EFFECT OF BROCCOLI BIOACTIVES ON PROSTATE CANCER CELL INVATION IN VITRO

BY

CHING-YU HUANG

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Master’s Committee:
Professor John W. Erdman, Jr.
Professor Elizabeth H. Jeffery
Professor Matthew A. Wallig
Abstract

Previous studies have shown that broccoli bioactives may have anti-cancer activity. In this work, we hypothesized that broccoli bioactive components inhibit invasion of human prostate cancer cell line, DU145, in an in vitro model system. DU145 cells were plated into Matrigel invasion chambers placed in 24-well plates with complete growth medium as a chemo-attractant. In the chambers, cells were treated with sulforaphane (SF), indole-3-carbinol (I3C), hydrolyzed neoglucobrassicin (hNGB), and quercetin (Q) individually or in selected combinations. After a total of 48 hour treatment exposure, with the treatments being renewed at the 24th hour, the invading cells were fixed, stained, and counted. The results of Matrigel invasion assay indicate that the invasive ability of DU145 cells was significantly inhibited by the physiologically relevant levels of SF (3-5 µM) and I3C (120-200 nM), but not NGB (1-50 µM) or Q (1-5 µM). We further performed the MTT assay to examine whether the reduction of invading cells were attributed to the decreased invasive ability or cell death in the condition of the Matrigel invasion assay. DU145 cells were plated in 96-well plates and followed the treatment and incubation procedure similar to the Matrigel invasion assay, with invasion-suppressive concentrations of SF (3-5 µM) or I3C (160 and 200 nM). At the end of 48 hours, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each well, followed by incubation for 4-6 hours. The 96-well plates were analyzed at the absorbance of 540nm. The result of MTT assay shows that invasion-suppressive concentrations of SF and I3C did not decrease cell viability, meaning that SF and I3C inhibited DU145 cells not by causing cell death but by other regulatory pathways that may suppress invasion. The results of this study provide the support for broccoli consumption by
prostate cancer patients. Further work will be needed to evaluate whether broccoli bioactives reduce metastasis of prostate cancer to extra-prostatic sites.
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Chapter I

Literature Review

Introduction

In 2014, prostate cancer is estimated to be the leading type of cancer diagnosed and the second leading cause of cancer-related deaths in men in the United States (Siegel et al., 2014). According to the five-year survival rate study (between 2002-2008) based on the stage at diagnosis, 100% of the patients diagnosed with the localized or regional prostate cancer survived; however, only 28% of the patients with metastasized prostate cancer survived at the end of the five years (Siegel et al., 2014). In other words, metastatic disease is the main cause of death for prostate cancer. Unfortunately, it has been reported that one out of seven prostate cancer patients would develop metastatic prostate cancer (Siegel et al., 2014). Therefore, it is important to decrease the incidence of metastasis, in order to increase the survival rate in men with prostate cancer.

Risk Factors of Prostate Cancer

Age

The incidence of prostate cancer is age-related. Men aged less than 40 years are rarely diagnosed with prostate cancer; while those aged around 70 years are commonly diagnosed with prostate cancer (Bostwick et al., 2004). Therefore, the American Cancer Society recommends that men should have prostate cancer screening at the age starting at 50 (“American Cancer Society Guidelines for the Early Detection of Cancer,” 2013).
Race

Race plays an important role in both incidence and mortality rate of prostate cancer. Siegel and colleagues (2014) reported that African American men have higher incidence and mortality rate of prostate cancer than non-Hispanic white, Asian American, and Pacific islanders in the United States. Since all the cases in this analysis were investigated in the United States during 2006-2010, the differences between races should not be due to the geographic impact, but due, at least in part, to ethnicity. Therefore, race could impact the incidence and mortality rate of prostate cancer (Siegel et al., 2014).

Region of Residency

Environmental factors vary in different regions of residency, where the interactions of climate, life style, culture, health care system, and others could have influence on the incidence and mortality rate of prostate cancer. Based on a continental statistics, men in North America are more likely to have prostate cancer; whereas those in Asia have a 25.5-time lower incidence rate than in North America. As for the mortality rate, on the other hand, Oceania has the highest death rate, and Asia has the lowest death rate of prostate cancer (Kamangar, 2006).

Sun exposure—another factor related to the latitude of residency—could decrease prostate cancer risk and mortality rate. The John group (2007) suggested that the prostate cancer risk was inversely related to sun exposure (John et al., 2007). Similarly, the Hanchette group (1992) found that the increase in the mortality rate of prostate cancer was correlated to the decrease in the UV exposure (Hanchette & Schwartz, 1992). Since sun exposure also directly affects Vitamin D formation in the human body, both groups related that prostate cancer risk
and mortality rate could be altered by vitamin D formation stimulated by sun exposure. In conclusion, region of residency may change the risk of prostate cancer, and sunlight—a trigger of vitamin D production—has been considered to be one of important environmental factors.

**Family History**

Family history is believed to be one of the primary risk factors of prostate cancer. Frank (2014) introduced the population attributable fraction (PAF)—the ratio of a certain disease that could be prevented if freed from the specific risk factor—to examine the influence of familial factors on prostate cancer. He suggested that prostate cancer has the highest PAF of 13.9% in comparison with other types of cancer, such as breast (7.46%), and colorectum (6.78%), based on the database of 8,148,737 individuals in Swedish Family-Cancer center. Hence, family history may be an indicator of prostate cancer incidence (Frank et al., 2014).

**Diet**

Different dietary patterns influence health outcomes, including the incidence of prostate cancer. Micronutrients, bioactives, or carcinogens in the diet have been thought to be important factors that affect prostate cancer occurrence. Indeed, epidemiological studies have found that high consumption of animal fat and red meat is related to increased risk of prostate cancer (Kolonel, 2001), whereas high consumption of fruit and vegetable is related to reduced risk of prostate cancer (reviewed in Nelson et al., 2014; Giovannucci et al., 2003). Thence, diet may alter the risk of prostate cancer occurrence.
Metastasis

Metastasis is a group of sequential events that result in the spread of cancer cells from the primary organ to distant parts of the body. This process (figure 1.1) begins with epithelial-mesenchymal transition (EMT) and invasion of extracellular matrix (or basement membrane in figure 1.1), followed by the escape of cancer cells into the circulation system, and finally ends with the extravasation of the tumor cells into the metastatic sites where the metastatic cells undergo mesenchymal-epithelial transition (MET) and re-colonization (Arya et al., 2006).

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition, or EMT, is an essential initiation process before tumor cell invasion. Tumor cells undergo the morphological changes from epithelial phenotype to mesenchymal phenotype, where they lose cell-to-cell adhesion, and gain mobility, allowing them to become free from the original sites. The cell-cell adhesion is mediated by classes of cell adhesion molecules, or CAMs. The most well-observed CAMs in cancer studies are E-cadherin and N-cadherin, calcium-dependent cadherins (reviewed in Hanahan & Weinberg, 2000; Arya et al., 2006). The loss of E-cadherin expression and the increase in N-cadherin expression are believed to be the hallmarks of EMT. The Tomita group (2000) showed that lowered E-cadherin and increased N-cadherin expression was found in undifferentiated prostate tumors and in metastases from the prostate tissues obtained by radical prostatectomy or transurethral resection. They also found that normal prostate tissue obtained from cystoprostatectomy did not express N-cadherin; whereas high-grade prostate cancer and prostate cancer metastases
were reported to have increased N-cadherin level (Tomita et al., 2000). Collectively, the results indicated that decreased expression of E-cadherin with increased expression of N-cadherin is a sign of prostate cancer progression. Besides, the Gravdal group (2007) also examined the prostate tissue from 104 men treated by radical prostatectomy during 1988-1994. They suggested that low E-cadherin expression is significantly associated with seminal vesicle invasion, clinical recurrence, skeletal metastasis, and cancer-specific death; and high expression of N-cadherin is related to seminal vesicle invasion, clinical recurrence, and skeletal metastasis (Gravdal et al., 2007). Taken together, up-regulated N-cadherin together with down-regulated E-cadherin plays an essential role in the initiation of prostate cancer progression.

**Invasion**

To accomplish the process of invasion, epithelial-mesenchymal transition is needed to help cancer cells migrate, and degradation of extracellular matrix (ECM) is essential to release the migratory cells from their original site. The degradation of ECM is regulated or executed by a wide variety of proteinases, including commonly studied matrix metalloproteinases (MMPs) and urokinase-type plasminogen (uPA) (reviewed in Hanahan & Weinberg, 2000; Arya et al., 2006).

Matrix metalloproteinases (MMPs) are zinc-dependent proteinases responsible for the degradation of collagen, including extracellular matrix (ECM). Up-regulation of MMPs could result in progression of cancers (Page-McCaw et al., 2007). The Lichtinghagen group (2002) found significant increase in MMP-9 protein expression and unchanged MMP-2 level in cancerous parts of 17 prostate samples from radical prostatectomy (Lichtinghagen et al., 2002).
The Xiao group (2012) indicates that inhibition of MMP-2 or MMP-9 by siRNA led to inhibited invasiveness of PC-3 cells (Xiao et al., 2012). Reis and others (2012) analyzed the prostate tissues from 79 prostate cancer patients who underwent radical prostatectomy. They found that prostates with higher Gleason Scores (≥7, more aggressive) had higher mRNA expression of MMP-2 and MMP-14 (Reis et al., 2012). Overall, the previous studies have shown up-regulation of certain kinds of MMPs was related to malignancy of prostate cancer and inhibition of MMP-2 and MMP-9 resulted in decreased invasiveness in prostate cancer cells, meaning that MMP proteins may be involved in cancer progression in prostate.

