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1. INTRODUCTION:

My long-term career goal is to be a leader in basic and translational breast cancer research, and have an impact on the prevention of breast cancer. To achieve the goal of this journey, a valuable postdoctoral training in breast cancer research in a setting with experienced, well established investigators is very important. The BC103928 Postdoc Fellowship Award granted me the opportunity to deepen my understanding of the mechanisms and underlying role of estrogens in the process of carcinogenesis leading to breast cancer. Elevated levels of estrogens have been recognized as a determinant of risk of breast cancer. Two separate but complementary mechanisms for estrogen carcinogenesis have been advanced. One is the estrogen receptor(ER)dependent pathway, by which Estradiol (E₂) can bind to ER-alpha, leading to altered gene expression and increased cell proliferation accompanied by acquisition of spontaneous mutations. The other one is an ER-independent pathway, by which the estrogen oxidation product, catechol estrogen-3,4-quinone (CE-3,4-Q), can bind to DNA to form adenine or guanine adducts and cause depurination, leading to critical mutations initiating breast carcinogenesis. My research mainly focuses on estrogen carcinogenesis via ER-independent pathway. During my PhD training in Dr. Eleanor Rogan's group, I did several breast cancer case-control studies. I found higher levels of depurinating estrogen-DNA adducts in urine and serum from women at high risk for or diagnosed with breast cancer compared to women at normal risk. Such studies indicate that CE-3,4-Q plays very important role in the initiation and progression of breast cancer. The findings support that the depurinating estrogen-DNA adducts could be biomarkers of risk of developing breast cancer. The goal of the study is to target formation of depurinating estrogen-DNA adducts for prevention of breast cancer. Levels of depurinating estrogen-DNA adducts are strongly influenced by the balance of enzymes involved in their bioactivation to reactive intermediates and their detoxification. glutathione-S-transferases Detoxication enzymes such as (GSTs) and NAD(P)H:guinone oxidoreductase1 (NQO1) can decrease steady-state levels of CE-3,4-Q and, thus, the resultant depurinating estrogen-DNA adducts (Fig.1). Sulforaphane (SFN), an isothiocvanate found in cruciferous vegetables, is the ideal candidate for blocking estrogen-DNA adduct formation as SFN is an inducer of detoxication enzymes such as NQO1 and GST through activation of the antioxidant response element (ARE) -Keap1- Nrf2 signaling pathway. To evaluate the hypothesis that SFN may alter estrogen metabolism and thus protect against estrogen-mediated DNA damage and carcinogenesis, cell model, animal model and human samples from clinical settings have been used for the study.



Fig. 1. Pathway for formation of estrogen depurinating DNA adducts. E_2 or E_1 can be oxidized to CE-3,4-quinone, which can bind to DNA to form 4-OHE_{1/2}-1- N3Adenine or 4-OHE_{1/2}-1-N7Guanine adducts. NQO1 reduces CE-3,4-

quinones back to catechols and GST catalyzes the conjugation of CE-3,4-quinones with glutathione, whereas COMT catalyzes the methylation of $4-OHE_{1/2}$ to $4-OCH_3E_{1/2}$.

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

2. KEYWORDS:

Sulforaphane; NQO1; GST; depurinating estrogen-DNA adducts; Keap1-Nrf2; breast cancer; chemoprevention; UPLC-MS/MS.

3. OVERALL PROJECT SUMMARY:

In summary of the overall projects that I have conducted during the award period (from Sep., 15, 2011 to Sep., 14, 2014) at University of Pittsburgh, I have completed majority of the research tasks and completed all the training tasks. For some of the research tasks, following the guidance from my Postdoctoral mentor, Dr. Thomas W. Kensler, I have modified it according to availability of standards or the data I have obtained. The results from cell and animal studies indicate that SFN alters estrogen metabolism and thus protect against estrogen-mediated DNA damage and carcinogenesis. SFN is a promising chemoprevention agent against breast cancer. SFN modulates not only estrogen protective pathway enzymes including NQO1 and GST, but also estrogen oxidation pathway enzymes such as CYP1A1 and CYP1B1. The study indicates that Keap1-Nrf2 pathway may account for some, but not all of the protective effects of SFN against estrogen-mediated DNA damage.

3.1 Summary of the Major Research Tasks

3.1.1 Major Research Task 1

The original major research task 1 was to validate methodologies for quantifying estrogen metabolites and depurinating DNA adducts using isotope dilution mass spectrometry in estradiol (E_2) - treated MCF-10A cells undergoing pharmacologic or genetic manipulation to alter estrogen quinone metabolism (month 1-18).

For this task, I have to modify it due to the limitation of the availability of isotope labeling depurinating estrogen-DNA adducts. I have completed all three sub-tasks in task1 and obtained results. The manuscript from the results of task1a is under preparation and expected to submit in February 2014. The results of task 1b and 1c have been published in Carcinogenesis in 2013 (please see reference1).

Summary of Current Objectives of Task1a. Obtain or synthesize heavy isotope estrogen adduct standards and optimize solid phase extraction methods for enrichment of estrogen metabolites and depurinating DNA adducts from media and cells. (month 1-<u>36</u>)

For Task 1a, I have obtained estrogen metabolites and depurinating DNA adducts from my previous PhD mentor, Dr. Eleanor Rogan's group at University of Nebraska Medical Center at the beginning of the award in September 2011. However, isotope labeling depurinating estrogen-DNA adducts are not available from Dr. Rogan's group, neither commercially available. I contacted with several companies regarding the possibility to synthesize heavy isotope labeling estrogen DNA adducts and got the feedback that either they have no capability of synthesizing these adducts or they offer very expensive price with long term (around 1-2 years), in addition, they can't guarantee the success. So my Postdoctoral mentor, Dr. Thomas W. Kensler suggests me to start the project by using non-isotope labeling estrogen DNA adducts to establish ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) methodologies in month 1-4. During this period, I have optimized solid phase extraction methods for enrichment of estrogen metabolites and depurinating DNA adducts from cell culture media (see reference 1). I used this methodology for completion of the task 1b and 1c in month 5 – 18 (Jan., 2012 - March, 2013) according to the timeline.

In 2013, with the collaboration with Dr. Steven Woodcock, who is an organic chemist in Dr. Bruce Freeman's group at University of Pittsburgh, I start synthesizing heavy isotope labeling estrogen DNA adducts. With the support from my Postdoctoral mentor – Dr. Thomas W. Kensler and our department chair, Dr. Bruce Freeman, I and Dr. Woodcock have successfully synthesized all four depurinating estrogen-DNA adducts. We have successfully synthesized depurinating estrogen-DNA adducts and heavy isotope labeling estrogen-DNA adducts. The synthesizing project in task 1a lasted total 14 months from July, 2013 to September 2014.

To establish and optimize the synthesis procedure, we firstly synthesized four major depurinating estrogen-DNA adducts including $4-OHE_{1/2}-1-N3Ade$ and $4-OHE_{1/2}-1-N7Gua$ without stable isotope labeling. The methods are the followings:

Summary of the Methods of Task1a:

Synthesis of 4-OHE_{1/2}; CE-3,4-Q and 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua

Catechol estrogens including 4-hydroxyestrone (4-OHE₁) and 4-hydroxyestradiol (4-OHE₂) were firstly synthesized by using 2-iodoxybenzoic acid (IBX) as the oxidizing agent and E₁ or E₂ [2]. Then estrone 3,4-quinone (E₁-3,4-Q) and 17 β -estradiol 3,4quinone (E₂-3,4-Q) were synthesized using activated manganese dioxide (MnO₂) as oxidizing agent. The protocol was modified according to the publication [3]. A suspension of 4-OHE₁ or 4-OHE₂ (0.17mmole) in 5mL of acetonitrile (CH₃CN) was cooled to 0 °C prior to the addition of activated MnO₂ (1.15 mmol). The suspension was stirred for 10 min and then filtered directly into a stirred solution of adenine (Ade) (1.11 mmol) or deoxyguanosine (dG) (0.56 mmol) in a solvent mixture of 10 mL of acetic acid (CH₃CO₂H) / water (H₂O) (1:1, v/v). Aliquots were removed for Thin-Layer Chromatography (TLC) analysis at 1, 2, 5 and 12h to monitor the course of the reaction. TLC was performed on pre-coated silica gel F-254 plates and visualized by 254nm UV light. After overnight (12h) at room temperature, the solvents were removed under reduced pressure and the crude product is dissolved in methanol (CH₃OH). The product was then isolated via preparative reverse phase High Performance Liquid Chromatography (HPLC) to afford 4-OHE₁-1-N3Ade, or 4-OHE₂-1-N3Ade, or 4-OHE₁-1-N7Gua or 4-OHE₂-1-N7Gua.

Purification of 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua by preparative HPLC

Separation of target compounds was performed after synthesis. Crude reaction mixtures were purified by preparative reverse phase HPLC using a Waters (Milford, MA)

preparative LC system equipped with a Waters 2487 Dual λ absorbance detector. For detecting 4-OHE₁-1-N3Ade or 4-OHE₂-1-N3Ade, 270nm was used to monitor the eluent. For detecting 4-OHE₁-1-N7Gua or 4-OHE₂-1-N7Gua, 290 nm was used to monitor the eluent. A YMC ODS-AQ, 120Å, 5µ column (250×20mm, Allentown, PA) was eluted with 95% water containing 0.1% acetic acid and 5% acetonitrile containing 0.1% acetic acid at the beginning, followed by a 35-min linear gradient to 45% water, then followed by a 25 min linear gradient to 100% acetonitrile at a flow rate of 20 mL/min. The fraction eluents with target compound were collected and identified by Nuclear magnetic resonance (NMR) and UPLC-MS/MS, then dried with N₂ gas.

<u>Characterization of 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua by NMR and UPLC-MS/MS spectroscopy</u>

The parameters of characterization of $4-OHE_{1/2}-1-N3Ade$ and $4-OHE_{1/2}-1-N7Gua$ by NMR will follow references [2, 4]. For mass spectroscopy, the following parameters are used:

4-OHE₂-1-N3Ade, MS, $[M + H]^+$, $C_{23}H_{27}N_5O_3$: calculated *m/z* 422.22. 4-OHE₁-1-N3Ade, MS, $[M + H]^+$, $C_{23}H_{25}N_5O_3$: calculated *m/z* 420.21. 4-OHE₂-1-N7Gua, MS, $[M + H]^+$, $C_{23}H_{28}N_5O_4$: calculated *m/z* 438.21. 4-OHE₁-1-N7Gua, MS, $[M + H]^+$, $C_{23}H_{26}N_5O_4$: calculated *m/z* 436.20.

Synthesis of stable isotope labeled 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua.

We synthesized ¹⁵N5 and ¹³C5 DNA adduct standards using methods without labeling listed above. The mass difference of 10 will make it easy to quantify both internal standard and biological analytes in the bio-samples.

Methods of synthesis of stable isotope labeled 4-OHE_{1/2}-1-N3Ade

Adenosine 5'-monophosphate (AMP) (U-¹³C-10; U-¹⁵N-5) was obtained from Cambridge Isotope Laboratories, Inc (Tewksbury, MA). 4-OHE₁ and 4-OHE₂ were either synthesized by Dr. Woodcock or obtained from Steraloids. To convert stable isotope labeled AMP to the free stable isotope labeled Ade base, U-¹³C-10; U-¹⁵N-5 AMP 60mg were hydrolyzed in 12M HCl for 3h at room temperature. The solvent was removed under reduced pressure with a SpeedVac. The hydrolyte was reconstituted in 1mL of water and purified by preparative HPLC. Then the synthesis of ¹³C-5; ¹⁵N-5 labeled 4-OHE_{1/2}-1-N3Ade will be carried out on the basis of above unlabeled method and publication [5]. The mass shift is 10, as what we expected. [M+1] for heavy isotope labeled 4-OHE₁-1-N3Ade is 430; [M+1] for heavy isotope labeled 4-OHE₂-1-N3Ade is 432.

Methods of synthesis of stable isotope labeled 4-OHE_{1/2}-1-N7Gua

2'-Deoxyguanosine (¹³C-10; ¹⁵N-5) was obtained from Cambridge Isotope Laboratories. 2'-Deoxyguanosine- 5'-monophosphate sodium salt (¹³C-10; ¹⁵N-5) was obtained from Sigma Aldrich (St. Louis, MO). 4-OHE₁ and 4-OHE₂ were either synthesized by Dr. Woodcock or obtained from Steraloids. The synthesis of ¹³C-5; ¹⁵N-5 labeled 4-OHE_{1/2}-1-N7Gua were carried out on the basis of above unlabeled method and publication [6]. The mass shift is 10, as what we expected. [M+1] for stable isotope labeled 4-OHE₁-1-N7Gua is 446; [M+1] for heavy isotope labeled 4-OHE₂-1-N7Gua is 448.

Methods of solid phase extraction for purification of DNA adducts from cell culture medium

During Sep. 15, 2011 – Sep. 14, 2012, I have developed a solid phase extraction method to partially purify estrogen metabolites and depurinating DNA adducts from cell culture medium. After adjusting the pH to 7, cell culture media (10 mL/sample) was loaded onto phenyl cartridges (Agilent Technologies) that were preconditioned with methanol and water. Extracts were eluted, lyophilized, redissolved in a methanol:water 50:50 mixture containing 0.1% formic acid and finally subjected to UPLC-MS/MS analysis. (Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

Summary of the Results, Progress and Accomplishments of Task1a

In summary of Task1a, heavy isotope estrogen adduct standards were obtained and synthesized. Solid phase extraction methods for enrichment of estrogen metabolites and depurinating DNA adducts from media and cells were optimized. However, the time line of this aim has been modified from month 1-4 to month 1-36. Although four estrogen-DNA adducts have been successfully synthesized, the yield of the synthesis still needs to be further optimized after the ending of this award. The manuscript of this project is under preparation and is expected to be submitted in February 2015. The personnel involved in the synthesis project are Dr. Li Yang, Dr. Steven Woodcock (Research Associate in Dr. Bruce Freeman's group) and Miss Sonia Salvatore (technician in Dr. Bruce Freeman's group), Dr. Thomas W. Kensler and Dr. Bruce Freeman in department of Pharmacology & Chemical Biology at University of Pittsburgh. The examples of full scan MS/MS daughter ion mass spectra of adducts are in the Fig.2A and 2B.



Fig.2. Full scan MS/MS daughter ion mass spectra of $4-OHE_{1/2}-1-N3Ade$. A) $4-OHE_2-1-N3Ade [M + H]^+ = 422.2$; B) $4-OHE_1-1-N3Ade$. [M + H]⁺=420.2.

Summary of the Current Objectives of Task1b. <u>Measure estrogen metabolites and</u> depurinating DNA adducts in culture media from MCF-10A cells treated with vehicle or sulforaphane (SFN) and E₂. Keap1, Nrf2, NQO1 and GST levels/activities and mRNA

transcript expression will be assayed for pharmacodynamic profiling of SFN (month 5-12)

Summary of the Current Objectives of Task1c. <u>Measure estrogen metabolites and</u> depurinating DNA adducts in culture media from MCF-10A cells, in which expression of Keap-1, Nrf2, or NQO1 have been knocked down using siRNA. In addition, Keap1, Nrf2, NQO1 and GST levels/activities and mRNA transcript expression will be assayed and related to estrogen biomarker levels. (month 12-18)

For Task1b and 1c, I have completed the project according to the original plan. I have used pharmacological approach - SFN to modulate estrogen metabolism and estrogen depurinating DNA adducts in MCF-10A cell model. The results show that SFN modulates estrogen metabolism leading to diminished formation of estrogen-DNA adducts by up-regulating antioxidant genes including *NQO1* and *GST*. NQO1 reduces the carcinogenic estrogen metabolite, CE-3,4-Q, to catechols, while GSTs detoxify it through nucleophilic addition. CE-3,4-Q can bind with DNA to form depurinating DNA adducts, leading to DNA damage via an ER independent pathway. Thus, the results from cell study suggest that SFN can be an ideal chemoprevention agent to block estrogen-mediated carcinogenesis.

It has been reported that SFN releases Nrf2 from the fate of degradation by disrupting the association of Cul3 ubiquitin ligase with KEAP1, leading to Nrf2's translocation into the nucleus to induce the transcription of series antioxidant genes such as *NQO1* and *GST*.

To further confirm the above findings that KEAP1-Nrf2 pathway might play a role in modulation of estrogen metabolism and reducing estrogen depurinating DNA adducts, I have continued the project by using a genetic approach – siKEAP1 knock down in MCF-10A cells during Sep. 15, 2012 – Sep. 14, 2013. MCF10A cells were treated with either scrambled or siKEPA1 knock down and E_2 (10µM) or its proximate carcinogen 4-OHE₂ (10µM). With Dr. Thomas W. Kensler's approval, I have added 4-OHE₂ treatment into the study because we can escape the metabolism of E_2 to 4-OHE₂ to observe the modulation of estrogen DNA adducts in more direct way and also the levels of DNA adducts are also higher.

Methods of task1b and 1c

The timeline for treatments of MCF-10A cells in the pharmacological and genetic approaches is the in the Figure 3.



Fig.3. Timeline for treatment of MCF-10A

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041)

Cell culture

MCF-10A cells were obtained from the American Type Culture Collection and cultured in estrogen-free medium at 37°C in a humidified incubator containing 5% CO₂. Cell culture medium was prepared by a phenol red-free mixture of Dulbecco's modified Eagle's media and Ham's nutrient mixture F-12 media (Mediatech, Manassas, VA) containing 20 ng/ml epidermal growth factor, 10 µ g/ml insulin, 0.5 µ g/ml hydrocortisone, 100 ng/ml cholera toxin and 5% charcoal-stripped fetal bovine serum (Invitrogen). For SFN treatment, cells were seeded in 10 cm dishes $(1 - 2 \times 10^6 \text{ cells}/10)$ ml media/dish) overnight and then treated with either vehicle or SFN for 24 h. After discarding the media, all plates were fed with fresh cell culture media containing 10 µ M E₂ or 4-OHE₂ for 24 h and then retreated with vehicle or SFN for another 24 h without changing cell culture media. SFN and E₂ were dissolved in dimethyl sulfoxide and 4-OHE₂ was dissolved in ethanol; the final vehicle concentration in the media was <0.05% (v/v). For siRNA knockdown of KEAP1, cells were seeded in 10 cm dishes $(1-2 \times 10^6)$ cells/10 ml medium/dish) overnight and were transfected at 30-40% confluence with siRNA human KEAP1(J-012453-07) or scrambled siRNA control(D-001810-01) from Thermo Scientific (Dharmacon, Lafayette, CO) with lipofectamine 2000 for 48 h following the manufacturer's protocol (Figure 2). Confirmatory experiments were conducted using a shRNA construct for KEAP1 (shRNA against KEAP1 was provided by Dr. Yong Liao). The cell culture media were collected with 2 mg/mL ascorbic acid added and either processed immediately or frozen at -80°C prior to assay for estrogen metabolites and depurinating DNA adducts. Cells were harvested for RNA, protein and activity assays.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) or 5 PRIME PerfectPure RNA Cell & Tissue Kit (5PRIME, Gaithersburg, MD), from which complementary DNA was synthesized using the qScript[™] cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD). PCR was carried out in a 20 µl volume including each target primer, complementary DNA and iQ[™] SYBR® Green Supermix (Bio-Rad, Hercules, CA) and run in a Bio-Rad Thermal Cycler (Bio-Rad, Hamburg, Germany). Foldchange values were determined using the 2- \triangle \triangle Ct relative quantification method. The amplified products were electrophoresed on agarose gel and stained with ethidium bromide. PCR primers were as follows: forward 5 ' -TGACAATGAGGTTTCTTCGG-3' and reverse 5' -TCTGTCAGTTTGGCTTCTGG-3' for human Nrf2, forward 5' -ACGTCCTTGGAGGCTATGAT-3' and reverse 5' -TCTGCTGGTCAATCTGCTTC-3 for KEAP1, 5 ′ human forward CGCTTCTCTTGGAGGA ATGT-3' and reverse 5' -TCCACCACCTCCCTGTATTC-3 for humanCOMT, forward 5' -TTCCGGAGTAAGAAGGCAGT-3' and reverse 5' -GGAGTGTGCCCAATGCTAT-3 for human NQ01. forward 5 TAAAGGAGAGAGCCCTGATTG-3 / and reverse 5 ' -TTCAAAGGCAGGGAAGT AGC-3' for human GSTA1 and forward 5' -GGACTCATGACCACAGTCCA-3' and reverse 5 ' -CTGCTTCACCACCTTCTTGA-3 ' for glyceraldehydes 3-phosphate dehydrogenase.

