

Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast

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Consumers of higher levels of *Brassica* vegetables, particularly those of the genus *Brassica* (broccoli, Brussels sprouts and cabbage), reduce their susceptibility to cancer at a variety of organ sites. *Brassica* vegetables contain high concentrations of glucosinolates that can be hydrolyzed by the plant enzyme, myrosinase, or intestinal microflora to isothiocyanates, potent inducers of cytoprotective enzymes and inhibitors of carcinogenesis. Oral administration of either the isothiocyanate, sulforaphane, or its glucosinolate precursor, glucoraphanin, inhibits mammary carcinogenesis in rats treated with 7,12-dimethylbenz[*a*]anthracene. In this study, we sought to determine whether sulforaphane exerts a direct chemopreventive action on animal and human mammary tissue. The pharmacokinetics and pharmacodynamics of a single 150 μ mol oral dose of sulforaphane were evaluated in the rat mammary gland. We detected sulforaphane metabolites at concentrations known to alter gene expression in cell culture. Elevated cytoprotective NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1) gene transcripts were measured using quantitative real-time polymerase chain reaction. An observed 3-fold increase in NQO1 enzymatic activity, as well as 4-fold elevated immunostaining of HO-1 in rat mammary epithelium, provides strong evidence of a pronounced pharmacodynamic action of sulforaphane. In a subsequent pilot study, eight healthy women undergoing reduction mammoplasty were given a single dose of a broccoli sprout preparation containing 200 μ mol of sulforaphane. Following oral dosing, sulforaphane metabolites were readily measurable in human breast tissue enriched for epithelial cells. These findings provide a strong rationale for evaluating the protective effects of a broccoli sprout preparation in clinical trials of women at risk for breast cancer.

Introduction

Cancer chemoprevention entails the use of natural or synthetic agents to retard, block or reverse any of the multiple stages of the carcinogenic process (1–3). It is probable that a combination of approaches will be required to reduce the risk of the major types of human cancers. Current chemopreventive strategies have been directed at individuals in high-risk groups, and may not be suitable for those at moderate or lower risk. This has been observed in the case of breast cancer chemoprevention where the use of tamoxifen, a selective estrogen receptor modulator to prevent hormone receptor-positive breast cancer, has been limited even in women at high risk for breast cancer due to the perception of an unfavorable side-effect profile. As

Abbreviations: BPP, Brassica Protection Products LLC; DTC, dithiocarbamate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; NRF2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase; PCR, polymerase chain reaction.

breast cancer remains a global public health challenge affecting one in eight North American and Northern European women, with an increasing incidence in developing countries, the need for non-toxic and inexpensive preventive agents that have broad application is great.

In addition to inherited genetic alterations and estrogen burden, environmental exposures are believed to play a significant role in the development of breast cancer through the cumulative accumulation of mutagenic events over a woman's lifetime (4). Extent of damage to DNA reflects a molecular equilibrium between activating and detoxifying reactions as well as DNA repair capacity (5,6). This balance can be altered by many factors including drugs and phytochemicals (7). Environmental carcinogens are often first metabolically activated via phase 1 enzymes (principally cytochrome P450) into reactive intermediates (8). Fortunately, at the cellular level, there exist competing phase 2 enzymes [e.g. glutathione S-transferases and NAD(P)H:quinone oxidoreductase (NQO1)] which facilitate the elimination of the reactive forms of carcinogens through biotransformation reactions including sulfation, acetylation, quinone reduction and glutathione conjugation (9). As a consequence, a promising strategy for cancer chemoprevention involves the use of agents to favorably shift the balance between phase 1 and 2 enzymes (3,5,10).

