Capsaicin as a Novel Chemopreventive and Therapeutic Option for Prostate Cancer

by

Natalie Anna Venier

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

© Copyright by Natalie Anna Venier 2015

Natalie Anna Venier

Capsaicin as a Novel Chemopreventive and Therapeutic Option for Prostate Cancer

Doctor of Philosophy

Institute of Medical Science
University of Toronto

2015

Abstract

Capsaicin, the active compound in chilli peppers, has been reported to have anti-carcinogenic properties. In the following thesis, the chemopreventive and therapeutic potential of capsaicin on prostate cancer has been investigated.

To examine the chemopreventive properties of capsaicin, *in vitro* studies were carried out using a panel of prostate cancer cell lines. Here, it was deduced that capsaicin alone and in combination with the antioxidant lycopene significantly reduced the proliferation and induced apoptosis in a dose-dependent manner. Detailed mechanistic studies revealed that these effects were predominately mediated though intrinsic apoptosis. Based on these studies, the effect of capsaicin was assessed using a transgenic chemoprevention model of prostate cancer. Long-term oral administration of capsaicin reduced the tumour grade and metastatic burden in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, compared to control mice.

To understand the therapeutic value of capsaicin, prostate cancer radio-sensitivity studies were carried out using various pre-clinical models. A dose-dependent growth inhibition in colony survival was demonstrated in prostate cancer cell lines following exposure to capsaicin with radiotherapy. Using the LNCaP xenograft mouse model, capsaicin and radiation therapy reduced the growth of prostate cancer tumours over time, greater than either monotherapy. A number of mechanisms were involved in mediating these effects, including the suppression of the NFκB

pathway. Interestingly, the TRP-V1 capsaicin receptors were present on prostate cancer cells; however, they were not found to predominately mediate the anti-cancer effects of capsaicin.

Evidence from this thesis has formed the basis for a clinical trial that is currently examining the effect of capsaicin supplementation on prostate cancer patients. If this trial proves to show benefit of capsaicin supplementation on prostate cancer, it may be integrated into the management and treatment strategies for prostate cancer, thus reducing the morbidity and mortality of the disease.

Acknowledgments

First and foremost, I would like to thank my primary supervisor Dr. Vasundara Venkateswaran and co-supervisor Dr. Laurence Klotz. They both helped me identify my passion for scientific research and inspired me to pursue my doctorate degree. Furthermore, they took a chance on me when I was a naive undergraduate student with huge aspirations and not much to show for it. They have mentored and supported me throughout my degree, for which I will be forever grateful.

I would like to acknowledge my fellow lab members (past and present). Latha Jacob who first taught me basic science techniques that allowed me to be successful in the first year of my degree. Dr. Alexandra Colquhoun who served as a mentor, scientific collaborator, and good friend for a significant portion of my degree. I would also like to thank my other lab members Avi Vandersluis, Benjamin Mora, Xianfeng Ge, Ricky Besla, Dr. Hiroshi Sasaki, Dr. Toshihiro Yamamoto, Michelle Mayer, Min Hou, and Suresh who have positively influenced me in some way throughout my degree.

I would also like to thank my Program Advisory Committee, particularly Dr. Bharati Bapat, Dr. Stanley Liu, and Dr. Neil Fleshner, whose advice and ongoing support have been integral to the completion of my degree. I would also like to recognize my external examiners Dr. Urban Emmenegger, Dr. David Ma, Dr. Xiao-Yan Wen and Dr. Sanjay Gupta who took the time to review and edit my thesis.

I would like to thank my fellow collaborators who have helped me successfully complete various aspects of my degree. Dr. Linda Sugar who has reviewed all my slides for a number of manuscripts. Dr. Alex Kiss who has helped with statistical analysis for various *in vivo* projects. Dr. Daniel Dumont who kindly provided TRAMP mice for work outlined in chapter four. Dr. Rajiv Chopra who served as the external examiner for my PhD transfer exam.

I would also like to acknowledge the IMS Department for their support in helping me complete my degree and develop the *IMS Magazine*. Particularly, I would like to acknowledge the former IMS directors Dr. Ori Rotstein and Dr. Allan Kaplan, the former associate directors, Dr. Karen

Davis, Dr. MingYau Liu, and the outstanding graduate coordinators Dr. Howard Mount, Dr. Mary Seeman, Dr. Carol Westall, Dr. Brenda Toner, Dr. Cindi Morshead, and Dr. Vasundara Venkateswaran. I would also like to thank Kamila Lear, Hazel Pollard, Marika, Kaki, Elena, and the entire IMS community for allowing me the opportunity and or their support throughout my degree.

Last, but certainly not least, I would like to thank my loving parents who have worked very hard to give me a life filled with unconditional love, continuous encouragement, and endless opportunity; my brothers, Adrian and Stefan, who have been my support pillars throughout the my life, and have inspired me to be the best I can be; and to my and wonderful friends who have supported me throughout my degree, in particular, I would like to thank my partner in crime, Dr. Amanda Ali, for her help getting me through some of the toughest parts of my thesis.

Contributions

With the continuous guidance and support of my supervisors Dr. Vasundara Venkateswaran and Dr. Laurence Klotz, I have conceptualized, designed, and performed experiments, interpreted results, and published my findings in multiple peer-reviewed publications. In each chapter, a number of individuals contributed to the progression of my project and ensured that my project stayed on track, remained clinically relevant, and scientifically sound. The details of each chapter are described below.

In chapter three, all experimental procedures were performed independently with the guidance of the lab technician, Latha Jacob. There were significant intellectual contributions by Dr. Alexandra Colquhoun, Dr. Neil Fleshner, as well as both my supervisors, which allowed for most of the results to be published in the Journal of Cancer Therapeutics and Research (2012) 1:30.

The results obtained in chapter four have been written, compiled and submitted to Cancer Prevention Research Journal (2014). Results presented in this manuscript were obtained with the contribution of a number of individuals. Dr. Toshihiro Yamamoto assisted with *in vivo* procedures. Dr. Linda Sugar contributed to histopathological review of all animal tissues. Hans Adomat performed all HPLC analysis on animal serum. Results were interpreted with the assistance of Dr. Neil Fleshner, Dr. Laurence Klotz, and Dr. Vasundara Venkateswaran.

In chapter five, Dr. Alexandra Colquhoun assisted in the conceptualization of the project as well as a number of experiments including clonogenic assays, Western blots, and the *in vivo* procedures. Dr. Hiroshi Sasaki assisted in Western blot experiments. Dr. Linda Sugar, pathologist at Sunnybrook Hospital, reviewed xenograft tumours and liver samples obtained from the in vivo component of the project. Hans Adomat used HPLC techniques to analyze serum obtained from the xenograft experiment for capsaicin levels at the Vancouver Prostate Cancer Centre. Dr. Alex Kiss carried out all statistical analysis for the in vivo component of the project. All results were interpreted with my supervisors, Dr. Vasundara Venkateswaran and Dr. Laurence Klotz, Dr. Stanley Liu, Dr. Alexandra Colquhoun, and Dr. Neil Fleshner. The final manuscript was published in the Prostate Journal (2014).

Table of Contents

i.	Abstract	ii
ii.	Acknowledgements	iii
iii.	Contributions	v
iv.	Table of Contents	xi
v.	List Abbreviations	xii
vi.	List of Tables	xiv
vii.	List of Figures	xv
viii.	List of Appendices	xix

CHAPTER ONE: INTRODUCTION **01**

1.1 Prostate Anatomy		01
1.2 Prostate Cancer Pathophysiology		01
1.3 Gleason Grading System		02
1.4 Prostate Specific Antigen Test		03
1.5 Digital Rectal Exam		04
1.6 Epidemiological Studies		04
1.7 The Genetics and Epigenetics of Prostate Cancer		05
1.8 Management and Treatment Strategies		07
1.8.1	Active Surveillance	07
1.8.2	Androgen Deprivation Therapy	07
1.8.3	Radiotherapy	07
1.8.4	Radio-sensitizing agents	08
1.8.5	Neo-Adjuvant Therapies	08
1.8.6	Castrate Resistant Prostate Cancer	09
1.8.7	Chemoprevention Strategies	09
1.9 Capsaicin		10
1.9.1	Structure of Capsaicin	11
1.9.2	Consumption and Metabolism	12
1.9.3	Scoville Heat Units	14

1.10 Molecular Targets of Capsaicin 14
1.10.1 The Vanilloid Receptors: TRP-V1 14
1.10.2 Cell-Cycle Regulation 15
1.10.3 ER Stress Pathway 17
1.10.4 Inflammatory Pathway 19
1.10.5 Androgen Signalling 21
1.10.6 Apoptosis 23
1.10.6.1 Intrinsic Pathway 23
1.10.6.2 Extrinsic Pathway 25
1.10.7 *In Vivo* Studies 26
1.11 Commonly Investigated Chemoprevention Agents 27
1.11.1 5α-Reductase Inhibitors 27
1.11.2 Aspirin 27
1.11.3 Lycopene 28
1.11.4 Vitamin E 30
1.11.5 Selenium 31
1.11.6 Combination Studies 33
1.12 Translating *in vitro* to *in vivo* Studies into Clinical Trials 35
1.13 Pre-Clinical Models of Prostate Cancer 35
1.13.1 *In Vitro* Models 35
1.13.2 *In Vivo* Models 36
1.13.2.1 Xenograft Mouse Models 37
1.13.2.2 Transgenic Models 38
1.13.2.3 TRAMP Model 38
1.13.2.4 *Lady Transgenic* Model 39
1.13.2.5 c-MYC 40
1.13.2.6 NKX3.1 40
1.13.2.7 PTEN Model 41

CHAPTER TWO: STUDY RATIONALE AND SPECIFIC AIMS 43

2.1 Study Rationale 44

2.2 Overall Hypothesis 45
2.3 Aims and Objectives 45
2.4 Overall Relevance 47

CHAPTER THREE: UNDERSTANDING THE ANTI-CANCER EFFECTS OF CAPSAICIN ALONE AND IN COMBINATION WITH OTHER MICRONUTRIENTS 48

3.1 Abstract 49
3.2 Introduction 50
3.3 Materials and Methods 51
3.4 Results 55
3.4.1 Differential growth inhibitory effect of capsaicin on prostate cell lines 55
3.4.2 Combinatorial Studies suggest that capsaicin and lycopene are most effective at reducing proliferation in prostate cancer cells. 56
3.4.3 Combinatorial Studies suggest that the combination of capsaicin and lycopene is most effective at altering proteins involved in prostate cancer growth, proliferation and apoptosis 58
3.4.4 Treating cells with a combination of capsaicin and lycopene reduces cell proliferation 60
3.4.5 Capsaicin and lycopene in combination alters the percentage of cells in the S-phase of the cell cycle 63
3.4.6 Treatment with capsaicin and lycopene reduce Androgen Receptor (AR) and Prostate Specific Antigen (PSA) expression in LNCaP Cells 64
3.4.7 A combination of capsaicin and lycopene promote apoptosis in androgen-sensitive cells 65
3.4.8 The TRP-V1 antagonist, capsazepine (CZP), does not inhibit the anti-proliferative effects of

capsaicin 67

3.5 Discussion 68
3.6 Conclusions 72
3.7 Caveats and Pitfalls 73

CHAPTER FOUR: CAPSAICIN REDUCES THE METASTATIC BURDEN IN THE TRANSGENIC ADENOCARCINOMA OF THE MOUSE PROSTATE (TRAMP) MODEL 75

4.1 Abstract 76
4.2 Introduction 77
4.3 Materials and Methodology 79
4.4 Results 86
4.4.1 Long-term oral administration of capsaicin is well tolerated with no evidence of associated toxicities 86
4.4.2 Animals administered capsaicin had a lower overall tumour grading 89
4.4.3 TRAMP mice treated with capsaicin had a higher expression of androgen receptor and lower expression of neuroendocrine markers. 90
4.4.4 Mice consuming capsaicin had a significantly lower metastatic burden compared to those receiving vehicle alone 92
4.4.5 Capsaicin treatment reduces the migration and invasion capacity of androgen-resistant prostate cancer cells. 94
4.4.6 Serum from capsaicin treated animals reveal the presence of capsaicin as well as a metabolite 95
4.5 Discussion 97
4.6 Conclusions 101
4.7 Caveats and Pitfalls 101

CHAPTER FIVE: CAPSAICIN: A NOVEL RADIO-SENSITIZING AGENT FOR PROSTATE CANCER 103

5.1 Abstract 104
5.2 Introduction 105
5.3 Methodology 106
5.4 Results 115
5.4.1 Capsaicin treatment reduces colony formation in prostate cancer cells 115
5.4.2 Capsaicin treatment sensitizes prostate cancer cells to radiation 117
5.4.3 Capsaicin treatment alters the cell cycle distribution in LNCaP cells 119
5.4.4 Capsaicin treatment suppresses NFκB expression in LNCaP cells by Western blot analysis 121
5.4.5 Capsaicin treatment sensitizes cells to radiation independent of the vanilloid receptor, TRP-V1 123
5.4.6 Capsaicin and radiation treatment reduced tumour growth in a mouse xenograft model 125
5.4.7 Tumours of mice treated with capsaicin and radiation display markers of persistent DNA damage (yH2AX), reduction in proliferative marker, Ki67, and NFκB 126
5.5 Discussion 129
5.6 Supplementary Experimental Work Evaluating the Role of the Transient Receptor Potential (TRP) Proteins and Capsaicin in Prostate Cancer 133
5.7 Caveats and Pitfalls 140

CHAPTER SIX: DISCUSSSION AND CONCLUSIONS 141

6.1 Discussion and Conclusions 141

CHAPTER SEVEN: FUTURE DIRECTIONS **143**

7.1 Future Directions 143

REFERENCES **154**

APPENDICES **179**

List of Abbreviations

ADT – Androgen deprivation therapy
Akt – Protein kinase B
ANOVA – One-Way Analysis of Variance
AR – Androgen Receptor
5ARi –5-alpha reductase inhibitors
AS – Active Surveillance
BAX – Bcl-2-associated X protein
BCL-2 – B-cell lymphoma 2
BrdU – BromodeoxyUridine
CAP – Capsaicin
CZP – Capsazepine
DHT –Dihydrotestosterone
DMSO -- Dimethyl Sulfoxide
DU-145 – Prostate Cancer Cell Line
E2F – Transcription Factors in Higher Eukaryotes
eIF2α – Eukaryotic Translation Initiation Factor 2 Subunit Alpha
ER Stress – Endoplasmic Reticulum Stress
FITC – Fluorescein Isothiocyanate
GADD34 – Growth Arrest and DNA Damage-Inducible Protein
H/E – Hematoxylin/Eosin
H2AX – H2A Histone Family, Member X
HGPIN – High-Grade Prostate Intraepithelial Neoplasia
HMT – Histone Methyltransferases
HPLC – High-Performance Liquid Chromatography
IκBα – Inhibitory Subunit Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, alpha
IGF1 – Insulin-like growth factor 1
IGFBP – Insulin-like growth factor 1 Binding Protein
IMS – Institute of Medical Science
IRE-1alpha – Inositol-Requiring Kinase 1
LGPIN – Low-Grade Prostate Intraepithelial Neoplasia
LHRH – Luteinizing-Hormone- Releasing Hormone
LNCaP – 'Lymph Node Carcinoma of the Prostate' Prostate Cancer Cell Line
Lyco – Lycopene
MD – Moderately Differentiated
NFκB – Nuclear Factor Kappa B
PARP – Poly ADP Ribose Polymerase
PB – ProBasin
PBS – PreBuffered Saline
PC3 – Prostate Cancer Cell Line
PCa – Prostate Cancer
PCNA – Proliferating Cell Nuclear Antigen
PD – Poorly Differentiated
PERK – Protein Kinase RNA-like Endoplasmic Reticulum Kinase

PI – Propidium Iodide
PI3K – Phosphatidylinositide 3-Kinases
PRSC – Prostate Cancer Stromal Cells
PSA – Prostate Specific Antigen
PTEN – The Phosphatase and Tensin Homolog Deleted on Chromosome Ten
RB – Retinoblastoma
RFX – Resinferatoxin
ROS – Reactive Oxygen Species
RT – Radiotherapy
SBRT – Stereotactic Body Radiotherapy
Sel – Selenium
SELECT – Selenium and Vitamin E Cancer Prevention Trial
SHU – Scoville Heat Units
SV40 TAg – Small Simian Virus 40 t Antigen
TNF-α – Tumour Necrosis Factor - alpha
TRAMP – Transgenic Adenocarcinoma Model of the Mouse Prostate
TRP-V1 – Transient Receptor Potential-Vanilloid Receptor -1
TRP-V6 – Transient Receptor Potential-Vanilloid Receptor -6
UPR – Unfolded Protein Response
Vit E – Vitamin E
WD – Well Differentiated

List of Tables

Table 1: General Characteristics of DU-145, PC3, and LNCaP Cell Lines.

Table 2: Capsaicin Serum Analysis.

Table 3: Capsaicin and Radiation Alter Cell Cycle Distribution in LNCaP Cells.

List of Figures

Figure 1: The Prostate Anatomy.

Figure 2: The Carcinogenesis Process.

Figure 3: Chemical Structure of Capsaicin.

Figure 4: Cell Cycle Regulation by Capsaicin.

Figure 5: Endoplasmic Reticulum Stress Pathway.

Figure 6. Androgen Signaling and Inflammatory Pathway Signaling.

Figure 7: Intrinsic and Extrinsic Regulation of Apoptosis.

Figure 8: Chemical Structure of Antioxidants Implicated in Prostate Cancer Chemoprevention.

Figure 9: Stages of Prostate Cancer Progression and Mouse Models Representing the Corresponding Stages of Disease.

Figure 10: A Summary of Thesis Objectives

Figure 11: Differential growth inhibitory effect of capsaicin on prostate cancer cell lines.

Figure 12: Proliferative Studies on LNCaP Cells Treated with Vitamin E, Selenium, and Lycopene with or without capsaicin.

Figure 13: Morphological representation of LNCaP Cells Treated with capsaicin for 24 hours

Figure 14: Proliferative Studies on LNCaP Cells Treated with Vitamin E, Selenium, and Lycopene with or without capsaicin.

Figure 15: Western Blot Analysis examining Cell Cycle Marker, $p27^{Kip1}$, Apoptosis Marker, PARP, and Prostate Specific Antigen (PSA) in LNCaP Cells treated with various micronutrients including capsaicin (Cap), Selenium (Sel), Lycopene (Lyco), and Vitamin E (Vit E).

Figure 16: Effect of capsaicin (CAP) and/or lycopene (Lyco) on proliferation of LNCaP, PC3, PC3-AR2, DU145, Prostate Cancer Stromal (PCSC) cell lines.

Figure 17: Alterations in the cell cycle distribution (G1, S, G2/M) in LNCaP cells treated with capsaicin and lycopene.

Figure 18: Western Blot analysis demonstrates the changes in the expression of a) Prostate Specific Antigen (PSA), Androgen Receptor (AR), Proliferating Cell Nuclear Antigen (PCNA), and apoptotic indicator, Cleaved PARP in LNCaP cells treated with capsaicin (100μM) and/or lycopene (10μM); b) Corresponding densitometric analysis.

Figure 19: Western blot analysis reveals that a combination of capsaicin and lycopene induces the expression of cleaved PARP, in a time-dependent manner.

Figure 20: Capsaicin treatment alters the Bax:Bcl-xL ratio and reduces total caspase-3 in LNCaP cells after 24 hours.

Figure 21: TRP-V1 is not mediating the anti-proliferative effect of capsaicin.

Figure 22: Hypothesized mechanism for the induction of apoptosis by capsaicin and lycopene in androgen-sensitive cells.

Figure 23: Histopathological Scoring of TRAMP Tumours.

Figure 24: Matrigel Invasion Assay

Figure 25: Scratch Migration Assay

Figure 26: Body Weight Overtime of C57Blk Mice consuming capsaicin.

Figure 27: Body Weight Overtime in TRAMP Mice Consuming Capsaicin.

Figure 28: TRAMP Tumour Grading Analysis.

Figure 29: Proportion of Metastatic Tumours.

Figure 30: Immunohistochemical Analysis of TRAMP Tumours.

Figure 31: Migration and Invasion Studies.

Figure 32: HPLC Analysis of Capsaicin in Mouse Serum.

Figure 33. Proposed Mechanism of Reduced Tumour Burden in TRAMP Model.

Figure 34. Clonogenic Assay Protocol.

Figure 35: Protocol for Inoculation and Irradiation of Mice.

Figure 36: Capsaicin and Radiation Xenograft Experiment Timeline.

Figure 37: Capsaicin reduced the colony formation of Prostate Cancer cells in a dose-dependent manner.

Figure 38: Low doses capsaicin sensitizes prostate cancer cells to radiation.

Figure 39: Capsaicin and radiation increase yH2AX expression in LNCaP and PC3 Cells.

Figure 40: Capsaicin and Radiation Reduce the Percentage of Cells in the S-phase of the cell cycle maximally at 24 hours.

Figure 41: Capsaicin suppresses radiation-induced pNfκB activity in LNCaP Cells.

Figure 42: Capsaicin does not sensitize cells predominately through the TRP-V1 receptor

Figure 43: Capsaicin and Radiation significantly reduce tumour growth rate.

Figure 44: Immunohistochemical analysis of LNCaP xenograft tumours reveal that capsaicin and radiation alter the expression of Ki67, γH2AX, and NFκB

Figure 45: The Transient Receptor Potential – Vanilloid Receptor-1

Figure 46: TRP-V1 is present on LNCaP prostate cancer cells in both the nuclear and cytoplasmic compartments.

Figure 47: Chemical Structure of TRP-V1 antagonist, Capsazepine.

Figure 48: Chemical Structure of the TRP-V1 Agonist, Resinfertoxin.

Figure 49: Resinferatoxin (RFX) Reduces the proliferation of LNCaP Cells in a Dose-Dependent Manner.

Figure 50: Resinferatoxin Reduces the proliferation of PC3 Cells at high concentrations.

Figure 51: Capsazepine (CZP) does not reduce the anti-proliferative effects of capsaicin at high doses in PC3 Cells.

Figure 52: Capsazepine does not reduce the anti-proliferative effects of capsaicin at high doses in LNCaP Cells.

Figure 53: Summary the Potential Mechanisms Exerted by Capsaicin on Prostate Cancer.

Figure 54: Immunocytochemical images of untreated LNCaP cells stained for TRP-V6 and TRP-V1.

Figure 55: Immocytochemistry Images of LNCaP Cells treated with capsaicin (10uM).

Figure 56: Western Blot analysis reveals an increase in TRP-V1 and TRP-V6 expression in LNCaP cells following 24-hour treatment with capsaicin (10μM).

Figure 57: TRP-V6 expression is increased in a time-dependent manner in prostate cancer cells.

Figure 58: Hypothesized role of TRP-V6 in prostate cancer.

Figure 59: Schematic representation of the effect of capsaicin on prostate cancer cells.

Figure 60: Schematic Representation of CAPSAICIN Trial evaluating the effect of capsaicin on prostate cancer patients.

Figure 61: Primary and Secondary Outcome measures from CAPSAICIN Trial.

List of Appendices

Appendix 1: Micronutrients Review

Appendix 2: CAPSAICIN Clinical Trial Protocol

Appendix 3: Copyright Permission

CHAPTER ONE:
Introduction

1.1 Prostate Anatomy

The prostate is a walnut-shaped exocrine gland, located between the bladder and rectum, surrounding the urethra (see Figure 1). It functions mainly to support fertility and continence.

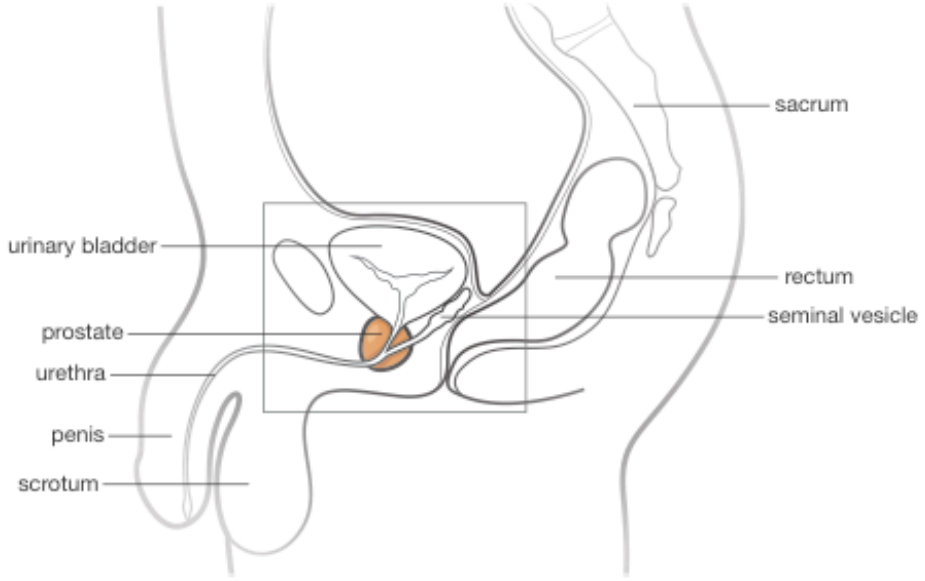


Figure 1: The Prostate Anatomy. *The above image was obtained with permission from the IMS Magazine, 2011.*

1.2 Prostate Cancer Pathophysiology

Like most cancers, prostate cancer develops when cells within the prostate grow uncontrollably

and form malignant tumours. This uncontrollable growth is characterized by the following hallmarks: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction, all of which are elegantly reviewed by Hanahan and Weinberg (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011).

In most cases, prostate cancer can progress in three stages: initiation, promotion, and progression. In the initiation phase, normal cells become mutated by some external insult (e.g. radiation, exposure to a carcinogen, virus, etc.). Growth and replication of cells in this phase occurs relatively slowly. In fact, it usually takes years for tumours to become large enough to be detectable and enter the promotion phase. In the promotion phase, cells become activated by a promoting agent, which causes them to progress and become a malignant tumour. In this progression phase, tumours can remain localized to the prostate or metastasize to other sites of the body. (See Figure 2)

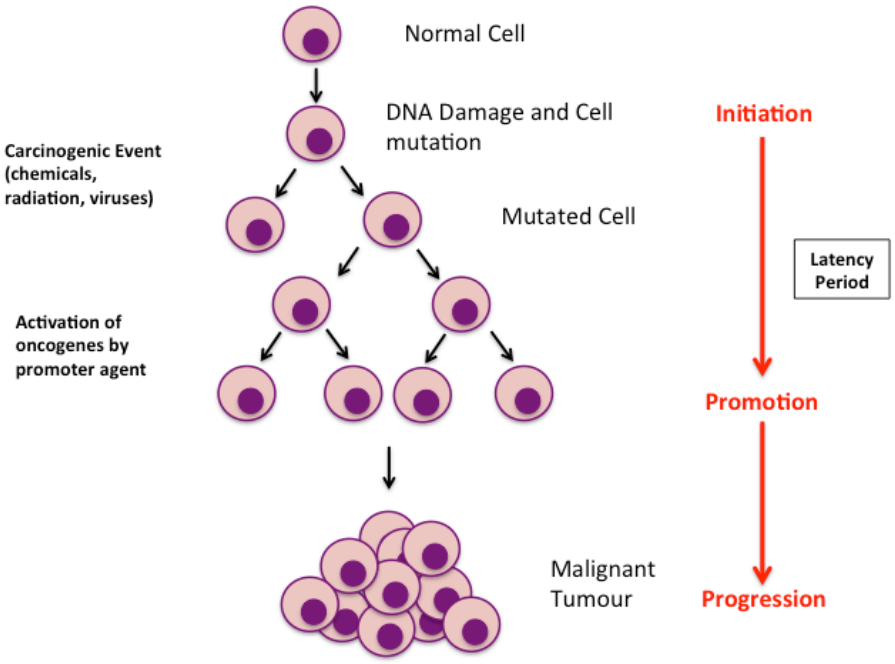


Figure 2: The Carcinogenesis Process. *In most cases, prostate cancer can progress in three stages: initiation, promotion, and progression. One unique characteristic of prostate cancer is its prolonged latency of multifocal disease which can last anywhere from 1 to 20 years.*

1.3 Gleason Grading System

Pathological analysis of prostate tissue can determine the Gleason Score through the use of the Gleason Grading System. Depending on the cancerous changes in the prostate, a score of 1-5 can be applied, where 1 is closest to normal tissue and 5 is very irregular pathology (Epstein 2010). Gleason Score 1 is comprised of simple round glands, closely-packed with round masses with well-defined edges, Gleason Score 2 is simple round glands, loosely packed, Gleason Score 3 is comprised of medium sized glands with irregular shape with irregular shaping, Gleason Score 4 is comprised of small, medium, or large glands fused into chords or chains, Gleason Score 5 is papillary and cribriform epithelium in smooth, rounded masses (Gleason 1992).

The Gleason Score is a compilation of two tissue grades, the first being the "primary pattern" which is the grade representing the greatest amount of tumour tissue and the "secondary pattern" which is the smaller amount of tumour. The Gleason Score can range from 2 (1+1) to 10 (5+5). The Gleason Score is an important factor in determining prognosis and for planning management strategies for prostate cancer (Epstein 2010).

1.4 Prostate Specific Antigen Test

The Prostate Specific Antigen (PSA) is a protein produced by the cells of the prostate gland (Hayes and Barry 2014). The PSA levels can be measured from a blood sample in a standard laboratory. The PSA test is generally expressed as nanograms per millilitre (ng/mL) of blood.

The PSA test can be used to monitor the progression of disease after prostate cancer diagnosis. In general, higher PSA levels are found in patients with higher tumour burden.

Historically, the PSA test was also used as a prostate cancer screening marker in conjunction with other screening tests (e.g. the Digital Rectal Exam) to aid in the diagnosis of disease ((NCI) 2012). More recently, the PSA test has come under scrutiny as a screening tool as the PSA level can be elevated by a number of factors that are not related to cancer (e.g. prostatitis), which can increase the risk of a false-positive diagnosis (Hayes and Barry 2014). The PSA cut-off level is a debatable issue internationally, details of the cutoff levels of PSA are discussed in a review by Van der Meer et al. in 2014 (Van der Meer, Löwik et al. 2012).

1.5 Digital Rectal Exam

The Digital Rectal Exam (DRE) is a simple assessment performed by a physician, whereby a lubricated, gloved finger is inserted to the rectum to feel for any abnormalities. The DRE can aid in diagnosing PCa and determining its clinical stage.

1.6 Epidemiological Studies

Prostate Cancer (PCa) has a global variation with high-risk regions, such as the US, Canada, Australia, and Europe displaying a 10-fold higher disease-specific mortality than low-risk nations including China and Japan (Center, Jemal et al. 2012). Despite these wide variations in disease incidence and mortality, autopsy studies suggest the highest risk factor for prostate cancer is age (Stangelberger, Waldert et al. 2008). The question of the relative contribution of genetics versus environment is raised by the global variation data. Migrating populations from areas of low-risk to areas of high-risk gain increased risk if they migrate within 11 years and their offspring have virtually the same rates of PCa as seen in high-risk countries (Matikaine, Pukkala et al. 2001). This is suggestive of the fact that environmental factors are responsible for prostate carcinogenesis (Fleshner and Zlotta 2007). These data are validated by twin studies assessing the impact of heredity on PCa among identical twins (Page, Braun et al. 1997). Given that PCa starts histologically among men in their 30's, these data also suggest that early PCa events are likely similar regardless of environmental factors, and that intraprostatic progression to clinically detectable disease and metastases is driven by unfavorable environmental conditions such as diet, oxidative stress, or androgenic effects (Fleshner and Zlotta 2007). It must also be recognized that an effective strategy that can prevent PCa should also be studied as a complementary therapy for clinical PCa (Fleshner and Zlotta 2007).

