THE CELLULAR AND MOLECULAR PROPERTIES OF FLAVONOIDS IN PROSTATE CANCER CHEMOPREVENTION

by

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ABSTRACT

Flavonoids are a large class of dietary polyphenols that have emerged as candidate agents for chemoprevention in prostate cancer. Despite the large number of known flavonoids (over 9000), only a few have been studied in prostate cancer to date. The work presented in this thesis describes the identification of novel anti-proliferative flavonoids, their molecular effects on cell cycle and related proliferation and survival pathways, and their chemopreventive properties in a murine model of prostate carcinogenesis.

We identified several novel flavonoids with potent anti-proliferative effects in human prostate cancer cells *in vitro.* Non-prostate cell lines were generally resistant to the effect of these flavonoids. Two of the most potent flavonoids identified, 2,2-dihydroxychalcone (DHC) and fisetin, induced S and G2 phase cell cycle arrest in LNCaP and PC3 prostate cancer cells. Gene expression studies employing oligonucleotide microarray demonstrated profound down-regulation in gene expression of 75 key cell cycle (predominantly G2 and M phase) genes by DHC and fisetin, and the enhanced expression of 50 stress-response genes with important roles in cell proliferation and survival. DHC and fisetin induced apoptosis, but not accelerated senescence, in prostate cancer cells.

The chemopreventive effect of 4 flavonoids identified from the *in vitro* studies was examined in an autochthonous murine model of prostate cancer (TRAMP). Mice were administered diets supplemented with 1% DHC, 1% fisetin or a combination of flavonoids (0.25% DHC, 0.25% fisetin, 0.25% quercetin, 0.25% luteolin) for 32 weeks. We demonstrated a significant reduction in genitourinary weight, and a reduction in prostate cancer grade in mice administered 1% DHC and combination diets. Flavonoid supplementation was, however, associated with gastrointestinal toxicity in some mice. Liquid chromatography-mass spectrometry demonstrated the accumulation of high levels of flavonoid in the prostates of TRAMP mice. These findings lay the foundation for further studies of flavonoids in clinical chemoprevention trials.

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My search for a supervisor led me across the Atlantic to the laboratory of Dr.Laurence Klotz in Toronto. I essentially arrived with only a strong yearning to succeed in this field but with no prior basic science experience. Dr. Klotz took the brave and not so trivial decision to take me on as a graduate student. I am deeply indebted and grateful that Dr. Klotz saw in me the potential to succeed in this endeavour. Without Dr. Klotz's guidance and infectious enthusiasm for research, I would not have been able to endure the rigours of the journey to a PhD.

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My prayers and thanks to God Almightly for helping me succeed in this voyage and for the health and happiness He has provided me.

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List of Abbreviations

ANLN	Anillin
AR	Androgen Receptor
ATCC	American Type Culture Collection
ATF3	Activated Transcription Factor 3
ATM	Ataxia Telangectasia Mutated
ATR	Ataxia Telangectasia And Rad 3 Related
AURK	Aurora Kinase
BPH	Benign Prostatic Hypertrophy
BSA	Bovine Serum Antigen
CAK	Cdk Activating Kinase
CBC	Complete Blood Count
CCNA	Cyclin A
CDC2	Cell Division Cell Cycle 2
CDE-CHR	Cell Cycle Dependent-Cell Cycle Homology Region
CDK	Cyclin Dependent Kinase
CENP	Centromere Protein
CHC	Chromosome Condensation
CHK	Checkpoint Kinase
DAPI	4',6-Diamidino-2-Phenylindole
DDIT	DNA Damage Inducible Transcript
DHC	2,2'-Dihydroxychalcone
DLG7	Discs, Large Homolog 7
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DRE	Digital Rectal Exam
EGC	Epigallocatechin
EGCG	Epigallocatechin Gallate

ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen Receptor
FACS	Fluorescent Activated Cell Sorting
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GADD	Growth Arrest And DNA Damage Inducible Protein
GO	Gene Ontology
H&E	Hematoxylin And Eosin
H2AX	H2A Histone Family, Member X
H3P	Phosphorylated Histone 3
HCT	Hematocrit
HF	High Fat
HGPIN	High Grade Prostatic Intraepithelial Neoplasia
HIF	Hypoxia Inducible Factor
HMGA	High Mobility Group AT-Hook
IAP	Inhibitor Of Apoptosis
IGF	Insulin-Like Growth Factor
IGF-BP	Insulin-Like Growth Factor Binding Protein
iNOS	Inducible Nitric Oxide Synthase
IR	Ionizing Irradiation
IRS	Insulin Receptor Substrate
ISLQ	Isoliquiritigenin
JNK	Jun N-Terminal Kinase
JUNB	Jun B Proto-Oncogene
KIF	Kinesin Family Member
KNTC	Kinetochore Associated
LC-MS	Liquid Chromatography- Mass Spectrometry
LDL	Low Density Lipoprotein
LOWESS	Locally Weighted Scatterplot Smoothing

LUT	Luteolin
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MYT	Myelin Transcription Factor
NASP	Nuclear Autoantigenic Sperm Protein
NEK	Never In Mitosis Genes
NUBP1	Nucleotide Binding Protein 1
NUSAP	Nucleolar And Spindle Associated Protein
PARP	Poly (ADP-Ribose) Polymerase
PB-Tag	Probasin- Large T Antigen
PBS	Phosphate Buffered Saline
Pca	Prostate Cancer
PCNA	Proliferating Cell Nuclear Antigen
PCPT	Prostate Cancer Prevention Trial
PI	Propidium Iodide
PI3K	Phosphoinositide-3-Kinase
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasia
PLK	Polo-Like Kinase
PPAR	Peroxisome Proliferator Activated Receptor
PSA	Prostate Specific Antigen
PVDF	Polyvinylidene Fluoride
QPCR	Quantitative Real Time PCR
RANBP9	RAN Binding Protein 9
RB	Retinoblastoma
RFC3	Replicator Factor C 3
ROS	Reactive Oxygen Species
RPA2	Inducible Nitric Oxide Synthase
SA-Beta Gal	Senescence Associated Beta Galactosidase

SDS	Sodium Dodecyl Sulfate
SELECT	Selenium And Vitamin E Chemoprevention Trial
SHBG	Sex Hormone Binding Globulin
STAT3	Signal Transducer And Activator Of Transcription 3
STMN	Stathmin
SV40	Simian Virus 40
TERT	Telomerase Reverse Transcriptase
TOPO	Topoisomerase
TRAIL	Tumor Necrosis Factor Ligand
TRAMP	Transgenic Adenocarcinoma Of The Mouse Prostate
TYMS	Thymidylate Synthase
UBE2C	Ubiquitin Conjugating Enzyme 2C
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

1.1 Prostate Cancer Overview

Prostate cancer is the most common non-cutaneous malignancy in North America accounting for 27-29% of all new cases of cancer in males (1). It is also estimated to be the second (United States) or third (Canada) leading cause of cancer mortality (1, 2). The Canadian Cancer Society estimates that 1 in 8 men will develop prostate cancer in their lifetime (2). Prostate cancer therefore constitutes a significant public health burden, and like many other cancers, the burden is likely to increase due to the continued growth in the aging population.

Despite technological and scientific advancements, prostate cancer management remains a challenging field. Once the diagnosis of prostate cancer is made, both patient and physician are faced with complicated risk-benefit choices that range from conservative approaches such as active surveillance to invasive surgical and radiation therapy based treatments (3, 4). Definitive therapy with radical prostatectomy or radiotherapy for localized disease are in many cases not curative, with only 80% of patients remaining free of biochemical recurrence at 10 years (5-7). Most patients who present with advanced and metastatic disease end up dying of prostate cancer (8). Furthermore, treatment modalities often impact significantly on quality of life. Complications of therapy such as incontinence and sexual dysfunction occur in up to 70% of patients post radical prostatectomy and occur even following less invasive therapies such as brachytherapy (9).

Treatment of prostate cancer has been further affected by the widespread use of prostate specific antigen (PSA) screening by physicians despite the absence of clear evidence that such screening positively affects survival. Prostate cancer is unique in its natural history in that many cancers diagnosed on prostate biopsy are not destined to develop into clinically aggressive tumors, and therefore the concern with widespread use of PSA is the problem of over-diagnosis. In a study of patients diagnosed with prostate cancer in the pre-PSA era, Adolfson et al

demonstrated that under half of patients diagnosed died of the disease (10). The high incidence of indolent cancers is also highlighted in autopsy studies where almost 85% of men in their eighties have microfoci of prostate cancer, yet the majority will have died of other causes. Therefore, unlike many other cancers, early detection in prostate cancer has not yet been shown to be of survival benefit and may actually be of detriment (because of over-treatment) to those patients with indolent cancers. Even if managed conservatively, such patients now bear the 'survivor' label, with its attendant anxiety, need for close follow up, and potential for reduced quality of life. These complexities in the management of prostate cancer and the lack of low-morbidity treatment options have necessitated the development of alternative strategies to tackling this highly prevalent disease.

1.2 Prostate Cancer Chemoprevention

1.2.1 Types of prevention

Prevention has come to light as one approach to reduce the public health impact of prostate cancer. Various types of prevention approaches may be applied in prostate cancer including both primary and secondary prevention methods. Primary prevention (i.e. preventing the disease before it occurs), although the desired preventive strategy for chronic diseases with clearly defined and modifiable risk factors, such as lung cancer, is a less suitable approach in prostate cancer where the etiology is multi-factorial and risk factors are less well defined. Further research into the mechanism of initiation of prostate carcinogenesis is required in order to identify the specific molecular events and risk factors involved in prostate cancer transformation before primary prevention approaches can be appropriately applied.

Secondary prevention on the other hand refers to interventions that prevent or minimize the progression of a disease at an early stage, thereby limiting disability once the disease is diagnosed. Therefore, while the underlying prevalence of the disease may not be altered by secondary prevention measures, progression to clinically apparent disease may be reduced. The utility of this approach in prostate cancer is supported by epidemiologic observations. Studies of 'latent' prostate cancer, that is cancer detected at post-mortem examination, have shown that the incidence of small latent cancers does not vary with geographic region (11-13). In contrast, population studies of worldwide prostate cancer incidence and mortality rates suggest that certain regions impart a favorable risk to the development of prostate cancer. The incidence in China and Japan, for example is 80 times less than the incidence in high risk regions such as North America and Northern Europe (14). While differences in incidence have to be interpreted with caution since the widespread use of prostate cancer screening in North America undeniably accounts for the dramatically higher incidence, other indices such as mortality rate demonstrate the same overall pattern. The prostate cancer mortality rate, which is a more accurate reflection of prostate cancer risk, is 15 times lower in China and Japan compared to North America (14), although under-reporting in Asian countries could be a confounding factor in this difference. Since we know from autopsy studies that the prevalence of prostate cancer is the same worldwide, the observation of reduced prostate cancer mortality in low risk countries implies that a secondary prevention type of effect appears to be occurring, such that select environmental pressures appear to be affecting progression of the disease into clinically aggressive cancer.

1.2.2 Rationale for Chemoprevention in Prostate Cancer

Both genetic and environmental factors could account for the differences in worldwide prostate cancer mortality rates. The contribution of environmental factors is clearly substantiated

by epidemiologic migration studies that show a change in the prostate cancer mortality rate for men migrating from low to high-risk regions. For example, men migrating from Japan to the United States have been shown to acquire the high risk of the local population as early as one generation after migration. This provides the best evidence to date of the role of environmental and lifestyle factors in the risk of prostate cancer (14-19).

Certain biological features of prostate cancer further define it as a suitable target for secondary prevention. Prostate cancer is a slowly progressing disease that arises decades prior to diagnosis. The time between the onset of microscopic evidence of prostate cancer and clinically perceptible disease is believed to be 20 years or more in many cases. Autopsy studies have shown that 29% of men in their thirties harbor microfoci of latent prostate cancer (20), however, prostate cancer is rarely clinically apparent before the age of 40 and incidence reaches a peak between 50-70 years. The gradual progression of prostate cancer over many years provides an ideal window of opportunity for lifestyle modifications or for the use of chemo-preventive agents (21, 22).

The identification of proposed pre-malignant precursor lesions to prostate adenocarcinoma, such as prostatic intraepithelial neoplasia (PIN) or proliferative inflammatory atrophy (PIA), also holds promise for the identification of prevention agents capable of halting the multi-step progression of the disease. The natural evolution of prostate cancer from pre-neoplastic to adenocarcinoma to aggressive androgen independent disease and metastasis is hastened in high risk populations compared to low risk groups such as in South East Asia. The aim of secondary prevention is to emulate the natural history of the disease in low risk regions of the world and to maintain the status of the disease in the pre-cancerous or early steps prior to the development of an aggressive phenotype.

1.2.3 Clinical Chemoprevention Trials

The first trial to demonstrate the efficacy of prevention in any cancer was the Breast Cancer Prevention Trial that examined the effect of tamoxifen, a non-steroidal anti-estrogen on the prevention of breast cancer in high risk women (23). The study demonstrated a 49% reduction of breast cancers diagnosed in women in the tamoxifen group, however, this came at the cost of increased risk of endometrial cancer and thrombo-embolic events in this group. This landmark trial was soon followed by the Prostate Cancer Prevention Trial (PCPT) which provided the first level I evidence that prostate cancer, like breast cancer, is a preventable disease (24). The study randomized 18,882 men 55 years of age or older with a normal digital rectal examination (DRE) and a prostate-specific antigen (PSA) level of 3.0 ng per milliliter or lower to treatment with finasteride (5 mg per day) or placebo for seven years. Finasteride, a 5-alpha reductase inhibitor was hypothesized to reduce the prevalence of prostate cancer owing to its effect of inhibiting the conversion of testosterone to the more potent dihydrotestosterone. The study was designed to compare the biopsy proven prevalence of prostate cancer in the two groups. The primary results demonstrated a 24.8% reduction in the number of prostate cancers detected in the finasteride arm. While there was a reduction in prevalence, there was also an increase in the number of high grade cancers (Gleason grade 7 or more) in the finasteride group. This appears to be an artifact of the cytoreduction induced by finasteride, resulting in a) biopsies which more accurately identify high grade cancer, and b) improved performance of PSA as a screening test for high grade cancer (25, 26).

A number of questions remain unanswered with regards to the actual preventive effect of finasteride. The study accrued men 55 years of older with a PSA less than 3 and normal DRE. We know from previous studies that the incidence of histologically detectable prostate cancer is high in men less than 55 years old. Since no pre-study biopsy was performed, it is not clear

whether the cancers detected in the PCPT were actually present before the commencement of the trial. This is conceptually important because a reduction was seen in prevalence of disease, but it is not clear to what degree there was a reduction in numbers of new cancers (primary prevention), or rather a treatment effect of finasteride on pre-existing cancer (secondary prevention). It is impossible to determine this since there were no pre-study biopsies performed. Another point is that the effect of finasteride on morbidity or mortality has not been assessed. Although fewer cancers were identified in the finasteride arm, not all histologically diagnosed cancers are destined to clinical progression. Therefore, the long-term effects of finasteride on progression to clinically advanced disease and survival is not known. Fewer men in the finasteride group required biopsy for cause (e.g. because of elevated PSA), but this does not imply a reduction in disease progression. Most chemoprevention trials, however, are not designed to have mortality as an endpoint. This trial has demonstrated the utility of chemoprevention as a real option in controlling prostate cancer. However, long-term follow up is required before reaching definite conclusions about the efficacy of finasteride in prostate cancer prevention.

In summary, prevention of prostate cancer has become increasingly attractive owing to the significant limitations of current management options once prostate cancer has clinically progressed. Prevention is particularly favorable since most prostate cancer deaths occur after the sixth decade, so that even modest delay in the natural progression of the disease could impact significantly on mortality (27). Epidemiologic evidence suggests that although the underlying rates of initiation of prostate cancer in high and low risk populations are the same, clinical progression of the disease is much greater in high risk populations, and this may in large part be attributable to environmental factors. These observations support the rationale for secondary prevention of prostate cancer by demonstrating that under favorable conditions, prostate cancer progression can be inhibited or perhaps arrested. Prostate cancer is a disease that gradually

progresses over 20-30 years, and in this regard is ideally suited to prevention strategies with lifestyle modification or targeted chemical agents. The success of chemoprevention in prostate cancer has most recently been showcased by the outcome of the PCPT trial. Although finasteride was effective at reducing prostate cancer prevalence, the side effects of 5-alpha reductase inhibitors, and lingering uncertainty about the increase of grade associated with finasteride administration highlight the necessity for alternative chemoprevention agents. The epidemiologic evidence supporting the role of environment on the rate of progression of prostate cancer has paved the way for concerted research efforts to identify specific environmental, dietary and lifestyle factors that could account for the preventive effect described in epidemiologic studies.

1.3 Diet and Cancer

1.3.1 Background

The role of dietary influences in cancer has been studied since the 1940's when Tannenbaum and others demonstrated the tumor promoting effects of various dietary constituents, especially dietary fat, in animal models (28-30). Dietary factors have been estimated to contribute up to 30% to cancer progression and mortality, although this figure suggested by Doll et al is highly speculative (31). While the animal experiments of the early to mid 1900's set the scene for diet-cancer hypotheses, the emergence of strong epidemiological observations in the 1960's and 1970's linking dietary habits with cancer incidence world-wide reignited interest in the field of diet and cancer (32-40). Armstrong and Doll's study of correlations between dietary habits and cancer incidence and mortality in 32 countries probably received the greatest attention. The strongest associations in this study were meat consumption with colon cancer and fat

consumption with breast and uterine cancer, although weaker correlation of fat and prostate and other cancers was also demonstrated (32).

Many diet-cancer hypotheses were extrapolated from these epidemiologic observations. Since the strongest associations were from international geographical correlations where confounding variables are incompletely accounted for, and estimations of dietary consumption are often flawed, some argue that conclusions and dietary recommendations were inappropriately reached based purely on indirect epidemiologic evidence (41). Enstrom also questions the selective interpretation of data from migration studies where although an increased risk of breast and colon cancer is observed in Japanese migrants to the US, the total cancer rate for Japanese Americans is unchanged and overall mortality is actually lower than that of Japanese natives. The challenge in the field of nutrition and cancer is that testing these hypotheses in the setting of randomized controlled trials is often difficult due to poor patient compliance and the enormous expense required for such extensive trials.

Most research in the field in the last 20 years has focused on determining diet and cancer associations by well designed case-control and prospective cohort studies. The trend has been that case-control studies tend to show a correlation between a dietary component and cancer risk that is not evident when examined by prospective cohort studies. Part of the problem with case-control studies investigating an association of diet with cancer is the strong effect of both recall and selection bias on this type of study design (42). Recall of diet can be biased by the diagnosis of cancer in which case patients may unduly associate particular unhealthy dietary habits with their diagnosis (43). Case and control selection in these studies is often biased since although cancer patients have a high degree of participation, control participation is usually low. Furthermore, control subjects who do participate are usually more health conscious and have

healthier dietary habits, therefore accentuating the inverse association of healthy dietary habits with cancer in case-control studies (42).

A major complicating factor in our interpretations of epidemiologic studies of diet and prostate cancer is the confounding role of PSA screening on study outcomes (44). This is highlighted by the disparity in results of epidemiologic studies of lycopene and prostate cancer incidence conducted in the pre- and post-PSA era. A protective effect of lycopene was demonstrated in studies pre-PSA screening while the results of more recent studies have been negative. Giovannucci has proposed that this trend may be accounted for by the decreased likelihood of detecting a significant effect of lycopene in populations that are PSA screened (44). The inverse association of lycopene and prostate cancer incidence in pre-PSA era studies was strongest for metastatic disease. Since most PSA diagnosed cancers are early and organ-confined, the resulting dilution of the number of advanced cancers may mean that post-PSA era studies fail to show an effect on advanced disease (44). This is supported by findings of prospective studies such as the Health Professionals Study that spanned both the pre- and post-PSA eras.

Other sources of inaccuracy and bias in the design of nutritional studies have been in the inconsistencies associated with gathering accurate data on dietary habits. Willett et al have been leaders in designing validated food frequency questionnaires (45-47). Although their food questionnaires have been widely adopted, validation of this tool by weighing participant diets for one week and comparing to the questionnaire demonstrated correlation of 0.5-0.6 (47). The enormous variability in dietary intake on a day-to-day basis makes it extremely challenging to accurately estimate dietary intake. Although the application of food frequency questionnaires has been questioned, their efficacy has been demonstrated in studies highlighting the effects of diet in cardiovascular disease (48). Therefore, lack of positive studies using these questionnaires in

cancer studies is not solely due to the inaccuracy of the questionnaire, but rather could be due to issues related to cancer such as complexity of causation and latency (45).

When estimating the intake of particular nutrients or micronutrients, food frequency questionnaires can be quite misleading owing to the complexity of micro- and macro-nutrients in the diet, and the significant effect of food processing and cooking on the availability of these nutrients. When examining a heterogeneous group of micronutrients such as flavonoids or carotenoids, a further source of bias may be that the total micronutrient content of particular foods may not have been determined. For example, while the vitamin A content of many foods is known, the total carotenoid levels for these foods are not known. Thus, while β-carotene may not correlate with a particular disease, the same conclusions cannot be reached for total carotenoid intake. The United States Department of Agriculture flavonoid content has attempted to address this deficiency in our knowledge for flavonoid content of different foods, but this database is limited to only a few flavonoids (49).

Over the last decades a number of dietary associations with cancer risk have gained credibility, while at the same time many hypotheses have been refuted (50). Progress in this field has been slow to produce definite recommendations for dietary modifications for improving cancer risk not least for the difficulties mentioned above. Another reason, according to Giovannucci, for the slow progress in the field of nutrition and cancer, compared to the study of nutritional factors in cardiovascular disease has been the absence of intermediate biomarkers (such as blood pressure or lipid profile in cardiovascular disease) which means that cancer studies have to rely on long-term delayed outcome measures (51).

In conclusion, the studies examining dietary associations with cancer are highly inconsistent. The variety of approaches (case-control, cohort etc) and the differences in adjustment for confounding variables makes it difficult to reach firm conclusions on dietary

associations with cancer development. While retrospective studies have shown correlations of dietary factors with cancer incidence, prospective cohort studies have in many cases failed to support these results. The influence of PSA screening on the outcome of recent epidemiologic studies is another critical factor adding to the difficulty of demonstrating any dietary associations with prostate cancer risk. Below, I will address the most pertinent reports of the role of dietary factors and cancer risk, with a focus on dietary associations with prostate cancer.

1.3.2 Role of Macronutrients in Cancer

Possibly the most intensely studied of all dietary associations is that of fat and cancer. The availability of international age-adjusted mortality rates in the 1960's for various malignancies paved the way for analytic epidemiologic studies of dietary habits and cancer that highlighted the positive association of dietary fat consumption and cancer (52). Epidemiologic studies correlated the high intake of fat in developed countries with increased incidence of colon (34), breast (36), and ovary, endometrium and prostate (37). Epidemiologic studies of this nature fail to take into account major confounding variables that could influence disease risk, such as age of menarche or parity in breast cancer risk (53).

Data from case-control studies of fat intake and breast cancer has been inconsistent, with studies demonstrating both positive and negative correlations. Several prospective cohort studies have failed to show an association with fat intake and breast cancer, as did the Women's Health Initiative randomized control trial. The consensus, therefore, is that there is no association between fat intake and breast cancer risk (54). Most studies of fat intake and colon cancer risk similarly have shown no correlation, with the exception of the Nurse's Health Study that demonstrated a statistically significant increased risk (relative risk of 1.89) of colon cancer between women in the highest and lowest quintiles of fat consumption (55). Most analytic

epidemiologic studies of prostate cancer have failed to demonstrate a correlation with fat intake. Early case-control studies that did show a significant correlation failed to control for total energy intake (56). Interestingly the type of fat may be important, with alpha-linolenic acid in particular associated with increased cancer risk in several studies. Thus, while early population studies have correlated fat intake with cancers of the breast, colon, and prostate, prospective studies have largely failed to confirm these associations possibly reflecting the lack of suitable adjustment for confounding variables in population studies (57).

A difficulty arising from the assessment of dietary studies of fat and cancer risk is the inverse correlation of fat intake with carbohydrate consumption. Therefore, it can be difficult to determine the extent to which outcomes of studies examining alterations in fat intake are attributable to fat or carbohydrate variations (58). Carbohydrate intake in itself has been linked to cancer risk. In a review of 21 case-control studies of sugar and colorectal cancer, 7 demonstrated positive correlation between sugar consumption and colorectal cancer risk (59). Prospective cohort studies have failed to show significant correlations of sugar intake or glycemic load and colon cancer risk, although positive trends are apparent (58). Similarly for breast cancer, all prospective studies but one (60) failed to show a significant correlation with carbohydrate intake. Studies of the effect of carbohydrates in prostate cancer have been mostly limited to animal experiments that have shown the inhibition of prostate cancer xenograft growth in mice fed low carbohydrate diets compared to high carbohydrate diets (61, 62). This effect was associated with a reduction in levels of insulin, IGF-1 and its downstream target Akt. Insulin and IGF-1 are both growth hormones that have been associated with increased risk of prostate cancer (63, 64). A weak but significant positive correlation of fasting serum glucose levels was demonstrated in a large Icelandic cohort indirectly suggesting a possible relationship between carbohydrates in the diet and prostate cancer risk (65). In summary, while results of animal experiments are

promising, the association of carbohydrates with colorectal and breast cancer is minimal based on analytic epidemiologic studies, and the association with prostate cancer is yet to be adequately investigated in humans.

The effect of dietary protein intake on tumorigenesis has been studied for over 50 years. Animal experiments have demonstrated a reduction in onset of various types of malignancy in animals fed low protein (casein) compared to high protein diets (66-69). Verification of these observations in the human setting has been less widely studied than other macronutrients. A small case-control study demonstrated that high protein intake was associated with increased levels of growth factors including IGF-1 (70). An extensive report by the World Cancer Research Fund (WCRF) did not include protein in it's recommendations of dietary modifications based on extensive review of available literature (71). The intakes of meat and dairy products, both major sources of dietary protein have been extensively studied; however, since these also represent major sources of other nutrients such as saturated fat, correlations with cancer risk cannot be directly attributed to protein in these studies. Therefore, further research is needed to fully determine the effects of protein intake on carcinogenesis.

The evidence for meat is particularly strong for colorectal cancer, with a recent meta-analysis of several cohort studies by the WCRF concluding that 'red meat is a convincing cause of colorectal cancer' (71). This conclusion is debated by others who site the absence of correlation or indeed the inverse association of meat and colon cancer risk in several large cohort studies (72, 73). The major studies demonstrating increased colorectal cancer risk with meat are the Nurses' and Health Professional studies where a significant risk was associated with very high meat intake >140g per day. Thus, while there are conflicting reports in the literature, the consensus is that meat intake of >140g per day is detrimental (55, 74). Meta-analyses of cohort studies examining meat and breast cancer have shown a slight or no association (75, 76). Several

case-control and cohort studies have shown significant positive correlation of meat intake with prostate cancer (77). Therefore, the evidence for meat as an etiological factor in cancer appears to be strongest for colorectal and prostate cancer.

The WCRF study panel also concluded that milk and dairy products likely have a protective role in colon cancer, while the results of cohort studies in prostate cancer are inconsistent (71).

The emergence of obesity as a public health problem was highlighted as early as the early 1900's when large studies sponsored by the American life insurance industry demonstrated a positive association between premature mortality and body build (78). A century later and the association of obesity to mortality from all causes and cancer continues to be highlighted in epidemiologic studies (79-82). The International Association for Cancer Research (IARC) conducted an extensive review of the evidence linking obesity with cancer in 2002. This report suggested that the evidence supporting the association of obesity to cancer was greatest for colorectum, breast, endometrium, kidney and esophagus. The evidence for an association of obesity and prostate cancer was more inconsistent. This is despite the outcome of the largest study examining obesity and prostate cancer risk in a Norwegian cohort of 950,000 men with 33,000 incident prostate cancer cases that showed a significant association of obesity with incidence of prostate cancer in men aged 50-59 (RR 1.59) (83). Prospective cohort studies published after 2002 continued to support this conclusion for both prostate and non-prostate cancers (82, 84-89). Interestingly, while the data suggest a weak association of obesity with incidence of prostate cancer, the association is somewhat stronger for mortality from prostate cancer. The American Cancer Society Cancer Prevention Study I and II enrolled over 900,000 participants and demonstrated a significant positive association of obesity and prostate cancer mortality (RR 1.27) (90). This association was also confirmed in a prospective cohort study of 135,000 Swedish construction workers (91). This supports the notion that obesity has a role in

progression rather than initiation of prostate cancer. Thus, the evidence suggests that caloric intake, obesity and macronutrient consumption are all important factors in the pathogenesis of prostate cancer.

1.32. Effect of Micronutrients and phytochemicals in Cancer Prevention

Micronutrients, vitamins, minerals and trace elements are essential constituents of diets and are needed in small quantities. Phytochemicals are non-essential bioactive components derived from plant products. Thousands of phytochemicals exist in nature, and many have been developed into pharmaceutical agents in use today, while many more are currently under investigation. Examples of phytochemicals include flavonoids, stilbenes (e.g. resveretrol), isothiocyanates amongst many others. Many of these classes of compounds have been shown to possess anti-oxidant, anti-inflammatory, anti-microbial and anti-cancer effects. Fruits and vegetables, tea, and medicinal herbs are all rich sources of phytochemicals.

Fruits and vegetables have been used for medicinal purposes for millennia. The numerous case-control and cohort studies examining the relationship of fruit and vegetable consumption with cancer risk have recently been reviewed in a report by the WCRF (71). The authors examined evidence for non-starchy vegetables and fruits separately. The evidence for non-starchy vegetable consumption appears to be strongest for mouth, pharynx and larynx cancers where a large body of evidence has shown a dose-response effect, while the evidence in lung, stomach, colorectal, and ovarian cancer was limited. The evidence for the cancer preventive effect of fruit consumption was strongest for lung cancer, weaker for esophageal and mouth, pharynx and larynx cancer, and limited evidence existed for other cancers. Unfortunately, the WCRF report did not review the association of fruit or vegetable intake on prostate or breast cancer.

Several case-control and cohort studies have examined the link between fruit and vegetable consumption and prostate cancer (92-103). Early case-control studies revealed no association of fruit and vegetable consumption and PCa risk (92, 95-98). Larger cohort studies have recently corroborated these conclusions (99, 102, 103). The European Prospective Investigation into Cancer and Nutrition (EPIC), a cohort of over 130 000 men from seven European countries, reported no association (99). Similarly, in the multi-ethnic cohort study, comprising 90,000 participants and almost 4000 cases of prostate cancer, no significant association was found for prostate cancer incidence and fruit and vegetable intake (102). In a recent prospective study of diet and prostate cancer in almost 30,000 men, higher intake of vegetables was associated with a significant reduction only in high grade prostate cancer (RR 0.41), particularly cruciferous vegetables such as broccoli (RR 0.55) and cauliflower (RR 0.48) (103). However, other prospective cohort studies have failed to demonstrate a significant inverse association of cruciferous vegetable intake and PCa risk (96, 99-101).

Tomato products have demonstrated protective effects on prostate cancer development, although the data is somewhat conflicting. Tomatoes are high in lycopene, a potent free radical scavenger of the carotenoid family. In a recent meta-analysis, high versus low intake of tomatoes was associated with a 10% to 20% reduction in prostate cancer risk (104). The association was stronger for cooked versus raw tomato products. In a prospective cohort study, Giovannucci et al reported that tomato sauce intake was associated with a 35% decreased risk of incident advanced prostate cancer (105). Several studies have not shown an association of lycopene with prostate cancer risk and it has been proposed that the negative outcomes of three of these studies may have been due to the generally low intake of tomato products in the study populations (106). The recently conducted U.S. Food and Drug Administration's review of the evidence for an association between tomatoes, lycopene and prostate cancer reported very little evidence

supporting an association (107). The effect of PSA screening on the outcome of lycopene studies was proposed by Giovannucci to account for the lack of an association of lycopene and prostate cancer in recent epidemiologic studies, as was discussed earlier (44).

Other micronutrients with postulated effects in prostate cancer include vitamin E and selenium. The initial evidence for vitamin E emerged from the Alpha-Tocopherol Beta-Carotene cancer prevention trial that reported statistically significant reductions in prostate cancer incidence and mortality in men randomly assigned to alpha-tocopherol versus placebo (108). Similarly, gamma-tocopherol has also demonstrated an inverse association with prostate cancer risk in epidemiologic studies (109, 110). Several studies however have failed to show any association of alpha or gamma tocopherol intake and prostate cancer (111-114). The evidence for the effect of selenium is stronger, with a number of large prospective cohort studies and a randomized controlled trial demonstrating an inverse association of selenium consumption and prostate cancer (115-117). The chemopreventive effects of selenium and vitamin E are currently being examined in a randomized controlled trial (SELECT) of 32,400 men, the results of which are expected in 2013 (118).

In vitro, animal and epidemiological studies have pointed to the chemopreventive role of vitamin D in cancer. Schwartz et al. first put forward the vitamin D hypothesis for prostate cancer, postulating that various epidemiologic risk factors for prostate cancer (increasing age, Black race, Northern latitude) could be explained by relative vitamin D deficiency (119). Supporting this hypothesis are epidemiological studies that have demonstrated that solar ultraviolet-B radiation, the most important source of vitamin D, is associated with reduced risk of several malignancies including prostate cancer (120, 121). Furthermore, striking reductions in cancer mortality have been demonstrated for cancers diagnosed in summer months when vitamin D levels are highest, compared to winter (122, 123). The discovery that many cell types,

including prostate epithelial cells express vitamin D receptors, lends further rationale to this hypothesis (124). Prostate epithelial cells also express the vitamin D metabolizing enzyme 1-alpha-hydroxylase which converts circulating 25-hyroxyvitamin D (25-D) into the more potent 1,25 dihydroxyvitamin D (1,25-D) (125). 1,25-D has been shown to promote differentiation and inhibit proliferation and invasiveness *in vitro* (126, 127).

Epidemiological studies of the association of prostate cancer with serum vitamin D levels have been conflicting (128). Several small serum based case-control studies have shown no difference in serum vitamin D (both 25-OH and 1,25-OH vitamin D) levels between cases and controls (129-132), while others have demonstrated a protective effect of higher levels of 25-OH vitamin D in prostate cancer (133, 134). Dietary intake studies of vitamin D consumption or supplementation have not supported the role of vitamin D in prostate cancer risk (135, 136). This is in contrast to colorectal cancer where dietary vitamin D intake is more strongly associated with reduced cancer risk (137). It has been postulated that lower levels of 1-alpha hydroxylase in prostate cancer cells compared to colon cancer cells may account for the disparity in the effect of vitamin D in prostate and colon cancers (138).

Calcium intake has been shown to be associated with prostate cancer risk. Case-control and prospective cohort studies have shown positive associations between calcium intake and prostate cancer risk, particularly for advanced disease and at higher supplemental doses (135, 139, 140). In a meta-analysis of eleven case-control studies examining calcium intake and prostate cancer risk, the combined odds ratio was 1.68 (95% CI = 1.34–2.12) (141). A meta-analysis of prospective cohort studies demonstrated a relative risk of total prostate cancer of 1.11 (95% CI 1.00, 1.22; p=0.047) for the highest vs. the lowest intake categories of dairy products and 1.39 (95% CI 1.09, 1.77; p=0.018) for the highest vs. lowest intake categories of calcium (142). The hypothesis that increased calcium intake increases prostate cancer risk by virtue of

lowering 1,25-OH vitamin D levels is not supported by studies demonstrating that large variations in calcium intake are associated with small changes in 1,25-OH vitamin D levels (143). Thus, the effect of calcium on prostate cancer progression is most likely unrelated to the vitamin D pathway.

The detailed molecular effects of vitamin D, calcium and other micronutrients such as selenium, vitamin E, and lycopene are beyond the scope of this thesis and have been reviewed elsewhere (144-147). This thesis will focus instead on the cellular and molecular effects of another important class of antioxidant phytochemicals- the flavonoids.

1.4. Flavonoids in prostate cancer prevention

1.4.1 Definition, Classification & Chemistry

Phenolics are the most ubiquitous class of plant chemicals, and consist of compounds with a hydroxyl group (-OH) attached to an aromatic hydrocarbon. Flavonoids are a special class of polyphenolic plant secondary metabolites. The term 'flavonoids' encompasses a diverse range of compounds all of which are based on the C_6-C_3-C_6 (phenylbenzopyran) carbon skeleton. Flavonoids are located inside the cells or on the surface of various plant organs and have various functions in plants (148).

The underlying basic structure of all flavonoids consists of 2 aromatic rings, the 'A' and 'B' rings linked by a 3-carbon bridge (Fig 1.1). For most flavonoids, the 3-carbon bridge combines with an oxygen and the 'A' ring to form a third central ring structure, the 'C' ring (149). The central ring structure is known as a chromane ring and is a shared structure of flavonoids and tocopherols. Chalcones, a sub-class of flavonoids, lack the central 'C' ring. Flavonoids are categorized into several groups based on specific structural features: the major flavonoids, isoflavonoids, and neoflavonoids (148) (Fig 1.2). The major flavonoids, isoflavonoids

and neoflavonoids are isomers that differ according to the position of the aromatic B-ring moiety (Fig 1.2). Other variations of the flavonoid structures include the chalcone and aurone flavonoid families that are referred to as minor flavonoids.

The group of major flavonoids is further subcategorized based on differences in structure of the central chromane ring (Fig 1.3). Different degrees of saturation of the chromane ring occur, as shown in Fig 1.3, with flavanones and flav***a***nols having no carbon-carbon double bonds in the middle ring; flavones having one C-C double bond; and anthocyanidins possessing two C-C double bonds. Flavanones and flav***a***nols are differentiated based on the presence of a carbonyl (C=O) group at position 4 on the chromane ring of flavanones which is absent in flavanols. This C=O functional group is also seen in flavones. Flav***o***nols are very similar to flavones differing only by the addition of a hydroxyl at the 3- position on the central ring. Flav***a***nols usually have a hydroxyl at the 3-position and are therefore termed flavan-3-ols, helping to avoid confusion with the similar sounding flav***o***nols. Anthocyanidins are unique in that the oxygen atom of the chromane ring has a positive charge (Fig 1.3) (148, 150).

Up until the middle of the 20th century, flavonoids were believed to be waste products of plant primary metabolism, a notion that was soon abandoned based on research demonstrating the myriad functions of flavonoids in plant survival (151). We now know the complex metabolic pathways used to synthesize flavonoids in plants- pathways that have evolved over millennia to provide a survival advantage to plants. Plants, clearly do not have the luxury of mobility and have had to evolve elaborate chemical mechanisms in order to defend themselves from various insults (such as UV radiation) and in order to attract those that will assist with their reproduction (bees, birds) (152). Indeed, the complexity of the plant genome, which exceeds that of humans, is believed to have developed to maintain these immensely complex pathways of synthesis of novel chemicals (152).

Flavonoids are best known as the red, blue and purple pigments of flowering plants (due to the *anthocyanidin* sub-group) although the red pigment of some fruits can be due to carotenoids such as lycopene (153). These pigments and the yellow pigments of flavones and flavanols are also responsible for the fall leaves coloration (154). Because of the importance of color in pollinator attraction, flavonoids have an important role in plant reproduction. Flavonoids in plants also serve to protect from ultraviolet (UV) light owing to their high UV absorbance coefficients, and it has been suggested that this property of flavonoids was critical in the evolution of aquatic plants to a terrestrial existence (151). Flavonoids also possess other critical functions in defense against microorganisms and germination of pollen (155, 156).

Biosynthesis of flavonoids in plants is via a series of enzymatic steps starting with the aromatic amino acid phenylalanine and acetate (154). The flavonoid pathway is regulated in plants according to environmental stimuli that cause alterations in transcription of various enzymes in the pathway (157). This results in the generation of flavonoids with specific function according to the survival needs of the plant. The flavonoid pathway is one of the most clearly defined of all secondary metabolism pathways in plants. This has been partly attributed to the bright colors of flavonoids that greatly facilitate the monitoring of experiments in flavonoid biology. An intriguing possibility that has emerged with the understanding of flavonoid biochemical pathways has been the application of biotechnology methods to generate plants or even bacteria that are genetically engineered to produce high levels of a particular flavonoid for human use (158).

Each group of flavonoids possesses unique chemical properties and has a particular distribution in plants. Anthocyanins (glycosylated anthocyanidins) and proanthocyanidins (polymers that produce anthocyanidins when hydrolyzed) primarily provide color to flowering plants and fruit, and are therefore found in high concentrations in the skin of red grapes, red wine

and berries. Flavan-3-ols, such as catechin, epicatechin gallate are colorless and are found in high concentrations in green tea. Isoflavones are only found in legumes (e.g. soy) and are therefore consumed in high quantities in regions of the world with high soy consumption. Flavanones are found in high levels in citrus fruits, while flavones are present in green leafy spices such as parsley, and flavonols are ubiquitous and found in most fruits and vegetables consumed in the human diet (149, 159).

Over 9000 flavonoids have been discovered in nature (160). The number of theoretically possible flavonoids is even greater. This enormous diversity is due to the large number of possible substitutions (hydroxyl, methoxyl, methyl, isoprenyl, benzyl etc.) at the various positions on the flavonoid carbon framework. Furthermore, each hydroxyl group and some carbons can be substituted by a range of different sugars, which can be substituted themselves by various organic and aliphatic acids (160). Because of their common presence in plants they constitute an important part of the human diet and estimates of daily consumption range from 50-1000mg total flavonoid intake daily (161-164). The large differences in estimated flavonoid consumption is partly due to the estimation of different forms of flavonoids by different studies. Most flavonoids in nature are glycosylated, and therefore, the studies estimating flavonoid glycoside consumption quote flavonoid consumption at several fold higher than studies measuring aglycone intake. Regardless of the estimation, the intake of flavonoids even in the Western diet which is low in fruits and vegetables greatly surpasses the intake of other phytochemicals such as vitamin E and β-carotene (161). Owing to the considerable human consumption, it is imperative that we examine closely the safety and underlying effects of flavonoids on human health and disease.

Interest in the health benefits of flavonoids stemmed from early research in 1936 by the Hungarian scientist Szent-Gyorgyi, who also incidentally discovered vitamin C. He isolated a

substance, which he called citrin, from lemons that restored weakened capillaries to normal. This effect was not noted when vitamin C alone was administered. Citrin was later shown to be composed of the flavonoids hesperidin and eriodictyol (165). Although citrin was also called vitamin P, this name was dropped in the 1950s after it was concluded that flavonoids did not fit the strict definition of a vitamin (166). Despite not qualifying as vitamins, flavonoids have been shown in the last 72 years since Szent-Gyorgyi's initial discovery to affect various aspects of human health not limited to their beneficial effects on capillary wall integrity.

The diversity of effects of flavonoids on mammalian biological systems is nothing short of remarkable. The cellular processes modulated by flavonoids *in vitro* number in the hundreds. A few themes emerge from the many thousands of studies in this field. Firstly, the effects of flavonoids while remarkable *in vitro* are not conclusively translated in the *in vivo* situation. This can be accounted for by a number of factors such as poor absorption of flavonoids, extensive metabolism, and the complexity of the milieu *in vivo*, which is poorly replicated with *in vitro* experiments. Secondly, the literature points to the importance of flavonoid structure in their biological effects, such that certain structures (e.g. flavonols) have considerably greater biological effect than others (e.g. flavanones). One explanation for this latter effect may be the more planar structure of flavonols (owing to the double bond in the C-ring) that makes these molecules more likely to interact with active sites on enzymes. Many other mechanisms are likely to account for differences between individual flavonoids. Such generalizations that emerge from the literature allow us to put flavonoid research in perspective. Thus, we need to be aware that *in vitro* flavonoid effect should not be taken to immediately imply *in vivo* activity; and that the effects of one class of flavonoid does not necessarily apply to all flavonoid types.

1.4.2. The Epidemiology of Flavonoids in Relation to Cancer

A substantial epidemiologic literature supports the beneficial health effects of flavonoid consumption. The main areas of interest have been the anti-cancer and cardio-protective effects of flavonoids. Prostate cancer has gained special attention in this regard owing to observations of dramatic differences in mortality of prostate cancer between populations consuming high (China) and low (North America/Europe) levels of flavonoids. Epidemiological studies, including several case-control and cohort studies have broadly supported this hypothesis. Only 2 out of 7 case-control studies failed to show a protective effect of flavonoid consumption and prostate cancer risk (167, 168). Both these studies were conducted in European populations where flavonoid intake is low and may not reach chemopreventive levels even in the highest category of consumption. These two studies also differ in the relatively large sample size compared to those studies that demonstrated significant inverse association with flavonoid consumption. The chemopreventive effect of flavonoids in prostate cancer has been further supported by a number of large prospective cohort studies that have demonstrated an inverse association of cancer risk with flavonoid intake. In these studies, soy (RR 0.3 for consumption of soy milk more than once per day), isoflavone (RR~0.5 in men over 60 years of age) and green tea consumption (RR 0.52 for consumption of more than 5 cups per day) was significantly correlated to lower prostate cancer risk (169-171). However, one large cohort study conducted in Japan did not demonstrate an association of green tea intake with prostate cancer (172). This study differed from the other large Japanese cohort study examining the effect of green tea intake (171), by having a shorter follow-up time and a lower mean age of study participants, both of which may have influenced the likelihood of detecting a significant effect of green tea consumption. Two other cohort studies, both conducted on Finnish populations, did not report an association (173, 174). In a follow-up to one of these studies however, a significant inverse association was demonstrated for

consumption of the flavonol myricetin and prostate cancer risk. This highlights the effect of non-soy or green tea flavonoids in prostate cancer chemoprevention (175). Overall, the evidence for a protective effect of flavonoids in prostate cancer appears to be supported by the results of most case-control and cohort studies.

Flavonoids have been the subject of controversy regarding their potentially harmful phytoestrogenic effects in estrogen dependent malignancies such as breast and endometrial cancer. Despite this theoretical risk, the great majority of epidemiologic studies have failed to demonstrate an increased breast cancer risk, and most in fact show an inverse association with flavonoid intake. These studies have examined breast and endometrial cancer risk and the intake of soy and green tea (both rich sources of flavonoids), or the specific flavonoids such as isoflavones found in soy (genestein, daidzein), as well as flavonoids of other classes such as flavonols, flavones, anthocyanidins and catechins. Flavonoid intake was assessed in these studies by dietary questionnaire or urine/serum determination of flavonoid levels. Fourteen out of 19 case control studies demonstrated significantly lower flavonoid consumption in breast cancer patients compared to matched cancer free controls (176-193). Four case-control studies showed no association of flavonoid consumption to cancer risk, while one study demonstrated a slight increased risk (RR 1.2) with daidzein consumption in post-menopausal women with breast cancer. In contrast to the protective effect observed in case-control studies, five out of six cohort studies failed to show a significant association with flavonoid intake (194-198). Only one cohort study demonstrated a protective effect with high isoflavone consumption (RR 0.46). The absence of an association in other cohort studies may be explained by differences in study populations. The only positive study was conducted in Japan where isoflavone consumption is considerably higher than in the West (199). The five negative studies were conducted in European or American populations where isoflavone consumption is low and may not reach chemopreventive

levels even in the highest category of consumption. With regard to other estrogen-dependent cancers, 3 case control studies reported a significant inverse association of soy or isoflavone intake and endometrial cancer (200-202).

The beneficial effects of flavonoids in cancer are not limited to breast and prostate cancer. Results of prospective cohort studies of flavonoid intake have generally been mixed in colon (203-207), gastric (208-211) and pancreatic cancer (212, 213). One reason for inconsistencies between studies includes the variability of dietary screening tools for estimation of flavonoid intake. The results are more consistent in ovarian cancer where a risk reduction was noted for higher levels of isoflavone, black tea and flavonol (kaempferol and luteolin) intake (214-216). One large cohort study of oral and pharyngeal cancer in Japan showed significant inverse association with green tea consumption, but only in female subjects (217).

Although the anti-cancer effects of flavonoids predominate, one cancer type, bladder cancer, demonstrates an increased incidence with higher levels of flavonoid consumption. Three studies (one cohort and two case-control) have examined the intake of flavonols (quercetin, luteolin, kaempferol, myricetin), green tea intake or soy isoflavones with respect to bladder cancer risk (218-220). The largest of these studies was a cohort study conducted in Singapore (n=63257), which demonstrated a significantly increased risk of bladder cancer (RR= 2.32) with the highest quartile of soy consumption (220). This pro-carcinogenic effect may be due in part to the urinary excretion of metabolized soy isoflavones, resulting in high levels of modified pro-carcinogenic forms of the flavonoids in the urine. The authors also proposed that other factors in soy may be responsible for the perceived pro-carcinogenic effect, since total soy was more highly correlated with bladder cancer risk than isoflavone concentration (220).

A number of studies have examined the association of non-flavonoid phytoestrogens such as lignans (e.g. enterolactone) with prostate cancer risk and have generally highlighted a

protective effect of lignans. Since lignans are found in high levels in soy, positive epidemiologic associations of the soy flavonoids (genistein and daidzein) with cancer may indeed be a reflection of the confounding effect of lignans, or other as yet unidentified phytochemicals (168, 186).

1.4.3. Bioavailability and Metabolism of Flavonoids

The absorption, metabolism and excretion of flavonoids is a complex process involving various structural modifications to the ingested flavonoid in multiple tissues and cellular compartments. Determining the bioavailability of flavonoids (the proportion of flavonoid found in blood or target tissue after ingestion) is critical to understanding the effects of flavonoids as chemopreventive agents. Although flavonoids have been shown to undergo extensive metabolism, this does not necessarily equate to biological inactivation of the compound. In many cases in pharmacology, the metabolized product is often more active than the parent compound. A case in point is morphine whose glucuronated metabolite is known to be a more potent opiate than the parent compound (221).

Flavonoids are predominantly absorbed in the small intestine, with only small amounts absorbed via the gastric mucosa (222). The glycosylation state of the flavonoid greatly affects the mechanism of flavonoid absorption. Most flavonoids in nature exist as glycosides. Early studies suggested that flavonoid glycosides were not absorbed intact in the human gut (due to their high hydrophilicity). These findings have more recently been refuted (223). Studies have since demonstrated that quercetin glycosides are not only absorbed, but that their absorption is actually enhanced compared to quercetin aglycone (224). This absorption is believed to occur partly due to the action of sodium dependent glucose transporter (SGLT1) (225). More commonly, however, the first step in absorption of flavonoid-glycosides is usually hydrolysis of the sugar moiety in the gut resulting in generation of the flavonoid aglycone (226). This hydrolysis was initially assumed

only to occur in the colon by bacteria since humans lack the necessary enzymes to hydrolyze the β-glycoside linkages of flavonoid glycosides. However, recently is has become clear that a broad-specificity β -glucosidase enzyme in enterocytes and lactase phloridzin hydrolase in the small intestine brush border can hydrolyze these β-glycoside linkages (227). Hydrolysis of flavonoid-glycosides has also been shown to occur in the oral cavity (228). The flavonoid aglycones generated by hydrolysis of the sugar moiety are more lipophilic, and hence are more readily absorbed in the gut by passive diffusion. Flavonoids entering the colon undergo a similar hydrolysis by bacterial glucosidases (223). Flavonoids that reach the colon undergo ring scission of the aromatic ring after sugar hydrolysis, resulting in simple phenolic compounds, which accounts for the low levels of flavonoid absorption from the colon (229).

The type of sugar moiety is an important determinant of absorption efficiency, with flavonoid-glucosides much more readily absorbed than flavonoid-rutinosides (230). With respect to the flavonoid aglycones, methoxylated flavonoids as opposed to hydroxylated flavonoids are much more readily absorbed owing to their increased lipophicity, Flavonoids lack an active transporter, and as such they are absorbed via passive diffusion, a more efficient process in hydrophobic flavonoids (231). Other factors affecting flavonoid absorption include the protein content of food ingested with the flavonoid. Since flavonoids bind to proteins, flavonoid absorption will be attenuated until the protein is digested (232).

The biotransformation of flavonoids continues in the enterocytes. The main metabolic transformations include conjugation of glucuronic acid (glucuronidation), methylation and sulphation (233). These conjugations are essentially phase II detoxification reactions resulting in increased molecular mass and improved solubility of the compound which enhances excretion of the compound in bile and urine (234). Thus the enterocyte is an important site of flavonoid metabolism. Flavonoid aglycones that reach the circulation are bound to albumin. Interestingly,

binding to albumin does not affect the antioxidant ability of flavonoids, an important point in terms of the likely biological effect of absorbed flavonoids (235). Flavonoids entering the circulation subsequently undergo phase II detoxification in the liver. Other transformations in the liver include the formation of flavonoid-glutathione adducts also resulting in enhanced excretion of the flavonoid (233). Excretion of flavonoids occurs in urine as well as bile (236).

The kinetics of flavonoid absorption and metabolism in humans has been studied for quercetin and other flavonoids. Hollman et al demonstrated that peak plasma levels of quercetin were reached in 2.9 hours in subjects consuming a meal of 333 grams fried onion (237). The mean peak plasma level of quercetin was 196 ng/ml and the half-life of quercetin was 16.8 hours. This long half-life suggests that quercetin may accumulate with continued flavonoid administration. Importantly for purposes of chemoprevention, studies have also demonstrated the accumulation of flavonoids in various animal tissues (238).

Quercetin, a flavonol, is believed to have different absorption and kinetics to other flavonoid classes. Anthocyanins, in comparison, are absorbed poorly and rapidly excreted in urine. Citrus flavanones are well absorbed but have shorter plasma half lives. They also reach higher maximum concentrations than flavonols (239). A significant degree of variability is therefore expected in the pharmacokinetic properties of different flavonoids. As with other ingested compounds, inter-individual variability is another important factor accounting for the pharmacokinetic properties of flavonoids in humans.

Food preparation has variable effects on the bioavailability of flavonoids in the diet. Peeling, for example, greatly reduces flavonoid content since the peel contains a large proportion of flavonoids in fruits and vegetables (240). The effect of cooking on flavonoid content has been examined for quercetin content in onions after cooking. When onions are boiled, flavonoids diffuse out to enter the broth, making the broth a rich source of flavonoids. Frying onions for 40

minutes did not alter total quercetin content. An increase in quercetin is noted on microwaving due to increased extractability (241). Consumption of flavonoids and protein together, while postulated to reduce the absorption of flavonoids, has not been shown to have an effect (242). Thus, in general, flavonoid availability from food appears to be enhanced by cooking.

1.4.4. Are Flavonoids Safe for Human Consumption?

The availability of a diverse range of flavonoids to the general public as prescription-free supplements propels the issue of flavonoid safety to the forefront of public health concerns. A large number of studies have investigated the safety of flavonoids, particularly of the isoflavone genistein, the green tea catechin EGCG and the flavonol quercetin. The effect of these flavonoids has been investigated using various genotoxicity assays *in vitro* and *in vivo*, and the safety of long-term administration of these compounds in animals and humans has been assessed (243-266).

Studies examining the toxicity of EGCG demonstrated no mutagenicity in a salmonella mutagenicity assay *in vitro*, but at doses of 210 μM were clastogenic (248). When administered to rats, EGCG was non-toxic at 50mg/kg as an intravenous bolus and was non-toxic to rats after 13 weeks administration at doses of 500mg/kg/day. An oral dose of 2 grams/kg was lethal in rats (250). In order to determine reproductive toxicity, mice were administered 14000ppm EGCG during gestation (249). Although there was reduced pup growth and pup loss, there was no evidence of teratogenecity. 800mg/day of EGCG administered to humans for 4 weeks had only minimal side effects including mild gastrointestinal symptoms and muscle aches (244). There was no hematologic or serum chemistry abnormalities. Thus, EGCG appears to be non-mutagenic except at very high doses in vitro. EGCG should be avoided during pregnancy.

Genistein had no mutagenic effect in the Ames test, and although clastogenic *in vitro*, there was no increase in micronuclei in Wistar rats administered 2 grams/kg of genistein (256). Like EGCG, genistein administration during gestation (at 1gram/kg) resulted in increased pup mortality without being teratogenic (251). Studies examining effects of soy infant formula have not demonstrated any adverse effects in terms of growth, development or later reproduction (254). Genistein administered to humans at 600mg/day for several weeks did not induce micronucleus formation or rearrangement in MLL gene (267). McClain et al examined the effect of short and long term administration of genistein by rats at a dose of 500 mg/kg/day (256). This dose was toxic to rats as evidenced by reduction in body weight and poor feeding. Long term administration (52 weeks) was associated with increased liver gamma glutamyl transferase and minimal bile duct proliferation suggestive of liver toxicity. Effects on hormone sensitive tissues included ovarian atrophy, hyperplasia of endometrial epithelium and vacuolation of epididymal epithelium suggestive of a estrogenic effect of high dose genistein on these tissues (256). In summary, short term high dose (2 grams/kg) genistein is not genotoxic *in vivo*, despite being clastogenic *in vitro*. Toxic effects of genistein including estrogenic side effects are observed at doses of 500mg/kg. A safe dose in humans is 600mg/day, although the adverse effects on the fetus should preclude genistein use during pregnancy.

Quercetin has tested positive for mutagenicity in most salmonella mutagenicity assays (247). Quercetin also induces micronucleus formation and chromosomal aberrations *in vitro*. As for genistein and EGCG above, *in vivo* genotoxicity is not observed. The numerous *in vivo* studies examining quercetin toxicity have recently been reviewed (247). No toxic effects of quercetin at doses up to 1 gram/kg body weight/day have been demonstrated. In mice consuming 2000mg/kg/day quercetin for 2 years, an increased incidence of renal adenomas and

adenocarcinomas was noted, although this observation has not been noted in other long term studies of similar dose quercetin ingestion.

In summary, the degree of toxicity from flavonoids is dependent on dose. Very high doses (>1gram per kg) are likely to have toxic side effects. Since over-the-counter flavonoid supplements are recommended at doses of approximately 14mg per kg, side effects from flavonoid self-supplementation in humans are unlikely. Although high doses of flavonoids were not teratogenic in most studies, fetal loss was increased and pup weight was reduced. For these reasons, excess flavonoid intake should be avoided during pregnancy.

