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14. ABSTRACT 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, normal prostate cells and different stage of prostate cancerous cells were treated with 15µM SFN and harvest to test cell viability, cell cycle arrest, apoptosis, HDAC activity, transcription and protein level of HDACs and tumor suppressor genes. MTT cell viability showed that SFN inhibited prostate cancerous cell growth only. SFN also inhibited HDAC activity in prostate cancerous cell alone but not normal cells. SFN selectively induced cell cycle arrest and apoptosis in prostate cancer cells, but not normal prostate cells. SFN increased mRNA and protein level of tumor suppressor gene p21 in prostate cancerous cells. All the study showed that SFN is a HDAC inhibitor and protected against cancer development through epigenetic alteration in prostate cancer cells.						
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Table of Contents

	<u>Page</u>
Introduction.....	3
Body.....	3
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	9
References	9
Appendices.....	10

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 is 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. This work will also be a scientific foundation for future large –scale human clinical intervention studies which will benefit to reduce 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. Aberrant epigenetic events such as DNA hypermethylation and altered histone acetylation have been observed during prostate cancer. To control histone acetylation, a balance exists in normal cells between histone acetyltransferase (HAT) and

histone deacetylase (HDAC) activities, and when this balance is disrupted, cancer development can ensue. HDAC activity increases in metastatic cells compared with normal prostate cells, and global changes in acetylation pattern predicts prostate cancer risk and recurrence. In addition, DNA methyltransferase (DNMT) activity is elevated during prostate cancer and many genes involved in cell cycle arrest, DNA damage repair and carcinogen detoxification are commonly hypermethylated and silenced during prostate cancer. Both DNA hypermethylation and histone modifications are closely related. *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 and 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. Meanwhile, SFN is also working as a DNMT inhibitor, causing DNA demethylation and induction of cell cycle arrest/apoptosis, leading to cancer prevention.*

To test this hypothesis, we treated prostate epithelial cells ranging from normal to highly malignant with SFN and analyzed cell proliferation, HDAC activity and protein expression of HDACs, histone acetylation status acetylated histones associated with specific tumor suppressor genes. The results from our first year studies showed that SFN and its metabolites induce prostate cancerous cell death, Inhibit HDAC, and induced HDAC1, HDAC3 and acetylated histone H4 protein expression in prostate cancer cells. We also showed the preliminary data about SFN selectively induces cell cycle arrest in prostate cancer cells, but not normal prostate cells.

This year, a comprehensive study about the effect of SFN treatment on the normal and prostate cancer cell was conducted, including cell cycle arrest, apoptosis, HDACs activities, mRNA and protein level expression of HDACs, mRNA and protein level of tumor suppression gene expression. Another study about testing SFN as DNMT inhibitor was also conducted.

1. SFN induced cell death, cell cycle arrest, apoptosis and reduced HDAC activity specifically in prostate cancer cell but not in normal prostate epithelial cells.

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 cell viability 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 (androgen-independent carcinoma). Treatment with 15 μ M SFN caused more than 50% of cell death in prostate cancerous cells, while only around 20% of cell death in normal prostate cells (Figure 1). Treatment with 15 μ M SFN caused inhibition of HDAC activity (20-33%) on benign, early and later state of prostate cancerous cell. In contrast, no HDAC inhibition was seen in normal PrEC cells (Figure 2). We also detected that HDAC activity is 1.6 fold higher in PC3 cells compared to normal PrEC cells.

These data proved that SFN is chemoprevention agent in cell culture model and the inhibition effects are partially through inhibition of HDAC activity. The inhibition effects are only seen in prostate cancer cells not in normal cells, indicating that anti-proliferative effects are specific, similar to pharmacological HDAC inhibitor. To specific how SFN reduced cell viability, cell cycle arrest and apoptosis assay were performed. Addition of 15 μ M SFN caused cell cycle arrest in PC3 cells, with no effects in normal PrEC cells. As shown in Figure 3, 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. 48hr treatment of SFN also caused a significant increasing in apoptosis (about 2.5 folds) in PC3 cells (Figure 4).

