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Oral Sulforaphane increases Phase II antioxidant enzymes in the human upper airway

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Abstract

Background—Cellular oxidative stress is an important factor in asthma and is thought to be the principle mechanism by which oxidant pollutants such as ozone and particulates mediate their pro-inflammatory effects. Endogenous Phase II enzymes abrogate oxidative stress through the scavenging of reactive oxygen species and metabolism of reactive chemicals.

Objective—We conducted a placebo-controlled dose escalation trial to investigate the *in vivo* effects of sulforaphane, a naturally occurring potent inducer of Phase II enzymes, on the expression of glutathione-s-transferase M1 (GSTM1), glutathione-s-transferase P1 (GSTP1), NADPH quinone oxidoreductase (NQO1), and hemoxygenase-1 (HO-1) in the upper airway of human subjects.

Methods—Study subjects consumed oral sulforaphane doses contained in a standardized broccoli sprout homogenate (BSH). RNA expression for selected Phase II enzymes was measured in nasal lavage cells by RT-PCR before and after sulforaphane dosing.

Results—All subjects tolerated oral sulforaphane dosing without significant adverse events. Increased Phase II enzyme expression in nasal lavage cells occurred in a dose-dependent manner with maximal enzyme induction observed at the highest dose of 200 grams broccoli sprouts prepared as BSH. Significant increases were seen in all sentinel Phase II enzymes RNA expression compared to baseline. Phase II enzyme induction was not seen with ingestion of non-sulforaphane containing alfalfa sprouts.

Conclusion—Oral sulforaphane safely and effectively induces mucosal Phase II enzyme expression in the upper airway of human subjects. This study demonstrates the potential of antioxidant Phase II enzymes induction in the human airway as a strategy to reduce the inflammatory effects of oxidative stress.

Clinical Implications—This study demonstrates the potential of enhancement of Phase II enzyme expression as a novel therapeutic strategy for oxidant induced airway disease.

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Capsule Summary—A placebo-controlled dose escalation trial demonstrated that naturally occurring sulforaphane from broccoli sprouts can induce a potent increase in antioxidant Phase II enzymes in airway cells.

Keywords

oxidative stress; antioxidants; asthma; allergic inflammation; sulforaphane; Phase II enzymes; air pollution

INTRODUCTION

Recent years have seen a growing appreciation of the role of oxidative stress in the pathogenesis of asthma and allergy.^{1–2} Certain allergens such as ragweed pollen containing NADPH oxidase can generate reactive oxygen species (ROS) which at least in some murine models have been shown to be critical in the induction of a robust inflammatory response.³ Additionally, ROS generation is thought to be the principal pathways by which certain pollutants such as diesel exhaust particles (DEP) and secondhand tobacco smoke exert their pro-inflammatory and adjuvant effects.^{4–5}

Phase II (PII) enzymes are an important cellular defense mechanism against oxidative stress.^{6–7} Several studies have demonstrated they have important protective effects against xenobiotics such as DEP, ozone, and tobacco smoke. PII enzymes neutralize reactive oxygen species and metabolize xenobiotics.^{8–10} Overexpression of PII enzymes will ablate DEP-induced pro-inflammatory cytokine production from airway epithelial cells.¹¹ Thus, PII enzyme induction may be a valuable interventional strategy to prevent the effects of oxidative stress in asthma, especially with regard to OS induced by certain pollens or oxidant pollutants. Sulforaphane (SFN), is a naturally occurring isothiocyanate found in cruciferous vegetables, and is richest in broccoli sprouts.¹² It is the most potent inducer of PII enzymes identified to date and is thought to act via activation of the Nrf2 transcription factor and the Anti-oxidant Response Element (ARE).^{13–14} These studies, however, have been limited to either in vitro or animal models. In these systems SFN abrogates the pro-inflammatory and pro-allergic effects typically observed with cellular DEP exposure.¹⁵ SFN has therefore been proposed as a novel therapeutic approach. However, the efficacy of oral SFN in elevating Phase II enzymes in airway cells of humans has yet to be determined.

