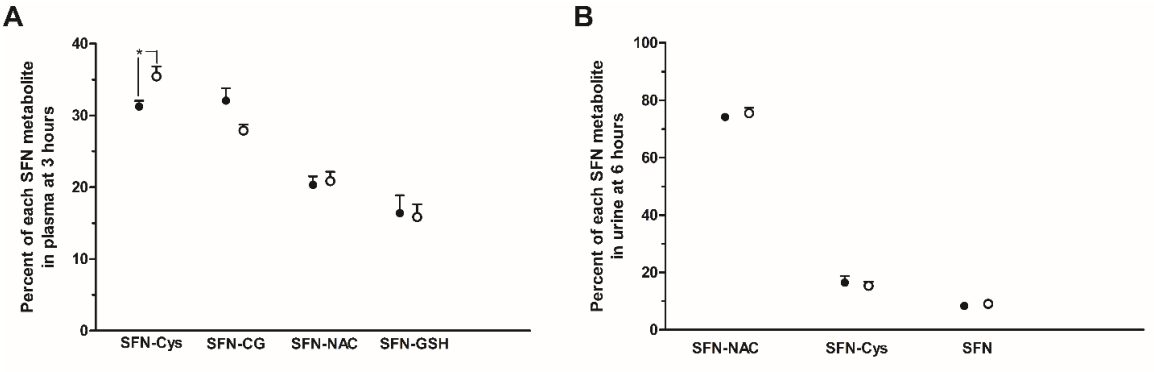

Figure 3.3. Distribution of individual sulforaphane (SFN) metabolites in plasma and urine at peak concentrations following consumption of broccoli sprouts and broccoli sprout extract (BSE) supplements. Relative percents (mean ± SEM) of SFN metabolites detected in A) plasma and B) urine at peak concentrations after consuming a single dose of 200 μmol SFN equivalents from broccoli sprouts (solid) or the BSE (open) (n = 10). SFN-CG in urine and free SFN in plasma were detected at < 2% of total SFN metabolite levels (not shown). *p < 0.05, **p < 0.01. Cys, cysteine; CG, cysteinylglycine; NAC, N-acetylcysteine; GSH, glutathione.

After 12 hours, the most abundant plasma metabolite was SFN-NAC, followed closely by SFN-CG. In a previous study, we detected SFN-CG as the major plasma metabolite in subjects 12 hours after consuming sprouts [96]. Some groups have reported the parent compound, SFN, to be more prevalent in plasma than its metabolites [141, 144]. The reasons for these discrepancies are unclear but may relate to differences in sample preparation, timing of measurements, analytical procedures, or perhaps variation in SFN metabolism among study populations [95, 101]. Prior to analyzing our samples, we conducted multiple spike-recovery experiments to ensure SFN and its metabolites were preserved through sample processing and analysis.

Table 3.6. Total levels and relative percents of sulforaphane (SFN) metabolites in plasma and urine after consumption of broccoli sprouts or the broccoli sprout extract (BSE). [a]Total levels (mean ± SEM) of SFN metabolites were compared at each time point between sprout and BSE groups (n = 10) using unpaired Student's t-tests. [b]Relative percent (mean percent ± SEM) that each SFN metabolite represents of the total level of SFN metabolites in plasma or urine (for those subjects where total SFN > 0). For indicated groups and time points, no SFN or SFN metabolites were detected in 10 of 10 subjects (n = 0 for % mean)[c], in 9 of 10 subjects (n = 1 for % mean)[d], in 5 of 10 subjects (n = 5 for % mean)[e], in 2 of 10 subjects (n = 8 for % mean)[f], or in 1 of 10 subjects (n = 9 for % mean)[g]. In plasma, SFN metabolites were not detected at 0 or 48 hours, and free SFN comprised < 2% of total SFN metabolites at 3 hours in the single-dose phase (not shown). *p<0.05, **p<0.01, ***p<0.001. Cys, cysteine; GC, cysteinylglycine; NAC, N-acetylcysteine; GSH, glutathione.

Table 3.6. Total levels and relative percents of sulforaphane (SFN) metabolites in plasma and urine after consumption of broccoli sprouts or the broccoli sprout extract (BSE).

PLASMA SINGLE DOSE		3 hours			6 hours			12 hours		
		Sprout	BSE	*p*	Sprout	BSE	*p*	Sprout	BSE[f]	*p*
SFN-GSH	µM[a]	0.3 ± 0.1	0.1 ± 0.0	*	0.2 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	***
	%[b]	16.4 ± 2.5	15.9 ± 1.8	0.5	13.4 ± 0.6	11.8 ± 1.8	0.9	13.2 ± 1.9	0.0 ± 0.0	***
SFN-CG	µM	0.6 ± 0.1	0.2 ± 0.0	***	0.3 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***
	%	32.0 ± 1.7	27.9 ± 0.8	0.1	28.4 ± 0.9	26.1 ± 0.6	0.1	28.3 ± 1.6	35.9 ± 11.2	0.2
SFN-Cys	µM	0.6 ± 0.0	0.2 ± 0.0	***	0.3 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***
	%	31.2 ± 0.8	35.4 ± 1.4	*	26.8 ± 0.8	29.6 ± 1.0	0.1	20.5 ± 3.5	0.0 ± 0.0	**
SFN-NAC	µM	0.4 ± 0.0	0.1 ± 0.0	***	0.4 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***
	%	20.3 ± 1.2	20.8 ± 1.3	0.9	31.3 ± 1.7	32.5 ± 1.5	0.9	38.1 ± 2.9	64.1 ± 11.2	**
Total	µM	1.9 ± 0.2	0.7 ± 0.1		1.2 ± 0.1	0.2 ± 0.0		0.2 ± 0.0	0.0 ± 0.0	

DIVIDED DOSE		3 hours			6 hours			12 hours		
		Sprout	BSE	*p*	Sprout	BSE	*p*	Sprout	BSE[c]	*p*
SFN-GSH	µM[a]	0.1 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	1.0
	%[b]	13.5 ± 0.7	13.7 ± 0.7	0.5	13.9 ± 0.4	5.6 ± 3.3	*	0.0 ± 0.0	n/a	n/a
SFN-CG	µM	0.2 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	***
	%	29.8 ± 1.0	26.7 ± 0.8	**	26.5 ± 0.7	31.2 ± 4.9	0.2	41.0 ± 5.2	n/a	n/a
SFN-Cys	µM	0.3 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	32.1 ± 0.9	36.9 ± 1.9	0.1	30.2 ± 0.7	14.0 ± 5.8	0.2	0.0 ± 0.0	n/a	n/a
SFN-NAC	µM	0.2 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	***
	%	24.6 ± 1.4	22.8 ± 2.2	0.5	29.4 ± 1.3	49.2 ± 6.9	**	59.0 ± 5.2	n/a	n/a
Total	µM	0.8 ± 0.1	0.3 ± 0.0		0.3 ± 0.0	0.1 ± 0.0		0.1 ± 0.0	0.0 ± 0.0	

Table 3.6. Total levels and relative percents of sulforaphane (SFN) metabolites in plasma and urine after consumption of broccoli sprouts or the broccoli sprout extract (BSE).

(Continued)

PLASMA SINGLE DOSE		24 hours		
		Sprout[f]	BSE[c]	*p*
SFN-GSH	µM[a]	0.0 ± 0.0	0.0 ± 0.0	1.0
	%[b]	2.2 ± 2.2	n/a	n/a
SFN-CG	µM	0.0 ± 0.0	0.0 ± 0.0	***
	%	44.0 ± 3.1	n/a	n/a
SFN-Cys	µM	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	n/a	n/a	n/a
SFN-NAC	µM	0.0 ± 0.0	0.0 ± 0.0	***
	%	53.9 ± 3.8	n/a	n/a
Total	µM	0.0 ± 0.0	0.0 ± 0.0	

DIVIDED DOSE		24 hours			48 hours		
		Sprout	BSE[e]	*p*	Sprout[d]	BSE[c]	*p*
SFN-GSH	µM[a]	0.0 ± 0.0	0.0 ± 0.0	*	0.0 ± 0.0	0.0 ± 0.0	1.0
	%[b]	8.2 ± 2.5	0.0 ± 0.0	0.1	0.0 ± 0.0	n/a	n/a
SFN-CG	µM	0.0 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	34.2 ± 2.8	43.5 ± 19.6	0.6	53.1	n/a	n/a
SFN-Cys	µM	0.0 ± 0.0	0.0 ± 0.0	*	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	15.3 ± 4.5	0.0 ± 0.0	0.1	0.0 ± 0.0	n/a	n/a
SFN-NAC	µM	0.0 ± 0.0	0.0 ± 0.0	***	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	42.4 ± 3.4	56.5 ± 19.6	0.7	46.9	n/a	n/a
Total	µM	0.1 ± 0.0	0.0 ± 0.0		0.0 ± 0.0	0.0 ± 0.0	

Table 3.6. Total levels and relative percents of sulforaphane (SFN) metabolites in plasma and urine after consumption of broccoli sprouts or the broccoli sprout extract (BSE).