1.4.5. The Mechanisms of Action of Flavonoids

1.4.5.1 Antioxidant Potential

Reactive oxygen species (ROS) are highly reactive molecules with both physiologic and pathologic roles. ROS can occur in the form of molecules with highly reactive unpaired electrons known as free radicals (e.g. superoxide, $O_2^{\bullet-}$), or as non-radicals that are highly liable to form free radicals (e.g. hydrogen peroxide, H_2O_2). They can exist in the body as a result of deliberate synthesis (e.g. production by macrophages for bacterial killing), or as a result of accidental production by metabolic processes such cellular respiration in mitochondria, or via exogenous insults such as smoking (268). Many ROS are not in themselves exceptionally reactive; however, in the presence of free heavy metal ions such as copper and iron they generate highly toxic radicals such as hydroxyl ions ($OH^{\bullet}$). ROS are highly damaging as they can attack lipids in cell membranes, proteins, carbohydrates, and DNA. The resulting oxidative damage may play a role in aging and chronic and degenerative diseases including cancer (269-272).

About 1-3% of the oxygen we breathe ultimately goes into making ROS, resulting in a huge burden of pro-oxidant free radicals that has to be effectively removed (273). The human body relies on both endogenous and exogenous (dietary) anti-oxidant systems to buffer the effect of the ROS constantly produced by metabolic processes. Endogenous antioxidant systems include enzymes such as superoxide dismutase, which converts $O_2^{-\cdot}$ into H_2O_2, and glutathione peroxidase and catalase, that serve to remove H_2O_2. Non-enzymatic endogenous defense mechanisms also have a significant anti-oxidant impact, including buffering by plasma urate and plasma protein thiols. Furthermore, the sequestration of heavy metal ions in binding proteins such as transferrin (iron) reduces the risk of formation of toxic hydroxyl radicals (274). Despite these many levels of protection against ROS damage, endogenous antioxidant systems are incompletely efficient in elimination of all ROS, particularly with the added insult of various environmental ROS from smoking, air pollution etc. Exogenous antioxidant supplementation, from dietary sources therefore has a critical role in the prevention of oxidative stress in human physiology (274, 275).

Antioxidant phytochemicals constitute some of the most important exogenous defense antioxidants in mammalian physiology. Up until the mid-1990's the dietary phytochemicals most prominently studied for their antioxidant properties were vitamin C, E and the carotenoids (276). Polyphenols, which constitute a major group of plant chemicals, only gained interest for their antioxidant effects in the last decade. Flavonoids, the largest group of polyphenols found in nature, appear to be particularly potent antioxidants *in vitro*. The flavonoids, however, are not all equally effective, with definite structural requirements necessary for the greatest antioxidant effect. The presence of a 2,3 double bond in the C-ring, a catechol structure in the B-ring, and hydroxylation at position 3 and 5 of the A ring appear to impart increased redox potential (277).

The redox potential of quercetin was similar to ascorbic acid and greater than the redox potential of uric acid (278).

Direct scavenging of free radicals is one of the major mechanisms of antioxidant activity by flavonoids. The resulting aroxyl radical (Flavonoid-O˙) is more stable than other ROS and gains further stability on reacting with a second radical to form a stable quinone structure (279). Several other mechanisms of antioxidant activity of flavonoids have been proposed including scavenging of transition metal ions (280), and inhibition of enzymes responsible for antioxidant production. In terms of the latter property, flavonoids have been shown to inhibit several pro-oxidant enzymes including xanthine oxidase (281), glutathione S-transferase (282), nitric oxide synthase (283), and NADH oxidase (284) amongst others.

The antioxidant effects of flavonoids *in vitro* are well established, and have been confirmed using several methodologies (285-287). However, studies examining the effects of flavonoids in humans have demonstrated conflicting results. Lotito et al recently reviewed studies examining the protective effects of consumption of flavonoid rich foods or extracts on oxidizability of LDL in human plasma (288). Studies reviewed included those examining green tea, black tea and red wine consumption. Conflicting results were shown for each of these flavonoid rich food products, with both positive and negative results. Differences between studies could be accounted for by differences in the amount and frequency of flavonoid-rich food consumption. Others dismiss the results of studies of this design because of the likelihood that flavonoids could be removed from LDL during the lipoprotein isolation and wash procedures, therefore yielding inaccuracies in the results (289). Despite inconsistencies when examining LDL oxidizability, studies examining total antioxidant capacity of human plasma after consumption of flavonoid rich diets have almost all noted significant increase (288).

Measuring markers of oxidative damage of lipids (F_2-isoprostane) and DNA (8-hydroxy-2'-deoxyguanosine) constitute alternate methodologies employed to determine the true antioxidant effect of flavonoids *in vivo*. Studies employing these methodologies have generally failed to demonstrate a protective antioxidant effect of flavonoid rich diets on lipid peroxidation or oxidative DNA damage (289). Therefore, while studies consistently demonstrate an increase of plasma antioxidant capacity with intake of flavonoid rich foods, studies measuring markers of oxidative lipid and DNA damage fail to demonstrate a protective effect. Virtually all the research in this field has focused on the antioxidant effect of flavonoid rich food or extracts, rather than pure flavonoids. Thus, conclusions about the antioxidant effect of flavonoids cannot be drawn from these studies, since food products such as those examined are also rich sources of non-flavonoid antioxidants.

Discrepancies between the strong antioxidant capacity of flavonoids *in vitro* and their antioxidant efficacy *in vivo* may be due to the poor absorption and extensive metabolization of flavonoids leading to poor bioavailability. The serum levels of other dietary antioxidants such as vitamins C and E are typically over 100 times greater than flavonoids even after the consumption of flavonoid rich foods (288). Therefore, in normal human physiology, the actual impact of flavonoids on the total antioxidant capacity of human serum is likely to be minimal. Therefore, despite flavonoids constituting the largest group of antioxidants in plants, a diet high in fruits and vegetables is more likely to provide actual antioxidant supplementation in the form of non-flavonoid compounds.

1.4.5.2. Pro-Oxidant Effects

While flavonoids are best known for their anti-oxidant properties, it has been shown that under certain conditions, flavonoids may be pro-oxidant. This property has been proposed to

account for several biological effects of flavonoids, such as apoptosis, that are induced in the setting of oxidative stress. These pro-oxidant effects have also been observed for other phenolic antioxidants including tocopherols, ascorbate, urate, curcumin and N-acetylcysteine. The balance between anti-oxidant and pro-oxidant effects of these compounds is dependent on several factors in the cellular environment, particularly on the presence of transition metal ions.

The hydroxyl groups of flavonoids account for much of their antioxidant effect. After scavenging ROS, flavonoids are themselves oxidized, with a hydroxyl group now containing a free radical known as a phenoxyl radical. Some flavonoids possess a catechol structure in the B-ring. Oxidation of these flavonoids can result in a semi-quinone radical. The flavonoid semi-quinone can undergo further oxidation resulting in flavonoid quinone. Therefore different types of oxidation of flavonoids occur depending on their exact structure. In addition to scavenging of ROS, flavonoids can also be oxidized in other ways. These include oxidation by cellular peroxidases, or by auto-oxidation in the presence of oxygen- a process greatly accelerated in the presence of transition metal ions.

The paradox of 'antioxidant' flavonoids is that in the process of scavenging ROS, they become pro-oxidant radicals themselves, albeit less reactive than the scavenged species. The flavonoid radicals never-the-less have undesirable properties. For example, in the presence of transition metals such as Cu2+, flavonoids undergo a series of redox reactions culminating in the genesis of damaging hydroxyl radicals (290). Another mechanism involves the flavonoid-quinones that are the products of oxidation in catechol containing flavonoids. These are highly reactive to thiol groups, and result in the formation of flavonoid conjugates with thiol containing proteins such as glutathione. Interestingly, flavonoids that do not form flavonoid-quinones have also been shown to form thiol conjugates, highlighting the complexity of flavonoid chemistry that remains to be fully elucidated (291). The pro-oxidant effects of flavonoids have been shown to

result in DNA damage and lipid peroxidation *in vitro*. Pro-oxidant radicals have been demonstrated for several flavonoids including myricetin (292), quercetin (293, 294), proanthocyanidins (295), green tea catechins (296, 297), daidzein (298) and baicalin (299).

The precise conditions promoting either the anti-oxidant or pro-oxidant effects of flavonoids *in vivo* remain to be addressed, and is an important area of future research. Most of the studies of the oxidant properties of flavonoids have been performed in cell free or cell culture systems. The dose of flavonoid used in these studies is often over 10 fold higher than is physiologically achievable by diet or flavonoid supplements. Cell culture models also suffer a number of deficiencies not least owing to the artificially high oxidative stress conditions in cell culture, and the presence of free metal ions that are known to induce pro-oxidant effects in flavonoids (300). Various protective features of human physiology make it unlikely that the pro-oxidant properties of flavonoids will have such dramatic effects in the human body. For example, flavonoid-quinone toxicity is rapidly prevented *in vivo* by dithiols (301). Flavonoids also undergo considerably less auto-oxidation *in vivo* as a result of the sequestration of free transition metal ions in carrier proteins such as transferrin. Thus, the pro-oxidant effects of flavonoids are expected to be considerably less pronounced *in vivo*.

1.4.5.3. Hormonal Properties

Together with the antioxidant effects of flavonoids, the hormonal, and particularly the estrogenic effects of flavonoids have garnered the greatest attention in flavonoid research over the past 50 years. The estrogenic properties of flavonoids first came to light in the 1950's when it was observed that sheep grazing on red clover pastures had reduced breeding rates (302). Red clover was found to contain several isoflavones and the estrogen-like properties of isoflavones were shown to account for fertility disturbances in animals feeding on red clover. As a result of

their estrogen-like properties, isoflavones are also classed as phytoestrogens. The high levels of isoflavones in red clover may serve as a defense mechanism limiting the population of grazers, and thereby providing a survival advantage to the plant. The reproductive effects of isoflavones are of considerable concern to human health because foods rich in isoflavones, such as soy products, are consumed in large amounts in the human diet. These and other safety issues of flavonoids were discussed in greater detail in section 1.4.4.

Isoflavones (genistein and formononetin) have been shown to displace radiolabeled estradiol from the estrogen receptor (ER) (303). Studies have also demonstrated the partial agonist properties of isoflavones on the estrogen receptor using transfected ER-element reporter assays (304, 305). Flavonoids from the flavone, flavonols, flavonone and chalcone classes are considerably weaker phytoestrogens than the isoflavones as determined by competitive binding assays (306). Of the two estrogen receptor isoforms, genistein has a 7-fold greater binding affinity to ERβ than ERα, although binding affinity is 20 and 3.7 fold less than 17-β estradiol (E_2) (307). Three-dimensional structure analysis confirmed that genistein bound to the ligand-binding site of ERβ is similar to the natural ligand E_2 (308). Furthermore, the recruitment of ER co-activators was modified by genistein differentially with the ERβ-genistein complex binding ER co-activators to a much greater degree than ERα-genistein (309).

The differential binding of phytoestrogens to ER isoforms is of importance, since each of the ER isoforms has been shown to have distinct functions in proliferation. At the promoters of certain proliferation genes, ERα and ERβ have opposite actions, with ERα being pro-proliferative and ERβ anti-proliferative (310). Despite the weak binding of ERα, genistein caused ERα activation at concentrations of 1μM in a luciferase-reporter assay (311). Therefore, at the physiologically achievable dose of 1μM, genistein is an activator of both ER isoforms. Genistein and other flavonoids such as quercetin display a biphasic proliferation pattern *in vitro,* such that

at doses <1μM these compounds cause an increase in proliferation in ER positive cell lines, and at >10μM they are anti-proliferative (312-314). The proliferative effect of flavonoids at low doses was shown to be dependent on ERα since the effect was abolished by the ERα antagonist hydroxytamoxifen. Interestingly, genistein activated ER mediated expression more in cells co-transfected with ERα and ERβ, than in cells transfected with ERα alone, suggesting that ERβ contributes to the agonistic effects of genistein, which goes against the predominantly anti-proliferative effect typically attributed to ERβ (315). The anti-proliferative effect of flavonoids at higher doses is seen in ER-negative cell lines, and is thus independent of both ER isoforms (314).

Taken together, the data suggest that at low doses, flavonoids may be pro-proliferative in an ERα and possibly ERβ dependent fashion, while neither ERα nor ERβ are critical for the anti-proliferative effect of flavonoids at higher doses. The precise physiologic effects of flavonoids mediated by their binding to ERα and ERβ are yet to be fully determined. This is an important area of future research because phytoestrogenic flavonoids are consumed in large amounts in the human diet. If the estrogenic effects of these compounds are predominantly pro-proliferative at low concentrations, flavonoids could potentially pose a health risk in terms of promoting hormone dependent cancers.

Flavonoids may also exert anti-estrogenic effects by various enzymatic mechanisms. Blocking the synthesis of estrogens by inhibiting aromatase is an established strategy in the treatment of breast cancer. Flavonoids have been shown to bind the active site of aromatase, and inhibit its function, with flavones and flavanones, rather than isoflavones having the greatest effect (316, 317). Other enzymes of note in estrogen metabolism include sulfatase and 17β-hydroxydteroid dehydrogenase, both of which result in activation on estradiol precursors, and which are inhibited by flavonoids (318). The levels of sex hormone binding globulin (SHBG) by flavonoids is also of importance, as excess SHBG can bind estrogen reducing its effect (319).

The estrogenic effects of isoflavones in humans is supported by studies demonstrating altered menstrual cycle length in women consuming daily soy protein, a product rich in isoflavones (320-322). Despite the undoubted estrogenic properties of flavonoids, epidemiologic studies have for the most part demonstrated a protective effect for high flavonoid intake and hormone dependent cancers (section 1.4.2.). The degree of dietary flavonoid intake, estrogenic properties of flavonoids and hormone dependent cancers is highly complex. The relationship cannot be explained solely by the estrogenic properties of flavonoids. Other mechanisms of action need to be considered in attempting to understand the actual effect of flavonoid intake on hormonal cancer risk in humans.

The similarity in structure of flavonoids to all steroid hormones raises the possibility of ligand binding of flavonoids to other members of the nuclear steroid receptor family. Flavonoids have been shown to bind and activate a number of nuclear receptors including androgen (323, 324), progesterone (323), thyroid (325), and peroxisome proliferator-activated receptor PPARγ (325). Flavonoids functionally activate androgen receptor mediated transcription, resulting in increased PSA, a major downstream androgen receptor regulated gene. Apigenin, a flavone, was the most effective flavonoid in upregulating PSA expression (326). Interestingly, in a related study other flavonoids were shown to have precisely the opposite effect and inhibit PSA production. It was concluded that unlike the estrogenic effects of flavonoids, the effects on PSA production did not follow a structure-function relationship (327). Genistein and quercetin were shown to induce AR activation, however, it is evident from these studies that the effect of flavonoids, like that of dihydrotestosterone is biphasic, with activation of AR occurring at low doses and AR inhibition at higher flavonoid doses (328-331).

In addition to their sex steroid effects, flavonoids affect several other hormonal pathways. For example, several mechanisms have been described for the potentiation of the vitamin D

pathway by genistein. This includes up-regulation of vitamin D receptor gene expression and activity (332), and inhibition of enzymes (CYP24) that convert 1,25-vitamin D into less active metabolites (333). The vitamin D pathway is increasingly implicated in chemoprevention of prostate cancer, and up-regulation of this pathway is a potentially useful synergistic property of flavonoids. Genistein has also been shown to have a stimulatory effect on insulin secretion *in vitro* (although this effect is not seen *in vivo)* and an inhibitory effect on leptin secretion in rats administered genistein (334). Flavonoids also inhibit corticosteroid secretion *in vitro* and *in vivo (334)*. Finally, the goitrogenic activity of soy is well documented, especially in the setting of iodine deficiency. This effect is believed to be secondary to inhibition of thyroid peroxidase, a major metabolizing enzyme in thyroxine biosynthesis (335).

Overall, the interactions of flavonoids with steroid hormone pathways are highly complex. However, the effect of flavonoids on these pathways has been shown to ultimately cause alterations resulting in beneficial effects such as the negative regulation of proliferative stimuli. As with much of flavonoid research, the *in vivo* effects of flavonoids on hormonal signaling require further study. This is highlighted in a recent review by Hamilton-Reeves et al, where the majority of intervention studies reviewed did not find a difference in circulating sex steroid hormone levels (336).

1.4.5.4. Cell Cycle Effects of Flavonoids

1.4.5.4.1. The cell cycle- background

The mammalian cell cycle is divided into a DNA synthesis (S phase) and mitotic phase (M-phase) that are preceded by two gap phases, G1 and G2 respectively (Fig 1.4). The main coordinators of the cell cycle are the cyclin dependent kinases (cdks). Cdks are activated by binding to specific cyclins, which results in down-stream alterations that enable the cell to

progress from one phase to another. Cdks are expressed throughout the cell cycle, while expression of cyclins fluctuates with the different stages of the cycle. The specific cyclin/cdk complexes of the G1 to S transition are cyclin D-cdk4/6, and cyclin E-cdk2. The main target of these kinase complexes is phosphorylation of the retinoblastoma protein (Rb). Phosphorylation of Rb causes it to dissociate from the E2F transcription factors which can then promote G1 to S transition by the transcriptional up-regulation of downstream genes. S-phase progression requires cyclin A-cdk2/cdk1(cdc2) complex and G2 to M requires cyclin B/cdk1(cdc2) activation. Cyclin-cdk complexes are regulated by members of the cip/kip family of pan-cdk inhibitory proteins ($p21^{cip1}$, and $p27^{kip1}$). These proteins bind to cdk resulting in inhibition of kinase activity.

Throughout the different stages of the cell cycle, a number of fail-safe mechanisms exist referred to as cell cycle checkpoints. These are signaling pathways necessary for the orderly and error free progression of the cell cycle (337). Cell cycle arrest occurs when checkpoints are activated by stresses such as genomic damage. Activation of checkpoints arrests the cell cycle thereby allowing time for DNA repair, or the activation of cell death pathways in cases of irreparable insult. The activation of cell cycle checkpoints leads to cell cycle arrest by several pathways both post-translational and transcriptional. These pathways invariably lead to a modulation of cyclin-cdk complexes- the critical and final determinants of cell cycle progression.

The DNA damage checkpoint is a classical example of the complex events that lead to checkpoint activation (Fig 1.5). ATM, a phosphatidylinositol 3-kinase like protein kinase, is an early sensor of DNA double strand breaks and a key player in the DNA damage checkpoint (338). ATM is mutated in the rare inherited disorder, ataxia telangectasia. This disease is characterized by cerebellar degeneration, immunodeficiency, cancer predisposition and genomic instability (339). ATM is activated by a variety of agents, such as ionizing radiation (IR), that induce DNA double strand breaks (340). This triggers autophosphorylation of ATM on serine

1981, and conversion of ATM from the inactive dimeric form to the active monomeric form (341). ATM phosphorylates a number of downstream proteins that result in cell cycle checkpoint activation, and arrest at G1/S and G2/M phases of the cell cycle. Amongst the downstream phosphorylation targets of ATM are p53, mdm2, chk2, and H2AX (342-344). ATM phosphorylates p53 on serines 9, 15, 20 and 46, resulting in increased stability and activity of p53 (345). Phosphorylation of the p53 negative regulator mdm2 on serine 395 is also ATM dependent (342). This phosphorylation has been shown to attenuate the actions of mdm2, thereby ensuring a dual mechanism whereby p53 is activated and is responsible for G1/S phase cell cycle arrest. p53 has also been shown to influence the G2/M checkpoint by inhibition of cyclin B1/Cdc2 activity and through transcriptional upregulation of additional downstream target genes that activate the G2/M checkpoint (346).

The G2/M checkpoint is regulated by the cyclin dependent complex, cdc2-cyclin B1. Cdc2 is maintained in an inactive form by phosphorylation of residues threonine 14 and tyrosine 15 by Wee1 and Myt1 kinases(347). Dephosphorylation of these residues by cdc25C activates cdc2 and is a prerequisite for mitosis(348). Further activation of cdc2 occurs by phosphorylation of threonine 161 by CDK-activating kinase (CAK). The checkpoint protein chk2 is a key player at the G2/M checkpoint, and is activated by IR in an ATM-dependent manner by phosphorylation at threonine 68. Chk2 is responsible for phosphorylating cdc25C at serine 216 causing inactivation of cdc25C by allowing 14-3-3σ binding, anchoring cdc25C in the cytoplasm(349). It is therefore clear that there is a close interplay between cell cycle proteins and upstream regulators in the form of DNA damage sensors such as ATM.

1.4.5.4.2 Cell cycle alterations by flavonoids

Most flavonoids studied to date have been shown to target the cell cycle. Flavonoid treatment results in a wide range of cell cycle alterations including inhibition of cyclin-dependent kinases and cyclins, or up-regulation of cdk-inhibitors of the cip/kip family. Alterations in the regulators of G1 to S transition including RB and E2F proteins have also been demonstrated. Flavonoids have also been shown to regulate cell cycle checkpoint pathways, in particular the DNA damage response pathway.

Several flavonoids have been studied in prostate cancer *in vitro.* Apigenin, a flavone, has been studied in 3 prostate cancer cell lines –PC3, LNCaP and DU145 (350-353). This flavone has been shown to induce G1 cell cycle arrest in all three cell lines. The molecular mechanisms of apigenin have been demonstrated and include down-regulation of cyclins D1, D2 and E, and inhibition of cdk2, 4, 6. This was associated with up-regulation of p21, p27 and a reduction in phosphorylated RB (350-352). The green tea catechins have also been studied in these cell lines and like apigenin, caused a G1 arrest in all three cell types(354-356). In addition, the authors demonstrated an up-regulation of p16 and p18 (356). In contrast, genistein, a soy isoflavone, caused G2 arrest in PC3 and DU145 cells (357-359), and G1 arrest in LNCaP (360). The G2 arrest of genistein is explained by inhibition of cyclin B1, cdk1 and up-regulation of p21 and p27 (359). Silibinin, a flavono-lignan from milk thistle, caused a G1 arrest in DU145 and LNCaP and a G1 and G2 arrest in PC3 (361, 362). Other flavonoids studied in prostate cancer include quercetin (G2/M in PC3 and LNCaP) (353, 363), baicalin (G1 in LNCaP) (364), acacetin (G1 in LNCaP and G1 and G2/M in DU145) (365). The effect of flavonoids on cell cycle is cell-type dependent. Apigenin, which arrested 3 prostate cancer cell lines in G1, has been shown to arrest bladder cancer cells in G2. Thus, prediction of the type of cell cycle arrest by flavonoids is

difficult, and probably depends on several factors such as p53 status of the cell lines being examined.

Modulation of the ATM dependent DNA damage checkpoint by flavonoids has been demonstrated in several cancer types, but has not been examined in prostate cancer. Genistein was shown to arrest hepatoma cells in G2/M, an effect mediated by decrease in cdc2 kinase activity and activation of the ataxia telangectasia mutated (ATM) gene (337). Up-regulation of the ATM DNA damage response pathway by genistein represents a constraint to the progression of genetic instability commonly seen in pre-malignant cells. Silibinin has also been shown to activate tumor suppressor p53 by the ATM-chk2 pathway (366). Kaempferol, apigenin and luteolin have all been shown to activate the ATM pathway in oral cancer cells *in vitro* (367). Flavonoids are known to inhibit DNA topoisomerase II, which is responsible for the clastogenic (DNA strand breaking) properties of flavonoids. The clastogenic properties of flavonoids occur at high micromolar concentrations and it is undetermined whether the relatively low doses of flavonoids in these studies causes DNA strand breaks. It remains to be determined whether ATM pathway activation by flavonoids is simply a result of the DNA damaging effects of flavonoids, or whether the activation of ATM by flavonoids occurs via a novel mechanism.

The activation of cell cycle checkpoints has been shown to occur early in the process of tumorigenesis. This has been proposed to act as a barrier against genetic instability and further malignant transformation (368). Flavonoids thus have a direct impact on key cell cycle regulatory mechanisms that ordinarily exist to suppress the proliferation of cells with tumorigenic potential. These properties of flavonoids may explain their chemopreventive effect highlighted in epidemiologic studies.

1.4.5.5. Apoptotic Effects as a Consequence of Flavonoid Treatment

In addition to the cell cycle and DNA damage pathways outlined above, flavonoids have been shown to induce apoptosis in a variety of cell types. Apoptosis occurs through two well-characterized pathways: the external pathway which is initiated by ligand binding to cell membrane death receptors, and the intrinsic pathway triggered by changes in internal cellular signals. Both pathways ultimately result in activation of the caspase cascade. Caspases are cysteine dependent proteases that initiate the sequence of events culminating in the apoptotic phenotype. In the intrinsic pathway, apoptogenic stimuli cause cytochrome c release from the mitochondria, an event inhibited by bcl-2 and promoted by bax (369).The ratio of bcl-2 to bax is an important factor in apoptosis progression. Once in the cytoplasm, cytochrome c binds to Apaf-1 which recruits ATP and caspase 9 to form the apoptosome. Caspase 9 recruits pro-caspase 3 to the apoptosome and activated caspase 3 mediates cell death (370). The apoptotic pathway is modulated by several other proteins including the inhibitor of apoptosis family (IAP).

Flavonoids have been shown to influence some aspects of this pathway. Apigenin was shown to activate the mitochondrial apoptotic pathway as evidenced by loss of mitochondrial Bcl-2 expression, mitochondrial permeability, cytochrome C release, and the cleavage of caspase 3 and 9 (371). Quercetin potentiated TRAIL induced apoptosis via the extrinisic pathway in DU145 and LNCaP prostate cancer cell lines, and induced apoptosis in a p53 independent fashion in PC3 cells, associated with an increase in Bax protein expression and a decrease in Bcl-x(L)and Bcl-2 protein (372, 373). EGCG on the other hand has been shown to cause apoptosis by a p53-dependent mechanism. EGCG mediated apoptosis in PC3 cells was attenuated by inhibition of p21 and bax by siRNA (355). Inhibition of transcription factor NFκB by EGCG leading to increased apoptosis has also been demonstrated (374). Other flavonoids shown to induce apoptosis via similar mechanisms in prostate cancer cells include genistein, isoliquiritigenin,

silibinin, baicalin, amongst others (375-378). A novel association of flavonoids and apoptosis has been the association of degree of apoptosis induced by flavonoids (EGCG) and their ability to inhibit fatty acid synthase (FAS) activity. FAS is a key lipogenic enzyme over-expressed in cancer cells (379). In summary, flavonoids induce apoptosis in cancer cells by several mechanisms regulating both the intrinsic and extrinsic pathway.

1.4.5.6. Flavonoids and Alterations in Signal Transduction Pathways

Flavonoids modulate several key elements of signal transduction pathways related to cellular growth and survival. These cover the full spectrum of the intracellular signaling network. Below I will summarize the major reported signaling effects of flavonoids outside of the cell cycle, apoptosis, hormonal and antioxidant pathways already discussed.

NFκB: The transcription factor NFκB has important functions in several cellular processes. Activation of NFκB has been shown to inhibit apoptosis. Gong et al have demonstrated the effect of genistein on abrogating NFκB DNA-binding activity. The effects of genistein were shown to be partly mediated by inhibition of the cell survival oncoprotein Akt (380). Similar findings were also shown for the tea flavonoid EGCG (381). Although genistein inhibits NFκB activity, it appears to increase NFκB gene transcription as evidenced by increased level of the p50 NFκB subunit in nuclear extracts of genistein treated cells (382).

Wnt/β-catenin: The Wnt pathway is a key pathway dysregulated in cancer. Flavonoids have been shown to inhibit this pathway at several levels. The nuclear co-factor β-catenin associates with T-cell factor (Tcf) transcription factor to activate genes needed for growth promotion and metastasis of tumors. Using reporter assays for β-catenin activation, Lee et al demonstrated the down-regulation of β-catenin signaling by the citrus flavanone naringenin (383). Park et al demonstrated that quercetin inhibits Wnt/ β-catenin signaling by disrupting the β-catenin/Tcf

complex and by inhibiting the binding of Tcf to promoter target sequences (384). Nuclear levels of β-catenin and its down-stream targets c-Myc and cyclin D1 were also shown to be reduced by apigenin administration *in vivo* (385).

STAT3: Signal-transducer-and activator-of-transcription-3 (STAT3) is a transcription factor often implicated in malignancy. STAT3 is activated constitutively in malignancy, induces transcription of several genes that mediate proliferation and promote angiogenesis (386). Flavonoids such as EGCG and the synthetic flavonoid flavopiridol have been shown to act as inhibitors of STAT3, disrupting STAT3/DNA interactions (375, 387). Future studies are likely to examine in greater detail the potential of flavonoids as STAT3 inhibitors.

Matrix metalloproteinases: Proteases secreted by cancer cells include urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs). These proteases are believed to lead to the facilitation of metastasis and promotion of angiogenesis by degredation of the extracellular matrix. Ho et al demonstrated the inhibition of invasion and migration of oral cancer cells *in vitro* by EGCG. This was associated with a reduction in levels of MMP2 and 9. Similar findings were shown for quercetin in PC3 prostate cancel cells (388). The membrane-type 1 matrix metalloproteinase (MT1-MMP) which activates pro-MMP2 was shown to be inhibited by tea polyphenols including EGCG (389).

VEGF and HIF-1α: In addition to suppression of matrix metalloproteinases, flavonoids have been shown to inhibit angiogenesis in several other ways. Liu et al demonstrated the effect of apigenin on VEGF expression in lung cancer cells. Apigenin was shown to inhibit transcription of VEGF through the hypoxia inducible factor binding site and by reduction of total HIF1alpha levels (390). Suppression of VEGF transcription and activity was also shown for EGCG in gastric cancer cells (390). Genistein was shown to be a potent inhibitor of angiogenesis in a xenograft model. It was shown to inhibit expression of VEGF, platelet derived growth factor, and up-

regulate anti-angiogenic factors such as endostatin and angiostatin (391). Thus by inhibition of VEGF and MMPs, and activation of anti-angiogenic factors, flavonoids may have an important role as anti-angiogenic agents in cancer. Not all studies, however, have demonstrated an anti-angiogenic effect of flavonoids. EGCG, for example, was shown to demonstrate strong activation HIF1alpha in human breast cancer cells. A similar up-regulation of HIF-1alpha was also noted for quercetin (392). The precise cause of the disparity in HIF-1alpha and VEGF expression between flavonoids is undetermined.

Anti-inflammatory: Inflammation is an established etiological factor for several cancer types including prostate cancer. Flavonoids have been shown to possess anti-inflammatory properties. Flavonoids have been shown to inhibit phospholipase A2, cyclooxygenases, and lipoxygenases resulting in reduction of levels of pro-inflammatory mediators. These alterations have partly been accounted for by inhibition of transcription of pro-inflammatory genes. Nitric oxide, an inflammatory mediator is induced by inducible nitric oxide synthase iNOS, which has been shown to be inhibited by flavonoids such as quercetin and luteolin.

IGF-1: Elevated levels of insulin-like-growth factor 1 (IGF1) have been associated with a number of cancer types including prostate cancer. In prostate cancer, progression to androgen independent disease has been associated with increased IGF-1 levels. The IGF-1 receptor has intrinsic tyrosine kinase activity, and phosphorylates adapter protein IRS-1. This in turn activates the PI3-kinase-AKT survival pathway. Fang et al demonstrated the effect of luteolin on IGF-1 signaling. Luteolin inhibited IGF-1 induced activation of IGF-1R and AKT in prostate cancer PC-3 and DU145 cells. Inhibition of AKT by luteolin resulted in decreased phosphorylation of its downstream targets (393). Genistein treatment has also been shown to reduce IGF-1R levels. This was associated with inhibition of phosphorylation of IRS-1 and AKT (394). Similar findings were shown for EGCG in colorectal cancer cells (395).

Telomerase: Human telomerase adds telomeric repeats to the 3'-end of telomeric DNA. Telomerase is expressed in tumors but is repressed in normal cells. Ouchi et al employed the PCR-based telomeric repeat amplification protocol (TRAP) assay to examine the activity of h-TERT the catalytic subunit of telomerase. Using this assay, the telomerase activity of both LNCaP and DU145 cells was reduced by genistein. mRNA levels of h-TERT were also reduced by genistein. Several other flavonoids have demonstrated telomerase inhibition including EGCG (396), silibinin (397) and other novel polyhydroxylated flavonoids (398).

In summary, the modulation of a large number of signaling pathways is a distinctive property of flavonoids. The multi-targeting of several pathways at once by a single compound is likely to be more effective at suppression of cancer cell proliferation than single target inhibition. The key to the suitability of flavonoids as chemopreventive agents lies in the translational potential of these *in vitro* findings to the human situation. Bridging the gap to the clinical application of flavonoids requires the use of suitable animal models to study the effects and safety of flavonoids *in vivo*.

1.4.6. The Translational Potential of Flavonoids

Testing the potential of flavonoids to prevent advanced prostate cancer *in vivo* is a priority of flavonoid research. Efforts to study the role of flavonoids on PCa *in vivo* have been hampered by the lack of available animal models. Over 100 studies have examined the effect of flavonoids in animal models of carcinogenesis. Most of these studies have employed mouse models wherein tumors have been induced by carcinogen administration or tumor cells injected into immunocompromised hosts (xenograft). Neither of these models resembles the true carcinogenesis process that occurs in human tumors. In prostate cancer in humans, the prostate tumor progresses through distinct phases of prostatic intraepithelial neoplasia, adenocarcinoma

and undifferentiated cancer. This natural progression is not represented in the carcinogen and xenograft model. Another drawback of the xenograft model is that although it is suitable for testing treament agents, it is not an adequate model for chemoprevention.

One of the best available models in terms of its resemblance to the human form of prostate cancer is the *tr*ansgenic *a*denocarcinoma of the *m*ouse *p*rostate (TRAMP) model developed by Greenberg et al (399). This model was generated using a construct consisting of the minimal rat probasin promoter driving expression of the SV40 early genes (T antigen; Tag). TRAMP mice develop progressive forms of PCa with lesions ranging from mild PIN to large multinodular malignant neoplasia and metastatic spread to lymph nodes and bone (400). Mice bred on a C57Bl/6 background strain develop severe hyperplasia and adenocarcinoma by 18 weeks of age. By 24-30 weeks of age, TRAMP males develop primary prostate cancer. Metastases are common in the lymph nodes and lungs.

A common misconception with the TRAMP model is that the undifferentiated tumors that develop in a proportion of the animals are purely neuroendocrine. In contrast to the 12T-10 "LADY" model where cancers originate within the neuroendocrine compartment of the prostate, neuroendocrine differentiation in TRAMP occurs as a stochastic event correlated with progression and the loss of differentiation (401). The fact that undifferentiated TRAMP tumors express AR further supports the non-neuroendocrine origin of this phenotype since neuroendocrine cells do not express AR (402). Finally, gene expression profiling shows distinct differences between true neuroendocrine cells and undifferentiated TRAMP samples (403). Therefore, undifferentiated tumors in TRAMP are a consequence of epithelial to neuroendocrine differentiation and are not pure neuroendocrine tumors, and mimic the human occurrence of neuroendocrine differentiation in advanced prostate cancer.

The TRAMP model has been implemented to test the effects of a number of preventative agents on PCa progression(404-406). Gupta et al were the first to report a chemopreventive effect of flavonoids in this model. They administered 0.1% green tea polyphenols in drinking water from 8 weeks of age. They demonstrated a 64% reduction in prostate weight compared to control and a reduction in IGF-1 serum levels. There was also a reduction in IGF-1 and an increase in IGF-BP3 and inhibition of phosphorylated Akt. This study did not quantify tumors on a histologic basis (407). In a study using 0.06% EGCG in tap water in TRAMP, EGCG was shown to inhibit the formation of tumors at the early time-point of 12 weeks, but not at 28 weeks (408). Mentor-Marcel et al studied the chemopreventive effect of genistein in TRAMP. They demonstrated a reduction in poorly differentiated prostate cancers in mice consuming 250mg or 500 mg genestein per day, improved survival and decrease in prostate levels of pro-metastatic osteopontin transcript (404, 409). Other flavonoids tested in this model include 0.1%-1% silibinin (410) and 50μg/day of apigenin (385). In the former, there was a significant increase in the number of PINs in the silibinin group. In the apigenin study, the treatment group had lower prostate weights, no metastasis and improved survival.

In summary, the TRAMP model is currently the best available murine model of prostate cancer. This model has been employed in chemoprevention studies of various phytochemicals and has been consistent in terms of tumor formation, and incidence of lymph node and distant metastases. There is a need for further investigation of the chemopreventive effect of flavonoids in this model.

1.5. Organization of Thesis

The studies presented in this thesis were designed to investigate the role of novel flavonoids in prostate cancer chemoprevention. The mechanism of action of flavonoids was examined using prostate cancer cell lines *in vitro*, while their *in vivo* chemopreventive effect and mechanisms were examined in an autochthonous transgenic model of prostate cancer. The organization of the thesis is outlined in Fig. 1.6.

Experiments were designed to investigate the following specific aims:

1. Identify novel flavonoids with potent effects on the inhibition of prostate cancer cell proliferation and determine the modalities of cell death induced by flavonoids
2. Determine the molecular effects of flavonoids on cell cycle regulation focusing on the modulation of cell cycle gene expression by flavonoids
3. Examine the effects of select flavonoids as prostate cancer chemopreventive agents in an *in vivo* transgenic model of prostate cancer (TRAMP).

Previous investigators have demonstrated the anti-proliferative effect of soy and green tea flavonoids in prostate cancer cell lines. Fewer studies have highlighted the role of non-soy and green tea flavonoids in prostate cancer. Since over 9000 flavonoids exist in nature, we postulated that novel flavonoids in this diverse group could have more potent anti-cancer effects than the more widely studied soy and green tea flavonoids. We tested this by screening a large number of flavonoids representative of the major flavonoid subgroups in PC3 and LNCaP prostate cancer cell lines. In order to determine the prostate cancer specificity of these flavonoids, we also

employed a benign prostate epithelial (BPH-1), prostate stromal (PrSC) and breast cancer (MCF-7) cell lines. Clonogenic assays were performed to determine the effect of select flavonoids on colony formation in PC3 cells (Chapter 2).

The mechanism of action of the most potent flavonoids identified in the screening experiments was explored in several ways. Firstly the modality of cytotoxicity of the flavonoids was examined to determine the relative importance of cell cycle arrest, apoptosis/necrosis, and accelerated senescence in flavonoid action. Cells were examined by flow cytometry of bromodeoxyuridine labeled cells to determine the cell cycle arrest pattern of flavonoid treated cells. Anti-phospho Histone 3 antibody (mitosis marker) was used to enable differentiation of G2 and mitotic phase cells on flow cytometry since both contain 4N DNA content. Nuclear morphology was assessed by microscopic analysis of Hoechst 33342 stained cells to examine for features of apoptosis. Accelerated senescence was determined by senescence associated beta galactosidase activity assay (Chapter 2)

The effect of flavonoids on key cell cycle regulators was determined at the mRNA and protein level. Oligonucleotide microarray was performed to test the effect of two of the most potent flavonoids from the screening experiments, 2,2'-dihydrocychalcone (DHC) and fisetin, on cell cycle gene expression. Oligonucleotide array also enabled us to examine gene expression effects of flavonoids on other proliferative and survival pathways. Quantitative real time PCR and Western blotting were used to further validate the alterations in gene expression and to investigate alterations of key cell cycle regulators modulated by DHC and fisetin that had not been identified on microarray (Chapter 3).

Having demonstrated the effects of flavonoids *in vitro*, we sought to perform translational studies to test their effect in an *in vivo* chemoprevention model. We employed the widely used transgenic adenocarcinoma of the mouse prostate (TRAMP) model for these studies. The

chemopreventive effect of 4 flavonoids was determined by administering TRAMP mice diets supplemented with flavonoids alone or in combination for 32 weeks from weaning. Alterations in genitourinary weight and histological grade were determined. Survival, body weight, dietary consumption, histological analysis of major body organs, and complete blood count were used to assess toxicity of diets. We examined alteration in cell cycle proteins (PCNA and p27) by Immunohistochemistry. Finally, since previous studies had not examined the bioavailability of flavonoid in prostate tissue, we employed liquid chromatography-mass spectrometry (LC-MS) to quantify the level of flavonoids in TRAMP prostate, serum, and other tissues (Chapter 4).

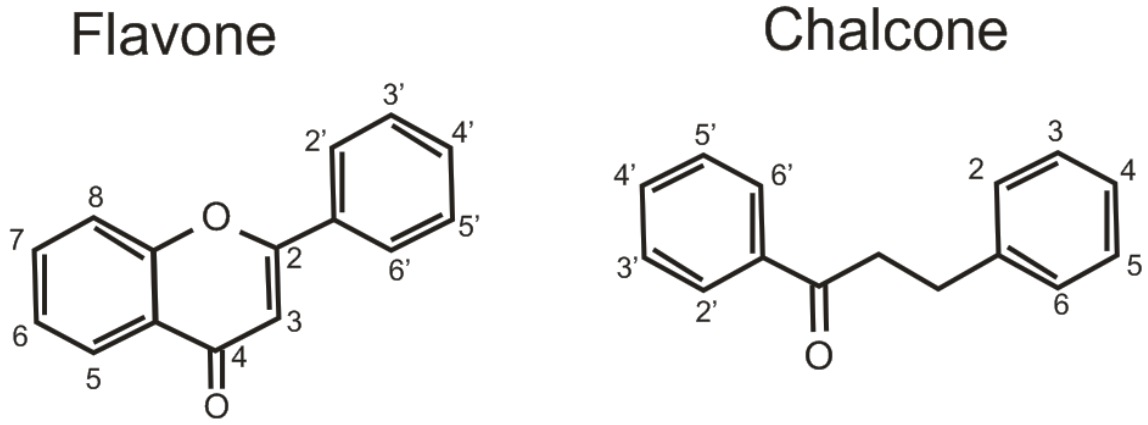
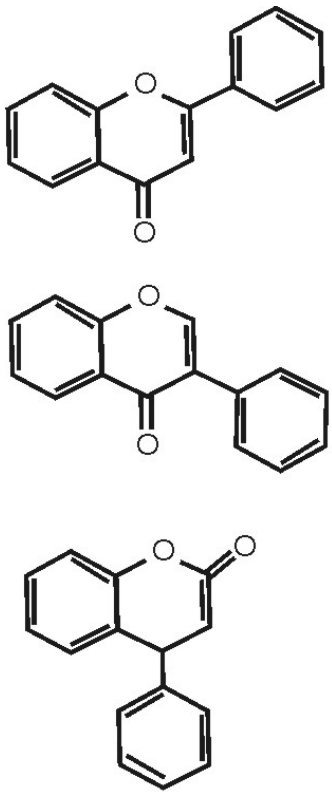


Fig 1.1. Flavonoid structure. The basic flavonoid carbon skeleton is shown. Flavonoids consist of 2 aromatic rings, the 'A' and 'B' rings, linked by a 3-carbon bridge. The connecting carbon bridge combines with an oxygen to form a heterocyclic central or C-ring for most flavonoids with the exception of chalcones in which the carbon bridge between the A and B rings is linear. The standard numbering scheme for flavonoids is shown. The numbering scheme for chalcones differs from 3-ring flavonoids in that the A ring, rather than the B ring carbons are labeled as prime.



Major Flavonoids

Isoflavonoids

Neoflavonoids

Fig 1.2. Flavonoids classification. Flavonoids are broadly classified based on the position of the B ring moiety into flavonoids, isoflavonoids or neoflavonoids.

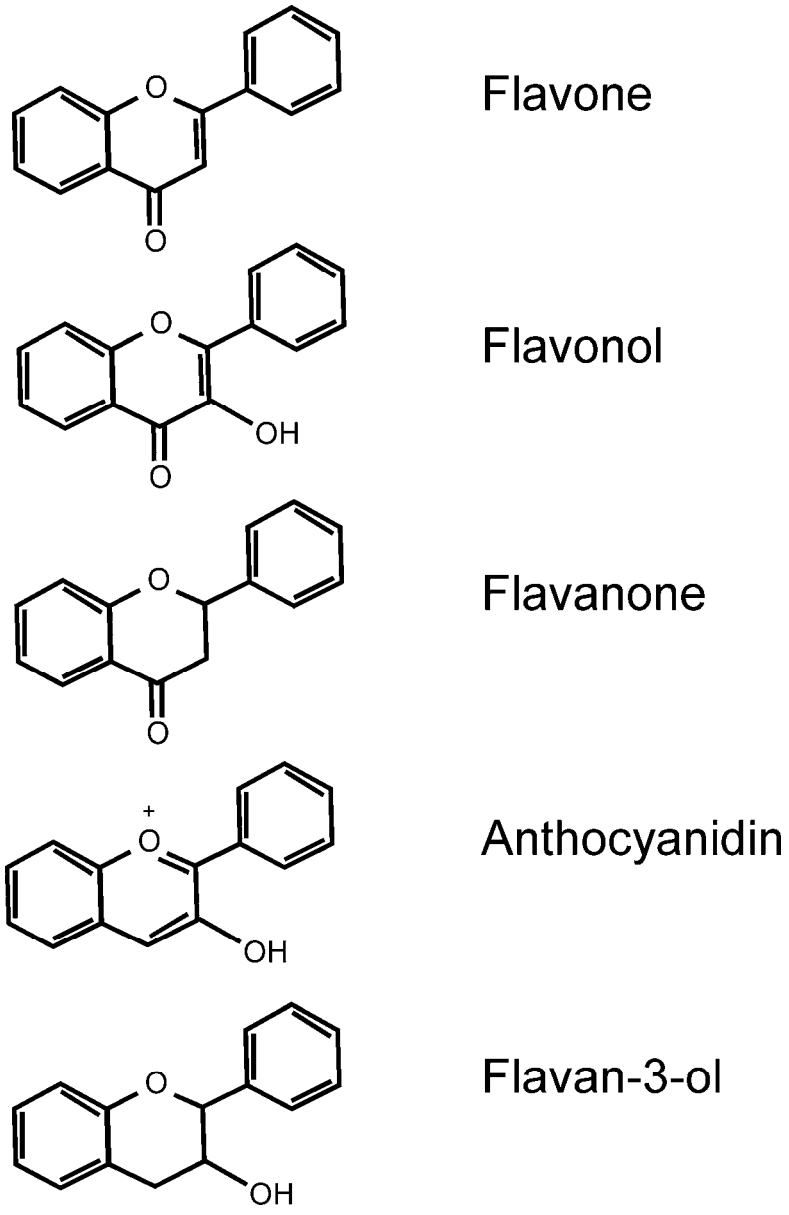


Fig 1.3. Subclasses of flavonoids. Flavonoids can be subclassified according to the structural variations of the C-ring. Flavones, flav***o***nols and flavanones possess a carbonyl (C=O) group at position 4 on the C-ring. Flav***o***nols are very similar to flavones differing only by the addition of a hydroxyl at the 3- position on the central ring. Flavanones and flav***a***n-3-ols lack a carbon-carbon double bond in the middle ring, while flavones and flavonols have one C-C double bond; and anthocyanidins possess two C-C double bonds. Anthocyanidins are unique in that the oxygen atom of the chromane ring has a positive charge.

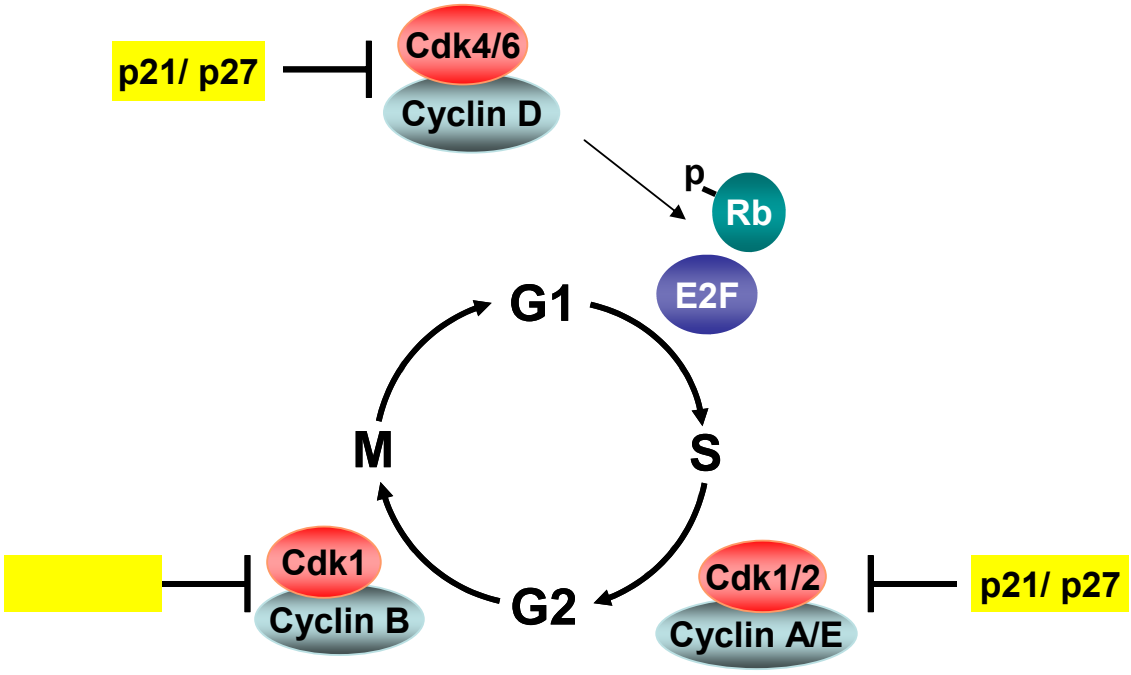


Fig 1.4. The Cell Cycle. The mammalian cell cycle is divided into synthesis (S) and mitosis (M) phases separated by two gap phases (G1 and G2). Progression through the cell cycle is regulated by cyclin dependent kinase/ cyclin complexes as shown above. In the G1 phase, cyclin D/cdk4/6 phosphorylate retinoblastoma protein (Rb) resulting in dissociation of E2F transcription factor from Rb. Transcription of E2F downstream targets results in progression from G1 to S phase. Cyclins A and E primarily regulate transition through S phase. The critical mediators of entry into mitosis from G2 are cyclin B and cdk1. p21 and p27 are pan-cyclin dependent kinase inhibitors that exert inhibitory control over the cell cycle.

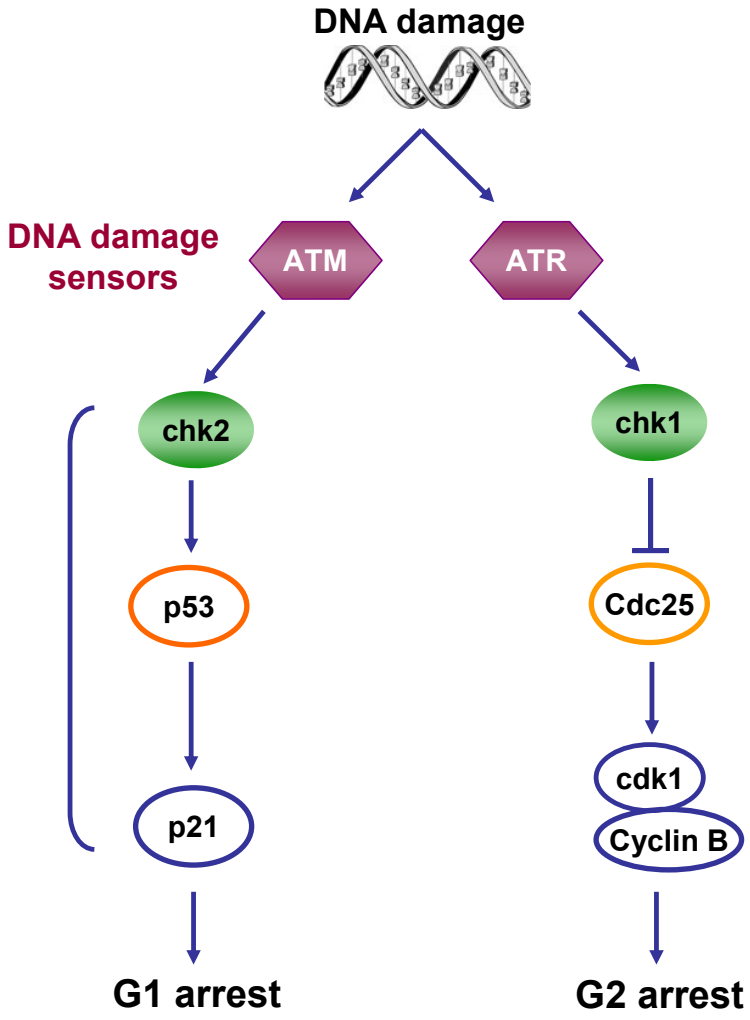


Fig 1.5. DNA damage response pathway. The DNA damage sensors ATM and ATR mediate the DNA damage checkpoint response following damage incurred by insults such as ionizing radiation (ATM) and ultraviolet radiation (ATR). Activation of ATM leads to phosphorylation of chk2 which subsequently leads to phosphorylation of tumor suppressor p53, transcription of p21 and G1 arrest. Activation of ATR results in phosphorylation of chk1 and phosphorylation of cdc25 resulting in its inactivation. This results in G2 arrest since cdc25 is necessary for removal of inhibitory phosphate group on cdk1 a pre-requisite to mitotic entry. Considerable overlap exists between the ATM and ATR pathways; however, this has not been shown for clarity.

Experimental Design

Discovery of novel anti-proliferative flavonoids

- ***In vitro*** **proliferation assay**
- **35 flavonoids**
- **Malignant and benign prostate cell lines, and non-prostate cell line**

Determine mechanism of action *in vitro* (DHC and Fisetin)

- **Clonogenic Assay**
- **Cell cycle arrest→ flow cytometry**
- **Apoptosis → Hoechst staining, morphology**
- **Gene expression alterations → oligoarray, quantitative real time PCR**
- **Cell cycle protein alterations → immunoblot**

Examine chemo-preventive effect *in vivo*

- **Dietary administration flavonoids TRAMP mice**
- **Effect of flavonoid diets on prostate cancer incidence and grade, genitourinary weight and survival**
- **Proliferation index (PCNA) and cell cycle inhibitor (p27)**
- **Serum and tissue levels of flavonoids→ mass spectrometry**

Fig. 1.6. Flowchart outlining overall design of experiments undertaken in thesis.

CHAPTER 2

The Induction of Cell Cycle Arrest and Apoptosis by Novel Anti-Proliferative Flavonoids in Prostate Cancer Cell Lines

This chapter incorporates a paper by Haddad AQ, Venkateswaran V, Viswanathan L, Teahan SJ, Fleshner NE, Klotz LH. entitled "Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines" published in Prostate Cancer and Prostatic Diseases. Prostate Cancer Prostatic Dis. 2006;9(1):68-76.

2.1 ABSTRACT

Epidemiologic studies have demonstrated an inverse association between flavonoid intake and prostate cancer (PCa) risk. The East Asian diet is very high in flavonoids and correspondingly, men in China and Japan have the lowest incidence of prostate cancer worldwide. There are thousands of different naturally occurring and synthetic flavonoids. However, only a few have been studied in PCa. Our aim was to identify novel flavonoids with anti-proliferative effect in prostate cancer cell lines, as well as determine their effects on cell cycle. We have screened a representative subgroup of 26 flavonoids for anti-proliferative effect on the human PCa (LNCaP and PC3), breast cancer (MCF-7), and normal prostate stromal cell lines (PrSC). Using a fluorescence based cell proliferation assay (Cyquant), we have identified 5 flavonoids, including the novel compounds 2,2'-dihydroxychalcone and fisetin, with anti-proliferative and cell cycle arresting properties in human PCa in vitro. Most of the flavonoids tested exerted anti-proliferative effect at lower doses in the PCa cell lines compared to the non-PCa cells. Flow cytometry was used as a means to determine the effects on cell cycle. PC3 cells were arrested in G2 phase by flavonoids. LNCaP cells demonstrated different cell cycle profiles,. Further studies are warranted to determine the molecular mechanism of action of DHC and fisetin in prostate cancer, and to establish their effectiveness *in vivo*.

2.2 INTRODUCTION

Diets rich in flavonoids have been associated with reduced incidence and mortality of prostate cancer (PCa). The lowest incidence of prostate cancer worldwide is seen in populations consuming the largest amount of flavonoids(411). In East Asian countries (China and Japan),

diets are up to 100 times more abundant in flavonoids than in the West, due in part to the consumption of soy and green tea(412, 413). Correspondingly, the incidence of PCa in China and Japan is 60-80 fold lower than in North America(414). Studies on Japanese migrants to the United States have shown that migrants born in Japan and living in the United States have a higher incidence of PCa compared to men living in Japan(16, 415). The incidence rates for Japanese Americans born in the United States increases further, approaching that of American white men. Although these studies are not definitive, they emphasize the importance of environmental, lifestyle and dietary factors on PCa incidence. A number of case-control studies have correlated increased flavonoid intake with a reduced incidence of a number of malignancies including prostate cancer(173, 175, 411, 416, 417).

Flavonoids comprise over 4000 structurally related polyphenols (150), which are ubiquitous in plants, and ingested to varying degrees in the diet. The estimated average daily intake of flavonoids is up to 1 gram (164). This by far exceeds the intake of other antioxidants such as vitamin E, and highlights the potential importance of flavonoids in the diet. Flavonoids have been shown to possess a wide range of biological activity , including antioxidant (greater than vitamin C) (418), anti-inflammatory, anti-thrombogenic, and anti-angiogenic activity (419). The anti-cancer properties of flavonoids have been demonstrated in a variety of cell types *in vitro* and *in vivo* (420). Despite the large number of flavonoids, studies have focused only on a select few. The flavonoids most intensely studied in PCa to date are the soy isoflavones (genestein/ daidzein) (421, 422), the green tea catechins (EGCG-epigallocatechin-3-gallate) (423, 424) and the milk thistle flavonones (silibinin/ silymarin) (425, 426). Little is known of the biological effect of most other flavonoids (427, 428).

