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CONTRACTING ORGANIZATION: Oregon State University
Corvallis, OR 97331-8507

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9. **ABSTRACT**  
The central hypothesis for this research project is that sulforaphane acts as an HDAC and DNMT1 inhibitor in the prostate, causing enhanced histone acetylation and DNA demethylation at specific promoters, and induction of cell cycle arrest/apoptosis in cancer cells, leading to cancer prevention. To test this hypothesis we characterized the effects of SFN in normal (PrEC), benign hyperplasia (BPH1) and cancerous (LnCap and PC3) prostate epithelial cells. We observed that SFN selectively induced cell cycle arrest and apoptosis in BPH1, LnCap and PC3 cells but not PrEC cells. SFN treatment also decreased HDAC activity, decreased specific HDAC proteins, increased acetylated histone H3 at the promoter for P21, induced p21 expression and increased tubulin acetylation. In PrEC cells, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. From these data we conclude that SFN exerts differential effects on HDAC activity and downstream targets in normal and cancer cells. We have also employed a rodent model of prostate cancer to determine the effects of SFN on the epigenetic endpoints mentioned above. Histopathology analysis revealed that SFN reduced the severity of prostate cancer in this rodent model.

10. **SUBJECT TERMS**  
apoptosis, chemoprevention, epigenetics, isothiocyanate, prostate cancer

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Introduction:
Prostate cancer is the second leading cause of cancer related death in American men, and the American Cancer Society estimates that in 2010 prostate cancer will be the most frequently diagnosed cancer in the U.S. Epidemiological data indicates that one third of the cancer related deaths expected to occur in 2008 will be linked to lifestyle, namely overweight or obesity, inactivity, and nutrition. Supporting the idea that diet can affect cancer risk, other epidemiologic studies suggest that cruciferous vegetable intake may lower overall risk of prostate cancer, particularly during the early stages. There is growing interest in identifying the specific chemoprotective constituents of cruciferous vegetables and their mechanisms of action. Sulforaphane (SFN) is a phytochemical found in cruciferous vegetables and is well known for its chemopreventive properties. One mechanism of SFN chemoprevention that has gained increasing attention is the ability of SFN to inhibit histone deacetylase (HDAC) enzymes. SFN mediated HDAC inhibition leads to an increase in acetylated histones, de-repression of cell cycle arrest and apoptosis genes, and ultimately cell death. Other closely structurally related phytochemicals act as both HDAC inhibitors and DNA methyltransferase inhibitors indicating that SFN may have similar capabilities. Interestingly, HDAC inhibitor mediated cell cycle arrest and apoptosis occurs preferentially in cancer cells but not normal cells. The central hypothesis for this research is that sulforaphane will act as an HDAC and DNMT1 inhibitor in the prostate, causing enhanced histone acetylation and DNA demethylation at specific promoters, and induction of cell cycle arrest/apoptosis in cancer cells, leading to cancer prevention.

Body:
Task 1: Previously PrEC and PC3 cells were treated with SFN or a vehicle control and harvested for chromatin immunoprecipitation (ChIP) using antibodies that recognize acetylated histone H3 and H4. Due to unforeseeable technical difficulties, the previously treated samples could not be used and a new experiment was required. Cells have been treated again and harvested for analysis using Solexa 1G Genome Analyzer processing.

Task 2: We characterized the effects of SFN in normal (PrEC), benign hyperplasia (BPH1) and cancerous (LnCap and PC3) prostate epithelial cells. We observed that SFN selectively induced cell cycle arrest and apoptosis in BPH1, LnCap and PC3 cells but not PrEC cells (Figures 1 and 2). SFN treatment also decreased HDAC activity (Figure 3A), decreased specific HDAC proteins (Figure 3B and 3C and Table I), increased acetylated histone H3 at the promoter for P21 (Figure 4A), induced p21 expression (Figure 4B) and increased tubulin acetylation (Figure 4C). In PrEC cells, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. From these data we conclude that SFN exerts differential effects on HDAC activity and downstream targets in normal and cancer cells.
Table I. Effect of SFN treatment on HDAC protein level (fold change)

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<td>0.72±0.05*</td>
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Data are mean ± SEM (n=3). Values are represented as fold change after SFN treatment at each time point for each HDAC. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 versus respective control using Student’s t-test.

