Online Supplementary Methods

Reagents

Withaferin A (WA; purity 97.9%) was purchased from ChromaDex (Irvine, CA). Other reagents were from the following sources: 3,3'-diaminobenzidine (DBC859 H, L10) was purchased from Biocare Medical (Concord, CA); mouse monoclonal anti-HER-2 (human epidermal growth factor receptor-2) antibody (e2-4001 + 3B5, 1:400 dilution) was purchased from ThermoFisher Scientific (Waltham, MA); mouse monoclonal anti-PCNA (proliferating cell nuclear antigen) antibody (PC10, 1:750 dilution) was purchased from Dako-Agilent Technologies (Carpinteria, CA); goat polyclonal anti-CD31 (platelet/endothelial cell adhesion molecule 1; M-20, 1:750 dilution) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibody against 8-hydroxy-2'-deoxyguanosine (8-OHdG; N45.1, 1:250 dilution) was purchased from JaICA-Japan Institute for the Control of Aging (Shizuoka, Japan); ApopTag® Plus Peroxidase *In Situ* Apoptosis detection kit (S7101) for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was purchased from EMD Millipore (Billerica, MA); D-sorbitol colorimetric assay kit (K631-100) was purchased from BioVision (Milpitas, CA); lactate assay kit (MAK064) was purchased from Sigma-Aldrich (St. Louis, MO).

WA Administration

The mice (5 mice/cage) were acclimated for 1 week and then placed on AIN-76A semi-purified diet (Harlan Laboratories, Indianapolis, IN) for 1 week before starting the treatments. The mice were maintained on the AIN-76A diet throughout the experimental duration. Control group of mice was injected intraperitoneal (i.p.) with 100 µL vehicle consisting of 10% dimethyl sulfoxide, 40% Cremophor EL (Sigma-Aldrich; 3:1 dilution with ethanol), and 50% phosphate-

buffered saline (PBS) three times/week (Monday, Wednesday, and Friday of each week). The WA treatment group of mice was administered i.p. with 100 µg WA (equates to about 4-5 mg WA/kg body weight) in 100 µL of the above mentioned vehicle three times/week (Monday, Wednesday, and Friday of each week). One mouse from the control group was removed from the study due to excessive body weight loss (final number of evaluable mice in the control group, n = 29). Three mice from the WA treatment group were removed from the study/analysis due to morbidity, injection-related lump or skin disorder (final number of evaluable mice in the WA treatment group, n = 32).

Tissue Collection and Processing

Plasma, mammary glands, and lung tissues were collected after sacrifice. The entire mammary tissue (all glands) from each mouse was submitted for histological evaluations in a single tissue block. Similarly, entire lung from each mouse was submitted for histological evaluations in a single tissue block. Both tissues were examined by a single 4-5 µm section. Serial sectioning was not performed. However, the method of histological examination was similar for both the test group and the control group. All mammary glands of every mouse and lung tissue from every mouse of both groups were examined by whole mount hematoxylin & eosin (H&E) staining. Mammary glands and lung tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and stained with H&E for microscopic examination. The tumor area (in 2 dimensions) was calculated on histological slides using on-screen drawing tool of the WebScope viewing software from Aperio (Vista, CA). The mean tumor area (tumor burden) was calculated from sum of the tumor area in an animal with microscopic tumor, and the average of tumor area over all the animals with microscopic tumors for each group. Lung metastasis count represents sum of metastatic foci in an animal with pulmonary metastasis, and that over all the animals with

metastasis for each group. Tumor tissues from mice with palpable tumors were snap-frozen in liquid nitrogen and stored at -80ºC for metabolomics and proteomics analyses, and measurement of complex III and complex IV activity. In some cases the tumor tissue was large enough to be used for multiple assays. If the tumor tissue was not large enough, only one assay was performed.

Pathological Characteristics of Mammary Lesions

The ductal carcinoma *in situ* (DCIS) lesions were characterized by ducts filled with monomorphic neoplastic epithelium showing one of the known patterns: cribriform, solid, micropapillary or papillary. The myoepithelium was identified (by H&E stain) in the duct periphery encircling the DCIS. Circumscribed papillary tumors (intracystic papillary carcinoma or encysted papillary carcinoma) lacked myoepithelial cells around the periphery of the lesion. Invasive carcinoma lesions were characterized by infiltration of neoplastic epithelial cells into normal breast tissue or mammary fat. These tumors lacked myoepithelium around the tumor cell nests.

