The Impact of Cruciferous Vegetable and Soy Phytochemicals on Prostate Cancer Cell Progression

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Research Distinction in Human Nutrition at The Ohio State University College of Education and Human Ecology

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June 2012

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I. Abstract

Background: Prostate cancer is a disease of aging, particularly in nations of affluence, with risk increasing several hundred-fold from age 40 to 75. Indeed, as one of the most common malignancies in Americans, it is a significant health care burden. In addition, prostate cancer treatment strategies result in significant negative impacts on quality of life for a growing number of senior citizens. Therefore, prevention strategies are critically needed. Accumulating research suggests that several dietary factors may reduce risk, such as consumption of cruciferous vegetables, soy, and tomatoes. Our laboratory work and others suggests that specific components of these foods demonstrate anticancer properties, such as inhibition of cancer cell proliferation and enhanced sensitivity to activation of cell death programs (apoptosis).

Objective: We hypothesize that bioactive phytochemicals can be combined in specific combinations within carefully designed functional foods that meaningfully contribute to the prevention of prostate cancer progression. We evaluated the combined effects of the bioactive components in arugula in cell-based studies, using erucin, sulphoraphane and genistein.

Design: Prostate cancer cells (PC3) were treated in vitro with 5-20 uM of sulforaphane, 5-20 uM of erucin, and 5-40 uM of genistein, alone and in specific combinations. Cell viability was measured at 24, 48 and 72 hours after treatment using the Sulforhodamine B method to investigate the combined contribution of proliferation and apoptosis. In addition, preliminary cell cycle analysis by flow cytometry was conducted to determine the impact of erucin and genistein alone and in combination on cell cycle progression.

Results: Cruciferous vegetables components at 20 uM such as sulforaphane (high in broccoli) and erucin (high in arugula) were equally effective at 72 hours with 75% vs 80% viability respectively. Genistein (20 uM) from soy foods was also modestly effective, reducing viability by 35%. Erucin and genistein display unique time and dose dependent profiles of inhibition.

Significance: Laboratory in vitro studies may help us define combinations of phytochemicals that have combined anticancer effects, helping us to design novel food products (e.g. a vegetable juice) for future human studies.
II. Introduction

Among U.S. men, prostate cancer (PCA) is the most common non-cutaneous malignancy, the leading cancer diagnosis and the second leading cause of death from cancer (Fowke, 2012). PCA is a disease of aging, particularly in nations of affluence, with risk increasing several hundred-fold from age 40 to 75. Fortunately, with screening the vast majority of prostate cancers can be detected and cured by radiation or surgery. Yet, treatment is associated with significant risk of morbidity, particularly sexual dysfunction, incontinence, and injury to the bladder or rectum that has a negative impact on quality of life for a growing number of men. Autopsy studies demonstrate pathologic diagnosis of prostate cancer as early as the third decade of life, prior to any clinically significant diagnostic symptoms (Sánchez-Chapado et al, 2003). Yet, the average age of diagnosis is 67 (Howlader et al, 2011).

Figure 1. Prevalence of prostate cancer by decades (Sánchez-Chapado et al, 2003).
Androgen production, which initiates during puberty, undoubtedly plays a significant role in the initiation and progression of prostate carcinogenesis (Wang et al, 2011). Therefore, for some, prostate cancer development occurs over decades, providing an ideal window of opportunity for dietary and nutrition based interventions to prevent and delay the progression of prostate cancer. The high prevalence and mortality, along with the length of tumor development time, make PCA a target for prevention, and strategies are critically needed.

Our laboratory has focused upon a number of preventive strategies related to chemopreventive pharmaceuticals, nutrients, or foods. To date, we have developed a novel tomato soy juice that has been used in a clinical trial of 60 men for four weeks prior to prostatectomy with excellent compliance. Preliminary data clearly demonstrates a dose dependent increase in tomato phytochemicals in the serum of these patients. Analysis is underway for soy phytochemicals in human blood, urine and prostate tissue samples. We aim to develop the next generation of vegetable juice and currently hypothesize that the addition of cruciferous vegetables and all that they provide may provide an added benefit for the anti prostate cancer activities.

