Published in final edited form as:

Toxicol Appl Pharmacol. 2013 July 1; 270(1): 60–69. doi:10.1016/j.taap.2013.02.016.

Differential modulation of dibenzo[*def,p*]chrysene transplacental carcinogenesis: Maternal diets rich in indole-3-carbinol versus sulforaphane

Lyndsey E. Shorey^{a,b,1}, Erin P. Madeen^{a,b}, Lauren L. Atwell^{b,c}, Emily Ho^{b,c}, Christiane V. Löhr^{b,d}, Clifford B. Pereira^e, Roderick H. Dashwood^{a,b}, and David E. Williams^{a,b,*}

^aDepartment of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, 97331 USA

^bLinus Pauling Institute, Oregon State University, Corvallis, OR, 97331 USA

^cSchool of Biological and Population Health Sciences, Oregon State University, Corvallis, OR, 97331, USA

^dCollege of Veterinary Medicine, Oregon State University, Corvallis, OR, 97331, USA

^eDepartment of Statistics, Oregon State University, Corvallis, OR, 97331, USA

Abstract

Cruciferous vegetable components have been documented to exhibit anticancer properties. Targets of action span multiple mechanisms deregulated during cancer progression, ranging from altered carcinogen metabolism to the restoration of epigenetic machinery. Furthermore, the developing fetus is highly susceptible to changes in nutritional status and to environmental toxicants. Thus, we have exploited a mouse model of transplacental carcinogenesis to assess the impact of maternal dietary supplementation on cancer risk in offspring. In this study, transplacental and lactational exposure to a maternal dose of 15 mg/Kg B.W. of dibenzo[*def,p*]chrysene (DBC) resulted in significant morbidity of offspring due to an aggressive T-cell lymphoblastic lymphoma. As in previous studies, indole-3-carbinol (I3C, feed to the dam at 100, 500 or 1000 ppm), derived from cruciferous vegetables, dose-dependently reduced lung tumor multiplicity and also increased offspring survival. Brussels sprout and broccoli sprout powders, selected for their relative abundance of I3C and the bioactive component sulforaphane (SFN), respectively, surprisingly enhanced DBC-induced morbidity and tumorigenesis when incorporated into the maternal diet at 10% wt/wt. Purified SFN, incorporated in the maternal diet at 400 ppm, also decreased the latency of DBC-dependent morbidity. Interestingly, I3C abrogated the effect of SFN when the two purified compounds were administered in equimolar combination (500 ppm I3C and 600 ppm SFN). SFN metabolites measured in the plasma of neonates positively correlated with exposure levels via the maternal diet but not with offspring mortality. These findings provide justification

© 2013 Published by Elsevier Inc.

*Corresponding author at: Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, 97331, USA. Fax: +1 541 737 0497. david.williams@oregonstate.edu (D.E. Williams)..

¹Current address: Division of Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Ave, Beaverton, OR, 97006.

Conflict of interest None declared.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2013.02.016>.

for further study of the safety and bioactivity of cruciferous vegetable phytochemicals at supplemental concentrations during the perinatal period.

Keywords

Transplacental; Indole-3-carbinol; Sulforaphane; T-cell lymphoblastic lymphoma; Polycyclic aromatic hydrocarbon

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are produced from the combustion of fossil fuels, and within this chemical class, DBC is among the most potent as a carcinogen in animal models (Cavalieri et al., 1991; Platt et al., 2004; Prahalad et al., 1997; Siddens et al., 2012) and a probable human carcinogen (IARC, 2010). The relative tissue concentration in neonates exposed transplacentally to PAHs is expected to be approximately 1% of the maternal burden (Shorey et al., 2012), yet Whyatt et al. (2001) demonstrated that levels of PAH-DNA adducts were higher in newborn white blood cells than in paired maternal samples, demonstrating the increased sensitivity of the neonate to environmental carcinogens. Various PAHs have been evaluated in preclinical rodent models as transplacental carcinogens including 3-methylcholanthrene (3-MC), 7,12-dimethylbenz[a]anthracene (DMBA), and benzo[a]pyrene (BaP) (Anderson et al., 1985, 1995; Miller et al., 1998). Our laboratory previously established that transplacental and/or lactational exposure to DBC in a murine model produces T-cell lymphoblastic lymphomas during early adulthood, in addition to lung tumors and liver tumors (predominantly in males) later in life (Castro et al., 2008a, 2008b, 2008c, 2009; Yu et al., 2006a, 2006b).

Although the etiology for acute lymphoblastic leukemia/lymphoma is not precisely known, it remains the most prevalent childhood cancer (NCI, 2011). Furthermore, polymorphisms in *CYP1A1* and *GSTM1*, two enzymes involved in the bioactivation and elimination of PAHs, have been associated with increased risk of childhood leukemia (Swinney et al., 2011; Vijayakrishnan and Houlston, 2010). Multiple lymphatic tissues, including bone marrow, spleen, and thymus, constitutively express *CYP1B1* during both fetal development and adulthood, the CYP isoform primarily responsible for immunotoxic and carcinogenic effects of specific PAHs (Choudhary et al., 2005; Miyata et al., 2001; Uno et al., 2006). For example, the preleukemic effects of DMBA are almost completely ablated in *Cyp1b1* null animals, whereas DBC-induced lymphomas in our transplacental model depend on *Cyp1b1* gene dosage (Castro et al., 2008a; Heidel et al., 2000).

High-heat cooking and preserving practices such as grilling, smoking, drying, or broiling introduce PAHs to foods, as does growth of vegetables in soils contaminated from atmospheric sources. Therefore, the diet is a primary route of exposure to PAHs among non-smokers, especially PAHs of large molecular weight (5–6 rings) having mutagenic properties (Ramesh et al., 2004). Conversely, evidence from epidemiological and animal studies suggests that modification of the diet to increase consumption of cruciferous vegetables reduces the occurrence of some common cancers (Bosetti et al., 2012; Davis et al., 2002; Keum et al., 2009; Richman et al., 2012). Broccoli, Brussels sprouts, mustard,

kale, cabbage, horseradish and arugula are vegetables in the Brassicaceae (Cruciferae) plant family and are rich sources of glucosinolates, substituted β -thioglucoside N-hydroxysulfates (Hayes et al., 2008). Breakdown products of glucosinolates, known as “indoles” and “isothiocyanates”, are widely accepted as contributing to the beneficial properties of crucifers. For example, indole-3-carbinol (I3C) is derived from the glucosinolate, glucobrassicin, abundant in Brussels sprouts, kale, and cabbage varieties, and has been widely studied for its anticancer properties (reviewed in Bradlow, 2008; Sarkar et al., 2009; Weng et al., 2008). Moreover, I3C supplemented to the maternal diet at 2000 ppm in our transplacental model of carcinogenesis reduces T-cell lymphoblastic lymphoma mortality and decreases lung tumor multiplicity in surviving offspring, and I3C is transplacentally bioavailable to the developing fetus (Yu et al., 2006a).

Interest in isothiocyanates as antineoplastic agents has been growing since the 1960s, and more recently, sulforaphane (SFN), derived from glucoraphanin and abundant in broccoli and broccoli sprouts, has become the most studied isothiocyanate due to its potency for induction of phase II enzymes. SFN has been demonstrated to inhibit CYP1A1 and CYP1A2 activity induced by the prototypic PAH, BaP, in MCF-7 cells at low micromolar concentrations achievable in vivo (Skupinska et al., 2009a, 2009b). SFN also decreased BaP-mediated AHR activation and CYP content while inducing phase II enzyme systems in the lungs of animals exposed to BaP (Kalpana Deepa Priya et al., 2011). These changes were associated with restored mitochondrial glutathione levels, reduced lipid peroxidation, and lessened alveolar hyperplasia (Priya et al., 2011a, 2011b).

Both I3C and SFN modulate a wide-range of biological targets and consequently may influence risk at all stages of cancer from initiation through metastasis. For example, I3C and SFN alter the expression and activity of phase I CYP enzymes and phase II detoxifying enzymes, cell signaling kinases (i.e. *MAPK*, *NF- κ B*), and histone deacetylase (HDAC) expression and activity (Aronchik et al, 2010; Clarke et al., 2008; Ho et al., 2009; Saw et al., 2011). Based on these findings and others, we hypothesize that I3C, at lower concentrations than previously tested, SFN, and their whole food sources will protect against DBC-induced transplacental carcinogenesis. To this end, we conducted a large preclinical animal study with multiple dietary regimens to compare the efficacy of these purified phytochemical components to whole food sources (Brussels sprouts and broccoli sprouts) and to one another.