Immunohistochemical Analyses

Mammary gland and lung sections were deparaffinized and rehydrated. The sections were then quenched with 3% hydrogen peroxide and blocked with normal goat serum (Dako-Agilent Technologies) diluted 1:67 in tris-buffered saline (TBS, Dako-Agilent Technologies) in a moist chamber at room temperature for 30 minutes. The sections were incubated for 60 minutes at room temperature with the desired primary antibody (anti-HER-2, anti-PCNA, anti-CD31, anti-8-OHdG) in PBS-based antibody diluting solution with background-reducing components (Dako-Agilent Technologies, S3022) and washed with TBS three times for 5 minutes each at room temperature. The sections were incubated for 30 minutes at room temperature with

appropriate biotinylated secondary antibody (ready-to-use LSAB2 horseradish peroxidase-conjugated streptavidin; Dako-Agilent Technologies, K1016). A characteristic brown color was developed with 3,3'-diaminobenzidine. Stained sections were examined under a Leica DC300F microscope at ×200 or ×400 magnifications. At least five nonoverlapping representative images were captured from each section, and analyzed using Image ProPlus 5.0 software (Media Cybernetics, Rockville, MD) for CD31-positive blood vessels or Aperio ImageScope v9.1 software (Aperio, Vista, CA) for HER-2 expression (using membrane intensity algorithm), PCNA (using nuclear algorithm), TUNEL-positive cells (using nuclear algorithm), and 8-OHdG level (using nuclear algorithm). Results for HER-2, PCNA, and 8-OHdG are expressed as H-score, which is a widely accepted method for semi-quantitative analysis. The H-score is based on intensity (0, 1+, 2+, and 3+) and % positivity (0-100%) and calculated using the formula: H-score = (% of negative cells × 0) + (% 1+ cells × 1) + (% 2+ cells × 2) + (% 3+ cells × 3). The TUNEL staining was performed as recommended by the supplier.

Measurement of Complex III and Complex IV Activity

Tumor tissues were homogenized in 200 µL STE buffer [250 mM sucrose, 10 mM Tris (pH 7.4), 1 mM ethylene glycoltetraacetic acid] at 4ºC and centrifuged at 1000×g for 3 minutes. The supernatant fraction was diluted 10-fold using STE buffer, and subjected to three consecutive cycles of freezing followed by thawing. Protein concentration was determined using the Bio-Rad colorimetric assay (Bio-Rad Laboratories, Hercules, CA). Activity of complex III (coenzyme Q:cytochrome *c* oxidoreductase or cytochrome bc_1 complex) was measured using oxidized cytochrome *c* and ubiquinol as substrates and by monitoring the reduction of cytochrome *c* at 550 nm. Diluted supernatant containing 10-30 µg protein was added to a reaction mixture containing 50 mM potassium phosphate (pH 7.4), 1 mM ethylenediaminetetraacetic acid, 5 mM

magnesium chloride, 2 mM potassium cyanide, 10 nM rotenone, and 15 µM oxidized cytochrome *c*. Reaction was initiated by the addition of 15 µM ubiquinol. The increase in absorbance at 550 nm was monitored for 3 minutes and the reduction of cytochrome *c* was expressed as *k*/min/mg protein. Activity of Complex IV (cytochrome *c* oxidase) was measured using reduced cytochrome *c* as the substrate and monitoring the oxidation of cytochrome *c* at 550 nm. Diluted supernatant containing 1-10 µg protein was equilibrated to 30ºC in 10 mM potassium phosphate (pH 7.0) and the reaction was initiated by the addition of 50 µM reduced cytochrome *c*. The decrease in absorbance at 550 nm was monitored for 3 minutes and the activity was expressed as *k*/min/mg protein.

Metabolomics

Each sample was accessioned into the Metabolon system, which was associated with the original source identifier only. Samples were handled on ice during preparation. Each tumor sample was weighed and placed in a 2 mL vial with two 3 mm zirconia beads, one 5/32" stainless steel ballcone, and one 1/8" ballcone for bead grinding. Water was added at .005 mL per mg tissue weight, caps were securely fastened, and tubes were transferred to a Genogrinder at 1350 strokes per minute for 5 minutes. The non-targeted metabolic profiling platform employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS^2) optimized for basic species, UHPLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed essentially as described previously (1,2). For each sample, 100 µL was used for analysis. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT) protein was precipitated from the plasma with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for

analysis on the three platforms. Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50 µL .1% formic acid in water (acidic conditions) or in 50 µL 6.5 mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC/MS/MS^2 analyses or derivatized to a final volume of 50 µL for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for one hour. In addition, three types of controls were analyzed in concert with the experimental samples: aliquots of a well-characterized human plasma pool served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Experimental samples and controls were randomized across platform run days.

For UHPLC/MS/MS^2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Milford, MA) and analyzed using an LTQ mass spectrometer (ThermoFisher Scientific) which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 *m/z* and alternated between MS and MS^2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (ThermoFisher Scientific) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range.

