ABSTRACT

Title of Document: THE EFFECTS OF CRUCIFEROUS VEGETABLE DERIVED BIOACTIVE COMPOUNDS ON THE MODULATION OF HUMAN PROSTATE CANCER

Arnetta Renee Fletcher, Doctor of Philosophy, 2015

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Prostate cancer is the most common non-skin cancer type in American males. Poor diet quality may have profound negative consequences on disease outcome. Diets consisting of low cruciferous vegetable and high red meat intake were found to be significant, yet modifiable risk factors. Currently, there are no effective cures for prostate cancer. As a result, improved diet quality through the promoted use of natural products is commonly sought after complementary health approach to disease prevention and cancer risk reduction. Phenethyl isothiocyanate (PEITC) and indole-3-carbinol (I3C), naturally occurring bioactive compounds in cruciferous vegetables, are considered ideal chemopreventive candidates.

This dissertation focused on examining biological efficacy and identifying distinctive biological mechanisms utilized by PEITC and I3C to modulate human prostate cancer growth in a xenograft mouse model.

When treated with PEITC, mice were found to be differentially responsive. Tissue analysis revealed a significant decrease in tumor burden with no observable toxic effects. PEITC exerted minimal effects on androgen responsive pathways within tumors. However, cell proliferation and macrophage presence were significantly

reduced and insulin-like growth factor binding protein-3 expression was increased in the treatment group fed PEITC.

Previous studies have shown an overlap in no effect and chemopreventive I3C dosage levels. Therefore, physiological and biological sensitivity to I3C in a commonly used xenograft model was examined. Mice were biologically responsive to doses exceeding 10 μmoles I3C/g diets. Viability was significantly affected at the highest concentration unless mice were switched to the control diet without I3C after first detection of stress. The intestine appeared to be the target of I3C toxicity as noted by changes in number and width of intestinal villi, proliferation, and apoptosis. Changes to xenobiotic metabolism were indicated within the livers of the treatment mice, supporting involvement of aryl hydrocarbon receptor-mediated pathway in the metabolism of I3C.

Biological efficacy of low, physiologically relevant doses of I3C was also examined. Following exposure to low concentrations of I3C, tumor volume, xenobiotic metabolism, and cell motility were altered in tumorigenic mice.

Collectively, findings from this study highlighted unique, yet complicated, pleiotropic responses of cruciferous-derived compounds in prostate cancer.

THE EFFECTS OF CRUCIFEROUS VEGETABLE DERIVED BIOACTIVE COMPOUNDS ON THE MODULATION OF HUMAN PROSTATE CANCER

By

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Dedication

To the faculty and staff within the Department of Nutrition and Food Science, College of Agriculture and Natural Resources, University of Maryland, College Park as well as the Beltsville Human Nutrition Research Center, Agricultural Research Service, United States Department of Agriculture (USDA).

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Introduction

Prostate cancer (PCa) is one of the leading causes of cancer-related deaths in American males. Factors associated with prostate cancer onset include age, genetics, and diet. Poor diet quality may drastically affect disease outcomes. Diets consisting of low cruciferous vegetables and high red meat intake were found to be significant, yet modifiable risk factors. Currently, there are no effective cures for PCa. As a result, improved diet quality and promoted use of natural products are commonly sought after as complementary health approaches to disease prevention and risk reduction. Phenethyl isothiocyanate (PEITC) and indole-3-carbinol (I3C), naturally occurring bioactive compounds in cruciferous vegetables, are considered ideal chemopreventive candidates.

This project focuses on identifying distinctive mechanisms of PEITC and I3C to modulate human prostate cancer growth. Three specific aims of this study addressed the biological mechanisms and efficacy of PEITC and I3C in a xenograft mouse model. The first aim of this study investigated the effects of PEITC along the macrophage axis and the insulin-like growth factor-1 pathway. Previous studies have shown an overlap in no effect and chemopreventive I3C dosage levels. Therefore, the second aim of this project was designed to assess for physiological and biological sensitivity to I3C in a commonly used xenograft model. Utilizing the same animal model, the final aim addressed the biological efficacy of low, yet physiologically relevant doses of I3C.

Findings from this study highlighted unique, yet complicated, pleiotropic responses of cruciferous-derived compounds in prostate cancer.

Chapter 1: Literature Review

Chronic Disease

Chronic diseases are long-lasting conditions that are generally slow to manifest and progress (Goodman, Posner, Huang, Parekh, & Koh, 2013). Monitoring and evaluating changes in mortality patterns has been crucial in the assessment of health status and well being in our nation. Age and sex are commonly identified as variables in mortality patterns and drivers in the likelihood of developing certain chronic diseases. In the United States, cardiovascular disease, malignant neoplasms, diabetes mellitus, and cerebrovascular diseases such as stroke are among the leading causes of death and contribute to greater than 50% of all deaths (Heron, 2013). Due to medical advances, mortality rates for heart disease and cancer are gradually declining; however, incidence rates of these two, amongst other chronic diseases, are steadily rising (Heron, 2013; "On the Rise Globally, Cancer Mortality Declines in U.S.," 2014).

Cancer, a general name given to more than 100 diseases, marks the development of abnormal and malignant cell growth within the body. A 2012 GLOBOCAN review of worldwide cancer incidence, mortality, and prevalence (within 5 years of diagnosis) projected 14.1 million new cancer cases, 8.2 million cancer-related deaths, and estimated 32.6 million were living with cancer. Overall age standardized cancer incidence rate (ASR) is 25% higher in men than women (International Agency for Research on Cancer, 2013). As of January 2012, approximately 13.7 million U.S. citizens were living in America with a history of cancer and approximately 1.7 million new cases, excluding carcinoma in situ, are expected to be diagnosed this year, as reported by the American Cancer Society (American Cancer Society, 2014).

This dissertation focuses on prostate cancer, a male prominent cancer type, with the intent of studying the barriers to finding a cure as well as possible means of successful prevention.

Chronic disease management takes a heavy toll on the economy. The cost associated with chronic disease affects the individual not only through direct medical

cost but also on a personal level by coping with severe disabilities, such as chronic neuropathic pain for those experiencing cancer. In 2012, the American Public Health Association reported heart disease, cancer, lung ailments, diabetes, and hypertension, as the top five most costly yet preventable diseases, which accounted for approximately $327 billion dollars or 30% of the total health spending nationwide. National Cancer Institute (NCI) 2020 cost projections for all sites of cancer in males were $6.6 billon dollars, a 35.6% increase from 2010 in initial costs of care. Likewise, initial costs of care for specific types such as prostate cancer, are expected to increase 51% by 2020.

CDC reports common causes of the growing epidemic of chronic disease as 1) lack of physical activity; 2) poor nutrition; 3) tobacco use; and 4) excessive alcohol consumption (Centers for Disease Control and Prevention, 2012). These modifiable risk factors heavily contribute to the development of the secondary illness & comorbidity, suffering, and decreased life expectancy associated with chronic disease. Thus, there is a critical need for effective public health interventions that target chronic disease, to promote a healthier population, and improve quality of life.

Prostate Cancer and Chronic Inflammation

Prostate Cancer

Prostate cancer (PCa) is the most common type of non-skin cancer and the second leading cause of cancer-related deaths in males (R. L. Siegel, Miller, & Jemal, 2015). In 2013, approximately 239,000 new cases and 30,000 deaths were projected to occur (Edwards et al., 2013). Prostate cancer is within the top 5 commonly diagnosed cancers and the fifth leading cause of cancer-related deaths in the world (International Agency for Research on Cancer, 2013). GLOBOCAN estimates that nearly 1.1 million men worldwide were diagnosed with PCa in 2012.

Common risk factors for prostate cancer include diet quality, inflammation of the prostate, weight classification (underweight, overweight, or obese status), age, race/ethnicity, nationality, family history, and genetic susceptibility. According to GLOBOCAN, mortality rates are highest in predominantly black populations

(Caribbean, 29 per 100,000 and sub-Saharan Africa, ASRs 19-24 per 100,000) and very low amongst Asian populations (2.9 per 100,000 in South-Central Asia) (International Agency for Research on Cancer, 2013). Rates are intermediate in the American regions (International Agency for Research on Cancer, 2013). In the U.S., the probability of developing invasive prostate cancer throughout an individual's lifetime is approximately 15% or 1 out of 7 males (DeSantis et al., 2014; R. L. Siegel et al., 2015). Moreover, prostate cancer incidence are disproportionately high in African Americans (230.8 per 100,000) and Caucasians (142.8 per 100,000) as compared to rates of Asian Americans or Pacific Islanders (79.7 per 100,000) (R. Siegel, Naishadham, & Jemal, 2012).

Exact mechanisms yielding to the transition from normal to cancerous prostate cancer cells are complicated and remain unclear. Somatic and acquired gene mutations are several reasons for the development of prostate cancer. These inherited gene mutations may contribute to up to 10% of prostate cancers ("Prostate Cancer: Do we know what causes prostate cancer?," 2014). Although, overall inherited DNA mutations contribute to a small amount of prostate cancer incidences, primary genes correlated with the increased susceptibility of PCa include Hereditary Prostate Cancer Gene 1 (HPCG1), Breast Cancer, Early Onset (BRCA) -1 and -2 (Agalliu et al., 2007). Underlying mechanisms for the acquired mutations include invading, and ultimately overriding DNA checkpoints during the cell replication process. Numerous factors that may contribute to the regulation of the cell replication process, checkpoint failures, and the replication of DNA mutations means that understanding and combatting this disease is increasingly difficult.

To date, there are no effective cures for PCa. Like other epithelial derived carcinomas, PCa has a higher survival rate than other adenocarcinomas. However, factors affecting prognosis include age, underlying health conditions, metastasis, morphology, and detection time. According to the American Cancer Society, men should undergo screening for prostate cancer by age 50 or 45 for African Americans and other higher risk populations. Two standard tests include the digital rectal exam (DRE) and prostate specific antigen (PSA) blood test, which was considered a gold standard but now have contradictory findings of effectiveness (Brawley, 2013; Drazer

et al., 2014; Edwards et al., 2013; Rodrigues et al., 2014; M. C. Wang et al., 1981). In general, high levels of PSA have been correlated with cancer incidence. However, benign conditions such as an enlarged and inflamed prostate can raise PSA levels and therefore cannot be used as a sole determinant for prostate cancer. The treatment of prostate cancer is complex and varies due to the disease etiology. Current methods used in the treatment of prostate cancer include surgery, radiation therapy, cryosurgery, and hormonal and chemotherapy.

All in all, there are various pathways and biomarkers involved in the initiation and progression of prostate cancer. Many of these pathways play an intricate and integral role in the regulation of cell properties and functions such as cellular division, differentiation, hormonal homeostasis, inflammation, immunity, compound activation, apoptosis, and proliferation (World Cancer Research Fund, 2007). As a result, diet and hormone levels may influence the rate in which these changes occur. Diet, as a modifiable risk factor, is an essential component in prostate cancer development and the regulation of secondary risk factors such as chronic inflammation (Arab et al., 2013; Gonzales et al., 2014).

Therefore, my research focuses specifically on evaluating prostate cancer-related endpoint biomarkers to elucidate antiproliferative regulatory mechanisms associated with cruciferous vegetable intake.

Chronic Inflammation

Inflammation is a protective mechanism that attenuates insults incurred in response to internal or external environmental stimulus within a host defense system. Persistent or chronic inflammation can prove harmful and may incite disease. In a review conducted by Aggarawal et al, chronic inflammation was a noted cause of cellular dysfunction and has been implicated in the promotion of chronic diseases such as cancer, diabetes mellitus, cardiovascular, pulmonary, and neurological diseases (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). Moreover, the diagnosis of inflammation and its biomarkers are not fully understood. However, the role of proinflammatory cytokines, chemokines, adhesion molecules, and

inflammatory enzymes have been linked with chronic inflammation (Aggarwal et al., 2006).

Cancer-driven Inflammation

Chronic inflammation is a well-known risk factor for most cancers (Anand et al., 2008). It has been linked to various steps in tumorigenesis including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Coussens & Werb, 2002; Mantovani, 2005). Inflammation plays a critical role within the prostate cancer microenvironment and exacerbates growth as a result of its close proximity to cancer sites. The National Institutes of Health (NIH) issued a consensus statement to clinically define prostatic inflammation (Krieger, Nyberg, & Nickel, 1999). Class III prostatitis is characterized as a state of chronic, intermittent inflammation (Krieger et al., 1999). As one of the most common forms, Class III prostatitis is detected in 90% of prostate cancer cases (Hua & Schaeffer, 2004; Lu, Ouyang, & Huang, 2006).

The tumor microenvironment encompasses an assortment of malignant and nonmalignant cell populations. Non-malignant populations consist of stromal cells, an expanding vasculature, and a leukocyte infiltrate. Macrophages comprise the dominant portion of the leukocyte population (Tarin, 2012).

The influence of macrophages have been studied in carcinoma cell lines (B. Wang et al., 2011). Notable findings indicate the presence of tumor associated macrophages in benign prostatic conditions such as prostatitis, prostatic hyperplasia, prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) (De Marzo, Marchi, Epstein, & Nelson, 1999). PIN is characterized as dysplasia of the prostate epithelial lining. Both PIA and PIN are considered precursors of prostate cancer and with characteristically high expression levels of inflammatory mediators (De Marzo et al., 2003). Molecular stress indicated in these conditions increase the likelihood of inflammatory cell invasion and inflammatory-mediated onset of prostate carcinogenesis (De Marzo et al., 2004).

Microvesicles have been connected to cancer progression since the 1970's (Friend et al., 1978). In studies conducted by Croce et al, the role of microvesicle

shedding in the promotion of cancer growth and metastasis was examined. Findings from these studies provided a mechanism: microvesicles shed from cancer cells and fuse to macrophages and other cells in the tumor microenvironment. Once enveloped into the cell, the miRNAs released from the microvesicles may bind to toll-like receptors (TLRs) and cause host cells to release pro-inflammatory cytokines, which facilitate tumor invasion and metastasis (Aggarwal et al., 2006; Fabbri, Paone, Calore, Galli, & Croce, 2013; Fabbri et al., 2012). Moreover, circulating miRNAs have been correlated with tumor progression in prostate cancer and may have a high clinical relevance as a potential biomarker in the early detection of cancers (Brase et al., 2011; Schwarzenbach, Nishida, Calin, & Pantel, 2014). Moreover, decreasing microvesicle density within tumor microenvironments through dietary intervention has been a priority interest among cancer prevention and immunology researchers.

Cytokines, anti-inflammatory and pro-inflammatory, have a major role in regulating biological responses including the regulation of apoptosis and suppression of proliferation, angiogenesis, invasion, and metastasis in prostate cancer. These genes include TNF, IL-1β, IL-6, IL-8, IL-18, chemokines, MMP-9, and VEGF. The expression of these genes is predominantly regulated by the transcription factor NF-κB. NF-κB is constitutively active in most tumors and is induced by xenobiotics including carcinogens, tumor promoters, chemotherapeutic agents, radiation and carcinogenic viral proteins (Aggarwal & Shishodia, 2006; Aggarwal et al., 2006).

Increased levels of cytokines have been found in blood, ascites, pleural effusions, and urine of individuals with cancer (Dunlop & Campbell, 2000). Interestingly, no single cytokine has been shown to be present in all individuals with cancer and the absence of clinically detectable cytokine levels does not exclude the possibility that they play a role in systemic effects (Dunlop & Campbell, 2000; Todorov et al., 1996). Moreover, crosstalk amongst cytokines like IL-6 with growth factor mediators such as IGFBP3 is possible (Hudson et al., 2012). Inflammation may lead to DNA damage directly or indirectly through crosstalk along other pathways, which may promote a tumorigenic environment within a cell.

In addition to inciting the tumor microenvironment, cytokine accumulation has been linked to adverse health effects such as wasting associated with cancer-

related cachexia in more advanced cancers. Cytokines have been shown to affect gastric motility and emptying by altering the signals that control satiety (Argilés, Moore-Carrasco, Busquets, & López-Soriano, 2003; Argilés, Moore-Carrasco, Fuster, Busquets, & López-Soriano, 2003). Consuming vegetables high in anti-inflammatory agents such as I3C and PEITC plays a critical role in reducing adverse health effects by suppressing NF-κB and NF-κB - regulated products and should be considered candidates in both the prevention and treatment of cancer (Aggarwal & Shishodia, 2006; Aggarwal et al., 2006)

Role of Diet in Prostate Cancer Development

The impact of diet quality on health has been an on-going issue in America. With overwhelming support indicating that Americans, males especially, are at risk of low vegetable intake, diet modification is a particularly novel application to improve nutritional status and thus, decrease the rate of individuals undergoing more advanced medical treatment. Nutritional recommendations for cancer patients, specifically those with hormonal cancers, should be cautionary as some dietary manipulation of certain macro-, micronutrients, and phytochemicals can stimulate tumor growth (Gonzales et al., 2014; Langen et al., 1993). However, overall intake of varied vegetables along with the reduction of red meat intake has been shown to improve prostate health (Michaud et al., 2001). One reason cruciferous vegetable intake counterbalances prostate cancer development may be due to their unique composition of biological compounds, which will be discussed in later sections.

Epidemiological, clinical, and preclinical studies have been vital in the assessment of diet quality in prostate cancer development. In an epidemiological literature review by Kristal and Lampe, studies indicate modest to strong support for the hypothesis that a high intake of Brassica vegetables reduces prostate cancer risk (Jain, Hislop, Howe, & Ghadirian, 1999; Kolonel et al., 2000; Kristal & Lampe, 2002). Moreover, in a population-based case-control study of men under 65 years of age indicated that consuming three or more servings of cruciferous vegetables per week was a significant factor in decreasing prostate cancer cases as compared to that of one serving or less per week (J. H. Cohen, Kristal, & Stanford, 2000). In a

multiethnic case control study of African-American, Caucasian, Japanese and Chinese men, intake of cruciferous vegetables was inversely related to prostate cancer, particularly in advanced cases (P for trend = 0.006), with consistent findings across ethnic groups (Kolonel et al., 2000). More recently, Men's Eating and Living (MEAL) study, a promising large scale randomized clinical trial, began assessing how dietary modification through increased cruciferous vegetable intake affects men with early stage localized cancer (Parsons et al., 2014).

However, the biochemical relationships between tumor-host interactions with cruciferous vegetable-derived bioactive compounds must be fully understood if nutrition intervention is to offer cancer preventive or therapeutic benefits.

Influence of Diet on Chronic Disease

The role of diet in the prevention, manifestation, progression, and premature mortality in association with chronic diseases such as cancer has been well established. In an early review by Doll et al, nutritional factors were suspected to be one of the leading factors in cancer onset and mortality (Richard Doll et al., 1966; R. Doll & Peto, 1981). Surprisingly, 30- 35% of all cancer-related deaths (500,000 at that time) were related to dietary factors (Willett, 2000). Scientists have continued to study the relationship between diet and health since this foundational study 48 years ago.

Both retrospective and prospective epidemiological studies have found dietary quality and lifestyle factors as the main determinants of chronic diseases (Ford, Jensen, Hartman, Wray, & Smiciklas-Wright, 2013; Fouwels, Bredie, Wollersheim, & Schippers, 2009; E. Liu et al., 2009; Samieri et al., 2013; Van Duyn & Pivonka, 2000). Findings from a review written by Ford et al. indicated a dietary component associated with increased burden of chronic diseases on aging Americans. Of the articles reviewed in this article, delayed mortality was associated with individuals that adhered to diets that were composed of a variety of wholesome fruits, vegetables, whole grains, and other healthful choices (Ford et al., 2013). Moreover, the protective role for vegetables in the prevention of coronary heart disease, stroke, diverticulosis, and cancer is substantial (Van Duyn & Pivonka, 2000).

Health Benefits of Cruciferous Vegetables

Benefits associated with healthful eating include decreased risk of chronic diseases, reduced overweight and obesity status, and diminished micronutrient deficiencies. Improvement of these three overlapping factors alone could significantly improve the health of Americans.

These conditions are heavily influenced by genetic variability and diet management. Genetic variability also plays an integral role in disease predisposition and manifestation (Brigelius-Flohé, 2006; Stearns; Weiss, 1993). Combining dietary and lifestyle factors with genetic variation complicates the identification and applicability of discrete roles of risk factors on disease development. However, an underlying conclusion from these studies is that nutrition plays a crucial role in disease manifestation and progression.

Poor nutrition and diet management can lead to increased susceptibility of disease. In terms of chronic diseases, cancer incidence and mortality are affected by an individual's environment (regional differences), food patterns and related behaviors, as well as medical conditions (International Agency for Research on Cancer. & International Association of Cancer Registries., 2010). Decreased rates of disease incidences, morbidity, and mortality can be achieved through an increased understanding of nutritional genomics and timely and effective dietary intervention.

In this dissertation, I will explore how one possible diet, a diet rich in cruciferous vegetables, can be used successfully to prevent chronic diseases such as prostate cancer.

Cruciferous vegetables, members of the *Cruciferae (Brassicaceae)* family, are often characterized as having four equal-sized petals in the shape of a cross. Cruciferous vegetables include broccoli, Brussels sprouts, cauliflower, collard greens, watercress, wasabi, horseradish, bok choy, and kale. Primary characteristics of the cruciferous vegetables are their dense distribution of fiber, carotenoids, vitamins A, C, E, folate, and minerals. Moreover, cruciferous vegetables are a rich source of glucosinolates, which are sulfur-containing compounds.

Diets rich in cruciferous vegetables play an important role in the management of chronic diseases (D. Li, 2014). The impact of cruciferous vegetable intake on cardiovascular disease, stroke, diverticulitis, and cancer are reviewed below.

Protection against Cardiovascular Disease

Diagnosed, unmanaged cardiovascular diseases (CVD) are one of the leading causes of death in the United States. Of these diseases, coronary heart disease is one of the most common and serious forms of cardiovascular disease. There has been a decline in the number of cardiovascular disease-related deaths. Extenuating circumstances such as overweight and obesity prevalence in adults and children are aggravating the incidence and invasiveness of these disorders.

Epidemiological evidence supports the protective role of dark green vegetable intake on coronary heart disease (Joshipura et al., 2009; S. Tanaka et al., 2013; X. Zhang et al., 2011) as well as reductions in mortality rates among individuals consuming daily servings of vegetables (Park et al., 2009). Moreover, decreased prevalence of myocardial infarctions and angina pectoris has been associated with high intake of raw, leafy, green vegetables and juice (Crowe et al., 2011; Shenoy et al., 2010). Diets consisting mostly of vegetables, grains, beans, fruits, and occasional dairy in the absence of animal products, in combination with lifestyle changes can reverse heart disease without need for medication (Wofford, Greenlund, Croft, & Labarthe, 2007).

Vegetables contain a variety of components that can be attributed to these health benefits. Soluble fiber, for example, composing the outer skin and inner pulp, has contributed to lowering homocysteine, a known risk factor and biochemical parameter commonly used in the assessment of cardiovascular disease (Eilat-Adar, Sinai, Yosefy, & Henkin, 2013; Threapleton et al., 2013; W. Xiao et al., 2014). Moreover, soluble fiber in vegetables may help to control serum cholesterol levels, which is another risk factor for cardiovascular disease.

Protection against Stroke and Diverticulitis

Stroke and diverticulitis are among other chronic diseases that affect millions of individuals each year. Stroke is the 4th leading cause of death in the U.S. In 2010, this disease claimed approximately 39.1/ 100,000 lives and nearly 800,000 people suffer from stoke each year (Go et al., 2014; Go et al., 2013). Vegetable consumption may reduce the risk of stroke. Studies conducted by Joshipura et al and Larsson et al have correlated a high vegetable intake with a 19% decrease in ischemic stroke and lower mortality rate (Joshipura et al., 1999; Larsson, Virtamo, & Wolk, 2013). In a review by Ness and Powles, findings from 3 ecological, 1 case-control, and 4 cohort studies revealed a protective effect of fruits and vegetables to be stronger against stroke than against coronary heart disease (Ness & Powles, 1997). Moreover, another prospective study revealed a 30% reduction in ischemic stroke among men and women who consume 6 daily servings of fruits and vegetables, particularly green, leafy cruciferous vegetables and citrus fruits (Joshipura et al., 1999; Joshipura et al., 2009; Ness & Powles, 1997). The beneficial effects mentioned in these studies can be attributed to the soluble fiber and complex matrix of bioactive compounds found in cruciferous vegetables. Not only do vegetables potentially prevent strokes, but they also have been shown to deter diverticulitis.

Diverticulitis is a chronic disease that primarily affects the inner lining of the colon and is characterized with acute and chronic inflammatory states. One cause of diverticulitis is low dietary intake of vegetables, which are specifically noted for their fiber content (Marlett, McBurney, Slavin, & Association, 2002). Diets rich in cruciferous vegetables significantly reduce the incidences of this disease (Aldoori & Ryan-Harshman, 2002; Korzenik & NDSG, 2008). Moreover, clinical studies has found that prostate and colon-rectum are among cancer types associated with patients diagnosed with diverticulitis (Stefánsson, Ekbom, Sparén, & Påhlman, 1995).

Protection against Cancer

The reduction of risk factors associated with the development of certain cancers has provided additional evidence for the health benefits of vegetable consumption (Van Duyn & Pivonka, 2000). World Cancer Research Fund and the

American Institute for Cancer Research (AICR) have published numerous reports exhibiting an extensive collection of global research that reviews this topic. In a World Cancer Research Fund review, strong epidemiological and experimental evidence supports the conclusion that diets "high in vegetables and fruits (more than 400 g/day) could prevent at least 20% of all cancer incidence" (*Nutritional Oncology*, 2006). Due to the extensive background on the cancer protective effects of vegetables on a host of cancer types, AICR established dietary intake recommendations of 400 to 800 grams of varied vegetables and fruits per day (World Cancer Research Fund, 2007).

Uncovering health benefits associated with the consumption of cruciferous vegetables and reduced risk of certain cancers has been of particular interest over the past few decades (Farnham, Wilson, Stephenson, & Fahey, 2004). Epidemiological-based studies have been essential in identifying diet-related issues in health outcomes, vegetable intake patterns, and cancer development. In an extensive review conducted by Verhoeven et al, 87 case-control studies were analyzed and nearly 67% reported an inverse association between cancer risk and the consumption of assorted cruciferous vegetables (Verhoeven, Goldbohm, van Poppel, Verhagen, & van den Brandt, 1996). Findings from another major review conducted by Hidgon et al, epidemiological data revealed the importance of exposure to indoles and isothiocyanates within cruciferous vegetables in the reduction of cancer risk. Moreover, results from this study indicated the possible influence of genetic variation on the cancer protective effects of cruciferous compounds (Higdon, Delage, Williams, & Dashwood, 2007).

Exactly how cruciferous vegetables demonstrate these beneficial effects in a variety of cell, animal, and human systems is a critical research area devoted to resolving cancer outcomes. Cruciferous vegetables, in both whole and purified, isolated forms, have been implicated in regulating a variety of anticarcinogenic mechanisms including the modification of chemical compounds by inducting enzymatic activity involved in the detoxification of carcinogens and other xenobiotics. Cruciferous vegetables are a rich source of glucosinolates, sulfur-containing compounds. These compounds have been shown to block tumor

production in cellular and animal studies and inhibit the binding to various cell receptors such as the aryl hydrocarbon and androgen receptors in prostate cancer (Van Duyn & Pivonka, 2000).

Glucosinolates within Cruciferous Vegetables

Naturally, human diets are diverse in cruciferous vegetable food sources such as Brussels sprouts, collard greens, watercress, and bok choy. Glucosinolates (GLS) are secondary plant metabolites found in cruciferous vegetables. Glucosinolates, over 100+, have been identified and classified into three main groups based on their side chain configuration: aliphatic with alkyl or alkenyl group such as sinigrin, progoitrin; aromatic with benzyl group such as gluconasturtiin; and heterocyclic with indolyl group such as glucobrassicin, neoglucobrassicin. The structural diversity of glucosinolates is due to substituted side-chain configurations.

The total content of glucosinolates as well as their relative abundance not only varies by vegetable type but can also vary by vegetable species. Glucosinolate content for Chinese cabbage may yield 20 mg/100 g fresh weight versus watercress, which may yield 389 mg/100 g fresh weight (McNaughton & Marks, 2003). Glucobrassicin, a type of glucosinolate, can account for 8-12% of total glucosinolates in broccoli and other dark green vegetable varieties (Fahey, Zhang, & Talalay, 1997; Farnham et al., 2004; Galletti et al., 2015; Kushad et al., 1999). Therefore, in addition to variety, growing conditions and preparation techniques may greatly influence the glucosinolate content variability (Fahey et al., 1997; Freeland-Graves & Nitzke, 2013; Kushad et al., 1999). These are among a myriad of factors complicating the development of dietary recommendations for nonessential bioactive constituents (Gaine et al., 2013). Nonetheless, estimated intake of glucosinolates was estimated to be between 12-16 mg person/day (Fenwick & Heany, 1983; Getahun & Chung, 1999). The biologically active byproducts of glucosinolates are studied for their role in cancer prevention (Cirignano & Morgan, 2014; Fahey et al., 2012; Royston & Tollefsbol, 2015).

Chemical and Physical Properties of Glucosinolates

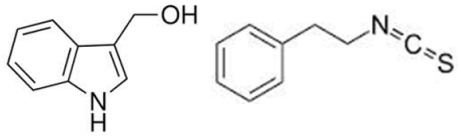
The structures of glucosinolates are diverse. However, the general structure of a glucosinolate consists of a β-D-thioglucose group, a sulphonated oxime moiety, and a variable side-chain derived from an amino acid. Examples of these amino acids include methionine for aliphatic glucosinolates, tryptophan for indole glucosinolates, phenylalanine for aromatic glucosinolates, and other branched chain amino acids such as isoleucine, alanine, valine, leucine) (Fahey, Zalcmann, & Talalay, 2001). There are 4 general categories of glucosinolates: 7 glucosinolates are derived directly from the amino acid side chains mentioned above. Other glucosinolates are synthesized from general ways, which include 1) chain-elongated forms of the amino acids, methionine and phenylalanine; 2) modified side chain structures after amino acid elongation and glucosinolate synthesis; and 3) complex side chains (such as o-(a -L - rhamnopylransoyloxy)-benzyl glucosinolate) (Mithen, Dekker, Verkerk, Rabot, & Johnson, 2000).

These phytochemicals are constitutively found in plant sources. Given their structure, glucosinolates may remain chemically stable within the cytoplasm until hydrolyzed by myrosinase upon tissue disruption. Upon hydrolysis, glucosinolates yield equimolar quantities of glucose, aglycone and sulphate molecules. The instability of the aglycones causes the compounds to be further metabolized to yield thiocyanates, nitriles and isothiocyanates. The structure of the side chain and reaction conditions will determine its final structure (Fahey et al., 2001; Mithen et al., 2000).