Such a favorable enzymatic induction strategy, in which cytoprotective enzymes are selectively elevated, has been demonstrated using *Brassica* vegetables that contain compounds known as glucosinolates (β -thioglucoside *N*-hydroxysulfates) (11). Glucosinolates are hydrolyzed to isothiocyanates by myrosinase (β -thioglucoside glucohydrolase), an enzyme released in the plant upon damage, such as by chewing (12). Sulforaphane, one such isothiocyanate whose precursor is abundant in 3-day-old broccoli sprouts, triggers the induction of many cytoprotective enzymes including NQO1 and heme oxygenase-1 (HO-1). NQO1 protects cells from oxidative damage by two-electron reduction of quinones, such as those derived from estrogens, thus suppressing oxidative cycling and reactive oxygen species generation (13). HO-1, an important enzyme in heme catabolism, leads to the production of biliverdin, which upon reduction forms the reactive oxygen scavenger bilirubin (14). Much of sulforaphane's cytoprotective enzyme induction is thought to occur via the actions of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) (15). Importantly, when sulforaphane or its precursor, glucoraphanin, was given to female rats treated with the known mammary gland carcinogen, 7,12-dimethylbenz[*a*]anthracene, the number, size and rate of development of mammary tumors were significantly reduced (16,17). Additional preclinical studies revealed the capacity of sulforaphane to diminish DNA adduct formation in normal mammary epithelial cells, favorably adjust cellular redox states, and inhibit mitosis and proliferation in mammary cancer cell lines (18–22). These laboratory findings are supported by some epidemiological data that suggest a protective association between increased intake of broccoli or Brussels sprouts and breast cancer (23–25).

The identification of biomarkers for efficacy and breast cancer risk is necessary to advance diet-derived chemopreventive agents in both animal and human studies (26). Several clinical studies using 3-day-old broccoli sprouts have already established their safety, described plasma pharmacokinetics, validated methods to monitor compliance and, most importantly, led to the development of standardized preparations with defined concentrations of sulforaphane or its precursor, glucoraphanin (27–31). We sought to establish whether orally administered sulforaphane reaches the mammary gland and increases the functional capacity of antioxidative and detoxification enzymes in this tissue.

Materials and methods

Animals and sulforaphane treatments

Female Sprague–Dawley rats (10 weeks of age, 208–215 g) were purchased from Harlan (Indianapolis, IN) and maintained on an AIN-76A diet without

ethoxyquin (Harlan Teklad, Madison, WI). Sprague–Dawley rats (10 weeks old) were used in this study, as the mammary glands at this age are quite mature, containing the highest known levels of the more differentiated alveolar buds (32,33). After 3 days of acclimatization, six rats were killed as controls whereas the remaining animals were gavaged with 150 μmol *R,S*-sulforaphane (LKT Laboratories, St Paul, MN) in 400 μl corn oil (Sigma–Aldrich, St Louis, MO). This dose of sulforaphane has been shown previously to inhibit the number, size and rate of development of mammary tumors in carcinogen-treated rats (16). At 0.5, 1, 2, 4, 8, 12, 24 and 48 h after treatment, three animals were killed (Figure 1A). From each rat, 4–5 ml of blood was obtained by cardiac puncture and transferred to a 15 ml conical polypropylene centrifuge tube containing disodium ethylenediaminetetraacetic acid for plasma sulforaphane determination. Mammary gland tissues were removed, immediately placed in liquid nitrogen and stored at -80°C for measurement of tissue sulforaphane levels and NQO1 enzymatic activity. Additional tissue was subsequently placed in RNAlater (Ambion, Austin, TX) for gene expression analyses. Three additional animals were killed at 12, 24 and 48 h and mammary gland tissue was removed and placed in buffered 10% formalin for immunohistochemical assays. All experiments were conducted in accordance with the standards established by the USA Animal Welfare Acts, set forth in National Institutes of Health guidelines and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee.

Quantitative measurements of dithiocarbamates in urine and plasma

Measurement of sulforaphane distribution via dithiocarbamate (DTC) metabolites in biofluids was carried out using an established, well-validated cyclocondensation reaction (29).

Pharmacokinetic analysis

The measured concentrations of DTC metabolites in the rat plasma were fit into a non-compartmental extravascular model (NCA Model 200) using WinNonlin software (Pharsight Corporation, Mountain View, CA). The area under the curve (AUC), maximum concentration (C_{max}), elimination rate constant (K_{el}) and elimination half-life ($0.693/K_{\text{el}}$) were obtained.