Materials and methods

Chemicals and diet

DBC was obtained from the NCI carcinogen repository at the Midwest Research Institute (Kansas City, MO) and was confirmed as >98% pure by HPLC. Custom diets and semi-purified control diets, AIN93G and AIN93M, were purchased from Research Diets (New Brunswick, NJ). Dietary additives were purchased from the following suppliers: I3C, Cat # 17256, Sigma Aldrich (St. Louis, MO); SFN, Cat # S699115, Toronto Research Chemicals (North York, Ontario); Brussels sprout powder, Cat # N54 and broccoli sprout powder, Cat # N216, Future Ceuticals (Mokena, IL). Custom diets were prepared with AIN93G diet base, adjusted for macronutrient content between diets by Research Diets (Table 1). Diets

were stored protected from light at -20°C throughout the feeding phase of the trial. Whole food powders were analyzed by American Analytical Chemistry Laboratories Corporation for I3C content and by Van Drunen Farms/Future Ceuticals for glucoraphanin content, and subsequently in-house as described below.

Glucoraphanin extraction and analysis from freeze-dried powders

Approximately 30 mg of powder (broccoli or Brussels sprout), in triplicate, was weighed into 2 mL vials. Glucotropaeolin (GTP) (Applchem cat # A5300,0020) was used as an internal standard, and 100% MeOH (375 μL) was added to each powder sample and sonicated for 10 min before centrifuging (Spectrafuge 24D at $16,300 \times g$ for 5 min) and transferring supernatant to a 15-mL conical tube. Three additional MeOH extractions were collected similarly and the four extracts pooled. Supernatants were centrifuged through 0.22- μm nylon Spin-X® filter columns (VWR, Radnor, PA), diluted with 0.1% (v/v) formic acid in H_2O , and stored at -80°C until analysis by HPLC–MS/MS. Ten μL was injected in duplicate and HPLC conditions were as follows: Phenomenex Synergi Hydro-RP column (80 $\text{\AA}$, 150×2 mm, 4 μm pore size), mobile phases of 0.1% (v/v) formic acid in acetonitrile and 0.1% (v/v) formic acid in H_2O , 0.25 mL/min flow rate at 25°C . An Applied Biosystems MDX Sciex 3200™ triple quadrupole mass spectrometer was used in negative ion mode to detect GFN ($436 > 97$) and GTP ($408 > 97$). Quantification was performed based on a 6-point calibration curve that spanned the extract concentrations and showed excellent linearity ($r^2 = 0.999$).

Sulforaphane extraction and analysis from freeze-dried powders

Six samples of approximately 30 mg were weighed into 2 mL vials and the weights recorded for each powder (broccoli or Brussels sprout). Three samples per powder type were supplemented with 0.64 units of exogenous *Sinapis alba* (white mustard) myrosinase (Sigma-Aldrich, St. Louis, MO) before incubation in 1 mL dH_2O at 60°C , in the dark, for 2 h. Hydrolyzed samples were centrifuged at $16,300 \times g$ for 5 min to pellet powder, and supernatants were filtered through 0.22- μm nylon Spin-X® filter columns, diluted with 0.1% (v/v) formic acid in H_2O , and stored at -80°C until analysis by HPLC–MS/MS. Deuterated SFN-NAC was used as an internal standard. Ten μL of extract was injected in duplicate and HPLC conditions were as follows: Phenomenex® Kinetex PFP column (100 $\text{\AA}$, 100×2.1 mm, 2.6 μm pore size), mobile phases of 0.1% (v/v) formic acid in acetonitrile and 0.1% (v/v) formic acid in H_2O , 0.25 mL/min flow rate at 40°C . An Applied Biosystems MDX Sciex 3200™ triple quadrupole mass spectrometer was used in positive ion mode to detect SFN ($178 > 114$) and deuterated SFN-NAC ($344.1 > 114$). Quantification was performed based on a 7-point calibration curve that spanned the extract concentrations and showed excellent linearity ($r^2 = 0.999$).

Indole-3-carbinol extraction and analysis from freeze-dried powders

Broccoli sprout/Brussels sprout powders were analyzed for I3C content with slight modification of a previously published method (Liu et al., 2009). In brief, powders were hydrolyzed in triplicate in H_2O (10 mg/mL) for 4 h at room temperature away from light, with periodic vortexing. Solutions were centrifuged at $3600 \times g$ for 5 min to pellet sprout

powder, and supernatant was extracted twice with 5 mL of dichloromethane. Extracts were pooled and evaporated under a stream of nitrogen gas before resuspending in 200 μ L of acetonitrile. Acetonitrile extracts were further centrifuged at 16,000 $\times$ g to remove any precipitates and 50 μ L of supernatant was injected onto a Polarity C₁₈ 5 μ m (4.6 $\times$ 250 mm) column for detection of I3C. A Waters 2695 separation module with a Waters 2996 photodiode array detector was utilized with mobile phase conditions as described previously (Stresser et al., 1995). Spiked extracts were analyzed to calculate recovery and to select an appropriate time period of hydrolysis. Quantification was performed based on a 13-point calibration curve that spanned the extract concentrations and showed excellent linearity ($r^2 = 0.997$).

Neonatal plasma SFN metabolite analysis

Whole blood was obtained from pups at various times after birth, spun at 16,000 $\times$ g 1 min, and plasma was acidified immediately with 10% (v/v) trifluoroacetic acid to stabilize SFN compounds and facilitate protein precipitation before storage at -80°C . To prepare samples for LC-MS/MS, plasma was thawed and centrifuged at 4°C for 5 min at 16,000 $\times$ g. Supernatants were centrifuged twice through 0.22- μ m nylon Spin-X® filter columns. Filtrates were frozen at -80°C until analysis by HPLC-MS/MS. Ten μ L of filtrate was analyzed in duplicate as described previously with the exception of mass spectrometer model. An Applied Biosystems MDX Sciex 4000™ triple quadrupole in positive ion mode detected SFN (178 > 114), SFN-Cys (299 > 114), SFN-CG (356 > 114), SFN-GSH (485 > 179), and SFN-NAC (341.1 > 114) (Clarke et al., 2011). Quantification was determined using calibration curves (6-points) for each metabolite and showed excellent linearity (average $r^2 = 0.994$, $SD_{r^2} = 0.005$).

Animals and treatment protocols

All protocols for the handling and treatment of mice were approved by the Oregon State University Institutional Animal Care and Use Committee. Seven- to eight-week-old B6129SF1 female and 129S1/ScImJ male mice (Jackson Laboratories, Bar Harbor, ME) were maintained in a pathogen-free environment in micro-isolator cages (Life Products, Inc., Seaford, DE) with CareFRESH bedding at $20 \pm 1^\circ\text{C}$ and $50 \pm 10\%$ humidity with a light/dark cycle of 12 h.

Experimental design

After acclimation for one-week, mice were provided AIN93G diet ad libitum and paired for breeding. Day 0 of gestation was based on appearance of the vaginal plug at which time males were removed to a separate cage and female weights recorded. On GD9, the maternal AIN93G diet was replaced with experimental or control pellets according to treatment group (Table 1), and maternal dietary supplementation continued until weaning (post-natal day 21 (PND21)). Diet was provided in pelleted form and changed daily in order to maintain stability of active ingredients. Doses and duration of dietary intervention with purified I3C were based on a previous study in this laboratory demonstrating transplacental chemoprevention (Yu et al., 2006b). Thus in this study we tested three levels of 1000, 500, and 100 ppm I3C which are roughly equivalent to human consumption of commercially

available dietary supplements of 72–720 mg/day. The safety and tolerability of I3C supplements has been assessed up to 1200 mg in non-pregnant healthy women (Reed et al., 2006). The COMBO diet was made up with equimolar levels of 500 (I3C) and 600 (SFN) phytochemicals. Pregnant dams were administered an oral gavage of 15 mg/kg (5 mL/kg body weight) DBC in corn oil or vehicle control on gestation day 17 (GD17) between 8:00 and 10:00 AM. Three litters from each treatment group having 7 or more pups were designated for future molecular work and SFN analysis, and the newborn pups (post-natal day 0 (PND0)) were immediately removed following delivery, decapitated and rapidly dissected. Blood was pooled within a litter and processed for SFN metabolite analysis as described above. Remaining litters were kept with the mother until weaning at which time male and female siblings were separated into new micro-isolator cages and given access to AIN93G control diet. Animals were monitored twice daily for signs of morbidity, pain, or distress, at which time animals were euthanized with an overdose of CO₂ followed by cervical dislocation. A full necropsy was conducted and thymus, heart, lung, liver, kidneys, spleen, digestive organs, and reproductive organs inspected for gross abnormalities before fixing tissues in 10% neutral-buffered formalin. Surviving animals at 10 months of age were euthanized and necropsied as above with the following exceptions: consistently located portions of lung and liver were preserved in RNA later for future analysis, and lungs were closely assessed for lesion multiplicity and size. A portion of ear from each animal was collected and frozen for DNA extraction and genotyping.

