

Award Number: W81XWH-07-1-0056

TITLE: Prostate cancer prevention by sulforaphane, a novel dietary histone deacetylase inhibitor

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REPORT DATE: January 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

*Form Approved*  
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<b>1. REPORT DATE</b> 31-01-2008			<b>2. REPORT TYPE</b> ANNUAL		<b>3. DATES COVERED</b> 1 JAN 2007 - 31 DEC 2007	
<b>4. TITLE AND SUBTITLE</b> Prostate cancer prevention by sulforaphane, a novel dietary histone deacetylase inhibitor					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-07-1-0056	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Yu Zhen					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Oregon State University Corvallis, OR, 97331					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b>  Prostate cancer is the second leading cause of cancer related death in men. To test Sulforaphane (SFN) as a novel histone deacetylases (HDAC) inhibitor and explore the mechanism of SFN protection against prostate cancer, different stage of prostate cancerous cells were treated with 15µM or 30 µM SFN and harvest 48hr later for MTT assay, HDAC activity and western blot assay. MTT cell proliferation showed that SFN and its metabolites inhibited PC3 prostate cancerous cell growth. SFN also inhibited HDAC activity in prostate cancerous cell alone but not normal cells. SFN selectively induced cell cycle arrest in prostate cancer cells, but not normal prostate cells. SFN treatment reduced the HDAC1 and HDAC3 protein level in PC3 cells and increased the acetylated histone H4. All the study showed that SFN is a HDAC inhibitor and protected against cancer development through epigenetic alteration in prostate cancer cells.						
<b>15. SUBJECT TERMS</b> Sulforaphane, prostate cancer, Histone deacetylases						
<b>16. SECURITY CLASSIFICATION OF:</b>				<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U	<b>19b. TELEPHONE NUMBER</b> (include area code)			
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## Introduction

Prostate cancer is the second leading cause of cancer related death in men. Preventive measures that target the various steps involved cancer initiation and progression could significantly reduce the incidence and mortality of prostate cancer. One novel group of chemopreventive agents are inhibitors of histone deacetylases (HDAC) which target epigenetic events that can occur at various stages of cancer development. Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables and is an effective chemoprotective agent. **This training grant is trying to establish SFN as a novel HDAC inhibitor and study the prevention mechanism of HDAC inhibitors using *in vitro* model.** The mechanism will be further confirmed in animal models in future study. This work will also be a scientific foundation for future large –scale human clinical intervention studies which will benefit to reduce the prostate cancer.

## Body

Epidemiological studies showed that dietary intake of cruciferous vegetables may be protected against the risk of prostate cancer(1-2). SFN is an isothiocyanate found in cruciferous vegetables and is especially high in broccoli and broccoli sprouts. SFN is an effective chemoprotective agent in carcinogen-induced animal models, as well as in xenograft models of prostate cancer (3-5). Traditionally, chemoprotective effects of SFN have been attributed to SFN’s ability to up-regulate Phase II detoxification systems in the initiation phase of cancer development. Recent studies are seeking other protection mechanism targeted also at later phase of cancer development. The reversible acetylation of nuclear histones is an important mechanism of gene regulation. In general, addition of acetyl groups to histones by histone acetyltransferases (HAT) results in an “open” chromatin conformation, facilitating gene expression by allowing transcription factors access to DNA. Removal of acetyl groups by histone deacetylases (HDACs) results in a “closed” conformation, which represses transcription.

\*

A tightly regulated balance exists in normal cells between HAT and HDAC activities, and when this balance is disrupted, cancer development can ensue. The *working hypothesis* is that epigenetic alterations, namely changes in HDAC activity, HDAC protein levels, histone acetylation status and acetylated histones associated with specific tumor suppressor genes, will accumulate and be detectable in prostate cell lines from various stages of the tumorigenic process. Our working hypothesis is that SFN will inhibit HDAC activity and induce accumulation of acetylated histones at all stages of prostate cancer; however, only in cancerous cells will downstream effects of HDAC inhibition, namely cell cycle arrest and apoptosis, be observed. To test this hypothesis, we treated different stages of prostate cells with SFN and analyzed cell proliferation, HDAC activity and protein expression. The results were shown below.