Task 3: The treatment phase of the TRAMP mouse study is complete. Tissues have been either flash frozen for epigenetic analysis or fixed for pathology. In this study, dietary SFN, fed as freeze-dried broccoli sprouts, increased SFN content in the prostate (Figure 5A) and decreased the severity of prostate cancer at 12 and 28 weeks of age (Figure 5B). Immunohistochemistry and western blot analysis for epigenetic endpoints in mouse prostates from this study is underway.

Key Research Accomplishments:
- PC3 and PrEC cell lines have been treated and DNA has been immunoprecipitated for epigenomic analysis. Samples are currently being processed.
- SFN is selectively cytotoxic for hyperplastic and cancerous prostate cells, not normal cells.
- SFN also selectively reduces HDAC activity and specific HDAC proteins with concomitant increases in targets for acetylation in the hyperplastic and cancerous cells but not in the normal cells.
- All mice have been treated and tissues have been collected for the dietary SFN study in the TRAMP mouse model. SFN reduced the severity of prostate cancer at 12 and 28 weeks of age. Immunohistochemistry for epigenetic endpoints is underway.
- Several manuscripts have either been accepted in peer-reviewed journals.
- Multiple conference presentations have been given regarding research accomplished under this training award.
Reportable Outcomes:

Publications (see Appendix 3 for full texts)

Conference poster presentation (see Appendix 4 for abstracts):

Conference oral presentation published abstract (see Appendix 5 for full presentation):

Presentations:
Nutrition and Exercise Sciences seminar (April 2010)
“Cancer Prevention with Broccoli – from Cellular to Human Studies”
Cancer chemoprevention seminar (December 2009)
“Dietary sulforaphane as an efficacious anti-cancer agent”

Awards:
Diet and Cancer Research Interest Group Poster Presentation Award Winner (2011)
Experimental Biology Conference
American Society for Nutrition
Dietary Bioactive Components Research Interest Group Poster Presentation Award Winner (2011)
Experimental Biology Conference
American Society for Nutrition
Oregon Lottery Graduate Scholarship (2010-2011)
Graduate School
Oregon State University
Betty E. Hawthorne Fellowship (2010-2011)
College of Health and Human Sciences
Oregon State University
Helen Charley Fellowship (2010-2011)
Nutrition and Exercise Sciences
Oregon State University
Office of Dietary Supplements Practicum Travel Award Winner (2010)
Conclusion:
The work performed under this training award is valuable on the merit of scientific and professional accomplishments thus far. The scientific accomplishments include characterizing the effects of SFN on epigenetic and cell fate endpoints in the context of multiple prostate cell lines, from normal to cancerous. Furthermore, SFN was able reduce the severity of prostate cancer. The professional accomplishments include the training I have received during the course of this project and the collaborations that have taken place. I have also been able to present the data at several meetings and network and collaborate with fellow scientists. The work performed here is significant because it provides further evidence that SFN and more generally cruciferous vegetables may help prevent prostate cancer through epigenetic mechanisms.
Appendix 1: Figures

Figure 1

[Bar chart showing multi-caspase activity (Fold change) comparison between Control and 15 μM SFN in 24 hr and 48 hr for different cell lines: PEC, BPH1, LNCap, and PC3.]

Figure 2

[Bar chart showing % cells in phase (G1, S, G2/M) comparison across 24 hr and 48 hr for different cell lines: PrEC, BPH1, LNCap, and PC3 under Control and 15 μM SFN conditions.]

Figure 3

A

![Graph showing HDAC activity for different cell types and time points.]

B

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Figure 5

(A) Individual metabolites

(B) 12 wks old (on diet for 8 wks) vs 28 wks old (on diet for 24 wks)

Cancer severity
- Standard diet
- 15% Broccoli sprouts diet
Appendix 2: Legends to figures

**Figure 1**: SFN preferentially induces apoptosis in BPH1, LnCap and PC3 cells. Cells were treated with DMSO (control) (white bars) or 15 μM SFN (black bars) for 24 and 48 h and harvested for apoptosis analysis. Flow cytometry assay for apoptosis as indicated by multicaspase activity. Data in graphs represent mean ± SEM (n=3). Statistical significance: **p<0.01 versus control using Student’s t-test.

