

ENRICHMENT OF TOMATOES AND BROCCOLI WITH SPECIFIC BIOACTIVES FOR  
THE REDUCTION OF PROSTATE CARCINOGENESIS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Nutritional Sciences  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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## ABSTRACT

Epidemiological studies have linked high consumption of tomatoes and cruciferous vegetables to decreased risk of prostate cancer. Several bioactive components isolated from cruciferous vegetables and tomatoes exhibit anti-cancer properties. Previous studies have evaluated the cancer preventive potential of these individual bioactives, but few have examined them within the context of a whole food.

In a pilot study to evaluate bioactivity of different tomato and broccoli powders, male Copenhagen rats were fed diets containing 10% standard tomato powder, tomato enriched with lycopene or total carotenoids, standard broccoli floret, broccoli sprouts, or broccoli enriched with indole glucosinolates or selenium for 7 days. All broccoli diets increased activity of colon quinone reductase (NQO1). Indole glucosinolate-enriched broccoli and selenium-enriched broccoli increased hepatic NQO1 and cytochrome P450 1A activity ( $P < 0.05$ ). Different tomato diets resulted in altered hepatic accumulation of lycopene, phytofluene, and phytoene. These results demonstrate that the bioactive content of vegetables affects both tissue content of bioactives and activity of detoxification enzymes. Enhancing bioactive content of tomatoes and broccoli may enhance efficacy in the prevention of prostate cancer.

Based on the results of this pilot study, our next objective was to determine if standard broccoli or the indole glucosinolate-enriched (IG) broccoli, would impact prostate carcinogenesis in the aggressive TRAMP model. Male mice were randomized into 3 diet groups at 5-7 weeks of age: AIN-93G control, 10% control broccoli powder, or 10% IG broccoli powder. Diets were fed throughout the study until termination at 20 weeks of age, with no differences in body weight or food intake observed between groups. There were no differences between groups in genitourinary tract weight, a surrogate marker of tumor volume, and no differences were found

in cancer grade upon histopathologic evaluation indicating that broccoli feeding did not impact cancer aggressiveness. The horticultural manipulation of broccoli to alter phytochemical concentration is a feasible approach to optimizing the potential for cancer prevention, yet optimal patterns of phytochemicals remain to be characterized.

To assess potential epigenetic effects of lycopene, we examined the effects of lycopene and its metabolite, apo-10'-lycopenal, on methylation of the GSTP1 promoter in LNCaP cells. GSTP1 is hypermethylated in >90% of prostate cancers, which results in complete silencing of the gene. Neither lycopene nor apo-10'-lycopenal altered mRNA expression or DNA methylation of GSTP1 indicating that lycopene is not an effective demethylating agent for this gene in this particular prostate cancer cell line. It remains to be seen if lycopene has epigenetic effects on other genes or in other cell lines.

Overall we have demonstrated that the bioactive content of tomatoes and broccoli can be altered through agronomic means, but optimal profiles for cancer prevention remain to be determined. Much remains to be learned about how tomatoes and broccoli alter cancer progression at different stages and the mechanisms through which they exert their effects.

## ACKNOWLEDGEMENTS

*In memory of Chia-ling Liu and Siu Feng Tsai*

Thank you to my advisor and mentor, Dr. John Erdman for all his support over the years.

Thank you to Dr. Elizabeth Jeffery for always pushing me out of my comfort zone and making me a better scientist.

Thank you to all the Erdman and Jeffery lab members past and present. Science is most definitely a team effort, and I would never have been able to do all this without your help and advice.

Most of all, thank you to my wonderful husband Ryan.

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# CHAPTER 1

## LITERATURE REVIEW

### Introduction

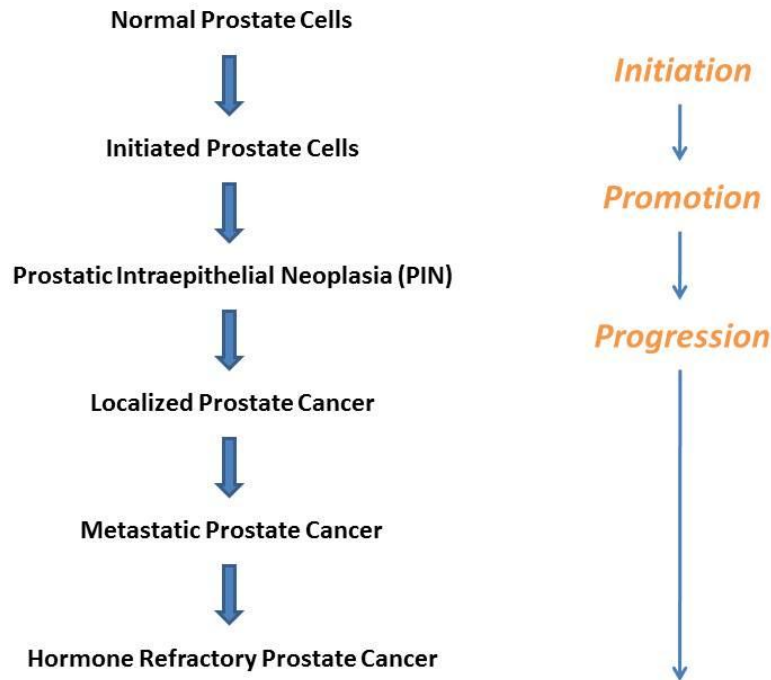
Cancer is a class of over 100 diseases characterized by uncontrolled cell growth. An individual's chances of developing cancer are governed by multiple risk factors including family history, age, race, diet, exercise, and environmental exposures. Many of these risk factors can be modified through individual behavior. In 1980, Doll and Peto estimated that approximately 35% of human cancer deaths could be prevented through dietary modifications (1). This work suggested that the food choices a person makes every day can have profound impacts on his or her risk of developing cancer over a lifetime.

Prostate cancer is the most commonly diagnosed cancer and second leading cause of male cancer deaths in United States with approximately 186,000 new cases diagnosed each year (2). Early diagnosis and intervention is important as metastatic disease accounts for the majority of deaths. Dietary interventions may play an important role in delaying prostate cancer growth, especially because it is a slow growing cancer that takes decades to develop.

### Development of Prostate Cancer

Prostate cancer is mainly a disease of aging with 63% of all prostate cancer cases diagnosed in men over the age of 65 (3). Between the ages of 40 and 65, the risk of developing prostate cancer increases 1000 times (4). However, the premalignant lesions that may lead to prostate cancer appear in men as early as their 20s suggesting that diet has the potential to influence risk from a young age (5).

The pathologic progression of prostate cancer can be classified as follows: normal prostate epithelium, proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN), localized prostate cancer, metastatic prostate cancer, androgen-independent prostate cancer (Figure 1.1).



**Figure 1.1.** Development of human prostate cancer.

PIA is a candidate precursor lesion to prostate cancer characterized by hyperproliferative glandular epithelium with the morphological appearance of atrophy and accompanied by inflammation (6). At this time, its relationship to prostate cancer development is speculative.

PIN is a precursor lesion to prostate cancer and is the earliest accepted stage in prostate carcinogenesis. It is a proliferation of irregular, atypical epithelial cells with progressive disruption of the basal cell layer in the absence of stromal invasion. Duct and gland architecture

remain intact. PIN is generally divided into low-grade and high-grade PIN. High-grade PIN is a standard clinical diagnosis that must be included in pathological evaluations of prostate biopsies. Low grade PIN is often not reported in pathologic evaluations because its utility is limited by high levels of inter-observer variability (7). Approximately 30-40% of men with high grade PIN will have developed prostate cancer upon repeat biopsy (7).

Over 90% of prostate cancers in the United States are discovered in the localized (confined to the prostate) and regional (extending into nearby surrounding tissues) stages where the 5-year survival rate approaches 100% (3). When distant site metastases are found, the 5-year survival rate drops to approximately 30% (2). Autopsy studies indicate that most men over the age of 85 have histological prostate cancer, but these small, localized cancers remain latent and unrecognized for many years (4). Overall 5-year survival rates for men with prostate cancer are 98%, suggesting that men are much more likely to die with prostate cancer than to die from prostate cancer.

Current treatment options for prostate cancer include expectant management, radiation therapy, and surgery. Expectant management (a.k.a. watchful waiting) is appropriate for patients with a life expectancy of less than 10 years and for men 65 years of age or older who have a low-volume, low-grade prostate cancer (8). Patients are followed closely with serum PSA testing and digital rectal exams every six months. Biopsies are performed at frequent intervals. Radiation therapy is an option for patients with localized prostate cancer with various degrees of severity. Radiation beams are focused to the prostate, or radioactive iodine-125 or palladium-103 seeds are implanted directly in the prostate. It is less invasive than surgery and tends to have fewer complications. Radical prostatectomy is a surgical procedure that involves removal of the entire prostate, seminal vesicles, and sufficient surrounding tissue. It can be performed through open



incisions or laproscopically. If the cancer becomes hormone refractory or metastatic, chemotherapy may be used. Dietary intervention would likely be most appropriate for men undergoing expectant management because of the low-grade of the cancer and it would not interfere with any medical therapies.

### **Fruit and Vegetable Consumption in the United States**

It is generally recognized that fruits and vegetables are important components of a healthy diet. The 2005 Dietary Guidelines for Americans recommends that people consume 2 servings of fruit and 3 servings of vegetables per day and that they choose a variety of different fruits and vegetables (9). In spite of this, only 14% of U.S. adults achieve the recommended levels of both fruits and vegetables (10). Tomatoes are the second most commonly consumed vegetable in the American diet second only to potatoes (11). In 2007, the average American consumed 19.2 pounds of fresh tomatoes and 68.8 pounds of processed tomato products. Broccoli is the tenth most consumed vegetable with the average American eating 5.6 pounds of fresh broccoli and 2.7 pounds of frozen broccoli products (total 8.3 pounds) annually. Whereas broccoli intake is substantially less than that of tomatoes, consumption has been gradually increasing since 1970 when the average American only ate 1.5 pounds of broccoli each year (12).

### **Epidemiological Associations Between Tomatoes, Broccoli, and Prostate Cancer**

In recent decades there has been a surge of interest in the role of bioactive phytochemicals in reducing chronic disease risk. Consumption of fruits and vegetables is

important for general health, but it has also been linked to reduced risk of chronic diseases such as cancer (13). However, the literature is not without controversy.

Interest in the role of tomatoes in prostate cancer blossomed after 1995 when Giovannucci et al. published a report from the Health Professional's Follow-up Study, a prospective cohort study of 50,000 American men (14). Their results demonstrated a significantly reduced risk of prostate cancer in men with high intakes of tomato sauce ( $P=0.001$ ), tomatoes ( $P=0.03$ ), and pizza ( $P=0.05$ ). However, a decade later, two different reviews of the epidemiological literature came to two very different conclusions on the role of tomato products in prostate cancer prevention.

In 2007, the FDA published a report of their evidence-based review for qualified health claims regarding tomatoes or lycopene for prostate cancer. After reviewing 81 observational studies examining the relationship between lycopene and various cancers and 64 studies examining the relationship between tomato products and various cancers, the FDA found “no credible evidence to support an association between lycopene intake and reduced risk of prostate cancer [and] very limited evidence to support an association between tomato consumption and reduced risk of prostate cancer” (15). Just a few months later, an international expert panel convened by the World Cancer Research Fund concluded in their report that “foods containing lycopene probably protect against prostate cancer” (13). Additionally they stated: “there is a substantial amount of consistent evidence, in particular on tomato products, both from cohort and case-control studies.” One of the key reasons for this discrepancy in interpretation of the same body of literature may be the lack of definitive randomized human trials. However, it also highlights the need for further research in this area to better address the role of tomato products in preventing prostate carcinogenesis.

In contrast to tomatoes, literature on the relationship between broccoli consumption and prostate cancer risk is quite sparse. The majority of studies look at the relationship between the family of cruciferous vegetables and cancer risk. A 2002 review by Kristal and Lampe evaluated the 12 published studies reporting on the associations between *Brassica* consumption and prostate cancer risk and concluded that “the epidemiological literature provides moderate support for the hypothesis that high intakes of *Brassica* vegetables reduce prostate cancer risk.” (16). More recently, a 2007 report from the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial found that men consuming >1 serving of broccoli per week had a 45% reduction in risk of developing aggressive prostate cancer compared to men consuming <1 serving of broccoli per month (17). While the literature is suggestive of a possible protective effect of cruciferous vegetable consumption, much more research is needed to come to a definitive consensus.

### **Intervention Studies with Tomatoes or Broccoli**

Consumption of tomato juice has been demonstrated to reduce oxidative DNA damage in leukocytes of healthy individuals (18). These results suggest that carotenoid-containing plant products may decrease DNA damage in humans and thus exert cancer-protective effects. To determine how tomato products might act in the prostate, it is first necessary to establish whether compounds of interest such as carotenoids accumulate in the prostate. Clinton et al. demonstrated that the lycopene and  $\beta$ -carotene are the predominant carotenoids found in the prostate, and  $\alpha$ -carotene, lutein, zeaxanthin,  $\alpha$ -cryptoxanthin, and  $\beta$ -cryptoxanthin are all consistently detectable in prostate tissue (19). Though there was large intra-individual variation, an array of carotenoids could be detected in the prostate.

Two small clinical trials have evaluated the effects of tomato-based products in men with prostate cancer. In a phase II clinical trial, Kucuk et al. randomized 26 men with clinically localized prostate cancer to receive either Lyc-O-Mato capsules or no supplement for 3 weeks prior to radical prostatectomy (20). The Lyc-O-Mato capsules contained 15 mg of lycopene, 2.5 mg of phytoene/phytofluene, and small amounts of other carotenoids found in tomatoes. In the intervention group, 73% of subjects had no involvement of surgical margins and/or extraprostatic tissues with cancer compared to 18% in the control group ( $P = 0.02$ ). These results suggest that lycopene may reduce the microscopic extension of prostate cancer and may be useful as an adjunct to standard prostate cancer treatments.

Chen et al. determined the effects of tomato sauce-based pasta dishes on oxidative DNA damage and PSA levels in 32 patients with localized prostate cancer (21). Subjects consumed one tomato sauce-based pasta dish containing approximately 30 mg lycopene per day for 3 weeks. After the intervention period, subjects had significantly increased serum and prostate lycopene levels, decreased leukocyte oxidative DNA damage, and decreased serum PSA levels. While these results appear promising, the study suffered from lack of a control group and a small sample size.

No studies thus far have evaluated the effects of broccoli or broccoli bioactives in prostate cancer patients. Trials have been performed to define the pharmacokinetics of certain broccoli bioactives such as sulforaphane and indole-3-carbinol (22, 23), but thus far no research has evaluated accumulation of these compounds in the prostate. One clinical trial has examined the effects of a broccoli-rich diet on global gene expression in the prostate of men with high grade PIN (24). Twenty-two men with a previous diagnosis of HGPIN (a precursor lesion to prostate cancer) were randomized to consume either 400 g broccoli or 400 g peas per week for

12 months, and prostate biopsies were taken at 6 and 12 months. Men on the broccoli diet had alterations in the TGF $\beta$ 1, EGF, and insulin signaling pathways after 12 months as measured via pathway analysis. Further research is needed to clarify how broccoli consumption alters these pathways and how that in turn affects prostate carcinogenesis.