(Continued)

URINE SINGLE DOSE		0 hours			3 hours			6 hours		
		Sprout[c]	BSE[d]	*p*	Sprout	BSE	*p*	Sprout	BSE	*p*
SFN	**µmols**[a]	0.0 ± 0.0	0.0 ± 0.0	1.0	7.2 ± 1.4	3.5 ± 0.8	0.1	15.3 ± 2.2	3.3 ± 0.5	***
	%[b]	n/a	20.6	n/a	9.4 ± 2.3	6.3 ± 1.3	0.4	8.3 ± 1.3	9.0 ± 1.3	0.6
SFN-CG	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	0.1 ± 0.0	0.0 ± 0.0	0.5	0.2 ± 0.0	0.0 ± 0.0	**
	%	n/a	0.0 ± 0.0	n/a	0.1 ± 0.0	0.1 ± 0.0	0.9	0.1 ± 0.0	0.1 ± 0.0	0.5
SFN-Cys	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	19.9 ± 6.7	14.8 ± 1.6	0.5	35.3 ± 5.6	6.2 ± 0.9	***
	%	n/a	28.2	n/a	22.4 ± 3.0	26.5 ± 2.5	0.3	17.4 ± 1.7	15.4 ± 1.4	0.4
SFN-NAC	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	62.2 ± 18.6	41.3 ± 6.4	0.7	143.3 ± 11.9	31.1 ± 4.2	***
	%	n/a	51.2	n/a	68.1 ± 3.2	67.2 ± 2.5	0.9	74.1 ± 1.3	75.5 ± 1.8	0.7
Total	**µmols**	0.0 ± 0.0	0.0 ± 0.0		92.3 ± 24.3	59.6 ± 7.4		194.4 ± 16.8	40.6 ± 4.9	

DIVIDED DOSE		0 hours			3 hours			6 hours		
		Sprout[d]	BSE[c]	*p*	Sprout	BSE	*p*	Sprout	BSE	*p*
SFN	**µmols**[a]	0.0 ± 0.0	0.0 ± 0.0	1.0	8.6 ± 1.1	2.7 ± 0.5	***	5.4 ± 1.0	1.7 ± 0.3	**
	%[b]	0.0 ± 0.0	n/a	n/a	13.0 ± 1.9	11.9 ± 1.7	0.6	11.7 ± 2.0	10.7 ± 1.8	0.7
SFN-CG	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	0.1 ± 0.0	0.0 ± 0.0	0.1	0.0 ± 0.0	0.0 ± 0.0	0.1
	%	0.0 ± 0.0	n/a	n/a	0.1 ± 0.0	0.1 ± 0.0	1.0	0.1 ± 0.0	0.1 ± 0.0	0.7
SFN-Cys	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	13.3 ± 1.8	5.6 ± 0.4	***	6.3 ± 0.8	2.6 ± 0.4	***
	%	100.0	n/a	n/a	19.3 ± 2.8	26.6 ± 2.5	0.1	13.4 ± 1.4	17.6 ± 2.2	0.1
SFN-NAC	**µmols**	0.0 ± 0.0	0.0 ± 0.0	1.0	51.9 ± 8.7	14.7 ± 2.6	***	35.8 ± 3.1	10.5 ± 0.7	***
	%	0.0 ± 0.0	n/a	n/a	67.5 ± 3.7	61.3 ± 2.7	0.2	74.8 ± 1.5	71.6 ± 2.0	0.2
Total	**µmols**	0.0 ± 0.0	0.0 ± 0.0		73.9 ± 9.0	23.0 ± 3.0		47.6 ± 3.5	14.8 ± 1.0	

Table 3.6. Total levels and relative percents of sulforaphane (SFN) metabolites in plasma and urine after consumption of broccoli sprouts or the broccoli sprout extract (BSE).

(Continued)

URINE SINGLE DOSE		12 hours			24 hours			48 hours		
		Sprout	BSE	*p*	Sprout	BSE	*p*	Sprout	BSE[g]	*p*
SFN	µmols[a]	2.5 ± 0.6	0.3 ± 0.1	***	0.5 ± 0.2	0.1 ± 0.0	**	0.3 ± 0.5	0.0 ± 0.0	**
	%[b]	7.3 ± 1.8	4.6 ± 0.8	0.5	5.8 ± 1.4	2.5 ± 0.8	0.1	4.7 ± 0.9	4.0 ± 3.0	0.1
SFN-CG	µmols	0.0 ± 0.0	0.0 ± 0.0	0.5	0.0 ± 0.0	0.0 ± 0.0	1.0	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	0.0 ± 0.0	0.0 ± 0.0	0.5	0.0 ± 0.0	0.0 ± 0.0	1.0	n/a	n/a	n/a
SFN-Cys	µmols	3.3 ± 0.5	0.8 ± 0.1	***	0.8 ± 0.1	0.2 ± 0.0	***	0.8 ± 0.1	0.0 ± 0.0	***
	%	8.7 ± 1.0	14.0 ± 2.2	0.1	10.9 ± 1.4	11.5 ± 2.2	0.5	12.2 ± 1.8	2.6 ± 2.6	**
SFN-NAC	µmols	39.2 ± 9.3	5.3 ± 1.3	***	6.1 ± 0.8	1.7 ± 0.2	***	5.5 ± 0.4	1.1 ± 0.2	***
	%	84.0 ± 2.3	81.3 ± 1.7	0.5	83.2 ± 1.5	86.0 ± 2.1	0.5	83.1 ± 2.1	93.4 ± 3.9	0.1
Total	µmols	45.0 ± 9.5	6.4 ± 1.5		7.4 ± 1.0	2.0 ± 0.2		6.6 ± 0.5	1.1 ± 0.2	

DIVIDED DOSE		12 hours			24 hours			48 hours		
		Sprout	BSE	*p*	Sprout	BSE	*p*	Sprout	BSE	*p*
SFN	µmols[a]	1.2 ± 0.3	0.4 ± 0.1	*	7.4 ± 1.4	2.1 ± 0.6	**	0.3 ± 0.1	0.0 ± 0.0	**
	%[b]	12.2 ± 2.7	10.9 ± 3.6	0.6	13.8 ± 2.5	8.1 ± 2.0	0.1	6.0 ± 1.3	2.5 ± 1.1	*
SFN-CG	µmols	0.0 ± 0.0	0.0 ± 0.0	1.0	0.0 ± 0.0	0.0 ± 0.0	0.7	0.0 ± 0.0	0.0 ± 0.0	1.0
	%	0.0 ± 0.0	0.0 ± 0.0	1.0	0.1 ± 0.0	0.1 ± 0.0	0.9	0.0 ± 0.0	0.0 ± 0.0	1.0
SFN-Cys	µmols	0.7 ± 0.1	0.3 ± 0.1	*	4.9 ± 0.9	3.0 ± 0.5	0.2	0.4 ± 0.2	0.1 ± 0.1	0.2
	%	8.2 ± 1.5	11.1 ± 1.7	0.3	8.5 ± 1.7	13.3 ± 1.4	*	6.6 ± 3.1	3.5 ± 2.3	0.5
SFN-NAC	µmols	7.0 ± 1.0	2.4 ± 0.5	***	52.8 ± 11.2	19.9 ± 3.5	**	4.1 ± 0.8	1.6 ± 0.2	***
	%	79.7 ± 2.0	78.0 ± 2.6	1.0	77.7 ± 3.3	78.5 ± 2.4	0.6	87.3 ± 3.9	94.1 ± 2.6	0.1
Total	µmols	8.8 ± 1.2	3.1 ± 0.6		65.1 ± 13.0	25.0 ± 4.1		4.7 ± 0.9	1.7 ± 0.2	

3.3 Effects of Intake Frequency

In the divided-dose phase, comparative assessments were performed on plasma concentrations of SFN metabolites 12 hours after consuming each dose to evaluate the effects of the twice-daily dosing regimen on achieving steady-state SFN concentrations. In sprout consumers (Fig. 3.2C inset), plasma concentrations were 2.4-fold higher after consuming the second dose than after the first dose. In BSE consumers, SFN metabolites were only detected after the second dose, and in only 5 of 10 subjects. We also compared

24-hour plasma concentrations of SFN metabolites following consumption of the single, 200-µmol dose and the divided dose to evaluate the potential for twice-daily dosing schedules to achieve higher steady-state concentrations than once-daily dosing schedules. In sprout consumers, mean plasma concentrations of total SFN metabolites were significantly higher following consumption of the divided dose compared to the single dose (Fig. 3.2A inset; 0.12 ± 0.02 µM vs. 0.04 ± 0.01 µM, $p < 0.001$). At the same time points in BSE consumers, SFN metabolites were only detected after consuming the divided dose.

3.4 Chemopreventive Targets

Sprout and BSE consumers had similar fluctuations in PBMC HDAC activity during the study (Fig. 3.4). Similar to what we have observed previously, HDAC activity decreased transiently, though non-significantly ($p = 0.097$), in most subjects 3 hours after consuming sprouts (7 of 9) or the BSE (6 of 10) [126]. Mean HDAC activity increased significantly at the 12-hour time point ($p < 0.010$) (Fig. 3.4A). One sprout consumer was unable to provide a PBMC sample for analysis at time 0 due to low volume of blood drawn; blood draws at other time points were obtained successfully. For p21 mRNA, the mean fold change from time 0 was greater in sprout consumers than in BSE consumers, but differences between the two groups did not reach statistical significance ($p = 0.071$; Fig. 3.5A).