Previous work employing broccoli sprout preparations administered to human subjects has delineated the basic pharmacokinetics and safety of oral SFN.^{16–19} Based on that preliminary data, we performed a placebo-controlled human study to investigate the safety, efficacy, and dose-effect of oral SFN for the induction on Phase II enzyme expression in upper airway cells.

METHODS

Subjects

Sixty-five human volunteers were recruited by advertisements and flyers posted at the University of California – Los Angeles (UCLA) campus and surrounding communities. Eligible subjects were clinically healthy nonsmokers ≥ 18 years of age. The use of inhaled, topical, or systemic corticosteroids or any other immune modulating medications was prohibited during the study period. At initial screening, a cheek scrape was performed to determine genotype for GSTM1 polymorphisms. Subjects were not selected or excluded based on genotypic status, however, subjects homozygous for *GSTM1 null* were excluded from analysis of GSTM1 expression. The Human Subject Protection Committee at UCLA approved all study activities.

Study Design

The study protocol schedule is summarized in Figure 1. Prior to starting the single-blind study, subjects were provided a list of sulforaphane-containing foods and instructed to avoid these foods during the study period. Baseline nasal lavage and blood samples were collected from each enrolled subject on day 1 to assess baseline Phase II enzyme expression. Subjects subsequently ingested a measured amount of broccoli sprout homogenate (BSH) once daily on days 1, 2, and 3. An escalating dose block design was employed to monitor for tolerability and toxicity associated with the treatment. A group of five subjects completed each dose level prior to enrolling subjects in the next dose level. New subjects were recruited for each dose block so that individuals completed only one dose regimen during the study, thus avoiding potential interference of previous BSH dosing on the measurement of Phase II enzyme expression. Following this design, doses of BSH prepared from 25, 50, 75, 100, 125, 150, 175, and 200 grams were completed. Once safety and tolerability were established, additional subjects were subsequently enrolled at doses of 125, 150, 175, and 200 grams to examine dose-response effect. Five subjects completed alfalfa sprout homogenate dosing at 200 grams as a control group. Alfalfa sprouts are similar in appearance and taste but do not contain significant amounts of sulforaphane glucosinolates. To assess Phase II enzyme expression following sprout homogenate doses, a repeat nasal lavage sample was collected on day 3 (2 hours after 3rd BSH dose). A blood sample was obtained on day 4 to evaluate serum SFN levels.

Nasal lavage and blood processing

Nasal lavage collection was performed in standard fashion as previously described.²⁰ After collection, cell pellets were isolated from nasal lavage samples, resuspended in 350µl lyses buffer, and stored at -80 degrees C until RT-PCR analysis. A blood sample was collected on day 4 to assess for serum SFN levels 24 ± 4 hours after the final BSH dose. Blood samples were collected, processed, and stored in a manner to avoid contamination with rubber materials known to interfere with the cyclocondensation assay. See additional Methods information in the Online Respository.