Compounds were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated

MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon, Inc. (3). At present more than 2500 commercially available purified standards are registered into system for distribution to both the LC and GC platforms for determination of their analytical characteristics. Compound abundance was quantified by calculating the area under the curve for the quantification ion of the compound. To aid data visualization, the raw area counts for each biochemical were rescaled by dividing each sample value by the median value for that specific biochemical. For statistical analyses, any missing values were assumed to be below the limits of detection and these values were imputed with the compound minimum (minimum value imputation). Initially, statistical analysis of log-transformed data was performed using "R" (http://cran.r-project.org/), which is a freely available, open-source software package. Welch's *t* tests were performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values (4). The metabolite levels are expressed as mean scaled intensities and their corresponding 95% confidence intervals (CI). Statistical significance of difference in metabolite levels between control group and WA treatment group was determined by two-sided Student's *t* test.

Two-Dimensional Gel Electrophoresis and Mass Spectrometry

Tumor tissues randomly selected from mice of control group (n = 3) and WA treatment group (n = 3) were used to determine treatment-related protein alterations by two-dimensional gel electrophoresis followed by MALDI-TOF/TOF (Applied Biomics, Hayward, CA). The sample size for the proteomics profiling was based on availability of tissue specimens and our previous experience and success with similar work using three mice per group (5,6). Tumor tissues were washed with the washing buffer (10 mM Tris-HCl, 5 mM magnesium acetate, pH 8.0) three times to remove the contaminating blood. To 10 mg of tumor tissue, 200 µL of 2-dimensional

cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) was added. Protein concentration was measured using Bio-Rad protein assay method. Gel layout was as follows: gel-1: animal #874 (control group), animal #762 (WA treatment group), and internal standard; gel-2: animal #875 (control group), animal #900 (WA treatment group), and internal standard; and gel-3: animal #880 (control group), animal #NT/B6 (WA treatment group), and internal standard. For each sample, 30 µg of protein was mixed with 1 µL of diluted CyDye, and kept in dark on ice for 30 minutes. Samples from each pair were labeled with Cy3 and Cy5, and the internal standard was labeled with Cy2 and run on every gel. The labeling reaction was stopped by adding 1 µL of 10 mM lysine to each sample, and incubating in dark on ice for additional 15 minutes. The labeled samples were then mixed together. The 2× sample buffer [8 M urea, 4% CHAPS, 20 mg/mL dithiothreitol (DTT), 2% pharmalytes and trace amount of bromophenol blue], 100 µL destreak solution and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT, 1% pharmalytes and trace amount of bromophenol blue) were added to the labeling mixture to make the total volume 250 µL. Samples were mixed and spun well before loading into the strip holder. Isoelectric focusing was performed in dark at 20°C. Immediately after sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% gels, the images were scanned using Typhoon TRIO (GE Healthcare). The images were analyzed with the use of Image Quant software (version 6.0, GE Healthcare), and subjected to in-gel analysis and cross-gel analysis with the use of DeCyder software (version 6.5, GE Healthcare). The cut-off criteria for the spot selection and protein identification were 1.3-fold difference in spot intensity (ratio of WA treatment group/control group) and a *P* value of .10 or less (two-sided Student's *t* test). A total of 65 spots from the 2-dimensional gel electrophoresis adhering to the above criteria were identified (Table 1). The spots of interest were picked using Ettan Spot Picker based on the

DeCyder analysis and spot picking design by DeCyder software. The picked spots were subjected to in-gel trypsin digestion, peptide extraction, and desalting followed by MALDI-TOF/TOF to identify the desired protein spot. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). MALDI-TOF mass spectra were acquired in positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues.

Cluster Analysis for Protein Alterations

Cluster analysis of identified proteins was performed using a public bioinformatics tool The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) (7). All the proteins entered into the analysis were first annotated into > 40 categories with gene ontology (GO) terms, including protein-protein interactions, protein functional domains, disease associations, bio-pathways, sequence general features, homologies, gene functional summaries, gene tissue expressions, and literatures. The software uses a novel algorithm to measure the relationships among the annotation terms based on the degrees of their co-association genes to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups.

References

1. Ohta T, Masutomi N, Tsutsui N, et al. Untargeted metabolomic profiling as an evaluative tool of fenofibrate-induced toxicology in Fischer 344 male rats. *Toxicol Pathol.* 2009;37(4):521-535.
2. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem.* 2009;81(16):6656-6667.
3. DeHaven CD, Evans AM, Dai H, Lawton KA. Organization of GC/MS and LC/MS metabolomics data into chemical libraries. *J Cheminform.* 2010;2(1):9.
4. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A.* 2003;100(16):9440-9445.
5. Powolny AA, Bommareddy A, Hahm ER, et al. Chemopreventative potential of the cruciferous vegetable constituent phenethyl isothiocyanate in a mouse model of prostate cancer. *J Natl Cancer Inst.* 2011;103(7):571-584.
6. Singh SV, Kim SH, Sehrawat A, et al. Biomarkers of phenethyl isothiocyanate-mediated mammary cancer chemoprevention in a clinically relevant mouse model. *J Natl Cancer Inst.* 2012;104(16):1228-1239.
7. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;4(1):44-57.