Histopathology

Tissues collected upon necropsy (heart, thymus, lung, spleen, liver, kidney, stomach, abnormal lymph nodes, testes or ovaries and colon) were fixed in 10% formalin. Early mortality samples (<10 weeks of age) and a balanced sub-set of samples collected at the termination of the study were stained with hematoxylin and eosin and examined by a veterinary anatomic pathologist as described previously to confirm gross observations (Castro et al., 2008a, 2008b, 2008c, 2009; Yu et al., 2006a, 2006b).

Genotyping for *Ahr*^{b-1} “responsive” and *Ahr*^d “non-responsive” alleles

DNA was isolated from a small portion of ear by digesting in a DirectPCR lysis reagent (Viagen Biotech, Inc. Los Angeles, CA) containing proteinase K at 55 °C until homogenous. Lysates were then heated to 85 °C to inactivate proteinase K, centrifuged to pellet unlysed material and 0.4 µl of supernatant used directly in a PCR reaction containing allele specific primers to permit single-tube genotyping of *Ahr* alleles as described previously (Yu et al., 2006a).

Statistical analyses

The experimental unit in transplacental studies is the mother. Pup level responses are therefore clustered within each litter/mother so that statistical methods require random litters be nested within treatments in order to match the experimental design. Pup level responses were compared between treatments, genders and genotypes (including interactions). Pup survival times were analyzed using Cox proportional hazards regression (SAS Phreg procedure) with random litters (frailty model). Treatment comparison results shown here are

for a model with additive cohort effects. It was found that randomization resulted in litter sizes that differed somewhat between treatments ($p = 0.02$ ANOVA). A more complex model with an adjustment for litter size indicated a somewhat increased hazard of dying with increased litter size, but did not change the conclusions regarding treatment differences because residuals revealed approximate normality with no evidence of increasing variance with the mean. Pup tumor multiplicity in survivors was analyzed with a linear mixed model (SAS mixed procedure) including random litter effects. Sex and genotype ratios per litter were analyzed with logistic regression. When the ratio of residual deviance (rdev) divided by residual degrees of freedom (rdf) was enough larger than one to affect inference, quasiliikelihood was used with the overdispersion parameter estimated by the ratio (rdev/rdf).

Results

Broccoli sprout and Brussels sprout powder characterization

We obtained freeze-dried sprout powders for maternal supplementation in this study, as this process maintains myrosinase activity, and therefore bioactivity (i.e. induction of colonic and hepatic NADPH-quinone oxidoreductase (Hwang and Jeffery, 2004; Liu et al., 2009)). Our study design was based on manufacturer-estimated concentrations of I3C and SFN in the purchased products. However, as these values were provided in some cases as a range of previously observed values, we thought it prudent to analyze in-house the concentration of SFN and I3C in the powders after we received them. As expected, SFN levels were significantly higher in the broccoli sprout powder (1775 ± 66.3 ppm), relative to the Brussels sprout powder (85.2 ± 1.5 ppm), based on HPLC–MS/MS analysis following hydrolysis with addition of exogenous myrosinase (Table 2). Our SFN measurements were markedly different than those estimated by the manufacturer as “SFN potential — 5000 ppm (broccoli sprout)”, which they based on glucoraphanin levels and a predicted hydrolysis conversion of 50%. Glucoraphanin was readily detected in broccoli sprout powder MeOH extracts (Table 2). The formation of isothiocyanates from glucosinolates depends on many experimental factors including temperature, pH, and the presence of ferrous ions. The reaction conditions and amount of exogenous myrosinase added during in-house experiments were based on the methods of Conaway et al. (2000) and Fahey et al. (1997) reported to allow for complete hydrolysis of glucosinolates. The formation of additional products from glucoraphanin, such as SFN-nitrile, was not measured and may account for the 3:1 molar ratio of GFN:SFN measured in broccoli sprout powder extracts. No GFN was detected in broccoli sprout powder extracts with added excess myrosinase, suggesting that complete hydrolysis of GFN was achieved (data not shown). I3C is not routinely measured by the sprout powder supplier; therefore, analysis was contracted to American Analytical Chemistry Laboratories prior to our purchase of the powder. The assay used for this analysis (UV–VIS absorbance at 280 nm) overestimates the actual concentration of I3C due to matrix components which also absorb light at this wavelength. Thus, after receipt of the powder, we coupled UV–VIS detection at 280 nm to HPLC to separate I3C from other analytes present in the extract using a previously established method (Stresser et al., 1995). We achieved outstanding linearity over a 13-point standard curve ($r^2 = 0.997$) ranging from 0.75 to 75 nmol in 50 μ l of extract (~25–2500 ppm adjusted powder concentration). Our extraction

efficiency was $60.3 \pm 3.4\%$ based on spike-recovery experiments, and this was accounted for in our quantification. In the Brussels sprout powder we detected 151 ± 10.7 ppm I3C, compared to 2100 ppm indicated by the UV–VIS method without HPLC separation. We detected no I3C in the broccoli sprout powder, although drum-dried sprouts from the same supplier and variety (*Brassica oleracea italica*) were reported to contain 5.88 ppm (Liu et al., 2009), which is below our method's limit of quantitation.

Detection of SFN metabolites in plasma of neonates exposed transplacentally to SFN

It has been previously demonstrated in adult ICR mice administered 20 μ mol SFN by oral gavage, roughly equivalent to the daily intake of dams given 600 ppm SFN diet in this study, that plasma levels of SFN metabolites peak at 15 μ M on average (Clarke et al., 2011). Thus, we sought to determine whether SFN and/or its metabolites cross the placenta and can be detected in neonatal plasma. As SFN is rapidly metabolized via the mercapturic acid pathway with a half-life of approximately 2 h (Hanlon et al., 2008), blood was collected immediately after delivery when possible and pooled within a litter before separating and acidifying plasma as described above. We observed that SFN indeed crossed the placenta during maternal dietary supplementation between GD9 and birth (GD19). Three out of four metabolites analyzed (SFN-GSH, SFN-Cys, and SFN-NAC) were quantifiable with sum concentrations of metabolites reaching roughly 2 μ M in the 600 ppm SFN + 500 ppm I3C treatment group (Fig. 1A). The variability between litters likely reflects the differences in the length of time from birth to sample collection (as some dams gave birth late at night) and also the variable time since the dam last fed. A dose-dependent relationship between maternal level of SFN intervention and neonatal plasma concentration of SFN metabolites was observed (Fig. 1B).

Population demographics and influence on statistical modeling

Litter sizes differed between treatments (Table 3), thus litter size was added to the Cox model as a linear covariate. There were strong litter effects on survival ($p = 0.04$), with some evidence to suggest that animals from larger litters were at increased risk of morbidity. Genotype ratios were overdispersed relative to binomial variation ($\text{dev/df} = 1.22$) and there was no evidence of treatment differences ($p > 0.18$). Sex ratios were not overdispersed relative to binomial variation and there was no evidence of treatment differences ($p > 0.10$).

Maternal consumption of I3C, SFN, or whole food sources variably modulates mortality in offspring from transplacental DBC-induced lymphoma

As previously demonstrated, transplacental and lactational exposure to the potent PAH carcinogen, DBC, resulted in significant offspring morbidity/mortality between 12 and 35 weeks of age (Castro et al., 2008a, 2008b, 2008c, 2009; Yu et al., 2006a, 2006b). This morbidity is generally the result of highly aggressive thymic lymphomas, previously confirmed to be of T-cell origin (Yu et al., 2006a). In over 80% of animals upon gross necropsy large solid thymic masses were observed and histological examination of a sub-set of animals in this study confirmed the previously described pathology. All animals from dams receiving control diet and vehicle in place of carcinogen survived until termination of the study at 10 months and displayed no signs of illness throughout the study or at necropsy.

