The Effects of Cranberry Phytochemicals on the Behaviour of Prostate Cancer *In Vitro* and *In Vivo*

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Abstract

With an estimated 24,600 cases, prostate cancer is one of the most prevalent cancers in Canada, and evidence suggests that the development of prostate cancer can be related to the consumption or avoidance of certain foods. Recently there has been considerable interest in the phytochemical components of the North American cranberry (*Vaccinium macrocarpon*), as they have been shown to affect certain behaviours of cancerous cells in an *in vitro* setting. The work in this thesis demonstrates the effects of whole cranberry extract (WCE) and proanthocyanidins (PACs) from the American cranberry on the development and behaviour of prostate cancer cells in an *in vitro* setting, and details the results of a pilot study of a cranberry-infused diet in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. Cranberry PACs were shown to affect the viability of DU145 human prostate cancer cells and decreased the activity of both MMP 2 and MMP 9, both of which are associated with the metastatic potential of prostate cancer *in vivo*. PACs also affected specific cellular signalling pathways which have been associated with the expression of MMPs 9 and 2. WCE, like PACs, affected the viability of DU145 cells. WCE also decreased the expression of cell cycle associated proteins, increased the expression of a cell cycle inhibitor protein, and decreased the movement of DU145 human prostate cancer cells through the cell cycle. The results of the TRAMP mouse pilot study detailing the effects of a cranberry-infused diet on the behaviour of prostate cancer demonstrated no appreciable effects on the size or weight of the prostate tumour and surrounding lower urogenital tract. There was also no significant different in the survival rates of TRAMP mice fed a 3% cranberry diet, nor changes in the expression of cell cycle associated proteins. Interestingly, a cranberry-infused diet decreased the weight of control mice during a four week period of time. Results from this study suggest that cranberry compounds can affect

the behaviour of prostate cancer cells and its affects should be characterised further, with additional modifications necessary to order to further investigate its affects *in vivo*.

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List of Abbreviations

AIPC	Androgen-Independent Prostate Cancer
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
AR	Androgen Receptor
AVC	Atlantic Veterinary College
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent Kinase
CKI	Cyclin-dependent Kinase Inhibitor
DMSO	Dimethyl Sulfoxide
ECM	Extra-cellular Matrix
EGCG	Epigallocatechin-3-gallate
EGF	Epithelial Growth Factor
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
FBS	Fetal Bovine Serum
G	Gravity
GSE	Grape Seed Extract
IGF-1	Insulin Growth Factor 1
LPS	Lipopolysaccharides
LUT	Lower Urogenital Tract
MAPK	Mitogen-activated Protein Kinase
MEK5/ERK5	Mitogen/extracellular-signal-regulated kinase kinase 5/extracellular signal-regulated kinase-5
MEM	Minimal Essential Media

MMP	Matrix Metalloproteinase
NBF	Neutral Buffered Formalin
NF-κß	**Nuclear Factor Kappa**-light-chain-enhancer of Activated **B** Cells
PACs	Proanthocyanidins
PAGE	Polyacrylamide Gel Electrophoresis
PANC-1	Pancreatic Cancer Cells
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PD	Poorly Developed
PI3K	Phosphatidylinositol 3-kinase
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
RB	Retinoblastoma
RECK	Reversion-inducing Cysteine-rich Protein With Kazal Motifs
SDS	Sodium Dodecyl Sulphate
SV40	Simian Virus 40
TNF-alpha	Tumour Necrosis Factor Alpha
TGHQ	2,3,5-*tris*-(glutathion-S-yl) Hydroquinone
TIMP	Tissue Inhibitors of Matrix Metalloproteinase
TNF-α	Tumour Necrosis Factor Alpha
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
WCE	Whole Cranberry Extract
WD	Well Developed
WHO	World Health Organization

CHAPTER ONE

Introduction and Literature Review

1.1. Introduction

For the last 40 years, incidences of prostate cancer in Canada have been steadily increasing, with over 24,000 estimated cases in 2010. Though more is known about this disease than ever before and treatment options have significantly improved, there are still over 4,000 deaths in Canada from prostate cancer every year (Canadian Cancer Society, 2010). As such, new strategies for the treatment and prevention of this disease are necessary. One interesting observation which has been made time and again is the correlation between the consumption of fruits and vegetables and a decreased risk of developing certain types of cancer (Block, Patterson et al. 1992; Riboli and Norat 2003; Kirsh, Peters et al. 2007). Because of these repeated observations, it has been suggested that there are families of compounds within fruits and vegetables which work alone or in tandem with each other to alter the behaviour and development of cancer *in vivo* (Liu 2004). One fruit which has a number of interesting and potentially anti-cancer compounds is the American cranberry (*Vaccinium macrocarpon).* In previous studies, whole cranberry extract (WCE) and fractions enriched with specific phytochemical families from this fruit have been shown to affect the behaviour of certain cancer cells by decreasing their cellular viability via apoptotic pathways, affecting the expression of specific pro-inflammatory proteins, and decreasing the expression of certain proteins associated with the metastatic potential of cancer (Neto 2007). The work presented in this thesis seeks to demonstrate some of the anti-cancer effects of WCE and proanthocyanidins (PACs) on DU145 human prostate cancer cells, as well as demonstrate the results of a pilot study examining the effects of a cranberry-infused diet on the behaviour of prostate cancer in a mouse model.

1.1.1. Research rational and general hypothesis

Previous research in our laboratory has demonstrated the effects of WCE on the behaviour of DU145 cells, including decreases in cellular viability and decreases in the expression of MMP 2 and 9, which are key proteins linked with the metastasis of cancer *in vivo* (Egeblad and Werb 2002). This work seeks to expand the work done previously by examining a specific family of compounds extracted from the cranberry, specifically the proanthocyanidins (PACs). Cranberry has also been shown in *in vitro* models to affect cancer cell viability, alter the expression of cell cycle proteins, and decrease the movement of cells through the cell cycle (Ferguson, Kurowska et al. 2004; Jie and Rui Hai 2006). Furthermore, it has been observed that phytochemical compounds have affected the expression of cell cycle proteins in *in vivo* prostate cancer models (Raina, Singh et al. 2007; Singh, Raina et al. 2009). As well, MMP expression in other cancer cell types after treatment with this family of compounds has been noted (Vayalil, Mittal et al. 2004; La, Howell et al. 2009) and other anti-cancer effects of WCE on prostate cancer behaviour have been demonstrated in our lab and elsewhere. Therefore, **it was hypothesized** that cranberry phytochemicals would affect the behaviour of prostate cancer cells both *in vitro* and *in vivo*.

The specific objectives of this thesis were threefold:

1) To determine at what time points and concentrations there were significant decreases in MMP activity after treatment with PACs on DU145 cells, and through what cellular pathways these decreases may be occurring.
2) To determine if WCE affects the expression of key cell cycle proteins, and whether WCE can affect the movement of human prostate cancer cells through the cell cycle.

3) To determine if a cranberry-enriched diet can affect the development and behaviour of prostate cancer *in vivo*. The Transgenic Adenocarcinoma of the Mouse Prostate model was used in this experiment.

1.2. Literature Review

1.2.1. Cancer: Etiology, Epidemiology and Treatment

Cancer has been studied in medicine for at least the last three millennia. The first written accounts in the Western world are thought to have been made by Hippocrates during the fifth century B.C., where he described many types of cancers including cancer of the breast, neck and uterus (Karpozilos and Pavlidis 2004). Interestingly, for "hidden" cancers (cancers unseen at the surface) he recommended no treatment, as treatment of this form of cancer was likely to decrease the lifespan of the patient rather than prolong it. Other ancient physicians such as Galen even suggested a link between the development of cancer and the environment / diet of the individual or population (Karpozilos and Pavlidis 2004). Though there were advances in cancer treatment in the ancient world, medical research and development were stalled after the downfall of the Roman Empire, evidenced by the fact that cancer treatment between the times of the Greeks and Romans and the middle ages did not change significantly.

Presently, cancer is the third leading cause of death in the world. In 2007, there were over 12 million new cases and an estimated 7.6 million cancer deaths. It is also expected that by 2030, the number of cancer cases diagnosed yearly will increase to 26 million. Much of this increase will likely occur in low to moderately developed countries which will stretch already inadequate health care resources (Thun, DeLancey et al. 2010). Although aging populations in developed and developing countries contribute significantly to these projected increases in world cancer incidence, modifiable risk factors also play an important part in the development of cancer. These risk factors include such things as smoking, physical inactivity and the adoption of a more "Western" diet (Thun, DeLancey et al. 2010). It has been estimated that as many as 90-95% of all cancer cases are preventable by diet and lifestyle modifications which include the

cessation of smoking, reduced intake of red meat and energy-dense foods and an increase in physical activity (Anand, Kunnumakara et al. 2008). Because of this fact, health organizations, including the World Health Organization (WHO), are focusing efforts on the prevention of cancer through promotion of a healthy lifestyle (Petersen 2009).

Cancer can be characterized as multiple different diseases originating from the same problem, the unmitigated division and growth of many separate cell types. It can be considered as multiple diseases since different cell types, once they have made the transition from normal cells to malignant cells, act dissimilarly from one another. Though many forms of cancer have similar genetic defects such as mutations in the p53 and pRB genes (Sherr and McCormick 2002), many specific cancers have unique genetic mutations. For example, men who develop prostate cancer are more likely to have genetic mutations in the BRCA1, BRCA2 and BRIP/FANCJ genes (Mitra, Fisher et al. 2008; Kote-Jarai, Jugurnauth et al. 2009). Though many oncogenes and tumour suppressor genes are shared between cancer cell types, each type of cancer follows its own path into becoming a malignant tumour. Though cancer acts as though it were multiple different diseases, all cancers have six characteristic hallmarks as described by Hanahan and Weinberg (2000). These hallmarks include the evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth factors, tissue invasion and metastasis, limitless replicative potential, and sustained angiogenesis. Because of these six different characteristics, it is important in any treatment of cancer to identify the disease at its earlier and smaller stages and eliminate it from the body. It is especially important to identify and eliminate small, well-defined tumours before they spread and metastasize to different regions of the body. Most solid tumours are not life-threatening in and of themselves; it is when they become metastatic and spread to other areas of the body when they become life threatening (Kauffman, Robinson et al. 2003). This is the case with many types of cancer, including breast, colorectal, and prostate

cancer (August, Ottow et al. 1984; Pinski and Dorff 2005; Mastro, Clavarezza et al. 2007). As such, one of the goals of cancer treatment or prevention is to stop or prevent the spread of cells throughout the body. Current cancer treatment against primary tumours and some distant site metastase, focuses on the elimination of fast-growing cells. This is usually accomplished with a combination of chemotherapy and radiation, as these forms of treatment eliminate fast-growing cells (Kalinsky, Ho et al. 2009). Unfortunately, these forms of treatment can often have adverse side effects including inducing nausea and vomiting, central nervous system and cardiovascular toxicity (Bovbjerg 2006; Carole, Damien et al. 2009; Renske, Patrick et al. 2009). Because of these negative side effects, new forms of prevention and treatment are required.

1.2.1.1. Diet and Cancer

During the last 25 years, a wealth of scientific knowledge has shown the implications of certain dietary choices and the development / prevention of cancers. The focus of much research has examined the role of specific foods and the intake of excess foods in the development of cancers of the colon, breast, pancreas, lung and prostate cancer. For example, it has been noted for almost a century that the reduction of caloric intake has decreased the development of tumours in animal models, most notably mammary tumours. An excess of fat intake, specifically animal fats, has been shown to increase the probability of developing specific cancers including cancers of the breast, colon and prostate. Fibre has also been hypothesized to reduce the risk of colon cancer by means of increasing the speed at which carcinogens leave the body and because of their ability to bind to carcinogenic substances (Willett 2000). Another observation which has been made is the relationship between fruit and vegetable intake and the development of cancer. The inverse relationship between a higher consumption of fruits and vegetables and developing cancer has been shown in over 200 different scientific studies, which includes cancers of the lung, stomach, cervix bladder, kidney and breast (Willett 2000). Though

the inverse relationship between consumption and cancer risk is clear, the exact mechanisms are unknown, though there have been many hypotheses suggested. Some of these potential mechanisms include: the anti-oxidant action of many natural compounds, anti-inflammatory compounds, the modulation of the immune system, anti-angiogenesis and the induction of specific anti-cancerous proteins (Tsuda, Ohshima et al. 2004). These protective factors in foods could be related to a number of different food constituents including carotenoids, vitamin C, flavonoids, phytoestrogens and isothiocyanates (Willett 2000). Other protective factors include specific antioxidants including lycopene, lutein and epigallocatechin-3-gallate (EGCG), probiotics, and vitamin D (Donaldson 2004).

1.2.2. Prostate Cancer: History, etiology and treatment

The identification and treatment of prostate cancer has been evaluated for at least the last four and a half centuries (Sriprasad, Feneley et al. 2009). Most forms of treatment in earlier times focused on the removal of the prostate gland, which is called a prostatectomy. The first recorded successful complete removal of a prostate was performed in 1895 by Eugene Fuller (Sriprasad, Feneley et al. 2009). Interestingly, the discovery of radium during this time period led to the development of a new form of prostate cancer treatment: radiation. X-rays were initially used to destroy the disease by inserting a radium tube into a catheter and exposing the prostate to the radiation (Lytton 2001). Though these treatments did show improvements in patient prognosis, one of the major advances in prostate cancer treatment in the 20th century occurred in 1941 when Dr. Charles Huggins first noted that castration and the injection of estrogens into patients with metastatic prostate cancer had a beneficial effect (Sharifi, Gulley et al. 2005). This procedure, in fact, lowered the amount of circulating androgens in the body. Today, androgen ablation is used in the treatment of prostate cancer and is the most common form of treatment

with the exception of prostatectomies (Lu-Yao, Albertsen et al. 2008). Androgen ablation works by eliminating circulating androgens in blood by either chemical or physical castration. Since prostate cells, and therefore prostate cancer cells, need androgen for both maintenance and growth, the elimination of this hormone reduces the number and the growth of prostate cancer cells and causes cells to undergo apoptosis (Galbraith and Duchesne 1997). Unfortunately, human prostate cancer is a heterogeneous disease in that not all of the cancer cells will be dependent on androgen in order to survive. The ability of human prostate cancer to transform into an androgen-independent disease makes it a lethal disease, as once it has progressed to androgen-independent prostate cancer (AIPC) there are no permanent treatment options (Feldman and Feldman 2001). The ability of prostate cancer to transform into AIPC has been hypothesized to occur by 5 different methods: hypersensitive androgen receptors (ARs) which respond to very low levels of androgens, mutated ARs which bind to a variety of circulating hormones, the promotion of growth factors which can activate genes normally activated by ARs (e.g. IGF-1), bypassing the AR pathway (e.g. increasing anti-apoptotic pathways), and by precursor epithelial stem cells already within the prostate which were never androgen sensitive to begin with (Feldman and Feldman 2001). Because of these mutations and behaviours which allow prostate cancer to bypass androgen ablation, it remains difficult to treat.

Though death rates for prostate cancer have decreased in the developed world, it is still the third leading cause of cancer deaths in men in Canada according to the Canadian Cancer Society (2010). There are a variety of possible reasons as to why this disease is more prevalent in the Western countries than in east Asia, which have the lowest rates of prostate cancer in the world (Grönberg 2003). The first reason that cancer rates are higher in the West is because of the nature of prostate cancer itself. Prostate cancer affects elderly men much more so than younger men. The average age of a diagnosis for prostate cancer occurs between the ages of 72-74 years,

and 0.1% of all prostate cancer cases occur in men who are less than 50 years of age (Grönberg 2003). Because men in the West have higher life expectancy rates than their counterparts in other areas of the world, proportionally more Western men are diagnosed. But, countries that have similar life expectancy rates than those in North America and Europe, including Japan and South Korea, have lower rates of prostate cancer. Because of this, is has been suspected that both ethnicity and environment play a part in differences in the rates of prostate cancer. Epidemiological evidence demonstrates that the highest rates of prostate cancer in the United States occur within the African American population, which has a prostate cancer diagnosis rate of 165.8 people / 100,000 population. This is in contrast to people of Caucasian descent who have a diagnosis rate of 105.5 people / 100,000 population (Baade, Youlden et al. 2009). Though rates of prostate cancer are lower for people of Asian descent, it has been observed that rates of prostate cancer for Asian men living in the United States are higher than those living in their countries of origin. In one study, rates of prostate cancer incidence in Japan were shown to be 68% lower than rates in the United States, but the rates of prostate cancer for Japanese men living in the United States were only 44% lower than the rest of the population. A similar trend was also seen for men of Chinese and Filipino descent in the same study (Cook, Goldoft et al. 1999). So, although clearly genetics play a role in prostate cancer development, environment has an influence as well.

1.2.2.1. Diet and Prostate Cancer

One of the suggested environmental factors affecting the difference in the rates of prostate cancer between men in North America and the East is diet (Watanabe, Nakayama et al. 2000; Crawford 2003). Due to this suggested link, there have been epidemiological studies examining the intake of certain types of foods and the risk of developing prostate cancer. Some

of these studies have provided clues to researchers and health professionals as to what types of foods can either prevent or promote the development of prostate cancer. Analyses examining dietary consumption and prostate cancer incidence in Western countries have found a correlation between the consumption of total meat, alcohol, cooking oils, dairy products and vegetable shortening (Grant 2004; Colli and Colli 2005; Ambrosini, Fritschi et al. 2008). Some of the reasons why these foods may cause prostate cancer include an increase of sex hormone consumption (as animal fat has a high concentration of sex hormones), fatty acid compounds and their metabolites causing an increase in both benign prostatic and prostate cancerous growth, and an increase in the production of free radical compounds which are known to increase the severity of prostate cancer (Willis and Wians 2003).

There are a number of epidemiological studies suggesting that there are many foods and specific compounds derived from diet, which can play a preventative role in the development of prostate cancer. Some of these foods and compounds include fruits and vegetables, especially cruciferous vegetables, selenium, vitamin E and lycopene (Clark, Dalkin et al. 1998; Cohen, Kristal et al. 2000; Kolonel, Hankin et al. 2000; Brooks, Metter et al. 2001; Giovannucci, Rimm et al. 2002; Zhang, Ni et al. 2002). Despite the positive evidence that some fruits, vegetables and compounds can prevent or delay the onset of prostate cancer, there are other epidemiological studies which find either weak links or no such links between certain foods, supplements and prostate cancer, suggesting that more research is necessary to determine the validity of including specific foods and/or supplements in a preventative diet (Clinton and Giovannucci 1998; Lawson, Wright et al. 2007). One recent study examining the effects of vitamin E and/or selenium supplementation on the development of prostate cancer in a large American cohort showed that there were no differences between the placebo and treatment groups (Lippman, Klein et al. 2009). Vitamin E consumption was weakly associated

with an increased risk of developing prostate cancer (P = 0.06) and selenium consumption was weakly associated with an increased risk of developing type II diabetes (P = 0.16) (Lippman, Klein et al. 2009). Because of this, more research is necessary to determine the etiology of prostate cancer and to determine possible mechanisms for the prevention of this disease.

1.2.3. The American Cranberry: *Vaccinium macrocarpon*

The American cranberry is a fruit native to the American northeast and naturally grows in bogs stretching from the Atlantic Canadian provinces to as far south as Tennessee. It grows in soils which have relatively low pH (4-5). It is an economically important crop in some US states and in Canada, which had over 10,000 acres dedicated to cranberry production in 2008 (Sandler and DeMoranville 2008).

The cranberry has been studied for decades for its potential health effects. In 1933, an analysis of *Vaccinium macrocarpon* demonstrated that it had relatively large amounts of vitamin C and iodine. Interestingly, the fruit also was also shown to contain a large amount of fiber, tannins, and "undetermined" compounds (Fellers 1933). Later research showed some of the potential health benefits of incorporating the cranberry into the diet, including decreasing pH which could help treat urinary tract infections and increasing calcium in the body (Mindell, Esselen et al. 1939; Kaye 1968). Recent research on the health effects of the cranberry have focused on a variety of perceived health benefits including cardiovascular benefits, decreased adherence of certain harmful bacteria in the body, the anti-oxidant capabilities of certain cranberry compounds, the reduction of LDL cholesterol, and its potential anti-cancer benefits (Leahy, Speroni et al. 2002; McKay and Blumberg 2007; Neto 2007).

In vitro research into the potential anti-cancer effects has progressed throughout the last 15 years. The first report of the potential anti-cancer activity of cranberries appeared in 1996

from researchers at the University of Illinois. This study found that extracts from cranberries and other berry fruits could decrease the expression of certain inflammatory proteins associated with cancerous development *in vitro* (Bomser, Madhavi et al. 1996). Other initial observations of the potential anti-cancer effects of the cranberry were associated with its anti-adherence properties. One such study examined the effects of cranberry extracts which contained high molecular weight compounds, and found that they inhibited the binding of *H. pylori* to gastric mucus and human cells *in vitro*, suggesting that cranberry compounds may be useful in fighting *H. pylori* infections which can lead to stomach cancer (Burger, Ofek et al. 2000). Another study examining cranberries and bacterial adherence was performed in 2005, in which one group of individuals were given 500 mL of cranberry juice daily and the other group was given a placebo. The cranberry-fed group had a lower levels of *H pylori* than the control group, suggesting that cranberries can inhibit the binding of this bacterium *in vivo* as well (Zhang, Ma et al. 2005).

The American cranberry's phytochemical constituents and its effects on cancer are being actively researched, as the cranberry has a diverse array of phytochemicals and has one of the highest levels of phenolics per serving size of any fruit (Vinson, Su et al. 2001). The main constituent phytochemical families of whole cranberry extract (WCE) includes the flavonols (21.96 mg per 100 g fruit), anthocyanins (91.57 mg per 100 g fruit) and proanthocyanidins (411.5 mg per 100 g fruit) (Neto, Amoroso et al. 2008). Much of the current research into the cranberry focuses on the effects of the cranberries' anti-oxidant and phenolic compounds in the search for potential anti-cancer compounds which can alter the behaviour of cancer cells directly (Neto 2007). A number of *in vitro* studies have been performed with cranberry and its extracts and have found some interesting results in a variety of different cancer cell lines. Cranberry extracts have been shown to be cytotoxic to human ovarian cancer cells sensitized with platinum (Singh, Singh et al. 2009). Cranberry PACs have also been found to decrease the

viability (via apoptosis) and decrease proliferation in human esophageal adenocarcinoma cells (Kresty, Howell et al. 2008). Cranberry extracts have also been shown to decrease cellular viability and progression through the cell cycle in human breast cancer cells (Jie and Rui Hai 2006). Cranberry has also shown interesting effects in prostate cancer cells. One study examined the effects of a botanical extract containing compounds from seven different sources, including cranberry, and showed that it decreased cellular growth and increased apoptosis *in vitro*, and it decreased the growth of PC-3 androgen refractory prostate cancer cells *in vivo* (Evans, Dizeyi et al. 2009). Further research by Ferguson and Kurowska (2004) demonstrated that a fraction of cranberry flavonoids had apoptotic effects on numerous types of cancer cells, including prostate cancer *in vitro*. Furthermore, studies with DU145 explant tumours showed that treatment with a flavonoid-rich fraction of cranberry slowed , and in two cases completely regressed the growth of these tumours (Ferguson, Kurowska et al. 2006). To date, this is the only published *in vivo* study of a prostate cancer model and the effects of a cranberry extract on tumour growth and development. Because of these findings both *in vitro* and *in vivo*, more research on the effects of cranberry on prostate cancer are needed.

Though the cranberry demonstrates anti-proliferative and apoptotic effects on a variety of cancerous cells, many questions remain regarding the molecular mechanism of *how* the cranberry and its constituent phytochemicals affect specific cellular pathways. This information would help investigators better understand the relationship between prostate cancer and diet. Studies have revealed that the phytochemicals in the cranberry act variously to decrease the growth and spread of prostate cancer (Neto 2007). Extracts from cranberries can decrease the expression and activity of matrix metalloproteinases (MMPs) *in vitro* (MacLean, Matchett et al. 2007). These proteins degrade compounds in the extra cellular matrix and have been linked to prostate cancer metastasis which will be discussed later in this review. WCE and other families

of phytochemicals from the cranberry decrease the viability of human prostate cancer cells and induce apoptosis (MacLean, Scott et al. 2010;(Hurta, Scott et al. 2010). Cranberry anthocyanins have also been shown to limit the growth of prostate cancer cells by decreasing the expression of tumour necrosis factor alpha (TNF-α) (Neto, Amoroso et al. 2008).

The American cranberry shares many families of phytochemical compounds with other fruits, including blueberries (another *Vaccinium* species), blackberries, chokeberries, strawberries, grape seeds, and a host of other assorted fruits (Hakkinen, Karenlampi et al. 1999; Gu, Kelm et al. 2004; Wu, Beecher et al. 2006). One phytochemical family of interest in the cranberry is the proanthocyanidin (PACs). Proanthocyanidins, or condensed tannins, are dimers, trimers, or oligomers of combinations of various flavan-3-ols including catechin, epicatechin and galloylated catechins, as shown in figure 1.1 (Neto 2007). PACs have been of particular interest in regards to their anti-cancer effects because of their potent and diverse effects. These molecules contain potent anti-oxidant and anti-inflammatory properties, and both of these processes are heavily involved with the development of cancer. PACs have been shown to affect various signalling pathways in cancerous cells, including the NF-κß, MAPK, and PI3K-AKT pathways, all of which are differentially expressed and/or activated in cancerous cells (Strek, Gorlach et al. 2007). PACs can also induce apoptosis and decrease the expression of factors associated with angiogenesis in cancerous cells, which is a critical process in tumour growth (Roy, Baliga et al. 2005; Akhtar, Meeran et al. 2009). Finally, grape seed PACs have also been shown to affect the expression of cell cycle proteins in human epithelial and prostatic cancerous cells (Nandakumar, Singh et al. 2008). An *in vivo* study examining the effects of grape seed extract (GSE), which has a high concentration of procyanidins (89.3% w/w), on the behaviour of prostate cancer found that GSE lowered the numbers of adenocarcinoma compared to control fed mice, increased the number of apoptotic cells and decreased the expression of cell cycle

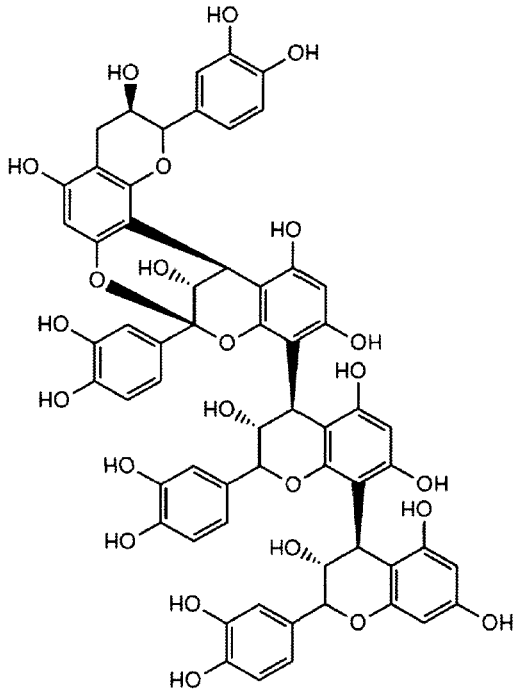


Figure 1.1: An example of a typical cranberry proanthocyanidin oligomer with epicatechin subunits with A-type linkages (Neto 2007).

proteins (Raina, Singh et al. 2007). In summary, cranberry and constituent phytochemical families found within the cranberry have shown interesting anti-cancer effects *in* vitro studies with various cancer cell lines and in studies using *in vivo* models.