1.7 The Genetics and Epigenetics of Prostate Cancer

Prostate cancer can arise from both genetic and epigenetic factors (Grönberg, Damber et al. 1994, Jerónimo, Bastian et al. 2011). It is estimated that the genetic background accounts for 5-10% of all prostate cancers (Kral, Rosinska et al. 2011). Early evidence supporting the role of genetics in the development of prostate cancer came from a large body of epidemiological studies on monozygotic and dizygotic twins. In one study examining World War II veterans, prostate cancer was diagnosed in 1009 men and the incidence was 27.1% and 7.1% in monozygotic twins and dizygotic twins respectively (Page, Braun et al. 1997, Kral, Rosinska et al. 2011). Similar results were also found in studies carried out by Gronberg in 1994 (Grönberg, Damber et al. 1994, Kral, Rosinska et al. 2011). Many linkage and association studies have been

carried out to identify high-penetrance genetic variants that may be responsible for an increased risk of developing prostate cancer (Ishak and Giri 2011). It is estimated that there are over 30 single-nucleotide polymorphisms associated with prostate cancer susceptibility (Catalona, Bailey-Wilson et al. 2011). Identifying genetic variants associated with prostate cancer aggressiveness is one of the most active areas of interest to date (Catalona, Bailey-Wilson et al. 2011).

Epigenetic mechanisms control the transcriptional regulation of several prostate cancer genes through DNA methylation, histone modification and miRNA regulation at several levels. These mechanisms play essential roles in normal development as well as in human carcinogenesis (Jerónimo, Bastian et al. 2011).

Aberrant DNA methylation of CpG sites is the earliest and most frequent alteration in any cancer. This modification is one of the best-studied and has importance in gene expression, especially when involving CpG-rich areas (CpG islands) located in the promoter regions of genes (Li, Carroll et al. 2005). Hypermethylation of CpG islands in the promoter region of tumour suppressor genes is an early event in cancer and correlates with gene silencing (Li, Carroll et al. 2005). Hence, markers of anomalous methylation hold promise particularly for monitoring the onset and progression of cancer.

Histone modifications often occur with acetylation or methylation changes which can either activate or inactive certain genes based on altering the DNA-histone binding affinity. Histone acetylation and methylation enzymes such as histone acetyltransferases (HATS) and histone methylatransferases (HMT) play an important role in controlling gene transcription and have also been associated with cancer development and progression.

MicroRNAs (miRNA/miR) are a class of small non-coding RNA (18-25 nucleotides in length), synthesized and processed in the nucleus before being exported to the cytoplasm. They bind to complementary mRNA complexes in the cytoplasm and alter their expression through recruiting an RNA-induced silencing complex. In cancer, microRNA expression is frequently altered which can promote or suppress tumour growth depending on the specific target genes (Jerónimo, Bastian et al. 2011).

An improved understanding of the genetic and epigenetic foundation is important for improving prostate cancer outcomes. Identifying genetic variants associated with an increased risk of prostate cancer will help contribute to early diagnosis of prostate cancer and improved treatment strategies (Li, Carroll et al. 2005, Jerónimo, Bastian et al. 2011). Furthermore, the identification of genetic markers of aggressive prostate cancer will aid in the classification of men likely to progress into lethal disease and promote appropriate intervention and management strategies.

1.8 Management and Treatment Strategies

Prostate cancer can be managed through a number of different strategies based on the tumour grade and staging. In the following section, the most commonly used management and treatment options are detailed.

1.8.1 Active Surveillance

Active Surveillance (AS) is a solution to the widely acknowledged problem of over-diagnosis and overtreatment of clinically insignificant disease that accompanies early detection of prostate cancer (PCa) using prostate-specific antigen (PSA) and biopsy (Klotz 2013, Klotz 2013, Klotz 2013). Many men enrolled in AS have micro-focal tumours that may never progress to a point where they will cause significant morbidity or mortality (Klotz 2013). However, a subset of these patients will go on to develop aggressive disease. In this situation, intervention with a chemopreventive therapy that may prevent or delay progression of disease and reduce the risk of radical treatment is very appealing (Chan, Gann et al. 2005).

1.8.2 Androgen Deprivation Therapy

Androgens play a significant role in the development and progression of prostate cancer (see genetics and epigenetics of prostate cancer). Targeting the androgen signalling pathway has been an important strategy to manage prostate cancer. Androgen deprivation therapy (ADT)—also

referred to as hormonal therapy or androgen suppression—is a therapy that reduces androgen levels to improve prostate cancer progression. This suppression in androgen levels was historically carried out by surgical castration, nowadays, pharmacological agents are used to induce similar effects by chemically blocking various androgen stimulating hormones (e.g. LHRH antagonists) (Sharifi, Gulley et al. 2005).

1.8.3 Radiotherapy

Radiotherapy (RT) is a very commonly used treatment for PCa (Gwede, Pow-Sang et al. 2005). Radiotherapy mediates its lethal effects on cells by inducing DNA damage (Zhou and Elledge 2000). Cells respond to this damage by either undergoing immediate apoptosis, by inhibiting cell cycle progression, and/or repairing the DNA damage. The conventional regimen for administering prostatic radiation involves 70-80Gy given on weekdays in 2Gy fractions. Alternative regimens under investigation include the use of hypofractionation. Hypofractionation means the administration of a full therapeutic dose of radiotherapy in fewer, larger sized fractions (Loblaw and Cheung 2006). The intent is to improve patient convenience, increase system capacity, and decrease costs while offering the same or greater tumour control with the same or reduced normal tissue toxicities. Radiation can be administered as external beam radiotherapy or brachytherapy, which involves the implantation of multiple radioactive seeds into the tumour site.

1.8.4 Radio-Sensitizing agents

Radio-sensitizing agents are chemical or pharmacologic compounds that sensitize cells to the lethal effects of radiotherapy. Successful radio-sensitizing agents achieve their effect by increasing tumour cell kill whilst minimizing adverse effects on normal tissues. Compounds occurring naturally in the human diet are ideal potential radio-sensitizing agents, given their lack of toxicity at conventional doses (Loblaw and Cheung 2006). Currently, there is no standard of care radio-sensitizer for prostate cancer.

1.8.5 Adjuvant and Neo-Adjuvant Therapies

Adjuvant therapy is the additional treatment given after the primary treatment has been given to prevent cancer from returning. In PCa, this can be a number of strategies including ADT after surgery or radical radiation (Lam and Glodé 2013).

Neo-adjuvant therapy is the administration of therapeutic agents prior to definitive treatment with the aim of down-staging disease and thus improving surgical outcome (Gonzalez, Laudano et al. 2008). For example, neo-adjuvant (and concomitant) hormonal manipulation is commonly used in patients with intermediate or high-risk prostate cancer treated with radical radiotherapy, with a view to enhancing the efficacy of the radiation treatment (Gonzalez, Laudano et al. 2008).

1.8.6 Castrate-Resistant Prostate Cancer Management

Castrate-Resistant Prostate Cancer is characterized by a rise in PSA levels or progressive disease with serum testosterone within the castrate range (Suzman and Antonarakis 2014). Since this advanced stage of disease does not respond to ADT, alternative palliative strategies are generally employed to manage disease. Ideally, patients with castrate-resistant prostate cancer should be managed and treated in a multidisciplinary team (Heidenreich, Bastian et al. 2014). Patients with asymptomatic or mildly symptomatic should be considered for abiraterone/prednisone treatment as it has been shown to have survival benefit (Heidenreich, Bastian et al. 2014). Patients with metastatic castrate-resistant prostate cancer should be considered for docetaxel every three weeks as it has also shown a significant survival benefit (Heidenreich, Bastian et al. 2014). Second line treatment, enzalutaminde, should be considered because of its benefit to overall survival, radiographic progression-free survival, and quality of life (Heidenreich, Bastian et al. 2014).

1.8.7 Chemoprevention Strategies

Chemopreventive agents are natural or pharmaceutical compounds used to prevent the development (primary chemoprevention) or delay the progression (secondary chemoprevention) of disease (Gupta, Ahmad et al. 2000, Adhami, Siddiqui et al. 2004, Aggarwal, Takada et al. 2004, Gupta, Adhami et al. 2004, Chan, Gann et al. 2005, Aggarwal and Shishodia 2006). Prostate cancer represents an ideal tumour system for studying chemopreventive agents due to its prolonged developmental phase and subsequent, commonly slow progression (Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012). Thus, an extended window of opportunity exists for administration of both primary (pre-tumour development) and secondary (post-diagnosis) chemoprevention (Chan, Gann et al. 2005, Rittmaster 2011, Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012).

The use of dietary agents (e.g. micronutrients) for the chemoprevention of prostate cancer has been intensely investigated (Gupta, Ahmad et al. 2000, Adhami, Siddiqui et al. 2004, Aggarwal, Takada et al. 2004, Gupta, Adhami et al. 2004, Aggarwal and Shishodia 2006, Ledesma, Jung-Hynes et al. 2011, Rittmaster 2011, Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012). While many agents have shown promising results in the pre-clinical arena, none have been successfully translated into chemopreventive strategies that are routinely implemented in clinical practice (Venier, Klotz et al. 2012). Potential explanations as to why micronutrients often fail to successfully translate into clinical use include: 1) lack of knowledge regarding optimum onset and required duration of intervention, 2) impaired bioavailability of the active component(s) present in the dietary agents and 3) lack of biologically plausible mechanisms of action (Johnson, Bailey et al. 2010, Ledesma, Jung-Hynes et al. 2011). Thus, potential successful chemopreventive agents are non-toxic dietary agents, which are readily absorbed/metabolised to active compounds and target specific pathways related to tumour development/progression. The focus of the following section is to examine the mechanisms of capsaicin and explore its potential translation into clinical trials for prostate cancer chemoprevention.

1.9 Capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the principal compound in plants from the genus *Capsicum* and is responsible for their unique potency upon ingestion. In addition to its

use as a major spice and food additive in most cuisines around the world, capsaicin has been also used for its medicinal and therapeutic potential in human health since the 16th century (Bode and Dong 2011). Its unique vanilloid structure allows capsaicin to bind and activate the Transient Receptor Potential-Vanilloid Receptor -1 (TRP-V1; also referred to as the capsaicin or vanilloid receptor), which elicits downstream effects.

When applied topically, capsaicin binds and activates the vanilloid receptors on sensory neurons causing the stimulation of the nerve fibres, and release of 'substance P', which in turn results in pain. The continuous stimulation of these fibers inhibits the release of 'substance P', and reduces the pain sensation in the affected areas through the desensitization of the neurons (Fraenkel, Bogardus et al. 2004, Luo, Peng et al. 2011). In this way, capsaicin is commonly used as an analgesic and anti-inflammatory agent for the treatment of various chronic pain syndromes such as rheumatoid arthritis or fibromyalgia (Fraenkel, Bogardus et al. 2004, Bode and Dong 2011).

Orally consumed capsaicin, usually in the form of chilli pepper extracts or supplements, has been commercially used as a weight management tool through appetite suppression and up-regulation of the thermoregulatory system (Chaiyasit, Khovidhunkit et al. 2009).

More recently, capsaicin has emerged as a promising anti-cancer agent. In this review, we summarize the mechanisms by which capsaicin has been reported to exert these anti-cancer effects, with a focus on prostate cancer.

1.9.1 Structure of Capsaicin

At room temperature, pure capsaicin is hydrophobic, colorless, odourless, and a crystalline-to-waxy solid. It belongs to a group of acid amides of vanillylamine and C8 to C13 fatty acids. It contains a vanilloid ring, a 3-methoxy-4-hydroxybenzylamine (vanilloid) ring, and an alkyl side chain (CO(CH2)4(CH)2CH(CH3)2) as depicted in Figure 3. It is biosynthesized predominately in the placenta of the chilli pepper by the condensation of fatty acids and vanillylamine (Thiele, Mueller-Seitz et al. 2008, Wahyuni, Ballester et al. 2013). Recently, it has been reported that the production of capsaicin can also take place using the fungus alternaria alternaria (Devari, Jaglan et al. 2013). There are a number other less-potent, related compounds in the capsaicinoid family

that also contribute to the potency of capsicum species, most notably, dihydrocapsaicin and nordihydrocapsaicin (Luo, Peng et al. 2011). These compounds are naturally synthesized in a similar manner, but have a shorter fatty acid chain which alters their ability to bind and activate the capsaicin receptor. Capsaicin binds to the vanilloid receptor with the highest affinity, making it the most pungent and potent agent in the capsicum family (Reilly and Yost 2006).

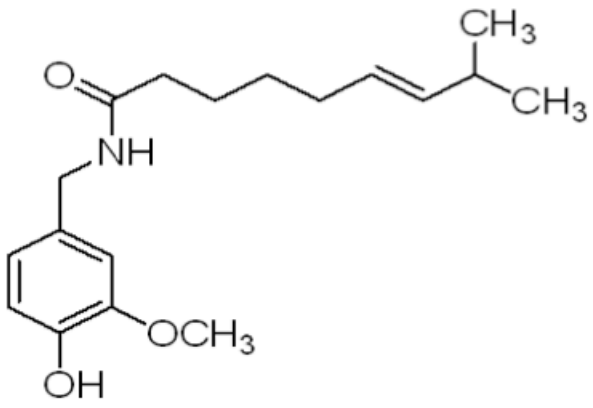


Figure 3: Chemical Structure of Capsaicin. *The structure of capsaicin contains a vanilloid ring, a 3-methoxy-4-hydroxybenzylamine (vanilloid) ring, and an alkyl side chain.*

1.9.2 Consumption and Metabolism

The chilli pepper plant is the most widely grown spice in the world (Kim, Park et al. 2014). Although the global consumption of capsaicin is not well documented in the literature, it is notably consumed though food-products and spices at high levels in certain areas of the world, particularly Mexico, South America, Ethiopia, India, Indonesia, Korea, Laos, Malaysia, Pakistan, Southwest China, Sri Lanka and Thailand (Perry, Dickau et al. 2007, Bode and Dong 2011).

Most of the literature focusing on the bioavailability and metabolism of capsaicin after oral ingestion is limited to animal studies. In rodents, orally administered capsaicin is absorbed in the stomach and undergoes the first-pass effect—reducing its bioavailability—and subsequent systemic absorption (Reilly and Yost 2006). It is transported to the portal vein through the GI tract by a non-active process and is partly metabolized during absorption (Reilly and Yost 2006). In the liver, capsaicin undergoes a number of modifications by P450 enzymes to decrease their ability to activate the TRP-V1 receptors, thus reducing their pharmacological and toxicological potency. Metabolism of capsaicinoids by P450 enzymes also produces reactive electrophiles

capable of modifying biological macromolecules as outlined in a review by Reilly and Yost (Reilly and Yost 2006).

Human bioavailability studies have found that human subjects had serum concentrations of capsaicin ranging from 13.4-16.3 ng/mL after consuming a chilli-containing meal. These measurements were analyzed using a modified HPLC procedure for analyzing the metabolism of orally ingested chilli. Of note, they also found measureable but significantly lower levels (range: 3.6 – 7.5ng/mL) of dihydrocapsaicin, an analogue of capsaicin found in lower levels in chilli peppers. This suggests that analogues of similar potency do not have an enhanced bioavailability compared to capsaicin (Hartley, Stevens et al. 2013). The only other study which has reported similar serum concentration by orally ingested capsaicin comes from a study out of Thailand, where oral administration of 5 g of chilli peppers resulted in serum concentrations of capsaicin averaging 8.1 nM (Chaiyasit, Khovidhunkit et al. 2009). Whether the bioavailability is enough to achieve therapeutic effectiveness is not clear. One promising case-study by Jankovic et al, describes a prostate cancer patient consuming 2.5 mL of habaneros chilli sauce 1-2 times weekly, which contained capsaicin (calculated to be 454 ug/mL). He had a stabilization in his prostate specific antigen (PSA) levels, suggesting that the therapeutic effects of capsaicin can be achieved by oral consumption of capsaicin (Jankovic, Loblaw et al. 2010). The serum concentration of capsaicin in this patient was not reported. Nonetheless, techniques to improve the bioavailability of capsaicin are slowly emerging in the literature: Chen et al have developed enteric-coated tablets called Capsaicin-Chitosan Microspheres (CCMS) that are able to sustain drug release into the intestine and a first-order release, allowing for increased bioavailability and serum concentration of capsaicin. How effective these CCMS are at increasing the bioavailability of capsaicin has yet to be reported in the literature (Chen, Huang et al. 2013).

As the limited data on capsaicin bioavailability in humans suggests that capsaicin is only present in nanomolar concentrations in serum, i.e. nearly 100-fold lower than concentrations studied *in vitro* and *in vivo,* a greater understanding of this discrepancy requires further investigation. One plausible explanation could be that capsaicin may deposit and concentrate in certain tissues (i.e the prostate), which has been reported with other micronutrients. Nevertheless, further investigation into the serum concentration required to achieve therapeutic benefits from oral capsaicin administration is essential for moving capsaicin into clinical trials for prostate cancer.

1.9.3 Scoville Heat Units

The Scoville Heat Units (SHU) scale was developed by Wilbur Scoville in 1912 as a way to quantify the relative potency or 'heat' of various chilli pepper plants. It is based on the number of times a chilli extract must be diluted in water to lose its 'heat' potential (Bode and Dong 2011). The SHU has been reported to be proportional to the amount of capsaicinoids in a plant by a factor of 15 000 SHU/microgram of capsaicinoids (Reilly and Yost 2006). It is important to note that although capsaicin is the focus of this review, there are a number of other less-potent compounds in the capsaicinoid family that also contribute to the SHU, most notably, dihydrocapsaicin and nordihydrocapsaicin (Thiele, Mueller-Seitz et al. 2008). Hence, the SHU of any particular chilli pepper plant can vary significantly based on the various capsaicinoid concentrations. In its pure form, capsaicin measures highest on the SHU scale with 16, 000, 000 SHU, followed by dihydocapsaicin and nordihydrocapsaicin as well as other capsaicinoid analogues. The SHU scale is typically used commercially to signify the potency or 'spiciness' of certain food-products, but rarely appears in scientific research (Thiele, Mueller-Seitz et al. 2008).

1.10 Molecular Targets Mediated by Capsaicin

In the past decade, capsaicin has emerged in the literature as a potent anti-cancer agent possessing anti-proliferative, apoptotic, and anti-metastatic properties. In this review, we review in detail some of the most notable chemopreventive mechanisms documented in the recent literature, and provide an integrated mechanism as to how capsaicin may be acting as a chemopreventive agent at a cellular level.

1.10.1 The Vanilloid Receptors: TRP-V1

The vanilloid receptors belong to the family of transient receptor potential (TRP) cation-selective channels that modulate the concentration of intracellular calcium (Prevarskaya, Zhang et al. 2007). Several vanilloid receptors have been reported to be up-regulated in prostate cancer (Czifra, Varga et al. 2009). TRP-V1 is a heat-activated, non-selective cation channel that belongs

to the family of transient receptor potential (TRP) cation-selective channels that modulate the concentration of intracellular calcium (Prevarskaya, Zhang et al. 2007). TRP-V1 was originally identified as the receptor for capsaicin. It is one of the most polymodal TRP channels as it is activated by heat, voltage, protons, as well as exogenous and endogenous vanilloids. Although it is mainly expressed in sensory neurons, TRP-V1 expression has also been described at the mRNA and protein level in human prostate cancer (Czifra, Varga et al. 2009). The expression of TRP-V1 mRNA is low or undetectable in healthy and benign prostate tissue, whereas there is an observed increase in expression with increasing tumour grade (Peng, Zhuang et al. 2001, Fixemer, Wissenbach et al. 2003, Wissenbach, Niemeyer et al. 2004, Czifra, Varga et al. 2009). The clear positive correlation of TRP-V1 with increasing degrees of malignancy suggests that TRP-V1 may serve as a prognostic factor and potential therapeutic target for PCa (Fixemer, Wissenbach et al. 2003, Bödding 2007, Gunthorpe and Szallasi 2008).

A recent review by Zigloli et al suggests that capsaicin may exert its effects through a TRP-V1-dependent and –independent manner (Ziglioli, Frattini et al. 2009). It was reported that capsazepine (CZP), a TRP-V1 antagonist, could not reverse the anti-proliferative effects of capsaicin in PC3 cells (Sánchez, Sánchez et al. 2006). Similar findings in LNCaP and DU145 cells have been reported using several other TRP-V1 antagonists (Mori, Lehmann et al. 2006). CZP has been found to reverse capsaicin-induced proliferation in LNCaP cells treated with low doses of capsaicin, thereby suggesting that TRP-V1 effects are partially responsible for proliferation in LNCaP cells. It has also been suggested that that TRP-V1 may be indirectly contributing to capsaicin-induced apoptosis through the increase of intracellular calcium and activation of calcium-dependent enzymes (i.e. endonucleases, proteases, transglutaminases), resulting in DNA injury, cytoskeleton damage and protein alterations (Ziglioli, Frattini et al. 2009). Further elucidation of the interaction between capsaicin and TRP-V1 in prostate cancer cells is warranted. Nonetheless, therapies targeting the TRP-V1 receptor provide a promising target for the chemoprevention of prostate cancer.

1.10.2 Cell-Cycle Regulation

Capsaicin has been found to reduce proliferation in a number of malignant cell lines. Thorough assessment of recent literature suggests that one of the main mechanisms by which proliferation is reduced is through the inhibition of cell cycle progression as depicted in Figure 4.

In prostate cancer cell lines, capsaicin has been found to reduce cell proliferation in a dose-dependent manner. Studies that have focused on the use of the human prostate cancer cell line PC3 (an androgen-independent cell line derived from prostatic adenocarcinoma metastatic to bone) have been relatively consistent in terms of anti-proliferative and apoptotic effects. In 2006, Sánchez et al. reported that capsaicin reduced the proliferation and induced apoptosis in PC3 cells, at concentrations ranging from 0.1-50 micromolar (Sánchez, Sánchez et al. 2006). Cell cycle analysis revealed that the population of cells in the G_0/G_1 phase increased and those in S phase decreased in a capsaicin dose-dependent manner (Sánchez, Sánchez et al. 2006). This increase corresponded to an increase in tumour suppressor proteins, p53 and p21. Similar effects were established in the androgen-independent DU145 cell line (Mori, Lehmann et al. 2006), and in pancreatic, breast, and bladder cancer cell lines (Thoennissen, O'Kelly et al. 2010).

Proliferation studies examining the androgen-dependent, p53-negative, LNCaP cells have suggest that capsaicin has anti-proliferative effects on this cell line as well. Mori et al. reported that capsaicin inhibited growth, induced apoptosis and reduced androgen receptor signalling (Mori, Lehmann et al. 2006). One isolated study by Malagarie-Cazenave et al. reported that treating LNCaP cells with higher doses of capsaicin than previously reported by Mori et al (Malagarie-Cazenave, Olea-Herrero et al. 2009). increased proliferation of LNCaP cells and up-regulated expression of prostate specific antigen (PSA) and androgen receptor (AR) (Malagarie-Cazenave, Olea-Herrero et al. 2009). It is to be noted that the two studies used different concentrations of capsaicin, which may have caused this differential effect. Hence, it is possible that capsaicin may interact with the androgen receptor (see discussion below). Lower concentrations of capsaicin stimulate growth, whereas higher doses of capsaicin inhibit growth and may induce apoptosis, an effect observed with other pharmaceutical and/or dietary agents that effect androgen signalling (Wetherill, Fisher et al. 2005).

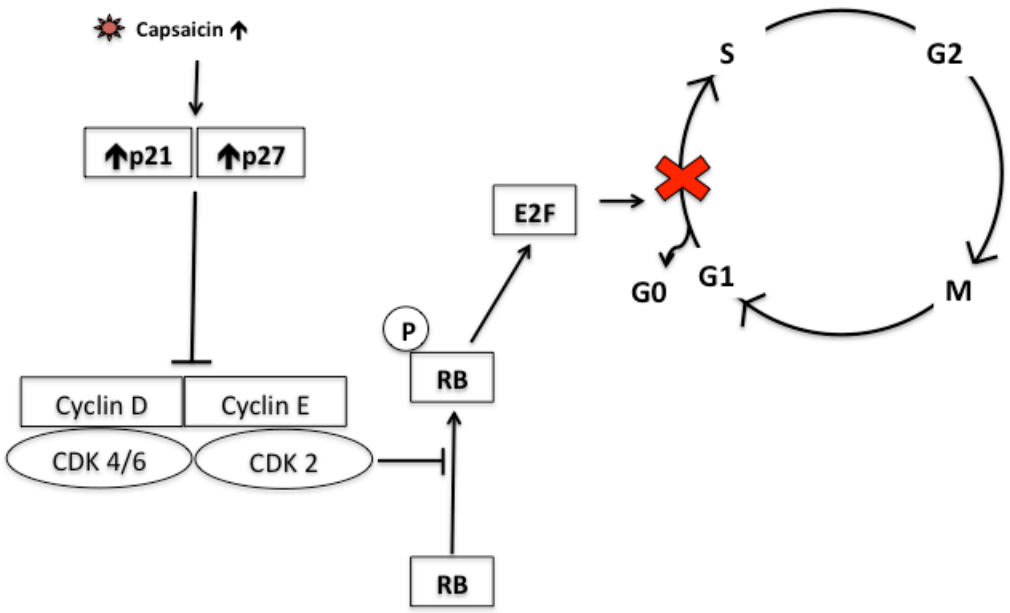


Figure 4: Cell Cycle Regulation by Capsaicin. *The above figure depicts the mechanism of cell cycle regulation by capsaicin. Capsaicin acts to inhibit cell cycle progression in the G0/G1 phase. Upregulation of p21 and* $p27^{Kip}$ *inhibits cycle D and cyclin E from forming complexes with cyclin-dependent kinase 4/6 and 2, thus inhibiting Retinoblastoma (RB) phosphorylation and E2F activation.*

1.10.3 ER Stress Pathway

The endoplasmic reticulum (ER) is responsible for protein folding and modification, lipid biosynthesis as well as calcium homeostasis. At the cellular level, a number of factors, including the generation of reactive oxygen species (ROS) and the disruption of calcium homeostasis, can cause the ER to become stressed and triggers the unfolded protein response (UPR), also referred to as the ER Stress Pathway, to preserve cell function (Tsai and Weissman 2010, Chuang, Ho et al. 2011). The UPR relieves ER stress through activating three main pathways, PERK, IRE1alpha, and ATF6, which cause a downstream signalling cascade leading to the following series of events: 1) the inhibition protein translation, 2) an increase in transcription and

translation of proteins which increases protein folding capacity, 3) enhanced clearance of unfolded proteins from the ER (i.e. heat-shock proteins) and 4) induction of apoptosis via CHOP and GADD34 upregulation (See Figure 5) (Tsai and Weissman 2010, Chuang, Ho et al. 2011).

Recent literature has suggested that the ER Stress Pathway is one of the main pathways activated by capsaicin *in vitro* that lead to apoptosis. In 2008, Sanchez et al. was the first group to report the involvement of the ER Stress Pathway in prostate cancer cells. They performed a microarray analysis of PC3 cells treated with capsaicin and detected significant alterations in several genes and proteins related to the ER Stress Pathway (Sánchez, Martínez-Botas et al. 2008). Several other groups have more thoroughly examined the role of capsaicin in the ER Stress Pathway in other malignant cell lines (Ip, Lan et al. 2012, Lin, Zhang et al. 2013, Krizanova, Steliarova et al. 2014). In nasopharyngeal cancer cell lines, ROS generated by capsaicin were reported to trigger the ER Stress Response and cause downstream apoptosis in a dose-dependent manner (Krizanova, Steliarova et al. 2014). Similarly, capsaicin caused apoptosis in nasopharyngeal carcinoma cells by triggering ER stress through promoting the production of ROS, and by increasing IRE-1, GADD153 and GRP78 (Ip, Lan et al. 2012). In a pancreatic cancer xenograft model, capsaicin prolonged the survival time and increased ER stress markers: GRP78, phosphoPERK, phospho-eIF2alph, ATF4, and GADD153 (Lin, Zhang et al. 2013).

A recent review elegantly describes how capsaicin may be implicated in the ER Stress Pathway, which may lead to downstream apoptosis in PC3 cells (Díaz-Laviada 2010). This theory is supported by Malagarie-Casenave et al. for prostate cancer as they hypothesize that in addition to ROS, ceramide production may contribute to the generation of ER stress and promote apoptosis (Malagarie-Cazenave, Olea-Herrero et al. 2009). The figure below outlines the proposed involvement of capsaicin in ER stress and apoptosis.

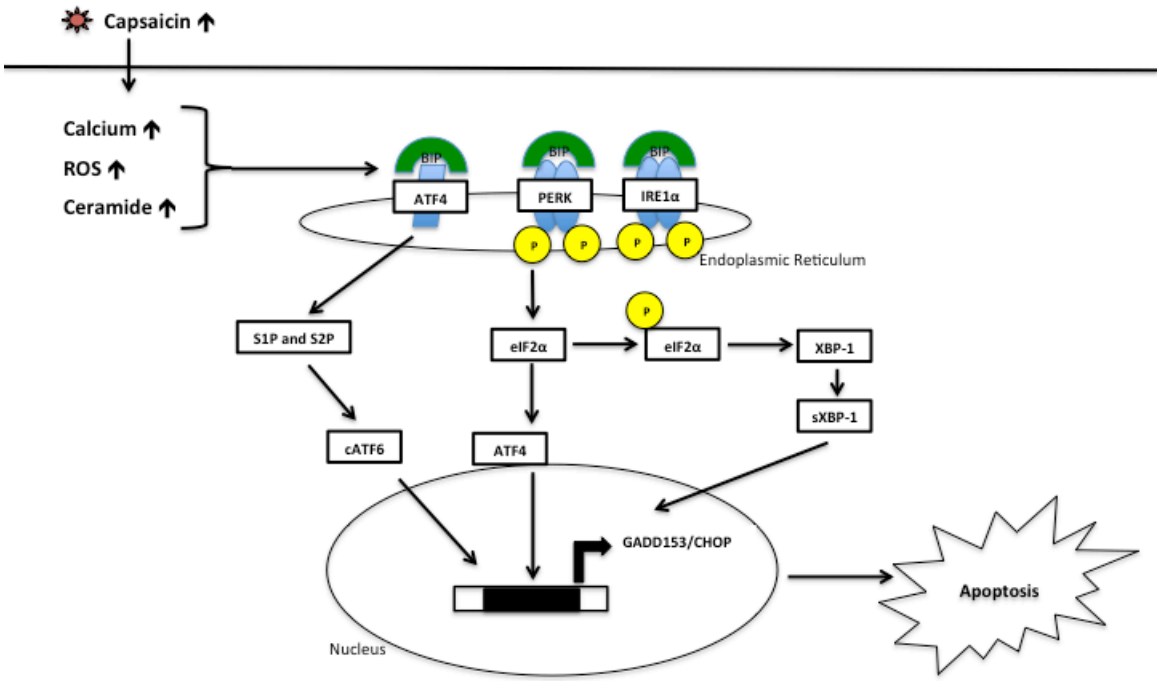


Figure 5: Endoplasmic Reticulum Stress Pathway: *The above figure depicts the impact of capsaicin on the Endoplasmic Reticulum (ER) Stress Pathway. The ER Stress response is activated by intracellular stressors induced by capsaicin such as calcium, reactive oxygen species (ROS), and ceramide accumulation. These stressors facilitate the dissociation of BiP binding proteins from AT4, PERK, and IRE1alpha, which activates a downstream signalling cascade that causes 1) inhibition of protein translation, 2) increased transcription and translation of proteins which increase protein folding capacity, 3) enhanced clearance of unfolded proteins from the ER and, 4) induction of apoptosis (via CHOP and GADD34 upregulation).*

1.10.4 Inflammatory Pathways

Inflammation is one of the most recognized hallmarks of cancer development and progression (Hanahan and Weinberg 2000, Balkwill and Mantovani 2001). Inflammation fosters a tumour-

promoting environment by altering factors that sustain proliferative signalling, survival factors, extracellular matrix-modifying enzymes (Balkwill and Mantovani 2001). Targeting these factors in the tumour microenvironment at early stages of cancer progression may represent a promising therapeutic approach for reducing cancer progression (Albini and Sporn 2007).