### **Animal Studies with Tomatoes or Broccoli**

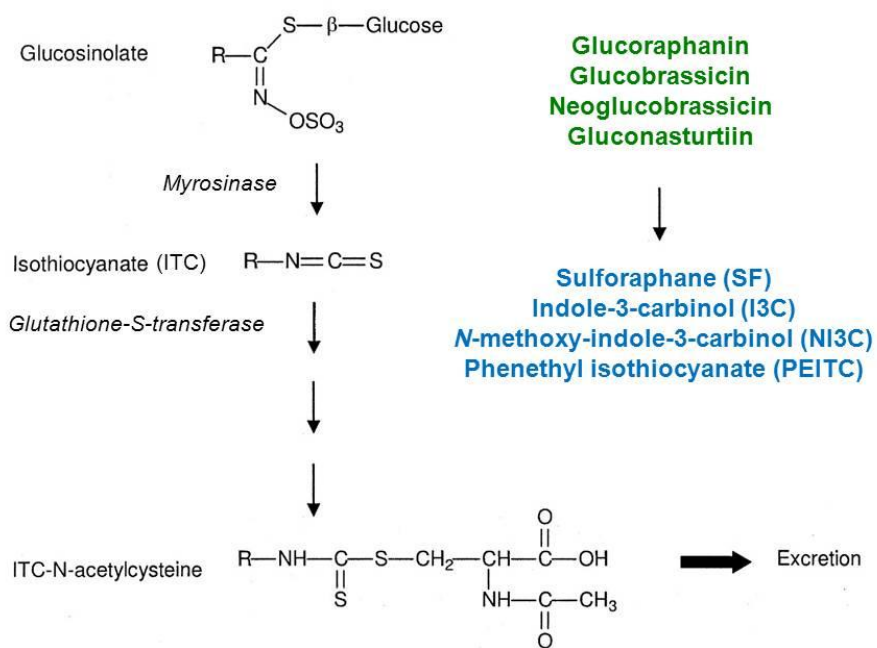
A limited number of animal studies have examined the effects of whole tomatoes or broccoli on prostate carcinogenesis. Our lab has previously shown that whole tomato powder is more effective than lycopene alone in two different animal models of prostate cancer. In a rat model of prostate cancer induced by testosterone and N-nitroso-N-methylurea, rats fed a 10% tomato powder diet lived significantly longer than rats fed control diet or lycopene beadlets (25). Similarly, in the Dunning transplantable tumor rat model, a 10% tomato powder diet significantly reduced tumor weights whereas both low and high levels of lycopene alone did not (26). In contrast, work by Konijeti et al. found that TRAMP mice fed lycopene beadlets had decreased incidence of prostate cancer whereas mice fed tomato paste did not (27). However work by a different group in the same model, found that feeding 10% tomato powder significantly delayed progression from PIN to adenocarcinoma (28). Adding a new insight into the tomato and prostate cancer story, Mossine et al. demonstrated that ketosamines, a class of carbohydrate derivatives present in dehydrated tomato products, may impact tumorigenesis in the highly metastatic rat prostate adenocarcinoma MAT-LyLu (29). The combination of FruHis and lycopene was the most effective in reducing *in vivo* tumor formation suggesting that ketosamines may be non-carotenoid components of tomatoes that contribute to the inhibition of carcinogenesis.

Thus far, only one study has examined the effects of whole broccoli on prostate cancer in an animal model. Canene-Adams et al. demonstrated that a diet containing 10% broccoli powder significantly reduced tumor weights compared to the control group (26). Numerous other studies have examined the effects of individual compounds such as sulforaphane and indole-3-carbinol.

### **Bioactives in Tomatoes and Broccoli**

The health benefits of functional foods are often associated with their bioactive components that are believed to deliver a health benefit beyond basic nutrition. Tomatoes contain carotenoids, which are a class of over 600 lipophilic compounds that impart color to fruits and vegetables. Lycopene is the most abundant carotenoid in tomatoes followed by its colorless precursors, phytoene and phytofluene. Whereas the bioactive properties of lycopene have been extensively studied, little is known about the biological roles of phytoene and phytofluene. Broccoli contains glucosinolates which are a class of phytochemicals abundant in *Brassica* vegetables. Intact glucosinolates are relatively biologically inactive, but when the plant tissue is disrupted glucosinolates undergo enzymatic hydrolysis by myrosinase. Glucosinolates can go on to form many different breakdown products, depending on the reaction conditions, with diverse biological activities (Figure 1.2). Some well-known glucosinolate hydrolysis products include sulforaphane and indole-3-carbinol.

Individual bioactives have been shown to have wide-ranging effects on biological processes. For instance, lycopene in tomatoes may help maintain prostate health, and sulforaphane (SF), a hydrolysis product of glucoraphanin from broccoli may enhance detoxification of carcinogens. The anti-carcinogenic properties of tomatoes and broccoli are often attributed to individual bioactives such as lycopene from tomatoes and sulforaphane and



**Figure 1.2.** Glucosinolate metabolism.

indole-3-carbinol from broccoli. Each of these bioactives has been reported to act through a variety of mechanisms including inhibition of cell cycle progression and induction apoptosis in various cancer cell lines (30-32). Additionally, lycopene is a potent antioxidant and some of its other purported mechanisms of action include increased gap-junctional communication, inhibition of androgen signaling, and inhibition of IGF-1 signal transduction (33). Sulforaphane increases the transcription of genes containing an antioxidant response element (ARE) including several phase II detoxification enzymes. It is also may have anti-inflammatory activities and may act as a histone deacetylase inhibitor (34). Indole-3-carbinol induces both phase I and phase II enzymes and may alter estrogen metabolism and transcription of estrogen responsive genes (35).

Much research has examined the putative health benefits of individual, isolated bioactive compounds. However, in many *in vitro* studies these bioactive compounds are provided at levels tens to hundreds of times greater than can be physiologically achieved through incorporation of whole foods into the diet. Furthermore, these compounds are typically tested as the aglycone form in cell culture, although they are present as glycosides in plant foods and conjugates in the body following absorption. Therefore it is also important to consider the impact of chemical form on bioavailability and bioactivity during dietary interventions *in vivo*. Once the efficacy of functional food components is verified, enhancing the concentrations of health-promoting compounds in foods may be beneficial in preventing and treating chronic diseases such as cancer.

Humans also normally consume tomatoes and broccoli as whole foods whether fresh, frozen, or processed. The whole vegetable contains many other nutrients and phytochemicals which may act additively or synergistically to produce a greater effect than can be accounted for by one bioactive alone. Indeed tomatoes contain high levels of other carotenoids, and they are good sources of potassium, folate, and vitamins A, C and E. Broccoli contains many different glucosinolates, and it is also a good source of dietary fiber, folate, and vitamins A, C and K. Previous work from our lab has shown that whole tomato powder is more effective than lycopene alone in reducing prostate cancer growth in rat models (25, 26).

### **Bioactive Enrichment/Biofortification of Tomatoes and Broccoli**

Biofortification has traditionally been considered the enhancement of micronutrient levels in staple crops through traditional breeding practices and/or genetic modification (36). The main goal has been to alleviate micronutrient deficiencies in the world population especially



for vitamin A, iron, and zinc. One of the most well known examples of a biofortified crop is golden rice, which has higher levels of  $\beta$ -carotene, a vitamin A precursor (37).

In recent years, researchers have begun to investigate whether enhancement of bioactive compounds in food crops can lead to reductions in chronic diseases such as cancer. For reducing the formation of aberrant crypt foci in a chemically-induced rat model of colon cancer, selenium-enriched broccoli was shown to be more effective than high doses of selenite alone or a combination of selenite and broccoli sprout powder low in selenium (38). “Super broccoli” has been bred to have high glucoraphanin levels (10.6 compared to 4.4  $\mu\text{mol/g}$  dry weight in some store-bought broccoli varieties), and it enhances transforming growth factor  $\beta$ 1 signaling which is important for controlling cell proliferation (24). Another study found that a purple anthocyanin-enriched tomato was more effective than regular red tomato in increasing the survival time of Trp53<sup>-/-</sup> mice which predominantly develop lymphomas (39). These studies provide encouraging evidence that enrichment of tomatoes and/or broccoli with bioactives may be beneficial in reducing cancer growth.

### **Mechanisms of Action: Epigenetic Modifications**

Epigenetic changes are defined as heritable changes in gene expression that take place without a change in DNA sequence. The two major types of epigenetic change are modifications to DNA, in the form of DNA methylation, and modifications to chromatin packaging, specifically histone modification. DNA methylation is critical for regulation of gene activity and structure of the cell nucleus. Aberrant DNA methylation patterns are commonly seen in human cancers and often consist of global hypomethylation of DNA accompanied by hypermethylation of specific genes including some tumor-suppressor genes (40). Currently, specific genes such as

glutathione S-transferase P1 (GSTP1) are being investigated to determine if their methylation status can serve as an early and specific biomarker of prostate cancer (41-43).

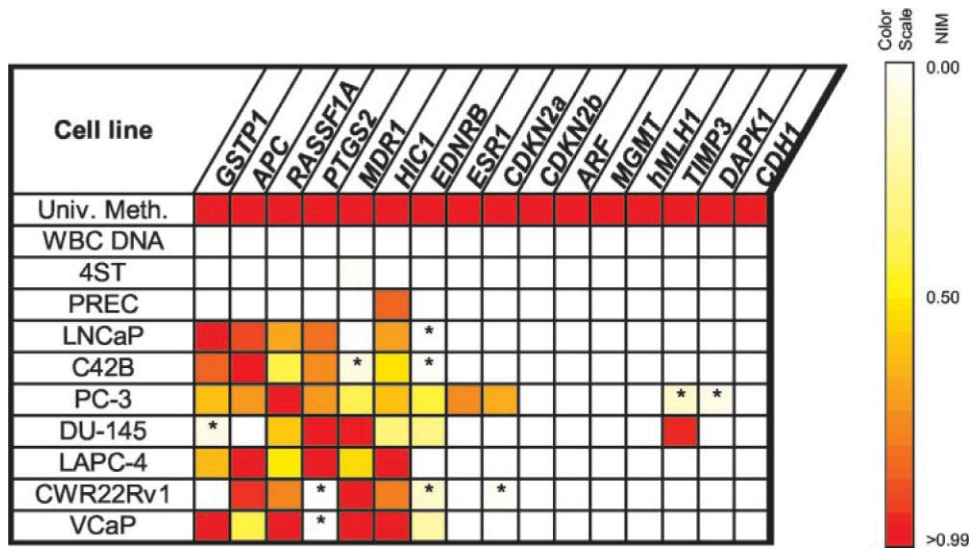
Glutathione S-transferases (GSTs) are a large family of detoxification enzymes which catalyze conjugation reactions with reduced glutathione. They play an important role in protecting DNA from reactive electrophilic compounds and oxidants. GSTP1 is hypermethylated in >90% of prostate cancer cases and 70% of prostatic intraepithelial neoplasia (PIN) lesions making it one of the earliest and most commonly found genome alterations in prostate cancer (43). Mice that are null for GSTP1 develop skin tumors at significantly increased rates compared to wild type mice when exposed to the carcinogen 7,12 dimethylbenz[a]anthracene (44). GSTP1 is completely silenced in LNCaP human prostate cancer cells due to hypermethylation (45). When these cells are exposed to PhIP, high levels of PhIP-DNA adducts are formed, but when the cells are modified to express GSTP1 they are resistant to the formation of PhIP-DNA adducts (46). These data demonstrate that GSTP1 may play a key role in the prevention of cancer formation.

Recent data suggest that certain dietary compounds may prevent cancer through epigenetic modifications in the cell (47, 48). In particular, lycopene has been shown to partially demethylate the GSTP1 promoter and restore GSTP1 expression in breast cancer cell lines (49). While this effect has not been examined in prostate cancer cells, these data suggest that lycopene may be a candidate for preventing or slowing prostate cancer growth through alterations in DNA methylation patterns.

## **Methylation Patterns in Prostate Cancer Cell Lines**

Aberrant DNA methylation patterns have commonly been associated with human cancers and may be one of the earliest genome alterations in prostate cancer. In studying prostate cancer, cell lines are often employed, but each line has its own unique characteristics since each was derived from an individual cancer. Yengnasubramanian et al. assessed the extent of hypermethylation at 16 CpG islands in seven prostate cancer cell lines, normal prostate epithelial cells, normal prostate stromal cells, primary prostate cancers, metastatic prostate cancers, and noncancerous prostate tissue (41). Each cell line had its own unique methylation pattern with regard to the 16 CpG islands examined (Figure 1.3). However, the CpG islands at GSTP1, APC, RASSF1a, PTGS2, and MDR1 were hypermethylated in >85% of prostate cancers and cancer cell lines, but not in normal prostate cells or tissues indicating that they might be particularly useful for distinguishing between cancerous and benign prostate tissue.

GSTP1 is known to be completely hypermethylated in LNCaP cells (45) and completely unmethylated in normal prostate epithelial cells (PrEC) and normal prostate stromal cells (4ST) (41).

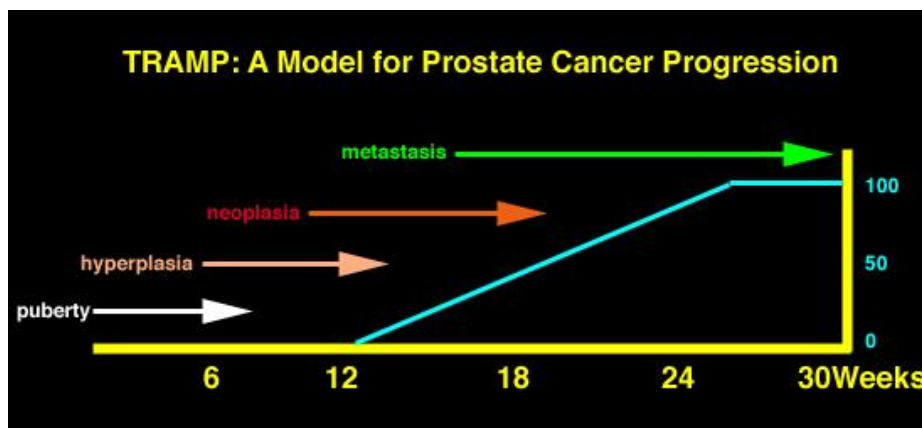


**Figure 1.3.** Normalized index of methylation (NIM) for prostate cancer cell lines and normal prostate and epithelial and stromal cells. The NIM was color scaled between white (representing no methylation detected) and red (indicating that >99% of bisulfite-converted input copies were methylated). \*, indicates NIM values <0.20 but greater than the calculated threshold for each CpG island (41).

### The TRAMP Model

The Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model was established in 1995 by Dr. Norman Greenberg (50). The model was generated in C57BL/6 mice by microinjection of a construct containing the simian virus 40 T antigen (SV40 Tag) under transcriptional control of a rat probasin promoter (PB). The PB gene encodes an androgen-regulated protein specific to the dorsolateral epithelium. This restricts expression of the transgene specifically to the prostate and results in activation of the transgene during puberty when androgen levels rise. The SV40 Tag is an oncoprotein which binds and abrogates function of retinoblastoma (Rb) and p53 tumor-suppressor proteins

TRAMP mice develop progressive prostate cancer similar to clinical prostate cancer (Figure 1.4). The cancer is invasive and capable of metastatic spread to the lungs and lymph nodes. Mice castrated at 12 weeks of age develop androgen-independent cancer which may serve as a model for hormone-refractory cancer (51). The main outcomes measured in this model include pathological tumor grade, genitourinary tract weight (a surrogate measure of tumor volume), and metastasis incidence (52).



**Figure 1.4.** Prostate cancer progression in the TRAMP model (52).

### **Aims of Dissertation**

Bioactive-enrichment of vegetables for the reduction of cancer growth is a relatively new field that has so far yielded promising results. Here we investigate whether bioactive-enriched tomatoes and broccoli can be produced through agronomic means, examine their effects on prostate carcinogenesis, and investigate possible mechanisms of action. The overall hypothesis of this work is that enhancing the bioactive content of tomatoes and broccoli will reduce prostate carcinogenesis. The specific aims of this work were to:

1. Obtain tomato and broccoli powders with enhanced levels on specific bioactives and screen them for bioactivity.
2. Test the efficacy of standard broccoli vs. methyl jasmonate-treated broccoli, which is high in indole glucosinolates, in reducing prostate carcinogenesis in the TRAMP model
3. Examine whether lycopene or apo-10'-lycopenal can restore expression of silenced tumor suppressor genes in prostate cancer cells through epigenetic modifications.