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041)

Western blots

After treatment, cells were harvested and then lysed in RIPA buffer with protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) and unlysed cellular debris removed by centrifugation. Protein concentrations were determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Western blot procedures followed the ABC protocol. Samples were electrophoresed on sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline with 0.05% Tween 20 with 5% non-fat milk, incubated with primary antibodies and then incubated with a peroxidaseconjugated secondary antibody after extensive washing. Dilutions of primary anti-KEAP1, Nrf2, NQO1 (Santa Cruz Biotechnology), CYP1B1 (Genetest, Bedford, MA), COMT, GSTA1 and β -actin (Sigma) antibodies were made in blocking solution (5% non-fat dry milk in Tris-buffered saline). The blots were incubated with Western Lightning® Plus-ECL solution (PerkinElmer, Waltham, MA) and visualized with X-ray film. Intensities of the bands were quantified by Bio-Rad Quantity One® software (Bio-Rad, Hercules, CA). The densitometry ratios for treated samples compared with controls were determined for three biological replicates and normalization was to β -actin.

NQO1 activity

MCF-10A cells were washed three times with 0.25 M sucrose/10 mM potassium phosphate (pH 7.2), collected from the plates by scraping, frozen in liquid nitrogen and stored at -80° C for assay as described previously (7).

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041)

UPLC-MS/MS

I have established a UHPLC-MS/MS method to separate and quantify estrogen metabolites and depurinating DNA adducts. Analyses were conducted using selected reaction monitoring with a triple stage quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific) by using heated electrospray ionization in positive ion mode. The mass spectrometer was interfaced to a UPLC system consisting of an Accela quaternary pump (Thermo Scientific) used for the chromatographic separation, and a Thermo Pal autosampler (HTC PAL, Zwingen, Switzerland). A Hypersil Gold column (1.9 µm, 100 × 2.1 mm, Thermo Electron) was used for separation at a flow rate of 0.5 ml/min. The gradient started with 95% A (0.1% formic acid in H₂O) and 5% B (0.1% formic acid in CH₃CN), changed to 80% A over 1 min, changed to 79% A over 4 min, followed by a 4 min linear gradient to 30% A, changed to 2% A for 2 min, then changed back to the original conditions with a 3 min hold, resulting in a total separation time of 14 min. For all the studies, a methanol:water (1:1) mixture with 0.1% formic acid was used as the carrier solution. A signal-to-noise ratio of 3 was used as the limit of detection for each compound. Experiments were performed by applying a capillary (ion transfer tube) temperature of 380°C, vaporizer temperature 398°C, sheath gas pressure (arbitrary units) 50, auxiliary gas pressure (arbitrary units) 20, spray voltage 3.98 kV and collision gas pressure 1.5 mTorr. The collision energy for each compound is listed in Table1. The coefficient of variance for all analytes was <4%. The Xcalibur software (Thermo Scientific) was used to process and quantify the acquired data of estrogen metabolites. The Mass spectrometry parameters for all the compounds are listed in Table1).

Name	Parent	Product	Collision energy	Limit of detection (fmol)
4-OCH ₃ E ₁	301.18	189.12	21	166.0
4-OCH ₃ E ₂	303.15	137.10	23	329.9
4-OHE ₁ -2-glutathione	592.16	317.06	23	84.4
4-OHE,-2-glutathione	594.17	319.08	24	168.3
4-OHE ₁ -1-N7Guanine	436.20	152.02	39	2.8
4-OHE,-1-N7Guanine	438.21	272.12	35	2.8
4-OHE ₁ -1-N3Adenine	420.20	296.09	44	2.9
4-OHE-1-N3Adenine	422.16	136.00	47	2.9
2-OHE, -1-N3Adenine	420.20	136.04	31	1.4
2-OHE-1-N3Adenine	422.22	136.02	28	1.4

Table1. Mass Spectrometry Parameters

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

Statistics

The data are presented as means \pm SE of at least three independent experiments. Comparisons between two groups were analyzed using the Student's t-test, and significance was established at P < 0.05 using Prizm 5 software.

Summary of the Results, Progress and Accomplishments of Task1b and 1c

The results show that both of SFN and siKEAP1 have similar effect to decrease the level of estrogen DNA adducts after treatment with either E_2 or 4-OHE₂. As what we expected, the levels of estrogen DNA adducts are higher in 4-OHE₂ treatment than E_2 treatment, which further support our conclusion that SFN can modulate estrogen DNA adducts by up-regulation of NQO1 and GST.

Treatment of MCF-10A cells with SFN led to induction of *GSTA1* and *NQO1* transcripts but no changes in expression levels of two other genes known to influence E_2 metabolism, namely *CYP1B1* and *COMT*, were observed (Figure 4A). These inductions exhibited a dose response, with minimal induction observed with 3 µM, moderate with 7 µM and near maximal with 10 µM SFN (data not shown). As expected, there were also no changes in the transcript levels of *KEAP1* or *NRF2*. SFN treatment significantly elevated NQO1 protein level 3.0-fold (P < 0.01; SFN treatment versus vehicle, Figure 4B) and its specific activity 2.7-fold (P < 0.01; SFN treatment versus vehicle, Figure 4C). Although no change of COMT messenger RNA level was detected, a significant 2.4-fold increase in COMT protein (P < 0.05; SFN treatment versus vehicle, Figure 4B) was observed. CYP1B1 protein was significantly decreased by 50% with SFN treatment (Figure 4B) (P < 0.05; SFN treatment versus vehicle). Thus, it appears that SFN influences the expression of E_2 metabolizing enzymes through both transcriptional and post-transcriptional mechanisms. As expected, SFN leads to increases in intracellular concentrations of reduced glutathione in MCF-10A cells (1.48-fold).



Fig. 4. Effects of SFN on transcript, protein and activities of enzymes metabolizing E2 or E1. (**A**) Effect of SFN on transcripts levels of estrogen metabolism enzymes. (**B**) Effect of SFN on protein levels of estrogen metabolism enzymes. (**C**) Effect of SFN on NQO1 activity. MCF-10A cells were treated with 10 μ M SFN as described in methods. Values are mean ± SE of three independent experiments. *Differs from vehicle control, *P* < 0.05.

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

Since SFN is a well-characterized activator of Nrf2 signaling in MCF-10A and other mammary cells (7, 8), the influence of siKEAP1 knockdown, and hence genetic activation of the pathway, was evaluated. As shown in Figure 6, transcript (Figure 5A), protein (Figure 5B) and specific activity of NQO1 (Figure 5C) significantly increased in the setting where *KEAP1* expression was significantly reduced by 80% (P < 0.01 siKEAP1 versus scrambled). Interestingly, no induction of *GSTA1* transcripts was detected, suggesting that the SFN-mediated induction of this gene is Nrf2 independent. Also unexpectedly, levels of *COMT* transcripts as well as COMT protein were significantly decreased 60–70% by the siKEAP1 treatment (P < 0.01for COMT protein level in siKEAP1 versus scrambled) (Figure 5A and 5B). Comparable results were seen using shKEAP1 knockdown (data not shown). *COMT* is not known to be a direct Nrf2-regulated gene and the mechanism underlying this response is not known. As expected, siKEAP1also lead to increases in intracellular concentrations of reduced glutathione in MCF-10A cells (1.71-fold).



Fig 5. Effects of siKEAP1 on transcript, protein and activities of estradiol metabolizing enzymes. A. Effect of siKEAP1on levels of estrogen metabolism enzyme transcripts. B. Effect of siKEAP1on protein levels of estrogen metabolism enzymes. Scr, scrambled; siKp, siKEAP1. C. Effect of siKEAP1 on NQO1 activity. Values are mean ± SE of 3 independent experiments. *, Differs from scrambled control, p < 0.05. (Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

The level of depurinating estrogen–DNA adducts and estrogen metabolites in the cell culture media were assayed. At 48 h after E₂ treatment, the summed levels of the depurinating adducts, 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine, in the culture media were significantly lower in SFN-treated cells compared with vehicle (0.03 ± 0.01 versus 0.07 \pm 0.02 pmol/10⁶ cells, *P*< 0.05) (Figure6A). Although E₂ was added to the cells, there was considerable conversion to E₁. Approximately half of the formed adenine and guanine adducts were derivatives of E_1 and the remainder were from E_2 . In contrast, levels of 4-OCH₃E_{1/2} increased significantly with SFN treatment (5.36 \pm 0.16 versus 1.81 \pm 0.20 pmol/10⁶ cells, P < 0.01) (Figure6B). More modest increases in the levels of 4-OHE_{1/2}-glutathione conjugates were measured following SFN treatment (1.54 \pm 0.37 versus 0.83 \pm 0.19 pmol/10⁶ cells, P < 0.05, Figure 6C). About 25-fold more methoxy conjugates were formed than glutathione conjugates in either the vehicle or SFN-treated cells. 2-OHE_{1/2} adducts were also measured but typically reflected only 2-3% of the level of the 4-OHE_{1/2} DNA adducts formed. Therefore, we did not characterize patterns of 2-OHE_{1/2}-derived metabolites.Addition of the proximate metabolite, 4-OHE₂, to cells led to 20-fold higher levels of depurinating estrogen-DNA adducts than seen with E_2 in vehicle-treated cells. In this instance, the majority was derived from E_1 . 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts were again significantly lower in SFN-treated cells compared with vehicle (0.59 \pm 0.11 versus 1.42 \pm 0.16 pmol/10⁶ cells, P < 0.01; Figure 6D). 4-OCH₃E_{1/2} levels increased 3.4-fold (195.00 ± 12.33 versus 58.05 ± 1.77 pmol/10⁶ cells, P < 0.01; Figure 6E), whereas 4-OHE_{1/2}-glutathione conjugates increased 5.1-fold following SFN treatment (4.44 ± 0.52 versus 0.87 ± 0.03 pmol/10⁶ cells, P < 0.01; Figure 6F). The methoxy conjugates were the dominant metabolites detected. Pretreatment of cells with siKEAP1 led to more substantial declines in levels of the depurinating estrogen–DNA adducts in the cell culture media. Following E₂ treatment, levels of the 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts dropped 70% in siKEAP1-treated cells compared with scrambled vector. 4-OCH₃E_{1/2} levels decreased 50% with siKEAP1 treatment (Figure6E), whereas levels of the 4-OHE_{1/2}-glutathione conjugates were not significantly different (Figure6F). Both outcomes are consistent with the effects of siKEAP1 on *COMT* and *GSTA1* transcripts (Figure6A). Similar results were seen following 4-OHE₂ treatment. 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts declined 90% in siKEAP1-treated cells compared with scrambled vector (Figure6D); 4-OCH₃E_{1/2} declined 60% (Figure6E), whereas there was no significant change in levels of 4-OHE_{1/2}-glutathione conjugates with siKEAP1 treatment (Figure6F).



Fig.6. Effect of pharmacologic or genetic perturbation of estrogen metabolism on estrogen-DNA adducts and metabolites in MCF-10A cells. Cell culture media were collected, partially purified by solid phase extraction, and analytes separated and quantified by UPLC-/MS/MS. A, B, C. Levels of estrogen-DNA adducts, 4-OHCH₃E_{1/2} or E_{1/2}-glutathione conjugates, respectively, following addition of E₂ to cells pretreated with either SFN or siKEAP1. D, E, F. Levels of estrogen-DNA adducts, 4-OHCH₃E_{1/2} or E_{1/2} glutathione conjugates, respectively, following addition of 4-OHE₂ to cells pre-treated with either SFN or siKEAP1. Veh, vehicle; Scr, scrambled vector. Values are mean of triplicate biological replicates \pm SE. * Differs from control, p<0.05. **, Differs from vehicle control, p < 0.01.

(Reference: Yang L. Zahid M, Liao Y., et al. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 2013 Jul 10. [Epub ahead of print] PMID: 23843041).

In summary, treatment with SFN or siKEAP1 has similar effects on inducing NQO1 at mRNA, protein and activity level. Treatment with SFN or siKEAP1 has similar effects on reduction of depurinating estrogen-DNA adduct levels following estrogen challenge. However, these pharmacologic and genetic approaches have different effects on estrogen metabolism to O-methyl and glutathione conjugates. Activation of the Nrf2 pathway, especially elevated NQO1, may account for some but not all of the protective effects of SFN against estrogen-mediated DNA damage. **The relevant data generated from Research Task1 have been published in Carcinogenesis in 2013** (2013 Aug 5. [Epub ahead of print] PMID: 23843041).

3.1.2 Major Research Task 2

The original major Research Task 2 was to determine the efficacy of sulforaphane action in vivo by measuring serial urinary and serum levels of key estrogen metabolites and depurinating DNA adducts collected from either (a) ACI rats or (b) wild type and Nrf2-disrupted mice treated chronically with E₂ and either sulforaphane and vehicle. (month 4-32). For this task, following my Postdoctoral mentor, Dr. Thomas W. Kensler's suggestion, I have modified Task2a and discontinued Task2b from the study. For Task2a, I have completed two short-term ACI rats experiments and discontinued longterm experiments. The main reason for the modification is based on the findings from MCF-10A cell study and short-term animal study. The results from MCF-10A cell study and short-term animal study (6-week) indicate that activation of the Nrf2 pathway may account for some but not all of the protective effects of SFN against estrogen-mediated DNA damage. SFN exert protective function by modulating not only protecting enzymes such as NQO1 and GST, but also oxidation pathways enzymes such as CYP1B1, CYP1A1, or COMT. Since the data suggests that Nrf2 pathway plays partial role for the chemoprevention effect of SFN, Dr. Kensler recommends me to discontinue the project in Task2b, which solely address the role to Nrf2 pathway in animal model. For the Task2a, since I have already obtained results from short-term study, which demonstrates that SFN can modulate estrogen metabolism by decreasing the estrogen DNA adducts formation in vivo, Dr. Kensler recommends to discontinue the long-term experiments (27weeks).

Here I summarize the progress, methodology and findings from Task2a – short term ACI rats experiments. The purpose of the current Task2a study is to test the hypothesis that SFN, Nrf2 activator, exerts as chemoprevention agent by shifting the balance of estrogen carcinogen metabolism toward deactivation, so that the electrophilic carcinogens can be prevented from reaching or reacting with critical target sites, spring DNA from carcinogen damage. Total two experiments were applied. One is one-week pretreatment of animals with SFN; another one is one-week pretreatment and six-week treatment animals with SFN. Female ACI rats are uniquely sensitive to induction of mammary adenocarcinomas upon chronic treatment with E2 [9,10], and are an ideal model for investigating the mechanism of estrogen mammary carcinogenesis and relevant chemoprevention strategies.

Summary of Current Objectives of Task2a

Task2a.1

Measuring serial urinary levels of key estrogen metabolites and depurinating DNA adducts collected from ACI rats treated chronically with E₂ and either SFN and vehicle for 6 weeks. The protein/activities and mRNA transcripts expression of key estrogen metabolism enzymes in the hepatic and mammary tissue will be assayed and related to estrogen biomarker levels.

Task2a.2

Measuring urinary levels of key estrogen metabolites and depurinating DNA adducts collected from ACI rats pre-treated with either SFN and vehicle for 1 week, and then E_2 for one day. The protein/activities and mRNA transcripts expression of key estrogen metabolism enzymes in the hepatic and mammary tissue were assayed and related to estrogen biomarker levels.

Summary of the Methods of Task2a:

Treatment of Animals

Animal experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set forth in NIH guidelines and the Policy and Procedures Manual of the University of Pittsburgh Animal Care and Use Committee (IACUC). Animal protocols were reviewed and approved by the US Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO) prior to initiation. Female ACI rats (4 weeks of age) were obtained from Harlan Sprague Dawley (Indianapolis, IN). After a two-week acclimatization to the vivarium and phytoestrogen-free AIN-76A diet, rats will be divided into four groups, each with 5 rats/group; i) Corn oil + placebo, ii) SFN + placebo, iii) Corn oil + E₂, and iv) SFN 150 µmole/kg + E₂. E₂ treatment dose (3 mg) was determined according to literature [10] as were SFN doses [11]. Rats were implanted subcutaneously with pellet containing either placebo or 3 mg E₂. 400 µL pure Corn oil or 400 µL Corn oil containing SFN were delivered by gavages on Monday-Wednesday-Friday (three times a week) for total seven weeks. Phytoestrogen-free AIN76A diet (Harlan Indianapolis, IN) were fed to animals. Urine samples were collected by housing rats in metabolism cages at baseline (6-week old) and 4 weeks after E_2 implantation; blood, liver and mammary tissue were collected at 6 weeks of treatment. Figure 7 and 8 shows the animal study design. Biosamples were kept in -80°C prior to analysis. Estrogen depurinating DNA adducts were analyzed in urine and will be analyzed in serum. The expression at mRNA and protein level of key estrogen metabolism enzymes including NQO1, GSTs, Nrf2 in the liver and mammary tissue were assayed by using RT-PCR and western blot. The activities of NQO1 and GST in the liver were assayed. While the activities of NQO1 and GST in the mammary gland were unable to be assayed due to the limitation of availability of tissue harvested. One rat in control group was dead with unknown reason and was removed from the study.



Fig.7. Timeline for treatment of ACI rats



E₂ dose: 3mg (release for 6 weeks)

Fig.8. Treatment of ACI rats

NQO1 and GST activity assay for animal tissue

Method of NQO1 activity assay for animal tissue it similar with cell study as described in Task1b and 1c. For GST activity assay, after taking out the liver and mammary tissue stored from -80 ° C freezer. 2-5 μ L(20 μ g protein) Tissue cytosol and microsomal was added to 50 μ L PBS(pH=6.5) in 96-well plate. Then 150 μ L reaction mixture (including PBS(pH=6.5) 9.8 mL, L-Glutathione 0.1 mL and 100mM 1-chloro-2,4-dinitrobenzene 0.1mL in total 10mL volume) was added into the well. Read the absorbance in the plate reader at 340 nm immediately after preparing the reaction tests, and every minute thereafter to obtain at least 6 time points. GST activity was expressed as nmole/min/mg.

UPLC-MS/MS analysis of estrogen metabolites and depurinating DNA adducts from urine.

Extraction of estrogen metabolites and depurinating DNA adducts from biosamples were modified from previously described procedures [12-14]. After adjusting the pH to 7, urine (duplicate 2mL/sample) were loaded onto phenyl cartridges (Agilent Technologies) that are preconditioned with methanol and water. Extracts were eluted as described [12-14], lyophilized, re-dissolved in a methanol: water 50:50 mixture containing 0.1% formic acid, and finally subjected to UPLC-MS/MS analysis. Analyses were conducted using selected reaction monitoring with a triple stage quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, USA) by using heated electrospray ionization in positive or negative ion mode. The details of the methodology is the same with the method section of Task1b and Task1c. The Xcalibur software (Thermo) were used to process and quantify the acquired data of estrogen metabolites. Values were normalized to creatinine levels in the urine samples. I have measured creatinine by UPLC-mass spectrometry as described [15].

Statistics

The data are presented as means \pm SE of four or five animals in each group. Comparisons between two groups were analyzed using the Student's t-test, and significance was established at P < 0.05 using Prizm 5 software.