Offspring were assessed twice daily throughout the course of the study, and animals were euthanized upon symptoms of distress or discomfort. All offspring from mothers receiving dietary supplementation were also exposed transplacentally to DBC. Fig. 2A depicts the survival of offspring born to mothers receiving dietary supplementation with varying concentrations of I3C versus no dietary intervention or no DBC exposure groups. Incorporation of I3C into the maternal diet appeared to reduce the DBC-related mortality of offspring (hazard ratio of 0.476 at 500 ppm), though to a lesser extent than in a previous study with diet supplementation at 2000 ppm, as expected (Yu et al., 2006b). Interestingly, survival protection by I3C in this study did not correlate with its concentration of incorporation to the diet ($p = 0.34$). The 500 ppm dietary concentration exhibited the most protection, although the reduction in mortality did not reach statistical significance (unadjusted $p = 0.24$) (Fig. 2A).

Surprisingly, in the SFN and whole food treatment groups, early and often asymptomatic mortality was observed in a subset of pups before 10 weeks of age, when the onset of lymphoma symptoms generally occurs with DBC exposure alone. Of the pups born to mothers receiving Brussels sprouts, 7% died before 10 weeks of age, as did 10.2% and 17.7% of animals from the broccoli sprout and SFN treatment groups, respectively (Fig. 2B). Histological examination of tissues collected from these early morbidities did not reveal any consistent etiology, and only one instance of lymphoma prior to 10 weeks of age was confirmed (Supplemental Table S1). Conversely, no early mortalities were observed in the vehicle control, DBC control, I3C, or I3C + SFN groups. Survival curves for offspring from broccoli sprout- and SFN-fed dams were strikingly similar ($p > 0.5$), suggesting that high levels of SFN in the broccoli may contribute to increased mortality (Fig. 2B). This is further supported by measurements of SFN and relative concentration in the broccoli powder compared to Brussels sprout powder (Table 2). Interestingly, when I3C was incorporated into the maternal diet in combination with an equivalent molar amount of SFN, no early mortalities occurred as in the SFN and whole food diet groups, and overall survival trends were more similar to treatment groups with I3C alone (Fig. 2A).

After adjusting for effects of varying litter sizes, the increased risk of mortality for offspring born to mothers receiving DBC and SFN versus SFN + I3C (COMBO) was 2.88 fold ($p = 0.004$); unadjusted, the effect was even more dramatic (hazard ratio of 3.00). Comparing all dietary treatments versus DBC alone increases the chance of so-called false positives (Type 1 error), and the Dunnett method of p -value adjustment takes this into consideration. None of the treatment groups were statistically different from DBC alone after p -value adjustment due in part to the high number of treatment groups and unstructured testing method, the small number of animals in the DBC group, and the fact that DBC results fell toward the middle of all treatments. However, without adjustment for multiple testing, broccoli treatment increased risk of morbidity by a factor of 2.07 ($p = 0.0846$) and SFN treatment by 2.50-fold ($p = 0.030$).

Evidence that gender, but not Ahr genotype, influences survival from transplacental DBC-induced lymphoma

Previously, we have identified Ahr status of mothers and offspring as influencing offspring survival following treatment of the mother with DBC (15 mg/kg on GD17). Pups born to mothers having a responsive phenotype (*Ahr*^{b-1/d}) were less susceptible compared to those born to mothers homozygous for the non-responsive allele (*Ahr*^{d/d}). Conversely, if the pup was genotyped as *Ahr*^{b-1/d}, the lymphoma mortality and lung tumor multiplicity were enhanced (Yu et al., 2006a). Interestingly, the *Ahr* phenotype had no impact on the degree of I3C chemoprevention when added to the maternal diet during gestation (Yu et al., 2006b) even though a long-proposed mechanism of I3C cancer chemoprevention is through modulation of Ahr-dependent gene expression (Park and Bjeldanes, 1992). In this study, we found no evidence of genotype or cohort being the primary effects on survival ($p > 0.5$ and $p = 0.4$, respectively); however, there was some evidence to suggest an interaction between gender and treatment related outcome ($p = 0.09$). After partitioning the data to evaluate gender, it is obvious that the majority of treatment effects are attributed to males, as evidenced by the larger spread in survival curves among this group (male treatment effect: $p = 0.0002$ versus female effect: $p = 0.07$). The survival curves for males (with the exception of SFN and whole food treatments) dropped between 91 and 168 days (13–24 weeks), after which time the slope of the curves appear to plateau, while female survival curves steadily decline over the entire study period (Fig. 3). Interestingly, in female offspring alone the I3C treatment survival curves appear to follow a dose-dependent pattern, although this effect was not statistically significant due to the small sample size after partitioning by gender.

Maternal consumption of I3C but not SFN or whole food sources reduces DBC-dependent transplacental lung cancer in surviving offspring at 10 months

In this established model of transplacental carcinogenesis, mice surviving until termination of the study (10 months of age) exhibit a 100% incidence of lung tumors with varying degrees of progression (hyperplasia, adenoma, adenoma with progression, and carcinoma) (Yu et al., 2006a). Animals euthanized due to lymphoma-dependent morbidity prior to 10 months in this study often exhibited lung lesions, and a previous report by our laboratory has documented increasing lung tumor multiplicity as a function of age (Castro et al., 2008c). We closely examined lungs of all animals surviving to study termination for tumor multiplicity and tumor diameter before preserving the tissues for histology. A similar pattern of treatment-related effect (compared with survival) was revealed following analysis of lung tumor multiplicity, with the exception of I3C treatments (Table 4). An inverse trend for tumor multiplicity was observed with increasing concentrations of I3C ($p = 0.019$). Neither the SFN nor whole food treatment groups (broccoli sprouts or Brussels sprouts) reduced the number of lung lesions per animal, yet the SFN + I3C group (COMBO) markedly reduced lung tumor multiplicity in surviving animals (simple mean = 16.2 DBC versus 10.4 in COMBO, unadjusted $p = 0.046$), suggesting a protective effect of I3C even in the presence of SFN.

Discussion

There are currently over 40 clinical trials listed on www.clinicaltrials.gov utilizing cruciferous vegetable extracts or derived phytochemicals (I3C, SFN, or 3,3'-diindolylmethane), highlighting the potential for cruciferous vegetable-based disease prevention and therapy. Although a greater number of preclinical studies have been conducted to examine effectiveness, safety, pharmacokinetics, and mechanisms of action for these compounds, there are still many challenging questions to be asked. One particular gap in the knowledge base pertains to the critical neonatal window, where developmental programming is modifiable by environmental and nutritional factors and may determine disease risk later in life (Calkins and Devaskar, 2011). This prompted us to investigate the effects of maternal dietary supplementation with phytochemicals derived from cruciferous vegetables either in purified form or as part of the whole-food matrix.

Our results show that I3C, abundant in Brussels sprouts and cabbage, modestly protects against DBC-induced T-cell lymphoma, and more dramatically, lung tumor multiplicity. The dose-dependent protective effect of I3C on lung tumorigenesis is consistent with results in A/J mice initiated with a mixture of carcinogens found in tobacco smoke (NNK and BaP) (Kassie et al., 2008). We have previously observed a greater protective effect of maternal dietary supplementation with 2000 ppm I3C on offspring survival (Yu et al., 2006b). The lack of statistically significant protection against morbidity in this study is possibly due to the large study design, with multiple comparisons and an insufficient number of offspring in the control group, in addition to the lower level of I3C in the diet. Further, we identified an impact of litter size on survival and evidence for larger treatment effects in males than females (Fig. 3), which may have been influenced by symptoms unrelated to lymphoma. Upon necropsy, we frequently observed lesions of the reproductive tract including granulosa cell tumors of the ovary and endometrial hyperplasia in females and a spectrum of abnormalities in males (atrophy or dilation of seminiferous tubules, sperm granuloma, Leydig cell hyperplasia or atrophy, severe hemorrhage, etc.). No diet-specific patterns were identified, and none of the corn oil control animals exhibited these abnormalities, confirming that DBC is also a reproductive hazard in this model.