### 1. SFN and its metabolites induce prostate cancerous cell death. SFN and its metabolites also inhibit HDAC activity in PC3 cells.

SFN is metabolized via the mercapturic acid pathway, starting with glutathione (GSH) conjugation by glutathione s-transferase (GST) and subsequent steps generate SFN-cysteine (SFN-Cys) followed by SFN-N-acetylcysteine (SFN-NAC). MTT assay was performed to detect the cell viability after SFN and its metabolites treatment on PC3 cells. Up to 50% cells proliferation was inhibited after the SFN and its metabolites treatment (Figure 1). HDAC activity assay also showed the inhibition effects of SFN and its metabolites on prostate cancerous cells (Figure 2).

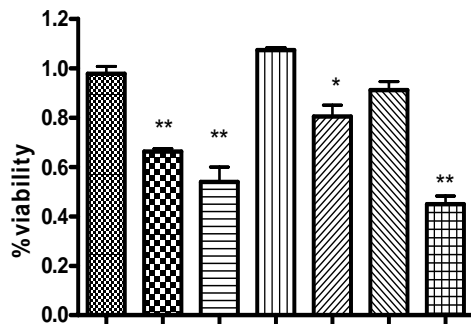


Figure 1. MTT assay for PC3 cells treated with SFN & metabolites. SFN & Metabolites: 15 & 30 µM, treatment period: 48hrs. \* P<0.05, \*\*, p<0.01, t test compared to PC3 cells alone.

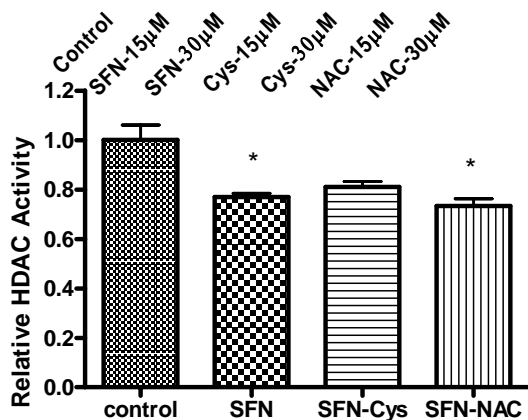


Figure 2 SFN and metabolites inhibit HDAC activity in PC3 cells. PC3 cells were harvested 48hrs after treatment with SFN, SFN-Cys, SFN-NAC (15µM) and cell lysates were analyzed for HDAC activity. Results = mean ± SEM, n=3 \* p<0.05.

These data proved that SFN and its metabolites are chemoprevention agents in cell culture model and the inhibition effects are partially through inhibition of HDAC activity.

## 2. SFN inhibits HDAC activity in prostate cancerous cells only.

Reports showed that HDAC inhibitors such as SAHA are promising anti-cancer agents because they induce cell death in a broad spectrum of transformed cells, whereas normal cells are relatively resistant (6).

We also performed initial screening of effects of SFN on HDAC activity in several prostate epithelial cell lines, including normal prostate cells (PrEC), benign prostate hyperplasia (BPH-1), early stage LNCaP cells (androgen-dependent prostate cancer cells) and late stage PC3 and DU145 cells (androgen-independent carcinoma). Treatment with 15  $\mu$ M SFN caused inhibition of HDAC activity (20-33%) on benign and early and later state of prostate cancerous cell, but not on normal prostate cells (Figure 3). Meanwhile, we also detected that HDAC activity is 1.6 fold higher in PC3 cells compared to normal PrEC cells.