**Figure 2**: SFN preferentially induces cell cycle arrest in BPH1, LnCap and PC3 cells. Cells were treated with DMSO (control) or 15 μM SFN for 24 and 48 h and harvested for cell cycle analysis. Distribution of cells (in percentage) in the G1 (white bars), S (black bars), and G2/M (grey bars) phases of the cell cycle. Data in bar graphs represent mean ± SEM (n=3). Statistical significance: *p<0.05, **p<0.01, ***p<0.001 versus respective control using Student’s t-test.

**Figure 3**: SFN selectively reduces HDAC activity and several class I and class II HDAC proteins in BPH1, LnCap and PC3 but not normal PrEC prostate cells. Cells were treated with DMSO (control) (white bars) or 15 μM SFN (black bars) for the time indicated and harvested for HDAC activity or western blots. (A) HDAC activity was measured at 24 and 48 h in arbitrary fluorescence units (AFU) using a fluorescence kit as described in materials and methods. Western blot of class I (B) and class II (C) HDACs at 24 and 48 h (see Table I for densitometry). Data in bar graphs represent mean ± SEM (n=3). Statistical significance: *p<0.05, **p<0.01, ***p<0.001 versus respective control using Student’s t-test.

**Figure 4**: SFN treatment selectively induces histone H3 acetylation at the P21 promoter, p21 protein and tubulin acetylation in BPH1, LnCap and PC3 but not normal PrEC prostate cells. Cells were treated with DMSO (control) (white bars) or 15 μM SFN (black bars) for the time indicated and harvested for ChIP or western blots. (A) ChIP for acetylated histone H3 at the P21
promoter at 48 h. For normalization between samples the immunoprecipitated DNA was expressed as percentage of input DNA ($2^{\text{CT input} - \text{CT IP}} \times 10$) and shown as fold change compared to control treatments. TSA was included as a positive control for histone acetylation (data not shown). (B) Densitometry and western blots for p21 protein at 24 h. (C) Western blot and densitometry of acetylated tubulin and total tubulin at 24 and 48 h. Data in bar graphs represent mean ± SEM (n=3). Statistical significance: *p<0.05 versus respective control using Student’s t-test.

**Figure 5:** Dietary SFN results in SFN metabolites in prostate and slows the progression of prostate cancer in TRAMP mice. (A) Liver, kidney, colon and prostates were collected from mice fed standard diet and mice fed a 15% broccoli diet and processed according to the procedure in the Materials and Methods section (n=3). No SFN compounds were detected in the control mice. (B) Prostate cancer severity in mice fed standard diet and 15% broccoli diet (n=20 in each group). Range of cancer severity: 1) normal, 2) prostatic intraepithelial neoplasia, 3) cribriform, 4) early adenoma 5) moderate adenoma, 6) poorly differentiated adenoma.
Dietary Sulforaphane, a Histone Deacetylase Inhibitor for Cancer Prevention

Emily Ho,1,4,5,6 John D. Clarke,5,6 and Roderick H. Daykwood1,3,7

1Department of Nutrition and Exercise Sciences, 2Linus Pauling Institute, 3Molecular and Cellular Biology Program, and 4Department of Environmental & Molecular Toxicology Oregon State University, Corvallis OR 97331

Abstract

The reversible acetylation of histones is an important mechanism of gene regulation. During prostate cancer progression, specific modifications in acetylation patterns on histones are apparent. Targeting the epigenome, including the use of histone deacetylase (HDAC) inhibitors, is a novel strategy for cancer chemoprevention. Recently, drugs classified as HDAC inhibitors have shown promise in cancer clinical trials. We have previously found that sulforaphane (SNF), a compound found in cruciferous vegetables, inhibits HDAC activity in human colorectal and prostate cancer cells. Based on the similarity of SNF metabolites and other phytochemicals to known HDAC inhibitors, we previously demonstrated that sulforaphane acted as an HDAC inhibitor in the prostate, causing enhanced histone acetylation, derepression of P21 and Bax, and induction of cell cycle arrest, apoptosis, leading to cancer prevention. The ability of SNF to target aberrant acetylation patterns, in addition to effects on phase 2 enzymes, may make it an effective chemoprevention agent. These studies are important because of the potential to qualify or change recommendations for high-risk prostate cancer patients and thereby increase their survival through simple dietary changes incorporating easily accessible foods into their diet. These studies also will provide a strong scientific foundation for future large-scale human clinical intervention studies. J. Nutr. 139: 2383-2390, 2009.