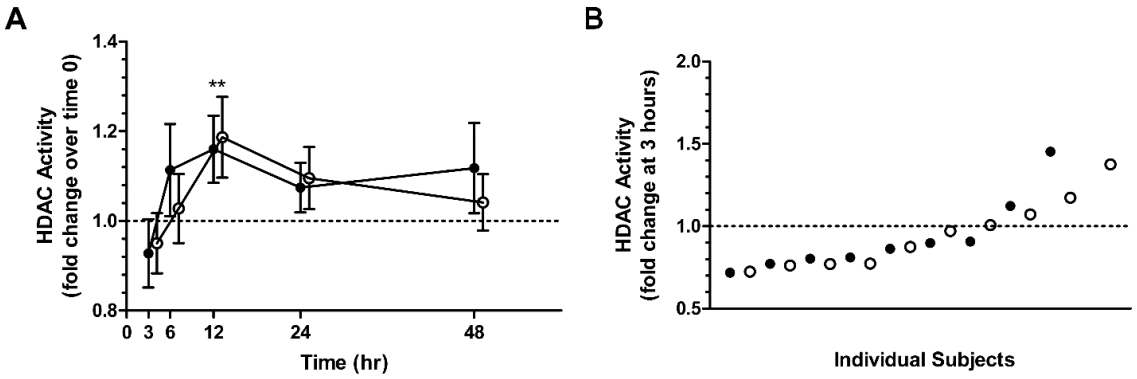

Figure 3.4. HDAC activity in PBMCs following consumption of broccoli sprouts or the broccoli sprout extract (BSE). A) Average fold changes (mean ± SEM) in PBMC HDAC activity after consuming a single dose of 200 µmol SFN equivalents from broccoli sprouts (solid, $n = 9$) or BSEs (open, $n = 10$). B) Individual fold changes in HDAC activity from time 0 to 3 hours. Note: Offsets for the BSE group are for visualization purposes only. $^{**}p < 0.01$. HDAC, histone deacetylase; PBMC, peripheral blood mononuclear cells.

In a pooled analysis of sprout and BSE groups, increasing fold changes were not statistically significant ($p = 0.185$). Sprout and BSE consumers experienced similar changes in plasma HO-1 protein levels with the exception of a single sprout consumer whose response curve was not similar to the other subjects ($p = 0.535$, excluding outlier; Fig. 3.5B). Inclusion of the outlier in the analysis did not change statistical conclusions. For HO-1 mRNA, the mean fold change from time 0 was greater in the sprout group than the BSE group, but this difference was not significant after accounting for lower baseline values in the sprout group ($p = 0.354$; Fig. 3.5C). In a pooled analysis of sprout and BSE groups, increases in HO-1 expression over time were not significant at the transcript ($p = 0.152$; Fig. 3.5C) or protein ($p = 0.287$; Fig. 3.5B) level following SFN consumption, though most subjects experienced some increase in HO-1 protein levels during the study (Fig. 3.6).

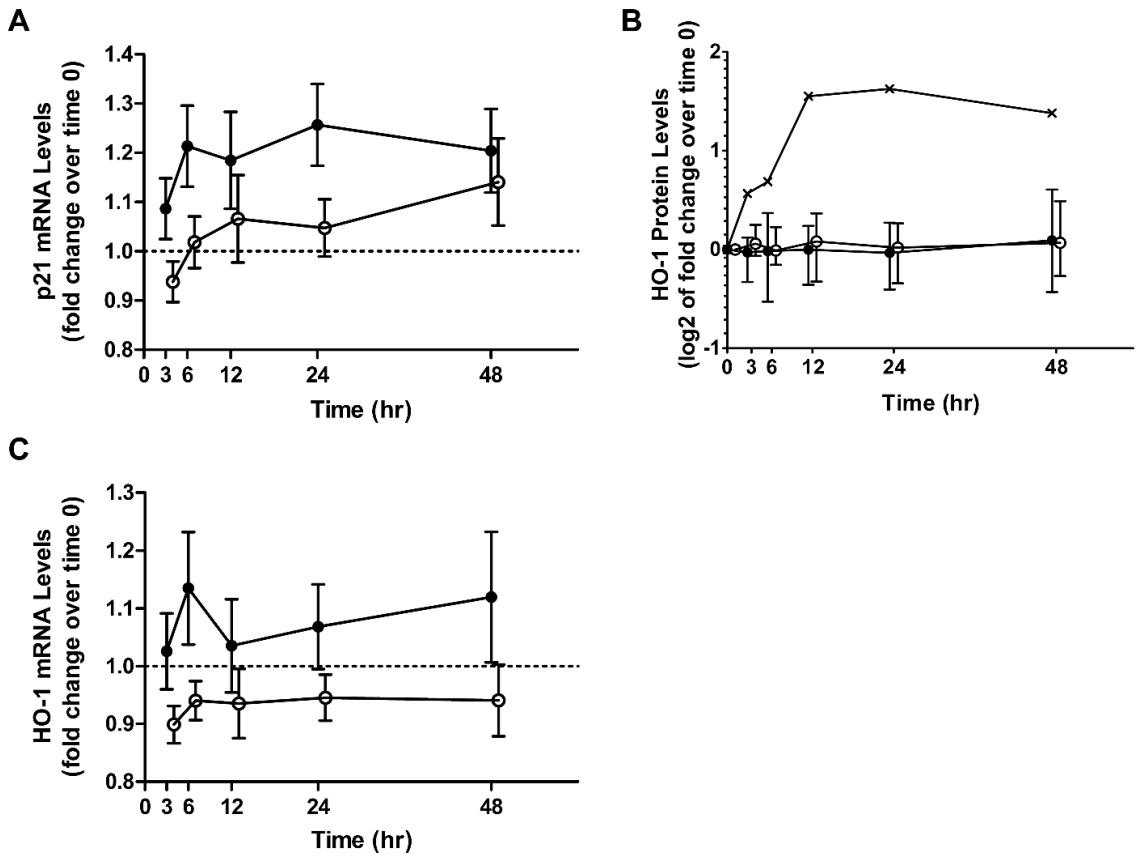

Figure 3.5. p21 and HO-1 expression following consumption of broccoli sprouts or the broccoli sprout extract (BSE). Changes in A) whole blood p21 mRNA levels, B) plasma HO-1 protein levels, and C) whole blood HO-1 mRNA after consuming a single dose of 200 µmol SFN equivalents from broccoli sprouts (solid) or BSEs (open). The sprout subject excluded from analyses is shown separately in the figure (-x-). Values represent mean ± SEM (A and B) and geometric mean ± SD (C). Note: Offsets for the BSE group are for visualization purposes only. HO-1, heme oxygenase-1.

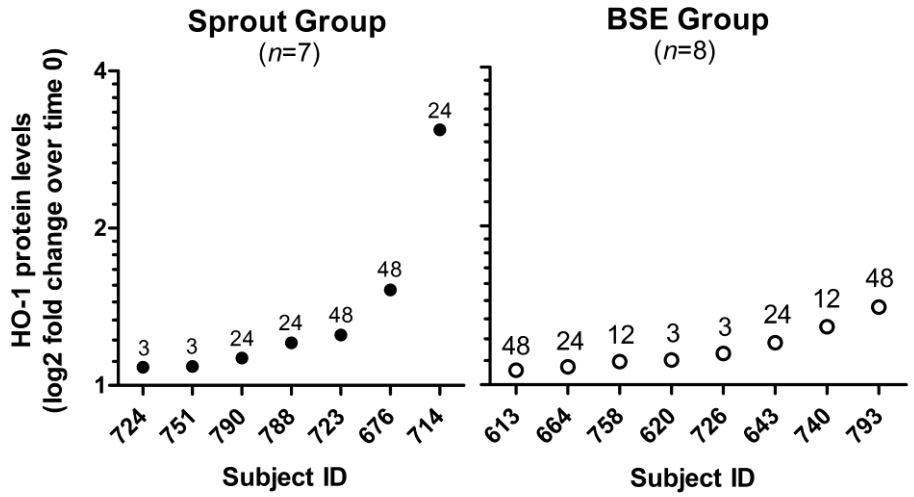

Figure 3.6. Maximum increases in plasma HO-1 protein levels in broccoli sprout and broccoli sprout extract (BSE) consumers during the single-dose phase. Maximum increasing fold changes in plasma HO-1 protein levels per subject in sprout (closed, $n = 7$) and BSE (open, $n = 8$) groups. Numbers above dots represent the time point (hour) when the maximum increasing fold change was observed for each subject. Values represent individual fold changes relative to time 0. HO-1, heme oxygenase-1.

4. Discussion

SFN, a phytochemical derived from cruciferous vegetables, has many cancer chemopreventive functions. Details of its molecular mechanisms in humans and its absorption from different dietary sources and forms are emerging. Rich, whole-food SFN sources include cruciferous vegetables, namely broccoli and broccoli sprouts. These foods contain GFN, which is hydrolyzed to yield SFN by the enzyme myrosinase, also found in cruciferous vegetables. Yet, GFN content within vegetables is highly variable, and conversion efficiency of GFN to bioactive SFN is affected by several factors, such as chewing intensity, glutathione-*S*-transferase (GST) genotype, and presence of gut microbes capable of hydrolyzing GFN when the plant enzyme is inactivated (as occurs with cooking) [95, 140, 145]. These factors complicate consistent SFN delivery from whole

foods in clinical trials, and dietary SFN supplements may be a feasible alternative. Yet, little is known about SFN bioavailability from supplements and how they impact biological processes compared to whole foods. This study is the first to provide evidence for the use of a myrosinase-treated BSE supplement to study cancer chemoprevention strategies involving SFN. We show that SFN absorption and excretion differ in individuals consuming whole foods (broccoli sprouts) and a SFN-containing BSE as well as when a dose is consumed at intervals rather than at a single time. We also describe the impact of these two SFN forms on specific chemopreventive targets.

