Sulforaphane Bioavailability and Chemopreventive Activity in Women Scheduled for Breast Biopsy

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Abstract

Epidemiologic 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 select biomarkers from blood and breast tissues. In a 2- to 8-week double-blinded, randomized controlled trial, 54 women with abnormal mammograms and scheduled for breast biopsy were randomized to consume a placebo or a glucoraphanin (GFN) supplement providing SFN (n = 27). 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, or invasive ductal carcinoma breast tissues. Within the supplement group, Ki-67 (P = 0.003) and HDAC3 (P = 0.044) levels significantly decreased in benign tissue. Pre-to-postintervention changes in these biomarkers were not significantly different between treatment groups after multiple comparison adjustment. GFN supplementation was associated with a significant decrease in PBMC HDAC activity (P = 0.04). No significant associations were observed between SFN and examined tissue biomarkers when comparing treatment groups. This study provides evidence that GFN 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. Cancer Prev Res; 8(12) 1184–91. ©2015 AACR.

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 (1). 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 (2, 3). 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 biologic mechanisms (4).

Ductal carcinoma in situ (DCIS) is a noninvasive form of breast cancer that accounts for about 20% of newly diagnosed cases of breast cancer (5, 6). 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 (6, 7). While there have been recent improvements in the treatment of breast cancer, epidemiologic studies have shown that women are more likely to change their lifestyle behaviors and medication use following diagnosis of DCIS (8). 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, obtained from a supplement containing its precursor glucosinolate, on molecular response biomarkers in blood and breast tissue (including tumor and nontumor) 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 chemoprevention 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 (9) 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 (10). SFN has been shown to be an effective chemopreventive agent in both in vitro and in vivo models for breast cancer where...
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Sulforaphane (SFN) is able to selectively induce apoptosis and slow tumor growth (11–14). Mechanistic studies have identified several targets of SFN, including cell-cycle proteins such as p21, which may be involved in its antitumor effects (15). SFN has also been shown to decrease levels of Ki-67, a marker of cell proliferation, in prostate tumor tissue and breast cancer cell xenografts (15, 16). Ki-67 is known as an important prognostic biomarker in women with breast cancer (17).

Recent work indicates that SFN targets epigenetic alterations and inhibits histone deacetylases (HDACs; refs. 18, 19). HDACs, along with histone acetyltransferases (HATs), facilitate an important mechanism of gene regulation that 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., p21) that are often silenced in cancer cells (20, 21). Pharmacologic HDAC inhibitors have demonstrated anticancer effects in breast cancer cells both in vitro and in vivo (22, 23). However, the adverse effects of these agents make them undesirable for long-term use in women with preinvasive disease, such as DCIS (24). Intake of cruciferous vegetables and dietary SFN are considered safe and have not been associated with any serious adverse side effects (25). Therefore, we aimed to evaluate the efficacy of consuming a broccoli sprout extract in altering HDAC activity and improving biomarkers for prognosis in women with benign disease or DCIS with or without a component of invasion.

Materials and Methods

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 + 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).

Study design
The study sample size flowchart is depicted in Fig. 1 following CONSORT guidelines (26). Consented subjects (N = 54)
were randomized to consume a minimum 2-week supply of either approximately 250 mg of a broccoli seed extract containing GFN (BroccoMax; n = 27), or matching placebos containing ingredients of microcrystalline cellulose (n = 27). Subjects were instructed to take 2 pills 3 times a day. BroccoMax supplements are reported to contain 30 mg GFN by manufacturers. SFN is rapidly cleared from the body, and multiple doses during the day may help maintain higher SFN plasma levels. Subjects always began supplementation following diagnostic biopsy and informed consent. For those 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 3 times a day. The total daily dose of GFN used in this study was 224 mg GFN (verified in-house), similar to the amount administered in our pilot study and other trials that achieved a significant increase in blood and urine ITC levels and reduced histone activity within one month with no reported adverse effects (25, 27, 28). All pills and containers were provided by Jarrow Formulas and 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 questionnaires, as well as two dietary history questionnaires: a modified NCI diet history questionnaire, and the Arizona cruciferous vegetable food frequency questionnaire (CVFFQ; ref. 29). 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 (≥ 80%) was determined by Research Pharmacy count of returned pills.

Sample collection

Two, nonfasting, 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. Blood was collected in BD lavender top tubes containing EDTA as the anticoagulant. Plasma was isolated by centrifugation, immediately acidified with 10% (v/v) precooled trifluoroacetic acid (TFA), and stored at −80°C. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma) separation, suspended in DMSO, and stored in liquid nitrogen until further analysis. Urine samples were acidified immediately with 10% (v/v) TFA and stored at −80°C. Diagnostic tissue specimens were formalin fixed, subjected to routine processing, and paraffin-embedded. All breast biopsy or surgical tissues were evaluated for the presence of DCIS and/or ADH or invasive ductal carcinoma (IDC) immediately after these procedures by board-certified pathologists.