1.3 A Brief Review of the cell cycle

All cells grow and divide, and this growth and division involves the replication of the cell's genetic components. In eukaryotic cells, the phases of growth and division are known as interphase and mitosis (M) and originally these were thought to be the only two divisions in the entire cell cycle. Newer molecular techniques demonstrated that there was other more intricate and elaborate steps occurring in interphase other than cellular growth. There are three steps in interphase: **G1** (the first gap after mitosis when the cell prepares for the synthesis of DNA), **S** (the DNA synthesis phase) and **G2**, when the cell prepares itself for mitosis (Vermeulen, Van Bockstaele et al. 2003).

In mammalian cells the process of cell division is governed by a series of proteins which regulate if and when each cell enters a specific point in the cell cycle, whether it is G1, G2, S or M. These proteins include the cyclins, the cyclin-dependent kinases (CDKs), and the two families of cyclin-dependent kinase inhibitors (CKIs) which include the INK4 family and the Cip/Kip family. The process of cellular division is a tightly regulated process, and most cells in organisms are in the G_0 phase, which is the resting phase of the cell cycle immediately following mitosis (Vermeulen, Van Bockstaele et al. 2003). Each cyclin and CDK is involved in the transition between different phases of the cell cycle. For example cyclins D1, D2 and D3 are involved in the transition from G_0 to G1, and CDK2 is involved in the transition of the cell from G1 to S. Cyclins and CDKs can be involved in one or more transition points/phases of the entire cycle. In order for the transition to proceed, an appropriate CDK (the catalytic subunit) needs to be activated and bind with an appropriately matched cyclin (the regulatory subunit) (Malumbres and Barbacid 2007). As such, to proceed from G_0 to the G1 phase CDK 4 or 6 needs to become activated and bind with one of the three subtypes of cyclin D, at which point the G1 phase of the cycle will proceed. An overview of this process is shown in Figure 1.2.

1.3.1 Dysregulation of cell cycle in cancer

Problems do occur during the cell cycle, and these are often the result of defects or mutations in cell cycle proteins. These defects and mutations are the result of cumulative genetic mutations that occur over the course of an organism's lifetime, and may eventually lead to constitutive signalling and deregulation of anti-mitogenic signalling that leads to uncontrolled reproduction of cells. Tumours may also develop genomic and chromosomal instability which leads to more genetic mutations and uncontrolled cellular division. These mutations often occur because of a lack of regulation of CDKs (Malumbres and Barbacid 2009). Cell cycle mutations have been noted in a large variety of human tumours, including over 70% of prostatic and bladder cancers, and over 80% of breast, lung, pancreatic, lung, and head/neck cancers. These mutations include defects in both cyclin dependent inhibitor families, the retinoblastoma (RB) gene, the cyclins and the CDKs (Malumbres and Barbacid 2001). Because of the discovery that the mutation of cell cycle proteins can cause cancer, research has focused on the prevention and treatment of cell cycle mutations. A number of different avenues, including the "druggability" of CDKs by both synthetic chemical analogues have been explored. Unfortunately, to date there have been no advancements with CDK drugs. Many CDK drugs involve the inhibition of this enzyme, and these drugs have been ineffective as they cause many cytotoxic effects during clinical development (Malumbres and Barbacid 2007).

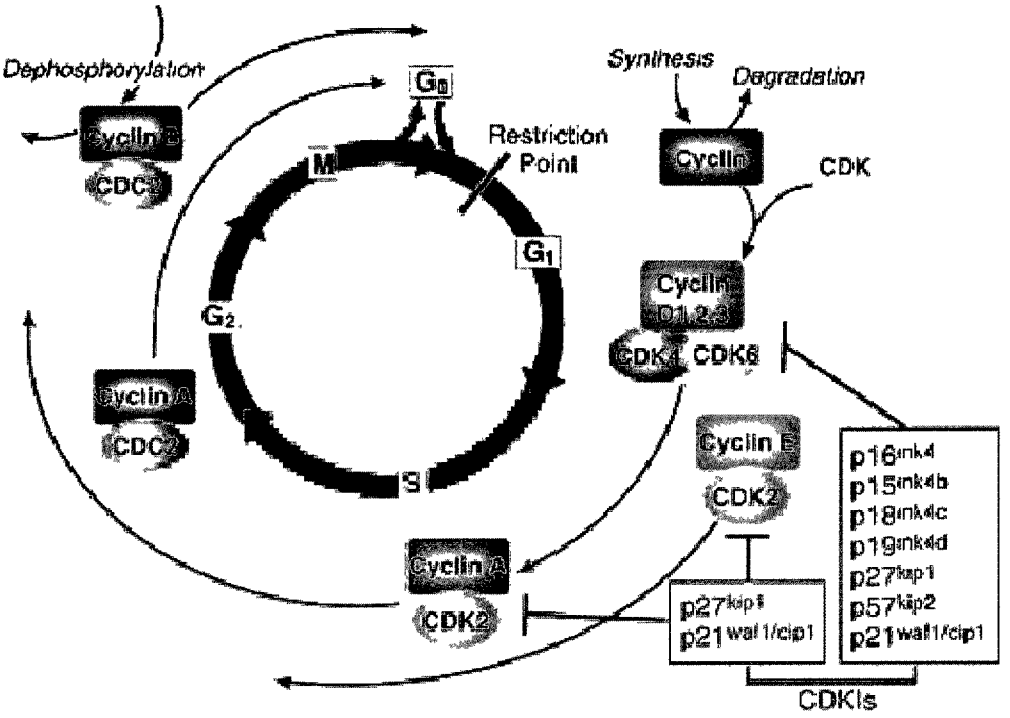


Figure 1.2: An overview of the key proteins involved in cell cycle progression and arrest (Schwartz and Shah 2005).

Although there have been few advancements with cell cycle inhibitory drugs, there has been progress examining the effects of diet and nutritional compounds on the expression of cell cycle proteins. *In vitro* studies have revealed considerable information on the effects of dietary agents on cell cycle regulation. Stilbenoids, which occur naturally in grapes and several other plants, have been shown to increase the expression of cell cycle inhibitory proteins and decrease the expression of cell cycle proteins in human lungs cells (Lee, Min et al. 2004). Genistein and daidzein, which are found in soy products, have been reported to decrease the expression of CDK related expression genes in three different human prostate cancer cell lines, including DU145 cells (Rabiau, Kossaï et al. 2010). *In vivo* studies examining cell cycle expression have also provided interesting findings. In this regard, the effects of an extract from *Antrodia camphorata*, which is a species of fungus, on the behaviour of an MDA-MB-231 cell xenograft in nude mice have been studied. It was demonstrated that this extract decreased tumour volume and decreased the expression of cyclin D1 within the tumour itself (Hseu, Chen et al. 2008). The effects of soy isoflavones on the behaviour of bladder cancer cells in a mouse model was examined a decrease in cellular proliferation and an increase in apoptosis was found (Zhou, Gugger et al. 1999). These studies suggested that certain compounds in diet can potentially have anti-proliferative effects on cancer cells *in vivo*, and that further research is warranted to determine the effects of other compounds and phytochemicals on the proliferation and expression of cell cycle proteins.

1.4 Prostate Cancer and Matrix Metalloproteinases

In order for prostate cancer to become a life-threatening disease, the cancer must spread beyond the boundaries of the prostate through the body's vasculature and metastasize to different sites, typically bone in advanced prostate cancer. The process of metastasis is highly

complex, and this review will focus on a specific aspect of prostate cancer metastasis: the escape and invasion of prostatic carcinoma through the extra-cellular matrix (ECM) and basement membrane surrounding the prostate and into vasculature which is a process known as intravasation. Intravasation of prostate cancer requires the cancerous cells to detach from the main tumour and navigate through the degraded stromal and basement tissues. This process involves many factors, but one of the most crucial of these factors is the matrix metalloproteinases (MMPs).

MMPs are a family of over 20 proteases which were first discovered by Gross and Lapiere (1962).They demonstrated a protein which could degrade collagen in tadpole tails. Further research explored the implications of this extracellular matrix cleavage. The cleaving of substrates releases growth factors, and some of these growth factors promote angiogenesis which is crucial for tumours in order to receive a blood supply and continue to proliferate (Foda and Zucker 2001).

All MMPs have two common features: they all contain a site in their catalytic domain that contains zinc in order to function, and they are all synthesized as inactive zymogens and which require cleavage in order to function. MMPs are actively involved with many homeostatic functions. These include tissue remodelling during embryonic development, wound healing, cartilage-to-bone transformation, and trophoblast invasion into the endometrial stoma during placental development (Rundhaug 2003). Overexpression of MMPs occurs in many different cancer cell tyles *in vivo* (Sang, Yonghao et al. 2006). Increases in MMP-2, also known as gelatinase A, have been shown to correlate with the malignancy of prostate cancer (Stearns and Stearns 1996). An increase in the expression of the MMP-7 gene has also been associated with prostate cancer (Pajouh, Nagle et al. 1991). Mitogen/extracellular-signal-regulated kinase kinase 5/extracellular signal-regulated kinase-5 (MEK5/ERK5) has also been shown to be upregulated in

prostate cancer as compared to benign prostate tissue, and MEK5 enhances the expression of MMP-9, also known as gelatinase B (Mehta, Jenkins et al. 2003). The expression of MMP-2 and MMP-9, and tissue inhibitors of matrix metalloproteinase 1 and 2 (TIMP-1 and TIMP-2) were found to be altered in patients with a low Gleason score (which is a measure of the development of prostate cancer). Such patients had increased levels of TIMP proteins. Conversely, patients with a high Gleason score (advanced prostate cancer) had moderately to highly elevated levels of both MMP-2 and MMP-9 (Wood, Fudge et al. 1997). The authors of this study suggested that MMPs, and especially the gelatinases (MMP-2 and MMP-9), are highly involved in the development of prostate cancer. The overexpression of MMPs in prostate cancer is related to their functions in normal tissues. MMPs can degrade gelatin in the ECM; gelatin is found in very high concentrations in the basement membranes of tissues. Since these MMPs can degrade this extracellular matrix substrate, they have the potential to degrade the ECM around lymph nodes or vasculature, which would allow prostate cancer cells an avenue to enter the blood stream and metastasize to different areas of the body. Another factor to consider is that MMPs degrade and liberate growth factors and pro-angiogenic factors, which provides for the growth of new blood vessels surrounding the primary or secondary tumour. This allows the tumour to facilitate its own growth.

1.4.1 MMP-2 and MMP-9 Expression and Regulation

MMP expression and regulation are governed by a number of different factors in untransformed and transformed (potentially-cancerous) cells. Some of these factors include specific transcriptional pathways, including the activator protein-1 (AP-1) pathway, the nuclear factor κB (NF-κB) pathway, the mitogen activated protein kinase (MAPK) pathway, and the PI-3/AKT pathway (Thant, Nawa et al. 2000; Reddy, Nabha et al. 2003; Zhang and Brodt 2003; Yan and Boyd 2007). MAPK regulates the production of MMP-9 in human breast cancer cells (Reddy,

Krueger et al. 1999). Decreasing the phosphorylation status of MAPK proteins decreases the expression of both MMP-9 and MMP-2 in prostate cancer cells *in vitro*, which affects the downstream regulation of AP-1 (Kim, Kim et al. 2004).

MMP expression can also be modified by a number of different proteins including extracellular matrix metalloproteinase inducer (EMMPRIN), an inducer of MMP production, and "reversion-inducing cysteine-rich protein with Kazal motifs" (RECK), an MMP expression inhibitor. EMMPRIN is a trans-membrane protein which can stimulate the production of a variety of different MMPs, including MMP-1, MMP-2, and MMP-3. Increased MMP-9 expression occurred in human head and neck cancer cells (FaDu cells) after transfection with EMMPRIN cDNA (Newman, Bohannon et al. 2008). Increased EMMPRIN expression has been shown in a large variety of malignant tumours and cancer cell lines including skin, breast, bladder, lung and prostate cancers (Gabison, Hoang-Xuan et al. 2005; Madigan, Kingsley et al. 2008). EMMPRIN increases not only the expression of MMPs in an autocrine manner, but can also affect the expression of MMPs in nearby endothelial cells, which can potentially lead to these cells inducing angiogenesis (Gabison, Hoang-Xuan et al. 2005). RECK, on the other hand, is an inhibitor of MMP expression and has been shown to decrease the expression of both MMP-2 and MMP-9, and has been shown to be intimately involved with angiogenesis (Oh, Takahashi et al. 2001). Decreased expression of RECK in patients with prostate cancer has been inversely correlated with the aggressiveness of prostate cancer and serum prostate specific antigen (PSA) levels (Rabien, Burkhardt et al. 2007).

Finally, the catalytic activity of MMPs can be regulated by "tissue inhibitors of matrix metalloproteinases" or TIMPs. This family of catalytic inhibitors includes TIMPs 1-4. TIMPs inhibit MMPs by binding its N-terminal domain to the catalytic domain of the appropriate MMP in a one to one stoichiometric and reversible fashion. Each TIMP binds preferentially to a specific

MMP. For example, TIMP-1 preferentially binds to MMP-9, and TIMP-2 preferentially binds to MMP-2 (Brew, Dinakarpandian et al. 2000). Since TIMPs play an important role in the inhibition of MMPs, studies of these proteins in relation to cancer development have been undertaken with interesting results. Pancreatic cancer cells (PANC-1) designed to overexpress the TIMP-1 gene were significantly less likely to metastasize and promote angiogenesis in immunodeficient mice, and also showed increased rates of apoptosis when compared to control (Bloomston, Shafii et al. 2002). TIMP-1 transfected human gastric cancer cells when injected into nude mice metastasized but showed a much lower rate of tumour growth after metastasis when compared to controls (Watanabe, Takahashi et al. 1996). Increases in TIMP-2 expression resulted in anti-metastatic effects in MB-231 and MCF-7 cells (Simeone, McMurtry et al. 2008). This and other evidence suggests that TIMPs can affect the growth of tumours *in vivo*. Interestingly, although the TIMPs bind and inhibit MMP activity, it has been noted in some studies that increases in TIMP expression correlate to increased metastatic potential and a worsened prognosis for the some cancer patients, which leaves open the question of the beneficial effects of increased TIMP expression in cancers (Grignon, Sakr et al. 1996; Remacle, McCarthy et al. 2000)

1.5 Models of Prostate Cancer

In prostate cancer research there are many models to evaluate the multiple characteristics of this disease and its potential response in humans to possible chemotherapeutic and chemopreventative mechanisms. These models can be divided into two different categories: *in vitro* based cell culture models of prostate cancer cells from human origin, and *in vivo* animal models examining the growth and spread of prostate cancer. Both methods of evaluating prostate cancer have their own unique advantages and in the subsequent paragraphs the characteristics of both approaches will be discussed.

1.5.1 *In Vitro* Models of Prostate Cancer

In order to study the effects of specific compounds on prostate cancer, *in vitro* prostate cancer cell cultures are often used. This method is used to examine the behaviour of one or a number of different attributes that potential anti-cancer compounds may possess, including the induction of apoptosis, the regulation or specific cell pathways, the inhibition of cellular growth, or potentially decreased metastatic potential amongst many others. There are a number of prostate cancer cell lines which are often used in the evaluation of natural compounds' effects on prostate cancer. Such cell lines include the androgen-sensitive LNCaP cells, and the androgen-insensitive cell lines PC-3 and DU-145 (Maarten, Leland et al. 1996). LNCaP cells are considered to be less "aggressive" than either DU-145 or PC-3 cells, as they still require the hormone androgen to grow. DU-145 cells and PC-3 cells are considered to be representative of the more "aggressive" form of the disease, being derived from a brain metastasis and a bone metastasis, respectively (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979). PC-3 cells are the most aggressive of the classic prostate cancer cell lines discussed here (Nair, Rao et al. 2004). Because DU-145 cells are considered moderately aggressive, express and excrete MMPs into the ECM, have been used in a large variety of phytochemical tests, and have been used as justification of clinical trials (Lippman, Klein et al. 2009), this cell line was selected for this study and was used in cell culture based experiments in this thesis.

1.5.2 *In Vivo* Models of Prostate Cancer

Prostate cancer has been much more difficult to represent in an animal model, as the development of spontaneous prostate cancer occurs in only a select few species, which includes rats, dogs, and humans (Lamb and Zhang 2005). Because of this, research into the causes of this disease has been an arduous process. In the previous few years, some of the obstacles relating

to *in vivo* models of prostate cancer have been overcome. Research into animal models of this disease has focused on xenographs, which are immunocompromised mice that have been transfected with human prostate cancer cells. The first successful xenograph model of prostate cancer was demonstrated in 1976 (Lamb and Zhang 2005). Mickey et al. (1977) successfully implanted DU-145 cells into nude mice and the tumours that developed after five weeks had similar characteristics as the original tumour from the patient.

Although these studies and related chemotherapeutic studies have revealed information about the development of tumours *in vivo*, these cancers did not develop spontaneously and do not give a good indication of what molecular steps occur during the preliminary steps of prostate cancer development. Also, they are not as effective as spontaneous tumours for research into chemopreventative studies as the cells transplanted into the model are already fully malignant cells. As such, new methods of studying prostate cancer were needed. In the mid 1990s, the first animal model designed to study prostate cancer was developed in the Greenberg laboratory (Greenberg, DeMayo et al. 1995). This model, known as the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP), was developed by constructing the rPB-SV40 Tag transgene. This transgene is specifically designed to promote the expression of an oncogene, simian virus 40 (SV40), in the epithelial cells of dorsolateral prostate (Greenberg, DeMayo et al. 1995). This oncogene acts by downregulating the expression of p53 and pRB, which are well known anti-oncogenic factors in cells. In this model, there is a well-documented developmental timeline of metastatic prostate cancer, which is reported to occur in 100% of animals by 28 weeks of age (Gingrich and Greenberg 1996). Between 8 and 12 weeks there is the development of prostatic intraepithelial neoplasia, or PIN. PIN in both mice and men is often a precursor state to prostate cancer. By 16 weeks of age, most mice develop "Well Developed (WD)" adenocarcinoma of the prostate. This stage at a cellular level is often associated with

increased levels of mitosis, apoptosis, and inflammation.(Kaplan-Lefko, Chen et al. 2003). By 20 weeks of age, mice display both focal (localized) and invasive adenocarcinoma (Greenberg, DeMayo et al. 1995). By 24 weeks, metastases can be found in this model in the lungs, bone, and lymph nodes, and by 28 weeks, all mice have metastases in at least the lungs and lymph nodes (Gingrich and Greenberg 1996). This model has been used in many chemopreventative studies with interesting results. For example, green tea extracts have been noted to decrease tumour burden and delay the onset of primary prostate cancer in this model (Gupta, Hastak et al. 2001). Grape seed extracts, composed primarily of proanthocyanidins, have also shown beneficial effects in this model (Raina, Singh et al. 2007). Because of the reliability of this model noted in the literature and multiple successful chemopreventative studies undertaken by other researchers, this model was selected for research into the chemopreventative effects of cranberry extracts in prostate cancer.

CHAPTER TWO

Compounds from the American Cranberry (*Vaccinium macrocarpon)* inhibit matrix metalloproteinase activity in DU-145 human prostate cancer cells[1]

2.1 Introduction

Prostate cancer is one of the most common forms of cancer in North American men, with over 210,000 diagnosed cases in 2008. Despite improvements in therapy and treatment over the last few decades, this disease still accounts for over 32,000 deaths per year in North America according to both the American and Canadian Cancer Society (2008). This disease reaches a critical point in development when it becomes metastatic and spreads to multiple sites in the body, primarily bone and lung tissue (Bubendorf, Schopfer et al. 2000). The earlier stages of prostate cancer are androgen-dependent, meaning that this cancer requires androgen in order to grow. This characteristic is often used as a treatment by means of androgen ablation by physical or chemical castration. However, the heterogeneity of cancerous cells allows for the development and spread of androgen-independent cancerous cells which do not respond to androgen deprivation therapy (Abate-Shen and Shen 2000). Because this form of treatment eventuality falters, new forms of prostate cancer treatments are required.

One of the critical steps during metastasis of prostate cancer is the penetration of the cancerous cells into vasculature, allowing it an avenue to spread to other parts of the body. A family of proteins called the matrix metalloproteinases (MMPs) allows cancerous cells to do just

[1]The results of this study have been published in the Journal of Cellular Biochemistry (Deziel, Patel et al. 2010). This chapter is an adaptation and reformatting of this publication.

that (Nemeth, Yousif et al. 2002). These proteins degrade the surrounding extracellular matrix and permit the cancer cells to access lymph tissue and vasculature. The gelatinases, specifically MMP 2 and MMP 9, degrade gelatin found in the basement membrane of tissues. The expression of these gelatinases has been reported to be up-regulated in a variety of different cancerous cells (Bachmeier, Beatrice et al. 2001; Gimeno-Garcia, Santana-Rodriguez et al. 2006), and this is a possible reason as to why these cancers are able to spread throughout the body (Bjorklund and Koivunen 2005).

One possible method of slowing or halting the metastasis of prostate cancer is by a change in diet. Certain foods have been associated with a decreased risk of developing cancer, notably fruits and vegetables (Steinmetz and Potter 1991; Cohen, Kristal et al. 2000). It has been shown that these foods contain many "bioactive" compounds which have profound effects on the behaviour of cancerous cells both *in vitro* and *in vivo*. Some of these effects include increasing rates of apoptosis, decreasing incidences of metastasis, and arresting the cell cycle (Liu, Wu et al. 2007; Gallo, Zannoni et al. 2008; Pan, Ghai et al. 2008).

The American cranberry (*Vaccinium macrocarpon*) has been reported to have many "bioactive" properties. The constituents of this food have been shown to act as powerful antioxidants (Vinson, Bose et al. 2008), and arrest the growth and adherence of potentially harmful bacteria (Lavigne, Bourg et al. 2008; Matsushima, Suzuki et al. 2008). Recently, researchers have been studying the potential anti-cancer properties of the American cranberry and its effects upon cancerous cells. These effects have included a reduction in proliferation of cancerous cells, inhibition of cyclooxygenase enzyme activity and expression, and an induction of apoptosis (Neto 2007). One family of "bioactive" compounds which are found in high concentrations in cranberries is flavonoids. These compounds have also been shown to affect

the behaviour of cancer cells. Recent reports have described the effects of flavonoids and different fractions of this family on the matrix metalloproteinase expression and activity *in vitro* (Vayalil, Mittal et al. 2004; Matchett, MacKinnon et al. 2005). Previously, the effects of cranberry flavonoid- containing fractions (whole cranberry extract) on matrix metalloproteinase-2 and -9 activity in DU145 human prostate adenocarcinoma cells *in vitro* have been described as well (MacLean, Matchett et al. 2007). The purpose of this current study is to examine the effect of a proanthocyanidin-enriched extract from the American cranberry (*Vaccinium macrocarpon)* on MMP activity in DU145 human prostate cancer cells.

2.2 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following antibodies were used: goat polyclonal anti-actin, anti-Akt 1, anti-pJNK, anti-JNK 1, anti-pIκBα, anti-histone H2B, rabbit polyclonal anti-p38, anti-p-p38, anti-p-ERK1/2, anti-ERK 1, anti-ERK 2, anti-p-Akt 1, PI-3- Kinase p110, anti-NFκB (p65), anti-JNK 2 and mouse monoclonal anti-IκBα and PI-3-Kinase p85α. All other chemicals and materials were purchased as indicated.

2.2.1 Preparation of Cranberry Extracts

The proanthocyanidin-enriched cranberry extracts used in this study were prepared by the laboratory of Dr. Catherine C. Neto (University of Massachusetts –Dartmouth, North Dartmouth, MA), and its extraction and preparation are detailed in Appendix A.

2.2.2 Cell Culture and Treatment with Proanthocyanidins

Human DU145 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in alpha minimal essential media

(MEM) (Gibco, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Sigma, Oakville, ON) and 1% antibiotic-antimycotic (Gibco, Burlington, ON). Cells (4×10^5) were cultured on the tissue culture dishes and were initially incubated for 24 hours at 37°C in the presence of 5% CO_2. After this 24 hour incubation period, the media was removed and replaced with serum free alpha MEM supplemented with 5 μg/ml of transferrin (Sigma) and 2.5μg/ml of insulin (Sigma). These plates were again incubated for 24 hours at 37°C in the presence of 5% CO_2. After this incubation period, cells were treated with various concentrations of PACs ranging from 1μg/ml to 25 μg/ml for times ranging from a half hour to six hours. PACs were dissolved in dimethyl sulfoxide as the vehicle prior to use. The control cells received only the vehicle. After treatment, the media was removed from the cells and stored at -80°C for further analysis. The cells were then washed with phosphate buffered saline (PBS), and were removed by trypsin (Sigma) diluted in PBS. The cells were re-suspended with alpha MEM supplemented with 10% FBS and were centrifuged for 4 minutes at 500 x g. After centrifugation, the cell pellet was re-suspended with PBS and was transferred to a microcentrifuge tube and was centrifuged at 500 x g for 4 minutes. After centrifugation, this cell pellet was then stored at -80°C until further analysis.

2.2.3 Zymography

Zymography, or gelatin-gel electrophoresis was performed to evaluate matrix metalloproteinase (MMP) activity as previously described (Matchett et al, 2005). Briefly, an aliquot of the conditioned media was mixed in a 4:1 ratio with sample buffer, which consisted of 10% sodium dodecyl sulphate (SDS) and 0.1% bromophenol blue in 0.3 M Tris–HCl (pH 6.8) (Sigma). Aliquots of each sample were loaded into wells of a 5% stacking gel and resolved by electrophoresis at a constant 35 milliamp current at ambient temperature for three hours. The

10% resolving gel contained 0.1% gelatin (Sigma). Following electrophoresis, the gel was then incubated with a solution containing 2% Triton 100X (Sigma) and 0.5 M Tris-HCl (Sigma) for one hour. After this first incubation, the gel was then placed in the second solution containing 0.05 M of Tris-HCl (Sigma) and incubated for 30 minutes. After this second incubation, the gel was placed in a solution containing 1% Triton 100x, 0.05 M Tris-HCl and 0.005M $CaCl_2$ (Sigma) and incubated at 37˚C for 24 hours. Following this incubation period, the gels were stained with a 0.1% Brilliant Blue R-250 stain containing 50% methanol, 40% distilled water and 10% acetic acid. Once stained, the gel was de-stained in a solution containing 75% distilled water, 20% methanol and 10% acetic acid. Pre-stained molecular weight markers (Biorad Mississauga, ON) were also resolved on the same gel. Gelatinase activity appeared as zones of clearing (due to gelatin degradation) against a blue background.