In an attempt to identify novel flavonoids with growth arresting properties in PCa cells, we have screened a number of compounds from each of the major flavonoid sub-groups (Table

2.1). We have examined their anti-proliferative effect on the prostate cancer cell lines PC3 (androgen independent) and LNCaP (androgen dependent), MCF-7 breast cancer cell line and a non-malignant prostate stromal cell line (PrSC). We have identified a number of novel flavonoids with anti-proliferative and cell cycle effects in human PCa cells *in vitro*. The compound identified with the greatest anti-proliferative effect was the synthetic flavonoid 2,2'-dihydroxychalcone (DHC).

2.3 MATERIALS AND METHODS

Chemicals and Antibodies

The flavonoids which have been included in this study are presented in Table 2.1. Quercetin, pinostrobin, kaempferol, pelargonidin, galangin, formononetin, prunetin, 5-methoxyflavone, acacetin, morin, 6-aminoflavone, 7,8-benzoflavone, epigallocatechin and epicatechin gallate were purchased from Sigma Aldrich (St. Louis, MO). All other flavonoids were procured from Indofine Chemical Co. (Hillsborough, NJ). Green tea catechin-epigallocatechin (EGC) was dissolved in water. All other flavonoids were dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 100mM. Working standards were made up in serum containing media. The final concentration of DMSO in culture did not exceed 0.2%. Flavonoids that were poorly soluble in DMSO were not studied further. These included the flavonoids prunetin, acacetin, formononetin, 7,8 benzoflavone, diosmin, and karanjin.

ß-Actin mouse monoclonal was purchased from Sigma Laboratories (St.Louis, MO). Anti-PARP (#9546) was purchased from Cell Signaling (Danvers, MA).

Tissue Culture

The human prostate cancer cell lines, LNCaP (mutated androgen receptor, P53 wild-type), PC3 (androgen receptor null, p53 null), and the estrogen receptor positive breast cancer cell line MCF-7 were obtained from the American Type Tissue Collection (ATCC), Rockville, MD. The non-malignant prostate stromal cell line, PrSC, was obtained from Cambrex, NJ. LNCaP cells were cultured in RPMI 1640 medium (Gibco, New York) supplemented with 10% foetal bovine serum (FBS) and 100IU/ml penicillin and 100µg/ml streptomycin. PC3 cells were cultured in DMEM/F12 medium with 10% FBS and antibiotics. MCF-7 cells were cultured in IMEM media supplemented with 5% FBS, antibiotics, and insulin. PrSC medium consisted of stromal cell basal media, and the manufacturer's growth factor supplements (Cambrex, NJ). All cells were cultured at 37ºC with 5% CO_2.

Cell Proliferation Assay

Proliferation was assessed using the CyQuant cell proliferation assay (Molecular Probes, OR). In this assay, the proprietary CyQuant dye binds to DNA, and the fluorescence emitted by the dye is linearly proportional to the number of cells in the well. Cells were plated in 96 well black fluorescence micro-titre plates, at a density of 4000 cells/well. 24 hours after plating, triplicate wells were treated with the appropriate flavonoid at a concentration of 10-150 µM. Control wells were treated with vehicle alone (DMSO 0.2%). After 72 hours of treatment at 37ºC, the media was discarded, and the plates frozen at -80ºC until use. On the day of the analysis, the plates with the adherent cells were thawed and incubated with the CyQuant dye for 5 minutes in the dark. Fluorescence was measured on a FL600 fluorescence micro-plate reader (Bio-Tek, VT) with filters set at 480nm excitation and 520nm emission. The IC_{50} for each flavonoid was

determined in the 4 cell lines tested (Tables 2 & 3). Each experiment was performed independently at least 3 times.

Clonogenic Assays

The effect of flavonoids on colony-forming ability was performed by clonogenic assay. 250 PC3 cells were plated in 6 well plates with 3ml of standard media. The experiment was performed in triplicate wells. Cells were allowed to attach overnight. Media was then replaced with flavonoid containing media. Flavonoid treatment was removed after 24 hours. Cells were gently washed in PBS and fresh media added. Cells were allowed to grow for 2 weeks without subsequent media changes. After 2 weeks of incubation, media was removed, cells washed gently in PBS and fixed in 4% paraformaldehyde for 15 minutes. Cells were washed again and stained with 0.05% Crystal violet solution. Colonies consisting of greater than 50 cells were counted in each treatment well.

Senescence Beta Associated Galactosidase Assay

Histochemical detection of beta-galactosidase activity at pH 6 was performed as a means to determine the proportion of cells undergoing cellular senescence. At pH 6, beta-galactosidase activity can only be detected in senescent cells. After treatment with DMSO or flavonoid for 72 hours, cells were washed in PBS and fixed in 4% paraformaldehyde for 15 minutes. Cells were then washed again, and incubated with the staining solution in the Senescence Beta Galactosidase staining kit overnight at 37 ^{o}C (Cell Signalling, Danvers, MA). Cells were then viewed under light microscope for development of blue color. The percentage of cells with positive staining was counted in a field of 200 cells. As a positive control, the assay was performed using staining

solution with pH adjusted to 4. Under these acidic conditions, activity of beta-galactosidase can be detected in all cells.

Hoechst staining

Percent apoptosis in flavonoid treated cells was determined by examining morphological alterations typical of apoptosis including cellular shrinkage, chromatin condensation, and nuclear fragmentation. Nuclear morphology was assessed by fluorescence microscope analysis of cells stained with Hoechst 33342. PC3 and LNCaP cells were plated in 6 well dishes. After 24 of adherence, cells were treated with either DMSO or flavonoid for 72 hours. After 72 hours floating cells were collected and adherent cells collected by trypsinization, washed in PBS and fixed in 4% paraformaldehyde for 15 minutes. Cells were then washed and incubated at room temperature with 1.2µg/ml of Hoechst 33342 for 20 minutes. Cells were then resuspended in 20ul PBS and spread onto glass slides for visualization by fluorescence microscopy using the DAPI filter set.

Flow Cytometry and Cell Cycle Analysis

Cell cycle arrest pattern and S phase enumeration were determined by flow cytometry on cells labelled with anti-BrdU FITC and propidium iodide. Asynchronously growing cells ($5x10^5$ cells/ plate) were plated in 10cm dishes and treated for 72 hours with the flavonoid at the IC_{50} concentration as determined earlier. Control plates were treated with vehicle alone (DMSO 0.2%). The cells were pulse labelled with bromodeoxyuridine (BrdU) for 2 hours prior to harvesting. As a negative control, a no-BrdU control was also included. Cells were trypsinized, fixed in ice cold 70% ethanol and stored at -20ºC until further analysis. Cells were then washed in buffer (PBS and 0.5% Tween20) and treated with 2N HCl for 20 minutes to expose labelled

DNA. Cells were incubated for one hour on ice with anti-BrdU conjugated fluorescein isothiocyanate (Becton Dickenson, San Jose, California). Cells were washed, centrifuged, and re-suspended in 10ug/ml propidium iodide, and allowed to incubate for 30 minutes on ice. Samples were filtered through a nylon mesh and cell cycle analysis performed on the FACSCalibur flow cytometer using the Cell Quest Pro software package (Becton Dickenson, California).

Viability Assay

Viability assays were performed in unfixed cells treated with DMSO control or flavonoid for 72 hours. Treated cells were trypsinized to detach and cells collected. The cell pellet was stained with 1mg/ml propidium iodide for 30 minutes and cells examined by flow cytometry as above. Cells incorporating propidium iodide were deemed to be non-viable.

Immunoblotting

LNCaP and PC3 cells were treated with 0.1% DMSO, 15-50μM DHC or 25-50μM fisetin for 48hours. Whole cell lysates were prepared in ice-cold NP40 lysis buffer (0.1% NP40, 50mM Tris pH 7.5, 150mM NaCl, and the Roche Complete Mini protease inhibitor cocktail). Lysates were sonicated and clarified by centrifugation. Proteins were quantified by Bradford analysis and 40 μg protein/lane resolved by electrophoresis on 10-12% SDS-PAGE. Resolved proteins were transferred onto PVDF membrane (Millipore, Billerica, MA) by semi-dry transfer in buffer (Tris 50mM, Glycine 40mM, 0.01% SDS, and 4% methanol) at 25volts for 30 minutes using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad, USA). Membranes were probed with primary antibody followed by HRP conjugated secondary antibody and detection by ECL chemi-illuminescence. All primary antibodies were used at concentrations of 1:200, except ß-Actin 1:10,000. Secondary antibodies were used at dilutions of 1:40,000.

Statistical Analysis

Cell proliferation was determined by Cyquant fluorescence and expressed as a percentage of untreated control. The percentage value for each treatment was obtained from 3 replicate experiments. The mean of the 3 experiments was plotted on a concentration-response curve. A best fit regression curve was determined by polynomial third order equation, and performed individually for each flavonoid (GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, California USA). We next determined (from the regression curves) the concentration of flavonoid at which there was a 50% reduction in cell number compared to the control (IC_{50}).

The unpaired t-test (two-tailed) was performed to determine statistical significance of S-phase alterations in flavonoid treated and control cells (GraphPad Prism version 4.03 for Windows).

2.4. RESULTS

Flavonoids exert an anti-proliferative effect on human prostate cancer cells *in vitro.*

The anti-proliferative effects of flavonoids were assessed in LNCaP and PC3 cells *in vitro*. A subset of flavonoids with anti-proliferative effect were also tested in the MCF-7 breast cancer cell line, and the non-malignant prostate stromal cell line PrSC. Proliferation studies were initially performed by the more commonly used MTT assay. As many flavonoids are coloured when in solution, the MTT assay was unsuitable as this interfered with the colorimetric nature of this assay. In addition, flavonoids are known to reduce the tetrazolium in the MTT solution, even in the absence of cells (429). The CyQUANT assay overcame these limitations and was used for the high throughput screening of flavonoids in all further experiments.

Concentration-response curves were generated for each flavonoid. We determined the concentration at which flavonoids caused 50% growth inhibition compared to control (IC_{50}) (Table 2.2). The five flavonoids with the greatest anti-proliferative effect in LNCaP were 2,2'-dihyroxychalcone, baicalein, isoliquiritigenin (ISLQ), luteolin and quercetin. In PC3, the most potent compounds were DHC, luteolin, fisetin, quercetin and ISLQ. DHC caused growth inhibition at low doses in all four cell lines tested, the lowest IC_{50} observed in PC3 and LNCaP (10.26 & 10.8 μM respectively), and the highest in MCF7 (23 μM) (Table 2.2). Fisetin had an IC_{50} of 22.65 μM in LNCaP and 32.5 μM in PC3. 50% reduction in cell number at the maximum concentration was not achieved in MCF-7 and PrSC. Similarly, ISLQ had greater anti-proliferative effect in PCa compared to non- PCa cell lines (Table 2.2). Luteolin demonstrated anti-proliferative activity in LNCaP (IC_{50} 18.22μM), PC3 (IC_{50} 28.84 μM) and MCF-7 cells (IC_{50} 29.13 μM) and to lesser degree on PrSC (IC_{50} 68.37 μM). Quercetin had IC_{50} of 33.41 μM and 19.44 μM in PC3 and LNCaP, with 50% reduction in cell numbers not reached in the prostatic stromal or breast cancer cell lines.

Other flavonoids shown to inhibit proliferation included 5-methoxyflavone, baicalin, baicalein, chrysin and kaempferol as detailed in Table 2.2. A greater effect on proliferation of LNCaP than PC3 cells were observed with 5-methoxyflavone, (IC_{50} 25.22 and 97.31 μM respectively). Baicalein had a lower IC_{50} than the glycosylated baicalin when tested in LNCaP, while baicalin seemed to be more potent in PC3. Kaempferol and chrysin had an IC_{50} above 40 μM in LNCaP and PC3 cells, a concentration unlikely to be achieved physiologically. Galangin inhibited proliferation in LNCaP only at very high concentrations, and the remainder of the flavonoids (6-aminoflavone, EGC, geraldol, gossypin, morin, myricetin, pinostrobin, and pelargonidin) did not inhibit proliferation at the doses tested.

The cytotoxic effect of DHC and fisetin were examined in clonogenic assay. PC3 cells were treated with flavonoids for 24 hours, media removed and fresh media added. Cells were allowed to proliferate for 2 weeks. The reduction in colonies by both DHC and fisetin occurred at lower doses compared to the IC_{50} determined in the Cyquant assay (Fig. 2.1).

Flavonoids cause alteration in cell cycle regulation in human prostate cancer cells *in vitro.*

Having identified the flavonoids that possessed greatest effect on proliferation, we next examined the effect of these compounds on the alterations in cell cycle profiles in LNCaP and PC3 cells. Cells were treated with flavonoids for 72 hours *in vitro*, and flow cytometry performed with dual labelling of cells with PI and anti-BrdU (Fig. 2.2 & 2.3). All five flavonoids (DHC, fisetin, ISLQ, luteolin, and quercetin) caused cell cycle arrest in PC3 and LNCaP cells. Interestingly, all of these flavonoids caused a similar pattern of cell cycle arrest, (G2/M) in PC3 cells (Fig 2.2), while LNCaP cells were arrested in both G1/S and G2/M (Fig.2.3). All of the flavonoids tested caused a reduction in the proportion of cells in the synthesizing (S-phase) of the cell cycle in both cell lines (Table 2.3). Time course studies performed demonstrated that the effect of flavonoid treatment on the cell cycle was observed as early as 24 hours of treatment (data not shown).

There was a 3 fold increase in cells in the G2/M phase in PC3 cells with flavonoid treatment compared to control cultures. Fisetin caused the greatest accumulation of cells in G2/M, with 66.5% (± 12.6) of cells in G2/M compared to 13.0% (± 2.8) in the control. A corresponding reduction of S and G1 phase cells was seen in PC3 cells. In terms of S-phase reduction in PC3 cells, the greatest effect was seen with quercetin, fisetin and luteolin (81.7%, 68.3% and 61.2% reduction respectively). DHC, the flavonoid with the greatest anti-proliferative

effect, caused a 53.4% reduction in S-phase, and a 3-fold rise in G2/M phase cells in PC3 (Fig.2.2). ISLQ caused a smaller reduction in S- phase cell numbers (42.9%) (Table 2.3).

A different pattern of cell cycle arrest was observed in LNCaP (Fig 2.3). Although LNCaP cells treated with flavonoid demonstrated an increase in G2/M phase cell numbers, indicative of a G2/M arrest, the percentage of cells in G1 phase did not decrease. This suggests that cells were being partially arrested in G1 phase. Compared to PC3, the increase in the proportion of G2/M cells was smaller in LNCaP, with most flavonoids causing around a 2-fold increase in G2/M phase distribution. However, as in PC3, all flavonoids caused a reduction in percentage of cells in S-phase in LNCaP cells. Fisetin, at a concentration of 25 μM caused the greatest reduction of cells in S-phase in LNCaP cells (76.6% reduction) with a concomitant rise in the proportion of cells in G2 phase (19.4% ± 4.9 compared to 8.7% ± 0.63 in control) and a non-significant increase in G1 phase (71.2% ± 6.2 in fisetin vs 69% ± 4.3 in control) (Table 2.3). LNCaP cells treated with, ISLQ, quercetin, luteolin and DHC also caused reductions in the percentage of cells in the S-phase compared to vehicle only control (71.1%, 63.2%, 57.3% and 47.2% respectively). In general, the proportion of cells in S-phase in flavonoid treated groups was significantly lower than the DMSO control, with few exceptions (Table 2.3).

Lack of induction of senescence phenotype by DHC and fisetin

In order to determine whether flavonoids induce cellular senescence in prostate cancer cells, we examined for act for beta-galactosidase activity at pH 6. At this pH, beta-galactosidase activity can only be detected in cells undergoing senescence. At the more acidic pH 4, beta galactosidase activity can be detected in all cells, and this was our positive control (Fig 2.4.A). There was no detectable beta-galactosidase activity in PC3 and LNCaP cells treated for 72 hours with DHC or fisetin (Fig 2.4.B). Thus, flavonoids did not induce cellular senescence.

DHC and fisetin induce apoptosis in prostate cancer cell lines

Determination of apoptosis by assessment of morphological changes remains the gold standard for determination of apoptosis. We examined morphology by Hoechst 33352 staining (nuclear dye) and fluorescence microscopy. Cells with typical features of apoptosis, including cellular shrinkage, chromatin condensation and nuclear fragmentation were counted, and the percentage apoptosis (apoptotic index) was determined from a field of 200 cells (Fig 2.5.A and 2.5.B).

Cleavage of PARP, a target of caspases, occurs in the final stages of apoptosis, and detection of cleaved PARP constitutes further eveidence for the occurrence of apoptosis. We determined PARP cleavage by Western blotting in cells treated with DHC and fisetin for 48 hours. PARP cleavage was observed in both DHC and fisetin treated prostate cancer cells (Fig 2.6).

Loss of viability in PC3 cells treated with DHC and fisetin

We performed **c**ell viability analysis in PC3 cells treated with varying doses of DHC and fisetin by staining unfixed cells with propidium iodide (PI) and analysing on flow cytometry. PI only stains those cells with compromised cell membranes as is typically seen in cells that undergo necrosis or in the late stages of apoptosis. Cells incorporating PI were therefore deemed non-viable. DHC and fisetin caused a dose-dependent increase in the proportion of cells incorporating PI (Fig 2.7).

2.5. DISCUSSION

In this study, we have screened a diverse group of flavonoids for their anti-proliferative effect on prostate cancer, breast cancer and normal prostate stromal cells *in vitro*. Most of the flavonoids screened are novel compounds as they that have not been previously evaluated. The flavonoids screened are representative of the major subgroups of flavonoids including flavones,

flavonols, flavonones, flavan-3-ols, anthocyanidins and chalcones. Both glycosylated flavonoids and flavonoid aglycones were tested. We have identified a number of flavonoids which cause growth arrest at low concentrations in PCa, and appear to be less effective on non-prostate cancer cell lines (MCF-7 and PrSC). We have also demonstrated that these flavonoids caused cell cycle arrest (G1/S and G2/M), with a reduction in the number of cells in the S-phase (81% reduction) (Figs 1&2).

As evident from Table 2.2, the flavonoid with the lowest IC_{50} concentration was DHC. This is a synthetic flavonoid belonging to the chalcone subgroup of flavonoids that are precursors in the flavone synthesis pathway in plants (430). The growth inhibitory effect of DHC was observed as early as 24 hours following treatment (data not shown). Whereas most of the flavonoids tested were minimally cytotoxic to the PrSC and MCF7 cell lines, DHC was unique in that it was effective at low concentrations on PrSC and MCF7 cells (IC_{50} 17.47μM and 22.99μM respectively). The apparent resistance of MCF-7 and PrSC cells to flavonoids may be a true effect, however, this is difficult to ascertain since all cell lines have been cultured in cell specific media, and differences in growth media differences may account for some of the observations.

Other flavonoids identified with anti-proliferative effects were fisetin, ISLQ, luteolin, quercetin, baicalein and 5-methoxyflavone (Table 2.2). Fisetin, isolated from the bark of *Rhus cotinus* (431), was anti-proliferative in both PC3 and LNCaP cells. Fisetin was less cytotoxic in MCF-7 and PrSC cells (IC_{50} >80μM). ISLQ is a chalcone found in liquorice root and is a constituent of a some herbal remedies (432). Luteolin is a flavone widely distributed in nature, and found in sources such as parsley, artichoke, and celery (163). Quercetin is the most prevalent flavonoid in the Western diet (433), and is ubiquitous in plants. It is the most widely studied flavonoid and has been shown to exert anti-proliferative effect on a number of cell lines (434-436). ISLQ, luteolin and quercetin appeared to have greater effect on proliferation in LNCaP than

in PC3 cells. In addition, two other flavonoids, 5-methoxyflavone (synthetic) and baicalein (component of PCSPES herbal remedy) had low IC_{50} in LNCaP but not PC3 (Table 2.2). A number of differences between PC3 and LNCaP cells may account for this difference in sensitivity. PC3 is a more aggressive cell line, and is null for p53 and androgen receptor (AR). LNCaP, a much less aggressive cell line possesses a wild type p53 and a mutated AR. ISLQ, luteolin and quercetin have a less anti-proliferative effect on MCF-7 and PrSC cells. Luteolin has a low IC_{50} in the MCF-7 breast cancer cell line, a property which merits further investigation (Table 2.2).

Despite the close similarities in the structure of the flavonoids, we observed a marked difference in their anti-proliferative activities (Table 2.1). For example, quercetin and gossypin differ by a single hydroxyl substitution at the C-8 position. While quercetin caused growth arrest in LNCaP and PC3 cells, gossypin had no such action despite high concentrations. Microscopically, at the highest doses tested (100-150 μM) some of the flavonoids such as EGC caused minimal cell toxicity leading to a high rate of cell survival. Treatment at high concentration with the more toxic flavonoids such as DHC, resulted in morphological changes (viz., rounding and cell detachment). However, even with doses as high as 500μM of DHC, a small number of cells (500-600) remained adherent as detected by the CyQuant assay (data not shown).

Several flavonoids such as myricetin, pelargonidin and epigallocatechin (EGC) previously identified in epidemiological studies (as being linked to reduced incidence of PCa) (424, 437-442) did not display anti-proliferative activity in our experiments. These studies however, were based on retrospective dietary assessments and numerous problems arise while attempting to examine the effect of flavonoids by this methodology. The *in vivo* anti-cancer properties of flavonoids cannot be accurately determined from epidemiologic studies partly due to the

confounding effects of the hundreds of other flavonoids present in the diet. Flavonoids examined in previous *in vitro* studies include EGC (IC_{50} 88μM in DU145 prostate cancer cell line), and ISLQ (IC_{50} 13μM in DU145). In our study, both of these compounds did not inhibit cell growth (50% growth inhibition) in LNCaP or PC3 cells, at concentration up to 150μM.

In previous studies flavonoids have been shown to cause alterations in cell cycle regulation in a number of cell lines. We performed FACS analysis to confirm the alterations in cell cycle regulatory properties of the key flavonoids identified in this study (Fig. 2.2, 2.3). Results indicate that DHC, fisetin, ISLQ, luteolin and quercetin all caused a G2/M arrest in both LNCaP and PC3 cells. The percentage of cells in G2 increased with corresponding decrease in the percentage of cells in the S phase cells for all flavonoids in both LNCaP and PC3cells. In PC3 cells the percentage of cells in the G1 phase decreased, while in LNCaP, the proportion of cells in G1 remained unchanged compared to the control. The p53 status may partly explain the different cell cycle arrest pattern observed between cell lines. As mentioned earlier, PC3 cells are p53 null, and as a result may be defective in G1 checkpoint control, explaining the lack of a G1 arrest in this cell line. This hypothesis remains to be proven. Previous studies have shown that quercetin and ISLQ cause cell cycle arrest in PCa cell lines(353, 363, 443). However, there have been no reports to date on the cell cycle regulatory effects of DHC, fisetin and luteolin.

The two flavonoids DHC and fisetin, were selected for further studies examining the mechanism of cytotoxicity induced by flavonoids. We demonstrated that both compounds had profound effects of colony formation in clonogenic assay (Fig 2.1), and caused a dose-dependent decrease in viability of prostate cancer cells as determined by propidium iodide incorporation (Fig. 2.7). The pathway of senescence was not induced by DHC or fisetin as determined by the lack of expression of senescence associated beta-galactosidase (Fig 2.4.A and 2.4.B). Apoptosis was observed in prostate cancer cells as evidenced by typical changes in cellular and nuclear

morphology (Fig. 2.5.A and 2.5.B) and PARP cleavage (Fig 2.6). Maximal apoptosis occurred at higher flavonoid doses compared to the doses required to induce cell cycle arrest. Thus, at the IC_{50} values determined in this study, flavonoids exert cell cycle arrest without senescence induction, and at higher doses induce programmed cell death.

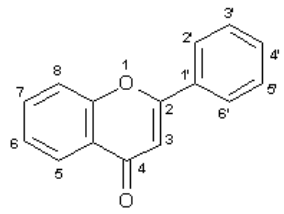
The precise mechanism by which the flavonoids identified in this study exert their action remains to be determined. Flavonoids have also been shown to alter a number of key proteins implicated in growth and differentiation. These include induction of cyclin dependent kinase (cdk) inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ (359, 363), inhibition of phosphorylation of retinoblastoma (Rb) protein (444), decrease in levels of cyclins B, D and E and cdk 2,4 and 6 (356, 377, 445), induction of apoptosis (364, 446), inhibition of topoisomerase II, and alterations to the MAPkinase pathway (447). Genestein, a soy isoflavone, enhances the anti-proliferative effects of vitamin D by up-regulating vitamin D receptor and $p21^{Cip1/WAF1}$ protein levels (448). This synergistic effect with vitamin D may contribute to the reduced incidence of prostate cancer associated with diets that are rich in both vitamin D and flavonoids, such as the traditional Asian diet. In the present study, anti-proliferative effects were seen in both AR dependent and independent human prostate cancer cell lines, suggesting that the androgen receptor is not a critical component for mediating the growth arresting properties of flavonoids.

Preliminary animal studies investigating the *in vivo* effects of flavonoids have been promising, with a number of flavonoids having demonstrated anti-PCa activity(404),(407). For example, TRAMP mice (transgenic adenocarcinoma of the mouse prostate) fed genestein showed a reduction in the incidence prostate carcinomas(404). Gupta et al. have shown that TRAMP mice placed on a green tea catechin rich extract had reduced liver metastases(407). Other investigators have confirmed the effect of selected flavonoids in PCa employing xenograft models(426, 449, 450). In these experiments, there have been no reports of flavonoid induced

systemic toxicity (a desirable property of an anti-cancer agent). Since we have shown DHC, fisetin, luteolin and quercetin to have greater *in vitro* activity than genestein and catechins, we feel it would be necessary to examine the effect of these compounds *in vivo*. DHC and fisetin have demonstrated no toxicity at the concentrations used in this experiment (12-14μM) when tested on isolated rat hepatocytes(451, 452), and minimal toxicity would be expected *in vivo*.

Flavonoids account for many of the beneficial effects observed with diets rich in fruits and vegetables. We have identified several anti-proliferative flavonoids that cause cell cycle arrest in prostate cancer *in vitro*. Further studies are underway to explore the molecular mechanisms of the novel flavonoids identified in this study, and to determine their properties *in vivo*.

Table 2.1. Chemical Composition of the Flavonoids Tested.

Subgroup	Flavonoids	Flavonoid Side Chain Position										
												
		2'	**3'**	**4'**	**5'**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flavones	Acacetin			OMe*					OH		OH	
	6-Aminoflavone									NH_2†		
	Baicalein								OH	OH	OH	
	Baicalin								OH	OH	O-glu‡	
	7,8-Benzoflavone										benz§	benz§
	Chrysin								OH		OH	
	Diosmin		OH	OMe					OH		O-glu	
	Karanjin						OMe				furan‖	
	Luteolin		OH	OH					OH		OH	
	5-Methoxyflavone								OMe			
	Primuletin								OH			
Flavonols	Fisetin		OH	OH			OH				OH	
	Galangin						OH		OH		OH	
	Geraldol		OMe	OH			OH				OH	
	Gossypin		OH	OH			OH		OH		OH	OH
	Kaempferol			OH			OH		OH		OH	
	Morin	OH		OH			OH		OH		OH	
	Myricetin		OH	OH	OH		OH		OH		OH	
	Quercetin		OH	OH			OH		OH		OH	
Isoflavonoids	Formononetin			OMe							OH	
	Prunetin			OH					OH		OMe	
Chalcones	DHC	OH				OH						
	Isoliquiritigenin	OH		OH				OH				
Flavonones	Pinostrobin								OH		OMe	
Catechins	Epigallocatechin		OH	OH	OH		OH		OH		OH	
Anthocyanidins	Pelargonidine			OH			OH		OH		OH	

*Methoxyl group, † amino group, ‡ glucoronide, § benzene ring, ‖ furanone.

Table 2.2. Anti-proliferative effect of flavonoids. IC_{50} (in μM) for flavonoids in human prostate cancer (PC3 and LNCaP), breast cancer (MCF-7) and prostate stromal cells (PrSC).

Full name	Common name or abbreviation	LNCaP	PC3	MCF7	PrSC	BPH-1
2,2'-dihydroxychalcone	DHC	10.8	10.26	22.99	17.47	37.313
2',4,4',6'-tetramethoxychalcone		16.064	41.68			>50
5,6,7-trihydroxyflavone	Baicalein	16.78	64.23	>80	>80	
4,2',4'-trihydroxychalcone	Isoliquiritigenin	17.88	34.43	67.17	45.88	>50
5,7,3',4'-tetrahydroxyflavone	Luteolin	18.22	28.84	29.13	68.37	>50
3,5,7,3',4'-Pentahydroxyflavone dihydrate	Quercetin	19.44	33.41	>80	>80	>50
3,7,3',4'-tetrahydroxyflavone	Fisetin	22.65	32.5	>80	>80	>50
5,3'-dimethoxyflavone		24.75	>50			>50
5-methoxyflavone		25.22	97.31	>80	>80	
5,7,3',4'-tetrahydroxyflavanone	Eriodictol	43.276	>50			>50
3,5,7,4'-tetrahydroxyflavone	Kaempferol	52.24	41.98			
Baicalein 7-O-glucuronide	Baicalin	52.85	51.58	>80	>80	
5,7-dihydroxyflavone	Chrysin	56.81	>100			>50
3,5,7-trihydroxyflavone	Galangin	94.92	>100			
7,8-benzoflavone		98.04	>100			
6-aminoflavone		>100	>100			
Epigallocatechin	Epigallocatechin	>100	>100			
3,7,4'-Trihydroxy-3'-methoxyflavone	Geraldol	>100	>100			
3,3',4',5,7,8-hexahydroxyflavone 8-glucoside	Gossypin	>100	>100			
3,5,7,2',4'-pentahydroxyflavone	Morin	>100	>100			
3,5,7,3',4',5'-hexahydroxyflavone	Myricetin	>100	>100			
3,5,7,4'-Tetrahydroxyflavylium chloride	Pelargonidin	>100	>100			
5-hydroxy-7-methoxyflavanone	Pinostrobin	>100	>100			
2'-hydroxy-2,4,4',5,6'-pentameyhoxy-chalcone		>50				
5,7,3'-trihydroxy-4'-methoxyflavanone	Hesperetin	>50				
3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside	Diosmin	>50				
5-hydroxyflavone		>50				
6,2',4'-trimethoxyflavanone		>50				
3-methoxy-furano-(2',3':4',3) flavone	Karanjin	>50				
3-hydroxy-7,3',4',5-tetramethoxy-flavone		>50				
2'-hydroxy-2,4,4',5,6'-pentamethoxy-chalcone		Nsolub				
3-hydroxy-7,3',4',5-tetramethoxy-flavone		NSolub				
4',5-dihydroxy-7-methoxyisoflavone	Prunetin	Nsolub				
5,7-Dihydroxy-4'-methoxyflavone	Acacetin	Nsolub				
7-Hydroxy-4'-methoxyisoflavone	Formononetin	Nsolub				

Table 2.3. S-phase reduction. The percentage of cells in S-phase (± standard deviation) –mean of 3 experiments in control and flavonoid treated cells at 72 hours. The percentage reduction in flavonoid treated compared to vehicle treated control cells was calculated and unpaired t-test performed to compare each treatment with the control.

* $p<0.05$, † $p<0.1$

Flavonoid	LNCaP	% reduction	PC3	% reduction
Vehicle treated control	22.08 ± 4.93*		21.81 ± 5.50	
DHC	5.16 ± 3.08 *	47.18	10.16 ± 6.98†	53.44
Fisetin	9.43 ± 1.18 *	76.63	6.93 ± 3.85 *	68.25
Isoliquiritigenin	6.39 ± 0.27 *	71.07	12.45 ± 6.49	42.93
Luteolin	11.66 ± 1.31 †	57.31	8.475 ± 1.72*	61.15
Quercetin	8.13 ± 2.04 *	63.19	4.0 ± 2.61*	81.66

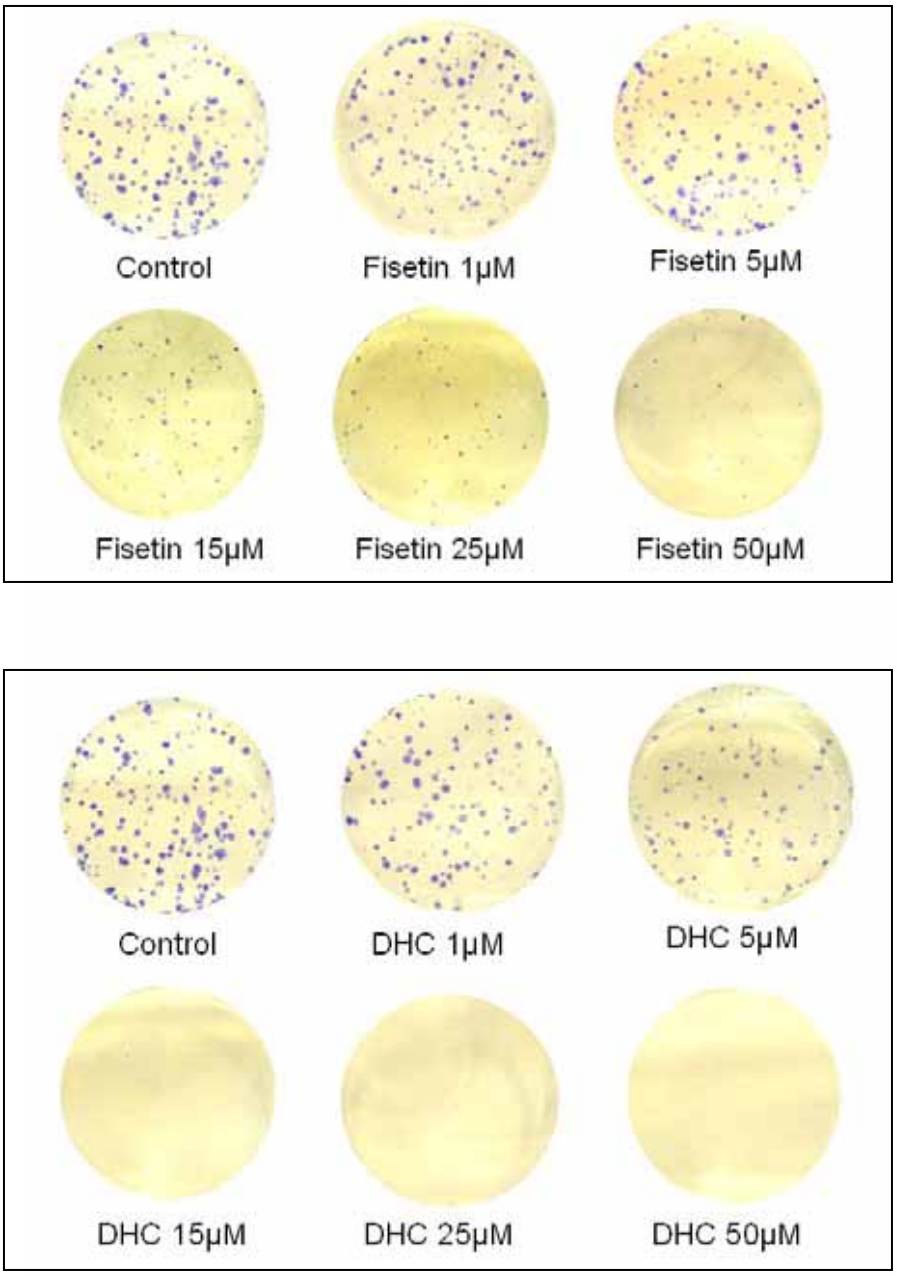
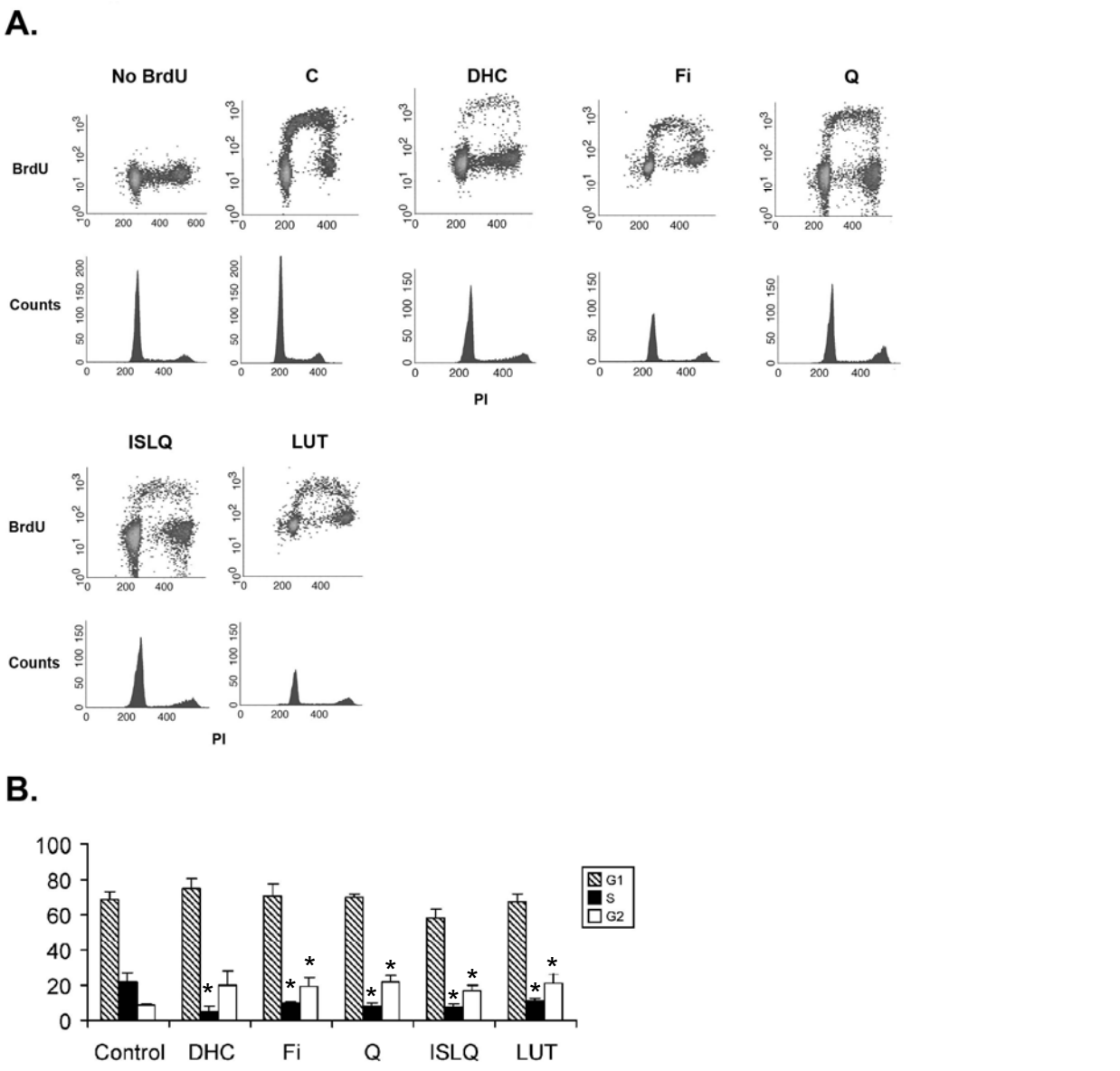


Fig 2.1 Clonogenic Assay. PC3 cells seeded in 6 well plates were treated with flavonoids for 24 hours. Cells were then allowed to grow in flavonoid free media for 2 weeks. A dose-dependent reduction in colonies formed in fisetin and DHC treated cells is evident.



<u>Fig 2.2</u>. Flow cytometric analysis – LNCaP. Cells were treated for 72 hours with flavonoid or vehicle alone. ***(A)*** Representative FACS plots are shown for the flavonoids identified. For each flavonoid 2 graphs are shown. The top panel represents BrdU (y-axis) vs PI (x-axis), while the bottom panel shows the PI histogram. **(*B)*** Quantification of cell cycle phases determined for each sample is shown in the bar chart. The mean value of 3 experiments is shown.

(no-BrdU) control without incorporation of BrdU; *(C)* vehicle only (0.2% DMSO) control incorporating Brdu; *(DHC)* 2,2'-dihydroxychalcone; *(Fi)* fisetin; *(Q)* quercetin; *(ISLQ)* isoliquiritigenin; and *(LUT)* luteolin. *$p<0.05$ (compared to corresponding cell cycle phase of DMSO control).

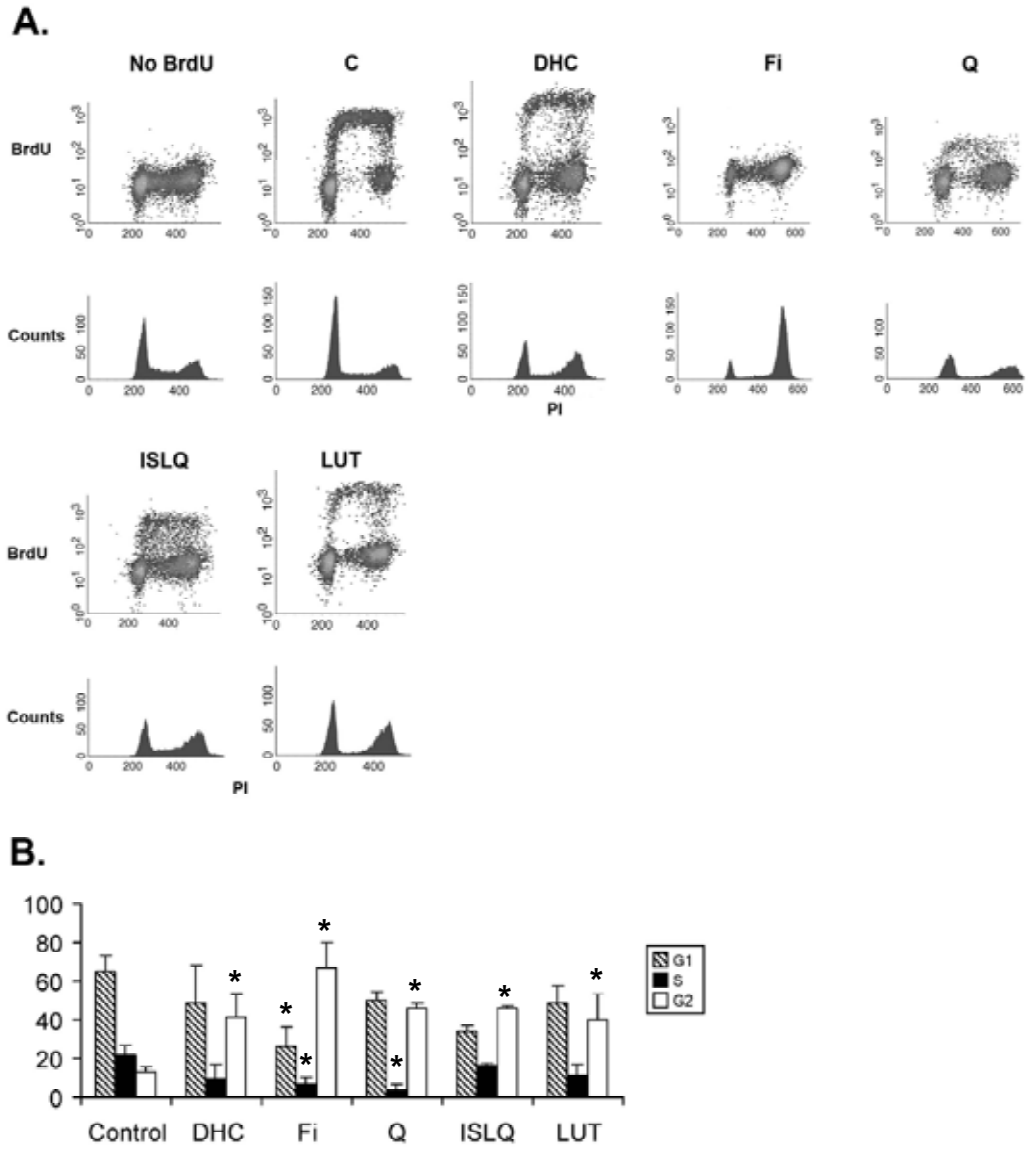


Fig 2.3. Cell cycle analysis – PC3. ***(A)*** Representative FACS plots for flavonoid treated cells- BrDU vs PI (*top panel*) and PI histogram (*bottom panel*). ***(B)*** Quantification of cell cycle parameters as mean of 3 experiments. *(no-BrdU)* control without incorporation of BrdU; *(C)* vehicle only (0.2% DMSO) control incorporating Brdu; *(DHC)* 2,2'-dihydroxychalcone; *(Fi)* fisetin; *(Q)* quercetin; *(ISLQ)* isoliquiritigenin; and *(LUT)* luteolin. *$p<0.05$ (compared to corresponding cell cycle phase of DMSO control).

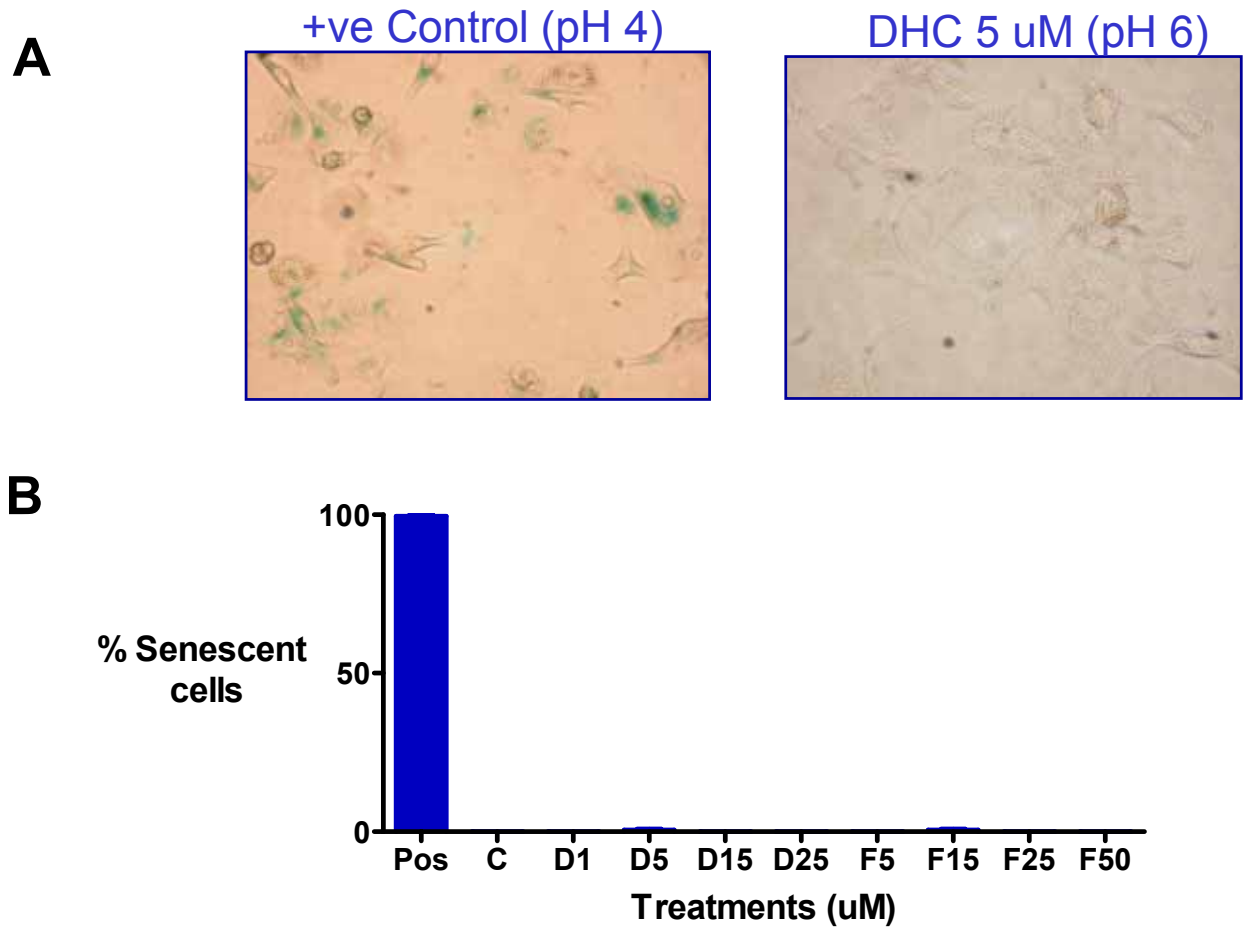


Fig 2.4. Lack of senescence induction by flavonoids. A) Micrograph of beta-galacosidase staining in LNCaP cells. Positive control staining (left) is compared to a representative micrograph demonstrating lack of beta-galactosidase activity in flavonoid treated cells (right). B) Quantification of number of cells (LNCaP) staining positively for beta-galactosidase activity in a field of 200 cells. D=DHC, F=Fisetin.

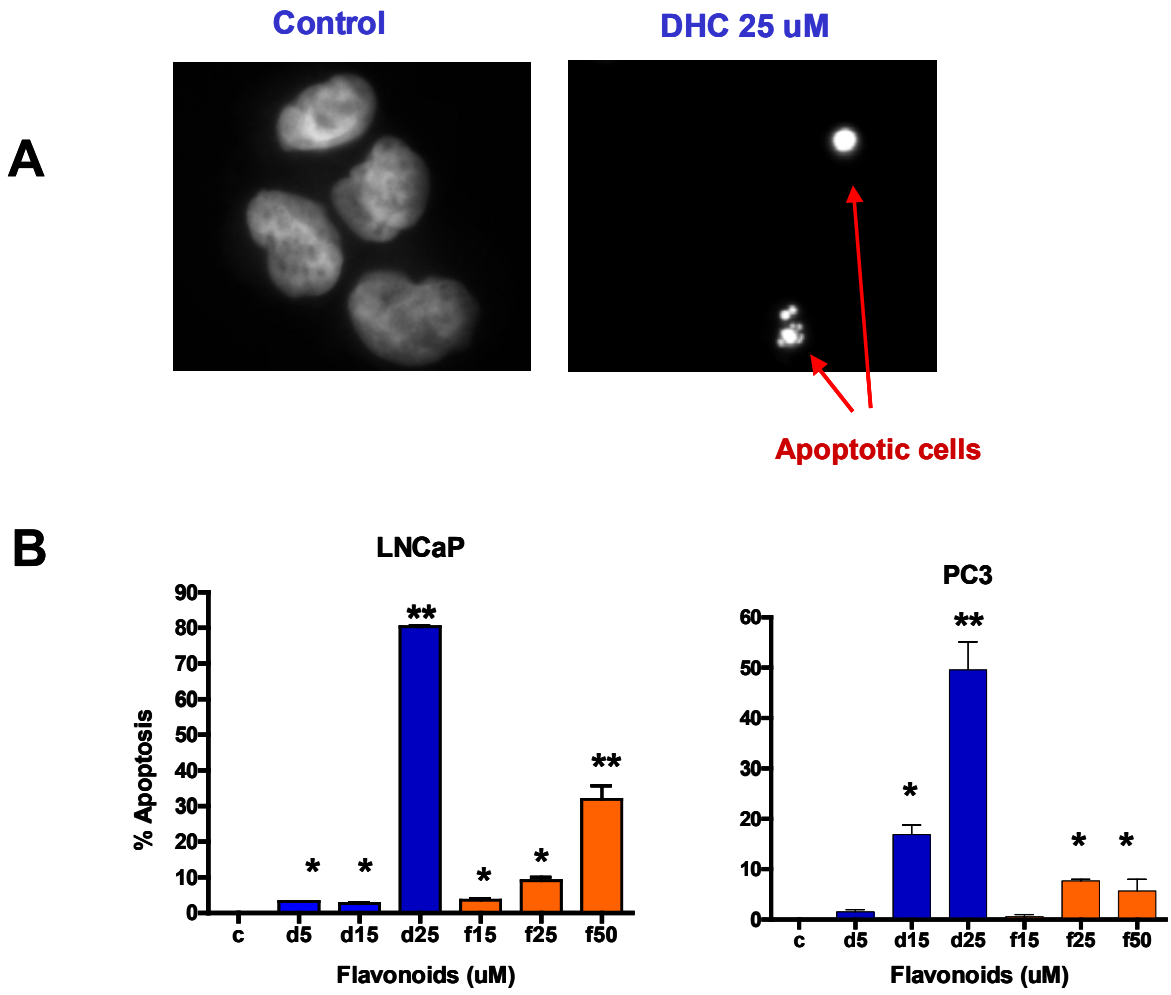


Fig 2.5. Apoptosis induction by flavonoids. A) Representative micrographs demonstrating nuclear morphology by Hoechst staining of control (left) and flavonoid treated cells (right). The apoptotic morphology induced by flavonoids is demonstrated with nuclear condensation and fragmentation evident. B) The percent apoptosis was determined for flavonoid treated PC3 and LNCaP cells by counting the number of cells with typical apoptotic morphology in a field of 200 cells. Dose dependent increase in proportion of apoptotic cells is shown. *$p<0.05$, **$p<0.001$. D=DHC, F-Fisetin.

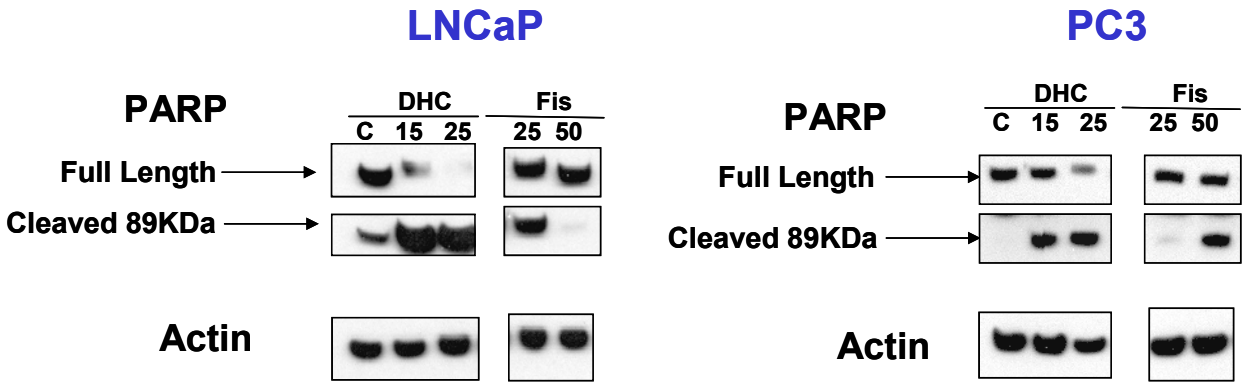


Fig 2.6. Induction of PARP cleavage by DHC and fisetin. Western blot demonstrating increased PARP cleavage by DHC and fisetin in LNCaP and PC3 cell lines.

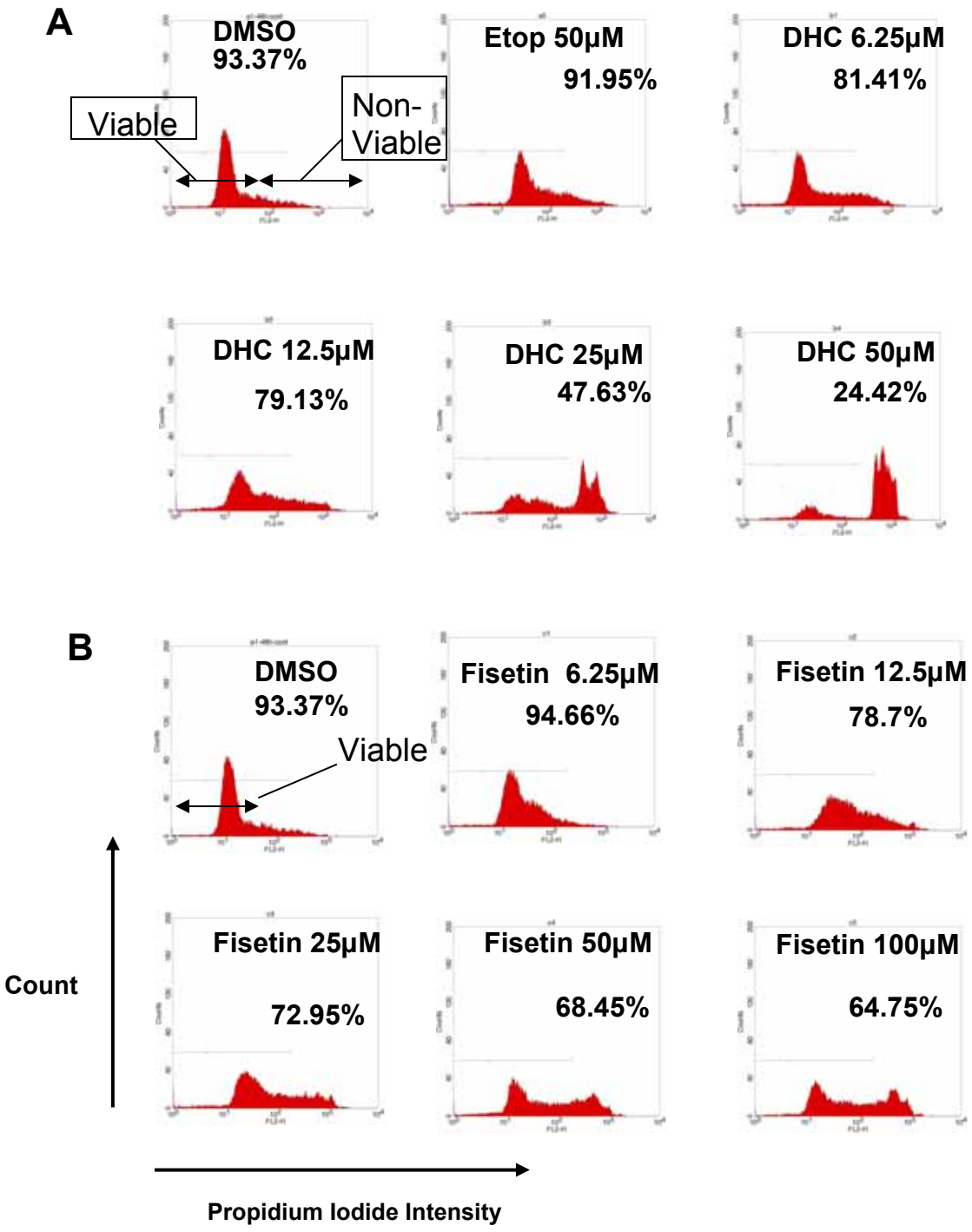


Fig 2.7. Viability assay. Unfixed PC3 cells treated with flavonoids at the concentrations indicated for 72hours were stained with propidium iodide and examined by flow cytometry. The increase in the proportion of nonviable cells treated with flavonoid treatment is evident. Percentage of viable cells is shown A) DHC, B) Fisetin.