Summary of the Results, Progress and Accomplishments of Task 2a.1

For transcripts assay of *NRF2*, *GSTA1*, *NQO1*, *CYP1B1*, *CYP1A1* and *COMT* in the liver and mammary tissue, there were no statistical significant difference detected between group i (placebo+ Corn oil) and group ii (placebo +SFN); between group iii)

(E₂+Corn oil), and iv (E₂+SFN) (data not shown). The collection time of tissue was 24 hours after the last SFN treatment, which might contribute to the fast degradation of transcripts of these genes. It has been reported that the peak expression level of transcript of *NQO1* in the animal was 5 hours after treatment, and decrease quickly within 24 hours treatment (16).

The proteins expression level of key estrogen metabolism including CYP1B1, CYP1A1 and NQO1in the liver have been assayed.

The results show that there were no statistical significant difference of levels of CYP1B1 between group i (placebo+ Corn oil) and group ii (placebo +SFN); between group iii (E_2 +Corn oil) and iv (E_2 +SFN) (Figure 9A and 9B). This indicates that treatment of 150 µmole/kg SFN for 7 weeks (3times/week) in female ACI rats has no effect on the expression level of CYP1B1.

There was statistically significant decreased level of CYP1A1 in group iv (E_2+SFN) comparing with group iii (E_2+Corn oil). However, there was no similar observation detected between the group i (placebo+ Corn oil) and group ii (placebo +SFN). The explanation of this observation is that the dosage of 150 µmole/kg SFN treatment is pretty low and has no effect on the expression level of CYP1A1 in healthy animals. However, when the levels of estrogen have been increased in animals after implantation of 3mg E_2 pellet, which significantly up-regulated the level of CYP1A1, then the SFN treatment (150 µmole/kg) exerts the prevention function by inhibiting abnormal high levels of estrogen oxidation pathway enzyme CYP1A1 (Figure 9A and 9C).

There was also statistically significant increased level of NQO1 in group iv (E_2 +SFN) comparing with group iii (E_2 +Corn oil). However, there was no similar observation detected between the group i (placebo+ Corn oil) and group ii (placebo +SFN). The results indicate that SFN has the function of preventing estrogen DNA damage by upregulating NQO1 expression after E_2 challenge in the ACI rats. However, this dosage has no impact on the ACI rats, which were treated with placebo (Figure 9A and 9D).



Fig. 9. Effects of SFN on protein expression of enzymes metabolizing E_2 or E_1 in the liver (**A**) Effect of SFN on protein levels of estrogen metabolism enzymes. (**B**) Effect of SFN on CYP1B1 protein levels. (**C**) Effect of SFN on CYP1A1 protein levels. (**D**) Effect of SFN on NQO1 protein levels. The animals numbers are 5 for each group, except placebo+veh (n=4). Values are mean \pm SE of four or five ACI rats. *Differs from vehicle control, P < 0.05.student t-test, two-way. Please note that the western blot band is representative of one animal from each group, which might not reflect the statistical data in B, C and D.

For the NQO1 and GST activity assay, there were significantly higher levels of NQO1 and GST activity in liver in group iv (E_2 +SFN) comparing with group iii (E_2 +Corn oil). However, there was no similar observation detected between the group i (placebo+ Corn oil) and group ii (placebo +SFN) (Figure 10). The results indicate SFN has the function of preventing estrogen DNA damage by up-regulating NQO1 and GST activity in the liver after E_2 challenge in the ACI rats. However, this dosage has no impact on the ACI rats, which were treated with placebo (Figure 10A and 10B).



Fig. 10. Effects of SFN on protein activity of key enzymes metabolizing E_2 or E_1 in the liver (**A**) Effect of SFN on NQO1 activity (**B**) Effect of SFN on GST activity. The animals numbers are 5 for each group, except placebo+veh (n=4). *Differs from vehicle control, P < 0.05. student t-test, two-way.

The level of depurinating estrogen–DNA adducts and estrogen metabolites in the urine from ACI rats were assayed. The summed levels of the depurinating adducts, 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine in the group treated with SFN + E₂ were significantly lower compared with vehicle (Corn oil+ E₂) (P< 0.05) (Figure11A). There is around 75% inhibitory effect of the SFN treatment. However, for the summed levels of the depurinating adducts, 2-OHE_{1/2}-1-N3Adenine, in the group treated with SFN + E₂, were not significantly changed compared with vehicle (Corn oil+ E₂) (Figure 11B). Although there is the trend that SFN treatment decreases the levels of 2-OHE_{1/2}-1-N3Adenine comparing with vehicle, however, there is no statistical significance.

There is no inhibitory effect of SFN in the group treated with placebo + SFN comparing with the group treated with placebo + corn oil. The data is consistent with the above observations that SFN has no impact on estrogen metabolism enzymes in the group of placebo + SFN comparing with group treated with vehicle.



Fig. 11. Effect of pharmacologic perturbation of estrogen metabolism on estrogen-DNA adducts in ACI rats. Urine specimens were collected, partially purified by solid phase extraction, and analytes separated and quantified by UPLC-/MS/MS. A, Levels of the sum of $4\text{-OHE}_{1/2}$ -1-N3Ade and $4\text{-OHE}_{1/2}$ -1-N7Gua in the urine (unit: pmole/mg creatine) in four animal groups. B. Levels of the sum of $2\text{-OHE}_{1/2}$ -1-N3Ade in the urine (unit: pmole/mg creatine) in four animal groups. The number of the animals in each group is 5 rats / group except group of placebo+Vehicle (4 rats / group) * Differs from control, p<0.05. NS, no significance. Student t-test.

Summary of the Results of Task 2a.2

After reviewing the data from the above 7 weeks experiment, Dr. Kensler recommends me to continue another short-term experiment. In this study, total 20 ACI rats were used and there were four groups with the same treatment as the above 7 weeks experiment. After pre-treatment of one week SFN (150 μ mole/kg), then the animals were treated with E₂, one day later, the animals were sacrificed. The urine, liver and mammary tissue were collected at the end of the study (1wk). The results show that there is no impact on the key estrogen metabolism enzymes including NQO1, GST, CYP1A1, CYP1B1 and COMT at transcript, protein and activity level by the SFN pre-treatment. Also there is no effect on the estrogen DNA adducts level in the SFN treatment comparing with the vehicle (data not shown). The results from this study indicate that short-term, low dosage of SFN treatment has no the chemoprevention effect in ACI rats.

In summary of the Task2, treatment of ACI female rats with SFN (dose 150µmole/kg; pre-treatment 1 wk + treatment 6 wks; 3 times/week led to induction of key estrogen protective enzyme NQO1 at protein and activity level; and GST activity level in the liver and significantly inhibit the formation of estrogen-DNA adducts. The findings from the animal study provide strong evidence that SFN is a promising chemoprevention agent against breast cancer by modulating of estrogen DNA adducts. One 1st authorship manuscript is under preparation and expected to send out in March 2015.

Major Research Task 3

The original major Research Task 3 was to utilize samples collected from completed and ongoing intervention trials with broccoli sprouts rich in either sulforaphane or its precursor glucoraphanin to establish proof-of-principle evaluations of the pharmacodynamic action of sulforaphane on depurinating estrogen-DNA adducts in human (month 12-36).

Task3a. Measure key estrogen metabolites and depurinating DNA adducts in daily urine samples collected from 33 female participants days 1-5 before and then during a 7-day intervention with either sulforaphane-rich (150 µmole) or glucoraphanin-rich (800 µmole) broccoli sprout preparations. Measures during the run-in phase will determine the extent of intra- and inter-individual variability of the estrogen-DNA adducts in this cohort of Chinese women, while the measures at the end of the 7-day intervention will assess efficacy. (month 12-18)

Task3b. <u>Measure key estrogen metabolites and depurinating DNA adducts in serial,</u> <u>biweekly urine samples collected from approximately 200 female participants during a</u> <u>3-month randomized placebo-controlled intervention of a blended sulforaphane-rich (40</u> <u>µmole)-glucoraphanin-rich (800 µmole) broccoli sprout preparation. Serial serum and</u> <u>urine samples will be assayed to determine the extent and sustainability of diminution of</u> <u>estrogen –DNA adducts in this intervention cohort. (month 18-36)</u>

Since the projects in Task3a and 3b will use clinical samples, which are very important, Dr. Kensler suggests me to focus on project to synthesize the heavy isotope labeling estrogen DNA adducts. Then I can use the isotope labeling estrogen DNA adducts for the human urine samples assay. So I was unable to complete the Task 3a and 3b due to the limitation of the availability of these standards for the proposed time (month 12-36). However, I will keep doing the projects of Task3a and Task3b from January of 2015. Also Dr. Kensler also was awarded a breast cancer foundation grant, which will support the projects focusing on Task3a and 3b.

Although I didn't conduct the Task 3a and 3b, with the recommendation and guidance from Dr. Kensler, I have completed another small project with the purpose of testing whether one 12-hour urine sample assay can be representative to reflect the estrogen metabolism in the body, a preliminary study has been conducted. The urine samples

were collected for three continuous days from 14 female subjects in Qidong, China. The results show that there are no significant differences among day 1, day 2 and day 3 for the sum of $4\text{-OHE}_{1/2}$ -1-N3Ade and $4\text{-OHE}_{1/2}$ -1-N7Gua adducts in the urine (Kruskal-Wallis with Dunn's Multiple Comparison Test, p>0.05). From this data, one 12-hour urine assay could reasonably reflect current estrogen metabolism.

3.2 Summary of the Major Training Tasks

I have completed all the major training tasks according to the original plan. I have completed course of "TSQ operation MASS Spectrometry advanced training" at Thermo Scientific (month 3) in Oct., 2011 in Florida. I have audited the course of "Clinical Trials: Methods and Practice" at Graduate School of Public Health at University of Pittsburgh in from January to May 2012. I have attended the course of "Scientific Management & Leadership" at University of Pittsburgh on April 18 and April 19, 2013. I have provided the scientific guidance and training to the rotation student in Dr. Kensler's lab (Tao Xue). I also was the teaching assistant of the course of "Cancer Biology & Therapeutics", which is directed by Dr. Kensler in 2013. With Dr. Kensler's support, I have applied K99-R00 grant application to National Cancer Institute in February, 2014, Though I didn't get the K99 funding and I also can't resubmit it due to the rule of 4-year postdoctoral training, I am gaining the experience for the grant application. In addition, I have participated workshop and seminars, which provide diverse trainings such as how to improve the grantship and how to enhance career development at University of Pittsburgh. I have attended local, national and international meetings to communicate with researchers outside of the lab and improve my communication skills.

In summary the major research tasks I have completed during the award period, I have learned a lot from cell culture model, animal model and synthesis project. I further deepen my knowledge of UPLC-MS/MS for the methodology establishment and maintenance also. Though I was not able to complete the major research task3 because of the limitation of availability of heavy isotope labeling estrogen DNA adducts during the award period, I am looking forward to assay these samples staring from January 2015. By completion of the major training tasks, my eyesight as a breast cancer researcher has been broadened and deepened. With the support from this award and substantial support from my Postdoctoral mentor, Dr. Thomas Kenser; my co-mentor, Dr. Nancy Davidson; and chair of our department, Dr. Bruce Freeman, and other researchers in our department, I am making solid progress in the science. I expect to have at least three first authorship publications in 2015. Not only gaining knowledge in science, I also improve my capability in teaching skills, training skills, and administration skills. I believe that I am ready to be an independent principal investigator in breast cancer research area.

4. KEY RESEARCH ACCOMPLISHMENTS:

In the MCF-10A cell study, I found that SFN and KEAP1 knock down can both modulate estrogen metabolism leading to diminished formation of estrogen-DNA adducts by up-regulating NQO1 and GST via Keap1-Nrf2 pathway. The manuscript has been published in Carcinogenesis in 2013. This is the first study to investigate the modulation of estrogen DNA adducts by SFN via Keap1-Nrf2 pathway. Most interestingly, the findings from this study indicate that pharmacologic and genetic approaches have different effects on estrogen metabolism to O-methyl and glutathione conjugates. The cell study supports that SFN exerts chemoprevention effect against breast cancer by modulating multiple key estrogen metabolism enzymes. After the findings of this study were published, other researchers were also attracted to the field to investigate the effect of broccoli sprout on the modulation of estrogen metabolism.

- In the ACI rat study, I found that long-term (at least 7 weeks) low dose of SFN treatment can modulate estrogen metabolism leading to diminished formation of estrogen-DNA adducts by up-regulating NQO1 and GST via Keap1-Nrf2 pathway. However, Keap1-Nrf2 pathway might play partial role of the SFN effect considering the findings that SFN modulates other estrogen metabolism enzymes such as CYP1A1 and CYP1B1. To date, there is no publication reported regarding the role of SFN in the modulation of estrogen metabolism and depurinating DNA adducts in rodent models. Nor is there literature on the role of the Nrf2 pathway in this setting. The study was characterized the efficacy of SFN action in vivo and the role of Nrf2 pathway in the underlining molecular mechanism of SFN against ER-independent breast carcinogenesis. The findings of this study provide strong evidence that SFN is a promising chemoprevention agent against breast cancer by modulating of estrogen DNA adducts. One 1st authorship manuscript is under preparation and expected to send out in March 2015.
- In the human urine samples project, I found that there is no significant difference for the estrogen depurinating DNA adducts in the urine of the same subject who has been assayed continuously three days. So one 12-hour urine sample can be considered to be representative to reflect the extent of estrogen metabolism and DNA damage caused by estrogen oxidative metabolites in the body
- Four heavy isotope estrogen adduct standards were successfully synthesized and will be utilized into the clinical trial samples. The establishment of methodology to assay estrogen DNA adducts by using golden standard – steady isotope labeling compounds will have significant impact to the research field including translational, chemoprevention, and epidemiology area. This is a very important milestone for the discovery of biomarker to assess the risk of developing breast cancer and also can be utilized into the chemoprevention research field. Currently there is no any publication regarding the utilization of heave isotope labeling estrogen DNA adducts as biomarker of cancer. If this method succeeds in assaying clinical trial samples in the original Research Task 3a and 3b in 2015, then there is substantial impact to push the estrogen carcinogenesis research field. The manuscript of this project is under preparation and expected to submit in February 2015.

5. CONCLUSION:

SFN can modulate estrogen metabolism leading to diminished formation of estrogen-DNA adducts via partial Keap1-Nrf2 pathway in breast epithelial cell model and animal model. The findings support that SFN, a food-derived natural product, could be a novel breast cancer chemoprevention agent. The studies not only markedly advance knowledge into the fundamentals of ERindependent pathway and open an entirely new area of exploration in food-based breast cancer chemoprevention strategies in clinical settings. The study has the following innovations:

Conceptual innovations:

1). Identify and targeting estrogen oxidative metabolism and DNA adducts as novel targets for BC

2). Investigate SFN, an effective, simple, safe, and inexpensive approach, to reduce risk of BC through diversion of the estrogen oxidation pathway away from DNA damaging intermediates.

Technical innovation

Development of a novel analytical method to measure low abundance analytes in complex biological matrices with high accuracy and precision. This is the first study to use stable isotope labeled mass spectrometry method to detect estrogen-DNA adducts in human specimens, which opens a new door to the estrogen oxidative metabolism research area.

Therapeutic/Translational Innovation:

The ultimate targets include prevention of ER-negative breast cancer, in as much as they account for 25% of BC patients. They face poor prognosis and limited chemoprevention options.

After completion of the comprehensive training supported by this DoD Postdoctoral Fellowship, I plan to continue the research focusing on estrogen carcinogenesis and chemoprevention of breast cancer by using food-based components. I also plan to apply assistant professor position in academic settings in the near future.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. Manuscripts and publications

- 1. Lay Press: (Nothing to report)
- 2. Peer-Reviewed Scientific Journals:

Yang L. Zahid M, Liao Y., Cavalieri E., Rogan E., et al. (**2013**). Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 34(11): 2587-92. PMID: 23843041).

Yang L. Liao Y., Zahid M, Cavalieri E., Rogan E, Kensler T, et al. Pharmacological activation of Nrf2 signaling in ACI rat for modulation of estrogen depurinating DNA adducts (in preparion).

Woodcock S*, **Yang L***. Salvatore S. Kensler T., Freeman B. Development of a liquid chromatography electrospray ionization tandem mass spectrometry method for analysis of stable 4-hydroxyestrogen-DNA adducts in human urine (in preparation).(* equal first authorship).

3. Invited Articles:

Yang L, Palliyaguru D, Kensler T. Targeting Nrf2 for cancer chemoprevention with naturally-occurring, dietary agents. (in preparation; submit to Seminars in Oncology)

Yang L, Liao Y. D, Kensler T. Detection of estrogen DNA adducts from bio-samples: implication of biomarkers in the diagnosis and treatment of Breast Cancer. (in preparation; submit to BioMed Research International in Jan 2015).

- 4. Abstracts:
- AACR Annual Meeting 2013. April 6-10, 2013. Washington, DC. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 3679). **Yang, L**; Cavalieri, E.; Rogan, E., Kensler T. et al.

b. Presentations:

- First annual women's cancer and research center retreat at UPMC May 6-7, 2011, Pittsburgh, PA. (Poster 16). The role of estrogen metabolism in risk of developing breast cancer: Detection of novel serum biomarkers from a case-control study. Yang L, Kensler T., Rogan E. G. et al.
- University of Pittsburgh Cancer Institute Satellite Conference 2012 (Cancer Epidemiology, Prevention and Control Program) June 20, 2012. Greensburg. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. (Poster).
 Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- 24th University of Pittsburgh Cancer Institute Scientific Retreat 2012 June 21 22, 2012. Greensburg. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. (Poster). Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- Metabolomics Society 8th Annual Meeting 2012 June 25-28, 2012. Washington, DC. Determination of serum estrogen depurinating DNA-adducts as potential biomarker for breast cancer risk: results from a case-control study. (Abstract 402). Yang, L; Kensler, T; Cavalieri, E.; Rogan, E. et al.
- 2nd Annual Women's Cancer Research Center (WCRC) Retreat 2012 Sep. 7-8, 2012. Farmington, PA. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via Pharmacological and genetic activation of Nrf2 signaling in human breast epithelial cells. (Poster and Oral). Yang, L; Zahid, M., Kensler T., et al.
- AACR Annual Meeting 2013. April 6-10, 2013. Washington, DC. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 3679). **Yang, L**; Cavalieri, E.; Rogan, E., Kensler T. et al.
- 25th University of Pittsburgh Cancer Institute Scientific Retreat 2013 June 14. Pittsburgh. Modulation of estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 101). Yang, L; Zahid, M., Liao Y. et al.
- Department of Pharmacology & Chemical Biology 2013 Annual Retreat Sep. 27. Pittsburgh. Reduced formation of depurinating estrogen-DNA adducts by

sulforaphane: Mechanisms and opportunities for clinical translation. (Oral presentation). **Yang L**.

AE-SOT 28th Annual Meeting 2014. May 15 – 16, 2014. Morgantown, WV. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster). **Yang, L**; Liao, Y., Zahid, M., et al.

7. INVENTIONS, PATENTS AND LICENSES: (Nothing to report)

8. REPORTABLE OUTCOMES:

- Manuscript titled with "Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells" has been published in Carcinogenesis. 34(11): 2587-92. PMID: 23843041.
- Manuscript titled with "Detection of estrogen DNA adducts from bio-samples: implication of biomarkers in the diagnosis and treatment of breast cancer" is under preparation and expected to submit to BioMed Research International in Jan 2015.
- Manuscript titled with "Development of a liquid chromatography electrospray ionization tandem mass spectrometry method for analysis of stable 4hydroxyestrogen-DNA adducts in human urine" is under preparation and expected to submit in Feb. 2015.
- Manuscript titled with ":Pharmacological activation of Nrf2 signaling in ACI rat for modulation of estrogen depurinating DNA adducts" is under preparation and expected to submit in March 2015.
- Manuscript titled with "Targeting Nrf2 for cancer chemoprevention with naturallyoccurring, dietary agents" is under preparation and expected to be published in Seminars in Oncology in April 2015.
- Poster presentation in AACR Annual Meeting 2013. April 6-10, 2013. Washington, DC. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 3679). Yang, L; Cavalieri, E.; Rogan, E., Kensler T. et al.
- Poster presentation in 24th University of Pittsburgh Cancer Institute Scientific Retreat 2012 – June 21 – 22, 2012. Greensburg. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. (Poster). Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- Poster presentation in University of Pittsburgh Cancer Institute Satellite Conference 2012 (Cancer Epidemiology, Prevention and Control Program) – June 20, 2012. Greensburg. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- Poster presentation in First annual women's cancer and research center retreat at UPMC – May 6-7, 2011, Pittsburgh, PA. (Poster 16). The role of estrogen

metabolism in risk of developing breast cancer: Detection of novel serum biomarkers from a case-control study. Yang L, Kensler T., Rogan E. G. et al.