One of the main mediators associated with chronic inflammation is Nuclear Factor κB (NFκB) (Aggarwal and Shishodia 2004, Luqman and Pezzuto 2010). This is a ubiquitously expressed transcription factor involved in regulating the expression of genes implicated in inflammation, transformation, survival, proliferation, invasion, stress response and many other physiological processes (Gupta, Prasad et al. 2010). In its inactivated form, NFκB resides in the cytoplasm as a heterodimer consisting of subunits p50, p65, and the inhibitory subunit nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). Upon activation, the IκBα protein undergoes phosphorylation, ubiquitination and degradation, releasing P50 and p65 causing NFκB to translocate to the nuclear compartment and initiate transcription. Active NFκB is aberrantly expressed in prostate cancer tissue correlating with disease progression and has been identified as a potential therapeutic target (Luqman and Pezzuto 2010). Over the past two decades, it has been reported that certain nutraceuticals exert their anti-cancer effects by suppressing NFκB signalling. Using PC3 cells transfected with NF-κB-Luc, Mori et al. demonstrated that capsaicin inhibited the activation and nuclear translocation of NFκB through the inhibition of TNF-α (tumour necrosis factor)-stimulated degradation of IκBα, which was associated with the inhibition of proteasome activity (Mori, Lehmann et al. 2006).

Another well-studied cytokine that is released in the presence of inflammation is Interleukin-6 (IL-6). Interleukin-6 has a number of physiologic roles and has been implicated in a number of pathophysiologic processes, including prostate cancer progression (Smith, Hobisch et al. 2001). Serum levels of IL-6 correlate with prostate tumour burden and patient morbidity (Smith, Hobisch et al. 2001, Aggarwal and Shishodia 2006). Various capsinoids, (compounds structurally related to capsaicin) have been shown to cause calcium- dependent production of IL-6 (Smith and Keller 2001, Malagarie-Cazenave, Olea-Herrero et al. 2011). Recently, Malagarie-Cazenave et al. have found that treating PC-3 cells with capsaicin increased both the synthesis and secretion of IL-6 (Malagarie-Cazenave, Olea-Herrero et al. 2011). In addition, this effect was reversed by capsazepine (the TRPV1 antagonist), as well as by inhibitors of Protein Kinase C – a (PKC-a), phosphoinositol-3 phosphate kinase (PI-3K), Akt and extracellular signal-

regulated protein kinase (ERK). This suggests that Akt, PI3K, TRPV1 and TNF-a all play a role in IL-6 secretion in PCa cells (Malagarie-Cazenave, Olea-Herrero et al. 2011). Other sites have also found that capsaicin effects various immunological factors. In 2012, Anandakumar et al. reported that capsaicin administered to lung-cancer challenged Swiss albino mice administered benzo(a)pyrene resulted in a significant reduction in TNF-alpha, IL-6, COX-2, and NF-KB in serum (Anandakumar, Kamaraj et al. 2012). Ghosh et al. reported that capsaicin injection alters the tumour microenvironment by reducing Treg cells, as well as the cytokine milieu, at the site of the tumour potentially sensitizing colon cancer cells to undergo apoptosis (Ghosh and Basu 2012). Hence, capsaicin may modify inflammatory signalling, which may contribute to its chemopreventive potential in prostate cancer.

1.10.5 Androgen Signalling

Androgen receptor (AR) signalling plays an important role in the development and progression of prostate cancer by working through the regulation and transcription of androgen-responsive genes, such as prostate specific antigen (PSA) as depicted in Figure 6 (Dehm and Tindall 2006). Some of the most well characterized chemoprevention agents for early-stage prostate cancer are 5-alpha reductase inhibitors (5ARi), which inhibit the conversion of testosterone to its more potent isoform, dihydrotestosterone (DHT), thereby inhibiting the androgen-signalling pathway (Dehm and Tindall 2006, Dehm and Tindall 2007). Recent clinical trials suggest that 5ARi may be only effective at reducing the risk of low-grade PCa and are associated with a number of side effects. Nevertheless, non-toxic, natural agents with 5ARi properties may be beneficial in prostate cancer prevention.

Several pre-clinical studies have found that capsaicin alters androgen signalling in androgen-sensitive prostate cancer cells (Mori, Lehmann et al. 2006). In a detailed study examining the anti-androgenic properties of capsaicin, Mori et al. examined the transcriptional activity of PSA using LNCaP cells transfected with a PSA promoter/enhancer- luciferase reporter vector (PSA P/E-Luc) (Mori, Lehmann et al. 2006). Here, capsaicin was shown to inhibit the promotional effect of DHT (60-fold increase) and reduce AR and PSA expression at the protein and RNA level, corresponding to a dose-dependent decrease in cell growth (Mori, Lehmann et al. 2006). In

contrast, Malagarie-Cazenave et al. reported the opposite effects with an increase in the expression of AR in LNCaP cells treated with capsaicin corresponding to an increase in proliferation (Malagarie-Cazenave, Olea-Herrero et al. 2009). It is important to note here that the concentrations used in the study by Malagarie-Cazenave et al. were more than 100-fold lower compared to the studies by Mori et al., suggesting a biphasic effect with capsaicin treatment in the androgen-sensitive LNCaP cell line. This biphasic effect (i.e. stimulation of growth with low-dose treatment and inhibition of growth with high-dose treatment) is commonly reported in the literature with compounds that interact with the androgen receptor (Wetherill, Fisher et al. 2005). Nevertheless, an in-depth analysis of the effect of physiologically relevant concentrations of capsaicin on androgen-signalling pathway is warranted to develop a better understanding of the chemopreventive effects of capsaicin.

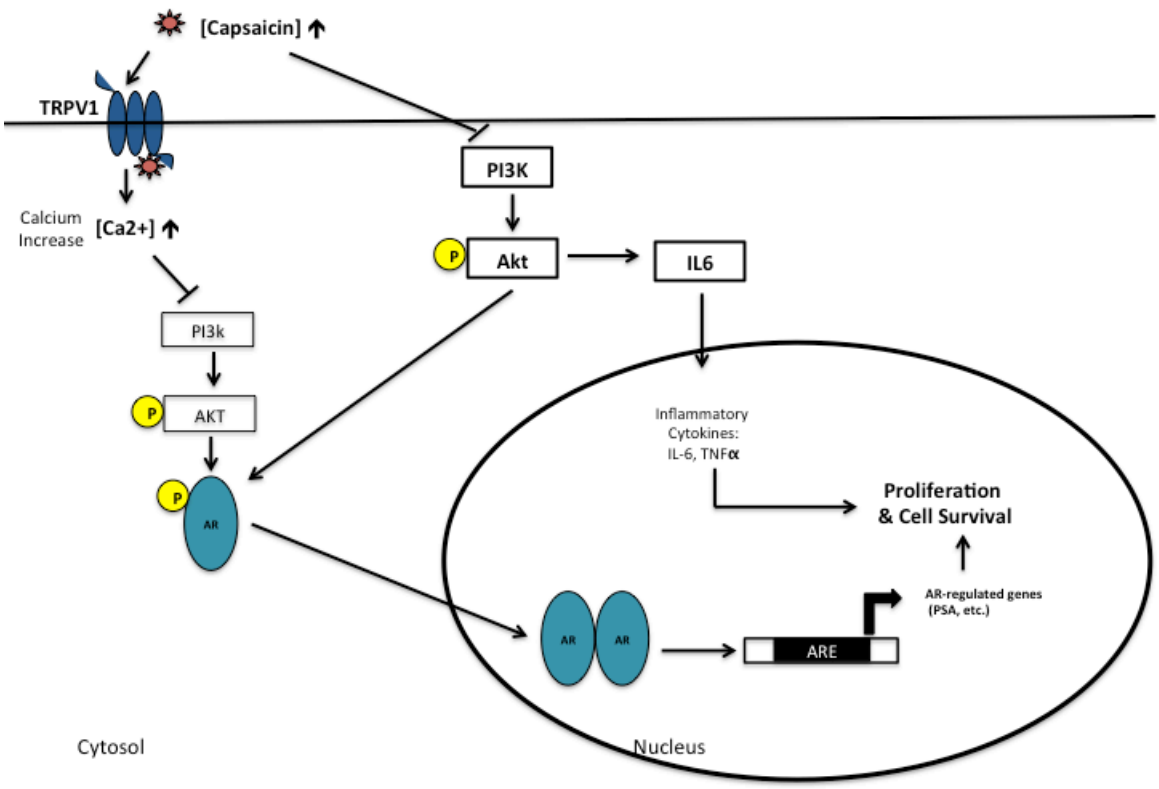


Figure 6. Androgen Receptor and Inflammatory Pathway Signalling: *Capsaicin reduces proliferation and induces apoptosis through the inhibition of PI3 Kinase directly and indirectly, via TRP-V1 signaling, causing down-regulation of androgen dimerization and translocation to the nucleus.*

1.10.6 Apoptosis

1.10.6.1 Intrinsic Pathway

The intrinsic mechanism of apoptosis is characterized by intracellular activation of apoptosis through intracellular stressors, most notably reactive oxygen species (ROS) and excess intracellular calcium. This pathway (illustrated in Figure 7) is initiated with disruption of the mitochondrial membrane and alterations in bcl-2, Bax, bcl-xL, and bad, causing release of

cytochrome c, activation of the caspase cascade, leading eventually to apoptosis (Kim and Moon 2004, Mori, Lehmann et al. 2006, Zhang, Humphreys et al. 2008).

The majority of studies examining the pro-apoptotic effects of capsaicin on prostate and other malignant cell lines, in the recent literature, have reported apoptosis linked to intrinsic mechanisms due to an increase in ROS. Using fluorescent probes, Sanchez et al. reported that both capsaicin and capsazepine increased ROS production in human prostate cancer PC3 cells in a biphasic manner (Sánchez, Sánchez et al. 2006). Utilizing mitochondrial complex inhibitors, they found that the initial acute increase in ROS was generated from the plasma membrane, and the peak detected at a later time resulted from the mitochondria. Furthermore, the generation of ROS induced by capsaicin correlated with the dissipation of the inner mitochondrial transmembrane potential and the release of cytochrome-c into the cytosol (Kryston, Georgiev et al. 2011). Activation of the caspase-3 cascade resulted in the cleavage of PARP and resultant apoptosis (Mori, Lehmann et al. 2006, Kryston, Georgiev et al. 2011). Similar findings have been reported in human pharyngeal cancer, nasopharyngeal carcinoma, and breast cancer cells, among others (Chang, Chen et al. 2011, Ip, Lan et al. 2012, Le, Jin et al. 2012). *In vitro* and *in vivo* analysis of colon cancer models revealed a dose- and time-dependent increase in ROS and calcium then a subsequent decrease in mitochondrial membrane activity followed by dysregulation of mitochondrial proteins and release of cytochrome c (Lu, Chen et al. 2010).

How capsaicin induces mitochondrial damage (and induces intrinsic apoptosis) is unclear at this point; however, recent reports suggest that it may be related to disruption in antioxidant enzymes and/or activation of the vanilloid receptors. In 2011, Pramanik et al demonstrated that capsaicin inhibited the enzymatic activity of antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione reductase causing oxidative stress resulting in mitochondrial complex-I and –III disruption (Pramanik, Boreddy et al. 2011). Subsequently, this led to capsaicin-mediated ROS generation and a decrease in anti-oxidant levels, resulting in severe mitochondrial damage and apoptosis in pancreatic cancer cells (Pramanik, Boreddy et al. 2011). Several other investigations of various malignant cell lines have supported this hypothesis.

The vanilloid receptor, TRP-V1 (discussed in greater detail in section 2.6), is activated by capsaicin and causes an influx of intracellular calcium. This influx of intracellular calcium has

been reported to be responsible (at least partially) for intracellular stress activating apoptosis in various cell lines, namely prostate cancer (Kryston, Georgiev et al. 2011).

It is to be noted, that ROS generation and increases in intracellular calcium have also been attributed to activation of the endoplasmic reticulum (ER) stress pathway. Hence, it is more than likely that a number of various mechanisms are interdependently working to cause prostate cancer cell death.

1.10.6.2 Extrinsic Pathway

The extrinsic mechanism of apoptosis is characterized by activation of the external cell surface receptors, namely TRAIL and DR, leading to downstream caspase-mediated apoptosis (Khan, Blanco-Codesido et al. 2014). These receptors can be activated upon contact or insult by death signalling molecules. In the past decade, only a few studies have reported apoptosis by capsaicin through extrinsic mechanisms. In kidney cancer cells, capsaicin induced the surface expression of TRAIL-receptor D5 through the activation of SP1 due to a calcium influx-dependent Sp1 (Moon, Kang et al. 2012). In multiple malignant glioma cells, subtoxic concentrations of capsaicin sensitized TRAIL-induced apoptosis mediated through ER Stress proteins CHOP/GADD153. DR5 and surviving contribute to amplification of the caspase cascade, thus restoring TRAIL sensitivity (Kim, Kim et al. 2010). There have been no reports on extrinsic-mediated apoptosis in prostate cancer cells.

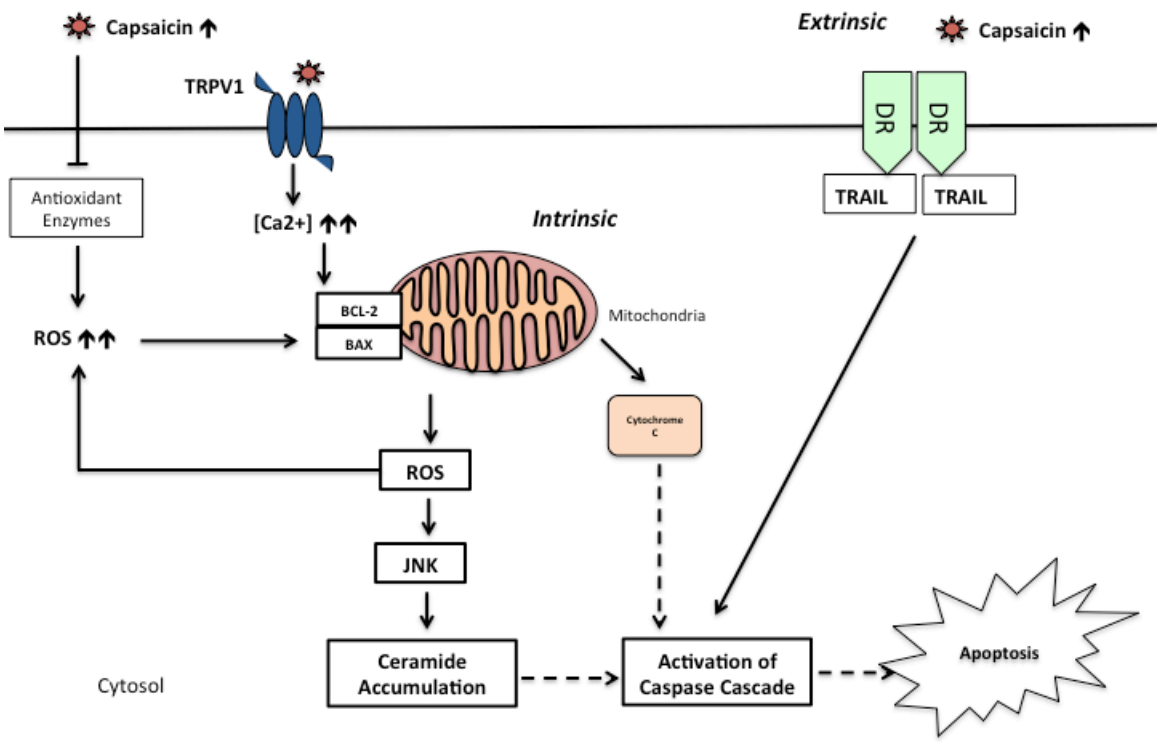


Figure 7: Intrinsic and Extrinsic Regulation of Apoptosis: *Capsaicin acts directly on the Death Receptors (DR) to trigger activation of extrinsically mediated apoptosis. The intrinsic mechanism is initiated intracellular by an influx of extracellular calcium (via TRP-V1 receptors) and Reactive Oxygen Species (ROS) generation through inhibition of antioxidant enzymes. These triggers destabilization of the mitochondria and downstream activation of the caspase cascade and subsequent apoptosis.*

1.10.7 Pre-clinical Studies

The few studies that have examined the effect of capsaicin *in vivo* are quite promising. Investigators including Mori et al. and Sanchez et al. have found that administering capsaicin either by intraperitoneal injection or by gavage suppressed the growth of xenograft tumours (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006). Neither of the studies could delineate a mechanism of action, although the *in vitro* studies by the same groups have suggested several

potential mechanisms of action including the involvement of the TRP-V1 and AR pathway. In pancreatic xenograft tumours, capsaicin inhibited the growth and development of tumours and prolonged the survival time by ER-stress-mediated mechanism (Lin, Zhang et al. 2013). Similar results in the same model reported alterations in the PI3 kinase, p85, and the Akt pathway (Zhang, Lai et al. 2013).

1.11 Other Commonly Investigated Chemoprevention Agents

Over the past decade, a number of pharmaceutical and dietary agents have been investigated for their chemopreventive properties on prostate cancer. Some of the most intensely studied agents include the 5-alpha Reductase Inhibitors (5ARi), lycopene, Vitamin E, and selenium. In the following sections we discuss literature on the role of these antioxidants.

1.11.1 5α-Reductase Inhibitors

The pharmacological agents most widely studied for the chemoprevention of PCa are the 5**α-**reductase inhibitors (5ARi), namely dutasteride and finasteride. The 5ARi inhibit the 5**α-**reductase enzyme which acts to convert testosterone into the more potent androgen dihydrotestosterone (DHT), which has a higher affinity for the androgen receptor (Lacy and Kyprianou 2014). Clinically, 5ARi are actively used to treat benign prostatic hyperplasia by reducing the volume of the prostate.

Two large randomized controlled trials have been carried out to investigate the chemoprevention potential of both dutasteride and finasteride. The Prostate Cancer Prevention Trial (PCPT) examined the whether finasteride could lower circulating androgen levels and reduce the risk of PCa (Lacy and Kyprianou 2014). Here, 18 882 men (≥55 years of age) with PSA ≤ 3.0ng/mL, and normal DRE were randomized to receive either placebo or finasteride (Thompson, Goodman et al. 2003). Results from this study revealed a significant reduction in the prevalence of PCa in the finasteride-treated group compared to the placebo group (18.3 vs. 24.4%, P<0.001), and a significant increase in high-grade tumours (Gleason 7-10) in the finasteride-treated group

controlled to placebo (37 vs. 22%. P<0.001) (Thompson, Goodman et al. 2003, Lacy and Kyprianou 2014).

The subsequent chemoprevention trial, called the Reduction of Prostate Cancer Events (REDUCE) Trial examined whether dutasteride could reduce the risk of PCa, as detected by TRUS biopsy. Here, 6729 men (50-70 years of age) with a PSA ranging from 2.5 to 10 ng/mL and a negative biopsy were randomized to either dutasteride or placebo over a 4-year period (Andriole, Bostwick et al. 2010). Results from this study revealed a 22.8% relative risk reduction in the dutasteride-treated group compared to the placebo, and a significant increase in high-grade tumours (similar to the PCPT) (Lacy and Kyprianou 2014).

Taken together, both the PCPT and REDUCE trials provide evidence that 5ARi can decrease the incidence of PCa. However, both trials also revealed that they increase the risk of high-grade disease, raising significant concern about using these agents as chemoprevention agents for men (Lacy and Kyprianou 2014). A number of criticisms have been raised about both trial designs, suggesting more research is necessary to understand the impact of 5ARi.

1.11.2 Aspirin

Aspirin has been used for nearly a century as an analegesic and anti-inflammatory agent. In the early 1980s, it was discovered to have anti-platelet and anti-thrombotic effects, which sparked its use for the prevention of cardiovascular disease. Recently, evidence has emerged from randomized controlled trials that aspirin may have chemopreventive properties on a number of malignancies, including the prostate (Thorat and Cuzick 2013). The chemopreventive mechanisms are not clear and require further investigation.

1.11.3 Lycopene

Lycopene is the lipophillic carotenoid and red pigment found most abundantly in tomatoes (van Breemen and Pajkovic 2008). It is a linear hydrocarbon, containing a series of conjugated and unconjugated double bonds, allowing for it to have a high affinity for quenching free radical

singlet oxygen molecules (Guns and Cowell 2005, van Breemen and Pajkovic 2008). Unlike β-carotene, lycopene is acyclic and lacks the essential pro-vitamin A activity, and consequently is not physiologically required to be consumed in the diet (van Breemen and Pajkovic 2008). Several animal studies involving lycopene supplementation have shown this agent to have a uniquely ability to deposit in androgen-sensitive regions of the body, particularly in the prostate (Ferreira, Yeum et al. 2000, Guns and Cowell 2005). In 2005, a study by Herzog et al. demonstrated that Copenhagen rats supplemented with lycopene for 8 weeks demonstrated a significant accumulation of lycopene in "*all-trans* form" in each of the four lobes of the prostate (Herzog, Siler et al. 2005). This group also found that lycopene acted to reduce local androgen signalling, IGF-1 expression and basal inflammatory signals in normal prostate tissue (Herzog, Siler et al. 2005). Several other *in vivo* and *in vitro* studies including our own have highlighted lycopene as a potential candidate for PCa chemoprevention (Venkateswaran, Fleshner et al. 2004, Guns and Cowell 2005, Venkateswaran, Klotz et al. 2009). *In vitro* investigations have shown that lycopene has the ability to induce cell cycle arrest and apoptosis in a dose-dependent manner on several human PCa cell lines (Hantz, Young et al. 2005, Ivanov, Cowell et al. 2007). The optimal effective dose of lycopene still remains controversial and debatable amongst researchers, as its chemical structure can be unstable in nature and oxidize in the presence of light (Guns and Cowell 2005, Hantz, Young et al. 2005, Ivanov, Cowell et al. 2007, Kanagaraj, Vijayababu et al. 2007, van Breemen and Pajkovic 2008).

In addition to the ability of lycopene to quench free radicals, this compound has been shown to down-regulate the IGF pathway by increasing the concentration of IGFBP, a critical factor that has been implicated to stimulate proliferation in PCa cells (Kanagaraj, Vijayababu et al. 2007). Liu et al. demonstrated the reversal of the stimulatory effect of DHT and IGF-1 mediated pathway with lycopene treatment in a co-culture system with human prostate stromal and epithelial cells (Liu, Allen et al. 2008). Using the *Lady* transgenic model, Venkateswaran et al. have shown a dramatic reduction in the incidence of PCa *in vivo,* when mice were placed on a diet supplemented with lycopene in combination with vitamin E and selenium (Venkateswaran, Klotz et al. 2009). More recently, our group has also specifically highlighted the importance of lycopene as a key component of this combination study (Venkateswaran, Klotz et al. 2009). It is important to note that although the favourable effects of lycopene have been well documented in

preclinical models, its chemopreventive nature remains highly controversial and should be reviewed with caution (Ablin 2005).

1.11.4 Vitamin E

Vitamin E is a fat-soluble vitamin that is obtained from a variety of dietary sources including: vegetable oils, nuts and egg yolks to name a few (Ni and Yeh 2007). Vitamin E belongs to the family of tocopherols and tocotrienols, which can classified into α-,β-,γ- and δ- isoforms (Ni and Yeh 2007). Dietary isoforms of this antioxidant exist predominately as γ-tocopherol, while supplementation forms are typically in the form of α-tocopherol which is the biologically active form of vitamin E in the body (Ni and Yeh 2007). Vitamin E has long been considered a potential candidate for PCa chemoprevention supported by numerous *in vitro* and *in vivo* animal studies and clinical trials. *In vitro* experiments have demonstrated that vitamin E in the form of α-tocopherol succinate can induce cell cycle arrest in human PCa cells (Venkateswaran, Fleshner et al. 2002, Conte, Floridi et al. 2004). Flow cytometry confirmed the effect of α-tocopherol depicting cell cycle arrest, impeding cells in the G1 and G2/M phase in LNCaP and PC3 cells respectively (Conte, Floridi et al. 2004). However, the mechanism by which vitamin E has taken effect has not been clearly delineated. *In vivo* studies have suggested that the anti-cancer effects of PCa may be due to disruption of the PI3K pathway and/or the sphingolipid synthesis (Jiang, Wong et al. 2004, Ni, Wen et al. 2005, Syed, Suh et al. 2008). Several *in vivo* studies using vitamin E in combination with other micronutrients have supported *in vitro* studies. Fleshner et al. have shown that the tumour promoting effect of a high-fat diet can be inhibited with vitamin E in a nude mouse model (Fleshner, Fair et al. 1999). While Venkateswaran et al. have shown that a combination of vitamin E with selenium and lycopene is more effective in reducing the incidence of PCa and liver metastasis than either of these micronutrients alone (Venkateswaran, Klotz et al. 2009). These studies carried out in the *Lady* transgenic model that spontaneously develops localized prostatic adenocarcinoma demonstrated a 4-fold reduction in the incidence of PCa relative to the control with the administration of a combination of vitamin E, selenium and lycopene (Venkateswaran, Fleshner et al. 2004, Venkateswaran, Klotz et al. 2009).

1.11.5 Selenium

Selenium is a trace mineral that is biologically essential for the proper functioning of various antioxidant enzymes and proteins in the body. Selenium exists naturally in foods predominately in the organic form as selenomethionine, selenocystine, and selenocysteine as shown in Figure 8. Some vegetables and animal feed may also contain the inorganic form of selenium as selenite and selenate (Gropper, Smith et al. 2005). Many studies have suggested selenium to play a beneficial role in the chemoprevention of PCa (Nomura, Lee et al. 2000, Yoshizawa, Ascherio et al. 2003). *In vitro* studies carried out by Venkateswaran et al. have shown that selenium has a significant effect on PCa cell lines containing a functional androgen-receptor (Venkateswaran, Klotz et al. 2002, Yoshizawa, Ascherio et al. 2003). Cells treated with seleno-DL-methionine for up to 72 hours exhibited a dose-dependent reduction in proliferation. Results from flow cytometry analysis of LNCaP cells demonstrated that selenium caused a G1 cell cycle arrest with an 80% reduction in the cells in the S phase, with no effect on PC3 cells (Venkateswaran, Klotz et al. 2002). However, PC3 cells transfected with a functional androgen-receptor (PC3-AR2) regained sensitivity to selenium, arresting cells in the G2/M phase, suggesting that selenium chemopreventative effects may be mediated by the presence of a functional androgen receptor (Venkateswaran, Klotz et al. 2002). In a similar *in vitro* experiment, treatment of LNCaP cells with sodium selenite resulted in apoptosis (Zhao, Xiang et al. 2006). It is suggested that selenite induced apoptosis through the production of superoxide, which mediates the translocation of p53 to the mitochondria, thereby increasing the production of superoxide and eventually causing apoptosis (Zhao, Xiang et al. 2006). It has also been suggested that certain selenium metabolites may in part mediate the anticancer effect of selenium (Wu, Zhang et al. 2005). Several mechanisms have been proposed for the induction of programmed cell death. A study by Wu et al. suggests that methylseleninic acid (MSA), a monomethylated metabolite of selenium, may stress the endoplasmic reticulum causing an accumulation of misfolded proteins triggering apoptosis in PC3 cells (Wu, Zhang et al. 2005). The effect of MSA on PC3 cells have also been shown to alter the PI3K/Akt pathway, a pathway commonly affected in several cancers (Wu, Zu et al. 2006). It is presumed that the status of phospho-Akt is affected by selenium-mediated dephosphorylation of Akt through calcineurin (Wu, Zu et al. 2006). The anti-cancer effect of selenium supplementation *in vivo* has also been studied in depth in a variety of preclinical models (Combs, Clark et al. 1997, Wu, Zu et al. 2006). Studies have also assessed the effect of

selenium in pre-clinical models, whereby selenium has been shown to act optimally when combined with other micronutrients, specifically lycopene and vitamin E (Venkateswaran, Klotz et al. 2009).

Although antioxidants have been generally supported for their chemopreventive potential, it has been suggested to avoid using them during chemotherapy and radiotherapy. A recent paper by D'Andrea proposes that the protective effect of antioxidants may in fact reduce the effectiveness of conventional therapies for PCa (D'Andrea 2005). As chemotherapy and radiotherapy patients may often improve their antioxidant intake to prevent toxicity to their body, these antioxidants may play a role in diminishing the cytotoxic effect on cancerous cells (Tabassum, Bristow et al. 2010). Since this data is still is uncertain more studies should be carried out to determine the interaction between conventional therapies and antioxidant intake.

One of the most promising clinical studies of the anti-carcinogenic effect of selenium was a randomized controlled clinical trial by the Nutritional Prevention of Cancer Study Group. Between 1983 and 1991 over 1300 patients with a history of basal cell or squamous cell carcinomas were randomized to receive 200µg/day of selenium or the placebo (Clark, Combs et al. 1996). In this study there was no significant impact on non-melanoma skin cancer however there were significant reductions in prostate (63%), lung (46%), and colorectal cancer (58%) incidence as well as an overall reduced incidence of cancer (53%) (Clark, Combs et al. 1996). Conversely the SELECT trial discussed in further detail below, found that men supplemented with selenium alone did not have any beneficial effect on PCa warranting further examination of this dietary agent (Clark, Combs et al. 1996, Lippman, Klein et al. 2009).