4.1 Sulforaphane Absorption and Excretion

Differences in SFN bioavailability among ingested forms of broccoli have largely been attributed to differences in myrosinase activity. Subjects consuming raw broccoli or broccoli sprouts containing intact myrosinase were reported to have relatively high percent recoveries of SFN and SFN metabolites (32-80%). Dietary forms with inactivated myrosinase (*e.g.*, cooked cruciferous vegetables, GFN supplements available over-the-counter) had much lower percent recoveries (10-12%) [93, 96, 146-148]. In a cross-over study (n = 8), Vermeulen *et al.* [93] observed 11% higher excretion of SFN metabolites following consumption of raw versus cooked broccoli. Also, less SFN was absorbed and excreted from GFN supplements containing inactive myrosinase compared to fresh broccoli sprouts [96]. Supplements containing SFN, such as the BSE used in this study, may deliver higher and more consistent SFN doses than supplements containing GFN and inactive myrosinase. We observed that the myrosinase-treated BSE provided more SFN than previously tested GFN supplements, but SFN absorption was still significantly lower than from fresh broccoli sprouts (Fig. 3.2A). Validation experiments dismissed inequality of SFN dosing from sprouts and the BSE as an explanation for differences in SFN absorption from the two dietary forms. Our observations suggest that GFN conversion to SFN is not the only factor influencing SFN absorption.

Food matrix and meal composition may have affected SFN absorption from sprouts and the BSE. Whole broccoli sprouts contain nutrients, minerals and phytochemicals possibly lost during BSE manufacturing which may enhance SFN transport across cell membranes. Compared to the BSE, raw sprouts likely contain more fiber, which can slow gut transit and increase contact time between SFN and absorptive surfaces in the proximal gut. Compounds derived from other ingested foods and beverages could similarly impact SFN absorption. However, all participants consumed similar breakfast meals along with the given dose of sprouts or the BSE. Although composition of subjects' meals varied slightly, the foods provided did not contain SFN or compounds known to influence SFN absorption or the molecular targets we evaluated. Macronutrient content of subjects' diets during the study was similar. Despite the many factors possibly influencing SFN absorption, we observed relatively little variability in SFN metabolite levels and percentages within each treatment group. Enzymes involved with SFN absorption and metabolism, such as GSTs, are also involved with absorption and metabolism of other nutrients and xenobiotics and, thus, may be tightly regulated [149]. Variability was further minimized by standardizing breakfast meals and ensuring fasting status prior to morning SFN doses.

Calculated SFN bioavailability from broccoli sprouts exceeded 100% (Table 3.5), which likely reflects a combination of high SFN bioavailability and interconversion of erucin to SFN *in vivo* [96]. Interconversion between erucin and SFN may also explain the elevated half-life estimated for SFN absorbed from broccoli sprouts. Glucoerucin levels were not measured in the sprout formulations, and erucin metabolites were not measured in plasma or urine samples, thus their influence on SFN bioavailability and elimination kinetic estimates could not be determined.

This is the first study to report the effects of consuming SFN twice in a single day toward the possibility of achieving higher steady-state SFN levels in humans than consuming an equivalent dose all at once. In the body, SFN is rapidly metabolized, and plasma concentrations of SFN metabolites reach extremely low levels 12-24 hours after consuming a single dose [96, 98, 150, 151]. This study demonstrated that 12-hour dosing,

compared to consuming a single dose, resulted in higher plasma concentrations of SFN metabolites 24 hours post-consumption, which was expected based on pharmacokinetic patterns commonly observed with multiple dosing [152]. While the higher concentrations observed at 24 hours in the divided-dose phase are likely related to residual SFN metabolites derived from the first divided dose, the increase was not proportional to the amount of residual SFN metabolites expected at the 24-hour time point based on previous reports and our observations of SFN kinetics following a single dose [96, 98, 141]. Based on the data, it is unclear what other factors may have contributed to the higher 24-hour plasma concentrations observed with the twice-daily dosing schedule. Additional studies are needed to fully understand the impact of consuming multiple daily doses of SFN on SFN absorption and metabolism in humans.

4.2 Chemopreventive Targets

SFN is implicated in both genetic and epigenetic mechanisms important for cancer prevention [13, 112, 126]. To identify mechanisms involved with SFN's ability to prevent carcinogenesis in humans, we evaluated the response of three chemopreventive, mechanistic targets of SFN (*i.e.*, HO-1, HDAC, p21) in healthy adults following consumption of sprouts or the BSE. Despite existing reports that SFN increases expression of Nrf-2 targets, such as HO-1, we did not observe any significant increases in HO-1 expression in whole blood or plasma following sprout or BSE consumption [67, 153, 154]. Regardless of the SFN form consumed, subjects experienced a large degree of inter-individual variability in HO-1 responses, which could relate to its biological function as an inducible component of cytoprotective mechanisms and prevalence of promoter polymorphism [155, 156]. SFN doses consumed in this study may have been too low or too rapidly metabolized in cells to induce HO-1 to degrees observable in the circulation. Importantly, our observations in whole blood may not represent changes occurring in tissues. Cornblatt *et al.* [67] observed a 10-fold induction of HO-1 in mammary tissue from

rats gavaged with 150 µmol SFN over control animals. Additional studies are needed to characterize molecular target responses to consuming SFN from sprouts and the BSE in target organs.

SFN may also prevent cancer through epigenetic mechanisms by functioning as an HDAC inhibitor [157]. Though sprout and BSE subjects consumed similar SFN doses as in previous human studies that have reported decreases in HDAC activity, neither of the two SFN forms produced a significant decrease in HDAC activity. Yet, the trends for decreased activity observed at the 3-hour time point in sprout and BSE consumers were similar in magnitude to those reported by Clarke *et al.* (Fig. 3.4A) [126]. Larger decreases in HDAC activity may occur in transformed cells or tumors with underlying aberrant HDAC regulation, rather than in non-transformed PBMCs obtained from healthy subjects, as in this study [14, 158]. Higher SFN doses or repeated intake may also result in greater or more sustained decreases in HDAC activity [126]. Increases in HDAC activity observed 12 hours after SFN consumption may relate to a homeostatic response to the slight decrease observed 9 hours earlier. Rhythmic HDAC expression is not expected as the cause of these increases, as we previously observed no change in HDAC activity over time in healthy adults consuming placebos and avoiding intake of other known dietary HDAC inhibitors (unpublished data). As with HO-1, high inter-individual variability was observed in HDAC responses regardless of the SFN form consumed. This variation could relate to biological variation, basal activity levels, or effects on specific HDAC isoforms not influenced by SFN, as we assessed measures of total HDAC activity [159]. Although sprout and BSE consumers did not experience different mean HDAC responses at the doses provided in this study, further evaluation is needed to clarify dose-response relationships between HDAC activity and SFN consumed from sprouts and the BSE as well as tissue-specific effects of SFN on HDAC regulation and downstream targets such as p21.

Despite having lower SFN bioavailability compared to broccoli sprouts, the myrosinase-treated BSE may be acceptable SFN sources for use in clinical trials to study certain chemopreventive mechanisms of SFN. Further, we demonstrated maintenance of plasma SFN levels using a twice-daily dosing schedule, which may be important for

increasing or prolonging certain chemopreventive benefits associated with SFN consumption. Together, these data provide important information for using SFN supplementation strategies in clinical studies of cancer chemoprevention.

CHAPTER 4

UNTARGETED METABOLOMIC SCREEN REVEALS CHANGES IN HUMAN PLASMA METABOLITE PROFILES FOLLOWING CONSUMPTION OF FRESH BROCCOLI SPROUTS

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Abstract

Several lines of evidence suggest that the consumption of cruciferous vegetables is beneficial to human health and disease prevention. However, precise mechanisms for how cruciferous vegetables exert protective benefits in humans are unclear. To this end, this study aimed to detect and characterize changes in metabolomic profiles in human plasma following consumption of fresh broccoli sprouts, a rich dietary source of the bioactive compound, sulforaphane (SFN). Ten healthy adults consumed fresh broccoli sprouts containing 200 μmol sulforaphane equivalents at time 0 and provided blood samples at times 0, 3, 6, 12, 24 and 48 hours. An untargeted metabolomics screen revealed changes in several plasma metabolites following sprout consumption, including several fatty acids (14:0, 14:1, 16:0, 16:1, 18:0, 18:1), glutathione (GSH), glutamine, cysteine, dehydroepiandrosterone, and deoxyuridine monophosphate. This investigation identified several potential molecular targets of SFN that may aid in studying established and novel health benefits of cruciferous vegetable consumption. We detected changes in several fatty acids and metabolites associated with GSH biosynthesis, steroid metabolism, and nucleotide metabolism. These data provide critical information for evaluating the health impact of consuming dietary sources of SFN in human studies.