Tissue DTC analysis

The cyclocondensation reaction used to measure plasma DTCs was modified slightly for tissue analysis. Rat tissue (100 mg) was homogenized in 1 ml of 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4) with a Duall grinder (Kontes Glass Co., Vineland, NJ) at 4°C for 2 min. Up to 0.6 ml of the crude homogenate was added directly to the cyclocondensation reaction mixtures and incubated as described previously (29). After incubation, the reaction mixture was centrifuged at low speed to remove insoluble materials. A 2 ml portion of the supernatant fraction was mixed with 2 ml water and loaded on a 50 mg Sep-Pak C_{18} Cartridge (Waters Corp., Milford, MA). The cartridge was then washed twice with 1 ml portions of 25% acetonitrile in water and subsequently eluted with 0.3 ml acetonitrile. The eluate was mixed with 0.3 ml water, and a 200 μl aliquot of the mixture was automatically injected (Waters Autosampler, Model 717 Plus) onto a reverse phase high performance liquid chromatography (HPLC) column (Partisil 10 ODS-2, 4.6×250 mm, Whatman, Clifton, NJ). The cyclocondensation product (1,3-benzodithiole-2-thione) was detected by a Waters Model 996 photodiode array detector at 365 nm. The DTC level of the tissue was adjusted to tissue weight.

Gene expression analysis

Total RNA was isolated using the RiboPure™ kit (Ambion, Austin, TX) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). TaqMan polymerase chain reaction (PCR) assays were performed in triplicate on cDNA samples in 96-well optical plates on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System for either *NQO1* or *HO-1*. For each 20 μl TaqMan reaction, 2 μl cDNA (10 ng) was mixed with 1 μl 20 \times TaqMan Gene Expression Assay Mix (Applied Biosystems, Foster City, CA), 10 μl 2 \times iQ Supermix (Bio-Rad Laboratories) and 7 μl nuclease-free water (Promega Corporation, Madison, WI). Parallel assays for each sample were performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. The reaction was carried out using the following parameters: 50°C for 2 min, 95°C for 5 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were prepared for each target using serial dilutions of a single sample. All TaqMan PCR data were captured using the MyiQ Optical System Software (Bio-Rad Laboratories). Relative quantification of gene expression changes in the rat was determined using the $2^{-\Delta\Delta C_t}$ method and an induction ratio was ultimately obtained by normalizing the treated animals to the controls (34).

NQO1 enzyme activity analysis

Rat tissue (100 mg) was homogenized in 1 ml of 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4) with a Duall grinder (Kontes Glass Co.) at 4°C for 2 min.