Together, our data showed that SFN is protective against prostate cancer through inhibition of HDAC activity, selectively induced cell cycle arrest and apoptosis in cancer cell only, similar to pharmacological HDAC inhibitors.

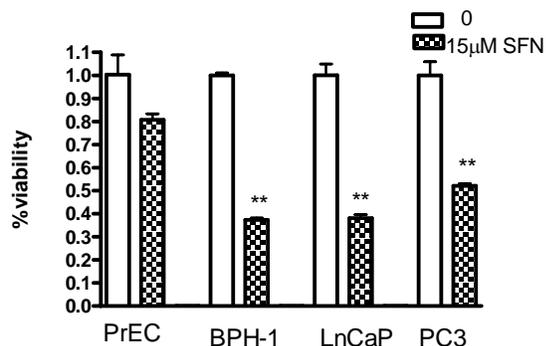


Figure 1. MTT assay for normal and cancerous prostate cells treated with 15µM SFN for 48hrs. Results = means ± SEM, n=3. ** p<0.01. The % of viability is compared to different control treatment cells, respectively.

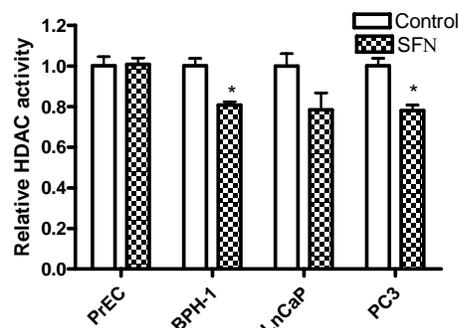


Figure 2. SFN inhibits HDAC activity in prostate cells. Cells were harvested 48hrs after treatment with SFN (15µM) and cell lysates were analyzed for HDAC activity. Results = mean ± SEM, n=3. * p < 0.05. The HDAC activity is compared to different control treatment cells, respectively.

2. SFN treatment reduced HDAC6 mRNA level and increase p21 in mRNA and protein levels in prostate cancer cells.

To exam the SFN effect on HDACs expression in prostate cancer cells and normal cells, we compared the mRNA levels of HDACs in PC3 and PrEC cells. For the overall HDACs expression, HDAC1 and HDAC2 are most abundant, HDAC3 is less and HDAC6 is the least (data not shown). However, only HDAC6 showed significant higher in PC3 compared to PrEC cells (p=0.015). SFN treatment reduced the HDAC6 mRNA levels in both PrEC and PC3 cells (Figure 5). Protein analysis also showed that SFN decreased HDAC6 level in PC3 cells (data not shown).

The cyclin-dependent kinase inhibitor p21 functions as a regulator of cell cycle progression at S phase. Induction of p21 may cause cell cycle arrest. The normal PrEC cell has 11 folds higher P21 level compared to prostate cancer PC3 cells. SFN treatment induced p21 expression in PC3 cells (Figure 6). Chromatin-immunoprecipitation (ChIP) assays identified alterations in acetylated histones at specific promoter sites of P21 (data not shown).

From these data we conclude that SFN, similar to other HDAC inhibitors, exerts differential effects on HDAC activity and downstream targets in normal and cancer cells, and subsequently exerts selective toxicity in cancer cells.