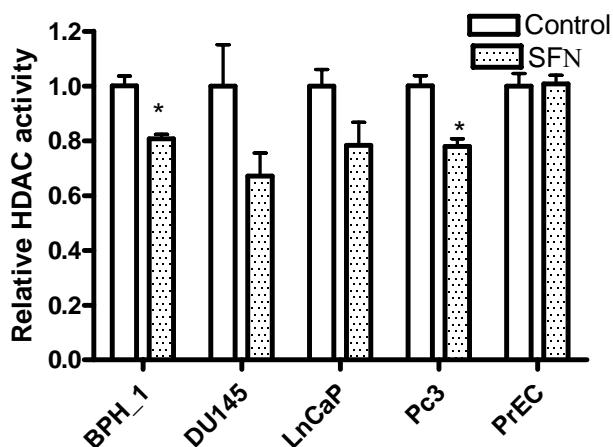


Figure 3. SFN inhibits HDAC activity in prostate epithelial cells. Cells were harvested 48hrs after treatment with SFN (15 $\mu$ M) and cell lysates were analyzed for HDAC activity. Results = mean  $\pm$  SEM, n=3. \* P < 0.05. The HDAC activity is compared to different control cells, respectively.

## 3. SFN selectively induces cell cycle arrest in prostate cancer cells, but not normal prostate cells.

To examine downstream consequences of HDAC inhibition in normal and prostate cancer cells, we examined cell cycle alterations following SFN treatment in both cell types.

The anti-proliferative effects of SFN were also specific to cancer cells. Addition of 15  $\mu$ M SFN caused cell cycle arrest in PC3 cells, with no effects in normal PrEC cells. As shown in Figure 4, SFN treatment caused a significant loss of the G1 peak, with accumulation in G2, suggesting a G2/M arrest with SFN treatment in PC3 cells. In contrast, no effect on cell cycle was seen in normal prostate epithelial cells.

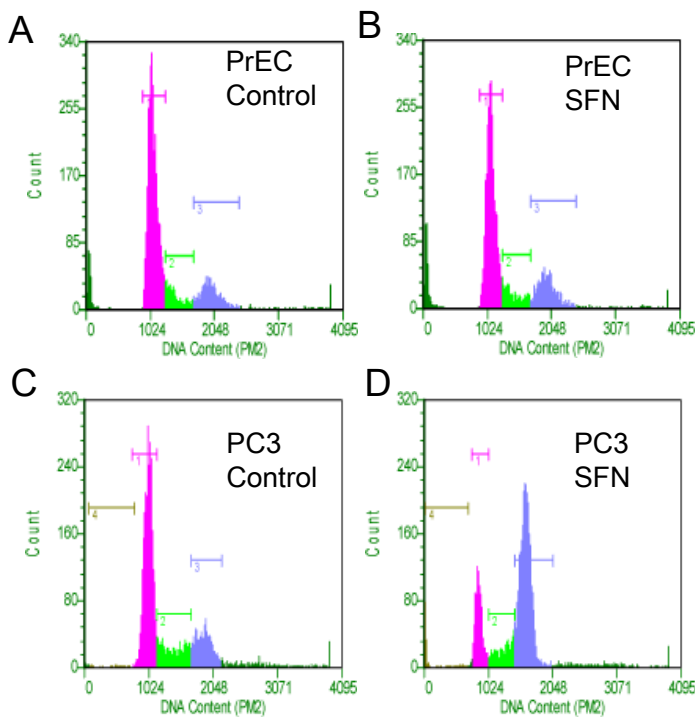


Figure 4. SFN induces cell cycle arrest in PC3 cancer cells, but not in normal prostate epithelial cells (PrEC). PrEC and PC3 cells were treated with 0 or 15  $\mu$ M SFN for 48 hrs. Cells were harvested, fixed, and stained for cell cycle. No change in cell cycle was seen in PrEC cells treated with DMSO vehicle (A) or SFN (B). In contrast a marked G2/M arrest was apparent in PC3 cells treated with SFN (D) compared to control (C). Histograms are representative of findings from three separate experiments.