Urokinase-type plasminogen activator, or uPA, is a serine proteinase catalyzing plasminogen to a potent pro tease, plasmin, which participates in the signaling and execution of ECM degradation (reviewed in Arya et al., 2006). Rats implanted with adenocarcinoma overexpressing uPA were found to have more wide spread metastases than the control rats implanted with adenocarcinoma producing basal level of uPA. Conversely, rats inoculated with adenocarcinoma underexpressing uPA showed decreased metastases than the control rats (Achbarou et al., 1994). In addition, induction of siRNAs of uPA and/or uPA receptor (uPAR) to highly invasive prostate cancer cell line, PC-3, significantly inhibited the invasive ability of cells (Pulukuri et al., 2005). Therefore, uPA may be crucial to trigger invasion of prostate cancer.

Invasion is a critical step for cancer metastasis. Many studies have indicated that MMPs, especially MMP-2, MMP-9 and MMP-14, and uPA play important roles in degrading ECM and help prostate cancer invasion. Therefore, these proteins can be the biomarkers to determine the invasiveness of prostate cancer.
The current thesis project is focused upon the effects of broccoli bioactives on invasion of a common-studied human derived prostate cancer cell line, DU145 cells, *in vitro*. A Matrigel model system (Baba et al., 2000) was chosen for this work. The model mimics the invasion stage shown in figure 1.1.

*Seed-and-Soil Hypothesis*

In human, prostate cancer tends to metastasize to bone, lung, and liver (Bubendorf et al., 2000). The organ preference of cancer metastasis can be illustrated by the seed-and-soil hypothesis, which was firstly proposed by Paget in 1889, based on 735 cases of fatal breast cancer. Paget (1889) found that the odds of distant organs developing cancer metastases were not equal. Thus, he suggested that the spreading of metastatic tumor cells can be explained by the analogy of that seeds can spread widely, but only those staying in the favorable soil (preferred organ microenvironment) can grow (Paget, 1889). This hypothesis has been well-used to explain the preference of cancer metastasis.

Over decades of investigation with supportive evidence, Fidler (2003) introduced three principles to novel seed-and-soil hypothesis. He firstly suggested that the primary neoplasms consist of tumor cells with various genotypes and phenotypes. Some genotypes and their corresponding phenotypes help tumor cells overcome the obstacles that may encounter during metastasis. However, most tumor cells do not have all of the genotypes and associated phenotypes needed for metastasis, so most of them are unable to survive. Fidler further indicated that metastases are tumor colonies formed by individual tumor cells that survived the
sequential steps of metastasis—from invasion to re-colonization. Finally, he stated that the
development of metastases depends on the microenvironment of different organs, where
growth factors, together with associated receptors on the cell surface, mediate the formation of
metastases (Fidler, 2003).

Seed-and-soil hypothesis has been used frequently to illustrate metastasis since 1889.
Although it cannot explain all the cases of metastases, it is still one of the fundamental ideas that
is worthwhile to understand.

**Cruciferous Vegetables**

Crucifers are the vegetables belonging to the family of Brassicaceae. These
vegetables—such as broccoli, cauliflower, cabbage, and kale—are widely cultivated and
consumed around the world. Some epidemiological trials have found that increasing
consumption of cruciferous vegetables is related to decreasing risk of prostate cancer (Jain et al.,
1999; Cohen, Kristal, & Stanford, 2000; Kolonel et al., 2000); while other studies have not
(Giovannucci et al., 2003; Key et al., 2004). Although epidemiological studies project inconsistent
health outcomes of crucifer consumption, researchers continue to evaluate whether cruciferous
vegetables are potentially protective against prostate cancer.

**Broccoli**

Broccoli (*Brassica oleracea var. italica*), one of the cruciferous vegetables, is commonly
consumed in the United States. The per capita total consumption of broccoli in 2013 was 9.2
pounds, which is almost six times more than the total consumption was in 1970 (figure 1.2) (USDA, 2014). Fresh broccoli is low in fat and devoid of cholesterol; and high in fiber, vitamin C, and vitamin K (USDA, 2013) (Table 1.1). Moreover, various broccoli bioactive components—such as isothiocyanates, indoles, and flavonols—are thought to have anti-cancer activity. Research results from the University of Illinois indicated that 10% broccoli diet lowered prostate tumor weight in a xenograft rat model (Canene-Adams et al., 2007). Additionally, a broccoli-containing diet, combined with tomato, alleviated the prostatic damage induced by a carcinogen, PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-B]pyridine), in a rat model (Canene-Adams et al., 2013). However, another study from our lab failed to find any significant effect of either 10% standard broccoli or glucosinolate-enriched broccoli diets on progression of prostate lesions in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (Liu et al., 2014, in press).

Canene-Adams’ studies suggested that broccoli may reduce the severity of prostate cancer in two different animal models with prostate tumors introduced exogenously, yet Liu’s study was not significant using a mouse model that spontaneously develops adenocarcinoma. Different outcomes from three studies may be due to different characteristics of animal models used among the three studies. Although Liu’s study did not show significance, we are still interested in whether broccoli bioactive components can affect the stages of metastatic prostate cancer, especially, invasion. Therefore, we examined four bioactive components, sulforaphane, indole-3-carbinol, neoglucobrassicin, and quercetin, individually or in selected combinations, and evaluated if these bioactives could affect prostate cancer invasion in vitro.
Glucosinolates

Glucosinolates are a class of sulfur-containing glucosides that contribute to the bitter and spicy tastes of cruciferous vegetables. In nature, when cruciferous plants are injured either by pathogens or by pests, the damaged tissue will release an enzyme, myrosinase, to hydrolyze glucosinolates into bioactive products (figure 1.3), which can mediate the insect-plant interactions, and suppress fungal growth (reviewed in Mithen, 1992). Researchers have hypothesized that these bioactive products derived from the side chain cleavage of glucosinolates are potentially protective against prostate cancer. Therefore, we investigated three hydrolyzed products of glucosinolates—glucoraphanin, glucobrassicin, and neoglucobrassicin—and evaluated the effects on prostate cancer cell invasion, in vitro.

Sulforaphane

Sulforaphane (SF) is an isothiocyanate (ITC) derived from the particular glucosinolate, glucoraphanin, through the hydrolysis reaction catalyzed by myrosinase released from injured tissue of crucifers (figure 1.4). SF has been considered a chemopreventive agent for cancer due to its special role in activation of nuclear factor erythroid 2-related factor 2, or Nrf2 (Reviewed in Fahey et al., 1997; Thimmulappa et al., 2002; Juge et al., 2007). Nrf2 is a transcription factor that mediates the expression of phase II detoxification enzymes and antioxidant proteins (reviewed in Niture et al., 2010). Under normal conditions, Nrf2 is inactivated and retained in the cytosol due to binding to actin-bound Kelch-like ECH-associated protein 1, or Keap1, which is responsible for signaling the degradation of Nrf2 (reviewed in Kobayashi & Yamamoto, 2006). When a cell is exposed to xenobiotics, antioxidants, heavy metals, radiation, electrophiles, or reactive oxygen
species (ROS), Nrf2 is released from Keap1, and translocates to nucleus, where Nrf2 binds to the DNA sequence termed the antioxidant responsive element, or ARE, triggering the expression of enzymes and other proteins involved in detoxification, antioxidation, or ubiquitination, protecting the cell against the damage caused by the exposure of stresses (figure 1.5) (reviewed in Motohashi & Yamamoto, 2004; Niture et al., 2010). For example, upon the activation of Nrf2, several proteins will be expressed, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), glutathione peroxidase and reductase, γ-glutamate cysteine ligase (γ-GCL), heme oxygenase (HO-1), multidrug resistance-associated proteins (MRPs), and others (reviewed in Niture et al., 2010). NQO1 is a two-electron reductase that is capable of detoxifying and reducing the quinones that would otherwise develop into reactive oxygen species, or ROS (reviewed in Ross et al., 2000). GST catalyzes the conjugation reaction of glutathione with xenobiotics to form less toxic and more hydrophilic products (reviewed in Townsend & Tew, 2003). Glutathione peroxidase is involved in the reduction of hydrogen peroxide and alkyl hydroperoxides by transferring reducing power from reduced glutathione, leaving oxidized glutathione, which is then recycled through reduction by glutathione reductase (reviewed in Jurkovič et al., 2008). γ-GCL is the rate-limiting enzyme in glutathione synthesis (Griffith, 1999). HO-1 is responsible for breakdown of heme, and one of the metabolites, bilirubin, is a potent antioxidant in the human body (reviewed in Morse & Choi, 2005). MRPs participate in drug transport (Borst et al., 2000). Taken together, these proteins, activated by sulforaphane-induced Nrf2-ARE signaling pathway, are able to help protect the cell from oxidative stress and toxicity.
In addition to the induction of the Nrf2-ARE signaling pathway, sulforaphane is capable of triggering apoptosis, or programmed cell death, which is a series of events leading to cell death that can be induced by cytotoxic substances, DNA damage, or oxidative stress. The typical hallmarks of apoptosis are shrinkage of cell and the nucleus, condensation of nuclear chromatin, orderly degradation of DNA, and formation of apoptotic bodies, which are then engulfed by macrophages (reviewed in Saraste & Pulkki, 2000; Juge et al., 2007). With apoptosis, the human body would be less likely to develop abnormal or cancerous cells.