1. Introduction

Consuming cruciferous vegetables is associated with many health benefits including cancer prevention [1, 34, 160]. Many of these health effects have been attributed to the phytochemical, sulforaphane (SFN), which is derived from crucifers such as broccoli. Cruciferous vegetables contain the glucosinolate precursor of SFN, glucoraphanin (GFN), which is hydrolyzed to yield SFN by plant myrosinase enzymes upon tissue damage, such as from chopping or chewing the vegetable, or from myrosinase-like activity produced by certain gut microbes. After SFN is formed and ingested, it is rapidly absorbed and

metabolized via the mercapturic acid pathway, yielding several additional metabolites that are thought to be important for some of the biological activity of the parent compound [161]. Cruciferous vegetables are known to deliver high amounts of SFN when consumed raw or lightly cooked. Fresh broccoli sprouts are a good source of SFN, because they contain high levels of GFN (up to 50x higher than mature broccoli) and intact myrosinase [64].

SFN has largely been studied for its roles in cancer chemoprevention, and emerging evidence suggests that consuming dietary sources of SFN may be beneficial in the prevention or management of other morbidities as well, such as cardiovascular disease and diabetes [160, 162, 163]. For example, Bahadoran *et al*. [164] demonstrated improved lipid profiles in patients with type 2 diabetes after 4 weeks of consuming a broccoli sprout powder. Several molecular mechanisms of SFN have been characterized in cultured cells and animal models, including the identification of both genetic and epigenetic targets. Perhaps the most well-studied targets include those under the transcriptional control of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), such as quinone reductase [64]. There is also evidence to suggest that SFN influences epigenetic landscapes by altering the expression and/or activity of epigenetic modifying enzymes, such as histone deacetylases (HDAC) and DNA methyltransferases (DNMT) [19, 126]. Observations from studies that have evaluated these effects of SFN in humans following consumption of dietary sources of SFN suggest that there may be differences in how humans respond compared to cultured cells and animals exposed to high doses of SFN and a purified chemical source [136, 165]. Thus, knowledge of SFN action acquired from mechanistic studies may not sufficiently encompass the breadth of health benefits that result from dietary SFN exposure. Establishing the molecular targets and molecular events that are associated with exposure to diet-relevant doses of SFN is critical to understanding the health benefits of cruciferous vegetable consumption. To this end, we sought to perform an unbiased metabolomics screen in humans that consumed fresh broccoli sprouts to identify potential molecular targets of SFN and to improve understanding of how SFN and cruciferous vegetables confer protection against disease in humans.

Metabolomics is emerging as a powerful approach to screen for physiological responses to diet, drugs and disease [166]. This analysis detects small molecule metabolites in blood or tissues, and changes in these metabolites provide information on the functioning of biochemical pathways upstream. Using an untargeted metabolomics approach, we examined plasma metabolomic profiles in healthy adult subjects before and after consuming fresh broccoli sprouts to provide an initial screen for changes in plasma metabolites. This approach allowed us to conduct an unbiased screen of physiological responses to consuming broccoli sprouts, since evaluation was not limited to previously recognized molecular targets of SFN. This investigation was an attempt to identify potential, novel molecular targets that could be used to study the molecular mechanisms that underlie the health benefits of consuming crucifers using more targeted approaches.

2. Materials and Methods

2.1 Participants

This human feeding study included ten healthy adults, ages 19-50 years, recruited in Corvallis, Oregon and was conducted in the Moore Family Center metabolic kitchen and clinical collection laboratory at Oregon State University (OSU). Subjects were excluded for smoking, having a BMI < 18.5 or > 30 kg/m^2, consuming a vegetarian diet, or using drugs known to alter lipid metabolism. All participants provided informed, written consent. The Institutional Review Board (IRB) at OSU approved all study protocols (OSU IRB #4995).

2.2 Study Design

Following an overnight fast, subjects consumed a single dose of fresh broccoli sprouts, providing 200 µmol SFN equivalents, at 8 AM on the first day of the study. Along with the SFN dose, subjects consumed a provided breakfast meal, which included bagels, cream cheese, orange juice, milk or coffee. Subjects consumed the same breakfast on all days of the study, but only consumed the SFN dose on day 1. Sprouter's Northwest (Kent, WA) provided fresh broccoli sprouts. Subjects avoided consumption of cruciferous vegetables and/or isothiocyanates (ITC) 1 week prior to and during the entire 3-day study. Subjects maintained 3-day diet records during the study period, following instruction by a registered dietitian. Diet records were analyzed using Food Processer® SQL (ESHA, Salem, OR).

2.3 Sample Collection and Processing

Whole blood (20 ml) was collected into EDTA vacutainers (VWR, Radnor, PA) before consumption of sprouts and following consumption at 3, 6, 12, 24 and 48 hours. Plasma was taken from the plasma layer of Ficoll preps which were prepared as described by Clarke, *et al.* [126]. Plasma was snap frozen in liquid nitrogen and stored at -80°C to await further processing for metabolomics. To prepare plasma extracts for untargeted metabolomics analysis, plasma was thawed on ice, mixed with 80 µl of ice-cold 50:50 (v/v) methanol:ethanol, vortexed, and spun 15 min at 13,000 x *g* at 4°C. The supernatant was collected and analyzed by HPLC-MS/MS. Extraction and HPLC-MS/MS methods were developed to capture and detect plasma metabolites associated with a variety of biological pathways and were identical to those described by Kirkwood, *et al.* except where differences are described [167].

2.4 Measuring Sulforaphane Content of Broccoli Sprouts

SFN content of fresh broccoli sprouts was determined using HPLC-MS/MS as described previously [136]. The SFN standard was purchased from LKT Laboratories, Inc. (St.Paul, MN). Standard dilutions were prepared in 0.1% (v/v) formic acid in water. Approximately 215 mg fresh sprouts were homogenized in 1 ml water, then incubated with 2 mg *Sinapis alba* thioglucosidase (Sigma-Aldrich, St. Louis, MO) at 60°C in the dark. After incubation, homogenates were filtered with 0.22-µm nylon Spin-X® centrifuge tube filters (VWR, Radnor, PA) by centrifugation at 15,000 x g for 5 min at 25°C. Supernatants were diluted 20-fold using 0.1% (v/v) formic acid in water. Extracts were immediately stored at -80°C to await analysis by HPLC-MS/MS.

2.5 LC-MS/MS-based Metabolomics

Plasma supernatants were analyzed by HPLC-MS/MS using methods described previously [168]. Briefly, HPLC was carried out using a Shimadzu Nexera system (Shimadzu, Columbia, MD) coupled to a TripleTOF™ 5600 mass spectrometer (AB SCIEX, Framingham, MA). Compounds were separated in positive and negative ion modes using an Inertsil phenyl-3 column (150 x 4.6 mm, 5 µm, MetaChem Technologies, Torrence, CA). The column was held constant at 70°C utilizing a flow rate of 0.4 ml/min with mobile phases of water and methanol, both with 0.1% formic acid. All injections had a volume of 10 µl. For mass spectrometry, the TripleTOF™ 5600 was connected to an electrospray ionization (ESI) source and operated in the information-dependent MS/MS (IDA-MS) acquisition mode. Exact settings were used as in [168]. Quality control samples to evaluate system and biological variance were prepared by combining 15 µl aliquots from all study samples (n = 20) and injected at random intervals throughout the batch.

2.6 Data Processing and Statistical Analysis

Raw HPLC-MS/MS data were imported into MarkerView™ software (AB SCIEX) to perform feature detection, peak alignment, peak integration, principal component analysis (PCA), and discriminant analysis (DA). Here, a feature is defined as having any m/z value with a unique retention time. PCA-DA determined degree of separation based on features detected in the positive and negative ion modes (separately). This analysis shows if there are changes in the amount or types of features present over time. Features representing SFN metabolites were identified and excluded from the data set prior to performing PCA-DA analysis to avoid falsely observing differences in metabolite patterns over time, since these compounds are known to change in plasma following SFN intake [96, 136]. To identify significantly altered features, paired *t* tests comparing each time point to time 0 were performed on non-normalized data using R, and significance was determined at $p < 0.05$. Fold changes were determined after normalizing values to the group mean at time 0.

2.7 Metabolite Identification

Raw metabolomics data (wiff files) were imported into PeakView® software (AB SCIEX) for identification of features using an established in-house MS database [167, 168]. This database was created by analyzing purchased compound standards with the same analytical protocols as used in this study to detect and characterize features in plasma samples. Database entries contain information on each metabolite including exact mass, isotope distribution, MS/MS fragmentation patterns, and retention time [167, 168]. In the present investigation, features were compared to metabolite entries in this database and matched based on mass and retention time. Any feature positively matching to a metabolite within the database was included in this report as long as the feature was also significantly altered at one of the five time points following sprout intake compared to time 0. Features were also examined using online metabolite databases, including METLIN: Metabolite and

Tandem MS/MS Database and the Human Metabolome Database (HMDB). This report focused on endogenous metabolites.

3. Results

3.1 Subject Characteristics

The study population included 4 adult males and 6 adult females with an average age of 30.5 ± 2.9 years. Average BMI was 23.0 ± 0.9 kilograms/meters^2. We experimentally confirmed that subjects avoided confounding food items the week before and during the study by analyzing dietary records and plasma and urinary SFN ITC levels during the study by HPLC-MS/MS (data not shown)[136].