Bioactive Components within Cruciferous Vegetables

Indoles and isothiocyanates have been identified as active components present in Brassica vegetables with protective effects against prostate cancer (Beecher, 1994; Talalay & Fahey, 2001; Y. Zhang & Talalay, 1994). Glucosinolates are hydrolytically cleaved by myrosinase, an endogenous plant enzyme that is activated upon mechanical injury and yields bioactive components such as indoles and isothiocyanates. Interestingly, if ingested glucosinolates bypass myrosinase conversion, they have been shown to undergo conversion to indoles and isothiocyanates *in vivo* from intestinal bacteria. Human and animal microflora has

been shown to exhibit myrosinase activity (Fahey et al., 2012; Getahun & Chung, 1999; Kensler et al., 2005; Shapiro, Fahey, Wade, Stephenson, & Talalay, 1998). The bioactive phytochemicals released from glucosinolates have gained a significant amount of attention for their anti-cancer activity. Controlled experimentation with glucosinolate derivatives, such as indole-3-carbinol (I3C) and phenethyl isothiocyanate (PEITC) have helped to characterize inhibitory and cytotoxic activity in prostate cancer cells and animal model systems and have provided a mechanistic explanation for how crucifers are causative in lowering cancer risk.



Indole-3-carbinol Phenethyl isothiocyanate

Figure 1 Chemical Structures of indole-3-carbinol and phenethyl isothiocyanate

Indole-3-carbinol, a Glucobrassicin-derived bioactive compound

Indole-3-carbinol (I3C) is a diet-derived bioactive compound generated by the hydrolysis of glucobrassicin from cruciferous vegetables (Fig.1, adapted from (Broadbent & Broadbent, 1998a)) . Glucobrassicin yields unstable intermediate aglycone products when combined with myrosinase, a plant defense enzyme activated upon mechanical injury such as chewing. I3C is a major indolic compound that is vigorously studied for its chemopreventive properties. Inhibitory effects of I3C lies within its ability to yield aromatic hydrocarbon byproducts such as 3 -, 3' - diindolylmethane (DIM) under acidic aqueous conditions (Bjeldanes, Kim, Grose, Bartholomew, & Bradfield, 1991). Formation of these metabolites is dependent upon environmental pH. Typically, DIM's highest formation levels/ yields occur between a pH of 4 to 5, similar to the pH range of mammalian stomach acid.

Furthermore, *in vitro* studies have shown that I3C does not induce cytotoxicity, is not mutagenic, and can be tolerated in doses up to 1,200 mg/d when consumed in controlled human subject testing (Meng et al., 2000; Reed et al., 2006; Reed et al., 2005; N. Takahashi, Dashwood, Bjeldanes, Williams, & Bailey, 1995; T. T. Wang, Schoene, Milner, & Kim, 2012). The effects of I3C in patients with PSA

recurrence following prostate cancer surgery have also been assessed. Participants were given a placebo, 2 servings of Brassica vegetables, or an I3C supplement for 6 months, however, results from this study have not been published (Clinical Trial ID: NCT00607932) Part of I3C's benefits lies within its ability to induce phase 1 and 2 drug metabolizing gene expression, which enhances modification and excretion of harmful substances. Moreover, I3C has been noted for its antiangiogenic effects on tumor cells lines and its elicited effects on immune response (Tsai, Liu, & Chen, 2010; T. T. Wang et al., 2012).

Amounts of I3C found in diets vary due to its chemical instability within individual vegetables. However, I3C has been found in concentrations of approximately 50 μg (339.7 μmol)/g dried vegetable diet (Bradfield & Bjeldanes, 1987). Estimated intake of I3C in the U.S. may be less than 2.64 mg/day based on an estimated vegetable intake of 2 ounces per day (Broadbent & Broadbent, 1998a).

Phenethyl isothiocyanate, a Gluconasturtiin-derived bioactive compound

Phenethyl isothiocyanate (PEITC) is another example of a diet-derived bioactive compound derived from the hydrolysis of gluconasturtiin in *Brassica* vegetables (Fig. 1, adapted from (Moon, Brazeau, & Morris, 2011)). PEITC and its derivatives are also extensively studied for their chemopreventive properties. Depending on the reactive conditions, phenethyl isothiocyanate is yielded from glucosinolates via the mercapturic pathway. PEITC-NAC and several other thiol conjugates of isothiocyanates have been reported as primary metabolites of PEITC. Amounts of PEITC found in the diet may vary based on vegetable composition. Watercress, for example, has been shown to yield approximately 12 mg PEITC/57 g wet weight (F. L. Chung, Morse, Eklind, & Lewis, 1992). A clinical trial in progress is assessing the effects of watercress juice (55 g watercress) on oral cancer of patients considered heavy smokers (ClinicalTrial.gov ID: NCT01790204).

Biological activities of Glucosinolate Compounds, I3C and PEITC

The potent chemopreventive activity of isothiocyanates has been demonstrated in many experimental models as well as in humans. Examples of these

models include hepatocarcinoma, mammary gland and other breast cancer cell lines, esophageal, pancreatic, gastrointestinal, and urinary and bladder of mice, rats, and other rodents and prostate, breast, colon cancer in humans (F. L. Chung et al., 1992; Hecht, 1995; Khor, Cheung, Prawan, Reddy, & Kong, 2008; Y. S. Kim & Milner, 2005; Moy et al., 2008).

Moreover, I3C and PEITC have attenuated growth in various cell models including the classic LNCaP, PC3, and DU145 lines. Moreover, these compounds are unique in their ability to differentially regulate cell growth. I3C, for example, has been shown to act as a regulator of the androgen responsive pathway. On the other hand, PEITC has been shown to inhibit growth independent of the androgen pathway through attenuation of angiogenesis and other biological pathways (Firestone & Sundar, 2009).

Initially, isothiocyanates' specific modes of action were thought to be through the regulation of Phase 1 and Phase 2 detoxification enzymes within carcinogenesis models. Findings from Chiao et al indicated that isothiocyanates and their metabolites mediate other functions including growth inhibition and apoptosis induction in prostate cancer cells (Chiao et al., 2000; Shapiro et al., 1998). Results from this study indicated the effectiveness of isothiocyanates at the post-initiation and progression stages of carcinogenesis (Moy et al., 2008; Rose, Faulkner, Williamson, & Mithen, 2000).

In addition to genetic alterations, the regulation of epigenetic mechanisms is an important factor contributing to the development and progression of prostate cancer. Epigenetic regulation is considered heritable changes (DNA hypermethylation and hypomethylation, and histone modifications) in gene expression that occur without changes in DNA sequence (W Watson, M Beaver, E Williams, H Dashwood, & Ho, 2013).

Anti-proliferation

I3C and PEITC have been well studied for their anticarcinogenic properties in *in vitro* cell model experiments and *in vivo* animal and human subject studies. Exposure to I3C and PEITC prior to carcinogenic insult has demonstrated these

protective properties as evidenced by inhibited tumor growth. Findings have shown that I3C and PEITC may also enhance cancer progression if exposed following initiation (D. J. Kim et al., 1997). However, the majority of reports overwhelmingly supports the anticarcinogenic effects of these compounds and supports their usage as candidate chemopreventive agents.

I3C plays a critical role in regulating prostate cancer cell growth. Previous studies have shown that I3C can inhibit cell growth of androgen-dependent and –independent cell lines including LNCaP, DU145, as well as PC-3, a poorly differentiated PCa cell line via the induction of G_1 cell arrest and apoptosis following exposure to 30 to 100 μM of I3C (Chinni, Li, Upadhyay, Koppolu, & Sarkar, 2001; Chinni & Sarkar, 2002; Nachshon-Kedmi, Yannai, Haj, & Fares, 2003; Yeh et al., 1999). Proposed mechanisms for I3C growth inhibition in androgen independent cell lines may be through the inactivation of AKT and NF-κB (Aggarwal & Ichikawa, 2005; Jin et al., 2008; J. Zhang, Hsu B A, Kinseth B A, Bjeldanes, & Firestone, 2003). In a study conducted by Souli et al, I3C (20 mg/kg body weight) was administered before and after transplanting TRAMP-C2 prostate cancer cells, which inhibited xenograft tumor growth by 78%, decreased proliferation (3 folds lower Ki67 staining), and promoted apoptosis (caspase 3 staining) as well as decreased angiogenesis by modulating microvessel density (CD 31 endothelial marker). These results were also correlated with the author's *in vitro* I3C treatment with the TRAMP-C2 cell line (Souli, Machluf, Morgenstern, Sabo, & Yannai, 2008).

PEITC has been shown to inhibit growth of locally isolated and metastatic prostate cancer cell growth. Proposed mechanisms for PEITC-induced apoptosis in advanced prostate cancer may be a result of caspase-3-dependent mechanisms as well as through the inhibition cell survival that is mediated by the activation of extracellular signal-regulated kinases (ERK 1 and -2) or phosphorylation of IKK-α and IKK-β (Hudson et al., 2012; Powolny et al., 2011; D. Xiao et al., 2006).

Chemopreventive effects of PEITC have also been studied in animal models. Rodents fed low concentrations of PEITC (≤ 5 μM), had significantly lower tumor growth within weeks of cancer initiation (F. L. Chung et al., 1992; Powolny et al., 2011; Staretz, Koenig, & Hecht, 1997). In a study conducted by Powolny et al,

treatment with 3 μmol PEITC/g diet using a more advanced prostate cancer xenograft model was not associated with a decrease in cell proliferation, apoptosis induction or select neogenesis biomarkers. Instead, the proposed pathways for the 26.48% decrease in tumor burden may have been due to the induction of autophagy. Conclusions from these studies highlight the complexity of tumor modulation and importance of examining physiologically-relevant dosing in various animal and cell models (Hudson et al., 2012).

Anti-inflammation

Glucosinolates are ideal inflammatory regulators due to their low side effects at physiologically relevant doses. I3C's and PEITC's anti-inflammatory effects have been demonstrated through the inhibition of cytokine production. Moreover, dietary supplementation with I3C and PEITC inhibited *in* vivo tumor growth through the suppression of anti-inflammatory biomarkers such as CD31 and VEGF (Hsu et al., 2005; Hudson et al., 2012; Souli et al., 2008). I3C (50 μM) and PEITC (5 μM) have been showed to moderately inhibit the production and release of cytokines such as IL-10 (anti-inflammatory) and TNF-α (proinflammatory) in macrophages under *in vitro* conditions. These results suggest that inhibition of these cytokines may rely on suppression of gene expression via NF-κB pathway (Aggarwal & Ichikawa, 2005; Tsai et al., 2010).

I3C and PEITC may differentially regulate cytokine production within prostate tumor cells. I3C has been shown to regulate monocyte/macrophage attraction in LNCaP, human prostate cancer cells. In a study conducted by Kim et al, I3C and its metabolites significantly inhibited promotional effects of DHT on CCL2 expression and monocyte migration (E. K. Kim, Kim, Milner, & Wang, 2013). PEITC has been shown to retard tumor growth independently of androgen responsive pathways. One mechanism lies in PEITC's ability to regulate inflammation within the tumor microenvironment. PEITC has also been studied for its effects on proinflammatory cytokines in macrophages (Tsai et al., 2010). Hudson et al, further demonstrated the ability of low dose PEITC to obstruct angiogenic properties by reducing tumor growth and VEGF gene expression (Hudson et al., 2012). Moreover,

results from this study indicated that PEITC had little effect on cell proliferation, cell cycle, and androgen-dependent pathways. More importantly, transcriptomic analysis of this mouse population treated with 3 μmol/g diet PEITC revealed significant changes along other pathways such as inflammatory status that may affect tumor growth (R. W. Li, Li, & Wang, 2013).

Due to the complexity of cytokine interactions, which may act synergistically or additively, results may differ within animal and human models and warrant further investigation (Gannon, Malone, & Napolitano, 2001; Pruett, Fan, Zheng, & Schwab, 2005).

Xenobiotic Clearance

Carcinogenic inhibitory properties of I3C and PEITC has been demonstrated through the alteration of xenobiotic metabolism within carcinogenic and xenograft models.

I3C and its metabolites have been shown to modulate xenobiotic metabolism when administered through the diet. The ability of I3C to induce CYP1 family, such as CYP1A1, and regulate ligand-activated aryl hydrocarbon receptor (AhR) has been extensively studied in human cell lines and animal models. Following ligand binding, the AhR translocates into the nucleus and forms a heterodimer with ARNT, a member of basic helix-loop-helix/Per-ARNT-Sim family. The heterodimer then binds to xenobiotic response elements (XREs) in the promoter and enhancer regions of targets genes to regulate their transcription (Hestermann & Brown, 2003). The expression of phase 1 enzymes, which may activate procarcinogens, is responsible for modifying the structure and functionality of the xenobiotic by adding a polar group (Hestermann & Brown, 2003; Yu & Kong, 2007).

These enzymes are mainly regulated through xenobiotic sensing nuclear receptors such as AhR, constitutive andostane receptor (CAR), pregnane-X receptor (PXR), and retinoid-x receptor (RXR). Human subfamilies primarily involved in xenobiotic metabolism include CYPs 1A, -2C, -2D, -2E, and -3A, with varied but overlapping substrate-specificity (Kirchmair et al., 2012). Phase 2 enzymes, including UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1) and NAD(P)H

dehydrogenase, quinone 1 (NQO1), are known to be induced by I3C and are responsible for facilitating complex excretion processes such as glucoronidation of modified xenobiotics (Shertzer & Sainsbury, 1991a). As a result, these enzymes may catalyze the conjugation of carcinogens and other substances. Generally, these enzymes are transcriptionally controlled by the Nrf2/ARE signaling pathways.

The signaling pathways regulate the expression of many detoxifying enzymes and the ability to inhibit or activate the expression these enzymes are a major target for dietary compounds. The chemopreventive effects elicited by these compounds are determined by a network composed of known and unknown molecular targets, their signaling pathways and its interaction with various xenobiotics, including carcinogens, drugs, and other dietary factors (Kensler et al., 2005; Talalay & Fahey, 2001; Yu & Kong, 2007). Thus, the exact mechanisms underlying the ability of I3C, PEITC, or their metabolites to induce Phase 1 and 2 xenobiotic metabolizing enzymes via the aryl hydrocarbon receptor are unclear.

Research Methodology

Chemoprevention refers to the use of naturally found, bioactive compounds in the prevention of prostate cancer, as adapted from (Sporn, 2011). Highly standardized analytical, practical, and efficient techniques, such as real-time PCR, Western Blot, and ELISA have been promoted as important research methodology for determining the efficacy of cruciferous vegetable-derived compounds on prostate cancer and inflammation encompassing the tumor microenvironment.

A variety of methods are employed to assess molecular sensitivities within the prostate cancer microenvironment following exposure to cruciferous vegetable-derived biological compounds. Utilizing cell-based assays have been a common practice to probe for bioactivity potential and chemopreventive effects of phytochemicals. These assays serve as swift, inexpensive means of analysis prior to initiating *in vivo* studies, which can provide a more physiologically relevant response.

Under *in vitro* conditions, growth inhibition, anti-proliferation, and cell adhesion assays were used to evaluate changes in cell viability and proliferation, which are indicators of sustained growth and evasion of apoptosis, key hallmarks in

mammalian cancer progression. Moreover, cell proliferation and growth assays are well established methods used to distinguish effective dosage from no effect levels and assess the responses of these concentrated phytochemicals on cell viability. Cell adhesion involves a variety of molecular processes such as receptor-ligand binding and changes in intracellular pathways. Cell adhesion assays provide insight on the changes in a cell and its extracellular adhesion proteins as well as other cellular events. The adhesion assay in this study has been commonly used to examine the sensitivity of a specific cell line and its substrate in the presence of an inhibitor, which in our case, are the candidate phytochemicals. All three assays are analyzed using the sulforhodamine B (SRB) method. SRB assay, discussed below, is another well-established method used in the quantification of adherent cell lines to an immobilized substrate.

Changes in cancer status are reflected by developmentally programmed gene expression patterns. The responsiveness of cell lines with dysregulated genes assist in facilitating the evasion of antineoplastic controls, including apoptosis, and cell cycle checkpoints. Analytical methods used to assess *in vitro* as well as *in vivo* changes include SRB method, to quantify changes in cell number, RT-PCR, to quantify relative changes in genomic expression levels.

SRB is a commonly used screening method to measure the loss of cell viability. It is often used a first-step assay to determine whether a compound has the ability to cause decreased cell proliferation, survival, or cell death at a single intracellular end-point before more intensive methods are used. SRB is an anionic aminoxanthene dye, which forms an electrostatic complex with basic amino acid residues of proteins (Skehan et al., 1990). The uses of this assay are numerous. It is commonly employed in a number of high-volume screening applications, is mostly known for its sensitive linear response, rapid color development and stable sensitivity when read at absorbances between 560 and 580 nm (Skehan et al., 1990).

Polymerase Chain Reaction (PCR) is an exquisitely sensitive, powerful, and central molecular biological method to detect amplified template nucleic acid PCR products. The use of real time, Reverse-Transcription Polymerase Chain Reaction (RT-PCR) can be used to correlate the cytotoxic potency of a compound

demonstrated in cell viability assays with a more comprehensive analysis of gene expression data yielding information on target regulators of signaling pathways involved in cell survival, proliferation, xenobiotic metabolism, and inflammation (Provenzano & Mocellin, 2007). The principles and practical uses of RT-PCR have been extensively review (Jozefczuk & Adjaye, 2011).

Techniques utilized in the detection of protein are manifold. For this reason, the selection of suitable approaches to optimally measure changes in expression levels according to the biological question, sample type and other methodical requirements is critical. Western blot, enzyme-linked immunoabsorbent assays, and immunohistochemistry are well within the most distinguished and desirable practices in scientific research and medical diagnosis within clinical disciplines.

Western blot, developed nearly four decades ago, is a fairly common detection method used to identify the nature or epitope of a protein. Detection of a desired protein occurs following gel electrophoresis separation, which spans transferred proteins across a nitrocellulose membrane prior to being exposed to antibodies against proteins of a desired molecular weight. In our study, the proteins of interested were revealed via infrared fluorescent detection, a relatively new detection method.

Immunoassays measure antibodies with varying degrees of sensitivity. Enzyme-linked immunoabsorbent assay (ELISA) may detect less than 0.001 ng/mL. This assay has been used in a variety of molecular processes to sensitively, quantitate levels of protein by an approach termed “sandwich enzyme immunoassay”. In our studies, this technique was used with sequential mouse monoclonal antibodies. When the immunocomplexes are assayed with an appropriate substrate such as peroxidase, the levels of the product can be quantitated colorimetrically and a directly proportional to the degree of protein product induced or reduced by the test compounds. ELISA is a highly sought-after method because of its lower cost, safety, speed, and simplicity in comparison to radioimmunoassays.

Diagnostic immunohistochemistry (IHC) has been a rapidly evolving field. However, foundational principles of IHC still operate under the notion that the distribution of antigens of interest can be detected in tissues often times using mono- or polyclonal, enzyme-linked antibodies (Coons & Kaplan, 1950). Different from WB

and ELISA, IHC enables pathologists and other researchers to visually assess and grade changes of a protein of interest within a particular cell or tissue (Duraiyan, Govindarajan, Kaliyappan, & Palanisamy, 2012). Thereby deeming this practice a valuable, gold standard tool in the diagnosis of cancers. IHC plays a fundamental role in the gross assessment of tissue functionality or changes in up- or down-regulation of specific tumor antigens (E. Rubin & Reisner, 2014). Spectroscopy such as light microscopy frequently serves to identify the antigen, which occurs when the substrate is degraded by immunoperoxidase to yield a colored insoluble substrate at the location of the antibody-antigen complex.

Staining methods provide immunohistochemistry with a high degree of functionality. Hematoxylin and Eosin (H & E) staining is a widely used, principle stain in histology and is considered a gold standard diagnostic tool (Bancroft & Gamble, 2008). Among a variety of practices, this stain is usually involved in the examination of a biopsy to detect suspected cancer (R. Rubin, Strayer, Rubin, & McDonald, 2008). Since most cells are transparent, this technique has been used to distinguish cell constituents by staining basic components and counterstaining acidic components based on their affinity for eosin and hematoxylin dyes (Bancroft & Gamble, 2008). Eosin is an acidic dye used to stain basic or acidophilic components such as the cytoplasm a red or pink color. Hematoxylin is a basic dye, which stains acidic or basophilic components such as DNA: heterochromatin and nucleolus within the nucleus and parts of the cytoplasm that contains RNA: ribosomes, and rough endoplasmic reticulum, a violet or blue color. Digital light microscopy is considered an easy, cost-effective, and efficient method to conduct manual histopathological analysis (Bancroft & Gamble, 2008).

KI-67 staining is a diagnostic tool routinely used in the determination of cellular proliferation and in conjunction with other laboratory methods. Assessing for changes in growth fraction is important when measuring effectiveness of a treatment compound used to recede cancer progression. Overexpression of KI-67 is associated with a greater risk of adverse outcomes in prostate cancer (Pollack et al., 2004). As with other IHC methods, KI-67 usage extends beyond cancer detection and can be used to confirm viability within healthy tissue. The World Health Organization has

included KI67's reactivity in the organization's grading system for assessment of the gastrointestinal tract (Klöppel, Perren, & Heitz, 2004). KI67 staining is considered a superior proliferative marker to PCNA in the colon (Holt, Moss, Kapetanakis, Petrotos, & Wang, 1997). In such cases, over expression of KI67 is also associated with decreased survival in human and animals (J. Kim et al., 2015; Nishimukai et al., 2014).

Evasion of apoptosis is another hallmark of cancer progression. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) is a commonly used assay to detect DNA fragmentation in apoptotic cells (Botelho et al., 2014; Z. Wang, Yang, Jacob, Aziz, & Wang, 2015). The terminal deoxynucleotidyl transferase (TdT) adds dNTPs to the 3' end of DNA from endonuclease cleavage products in the absence of a template. Initially, TUNEL signaling is weak, however, within an hour following binding to a strong uniform labeling, intensity increases proportionate to nicks in the DNA (Saraste, 1999). Thus, this very sensitive assay can quantitate DNA damage from a single to hundreds of cells, including those within the gastrointestinal tract (Akpek et al., 2003; C. Gao & Wang, 2009). False positives within the assays indicate necrotic cells or cells undergoing DNA repair and gene transcription.

Despite the revolutionized management of this disease from the development of biomarkers for prostate cancer screening and detection, the exact mechanisms of indole-3-carbinol and phenethyl isothiocyanate on human prostate cancer growth inhibition remains elusive. Further validation of existing biomarkers as well as the discovery of new markers to identify men at-risk or currently with undiagnosed aggressive forms of prostate cancer is necessary.

Summary

Overall diet quality heavily influences chronic disease development. Diets rich in cruciferous vegetables have an essential role in improving health outcomes by lessening the manifestation and progression of disease. Bioactive compounds from cruciferous vegetables, specifically I3C and PEITC, are ideal chemopreventive agents

due to their accessibility, bioavailability, and seemingly low side effects. In prostate cancer, PEITC and I3C may modulate tumor growth and its surrounding environment. Because of their perceived safety and health benefits, increased interest has been placed on the use of natural products to prevent or minimize chronic disease outcomes. PEITC and I3C have the potential to reduce the incidence of prostate cancer and associated poor outcomes by decreasing biological dysfunction and thus, minimizing clinical manifestation and advancement. However, the regulatory mechanisms in which these compounds elicit these beneficial responses are elusive and merit further investigation.

This dissertation addresses this issue by characterizing deregulated pathways related to apoptosis, proliferation, xenobiotic metabolism, androgen -response, and inflammation.

Chapter 2: Inhibition of LNCaP, Human Prostate Carcinoma, Xenograft Tumor Growth by Dietary Administration of Phenethyl Isothiocyanate is associated with Inhibition of Macrophage Infiltration and Insulin-like Growth Factor-1 Pathway

Abstract

Phenethyl isothiocyanate (PEITC), a bioactive constituent of cruciferous vegetables, is known for its risk-reducing effects against cardiovascular diseases, cancer, and secondary contributors such as chronic inflammation. However, its mechanism(s) in regulating tumor growth remains unclear. In the present study, chemopreventive activity of 5 μmol PEITC/g diet was examined in immune-deficient BALB/C nu/nu male mice (5 to 7 weeks old), inoculated with androgen-dependent human prostate cancer cell line. Mice were found to be differentially responsive to 5 μmoles PEITC/g diet and alterations in gene expression were assessed in tumor samples. Tissue analysis revealed a 41.6% decrease in tumor burden, as compared to control group, with no observable toxic effects. Consistent with previous in vivo findings, PEITC had minimal effects on prostate-specific antigen (PSA), an androgen-responsive gene in tumors. Significant reduction of KI67 (11.4%), a marker of cell proliferation, was observed in the PEITC treatment group. Moreover, reduced expression of EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (Emr1 or F4/80) (-38.8%), a macrophage marker was observed. A 49% increase in insulin-like growth factor binding protein 3 (IGFBP3) expression, responsible for forming a complex with insulin-like growth factor (IGF) (a prostate cancer-associated proliferative hormone), was also significantly altered in tumors of mice supplemented with a 5 μmol PEITC/g diet. In summary, dietary administration of PEITC in this xenograft model assuaged prostate cancer progression by reducing proliferation, macrophage infiltration, and inhibiting IGF-1-mediated pathways.

Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer in U.S. men. In 2014, there were an estimated 233,000 new cases and 29,480 deaths from prostate cancer (DeSantis et al., 2014). While prostate cancer is most frequently diagnosed in men 65 years or older, the process of carcinogenesis often takes 10 to 30 years to develop from initiated cells to malignant cancer (Howlader, Mariotto, Woloshin, & Schwartz, 2014). The presence of prostatic intraepithelial neoplasia and proliferative inflammatory atrophy, precursors of prostate cancer, further complicate matters by contributing to the long latency period of 10 or more years before invasive carcinomas are detected (J. J. Liu et al., 2014; R. Siegel, DeSantis, et al., 2012).

Delays in neoplastic development and tumor incidence may be achieved through pharmacological, hormonal, or nutritional intervention. Early onset prostate cancer comes with a higher risk of mortality; therefore cancer prevention and early detection are critical (Karchner, Powell, & Hahn, 1999; Kent et al., 2014). In addition, there is currently no effective treatment for advanced prostate cancer, which illustrates a need to identify and utilize cancer preventive strategies. Recently, published work has alluded to the consumption of cruciferous vegetables to have a significant impact on prostate cancer eradication (Chan, Van Blarigan, & Kenfield, 2014; W. W. Zhang, Feng, & Narod, 2014). As a result, dozen of classes of natural and synthetic chemotherapeutic agents are undergoing clinical evaluation to assess their ability in reducing the incidence, morbidity, and mortality of the disease. Phenethyl isothiocyanate, as one of these agents, holds promise for the prevention of clinically aggressive prostate cancer.

Phenethyl isothiocyanate (PEITC) is a well-documented isothiocyanate, derived from the hydrolysis of gluconasturtiin, a glucosinolate commonly found in cruciferous vegetables (P. Gupta, Wright, Kim, & Srivastava, 2014; Minarini et al., 2014). PEITC is generally regarded as safe and has displayed non-toxic effects in both animal and human studies when administered in micromole dosages. Moreover, the effects of isothiocyanates (ITCs) have been shown to display tumor-specific effects. In a recent study conducted by Clarke et al., ITCs selectively induced apoptosis and cell cycle arrest in benign hyperplasia, LNCaP and PC3, which are androgen-dependent and androgen-independent cell lines, respectively. But did not affect PrEC, which is a primary, normal prostate epithelial cell line (Clarke, Hsu, Yu, Dashwood, & Ho, 2011). Recent preclinical and clinical studies report enhanced tumor resistance within various cancer types following treatment with PEITC (P. Gupta et al., 2014). Moreover, PEITC has been shown to mitigate post-initiation events leading to the progression of prostate cancer. Proposed mechanisms suggest PEITC and its metabolites target the induction of multiple pathways related to autophagy, apoptosis, and reduction of angiogenesis, proliferation as well as cell attachment in androgen-dependent and independent cell lines. Covalent binding of ITC to protein targets may play an important role in inducing apoptosis. However,

how ITCs start intracellular signaling leading to tumor inhibition is not clearly understood. Thus, this research plays significant roles in examining the reduction of growth stimulation and sustained survival of prostate tumor cells.

Stimulated proliferation and enhanced probability of malignant transformation of epithelial cells has been attributed to reduced integrin expression, insulin-like growth factors (IGFs) and its counterparts. Low integrin gene expression may mediate ECM disruption and hinder cell attachment. Studies indicate that insulin-like growth factor binding proteins (IGFBPs) regulate cell survival activity (M. Schwartz, 2014; M. A. Schwartz, Schaller, & Ginsberg, 1995; Waterston et al., 2002). However, the balance between growth factors and receptors within the tumor microenvironment influences the presence of high affinity IGFBPs. Prostate cancer has a characteristically low expression level of IGFBP3 found in the plasma. Low IGFBP3 levels may be partly due to prostate specific antigen (PSA), which has been shown to reduce IGFBP3 affinity for free IGF (Takada, Ye, & Simon, 2007). This protein forms a ternary complex with IGF-I or –II and an acid-labile unit. IGFs within this complex are sequestered and unable to interact with IGFBPs or IGF receptors along the prostate cancer cell surface. Additionally, IGFBP3 may act independently of IGF to inhibit cell growth. In response to cellular stress, IGFBP3 has been shown to induce apoptosis (Bonkhoff, Stein, & Remberger, 1993; Fornaro, Manes, & Languino, 2001; T. H. Lin et al., 2013; Mizejewski, 1999). The anti-tumorigenic effects of IGFBP3 plasma proteins may support its usage as a potential end point biomarker for prostate cancer. Recent findings from our lab have shown that IGFBP3 gene expression is significantly increased following dietary exposure to PEITC in advanced human prostate cancer xenograft models (Hudson et al., 2012; R. W. Li et al., 2013). The full identification of molecular targets and exact efficacy and biological mechanisms of PEITC's anti-carcinogenic effects *in vivo* are still being established.

The present study examines the effect of dietary administered PEITC on LNCaP, human prostate cancer growth in a nude athymic mouse model. This study seeks to answer whether PEITC prevents or inhibits the growth of advanced prostate carcinoma *in* vivo; and if its *in vivo* anti-tumorigenic effect is correlated with up-

regulation of biomarkers related to proliferation, including the up-regulation of IGFBP3 and down-regulation of integrins and inflammatory biomarkers. Results from this study intend to shed light on the biological significance and elucidation of possible mechanisms affected by dietary administered and biologically achievable concentrations of PEITC.

Materials and Methods

Cell Adhesion Assay

LNCaP, prostate carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were incubated at 37^{o}C in the presence of CO_2-air (5:95, v/v).