Apoptosis is elaborately regulated by a wide variety of proteins, such as caspases, cytochrome c, apoptosis protease-activating factor (Apaf-1), bcl-2 family, bax, and others. Up-regulation of caspases, cytochrome c, Apaf-1, and bax; and down-regulation of bcl-2, are thought to stimulate apoptosis (reviewed in Saraste & Pulkki, 2000; Singh et al., 2004; Juge et al., 2007). Research done by Chiao (2002) showed that the apoptotic cells of human prostate cancer cell line, LNCaP, were dramatically increased with the treatment of 9µM and 50µM SF. Furthermore, the caspase positive cells were much more in the SF-treated group than in control group (Chiao et al., 2002). Singh (2004), based on their in vitro study of PC-3 human prostate cancer cells, indicated that 20µM and 40µM SF treatments significantly induced DNA fragmentation in PC-3 cells. Additionally, 20µM SF treatment significantly down-regulated bcl-2 and up-regulated bax protein expression in PC-3 cells, which is considered to trigger apoptosis. The same study also investigated the involvement of caspases, and it was found that caspases 3 and 9 were up-regulated after 16 hours of SF (20µM) treatment in PC-3 cells, meaning that the activity of apoptosis was increased by sulforaphane treatment (Singh et al., 2004). Singh (2009) also found similar results of DNA fragmentation, lowered bcl-2, and increased bax expression in the TRAMP-
C1 cell (the mouse prostate cancer cell line derived from transgenic adenocarcinoma mouse of model, or TRAMP) treated with 20µM SF (Singh et al., 2009). Choi (2007) observed increased apoptosis along with elevated expression of apoptosis protease activation factor-1 (Apaf-1), a triggering factor that may lead to apoptosis, in both PC-3 and LNCaP cells treated with SF (10-40µM) (Choi et al., 2007). Taken together, these in vitro studies suggest that sulforaphane can induce apoptosis through regulation of various proteins participating in apoptosis, which then protect the body against carcinogenesis (figure 1.6).

The anti-cancer activity of sulforaphane is supported by animal studies. The Singh group (2004) used a xenograft mouse model implanted with human prostate cancer, PC-3, and found that mice gavaged with 5.6µmol SF three times per week had significantly lower prostate tumor volumes than the control mice gavaged with the vehicle (Singh et al., 2004). The same group further reported that mice fed with 6 µmol SF three times per week through gavage have lower incidence of prostatic intraepithelial neoplasia (PIN) and well-differentiated carcinoma, and lower incidence of metastases in lung in TRAMP mice than control mice gavaged with vehicle (Singh et al., 2009).

Although many studies have reported that high levels of SF significantly inhibited cancer development, little work has been done using more physiological doses of SF. Therefore, in this work, we investigated the influence of physiologically-relevant levels of SF on invasion of prostate cancer cell line, DU145, in vitro.
Indole-3-Carbinol

Indole-3-carbinol (I3C) is a hydrolysis product from glucobrasscin catalyzed by myrosinase (figure 1.7). Under acidic conditions (e.g. stomach), I3C spontaneously undergoes condensation reaction, forming polymeric products, mainly 3,3'-diindolylmethane (DIM) (figure 1.8). Both I3C and DIM are chemopreventive agents of prostate cancer by inducing detoxification and suppressing cell growth. Li (2003) indicates that 100 µM I3C and 60 µM DIM significantly suppressed PC-3 cell growth in the first 24 hour; while lower concentrations of I3C (30 µM) and DIM (30 µM) showed cell growth-suppressive ability at both 48 and 72 hour. Furthermore, the gene expression profile of PC-3 cells treated with 60 µM I3C or 40 µM DIM suggested that both I3C and DIM were able to up-regulate gene expression related to phase I and phase II detoxification enzymes, and down-regulated gene expression that was responsible for the regulation of cell growth, proliferation, and apoptosis (Li et al., 2003). Obviously, DIM appeared to be more potent than I3C in this study, as DIM was effective in lower concentrations.

Nachshon-Kedmi (2003) examined the bioactivity of I3C and DIM in three human prostate cancer cell lines: PC-3, LNCaP, and DU145. The IC₅₀ values of cell viability with I3C were 150 µM for LNCaP, 160 µM for DU145, and 285 µM for PC-3; with DIM were 40 µM for LNCaP, 20 µM for DU145, and 40 µM for PC-3, providing more support for the hypothesis that DIM indeed is a more potent chemopreventive agent than I3C (Nachshon-Kedmi et al., 2003).

The bioactivity of I3C and DIM may be also related to induction of apoptosis. Chinni (2001) found that rather high I3C treatments (60µM and 100µM) in PC-3 human prostate cancer cells induced G₁ cell cycle arrest (100µM), and up-regulation of bax and down-regulation of bcl-2, which led to apoptosis (Chinni et al., 2001). The Nachshon-Kedmi group in 2004 reported that
DIM (75µM) induced activities of caspases -3, -6, and -9 as well as increasing cytosolic cytochrome c in PC-3 cells, which, taken together, leads to apoptosis (figure 1.9). It was reported that DIM can trigger apoptosis through inactivation of the PI3K/Akt pathway. PI3K is the kinase that phosphorylates and activates Akt. Akt, or protein kinase B, is responsible for inhibition of apoptosis. Phosphorylated activation of Akt can stimulate apoptosis. The Li group indicates that DIM-treated PC-3 cells have lower PI3K activity, which can also inhibit Akt activity, and leads to apoptosis (figure 1.9) (Li et al., 2005). Since apoptosis is a natural defensive mechanism to prevent cancer formation, I3C and DIM, which potentially trigger apoptosis of prostate cancer cells, can be considered chemopreventive agents against prostate cancer.

Many studies have examined the bioactive effect of I3C or DIM on cell growth or apoptosis. However, there is a lack of literature looking at the effect of I3C on prostate cancer invasion. Therefore, we performed a trial examining the potential bioactivity of I3C on suppressing prostate cancer cell invasion, in vitro.

**Neoglucobrassicin**

Neoglucobrassicin (NGB) is one of indole glucosinolates present in broccoli. The level of NGB in broccoli changes during plant development. Broccoli sprouts contains relatively lower level of NGB (Shapiro et al., 2001) in comparison to mature broccoli, when NGB becomes predominant (Hansen et al., 1995). Similar to other indole glucosinolates, NGB can be hydrolyzed by myrosinase into its corresponding cabinol, N-methoxyindole-3-carbinol (NI3C) (figure 1.10). There has been a lack of studies about the biological effects of NGB and its hydrolyzed product.
Vo (2014) found that synthetic NGB significantly inhibited lipopolysaccharide-induced inflammation through decrease in TNF-α expression in the THP1 Human monocyte cell line with the levels of 10µM and 15µM (Vo et al., 2014). However, Haack (2010) observed that the hydrolyzed product of NGB, hNGB, decreased NQO1 activity at the concentration of 7.5µM, and weakened the anti-cancer properties of the hydrolyzed product of glucoraphanin, SF. For example, hNGB reduced SF-mediate Gpx2 gene expression, SF-stimulated Gpx2 promoter activity, and SF-induced NQO1 activity (Haack et al., 2010).

Since only a few studies of NGB have been completed and the literature is limited, the function and the effect of NGB remains unclear and needs more investigation. Overall, the limited work might suggest that NGB may not have any ability to inhibit prostate cancer metastasis.

**Flavonols**

Flavonols are a class of flavonoids composed of a 3-hydroxyflavone backbone (figure 1.11A) with phenolic hydroxyl groups in different positions that determine the activity of these compounds (figure 1.11B, 1.11C). Flavonols exist commonly in a wide variety of fruits and vegetables, including broccoli (Bhagwat et al., 2014). In plants, flavonols act as the UV-B filter to protect plants from sunlight damage (Agati et al., 2011), and also behave as antioxidants to scavenge free radicals induced by sunlight exposure (Agati et al., 2009). In humans, high consumption of flavonols is related to lower incidence of multiple cancers, including prostate cancer (Knekt et al., 2002). Therefore, we were interested to determine if quercetin, the most abundant flavonol in vegetables and fruits, could suppress prostate cancer cell invasion, *in vitro*. 
**Quercetin**

Quercetin (Q), a flavonol present in a wide variety of vegetables and fruits (table 1.2), predominately exists in the form of quercetin-3-O-sophoroside in broccoli (figure 1.12) at levels estimated to be approximately 6.5mg/100g broccoli (Vallejo et al., 2004). The typical intake of Q ranges from less than 5mg/day to ~40mg/day; however, high-end consumers of non-peeled vegetables or fruits could obtain 200-500 mg/day of Q (reviewed in Harwood et al., 2007). Q is believed to have antioxidant properties that can scavenge reactive oxygen species (ROS) (Afanas’ev et al., 1989). ROS are oxygen-centered free radicals what the body can be exposed to either exogenously, through environmental stress, or endogenously, through respiration. Common ROS, including superoxide (O$_2^-$), peroxyl (ROO•), alkoxyl (RO•), hydroxyl (HO•), and nitric oxide (NO•), are so reactive that once generated, they can immediately oxidize nearby cell membranes, proteins or enzymes, or DNA, resulting in cell membrane damage, protein or enzyme structural modifications, and/or gene mutations. The accumulation of this damage is considered to be part of the reason for aging, inflammation, and several degenerative diseases, such as cardiovascular disease, hepatic disease, and cancer (Pietta, 2000; Blake, 1995). Q is thought to be capable of neutralizing oxidative stress by chelating the transition metals involved in the generation of ROS. Additionally, the phenolic hydroxyl groups of Q may act as electron donors to scavenge free radicals (Bors et al., 1990). Moreover, a study done by Tanigawa (2007) using HepG2 liver cancer cell line indicated that Q increased ARE activity by decreasing KEAP1 protein levels, increasing Nrf2 mRNA expression, stabilizing Nrf2 from being degraded as well as triggering Nrf2 to translocate from cytosol to nucleus, where Nrf2 binds to ARE and stimulate the
expression of downstream gene such as NQO1, a protein that helps scavenge of ROS (Tanigawa et al., 2007). Thus, Q apparently is a good dietary antioxidant that can slow down the accumulation of oxidative stress in the body.