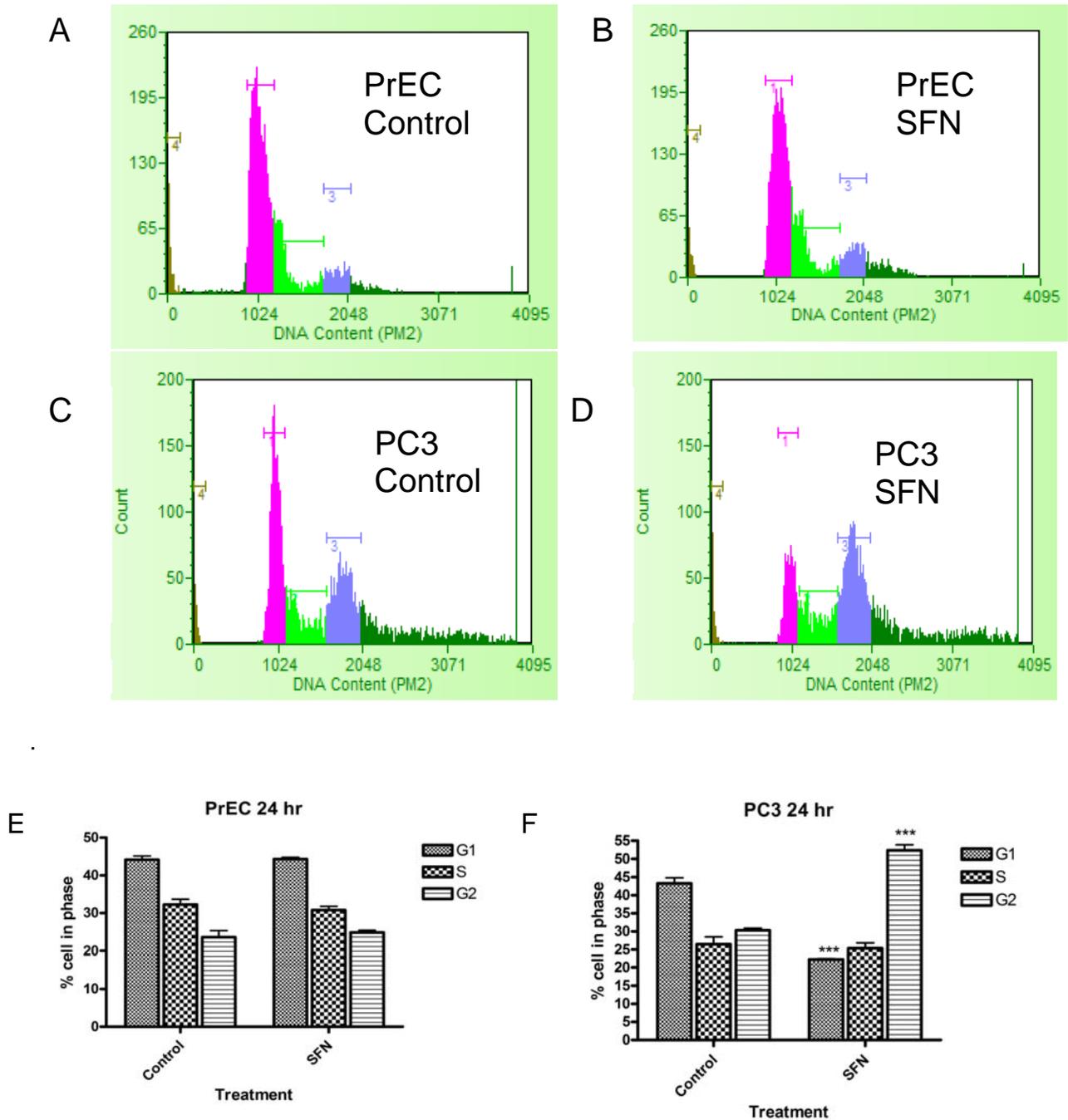


Figure 3. SFN induces cell cycle arrest in PC3 cancer cells, but not in normal prostate epithelial cells (PrEC). PrEC and PC3 cells were treated with 0 or 15 μ M SFN for 24 hrs. Cells were harvested, fixed, and stained for cell cycle. No change in cell cycle was seen in PrEC cells treated with DMSO vehicle (A) or SFN (B) and (E). In contrast a marked G2/M arrest was apparent in PC3 cells treated with SFN (D) compared to control (C) and (F). Histograms are representative of findings from three separate experiments.