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. and Toronto Research Chemicals (Canada), respectively. Deuterated SFN-NAC (SFN-NAC-D3) and SFN-cysteinylglycine (SFN-CG) were prepared in-house as previously described (30). GFN and glucotropaeolin (GTP) were purchased from The Royal Veterinary School of Denmark and AppliChem, respectively. All final standard dilutions were prepared in 0.1% (v/v) formic acid (FA) in H2O. Consistent and high (>80%) recoveries of ITC and glucosinolate standards from both biologic matrices and 0.1% (v/v) FA in water were confirmed through a series of spike and recovery experiments using available internal standards.

Analysis of GFN content within broccoli seed extract supplements

Each batch of BroccoMax supplements administered to subjects during the trial was analyzed in duplicate for GFN content. No significant batch-to-batch variation was detected. Our method was adapted from Tian and colleagues (31). BroccoMax powder (~450–480 mg) was dissolved into 100% methanol and homogenized 5 min with an Omni homogenizer (Omni International). Mixtures were centrifuged (5 minutes, 25°C, 10,000 g). Methanol extracts (3 times/extract) were performed using supernatants. 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) by centrifugation (3 minutes, 25°C, 10,000 g). Filtrates were diluted with 0.1% (v/v) FA in H2O to final concentrations of 250 μM/L GFN and stored at −20°C. 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 (32), except that analysis was performed in negative ion mode using a 4 μM Synergi Hydro-RP, 80 A, 150 x 20 mm reversed phase column (Phenomenex) with 0.2 μm guard column (Optimize Technologies, Inc.). The following precursor and product ions were used for detection: GFN (436 > 96/97), GTP (408 > 166). The final calculated GFN dose provided to participants was 224 mg GFN per day.

Isothiocyanate analysis in urine and plasma

Following protein precipitation by centrifugation (3,184 g, 2 minutes, 2°C), plasma and urine supernatants were filtered twice through Spin-X centrifuge tube filters (12,000 x g, 3.5 minutes, 2°C). Plasma filtrates were stored at −80°C. 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 (32), except SFN metabolites were detected with an Applied Biosystems MDS Sciex 4000 Q TRAP HPLC/MS-MS system.

Urinary creatinine analysis

Creatinine concentrations were determined using the Jaffe reaction method as previous described (33). SFN and SFN metabolite concentrations in urine were normalized using creatinine concentrations to control for differences in urine volume.
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 (19). Substrates and standards for the assay were custom synthesized by AAPPTEc, LLC. HDAC activity is expressed relative to PBMC protein content and negative control (DMSO).

Immunohistochemistry staining

Immunohistochemistry was performed on paraﬃn-embedded, breast biopsy samples preintervention and surgical samples postintervention as described by Elsheikh and colleagues (34). Briefly, slides of paraffin-embedded breast tissue specimens were deparaffinized in xylene (3 × 3 minutes), rehydrated with graded alcohols, washed 10 minutes in Tris-buffered saline (pH 7.2–7.6), heated 10 minutes in a Russell-Hobbs programmable pressure cooker in 0.01 mol/L citrate buffer (pH 6.0), treated 5 minutes with 3% aqueous H2O2 solution, blocked for 1 hour at 25°C in 3% goat serum, incubated for 1 hour at 25°C with primary antibodies for acetylated histone H3 lysine 9 (H3K9; 1:1,000) and 18 (H3K18; 1:2,000), p21 and Ki-67 (Abcam), and HDAC6 and HDAC3 (Santa Cruz Biotechnology, Inc.) followed by mouse Envision (Dako), counterstained for 1 minute with Gill hematoxylin, rinsed, dehydrated, and coverslipped using Permount. The primary outcomes examined include isothiocyanate levels, urinary and blood SFN metabolites, and PBMC HDAC activity within each treatment 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 postintervention). NSAIDs use was included as a covariate in all models due to the baseline difference between treatment groups. Length of intervention was also included in the mixed effect model. To adjust for multiple comparisons of the primary endpoints, we applied the method of false discovery rate (FDR; ref. 35). 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 χ2 tests or Fisher exact test, as appropriate. Tests of statistical signiﬁcance were conducted using two-sided tests, and a P ≤ 0.05 was considered statistically signiﬁcant unless otherwise noted.

Results

Patient characteristics and adverse events

From December 23, 2008, to March 27, 2013, a total of 54 participants ages 25 to 83 years (54 ± 12) were randomized into this trial at OHSU. The mean length of the intervention was 37 days (SD, 19 days). With regard to the number of women across the number of weeks, 34 (63%) of the women had intervention period between 2 and 8 weeks, 13 (24%) had intervention period < 2 weeks, and 7 (13%) had intervention period > 8 weeks. Supplementary Table S1 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 (Supplementary Table S2). In addition, no statistically signiﬁcant difference was observed in terms of compliance to the treatment plan between the two treatment groups (P = 0.88).