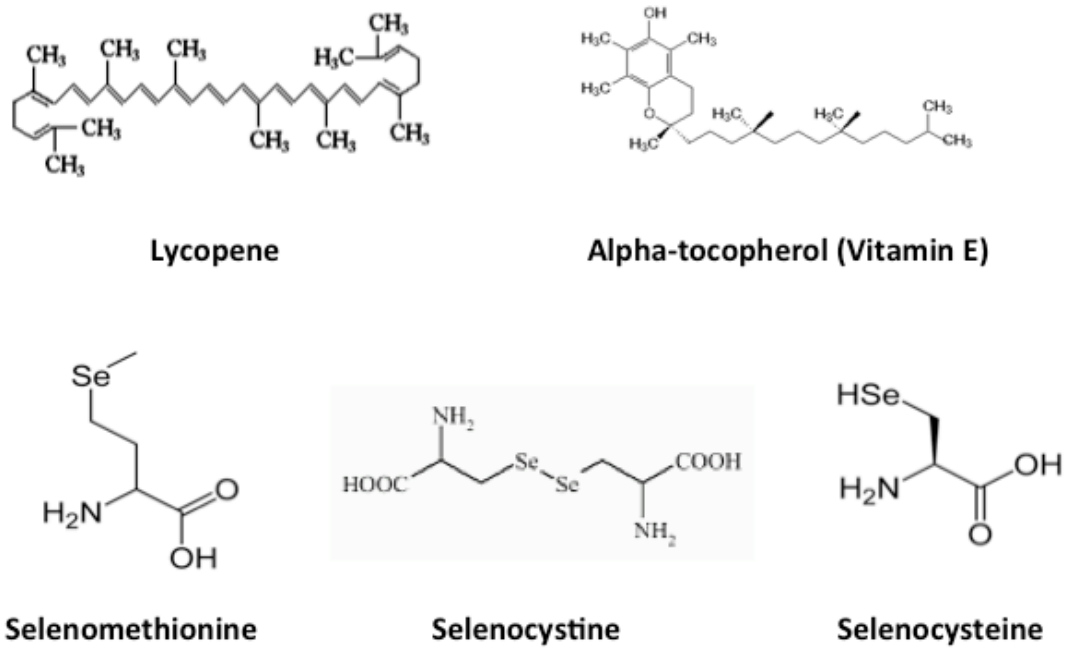


Figure 8: Chemical Structure of Antioxidants Implicated in Prostate Cancer Chemoprevention. *The above figure was adapted from Venier et al (Venier, Klotz et al. 2012). It demonstrates the chemical structure of lycopene, vitamin E, and three forms of selenium (i.e. selenomethionine, selenocystine, and selenocysteine).*

1.11.6 Combination Studies

With the plethora of data describing the effects of each individual micronutrient, combinatorial studies have begun to emerge assessing the effect of several dietary agents on PCa. Through using more than one micronutrient, there is the potential to have a greater effect (i.e. additive or synergistic) than a single agent alone. In the following section we analyze the SELECT Trial, the largest clinical study examining micronutrients alone and in combination.

Selenium and Vitamin E Cancer Prevention Trial (SELECT): One of the most ambitious cancer prevention studies stemming from the large body of evidence supporting the chemopreventive properties of vitamin E and selenium was the SELECT study (Clark, Combs et al. 1996, Lippman, Klein et al. 2009). In this randomized, placebo-controlled trial over 35,000 men were recruited from Canada, United States, and Puerto Rico, to test the efficacy of selenium and vitamin E (Klein 2003, Lippman, Klein et al. 2009). The study was initiated in 2001, and was anticipated to last 7-12 years. Men with a PSA reading of 4ng/mL or lower and a non-suspicious digital rectal exam (DRE) were recruited in a randomized fashion, and given either selenium (200μM in the form of L-selenomethionine) and/or vitamin E (400 IU in the form of All-*rac*-α-tocopheryl acetate) supplements (Lippman, Klein et al. 2009). Subjects were asked to discontinue taking supplements in 2008, after an estimated 5.46 years, based on evidence from the independent data and safety monitoring committee (Lippman, Goodman et al. 2005, Lippman, Klein et al. 2009). There was no evidence for a benefit of consuming vitamin E and/or selenium supplements on prostate cancer prevention. On the other hand there was a trend in participants taking vitamin E alone for an increased prostate cancer risk, and patients on selenium supplementation alone showed a non-significant increase in the risk of type-2 diabetes (Lippman, Klein et al. 2009).

With the substantial support of many clinicians and scientists the SELECT trial has stirred much debate with respect to the intensely investigated micronutrients selenium and vitamin E. A recent article by Hatfield et al. attributes the ineffectiveness of selenium to the form of L-selenomethionine, as well as high levels of selenium in the participants prior to the start of the trial (Hatfield and Gladyshev 2009). This study also stressed the need for further investigation into the biology of selenium or specifically targeting a subpopulation that would most likely benefit from such supplementation. In addition, Venkateswaran et al. has recently published data that highlights the importance of combinatory treatments in appropriate pre-clinical models to assess the effectiveness of certain pre-clinical therapies (Venkateswaran, Klotz et al. 2009). This newly released data demonstrated that selenium and vitamin E alone or in combinations are not effective in the *Lady* transgenic model; however, they have shown that they may be effective in reducing liver metastasis when treated with a combination of lycopene, selenium, and vitamin E in the same model system (Venkateswaran, Klotz et al. 2009). It is indisputable that additional studies looking at the specific forms and biology of vitamin E and selenium, as well as

alternative combinations are warranted to delineate, in appropriate pre-clinical models before initiating further clinical trials.

1.12 Translating *in vitro* to *in vivo* Studies into Clinical Trials

With the large body of existing *in vitro* and corresponding *in vivo* data, the translation into clinical trials is the next logical step in the development of effective chemopreventive strategies. Unfortunately based on the unsuccessful results of several large-scale clinical trials, it seems necessary to re-examine the approach that is required on the use of appropriate pre-clinical studies and/or model systems. Likewise, many studies translating *in vitro* results into *in vivo* model systems have been far from ideal, re-enforcing the need for additional rigorous investigation using appropriate pre-clinical models (Venkateswaran, Klotz et al. 2009). One such proposal by Scott et al. sketches a logical design for the development of dietary chemopreventive agents, which includes a series of steps that begin with several *in vitro* and *in vivo* studies, followed by translating them into clinical pilot studies and clinical trials (Scott, Gescher et al. 2009). Scott et al. have developed a strategy to identify appropriate clinical doses of micronutrients through the use of suitable long-term preclinical studies with attainable and realistic concentrations achievable in humans (Scott, Gescher et al. 2009). Furthermore, this proposal highlights the importance of devising sophisticated strategies, that include the utilization of appropriate preclinical models, required to successfully implement the use of chemopreventive agents in PCa.

1.13 Pre-Clinical Models of Prostate Cancer

1.13.1 *In Vitro* Models

Primary and immortalized PCa cell lines are the main two types of cell systems that are most predominately studied *in vitro*. Primary cell lines are isolated and cultured from fresh tissue, and can be propagated a limited number of times. Established cell lines, frequently studied in this thesis, are established cell lines that have been propagated for many generations and are

publically available. The original immortalized cell lines in basic prostate cancer research are LNCaP, PC3, and DU-145 (Sobel and Sadar 2005). These cell lines were all derived from human prostate cancer metastases (see table below) and have specific mutations that make them excellent tools for mechanistic studies. Over the years, LNCaP, PC3, and DU-145 cell lines have been modified and mutated into clonal derivatives by various investigators, which is described in a two-part review by Sobel and Sadar (see details in Table 1) (Sobel and Sadar 2005, Sobel and Sadar 2005).

General Characteristics of DU-145, PC3, and LNCaP Cell Lines			
	LNCaP	**PC3**	**DU-145**
Androgen Sensitive	Yes	No	No
PSA Expression	Yes	No	No
p53	Wild-type (with point mutation)	Wild-type	Mutated
Metastasis Site	Lymph Node	Vertebrae	Brain
Number of Clonal Derivatives *	63	11	1
***Note: The above table was derived from the review by Sobel and Sadar in 2005 (Sobel and Sadar 2005)**			

Table 1: General Characteristics of DU-145, PC3, and LNCaP Cell Lines

1.13.2 *In Vivo* Models

There are a number of different animal models studied for prostate cancer research. In nature, rats and dogs are the only species that can develop prostate cancer spontaneously. Investigators

have used this knowledge to investigate new genetically modified animals. In rats, the Dunning, Copenhagen, and Lobound-Wistar rat models have been well established based on genetic modifications, and are commonly used in therapeutic research. Canines on the other hand are difficult to manipulate genetically as they lack a functional androgen receptor (AR), do not consistently develop tumours, and are expensive to maintain. For these reasons, they are less commonly studied (Jeet, Russell et al. 2010).

The most common animal model used in prostate cancer research is the mouse. Mice are affordable, have a short gestation period, a relatively short lifespan, and genes that are structurally homologous to humans that can be easily manipulated, making them excellent experimental models for prostate cancer (Jeet, Russell et al. 2010). Some of the drawbacks of mouse research are the small size, prostate anatomy (four distinct lobes compared to a confined organ), as well as the development process (non-spontaneous in mice and spontaneously in humans). There are a wide variety of murine models that are used in prostate cancer research that are summarized in the sub-sections below:

1.13.2.1 Xenograft Mouse Models

The xenograft procedure involves the implantation of "xeno" (Greek meaning: foreign) cells, tissues, or organs from one species into a different species. Xenografted mice are generally immune compromised, athymic mice lacking mature T-cells, which allows for cross-species implantation. LNCaP, DU145, and PC3 are the most common cell lines used in prostate cancer xenograft models (Wu, Gong et al. 2013). The cell implantations can be supplemented with various growth factors and collagen matrix to help aid in xenograft formation, however this is not required. The location of the xenograft inoculation can vary in the mice. Subcutaneous xenografts (commonly inoculated in the lower flank area) are easily identified and measured overtime, which makes them ideal for radio-sensitizing studies as they can be targeted for radiotherapy and measured over time. However, the tumours lack the ability to metastasize from the subcutaneous space (Wu, Gong et al. 2013). Orthotopic grafting occurs when the xenograft is implanted in the prostatic (orthotopic) region of an immune compromised mouse. When carried out with certain prostate cancer cell lines (i.e. LNCaP, PC3), this procedure allows for primary

tumours to metastasize to various sites, including the lymph nodes, lung, and bone (Stephenson, Dinney et al. 1992, Rembrink, Romijn et al. 1997, Wu, Gong et al. 2013). Co-xenograft models consist of implanting both prostate cancer cell lines as well as fibroblast cell lines into an immune compromised mouse. This technique is generally used to study the tumour microenvironment, particularly the tumour-host cell and stromal interaction. These co-xenograft models have also been reported to have a shortened latency period and an accelerated growth rate (Gleave, Hsieh et al. 1991, Craig, Ying et al. 2008, Wu, Gong et al. 2013). A less commonly reported technique is the subrenal capsular xenografting model, which can be used to study growth and alternative phenotypes by inoculating a combination of prostate cancer cells with rat urogenital sinus mesenchymal cells under the kidney capsule in an immune compromised mouse (Xin, Ide et al. 2003, Wu, Gong et al. 2013). One emerging xenograft methodology is the use of *patient-derived tumour xenografts (PDX)* or avatars (Hidalgo, Amant et al. 2014). PDX xenografts offer an opportunity to retain both the histologic and genetic background of tumours, however, they are not easily established, particularly for prostate cancer tumours (Hidalgo, Amant et al. 2014).

1.13.2.2 Transgenic Models

Transgenic mouse models offer researchers the opportunity to study prostate cancer in a model that examines the tumour biology in a mouse with an intact immune system. Advances in genetically engineering techniques (e.g. CRE-LOX P recombination system), as well as identification of key regulatory genes involved in prostate cancer progression, has allowed for manipulation of the mouse genome and the creation of transgenic mouse models that more closely resemble the development of prostate cancer in humans (Jeet, Russell et al. 2010). In the following subsections we will discuss a few of the most commonly studied prostate cancer transgenic mouse models.

1.13.2.3 TRAMP Model

The Transgenic Adenocarcinoma Model of the Mouse Prostate (TRAMP) model was the first murine model developed by Gingrich in 1996 using the Probasin system (PB) targeting heterologous gene expression specifically to the prostate epithelium (Gingrich, Barrios et al. 1996, Gingrich and Greenberg 1996, Wu, Gong et al. 2013). The Probasin promoter regulates the expression of the both the large and small simian virus (SV40) t antigen, which is spatially restricted to the dorsolateral and ventral lobes of the prostate (Gingrich, Barrios et al. 1996, Gingrich and Greenberg 1996, Wu, Gong et al. 2013). The large t antigen inhibits both tumour suppressor genes, p53 and Retinoblastoma (Rb), while the small t antigen abolishes the function of protein phosphatase 2, causing the inhibition of growth and survival by the inhibition of MAP kinase activity (Gingrich and Greenberg 1996, Jeet, Russell et al. 2010). Targeting this gene results in the development of prostate cancer in a way that models human disease progression. TRAMP mice generally display high-grade prostate intraepithelial neoplasia (HGPIN) by 12 weeks of age, followed by poorly differentiated and invasive carcinoma by 18 weeks of age, and 100% metastasis at 24 weeks (6 months) (Gingrich, Barrios et al. 1996, Gingrich and Greenberg 1996, Jeet, Russell et al. 2010). Following castration, TRAMP mice display androgen-independent disease with neuroendocrine changes, similarly found in some men with prostate cancer (Gingrich, Barrios et al. 1996, Gingrich, Barrios et al. 1997, Huss, Gray et al. 2007). The TRAMP model has been an important tool for understanding the biology of tumour progression in an animal system, as well as studying the effect of diet, micronutrients, and chemoprevention agents on prostate cancer progression (Gupta, Ahmad et al. 2000, Adhami, Siddiqui et al. 2004, Gupta, Adhami et al. 2004, Shukla, MacLennan et al. 2007, Wu, Gong et al. 2013, Shukla, Bhaskaran et al. 2014). It has also inspired the development of similar model systems (i.e. the *Lady* transgenic model) as described below.

1.13.2.4 *Lady Transgenic* Model

Greenberg and others established the *Lady* Transgenic Mouse Model (also referred to as the 12-T10 model) using the same PB promoter system as previously described in the TRAMP Model (Greenberg 1996). It differs from the TRAMP model as it targets only the SV40 large t antigen using the large PB promoter (Kasper, Sheppard et al. 1998, Masumori, Thomas et al. 2001). This system allows for a less aggressive prostate cancer phenotype than the TRAMP model. The

development of prostate cancer is also more gradual, as the Lady transgenic model develops glandular PIN at 16 weeks, and high-grade epithelial and poorly undifferentiated adenocarcinoma and metastasis (88%) by 36 weeks of age (9 months) (Kasper, Sheppard et al. 1998, Masumori, Thomas et al. 2001, Jeet, Russell et al. 2010). Similar to the TRAMP model, Lady transgenic mice develop androgen-independent disease with neuroendocrine changes following castration (Jeet, Russell et al. 2010). The gradual development of prostate cancer progression in the Lady transgenic model makes it ideal for examining various time point interventions (i.e. early-stage disease, local disease, etc.) of various micronutrients and chemoprevention agents (Venkateswaran, Fleshner et al. 2004, Venkateswaran, Klotz et al. 2009).

1.13.2.5 c-MYC

Approximately 30% of prostate cancers cases report some degree of overexpression of the oncogene, c-myc (Taylor, Schultz et al. 2010, Irshad and Abate-Shen 2013). As such, mouse models with the c-myc transgene have been developed in an attempt to mimic human disease at high (Hy-myc) and low (Lo-myc) levels under the control of the PB promoter. Mice expressing Hy-Myc generally express PIN at 2 weeks of age, followed by invasive carcinoma at 3-6 months, while Low-myc transgenic mice develop PIN lesions at approximately 4 weeks, and invasive disease at 10-12 months (Iwata, Schultz et al. 2010, Wu, Gong et al. 2013). A key feature of the c-myc model is that it develops adenocarcinoma rather than neuroendocrine tumours (Irshad and Abate-Shen 2013).

1.13.2.6 NK3 Homeobox (1NKX3.1) Model

The NKX3.1 transcription factor is involved in the development and function of the urogenital tract, and is often mutated in early stages of prostate cancer (i.e. PIN and hyperplasia) (Sciavolino, Abrams et al. 1997, Kim, Cardiff et al. 2002, Grabowska, Degraff et al. 2014). In NKX3.1 knockout models, mice develop hyperplasia and PIN, but not prostate cancer (Kim, Cardiff et al. 2002).

1.13.2.7 The Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN) Model

PTEN is a commonly mutated tumour suppressor in prostate cancer (Yoshimoto, Cutz et al. 2006, Grabowska, Degraff et al. 2014). It is implicated in many different molecular pathways, but it mainly acts to regulate cell growth and survival through the activation of Akt and PDK.1, with a number of molecular pathways implicated in carcinogenesis (Cantley and Neel 1999, Grabowska, Degraff et al. 2014). As such, a number of transgenic models targeting the PTEN gene have been established in an effort to model disease progression. The homozygous PTEN knockout model is lethal, however heterozygous PTEN knockout models are slow to develop tumours, and demonstrate a wide spectrum of prostate cancer phenotypes (Podsypanina, Ellenson et al. 1999, Grabowska, Degraff et al. 2014). Lowering PTEN levels through inserting the hypomorphic allele promotes early progression of cancer from hyperplasia to PIN (Podsypanina, Ellenson et al. 1999). In 2003, Wang et al. was the only group able to generate a PTEN knockout model that demonstrated the whole range of prostate cancer progression from PIN to metastasis (Wang, Gao et al. 2003). When combined with other gene mutations, the PTEN knockout model can model all the various phenotypes of prostate cancer progression in humans. These phenotypes are depicted in Figure 9 which is modified from a detailed review by Irshad and Abate-Shen (Irshad and Abate-Shen 2013).

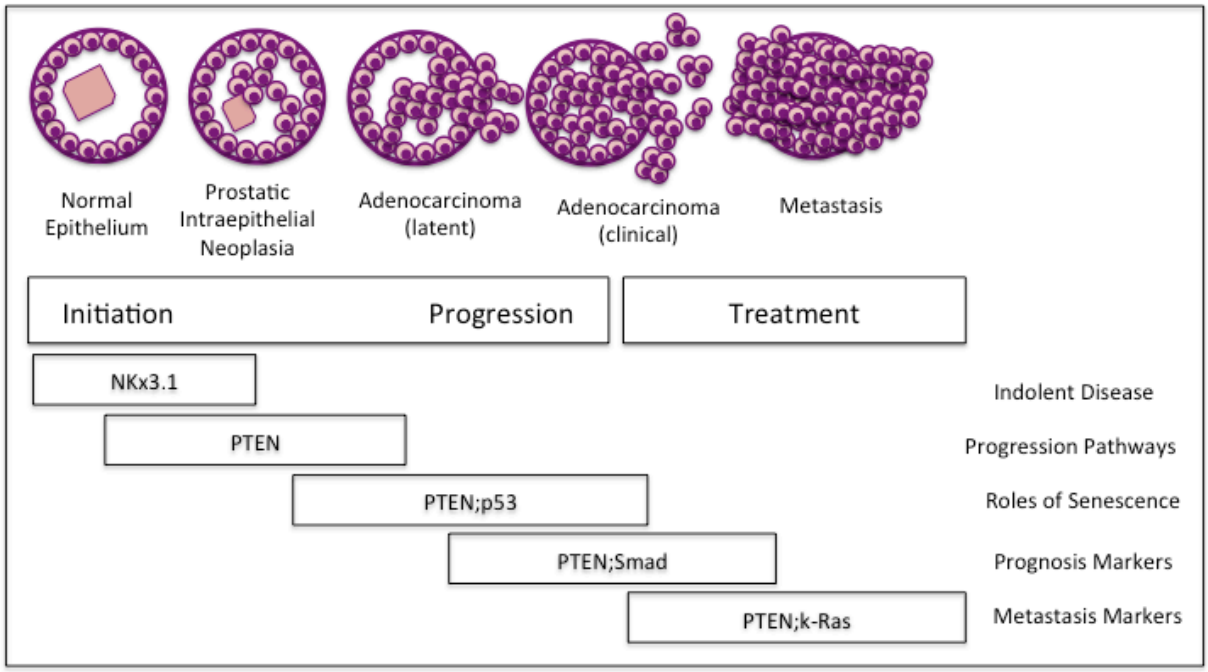


Figure 9: Stages of Prostate Cancer Development and Mouse Models represent Corresponding to the various Stages of Disease.

CHAPTER TWO
Study Rationale and Specific Aims

PCa is the most commonly diagnosed malignancy in men (Siegel, Ma et al. 2014). In 2014, Statistics Canada reported that one in eight men will develop PCa during their lifetime and one in twenty-eight will die from the disease (American Cancer Society 2014). Broadly speaking, there are two means of improving outcome in prostate cancer: using chemopreventive strategies or enhancing current therapeutic regimens. This thesis aims to evaluate both the chemopreventive and therapeutic role of capsaicin in prostate cancer.

Capsaicin as a Chemoprevention Agent

Chemopreventive agents are natural or pharmaceutical compounds used to prevent the development (primary chemoprevention) or delay the progression (secondary chemoprevention) of disease (Chan, Gann et al. 2005). Prostate cancer represents an ideal tumour system for studying chemopreventive agents, given its prolonged developmental phase and subsequent, commonly slow, progression (Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012). Thus, an extended window of opportunity exists for administration of both primary (pre-tumour development) and secondary (post-diagnosis) chemoprevention (Chan, Gann et al. 2005, Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012).

The use of dietary agents for the chemoprevention of prostate has been intensely investigated (Johnson, Bailey et al. 2010, Schmid, Fischer et al. 2011, Venier, Klotz et al. 2012). While many agents have shown promising results *in vitro* and in the pre-clinical arena, none have successfully translated into chemopreventive strategies routinely used in clinical practise (Venier, Klotz et al. 2012). Potential explanations as to why micronutrients often fail to successfully translate include: lack of knowledge regarding optimum onset and required duration of intervention, impaired bioavailability of the active component present in the compound, and a lack of biologically plausible mechanisms of action (Johnson, Bailey et al. 2010, Ledesma, Jung-

Hynes et al. 2011). Thus potential successful chemopreventive agents are dietary agents that are non-toxic, are readily absorbed/metabolised to active compounds, and target specific pathways related to tumour development/progression. We believe capsaicin fulfills all of these criteria.

Capsaicin as a Therapeutic Agent

Several treatment options exist for managing localized prostate cancer, including surgery and radiotherapy. Although both these strategies are therapeutically effective, they are often associated with significant side effects. One means of minimizing the adverse effects of curative treatment involves the use of therapeutic agents that sensitize prostate cancer cells to the effect of definitive treatment, i.e. the use of a radio-sensitizing agents combined with ionizing radiation or down-staging neo-adjuvant therapies prior to surgical intervention.

Capsaicin as a Radio-Sensitizing Agent

Radio-sensitizing agents are chemical or pharmacologic compounds that sensitize cells to the lethal effects of radiation therapy (RT). Successful radio-sensitizing agents achieve their effect by increasing tumour cell kill while minimizing adverse effects on normal tissues (Girdhani, Bhosle et al. 2005). Compounds occurring naturally in the human diet are ideal potential radio-sensitizing agents, given their lack of toxicity at conventional doses (Girdhani, Bhosle et al. 2005). As treatment with radiation and the use of capsaicin both promote apoptosis and reduce proliferation through distinct pathways, we rationalize that combining these two therapeutic regimes will achieve an ‘at least’ additive effect.

2.1 Study Rationale:

Capsaicin is a promising dietary agent for prostate cancer chemoprevention and management. It has the potential to bind to specific receptors present on prostate cancer cells, thereby exerting potential anti-neoplastic and radio-sensitizing effects on prostate cancer. Herein, the chemopreventive and therapeutic roles of capsaicin will be investigated in *in vitro* and *in vivo* model systems.

2.2 Overall Hypothesis:

Capsaicin exhibits chemopreventive and therapeutic properties in pre-clinical models of prostate cancer.

2.3 Objectives and Aims

Chapter 3:

Main Objective: Determine the anti-cancer properties of capsaicin alone, and in combination with other micronutrients in a PCa *in vitro* model.

Aim 1: To assess the proliferative properties of capsaicin on various prostate cancer cells lines *in vitro*.

Aim 2: To assess the effect of capsaicin in combination with other micronutrients.

Aim 3: To investigate the mechanism of action of capsaicin treatment on prostate cancer cells *in vitro.*

Chapter 4:

Main Objective: Determine the chemopreventive properties of capsaicin in a transgenic model of prostate cancer.

Aim 1: To determine the chemopreventive effects of capsaicin in the TRAMP model, a transgenic model of prostate cancer.

Aim 2: Investigate mechanisms related to the invasion and migratory potential of capsaicin of prostate cancer *in vitro* and *in vivo*.

Chapter 5:

Main Objective: ***Assess whether capsaicin can enhance the effect of existing therapeutic strategies for prostate cancer.***

Aim 1: To assess whether capsaicin can enhance existing PCa therapies (i.e. radiation and chemotherapy) in an *in vitro* and *in vivo* model of prostate cancer.

Aim 2: To determine the radio-sensitizing effects of capsaicin in a xenograft model.

Aim 3: To delineate a mechanism of action for capsaicin and radiation**,** with emphasis on the role of the transient receptor potential (TRP) family of proteins.

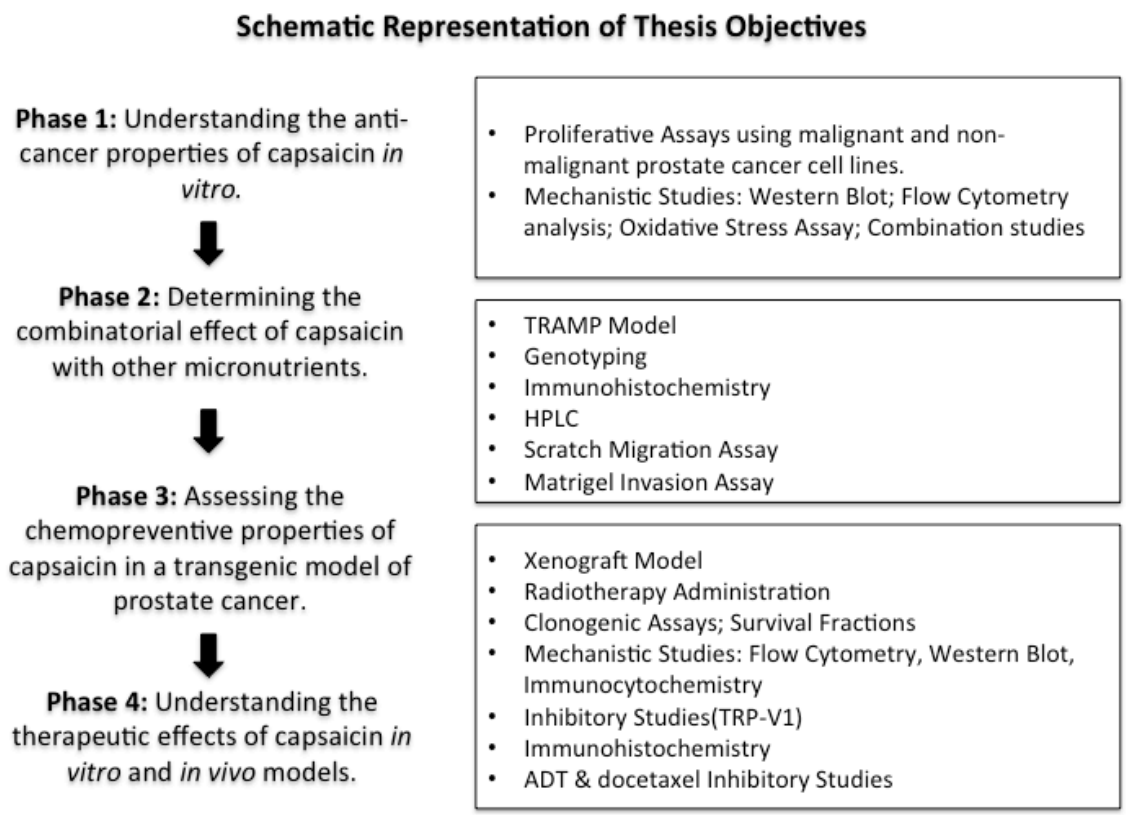


Figure 10: A Summary of Thesis Objectives

2.4 Overall Relevance

In the following thesis, capsaicin was shown to be effective as a chemoprevention and therapeutic agent in pre-clinical model systems. Promising *in vitro* data suggests that capsaicin may also act as a radio-sensitizing agent for prostate cancer. Taken together, this information serves as solid evidence supporting the use of capsaicin for future clinical trials.

CHAPTER THREE

Understanding the Anti-Cancer Effects of Capsaicin Alone, and in Combination with Other Micronutrients.

This chapter is modified from the following:

Venier N., Colquhoun AJ, Klotz LH., Fleshner NE., and Venkateswaran, V. Lycopene enhances the anti-proliferative and pro-apoptotic effects of capsaicin in prostate cancer in vitro. *Journal of Cancer Therapeutics and Research* 2012, 1. (Herbert Publications Ltd).

Published in the *Journal of Cancer Therapeutics and Research (2012) 1:30*

http://www.hoajonline.com/jctr/2049-7962/1/30

3.1 Abstract

Background: Increasing evidence suggests dietary factors influence the development and progression of prostate cancer (PCa). The chemopreventive properties of lycopene, the antioxidant found in tomatoes, have been extensively studied for PCa; however, the effect of lycopene, as a single agent, remains unclear. Studies that are more promising involve using lycopene in combination with other dietary agents. Capsaicin, the active compound in chilli peppers, is reported to exert potent anti-cancer effects in both *in vitro* and *in vivo* PCa models. We have investigated whether lycopene enhances the anti-proliferative and apoptotic effects of capsaicin using *in vitro*.

Methods: Using the MTS Cell Proliferation Assay we assessed the anti-proliferative effect of capsaicin alone, or in combination with lycopene in human PCa cell lines (LNCaP, PC3, PC3-AR2, DU145) and prostate stromal cells. Flow cytometry and Western Blotting techniques were used to assess potential mechanisms of interaction.

Results: Capsaicin exhibited anti-proliferative and pro-apoptotic effects, which were significantly enhanced with the addition of lycopene in PCa cell lines. The greatest effects were observed in androgen-sensitive cell lines. Detailed mechanistic studies revealed this combination may be interrupting the androgen-signaling pathways; independent of TRP-V1 signaling. Cell cycle analysis revealed that capsaicin and lycopene induce a cell cycle arrest, corresponding to alterations in tumour-suppressor proteins. Treating LNCaP cells with capsaicin and lycopene also altered proteins involved the apoptosis signaling pathway including, cleaved PARP, Caspase-3 and Bax/Bcl-xL expression.

Conclusions: Our results suggest that lycopene enhances the anti-cancer properties of capsaicin. Understanding micronutrients interactions may help improve current chemoprevention strategies.

3.2 Introduction

Prostate cancer remains the most commonly diagnosed cancer in men living in the Western world. There is increasing evidence that dietary factors play a role in the development and progression of prostate cancer. It is estimated that at least 30% of all prostate cancer patients use complementary and alternative medicine, which includes the consumption of micronutrient supplements (Bishop, Rea et al. 2011). Many dietary agents have been studied for the protective effects on prostate cancer (Venkateswaran and Klotz 2010, Venier, Klotz et al. 2012). Although a clear understanding of how these micronutrients interact with each other is largely unknown, many micronutrient supplements often contain a variety of dietary agents. Hence, studies examining the interaction of these dietary agents are essential for more effective chemopreventive regimens.

Lycopene, a potent antioxidant and carotenoid found in high concentrations in tomatoes, is one of the most extensively studied micronutrients for the chemoprevention of prostate cancer (Hantz, Young et al. 2005, van Breemen and Pajkovic 2008). Despite numerous studies, the protective effect of lycopene, as a single agent, on prostate cancer remains unclear (Ablin 2005). In 2004, Hantz et al. reported that lycopene induced apoptosis in androgen-sensitive prostate cancer cells, mediated through the disruption of the mitochondrial membrane and the generation of reactive oxygen species (ROS) (Hantz, Young et al. 2005). Subsequent studies however have been unable to successfully replicate these findings (Ablin 2005). More recent studies suggest the protective effects of lycopene to be most apparent in combination studies. Recently, our group has found that combining lycopene with vitamin E and selenium resulted in significant inhibition of the development of prostate tumours in the *Lady* transgenic mouse model compared to vitamin E and selenium alone, demonstrating that lycopene was necessary for effective combination regimens (Venkateswaran, Klotz et al. 2009). Lycopene has also been found to enhance the effect of other therapies (Tang, Parmakhtiar et al. 2011).