LNCaP cells were trypsinized with 0.25% Trypsin-EDTA solution (Invitrogen) then seeded in conditioned media with PEITC (0, .1, 1, 2.5, and 5 µmol/L media) at a density of 0.625 x 10^5 cells/well in 24-well plates (Corning Inc., Corning, NY, USA). Cell plating efficiency was defined as cells attached to culture plate surface and was determined by sulforhodamine B (SRB) assay (Hudson et al., 2012; Skehan et al., 1990)

Diet Composition

During the course of the study, mice were provided powdered AIN-93M diets in the absence or presence of purified phenethyl isothiocyanate (5 µmol/g diet). Diets were formulated by Research Diets (New Brunswick, NJ) and stored at -20°C until treatment initiation. Formulation for control and treatment diets were based upon previous experiments that assessed quality and quantity of I3C in rodent diets (Kassie, Matise, Negia, Upadhyaya, & Hecht, 2008).

Dosage Considerations

Animals were fed 0 and 5 μmol PEITC/g diet for the duration of this study. The selected dosage for this study was based upon previous studies reporting beneficial effects of this dosage range in both *in vitro* and *in vivo* mouse models, without any report of adverse effects. A dose of 5 μmol PEITC/g diet in mice is roughly equivalent to a human equivalent dose (HED) of 16.54 mg PEITC/kg body weight. Moreover, in a clinical oncology study, administration of PEITC have been provided in doses of ~14.4 mg/57 g watercress/day in male and female cancer patients (Clinical Trial ID: NCT01790204) (F. L. Chung et al., 1992). Therefore, the dose selected for this study assesses the effects of tumorigenesis on PEITC concentration similar to amounts consumed in the diet as well as amounts supported on a low dose chemopreventive range. Moreover in preclinical tumorigenesis studies, mice exhibited cancer protective effects following dietary and orally administered concentrations of PEITC exceeding 12 μmol/day, ranges that were 2.5 times higher than concentrations used in this study (N. Gao et al., 2011; D. Xiao et al., 2006). Thereby, deeming the concentration of 5 μmol PEITC/g diet used in this study, safe, cautious, and physiologically relevant dosage.

Tumor Xenograft Model

LNCaP, an androgen-dependent human prostate carcinoma cell line were be used to model response to dietary administered PEITC (ATCC, Manassas, Virginia). Prior to inoculation, LNCaP cultures were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin purchased from Invitrogen (Carlsbald, CA). Cultures were incubated in an atmosphere composed of 5% CO_2 at 37°C.

All experimental protocols were in accordance with the National Institutes of Health guidelines and were approved by the USDA Animal Research Advisory Committee (BAACUC Protocol No. 12-001). Male athymic nude mice (BALB/c nu/nu, 20-22 g, 5-7 weeks old) were purchased from Charles River (Frederick, MD) and were individually housed in filter-top cages at the USDA BHNRC animal facility. Room conditions were carefully monitored and held at a constant temperature

of 22 ± 1°C with a 12 hour rotating day and night cycle. Animals consumed food and filtered water *ad libitum*. Food consumption and body weights were recorded weekly. An acclimation period of 1 week prior to treatment feedings enabled mice to adapt to control AIN-93M diet and environment. Animals were then randomized into two experimental feeding groups- (i) control diet, (ii) control diet with 5 µmol PEITC/g diets (n = 10/group). Two weeks following treatment initiation, all mice were inoculated via subcutaneous injection into the right and left flanks with LNCaP cell suspension. Cell suspension consisted of LNCaP cells at a density of 2×10^6 with 50 µl of phosphate-buffered saline (PBS) and an equal volume of Matrigel (BD Biosciences, Mansfield, MA) (Kleinman & Martin, 2005). Injection sites and tumor volume (cm^3) were monitored and measured weekly for tumor growth and biological efficacy: (cm^3) = 0.523* x [length (cm) x width^2 (cm^2)] (Hudson et al., 2012; Tomayko & Reynolds, 1989). Mice remained on their respective diets for 7 weeks following inoculation.

Study Termination

At the end of the study, mice were euthanized using a CO_2 chamber and necropsy was immediately performed. Blood was collected, stored in vials coated with potassium EDTA solution (15% w/v), and placed on dry ice until plasma separation. Plasma was separated via centrifugation at 1500 rpm for 30 min at 4°C. Soft tissue including tumor, liver, kidneys, and spleens were collected and subdivided. Samples were immediately frozen in liquid nitrogen for protein analysis or in RNA*later*, an RNA stabilizing agent purchased from AMBION Inc. (Austin, Texas). Samples were stored in an -80°C freezer until further analysis.

in vivo RNA Isolation and Reverse-transcription (RT)-PCR

For determination of mRNA expression in tumor and liver samples, total RNA was isolated and purified using RNeasy (Qiagen Inc., Valencia, CA), according to manufacturer's protocol. StrataScript First Strand complementary DNA Synthesis kit from Stratagene (Santa Clara, CA) were used to reverse transcribe complementary DNA. Real-time PCR was performed on Applied Biosystems 7900HT Real-Time

PCR System using TaqMan® Gene Expression Assay (Carlsbad, CA). Relative mRNA levels for analysis of gene expression changes were quantified using ΔCt method, a previously described TaqMan real-time RT-PCR method (E. K. Kim et al., 2013; Yuan, Reed, Chen, & Stewart, 2006). TATA box binding protein (TBP), a housekeeping gene, was used to calculate relative gene expression levels in soft tissue samples.

Marker genes for pathways related to carcinogenesis are listed in Table 1.

Table 1

Pathway	Human	Mouse
Androgen Response	PSA/KLK_3, NKX3.1	
Proliferation	Ki67	
Growth Factor	IGFBP3	
Inflammation	IL-6	I1-6, Il-1β
Macrophage		Emr1/ F4/80
ECM	Integrins, FN1	

Protein Analysis

In order to identify post-translational changes of mRNA level gene changes, two protein analysis methods were utilized. Immunohistochemistry analysis was used to examine gross morphological changes in soft tissue samples. ELISA was utilized to analyze circulating inflammatory-related protein (interleukin-6) changes in isolated plasma. Assays were performed using a commercial kit according to manufacturer's protocols (eBioscience, San Diego, CA).

For Western blot analysis, tumor samples from 7 mice in each dietary treatment group were used. Western analysis was performed according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Anti-β-actin was used as a loading control and LNCaP lysate was used as a positive control. Immunoreactive bands were fluorescently quantified using LiCor Odyssey software.

Statistical Analysis

The 10 animals used per group give >95% power to detect effects with a magnitude of at least 10%. Mean body weights, food consumption, and tumor growth was used in the preliminary assessment of treatment groups. All end point assays were conducted in triplicate and averaged for group analysis. Means were reported as mean ± SEM. One-Way Analysis of Variance (ANOVA) and Bonferroni test were conducted to determine the differences among means. Statistical significance was declared at $p < 0.05$ using SPSS for Windows (version rel. 21.0, SPSS Inc., Chicago, IL) and GraphPad Prism 5 for Windows (version rel. 5.01, 2009, GraphPad Software, Inc., La Jolla, CA). Power calculations were completed using SAS 9.3 (version 6.1.7601, 2010).

Results

Effects of PEITC Intake on Body Weight and Food Consumption

Phenethyl isothiocyanate is a candidate chemopreventive agent. During the course of this study mice exposed to 5 µmol PEITC/g diets were assessed for physical and molecular changes at a concentration comparable to an average consumption amount of 204.05 mg PEITC/kg BW/day.

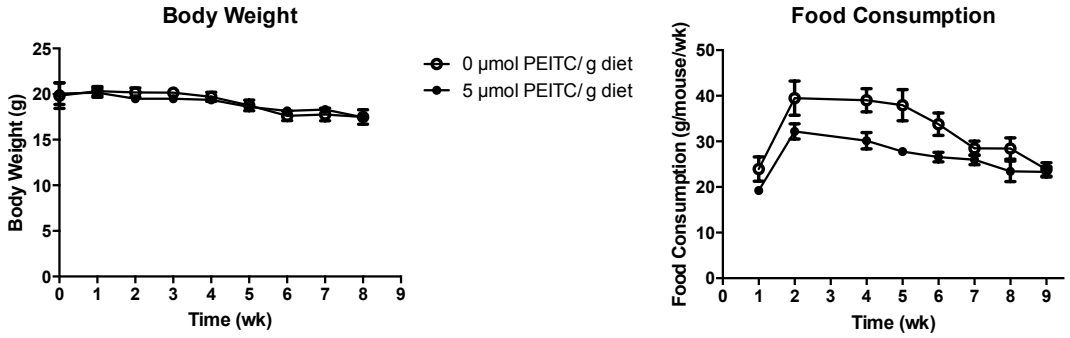


Figure 2 Body weight and food consumption patterns in athymic mice. Mice were randomized into two groups and fed 0 and 5 µmol PEITC/g diet for 9 weeks. Average A) body weights and B) food intake were recorded weekly and reported in g. Values are reported as means ± SD. n = 7 animals/control and 8 animals/5 µmol

PEITC/g diet treatment groups. (*) represents significant differences between mice fed control and treatment diets at $p < 0.05$.

Over the course of the treatment, mice were weighed and changes in body weight and food consumption were monitored. No significant differences in body weight or food consumption were observed in any treatment groups. Relative body weight remained constant during the course of treatment despite significant increases in tumor weight. Average weight and food consumption within treatment groups slightly decreased during the final weeks of treatment, which may be attributed to cancer-related cachexia in mice. PEITC may induce palatability changes given that the structure of the compound may elicit a sour taste.

Effect of PEITC on Xenograft Tumor Growth

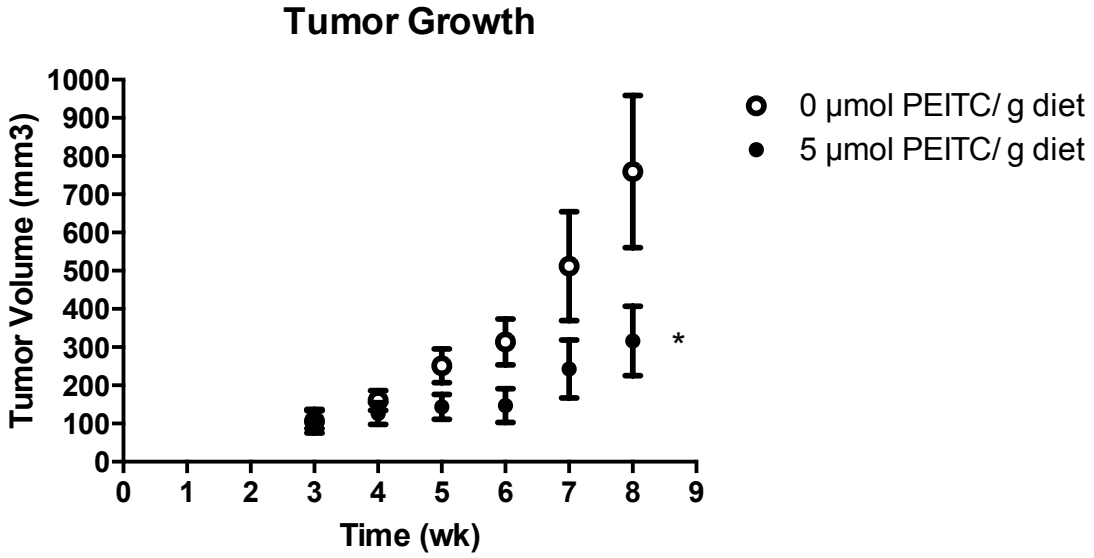


Figure 3 Tumor Growth and Formation in Treatment Mice. Tumor growth and formation in PEITC treatment mice. LNCaP human prostate cancer cell xenografts was established in athymic nude mice. Tumor volume was measured weekly and caluclated in described Materials and Methods section. Mean tumor volume in control and treatment mice during 9 week treatment period. Values are reported as means ± SD. n = 7 animals/control and 8 animals/5 µmol PEITC/g diet treatment groups. (*) represents significant differences between control and treatment diets at $p < 0.05$.

Changes in temporal tumor growth were assessed in 0 and 5 µmol PEITC/g diet treatment groups. During the final weeks (7 and 8) of treatment, tumor growth was 41.6% lower in mice fed 5 µmol PEITC/g diet. In our xenograft model, we found tumor growth to be inhibited within weeks following inoculation. Therefore, we further characterized the tumor and surrounding microenvironment utilizing a deductive approach.

Effect of PEITC on Androgen-responsive, Proliferative, and Inflammatory Pathways

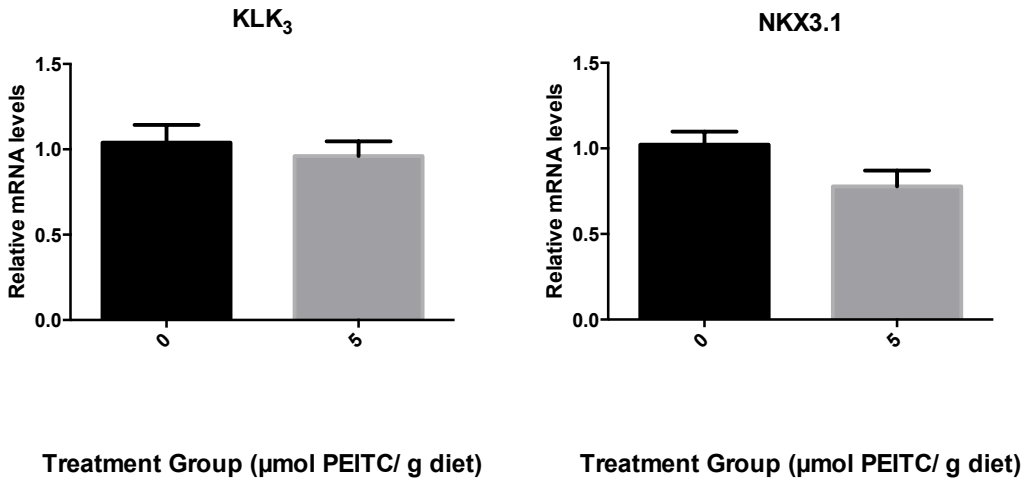


Figure 4 PEITC did not significantly affect androgen responsive genes in LNCaP xenografts. A) PSA/ KLK_3 and B) NKX3.1 expressions in LNCaP tumor xenograft model. Relative mRNA expression of PSA/ KLK_3 and NKX3.1 was assessed in LNCaP prostate cell tumor samples from control and PEITC-treated mice as described previously in Materials and Methods section. n = 7 animals for control and 8 animals for PEITC treatment groups. Results are reported as means ±SE. (*) represents $p < 0.05$.

Elevated androgen levels are a known risk factor for prostrate cancer and are associated with increased cell proliferation and evasion of apoptosis signals. In a previous study from our lab, PEITC significantly inhibited tumor growth without significantly altering PSA/ KLK_3 on transcription or protein levels (T. T. Wang et al., 2008). In this study, PSA/ KLK_3 and NKX3.1, androgen-responsive genes, were chosen as relevant endpoint biomarkers. There were no significant changes in KLK_3 and NKX3.1 expression levels following treatment with PEITC.

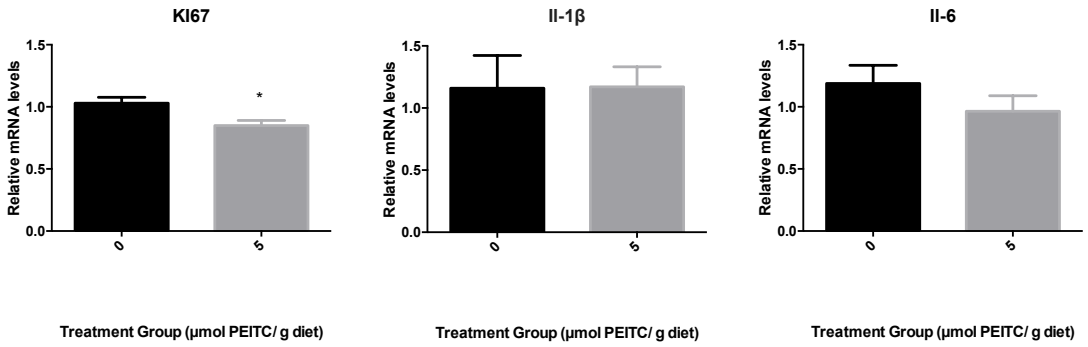


Figure 5 PEITC reduced tumor cell proliferation but did not significantly affect *in vivo* inflammatory markers. Real-time PCR analysis of mRNA expression of proliferative (KI67) and inflammatory markers (Il-1β and Il-6) in control and PEITC-treated LNCaP xenograft model. Real-time PCR was performed as described in Materials and Methods section. n = 7 animals for control and 8 animals for PEITC treatment groups. Results are reported as means ±SE. (*) represents $p < 0.05$

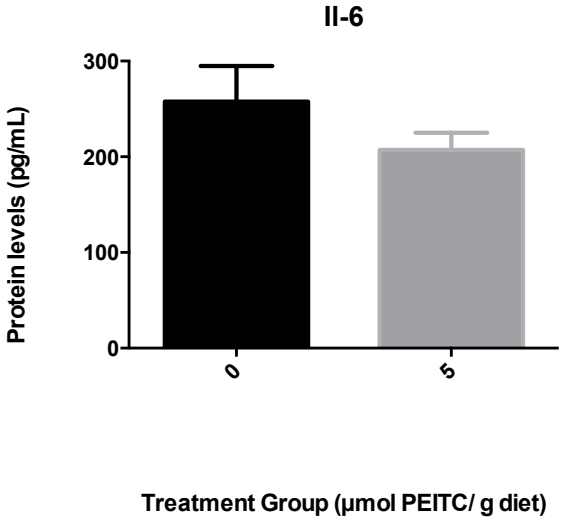


Figure 6 PEITC did not significantly affect circulating Il-6 levels. Circulating Il-6 protein levels in plasma of mice treated with control and 5 µmol PEITC/g diet was assessed using ELISA as previously described in Materials and Methods section. n = 7 animals/treatment group. Results are reported as means ±SE. (*) represents $p < 0.05$.

Inflammation was assessed for possible reductions following PEITC treatment. However, there were no significant changes in relative gene expression of Il-1β or Il-6 levels (Fig. 5). Although not significant, slight declines were observed in circulating Il-6 protein levels within the plasma (Fig. 6).

KI67 is a proliferation marker due to its presence in the G_1, S, and G_2 cell cycle phases and absence in quiescent G_0 cells. KI67 gene expression in mice treated with 5 µmol PEITC/g diet was significantly inhibited by 11.4% following treatment.

Effect of PEITC on Growth Factor Binding and Macrophage Infiltration

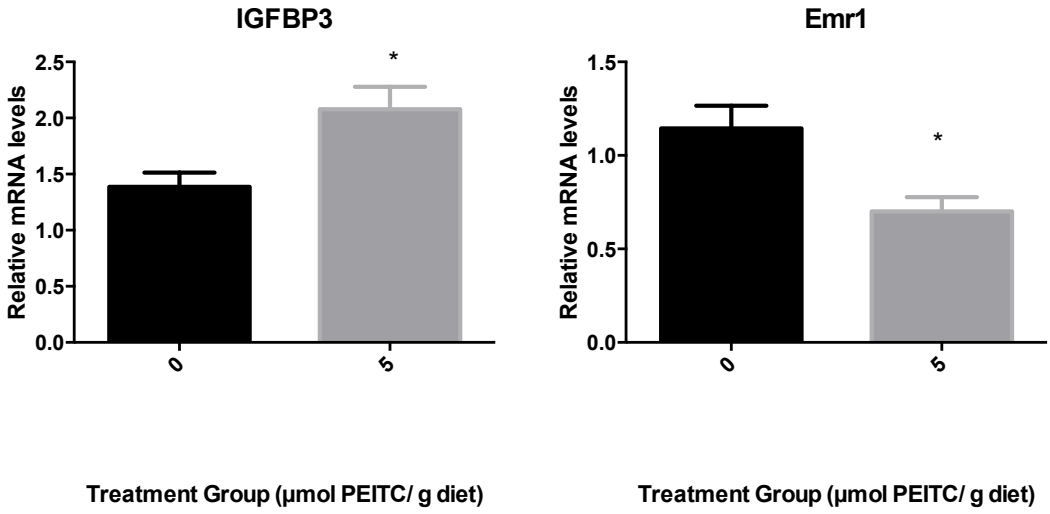


Figure 7 PEITC treatment significantly affected IGF pathway and macrophage infiltration. A) IGFBP3 and B) F4/80 expressions in LNCaP tumor xenograft model. Relative mRNA expression of IGFBP3 and F4/80 was assessed in LNCaP prostate cell tumor samples from control and PEITC-treated mice as described previously in Materials and Methods section. n = 7 animals for control and 8 animals for PEITC treatment groups. Results are reported as means ±SE. (*) represents $p < 0.05$.

Insulin-like growth factor 3 (IGFBP3), insulin-like growth factor 1 binding protein 3 was identified as a novel gene up-regulated by PEITC (R. W. Li et al., 2013). This gene plays a crucial role in the regulation of cell growth. IGFBP3 forms a ternary complex with insulin-like growth factor 1 or -2 and insulin-like growth factor acid-labile subunit (IGFALS) (Gu et al., 2010; Johansson et al., 2009). However, LNCaP, inherently under-expresses IGFBP3 receptors and enables IGF1 to freely circulate. As one of the most abundant IGF binding proteins, its effects on intrinsic growth regulation have conjured interest as a possible prognostic biomarker. Since low IGFBP3 levels are observed clinically, dietary modulation of its expression is of

interest. We found a significant induction (49%) in IGFBP3 expression following treatment with 5 µmol/PEITC (Fig. 7).

EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1) and F4/80, its mouse homologue, is derived from the B cell, myeloid lineage (Xu et al., 2013). It is a transmembrane protein that is expressed on the cell surface of human macrophages. It is commonly used as a mature macrophage infiltration marker in mouse and human models (Bucana, Fabra, Sanchez, & Fidler, 1992; Ikehara, Maeda, Kimura, Saito, & Ochiai, 2012). In this study, F4/80 gene expression was significantly reduced by 38.8% within tumors of mice treated with 5 µmol PEITC/g diet.

Overall, decreased macrophage infiltration and increased IGFBP3 gene expression observed in this study suggests that that an inflammatory- suppressed tumor environment may contribute to decreased tumor growth in PEITC fed mice. Although significant differences in tumors of control and PEITC fed mice could not be confirmed on a protein level (Fig. 8).

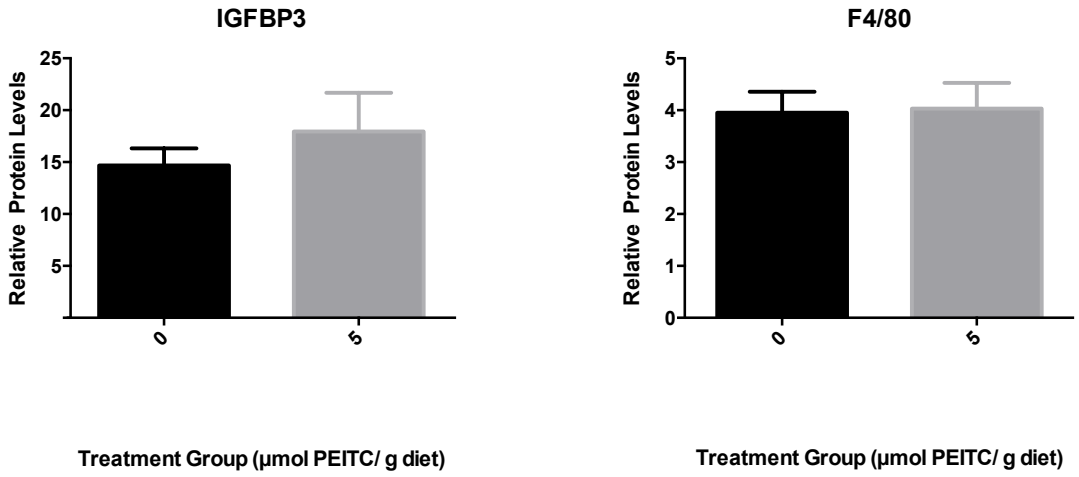


Figure 8 Effects of PEITC treatment on IGFBP3 and F4/80 protein levels. Changes in protein levels were assessed in tumor samples of control and PEITC fed mice using Western Blot analysis as described previously in Materials and Method sections. n = 7 animals/treatment group. Results are reported as means ±SE. (*) represents $p < 0.05$.

Effect of PEITC on Cell Adhesion and *in vitro* Proliferation

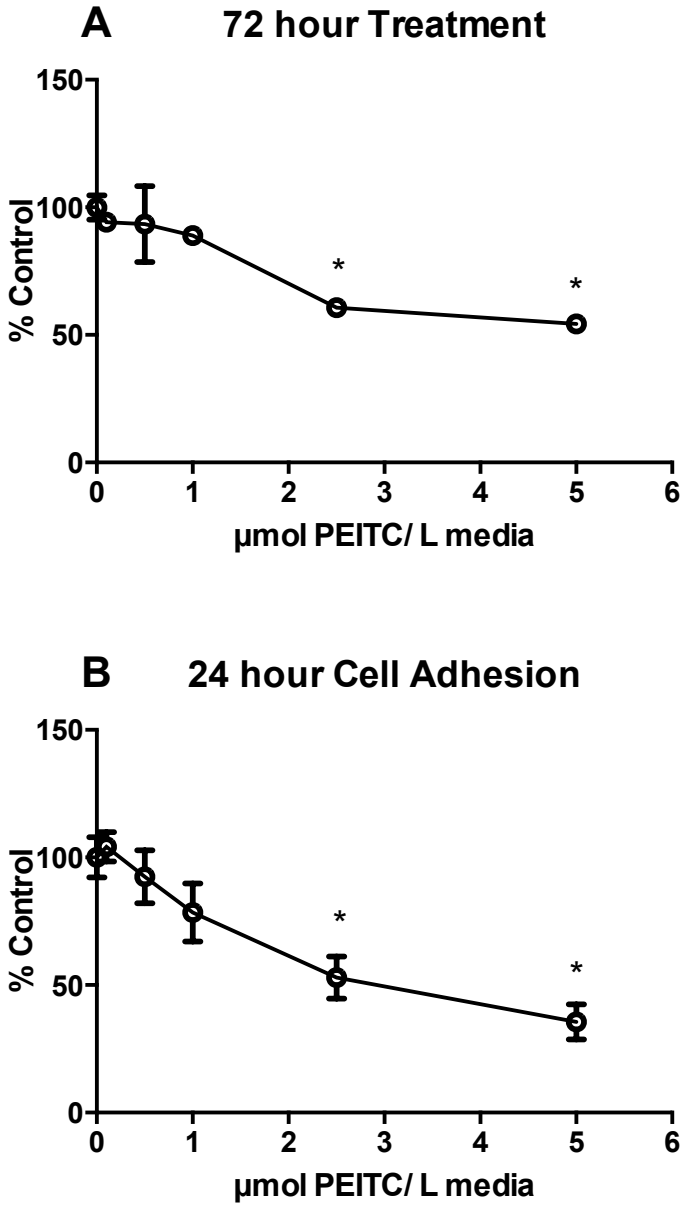


Figure 9 Effects of PEITC on LNCaP prostate cancer cell proliferation and cell adhesion *in vitro*. A)72 hr proliferation assay. LNCaP cells were plated in 24-well plates and after 24 hr plating period, cells were treated with 0, 0.1, 1, 2.5, and 5 µmol PEITC/L media for 72 hours. B) Cell Adhesion assay. LNCaP cells were plated in 24-well plates and were treated in the presence of 0, 0.1, 1, 2.5, and 5 µmol PEITC/L media for 24 hours. All treatment plates were analyzed via SRB method as described in Materials and Method section. n = 4 per treatment group. Results are reported as means ±SE. (*) represents $p < 0.05$.

Small but significant reductions in KI67 gene expression *in vivo* lead to further assessment of PEITC on LNCaP growth under *in vitro* conditions. When treated with concentrations greater than 2.5 µmol PEITC/L media, cell growth was significantly affected after 72 hour treatment (Fig. 9A). Moreover, cell plating efficiency was also assessed in LNCaP cell line. These results correspond with previous lab findings (Hudson et al., 2012). Response to PEITC on cell adhesion was assessed in LNCaP human prostate cell line. Interestingly, we found that in the presence of PEITC in the media led to a significant reduction in LNCaP plating efficiency, which was observed at relatively low (2.5 µmol/L) concentrations (Fig.9B).

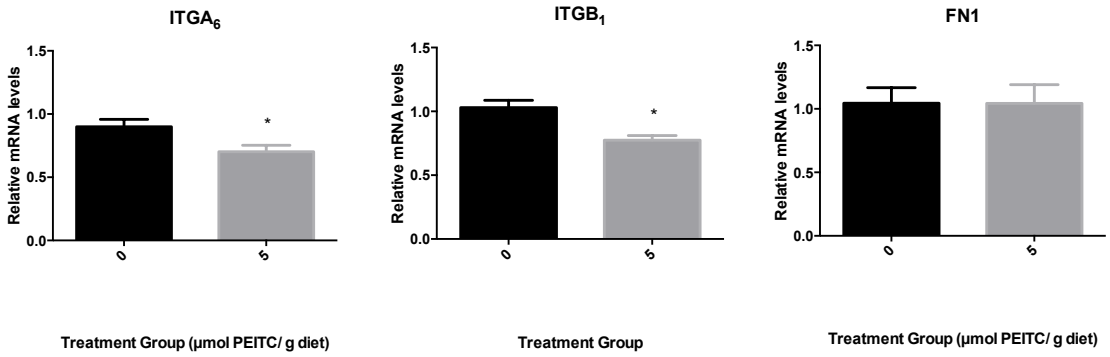


Figure 10 PEITC significantly affects integrins with LNCaP xenograft. Levels of A) $ITGA_6$, B) $ITGB_1$, and C) FN1 mRNA relative to TBP were quantified by RT-PCR as described in Materials and Methods section. n = 7 animals and 8 animals in control and 5 µmol PEITC treatment groups, respectively. Results are reported as means ±SE. (*) represents $p < 0.05$.

Moreover, we assessed PEITC's role in altering integrin-mediated cell adhesion under *in vivo* conditions. mRNA expression of integrins A_6 and B_1 were significantly inhibited by 21.9 and 24.8%, respectively, following PEITC treatment (Fig. 10). There were no significant changes found in FN1 expression, which is a commonly used marker of cell motility (Fig. 10).

Discussion

In the current study, we demonstrated that PEITC is capable of inhibiting tumor growth in an athymic mouse model. Key findings from this study include safe

administration of this compound without negatively affecting food consumption patterns and body weight, altered genomic regulation of proliferation pathways within the tumor; and significant changes to IGF-1, tumor macrophage infiltration, and cell adhesion pathways within the surrounding microenvironment.