2.2.4 Cytosolic Protein Fraction and Nuclear Protein Fraction Isolation

Protein expression was determined in the whole cytosolic protein fraction. Briefly, the cell pellets were removed from -80˚C and placed on ice. The pellets were then reconstituted in 100 µl of 10mM Tris-HCl buffer (7.4 pH) containing 0.5 mM PMSF (Sigma). Once reconstituted, the cells were briefly sonicated. The cell lysates were then centrifuged for 10 minutes at 9,300 x g at 4˚C. Following this centrifugation, the supernatant was removed from the pellet and subsequently evaluated. The total protein content of the supernatant was determined by Biorad protein assay (Biorad) as per manufacturer's instructions.

This nuclear isolation procedure was essentially as previously described (Wang, Gong et al. 2009). Briefly, the previously prepared cell samples were removed from -80˚C and placed on ice. These cells were then incubated on ice in a buffer containing 10mM HEPES (pH 7.9), 1.5mM

$MgCl_2$, 10mM KCl, 0.5mM DTT and 0.5 mM PMSF for 10 minutes. After 10 minutes, the cells were centrifuged at 10,000 x g for 10 minutes at 4˚C. Once centrifuged, the supernatant containing the cytosolic proteins was removed from the pellet and stored at -80˚C. The remaining pellet was then re-suspended in a buffer containing 20mM HEPES, 25% (v/v) glycerol, 0.42M NaCl, 1.5 mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and 0.5 mM PMSF. The contents were then incubated on ice for 40 minutes with vigorous shaking. Once 40 minutes had passed, the contents were centrifuged at 10,000 x g for 15 minutes at 4˚C. After centrifugation, the supernatant (containing the nuclear proteins) was removed and stored at -80˚C until used. The total protein content of the cytosolic and nuclear fractions was determined by Biorad protein assay.

2.2.5 Immunoblot Analyses

Equal amounts of protein extracts from whole, cytosolic, and nuclear fractions were mixed in a 3:1 ratio with standard Laemmili buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS , 0.1% bromophenol blue, 10% glycerol and 100mM beta-mercaptoethanol. Once mixed, these samples were boiled for 3 minutes. Once boiled, they were resolved by electrophoresis through 10% SDS-PAGE gels and were transferred onto nitrocellulose membranes (Biorad). After transfer, the membranes were incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution for one hour at room temperature. Then, the membranes were incubated with primary antibodies diluted to a 1:200 ratio (v/v). The membranes were incubated in the presence of the primary antibody for 24 hours at 4˚C. After incubation the membranes were then washed three times with TBS-Tween (0.05%) for ten minutes and were then incubated in alkaline phosphatase-conjugated secondary antibodies (1:2000 dilution) for 1 hour. After incubation, the membranes were again washed three times with TBS-Tween (0.05%) for ten

minutes, washed briefly with distilled water, and then exposed to SigmaFast BCIP-NBT tablets (Sigma) in solution to visualize protein expression levels. Western blots were then analyzed using Infinity Capture software (Lumenera Corp., Ottawa, ON), and densitometry was performed with ImageJ software (National Institute of Health, Bethesda, MA).

2.2.6 Alamar Blue Cytotoxicity Assay

The Alamar Blue assay (Invitrogen, Burlington, ON) was performed as per manufacturer's instructions to determine cellular viability after treatment with PACs at 25ug/ml at 3 and 6 hours of treatment. Briefly, DU145 cells were subcultured into a 96 well plate at 5,000 cells/well in αMEM with 10% FBS and 1% antibiotic/antimycotic. After 24 hours of incubation, the media was replaced with 100µl of serum-free media and was once again incubated for 24 hours. After this incubation, the cells were treated with PACs for either 3 or 6 hours, with a final concentration of dimethyl sulfoxide (DMSO) within each well of 1%. The control for this experiment was DU145 cells treated with DMSO at a total concentration of 1% for 6 hours. Following treatment, 10µl of Alamar Blue was added to each well which resulted in a final volume of 10% Alamar Blue in each well. The cells were incubated for 3 hours post exposure of the cells to Alamar Blue. After this 3 hour incubation period, the resulting fluorescence was read with an FLx800 fluorescence microplate reader (BioTek, Winooski, VT) with an excitation wavelength of 528 nm and an emission wavelength of 590 nm.

2.2.7 Statistical Analysis

The statistical analysis for each assay was done using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diego, CA). Results from each zymogram and cytotoxicity assay were compared using a one way ANOVA with a Tukey's Posthoc test, and results were considered statistically significant at $p < 0.05$. Results from the densitometric analyses of each

Western blot were compared using an unpaired t-test, and results were considered statistically significant at $p < 0.05$.

2.3 Results

2.3.1 Cranberry Proathocyanidins Significantly Decrease DU145 Cell Viability at 25 µg/ml after 6 Hours, but not after 3 Hours

A cytotoxicity assay using Alamar Blue to assess cellular viability in DU145 prostate cancer cells in response to treatment with PACs was performed. As shown in Figure 1, treatment of DU145 cells with PACs (25 µg/ml) for 6 hours resulted in 30% inhibition of cellular viability (which was significantly different from control [vehicle–treated] cells, $p < 0.001$). However, after only 3 hours of treatment with PACs, cell viability decreased by only 8%, which was not statistically significant when compared to control ($P > 0.05$) (Figure 2.1).

2.3.2 Cranberry Proanthocyanidins Decrease MMP-2 and MMP-9 Activity in DU145 Cells *in vitro* in both a Time and Dose-Dependent Manner.

In order to investigate the effects of PACs upon MMP activity, gelatin gel electrophoresis was performed with the cell media after treatment with PACs, with doses ranging from 0-25 µg/ml and from times ranging from a half hour to 6 hours. As shown in Figure 2.2 A, MMP-2 and MMP-9 activity decreased after 6 hour treatments with PACs at concentrations of 10 and 25 µg/ml, respectively. After performing a dose-dependent experiment, a time-dependent experiment was performed to determine how early the inhibition of MMP activity occurs after treatment with PACs. DU145 cells were treated with cranberry PACs at a concentration of 25 µg/ml for a half hour, 1 hour, and 3 hours. As shown in Figure 2.2 B, decreases in MMP-2 and MMP-9 activity were seen as early as 30 minutes post treatment with PACs.

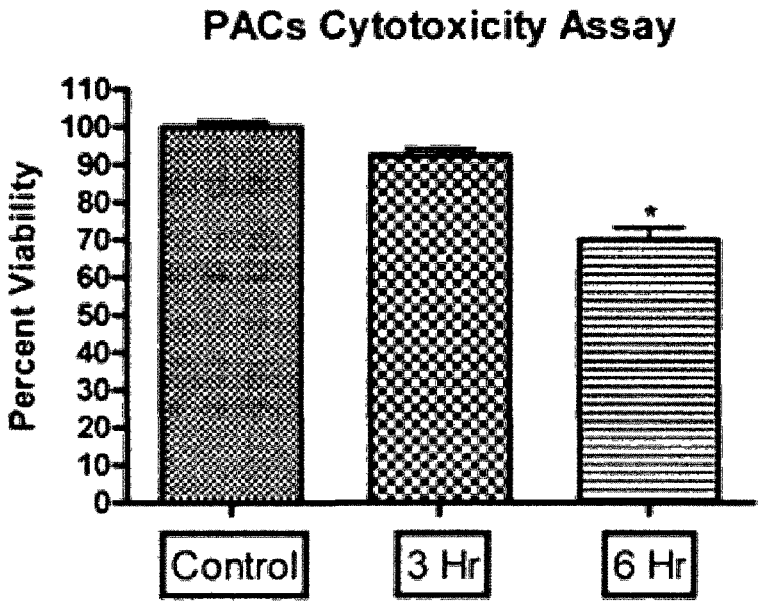


Figure 2.1: PACs Treatment of DU145 Cells Can Affect Cellular Viability. DU145 cell viability was evaluated by Alamar Blue assay following treatment of cells with 25 μg/ml of PACs for 3 and 6 hours, respectively. Control cells received 1% DMSO (vehicle) alone for 6 hours. Fluorescence was read at 528 excitation and 590 emission wavelengths. Control cells were standardized to represent 100% viability. Treatment of DU145 cells with 25 μg/ml of cranberry PACs for 6 hr significantly decreased the viability of DU145 cells relative to control cells ($p < 0.05$). The results presented represent values obtained from N = 8.

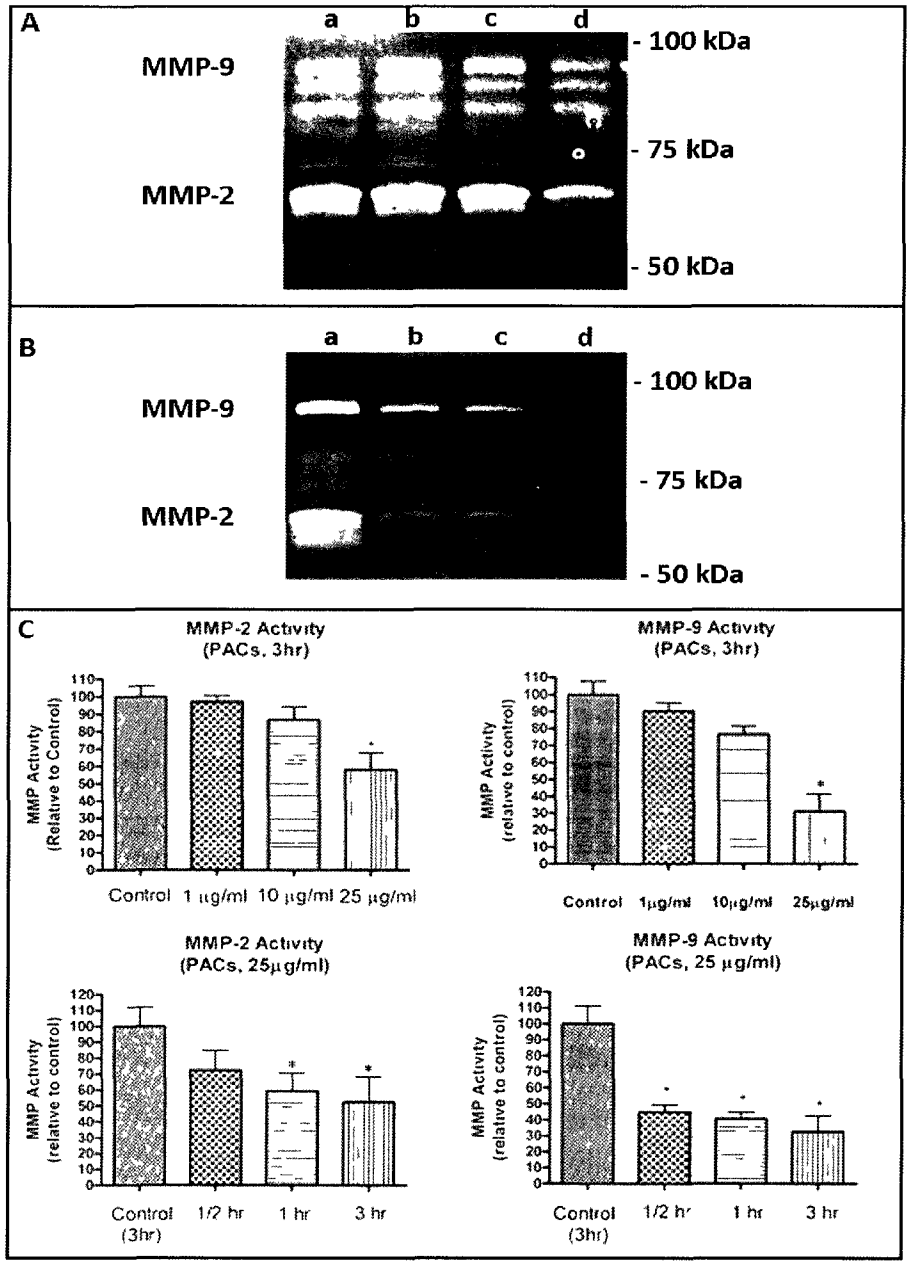


Figure 2.2: Cranberry PACs decrease MMP-2 and MMP -9 activity in a dose- and time-dependent manner. Gelatin gel electrophoresis was performed on aliquots of conditioned medium obtained from DU145 cells following exposure to PACs. Results obtained are representative of 3 independent experiments with assay duplicates. A: MMP-2 and MMP-9 activity decreases in a dose-dependent manner in response to PACs treatment: control cells (1 % DMSO for 6 hours) (a), and cells exposed to PACs (1 µg/ml) for 6 hours (b), to PACs (10 µg/ml) for 6 hours (c), and to PACs (25 µg/ml) for 6 hours (d). B: MMP-2 and MMP-9 activity decreases in a time dependent manner in response to PACs (25µg/ml): control cells (cells treated with 1% DMSO for 3 hours) (a), cells treated with 25 µg/ml PACs for 30 minutes (b), is cells for 1 hour (c), and for 3 hours (d). C: Histograms representing densitometric analyses of MMP-2 and MMP -9 activity levels. Asterisks denote a statistically significant difference from control ($P < 0.05$). Results presented are representative of findings from N = 3 with assay duplicates.

2.3.3 Cranberry Proanthocyanidins Decrease the Expression of MMP Inducer EMMPRIN Increase the Expression of TIMP-1, and Increase the Expression of TIMP-2

Having shown that treatment of DU145 cells with PACs results in an inhibition of both MMP-2 and MMP -9 activities, the effects of PACs on the expression of a number of key factors known to regulate the expression of MMP-2 and -9 were evaluated. These regulatory factors included the extracellular matrix metalloproteinase inducer (EMMPRIN) and two tissue inhibitors of matrix metalloproteinases (TIMPs), TIMP-1 and TIMP-2. As shown in Figures 2.3 A and 2.3 B, there was a statistically significant increase in the protein expression levels of TIMP-2 and a statistically significant decrease in the protein expression levels of both TIMP-1 and EMMPRIN, respectively after treatment of DU145 cells with cranberry PACs (25 μg/ml) for 3 hours.

2.3.4 Cranberry Proanthocyanidins Affect the Expression of MAPK's Pathway Proteins and the Expression of PI3-Kinase Pathway Proteins

In order to further characterize the mechanisms whereby cranberry PACs can inhibit MMP-2 and -9 expression levels, the effects of PACs on key cellular signal transduction proteins were determined. A number of cellular signalling proteins including those associated with the mitogen activated protein kinase (MAPKs) pathway and the phosphatidylinositol-3 kinase (PI3-kinase) pathway which subsequently activate AP-1 and NFκB have been implicated in the regulation of the expression of MMP levels (Lee et al., 2009). Western blot analyses were performed with whole cell lysates from DU145 cells following 3 hours treatment with PACs (25 μg/ml) and protein expression levels of a number of proteins were evaluated. As shown in Figures 2.4 A, results indicated that after treatment of DU145 cells with PACs, the

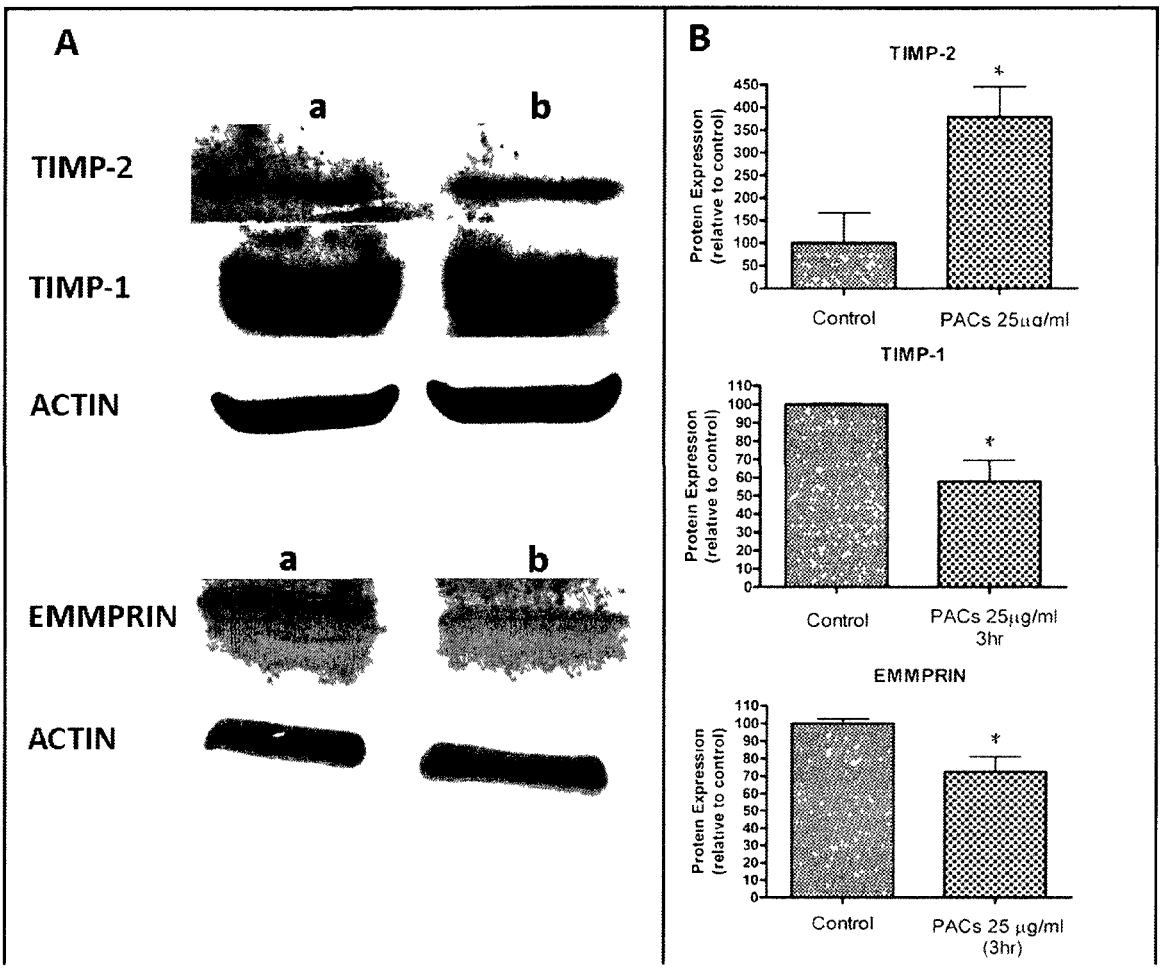


Figure 2.3: Cranberry PACs increase TIMP-2 protein expression levels and decrease TIMP-1 and EMMP2RIN protein expression levels in DU145 cells. A: TIMP-2, TIMP-1, EMMPRIN and actin protein levels, respectively, in vehicle-treated cells (a) and in PACs (25µg/ml)-treated (3 hours duration) cells (b). Actin was used as a loading control. Each blot shown is representative of results obtained from N = 3 with assay duplicates. B. Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($p < 0.05$).

phosphorylation status of p38, ERK1 and ERK2 increased. These increases were statistically significant.

Additionally, as shown in Figure 2.4B, increased JNK1 and decreased JNK2 protein expression levels occurred in response to PACs with corresponding alterations in both pJNK1 and pJNK2.

To further investigate the nature of the cellular signalling proteins involved in the PACs-mediated inhibition of MMP-2 and -9, the expression of proteins in the PI-3 kinase pathway was evaluated. As such, Western blot analyses were performed and Akt (a protein in the PI-3 kinase pathway) protein levels were determined. As shown in Figure 2.5 C and 2.5 D, PACs treatment of DU145 cells resulted in a statistically significant decrease in both phosphorylated Akt and Akt proteins levels. To further investigate the mechanisms by which PACs are acting, Western blot analyses were performed to examine the expression of PI-3 kinases (p85 and p110 proteins). It was observed that the expression of both PI-3 kinase p85 and PI-3 kinase p110 protein levels decreased in DU145 cells in response to treatment of these cells with PACs (Figures 2.5 A and 2.5 B). The decreases which occurred were statistically significant.

2.3.5 Cranberry Proanthocyanindins Decrease the Translocation of p65 to the Nucleus and Decrease the Phosphorylation Status of IκBα

Since the expression of matrix metalloproteinases can also be affected by the NFκB pathway and since NFκB has been suggested to be a pivotal transcription factor in

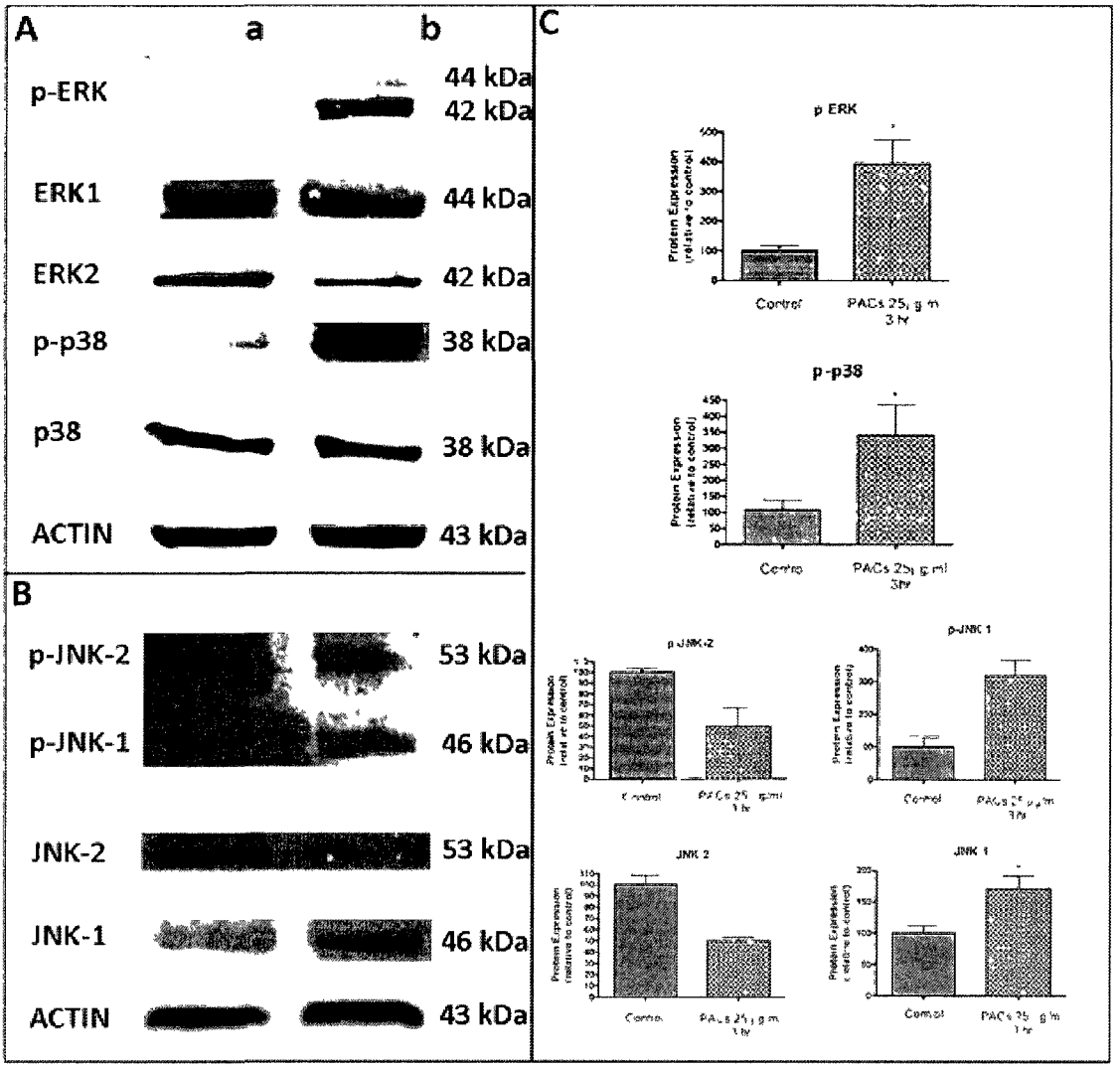


Figure 2.4: Cranberry PACs affect the expression of MAPKs pathway proteins. A: p-ERK, ERK1, ERK2, p-p38, p38 and actin protein levels, respectively in vehicle-treated control cells (a) and in PACs (25 µg/ml) (for 3 hours) - treated cells (b). Actin was used as a loading control B: p-JNK-2, p-JNK-1, JNK-2, JNK-1 and actin protein levels, respectively in vehicle-treated control cells (a) and in PACs (25 µg/ml) (for 3 hours) – treated cells (b). Each blot shown is representative of results obtained from N = 3 with assay duplicates. C: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($p < 0.05$).

prostate cancer metastasis to bone (Andela et al, 2003) whether or not PACs may be also acting via this pathway to affect MMPs' expression levels in DU145 cells was investigated. Therefore, to determine the amount of NFκB (p65) protein in the nucleus, Western blot analyses were performed with nuclear extracts from untreated DU145 cells and from DU145 cells treated with PACs (25 μg/ml) for 3 hours. To verify the integrity of the nuclear fraction extract prepared, the expression of a nuclear protein, H2B, which is a core histone protein, was evaluated. Results indicated that there was a marked expression of histone H2B protein in the isolated nuclear fraction preparation and none detected in the cytosolic fraction which was also prepared. This result as shown in Figure 2.6 A suggests that there is an enrichment of this protein in this fraction verifying the successful preparation of a nuclear fraction. As shown in Figure 2.6 B (and Figure 2.6 D), it was observed that there was a statistically significant decrease in the translocation of NFκB (p65) protein to the nucleus after treatment with PACs. In order to ascertain why this decreased expression level of NFκB (p65) protein occurred in response to PACs treatment, Western blot analyses were performed and the protein expression levels of IκBα, which is an inhibitor of p65 translocation, were determined. As shown in Figures 2.6 C and 2.6 D, results indicate no change in the amount of IκBα, but there was a statistically significant decrease in phosphorylated IκBα after treatment of cells with PACs.