Capsaicin has recently emerged as a potent anti-cancer agent, exhibiting anti-proliferative and pro-apoptotic properties in several different prostate cancer model systems (Díaz-Laviada 2010). It is widely consumed as a food additive throughout the world, particularly in areas with a low incidence of prostate cancer, such as South East Asia. The use of capsaicin *in vitro* has been

reported to induce apoptosis through the generation of ROS, dissipation of the mitochondrial inner transmembrane potential, and downstream activation of the caspase-3 cascade (Kim and Moon 2004, Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006, Sánchez, Malagarie-Cazenave et al. 2007, Zhang, Humphreys et al. 2008). It also demonstrates efficacy *in vivo* by suppressing the growth of prostatic tumour xenografts in nude mice (Mori, Lehmann et al. 2006). A recent case study reported by Jankovic et al. describes a 66-year-old patient with prostate cancer who experienced significant improvement in his PSA levels upon daily consumption of capsaicin (Jankovic, Loblaw et al. 2010). Cessation of the capsaicin was followed by a sharp increase in his PSA levels (challenge/re-challenge effect) and PSA trends suggest a dose-response relationship (Jankovic, Loblaw et al. 2010).

In our present study, we have investigated for the first time, whether lycopene enhances the effect of capsaicin using *in vitro* prostate cancer models. Through proliferation studies, flow cytometry and protein analysis techniques; we have found that the anti-proliferative and pro-apoptotic effects of capsaicin are greater when it is combined with capsaicin. Our *in vitro* analysis suggests these anti-proliferative effects may be mediated through androgen-signalling pathways, independent of the capsaicin receptor, TRP-V1.

3.3 Materials and Methods

Cell Culture

Three human prostate cancer cell lines (DU145, LNCaP and PC3) and the prostate stromal cell line (PRSC) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). PC3-AR2 cells (generous gift from Dr Ted Brown, Mount Sinai Hospital, Toronto, ON, Canada) are PC3 cells transfected with a full-length functional androgen receptor (AR). Cells were cultured at 37ºC in a 5% CO_2 incubator in the following media: LNCaP cells, RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin (Invitrogen, Burlington, ON, Canada); PC3 and DU145 cells, Dulbecco's minimal essential medium/F12 (Invitrogen, Burlington, ON, Canada) with 10% FBS supplemented with

0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin; PC3-AR2 cells, RPMI 1640 medium supplemented with 5% FBS, 0.3mg/ml l-glutamine, 100IU/ml penicillin and 100µg/ml streptomycin, Fungizone (250 µg/ml amphotericin B and 250 µg/ml deoxycholate, Invitrogen, Burlington, ON, Canada) and 100 µg/ml hygromycin B (Invitrogen, Burlington, ON, Canada). PRSC were maintained in RPMI 1640 media as per LNCaP cells. All cells were grown under sterile conditions.

Chemicals

Capsaicin (*trans-8-methyl-N-vanillyl-6-nonenamide*) was obtained from Tocris (Bristol, UK) and Capsazepine (CZP) was obtained from Santa Cruz Biotechnology (CA, USA). Both capsaicin and CZP were dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to create a stock concentration of 0.1M and stored at -20°C. Lycopene was obtained from Sigma (USA) and was dissolved in tetrahydrofuran (THF) to create a 0.01M stock solution. Lycopene was stored in a dark area at -80°C. Stock solutions of capsaicin (10-200µM), capsazepine (10µM) and lycopene (10µM) were diluted in appropriate medium and prepared fresh daily prior to treatment. All compounds were prepared and stored with minimal exposure to light to avoid oxidation. All other chemicals were purchased from Sigma unless otherwise specified.

Cell Proliferation Assay

Proliferation was assessed using the CyQuant MTS cell proliferation assay (Molecular Probes, OR, USA). Cells were plated in 96-well micro-titre plates, at a density of 500 (PRSC) or 5000 (LNCaP, PC3, PC3-AR2, DU145) cells per well. Cells were left to adhere for 24 hours and then treated accordingly. To determine the optimum concentration of capsaicin for treating prostate cancer cells, standardization were carried out using a range of doses of capsaicin from 0µM – 200µM on LNCaP and PC3 cells.

After establishing capsaicin treatment concentrations, a panel of micronutrients were screened for proliferative differences alone and in combination with capsaicin in both LNCaP and PC3

cells. Micronutrients were selected based on previous literature (Venkateswaran, Fleshner et al. 2004, Venkateswaran, Fleshner et al. 2004, Venkateswaran, Klotz et al. 2009).

Vitamin E (alpha-tocopherol succinate) was reconstituted in 95% ethanol (Venkateswaran, Fleshner et al. 2004), selenium (seleno-DL-methionine) was dissolved in 0.005N HCl, and lycopene was dissolved in tetrahydrofuran (THF) to create a 0.01M stock solution based on previous studies (Venkateswaran, Fleshner et al. 2004). Cells were treated with the reported IC50 concentrations each micronutrient (20µg/ml of Vitamin E, 150 µM of selenium, and 10 µM of lycopene) alone or in combination with capsaicin (100µM) (Venkateswaran, Fleshner et al. 2004). All compounds were prepared immediately before applying treatment to avoid exposure to light.

Based on micronutrient screening studies, the most effective combination at reducing proliferation was capsaicin and lycopene, in combination. Hence, further proliferative studies were carried out in LNCaP, PC3, PC3-AR2, DU145, and prostate stromal cells (PRSC). Combination studies were carried out using 100µM concentration of capsaicin, and 10µM of lycopene.

Antagonist studies using CZP and/or capsaicin, cells were treated with a range of doses of capsaicin (0-100 µM) with or without CZP (10 µM); treatments were applied at the same time. All experiments were carried out in triplicates and repeated a minimum of three times. Control wells were treated with vehicle alone (DMSO – 0.02% and THF – 0.01%). Cells were then incubated for 24 hours post treatment. At the end of the treatment period, 20µL of tetrazolium dye was added to each well and cells incubated for 2 hours. Absorbance was measured using a 96-well plate reader at 490nm.

Western Blot Analyses

LNCaP, PC3, or PC3AR2 cells were plated in petri dishes at a density of $1x10^6$ cells per plate. After 24 hours, adherent cells were incubated with lycopene (10µM) and/or capsaicin (100µM). Control wells were treated with vehicle alone (DMSO – 0.02% and THF – 0.01%). After 24 hours cells were lysed (NP-40 lysis buffer with inhibitors (leupeptin/pepstatin, aprotinin and

phenylmethanesulfonylflouride), sodium dodecyl sulfate (SDS), deoxychalate and ethylenediaminetetraacetic acid). In our time-point analysis, LNCaP cells were lysed at 3, 6, 12, 24, and 48 hours. Protein was quantified using the Bradford protein assay technique prior to loading into 12% SDS gels for electrophoresis. Following protein transfer, membranes were probed to assess; i) androgen receptor expression levels (AR, Santa Cruz), ii) prostate specific antigen (PSA, Santa Cruz), iii) markers of apoptosis (BAX, Bcl-2 and caspase-3 from Cell Signaling), proliferation (PCNA, Santa Cruz) and cell cycle regulation (p21 and p27 from Cell Signaling and p53 from Santa Cruz). Image quantification software (ImageJ, US National Institute of Health, Bethesda, MD, USA) was used to semi-quantitatively determine protein expression levels, relative to β-actin.

Flow Cytometry

Cell cycle arrest pattern and alterations in the percentage of cells in the S phase enumeration were determined in LNCaP cells by flow cytometry on cells labelled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and counterstained with propidium iodide (PI). Asynchronously growing cells (5 X 10^5 cells/plate) were plated in 10cm petri dishes and left to adhere for 24 hours. Subsequently, cells were treated for 24 hours with lycopene (10 μM) and/or capsaicin (0.1μM -100μM). Control plates were treated with vehicle alone (DMSO – 0.02% and THF – 0.01%). Cells were pulse labeled with BrdU for 2 hours, washed with PBS (Invitrogen), trypsinized, fixed in ice cold 70% ethanol and stored at -20˚C until further analysis. Cells were washed in buffer (PBS, Invitrogen, (Burlington, ON, Canada) and 0.5% Tween-20) and treated with 2N HCl for 20 mins to expose labeled DNA and neutralized with $Na_2B_4O_7$ (pH 8.5) for 2 minutes. Cells were incubated for 1hr on ice with anti-BrdU conjugated FITC (DAKO, Burlington, ON, Canada). Cells were washed, centrifuged, and resuspended in 10μg/ml PI, and allowed to incubate for 30 min on ice. Samples were filtered through a nylon mesh and cell cycle analysis performed with the FACSCalibur flow cytometer using the Cell Quest Pro software package (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data are presented as the mean ± S.D. of three experiments or more. Statistical comparisons among groups were made with a Student's t test or the one-way analysis of variance (ANOVA) test. The level of significance tests was set at $p<0.05$ or $p<0.001$, marked as * or ** respectively.

3.4 Results

3.4.1 Differential growth inhibitory effect of capsaicin on prostate cell lines

Using the MTS Cell Proliferation Assay, we assessed the effect of capsaicin on the growth of several prostate cancer cell lines and a non-malignant prostate stromal cell line. Results reveal that treatment of capsaicin at a concentration of 0-200 μM inhibited the growth of LNCaP, PC3 and PC3AR2 cells in a dose-dependent manner. The androgen-insensitive cell line, DU145, was unaffected by capsaicin at any of the concentrations. The non-malignant prostate stromal cells (PRSC) had a reduction in proliferation when treated with capsaicin at 100μM concentration; however, this inhibition was not dose-dependent. Lycopene (0-50 μM) did not reduce the proliferation of any cell line, except for LNCaP cells (as depicted in Figure 11). Based on these findings, a 10μM concentration of lycopene, (minimal dose at which had an affect), and a 100μM concentration of capsaicin, were used for subsequent combination studies.

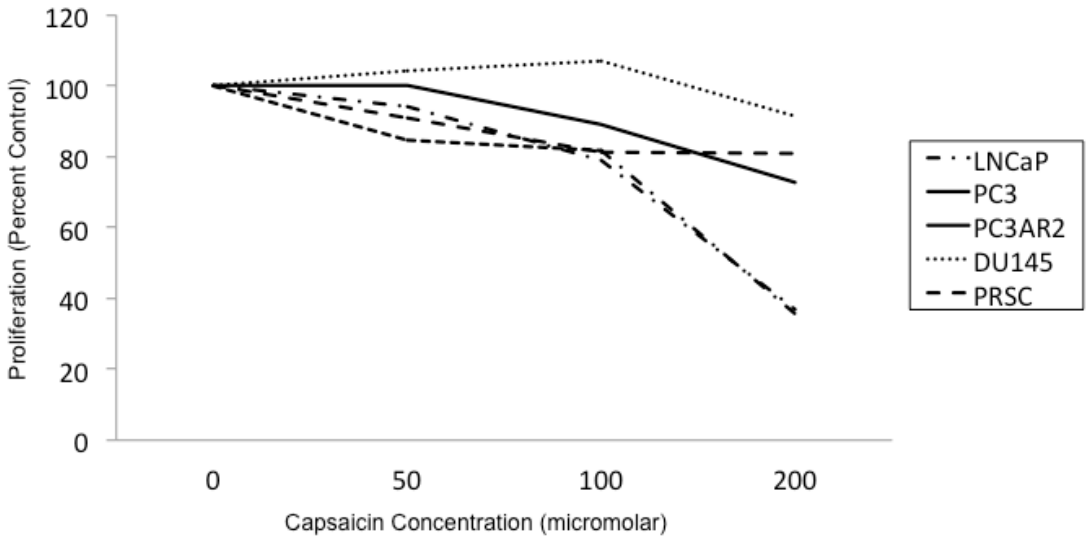


Figure 11: Differential growth inhibitory effect of capsaicin on prostate cell lines. *The above line graph demonstrates the proliferative changes in androgen-sensitive prostate cancer cell lines (LNCaP and PC3-AR2), androgen-resistant prostate cancer cell lines (PC3 and DU145), as well as prostate stromal cell line (PRSC).*

3.4.2 Combinatorial Studies suggest that capsaicin and lycopene are most effective at reducing proliferation in prostate cancer cells.

Results from the MTS Cell Proliferation Assay suggest that LNCaP cells treated with capsaicin, Vitamin E, and selenium treatment alone reduces the proliferation of LNCaP cells; and combining capsaicin with vitamin E, selenium, and or/lycopene significantly reduces the proliferation of LNCaP cells (Figure 12). The greatest reduction was observed in cells treated with capsaicin and lycopene. We also observed morphological changes that are depicted in the Figure 13.

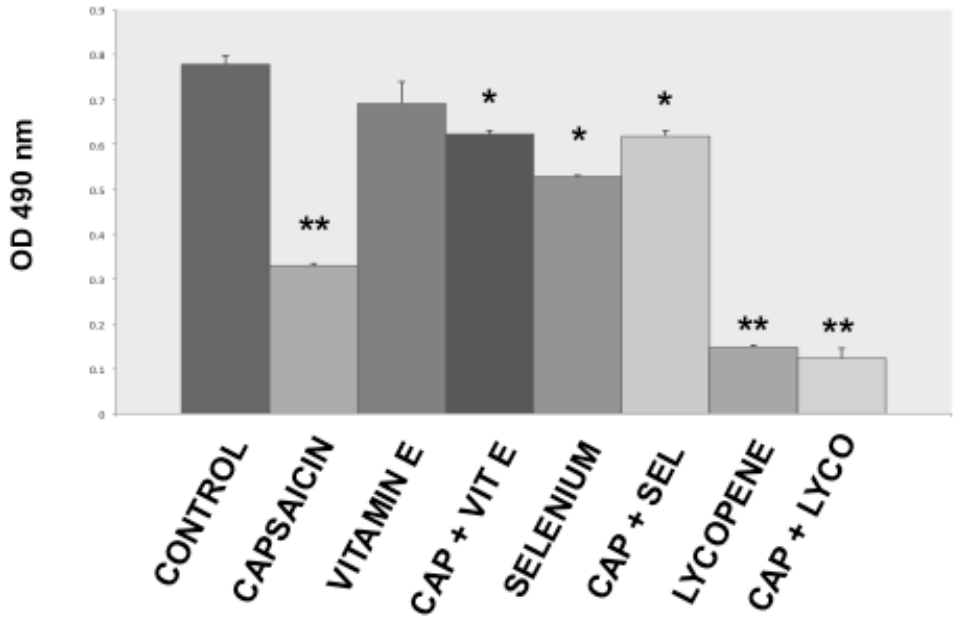


Figure 12: Proliferative Studies on LNCaP Cells Treated with Vitamin E, Selenium, and Lycopene with or without capsaicin. *The above histogram highlights alterations in proliferation in androgen-sensitive LNCaP cells treated with Vitamin E (20µg/mL), selenium (150µM) and lycopene (10µM), alone and in combination with capsaicin (100µM). Differences in optimal density (OD) that reach significance $p<0.05$ is denoted with an asterisk (*), and $p<0.01$ is denoted with double asterisk (**) relative to control.*

Figure 13: Morphological representation of LNCaP Cells Treated with capsaicin (100µM) for 24 hours.

Similar experiments were carried out on PC3 cells shown in Figure 14. Results demonstrated a significant reduction in the proliferation with capsaicin or lycopene alone, as well as the combination of capsaicin and selenium, capsaicin and vitamin E, and capsaicin and lycopene. Here again, capsaicin and lycopene had the greatest reduction.

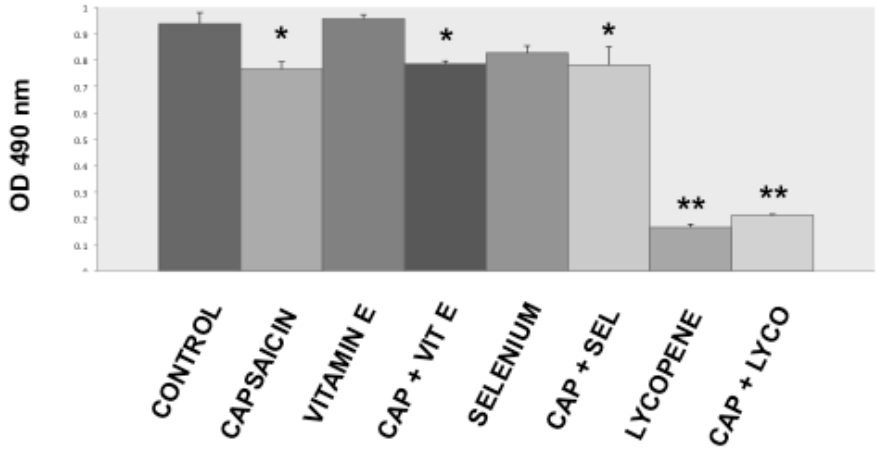


Figure 14: Proliferative Studies on PC3 Cells Treated with Vitamin E, Selenium, and Lycopene with or without capsaicin. *The above histogram highlights alterations in proliferation in androgen-resistant PC3 cells treated with Vitamin E (20µg/mL), selenium (150µM) and lycopene (10µM), alone and in combination with capsaicin (100µM). Differences in optimal density (OD) that reach significance $p<0.05$ is denoted with an asterisk (*), and $p<0.01$ is denoted with double asterisk (**) relative to control.*

3.4.3 Combinatorial Studies suggest that the combination capsaicin and lycopene is most effective at altering proteins involved in prostate cancer growth, proliferation and apoptosis

Western Blot studies examining the mechanism of effect of capsaicin with other micronutrients were performed to assess which treatment was most effective at reducing proliferation and inducing apoptosis. We first examined the tumour suppressor protein, $p27^{Kip1}$, which also acts to

inhibit the cell cycle. Here I found that vitamin E, capsaicin and vitamin E, as well as the combination of capsaicin and lycopene increased the expression of $p27^{kip1}$.

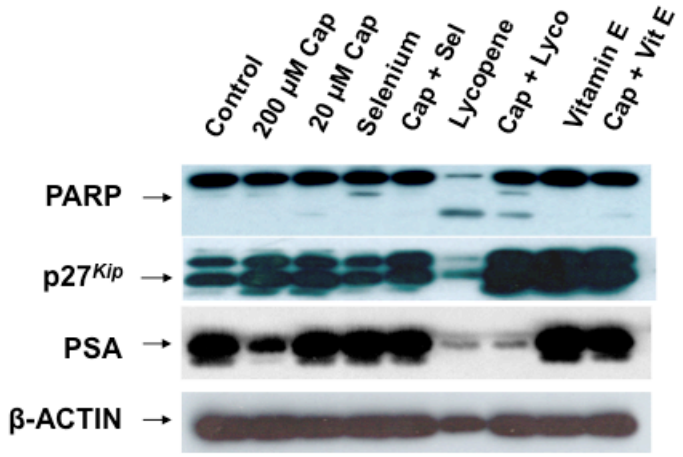


Figure 15: Western Blot Analysis examining Cell Cycle Marker, $p27^{Kip1}$, Apoptosis Marker, PARP (and cleaved PARP), and Prostate Specific Antigen (PSA) in LNCaP Cells treated with various micronutrients including capsaicin (Cap), Selenium (Sel), Lycopene (Lyco), and Vitamin E (Vit E) alone and in combination.

To understand whether these micronutrients could induce apoptosis, we looked at the apoptotic marker PARP (see Figure 15). In general, the cleavage of PARP is indicative of the final stage of apoptosis prior to DNA fragmentation. In our studies, we have found that when LNCaP cells are treated with capsaicin and lycopene they express cleaved PARP, suggesting early stages of apoptosis.

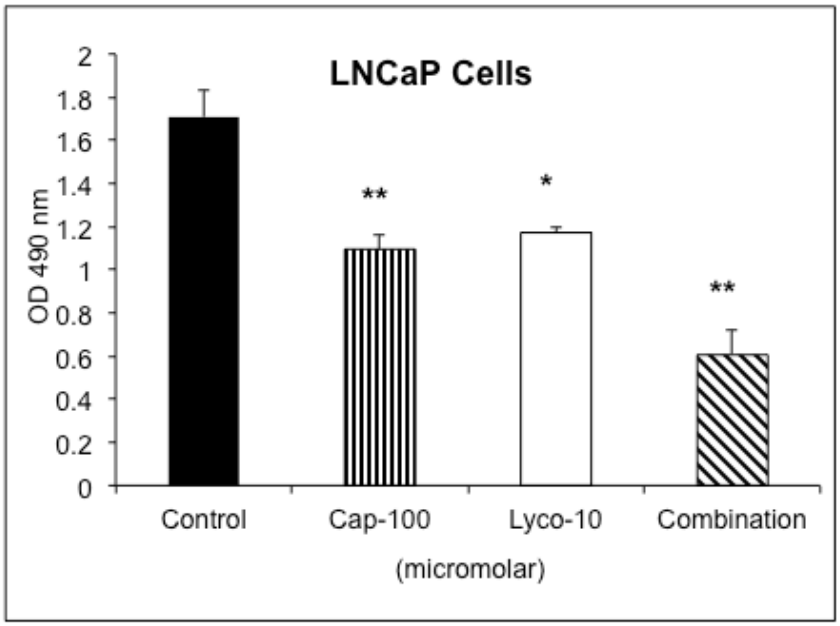
We also examined the expression of prostate specific antigen (PSA) to understand whether micronutrients can alter the androgen-signaling pathway. We found that capsaicin and lycopene reduced the expression of PSA.

Since we observed such predominate effects with capsaicin and lycopene, we went on to carry out further detailed experiments with this combination.

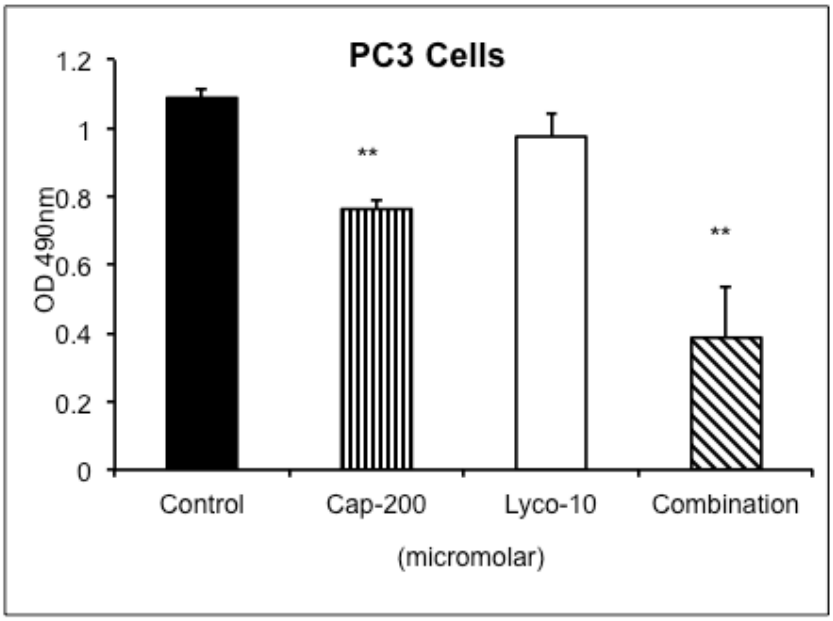
3.4.4 Treating cells with a combination of capsaicin and lycopene reduces cell proliferation

To determine whether the anti-proliferative effect of capsaicin could be enhanced by lycopene, we treated cells with capsaicin (100µM) along with a low-dose lycopene (10µM). Lycopene used in monotherapy significantly reduced cell growth only in LNCaP cells ($p<0.05$). Interestingly, treating cells with a combination of capsaicin and lycopene significantly reduced the proliferation of all prostate cancer cell lines (LNCaP, PC3, PC3AR2 ($p<0.001$; DU-145, $p<0.05$) as depicted in Figure 16. The combination of capsaicin and lycopene reduced the proliferation of PRSC; no significance was observed with either agent alone (Figure 16d)

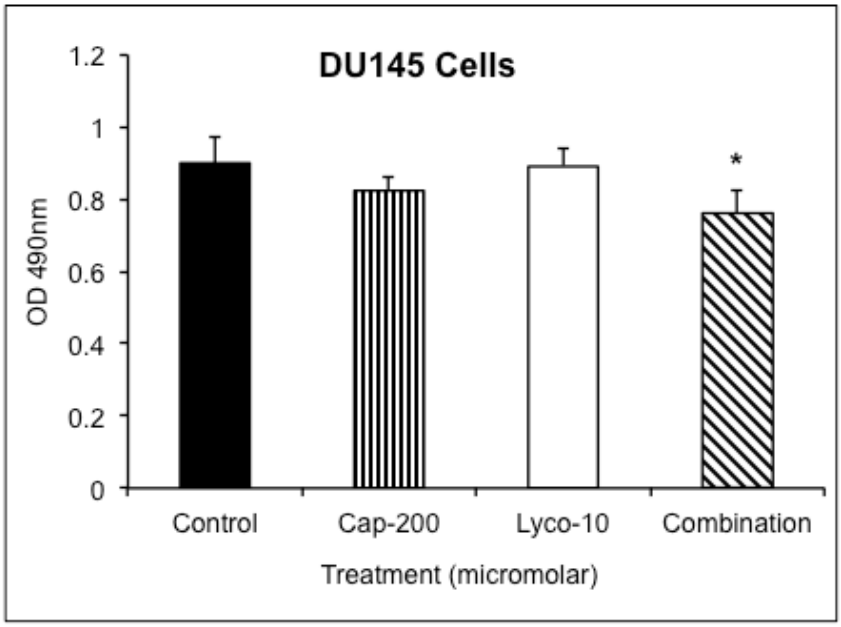
a)



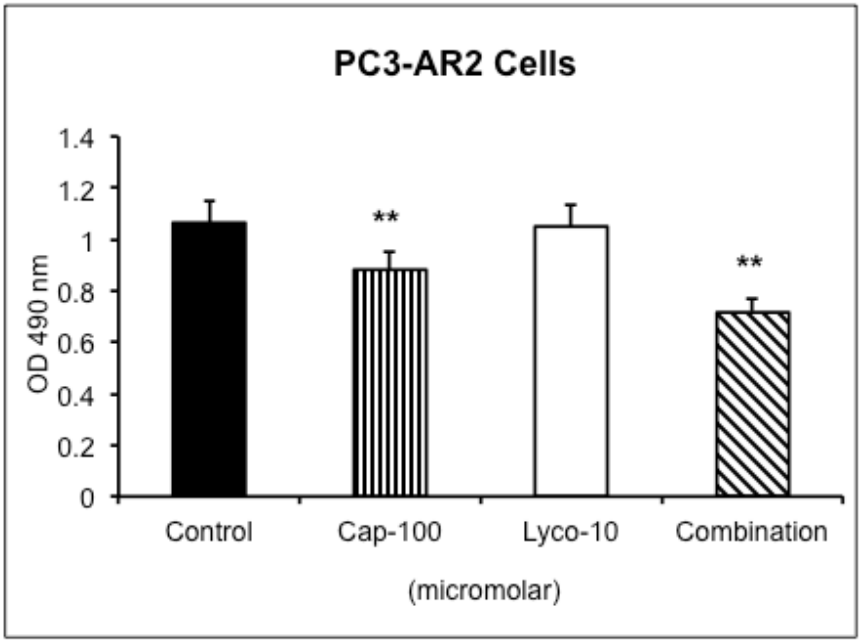
b)


PC3 Cells
1.2
1
0.8
0.6
0.4
0.2
0
OD 490nm
**
**
Control
Cap-200
Lyco-10
Combination
(micromolar)

c)


DU145 Cells
1.2
1
0.8
0.6
0.4
0.2
0
OD 490nm
*
Control
Cap-200
Lyco-10
Combination
Treatment (micromolar)

d)



e)

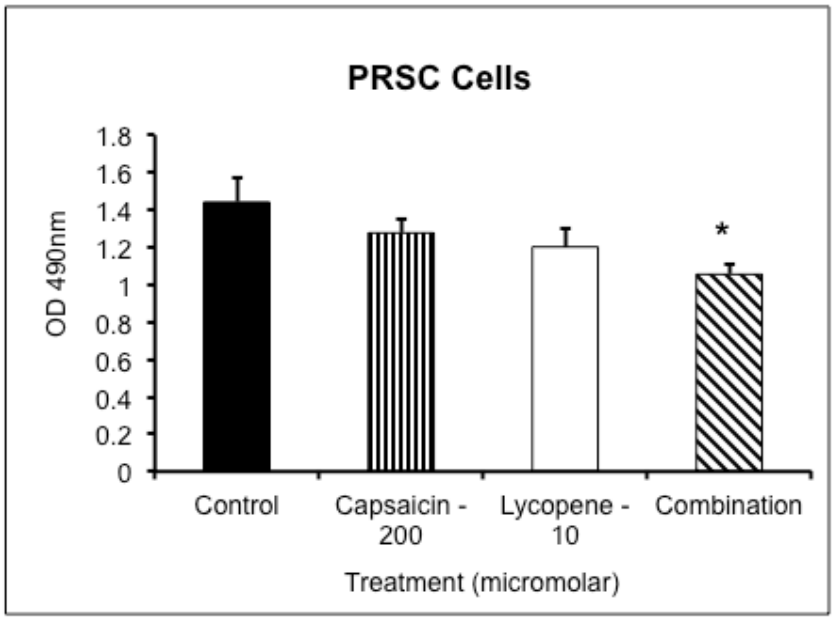


Figure 16: Effect of capsaicin (CAP) and/or lycopene (Lyco) on proliferation of LNCaP (a), PC3 (b), DU145 (c), PC3-AR2 (d), Prostate Cancer Stromal (PCSC) (e) cell lines. *Differences in optimal density (OD) that reach significance $p<0.05$ is denoted with an asterisk (*), and $p<0.01$ is denoted with double asterisk (**) relative to control.*

3.4.5 Capsaicin and lycopene in combination alters the percentage of cells in the S-phase of the cell cycle

To determine the minimal concentration of capsaicin and lycopene that resulted in a significant reduction in the percentage of cells in the S-phase of the cell cycle, flow cytometric analysis was performed on LNCaP cells with a low dose of lycopene (10μM) and increasing concentrations of capsaicin (0-100μM). Lycopene treatment alone was not effective at alternating the percentage of cells in the S-phase. Low concentrations of capsaicin (0-10μM) did not reduce the percentage of cells in the S-phase of the cell cycle. At a concentration of 50μM, the combination of capsaicin and lycopene was effective at reducing the percentage of cells in the S-phase at a 10μM concentration of lycopene, greater than either agent alone. Similar effects were seen at 100μM concentration of capsaicin. Concentrations of capsaicin greater than 100μM resulted in a significant reduction ($p<0.05$) in the percentage of cells in the S-phase compared to control. At these concentrations, there were no significant differences between capsaicin alone or in combination with lycopene (see Figure 17).