#### 4. SFN treatment reduced the HDAC1 and HDAC3 protein level in PC3 cells and increased the acetylated histone H4.

SFN treatment not only decreased the HDAC activity but also reduced the HDAC1 and HDAC3 protein level and increased the acetylated histone H4 (Figure 5). However, it was hard to detect the downstream tumor suppression gene P21 in PC3 cells (data not shown).

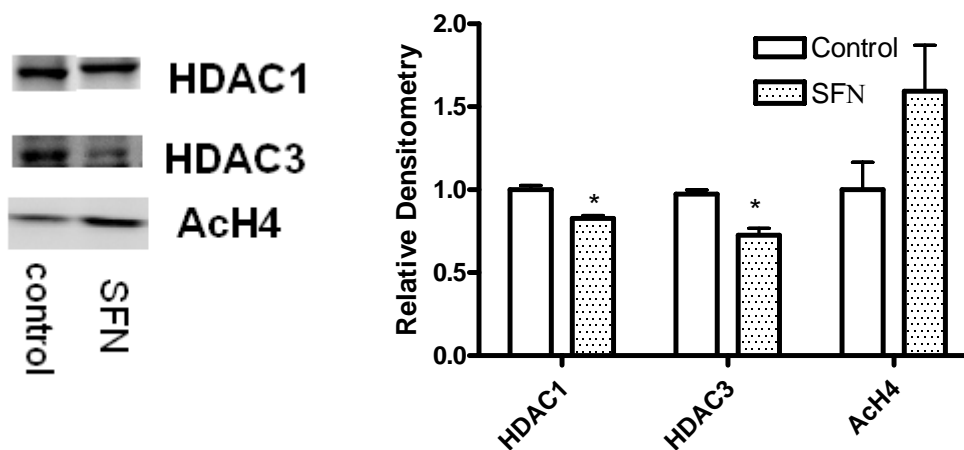


Figure 5. SFN inhibits the HDAC1 and HDAC3 expression in PC3 cells and increases acetylated histone H4. Cells were treated with 15 $\mu$ M SFN and harvest 48hr later and cell lysate were analyzed for westernblot. Results = mean  $\pm$  SEM, n=3. \* P < 0.05.

## 5. Development of HDAC1 over-expression cell line. No effects of HDAC1 over-expression on the ability of SFN to induce cell death.

Increased HDAC activity and expression is common in many cancer malignancies. In prostate cancer, increased expression of HDACs have been reported (7-9). Our hypothesis is that HDAC inhibition is an important mechanism for SFN to induce cell cycle arrest and apoptosis, and to exert chemopreventive effects in the prostate. To examine the requirement of HDAC activity in SFN-induced cell cycle arrest and apoptosis, we use HDAC over-expression strategies in prostate cancer cells to overcome HDAC inhibitory effects with SFN.

First, we built a HDAC1 over-expression cell line: prostate cancer cells were transfected with HDAC1 plasmids (kind gift from Dr. E. Verdin, UCSF) using Lipofectamine LTX reagents (Invitrogen) and empty pcDNA3.1 was used as control. Following transfection, stable transfectants were selected by serial dilutions in the presence of neomycin. Transfection efficiency of HDAC constructs was monitored by flow cytometry and the expression level was measured by Western blot, using Flag-tag antibodies (Upstate, Charlottesville, VA). We had tried different cell lines (BPH-1, LNCaP, PC3, DU145) and different transfection agents. It's turned out that PC3 is the best cell line for transfection and Lipofectamine LTX is the best transfection reagent.

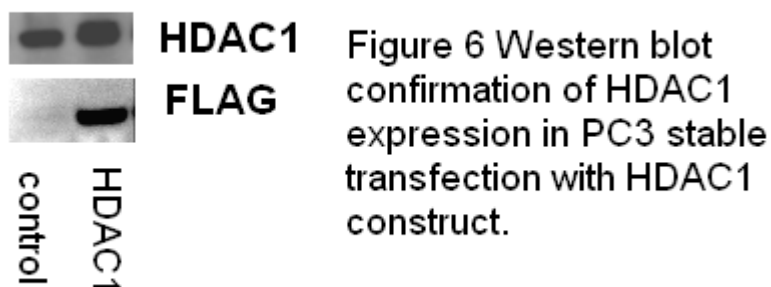


Figure 6 Western blot confirmation of HDAC1 expression in PC3 stable transfection with HDAC1 construct.