Cruciferous vegetables include broccoli, broccoli sprouts, cauliflower, and arugula among others. These vegetables are known for their rich source of phytochemicals with bioactivity on a number of processes associated with carcinogenesis. We are particularly interested in arugula, which is a rich source of the isothiocyanate (ITC) erucin, with the ultimate objective of adding an extract of arugula to our tomato-soy juice that is now in clinical trials. Our hypothesis is that erucin will enhance the anticancer properties of other phytochemicals. To date, no studies have
investigated the influence of arugula extracts and erucin in models of prostate cancer. Data from cell culture and rodent models of prostate cancer have suggested anticancer properties of the isothiocyanate sulforaphane and broccoli extracts. We tested the impact of erucin compared to sulforaphane, alone and in combination with other phytochemicals from soy, on the growth of prostate cancer cells in vitro.

Bioactive Phytochemicals and Nutrients:

Myrosinase hydrolyzes glucosinolates (GSLs) to isothiocyanates (ITCs) and other products during food processing or when the plant is broken down. The breakdown products of GSLs may play roles as antioxidants, which in part contribute to the protective effects on cancer (Kim et al, 2004). Myrosinase enzymes catalyze the conversion of inactive glucosinolate precursors to the active isothiocyanate form, such as the conversion of the GSL glucoerucin to the active isothiocyanate erucin (ER). When consuming cruciferous vegetables, myrosinase is released during chewing to hydrolyze GSLs into isothiocyanates (Melchini & Traka, 2010). Figure 2 shows this mechanism, along with the oxidation of ER to sulforaphane (SFN).
Studies investigating the compounds found in cruciferous vegetables have demonstrated significant anti-carcinogenic effects. *Eruca sativa*, also known as arugula or rocket salad, is rich not only in fiber, iron, and vitamins A and C, but also sulforaphane and erucin (Kim et al, 2007). One of the most prominent glucosinolates (GSLs) present in arugula leaves is glucoraphanin, which is converted to sulforaphane upon hydrolysis. In arugula leaves, at least 12 GSLs have been identified, including glucoerucin at a signal intensity of deprotonated molecules ratio of 420 m/z, and glucoraphanin at 436 m/z. It has been hypothesized that glucoraphanin levels are slightly higher in arugula than glucoerucin due to the oxidation of glucoerucin to form glucoraphanin. In a seed extract of arugula, only 3 GSLs have been identified, and glucoerucin is overwhelmingly the

**Figure 2.** The 4-(methylthio)butyl isothiocyanate, erucin (ER), is a reduced analog of the 4-(methylsulfinyl)butyl isothiocyanate, sulforaphane (SF), and its formed both from enzymatic hydrolysis of glucoerucin, a glucosinolate found at high levels in rocket species (*Eruca Sativa*) and in vivo reduction of SF, derived from broccoli (*brassica oleracea*) (Melchini & Traka, 2010).
Glucosinolates can be derived from amino acids like methionine, phenylalanine, and tryptophan. The bitter flavor characteristic of cruciferous vegetables comes from the presence of GSLs and their breakdown products (Kim et al, 2007). These compounds have clear effects on cell proliferation in vitro. For example, sulforaphane induced G2/M phase cell cycle arrest in prostate cancer cells by inhibiting a checkpoint kinase 2-mediated phosphorylation during cell division (Kim & Singh, 2009). For in vivo studies, ITCs must be provided in doses that are safe and provide bioavailable concentrations of the bioactive phytochemicals. Oral administration of 5.6 μM SFN three times a week retarded PC-3 growth in mice, and this amount of SFN is easily generated through diet as 100g of broccoli contains up to 40 μM SFN (Singh, 2004). Previous studies have shown that exposure of human prostate cancer cells to SFN results in G2/M phase cell cycle arrest via checkpoint kinase 2-mediated phosphorylation of cell division (Singh et al, 2009).

Proposed mechanisms of action for the anti-cancer activity of these ITCs include epigenetic modifications to receptor mediated alterations in transcriptional activity. SFN may inhibit testosterone induced cancer progression by inhibiting the expression of androgen receptor (AR) and its transcriptional activity in human prostate cancer cells (Kim & Singh, 2009). Epigenetics is the study of heritable changes in gene activity, and in recent years studies have shown that genes can be turned on and off, making the epigenome as important as changes in DNA. The ability of isothiocyanates like sulforaphane to target epigenetic patterns may make it an effective agent of
chemoprevention in carcinogenesis (Ho et al 2009). Reversing the acetylation of histones is one mechanism that may be involved. Histone deacetylase inhibitors (HDAC) are a cancer chemoprevention strategy (Sargeant et al, 2008), and SFN from cruciferous vegetables has been shown to inhibit HDAC activity in prostate cancer cells (Ho et al 2009).