- Oral presentation in 2nd Annual Women's Cancer Research Center (WCRC) Retreat 2012 – Sep. 7-8, 2012. Farmington, PA. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via Pharmacological and genetic activation of Nrf2 signaling in human breast epithelial cells. (Poster and Oral). Yang, L; Zahid, M., Kensler T., et al.
- Oral presentation in Department of Pharmacology & Chemical Biology 2013 Annual Retreat – Sep. 27. Pittsburgh. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane: Mechanisms and opportunities for clinical translation. (Oral presentation). Yang L.
- Review papers for the following journals: Biomarker Insights; Carcinogenesis; Breast cancer: Basic and Clinical Research; Nutrition and Metabolic Insights; Clinical Medicine Insights: Women's Health; Risk Analysis; Cancer Prevention Research; The Scientific World Journal; Chemical Research in Toxicology; PLOS ONE; BioMed Research International; Biochemistry Research International.

9. OTHER ACHIEVEMENTS:

- 2012, Award for the 3rd place best oral presentation, 2nd Annual Women's Cancer Research Center Retreat, Farmington, PA.
- 2012, Award for the 1st place best poster presentation for clinical study, 24th University of Pittsburgh Cancer Institute Scientific Retreat 2012, Greensburg, PA.
- 2012, Award for the 2nd place best poster presentation, University of Pittsburgh Cancer Institute Satellite Conference 2012, Greensburg, PA.

10. REFERENCES:

1. Yang L, Zahid M, Liao Y, Rogan EG, Cavalieri EL, Davidson NE, Yager JD, Visvanathan K, Groopman JD, Kensler TW, Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. (2013) Jul 10. [Epub ahead of print] PMID: 23843041.

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10. Stakhiv, T.M., Mesia-Vela, S., Kauffman, F.C. (2006).*Phase II antioxidant enzyme activities in brain of male and female ACI rats treated chronically with estradiol.* Brain Research. 104:80-91.

11. Veeranki OL, Bhattacharya A, Marshall JR, Zhang Y. (2013). Organ-specific exposure and response to sulforaphane, a key chemopreventive ingredient in broccoli: implications for cancer prevention. Br J Nutr. Jan 14;109(1):25-32. doi: 10.1017/S0007114512000657.

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15. Takahashi, N.B., G. Li, F. Li, Y. Swenberg, J. A., *Tandem mass spectrometry measurements of creatinine in mouse plasma and urine for determining glomerular filtration rate.* Kidney Int, 2007. **71**(3): p. 266-71.

11. APPENDICES: (See the attached)

CURRICULUM VITAE

Li Yang, Ph.D.

Molecular Toxicologist Dedicated to Environmental carcinogenesis

BUSINESS ADDRESS

Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, E1316, BST, 200 Lothrop Street, Pittsburgh, PA, 15261

E-mail: liyang@pitt.edu

PART I: TRAINING AND PROFESSIONAL EXPERIENCE

A. ACADEMIC TRAINING

2004.8-2010.5 Ph.D. in Environmental Toxicology

Department of Environmental, Agricultural and Occupational Health, College of Public Health, University of Nebraska Medical Center (UNMC), Omaha, NE

PhD Dissertation:

Association of estrogen metabolism and risk of breast or prostate cancer or non-Hodgkin lymphoma: Detection of novel biomarkers from casecontrol studies

1999.9-2002.7 M.S. in Biology

Department of Biology Science, College of Biology, China Agricultural University, Beijing, PRC

1991.9-1995.7 B.S. in Plant Protection

Department of Horticulture, College of Agronomy, Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, PRC

B. ACADEMIC AND PROFESSIONAL APPOINTMENT

2010.6- present Postdoctoral / Research Associate

Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA,

2004.9-2010.5 Graduate Research Associate

Department of Agricultural, Occupational and Environmental Toxicology, College of Public Health, UNMC, Omaha, NE

2002.9-2004.8 Research Associate (Pesticide analysis)

Department of Applied Chemistry, College of Science, China Agricultural University, Beijing, PRC

1999.9-2002.7 Graduate Research Associate

Department of Biology Science, College of Biology, China Agricultural University, Beijing, PRC

C. OTHER PROFESSIONAL TAINING

2014.11.04 – 2014.11.04 **Maintaining ACQUITY UPLC Systems,** Waters Corporation, Milford, MA

2014.08.11 – 2014.08.15 **Advanced Comprehensive Toxicology**, American College of Toxicology, Cincinnati, Ohio

2013.04.18 – 2013.04.19 Course in Scientific Management and Leadership;

University of Pittsburgh Schools of the Health Sciences. Pittsburgh, PA

2012.01.05 – 2012.04.28 **Clinical Trials: Methods and Practice;** University of Pittsburgh School of Public Health, Pittsburgh, PA

2011.10. 24 – 2011.10.27 **TSQ Family Operations Mass Spectrometry training;** Thermo Scientific; Florida;

D. PROFESSIONAL ORGANIZATION AND SOCIETY

2014.9 - present Member, American College of Toxicology (ACT)

2011.1 - present Member, American Association of Cancer Research (AACR)

2011.1 - present **Member**, American Society for Mass Spectrometry

2010.1 - present **Member**, Women in Toxicology Special Interest Group (SIG)

2008.1 - present Member, Society of Toxicology (SOT)

PART II: SCHOLARLY ACCOMPLISHMENTS

A. RESEARCH GRANT AND CONTRACT SUPPORT

DoD Breast Cancer Research Program (BCRP) Postdoctoral Fellowship BC103928, Yang, L (PI) 09/15/2011-09/14/2014

Title of Grant: Modulation of Estrogen-Depurinating DNA Adducts by Sulforaphane for Breast Cancer chemoprevention. Total cost \$ \$448,770.00. Role: PI (100%)

B. HONORED ACTIVITIES, INVITED LECTURES AND PRESENATIONS Honors/Awards

- 2012, Award for the 3rd place best oral presentation, 2nd Annual Women's Cancer Research Center Retreat, Farmington, PA.
- 2012, Award for the 1st place best poster presentation for clinical study, 24th University of Pittsburgh Cancer Institute Scientific Retreat 2012, Greensburg, PA.
- 2012, Award for the 2nd place best poster presentation, University of Pittsburgh Cancer Institute Satellite Conference 2012, Greensburg, PA.
- 2011 2014, DOD breast Cancer Research Program Postdoctoral Fellowship Award
- 2007-2010, UNMC graduate school, Fellowship Award

- 2009, Award for the 2nd place best abstract, American Association of Chinese in Toxicology (AACT), Baltimore, Maryland.
- 2009 Award for Graduate Student Travel Support for 2009 National Environmental Public Health Conference, Atlanta, GA.
- 2008, Award for the 3rd place poster presentation, 39th Midwest Student Biomedical Research Forum, Omaha, Nebraska.
- 2008, Award for Graduate Student Travel Support for 2008 National Toxicology Conference. Society of Toxicology (SOT). Seattle, Washington.
- 2008, Representative of UNMC graduate students for International Student Research Forum. Omaha, Nebraska.
- 2007, Award for best poster presentation, Central States Society of Toxicology Meeting. Iowa City, Iowa.
- 2004, Special award for contribution to the pesticides research, Society of Pesticide Res., Beijing, China.

Invited Speakers

- 2013. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane: Mechanisms and opportunities for clinical translation. Department of Pharmacology & Chemical Biology Annual Retreat. Pittsburgh, PA.
- 2012. Modulation of estrogen depurinating DNA adducts by SFN via Keap1/Nrf2 pathway in human epithelia cells. Women's Cancer Research Center, Farmington, PA.
- 2012. Pharmacological and genetic activation of Nrf2 signaling in MCF-10A cell for modulation of estrogen depurinating DNA adducts. University of Pittsburgh Cancer Institute. Pittsburgh, PA.
- 2011 Association of estrogen metabolism and risk of non-hodgkin lymphoma: detection of novel biomarkers from case-control study. Society of Toxicology 50th Annual Meeting. Platform presentation. Washington, D.C.
- 2008. Novel serum biomarkers for assessing breast cancer risk: results from a casecontrol study. For Breast Cancer Training Program at Eppley Cancer Center, UNMC, Omaha, NE.
- 2008. The role of estrogen metabolism in the initiation of breast cancer: Detection of potential early biomarkers from case-control studies. Society of Toxicology 47th Annual meeting. Special session for visiting students, Seattle, Washington.

Research Presentations

- AE-SOT 28th Annual Meeting 2014. May 15 16, 2014. Morgantown, WV. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster). Yang, L; Liao, Y., Zahid, M., et al.
- Department of Pharmacology & Chemical Biology 2013 Annual Retreat Sep. 27. Pittsburgh. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane: Mechanisms and opportunities for clinical translation. (Oral presentation). **Yang L**.

- 25th University of Pittsburgh Cancer Institute Scientific Retreat 2013 June 14. Pittsburgh. Modulation of estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 101). Yang, L; Zahid, M., Liao Y. et al.
- AACR Annual Meeting 2013. April 6-10, 2013. Washington, DC. Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells. (Poster 3679). **Yang, L**; Cavalieri, E.; Rogan, E., Kensler T. et al.
- 2nd Annual Women's Cancer Research Center (WCRC) Retreat 2012 Sep. 7-8, 2012. Farmington, PA. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via Pharmacological and genetic activation of Nrf2 signaling in human breast epithelial cells. (Poster and Oral). Yang, L; Zahid, M., Kensler T., et al.
- Metabolomics Society 8th Annual Meeting 2012 June 25-28, 2012. Washington, DC. Determination of serum estrogen depurinating DNA-adducts as potential biomarker for breast cancer risk: results from a case-control study. (Abstract 402). Yang, L; Kensler, T; Cavalieri, E.; Rogan, E. et al.
- 24th University of Pittsburgh Cancer Institute Scientific Retreat 2012 June 21 22, 2012. Greensburg. Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. (Poster). Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- University of Pittsburgh Cancer Institute Satellite Conference 2012 (Cancer Epidemiology, Prevention and Control Program) June 20, 2012. Greensburg.
 Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells. (Poster).
 Yang, L; Zahid, M., Cavalieri, E.; Rogan, E., Kensler T.
- First annual women's cancer and research center retreat at UPMC May 6-7, 2011, Pittsburgh, PA. (Poster 16). The role of estrogen metabolism in risk of developing breast cancer: Detection of novel serum biomarkers from a case-control study. Yang L, Kensler T., Rogan E. G. et al.
- Society of Toxicology 50th Annual meeting 2011 March 6-10, 2011, Washington D.C. Platform presentation. (Abstract 880). Association of estrogen metabolism and risk of non-hodgkin lymphoma: detection of novel biomarkers from case-control study. Yang L, Gaikwad W. N., Cavalieri E. L. and Rogan E. G. et al.
- Society of Toxicology 49th Annual meeting 2010 March 7-11, 2010, Salt Lake City, Utah. Poster presentation. (1885 Poster Board -440). Component analysis of novel serum biomarker of breast cancer: results from case-control studies. Yang L, Cavalieri E. L. and Rogan E. G. et al.
- The 41st Midwest Student Biomedical Research Forum February 20, 2010, Omaha, NE. Oral Presentation. Association between novel serum biomarkers for assessing breast cancer risk and Gail model score: Results from a case-control study. **Yang L**., Rogan E.G., Cavalieri E. L., and Pruthi S.
- Society of Toxicology 48th Annual meeting 2009 March 15-19, 2009, Baltimore, Maryland. Poster presentation (abstract 1605 at poster board 514): Novel urinary biomarkers for risk of prostate cancer: results from a case-control study. **Yang L**, Cavalieri E. L. and Rogan E. G.

- The 40th Midwest Student Biomedical Research Forum February 28 29, 2009, Omaha, NE. Oral Presentation: Novel serum biomarkers for assessing breast cancer risk: results from a case-control study in USA. **Yang L**., Rogan E.G., Cavalieri E. L.
- International Student Research Forum. June 1-3, 2008, Omaha, NE. Oral presentation: Estrogen metabolism and risk of breast cancer: detection of novel biomarkers from case-control study. **Yang L**, Rogan E. G and Cavalieri E. L.
- Society of Toxicology 47th Annual meeting 2008– March 16-20, 2008, Seattle, Washington. Poster presentation #1735&VSP#3009: The role of estrogen metabolism in the initiation of breast cancer: Detection of potential early biomarkers from case-control studies. **Yang L**, Cavalieri E. L. and Rogan E. G. et al.
- The 39th Midwest Student Biomedical Research Forum February 29- March1, 2008. Omaha. Poster Presentation P-50: The role of estrogen metabolism in the risk of developing breast cancer: Detection of novel serum biomarkers from a case-control study". Yang L, Rogan E. G and Cavalieri E. L.
- Central States Society of Toxicology Meeting September 20-21, 2007, University of Iowa Medical Center. Poster Presentation: The role of estrogen metabolism in the initiation of breast cancer: Detection of potential early serum biomarkers from a case-control study. **Yang L**, Rogan E. G and Cavalieri E. L.
- The 38th Midwest Student Biomedical Research Forum February 23, 2007. Omaha. Poster Presentation P-47: "Mass spectrometry detection of a novel biomarker of breast cancer in human serum". **Yang L**, Rogan E. G and Cavalieri E. L.
- The 37th Midwest Student Biomedical Research Forum February 18, 2006. Omaha. Poster Presentation P-68: "Mass spectrometric analysis of potential biomarkers of breast cancer in human serum". **Yang L**, Gaikwad N, Rogan E. G and Cavalieri E. L.

Note: Even though the title of the above posters might be the same or similar, the contents of above posters are different because the sample size increased with time and all the data were presented with updated data.

PARTIII: TEACHING AND MENTORING CREDIT

A. COURSES TAUGHT

Cancer Biology and Therapeutics (Teaching Assistant; Credit: 3; Aug. 2013-Dec. 2013; Graduate Course; Students Number: 13. University of Pittsburgh School of Medicine)

Plant Cell Anatomy (Teaching Assistant; Credit: 6; Sep. 2000 – May, 2001; Undergraduate Course; Students Number: 28. China Agricultural University College of Biology)

English (Director of course; Credit: 6; Aug. 2000. – May, 2001; Undergraduate Course; Student Number: 26; Peking PeiLi ZhiYe College)

B. GRADUATE STUDENT TRAINED

Tao Xue (PhD rotation student; 2012; provide scientific guidance and mentoring)

PART IV: SERVICE CREDITS

A. JOURNAL REVIEWERS

- 1. Biomarker Insights
- 2. Carcinogenesis
- 3. Breast cancer: Basic and Clinical Research
- 4. Nutrition and Metabolic Insights
- 5. Clinical Medicine Insights: Women's Health
- 6. Risk Analysis
- 7. Cancer Prevention Research
- 8. The Scientific World Journal
- 9. Chemical Research in Toxicology
- 10. PLOS ONE
- 11. BioMed Research International
- 12. Biochemistry Research International

B. INVITED JUDGE

2012. Intel International Science & Engineering Fair 2012. Pittsburgh, PA, USA.

C. PROJECTS INVOLVED

2004. Identification and quantification of multiple pesticides' residues from agricultural products including variety of vegetables in the whole production chain in Hohhot, Inner Mongolia, P.R. China. (Department of Agriculture, P. R. China)

PART V: PUBLICATIONS (ORIGINAL, PEER REVIEWED ARTICLES)

- **Yang L.** Liao Y.,Kensler T, et al. Pharmacological and genetic activation of Nrf2 signaling in ACI rat for modulation of estrogen depurinating DNA adducts (in preparion).
- **Yang L,** Kensler T, et al. Targeting Nrf2 for cancer chemoprevention with naturallyoccurring, dietary agents. (in preparation)
- Yang L. Liao Y., Jager J., Kensler T., et al. Quantitative determination of estrogen depurinating DNA adducts and metabolites across the estrus cycle in ACI rat (in preparation).
- Yang L. Zahid M, Liao Y., Cavalieri E., Rogan E., et al. (2013). Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. Carcinogenesis. 34(11): 2587-92. PMID: 23843041).
- Pruthi S.*, Yang L *, Ingle J., Sandhu N., Suman V., Cavalieri E. L., Rogan E. G. (2012). Evaluation of serum estrogen-DNA adducts as potential biomarkers for breast cancer risk. J Steroid Biochem Mol Biol. <u>(* equal contribution as first author)</u>. 132(1-2):73-9. 2012 Feb 24. [Epub ahead of print]. PMID: 22386952.
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- 4. Gaikwad NW, **Yang L**, Weisenburger DD, Vose J, Beseler C., Rogan E.G. and Cavalieri E. L. (**2009**) Urinary biomarkers suggest that estrogen-DNA adducts may play a role in the aetiology of non-Hodgkin's lymphoma. Biomarkers. 14(7): 502-12.
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- 6. **Yang L**, Gaikwad NW, Cavalieri EL, Muti P, Trock B, Rogan EG. (**2009**) Novel biomarkers for risk of prostate cancer: Results from a case-control study. The Prostate. 69:41-48.
- Gaikwad NW, Yang L, Pruthi S., Ingle J., Rogan E.G. and Cavalieri E. L.(2009) Urinary biomarker of risk in the molecular etiology of breast cancer. Breast cancer: basic and clinical research. 3:1-8.
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- Gaikwad NW, Yang L, Muti P., Meza J., Ingle J., Pruthi S., Rogan E.G. and Cavalieri E. L. (2008). The molecular etiology of breast cancer: evidence from biomarkers of risk. Int J Cancer. 122(9):1949-57.
- Zahid M, Gaikwad NW, Ali MF, Lu F, Saeed M, Yang L, Rogan EG, Cavalieri EL. (2008). Prevention of estrogen-DNA adduct formation in MCF-10F cells by resveratrol. Free Radic Biol Med. 45(2):136-45.
- 11.Tian Q, Zhou Z, Ren L, Jiang S, **Yang L.** (2005). Research Development of Pesticides Photodegradation in Water. Pesticides. 44(6):247-250.
- Yang L., Liao Y., Wang P., Bi C.L., Zhou Z.Q., Jiang S.R. (2004) Direct optical resolution of chiral pesticides by high performance liquid chromatography on cellulose tris-3, 5-dimethylphenyl carbamate stationary phase under reversed phase conditions. Journal of Liquid Chromatography & Related Technologies. 27(18): 2935-2944.
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- 14. Yang L., Jiang S.R., Liao Y., Wang P., Tian Q., Zhou Z.Q. (2004) Chiral separation of hexaconazole by reversed phase HPLC with β-cyclodextrin as a mobile phase and normal phase HPLC with CDMPC as chiral stationary phase. Journal of Instrumental Analysis. 23(5):133 -135.
- 15. Wang P., Zhou Z.Q., Jiang S.R., **Yang L**. (**2004**) Chiral resolution of cypermethrin on cellulose-tris(3,5-dimethylphenyl-carbamate) chiral stationary phase. Chromatographia. 59 (9-10): 625-629.
- Zhao H.X., Qiu Y.M., Wang L.P., Zhou Z.Q., Yang L. (2004) Research development of the determination of barbiturates. Animal Science & Veterinary Medicine (Chinese) Vol.21 No.03 P.29-30.
- 17.Zhou Z.Q., Wang P., Jiang S.R., Wang M., Yang L.(2003). Preparation of polysaccharide-based chiral stationary phases and the direct separation of six chiral pesticides and related intermediates. Journal of Liquid Chromatography & Related Technologies. 26(17): 2873-2880.
- Wang P., Zhou Z.Q., Jiang S.Q., Yang L., Zhang H.J. (2003) The preparation of two coated cellulose-based chiral stationary phases and the direct resolution of chiral pesticides and related intermediates. 2nd International Academic Symposium on Pesticides and Environmental Safety (Beijing) 175-180.