Flavonoid glycosides are generally poorly absorbed by the enterocytes in the intestine. Therefore, they must be hydrolyzed, by enterobacteria, into the aglycone form to facilitate the absorption, which mostly happens in the colon, where the flavonoid may also be degraded (Murota & Terao, 2003). However, Q may be different than other flavonoids. Hollman (1995) suggested that Q was better absorbed in glycoside forms than in the aglycone form in ileostomy volunteers who do not have colonic bacteria (Hollman et al., 1995). To elucidate Hollman’s (1995) case, researchers based on their studies indicate that the bioavailability of Q depends on the different position and variety of sugar moiety attached, as the glucose transporter system may be responsible for Q glycosides absorption (Hollman et al., 1997; Arts et al., 2004). Different sugar attached would also change lipophilicity of the entire compound, and affect absorption (Murota & Terao, 2003). While part of the Q glycosides are absorbed in small intestine, the rest may be hydrolyzed or degraded into metabolites by bacteria in colon prior to absorption (Crozier et al., 2009). After consumption of Q form dietary sources, such as onions, only quercetin glucuronides and quercetin sulfides can be found in human plasma (Wittig et al., 2001; Janisch et al., 2004), Moon et al. (2001) further examined the antioxidant ability of some quercetin glucuronides, and found that these quercetin glucuronides still have antioxidant ability to scavenge free radicals (Moon et al., 2001).

The epidemiological study suggests that health outcomes were related to quartiles of Q consumption per day. Q intake was inversely associated with mortality of ischemic heart disease
(4th quartile), risk of diabetes (2nd, 3rd quartiles) and all cancers (4th quartile), especially lung cancer (2nd, 3rd, 4th quartiles) (Knekt et al., 2002). Animal studies showed that the Q-containing diet (200mg/kg body weight) significantly reduced the incidence of prostatic tumor induced by the combination of a carcinogen (N-methyl-N-nitrosourea, or NMU) and testosterone in a rat model (Sharmila et al., 2014). In addition, Pratheeshkumar (2012) found that a diet containing Q (20mg/kg/day) significantly inhibited prostate tumor growth in a xenograft mouse model (Pratheeshkumar et al., 2012). The anti-cancer property of Q was also seen in cell culture studies. Q was able to suppress cell viability of PC-3 human prostate cancer cells (treated with 20µM-50µM Q) (Pratheeshkumar et al., 2012), and inhibited colony formation of highly invasive cell lines, DU145 and PC-3 (treated with 25µM and 50µM Q), but not in the non-invasive cell line, LNCaP (Nair et al., 2004). The result from Nair’s study (2004) indicates that Q may particularly target invasive prostate cancer. On the other hand, Pratheeshkumar treated highly progressive prostate cancer cell line, PC-3, with Q (30µM and 40µM), and detected decreased secretion of vascular epithelial growth factor (VEGF), which is involved in cancer progression (Pratheeshkumar et al., 2012). Furthermore, Senthilkumar found reduced migration/invasion-related mRNA expression, such as urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), epidermal growth factor (EGF), and EGF receptor (EGFR) in PC-3 cells (Senthilkumar et al., 2011). Collectively, literature suggests that Q is one of the components in broccoli that may potentially suppress prostate cancer invasion. Previous studies mainly investigated effects of supplement Q at high levels, but little study has been done with physiologically-relevant levels. Thus, in this work we examined the effect of Q on prostate cancer cell invasion in vitro at levels
that might be expected in blood following dietary intake of Q-containing foods or low dose supplements.
Figures and Tables

Figures

Figure 1.1. The scheme of metastasis (Weinberg, 2014). EMT represents epithelial-mesenchymal transition, and MET represents mesenchymal-epithelial transition.

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**Figure 1.3.** The structure of glucosinolate.

**Figure 1.4.** The hydrolysis reaction of glucoraphanin into sulforaphane.

**Figure 1.5.** The activation of Nrf2-ARE signaling by sulforaphane according to previous studies.
Figure 1.6. The possible apoptosis cascade induced by sulforaphane according to previous studies.

Figure 1.7. The hydrolysis reaction of glucobrassicin into indole-3-carbinol.
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Figure 1.10. The hydrolysis reaction of neoglucobrassicin into N-methoxyindole-3-carbinol.

Figure 1.11. A. The structure of 3-hydroxyflavone backbone in flavonols. B. The structure of flavonols. C. Examples of different flavonols with various R₁ and R₂ groups, such as myricetin, kaempferol, isorhamnetin, and luteolin (Crozier et al., 2000).
Figure 1.12. The hydrolysis reaction of quercetin-3-O-sophoroside into quercetin aglycone.

Table 1.1. The nutrition content of raw broccoli (edible portion). Data were obtained from USDA National Nutrient Database for Standard Reference, Release 26 (2013).

<table>
<thead>
<tr>
<th>Nutritional Value in 100 g (3.5 oz)</th>
<th>Amount</th>
<th>%DV</th>
<th>Amount</th>
<th>%DV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>34kcal</td>
<td>2%</td>
<td>Zinc</td>
<td>0.4mg</td>
</tr>
<tr>
<td>Water</td>
<td>89.3g</td>
<td></td>
<td>Manganese</td>
<td>0.2mg</td>
</tr>
<tr>
<td>Protein</td>
<td>2.8g</td>
<td>6%</td>
<td>Selenium</td>
<td>2.5µg</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.0mg</td>
<td>0%</td>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td>Total fat</td>
<td>0.4g</td>
<td>1%</td>
<td>Vitamin A</td>
<td>623 IU</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>6.6g</td>
<td>2%</td>
<td>Vitamin C</td>
<td>89.2mg</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>2.6g</td>
<td>10%</td>
<td>Vitamin E</td>
<td>0.8mg</td>
</tr>
<tr>
<td>Sugar, total</td>
<td>1.7g</td>
<td></td>
<td>Vitamin K</td>
<td>102µg</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
<td></td>
<td>Thiamin (B₁)</td>
<td>0.1mg</td>
</tr>
<tr>
<td>Calcium</td>
<td>47mg</td>
<td>5%</td>
<td>Riboflavin (B₂)</td>
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</tr>
<tr>
<td>Iron</td>
<td>0.7mg</td>
<td>4%</td>
<td>Niacin (B₃)</td>
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<tr>
<td>Magnesium</td>
<td>21.0mg</td>
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<tr>
<td>Phosphorous</td>
<td>66mg</td>
<td>7%</td>
<td>Folate</td>
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</tr>
<tr>
<td>Potassium</td>
<td>316mg</td>
<td>9%</td>
<td>Pantothenic Acid (B₅)</td>
<td>0.6mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>33mg</td>
<td>1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. Quercetin content in fruits and vegetables. Data were obtained from USDA Database for the Flavonoid Content of Selected Foods, Release 3.1 (2014).