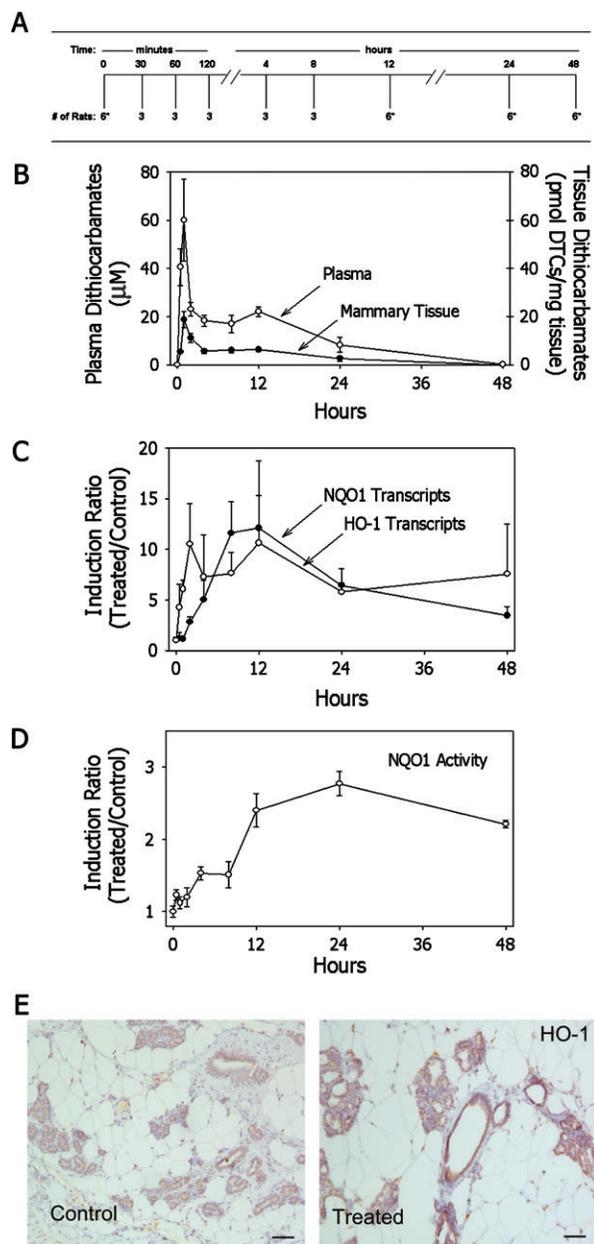


Fig. 1. (A) Rat experiment design. Plasma and mammary glands were removed from three female Sprague–Dawley rats per time point following 150 μmol *R,S*-sulforaphane gavage at time 0. At select time points (*), mammary glands were removed from three additional animals for slide preparation. (B) Pharmacokinetics of sulforaphane in rat plasma and mammary gland. Shown are concentrations of DTCs (sulforaphane metabolites) versus time following a gavage of a single dose of 150 μmol *R,S*-sulforaphane in female 10-week-old Sprague–Dawley rats. The DTC level in the mammary gland was adjusted for tissue weight. Every time point between 0 and 48 h is significantly different from control animals by *t*-tests ($P < 0.05$). Bars represent SEM ($n = 3$). (C) Quantitative real-time PCR based measurements of rat mammary gland *NQO1* and *HO-1* transcripts. Relative quantification of gene expression changes was determined using the $2^{-\Delta\Delta C_t}$ method (34). Induction ratios are the ratios of treated to the control values. All time points > 2 h (*NQO1*) or > 1 h (*HO-1*) are significantly different from controls by *t*-tests ($P < 0.05$). Bars represent SEM ($n = 3$). (D) NQO1-specific enzymatic activity induction in the mammary gland (43,44) adjusted for protein concentration determined by the bicinchoninic acid assay (38). Activities from treated animals were normalized to controls to obtain an induction ratio. Time points > 4 h are significantly different from controls as determined by *t*-tests ($P < 0.05$). Bars represent SEM ($n = 3$). (E) HO-1 immunostaining in the rat mammary gland after treatment with sulforaphane. Control (0 h) and treated (24 h). Original magnification, $\times 100$. Scale bar = 50 μm .

The homogenates were centrifuged at 14 000 r.p.m. for 20 min at 4°C. The NQO1 activity in the supernatant fractions was determined in a 250 µl assay in 96-well microtiter plates (35–37); 10, 20 and 40 µl of supernatants were added to the well and brought up to 50 µl with 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4). In order to assess non-specific enzymatic activity, 50 µl of 0.3 mM dicumarol (NQO1 inhibitor) was added to parallel wells. To each well 200 µl assay solution containing 25 mM Tris–HCl pH 7.4, 0.5% bovine serum albumin, 0.025% Tween 20, 5 µM flavin adenine dinucleotide, 30 µM nicotinamide adenine dinucleotide phosphate, 1 µM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase, 0.3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 25 µM menadione was added. The reaction rates were immediately measured at 610 nm for 5 min at room temperature by the microtiter plate reader. For each sample, the specific activity of NQO1 was derived by subtracting the initial reaction rates in wells containing no dicumarol from those in wells containing dicumarol. The protein level of the supernatant was measured by using the bicinchoninic acid assay (38). Activities from treated animals were normalized to controls to obtain an induction ratio.

Immunohistochemical staining and scoring

Staining for HO-1 was carried out using the DakoCytomation EnVision+ System (DakoCytomation, Carpinteria, CA). Briefly, deparaffinized slides were dehydrated and steamed, and endogenous peroxidase activity was quenched by incubation with Dako's dual endogenous enzyme block. Slides were washed and incubated with Stressgen's (Stressgen Bioreagents, Victoria, BC Canada) rabbit anti-HO-1 polyclonal antibody (1:2000 dilution) followed by a secondary anti-rabbit antibody-coated polymer peroxidase complex. Substrate/chromogen (3,3'-diaminobenzidine) was applied and slides were counterstained with Gill-2 hematoxylin. Slides were scanned and six regions per slide were quantitatively scored using the ACIS II Automated Cellular Imaging System (Clariant, Aliso Viejo, CA) as described previously (39). Negative controls utilizing rabbit serum and secondary antibody alone revealed no immunostaining in any tissues examined.