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## CHAPTER 2

### FEEDING TOMATO AND BROCCOLI POWDERS ENRICHED WITH BIOACTIVES IMPROVES BIOACTIVITY MARKERS IN RATS<sup>1,2</sup>

#### ABSTRACT

Many studies have evaluated the cancer preventive potential of individual bioactives from tomatoes and broccoli, but few have examined them within the context of a whole food. Male Copenhagen rats were fed diets containing 10% standard tomato powder, tomato enriched with lycopene or total carotenoids, standard broccoli floret, broccoli sprouts, or broccoli enriched with indole glucosinolates or selenium for 7 days. All broccoli diets increased activity of colon quinone reductase (NQO1). Indole glucosinolate-enriched broccoli and selenium-enriched broccoli increased hepatic NQO1 and cytochrome P450 1A activity ( $P < 0.05$ ). Standard broccoli and lycopene-enriched tomato diets down-regulated prostatic glutathione-S-transferase P1 mRNA expression. Different tomato diets resulted in altered hepatic accumulation of lycopene, phytofluene, and phytoene. These results demonstrate that the bioactive content of vegetables affects both tissue content of bioactives and activity of detoxification enzymes. Enhancing bioactive content of tomatoes and broccoli may enhance efficacy in the prevention of prostate cancer.

#### INTRODUCTION

The health benefits of functional foods are often associated with their bioactive components that are believed to deliver a health benefit beyond basic nutrition. For instance, lycopene in tomatoes may help maintain prostate health, and sulforaphane (SF), a hydrolysis

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<sup>1</sup> This chapter was the result of a collaboration between the following authors: Ann G. Liu, Sonja E. Volker, Elizabeth H. Jeffery, and John W. Erdman, Jr.

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product of glucoraphanin from broccoli may enhance detoxification of carcinogens. Much research has examined the putative health benefits of individual, isolated bioactive compounds. However, in many *in vitro* studies these bioactive compounds are provided at levels tens to hundreds of times greater than can be physiologically achieved through incorporation of whole foods into the diet. Furthermore, these compounds are typically tested as the aglycone form in cell culture, although they are present as glycosides in plant foods and conjugates in the body following absorption. Therefore it is also important to consider the impact of chemical form on bioavailability and bioactivity during dietary interventions *in vivo*. Once the efficacy of functional food components is verified, enhancing the concentrations of health-promoting compounds in foods may be beneficial in preventing and treating chronic diseases such as cancer.

In 1981, Doll and Peto estimated that approximately 35% of human cancer deaths could be prevented through dietary modifications (1). This led to an extensive study of fruit and vegetable intake as a modifiable risk factor. Epidemiological studies have linked higher consumption of tomatoes and cruciferous vegetables with a decreased risk of prostate cancer (2, 3). The efficacy of these vegetables is often associated with the anti-cancer actions of individual bioactives such as lycopene, SF, indole-3-carbinol (I3C, hydrolysis product of the indole glucosinolate glucobrassicin), and selenium. While these bioactives have been shown to have individual anti-cancer effects, they may also act additively or synergistically with the many other vitamins, minerals, and bioactive phytochemicals in tomatoes and broccoli to reduce tumor growth.

A few previous studies have examined the effects of increasing the bioactive content of tomatoes and broccoli on the reduction of cancer growth. For reducing the formation of aberrant

crypt foci in a chemically-induced rat model of colon cancer, selenium-enriched broccoli was shown to be more effective than high doses of selenite alone or a combination of selenite and broccoli sprout powder low in selenium (4). “Super broccoli” has been specifically bred to have high glucoraphanin levels (10.6 compared to 4.4  $\mu\text{mol/g}$  dry weight in store-bought broccoli), and it enhances transforming growth factor  $\beta 1$  signalling which is important for controlling cell proliferation (5). Another study found that an anthocyanin-enriched tomato was more effective than regular red tomato in increasing the survival time of Trp53<sup>-/-</sup> mice which predominantly develop lymphomas (6). These studies provide encouraging evidence that enrichment of tomatoes and/or broccoli with bioactives may be beneficial in reducing cancer growth.

Dietary interventions may play an important role in delaying prostate cancer growth especially since it is a slow-growing cancer that takes decades to develop. Optimizing the content of bioactive components within tomatoes and broccoli may provide an important boost to the effectiveness of these vegetables, especially considering that less than half of the US population meets recommended intake levels for fruits and vegetables (7).

The purpose of this study was to evaluate the effects of pre-harvest enrichment of tomato and broccoli with different bioactives on tissue accumulation and bioactivity markers in male Copenhagen rats in a 7-day screening trial for a future prostate cancer study. Different tomato and broccoli powders were produced agronomically, and profiled for their bioactive content including levels of carotenoids, glucosinolates, isothiocyanates, and selenium. *In vivo* exposure to bioactives was assessed by measurement of tissue carotenoid content and urinary SF metabolites. Our primary measure of bioactivity was phase I and phase II detoxification enzyme activity. We chose the phase I enzyme ethoxyresorufin O-deethylase (EROD) as reflective of any upregulation of cytochrome P450 1A and NAD(P)H-quinone oxidoreductase 1 (NQO1) as



reflective of any Nrf2-dependent upregulation of phase II enzymes (8) We also measured the phase II enzyme glutathione S-transferase P1 (GSTP1), which is associated with appearance of pre-neoplastic foci and has previously not always responded in concert with other Nrf2-dependent phase II enzymes (9). Markers of androgen metabolism were also assessed because androgens play a key role in the development and progression of prostate cancer.

## **MATERIALS AND METHODS**

**Tomato and broccoli powders.** Standard tomato powder, lycopene-enriched tomato powder, standard broccoli floret powder (referred to as “standard broccoli”), and broccoli sprout powder were obtained from FutureCeuticals (Momence, IL). Tomato powders were produced by grinding and pureeing the tomatoes, drum drying, and grinding the powder through a USA #20 screen (equivalent to particle size of 0.85 mm). To produce the broccoli floret powder, broccoli (*Brassica oleracea* var. Monaco) was cut and diced to produce a floret, blanched, freeze dried, and ground through a USA #20 screen. For the broccoli sprout powder, 3-day old broccoli sprouts (*Brassica oleracea* var. Calabrese) were blanched, macerated and ground, drum dried, and ground through a USA #20 screen. In addition, carotenoid-enriched tomatoes, indole glucosinolate-enriched broccoli, and selenium-enriched broccoli were grown by Dr. Gary Bañuelos at the USDA-ARS research facility in Parlier, CA from March to July, 2007. For the California-grown crops, normal agronomic management practices were applied on the test plots including pre-plant application of fertilizer, insect and animal control, and hand weeding. Broccoli (*Brassica oleracea* var. Magestic) and tomatoes (*Lycopersicum esculentum* var. APT 410) were started and maintained in a commercial hot house. Six to eight weeks later, tomato and broccoli seedlings were transplanted into experimental beds. To produce indole glucosinolate-enriched broccoli,

treatment consisted of the plant hormone methyl jasmonate (1 mg/mL solution) in a 2% ethanol 0.1% Triton x-100 aqueous solution sprayed on individual plants 7 days prior to harvest. To produce selenium-enriched broccoli, selenium was applied as a sodium selenate solution of 10 mg Se/L to the soil beneath each plant every two days beginning at day 45 after transplanting. Both broccoli, harvested 2 inches below the floret, and tomatoes, selected for bright red fruit, were dehydrated at a commercial dehydrator where they were blanched 4-5 minutes, cut, and dried at 110°C for 24 h to a moisture level of 5%. The product was then shipped to the University of Illinois in dry, opaque plastic bags. Upon receipt, the dried vegetables were stored at 4°C, and then processed into a powder using a hammermill (model 10; C.S. Bell Co., Tiffin, OH).

**Glucosinolate Analysis.** Intact glucosinolates were analyzed by modification of the method of Kushad et al. (10). Freeze-dried broccoli powder (0.2 g) was placed in an Oak Ridge tube (Nalgene, Rochester, NY) and heated on a heating block at 95°C for 10 min. To each tube, 2 mL of boiling 70% methanol were added and heated for an extra 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added, mixed, and centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant (extract) was saved and the pellet was re-extracted with 2 mL 70% methanol and the extracts combined. A sample (1 mL) from each pooled extract was transferred into a glass tube. Protein was precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. Each sample was then loaded onto a DEAE Sephadex A-25 column for desulfation with arylsulfatase for 18 h. Desulfated glucosinolates were eluted from the column with water and injected onto a Lichosphere RP-18 column (Alltech Inc., Springfield, KY). Desulfoglucosinolates were eluted using a linear gradient of 1-40% acetonitrile in water at a flow

rate of 1 mL/min over 40 minutes. Benzylglucosinolate (C2 Bioengineering Company, Denmark) was used as an internal standard.

**Isothiocyanate extraction and analysis.** Broccoli powders were suspended in water (50 mg/mL) for 24 h at room temperature away from light to facilitate glucosinolate hydrolysis. Slurries were centrifuged at 16,000 x g for 10 min and supernatants were filtered through 0.45 µm nylon membranes. Benzyl isothiocyanate (0.1 mg/mL DMSO) or 4-methoxyindole (0.1 mg/mL DMSO) was used as an internal standard to a final concentration of 2 µg/mL for analysis of SF or I3C, respectively. Each supernatant was extracted with an equal volume of dichloromethane. One volume of DMSO was added to 5 volumes of dichloromethane extract, which was removed under a stream of nitrogen gas, leaving the sample in DMSO.

SF and I3C were analyzed by reverse-phase HPLC with UV detection at wavelengths of 254 and 280 nm, respectively (Waters, Milford, MA). Fifty µL of the extract in DMSO sample was injected onto a Luna reverse-phase C18 column (5 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA). At a flow rate of 1 mL/min, 20% acetonitrile in water was increased linearly to 100%, over 15 min. The mobile phase was then maintained for 5 min, and returned to initial conditions at which the column was re-equilibrated for 10 min. Solvents contained 1% (v/v) glacial acetic acid. Using Empower Pro software (Waters, Milford, MA), quantification was performed by comparison to SF and I3C standards (LKT Laboratories, St. Paul, MN).

**Selenium Analysis.** Selenium content of vegetable powders was measured by instrumental neutron activation analysis at the University of Missouri-Columbia Research Reactor Center by inducing Se-77m and measuring photon emissions associated with its decay. A standard reference material (SRM 1577, Bovine Liver), certified for selenium at  $1.1 \pm 0.1$  ppm

and supplied by the National Institutes for Standards and Technology, was analyzed in replicate as a quality control material.

**Carotenoid extraction and quantification.** Carotenoid extraction and analysis was performed as previously described (11). Briefly, 0.1 g powder or 0.1 g tissue were combined with 6 mL KOH/ethanol solution (1:5) containing 0.1% BHT. Samples were saponified at 60°C for 30 min. Samples were then placed on ice and deionized water was added. Powder and tissue carotenoids were extracted 3 times with the addition of 6 mL of hexane. Hexane extracts were dried in a Speedvac evaporator (model AS160; Savant, Farmingdale, NY), flushed with argon, and stored at -20°C for  $\leq 48$  h prior to HPLC-PDA analysis. All carotenoid extracts were kept on ice and under yellow lights throughout the extraction process.

Carotenoid concentrations in powders and tissues were analyzed by a reverse-phase HPLC-PDA system previously described (12). All analyses were performed in duplicate, and the quantification of carotenoid isomers was carried out by comparing the UV spectra and retention times to analytical standards of phytoene, phytofluene, lycopene, and  $\beta$ -carotene (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany).

**Animals and Experimental Design.** The University of Illinois Animal Care and Use Committee approved the animal protocol. Sixty-four male Copenhagen rats (Cop 2331; Harlan, Indianapolis, IN) were obtained at ~ 8 weeks of age and individually housed in wire-bottom cages under controlled conditions (12 h light-dark cycle, 22°C, 60% humidity). Rats were weighed daily throughout the study. Rats were provided with AIN-93G powdered diet and acclimated for 7 days. On day 7, rats were randomly assigned to 8 experimental groups (n=8). AIN-93G based experimental diets included: AIN-93G control, 10% standard tomato powder, 10% lycopene-enriched tomato powder, 10% carotenoid-enriched tomato powder, 10% standard

broccoli floret powder, 10% broccoli sprout powder, 10% IG-enriched broccoli powder, or 10% selenium-enriched broccoli powder. Diets were balanced for protein, fat, energy, and fiber. Diet formulations are shown in Table 2.1. Food intake was measured daily. Animals were pair-fed experimental diets for 7 days.

**Urinary analysis of sulforaphane conjugates.** For the last 24 h of experimental feeding, urine was collected into 50 mg ascorbic acid and stored at -80 °C until analyzed. SF and erucin N-acetylcysteine conjugates were quantified by HPLC-UV at a wavelength of 254 nm, as previously described (13). Intake of glucoraphanin was calculated from broccoli hydrolysate analysis and average food intake ( $14.5 \pm 0.4$  g per animal per day).

**Preparation of microsomal and cytosolic fractions.** Rats were euthanized by CO<sub>2</sub> asphyxiation and tissues were taken. Livers were perfused via the portal vein with cold 1.15% KCl. Colons were flushed with cold 1.15% KCl and mucosal scrapings of the proximal 5 cm were collected. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C until use. Liver and colon mucosal samples were thawed on ice, homogenized in 3 and 1 mL cold 0.05 M Tris-HCl buffer (pH 7.4), respectively, and centrifuged for 20 min at 10,000 x g at 4 °C. Colonic mucosal supernatant was stored at -80°C until use; liver supernatant was further centrifuged for 60 min at 100,000 x g at 4 °C. The supernatant (cytosolic fraction) was stored at -80°C until use and the pellet (microsomal fraction) was resuspended in 1 mL cold 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, then stored at -80°C until use.

**Detoxification enzyme activity.** Activity of phase I enzyme CYP1A was measured in the microsomal fraction as ethoxyresorufin O-deethylase (EROD) activity (14) with slight modification (15). Activity of cytosolic phase II enzyme NAD(P)H-quinone oxidoreductase 1

(NQO1) was measured according to the method of Prochaska and Santamaria (16) with modification (15).

**Serum testosterone measurements.** Serum testosterone was quantified using a coated tube radioimmunoassay kit (Diagnostic Systems Laboratory Inc., Webster, TX).

**Real-time quantitative PCR.** Total RNA was prepared from tissues using Trizol (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Reactions were monitored by an ABI Prism 7900HT. Primer pairs were designed to measure steroid 5- $\alpha$ -reductase 1 (SRD5A1) (17), 5- $\alpha$ -reductase 2 (SRD5A2) (18), glutathione-S-transferase  $\pi$  1 (GSTP1, NM\_012577) forward-5'-CCTATGTGGCTCGCCTCAGT-3' and reverse-5'-GATGGGACGGTTCAAATGGT-3' and ribosomal protein L7a (RPL7A, NM\_001114391) forward-5'-GAGGCCAAAAGGTGGTCAATCC-3' and reverse-5'-CCTGCCCAATGCCGAAGTTCT-3'. A validation experiment was performed on each set of primers (MWG Biotech, Huntsville, AL) to confirm efficiency and product specificity. RPL7A was used as a housekeeping gene (19). Relative mRNA abundance was determined using the comparative critical threshold method.

**Statistical analysis.** Data were compared among treatments by two-tailed analysis of variance, using SAS Statistical Software (version 9.1; SAS Institute, Cary, NC), and values were considered different from controls at  $P < 0.05$  using Fisher's least significant difference procedure.

## RESULTS AND DISCUSSION

**Powder Characterization.** Here we demonstrate that tomato and broccoli powders with differing levels of bioactives can be readily produced by agronomic means and that this leads to altered uptake of bioactives *in vivo*. We obtained tomato powders with enhanced levels of lycopene or total carotenoids, and broccoli powders with enhanced levels of glucosinolates or selenium. Broccoli powders were analyzed for glucosinolate, SF, I3C, and selenium content (Table 2.2). Glucoraphanin levels were similar among all broccoli except the broccoli sprout powder, which were 7 times greater. As expected, SF content from dietary glucoraphanin was highest from the broccoli sprouts, being 23 times greater than in standard broccoli. Glucobrassicin was significantly higher in the indole glucosinolate-enriched broccoli, as expected. The indole glucosinolate-enriched broccoli also had increased neoglucobrassicin levels. Surprisingly, neoglucobrassicin levels were dramatically increased in the selenium-enriched broccoli, by approximately 6 times compared to standard broccoli powder. Broccoli sprouts had a low indole glucosinolate content. Following hydrolysis, indole glucosinolate-enriched broccoli contained slightly lower levels of I3C than standard broccoli ( $P < 0.05$ ).