<table>
<thead>
<tr>
<th>Food</th>
<th>Content (Mean)</th>
<th>Food</th>
<th>Content (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capers, raw</td>
<td>233.84</td>
<td>Blueberries, raw</td>
<td>7.67</td>
</tr>
<tr>
<td>Radish leaves, raw</td>
<td>70.37</td>
<td>Oregano, fresh</td>
<td>7.30</td>
</tr>
<tr>
<td>Red onions, raw</td>
<td>31.77</td>
<td>Chives, raw</td>
<td>4.77</td>
</tr>
<tr>
<td>Watercress, raw</td>
<td>29.99</td>
<td>Pears, raw</td>
<td>4.24</td>
</tr>
<tr>
<td>Elderberries, raw</td>
<td>26.77</td>
<td>Spinach, raw</td>
<td>3.97</td>
</tr>
<tr>
<td>Kale, raw</td>
<td>22.58</td>
<td>Blackberries, raw</td>
<td>3.58</td>
</tr>
<tr>
<td>Onions, raw</td>
<td>21.40</td>
<td>Broccoli, raw</td>
<td>3.26</td>
</tr>
<tr>
<td>Okra, raw</td>
<td>20.97</td>
<td>Bay leaves, fresh</td>
<td>3.19</td>
</tr>
<tr>
<td>Bee pollen</td>
<td>20.95</td>
<td>Cherry tomatoes, raw</td>
<td>2.76</td>
</tr>
<tr>
<td>Apple, skin only</td>
<td>19.36</td>
<td>Green tea, brewed</td>
<td>2.49</td>
</tr>
<tr>
<td>Chia seeds, raw</td>
<td>18.42</td>
<td>Grapes, black</td>
<td>2.08</td>
</tr>
<tr>
<td>Sweet potato leaves, raw</td>
<td>16.94</td>
<td>Chinese cabbage, raw</td>
<td>2.12</td>
</tr>
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<td>Cranberries, raw</td>
<td>14.84</td>
<td>Brussels sprouts, raw</td>
<td>1.92</td>
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<tr>
<td>Asparagus, raw</td>
<td>13.98</td>
<td>Garlic, raw</td>
<td>1.74</td>
</tr>
</tbody>
</table>
Chapter II

Effect of Broccoli Bioactives on Prostate Cancer Invasion in vitro

Abstract

Prostate cancer is the second leading cause of cancer-related deaths in males in the United States. Epidemiological studies suggest that high intake of cruciferous vegetables is associated with lower incidence and lower progression of prostate cancer. It has been found that these crucifers, especially broccoli, contain abundant bioactive components that may possess anti-cancer properties. However, little research has been completed regarding which components play significant roles in suppression of prostate cancer development, from progression to metastasis. Here, we performed the Matrigel invasion assay to examine effects of four abundant bioactive components in broccoli: sulforaphane (SF), indole-3-carbinol (I3C), hydrolyzed neoglucobrassicin (hNGB), and quercetin (Q) individually, or in selected combinations. DU145 invasive human prostate cancer cells were plated into the Matrigel invasion chambers inserted in 24-well plates, together with a chemo-attractant. The suspension medium in the chambers was replaced with treatments after cells attached to the Matrigel. After a total of 48 hour treatment exposure, with the treatments renewed at the 24th hour, invading cells were fixed, stained, and counted. Results show that 3µM-5µM SF or 160-200nM I3C significantly suppressed DU145 cell invasion; whereas NGB (1-50µM) and Q (1-5µM), did not. In the study combining 5µM SF with 120nM I3C shows significant inhibition of DU145 cell invasion. However, there was no significant difference between the combination treatments and 5µM SF alone, suggesting that the combination of SF and I3C did not synergistically or additively affect DU145 cell invasion. To verify whether the reduction of invading cells caused by SF and I3C,
individually, was due to the suppression of invasiveness or cell death, we further performed a test for cell viability using the MTT assay. Cells were seeded in 96-well plates, followed by the treatment procedure as described in the Matrigel invasion assay. After 48 hour incubation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each well, followed by 4-6 hour incubation. The result indicated that the invasion-suppressive concentrations of SF and I3C did not decrease cell viability, meaning that SF and I3C suppressed cell invasion not by causing cell deaths, but by inhibiting the invasive ability of DU145 cells. The mechanism of how SF and I3C suppressed DU145 cell invasion needs to be further studied in the future.

Introduction

Prostate cancer has been estimated to be the second leading cause of cancer-related deaths in men in the United States in 2014 (Siegel et al., 2014). The five-year relative survival rate of men with localized or regional prostate cancer is 100%; however, for those with metastatic prostate cancer, was only 28% (Siegel et al., 2014). Several environmental factors have been found to be influential in prostate cancer incidence and mortality. For example, age (Bostwick et al., 2004), race (Siegel et al., 2014), region of residency (Kamangar, 2006; John et al., 2007), sun exposure (Hanchette & Schwartz, 1992), family history (Frank et al., 2014), and diet (Kolonel, 2001; Giovannucci et al., 2003; Nelson et al., 2014). One of the manageable factors is diet. Epidemiological studies have suggested that high intake of meat products may increase the risk of prostate cancer (Kolonel, 2001); while high intake of fruits and vegetables may reduce the risk of prostate cancer (Giovannucci et al., 2003; Nelson et al., 2014). Cruciferous vegetables, or Brassicaceae plants, contain abundant bioactive compounds that are considered to make a
contribution to the decrease in the risk of prostate cancer (Jain et al., 1999; Cohen et al., 2000; Kolonel et al., 2000). Broccoli, one of the crucifers with high levels of glucosinolates and flavonols, is thought to be a cancer-preventive vegetable. Although Liu (2014) failed to find any effect of a broccoli supplemented diet on prostate tumor growth in a transgenic mouse model that develops prostate cancer spontaneously (Liu et al., 2014, in press), Canene-Adams observed that a 10% broccoli diet significantly lowered prostate tumor weight in rats that were implanted with prostate tumors (Canene-Adams et al., 2007). Additionally, Canene-Adams indicated that a diet containing broccoli and tomato significantly reduced prostate lesions in rats fed carcinogen (Canene-Adams et al., 2013). Overall, the data support a hypothesis that broccoli could be preventive against prostate cancer.

Although broccoli has been shown to suppress prostate cancer growth in some animal models, the effect of the main bioactive broccoli components on inhibition of prostate cancer invasion needs investigation. The purpose of this study was to examine effects of four bioactives abundantly present in broccoli—sulforaphane (SF), indole-3-carbinol (I3C), hydrolyzed neoglucobrassicin (hNGB), and quercetin (Q)—on prostate cancer cell invasion. The Matrigel invasion assay was performed to investigate suppressive abilities of these four bioactives, individually, or in combination (Baba et al., 2000). MTT assay was also conducted to confirm whether the decrease in invading cells was caused by the invasion-suppressive ability or by the cytotoxicity of these bioactives. The invasive human prostate cancer cell line, DU145, was used as the experimental model.
Materials and Methods

Cell Culture

In this study, DU145 cells were used as an *in vitro* prostate cancer model. DU145 is a metastatic prostate cancer cell line derived from the brain tissue of a 69-year-old male Caucasian (Stone et al., 1978). Previous studies have found that this cell line has a high invasive ability and adherent growth properties (Hoosein et al., 1991). The doubling time of DU145 cells ranges from 23.8 to 40.3 hours (Ware et al., 2007). Because of its characteristics—high invasive ability, adherent growth pattern, and adequate doubling time—DU145 was selected to examine the potency of broccoli bioactive compounds on inhibition of prostate cancer invasion.

DU145 cells (American Type Culture Collection, or ATCC) were cultured in 75 cm² cell culture flasks (Corning) in Eagle’s Minimum Essential Medium (EMEM) (ATCC), with 10% fetal bovine serum (FBS) (BenchMark), and 1% antibiotics composed of 100U/mL penicillin (Sigma-Aldrich) and 100mg/mL streptomycin (Sigma-Aldrich). The cell culture medium was renewed every 2-3 days. Once reaching 80%-90% of confluence, cells were harvested with 0.25% trypsin/0.02% EDTA solution, and passed to new flasks at the ratio of 1:5 to 1:10. All cell culture flasks were incubated in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. Prior to any study in this work, DU145 cells were starved with EMEM/0.1%BSA for 24 hours, in order to synchronize the cell cycle of stock cell, and minimize the variability caused by different stages of the cell cycle when cells grow.
Treatment Preparation

Sulforaphane (LKT laboratories), indole-3-carbinol (Sigma-Aldrich), 3,3’-diindolylmethane (Sigma-Aldrich), and quercetin (Sigma-Aldrich) were freshly made, dissolved in DMSO, and stored at -20°C, 4°C, and room temperature, respectively. Hydrolyzed neoglucobrassicin (extracted and purified by Dr. Berhow; ARS, USDA) was also freshly made by directly dissolving neoglucobrassicin in the EMEM with 20mU/mL myrosinase (Sigma-Aldrich).

Cell Counting Procedure for Plating

To count cells, duplicate cell counting solutions were made by adding 100 µL of well-suspended cells to each Eppendorf tube containing 570 µL of PBS and 330 µL of Trypan Blue. After mixing several times with a Pasteur pipet, the solution was placed into a hemocytometer. Cells in five fields (the shaded squares in Figure 2.1) were counted using a microscope. Each sample of cells was counted five times in separate hemocytometers. The calculation of cell number is displayed in calculation 2.1. All results from the duplications were averaged. Based on the averaged result, cell suspensions were diluted, using medium, to the desired concentration (5×10^4 cells/mL for Matrigel invasion assay and 1.25×10^5 cells/mL for the MTT assay).

Determine a Chemoattractant for Matrigel Invasion Assay

In order to choose a suitable chemoattractant that stimulates DU145 cell invasion, the Matrigel invasion assay was performed as previously described (Baba et al., 2000), with
modifications. After 24-hour starvation with EMEM/0.1% BSA, 2.5×10^4 cells (subcultured no more than 10 generations) were plated in Matrigel chambers (BD Bioscience) placed in 24-well plates with 100ng/mL epidermal growth factor (EGF) dissolved in EMEM or complete growth medium (EMEM/10% FBS) as the chemoattractant in the bottom wells, followed by a 48-hour incubation in an incubator with a humidified atmosphere with 5% CO₂ and 95% air at 37°C. After incubation, invading cells were fixed, stained, and observed under a microscope at the magnification of 200X. Seven light fields of each membrane were randomly selected, and cells in the light fields were counted.