Human pilot study design

Eight women with no prior history of cancer, recent antibiotic, aspirin, non-steroidal anti-inflammatory drugs (NSAID) or multivitamin use or with smoking history, who were scheduled to undergo elective reduction mammoplasty, were enrolled in a pilot clinical study. They had to complete a baseline dietary and risk factor questionnaire, be on a cruciferous-free diet for 3 days prior to surgery, complete a diet checklist of foods to avoid each day and collect their urine for the 12 h period prior to surgery. An hour before surgery, 10 ml of blood was drawn and the women were asked to drink 20 ml of a filtered broccoli sprout preparation containing sulforaphane. For this preparation, 3-day-old broccoli sprouts were grown by Sprouter Northwest (Kent, WA), extracted with hot water, treated with daikon and freeze dried under good manufacturing practice guidelines to produce a powder containing ~200 µmol of sulforaphane/g (Oregon Freeze Dry, Albany, OR). This powder was then stored in a freezer at –80°C. The amount of sulforaphane was confirmed prior to the preparation of each dose by a cyclocondensation assay (29). Blood and urine were also collected intra-operatively at the time when breast tissue was removed from the first breast. Breast tissue removed from both breasts was accessioned and reviewed in the pathology department before macroscopically enriched epithelial/stromal tissue was collected. The tissue was then placed in liquid nitrogen for measurement of sulforaphane distribution and NQO1 enzymatic activity or in RNAlater for gene expression analyses using methods described above. For tissue DTC levels, 80 mg of epithelial-enriched tissue from each breast was used. The clinical study was approved by the Johns Hopkins Bloomberg School of Public Health institutional review board and written informed consent was obtained from each subject.

Statistical analysis

Means and standard errors were calculated for DTC levels in plasma, urine and tissue in both the rat and human samples. *t*-tests were done to identify the minimal time to statistically significant induction and time of maximal induction for all transcript and enzymatic activity data. Analysis of variance was performed to assess significance of immunohistochemical scoring.

Results

Sulforaphane metabolite distribution in the rat mammary gland

In the rat mammary gland, DTC concentrations peaked at 18.8 pmol/mg tissue 1 h after oral administration of 150 µmol sulforaphane (Figure 1B). Similarly, DTC concentrations in the plasma peaked at 60 µM 1 h after dosing, declined rapidly and exhibited a minor second peak of 22 µM at 12 h (Figure 1B). The basis for this second peak is unclear but may relate to accumulation of glutathione conjugates

(40,41). Pharmacokinetic parameters were generated using WinNonlin by fitting the plasma data to a non-compartmental extravascular input model (NCA Model 200) yielding an AUC of 491 h µmol/l, C_{max} of 60 µM, K_{el} of 0.1 h⁻¹ and an elimination half-life of 6.7 h. The peak plasma concentration was 3-fold higher than that reported by Hu *et al.* (42) following oral administration of 50 µmol sulforaphane to rats. These investigators also reported a shorter elimination half-life (2.2 h) perhaps reflecting measures of sulforaphane itself, rather than all of its biotransformation products (DTCs).

Induction of cytoprotective gene transcripts in the rat mammary gland

Quantitative real-time PCR assays were used to measure levels of *NQO1* and *HO-1* transcripts to determine the extent of the pharmacodynamic action of sulforaphane in the rat mammary gland. A maximal 12-fold induction of *NQO1* transcripts was observed in the mammary gland 12 h after dosing and there was significant induction as early as 2 h ($P = 0.0001$) (Figure 1C). A biphasic pattern of *HO-1* transcript induction was observed, with an initial peak at 2 h followed by a subsequent peak at 12 h (Figure 1C). The minimal time to statistically significant *HO-1* induction was 1 h ($P = 0.0001$). Transcript levels of *GAPDH* did not significantly change in any of the tissues and therefore served as an appropriate control.

Induction of NQO1 enzymatic activity in the rat mammary gland

A microtiter plate assay was used to determine whether a single dose of sulforaphane leads to measurable induction of NQO1 enzymatic activity in the rat mammary gland (43,44). A maximal 2.8-fold induction of NQO1 enzymatic activity in the mammary gland was observed 24 h ($P = 0.0001$) after dosing with minimal time to statistically significant induction at 4 h ($P = 0.0001$) (Figure 1D).

Localization of increased HO-1 immunostaining to rat mammary epithelial cells

Immunostaining assays for HO-1 were used to localize the induction of cytoprotective enzymes. Minimal HO-1 immunostaining was noted throughout the control samples. Strong immunostaining for HO-1 was observed in the mammary gland 24 h after a single sulforaphane dose with considerable staining evident in mammary ductal and glandular epithelial cells (Figure 1E). Using the ACIS II Automated Cellular Imaging System the slides were scanned and six regions per slides were scored with 4-fold increased HO-1 staining confirmed in the treated mammary glands compared with controls [mean (SD) ACIS percentage × intensity score, 8640 (3662) versus 2168 (1121), $P = 0.002$].