Tomato powders were analyzed for carotenoid content (Table 2.3). Levels of lutein, zeaxanthin,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin were all below 5 nmol/g (data not shown). Lycopene-enriched tomato powder had similar levels of phytoene, phytofluene, and  $\beta$ -carotene compared to standard tomato powder, but it also had approximately 2.6 times more lycopene than the standard tomato powder. The carotenoid-enriched tomato powder contained total carotenoid levels approximately 3.4 times greater than the standard tomato powder and 2 times greater than lycopene-enriched tomato powder.

**Diet and Weight Gain.** The group of rats receiving the selenium-enriched broccoli diet had the lowest food intake. As a result, all other animals were pair-fed to this group. Each day other diet groups received the amount of diet equal to the average amount consumed by the selenium-enriched broccoli group the day before. The average amount of food consumed daily was  $14.5 \pm 0.4$  g. Rats gained weight as expected throughout the study, and there were no significant differences in final body weight between groups. The estimated daily intake of glucoraphanin from the broccoli powders was 4.4, 31.3, 3.6 and 4.9  $\mu\text{mol}$  for standard broccoli, broccoli sprouts, indole glucosinolate-enriched broccoli and selenium-enriched broccoli, respectively (Figure 2.1). The estimated daily intake of lycopene from the tomato powders was 249, 647, and 879 nmol for standard tomato, lycopene-enriched tomato, and carotenoid-enriched tomato, respectively.

**Sulforaphane Metabolism.** Sulforaphane is metabolized primarily via the mercapturic acid pathway (20), and SF-NAC is used as a marker for fractional absorption of this isothiocyanate (21). Urinary levels of sulforaphane N-acetylcysteine conjugates (SF-NAC) are shown in Figure 2.1. The reduced form, erucin N-acetylcysteine, was below the level of detection. Recovery of SF-NAC was significantly greater in the broccoli sprout group. Percent recovery of SF-NAC from ingested glucoraphanin was 4% for standard broccoli, 49% for broccoli sprouts, 28% for indole glucosinolate-enriched broccoli, and 12% for selenium-enriched broccoli. The enhanced urinary content of SF from sprouts is consistent with individual data for sprouts and standard broccoli from the literature (13, 22). However, early hydrolysis before digestive enzymes destroy broccoli myrosinase cannot explain this, since in the past pre-hydrolyzing broccoli did not enhance SF-NAC yield (13). Blanching of the sprouts may have destroyed the myrosinase cofactor that directs glucoraphanin hydrolysis to nitrile formation in



place of SF (23). This idea is consistent with the far greater yield of SF from glucoraphanin in sprouts compared to standard broccoli (Table 2.2).

**Hepatic Carotenoid Accumulation.** Liver carotenoid content is shown in Table 2.4. Interestingly, hepatic carotenoid accumulation did not strictly follow the patterns seen in the tomato powder carotenoid profiles. The carotenoid-enriched tomato powder had carotenoid levels 2-3 times greater than standard tomato powder, but only the hepatic accumulation of phytoene and  $\beta$ -carotene was significantly increased compared to standard tomato powder. The lycopene-enriched tomato group had the expected significantly higher levels of lycopene but also significantly lower levels of phytofluene and phytoene compared to standard and carotenoid-enriched tomato powders.

The lower than expected accumulation level of lycopene and phytofluene from feeding the carotenoid-enriched tomato powder may have been due to differences in processing method. The commercial tomato powders (standard and lycopene-enriched) were more finely ground than powders grown in California which were processed using a hammermill (carotenoid-enriched tomato powder), possibly leading to an increase in hepatic accumulation. Alternatively, there is literature indicating that supplementation of a normal diet with  $\beta$ -carotene supplements or  $\beta$ -carotene rich foods may lead to a decrease in serum lycopene (24, 25). The higher levels of phytoene and  $\beta$ -carotene in the carotenoid-enriched tomato powder could compete with lycopene for absorption and transport. Lycopene-enriched tomato powder feeding resulted in significantly higher hepatic levels of lycopene than standard tomato powder, but it also resulted in significantly reduced uptake of phytoene and phytofluene. These data support the possibility that there may be competition between carotenoids for absorption pathways.

**Indole Glucosinolate Metabolism.** High doses of purified I3C are rapidly degraded in the body. Serum levels of I3C have been reported to fall below detectable levels (0.05 µg/mL measured by HPLC) within an hour after oral administration to mice (250 mg/kg) (26). Similarly in humans, I3C was not detectable in serum one hour after oral administration (400 mg) with a limit of detection of 1.0 ng/mL (measured by HPLC-MS) (27). The dose provided here by a 10% indole glucosinolate-enriched broccoli diet is approximately 1000-fold lower than the levels used in the above pharmacokinetic studies and would likely not be detectable, particularly since rats were fed *ad libitum* rather than a bolus dose of the compound.

**Detoxification Enzyme Activity.** Broccoli and its glucosinolate hydrolysis products have been shown to induce phase I and phase II drug-metabolizing enzymes (13). Detoxification enzymes in the colon and liver play an important role in the metabolism and clearance of carcinogens. Xenobiotics are oxidatively metabolized by phase I enzymes generating products which are potentially carcinogenic. Phase II conjugating enzymes are then able to add polar molecules (glutathione, glucuronide, sulfate, etc.) to the metabolite, enhancing water solubility. These products are more easily eliminated from the body. Therefore the balance between activating (phase I) and detoxifying (phase II) reactions is an indication of the risk of ultimate carcinogen available for reaction with cellular DNA. Here we demonstrate that broccoli enriched with bioactive components through agronomic practices enhance both CYP1A and NQO1 activity in liver and colon to a greater extent than standard broccoli.

Tissue enzyme activities of EROD and NQO1 are shown in Figure 2.2A, B, and C. The standard broccoli diet did not alter EROD or NQO1 in liver but did increase NQO1 in the colon. SF-rich broccoli sprouts showed no hepatic EROD induction, but increased NQO1 1.9 times in the liver and 2.3 times in the colon ( $P < 0.05$ ). Indole glucosinolate-enriched broccoli increased

hepatic EROD and NQO1 activity 1.4 and 1.3 times, respectively, and colon NQO1 1.6 times ( $P<0.05$ ). Selenium-enriched broccoli induced hepatic EROD 1.8 times; NQO1 was elevated 2.2 and 1.8 times in the liver and the colon, respectively ( $P<0.05$ ). No differences in EROD or NQO1 were observed between groups receiving the tomato powder diets. Lycopene-enriched tomato and standard broccoli diets decreased glutathione S-transferase P1 (GSTP1) mRNA levels by 23.3% and 31.9%, respectively compared to the control diet ( $P<0.05$ ) (Figure 2.3).

Glucobrassicin and neoglucobrassicin, as well as their respective hydrolysis products I3C and N-methoxyindole-3-carbinol (NI3C), are known to induce CYP1A (28-31). Although neoglucobrassicin is a weaker inducer than glucobrassicin *in vivo*, a mixture containing both glucosinolates showed strongest induction of hepatic CYP1A (29). In concordance, indole glucosinolate-enriched broccoli and selenium-enriched broccoli increased hepatic CYP1A-dependent EROD activity. Neither standard broccoli nor broccoli sprouts altered hepatic CYP1A activity, which correlates with their low levels of neoglucobrassicin and I3C.

Broccoli sprouts, indole glucosinolate-enriched and selenium-enriched broccoli increased NQO1 in both liver and colon. Standard broccoli effectively upregulated NQO1 in the colon, but it did not significantly upregulate enzymes in the liver, possibly because the dose of SF was insufficient. This tissue-specific effect suggests incomplete myrosinase-dependent hydrolysis of glucoraphanin in the upper gut, resulting in hydrolysis in the lower gut and subsequent local increase in NQO1 (15).

The relative amounts of SF in standard broccoli, broccoli sprout and indole glucosinolate-enriched broccoli (1.0, 22.8 and 1.7  $\mu\text{mol SF/g}$  dry weight, respectively) elicited an increase in NQO1 activity in liver (20, 90 and 30%, respectively) and colon (20, 130 and 60%, respectively) that was proportional to the SF dose. Selenium-enriched broccoli induced enzyme activity to a

greater extent than can be ascribed to its SF level. Broccoli accumulates selenium primarily as Se-methylselenocysteine (32), which alone or in synergy with SF may account for the increase in NQO1 activity. These data are in contrast to those of Robbins et al. (33), who reported a suppression of SF levels in broccoli treated with a sodium selenate solution (100 or 10,000 ppm). This resulted in very high levels of selenium in broccoli (up to 800 ppm). The freeze-dried broccoli used here had 4 ppm Se, which would have been ~0.4 ppm in the fresh plant.

In contrast to feeding standard broccoli, which resulted in greater increases in NQO1 in the colon than in the liver, feeding selenium-treated broccoli resulted in larger increases in NQO1 in liver than in colon. One possible cause for this is that hepatic metabolism permitted the formation of a more potent metabolite.

Prostate cancer is characterized by loss of GSTP1 expression, regardless of the grade or stage of the disease (34). Unexpectedly, broccoli florets and lycopene-enriched tomato significantly reduced GSTP1 expression by 32% and 23%, respectively. These results appear to contradict previous evidence showing that lycopene partially demethylates the GSTP1 promoter and restores expression in breast cancer cells (35) and that oral intake of SF increases GSTP1 expression in nasal mucosa (36). The family of GST enzymes are not all regulated similarly and GSTP1 has previously been found to lack the inductive response to dietary crucifers seen for GSTM1 and GSTA1 (9).

**Androgen Metabolism.** Previous reports suggest that androgen metabolism might be altered by tomato and/or lycopene feeding. Our own lab has shown that 4-day feeding of lycopene or tomato powder resulted in significantly reduced levels of serum testosterone in Fisher 344 rats (18). It has also been reported that lycopene feeding decreased prostatic mRNA levels of SRD5A2 (37), which is a key enzyme in the conversion of testosterone to the more

potent androgen, dihydroxytestosterone. In the current study, no significant alterations were seen in either serum testosterone levels (values ranged from 0.54 – 1.78 ng/mL) or mRNA levels of SRD5A1 or SRD5A2 (data not shown). The lack of significant reduction in serum testosterone level may be due to the longer feeding period (7 vs. 4 days) or the strain of rat used. Reduction in expression of SRD5A2 has been seen in Copenhagen rats of a similar age (37). However, in that study rats were fed supplemental lycopene for a period of 8 weeks, resulting in a much longer and higher dose lycopene exposure than the present study. As noted above, alterations in serum testosterone were reported for Fisher 344 rats after 4 days of 10% tomato powder feeding (18), which is shorter than the 7 day feeding period used in this study, suggesting that the effects of tomato on serum testosterone levels could be a transient effect, or that the dose-response varies with strain. None of the broccoli treatments had significant effects on androgen metabolism endpoints. To our knowledge the effects of broccoli on testosterone metabolism have not been previously investigated, and these data demonstrate that broccoli feeding does not alter serum testosterone levels or expression of two key metabolizing enzymes.

In summary, we have demonstrated that levels of bioactive components in tomatoes and broccoli can be altered through agronomic means. This increase in bioactives in the vegetable can translate to increased tissue content and increased bioactivity *in vivo*. These encouraging findings suggest that bioactive-enriched vegetables should be tested for cancer prevention.

## **ACKNOWLEDGMENTS**

We thank Shawn Evers at FutureCeuticals for donating tomato and broccoli powders; Seminis for donating tomato seeds (var. APT 410); Dr. Gary Bañuelos at the USDA-ARS Water

Management Research Unit in Parlier, CA for overseeing the production of tomatoes and broccoli; and Ning Zhu at the University of Illinois for performing glucosinolate analysis.

**Table 2.1.** AIN-93G based diet formulations.

	g/100g total diet			
	Control	Tomato (Std, Lyc, Car)	Broccoli (Std, IG, Se)	Broccoli (Sprout)
Cornstarch	39.7	33.2	36.6	38.0
Casein	20.0	18.7	16.8	16.9
Maltodextrin	13.2	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0	10.0
Fiber*	5.0	3.3	1.6	3.4
Mineral mix**	3.5	3.5	3.5	3.5
Vitamin mix***	1.0	1.0	1.0	1.0
L-Cystine	0.3	0.3	0.3	0.3
Choline bitartrate	0.3	0.3	0.3	0.3
Soybean oil	7.0	6.5	6.7	5.3
Tomato powder		10.0		
Broccoli powder			10.0	10.0

\*Non-nutritive cellulose

\*\*AIN93M-MX formulation

\*\*\*AIN93-VX formulation

**Table 2.2.** Glucosinolate, isothiocyanate, and selenium profile of broccoli powders.

	$\mu\text{mol/g}$ dry weight			
	Standard Broccoli	Broccoli sprout	IG-enriched Broccoli	Se-enriched Broccoli
Glucoraphanin	$3.05 \pm 0.36^b$	$21.60 \pm 4.30^a$	$2.46 \pm 0.36^b$	$3.36 \pm 0.55^b$
Glucobrassicin	$2.17 \pm 0.06^b$	$0.05 \pm 0.00^d$	$3.27 \pm 0.17^a$	$0.73 \pm 0.05^c$
Neoglucobrassicin	$1.97 \pm 0.06^c$	$0.06 \pm 0.00^d$	$3.74 \pm 0.02^b$	$11.99 \pm 0.75^a$
Sulforaphane	$0.98 \pm 0.02^b$	$22.78 \pm 1.30^a$	$1.73 \pm 0.11^b$	$1.75 \pm 0.13^b$
Indole-3-Carbinol	$0.10 \pm 0.01^a$	$0.04 \pm 0.00^c$	$0.08 \pm 0.00^b$	$0.10 \pm 0.01^a$

Selenium	ppm			
	0.25	0.06	0.06	4.28

Values are means  $\pm$  SEM, n=2-4 (where n=2, SEM is not reported). Different superscript letters within rows indicate significant differences between treatments ( $P<0.05$ ).

**Table 2.3.** Carotenoid profile of tomato powders.

	nmol/g		
	Standard Tomato	Lycopene-enriched Tomato	Carotenoid-enriched Tomato
Phytoene	$102 \pm 7^b$	$92 \pm 1^b$	$347 \pm 6^a$
Phytofluene	$74 \pm 7^b$	$71 \pm 1^b$	$224 \pm 3^a$
$\beta$ -carotene	$13 \pm 1^b$	$13 \pm 0^b$	$50 \pm 2^a$
cis lycopene	$80 \pm 9^b$	$213 \pm 9^a$	$116 \pm 21^b$
all-trans & 5-cis lycopene	$92 \pm 11^c$	$233 \pm 16^b$	$490 \pm 66^a$
Total lycopene	$172 \pm 21^b$	$446 \pm 24^a$	$606 \pm 87^a$
Total carotenoid* ( $\mu\text{g/g}$ )	$195 \pm 19^c$	$335 \pm 14^b$	$663 \pm 52^a$

Values are means  $\pm$  SEM, n=3. Different superscript letters within rows indicate significant differences between treatments ( $P<0.05$ ).

\*Total carotenoid = phytoene + phytofluene +  $\beta$ -carotene + total lycopene



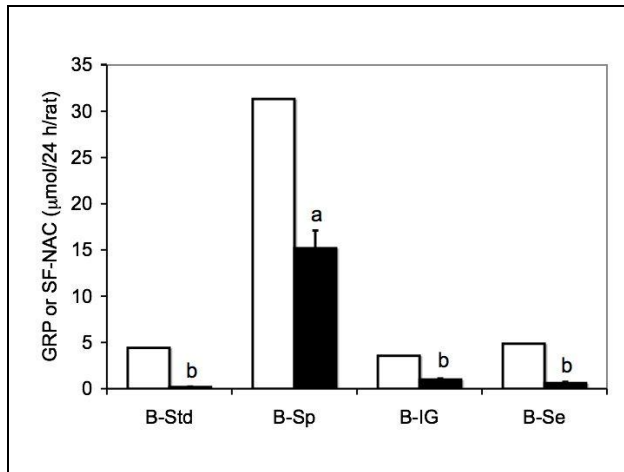
**Table 2.4.** Hepatic Carotenoid Content.