Broccoli sprout homogenate preparation

Sulforaphane was administered to subjects using broccoli sprout homogenate. Broccoli sprouts contain high concentrations of sulforaphane glucosinolates (SGS), the inert precursors of sulforaphane.²¹ Sulforaphane is released when SGS undergoes hydrolysis by myrosinase released from plant tissues during the chewing process. Based on procedures previously described by Talalay et al.,¹⁷ Broccosprouts® (Brassica Protection Products LLC, Baltimore) from a single production lot were used for the protocol. Broccosprouts® are a patented strain of broccoli sprout shown to contain > 6 µmol glucosinolates/gram by routine cyclocondensation and HPLC assays conducted by the supplier. BSH was prepared from a single lot of fresh BroccoSprouts® shipped directed from California Sprouts (Tracy, CA) a certified grower for Brassica Protection Products LLC (Baltimore, MD). The basic steps for BSH preparation are depicted in Figure 2. Sprouts were processed in a designated food product preparation area immediately upon arrival and prior to the expiration date printed on the packaging. Proper hand-washing procedures and vinyl gloves were used by staff to prevent contamination during processing. Sprouts were combined with sterile WFI water purchased from Mediatech Inc. (Herndon, VA) in 1:1.2, w: w proportions. This mixture was homogenized in 1 liter aliquots in a clean blender (600-watt Cuisinart Smartpower™ Series Blender Model CBT-500) to eliminate the variable of chewing by subjects. The aliquots of homogenate were pooled and mixed with daikon sprouts purchased fresh from Friends Fields, Inc. (Arvada, CO). This was accomplished by adding 2% daikon (compared to broccoli sprout mixture based on fresh weight) and homogenizing the preparation in the blender once again. The daikon/broccoli sprout mixture was incubated at 37 degrees C for 2 hours. This process added excess

myrosinase to maximally convert the free glucosinolates of the broccoli sprouts to sulforaphane, the biologically active compound. After incubation, the isothiocyanate mixture was aliquoted into 50 ml sterile Falcon tubes and stored at -20°C using dedicated freezer storage space. The broccoli sprout homogenate was thawed at 4°C 12–24 hours prior to dosing and was not administered if thawed for greater than 24 hours. Alfalfa sprout homogenate was prepared using the same procedures, substituting locally purchased alfalfa sprouts for BroccoSprouts[®].

Measurement of glucosinolate and isothiocyanate content

Analysis of BSH for glucosinolate (SGS) and isothiocyanate (ITC) content was performed by the Brassica Laboratory at Johns Hopkins University. Previous studies have demonstrated that the primary glucosinolate in broccoli sprouts is sulforaphane glucosinolate or glucoraphanin.¹⁹ Glucosinolate levels were determined using HILIC assay.²² Isothiocyanate content of BSH is determined by cyclocondensation assay.²³ Plasma sulforaphane levels were also determined using the cyclocondensation procedure described, though for plasma, the procedure was modified slightly.¹⁹ See additional Methods information in the Online Respository.

Measurement of Phase II enzyme gene expression

Gene expression was detected and measured by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using a method similar to that described by Wan et al.⁸ See additional Methods information in the Online Respository. For each individual, gene expression at baseline (pre-challenge) was considered the calibrator and was given an arbitrary figure of 1.0 and relative increase in gene expression following DEP challenge was calculated in reference to this value. 0.3–1.0 μg RNA was routinely extracted from a nasal lavage sampling. Typically 20 ng of RNA in a 25 μL reaction volume was used for each PCR and measurements were performed in triplicate.

Statistics

Statistical analysis was performed using Medrio (Oakland, CA) Clinical Study Software and SAS (Cary, NC) Statistical Software. Comparison of PII enzyme induction between BSH 200 g and placebo (Alfalfa 200 g) was performed using Wilcoxon rank sum test. We fit a linear regression model to approximate the BSH Dose-Response relationship. The slope of the linear regression model estimates the amount of increase in response by increasing one unit of dose, and the significance of the slope indicates significant dose dependent increase on the response.

RESULTS

Oral Sulforaphane is well tolerated and produced no serious adverse events

Fifty-seven subjects completed the study protocol. Subject demographics are reported in Table I. Eight subjects discontinued the protocol due to inability to keep the required visits, but none reported dose intolerance or side effects as a factor. No serious adverse events were reported. Five of sixty subjects reported mild events during BSH dosing of which 4 were gastrointestinal events. (Table II)

