Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** University of Pittsburgh – Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 – 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Margaret C. McDonald, PhD

4. **Grant Contact Person's Telephone Number:** 412-383-7474

5. **Grant SAP Number:** 4100050913

6. **Project Number and Title of Research Project:** 02 - Targeting Nrf2 for Cancer Prevention

7. **Start and End Date of Research Project:** 1/1/2010 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Thomas W. Kensler, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $1,382,809

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
None			

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Kensler, Thomas	Professor	10/10/10/10
Wakabayashi, Nobunao	Assistant Scientist	10/10/10/10
Yang, Li	Postdoctoral Associate	25/25/25/25

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Thermo Vantage & Orbitrap mass spectrometers	These instruments provide the University with additional capabilities to measure small molecules in biological matrices as well as peptides	1,000,000

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____X____ No_____

If yes, please indicate the source and amount of other funds:

NIH R01 CA39416 $200,000 yr direct costs

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Modulation of Estrogen-Depurinating DNA Adducts by Sulforaphane for Breast Cancer 9/15/2011-9/14/2014	☐NIH X Other federal (specify: Dept of Defense) ☐ Nonfederal source (specify:_)	Dec 2010	$448,770	$448,700

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

We plan to submit an R01 application to NIH to continue our characterization of the molecular pathogenesis of aflatoxin-induced hepatocarcinogenesis. We have recently submitted an R21 application to continue our work on the use of estrogen-DNA adducts as risk biomarkers in humans.

12. Future of Research Project. What are the future plans for this research project?

We hope to continue elements of this study through funding mechanisms outlined above.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No___X_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This project greatly enhanced our ability to measure with precision and sensitivity biomarkers of carcinogen-DNA damage in our cell culture and animal models. These tools will now be applied to human biomonitoring studies.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____X_____ No_____

If yes, please describe the collaborations:

Our ability to develop mass spectrometry methods for the analysis of estrogen-DNA adducts has led to collaborations with oncologists and epidemiologists at Johns Hopkins University.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending

publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Modulation of aflatoxin hepatocarcinogenesis. Initially, we began to use isotope-dilution mass spectrometry for the quantitation of biomarkers of the human carcinogen aflatoxin in liver and urine of rats challenged with a single dose of aflatoxin, as depicted in Figure 1. Male F344 rats, a strain exquisitely sensitive to aflatoxin hepatocarcinogenicity, were dosed by gavage with 20 µg AFB_1, placed in metabolism cages, and sacrificed 24 h later. Hepatic burden of DNA adducts (AFB-N7-guanine and formamidopyrimidine [FAPyr] were measured along with the urinary excretion of the N7-guanine adduct and the epoxide-derived detoxication product, aflatoxin mercapturide (AF-NAC). Animals were either pretreated with vehicle or the triterpenoid CDDO-Im [1-(2-cyano-3,12-dioxooleana-1,9{11}-dien-28-oyl)imidazole] (30 µmole/kg body weight) q.o.d. x3 prior to carcinogen challenge. Substantive levels of the DNA adducts were detected in both the liver and urine of vehicle-AFB_1 treated rats. The appearance of DNA adducts in the urine reflects both spontaneous depurination and enzyme-mediated repair of the hepatic adducts. Pretreatment of the rats with the potent small molecule activator of Nrf2 signaling, CDDO-Im, leads to dramatic reductions in the formation of aflatoxin DNA adducts in the target organ and in its subsequent elimination in urine. Equally striking, the pretreatment with CDDO-Im leads to a 20-fold increase in the elimination of the aflatoxin mercapturide. This profoundly altered disposition of the reactive epoxide intermediate likely reflects Nrf2-mediated induction of glutathione transferases (GSTs), enzymes key to the formation of mercapturic acids. Shunting of the aflatoxin toward the mercapturic acid pathway spares DNA from electrophilic modification.

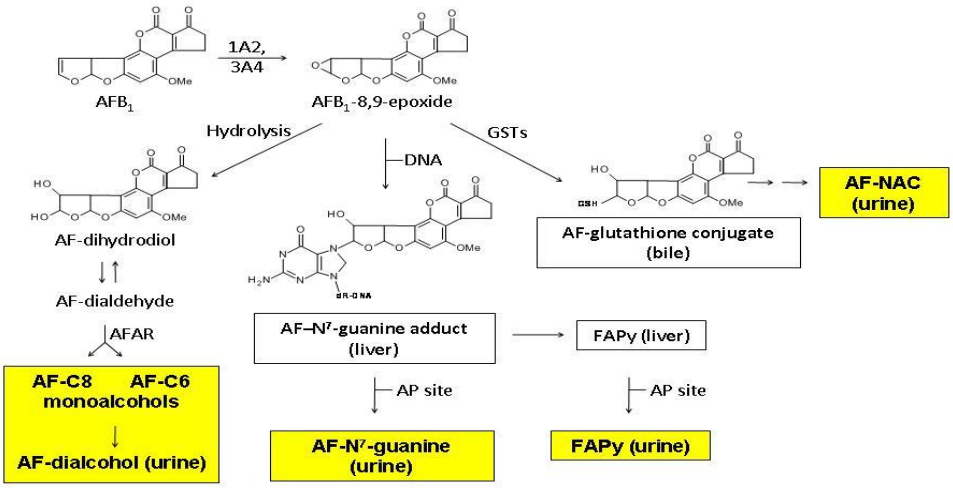


FIGURE 1. Scheme for the metabolism of the hepatocarcinogen aflatoxin B_1 (AFB_1). Metabolites labeled in boxes have been quantified by isotope dilution mass spectrometry in the target organ liver as well as in biofluids.

Subsquently we have conducted a chronic treatment protocol in which 43 F344 rats were randomized to receive 250 µg AFB_1 per kg body weight daily for 28 days. Twenty of these animals were additionally treated three times a week with CDDO-Im while the others received vehicle. Twenty-four-hour urine samples were collected weekly and blood samples monthly from all animals. All rats were euthanized and necropsied when clinical observations indicated that the rat was in pain or would likely not survive longer than 12 hours. The latter criteria were substantial (>15%) and rapid loss of body weight, failure to groom, and/or inability to ambulate. Standardized sections of normal hepatic tissue and all abnormal tissues, including all hepatic tumors, were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Lifetime cancer bioassay. Profound differences in HCC incidence were seen between treatment groups. In the AFB_1 group, the first HCC appeared in a rat that died at 44 weeks of age. The incidence of HCC in the AFB_1 group was 96% (22 of 23 rats), with the majority of the rats presenting with multiple HCCs that often appeared to coalesce into one larger tumor mass obliterating the normal gross hepatic architecture. The one animal in the AFB_1 group that was not diagnosed with HCC had five large preneoplastic foci, with an average focal transactional diameter of 1.3 mm, when it died, relatively young, at 35 weeks of age. The histopathological picture was radically different in the AFB_1 + CDDO-Im group as no HCCs were found. The most advanced AFB_1-related lesions were putative preneoplastic foci detected in only three of the 20 rats. These three rats died at 93, 95, and 111 weeks of age; and the foci were less than half the size of foci seen in the AFB_1-treated rat that died at 35 weeks of age.