	$\mu\text{g/g}$		
	Standard Tomato	Lycopene-enriched Tomato	Carotenoid-enriched Tomato
Phytoene	$13.0 \pm 0.7^b$	$8.7 \pm 0.6^c$	$19.4 \pm 0.7^a$
Phytofluene	$13.0 \pm 0.4^a$	$9.8 \pm 0.7^b$	$13.4 \pm 0.5^a$
$\beta$ -carotene	$0.2 \pm 0.0^b$	$0.2 \pm 0.0^b$	$0.3 \pm 0.0^a$
Lycopene	$18.4 \pm 1.5^b$	$26.7 \pm 2.4^a$	$19.1 \pm 0.9^b$
Total carotenoid*	$44.4 \pm 2.1^b$	$45.1 \pm 3.1^b$	$52.0 \pm 1.5^a$

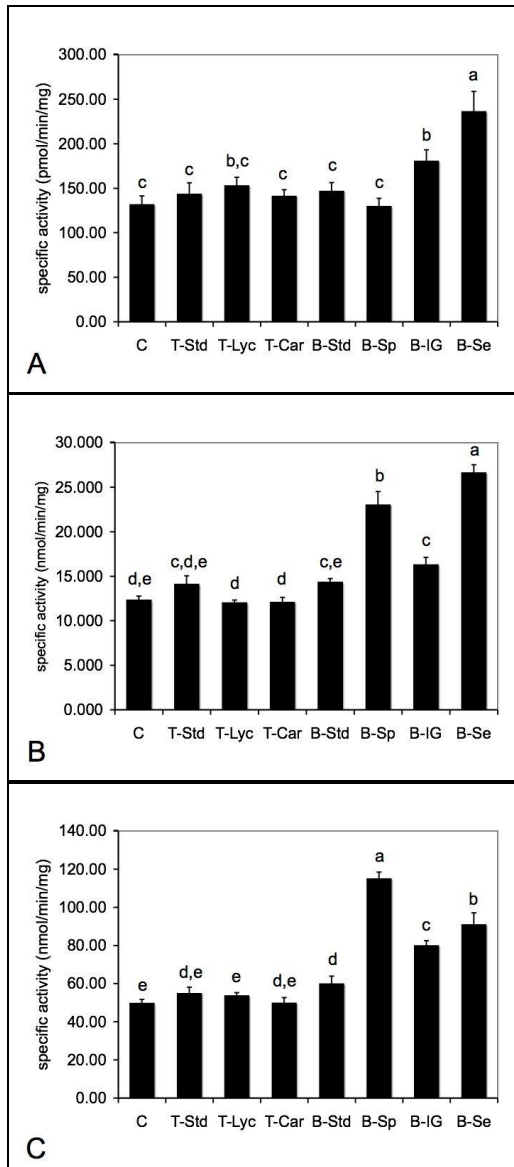
Values are means  $\pm$  SEM, n=8. Each sample was run in duplicate.

\*Total carotenoid = lycopene + phytofluene + phytoene +  $\beta$ -carotene.

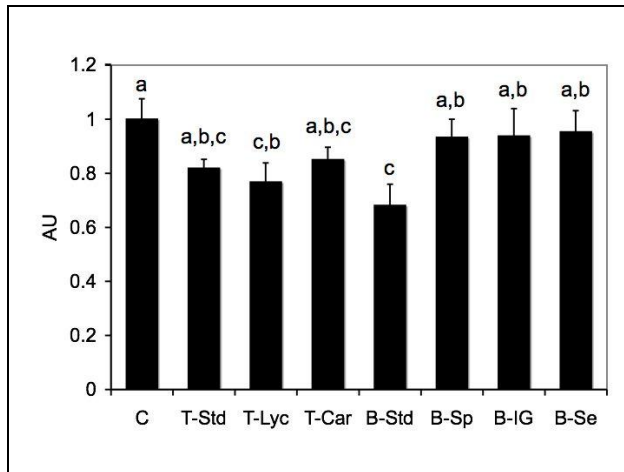
Different superscript letters within a row indicate significant differences between treatments ( $P < 0.05$ ).



**Figure 2.1.** Estimated daily glucoraphanin (GRP) intake (□) and 24 hour urinary SF-NAC excretion (■) on the last day of a 7 day feeding period. Experimental groups are as follows: B-Std = standard broccoli, B-Sp = broccoli sprout, B-IG = indole glucosinolate-enriched broccoli, B-Se = selenium-enriched broccoli. Results shown are means  $\pm$  SEM, n=8. Samples were run in triplicate. Different letters above bars indicate significant differences between treatments ( $P < 0.05$ ).



**Figure 2.2.** A: Liver EROD, B: Liver NQO1, C: Colon NQO1 after 7-day feeding period. Experimental groups are as follows: C = control, T-Std = standard tomato, T-Lyc = lycopene-enriched tomato, T-Car = carotenoid-enriched tomato, B-Std = standard broccoli, B-Sp = broccoli sprout, B-IG = indole glucosinolate-enriched broccoli, B-Se = selenium-enriched broccoli. Results shown are means  $\pm$  SEM, n=8. Samples were run in triplicate. For each enzyme, different letters above bars indicate significant differences between treatments ( $P < 0.05$ ).



**Figure 2.3.** Gene expression of prostatic GSTP1 after 7-day feeding. AU = arbitrary units.

Experimental groups are as follows: C = control, T-Std = standard tomato, T-Lyc = lycopene-enriched tomato, T-Car = carotenoid-enriched tomato, B-Std = broccoli, B-Sp = broccoli sprout, B-IG = indole glucosinolate-enriched broccoli, B-Se = selenium-enriched broccoli. Results shown are means  $\pm$  SEM, n=8. Different letters above bars indicate significant differences between treatments ( $P < 0.05$ ).

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## CHAPTER 3

### METHYL JASMONATE-TREATED BROCCOLI AND PROSTATE CARCINOGENESIS IN TRAMP MICE<sup>1</sup>

#### ABSTRACT

Broccoli is rich in bioactive components that may impact cancer risk, such as sulforaphane and indole-3-carbinol. The glucosinolate profile of broccoli can be manipulated through treatment with the plant stress hormone, methyl jasmonate (MeJa) to increase levels of indole glucosinolates. Our objective was to determine if feeding standard broccoli (*Brassica oleracea* var. Green Magic), or the same variety of broccoli treated with MeJa during growth, would impact prostate carcinogenesis in the aggressive TRAMP model. MeJa treatment significantly increased levels of glucobrassicin, neoglucobrassicin, and gluconasturtiin ( $P < 0.05$ ). Male TRAMP mice (n=99) were randomized into 3 diet groups at 5-7 weeks of age: AIN-93G control, 10% standard broccoli powder, or 10% MeJa broccoli powder. Diets were fed throughout the study until termination at 20 weeks of age. Neither broccoli treatment significantly altered genitourinary tract weight, pathologic score, proliferation or apoptosis indicating that broccoli feeding did not reduce prostate carcinogenesis in the TRAMP model.

#### INTRODUCTION

Epidemiological studies provide moderate support for the hypothesis that consumption of cruciferous vegetables reduces the risk of developing prostate cancer (1). Broccoli is the most commonly consumed cruciferous vegetable in the United States with the average American eating 8.6 pounds of fresh broccoli and broccoli products each year (2). Broccoli contains many

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<sup>1</sup> This chapter was the result of a collaboration between the following authors: Ann G. Liu, Lisa D. Berman-Booty, Steven K. Clinton, Elizabeth H. Jeffery, and John W. Erdman, Jr.

glucosinolates including glucoraphanin, the precursor to the anti-carcinogenic compound, sulforaphane, glucobrassicin, which is hydrolyzed to indole-3-carbinol (I3C) in the gastrointestinal tract, and neoglucobrassicin, which is hydrolyzed to N-methoxy-I3C. Sulforaphane and I3C are known to have anti-carcinogenic properties (3, 4), but studying these compounds in isolation may obscure and additive or synergistic actions that might take place when a whole vegetable is consumed.

Our lab had previously shown that feeding whole tomato or broccoli powder significantly reduced tumor growth in a rat transplantable tumor model of prostate cancer whereas pure lycopene alone did not, suggesting that the whole vegetable is indeed more effective than an individual bioactive (5). In a previous study, our lab also demonstrated that we could increase the levels of specific bioactives within whole tomatoes and broccoli using agronomic means (6). Finley et al. demonstrated that selenium-enriched broccoli was more effective than high doses of selenite alone or a combination of selenite and broccoli sprout powder for reducing the formation of aberrant crypt foci in a rat model of colon cancer (7). That study lends support to the hypothesis that optimizing the bioactive content of whole broccoli may enhance potential for cancer prevention.

Glucosinolates are divided into three classes based on the amino acid precursors from which they originate. Aliphatic glucosinolates are derived from methionine, indole glucosinolates from tryptophan, and aromatic glucosinolates from phenylalanine. These phytochemicals are constitutively present in cruciferous plant tissues, but they can also be rapidly induced by stresses such as wounding, pathogens, or herbivore attacks. These stress conditions lead to the endogenous biosynthesis of jasmonates, which induce the synthesis of indole glucosinolates (8). External application of methyl jasmonate, a volatile methylester of

jasmonate, has been shown to induce production of indole glucosinolates in *Brassica* species (9, 10). Methyl jasmonate treatment offers a way of altering the phytochemical profile of broccoli to increase concentrations of potentially anti-carcinogenic bioactives.

Here we examined the effects of standard broccoli and MeJa-treated broccoli on prostate carcinogenesis in TRAMP mice. We hypothesized that both broccoli treatments would reduce prostate carcinogenesis and that MeJa-treated broccoli would further reduce carcinogenesis compared to standard broccoli. To our knowledge, this is only the second study to examine the effects of whole broccoli in an animal model of prostate cancer; the first utilized the Dunning rat model (5).

## **MATERIALS AND METHODS**

**Broccoli Powder Production.** Broccoli (*Brassica oleracea* var. Green Magic) was grown on the University of Illinois campus in Urbana, IL from May through August 2009. Broccoli plants were started and maintained in a commercial hot house for 3 weeks. Before transplanting to experimental fields, broccoli seedlings were hardened outside for one week. To produce indole glucosinolate-enriched broccoli, treatment consisted of the plant hormone methyl jasmonate (250  $\mu$ M) in a 0.1% Triton X-100 aqueous solution sprayed on individual plants 4 days prior to harvest. Plant surfaces were fully saturated with MeJa, allowed to dry, and then received an additional application of MeJa. Broccoli was harvested at commercial size with some stem, frozen in liquid nitrogen, and stored at -20°C until freeze-dried. After freeze-drying, broccoli samples were finely ground using a commercial coffee grinder.

**Glucosinolate Analysis.** Intact glucosinolates were analyzed by modification of the method of Kushad et al. (11). Freeze-dried broccoli powder (0.2 g) was placed in an Oak Ridge

tube (Nalgene, Rochester, NY) and heated on a heating block at 95°C for 10 min. To each tube, 2 mL of boiling 70% methanol were added and heated for an extra 10 min. After cooling on ice, 0.5 mL benzylglucosinolate (1 mM) was added, mixed, and centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant (extract) was saved and the pellet was re-extracted with 2 mL 70% methanol and the extracts combined. A sample (1 mL) from each pooled extract was transferred into a glass tube. Protein was precipitated with 0.15 mL of a 1:1 mixture of 1 M lead acetate and 1 M barium acetate. Each sample was then loaded onto a DEAE Sephadex A-25 column for desulfation with arylsulfatase for 18 h. Desulfated glucosinolates were eluted from the column with water and injected onto a Lichosphere RP-18 column (Alltech Inc., Springfield, KY). Desulfoglucosinolates were eluted using a linear gradient of 1-40% acetonitrile in water at a flow rate of 1mL/min over 40 minutes. Benzylglucosinolate (C2 Bioengineering Company, Denmark) was used as an internal standard.

**Animals and Experimental Design.** The University of Illinois Animal Care and Use Committee approved the animal protocol. Ninety-nine male TRAMP mice on a pure C57BL/6 background (C57BL/6-Tg(TRAMP)8247Ng/J; The Jackson Laboratory, Bar Harbor, ME) were obtained at 5-7 weeks of age and individually housed in shoe-box cages under controlled conditions (12 h light-dark cycle, 22°C, 60% humidity). Mice were weighed weekly throughout the study. Mice were randomly assigned to 3 experimental groups (n=33). AIN-93G based experimental diets included: AIN-93G control, 10% control broccoli powder, or 10% MeJa-treated broccoli powder. Diets were balanced for protein, fat, energy, and fiber. Diet formulations are shown in Table 3.1. Food was replaced and intake was measured every other day. Fresh diets were prepared monthly. Mice were sacrificed at 20 wks of age. At the conclusion of the study, mice were asphyxiated by CO<sub>2</sub> and blood was collected by cardiac

puncture. Lungs, liver, and genitourinary tract were removed and weighed. The prostate was microdissected when possible and individual lobes were weighed and preserved in 10% formalin for histology. Lungs and sections of each liver lobe were also preserved in 10% formalin for histology.

**Preparation of microsomal and cytosolic fractions.** Livers were perfused via the portal vein with cold 1.15% KCl. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C until use. Liver samples were thawed on ice, homogenized in 3 and 1 mL cold 0.05 M Tris-HCl buffer (pH 7.4), respectively, and centrifuged for 20 min at 10,000 x g at 4 °C. Liver supernatant was further centrifuged for 60 min at 100,000 x g at 4 °C. The supernatant (cytosolic fraction) was stored at -80°C until use and the pellet (microsomal fraction) was resuspended in 1 mL cold 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, then stored at -80°C until use.

**Detoxification enzyme activity.** Activity of phase I enzyme CYP1A was measured in the microsomal fraction as ethoxyresorufin O-deethylase (EROD) activity (12) with slight modification (13). Activity of cytosolic phase II enzyme NAD(P)H-quinone oxidoreductase 1 (NQO1) was measured according to the method of Prochaska and Santamaria (14) with modification (13).

**Tissue Histology and Immunohistochemistry.** A section of each prostate lobe was preserved in 10% formalin for 24 hrs and then transferred to 70% ethanol. Formalin-fixed tissues were embedded in paraffin, sectioned, and transferred to slides. 4 µm sections for histological evaluation were stained with hematoxylin/eosin.

Pathologic grading was performed by trained pathologists at the Ohio State University who have extensive experience with transgenic mouse models of prostate cancer. Each of the

four prostate lobes (dorsal, ventral, lateral, anterior) of C57BL/6 TRAMP mice was assessed individually and were assigned two grades each ranging from 0 to 7 (Table 3.2). The first grade represented the most severe lesion within that lobe, and the second grade identified the most common lesion in the lobe. Regarding the individual grade, grade 0 was normal prostate. Grades 1, 2, and 3 represented low, moderate, and high grade PIN, respectively. Grade 4 included papillary adenomas and phyllodes-like lesions. Grades 5 and 6 represented well and moderately differentiated adenocarcinomas, respectively. Grade 7 was poorly differentiated carcinoma. The distributions of each of these lesions within the individual lobe were determined and described as focal, multifocal, or diffuse. Adjusting the lesion grades (0-7) to include an indication of distribution provided two adjusted scores, one for the most severe lesion and the other for the most common, ranging from 0 (normal) to 21 (diffuse poorly differentiated carcinoma). These adjusted scores were then added to obtain a sum that reflected the most severe lesion and its distribution and the most common lesion and its distribution (sum of the adjusted lesion scores).

Four  $\mu\text{m}$  sections were stained for PCNA (Abcam, Cambridge, MA) and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA). Images were captured and quantified as previously described (5) with the researchers blinded to treatments. Two representative images without necrosis or artifacts were captured for the dorsal and lateral lobes of the prostate. For PCNA, a proliferative index percentage, (PCNA positive/total nuclei counted) x 100, was calculated. For cleaved caspase-3 the number of apoptotic nuclei were counted at 400x magnification.