Introduction: Epigenetics and cancer development

Epigenetics is the study of the regulation of gene activity that is not dependent on nucleotide sequence; this may include heritable changes in gene activity and expression but also long-term alterations in the transcriptional potential of a cell that are not heritable. These features are potentially reversible and may affect genomic stability and expression of genes. In recent years, epigenetics researchers have made great strides in understanding the many molecular sequences and patterns that determine which genes can be turned on and off. This work has made it increasingly clear that in addition to genetic changes, the epigenome is just as critical as the DNA to healthy human development. More importantly, dietary factors and specific nutrients can modulate epigenetic alterations and alter susceptibility to disease. The classic view of cancer etiology is that genetic alterations (via genotoxic agents) damage DNA structure and induce mutations resulting in nonfunctional proteins that lead to disease progression. More recently, the role of epigenetic alterations during development and chronic disease development has gained increasing attention and has resulted in a paradigm shift in our understanding of mechanisms leading to disease susceptibility. A major focus in this review is the identification of dietary agents that target histone modifications and the mechanisms leading to cancer prevention.

Use of histone deacetylase inhibitors in prostate cancer

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 (Fig. 1). A tightly regulated balance exists in normal cells between HAT and HDAC activities, and when this balance is disrupted, cancer development can ensue. The HDACs can be divided into 3 types...
RESEARCH ARTICLE

Differential effects of sulforaphane on histone deacetylases, cell cycle arrest and apoptosis in normal prostate cells versus hyperplastic and cancerous prostate cells

John D. Clarke1,2, Anna Hsu2, Zhen Yu2, Roderick H. Dashwood3,4 and Emily Ho2,3

1Molecular and Cellular Biology Program Oregon State University, Corvallis, OR, USA
2Department of Nutrition and Exercise Sciences Oregon State University, Corvallis, OR, USA
3Linus Pauling Institute, Oregon State University, Corvallis, OR, USA
4Department of Environmental and Molecular Toxicology Oregon State University, Corvallis, OR, USA

Scope: Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables such as broccoli. The ability of SFN to inhibit histone deacetylase (HDAC) enzymes may be one mechanism by which it acts as a chemopreventive agent. The ability of a chemopreventive agent to specifically cause cytostasis in cancer and not normal cells is an important factor in determining its safety and clinical relevance.

Methods and results: We characterised the effects of SFN in normal (PrEC) benign hyperplasia (BPH1) and cancerous (LNCap and PC3) prostate epithelial cells. We observed that 15 μM SFN selectively induced cell cycle arrest and apoptosis in BPH1, LNCap and PC3 cells but not PrEC cells. SFN treatment also selectively decreased HDAC activity, and Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for p21, induced p21 expression and increased tubulin acetylation in prostate cancer cells. HDAC6 over-expression was able to reverse SFN-induced cytotoxicity. In PrEC cells, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. The differences in sensitivity to SFN in PrEC and PC3 are likely not due to differences in SFN metabolism or differences in phase 2 enzyme induction.

Conclusion: SFN exerts differential effects on cell proliferation, HDAC activity and downstream targets in normal and cancer cells.

Keywords:
Apoptosis / Chemoprevention / Epigenetics / Isothiocyanate / Prostate cancer

1 Introduction

Epidemiologic studies suggest that cruciferous vegetable intake may lower the overall risk of prostate cancer [1, 2]. Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables such as broccoli and broccoli sprouts [3]. The majority of chemoprevention studies have focused on the ability of SFN to act as a potent phase 2 enzyme inducer via Keap1-Nrf2 signaling and antioxidant response element (ARE)-driven gene expression. Additional evidence also suggests that SFN suppresses tumor development during the “post-initiation” phase of cancer via induction of cell cycle arrest and apoptosis [4, 5]. Recently, a novel suppression mechanism involving the ability of SFN to inhibit histone deacetylase (HDAC) enzymes, alter histone acetylation and affect gene regulation has been reported [6–8].

Lysine acetylation and deacetylation is a dynamic process executed by histone acetyltransferases and HDACs that can
Appendix 4: IMPACT Conference poster presentation abstract:
a) Background- Sulforaphane (SFN) is a phytochemical found in cruciferous vegetables and is well known for its chemopreventive properties. Epidemiological data indicate that a diet high in cruciferous vegetables may lower the overall risk of prostate cancer. One mechanism of SFN chemoprevention that has gained increasing attention is the ability of SFN to inhibit histone deacetylase (HDAC) enzymes. Pharmacological HDAC inhibitors are reported to preferentially induce cell cycle arrest and apoptosis in cancer cells but not normal cells.