PART VI: SPECIALTIES

A. RESEARCH AND LABORATORY SKILLS

- Quantitative analysis of drug metabolism: UPLC/MS-MS (maintenance of UPLC/MS-MS; method development for separating and identifying small molecules including drug metabolites in bio-samples such as blood, urine and tissues; running samples for drug metabolistes analysis; acquiring and processing data; report generating); HPLC/UPLC (maintenance; method development; and Chiral pesticides separation); MALDI-TOF
- Purification of biomarkers from biological fluid samples (including serum, urine, cell culture medium and tissue); Solid phase extraction technique; risk assessment
- Analysis of multiple pesticides' residues from agricultural products such as vegetables/fruits (HPLC/GC)
- Biology and molecular Biology: Cell culture; western blotting; PCR; real-time PCR; siRNA knock down gene technique; shRNA knock down gene technique; Confocal; Fluorescence microscope
- Animal model (including trans-genetic mice and rats): investigate molecular mechanisms of estrogen carcinogenesis in breast cancer initiation and progression; chemoprevention strategies
- Synthesis of depurinating estrogen-DNA adducts
- Translational study design and execution
- Statistical software: PAWS; SPSS; SAS; Prizm

B. INFORMATION TECHNOLOGY SKILLS

- Molecular Modeling software
- Microsoft Word, Excel, and PowerPoint
- Basic Internet skills

Reduced formation of depurinating estrogen–DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells

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Sulforaphane (SFN) is a potent inducer of detoxication enzymes such as NAD(P)H:quinone oxidoreductase 1 (NOO1) and glutathione-S-transferase (GST) via the Kelch-like erythroid-derived protein with CNC homology-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) signaling pathway. NQO1 reduces the carcinogenic estrogen metabolite, catechol estrogen-3,4-quinone, whereas GSTs detoxify it through conjugation with glutathione. These 3,4-quinones can react with DNA to form depurinating DNA adducts. Thus, SFN may alter estrogen metabolism and thus protect against estrogen-mediated DNA damage and carcinogenesis. Human breast epithelial MCF-10A cells were treated with either vehicle or SFN and either estradiol (E₂) or its metabolite 4-hydroxyestradiol (4-OHE2). 4-Hydroxy-derived estrogen metabolites and depurinating DNA adducts formed from E₂ and its interconvertable metabolite estrone (E1) were analyzed by mass spectrometry. Levels of the depurinated adducts, 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine, were reduced by 60% in SFN-treated cells, whereas levels of $4\text{-OCH}_3\text{E}_{1/2}$ and $4\text{-OHE}_{1/2}$ -glutathione conjugates increased. To constitutively enhance the expression of Nrf2-regulated genes, cells were treated with either scrambled or siKEAP1 RNA. Following E₂ or 4-OHE₂ treatments, levels of the adenine and guanine adducts dropped 60-70% in siKEAP1treated cells, whereas 4-OHE_{1/2}-glutathione conjugates increased. However, 4-OCH₃E_{1/2} decreased 50% after siKEAP1 treatment. Thus, treatment with SFN or siKEAP1 has similar effects on reduction of depurinating estrogen-DNA adduct levels following estrogen challenge. However, these pharmacologic and genetic approaches have different effects on estrogen metabolism to O-methyl and glutathione conjugates. Activation of the Nrf2 pathway, especially elevated NQO1, may account for some but not all of the protective effects of SFN against estrogen-mediated DNA damage.

Introduction

Elevated levels of estrogens have been recognized as an important determinant of the risk of breast cancer (1). Studies in experimental

Abbreviations: 4-OHE2, 4-hydroxyestradiol; COMT, catechol-*O*-methyltransferase; CYP1B1, cytochrome P450 1B1; E1, estrone; E2, estradiol; ER, estrogen receptor; GST, glutathione-S-transferase; Keap1, Kelch-like erythroid-derived protein with CNC homology-associated protein 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2-related factor 2; SFN, sulforaphane; siRNA, small interfering RNA. animal models demonstrate that estradiol (E_2) and estrone (E_1) are carcinogenic (2) and studies in cultured human cells (3,4) provide a mechanistic basis for this effect. Observational studies and clinical trials consistently support the contention that sustained exposure to endogenous estrogens is associated with the development of sporadic breast cancer. Two complementary pathways are likely required for estrogen carcinogenicity (2). One involves signaling through the estrogen receptor (ER) leading to altered gene expression and increased proliferation accompanied by spontaneous mutations (5). The other pathway, outlined in Figure 1, involves the oxidative metabolism of E_1 or E_2 to catechol estrogens and then reactive quinone metabolites. These metabolites can then directly and/or indirectly cause DNA damage and mutations responsible for the initiation and progression to breast cancer.

Metabolism of estrogens is characterized by a balanced set of activating and deactivating pathways. Aromatization of androstenedione and testosterone by aromatase (CYP19) yields E₁ and E₂, respectively. E_1 and E_2 are interconverted by 17\beta-hydroxysteroid dehydrogenase, and they are metabolized at the 2- or 4-position to form $2-OHE_{1/2}$ or 4-OHE_{1/2}, respectively. Cytochrome P450 1A1 preferentially hydroxvlates E_1 and E_2 at C-2, whereas cytochrome P450 1B1 (CYP1B1) almost exclusively catalyzes the formation of 4-OHE_{1/2} (6). The most common pathway of conjugation of estrogens in extrahepatic tissues is O-methylation, catalyzed by catechol-O-methyltransferase (COMT). If the activity of COMT is low, CYP or peroxidases can catalyze competitive oxidation of the catechol estrogens to E1/2-2,3-quinones and E1/2-3,4-quinones. Higher levels of depurinating DNA adducts are formed by $E_{1/2}$ -3,4-quinones compared with $E_{1/2}$ -2,3-quinones due to different mechanisms of adduction (7). The $E_{1/2}$ -3,4-quinones react via a 1,4-Michael addition, whereas the $E_{1/2}$ -2,3-quinones rearrange to p-quinone methides, which react via a 1,6-Michael addition (8). These adducts generate apurinic sites that can be converted into mutations by error-prone repair, which in turn may initiate breast carcinogenesis (9). Consistent with these actions, $E_{1/2}$ -3,4-quinone produced A-T to G–C mutations in the DNA of the mammary gland of ACI rats (10).

Although the estrogen oxidation pathway is detrimental to the integrity of DNA, several protective pathways in cells control the homeostasis of estrogen metabolism to avoid DNA damage. Catechol estrogens can be detoxified by COMT, and the $E_{1/2}$ -3,4-quinones by conjugation with glutathione or by reduction back to catechol estrogens, catalyzed by NAD(P)H:quinone oxidoreductase 1 (NQO1) (8) (Figure 1). Diminished expression of detoxication enzymes and/or upregulation of enzymes of the oxidation pathway can disrupt this homeostasis. For example, higher expression of CYP19 and CYP1B1, or lower expression of COMT and NQO1, in breast tissues is associated with elevated risk of breast cancer (11). Gaikwad et al. (12,13) and Pruthi et al. (14) have reported that there is a significantly higher ratio of depurinating DNA adducts to other estrogen metabolites when comparing women at high risk for breast cancer or diagnosed with the disease to controls, indicating that formation of depurinating estrogen-DNA adducts likely plays key roles in breast cancer development.

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables with particularly high levels in 3-day-old broccoli sprouts (15). It is converted by hydrolysis of the glucosinolate, glucoraphanin, by the enzyme, myrosinase, found in plants or by β -thioglucosidases found in the gut microflora. SFN is an attractive chemopreventive agent since it is safe and can be distributed widely as broccoli sprout preparations. Moreover, SFN and broccoli sprout preparations are effective chemopreventive agents in rodent models of mammary carcinogenesis (15,16) and initial pharmacokinetic studies indicate that pharmacologically relevant concentrations of SFN metabolites can be detected in the mammary epithelium of women consuming broccoli sprout-derived beverages (17). An important, but far from unilateral, mechanism of action for SFN is



Fig. 1. Pathway for formation of estrogen depurinating DNA adducts. E_2 or E_1 can be oxidized to $E_{1/2}$ -3,4-quinone, which can bind to DNA to form 4-OHE_{1/2}-1-N3Adenine or 4-OHE_{1/2}-1-N7Guanine adducts. NQO1 reduces $E_{1/2}$ -3,4-quinones back to catechols and GST catalyzes the conjugation of $E_{1/2}$ -3,4-quinones with glutathione, whereas COMT catalyzes the methylation of 4-OHE_{1/2} to 4-OCH₃E_{1/2}.

the induction of carcinogen detoxication enzymes such as NQO1 and glutathione-S-transferases (GSTs). SFN is an activator of the antioxidant response element Kelch-like erythroid-derived protein with CNC homology-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) signaling pathway regulating the expression of these and many other genes (18). Under normal cellular conditions, Nrf2 binds to Keap1 in the cytoplasm, resulting in ubiquitination of Nrf2 and its subsequent proteasomal degradation (19). SFN can modify cysteine 151 in Keap1 to disrupt the association of Cul3 ubiquitin ligase with Keap1, allowing Nrf2 to escape degradation. Thus, Nrf2 is stabilized and translocates into the nucleus to induce the transcription of its target genes such as NQO1 and GST (3,20). Using transcriptomic and proteomic profiling, we have shown previously that SFN induces Nrf2-regulated genes in ERa negative, non-tumorigenic human breast epithelial MCF-10A and MCF-12A cells (20) and primary cultures of human mammary epithelial cells (21). Interestingly, these profiles were similar to those provoked by treatment of the MCF-10A cells with small interfering RNA (siRNA) vectors for KEAP1 knockdown (20). In the present study, we have evaluated the efficacy of SFN to alter estrogen metabolism away from the formation of DNA adducts through induction of detoxication genes and to determine the extent to which such protection can be mimicked by genetic amplification of the Nrf2 signaling pathway through KEAP1 knockdown.

Materials and methods

Chemicals and reagents

E₂, 4-hydroxyestradiol (4-OHE₂) and 4-OCH₃E₁ (E₂) were obtained from Steraloids (Newport, RI). Standards of the glutathione conjugates of 4-OHE₂ and the depurinating DNA adducts were synthesized by published procedures (7,22–24). A mixed standard solution of 10 µg/ml was obtained by mixing 10 µl of individual stock solutions (1 mg/ml) and diluting to 1 ml with methanol/water 50:50 with 0.1% formic acid. Serial dilutions of this solution were used as standards for the preparation of calibration curves. Stock standard solutions and mixed standard solutions were stored at -80°C. SFN (1-isothiocyanato-4-(methylsulfinyl)-butane) was purchased from LKT Laboratories (St Paul, MN). The glutathione (reduced) quantification kit was purchased from Dojindo Molecular Technologies (Rockville, MD). All other chemicals were of the highest quality obtain able commercially.

Cell lines and cell culture

MCF-10A cells were obtained from the American Type Culture Collection and cultured in estrogen-free medium at 37°C in a humidified incubator containing 5% CO₂. Cell culture medium was prepared by a phenol red-free mixture of Dulbecco's modified Eagle's media and Ham's nutrient mixture F-12 media (Mediatech, Manassas, VA) containing 20 ng/ml epidermal growth factor, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin and 5% charcoal-stripped fetal bovine serum (Invitrogen) (25).

For SFN treatment, cells were seeded in 10 cm dishes $(1-2 \times 10^6 \text{ cells}/10 \text{ ml media/dish})$ overnight and then treated with either vehicle or SFN for 24 h. After discarding the media, all plates were fed with fresh cell culture media containing 10 μ M E₂ or 4-OHE₂ for 24 h and then retreated

with vehicle or SFN for another 24 h (Figure 2) without changing cell culture media. SFN and E₂ were dissolved in dimethyl sulfoxide and 4-OHE₂ was dissolved in ethanol: the final vehicle concentration in the media was <0.05% (v/v). For siRNA knockdown of KEAP1, cells were seeded in 10 cm dishes $(1-2 \times 10^6 \text{ cells}/10 \text{ ml medium/dish})$ overnight and were transfected at 30-40% confluence with siRNA human KEAP1(J-012453-07) or scrambled siRNA control(D-001810-01) from Thermo Scientific (Dharmacon, Lafayette, CO) with lipofectamine 2000 for 48 h following the manufacturer's protocol (Figure 2). Confirmatory experiments were conducted using a shRNA construct for KEAP1. The target sequence selected, 5'-GTGGGCGTGGCTGTCCTCAAT-3' (sense), corresponds to a region 1572-1592 bp of the Keap1 gene. The sh-Scramble sequence was selected as 5'-GGACGGAGCAGTCAAGTACAA-3'. The targeted and scrambled sequences were subcloned into entry vector pENTR/H1 plasmid (Invitrogen), pENTR/H1/Keap1 or scramble was recombined into pAD/ Dest. Then pAd/Keap1 or scramble was transfected into the adenovirus packaging cell line. After generation, amplification and titer measuring, the adenoviruses were then incubated with MCF-10A cells at 37°C. The cell culture media were collected with 2 mg/ml ascorbic acid added and either processed immediately or frozen at -80°C prior to assay for estrogen metabolites and depurinating DNA adducts. Cells were harvested for RNA, protein and activity assays.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) or 5 PRIME PerfectPure RNA Cell & Tissue Kit (5PRIME, Gaithersburg, MD), from which complementary DNA was synthesized using the qScriptTM cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD). PCR was carried out in a 20 µl volume including each target primer, complementary DNA and iQTM SYBR® Green Supernix (Bio-Rad, Hercules, CA) and run in a Bio-Rad Thermal Cycler (Bio-Rad, Hamburg, Germany). Fold-change values were determined using the $2^{-\Delta \Delta CI}$ relative quantification method (26). The amplified products were electrophoresed on agarose gel and stained with ethidium bromide.

PCR primers were as follows: forward 5'-TGACAATGAGGTTTCTTCGG-3' and reverse 5'-TCTGTCAGTTTGGCTTCTGG-3' for human Nrf2, forward 5'-ACGTCCTTGGAGGCTATGAT-3' and reverse 5'-TCTGCTGGTCA ATCTGCTTC-3' for human KEAP1, forward 5'-CGCTTCTCTTGGAGGA ATGT-3' and reverse 5'-TCCACCACCTCCCTGTATTC-3' for human COMT, forward 5'-TTCCGGAGTAAGAAGGCAGT-3' and reverse 5'-GGAGTGTGCCCAATGCTAT-3' for human NQ01, forward 5'-TAAA GGAGAGAGCCTGATTG-3' and reverse 5'-TTCAAAGGCAGGAAGT AGC-3' for human GSTA1 and forward 5'-GGACTCATGACCACAGGCACA-3' and reverse 5'-CTGCTTCACCACCTTCTTGA-3' for glyceraldehyde 3-phosphate dehydrogenase.



Fig. 2. Timeline for treatments of MCF-10A cells.

Western blots

After treatment, cells were harvested and then lysed in RIPA buffer with protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) and unlysed cellular debris removed by centrifugation. Protein concentrations were determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Western blot procedures followed the ABC protocol. Samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline with 0.05% Tween 20 with 5% non-fat milk, incubated with primary antibodies and then incubated with a peroxidaseconjugated secondary antibody after extensive washing. Dilutions of primary anti-KEAP1, Nrf2, NQO1 (Santa Cruz Biotechnology), CYP1B1 (Genetest, Bedford, MA), COMT, GSTA1 and β-actin (Sigma) antibodies were made in blocking solution (5% non-fat dry milk in Tris-buffered saline). The blots were incubated with Western Lightning® Plus-ECL solution (PerkinElmer, Waltham, MA) and visualized with X-ray film. Intensities of the bands were quantified by Bio-Rad Quantity One® software (Bio-Rad, Hercules, CA). The densitometry ratios for treated samples compared with controls were determined for three biological replicates and normalization was to β-actin.

NQO1 activity

MCF-10A cells were washed three times with 0.25 M sucrose/10 mM potassium phosphate (pH 7.2), collected from the plates by scraping, frozen in liquid nitrogen and stored at -80° C for assay as described previously (27).

Ultra-performance liquid chromatography-tandem mass spectrometry analysis of estrogen metabolites and depurinating DNA adducts

Extraction of estrogen metabolites and depurinating DNA adducts from cell culture media was modified from previously described procedures (12). After adjusting the pH to 7, cell culture media (10 ml/sample) was loaded onto phenyl cartridges (Agilent Technologies) that were preconditioned with methanol and water. Extracts were eluted as described (12), lyophilized, redissolved in a methanol:water 50:50 mixture containing 0.1% formic acid and finally subjected to ultra-performance liquid chromatography-tandem mass spectrometry analysis. Mass spectrometer parameters are presented in Table I. Analyses were conducted using selected reaction monitoring with a triple stage quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific) by using heated electrospray ionization in positive ion mode. The mass spectrometer was interfaced to a ultra-performance liquid chromatography system consisting of an Accela quaternary pump (Thermo Scientific) used for the chromatographic separation, and a Thermo Pal autosampler (HTC PAL, Zwingen, Switzerland). A Hypersil Gold column $(1.9 \,\mu\text{m}, 100 \times 2.1 \,\text{mm}, \text{Thermo Electron})$ was used for separation at a flow rate of 0.5 ml/min. The gradient started with 95% A (0.1% formic acid in H₂O) and 5% B (0.1% formic acid in CH₃CN), changed to 80% A over 1 min, changed to 79% A over 4 min, followed by a 4 min linear gradient to 30% A, changed to 2% A for 2 min, then changed back to the original conditions with a 3 min hold, resulting in a total separation time of 14 min. For all the studies, a methanol:water (1:1) mixture with 0.1% formic acid was used as the carrier solution. A signal-to-noise ratio of 3 was used as the limit of detection for each compound. Experiments were performed by applying a capillary (ion transfer tube) temperature of 380°C, vaporizer temperature 398°C, sheath gas pressure (arbitrary units) 50, auxiliary gas pressure (arbitrary units) 20, spray voltage 3.98 kV and collision gas pressure 1.5 mTorr. The collision energy for each compound is listed in Table I. The coefficient of variance for all analytes was <4%. The Xcalibur

Table I. Mass spectrometry parameters				
Name	Parent	Product	Collision energy	Limit of detection (fmol)
4-OCH ₃ E ₁	301.18	189.12	21	166.0
4-OCH ₃ E ₂	303.15	137.10	23	329.9
4-OHE ₁ -2-glutathione	592.16	317.06	23	84.4
4-OHE ₂ -2-glutathione	594.17	319.08	24	168.3
4-OHE ₁ -1-N7Guanine	436.20	152.02	39	2.8
4-OHE ₂ -1-N7Guanine	438.21	272.12	35	2.8
4-OHE ₁ -1-N3Adenine	420.20	296.09	44	2.9
4-OHE ₂ -1-N3Adenine	422.16	136.00	47	2.9
2-OHE ₁ -1-N3Adenine	420.20	136.04	31	1.4
2-OHE ₂ -1-N3Adenine	422.22	136.02	28	1.4

software (Thermo Scientific) was used to process and quantify the acquired data of estrogen metabolites.

Statistics

The data are presented as means \pm SE of at least three independent experiments. Comparisons between two groups were analyzed using the Student's *t*-test, and significance was established at P < 0.05 using Prizm 5 software.