Sulforaphane metabolite distribution in human breast tissue

To determine whether sulforaphane is bioavailable in human breast tissues, eight women undergoing reduction mammoplasty were enrolled in a proof of principle pilot study (Figure 2). All eight women were African American. Their average age was 30 years (21–50) and seven were pre-menopausal. Their mean pre-operative weight was 99 kg (68.3–158.8). They all consumed a broccoli sprout preparation containing 200 µmol of sulforaphane on average 50 min (27–75) prior to the start of surgery. As expected, the broccoli sprout preparation was well tolerated. This dose and schedule were chosen because previous work demonstrated a peak plasma concentration of 2.0 µM DTCs at 1 h after ingestion (29). The average time between the dose and removal of the right and left breast tissue was similar, 93 ± 17 and 103 ± 33 min, respectively. The mean epithelial/stromal-enriched breast tissue DTC concentration was 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue for the right and the left breast, respectively (Table I). The mean pre-dose urinary DTC concentration was 4.07 ± 5.22 µM with a level of 158.85 ± 93.89 µM obtained at the time of tissue removal, consistent with isothiocyanate ingestion. The mean pre-dose plasma DTC concentration was negligible (0.01 ± 0.02 µM), with a post-dose level of 0.92 ± 0.72 µM.

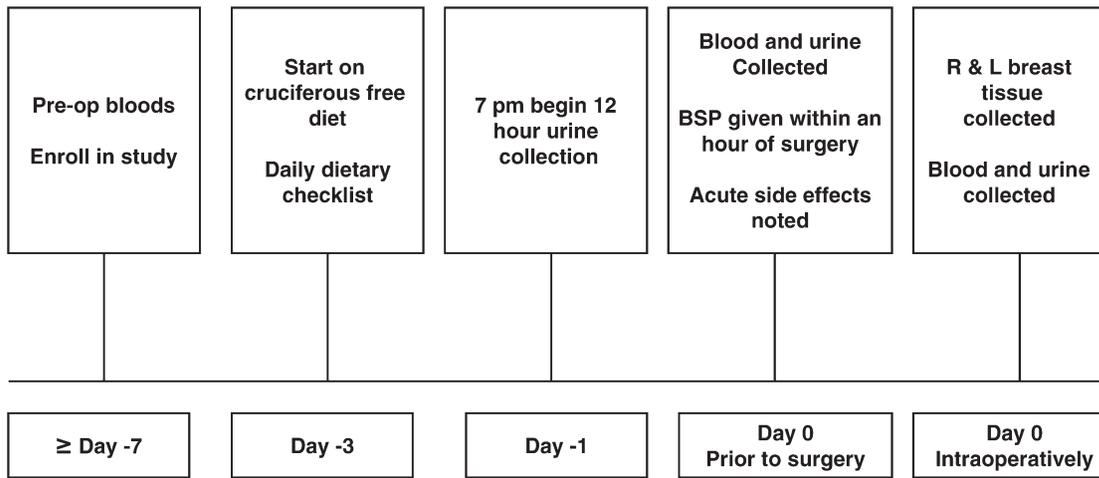


Fig. 2. Human trial design. Single-arm non-randomized clinical trial in eight healthy women undergoing reduction mammoplasty. Women were given a broccoli sprout preparation containing 200 μmol sulforaphane 1 h prior to surgery.

Measurement of cytoprotective gene transcripts and NQO1 enzymatic activity in human breast tissue

It was not possible to evaluate pharmacodynamic effect in breast tissue because of the short interval between ingestion of the broccoli sprouts preparation and breast resection. Nonetheless, it was possible to detect *NQO1* and *HO-1* transcripts and *NQO1* enzymatic activity in the mammary tissue of all subjects. For *NQO1*, a mean ΔC_t (*NQO1*–*GAPDH*) of 4.50 ± 0.70 in the right breast and 4.23 ± 0.63 in the left breast was determined. For *HO-1*, a mean ΔC_t (*HO-1*–*GAPDH*) of 6.30 ± 0.41 in the right breast and 6.26 ± 0.34 in the left breast was determined. The transcript levels were highly correlated (correlation coefficient = 0.78) between breasts for both *NQO1* and *HO-1*. Mean *NQO1* enzymatic activity was determined with levels of 0.21 ± 0.1 mOD/ $\mu\text{g}/\text{min}$ in the right breast and 0.20 ± 0.1 mOD/ $\mu\text{g}/\text{min}$ in the left breast.