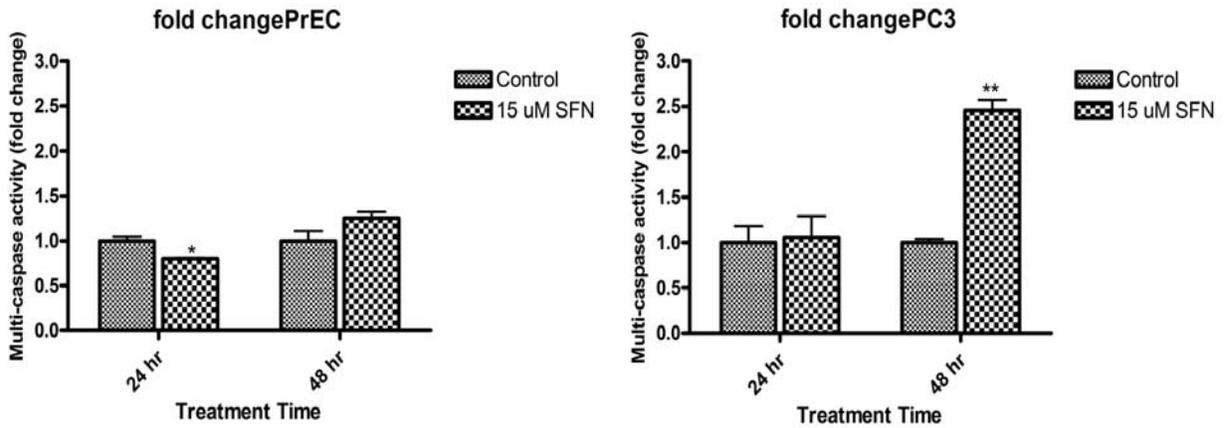


Figure 4. SFN induced apoptosis in PC3 cells. Cells were harvest 24hr and 48hr after treatment with 0 or 15 μ M SFN and attached cells were examined for multi-caspase activity using Guava PCA. Results represent mean \pm SEM, n=3. *, p<0.05; **, p<0.01.

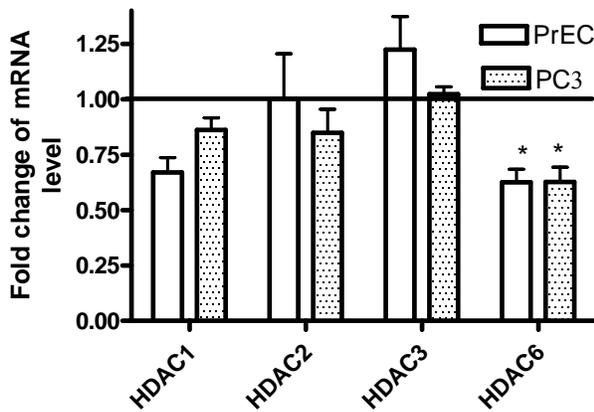


Figure 5. Fold changes of mRNA level of HDACs levels of SFN treated PrEC and PC3 (15 μ M, 12hr) compared to control (DMSO, level set as 1.0). Results = mean \pm SEM, n=3. * p< 0.05.