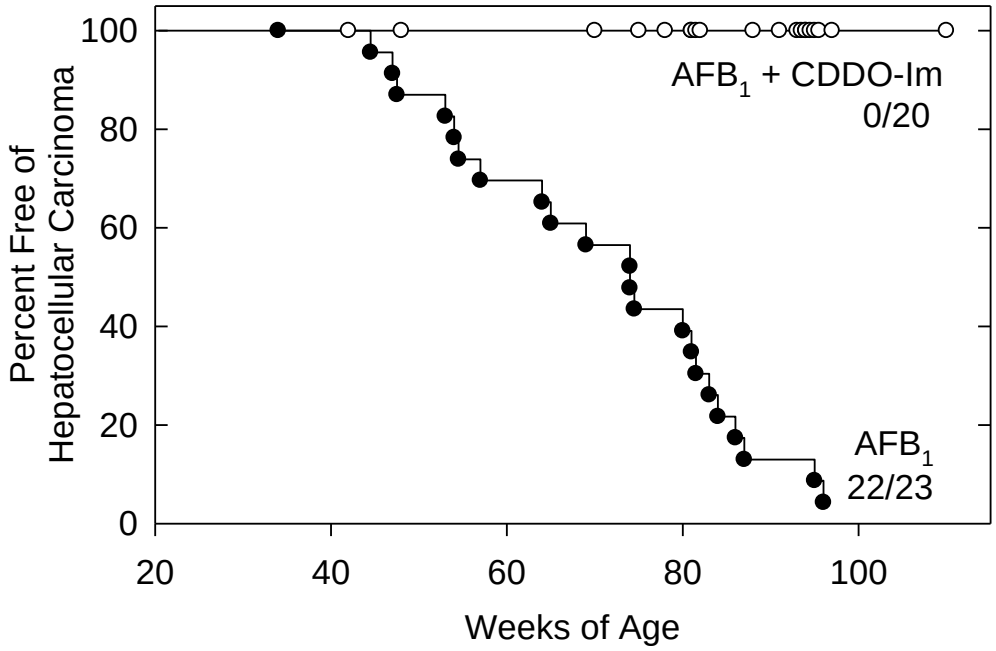


FIGURE 2. Kaplan Meier curves for proportion of rats free of hepatocellular carcinoma after receiving (●) AFB_1 or (○) AFB_1 + CDDO-Im.

The median age of death in the AFB_1 group was 74 weeks compared to the AFB_1 + CDDO-Im group median of 90 weeks ($p < 0.01$). The overwhelmingly positive impact of CDDO-Im on prevention of HCC and ultimately survival against a large exposure to AFB_1 is shown in Figure 2. Aged control F344 rats largely died of mononuclear cell leukemia or marked bilateral chronic progressive nephropathy. In the AFB_1 + CDDO-Im group, the incidence of nephropathy was 75% (15/20) and leukemia was 50% (10/20), with the majority having both pathologies. In the AFB_1 group, the prevalence of these lesions was much lower: 17% (4/23) had nephropathy and 30% (7/23) had leukemia. The burden of HCC was almost certainly the major contributing factor to the clinical decline and death of these AFB_1 rats rather than these comorbidities.

AFB_1 DNA adducts and mercapturic acid. Urinary biomarkers of aflatoxin were measured in the lifetime bioassay rats during the 28-day AFB_1-treatment period to determine relationships with protection by CDDO-Im. All animals had biomarker levels well above the analytical limit of determination. Figure 3 depicts the urinary excretion of aflatoxin-N_7-guanine, a biomarker of the biological effective dose of AFB_1. Also shown is the average administered daily dose of AFB_1. The AFB_1 + CDDO-Im rats excreted significantly less aflatoxin-N_7-guanine than the AFB_1 rats (overall, 34% of the AFB_1 group) despite receiving a cumulative 20% higher dose of AFB_1. At the end of the last week of the dosing, the AFB_1 rats had more than seven times as much aflatoxin-N_7-guanine per day in urine as did the protected group. A major route of

detoxication of AFB_1 is through conjugation of the aflatoxin-8,9-epoxide with glutathione by GSTs and, ultimately, excretion in the urine as a mercapturic acid.

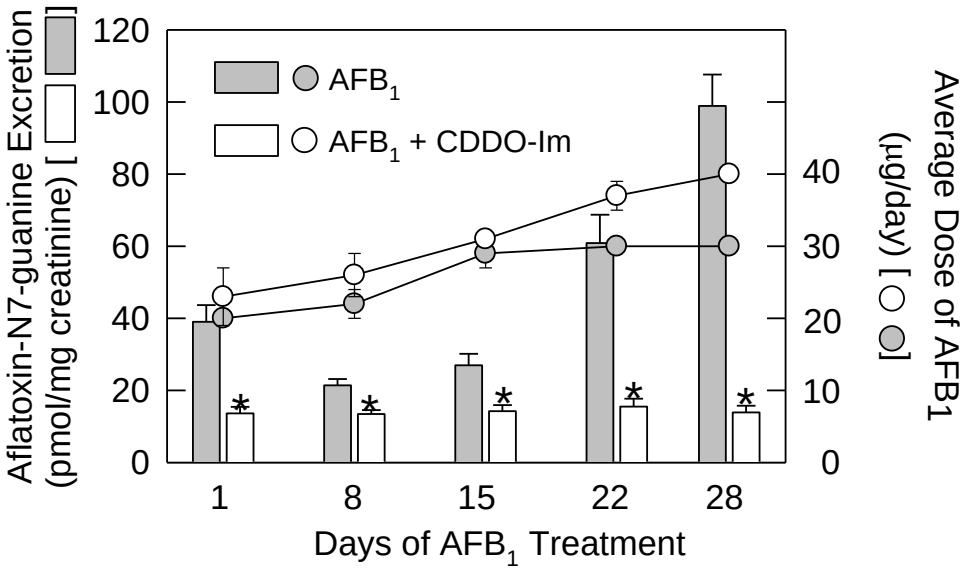


FIGURE 3. Urinary excretion of aflatoxin-N7-guanine during dosing phase in rats maintained on lifetime bioassay (*bars*). *Circles* indicate the mean dose of AFB_1 administered as calculated at weekly intervals during the dosing period.

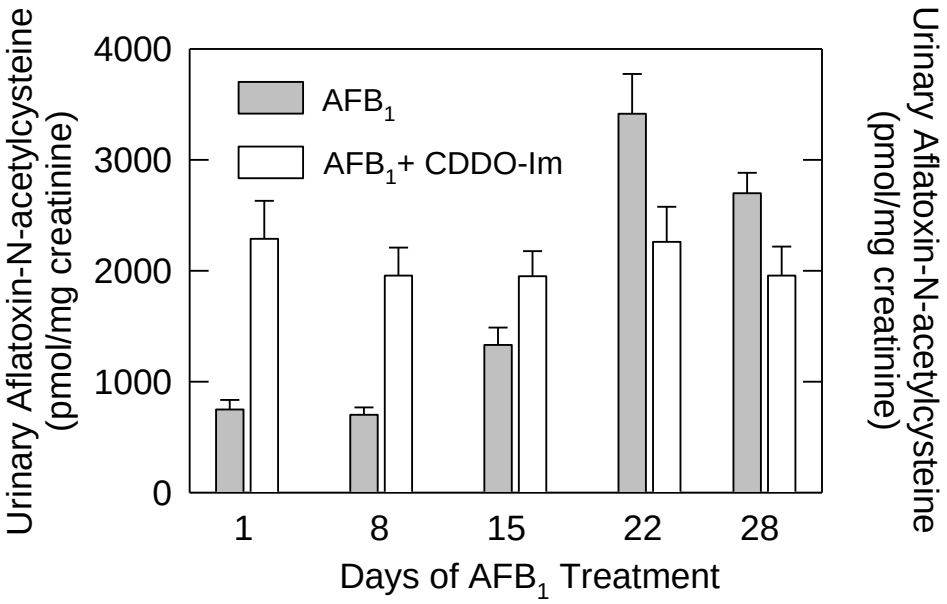


FIGURE 4. Urinary excretion of aflatoxin-N-acetylcysteine (mercapturic acid) during dosing phase in rats maintained on lifetime bioassay. Values are mean ± SE (n=10).

Elimination of aflatoxin-N-acetylcysteine in the AFB_1 + CDDO-Im rats was initially three-fold higher than in the AFB_1 group and remained at this elevated rate for the duration of the dosing period (Figure 4). An adaptive response in the AFB_1 rats was an increase in aflatoxin-N-acetylcysteine formation and elimination beginning in the second week of dosing and thereafter increasing to levels greater than in the AFB1 + CDDO-Im rats.