CHAPTER 3

TRANSCRIPTIONAL INHIBITION OF KEY CELL CYCLE GENES BY THE FLAVONOIDS 2,2'-DIHYDROXYCHALCONE AND FISETIN IN PROSTATE CANCER CELL LINES

The contents of this chapter have been submitted as a manuscript to the journal 'Carcinogenesis'. I performed the microarray studies reported in this chapter at the Jack Bell Prostate Cancer Institute at Vancouver General Hospital, BC in collaboration with Dr. Colleen Nelson who kindly provided training and materials.

3.1. ABSTRACT

Flavonoids are a large group of dietary polyphenols implicated in prostate cancer prevention. They have been shown to inhibit the cell cycle by inhibition of cyclin dependent kinase (cdks) activity and/or reduction of total levels of key cyclins and cdks. The effect of flavonoids on cell cycle gene transcription is less clear. In prior studies, we identified two novel flavonoids, 2,2'-dihydroxychalone (DHC) and fisetin, with anti-proliferative and cell cycle inhibitory effects in prostate cancer cell lines. In this study, we hypothesize that the effect of these flavonoids on cell cycle is secondary to inhibition of cell cycle gene transcription. We employed oligo-array and quantitative real-time PCR (qPCR) to examine the gene expression effects of both compounds in LNCaP and PC3 human prostate cancer cell lines. A group of 75 genes with key roles in cell cycle regulation were inhibited by both molecules. The major sub-categories within the group of inhibited cell cycle genes were G2/mitosis (28 genes) and DNA synthesis/ chromosome organization (20 genes). As expected from the microarray results, flow cytometry demonstrated the induction of S and G2 arrest by flavonoids. Alterations in other important cell cycle genes were studied by qPCR and immunoblot. We demonstrated reductions of G1 cyclin dependent kinases (cdk2), and up-regulation of cdk inhibitors ($p21^{waf1/cip1}$ and $p27^{kip1}$). DHC and fisetin also induced the expression of 50 stress response genes many of which are known to modulate cell cycle. The results of this study highlight the importance of gene expression alterations in the mechanism of action of flavonoids.

3.2. INTRODUCTION

Prostate cancer is the most common malignancy in men in North America, and the second leading cause of cancer death (453). Current definitive treatment modalities for prostate cancer

suffer from intolerably high incidence of side effects, and unacceptable cancer recurrence rates (5, 9). Attention has therefore turned to prevention strategies to limit the public health burden of this highly prevalent disease. The rationale for chemoprevention is supported by the prolonged natural progression of prostate cancer (over 20 years) that presents an ideal window for the action of preventive agents (21). Various chemoprevention agents have come to light, and some have proven their efficacy in randomized clinical trials (24). Dietary constituents have increasingly emerged as putative chemopreventive agents and are believed to account for the dramatic differences in mortality in prostate cancer worldwide. Prostate cancer mortality in China, for example is 16-fold lower than North America. The components of the Asian diet responsible for the observed chemopreventive effects are the subject of intense investigation (106).

Flavonoids, a diverse group of polyphenols found in all fruits, vegetables and plants, have been postulated to account for the beneficial effects of the Asian diet (454). They are consumed in particularly high amounts due to the intake of green tea and soy products. An increasing body of evidence has demonstrated the anti-cancer effects of flavonoids in pre-clinical animal models of carcinogenesis, and recent reports from clinical trials have been promising (404, 407, 455). Over 5000 flavonoids exist in nature. All are based on the basic C6-C3-C6 polyphenolic structure. The diversity is accounted for by the variation in functional side groups. The large number and biological activity of flavonoids has triggered a search for novel compounds within this group with anti-neoplastic effects. In previous screening experiments we identified several flavonoids including 2,2'-dihydroxychalcone (DHC) and fisetin (3,7,3',4'-tetrahydroxyflavone) with anti-proliferative and cell cycle inhibitory effects in prostate cancer cell lines (456). DHC, a synthetic compound based on the chalcone precursor to flavonoids, demonstrated the greatest potency in all cell lines tested. Fisetin, a ubiquitous flavonol from diverse natural sources such as

strawberries and apples (457), was of interest owing to its apparent prostate selective effect. The structures of DHC and fisetin are shown in Fig. 3.1A.

The molecular effects of DHC are poorly understood, however, its detoxification and anti-inflammatory effects have been demonstrated (458, 459). Owing to the different numbering schemes that exist for labeling the chalcone carbon skeleton, certain instances in the literature referring to 2,2'-dihydroxychalcone actually describe a different compound (2',6-dihydroxychalcone) according to our numbering scheme (460). Fisetin on the other hand has been extensively studied and has been shown to possess a remarkably diverse range of biological effects including anti-inflammatory, anti-angiogenic, anti-bacterial, and neuro-protective properties (461-464). Fisetin has been shown to induce apoptosis in colon and hepatocellular cell lines in a caspase 3 dependent manner (446, 465). Fisetin was also among several flavonoids shown to inhibit the activity of 5α-reductase, the enzyme that converts testosterone into the more potent 5α-dihydrotestosterone known to promote prostate carcinogenesis (466). These varied properties highlight the potential of flavonoids such as fisetin to target various pathways of importance in the carcinogenesis process.

The cell cycle pathway is frequently interrupted by flavonoids. In addition to direct binding and inhibition of cyclin dependent kinases (cdks), flavonoids such as fisetin have been shown to cause a decrease in total levels of cyclins and cdks (467, 468). Fisetin has also been shown to inhibit topoisomerase II, a key enzyme in DNA replication and cell cycle (469). Similar down-regulation of cell cycle regulatory proteins has been observed by other flavonoids, including apigenin, silymarin, the soy flavonoid genestein (362, 363, 445).

Despite several studies demonstrating a reduction in the total levels of cyclins and cdks, the effect of flavonoids on transcriptional modulation of cell cycle gene expression is poorly understood. We hypothesize that reduction in cyclins and cdks by flavonoids is secondary to

transcriptional inhibition. Using oligonucleotide microarray, we studied alterations in gene expression associated with DHC and fisetin treatment in human prostate cancer cells. These findings were validated by quantitative real-time PCR (qPCR). Alterations in cell cycle parameters were delineated by flow cytometric analysis. Immunblotting was performed to further elucidate mechanisms of cell cycle arrest by flavonoids. We detail the broad ranging effects of DHC and fisetin on cell cycle gene transcription, which could be a mechanism of action contributing to the preventive properties of flavonoids in human prostate cancer.

3.3. MATERIALS AND METHODS

Flavonoids and reagents

Fisetin (3,7,3',4'-tetrahydroxyflavone) and 2,2'-dihydroxychalcone were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Flavonoids were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mM. Working standards were made up in serum containing media to a final concentration of 15μM DHC and 25μM fisetin. The final concentration of DMSO in culture did not exceed 0.1%. RPMI 1640 and DMEM/F12 media and penicillin/streptomycin were from Gibco (Invitrogen Corporation, Carlsbad, CA). Fetal bovine serum (FBS), Tris, SDS, NaCL, bovine serum albumin, and 5-bromo-2'-deoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO). Nonidet-P40 was obtained from Bioshop (Burlington, ON). Complete Mini, EDTA-Free protease inhibitor cocktail was from Roche Molecular Biochemicals (Mississauga, ON). RNeasy minikit was purchased from Qiagen Inc (Valencia,CA) and universal human RNA from Stratagene (La Jolla, CA). Superscript II reverse transcriptase and random primers were obtained from Invitrogen. Primers for quantitative real time PCR were the pre-designed TaqMan® Gene Expression Assays from Applied Biosystems

(Foster City, CA). TaqMan Universal PCR Master Mix was also obtained from Applied Biosystems.

Antibodies

ß-Actin mouse monoclonal was purchased from Sigma Laboratories. Anti-cdk2 (H-298); anti-p21 (C-19); anti-p27 (C-19); and anti-cyclin A (H-432) rabbit polyclonal antibodies were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cdc2 (Ab-1) was procured from Calbiochem (San Diego,CA). Anti-rabbit/mouse/goat IgG HRP conjugates were all from Promega (Madison, WI). Anti-BrdU-fluorescein isothiocyanate (FITC) conjugate for flow cytometric analysis was acquired from Becton Dickenson (San Jose, CA) and anti-phospho-histone H3 (ser10) from Cell Signaling (Danvers, MA). Fluorescein conjugated donkey anti-rabbit IgG for flow cytometry was purchased from Jackson immunoresearch (West Grove, PA).

Cell lines and culture conditions

The androgen sensitive LNCaP and androgen independent PC3 cell lines were obtained from the American Type Tissue Collection (ATCC), Rockville, MD. LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100IU/ml penicillin and 100µg/ml streptomycin. PC3 cells were cultured in DMEM/F12 medium with 10% FBS and antibiotics as for LNCaP.

Microarray sample preparation and hybridization

To determine global gene expression alterations associated with flavonoid treatment, oligonucleotide-array analysis was performed. PC3 and LNCaP cells were treated with 15µM DHC, 25µM fisetin or 0.1%DMSO (vehicle only control) for 6, 12 and 24 hours (Fig 3.1B). Two independent replicate experiments were performed. Total RNA was isolated for each sample using the RNeasy minikit as per the manufacturers instructions. The quality of the RNA was

measured using the Agilent 2100 bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA). RNA quantity was assessed with the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). 10 μg RNA was reverse transcribed using Superscript II reverse transcriptase and random primers and the resultant cDNA was used in the oligoarray and quantitative real time PCR (qPCR) experiments. Human Operon v.2 (21K) glass microarrays were produced (based on human 70mers from Operon, Huntsville, AL) by the Microarray Facility of the Prostate Centre at Vancouver General Hospital (Vancouver, Canada). cDNA samples were labeled with either Cy3 or Cy5 (see below)(Genisphere, Hatfield, PA) and cohybridized to the microarrays. Following overnight hybridization and washing, arrays were imaged using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Signal quality and quantity were assessed using Imagene 8.0 (BioDiscovery, El Segundo, CA). Further analysis was performed using GeneSpring 7.3.1 (Agilent Techologies, Palo Atlo, CA).

Microarray experiment design and data analysis

The experimental design for the microarray experiments was based on the previously described 'reference design' which allows for normalization of all slides based on a common reference RNA sample (470). We used universal human RNA (UHRR) from Stratagene (La Jolla, CA) as the reference sample. Reference cDNA was labeled with Cy5 and test cDNA (DMSO, DHC or fisetin) labeled with Cy3. Expression values for all genes on the microarray were determined as a ratio of cy3/cy5. Using this design, a single DMSO control sample can be compared to multiple flavonoid treated samples.

Genes with expression <0.01 or >1000 were eliminated. Per spot and per chip intensity-dependent (LOWESS) normalization was performed to adjust for dye bias. Flavonoid treated samples were compared to the corresponding DMSO control (Fig 3.1B). We identified genes that were greater than 2-fold altered and were statistically significantly different ($p<0.01$) in flavonoid

treated samples compared to DMSO control. The gene list obtained was then classified according to biological function as determined by annotation in the gene ontology database (471). Fisher's exact t-test was used to identify over-represented gene classes based on the statistical likelihood that they are more highly enriched in our genelist compared to the genome as a whole. Clustering analysis of genes within functional groups was performed to discover genes with similar expression profiles and to allow a visual comparison of gene expression across multiple treatments. Clustering was performed using k-means analysis in Cluster and Treeview software packages (472). All cluster analysis is represented as a fold-change (expression in flavonoid sample/ expression in DMSO sample) rather than on absolute expression values. All fold-change values were log transformed (base 2) prior to clustering.

Quantitative Real Time PCR (qPCR)

The expression of 12 genes was determined by qPCR to validate the microarray results. Commercial TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) containing pre-designed probes were used in the qPCR reactions, with beta actin as an internal control. For each PCR reaction, a master mix was prepared containing Taqman probes for the gene of interest in 1× TaqMan Universal PCR Master Mix, and 0.5 µl of cDNA template. The qPCR was reaction was run on the ABI7000 detection system (Applied Biosystems, Foster City, CA) at 40 cycles with denaturing and annealing/extension temperatures of 95°C (15 s) and 60°C (1 min), followed by one cycle at 72°C for 5 min. Amplification curves were analyzed using the relative standard curve method as outlined in the manufacturer's instructions.

Flow Cytometry

Cell cycle parameters were analyzed by flow cytometry to correlate gene expression changes with functional cell cycle alterations. BrdU labeling was performed as previously described (456) in asynchronously growing PC3 and LNCaP cells treated for 6, 12 and 24 hours

with 0.1% DMSO, 15μM DHC, or 25μM fisetin. Anti-phospho histone H3 (ser 10) labeling was performed in LNCaP cells treated with 0.1% DMSO, 5-25μM DHC and 5-50μM fisetin for 48 hours. Cells were fixed in 70% ethanol as above and permeabilized with 0.25% ice cold Triton X100 in PBS for 15 minutes. Cells were then washed in 1% BSA in PBS and incubated with 1:100 dilution of anti-H3P antibody for 2 hours. After further washes in 1% BSA/PBS, cells were incubated in 1:50 dilution of FITC-anti-rabbit IgG. Cells were stained with propidium iodide for 30 minutes prior to analysis on flow cytometer. Flow cytometry was performed on the FACSCalibur flow cytometer using the Cell Quest Pro (Becton Dickenson, California) and Flowjo (Treestar Inc, OR) software packages.

Immunoblotting

PC3 cells were treated with 0.1% DMSO, 15μM DHC or 25μM fisetin for 6-48hours. Whole cell lysates were prepared in ice-cold NP40 lysis buffer (0.1% NP40, 50mM Tris pH 7.5, 150mM NaCl, and the Roche Complete Mini protease inhibitor cocktail). Lysates were sonicated and clarified by centrifugation. Proteins were quantified by Bradford analysis and 40 μg protein/lane resolved by electrophoresis on 10-12% SDS-PAGE. Resolved proteins were transferred onto PVDF membrane (Millipore, Billerica, MA) by semi-dry transfer in buffer (Tris 50mM, Glycine 40mM, 0.01% SDS, and 4% methanol) at 25volts for 30 minutes using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad, USA). Membranes were probed with primary antibody followed by HRP conjugated secondary antibody and detection by ECL chemi-illuminescence. All primary antibodies were used at concentrations of 1:200, except ß-Actin 1:10,000. Secondary antibodies were used at dilutions of 1:40,000.

3.4. RESULTS

The aim of this study was to examine alterations in cell cycle gene expression by DHC and fisetin in prostate cancer cells. The study design is outlined in Fig 3.1.B. The concentration of DHC and fisetin used in the experiments corresponds to the IC_{50} values ascertained by proliferation studies (456). The time points were chosen in order to enable us to determine the maximum number of gene expression changes caused by DHC and fisetin within the first 24 hours of incubation. We have focused on gene expression trends and common patterns over time and treatments. All microarray results are presented as fold change values (expression in flavonoid sample/ expression in DMSO control).

Fisetin and DHC alter expression of genes in several cancer related pathways

We employed oligo-based microarray to determine gene expression alterations in 21,000 genes by flavonoids. Only those genes that had greater than 2-fold alteration and statistically significantly different ($p<0.01$) compared to corresponding DMSO control were selected. This resulted in1051 genes that were identified using these criteria. Overall, there were slightly fewer genes up-regulated compared to those that were down-regulated (46% vs. 54%).

The number of genes identified in each treatment group (e.g. PC3 cells treated with fisetin for 6,12,24 hours) was compared (Fig. 3.2). There was little overlap in the genes identified between DHC and fisetin treated groups or between PC3 and LNCaP groups. This suggests that DHC and fisetin elicit different gene expression effects in these cell lines, and that the response is different between cell lines. This is true, however, if we only consider genes that passed our statistical criteria of >2 fold change and $p<0.01$ difference from control. When alterations less than 2-fold altered are examined, as is seen in the clustergrams (figs 3.3 and 3.6), a more consistent gene expression pattern between flavonoids and cell lines is observed.

Genes were categorized according to their functional classification as determined by the Gene Ontology (GO) database (471). Out of 1051 significantly altered genes, 700 genes had a defined biological function recorded in the GO database. We performed functional enrichment analysis to determine whether any particular functional class of genes was over-represented in the final gene list of 700 genes. There was a highly significant over-representation of cell cycle genes and genes in the related pathways of chromosome segregation and DNA metabolism (Table 3.1). Other enriched categories included signaling pathways involved in cell growth and survival such as RNA processing, translation, kinase regulation, apoptosis and stress response.

Fisetin and DHC down-regulate the expression of key cell cycle genes

Flavonoid treatment resulted in significant gene expression alteration of 82 genes classified as cell cycle related (Fig 3.3.). Cluster analysis enabled us to examine genes based on expression patterns across multiple treatments and time-points. Cell cycle genes clustered into two main expression groups: those that were predominantly up-regulated in all treatments and at various time-points (27 genes) and a larger group (55 genes) that were down-regulated at all treatments and different time-points (Fig.3.3). Genes with functions in G2 and mitosis (M) phases of the cell cycle comprised 62% of the repressed cell cycle genes. These included regulators of the mitotic spindle/microtubule (AURKA, AURKB, PLK1, DLG7, KIF2C, KIF22, STMN, CENP-E, CENP-F, CHC1, EML4, NUSAP, RANBP9), centrosome (NUBP1, CENPJ, NEK1, NEK2) and kinetochore (KNTC2) (Table 3.2). While mRNA inhibition was noted for these genes as early as 6 hours of treatment, immunoblot of cyclin A, cdc2 and plk1 (all of which were down-regulated by DHC and fisetin on microarray) showed inhibition of protein levels at 24 hours or later (Figs 3.3, 3.4 and 3.5).

Closely associated with the cell cycle category are the categories of chromosome organization (36 genes) and DNA metabolism (51 genes). Many genes in these two categories were already classified as cell cycle genes. In order to limit redundancy, we combined these two categories and eliminated genes that we had already identified as cell cycle, resulting in 36 unique genes in the combined category (Fig.3.3). Among this group there were 20 genes that were inhibited in all treatment conditions, in a manner similar to the cell cycle category (Fig.3.3). This group included genes involved in DNA replication (PFS2, RFC3, RPA2, TYMS, TOPO2A, TOPO2B) as well as chromatin regulation and metabolism (NASP, SMARCA5, CHFR, CSPG6, SMC4L1, HCAPG, HIST1H4C, HMGA2).

Multiple cell cycle checkpoints were activated by DHC and fisetin

We examined the effect of the transcriptional alterations described above on cell cycle parameters by flow cytometry (Table 3.3). Asynchronously growing LNCaP and PC3 cells were treated with 15 μM DHC, 25μM fisetin or 0.1% DMSO control, labeled with anti-BrdU antibody and propidium iodide, and examined for alterations in distribution of cells in the cell cycle. While differences in treatment response were observed between the two prostate cancer cell lines, the effect of DHC or fisetin was essentially the same. This corresponds to the similar effects of both DHC and fisetin noted on microarray. PC3 cells demonstrated a marked G2/M checkpoint arrest and an absence of G1 arrest with flavonoid treatment. In LNCaP cells however, flavonoids induced both G1 and G2 phase arrest (Table 3.3). There was a reduction in the number of cells in S-phase in both cell lines with a proportion of cells in both cell lines arrested in S-phase following flavonoid treatment. These cells contained between 2N and 4N DNA, which is typical of S-phase cells, yet they were negative for BrdU incorporation, suggesting that active synthesis of DNA was halted, and cells were arrested in this phase (Table 3.3).

To further elucidate the nature of the G2/M arrest induced by flavonoids in both cell lines, we employed labeling with a mitosis marker (anti-H3P ser 10) to distinguish between G2 and M cell populations, both of which have 4N DNA content and are indistinguishable on standard cell cycle flow protocols. Results reveal that there was a dose-dependent reduction in H3P labeling in DHC and fisetin treated cells (Table 3.4). Thus, the G2/M arrest induced by flavonoids is predominantly due to cells arrested in G2 phase prior to mitosis.

Flavonoids suppress G1 phase regulators.

Although flavonoids induced a G1 arrest in LNCaP cells, alterations of G1 regulatory genes were not evidenced on microarray. We therefore explored alterations in key G1 checkpoint regulators by qPCR and Western blot analysis by flavonoids in PC3 cells (Fig 3.4 and 3.5). The G1 regulatory kinase cdk2 was inhibited on immunoblot by flavonoids (Fig 3.5). The cip/kip family of cyclin dependent kinase inhibitors including $p21^{waf1/cip1}$ and $p27^{kip1}$ were however induced by flavonoid treatment as shown by qPCR and/or immunoblot (Fig 3.4 and 3.5). Therefore, in addition to their pronounced G2/M effects, DHC and fisetin also exert negative effects on the G1 phase of the cell cycle.

Genes belonging to the stress response pathway were induced by flavonoids.

Several other cancer-related pathways were altered on microarray including a large group of genes involved in stress response (76 genes, $p=4.5x10^{-2}$) (Fig 3.6). Many genes in this pathway directly influence cell cycle regulation. The majority (72%) of genes in the stress response pathway were up-regulated by DHC and fisetin. We observed enhanced expression of several endoplasmic reticulum stress genes, hypoxic stress response genes, and stress-induced genes in the MAPK pathway. Six heat shock proteins were also altered viz., heat shock 70kDa protein (HSP70) 1A, 1B and 8, Hsp90kDa protein 1, Hsp105kDa and Hsp40kDa (Fig.3.6.).

Correlative studies using quantitative real time PCR (qPCR)

Results obtained from microarray were verified by qPCR (Fig 3.4.). Using the same cDNA as for the microarray experiment, we performed qPCR for 12 genes, comparing gene expression levels in all flavonoid treated samples. Data are represented as fold-change values (flavonoid expression/DMSO expression) (Fig. 3.4). The overall Pearson correlation between microarray and qPCR expression levels was 0.879

3.5. DISCUSSION

The purpose of this study was to investigate the effect of two flavonoids on cell cycle gene transcription. We report the inhibition of gene expression in 55 cell cycle genes and 20 chromosome organization and DNA metabolism genes by both compounds. These alterations occurred at multiple time points and in both androgen receptor dependent and sensitive prostate cancer cells (Fig 3.3). The cell cycle group consisted of genes with diverse functions throughout the cell cycle, particularly mitosis, G2/M checkpoint and DNA/chromatin metabolism (S-phase).

Mitosis genes constituted the largest sub-group amongst the 75 inhibited cell cycle/ chromosome organization genes in this study (Table 3.2). The inhibited mitosis genes consisted of critical regulators of various aspects of the mitotic machinery including the mitotic spindle, centrosome and kinetochore (KNTC2). Recent studies by Carter et al (473) have shown that many of the suppressed mitosis genes identified in our study are abnormally expressed in cancer, and several are amongst the most frequently associated genes with chromosomal instability. Targeted inhibitors for cancer chemotherapeutics are currently being investigated for several of the mitotic kinases in this group including aurora kinase (AURK), polo-like kinase (PLK) and NIMA-related kinase (NEK) (474). Despite the broad ranging inhibition of mitosis genes by DHC and fisetin, it is interesting to note that cells were not arrested in mitosis (Table 3.4). This

can be explained by the fact that the activation of G2 checkpoint by DHC and fisetin is limiting the number of cells entering the mitotic phase (Table 3.3).

Accurate DNA replication during S-phase is a critical step in the cell cycle, and targeting DNA synthesis is a common mechanism of anti-cancer drugs. We report that DHC and fisetin inhibit the transcription of several S phase genes classified under the categories of 'DNA metabolism' or 'chromosome organization' (Fig 3.3). This group included genes involved in DNA replication and genes with functions in chromatin regulation and metabolism. Abnormal expression of these genes is also frequently associated with malignancy. For example, over-expression of topoisomerase IIa (TOPO2A) and thymidylate synthase (TYMS) is associated with poor outcome in prostate cancer (475, 476). These genes are also the targets of common chemotherapeutic drug targets such as etoposide (topoisomerase II) and 5-fluorouracil (thymidylate synthase).

Cyclins and cyclin dependent kinases (cdks) regulate the transition through all the major phases (G1, S, G2) of the cell cycle, and are often abnormally expressed in cancer. This has led to concerted efforts to develop novel target cyclin and cdk inhibitors (477). Flavonoids have been shown to inhibit various cyclins and cdks in cancer cells, both by direct competition with the ATP binding site of cdks and by inhibition of total cyclin/cdk levels (362, 363, 445, 467). However, the effect of flavonoids on transcription of cyclins and cdks is less clear. We have shown, by oligo-array and qPCR, that DHC and fisetin transcriptionally inhibited three key cyclins, cyclin A2 (CCNA2), cyclin B1 and B2 (CCNB1 and 2) and two cdks, CDC2 and CDK5 (Fig 3.3 and 3.4). The A-type and B-type cyclins are the major determinants of progression through S-phase and G2 respectively (477). Cylin A interacts with cdk2 (also inhibited by DHC/fisetin as shown on immunoblot) to promote progression from S to G2 with cyclin B-cdc2 complex is essential for mitotic entry. DHC and fisetin therefore inhibited the expression of the

main cyclin and cdk regulators of S and G2 phases of the cell cycle. As expected, this was associated with S and G2 phase arrest in both PC3 and LNCaP cell lines (Table 3.3). CDK5, while classified as a cdk, does not have a role in cell cycle regulation. Rather it has been shown to be involved in cytoskeletal dynamics and its activity is associated with increased metastasis in prostate cancer (478).

Other genes with functions at G2 that were inhibited by DHC and fisetin include CDC25A, MYT1 and 14-3-3 (YWHAQ) (Fig 3.3 and 3.4). Cdc25A is over-expressed in several malignancies and is a key phosphatase that serves to activate cdc2 at the G2 checkpoint (479). Inhibition of cdc25A by flavonoids therefore serves to enhance the overall cell cycle inhibitory effect of these compounds. 14-3-3 proteins and myt both regulate cdc2 resulting in the inhibition of cdc2 activity (480, 481). Therefore down-regulation of 14-3-3 (YWHAQ) and MYT1 would serve to increase cdc2 activity and abrogate G2 arrest, a potentially undesirable effect.

In addition to the S and G2 arrest, DHC and fisetin induced G1 arrest in androgen sensitive cells (LNCaP), but not the androgen independent PC3 cell line (Table 3.3). The lack of G1 arrest in PC3 cells likely is a result of the p53 null status of this cell line that could render the G1 checkpoint inactive. RB1, a major tumor suppressor of G1 to S progression was paradoxically down-regulated by flavonoid (Fig 3.3). However, G1 arrest occurred regardless, possibly owing to the effect of flavonoids on other mediators of G1, such as inhibition of the G1 kinase cdk2 as shown on immunoblot (Fig 3.5). We also demonstrated induction of $p21^{waf1/cip1}$ and $p27^{kip1}$, pan-cdk inhibitors of the cip/kip family, by immunoblot and/or qPCR (Fig 3.4 and 3.5). Low expression of cip/kip proteins correlates with high tumor grade in several cancers (482). Induction of $p21^{waf1/cip1}$ and $p27^{kip1}$ by flavonoids therefore could contribute to the cell cycle arrest and overall anti-tumor properties of these flavonoids.

While the cell cycle gene expression effects of DHC and fisetin predominated, alterations in other cancer related pathways were also demonstrated (Table 3.1). Over 70 stress response genes were altered by flavonoids, of which 76% were upregulated (Fig.3.6). Within this category, several MAP kinase genes were up-regulated. JUNB and JUND have been shown to have anti-proliferative or tumor suppressor effects (483), while JNK is an activator of mitochondrial induced apoptosis (484). Expression of endoplasmic reticulum (ER) stress response genes was also enhanced by flavonoids (e.g ATF4, ATF3, DDIT3/CHOP, asparagine synthase and selenoprotein S). The ER stress response is closely linked to the apoptotic pathway via mediators such as DDIT3/CHOP, and has been shown to synergize with DNA damaging agents such as cisplatin (485). In addition to cell cycle gene alteration, activation of stress response genes therefore constitutes another mechanism of DHC and fisetin in prostate cancer. Pathway analysis examining the interaction of stress response genes identified in this study with the cell cycle genes altered in this study reveal a close interaction between several members of both groups (Fig. 3.7).

Previous microarray studies on the effect of soy and green tea flavonoids did not demonstrate the broad ranging cell cycle effects shown for DHC and fisetin (486, 487). However, flavopiridol, currently in clinical trials as a cyclin-dependent kinase inhibitor, demonstrated down regulation of a number of cell cycle genes that were also inhibited in this study, including cdc2, cyclin B2 and aurora kinase B (488). This supports the concept of common mechanisms of action of diverse flavonoids. Although the precise mechanism accounting for the transcriptional inhibition by DHC and fisetin is unknown, the effect of the compounds on a common up-stream regulator in the cell cycle pathway is a possibility, especially since several inhibited genes contain a common promoter element (489).

In summary, we have demonstrated the transcriptional inhibition by DHC and fisetin of 75 genes with critical roles throughout the cell cycle. Many of the genes identified are over-expressed in various malignancies and are the targets of current or experimental therapeutic agents. The underlying mechanism for the transcriptional inhibition of cell cycle genes by DHC and fisetin has not yet been determined and warrants further investigation. Cell cycle checkpoint activation has been described as a barrier to carcinogenesis in the early stages of cancer. Induction of cell cycle arrest by a mechanism of transcriptional inhibition may therefore account for the chemo-preventive effect of flavonoids demonstrated in epidemiological studies of prostate cancer.

Table 3.1- Enriched functional categories

Enrichment of gene ontology (GO) categories was determined by Fisher's exact test comparing abundance in genome to abundance in our final genelist (see text for details). Functional categories are listed in order of p-value. Smaller p-values imply over-representation of functional group in the list of altered genes in the study.

Gene ontology class	Gene count	p-value
Cell cycle (M phase)	82	1.3E-10
chromosome organization	36	2.E-05
RNA processing	42	1.3E-04
DNA metabolism	51	8.8E-4
translation	36	1.5E-03
Kinase/ phosphatase	62	1.6E-03
transport	66	2.1E-03
exocytosis	10	5.4E-03
mitochondrial organization	5	6.8E-03
transcription	84	8.2E-03
apoptosis	42	8.7E-03
secretion	20	1.7E-02
stress response	76	4.5E-02

Table 3.2- Down-regulated G2/M genes.

Genes with known function in G2 or mitosis phase of the cell cycle that were inhibited by both DHC and fisetin are listed. These constituted the most enriched functional category in the study. Average fold change in all experimental conditions is shown.

Gene	**Full Name**	**Function**	**Fold change**
KNTC2	kinetochore associated 2	M	-2.27
CENPE	centromere protein E	M	-2.01
DLG7	discs large homolog 7	M	-1.79
TTK	TTK protein kinase	M	-1.75
NEK2	NIMA (never in mitosis gene a)-related kinase 2	M	-1.72
ANLN	anillin, actin binding protein	M	-1.72
PLK1	polo-like kinase	M	-1.71
CENPF	centromere protein F (350/400kD)	M	-1.70
AURKB	aurora kinase B	M	-1.64
KIF22	kinesin family member 22	M	-1.63
KIF4A	kinesin family member 4	M	-1.60
HCAP-G	chromosome condensation protein G	M	-1.58
CDC23	cell division cycle protein 23	M	-1.57
STK6	serine/threonine protein kinase 6	M	-1.54
NUSAP1	nucleolar and spindle associated protein 1	M	-1.52
KIF2C	kinesin family member 2C	M	-1.40
CHC1	regulator of chromosome condensation 1	M	-1.31
STMN1	stathmin 1	M	-1.21
NEK1	hypothetical protein	M	-1.17
CHFR	checkpoint with forkhead and ring finger domains	M	-1.11
RANBP9	RAN binding protein 9	M	-1.11
CDC2	cell division cycle 2 protein isoform 1	G2/M	-1.09
CCNB1	cyclin B1	G2/M	-2.22
CCNB2	cyclin B2	G2/M	-2.02
CCNA2	cyclin A	G2/M	-1.81
PKMYT1	protein kinase Myt1 isoform 1	G2/M	-1.74
UBE2C	ubiquitin-conjugating enzyme E2C isoform 1	G2/M	-1.68
YWHAQ	14-3-3 theta	G1, S, G2/M	-1.22

Table 3.3. Cell cycle distribution of PC3 and LNCaP cells treated with 0.1% DMSO (control), 15μM DHC or 25 μM fisetin for 6,12, and 24 hours. Cells were pulse labeled with BrdU prior to harvesting. Fixed cells were stained with propidium iodide (DNA content) and anti-BrdU-FITC conjugate antibody (s-phase) and analyzed by flow cytometry. DHC and fisetin both had similar effects on cell cycle, inducing S,G2/M arrest in PC3 and G1,S,G2/M arrest in LNCaP. S+ (S-phase cells incorporating BrdU), S- (S-phase cells not incorporating BrdU thus arrested in S-phase).

		LNCaP				PC3			
Treatment	**Time**	**G1**	**G2**	**S+**	**S-**	**G1**	**G2**	**S+**	**S-**
0.1% DMSO	**6h**	65.4	6.75	26.7	0.54	49.3	6.83	32.1	0.38
	12h	63	7.15	28.5	0.43	52.3	15.1	31.5	0.43
	24h	70.3	6.59	22.6	0.23	44.6	13.5	40.6	0.19
15μM DHC	**6h**	73.8	13.5	8.25	2.32	31.1	41	22.8	1.76
	12h	72.1	20.7	3.35	2.21	33.8	44.1	18.2	0.79
	24h	74.6	19	0.47	4.69	36.4	49.5	1.21	9.06
25 μM fisetin	**6h**	72.3	9.18	16.3	0.82	37	39.4	14.7	4.25
	12h	75.4	12.3	8.81	1.3	37.1	42.2	9.75	5.99
	24h	71.4	19.5	3.16	2.06	31	44.9	11.8	5.78

Table 3.4- Reduction in mitosis (M) phase population by DHC and fisetin

LNCaP cells treated with 0.1% DMSO control, 5-25μM DHC or 5-50μM fisetin for 48 hours were labeled with mitosis marker (phospho-histone 3 ser 10) and examined by flow cytometry. A dose-dependent reduction in percent of cells in M-phase was demonstrated in flavonoid treated samples.

Treatment	% H3P positive cells
0.1% DMSO	2.28
5 μM DHC	1.22
15 μM DHC	0.79
25 μM DHC	0.09
5 μM fisetin	1.79
15 μM fisetin	1.44
25 μM fisetin	1.13
50 μM fisetin	0.16

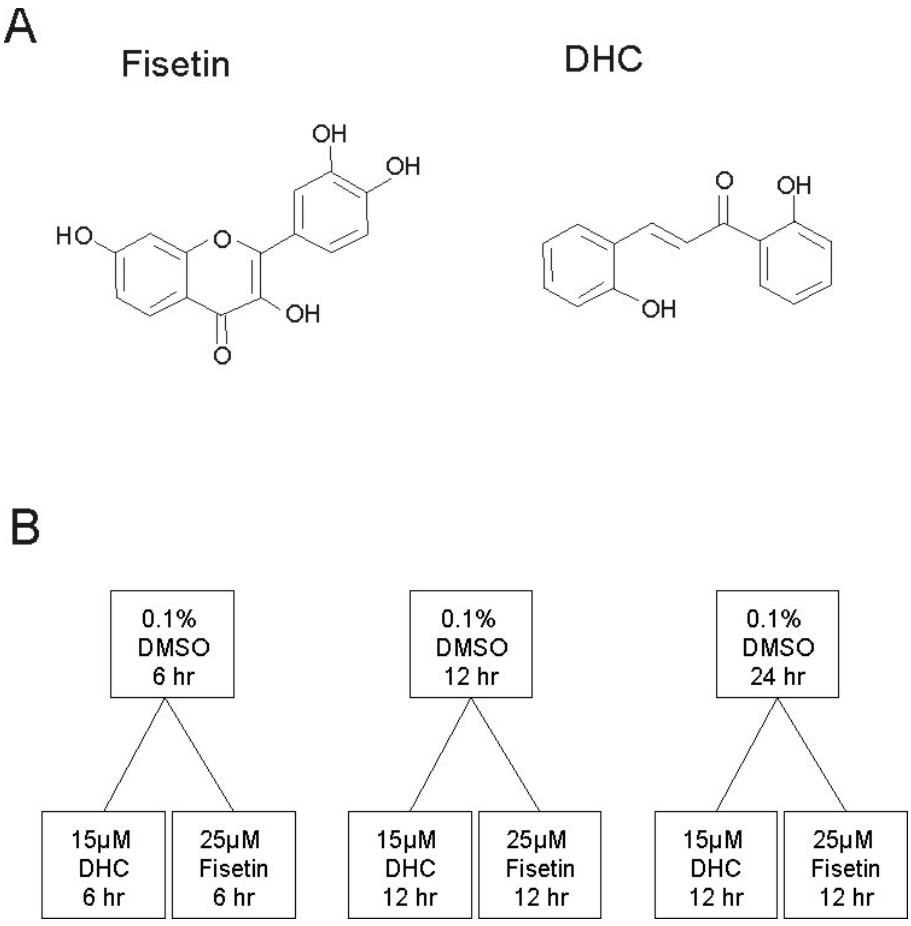


Fig 3.1. A) Structure of flavonoids used in the experiments. B) Microarray Study design. The basic design of the microarray experiment follows the previously described 'reference design'. In this technique, control and test samples are hybridized onto separate microarray slides allowing one control slide to be compared to multiple test slides. The figure shows an outline of the individual hybridizations and the lines represent comparisons made between control and test samples. The experiment was performed as shown in both PC3 and LNCaP prostate cancer cell lines.

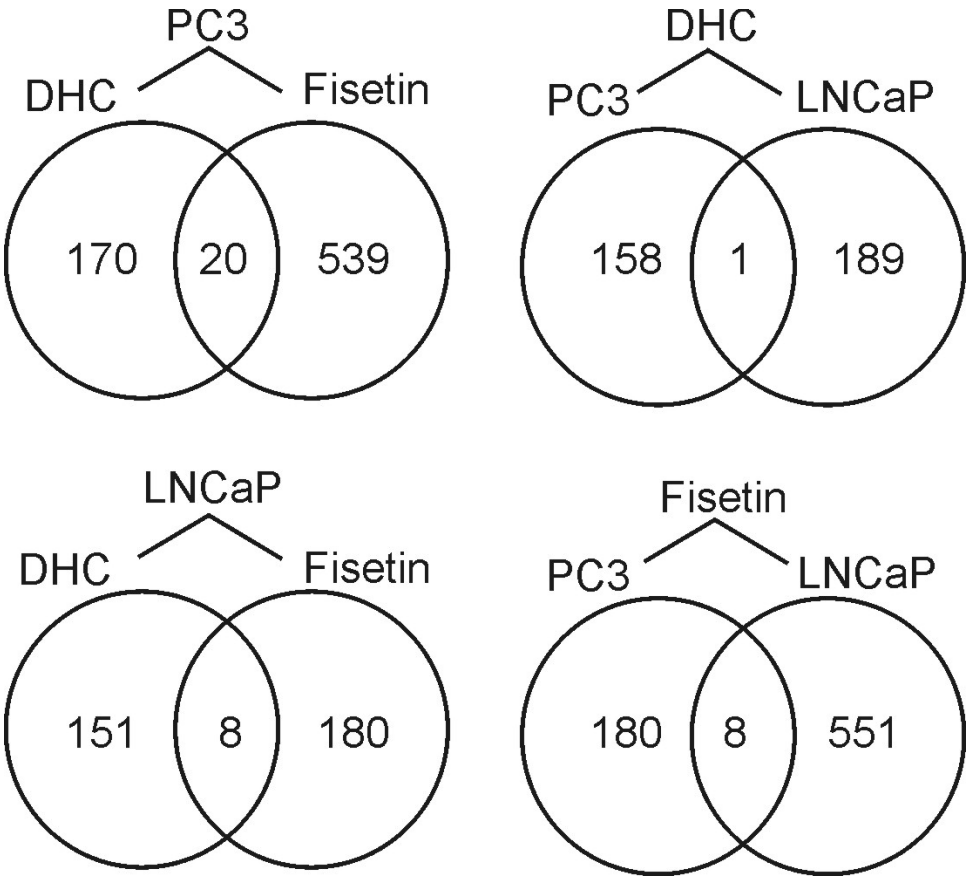


Fig 3.2. Venn diagrams representing numbers of significant genes identified in each treatment condition. For the purposes of this chart, 6,12, and 24 hour time points have been combined. The minimal overlap suggests that there are considerable differences in the gene expression alterations of DHC and fisetin, and in the response of PC3 and LNCaP cell lines.

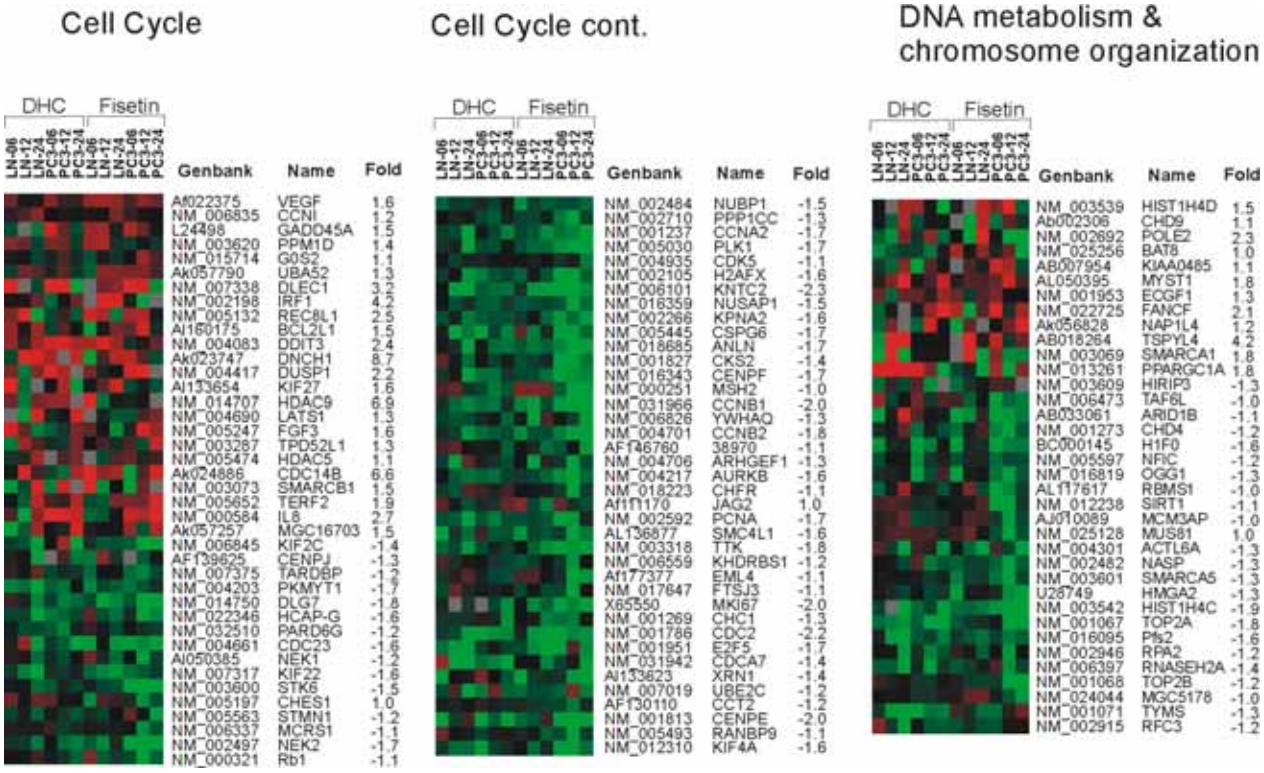


Fig 3.3. **Cluster analysis of cell cycle and chromosome organization/ DNA metabolism genes identified in the study.** LNCaP and PC3 cells were treated at 6-24 hours with 0.1% DMSO control, 15 uM DHC or 25μM fisetin, and gene expression alterations were examined by oligo-array. Gene expression levels in flavonoid treated samples (relative to control) were determined. K-means clustering was performed as discussed in 'materials and methods'. Individual genes are shown in rows and treatment conditions in columns. Up-regulated genes are shown as red while down-regulated genes are green. A large group of suppressed genes in cell cycle and chromosome organization categories, and a smaller group of genes that were up-regulated is demonstrated. Both DHC and fisetin appeared to have broadly similar effects on cell cycle gene expression. DHC= 2,2'-dihydroxychalcone, LN= LNCaP.

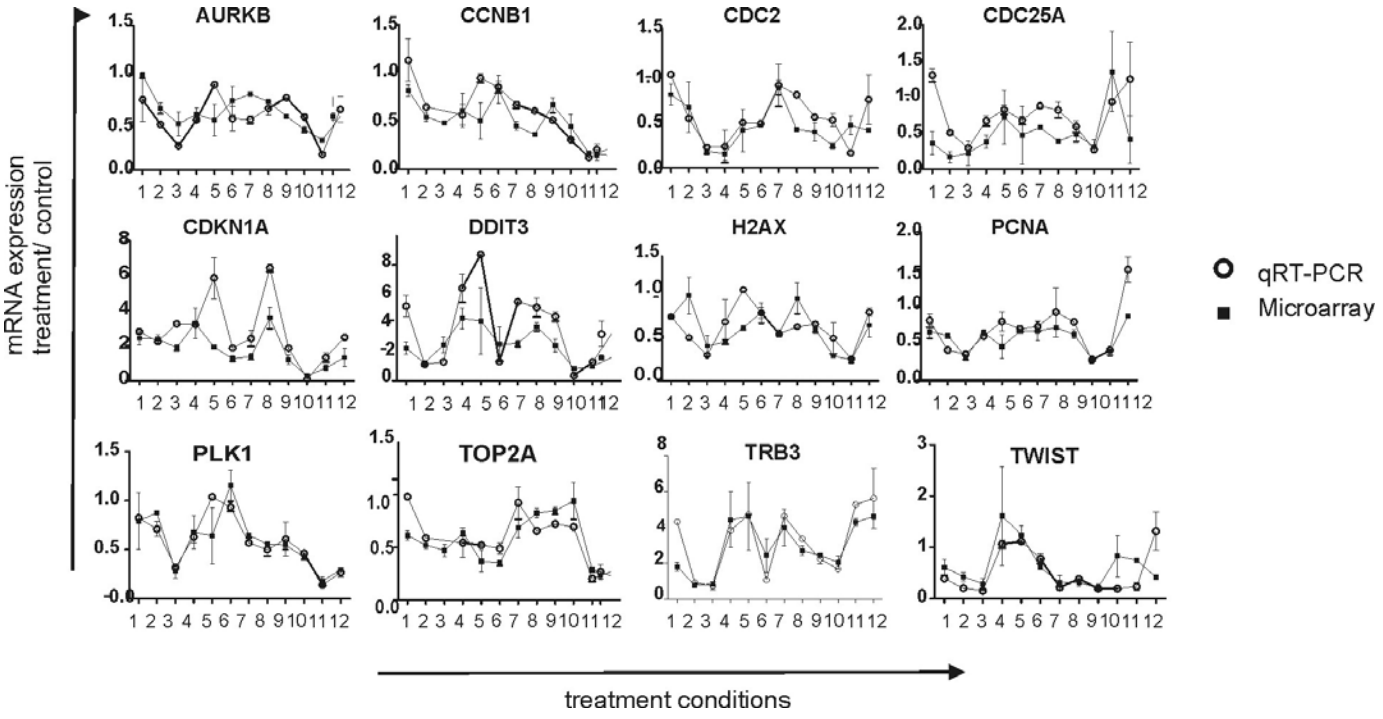


Fig 3.4. Quantitative real time PCR (qPCR) validation of microarray and immunoblotting by qPCR for 12 genes in the study corresponding to the treatment conditions shown in Fig 3.1A. The graph depicts fold change values for the genes as determined by microarray (solid square) and qRTPCR (open circle). Overall correlation of the two methods was 0.879. The labels 1-12 on the x-axis represent different treatment conditions as follows: 1) LNCaP,DHC,6h; 2) LNCaP,DHC,12h; 3)LNCaP,DHC,24h; 4)PC3,DHC,6h; 5)PC3,DHC,12h; 6)PC3,DHC,24h; 7) LNCaP, fisetin, 6h; 8) LNCaP, fisetin,12h; 9) LNCaP,fisetin,24h; 10)PC3,fisetin,6h; 11)PC3,fisetin,12h; 12)PC3,fisetin, 24h.

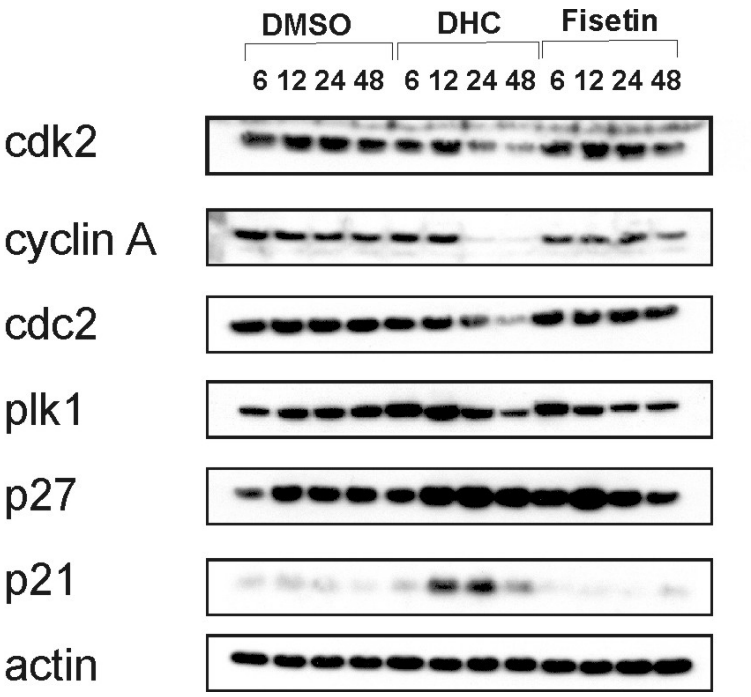
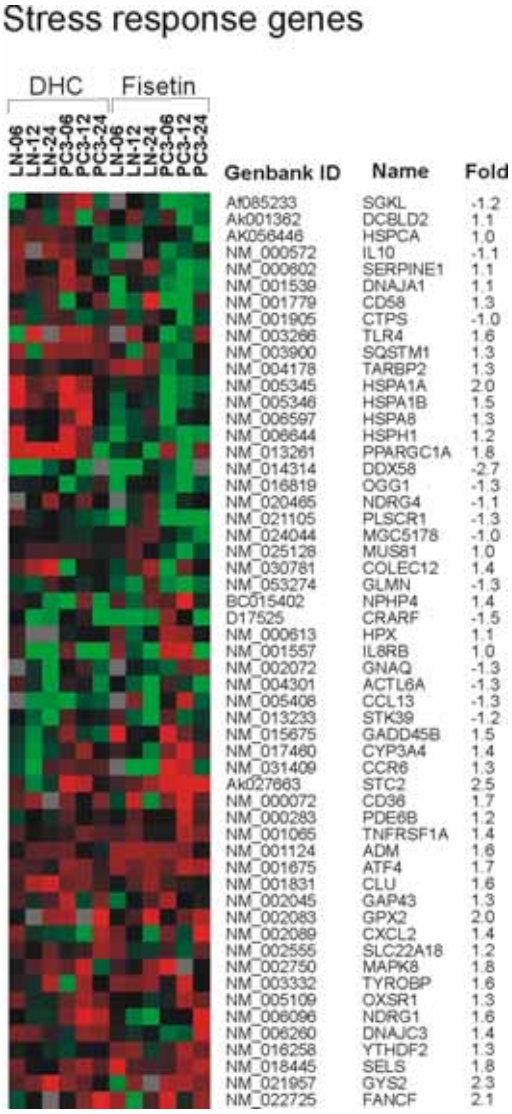
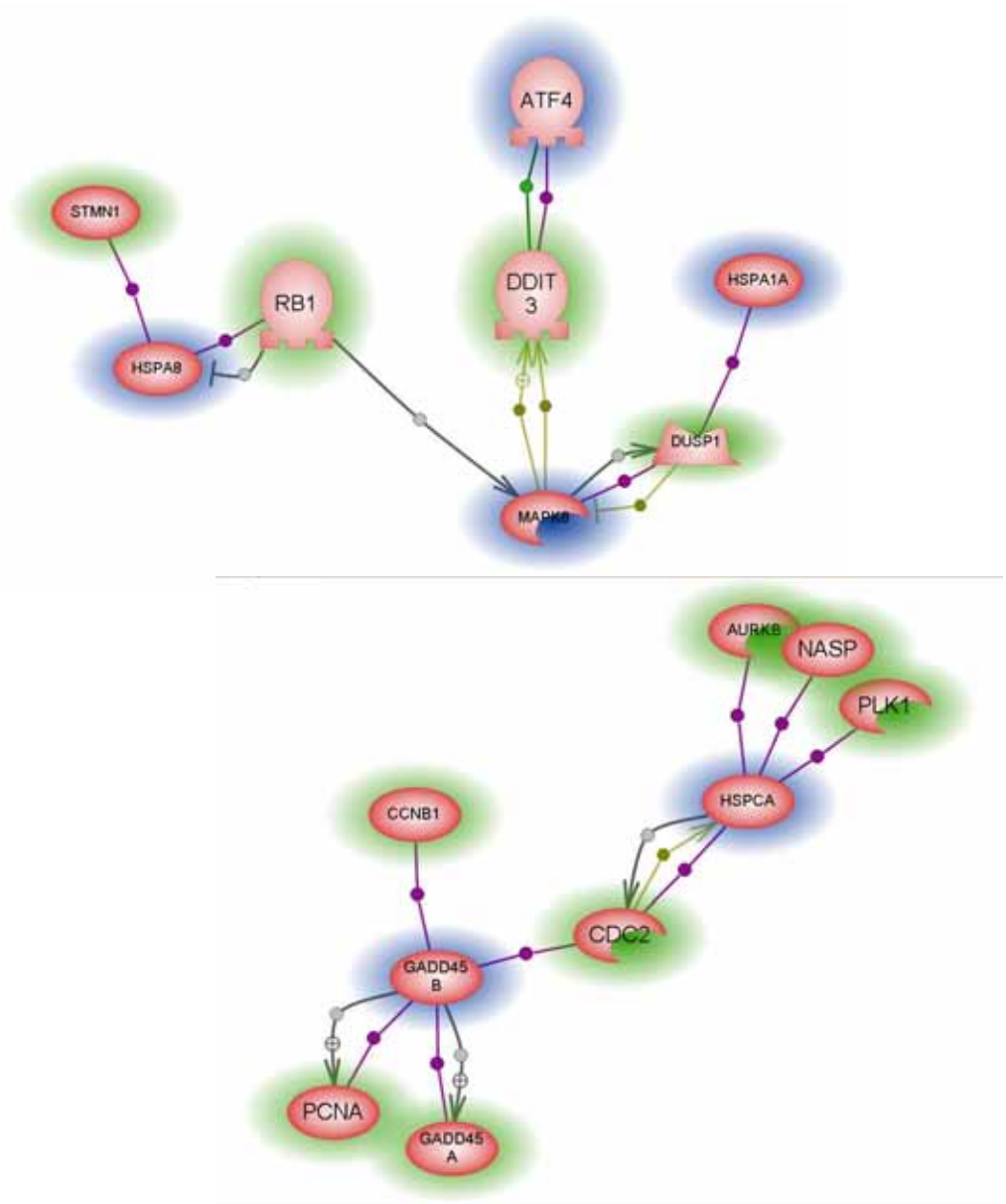


Fig 3.5. Immunoblot demonstrating alterations in cell cycle proteins by flavonoids. Cells were treated with DMSO, DHC or fisetin for 6-48 hours and immunoblotted for alterations in key cell cycle proteins. Down-regulation of G2/M proteins (cyclin A, cdc2, plk1) was demonstrated, although this down-regulation occurred at later time points to the inhibition of corresponding mRNA levels noted on microarray. G1 proteins cdk2 was not noted on microarray, however, immunoblot suggests that DHC and fisetin inhibit levels of this kinases. The cyclin dependent kinase inhibitors p21waf1/cip1 and p27kip1 were both up-regulated by flavonoids.



<u>Fig. 3.6.</u> mRNA fold-change alteration of stress response genes in DHC and fisetin treated cells.

Fold change values (treatment/control) for each gene were clustered using the K-means algorithm and represented as a clustergram. Up-regulated genes are shown as red while down-regulated genes are green. Columns on the clustergram are the various experimental conditions, while rows are individual genes. Induction of a large number of stress response genes is observed.



<u>Fig 3.7.</u> Pathway analysis demonstrating the interaction of cell cycle (green shading) and stress response (blue shading) genes altered by flavonoids. This demonstrates the interaction of stress response genes with the cell cycle pathway.