**Invasive Ability of DU145 by Matrigel Invasion Assay**

The invasive ability of DU145 cells was examined by an invasion assay (Figure 2.2), which was carried out as previously described (Baba et al., 2000), with some modifications. Basically, DU145 cells underwent serum depletion with EMEM/0.1% BSA for 24 hours prior to the invasion assay. Cells were then harvested with 0.25% trypsin/0.02% EDTA solution and plated, at a concentration of 5×10^4 cells/mL, into Matrigel invasion chambers with the Matrigel layer on the porous membrane (pore size, 0.8µM). The Matrigel invasion chambers were placed in the 24-well plate with complete growth medium as the chemoattractant in each well. After incubation for 12, 24, 36, and 48 hours, non-invading cells were removed by swabbing with cotton swabs, while invading cells were fixed with anhydrous methanol blue (Sigma-Aldrich), stained with 1% toluidine blue (Sigma-Aldrich), and counted in 7 randomly selected microscope light areas at the magnification of 200X. The sum of 7 fields was averaged, and is shown in the result.
**Effect of Individual Broccoli Bioactives on DU145 Cell Invasion**

The goal of the invasion assay was to determine the ability of bioactives to suppress DU145 invasion. DU145 cells were starved, harvested, and plated as previously described. After cells attached to the Matrigel, medium in the chambers was randomly replaced with treatments (SF, I3C, DIM, and Q in DMSO, diluted by EMEM/0.1% BSA to the desired concentrations, or NGB, diluted by EMEM/0.1% BSA with 20Mu/mL myrosinase to the desired concentrations) or control solutions (EMEM/0.1% BSA with DMSO, for trials of SF, I3C, DIM, and Q; or EMEM/0.1% BSA with 20mU/mL myrosinase, for the trial of NGB). Plates were then incubated for 24 hours in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. After 24 hours, all treatments were renewed, and cells were incubated for another 24 hours to reach a total of 48 hour exposure time. Finally, Matrigel along with non-invading cells were gently removed by swabbing with cotton tips; and invading cells on the bottom of the membrane were fixed with methanol, stained with toluidine blue, and counted in 7 randomly chosen microscope light fields at a magnification of 200×. The results were then standardized by the mean of DMSO or control groups, and shown in the form of percent invasion.

**Effect of Broccoli Bioactives in Combination on DU145 Cell Invasion**

The purpose of this experiment was to investigate whether broccoli bioactives in selected combinations additively or synergistically affect DU145 cell invasion. The Matrigel invasion assay was performed as previously described (Baba et al., 2000); except the treatments were the selected bioactives in combinations (fixed level of SF (2µM) combined with increasing levels of
I3C (80, 120, 160, and 200nM), or sequential levels of SF (2, 3, 4, 5 µM) combined with fixed level of I3C (120nM)). At the end of the 48 hour treatment exposure, with treatments renewed at the 24th hour, non-invading cells were removed by cotton swabs, and invading cells were fixed, stained, and counted. The results were shown as the percent invasion.

**Cell Viability: MTT Assay**

To examine whether the decrease in the number of invading cells was influenced by cell viability, the MTT assay was used to test the cytotoxicity of effective treatments. The principle of MTT assay is based on the color change of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) caused by mitochondrial activity in living cells, where MTT is converted into insoluble purple formazan crystals, which can dissolve in solubilization solutions, such as acidified isopropanol, and form colored complex that are further analyzed by spectrometer (van Meerloo et al., 2011).

In this work, we performed the MTT assay according to previous studies (van Meerloo et al., 2011; Uslu et al., 1997), with modifications to mimic the procedure described in the Matrigel invasion assay. Prior to the MTT assay, DU145 cells were starved with EMEM/0.1% BSA for 24 hours. Then 12,500 of DU145 cells in 100µL of EMEM/0.1% BSA were seeded into 96-well flat bottom plates with 150µL of EMEM/10% FBS, and incubated for 2-3 hours. After cells attached to the bottom, suspension medium was replaced by 200µL of treatments (3µM, 4µM, 5µM, 10µM SF; and 160nM, 200nM, 1µM I3C dissolved in DMSO, with the concentration of DMSO not exceeding 0.1% of the final solution) dissolved in the solution containing 60% complete medium,
and 40% EMEM/0.1% BSA, which simulates the composition of the chemoattractant in the bottom wells and the suspension medium in upper inserts. All the treatments were conducted in triplicate. Medium alone served as the blank (n=6); cells without any treatment served as positive control (n=6); while treatments without cells served as reagent control (n=3), which were subtracted from the result to minimize the effects of treatments and reagents on absorbance reading. The 96-well plate, then, was incubated in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. In order to simulate the conditions used for the Matrigel invasion assay in our study previously described, all the treatments were renewed at the 24th hour, and incubated for another 24 hours. After a total of 48 hour treatment exposure, 20µL MTT was added to each well, and the plate was shaken for 5 minutes before incubation for 4-6 hours. Then unreactive supernatant was replaced by 150µL of acidified isopropanol, which dissolves the formazan crystals, and forms a colored solution. All the samples, controls, and blanks were moved to a new 96-well plate. Finally, the new plate with samples, controls, and blanks was analyzed using a plate reader (µQuant, MQX200, Bio-Tek Instruments, Inc., Winooski, Vermont) at 540nm and 720nm. The OD_{720\text{nm}} values were subtracted from OD_{540\text{nm}} values to minimize the background noise. The OD_{720\text{nm}-540\text{nm}} values were further adjusted by the blank and reagent controls.

Statistical Analysis

The results were statistically analyzed by SAS 9.3 (Cary, NC) using one-way ANOVA followed by LSD test with α=0.05. Significant differences are displayed with different letters
when sample sets have the same N, or with an asterisk when sample sets have different N. All the results are displayed as mean ± SEM. Linear regression analysis by SAS 9.3 (Cary, NC) was used for the study of I3C to determine the relationship between dose and percent invasion.

**Results**

*EMEM/ 10% FBS was selected as the Chemoattractant for the Matrigel Invasion Assay*

To select a suitable chemoattractant for the Matrigel invasion assay, epidermal growth factor (EGF) and complete growth medium (EMEM/10% FBS) were tested by the Matrigel invasion assay. The DU145 cell line was reported to have androgen receptors (AR) on the cell membrane. EGF is one of the ligands that can trigger cell invasion through AR (Alimirah et al., 2006). Therefore, EGF was chosen as one of the candidate chemoattractants. On the other hand, several previous studies used complete growth medium as a chemoattractant to stimulate cell invasion (Shen et al., 2010; Petersen et al., 2012; Guo et al., 2013), so we also selected it as another candidate chemoattractant.

In this study, 2.5×10^4 cells were seeded in Matrigel invasion chambers placed in the 24-well plates with 100ng/mL EGF in EMEM or 10% FBS in EMEM. After 48 hour incubation, invading cells were fixed, stained and counted. The result (Figure 2.3) shows that EMEM with 10% FBS was better able to induce cell invasion. Therefore, we used EMEM with 10% FBS as the chemoattractant in the following studies.
48 Hour Treatment Exposure was selected for Matrigel Invasion Assay

To examine the invasive ability of DU145 cells, cells were seeded into Matrigel invasion chambers after 24-hour starvation. After incubation for 12, 24, 36 and 48 hours, cells were fixed, stained, and counted. The suspension medium of samples incubated for more than 24 hours was refreshed at the 24th hour. Each time point had 6 replications. The result (Figure 2.4) shows that only a few cells invaded by the first 24 hours, but after 36 hours, there was more than a 10-fold increase in cell invasion. Since the goal of the work was to test the potency of broccoli bioactives to inhibit cell invasion, we desired high enough cell invasion to show the suppressive ability of those bioactives, so 12 and 24 hour were not considered. 36 hour was not considered as well, for this time point may be the initiation stage of log phase, where cell growth is not stable and may cause high variability. Therefore, 48 hour was selected as the exposure time in further experiments.

Sulforaphane and Indole-3-Carbinol Suppressed Prostate Cancer Cell Invasion

To determine whether SF and/or I3C could suppress prostate cancer cell invasion, we performed the Matrigel invasion assay with DU145 cells treated with different concentrations of SFN (1, 2, 3, 4, 5 µM in 0.1% DMSO), or I3C (40, 80, 120, 160, 200 nM in 0.1% DMSO), using 0.1% DMSO as a negative control. All the treatments were diluted by EMEM/0.1% BSA to the desired concentrations. Each treatment had 5 replications.

All the 24-well plates with Matrigel invasion chambers were incubated for 24 hours, and treatments as well as DMSO control were refreshed at the 24th hour in order to mimic the
condition of daily broccoli consumption. After refreshing treatments, cells were then incubated for another 24 hours to achieve a total exposure time of 48 hours.

SFN and I3C were able to suppress cell invasion at the concentration as low as 3µM (Figure 2.5) and 120nM (Figure 2.6A), respectively. Since I3C suppresses prostate cancer cell invasion at lower concentration compared to SFN, we can conclude that under the condition of the assay that I3C is a more potent bioactive than SFN. In addition, we noted a dose response for I3C. Although I3C only shows significance with the concentrations higher than 120nM, linear regression analysis (Figure 2.6B) illustrates that as the concentration of I3C increases, the invading cell number decreases, with $R^2=0.93$, $p<0.01$. Therefore, it appeared that the main bioactives in broccoli, SFN and I3C, may have the ability to suppress prostate cancer invasion.