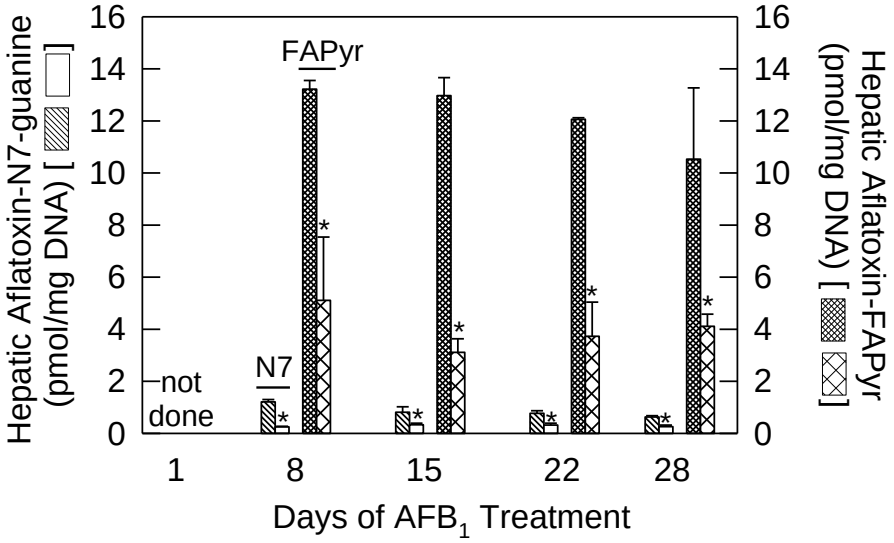


FIGURE 5. Hepatic levels of aflatoxin-N^7-guanine (N7) or FAPyr in rats receiving AFB_1 or AFB_1 + CDDO-Im (FAPyr). DNA was isolated 24 hr after the most recent dose of AFB_1 over a one to four week dosing period. Values are mean ± SE (n=3-7).

In a separate cohort of animals treated identically, livers were collected 24 hours post-AFB_1 dose at weekly intervals over four weeks and analyzed for the hepatic burden of aflatoxin-DNA adducts. The major and stable aflatoxin-N^7-guanine-derived adduct in liver is 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxyaflatoxin B_1 (FAPyr). As shown in Figure 5, hepatic FAPyr levels remained two-to three-fold higher in the AFB_1 group than in the AFB_1 + CDDO-Im group. Although initially the primary adduct formed is aflatoxin-N^7-guanine, by 24 hours aflatoxin-N_7-guanine has either undergone repair, depurinated, or ring-opened to form FAPyr. Overall, aflatoxin-N^7-guanine adducts were 53% lower in the AFB_1 + CDDO-Im livers compared to those treated with AFB_1; for FAPyr, the reduction was 69%.

Putative preneoplastic lesions. The hepatic burdens of GST-P positive foci were analyzed from histopathological sections obtained from the serially sacrificed animals of the second cohort. GST-P positive foci were not present following the first dose of AFB_1 but were histologically obvious at eight days and increased over the course of the 28-day experiment in the AFB_1 group. In the AFB_1 group, the GST-P positive focal volume percent was 0.01, 0.25, 3.22, and 13.81 at eight, 15, 22, and 28 days, respectively. In the AFB_1 + CDDO-Im group, foci were totally absent at eight and 15 days, and at 22 and 28 days the focal volume percent was 0.02 and 0.01, respectively. Only one animal in each of the day 22 and 28 groups had any foci, whereas all AFB_1 treated rats had multiple foci from day 15 onward. Clearly, the CDDO-Im intervention

largely prevented the formation of these putative preneoplastic, microscopic foci commonly seen after aflatoxin exposure.

Conclusions. The inclusion of risk-reduction cohorts in animal bioassays allows not only the evaluation of novel preventive agents but provides a unique perspective to assess possible genotoxicity thresholds and to validate predictive molecular signatures of carcinogenesis. This study demonstrates the remarkable efficacy and extreme potency of a synthetic oleanane triterpenoid as an inhibitor of carcinogenesis, in this instance induced by a known human carcinogen. The complete ablation of liver cancer development coupled with extended survivorship by CDDO-Im in a model in which aflatoxin induces a 96% incidence of liver cancer is unparalleled, irrespective of the dose or chemical class of chemopreventive agent used. Decades of mechanistic studies on aflatoxin hepatocarcinogenesis in rats provide a clear perspective on the roles of carcinogen metabolism, DNA damage, and hepatotoxicity on this pathogenesis. Previous studies by our group have indicated that the protection provided by CDDO-Im in this model is achieved largely through interaction with signaling pathways mediated by the transcription factor Nrf2. Hepatic expression of Nrf2 target genes known to be involved in aflatoxin detoxication, namely aldo-keto reductase 7A1 and GSTs, are elevated by CDDO-Im. The current results in which hepatic and urinary levels of aflatoxin-DNA damage products are substantively, but incompletely, reduced by CDDO-Im treatment during the period of AFB_1 dosing are consistent with this view. Moreoever, our data point to a view that substantial aflatoxin-DNA damage is not sufficient for development of HCC. The absence of cancer in this experimental setting supports the concept of a threshold for biological mode of action that links DNA damage to the development of liver cancer.

Mechanisms of protection against estrogen carcinogenesis. Breast cancer is a significant concern in public health since it is the second leading cause of cancer death among women and is the second most frequently diagnosed cancer. It is important to investigate the etiology and risk factors of breast cancer so that targeted prevention strategies can be provided. Exposure to elevated levels of estrogen has been recognized as an important determinant of the risk of breast cancer. Studies in experimental animal models demonstrate that estradiol (E_2) and estrone (E_1) are carcinogenic, and studies in cultured human cells 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. One involves signaling through the estrogen receptor (ER) leading to altered gene expression and increased proliferation accompanied by spontaneous mutations. The other pathway, outlined in Figure 6, 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.

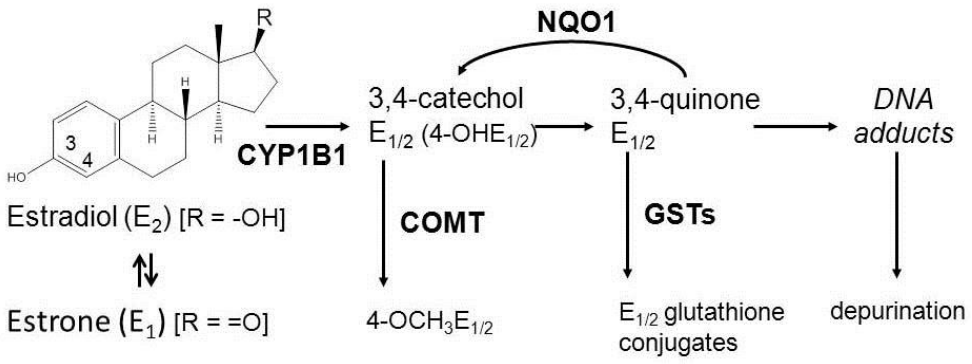


FIGURE 6. Pathway for formation of estrogen depurinating DNA adducts. Estradiol (E_2) or estrone (E_1) can be oxidized to $E_{1/2}$ -3,4-quinone, which can bind to DNA to form 4-$OHE_{1/2}$-1-N3Adenine or 4-$OHE_{1/2}$-1- N7Guanine adducts. As prevention strategies, NQO1 reduces $E_{1/2}$ - 3, 4- quinones back to catechols; GST catalyzes the conjugation of $E_{1/2}$ -3,4-quinones with glutathione while COMT catalyzes the methylation of 4-$OHE_{1/2}$ to 4-$OCH_3E_{1/2}$.