CHAPTER 4

PROSTATE CANCER CHEMOPREVENTION BY ORAL INGESTION OF FLAVONOID SUPPLEMENTED DIETS IN TRAMP MICE

The contents of this chapter will be submitted for publication in the journal 'Nutrition and Cancer'. The quantification of flavonoid levels in the serum and tissue of mice was performed by Dr. Emma Guns at Vancouver General Hospital, Vancouver, BC. Histological assessment of TRAMP prostate tissue was performed in collaboration with Dr. Linda Sugar, pathologist, Sunnybrook Health Sciences Centre, Toronto, ON.

4.1. ABSTRACT

Flavonoids are a class of naturally occurring polyphenols. Epidemiological studies have demonstrated that they are associated with reduced risk of prostate cancer. This has led to interest in the use of flavonoids as chemopreventive agents in prostate cancer. Despite a large body of in vitro data, there have been limited reports on the in vivo efficacy of flavonoids in prostate cancer prevention. In this study, we employ an autochthonous transgenic murine model of prostate cancer (TRAMP) to investigate the chemopreventive effect of 4 previously identified flavonoids: 2,2'-dihydroxychalcone (DHC), fisetin, quercetin and luteolin. TRAMP mice were randomized to a flavonoid-free diet (AIN76A) or AIN76A supplemented with 1% w/w DHC, 1% w/w fisetin, or a combination flavonoid diet (0.25% DHC, 0.25% fisetin, 0.25% quercetin, 0.25% luteolin). Animals were provided ad libitum access to diets from 4 to 36 weeks of age. Our findings demonstrate that TRAMP mice on DHC or combination flavonoid diets had smaller genitourinary tumor weight than control animals (58.1% reduction, p=0.008; and 38.1% reduction, p=0.064 respectively). There was a (non-significant) reduction in prostate cancer incidence, determined histologically, in DHC and combination flavonoid groups, mainly due to a decrease in the number of low grade tumors. This was associated with a corresponding increase in the number of mice with prostate intraepithelial neoplasia (PIN), a precursor lesion for prostate cancer. Immunohistochemistry demonstrated a non-significant reduction in the expression of proliferation marker PCNA in DHC (38.7% reduction, p=0.083) and combination flavonoid (42.9% reduction, p=0.087) groups. Bioavailability of the flavonoids was demonstrated by liquid chromatography-mass spectrometry, which demonstrated the accumulation of high levels of unchanged flavonoid in the prostates of TRAMP mice. Flavonoid supplementation was, however, associated with gastrointestinal toxicity, particularly in the DHC and combination flavonoid groups. The observations support a protective effect of DHC and combination flavonoid diets on

prostate cancer progression. However, the observed toxicity of the flavonoids at the concentrations used means that future studies are needed to confirm their efficacy and safety at lower doses.

4.2. INTRODUCTION

Treatment of prostate cancer poses a significant challenge despite advances in surgical and non-surgical approaches to the management of the disease. Current treatment modalities in patients with localized disease include radical prostatectomy and/or radiation therapy (4). Both treatment options suffer from high rates of complications (erectile dysfunction and incontinence post-surgery, erectile dysfunction and proctitis post irradiation) and high cancer recurrence rates (5, 9). With the aging population, the public health burden of prostate cancer will increase. The burden is compounded by the widespread use of prostate specific antigen (PSA) as a screening tool, which has resulted in the diagnosis of large numbers of cancers many of which are not destined to progress to clinically aggressive disease.

The failure of currently available treatment methods has led to the emergence of prevention strategies to limit the progression of prostate cancer in its early stages. Prostate cancer is an ideal target for prevention owing to the long period between the initial appearance of histologically diagnosed (latent) cancers and clinically apparent disease. Autopsy studies have demonstrated that 29% of males in their thirties harbor latent foci of prostate cancer (20). Since most prostate cancer deaths occur much later in life, the natural course of the disease spans some 20 years in typical cases. The goal of chemoprevention (prevention by chemical agents) is to reduce the initiation of cancer foci or to slow the progression of these latent cancers once they occur.

Flavonoids are a group of over 9000 naturally occurring compounds that share a common C6-C3-C6 carbon skeleton (490). They occur throughout the plant kingdom and constitute a significant part of dietary anti-oxidant intake. Interest in flavonoids as chemoprevention agents in prostate cancer has intensified. Although the data is conflicting, several epidemiologic studies have associated the consumption of certain flavonoids with a reduced risk of prostate cancer (175, 411, 413, 417, 491, 492). Flavonoids posses a remarkable array of biological properties including, but not limited to, anti-oxidant, anti-inflammatory, anti-microbial, cardio-protective and anti-cancer effects (490). Despite the large number of different flavonoids, only a few flavonoids (in particular the green tea and soy flavonoids) comprise the bulk of flavonoid research in humans. In previous cell culture studies in prostate cancer cells, we identified 4 flavonoids (2,2'-dihydroxychalcone (DHC), fisetin, quercetin and luteolin) with greater anti-proliferative potency than the more widely studied green tea and soy flavonoids (456). These compounds had greater effect in prostate cancer cell (LNCaP and PC3) than in the benign prostate epithelial cell line BPH-1 or the breast cancer cell line MCF-7. They were all shown to arrest the cell cycle in S and G2 phase in PC3 and G1, S and G2 phase in LNCaP cells. The most potent flavonoid identified in this screen was DHC, a synthetic compound from the chalcone family of flavonoids. The molecular effects of DHC are poorly understood, however, its detoxification and anti-inflammatory effects have been demonstrated (458, 459).

Quercetin, luteolin and fisetin are naturally occurring flavonoids belonging to the flavonol subgroup. In contrast to DHC, the molecular properties of quercetin, fisetin and luteolin have been extensively investigated in the literature. All three possess antioxidant and anti-inflammatory properties(493-497). Fisetin, luteolin and quercetin cause inhibition of cancer cell proliferation *in vitro* and induction of cell cycle arrest and apoptosis (465, 498). They share common molecular properties such as reduction in levels of cyclin dependent kinases (cdk),

upregulation of cdk inhibitors of the cip/kip family, activation of the caspase 3 cascade, inhibition of topoisomerase II (499), attenuation of the NF-kappaB signaling pathway (461), and inhibition of PI3-kinase (500). Fisetin has been shown to interact directly with cyclin dependent kinases resulting in suppression of kinase activity (467). They have also been shown to inhibit androgen receptor mediated signaling by inhibiting AR expression or inhibiting 5-alpha-reductase, the key enzyme that converts testosterone to its active metabolite (326, 501). These mechanisms highlight the potential of these flavonoids to target multiple pathways of critical importance in cancer.

Until recently, *in vivo* studies of the chemopreventive effect of flavonoids in prostate cancer were limited to investigations of the effect of flavonoids on models of carcinogen induced malignancy or xenograft models of human prostate cancer cells transplanted into immunocompromised hosts. Several flavonoids, including silymarin (502), soy isoflavones (503), genestein (504) have been reported to inhibit prostate cancer in carcinogen initiated cancer models. Other flavonoids have been shown to inhibit growth of prostate cancer xenografts, including luteolin, which inhibited LNCaP and PC3 xenografts, and quercetin, which inhibited CWR22 prostate xenograft growth (393, 505, 506). Although the xenograft model is suitable in demonstrating inhibition of tumor progression, it does not adequately address the chemoprevention situation where the aim is to inhibit tumor initiation and/or promotion. To our knowledge, the anti-cancer effects of fisetin and DHC have not been previously studied in animal models.

The development of transgenic autochthanous models of prostate cancer has recently afforded the opportunity to more adequately assess the chemopreventive effect of flavonoids. Amongst the most widely used models is the Transgenic adenocarcinoma of the mouse prostate (TRAMP) model (399). This transgenic autochthonous model for prostate cancer employs the -

426/+28 fragment of the rat probasin (PB) gene fused to the SV40 T antigen (Tag). Expression of the PB-Tag transgene is restricted to the prostate epithelial cells of the prostate. Development of prostate cancer in this model is also dependent on the background strain of the mice expressing the transgene. In the C56/Bl6 background, TRAMP mice develop hyperplastic lesions in the prostate by 12 weeks of age, progressing to severe hyperplasia, prostate intraepithelial neoplasia (PIN), and adenocarcinoma in all mice by 24-30 weeks. Metastasis to lymph nodes and lungs is a common feature of this model (399).

The TRAMP model has successfully been employed to study the chemopreventive effect of several flavonoids. Green tea administered in the drinking water from 8 weeks of age has been shown to reduce prostate tumor size (65-80% reduction) in this model (407, 507). The pure compound epigallocatechin gallate (EGCG), a green tea catechin, however, failed to inhibit prostate tumorigenesis at 28 weeks when administered at a concentration of 0.06% (408).Other flavonoids examined in this model include silibinin (410), apigenin (385)and genestein (409).

In this study we examine the effects of the flavonoids DHC, fisetin, quercetin and luteloin in prostate cancer prevention in TRAMP mice. We hypothesized that these flavonoids administered individually or in combination would attenuate the progression of prostate lesions in TRAMP mice.

4.3. MATERIALS AND METHODS

Transgenic animals

Animal care and treatments were conducted in accordance with established guidelines at our institution and guidelines of the Canadian Council on Animal Care. Female TRAMP mice maintained on a pure C57BL/6 background and wild type male C57BL/6 mice were purchased

from The Jackson Laboratory (Bar Harbor, ME). Wild type males were bred with TRAMP females to generate heterozygous TRAMP males. TRAMP males were not used in breeding owing to cancer involvement of the prostate. Tail DNA obtained at 3-4 weeks was screened for probasin-SV40 T antigen (PB-Tag) by PCR.

Study design and flavonoid administration

DHC, fisetin, quercetin and luteolin were obtained from Indofine Chemical Company (Hillsborough, NJ). Flavonoid diets were manufactured by mixing the required amount of flavonoid with AIN-76A base diet (flavonoid-free) and formation of pellets. TRAMP males were divided into 4 groups consuming the following diets: 1) AIN-76A diet, 2) 1% w/w fisetin diet, 3) 1% w/w DHC diet, and 4) Flavonoid combination diet (0.25% w/w fisetin + 0.25% w/w DHC + 0.25% w/w quercetin + 0.25% w/w luteolin). Diets were obtained from Harlan Teklad Custom Diets (Madison, WI). The flavonoid doses were comparable to those used previously for other flavonoids in the TRAMP model (410). Animals were given ad libitum access to drinking water and experimental diets. Dietary treatment with flavonoids commenced at 4 weeks of age until sacrifice at 36 weeks of age. Diet consumption was determined weekly and animal body weight was recorded every other week. Animal health was monitored daily.

Serum and tissue collection

At the study endpoint (36 weeks), the animals were anesthetized by isofluorane and blood sample obtained by direct heart puncture prior to euthanization. Serum was separated from the blood by centrifugation, alequoted and stored at –80°C. In some animals whole blood was collected in EDTA bottles for complete blood count (CBC) determination. Animals were examined for gross organ abnormalities at necropsy. The lower urogenital tract including bladder, seminal vesicles, and prostate was removed *en bloc* and weighed. The bladder was emptied of urine prior to weighing. The dorsolateral and ventral lobes of the prostate were harvested and one

portion of each fixed in 10% (v/v) buffered formalin and another portion snap frozen in liquid nitrogen and stored at –80°C (for later determination of tissue flavonoid levels). Tissue was also collected from liver, spleen, kidney, heart, lung and colon and portions formalin fixed and snap frozen.

Histologic grading of tumors

Formalin fixed tissues were embedded in paraffin and five micrometer sections were obtained and mounted on slides. H&E staining was routinely performed on all sections, and histopathological evaluation performed by one pathologist (LS). Prostate histology was classified as benign, PIN, well differentiated adenocarcinoma or poorly differentiated. Since prostate cancer can be multifocal, we re-sectioned those prostates that were histologically benign or had PIN in order to minimize the risk of missing a focus of prostate cancer.

Immunohistochemistry

Expression of $p27^{Kip1}$ and proliferating cell nuclear antigen (PCNA) proteins were determined immunohistochemically. Paraffin sections were deparaffinized with xylene, rehydrated and boiled for 10 min in citrate buffer (pH 7.0) to expose antigenic sites. Sections were blocked with 0.3% hydrogen peroxide in methanol followed by normal serum and then incubated overnight at 4°C with the primary antibody followed by biotin-labeled anti-rabbit IgG secondary antibody. Slides were then incubated with peroxidase labeled streptavidin (Vector Laboratories) and visualized using diaminobenzidine tetrahydrochloride (Vector Laboratories) as the peroxidase substrate. Primary antibodies used were anti-$p27^{Kip1}$ rabbit polyclonal antibody (1:100; Santa Cruz Biotechnology) and PCNA rabbit polyclonal antibody (1:50; Santa Cruz Biotechnology). Sections were counterstained with hematoxylin, dehydrated, and mounted. Intensity of staining was scored 0-3 by a single pathologist (0, no staining; 1, <25% staining intensity; 2, 25%-75% staining intensity; 3, >75% staining intensity).

Tissue extraction for LCMS

Flavonoid serum and tissue levels were analyzed in a blinded fashion by liquid chromatography-mass spectrometry (LCMS). 100-150mg or the entire organ for smaller tissue samples was used. 380µl of 18 MΩ water and 20µl of internal standard was added prior to homogenization. The internal standard (IS) solution contained 4µg/ml Finasteride (Merck Frosst) and was prepared in 50% methanol (Fisher Optima). This mixture was homogenized on ice with a PowerGen 125 homogenizer (Fisher). 400µl of homogenate was transferred to a 2ml eppendorf tube and 1400µl of acetonitrile added. The sample is then vortexed for ~15 seconds and placed into a rotating mixer at room temperature for an additional 15 minutes. The extract mixture was centrifuged for 5min at 14000g and 1500µl of clear supernatant transferred to a fresh 2ml eppendorf that was dried in a centrifugal evaporator (Centrivap Concentrator, Labconco) at 30°C. The dried residue was reconstituted with 100µl methanol (Fisher Optima), vortexed 30sec, sonicated 10min (VWR model 150T bath) and centrifuged for 5min at 14000g to pellet any undissolved residues.

Serum extraction for LCMS

100µl of serum was transferred to an eppendorf tube and diluted with 90µl water. 10µl of IS was added and the sample vortexed. 700µl of acetonitrile was then added and the sample was again vortexed for ~15 seconds and placed into a rotating mixer at room temperature for an additional 15 minutes. The extract mixture was centrifuged for 5min at 14000g and 750µl of clear supernatant transferred to a fresh eppendorf that was dried in the centrifugal evaporator at 30°C.

LC-MS Analysis

The system used for analysis consisted of an integrated Alliance 2695 solvent delivery system coupled with a 996 photodiode array detector (PDA) and a Quattro Micro triple quadrupole mass spectrometer (MS) (Waters Corp., Milford, MA) controlled with MassLynx (V4.0 SP4) software. Chromatography was carried out with a Sunfire C18 column (3.5 μm, 150 mm x 2.1 mm; Waters, Milford, Massachussets) held at 40°C using a water - acetonitrile (ACN) gradient run at 0.3ml/min with 0.1% formic acid present throughout the separation. The following - time(min), %CAN - pairs define the gradient - 0, 10 - 0.2, 10 - 1, 25 - 7.5, 50 - 10, 85 - 10.2, 100 - 12.5, 100 - 13, 10 - 20, 10. The column eluate passed sequentially through the PDA to the MS. The PDA was programmed to collect 210-600nm wavelength data. All MS data was collected by segmented multiple reaction monitoring (MRM) in ES+ mode with the following instrument parameters: capillary, 3kV; source, 120°C; desolvation gas, N2, 350°C, 500L/hr; ion energies, 0.5 & 3V; multiplier, 650V and resolution set less than unity to increase sensitivity. Chromatographic and infusion data from standards was used to identify reactions to monitor and optimal cone voltage and collision energy (CE) for each. Those used for the quantitative analysis are listed in the table below.

Analyte	RT (min)	Reaction	Cone V	CE (V)	Wavelength (nm)
Luteolin	8.7	287>153	45	30	345
Quercetin	8.8	303>153	45	35	375
Fisetin	7.6	287>137	40	30	360
2,2'-DHC	13.6	241>121	15	15	365
Finasteride	12.5	373>317	50	20	---

The channels in the table were collected with 0.2-0.3sec dwell times and were divided into 4 segments. No interference was observed between analytes using the parameters in the table or with additional secondary MRM's also collected (0.1sec dwell, 287>135, 303>153, 287>185 and 241>199 respectively for the flavonoids in the table). 10nm bandwidth OD traces were extracted from the PDA data at the noted wavelengths as well, which could be used for comparison with MS results in some samples with high analyte levels and no spectral interference. 2-3mg/ml stock solutions of luteolin, quercetin, fisetin & DHC were made up in methanol from which a pooled standard stock containing all 4 flavonoids was prepared. A 5mg tablet of Proscar (Merck) was extracted with 10ml methanol and clarified by centrifugation to prepare the IS stock solution. This IS was chosen for its LC retention relative to the flavonoid analytes. No interference from co-extracted excipients was evident in this preparation. Calibration was performed using a series of samples containing 400ng/ml IS and 0, 5, 20, 50, 100, 200, 400 and 1000ng/ml mixed standard in methanol. Matrix effects were assessed by diluting a 10x solution of standard 10 fold with blank liver extract (1:3 liver:water) and blank serum extracts. Extraction efficiency was assessed by spiking blank liver homogenates or blank serum and comparing with standards and the above post spiked extracts. QC samples were similarly prepared using blank liver homogenates or serum and run every ~10 injects, as were calibration samples to assess ongoing assay and instrument performance. QuanLynx (Ver 4.0 SP4, Waters) was used to process chromatographic traces.

Statistical Analysis

Comparison of differences in cancer incidence of treatment groups compared to control was performed using Fisher's Exact test (Graphpad Prism). Statiscal significance of differences in wet tumor weight, CBC parameters and serum/tissue flavonoid levels between groups was

determined by Student's t-test. Survival curves were generated by the Kaplan-Meier method and comparison of curves performed by the log-rank test.

4.4 RESULTS

Effect of flavonoid consumption in prostate of TRAMP mice

To investigate the effect of DHC, quercetin, fisetin and luteolin on growth of prostate cancer in the TRAMP model, mice were divided into groups fed AIN76A flavonoid-free control or one of three flavonoid supplemented diets: 1% DHC, 1% fisetin or 1% combination (0.25% DHC, 0.25% fisetin, 0.25% quercetin, 0.25% luteolin). Diets were consumed ad libitum from 4-36 weeks of age. At autopsy, gross prostate morphology was examined, genitourinary weight determined and all prostates collected for histological examination.

Gross macroscopic features (Fig 4.1) of the prostates from animals in this study correlated with the histological findings (Fig 4.2). In particular, tumors that were shown to be high grade (undifferentiated) on microscopic examination were associated with a well circumscribed, spherical shaped tumor originating from the dorsolateral lobes on gross examination (Fig 4.1.A). Low grade tumors (adenocarcinoma) on the other hand were associated with a poorly demarcated, and less pronounced enlargement of the dorsolateral lobes of the prostate (Fig 4.1.B). The one exception was an animal in the combination flavonoid group with a high grade tumor microscopically that did not have the gross morphology generally associated with this tumor grade. Histology of the ventral prostate was either of the same or lower grade than tumors of the dorsolateral prostate, but never of higher grade. Therefore, in this study, we only report the histology of the dorsolateral prostate as this reflects the site of most aggressive tumors.

Average wet weight of the genitourinary (GU) tract (prostate, bladder, seminal vesicles) in the control arm was 4.7g (SD, 3.1g). There was a statistically significant reduction in GU wet weight in the DHC group (58.1% reduction, p=0.0082), and a non-significant reduction in the combination flavonoid group (38.1% reduction, p=0.0645) (Fig 4.3.A). No difference was noted in GU weight between the fisetin and control groups. Comparing high grade tumors in different groups, we demonstrated a reduction in tumor weight by approximately 50% in both DHC (p= 0.04) and combination flavonoid groups (p= 0.038), highlighting the effect of flavonoids even on this aggressive phenotype (Fig 4.3.B). A reduction in the weight of low grade cancers was observed only in prostates of mice in the DHC group (65.4% reduction, p=0.006).

Tumor formation was noted in 16 of 17 animals in the control group (Table 4.1). The one animal that did not develop cancer in this group had prostate intraepithelial neoplasia (PIN), a presumed precursor lesion to prostate cancer. The majority of tumors in the control group were low grade (61.1% compared to 33.3% high grade). There was a non-significant trend of reduction in the incidence of prostate cancer in the combination (25.8% reduction, p=0.077), DHC (18.6% reduction, p=0.151) and fisetin (9.2% reduction, p=0.40) groups (Table 4.1). This reduction of tumor incidence was due to a decrease in low grade cancers, with 37% and 51% fewer low grade cancers noted in DHC and combination flavonoid groups respectively (Table 4.1). There was an associated increase in the number of mice diagnosed with PIN in these groups, with 17% and 24% more mice with PIN in the DHC and combination flavonoid groups respectively compared to control. The number of high grade tumors increased slightly in DHC and combination flavonoid groups. Mice on the 1% fisetin diet demonstrated a reduction in high grade cancers despite having no effect on the number of low grade cancers (Table 4.1).

Few TRAMP mice developed liver metastases (1 out of 17 in the control group). Lymph node metastases were more commonly observed than liver metastases (5 out of 17 in the control

group). A non-significant increase in rates of liver metastasis was noted in the fisetin and combination flavonoid groups. No liver metastasis was observed for mice in the DHC group. Small, non-significant reductions in lymph node metastasis rates were noted in all the flavonoid treatment groups compared to control. The size of the prostate tumor did not correlate with the incidence of metastasis, with small tumors associated with lymph node or liver metastases in some animals.

Reduction in expression of proliferative marker (PCNA) in the prostate of TRAMP mice administered flavonoids.

Immunohistochemical staining for proliferation marker, PCNA, was performed (Fig 4.4A). Non-significant reductions in PCNA expression were noted in prostate tissue of TRAMP mice in the DHC (38.7% reduction, p=0.083) and combination flavonoid (42.9% reduction, p=0.087) groups. No reduction in PCNA expression was observed in the fisetin group. While 25% of mice in the control arm had maximal PCNA intensity score of 3, none of the animals in the DHC or combination flavonoid groups, and only 10% of animals in the fisetin group demonstrated this level of expression.

Expression of prognostic tissue marker p27kip1 in the prostate of TRAMP mice supplemented with flavonoids.

Immunohistochemical analysis of prostate tissue was performed to examine expression of the cell cycle inhibitory protein p27 (Fig 4.4.B). Non-significant alterations in the expression of p27 were noted in the DHC (33.3% increase, p=0.3) and fisetin (48.7% increase, p=0.102) groups. A non-significant reduction in p27 expression was noted in the combination flavonoid group compared to control (25% reduction, p=0.63).

Flavonoids accumulate at high concentrations in prostates of TRAMP mice

LC-MS analysis was employed to quantify serum and tissue levels of flavonoids in the control and flavonoid supplemented animals (Fig 4.5 and 4.6). Flavonoids were essentially undetectable in the serum and tissue of TRAMP mice on the control diet. In mice consuming flavonoid supplemented diets, high levels of flavonoid accumulated in the tissues analyzed. The highest levels of flavonoids were observed in the prostate samples, followed by kidney, liver and finally serum. DHC in particular accumulated to very high levels in the prostates of some mice, with a maximum concentration of 34410 ng per gram of tissue noted in one animal on the1% DHC diet. Fisetin did not accumulate to such a high degree, with the highest level of fisetin reached being 11030 ng per gram of tissue. However, in the combination flavonoid group, where the diet contained equal amounts of fisetin and DHC (0.25% w/w), higher concentrations of fisetin than DHC were observed in the prostate, although this was not statistically significant (mean 2866.8 ng/g vs 650.7 ng/g, p=0.32). Relatively low levels of quercetin were observed in prostates of animals on combination flavonoid diet (containing 0.25% quercetin), with mean quercetin levels in prostate tissue in the combination flavonoid group 367.2 ng/g. Luteolin (mean 1112.3 ng/g) accumulated to a higher degree than both quercetin and DHC in the combination flavonoid group (Fig. 4.5).

There was a dose dependent effect with mice consuming 1% DHC having significantly higher DHC prostate levels than mice on combination flavonoid diet consuming 0.25% DHC (means 9736.7 ng/g vs 650.7 ng/g respectively). Similar dose dependent accumulation was noted for fisetin (means 4088.2 ng/g vs. 2866.8 ng/g in 1% fisetin and combination flavonoid groups respectively). There was no apparent correlation between the levels of flavonoids in the prostate or serum of individual TRAMP mice and the histological prostate tumor grade (data not shown).

Body weight and dietary consumption differences between groups

Animal weights were measured biweekly (Fig 4.7). Differences in body weight became apparent as early as 3 weeks after commencement of the diets. TRAMP mice on the 1% DHC diet consistently had the lowest body weights than mice in any other group. Mice in the combination flavonoid group also had lower body weight compared to control, although the decrease was not as pronounced as in the DHC group. Mice consuming 1% fisetin gained more body weight than control animals with differences becoming apparent around 12 weeks after test diets were initiated. At 24 weeks of age, mice in the DHC group weighed 7.16g less than control mice ($p=0.015$), while mice consuming combination flavonoids weight 4.05g less than control ($p=0.076$). Animals in the fisetin group weighed 5.85g more than control mice at 24 weeks ($p=0.041$).

Differences in dietary consumption were also noted between groups. TRAMP mice administered the DHC diet had slightly lower dietary consumption than control (1.1g vs 1.27g diet per animal per day respectively, $p=0.66$). In contrast, mice on fisetin consumed more diet than mice on control (1.66g, $p=0.24$), as did mice in the DHC group (2.11g, $p=0.015$).

Hematological parameters of TRAMP mice administered flavonoid supplemented diets

To examine for potential hematological toxicity, whole blood was collected at time of necropsy (36 weeks) from several animals in each group and complete blood count (CBC) measurements performed (Table 4.2). Mice in the DHC group had slightly higher red blood cell count compared to control (9.76 vs 8.72 respectively, $p=0.054$) and lower mean corpuscular volume (44.65 vs 46.9 respectively, $p=0.003$). All other parameters were otherwise normal in the DHC group. No abnormalities in CBC were noted in the fisetin and combination flavonoid groups.

Effect of Flavonoid supplementation on Survival in TRAMP Mice

Kaplan Meier analysis was performed to compare survival of TRAMP mice on flavonoid enriched and control diets (Fig 4.8). Log-rank test did not demonstrate a survival advantage with any of the flavonoid diets. At 36 weeks of age (study endpoint), 55.56% of mice in the control group were still alive compared to 62.5% in DHC, 68.75% in Fisetin and 69.23% in the combination flavonoid group. However, this alteration in survival at 36 weeks was non-statistically significant ($p>0.05$).

Causes of premature death in the study included deaths from both prostate cancer and non-prostate cancer related causes. In order to examine the effect of the diets on mortality specifically from prostate cancer, we performed survival analysis excluding those mice that died of non-prostate cancer related causes (data not shown). No significant differences in survival were noted between groups in this sub-analysis (log-rank test).

Causes of non-prostate cancer deaths are shown in table 4.3. There were no non-prostate cancer related deaths in the control group. In contrast, there were 3 non-prostate cancer related deaths in the DHC group, 2 in fisetin and 3 in the combination flavonoid group. The non-prostate cancer deaths in the DHC group included two animals that presented with poor feeding, dehydration, weight loss, and lack of grooming. Autopsy performed on these animals revealed a distended small and large bowel, impacted with fecal matter, without any obvious cause of intestinal obstruction. Histological examination of the large bowel revealed a thin intestinal mucosal layer with mild acute inflammatory infiltrate and undigested matter in the lumen. Histology of major organs including liver, kidney, brain, heart, lung, stomach and spleen were normal. Therefore, the precise cause of death in these mice was unclear, but the findings of autopsy are suggestive of an intestinal pathology. The third premature death in the DHC group

was of undetermined cause as the mouse was found dead in its cage with no obvious signs of illness prior to death.

The premature deaths in the fisetin group included one animal that presented with loss of balance and severely abnormal gait suggestive of a neurological abnormality. Autopsy findings in this mouse did not reveal any gross abnormalities and CBC and histological examination of major organs including brain were normal. The second death in the fisetin group occurred in a mouse with general signs of illness including poor feeding, withdrawn behavior, and weight loss. The cause of death in this animal was unknown with no abnormality revealed at autopsy, and histology of major organs was normal. The premature deaths in the combination flavonoid group included one mouse that had to be sacrificed (age 28 weeks) due to a large kidney tumor that was subsequently shown to be of adenocarcinoma origin. The prostate in this mouse was histologically benign. The other 2 deaths in the combination group occurred in mice that presented in the same manner as the DHC mice described above with signs of intestinal distension and inflammation.

4.5. DISCUSSION

Despite the substantial literature on the properties of flavonoids in prostate cancer *in vitro*, relatively few studies have examined the effect of flavonoids in animal models of prostate cancer. In particular, the chemopreventive properties of flavonoids have not been fully addressed, partly due to the lack of suitable prostate cancer animal models for testing putative chemopreventive agents. The TRAMP model has emerged as a valuable tool to study prostate cancer chemopreventive agents. TRAMP mice spontaneously develop gradually worsening malignancy of the prostate epithelium closely represents the human progression of the disease.

In this study, the TRAMP model was employed to examine the effects of 3 previously untested flavonoid diets on prostate cancer chemoprevention. The flavonoids were selected on the basis of previous *in vitro* proliferation assays conducted to screen a diverse group of flavonoids. The flavonoids were used alone (1% w/w DHC and 1% w/w fisetin) or in combination (0.25% DHC, 0.25% fisetin, 0.25% quercetin, 0.25% luteolin). Our findings are consistent with a chemopreventive effect of the 1% DHC and combination flavonoid diet, but not the 1% fisetin diet in prostate cancer of TRAMP mice. This was evidenced by a reduction in prostate tumor size in DHC and combination flavonoid groups (Fig 4.4A and B), and a reduction (albeit non-significant) in the number of mice with histologically defined prostate cancer in these treatment groups (Table 4.1). A desirable property of a chemopreventive agent in addition to reducing the initiation of cancers (primary prevention) is to reduce the progression of these cancers (secondary prevention). The findings of our study are suggestive of a secondary prevention effect of flavonoids in TRAMP mice. There was a slowing of tumor progression in the DHC and combination flavonoid groups as shown by a) the reduction in low grade cancers and increase in pre-malignant PIN lesions in DHC and combination groups, and b) the smaller size of tumors of similar histological classification in the DHC and fisetin groups compared to control. Consistent with these findings, we demonstrated a reduction in proliferation marker PCNA in DHC and combination flavonoid groups ($p=0.083$ and 0.087 respectively) but not in the fisetin group. The absence of an effect on high grade tumors with any of the diets (Table 4.1) suggests that this tumor phenotype is highly aggressive and unlikely to be affected by the administration of flavonoids.

The cell cycle is an important target of flavonoids and multiple mechanisms have been described *in vitro* for the cell cycle inhibitory properties of flavonoids. Previous studies have highlighted an important role for the cyclin dependent kinase inhibitor p27 in the prognosis of

prostate cancer (508). Flavonoids have been shown to up-regulate p27 *in vitro* (362, 445). Although we did not demonstrate a significant alteration in p27 in this study, there was a trend towards up-regulation of this protein in DHC and fisetin groups but not in the combination flavonoid groups.

A major uncertainty surrounding the use of flavonoids in the prevention or treatment of disease in humans is the degree of bioavailability of flavonoids in target tissues. Flavonoids are generally thought to be poorly absorbed, and undergo significant metabolization in the liver resulting in potential inactivation of the compound (509). In this study we addressed this issue by collecting serum and multiple tissue specimens for analysis of flavonoid content by LC-MS. Remarkably, our findings suggest that not only were the administered flavonoids detected in serum and tissue, but also that the prostate gland accumulated doses of flavonoid more than liver or kidney, and several fold more than levels observed in serum. In particular, DHC accumulated to very high levels in prostate reaching a concentration of over 100μM in one animal. The study animals consumed the flavonoid supplemented diets for a prolonged period of time (32 weeks) which may have led to accumulation of the compound in tissues. High variability of the LC-MS measurements can be accounted by the fact that serum samples were acquired from mice at different times from their last feed. When extrapolating these findings to humans, the fact that absorption and metabolism of flavonoids is likely species dependent must be taken into consideration.

A significant level of toxicity of the flavonoid diets was noted in this study (Table 4.3). Several mice (17.6%) in the 1% DHC group developed signs of small and large bowel acute inflammation and atrophy leading to intestinal distention and lack of function. A similar side effect was noted in the combination flavonoid group. Since the combination group also contains DHC at 0.25% w/w, toxicity in this group may have been attributed to the DHC component of

this diet, although the effect of other components such as quercetin and fisetin (both 0.25% w/w) cannot be excluded. Chemotherapeutic agents often adversely affect the highly mitotic epithelial lining of the intestine, and a similar mechanism may account for the toxicity of flavonoids in this study. CBC performed on one of the animals in the combination diet group experiencing this side effect demonstrated a leukocytopenia (WBC 2.0), which indicates a potential toxic effect of this diet on the bone marrow. However, a low WBC count was noted in other animals in the study that survived to the study endpoint without signs of toxicity (Table 4.2). These findings highlight the need to exercise caution in the administration of flavonoids to humans and the need for further assessment of the safety profile of these compounds. This also raises concerns of the safety of flavonoids available as prescription-free 'natural supplements' although no reports of major adverse effects of these supplements have been reported. These supplements are usually consumed at the dose of 1 gram per day, which is a 100 times lower dose than used in this study.

The safety of flavonoids has been the subject of significant controversy for many years with early studies demonstrating a genotoxic effect of flavonoids *in vitro* (243). These studies employed very high doses of flavonoids (>100μM), concentrations that are unlikely to be achieved physiologically with normal dietary intake of flavonoids. However, in this study we have shown the dramatic accumulation of high levels of flavonoids in body tissues with chronic intake of high levels of flavonoids. The 1% w/w flavonoid concentration employed in this study would not be contemplated in humans and was employed here because our study was designed as a proof of principle. Previous studies employing 1% flavonoid diets did not demonstrate toxicity (410). The fact that tumor inhibition in our study was often associated with a low-moderate (<30μM) level of flavonoid in the prostate (data not shown) suggests that a lower dose may be equally efficacious without the toxic side effects.

In conclusion, we have demonstrated the inhibition of prostate cancer progression by 1% DHC and a combination flavonoid diet. Although these diets were toxic to some animals, their observed prostate cancer chemopreventive effects warrant future studies examining the chemopreventive effect of these flavonoids at lower doses. The tumor inhibitory effects of flavonoids demonstrated in this study support a growing body of data on several flavonoids and prostate cancer prevention in TRAMP mice. Finally, the TRAMP model is an aggressive model of prostate cancer. The chemopreventive effect demonstrated for flavonoids in this study may be more pronounced in humans where the natural history of prostate cancer is much more indolent.

TABLE 4.1: Histologic grade of TRAMP prostate tumors

	Number of animals			
	PIN (%)	**Low Grade (%)**	**High Grade (%)**	**Total**
Control	1 (5.6)	11 (61.1)	6 (33.3)	18
DHC	3 (23.1)	5 (38.5)	5 (38.5)	13
Fisetin	2 (14.3)	8 (57.1)	4 (28.6)	14
Combination	3 (30)	3 (30)	4 (40)	10

TABLE 4.2: Hematological parameters in TRAMP mice administered flavonoids

	WBC	RBC	HB	HCT	MCV	PLT
Control	5.04 (2.4)	8.72 (0.55)	131.43 (12.55)	0.42 (0.04)	46.9 (1.14)	1061.29 (241.87)
DHC	7.79 (4.33)	9.76 (1.39)	134.5 (18.17)	0.43 (0.06)	44.65 (1.53)	949.9 (257.66)
Fisetin	3.75 (2.3)	8.59 (1.16)	127.63 (15.47)	0.41 (0.04)	47.9 (2.93)	860.5 (333.23)
Combination	6.07 (3.3)	8.58 (2.7)	124 (36.5)	0.39 (0.11)	46.13 (2.51)	964.33 (285)

Mean values (Standard deviation).

Table 4.3: Causes of Non-prostate cancer mortality in TRAMP mice on flavonoid supplemented diets

ID #	**Diet**	Clinical signs	**Cause of death**
140	DHC	poor feeding, dehydration, weight loss, distended small and large bowel	Intestinal toxicity
142	DHC	poor feeding, dehydration, weight loss, distended small and large bowel	Intestinal toxicity
146	DHC	poor feeding, dehydration, weight loss, distended small and large bowel	Intestinal toxicity
149	DHC	None	Unknown
160	Fisetin	poor feeding, dehydration, weight loss,	Unknown
224	Fisetin	Loss balance	Unknown
217	mixed	poor feeding, dehydration, weight loss, distended small and large bowel	Intestinal toxicity
220	mixed	poor feeding, dehydration, weight loss, distended small and large bowel	Intestinal toxicity
244	mixed	Kidney tumor	Adenocarcinoma unknown origin

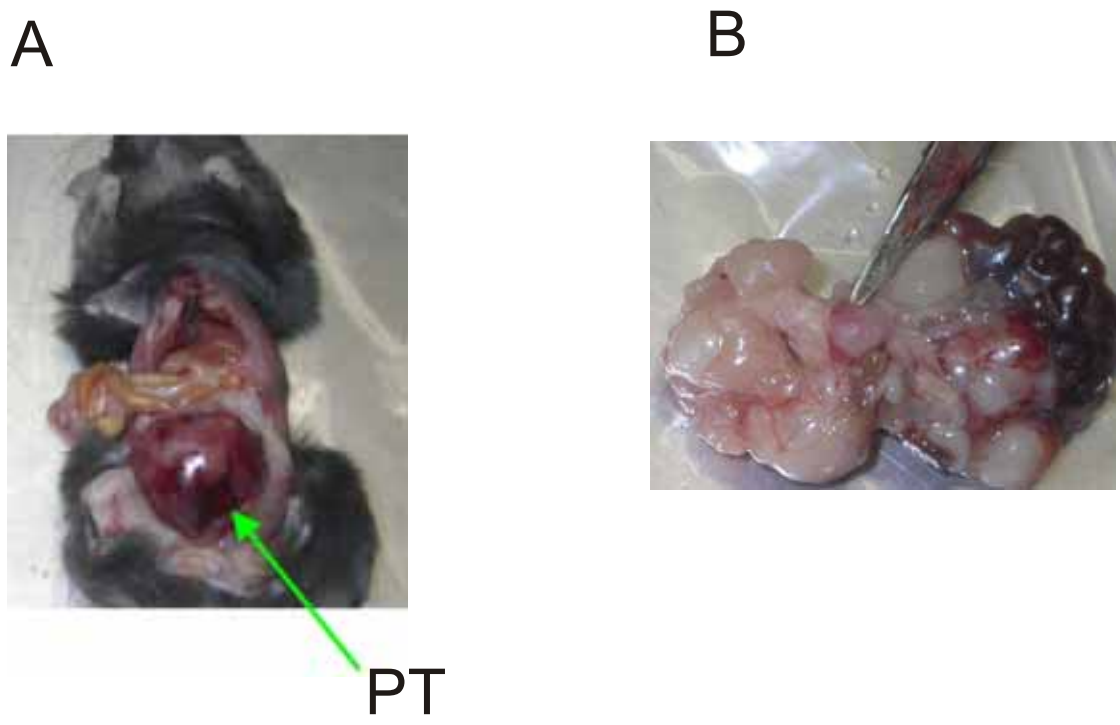


Figure 4.1. Gross morphological appearance of tumors arising in the dorsolateral prostates of TRAMP mice on control diet at 36 weeks of age. Two type of tumor developed: A) a large, well encapsulated, spherical shaped adenocarcinoma and B) a less well circumscribed, locally invasive adenocarcinoma.

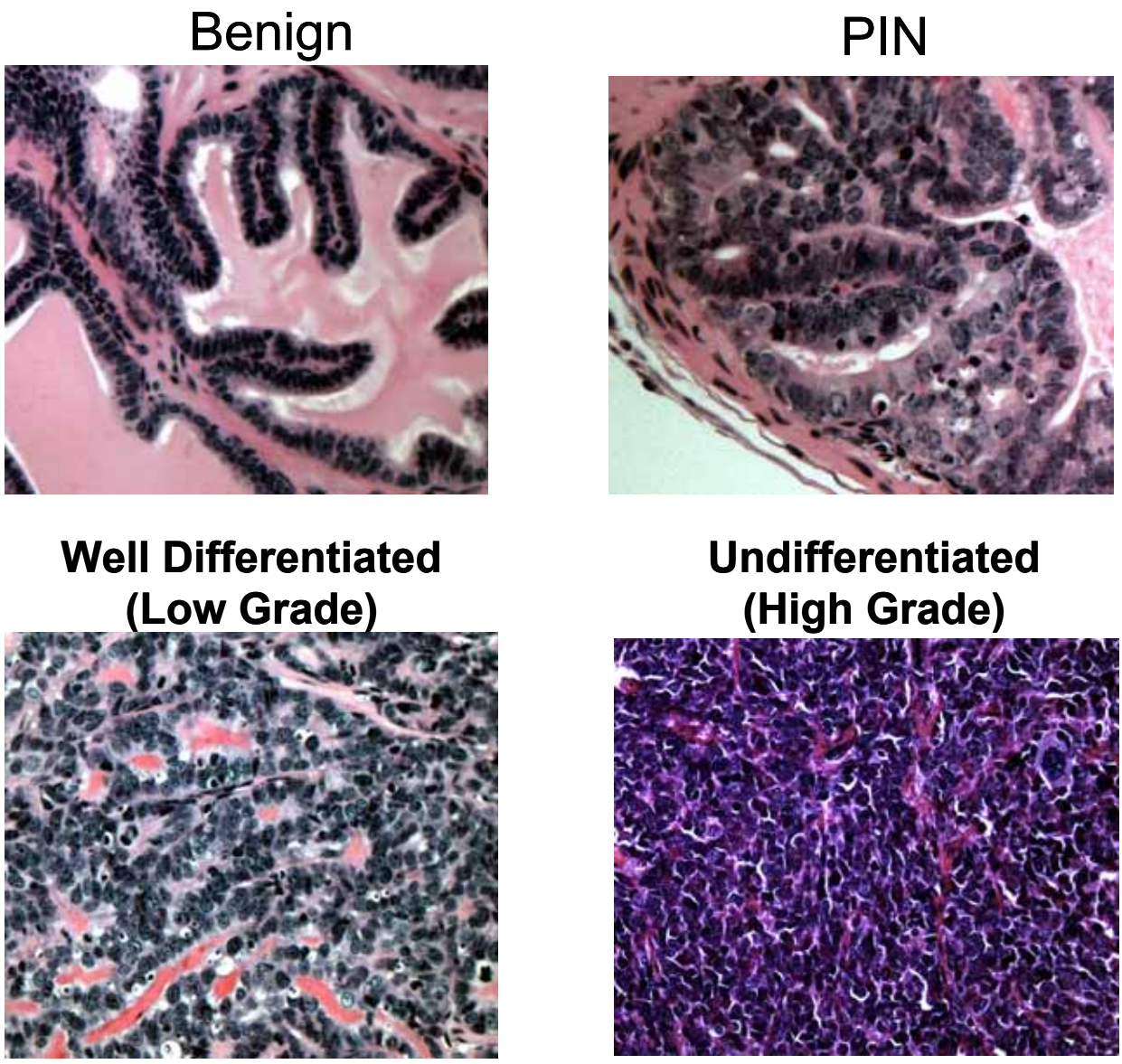


Figure 4.2. Histological appearance of TRAMP dorsolateral prostate tissue (H&E stain). Prostate tumors in TRAMP mice in the study demonstrated 3 main histological types: prostatic intraepithelial neoplasia (PIN), well differentiated adenocarcinoma and undifferentiated. A benign specimen from a CD57-BL mouse lacking the SV40-T antigen is shown for comparison. None of the TRAMP mice in the study had benign tumors at 36 weeks of age.

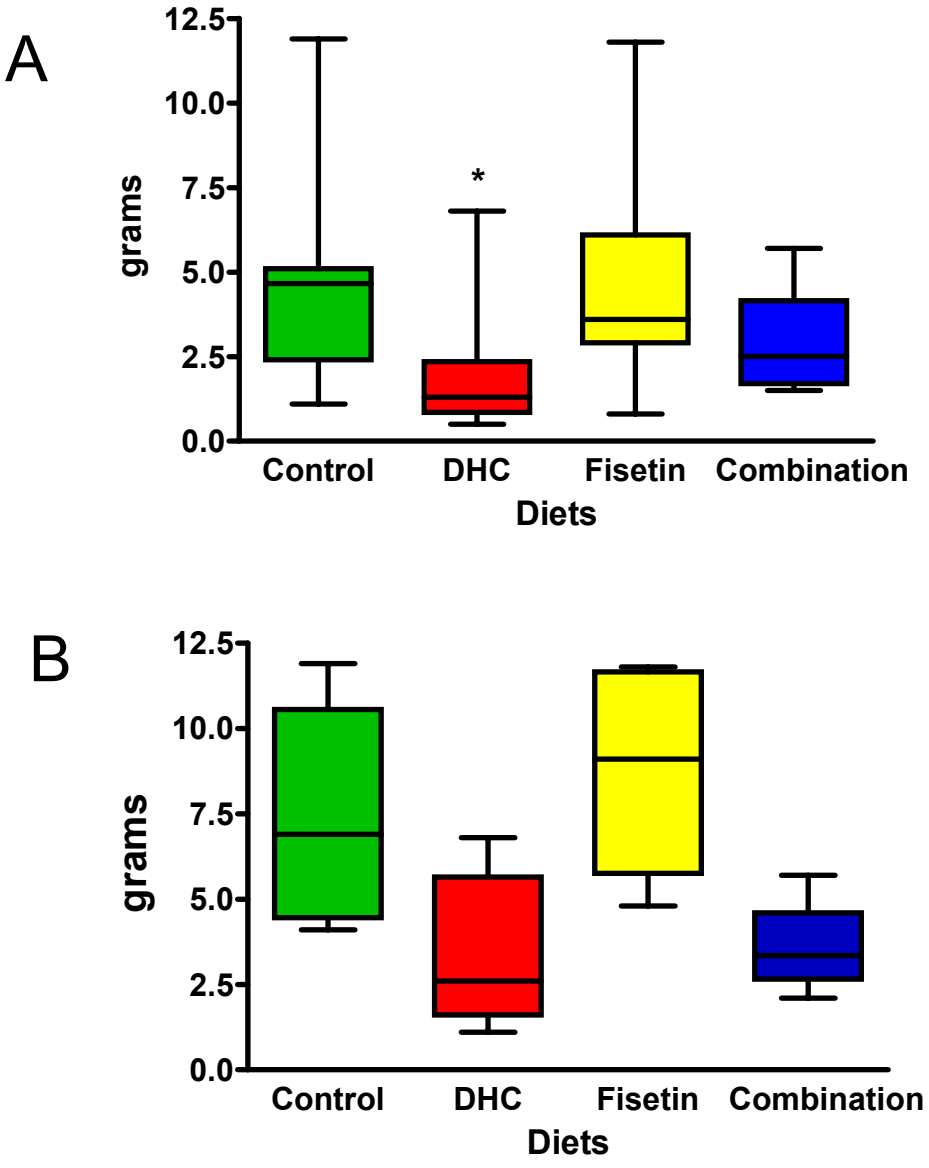


Fig 4.3. Genitourinary weight (prostate, seminal vesicles, empty bladder) in TRAMP mice in each dietary group. A) All tumors, B) Undifferentiated/ high grade tumors only. * C vs DHC $p<0.05$.

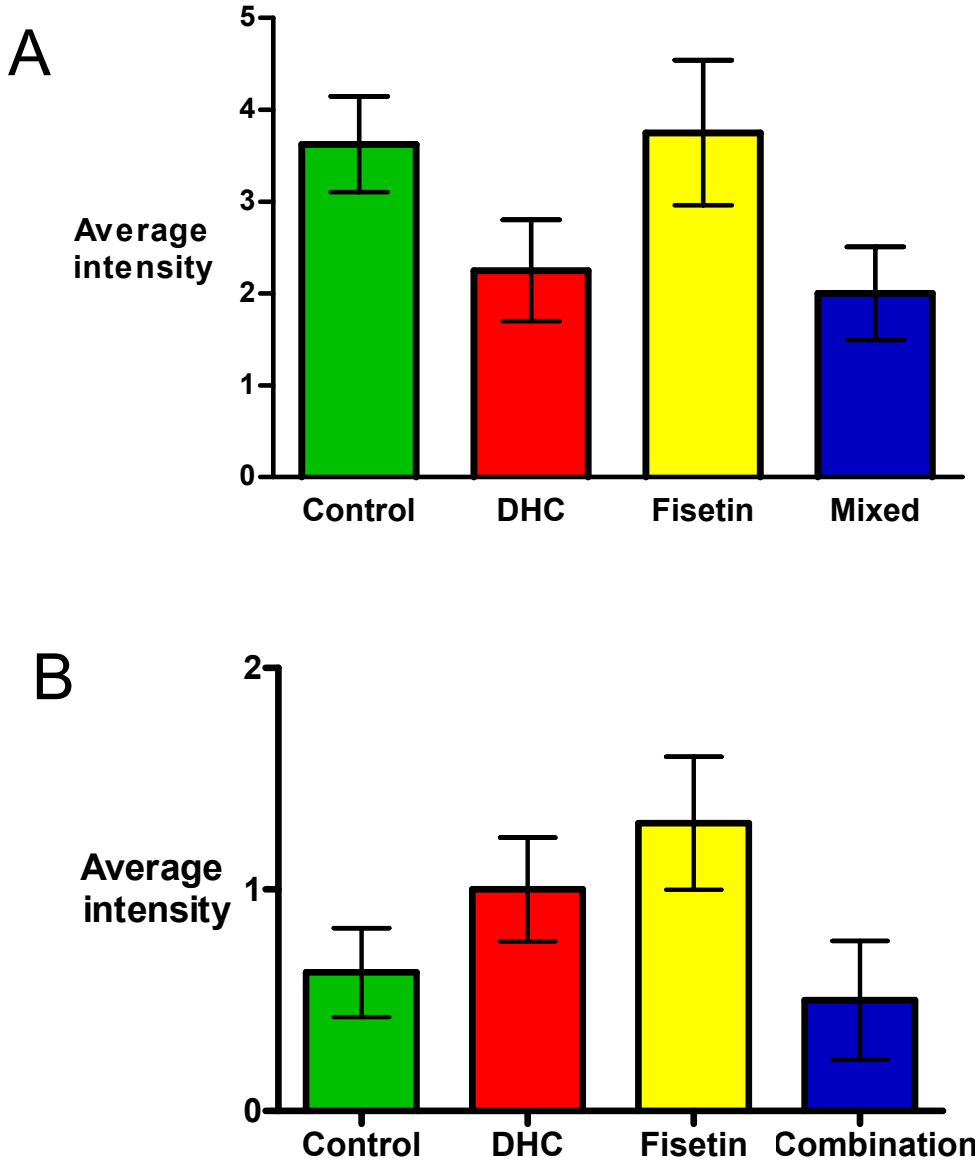


Fig 4.4. Immunohistochemical analysis of cell cycle markers A) PCNA and B) p27 in dorsolateral prostate tumors of TRAMP mice in each treatment group. A non-statistically significant down-regulation of PCNA expression is noted in DHC and mixed flavonoid groups. Non-significant increases in p27 expression are noted in DHC and fisetin groups.

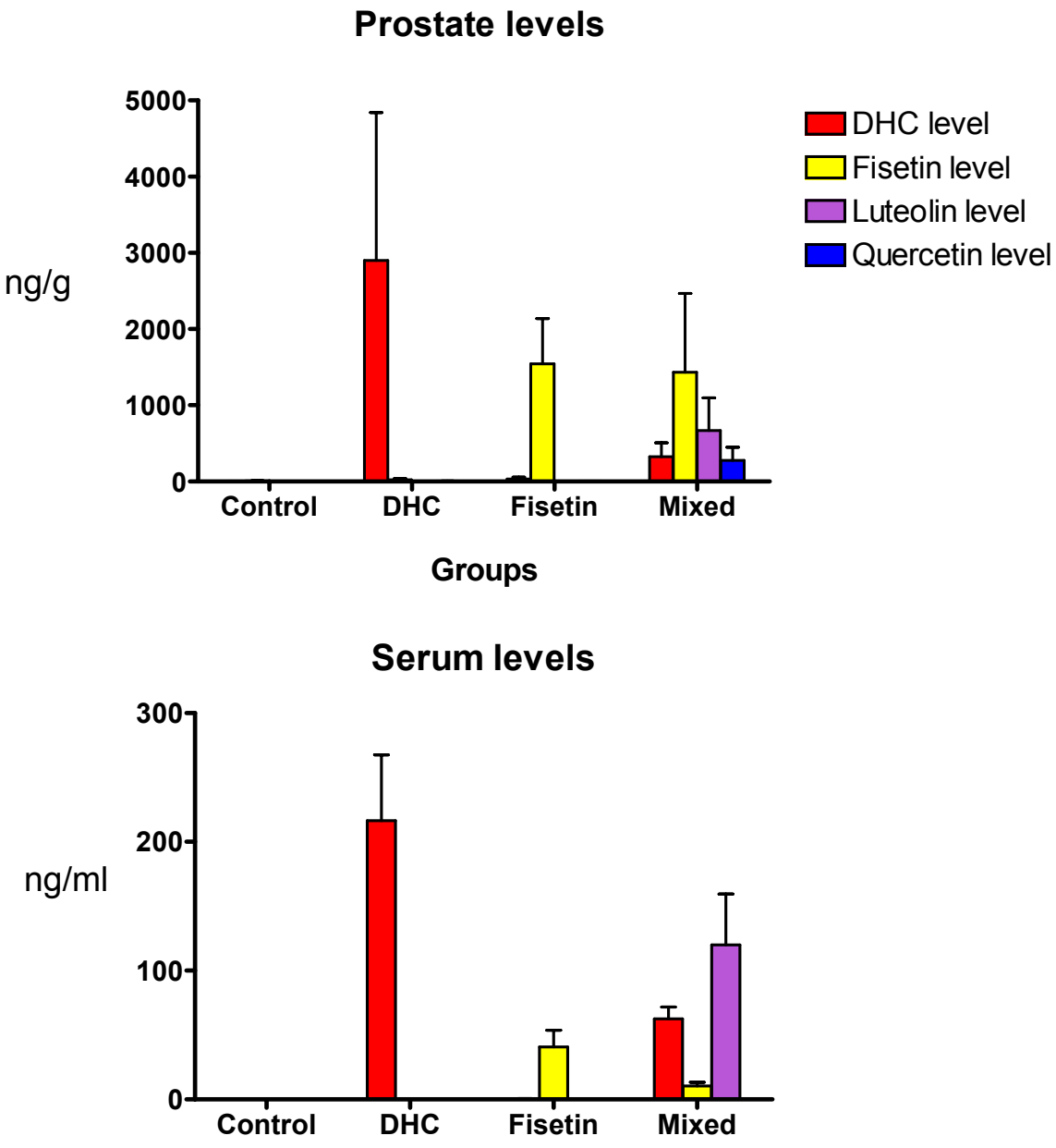


Figure 4.5. Liquid chromatography-mass spectrometry analysis of flavonoid levels in dorsolateral prostate tissue and serum collected from TRAMP mice at 36 weeks of age. The highest accumulation of flavonoids in all tissues tested was observed in prostate. Tissue levels expressed as nanograms per gram of tissue.

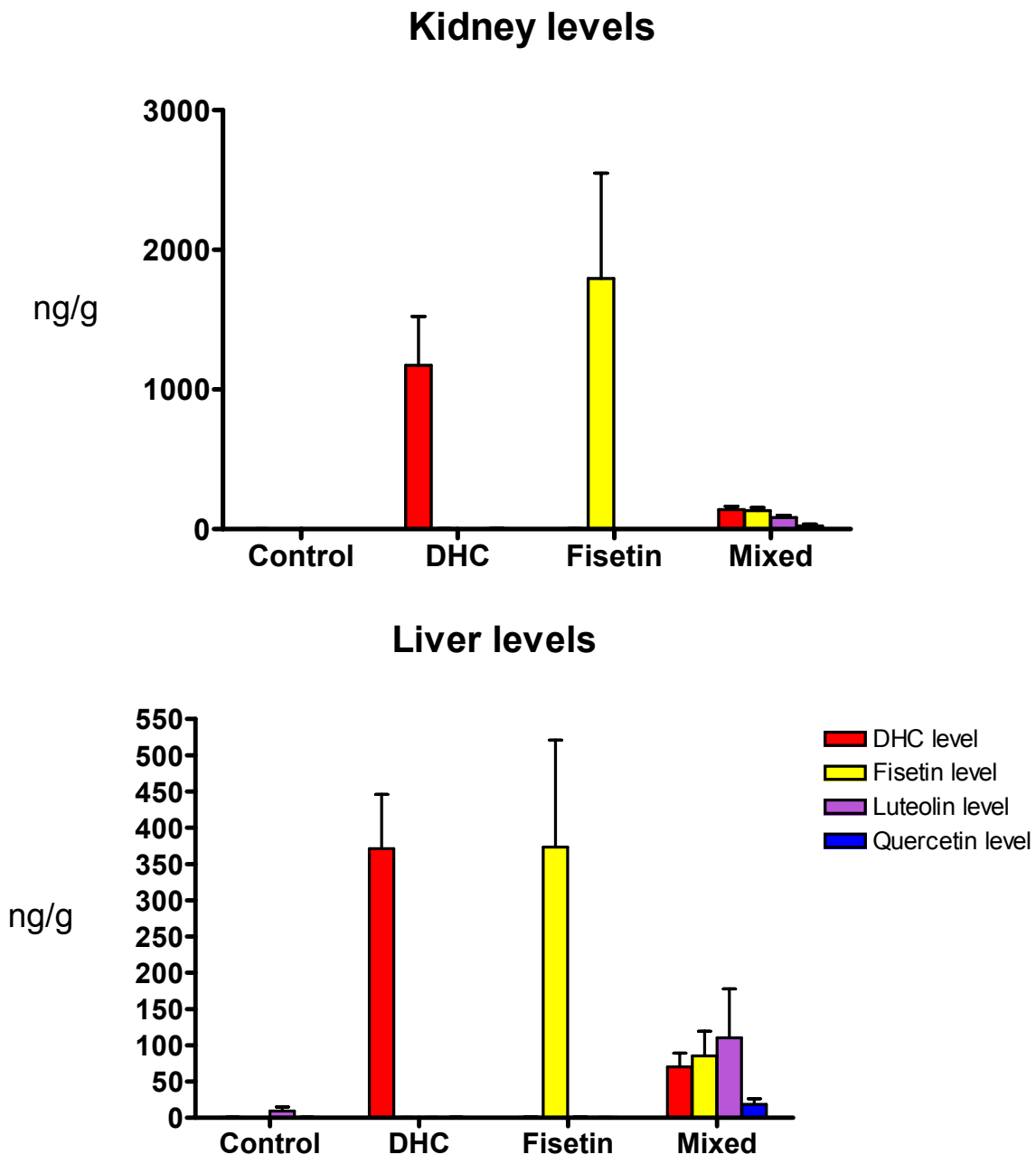


Figure 4.6. Liquid chromatography- mass spectrometry analysis of flavonoid levels in liver and kidney tissue of animals collected at 36 weeks of age. Kidney accumulated flavonoids at higher levels than liver for all flavonoids tested. Tissue levels expressed as nanograms per gram of tissue.