Figure 6 is the western blot confirmation of the over-expression of HDAC1 in PC3 cells. However, the HDAC activity was not changed in HDAC1 over-expression cells. When the HDAC1-transfected cell treated with 15  $\mu$ M SFN and 30  $\mu$ M SFN or its metabolites, the ratio of cell death was not changed, either (Figure 7). Stable transfection with HDAC1 construct caused no changes in HDAC activity (Figure 8) and no protection against SFN-induced cell death. We had failed to build a cell line stable transfection with HDAC3 construct even the transient transfection is successful.

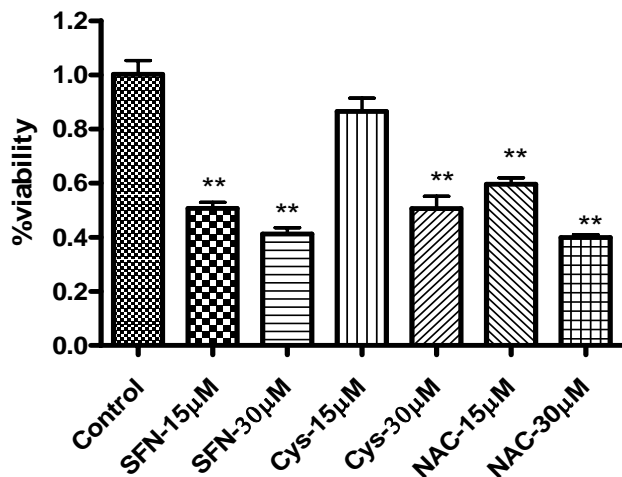


Figure 7. MTT assay for HDAC1-PC3 cells treated with SFN & metabolites. SFN & Metabolites: 15 & 30 µM, treatment period: 48hrs. \* P<0.05, \*\*, p<0.01, t test compared to HDAC1-PC3 cells alone.

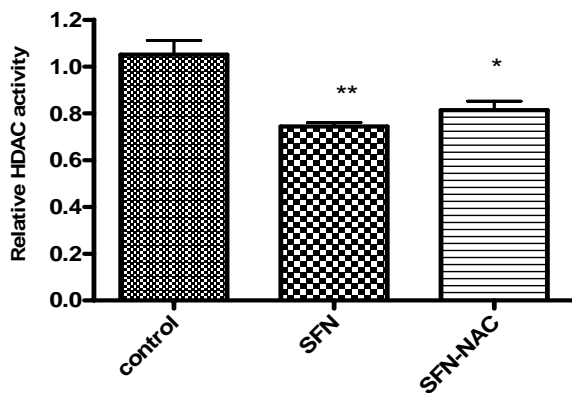


Figure 8. SFN inhibits HDAC activity in PC3 cells. Cells were harvested 48hrs after treatment with SFN & SFN-NAC(15µM) and cell lysates were analyzed for HDAC activity. Results = mean ± SEM, n=3. \* P < 0.05.

### Key Research Accomplishment

1. SFN and its metabolites induce prostate cancerous cell death. SFN and its metabolites also inhibit the HDAC activity in PC3 cells.
2. SFN inhibits HDAC activity in prostate cancerous cells only.
3. SFN selectively induces cell cycle arrest in prostate cancer cells, but not normal prostate cells.
4. SFN treatment reduced the HDAC1 and HDAC3 protein level in PC3 cells and increased the acetylated histone H4.
5. Development of HDAC1 over-expression cell line. No effects of HDAC1 over-expression on the ability of SFN to induce cell death.

### Reportable outcomes

The results will be presented at Carcinogenesis and Chemoprevention group meeting of Oregon State University on May, 2008.