Modulation of transcripts, protein expression, and activity of estrogen metabolism enzymes by SFN treatment or *KEAP1* knockdown. Metabolism of estrogens is characterized by a balanced set of activating and deactivating pathways. Aromatization of androstenedione and testosterone by aromatase (CYP19) yields E_1 and E_2, respectively. E_1 and E_2 are interconverted by 17β-hydroxysteroid dehydrogenase, and they are metabolized at the 2- or 4-position to form 2-$OHE_{1/2}$ or 4-$OHE_{1/2}$, respectively. CYP1A1 preferentially hydroxylates E_1 and E_2 at C-2, whereas CYP1B1 almost exclusively catalyzes the formation of 4-$OHE_{1/2}$. 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 $E_{1/2}$-2,3-quinones and $E_{1/2}$-3,4-quinones. Higher levels of depurinating DNA adducts are formed by $E_{1/2}$-3,4-quinones compared to $E_{1/2}$-2,3-quinones due to different mechanisms of adduction. 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. These adducts generate apurinic sites that can be converted into mutations by error-prone repair, which in turn may initiate breast cancinogenesis.

While the estrogen oxidation pathway is detrimental to the integrity of DNA, several protective pathways in cells control the homeostasis of estrogen metabolism and 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) (FIGURE 6). 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. There are reports of a significantly higher ratio of depurinating DNA adducts to other estrogen metabolites when comparing breast cancer cases to controls, indicating that formation of depurinating estrogen-DNA adducts likely plays a key role in the breast cancer development.

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables, with particularly high levels in three-day old broccoli sprouts. SFN is an attractive chemopreventive agent because it is safe and can be distributed easily and widely as a broccoli sprout extract. Moreover, SFN and broccoli sprout preparations are effective chemopreventive agents in rodent models of mammary carcinogenesis; and initial pharmacokinetic studies indicate that pharmacologically relevant concentrations of sulforaphane metabolites can be detected in the mammary epithelium of women consuming broccoli sprout derived beverages. An important, but far from unilateral, mechanism of action for SFN is the induction of carcinogen detoxication enzymes such as NQO1 and glutathione S-transferases (GSTs). SFN is an activator of the antioxidant response element - Keap1-Nrf2 signaling pathway regulating the expression of these and many other genes. Under normal cellular conditions, Nrf2 binds to Keap1 in the cytoplasm, resulting in ubiquitination of Nrf2 and its subsequent proteasomal degradation. SFN can modify cysteine 151 in Keap1 to disrupt the association of Cul3 ubiquitin ligase with Keap1, allowing Nrf2 to escape degradation. Thus, Nrf2 becomes stabilized, translocates into the nucleus, and induces the transcription of its target genes such as *NQO1* and *GST*. These genes, in turn, may modify estrogen metabolism to DNA damaging species.

Treatment of MCF-10A cells with SFN led to induction of *GSTA1* and *NQO1* transcripts treatments (Figure 7A). No changes in expression levels of two other genes known to influence E_2 metabolism, namely *CYP1B1* and *COMT*, were observed. 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) and its specific activity 2.7-fold ($p<0.01$; SFN treatment versus vehicle) in these cells (FIGURES 7B & 7C). Although no change at the mRNA level has been detected with *COMT*, a 2.4-fold increase in COMT protein ($p<0.05$; SFN treatment versus vehicle) was significantly detected. CYP1B1 protein was significantly decreased 50% with SFN treatment (Figure 7B) ($p<0.05$; SFN treatment versus vehicle). Thus, it appears that SFN influences the expression of E_2 metabolizing enzymes through transcriptional and post-transcriptional mechanisms.

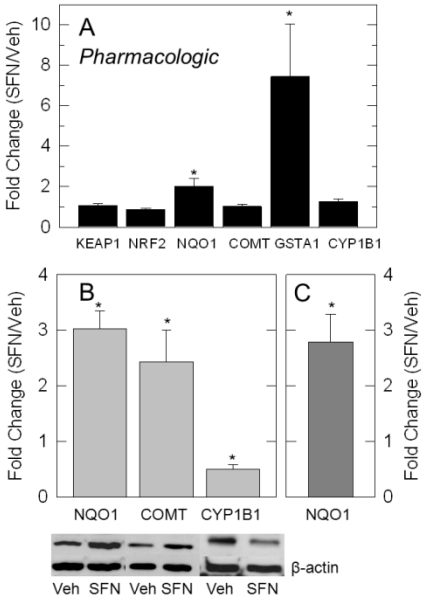
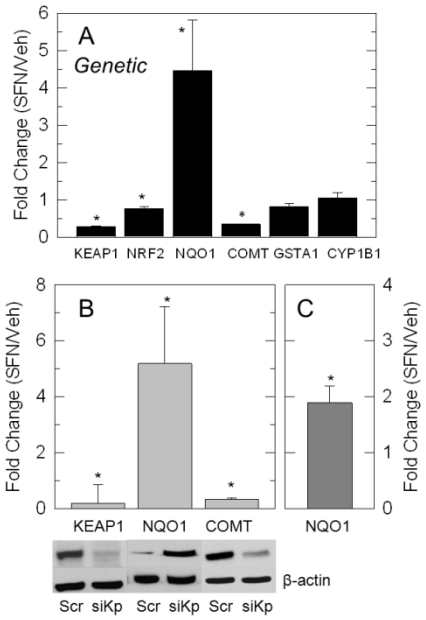


FIGURE 7. Effects of sulforaphane on transcript, protein, and activities of enzymes metabolizing estradiol or estrone. **A**. Effect of SFN on transcript levels of estrogen metabolism enzymes**. B**. Effect of SFN on protein levels of estrogen metabolism enzymes**. C**. Effect of SFN on NQO1 activity. MCF-10A cells were treated with 10 µM SFN as described in Figure 2. Values are mean ± SE of 3 independent experiments. *, Differs from vehicle control, $p < 0.05$. **, Differs from vehicle control, $p < 0.01$.

Since SFN is a well characterized activator of Nrf2 signaling in MCF-10A and other cells, the influence of siKEAP1 knockdown and, hence, genetic activation of the pathway was evaluated. Shown in Figure 8, transcript, protein, and specific activity of NQO1 significantly increased ($p<0.01$ for NQO1 protein level in siKEAP1 versus Scrambled treatment) 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 unexpected, transcript levels of *COMT* as well as COMT protein were significantly decreased 60-70% by the siKEAP1 treatment ($p<0.01$ for COMT protein level in siKEAP1 versus Scrambled treatment) (Figure 8A and 8B). *COMT* is not known to be a Nrf2 regulated gene, and the mechanism underlying this response is not known.

FIGURE 8 . Effects of siKEAP1 on transcript, protein, and activities of enzymes metabolizing estradiol. **A**. Effect of siKEAP1on transcript levels of estrogen metabolism enzymes**. B**. Effect of siKEAP1on 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 ± SE of 3 independent experiments. *, Differs from scrambled control, $p < 0.05$. **, Differs from vehicle control, $p < 0.01$.



Modulation of levels of estrogen depurinated DNA adducts and estrogen metabolites by SFN or siKEAP1 treatment. We have used mass spectrometry to determine the effects of pretreatment of cells with sulforaphane on the formation of estrogen DNA adducts as well as COMT and GST derived metabolites. MCF-10A cells were challenged with either E_2 or 4-OH E_2 and these adducts and metabolites quantified 48 h later. As shown in Figures 9A and 9B, pretreatment of these human mammary epithelial cells with 10 μM SFN dramatically reduced levels of depurinating estrogen adducts, signaling a likely protective effect. Companion studies in which we use the genetic approach of amplifying Nrf2 signaling with siKeap1 RNA are also shown.