Oral Sulforaphane induces Nasal Phase II enzyme induction

Expression of PII enzymes significantly increased in NLF samples at BSH doses > 100 g daily. Table III shows the NLF data for individual PII enzymes. At BSH doses > 100 g daily, NQO1 consistently showed the greatest induction followed by HO-1. In contrast, no significant change in PII enzyme expression was observed for subjects completing the protocol with oral alfalfa sprouts. Significant differences in PII enzyme expression were evident for BSH 200 g dosing compared to placebo (alfalfa 200 g) by Wilcoxon Rank sum test ($p \leq 0.004$). A linear regression

model demonstrated the highly significant dose-response effect of BSH on mucosal PII enzyme induction with p-values less than or equal to 0.001 for all measured PII enzymes. A strong positive correlation was evident for the induction of individual Phase II enzymes as demonstrated in Figure 3 for GSTP1 and NQO1. This linear correlation was apparent for all of the measured PII enzymes, consistent with the common pathway of induction by SFN, i.e. Nrf-2 activation.

Analysis of glucosinolate and isothiocyanate levels

Analysis of BSH demonstrated undetectable levels of SGS, supporting effective and complete conversion of glucosinolates to isothiocyanates. Cyclocondensation assay demonstrated SFN content of 0.283 $\mu\text{mol/ml}$ BSH. As the preparation process yielded a mass (g fresh sprouts) to volume (ml BSH) conversion factor of 1.8 (i.e. 25 g sprouts = 45 ml BSH), the 175 mg and 200 mg BSH doses delivered 89 and 102 μmol of SFN per dose respectively.

Oral Sulforaphane is bioavailable as assessed by serum SFN levels

Serum trough SFN levels were measured in selected subjects. Baseline serum SFN levels by cyclocondensation assay were undetectable in all measured samples, but reached levels of 0.0115–0.0121 nmol at 24 hours after the final BSH dose.

DISCUSSION

This is the first clinical study to demonstrate that oral sulforaphane can enhance Phase II antioxidant enzyme expression in human airway cells. The induction of Phase II enzymes by SFN and other Nrf-2 activators has previously been shown using in vitro and animal models, 8, 11, 14, 24–25 however this is the first data to clearly demonstrate this biological effect of SFN in vivo in the human airway. Previous investigation has confirmed that Phase II enzyme mRNA expression as measured by RT-PCR correlates with protein and enzyme activity levels. This has been demonstrated with NQO1 protein expression by Western blot in human lymphocytes⁸ and GST enzyme activity correlation with GSTM1 mRNA expression in normal human bronchial epithelial cells.¹¹ This evidence supports the potential biologic significance of the marked Phase II enzyme mRNA induction now described with SFN administration in human subjects. The up regulation of endogenous Phase II enzymes was accomplished without apparent toxicity using the administration of oral BSH, a natural readily available source of SFN which was well-tolerated by study subjects. This finding may have important therapeutic implications with regard to asthma and other conditions associated with airway OS.

Cellular OS is believed to be an important underlying pathologic mechanism in the inflammation associated with asthma. Such OS may result from the cellular inflammation seen in asthma, but there is strong evidence that environmental respiratory exposures to air pollutants (i.e. diesel exhaust particles, ozone, NO₂) and pollen fragments cause OS in the airways which subsequently leads to tissue inflammation and clinical symptoms.^{3, 26–28} Cellular exposure to pollutant reactive chemicals or pollens generates reactive oxygen species (ROS) capable of oxidizing proteins, lipids, and DNA. This results in subsequent glutathione depletion and the activation of intrinsic cellular antioxidant defenses via Nrf2 signaling.²⁹ A primary mechanism of this pathway is the activation of the antioxidant response element (ARE) and induction of Phase II metabolizing enzymes.³⁰ This group of enzymes includes glutathione transferases (GSTM, GSTP), heme-oxygenase (HO1), and NAD(P)H:quinone oxidoreductase (NQO1) as well as other enzymes that serve to metabolize xenobiotic chemicals and neutralize ROS.^{31–32} If this cytoprotective antioxidant response is insufficient or overwhelmed by heavy oxidative burden, glutathione depletion continues resulting in MAPK and NF κ B signaling and gene transcription for a cascade of inflammatory cytokines such as IL-4, IL-5, IL-13, TNF- α as well as chemokines and cellular adhesion molecules.³³ In addition to these pro-

inflammatory effects of environmentally-induced OS, there is compelling evidence that OS also serves as a potent adjuvant to enhance Th2-responses to allergens and may initiate immune sensitization to common environmental allergens.³⁴⁻³⁶