As stated previously, we are ultimately interested in the ability of isothiocyanates to enhance the anti-cancer activity of other food constituents in the modification of prostate cancer risk. Genistein is the most abundant isoflavone in soy and one of the more potent bioactives in soy. Genistein has the ability to inhibit growth in vitro of both androgen-dependent and independent prostate cancer cells (Zhao et al, 2009). Epidemiological studies have shown that Asian men who consume a diet high in soy isoflavones have a lower incidence of prostate cancer (Zhao et al, 2009). The mechanism of genistein’s anti-carcinogenic activity includes the induction of apoptosis and inhibition of angiogenesis. Genistein resulted in cell death in as low of a dose as 5 uM and treatment with 10 uM of genistein resulted in a 27% increase in PC3 cells in the G2/M phase. (Zhao et al, 2009). One of the objectives of this study is to determine if the combination of bioactive soy phytochemicals with cruciferous vegetable ITC would have enhanced anti-carcinogenic activities.

**Benefits of Functional Food Based Cancer Prevention Strategies:**

Nutrition based interventions are potentially useful strategies to prevent and delay the progression of prostate cancer. There is a significant period of time during which dietary and nutrition based interventions may impact the carcinogenesis process, and combinations of bioactive rich foods consumed over extended periods of time have the
potential to improve prognosis. Much of what we know about nutrition and cancer prevention comes from epidemiological studies or preclinical laboratory models. However, our team is developing functional foods which provide specific combinations of bioactive phytochemicals in a safe palatable form. Our ultimate goal is to develop functional foods integrating arugula with tomatoes and soy for prostate cancer prevention clinical trials.

To date, there are no studies evaluating the combined anti-prostate cancer effects of cruciferous vegetable phytochemicals with soy phytochemicals. Therefore, before proceeding with the development of a second generation vegetable juice adding cruciferous vegetables to the existing novel tomato soy juice, we aim to test the ability of these phytochemicals to enhance the activity of genistein in a pre-clinical model of prostate cancer.

III. Methods

We treated the prostate cancer cell line PC3 with sulforaphane, erucin, genistein, and a control (DMSO) in a factorial design. The effects of these treatments on the PC3 cells were evaluated in a time and dose dependent manner. The growth of these cells was measured to determine how sulforaphane, erucin and genistein affect cell viability and cell cycle progression. Cell viability was quantified by Sulforhodamine B (SRB) assay after 24, 48 and 72 hours of exposure to sulforaphane, erucin, and genistein. Cells were seeded in 96-well flat-bottomed plates for 24h then treated with erucin at concentrations between 5-20 uM, genistein at 5-40 uM, and sulforaphane at 5-20
uM, with three replicates each. For pre-treatment studies, cells were treated for 24h for pre-treatment, then media exchanged with new treatment. Viability was quantified by fixing cells and staining with Sulforhodamine B solution (In vitro toxicology assay, Sigma Aldrich) to determine the protein content of the treated cells was measured, which yielded growth curves that show the effects of these compounds on cell proliferation.

Cell cycle analysis was performed on cells plated for 24 hours prior to treatment, then cultured for 24, 48, and 72h in the presence of 10uM erucin and 10um Genestein alone and in combination, with treatments replenished daily. Cells were collected, rinsed, fixed with ice-cold 70% ethanol and stained with 50ug/ml propidium iodide in PBS containing 0.1% Triton-X and 0.2mg/ml RNAse A for 30 minutes at room temperature. Flow cytometry was conducted on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) instrument and cell cycle modeling analysis by FlowJo (Tree Star, Inc, Ashland, OR)
IV. Results