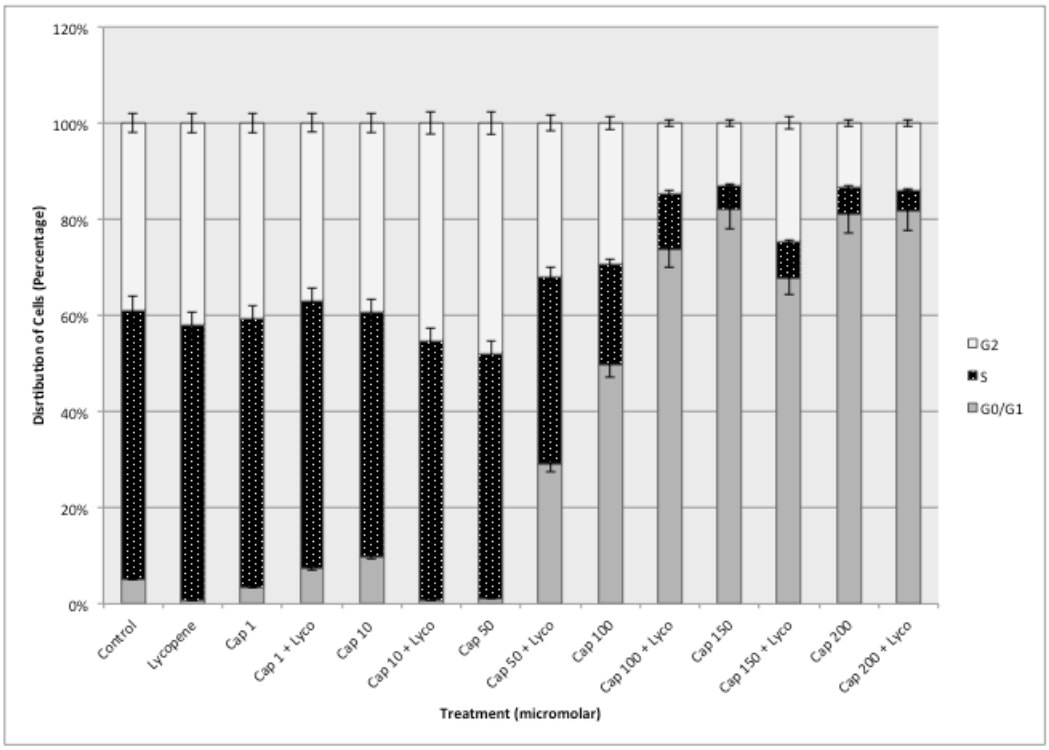


Figure 17: Alterations in the cell cycle distribution (G1, S, G2/M) in LNCaP cells treated with capsaicin and lycopene. *Cells treated with capsaicin demonstrated a dose-dependent reduction in the percentage of cells in the S-phase.*

3.4.6 Treatment with capsaicin and lycopene reduce Androgen Receptor (AR) and Prostate Specific Antigen (PSA) expression in LNCaP Cells

To better understand the effect of capsaicin and/or lycopene on androgen-sensitive cells, we examined the expression levels of AR and PSA. Capsaicin treatment alone caused a marked reduction in AR expression in LNCaP cells. Treatment of LNCaP cells with lycopene monotherapy did not affect AR or PSA expression (see Figure 18). Interestingly, treating cells with a combination of capsaicin and lycopene resulted in a reduced expression of PSA and AR, greater than either individual treatment.

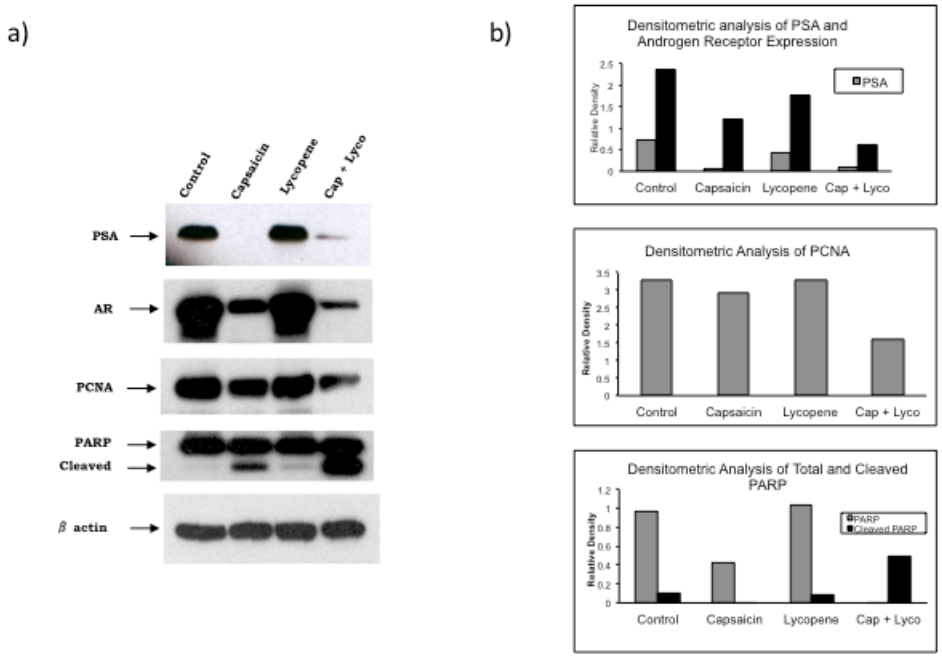


Figure 18: Western Blot analyses demonstrates the changes in the expression of a) Prostate Specific Antigen (PSA), Androgen Receptor (AR), Proliferating Cell Nuclear Antigen (PCNA), and apoptotic indicator, Cleaved PARP in LNCaP cells treated with capsaicin (100µM) and/or lycopene (10µM); b) Corresponding densitometric analysis.

3.4.7 A combination of capsaicin and lycopene promote apoptosis in androgen-sensitive cells

Based on my cell cycle profiles obtained by flow cytometry, we examined proteins implicated in the apoptotic pathway using Western Blot analysis. Lycopene treatment alone did not significantly alter the apoptotic pathway. We found a time-dependent increase in the expression of cleaved PARP (Poly ADP ribose polymerase) to a greater extent than either agent alone, maximal at 24 hours (see Figure 19). At the 24 hour time point, we found an increase of the BAX:BCL-xL ratio and reduction in total Caspase 3 in androgen-sensitive cell lines, LNCaP and

PC3AR2; this effect was not found in the androgen-resistant, PC3 cell line. These findings suggest that lycopene is enhancing the pro-apoptotic effect of capsaicin (see Figure 20).

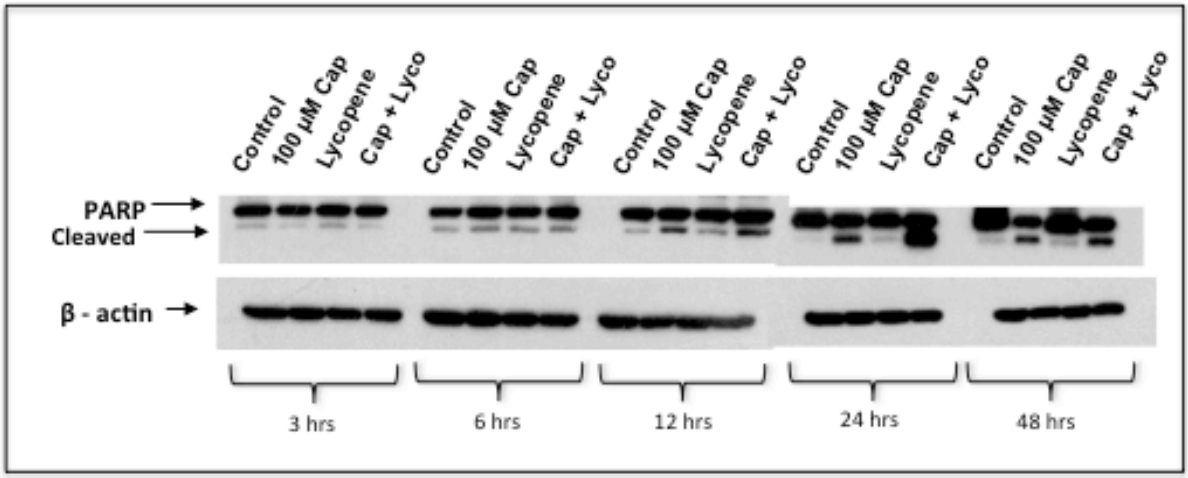


Figure 19: Western blot analysis reveals that a combination of capsaicin and lycopene induces the expression of cleaved PARP, in a time-dependent manner.

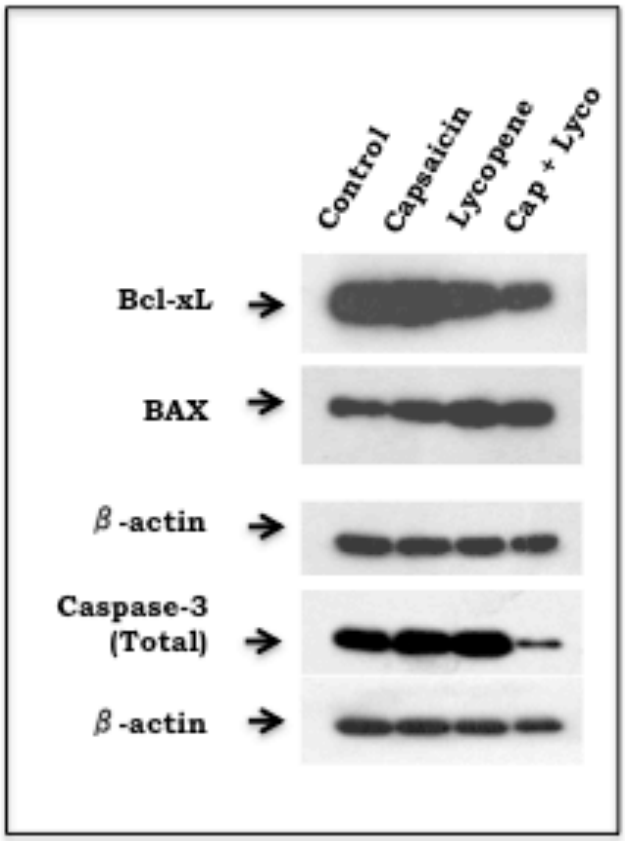


Figure 20: Capsaicin treatment alters the Bax:Bcl-xL ratio and reduces total caspase-3 in LNCaP cells after 24 hours.

3.4.8 The TRP-V1 antagonist, capsazepine (CZP), does not inhibit the anti-proliferative effects of capsaicin

Using the TRP-V1 antagonist, CZP, we assessed whether the anti-proliferative effects of capsaicin could be reversed. We observed no significant changes in proliferation with CZP and capsaicin, indicating that capsaicin's effects were not mediated through the TRP-V1 receptor suggesting an alternative mechanism for proliferation (as depicted in Figure 21).

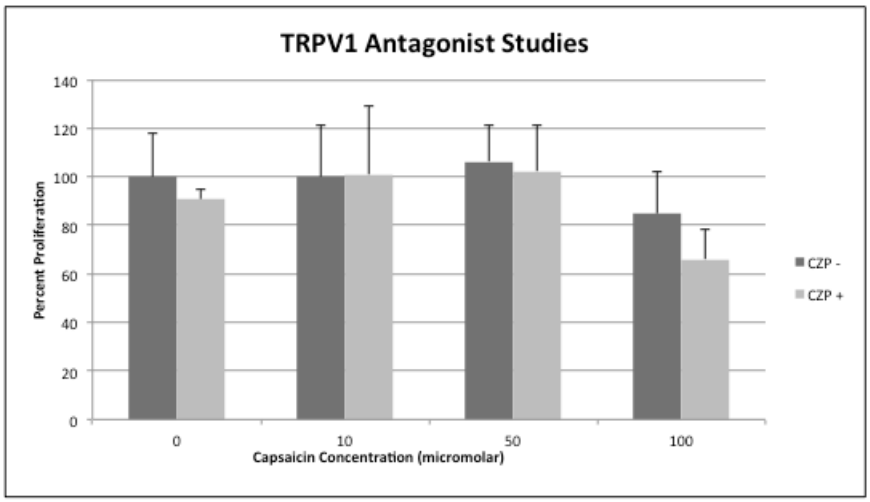


Figure 21: TRP-V1 is not mediating the anti-proliferative effect of capsaicin in the LNCaP cell line.

3.5 Discussion

In this study, we report for the first time that lycopene, a micronutrient well studied for prostate cancer prevention, enhances the anti-proliferative and pro-apoptotic effects of capsaicin in prostate cancer cells. Previous *in vivo* reports published from my group have shown that lycopene, in combination with antioxidants, can significantly reduce the incidence and progression of prostate tumours in the *Lady* transgenic mouse model (Venkateswaran, Klotz et al. 2009). Lycopene has also been described as a necessary component of combinatorial studies. Recently, Tang et al. has reported the chemosensitizing effect of lycopene in combination with other chemotherapeutic agents, including docetaxel (Tang, Parmakhtiar et al. 2011).

To understand which micronutrient combination was most effective at reducing proliferation, a panel of micronutrients were screened for proliferative differences alone and in combination with capsaicin in LNCaP and PC3 cells. Micronutrients were selected based on previous literature supporting their chemopreventive effects on the prostate cancer (Venkateswaran, Fleshner et al. 2004, Venkateswaran, Fleshner et al. 2004, Venkateswaran, Klotz et al. 2009). Results from these preliminary screening analyses using the MTS proliferation assay and Western blotting suggested that lycopene demonstrated potent anti-cancer effects compared to vitamin E and selenium. Based on these results, further analysis on lycopene and capsaicin was undertaken.

There is a lack of consensus in the literature about the anti-proliferative effects of lycopene as a PCa preventive single agent that has yet to be resolved. A few studies report that lycopene reduces cell proliferation *in vitro*; however, many have been unable to successfully translate these findings in related preclinical/clinical model systems (Ablin 2005, van Breemen and Pajkovic 2008). In this present study, we have demonstrated that lycopene, as a single agent, does not have the potential to reduce the proliferation in several prostate cancer cell lines, except in the androgen-sensitive LNCaP cells. This differential effect of lycopene on androgen-sensitive cell lines has been previously described (Ivanov, Cowell et al. 2007). Cell cycle distribution analysis on LNCaP cells revealed that treatment with lycopene resulted in a non-significant reduction of the percentage of cells in the S-phase. However, when combined with capsaicin, this reduction in the percentage of cells in the S-phase was significantly greater than either monotherapy, indicating that cell-cycle arrest induced by lycopene may enhance the anti-proliferative effects induced by capsaicin. Alterations in protein expression, particularly a reduction in PCNA and the up-regulation of the tumour suppressor proteins, $p21^{cip1}$ and $p27^{kip1}$ support these findings, suggesting that the inhibition of the cell cycle is one possible way in which lycopene is making cells more susceptible to the inhibitory effects of capsaicin. The reduction of $p21^{cip1}$ and $p27^{kip1}$ by the combination of capsaicin and lycopene suggests that alternative anti-proliferative mechanisms and/or apoptotic mechanisms may play a role in understanding how these micronutrients are acting in combination (Geng, Rademacher et al. 2010).

Androgen signalling plays a key role in prostate cancer growth and development (Suzuki, Ueda et al. 2003). Some of most promising chemopreventive agents for prostate cancer include the 5-alpha reductase inhibitors, which block the conversion of testosterone to the more potent dihydrotestosterone (DHT), thereby inhibiting prostate cancer growth (Kramer, Hagerty et al. 2009). The inhibition or activation of the AR causes alterations in the growth and proliferation of prostate cancer cells (Lee, Sutkowski et al. 1995). Our findings suggest that capsaicin and lycopene may reduce proliferation through the inhibition of androgen signalling. The effect of capsaicin on androgen signalling has been previously reported (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006). Studies carried out by Mori et al. have found that capsaicin can cause a significant down-regulation in the expression of androgen-receptor and PSA in androgen-sensitive cells (Mori, Lehmann et al. 2006). In this study, we have successfully replicated these findings and demonstrated that androgen-sensitive cell lines have an enhanced sensitivity to the anti-cancer effects of capsaicin in combination with lycopene.

We have compared both androgen-sensitive and –insensitive cell lines and determined androgen-sensitive cells are most responsive to the anti-proliferative effects of capsaicin and lycopene. In particular, we have compared the PC3 and PC3-AR2 cell lines. We demonstrated that the incorporation of a functional AR to the PC3 cell line causes cells to have an increased sensitivity to the anti-proliferative effects of capsaicin and lycopene. This sensitivity is comparable to the LNCaP cell line, suggesting that a functional AR is an important determinant for inhibition of prostate cancer cell proliferation through the use of capsaicin and/or lycopene. Secondly, we have demonstrated that capsaicin and lycopene can reduce protein expression levels of AR and PSA. We have observed that treatment with a combination of both lycopene and capsaicin reduces the expression of AR, this effect being significantly greater than with either agent alone, indicating that these compounds may be acting through the androgen-signalling pathway. It is likely that the inhibition of PSA is mediated at least partly through the down-regulation of the AR. Whether the down-regulation of PSA is enhanced by lycopene is not clear in our studies. Although the combination of capsaicin and lycopene enhances the down-regulation of AR, it does not further reduce PSA, suggesting that PSA expression may be mediated in an androgen-independent manner.

The role of the transient receptor vanilloid-1 receptor (TRP-V1), commonly referred to as the capsaicin receptor, in prostate cancer chemoprevention has not been clearly elucidated. In our

studies, we have found that the capsaicin antagonist, capsazepine, does not reverse the anti-proliferative effects of capsaicin. Furthermore, there is no significant difference in the expression of this receptor, suggesting that the effects of capsaicin are not TRP-V1 dependent.

Accumulated data clearly indicate that the induction of apoptosis is an important event for the chemoprevention of prostate cancer by naturally occurring dietary agents (Khan, Adhami et al. 2010). Capsaicin has been reported to induce apoptosis through a number of pathways in prostate cancer cells (Kim and Moon 2004, Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006, Zhang, Humphreys et al. 2008). In our present studies, we have found that the apoptotic and anti-proliferative effects of capsaicin are enhanced by treatment with lycopene (See Figure 22). Several studies have found that capsaicin alone can up-regulate the generation of ROS and induce apoptosis. We demonstrate that a combination of capsaicin and lycopene induces apoptosis through alterations in the Bax:Bcl-xl ratio, with activation of the caspase-3 cascade in androgen sensitive cell lines, but not the androgen-insensitive cell lines. It is likely that these effects are downstream of ROS generation and mitochondrial destabilization, which have been previously reported in studies using capsaicin and lycopene independently *in vitro* (Hantz, Young et al. 2005, Zhang, Humphreys et al. 2008).

The dose of capsaicin and lycopene used in our studies were selected on the basis of our dose-standardization studies and previously reported studies (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006). These concentrations do not reflect the physiological concentration of capsaicin, a major caveat of this study. There is a lack of data detailing the physiological serum concentration of capsaicin and amount of capsaicin in the prostate tissue; however, whether these concentrations are physiologically relevant using pre-clinical models is currently being investigated. Based on the *in vitro* findings, the effect of capsaicin should be examined in an androgen-sensitive xenograft/transgenic model. Previous studies have found that capsaicin alone can reduce the growth of androgen-resistant, PC-3 tumours. Replicating these findings using an androgen-sensitive model (i.e. LNCaP) with the administration of a combination of capsaicin and lycopene will provide a better understanding of this potential novel therapeutic strategy against AR-dependent prostate cancer.

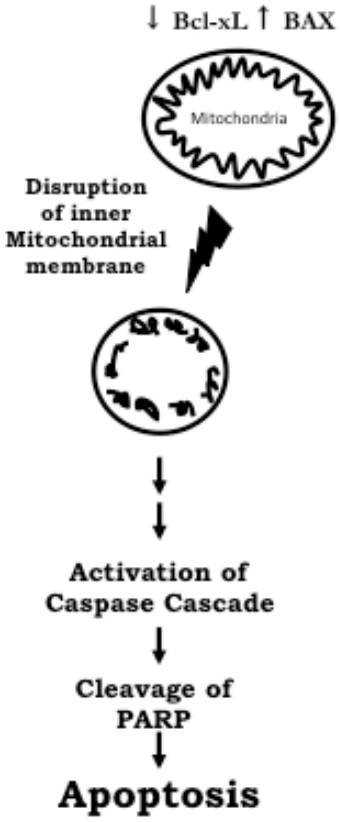


Figure 22: Hypothesized mechanism for the induction of apoptosis by capsaicin and lycopene in androgen-sensitive cells.

3.6 Conclusions

In summary, combination studies using dietary agents are promising new approaches to developing effective chemoprevention strategies. Decoding the exact mechanism of action of each agent alone and in combination is the key to a better understanding of the effect of micronutrients on prostate cancer. In this study, we have described for the first time the chemopreventive interaction of capsaicin and lycopene, two promising dietary agents. Through understanding the relationships of these dietary agents, We can postulate the anti-cancer effects of existing, widely consumed supplements and aid in the development of supplements that can prevent or even delay the progression of prostate cancer.

3.7 Caveats and Pitfalls

In Chapter 3, *Understanding the Anti-Cancer Effects of Capsaicin Alone, and in Combination with Other Micronutrients*, capsaicin demonstrates potent anti-cancer effects on prostate cancer cells, which were enhanced by the micronutrient lycopene. As with any experiment, there were a number of limitations to this study. There are a number of caveats and pitfalls associated with this project that are outlined in the following section. Future research investigating capsaicin and other micronutrients should take these limitations into account when designing experiments.

Unfortunately, the anti-proliferative studies with both capsaicin and lycopene were carried out using very high concentrations of each agent. Although this was beneficial to understand their anti-cancer effects of each agent, it was very difficult to assess the impact of each micronutrient, and whether these effects were additive or synergistic. Furthermore, using the high concentrations of each agent may have caused apoptosis to be the predominate mechanism and we may have been unable to accurately assess other mechanisms, such as the impact of TRP-V1.

In vitro studies were only carried out in prostate cancer and prostate stromal cells. Studies examining other types of malignant cell lines (e.g. MCF-7 breast cancer cells) would have been beneficial to understand the specificity of capsaicin.

The apoptotic effects of capsaicin and lycopene were nicely outlined. However, it would have been beneficial to include more detailed mechanistic studies such as alterations in calcium levels, changes in oxidative stress, or an apoptosis assay (ex. COMET assay or yH2AX). Furthermore, it was very interesting to find that AR-positive cells had a greater response than AR-negative cell lines. More emphasis on this pathway through assessing the effects of androgens (i.e. DHT) and anti-androgenic agents, with or without capsaicin, could strengthen the studies.

The role of the capsaicin receptor, TRP-V1, was not reported to be the predominate mechanism mediating the anti-cancer effects of capsaicin. This was demonstrated using the TRP-V1 antagonist, capsazepine. It is possible that capsazepine may act to antagonize a number of receptors, and other TRP-V1 antagonists/techniques (i.e. siRNA) could demonstrate more

specific results. Furthermore, comparative studies using TRP-V1 positive and negative cell lines (i.e. HEK 296) would have been informative to better understand this pathway.

Lastly, the half-life of capsaicin and lycopene in cell culture is not clear. Experiments analyzing the content, concentrations, and metabolites of both these agents in media would be beneficial for better understanding of what form and concentration of these agents is having the effect on prostate cancer cells, which could aid in the translation into *in vivo* models.

CHAPTER FOUR

Capsaicin Reduces the Metastatic Burden in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model.

4.1 Abstract

Capsaicin, the active compound in chilli peppers, has demonstrated anti-carcinogenic properties *in vitro* in a number of malignancies, including prostate cancer. In the present study, we investigate the chemopreventive potential of capsaicin on prostate cancer using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, a murine model that resembles the progression of human disease. Thirty-five 6-week-old TRAMP x C57Blk mice were randomized between treatment with capsaicin (5 mg/kg body weight) or control (saline) three times a week by oral gavage until 30 weeks of age. Mice in the control group expressed an overall trend of higher-grade disease with 37.5% poorly differentiated (PD), 18.75% moderately differentiated (MD), and 44% of well differentiated (WD) adenocarcinoma, compared to the capsaicin-treated group with only 27.7 % PD, 61.0% of WD, and 11.1 % of intraepithelial neoplasia (PIN). The capsaicin group demonstrated a higher incidence of noncancerous PIN lesions compared to the control group. The capsaicin group also demonstrated a significant reduction ($P<0.05$) in the metastatic burden compared to the controls, which correlated to a reduction in $p27^{Kip1}$ expression and neuroendocrine differentiation in prostate tumors. Furthermore, there were no differences in body weight between the groups overtime, and no pathological toxicities in the liver or gastrointestinal tract were detected with capsaicin consumption. *In vitro* studies revealed a dose-dependent reduction in the invasion and migration capacity of PC3 cells. In conclusion, this study provides evidence supporting the safety and chemopreventive effects of capsaicin in the TRAMP model.

4.2 Introduction

Prostate cancer is the most common internal malignancy in North American men. Over the course of their lifetime, it is estimated that one in 7 men will develop prostate cancer, and approximately one in 36 men in the United States will die from the disease (American Cancer Society 2014). Most prostate cancer is localized and confined to the prostate and will remain low-risk, however a subset of prostate cancer cases will progress into aggressive disease causing significant morbidity and mortality. Hence, strategies to reduce the development and progression of high-risk disease are essential to improve the outcome of men with prostate cancer.

Over the years a number of botanical and dietary agents have been studied for their chemopreventive effects on prostate cancer (Palmer, Venkateswaran et al. 2008, Venier, Klotz et al. 2012, Zuniga, Clinton et al. 2013). Capsaicin, the active compound in chilli peppers responsible for their characteristic pungency upon ingestion, has more recently demonstrated a plethora of anti-cancer effects in pre-clinical studies of various malignancies, including prostate cancer (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006, Wu, Starzinski-Powitz et al. 2008, Zhang, Humphreys et al. 2008). *In vitro* studies have revealed that capsaicin reduces proliferation and induces apoptosis in a number of prostate cancer cell lines including PC3, DU145, and LNCaP (Mori, Lehmann et al. 2006). These effects are mediated through a number of mechanisms including cell cycle inhibition, with up-regulation of tumour suppressor proteins, such as $p21^{cip1}$ and $p27^{Kip1}$; inhibition of nuclear factor kappa B signalling pathway; and the inhibition of androgen signalling (Mori, Lehmann et al. 2006, Malagarie-Cazenave, Olea-Herrero et al. 2009, Venier, Colquhoun et al. 2012). Published studies from my laboratory and several other groups have reported a reduction in the expression of androgen receptor and prostate specific antigen (PSA), an androgen-regulated protein, in the LNCaP cell line following treatment with capsaicin (Venier, Colquhoun et al. 2012). The pro-apoptotic effect of capsaicin on cancer cells has been more consistently documented in the recent literature across various cancer sites, including the prostate (Mori, Lehmann et al. 2006, Zhang, Humphreys et al. 2008, Ip, Lan et al. 2012). The majority of these studies have reported that capsaicin acts predominately by inducing intrinsic apoptosis through the generation of reactive oxygen species (ROS), disruption of the mitochondrial membrane, alterations in bax to bcl-2 ratio, release of

cytochrome C, and activation of the caspase-cascade (Sánchez, Sánchez et al. 2006, Venier, Colquhoun et al. 2012).

Pre-clinical *in vivo* studies examining the role of oral capsaicin administration have been limited to xenograft studies using immune compromised animals. They reported that capsaicin can reduce tumour-growth rate and enhance existing therapies (i.e. radiation) through sensitizing tumours to undergo apoptosis (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006). Studying models with an intact immune system that more closely mimic human prostate cancer progression provides the opportunity to gain a better understanding of the chemopreventive properties of capsaicin.

Unfortunately, there are also very few clinical or epidemiological studies that examine the effect of orally ingested capsaicin on cancer progression. However, one promising case-study by Jankovic et al. describes a prostate cancer patient consuming 2.5 mL of habaneros chilli sauce, containing capsaicin (calculated to be 454 ug/mL), twice weekly experienced a stabilization in his prostate specific antigen (PSA) levels. This suggests that the therapeutic effects of capsaicin on prostate cancer can be achieved by oral consumption of capsaicin (Jankovic, Loblaw et al. 2010). The serum concentration of capsaicin in this patient was not reported in this case study. Nevertheless, more epidemiological and large-scale clinical trials are warranted to provide substantial evidence supporting the use of capsaicin as a chemoprevention agent.

In the present study, we have investigated the effect of long-term oral administration of capsaicin as a chemopreventive agent for prostate cancer using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model. The TRAMP model is a transgenic mouse model of prostate cancer first described by Gingrich et al. in 1996 (Gingrich, Barrios et al. 1996). It is characterized by a mutation in the Probasin-SV40 T-antigen (PB SV40-Tag) that results in the development of spontaneous autochthonous disease, closely modeling human disease progression. At 8-10 weeks, mice develop mild to severe hyperplasia, followed by localized neoplastic lesions at 12 weeks, and eventually metastatic cancer by 24-28 weeks of age (Gingrich, Barrios et al. 1996, Gingrich, Barrios et al. 1999).

The goal of this study was to examine whether long-term oral administration of capsaicin could reduce the progression of disease in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.

4.3 Materials and Methodology

Capsaicin: Preparation and Storage

Capsaicin *([(E)-N-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide])* was obtained from Tocris Bioscience (Bristol, UK). To prepare capsaicin for *in vivo* studies, capsaicin was dissolved at a concentration of 0.2M in ethanol and stored at -20°C. On the day of feeding, capsaicin was prepared fresh by diluting the stock solution of capsaicin in saline (Invitrogen, Canada) to a final concentration for 5mg/kg body weight administration.

To conduct invasion and migration studies *in vitro*, capsaicin was dissolved in DMSO at a concentration of 0.1M and stored at -20°C. The stock solution of capsaicin was then dissolved in the appropriate growth medium prior to *in vitro* studies. Capsaicin was also prepared in minimal lighting to avoid degradation of the compound due to its light sensitive properties.

Preliminary Studies to Evaluate Toxicity

Five four-week-old C57Blk/6 male mice were administered capsaicin at a dose of 5mg/kg body weight over the course of 26 weeks. Body weight was measured thrice weekly and any changes in behaviour were documented. At thirty-six weeks, capsaicin treatment was stopped and mice were asphyxiated by CO_2 and blood was obtained by cardiac puncture. Liver and gastrointestinal tract (including stomach and oesophagus) were fixed in 10% formalin, embedded in paraffin and sections obtained for pathological analysis.

Transgenic Model

Animal care approval for the *in vivo* component of this work was obtained from the University of Toronto Animal Research Ethics Board and all work was conducted in accordance with established guidelines and protocols approved by the Canadian Council on Animal Care (CCAC). Heterozygous female mice were bred with C57Blk/6 male mice according to CCAC and offspring were used for the study. At three weeks, mouse-tail DNA was isolated and extracted using Qiagen's DNeasy Blood and Tissue Kit (Cat# 69504). Genotyping was carried out using a modified protocol adapted from the "Genotyping Protocol for TgN(Sv)&Bri, TgN(RipTAg)1Lt, TgN(TRAMP)8247NG" Updated May 29, 2003 by The Jackson Laboratory: briefly, DNA was extracted from mouse tail tissue. Once extracted, PCR was performed using the Qiagen's *Taq Polymerase* (Cat#: 201203) with the following primers: TRAMP fwd: 5'- CAG AGC AGA ATT GTG GAG TGG –3' and TRAMP rvs: 5'- GGA CAA ACC ACA ACT AGA ATG CAG TG –3'. At four weeks, mice were randomized into two groups: capsaicin (5mg/kg body weight) and control (saline vehicle). All mice received treatment or vehicle thrice weekly by oral gavage using plastic gavage tubes (Solomon Scientific). Body weight was recorded thrice weekly, all through the length of the study.

At 30 weeks, treatment was stopped and mice were asphyxiated by CO_2 and blood was obtained by cardiac puncture. All animals were carefully examined for gross organ abnormalities, photographed, and documented. The genitourinary (GU) system was excised and weighed. The prostate and tumour tissue was then carefully microdissected along with the liver, lungs, seminal vesicles, enlarged lymph nodes, gastrointestinal tract (including stomach and oesophagus), pancreas, and kidneys. The tissues were fixed in 10% formalin for 24 hours prior to processing for pathological analysis. A total of two TRAMP mice in each group were sacrificed at 12 weeks and sent for pathological analysis to assess disease progression and any capsaicin-related toxicities (i.e. histopathological liver changes).