Potential chemopreventive effects of bioactive compounds derived from cruciferous vegetables against prostate cancer are well established (Hecht, 1995; Moon et al., 2011; Powolny et al., 2011; D. Xiao et al., 2006). But the exact regulatory mechanisms of these compounds are not fully known. Physiologically relevant exposure of PEITC was administered to these animals in dosage equivalent 204.05 mg/kg BW/day and the modulations of multiple molecular pathways were evaluated. Significant modulation of proliferation, IGF-1, tumor macrophage infiltration, and cell adhesion pathways was identified at transcriptional level. Protein analysis of F4/80 and IGFBP3 did not reveal any significant changes, which may be due to specific targeting of molecular genes that are not expressed post-transcriptionally (Ozsolak & Milos, 2011). Another possible reason may be due to normalization differences in relative mRNA expression and protein analysis, for example, in PCR mouse TBP is specifically used as a housekeeping gene to analyze F4/80 levels and total protein was used in Western blot analysis. Immunohistochemical analysis of tissues stained with F4/80 may provide a more accurate account of the presence of macrophages and should be explored in future studies.

Integrins, as important therapeutic targets in prostate cancer, are substantially modulated by PEITC. In a recent study conducted by Hudson et al., PEITC significantly altered integrin-signaling pathways by reducing the expression of major integrins such as $ITGA_2$, $ITGA_6$, and $ITGB_1$ in LNCaP cell line (Hudson et al., 2012). Our findings further support PEITC's ability to regulate integrin-mediated cell adhesion through the modulation of $ITGA_6$ and $ITGB_1$ under *in vivo* conditions. These findings have the potential to be of great clinical importance as changes in integrin expression have been correlated with decreased cell motility and thus, a less pronounced and progressive cancer state.

The results of our study are particularly interesting when compared to *in vitro* exposure to PEITC on LNCaP cell models. Treatment under similar conditions at a lower concentration of PEITC indicated that integrin and IGF pathways were affected by PEITC *in vivo*; our study further supports these findings and may indicate a compensatory role under *in vivo* conditions that requires further investigation.

Conclusion

In conclusion, when PEITC is administered in low doses, tumor growth in animals was significantly inhibited weeks after inoculation of LNCaP, human prostate cancer cells. PEITC displays pluripotent, and potential chemopreventive effects within the tumor microenvironment by significantly reducing proliferation, inflammation and tumor macrophage infiltration. Moreover, our study demonstrated potentially chemopreventive effects of diet-derived PEITC by regulating key genes related to cell adhesion pathways. Results from our study supports previous findings demonstrating beneficial effects of PEITC under *in vitro* conditions as well as demonstrates unique sensitivities among various animal strains. The mechanistic effects of PEITC within the tumor microenvironment require further examination.

Chapter 3: Toxic Effects of Dietary Indole-3-Carbinol: Intestine as the Target Tissue

Abstract

Indole-3-carbinol (I3C), a cruciferous vegetable-derived bioactive compound, is known for its potentially protective effects against chronic diseases. These benefits fostered its commercial availability as a dietary supplement. However, the safety of orally consumed I3C remains largely unresolved. In this study, immune-deficient BALB/c nu/nu male mice were fed an AIN-93 diet supplemented with 0- 100 μmoles I3C/g. Mice were biologically responsive to 10-100 μmoles I3C/g diets, concentrations used in previous studies. Mice fed 100 μmoles I3C/g diet were not viable after seven days. However, switching mice to the control diet without I3C after first detection of stress resulted in a 75% recovery rate. Mice fed 10-50 μmoles I3C/g survived but show concentration-dependent physical effects including lethargy, turgid skin, as well as blood within stool collections. The intestine appeared to be the target of I3C toxicity. Number and width of intestinal villi were significantly altered by I3C. Molecular analysis using Ki67, a proliferative marker and TUNEL assay, an apoptosis marker indicated a dose-dependent reduction in cell proliferation and increase in apoptosis, respectively. Other molecular effects of I3C include significant induction of CYP1A1 expression (200 fold) and other xenobiotic enzymes within livers of mice treated with 50 μmol I3C/g diet. These findings were similar to that found using human HepG2 liver cell culture model, further supporting involvement of aryl hydrocarbon receptor-mediated pathway in the metabolism of I3C. This study is the first report of hazardous effects of I3C that are specific to the gastrointestinal tract and should serve as a caution for excessive use of I3C.

Introduction

Indole-3-carbinol (I3C) is a diet-derived, bioactive compound from cruciferous vegetables such as broccoli, Brussels sprouts, and cauliflower, generated from the hydrolysis of glucobrassicin by myrosinase (Broadbent & Broadbent, 1998b). Due to the perceived safety of I3C, increased interest has been placed on the

use of this natural dietary product to prevent or minimize chronic diseases such as cancer, inflammatory bowel disease, or to suppress secondary complications such as chronic inflammation (Meng et al., 2000; Reed et al., 2006; N. Takahashi et al., 1995; T. T. Wang et al., 2012). *In vitro* studies have shown I3C and its metabolites to have anti-tumorigenic properties by acting as aryl hydrocarbon receptor (AhR) ligands, inhibiting androgen receptor-mediated pathways and prostate cancer cell growth, anti-angiogenic abilities to reduce tumor viability, as well as elicit effects specific to immune response and apoptosis (Tsai et al., 2010; T. T. Wang et al., 2012; Weng et al., 2007). Ability to activate or inhibit the expression of these biological pathways are thought to be major mechanisms exerted by I3C. However, the precise contrivances underlying the ability of I3C to promote healthful outcomes and prevent chronic diseases remain unclear.

The perceived benefits of I3C on health have fostered its availability as a commercially available dietary supplement. However, controversy exists between effective I3C concentrations on clinical and preclinical levels and the outcomes associated with consuming high quantities warrant further investigation. Clinical studies have shown tolerability of doses up to 1,200 mg I3C/day before adverse effects were evident (Reed et al., 2006). Phase 1 trials in women provided single and multi-doses of I3C supplements revealed no additional, observable benefits in doses greater than 1000 mg (Reed et al., 2006). However, there is a wide range of I3C doses used in rodent models ("Clinical development plan: indole-3-carbinol," 1996; Shertzer & Sainsbury, 1991b; Verschoyle, Steward, & Gescher, 2007). Overlaps in I3C's effective health-promoting and toxic doses have made it difficult to distinguish no effect levels (NOELs) from chemopreventive ranges ("Clinical development plan: indole-3-carbinol," 1996). Genetic variability among subjects and metabolic efficiency among rodent species are contributing factors to the differential responses of I3C. This has led to the need to gauge short and long term effects of dietary consumption of I3C on various models by determining upper thresholds or limits (ULs) and relevance to humans (*Bioactive Compounds and Cancer*, 2010; Meyer, 1996).

Given that effective doses of I3C remain elusive, as a prelude to tumorigenesis study, we first evaluated the effects of dietary exposure to I3C utilizing immune-deficient BALB/C nu/nu male mice, a rodent model commonly used in tumorigenesis studies (Hudson et al., 2012; R. W. Li et al., 2013; T. T. Wang et al., 2008). A range of 0 to 100 μmol I3C/g diet was chosen based on concentrations used in a previous study (Kassie et al., 2008). We hypothesized that I3C consumption up to 100 μmol/g diet could be safely administered based on previous lab findings. Unexpectedly, I3C was found to be toxic. We further evaluated and characterized the pathophysiological and molecular effects of I3C. Qualitative and quantitative effects of dose-dependent consumption and sensitivity to I3C on the spleen, liver, kidney, and intestine of mice as well as physiological and molecular parameters were also examined to elucidate tissue targets and mechanisms.

Materials and Methods

Chemicals and Diets

I3C (99% purity), dimethyl-sulfoxide (DMSO), and 10% neutral formalin buffer were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Nutritionally-complete powdered AIN-93M diets with and without I3C (100 μmol/g diet) were prepared by Research Diets (New Brunswick, NJ) and stored at -20°C until weekly feedings. Formulation for control and treatment diets were based upon previous experiments that assessed quality and quantity of I3C in rodent diets (Kassie et al., 2008). The diets containing 10 μmoles/g and 50 μmoles/g I3C were obtained using a 1:10 and 1:2 dilution of 100 μmoles/g stock with 0 μmoles/g I3C control stock. All diets were thoroughly mixed before administration to the animals.

Cells and Cell Culture

HepG2, human hepatocellular carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 100 U/ml penicillin,

100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in the presence of CO_2-air (5:95, v/v).

in vivo RNA Extraction and Reverse Transcription (RT)-PCR Analysis

HepG2 cells were trypsinized with 0.25% Trypsin-EDTA solution (Invitrogen). Then, seeded in conditioned media at a density of 0.25 x 10^6 cells/well in 6-well plates (Corning Inc., Corning, NY, USA), one day prior to treatment initiation. Fresh medium was replaced daily with treatment media containing 0, 1, 5, 25 or 50 μM I3C. Cells were harvested for total RNA isolation after 48 hours using the TRIzol® method (Invitrogen) as described previously (Hudson et al., 2012). Real time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify gene expression levels of human cytochrome P450 family 1, subfamily A, polypeptide 1 (CYP1A1), cytochrome P450 family 1, subfamily B, polypeptide 1 (CYP1B1), cytochrome P450 family 2, subfamily D, polypeptide 6 (CYP2D6), cytochrome P450 family 2, subfamily C, polypeptide 9 (CYP2C9), and cytochrome P450 family 3, subfamily A, polypeptide 4 (CYP3A4). Human TATA binding protein (TBP) was used as a housekeeping gene for calculation of relative expression levels.

Experimental Design and Animals

All experimental protocols were in accordance with the National Institutes of Health guidelines and were approved by the USDA Animal Research Advisory Committee (BAACUC protocol # 12-030). Male athymic nude mice (BALB/c nu/nu, 20-22 g, 5-7 weeks old) were purchased from Charles River (Frederick, MD) and were individually housed in filter-top cages at the USDA BHNRC animal facility. Room conditions were carefully monitored and held at a constant temperature of 22 ± 1°C with a 12 hour rotating day and night cycle. Animals consumed food and filtered water *ad libitum*. Food consumption and body weights were recorded weekly. An acclimation period of 1 week prior to treatment feedings enabled mice to adapt to control AIN-93M diet and environment.

Animals assigned to one of four experimental feeding groups- (i) control diet, (ii) control diet with 10, (iii) 50, and (iv) 100 μmol I3C/g diets (n = 5/group). Mice remained on their respective diets for 4 weeks. The animals were then euthanized and necropsy was immediately performed.

Animal Observations and Survival

Animals were observed 2 times/week for changes in physical appearance, activity levels, food consumption and the presence of fecal blood. A 4-level scale was arbitrarily constructed to categorize the varying degree of activity levels into groups, as adapted from previously used methods (Morton, 2000; Nunamaker, Anderson, Artwohl, Lyubimov, & Fortman, 2013; Nunamaker, Artwohl, Anderson, & Fortman, 2013; Paster, Villines, & Hickman, 2009; van Vlissingen, 1999). An activity level of ++++ represents very active, normal animal behavior. +++, represents a slightly reduced, active activity level or with mild gait. ++ represents an abnormal activity level, in which the animal is moving very slowly or with a severely altered gait and + represents lethargy or very reluctant to move more than 3 or 4 steps. 0, represents complete inactivity or animals were awake but did not move. Animals were monitored by trained animal care staff as appropriate for species-specific signs of pain and distress, in accordance with the National Institutes of Health guidelines and were approved by the USDA Animal Research Advisory Committee. Cage-side observations were completed by a primary observer and confirmed by secondary observers of trained research staff throughout the duration of the study.

in vivo RNA Isolation and RT-PCR

Livers were harvested and stored in RNA*later* (AMBION Inc., Austin, TX) immediately following necropsy and frozen until analysis. For determination of mRNA expression in liver samples, total RNA was isolated and purified using RNeasy kit (Qiagen Inc., Valencia, CA), according to manufacturer's protocol. Gene expression was quantified using a previously described TaqMan real-time RT-PCR method (Y. Takahashi et al., 2006). TATA box binding protein (Tbp), cytochrome P450 family 1, subfamily A, polypeptide 1 (Cyp1a1), cytochrome P450 family 2,

subfamily C, polypeptide 55 (Cyp2c55), cytochrome P450 family 2, subfamily D, polypeptide 10 (Cyp2d10), cytochrome P450 family 3, subfamily A, polypeptide 11 (Cyp3a11), UDP glucoronosyltransferase 1 family, polypeptide A1 (Ugt1a1), NAD(P)H dehydrogenase, quinine 1(Nqo1), and TaqMan gene expression assay primers and probes were purchased from Applied Biosystems (Foster City, CA). TBP, a housekeeping gene, was used to calculate relative gene expression levels in mouse liver samples.

Immunohistochemical (IHC) Preparation and Analysis of Results

Tissue samples were prepared for *in situ* immunohistochemical analysis according to a previously discussed method (T. T. Wang et al., 2008). Liver, kidney, spleen, and proximal, middle, and distal intestinal sections were immediately removed following euthanization and placed in a 10% neutral buffered formalin fixative agent. Spleens were harvested from all treatment groups with the exception of animals treated with 100 μmol I3C/g diet. Tissues were stored in formalin for approximately 72 hours prior to slide preparation. Cross-sectional and longitudinal tissue sections were cut approximately 4-5 microns thick and stained with hematoxylin and eosin, or colorimetric Ki-67 proliferation marker. TUNEL assay was performed with modifications using an ApoTag kit (Millipore), as previously described (Berry & Baehrecke, 2007; Lee & Baehrecke, 2001; S. L. Wang, Hawkins, Yoo, Müller, & Hay, 1999). Slides were then cover slipped with mounting medium and stored in a dark location at room temperature until analysis.

All IHC slides were viewed on an Olympus light microscope (Olympus Optical Co., Japan) and images were acquired using Nikon D7000 digital SLR camera (Nikon Inc., Melville, NY). Images were viewed with Nikon Camera Control Pro 2 software, Version 2.8.0 (Nikon Inc., Melville, NY). Parameters for image analysis include: (1) all images were taken at a constant exposure; (2) images were acquired pseudo randomly with no area overlapping; (3) all images were acquired using 400x magnification (or 40x magnification for TUNEL and Ki67 analysis); (4) images of 10 fields were randomly taken for each slide and average data is reported. No modifications were made to the images to improve color intensity of samples.

Intestinal villi data was recorded as raw area in inches squared and relative percent composition of each treatment group was used for analysis.

Statistical Analysis

The 5 animals per treatment group provide at least 85% power to detect effects and provide > 95% to detect differences as small as 10%. Statistical One-Way Analysis of Variance (ANOVA) followed by *post hoc* analysis using Bonferoni's Multiple Comparison Test were conducted to determine the differences among means using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Means were reported as mean ± SEM. Statistical significance was declared at $p < 0.05$. Kaplan-Meier analysis was used to calculate survival rates among treatment using GraphPad Prism 5 for Windows (version rel. 5.01, 2007, GraphPad Software, Inc., La Jolla, CA) and results were displayed as percentages. Power calculations were completed using SAS 9.3 (version 6.1.7601, 2010).

Results

I3C at 100 µmol/g is Toxic

Treatment Group	General Appearance	Activity Level	Fecal Blood
Control	Pink skin, clear eyes	Very active (++++)	None
10 µmol I3C	Pink skin, clear eyes	Very active (++++)	Yellow with red tinge
50 µmol I3C	Dull pink skin	Somewhat active (++)	Dark red, dense
100 µmol I3C	Pale, dull skin	Lethargic/inactive (+)	Dark, dense, covered majority of cage
Recovery	Pink skin, clear eyes	Active (+++)	None present following week 1 on control diet

Table 2 Physical observations of athymic mice fed control and treatment (10- 100 µmol I3C/g) diets. Mice were observed intermittently 2 times per week. Final results are recorded at week 5 of study. Results are reported as average observation per treatment group. n = 5 animals/treatment group. Activity level: ++++, very active; +++, active; ++, somewhat active; +, lethargic/inactive

Changes in physical appearance, activity levels, and stool abnormalities were found in animals consuming ≥ 50 µmol I3C/g diet. The most severe phenotypic effects occurred in mice fed 100 µmol I3C/g diets and the deleterious effects of I3C were observed within 24 hours of treatment initiation (Table 2). After 72 hours of treatment, animals fed the highest dose had visible skin flush, lethargy, and blood discharge. These animals were not viable within seven days post treatment initiation (Fig.11).

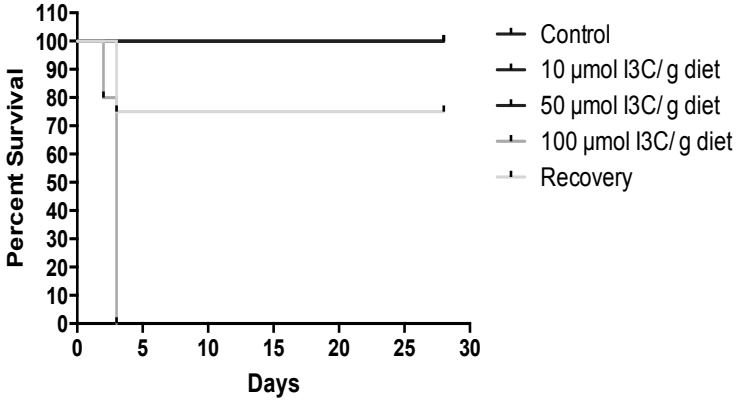


Figure 11 Survival Rates of Athymic Mice Treated with 0- 100 µmoles I3C/g diet. Results were reported as percentages. n = 5 animals/treatment group

Effect of Lower I3C Concentrations on Animal Survival

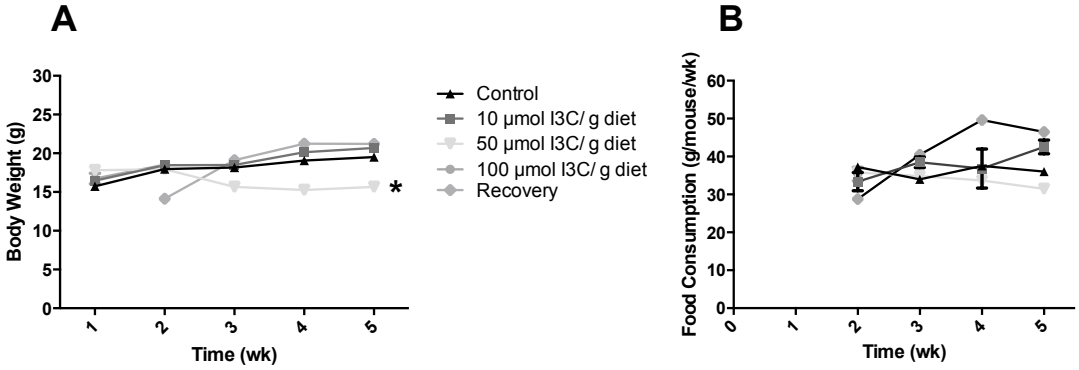


Figure 12 Body Weight Measurements and I3C-powdered Diet Intake in Athymic Mice. Mice were randomized into two groups and fed either control or 100 µmol I3C/g diets for 1 week. Mice originally fed with

100 µmol I3C/g diet were switched to control diet and animals fed control diet were subdivided into 4 experimental groups- control, 10, 50, and 100 µmol I3C/g diet, with 5 animals per group. Mice remained on their respective diets for 4 additional weeks. Body weight (g) and food consumption (g) were recorded weekly. A) Mean weekly body weights of mice fed control or treatment diets. B) Mean weekly food consumption of mice fed control or treatment diets. Values are reported as means ± SD. (*) represents significant differences between control and treatment groups at $p < 0.05$.

Animals fed 10 and 50 µmol I3C/g diets survived through the entirety of the study. No significant differences were found in the amount of food consumed (g) between mice fed control and I3C- containing diets (Fig. 12). However, significant weight loss occurred in mice fed 50 µmol I3C/g diet compared to animals assigned to control group (Table 2).

Changes in physical appearance, activity levels, and stool abnormalities were also found in animals fed I3C containing diets. Mice consuming 50 µmol I3C diets exhibited pale skin color and blood in the feces. With the exception of slight rusty red color in urine/ fecal matter, mice fed 10 µmol I3C/g diet had no overt differences in appearance compared to animals fed control.

Toxicity Effect of I3C can be Reversed

Discontinuing high concentration diets (100 µmol/g) upon detecting stress (at 72 hours) and switching mice to the control diet resulted in recovery with a 75% survival rate (Fig. 11). After 4 weeks of continuous feeding on control diet, animals showed similar activity as well as phenotypic (skin color, fecal color) signs as the animals fed control diet.

Toxic Effect of I3C is due to Intestinal Damage

To elucidate the mechanisms, hematoxylin and eosin staining of intestine, spleen, kidney, and liver revealed the gastrointestinal tract as the primary affected tissue. No obvious differences were found in spleen, kidney, or liver morphology in mice fed control or I3C-containing diets.

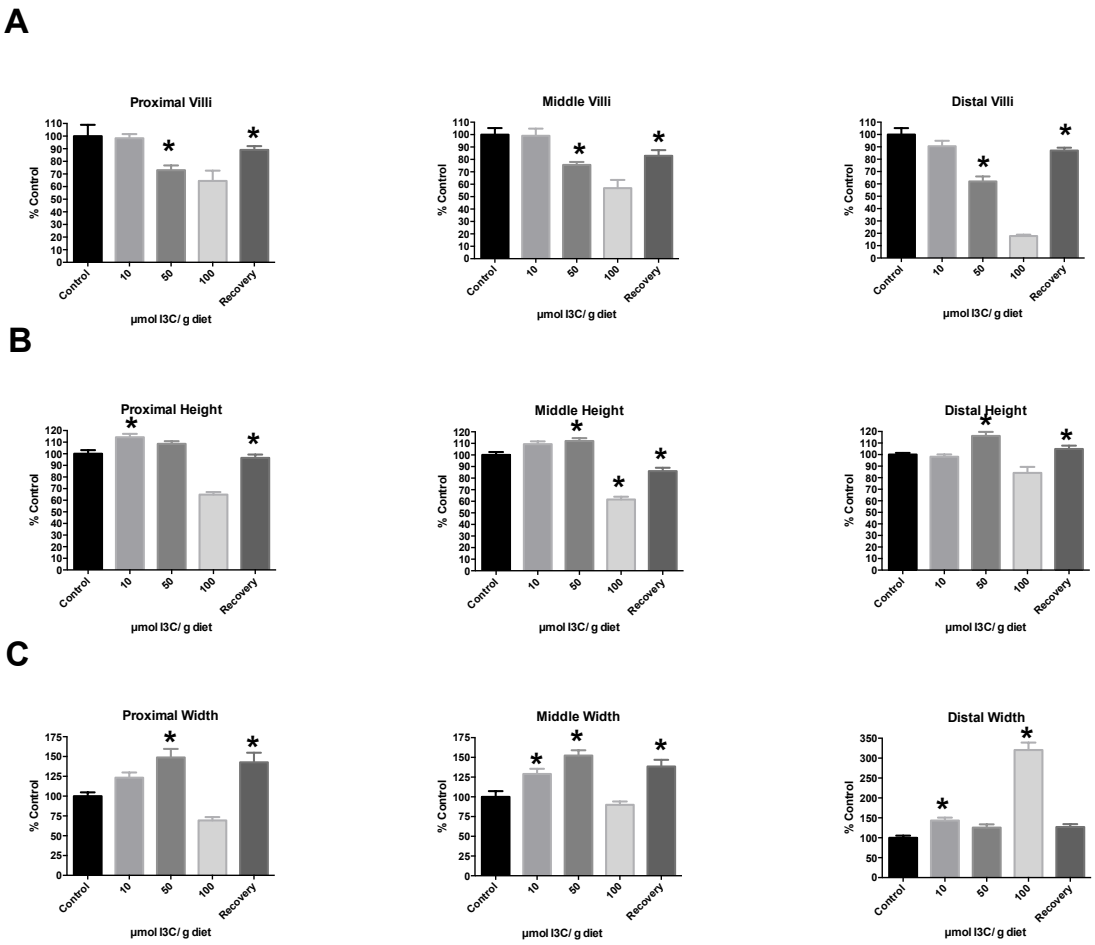


Figure 13 Intestinal Villi of Athymic Mice. Changes in intestine villi of mice treated with 0 to 100 µmol I3C/g diet were examined by immunostaining with hematoxylin and eosin at x4 magnification (a 0.17 mm^2 field). Proximal, middle, and distal intestinal villi A) number, B) height, and C) width of control and treatment mice. Villi number values are reported as mean ± SE. Differences in villi height and width were reported as % control. n= 3 animals per treatment group. (*) represents significant differences between control and treatment groups at $p < 0.05$

In the gastrointestinal tract, animals fed I3C had a significantly lower number of villi. A dose-dependent reduction in average villi number was observed in mice fed 10, 50, and 100 µmol I3C/g diets compared to control treatment group. Compared to the control, animals treated with the 10 µmol I3C/g diet had an average of 1.7 ± 3.3, 1 ± 5.7, and 9.5 ± 4.4% less villi in the proximal, middle, and distal intestinal regions, respectively. Our analysis also revealed significant decreases in intestinal villi number of 27 ± 3.8% in the proximal region, 24.5 ± 2.4% in the middle region, and

38 ± 4.1% in the distal regions of mice treated with 50 μmol I3C/g diet. Additionally, those treated with the 100 μmol I3C/g, incurred a 35.5 ± 8.1%, 43.1 ± 6.6% and 44.1 ± 13.6% loss of villi in the proximal, middle, and distal regions, respectively (Fig. 13).

A dose-dependent increase in villi height and significantly larger and wider villi were also observed in mice fed I3C-containing diets than villi of mice fed control diets (Fig. 13). Animals fed 10 μmol I3C/g diet had average villi heights of 5.8 ± 4.77% and 5.4 ± 3.6% longer than control animals in the proximal and middle regions, respectively. A 5 ± 3.3% average villi shortening in the distal region of mice fed 10 μmol I3C/g diet. 6.3 ± 4.3% and 12.5 ± 4.7% increases in height were observed in the proximal and middle regions with an 8.8 ± 6% shortening in the distal intestinal region were found in mice treated with 50 μmol I3C/g diet. Moreover, an average increase in width by 45.4 ± 9.1%, 20.7 ± 12.1, and 55.8 ± 13% was found in the proximal through distal regions in mice fed 10 μmol I3C/g diet. For animals provided 50 μmol I3C/g diet, intestinal widths were 91.7 ± 12.2%, 46.6 ± 11.7, and 32.7 ± 13.5% larger in the proximal, middle, and distal regions, respectively, than the control group. Those fed the highest dose had villous shortening of 42.7± 3.4% within the proximal region, 44 ± 5.4% in the middle region, and 3.6 ± 5% in the distal region as compared to the control diet animals. Moreover, those animals fed the highest dose had a decrease in villi width of 22.2 ±13.6% in the proximal region, 25 ± 10.3% in the middle region compared to control diet (Fig. 13). Width changes in distal region could not be detected due to severe morphological changes.

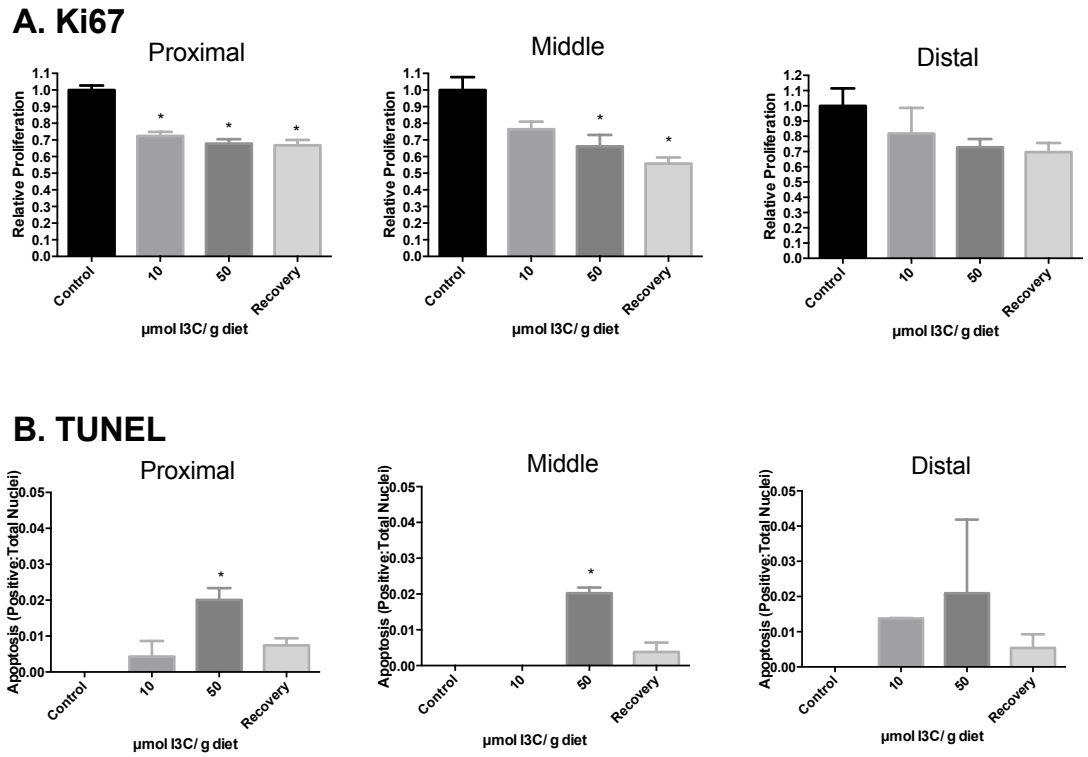


Figure 14 Ki67 and TUNEL analysis of Athymic Mice Intestine. Apoptotic and proliferative changes in intestine villi of mice treated with 0 to 50 µmol I3C/g diet were examined by immunostaining with Ki67 and TUNEL assay at 40x magnification (a 0.17 mm^2 field). A) Ki67 quantification of proximal, middle, and distal intestinal villi. B) TUNEL analysis of proximal, middle, and distal intestinal villi of control and treatment mice. Differences in Ki67 positive staining were reported as a relative proliferative index. Values are reported as mean ± SE. n= 3 animals per treatment group. (*) represents significant differences between control and treatment groups at $p < 0.05$.

The effects of indole-3-carbinol on apoptosis and proliferation within the gastrointestinal tract of mice were also examined to provide molecular mechanisms that lead to changes in intestinal villi. Gradient changes in positive Ki67-stained nuclei, a proliferation marker, were observed in the proximal and middle intestinal regions of mice fed 10 and 50 µmol I3C/g diet (Fig. 14A). TUNEL method was used to detect apoptosis-mediated DNA breaks and DNA fragmentation. We found significant increases in TUNEL positive nuclei in the proximal and middle intestine regions of the highest treatment group (Fig. 14B).