3.2 Discovery of Metabolites and Identification

PCA-DA analysis revealed significant differences in metabolite profiles over time following sprout consumption (Fig. 4.1). Separation was not driven by SFN ITC changes over time, since they were removed from the data set prior to this analysis. The 3-hour samples clustered further away from the 0-hour samples than any other time point. Six-hour samples clustered near 3-hour samples, with the 12-, 24-, and 48-hour samples clustering progressively back toward 0-hour samples. Importantly, metabolite profiles at 0 and 24 hours were clearly separated on PCA-DA plots suggesting changes induced by broccoli sprout consumption beyond a basic time-of-day effect. Analysis of quality control samples confirmed low system variance and absence of time drifts (data not shown).

Eleven significantly altered features were matched to endogenous metabolite entries in the in-house metabolite database. These metabolites are shown in Table 4.1 and are organized by ontology in Figure 4.2. Furthermore, the profiles of each metabolite in

the plasma following sprout intake are shown in Figure 4.3. We observed significant decreases in glutathione (GSH) at 6, 12 and 24 hours following sprout intake. Two of its precursors, glutamine and cysteine, were also decreased at 3 and 24 hours, and 12 and 24 hours, respectively (Fig. 4.3). This investigation also revealed significant decreases in several fatty acids (FA), including FA 14:0, FA 14:1, FA 16:0, FA 16:1, FA 18:0 and FA 18:1, at multiple time points following sprout intake (Fig. 4.3). Additionally, we observed significant decreases in dehydroepiandrosterone (DHEA), specifically at 3, 6 and 12 hours. This was one metabolite identified that was associated with steroid metabolism. The only metabolite that increased significantly following sprout consumption was deoxyuridine monophosphate (dUMP). dUMP was significantly increased at 12 hours (Fig. 4.3).

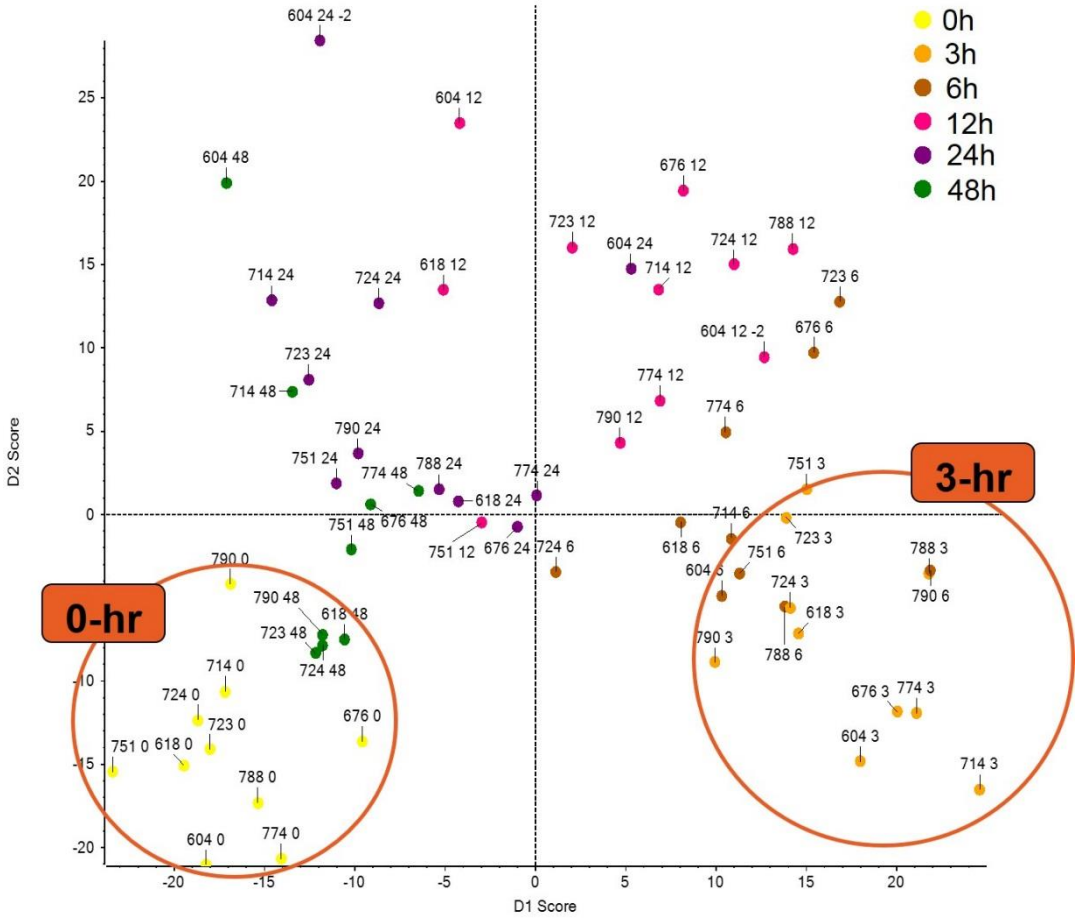

Figure 4.1. Human plasma metabolomic profiles differ over time following broccoli sprout consumption. Principal components analysis – discriminant analysis (PCA-DA) was conducted on all features detected in plasma of subjects with the exception of SFN and SFN metabolites (erucin and other isothiocyanates not detected). Each dot represents a different subject at a specific time point (times denoted by different symbols and indicated by the number following the dash (-) in the plot). *n* = 10 for all time points except 48h, where *n* = 9.

Table 4.1. Endogenous metabolites altered following consumption of fresh broccoli sprouts in human plasma.

Metabolite	Formula	Adduct	Theoretical m/z[a]	Experimental m/z	Mass Error (ppm)
Glutathione	$C_{10}H_{17}N_3O_6S$	+ H	308.0911	308.0914	1
Cysteine	$C_3H_7NO_2S$	+ H	122.0270	122.0266	– 3.4
Glutamine	$C_5H_{10}N_2O_3$	+ H	147.0764	147.0765	0.4
DHEA	$C_{19}H_{28}O_2$	+ H	289.2162	289.2154	– 2.7
dUMP	$C_9H_{13}N_2O_8P$	+ H	309.0482	309.0485	0.9
FA 14:0	$C_{14}H_{28}O_2$	– H	227.2017	227.2019	1.2
FA 14:1	$C_{14}H_{26}O_2$	– H	225.1860	225.1856	– 1.6
FA 16:0	$C_{16}H_{32}O_2$	– H	255.2330	255.2328	– 0.7
FA 16:1	$C_{16}H_{30}O_2$	– H	253.2173	253.2172	– 0.6
FA 18:0	$C_{18}H_{36}O_2$	– H	283.2643	283.2643	0.1
FA 18:1	$C_{18}H_{34}O_2$	– H	281.2486	281.2486	– 0.2

[a] Values acquired from database entries. m/z, mass-to-charge ratio.

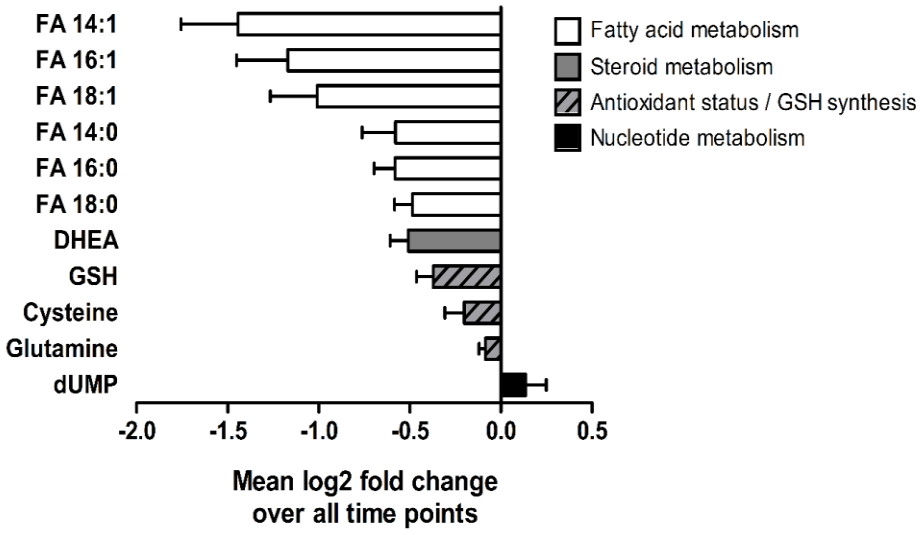

Figure 4.2. Endogenous metabolites altered following broccoli sprout consumption in human plasma are associated with multiple biochemical pathways. Values represent mean log2 fold change across all time points ± SEM. *n* = 10 for all time points except 48h, where *n* = 9.

Figure 4.3. Time course profiles of metabolites altered following broccoli sprout consumption in human plasma show significant changes over time. Fold change plotted on log2 scale. Fold changes are normalized to the group mean value at time 0. Values represent mean fold change ± SEM. *n* = 10 for all time points except 48h, where *n* = 9. hr, hour.