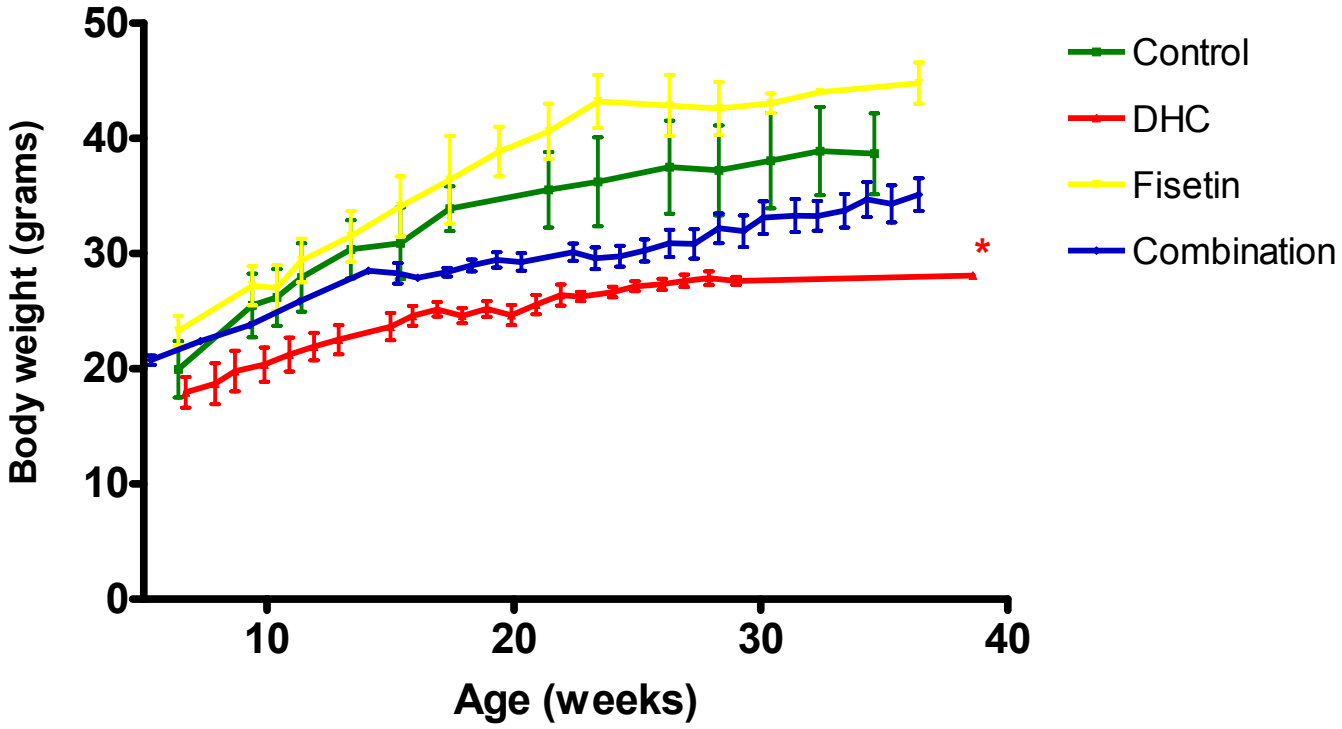


Fig 4.7. Average body weight of TRAMP mice in each dietary group. A significant reduction in the body weight of mice in the DHC group vs control is observed (*$p<0.01$).

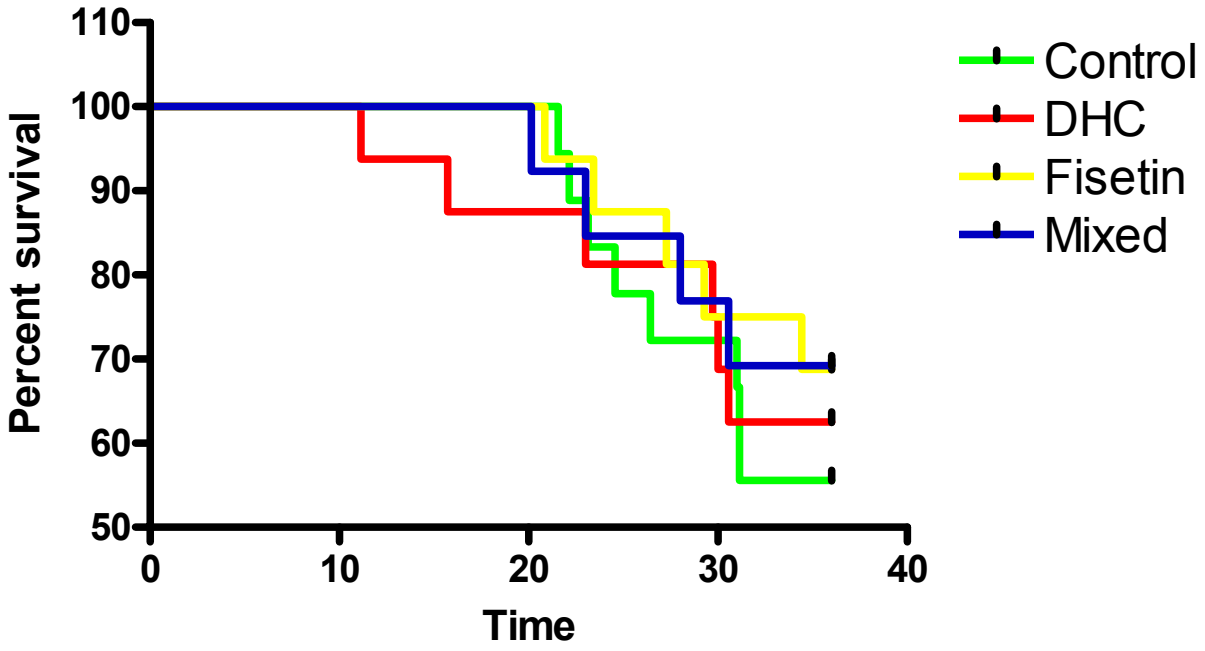


Figure 4.8. Overall survival of TRAMP mice in study. No statistically significant difference in survival was noted in any of the flavonoid treatment groups compared to control.

CHAPTER 5

GENERAL DISCUSSION & FUTURE DIRECTIONS

5.1 Summary of experimental work

Flavonoids are a class of phytochemicals with remarkable chemical and biological properties. Since ancient times humans have exploited the therapeutic properties of medicinal plants, many of which have been found to have flavonoids as their primary active constituent. Research on the health benefits of flavonoids over the last century has elucidated the effects of flavonoids as antioxidant, anti-inflammatory, anti-microbial, cardioprotective, neuroprotective and anti-cancer compounds.

Their proposed chemopreventive properties in prostate cancer have been the focus of considerable interest stemming from epidemiological observations of their protective effect in prostate cancer. Population studies have correlated the low incidence of prostate cancer in regions of the world such as China and Japan with high flavonoid consumption in these populations (mainly due to the intake of soy and green tea). The outcomes of numerous case-control and cohort studies further support the association of flavonoid intake and reduced prostate cancer risk.

Current dilemmas and deficiencies in the medical and surgical treatment of prostate cancer have necessitated the utilization of prevention strategies to handle the public health burden of this common malignancy. The slow progression of prostate cancer over many decades provides a considerable window of opportunity for the action of chemoprevention drugs. A recent randomized controlled trial has underscored the suitability of this approach in prostate cancer, although the agent used in this ground-breaking study (finasteride) is limited by a number of side effects, and the cause of the observed increase in high grade cancers in the study is an issue that remains controversial (24). Thus the door is open for the use of novel agents in the chemoprevention of prostate cancer. Flavonoids hold considerable promise in this regard,

supported by both epidemiologic and experimental evidence of their putative anti-cancer properties.

Over 9000 flavonoids have been identified in plants and many more variants on the basic flavonoid structure can be chemically manufactured. Despite the tremendous abundance of unique flavonoids, research into the health benefits of flavonoids has focused on a remarkably select group of flavonoids. As a proportion of the huge number of structurally diverse flavonoids, the molecules that have been studied is trivial. This diversity presents great possibilities for the identification of novel compounds with unique biological properties. There is an enticing opportunity to discover flavonoids with greater potency than those currently under investigation.

The research undertaken in this thesis was designed to:

1. Identify novel flavonoids with potent effects in the inhibition of prostate cancer cell proliferation and determine the modalities of cell death induced by flavonoids
2. Determine the molecular effects of flavonoids on cell cycle regulation focusing on regulation of cell cycle gene expression by flavonoids
3. Examine the effects of select flavonoids as prostate cancer chemopreventive agents in an *in vivo* transgenic model of prostate cancer (TRAMP).

Chapter 3 detailed the cellular proliferation dose-response of a group of 35 flavonoids, whose members encompass all major flavonoid groups. Proliferation assays were conducted in prostate cancer and non-prostate cancer cell lines. We included flavonoids that had been examined by others in prostate cancer as controls. Dramatic differences in cytotoxicity between

flavonoids were noted despite only minor structural alterations. Although the screen was not large enough to establish a comprehensive structure-activity relationship, a few structural features were apparent that clearly influenced the ability of the flavonoids to alter proliferation. Several compounds were identified that inhibited prostate cancer cell proliferation with IC_{50}<20μM, a physiologically achievable dose. Many of these compounds exerted their effect selectively in prostate cancer cells, and did not demonstrate growth arrest in non-prostate cancer cell lines.

Two novel flavonoids identified were selected for further mechanistic studies: 2,2'-dihydroxychalcone (DHC) and fisetin. DHC was the most potent flavonoid identified in the proliferative screen, while fisetin, although less potent than DHC, was of interest owing to its apparent selective effect on prostate cancer cell lines. Both compounds at their IC_{50} dose were effective at inhibiting clonogenicity of prostate cancer cells in culture. Determination of the mechanisms of action of DHC and fisetin was achieved by a variety of methods including assessment of morphological alterations, cell cycle alterations by flow cytometry and immunoblot, and alterations in transcription of cell cycle genes by microarray and quantitative real-time PCR (qRT-PCR).

Analysis of the morphology of flavonoid treated prostate cancer cells (LNCaP and PC3) suggested a dose and time-response effect. At doses around the IC_{50} concentration, flavonoid treated cells underwent morphological changes such as chromatin condensation, cellular shrinkage and detachment suggestive of apoptosis. The long term nature of the growth arrest induced by flavonoids was demonstrated on clonogenic assay. Expression of SA-βGAL (senescence associated beta galactosidase) was performed to elucidate the activation of accelerated senescence pathways. Growth arrest was associated with various alterations of cell cycle parameters based on FACS analysis. Flavonoids caused a significant reduction in active DNA synthesis (S-phase reduction), and arrest in G1, S and G2 phase in LNCaP, and S and

G2/M in PC3. Alteration in cell cycle phenotype was noted as early as 6 hours after flavonoid treatment. By labeling cells with anti-phospho-histone 3 (anti-H3P) we were able to distinguish between cells in G2 and mitosis, both of which have 4N DNA content. The G2/M arrest induced by flavonoids was primarily due to cells arrested in G2 and not mitosis. Interestingly, all the flavonoids tested appeared to share a common mechanism of action in terms of cell cycle phenotype, as evidenced by very similar alterations of cell cycle profile on flow cytometry.

Chapter 4 built on these findings by determining the molecular aspects underlying the cell cycle effects of DHC and fisetin. Oligonucleotide array was employed to examine the alterations in global gene expression at 6-24 hours of treatment in PC3 and LNCaP cells. We noted a dramatic inhibition of cell cycle gene transcription by both DHC and fisetin. These flavonoids inhibited the expression of over 50 cell cycle genes at 6-24 hours of treatment. The similarity of effect of DHC and fisetin corroborates the similarity of effect of these compounds on cell cycle phenotype demonstrated earlier. Most of the genes that were inhibited have functions in G2 or mitosis phases of the cell cycle. The broad ranging inhibition of cell cycle genes was not part of a generalized inhibition of gene transcription because 50% of genes on the microarray were up-regulated. qRT-PCR was used as a gold standard to confirm the accuracy of the microarray findings. The correlation of qRT-PCR and microarray was 0.8. Western blotting demonstrated inhibition of cell cycle protein levels for some (e.g. cdc2, cyclin A), but not all inhibited cell cycle genes identified in the microarray study. This may be a result of different half-life of the proteins examined. Other pathways of importance in proliferation and survival were altered by flavonoids. A large group of stress-response genes were up-regulated by flavonoids, as were several MAP-kinase genes. Complex alterations in metabolism gene transcription were also identified. Chapter 4 therefore successfully identified the role of transcriptional regulation of several cellular pathways in the action of flavonoids. In particular, these studies demonstrated

that the inhibition of cell cycle gene transcription is a major mechanism of action of flavonoids in prostate cancer *in vitro*.

In chapter 5, we studied the effect of flavonoids as chemopreventive agents in an animal model of prostate cancer. We examined the effects of 3 flavonoid diets: 1% DHC, 1% fisetin, and 1% combination flavonoids (containing 0.25% each of DHC, fisetin, quercetin and luteloin) and flavonoid free control. The flavonoids were selected from our initial *in vitro* screening experiments. The experimental model used was the TRansgenic Adenocarcinoma of the Murine Prostate- (TRAMP). This autocanthous model of prostate cancer developed by Greenberg et al employs the SV40 large T antigen fused to the rat probasin promoter (for prostate specific expression) in mice of C57Bl/6 background. The model has been used extensively to study the effects of various chemopreventive agents. Mice were administered the diets from weaning, and at 36 weeks of age mice were sacrificed and autopsy and tissue collection performed.

We noted very differing responses of the three flavonoid-enriched diets in prostate tumor progression in our study. DHC, which was the most potent flavonoid identified *in vitro,* demonstrated non-significant reductions in incidence of TRAMP prostate tumors. The effect of this diet may however have been attenuated as a result of toxicity of this flavonoid at the 1% w/w dose as evidenced by loss of weight and increased mortality in these animals. There was a significant reduction in genitourinary weight, and weight of high grade tumors in this group. Similarly, the combination flavonoid diet demonstrated significant reduction of prostate tumor size and a non-significant reduction in tumor incidence histologically. Dietary supplementation with 1% fisetin was ineffectual but did not demonstrate signs of systemic toxicity. We did not identify significant alterations of cell cycle markers PCNA and cyclin A. Critically, we demonstrated the presence of flavonoid in target tissue. High performance liquid chromatography

and mass spectrometry analysis confirmed high levels of unaltered flavonoid in TRAMP prostate tissue.

In summary, in this research project we have identified novel flavonoids that at physiologically achievable concentrations induce cell cycle and prolonged growth arrest and apoptosis in prostate cancer cells *in vitro*. This is associated with gene expression alterations in several key proliferative pathways, in particular, the inhibition of a large number of cell cycle genes with functions in G2 and mitosis phases of the cell cycle. These alterations are also reflected at the protein level. In the *in vivo* studies, we showed that dietary consumption of flavonoids by TRAMP mice resulted in high levels of flavonoid in target tissue (prostate). Finally, consumption of a diet enriched with a combination of flavonoids from age 4 weeks was successful in inhibiting prostate cancer progression in TRAMP mice.

5.2 Implications of Experimental Findings

5.2.1 Implications of *in vitro* and *in vivo* findings to prostate cancer chemoprevention in humans

A shortcoming of the studies presented in this thesis is the use of *in vitro* models that may not necessarily replicate the effect of flavonoids in humans, and that may not be ideal models for the study of the effects of flavonoids as chemopreventive agents. The rationale for employing a cell culture model was the necessity for a convenient method to screen a large number of compounds in several cell types. While such an analysis could theoretically be performed in an animal model, the large numbers of animals needed would be restrictive. We therefore deemed that it would be reasonable to initially perform basic mechanistic studies on prostate cancer cells grown in culture. Since prostate cancer is a disease that develops at a young age but is not

clinically apparent until later in life, it could be argued that chemopreventive agents for prostate cancer are required to inhibit the growth of established malignancy. This further justifies the use of malignant cell lines in our studies.

Testing flavonoids *in vitro* does have several limitations. Firstly, flavonoids are extensively metabolized in humans. Ingestion of a flavonoid will result in multiple modifications and the compound eventually reaching the target tissue may be dramatically different from the compound ingested. Thus, even though a flavonoid may have considerable biological effect *in vitro*, the same compound may have no effect *in vivo* owing to the extensive modification of the compound *in vivo*. In the same light, an ingested flavonoid may also gain activity by undergoing metabolization, although this is not expected to be common since most modifications are performed by detoxifying enzymes with the aim of reducing the effect of flavonoids which are seen by the body as a toxic substances. Secondly, since the anti-/pro-oxidant effects of flavonoids are believed to account for the effects of flavonoids *in vivo*, it is important to note that the redox environment in cell culture is dramatically different from that seen *in vivo*.

The disparity between the effect of flavonoids *in vitro* and in the human situation is highlighted to a certain extent by findings in our studies. The case in point is the flavonoid myricetin which was shown to lack significant anti-proliferative effect against LNCaP and PC3 cells in vitro. In contrast, this flavonoid is one of a few flavonoids that have been associated with reduced prostate cancer risk in epidemiologic studies. Hertog et al demonstrated the association of myricetin with reduced prostate cancer risk in a Scandinavian population. In their study, flavonoid consumption was estimated by food frequency questionnaire and not by serum or tissue analysis. Without direct measurements in target tissue of interest, the direct effect of myricetin cannot be confirmed. Since flavonoids are extensively metabolized, a metabolite of myricetin may have accounted for the chemoprevention effect. Also, since Hertog et al only analyzed a

limited number of flavonoids, myricetin may simply be a marker for a more active flavonoid in the diet. These factors may have all contributed to the different effects of myricetin noted in different models.

5.2.2 Structure-activity relationships of flavonoids

A key question with respect to flavonoids is the relationship between structure and activity. Are the different flavonoids likely to act by different pathways/mechanisms, or by a common pathway? If the latter, the search for different flavonoids with activity is less likely to be fruitful, since ingesting one widely available flavonoid like quercetin, would be as effective as ingesting another. In fact, the answer is rather complex, with evidence suggesting that flavonoids have both similar and differing actions depending on structure. Flavonoids of a broadly similar structure act through similar pathways. On the other hand, flavonoids with major differences in functional side-groups have widely varying mechanisms of action. For example, most flavonols that possess only hydroxyl functional groups modulate cell cycle in a fashion that is broadly similar and dependent more on the cell line than the flavonoid tested. Although the type of cell cycle response in this instance is similar between flavonoids, the potency of each flavonoid in eliciting the response is as varied as their structures. However, we noted considerable differences in morphology in cells treated with closely related flavonoids that otherwise had similar cell cycle effects (e.g. quercetin and fisetin). This suggests that differences in as yet unidentified pathways may exist between these closely related compounds. These flavonoids may differ in pathways that have not yet been examined. Much like pharmacological agents, slight differences in flavonoid structure may translate into important biological differences *in vivo*.

By screening a large number of flavonoids for anti-proliferative effect *in vitro*, we were able to discern certain structural features of flavonoids that influence their degree of activity in

prostate cancer cells. Structural features that were of importance included the presence of C2-C3 double bond in the C-ring, which was necessary for cytotoxic effect in our model. Compounds that lack this double bond, such as the flavanones (e.g. naringenin), had only weak cytotoxic effect if any against prostate cancer cells. This is consistent with previous reports of the lack of activity of flavanones as enzyme inhibitors or anti-inflammatory agents.

Another interesting finding was the lack of activity of glycosylated flavonoids. This has been noted by others, and glycosylated flavonoids, broadly speaking appear to lack biological activity until they are deglycosylated. The significance of this finding lies in the fact that almost all flavonoids in nature are glycosylated. This builds on previous reports demonstrating the effect of glycosylation on reducing the biological activity of flavonoids. Glycosylation in plants is proposed as a protective mechanism that allows high levels of flavonoids to accumulate without causing toxicity in plant organs. This further highlights the effect of this modification in altering the activity of flavonoids. The fact that virtually all flavonoids in nature are glycosylated does not conflict with the biological activity of flavonoids in humans since it has been demonstrated that the sugar moiety of flavonoid glycosides is usually removed in the digestive tract prior to absorption. The type of glycosylation is important, as it has been shown that flavonoid-rutinosides as opposed to glucosides are poorly deglycosylated in the human gastrointestinal tract, and may therefore have considerable influence on bioavailability. Thus, most flavonoids are absorbed as aglycones, which according to our findings, is the active form of flavonoids in mammalian cells.

Interestingly, 3 of the 4 most potent flavonoids identified in the proliferation screen were chalcones. These compounds are precursors to other flavonoids and lack the central ‘C’-ring. Thus the presence of a ‘C’ ring does not appear to be crucial to the anti-proliferative effect of flavonoids, and indeed may reduce the activity of the compound. The only chalcone that did not

have significant anti-proliferative effect was the highly methoxylated and hydroxylated compound 2'-hydroxy-2,4,4',5,6'-pentamethoxy-chalcone. In contrast, another highly methoxylated chalcone, 2',4,4',6'-tetramethoxychalcone, was actually the second most potent against the LNCaP cell line. This illustrates how very slight modifications in flavonoid structure can have remarkable effects on biological effect of the compound. Methoxylation also affected solubility in DMSO, such that many methoxylated flavonoids were untestable due to poor solubility.

Hydroxylated flavones and flavonols, which are ubiquitous in nature, such as quercetin, luteolin and fisetin, tended to have moderate anti-proliferative effects (IC_{50} 20-40μM). However, greater than 5 hydroxyl groups appeared to lower their anti-proliferative effects. As mentioned earlier, the most widely studied flavonoids include the soy isoflavones (genestein), the green tea catechins, milk thistle flavonoid (silibinin), and red wine anthocyanidins. Previous studies have examined the effect of genestein and silibinin in LNCaP and PC3. The IC_{50} in these studies (72hrs treatment) were 40μM (genestein in LNCaP), 43μM (genestein in PC3), >60μM (silibinin in LNCaP) and >30μM (silibinin in PC3). In our study, we identified 9 flavonoids with IC50<30 all of which therefore demonstrate greater cytotoxicity *in vitro* than genestein and silibinin. In our study, we also examined the effect of the green tea flavonoid epigallocatechin (EGC). This compound has weak cytotoxic effect- IC_{50} not reached at up to 100μM of EGC. Thus, while green tea flavonoids have received great attention for their presumed chemopreventive effects, the in vitro effects of EGC did not indicate a direct cytotoxic mechanism. Another flavonoid with poor in vitro activity was pelargonidin. This dark purple anthocyanin is a component of berries and red wine. Despite the presumed chemopreventive effect of wine anthocyanidins, the in vitro effect of pelargonidin was lacking. A possible explanation is the different form of anthocyanidins

occurring in nature. Anthocyanidins are usually found as polymers known as pro-anthocyanidins that release anthocyanidin on hydrolysis. We did not test any pro-anthocyanidins in our study.

5.2.3 Cell cycle checkpoint activation by flavonoids as a cancer preventive mechanism

The role of flavonoids in the activation of cell cycle checkpoints is clearly outlined in our studies. Both DHC and fisetin had similar effects on cell cycle gene transcription and cell cycle arrest/checkpoint activation. Evidence presented for other flavonoids including quercetin and luteolin indicate a similar pattern of cell cycle arrest, pointing towards a shared mechanism of action of flavonoids on cell cycle modulation.

The mammalian cell cycle consists of a series of checkpoints, which are essentially signaling pathways that are activated in response to genetic stress such as DNA damage. Activation of these pathways results in various post-translational alterations of cyclins and cyclin dependent kinases leading to arrest of cell cycle progression. Multiple checkpoints can be activated by a single stimulus. DNA damage, for example, can result in the activation of G1, intra-S, and G2/M checkpoints.

In Chapter 4, we described the transcriptional inhibition of over 50 cell cycle genes by flavonoids. The inhibited genes have functions primarily in G2 and mitosis phases of the cell cycle. Low levels of key cyclins and cyclin dependent kinases will likely result in slowing or arrest of cell cycle progression. While this mechanism is different from the activation of cell cycle checkpoints by DNA damage, which results in post-translational alterations of cyclins and cdks, the ultimate effect of both is slowing or arrest of cell cycle progression. Although transcriptional inhibition may partially account for the cell cycle arrest induced by flavonoids, other mechanisms cannot be excluded.

Flavonoids, and other planar aromatic compounds are known to intercalate DNA (i.e. bind into the space between 2 adjacent DNA base pairs). DNA intercalation results in unwinding of the DNA helix and can have clastogenic (DNA break inducing) effects (510). Flavonoids are also known to act as poisons of DNA topoisomerase II, a key enzyme in DNA replication. DNA topoisomerase II regulates DNA topology and unwinding during replication by inducing transient DNA breaks. DNA topoisomerase II poisons result in high numbers of cleaved DNA complexes and permanent DNA strand breaks. These mechanisms of DNA strand break induction by flavonoids can result in classical cell cycle checkpoint activation. Thus, while we propose transcriptional inhibition of cell cycle genes as a mechanism for cell cycle arrest, DNA damage checkpoint activation by flavonoids may also be a factor.

Cell cycle checkpoints exist to maintain the fidelity of DNA replication and ensure error-free transmission of DNA from mother to daughter cells. Checkpoint activation delays cell cycle progression to provide time for DNA repair mechanisms to take effect. In normal cells cell cycle checkpoints are not normally activated. However, in pre-neoplastic lesions the activation of DNA damage checkpoints is more pronounced (368). This is associated not only with DNA damage, but also with various oncogenic stimuli such as abnormal expression of cyclin E and retinoblastoma (Rb) protein (368). The activation of DNA damage checkpoints was therefore proposed as a barrier to progression of preneoplastic lesions and genetic instability. In our studies, we did not specifically examine the DNA damage checkpoint, however, we did demonstrate cell cycle arrest at multiple phases of the cell cycle secondary to the molecular alterations in transcription of cell cycle genes. We propose that activation of cell cycle checkpoints by flavonoids may act as a barrier to progression of early prostate cancer lesions. The cell cycle arrest induced by flavonoids acts as a safety mechanism to allow for DNA repair mechanisms to correct abnormalities in DNA such as double strand breaks that are commonly

seen in the progression from normal to pre-malignant transformation. This mechanism may account for the chemopreventive effect of flavonoids seen in populations consuming high levels of flavonoids.

5.2.4 The role of p53 in the cell cycle effects of flavonoids

The tumor suppressor protein p53 is a key mediator of DNA damage response and cellular stress response pathways. p53 is phosphorylated and stabilized by ATM/ATR and CHK1/CHK2 kinases in response to DNA damage which leads to induction of p53 target p21, a key cdk inhibitor protein. p21 accumulation results in inhibition of cyclin-cdk complexes necessary for G1 to S transition which ultimately leads to G1 arrest. p53 can also induce intra-S and G2 checkpoints, however unlike the G1 checkpoint, a functioning p53 is not a requirement for these checkpoints. Several non-p53 related factors are involved in the intra-S and G2 checkpoint.

Although our studies did not specifically examine the role of p53 in flavonoid induced cell cycle arrest, and p53 alteration was not noted on microarray, several lines of evidence from our findings point to a possible role of p53 in the mechanism of action of flavonoids. Firstly, differences in the cell cycle arrest pattern induced by flavonoids in LNCaP compared to PC3 cells can be explained by differences in p53 status of these cell lines. Flavonoids failed to induce a G1 arrest in the p53-nonfunctioning cell line, PC3, whereas a clear G1 arrest was noted in LNCaP cells expressing wild-type p53. Since p53 is a critical mediator of G1 checkpoint activation, the G1 arrest induced by flavonoids in LNCaP could conceivably be a p53-dependent effect. The induction of S and G2/M arrest in PC3 cells expressing non-functioning p53, demonstrates that induction of these checkpoints by flavonoids is p53-independent. Secondly, we demonstrated up-regulation of a number of genes known to be down-stream p53 transcriptional targets including

p21, Gadd45A, and 14-3-3. However, flavonoids have been shown to induce some of these genes in both a p53-dependent and independent manner. In summary, p53 activation by flavonoids is probably important in G1 arrest but is not necessary for the S and G2/M arrest and other cytotoxic effects of flavonoids. Precisely how flavonoids activate p53 is unknown, but it may simply be a response to DNA damage induced by flavonoids.

Findings from our gene expression analysis highlighted the importance of other stress-response genes in flavonoid action. Several genes involved in endoplasmic reticulum stress, hypoxic stress and heat-shock were up-regulated by DHC and/or fisetin. The direct association between these stress response genes and cell cycle arrest is far from clear since the cell cycle effects of most stress response genes are poorly understood. However, previous studies stress response genes such as GADD45 and GADD153 that have shown direct regulatory action on the cell cycle point to the possible role of other stress-response genes in cell cycle regulation.

5.2.5 Terminal Growth Arrest and Chemoprevention by Flavonoids

Closely related to the processes of cell cycle arrest and checkpoint activation is the phenomenon of terminal growth arrest. Both DHC and fisetin were shown to induce a prolonged growth arrest lasting up to 2 weeks in PC3 cells growing in colony forming assay. Accelerated cellular senescence is a related phenomenon and the distinction between prolonged cell cycle arrest and senescence is rather vague with both processes sharing a number of common molecular features. The defining features of senescence are: permanent cell cycle arrest, lack of response to mitogenic stimuli, dramatic changes in chromatin structure and gene expression, enlarged and flattened morphology with increased adherence, and expression of senescence associated beta galactosidase (SA-βgal). Expression of SA- βgal was not induced by flavonoids in our study.

Similar to apoptosis, terminal growth arrest is a cellular defense mechanisms that limit further proliferation of stressed or damaged cells. While apoptosis has gained tremendous interest as anti-cancer process, terminal growth arrest and cellular senescence are emerging as equally critical mechanisms in the body's defenses against tumorigenesis. Many chemotherapeutic drugs have been shown to activate the senescence phenotype in cancer cells. In this study, cell cycle arrest, prolonged growth arrest and apoptosis were shown to be the mechanisms of cytotoxicity induced by flavonoids. Future studies will need to determine the effect of flavonoids on these cytotoxic pathways *in vivo*. Terminal growth arrest by flavonoids early in the carcinogenic process is likely to translate into effective and beneficial prostate cancer prevention effects. Future human studies should be designed to examine this possibility.

5.2.6 Mitotic kinases as cancer targets- inhibition by flavonoids

Dysfunction of mitotic regulatory genes leads to chromosome instability and is one of the most common abnormalities in cancer cells. Targeted drug inhibition of mitotic kinases is an increasingly adopted strategy of cancer therapy. Modulation of mitotic regulatory genes by flavonoids was noted in our studies. Mitotic regulatory genes constituted the largest sub-group of cell cycle genes whose transcription was inhibited by DHC and fisetin. Despite the inhibition of several key mitotic genes by both compounds, cell cycle analysis by anti-H3P labeling and flow cytometry did not demonstrate mitotic arrest. Rather, cells were arrested in S and G2 phases (and G1 in LNCaP). The effective inhibition of these cell cycle phases by flavonoids may have limited the progression of cells into mitosis and may account for the absence of mitotic arrest. Future studies can explore whether mitotic arrest is induced if cells are forced to exit pre-mitotic phases of the cell cycle. The down-regulation of key mitotic genes is a further mechanism inhibiting proliferation of flavonoid treated cells.

5.2.7 Prostate cancer specificity of flavonoids

The prostate cancer specificity of flavonoids demonstrated in our studies is a desirable effect. The mechanism of the apparent predisposition of prostate cancer cells to flavonoid induced cytotoxicity remains unexplained. Non-malignant cell lines in our study were generally resistant to flavonoids. Although DHC did have cytotoxic effect in non-cancer cell lines, the IC_{50} was higher than that seen in prostate cancer cells. The prostate specificity of the flavonoids tested cannot be definitely concluded, as this would require testing the flavonoids against a larger array of cell lines. Furthermore, since the media used to grow the prostate and non-prostate cells differed, effects of components in the media may have accounted for the apparent lack of effect on non-prostate cells. However, the same medium was used to grow BPH-1 (benign) and LNCaP (prostate cancer) thereby excluding medium effects in comparing those cell lines. A final factor to consider is the doubling time of different cell lines. PC3 is a much more aggressive cell line compared to LNCaP with doubling time of 18hrs vs 26hrs. Differences in doubling time could account for differences in dose needed to achieve the same anti-proliferative effect.

Prostate specificity was also noted in the animal studies we performed, where the highest levels of flavonoids in body tissues was noted in the prostate. We can only speculate that the prostate may contain unique proteins or receptors that constitute binding sites for flavonoids. The low levels of flavonoids in other tissues such as the liver and kidney may have accounted for the lack of toxicity in these organs in the mice consuming the mixed flavonoid diet.

The proposal that flavonoids be administered over many years as a chemoprevention program means that both normal and early malignant cells will be exposed to moderate levels of flavonoids over a prolonged period. It is possible that the mechanisms of cell cycle arrest, apoptosis, stress response, and transcriptional inhibition may be deleterious to normal

proliferating cells. What is critical to determine is whether cells that have already accumulated DNA damage and have initiated the carcinogenic process are in any way more susceptible to flavonoid action. Several changes to the normal physiology such as increased membrane permeability, pro-oxidant or genotoxic stress may make these transformed cells more likely than normal cells to undergo cell cycle arrest in response to flavonoid administration. Although flavonoids at low doses are non-toxic, safety of these compounds has not been determined for consumption over a period longer than a few months. It will be important to establish doses of flavonoid that induce growth arrest in pre-neoplastic cells while not interfering with the normal cell cycle of non-malignant cells.

5.3. Conclusions and future directions

Attempting to decipher the mechanism of action of flavonoids in cancer and the utility of flavonoids in human cancer chemoprevention is a challenging task. A multitude of chemical and biological properties of flavonoids may together collaborate in the anti-cancer effects of these compounds. The findings outlined in this thesis provide the basis for piecing together a mechanism explaining the properties of flavonoids in prostate cancer cells.

We have demonstrated the ability of flavonoids to induce a prolonged cell cycle arrest and apoptosis in PC3 and LNCaP cells. The transcriptional inhibition of a large number of cell cycle genes by flavonoids has been demonstrated. The precise mechanism underlying this transcriptional inhibition remains to be determined. Possible mechanisms include inhibition of a common upstream transcription factor, generalized inhibition of the transcriptional machinery, or inhibitory effects on common promoter cis-acting elements. Since most of the transcriptionally inhibited genes in the study had functions in G2 and M phase, possible targets of flavonoids

could include the NF-Y- CDE-CHR cis-promoter complex which is commonly shared between G2/M genes. Future studies should be designed to explore these possibilities.

A prominent cellular stress response was induced by flavonoids *in vitro*, as evidenced by the up-regulation of over 40 genes with key roles in various stress response pathways. Terminal growth arrest is a form of protective response that prevents cells that are critically damaged from ever replicating. Various stress stimuli including oncogene expression and DNA strand breaks have been shown to induce terminal growth arrest and cellular senescence. Flavonoids can induce cellular stress by several mechanisms including DNA intercalation resulting in DNA breaks, pro-oxidant activity also leading to genotoxic stress and inhibition of key enzymes regulating DNA synthesis such as DNA topoisomerase II. The large number of endoplasmic reticulum stress genes induced by flavonoids also highlights the non-genotoxic stress mechanisms induced by flavonoids. Flavonoids are also potent inhibitors of cellular kinases, and the key role of these enzymes in cellular signaling and metabolism can also lead to dramatic disruptions of normal cellular physiology. The multitude of cellular stress responses induced by flavonoids may together contribute to the generation of signals that activate the fail-safe mechanism of terminal growth arrest.

What directly links the cell stress response to cell cycle arrest is not known. The wide-ranging inhibition of cell cycle gene expression in our studies could plausibly be a response to the stress stimulus, although other mechanisms may also account for this transcriptional effect. Whether flavonoid-induced cell cycle arrest is the direct result of down-regulation of cell cycle gene transcription is presently unknown. Other mechanisms for the cell cycle arrest by flavonoids could be activation of classical checkpoint pathways as would be seen in response to DNA double strand breaks induced by flavonoids. Indeed flavonoids have been shown to activate DNA damage response pathways by activation of key regulators of these signaling cascades such as

ATM and Chk2. Tumor suppressor p53 is a key regulator of DNA damage response pathways. The importance of this pathway is diminished however when we consider that cell cycle arrest was caused by flavonoids even in p53 null cells in our study. A number of mechanisms may converge in the growth arrest brought about by flavonoids, and the relative importance of one pathway over another remains to be established.

The design of the majority of studies in the field have focused on the effects of flavonoids in cell culture, where high doses of flavonoids are administered for prolonged periods of time in a non-physiologic redox environment. This is far removed from the scenario in humans where relatively low doses of flavonoids have been associated with reduced cancer risk in epidemiologic studies. The cell cycle gene or protein expression profile has not been compared in men from populations with varying flavonoid consumption levels. The low doses of flavonoids consumed in the diet are presumably less likely to induce dramatic cellular stress responses. The results of animal studies have demonstrated alteration in expression of cell cycle protein in tumor cells of animals consuming flavonoid supplemented diets. This supports the notion that the cell cycle may be an important target of flavonoids in vitro as well as in vivo. In the animal experiments described in this thesis, we did not demonstrate significant alterations of cell cycle proteins in prostate tissue despite high flavonoid levels detected in prostate samples of mice consuming flavonoid enriched diets. Since there was significant tumor reduction in animals consuming flavonoids, alterations in cell cycle markers may have been missed due to increased apoptosis or cell death occurring before tissue staining.

In many ways, the cell cycle arrest induced by flavonoids is analogous to cell cycle arrest and DNA damage checkpoint activated by radiation therapy treatment and chemotherapeutic agents. These modalities induce genotoxicity such as double strand breaks that activate cell cycle checkpoints leading to growth arrest and senescence. Flavonoids may induce similar pathways

also by causing DNA damage, however, in this thesis we have described other mechanisms that may be important in cell cycle arrest caused by flavonoids. The broad-ranging cell cycle transcriptional inhibition by flavonoids has not been shown for other chemotherapeutic agents, and represents a novel strategy that can supplement the current armamentarium of cancer drugs. The overlapping mechanisms of flavonoids and other treatment agents supports the rationale for employing these agents together with flavonoids to promote synergistic mechanisms.

Although the use of flavonoids as chemotherapeutics is an exciting future prospect for flavonoids, even more intriguing is the potential application of flavonoids as chemopreventive agents. In the animal chemoprevention study described in this thesis, relatively high doses of flavonoids were associated with a reduction of prostate cancer progression. These high doses were associated with gastrointestinal toxicity for the most efficacious diets. Future studies should examine the effect of these flavonoids at lower doses. The lack of toxicity of flavonoids at low doses is an attractive property that encourages their use over the long-term. The inhibition of progression of pre-malignant lesions to cancer by activation cell cycle checkpoints, growth arrest and apoptosis by flavonoids is worth exploring further in clinical studies. Human research is required to determine whether doses of flavonoid achieved by supplementation can successfully activate these protective cellular mechanisms, and to establish whether indeed this is a relevant mechanism in the chemopreventive effect of flavonoids in prostate cancer.

Although the various cellular and molecular events described above are important, flavonoids also modulate other physiologic parameters outside the cancer cell. The role of the stroma and extracellular environment are key to understanding the full complexity of cancer. Flavonoids possess numerous beneficial properties in this regard such as their proven anti-inflammatory and anti-angiogenic effects. The hormonal effects of flavonoids constitute a dilemma since at low doses flavonoids appear to activate steroid hormone receptors, a potentially

detrimental effect of flavonoids. Dose determination will be key in future human studies since at higher doses, the growth arresting properties of flavonoids will dominate. In conclusion, flavonoids possess a multitude of properties, both on the cancer cell and the surrounding milieu, that make them exciting prospects as both chemotherapeutic and chemopreventive agents in prostate cancer.

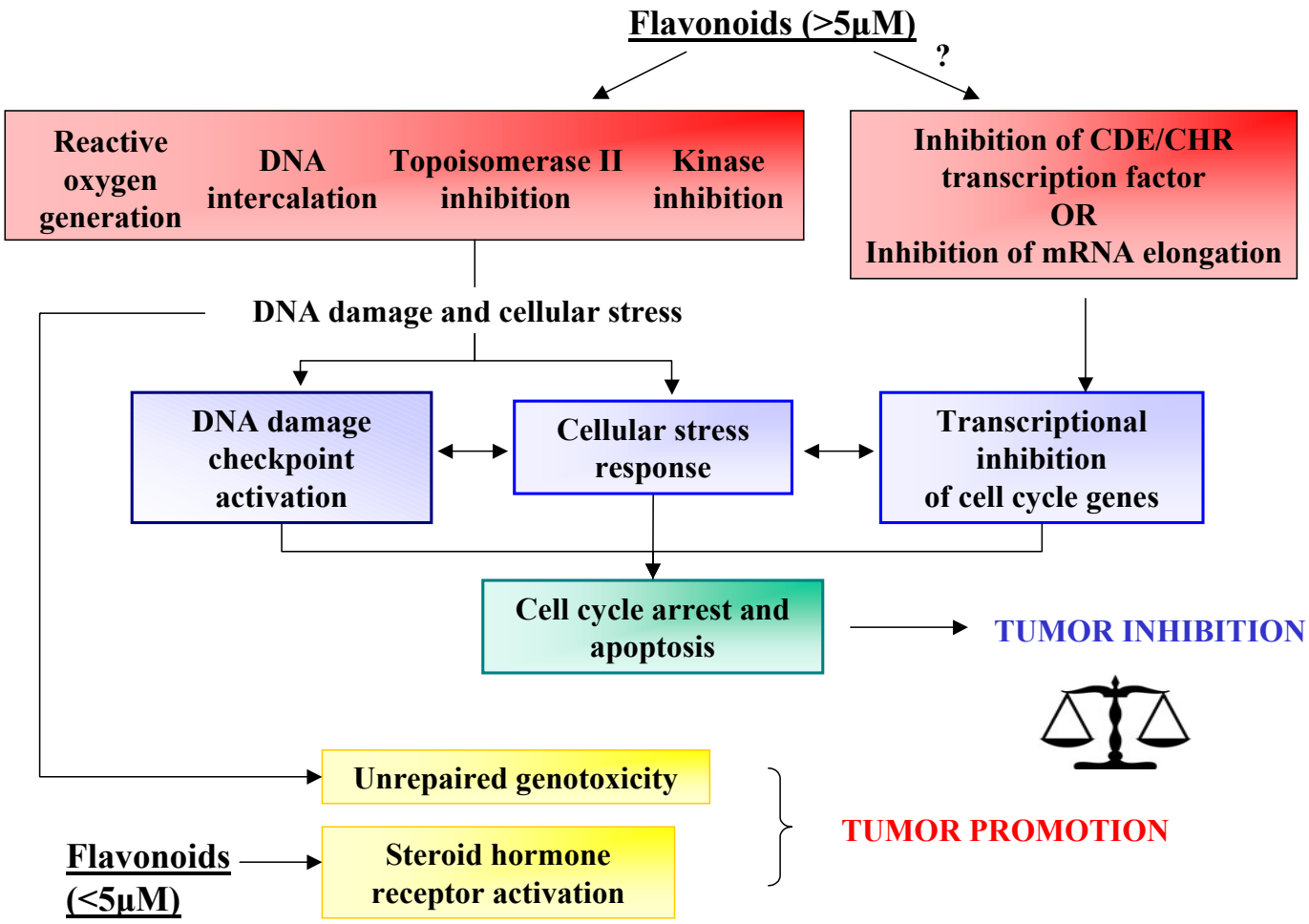


Figure 5.1. Proposed mechanism for the cytotoxic action of flavonoids in prostate cancer. A differential proliferative response is noted for low dose and high dose flavonoids. At low doses (<5μM), flavonoids are growth stimulatory in culture, mediated by their 'phytoestrogenic' effects on the estrogen receptor. At higher doses (>5 μM), flavonoids are cytotoxic. Various mechanisms for this cytotoxic effect have been demonstrated in this thesis including cell cycle arrest secondary to inhibition of gene expression and activation of a stress response, and induction of apoptosis by flavonoids.

APPENDIX 1

ASSOCIATION OF DIET INDUCED HYPERINSULINEMIA WITH ACCELERATED GROWTH OF PROSTATE CANCER (LNCAP) XENOGRAFTS

This appendix consists of a paper by Vasundara Venkateswaran, Ahmed Q. Haddad, Neil E. Fleshner, Rong Fan, Linda M. Sugar, Rob Nam, Laurence H. Klotz, and Michael Pollak entitled "Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer (LNCaP) xenografts" which was published in the Journal of the National Cancer Institute. 2007 Dec 5;99(23):1793-800. Epub 2007 Nov 27.

A.1.1 ABSTRACT

Background: Prior research suggested that energy balance and fat intake influence prostate cancer progression, but the influence of dietary carbohydrate on prostate cancer progression has not been well characterized. We hypothesized that hyperinsulinemia resulting from high intake of refined carbohydrates would lead to more rapid growth of tumors in the murine LNCaP xenograft model of prostate cancer.

Methods: Athymic mice were injected subcutaneously with LNCaP human prostate cancer cells and when tumors were palpable, were randomly assigned (n=20/group) to high carbohydrate-high fat (HC-HF) or low carbohydrate-high fat (LC-HF) diets. Body weight and tumor volume was measured weekly. After 9 weeks, serum levels of insulin and insulin-like growth factor 1 (IGF-1) were measured by enzyme immunoassay. AKT activation and the levels of the insulin receptor in tumor cells were determined by immunoblotting. The *in vitro* growth response of LNCaP cells to serum from mice in the two treatment groups was measured based on tetrazolium compound reduction. All statistical tests were two-sided.

Results: After 9 weeks on the experimental diets mice on the HC-HF diet were heavier (mean body weight of mice on the HC-HF diet = 34 g versus 29.1 g on the LC-HF diet; difference = 4.9 g, 95% CI = 3.8 to 6.0 g; $P < 0.05$), experienced increased tumor growth (mean tumor volume in mice on HC-HF diet = 1695 mm^3 versus 980 mm^3 on LC-HF diet; difference = 715 mm^3, 95% CI = 608 to 822 mm^3; $P < .001$), and experienced a statistically significant increase in serum insulin and IGF-1 levels. Tumors from mice on the HC-HF diet had higher levels of activated AKT and modestly higher insulin receptor levels than tumors from mice on the LC-HF diet. Serum from mice on the HC-HF diet was more mitogenic for LNCaP cells *in vitro* than serum from mice fed the LC-HF diet.

Conclusion: A diet high in refined carbohydrates is associated with increased tumor growth and with activation of signaling pathways distal to the insulin receptor present in a murine model of prostate cancer.

A1.2 INTRODUCTION

Obesity is associated with increased cancer mortality (82), and its increasing prevalence (511) may hinder progress in cancer control. Recent studies focusing on prostate cancer have shown that obesity is associated with only a small increase in the risk of this disease but that it is an important adverse prognostic factor (87, 512-517). The mechanisms that link obesity to prostate cancer prognosis are incompletely characterized. It is possible that the association of obesity with prostate cancer prognosis is mediated by adipokines (signaling molecules produced by adipocytes) and that the amount of adipose tissue is therefore of key importance (518). However, it has also been suggested that it is the hormonal profile associated with excess energy intake over expenditure that mediates the effects of obesity on prostate cancer prognosis (194, 519-521), and that obesity is associated with prostate cancer prognosis because it is a surrogate for excess energy intake.

Recent evidence from population studies indicates that hyperinsulinemia is also related to adverse outcome in prostate cancer (63, 522-525). This relationship is plausible because insulin and IGF-I receptors activate downstream signalling pathways that involve AKT, mTOR, and other molecules that positively regulate protein translation and proliferation, and inhibit apoptosis (526). Insulin levels, like obesity, increase with greater energy intake, particularly in the form of rapidly absorbed carbohydrates (527). However, the influence of diet-induced hyperinsulinism on experimental prostate cancer models has not been examined. In this report,

we examine the relationship between dietary carbohydrate intake and insulin-mediated signaling and prostate cancer progression using a xenograft murine model of human prostate cancer progression.

A.1.3. MATERIALS AND METHODS

Establishment of the LNCaP xenograft Model of prostate cancer

Mice were maintained in a sterile and pathogen-free facility, with cages, bedding and water autoclaved before use. Animal care conformed to institutional guidelines including the Care and Use of Experimental Animals guidelines of the Canadian Council on Animal Care. Human prostate cancer cells (LNCaP) (American Type Culture Collection, Manassas, VA) were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% Fetal Bovine Serum (FBS). Using a 27-guage needle, we injected 1 x 10^6 LNCaP cells in 0.2 ml Matrigel into the flanks of 6-8 week old athymic male Swiss nu/nu mice (Taconic, NY), that had been anaesthetized with methoxyfluorane. Two weeks after injection, all mice, all with palpable tumors, were randomly assigned to one of the two diets, low carbohydrate-high fat (LC-HF) or high carbohydrate-high fat (HC-HF) diets (n = 20/group).

Diet Formulation and Treatment

The HC-HF diet included 40% carbohydrate, 45% fat, and 15% casein. The LC-HF diet consisted of 10% carbohydrate, 45% fat and 45% protein in the form of casein. (Table A.1.1). Protein levels were adjusted in the diets to ensure an equivalent amount of calories (Table A.2.2), such that mice were fed 4.76 and 4.58Kcal/g in the HC-HF and LC-HF diets respectively for a period of 9 weeks. The high-carbohydrate diet was created by the addition of dextrin (30.5%) and sucrose (17%). Diets were stable for 6 months (as determined by the manufacturer (Purina Mills Test Diets, Richmond, IN, who reported that they were free of phytoestrogens), stored at 4°C, and sterilized by irradiation prior to administration. Mice were allowed ad-libitum access to food and water throughout the treatment period and were sacrificed at the end of the treatment or when

a tumor reached maximum permissible tumor diameter (17 mm), in accordance with the Canadian Council on Animal Care and Cancer Endpoint Guidelines.

Assessment of Body Weight and Tumor Volume

Body weight and tumor measurements were recorded weekly. Tumor size was assessed by measuring the (longest and shortest) two tumor diameters with a caliper. Tumor volume was calculated by the formula (short length^2 x long length / 2).

Preparation and Analysis of Blood and Tissue

At necropsy, blood was drawn from all mice by direct heart puncture, serum was separated, and aliquots of serum were stored at -80^{o}C. Tumors were excised, weighed, and processed for histopathologic studies or immunoblotting as described below. Tissue for histopathology was fixed in 10% v/v buffered formalin. Sections (5 μm thick) were cut from the paraffin-embedded tissue, mounted on slides, and stained with hematoxylin and eosin.

Immunohistochemical analysis of proliferative marker (Ki67) in tumor tissue

Immunostaining was performed on primary tumor sections using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, paraffin-embedded tissues were deparaffinized with xylene and dehydrated using washes of decreasing ethanol concentration (100%, 95%, and 80%). Antigen retrieval was performed by boiling the sections in 10 mM citrate buffer for 10 minutes. Sections were then blocked with diluted normal goat serum (Blocking serum in Vectastain Elite ABC kit) and incubated overnight with primary antibody, Ki-67 (rabbit polyclonal antibody; 1:25 dilution; Abcam, Cambridge, MA). Endogenous peroxidase was blocked and sections incubated with secondary antibody (biotin-labeled anti-rabbit IgG, Vector Laboratories) according to the manufacturer's instructions followed by incubation with streptavidin–peroxidase and 3, 3′-diaminobenzidine. Sections were counterstained with hematoxylin. The Ki-67 labeling index was determined by counting 1000 tumor cells at × 40X

magnification in 10 microscopic fields. Brown, granular nuclear staining was considered positive for Ki-67. Labeling indices were calculated as the percentages of tumor cells with positive nuclear staining. Immunostaining was performed blindly and scored on prostate tumor tissue sections from each mouse (n = 10/group; 2 sections from each tumor) by an independent pathologist.

Plasma Insulin Measurements

Serum insulin was measured in duplicate n = 20/treatment group) using a rat insulin ELISA kit (Crystal Chem Inc., IL) according the manufacture's instructions. Insulin in the sample was bound to guinea pig anti–rat insulin antibody coated on microplate wells, and unbound materials were removed by washing with the washing buffer provided by the manufacturer. Horseradish peroxidase–conjugated anti–guinea pig antibody was then added to the insulin complex immobilized on the microplate well and excess peroxidase was removed with washing buffer. The peroxidase conjugate on the microplate well was detected using 3,3',5,5'-tetramethylbenzidine substrate solution. The enzymatic reaction was stopped by the addition of 100 μl of enzyme reaction stopping solution (provided in the kit) and absorbance measured by a plate reader within 30 min (measuring wavelength: 450 nm, background wavelength 530 nm). Appropriate insulin standards ranging in concentration from 0-6.4 ng/ml) and internal controls (provided by the manufacturer) were used.

Plasma Insulin-like Growth Factor 1 Measurements

IGF-1 was measured in duplicate by Enzyme Immunoassay (Diagnostic Systems Laboratories, Inc., TX), in serum samples from all mice in the two treatment groups. Samples were incubated for 1 hour with biotin-labeled mouse IGF-1 and goat anti-mouse IGF-1 antiserum in microtitration wells coated with rabbit anti-goat gamma globulin (according to manufacturer's instructions). After incubation and washing with washing solution (saline solution with a non-

ionic detergent; provided in the kit), the wells were incubated with streptavidin-horseradish peroxidase, which binds to biotinylated mouse IGF-1. Unbound peroxidase was washed with washing buffer and the wells incubated with tetramethylbenzidine. An acidic stopping solution containing 0.2M sulfuric acid was then added and the degree of enzymatic turnover of the substrate determined. Absorbance was measured by a plate reader within 30 min (measuring wavelength: 450 nm, background wavelength 530 nm) Appropriate standards ranging in concentration from 0-4000 ng/mL and internal controls (provided by the manufacturer) were used.

Immunoblotting

Tumor tissues from five mice in each group were cut into 1 mm pieces and homogenized separately in ice-cold RadioImmuno Precipitation Assay lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonylfluoride and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin; Sigma Chemical Company, St. Louis, MO). Protein amounts were determined by Bradford analysis. Protein (40 μg suspended in lysis buffer was loaded in lanes of SDS-containing polyacrylamide gels, electophoresed and transferred to membranes (Immobilon transfer membrane, Millipore, Bedford, MA). Blots were blocked for 1 hour with blocking buffer (5% nonfat dry milk in phosphate-buffered saline containing 0.2% tween-20) followed by sequential incubation with primary and secondary antibodies. The following primary antibodies were used: β-actin mouse monoclonal antibody (Sigma) at 1:20,000 dilution, AKT antibody (recognizing phosphorylated and unphosphorylated forms) at 1:1000 dilution (Cell Signaling, Danvers, MA) phospho-AKT (Ser^{473}) polyclonal antibody (Cell Signaling) at 1:1000 dilution, and mouse anti–insulin receptor (β-subunit) monoclonal antibody

(Chemicon, Temecula, CA) at 1:200 dilution. Secondary antibodies were horseradish peroxidase-labeled anti-mouse IgG, 1:5000 dilution or horseradish peroxidase-labeled anti-rabbit IgG, 1:1000 dilution. Antibody–protein complexes were visualized by electrochemiluminescence.

***In Vitro* Mitogenicity Assay** LNCaP cells ($5x10^3$/well) were plated in 96-well plates. After 24 hours, cells were washed twice with phosphate-buffered saline (to remove serum) and treated (6 wells per treatment) with serum free media for an additional 24 hours. Then cells were treated for 72 hours with serum (1% in media) obtained at necropsy from individual mice on the different diets (n = 20/group). The MTS {3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} method was used to measure cell proliferation as previously described (528).

Statistical Analysis The statistical significance of differences between dietary groups with respect to body weight, tumor volume, tumor wet weight, serum insulin levels, serum IGF-1 levels, serum *in vitro* mitogenicity data were calculated using the two-tailed Student's *t*-Test. Results were considered statistically significant at $P < .05$. All statistical tests were two-sided.

A.1.4. RESULTS

Influence of diet on body weight

To investigate the influence of diet on body weight, mice bearing palpable tumors were randomly assigned to a high- or low-carbohydrate diet for 9 weeks and food consumption was recorded. Diets were well tolerated. There was no statistically significant difference in grams of food consumed per day between the groups (4.77 g in the high-carbohydrate group versus 4.83 g in the low-carbohydrate, difference = -0.06 g, 95% CI = -1.02 to 0.90 g; $P = .893$). The total calories consumed were also similar (Table A.2.2), but mice on the high-carbohydrate diet obtained fewer calories from protein as expected**.** Mice on the HC-HF diet began to gain weight relative to the

mice LC-HF on the diet after approximately 5 weeks of treatment (Fig A.1.1), although differences in body weight between the treatment groups did not become statistically significant until the conclusion of the experiment (after 9 weeks of treatment) at which time mice on the high-carbohydrate diet were 15% heavier than mice on the low-carbohydrate diet (mean body weight of mice on a HC-HF diet = 34 g versus 29.1 g on the LC-HF diet; difference = 4.9 g, 95% CI = 3.8 to 6.0 g, P = .003, Fig A.1.1).