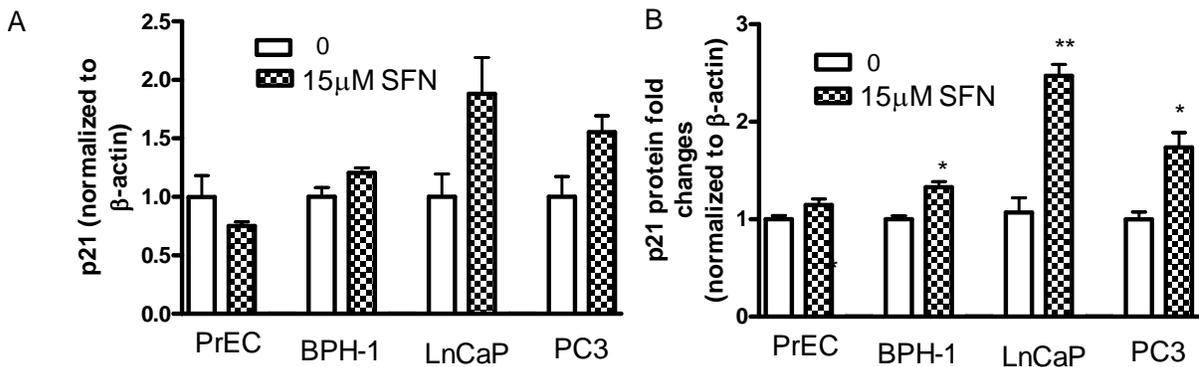


Figure 6. SFN increased p21 mRNA (A) and protein (B) level in prostate cancer cells only. Cells were treated with 15mM SFN for 12hr and isolated for RT-PCR analysis, 24hr later (BPH-1: 48hr later) for westernblot. Results = mean \pm SEM, n=3. mRNA and protein level is compared to different control treatment cells, respectively. * p<0.05; ** p<0.01.

3. SFN decreased dnmt1 expression in PC3 cells.

DNA methyltransferase (DNMT) activity is elevated during prostate cancer and many genes involved in cell cycle arrest, DNA damage repair and carcinogen detoxification are commonly hypermethylated and silenced during prostate cancer. Both DNA hypermethylation and histone modifications are closely related. To determine whether SFN is a DNMT inhibitor, we treated normal prostate cells (PrEC) and cancerous prostate cells (PC3) with SFN. DNMT1 is highly expressed in PC3 compared to PrEC cells (> 3 folds expression), which was also seen in prostate cancer tissue. SFN treatment significantly reduced the DNMT1 expression in PC3 cells. The mRNA expression of dnmt1 was down to similar level of normal cells (Figure 7). We also noticed that transcription level of dnmt 3a and dnmt 3b also decreased by SFN (data not shown). The identification of dietary agents, such as SFN, that target HDAC inhibitor and/or DNA methylation, with few side effects could make a significant impact on prostate chemoprevention.