The recognized importance of OS in respiratory inflammation has spurred recent interest in therapeutic measures to prevent or reduce OS. At present, the effects of current allergy and asthma therapy on OS are unclear.³⁷⁻⁴¹ As current standard treatment may not adequately address respiratory OS burden induced by environmental exposures, additional therapies protecting against OS may represent a significant step in reducing the morbidity of allergic respiratory disease.

Our current investigation explores a unique strategy to enhance an important endogenous cytoprotective response designed to prevent cellular damage from ROS. The Nrf-2 signaling pathway is a negative regulator of inflammation through induction of many antioxidant, cytoprotective, and detoxification enzymes.⁴² These inducible Phase II enzymes represent an early and sensitive response to OS by scavenging ROS and metabolizing xenobiotics such as air pollutants. SFN, as used in our investigation, has a number of attractive qualities with regard to human use. Glucosinolate precursors of SFN are naturally found in cruciferous vegetables with previous studies demonstrating bioavailability and basic pharmacokinetics with human consumption. A number of human studies have demonstrated the safety and tolerability of glucosinolates and SFN, findings further confirmed by our recent study data.¹⁶⁻¹⁹ In vitro, animal, and human studies have supported the beneficial biological effects of SFN, with Phase II enzyme induction believed to be an important mechanism of action for these observed effects.⁴³⁻⁴⁶ With regard to respiratory inflammation, recent in vitro investigations have shown Phase II enzyme induction with SFN to be effective in blocking the pro-allergic effects of DEP on human B-cells as DEP-induced IgE enhancement is inhibited by preculture with SFN.⁸ Additional studies using human BEC confirm this strategy to be protective against DEP extract oxidative effects.¹¹ SFN effectively upregulates Phase II enzyme expression and blocks DEP extract induced IL-8, GM-CSF, and IL-1 β production by human BEC. Thus, while the protective antioxidant effects of PII enzyme induction have yet to be established in human clinical studies, compelling data exists to support the potent anti-inflammatory effects of this strategy in the setting of DEP-induced OS.

Our placebo-controlled study is the first to examine the in vivo effects of SFN administration on PII enzyme expression in the human airway. At BSH doses > 100 grams (51 μ mol SFN) daily, we observed a significant dose-response effect in Phase II enzyme expression as measured by RT-PCR using cells recovered from nasal lavage. Our NL collection yields predominantly respiratory epithelial cells making such findings congruent with SFN-mediated PII enzyme induction seen in previous studies using human BEC. We did not observe SFN-induced changes in NL cell counts or differentials, yet the increase in mean expression of PII enzymes ranged from 101% for GSTP1 to 199% for NQO1 at the highest dose of BSH administration. This represents a doubling to tripling of baseline enzyme expression rates. Also significant is the observation that measured increases in individual sentinel PII enzymes were strongly associated with increases in all other PII enzymes. This positive linear-correlation is consistent with our current understanding of a common mechanism of induction for these enzymes, i.e. Nrf-2 signaling with resultant ARE activity promoting transcription of numerous PII enzymes.

While our study was not designed to examine the pharmacokinetics (pK) of oral SFN, the results appear to confirm previously described human pK results. SFN has an apparent volume of distribution of 59.9 ± 7.0 L, consistent with total body water, a mean half-life of 1.77 ± 0.13 hours, and first-order kinetics.¹⁹ Previous dosing of 200 μ mol SFN has yielded peak plasma levels of 2.00 ± 0.30 μ mol/l, suggesting 60% bioavailability.¹⁷ Our results are consistent with

these previous observations. Using the aforementioned pK parameters, the 200 gram BSH dose (102 μmol SFN) used in our study would be expected to give a peak serum concentration of 1.02 $\mu\text{mol/l}$. Expected serum concentration 24 hours later (14 $t_{1/2}$) would be 0.0622 nmol/l. For samples collected nearer the 28 hour mark, the expected concentration is 0.0155 nmol/l. Our serum samples analyzed for SFN content 24 ± 4 hours after the 200 gram BSH dose are within this range (0.0115–0.0121 nmol).