Influence of diet on tumor volume

Tumors were measured weekly. Statistically significant differences in tumor volume were observed from week five (mean tumor volume of mice on the high-carbohydrate diet = 901 mm^3 versus 588 mm^3 for mice on the low-carbohydrate diet, difference = 313 mm^3, 95% CI = 219 to 407 mm^3; P = 0.035, Fig A.1.2 A) By the end of the 9-week treatment period, there was a statistically significant increase (45%) in tumor volume in the mice on a high-carbohydrate diet relative to animals on a low-carbohydrate diet. Mean tumor volume in mice at on a high-carbohydrate diet was 1695 mm^3 versus 980 mm^3 in the low-carbohydrate diet (difference = 715 mm^3, 95% CI = 608 to 822 mm^3; P = .001).

There was also an increase in the ratio of the mean tumor volume to body weight in the mice on a high-carbohydrate compared to the low-carbohydrate group (Fig A.1.2 C). The mean tumor volume to body weight ratio at week 9 was 49.9 and 30.6 in the high-carbohydrate and low-carbohydrate group respectively (difference = 19.3, 95% CI = 15.9 to 22.7; P = .001). At the end of the 9-week treatment period, mice were sacrificed and tumors were excised and weighed. Tumor weights ranged from 0.6-1.4 g in mice on the high-carbohydrate diet and from 0.2-0.8 g in mice on the low-carbohydrate diet. Mean tumor weight in mice on the high-carbohydrate diet was 0.88 g versus 0.51 g in the low-carbohydrate diet (difference = 0.37 g, 95% CI = 0.13 to 0.61 g; P = .04, Fig. A.1.2 B).

Expression of Ki67 proliferation marker on LNCaP xenografts

To better understand the basis for the effect of diet on tumor volume, we studied markers of proliferation in the xenograft tumors, as well as circulating insulin and IGF-1 levels in tumor-bearing mice. Immunohistochemical analysis of a proliferation marker (Ki67) was performed on paraffin-embedded prostate tumor tissues from 10 mice / treatment group; 2 sections were examined from each tumor. There was a substantial increase in the percentage of positively stained nuclei in tumors from the high-carbohydrate group (27 %) compared to that in tumors from the low-carbohydrate group (5.7 %) (difference = 21.3 %, 95% CI = 13.6 to 28.8 %; *P* =.0002, Fig A.1.3).

Influence of diet on serum insulin and IGF-I levels

In order to explore dietary effects on insulin and IGF-1 levels, blood samples were collected from mice in both groups by direct heart puncture, serum separated, and aliquots of serum stored at -80^{o}C. The mean insulin level in serum samples of mice on the high-carbohydrate diet was statistically significantly higher than that of mice on the low-carbohydrate diet (1.45 ng/ml versus 0.45 ng/ml, difference = 1 ng/ml, 95% CI = 0.95 to 1.06 ng/ml; *P* = .039, Fig. A.1.4 A). Serum from mice on the high-carbohydrate diet had a 21.4% higher level of IGF-1 compared with that in mice on the low-carbohydrate diet (1868.5 ng/ml versus 1468.5 ng/ml, difference = 400 ng/ml, 95% CI = 358.3 to 441.7 ng/ml; *P* = .022, Fig. A.1.4 B).

Influence of diet on phosphorylation of AKT in prostate cancer tissue In view of the diet-induced changes in insulin and IGF-1 levels, we next investigated if the diets influenced signaling pathways in the experimental tumors. First, we measured phosphorylation of AKT, a signaling molecule that is downstream of the insulin and IGF-1 receptors (526), in lysates from tumor tissues (n = 5/group). The amount of phosphorylated AKT was below the detection limit in tumor tissue lysate from the mice on the low-carbohydrate diet, but a band corresponding to the

phosporylated kinase was clearly visible in the immunoblots obtained from tumor tissue lysates from the mice on high-carbohydrate diet (Fig A.1.5). Thus, the high-carbohydrate diet increased AKT activation in the LNCaP xenograft tissue. Insulin receptor levels were also higher in the neoplastic tissue of the animal on the high-carbohydrate diet (n = 5/group) (Fig A.1.5).

Mitogenicity of mouse serum *in vitro*: In view of the pronounced changes in hormone-mediating signaling we wished to investigate if serum from animals on the carbohydrate diets altered the growth of human prostate cancer cells *in vitro***.** LNCaP cells were treated with serum obtained from mice on the high- and low-carbohydrate diet (n = 20/group). The MTS method was used to measure cell proliferation as previously described (528). LNCaP cells treated with 1% serum from mice on the high-carbohydrate diet showed greater proliferation than cells treated with serum from mice on the low-carbohydrate diet (optical density at 490nm from MTS cell proliferation assay = 0.64 versus 0.29; difference = 0.35, 95% CI = 0.3 to 0.4; P = .005).

A.1.5. DISCUSSION

We examined the relationship between dietary carbohydrate intake and prostate cancer behavior using the LNCaP model and observed that increasing carbohydrate intake (without altering fat intake or total calories) augmented the growth rate of prostate cancer xenografts. The high-carbohydrate diet was also associated with a major increase in host insulin levels, a modest increase in host IGF-I levels, and, in neoplastic tissue, an increase in insulin receptor levels, AKT activation, and the rate of cell proliferation. Most prior studies of modulation of signaling at critical nodes such as AKT (which is downstream from receptors of the tyrosine kinase class and upstream of mTOR) involved pharmacologic interventions (529); our data provide evidence for an effect of macronutrient composition on AKT activation in LNCAP xenografts.

There was a small difference in the weight gain of mice between the dietary groups (Fig. A.1.1), although the magnitude of the difference in tumor weight was greater than the difference in body weight. It is possible that mice on the high-carbohydrate diet were slightly heavier due to fluid retention because insulin has been reported to have anti-natriuretic properties (530).

Rapidly absorbed sugars are an important sources of carbohydrate in modern American diets and evoke high surges in insulin levels (531-533). However, our results do not establish that the effect of host diet on tumor behaviour was attributable to effects of diet on insulin. Insulin is nevertheless a candidate mediator of the effect of the high-carbohydrate diet on prostate cancer behaviour, in view of: 1) the well-known association of diets high in sugars with higher insulin levels, 2) the detection of insulin receptors in the LNCAP xenografts, 3) enhanced activation of AKT (a signaling protein that is downstream of the insulin receptor) in tumors of mice on the high-carbohydrate diet, and 4) our observation of increased proliferation *in vitro* of human prostate cancer cells that were exposed to serum from mice on the high-carbohydrate diet compared to that of prostate cancer cells that were exposed to serum from mice on the low-carbohydrate diet. These results of our study are consistent with an effect of hyperinsulinemia related to the ingestion of sugars, and they might not have been observed if an isocaloric starch-based carbohydrate diet had been used.

One limitation of our study is that we cannot exclude the possibility that some of our observations are related to decreased protein intake. To maintain caloric equivalence, the diet containing higher sugar levels had lower protein levels. Although it would also be of interest to compare the effects of isocaloric, iso-protein diets that vary in the nature of the carbohydrate (sugar versus starch), this is technically challenging because of differences in palatability that could lead to differences in consumption.

Our findings are consistent with population studies that showed an association between higher levels of insulin or c-peptide (a surrogate marker of insulin levels that can be conveniently measured in serum, especially when fasting samples are not available) with adverse outcome in prostate cancer (523, 525). Further studies will be required to determine if insulin directly mediates the effect of the diet, indirectly mediates the effect through an insulin-regulated host factor that acts on neoplastic cells, or if insulin is simply a surrogate for another effector molecule. However, prior circumstantial evidence consistent with a role for insulin in neoplasia includes 1) the association of hyperglycemia with cancer risk and prognosis (534), 2) the finding, over 20 years ago, that insulin deficiency is associated with reduced tumor growth (535), and 3) the observation that transplantation of pancreatic islets to an ectopic site can lead to carcinogenesis in that site (536). Regardless of the exact role of insulin signaling in regulating tumor growth, it will be important to describe more comprehensively the dietary induced changes in signaling pathways in neoplastic tissue and to determine if the activation state of IRS (Insulin receptor substrate) family members, m-Tor (mammalian target of rapamycin), and S6-kinase are altered by the dietary manipulation.

We also observed that the higher IGF-I levels in mice on the high-carbohydrate diet were associated with more rapid tumor growth. These experimental results are consistent with epidemiologic data (537, 538) showing that increased risk of prostate cancer, particularly aggressive prostate cancer is associated with higher serum IGF-I concentration. IGF-1-mediated signaling has received more attention in the context of neoplasia than insulin signaling, but our data are compatible with the hypothesis that insulin itself may play a role in mediating the effect of macronutrient composition of diets on tumor growth (526, 539).

The association of the high-carbohydrate diet with elevated circulating insulin levels was expected based on previous results (527, 540, 541) but the increased levels of insulin receptors in

the neoplastic tissue was an unexpected finding for which the mechanism is unknown. Although IGF-I receptors on prostate cancer cells have been studied extensively (542-544), there is only limited evidence that prostate cancer cells display insulin receptors (545), and our results justify further research to better characterize insulin and insulin/IGF hybrid receptors in prostate cancer using both laboratory models and tissue microarrays of human tumors.

Androgen deprivation therapy for prostate cancer, which is effective and in widespread use, induces hyperinsulinemia in a substantial proportion of men (546, 547). This adverse effect has been discussed in terms of its effect on quality of life and cardiovascular health in long-term prostate cancer survivors (548, 549). However, it is possible that the hyperinsulinemic state also influences risk of progression to aggressive, androgen-independent disease. This possibility is being addressed by ongoing studies that seek to determine if inter-individual differences in degree of castration-induced hyperinsulinism are related to time to emergence of androgen independence.

Our results provide support for the concept that diets associated with reduction in insulin level may have benefits for prostate cancer patients (550), particularly for the subset of patients who are hyperinsulinemic. It should be noted that although obese men tend to be hyperinsulinemic, some individuals described as "metabolically obese, normal weight" (551) are also hyperinsulinemic, and cancer patients in this group (as well as obese men) may benefit from strategies to reduce insulin levels. Our experimental data justify clinical research to determine if optimization of macronutrient intake to meet, but not exceed, nutritional requirements, and to minimize insulin levels, may lower prostate cancer risk and/or improve prostate cancer prognosis. Pharmacologic agents such as metformin, which reduce hyperinsulinemia and associated metabolic abnormalities, may also have a role to play in the treatment of metabolically defined subsets of prostate cancer patients.

Table A.1.1: Composition of low-carbohydrate and high-carbohydrate diets*

Dietary Parameters	High-Carbohydrate	Low-Carbohydrate
Composition of diet (% weight)		
Carbohydrate (Dextrin + Sucrose)	47.5	11.4
Fat	23.8	22.8
Protein	17.9	51.5
Others (Minerals)	10.8	14.3
Energy cntribution (%)		
Carbohydrate (Dextrin + Sucrose)	40	10
Fat	45	45
Protein	15	45

. *These data are provided in the specification sheets of the manufacturer.

Table A.1.2. Calculated daily intakes and the caloric value for the treatment groups

Variable	High -Carbohydrate	Low-Carbohydrate
Food consumed (g) /day / animal	4.77	4.83
Total Energy Value of diets /gm (Kcals/g) *	4.76	4.58
† Total energy consumed (Kcals) / day / animal	22.7	22.1
Grams Consumed / day / animal of ±		
Carbohydrate	2.27	0.55
Fat	1.14	1.1
Protein	0.85	2.49
Energy (Kcal) from dietary constituent / day / animal §		
Carbohydrate	9.08	2.2
Fat	10.2	9.9
Protein	3.4	9.96

* Data provided in the specification sheets of the manufacturer
† Total energy value X g of diet consumed
± Composition X g consumed / 100
§ Calculated as grams of diet consumed / day / animal x physiologic fuel value (Carbohydrate,

Fat and Protein are approximately 4, 9, 4 Kcal/g respectively)

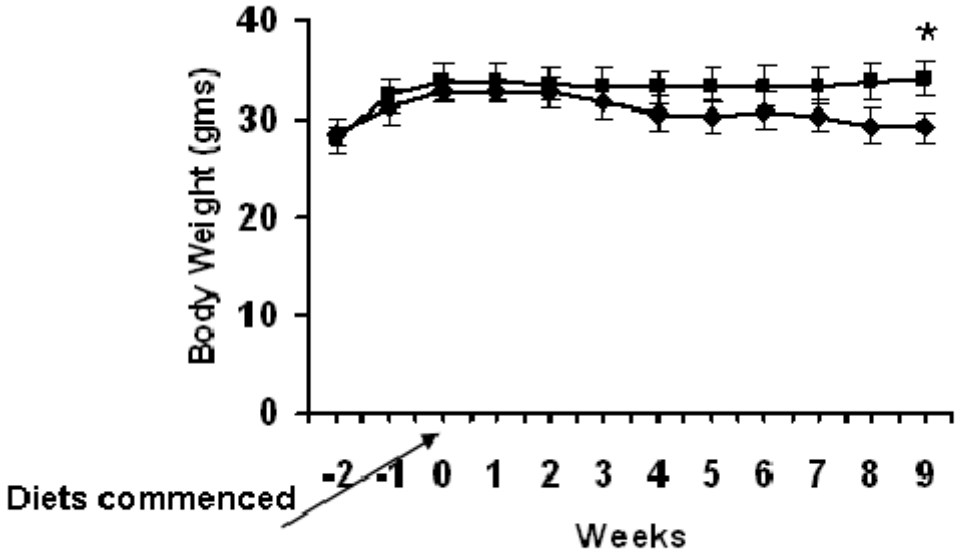


Fig A.1.1. Effect of high carbohydrate-high fat and low carbohydrate-high-fat diets on body weight. Mice (n = 20/group) injected with LNCaP human prostate cancer cells were confirmed to have tumors after 2 weeks and assigned to either the high-carbohydrate, high-fat (HC-HF) diet (Black Square) (40% carbohydrate, 45% fat, and 15% casein) or the low carbohydrate-high fat (LC-HF) diet (Black Diamond) (10% carbohydrate, 45% fat, and 45% protein in the form of casein). Body weight was measured weekly over a period of 9 weeks. Error bars correspond to upper the 95% confidence interval of the mean. Means were statistically significantly different based on the two-sided Student's *t* Test (* $P < .05$).

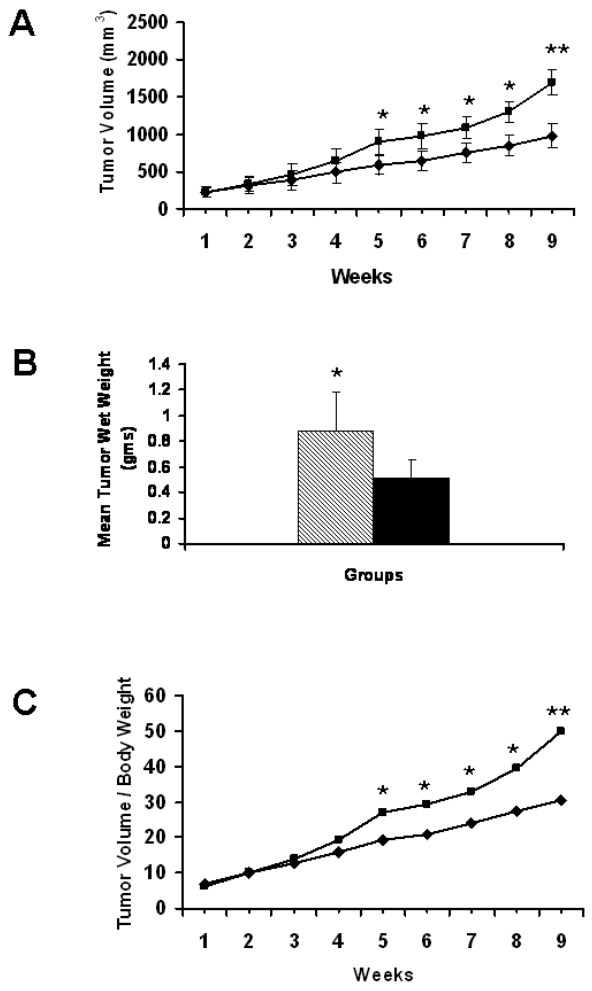


Fig A.1.2. Effect of diet on tumor growth. A) Tumor volume of mice injected with LNCaP human prostate cancer cells and assigned (n = 20/group) 2 weeks later to the high carbohydrate-high fat (HC-HF) (Black Square) or low carbohydrate-high fat (LC-HF) (Black Diamond) diet was measured using calipers and recorded weekly over the 9 week treatment period. Error bars correspond to the 95% confidence interval (CI) of the mean. **B)** At the end of the treatment period, mice on HC-HF (Shaded Bar) and LC-HF (Black Bar) diets were sacrificed and tumors excised and weighed. Error bars correspond to the upper 95% CI of the mean.

C) Ratio of mean prostate tumor volume to body weight. The weekly change over the treatment period in the ratio of the mean tumor volume to body weight for mice on the HC-HF (Black Square) and LC-HF (Black Diamond) diets is shown. Means were statistically significantly different based on the two-sided Student's *t* Test (* $P < .05$; ** $P < .001$).

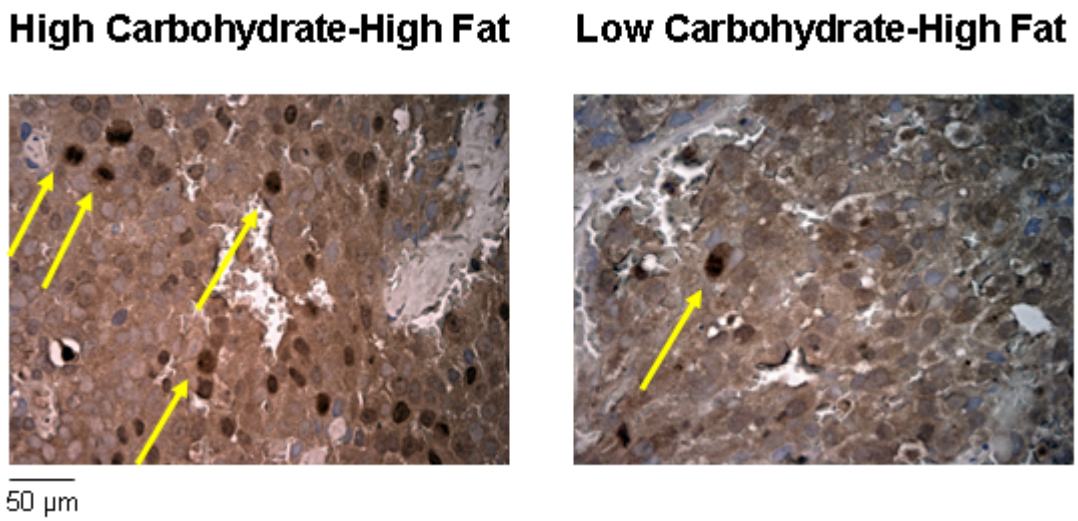


Fig A.1.3. Imunohistochemistry of proliferation marker Ki67 in tumor tissue from mice on low-fat, high-carbohydrate and high-fat, high-carbohydrate diets. Immunostaining was performed on paraffin-embedded tumor sections (n = 10/group; 2 sections from each tumor) using the Vectastain Elite ABC kit (Vector Laboratories). Cells with brown, granular nuclear staining (indicated by arrows) were considered positive for Ki-67. A section of a tumor from each treatment group is shown.

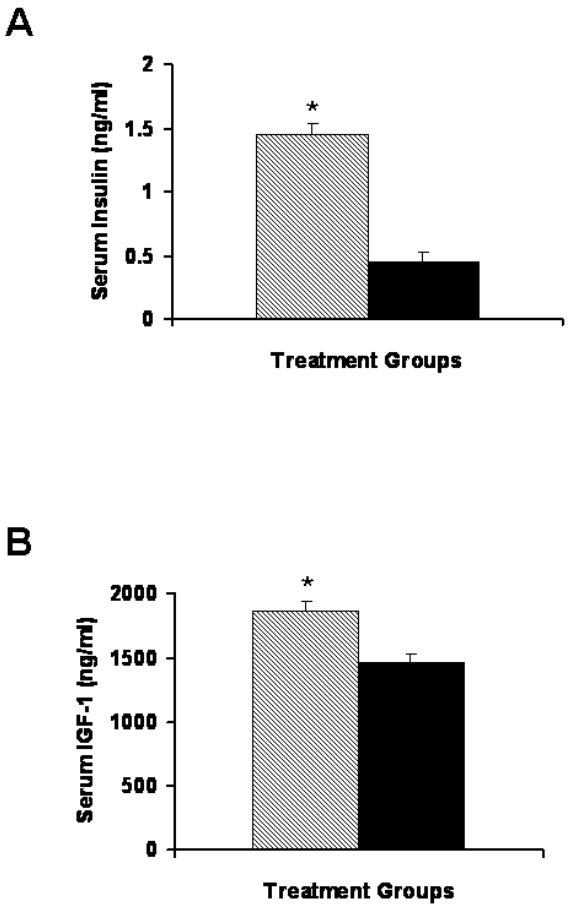


Fig A.1. 4. Serum insulin and insulin-like growth factor levels in mice on high and low-carbohydrate diets. A) Serum insulin in mice (n = 20/group) that had been assigned to high carbohydrate-high fat (HC-HF) (Shaded Bar) and low carbohydrate-high-fat (LC-HF) (Black Bar) diets was measured after 9 weeks. Means were statistically significantly different based on the two-sided Student's *t* Test. Error bars correspond to the upper 95% CI of the mean. **B)** Serum IGF-1 in mice (n = 20/group) that had been assigned to high carbohydrate-high fat (HC-HF) (Shaded Bar) and low carbohydrate-high-fat (LC-HF) (Black Bar) diets was measured after 9 weeks was measured using an immunoassay. The degree of enzymatic turnover of the substrate was determined by dual absorbance at 450 and 620 nm. Difference was statistically significantly different based on the two-sided Student's *t* Test. Error bars correspond to the upper 95% CI of the mean (* $P < .05$).

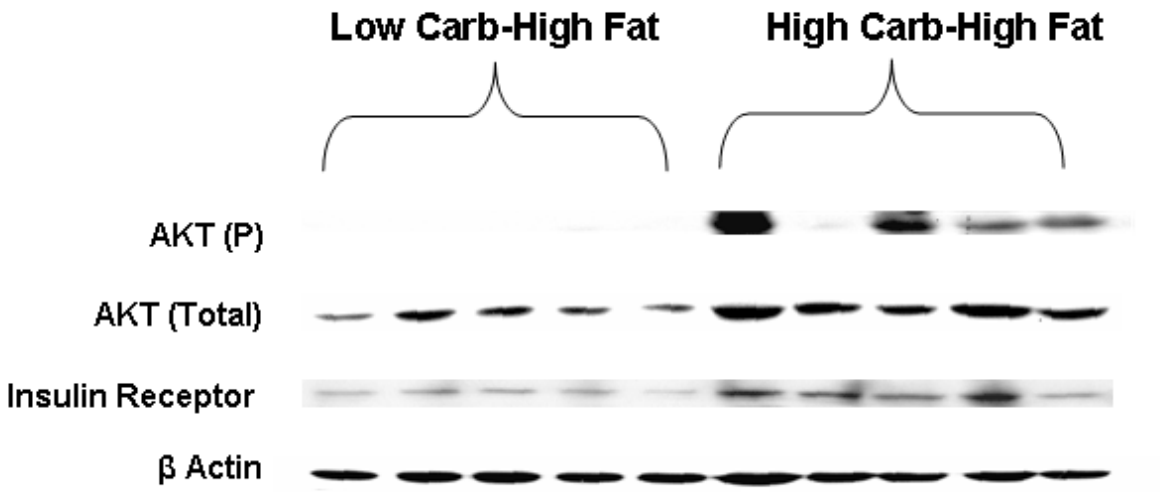


Fig A.1.5. Comparison of activated AKT and insulin receptor levels in tumors from mice on low-carbohydrate, high-fat and high-carbohydrate, high-fat diets. Total AKT, phosphorylated AKT, and insulin receptor in individual lysates (5/group) prepared from tumors excised at the end of the dietary treatment were detected on immunoblots by electrochemiluminescence using antibodies specific for the insulin receptor and the phosphorylated form of AKT. Detection of β actin was used as an internal control for protein loading and transfer.

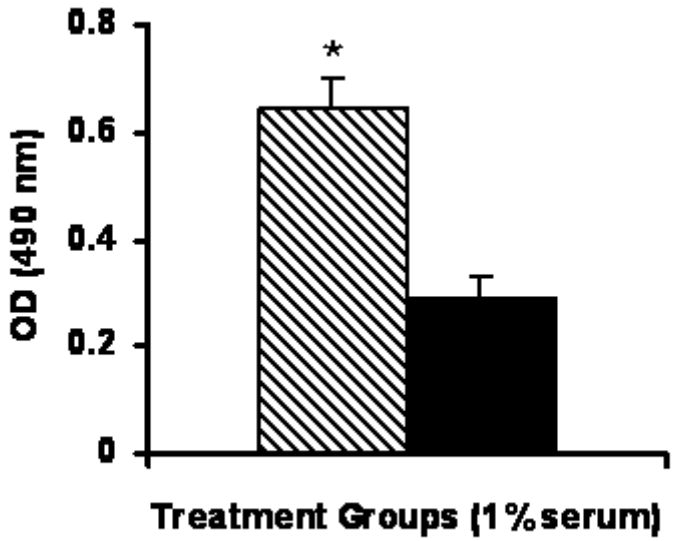


Fig A.1.6. ***In vitro*** **mitogenicity of serum from mice on low-carbohydrate, high-fat and high-carbohydrate high-fat diets.** LNCaP cells were plated at $5x10^3$/well and treated for a period of 72 hours with serum (1% in media) obtained from individual mice on the different diets (n = 20/group). High carbohydrate-high fat (HC-HF) (Shaded Bar) and low carbohydrate-high-fat (LC-HF) (Black Bar). The MTS method was used to measure cell proliferation. Difference was statistically significantly different based on the two-sided Student's *t* Test. Error bars correspond to the upper 95% CI of the mean (* $P < .05$).

CHAPTER 6

REFERENCES

1. Cancer Facts and Figures. Atlanta: American Cancer Society; 2007.
2. Canadian Cancer Statistics 2007. Toronto: Canadian Cancer Society/National Cancer Institute of Canada; 2007.
3. Klotz L. Active surveillance with selective delayed intervention for favorable risk prostate cancer. Urologic oncology 2006;24(1):46-50.
4. Pomerantz M, Kantoff P. Advances in the treatment of prostate cancer. Annual review of medicine 2007;58:205-20.
5. Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC. Natural history of progression after PSA elevation following radical prostatectomy. Jama 1999;281(17):1591-7.
6. Jhaveri FM, Zippe CD, Klein EA, Kupelian PA. Biochemical failure does not predict overall survival after radical prostatectomy for localized prostate cancer: 10-year results. Urology 1999;54(5):884-90.
7. Han M, Partin AW, Pound CR, Epstein JI, Walsh PC. Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. The Urologic clinics of North America 2001;28(3):555-65.
8. Wirth MP, Hakenberg OW, Froehner M. Optimal treatment of locally advanced prostate cancer. World journal of urology 2007;25(2):169-76.
9. Quek ML, Penson DF. Quality of life in patients with localized prostate cancer. Urologic oncology 2005;23(3):208-15.
10. Adolfsson J, Tribukait B, Levitt S. The 20-Yr outcome in patients with well- or moderately differentiated clinically localized prostate cancer diagnosed in the pre-PSA era: the prognostic value of tumour ploidy and comorbidity. Eur Urol 2007;52(4):1028-35.
11. Breslow N, Chan CW, Dhom G*, et al.* Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France. International journal of cancer 1977;20(5):680-8.

12. Shiraishi T, Watanabe M, Matsuura H, Kusano I, Yatani R, Stemmermann GN. The frequency of latent prostatic carcinoma in young males: the Japanese experience. In Vivo 1994;8(3):445-7.

13. Yatani R, Shiraishi T, Nakakuki K*, et al.* Trends in frequency of latent prostate carcinoma in Japan from 1965-1979 to 1982-1986. Journal of the National Cancer Institute 1988;80(9):683-7.

14. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA: a cancer journal for clinicians 2005;55(2):74-108.

15. Grulich AE, McCredie M, Coates M. Cancer incidence in Asian migrants to New South Wales, Australia. British journal of cancer 1995;71(2):400-8.

16. Maskarinec G, Noh JJ. The effect of migration on cancer incidence among Japanese in Hawaii. Ethn Dis 2004;14(3):431-9.

17. Lee J, Demissie K, Lu SE, Rhoads GG. Cancer incidence among Korean-American immigrants in the United States and native Koreans in South Korea. Cancer Control 2007;14(1):78-85.

18. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. British journal of cancer 1991;63(6):963-6.

19. Muir CS, Nectoux J, Staszewski J. The epidemiology of prostatic cancer. Geographical distribution and time-trends. Acta Oncol 1991;30(2):133-40.

20. Sakr WA, Grignon DJ, Crissman JD*, et al.* High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. In Vivo 1994;8(3):439-43.

21. Klein EA. Can prostate cancer be prevented? Nature clinical practice 2005;2(1):24-31.

22. Neill MG, Fleshner NE. An update on chemoprevention strategies in prostate cancer for 2006. Current opinion in urology 2006;16(3):132-7.

23. Fisher B, Costantino JP, Wickerham DL*, et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute 1998;90(18):1371-88.

24. Thompson IM, Goodman PJ, Tangen CM*, et al.* The influence of finasteride on the development of prostate cancer. The New England journal of medicine 2003;349(3):215-24.

25. Fleshner N, Kulkarni G. Should finasteride be used to prevent prostate cancer? Current treatment options in oncology 2006;7(5):346-54.
26. Kulkarni GS, Al-Azab R, Lockwood G*, et al.* Evidence for a biopsy derived grade artifact among larger prostate glands. The Journal of urology 2006;175(2):505-9.
27. Thompson IM. Chemoprevention of prostate cancer: agents and study designs. The Journal of urology 2007;178(3 Pt 2):S9-S13.
28. Silverstone H, Tannenbaum A. The effect of the proportion of dietary fat on the rate of formation of mammary carcinoma in mice. Cancer research 1950;10(7):448-53.
29. Boutwell RK, Brush MK, Rusch HP. The stimulating effect of dietary fat on carcinogenesis. Cancer research 1949;9(12):741-6.
30. Tannenbaum A, Silverstone H. The influence of the degree of caloric restriction on the formation of skin tumors and hepatomas in mice. Cancer research 1949;9(12):724-7.
31. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. Journal of the National Cancer Institute 1981;66(6):1191-308.
32. Armstrong B, Doll R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. International journal of cancer 1975;15(4):617-31.
33. Lea AJ. Dietary factors associated with death-rates from certain neoplasms in man. Lancet 1966;2(7458):332-3.
34. Wynder EL. The epidemiology of large bowel cancer. Cancer research 1975;35(11 Pt. 2):3388-94.
35. Wynder EL. Nutrition and cancer. Federation proceedings 1976;35(6):1309-15.
36. Carroll KK, Gammal EB, Plunkett ER. Dietary fat and mammary cancer. Canadian Medical Association journal 1968;98(12):590-4.
37. Correa P. Epidemiological correlations between diet and cancer frequency. Cancer research 1981;41(9 Pt 2):3685-90.
38. Berg JW. Can nutrition explain the pattern of international epidemiology of hormone-dependent cancers? Cancer research 1975;35(11 Pt. 2):3345-50.
39. Phillips RL. Role of life-style and dietary habits in risk of cancer among seventh-day adventists. Cancer research 1975;35(11 Pt. 2):3513-22.

40. Walker AR, Burkitt DP. Colonic cancer--hypotheses of causation, dietary prophylaxis, and future research. The American journal of digestive diseases 1976;21(10):910-7.

41. Enstrom JE. Reassessment of the role of dietary fat in cancer etiology. Cancer research 1981;41(9 Pt 2):3722-3.

42. Willett WC. Diet and cancer: an evolving picture. Jama 2005;293(2):233-4.

43. Giovannucci E, Stampfer MJ, Colditz GA*, et al.* A comparison of prospective and retrospective assessments of diet in the study of breast cancer. American journal of epidemiology 1993;137(5):502-11.

44. Giovannucci E. Does prostate-specific antigen screening influence the results of studies of tomatoes, lycopene, and prostate cancer risk? Journal of the National Cancer Institute 2007;99(14):1060-2.

45. Willett WC, Hu FB. Not the time to abandon the food frequency questionnaire: point. Cancer Epidemiol Biomarkers Prev 2006;15(10):1757-8.

46. Willett WC, Hu FB. The food frequency questionnaire. Cancer Epidemiol Biomarkers Prev 2007;16(1):182-3.

47. Willett WC, Sampson L, Stampfer MJ*, et al.* Reproducibility and validity of a semiquantitative food frequency questionnaire. American journal of epidemiology 1985;122(1):51-65.

48. Hu FB, Willett WC. Optimal diets for prevention of coronary heart disease. Jama 2002;288(20):2569-78.

49. USDA Database for the Flavonoid Content of Selected Foods. 2003 [cited; Available from: http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html

50. Milner JA. Molecular targets for bioactive food components. J Nutr 2004;134(9):2492S-8S.

51. McCullough ML, Giovannucci EL. Diet and cancer prevention. Oncogene 2004;23(38):6349-64.

52. Segi M. Formal discussion: epidemiology of cancer: spatial-temporal aggregation. Cancer research 1965;25(8):1375-9.

53. Willett WC. Diet and cancer: one view at the start of the millennium. Cancer Epidemiol Biomarkers Prev 2001;10(1):3-8.

54. Kushi L, Giovannucci E. Dietary fat and cancer. The American journal of medicine 2002;113 Suppl 9B:63S-70S.

55. Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. The New England journal of medicine 1990;323(24):1664-72.

56. Kolonel LN, Nomura AM, Cooney RV. Dietary fat and prostate cancer: current status. Journal of the National Cancer Institute 1999;91(5):414-28.

57. Moyad MA. Dietary fat reduction to reduce prostate cancer risk: controlled enthusiasm, learning a lesson from breast or other cancers, and the big picture. Urology 2002;59(4 Suppl 1):51-62.

58. Key TJ, Spencer EA. Carbohydrates and cancer: an overview of the epidemiological evidence. European journal of clinical nutrition 2007;61 Suppl 1:S112-21.

59. Burley VJ. Sugar consumption and cancers of the digestive tract. Eur J Cancer Prev 1997;6(5):422-34.

60. Holmes MD, Liu S, Hankinson SE, Colditz GA, Hunter DJ, Willett WC. Dietary carbohydrates, fiber, and breast cancer risk. American journal of epidemiology 2004;159(8):732-9.

61. Freedland SJ, Mavropoulos J, Wang A*, et al.* Carbohydrate restriction, prostate cancer growth, and the insulin-like growth factor axis. The Prostate 2008;68(1):11-9.

62. Venkateswaran V, Haddad AQ, Fleshner NE*, et al.* Association of Diet-Induced Hyperinsulinemia With Accelerated Growth of Prostate Cancer (LNCaP) Xenografts. Journal of the National Cancer Institute 2007;99(23):1793-800.

63. Hsing AW, Chua S, Jr., Gao YT*, et al.* Prostate cancer risk and serum levels of insulin and leptin: a population-based study. Journal of the National Cancer Institute 2001;93(10):783-9.

64. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004;363(9418):1346-53.

65. Tulinius H, Sigfusson N, Sigvaldason H, Bjarnadottir K, Tryggvadottir L. Risk factors for malignant diseases: a cohort study on a population of 22,946 Icelanders. Cancer Epidemiol Biomarkers Prev 1997;6(11):863-73.

66. Silverstone H, Tannenbaum A. Proportion of dietary protein and the formation of spontaneous hepatomas in the mouse. Cancer research 1951;11(6):442-6.
67. Madhavan TV, Gopalan C. Effect of Dietary Protein on Aflatoxin Liver Injury in Weanling Rats. Archives of pathology 1965;80:123-6.
68. Green JW, Jr., Benditt EP, Humphreys EM. The effect of protein depletion on the host response to transplantable rat tumor Walker 256. Cancer research 1950;10(12):769-74.
69. Campbell TC. Dietary protein, growth factors, and cancer. The American journal of clinical nutrition 2007;85(6):1667.
70. Fontana L, Klein S, Holloszy JO. Long-term low-protein, low-calorie diet and endurance exercise modulate metabolic factors associated with cancer risk. The American journal of clinical nutrition 2006;84(6):1456-62.
71. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: World Cancer Research Fund / American Institute for Cancer Research.; 2007.
72. Hill M. Meat, cancer and dietary advice to the public. European journal of clinical nutrition 2002;56 Suppl 1:S36-41.
73. Truswell AS. Meat consumption and cancer of the large bowel. European journal of clinical nutrition 2002;56 Suppl 1:S19-24.
74. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Intake of fat, meat, and fiber in relation to risk of colon cancer in men. Cancer research 1994;54(9):2390-7.
75. Boyd NF, Stone J, Vogt KN, Connelly BS, Martin LJ, Minkin S. Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. British journal of cancer 2003;89(9):1672-85.
76. Missmer SA, Smith-Warner SA, Spiegelman D*, et al.* Meat and dairy food consumption and breast cancer: a pooled analysis of cohort studies. International journal of epidemiology 2002;31(1):78-85.
77. Kolonel LN. Fat, meat, and prostate cancer. Epidemiologic reviews 2001;23(1):72-81.
78. Rothstein WG. Dietary fat, coronary heart disease, and cancer: a historical review. Preventive medicine 2006;43(5):356-60.
79. Manson JE, Willett WC, Stampfer MJ*, et al.* Body weight and mortality among women. The New England journal of medicine 1995;333(11):677-85.

80. Willett WC, Manson JE, Stampfer MJ*, et al.* Weight, weight change, and coronary heart disease in women. Risk within the 'normal' weight range. Jama 1995;273(6):461-5.

81. Calle EE, Thun MJ, Petrelli JM, Rodriguez C, Heath CW, Jr. Body-mass index and mortality in a prospective cohort of U.S. adults. The New England journal of medicine 1999;341(15):1097-105.

82. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. The New England journal of medicine 2003;348(17):1625-38.

83. Engeland A, Tretli S, Bjorge T. Height, body mass index, and ovarian cancer: a follow-up of 1.1 million Norwegian women. Journal of the National Cancer Institute 2003;95(16):1244-8.

84. Jee SH, Sull JW, Park J*, et al.* Body-mass index and mortality in Korean men and women. The New England journal of medicine 2006;355(8):779-87.

85. Merry AH, Schouten LJ, Goldbohm RA, van den Brandt PA. Body mass index, height and risk of adenocarcinoma of the oesophagus and gastric cardia: a prospective cohort study. Gut 2007;56(11):1503-11.

86. Rapp K, Schroeder J, Klenk J*, et al.* Obesity and incidence of cancer: a large cohort study of over 145,000 adults in Austria. British journal of cancer 2005;93(9):1062-7.

87. Rodriguez C, Freedland SJ, Deka A*, et al.* Body mass index, weight change, and risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiol Biomarkers Prev 2007;16(1):63-9.

88. Samanic C, Chow WH, Gridley G, Jarvholm B, Fraumeni JF, Jr. Relation of body mass index to cancer risk in 362,552 Swedish men. Cancer Causes Control 2006;17(7):901-9.

89. Zhang M, Xie X, Lee AH, Binns CW, Holman CD. Body mass index in relation to ovarian cancer survival. Cancer Epidemiol Biomarkers Prev 2005;14(5):1307-10.

90. Rodriguez C, Patel AV, Calle EE, Jacobs EJ, Chao A, Thun MJ. Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States. Cancer Epidemiol Biomarkers Prev 2001;10(4):345-53.

91. Andersson SO, Wolk A, Bergstrom R*, et al.* Body size and prostate cancer: a 20-year follow-up study among 135006 Swedish construction workers. Journal of the National Cancer Institute 1997;89(5):385-9.

92. Severson RK, Nomura AM, Grove JS, Stemmermann GN. A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. Cancer research 1989;49(7):1857-60.

93. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. Journal of the National Cancer Institute 2000;92(1):61-8.

94. Bosetti C, Micelotta S, Dal Maso L*, et al.* Food groups and risk of prostate cancer in Italy. International journal of cancer 2004;110(3):424-8.

95. Tzonou A, Signorello LB, Lagiou P, Wuu J, Trichopoulos D, Trichopoulou A. Diet and cancer of the prostate: a case-control study in Greece. International journal of cancer 1999;80(5):704-8.

96. Hsing AW, McLaughlin JK, Schuman LM*, et al.* Diet, tobacco use, and fatal prostate cancer: results from the Lutheran Brotherhood Cohort Study. Cancer research 1990;50(21):6836-40.

97. Talamini R, Franceschi S, La Vecchia C, Serraino D, Barra S, Negri E. Diet and prostatic cancer: a case-control study in northern Italy. Nutr Cancer 1992;18(3):277-86.

98. Lee MM, Wang RT, Hsing AW, Gu FL, Wang T, Spitz M. Case-control study of diet and prostate cancer in China. Cancer Causes Control 1998;9(6):545-52.

99. Key TJ, Allen N, Appleby P*, et al.* Fruits and vegetables and prostate cancer: no association among 1104 cases in a prospective study of 130544 men in the European Prospective Investigation into Cancer and Nutrition (EPIC). International journal of cancer 2004;109(1):119-24.

100. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of cruciferous vegetables and prostate cancer. Cancer Epidemiol Biomarkers Prev 2003;12(12):1403-9.

101. Schuurman AG, Goldbohm RA, Dorant E, van den Brandt PA. Vegetable and fruit consumption and prostate cancer risk: a cohort study in The Netherlands. Cancer Epidemiol Biomarkers Prev 1998;7(8):673-80.

102. Stram DO, Hankin JH, Wilkens LR*, et al.* Prostate cancer incidence and intake of fruits, vegetables and related micronutrients: the multiethnic cohort study* (United States). Cancer Causes Control 2006;17(9):1193-207.

103. Kirsh VA, Peters U, Mayne ST*, et al.* Prospective study of fruit and vegetable intake and risk of prostate cancer. Journal of the National Cancer Institute 2007;99(15):1200-9.
104. Etminan M, Takkouche B, Caamano-Isorna F. The role of tomato products and lycopene in the prevention of prostate cancer: a meta-analysis of observational studies. Cancer Epidemiol Biomarkers Prev 2004;13(3):340-5.
105. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of tomato products, lycopene, and prostate cancer risk. Journal of the National Cancer Institute 2002;94(5):391-8.
106. Chan JM, Gann PH, Giovannucci EL. Role of diet in prostate cancer development and progression. J Clin Oncol 2005;23(32):8152-60.
107. Kavanaugh CJ, Trumbo PR, Ellwood KC. The U.S. Food and Drug Administration's evidence-based review for qualified health claims: tomatoes, lycopene, and cancer. Journal of the National Cancer Institute 2007;99(14):1074-85.
108. Heinonen OP, Albanes D, Virtamo J*, et al.* Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. Journal of the National Cancer Institute 1998;90(6):440-6.
109. Helzlsouer KJ, Huang HY, Alberg AJ*, et al.* Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer. Journal of the National Cancer Institute 2000;92(24):2018-23.
110. Huang HY, Alberg AJ, Norkus EP, Hoffman SC, Comstock GW, Helzlsouer KJ. Prospective study of antioxidant micronutrients in the blood and the risk of developing prostate cancer. American journal of epidemiology 2003;157(4):335-44.
111. Chan JM, Stampfer MJ, Ma J, Rimm EB, Willett WC, Giovannucci EL. Supplemental vitamin E intake and prostate cancer risk in a large cohort of men in the United States. Cancer Epidemiol Biomarkers Prev 1999;8(10):893-9.
112. Gann PH, Ma J, Giovannucci E*, et al.* Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. Cancer research 1999;59(6):1225-30.
113. Goodman GE, Schaffer S, Omenn GS, Chen C, King I. The association between lung and prostate cancer risk, and serum micronutrients: results and lessons learned from beta-carotene and retinol efficacy trial. Cancer Epidemiol Biomarkers Prev 2003;12(6):518-26.

114. Nomura AM, Stemmermann GN, Lee J, Craft NE. Serum micronutrients and prostate cancer in Japanese Americans in Hawaii. Cancer Epidemiol Biomarkers Prev 1997;6(7):487-91.
115. Duffield-Lillico AJ, Dalkin BL, Reid ME*, et al.* Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. BJU international 2003;91(7):608-12.
116. Nomura AM, Lee J, Stemmermann GN, Combs GF, Jr. Serum selenium and subsequent risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 2000;9(9):883-7.
117. Yoshizawa K, Willett WC, Morris SJ*, et al.* Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. Journal of the National Cancer Institute 1998;90(16):1219-24.
118. Klein EA, Thompson IM, Lippman SM*, et al.* SELECT: the next prostate cancer prevention trial. Selenum and Vitamin E Cancer Prevention Trial. The Journal of urology 2001;166(4):1311-5.
119. Schwartz GG, Hulka BS. Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). Anticancer research 1990;10(5A):1307-11.
120. Grant WB. An estimate of premature cancer mortality in the U.S. due to inadequate doses of solar ultraviolet-B radiation. Cancer 2002;94(6):1867-75.
121. Colli JL, Grant WB. Solar ultraviolet B radiation compared with prostate cancer incidence and mortality rates in United States. Urology 2008;71(3):531-5.
122. Lagunova Z, Porojnicu AC, Dahlback A, Berg JP, Beer TM, Moan J. Prostate cancer survival is dependent on season of diagnosis. The Prostate 2007;67(12):1362-70.
123. Porojnicu A, Robsahm TE, Berg JP, Moan J. Season of diagnosis is a predictor of cancer survival. Sun-induced vitamin D may be involved: a possible role of sun-induced Vitamin D. The Journal of steroid biochemistry and molecular biology 2007;103(3-5):675-8.
124. Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer research 1994;54(3):805-10.
125. Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL, Holick MF. Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3. Cancer Epidemiol Biomarkers Prev 1998;7(5):391-5.

126. Skowronski RJ, Peehl DM, Feldman D. Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. Endocrinology 1993;132(5):1952-60.

127. Schwartz GG, Wang MH, Zang M, Singh RK, Siegal GP. 1 alpha,25-Dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells. Cancer Epidemiol Biomarkers Prev 1997;6(9):727-32.

128. Giovannucci E. The epidemiology of vitamin D and cancer incidence and mortality: a review (United States). Cancer Causes Control 2005;16(2):83-95.

129. Gann PH, Ma J, Hennekens CH, Hollis BW, Haddad JG, Stampfer MJ. Circulating vitamin D metabolites in relation to subsequent development of prostate cancer. Cancer Epidemiol Biomarkers Prev 1996;5(2):121-6.

130. Nomura AM, Stemmermann GN, Lee J*, et al.* Serum vitamin D metabolite levels and the subsequent development of prostate cancer (Hawaii, United States). Cancer Causes Control 1998;9(4):425-32.

131. Jacobs ET, Giuliano AR, Martinez ME, Hollis BW, Reid ME, Marshall JR. Plasma levels of 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D and the risk of prostate cancer. The Journal of steroid biochemistry and molecular biology 2004;89-90(1-5):533-7.

132. Platz EA, Leitzmann MF, Hollis BW, Willett WC, Giovannucci E. Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer. Cancer Causes Control 2004;15(3):255-65.

133. Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control 2000;11(9):847-52.

134. Tuohimaa P, Tenkanen L, Ahonen M*, et al.* Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a longitudinal, nested case-control study in the Nordic countries. International journal of cancer 2004;108(1):104-8.

135. Chan JM, Giovannucci E, Andersson SO, Yuen J, Adami HO, Wolk A. Dairy products, calcium, phosphorous, vitamin D, and risk of prostate cancer (Sweden). Cancer Causes Control 1998;9(6):559-66.

136. Kristal AR, Cohen JH, Qu P, Stanford JL. Associations of energy, fat, calcium, and vitamin D with prostate cancer risk. Cancer Epidemiol Biomarkers Prev 2002;11(8):719-25.

137. Grant WB, Garland CF. A critical review of studies on vitamin D in relation to colorectal cancer. Nutr Cancer 2004;48(2):115-23.
138. Hsu JY, Feldman D, McNeal JE, Peehl DM. Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition. Cancer research 2001;61(7):2852-6.
139. Giovannucci E, Liu Y, Stampfer MJ, Willett WC. A prospective study of calcium intake and incident and fatal prostate cancer. Cancer Epidemiol Biomarkers Prev 2006;15(2):203-10.
140. Rodriguez C, McCullough ML, Mondul AM*, et al.* Calcium, dairy products, and risk of prostate cancer in a prospective cohort of United States men. Cancer Epidemiol Biomarkers Prev 2003;12(7):597-603.
141. Qin LQ, Xu JY, Wang PY, Kaneko T, Hoshi K, Sato A. Milk consumption is a risk factor for prostate cancer: meta-analysis of case-control studies. Nutr Cancer 2004;48(1):22-7.
142. Gao X, LaValley MP, Tucker KL. Prospective studies of dairy product and calcium intakes and prostate cancer risk: a meta-analysis. Journal of the National Cancer Institute 2005;97(23):1768-77.
143. Bonjour JP, Chevalley T, Fardellone P. Calcium intake and vitamin D metabolism and action, in healthy conditions and in prostate cancer. The British journal of nutrition 2007;97(4):611-6.
144. Ip C, Dong Y, Ganther HE. New concepts in selenium chemoprevention. Cancer metastasis reviews 2002;21(3-4):281-9.
145. Klein EA. Selenium: epidemiology and basic science. The Journal of urology 2004;171(2 Pt 2):S50-3; discussion S3.
146. Fleshner NE. Vitamin E and prostate cancer. The Urologic clinics of North America 2002;29(1):107-13, ix.
147. Guns ES, Cowell SP. Drug Insight: lycopene in the prevention and treatment of prostate cancer. Nature clinical practice 2005;2(1):38-43.
148. J.P.J. Marais BD, R.A Dixon, and D. Ferreira. The Stereochemistry of Flavonoids. In: Grotewold E, editor. The Science of Flavonoids: Springer Science; 2006.
149. Beecher GR. Overview of dietary flavonoids: nomenclature, occurrence and intake. J Nutr 2003;133(10):3248S-54S.

150. Bohm BA. Structural variation and the flavonoid literature. Introduction to Flavonoids: Harwood Academic; 1998. p. 5-7.

151. Øyvind M. Andersen KRM. Flavonoids: Chemistry, Biochemistry and Applications: CRC Press; 2006.

152. Prasain JK, Barnes S. Metabolism and Bioavailability of Flavonoids in Chemoprevention: Current Analytical Strategies and Future Prospectus. Mol Pharm 2007;4(6):846-64.

153. Timberlake CF, Henry BS. Plant pigments as natural food colours. Endeavour 1986;10(1):31-6.

154. Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant physiology 2001;126(2):485-93.

155. Taylor LP, Grotewold E. Flavonoids as developmental regulators. Current opinion in plant biology 2005;8(3):317-23.

156. Treutter D. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant biology (Stuttgart, Germany) 2005;7(6):581-91.

157. Winkel-Shirley B. Biosynthesis of flavonoids and effects of stress. Current opinion in plant biology 2002;5(3):218-23.

158. Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes. Biotechnology journal 2007;2(10):1235-49.

159. Harnly JM, Doherty RF, Beecher GR*, et al.* Flavonoid content of U.S. fruits, vegetables, and nuts. Journal of agricultural and food chemistry 2006;54(26):9966-77.

160. Williams CA, Grayer RJ. Anthocyanins and other flavonoids. Natural product reports 2004;21(4):539-73.

161. Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 1993;20(1):21-9.

162. Johannot L, Somerset SM. Age-related variations in flavonoid intake and sources in the Australian population. Public health nutrition 2006;9(8):1045-54.

163. Sampson L, Rimm E, Hollman PC, de Vries JH, Katan MB. Flavonol and flavone intakes in US health professionals. Journal of the American Dietetic Association 2002;102(10):1414-20.

164. Kuhnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. World review of nutrition and dietetics 1976;24:117-91.
165. Bentsath A, St. Rusznyak I, Szent-Gyorgyi A. Vitamin nature of flavones. Nature 1936;138.
166. Harborne J. The Flavonoids: Advances in Research Since 1986: CRC Press; 1994.
167. Bosetti C, Bravi F, Talamini R*, et al.* Flavonoids and prostate cancer risk: a study in Italy. Nutr Cancer 2006;56(2):123-7.
168. Hedelin M, Klint A, Chang ET*, et al.* Dietary phytoestrogen, serum enterolactone and risk of prostate cancer: the cancer prostate Sweden study (Sweden). Cancer Causes Control 2006;17(2):169-80.
169. Jacobsen BK, Knutsen SF, Fraser GE. Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). Cancer Causes Control 1998;9(6):553-7.
170. Kurahashi N, Iwasaki M, Sasazuki S, Otani T, Inoue M, Tsugane S. Soy product and isoflavone consumption in relation to prostate cancer in Japanese men. Cancer Epidemiol Biomarkers Prev 2007;16(3):538-45.
171. Kurahashi N, Sasazuki S, Iwasaki M, Inoue M, Tsugane S. Green tea consumption and prostate cancer risk in Japanese men: a prospective study. American journal of epidemiology 2008;167(1):71-7.
172. Kikuchi N, Ohmori K, Shimazu T*, et al.* No association between green tea and prostate cancer risk in Japanese men: the Ohsaki Cohort Study. British journal of cancer 2006;95(3):371-3.
173. Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). Cancer Causes Control 2001;12(9):789-96.
174. Knekt P, Jarvinen R, Seppanen R*, et al.* Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. American journal of epidemiology 1997;146(3):223-30.
175. Knekt P, Kumpulainen J, Jarvinen R*, et al.* Flavonoid intake and risk of chronic diseases. The American journal of clinical nutrition 2002;76(3):560-8.
176. Bosetti C, Spertini L, Parpinel M*, et al.* Flavonoids and breast cancer risk in Italy. Cancer Epidemiol Biomarkers Prev 2005;14(4):805-8.
177. den Tonkelaar I, Keinan-Boker L, Veer PV*, et al.* Urinary phytoestrogens and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 2001;10(3):223-8.

178. dos Santos Silva I, Mangtani P, McCormack V, Bhakta D, McMichael AJ, Sevak L. Phyto-oestrogen intake and breast cancer risk in South Asian women in England: findings from a population-based case-control study. Cancer Causes Control 2004;15(8):805-18.
179. Fink BN, Steck SE, Wolff MS*, et al.* Dietary flavonoid intake and breast cancer survival among women on Long Island. Cancer Epidemiol Biomarkers Prev 2007;16(11):2285-92.
180. Fink BN, Steck SE, Wolff MS*, et al.* Dietary flavonoid intake and breast cancer risk among women on Long Island. American journal of epidemiology 2007;165(5):514-23.
181. Grace PB, Taylor JI, Low YL*, et al.* Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. Cancer Epidemiol Biomarkers Prev 2004;13(5):698-708.
182. Hirose K, Imaeda N, Tokudome Y*, et al.* Soybean products and reduction of breast cancer risk: a case-control study in Japan. British journal of cancer 2005;93(1):15-22.
183. Horn-Ross PL, John EM, Lee M*, et al.* Phytoestrogen consumption and breast cancer risk in a multiethnic population: the Bay Area Breast Cancer Study. American journal of epidemiology 2001;154(5):434-41.
184. Linseisen J, Piller R, Hermann S, Chang-Claude J. Dietary phytoestrogen intake and premenopausal breast cancer risk in a German case-control study. International journal of cancer 2004;110(2):284-90.
185. Murkies A, Dalais FS, Briganti EM*, et al.* Phytoestrogens and breast cancer in postmenopausal women: a case control study. Menopause (New York, NY 2000;7(5):289-96.
186. Piller R, Chang-Claude J, Linseisen J. Plasma enterolactone and genistein and the risk of premenopausal breast cancer. Eur J Cancer Prev 2006;15(3):225-32.
187. Thanos J, Cotterchio M, Boucher BA, Kreiger N, Thompson LU. Adolescent dietary phytoestrogen intake and breast cancer risk (Canada). Cancer Causes Control 2006;17(10):1253-61.
188. Torres-Sanchez L, Lopez-Carrillo L, Lopez-Cervantes M, Rueda-Neria C, Wolff MS. Food sources of phytoestrogens and breast cancer risk in Mexican women. Nutr Cancer 2000;37(2):134-9.
189. Verheus M, van Gils CH, Keinan-Boker L, Grace PB, Bingham SA, Peeters PH. Plasma phytoestrogens and subsequent breast cancer risk. J Clin Oncol 2007;25(6):648-55.