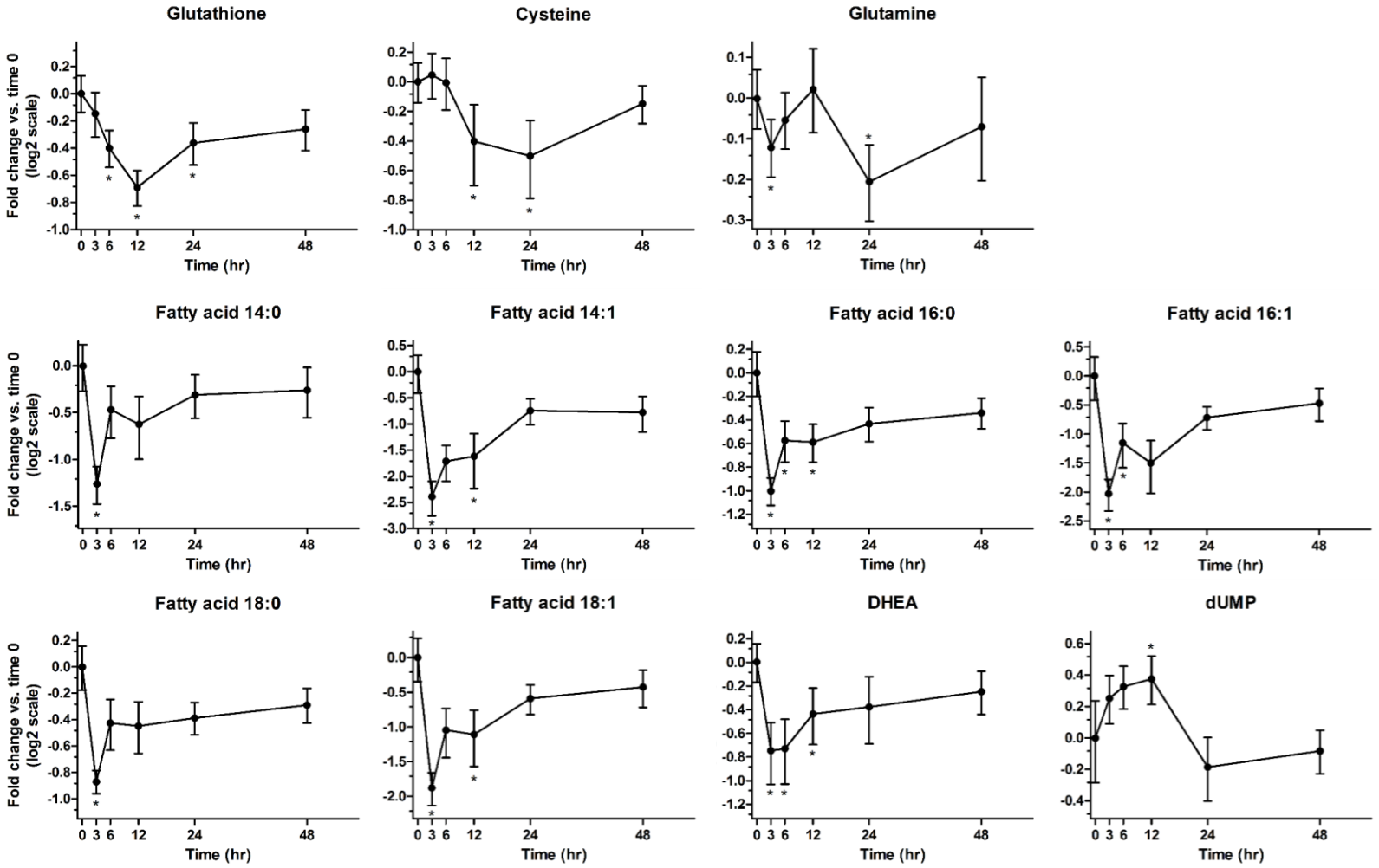

Figure 4.3. Time course profiles of metabolites altered following broccoli sprout consumption in human plasma show significant changes over time.

4. Discussion

We used untargeted metabolomics to screen for metabolomic phenotypes associated with broccoli sprout consumption. A major strength of this approach is the ability to discover novel targets with health implications from dietary exposures. This screen identified several metabolites that were altered and detectable in human plasma following broccoli sprout intake. This information can be used to study both known and novel health benefits of cruciferous vegetable consumption and dietary exposure to SFN. Future studies employing larger study populations and targeted approaches are needed in each of these areas to confirm the effects of broccoli spout consumption on the pathways identified from this screen and how they may be affected in different populations and disease conditions.

Among the metabolites we detected, GSH is a major intracellular antioxidant and conjugates with SFN when SFN enters the cell. Transient decreases of intracellular GSH have previously been reported to occur upon exposure to SFN and are thought to be necessary to activate certain cellular antioxidant responses [169]. We observed decreases in plasma GSH in human subjects following consumption of broccoli sprouts, suggesting this antioxidant response mechanism may also occur in humans that consume dietary sources of SFN. We also observed decreases in two precursors of GSH, glutamine and cysteine, following SFN exposure. Decreases in these precursors may reflect rebounding GSH biosynthesis [169, 170].

dUMP is a precursor of the dinucleotide, DNA thymine (dTTP), which is one of the building blocks of DNA. We observed a transient increase in dUMP in subjects following broccoli sprout intake, suggesting alterations in DNA synthesis or one-carbon metabolism. SFN is well-known to alter the cell cycle and cell proliferation, events that certainly impact DNA synthesis [34, 171]. Specifically, SFN has been shown to alter levels of cyclins and cyclin kinases, as well as a variety of other proteins, that govern progress through different stages of the cell cycle [171]. Our observations support the possibility that consuming dietary sources of SFN, such as broccoli sprouts, may also impact cell

proliferation in humans. Additional studies are needed to clarify mechanisms underlying changes in DNA synthesis and related molecular events following dietary SFN exposure.

This study also revealed altered levels of DHEA following consumption of broccoli sprouts, which suggests alterations in steroid metabolism or activity. DHEA is a steroid that is converted to estrogen and/or testosterone. It is synthesized mostly in the adrenal glands, with smaller amounts produced in the testes and brain [172]. A recent study in human breast cancer cell lines revealed evidence that metabolic derivatives of DHEA, such as 7β-hydroxy-epiandrosterone, may have anti-cancer effects by serving as an anti-estrogenic agent and preventing cell proliferation [173]. In the central nervous system, DHEA has also been shown to bind neuroreceptors that activate apoptotic signaling pathways [174]. Studies have reported altered levels of sex steroid receptors following SFN exposure, but direct evidence of an effect of SFN on DHEA levels is limited [175]. Due to the major roles of DHEA in human health and possible links to cancer development, studying the impact of cruciferous vegetable consumption on steroid metabolism and DHEA levels will be an interesting area of future research.

The effects of SFN exposure on lipid metabolism have received less attention, but recent studies suggest that the consumption of dietary sources of SFN could aid in the prevention or management of diseases characterized by dyslipidemia. In a recent clinical trial of patients with diabetes, daily consumption of a broccoli sprout powder for 4 weeks was shown to beneficially modulate circulating lipid profiles by reducing triglycerides and improving cholesterol ratios [164]. Similar effects were observed in healthy adult subjects that consumed fresh broccoli sprouts daily for 1 week [176]. Another study found evidence of decreased adipogenesis in high-fat diet-induced obese mice when SFN was added to the diet [177]. The decreases in fatty acids observed in this study are consistent with these previous findings and suggest that even a single dose of broccoli sprouts may alter plasma lipids in healthy adult populations. More work is needed to further test this potential health benefit of broccoli sprouts and elucidate the mechanisms by which broccoli sprout consumption alters lipid metabolism and related health outcomes.

This study aimed to examine endogenous metabolite profiles in human plasma following consumption of broccoli sprouts, a rich, bioavailable source of dietary SFN. To our knowledge, we are the first to apply untargeted metabolomics to screen for the physiological effects of broccoli sprout consumption, and this work has provided new insights into potential health benefits of crucifers. We detected changes in several fatty acids and metabolites associated with GSH biosynthesis, steroid metabolism, and nucleotide metabolism (Figs. 4.2 and 4.3). These data provide critical information for evaluating the health impact of consuming dietary sources of SFN in human studies.

Limitations of this study include having a small sample size and lack of a comparison group that did not consume any SFN. It is possible that changes in each of these metabolites were due to consumption of other food items provided at breakfast or an effect of circadian rhythms. To rule out this possibility, we compared the peak changes of each metabolite to its levels in a control group that consumed the same breakfast with a broccoli sprout extract supplement (Fig. 4.4). We have previously shown that this supplement delivers 3x lower amounts of SFN compared to fresh broccoli sprouts [136]. From this data, it is clear that 10 of the 11 metabolites were differentially altered between the two groups, where broccoli sprouts consistently had a larger effect. Glutamine was the only exception. Since changes in glutamine levels were similar between the two groups, we cannot rule out an influence of the other breakfast food items consumed on this metabolite. However, since there was a significant difference in glutamine levels at 0 and 24 hours following sprout consumption, we can conclude that a circadian rhythm was not the only contributing factor to the changes observed.

In conclusion, many of the metabolites identified in this study offer new insights into SFN mechanisms in humans and how the consumption of dietary SFN can impact human health. In the future, the identities of the metabolites reported in this study should be confirmed by evaluating isotopic distributions and MS/MS fragmentation patterns alongside those of analytical standards. Future metabolomics studies that include larger sample sizes and evaluate changes in tissues will be important for understanding the role of consuming dietary SFN and cruciferous vegetables in disease prevention.