This study demonstrating the potent biological effects of oral SFN on PII enzyme expression in the human upper airway provides vital information for planning additional clinical trials. Future human studies will be necessary to thoroughly investigate the potential beneficial effects of Phase II enzyme induction on environmentally-induced oxidative stress and associated allergic airway inflammation. Currently, it is unknown whether the observed increase in PII enzyme expression will be sufficient to prevent or reduce respiratory OS in the human airway. Also unknown is whether higher doses of SFN will lead to further increases in PII enzyme expression or whether toxicity will be dose-limiting. Our experience, and those of other investigators, is that the currently reported doses are well-tolerated and non-toxic. Additionally, genetic polymorphisms affecting PII enzymes will be an important consideration in future studies examining this therapeutic strategy. We excluded GSTM1 null subjects from our analysis so as to have a clear picture of the induction effects of SFN on PII enzymes. However, with regard to the clinical value of SFN, the presence or absence of specific functional enzymes may be quite important in the resulting therapeutic or nominal effect. Given the complex network of PII antioxidant enzymes, gene-gene-environment interactions are likely to have a strong influence on the observed responses to specific antioxidant therapies.^{47–48} Careful selection of susceptible target populations based on genetic polymorphisms will be an important consideration for future interventional studies aimed at reducing pollutant-induced oxidative stress.

In summary, our placebo-controlled human study has demonstrated that oral SFN contained in BSH can significantly induce PII enzyme expression in the human airway. This data allows for future clinical studies to examine the potential benefit of SFN in abrogating allergic respiratory inflammation from oxidant stimuli and demonstrates proof-of-concept for Nrf2-activation as a mechanism of PII enzyme upregulation in the human respiratory tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARE	antioxidant response element
BEC	bronchial epithelial cells
BSH	broccoli sprout homogenate

DEP	diesel exhaust particles
GSTM1	glutathione-s-transferase M1
GSTP1	glutathione-s-transferase P1
HO1	hemeoxygenase-1
ITC	isothiocyanate
MAPK	mitogen activated protein kinase
NF-κB	nuclear factor kappa B
NLF	nasal lavage fluid
NQO1	NADPH quinone oxidoreductase 1
Nrf-2	Nuclear erythroid 2 p45-related factor 2
OS	oxidative stress
PII	phase II
ROS	reactive oxygen species
RT-PCR	real-time quantitative reverse transcriptase-polymerase chain reaction
SFN	sulforaphane
SGS	sulforaphane gluconsinolates
WFI	water for injection

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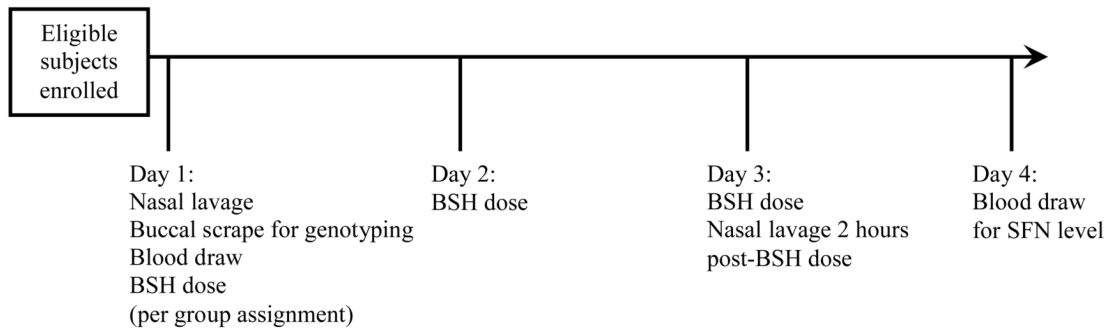