Results

Modulation of transcripts, protein expression and activity of estrogen metabolism enzymes by SFN treatment or KEAP1 knockdown

Treatment of MCF-10A cells with SFN led to induction of *GSTA1* and *NQO1* transcripts but no changes in expression levels of two other genes known to influence E_2 metabolism, namely *CYP1B1* and *COMT*, were observed (Figure 3A). These inductions exhibited a dose response, with minimal induction observed with 3 μ M, moderate with 7 μ M and near maximal with 10 μ M SFN (data not shown). As expected, there were also no changes in the transcript levels of *KEAP1* or *NRF2*. SFN treatment significantly elevated NQO1 protein level 3.0-fold (*P* < 0.01; SFN treatment versus vehicle, Figure 3B) and its specific activity 2.7-fold (*P* < 0.01; SFN treatment versus vehicle, Figure 3C). Although no change of COMT messenger RNA level was detected, a significant 2.4-fold increase in COMT protein (*P* < 0.05; SFN treatment versus vehicle, Figure 3B) was observed. CYP1B1 protein was significantly decreased by 50% with SFN treatment (Figure 3B) (*P* < 0.05; SFN treatment versus vehicle). Thus, it appears that SFN influences the



Fig. 3. Effects of SFN on transcript, protein and activities of enzymes metabolizing E_2 or E_1 . (A) Effect of SFN on transcripts levels of estrogen metabolism enzymes. (B) Effect of SFN on protein levels of estrogen metabolism enzymes. (C) Effect of SFN on NQO1 activity. MCF-10A cells were treated with 10 µM SFN as described in Figure 2. Values are mean ± SE of three independent experiments. *Differs from vehicle control, P < 0.05.

expression of E_2 metabolizing enzymes through both transcriptional and post-transcriptional mechanisms. As expected, SFN and siKEAP1 also lead to increases in intracellular concentrations of reduced glutathione in MCF-10A cells (1.48- and 1.71-fold, respectively).

Since SFN is a well-characterized activator of Nrf2 signaling in MCF-10A and other mammary cells (20,21), the influence of siKEAP1 knockdown, and hence genetic activation of the pathway, was evaluated. As shown in Figure 4, transcript (Figure 4A), protein (Figure 4B) and specific activity of NQO1 (Figure 4C) significantly increased in the setting where *KEAP1* expression was significantly reduced by 80% (P < 0.01 siKEAP1 versus scrambled). Interestingly, no induction of *GSTA1* transcripts was detected, suggesting that the SFN-mediated induction of this gene is Nrf2 independent. Also unexpectedly, levels of *COMT* transcripts as well as COMT protein were significantly decreased 60–70% by the siKEAP1 treatment (P < 0.01for COMT protein level in siKEAP1 versus scrambled) (Figure 4A and B). Comparable results were seen using shKEAP1 knockdown (data not shown). *COMT* is not known to be a direct Nrf2-regulated gene and the mechanism underlying this response is not known.

Modulation of levels of depurinating estrogen–DNA adducts and estrogen metabolites by SFN or siKEAP1 treatment

At 48 h after E_2 treatment, the summed levels of the depurinating adducts, $4\text{-OHE}_{1/2}$ -1-N3Adenine and $4\text{-OHE}_{1/2}$ -1-N7Guanine, in the



Fig. 4. Effects of siKEAP1 on transcript, protein and activities of E_2 metabolizing enzymes. (A) Effect of siKEAP1 on levels of estrogen metabolism enzyme transcripts. (B) Effect of siKEAP1 on protein levels of estrogen metabolism enzymes. Scr, scrambled; siKp, siKEAP1. (C) Effect of siKEAP1 on NQO1 activity. MCF-10A cells were treated with siKEAP1 as described in Figure 2. Values are mean \pm SE of three independent experiments. *Differs from scrambled control, P < 0.05.

culture media were significantly lower in SFN-treated cells compared with vehicle $(0.03 \pm 0.01$ versus 0.07 ± 0.02 pmol/10⁶ cells, P < 0.05) (Figure 5A). Although E₂ was added to the cells, there was considerable conversion to E₁. Approximately half of the formed adenine and guanine adducts were derivatives of E₁ and the remainder were from E₂. In contrast, levels of 4-OCH₃E_{1/2} increased significantly with SFN treatment (5.36 ± 0.16 versus 1.81 ± 0.20 pmol/10⁶ cells, P < 0.01) (Figure 5B). More modest increases in the levels of 4-OHE_{1/2}-glutathione conjugates were measured following SFN treatment (1.54 ± 0.37 versus 0.83 ± 0.19 pmol/10⁶ cells, P < 0.05, Figure 5C). About 25-fold more methoxy conjugates were formed than glutathione conjugates in either the vehicle or SFN-treated cells. 2-OHE_{1/2} adducts were also measured but typically reflected only 2-3% of the level of the 4-OHE_{1/2} DNA adducts formed. Therefore, we did not characterize patterns of 2-OHE_{1/2}-derived metabolites.

Addition of the proximate metabolite, 4-OHE₂, to cells led to 20-fold higher levels of depurinating estrogen–DNA adducts than seen with E₂ in vehicle-treated cells. In this instance, the majority was derived from E₁. 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts were again significantly lower in SFN-treated cells compared with vehicle (0.59 ± 0.11 versus 1.42 ± 0.16 pmol/10⁶ cells, P < 0.01; Figure 5D). 4-OCH₃E_{1/2} levels increased 3.4-fold (195.00 ± 12.33 versus 58.05 ± 1.77 pmol/10⁶ cells, P < 0.01; Figure 5E), whereas 4-OHE_{1/2}-glutathione conjugates increased 5.1-fold following SFN treatment (4.44 ± 0.52 versus 0.87 ± 0.03 pmol/10⁶ cells, P < 0.01; Figure 5F). The methoxy conjugates were the dominant metabolites detected.

Pretreatment of cells with siKEAP1 led to more substantial declines in levels of the depurinating estrogen–DNA adducts in the cell culture media. Following E_2 treatment, levels of the 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts dropped 70% in siKEAP1-treated cells compared with scrambled vector. 4-OCH₃E_{1/2} levels decreased 50% with siKEAP1 treatment (Figure 5E), whereas levels of the 4-OHE_{1/2}-glutathione conjugates were not significantly different (Figure 5F). Both outcomes are consistent with the effects of siKEAP1 on *COMT* and *GSTA1* transcripts (Figure 4A). Similar results were seen following 4-OHE₂ treatment. 4-OHE_{1/2}-1-N3Adenine and 4-OHE_{1/2}-1-N7Guanine adducts declined 90% in siKEAP1-treated cells compared with scrambled vector (Figure 5D); 4-OCH₃E_{1/2} declined 60% (Figure 5E), whereas there was no significant change in levels of 4-OHE_{1/2}-glutathione conjugates with siKEAP1 treatment (Figure 5F).

Discussion

The natural and synthetic estrogens E₁, E₂, diethylstibestrol and hexestrol induce kidney tumors in Syrian golden hamsters (28). Additionally, the proximate estrogen metabolites, $4-OHE_{1/2}$, are carcinogenic in hamsters and mice, but 2-OHE_{1/2} metabolites are not (29–31). 4-OHE₂ and E_2 -3,4-quinone are mutagenic in mouse skin and rat mammary gland (9,32). The genotoxicity of 4-OHE₂ and E₂-3,4-quinone was also demonstrated in the Big Blue rat embryonic cell line, but no mutations were observed after treatment of the cells with 2-OHE₂ (33). The much greater carcinogenic activity of 4-OHE_{1/2} compared with 2-OHE_{1/2} likely reflects the far greater propensity of E1/2-3,4-quinones to form estrogen-DNA adducts compared with $E_{1/2}$ -2,3-quinones (7). Transgenic mice with ER α knocked out (ERKO/wnt-1 mice) provide another model that demonstrates the critical role of estrogen genotoxicity in carcinogenesis. The wnt-1 transgene induced mammary tumors in female ERKO/wnt-1 mice, despite the lack of ER α (34). Mammary tumors developed in these mice even when ovariectomized mice were implanted with both E2 and the antiestrogen ICI-182,780 (35) suggesting that non-ER pathways such as metabolism to the genotoxic quinones that form the adenine and guanine adducts contribute to mammary tumor development.

MCF-10A cells are an immortalized mammary epithelial cell line lacking ER α . Treatment of MCF-10A cells with E₂ or 4-OHE₂ generates the depurinating estrogen–DNA adducts and transformation of



Fig. 5. Effect of pharmacologic or genetic perturbation of estrogen metabolism on estrogen–DNA adducts and metabolites in MCF-10A cells. Cell culture media were collected, partially purified by solid-phase extraction, and analytes separated and quantified by ultra-performance liquid chromatography–tandem mass spectrometry. (**A**–**C**) Levels of estrogen–DNA adducts, 4-OHCH₃E_{1/2} or E_{1/2}⁻glutathione conjugates, respectively, following addition of E₂ to cells pretreated with either SFN or siKEAP1. (**D**–**F**) Levels of estrogen–DNA adducts, 4-OHCH₃E_{1/2} or E_{1/2}⁻glutathione conjugates, respectively, following addition of 4-OHE₂ to cells pretreated with either SFN or siKEAP1. Veh, vehicle; Scr, scrambled vector. Values are mean of triplicate biological replicates ± SE. *Differs from control, P < 0.05.

the cells, as detected by their ability to form colonies in soft agar (36). This transformation can also occur in the presence of the antiestrogens, tamoxifen or ICI-182,780 (37). Thus, DNA damage and cell transformation by estrogens do not require a functional ER α signaling pathway. Consequently, strategies to prevent the formation of estrogen–DNA adducts may alter the initiation and development of both ER α positive and negative cancers.

The depurinating estrogen-DNA adducts that efflux from cells and tissues are excreted in urine, allowing their identification and quantification as biomarkers of risk of developing breast cancer (7,12-14). High levels of estrogen-DNA adducts have been observed in analyses of urine and serum from women at high risk for or diagnosed with breast cancer compared with women at normal risk (12-14). Levels of DNA adducts are strongly influenced by the balance of enzymes involved in their bioactivation to reactive intermediates and their detoxication. Protective enzymes such as COMT, GSTs and NQO1 can decrease steady-state levels of E1/2-3-4-quinones and the resulting depurinating estrogen-DNA adducts in cell culture models (Figure 1). For example, inhibition of COMT led to an increase in oxidative DNA damage in human breast cancer-derived MCF-7 cells (38) and formation of $E_{1/2}$ -3-4-quinone adenine and guanine DNA adducts in MCF-10F cells (39). Conjugation of the quinones with glutathione catalyzed by GSTs and reduction by NQO1 of the quinones back to catechol estrogens can be envisioned to mitigate the formation of depurinating adducts. A series of isogenic MCF-10F cells stably expressing polymorphic variants of NQO1 with lower capacity to reduce estrogen quinones compared with wild-type NQO1 consistently led to increased formation of estrogen-DNA adducts from E2-3,4-quinone (40).

As seen in an earlier study using the phytochemical resveratrol in MCF-10F cells, it is possible to reduce the formation of estrogen–DNA adducts and to decrease E_2 -induced transformation to anchorage-independent growth (4). We show here that the broadly effective chemopreventive agent isolated from broccoli, SFN, also exerts strong protective effects against the DNA damaging actions of estrogens in the MCF-10A cells. The enzymes associated with protection are inducible by SFN in many human cell types, including MCF-12A (20) and primary mammary epithelial cells (21). Given the current preclinical and clinical evaluation of SFN (typically as an enriched component of broccoli sprout extracts; see ClinicalTrials.gov), there is interest in targeting this agent toward breast cancer prevention.

Protection against the genotoxicity of estrogens in the MCF-10A cells can be induced by treatment with SFN or genetic activation of Nrf2 signaling. However, the underlying metabolic changes are distinct, indicating that upregulation of Nrf2 signaling may not account for the full effects of SFN on this endpoint. Following treatment with E₂, the SFN pretreated cells exhibit significant increases in the production of 4-OCH₃ and glutathione conjugates. However, these detoxication metabolites were not elevated by the pretreatment of cells with siKEAP1. Thus, the effects of SFN on COMT and GSTA1 appear to occur independently of Nrf2 signaling. Indeed, siKEAP1 led to a decrease in COMT protein levels and decline in 4-OCH₃ formation while nonetheless exhibiting protection. Whether Nrf2 exerts negative regulation on COMT is not known although neither microarray nor ChIP-Seq studies indicate it to be a direct Nrf2 target gene (41,42). Presumably then, SFN-mediated upregulation of COMT levels at the protein, but not transcript level, reflects a distinct, Nrf2-independent mode of action. Given the protective actions of both the pharmacologic and genetic interventions, and the common induction of NQO1 by both modalities, it is likely that reduction of the $E_{1/2}$ -3,4-quinones back to catechol estrogens is central to the effect. SFN also reduces levels of CYP1B1 protein in these cells, which could dampen formation of the catechol estrogens. However, the protection by SFN is also seen when 4-OHE₂ is administered to cells, a metabolite that is a product of CYP1B1 action. Thus, it is unlikely that inhibition of estrogen bioactivation is critical to protection in this setting. That bioactivation of E_2 is critical to its genotoxicity is underscored by the observation that 20-fold higher levels of depurinating estrogen-DNA adducts are detected following administration of 4-OHE₂ than with E₂. It should also be noted that considerably more catechol-O-methyl conjugates are formed than glutathione conjugates (~200 pmol/106 cells versus 4 pmol/10⁶ cells), indicating that GST catalyzed detoxication plays a minor role in overall protection against estrogen genotoxicity. Moreover, the absence of induction of GSTA1 by siKEAP1, which is nonetheless protective, also signals that GSTs are not critical in modulating estrogen-DNA adduct burden. Elevation of intracellular glutathione, observed in both the pharmacologic and genetic manipulations, did not lead to elevated 4-OH-estrogen-glutathione conjugates in the siKEAP1-treated cells, suggesting that nonenzymatic conjugation with glutathione too has no protective role.

In conclusion, SFN is the embodiment of phytochemical polypharmacy in a single molecule. It interacts with many molecular targets in cells and exerts its chemopreventive actions through actions on multiple pathways (43). The protective effect of SFN against estrogen-mediated DNA damage further highlights its possible role in chemoprevention of mammary carcinogenesis and illustrates that multiple mechanisms likely account for this outcome. Induction of the Nrf2-regulated detoxication gene, NQO1, would seem to be central to the protective alterations in metabolite distribution. At the same time, Nrf2-independent actions of SFN on COMT and GSTA1 are likely to contribute to enhanced protection of the genome.

Funding

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Conflict of Interest Statement: None declared.

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Training record:

From:	us.training.analyze@thermofisher.com <us.training.analyze@thermofisher.com></us.training.analyze@thermofisher.com>
Subject:	Thermo Scientific Enrollment Confirmation
Date:	Fri, October 7, 2011 1:15 pm
To:	liyang@pitt.edu <liyang@pitt.edu></liyang@pitt.edu>

Dear LI YANG:

This is your course confirmation! We would like to thank you for enrolling in the following course(s): TSQ Family Operations 10/24/2011: 9:00 AM - 5:00 PM 10/25/2011: 9:00 AM - 5:00 PM 10/26/2011: 9:00 AM - 5:00 PM 10/27/2011: 9:00 AM - 5:00 PM

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Class content

BIOSTATISTICS 2062

Clinical Trials: Methods and Practice

Lecture Schedule Spring Term, 2012 (12-2) Class Times: Thursday, 9:00 am - 11:50 am Room: A425 Crabtree Hall, GSPH

Date 1/05/12	<u>Topics</u> Introduction and History of Clinical Trials Salk Vaccine Clinical Trial	Instructor Dr. Redmond Dr. Redmond
1/12/12	Ethical Considerations Clinical Trials Organization and Protocol Development	Dr. Redmond Dr. Redmond
1/19/12	Translational Studies	Dr. Redmond
	Assessment	Dr. Redmond
2/02 /12	Randomization and Treatment Allocation Methods First Examination	Dr. Redmond
2/09/12	Bias, Precision, and Generalizability Overview of Clinical Trial Designs	Dr. Rockette Dr. Rockette
2/16/12	Comparative Trials: Parallel Group Designs Comparative Trials: Factorial and Crossover Design	Dr. Redmond Dr. Redmond
2/23/12	Comparative Trials: Cluster Randomized Designs Comparative Trials: Equivalence Trials	Dr. Redmond Dr. Redmond
2/26/12	Phase II - Activity/Efficacy Studies Sample Size Considerations	Dr. Redmond Dr. Redmond
3/01/12	Multiple Comparisons and Multiplicity Collaborative Group Projects: Introduction	Dr. Rockette Dr. Redmond

Spring Break - March 5-9, 2012

Date 3/15/12	<u>Topics</u> Time to Event Outcomes – Life Table Methods Time to Event Outcomes – Regression Models	Instructor Dr. Rockette Dr. Rockette
3/22 /12	General Analysis Considerations; Continuous and Discrete Outcomes Second Examination	Dr. Redmond Dr. Redmond
3/29/12	Repeated Measures in Clinical Trials Controlling for Confounding; Testing for Interaction	Dr. Redmond Dr. Redmond
4/05/12	Intention-to-Treat Analysis: Rationale and Approaches Interim Data Analysis	Dr. Redmond Dr. Redmond
4/12/12	Final Analysis and Reporting Guidelines Meta-analytic Methods	Dr. Redmond Dr. Redmond
4/19/12	Collaborative Group Presentations	Students
rom: Subject: Date: To:	"Redmond, Carol K" <ckr3@pitt.edu> RE: hello from li yang Fri, April 27, 2012 3:49 pm "Yang, Li" <liyang@pitt.edu></liyang@pitt.edu></ckr3@pitt.edu>	

Dear Li Yang,

It was a pleasure to have you in the class. Your contributions as an auditor were important. I hope that you will keep in touch and let me know about how your career and other activities progress over time. If you should need a statistical collaborator or consultant at some time in the future, please feel free to contact me. If I am not able to assist directly, I will try to recommend someone to you.

Best wishes and warm regards,

Carol Redmond

Dr. Carol K. Redmond

Distinguished Service Professor of Public Health

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Pittsburgh, PA 15261 Telephone: 1-412-624-0765 Fax: 1-412-624-2183 http://www.biostat.pitt.edu/redmond.htm http://www.wiley.com/legacy/products/subject/reference/bct_articles.html

Abstract acceptance record

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Welcome

Dear WOMEN'S CANCER RESEARCH CENTER MEMBERS:

We are pleased to invite you to attend the **2nd Annual WCRC Retreat** on September 7 and 8, 2012 at Nemacolin Woodlands Resort (1001 Lafayette Drive, Fa of the Retreat are to showcase exciting research which is currently performed in labs of WCRC members, to further enhance interaction, and foster collaboration stimulating program in the area of women's cancer research, covering basic, clinical, and translational areas of investigations.

The Retreat will begin Friday September 7th at noon, with a presentation by Dr Lee, Director of the WCRC, entitled *"Two years of WCRC - what have we acco done?"*. For the afternoon, we have scheduled presentations on breast and ovarian cancer topics (including presentations by last year's WCRC Pilot grant award conclude with dinner and entertainment. The Keynote Address will be given by Dr Bill Hahn, Associate Professor, Department of Medicine, Harvard Medical S Cancer Genome Discovery, Dana-Farber Cancer Institute. Dr Hahn is an expert in cancer genomics (including breast and ovarian cancer), and high-throughput for Saturday session will include a short morning session for all and then interested Faculty can participate in a brain-storming session dealing with programmatic in the store of the store.

We very much look forward to your attendance and participation in this retreat. We especially encourage you to bring students and fellows, as our trainees are so current and future research endeavors! We will need to limit the number of attendees at the Retreat, and therefore encourage you to register soon. Should we rear Retreat committee will make decisions based on active participation in WCRC activities.

All information, including a preliminary program, and forms regarding the WCRC Retreat can be found at http://www.upci.upmc.edu/WCRC/retreat. Please use abstract submission forms for this year's event. The deadline for registration (including abstract submission) will be July 10th at 5 pm. For questions regar Steffi Oesterreich, oesterreichs@upmc.edu.

Adrian V Lee, PhD Director, WCRC

Bob Edwards, MD Co-Director, WCRC

IN PARTNERSHIP WITH:



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From: kpater@magee.edu

Subject: Notice of Acceptance: 2012 WCRC Retreat Abstract Submission

Date: Tue, July 10, 2012 9:54 am

To: liyang@pitt.edu

Congratulations! Your abstract has been accepted for display and presentation at the Women's Cancer Research Center Retreat on September 7, 2012. You will be notified of your poster number the week before the retreat.