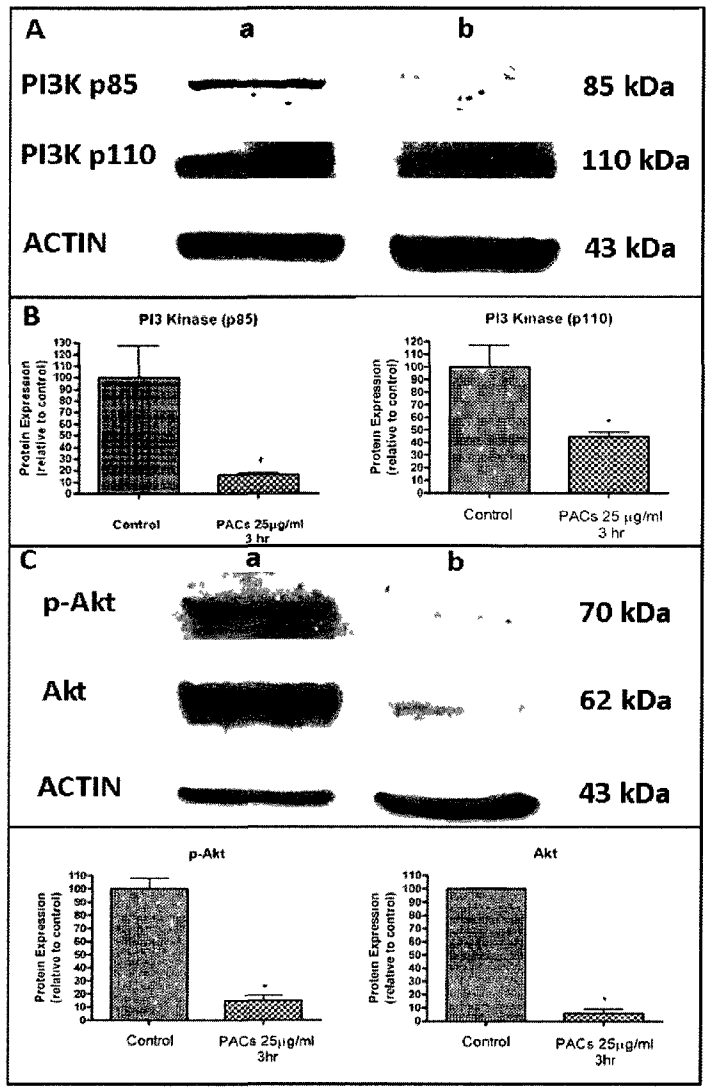


Figure2.5: Cranberry PACs affect the expression of PI-3 kinase pathway proteins. A: Western blot analysis of PI-3K p85 , PI-3K p110 , and actin protein expression levels in vehicle-treated cells (a) and in PACs (25 µg/ml)-treated (for 3 hours) cells (b) Each blot shown is representative of results obtained from N = 3 with assay duplicates. B: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$). C: Western blot analysis of p-Akt, Akt, and actin protein expression levels in cells as described above. Actin was used as a loading control. Each blot is representative of results obtained from N = 3 with assay duplicates. D: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$).

2.4 Discussion:

In this study the effects of cranberry PACs upon MMP activity in DU145 human prostate cancer cells were examined. Cranberry PACs were able to inhibit MMP-2 and MMP-9 activity in a dose and time-dependent manner. This inhibition was part of the cytotoxic response associated with PACs treatment of DU145 cells. Cranberry PACs were shown to be cytotoxic to these cells post 6 hours of treatment. However, most interestingly, PACs also were able to inhibit MMP-2 and MMP-9 activity in these cells at earlier times which occurred in the absence of cellular cytotoxicity in response to PACs treatment. This finding suggests that cranberry PACs may have the ability to modify MMPs' behaviour directly. The findings that cranberry PACS can inhibit MMPs in DU145 cells are in agreement with studies which have shown that grape seed proanthocyanidins can also inhibit MMPs in DU145 cells (Vayalil, Mittal et al. 2004). Similarly, cranberry PACs have been shown to inhibit MMP production and activity in macrophages stimulated with lipopolysaccharides (LPS) (La, Howell et al. 2009). Interestingly, in that study, the macrophages did not exhibit any significant changes in cellular viability after 24 hour treatment with PACs at doses of 100 μg/ml (La, Howell et al. 2009). This difference in cytotoxicity between cancer cells and untransformed cells suggests that cranberry proanthocyanidins may not be cytotoxic to untransformed cells, although further research is needed to determine the effects of cranberry phytochemicals upon untransformed human prostate cells. Cocoa procyanidins have also been shown to inhibit the expression and activation of MMP-2 in vascular smooth muscle cells by direct inhibition of MEK and MT1-MMP activities (Lee, Kang et al. 2008).

The cranberry PACs were able to inhibit the MMP-2 and MMP-9 activities, potentially by affecting the biological signalling within these DU145 cells. PACs treatment affected the

expression of a number of proteins in the MAPKs and PI-3 kinase signal transduction pathways and also disrupted the translocation of NFκB to the nucleus. Different flavonoids and their effects upon MMP activity have been recently examined, and these studies have provided evidence that various flavonoids limit the activity of MMPs (Ende and Gebhardt 2004). This study suggests that the decrease in MMP activity seen in response to cranberry PACs treatment occurs in a multi-factorial manner. One of these factors is the decrease in translocation of NFkB to the nucleus, leading to a proposed decrease in the transcription rate of NFkB mediated genes. When IκBα becomes phosphorylated, it is no longer able to inhibit the translocation of p65 to the nucleus. We found no change in the amount of IκBα; but, there was a pronounced decrease in the amount of phosphorylated IκBα following treatment of DU145 cells with cranberry PACs. Such a decrease in phosphorylated IκBα in the cytosol in response to cranberry PACs treatment could potentially decrease the ability of p65 to translocate to the nucleus. In this regard, the effects of flavonoids upon various cancer cell lines, including DU145 human prostate cancer cells, have been examined and these studies have found a decrease in both NFκB translocation and DNA binding after treatment with this class of compounds (Woo, Pokharel et al. 2006; Yun, Afaq et al. 2009; Shen, Hung et al. 2010). Recently, La et al. (2009) have shown that PACs from cranberries can affect both the expression and activity of select MMPs *in vitro,* and cranberry - derived PACs were able to inhibit the activation of p65 in human macrophages.

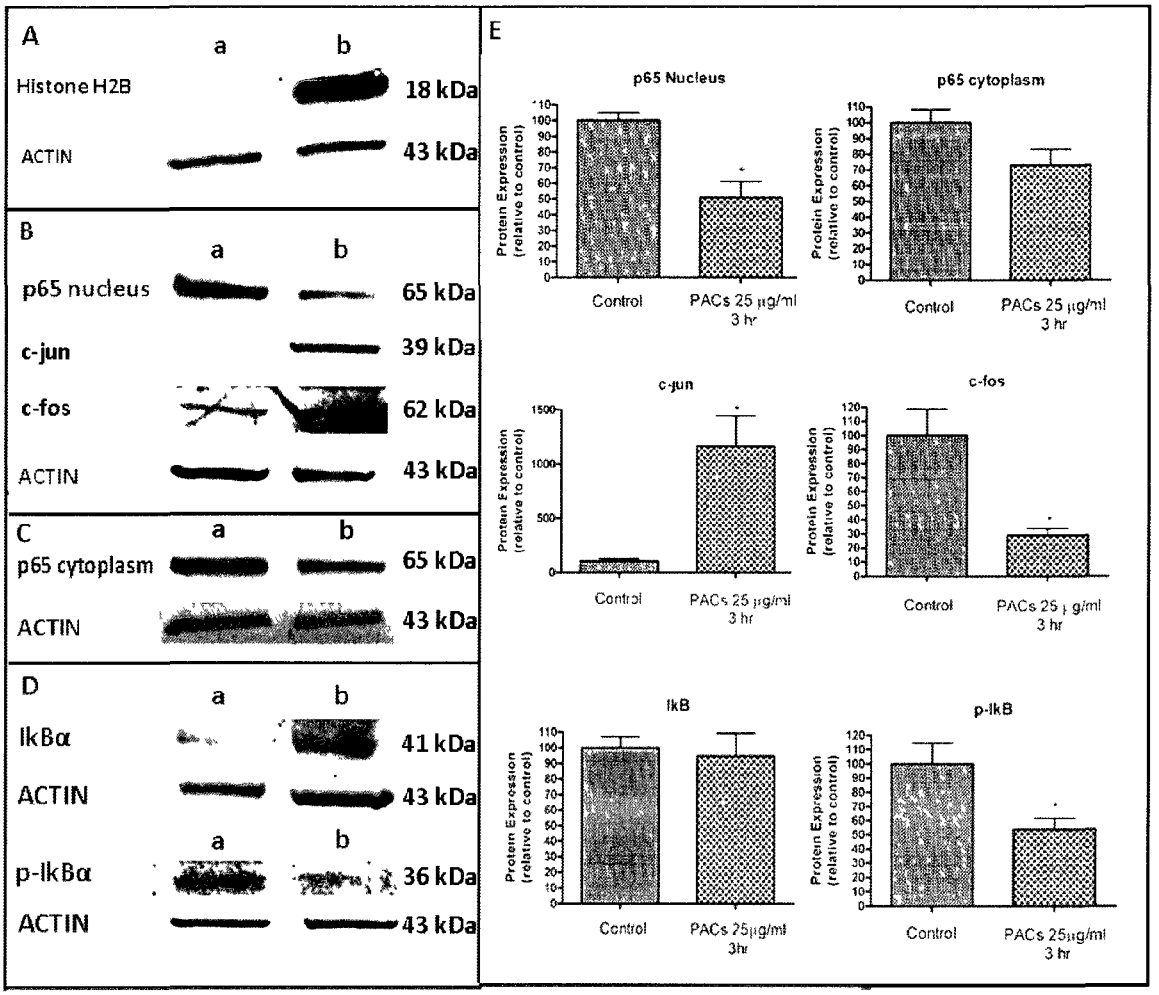


Figure 2.6: Cranberry PACs affect protein expression levels of proteins in the NFκB pathway. A: Western blot analysis of histone H2B protein expression levels in cytosolic fraction (a) and in nuclear fraction (b) from DU145 cells treated with 25 µg/ml of PACs for 3 hours. Nuclear-enriched cell lysates and cytosolic cell lysates (10 µg of protein each) were electrophoresed through a 10% SDS-PAGE gel and immunoblotted with anti-histone H2B. Actin was used as the loading control. Each blot is representative of three separate experiments with assay duplicates. B: Western blot analysis of p65 protein expression levels in the nuclear enriched cell lysate isolated from vehicle treated control cells (a) and isolated from DU145 cells treated with 25 µg/ml PACs for 3 hours (b). Actin was used as a loading control. Each blot shown is representative of N = 3 with assay duplicates. C: Western blot analysis of IκBα and pIκBα protein expression levels in cytosolic cell lysates isolated from vehicle treated control cells (a) and isolated from DU145 cells treated with 25 µg/ml of PACs for 3 hours (b). Actin was used as a loading control. Each Western blot shown is representative of results obtained from N = 3 with assay duplicates. D: Histograms representing densitometric analysis of each of the Western blots of p65, pIκBα and IκBα with the asterisk denoting a statistically significant difference from control ($P < 0.05$).

Interestingly, a study by Vayalil et al (2004), which found that grape seed proanthocyanidins decreased MMP-2 and -9 activities in DU145 cells, showed that this effect was linked to decreases in both NFκB translocation and DNA binding which is agreement with the findings reported herein with cranberry PACs. The effects of grape seed PACs were also attributed to decreases in phosphorylation of three MAP kinase pathway proteins (Vayalil, Mittal et al. 2004). In the current study, the cranberry PACs-mediated inhibition of MMP-2 and -9 activities were associated with increases in the phosphorylation status of p38 and ERK 1/2. This difference may be due to the fact that fractions of proanthocyanidins from different sources may simply contain different compounds and consequently, exert their effects upon the same cell line differently. For example, grape seed proanthocyanidins have B-type linkages between their monomers, whereas cranberries more commonly have A- type linkages (Neto 2007). Acacetin, a flavonoid, has been shown to inhibit the invasion and migration of DU145 cells via inactivation of the p38 MAPK signalling pathway (Shen, Hung et al. 2010). Alternatively, cranberry PACs may be inducing cellular stresses upon the cell earlier than grape seed proanthocyanidins, and thereby causing an initial increase in the phosphorylation of p38, which is activated during periods of cellular stress (Krishna and Narang 2008). The activation of p38 has been associated with programmed cell death, and in this case cranberry PACs may be activating p38 in order to induce apoptosis (Chen and Wong 2008; Kim, Oh et al. 2008). Previous research in our laboratory has also demonstrated that at 25 μg/ml concentrations of PACs from the American cranberry have caused DU145 cells to undergo apoptosis (Scott 2010).The activation of ERK 1/2 in DU145 cells in response to cranberry PACs treatment is also very interesting as this pathway is commonly associated with cellular proliferation and growth. It is suggested that the activation of this particular pathway is due, in part, to some of the cellular stresses imposed upon the cell by the cranberry PACs treatment, and that activation of these proteins may be part

of a compensatory response whereby the cells are attempting to maintain homeostasis. For example, in response to 2,3,5-*tris*-(glutathion-S-yl) hydroquinone (TGHQ), a compound which after treatment results in oxidative stress, the ERK1/2 pathway was induced in non-transformed epithelial cells (Ramachandiran, Huang et al. 2002). The ERK1/2 activation by TGHQ was suggested to be a compensatory mechanism exerted by cells since blocking this pathway with a selective inhibitor prevented the cytoprotective effects of ERK 1/2 activation and resulted in cell death (Ramachandiran, Huang et al. 2002). Cranberry PACs treatment of DU145 cells also resulted in alterations in the expression levels of phosphorylated JNK1/2. The antimetastatic potential of fisetin, a naturally occurring flavonoid, involves inactivation of the PI-3 kinase/Akt pathway and JNK signalling pathways with a concomitant down-regulation of MMP-2 and -9 expressions in PC-3 prostate cancer cells (Chien, Shen et al. 2010). Grape seed extract has been shown to inhibit epithelial growth factor (EGF)-induced and constitutively active mitogenic signalling but activate JNK in DU145 cells, and this was suggested to have a possible role in anti-proliferation and apoptosis in these cells (Tyagi, Agarwal et al. 2003).

The Akt pathway is commonly associated with cellular survival and the evasion of apoptosis and the over-expression of these proteins has been found to occur in many patients with prostate cancer (Le Page, Koumakpayi et al. 2006). In this study, DU145 cells treated with cranberry PACs, affected both the phosphorylation status of Akt and expression of Akt along with a decrease in expression of their upstream mediators, the PI-3 kinases. A decrease in the phosphorylation of PI-3K/Akt proteins has been shown to cause human prostate and colon cancer cells to undergo apoptosis (Hsu, Ching et al. 2000; Engelbrecht, Mattheyse et al. 2007). Quercetin, a flavonoid present in red wine has been shown to affect the tumor necrosis factor α–mediated upregulation of MMP-9 expression by affecting the expression of PI-3 kinase (Hwang, Song et al. 2009). The decrease in expression of PI-3-kinases by cranberry

proanthocyanidin treatment seen in this study may, in part, offer an explanation why the viability of the DU145 cells diminishes substantially after 6 hours of treatment with cranberry PACs, as a dramatic decrease in expression of PI-3K/Akt proteins could cause human prostate cancer cells to undergo apoptosis. In this regard, in a study examining the effects of grape skin extract treatment on human prostate cancer cells, it was observed that the expression of phosphorylated Akt and Akt decreased (Hudson, Hartle et al. 2007). Additionally, these authors suggested another possible explanation which was that treatment with grape skin extract could be affecting the ubiquitination and the subsequent degradation of this protein. Whether or not cranberry PACs affects the ubiquitination status of cellular proteins in DU145 cells remains to be determined. Cranberry PACs treatment of DU145 cells also resulted in alterations in the expression levels of phosphorylated JNK1/2. In this regard, the antimetastatic potential of fisetin, a naturally occurring flavonoid, involves inactivation of the PI-3 kinase/Akt pathway and JNK signalling pathways with a concomitant down-regulation of MMP-2 and -9 expressions in prostate cancer PC-3 cells (Chien, Shen et al. 2010).

Many different types of cancerous cells over express the MMP inducer EMMPRIN, or extracellular matrix metalloproteinase inducer (Riethdorf, Reimers et al. 2006), and the modulation of expression of this protein has been suggested as a potential target for the treatment of cancer (Dean, Newman et al. 2009). Conversely, the increased expression of certain inhibitors of MMP activity (notably the TIMPs, or tissue inhibitors of matrix metalloproteinases) has been associated with decreased vascular formation and decreased cellular migration and invasion within cancerous cells (Valente, Fassina et al. 1998; Ramer and Hinz 2008). This study also showed that cranberry PACs can affect the expression of MMP-2 and MMP-9 activity by affecting the expression of known cellular modulators of MMP activity. In this regard, the expression of EMMPRIN (a documented activator of MMPs) decreased and the

expression of TIMP-2 (a documented inhibitor of MMP activity) increased in DU145 cells in response to cranberry PACs. Such changes would affect and regulate the behaviour of MMP-2 and MMP-9 in response to cranberry PACs. Unexpectedly, the expression of TIMP-1 decreased after treatment with cranberry PACs. TIMP-1 is an inhibitor of MMP-9 activity and as such these findings suggest that the cranberry PACs-mediated inhibition of MMP-9 does not apparently involve an activation of TIMP-1 and occurs apparently independent of this fact. Alternatively, the decreases in TIMP-1 protein expression levels in response to cranberry PACs treatment may represent part of the cell's attempts to maintain homeostasis in response to the cellular stresses imposed upon the cell by cranberry PACs. However, this remains to be determined.

Treatment of cells with proanthocyanidins from the Japanese quince have also been shown to affect the activity of both MMP-2 and -9 in a dose-dependent manner (Strek, Gorlach et al. 2007). Although rather than looking into the molecular mechanisms as to why this may occur, this particular study hypothesized that PACs isolated from the Japanese quince may be directly binding with the MMPs, therefore rendering them inactive. Baxter et al (1997)have suggested that polyphenols can directly bind to proteins which have proline-rich regions in their structure. Whether or not cranberry PACs can affect MMPs' activity in this manner is not known.

In summary, this study has demonstrated and characterized a relationship between cranberry proanthocyanidins and the expression and regulation of matrix metalloproteinases in DU145 human prostate cancer cells. DU145 cells are hormone refractory and as such the response demonstrated may be reflective of a cell type-specific response. As such, future studies should be directed to examining the effects of cranberry PACs on the expression and regulation of MMPs in hormone sensitive prostate cancer cells such as LNCaP cells. It is possible that the response which occurs in such cells may be different or more pronounced. The DU145

cell line is one of the more aggressive prostate cancer cell lines as it is androgen-independent. But, since prostate cancer is a relatively slow growing tumour, it may be more beneficial to gain insight into how cranberry PACs could affect less aggressive or androgen -sensitive/-dependent prostate cancer cells. Previous studies have shown that a whole cranberry extract is capable of affecting the expression of MMPs in DU145 cells (MacLean, Matchett et al. 2007). This current study has extended these previous observations and has identified the specific nature of some of the compounds found in cranberry which can account for cranberry's effects on MMPs. It is possible that the PACs are not the only compounds found in cranberry which are capable of affecting MMP expression. As such, studies are ongoing to examine the effects of other enriched fractions isolated from cranberry on the expression and regulation of MMPs in DU145 cells. It is possible that the PACs fraction may be responsible entirely for the behaviours noted or alternatively the PACs may act in concert with some or all of these other fractions in either an additive or synergistic manner to further affect/ modify the expression of MMPs in DU145 cells. Whether this occurs or not requires further investigation. The effect of cranberry extract and its constituents on the behaviour of prostate cancer cells in an *in vivo* system, to the best of our knowledge, has not been reported and remains to be determined. Finally, this present study contributes to our understanding of how a naturally occurring "bioactive" is capable of regulating the behaviour of prostate cancer cells and as such, provides further evidence in support of the potential health benefits associated with the American cranberry. This further provides support for the inclusion of cranberries as part of a healthy diet because of its possible chemo-preventative and its possible chemo-protective properties against prostate cancer.

CHAPTER THREE

Extracts from the American Cranberry (*Vaccinium Macrocarpon*) decrease the expression of cell cycle proteins and decrease the movement of DU145 human prostate cancer cells through the cell cycle

3.1 Introduction

Prostate cancer is one of the most prevalent cancers in the Western world with the United States having the highest rate of prostate cancer incidence of any country. Though mortality rates for this disease have been decreasing in developed nations in recent years, in 2009 alone prostate cancer claimed the lives of over 36,000 men in North America (Baade, Youlden et al. 2009). As such, more research into the etiology and treatment of prostate cancer is necessary. Though there is certainly a genetic component to a person's risk of developing some cancers, including prostate cancer, environmental factors are also known to play a key role (Alberti 2010). For example, rates of prostate cancer are much lower in East Asian countries as compared to North America (Hsing and Chokkalingam 2006). But, when populations of East Asian men move to North America their risk of developing prostate cancer increases for both themselves and their descendents, suggesting an environmental role for the development of this neoplasm (Cook, Goldoft et al. 1999). Further, it has been suggested that diet plays an important role in the development of prostatic cancer (Shirai, Asamoto et al. 2002) (Sonn, Aronson et al. 2005).

Recently, there has been interest in examining the effects of whole extracts and their constituent phytochemicals from a wide variety of different foods and beverages, on the

behaviour of cancer both *in vitro* and *in vivo*. It has been shown that a large variety of phytochemicals have profound effects on the behaviour of prostate cancer cells including apoptosis, cell cycle arrest, and the modulation of mitogenic signalling pathways (Neehar and Rajesh 2001; Catherine, Jon et al. 2008; Neto, Amoroso et al. 2008; Dixan, Marcela et al. 2009; Thomas, Norberta et al. 2010).

Our research focuses on the fruit from the American cranberry (*Vaccinium macrocarpon*). Previous research in our laboratory has demonstrated that constituents from the cranberries can induce apoptosis and affect the activity of certain proteins associated with the metastatic potential of cancer in androgen-insensitive human prostate cancer cells (MacLean, Matchett et al. 2007) (Déziel, Patel et al. 2010). Due to its ability to affect apoptosis, it was hypothesized that whole extracts from the American cranberry could cause cell cycle arrest in DU145 human prostate cancer cells by affecting specific signalling mechanisms. In this study we examined the effects of increasing doses of whole cranberry extract (WCE) on cellular viability, on the expression of specific cell cycle proteins, and the progression of DU145 cells through the cell cycle.

3.2 Materials and Methods

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following antibodies were used: goat polyclonal anti-actin, and rabbit polyclonal anti-Cdk2, anti-Cdk4, anti-Cyclin A, anti-Cyclin B1, anti-Cyclin D1, anti-Cyclin E, anti-p21 and anti-p27. All other chemicals and materials were purchased as indicated.

3.2.1 Preparation of Cranberry Extract

The cranberry extracts used in this study were prepared by the laboratory of Dr. Catherine C. Neto (University of Massachusetts –Dartmouth, North Dartmouth, MA), and their extraction and preparation are detailed in Appendix A.

3.2.2 Cell Culture and Treatment with Whole Cranberry Extracts

Human DU145 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in alpha MEM (Gibco, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Sigma, Oakville, ON.) and 1% antibiotic-antimycotic (Gibco). Cells (4×10^5) were cultured on the tissue culture dishes and were initially incubated for 24 hours at 37˚C in the presence of 5% CO_2. After this 24 hour incubation period, the media was removed and replaced with serum free alpha MEM supplemented with 5 µg/ml of transferrin (Sigma) and 2.5µg/ml of insulin (Sigma). These plates were again incubated for 24 hours at 37˚C in the presence of 5% CO_2. After this incubation period, cells were treated with various concentrations of whole cranberry extract (WCE) ranging from 1µg/ml to 50 µg/ml six hours. WCE was dissolved in 100% methanol as the vehicle prior to use. The control cells received only the vehicle. After treatment, the media was removed from the cells and stored at -80˚C for further analysis. The cells were then washed with PBS, and were removed by trypsin (Sigma) diluted in PBS. The cells were re-suspended with alpha MEM supplemented with 10% FBS and were centrifuged for 4 minutes at 500 x g. After centrifugation, the cell pellet was re-suspended with PBS and was transferred to a micro centrifuge tube and was centrifuged at 500 x g for 4 minutes. After centrifugation, this cell pellet was then stored at -80˚C until analyzed further.

3.2.3 Alamar Blue Cytotoxicity Assay

The Alamar Blue assay (Invitrogen, Burlington, ON) was performed to determine cellular viability after treatment with WCE and was performed as per manufacturer's instructions. Briefly, DU145 cells were subcultured into a 96 well plate at 5,000 cells/well. After 24 hours of incubation, the media was replaced with 100µl of serum-free media and was once again incubated for 24 hours. After this incubation, the cells were treated with WCE for 6 hours, with a final concentration of methanol within each well of 1%. The control for this experiment was DU145 cells treated with methanol at a total concentration of 1% for 6 hours. Following treatment, 10µl of Alamar Blue was added to each well. The cells were incubated for 3 hours post exposure of the cells to Alamar Blue. After this 3 hour incubation period, the resulting fluorescence was read with an excitation wavelength of 528 nm and an emission wavelength of 590 nm.

3.2.4 Immunoblot Analyses

Equal amounts of protein extracts from whole, cytosolic, and nuclear fractions were mixed in a 3:1 ratio with standard Laemmili buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% SDS , 0.1% bromophenol blue, 10% glycerol and 100mM beta-mercaptoethanol. Once mixed, these samples were boiled for 3 minutes. Once boiled, they were resolved by electrophoresis through 10% SDS-PAGE gels and were transferred onto nitrocellulose membranes (Biorad). After transfer, the membranes were incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution for one hour at room temperature. Then, the membranes were incubated with primary antibodies diluted to a 1:200 ratio (v/v). The membranes were incubated in the presence of the primary antibody for 24 hours at 4˚C. After incubation, the membranes were then washed three times with TBS-Tween (0.05%) for ten minutes and were then incubated in alkaline phosphatase-conjugated secondary antibodies (1:2000 dilution) for 1 hour. After

incubation, the membranes were again washed three times with TBS-Tween (0.05%) for ten minutes, washed briefly with distilled water, and then exposed to SigmaFast BCIP-NBT tablets (Sigma) in solution to visualize protein expression levels. Such Western blots were then analyzed using Infinity Capture software (Lumenera Corp., Ottawa, ON), and densitometry was performed with ImageJ software (National Institute of Health, Bethesda, MA).

3.2.5 Flow cytometry for Cell Cycle Analysis

DU145 cells treated with 1% methanol (control) or treated with 1, 10, 25, or 50 µg/ml for 6 hours were harvested with trypsin and then washed with alpha MEM containing 10% FBS. The cells were then centrifuged for 5 minutes at 200 x g at room temperature. Once centrifuged, the media was removed and the cells were re-suspended in 25 µl of PBS, which were then placed in 225 µl of 70:30 ethanol:PBS mixture and allowed to fix for 2 hours on ice. The fixed cells were then centrifuged for 5 minutes at 200 x g at 4˚C, and were then re-suspended in 250 µl of ice-cold PBS for 5 minutes. After 5 minutes had passed, the cells were then re-centrifuged for 5 minutes at 200 x g at 4˚C and the cell pellet was then suspended in a PBS propidium iodide (.02 mg/mL) (Sigma) staining solution containing .088% Triton-X (Sigma) and .4 mg/mL of RNAse (Sigma) and were incubated for 30 minutes at room temperature in the dark. Once incubated, the cells were transferred into a 96 round bottom well plate and were analysed using a BD FACSARRAY bioanalyzer equipped with BD FACSARRAY system software version 1.0.3 (BD Biosciences, Mississauga, ON). The results of this analysis were processed using software version 2.9 (The Scripps Institute, La Jolla, CA).

3.2.6 Statistical Analysis

Statistical analyses for each assay were done using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diego, CA). Results from the cytotoxicity assay, flow cytometry

and Western blots were compared using a one way ANOVA with a Tukey's Posthoc test, and results were considered statistically significant at $p < 0.05$.