Histopathology

After fixation, tumour, prostate, liver, lungs, seminal vesicles, enlarged lymph nodes, gastrointestinal tract (including stomach and oesophagus), pancreas, kidneys, and other tissue were embedded in paraffin 5μM, mounted, and stained with hematoxylin and eosin for pathological grading. Prostate tumours were carefully graded by my in-house pathologist (Dr. Linda Sugar) in a blinded fashion using the modified grading classification from Hurwitz AA, et al. (Hurwitz, Foster et al. 2001) (detailed in Figure 23). Normal prostate was numerically classified as 1; both low-grade and high-grade prostate intraepithelial neoplasia (PIN) characterized by in-folding and a change in the nuclear-cytoplasm ratio was numerically classified as 2; well-differentiated adenocarcinoma, characterized by invasion into the basement membrane was numerically classified as 3; moderately-differentiated adenocarcinoma, which displayed more solid growth pattern with smaller less formed lumen was numerically classified as 4; and poorly-differentiated adenocarcinoma which often contained neuroendocrine-like changes was numerically classified as 5. Other tissues were analyzed for abnormalities or evidence of toxicity as determined by changes in body weight, gross anatomy and histopathological observations.

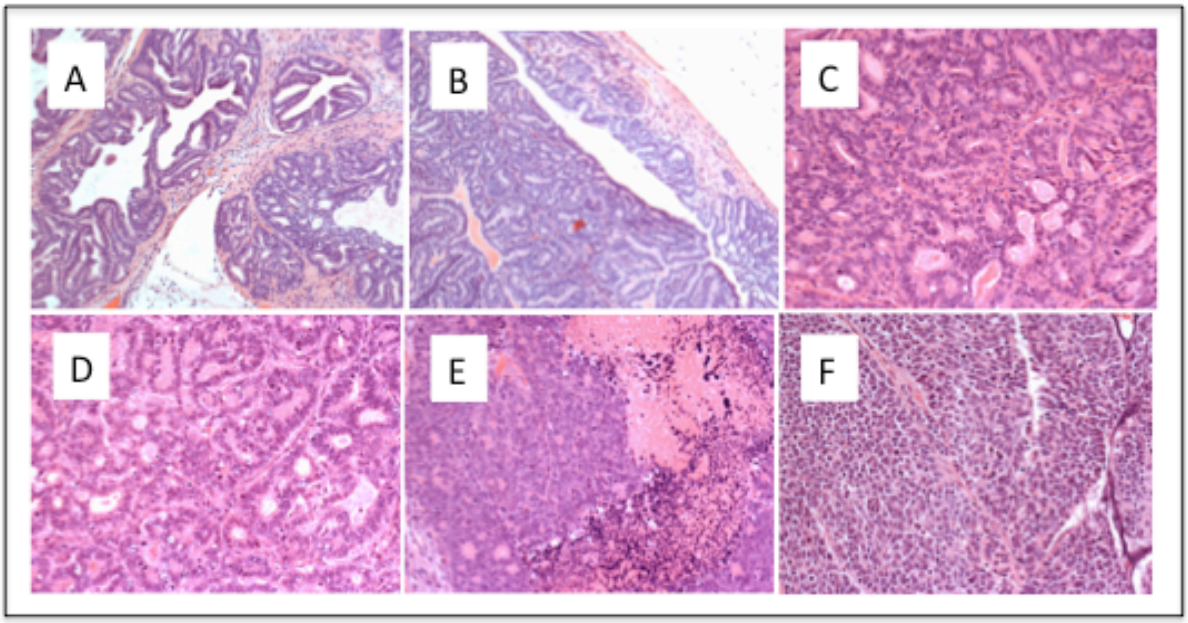


Figure 23: Histopathic Scoring of TRAMP Tumours. *The above panel depicts the scoring system with representative H/E stained prostate tumour sections based on the grading classification by Hurwitz et al. The first image demonstrated a normal prostate epithelium (A) (numerical classification = 1); the second image depicts intraepithelial neoplasia (B) with extensive infolding and a change in the nuclear-cytoplasm ratio (numerical classification = 2); the third image represents well differentiated adenocarcinoma (C) with invasion into the basement membrane (numerical classification = 3); the fourth image, moderately differentiated adenocarcinoma (D) displays more solid growth pattern with smaller less formed lumen (numerical classification = 4); the final two images represent poorly differentiated adenocarcinoma (E-F) with neuroendocrine-like changes (numerical classification =5)*

Cell Lines

Human prostate cancer cell line PC3 was obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Cells were cultured in Dulbecco's minimal essential medium/F12 (Invitrogen, Burlington, ON, Canada) with 10% FBS supplemented with 0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin, and maintained at 37ºC in a 5% CO_2 incubator under sterile conditions.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described (Venkateswaran, Fleshner et al. 2004). Briefly, deparaffinized sections were incubated with either the primary antibodies anti-$p27^{Kip1}$ (rabbit polyclonal, Santa Cruz Biotechnology) diluted 1:100 (200 μg/mL) in PBS; or androgen receptor rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:100 (200 μg/mL) in PBS; or anti-Chromogranin A (Santa Cruz Biotechnology); or SV40 TAg monoclonal anti- body (BD BioSciences) diluted 1:100 (200 μg/mL) in PBS respectively, overnight at 4°C. Tissue sections were then incubated with appropriate biotin-labeled secondary antibodies: anti-rabbit IgG/anti-mouse IgG/anti-goat IgG and incubated with preformed avidin-biotin peroxidase complex (Vector Laboratories). Metal-enhanced diaminobenzidine substrate (Vector Laboratories) was added and sections were briefly counterstained with hematoxylin.

Matrigel Invasion Assay

The effect of capsaicin on PC3 cell invasion was determined using BD BioCoat™ Matrigel™ Invasion Chamber 8.0 Micron, obtained from BD Biosciences (Mississauga, ON, Canada). As depicted in Figure 24, PC3 (1×10^5 cells/well) cells were seeded into the upper chamber/insert, using 12-well plates, and cultured for 24 h at 37 °C. Mitomycin C (0.5uM) was added 2 hours prior to capsaicin treatment (0.1-100 μM) to the bottom wells. After 24 h, the non-migrating cells were removed from the upper chamber with a cotton swab. The inserts were fixed with methanol and stained with 0.1% crystal violet solution. The number of migrated cells was quantified by counting the stained cells (cells per four fields) with a microscope. Each experiment was carried out in duplicate wells and repeated three times.

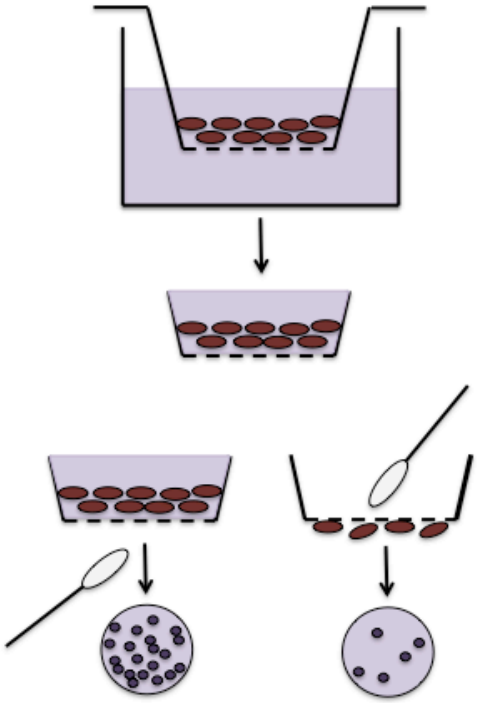


Figure 24: Matrigel Invasion Assay: *The Matrigel Invasion Assay is an assay performed to assess the ability of cells to migrate across the Matrigel matrix. Briefly, the plate is rehydrated using cell media, cells are plated, treated with mitomyocin C (to inhibit proliferation). Next, the treatment is added to the lower chamber and cells are left to migrate overnight. After 24 hours migrating cells are fixed, stained with crystal violet, and counted.*

Wound-Healing (Scratch) Assay

Cell migration was assessed in PC3 cells using a wound-healing assay as depicted in Figure 25. Approximately 1.0 x 10^6 cells/well were grown using a 24-well plate until the cells reached100% confluence. A vertical scratch across the well was made with a 10 µL pipette tip, followed by two washes with PBS. Capsaicin (0.1-100 µM) was then added to each well and left for 24 hours. Microscopy images were taken at 0 and 24 hours to visually assess cell migration. Each experiment was carried out in duplicate wells and the experiments were repeated three times.

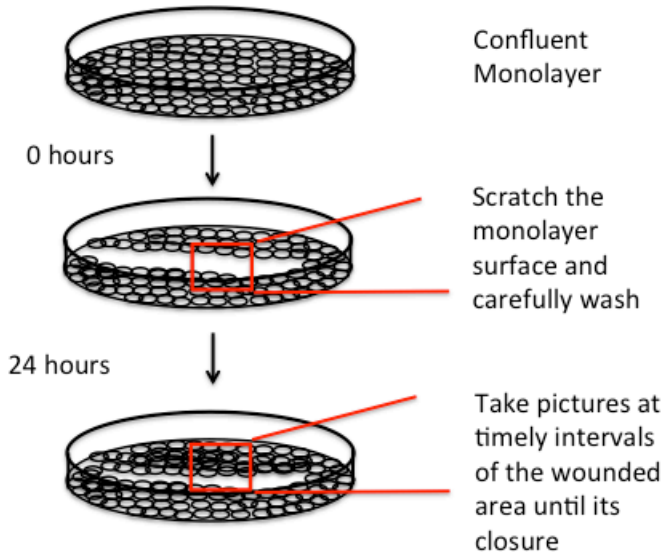


Figure 25: Scratch Migration Assay: *PC3 cells are plated in a 6-well plate and left to grow. Once a confluent monolayer is formed on the surface of the well a scratch is made using a 10uM pipette tip. At this point a photograph of the scratch is taken and cells are treated appropriately (treatment and vehicle control), after 24 hours another photograph of the scratch is taken and assessed using the rating scale for cell migration.*

Serum Analysis by Liquid Chromatography–Mass Spectrometry

Twenty microliters of serum from each sample of the *in vivo* studies was thawed and transferred to individual Eppendorf tubes. 5µL of 1µg/ml deuterated testosterone (d3T, C/D/N Isotopes) was then added, followed by 50 µL of acetonitrile, after which samples were vortexed for 5-10 sec and centrifuged for 5 min at 20,000 g to sediment precipitated protein. The clarified supernatant was transferred to LC vials for analysis. Standards were prepared in 50% methanol, and QC samples with capsaicin spiked blank mouse serum were prepared similar manner to standards. Optima grade (Fisher) solvents and 18 MΩ water (Millipore) were used for sample preparation and subsequent LC-MS analysis.

Analysis was carried out with an Acquity UPLC coupled with a Quattro Premier (Waters). A 100 mm BEH C18 1.7µ column (Waters) was used for separations with a 40-85% acetonitrile (ACN) gradient from 0.2-3 min, ramped 0.5 min to 98% ACN for flushing (2.5min) followed by a 2 min re-equilibration for an 8 minute run length (0.1% formic acid present throughout). All MS data was collected in ES+ at unit resolution with the following instrument parameters: capillary, 3.0 kV; extractor and RF lens, 3V and 0V; source and desolvation temperatures, 120 ºC and 300 ºC; desolvation and cone gas (N_2), 1000 L/hr and 50 L/hr; collision gas (Ar), 0.15 ml/min (6.2e-3 m bar). Compounds were detected using multiple reaction monitoring (MRM) with m/z 306>137 and 306>182 for capsaicin and m/z 292>97 for d3T (22V/17V, 22V/11V and 32V/21V cone/collision volt combinations used respectively for the three transitions) with 0.1 sec dwell each. Retention times (RT) for d3T and capsaicin were 2.35 and 2.8 min, respectively.

Quanlynx (Waters) was used for analysis of data using peak area ratios of capsaicin/d3T for calibration and quantification. Calibration standards ranged from 0.002 to 1µg/ml (6 points) with R2 >0.99 and all % deviation from nominal < 15% above 0.01µg/ml (omitting 1µg/ml due to curvature). Comparison of spiked serum with QC samples indicated little to no matrix interference with extraction efficiencies of 95%.

Statistical Analysis

Analyses of the results were performed using either Student's t-testing or repeated measures ANOVA techniques. Statistical analysis was performed using SAS software, version 8 (SAS Institute Inc., Cary, NC, USA).

4.4 Results

4.4.1 Long-term oral administration of capsaicin is well tolerated with no evidence of associated toxicities

Since the safety of long-term oral administration of capsaicin has not been previously reported in the literature, we carried out preliminary standardization studies to better understand if long-term administration of capsaicin was well tolerated in C57Blk male mice prior to commencing the TRAMP study. Here we found, for the first time, that long-term oral gavage administration of capsaicin at a concentration of 5mg/kg body weight was well tolerated with no evidence of toxicity (see Figure 26 for changes in body weight overtime) over the course of their lifespan. Animals administered capsaicin demonstrated a normal body weight gain over time, similar to those receiving the vehicle alone. Gross anatomy and histopathological analyses of tissue samples including the liver and GI tract were reported to be normal. No other physical abnormalities were observed over the course of the entire thirty weeks of study.

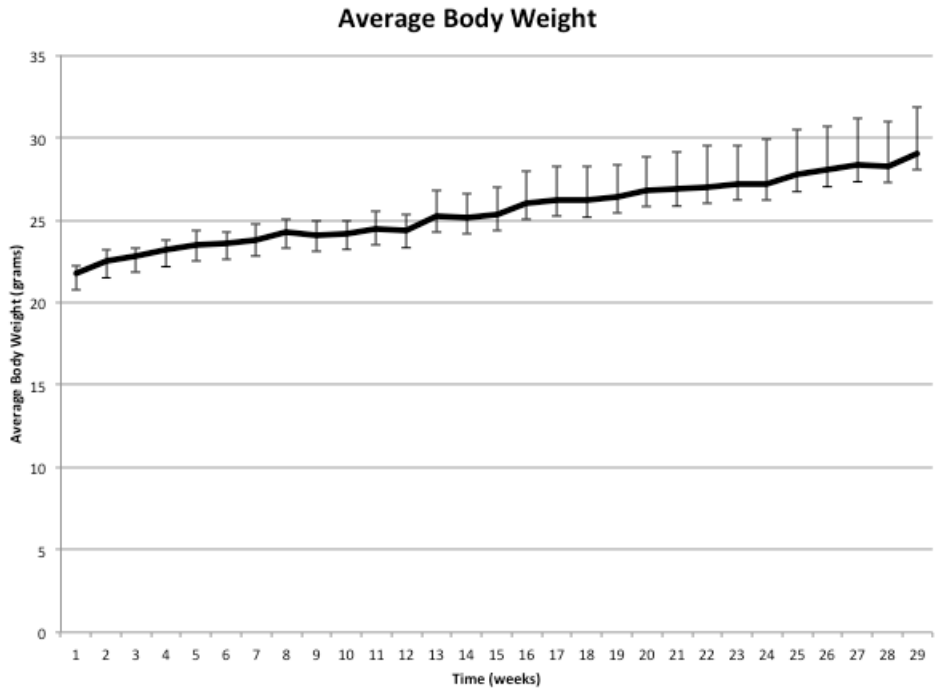


Figure 26: Body Weight Overtime of C57Blk Mice consuming capsaicin. *The above figure depicts the average body weight of C57Blk mice receiving capsaicin overtime. The gradual increase in body weight suggests that capsaicin was well tolerated and did not cause changes in bogy weight overtime.*

Based on the previous standardization studies examining tolerance of long-term capsaicin ingestion, we were confident that long-term administration of capsaicin was well tolerated with no toxicities. Therefore, we went on to administer capsaicin to animals that tested positive for the presence of the TRAMP transgene. Similar to the standardization analysis, TRAMP mice were administered capsaicin over the course of their life-span had no associated toxicities, as there were no significant differences in body weight between the capsaicin group and the control group (Figure 27). The gross anatomy and histopathological analysis of the gastrointestinal tract (including stomach and oesophagus) and liver were identified to be normal.

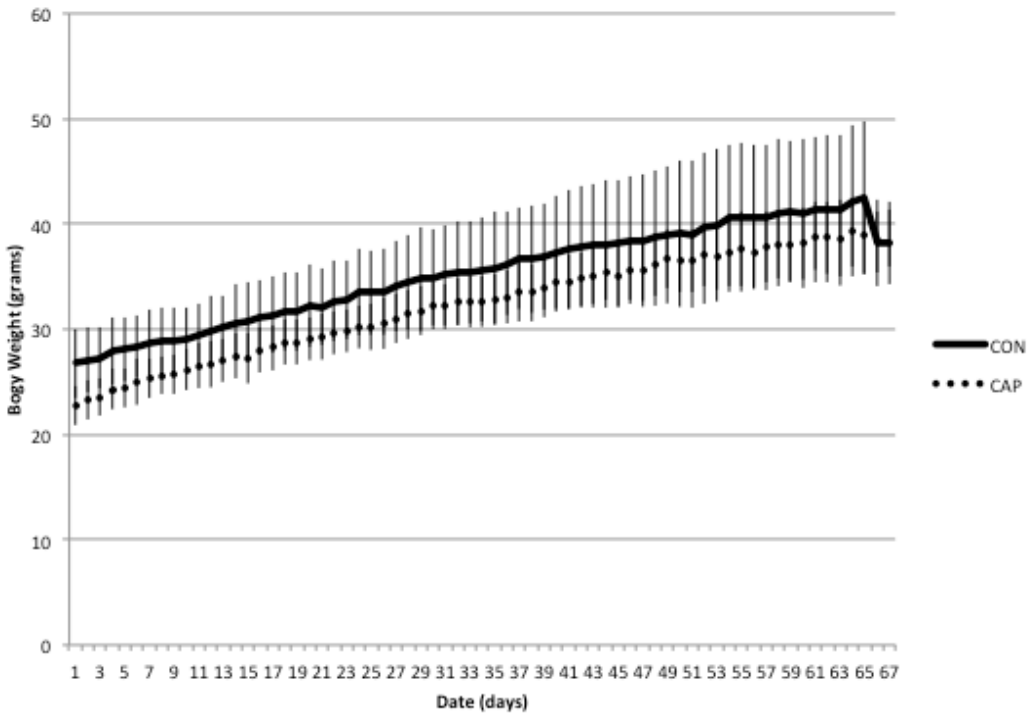


Figure 27: Average Body Weight Overtime in TRAMP Mice Consuming Capsaicin. *The above line graph depicts the average body weight of TRAMP mice overtime in the control group compared to the capsaicin group. No significant difference was found between the groups overtime.*

4.4.2 Animals administered capsaicin had a lower overall tumour grading

At the time of necropsy, all TRAMP mice expressed the various stages of prostate cancer progression as described by the grading classification from *Hurwitz AA et al.* At 12 weeks of age, the pathological grading of the prostate in the untreated and treated animals ranged from normal prostate to low-grade and high-grade prostate intraepithelial neoplasia (PIN), characterized by extensive infolding and a change in the nuclear-cytoplasm ratio (Figure 23). At the termination of the study, animals in the untreated arm expressed well-differentiated adenocarcinoma (WD), moderately differentiated (MD) adenocarcinoma, or poorly differentiated adenocarcinoma (PD). Representative images of the range of pathological progression are depicted in Figure 28. An on-site pathologist graded all tissue samples independently, in a blinded fashion.

Mice in the control group expressed an overall trend of higher-grade disease compared to the capsaicin group. They had a higher proportion of PD tumours (37.5%) compared to the capsaicin group (27.7 %). Mice in the control group also demonstrated a higher proportion of MD tumours (18.75%) compared to the capsaicin group, which had no MD tumours. Interestingly, there was a lower proportion of WD adenocarcinoma in the control group compared to the capsaicin group (44% vs 61.0% respectively). Furthermore, 11.1 % of tumours in the capsaicin group expressed PIN lesions and no adenocarcinoma, suggesting an inhibition of tumour progression at the early stages of disease.

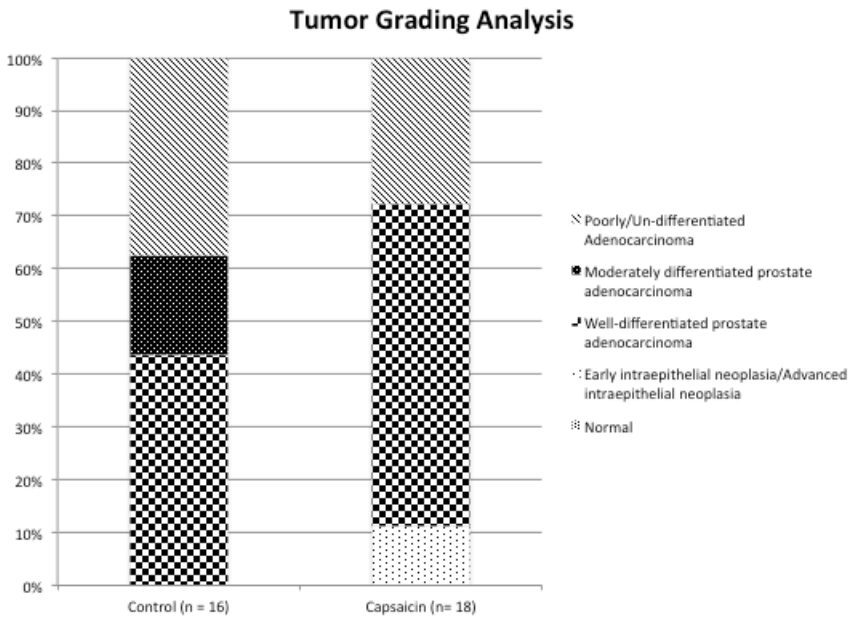


Figure 28: TRAMP Tumour Grading Analysis. *Prostate tumours were classified based on the scoring system described in Figure 23. Tumour samples were taken from each individual TRAMP mouse, stained with H/E, and classified by an in-house pathologist. The control group had a large proportion of tumours in the well-differentiated group, followed by poorly differentiated tumours, and a smaller proportion of moderately differentiated tumours. Compared to the control group, the capsaicin group had some normal tumours and a greater percentage of well-differentiated tumours.*

4.4.3 Mice consuming capsaicin had a significantly lower metastatic burden compared to those receiving vehicle alone

To determine whether TRAMP mice developed metastases from the primary tumour, tissue from the lung, liver, kidneys, enlarged lymph nodes, and seminal vesicles were processed at the time of necropsy. The metastatic burden observed in TRAMP mice receiving capsaicin was

significantly lower (27.7%) (p = 0.031) than in the control group (43.8%) (Figure 29a). The sites of the metastasis varied between the two groups. Mice in the control group developed metastases predominately in the liver followed by the lung, lymph, seminal vesicles, and pancreas. However mice in the treated group developed metastases predominately in the lung and pancreas, followed by metastasis at the site of the lymph nodes, seminal vesicles, and kidney (Figure 29b). All metastases were processed, and a subset form each group were randomly selected and analyzed by immunohistochemistry for the presence of SV40 TAg transgene to determine the origin of the metastases. All metastases stained positive for SV40 TAg transgene, suggesting that they originated from the primary tumour. A representative image of a liver tumour metastasis is depicted in Figure 29c, suggesting that the original tumours were derived from the primary site.

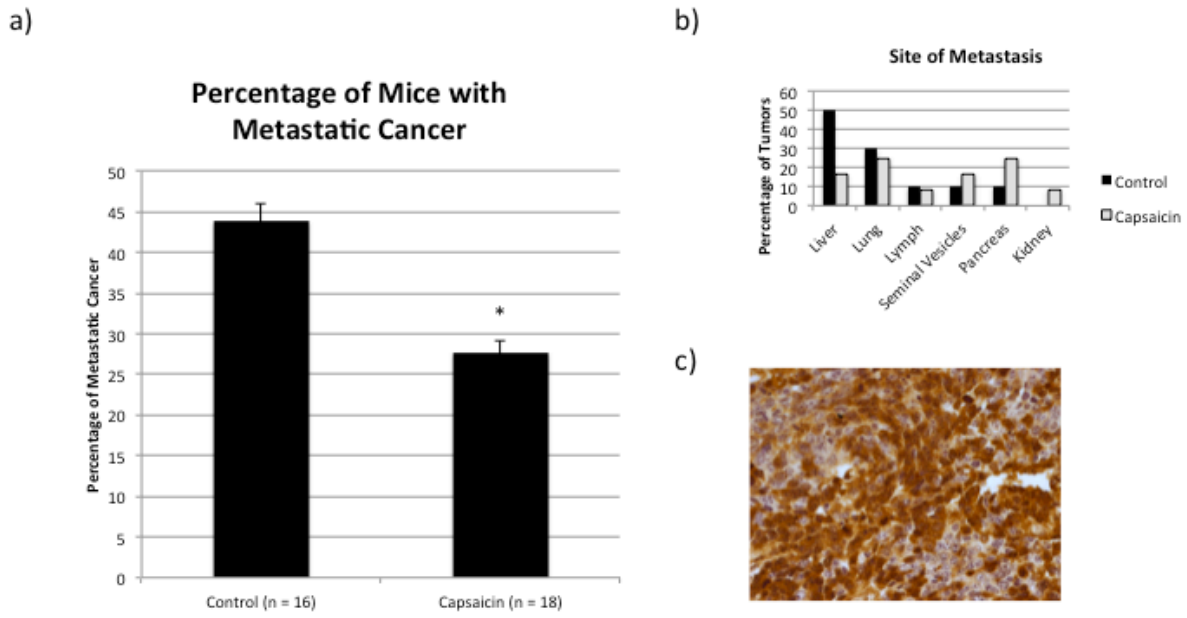


Figure 29: Proportion of Metastatic Tumours. *TRAMP mice that received long-term oral administration of capsaicin had a significantly lower proportion of metastases compared to the control group which received vehicle alone (p = 0.031) (A). The histogram is a graphical representation of the various sites of tumour metastasis in the control and capsaicin group (B). A representative histological image of a TRAMP liver metastasis from the control group that is stained positive for the SV40 TAg transgene (C).*

4.4.4 TRAMP mice treated with capsaicin had a higher expression of androgen receptor and lower expression of neuroendocrine markers.

Immunohistochemical analysis of prostate tumours from both the control and the capsaicin group was performed to assess molecular changes within the prostate tumours (Figure 30). All TRAMP tumours expressed the SV40 transgene, as expected for the TRAMP model system. Tumours from the control group expressed high levels of the neuroendocrine marker Chromagranin A (ChA) and very low levels of the androgen receptor (AR), consistent with neuroendocrine changes in advanced stage prostate cancer. On the contrary, tumours of the capsaicin treated group showed low ChA and higher AR expression, suggesting that capsaicin may be inhibiting or delaying progression towards neuroendocrine differentiation.

We also examined the expression of $p27^{Kip1}$, the tumour suppressor. Mice treated with capsaicin exhibited tumours with a higher expression of $p27^{Kip1}$ compared to the control group.

Taken together, tumours in the control group demonstrated higher grade disease with lower expression of $p27^{Kip1}$ and more pronounced neuroendocrine differentiation compared to the tumours in the capsaicin treated group, which showed a significantly reduced tumour burden.

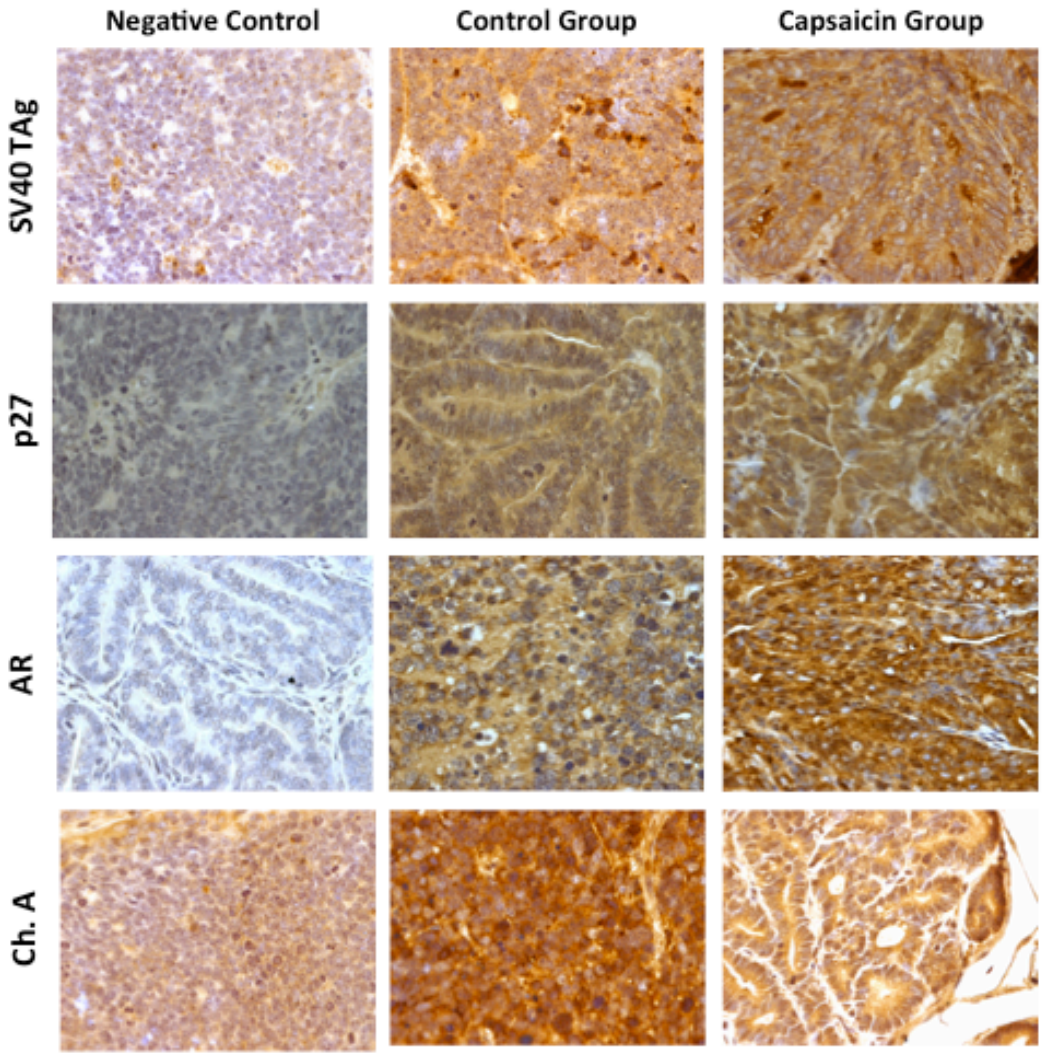


Figure 30: Immunohistochemical Analysis of TRAMP Tumours. *The above panel displays representative tumour samples of the immunohistochemical staining for TRAMP mice in the control group and the capsaicin group. The SV40 T Antigen (SV40 TAg) stained positive in both groups. The* $p27^{KiP1}$ *expression was higher in the capsaicin groups compared to the control group. The Androgen Receptor (AR) is strongly expressed in the capsaicin group and weakly expressed in the control group. This corresponds to the high expression of the neuroendocrine marker Chromogranin A (ChA) in the control group and lower expression of ChA in the capsaicin group.*

4.4.5 Capsaicin treatment reduces the migration and invasion capacity of androgen-resistant prostate cancer cells.

Since capsaicin treatment reduced the metastatic burden in TRAMP animals, we investigated the migratory and invasive potential of capsaicin using the androgen-resistant PC3 cell line (Figure 31). The wound-healing assay was performed to test whether capsaicin treatment could alter the migration of PC3 cells. Results of this assay clearly indicated that capsaicin had a great capacity to reduce the migration of PC3 cells in a dose-dependent manner. There was no significant reduction in migration at 0.1μM of capsaicin, however there was significant reduction in migration of PC3 cells treated with 1μM - 100μM ($p<0.05$), with the maximal reduction in migration at 100μM of capsaicin ($p<0.001$).