If you have any questions regarding your abstract submission or the UPCI Scientific Retreat, please contact Kathleen Pater at kpater@magee.edu

From: "Bakkenist, Christopher J" <bakkenistcj@upmc.edu>

Subject: UPCI seminar for UPCI retreat poster winners (Clinical Science)

Date: Fri, July 6, 2012 2:04 pm

- To: "nakajima.erica@medschool.pitt.edu" <nakajima.erica@medschool.pitt.edu>,"rws9@pitt.edu" <rws9@pitt.edu>,"Van Houten, Bennett" <vanhoutenb@upmc.edu>,"tkensler@pitt.edu" <tkensler@pitt.edu>,"liyang@pitt.edu" <liyang@pitt.edu>,"Fang, Qingming" <fangq@upmc.edu>,"Thompson, Lola" <thompsonla3@upmc.edu>,"George, Lisa (UPCI)" <georgel@upmc.edu>
- Cc: "saumen@pitt.edu" <saumen@pitt.edu>,"freerad@pitt.edu" <freerad@pitt.edu>,"Singh, Shivendra" <singhs@upmc.edu>,"jsk5@pitt.edu" <jsk5@pitt.edu>

Dear Li, Erica and Qingming,

As the winners of the Clinical Science Poster competition that was held during this year's UPCI retreat, Dr. Nancy Davidson invites you to participate in our UPCI seminar series. You are each invited to deliver a 15 minute oral presentation at noon on 25th September 2012.

Dr. Davidson will introduce you personally. I suggest we use the following order:

12.00-12.15 1st place: Li Yang et al. "Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in breast epithelial cells" (senior author Dr. Thomas Kensler)

12.20-12.35 2nd place: Erica Nakajima et al. "Diverse metabolite consumption by head and neck squamous cell carcinoma cells" (senior author Dr. Ben Van Houten)

12.40-12.55 3rd place: Qingming Fang et al. "Complex formation regulates the stability and degradation of PolB and XRCC1" (senior author Dr. Robert Sobol)

I am copying Dr. Freeman because I note that the Department of Pharmacology and Chemical Biology is well represented, to say the least, in these winning posters.

Please save this date for your seminar presentation.

Sincerely,

Chris Bakkenist

Invited review Records:

From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	Your Completed Peer Review
Date:	Mon, July 9, 2012 7:00 pm
To:	liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Combining mTOR Inhibitors With Chemotherapy and Other Targeted Therapies in Advanced Breast Cancer: Rationale, Clinical Experience, and Future Directions.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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Regards, Jan

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From:	"Jan Mclver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You Are Invited to Review a Paper [10071]
Date:	Tue, June 26, 2012 10:47 pm
To:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake a peer review of "Combining mTOR Inhibitors With Chemotherapy and Other Targeted Therapies in Advanced Breast Cancer: Rationale, Clinical Experience, and Future Directions", a manuscript submitted by Dr Yardley to Breast Cancer: Basic and Clinical Research.

If you are able to do this I need to receive your review comments by 09 July 2012. Please go to http://la-press.com/review.php?I=YDoXDA7oClKuIYZAnBdPA6YS71575 to accept or decline to undertake this review. Please also read the peer review guidelines in this email. The guidelines may be different to those of other journals.

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the link given above. If you have any questions please email me for assistance.

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Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Insulin-like growth factor 1gene polymorphism and breast cancer risk among Arab Omani women: A case-control study.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

Completing the peer reviewer survey takes less than one minute of your time. Your responses will be anonymous and will be used to enhance our services.

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Regards, Jan

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You Are Invited to Review a Paper [9784]
Date:	Thu, April 19, 2012 8:01 pm
То:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake a peer review of "Insulin-like growth factor 1gene polymorphism and breast cancer risk among Arab Omani women: A case-control study", a manuscript submitted by Professor Al-moundhri to Breast Cancer: Basic and Clinical Research.

If you are able to do this I need to receive your review comments by 04 May 2012. Please go to http://la-press.com/review.php?I=YDoXDA7oClKuIYZAnBdPA6YS71575 to accept or decline to undertake this review. Please also read the peer review guidelines in this email. The guidelines may be different to those of other journals.

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From:	"Jan McIver" <jan.mciver@la-press.com< th=""></jan.mciver@la-press.com<>
Subject:	Your Completed Peer Review
Date:	Wed, April 4, 2012 1:37 pm
To:	liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Circulating immune complex levels are associated with disease severity and seasonality in children with malaria from Mali.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You Are Invited to Review a Paper [9624]
Date:	Wed, March 21, 2012 6:20 pm
То:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake a peer review of "Circulating immune complex levels are associated with disease severity and seasonality in children with malaria from Mali", a manuscript submitted by Dr Bolaji N. Thomas to Biomarker Insights.

If you are able to do this I need to receive your review comments by 05 April 2012. Please go to http://la-press.com/review.php?I=YDoXDA7oClKuIYZAnBdPA6YS71575 to accept or decline to undertake this review. Please also read the peer review guidelines in this email. The guidelines may be different to those of other journals.

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From:"Jan McIver" <jan.mciver@la-press.com>Subject:Your Completed Peer ReviewDate:Mon, March 5, 2012 3:49 pm

To: liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for The prognostic value of suPAR compared to other inflammatory markers in patients with severe sepsis.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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Regards, Jan

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	Your Completed Peer Review
Date:	Mon, March 5, 2012 3:49 pm
То:	liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for The prognostic value of suPAR compared to other inflammatory markers in patients with severe sepsis.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You Are Invited to Review a Paper [9460]
Date:	Thu, February 23, 2012 4:55 pm
То:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake a peer review of "The prognostic value of suPAR compared to other inflammatory markers in patients with severe sepsis", a manuscript submitted by Mrs Gustafsson to Biomarker Insights.

If you are able to do this I need to receive your review comments by 09 March 2012. Please go to http://www.la-press.com/review.php?l=YDoXDA7oClKulYZAnBdPA6YS71575 to accept or decline to undertake this review. Please also read the peer review guidelines in this email. The guidelines may be different to those of other journals.

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	Your Completed Peer Review
Date:	Tue, January 10, 2012 12:31 am
То:	liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Evaluation of ischemia-modified albumin and C-reactive protein in type 2 diabetics with and without ketosis.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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From:	"Jan Mciver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You are invited to review a paper [9060]
Date:	Tue, December 20, 2011 8:59 pm
To:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake the peer-review of "Evaluation of ischemia-modified albumin and C-reactive protein in type 2 diabetics with and without ketosis". This paper has been submitted by Dr Ma to Biomarker Insights.

If you are able to do this I need to receive your review comments by 09 January 2012.

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Regards, Jan

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From:	"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	Your Completed Peer Review
Date:	Wed, December 7, 2011 3:45 pm
То:	liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Safety profile of a dietary supplement containing 1,3-dimethylamylamine: a 10-week intervention study.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

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From:	"Jan Mciver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You are invited to review a paper [8885]
Date:	Thu, November 24, 2011 4:58 pm
То:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake the peer-review of "Safety profile of a dietary supplement containing 1,3-dimethylamylamine: a 10-week intervention study". This paper has been submitted by Dr Bloomer to Nutrition and Metabolic Insights.

If you are able to do this I need to receive your review comments by 07 December 2011.

Please go to http://www.la-press.com/review.php?I=YDoXDA7oCIKuIYZAnBdPA6YS71575 to accept or decline to undertake this review.

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"Jan McIver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Your Completed Peer Review
Tue, October 18, 2011 10:20 pm
liyang@pitt.edu

Dear Dr liyang

On behalf of the Editor in Chief as well as the authors, I would like to thank you for completing your review for Sustainable long-term delivery microRNA with polylysine nanoparticles for inhibition of breast cancer invasion.

The effort you have put into this is most appreciated by us and will be of great value to the authors.

Completing the peer reviewer survey takes less than one minute of your time. Your responses will be anonymous and will be used to enhance our services.

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From:	"Jan Mciver" <jan.mciver@la-press.com></jan.mciver@la-press.com>
Subject:	You are invited to review a paper [8513]
Date:	Mon, October 3, 2011 5:36 pm
То:	liyang@pitt.edu

Dear Dr liyang,

I would like to invite you to undertake the peer-review of "Sustainable long-term delivery microRNA with polylysine nanoparticles for inhibition of breast cancer invasion". This paper has been submitted by Dr Jin to Breast Cancer: Basic and Clinical Research .

If you are able to do this I need to receive your review comments by 18 October 2011.

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From:carcinogenesis.editorialoffice@oup.comSubject:Thank you for submitting your review of manuscript ID CARCIN-2011-00715.R1 for CarcinogenesisDate:Mon, October 31, 2011 11:25 amTo:liyang@pitt.edu

31-Oct-2011

Dear Ms Yang

Thank you for reviewing manuscript CARCIN-2011-00715.R1 entitled "Catechol-<i>O</i>-methyltransferase-mediated metabolism of 4-hydroxyestradiol inhibits the growth of human renal cancer cells through the apoptotic pathway" for Carcinogenesis.

On behalf of the Editors of Carcinogenesis, we appreciate the voluntary contribution that each reviewer gives to the Journal. We thank you for your participation in the online review process and hope that we may call upon you again to review future manuscripts.

Yours sincerely Dr Thomas Kensler Editor Carcinogenesis

From:	carcinogenesis.editorialoffice@oup.com
From:	carcinogenesis.editoriaioffice@oup.com

Subject: Carcinogenesis MS - CARCIN-2011-00715

Date: Tue, August 30, 2011 1:41 pm

To: liyang@pitt.edu

Dear Li:

A manuscript entitled "Catechol-<i>O</i>methyltransferase-mediated metabolism of 4-hydroxyestradiol inhibits the growth of human renal cancer cells through the apoptotic pathway," with Dr Yuichiro Tanaka as corresponding author, has been submitted to Carcinogenesis, and we are writing to ask whether you could assess it for us. The abstract is shown below. We appreciate that there are many demands on your time, but would greatly value your evaluation of this article.

Could you please let us know whether you would be willing to review this paper for Carcinogenesis, bearing in mind that we would hope to receive the assessment within 2-3 weeks? If you are unable to review this manuscript, we would be grateful for any suggestions for suitable alternative referees, including senior members of your laboratory.

To record your reply automatically, click on the appropriate link below. Details of how to view the manuscript and submit your review will be e-mailed to you as soon as you have agreed.

Agreed: http://mc.manuscriptcentral.com/carcin?URL_MASK=CQyQyR5cFmCRRqGn5T8j

Declined: http://mc.manuscriptcentral.com/carcin?URL MASK=stXKd5K6D9FJnHdfQ8PF

Alternatively, please reply by return e-mail to carcinogenesis.editorialoffice@oup.com

within 24 hours if at all possible, so that we can continue to provide a timely review process for the research community.

Yours sincerely

Dr Thomas Kensler Editor Carcinogenesis

Catechol-<i>O</i>-methyltransferase-mediated metabolism of 4-hydroxyestradiol inhibits the growth of human renal cancer cells through the apoptotic pathway -CARCIN-2011-00715 Corresponding Author: Dr Yuichiro Tanaka Contributing Authors: Chang, Inik; Liu, Jan; Majid, Shahana; Saini, Sharanjot; Zaman, Mohd; Yamamura, Soichiro; Shahryari, Varahram; Chiyomaru, Takeshi; Deng, Guoren; Dahiya, Rajvir; Tanaka, Yuichiro

Abstract

Long-term exposure to estrogen and its metabolites may play an important role in renal cell carcinogenesis. Catechol-<i>O</i>-methyltransferase (COMT) participates in the estrogen metabolism pathway by neutralizing toxic substances. Although reduced COMT activity has been suggested to be a risk factor for estrogen-associated cancers, no studies have investigated the biological significance of COMT in the pathogenesis of human renal cell cancers (RCC). We initially found that COMT levels are significantly decreased in human RCC tissues and cells suggesting it plays a suppressive role in tumor development. However, transient over-expression of COMT has no functional effect on RCC cell lines. In contrast, when cells over-expressing COMT are treated with its substrate 4-hydroxyestradiol (4-OHE₂). growth is inhibited by apoptotic cell death. We also found that COMT over-expression combined with 4-OHE₂ induces up-regulation of growth arrest- and DNA damage-inducible protein α (GADD45 α). We further show that down-regulation of GADD45 α by a siRNA-mediated approach inhibits cell death, indicating the essential role of GADD45α in the underlying mechanism of COMT action in response to 4-OHE₂. Finally, 4-methoxyestradiol (4-ME) fully reproduces the anti-proliferative function of COMT with 4-OHE₂ prevents RCC cell proliferation by enhancing apoptosis, and that GADD45a plays a critical role in the COMT-mediated inhibition of RCC.

From: "Intel ISEF Grand Awards Judge Chair" < judging@societyforscience.org>

Subject: ISEF Category Judge Information

Date: Tue, April 24, 2012 6:52 am

To: "Dr. Li Yang" <liyang@pitt.edu>

Thank you for agreeing to serve as a Grand Awards judge at the Intel International Science & Engineering Fair 2012. Please allow this email to serve as a reminder of your commitment as well as general information about the logistics of Tuesday, May 15 and Wednesday, May 16.

SCHEDULE:

Please remember that the time commitment for Grand Awards judging is from Tuesday, May 15, through the evening of Wednesday, May 16. We need you to register in the David L. Lawrence Convention Center no later than 5:00 pm on Tuesday.

Once registered, you should check-in at your category room, pick-up a category ribbon and then spend the time prior to 5:30 p.m. reviewing the projects within your category on the exhibit hall floor. The exhibit hall opens at noon with no finalists present. At 5:30 p.m. all judges will be asked to go back to their respective category rooms for a buffet dinner and brief orientation by the category co-chairs. There will then be a Welcome and training session for all judges from 6:30 - 8:00 p.m. in Hall A. Following this session, the co-chairs will meet their judges in the exhibit hall within their category area to distribute the specific judging interview schedules for the next morning.

On Wednesday, the Exhibit Hall will be open to judges at 7:00 a.m. and all judges are asked to report to their category rooms by 8:30 for final attendance verification and to receive their score cards. Judging and post-interview category deliberations will occupy all of Wednesday. The detailed schedule for your judging as well as the Judging Guide that explains the complete process is available on the SSP site at http://www.societyforscience.org/page.aspx?pid=290

PARKING:

Parking has been arranged for judges and volunteers on Tuesday and Wednesday at the Grand Street Parking Lot between 11th and 12th Streets and Penn and Liberty. A map is available at the website address above. Those that park will need to ask their category co-chair for a chaser card to provide when exiting the garage each day.

MEALS: All meals will be provided from dinner Tuesday, breakfast-lunch-dinner Wednesday. These will be in the judge`s meeting area.

QUESTIONS:

Send all inquiries to: Judging@societyforscience.org Thank you again for your commitment to science education and young scientists. We look forward to working with you.

Sincerely, Chuck



24th Annual UPCI Scientific Retreat

POSTER ABSTRACTS

June 21-22, 2012

University of Pittsburgh at Greensburg



Breast Cancer Translational Science Poster No. 33.

Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells

Li Yang(1), Muhammad Zahid(2), Eleanor G. Rogan(2,3), Ercole L. Cavalieri(3), Thomas W. Kensler(1*)

1)Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213; 2) Environmental, Agricultural and Occupational Health, College of Public Health, and 3)Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198.

Sulforaphane (SFN) is a potent inducer of detoxification enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-S-transferase (GST) via the Kelch-like ECH-associated protein 1 (Keap1) - Nuclear Factor- E2-related factor (Nrf2) signaling pathway. NOQ1 reduces the carcinogenic estrogen metabolite, Catechol Estrogen-3,4-Quinone (CE-3,4-Q), to catechols while GSTs detoxify it through nucleophilic addition. CE-3,4-Q can bind with DNA to form depurinating DNA adducts, leading to DNA damage via an estrogen receptor (ER) independent pathway. Thus, SFN, a bioavailable phytochemical found in young broccoli plants, may be an ideal chemoprevention agent to block estrogen-mediated carcinogenesis. For this study we used the ER-negative, nontumorigenic human breast epithelial MCF10A cell line. MCF10A cells were treated with either vehicle or SFN (10 µM) and E2 or 4-OHE2. NQO1 was up-regulated at the mRNA (~2fold), protein (~3fold) and activity levels (~3fold) by SFN treatment. Estrogen metabolites and depurinating DNA adducts in the cell culture medium were partially purified by solid phase extraction and then analyzed by HPLC- ESI-MS/MS. . Following E2 treatment, the depurinated adducts 4-OHE1/2-1-N3Ade and 4-OHE 1/2-1-N7Gua were significantly lower in SFN treated cells compared to vehicle (0.03±0.01 versus 0.07±0.02 pmole/106cell, p=0.0294); 4-OHE1/2- glutathione conjugates were significantly higher following SFN treatment (1.54±0.37 versus 0.83±0.19 pmole/106cell, p=0.0015) as were 4-OCH3E1/2 (5.36 ± 0.16 versus 1.81±0.20pmole/106cell,p<0.0001) levels. Following treatment with the proximate metabolite 4-OHE2, 4-OHE1/2-1-N3Ade and 4-OHE 1/2-1-N7Gua were again significantly lower in SFN treated cells compared to vehicle (0.59±0.11 versus 1.42±0.16 pmole/106cell, p=0.0028) while 4-OHE1/2-glutathione-conjugates (4.44±0.52 versus 0.87±0.03 pmole/106cell,p=0.0001) and 4-OCH3E1/2 levels were significantly higher (195.00±12.33 versus 58.05±1.77pmole/106cell, p<.00001). Follow-up studies are examining the effects of genetic activation of Nrf2 signaling though disruption of Keap1 as well as targeted silencing of key metabolic genes. In conclusion, SFN can modulate estrogen metabolism leading to diminished formation of estrogen-DNA adducts.

Supported by DOD BCRP Postdoctoral Fellowship103928.

24th Annual UPCI Scientific Retreat Poster Abstracts





Annual UPCI Scientific Retreat Satellite Conference

POSTER ABSTRACTS

June 20, 2012

University of Pittsburgh at Greensburg



twe have ZOMi Cancer Epidemiology, Prevention and Control **Translational Science**

Abstract No. 17.

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Modulation of estrogen metabolism and estrogen depurinating DNA adducts via activation of Nrf2 signaling by sulforaphane in human breast epithelial cells

exercise

Li Yang (1), Muhammad Zahid (2), Eleanor G. Rogan (2,3), Ercole L. Cavalieri (3), Thomas W.

1) Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213; 2) Environmental, Agricultural and Occupational Health, College of Public Health, and 3) Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198

Sulforaphane (SFN) is a potent inducer of detoxification enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-S-transferase (GST) via the Kelch-like ECH-associated protein 1 (Keap1) - Nuclear Factor- E2-related factor (Nrf2) signaling pathway. NOQ1 reduces the carcinogenic estrogen metabolite, Catechol Estrogen-3,4-Quinone (CE-3,4-Q), to catechols while GSTs detoxify it through nucleophilic addition. CE-3,4-Q can bind with DNA to form depurinating DNA adducts, leading to DNA damage via an estrogen receptor (ER) independent pathway. Thus, SFN, a bioavailable phytochemical found in young broccoli plants, may be an ideal chemoprevention agent to block estrogen-mediated carcinogenesis. For this study we used the ER-negative, nontumorigenic human breast epithelial MCF10A cell line. MCF10A cells were treated with either vehicle or SFN (10 µM) and E2 or 4-OHE2. NQO1 was up-regulated at the mRNA (~2fold), protein (~3fold) and activity levels (~3fold) by SFN treatment. Estrogen metabolites and depurinating DNA adducts in the cell culture medium were partially purified by solid phase extraction and then analyzed by HPLC- ESI-MS/MS. Following E2 treatment, the depurinated adducts 1211-12-1-N3Ade and 4-0HE-1/2-1-N7Gua were significantly lower in SFN treated cells compared to vehicle (0.03-010/aresus 9:07+0:02-pmole/106cell-to-00020); 4-OHE1/2- glutathione conjugates were significantly higher following SFN treatment (1.54±0.37 0.93-0-19-anole/106cell p=0.00 5) as were 4-OCH3E1/2 (5-36-2-46-versus 1.81±0 ... notice 105cell.p<0.000() levels. Following treatment with the proximate metabolite 4-OHE2, 4-OHE1/2-1-N3Ade and 4-OHE 1/2-1-N7Gua were again significantly lower in SFN treated cells compared to vehicle (0.59+0.44 wersus 1.42±0.16 proto/06660 proto/066600 proto/06660 proto/06 4-OHE1/2-glutathione-conjugates (2000 versus 0107-0003 pmole/106cell, p-02000) and 4-OCH3E1/2 levels were significantly higher (195.00+12-33 versus 50.061+77pmole/196cell p<00001). Follow-up studies are examining the effects of genetic activation of Nrf2 signaling though disruption of Keap1 as well as targeted silencing of key metabolic genes. In conclusion, SFN can modulate estrogen metabolism leading to diminished formation of estrogen-DNA Const

Supported by DOD BCRP Postdoctoral Fellowship 103928.