Immunohistochemical results of the recovery treatment group, which was initially fed 100 μmol I3C/g diet for 72 hours then switched to control diet for the remainder of the feeding study, were examined. At the end of treatment, there were no significant differences in spleen, kidney, liver morphology when compared to control group. Overall, investigation of gastrointestinal tract stained with hematoxylin and eosin revealed slight but significant changes in the number, length, or width of villi in comparison to those fed control diets for the entirety of the study. Assessing for changes against Ki-67 marker noted significant decreases in proliferation by 33.3 ± 3.2 and 44.3 ± 3.7% of the proximal and middle intestinal regions, respectively. No significant proliferative differences were found in the distal region nor were significant changes in apoptotic rates of intestinal cells observed. While the recovery group resembled physical appearances of the control group, their intestinal changes closely resembled results of either the 10 or 50 μmol treatment groups. Taken together, these results further support findings of intestinal damage reversal as a result of acute, high dose I3C exposure.

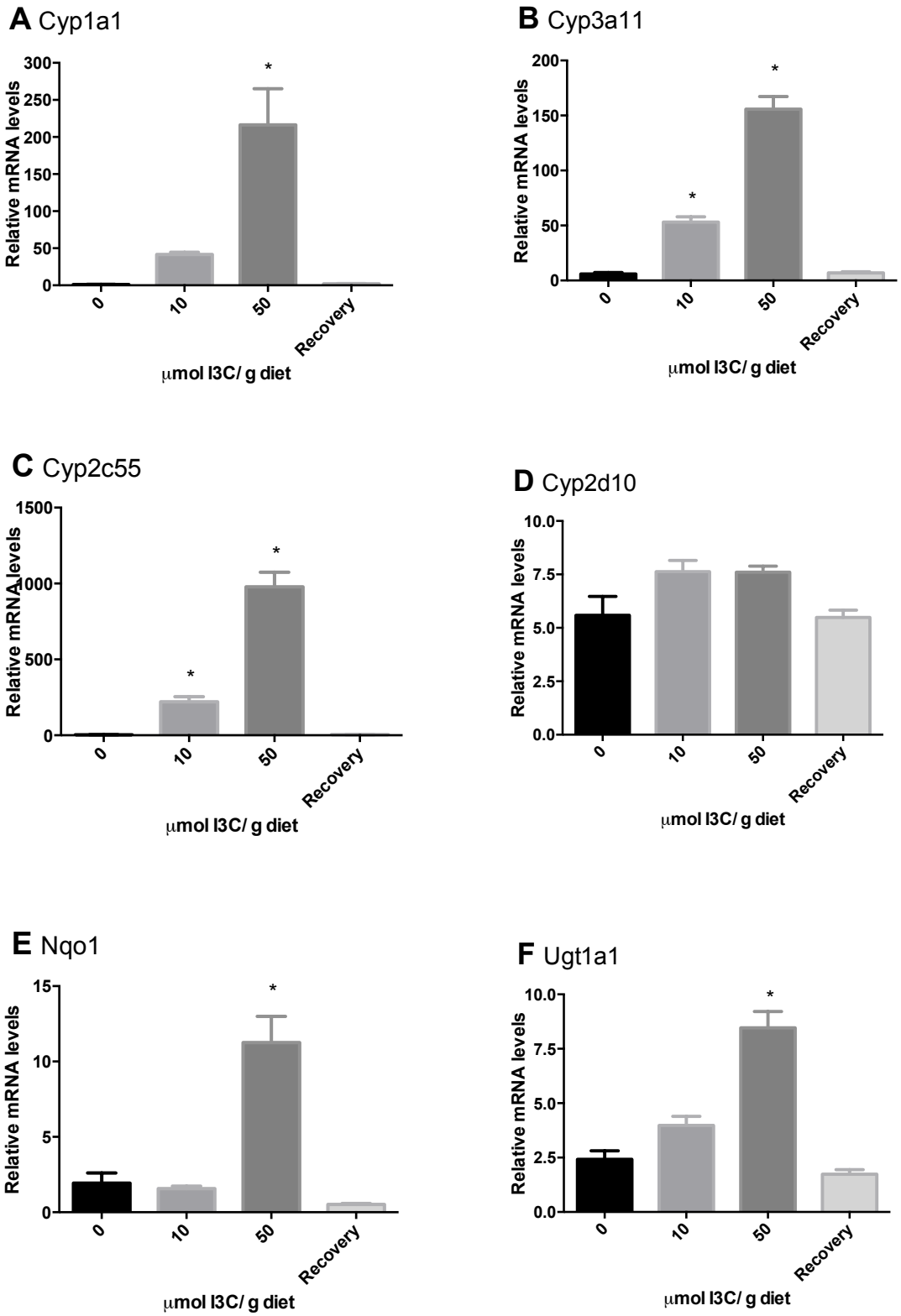

A Cyp1a1
Relative mRNA levels
300
250
200
150
100
50
0
*
0
10
50
Recovery
μmol I3C/ g diet
B Cyp3a11
Relative mRNA levels
200
150
100
50
0
*
*
0
10
50
Recovery
μmol I3C/ g diet
C Cyp2c55
Relative mRNA levels
1500
1000
500
0
*
*
0
10
50
Recovery
μmol I3C/ g diet
D Cyp2d10
Relative mRNA levels
10.0
7.5
5.0
2.5
0.0
0
10
50
Recovery
μmol I3C/ g diet
E Nqo1
Relative mRNA levels
15
10
5
0
*
0
10
50
Recovery
μmol I3C/ g diet
F Ugt1a1
Relative mRNA levels
10.0
7.5
5.0
2.5
0.0
*
0
10
50
Recovery
μmol I3C/ g diet

Figure 15 Effects of Indole-3-carbinol on xenobiotic metabolizing enzyme gene expression in mice liver tissue. Gene expression of Phase 1 and -2 xenobiotic metabolizing enzymes, A) Cyp1a1; B) Cyp3a11; C) Cyp2c55; D) Cyp2d10; E) Nqo1; and F) Ugt1a1, was assessed. RT-PCR analysis was conducted on stabilized RNA from livers of mice treated with 0- 50 µmol I3C/g diet and Recovery treatment groups. Recovery treatment group consisted of animals given 100 µmol I3C/g diet and subsequently fed control diet for the remainder of the study. Relative mRNA levels of each group are expressed as means ± SE (n=5 per treatment group). (*) represents significant differences between control and treatment groups at $p < 0.05$.

Effects of I3C on mRNA levels of phase-1 enzymes Cyp1a1, Cyp3a11 and Cyp2c55, mouse homologs of CYP3A4 and CYP2C9, as well as phase-2 enzymes, Ugt1a1 and Nqo1 were determined to further understand the metabolic pathway activated by consuming I3C. Significant gene induction for all Phase 1 enzymes in mouse liver was seen at doses as low as 10 µmol I3C/g diet (Fig. 15). Significant Phase 2 induction was observed in livers of mice treated with 50 µmol I3C/g diet at 11.06 ± 2.69 and 11.25 ± 1.74 fold changes for Ugt1a1 and Nqo1, respectively. There were no induction of any Phase 1 and 2 xenobiotic enzymes in the recovering group (Fig. 15).

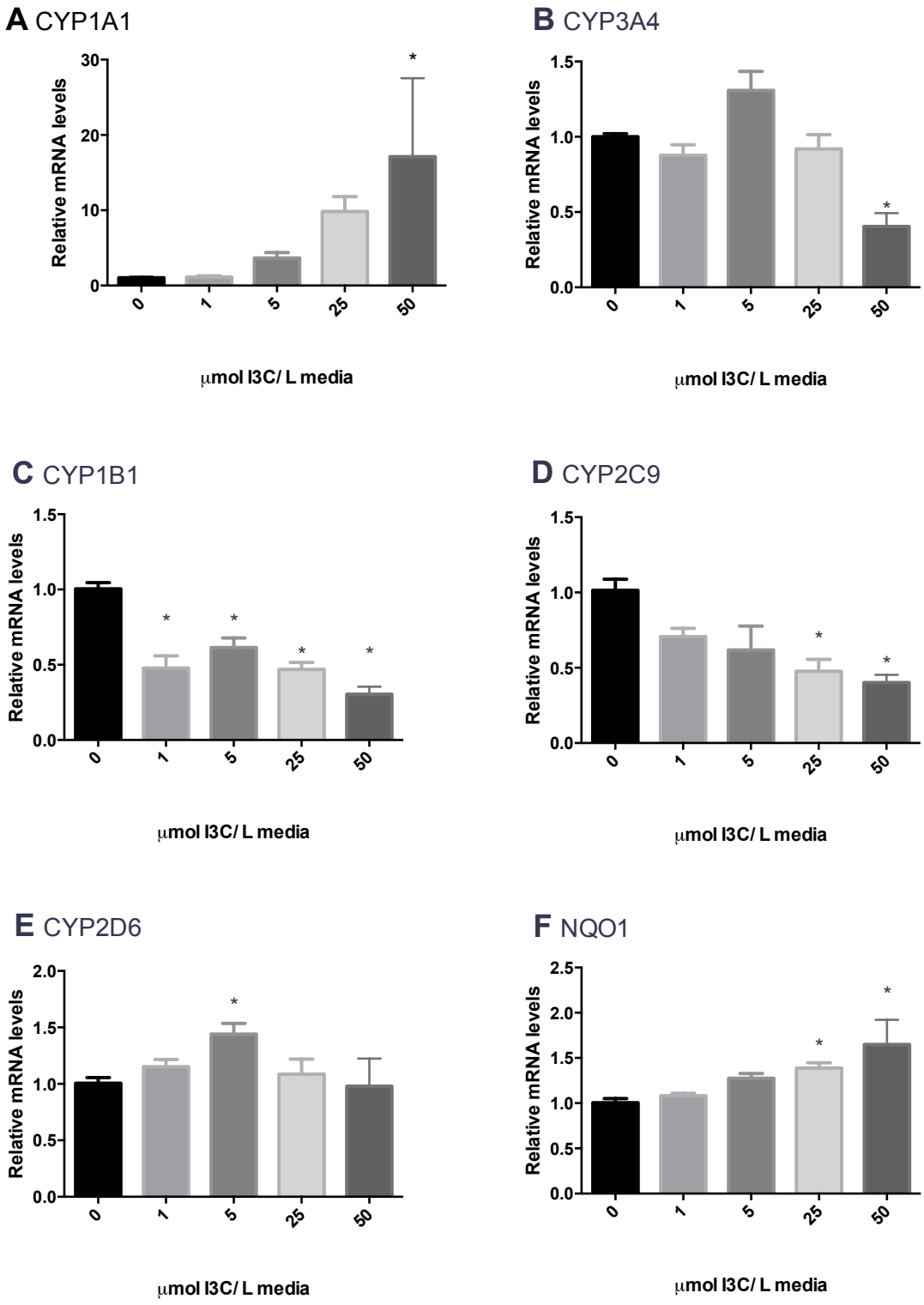


Figure 16 Effects of Indole-3-carbinol on xenobiotic metabolizing enzyme gene expression in HepG2 cells.

RT-PCR analysis was conducted on stabilized RNA from HepG2, human hepatocarcinoma cells treated with 0- 50

μmol I3C/L media for 48 hours. Gene expression of Phase 1 and -2 xenobiotic metabolizing enzymes, A) CYP1A1; B) CYP3A4; C) CYP1B1; D) CYP2C9; E) CYP2D6; and F) NQO1, was assessed. Relative mRNA levels of each treatment group are expressed as means ± SE (n=6 per treatment group). (*) represents significant differences between control and treatment groups at $p < 0.05$.

Gene regulation of drug metabolizing enzymes was also assessed in HepG2, a human hepatocarcinoma cell line as a comparison to the mouse data. For CYP1A1, there was a concentration-dependent induction of CYP1A1 mRNA level and the effect occurred at concentrations as low as 5 μM (Fig. 16).

In HepG2, a significant dose-dependent inhibition of Phase 1 enzymes, CYP1B1 and CYP2C9, was observed at a dose of 1 μM (Fig. 16). Additionally, inhibition of CYP3A4 mRNA expression occurred at 50 μmol/L while CYP2D6 gene expression was not substantially altered (Fig. 15). Small but significant induction of NQO1 mRNA expression was observed in HepG2 by 1.4 ± 0.1 and 1.6 ± 0.1, when treated with 25 and 50 μM I3C (Fig. 16).

Discussion

In this study, we demonstrate that I3C exerts tissue-specific effects on an athymic mouse model. Key findings from this study including sensitivities of mouse fed I3C; tissue damage primarily localized to the intestine; and changes in cell proliferation and apoptosis in intestine, appeared to be the mechanisms. Additionally, differential induction of xenobiotic metabolizing enzymes occurred in livers of athymic mice as a result of consuming dietary I3C was observed.

Contrary to studies utilizing similar dosage of I3C, our data indicates significant changes in weight (Fig. 12) (Kassie et al., 2008). Weight loss in our study may be due to palatability changes, perturbed metabolic functioning (Kassie et al., 2008), but more importantly toxic effect (Table 2, Figs. 11, 13-14). In the present

study, the amount of I3C administered to the animals ranged from approximately 202.7 μmol to 2.03 mmol I3C/kg body weight Human Equivalent Doses (HED), which is similar to amounts used in other experimental animal studies (Dagne et al., 2011; Kassie et al., 2010; Reagan-Shaw, Nihal, & Ahmad, 2007). At these administered levels, changes in physical activity and the presence of fecal blood were dose-dependent and linear. These observations are consistent with previous studies that report severe lethargy following I3C feedings, as indicated by hunched postures and decreased movement compared to animals fed on control diets (Kantachuvesiri et al., 2001; Mitchell et al., 2006; Patterson, Mullins, & Mitchell, 2008; Prieto et al., 2011). The most substantial changes and signs of lethargy were observed in animals fed 50-100 μmol/g diets (Table 2). Moreover, fecal blood was observed in I3C as low as 10 μmoles/g, a concentration comparable to orally administered doses of I3C in Phase 1 clinical trials (Dagne et al., 2011; Reed et al., 2006; Reed et al., 2005). Our results suggest potential hazards in consuming I3C. The reasons underlying the disparity in results from our study versus published literature that did not report toxic effects are unclear. However, it is possible that these differences are also influenced by mouse strain variance.

There are limited documented studies on direct intestinal damage of I3C. Our immunohistochemical analysis revealed morphological changes that were localized to the intestine and not grossly detected in other tissues such as the spleen, liver, or kidney of the I3C treated mice. Significant yet reversible, general villous atrophy occurred in all intestinal regions after 4 weeks (Fig. 13). Mechanistically, the effects of I3C appeared to be associated with decreased proliferation and increased apoptosis

as indicated by TUNEL and Ki67 staining (Fig. 14). These changes are also consistent with increased intestinal villi width and height (Fig. 13).

In general, dietary exposure to I3C in moderate to high doses may induce certain phase 1 and 2 xenobiotic metabolizing enzymes (Bonnesen, Eggleston, & Hayes, 2001; Nho & Jeffery, 2001). *In vivo* results from this study revealed a significant dose dependent increase in homologue gene expression of Cytochrome P450 1A1, -3A4, -2C9, -2D6 as well as Phase 2 enzymes, UGT1A1 and NQO1 upon dietary administration of I3C (Fig. 15). This panel represents genes primarily involved in approximately 90-95% of all xenobiotic metabolism in humans as related to the detoxification polycyclic aromatic hydrocarbons from chemical waste and other organic materials as well as steroid hormones (Sangar, Bansal, & Avadhani, 2010). Conversely, we found that I3C significantly altered the HepG2, human liver cancer cell line expression patterns of both Phase 1 and 2 homologue genes (Fig. 16). We found similar *in vitro* and *in vivo* induction of CYP1A1 but not other enzymes. The differences in gene expression could be due to the distinct biology of the liver cancer cells for the *in vitro* studies versus mouse liver cells; and the duration of compound administration (short term exposure in *in vitro* models versus chronic dosing in the animal study) (Kassie et al., 2010). Further studies are necessary to elucidate the significance of CYP1A1 gene induction in the metabolism of I3C.

Conclusion

In summary, we found intestinal damage in animals receiving I3C supplementation as low as 10 μmol/g diet. This is a concentration comparable to orally administered doses in humans (Reed et al., 2006; Reed et al., 2005). Our study

demonstrated that the potentially toxic effects of diet-derived indole-3-carbinol appeared to be specific to the gastrointestinal tract. Our study should serve as a caution for excessive use of I3C in human and future studies with I3C on experimental mouse models. The safety of orally consumed I3C in human requires further validation.

Chapter 4: Inhibition of LNCaP, Human Prostate Carcinoma Xenograft Tumor Growth by Dietary Administration of Indole-3-carbinol is associated with Inhibition of Xenobiotic Metabolism and Integrin Pathways

Abstract

Indole-3-carbinol (I3C), a bioactive compound found in cruciferous vegetables, has been shown to possess protective properties against chronic diseases and associated conditions. In previous studies, I3C has exhibited suppressive effects within the tumor microenvironment by diminishing growth and reducing surrounding inflammation. However, the exact mechanism in regulating tumor growth remains unclear. In the present study, tumor volume of androgen-dependent, LNCaP, human prostate carcinomas was significantly reduced in immunodeficient mice (6- 8 weeks old, male, Balb c/c nu/nu) fed physiologically relevant dosages of I3C (0.1 and 1 μmoles I3C/g AIN-93 diet). Tumor analysis revealed a 49.7% decrease in tumor volume within the groups fed 0.1 and 1 μmoles I3C/g diet. Alterations in gene expression were assessed in tumor and liver samples. Consistent with previous results, treatment with I3C significantly upregulated Phase 1 xenobiotic metabolizing enzymes. Moreover, reduced expression of integrin genes were observed in mice fed 1 μmole I3C/g. Results from this study has shown that I3C may reduce the likelihood of prostate cancer progression by stimulating xenobiotic metabolism and modulating tumor adhesion properties.

Introduction

Prostate cancer is the most common, non-skin cancers in America, in which 1 of every 6 men are affected (Edwards et al., 2013). Many lifestyle factors, such as diet and exercise, contribute to cancer incidence. Epidemiological studies focusing on dietary intake have shown a strong correlation between cancer risk and lack of vegetable consumption and variety (P. Gupta et al., 2014; Verhoeven et al., 1996; Zanini, Marzotto, Giovinazzo, Bassi, & Bellavite, 2014). This evidence supports the notion that diet quality influences chronic disease manifestation. Low consumption of

cruciferous vegetables is inversely proportionate to prostate cancer development (Arab et al., 2013; Kristal & Lampe, 2002). Moreover, the normal function of urinary, bowel, and sexual reproductive structures may be affected and disrupted by prostate cancer and its current treatment options (Diefenbach et al., 2012; National Cancer; Wilt, 2002). With the lack of effective treatments available for prostate cancer, the identification and use of natural, anti-carcinogenic bioactive compounds found in cruciferous vegetables have gained popularity.

Indole-3-carbinol (I3C) is a dietary compound derived from glucobrassicin, a glucosinolate found in cruciferous vegetables. I3C is well known for its chemopreventive effects and has undergone various testing under clinical and preclinical settings (Huang, Jeffery, & Erdman, 2014; Souli et al., 2008). I3C has been shown to inhibit the development of epithelial tumors within the colon, lung, liver, breast, and prostate of humans in cell and animal models (Firestone & Sundar, 2009). Moreover, I3C has been well tolerated in clinical studies with few adverse effects reported in patients taking pharmacologically-relevant dosage of I3C (McAlindon et al., 2001; Rosen, Woodson, Thompson, Hengesteg, & Bradlow, 1998; Wong et al., 1997).

I3C exerts several effects that may contribute to cancer prevention (E. K. Kim et al., 2013). One mechanism involves the modulation of androgen-responsive proteins such as prostate specific antigen (PSA), which are key growth regulators. In cell culture, I3C modulates androgen metabolism and significantly suppresses the androgen receptor (AR) signaling via a ligand-dependent mechanisms (Hsu et al., 2006; Hsu et al., 2005). I3C may also induce phase 1 and 2 xenobiotic enzymes and is considered a first line of defense against toxins within humans and animals (Aggarwal, Danda, Gupta, & Gehlot, 2009; Aggarwal & Ichikawa, 2005; Aggarwal & Shishodia, 2006). These changes may lead to increased catabolism and excretion of carcinogens and steroid hormones shown to promote cancer. Additionally, the role of integrins within the extracellular matrix of prostate cancer cells in growth, migration, and metastasis has been of particular interest (Fornaro et al., 2001; Goel, Li, Kogan, & Languino, 2008). Activation of integrins occurs by local stimuli like soluble mediators such as hormones, cytokines, growth factors (Mizejewski, 1999).

Therefore, the potential of I3C to reduce integrin expression in androgen-dependent cell lines warrants further investigation.

In recent studies from our lab, I3C has been shown to regulate androgen-responsive gene expression under *in vitro* conditions (E. K. Kim et al., 2013; T. T. Wang et al., 2012). However, I3C has variable effects on multiple cell and animal models (Garikapaty, Ashok, Tadi, Mittelman, & Tiwari, 2006; Y. Li, Ahmad, Kong, Bao, & Sarkar, 2014; J. Zhang et al., 2003). Earlier research has focused on testing physiologically-achievable dosage of I3C in a lung cancer xenograft mouse model (Kassie et al., 2008). The exact mechanisms that underlie I3C-modulated events *in vivo* remains unclear. The purpose of this study is to take an initial step towards examining the physiological and biological effects of indole-3-carbinol on prostate cancer development in an athymic mouse androgen-dependent human prostate tumor xenograft model. The overall goal of this research is to determine if I3C can provide protection against prostate cancer, if consumed naturally through the diet. A secondary goal is to identify biomarkers and surrogate endpoints sensitive to the presence of I3C. The significance of this research could potentially enhance the utilization of this compound within whole food sources to improve the health status of the human population while enhancing the market value and variety of cruciferous vegetables within the agricultural industry

Materials and Methods

Cell Adhesion Assay

LNCaP, prostate carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were incubated at 37^{o}C in the presence of CO_2-air (5:95, v/v). LNCaP cells were trypsinized with 0.25% Trypsin-EDTA solution (Invitrogen) then seeded in conditioned media with I3C (0, 0.1, 1, 5, 25, and 50 µmol/L media) at a density of 0.625 x 10^5 cells/well in 24-well plates (Corning Inc., Corning, NY, USA). Cell plating efficiency was defined as cells attached to culture

plate surface and was determined by sulforhodamine B (SRB) assay (Hudson et al., 2012; Skehan et al., 1990).

Treatment Diet Composition

During the course of the study, mice were provided powdered AIN-93M diets in the absence or presence of purified indole-3-carbinol (0.1 or 1 μmol/g diet). Diets were purchased from Research Diets (New Brunswick, NJ) and stored at -20°C until treatment initiation, as per manufacturer's instructions. Formulation for control and treatment diets were based upon previous experiments that assessed quality and quantity of I3C in rodent diets (Kassie et al., 2008).

Animals were fed 0, 0.1, and 1 μmol I3C/g diet for the duration of this study. A dose of 1 μmol I3C/g diet in mice equates to a dietary intake of 2.98 mg I3C/kg body weight. A dose of .1 μmol I3C/g diet in mice equates to a human equivalent dose of 2.98 mg I3C/kg body weight. If estimating for a 60 kg man, the amount of I3C provided in the lower dose may correspond with consuming approximately 1.72 cups/ cruciferous vegetable. Moreover, in a clinical study, administration of I3C supplements was tolerated in doses up to 1,200 mg/day in male and female cancer patients (Reed et al., 2005). Therefore, the two doses selected for this study assessed the effects of tumorigenesis on I3C concentration similar to amounts consumed in the diet as well as amounts supported on a low dose chemopreventive range. In preclinical tumorigenesis studies, mice showed protection against cancer in diets supplements with 0- 100 μmol I3C, ranges that were considered approximately, 1000 and 100 times higher than the concentrations used in this study, thereby, deeming the concentrations of 0.1 and 1 μmol I3C/g diet used in this study, as safe, cautious, and physiologically relevant dosages.

Tumor Xenograft Model

LNCaP (American Type Culture Collection, Manassas, Virginia), an androgen-dependent human prostate carcinoma cell line is used to model response to dietary administered I3C. Prior to inoculation, LNCaP cultures were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100

µg/ml streptomycin purchased from Invitrogen (Carlsbald, CA). Cultures were incubated in an atmosphere composed of 5% CO_2 at 37°C.

All experimental protocols were in accordance with the National Institutes of Health guidelines and were approved by the USDA Animal Research Advisory Committee. Male athymic nude mice (BALB/c nu/nu, 20-22 g, 6-8 weeks old) were purchased from Charles River (Frederick, MD) and were individually housed in filter-top cages at the USDA BHNRC animal facility. Room conditions were carefully monitored and held at a constant temperature of 22 ± 1°C with a 12 hour rotating day and night cycle. Animals consumed food and filtered water *ad libitum*. Food consumption and body weights were recorded weekly. An acclimation period of 1 week prior to treatment feedings enabled mice to adapt to control AIN-93M diet and environment. Animals were then, randomized into three experimental feeding groups- (i) control diet, (ii) control diet with .1, and (iii) 1 µmol I3C/g diets (n = 15/group). Two weeks following treatment initiation, all mice were inoculated via subcutaneous injection into the right and left flanks with LNCaP cell suspension. Cell suspension consisted of LNCaP cells at a density of 2 x 10^6 with 50 µl of phosphate-buffered saline (PBS) and an equal volume of Matrigel (BD Biosciences, Mansfield, MA). Injection sites and tumor volume (cm^3) were monitored and measured weekly for palpable tumor growth and biological efficacy: (cm^3) = 0.523 x [length (cm) x width^2 (cm^2)] (Hudson et al., 2012; Kleinman & Martin, 2005). Mice remained on their respective diets for 7 weeks following inoculation.

Plasma and Tissue Collection

At the end of the study, mice were euthanized using a CO_2 chamber and necropsy was immediately performed. Blood was collected, stored in vials coated with potassium EDTA solution (15% w/v), and placed on dry ice until plasma separation. Plasma were separated via centrifugation at 1500 rpm for 30 min at 4°C. Soft tissue including tumor, liver, kidneys, and spleens were collected and subdivided. Subdivided samples were immediately frozen in liquid nitrogen for protein analysis or in an RNA*later*, an RNA stabilizing agent purchased from

AMBION Inc. (Austin, Texas). Samples were stored in an -80°C freezer until further analysis.

RNA Isolation and Reverse-transcription (RT)-PCR

For determination of mRNA expression in tumor and liver samples, total RNA was isolated and purified using RNeasy (Qiagen Inc., Valencia, CA), according to manufacturer's protocol. StrataScript First Strand complementary DNA Synthesis kit from Stratagene (Santa Clara, CA) was used to reverse-transcribe mRNA to complementary DNA. Real-time PCR were performed on Applied Biosystems 7900T Real-Time PCR System using TaqMan® Gene Expression Assay (Carlsbad, CA). Relative mRNA levels for analysis of gene expression changes were quantified using ΔCt method, as previously described TaqMan real-time RT-PCR method (E. K. Kim et al., 2013; Yuan et al., 2006). TATA box binding protein (TBP), a housekeeping gene, was used to calculate relative gene expression levels in soft tissue samples.

Table 3 Marker genes for pathways related to carcinogenesis

Pathway	Human	Mouse
Proliferation	KI67	
Cell Cycle	CDKN1a, CDKN1b	
Androgen	PSA	
Inflammation	IL1RN	Il-1β
Monocyte Attraction	CCL2	
Xenobiotic Metabolism		Cyps, Nqo1, Ugt1a1
Cell Adhesion	Integrins, FN1	

Protein Analysis

In order to confirm or identify post-translational changes of mRNA level gene changes, two protein analysis methods were utilized. Immunohistochemistry was used to identify protein level changes in soft tissues. ELISA was utilized to analyze protein changes in isolated plasma. Circulating Il-1β levels within plasma samples

was determined using ELISA. Assays were performed using a commercial kit according to manufacturer's protocols (eBioscience, San Diego, CA).

Hypothetical Tumor Size Analysis

Collected tumor volume data was fitted into an exponential growth equation, previously used by Li., et al (R. W. Li et al., 2013). The best fit values of START and K from the equation, Y = START x EXP (K x Y), was used to generate hypothetical tumor growth curves to depict stimulatory growth following 20 weeks of treatment. All data calculations were performed using Prism 6 software (GraphPad Software, San Diego, CA).

Statistical Analysis

The 15 animals used per group give >95% power to detect effects with at least a 10% magnitude. Mean body weights, food consumption, and tumor growth were used in the preliminary assessment of treatment groups. All end point assays were conducted in triplicate and averaged for group analysis. Means were reported as mean ± SEM. One-Way Analysis of Variance (ANOVA) and Bonferroni test were conducted to determine the differences among means. Statistical significance was declared at $p < 0.05$ using SPSS for Windows (version rel. 21.0, SPSS Inc., Chicago, IL) and GraphPad Prism 5 for Windows (version rel. 5.01, 2009, GraphPad Software, Inc., La Jolla, CA). Power calculations were completed using SAS 9.3 (version 6.1.7601, 2010).

Results

Effects of I3C on Body Weight and Food Consumption

Prior to tumor injection, diet tolerance was assessed. Animals were observed for gross signs of diet-related toxicity, and a baseline line of food consumption was established. Overall, there were no significant differences in body weight or food consumption over the course of I3C administration (Fig. 17). However, trends in body weight and food consumption slightly decreased during the final weeks as tumor

significantly grew in size. Changes in weight may be due to cancer-related cachexia as tumor burden increased for the animals.