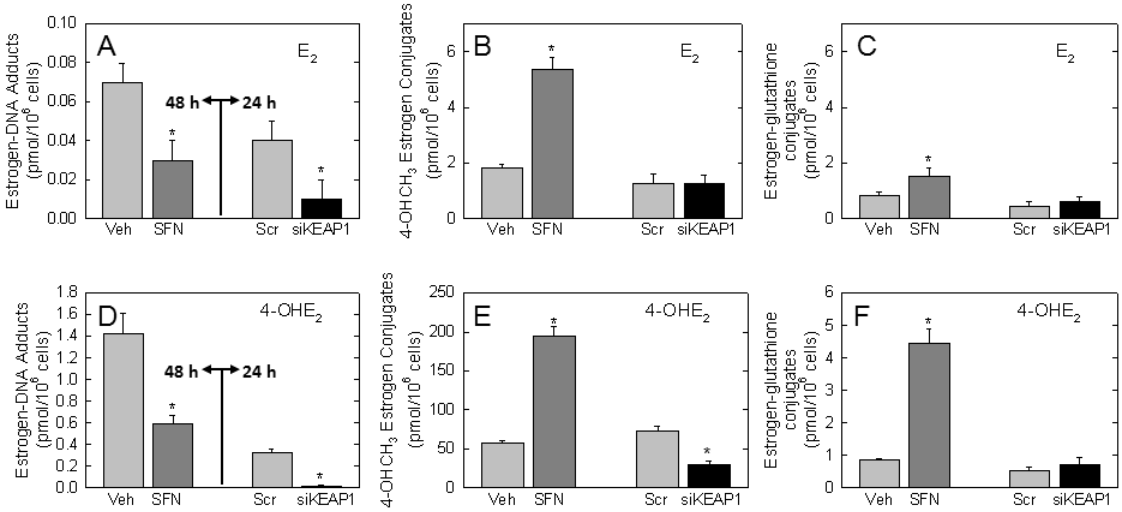


FIGURE 9. Effects of treatment of MCF-10A human mammary epithelial cells with sulforaphane (10 μM) on estradiol (E_2) (panels **A-C**) and 4-hydroxy-Estradiol (4-OH E_2) (panels **D-F**) metabolism as measured by mass spectrometry.Values are mean ±SEM of 3-4 independent experiments. * $p < 0.01$

At 48 hr after E_2 treatment, the levels of depurinated 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 to vehicle (0.03 ± 0.01 versus 0.07 ± 0.02 pmole/10^6cell, $p<0.05$) (FIGURE 9A). Although E_2 was added to the cells, there was considerable conversion to E_1. Approximately half of the formed adenine and guanine adducts were derivatives of E_1, the remainder being from E_2. By contrast, levels of 4-OCH$_3$E$_{1/2}$ increased substantively with SFN treatment (5.36 ± 0.16 versus 1.81 ± 0.20 pmole/10^6cell, $p<0.01$) (FIGURE 9B). More modest increases in levels of 4-OHE$_{1/2}$- glutathione conjugates were measured following SFN treatment (1.54 ± 0.37 versus 0.83 ± 0.19 pmole/10^6cell, $p<0.05$) (FIGURE 9C). About 25-fold more methoxy conjugates were formed than glutathione conjugates in either the vehicle or SFN treated cells.

Addition of the proximate metabolite 4-OHE$_2$ to cells led to 20-fold higher levels of estrogen depurinated adducts than seen with E_2 in vehicle treated cells. In this instance, the majority were 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 to vehicle (0.59 ± 0.11 versus 1.42 ± 0.16 pmole/10^6cell, $p<0.01$) (FIGURE 9D). 4-OCH$_3$E$_{1/2}$ levels increased 3.4-fold (195.00 ± 12.33 versus 58.05 ± 1.77 pmole/10^6cell, $p<0.01$) (FIGURE 9E) while 4-OHE$_{1/2}$-glutathione-conjugates increased 5.1-fold following SFN treatment (4.44 ± 0.52 versus 0.87 ± 0.03 pmole/10^6cell, $p<0.01$) (FIGURE 9F). The methoxy conjugates were the dominant metabolites detected.

Conclusions. The depurinating estrogen-DNA adducts efflux from cells and tissues and are excreted in urine, allowing their identification and quantification as biomarkers of risk of developing breast cancers. High levels of estrogen-DNA adducts have been observed in analyses of urine and serum from women at high risk for breast cancer or having the disease compared to women at normal risk. 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 E$_{1/2}$-3-4-quinones and the resulting estrogen quinone-DNA adducts in cell culture models. SFN is the embodiment of phytochemical poly-pharmacy in a single molecule. It touches many molecular targets in cells and exerts its chemopreventive actions through actions on multiple pathways. The protective effect of SFN against estrogen-mediated DNA damage further highlights its possible role in chemoprevention of mammary carcinogenesis but also 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.

Note: Central to this project was the installation of a Thermo TSQ Vantage mass spectrometer with an ion max source and H-ESI-II probe, which was coupled to an Accela ultra-HPLC system with autosampler and Quick Quan software late in the first year of the funding period. Laboratory personnel were subsequently trained on the instrument and pursued the aims related to this project.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells.	1. Yang, L., Zahid, M., Liao, Y., Rogan, E.G., Cavalieri, E.L., Davidson, N.E., Yager, J.D., Visvanathan, K., Groopman, J.D., Kensler, T.W.	*Carcinogenesis* 34:2587-92.	Feb 2013	☐Submitted ☐Accepted X Published
2. Complete protection against aflatoxin B1-induced liver cancer with a triterpenoid: DNA adduct dosimetry, molecular signature and genotoxic threshold.	Johnson, N.M., Egner, P.A., Baxter, V.A., Sporn, M.B., Wible, R.S., Sutter, T.R., Groopman, J.D., Kensler, T.W. and Roebuck, B.D.	*Cancer Prevention Res.*	Dec 2013	☐Submitted X Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

We plan to develop a manuscript describing the effects of the triterpenoid intervention against aflatoxin hepatocarcinogenesis on biomarker levels in the sera of these animals.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If

there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The results from this project highlight the important role that targeting the Nrf2 cytoprotective pathway has in the prevention of the actions of endogenous and exogenous carcinogens of importance to cancer risk in humans, namely, aflatoxins and estrogen. While the efficacy of upregulating this pathway is unequivocal in the preclinical models we employed, subsequent studies to translate these findings to humans is critical. We are currently conducting several clinical trials of preparations from broccolis sprouts, rich in the Nrf2 inducer sulforaphane, to probe this possibility.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Thomas W. Kensler

POSITION TITLE
Professor

eRA COMMONS USER NAME (credential, e.g., agency login)
tkensler

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

Hamilton College, Clinton, NY	A.B.	06/70	Biology
Massachusetts Institute of Technology,	MA Ph.D.	05/76	Toxicology
McArdle Laboratory, Univ. Wisconsin, Madison	PDF	08/78	Oncology

A. Personal Statement Health reflects the ability of an organism to adapt to stress. Stresses—metabolic, proteotoxic, mitotic, oxidative and DNA-damage stresses—not only contribute to the etiology of cancer and other chronic degenerative diseases but are also hallmarks of the cancer phenotype. Our work demonstrates that activation of the Kelch-like ECH-associated protein 1 (KEAP1)–NF-E2-related factor 2 (NRF2)-signaling pathway is an adaptive response to environmental and endogenous stresses and serves to render animals resistant to chemical carcinogenesis, other forms of toxicity, and inflammation whilst disruption of the pathway exacerbates these outcomes. Further, we have demonstrated that protection against these stresses is manifest in multiple ways: (i) prevention of macromolecular damage through induction of electrophile detoxication and antioxidative enzymes, as well as dampening of inflammatory processes, (ii) induction of macromolecular damage repair/removal systems including the proteasome and DNA repair, and (iii) activation of tissue repair/regeneration pathways. These cytoprotective effects of Nrf2 reflect responses mediated by direct activation of downstream effector genes and through cross-talk with other signaling networks contributing to cellular plasticity including aryl hydrocarbon receptor, NF-kB, p53 and Notch1. The Keap1-Nrf2 pathway can be induced by thiol-reactive small molecules including dithiolethiones (e.g., oltipraz), isothiocyanates (e.g., sulforaphane) and triterpenoids (e.g., CDDO-Im) that demonstrate protective efficacy in preclinical chemoprevention models and in clinical trials. We view that targeting the pathway may provide important opportunities for disease prevention. Translational studies in the form of longitudinal surveys and prospective case-control studies in Qidong, People's Republic of China demonstrate consistent exposure of individuals in this region to aflatoxins, and indicate a prime role for aflatoxin in the etiology of liver cancer, respectively. Dr. Kensler and his co-workers continue to utilize these cohorts for discovering, validating and utilizing markers of disease progression and as novel biomarkers for assessing intervention efficacy. He has lead several clinical trials of small molecule activators of the Nrf2 pathway in the Qidong region.