3.3 Results

3.3.1 WCE causes cytotoxicity to DU145 cells at 10, 25 and 50 µg/ml at 6 hours

The effects of WCE on DU145 human prostate cancer cells were measured by Alamar Blue assay. As shown in figure 3.1, WCE significantly decreased the viability of these cells at 10, 25 and 50 µg/ml of treatment. Viability decreased by 25.9%, 31.8% and 46.0% at 10, 25 and 50 µg/ml of WCE, respectively.

3.3.2 WCE decreases the expression of cell cycle dependent proteins, and increases the expression of p27, a cell cycle inhibitor

As shown in figure 3.2, the expression of CDK 4, cyclin A, cyclin B1 and cyclin E significantly decreased at 50 µg/ml of treatment for 6 hours. The expression of cyclin D1 significantly decreased at 10, 25 and 50 µg/ml of WCE treatment. To further explore why the expression of these proteins may have been affected, the expression of two cell cycle inhibitors, p21 and p27, was also examined. After treatment with 1, 10, 25, and 50 µg/ml of WCE for 6 hours, the expression of p21 remained unchanged when compared to vehicle control, but the expression of p27 increased significantly at 50 µg/ml. Figure 3.3 represents the densitometric analysis of each Western blot, which represents the averages of 3 separate replicates.

3.3.3 WCE decreases the number of DU145 cells in the G2-M phase, and increases the number of DU145 cells in the G1 phase of the cell cycle.

To further investigate the effects of WCE on DU145 cells, flow cytometric analysis was conducted to ascertain if WCE affects cell cycle progression. As shown in figure 3A and B, after treatment with 25 and 50 μg/ml for 6 hours, WCE significantly decreased the number of cells in the G2-M phase of the cell cycle, and increased the number of cells in the G1 phase of the cell cycle. The first image in figure 4A represents one experiment with vehicle treated cells (1% methanol for 6 hours), and the second image represents one experiment with DU145 cells treated with 50 μg/ml WCE for 6 hours. Each flow cytometric experiment was repeated in triplicate, and the averaged results of each experiment are shown in figure 4B.

3.4 Discussion

In this study, we examined the effects of WCE on the behaviour of human prostate cancer cells. WCE was able to induce cytotoxicity at 10, 25 and 50 μg/ml after 6 hours, inhibit the expression of cell cycle proteins, and slow the progression of DU145 cells through the cell cycle. Cell cycle deregulation is a common occurrence in cancer (Katrien, Dirk et al. 2003), as one of the main hallmarks of cancer is uncontrolled and unmitigated cell growth (Hanahan and Weinberg 2000). Deregulation of cell cycle proteins allows cancerous cells the ability to grow and divide with little restriction. There has been interest in examining the ability of phytochemicals from fruits and vegetables to affect the expression of cell cycle proteins, as this may be a method of controlling or preventing the growth of cancer (Agarwal 2000) (Le Marchand 2002).

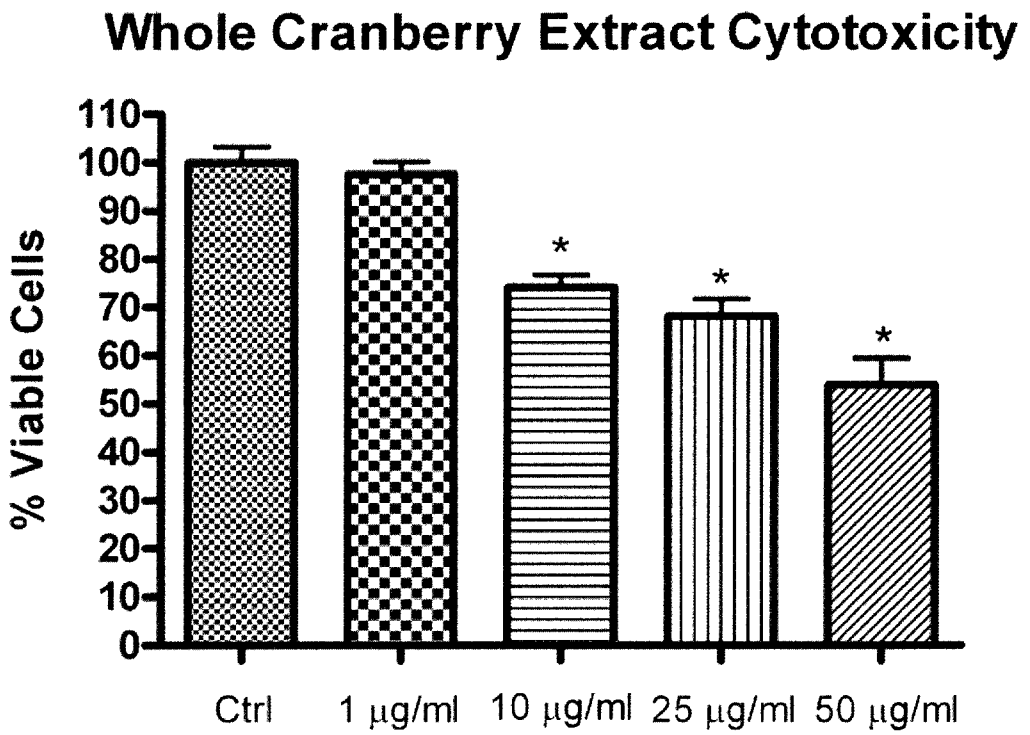


Figure 3.1 WCE treatment of DU145 cells affects cellular viability. DU145 cell viability was evaluated by Alamar Blue assay following treatment with 1 μg/ml, 10μg/ml, 25 μg/ml and 50 μg/ml of WCE for 6 h. Control cells received 1% methanol (vehicle) for 6h. Fluorescence was read at 528 excitation and 590 emission wavelengths. Control cells were set to 100% viability. Treatment with WCE for 6 h significantly decreased the viability of DU145 cells at 10 μg/ml, 25 μg/ml and 50 μg/ml relative to control cells ($p < 0.05$). The results presented represent values obtained from N = 8.

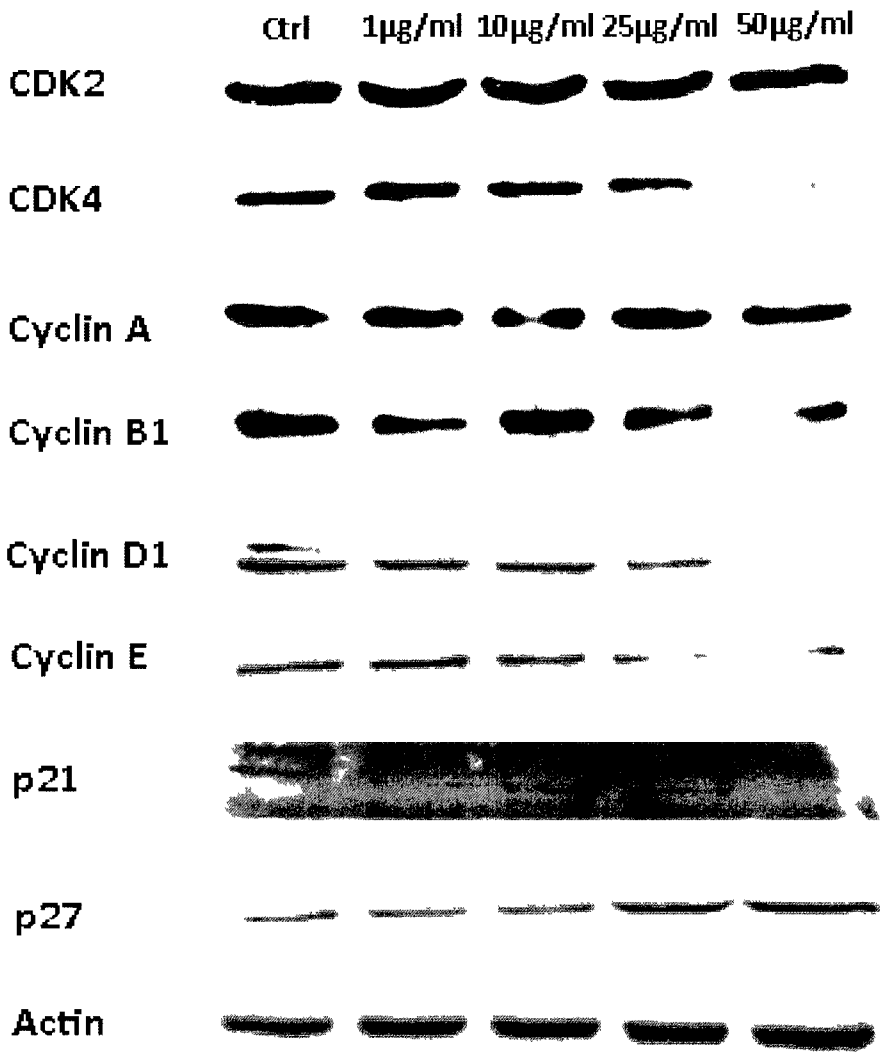


Figure 3.2 WCE affects the expression of cell cycle proteins in DU145 cells. Cells were treated with vehicle (1% methanol) 1 µg/ml, 10 µg/ml, 25 µg/ml and 50µg/ml for 6 h. Actin was used as a loading control. Each blot shown is representative of results obtained from N = 3 with assay duplicates.

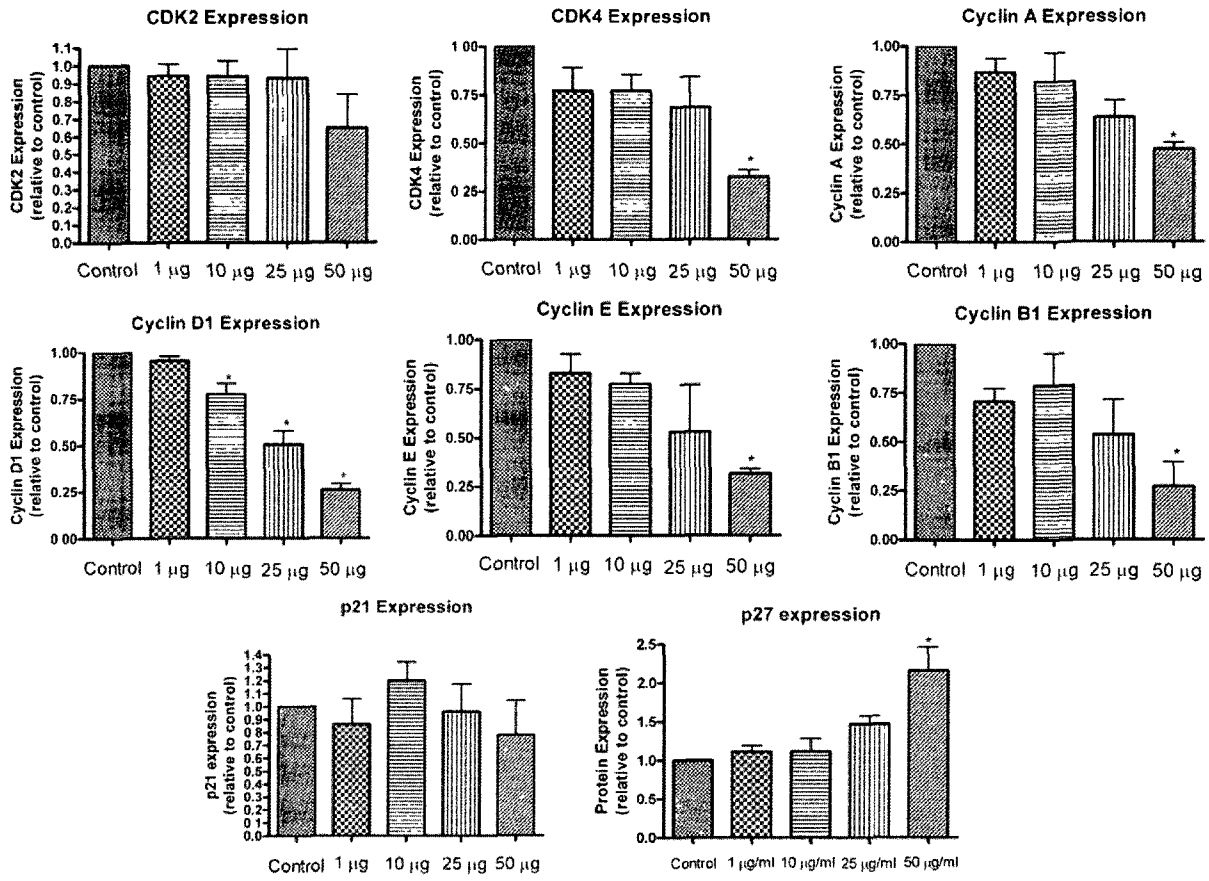


Figure 3.3 Histograms representing the densitometric averages of N = 3 of each Western blot analysis shown in figure 3.2. Asterisks denote a statistically significant difference from control ($p < 0.05$).

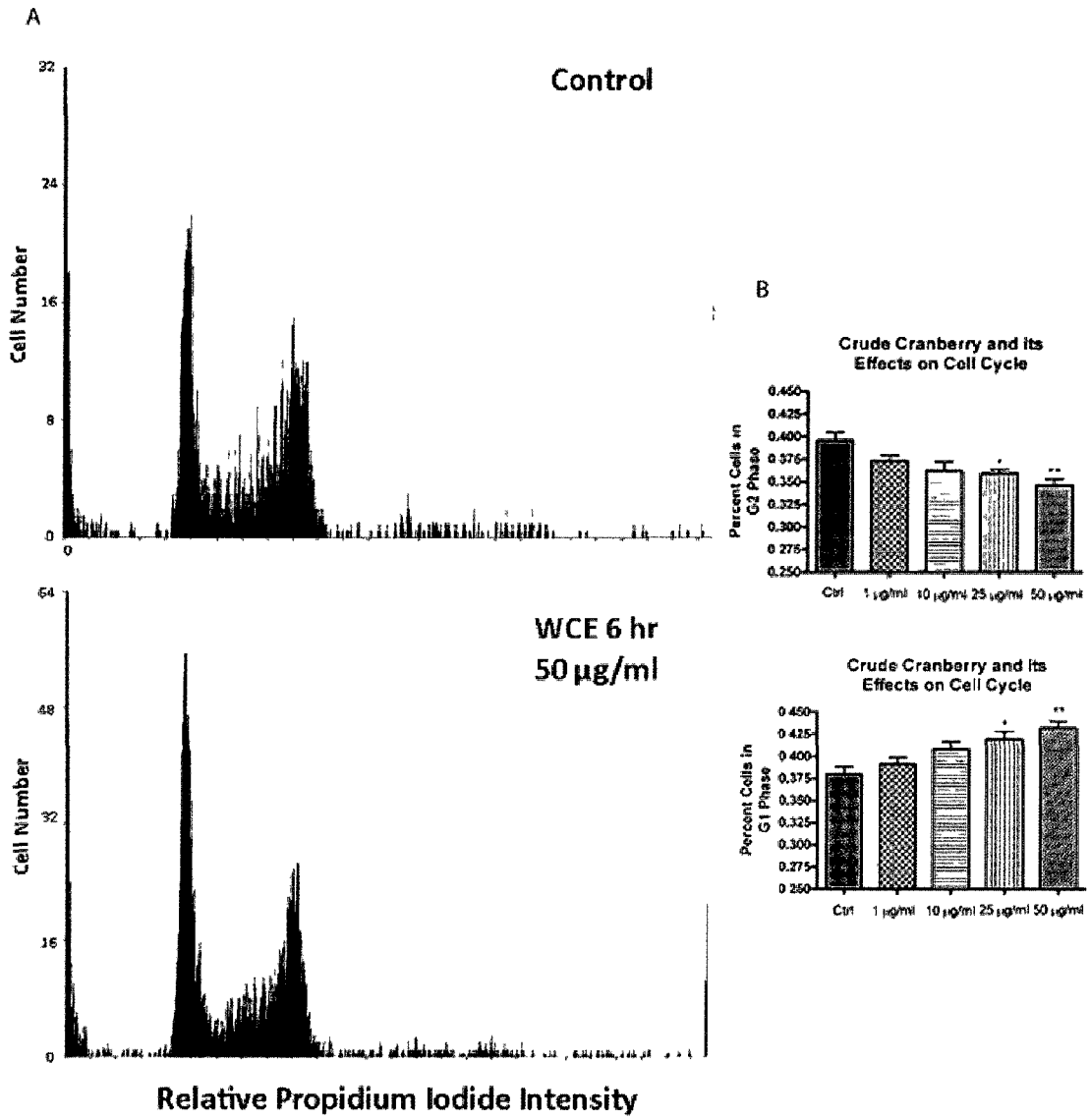


Figure 3.4A and 3.4B: Flow cytometric analysis of DU145 cells treated with vehicle control (1% methanol) and 50 µg/ml of WCE for 6 hours. WCE significantly decreased the number of cells in the G2 phase of the cell cycle and increased the number of cells in the G1 phase of the cell cycle at 25 and 50 µg/ml. Data in figure 3.4A is a representative experiment repeated in tripliacate. Figure 3.4B includes histograms from data averaged from N = 3 experiments. One asterisk denotes a statistically significant difference from control with a ($P < 0.05$), and two asterisks denote a statistically significant difference from control with a ($P < 0.01$).

The present study is consistent with other reports in the literature regarding changes in the expression of cell cycle proteins with whole food extracts and/or constituent phytochemicals. For example, Agarwal and Bhatia (2001) found that treating cells with silymarin, genistein and EGCG decreased or completely inhibited cell growth, decreased the expression of specific cell cycle proteins and caused significant cytotoxic effects in DU145 cells at doses ranging from 100 to 200 μM. Interestingly, in their study CDK 2 levels changed only after treatment with silymarin and not with the other two compounds. This result is similar to our observations wherein CDK 2 was the only cell cycle protein which was not affected by treatment with WCE. The effects of cranberry extract on the induction of apoptosis and cell cycle arrest in MCF-7 human breast cancer cells revealed similar results. In their study, the concentrations of cranberry were much higher than ours, in the order of approximately 1000 fold, suggesting that perhaps the extracts used in our study are more concentrated or have higher anti-cancer activity. The cranberry extracts used in their study also appeared to increase the number of cells in the G2-M phase of the cell cycle, whereas our WCE decreased cells in this phase. This could be attributed to the different cell line used and a potentially different extraction process for obtaining our cranberry extract.

The induction of p27 and not p21 is an intriguing finding, as some studies examining the effects of phytochemicals on cell cycle proteins often find that both proteins increase in expression (Aggarwal, Banerjee et al. 2007) (Yoon, Jeong et al. 2007). However, other studies show that p21 and p27 expressions are not in sync after treatment with phytochemicals (Lu, Arteaga et al. 2005) (Shi, Lin et al. 2008). The study by Lu, Arteaga et al. specifically examined the effects of avocado carotenoids on the androgen-sensitive and insensitive human prostate cancer lines LNCaP and PC-3, respectively. Their results indicated that these compounds decrease cell proliferation and increase p27 expression at concentrations similar to ours.

However, after 72 hours of treatment they had an accumulation of cells in the G2-M phase of the cell cycle, indicating that WCE potentially acts differently than avocado carotenoids, though this difference may be in part due to the different time frame used in the study (72 hours) and different cell lines.

The other family of cell cycle inhibitors, which was not examined in this study, is the INK4 family. This protein family specifically targets the cyclin-dependent kinases necessary for progression through the G1 phase of the cell cycle (Vermeulen, Bockstaele et al. 2003). In one study, the expression of p18, an INK4 family member, increased in lung cancer cells after treatment with silibinin, a phytochemical found in milk thistle. (Samiha, Alpna et al.). This was found in conjunction with increases in p21 and p27 inhibitory proteins. Another study examined the effects of combined selenium and whole extracts from *Spirulina platensis*, which are microalgae used for nutritional supplements. These combined extracts with selenium were able to arrest MCF-7 breast cancer cells in the G1 phase and increase the expression of p15, an INK4 protein, and p21 (Chen, Wong et al. 2010). Future direction in regards to research with WCE could focus on the effects of cranberry on the expression of this family of inhibitor proteins.

Another potential inhibitor of cell cycle progression which was not examined in this study is pRB. This phosphorylated protein product is required for the transition from G1 to S, and this protein's regulation is often uncontrolled or mutated in cancer cells (Bartek, Bartkova et al. 1996). Decreased phosphorylation of this protein has been demonstrated after treatment with many novel phytochemical extracts (Vivar, Lin et al. 2009) (Kwon, Hong et al. 2010), and we suggest that pRB phosphorylation may be altered after treatment with WCE as well, though more research is necessary to determine the effects, if any, of WCE on pRB phosphorylation.

In conclusion, this study demonstrates the cell cycle inhibitory effects of WCE on hormone-refractory human prostate cancer cells. To elucidate the effects of WCE further, future studies should examine the effects of WCE on different prostate cancer cell lines to determine if the effects seen in this study are cell line specific. For example, LNCaP cells represent a less aggressive form of prostate cancer as they are hormone-sensitive, and future research may focus on this cell line in order to determine if the effects seen with DU145 are similar or perhaps more pronounced with LNCaP cells. Also, there are specific phytochemical families found within the cranberry which may be having direct effects on the expression of cell cycle proteins. As such, future directions will focus on the effects of certain families of compounds found with the fruit on DU145 cells in an attempt to determine which families affect cell cycle behaviour. Such studies are currently ongoing. Finally, this study reveals many novel effects of WCE on DU145 cells, which we suggest is a potential source of new phytochemical anti-cancer agents. More research is necessary to determine the mechanisms by which cranberry exhibits its anti-proliferative effects within human prostate cancer cells.

CHAPTER FOUR

The results of an *in vivo* dietary intervention prostate cancer pilot study: the TRAMP mouse trial

4.1 Introduction

Prostate cancer is a disease which is only commonly found in humans, so as such it has been difficult to develop an animal model for this disease in order to study disease etiology and progression, to examine possible treatment options, and to also conduct research regarding possible chemo-therapeutic properties associated with natural products. Though prostate cancer xenograph animals have provided researchers with some information about this disease, there has been a need for a model which develops prostate cancer spontaneously so that intervention and preventative measures could be tested. Because of this need, the TRAMP mouse model was developed. The TRAMP mouse is a genetically altered mouse which has been reported to develop prostate cancer 100% of the time in a predictable and stepwise manner. This model is especially advantageous for feeding studies because it allows for dietary intervention to commence even before the animal has developed a primary tumour, prostatic intraepithelial neoplasia (PIN), or hyperplasia of the prostate.

Recent research into the effects of dietary components on the development of prostate cancer in humans has demonstrated interesting results which suggest that some components of the diet may promote the development of this disease, while others potentially decrease the risk of developing this form of cancer (Sonn, Aronson et al. 2005) (Aronson, Barnard et al. 2010). Many studies have shown that the consumption of certain fruits and vegetables have been associated with decreased risk of developing cancer (Kirsh, Peters et al. 2007; Yan and Spitznagel

2009), and because of this there have been a variety of nutritional studies examining the effects of select fruits and vegetables on the development of prostate cancer in the TRAMP mouse model. For example, a recent study demonstrated that grape seed extracts, which were for the most part made up of proanthocyanidins, decreased the expression of proliferating cell nuclear antigen (PCNA), the expression of cell cycle related proteins, and the incidence of adenocarcinoma in the TRAMP mouse (Raina, Singh et al. 2007). Another TRAMP mouse study examined the effects of tocotrienols, which are phytochemicals commonly found in palm oil, and found that family of compounds decreased the expression of cell cycle proteins and increased the expression of cell cycle inhibitors p21 and p27 in the dorsolateral prostate (Barve, Khor et al. 2010).

Because of previous research in our laboratory demonstrating the diversity of effects of cranberry and its related phytochemicals on prostate cancer cells *in vitro*, it was the purpose of this pilot study to examine the effects of freeze-dried whole cranberry in an animal model, the TRAMP mouse. It was hypothesized that dietary intervention with cranberries, at 3% of dietary intake (w/w) would affect the behaviour of prostate cancer *in vivo*. Specifically, it was hypothesized that cranberry feeding would decrease tumour growth, cell cycle expression within the prostate, and mortality of the mice.

4.2 Materials and Methods

4.2.1 Antibodies

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following antibodies were used : goat polyclonal anti-actin, anti-rabbit polyclonal anti-Cdk2, anti-Cdk4, anti-cyclin A, anti-cyclin B1, anti-cyclin D1, anti-cyclin E, anti-

PSP94 (M-113) and anti-PSP94 (R-113). All other chemicals and materials were purchased as indicated.

4.2.2 Diet Preparation

All ingredients used to make the AIN-93 purified diet were purchased from Dyets (Bethlehem, PA). In this study, two experimental diets were prepared. The first was a control AIN-93 diet, and the second was an AIN-93 diet supplemented with 3% cranberry (w/w). The ingredient composition of each of these diets is listed in Table 4.1.

4.2.3 Animal Care and Husbandry

All procedures involving animals were overseen by the University of Prince Edward Island Animal Care Committee.

Twenty male TRAMP mice (TRAMPC57BL/6-Tg) and twenty control mice (C57BL/6J) were purchased at 8 weeks of age from Jackson Laboratories (Ann Arbor, ME). After arrival at the Atlantic Veterinary College (AVC), all mice were housed individually and weighed. Each mouse was then randomly assigned to one of two separate feeding groups. One group of 20 mice (10 control and 10 TRAMP) received the control AIN-93 diet, and the other 20 mice received the 3% cranberry-supplemented diet. The mice were acclimated to the AIN-93 diet for one week beginning at 8 weeks of age, and the study began after after the mice reached 9 weeks of age. Each mouse was allowed to consume food and water *ad libitum*. Their food intake was measured daily, and each mouse was weighed on a weekly basis. If any individual animal showed signs of lowered food or water intake, a reduction in weight of 10% or more or significant changes in behaviour including increased aggression, decreased grooming, or difficulties during movement, they were monitored with greater scrutiny. If these symptoms

persisted, the animal was anaesthetized, euthanized, and tissues collected according to the procedure found in section 4.2.4.

4.2.4 Evaluation of Tumour Development by Ultrasound

Beginning at week 20 and every subsequent week, an ultrasound was performed on each TRAMP mouse in order to evaluate the growth of the prostatic tumour. This procedure was performed by the senior veterinarian of the animal facility. Briefly, each mouse was restrained and a solution of 70% ethanol was applied to each animal's abdomen which was then ultrasounded for approximately 20 – 30 seconds using a LOGIQ® E ultrasound device (GE Healthcare, Buckinghamshire, UK). After scanning, each image was examined for the presence / absence of a visible tumour, located in proximity to the bladder. The bladder was used as a point of reference in each scan. Two dimensional measurements of the size of each visible tumour were taken and measurements were taken for each subsequent week until the animals reached 33 weeks of age.