Award for Scientific Excellence Cancer Epidemiology Prevention and Control Program

> University of Pittsburgh Cancer Institute 2012 Satellite Conference

> > 2ND PLACE AWARD

Li Yang

June 20, 2012
0 Office of Academic Career Development Associate Professor of Psychiatry Anne Germain, PhD, Course Director Darlene F. Zellers, PhD, Director **Course in Scientific Management and Leadership** 1 A WA Schools of the Health Sciences University of Pittsburgh has completed the requirements of the This document recognizes that LI YANG, PHD April 18-19, 2013 Date Date 4-19-13 A. 19.13 0 0



ANNUAL MEETING 2013 April 6-10, 2013, Walter E. Washington Convention Center, Washington, DC

Presentation Abstract

Abstract Number:	3679
Presentation Title:	Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells
Presentation Time:	Tuesday, Apr 09, 2013, 1:00 PM - 5:00 PM
Location:	Hall A-C, Poster Section 10
Poster Board Number:	25
Author Block:	Li Yang ¹ , Muhammad Zahid ² , Eleanor G. Rogan ² , Ercole L. Cavalieri ² , James D, Yager ³ , Kala Visvanathan ³ , John Groopman ³ , Nancy E. Davidson ⁴ , Thomas W. Kensler ¹ . ¹ University of Pittsburgh, Pittsburgh, PA; ² University of Nebraska Medical Center, Omaha, NE; ³ Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; ⁴ University of Pittsburgh Cancer Institute and UPMC Cancer Center, Pittsburgh, PA
Abstract Body:	Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells Sulforaphane (SFN) is a potent inducer of detoxication enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-S-transferases (GST) via the Kelch-like ECH-associated protein 1 (Keap1) - Nuclear Factor- E2-related factor (Nrf2) signaling pathway. NQO1 reduces the carcinogenic estrogen metabolite, catechol estrogen-3,4-quinone (CE-3,4-Q), while GSTs detoxify it through nucleophilic addition. CE-3,4-Q can bind with DNA to form depurinating DNA adducts. Thus, SFN, a isothiocyanate found in broccoli, may alter estrogen metabolism to protect against estrogen-mediated carcinogenesis. MCF10A cells were treated with either vehicle or SFN (10 µM) and either estradiol (E2) or 4-OHE2 (10 µM). NQO1 activity increased 3.2-fold by SFN treatment compared to vehicle. Estrogen metabolites and depurinating DNA adducts in the cell culture medium were partially purified by solid phase extraction and then analyzed by UHPLC-MS/MS. Following E ₂ treatment, 4-OHE _{1/2} -1-N3Ade and 4-OHE _{1/2} -1-N7Gua adducts were reduced by 60% in SFN treatment; 4-OHE _{1/2} - glutathione conjugates increased 1.9 while 4-OCH ₃ E _{1/2} increased 3.0 fold with SFN. Following treatment with the proximate metabolite 4-OHE ₂ , 4-OHE _{1/2} -glutathione-conjugates increased 5.0-fold and 4-OCH ₃ E _{1/2} levels were 3.4-fold higher. To constitutively enhance the expression of Nrf2-regulated genes including NQO1, cells were treated with either scrambled or siKEAP1 RNA and E ₂ or 4-OHE _{1/2} -1-N7Gua adducts dropped 70% in SIKEAP1 treatment. Following E ₂ treatment, 4-OHE _{1/2} -glutathione conjugates increased 1.4-fold while 4-OCH ₃ E _{1/2} decimed 60% with siKEAP1 treated cells compared to scrambled; 4-OHE _{1/2} -1-N3Ade and 4-OHE _{1/2} -1-N7Gua adducts dropped 70% in siKEAP1 treated cells compared to scrambled; 4-OHE _{1/2} -glutathione conjugates increased 1.4-fold while 4-OCH ₃ E _{1/2} declined 60% with siKEAP1 treatment. Following 4-OHE _{1/2} -glutath

American Association for Cancer Research 615 Chestnut St. 17th Floor Philadelphia, PA 19106



25th Annual UPCI Scientific Retreat

POSTER ABSTRACTS

June 14, 2013

Petersen Events Center Oakland

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UPMC CancerCenter Partner with University of Pittsburgh Cancer Institute Pre-Clinical Science Breast Cancer Poster No. 101.

Modulation of estrogen depurinating DNA adduct by sulforaphane or KEAP1 knockdown in human breast epithelial cells

Li Yang¹, Muhammad Zahid², Yong Liao³, Eleanor G.Rogan², Ercole L.Cavalieri⁴, Nancy E.Davidson^{1,5}, James D.Yager⁶, Kala Visvanathan⁷, John D.Groopman⁶, Thomas W.Kensler^{1,5,6*}

¹ Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213

² Environmental, Agricultural and Occupational Health, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198

³ Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213
⁴ Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198

⁵ University of Pittsburgh Cancer Institute and UPMC Cancer Center

⁶ Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205

⁷ Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205

Background: Sulforaphane (SFN) is a potent inducer of detoxication enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) and glutathione-S-transferases (GST) via the Kelch-like ECH-associated protein 1 (Keap1) - Nuclear Factor- E2-related factor (Nrf2) signaling pathway. NQO1 reduces the carcinogenic estrogen metabolite, catechol estrogen-3,4-quinone (CE-3,4-Q), while GSTs detoxify it through nucleophilic addition. CE-3,4-Q can bind with DNA to form depurinating DNA adducts. Thus, SFN may alter estrogen metabolism to protect against estrogen-mediated carcinogenesis.

Methods: MCF10A cells were treated with either vehicle or SFN (10 μ M) and either estradiol (E₂) or 4-OHE₂ (10 μ M). Estrogen metabolites and depurinating DNA adducts in cell culture medium were partially purified by solid phase extraction and then analyzed by UHPLC-MS/MS.

Results: NQO1 activity increased 3.2-fold by SFN treatment compared to vehicle. Following E₂ treatment, 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua adducts were reduced by 60% in SFN treatment; 4-OHE_{1/2}- glutathione conjugates increased 1.9 while 4-OCH₃E_{1/2} increased 3.0 fold. Following treatment with the proximate metabolite 4-OHE₂, 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua adducts also decreased 60% in SFN treated cells compared to vehicle; while 4-OHE_{1/2}-1-glutathione-conjugates increased 5.0-fold and 4-OCH₃E_{1/2} 3.4-fold.

NQO1 activity increased 2.2-fold with siKEAP1 treatment. Following E₂ treatment, 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua adducts dropped 70% in siKEAP1 treated cells compared to scrambled vector; 4-OHE_{1/2}-glutathione conjugates increased 1.3-fold; however, 4-OCH₃E_{1/2} decreased 50% with siKEAP1 treatment. Following 4-OHE₂ treatment, the 4-OHE_{1/2}-1-N3Ade and 4-OHE_{1/2}-1-N7Gua adducts declined 60% in siKEAP1 treated cells compared to scrambled vector; 4-OHE_{1/2}-1-N7Gua adducts declined 60% in siKEAP1 treated cells compared to scrambled vector; 4-OHE_{1/2}-glutathione conjugates increased 1.4-fold while 4-OCH₃E_{1/2} declined 60%.

Conclusions: SFN or siKEAP1 have similar effects on up-regulation of NQO1 transcripts, protein expression, activity levels and on diminution of depurinating estrogen DNA adducts following E₂ or 4-OHE₂ challenge. However, pharmacologic and genetic approaches have different effects formation of 4-OCH₃E_{1/2}. Activation of Nrf2 pathway may account for some but not all of protective effects of SFN against estrogen-mediated DNA damage.

Supported by DOD BCRP Postdoctoral Fellowship103928.

- 108 -

From:	fwu@eoh.pitt.edu
Subject:	Risk Analysis - RA-00474-2012
Date:	Wed, January 16, 2013 5:53 pm
To:	liyang@pitt.edu

Dear LI:

Thank you for agreeing to review the paper entitled "Risk Assessment of Multistate Progression of Breast Tumor with State-dependent Genetic and Environmental Covariates."

To access the manuscript, log in to the Risk Analysis - Manuscript Central site at http://mc.manuscriptcentral.com/riskanalysis . Your case-sensitive USER ID is liyang@pitt.edu

. For security purposes your password is not listed in this email. If you are unsure of your password, you may click the link below to set a new password.

http://mc.manuscriptcentral.com/riskanalysis?URL_MASK=fFKs58f9hCr3rRc6bw2P

Click on the Reviewer Center and find the assigned manuscript. Click on the title of the manuscript to view and print it.

Alternately, you may click on

http://mc.manuscriptcentral.com/riskanalysis?URL_MASK=DNwXJy5GBZh7rPsx4DRw to go directly to the reviewer instructions, details and scoresheet for this manuscript.

When you have completed your review, please go to the site and complete and submit the scoresheet. We ask that you complete your review within 4 weeks.

Best regards,

Felicia

From: Subject:	few8@pitt.edu Thank you for submitting your review for Risk Analysis
Date:	Thu, February 7, 2013 4:53 pm
To:	līyang@pitt.edu
Cc:	klowrie@rci.rutgers.edu

07-Feb-2013

Dear Reviewer,

Thank you for reviewing manuscript # RA-00474-2012 entitled "Risk Assessment of Multistate Progression of Breast Tumor with State-dependent Genetic and Environmental Covariates" for Risk Analysis.

On behalf of the Editors, we appreciate the voluntary contribution that each reviewer gives to the Journal. We thank you for your participation in the review process and hope that we may call upon you again to review future manuscripts.

Sincerely, Dr. Felicia Wu Area Editor, Risk Analysis few8@pitt.edu

From: Subject:	alanpaine@hotmail.com BiOMARKERS - Invitation to Review Manuscript ID TBMK-2013-0101
Date:	Mon, April 29, 2013 2:35 pm
To:	liyang@pitt.edu

29-Apr-2013

Dear Dr Yang Li

I hope you can help review this submission please?

Best wishes Alan Paine BiOMARKERS

The above manuscript, entitled "PPARy and PPARG1CB polymorphisms modify the association between phthalate metabolites and breast cancer risk." with Dr Cebrián as contact author has been submitted for consideration for publication in BIOMARKERS.

Aware of your expertise in the area, I would be very grateful if you would kindly agree to act as a reviewer for this manuscript. The abstract appears at the end of this letter, along with the names of the authors. Ideally, the deadline for submission of the review would be in one month's time.

Please let me know as soon as possible if you will be able to accept this invitation and request to review. To do this please either click the appropriate link below to automatically register your reply with our online manuscript submission and review system, or e-mail me with your reply.

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Unavailable: http://mc.manuscriptcentral.com/tbmk?URL_MASK=2DMjtNmn6tmNq6rX7ZXq

Should you accept my invitation to review this manuscript, you will be notified via e-mail about how to access ScholarOne Manuscripts, our online manuscript submission and review system. You will then have access to the manuscript and reviewer instructions in your Reviewer Centre.

I realise that our expert reviewers greatly contribute to the high standards of the Journal, and I thank you for your present and/or future participation.

Sincerely,

Professor Alan Paine BiOMARKERS Editorial Office alanpaine@hotmail.com

http://www.informahealthcare.com/bmk

MANUSCRIPT DETAILS

TITLE: PPARy and PPARG1CB polymorphisms modify the association between phthalate metabolites and breast cancer risk.

AUTHORS: Martínez-Nava, Gabriela; Burguete-García, Ana; López-Carrillo, Lizbeth; Hernández-Ramírez, Raúl; Madrid-Marina, Vicente; Cebrián, Marlano

ABSTRACT: Context: Breast cancer (BC) risk has been differentially associated with

urinary levels of some phthalate metabolites. Objective: To investigate whether PPARy and PPARGC1B polymorphisms modulate these associations.

Materials and methods: 208 BC cases were age-matched with 220 population controls. Phthalate metabolites were determined by HPLC-MS. PPARy Pro12Ala (rs1801281) and PPARGC1B Ala203Pro (rs7732671) and Val279Ile (rs17572019) were genotyped. Results: The associations between di(2-ethylhexyl) phthalate (DEHP) metabolites and BC risk were positively modified in PPARy Pro12Ala C carriers. The association with mono-iso-butyl phthalate (MIBP) in PPARGC1B Ala203Pro G carriers was negatively modified.

Conclusion: PPARy and PPARG1CB polymorphisms modulate the association between phthalate exposure and BC risk.

Agreeing to review an article for this Journal implies that you as the reviewer will adhere to the accepted ethical standards of scientific, medical and academic publishing. Material submitted for peer review is a privileged communication that should be treated in confidence. Material under review should not be shared or discussed with anyone outside the designated review process, unless approved by the editor. All communications relating to the paper in review should also be treated in confidence. Any breach of confidentiality in the review process is taken seriously by the journal and will be investigated according to the advice of COPE (http://publicationethics.org). Any conflict of interest, suspicion of duplicate publication, fabrication of data, plagiarism or other ethical concerns must immediately be reported to the Editor.

By agreeing to review this manuscript, you are stating that you are the person completing this review. If you wish to collaborate with a colleague and/or trainee to perform this review, or wish to assign this review to a trainee for completion under your guidance, please contact the Editor for permission before sharing the manuscript. If the Editor agrees please provide the name, affiliation and e-mail address for the trainee/ colleague so he or she may be assigned as a reviewer directly.

If you have any conflict of interest (for example, collaborate with the author(s) or are currently working on a similar study), please decline to review this manuscript and, if possible, suggest appropriate alternate reviewers.

From:	alanpaine@hotmail.com
Subject:	Thank you for submitting your review of Manuscript ID TBMK-2013-0101 for BIOMARKERS
Date:	Mon, May 20, 2013 5:33 pm
To:	liyang@pitt.edu

20-May-2013

Dear Dr Yang Li:

Thank you for reviewing the above manuscript, entitled "PPARy and PPARG1CB polymorphisms modify the association between phthalate metabolites and breast cancer risk." for BiOMARKERS.

We greatly appreciate the voluntary contribution that each reviewer gives to the Journal. We hope that we may continue to seek your assistance with the refereeing process for BiOMARKERS, and hope also to receive your own research papers that are appropriate to our aims and scope.

Sincerely, Professor Alan Paine Editor In Chief, BiOMARKERS alanpaine@hotmail.com

Visit www.informahealthcare.com and sign up for free eTOC alerts to all Informa Pharmaceutical Science journals

From: Subject:	Stephanie.Seymour@aacr.org Request to Review Cancer Prevention Research Manuscript: CAPR-13-0296 The Sentember 3 2013 1:21 pm
Date:	The, Depiction of 2010 1721 pin
To:	liyang@pitt.edu

Dear Dr. Yang:

We request your assistance as a reviewer for the above-referenced submission to the AACR journal CANCER PREVENTION RESEARCH. AACR is happy to announce that Cancer Prevention Research reviewers may now be eligible to claim up to 3 Continuing Medical Education (CME) Credits for highly rated, timely reviews. If your review meets these criteria (on time and judged by the editors to be of satisfactory quality), you will receive an email with instructions for claiming credit. Please use the link below to access the AACR CME Accreditation Statement: http://capr.msubmit.net/html/CME_Accreditation_Statement.html

The name of the assigned editor, title, corresponding author, and abstract are listed below for your reference. If you agree to review this manuscript, you would be given two weeks to complete your review.

Please note that if you agree to review this manuscript, the manuscript and its contents must be kept confidential. If you wish to have another expert co-review the manuscript with you, you must first obtain permission from the journal office.

EDITOR Thomas Kensler

MANUSCRIPT NUMBER CAPR-13-0296

MANUSCRIPT TITLE

Benzothiophene SERMs act as breast cancer chemopreventive agensts by modulating estrogen oxidative metabolism in human mammary epithelial cells

MANUSCRIPT TYPE Research Article

AUTHOR

Dr. Thatcher

ABSTRACT

The risk of developing hormone-dependent cancers with long-term exposure to estrogens is attributed both to proliferative, hormonal actions at the estrogen receptor (ER), and chemical carcinogenesis elicited by genotoxic, oxidative estrogen metabolites. Non-tumorigenic MCF-10A human breast epithelial cells are classified as ER(-) and undergo estrogen-induced malignant transformation. Selective estrogen receptor modulators (SERMs), in use for breast cancer chemoprevention and for post-menopausal osteoporosis, were observed to inhibit malignant transformation, as measured by anchorage-independent colony growth. This chemopreventive activity was observed to correlate with reduced oxidative estrogen metabolism, as measured by LC/MS-MS, and attenuated cellular ROS and DNA oxidation. The ability of raloxifene, desmethylarzoxifene (DMA), and bazedoxifene to inhibit this chemical carcinogenesis pathway was not shared by 4-hydroxytamoxifen. Regulation of Phase 2 rather than

metabolic enzymes was implicated mechanistically: raloxifene and DMA were observed to upregulate sulfotransferase (SULT 1E1) and glucuronidase (UGT 1A1). Estrogen metabolite formation on co-treatment with DMA analogues did not correlate with classical ERα and ERβ mediated activity. The results support upregulation of Phase 2 metabolism, in particular via SULT 1E1, in detoxification of catechol estrogen metabolites leading to attenuated ROS formation as a mechanism for inhibition of malignant transformation by selected clinically important SERMs.

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Thank you for your consideration and support of CANCER PREVENTION RESEARCH.

From:	"Gary A. Lorigan" <bri@hindawi.com></bri@hindawi.com>
Subject:	591059: Review Request
Date:	Mon, November 24, 2014 7:19 am
To:	liyang@pitt.edu

Dear Dr. Yang,

Biochemistry Research International has received a Research Article titled "The Protective Effects of Vitamin C and Vitamin E ffrom Cell Apoptosis Induced by Intermitnent 50Hz ELF-EMF Radiation," by Zhen Ding, Mohammadreza Mohammadzad Mephryar, Fan Li, Jintao Li, Minglian Wang, Wu Shuicai, Chen Zhang, Jianzhe Liu and Yi Zeng, submitted for possible publication in the journal. As the editor in charge of this manuscript, I would be grateful if you can review it and submit a review report by December 15, 2014. You can view the manuscript details and decide whether or not you will be able to review the manuscript using the following URL:

http://mts.hindawi.com/reviewer/1916441406204610/

Once you agree to review the manuscript, you will be able to view the PDF file of the manuscript.

With many thanks and best regards,

Gary A. Lorigan garylorigan@muohio.edu

From:"BioMed Research International" <marwa.rashed@hindawi.com>Subject:425380: (Thank you)Date:Fri, August 8, 2014 1:00 pmTo:liyang@pitt.edu

Dear Dr. Yang,

Thank you for submitting your review report on the Research Article 425380 titled "Curcumin mitigates accelerated aging after irradiation in Drosophila," by Ki Moon Seong, Mira Yu, Kyu-Sun Lee, Young Woo Jin and Kyung J. Min, and for taking the time and effort to review this manuscript.

Best regards,

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