190. Wu AH, Wan P, Hankin J, Tseng CC, Yu MC, Pike MC. Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. Carcinogenesis 2002;23(9):1491-6.
191. Wu AH, Yu MC, Tseng CC, Hankin J, Pike MC. Green tea and risk of breast cancer in Asian Americans. International journal of cancer 2003;106(4):574-9.
192. Zhang M, Holman CD, Huang JP, Xie X. Green tea and the prevention of breast cancer: a case-control study in Southeast China. Carcinogenesis 2007;28(5):1074-8.
193. Zheng W, Dai Q, Custer LJ*, et al.* Urinary excretion of isoflavonoids and the risk of breast cancer. Cancer Epidemiol Biomarkers Prev 1999;8(1):35-40.
194. Adebamowo CA, Cho E, Sampson L*, et al.* Dietary flavonols and flavonol-rich foods intake and the risk of breast cancer. International journal of cancer 2005;114(4):628-33.
195. Horn-Ross PL, Hoggatt KJ, West DW*, et al.* Recent diet and breast cancer risk: the California Teachers Study (USA). Cancer Causes Control 2002;13(5):407-15.
196. Keinan-Boker L, van Der Schouw YT, Grobbee DE, Peeters PH. Dietary phytoestrogens and breast cancer risk. The American journal of clinical nutrition 2004;79(2):282-8.
197. Touillaud MS, Thiebaut AC, Niravong M, Boutron-Ruault MC, Clavel-Chapelon F. No association between dietary phytoestrogens and risk of premenopausal breast cancer in a French cohort study. Cancer Epidemiol Biomarkers Prev 2006;15(12):2574-6.
198. Travis RC, Allen NE, Appleby PN, Spencer EA, Roddam AW, Key TJ. A prospective study of vegetarianism and isoflavone intake in relation to breast cancer risk in British women. International journal of cancer 2008;122(3):705-10.
199. Yamamoto S, Sobue T, Kobayashi M, Sasaki S, Tsugane S. Soy, isoflavones, and breast cancer risk in Japan. Journal of the National Cancer Institute 2003;95(12):906-13.
200. Goodman MT, Wilkens LR, Hankin JH, Lyu LC, Wu AH, Kolonel LN. Association of soy and fiber consumption with the risk of endometrial cancer. American journal of epidemiology 1997;146(4):294-306.
201. Horn-Ross PL, John EM, Canchola AJ, Stewart SL, Lee MM. Phytoestrogen intake and endometrial cancer risk. Journal of the National Cancer Institute 2003;95(15):1158-64.
202. Xu WH, Zheng W, Xiang YB*, et al.* Soya food intake and risk of endometrial cancer among Chinese women in Shanghai: population based case-control study. BMJ (Clinical research ed 2004;328(7451):1285.

203. Lin J, Zhang SM, Wu K, Willett WC, Fuchs CS, Giovannucci E. Flavonoid intake and colorectal cancer risk in men and women. American journal of epidemiology 2006;164(7):644-51.

204. Oba S, Nagata C, Shimizu N*, et al.* Soy product consumption and the risk of colon cancer: a prospective study in Takayama, Japan. Nutr Cancer 2007;57(2):151-7.

205. Sun CL, Yuan JM, Koh WP, Lee HP, Yu MC. Green tea and black tea consumption in relation to colorectal cancer risk: the Singapore Chinese Health Study. Carcinogenesis 2007;28(10):2143-8.

206. Suzuki Y, Tsubono Y, Nakaya N*, et al.* Green tea and the risk of colorectal cancer: pooled analysis of two prospective studies in Japan. Journal of epidemiology / Japan Epidemiological Association 2005;15(4):118-24.

207. Yang G, Shu XO, Li H*, et al.* Prospective cohort study of green tea consumption and colorectal cancer risk in women. Cancer Epidemiol Biomarkers Prev 2007;16(6):1219-23.

208. Hoshiyama Y, Kawaguchi T, Miura Y*, et al.* A prospective study of stomach cancer death in relation to green tea consumption in Japan. British journal of cancer 2002;87(3):309-13.

209. Nagata C, Takatsuka N, Kawakami N, Shimizu H. A prospective cohort study of soy product intake and stomach cancer death. British journal of cancer 2002;87(1):31-6.

210. Sasazuki S, Inoue M, Hanaoka T, Yamamoto S, Sobue T, Tsugane S. Green tea consumption and subsequent risk of gastric cancer by subsite: the JPHC Study. Cancer Causes Control 2004;15(5):483-91.

211. Tsubono Y, Nishino Y, Komatsu S*, et al.* Green tea and the risk of gastric cancer in Japan. The New England journal of medicine 2001;344(9):632-6.

212. Luo J, Inoue M, Iwasaki M*, et al.* Green tea and coffee intake and risk of pancreatic cancer in a large-scale, population-based cohort study in Japan (JPHC study). Eur J Cancer Prev 2007;16(6):542-8.

213. Nothlings U, Murphy SP, Wilkens LR, Henderson BE, Kolonel LN. Flavonols and pancreatic cancer risk: the multiethnic cohort study. American journal of epidemiology 2007;166(8):924-31.

214. Chang ET, Lee VS, Canchola AJ*, et al.* Diet and risk of ovarian cancer in the California Teachers Study cohort. American journal of epidemiology 2007;165(7):802-13.

215. Gates MA, Tworoger SS, Hecht JL, De Vivo I, Rosner B, Hankinson SE. A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. International journal of cancer 2007;121(10):2225-32.
216. Larsson SC, Wolk A. Tea consumption and ovarian cancer risk in a population-based cohort. Archives of internal medicine 2005;165(22):2683-6.
217. Ide R, Fujino Y, Hoshiyama Y*, et al.* A prospective study of green tea consumption and oral cancer incidence in Japan. Annals of epidemiology 2007;17(10):821-6.
218. Garcia R, Gonzalez CA, Agudo A, Riboli E. High intake of specific carotenoids and flavonoids does not reduce the risk of bladder cancer. Nutr Cancer 1999;35(2):212-4.
219. Lu CM, Lan SJ, Lee YH, Huang JK, Huang CH, Hsieh CC. Tea consumption: fluid intake and bladder cancer risk in Southern Taiwan. Urology 1999;54(5):823-8.
220. Sun CL, Yuan JM, Arakawa K, Low SH, Lee HP, Yu MC. Dietary soy and increased risk of bladder cancer: the Singapore Chinese Health Study. Cancer Epidemiol Biomarkers Prev 2002;11(12):1674-7.
221. Wittwer E, Kern SE. Role of morphine's metabolites in analgesia: concepts and controversies. The AAPS journal 2006;8(2):E348-52.
222. Piskula MK, Yamakoshi J, Iwai Y. Daidzein and genistein but not their glucosides are absorbed from the rat stomach. FEBS letters 1999;447(2-3):287-91.
223. Griffiths LA, Barrow A. Metabolism of flavonoid compounds in germ-free rats. The Biochemical journal 1972;130(4):1161-2.
224. Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. The American journal of clinical nutrition 1995;62(6):1276-82.
225. Walgren RA, Lin JT, Kinne RK, Walle T. Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. The Journal of pharmacology and experimental therapeutics 2000;294(3):837-43.
226. Spencer JP, Chowrimootoo G, Choudhury R, Debnam ES, Srai SK, Rice-Evans C. The small intestine can both absorb and glucuronidate luminal flavonoids. FEBS letters 1999;458(2):224-30.
227. Day AJ, Gee JM, DuPont MS, Johnson IT, Williamson G. Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin

hydrolase and the sodium-dependent glucose transporter. Biochemical pharmacology 2003;65(7):1199-206.

228. Walle T, Browning AM, Steed LL, Reed SG, Walle UK. Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. J Nutr 2005;135(1):48-52.

229. Rice-Evans C. Flavonoid antioxidants. Current medicinal chemistry 2001;8(7):797-807.

230. Hollman PC, Bijsman MN, van Gameren Y, Cnossen EP, de Vries JH, Katan MB. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. Free radical research 1999;31(6):569-73.

231. Walle T. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? Seminars in cancer biology 2007;17(5):354-62.

232. Prasain JK, Barnes S. Metabolism and bioavailability of flavonoids in chemoprevention: current analytical strategies and future prospectus. Mol Pharm 2007;4(6):846-64.

233. Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. Archives of biochemistry and biophysics 2004;423(1):148-61.

234. Williamson G, Day AJ, Plumb GW, Couteau D. Human metabolic pathways of dietary flavonoids and cinnamates. Biochemical Society transactions 2000;28(2):16-22.

235. Janisch KM, Williamson G, Needs P, Plumb GW. Properties of quercetin conjugates: modulation of LDL oxidation and binding to human serum albumin. Free radical research 2004;38(8):877-84.

236. Nielsen IL, Williamson G. Review of the factors affecting bioavailability of soy isoflavones in humans. Nutr Cancer 2007;57(1):1-10.

237. Hollman PC, vd Gaag M, Mengelers MJ, van Trijp JM, de Vries JH, Katan MB. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. Free radical biology & medicine 1996;21(5):703-7.

238. Chu KO, Wang CC, Chu CY, Rogers MS, Choy KW, Pang CP. Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. Journal of chromatography 2004;810(2):187-95.

239. Manach C, Donovan JL. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free radical research 2004;38(8):771-85.

240. Gennaro L, Leonardi C, Esposito F*, et al.* Flavonoid and carbohydrate contents in Tropea red onions: effects of homelike peeling and storage. Journal of agricultural and food chemistry 2002;50(7):1904-10.

241. Nemeth K, Piskula MK. Food content, processing, absorption and metabolism of onion flavonoids. Critical reviews in food science and nutrition 2007;47(4):397-409.

242. Hollman PC, Van Het Hof KH, Tijburg LB, Katan MB. Addition of milk does not affect the absorption of flavonols from tea in man. Free radical research 2001;34(3):297-300.

243. Brown JP. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. Mutation research 1980;75(3):243-77.

244. Chow HH, Cai Y, Hakim IA*, et al.* Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clin Cancer Res 2003;9(9):3312-9.

245. Dunnick JK, Hailey JR. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. Fundam Appl Toxicol 1992;19(3):423-31.

246. Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free radical biology & medicine 2004;37(3):287-303.

247. Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. Food Chem Toxicol 2007;45(11):2179-205.

248. Isbrucker RA, Bausch J, Edwards JA, Wolz E. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 1: genotoxicity. Food Chem Toxicol 2006;44(5):626-35.

249. Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats. Food Chem Toxicol 2006;44(5):651-61.

250. Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies. Food Chem Toxicol 2006;44(5):636-50.

251. McClain RM, Wolz E, Davidovich A, Edwards J, Bausch J. Reproductive safety studies with genistein in rats. Food Chem Toxicol 2007;45(8):1319-32.

252. McClain RM, Wolz E, Davidovich A, Pfannkuch F, Bausch J. Subchronic and chronic safety studies with genistein in dogs. Food Chem Toxicol 2005;43(10):1461-82.
253. Mennen LI, Walker R, Bennetau-Pelissero C, Scalbert A. Risks and safety of polyphenol consumption. The American journal of clinical nutrition 2005;81(1 Suppl):326S-9S.
254. Merritt RJ, Jenks BH. Safety of soy-based infant formulas containing isoflavones: the clinical evidence. J Nutr 2004;134(5):1220S-4S.
255. Michael McClain R, Wolz E, Davidovich A, Bausch J. Genetic toxicity studies with genistein. Food Chem Toxicol 2006;44(1):42-55.
256. Michael McClain R, Wolz E, Davidovich A, Pfannkuch F, Edwards JA, Bausch J. Acute, subchronic and chronic safety studies with genistein in rats. Food Chem Toxicol 2006;44(1):56-80.
257. Miltyk W, Craciunescu CN, Fischer L*, et al.* Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein, and glycitein) in 20 patients with prostate cancer. The American journal of clinical nutrition 2003;77(4):875-82.
258. Nahas EA, Nahas-Neto J, Orsatti FL, Carvalho EP, Oliveira ML, Dias R. Efficacy and safety of a soy isoflavone extract in postmenopausal women: a randomized, double-blind, and placebo-controlled study. Maturitas 2007;58(3):249-58.
259. Rozman KK, Bhatia J, Calafat AM*, et al.* NTP-CERHR expert panel report on the reproductive and developmental toxicity of genistein. Birth defects research 2006;77(6):485-638.
260. Shoji T, Akazome Y, Kanda T, Ikeda M. The toxicology and safety of apple polyphenol extract. Food Chem Toxicol 2004;42(6):959-67.
261. Skibola CF, Smith MT. Potential health impacts of excessive flavonoid intake. Free radical biology & medicine 2000;29(3-4):375-83.
262. Stopper H, Schmitt E, Kobras K. Genotoxicity of phytoestrogens. Mutation research 2005;574(1-2):139-55.
263. Verschoyle RD, Greaves P, Cai H*, et al.* Preliminary safety evaluation of the putative cancer chemopreventive agent tricin, a naturally occurring flavone. Cancer chemotherapy and pharmacology 2006;57(1):1-6.
264. West MC. The impact of dietary oestrogens on male and female fertility. Current opinion in obstetrics & gynecology 2007;19(3):215-21.

265. Wuttke W, Jarry H, Seidlova-Wuttke D. Isoflavones--safe food additives or dangerous drugs? Ageing research reviews 2007;6(2):150-88.

266. Yamakoshi J, Saito M, Kataoka S, Kikuchi M. Safety evaluation of proanthocyanidin-rich extract from grape seeds. Food Chem Toxicol 2002;40(5):599-607.

267. Miltyk W, Craciunescu CN, Fischer L*, et al.* Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein, and glycitein) in 20 patients with prostate cancer. The American journal of clinical nutrition 2003;77(4):875-82.

268. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344(8924):721-4.

269. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. Free radical biology & medicine 2007;43(4):477-503.

270. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Molecular and cellular biochemistry 2004;266(1-2):37-56.

271. Nagy IZ. On the true role of oxygen free radicals in the living state, aging, and degenerative disorders. Annals of the New York Academy of Sciences 2001;928:187-99.

272. Halliwell B. Oxidative stress and cancer: have we moved forward? The Biochemical journal 2007;401(1):1-11.

273. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science (New York, NY 1996;273(5271):59-63.

274. Halliwell B. Antioxidants and human disease: a general introduction. Nutrition reviews 1997;55(1 Pt 2):S44-9; discussion S9-52.

275. Seifried HE, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. J Nutr Biochem 2007;18(9):567-79.

276. Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. The American journal of clinical nutrition 2005;81(1 Suppl):215S-7S.

277. Bors W, Heller W, Michel C, Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods in enzymology 1990;186:343-55.

278. Jovanovic SV, Simic MG. Antioxidants in nutrition. Annals of the New York Academy of Sciences 2000;899:326-34.

279. Pietta PG. Flavonoids as antioxidants. Journal of natural products 2000;63(7):1035-42.

280. Mira L, Fernandez MT, Santos M, Rocha R, Florencio MH, Jennings KR. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free radical research 2002;36(11):1199-208.

281. Van Hoorn DE, Nijveldt RJ, Van Leeuwen PA*, et al.* Accurate prediction of xanthine oxidase inhibition based on the structure of flavonoids. European journal of pharmacology 2002;451(2):111-8.

282. van Zanden JJ, Geraets L, Wortelboer HM, van Bladeren PJ, Rietjens IM, Cnubben NH. Structural requirements for the flavonoid-mediated modulation of glutathione S-transferase P1-1 and GS-X pump activity in MCF7 breast cancer cells. Biochemical pharmacology 2004;67(8):1607-17.

283. Raso GM, Meli R, Di Carlo G, Pacilio M, Di Carlo R. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life sciences 2001;68(8):921-31.

284. Morre DJ, Bridge A, Wu LY, Morre DM. Preferential inhibition by (-)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. Biochemical pharmacology 2000;60(7):937-46.

285. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free radical biology & medicine 1996;20(7):933-56.

286. Pryor WA, Cornicelli JA, Devall LJ*, et al.* A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. Journal of Organic Chemistry 1993;58:3521-32.

287. Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic M. Flavonoids as Antioxidants. Journal of the American Chemical Society 1994;116(11):4846-51.

288. Lotito SB, Frei B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free radical biology & medicine 2006;41(12):1727-46.

289. Halliwell B, Rafter J, Jenner A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? The American journal of clinical nutrition 2005;81(1 Suppl):268S-76S.

290. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free radical biology & medicine 1997;22(5):749-60.

291. Michels G, Haenen GR, Watjen W, Rietjens S, Bast A. The thiol reactivity of the oxidation product of 3,5,7-trihydroxy-4H-chromen-4-one containing flavonoids. Toxicology letters 2004;151(1):105-11.

292. Hodnick WF, Kung FS, Roettger WJ, Bohmont CW, Pardini RS. Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study. Biochemical pharmacology 1986;35(14):2345-57.

293. Metodiewa D, Jaiswal AK, Cenas N, Dickancaite E, Segura-Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free radical biology & medicine 1999;26(1-2):107-16.

294. Canada AT, Giannella E, Nguyen TD, Mason RP. The production of reactive oxygen species by dietary flavonols. Free radical biology & medicine 1990;9(5):441-9.

295. Shao ZH, Vanden Hoek TL, Xie J*, et al.* Grape seed proanthocyanidins induce pro-oxidant toxicity in cardiomyocytes. Cardiovascular toxicology 2003;3(4):331-9.

296. Azam S, Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicol In Vitro 2004;18(5):555-61.

297. Raza H, John A. Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. Toxicol Appl Pharmacol 2005;207(3):212-20.

298. Choi EJ. The prooxidant, rather than antioxidant, acts of daidzein in vivo and in vitro: daidzein suppresses glutathione metabolism. European journal of pharmacology 2006;542(1-3):162-9.

299. Woo AY, Cheng CH, Waye MM. Baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism. Cardiovascular research 2005;65(1):244-53.

300. Halliwell B. Oxidative stress in cell culture: an under-appreciated problem? FEBS letters 2003;540(1-3):3-6.

301. Boots AW, Balk JM, Bast A, Haenen GR. The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity. Biochem Biophys Res Commun 2005;338(2):923-9.

302. Bennets HW, Underwood FL, Shier A. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust Vet J 1946;22:124-9.

303. Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978;103(5):1860-7.
304. Mayr U, Butsch A, Schneider S. Validation of two in vitro test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. Toxicology 1992;74(2-3):135-49.
305. Makela S, Davis VL, Tally WC*, et al.* Dietary Estrogens Act through Estrogen Receptor-Mediated Processes and Show No Antiestrogenicity in Cultured Breast Cancer Cells. Environmental health perspectives 1994;102(6-7):572-8.
306. Kuiper GG, Lemmen JG, Carlsson B*, et al.* Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139(10):4252-63.
307. Kuiper GG, Carlsson B, Grandien K*, et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997;138(3):863-70.
308. Pike AC, Brzozowski AM, Hubbard RE*, et al.* Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. The EMBO journal 1999;18(17):4608-18.
309. Routledge EJ, White R, Parker MG, Sumpter JP. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 2000;275(46):35986-93.
310. Heldring N, Pike A, Andersson S*, et al.* Estrogen receptors: how do they signal and what are their targets. Physiological reviews 2007;87(3):905-31.
311. An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 2001;276(21):17808-14.
312. van der Woude H, Ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM. The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. Molecular nutrition & food research 2005;49(8):763-71.
313. Wang TT, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 1996;17(2):271-5.
314. Maggiolini M, Bonofiglio D, Marsico S*, et al.* Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. Molecular pharmacology 2001;60(3):595-602.

315. Pettersson K, Delaunay F, Gustafsson JA. Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 2000;19(43):4970-8.
316. Hackett JC, Kim YW, Su B, Brueggemeier RW. Synthesis and characterization of azole isoflavone inhibitors of aromatase. Bioorganic & medicinal chemistry 2005;13(12):4063-70.
317. Kao YC, Zhou C, Sherman M, Laughton CA, Chen S. Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: A site-directed mutagenesis study. Environmental health perspectives 1998;106(2):85-92.
318. Basly JP, Lavier MC. Dietary phytoestrogens: potential selective estrogen enzyme modulators? Planta medica 2005;71(4):287-94.
319. Pino AM, Valladares LE, Palma MA, Mancilla AM, Yanez M, Albala C. Dietary isoflavones affect sex hormone-binding globulin levels in postmenopausal women. The Journal of clinical endocrinology and metabolism 2000;85(8):2797-800.
320. Cassidy A, Bingham S, Setchell KD. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. The American journal of clinical nutrition 1994;60(3):333-40.
321. Jakes RW, Alexander L, Duffy SW, Leong J, Chen LH, Lee WH. Dietary intake of soybean protein and menstrual cycle length in pre-menopausal Singapore Chinese women. Public health nutrition 2001;4(2):191-6.
322. Kurzer MS. Hormonal effects of soy in premenopausal women and men. J Nutr 2002;132(3):570S-3S.
323. Beck V, Unterrieder E, Krenn L, Kubelka W, Jungbauer A. Comparison of hormonal activity (estrogen, androgen and progestin) of standardized plant extracts for large scale use in hormone replacement therapy. The Journal of steroid biochemistry and molecular biology 2003;84(2-3):259-68.
324. Fang H, Tong W, Branham WS*, et al.* Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. Chemical research in toxicology 2003;16(10):1338-58.
325. Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF. Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. J Nutr Biochem 2005;16(6):321-30.

326. Zand RS, Jenkins DJ, Diamandis EP. Steroid hormone activity of flavonoids and related compounds. Breast cancer research and treatment 2000;62(1):35-49.
327. Rosenberg Zand RS, Jenkins DJ, Brown TJ, Diamandis EP. Flavonoids can block PSA production by breast and prostate cancer cell lines. Clinica chimica acta; international journal of clinical chemistry 2002;317(1-2):17-26.
328. Maggiolini M, Vivacqua A, Carpino A*, et al.* The mutant androgen receptor T877A mediates the proliferative but not the cytotoxic dose-dependent effects of genistein and quercetin on human LNCaP prostate cancer cells. Molecular pharmacology 2002;62(5):1027-35.
329. Gao S, Liu GZ, Wang Z. Modulation of androgen receptor-dependent transcription by resveratrol and genistein in prostate cancer cells. The Prostate 2004;59(2):214-25.
330. Morris JD, Pramanik R, Zhang X*, et al.* Selenium- or quercetin-induced retardation of DNA synthesis in primary prostate cells occurs in the presence of a concomitant reduction in androgen-receptor activity. Cancer Lett 2006;239(1):111-22.
331. Ren F, Zhang S, Mitchell SH, Butler R, Young CY. Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. Oncogene 2000;19(15):1924-32.
332. Gilad LA, Tirosh O, Schwartz B. Phytoestrogens regulate transcription and translation of vitamin D receptor in colon cancer cells. The Journal of endocrinology 2006;191(2):387-98.
333. Farhan H, Wahala K, Cross HS. Genistein inhibits vitamin D hydroxylases CYP24 and CYP27B1 expression in prostate cells. The Journal of steroid biochemistry and molecular biology 2003;84(4):423-9.
334. Szkudelska K, Nogowski L. Genistein--a dietary compound inducing hormonal and metabolic changes. The Journal of steroid biochemistry and molecular biology 2007;105(1-5):37-45.
335. Doerge DR, Sheehan DM. Goitrogenic and estrogenic activity of soy isoflavones. Environmental health perspectives 2002;110 Suppl 3:349-53.
336. Hamilton-Reeves JM, Rebello SA, Thomas W, Slaton JW, Kurzer MS. Isoflavone-rich soy protein isolate suppresses androgen receptor expression without altering estrogen receptor-beta expression or serum hormonal profiles in men at high risk of prostate cancer. J Nutr 2007;137(7):1769-75.

337. Chang KL, Kung ML, Chow NH, Su SJ. Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulation of p21waf1/cip1 and Wee1. Biochem Pharmacol 2004;67(4):717-26.

338. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2001;27(3):247-54.

339. Shiloh Y, Kastan MB. ATM: genome stability, neuronal development, and cancer cross paths. Adv Cancer Res 2001;83:209-54.

340. McGowan CH, Russell P. The DNA damage response: sensing and signaling. Curr Opin Cell Biol 2004;16(6):629-33.

341. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421(6922):499-506.

342. Maya R, Balass M, Kim ST*, et al.* ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev 2001;15(9):1067-77.

343. Fei P, El-Deiry WS. P53 and radiation responses. Oncogene 2003;22(37):5774-83.

344. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 2001;276(45):42462-7.

345. Saito S, Goodarzi AA, Higashimoto Y*, et al.* ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem 2002;277(15):12491-4.

346. Park M, Chae HD, Yun J*, et al.* Constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated G2-M arrest. Cancer Res 2000;60(3):542-5.

347. Fattaey A, Booher RN. Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. Prog Cell Cycle Res 1997;3:233-40.

348. Busino L, Chiesa M, Draetta GF, Donzelli M. Cdc25A phosphatase: combinatorial phosphorylation, ubiquitylation and proteolysis. Oncogene 2004;23(11):2050-6.

349. Ahn J, Urist M, Prives C. The Chk2 protein kinase. DNA Repair (Amst) 2004;3(8-9):1039-47.

350. Shukla S, Gupta S. Apigenin-induced cell cycle arrest is mediated by modulation of MAPK, PI3K-Akt, and loss of cyclin D1 associated retinoblastoma dephosphorylation in human prostate cancer cells. Cell cycle (Georgetown, Tex 2007;6(9):1102-14.

351. Shukla S, Gupta S. Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther 2006;5(4):843-52.
352. Gupta S, Afaq F, Mukhtar H. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. Biochem Biophys Res Commun 2001;287(4):914-20.
353. Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. Flavonoids suppress androgen-independent human prostate tumor proliferation. Nutr Cancer 2000;38(1):116-22.
354. Gupta S, Ahmad N, Nieminen AL, Mukhtar H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicol Appl Pharmacol 2000;164(1):82-90.
355. Hastak K, Agarwal MK, Mukhtar H, Agarwal ML. Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate. Faseb J 2005;19(7):789-91.
356. Gupta S, Hussain T, Mukhtar H. Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. Archives of biochemistry and biophysics 2003;410(1):177-85.
357. Oki T, Sowa Y, Hirose T*, et al.* Genistein induces Gadd45 gene and G2/M cell cycle arrest in the DU145 human prostate cancer cell line. FEBS letters 2004;577(1-2):55-9.
358. Xiang H, Schevzov G, Gunning P, Williams HM, Silink M. A comparative study of growth-inhibitory effects of isoflavones and their metabolites on human breast and prostate cancer cell lines. Nutr Cancer 2002;42(2):224-32.
359. Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. Jpn J Cancer Res 2000;91(2):164-73.
360. Shen JC, Klein RD, Wei Q*, et al.* Low-dose genistein induces cyclin-dependent kinase inhibitors and G(1) cell-cycle arrest in human prostate cancer cells. Molecular carcinogenesis 2000;29(2):92-102.
361. Zi X, Agarwal R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate

cancer intervention. Proceedings of the National Academy of Sciences of the United States of America 1999;96(13):7490-5.

362. Deep G, Singh RP, Agarwal C, Kroll DJ, Agarwal R. Silymarin and silibinin cause G1 and G2-M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture silymarin. Oncogene 2006;25(7):1053-69.

363. Kobayashi T, Nakata T, Kuzumaki T. Effect of flavonoids on cell cycle progression in prostate cancer cells. Cancer Lett 2002;176(1):17-23.

364. Ikezoe T, Chen SS, Heber D, Taguchi H, Koeffler HP. Baicalin is a major component of PC-SPES which inhibits the proliferation of human cancer cells via apoptosis and cell cycle arrest. Prostate 2001;49(4):285-92.

365. Singh RP, Agrawal P, Yim D, Agarwal C, Agarwal R. Acacetin inhibits cell growth and cell cycle progression, and induces apoptosis in human prostate cancer cells: structure-activity relationship with linarin and linarin acetate. Carcinogenesis 2005;26(4):845-54.

366. Tyagi A, Singh RP, Agarwal C, Agarwal R. Silibinin activates p53-caspase 2 pathway and causes caspase-mediated cleavage of Cip1/p21 in apoptosis induction in bladder transitional-cell papilloma RT4 cells: evidence for a regulatory loop between p53 and caspase 2. Carcinogenesis 2006;27(11):2269-80.

367. O'Prey J, Brown J, Fleming J, Harrison PR. Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells. Biochemical pharmacology 2003;66(11):2075-88.

368. Bartkova J, Horejsi Z, Koed K*, et al.* DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434(7035):864-70.

369. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2(9):647-56.

370. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269-90.

371. Gupta S, Afaq F, Mukhtar H. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene 2002;21(23):3727-38.

372. Kim YH, Lee YJ. TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. Journal of cellular biochemistry 2007;100(4):998-1009.
373. Vijayababu MR, Kanagaraj P, Arunkumar A, Ilangovan R, Dharmarajan A, Arunakaran J. Quercetin induces p53-independent apoptosis in human prostate cancer cells by modulating Bcl-2-related proteins: a possible mediation by IGFBP-3. Oncology research 2006;16(2):67-74.
374. Hastak K, Gupta S, Ahmad N, Agarwal MK, Agarwal ML, Mukhtar H. Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. Oncogene 2003;22(31):4851-9.
375. Agarwal C, Tyagi A, Kaur M, Agarwal R. Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. Carcinogenesis 2007;28(7):1463-70.
376. Chan FL, Choi HL, Chen ZY, Chan PS, Huang Y. Induction of apoptosis in prostate cancer cell lines by a flavonoid, baicalin. Cancer Lett 2000;160(2):219-28.
377. Davis JN, Singh B, Bhuiyan M, Sarkar FH. Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. Nutr Cancer 1998;32(3):123-31.
378. Jung JI, Lim SS, Choi HJ*, et al.* Isoliquiritigenin induces apoptosis by depolarizing mitochondrial membranes in prostate cancer cells. J Nutr Biochem 2006;17(10):689-96.
379. Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem 2005;280(7):5636-45.
380. Gong L, Li Y, Nedeljkovic-Kurepa A, Sarkar FH. Inactivation of NF-kappaB by genistein is mediated via Akt signaling pathway in breast cancer cells. Oncogene 2003;22(30):4702-9.
381. Sen P, Chakraborty PK, Raha S. Tea polyphenol epigallocatechin 3-gallate impedes the anti-apoptotic effects of low-grade repetitive stress through inhibition of Akt and NFkappaB survival pathways. FEBS letters 2006;580(1):278-84.
382. Borras C, Gambini J, Gomez-Cabrera MC*, et al.* Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB. Faseb J 2006;20(12):2136-8.

383. Lee JH, Park CH, Jung KC, Rhee HS, Yang CH. Negative regulation of beta-catenin/Tcf signaling by naringenin in AGS gastric cancer cell. Biochem Biophys Res Commun 2005;335(3):771-6.

384. Park CH, Chang JY, Hahm ER, Park S, Kim HK, Yang CH. Quercetin, a potent inhibitor against beta-catenin/Tcf signaling in SW480 colon cancer cells. Biochem Biophys Res Commun 2005;328(1):227-34.

385. Shukla S, MacLennan GT, Flask CA*, et al.* Blockade of beta-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice. Cancer research 2007;67(14):6925-35.

386. Aggarwal BB, Sethi G, Ahn KS*, et al.* Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. Annals of the New York Academy of Sciences 2006;1091:151-69.

387. Lee YK, Isham CR, Kaufman SH, Bible KC. Flavopiridol disrupts STAT3/DNA interactions, attenuates STAT3-directed transcription, and combines with the Jak kinase inhibitor AG490 to achieve cytotoxic synergy. Mol Cancer Ther 2006;5(1):138-48.

388. Vijayababu MR, Arunkumar A, Kanagaraj P, Venkataraman P, Krishnamoorthy G, Arunakaran J. Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3). Molecular and cellular biochemistry 2006;287(1-2):109-16.

389. Oku N, Matsukawa M, Yamakawa S*, et al.* Inhibitory effect of green tea polyphenols on membrane-type 1 matrix metalloproteinase, MT1-MMP. Biological & pharmaceutical bulletin 2003;26(9):1235-8.

390. Liu LZ, Fang J, Zhou Q, Hu X, Shi X, Jiang BH. Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer. Molecular pharmacology 2005;68(3):635-43.

391. Su SJ, Yeh TM, Chuang WJ*, et al.* The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochemical pharmacology 2005;69(2):307-18.

392. Park SS, Bae I, Lee YJ. Flavonoids-induced accumulation of hypoxia-inducible factor (HIF)-1alpha/2alpha is mediated through chelation of iron. Journal of cellular biochemistry 2007.

393. Fang J, Zhou Q, Shi XL, Jiang BH. Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells. Carcinogenesis 2007;28(3):713-23.

394. Kim EJ, Shin HK, Park JH. Genistein inhibits insulin-like growth factor-I receptor signaling in HT-29 human colon cancer cells: a possible mechanism of the growth inhibitory effect of Genistein. Journal of medicinal food 2005;8(4):431-8.

395. Shimizu M, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. Biochem Biophys Res Commun 2005;334(3):947-53.

396. Sadava D, Whitlock E, Kane SE. The green tea polyphenol, epigallocatechin-3-gallate inhibits telomerase and induces apoptosis in drug-resistant lung cancer cells. Biochem Biophys Res Commun 2007;360(1):233-7.

397. Thelen P, Wuttke W, Jarry H, Grzmil M, Ringert RH. Inhibition of telomerase activity and secretion of prostate specific antigen by silibinin in prostate cancer cells. The Journal of urology 2004;171(5):1934-8.

398. Menichincheri M, Ballinari D, Bargiotti A*, et al.* Catecholic flavonoids acting as telomerase inhibitors. Journal of medicinal chemistry 2004;47(26):6466-75.

399. Gingrich JR, Barrios RJ, Morton RA*, et al.* Metastatic prostate cancer in a transgenic mouse. Cancer research 1996;56(18):4096-102.

400. Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM. Androgen-independent prostate cancer progression in the TRAMP model. Cancer Res 1997;57(21):4687-91.

401. Huss WJ, Gray DR, Tavakoli K*, et al.* Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model. Neoplasia (New York, NY 2007;9(11):938-50.

402. Kaplan-Lefko PJ, Chen TM, Ittmann MM*, et al.* Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. The Prostate 2003;55(3):219-37.

403. Hu Y, Ippolito JE, Garabedian EM, Humphrey PA, Gordon JI. Molecular characterization of a metastatic neuroendocrine cell cancer arising in the prostates of transgenic mice. J Biol Chem 2002;277(46):44462-74.

404. Mentor-Marcel R, Lamartiniere CA, Eltoum IE, Greenberg NM, Elgavish A. Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP). Cancer research 2001;61(18):6777-82.

405. Huss WJ, Lai L, Barrios RJ, Hirschi KK, Greenberg NM. Retinoic acid slows progression and promotes apoptosis of spontaneous prostate cancer. Prostate 2004;61(2):142-52.
406. Gupta S, Ahmad N, Marengo SR, MacLennan GT, Greenberg NM, Mukhtar H. Chemoprevention of prostate carcinogenesis by alpha-difluoromethylornithine in TRAMP mice. Cancer Res 2000;60(18):5125-33.
407. Gupta S, Hastak K, Ahmad N, Lewin JS, Mukhtar H. Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. Proceedings of the National Academy of Sciences of the United States of America 2001;98(18):10350-5.
408. Harper CE, Patel BB, Wang J, Eltoum IA, Lamartiniere CA. Epigallocatechin-3-Gallate suppresses early stage, but not late stage prostate cancer in TRAMP mice: mechanisms of action. The Prostate 2007;67(14):1576-89.
409. Mentor-Marcel R, Lamartiniere CA, Eltoum IA, Greenberg NM, Elgavish A. Dietary genistein improves survival and reduces expression of osteopontin in the prostate of transgenic mice with prostatic adenocarcinoma (TRAMP). J Nutr 2005;135(5):989-95.
410. Raina K, Blouin MJ, Singh RP*, et al.* Dietary feeding of silibinin inhibits prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate model. Cancer research 2007;67(22):11083-91.
411. Ganry O. Phytoestrogens and prostate cancer risk. Preventive medicine 2005;41(1):1-6.
412. Morton MS, Chan PS, Cheng C*, et al.* Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. The Prostate 1997;32(2):122-8.
413. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 1993;342(8881):1209-10.
414. J. Ferlay FB, P. Pisani and D.M. Parkin. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. Lyon: IARCPress; 2004.
415. Lyn-Cook BD, Rogers T, Yan Y, Blann EB, Kadlubar FF, Hammons GJ. Chemopreventive effects of tea extracts and various components on human pancreatic and prostate tumor cells in vitro. Nutr Cancer 1999;35(1):80-6.
416. Neuhouser ML. Dietary flavonoids and cancer risk: evidence from human population studies. Nutr Cancer 2004;50(1):1-7.

417. Lee MM, Gomez SL, Chang JS, Wey M, Wang RT, Hsing AW. Soy and isoflavone consumption in relation to prostate cancer risk in China. Cancer Epidemiol Biomarkers Prev 2003;12(7):665-8.

418. Noroozi M, Angerson WJ, Lean ME. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am J Clin Nutr 1998;67(6):1210-8.

419. Valachovicova T, Slivova V, Sliva D. Cellular and physiological effects of soy flavonoids. Mini Rev Med Chem 2004;4(8):881-7.

420. Le Marchand L. Cancer preventive effects of flavonoids--a review. Biomed Pharmacother 2002;56(6):296-301.

421. Sarkar FH, Li Y. Soy isoflavones and cancer prevention. Cancer Invest 2003;21(5):744-57.

422. Castle EP, Thrasher JB. The role of soy phytoestrogens in prostate cancer. Urol Clin North Am 2002;29(1):71-81, viii-ix.

423. Park OJ, Surh YJ. Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. Toxicol Lett 2004;150(1):43-56.

424. Adhami VM, Ahmad N, Mukhtar H. Molecular targets for green tea in prostate cancer prevention. J Nutr 2003;133(7 Suppl):2417S-24S.

425. Agarwal R. Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. Biochem Pharmacol 2000;60(8):1051-9.

426. Tyagi AK, Singh RP, Agarwal C, Chan DC, Agarwal R. Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. Clin Cancer Res 2002;8(11):3512-9.

427. Manthey JA, Guthrie N. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. J Agric Food Chem 2002;50(21):5837-43.

428. Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. Antiproliferative activity of flavonoids on several cancer cell lines. Biosci Biotechnol Biochem 1999;63(5):896-9.

429. Bruggisser R, von Daeniken K, Jundt G, Schaffner W, Tullberg-Reinert H. Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. Planta Med 2002;68(5):445-8.

430. Bohm BA. Biosynthesis and Genetics. Introduction to Flavonoids: Harwood Academic; 1998. p. 299-306.

431. Chas. E. Sando HHB. The Flavones of Rhus. American Journal of Botany 1918;5(3):105-11.

432. Takahashi T, Takasuka N, Iigo M*, et al.* Isoliquiritigenin, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development. Cancer Sci 2004;95(5):448-53.

433. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary flavonoids and cancer risk in the Zutphen Elderly Study. Nutr Cancer 1994;22(2):175-84.

434. Trzeciak A. [Quercetin: significance in mutagenesis and carcinogenesis]. Postepy Biochem 2001;47(4):299-306.

435. van der Woude H, Gliszczynska-Swiglo A, Struijs K, Smeets A, Alink GM, Rietjens IM. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett 2003;200(1):41-7.

436. Choi JA, Kim JY, Lee JY*, et al.* Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int J Oncol 2001;19(4):837-44.

437. Galvano F, La Fauci L, Lazzarino G*, et al.* Cyanidins: metabolism and biological properties. J Nutr Biochem 2004;15(1):2-11.

438. Xue H, Aziz RM, Sun N*, et al.* Inhibition of cellular transformation by berry extracts. Carcinogenesis 2001;22(2):351-6.

439. Paschka AG, Butler R, Young CY. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. Cancer Lett 1998;130(1-2):1-7.

440. Brusselmans K, De Schrijver E, Heyns W, Verhoeven G, Swinnen JV. Epigallocatechin-3-gallate is a potent natural inhibitor of fatty acid synthase in intact cells and selectively induces apoptosis in prostate cancer cells. Int J Cancer 2003;106(6):856-62.

441. Saleem M, Adhami VM, Siddiqui IA, Mukhtar H. Tea beverage in chemoprevention of prostate cancer: a mini-review. Nutr Cancer 2003;47(1):13-23.

442. Chung LY, Cheung TC, Kong SK*, et al.* Induction of apoptosis by green tea catechins in human prostate cancer DU145 cells. Life Sci 2001;68(10):1207-14.

443. Kanazawa M, Satomi Y, Mizutani Y*, et al.* Isoliquiritigenin inhibits the growth of prostate cancer. Eur Urol 2003;43(5):580-6.

444. Tyagi A, Agarwal C, Agarwal R. Inhibition of retinoblastoma protein (Rb) phosphorylation at serine sites and an increase in Rb-E2F complex formation by silibinin in

androgen-dependent human prostate carcinoma LNCaP cells: role in prostate cancer prevention. Mol Cancer Ther 2002;1(7):525-32.

445. Shukla S, Gupta S. Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells. Molecular carcinogenesis 2004;39(2):114-26.

446. Chen YC, Shen SC, Lee WR*, et al.* Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. Archives of toxicology 2002;76(5-6):351-9.

447. Siddiqui IA, Adhami VM, Afaq F, Ahmad N, Mukhtar H. Modulation of phosphatidylinositol-3-kinase/protein kinase B- and mitogen-activated protein kinase-pathways by tea polyphenols in human prostate cancer cells. J Cell Biochem 2004;91(2):232-42.

448. Rao A, Coan A, Welsh JE, Barclay WW, Koumenis C, Cramer SD. Vitamin D receptor and p21/WAF1 are targets of genistein and 1,25-dihydroxyvitamin D3 in human prostate cancer cells. Cancer Res 2004;64(6):2143-7.

449. Rice L, Samedi VG, Medrano TA*, et al.* Mechanisms of the growth inhibitory effects of the isoflavonoid biochanin A on LNCaP cells and xenografts. Prostate 2002;52(3):201-12.

450. Singh RP, Sharma G, Dhanalakshmi S, Agarwal C, Agarwal R. Suppression of advanced human prostate tumor growth in athymic mice by silibinin feeding is associated with reduced cell proliferation, increased apoptosis, and inhibition of angiogenesis. Cancer Epidemiol Biomarkers Prev 2003;12(9):933-9.

451. Moridani MY, Galati G, O'Brien PJ. Comparative quantitative structure toxicity relationships for flavonoids evaluated in isolated rat hepatocytes and HeLa tumor cells. Chem Biol Interact 2002;139(3):251-64.

452. Sabzevari O, Galati G, Moridani MY, Siraki A, O'Brien PJ. Molecular cytotoxic mechanisms of anticancer hydroxychalcones. Chem Biol Interact 2004;148(1-2):57-67.

453. Jemal A, Tiwari RC, Murray T*, et al.* Cancer statistics, 2004. CA: a cancer journal for clinicians 2004;54(1):8-29.

454. Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. Environmental health perspectives 1995;103 Suppl 7:103-12.

455. Bettuzzi S, Brausi M, Rizzi F, Castagnetti G, Peracchia G, Corti A. Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade

prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. Cancer research 2006;66(2):1234-40.

456. Haddad AQ, Venkateswaran V, Viswanathan L, Teahan SJ, Fleshner NE, Klotz LH. Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines. Prostate cancer and prostatic diseases 2006;9(1):68-76.

457. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr 2000;130(9):2243-50.

458. Foresti R, Hoque M, Monti D, Green CJ, Motterlini R. Differential activation of heme oxygenase-1 by chalcones and rosolic acid in endothelial cells. The Journal of pharmacology and experimental therapeutics 2005;312(2):686-93.

459. Hsieh HK, Tsao LT, Wang JP, Lin CN. Synthesis and anti-inflammatory effect of chalcones. The Journal of pharmacy and pharmacology 2000;52(2):163-71.

460. Dinkova-Kostova AT, Abeygunawardana C, Talalay P. Chemoprotective properties of phenylpropenoids, bis(benzylidene)cycloalkanones, and related Michael reaction acceptors: correlation of potencies as phase 2 enzyme inducers and radical scavengers. Journal of medicinal chemistry 1998;41(26):5287-96.

461. Sung B, Pandey MK, Aggarwal BB. Fisetin, an inhibitor of cyclin-dependent kinase 6, down-regulates nuclear factor-kappaB-regulated cell proliferation, antiapoptotic and metastatic gene products through the suppression of TAK-1 and receptor-interacting protein-regulated IkappaBalpha kinase activation. Molecular pharmacology 2007;71(6):1703-14.

462. Fotsis T, Pepper MS, Aktas E*, et al.* Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer research 1997;57(14):2916-21.

463. Arima H, Ashida H, Danno G. Rutin-enhanced antibacterial activities of flavonoids against Bacillus cereus and Salmonella enteritidis. Bioscience, biotechnology, and biochemistry 2002;66(5):1009-14.

464. Maher P, Salgado KF, Zivin JA, Lapchak PA. A novel approach to screening for new neuroprotective compounds for the treatment of stroke. Brain research 2007;1173:117-25.

465. Kuntz S, Wenzel U, Daniel H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. Eur J Nutr 1999;38(3):133-42.

466. Hiipakka RA, Zhang HZ, Dai W, Dai Q, Liao S. Structure-activity relationships for inhibition of human 5alpha-reductases by polyphenols. Biochemical pharmacology 2002;63(6):1165-76.

467. Lu H, Chang DJ, Baratte B, Meijer L, Schulze-Gahmen U. Crystal structure of a human cyclin-dependent kinase 6 complex with a flavonol inhibitor, fisetin. Journal of medicinal chemistry 2005;48(3):737-43.

468. Lu X, Jung J, Cho HJ*, et al.* Fisetin inhibits the activities of cyclin-dependent kinases leading to cell cycle arrest in HT-29 human colon cancer cells. J Nutr 2005;135(12):2884-90.

469. Olaharski AJ, Mondrala ST, Eastmond DA. Chromosomal malsegregation and micronucleus induction in vitro by the DNA topoisomerase II inhibitor fisetin. Mutation research 2005;582(1-2):79-86.

470. Kerr MK, Churchill GA. Experimental design for gene expression microarrays. Biostatistics 2001;2(2):183-201.

471. Ashburner M, Ball CA, Blake JA*, et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25(1):25-9.

472. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95(25):14863-8.

473. Carter SL, Eklund AC, Kohane IS, Harris LN, Szallasi Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nature genetics 2006;38(9):1043-8.

474. Malumbres M, Barbacid M. Cell cycle kinases in cancer. Current opinion in genetics & development 2007;17(1):60-5.

475. Hughes C, Murphy A, Martin C*, et al.* Topoisomerase II-alpha expression increases with increasing Gleason score and with hormone insensitivity in prostate carcinoma. Journal of clinical pathology 2006;59(7):721-4.

476. Li Y, Mizutani Y, Shiraishi T*, et al.* Prognostic significance of thymidylate synthase expression in patients with prostate cancer undergoing radical prostatectomy. Urology 2007;69(5):988-95.

477. Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 2006;24(11):1770-83.

478. Strock CJ, Park JI, Nakakura EK*, et al.* Cyclin-dependent kinase 5 activity controls cell motility and metastatic potential of prostate cancer cells. Cancer research 2006;66(15):7509-15.

479. Boutros R, Lobjois V, Ducommun B. CDC25 phosphatases in cancer cells: key players? Good targets? Nature reviews 2007;7(7):495-507.

480. Hermeking H, Benzinger A. 14-3-3 proteins in cell cycle regulation. Seminars in cancer biology 2006;16(3):183-92.

481. Wang Y, Decker SJ, Sebolt-Leopold J. Knockdown of Chk1, Wee1 and Myt1 by RNA interference abrogates G2 checkpoint and induces apoptosis. Cancer biology & therapy 2004;3(3):305-13.

482. Tsihlias J, Kapusta L, Slingerland J. The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. Annual review of medicine 1999;50:401-23.

483. Passegue E, Wagner EF. JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. Embo J 2000;19(12):2969-79.

484. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol 2007;19(2):142-9.

485. Mann MJ, Hendershot LM. UPR activation alters chemosensitivity of tumor cells. Cancer biology & therapy 2006;5(7):736-40.

486. Li Y, Sarkar FH. Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett 2002;186(2):157-64.

487. Wang SI, Mukhtar H. Gene expression profile in human prostate LNCaP cancer cells by (--) epigallocatechin-3-gallate. Cancer Lett 2002;182(1):43-51.

488. Lu X, Burgan WE, Cerra MA*, et al.* Transcriptional signature of flavopiridol-induced tumor cell death. Mol Cancer Ther 2004;3(7):861-72.

489. Blagosklonny MV. Flavopiridol, an inhibitor of transcription: implications, problems and solutions. Cell cycle (Georgetown, Tex 2004;3(12):1537-42.

490. Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacology & therapeutics 2002;96(2-3):67-202.

491. Vastag B. Soy and prostate cancer study results mixed. Journal of the National Cancer Institute 2007;99(18):1364-5.

492. Moyad MA. Soy, disease prevention, and prostate cancer. Seminars in urologic oncology 1999;17(2):97-102.

493. Romanova D, Vachalkova A, Cipak L, Ovesna Z, Rauko P. Study of antioxidant effect of apigenin, luteolin and quercetin by DNA protective method. Neoplasma 2001;48(2):104-7.

494. Kampkotter A, Gombitang Nkwonkam C, Zurawski RF*, et al.* Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism Caenorhabditis elegans. Archives of toxicology 2007;81(12):849-58.

495. Park HH, Lee S, Oh JM*, et al.* Anti-inflammatory activity of fisetin in human mast cells (HMC-1). Pharmacol Res 2007;55(1):31-7.

496. Odontuya G, Hoult JR, Houghton PJ. Structure-activity relationship for antiinflammatory effect of luteolin and its derived glycosides. Phytother Res 2005;19(9):782-6.

497. Garcia-Mediavilla V, Crespo I, Collado PS*, et al.* The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. European journal of pharmacology 2007;557(2-3):221-9.

498. Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N, Martinez-Irujo JJ. Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. Nutr Cancer 2004;50(1):90-100.

499. Cantero G, Campanella C, Mateos S, Cortes F. Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin. Mutagenesis 2006;21(5):321-5.

500. Agullo G, Gamet-Payrastre L, Manenti S*, et al.* Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochemical pharmacology 1997;53(11):1649-57.

501. Xing N, Chen Y, Mitchell SH, Young CY. Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Carcinogenesis 2001;22(3):409-14.

502. Kohno H, Suzuki R, Sugie S, Tsuda H, Tanaka T. Dietary supplementation with silymarin inhibits 3,2'-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in male F344 rats. Clin Cancer Res 2005;11(13):4962-7.

503. Hikosaka A, Asamoto M, Hokaiwado N*, et al.* Inhibitory effects of soy isoflavones on rat prostate carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Carcinogenesis 2004;25(3):381-7.

504. Wang J, Eltoum IE, Lamartiniere CA. Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. Cancer Lett 2002;186(1):11-8.

505. Chiu FL, Lin JK. Downregulation of androgen receptor expression by luteolin causes inhibition of cell proliferation and induction of apoptosis in human prostate cancer cells and xenografts. The Prostate 2008;68(1):61-71.

506. Ma ZS, Huynh TH, Ng CP, Do PT, Nguyen TH, Huynh H. Reduction of CWR22 prostate tumor xenograft growth by combined tamoxifen-quercetin treatment is associated with inhibition of angiogenesis and cellular proliferation. International journal of oncology 2004;24(5):1297-304.

507. Caporali A, Davalli P, Astancolle S*, et al.* The chemopreventive action of catechins in the TRAMP mouse model of prostate carcinogenesis is accompanied by clusterin over-expression. Carcinogenesis 2004;25(11):2217-24.

508. Li R, Wheeler TM, Dai H*, et al.* Biological correlates of p27 compartmental expression in prostate cancer. The Journal of urology 2006;175(2):528-32.

509. Walle T. Absorption and metabolism of flavonoids. Free radical biology & medicine 2004;36(7):829-37.

510. Ferguson LR, Denny WA. Genotoxicity of non-covalent interactions: DNA intercalators. Mutation research 2007;623(1-2):14-23.

511. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. JAMA 2006;295(13):1549-55.

512. Amling CL, Riffenburgh RH, Sun L*, et al.* Pathologic variables and recurrence rates as related to obesity and race in men with prostate cancer undergoing radical prostatectomy. JClinOncol 2004;22(3):439-45.

513. Amling CL. Obesity and Prostate Cancer: What is the relationship. Semin Prev Altern Med: Saunders Elsevier; 2005. p. 13-7.

514. Freedland SJ, Aronson WJ. Examining the relationship between obesity and prostate cancer. RevUrol 2004;6(2):73-81.

515. Freedland SJ. Obesity and prostate cancer: a growing problem. ClinCancer Res 2005;11(19 Pt 1):6763-6.

516. Rohrmann S, Roberts WW, Walsh PC, Platz EA. Family history of prostate cancer and obesity in relation to high-grade disease and extraprostatic extension in young men with prostate cancer. The Prostate 2003;55(2):140-6.

517. Strom SS, Wang X, Pettaway CA*, et al.* Obesity, weight gain, and risk of biochemical failure among prostate cancer patients following prostatectomy. ClinCancer Res 2005;11(19 Pt 1):6889-94.

518. Trayhurn P, Wood IS. Signalling role of adipose tissue: adipokines and inflammation in obesity. BiochemSocTrans 2005;33(Pt 5):1078-81.

519. Hsieh LJ, Carter HB, Landis PK*, et al.* Association of energy intake with prostate cancer in a long-term aging study: Baltimore Longitudinal Study of Aging (United States). Urology 2003;61(2):297-301.

520. Huffman DM, Johnson MS, Watts A, Elgavish A, Eltoum IA, Nagy TR. Cancer progression in the transgenic adenocarcinoma of mouse prostate mouse is related to energy balance, body mass, and body composition, but not food intake. Cancer Res 2007;67(1):417-24.

521. Platz EA, Leitzmann MF, Michaud DS, Willett WC, Giovannucci E. Interrelation of energy intake, body size, and physical activity with prostate cancer in a large prospective cohort study. Cancer Res 2003;63(23):8542-8.

522. Hammarsten J, Hogstedt B. Clinical, haemodynamic, anthropometric, metabolic and insulin profile of men with high-stage and high-grade clinical prostate cancer. Blood Press 2004;13(1):47-55.

523. Hammarsten J, Hogstedt B. Hyperinsulinaemia: a prospective risk factor for lethal clinical prostate cancer. EurJCancer 2005;41(18):2887-95.

524. Lehrer S, Diamond EJ, Stagger S, Stone NN, Stock RG. Increased serum insulin associated with increased risk of prostate cancer recurrence. The Prostate 2002;50(1):1-3.

525. Ma J, Li H, Pollak M, Kurth T, Giovannucci E, Stampfer MJ. Prediagnostic plasma c-peptide levels and prostate cancer incidence and survival. Proceedings AACR Frontiers in Cancer Prevention Research Meeting 2006;A 204.

526. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. NatRevCancer 2004;4(7):505-18.

527. Fernandez ML. The metabolic syndrome. NutrRev 2007;65(6 Pt 2):S30-S4.

528. Venkateswaran V, Klotz LH, Fleshner NE. Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. Cancer Res 2002;62(9):2540-5.

529. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. NatRevDrug Discov 2005;4(12):988-1004.

530. Friedberg CE, van Buren M, Bijlsma JA, Koomans HA. Insulin increases sodium reabsorption in diluting segment in humans: evidence for indirect mediation through hypokalemia. Kidney Int 1991;40(2):251-6.

531. Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. HormMetab Res 2003;35(11-12):694-704.

532. Wu T, Giovannucci E, Pischon T*, et al.* Fructose, glycemic load, and quantity and quality of carbohydrate in relation to plasma C-peptide concentrations in US women. AmJClinNutr 2004;80(4):1043-9.

533. McCarty MF. Insulin and IGF-I as determinants of low "Western" cancer rates in the rural third world. IntJEpidemiol 2004;33(4):908-10.

534. Jee SH, Ohrr H, Sull JW, Yun JE, Ji M, Samet JM. Fasting serum glucose level and cancer risk in Korean men and women. JAMA 2005;293(2):194-202.

535. Pavelic K, Slijepcevic M, Pavelic J*, et al.* Growth and treatment of Ehrlich tumor in mice with alloxan-induced diabetes. Cancer Res 1979;39(5):1807-13.

536. Dombrowski F, Mathieu C, Evert M. Hepatocellular neoplasms induced by low-number pancreatic islet transplants in autoimmune diabetic BB/Pfd rats. Cancer Res 2006;66(3):1833-43.

537. Chan JM, Stampfer MJ, Giovannucci E*, et al.* Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science (New York, NY 1998;279(5350):563-6.

538. Wolk A, Mantzoros CS, Andersson SO*, et al.* Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study. JNatlCancer Inst 1998;90(12):911-5.

539. Kurmasheva RT, Houghton PJ. IGF-I mediated survival pathways in normal and malignant cells. BiochimBiophysActa 2006;1766(1):1-22.

540. McKeown NM, Meigs JB, Liu S, Saltzman E, Wilson PW, Jacques PF. Carbohydrate nutrition, insulin resistance, and the prevalence of the metabolic syndrome in the Framingham Offspring Cohort. Diabetes Care 2004;27(2):538-46.

541. Yang EJ, Kerver JM, Park YK, Kayitsinga J, Allison DB, Song WO. Carbohydrate intake and biomarkers of glycemic control among US adults: the third National Health and Nutrition Examination Survey (NHANES III). AmJClinNutr 2003;77(6):1426-33.

542. Krueckl SL, Sikes RA, Edlund NM*, et al.* Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. Cancer Res 2004;64(23):8620-9.

543. Hellawell GO, Turner GD, Davies DR, Poulsom R, Brewster SF, Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. Cancer Res 2002;62(10):2942-50.

544. Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. JCell Biochem 2006;99(2):392-401.

545. Pandini G, Mineo R, Frasca F*, et al.* Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res 2005;65(5):1849-57.

546. Basaria S, Muller DC, Carducci MA, Egan J, Dobs AS. Hyperglycemia and insulin resistance in men with prostate carcinoma who receive androgen-deprivation therapy. Cancer 2006;106(3):581-8.

547. Smith MR, Lee H, Nathan DM. Insulin sensitivity during combined androgen blockade for prostate cancer. JClinEndocrinolMetab 2006;91(4):1305-8.

548. Braga-Basaria M, Dobs AS, Muller DC*, et al.* Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. JClinOncol 2006;24(24):3979-83.

549. Keating NL, O'Malley AJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. JClinOncol 2006;24(27):4448-56.

550. Mavropoulos JC, Isaacs WB, Pizzo SV, Freedland SJ. Is there a role for a low-carbohydrate ketogenic diet in the management of prostate cancer? Urology 2006;68(1):15-8.

551. St Onge MP, Janssen I, Heymsfield SB. Metabolic syndrome in normal-weight Americans: new definition of the metabolically obese, normal-weight individual. Diabetes Care 2004;27(9):2222-8.