Discussion

This study was carried out to investigate the pharmacokinetics and pharmacodynamics of sulforaphane in the rat mammary gland with hopes of identifying biomarkers that can be used to assess the chemopreventive efficacy of broccoli sprouts in human clinical trials. For the first time, we measured sulforaphane metabolites in mammary tissue from 10-week-old female Sprague–Dawley rats as early as 30 min following a single oral 150 μmol dose of sulforaphane. This was followed by significant induction of *NQO1* and *HO-1* cytoprotective gene transcripts in the rat mammary gland. This response is consistent with prior studies examining NRF2-mediated transcripts induced by sulforaphane in the rodent small intestine and liver (42,45). The pattern and timing of induction varied between transcripts. The more rapid and biphasic induction of *HO-1* transcripts observed in the mammary gland has been described previously in rat heart and liver. The initial phase is likely mediated by the transcription factor AP-1 (46,47), whereas the subsequent induction, which peaked at 12 h, is probably NRF2 mediated in conjunction with the catalytic subunit of the SW12/SNF2-like chromatin-remodeling complex, BRG1 (48). A significant induction of mammary gland *NQO1* enzymatic activity was also observed, peaking 12 h after maximal *NQO1* transcripts were measured. The induction of *NQO1* activity by sulforaphane was originally established in cultured murine hepatoma cells by Zhang *et al.* (11) and recently further validated *in vivo* in the small intestine of mice (45). The unimodal induction pattern of *NQO1* is typical of NRF2-regulated genes (49).

Of great importance is the necessity for any chemopreventive agent to cause beneficial molecular changes not only in target tissues generally but also in at-risk cell populations specifically of these tissues.

Table I. Human sulforaphane pharmacokinetics

Specimens (units)	Pre-dose	Post-dose
Breast tissue (pmol/mg tissue)	—	Left: 2.00 ± 1.95 ; Right: 1.45 ± 1.12
Urine (μM)	4.07 ± 5.22	158.85 ± 93.89
Plasma (μM)	0.01 ± 0.02	0.92 ± 0.72

Data, mean ($n = 8$) \pm SE.

In humans, >85% of invasive breast cancers originate in ductal epithelial cells (50). Using immunostaining assays, we were able to detect significant HO-1 induction in rat mammary ductal and glandular epithelial cells 24 h after dosing. HO-1 was chosen as several studies revealed little to no immunostaining in both normal human breast tissue (<http://www.proteinatlas.org>) and established breast epithelial cell lines (51). While immunostaining of *NQO1* would have been desirable, published data and pilot studies showed substantial *NQO1* staining in untreated rat and human mammary ductal epithelial cells (52) minimizing the capacity to detect induction.

In a proof of principle clinical study, we were also able to demonstrate the presence of DTCs in human breast tissue after a single dose of a broccoli sprout preparation containing 200 μmol of sulforaphane. In addition, we have been able to measure *NQO1* and *HO-1* transcripts, as well as *NQO1* enzymatic activity in human breast tissue. Together, our studies demonstrate that sulforaphane distributes to the breast epithelial cells *in vivo* and exerts a pharmacodynamic action in these target cells consistent with its mechanism of chemoprotective efficacy. Such efficacy, coupled with earlier randomized clinical trials revealing the safety of repeated doses of broccoli sprout preparations (27–31), supports further evaluation of broccoli sprouts in the chemoprevention of breast and other cancers.

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P.T., J.W.F. and the Johns Hopkins University are founders, unpaid consultants and equity holders in Brassica Protection Products LLC (BPP), a company that is licensed by Johns Hopkins University to produce broccoli sprouts. These parties may be entitled to royalty payments, and their equity in BPP is managed according to university policy. P.T.'s son is the Chief Executive Officer of BPP. A portion of any proceeds of BPP from broccoli sprout sales are used to support cancer research, but no such funds were provided to support this study.

Conflict of Interest Statement: None declared.

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