**Statistical analysis.** Data were compared among treatments by two-tailed analysis of variance, using the Mixed Models procedure of SAS Statistical Software (version 9.2; SAS

Institute, Cary, NC). Values were considered different from controls at  $P < 0.05$  using Tukey's procedure.

## RESULTS AND DISCUSSION

**Broccoli Powder Characterization.** Broccoli powders were analyzed for glucosinolate content (Table 3.3). MeJa treatment significantly increased levels of glucobrassicin, neoglucobrassicin, and gluconastrutin ( $P < 0.05$ ). Strikingly, neoglucobrassicin levels were over 7 times higher in the MeJa broccoli than in the control broccoli. This induction was much greater than what we had previously observed with a different variety of broccoli (*Brassica oleracea* var. Marathon) (6). Glucoraphanin levels were similar in both broccoli powders. However, these levels are much lower than those found in broccoli sprouts which have very high levels of glucoraphanin and minor amounts of other glucosinolates (15).

**Diet and Weight Gain.** Diets were well tolerated and there were no significant differences among groups in food intake or body weight (Table 3.4). Two animals had to be put down prior to completion of the study for health reasons unrelated to cancer development. On average mice consumed  $5.1 \pm 0.1$  g per day. The estimated daily intakes of glucoraphanin, glucobrassicin, and neoglucobrassicin per mouse were 1.2, 1.3, and 2.6  $\mu\text{mol}$  for standard broccoli, and 1.8, 1.5, and 19.9  $\mu\text{mol}$  for MeJa broccoli, respectively.

**Detoxification Enzyme Activity.** Broccoli and its glucosinolate hydrolysis products have been shown to induce phase I and phase II detoxification enzymes (16). Glucobrassicin and neoglucobrassicin, as well as their respective hydrolysis products I3C and N-methoxyindole-3-carbinol (NI3C), are known to induce CYP1A. As expected, we observed a dose-dependent increase in CYP1A activity with increasing levels of indole glucosinolates in broccoli (Figure



3.1A). MeJa broccoli feeding resulted in hepatic EROD activity 2.5 times greater than the control group ( $P<0.05$ ). Standard broccoli also significantly increased hepatic EROD activity but to a lesser magnitude, 1.5 times greater than control ( $P<0.05$ ).

Surprisingly, neither broccoli treatment increased hepatic NQO1 activity (Figure 3.1B). Sulforaphane is known to induce NQO1 activity, and broccoli has previously been documented to increase hepatic NQO1 activity in other studies (6, 16). This lack of induction may have been due to the length of the study. Previous studies showing hepatic induction of NQO1 were 7 days or fewer in length. In contrast, the present study was approximately 15 weeks in length. Previous work in our lab found no alterations in hepatic NQO1 activity after male Copenhagen rats were fed a 10% broccoli powder diet for 22 weeks (unpublished data), suggesting that induction of NQO1 may be lost following long-term feeding.

**Tissue weights.** Liver and genitourinary tract weights were not different among groups. Eleven animals developed visible prostate tumors, which engulfed the genitourinary tract: one in the control group, six in the standard broccoli group, and four in the MeJa broccoli group. Mean genitourinary tract weights were 1.00, 1.25, and 1.25 g for control, standard broccoli, and MeJa broccoli groups, respectively (Figure 3.2). Because genitourinary tract weight is a surrogate marker of tumor volume, these results indicate that broccoli feeding did not alter tumor growth. These results are in contrast to work by Keum et al. who demonstrated a significant decrease in genitourinary tract weight feeding an 8% broccoli sprout diet (17).

When possible, the prostate was microdissected into four individual lobes (ventral, lateral, dorsal, anterior) and each lobe was weighed. Total prostate weight is the sum of these weights, and we observed a small but significant decrease in animals fed either broccoli powder (data not shown). Mean prostate weights were: 0.136 g in the control group, 0.119 g in the

broccoli group ( $P < 0.05$  vs. control), and 0.118 g in the MeJa broccoli group ( $P < 0.05$  vs. control,  $P = 0.99$  vs. broccoli). However, these results are inconclusive because our sample was biased by the removal of animals that developed prostate tumors which engulfed the genitourinary tract. Because more animals were removed from the two broccoli groups, this would likely skew our sample to animals with smaller prostates.

**Proliferation and apoptosis.** There were no significant differences among groups in prostatic proliferation or apoptosis as measured via PCNA and cleaved caspase-3 (Figure 3.3). These findings are not surprising in light of our data showing no alterations in genitourinary tract weight. Our values are within the normal range of proliferative index and apoptotic index values observed in TRAMP mice. Previous studies have reported decreased proliferation when sulforaphane or DIM were administered via oral gavage (18, 19).

**Histology.** Prostate cancer progression varied by lobe as expected (20). The lateral prostate had mostly diffuse well differentiated carcinomas while the dorsal prostate had predominantly diffuse high grade PIN. Within each prostate lobe, no significant differences in adjusted lesion score were observed, indicating that broccoli feeding did not impact prostate cancer progression (Table 3.5). This was surprising given that studies with sulforaphane, 3,3'-diindolylmethane (DIM), PEITC, and broccoli sprouts had all shown reduced cancer progression in the TRAMP model (18, 19, 21)(E. Ho, personal communication, April 16, 2011). Singh et al. observed reductions in the incidence of PIN and well differentiated (WD) carcinomas when TRAMP mice were gavaged with 6  $\mu\text{mol}$  sulforaphane (18). Cho et al. gavaged TRAMP mice with 3  $\mu\text{mol}$  of DIM and saw increased incidence of PIN and decreased incidence of WD carcinoma, indicating a slowing of prostate cancer progression (19). Powlony et al. reported decreased incidence of poorly differentiated (PD) cancers when TRAMP mice received

approximately 15  $\mu\text{mol}$  of PEITC per day in their diet (21). Additionally, an unpublished study from Dr. Emily Ho's laboratory found that feeding a 15% broccoli sprout diet decreased incidence of WD carcinomas and increased incidence of PIN (personal communication, April 16, 2011). All studies varied in the treatment, delivery route, background strain, and age of mice at study termination making it difficult to directly compare results (Table 3.6 and 3.7). However, the literature thus far seems to be supportive for the role of broccoli sprouts or sulforaphane in reducing prostate carcinogenesis, perhaps suggesting that high sulforaphane levels may be key to the beneficial effects of crucifers with indole glucosinolates being less influential. Work by Cho et al. demonstrates that DIM, a breakdown product of the indole glucosinolate glucobrassicin can reduce prostate carcinogenesis (19), suggesting that indole glucosinolates can be effective, but it is unknown how the level of this pure compound compares to the dose formed upon consumption of whole broccoli.

In the current study, mice received whole glucosinolates in the broccoli powder matrix. However, other studies have used pure glucosinolate hydrolysis products such as sulforaphane, DIM, and PEITC. The formation of these glucosinolate hydrolysis products from glucosinolates depends on several factors including levels in the plant material, myrosinase activity, pH, and the stability of the compounds (22). Thus it is difficult to directly compare glucosinolate levels with those of a pure compound, however we would speculate that conversion is not one hundred percent efficient. The amount of glucosinolates consumed per day by the animals in this study are similar to levels in our previous study in the Dunning model (5). However they are 2-8 times lower than the corresponding dose of glucosinolate hydrolysis product received by TRAMP mice in other reports (Table 3.7). It is possible that there may be low conversion of glucosinolates to isothiocyanates from the broccoli powder used in this study, and it would be useful to examine

hydrolysis product formation. These discrepancies highlight the importance of considering dietary levels and the impact of chemical form when conducting studies with dietary bioactives.

This is only the second study to evaluate the effects of whole broccoli on prostate cancer in an animal model. In summary, neither broccoli treatment significantly reduced prostate carcinogenesis in TRAMP mice. However, we had previously reported that whole broccoli with similar levels of glucosinolates was effective in reducing tumor growth in the Dunning R3327-H model of prostate carcinogenesis (5). This difference may be due to the aggressive nature of the TRAMP model. Optimal patterns of phytochemicals for reducing prostate carcinogenesis remain to be characterized.

#### **ACKNOWLEDGMENTS**

We thank Dr. Jack Juvik and Kang Mo Ku at the University of Illinois for overseeing the production of broccoli and performing glucosinolate analysis; Dr. Matt Wallig for providing immunohistochemistry expertise; Sonja Volker for performing liver detoxification enzyme assays.

**Table 3.1.** AIN-93G based diet formulations.

	g/100g total diet		
	Control	Broccoli (Control)	Broccoli (MeJa)
Cornstarch	39.7	36.6	36.6
Casein	20.0	16.8	16.8
Maltodextrin	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0
Fiber*	5.0	1.6	1.6
Mineral mix**	3.5	3.5	3.5
Vitamin mix***	1.0	1.0	1.0
L-Cystine	0.3	0.3	0.3
Choline bitartrate	0.3	0.3	0.3
Soybean oil	7.0	6.7	6.7
Control Broccoli powder		10.0	
MeJa Broccoli powder			10.0

\*Non-nutritive cellulose

\*\*AIN93M-MX formulation

\*\*\*AIN93-VX formulation

**Table 3.2.** Grading scheme for adjusted lesion score.

<b>Pathology</b>	<b>Grade</b>	<b>Distribution</b>	<b>Adjusted Score</b>
Normal	0	Diffuse	0
Low grade PIN	1	Focal	1
	1	Multifocal	2
	1	Diffuse	3
Moderate grade PIN	2	Focal	4
	2	Multifocal	5
	2	Diffuse	6
High grade PIN	3	Focal	7
	3	Multifocal	8
	3	Diffuse	9
Phylloides-like tumor	4	Focal	10
	4	Multifocal	11
	4	Diffuse	12
Well differentiated carcinoma	5	Focal	13
	5	Multifocal	14
	5	Diffuse	15
Moderately differentiated carcinoma	6	Focal	16
	6	Multifocal	17
	6	Diffuse	18
Poorly differentiated carcinoma	7	Focal	19
	7	Multifocal	20
	7	Diffuse	21

Total adjusted lesion score = score of most severe lesion + score of most common lesion

**Table 3.3.** Glucosinolate profile of broccoli powders.

	$\mu\text{mol/g}$ dry weight	
	Standard Broccoli	MeJa Broccoli
Glucoraphanin	$2.49 \pm 0.59$	$3.64 \pm 0.59$
Glucobrassicin	$2.61 \pm 0.07$	$3.06 \pm 0.07^*$
Neoglucobrassicin	$5.23 \pm 0.67$	$39.77 \pm 0.67^*$
Gluconastrurtiin	$0.87 \pm 0.19$	$3.79 \pm 0.19^*$
Glucoiberin	$0.32 \pm 0.08$	$0.49 \pm 0.08$
Progoitrin	$0.46 \pm 0.07$	$0.49 \pm 0.07$
Sinigrin	$0.24 \pm 0.02$	$0.22 \pm 0.02$
Gluconapin	$0.50 \pm 0.03$	$0.41 \pm 0.03$

Values are means  $\pm$  SEM, n=3.

\* within rows indicate significant differences between treatments ( $P < 0.05$ ).

**Table 3.4.** Final body weight, food intake, and weight gain.

<b>Treatment Group</b>	<b>Final Body Weight</b>	<b>Food Intake</b>	<b>Weight Gain</b>
Control	34.7 ± 0.7	5.1 ± 0.1	14.6 ± 0.7
Standard Broccoli	35.4 ± 0.8	4.8 ± 0.1	15.6 ± 0.7
MeJa Broccoli	34.8 ± 0.7	4.9 ± 0.1	14.7 ± 0.7

Values are means ± SEM, n=32-33.



**Table 3.5.** Adjusted lesion scores of prostate lobes.

<b>Treatment Group</b>	<b>Ventral</b>	<b>Lateral</b>	<b>Dorsal</b>	<b>Anterior</b>
Control	11.5 ± 1.6	25.9 ± 1.1	18.1 ± 0.2	9.0 ± 0.5
Standard Broccoli	15.4 ± 2.4	29.5 ± 1.3	22.2 ± 1.6	10.4 ± 1.2
MeJa Broccoli	12.5 ± 2.1	26.2 ± 1.5	20.7 ± 1.5	8.8 ± 0.6

Values are means ± SEM, n=31-33. Scale ranges from 0 (normal prostate tissue) to 42 (diffuse poorly differentiated carcinoma).

**Table 3.6.** Summary of studies with broccoli or broccoli compounds in the TRAMP model.

Study	Compound	Delivery Route	Diet	Age at sacrifice	Background strain	Outcome
Liu (current study)	Broccoli	10% powder in diet	AIN-93G	20 wks	C57BL/6	No effect
Singh 2009 (18)	Sulforaphane	6 $\mu$ mol gavage every other day	Chow	24 wks	C57BL/6 X FVB	Decreased PIN, WD carcinoma, and pulmonary metastases
Keum 2009 (17)	Broccoli sprout	8% powder in diet	AIN-76A	24 wks	C57BL/6	Decreased GU tract weight
Ho (unpublished)	Broccoli sprout	15% powder in diet	---	12 and 28 wks	C57BL/6	Decreased WD carcinoma and increased PIN at 28 wks
Cho 2010 (19)	3,3'-diindolylmethane	1.5 or 3.0 $\mu$ mol gavage every other day	Chow	22 wks	C57BL/6	Decreased WD carcinoma, increased PIN at the 3.0 $\mu$ mol dose level
Powolny 2010 (21)	Phenethyl isothiocyanate	3 $\mu$ mol/g in diet	AIN-76A	24 wks	C57BL/6 X FVB	Decreased PD carcinoma

GU: genitourinary tract

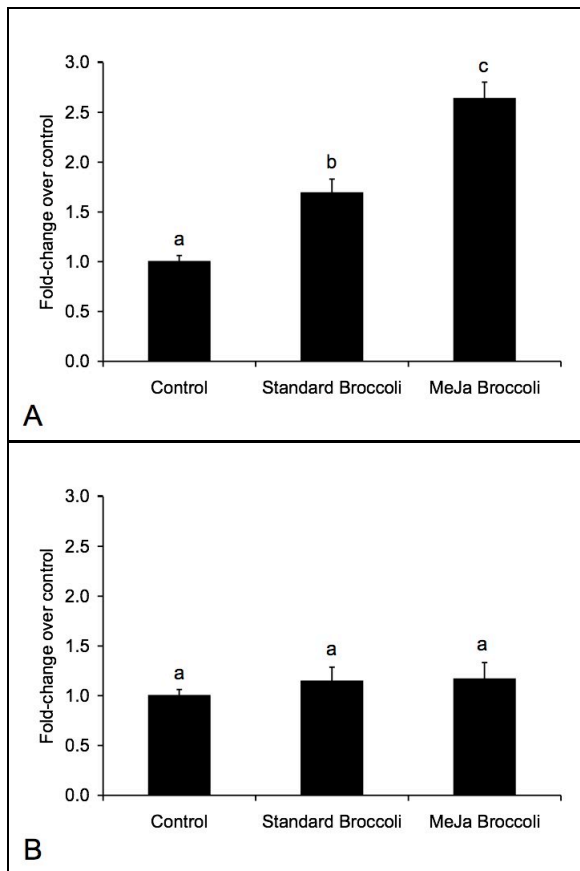
PIN: prostatic intraepithelial neoplasia