Figure 1. Protocol schedule for oral broccoli sprout homogenate dosing study

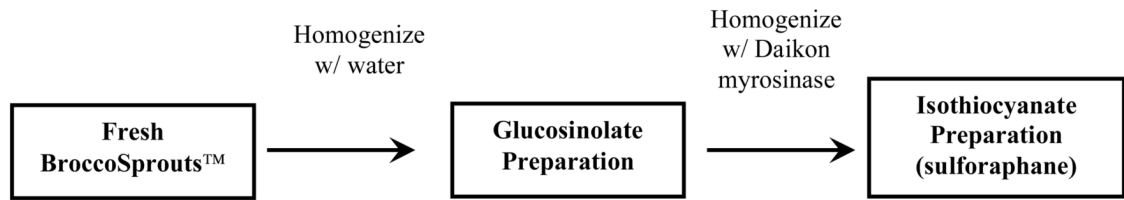


Figure 2. Steps in Broccoli Sprout Homogenate (BSH) preparation

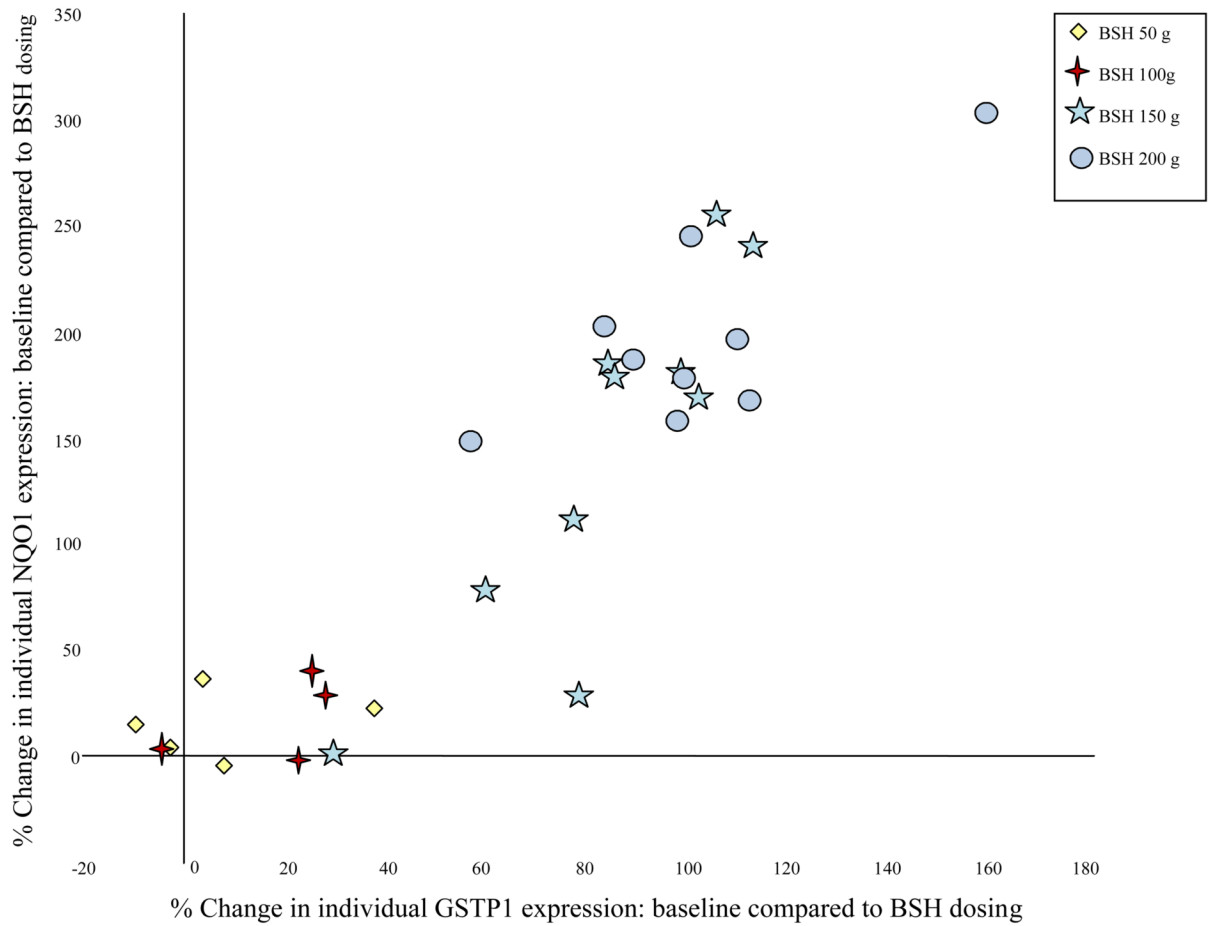


Figure 3. Induction of individual Phase II enzymes by oral sulforaphane is strongly correlated
Expression of *GSTP1* vs. *NQO1* in nasal lavage cells following consumption of oral sulforaphane in each of the subjects.

Table 1

Demographics of Subjects Completing Protocol

Dose (g)	25 BSH	50 BSH	75 BSH	100 BSH	125 BSH	150 BSH	175 BSH	200 BSH	200 Alfa
Age (years), mean \pm SD	34.0 \pm 5.0	35.3 \pm 10.3	33.3 \pm 4.0	28.0 \pm 1.7	33.0 \pm 7.1	41.8 \pm 16.7	36.4 \pm 12.4	32.2 \pm 10.4	31.6 \pm 8.0
Gender (F/M)	4/1	4/1	2/2	2/2	4/4	8/2	7/0	3/6	2/3
Number of Subjects Discontinued	0	0	1	1	2	0	3	1	0

Table II

Safety and Tolerability of Oral Sulforaphane*

Dose	Description	Medication Required	Duration
150 grams BSH	Large Bowel Movement	No	1 day
175 grams BSH	Nasal Congestion	No	1 day
200 grams BSH	Soft Stool	No	1 day