4.2.5 Tissue Collection and Necropsy

At 33 weeks of age, each of the remaining animals were weighed a final time and anaesthetized with a solution of 65mg/kg of sodium pentobarbital. Blood (approximately 5 ml) was collected by cardiac punction and the animals were euthanized by exsanguinations. The blood was then placed into microcentrifuge tubes and allowed to coagulate for one hour at room temperature. These tubes were then stored on ice for approximately one hour and then these tubes were centrifuged for 10 minutes at 1700 x g. This centrifugation allowed for the collection of serum, which was then stored in a separate tube. Both the remaining blood and serum were stored at -80˚C. During necropsy the lower urogenital tract (LUT), which included the bladder, prostate, and seminal vesicles, was removed, drained, and weighed.

Table 4.1 AIN-93G Diet Composition

Ingredient	Amount (g/kg diet)
Casein, High Nitrogen, 80 mesh	200.0
Cornstarch	397
Dyetrose[1]	132
Sucrose	100
Cellulose	50
Cranberry Extract[2]	0
Soybean Oil + TBQ	70
Mineral Mix[3]	35
Vitamin Mix[4]	10
L-cystine	3
Choline bitartrate	2.5

[1] 90% tetrasaccharides and higher.

[2] To formulate 3% cranberry diet, 30 g of cranberry was added in lieu of cornstarch.

[3]Mineral mix supplied the following concentration of minerals in g/kg mix: calcium carbonate, 357.0; KH_2PO_4, 196.0; K Citrate·H_20, 70.78; NaCl, 74.0; K_2SO_4, 46.6; MgO, 24.3; Fe citrate, 6.06; zinc carbonate, 1.65; $MnCO_3$, 0.63; $CuCO_3$, 0.31; KIO_3, 0.01; Na_2SeO_4, 0.01025; $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.00795; $Na_2SiO_3·9H_2O$, 1.45; $CrK(SO_4)_2·12H_2O$, 0.275; LiCl, 0.0174; H_3BO_3, 0.0815; NaF, 0.0635; Nickel carbonate, hydroxide, tetrahydrate, 0.0318; NH_4VO_3, 0.0066.

[4] Vitamin mix supplied the following concentration of vitamins in g/kg mix: thiamin HCL, 0.6; riboflavin, 0.6; pyridoxine HCL, 0.7; nicotinic acid, 3.0; Ca pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; vitamin B_{12} (0.1% in mannitol), 2.5; vitamin A palmitate (500 000 IU/g), 0.8; dl -α-tocopheryl acetate (500 IU/g), 15; vitamin D_3 (400 000 IU/g), 0.25; vitamin K/Dextrose 10 mg/g (phylloquinone), 7.5.

After this, approximately half of the dorso-lateral prostate (or half of any visible prostatic tumour) was removed and stored at -80˚C. The rest of the LUT was stored in 10% neutral buffered formalin (NBF). Other tissues which were stored in 10% NBF included the spleen, lungs, the left kidney, and right femur. Other tissues that were stored at -80˚C were the liver and right kidney.

4.2.6 Histology

Upon fixation within 10% NBF, the LUT from each of the TRAMP mice was removed from formalin and the seminal vesicles, anterior prostate, and any visible section of prostatic tumour were pre-sectioned appropriately. These tissues were then embedded in paraffin, sectioned and routinely stained with haematoxylin and eosin by the AVC histology department. Each section was then examined by a pathologist from the AVC who was blind to which tissues came from which treatment group. The pathologist evaluated the seminal vesicles and anterior lobe of the prostate similarly, with slight modifications, to according to the procedure of Kaplan-Lefko, Chen et al. (2003). Briefly, the seminal vesicles and anterior lobe were evaluated on a grading scheme and each sample was assigned a grade ranging from "Normal", "PIN", "Well Developed Adenocarcinoma", "Poorly Developed Adenocarcinoma" and "Phyllodes-like Regions". These types of tumours in humans are a relatively rare development within the seminal vesicles during prostatic cancer progression, which have a variety of clinical outcomes (Kaplan-Lefko, Chen et al. 2003).

The lungs were also sectioned and stained in the same manner and were evaluated for the presence or absence of metastases.

4.2.7 Prostate Tissue Preparation

From four randomly selected control mice and four 3% cranberry-fed mice, sections of the aforementioned frozen prostatic tissue were removed from the -80°C freezer and thawed on ice. They were then homogenized using the Retsch MM400 Mixer mill (Newtown, PA) for 2 minutes at a frequency of 30 Hz. Once homogenized, the tissues were then kept on ice and sonicated for approximately 3 seconds thrice. The tissues were then centrifuged at 9300 x g at 4°C for 20 minutes. Once centrifuged, the supernatant was removed, placed into a new Eppendorf tube, and evaluated by Bradford assay using Biorad protein assay (Biorad) as per manufacturer's instructions.

4.2.8 Prostate Tissue Western Blots

Equal amounts of protein extracts from four prostate tissue samples from the control mice and four samples from the 3% cranberry-fed mice were mixed in a 3:1 ration with standard Laemmili buffer, the contents of which are detailed in chapter two, section 2.2.5. Once mixed, these samples were boiled for 3 minutes. Once boiled, they were resolved by electrophoresis through 10% SDS-PAGE gels and were transferred onto nitrocellulose membranes (Biorad). After transfer, the membranes were incubated in the presence of a 1% BSA (w/v) TBS-Tween (0.05% v/v) solution for one hour at room temperature. Then, the membranes were incubated with primary antibodies diluted to a 1:200 ratio (v/v). The membranes were incubated in the presence of the primary antibody for 1 hour at room temperature. After incubation the membranes were then washed three times with TBS-Tween (0.05%) for ten minutes and were then incubated in alkaline phosphatase-conjugated secondary antibodies (1:2000 dilution) for 1 hour. After incubation, the membranes were again washed three times with TBS-Tween (0.05%) for ten minutes, washed briefly with distilled water, and then exposed to SigmaFast BCIP-NBT

tablets. Once development had finished, the blots were then rinsed with distilled water twice and then stored in distilled water until imaged.

4.2.9 Imaging and Statistical Analysis

Imaging for each Western blot was performed by Infinity Capture software (Lumenera Corp., Ottawa, ON), and densitometry was performed with ImageJ software (National Institute of Health, Bethesda, MA). All statistical analyses were performed by using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc. San Diego, CA) and results for each analysis were considered significant at $P < 0.05$.

4.3 Results

4.3.1 Control Mice Fed A 3% Cranberry Diet Weighed Significantly Less than Control During A Period of 4 Weeks

A 3% cranberry diet appears to affect weight gain in control mice. As shown in Figure 4.01, mice fed a cranberry-supplemented diet were significantly lighter than those in the control group from week 14 to week 17. Once the mice reached the 18 week mark of the study (at which time they were 27 weeks of age), the weights of the mice in the cranberry diet were no longer statistically significantly from those in the control group. This effect was not seen in the TRAMP mice (Figure 4.02).

Feed efficiently for both the control and the TRAMP mice was calculated by dividing the total weight gained by each animal by the average food consumed for each animal per day. For both the control group and TRAMP group, the feed efficiencies between both diets were not statistically different as shown in Figures 4.05a and 4.05b.

4.3.2 Ultrasounds of the TRAMP mouse provide a reliable indicator of the size of the prostatic tumour

Ultrasounds performed on TRAMP mice once per week were able to detect large prostatic masses of at least .5 cm in diameter and were able to detect changes in size after each subsequent week. A series of example ultrasounds is shown in Figures 4.06a through 4.06d for a representative animal. Upon euthanization of mouse, the tumour's size was measured as approximately 2 cm x 2 cm x 1.4 cm, approximating what was measured during the ultrasound.

During each necropsy the size of any discernable mass was measured and compared to what was detected at the time of the last ultrasound before euthanization. A summary of these results is listed in appendix B.

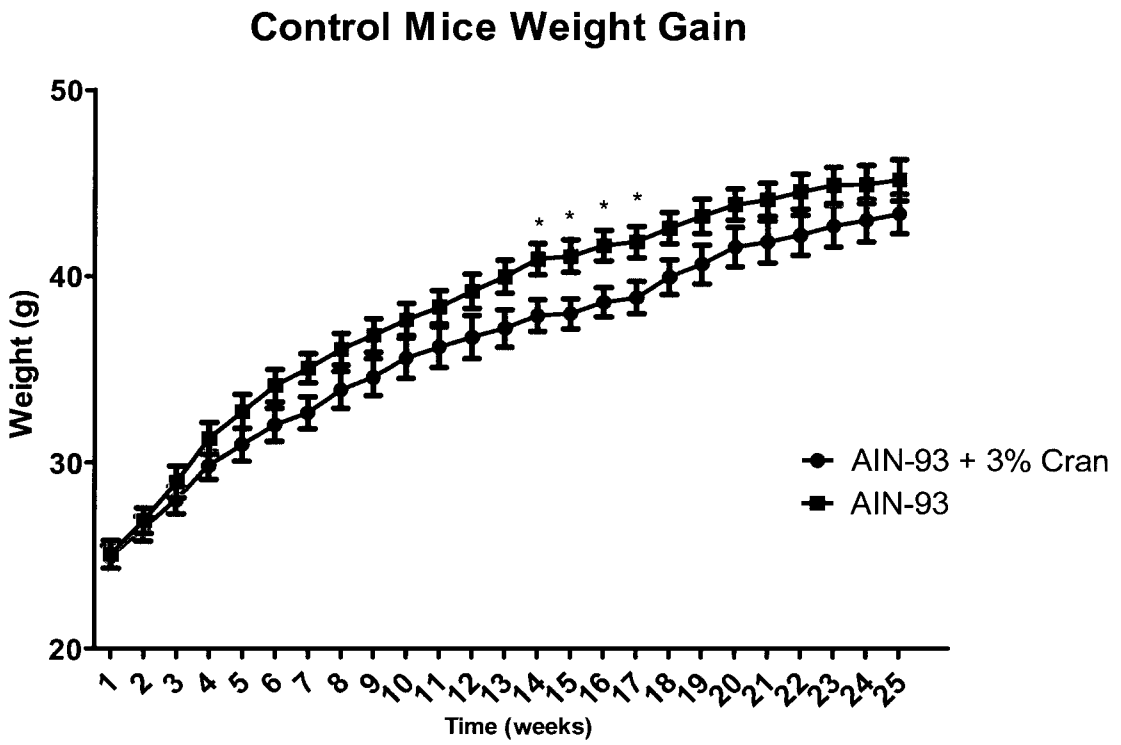


Figure 4.01 Weights of both groups of control mice for the 24 weeks of the study. Data were analyzed by t-test for each separate week and results were considered significant at $p < 0.05$, as represented by an asterisk over each week that was significant.

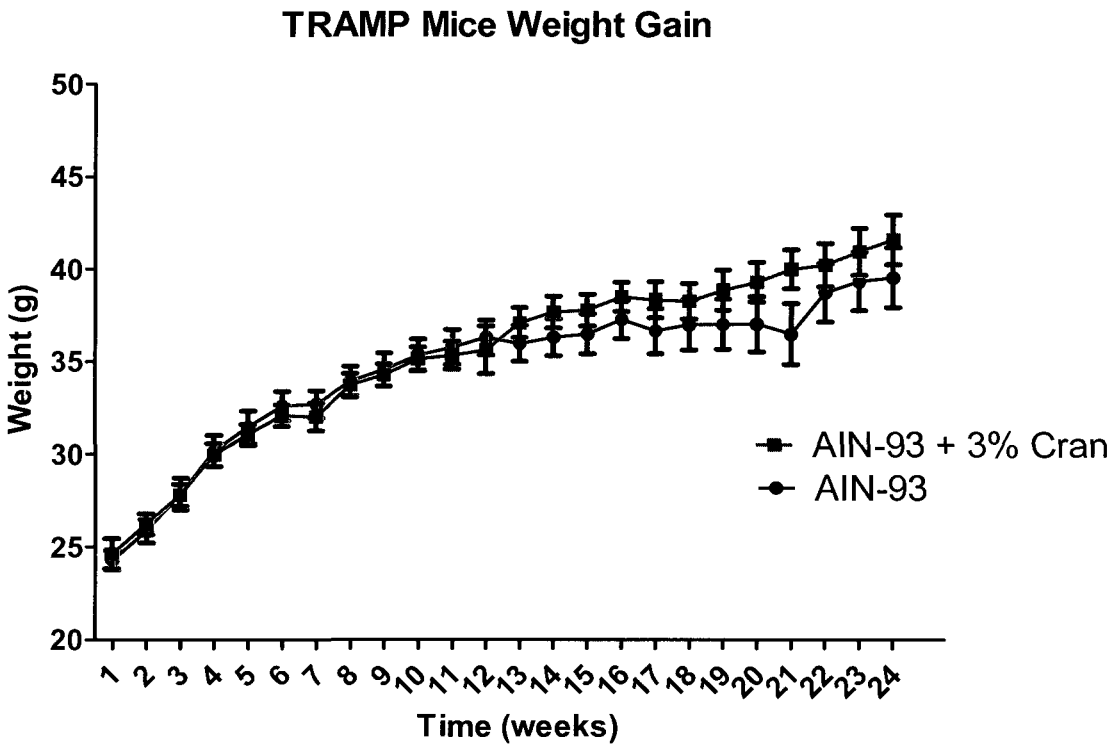


Figure 4.02 Weights of both groups of TRAMP mice for the 24 weeks of the study. Data were analyzed by t-test for each separate week and results were considered significant at $p < 0.05$. There were no weeks where the weights between either group were significantly different.

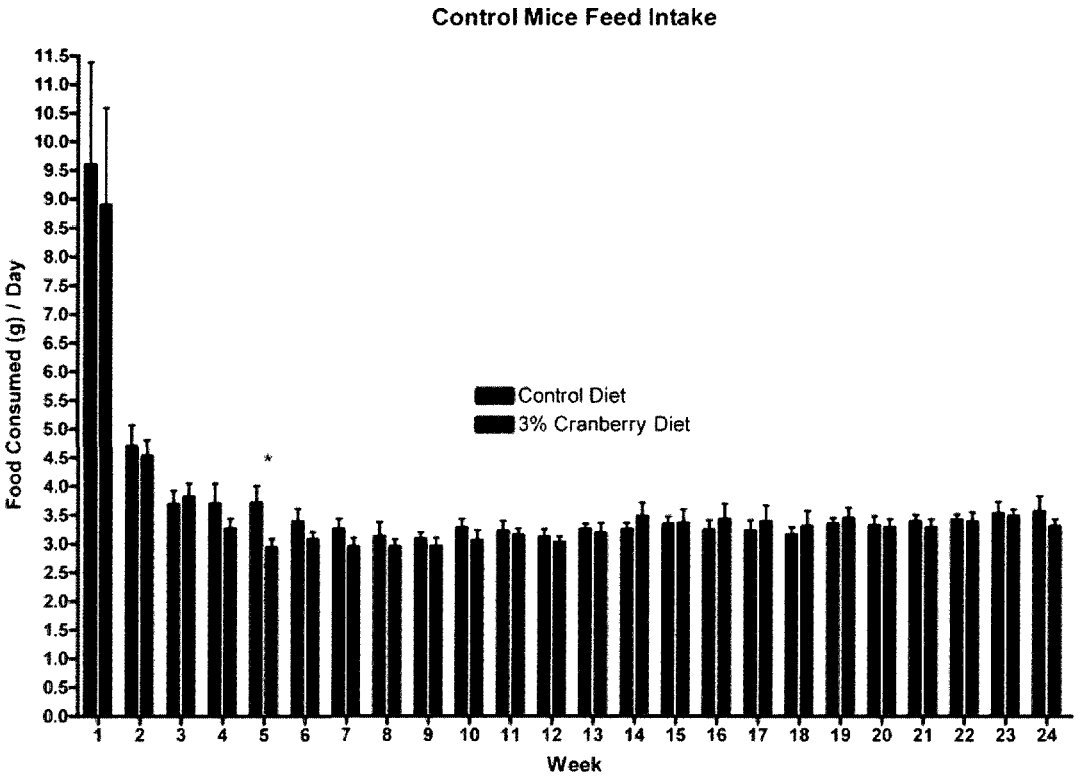


Figure 4.03 Average feed intakes of both groups of control mice for the 24 weeks of the study. Data were analyzed by t-test for each separate week and results were considered significant at $p < 0.05$. The sole week at which there was a significant difference between groups was at week 5, wherein the control mice ate significantly more than 3% cranberry-fed mice.

*Note, for the first week of experiments the weight of food 'consumed' averages approximately 9 grams. This discrepancy from the other data was due to optimization of food delivery, as the mice were not truly consuming 9 grams of food per day. During the first week the mice would physically displace the food from within their feeding jars resulting in larger intake values.

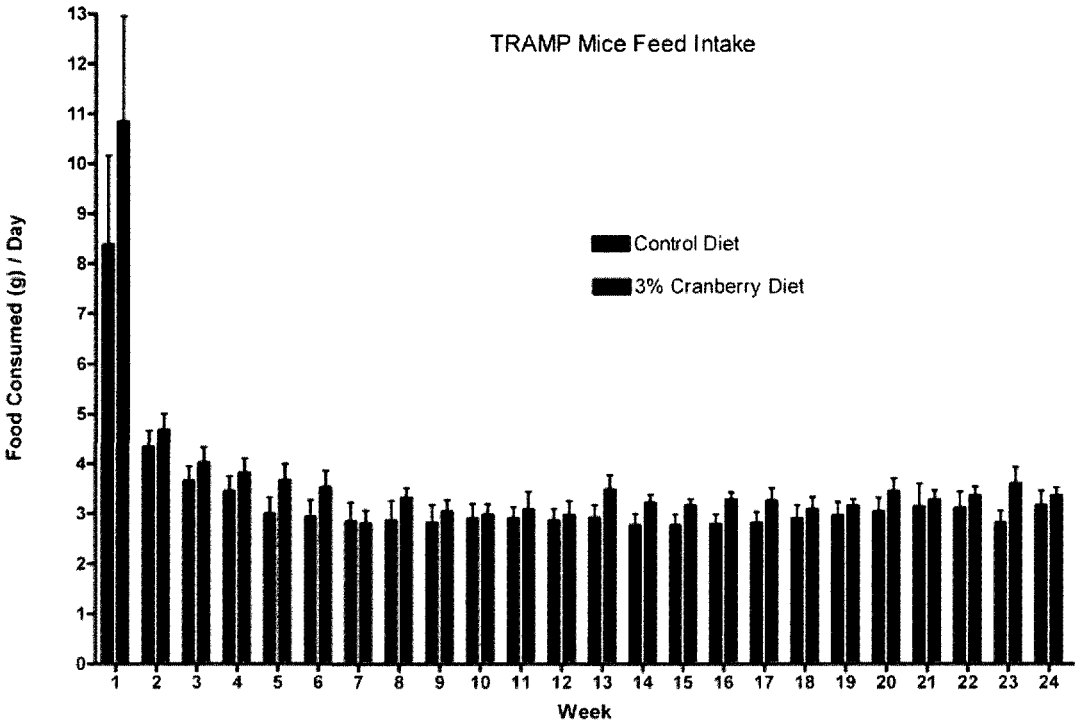
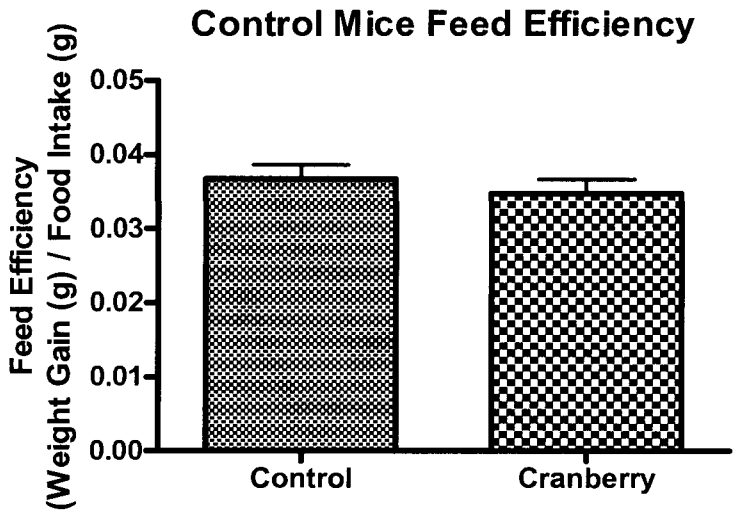
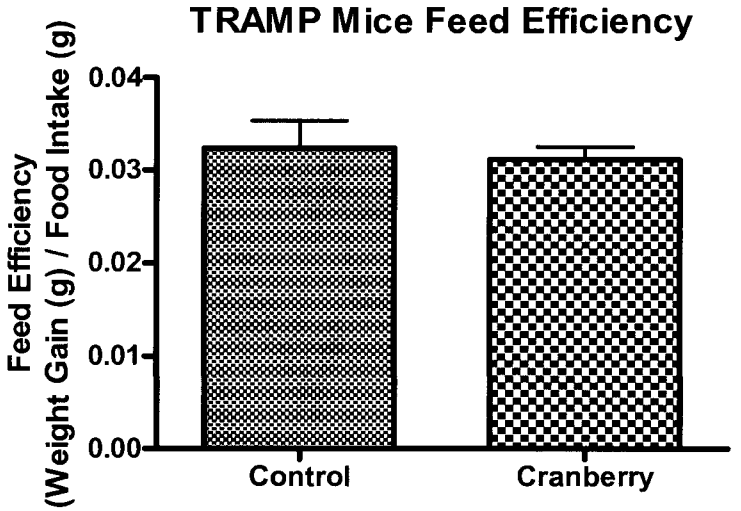


Figure 4.04 Average weekly feed intakes of both groups of TRAMP mice for the 24 weeks of the study. Data were analyzed by t-test for each separate week and results were considered significant at $p < 0.05$. There were no weeks where feed intakes between either group were significantly different.

*Note, for the first week of experiments the weight of food 'consumed' averages approximately 9 grams. This discrepancy from the other data was due to optimization of food delivery, as the mice were not truly consuming 9 grams of food per day. During the first week the mice would physically displace the food from within their feeding jars resulting in larger intake values.





Figures 4.05a and 4.05b Feed efficiencies of both the control mice (Figure 4.05a) and TRAMP mice (Figure 4.05b). Feed efficiency was calculated by taking the total weight gained by each animal and dividing this by the total food consumed by each animal. A t-test was performed between the average feed efficiency of the control diet vs. the average feed efficiency of the cranberry-infused diet. There were no statistical differences in feed efficiencies with respect to both the control mice and the TRAMP mice.

Figure 4.06a

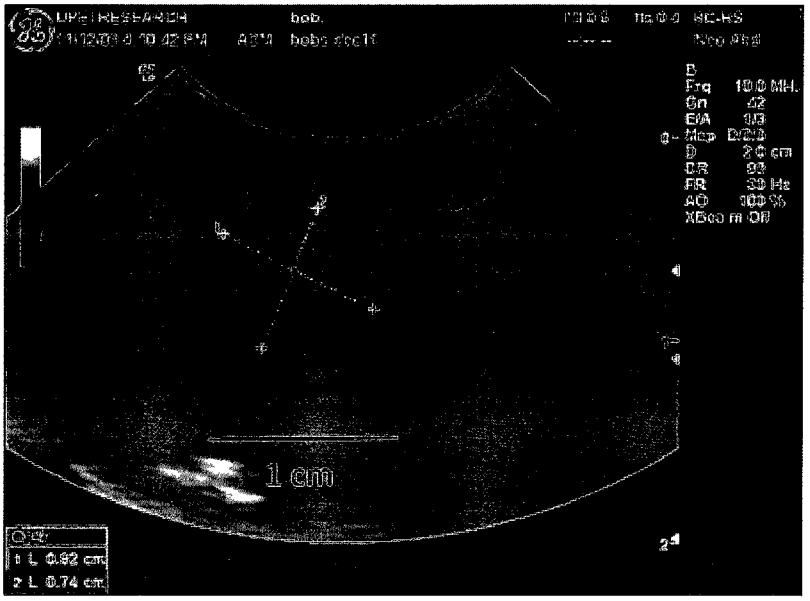
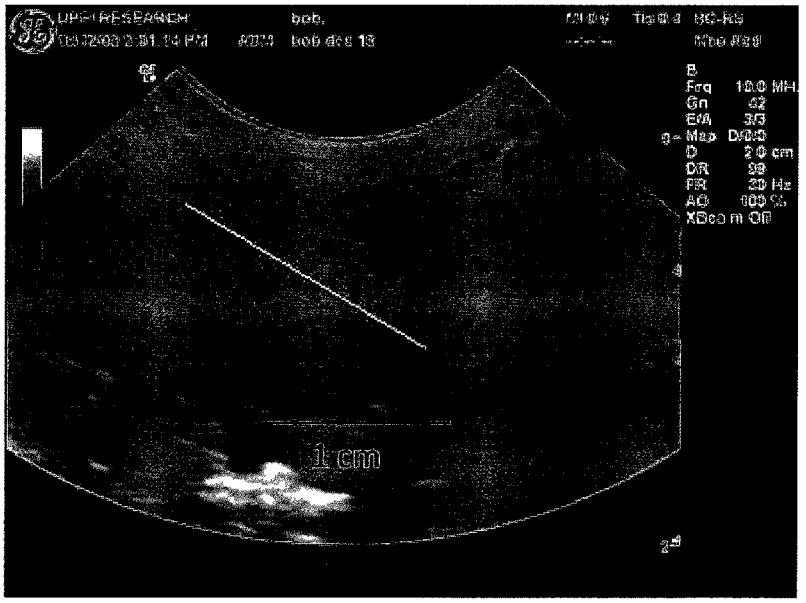


Figure 4.06b



Figures 4.06a-b Ultrasound pictures of subject 9A over a period of 4 weeks. There was an incremental increase in the size of the detectable tumour, from approximately 0.8 cm x 0.7 cm (figure 4.06a) to 2.1 cm x 1.1 cm (figure 4.06d, shown on following page). After the tumour reached this size the mouse was euthanized due to the excessive burden of the tumour.