Previous studies have shown that SFN inhibits the viability of prostate cancer cells. We first aimed to compare the effectiveness of ER and SFN on PC-3 cells (Kim and Singh, 2009). Both ER and SFN significantly inhibited cell viability in a time and dose dependent manner. We found that time and dose had a p-value <0.001, meaning that there was a statistical difference in the cell viability between 24, 48 and 72 hours, as well as a statistically significant decrease in viability between different doses. There was no difference between drugs overall, with a p>0.05, meaning ER and SFN were equally effective. All doses were significant for each drug at 48 and 72 hours. At 24, 48 and 72 hours, 20 uM ER reduced cell viability by 39.3%, 56.2% and 78.6% respectively. At 24, 48 and 72 hours, 20 uM SFN reduced cell viability by 35.4%, 60% and 74.6% respectively. Figure 3 shows ER and SFN in a time and dose dependent manner at 24, 48 and 72 hours. Since SFN and ER were equally effective at these time points and doses, we chose to use ER in the remainder of our studies, because it is more abundant in arugula.
Figure 3. Erucin and sulforaphane equally inhibit prostate cancer cell viability. Both erucin and sulforaphane inhibit cell viability in a dose and time dependent manner (p<0.001). At equal concentrations, there is not a significant difference between the two compounds. *indicates significance (p<0.05) from control.
We next tested the ability of addition of 10 uM GEN to enhance the dose dependent inhibition of ER on viability. The PC-3 cells were treated with a control of DMSO, 10uM GEN alone, 10uM GEN in combination with 5, 10 and 20uM ER, and 5 and 20uM ER alone. All of these different treatments were significantly inhibited viability compared to control (P <0.001). The main effect of time was also significant with a p=0.013. At 24, 48 and 72 hours, 20 uM ER in combination with 10 uM GEN decreases viability by 45.3%, 58% and 67.1% respectively, compared to 20 uM ER alone, which decreased viability by 53.3%, 61% and 68.5%. Figure 4 shows these relationships in a dose dependent manner over 24, 48 and 72 hours.
Figure 4. Genistein does not enhance the inhibitory activity of erucin.
Genistein, at a 10uM concentration in vitro did not further enhance the inhibitory activity of erucin. Each dose of treatment was significant (P<0.001) compared to the control, but treatment of erucin alone was not significantly different from treatment in combination with 10uM genistein at any dose or timepoint (p>0.050). *indicates significance (p<0.050) from control.
We next tested the dose dependent effect of Genistein on viability and if erucin at 10 uM enhances this effect. We kept ER constant at 10 uM and added GEN in 5, 10, 20, and 40 uM. The main effects of time, addition of ER and GEN dose response were significant (p<0.001). At 24, 48 and 72 hours, 20 uM GEN in combination with 10 uM ER decreased cell viability by 39.8%, 46.7%, and 56.4% respectively, compared to 20uM GEN alone which decreased viability by 17%, 32%, 45.3% at 24, 48 and 72 hours. At 24, 48 and 72 hours, 40 uM GEN in combination with 10 uM ER decreased cell viability by 45.8%, 49.5%, and 64.3% respectively, compared to 40 uM GEN alone which decreased viability by 27.6%, 45.7%, and 54.8% at 24, 48 and 72 hours. Figure 5 shows the relationship over 24, 48 and 72 hours.
Figure 5. Genistein has a modest dose and time dependent inhibitory effect on prostate cancer viability that is enhanced by the addition of Erucin.

Genistein, alone, displays a significant main effect of time and dose dependent inhibition in viability (p<0.001). Erucin (10uM) enhances the activity of genistein (p<0.001) and there is a modest interaction between erucin and genistein at 72h (p=0.036). *indicates significance (p<0.050) from no genistein; # indicates significance (p<0.05) from no erucin.
We conducted a cell cycle analysis by flow cytometry on PC-3 cells treated daily with a control of DMSO, 10 uM ER, 10 uM GEN, and a combination of 10 uM ER and 10 uM GEN for 24 hours, 48 hours, and 72 hours. This technique identifies changes in the proportion of cells in each of the phases of the cell cycle as demonstrated in Figure 6 adapted from (Ormerod, 2008). First, ER treated samples demonstrated a greater proportion of cells in the G2/M and S phases, most robustly after 72 hours of treatment (Figure 7). There were no dramatic differences between the GEN treated cells and control at any of the time points tested. The combination of ER and GEN demonstrated a similar pattern of accumulation in the G2/M and S phases as ER alone.
**Figure 6.** Profile of flow cytometry histogram with phases of the cell cycle (Adapted from Flow Cytometry: A Basic Introduction ed. Michael G. Omerod)