B. Positions and Honors
Positions and Employment

1978-1980 Staff Fellow, Biochemistry Section. Laboratory of Toxicology, DTP, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD
1980-1983 Research Associate, Division of Toxicology, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene & Public Health, Baltimore, MD
1983-1986 Assistant Professor of Toxicology, Johns Hopkins School of Hygiene & Public Health

1985- Joint Appointment: Department of Pharmacology & Molecular Sciences, Johns Hopkins University School of Medicine
1986-1992 Associate Professor of Toxicology, Johns Hopkins University School of Hygiene & Public Health
1992- Professor of Toxicology
1995- Joint Appointment: Department of Oncology/Johns Hopkins Oncology Center
1998- Joint Appointment: Department of Biochemistry and Molecular Biology
2000-2006 Director, Division of Toxicological Sciences, Johns Hopkins School of Public Health
2010- Professor, Department of Pharmacology & Chemical Biology, University of Pittsburgh

Other Experience and Professional Memberships
1983-2013 Diplomate American Board of Toxicology (recertified 1987, 1993, 1999, 2004, 2009)
1987-1991 Member, Chemical Pathology Study Section, NIH
1994-1997 Editorial Board, *Free Radical Biology & Medicine*
1995-1997 Editorial Board, *Chemical Research in Toxicology*
2001- Editorial Board, *Reviews in Mutation Research*
2007-2011 Editor for Cancer Prevention, *Carcinogenesis*
2007-2011 Member, Chemical-Dietary Prevention Study Section NIH (Chair: 2009-11)
2012- Senior Editor, *Cancer Prevention Research*

Honors
1987-1992 Research Career Development Award, NCI
2007 AACR-American Cancer Society Award for Research Excellence in Cancer Epidemiology and Prevention
2009 Society of Toxicology Translational Impact Award
2009 Golden Apple Award for Excellence in Teaching in Public Health Studies, Johns Hopkins Krieger School of Arts and Sciences (undergraduates)
2011 National Friendship Award, Beijing, People's Republic of China (highest foreign civilian award)
2012 Oxygen Club of California – Jarrow Formulas Health Science Prize (shared with Yamamoto)

C. Selected Peer-reviewed Publications (Selected from >360 publications)
Most relevant to the current application
1. **Kensler, T.W.,** Chen, J-G., Egner, P.A., Fahey, J.W., Jacobson, L.P., Stephenson, K.K., Ye, L., Coady, J.L., Wang, J-B., Wu, Y., Sun, Y., Zhang, Q-N., Zhang, B-C., Zhu, Y-R., Qian, G-S., Carmella, S.G., Hecht, S.S., Benning, L., Gange, S.J., Groopman, J.D. and Talalay, P. (2005) Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthene tetraols in a randomized clinical trial in He Zuo Township, Qidong, PRC. *Cancer Epidemiol. Biomarkers Prev*. 14:2605-2613. PMCID: none.
2. **Kensler, T.W.**, Wakabayashi, N., and Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Ann. Rev. Pharmacol. Toxicol*. 47: 89-116. PMCID: 16968214
3. Osburn, W.O., Yates, M.S., Dolan, P.M., Chen, S., Liby, K.T., Sporn, M.B., Taguchi, K., Yamamoto, M. and **Kensler, T.W.** (2008) Genetic or pharmacologic amplification of Nrf2 signaling inhibits acute inflammatory liver injury in mice. *Toxicological Sciences* 104: 218-227. PMCID: 18417483
4. Sussan, T.E., Rangasamy, T., Blake, D.J., Malhotra, D., El-Haddad, H., Bedja, D., Yates, M.S., Yamamoto, M., Liby, K.T., Sporn , M.B., Gabrielson, K.L., Champion , H.C., Tuder, R.M., **Kensler, T.W**. and Biswal, S. (2009) Targeting Nrf2 with the triterpenoid CDDO-imidazolide

attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proc. Natl. Acad. Sci. (USA)* 106:250-255. PMCID: 19104057
5. Yates, M.S., Tran, Q.T., Dolan, P.D., Osburn, W.O., Shin, S., McCulloch, C.C., Silkworth, J.B., Taguchi, K., Yamamoto, M., Williams, C.R., Liby, K.T., Sporn, M.B., Sutter, T.R., and **Kensler, T.W.** (2009) Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid treated mice. *Carcinogenesis* 30: 1024-1031. PMCID: 19386581
6. Wakabayashi, N. Shin, S., Slocum, S., Agoston, E.S., Wakabayashi, J., Kwak, M.K., Misra, V., Biswal, S., Yamamoto, M. and **Kensler, T.W.** (2010) Regulation of Notch1 signaling by Nrf2: implications for tissue regeneration. *Science Signaling* 3: ra52. PMCID 20628156
7. **Kensler, T.W.** and Wakabayashi, N. (2010) Nrf2: friend or foe for chemoprevention? *Carcinogenesis* 31: 90-99. PMCID 19793802
8. Agyeman, A., Chaerkady, R., Shaw, P., Davidson, N.E., Visvanathan, K., Pandey, A., and **Kensler, T.W.** (2011) Transcriptomic and proteomic profiling of KEAP1 disrupted and sulforaphane treated human breast epithelial cells reveals common expression profiles. *Breast Cancer Res. Treat*. 172:175-187. PMCID pending.
9. Davidson, N.E. and **Kensler, T.W.** (2011) “MAPping” the course of breast cancer chemoprevention. *N Engl. J Med* 364: 2460-2461.
10. **Kensler, T.W.,** Ng, D., Carmella, S.G., Chen, M., Jacobson, L.P., Muñoz, A., Egner, P.A., Chen, J.G., Qian, G.S., Chen, T.Y., Fahey, J.W., Talalay, P., Groopman, J.D., Yuan, J.M. and Hecht, S.S. (2012) Modulation of the metabolism of airborne pollutants by glucoraphanin-rich and sulforaphane-rich broccoli sprout beverages in Qidong, China. *Carcinogenesis* 33: 101-107. PMCID 3276337.
11. Fahey, J.F., Talalay, P. and **Kensler, T.W.** (2012) MiniReview. Notes from the field: “green” chemoprevention as frugal medicine. *Cancer Prev. Res*. 5: 179-188. PMCID 3273844.
12. Fahey, J.W., Wehage, S.L., Holtzclaw, W.D., **Kensler, T.W**., Egner, P.A., Shapiro, T.A. and Talalay, P. (2012) Protection of humans by plant glucosinolates: efficiency of conversion of glucosinolates to isothiocyanates by the gastrointestinal microflora. *Cancer Prev. Res* 5: 603-611. PMCID: pending
13. Yang, L., Zahid, M., Liao, Y., Rogan, E.G., Cavalieri, E.L., Davidson, N.E., Yager, J.D., Visvanathan, K., Groopman, J.D., **Kensler, T.W.** (2013) Diminished formation of estrogen depurinating-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells. *Carcinogenesis* 34:2587-2592. PMCID pending
14. Chen, J.G., Egner, P.A., Ng, D., Jacobson, L.P., Muñoz, A., Lu, J.H., Zhu, Y.R., Qian, G.S., Chen, T.Y., Wu, F., Yuan, J.M., Groopman, J.D. and **Kensler, T.W.** (2013) Reduced aflatoxin exposure presages decline in liver cancer mortality in an endemic region of China. *Cancer Prev. Res*. 6:1038-1045. PMCID: 3800239.
15. Chen, J.G. and **Kensler, T.W.** (2014) Perspectives on Statistical Trends: Changing rates for liver and lung cancer in Qidong, China. *Chem. Res. Toxicol.,* in press.