An abstract will be submitted to AACR cancer epigenetics meeting, May 28-31,2008, Boston, MA.

### Conclusion



We have proved that SFN is a HDAC inhibitor and inhibit cell proliferation in prostate cancer cell. However the mechanism is still not quite clear. Our hypothesis is that SFN will inhibit the HDAC activity and decrease the HDAC protein level, increase the acetylated histones expression and increase the acetylated histones associated with specific tumor suppressor genes expression. We found that SFN inhibited HDAC activity, decreased the protein level of HDAC1 and HDAC3, and increased the acetylated histone H4 level. However, we could not detected the change of the specific tumor suppressor genes P21 and BAX. Our next direction will be studying the different proteins related to cell cycle and apoptosis, for example, CDK1, cyclin B (10). Protein array will also be another option. The Panorama Antibody Microarray (Sigma) is designed for studying protein expression in cell or tissue extracts. The ability to identify multiple proteins simultaneously allows global molecular characterization of biological samples. We'll have a better understanding of the overall pathway that SFN affects and relates to HDAC, cell cycle and apoptosis.

There may be other HDACs which contribute more to the HDAC activity. For example, HDAC5 and HDAC7 had almost 2- fold higher transcript level compared to HDAC1 in PC3 cells (11). Our lab also detected decrease of HDAC5 and HDAC7 activity by treatment of SFN. Building another HDAC5 or HDAC7 over-expression cell line to study the mechanism of SFN as HDAC inhibitor in cancer development will conduct.

This work will also be the foundation for future *in vivo* study and human trials. Our lab had already begun the study of effect of dietary SFN on tumor development in mice model and also a short-term SFN consumption study on human subject based on the *in vitro* study results.

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Castro, DJ, **Yu, Z**, Dashwood, RH, Bailey, GS, Baird, WM and Williams, DE (2008) A Model for the Study of Maternal Dietary Inhibition of Transplacental Carcinogenesis. In *Recent Advances in Carcinogenesis*, (R.M. Mohan, Ed.), in press.

Castro DJ, **Yu Z**, Loehr CV, Pereira CB, Giovanini J, Fische KA, Orner GA, Dashwood RH, Williams DE. (2008) **Chemoprevention of Dibenzo[a,l]Pyrene Transplacental Carcinogenesis in Mice Born to Mothers Administered Green Tea: Primary Role of Caffeine**. *Carcinogenesis*, in press.

Castro DJ, William MS, **Yu Z**, Loehr CV, Pereira CB, Giovanini J, Fische KA, Orner GA, Dashwood RH, Williams DE. (2008) **Fetal Mouse Cyp1b1 and Transplacental Carcinogenesis from Maternal Exposure to Dibenzo[a,l]pyrene**. *Cancer Prevention Research*, in press.

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## **Presentations**

**Yu Z, Mahadevan B, Siddens LK, Albershardt DJ, Kureger SK, Louderback M, Baird WM, Williams DE. (2005) Transplacental genotoxicity of dibenzo[a,l]pyrene (DBP) and the effect of indole -3-carbinol (I3C) in the maternal diet.** Society of Toxicology Annual Meeting, New Orleans, LA.

**Yu Z, Louderback MA, Loehr CV, Fischer KA, Kureger SK Williams DE. (2005) Chemoprotection against dibenzo[a,l]pyrene (DBP) transplacental carcinogenesis by maternal dietary exposure to indole-3 carbinol (I3C): Role of the aryl hydrocarbon receptor (Ahr).** American associates of Cancer Research Annual Meeting. Aneheim, CA.

**Yu Z, Mahadevan B, Albershardt DJ, Castro DJ, LaRonge V, Baird WM, Williams DE.(2006) Mechanism of transplacental carcinogenesis of dibenzo[a,l]pyrene and chemoprotection of indole-3-carbinol in the maternal diet.** American associates of Cancer Research Annual Meeting. Washington DC.