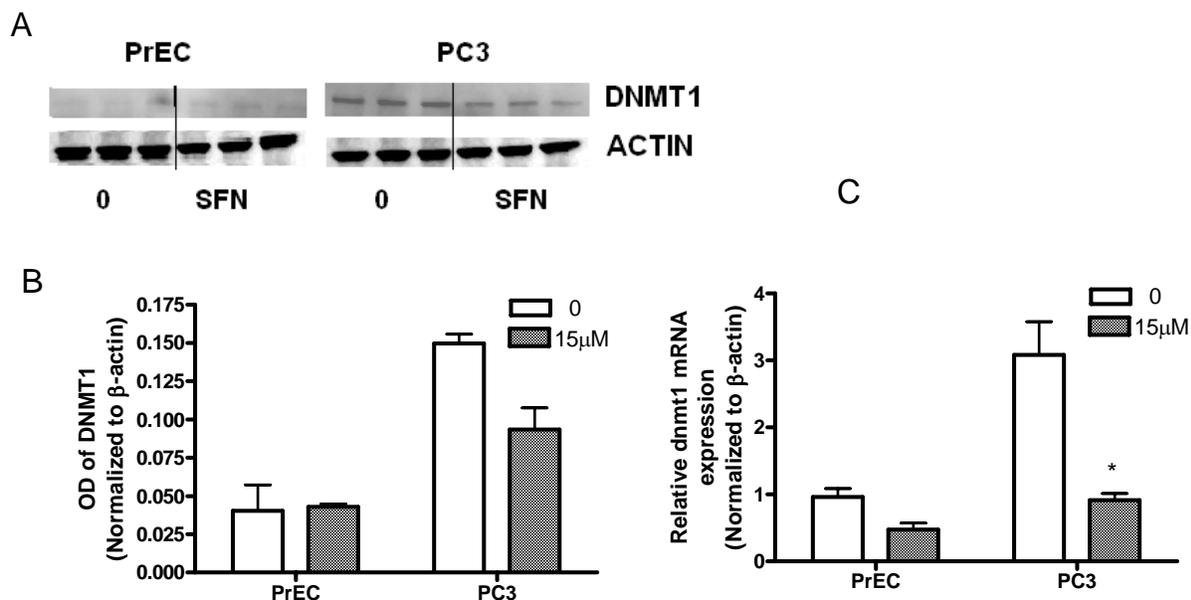


Figure 7. SFN decrease the DNMT1 expression in PC3 cells. (A+B), PrEC and PC3 cells were treated with 15 μM SFN and harvested 48hr later and cell lysates were analyzed by western blot. (C), real-time PCR results for dnmt1 mRNA expression after 12hr treatment. Results = mean ± SEM, n = 3. * p < 0.05.

Key Research Accomplishment

1. SFN induced cell death, cell cycle arrest, apoptosis and reduced HDAC activity specifically in prostate cancer cell but not in normal prostate epithelial cells.
2. SFN treatment reduced HDAC6 mRNA leveling both normal and prostate cancer cells. SFN causes specific increasing of p21 in mRNA and protein levels in prostate cancer cells.
3. SFN decreased dnmt1 expression in PC3 cells.

Reportable outcomes

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

Poster presentation on AICR annual research conference on Food, Nutrition, Physical activity, and Cancer. November 6-7, 2008 Washington DC.

Poster presentation on NCI Translational Science Meeting. November 7-9, 2008, Washington DC.

Data from this grant has been used for preliminary data for additional pending PO1 research grant "Comparative mechanism of chemoprevention" to be submitted June 2009.

Conclusion

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. Our data support our hypothesis. SFN, similar to other HDAC inhibitors, exerts differential effects on HDAC activity and downstream targets in normal and cancer cells, and subsequently exerts selective toxicity in cancer cells. The next step will be further exploration the mechanistic consequences of HDAC inhibition by SFN. We'll also want to investigate the effect of SFN on DNA methyltransferase expression and aberrant hypermethylation patterns in prostate cancer cells and the potential coordination between DNA methylation and HDAC inhibition by SFN leading to depression of gene expression.

One of the specific aims of this proposal is to test the hypothesis that dietary SFN mediates epigenetic alterations and suppresses prostate cancer development in a novel APC-mutant mouse model of prostate carcinogenesis. We noticed that this model does not closely mimic the human condition, as mutations in Apc are rare in human prostate cancer. Thus, we have modified the experimental design to use the Transgenic Adenocarcinoma Model for Prostate cancer (TRAMP) model. The experiments are conducting at the OHSU by our collaborators. Due to breeding difficulty and time limitations, we don't have any data in hand now. However, cohorts of animals are currently being fed SFN diet.

Another specific aim is to test the hypothesis that SFN inhibits HDAC activity and increases accumulation of acetylated histones in humans following dietary consumption of broccoli sprouts. We have recruited the subjects and conducted the study. The samples are under the analysis.

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Appendices

Curriculum vitae of Zhen Yu

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Thesis title: Antimutagenic potency of wheat grain and berry extracts *in vitro* and anticarcinogenicity of wheat grain *in vivo*

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Professional positions

2007 – present Postdoctoral researcher, Department of Nutrition and Exercises, Oregon State University

2006- 2007 Postdoctoral researcher, Department of Environmental and Molecular Toxicology, Oregon State University

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1996-1999 Graduate Research Assistant, State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Science, Chinese Academy of Sciences. Focus on the etiologic hypothesis of Kashin-Beck Disease, an endemic osteoarthrophy in China.

1994-1996 Laboratory Technician and Assistant Environmental Engineer, Environmental Protection Agency (EPA) of Yantai, Shandong Province, P. R. China.