Surprisingly, we observed increased morbidity and no reduction in lung tumorigenesis in offspring born to mothers supplemented with dietary SFN or its primary whole food source, broccoli sprouts, converse to many reports of chemoprotection in adult animal models (Kalpana Deepa Priya et al., 2011; Keum et al., 2009; Zhang et al., 1994). Importantly, we show in this study that maternal dietary consumption of broccoli sprouts, or pure SFN, results in measurable neonatal plasma levels of SFN-mercapturic acid metabolites, suggesting that the fetus may be capable of metabolic activation of SFN although it is also possible that the origin was from maternal metabolism. The ATP-binding cassette transporter, mrp2, is expressed in placenta and capable of transporting glutathione conjugates (Gerk and Vore, 2002). The highest mean SFN metabolite concentration (~2 μ M) observed in plasma was in the offspring from mothers receiving 600 ppm SFN in combination with 500 ppm I3C (Fig. 1). These levels are significant given the short half-life of SFN, route of exposure, and biological activity of SFN and/or SFN-metabolites at low micromolar levels in vitro (Matusheski and Jeffery, 2001; Myzak et al., 2004). Myzak et al.

(2004) demonstrated that SFN-NAC and SFN-Cys, but not parent SFN, produced concentration-dependent decreases in HDAC activity. An earlier report has established that maternal *i.p.* administration of SFN on the last day of pregnancy, in a genetic mouse model of epidermolysis bullosa, increased Nrf2-mediated induction of specific keratins in neonatal epidermis (Kerns et al., 2007). In another study, feeding broccoli sprouts containing glucoraphanin, the precursor of SFN, to pregnant hypertensive stroke-prone rats (SHRsp) reduced oxidative stress in the dams and significantly lowered blood pressure and tissue inflammation in adult offspring (Noyan-Ashraf et al., 2005). We estimate that the human equivalent dose would be about 1 serving of broccoli in the Noyan-Ashraf et al. study, whereas, depending upon group, the present study gave 3–22 human equivalent servings of broccoli to the pregnant mouse. Thus, future work is necessary to determine the tissue-specific distribution of SFN-metabolites to offspring following maternal oral consumption, and the effects on normal tissue development through classical Nrf2-mediated pathways and also through modification of the epigenome. No early mortalities were observed in these offspring, and survivors were similarly protected compared to those exposed only to I3C, suggesting that fetal plasma concentration of SFN alone does not equate with toxicity. Of note, we selected concentrations of SFN and/or broccoli sprouts that have been used with successful efficacy in preclinical animal models of cancer protection, and these levels far exceed typical exposure to SFN in the human population through whole foods. Future work is necessary to address the effects of dietarily and physiologically achievable concentrations of these phytochemicals on the developing fetus, in the presence and absence of potent carcinogens such as DBC.

We have previously shown, using radiolabeled DBC, that approximately 1% of the 15 mg/kg dose given orally to pregnant B6129 female mice reaches the fetus as DBC or DBC metabolites (Shorey et al., 2012). However, PAH-DNA adduct levels are higher in newborns compared with paired maternal samples, likely due to decreased metabolic and repair capacity (Whyatt et al., 2001). The carcinogenic potential of DBC depends primarily upon metabolic activation by CYP1B1 and epoxide hydrolase to the *trans*-11,12-dihydrodiol and, subsequently, to the ultimate carcinogen and DNA binding species, the 11,12-*trans*-dihydrodiol-13,14-epoxide (DB[*def,p*]CDE) (Buters et al., 2002; Castro et al., 2008a; Luch et al., 1998). A well-supported mechanism of chemoprevention by SFN in adult animal models is its potent induction of phase II enzymes involved in detoxification to favor carcinogen deactivation (Zhang et al., 1992). SFN has also been shown to inhibit the activity of CYP enzymes and to reduce CYP1A1 and 1A2 activity induced by the prototypical PAH, BaP (Kalpana Deepa Priya et al., 2011; Skupinska et al., 2009b).

Conversely, I3C is termed a bi-functional inducer in that both phase I and phase II enzyme systems are induced upon oral exposure (Steinkellner et al., 2001). For example, oral pretreatment of Sprague-Dawley rats with I3C dramatically induced EROD activity in the small intestine, thereby decreasing systemic exposure to BaP and DNA adduct levels in the lung, while intraperitoneal injection of I3C did not (Park and Bjeldanes, 1992). Small intestine CYP activity was further demonstrated to be critical in order to decrease systemic exposure to BaP through tissue-specific knock-out of either *Cyp1a1* or its electron donor, NADPH CYP oxidoreductase (Fang and Zhang, 2010; Shi et al., 2010). Inhibition of CYP

activity by SFN in the small intestine could potentially increase systemic circulation of DBC in the pregnant female and increase exposure to the developing fetus, possibly explaining the higher incidence of mortality observed in the SFN and broccoli sprout groups. The small intestine will be a major site of exposure to SFN either ingested intact or formed in vivo from glucoraphanin (in the case of broccoli sprouts) and have the highest concentration of SFN metabolites after oral exposure in adult ICR mice (Clarke et al., 2011).

In a neonatal carcinogenesis assay, acute toxicity was observed in 100% of animals at a cumulative dose of 400 nmol of the *trans*-DBC-11,12-dihydrodiol species (~12 mg/kg assuming a 10 g pup) and in ~50% of animals administered only 0.4 nmol of the *anti*-DB[*def,p*] CDE diastereomer (Platt et al., 2004). The authors also noted chronic toxicity with parent DBC exposure or its metabolites, potentially through an immunosuppressive mechanism. Oral BaP administered to *Cyp1a1* knockout mice produces lethality following dramatic immunosuppression, evidenced by wasting and atrophy of hematopoietic tissues (thymus, bone marrow, liver, and spleen) (Uno et al., 2004, 2006). Indeed, we did observe one incidence of bacterial sepsis in the early mortality cases from the SFN treatment group (Supplemental Table S1). Furthermore, early mortalities were in some cases clustered by cage or litter, which may indicate the presence and spread of infection (data not shown). Thus, one possible explanation for the increased mortality in offspring born to dams supplemented with SFN or whole foods rich in SFN is immunotoxicity as a function of increased DBC exposure. Follow-up studies are warranted in order to test this hypothesis and also to determine whether this effect is replicated at dietary/physiological levels of maternal supplementation.

Isothiocyanates at high concentrations (10 μ M) are genotoxic, possibly due to their electrophilic reactivity or induction of oxidative stress (reviewed in Fimognari et al., 2011). As SFN and related compounds are metabolized via the mercapturic acid pathway, they deplete the cellular pool of glutathione, although this is rapidly restored through a feedback mechanism involving Nrf2-ARE (Pappa et al., 2007). Glutathione is also important for the conjugation and detoxification of PAH-epoxide and diol-epoxide metabolites (Shimada, 2006), and depletion of GSH resulted in increased testicular toxicity following transplacental exposure to BaP (Nakamura et al., 2012). Thus, altered maternal metabolism to decrease pre-systemic DBC clearance is only one possible explanation for the adverse effects observed in SFN-exposed offspring. However, the lack of early mortality, reduced morbidity and tumorigenesis, and highest neonatal plasma concentrations of SFN metabolites in the COMBO treatment group (SFN in the presence of I3C), suggests that SFN itself is not responsible for the adverse effects seen in these groups. This combination did not appear to increase protection relative to I3C alone; interestingly, I3C and SFN have been reported to be antagonistic or synergistic in activating Nrf2-ARE activity in vitro, depending on the concentration tested (Saw et al., 2011).

Testing the safety of SFN and broccoli sprout supplements during pregnancy is warranted given the commercial availability of such supplements and the observations in this study. However, it is important to note that the concentrations used here far exceed what would be achieved with typical consumption of fresh vegetables or recommended doses of available supplements. Further work is necessary to elucidate the molecular pathways altered by DBC

alone and in combination with these dietary supplements, and is ongoing. Transplacental animal models of carcinogenesis provide an opportunity to investigate cancer risk in the context of neonatal nutrition and furthermore to test the safety of nutritional supplements in this sensitive population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the staff of the Laboratory Animal Resource Center and the Cancer Chemoprevention Core Labs at Oregon State University. We greatly appreciate the experimental guidance provided by Lisbeth Siddens and the assistance of David Strickland, Rachel Azevedo, Elyssa Ridinger, and David Sampson with animal husbandry and sampling. This study was made possible, in part, by contributions from The Linus Pauling Institute at Oregon State University.

Funding This work was supported primarily by PHS through the National Institutes of Health grant P01 CA90890; L.E.S. was also supported through T32 ES07060; E.P.M. was also supported by the T32 grant and by P42 ES016465. This study utilized Core facilities made available through the NIEHS Environmental Health Science Center, P30 ES00210, and the NIEHS Superfund Research Program, P42 ES016465.