**Figure 7.** Erucin, alone and in combination with Genistein impacts cell cycle progression. Cell cycle profiles of cells treated daily suggest that Erucin (10uM) alone and in combination with Genistein (10um) induces accumulation in the S and G2/M phases of the cell cycle.
Because of the unique pattern of time and dose responses between ER and GEN and the response with co-treatment, we tested the sustained response of ER and GEN using a pre-treatment approach. Cells were plated for 24 hours then pre-treated for 24 hours with 10uM ER. After the 24 hour pre-treatment, media was removed and replaced with media containing 0, 5, 10 and 20uM GEN. Cell viability was analyzed at 24, 48, and 72 hours after the initiation of GEN treatment. As anticipated, there was a time and GEN dose dependent decrease in viability (p<0.001). Interestingly, pre-treatment with ER resulted in significantly less viability than with any GEN dose alone (p<0.001). Pre-treatment with 10 uM ER and 20uM GEN decreased cell viability by 42.6%, 55.6%, and 55.7% at 24, 48 and 72 hours. Without pre-treatment, 20 uM GEN decreased cell viability by 7.7%, 32.6%, and 24.2% at 24, 48 and 72 hours. Figure 8 shows pre-treatment with erucin at 24, 48 and 72 hours.
Figure 8. Pre-treatment with 10 uM Erucin causes sustained inhibition in viability maintained through 72h.

Pre-treatment with Erucin for 24h before treatment with Genistein significantly decreased cell viability compared to treatment of Genistein alone. Main effects of time, pre-treatment and doses of genistein significant (p<0.001). *indicates significance (p<0.050) from no genistein; # indicates significance (p<0.05) from no erucin pre-treatment.
To investigate the sustained influence of pre-treatment with GEN on the dose and time dependent response to ER, we pretreated PC-3 cells with 10 uM GEN for 24 hours, followed by treatment with 5, 10 and 20 uM ER for 24, 48 and 72 hours. As anticipated, the main effect for time and dose of ER was significant with $p<0.001$. Alternative to the effect of pre-treatment with ER, pre-treatment with GEN only modestly decreased viability after 24h, and was significant with $p=0.006$. At 24, 48 and 72 hours, cells pre-treated with 10 uM GEN and 20 uM ER decreased in viability by 34.4%, 50.6%, and 64.3% respectively. Without pre-treatment, 20 ER alone at 24, 48 and 72 hours decreased viability by 40.7%, 49.3%, and 57.2% respectively. Figure 9 shows pre-treatment with genistein at 24, 48 and 72 hours.
Figure 9. Pre-treatment with 10 μM Genistein modestly enhances the inhibitory activity of genistein. Pre-treatment with Genistein before Erucin was only modestly different at 48h when combined. The main effect of time and doses of Erucin were significant (p<0.001) and pre-treatment modestly significant at 48h (p=0.002) and 72h (p=0.032). *indicates significance (p<0.05) from no erucin; # indicates significance (p<0.05) from no genistein pre-treatment.
V. Discussion

These studies were designed to investigate the anti prostate cancer activity of known bioactive phytochemicals in cruciferous vegetables, including arugula and broccoli alone and in combination with the bioactive soy phytochemical, GEN. The results of these studies clearly demonstrate that in the PC-3 \textit{in vitro} model of prostate cancer, SFN, ER, and GEN exhibit dose dependent decreases in viability that become more robust over time.

Both SFN and ER resulted in significant inhibition in viability within 24 hours of treatment. Treatment with 5 uM of either SFN or ER significantly reduced viability at 24. This data is consistent with the work of Melchini et al, 2009 demonstrating equal inhibition of cell viability by SFN and ER on the A549 human lung adenocarcinoma cell line. In the lung cancer model, this inhibition was associated with increases in p53 and p21 expression and induction of apoptosis as detected by PARP cleavage. In Caco-2 human colon adenocarcinoma cells, ER was more effective than SFN at inducing phase II enzyme transcriptional activation, inducing G2/M cell cycle arrest, and apoptosis (Jakubikova et al, 2005). Therefore, based upon these published comparisons of ER and SFN, we focused on ER for subsequent analyses investigating the effect of combining genistein to an ITC.

First, genistein alone inhibited viability in a time and dose dependant manner, however, it was less robust and delayed compared to either ER or SFN. Treatment with 40 uM reduced viability first after 48 hours of treatment, and 10, 20 and 40uM reduced viability only after 72 hours of treatment. It is suggested that in prostate cancer cells, genistein treatments result in altered signaling pathways, such as the tyrosine kinase
Clearly, the addition of ER to GEN enhanced the modest but significant inhibitory activity of GEN. However, the addition of GEN to different doses of ER were not significantly different, possibly due to the different mechanisms of ER and GEN on inhibiting cell proliferation, as well as the difference in the length of treatment time before there is a significant impact on viability. GEN, without ER, significantly inhibited cell viability at a 10uM dose after 72h. This is the first set of experiments to combine ER with GEN and it is clear that the mechanism of action of these two compounds are quite different and further studies are under way to investigate the underlying mechanisms alone and in combination in a model of prostate cancer.