Figure 4.06c

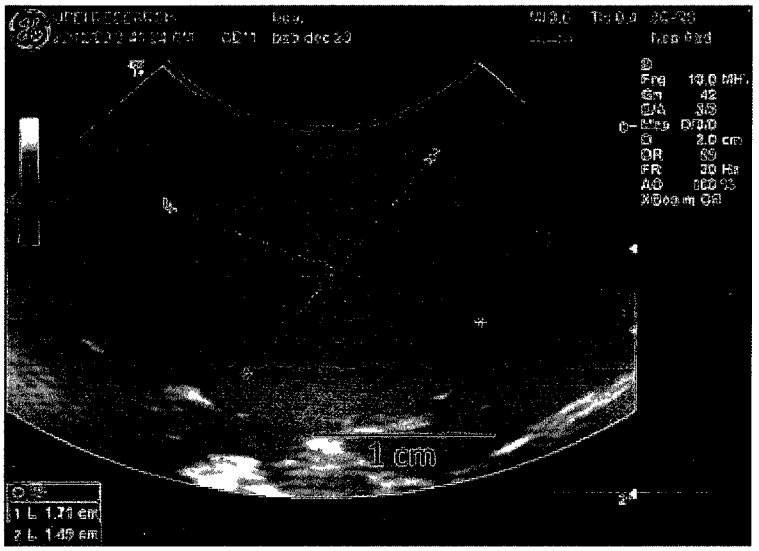
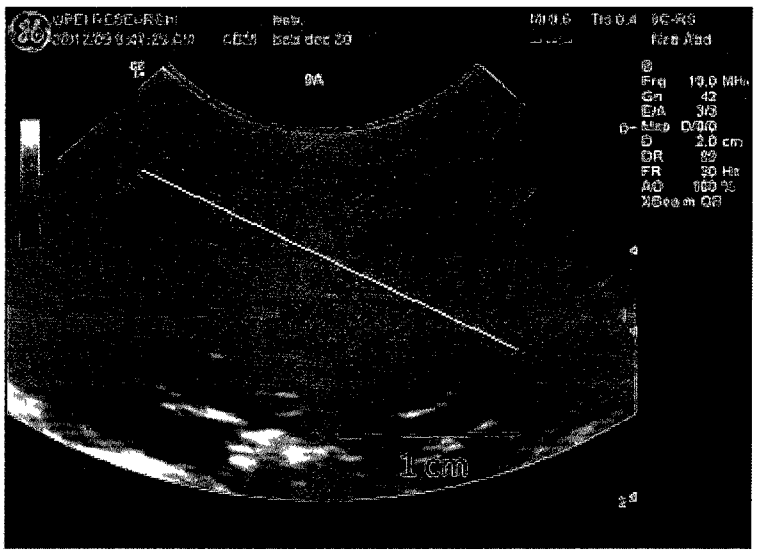


Figure 4.06d



Figures 4.06c-d Ultrasound pictures of subject 9A over a period of 4 weeks. There was an incremental increase in the size of the detectable tumour, from approximately 0.8 cm x 0.7 cm (figure 4.06a shown on previous page) to 2.1 cm x 1.1 cm (figure 4.06d). After the tumour reached this size the mouse was euthanized due to the excessive burden of the tumour.

4.3.3 A 3% cranberry dietary intervention does not significantly increase the lifespan of TRAMP mice

After the TRAMP mouse trial concluded, a statistical analysis was performed comparing the survival ratios between the TRAMP mouse cranberry group and the TRAMP mouse control group. As shown in Figure 4.07 there was no statistical difference in survival between each group.

4.3.4 LUT Weights between TRAMP control mice and TRAMP cranberry mice were not statistically different

As the weight of the LUT can be a marker of tumour burden in this model, the weights of each of the LUTs from the animals surviving 33 weeks was recorded and statistically analysed. As shown in Figure 4.08, there was no statistically significant difference between either group.

4.3.5 Histological examination and grading of the anterior lobe of the prostate, seminal vesicles, and lungs

A summary of the results of the histological grading of the anterior lobe of the prostate the seminal vesicles, and the lungs are shown in Figures 4.09 through 4.12.

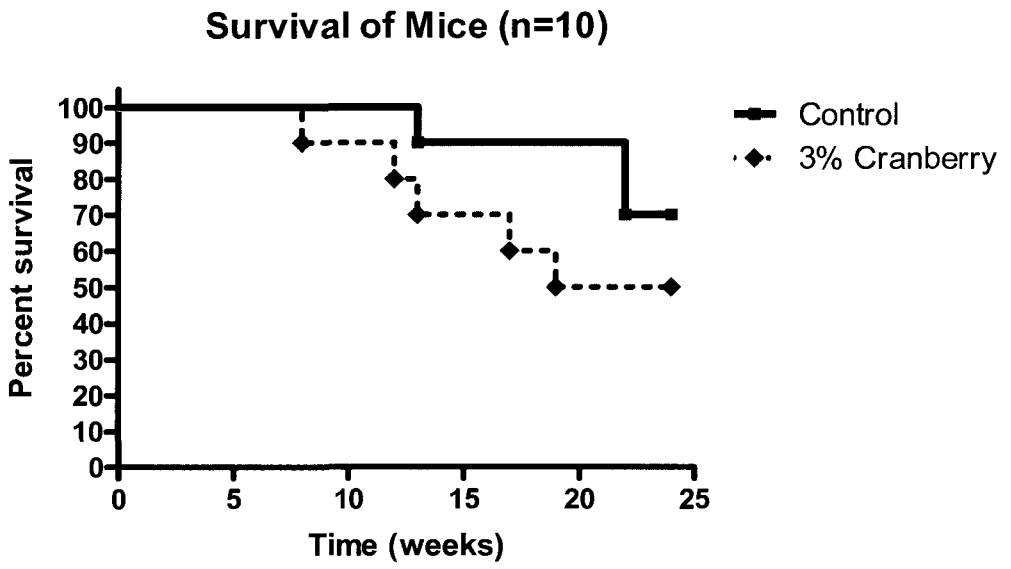


Figure 4.07 3% Cranberry dietary intervention does not significantly affect survival of TRAMP mice. The percentage survival of each group was graphed and a logrank test was performed on the data, which did not show significant different between either group ($p > 0.05$).

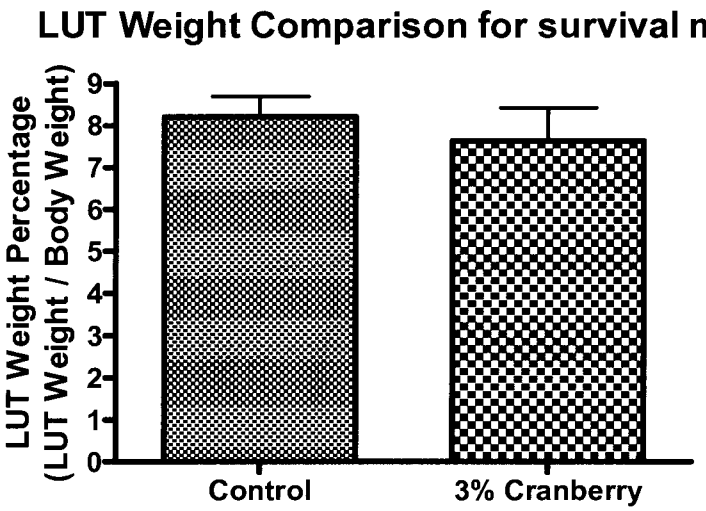


Figure 4.08 LUT weights between mice that survived in the control group (N = 7), and the 3% cranberry-fed group (N = 5). The Y-axis represents the weight of the LUT as a percentage versus the rest of the body. A t-test was performed on the data which showed no statistically significant difference between the control and the 3% cranberry-fed group.

Figure 4.09a

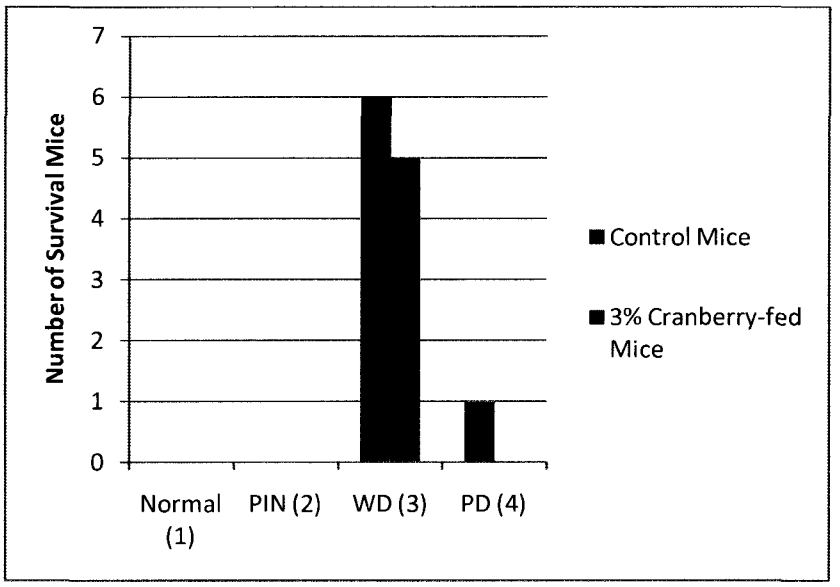
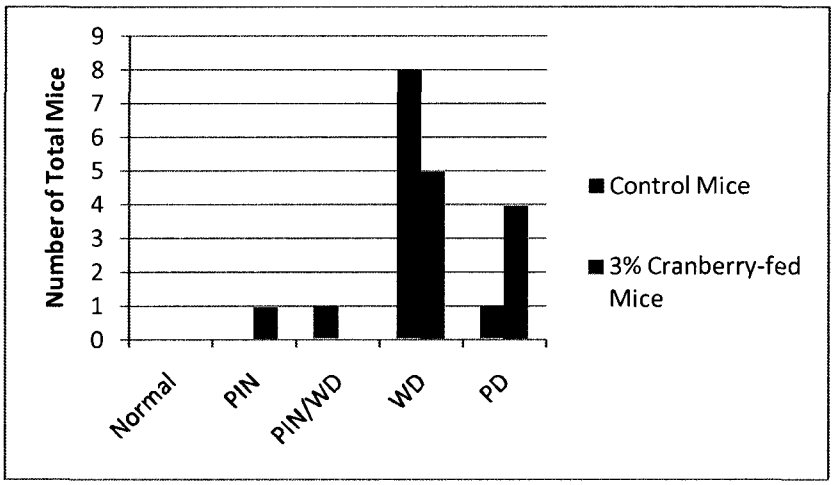


Figure 4.09b



Figures 4.09a and 4.09b The degree of severity of the tumours found within the anterior lobe of the prostate. Figure 4.09a represents the mice that survived the entire length of the trial (33 weeks of age), and figure 4.09b represents all mice. "Normal" indicates no tumour present, PIN represents the presence of prostatic intraepithelial neoplasia, PIN/WD indicates a state between PIN and a well developed adenocarcinoma, WD is a well developed adenocarcinoma, and PD is a poorly developed adenocarcinoma.

Figure 4.10a

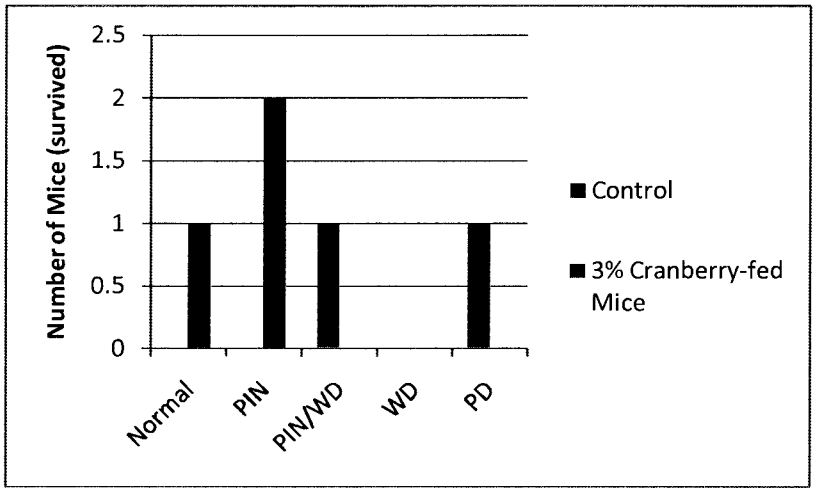
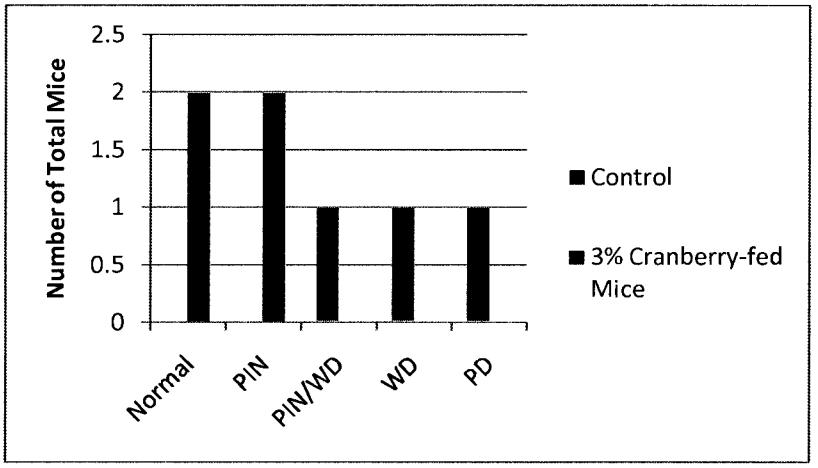


Figure 4.10b



Figures 4.10a and 4.10b The degree of severity of the tumours found within the seminal vesicles of the TRAMP mice, excluding those which had phyllodes-like regions. Figure 4.12a represents the mice that survived the entire length of the trial (33 weeks of age), and figure 4.12b represents all mice. "Normal" indicates no tumour present, PIN represents the presence of prostatic intraepithelial neoplasia, PIN/WD indicates a state between PIN and a well developed adenocarcinoma, WD is a well developed adenocarcinoma, and PD is a poorly developed adenocarcinoma.

Figure 4.11a

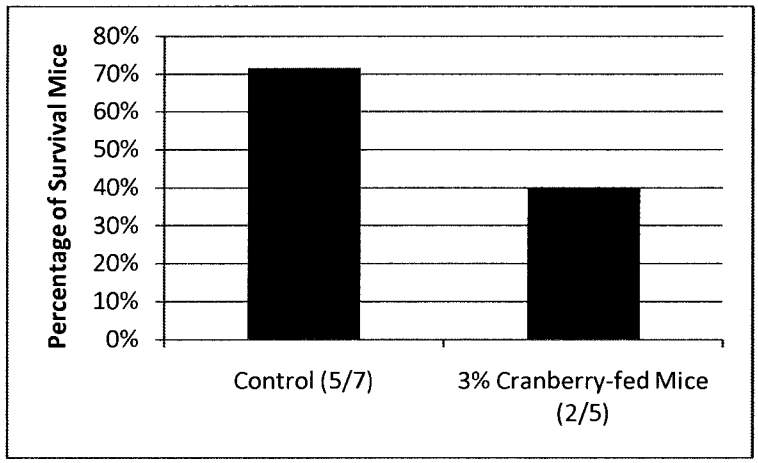
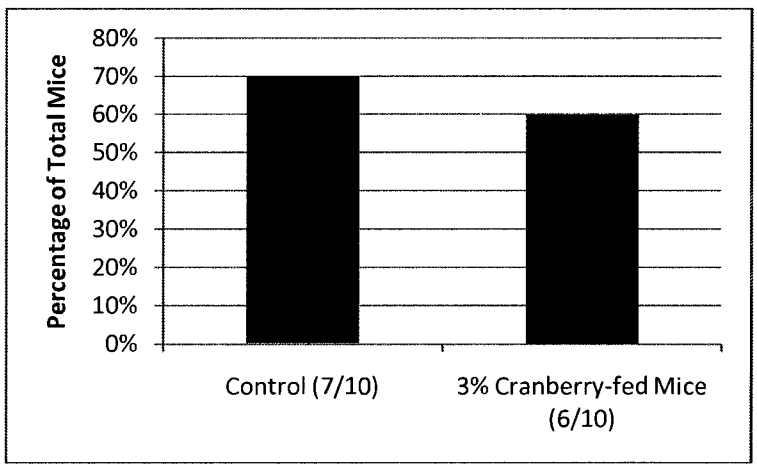


Figure 4.11b



Figures 4.11a and 4.11b The presence of phyllodes-like regions within the seminal vesicles. Figure 4.11a represents the percentage of mice which survived the length of the study (33 weeks of age) which had phyllodes-like regions within the seminal vesicles, and figure 4.11b represents the total percentage of mice which had phyllodes-like regions within the seminal vesicles. A Mann-Whitney U test was performed on the data in figure 4.11a, which showed no significant differences between both groups ($P > 0.05$).

	Lung Metastases (mice that survived 33 weeks)	Lung Metastases (all mice)
Control Mice	0/7	1/10
3% Cranberry-fed Mice	1/5	2/10

Figure 4.12 Lung metastases in control and 3% cranberry-fed mice. Only one metastasis was noted in the cranberry-fed mice, and no mice in the control group had lung metastases. Each tissue was evaluated by a trained pathologist whom was blind to the grouping of the mice. Histological grading was performed by Dr. Bourque at the Atlantic Veterinary College.

4.3.6 Immunoblot analyses detected no significant difference in cell cycle associated proteins between TRAMP control fed group and 3% cranberry-fed group

In order to determine if a cranberry-infused diet had any effect on the expression of specific cell cycle regulators *in vivo,* prostatic tissue from four mice from each feeding group which survived the length of the study were homogenized and the resulting proteins were immunoblotted for the presence of key cyclins and CDKs associated with cell proliferation. As shown in Figures 4.13a and 4.13b, a cranberry-infused diet appeared to have no effects on the expression of the six cell cycle proteins examined in this study.

Figure 4.13a

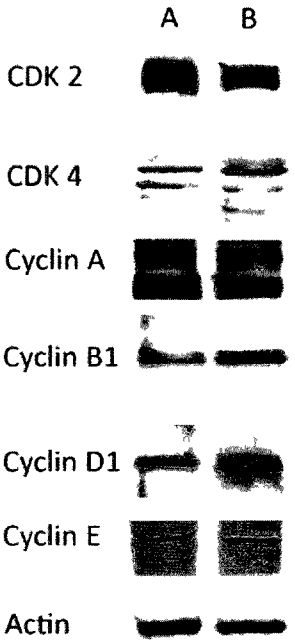
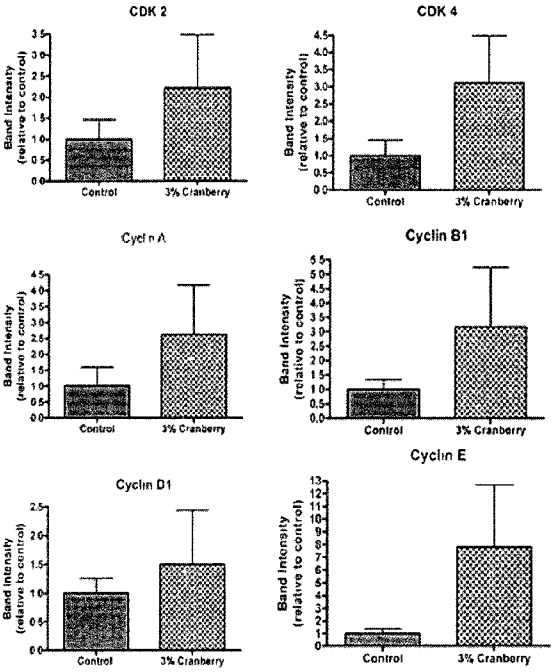


Figure 4.13b



Figures 4.13a and 4.13b: A 3% cranberry-infused diet does not affect the expression of specific cell regulators in the TRAMP mouse prostate. Prostatic tissue from four 33 week old cranberry fed mice and four 33 week old control fed mice were homogenized, sonicated, and immunoblotted for the presence of CDK2, CDK4, Cyclin A, Cyclin B1, Cyclin D1 and Cyclin E. Representative immunoblots are shown in figure 4.13a. Column A represents control mice, and Column B represents cranberry-fed mice. Histograms representing pooled densitometry data from four separate biological replicates (with assay duplicates) are represented in Figure 4.13b. The results demonstrate no statistical significant differences in the expressions of each of the 6 different proteins assayed ($P > 0.05$)

4.4 Discussion

The present chapter detailed a pilot study examining the effects of a 3% cranberry-supplemented diet on the effects of tumour growth, the expression of cell cycle proteins within the tumour, the invasion of prostatic tumours within the seminal vesicles and lungs, the weight of the LUT, and the overall survival of TRAMP mice fed a 3% cranberry diet. Overall, the effects of a 3% cranberry-infused diet on the behaviour of prostate cancer in this mouse model were negligible as there was no change in survivability, histological grade of the prostatic tumour, and invasion into different areas of the body.

Though there was no statistical difference in the survivability of animals fed a 3% cranberry-supplemented diet (figure 4.07), this could be the result of too few animals being used in the study. For example, in a study performed by Suttie et al (2005) which evaluates survival ratios in late-onset dietary restriction in TRAMP mice, a total of 40 mice were assessed in their treatment group, and 69 mice were assessed in their control group, which would potentially allow for less statistical variability. Another experimental variable which would affect survival ratios is the amount of time before euthanasia. If a larger cohort of animals were to be allowed to survive for 45-50 weeks, this would allow for more time for the disease to take its course and for the potentially cancer-mitigating effects of a cranberry diet to occur. Interestingly in this study, after 33 weeks of age there were fewer 3% cranberry-fed mice that survived when compared to control. Though this result was not significant ($p > .05$), survival of mice fed a cranberry-infused diet should be evaluated in future studies with increased numbers of animals.

Ultrasound data for this project provided fascinating results throughout the length of this study. Beginning at 20 weeks each mouse was scanned and examined for the presence or absence of a visible tumour. Though there were false positive readings taken from the

ultrasound data, this was more than likely due to user inexperience with this model and the behaviour of the animals themselves. Often during ultrasound, the presence of any notable circular feature with somewhat ambiguous borders was recorded and evaluated the next week, which was in order not to miss the development of a tumour in its initial stages.

The physical behaviour of the mice themselves often prevented an accurate reading. This was due to using the bladder as a central location to begin the ultrasound, as the bladder was a very well defined region in an ultrasound. When the bladder is filled with fluid it appears as an overt dark area. But, during the first three to four weeks of performing ultrasounds the animals would urinate, due likely to stress, and the bladder would become indistinguishable from other surrounding tissues. It is suggested that in future studies performing ultrasounds on this model that the animals become acclimatized to the procedure by performing ultrasounds at 16 weeks of age. This would aid in the reliability of each result in two ways. Firstly, the user would be more accustomed to working with the TRAMP mouse model and would be better able to distinguish tumours from non-tumours. Secondly, this would better acclimate the animal to the procedure. The approximate age that TRAMP mice develop a prostatic tumour is between 18 – 24 weeks (Gingrich, Barrios et al. 1996), so by performing this procedure earlier than 18 weeks of age may decrease the number of mice which urinate, which would increase the reliability of the results. Overall, performing ultrasounds on the TRAMP mouse model was able to reliably detect tumours of approximately .5 cm in size and by performing an ultrasound each subsequent week was able to provide an accurate measurement within the animal.

Though the prostate tumour originates within the dorso-lateral area of the prostate, this study examined grade of the tumour within the anterior lobe of the prostate. This procedure was performed within the anterior lobe as it was the area which could most consistently be

evaluated. During dissection, the dorso-lateral area of the prostate is malformed and often cannot be properly micro dissected from other areas of the prostate or seminal vesicles. The anterior lobe, in contrast, is located close to the seminal vesicles and can be much more easily distinguished from other structures. The anterior lobe was used for histological evaluation in this study as previous studies have examined this area in the TRAMP mouse in dietary intervention models (Nyska, Suttie et al. 2003; Harper, Cook et al. 2009), and because it was able to be sampled in a consistent manner. Though there was no appreciable difference between the grades of tumours within the anterior lobes of the prostate between the two feeding groups (figure 4.09a), this could be due to part of the experimental design of the project. By 24 weeks of age nearly 100% of control-fed mice should have tumours within prostatic tissue. Thus, by looking at prostatic tissue at 33 weeks of age any potentially advantageous effect of cranberry phytochemicals or its derivatives could have been unnoticeable due to the aggressive behaviour of prostate cancer within this model. For example, another study which documented the chemopreventative effects of two separate phytochemicals, curcumin and phenyethylisothiocyanate, observed differences in the development of tumour formation within the anterior lobe of the prostate after 24 weeks (Barve, Khor et al. 2008). Future studies examining the effects of a cranberry diet on the behaviour of prostate cancer in the TRAMP model should begin dietary intervention beginning at an earlier age, and should end the study between 24 and 28 weeks. This would allow for a better examination of the dorso-lateral area of the prostate, and would give a better indication of whether a cranberry diet has effects on the development of prostate cancer.

Unfortunately, it is difficult to examine both the survivability data and proper histological data within the same study. This is because most of the potential differences in a chemoprevention study will be most obvious before and during the formation of the primary

tumour, which occurs between 12-24 weeks in this model, and it has been reported in a previous study that 60% of TRAMP mice survived past 26 weeks (Majeed, Blouin et al. 2005). Therefore, it is suggested for future studies that a larger cohort of animals be used. The first cohort of animals should be used specifically for determining whether a cranberry-supplemented diet affects tumour development and progression, and the second cohort should be used solely to determine whether cranberry chemoprevention lengthens the lifespan of TRAMP mice versus being fed a control diet.

Though a 3% cranberry diet demonstrated no effects on the expression of specific cell cycle proteins found within the dorso-lateral area of the prostate (Figures 4.13a and 4.13b), this could be due to insufficient amounts of cranberry fed to the animal and by perhaps increasing this percentage within the diet could potentially affect the expression of these proteins within the prostate (Barve, Khor et al. 2010) *in vivo*. Future studies examining cell cycle expression within the prostate could also focus on specific cell cycle inhibitors, most notably p21 and p27. If changes in the expression of cell cycle proteins are noted in future experiments with the TRAMP mouse, assessing the expression of cell cycle inhibitors may be beneficial as increases in these proteins has been noted in a previous chemopreventative study (Barve, Khor et al. 2010).

Phyllodes-like regions found within the seminal vesicles were an interesting finding within this model. Though the presence of these regions have been well-documented within the TRAMP mouse (Tani, Suttie et al. 2005), the clinical value of these lobes is unclear. In humans, some patients with phyllodes-like regions within the prostate can go on to develop metastases, and in some patients after treatment there may be no recurrence. However, in this model the phyllodes-like regions within the seminal vesicles are suspected of being from seminal vesicle origin, not from metastatic tissue from the prostate as this tissue does not express the SV-40 Tag

protein, which is found within prostatic tissue (Tani, Suttie et al. 2005). In this study it was noted that there were less phyllodes-like tumours found within the seminal vesicles within control-fed mice (5/7) versus cranberry-fed mice (2/5). Future studies examining the effects of phyllodes-like tumour formation after consumption of a cranberry-infused diet should examine the effects within both prostate tissue and within the seminal vesicles.

Unexpectedly, this study provided evidence that a cranberry-supplemented diet has a significant effect on the weight gain of control mice, but not within the cranberry-fed TRAMP mice. This is despite the fact that there was no significant difference in the total food consumed between the control-fed and cranberry-fed mice, with the exception of week 5, within the control mice (Figure 4.03). Other *in vivo* studies have demonstrated that diets which contain certain phytochemicals can modulate weight gain (Wolfram, Wang et al. 2006; Hsu and Yen 2008), and previous research in our lab has demonstrated anti-obesity effects of cranberry (data not published). Though the anti-obesity effects of the American cranberry go beyond the scope of the research presented in this chapter, the observation that a cranberry diet can affect weight gain deserves further attention.

This pilot study focused only on a few areas of interest and was limited in scope due to the number of animals used during the study. Future direction and recommendations for utilizing the TRAMP mouse for cranberry chemopreventative research could focus on a variety of different areas, including:

1) Increasing the amount of cranberry within the diet.
2) Increasing the number of animals used within the study.
3) Commencing the study once the animals are weaned at 3 weeks of age, and decreasing the total length of the study to approximately 24 to 28 weeks. This

would allow for a better examination of prostatic tissues, including the dorso-lateral prostate.