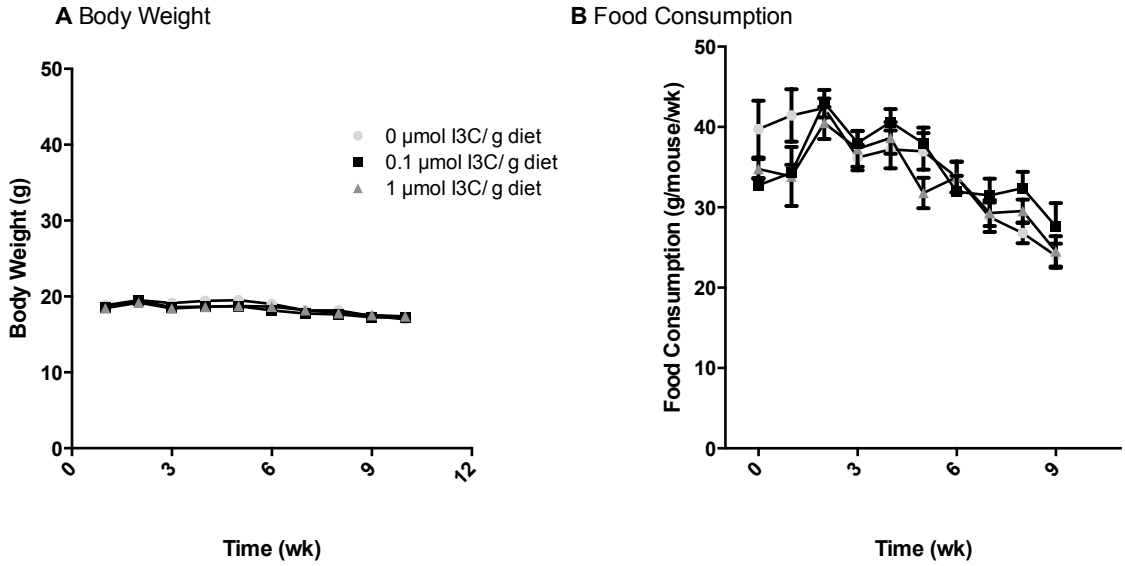
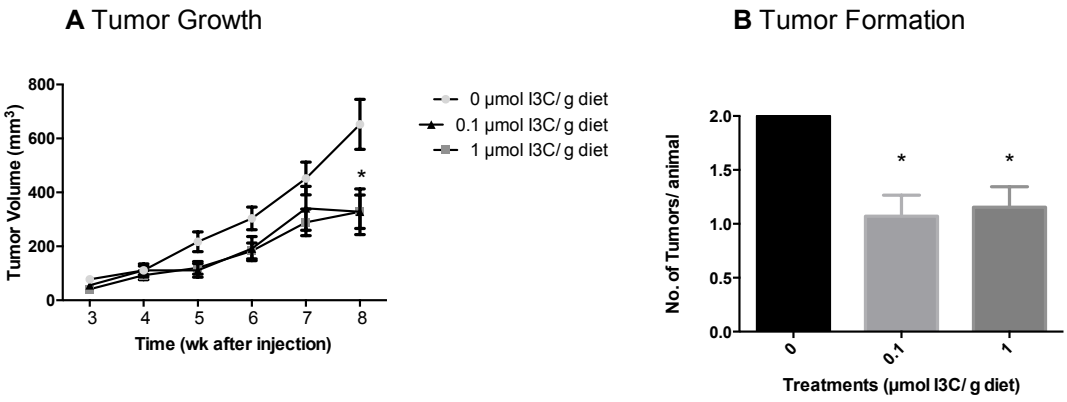


Figure 17 Body weight and dietary intake of treated mice. Mice were randomized into three groups and fed 0, 0.1, and 1 µmol I3C/g diet. Mice remained on their respective diets for 10 weeks. Body weight (g) and food consumption (g) were recorded weekly. A) Mean body weights of mice fed control or treatment diets. B) Mean weekly food consumption of mice fed control or treatment diets. Values are reported as means ± SD. (*) represents significant differences between control and treatment diets at $p < 0.05$.

I3C Intake Perturbs Tumor Formation and Growth



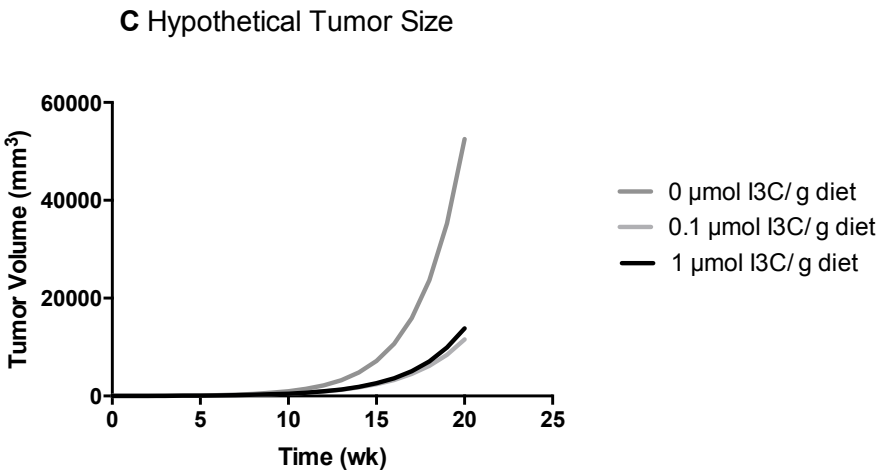


Figure 18 Tumor growth and formation in treatment mice. LNCaP human prostate cancer cell xenografts was established in athymic nude mice. Tumor volume was measured weekly and calculated in described Materials and Methods section. A) Mean tumor volume and B) mean number of tumors formed in control and treatment mice during 10 week treatment period. C) Hypothetical tumor size after 20 week treatment period using tumor growth (Fig. 18A). Growth through 20 weeks was simulated for treatment groups using exponential growth equation in Materials and Methods section. Values are reported as means ± SD. (*) represents significant differences between control and treatment diets at $p < 0.05$.

Significant decreases in tumor load were observed at week 8, in which tumor volume was 49.7% lower in the 0.1 and 1 µmol/g diet treatment groups (Fig. 18A). It is also possible that I3C treatment inhibited tumor formation prior to 3 weeks following LNCaP injection. Average tumors formed by the end of the study was 46.4 and 42.3% lower in mice fed 0.1 and 1 µmol/g diets, respectively (Fig. 18B).

Effect of I3C on androgen-responsive, proliferation, cell cycle, and inflammatory pathways

Previous studies have demonstrated I3C's role in modulating androgen-responsive, proliferation, or inflammation pathways. Thus, we evaluated whether delayed tumor growth would affect related biomarkers (PSA, KI67, IL1RN, IL1β, and CCL2) following I3C treatment.

Although there are appreciable trends in the data suggesting a significant role in the regulation of these pathways, treatment with I3C did not significantly affect these biomarkers with the tumors or circulating levels of Il-1β in the plasma (Figs.

19-20). One reason for these results may be due to I3C has been shown to affect protein expression without altering gene transcription. Other reasons may involve the complexity of the tumor environment since previous studies have demonstrated significant results in these genes in *in vitro* experiments or at higher concentrations of administered I3C *in vivo*. Therefore, further analysis can be done to identify whether other related genes were affected by treatment and if post-translational modifications could affect protein-level changes of the tested genes and contribute to the overall tumor attenuation.

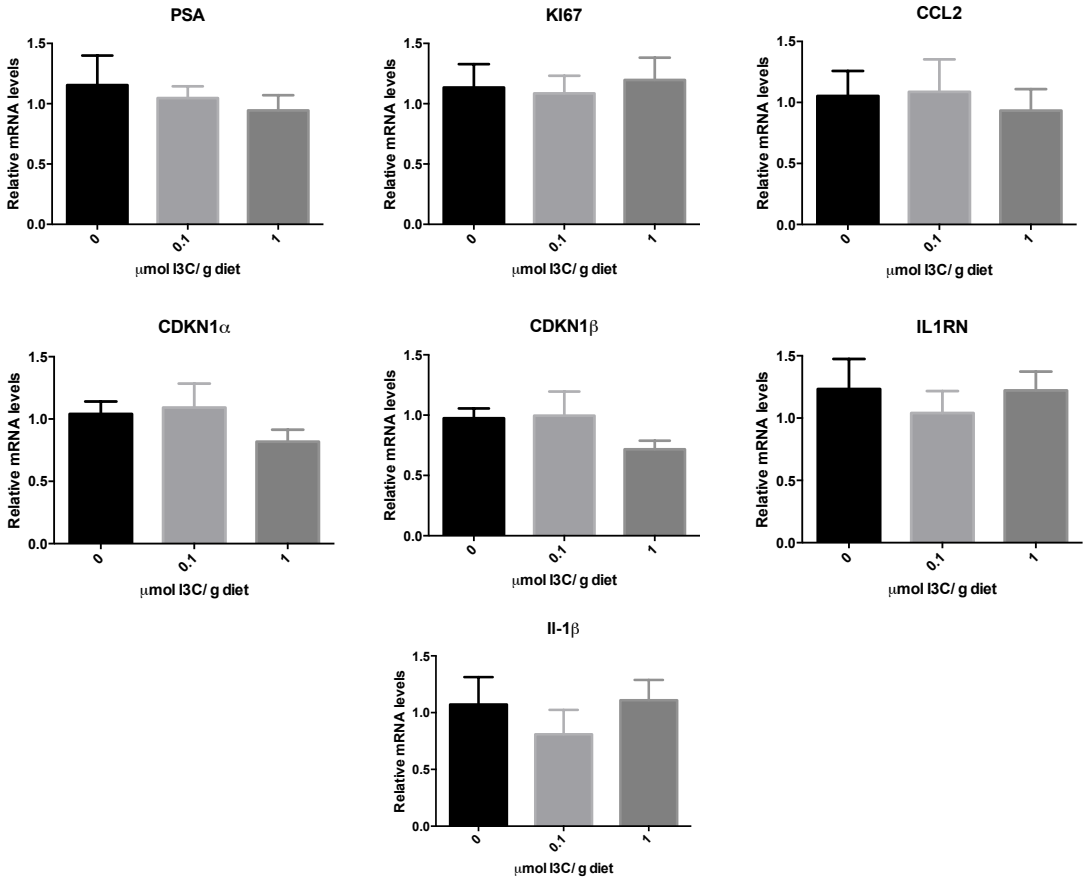


Figure 19 Various genes related to androgen responsive, proliferation, and inflammatory pathways assessed in athymic nude mouse model. Changes in mRNA expression of androgen-responsive, cellular proliferation, and inflammatory pathways were assessed in LNCaP prostate cancer cell xenografts in athymic nude mice. Real-time PCR analysis of PSA, KI67, Ccl2, CDKN1α, CDKN1β, IL1RN, and IL-1β was performed as described in the Materials and Methods section. Values are reported as means ± SD. (*) represents significant differences between control and treatment diets at $p < 0.05$.

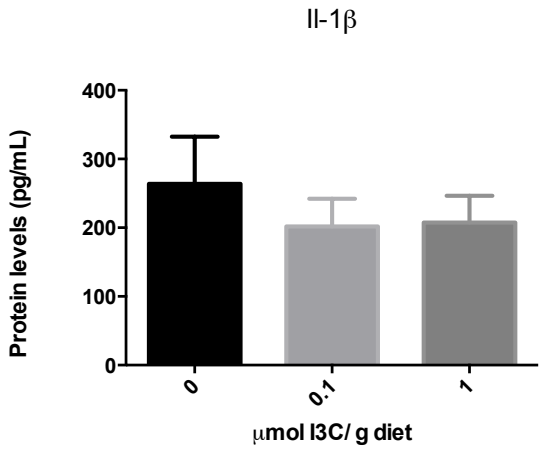


Figure 20 Effect of indole-3-carbinol on circulating Il-1β levels. Circulating Il-1β protein levels in plasma of mice treated with 0, 0.1, and 1 μmol I3C/g diet was assessed using ELISA as previously described in Materials and Methods section. n = 7 animals/treatment group. Results are reported as means ±SE. (*) represents $p < 0.05$.

Effect of I3C on Xenobiotic Metabolism

The effects of low dose I3C treatment on hepatic xenobiotic clearance were assessed. I3C has been previously shown to induce gene expression of Phase 1 and 2 xenobiotic enzymes. Cyp1a1, Cyp2c55, Cyp3a11, Cyp2d10, and Phase 2 enzymes Ugt1a1 and Nqo1 were examined. These Phase 1 enzymes are mouse homologues of a human panel representative of at 90-95% of genes related to clearance of xenobiotics.

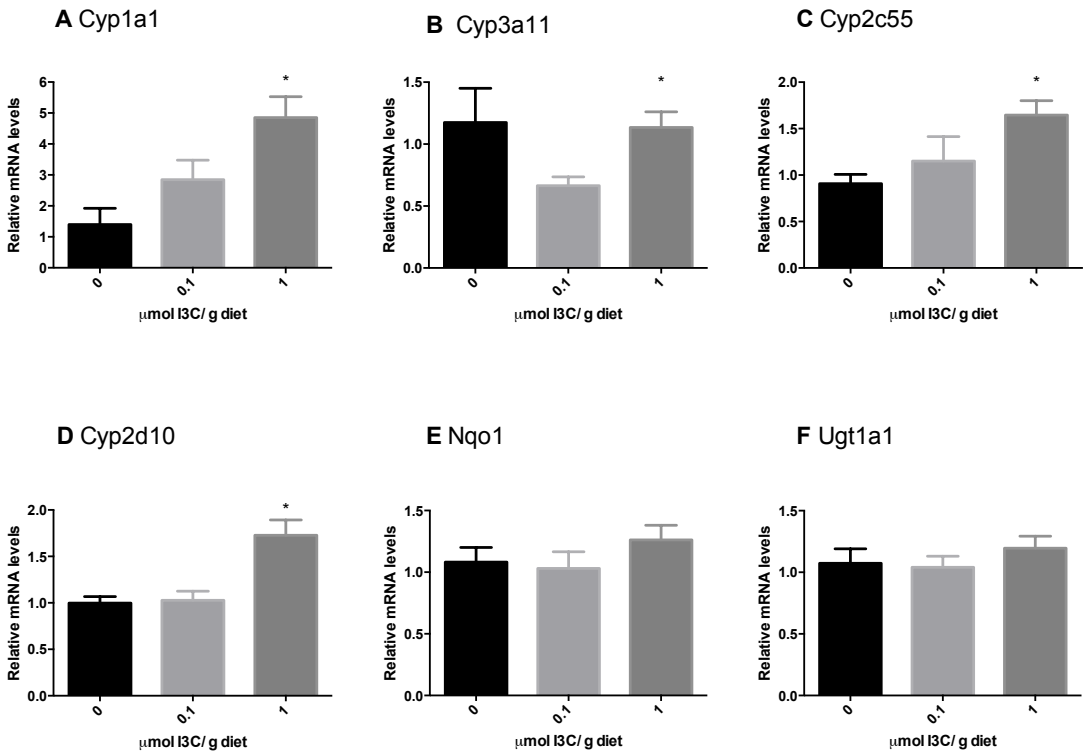


Figure 21 Xenobiotic metabolizing enzyme gene changes in livers of athymic nude mice. Changes in mRNA expression of Phase 1 and -2 xenobiotic metabolizing enzymes were assessed in LNCaP prostate cancer cell xenografts in athymic nude mice provided dietary administered I3C. Real-time PCR analysis of A) Cyp1a1, B) Cyp3a11, C) Cyp2c55, D) Cyp2d10, E) Nqo1, and F) Ugt1a1 was performed as described in the Materials and Methods section. Values are reported as means ± SE. (*) represents significant differences between control and treatment diets at $p < 0.05$.

Treatment with 1 μmol I3C/g diet was able to significantly induce expression of Phase 1 xenobiotic metabolizing enzymes (Fig. 21). Cyp1a1, -2c55, and -2d10 expression was significantly altered following treatment with low dose I3C. As compared to the control group, Cyp1a1 expression was 2 ± .6 and 3.5 ± .7 times higher in the 0.1 and 1 μmol/g treatment groups, respectively. Average change for Cyp2c55 in the 0.1 and 1 μmol/g treatment group was Cyp2d10 expression was 1 and 1.73 ± .2 times higher in the 0.1 and 1 μmol/g diet groups. UGT1A1 and NQO1 expression levels were reduced but not significantly inhibited by I3C treatment. Low

phase 2 enzyme induction potential has been seen in indole glucosinolates (Fahey et al., 1997; Shapiro et al., 1998).

I3C Attenuates Cell Adhesion *in vitro*

I3C's ability to alter cell adhesion was assessed under *in vitro* and *in vivo* conditions. The results of the *in vitro* cell adhesion assay indicated linear, dose-dependent inhibition with increasing concentration of I3C (Fig. 22). Significant decreases in cell adhesion were observed at concentrations of 25 µmol/L or greater. Next, cell adhesion was assessed on transcriptional level in LNCaP human prostate cancer cell line.

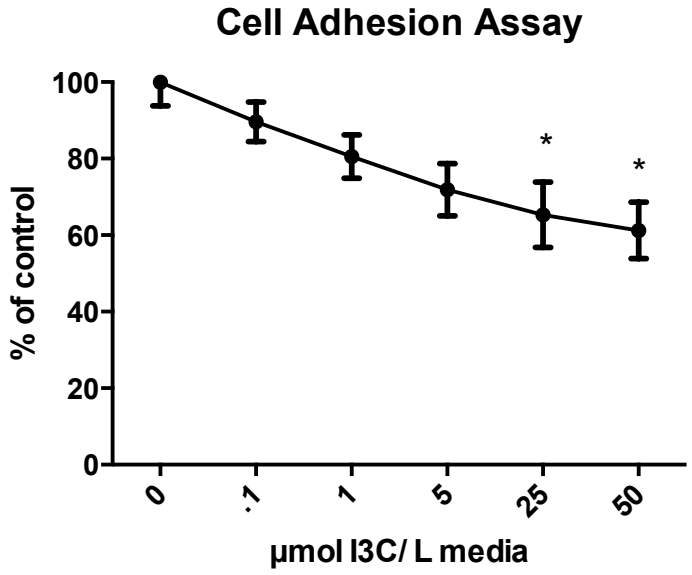


Figure 22 Changes in LNCaP cell adhesion following I3C treatment *in vitro*. LNCaP cells were plated in 24-well plates in the presence of 0, 0.1, 1, 5, 25, and 50 µmol I3C/L media. After overnight incubation, cell attachment to the plate was assessed using SRB method as described in Materials and Methods section. Values are reported as means ± SE. (*) represents significant differences between control and treatment diets at $p < 0.05$.

Integrin changes were also assessed under *in vitro* conditions. There were no significant changes in integrins A_2, A_5, and B_1 (Fig. 23). However, significant 30% reduction in integrin A_6 gene expression was observed in doses ≥ 25 µmol I3C/L

media. Integrin gene changes were also assessed *in vivo*. Following treatment with low dose I3C, $ITGB_1$ expression was significantly inhibited by 26.4% in tumors of mice treated with 1 μmol I3C/g diet (Fig. 24).

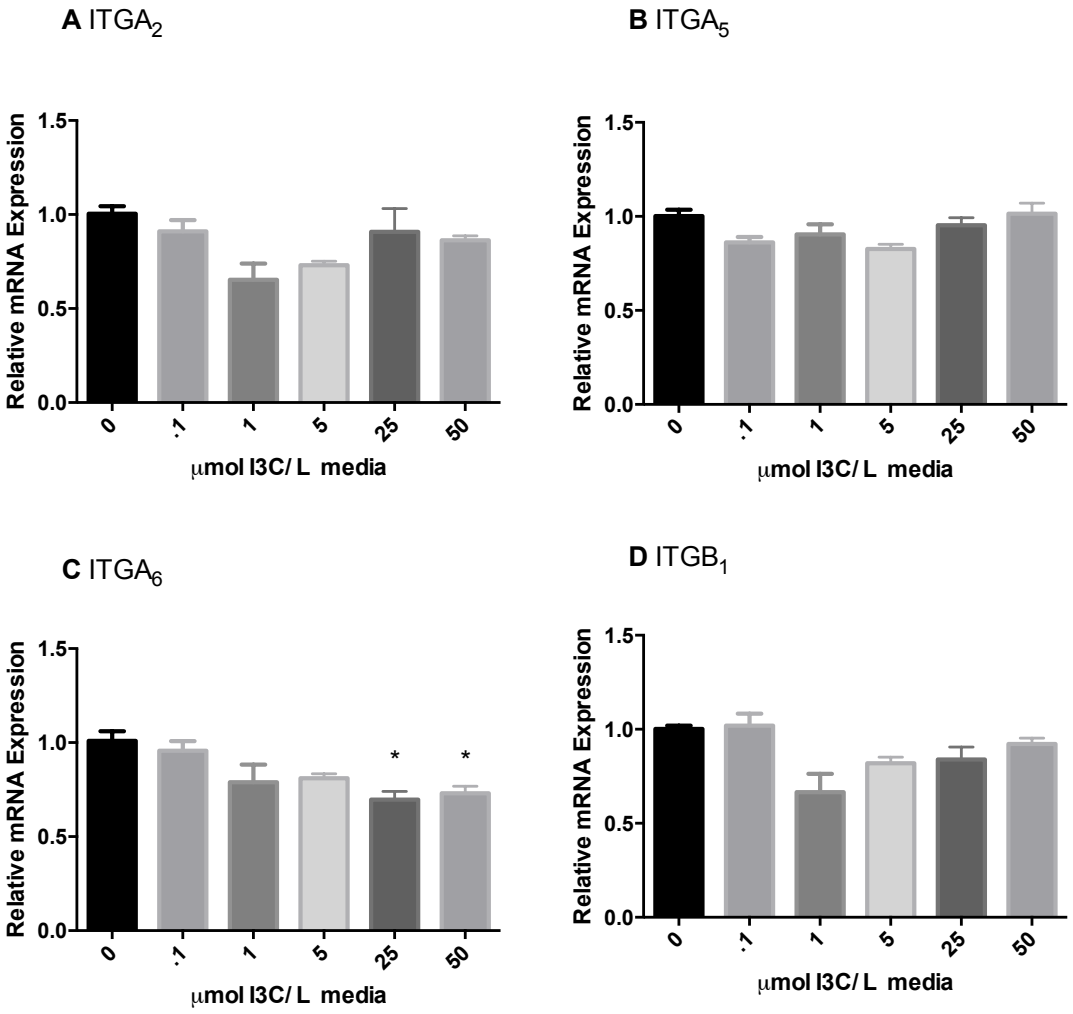


Figure 23 Changes integrin gene expression within LNCaP cells following *in vitro* treatment with I3C. LNCaP cells were plated in 6-well plates in the presence of 0, 0.1, 1, 5, 25, and 50 μmol I3C/L media. After overnight incubation, cells were harvested and RNA was harvested. Levels of $ITGA_2$, $ITGA_5$, $ITGA_6$, and $ITGB_1$ mRNA was quantified relative to TBP by RT-PCR as described in Materials and Methods section. Values are reported as means ± SE. (*) represents significant differences between control and treatment diets at $p < 0.05$.

The results of integrin gene expression led to further exploration into tumor interactions with the ECM. In the plasma, fibronectin, a glycoprotein, involved in cell adhesion and migration, is present in a soluble dimeric form. It is also found in

multimeric form at the cell surface and in extracellular matrix, and serves a ligand for the integrins. Fibronectin 1(FN1) is responsible for binding to cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Indole-3-carbinol and PEITC has been shown to inhibit cell adhesion and until fibronectin expression. Therefore, FN1 was characterized. However, there were no significant differences were observed in FN1 gene expression (Fig. 24E).

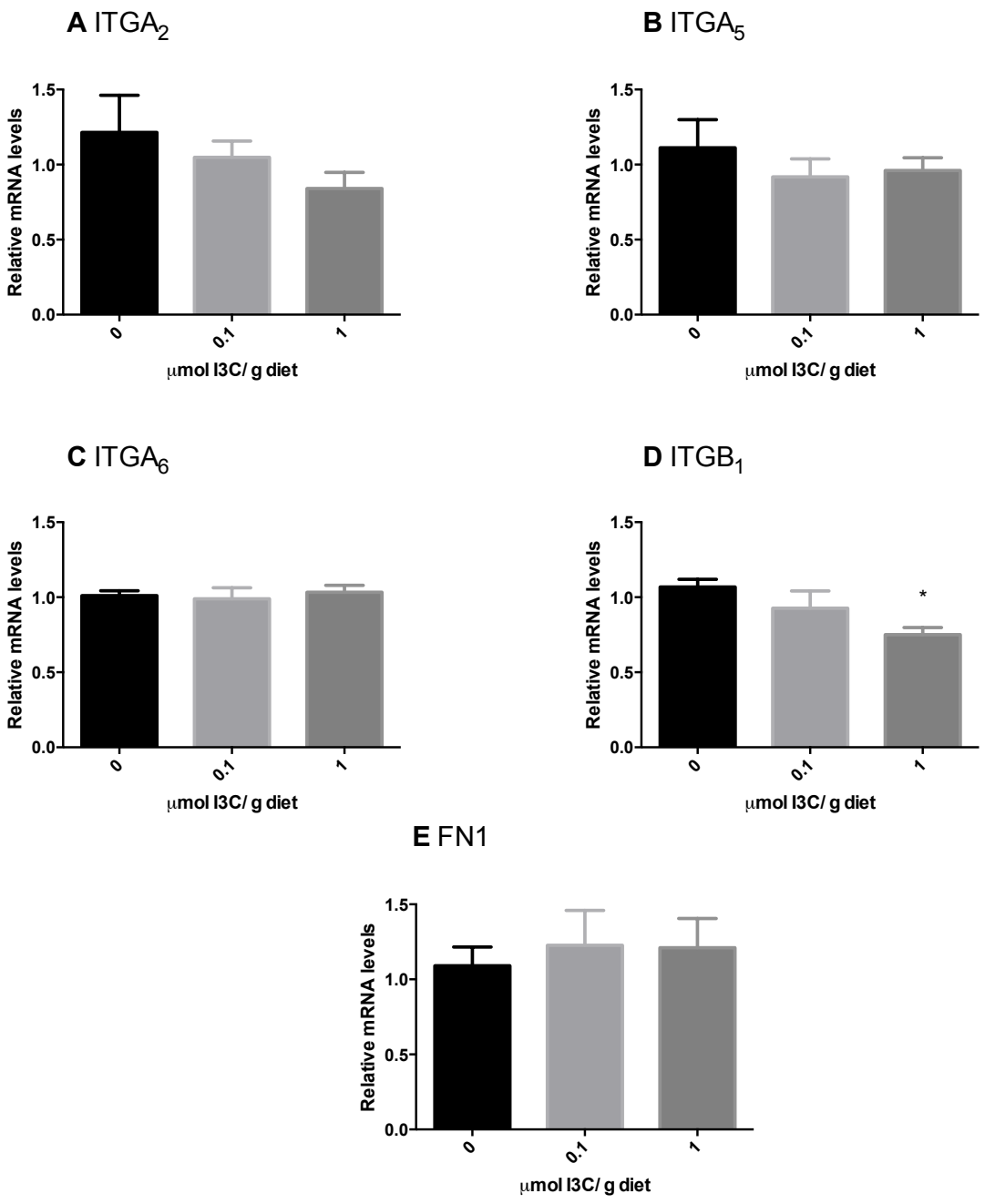


Figure 24 Integrin and FN1 gene changes in LNCaP xenografts in athymic nude mice. following treatment with I3C. Changes in mRNA expression of integrin and FN1 genes were assessed in LNCaP prostate cancer cell xenografts in athymic nude mice provided dietary administered I3C. Real-time PCR analysis of A) $ITGA_2$, B) $ITGA_5$, C) $ITGA_6$, D) $ITGB_1$, and E) FN1 was performed as described in the Materials and Methods section. Values are reported as means ± SE. (*) represents significant differences between control and treatment diets at $p < 0.05$.

Discussion

In the present study, we demonstrated that I3C is capable of inhibiting tumor growth in an athymic mouse model. Key findings from this study revealed a significant reduction in tumor formation and growth, and altered expression patterns of genes interconnected with adhesion and xenobiotics defense pathways within the tumor and liver of mice fed diets containing 1 μmol I3C/g diet.

Beneficial effects of cruciferous vegetable intake against prostate cancer have been demonstrated in previous studies (Aggarwal & Ichikawa, 2005; Souli et al., 2008). However, the exact regulatory mechanisms in which I3C renders its cancer-reducing effects have not been resolved under physiological conditions. To elucidate these mechanisms, physiologically relevant of I3C dosage was administered to animals in an amount up to 2.98 mg/ kg human body weight/ day. We did not find any major differences food consumption and body weight in animals fed control and I3C-containing diets. Despite the limited differences in phytochemical composition, significantly lower tumor burden, in terms of volume and formation were observed in the I3C-treated animals over time. Based on the findings of our studies, I3C may be a component within cruciferous vegetables that can be contributed to the prevention or regression of prostate cancer. The specific components attributed to prostate cancer regression are not known. Thus, future studies are of interest to uncover the associations between specific derivatives of indole-3-carbinol and tumor progression.

Understanding possible gene-response relationships between cytochrome P450 enzymes and natural compounds and the resulting therapeutic efficacy is of clinical importance (Zhou, Liu, & Chowbay, 2009). Earlier studies have reported

increased expression of members of the 1, 2, and 3 families in the human CYP superfamily following exposure to I3C and its metabolites within xenograft models (Bjeldanes et al., 1991; Crowell, Page, Levine, Tomlinson, & Hebert, 2006; Leibelt, Hedstrom, Fischer, Pereira, & Williams, 2003). Therefore tissue-specific effects on key phase 1 xenobiotic metabolizing enzymes are of considerable interest. In line with the previously mentioned studies, our results indicate that dietary administration of I3C can upregulate xenobiotic pathways within the livers of human prostate cancer xenograft mouse model. Importantly, the diets in our study were *ad libitum,* at relatively low doses, and caused significantly increased expression of CYP enzymes compared with diet used in previous studies at much higher concentrations. Thus, xenobiotic metabolism regulation can be achieved with low I3C concentrations available through the diet.

Our third major finding was the reduced expression of integrin genes following treatment with I3C. Previous studies suggest that increased integrin expression is a hallmark of cancer progression as related to the dysregulation of the extracellular matrix and invasion into other regions of the affected tissue or to different organs (Fornaro et al., 2001; Goel et al., 2008). Modulating integrins $-A_6$ and $-B_1$ gene expression led to a decrease in prostate cancer tumor growth (Goel et al., 2005; King et al., 2008). Moreover, our study demonstrated that I3C may specifically regulate integrin genes. When tested against a panel of integrin genes under *in vivo* conditions, only $ITGB_1$ was significantly downregulated. These results are unique in comparison to expression patterns against the same panel following I3C treatment in a LNCaP cell model. Under *in vitro* conditions, changes in cell adhesion may be an outcome of diminished $ITGA_6$ expression. Results may indicate a compensatory role of integrins under *in vivo* conditions. Therefore, the reduced integrin expression observed following treatment with I3C may reflect a long-term protective adaptation that counteracts deregulated growth in prostate cancer.
The factors mediating the changes in xenobiotic metabolism and integrin expression are unclear. But, indole-3-carbinol is an ideal candidate, which can drive diet-induced regulation of xenobiotic metabolism and cellular progression, including Cyp1a1, -3a11, -2c55, -2d10 and $ITGA_6$, $-B_1$ activity.