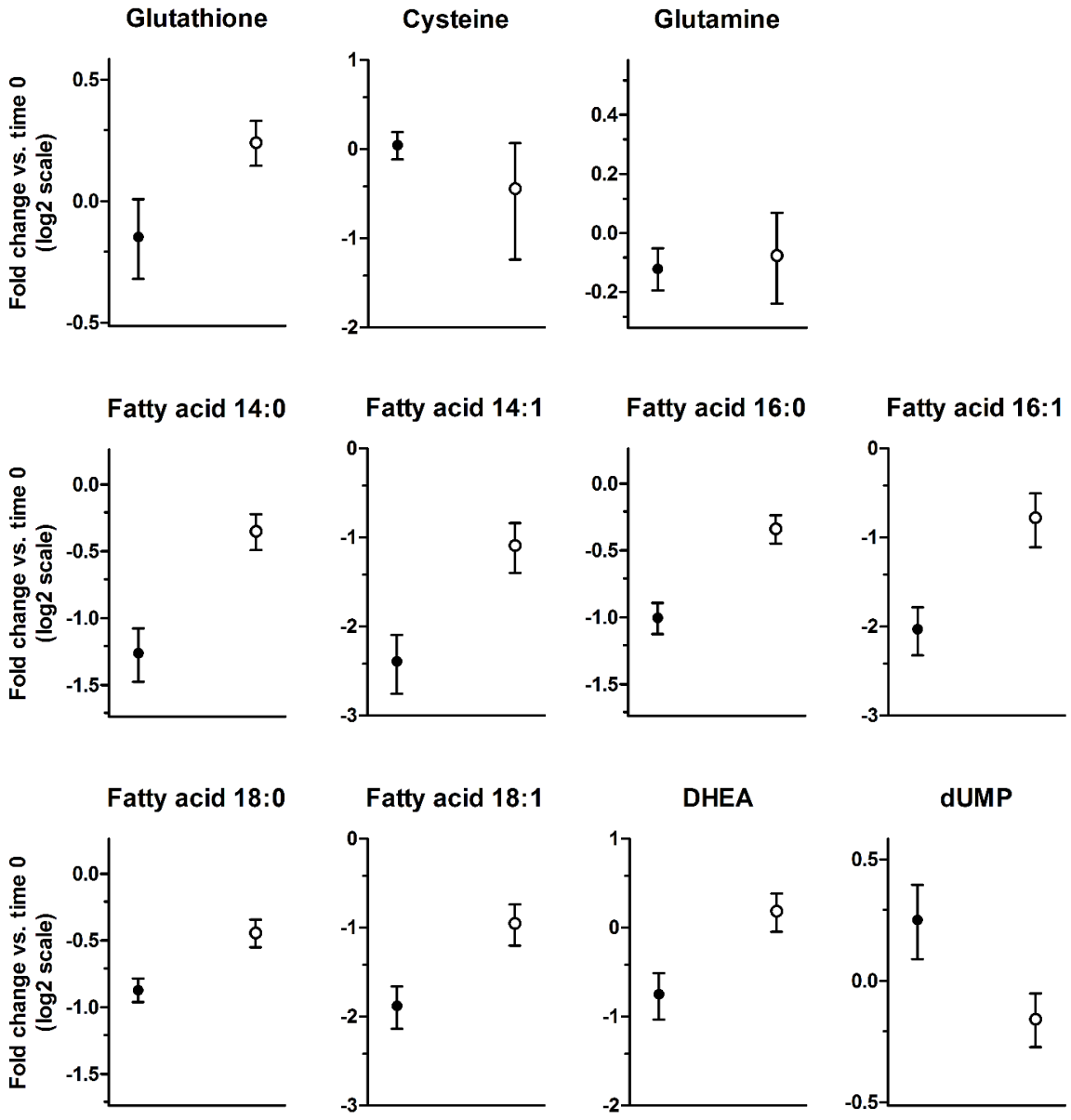

Figure 4.4. Metabolite fold changes following sprout intake differed at peak time points compared to those observed in supplement consumers. Fold change plotted on log2 scale. Fold changes are normalized to the group mean value at time 0. Values represent mean fold change ± SEM (*n* = 10) in sprout consumers (closed circles) and supplement consumers in comparison group (open circles).

CHAPTER 5

CONCLUSIONS

The majority of existing knowledge regarding SFN and health has arisen from studies conducted in cultured cells and animal models using high doses of SFN and purified chemical forms. Observations from these *in vitro* and model studies may not reflect events that occur in humans who obtain SFN from dietary sources, such as fresh broccoli sprouts. Only a few studies have been conducted to evaluate the effects of SFN in humans, and results sometimes vary from those seen in preclinical studies. Even fewer studies have evaluated effects of SFN in human tissue. Additionally, there is limited understanding of how dietary form (*i.e.*, food form versus supplemental form), dose, and dosing regimens may differ with respect to bioavailability and biochemical target. These differences could impact their ability to elicit specific health benefits in humans. The purpose of this dissertation was to translate mechanistic work with SFN into humans by evaluating responses of genetic and epigenetic molecular targets in human subjects following consumption of controlled doses of dietary SFN. We also evaluated the impacts of dietary form and dosing schedule on SFN absorption and metabolism in humans. Overall, our results present several new opportunities for the expansion of research relating to the roles of consuming dietary SFN and cruciferous vegetables in human health and disease prevention.

In this body of work, we demonstrated the ability of SFN to alter epigenetic mechanisms in humans, not only in circulating blood cells, but also in human breast tissue. This is also the first report of decreases in Ki-67 expression, a marker of cell proliferation, in human breast tissue following SFN exposure. These observations support a role of consuming dietary sources of SFN in the prevention of breast cancer. In this trial, our results were promising, yet somewhat modest. Future investigations that use dietary supplements with higher SFN bioavailability, such as a myrosinase-treated broccoli sprout extract (BSE) (Chapter 3), could deliver higher levels of SFN and may achieve larger

responses in tissue biomarkers. Additionally, having a larger sample size and specifying, a priori, the number of tissue samples needed for each disease category (*e.g.*, benign, DCIS, IDC) would improve power to detect changes following SFN supplementation. To allow for quantitative and more sensitive evaluation of molecular targets in future studies, investigators should consider freezing tissue cores, rather than or in addition to preparing formalin-fixed specimens. Together, these strategies would help to further characterize SFN effects on tumor biomarkers in human tissues.

Results from metabolomic screening identified new potential mechanisms that may mediate the health effects of broccoli sprouts and SFN in cancer prevention as well as in processes related to other chronic diseases, such as those that involve dyslipidemia. As a next step, our observations should be validated by detecting and measuring levels of these metabolites in our plasma samples using targeted approaches, such as enzymatic assays or mass spectrometry. Additional studies in humans and animal models should confirm responses of these metabolites to consuming broccoli sprouts and SFN. To verify that the changes we observed were related to the consumption of broccoli sprouts, and to distinguish effects specifically related to SFN consumption, one could conduct a controlled feeding study in mice where one arm is fed broccoli sprouts, a second arm is fed purified SFN, and a third arm is fed a control diet. Eventually, it will also be important to conduct controlled feeding studies in humans designed specifically for quantifying these metabolites in circulation and in tissues following consumption of broccoli sprouts or sprout formulations. All of these studies should employ a control group that does not consume SFN (*e.g.*, placebo or sprouts lacking glucoraphanin and glucoerucin) in order to differentiate among the effects of sprouts or SFN and other potential confounders.

Additionally, we demonstrated that a broccoli sprout extract containing SFN delivered detectable levels of SFN to human plasma and may be acceptable for use in controlled-dose human studies where the use of whole foods is logistically challenging. Though molecular responses, including HDAC activity, and p21 and HO-1 expression, were not different between sprout and BSE consumers despite the two formulations having a different relative SFN bioavailability, we did observe differential effects of the two forms

of broccoli sprouts using metabolomics analysis (Chapter 4). This information is critical for relating results from clinical studies using broccoli sprout extracts and supplemental SFN forms to effects that may be obtained with the whole food.

When analyzing the metabolomics data, we mainly focused on changes that occurred over time following intake of broccoli sprouts, but additional analyses to compare metabolites that were differentially altered by the two sprout forms would help establish key differences between consumption of the whole food and extract.

We also reported that a twice-daily dosing schedule maintained higher plasma levels of SFN metabolites at later time points compared to a once-daily dosing regimen. One explanation for this observation could be that the second daily dose may have been more slowly absorbed and metabolized due to lower GSH levels. GSH, which is an important determinant of SFN absorption, decreases in the evening. To determine how the time of day influences SFN bioavailability and metabolism, one could conduct a human feeding study that includes a third study phase (in addition to the two study phases we used; Chapter 3) in which subjects would consume a single SFN or sprout dose in the evening. A finding that SFN was more slowly absorbed and metabolized from evening doses compared to morning doses would warrant further investigation into the optimal timing of intake.

Taken together, this work makes several important and unique contributions to the understanding of SFN bioavailability and its molecular targets in humans. Future work in these and related areas will help to refine our knowledge of how best to achieve the health benefits of consuming cruciferous vegetables.

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