4) Implementing a study examining solely the potential effects of a cranberry-infused diet increasing the survival time of TRAMP mice.
5) Examining the potential relationship between the development of phyllodes-like tumours within the seminal vesicles and the consumption of a cranberry-infused diet.

In conclusion, though no potential chemopreventative effects of cranberry were demonstrated in this pilot study, it is recommended that further research investigates the effects of a cranberry-infused diet using a larger number of animals, beginning and ending the trial within a smaller timeframe, and increasing the total cranberry used within the diet.

CHAPTER FIVE

General Discussion and Future Directions

5.1 General Discussion

The results obtained through the experiments conducted and reported in this thesis indicate that cranberry can affect the behaviour of prostate cancer cells in *in vitro* models through multiple different mechanisms. Furthermore, although the results from the *in vivo* pilot study demonstrated no specific indications that cranberry can affect prostate cancer, it is suggested that this is due to the design of the study rather than the compounds of interest. For example, the dose of cranberry given to each mouse (3% of the total diet), the time frame of the study, and the number of animals within the study itself could be insufficient to produce significant changes in cancer incidence.

As shown in chapters two and three, cranberry compounds can affect prostate cancer cells by affecting the expression of certain MMPs, decreasing the expression of specific cell cycle proteins, and lowering the number of cells entering the cell cycle. Cranberry proanthocyanidins decreased MMP expression, which supports previous research from our laboratory that showed other fractions from the cranberry, including whole cranberry, can affect MMP expression (MacLean, Matchett et al. 2007; Scott 2010). Further, it was demonstrated that whole cranberry can affect regulation of the cell cycle in DU145 cells, suggesting a need for future studies of the cell cycle with enriched fractions of specific families of compounds from the cranberry. This approach would help identify the compound(s) of interest within the cranberry. Flavonols and proanthocyanidins appear to have potent effects on the expression of MMPs, and it is suggested that these compounds be tested for their effects on the expression of cell cycle proteins. Such studies have already commenced in our laboratory.

It is also interesting to note that the NF-Kß and AP-1 pathways have been reported to be involved in the expression of MMPs and cell cycle proteins (Moon, Kim et al. 2004). Future studies examining the effects of enriched fractions and specific compounds from the cranberry on the expression of cell cycle proteins and progression of the cell through the cell cycle could examine their effects on the regulation of NF-Kß and AP-1 proteins, and establish whether cranberry phytochemicals mediate cell cycle protein expression by affecting these pathways.

Multiple studies have demonstrated many beneficial effects of specific phytochemical compounds on the behaviour of prostate cancer cells both *in vitro* and *in vivo* (Berggren, Sittadjody et al. 2009; Constantinou, Hyatt et al. 2009; Barve, Khor et al. 2010; Konijeti, Henning et al. 2010). In contrast, this study examining a cranberry-supplemented diet in a prostate cancer mouse model appeared to have no beneficial effect on the expression of cell cycle proteins, despite the fact that whole cranberry extract decreased the expression of cell cycle proteins in human prostate cancer cells grown *in vitro* as shown in chapter three. This discrepancy may be explained in different ways. Firstly, each model was representative of prostate cancer from a different species. The *in vitro* project examined human prostate cancer cells, whereas the *in vivo* study was performed in mice which under normal conditions do not normally develop prostate cancer but must be transgenically modified for this cancer to occur. Secondly, in *in vitro* studies, cranberry phytochemicals are applied directly to prostate cancer cells without any modification. In studies using animal models, cranberry phytochemicals are consumed and digested within a living organism, potentially modifying the chemical structure and ultimately the biological effects of the cranberry. Thirdly, *in vitro* studies examined a high-dose of a select compound over a relatively short period of time. Conversely, this *in vivo* study examined consumption of cranberry supplemented diet over a relatively long period of time. Each of these variables could explain the discrepancies between the *in vitro* and *in vivo* results.

Though cranberry did not appear to affect the behaviour of prostate cancer *in vivo*, the pilot study was a success in that it provided a direction for future researchers to concentrate their efforts. For example, though a 3% cranberry diet may not have been effective in preventing the growth of prostate cancer, a larger dietary percentage of cranberry extract may prove beneficial. Although previous attempts to image tumours in the TRAMP mouse model have successfully utilized MRI techniques to detect and characterize tumour development (Degrassi, Russo et al. 2007), this was not technically feasible during the duration of this project. However, this project was able to successfully ultrasound relatively large tumours with the abdomen of the TRAMP mouse, demonstrating that this specific technique can be used in future studies.

In chapters two and three, two broad and separate characteristics of DU145 cells were evaluated before and after treatment with cranberry phytochemicals. These characteristics included the ability to excrete MMPs into the extra cellular matrix, and the growth of DU145 cells after treatment with increasing doses of WCE. In each case, cranberry phytochemicals had a marked effect. PACs were demonstrated to decrease the expression of MMPs into the extracellular matrix and affected the expression and phosphorylation of a variety of cellular pathways. WCE decreased the number of cells continuing in the cell cycle and affected the expression of certain cell cycle regulators. Although these two general characteristics, MMP expression and cellular growth, are separate, there is much cross talk which occurs between some the pathways affecting MMP expression and cellular growth. For example, pharmacologically-induced over-expression of CDK9 in human lung adenocarcinoma cells has been demonstrated to induce the expression of MMP9, and inhibition of this kinase affected the expression of TNF-alpha which in turn decreased MMP9 expression (Shan, Zhuo et al. 2005). Curcumin, a phytochemical derived from turmeric, has been demonstrated to affect the NF-kB

pathway and decreased the expression of both MMP1 and cyclinD in breast cancer cells(Liu, Loo et al. 2009). This suggests that future studies examining the effects of cranberries in regards to cyclin and CDK expression could examine the NF-kB pathway in order to if whole cranberry exerts the same affects as PACs in prostate cancer cells. If it does, than this may give another explanation as to why there was a marked decrease in the expression of cyclins and CDKs in DU145 cells. Other research has suggested that the over-expression of cyclin D1 in malignant glioma cells directly affects the expression of both MMP9 and MMP2 by increasing the expression of MMP3, a potent activator of MMP9 expression (Arato-Ohshima and Sawa 1999). So, though these two characteristics of DU145 cells (MMP expression and increased growth) are somewhat separate, there can be considerable cross-talk between cellular pathways which can be further evaluated in future studies. An overview of the affected pathways is given in Figure 5.1.

5.2 Future Directions

While this project examined many of the effects that cranberry phytochemicals can have on prostate cancer, there are a number of different experiments which can be undertaken to further elaborate these findings. Previous research in our laboratory demonstrated that blueberries (*Vaccinium angustifolium)*, which are a close relative to cranberries also affect MMP expression (Matchett, MacKinnon et al. 2005). It is suggested that blueberries also be examined for their potential to affect the expression of cell cycle proteins in human prostate cancer cells. If these compounds also affect the *in vitro* expression of cell cycle proteins, an *in vivo* study with blueberry extracts may be advisable.

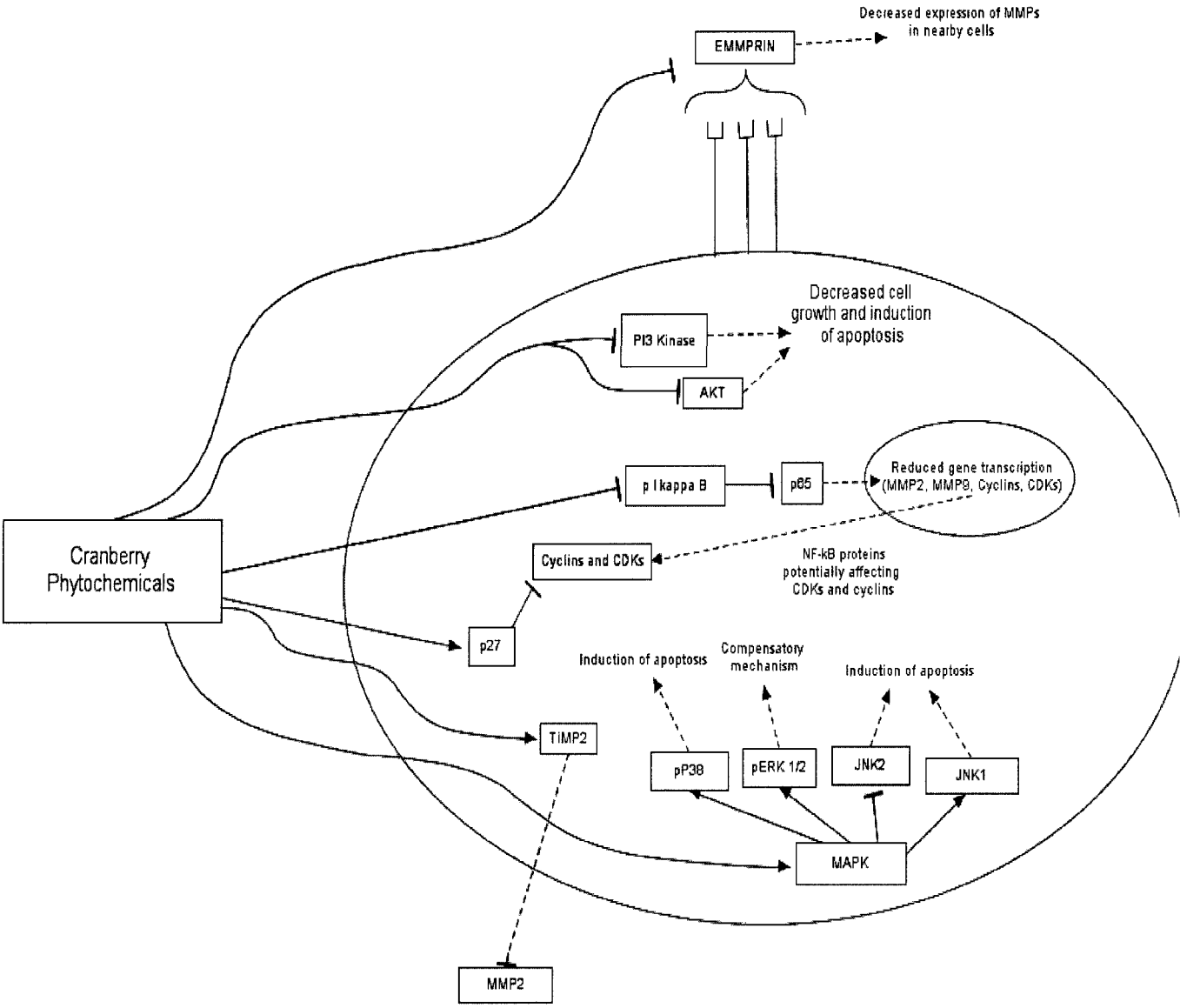


Figure 5.1 An overview of the pathways examined and affected as demonstrated in chapters two and three.

Though proanthocyanidins have been shown to have affects on the expression of MMPs, it may be beneficial to examine the effects of other families of compounds from the cranberry on MMP activity. Previous research by our lab has shown that cranberry flavonols can affect certain behaviours of DU145 human prostate cancer cells, including MMP expression (unpublished observations). In chapter two of this thesis the specific molecular pathways of MMP expression were examined, and it may be beneficial to examine how flavonols affect MMP expression, whether it be through the MAPK, PI3K, NF-κß, or AP-1 pathway. As well, proanthocyanidins are composed of a series of bonded flavan-3-ols (Fine 2000), so it may be beneficial to examine the effects of monomeric flavan-3-ols on the behaviour of human prostate cancer cells as well.

In both chapter two and three, the effects of cranberry phytochemicals were tested on DU145 human prostate cancer cells. Further research should attempt to elaborate the effects of these compounds on other prostate cancer cell lines, including PC-3 (androgen insensitive)and LNCaP cells (androgen sensitive) as these differ in their respective aggressiveness versus DU145 cells. It would also be beneficial to examine non-transformed human epithelial cells in an attempt to ascertain the effects on the expression of cell cycle proteins of these compounds in non-cancerous cells. This would allow future researchers to distinguish the cranberry's effects on cancerous versus non-cancerous cells and to determine if cranberry phytochemicals specifically affect cancerous cells.

Another interesting avenue which could be reviewed in future studies is the effects of cranberry phytochemicals on the expression of insulin growth factor 1 (IGF-1). It has been noted that the over-expression of this protein and its abundance in circulating serum is associated with prostatic cancer cell growth (Roberts 2004), and the modulation of IGF-1 by phytochemicals can

have beneficial effects *in vivo* (Konijeti, Henning et al. 2010). IGF-1 has also been implicated in the expression of MMP2 in prostate cancer cells (Papatsoris, Karamouzis et al. 2005), suggesting that its over-expression could be involved in prostate cancer metastasis. Studies could examine cranberry's effects on IGF-1 expression *in vitro*, and if there is evidence that IGF-1 is affected in prostatic cell lines further studies could examine a high-dose cranberry-supplemented diet on the expression of IGF-1 in the serum of TRAMP mice, and whether decreases in circulating IGF-1 are associated with potentially beneficial effects including decreased tumour and LUT weight, delayed growth of the tumour, and the survival of these mice.

In conclusion, the work in this thesis demonstrates that whole cranberry extracts and a specific phytochemical family (PACs) from the cranberry can affect the behaviour of human prostate cancer cells. Further, it was demonstrated that it is feasible to conduct an *in vivo* study examining these effects in a prostate cancer model. Though there were no significant effects of a cranberry-supplemented diet in the TRAMP mouse, future research investigations taking into account the recommendations explained in this chapter and in chapter four should be considered. As cranberry phytochemicals can clearly affect the behaviour of prostate cancer cells as described in chapters two and three, more *in vivo* research into these effects is warranted.

CHAPTER SIX

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Appendix A

Preparation and Analysis of Cranberry Extracts by Dr. Neto's Laboratory

Cranberry fruit (*Vaccinium macrocarpon*) was harvested in November 2006 at the State Bog in Wareham, Massachusetts. The fruit was flash-frozen in liquid nitrogen and stored at -20^0 C until use. For preparation of whole cranberry extract polyphenolic extract, 1.06 kg of fruit was extracted several times with 300 mL aliquots of 40/40/19/1 methanol/acetone/water/formic acid at room temperature, each time pulsing with a Waring blender for 5 minutes, filtering after 30 minutes, and collecting the filtrate. This was repeated with the pulp until most of the color was gone. The combined filtrates were concentrated *in vacuo* and then freeze-dried. Free sugars were removed to produce a concentrated whole crude extract (WCE) using chromatography on a Diaion HP-20 column (4.5 X 30 cm). The sample was then applied to the column, allowed to adsorb, then washed with several column volumes of distilled water to remove free sugars. The column was then eluted with methanol until no pink color remained and then rinsed with acetone. The methanol and acetone extracts were combined, concentrated and freeze-dried to produce 10.5 g of WCE.

Extracts and fractions were analyzed with a Waters HPLC chromatography system equipped with two pumps, an ultraviolet-visible photodiode array detector (PDA) and Millenium software. The WCE was analyzed using a Waters Symmetry C18 reversed phase column (4.6 X 250 mm) and gradient elution program employing solvent A (4% aqueous acetic acid) and solvent B (4% acetic acid in methanol) (figure A.1). Samples were dissolved in 100% solvent A at a concentration of 20 mg/mL for crude extract. Linear gradient elution at a flow rate of 0.80

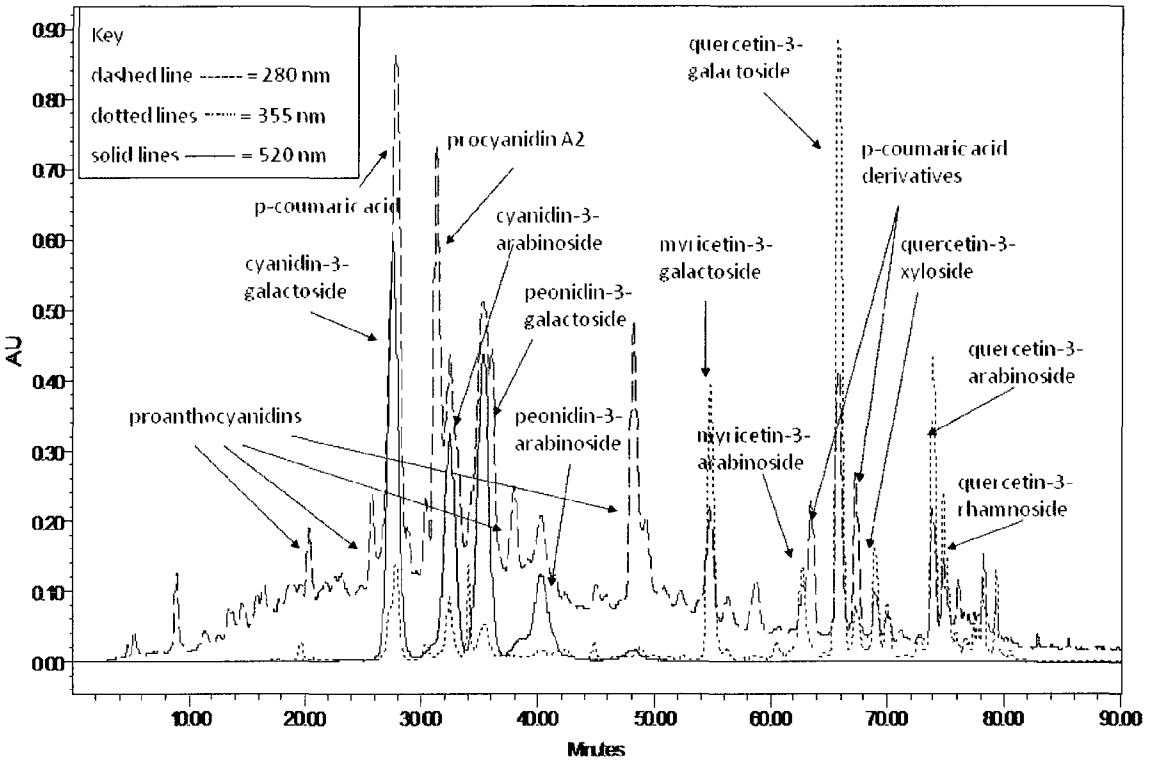

Figure 1: HPLC profile of whole cranberry polyphenolic extract (WCE). Anthocyanin glycosides were detected at 520 nm, flavonol glycosides at 355 nm and epicatechin-based proanthocyanidins at 280 nm. p-coumaric acid and derivatives showed absorbance maxima at 310 nm

mL/min began at 0 minutes with 99% solvent A with a gradient to 80% solvent A over 30 minute , a gradient of 70% solvent A at 70 minutes and finally a gradient of 100% solvent B at 90 minutes. Anthocyanin glycosides are detected at 520 nm, flavonol glycosides at 355 nm and proanthocyanidin at 280 nm. Content of the fractions was quantified by the standard curve method employing a commercial standard of cyanidin-3-galactoside and using the above program with peak absorbances measured at 520 nm. The flavonol-enriched fraction (FLAV) was analyzed with diode-array detection on a Waters Symmetry C18 reverse- phase column (3.0 X 150 mm). The HPLC program used gradient elution employing solvent A (2% aqueous acetic acid) and solvent B (2% acetic acid in methanol). Samples were prepared with 50/50 solvent A and B at 3 mg/mL concentration and analyzed using a linear gradient beginning at 0 minutes with 95% solvent A, a gradient to 75% solvent A over 5 minutes, a gradient to 65% solvent A at 25 minutes , a gradient to 60% solvent A at 35 minutes , and a gradient to 5% solvent A at 45 minutes. Flavonol glycosides were quantified as quercetin-3-galactoside by the standard curve method, measuring peak absorbance at 355 nm. The proanthocyanidin-enriched fraction (PAC) was analyzed by HPLC using a Waters Xterra C8 column (4.6 X 250 mm) to verify the presence of proanthocyanidins and the absence of contaminants . The program used gradient elution with solvent A (2% aqueous acetic acid) and solvent B (methanol). Samples were prepared in 50/50 solvent A and B at 10 mg/mL concentration and analyzed using a gradient with a flow rate of 0.8 mL.min , beginning at 0 minutes with 100% solvent A , with a gradient to 100% solvent A at 5 minutes, then a linear gradient to 100% solvent B at 60 minutes, holding until 80 minutes. Proanthocyanidins were detected by characteristic peak elution patterns at 280 nm (which is the absorbance maximum for epicatechin-based proanthocyanidins). Procyanidin A2 was identified in the extracts based on comparison to a commercial standard. The composition of the

proanthocyanidin fraction was verified by MALDI-TOF MS analysis by Dr. Christian Krueger at the University of Wisconsin using methods previously described (Neto, Krueger et al. 2006).

Appendix B

Supplemental Ultrasound Information

	Measurement of Tumour During Necropsy	Measurement of Tumour During Last Ultrasound
Mouse 1 (age 22 weeks)	N/A	N/A
Mouse 2 (age 33 weeks)	N/A	N/A
Mouse 3 (age 33 weeks)	Left SV Tumour .5 cm x .5 cm x .2 cm	N/A
Mouse 4 (age 33 weeks)	N/A	"Possible" 1.1 cm x .5 cm Mass
Mouse 5 (age 25 weeks)	P Tumour 2 cm x 2 cm x 2 cm	2.2 cm x 1.1 cm mass
Mouse 6 (age 33 weeks)	N/A	N/A
Mouse 7 (age 20 weeks)	P Tumour 2 cm x 1.5 cm x 1.3 cm	2.2 cm mass
Mouse 8 (age 33 weeks)	Left SV Tumour .3 cm	N/A
Mouse 9 (age 27 weeks)	P Tumour 2 cm x 2 cm x 1.4 cm	2.1 x 1.1 cm mass
Mouse 10 (age 20 weeks)	P Tumour 1.3 cm x 1.3 cm x 1.0 cm	2.2 cm mass
Mouse 11 (age 33 weeks)	N/A	N/A
Mouse 12 (age 33 weeks)	N/A	"Possible" SV Disseminated tumour noted
Mouse 13 (age 30 weeks)	N/A	N/A
Mouse 14 (age 16 weeks)	N/A	N/A
Mouse 15 (age 33 weeks)	P Tumour .7 cm x .7 cm x .5 cm	.8 cm x .6 cm mass
Mouse 16 (age 33 weeks)	N/A	N/A
Mouse 17 (age 33 weeks)	N/A	N/A
Mouse 18 (age 33 weeks)	N/A	N/A
Mouse 19 (age 33 weeks)	P Tumour 1.6 cm x 1.3 cm x .5 cm	1 cm x .7 cm mass
Mouse 20 (age 30 weeks)	Right SV Tumour .8 cm x .4 cm x .4 cm	N/A

Figure B.1 A summary of the findings of each necropsy versus the final ultrasound. The first column represents each mouse and the age of each mouse during the necropsy. For both of the subsequent columns, "N/A" refers to no discernable prostatic or seminal vesicle mass detected. "P Tumour" refers to a prostatic tumour, "right SV" refers to a tumour found on the right seminal vesicle, and "left SV" refers to a tumour found on the left seminal vesicle.

Figure B.2a

	Week 20	Week 21	Week 22	Week 23	Week 24	Week 25	Week 26
Mouse 1	ND	Large Mammary Tumour (2+ cm)	M	M	M	M	M
2	ND	ND	ND	Possible tumour noted	Possible tumour noted	ND	ND
3	ND	ND	ND	ND	ND	ND	ND
4	ND	Possible tumour noted	ND	Possible tumour noted	Possible tumour noted .4 cm	Possible tumour noted .4 x .3 cm	ND
5	ND	ND	ND	Tumour 1.2 cm x .7 cm	Tumour 1.8 cm x 1.0 cm	Tumour 2.2 cm x 1.1 cm	M
6	ND	ND	ND	ND	ND	ND	Possible tumour of .30 cm
7	Tumour 2.2 cm	M	M	M	M	M	M
8	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	Tumour .8 cm x .7 cm	Tumour 1.3 cm x .8 cm	Tumour 1.7 x 1.5 cm
10	Tumour	Tumour 2.2 cm	M	M	M	M	M
11	ND	ND	ND	ND	ND	ND	Possible tumour noted
12	ND	ND	ND	ND	ND	ND	ND
13	ND	ND	ND	ND	ND	ND	ND
14	M	M	M	M	M	M	M
15	ND	ND	ND	Possible tumour noted .29 x .52 cm	ND	ND	ND
16	ND	Possible tumour noted .4 x .2 cm	ND	ND	Possible tumour noted	ND	Possible tumour noted
17	ND	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	ND	ND	ND	Possible tumour noted .3 cm	ND
20	ND	ND	ND	Possible tumour noted .3 cm	ND	Possible tumour noted .3 cm	ND

Figure B.2a A summary of each ultrasound performed on each mouse between weeks 20-26. "ND" refers to no tumour detected; "M" represents a mortality. A possible tumour is noted when a small possible mass was located during the ultrasound.

Figure B.2b

	Week 27	Week 28	Week 29	Week 30	Week 31	Week 32	Week 33
Mouse 1	M	M	M	M	M	M	M
2	ND	Possible tumour noted	ND	Possible tumour noted .3 x .4 cm	Very Disseminated Tumour Likely	ND	ND
3	ND	ND	ND	Possible Mass located -> Cranial to Bladder	ND	Odd Mass Located By Kidneys	ND
4	ND	ND	ND	ND	ND	Very Large Seminal Vesicles	Possible Tumour Noted 1.1 x .5 cm
5	M	M	M	M	M	M	M
6	ND	ND	ND	ND	ND	Possible Tumour Noted, pushing bladder	ND
7	M	M	M	M	M	M	M
8	ND	ND	ND	ND	ND	ND	ND
9	Tumour 2.1 x 1.1	M	M	M	M	M	M
10	M	M	M	M	M	M	M
11	Possible tumour noted	ND	ND	Very Disseminated Tumour Likely	Tumour noted, .3 x .5 cm	ND	ND
12	ND	ND	ND	ND	ND	ND	Possible Disseminated Tumour Noted
13	ND	ND	ND	M	M	M	M
14	M	M	M	M	M	M	M
15	ND	Possible tumour noted	Possible tumour noted .9 x .5 cm	Possible Metastases to seminal vesicles/multiple tumours noted	ND	Tumour Noted .6 x .6 cm	Tumour noted .8 x .6 cm
16	ND	ND	ND	ND	ND	Possible Disseminated Tumour Noted	ND
17	ND	ND	ND	ND	ND	ND	ND
18	ND	Possible tumour noted	Fluid filled pocked noted	ND	ND	Tumour Noted, .6 x 1.0 cm, possible urinary obstruction	ND
19	Possible tumour noted .4 cm x .4 cm	ND	ND	ND	ND	Possible Tumour Noted	Tumour Noted 1 x .7 cm
20	ND	ND	Possible tumour noted .5 x .4 cm	ND	ND	ND	ND

Figure B.2b A summary of each ultrasound performed on each mouse between weeks 27-33. "ND" refers to no tumour detected; "M" represents a mortality. A possible tumour is noted when a small possible mass was located during the ultrasound.