Abbreviations

I3C	indole-3-carbinol
SFN	sulforaphane
DBC	dibenzo[<i>def,p</i>] chrysene
GFN	glucoraphanin
GSH	glutathione
CG	cysteine–glycine
Cys	cysteine
NAC	N-acetylcysteine
3-MC	3-methylcholanthrene
DMBA	7,12-dimethylbenz[a]anthracene
BaP	benzo[a]pyrene
PND	post natal day

References

- Anderson LM, Jones AB, Riggs CW, Ohshima M. Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. *Carcinogenesis*. 1985; 6:1389–1393. [PubMed: 4028336]
- Anderson LM, Ruskie S, Carter J, Pittinger S, Kovatch RM, Riggs CW. Fetal mouse susceptibility to transplacental carcinogenesis: differential influence of Ah receptor phenotype on effects of 3-methylcholanthrene, 7,12-dimethylbenz[a]anthracene, and benzo[a]pyrene. *Pharmacogenetics*. 1995; 5:364–372. [PubMed: 8747408]

- Aronchik I, Bjeldanes LF, Firestone GL. Direct inhibition of elastase activity by indole-3-carbinol triggers a CD40-TRAF regulatory cascade that disrupts NF-kappaB transcriptional activity in human breast cancer cells. *Cancer Res.* 2010; 70:4961–4971. [PubMed: 20530686]
- Bosetti C, Filomeno M, Riso P, Polesel J, Levi F, Talamini R, Montella M, Negri E, Franceschi S, La Vecchia C. Cruciferous vegetables and cancer risk in a network of case-control studies. *Annals Oncol.* 2012; 23:2198–2203.
- Bradlow HL. Review. Indole-3-carbinol as a chemoprotective agent in breast and prostate cancer. *In Vivo.* 2008; 22:441–445. [PubMed: 18712169]
- Buters JT, Mahadevan B, Quintanilla-Martinez L, Gonzalez FJ, Greim H, Baird WM, Luch A. Cytochrome P450 1B1 determines susceptibility to dibenzo [a, l]pyrene-induced tumor formation. *Chem. Res. Toxicol.* 2002; 15:1127–1135. [PubMed: 12230405]
- Calkins K, Devaskar SU. Fetal origins of adult disease. *Curr. Prob. Pediat. Adolesc. Hlth. Care.* 2011; 41:158–176.
- Castro DJ, Baird WM, Pereira CB, Giovanini J, Löhr CV, Fischer KA, Yu Z, Gonzalez FJ, Krueger SK, Williams DE. Fetal mouse Cyp1b1 and transplacental carcinogenesis from maternal exposure to dibenzo(a, l)pyrene. *Cancer Prev. Res.* 2008a; 1:128–134.
- Castro DJ, Yu Z, Löhr CV, Pereira CB, Giovanini JN, Fischer KA, Orner GA, Dashwood RH, Williams DE. Chemoprevention of dibenzo[a, l]pyrene transplacental carcinogenesis in mice born to mothers administered green tea: primary role of caffeine. *Carcinogenesis.* 2008b; 29:1581–1586. [PubMed: 18635525]
- Castro DJ, Löhr CV, Fischer KA, Pereira CB, Williams DE. Lymphoma and lung cancer in offspring born to pregnant mice dosed with dibenzo[a, l]pyrene: the importance of in utero vs. lactational exposure. *Toxicol. Appl. Pharmacol.* 2008c; 233:454–458. [PubMed: 18848954]
- Castro DJ, Löhr CV, Fischer KA, Waters KM, Webb-Robertson BJ, Dashwood RH, Bailey GS, Williams DE. Identifying efficacious approaches to chemoprevention with chlorophyllin, purified chlorophylls and freeze-dried spinach in a mouse model of transplacental carcinogenesis. *Carcinogenesis.* 2009; 30:315–320. [PubMed: 19073876]
- Cavalieri EL, Higginbotham S, RamaKrishna NV, Devanesan PD, Todorovic R, Rogan EG, Salmasi S. Comparative dose-response tumorigenicity studies of dibenzo[a, l]pyrene versus 7,12-dimethylbenz[a]anthracene, benzo[a]pyrene and two dibenzo[a, l]pyrene dihydrodiols in mouse skin and rat mammary gland. *Carcinogenesis.* 1991; 12:1939–1944. [PubMed: 1934274]
- Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Expression patterns of mouse and human CYP orthologs (families 1–4) during development and in different adult tissues. *Arch. Biochem. Biophys.* 2005; 436:50–61. [PubMed: 15752708]
- Clarke JD, Dashwood RH, Ho E. Multi-targeted prevention of cancer by sulforaphane. *Cancer Lett.* 2008; 269:291–304. [PubMed: 18504070]
- Clarke JD, Hsu A, Williams DE, Dashwood RH, Stevens JF, Yamamoto M, Ho E. Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice. *Pharm. Res.* 2011; 28:3171–3179. [PubMed: 21681606]
- Conaway CC, Getahun SM, Liebes LL, Pusateri DJ, Topham DK, Botero-Omary M, Chung FL. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutr. Cancer.* 2000; 38:168–178. [PubMed: 11525594]
- Davis CD, Zeng H, Finley JW. Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. *J. Nutr.* 2002; 132:307–309. [PubMed: 11823596]
- Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. U. S. A.* 1997; 94:10367–10372. [PubMed: 9294217]
- Fang C, Zhang QY. The role of small-intestinal P450 enzymes in protection against systemic exposure of orally administered benzo[a]pyrene. *J. Pharmacol. Exptl. Therap.* 2010; 334:156–163. [PubMed: 20400470]
- Fimognari C, Turrini E, Ferruzzi L, Lenzi M, Hrelia P. Natural isothiocyanates: genotoxic potential versus chemoprevention. *Mutat. Res.* 2011; 750:107–131. [PubMed: 22178957]
- Gerk PM, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J. Pharmacol. Exptl. Therap.* 2002; 302:407–415.

- Hanlon N, Coldham N, Gielbert A, Kuhnert N, Sauer MJ, King LJ, Ioannides C. Absolute bioavailability and dose-dependent pharmacokinetic behaviour of dietary doses of the chemopreventive isothiocyanate sulforaphane in rat. *Br. J. Nutr.* 2008; 99:559–564. [PubMed: 17868493]
- Hayes JD, Kelleher MO, Eggleston IM. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *Eur. J. Nutr.* 2008; 47(Suppl. 2):73–88. [PubMed: 18458837]
- Heidel SM, MacWilliams PS, Baird WM, Dashwood WM, Buters JT, Gonzalez FJ, Larsen MC, Czuprynski CJ, Jefcoate CR. Cytochrome P4501B1 mediates induction of bone marrow cytotoxicity and preleukemia cells in mice treated with 7,12-dimethylbenz[a]anthracene. *Cancer Res.* 2000; 60:3454–3460. [PubMed: 10910056]
- Ho E, Clarke JD, Dashwood RH. Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. *J. Nutr.* 2009; 139:2393–2396. [PubMed: 19812222]
- Hwang ES, Jeffery EH. Effects of different processing methods on induction of quinone reductase by dietary broccoli in rats. *J. Med. Food.* 2004; 7:95–99. [PubMed: 15117560]
- IARC. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC Monogr. Eval. Carcinog. Risks Hum. Suppl. 2010; 92:1–853.
- Kalpna Deepa Priya D, Gayathri R, Sakthisekaran D. Role of sulforaphane in the anti-initiating mechanism of lung carcinogenesis in vivo by modulating the metabolic activation and detoxification of benzo(a)pyrene. *Biomed. Pharmacother.* 2011; 65:9–16. [PubMed: 21177068]
- Kassie F, Matise I, Negia M, Upadhyaya P, Hecht SS. Dose-dependent inhibition of tobacco smoke carcinogen-induced lung tumorigenesis in A/J mice by indole-3-carbinol. *Cancer Prev. Res.* 2008; 1:568–576.
- Kerns ML, DePianto D, Dinkova-Kostova AT, Talalay P, Coulombe PA. Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:14460–14465. [PubMed: 17724334]
- Keum YS, Khor TO, Lin W, Shen G, Kwon KH, Barve A, Li W, Kong AN. Pharmacokinetics and pharmacodynamics of broccoli sprouts on the suppression of prostate cancer in transgenic adenocarcinoma of mouse prostate (TRAMP) mice: implication of induction of Nrf2, HO-1 and apoptosis and the suppression of Akt-dependent kinase pathway. *Pharm. Res.* 2009; 26:2324–2331. [PubMed: 19669099]
- Liu AG, Volker SE, Jeffery EH, Erdman JW Jr. Feeding tomato and broccoli powders enriched with bioactives improves bioactivity markers in rats. *J. Agric. Food Chem.* 2009; 57:7304–7310. [PubMed: 19650632]
- Luch A, Platt KL, Seidel A. Synthesis of fjord region tetraols and their use in hepatic biotransformation studies of dihydrodiols of benzo[c]chrysene, benzo[g]chrysene and dibenzo[a,l]pyrene. *Carcinogenesis.* 1998; 19:639–648. [PubMed: 9600349]
- Matusheski NV, Jeffery EH. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *J. Agric. Food Chem.* 2001; 49:5743–5749. [PubMed: 11743757]
- Miller MS, Leone-Kabler S, Rollins LA, Wessner LL, Fan M, Schaeffer DO, McEntee MF, O'Sullivan MG. Molecular pathogenesis of transplacentally induced mouse lung tumors. *Exptl. Lung Res.* 1998; 24:557–577. [PubMed: 9659583]
- Miyata M, Furukawa M, Takahashi K, Gonzalez FJ, Yamazoe Y. Mechanism of 7,12-dimethylbenz[a]anthracene-induced immunotoxicity: role of metabolic activation at the target organ. *Jpn. J. Pharmacol.* 2001; 86:302–309. [PubMed: 11488430]
- Myzak MC, Karplus PA, Chung FL, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer Res.* 2004; 64:5767–5774. [PubMed: 15313918]
- Nakamura BN, Mohar I, Lawson GW, Cortes MM, Hoang YD, Ortiz L, Patel R, Rau BA, McConnachie LA, Kavanagh TJ, Luderer U. Increased sensitivity to testicular toxicity of transplacental benzo[a]pyrene exposure in male glutamate cysteine ligase modifier subunit knockout (Gclm^{-/-}) mice. *Toxicol. Sci.* 2012; 126:227–241. [PubMed: 22253057]
- NCI. SEER Cancer Statistics Review, 1975–2008. National Cancer Institute; Bethesda, MD: 2011.