We tested the impact of ER and GEN, alone and in combination, on progression through the cell cycle. These results suggest that ER alone and ER + GEN both resulted in accumulation in G2/M and S phases. These accumulations in these phases of the cell cycle are supportive of prior studies demonstrating that treatment with ITCs including SFN and ER alter mechanisms, including epigenetic modifications, necessary for progression through the cell cycle, subsequently inhibit proliferation (Jakubikova et al, 2005) (Singh et al, 2009).

Because of the unique patterns in the time dependent response between ER and GEN, we tested the impact of pre-treatment of one followed by treatment with the other. Most interesting, pre-treatment with ER for 24 hours resulted in significant differences in viability that was maintained for 72 hours, even after ER was removed. It was surprising how modest the effect of pre-treatment with GEN for 24 hours was in comparison to pre-
treatment with ER. The decrease in viability with GEN pre-treatment was not maintained through 72 hours, unlike the pre-treatment with ER.

The robust response of prostate cancer cells to SFN and ER is supportive of their potential role in the reduction of risk and use in prevention based clinical trials. Whole foods contain many components including a multitude of ITCs and indole-3-carbinol that alone and in combinations have a collective impact on cancer. Therefore, the next step is to use cruciferous vegetable extracts to determine their impact on viability alone and with the more complex soy extract. We hypothesize that the whole food extracts would be more effective than the individual compounds due to the other components they provide.

This *in vitro* model, ideal for initial investigations of anti-cancer activity focusing upon proliferation and apoptosis and additional mechanisms, does not adequately model the impact of any intervention on cancer prevention. Therefore, future directions also include testing the individual compounds, unique combinations, and/or whole food extracts in models of carcinogenesis testing the impact on prevention. Some such models include the popular TRAMP, Pten, Nkx3.1 mouse models (Hensley and Kyprianou, 2012), many of which have been used in nutrition based intervention trials (Keum et al, 2009) (Pannellini et al, 2010).

It would be narrow minded to make the assumption that the only anti-cancer activity is on the cancer cells themselves. Ultimately, *in vivo*, these bioactive compounds have systemic effects including the tumor cells, the surrounding matrix, the vasculature supporting the growth of a tumor, and anti-cancer immunity. These studies presented here are supportive of moving forward with more complex food products (pure compounds vs
extracts), pre-clinical systems (animal models of prevention), and systems biology investigations (ie. Immune response and angiogenesis). In addition to investigating the mechanism of action, the ability to integrate cruciferous vegetables into novel food products providing a safe product of bioavailable phytochemicals at physiological doses in a palatable form is an additional layer of complexity under investigation.

VI. Significance

While common, prostate cancer is considered a disease of aging and for most men frequently involves a slow, indolent progression. However, for some men, the cancer develops aggressively. There are multiple, effective treatment strategies for prostate cancer yet these include dramatic side effects that impact the quality of life. Therefore, effective prevention strategies are needed. As the long term goal is to integrate cruciferous vegetables into a vegetable juice that can be used for prevention based clinical trials. We anticipate that this data will be used in our future studies to determine if arugula can be incorporated into our novel tomato soy juice that is designed for future cancer prevention clinical trials. These functional foods may provide overlapping mechanisms for prevention of carcinogenesis, which will need to be investigated further as these foods are developed. Although this study focused on prostate cancer cell progression, the phytochemicals in these vegetables have been shown to be effective in multiple malignancies, such as breast, colon and lung cancers (Melchini & Traka, 2010) and, therefore, the functional food product may be beneficial patients with for many types of cancer.
VII. Acknowledgements

To Dr. Steven Clinton and Dr. Jennifer Thomas-Ahner, whose knowledge and support helped me through the research process and to understand nutrition from a research prospective. Without their guidance, I would not have been able to complete my research, or my undergraduate honors thesis. I have gained such a solid research foundation and I am truly grateful for the time they dedicated my project that ultimately helped me succeed.

To Dr. Anne Smith, who was invaluable in my research, as she introduced me to Dr. Clinton’s research team and served on my research committee. Dr. Smith taught me the fundamentals in my first nutrition class, and is helping me apply that knowledge to disease states to help prepare me for graduate school in my last nutrition class at OSU. Her continual interest in my research and academic progress has helped me excel in the field of nutrition.

To my parents and friends, whose continual support helped me strive for academic and personal success these last four years. Their encouragement is the reason I am graduating from The Ohio State University with Honors, and I am thankful that I always have their love and support.