D. Research Support / Ongoing Research Support

University of Pittsburgh
5 R01 CA39416 Kensler (PI) 09/01/85-02/28/14 NIH/NCI
Molecular Mechanisms of Chemoprevention: NRF2 Signaling
This project, which entails a consortium between Pittsburgh, Johns Hopkins, Dartmouth and Memphis, investigates the role of Keap1-Nrf2 signaling in the chemopreventive activities of dithiolethiones and triterpenoids against aflatoxin B1-induced hepatocarcinogenesis in rats. Aims focus on developing and predicting biomarkers of individual risk to hepatocarcinogenesis; comparative genomics and proteomics with different classes of Nrf2 activators and effects on

aflatoxin biomarkers; and evaluation of post-initiation effects of Nrf2 activators on aflatoxin hepatocarcinogenesis in the rat.
Role: PI Overlap: None
1 R01 CA94076 Kensler (PI) 12/01/01-03/31/17 NIH/NCI
Role of Nrf2 Cross-Talk in Cancer Chemoprevention
This project seeks to evaluate the hypothesis that chemoprevention mediated by Nrf2 reflects both activation of its direct target genes but importantly cross-talk with other adaptive response signaling networks affecting cell fated, such as Notch1. The overall goals of the study are two-fold: to assess the underlying mechanisms and consequences of pathway cross-talk and to assess the functional significance and possible untoward effects of chronic induction of the Nrf22 response in order to facilitate the identification and utilization of safe, efficacious chemopreventive agents
Role: PI Overlap: None.

Johns Hopkins University
P01 ES06052 Groopman (PI) 04/07/93-06/30/14 (no cost extension) NIH/NIEHS
Molecular Biomarkers for Environmental Toxicants. Project IV: Biomarker Based Evaluation of Human Interventions
In a series of Phase I and II clinical trials, we are examining the effects of sulforaphane- or glucoraphanin-rich beverages prepared from broccoli sprouts on the urinary excretion of biomarkers of aflatoxin exposure (DNA adducts and metabolites) as well as mercapturic acid metabolites of air-borne toxics including 1,3-butadiene, benzene, acrolein, crotonaldehyde and ethylene oxide.
Role: Co-Investigator Overlap: None.
We seek to extend some of the work of this P01 in the current R0

BIOGRAPHICAL SKETCH

Nobunao Wakabayashi

POSITION TITLE
Research Assistant Professor

eRA COMMONS USER NAME (credential, e.g., agency login)
nwakabay

EDUCATION/TRAINING

Tohoku University, Sendai, Japan	B.A.	03/90	Agricultural Chemistry
Tohoku University Graduate School, Sendai, Japan	M.A.	03/92	Biochemistry
Tohoku University Graduate School, Sendai,Japan	Ph.D.	03/95	Molecular Biology
Osaka University, Research Inst. for Microbial Diseases	Postdoctoral	03/98	Molecular Embryology

A. Personal Statement

Loss of cellular homeostasis through exposures to endogenous (e.g., inflammation) and exogenous (e.g., carcinogens) stresses contributes to many diseases including carcinogenesis. The Keap1-Nrf2-ARE signaling pathway is the trunk to evoke an adaptive response to these stresses that serves to enhance cell survival. Through gene expression analyses of the signaling cascade linked to Nrf2, using both *Keap1*-and *Nrf2*-disrupted mice, we have shown that multiple signaling pathways intersect with Nrf2 signaling. Our research goals are to elucidate novel signaling crosstalk based on genes bearing functional ARE (Nrf2-sMaf recognition enhancer element) in the promoter of target genes and the underlying mechanistic roles of the Keap1-Nrf2 system in protecting against chronic degenerative diseases *in vivo*. Currently, our group has found that the role of Nrf2 signaling on liver tissue repair/regeneration is related to the expression of Notch-signaling. Furthermore, we found the elongated phenotype in small intestine, which consist of a lineage of reproducible epithelial cells fated by Notch-signaling, in the small intestine specific *Keap1* disrupted mice. The proposed study is to verify the cross talk between Keap1-Nrf2 and Notch signaling through analyzing the phenotypes in the liver regeneration and elongated small intestine using with various concerned genotyped mice and inducers which activate Nrf2 specific signal. *In vivo* and *in vitro* tools for verify them have already prepared and our group's productivity could expect the discovery and comprehension in tissue repair/regeneration area through the Keap1-Nrf2-ARE signaling pathway.

B. Positions and Honors

Positions and Employment

1998-1999 Research Fellow, Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, University of Tsukuba, Tsukuba, Japan

1999-2002 Research Fellow, the Ministry of Education, Science, Sports & Culture, University of Tsukuba, Tsukuba, Japan

2002-2005 Visiting Scholar, Division of Toxicological Sciences, Department of Environmental Health Sciences, Bloomberg School of Public Health, The Johns Hopkins University, Baltimore, MD

2005-2010 Research Associate, Division of Toxicological Sciences, Department of Environmental Health Sciences, Bloomberg School of Public Health, The Johns Hopkins University, Baltimore, MD

2010- Research Assistant Professor, Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA

Other Experience and Professional Memberships

1992- The Japan Society for Bioscience, Biotechnology, and Agrochemistry
1994- The Molecular Biology Society of Japan
1999- The Japan Biochemical Society
2008- American Association for Cancer Research
2013- American Society for Microbiology

Honors

2001 The JB Prize of The Japan Biochemical Society
2003 The 18th Aspen Conference Young Investigators through Aspen Cancer Conference Scientific Fellows Program
2009 Most Impressive Poster Presentation Award. The 3rd JST-International Symposium "Molecular Mechanism of Environmental Response to Food and Oxygen III"

C. Selected Peer-reviewed Publications (Selected from >46 peer-reviewed publications)
Most relevant to the current application

1. Itoh K., **Wakabayashi N.**, Katoh Y., Ishii T., Igarashi K., Engel J.D. & Yamamoto M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & Dev*. **13**, 76-86.
2. Dinkova-Kostova A.T., Holtzclaw W.D., Cole R.N., Itoh K., **Wakabayashi N.**, Katoh Y., Yamamoto M., & Talalay P. (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11908-11913
3. **Wakabayashi, N**, Itoh K., Wakabayashi J., Motohashi H., Noda S., Takahashi S., Imakado S., Kotsuji T., Otsuka F., Roop D.R, Harada T., Engel J.D., & Yamamoto M. (2003) *Keap1*-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* **35**, 238-245.
4. Shin, S., **Wakabayashi, N**., Misra, V., Biswal, S., Lee, G.H., Agoston, E.S., Yamamoto, M., & Kensler, T.W. (2007) NRF2 modulates AHR signaling: influence on adipogenesis. *Mol. Cell. Biol.* **27**: 7188-7197.
5. **Wakabayashi, N**. Shin, S., Slocum, S., Agoston, E.S., Wakabayashi, J., Kwak, M.K., Misra, V., Biswal, S., Yamamoto, M. & Kensler, T.W**.** (2010) Regulation of Notch1 signaling by Nrf2: implications for tissue regeneration. *Science Signaling* **3**: ra52.
6. **Wakabayashi N**., Skoko JJ, Chartoumpekis DV, Kimura S, Slocum SL, Noda K, Palliyaguru DL, Fujimuro M, Boley PA, Tanaka Y, Shigemura N, Biswal S, Yamamoto M, Kensler TW. Notch-Nrf2 axis: Regulation of Nrf2 gene expression and cytoprotection by Notch signaling.(2013 Dec 2) *Mol. Cell. Biol.* [Epub ahead of print] PMID:24298019

Additional recent publications of importance to the field (in chronological order)

1. Kwak M.K., **Wakabayashi N**., Itoh K., Motohashi H., Yamamoto M., & Kensler T.W. (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway: Identification of novel gene clusters for cell survival. *J. Biol. Chem.* **278,** 8135-8145.
2. **Wakabayashi N.**, Dinkova-Kostova A.T., Holtzclaw W.D., Kang M.L., Kobayashi A., Yamamoto M., Kensler T.W., & Talalay P. (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 2040-2045.
3. Dinkova-Kostova A.T., Holtzclaw W.D., & **Wakabayashi N**. (2005) Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. *Biochemistry* **44**, 6889-6899.
4. Osburn W.O., **Wakabayashi N**., Misra V., Nilles T., Biswal S., Trush M.A. & Kensler T.W. (2006) Nrf2 regulates an adaptive response protecting against oxidative damage following diquat-mediated formation of superoxide anion. *Arch. Biochem. Biophys*. **454**, 7-15.