Objective 1: Using cultured cells, determine if SFN targets epigenetic alterations specifically in cancer cells that lead to a decrease in cell proliferation. To test the hypothesis that SFN selectively targets cancer cells similar to pharmacological HDAC inhibitors, we characterized the effects of SFN in normal (PrEC), benign hyperplasia (BPH1) and cancerous (LnCap and PC3) prostate epithelial cells. Objective 2: Investigate the effects of diets rich in SFN on epigenetic markers in the prostate in vivo in a rodent model of prostate cancer.

(b) Methodologies- For objective 1, multiple prostate epithelial cell lines were used including prostate epithelial prostate cells (PrEC), benign prostate hyperplasia cells (BPH1), androgen responsive cells (LnCap) and androgen independent cells (PC3). Cells were treated with either SFN or a vehicle. Cell cycle arrest, apoptosis markers, HDAC activity and expression, p21 expression and chromatin immunoprecipitation (ChIP) were performed. For objective 2, weanling 4-week old transgenic adenoma of the mouse prostate (TRAMP) mice have been assigned into one of two dietary groups: Control-AIN93G diet or Broccoli-sprout supplemented diet (20%). At 12 and 28 weeks of age the following parameters will be evaluated: prostate cancer histopathology, HDAC activity, acetylated histone and p21 expression, in vivo ChIP, and apoptosis and cell proliferation markers.

(c) Results to date- We observed that 15 µM SFN selectively induced cell cycle arrest and apoptosis in BPH1, LnCap and PC3 cells but not PrEC cells. SFN treatment also decreased HDAC activity, decreased both Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for P21, induced p21 expression and increased tubulin acetylation. In PrEC cells, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. In vivo dietary chemoprevention studies in TRAMP mice are ongoing.

(d) Conclusions- From these data we conclude that SFN is an effective prostate chemoprevention agent and exerts selective cytotoxicity to prostate cancer cells. Mechanisms for selectivity may be related to differential effects on HDAC activity and downstream targets in normal and cancer cells.

(e) Impact- The use of epigenetic modulators in cancer treatment has become an intense area of research. HDAC inhibitors or other anti-cancer drugs have potent anti-cancer activity, but also have several associated side-effects. The identification of dietary HDAC inhibitors and their use either alone or in combination, may increase efficacy of anti-cancer therapies/prevention strategies, without ill side effects. Here an in-depth analysis of HDAC inhibition in response to the dietary agent SFN is being investigated. This work contributes to the growing body of evidence to indicate that a diet rich in cruciferous vegetable can reduce the risk of prostate cancer.

Experimental Biology Conference poster presentation abstract:
Epidemiological data indicate that a diet high in cruciferous vegetables may lower the overall risk of prostate cancer. The ability of sulforaphane (SFN), an isothiocyanate derived from cruciferous vegetables, to inhibit histone deacetylases (HDAC) may be one mechanism by which
it acts as a chemoprevention agent. Interestingly, the antiproliferative effects of pharmacological HDAC inhibitors are specific to cancer cells. The objective of this study was to compare the effects of SFN on HDAC inhibition and downstream molecular targets in both normal (PrEC) and cancerous (PC3) prostate epithelial cells. We hypothesize that SFN will act similar to pharmacological HDAC inhibitors and selectively target cancer cells. In PC3, SFN decreased HDAC activity, decreased HDACs 3, 6, and 8, increased acetylated histone H3 at the promoter for p21, induced p21 expression and increased tubulin acetylation. These data suggest that SFN targets both Class I and II HDACs in prostate cancer cells. In PrEC, SFN caused only a transient reduction in HDAC activity with no change in any other endpoints tested. SFN also induced selective cell cycle arrest and apoptosis in PC3 but not PrEC. We conclude that SFN exerts differential effects on HDAC activity and downstream targets in normal and cancer cells. Together, these data highlight the potential of SFN, a dietary HDAC inhibitor as an effective chemoprevention agent.
Differential Effects of Sulforaphane on Histone Deacetylases, Cell Cycle Arrest and Apoptosis in Normal and Cancerous Prostate Cells

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