- Noyan-Ashraf MH, Wu L, Wang R, Juurlink BHJ. Dietary approaches to positively influence fetal determinants of adult health. *FASEB J.* 2005; 20:371–373. [PubMed: 16354723]
- Pappa G, Bartsch H, Gerhauser C. Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line. *Mol. Nutr. Food Res.* 2007; 51:977–984. [PubMed: 17628879]
- Park JY, Bjeldanes LF. Organ-selective induction of cytochrome P-450-dependent activities by indole-3-carbinol-derived products: influence on covalent binding of benzo[a]pyrene to hepatic and pulmonary DNA in the rat. *Chem. Biol. Interact.* 1992; 83:235–247. [PubMed: 1516151]
- Platt KL, Dienes HP, Tommasone M, Luch A. Tumor formation in the neonatal mouse bioassay indicates that the potent carcinogen dibenzo[def, p]chrysene (dibenzo[a, l]pyrene) is activated in vivo via its trans-11,12-dihydrodiol. *Chem. Biol. Interact.* 2004; 148:27–36. [PubMed: 15223354]
- Prahalad AK, Ross JA, Nelson GB, Roop BC, King LC, Nesnow S, Mass MJ. Dibenzo[a, l]pyrene-induced DNA adduction, tumorigenicity, and Ki-ras oncogene mutations in strain A/J mouse lung. *Carcinogenesis.* 1997; 18:1955–1963. [PubMed: 9364006]
- Priya DK, Gayathri R, Gunassekaran G, Murugan S, Sakthisekaran D. Chemopreventive role of sulforaphane by upholding the GSH redox cycle in pre- and post-initiation phases of experimental lung carcinogenesis. *Asian Pacif. J. Cancer Prev.* 2011a; 12:103–110.
- Priya DK, Gayathri R, Gunassekaran GR, Sakthisekaran D. Protective role of sulforaphane against oxidative stress mediated mitochondrial dysfunction induced by benzo(a)pyrene in female Swiss albino mice. *Pulmon. Pharmacol. Therapeut.* 2011b; 24:110–117.
- Ramesh A, Walker SA, Hood DB, Guillen MD, Schneider K, Weyand EH. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. *Internatl. J. Toxicol.* 2004; 23:301–333.
- Reed GA, Arneson DW, Putnam WC, Smith HJ, Gray JC, Sullivan DK, Mayo MS, Crowell JA, Hurwitz A. Single-dose and multiple-dose administration of indole-3-carbinol to women: pharmacokinetics based on 3,3'-diindolylmethane. *Cancer Epidemiol. Biomarkers Prev.* 2006; 15:2477–2481. [PubMed: 17164373]
- Richman EL, Carroll PR, Chan JM. Vegetable and fruit intake after diagnosis and risk of prostate cancer progression. *Internatl. J. Cancer.* 2012; 131:201–210.
- Sarkar FH, Li Y, Wang Z, Kong D. Cellular signaling perturbation by natural products. *Cell. Signl.* 2009; 21:1541–1547.
- Saw CL, Cintron M, Wu TY, Guo Y, Huang Y, Jeong WS, Kong AN. Pharmacodynamics of dietary phytochemical indoles I3C and DIM: induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates. *Biopharmaceut. Drug Dispos.* 2011; 32:289–300.
- Shi Z, Dragin N, Galvez-Peralta M, Jorge-Nebert LF, Miller ML, Wang B, Nebert DW. Organ-specific roles of CYP1A1 during detoxication of dietary benzo[a]pyrene. *Molec. Pharmacol.* 2010; 78:46–57. [PubMed: 20371670]
- Shimada T. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metabol. Pharmacokin.* 2006; 21:257–276.
- Shorey LE, Castro DJ, Baird WM, Siddens LK, Löhr CV, Matzke MM, Waters KM, Corley RA, Williams DE. Transplacental carcinogenesis with dibenzo[def, p]chrysene (DBC): timing of maternal exposures determines target tissue response in offspring. *Cancer Lett.* 2012; 317:49–55. [PubMed: 22085489]
- Siddens LK, Larkin A, Krueger SK, Bradfield CA, Waters KM, Tilton SC, Pereira CB, Löhr CV, Arlt VM, Phillips DH, Williams DE, Baird WM. Polycyclic aromatic hydrocarbons as skin carcinogens: comparison of benzo[a]pyrene, dibenzo[def, p]chrysene and three environmental mixtures in the FVB/N mouse. *Toxicol. Appl. Pharmacol.* 2012; 264:377–386. [PubMed: 22935520]
- Skupinska K, Misiewicz-Krzeminska I, Lubelska K, Kasprzycka-Guttman T. The effect of isothiocyanates on CYP1A1 and CYP1A2 activities induced by polycyclic aromatic hydrocarbons in Mcf7 cells. *Toxicol In Vitro.* 2009a; 23:763–771. [PubMed: 19362136]