Previous studies have linked administration of I3C with the modulation of androgen responsive, proliferation, cell cycle regulation, inflammation, and monocyte attraction pathways. Therefore, inflammation surrounding the tumor site, for example, was investigated. Monocyte attraction to human prostate cells is responsible for the increased presence monocyte-derived macrophages and subsequent secretion of proinflammatory cytokines such as IL-1β within the tumor microenvironment. Previous studies have demonstrated the distinct ability of I3C to reduce monocyte attraction (E. K. Kim et al., 2013). However, results from this study did not uncover significant changes in IL-1β gene expression or protein levels under *in vivo* conditions. Differences from our study and the previously mentioned *in vitro* study include compound concentration amongst other factors. Interestingly, in light of the observed differences in tumor sizes following I3C exposure, tumor tissue assessed in our study did not exhibit any significant changes within the remaining pathways such as those that are androgen-responsive. Therefore, a more sensitive method to study the effects of I3C on human prostate cancer may be needed to reveal an association. This also suggests that other factors are linked to growth inhibition presented within tumors of animals fed I3C. Conversely, possible differences may be observed if interim samples are collected.

Importantly, our study has, to the best of our knowledge, the lowest exposure of I3C in which effects of dietary interventions on human prostate cancer regression has been reported. We consider this a major strength since dietary recommendations are given on a long-term basis. Recent data proposed that I3C derivatives, instead of the parent compound, are responsible for chemopreventive results as previously thought. Future studies using I3C derivatives are needed in order to estimate the direction of Phase 1 xenobiotic metabolizing enzymes and integrins *in vivo* after dietary interventions.

Conclusion

In summary, we found low doses of I3C to significantly inhibit tumor growth in animals weeks after inoculation of LNCaP, human prostate cancer cells. Administration of I3C resulted in significant reduction in cellular adhesion

mechanisms and increased xenobiotic metabolisms. Our study demonstrated potentially chemopreventive effects of diet-derived indole-3-carbinol by regulating defense responses in multiple organ systems. Our study supports previous findings demonstrating beneficial effects of I3C under *in vitro* conditions while highlighting sensitivities found among various animal strains. Moreover, testing should be done to identify novel alterations in endpoint markers leading to the observed decreased in tumor growth. The mechanistic effects of I3C along integrin-related pathways require further examination.

Chapter 5: Summary and Significance

Based on the conclusions of the three studies presented in this work, several future directions arise.

1. Influence of mouse strains on chemopreventive effectiveness of indole-3-carbinol

The second aim of our study was to assess the biological efficacy of I3C in a mouse model commonly used to assess prostate cancer. Genetic variation, for example, may affect the potency and effectiveness of biological compounds. Therefore, future work should be done to molecularly analyze and elucidate mechanisms of novel targets involved in the manifestation of prostate cancer. This would be to identify beneficial effects of indole-3-carbinol on a variety of mouse strains used as prostate cancer tumorigenic models. Understanding the effects on I3C on these mouse strains may provide insight on the influence of genetic variation on the sensitivity of this compound and its derivatives, more defined no effect levels (NOELs), optimal chemopreventive levels, and upper limits (ULs) and divulge the potential impact of its effectiveness as a chemopreventive agent on prostate cancer.

2. Direct measures of cruciferous vegetable-derived compounds within xenograft models

The third aim of our study determined that indole-3-carbinol possesses chemopreventive effects on prostate cancer when consumed through the diet. Previous studies have shown a positive correlation between the amount of I3C administrated through the diet, compound metabolism, and its retention within blood plasma and urine of mice (Anderton et al., 2004). Findings from the third study indicated that I3C significantly upregulated Phase 1 xenobiotic metabolizing enzymes. Therefore, it may be of interest to examine metabolic processes of indole-3-carbinol and phenethyl isothiocyanate. An example would include directly assessing for changes in Phase 1 and 2 xenobiotic metabolizing enzymes within prostate tumor following treatment with dietary compounds. Because indole-3-carbinol and phenethyl isothiocyanate may be immediately metabolized upon oral consumption, identifying novel compound derivatives that are primarily circulated through the

model's system and are found within the tumor microenvironment. Therefore, following consumption of I3C or PEITC, derivatives inducing Phase 1 xenobiotic metabolizing enzymes may differ from the derivatives responsible for inciting enzyme expression with the tumors. Potential of these results may not only be beneficial to the health status of the general population but also has an agriculture incentive. This research could aid researchers in developing optimal conditions that impact the nutritional quality and composition of cruciferous vegetables made available to the public.

3. Synergistic effects of indole-3-carbinol and phenethyl isothiocyanate

Results from our study determined that PEITC differentially affects prostate tumors and its surrounding microenvironment than I3C on an androgen-dependent cell line. Future work may further profile the role of macrophage presence or examine the synergistic effects of I3C and PEITC under *in vitro* and *in vivo* conditions. Moreover, prostate tumors are composed of androgen-dependent and androgen-independent variations. To our knowledge, there are no studies examining the role of PEITC and I3C in the athymic mouse model used in our study. Therefore, future work may also explore the effects of simultaneous exposure to PEITC and I3C on both androgen-dependent and androgen-independent xenograft models.

Appendices

7 Hallmarks of Cancer

There are several key steps in the transition from normal to cancerous epithelial cell growth: initiation, promotion, progression, and metastasis. The initiation phase is marked by the metabolism of cancer initiators such as carcinogens by xenobiotic metabolizing enzymes with the host system. If metabolized, the active initiators bind directly to DNA causing irreversible mutations that carry into daughter cells. The promotion phase is marked by abnormal cell growth as a result of cancer promoters and the production of daughter cells containing the same mutated genome. However, unlike the cancer initiators, promoters do not bind directly DNA or macromolecules within the cell. Instead, these promoter bind as ligands onto receptors on the cell surface in order to affect intracellular pathways that lead to increased cell proliferation. Moreover, it is in this phase where the promoted cells are recognized as benign tumors as they have slow growth patterns and adhesion properties with neighboring cells without invasion or metastasis. This phase is a critical point for dietary interventions (Surh, 2003). The third phase is progression, which is a stepwise transformation from benign tumor to malignant neoplasia. Characteristics of these progressive cancer cells are rapid cell growth, aggressive, invasive, and metastatic. Metastasis is the fourth phase and it is marked migration to secondary locations (other location in the organ or to other organs). Metastasis is accompanied by aggression. Characteristics of metastatic cells include loss of adhesion properties to neighboring cells, increased locomotion, destructive and

uncontrolled invasion, and possible re-education of the host immune system (Surh, 2003).

Prostate Anatomy

The prostate is the largest accessory sex gland in males. Located at the base of the bladder, a typical prostate gland may weigh approximately 50 grams and produces a secretion that aids in the production of semen and disintegration of cervical mucus. Of the accessory sex glands, the prostate is the most susceptible to cancer, despite all the sex glands having similar embryonic origination. There are four main regions within the prostate gland, which includes the central, transitional, periuretal, and peripheral zones. The epithelium within the peripheral zone is regulated by active androgen, dihydrotestosterone (DHT). Each of the four areas are susceptible to prostate cancer development. However, the region most affected by prostate cancer is the peripheral zone, which is the most susceptible to inflammation due to containing 70% of the prostate's glandular tissue.

Prostate Cancer

Prostate cancer development is complex. General consensus of the literature suggests that cancer begins from a single androgen sensitive cell and expands clonally acquiring mutations. The mutation then manifests into genetic alterations such as those leading to androgen independence. Low-growth fraction refers to the proportion of cells undergoing mitosis to cells in G_0, at any given time. Solid tumors, generally,

have a low growth fraction, which makes them relatively resistant to many of the traditional chemotherapeutic agents that are targeted at dividing cells (L. W. K. Chung, Isaacs, & Simons, 2001).

Two characteristics that make prostate cancer unique is its early stage: androgen sensitivity and relatively low growth fraction. Effective treatment for prostate cancer remains unknown. Androgen deprivation, through surgical or chemical castration, has been used for nearly 80 years for the regulation of prostate cancer. Results of this method have led to significant tumor regression by induction of programmed cell death in androgen responsive prostate cancer cells. However, by the time of clinical detection, these tumors may have heterogeneous populations ranging from androgen dependent to androgen sensitive and independent cells. Therefore, androgen ablation may result in significant androgen-dependent cell death, but it eventually selects for androgen sensitive and independent cancer cells (L. W. K. Chung et al., 2001).

The specific targeting of cancer cells has become a unifying theme supporting the development of novel therapeutic modes (Peng et al., 2008). Often, the targeting molecules are antibodies, or peptides, which bind to cell-surface membrane proteins that are specifically-, or over-expressed on malignant cells but not expressed on healthy cells. These innovative targeted therapeutic and diagnostic methods promise to increase both the specificity and efficacy of prostate tumor diagnosis and treatment while reducing the side effects (Adair, Parette, Altinoğlu, & Kester, 2010; Afnan & Tempany, 2010; Chellat, Merhi, Moreau, & Yahia, 2005; Jadvar, 2009). Using

dietary intervention as a preventive measure is ideal due its generally high availability and low side effects.

Sex Hormone

Role of Androgen in Prostate Cancer Development

Androgens play a multifaceted role in the development and maintenance of healthy prostate functioning as well as the progression to prostate cancer. However, the exact role androgens have in the transition from normal to cancerous conditions remains unresolved.

The "Androgen Hypothesis" published by Charles Huggins in 1941, determined that castration caused PCa regression in men with metastatic disease. Huggins concluded that testosterone proportionately activates PCa and enhances growth rate. More recent research suggests that high levels on testosterone may only affect long term risk (Muller et al., 2012). The Saturation Model, proposed by Adam Morgentaler, suggests that PCa cells utilize a certain level of androgens that supports optimal growth. The presence of androgen has a limited ability to stimulate benign or malignant prostate tissue, implicating cellular self-sufficiency, not the level of androgen, as the main drive of cancer progression (Morgentaler, 2012). However, there's overwhelming scientific evidence supporting the presence of androgen in the promotion and progression of prostate cancer.

Testicular synthesis of testosterone, prominent male sex steroid hormone, accounts for approximately 90% of the dihydrotestosterone (DHT), an active form of testosterone, found in the prostate. The remaining testosterone is secreted peripherally as adrenal androgens that are eventually excreted or recycled in the system. DHT

preferentially binds to the androgen receptor (AR) at an approximately 10x higher affinity than testosterone(*Drug Management of Prostate Cancer*, 2010).

The AR is a member of the steroid hormone superfamily, which includes estrogen, progesterone, and glucocorticoid receptors (Beato, 1989; Beato, Chalepakis, Schauer, & Slater, 1989; R. M. Evans, 1988). The AR serves as a ligand-binding transcription factor. These receptors exists in the nucleus and consists of three domains: 1) a carboxy-terminal hormone-binding domain; 2) an amino-terminal transactivation domain, and a 3) DNA-binding domain located between domains 1 and 2 (Beato et al., 1989; Jenster et al., 1991). After testosterone or DHT binds to the AR, an hormone binding domain, the receptor becomes phosphorylated, translocates into the nucleus, to subsequently, bind to AREs as dimers. Essentially, the genes whose expression is stimulated by androgen are flanked by specific DNA sequences, called androgen response elements (AREs), which are recognized by the DNA-binding domain of the AR (Jenster et al., 1991). Thus, stimulating transcription of androgen-inducible genes and ultimately, protein synthesis leading to the observed changes in cell proliferation, survival, and differentiation (*Drug Management of Prostate Cancer*, 2010; Jenster et al., 1991; Riegman, Vlietstra, van der Korput, Brinkmann, & Trapman, 1991).

Ultimately, the androgen signaling pathway plays a critical role in the progression of prostate cancer, especially in early stages. Elevation of testosterone may not be a sole determinant in the diagnosis of cancer due to the overall complexity of prostate cell development and heterogeneity within the tumor and surrounding environment ("Prostate-Specific Antigen (PSA)Test," 2012).

Influence of Estrogen on Prostate Cancer Development

Androgen in prostate cancer has specificity for estrogen in addition to androgen. Low levels of estrogen have been found in men. More importantly, I3C has been shown to modulate estrogen receptor amongst other nuclear transcription factors (Y. S. Kim & Milner, 2005).

Androgen Responsive Biomarkers: PSA and NKX3.1

Androgen Responsive Biomarkers: PSA and NKX3.1

Prostate specific antigen (PSA), also known as kallikrein-3 (KLK_3), is a serine protease secreted by prostate gland epithelial cells. The gene encoding PSA is a member of the kallikrein family and its expression is regulated by the presence of androgens. Prostate specific antigen is a glycoprotein with a molecular weight of 29 kDa and is commonly used as a marker for human prostate epithelial and prostate carcinoma cells growth (Papsidero et al., 1981; M. C. Wang et al., 1981).

Practical Approaches for PSA. PSA functions as an essential component for seminal fluid. Biopsies are recommended if abnormal PSA levels are found. United States Preventive Services Task Force considers PSA as gold standard biomarker but does not recommend PSA screening as a sole indicator of prostate cancer since elevated expression levels may not correlate with PCa risk (Ablin, 2012; Catalona, 2012). Moreover, results from a European Randomized Study of Screening for Prostate Cancer imply that there is around a 75% negative biopsy rate using PSA as a diagnostic marker (A. Gupta et al., 2010; Studer & Collette, 2010). These results confirm previous studies that reported a 30–50% false-negative biopsy rate in patients

with subsequently confirmed malignancy due to small and inconspicuous lesions (Rabbani, Stroumbakis, Kava, Cookson, & Fair, 1998).

In a Physician's Health Study, mRNA signatures were developed from patients with a Gleason score of 7, the most common grade. Conclusions from this study implicated the use of this signature to further estimate the risk of lethal prostate cancer and guiding therapy decisions to improve outcomes and reduce overtreatment (Penney, Sinnott, et al., 2011). Mixed findings from a clinical study conducted by Muller et al, suggests that variation in testosterone levels at the time of baseline readings affects PCa predictability. In this study, higher testosterone was associated with higher PCa detection but unrelated to PCa risk (Muller et al., 2012).

PSA remains present in prostate cancer cells after they become malignant. However, the role of PSA as sensitivity to androgenic growth hormones decreases and growth becomes independent of hormones remains unclear. Immunohistochemical analysis of PSA reveals weak staining due to the disruption of their normal functioning (Papsidero et al., 1981; M. C. Wang et al., 1981). Thus, individual prostate cancer cells may produce less PSA than healthy cells. But the raised serum levels in individuals with prostate cancer may be due to increased cell numbers not necessarily individual activity. However, in most cases of prostate cancer, the cells remain positive for the antigen, which can therefore be used to identify metastasis (*Manual of Diagnostic Cytology*, 2003). Since some high-grade prostate cancers may be entirely negative for PSA, however, histological analysis to identify such cases usually uses PSA in combination with other antibodies, such as PSAP and CD57 (*Manual of Diagnostic Cytology*, 2003).

NKX3.1 is a member of a large family of homeodomain transcription factors and helps regulate the production of secretory proteins (Bhatia-Gaur et al., 1999; He et al., 1997; Prescott, Blok, & Tindall, 1998). NKX3.1 can be found in high levels in the prostate and various organs including salivary glands, the kidney, and portions of the central nervous system (Sciavolino et al., 1997; M. Tanaka et al., 2000). NKX3.1 may be expressed in an androgen-dependent manner (Erbaykent-Tepedelen, Karamil, Gonen-Korkmaz, & Korkmaz, 2014; He et al., 1997; Prescott et al., 1998). Although NKX3.1 may not be essential, the loss of function of NKX3.1 might be associated with the development of prostate cancer. NKX3.1 is localized to chromosome 8p21 and its protein has a molecular weight of 26 kDa in humans (Voeller et al., 1997). Loss of heterogeneity (LOH) at this locus occurs in prostate cancer and is associated with more aggressive or invasive tumors, suggesting the presence of a tumor suppressor gene in the 8p21-8p22 interval (Bova et al., 1993; Kagan et al., 1995; MacGrogan et al., 1994; Suzuki et al., 1995). Thus, it is unclear whether this protein is a bona fide tumor suppressor gene in human prostate cancer, however, has been shown to elicit a DNA damage response (Erbaykent-Tepedelen et al., 2014; Voeller et al., 1997). However, haploinsufficiency occurs when a diploid organism may only have one functional copy of an allele due to the second copy being inactivated (possibly by a mutation). Findings from experimental mice studies have shown that a phenotypic consequence of loss of one allele of NKX3.1 may significantly increase the likelihood of haploinsufficiency of this gene in human tumors, which results in an inclination towards tumor formation or progression (Bhatia-Gaur et al., 1999; Thomas et al., 2013).

Progression of Prostate Cancer

Androgen dependent prostate cancer cells tend to be nonaggressive and remain within original tumor site. Androgen depletion from these cells will mostly lead to regression of growth leading to apoptosis. However, cancerous cells may evade cell death by going into a resting state and survive without cycling into the G0 phase (Bostwick et al., 2004). The tumor cells accumulate more mutations and lead to a more aggressive state, thus, increasing the likelihood of invasion and metastasis into other regions. This may be another reason how cells metastasize into bone marrow or other sites following clinical treatment (Hoimes & Kelly, 2010). However, the biological characteristics of androgen-dependent prostate cancer cells, especially in regards to their ability to adapt following clinical depletion of androgen, are not fully understood making it difficult to elucidate a safe, effective cure for prostate cancer. Malignancies of prostate cancer is fairly common and affects nearly 1 in 11 males (Landis et al, 1998 and Pisani et all, 1999). Prostate cancer preferentially metastasizes in the bone and has been report in approximately 75% of patients with advanced stage prostate cancer (Coleman, 1997). In general, these metastases are sclerotic, rigid and highly resistant to treatment (Bostwick et al., 2004).

Although difficult to determine how prostate cancer cells depend on androgen-mediated signaling for survival. In order to shift from androgen-dependent to androgen-independent growth, cells must adapt their survival mechanisms, leading to more resistant and aggressive state (Fowler, Lau, Ghosh, Mills, & Mounzer, 1988; Jarrard, Bussemakers, Bova, & Isaacs, 1995; Mansson, Adams, Kan, & McKeehan,

1989; Matuo et al., 1987; Matuo et al., 1988; Sitaras, Sariban, Bravo, Pantazis, & Antoniades, 1988).

Several Aspects that Differentiate Cancer from Normal Cell Development

There are several aspects that contribute to the transition of normal cells into cancerous cells and further enable the prostate cancer cells to transition from benign through malignant, androgen-independent conditions. The hallmarks of cancer include: self sufficiency in growth signals; insensitivity to anti growth signals; evading apoptosis; limitless reproductive potential; sustained angiogenesis; tissue invasion; and metastasis.

Loss of Density-dependent Cell Proliferation and Deregulation of Apoptosis

Normal cells are able to maintain a balance between proliferation and apoptosis signals. If mutations are allowed to evade checkpoints and repair mechanisms, the balance shifts favoring proliferation in preneoplastic or neoplastic growth states. In tumors, proliferative signaling pathways dominate paired with impaired apoptotic signaling, which differentiate them from their normal counterparts. KI67 and BCL2 were ideal biomarkers for proliferation and apoptosis, respectively.

Apoptosis is an active, inherent, programmed cell death that may be initiated by a multitude of environmental stimuli. The idea of apoptosis was introduced by J. Kerr, A. Wyllie, and A. Currie in 1972 (Kerr, Wyllie, & Currie, 1972). At that time,

apoptosis was noted to play both a complementary and opposing role to mitosis in the regulation of animal cell population. During normal functioning, cellular reproduction is homeostatic to cellular death. While in cases of toxicity, apoptosis may be considered the endpoint of toxic injury, which are comprised in various forms that are specific to causes and functions.

Apoptosis differs from necrosis, which involves violent environmental perturbation leading to the rapid incapacitation of major cell functions such as gene expression, ATP synthesis, and membrane potential.

Biomarkers: KI67 and BCL2

Ki67 is a nuclear protein encoded by marker of proliferation (MKI67). It is considered an indicator for cell proliferation and assesses for the growth fraction of a cell population. Ki67 expression is uniquely robust in its presence in all cell cycle phases except for G_0. Grading of prostate cancer does not consider the proliferation rate of cells so Ki67 can improve predictions of prostate cancer outcome based on standard factors alone in men treated conservatively or radically (G. Fisher et al., 2013; Jhavar et al., 2009). Moreover, KI67 protein expression directly reflects a certain physiological state of the cell, despite being little consensus in the choice of Ki67 cutoff points (G. Fisher et al., 2013).

B-cell lymphoma 2 (BCL2), is a part of a family of cytoplasmic proteins that regulate apoptosis through the induction or inhibition of apoptotic factors. The BCL-2 family members can form homo- and heterodimers through the BH3 domains. BCL2 forms heterodimers with its antagonistic counterpart, BAX leading to the inhibition of

apoptotic activity of BAX. Thus, the relative levels of pro-survival and pro-apoptosis BCL-2 family proteins determine cell survival or apoptosis, as in the case of cancer cells high expression BCL2 versus normal cells that highly express BAX or BAK. High expression of BCL2 has been implicated in advanced prostate cancer (Catz & Johnson, 2003). BAK and BAX often form homo- or heterodimers in the presence of apoptotic signals. This homodimer promotes the opening of a channel and promotes translocation of cytochrome c from mitochondria to the cytoplasm. If BAK or BAX dimers are neutralizes by the binding of BCL2, the channel cannot open and cytochrome c cannot translocate, which may inhibit the completion of apoptosis. Overexpression of survival proteins such as BCL2 has been implicated in prostate cancer progression. Elevated levels of BCL2 have frequently been associated with aggressive tumorigenesis and chemoresistance (Catz & Johnson, 2003; McDonnell et al., 1992). BCL2 and other oncogenes, through the products they encode, may be regulated by protein phosphorylation by protein kinases with tyrosine and threonine, as the substrate amino acids. Since BCL2 is overexpressed in prostate cancer cell lines, phytochemical treatment assists with maintaining BCL2/BAX balance by lowering BCL2 expression to allow apoptosis to occur in those lines.

Sustained Growth

Sustained growth is another hallmark characteristic of cancer progression. As prostate cancer becomes more resist, its ability to override and manipulate proliferation and survival pathways becomes more complex. Activations of these survival pathways may rely on cytokines and growth factors by inducing proliferation

and survival through signal transduction pathways. One such survival pathway is through PI3K/AKT.

IGF and EGF are potent mitogens known to influence proliferation and survival cascades.

Self-Regulation of Growth Factors

Impaired growth factor functionality and evasion of stop signals are hallmarks in uncontrolled tumor growth. Growth factors, such as epidermal growth factors (EGFs), vascular endothelial growth factors (VEGFs), keratinocyte growth factor (KGF), and platelet-derived growth factors (PDGF) are small molecules that have shown to be an integral part in the growth and development of cancer. Epidemiological studies have highlighted the importance of and provided evidence that has correlated increases in insulin-like growth factor (IGF-1) with an increased, associated risk for prostate cancer (Russell, Bennett, & Stricker, 1998).

There are two general types of growth factor signaling pathways. In the autocrine stimulatory pathway, cells are responsible for generating the growth factor and the ligand. On the other hand, the paracrine stimulatory pathway is one in which surrounding cells provide crucial growth factors to the cancer cell. Both pathways may to contribute to the progression of prostate cancer. For instance, in a study conducted by Scher et al, prostate cancer cells were shown to express the EGF receptor and its cognate ligand, transforming growth receptor factor alpha (Scher et al., 1995). On the other hand, the paracrine stimulatory pathway may promote the

progression of prostate cancer. Evidence of possible paracrine stimulation in prostate cancer progression comes from the observation both in the gland and at metastatic sites (Barrack, 1997).

The expression of IGF, EGF, and other peptide growth factors and their receptors may be regulated by androgen (Oosterhoff, Grootegoed, & Blok, 2005). The activation of specific growth factor pathways *in vitro* can enhance AR activity, even in the absence of androgens. These observations are critical in the connection of AR functionality to pathways involved in cancer progression. In a study conducted by Putz et al suggests that EGF, or IGF-1 stimulated cell growth and PSA secretion in DU145 cells that may be independent of androgen (Putz et al., 1999).

Androgen-dependent production of cell survival factors may contribute to repressed apoptosis in prostate cancer. Such factors include the production of secreted peptide survival factors like IGFs, platelet-derived growth factors (PDGF), and vascular endothelial growth factor (VEGF). For example, IGF-1 may bind and dimerize its transmembrane receptor (IGF-1R). Once dimerized, each monomer transphosphorylates the opposing monomer on specific tyrosine residues. This tyrosine transautophosphorylation functions to recruit intracellular signaling proteins to bind via their src homology 2 domains to specific phosphorylated tyrosines in the ligand-occupied dimeric receptor complex (Porter & Vaillancourt, 1998). This autophosphorylation initiates three major kinase-dependent signaling cascades. These include: (a) the ras/raf/Mek/srk cascade, (b) the phospholipase C(gamma)/ diacylglycerol/ inositol triphosphate (IP3)/ protein kinase C cascade, and (c) the phosphotidyl-inositol 3-kinase (PI3K)/protein kinase B kinase (PIBK)/ protein kinase

B (PKB aka AKT) (Kantoff, Carroll, & D'Amico, 2002). PI3K/Akt pathway is implicated in the regulation of prostate cell survival.

This PI3K/Akt signaling pathway begins with the binding of growth factors such as IGF-1 to the IGF-1 receptor (IGF-1R). Ligand binding results in receptor activation and recruitment of the PI3K complex to the plasma membrane. PI3K phosphorylates a specific type of lipid found in the plasma membrane, which generates a lipid product known as *phosphoinositide-3, 4,5-triphosphate* (PI-3,4,5-P3). PI-3,4,5-P3 is induced on growth stimulation by activating survival cascades (Kantoff et al., 2002). When present, PI-3,4,5-P3 recruits and activates a second kinase, known as Akt. There are three Akt kinases (Akt-1, -2, and -3), which are responsible for phosphorylating various downstream targets to sufficiently inhibit apoptosis and promote cell proliferation. Activation of this pathway has been associated in the regulation of cell adhesion and cell motility (Kantoff et al., 2002).

Numerous human studies have found increasing levels of plasma IGF-1 to be associated with the diagnosis of prostate cancer (Chen et al., 2009). In a population-based case control study conducted by Wolk et al, serum IGF-1 levels were positively associated with prostate cancer risk (Wolk et al., 1998). Furthermore, in the prospective Physicians' Healthy Study conducted by Penney et al, IGF-1 was also considered a strong risk factor for the development of prostate cancer (Penney, Sinnott, et al., 2011). Men in the highest quartile of plasma IGF-1 level were found to have a relatively higher risk of prostate cancer and elevated risk of cancer-related death when compared to men in the lower quartile. Conclusions from this study suggest that IGF-1 specifically contributes to the development of lethal tumors. While

IGF-1's roles in prostate cancer was proposed nearly two decades ago, more recent review of studies have shown the role of IGF-1 in androgen receptor regulation via PI3K/AKT pathway at low androgenic concentrations (Papatsoris, Karamouzis, & Papavassiliou, 2005; Pollak, Schernhammer, & Hankinson, 2004). Findings from these reviews suggest that IGF-1 expression is regulated by androgens through AREs in the IGF-1 promoter regions and the IGF-1 signaling pathway through the regulation of IGF-binding protein (IGFBP) expression.

Moreover, reduction of freely circulating IGF-1 in prostate cancer may also be associated with the reduced expression of its receptor. Wang et al demonstrated that treatment of I3C and DIM in LNCaP cells significantly reduced IGF-1 receptor expression as a means to inhibit IGF-promoted cell growth (T. T. Wang et al., 2012). Findings from this study warrant further exploration into the effects of I3C and PEITC on IGF and EGF growth modulation in *in vivo* systems

Biomarker: IGFBP3

Insulin-like binding protein 3 (IGFBP3) is a member of the IGFBP family. This protein has a high affinity and specificity to IGF-1, and is responsible for extending its serum half-life, circulating bioavailability, and modulating IGFs biological actions on target cells. Additionally, IGFBP3 may contribute to IGF-independent functions within the cells by inducing apoptosis or inhibiting cell growth.

IGFBP3 plays a critical role in the progression of prostate cancer (Gu et al., 2010). Proper regulation of IGFBP3 is accounts for a constant concentration of IGF-1. However, IGFBP3 may be cleaved and subsequently degraded by PSA, which

reduces its affinity to bind IGF-1 and increases amount of circulating IGF-1 (P. Cohen, Peehl, Graves, & Rosenfeld, 1994; Gu et al., 2010; Penney, Schumacher, et al., 2011). Therefore, dysregulation of hormones such as androgens play a significant role in modulating changes in cell growth during the early stages of prostate cancer.

Significantly, IGF-1 and IGFBP3 are regulated by dietary modification. I3C and PEITC have been shown in increase the expression of IGFBP3 gene, which may reduce bioavailability of circulating growth factors (R. W. Li et al., 2013). As one of the most abundant circulating carrier proteins, accounting for approximately 80% of all IGF-1 binding in human systems, IGFBP3 is considered an ideal prognostic biomarker and important antitumor agent targeting the down regulation of prostate cancer.

Cell Cycle Regulation

The cell cycle is regulated by networks of interacting proteins that receive signals from extra- or intracellular sources and integrate these responses into stimulatory and inhibitory pathways that decide the cell's fate. The cell cycle consist of 4 phases: G_0, G_1, S, G_2 and mitosis. Normal cells reach density-dependent inhibition and become quiescent, arrested in G_0 phase. G_1 is the first gap phase and is affected by growth factors, inhibiting factors, and nutrient supply. In this phase, the cell may move to the S phase, arrest in G_1 phase, or move into G_0 phase. Moving into the S phase, DNA synthesis, is a critical point for the cells. In this phase, DNA damage is detected and fixed via repair systems. Epithelial cells may override G_0 phase and continue to divide. However, continuous dividing may increase the chance

for error, which could contribute to cancer recurrence. Cancer cells overriding G_1/M and G_2/M checkpoints. CDKN1α and CDKN1β play an important role in the decision-making machinery in R-point process and have been chosen as cell cycle biomarkers.

Biomarkers: CDKN1α and CDKN1β

Cyclin-dependent kinase (CDK) such as CDK1 plays an important role in the regulation of procession into S phase. Cyclins complex with CDKs to activate CDKs' catalytic activity, which is a process mediated by extracellular signals and other mitogens. Cyclin D kinase inhibitors are another molecular target to prevent cell cycle progression and treat cancer. In prostate cancer, CDKN1α and -β have been shown to play a prominent role in cell cycle regulation by binding and inhibiting cyclins. This two inhibitors act as tumor suppressor genes to support cell growth arrest and progression at G1 and S phases.

In prostate cancer, Cyclin-D kinase Inhibitor 1-α (CDKN1α) is also known as P21, without inducing p53 and cyclin-D kinase Inhibitor 1- β (CDKN1β), also known as P27, are found at reduced levels. In a study conducted by Wang et al, I3C and its metabolite, DIM significantly induced cyclin inhibitors at concentrations ≥ 25 μmol/L media (T. T. Wang et al., 2012).