200 grams BSH	Increased Urination	No	3 days
200 grams BSH	Flatulence	Yes	3 days

* Events reported occurring during the Clinical Study

Table III

Group Mean Percent Change Mucosal Phase II Enzyme Expression* Compared to Baseline with Oral BSH

BSH Dose	GSTM1	GSTP1	HO-1	NQO1
25 g	-5.60 ± 6.29	1.53 ± 7.89	4.43 ± 7.18	-2.17 ± 4.97
100 g	5.78 ± 12.21	14.10 ± 15.07	15.32 ± 17.95	13.86 ± 19.43
125 g	30.53 ± 48.80	37.47 ± 27.97	37.80 ± 34.21	53.32 ± 41.86
150 g	74.31 ± 69.83	82.83 ± 24.81	105.06 ± 33.49	142.19 ± 86.30
175 g	89.24 ± 63.88	86.19 ± 28.30	106.56 ± 27.28	160.19 ± 65.67
200 g	118.81 ± 39.68	100.80 ± 27.33	120.86 ± 42.66	198.81 ± 48.36
Placebo (Alfalfa 200 g)	28.71 ± 34.01	11.00 ± 48.43	1.14 ± 18.40	1.69 ± 41.21
p-value for BSH 200 g vs. Placebo comparison (Wilcoxon Rank sum test)	0.002	0.004	0.001	0.001
p-value for Slope of BSH Dose-Response linear	<0.001	<0.001	<0.001	0.001

* Gene mRNA expression ± standard deviation relative to pre-treatment baseline, results for GSTM1 for GSTM1+ subjects only.