WD: well differentiated carcinoma

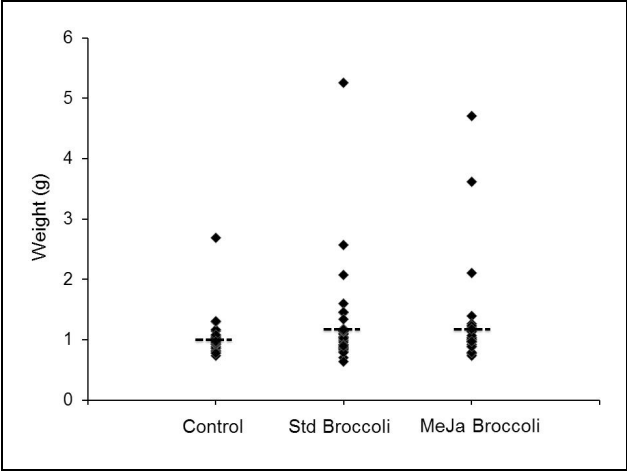
PD: poorly differentiated carcinoma

**Table 3.7.** Dose comparison with other studies.

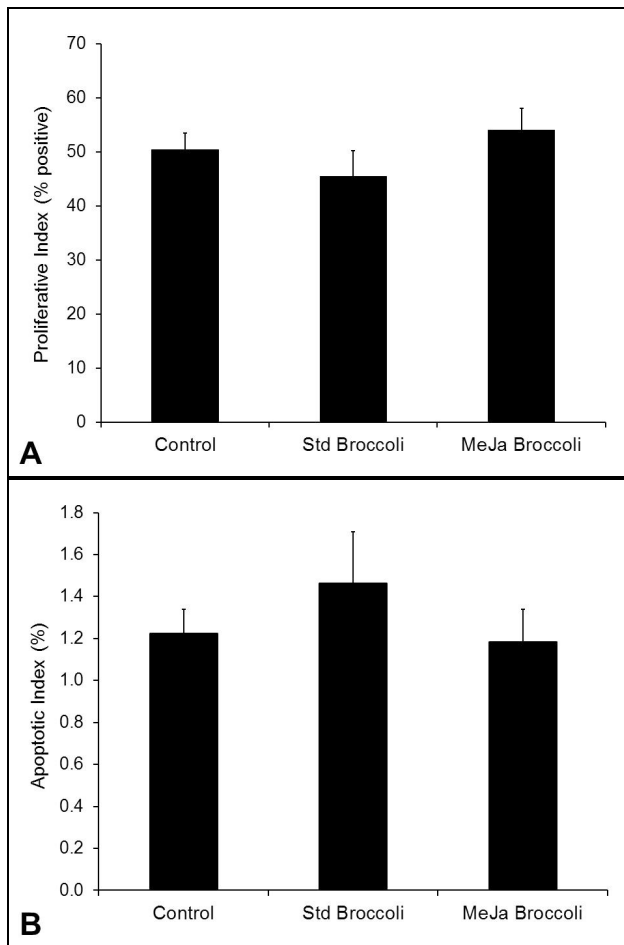
<i>Estimated daily intake (<math>\mu\text{mol}/\text{day}/\text{mouse}</math>), based on average food intake of 5.1 g/day</i>			
<b>Glucosinolate</b>	<b>Standard Broccoli</b>	<b>MeJa Broccoli</b>	<b>Other Studies</b>
Glucoraphanin	1.2	1.8	6 $\mu\text{mol}$ gavage of SF every other day (Singh 2009.) Estimate 11.3 $\mu\text{mol}$ SF/day from broccoli sprout diet (personal communication, E. Ho)
Glucobrassicin	1.3	1.5	3 $\mu\text{mol}$ gavage of DIM every other day (Cho 2009)
Neoglucobrassicin	2.6	19.9	
Gluconasturtiin	0.4	1.9	15 $\mu\text{mol}$ PEITC/day (Powolny 2010)



**Figure 3.1.** **A:** Liver ethoxyresorufin O-deethylase (EROD) activity, **B:** Liver NAD(P)H-quinone oxidoreductase 1 (NQO1) activity. Results shown are means  $\pm$  SEM, n=8. Samples were run in triplicate. For each enzyme, different letters above bars indicate significant differences between treatments ( $P < 0.05$ ).



**Figure 3.2.** Genitourinary tract weight. Dashed lines indicate mean values, n=32-33.



**Figure 3.3.** Proliferation (A) and apoptosis (B) rates of TRAMP prostates. Results are shown as means  $\pm$  SEM, n=14.

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## CHAPTER 4

### LYCOPENE AND APO-10'-LYCOPENAL DO NOT ALTER DNA METHYLATION OF GSTP1 IN LNCAP CELLS

#### ABSTRACT

DNA hypermethylation and silencing of tumor-suppressor genes are commonly seen in human cancers, and likely contribute to the process of carcinogenesis. A growing body of evidence suggests that dietary compounds may alter cancer risk through epigenetic modifications. Glutathione S-transferase P1 (GSTP1) is hypermethylated in >90% of prostate cancer cases making it one of the most common genome alterations in prostate cancer. LNCaP cells were treated either with lycopene or apo-10'-lycopenal for 7 days, and mRNA expression and DNA methylation of GSTP1 were evaluated. Neither compound significantly altered expression or methylation of GSTP1 while treatment with 5-azacytidine decreased methylation by 78%. These findings demonstrate that lycopene and apo-10'-lycopenal are not effective demethylating agents of GSTP1 in the human LNCaP cell line.

#### INTRODUCTION

Prostate cancer is the most commonly diagnosed male cancer and second leading cause of male cancer deaths in United States with approximately 186,000 new cases diagnosed each year (1). Environmental exposures can alter gene expression through epigenetic modifications and these modifications may in turn alter disease susceptibility (2). The two major types of epigenetic changes are modifications to DNA, in the form of DNA methylation, and modifications to chromatin packaging, specifically histone modification.

DNA methylation is critical for regulation of gene transcription and structure of the cell nucleus. Aberrant DNA methylation patterns are commonly seen in human cancers which often

consist of global hypomethylation of DNA accompanied by hypermethylation of specific tumor-suppressor genes (3). Currently, the methylation status of glutathione S-transferase P1 (GSTP1) is being investigated to determine if it can serve as an early and specific biomarker of prostate cancer (4, 5).

Glutathione S-transferases (GSTs) are a large family of detoxification enzymes which catalyze conjugation reactions with reduced glutathione. They play an important role in protecting DNA from reactive electrophilic compounds and oxidants. GSTP1 is hypermethylated in >90% of prostate cancer cases and 70% of prostatic intraepithelial neoplasia (PIN) lesions making it one of the earliest and most commonly found genomic alterations in prostate cancer (5). Mice that are null for GSTP1 develop skin tumors at significantly increased rates compared to wild type mice when exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (6). GSTP1 is completely silenced in LNCaP human prostate cancer cells due to hypermethylation (7). When these cells are exposed to the carcinogenic heterocyclic amine 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), high levels of PhIP-DNA adducts are formed, but when the cells are modified to express GSTP1 they are resistant to the formation of PhIP-DNA adducts (8). These data demonstrate that GSTP1 may play a key role in the prevention of cancer formation.

Recent data suggest that dietary compounds may prevent cancer through epigenetic modifications in the cell (9, 10). Tomatoes are the second most commonly consumed vegetable in the American diet second only to potatoes (11). In 2007, the average American consumed 19.2 pounds of fresh tomatoes and 68.8 pounds of processed tomato products (12). Epidemiological studies have linked higher consumption of tomatoes and tomato products with decreased risk of prostate cancer (13). In the Health Professionals Follow-Up Study, men who

consumed >2 servings of tomato sauce per week had a 23% reduction in risk of prostate cancer vs men who consumed <1 serving per month (RR = 0.77; 95% CI = 0.66 to 0.90;  $P_{\text{trend}} < .001$ ) (14). The anti-carcinogenic effects of tomatoes are often associated with lycopene, the major carotenoid found in tomatoes. Lycopene has been shown to be a potent antioxidant and exert several other anti-carcinogenic effects *in vitro* including induction of cell cycle arrest (15). However, the ability of lycopene to act through epigenetic alterations has thus far only been examined in one study where lycopene was shown to partially demethylate the GSTP1 promoter and restore GSTP1 expression in breast cancer cell lines (16). Thus lycopene may also be able to prevent or slow prostate cancer growth through alterations in DNA methylation patterns. Here we investigate whether lycopene or one of its reported metabolites, apo-10'-lycopenal, can restore expression of GSTP1 in prostate cancer cell lines through demethylation of the GSTP1 promoter.

## **MATERIALS AND METHODS**

**Cell lines and Culture Conditions.** The LNCaP cell line was originally isolated in 1977 by J.S. Horoszewicz, et al., from a needle aspiration biopsy of the lymph node of a 50-year-old Caucasian male with a confirmed diagnosis of metastatic prostate carcinoma (17). The cells are androgen receptor positive and responsive to dihydrotestosterone. LNCaP cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 media (Sigma-Aldrich, St, Louis, MO) containing 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 5.6 mg/ml amphotericin B. Cells were maintained at 37°C with 5% CO<sub>2</sub> and atmospheric oxygen partial pressure.

**Carotenoid Preparation.** Doses of lycopene and apo-10'-lycopenal were prepared fresh daily. For lycopene, water-soluble lycopene beadlets (5.7% pure lycopene, DSM Nutritional Products, Basel, Switzerland) were dissolved in deionized water and added to 10 mL of media. Apo-10'-lycopenal was dissolved in DMSO and added to 5 mL of media. The resulting stock solutions were sonicated and filtered through a 0.22  $\mu\text{m}$  sterile filter. Solubilization was confirmed through quick observation under a light microscope. Stock lycopene or apo-10'-lycopenal concentrations were determined by extracting 100  $\mu\text{L}$  of media and measuring absorbance via a spectrophotometer (lycopene  $\lambda_{\text{max}} = 472 \text{ nm}$ ,  $A_{1 \text{ cm}}^{1\%} = 3450$  in hexane; apo-10'-lycopenal  $\lambda_{\text{max}} = 460 \text{ nm}$ ,  $A_{1 \text{ cm}}^{1\%} = 2684$  in hexane). All carotenoid extracts were kept on ice and under yellow lights throughout the extraction process.

**Experimental Design.** In the first experiment, cells were dosed daily with lycopene at 1, 2, or 4  $\mu\text{M}$  for seven days. Placebo beadlets were used as a vehicle control. In the second experiment, cells were dosed daily with apo-10'-lycopenal at 0.01, 0.1, or 1  $\mu\text{M}$  for seven days, and 0.1 % DMSO was used as a vehicle control. In both experiments, media were changed daily to minimize carotenoid degradation. Three independent replicates were performed for each assay, with  $n=3$  per dose level.

5-azacytidine (Sigma-Aldrich, St. Louis, MO) is a known demethylating agent and was used as a positive control. Cells were dosed with 5  $\mu\text{M}$  5-azacytidine every other day (18). All experiments were carried out under dim light.

**Carotenoid Analysis.** Cells were rinsed with PBS and pelleted. For cell extraction, cells were resuspended in 100  $\mu\text{L}$  of water, and 500  $\mu\text{L}$  of acetone was added. Samples were sonicated for 10 min, centrifuged at 7000  $\times g$  for 5 min, and the supernatant was transferred to new tube. The acetone extract was dried down with argon and stored at  $-20^{\circ}\text{C}$  for  $\leq 24 \text{ h}$  prior to

HPLC-PDA analysis. For medium extraction, 1.0 mL ethanol containing 0.1% butylated hydroxytoluene was added to 1.0 mL medium and vortexed. Media samples were extracted 3 times with 2.0 mL hexane. Hexane extracts were dried in a Speedvac evaporator (model AS160; Savant, Farmingdale, NY), flushed with argon, and stored at -20°C for ≤24 h prior to HPLC-PDA analysis. All carotenoid extracts were kept on ice and under yellow lights throughout the extraction process.

Lycopene concentrations were analyzed by a reverse-phase HPLC-PDA system previously described (19). Apo-10'-lycopenal concentrations were analyzed using a similar system. The system consisted of two Ranin Dynamax pumps (Model SD-200; Varian, Walnut Creek, CA), a C30 column (4.6 × 250 mm, 3 μm, YMC, Wilmington, NC) with a guard column, an 18°C column oven and a photodiode array detector (Model 2996, Waters, Milford, MA) and Millennium<sup>32</sup> software (Waters). Mobile phase A (methanol: 15% ammonium acetate aqueous solution= 98:2, v/v) and mobile phase B (methanol: MTBE: 15% ammonium acetate aqueous solution= 8:90:2, v/v) were used, and the gradient profile was as follows: 0 min, 0%B; 30 min, 100%B; 40 min, 100%B; 45 min, 0%B; 50 min, 0%B. Samples were reconstituted in MTBE for injection, and the apo-10'-lycopenal was measured at 460 nm. All analyses were performed in duplicate, and the quantification of carotenoid isomers was carried out by comparing the UV spectra and retention times to analytical standards of lycopene or apo-10'-lycopenal (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany).

**Real-time quantitative PCR.** Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix

(Applied Biosystems, Foster City, CA). Reactions were monitored by an ABI Prism 7900HT. Primer pairs were designed to measure glutathione-S-transferase  $\pi$  1 (GSTP1) (8) and ribosomal protein L7a (RPL7A) (20). A validation experiment was performed on each set of primers (Integrated DNA Technologies, Coralsville, IA) to confirm efficiency and product specificity. A serial dilution was used to create a standard curve for quantification. RPL7A was used as a housekeeping gene (20).

**Methylation-specific PCR.** Total genomic DNA was extracted from cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). DNA was bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). MethPrimer (<http://www.urogene.com/methprimer>) was used to design primer pairs to measure methylated GSTP1 forward-5'-GGAAAGAGGGAAAGGTTTTTTC-3' and reverse-5'-GCCCTAAAATCCCCGAA-3' and unmethylated GSTP1 forward-5'-GGAAAGAGGGAAAGGTTTTTTT -3' and reverse-5'-AACACCCTAAAATCCCCAAA -3'. A serial dilution was used to create a standard curve for quantification. Reactions were monitored by an ABI Prism 7900HT.

**Statistical Analysis.** Dose-response effects were analyzed by linear regression, using the REG procedure of SAS Statistical Software (version 9.2; SAS Institute, Cary, NC), and values were considered different at  $P < 0.05$ . 5-azacytidine treated cells were compared among treatments by two-tailed analysis of variance, using the MIXED procedure. Values were considered different from controls at  $P < 0.05$  using Tukey's procedure.

## RESULTS AND DISCUSSION

**Cellular Uptake of Carotenoids.** Lycopene was provided at doses considered to be physiologically relevant (1 and 2  $\mu$ M) and super-physiological (4  $\mu$ M). Lycopene was taken up

by cells in a dose-dependent manner as shown in Figure 4.1 ( $P = 0.008$ ). The average lycopene content was 42, 61, and 78 pmol per million cells for 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , and 4  $\mu\text{M}$  dose levels, respectively. These levels are similar to those observed in previous studies with lycopene dosing of LNCaP cells (21).

When lycopene is enzymatically cleaved by carotenoid monooxygenase II, it yields one molecule of apo-10'-lycopenal. Concentrations of apo-10'-lycopenal are much lower in human serum than lycopene. In human subjects consuming a high tomato juice diet for 8 weeks, the average concentration of apo-10'-lycopenal was 0.28 nM (22). Even though the dose levels used in our study are relatively low compared to the concentrations sometimes used by others in cell culture experiments, our dose levels would still be considered super-physiological. The average apo-10'-lycopenal content of was 2.5 and 2.3 pmol per million cells for 1  $\mu\text{M}$  and 0.1  $\mu\text{M}$  dose levels, respectively, indicating very poor absorption by LNCaP cells. At the 0.01  $\mu\text{M}$  dose level, no apo-10'-lycopenal could be detected in the cells (data not shown). Thus the cellular concentration in LNCaP cells was considerably below what has been reported in human serum. There have been no reports of cellular levels of apo-10'-lycopenal, but our lab has previously identified apo-8'-lycopenal and apo-12'-lycopenal in the liver of rats following lycopene feeding (23).