Decreased Regulation by Cell-Cell and Cell-Matrix Interactions

E-caderin is a principle adhesion molecule in epithelial cells. The loss of E-caderin contributes to the malignancy and invasion of tumor cells. The epithelial-

mesenchymal transition (EMT) has had a crucial role in the regulation of tissue fibrosis and cancer pathogenicity. Under cancerous conditions, prostate epithelial cells may acquire mutations enabling them to loss differentiated adhesion properties in order to migrate and invade through the activation of EMT-related biological pathways. Moreover, inducible changes observed in EMT may be perturbed by the presence of androgens (E. K. Kim et al., 2013).

Insensitivity to Contact and Migration

Integrins, as cell adhesion receptors, may play a critical role in cell migration- a critical step in cancer progression. Epithelial cells that normally would not migrate may gain the ability to metastasize to other regions. This is also a critical step in differentiating benign from malignant cancer. The mechanism involved in cell motility is complicated and involves complex interactions between the cell and the extracellular matrix (ECM).

The interaction of integrins with the extracellular matrix is complicated. Integrins utilize an outside-in or inside-out signaling approach to regulate ligand binding and adhesion (Takada et al., 2007).The ligands for integrins are numerous and can be extracellular matrix ligands, cell-surface ligands, and soluble ligands. The ability of integrins to move relies on their ability to form transmembrane protein complexes on the surface of the cell. This is accomplished by forming alpha-beta heterodimers. Integrins have unique specificities, which allow them to be categorized into laminin-, collagen-, fibronectin-, leukocyte- binding integrins, with some overlap in each category. Other important ligands include intercellular adhesion molecules

(ICAMs), which are present on inflamed endothelial layers. These molecules promote ligand binding, which help integrins communicate.

Biomarkers: Integrin and FN1

Integrins are dysregulated in prostate cancer. $ITGA_{2, -5, -6}$ and $ITGB_1$ are aberrantly upregulated in human PCa and TRAMP (Goel et al., 2005; Goel et al., 2008). $-B_1$ associates with many alpha subunits and may predominantly associate with $-A_2$, $-A_5$, and $-A_6$ subunits to form heterodimeric complexes (Alam et al., 2007; Bonkhoff et al., 1993; Fornaro et al., 2001). $-B_1$ is also very important in the regulation of cell growth and previous studies have shown that $-B_1$ assists cell in anchorage-independent growth.

Fibronectin 1 (FN1) is an extracellular matrix glycoprotein, with a molecular weight of 220 kDa. This protein binds to integrins and may be responsible for the promotion of cell adhesion, growth, migration, and differentiation. The up-regulation of $-B_1$ and fibronectin ligand may promote prostate cancer cell progression (Goel et al., 2008).

Integrins can bind to ECM glycoproteins including collagens, fibronectins, laminins, and cellular receptors (VCAM-1 and ICAM) (Takada et al., 2007). $ITGA_2$ contains collagen and laminin receptors (Dickeson, Walsh, & Santoro, 1998). Integrin heterodimer, A_2B_1, may primarily contain collagen receptors (Ruggiero, Comte, Cabañas, & Garrone, 1996; M. A. Schwartz et al., 1995). Heterodimer, A_5B_1 is a major fibronectin receptors (van der Flier & Sonnenberg, 2001) and heterodimer, A_6B_1, may be a major laminin receptor (Tashiro et al., 1999).

The use of dietary phytochemicals such as I3C and PEITC to regulate cell adhesion cell migration has been explored. Treatment with PEITC disrupts cell adhesion properties under *in vitro* and *in vivo* conditions. In a study conducted by Hudson et al, treatment with doses up to 5 μmol/L significantly inhibited cell plating efficiency and down-regulated integrin expression in androgen-sensitive LNCaP cell line (Hudson et al., 2012). Moreover, Li et al demonstrated that dietary administered treatment with 3 μmol PEITC/g diet to significantly alters integrin expression in a LNCaP xenograft study (R. W. Li et al., 2013)

Properties that Affect Interactions with Other Tissue Components

A cancer cell's ability to micrometastasize depends on their ability to modulate and secrete angiogenic factors that may interact with the vascular endothelium and breakthrough the resistance of the endothelial-barrier. Controlling for factors that decrease the probability of micrometastasis has potentially high applications in nutrition-related cancer therapy and metastasis prevention. Due to the complexity of molecular mechanisms leading to micrometastasis, developing chemopreventive strategies to target prostate cancer utilizing dietary agents ideal but requires additional research.

Biomarkers: VEG-F and PECAM 1

Vascular endothelial growth factor (VEG-F) is expressed by a variety of tumor cells and infiltrating macrophages (Dvorak, 2002). Prostate cancer cells have been shown to make autocrine growth factors such as VEGF as well as its receptor. Therefore, VEGF has an integral role in the regulation of angiogenesis, and its gene

expression has been correlated with prostate cancer disease staging and prognosis (Ferrara, 2000). VEGF expressed by tumor cells or macrophages may directly stimulate endothelial cell proliferation and migration (Morgan, Forssmann, & Nakada, 2004).

Platelet/ endothelial cell adhesion molecule (PECAM-1) is also known as CD-31. Moreover, this gene is located on the surface of platelets, monocytes, neutrophils, and other T-cells as well as comprising a large proportion of the endothelial cell intercellular junctions. The most notable functions of PECAM-1 revolve around its role in regulating prostate cancer angiogenesis and integrin activation. In a clinical study conducted by Ding et al, PECAM-1 and VEGF were positively correlated with neoangiogenesis and tumor metastases in individuals with prostate cancer. Of the 34 cases, 44.12% were positive for VEGF mRNA expression, which as also inversely proportionate to the cancer stages I- IV (Ding, Li, Xu, Sun, & Tao, 2005). Moreover, in another clinical study conducted by Huss et al, VEGF and PECAM-1 were analyzed in TRAMP mouse model, with undergoes a distinct angiogenic switch that is consistent with the recruitment of new vasculature to lesions representative of PIN. Analysis of clinical samples within this study also validated the predictions of the TRAMP model, which may explain how anti-angiogenic dietary therapy may change the function of tumor progression (Huss, Hanrahan, Barrios, Simons, & Greenberg, 2001).

PEITC influences the expression rate of angiogenic factors in the early stages of prostate cancer. Low-dose exposure to PEITC (3 μmol/g diet) in mice xenografts

has significant attenuated the angiogenic potential in early stage, androgen-responsive human prostate cancer (Hudson et al., 2012).

Chronic Inflammation

Inflammatory cells, particularly macrophages, are heavily involved in the management of the tumor microenvironment, where they have been previously shown to influence the tumor metastasis and cell growth. However, the mechanisms involved in their cellular events are not fully understood (Morgan et al., 2004).

In normal inflammation, macrophages are an integral part in the M2 remodeling phase to help resolve a wound. However, different from normal environments where macrophages would disperse following wound repair, in a tumor microenvironment, they linger, remain in the M2 phase, and contribute to a state of chronic inflammation. In the M2 phase, they react with tumor cells, fibroblasts, and epithelial cells to promote angiogenesis and fibrosis.

Cytokine secretion from macrophages has a profound influence on tumor growth. Cytokines are soluble glycoproteins and low-molecular weight particles with multiple functions. However, they are mainly responsible for mediating intracellular interactions to regulate cell and tissue functions (Dunlop & Campbell, 2000). Cytokines contribute to the growth and spread of cancers by causing normal cells to produce additional cytokines that continue the malignant process (Dunlop & Campbell, 2000; Negus & Balkwill, 1996).

Cancer preventive techniques seeking to destroy the crosstalk interference and potentially interrupt cancer progression and metastasis are highly ideal. The use of

dietary agents or components naturally found in the diet are ideal due to the ease of acquirement, low side effects, and are key preventive measures utilized by health professionals.

Biomarkers of Inflammation

Secretion of IL-1β, an autocrine-produced cytokine, promotes cancer cell growth and confers with aggressive resistance against traditional therapeutic measures in LNCaP, human prostate cancer cells. High levels of IL-1β may also be responsible for the induction of angiogenic factors such as VEGF from the tumor and stromal cells that promote tumor growth through increased vascularization of human prostate cancer cells. Moreover, increased presence of IL-1 within the tumor microenvironment significantly enhances the expression of adhesion molecules in endothelial and malignant cells. This, in turn, facilitates malignant cell invasion, driving them into the circulatory system and promoting dissemination into distant tissues (Chirivi et al., 1996).

Interleukin 1 receptor antagonist (IL1RN) is a member of the interleukin 1 cytokine family that mediates the activity of IL-1 and IL-1-related immune and inflammatory responses. IL-1 receptor antagonist is encoded by the IL1RN gene in humans. Essentially, the IL-1 receptor binds IL-1(Apte & Voronov, 2008). However, IL1RA can also non-productively bind to the IL-1R, attenuating the effects of IL-1. Therefore, changes in IL1RN gene expression may be correlated with the inhibition of IL-1β. In prostate cancer, mutations have led to underexpressed levels of IL1RN so

regulation of IL1RN is ideal target for tumor growth reduction (Morgan et al., 2004; Rodríguez-Berriguete et al., 2013).

IL-6 is a multifunctional glycoprotein that is secreted by various cells(Guo, Xu, Lu, Duan, & Zhang, 2012). However, under normal conditions, its secretion is tightly controlled and its expression is relatively low. The role of IL-6 in prostate cancer is unresolved. Elevated levels of IL-6 have been linked to disease progression and morbidity in clinical studies (Michalaki, Syrigos, Charles, & Waxman, 2004; Wei et al., 2003). However, *in vitro* experiments have shown that IL-6 may stimulate or inhibit cancer growth. There are reports indicating that IL-6 may or may not be secreted in LNCaP cell line (T. D. Chung, Yu, Spiotto, Bartkowski, & Simons, 1999; P. C. Smith, Hobisch, Lin, Culig, & Keller, 2001). In general, studies indicate that elevated expressions levels play a critical role in prostate cancer development but understanding the role of IL-6 secreted by the prostate cancer cells and the role of exogenous IL-6 within the tumor microenvironment remains elusive. The pro-angiogenic effects of IL-6 expressed by macrophages have been attributed to the stimulation of VEGF expression(Tartour et al., 1994; Wei et al., 2003).

C-C Chemokine Ligand 2 (CCL2) is also known as monocyte chemoattractant protein (MCP-1), and has a significant role in perturbing prostate cancer progression. CCL2 presence within tumor cells initiates the activation of monocytes to macrophages by luring them to the surrounding inflamed microenvironment. Active binding of CCL2 to CCR2, a cognate ligand receptor, has been shown to stimulate prostate cancer development (Allavena, Sica, Solinas, Porta, & Mantovani, 2008). A recent study conducted by Kim et al, has demonstrated CCL2's ability to alter

behavior of androgen-dependent cell line. Results from the study also demonstrated I3C's ability to significantly decrease monocyte infiltration in LNCaP cell model. These results are further confirmed by other studies denoting the presence of CCL2 in more advanced prostate cancer cells lines such as C4-2B, PC-3, and VCaP (E. K. Kim et al., 2013; T. H. Lin et al., 2013; Roca, Varsos, & Pienta, 2009).

As another multifunctional cytokine, tumor necrosis factor-α (TNFα) plays a crucial role in the progression of cancer growth and metastasis. In a clinical study conducted by Michalaki et al, elevated TNFα serum levels with increased disease status. Serum levels for TNFα were higher in prostate cancer patients with metastatic disease and lower in those with localized disease (Michalaki et al., 2004).

Macrophages (MΦ) within Tumors

Macrophages are a diverse, heterogenous population that responds to environmental stimuli in order to maintain tissue homeostasis and remodeling under normal conditions. As inflammatory regulators, their protective feature can also promote disease progression by overstimulating inflammation and angiogenesis (Allavena et al., 2008; Condeelis & Pollard, 2006). Macrophages may infiltrate tumor tissues and promote all aspects of tumor initiation, growth, and development. Moreover, these inflammatory infiltrates are considered the dominant leukocyte population found within the microenvironment surrounding neoplastic tissue. Macrophages may differentiate from monocytes that are recruited into the tumor by chemokines and cytokines derived by tumors such as vascular-endothelial growth factor (VEGF). Disruption of cell homeostatic conditions such as by poorly

vascularized, hypoxic, and necrotic areas, promotes macrophage accumulation and further perturbs the tumor microenvironment (B. Wang et al., 2011). Moreover, after being activated, macrophages within tumors produce a variety of cytokines, growth factors, reactive oxygen species, proteases that modulate angiogenesis (Morgan et al., 2004). While macrophage infiltration at high levels may be associated with poor cancer prognosis, little is known about the exact role of the distinct pro- and antitumoral macrophages populations in cancer development (B. Wang et al., 2011). Therefore, defining and differentiating these subsets remains a challenging work in progress. Future studies in prostate cancer development that address the targeting macrophage plasticity necessary to mold an immunosuppressive population, the phenotypic heterogeneity of macrophages within tumors and their link in human prostate cancer will be advantageous.

Macrophage Biomarker

EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1) and its mouse homologue, F4/80, are members of the G Protein-Coupled Receptors (GPCR) superfamily, subfamily EGF-TM7. The molecular weight of F4/80 glycoprotein is approximately 160 kDa (Starkey, Turley, & Gordon, 1987). F4/80 is derived from mature B cell macrophage subpopulations such as splenic red pulp macrophages and microglia (Starkey et al., 1987). It is a transmembrane protein that is expressed on the cell surface of human macrophages (Gordon, Hamann, Lin, & Stacey, 2011). It is commonly used as a mature macrophage infiltration marker in mouse and human models. Moreover, EMR1 is regulated by EGF and an important

set of nuclear transcription factors (H. H. Lin, Stacey, Stein-Streilein, & Gordon, 2010).

Xenobiotic Metabolism

Xenobiotics are considered foreign substances within a mammalian system. These particles include dietary agents, carcinogens, and other substances. Since organisms are constantly exposed to xenobiotics, adaptive mechanisms have evolved to assist with resolving those challenges. In higher eukaryotes this mechanism includes an increase in its metabolism and clearance in response to the detection of an insulting biological compound. Clearance of these materials has been linked to xenobiotic metabolizing enzymes. These enzymes are generally classified into three groups, which include Phase 1, Phase 2, and Phase 3. Cytochrome P450s are diverse set of genes that composes approximately 70-80% of Phase 1 enzymes and are considered the first line of metabolic defense. These enzymes are involved in the transformation of lipophilic compounds into water-soluble compounds as well as the activation of procarcinogens to the active, electrophilic, carcinogens that disrupt functioning by binding to the DNA of normal cells (W. E. Evans & Relling, 1999). Tightly regulated induction of Phase 1 and 2 enzymes in response to the level of xenobiotic exposures has been considered a key characteristic of these enzymes. Moreover, characteristic has been associated with the increased clearance of carcinogens and overall health improvement. Thus, P450 enzymes have a key role in the regulation of carcinogenesis.

Biomarkers for Phase 1 and 2 Xenobiotic Metabolism

Functions of Phase 1 enzymes generally include catalyzing reactions to increase substrate hydrophilicity. This is done by introducing a functional group to modify the compound (G. Smith, Stubbins, Harries, & Wolf, 1998). In certain cases, xenobiotics including carcinogens may be activated during phase 1. However, these compounds may be further metabolized into less reactive products by Phase 2 redox-related proteins including NQO1 and UGT1A1 (G. Smith et al., 1998).

Cytochrome P450, family 1, member A1 (CYP1A1) is expressed predominately extrahepatically. One characteristic feature of CYP1A1 is that it has an aryl hydrocarbon receptor, which gives it the ability to bind to androgen receptors and nuclear factor- κβ. This binding capacity has an important role in carcinogenesis (Bassères & Baldwin, 2006; Currier et al., 2005). Cytochrome P450, family 1, member B1 (CYP1B1) is another well-studied xenobiotic metabolizing enzyme. However, some data suggests that certain CYP1B1 genotypes may have increased expression in patients with prostate cancer, in which single nucleotide point mutations were identified in cases of sporadic and hereditary prostate cancer cases. Differences in allele frequency as a result of these SNPs were marginally significant in the sporadic PCa cases than the control group (unaffected men) (Chang et al., 2003).Cytochrome P450, family 2, member C9 (CYP2C9). Cytochrome P450, family 3, member A4 (CYP3A4) may be considered one of the most important enzymes for drug metabolism as it accounts for approximately 50% of some currently utilized drugs and may be involved in the metabolism of a large number of carcinogens

(Singh & Michael, 2009). Cytochrome P450, family 2, member D6 (CYP2D6) has been heavily studied for its role in metabolism anticancer drugs (Singh & Michael, 2009). Taken together, these CYP enzymes and respective families are responsible for over 75% of metabolic actions with the human (Guengerich, 2008).

Use of natural dietary compounds such as I3C and PEITC to regulate xenobiotics is a widely studied subject. Both dietary administered I3C and PEITC upregulate phase 1 and 2 enzymes to facilitate the modification and excretion of carcinogens and has been implicated in the promotion of tumor regression (T. T. Wang et al., 2012). Moreover, PEITC has also been shown to inhibit CYP450 activity involved in carcinogen (DBMA and MKK)-induced cancers (Morse et al., 1990).

UDP glucoronosyltransferase 1 family, polypeptide A1 (UGT1A1) is an enzyme responsible for facilitating glucoronidation. Glucoronidation is a chemical process in which a glucoronic acid is conjugated to another substance, such as a modified carcinogen to aid with its excretion from the system. Moreover, glucoronidation is a main metabolic pathway for estrogen detoxification in the prostate and other steroid target tissues. Epidemiological and clinical studies assessed for associations between prostate cancer risk and UGT1A1 expression (Dalhoff, Buus Jensen, & Enghusen Poulsen, 2005; Karatzas et al., 2010; Tang et al., 2011).

NAD(P)H dehydrogenase, quinone 1 (NQO1) is a FAD-binding protein that is responsible for reducing quinones to hydroquinones. The gene encoding for NQO1 is highly inducible. The enzymatic activity of this protein inhibits the electron reduction of quinones and subsequently, controls the formation of radical species (National Center for Biotechnology Information, 2014). Mutations of NQO1 and others Phase 1

and 2 xenobiotic metabolism genes have been associated with increased susceptibility of cancer (Cunningham et al., 2007; Zhou et al., 2009). NQO1's role in the protection against reactive species contributes to cell stability by protecting against proteosomal degradation within the cell membrane (Dinkova-Kostova & Talalay, 2010).

Treatment with I3C and PEITC has been shown to significantly increase hepatic NQO1 gene expression in mammals (Abdull Razis & Noor, 2013; Barve et al., 2008; Razis et al., 2012). Increased production of NQO1 has been shown to have protective antioxidant functions against toxic and neoplastic effects of carcinogens and against oxidative species (Dinkova-Kostova & Talalay, 2010).

One overlapping theme with these Phase 1 and 2 xenobiotic metabolizing enzymes is the influence of genetic variation and correlation of the gene variants with prostate cancer (Chang et al., 2003; Zeigler-Johnson et al., 2004).

Overall, understanding the impact of dietary phytochemicals such as I3C and PEITC on the activity levels of various xenobiotic enzymes plays an important role in determining the efficacy of treatment in all stages of cancer. From a cancer prevention perspective, modulating rates of xenobiotic enzymes by modifying intake of glucosinolate-derived compounds may help provide a barrier of protection against carcinogen exposure.

Role of Aryl Hydrocarbon in I3C Metabolism

The ligand-activated aryl hydrocarbon receptor (AhR) is a transcription factor within the basic helix-loop-helix-PER/ARNT/SIM homology (bHLH-PAS) family(Karchner et al., 1999). AhR receptor is known to regulate the induction of xenobiotic metabolizing enzymes such as CYP1A1, -1B1, UGT1A1, and NQO1

through a classical pathway (Denison & Nagy, 2003; Denison, Soshilov, He, DeGroot, & Zhao, 2011). Moreover, AhR may play a role in the interference of nuclear receptor-mediated proliferation. In a review published by Okey, polymorphisms in nuclear receptors can dramatically modify particular xenobiotic actions on gene expression and subsequent toxicity in laboratory animals and possibly humans (A.B. Okey, 2005). Polymorphisms in the AR have been characterized for their effects on physiologic function and for possible roles in resistance to hormonal cancer therapy (A. B. Okey et al., 2005). Classical AhR ligands and CYP1A1 inducers are halogenated and polycyclic aromatic hydrocarbons such as 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BAP), and β-naphthoflavone. Nonclassical, naturally occurring AhR inducers include dietary compounds including I3C and its metabolite, DIM, flavonoids, and curcumin, that may act as exogenous ligands. In addition to its actions on androgen receptor, I3C has shown to accelerate the metabolism of xenobiotics by inducing P450 enzymes and AR degradation in a nonclassical pathway (Denison & Nagy, 2003; Ramadoss, Marcus, & Perdew, 2005).

Cell and Animal Model Used to Assess Prostate Cancer

Some of the major challenges in prostate cancer research has evolved around 1) the development of reliable screens that distinguish cancers that will remain indolent versus those that will become aggressive; and 2) to elucidate mechanisms that contribute to the prevention or control of disease progression. Laboratory and mechanistic studies are often employed as a better means to control confounding

factors. With these models, precise manipulation of experimental conditions enables researchers to have better environmental control within the studies.

Rodents are one of the most commonly used animals for laboratory research due to their relatively short life span, cost, and efficiency. Researchers are able to gather data in a comparatively fast manner in cancer studies through dynamic whole body system approaches that are suitable for a wide variety of tests. Moreover, *in vivo* studies, usually carried out in mice or rats, may depict how nutrients and other compounds affect various steps in the procession of cancer. Given limitations such as the reciprocal interpretations of rodent to human systems, the genetic similarity (~90%) still makes the use of rodents as ideal preclinical, laboratory models (Wiseman, 2008).

Cell Models

Prostate cancer cell models have been in use for decades. Classical models include LNCaP, DU145, and PC3. Early animal models of prostate cancer have suggested that prostate cancer, early in its development, is heterogenous and composed of both androgen-independent and androgen-dependent cells (Isaacs & Kyprianou, 1987; Kokontis, Hay, & Liao, 1998). Thus, there is a need for a wide variety of prostate cancer cell models in order to understanding the dynamic survival susceptibility of this disease. Suitable prostate cancer cell model with well-characterized phenotypes. LNCaP, PC-3, and DU145 are among some of the most widely used human cell lines in basic and preclinical research.

LNCaP cells are adherent, epithelial, human prostate cells were originally isolated from a lymph node metastasis. This model is not considered aggressive in *in*

vitro assays and tumorigenic in immunologically deficient animals (J. L. Fisher et al., 2002). Key characteristics of this cell line are its responsiveness to DHT and ability to stop cycling following the removal of androgen (Langeler, van Uffelen, Blankenstein, van Steenbrugge, & Mulder, 1993; Lim et al., 1993). Moreover, LNCaP contains a mutated androgen receptor, causing a point mutation (A-G) in codon 868 of a 910 codon (or its equivalent position 877 of a 919 codon) AR cDNA, which transitions threonine into alanine (Newmark et al., 1992; Veldscholte et al., 1992). As a result of this change, ligand specificity is decreased, enabling the AR to respond to steroid hormones such as estrogen and progesterone in addition to androgen (Olea, Sakabe, Soto, & Sonnenschein, 1990). This cell is positive for androgen and estrogen receptors and treatment of this cell line with relatively low levels of estrogen and progesterone results in enhanced growth, even in the absence of androgen (Newmark et al., 1992; Olea et al., 1990). With the recognition of an AR mutation in the prostate cancer cell line, LNCaP, it became important to search for mutations in the AR that might enhance or alter its function.

The C4-2 cells are derived from the LNCaP cells that were passaged in castrated mice. Since castration renders the mice, C4-2 cells are more invasive, less sensitive, and more characteristic of a human progressive prostate cancer with moderate tumorigenicity (Sobel & Sadar, 2005a, 2005b).

The underlying mechanism for adaption to androgen-independent state is not known. However, cells that survive androgen depletion by any means are theoretically available to repopulate metastatically, or even in the prostate at later stages in the illness. To better understand the mechanisms behind androgen-

independence, androgen independent cell lines such as DU145 and more advanced, PC-3 cell lines are used. The androgen-independent PC-3 cells were isolated from a bone metastasis in a patient with castration-resistant prostate cancer (CRPC) and consequently display a high tumorigenic potential. DU145 is a hypotriploid human prostate carcinoma cell line with an absent N13, abnormal Y chromosome, and X chromosome that may be present in a single copy. Significant features of this epithelial cell line is that it forms grade II adenocarcinomas consistent with primary prostatic tumors in nude mice (Stone, Mickey, Wunderli, Mickey, & Paulson, 1978). Moreover, DU145 cells lines are reported to be hormone insensitive and do not express prostate antigen (Papsidero et al., 1981). It is an adherent cell with observed microvilli, tonofilaments as revealed in ultrastructural analyses and moderate metastastic capabilities (Stone et al., 1978). Interestingly, while DU145 and PC-3 growth may be androgen-independent, both cell lines express the androgen receptor (Alimirah, Chen, Basrawala, Xin, & Choubey, 2006).

Animal Models

Genetic backgrounds of mice have been extensively studied. Its genome offers a high degree of homology with human counterparts, with greater than 99% of mouse genes being homologous to the human genome (Waterston et al., 2002).

Xenograft models are mainstays for immontherapy research. In xenograft models, the inoculated tumor cells and the target organs are not from the same species, which may preclude the development or onset of carcinogenesis. However, these models allow for assessment of human cell lines at specific cancer stages, which could not be ethically observed in human subjects (Hensley & Kyprianou, 2012). A

variety of human prostate cancer cell lines may be used in the establishment of xenografts within immune deficient or SCID mice. Common mouse models used in the assessment of prostate cancer include BALB/c nu/nu, C5BL/6, NOD SCID, and TRAMP mice (Irshad & Abate-Shen, 2013; Shirai et al., 2000).

The BALB/c nude mouse was originally developed in Japan by cross-breeding BALC/cABom-nu and BALB/cAnNCrj-nu. Notable characteristics in this mouse model include the absence of a thymus resulting in T-cell immunodeficiency, which makes this model an ideal hairless candidate for tumor biology and xenograft research. Average weight for these male mice species are approximately 15 g for 5 to 22 g for 7 week old mice at the time of purchase and 25 to 28 g at 15 weeks in age for mice purchased in the U.S. (Charles River).

The NOD SCID mice are another type of mouse model used in tumor biology and xenograft research. This white, albino mouse model have a SCID mutation. This mutation was transferred onto a non-obese diabetic background and have impaired T and B cell lymphocyte development (Ittmann et al., 2013). The mice model has also been reported to be deficient in natural killer (NK) cell function, which may explain why they have a slightly higher tumor implantation rate than Balb/c nude mice (Hensley & Kyprianou, 2012).

C57BL/6 is a black haired mouse model that is considered a general purpose laboratory mouse. This model has been used in tumor xenograft studies, however, it is more commonly used in diet-induced obesity, transgenic/knockout model development, safety and efficacy testing, and immunology studies.

Genetically engineered mouse models have taken front stage in terms of prostate cancer research. The transgenic adenocarcinoma of the mouse prostate (TRAMP) is a model generated on a C57BL/6 background and is used in the assessment of prostate cancer. The pathogenesis of prostate cancer in this model resembles the progression of human prostate cancer (Ittmann et al., 2013). However, tumors exhibit increased levels of nuclear TRP53 and decreased androgen receptor expression (Ittmann et al., 2013). In this model, the prostate cancer spontaneously develops as a consequence of SV40 T antigen expression (Hurwitz, Foster, Allison, Greenberg, & Kwon, 2001).

Expected timeline of prostate cancer is as follows:

- By 12 weeks of age- exhibit prostatic epithelial neoplasia (PIN)
- By 24 weeks of age- exhibit well differentiated adenocarcinomas; mostly located in the dorsal and lateral lobes of the prostate
- By 30 weeks of age- exhibit evidence of metastatic spread to lymph nodes, lungs, with phylloides appearance in some tumors, as well as seminal vesicle invasion in most mice

Diet Composition

Table 3 AIN-93M mature rodent diet formulation

Diet		
	gm%	***kcal%***
Protein	14	*15*
Carbohydrate	73	*76*
Fat	4	*9*
Total		*100*
kcal/gm	3.8	
Ingredient	**gm**	***kcal***
Casein	140	*560*
L-Cystine	1.8	*7.2*
Corn Starch	495.69	*1982.8*
Maltodextrin 10	125	*500*
Sucrose	100	*400*
Cellulose, BW200	50	*0*
Soybean Oil	40	*360*
t-Butylhydroquinone	0.008	*0*

Mineral Mix S10022M	35	*0*
Vitamin Mix V10037	10	*40*
Choline Bitartrate	2.5	*0*
Total	**1000**	***3850***

Table 4 AIN-93M mature rodent diet with 0.1, 1, and 100 µmol indole-3-carbinol per gram diet

Diet (µmol/g diet)	**0.1**		**1**		**100**	
	gm%	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***
Protein	14	*15*	14	*15*	14	*15*
Carbohydrate	73	*76*	73	*76*	73	*76*
Fat	4	*9*	4	*9*	4	*9*
Total		*100*		*100*		*100*
kcal/gm	3.8		3.8		3.8	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	140	*560*	140	*560*	140	*560*
L-Cystine	1.8	*7.2*	1.8	*7.2*	1.8	*7.2*
Corn Starch	495.69	*1982.8*	495.69	*1982.8*	495.69	*1982.8*
Maltodextrin 10	125	*500*	125	*500*	125	*500*
Sucrose	100	*400*	100	*400*	100	*400*
Cellulose, BW200	50	*0*	50	*0*	50	*0*
Soybean Oil	40	*360*	40	*360*	40	*360*
t-Butylhydroquinone	0.008	*0*	0.008	*0*	0.008	*0*

Mineral Mix S10022M	35	*0*	35	*0*	35	*0*
Vitamin Mix V10037	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2.5	*0*	2.5	*0*	2.5	*0*
Indole-3-carbinol	*0.014938*	*0*	*0.14938*	*0*	*14.938*	*0*
Total	**1000.01**	***3850***	***1000.14***	***3850***	***1014.94***	***3850***

Table 5 AIN-93M mature rodent diet with 5 µmol phenethyl isothiocyanate per gram diet

Diet		
	gm%	***kcal%***
Protein	14	*15*
Carbohydrate	73	*76*
Fat	4	*9*
Total		*100*
kcal/gm	3.8	
Ingredient	**gm**	***kcal***
Casein	140	*560*
L-Cystine	1.8	*7.2*
Corn Starch	495.69	*1982.8*
Maltodextrin 10	125	*500*
Sucrose	100	*400*
Cellulose, BW200	50	*0*
Soybean Oil	40	*360*
t-Butylhydroquinone	0.008	*0*

Mineral Mix S10022M	35	*0*
Vitamin Mix V10037	10	*40*
Choline Bitartrate	2.5	*0*
2-Phenylethyl Isothiocyanate	**0.816**	*0*
Total	**1000.8**	***3850***

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