Awards

1999-2001 Linus Pauling Institute Graduate Fellowship

2005 Society of Toxicology Graduate Travel Award

Professional Affiliations

Associate Member of Society of Toxicology

Associate Member of American Associates of Cancer Research

Publications

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.

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Presentations

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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. Anaheim, 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.

Appendices: Abstracts for the meeting

1. Clarke J., YU Z., and Ho E.. (2008) Differential effects on HDAC inhibition, cell cycle arrest and apoptosis by sulforaphane in normal and prostate cancer cells.

Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables that is particularly abundant in broccoli and broccoli sprouts. Epidemiological data indicates that a diet high in cruciferous vegetables may lower the overall risk of prostate cancer. A novel pathway by which SFN acts as a suppressing agent in prostate cancer chemoprevention is by targeting epigenetic alterations, namely the ability of SFN to inhibit histone deacetylase (HDAC) enzymes. Pharmacological HDAC inhibitors are being tested in clinical trials and have similar anticancer properties as SFN, such as changes in histone acetylation at promoters such as p21 and induction of apoptosis and cell cycle arrest. One characteristic of pharmacological HDAC inhibitors is that they are preferentially cytotoxic to cancer cells rather than normal cells, however the mechanisms for this selectivity is unclear. The goal of this study was to characterize effects of SFN on HDAC, cell cycle arrest and apoptosis in both normal prostate epithelial cells (PrEC) and prostate cancer cells (PC3). Similar to pharmacological HDAC inhibitors, we found an induction of cell cycle arrest and apoptosis in PC3 cells but no change in PrEC cells. In PC3 cells we also observed a reduction in HDAC activity and an induction of p21 mRNA and protein, with only a transient reduction in HDAC activity and no change in p21 levels in PrEC. Chromatin-immunoprecipitation (ChIP) assays were also performed to identify alterations in acetylated histones at specific promoter sites. From these data we conclude that SFN, similar to other HDAC inhibitors, exerts differential effects on HDAC activity and downstream targets in normal and cancer cells, and subsequently exerts selective toxicity in cancer cells. The identification of dietary HDAC inhibitors and their use either alone or in combination, may increase efficacy of anti-cancer therapies/prevention strategies for prostate cancer, without serious side effects.

2. Emily Ho and Roderick H. Dashwood (2008) Dietary histone deacetylase inhibitors for cancer prevention: from cells to mice to man.

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables, such as broccoli and broccoli sprouts. This anticarcinogen was first identified as a potent inducer of Phase 2 detoxification enzymes, but evidence is mounting that SFN acts through various other mechanisms. SFN has been shown to inhibit histone deacetylase (HDAC) activity in human colon and prostate cancer lines, with an increase in global and local histone acetylation status, such as on the promoter regions of *P21* and *bax* genes. SFN also inhibited the growth of prostate cancer xenografts and spontaneous intestinal polyps in mouse models, with evidence

for altered histone acetylation and HDAC activities in vivo. In human subjects, a single ingestion of 68 g broccoli sprouts inhibited HDAC activity in circulating peripheral blood mononuclear cells 3-6 h after consumption, with concomitant induction of histone H3 and H4 acetylation. These findings provide evidence that one mechanism of cancer chemoprevention by SFN is via epigenetic modulation of HDACs. Other dietary agents such as butyrate, biotin, lipoic acid, garlic organosulfur compounds, and metabolites of vitamin E have structural features compatible with HDAC inhibition. Pharmacological HDAC inhibitors have shown promise as anti-cancer agents and are currently in human clinical trials. The ability of dietary compounds to de-repress epigenetically-silenced genes in cancer cells, and to epigenetically prime these genes in normal cells, has important implications for cancer prevention and therapy using easily accessible foods with fewer side-effects as compared with potent HDAC inhibitor drugs. Supported by NIH grants CA107693, CA122906, CA090890, CA122959, and by Environmental Health Sciences Center grant P30 ES00210, from the National Institute of Environmental Health Sciences.