_Hydrolyzed Neoglucobrassicin and Quercetin Did Not Suppress Prostate Cancer Cell Invasions_

To investigate if hydrolyzed NGB (hNGB) and Q are able to inhibit prostate cancer invasion, the Matrigel invasion assay was carried out essentially as described above, except that NGB was dissolved in EMEM/0.1%BSA, and added directly to the medium with myrosinase. Trials with 1-5 µM and 10-50µM hNGB were performed in two separate experiments. The results indicate that hNGB (Figure 2.7A, 2.7B) treatments over a wide range of concentrations failed to significantly suppress prostate cancer cell invasion even at the high concentration of 50 µM, a serum/tissue level that is not achievable from diet. Quercetin also did not significantly inhibit prostate cancer cell invasion at the levels of 1 to 5 µM (figure 2.8).
**Sulforaphane Combined with Indole-3-Carbinol Did Not Have Additive Effects**

We examined SF and I3C, alone or in combination on DU145 cell invasion: 1) using a fixed level of SF (2µM), combined with increasing levels of I3C (80, 120, 160, and 200nM) in three separate trials, and 2) a fixed level of I3C (120nM), combined with increasing levels of SF (2, 3, 4, and 5 µM). In the first set of studies, no significant loss were seen in invasion between the treatment combinations and the DMSO control (n=15, 5 of each trial) (figure 2.9). In the second set of studies, 5 µM SF alone, or in combination with 120nM, significantly reduced cell invasion (figure 2.10). Yet, there was no statistical difference between 5 µM SF treatment and 5 µM SF in combination with 120nM I3C, meaning that the combination of SF and I3C did not additively or synergistically suppress DU145 cell invasion.

"Aged" I3C was More Potent Than Fresh-Made I3C and DIM

According to results testing I3C individually and I3C in combination with SF, we found that the outcomes were inconsistent. In the study treating cells with I3C individually had significantly lower invasion using 120-200nM treatments (figure 2.6); while in the study treating cells with the combination of I3C and SF did not result in a decrease in invasion even with the level of 200nM I3C alone (figure 2.9). The differences may be caused by the fact that we used the I3C that had been stored for months in the individual study, but used fresh-made I3C in the combination study. Since I3C is an unstable compound, it can spontaneously condense to potent polymeric products, mainly 3,3'-diindolylmethane (DIM). Therefore, we further compared the bioactivity of "aged" I3C, fresh-made I3C, and DIM (with levels equivalent to I3C). The result
(figure 2.13) shows that only “aged” I3C significantly inhibited DU145 cell invasion with levels of 120-200nM, which matches what we previously found. However, fresh-made I3C and DIM did not significantly decrease DU145 cell invasion. The “aged” I3C must widely contained DIM and additional polymeric products that may act in a synergistic manner to inhibit cell invasion.

**Cell Viability of DU145 was not Decreased by the Treatments of SF or I3C**

The MTT assay was used to determine whether the decrease in invading cells was caused by cell death. Therefore, the entire study simulated the condition of the Matrigel invasion assay. In short, DU145 cells were starved for 24 hours, followed by seeding, 2-hour incubation, treatment (3µM, 4µM, 5µM, and 10µM sulforaphane; and 160nM, 200nM, and 1000nM indole-3-carbinol), 24-hour incubation, treatment renew, and additionally 24-hour incubation. After a total of 48 hour treatment exposure, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each well, and incubated for 4 to 6 hours. The purple crystals formed by living cells were dissolved by acidified isopropanol, yielding the colored solution. Finally, the absorbance of the colored solution was read using a spectrometer.

The invasion-suppressive concentrations of SF (3µM, 4µM, and 5µM) and I3C (160nM and 200nM) did not reduce cell viability (figure 2.11 and figure 2.12). In other words, the decrease in invasion of cells treated with SF or I3C was not likely due to cell death, but due to the suppression of invasive ability of DU145 cells.
Discussion

Sulforaphane

Sulforaphane (SF) has been considered a potent chemopreventive agent for a number of cancer types. Most of previous studies have examined the property of SF using pharmaceutical levels for cancer prevention and apoptosis induction. Relatively few studies have looked at metastasis suppression, especially with physiologically-achievable levels. Our study indicated that 3-5 µM SF significantly inhibited invasion of DU145 cells without causing cell death. According to the literature, 3-5 µM SF in the human plasma can be achieved by the consumption of 136-227 grams of broccoli (Gasper et al., 2005).

We also examined the invasion-suppressive ability of SF combining with I3C. Figure 2.9 suggests that the combinations, with a fixed concentration of SF (2µM) and increasing concentrations of I3C (80nM, 120nM, 160nM, and 200nM), did not significantly inhibit prostate cancer cell invasion. This result was pooled from three separate trials, with no significance seen in the individual trail. Figure 2.10 demonstrated that 5 µM SF alone, or the combination of 5 µM SF and 120 nM I3C significantly inhibited invasion of DU145 cells. In our earlier trial testing SF individually, we saw significant decreases in invasion with 3-5 µM SF (figure 2.5). However, in this trial combining 3µM, 4µM, or 5 µM SF with 120nM I3C, only the combination of 5 µM with 120 nM significantly inhibited DU145 cell invasion. These surprising results may be due to experimental variability, which needs to be minimized in additional trials.
To understand more about how a mixture of broccoli bioactives may provide added benefit, effects of other combinations of bioactives on prostate cancer cell invasion need to be investigated in the future.

*Indole-3-Carbinol*

High levels of I3C has been reported to inhibit cell growth and induce apoptosis, yet few studies have been carried out regarding the effect of I3C on cancer cell invasion with far lower levels. The result of our study showed that 120-200 nM I3C significantly inhibited invasion of DU145 cells (figure 2.6). However, it is not clear whether the invasion-suppressive property was attributed to I3C or its polymeric products.

I3C is an unstable indole, especially in low pH environment such as stomach. Once generated, I3C tends to undergo a condensation reaction, forming polymeric products, mostly 3,3'-diindolylmethane, or DIM. Reed and others (2006) reported that 1 hour after I3C supplement intake (400mg and 1000mg), high levels of DIM (61ng/mL and 607ng/mL, respectively), but no I3C, could be detected in human plasma (Reed et al., 2006). Although cell culture has a different environment than in human bodies, Bradlow and Zeligs (2010) suggested that approximately 60% of I3C was converted into DIM at the end of a 24 hour incubation in cell culture medium, a pH-neutral environment (Bradlow & Zeligs, 2010). If I3C from broccoli was not converted into other compounds after consumption, 115g of broccoli consumption would theoretically achieve the level of 120nM in the plasma. Otherwise, 61ng/mL and 607ng/mL DIM
could be detected after supplementation of 400mg and 1000mg I3C, respectively. However, we do not know how much I3C was in the form of DIM in this study.

In our study investigating I3C individually, we treated cells with the I3C that had been stored for a few months, and the percent invasion of cells was significantly inhibited (figure 2.6). This “aged” I3C appeared to be very bioactive in these assay conditions, as we found no significant decrease in invasion of DU145 cells treated with 200nM fresh-made I3C in the study treating cells with SF and I3C, alone, or in combinations (figure 2.9). According to Bradlow and Zeligs (2010), fresh I3C in medium spontaneously condenses to DIM and other polymeric compounds in 24 hours. “Aged” I3C may potentially have a higher composition of DIM and other polymeric compounds in comparison to fresh-made I3C that we refreshed every 24 hours in the Matrigel invasion assay. Therefore, to compare the potency of fresh I3C, “aged” I3C, and DIM, and to verify if the bioactivity of “aged” I3C is mostly attributed to DIM, we conducted the Matrigel invasion assay with 80-200nM fresh I3C, 80-200nM “aged” I3C, and 40-100nM DIM (equivalent to 80-200nM I3C). In this study, we expected DIM would suppress invasion since DIM is the main compound formed during I3C condensation reactions. However, we found that “aged” I3C, but not fresh-made one, was able to suppress DU145 cell invasion with levels of 120-200nM (figure 2.13), the similar outcome as in the individual study with “aged” I3C (figure 2.6). The reason that “aged” I3C acting more potent than DIM could be that the complex of polymeric compounds has higher bioactivity than I3C or DIM alone. More studies need to be done to analyze compositions of “aged” I3C and interactions between polymeric products of I3C.

We also tested cell viability with fresh-made I3C using concentrations of 160 nM and 200 nM, and found that these two levels of I3C did not decrease cell viability of DU145. However, we
cannot conclude if the “aged” I3C would have the same result. Therefore, more investigation about cell viability by treating with the “aged” I3C or DIM alone needs to be completed.