- Skupinska K, Misiewicz-Krzeminska I, Stypulkowski R, Lubelska K, Kasprzycka-Guttman T. Sulforaphane and its analogues inhibit CYP1A1 and CYP1A2 activity induced by benzo[a]pyrene. *J. Biochem. Molec. Toxicol.* 2009b; 23:18–28. [PubMed: 19202560]
- Steinkellner H, Rabot S, Freywald C, Nobis E, Scharf G, Chabicovsky M, Knasmüller S, Kassie F. Effects of cruciferous vegetables and their constituents on drug metabolizing enzymes involved in the bioactivation of DNA-reactive dietary carcinogens. *Mutat. Res.* 2001; 480–481:285–297.
- Stresser DM, Williams DE, Griffin DA, Bailey GS. Mechanisms of tumor modulation by indole-3-carbinol. Disposition and excretion in male Fischer 344 rats. *Drug Metabol Dispos.* 1995; 23:965–975.
- Swinney RM, Beuten J, Collier AB III, Chen TT, Winick NJ, Pollock BH, Tomlinson GE. Polymorphisms in CYP1A1 and ethnic-specific susceptibility to acute lymphoblastic leukemia in children. *Cancer Epidemiol. Biomarkers Prev.* 2011; 20:1537–1542. [PubMed: 21586621]
- Uno S, Dalton TP, Derkenne S, Curran CP, Miller ML, Shertzer HG, Nebert DW. Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. *Molec. Pharmacol.* 2004; 65:1225–1237. [PubMed: 15102951]
- Uno S, Dalton TP, Dragin N, Curran CP, Derkenne S, Miller ML, Shertzer HG, Gonzalez FJ, Nebert DW. Oral benzo[a]pyrene in Cyp1 knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. *Molec. Pharmacol.* 2006; 69:1103–1114. [PubMed: 16377763]
- Vijayakrishnan J, Houlston RS. Candidate gene association studies and risk of childhood acute lymphoblastic leukemia: a systematic review and meta-analysis. *Haematol.* 2010; 95:1405–1414.
- Weng JR, Tsai CH, Kulp SK, Chen CS. Indole-3-carbinol as a chemopreventive and anti-cancer agent. *Cancer Lett.* 2008; 262:153–163. [PubMed: 18314259]
- Whyatt RM, Jedrychowski W, Hemminki K, Santella RM, Tsai WY, Yang K, Perera FP. Biomarkers of polycyclic aromatic hydrocarbon-DNA damage and cigarette smoke exposures in paired maternal and newborn blood samples as a measure of differential susceptibility. *Cancer Epidemiol. Biomarkers Prev.* 2001; 10:581–588. [PubMed: 11401906]
- Yu Z, Loehr CV, Fischer KA, Louderback MA, Krueger SK, Dashwood RH, Kerkvliet NI, Pereira CB, Jennings-Gee JE, Dance ST, Miller MS, Bailey GS, Williams DE. In utero exposure of mice to dibenzo[a, l]pyrene produces lymphoma in the offspring: role of the aryl hydrocarbon receptor. *Cancer Res.* 2006a; 66:755–762. [PubMed: 16424006]
- Yu Z, Mahadevan B, Löhr CV, Fischer KA, Louderback MA, Krueger SK, Pereira CB, Albershardt DJ, Baird WM, Bailey GS, Williams DE. Indole-3-carbinol in the maternal diet provides chemoprotection for the fetus against transplacental carcinogenesis by the polycyclic aromatic hydrocarbon dibenzo[a, l]pyrene. *Carcinogenesis.* 2006b; 27:2116–2123. [PubMed: 16704990]
- Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U. S. A.* 1992; 89:2399–2403. [PubMed: 1549603]
- Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc. Natl. Acad. Sci. U. S. A.* 1994; 91:3147–3150. [PubMed: 8159717]

Fig. 1.

Measurement of neonatal plasma SFN concentrations and correlation with maternal supplement levels. Values represent mean $\pm$ SEM for $n = 3$ litters per treatment. (A) Neonatal (PND0) plasma concentrations of SFN metabolites (GSH = glutathione, CG = cysteine-glycine, Cys = cysteine, NAC = N-acetylcysteine) were analyzed by LC-MS/MS. SFN-NAC was the dominant metabolite; SFN-CG and parent SFN were below detection. (B) Neonatal plasma concentrations of summed SFN metabolites positively correlated with levels of maternal dietary supplementation based on calculated diet concentrations (400 ppm SFN and COMBO: 600 ppm SFN + I3C) or measured concentration in whole broccoli sprout powder (Table 2).

Fig. 2.

Survival of offspring born to mothers given vehicle, DBC, or DBC with dietary supplementation. DBC was administered at 15 mg/kg by oral gavage on GD17 in corn oil. During gestation and lactation (GD9 – PND21), dams also received dietary supplementation as described in Materials and methods with (A) I3C-rich or (B) SFN-rich diets. “COMBO” signifies 500 ppm I3C + 600 ppm SFN. Treatment legends are arranged in descending order of survival at termination of the study.

Fig. 3.

Gender-specific survival of offspring exposed in utero and lactationally to DBC and dietary intervention. (A) I3C-rich diets (B) SFN-rich diets. “COMBO” signifies 500 ppm I3C + 600 ppm SFN. Treatment legends are arranged in descending order of survival at termination of the study. Number of pups per treatment is shown in Table 3.

Table 1

Designed composition of custom maternal diets based on AIN93G.

	CTRL	100 ppm I3C	500 ppm I3C	1000 ppm I3C	10% Brussels sprouts	10% broccoli sprouts	400 ppm SFN	500 ppm I3C + 600 ppm SFN "COMBO"
<i>Dietary composition (gram %)</i>								
Protein	20	20	20	20	17	16	20	20
Carbohydrate	64	64	64	64	61	61	64	64
Fat	7	7	7	7	7	6	7	7
<i>Base diet ingredient (gram/kg)</i>								
Casein	200	200	200	200	168	164	200	200
L-cysteine	3	3	3	3	3	3	3	3
Corn starch	397	397	397	397	375	388	397	397
Maltodextrin	132	132	132	132	132	132	132	132
Sucrose	100	100	100	100	93	98	100	100
Cellulose	50	50	50	50	22	34	50	50
Soybean oil	70	70	70	70	67	66.5	70	70
t-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Mineral mix S10022G	35	35	35	35	35	35	35	35
Vitamin mix V10037	10	10	10	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
<i>Active diet ingredient (gram/kg)</i>								
Indole-3-carbinol	0	0.1	0.5	1.0	0	0	0	0.5
N54 Brussels sprout powder	0	0	0	0	100	0	0	0
N216 broccoli sprout powder	0	0	0	0	0	100	0	0
Sulforaphane	0	0	0	0	0	0	0.4	0.6

Table 2

Whole food powder analysis of active ingredients.

	SFN ppm $\pm$ s.d. ^a	GFN ppm $\pm$ s.d. ^b	I3C ppm $\pm$ s.d. ^c
Broccoli sprout powder	1775 $\pm$ 66.3	12905 $\pm$ 223.8	n.d.
Brussels sprout powder	85.2 $\pm$ 1.5	n.d.	151 $\pm$ 10.7

^a SFN measured by HPLC–MS/MS following hydrolysis for 2 h with exogenous myrosinase source (0.64 units/mL).

^b Glucoraphanin measured by HPLC–MS/MS as described in Materials and methods.

^c Analysis by HPLC coupled to detection by UV–VIS (280 nm) and standard curve quantification.

Table 3

Demographics of offspring according to treatment group.

Treatment group	<u>Pups</u>	<u>Litters</u>	<u>Genotype</u>	<u>Gender</u>
	N	Mean size $\pm$ SEM (n)	<i>Ahr</i> ^{b-1/d} / <i>Ahr</i> ^{d/d} (sample size) ^a	Female/male
Corn oil CTRL	21	5.3 $\pm$ 1.4 (4)	1.10 (21)	1.63
DBC CTRL	26	5.2 $\pm$ 0.7 (5)	1.78 (25)	0.63
100 ppm I3C	30	5.0 $\pm$ 0.9 (6)	1.64 (29)	1.14
500 ppm I3C	46	7.7 $\pm$ 1.0 (6)	1.14 (45)	0.84
1000 ppm I3C	33	4.1 $\pm$ 0.9 (8)	0.83 (33)	2.00
10% Brussels sprouts	41	6.8 $\pm$ 0.7 (6)	0.54 (37)	1.28
10% broccoli sprouts	49	7.0 $\pm$ 0.3 (7)	1.15 (43)	0.69
400 ppm SFN	48	8.0 $\pm$ 0.5 (6)	0.95 (41)	1.40
500 ppm I3C + 600 ppm SFN ("COMBO") ^b	42	7.0 $\pm$ 1.1 (6)	0.56 (42)	1.33

^aSamples were not collected from a subset of animals due to mortality between health checks.^bThe COMBO group was made up so that I3C and SFN were equimolar.

Table 4

Lung tumor incidence and multiplicity in 10 month old survivors.

Treatment group	Incidence		Multiplicity		Un-adjusted p-value	Adjusted p-value ^a
	n	%	Range	Simple mean		
Corn oil CTRL	21	0	0	0.0	n/a	n/a
DBC CTRL	11	100	5–25	16.2	n/a	n/a
100 ppm I3C	10	100	4–30	17.6	0.488	0.961
500 ppm I3C	27	100	5–21	12.4	0.159	0.518
1000 ppm I3C ^b	16	100	5–28	11.3	0.111	0.398
10% Brussels sprouts	11	100	7–25	15.4	0.663	0.997
10% Broccoli sprouts	11	100	5–25	17.3	0.897	1.000
400 ppm SFN	10	100	4–35	14.9	0.649	0.996
500 ppm I3C + 600 ppm SFN	22	100	2–19	10.4	0.046	0.187

^a Adjusted (Dunnett method) for multiple pairwise comparisons of model estimated treatment means versus DBC CTRL.^b For the model estimated means for the 4 doses of I3C (0, 100, 500, 1000 ppm) there is evidence of a linear trend component ($p = 0.019$).