5. Kensler, T.W**.**, **Wakabayashi, N**., & Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Ann. Rev. Pharmacol. Toxicol*. 47: 89-116.
6. Shin S., Wakabayashi J., Yates M.S., **Wakabayashi N**, Dolan P.M., Aja S., Liby K.T., Sporn M.B., Yamamoto M. & Kensler T.W. (2009) Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-Imidazolide. *Eur J Pharmacol.* **620**, 138-144.
7. Singh A., Bodas M., **Wakabayashi N.**, Bunz F., & Biswal S. (2010) Gain of Nrf2 function in Non-Small-Cell Lung Cancer Cells Confers Radioresitance. *Antioxid. Redox Signal*. **13**, 1627-1637.
8. Malhotra D., Portales-Casamar E., Singh A., Srivastava S., Arenillas D., Happel C., Shyr C., **Wakabayashi N.**, Kensler T.W., Wasserman W.W. & Biswal S. (2010) Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res.* **38**, 5718-5734.
9. Kensler, T.W. & **Wakabayashi N.** (2010) Nrf2: friend or foe for chemoprevention? *Carcinogenesis* **31**: 90-99.
10. **Wakabayashi N**., Slocum, S.L., Skoko, J.L., Shin, S. & Kensler, T.W. (2010) When NRF2 talks, who's listening? *Antioxidant & Redox Signaling* **13**: 1649-1663.
11. Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway *in vivo*. Kawamura T, **Wakabayashi N**, Shigemura N, Huang CS, Masutani K, Tanaka Y, Noda K, Peng X, Takahashi T, Billiar TR, Okumura M, Toyoda Y, Kensler TW, Nakao A. *Am J Physiol Lung Cell Mol Physiol*. **304**, L646-656, 2013.
12. Inhibition of Nuclear factor-erythroid 2-related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence. Volonte D, Liu Z, Musille PM, Stoppani E, **Wakabayashi N**, Di YP, Lisanti MP, Kensler TW, Galbiati F. *Mol. Biol. Cell*. **24**, 1852-1862, 2013.
13. Nrf2 regulates miR-1 and miR-206 to drive Tumorigenesis. Singh A, Happel C, Manna S, Acquaah-Mensah G, Carretero J, Kumar S, Nasipuri P, Kraubsz K, **Wakabayashi N**, Dewi R, Boros L, Gonzalez F, Gabrielson E, Wong KK, Girnun G and Biswal S. *J. Clin Invest*. **123**, 2921-2934, 2013.

BIOGRAPHICAL SKETCH

Yang, Li

POSITION TITLE
Postdoctoral Associate

eRA COMMONS USER NAME (credential, e.g., agency login)
LIYANG5854

EDUCATION/TRAINING

Inner Mongolia Agricultural Unviersity	B.S.	07/1995	Plant Protection
China Agricultural University	M.S.	07/2002	Biology
University of Nebraska Medical Center	PhD	05/2010	Environmental Toxicology
University of Pittsburgh	PDF	current	Cancer Chemoprevention

A. Personal Statement

During my PhD study, I have been involved in four breast cancer case-control studies and two prostate cancer case-controls studies with the goal of detection of biomarker of the above cancers by analyzing urine and blood samples from cases and controls. I have mastered solid phase extraction techniques, which is one of the key steps to accomplish successful purification the estrogen metabolites from complex biosamples. I have utilized ultra performance liquid chromatography coupled to mass spectrometry (UPLCMS/ MS) to identify and quantify the estrogen metabolites, including the depurinating estrogen DNA adducts. To complement my analytical skills, during my PhD study, I completed courses in epidemiology courses,advanced statistics, and clinical methodologies, which have provided me with very strong background to develop a career in translational research. Since joining the Kensler lab as Postdoc Associate, I have obtained extensive training in methods development on the Thermo TSQ Vantage mass spectrometer in the lab and clinical trial study design. The goal of my current research is to investigate the hypothesis that sulforaphane, a bioactive constituent of cruciferous vegetables such as broccoli, prevents breast cancer through modulation of estrogen metabolism leading to diminished levels of depurinating estrogen-DNA adducts. I have discovered that sulforaphane reduces estrogen depurinating DNA adducts via Keap1-Nrf2 pathway in MCF-10A cell models. Currently, I plan to measure depurinating estrogen-DNA adducts and the other key estrogen metabolites from urine and blood from women enrolled in a series of placebo controlled clinical trials of broccoli sprouts preparations rich in sulforaphane. I have the expertise, leadership and motivation necessary to successfully carry out the proposed work..

B. Positions and Honors

Professional memberships

2008 -	Present Full Member, Society of Toxicology (SOT)
2009 -	Present Member, American Association of Chinese in Toxicology (AACT) SIG
2009 - 2010	Graduate Student Member, Association of Public Health Laboratories (APHL)
2010 -	Present Member, Women in Toxicology (WIT) SIG
2011 -	Present Active Member, American Association of Cancer Research (AACR)
2011 -	Present Postdoc Member, National Postdoctoral Association (NPA)
2011 -	Present Member, American Society for Mass Spectrometry

Honors

2004 Special award for contribution to the pesticides research, Society of Pesticide Res., Beijing, China

2007 Award for best poster presentation, Central States Society of Toxicology Meeting. Iowa City, Iowa
2008 Representative of UNMC graduate students for International Student Research Forum. Omaha, Nebraska
2008 Award for Graduate Student Travel Support for 2008 National Toxicology Conference. Society of Toxicology (SOT). Seattle, Washington
2008 Award for the 3rd place poster presentation, 39th Midwest Student Biomedical Research Forum, Omaha, Nebraska
2009 Award for the 2nd place best abstract, American Association of Chinese in Toxicology (AACT), Baltimore, Maryland
2007-2010 UNMC graduate school, Fellowship
2011-2014 DOD breast Cancer Research Program Postdoctoral Fellowship Award BC103928
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.

C. Selected Peer-reviewed Publications

1. 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.
2. **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.
3. **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.
4. **Yang L**., Liao Y., Zhou Z.Q., Jiang S.Q., Wang P. (**2004**) Separation of enantiomers of two pesticides using β-CD as a chiral mobile phase additive in high-performance liquid chromatography. *Chinese Journal of Pesticide Science.* 6(2): 90-92.
5. 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.
6. 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.* 122:1949-57. PMCID: 18098283
7. 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*. 45:136-45. PMCID: 18423413
8. **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. PMCID: 18816637
9. 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 Res.* 3:1-8. PMCID: 18816637

10. Maddalena Barba, **Yang L**, Francesca Sperati, Holger J. Schünemann, Sara Grioni, Saverio Stranges, Kim C. Westerlind, Michele Gallucci, Paola Muti. (**2009**) Urinary estrogen metabolites and prostate cancer: a case–control study and meta-analysis. *J Experimental & Clinical Cancer Res.* 28:135.
11. 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: 502-12. PMCID: 19863189
12. Gaikwad NW, **Yang L**, Rogan E.G. and Cavalieri E. L. (**2009**) Evidence for NQO2-mediated reduction of the carcinogenic estrogen ortho-quinones. *Free Radic Biol Med.* 46:253-62. PMID: 18996184
13. Zahid M, Saeed M, **Yang L**, Beseler C, Rogan EG, Cavalieri EL. (**2011**). Formation of dopamine quinone-DNA adducts and their potential role in the etiology of Parkinson's
14. 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. *(* equal contribution as first author*). 132(1-2):73-9. 2012 Feb 24. [Epub ahead of print]. PMID: 22386952.
15. **Yang L.** Zahid M, Liao Y., Cavalieri E., Rogan E., 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).

D. Research Support.
Dr. Yang is currently supported by institutional funds provided by the University of Pittsburgh to Dr. Kensler and DoD Breast Cancer Research