*Neoglucobrassicin*

The results of the studies with hydrolyzed neoglucobrassicin (hNGB) in the Matrigel invasion assay suggest that hNGB did not inhibit prostate cancer cell invasion (figure 2.7). There are limited studies of NGB reported in the literature, but most would support lack of bioactivity for this form of glucosinolate. According to the Haack group (2010), hNGB did not stimulate anti-cancer activity, such as Nrf2 translocation, glutathione peroxidase 2 (GPx2) protein expression, or GPx2 promoter activity in HepG2 (human liver cancer cells), indicating that hNGB did not have anti-cancer properties in the liver cancer cells. In addition, hNGB weakened the bioactivity of sulforaphane-mediated induction of the expression of NQO1 protein, GPx2 protein, and GPx2 promoter activity in HepG2 cells (Haack et al., 2010). Liu (2014) also reported that the TRAMP mice fed with diet containing glucosinolates-enhanced broccoli power—with 7.6 times more NGB than standard broccoli—did not reduce prostate cancer development in vivo (Liu et al., 2014, in press). Interestingly, Vo (2014) provided positive impact of NGB. They suggested that NGB, not hydrolyzed NGB, was able to decrease lipopolysaccharide-induced inflammation by reducing TNF-α expression in THP1, human monocyte cells (Vo et al., 2014).

The different outcomes from these three previous studies may be due to different experimental targets. Haack’s and Liu’s studies are focused on the cancers in liver and prostate, respectively; while Vo focused on leukocytes. Therefore, NGB and hNGB may affect differently in
these two distinct experimental models with distinct mechanisms. However, more studies need to be done to elucidate the mechanisms of NGB and hNGB in different model systems.

*Quercetin*

Quercetin (Q) is a good antioxidant that can scavenge reactive oxygen species (ROS), a class of reactive free radicals that may lead to carcinogenesis (Afanas’ev et al., 1989). Our study examined the invasion suppressive ability of Q on the human prostate cancer cell, DU145, but failed to find any significant decrease in invading cells over the concentration range from 1-5 µM (figure 2.8).

Previous studies suggested that 25µM and 50µM Q were capable of suppressing colony formation of two invasive human prostate cancer cell lines, PC-3 and DU145, but were not able to suppress growth of non-invasive cell line, LNCaP (Nair et al., 2004), indicating that Q may particularly target invasive cell lines of human prostate cancer, but at high concentrations. Senthilkumar and colleagues (2011) suggested that 50 and 100µM Q significantly reduced the invasiveness of PC-3 cells (Senthilkumar et al, 2011). They further examined the expression of mRNAs related to the induction of invasion in prostate cancer cells. The results show that 100 µM Q-treated PC-3 cells were found to have lower levels of uPA, uPAR, EGF, and EGF-R, meaning that the invasiveness of PC-3 may have been suppressed by Q through the inhibition of uPA, uPAR, EGF, and EGF-R (Senthilkumar et al, 2011).

Our study treated DU145 cells with much lower levels of Q than others had used. Thus, it may not be surprising to find lack of significance effects for Q on prostate cancer cell invasion.
Among the studies we have reviewed, 25µM Q is the lowest effective concentration that inhibited colony formation of DU145 cells (Nair et al., 2004). To achieve this level in the plasma, 4.3 grams of pure Q aglycone, or about 1.5 kg of onions containing 330 mg Q would need to be consumed. This also suggests that the absorption of Q from dietary sources is better than quercetin aglycone (reviewed in Manach et al., 2005). Although such invasion-suppressive levels of Q cannot be achieved by the consumption of broccoli, balanced selective plant foods consumed with some dietary supplements could reach the effective levels of Q by the intake of a wide variety of spices, fruits, and vegetables, particularly onions, where Q is present abundantly and can be better absorbed. Alternatively, it would be helpful to study the absorption of broccoli Q glycosides, mainly quercetin-3-O-sophoroside, in the future since these glycoside form of quercetin are rarely present in other plants.

**Future Study**

**Bioactive Components**

In this work, we focused on effects of broccoli bioactives on prostate cancer cell invasion in vitro using physiologically-achievable levels. Results show that SF and “aged” I3C were protective against DU145 prostate cancer cell invasion in the Matrigel invasion assay, and also indicate that invasion-suppressive concentrations of SF did not cause DU145 cell death in the MTT assay. However, the cytotoxicity of “aged” I3C still needs to be evaluated to determine if “aged” I3C would cause cell death.
Since DIM is the main product of I3C condensation reactions, we compared the potency of “aged” I3C, fresh-made I3C, and DIM on DU145 cell invasion. The result shows that “aged” I3C was able to suppress DU145 cell invasion, but fresh-made I3C and DIM did not. To understand what polymeric products in “aged” I3C play main roles in inhibiting DU145 cell invasion, a detailed analysis of “aged” I3C composition needs to be done in the future. The Matrigel invasion assay using combinations of these polymeric products would also help us know if these polymeric products act synergistically or additively.

RNA Expression

Invasion is the beginning stage of metastasis, a process of cancer dissemination from the primary organ to other parts of the body. Two major steps are involved in invasion: epithelial-mesenchymal transition (EMT) of tumor cells and degradation of extracellular matrix (ECM). The ability of cells to accomplish invasion is highly related by specific genes. Literature has shown that up-regulation of N-cadherin and down-regulation of E-cadherin is related to occurrence of EMT (Tomita et al., 2000; Gravdal et al., 2007); while up-regulation of urokinase-like plasminogen (uPA) (reviewed in Hanahan & Weinberg, 2000; Arya et al., 2006) and certain kinds of matrix metalloproteinases (MMPs) (Page-McCaw et al., 2007; Lichtinghagen et al., 2002; Xiao et al., 2012) is associated with degradation of ECM. Taken together, the RNA expression of these proteins can be biomarkers that determine invasive ability of prostate cancer cells. Therefore, knowing the RNA expression profile of these proteins may be helpful to know if cells treated with broccoli bioactives have less invasive ability.
Conclusion

In this work, we investigated the ability of broccoli bioactive compounds to suppress prostate cancer cell line, DU145, invasion individually, or in selected combinations using physiologically-achievable levels. Among several different broccoli bioactives, 3-5µM SF and 120-200nM “aged” I3C significantly inhibited DU145 cell invasion. Additionally, 5µM SF in combination with 120nM “fresh” I3C significantly inhibited invasion, but this combination was not significantly different from 5µM SF treatment alone, indicating that the combination of SF and fresh-made I3C treatment did not synergistically or additively suppress prostate cancer cell invasion. Other bioactive components—Q, hNGB, DIM—were also examined individually, but no significant impact on DU145 cell invasion was found even treating cells beyond physiologically-relevant levels of Q and hNGB.

We also examined cell viability by the MTT assay. Results show that 3-5µM SF did not significantly cause cell death, indicating that SF inhibited DU145 cell invasion not by causing cell death, but by inhibiting invasive ability of cells.

Further study of the MTT assay with “aged” I3C needs to be carried out in the future. In addition, invasion-related RNA expression of DU145 cells treated with SF or “aged” I3C should be examined as well.
Figures and Calculation

Figures

Figure 2.1. The shaded squares on the hemocytometer were chosen for cell counting.

Figure 2.2. The Matrigel invasion system is composed of a Matrigel chamber, with a layer of Matrigel on the porous membrane, placed in the 24-well plate with chemoattractant, which is able to attract cells in the chamber to invade toward the bottom of the porous membrane.
**Figure 2.3.** The ability of 100ng/mL EGF and 10% FBS to stimulate DU145 cell invasion (N=5; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.

**Figure 2.4.** Effect of incubation time on DU145 cell invasion assay (N=6; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.
Figure 2.5. The percent invasion of DU145 cells treated with sulforaphane (N=5; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.

Figure 2.6. The percent invasion of DU145 cells treated with “aged” indole-3-carbinol (N=5; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05, shown in chart A; and further analyzed by linear regression analysis, shown in chart B. Means with different letters are significantly different.
Figure 2.7. The percent invasion of DU145 cells treated with neoglucobrassicin (A: N=6; B: N=5; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.

Figure 2.8. The percent invasion of DU145 cells treated with quercetin (N=10; mean ± SEM). The assay were carried out in two separate trials with N=5 in each trial, and results were pooled and analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.
Figure 2.9. The percent invasion of DU145 cells treated with SF or I3C or in combination (N=15; mean ± SEM). The assay were carried out in three separate trials with N=5 in each trial, and results were pooled and analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.

Figure 2.10. The percent invasion of DU145 cells treated with SF or I3C or in combination (N=5; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Means with different letters are significantly different.
**Figure 2.11.** The cell viability of DU145 cells treated with sulforaphane (treatment: N=3, DMSO: N=6; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Significant differences are indicated by “*”.

**Figure 2.12.** The cell viability of DU145 cells treated with indole-3-carbinol (treatment: N=3, DMSO: N=6; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Significant differences are indicated by “*”.
Figure 2.13. The percent invasion of DU145 cells treated with “aged” I3C, fresh-made I3C, and DIM (DMSO: N=5, treatments: N=3; mean ± SEM). The result was analyzed by one-way ANOVA, followed by LSD test with alpha=0.05. Significant differences are indicated by “*”.

Calculation

Calculation 2.1. The calculation for cell number using a hemocytometer.

1. **Number of cells from chamber 1 + number of cells from chamber 2 = X**

2. \( \frac{X}{10} = the\ number\ of\ cells/square\ (Q) \)

3. \( Q \times 10\ \text{ (dilution factor)} \times 10^4 = number\ of\ cells/mL\ in\ the\ medium \)
Chapter III

Bibliography