**GSTP1 Expression and Promoter Methylation.** Expression of GSTP1 and DNA methylation of the GSTP1 promoter were not significantly altered by treatment with either lycopene or apo-10'-lycopenal (Figures 4.2 and 4.3). Treatment with 5-azacytidine resulted in significant demethylation and increased expression of GSTP1 as expected ( $P < 0.05$ ). These results indicate that lycopene is not an effective demethylating agent in LNCaP cells. It is possible that lycopene could have epigenetic effects in different prostate cancer cell lines or on



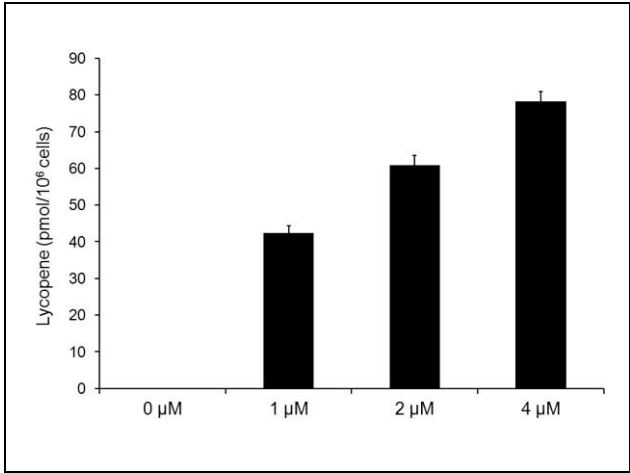
different genes. It is well known that the degree of hypermethylation of various genes varies by cell line (24). King-Batoon et al. demonstrated that a single dose of 2  $\mu$ M lycopene partially demethylated the GSTP1 promoter in MDA-MB-468 cells but not in MCF-7 cells. The effect was also specific to the GSTP1 as RAR $\beta$ 2, another gene hypermethylated in breast cancer, was not affected by lycopene treatment (16).

Using lycopene in cell culture can be difficult due to issues with stability and solubility. Multiple systems have been used to deliver carotenoids *in vitro*, and differences could potentially arise due to different delivery methods. Here we used water-soluble lycopene beadlets containing approximately 65% all-trans lycopene and 35% cis-isomers, and media was changed daily to minimize carotenoid degradation. However, King-Batoon et al. used tetrahydrofuran as a solvent and gave a single lycopene dose at the beginning of the 7-day study. Lycopene solubilized in THF degrades rapidly in cell culture conditions with a half-life of less than 2 hr (21). In contrast, when water-soluble lycopene beadlets are used as a delivery vehicle, approximately 80% of the original dose remains in the medium after 24 hours (25). This variation with *in vitro* techniques can make interpretation of the literature somewhat confusing.

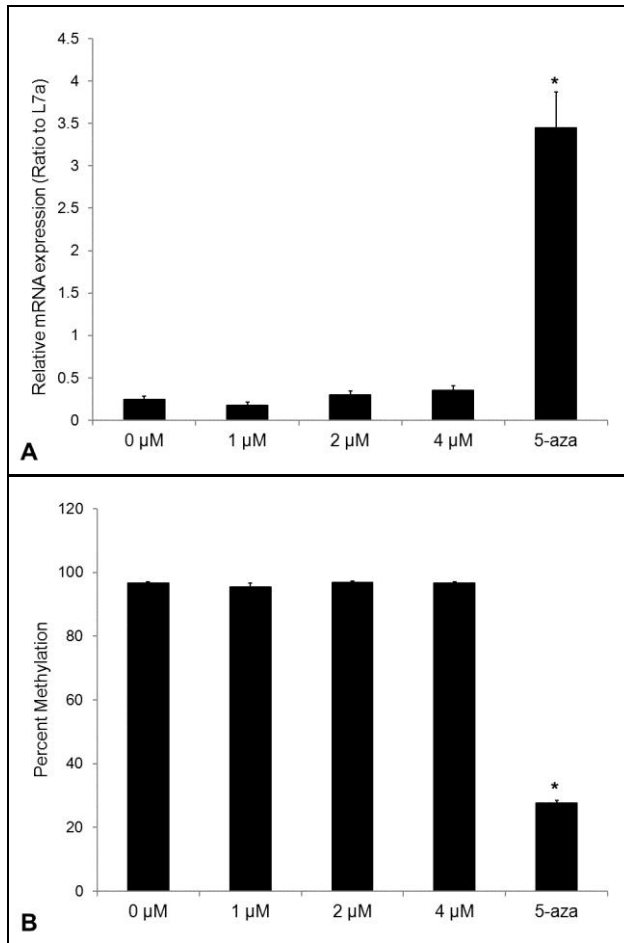
In summary, we have shown that lycopene and apo-10'-lycopenal are taken up by LNCaP cells but do not alter DNA methylation of GSTP1. Further research is needed to determine whether lycopene might exert epigenetic effects on other tumor suppressor genes in LNCaP cells or in other prostate cancer cell lines.

## ACKNOWLEDGMENTS

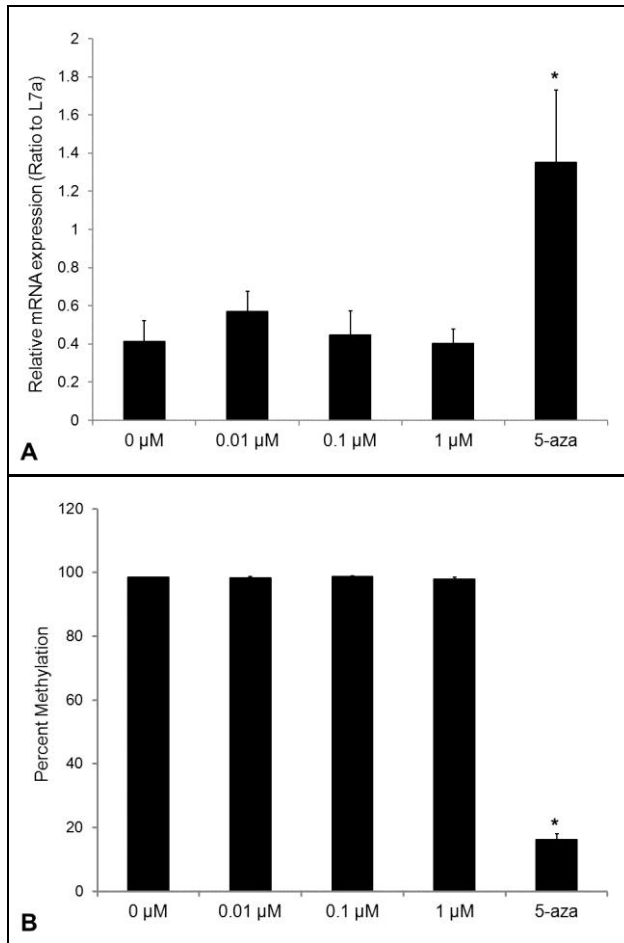
We thank Dr. Hong Chen and Rita Strakovsky for providing guidance on methylation analysis.



**Figure 4.1.** Lycopene concentration in LNCaP cells. Values are means  $\pm$  SEM, n=3.



**Figure 4.2.** GSTP1 mRNA expression (A) and GSTP1 promoter methylation (B) of LNCaP cells treated with lycopene. Values are means of three independent experiments  $\pm$  SEM, n=3. Asterisk indicates significant differences ( $P < 0.05$ ).



**Figure 4.3.** GSTP1 mRNA expression (A) and GSTP1 promoter methylation (B) of LNCaP cells treated with apo-10<sup>3</sup>-lycopenal. Values are means of three independent experiments  $\pm$  SEM, n=3. Asterisk indicates significant differences ( $P < 0.05$ ).

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## CHAPTER 5

### SUMMARY AND FUTURE DIRECTIONS

Prostate cancer is the most commonly diagnosed male cancer in the United States.

Dietary intervention could play an important role in delaying prostate cancer growth since it is a slow growing cancer that takes decades to develop. Although individual bioactives have been extensively studied for their anti-carcinogenic properties, most people consume these bioactives within the diet as part of a whole food. Recently, some studies have shown that increasing the content of specific bioactives with anti-carcinogenic properties in whole tomatoes or broccoli enhances their efficacy in reducing the development of various cancers. Increasing the cancer preventive potential of tomatoes and broccoli is especially of interest because over half the U.S. population eats less than the recommended 5 or more servings of fruits and vegetables per day.

The objective of study 1 (Chapter 2) was to obtain tomato and broccoli powders with enhanced levels of specific bioactives and screen them for bioactivity. We successfully obtained tomato powders with significantly increased levels of lycopene alone or total carotenoids as well as broccoli powders with increased levels of indole glucosinolates or selenium. When rats were fed diets containing these powders in a short-term feeding study, higher carotenoid content in the tomato powders resulted in greater hepatic carotenoid accumulation. Both indole glucosinolate-enriched broccoli and selenium-enriched broccoli significantly increased hepatic activity of phase I and phase II detoxification enzymes. These results indicate that the bioactive profile of tomatoes and broccoli can be altered through agronomic means, and these alterations can result in increased tissue accumulation and enhanced bioactivity *in vivo*.

The objective of study 2 (Chapter 3) was to test the efficacy of standard broccoli and MeJa-treated broccoli, which was high in indole glucosinolates, in reducing prostate



carcinogenesis in the TRAMP model. While MeJa treatment increased the level of indole glucosinolates 5 times compared to standard broccoli, neither broccoli treatment significantly reduced prostate carcinogenesis as measured via genitourinary tract weight and pathologic score. Prostatic proliferation and apoptosis were also unchanged by broccoli feeding. These findings were contrary to our hypothesis that broccoli feeding would result in reduced prostate carcinogenesis. It may be that our glucosinolate levels were too low for this aggressive transgenic model. Other studies in the same model, which reported reductions in prostate carcinogenesis, have used higher doses or pure compounds or fed broccoli sprouts, which have a different phytochemical profile compared to whole broccoli.

In study 3 (Chapter 4) we examined whether lycopene or apo-10'-lycopenal could epigenetically modify silenced tumor suppressor genes in LNCaP prostate cancer cells. Neither treatment altered DNA methylation or mRNA expression of GSTP1, one of the most commonly hypermethylated and silenced genes in prostate cancer. While this study does not rule out the possibility of epigenetic modifications by lycopene, further research is needed to determine whether lycopene might alter methylation of other tumor suppressor genes in LNCaP cells or in other cell lines.

Overall, we have demonstrated that horticultural manipulation of tomatoes or broccoli to alter phytochemical profiles is a feasible approach to optimize cancer prevention potential. However it remains to be determined what the optimal levels of phytochemicals are for reducing carcinogenesis. While neither broccoli treatment reduced prostate carcinogenesis in the TRAMP model, it is possible that either standard or MeJa broccoli could be effective in a less aggressive cancer model.

In the future it would be useful to utilize different models to examine the effects of tomatoes and/or broccoli on different stages of prostate carcinogenesis. The effect of broccoli on cancer initiation would be particularly interesting to look at because certain broccoli bioactives are known to induce phase I and phase II detoxification enzymes. These detoxification systems are critical both for activation of pro-carcinogens and for the removal of carcinogens from the body. The balance between competing activating and detoxifying reactions may play a critical role in the amount of carcinogen in the body available to react with targets such as DNA.

Whereas in the work presented here, there was no effect of broccoli on prostate carcinogenesis, previous work by our lab in the Dunning model demonstrated that broccoli feeding significantly reduced the growth of transplantable tumors indicating that whole broccoli powder can exert effects on prostate cancer progression (1). Thus current evidence is mixed on broccoli's ability to reduce prostate cancer progression possibly due to differences in models.

Finally, there is some evidence to indicate that broccoli may also be able to slow metastatic spread of prostate cancers. A prospective study of men in the screening arm of the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial found that men with higher broccoli intakes had reduced risk for developing stage III or IV tumors, which are cancers that have spread outside of the prostate (2). Additionally, Singh et al. found that dosing TRAMP mice with sulforaphane reduced incidence of pulmonary metastases (3). While this evidence is by no means definitive, it does suggest the possibility that broccoli may be able to reduce the spread of prostate cancer to other organs. We plan on determining metastasis incidence in the lungs and livers of animals from our study fed control, standard broccoli powder, or MeJa broccoli powder in collaboration with our colleagues at the Ohio State University.

Much remains to be determined with regards to possible epigenetic effects of tomato and broccoli compounds. Virtually nothing is known about whether tomatoes or individual carotenoids exert epigenetic effects. While one study has reported decreased methylation of GSTP1 in breast cancer cells with lycopene treatment, the effect was not consistent over all cell lines suggesting that effectiveness can vary widely depending on the type or subtype of cancer as well as on the specific gene being examined (4). It would be useful to define the effects of lycopene and other tomato constituents on several genes in several prostate cancer cell lines, or to perform in vivo work to examine this effect. However, the feasibility of in vivo work may be limited by model constraints. We considered examining GSTP1 methylation in our TRAMP study, but found that TRAMP mice do not develop GSTP1 hypermethylation as humans do. Thus, not all models may be appropriate for studying the hypermethylation of tumor suppressor genes in cancer.

While lycopene may alter DNA methylation in some cancers, sulforaphane has been shown to act through alterations in histone acetylation. Work by Myzak et al. clearly demonstrated that sulforaphane was inhibitor of histone deacetylase (HDAC), an enzyme which removes acetyl groups from chromatin leading to chromatin condensation and transcriptional repression (5). By inhibiting HDAC, sulforaphane relieves this transcriptional repression so that genes can be transcribed normally. Most of the work thus far has been done with pure sulforaphane, and it would be interesting to examine whether this inhibition of HDAC is seen with whole broccoli as well.

In the end, humans consume whole foods so it is important that research should reflect the foods that people actually consume at levels that are achievable through dietary consumption.

Much remains to be learned about how whole foods can impact the development of prostate cancer over a lifetime and possible mechanisms by which they may exert their effects.

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**Graduate Research Assistant**, *Division of Nutritional Sciences, Department of Food Science and Human Nutrition, University of Illinois*, (July 2006 – present).

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- Determined effects of standard broccoli and methyl jasmonate-treated broccoli on prostate carcinogenesis in the TRAMP model
- Analyzed effects of lycopene and apo-10'-lycopenal on tumor-suppressor gene expression and DNA methylation in prostate cancer cells
- Presented findings at national meetings and published results in scientific journals
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**Intern**, *Abbott Nutrition*, (August 2010 – present)

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## **PUBLICATIONS**

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Hagiwara, N, Yeh, M, **Liu, A**. Sox6 is required for normal fiber type differentiation of fetal skeletal muscle in mice. *Dev Dyn*. 2007 Aug;236(8):2062-76.

## **ABSTRACTS**

**Liu, AG**, Berman-Booty, LD, Clinton, SK, Jeffery, EH, Erdman Jr., JW. (2011) Methyl jasmonate-treated broccoli and prostate carcinogenesis in TRAMP mice. *FASEB J*. 25: 977.8. Presented as a poster presentation.

**Liu, AG**, Volker, SE, Jeffery, EH, Erdman Jr., JW. (2009) Bioaccumulation of carotenoids and sulforaphane conjugates from tomato and broccoli powders with differing bioactive profiles. *FASEB J*. 23: 104.1. Presented as an oral presentation.

Volker, SE, **Liu, AG**, Erdman Jr., JW, Jeffery, EH. (2009) Enrichment of broccoli and tomato with bioactive components: effects on bioactivity markers in vivo. *FASEB J*. 23: 561.2.

**Liu, A**, Nakamura, MT, Yudell, BE, Erdman Jr., JW. (2007) Short term consumption of lycopene and phytofluene decreases PPAR $\alpha$  and PPAR $\gamma$  expression in selected rat tissues. *FASEB J*. 21: 102.4. Presented as an oral presentation.

## **PROFESSIONAL COMMITTEES**

Nutritional Sciences Symposium Planning Committee, 2008, 2009, 2010

- Managed keynote speaker selection and recruitment
- Coordinated oral and poster competitions

Nutritional Sciences Graduate Student Association, Secretary, 2007

- Recorded and organized meeting minutes
- Helped plan various events including professional, social, and fundraising activities

## **HONORS AND AWARDS**

Division of Nutritional Sciences, Margin of Excellence Research Award, UIUC, 2008, 2010

Division of Nutritional Sciences, Margin of Excellence Travel Award, UIUC, 2007, 2009, 2011

Graduate College Travel Award, UIUC, 2009

Jonathan Baldwin Turner Graduate Fellowship, UIUC, 2006-2009

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