Urinary and blood biomarkers

Supplementary Table S3 presents all continuous outcomes of isothiocyanates from urine and plasma and HDAC activity in PBMCs. Pre- to postintervention 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 with the placebo group. In plasma, pre- to postintervention 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 postintervention PBMC HDAC activity levels within each treatment group. For the SFN group, the average change in HDAC activity from pre- to postintervention was a decrease of 80.39 pmol/min/mg protein (P = 0.11); for the placebo group, the average change from pre- to postintervention was an increase of

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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 posttreatments 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 postintervention). NSAIDs use was included as a covariate in all models due to the baseline difference between treatment groups. Length of intervention was also included in the mixed effect model. To adjust for multiple comparisons of the primary endpoints, we applied the method of false discovery rate (FDR; ref. 35). The FDR P values were provided in addition to the standard P values for the overall treatment comparisons.
In this analysis of 54 women who participated in this randomized, placebo-controlled trial, we found that 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 nonsignificant 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 and colleagues (38) 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 with untreated subjects. Similar to this report, we also did not observe changes in Ki-67 protein levels following SFN supplementation in benign tissue. It is possible that timing (predisease 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 (18, 28, 37–39). One study in healthy individuals reported that PBMC HDAC activity was lower after consuming BroccoMax supplements compared with a placebo, though changes from preintervention levels were not statistically significant (39). Another human study reported larger decreases in PBMC HDAC activity in healthy adults following consumption of broccoli sprouts (28). 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 and colleagues (38) reported decreased HDAC activity in multiple breast cancer cell lines, including the DCIS-like cell line, T-47D. Specific metabolites of SFN are thought responsible for SFN-associated effects on HDACs. One study using several cell lines showed that SFN-NAC and SFN-Cys had the greatest HDAC inhibition effects compared with SFN and other SFN metabolites (19). In this study, we observed a significant difference in changes in PBMC HDAC activity between intervention groups, suggesting that the
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Decreases observed may have been related to higher SFN exposure in the supplementation group. Because NSAIDs use was different between intervention groups, we compared changes in HDAC activity between groups among NSAIDs users and separately among NSAIDs nonusers. Among NSAIDs nonusers, mean fold change in HDAC activity was significantly different in supplement consumers compared with placebo consumers (Fig. 2). NSAIDs inhibit synthesis of prostaglandins, which could suppress regulatory protein expression via recruitment of HDACs (40). Increased recruitment of HDACs to chromatin may prevent inhibition associated with SFN consumption, which is one potential explanation for our observations. Future studies on SFN with larger sample sizes 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 (41). Clarke and colleagues (42) 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 decreases in total HDAC activity we observed. HDAC6 was not decreased with SFN supplementation. Rajendran and colleagues (41) 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 (43). Decreases in histone H3 or H4 acetylation (38). In this study, we did not observe increased H3K18ac or H3K9ac, despite decreases in HDAC3 expression and total HDAC activity. We did observe a significant decrease in H3K9ac in DCIS tissue among placebo consumers, which could be related to cancer progression (43). Though it cannot be determined from the current data, SFN supplementation may have mitigated decreases in histone acetylation.

p21 is a major cell-cycle regulator. Increased expression of p21 in breast cancer cells leads to cell-cycle arrest (46). SFN increased p21 expression in breast cancer cell lines (15), and regulation of p21 protein expression was 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 posttranslational modifications as reviewed recently (49). In addition, it is possible that regulation of p21 expression was altered in the tumors analyzed, altering responsiveness to changes in HDAC activity. Yet, 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 analyzed biomarkers from both breast tissues and PBMCs among pre- and postintervention samples. Second, we collected multiple types of breast tissue, including benign, DCIS and IDC, to examine potential effects of SFN in lesional and nonlesional 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 entire intervention period and approximately 30 days after participants' surgical or postintervention appointment. Study coordinators and subjects were both blinded to treatment assignment to minimize information bias. Study limitations include small sample size, limited tissue availability in some cases, and hospital-based study design. Importantly, most comparisons met the minimum sample size required to detect a biologically meaningful difference between intervention groups with ≥80% power.

In conclusion, this study provides evidence for chemopreventive activity of SFN in human breast tissue. We demonstrated effects of SFN on adverse events and changes to diet and medication use were well-tolerated, recent work indicated that other broccoli extract preparations may be more bioavailable and should be considered for use in future studies to enhance SFN absorption from these forms (33). Overall, this work provides important information for future larger population-based clinical trials investigating the impacts of consuming dietary sources of SFN.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P. Thuillier, E. Ho, J. Shannon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.L. Atwell, P.E. Farm, J.T. Vetto, A.M. Naik, K.Y. Oh, J. Shannon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.L. Atwell, Z. Zhang, M. Mori, J.T. Vetto, P. Thuillier, E. Ho, J. Shannon
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