The Matrigel invasion assay is an assay that assesses the ability of a cell to penetrate a Matrigel layer. we observed that capsaicin significantly inhibited the invasion of PC3 cells at 1-100μM ($p<0.05$).

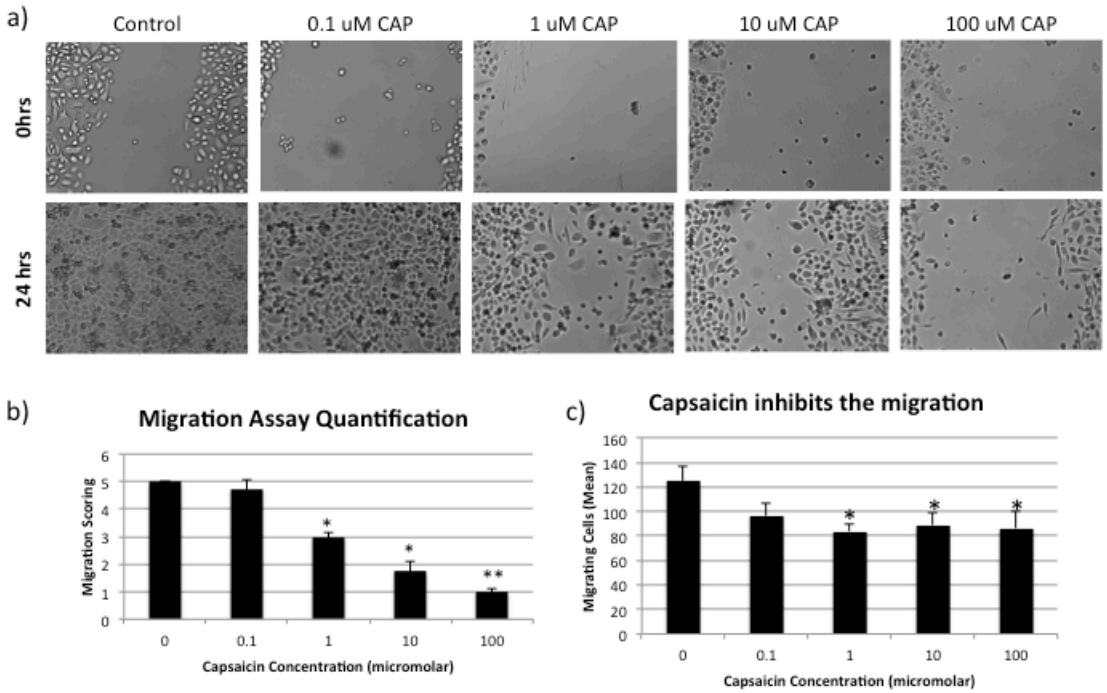


Figure 31: Migration and Invasion Studies. *A) Morphological representation of wound healing assay, where PC3 cells treated with capsaicin (0.1 – 100 µM) underwent a dose-dependent reduction in cell migration after 24 hours; B) Graphical representation of wound healing assay; C) Capsaicin reduced the invasion of PC3 cells at 24 hours as assessed by the Matrigel Invasion Assay. The * symbol denotes significance (p<0.05) relative to control; ** symbol denotes significance (p<0.001) relative to radiation alone.*

4.4.6 Serum from capsaicin treated animals reveals the presence of capsaicin, as well as its metabolite capsaicin glucuronide

To understand whether the capsaicin administered through oral gavage was metabolized and present in the serum of mice, we performed Liquid Chromatography–Mass Spectrometry (LC-MS) on serum obtained from mice at 25, 40, 60, and 75 minutes as well as 48 hours after administration. Results from LC-MS revealed that after 25-75 minutes of oral capsaicin administration, capsaicin, as well as its metabolite glucuronide, was present in the serum (Figure 32). Serum concentrations of capsaicin ranged from 0.001-0.002 µg/mL (Table 2). The maximal

capsaicin concentration was achieved at 60 minutes post-capsaicin administration. The serum concentration of capsaicin glucuronide ranged from 0.158-0.787 µg/mL, peaking at 60 minutes post-capsaicin administration. By 48 hours post-capsaicin administration, capsaicin and its metabolite were undetectable in serum.

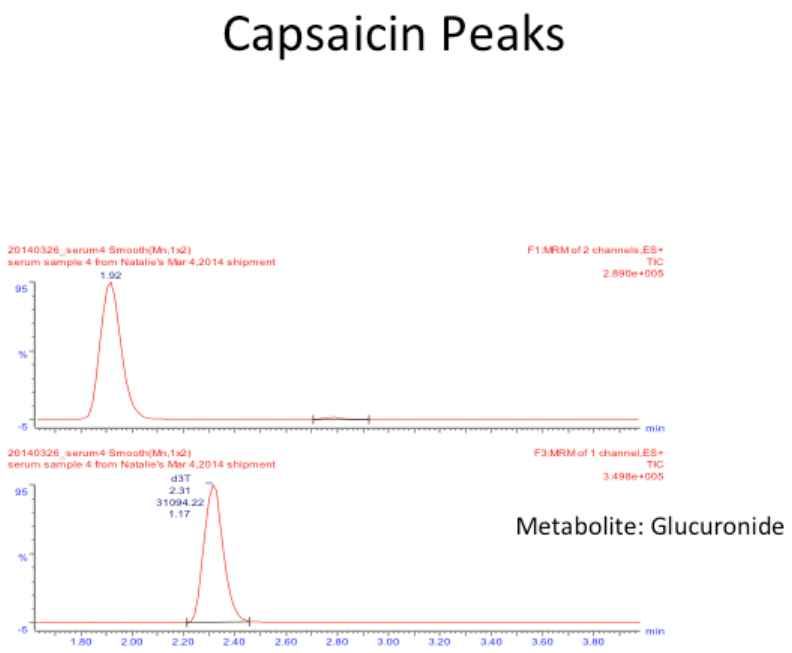


Figure 32: HPLC Analysis of Capsaicin in Mouse Serum. *The above figure depicts the presence of capsaicin peak (above) as well as its associated metabolite peak (below)*

Summary of Analysis

Sample	Time Post-capsaicin gavage (5mg/kg b.w.)	Serum Conc. of Capsaicin (ug/mL)	Serum Concentration of Glucuronide (ug/mL)
1	25 mins	0.0014	0.158
2	40 mins	0.0010	0.154
3	60 mins	0.0019	0.787
4	75 mins	0.0011	0.072
5	Negative Control	0	0

Table 2: Capsaicin Serum Analysis. *The above table provides a summary of the concentration of capsaicin (ug/mL), and its metabolite capsaicin glucuronide (ug/mL), at various time intervals (minutes) after oral administration of capsaicin by gavage.*

4.5 Discussion

We have examined the chemopreventive properties of capsaicin in a transgenic mouse model of prostate cancer. This is the first report that capsaicin reduces the metastatic burden and overall disease severity in the TRAMP model. We have demonstrated that long-term oral administration of capsaicin does not result in toxicity in TRAMP mice. Mechanistic studies suggest that capsaicin lowers the metastatic potential of the tumours by inhibiting migration, invasion, neuroendocrine differentiation, and by upregulation of the tumour suppressor protein $p27^{Kip1}$ (See Figure 33). Taken together, these studies provide a solid foundation of evidence for capsaicin as a safe and potent anti-cancer agent.

The most effective time to initiate chemopreventive interventions in transgenic models of prostate cancer still remains unclear. In the present study, we commenced capsaicin administration at a very early stage, prior to cancer development (i.e. 4 weeks of age). Extrapolated to humans, this would be equivalent to commencing capsaicin supplementation in

early adulthood as a strategy to prevent the development and progression of disease. Whether this is the most appropriate time to initiate capsaicin treatment remains unclear, however the administration of capsaicin from 4 weeks of age demonstrated a down-grading in disease staging, and a significant reduction in metastases.

Our laboratory has previously examined the effect of initiating Vitamin E, Selenium, and Lycopene supplementation at 4, 8, 20, and 36 weeks of age in *the Lady* transgenic model of prostate cancer, and reported that interventions at the 8 week period were most effective (Venkateswaran, Klotz et al. 2009). It is possible these chemopreventive therapies are more effective at targeting pre-malignant tissue, or perhaps initiating chemopreventive measures early in cancer development alters the microenvironment of the malignancy, affecting tumour progression. Nevertheless, future studies examining the effect of capsaicin supplementation at different stages of prostate cancer development would help gain a better understanding of whether capsaicin can have a therapeutic role in later stages of disease (Venkateswaran, Fleshner et al. 2004).

Over the past decade, capsaicin has been reported to have a number of chemopreventive properties in pre-clinical studies. However, the majority of these studies have been limited to cell lines and xenograft tumours grown in immunocompromised mice, focusing on the anti-proliferative and pro-apoptotic effects of capsaicin (Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006). Using the TRAMP model, which possesses an intact immune system, we have demonstrated for the first time that capsaicin reduces the metastatic disease burden.

The detailed mechanisms behind the anti-metastatic changes are still not clear. We conducted *in vitro* analyses and found that capsaicin had the potential to reduce both the migratory and invasive properties of prostate cancer cells. To date, such data has not been reported in prostate cancer cells. However, similar effects have been noted in other cancers wherein low doses of capsaicin inhibited the invasion and migration of B16-F10 melanoma cells without cytotoxicity (Shin, Kim et al. 2008). These effects were correlated with the down-regulation of phosphatidylinositol 3-kinase (PI3-K) and Akt (Shin, Kim et al. 2008). Anti-migratory effects of capsaicin have also been reported in cholangiocarcinoma and urothelial carcinoma (Caprodossi, Amantini et al. 2011, Wutka, Palagani et al. 2014). Although the effects of PI3-K and Akt have

not been investigated in this study, it is possible that modulation of this pathway could play a role in the anti-metastatic effects that was found.

In the present study, capsaicin treatment of TRAMP mice for 26 weeks resulted in down-staging in tumour grade, with only a small proportion of PD and MD tumours and presence of pre-neoplastic lesions, compared to the control group. Perhaps using a larger sample size might statistically power the current findings for future studies. Immuohistochemical results support the finding that inhibition of cancer progression may be through an upregulation of the tumour suppressor protein $p27^{Kip1}$. *In vitro*, an elevation of $p27^{Kip1}$ expression has been reported in a number of capsaicin studies contributing to cell cycle inhibition and a reduction in proliferation (Kuczyk, Machtens et al. 1999, Kuczyk, Bokemeyer et al. 2001). It is also possible that this upregulation contributed to attenuating cancer progression by impairing neuroendocrine differentiation (Kuczyk, Machtens et al. 1999, Kuczyk, Bokemeyer et al. 2001, Wei, Xu et al. 2012). Nevertheless, mechanistic changes demonstrated *in vitro* may differ from *in vivo* experiments as the concentration of capsaicin tested in cell culture may not be achieved in an animal model. Hence, further studies examining the pharmacokinetics and pharmacodynamics of capsaicin *in vivo* should be evaluated moving forward.

Androgens are important regulators of prostate cancer regulation and development, working through the activation of the Androgen Receptor (AR) (Dehm and Tindall 2006, Dehm and Tindall 2007). Early stages of castrate-resistant prostate cancer are often characterized by a lack of androgen response or re-activated AR signaling (Dehm and Tindall 2006).The transformation of androgen-responsive, to androgen-resistance, to neuroendocrine differentiated carcinoma is not well established. However, there are a number of neuroendocrine markers reported to identify changes including, Chromogranin A (Shariff and Ather 2006, Ather, Abbas et al. 2008, Palmer, Venkateswaran et al. 2008). In the present study, we observed an upregulation of AR expression and a reduction in the levels of Chromogranin A in tumours in the treatment group, suggesting that capsaicin played an integral role in preventing the neuroendocrine differentiation process. Unfortunately, how capsaicin may be inhibiting this process is not clearly understood. It is hypothesized that certain enzymes, including AKR1C3, may be involved in the transformation of cells undergoing the neuroendocrine differentiation process (Adeniji, Chen et al. 2013, Knuuttila, Yatkin et al. 2014). Future studies examining how capsaicin may be altering

these enzymes may be of relevance for developing therapeutic strategies for preventing the progression of neuroendocrine and castrate-resistant tumours (Huss, Gray et al. 2007).

There are only a few studies that have examined the pharmokinetics and bioavailability capsaicin, most of which have been carried out in rats (Bernard, Ubukata et al. 2010, Kodama, Masuyama et al. 2010, Shirai, Ueno et al. 2010). In humans, capsaicin is generally assessed through the consumption of chilli peppers and related spices (Khan, Adhami et al. 2010). One study reported serum concentrations of 8.1 nM after oral consumption of 5 g of chilli peppers, whereas other studies have reported concentrations ranging from 13.4-16.3 ng/mL after the consumption of a chilli-containing meal (Reilly and Yost 2006, Chaiyasit, Khovidhunkit et al. 2009). Whether the bioavailability of capsaicin is sufficient to achieve the desired therapeutic effectiveness is neither clear nor easy to conduct. However, the ranges reported in humans are comparable to the serum concentrations we found in my TRAMP studies, thereby suggesting that it may be possible to obtain a therapeutically relevant concentration of capsaicin through dietary means. Unfortunately, these concentrations are still 10 to 100-fold lower than the concentrations used herein and in reported *in vitro* analyses. It is still unclear whether capsaicin can accumulate in certain tissues (i.e. prostate tissue), as reported with other dietary agents (e.g. lycopene)(Venier, Colquhoun et al. 2012). It is also possible that the metabolite capsaicin glucuronide, which was identified, may possess anti-cancer properties. A similar glucuronide metabolite has been reported for the flavoinoid quercetin (Ruotolo, Calani et al. 2014). Interestingly, this metabolite is reported to have inhibitory properties on androgen-sensitive LNCaP cells (Stolarczyk, Piwowarski et al. 2013). Nevertheless, further investigation examining the effects of capsaicin and its metabolite are warranted.

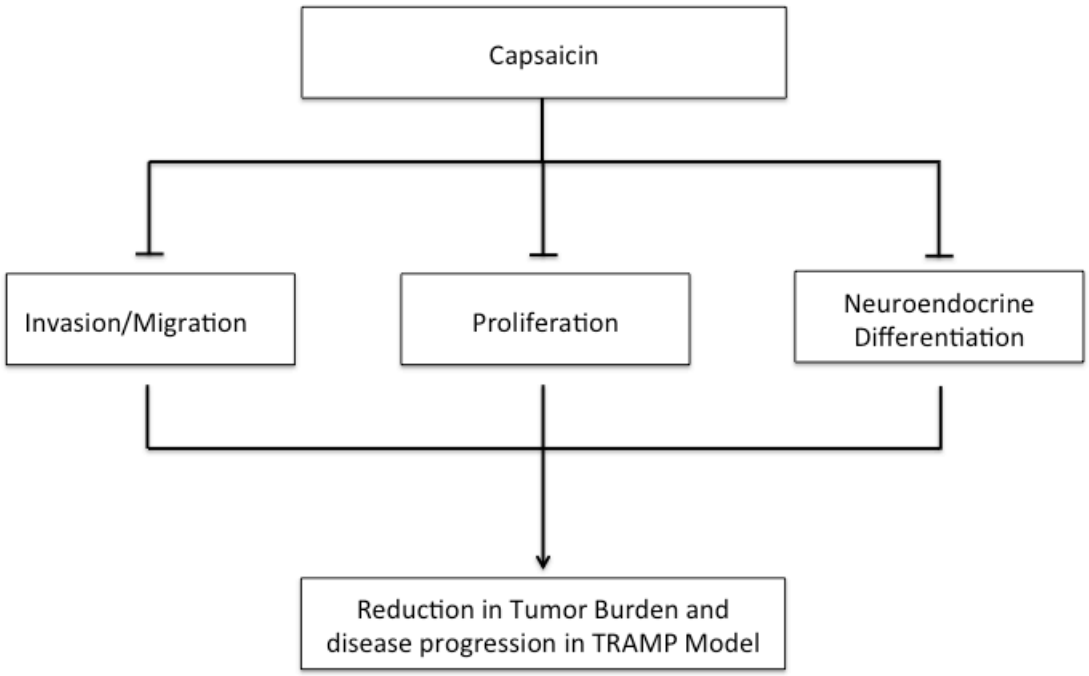


Figure 33. Proposed Mechanism of Reduced Tumour Burden in TRAMP Model. *The above flow chart visually depicts how capsaicin acts to reduce invasion and migration capacity, proliferation, and neuroendocrine differentiation to inhibit metastases in the TRAMP Model.*

4.6 Conclusions

Capsaicin has emerged as a promising anti-cancer agent in a number of pre-clinical studies. We report for the first time the anti-metastatic potential of capsaicin in a transgenic model of prostate cancer, and its long-term oral administration to be both safe and well tolerated. These studies form the basis for ongoing clinical trials in men with advanced prostate cancer, as well as in men with low-grade disease with the intent to reduce tumour progression.

4.7 Caveats and Pitfalls

In chapter 4, *Capsaicin Reduces the Metastatic Burden in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model*, long-term oral administration of capsaicin was well tolerated and exhibited anti-metastatic potential. The following section details some of the caveats to this study that should be mitigated in future studies.

In this study, we were able to identify a trend towards a reduction in overall tumour grading. Perhaps by using a larger sample size, this trend may have reached significance and supported the use of capsaicin as a chemoprevention agent for prostate cancer.

To better understand the mechanism by which capsaicin is exerting its effect *in vivo,* further mechanistic studies could involve more detailed analyses of neuroendocrine changes and extensive analyses of tumour suppressor and metastases pathways. The role of TRP-V1 was not addressed in this study. Perhaps additional control groups with a TRP-V1 agonist and antagonist with capsaicin may have been advantageous for better understanding of the role of TRP-V1.

In this study, we did not address the optimal timing of capsaicin administration. Future studies with mouse cohorts commencing capsaicin treatment at different stages of disease progression will be needed to provide further insight as to when capsaicin is most effective (e.g. prior to cancer development, after PIN lesions have developed, etc.) as considered in the Discussion. Furthermore, based on early combinatorial studies (Chapter 3), it would have been interesting to see the effect of capsaicin combined with other micronutrients, particularly lycopene, on prostate cancer progression.

While we were able to detect capsaicin along with its metabolite, capsaicin glucuronide, in the mouse serum, more detailed investigations about the bioavailability and pharmacokinetics of capsaicin may aid the understanding of the chemopreventive effects of capsaicin. Furthermore, studies examining the deposition of capsaicin or any of its metabolites in certain tissues or organs (i.e. the prostate) would provide valuable information to clinically translate these findings.

CHAPTER FIVE

Capsaicin: A Novel Radio-sensitizing Agent for Prostate Cancer

This chapter is modified from the following:

Venier, N. A., Colquhoun, A. J., Sasaki, H., Kiss, A., Sugar, L., Adomat, H., Fleshner, N. E., Klotz, L. H. and Venkateswaran, V. (In press), Capsaicin: A novel radio-sensitizing agent for prostate cancer. (Wiley Periodicals, Inc)

Published in *The Prostate* Journal *(2014)*

http://www.ncbi.nlm.nih.gov/pubmed/25307418

5.1 Abstract:

Introduction: Radio-sensitizing agents sensitize tumour cells to the lethal effects of radiotherapy (RT), allowing for use of lower doses of radiation to achieve equivalent cancer control while minimizing adverse effects to normal tissues. Given their limited toxicity and ability to easily integrate into the diet, compounds occurring naturally in the diet make ideal potential radio-sensitizing agents. In this study, we have examined whether capsaicin, the active compound in chilli peppers, can modulate the response to RT in preclinical models of prostate cancer (PCa).

Methods: The effects of RT (1-8 Gy) and/or capsaicin (1-10μM) on colony formation rates in human PCa cells were assessed using clonogenic assays. Mechanistic studies were performed by Western Blot, immunocytochemistry, and flow cytometry. Athymic mice (n=40) were inoculated with human LNCaP cells. Once tumours reached $100mm^3$, animals were randomized into 4 groups: control, capsaicin alone (5 mg/kg/d), RT alone (6Gy), and capsaicin and RT.

Results: Capsaicin reduced colony formation rates and radio-sensitized human PCa cells (Sensitizer enhancement ratio = 1.3) which corresponded to the suppression of NFκB, independent of TRP-V1 receptor activation. Cell cycle arrest occurred following RT and capsaicin treatment independently. *In vivo*, oral administration of capsaicin with RT resulted in a 'greater than additive' growth delay and reduction in the tumour growth rate, greater than capsaicin ($p<0.001$) or RT ($p<0.03$) alone. Immunohistochemical analysis revealed a reduction in proliferation and NFκB expression, and an increase in DNA damage.

Discussion: These findings suggest that capsaicin acts as a radio-sensitizing agent for PCa through the inhibition of NFκB signalling.

5.2 Introduction

Radiotherapy (RT) is a very commonly used treatment for prostate cancer (PCa) (Gwede, Pow-Sang et al. 2005, Loblaw and Cheung 2006). Conventionally RT is administered to the prostatic region on weekdays in 2 Gy fractions, resulting in a cumulative dose of 70-80Gy. Radiotherapy mediates its lethal effects on tumour cells by inducing DNA damage (Zhou and Elledge 2000). At a molecular level, cells respond to this damage by either undergoing cell death, and/or repairing the damaged DNA through the upregulation of repair proteins and survival factors, such as NF**κ**B (Palacios, Miyake et al. 2013). Targeting such repair pathways and survival factors, chemically or pharmacologically, can sensitize cells to the lethal effects of RT (Palacios, Miyake et al. 2013). If successful, such radiosensitizing agents achieve their effect by increasing tumour cell kill while minimizing adverse effects on normal tissues (Girdhani, Bhosle et al. 2005, Palacios, Miyake et al. 2013). Compounds occurring naturally in the human diet are ideal radio-sensitizing agents given their ability to be easily integrated into the diet and minimal toxicity at conventional doses (Girdhani, Bhosle et al. 2005).

Capsaicin (*trans-8-methyl-N-vanillyl-6-nonenamide*) is the natural compound found in the *Capsicum sp.* plants and is responsible for the burning sensation experienced upon contact with chilli peppers. It displays both analgesic and anti-inflammatory properties and has been widely used topically for treatment of various chronic pain syndromes, through the binding and activation of the vanilloid receptor, TRP-V1. More recently, capsaicin demonstrated anti-carcinogenic properties in several cancers, including PCa (Kim and Moon 2004, Mori, Lehmann et al. 2006, Sánchez, Sánchez et al. 2006, Zhang, Humphreys et al. 2008). These anti-cancer properties have been reported to act through a number of pathways, including NF**κ**B pathway inhibition (Mori, Lehmann et al. 2006, Luqman, Meena et al. 2011).

NF**κ**B resides in the nucleus in an inactive state bound to the IκB complex (Ahmed and Li 2008). In the classical NF**κ**B activation pathway, after the cell undergoes an insult (e.g. stress, radiation), the IKK complex enzyme degrades IκB proteins through the ubiquitinization process, causing the dissociation of the NF**κ**B-IKK complex. At this point, NF**κ**B can be phosphorylated and translocates into the nuclear compartment, where it can bind to various DNA binding sites. This results in the upregulation of genes involved in a number of processes including cell cycle

regulation, cell survival, and radio-resistance (Ahmed and Li 2008). Hence, targeting the NFκB pathway is thought to be an effective strategy to overcome radioresistance and improve radiation therapy (Kimura, Bowen et al. 1999, Pajonk, Pajonk et al. 1999, Li and Stark 2002, Ahmed and Li 2008).

In the present study, we have examined whether capsaicin, the active compound found in chilli peppers, can modulate the response to radiation through the inhibition of NFκB pathway in prostate cancer (PCa) in both *in vitro* and *in vivo* model systems. We observed capsaicin to enhance prostate cancer radio-sensitivity though the suppression of radiation-induced NFκB signalling in the *in vitro* model. The radio-sensitizing effect is independent of cell cycle inhibition and TRP-V1 activation. We also found that in the LNCaP xenograft model, capsaicin fed animals treated with radiotherapy experienced a significant delay in tumour growth. This effect was 'more than additive' compared to capsaicin or radiation alone.

5.3 Methodology

Cell lines

Three human prostate cancer cell lines DU145, LNCaP, and PC3 were obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland, USA). LNCaP cells were cultured in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin (Invitrogen, Burlington, ON, Canada). PC3 and DU145 cells were cultured in Dulbecco's minimal essential medium/F12 (Invitrogen, Burlington, ON, Canada) with 10% FBS supplemented with 0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin. PC3AR2 cells are PC3 cells which have been transfected with a full-length functional androgen receptor (AR). PC3AR2 cells were obtained as a generous gift from Dr. Ted Brown, Mount Sinai Hospital, Toronto, Ontario, Canada. Human prostate epithelial cells (RWPE-1) were purchased from ATCC, and cultured in Keratinocyte Serum Free Medium (K-SFM), supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF). All cell lines were subcultured at a ratio of 1:3 –

1:5, as described by the supplier. All cells were maintained at 37ºC in a 5% CO_2 incubator under sterile conditions.

Chemicals

Capsaicin *([(E)-N-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide]* was obtained from Tocris Bioscience (Bristol, UK). Capsaicin was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to create a stock concentration of 0.1M and stored at -20°C. All compounds were prepared and stored with minimal exposure to light to avoid oxidation. All other chemicals were purchased from Sigma unless otherwise specified.

Clonogenic assays

Prostate cancer cells were seeded at densities ranging from 400 to 4,000 cells per 10cm petri dish, depending on the cell line. Twenty-four hours after plating, cells were treated either with capsaicin alone (0.01 to 10.0 μM), radiation alone (0-8 Gy) or in combination. In the combination treatment regimen, cells were treated with capsaicin one hour prior to irradiation. The total treatment period was 5 days without media change. Capsaicin alone colony formation assays were mock irradiated. Colonies developed over 9-14 days and were stained with crystal violet and manually counted (See Figure 34). Each assay was internally controlled using untreated cells (cell media or 0.01% DMSO carrier). Relative plating efficiencies were expressed as percentages relative to the plating efficiency of untreated cells. The Sensitizer Enhancement Ratio (SER) was calculated by dividing Area Under the Curve (AUC) for the control (DMSO) by the AUC of the capsaicin treated cells. All experiments were performed in triplicates (at a minimum) and statistical analysis carried out as detailed below.

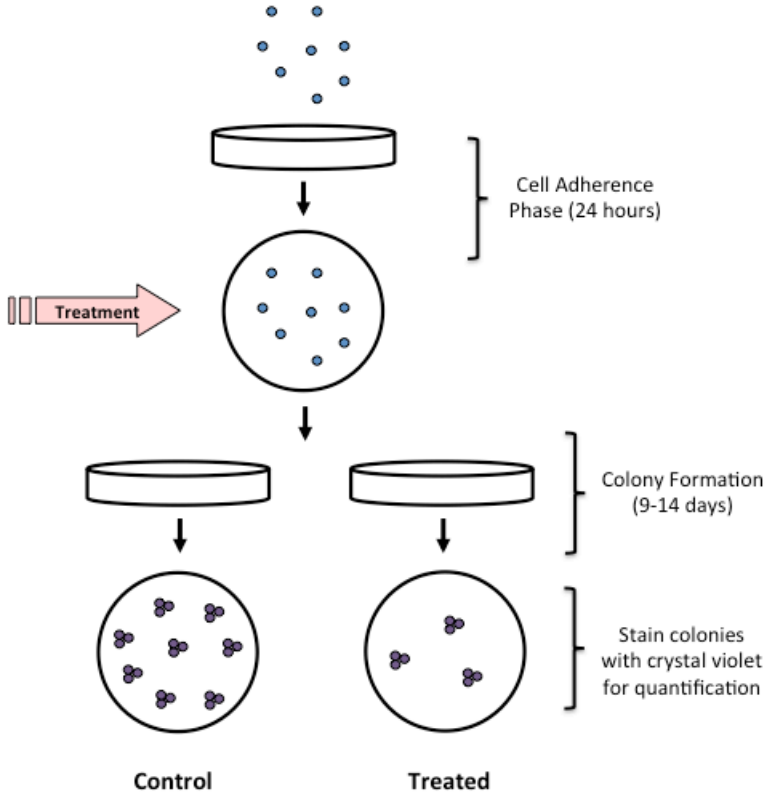


Figure 34. Clonogenic Assay Protocol. *To develop clonogenic assays, adherent cells are first plated in a petri dish and left to adhere for 24 hours. After adherence, cells are undergoing appropriate treatment (e.g. capsaicin and control) for 5 days consecutively. After the treatment period, cells are left untouched to allow for colony formation. At the end of the experiment, colonies are fixed and stained with crystal violet for quantification.*

Experimental design for TRP-V1 inhibitor experiments

Capsazepine (CZP), the TRP-V1 inhibitor, was obtained from Santa Cruz Biotechnology (CA, USA). CZP was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to create a stock concentration of 0.1M and stored at -20°C. Working solutions of CZP were prepared fresh in appropriate cell media. For clonogenic assay experiments, cells were treated with capsaicin and or CZP simultaneously as detailed in the clonogenic assay protocol. Clonogenic assay

experiments were carried out in duplicate at a minimum of three biological replicates per cell line.

Flow Cytometry

Cell cycle arrest patterns and S phase were determined by flow cytometry in LNCaP cells labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI) as per the published manufacturer protocol. Briefly, asynchronously growing cells (5 X 10^5 cells/plate) were plated using 10cm petri dishes and treated with capsaicin (10 μM) for 48 hours, followed by a dose of 2Gy of radiation. Control plates were treated with vehicle alone (cell culture media or 0.01% DMSO) and mock irradiated. Cells were pulse labeled with BrdU for 2 hours prior to harvesting. As a negative control, a no-BrdU control was included. Cells were trypsinised, fixed in ice cold 70% ethanol and stored at -20˚C until further analysis. Cells were subsequently washed with PBS buffer (Invitrogen, Burlington, ON, Canada) with 0.5% Tween-20 and treated with 2N HCl for 20 min to expose labeled DNA. Cells were incubated for 1hr on ice with anti-BrdU conjugated FITC (DAKO, Burlington, ON, Canada). Cells were washed, centrifuged, and resuspended in 10μg/ml PI, and allowed to incubate for 30 min 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 Dickinson, San Jose, CA, USA).

Western Blot Analysis

Western blot analyses of lysates from LNCaP treated cells were carried out as described (Venier, Colquhoun et al. 2012). Briefly, LNCaP cells were plated at a density of $1x10^6$ cells per 10 cm plate. Twenty-four hours after plating, adherent cells were treated with capsaicin (10μM) and irradiated (2Gy) after one hour of incubation. Control wells were treated with vehicle alone (DMSO 0.01%). After treatment for 24hrs, the cells were lysed using NP-40 lysis buffer containing inhibitors (leupeptin/pepstatin, aprotinin and phenylmethanesulfonylflouride), sodium dodecyl sulfate (SDS), deoxycholate and ethylenediaminetetraacetic acid (EDTA). Protein was

quantified using the Bradford protein assay technique prior to loading into 12% SDS gels for electrophoresis. Antibodies phosphor-NFκB p65 (Ser 536), NFκB p65, IκBα (L35A5), phosphor-IκBα (Ser 32) were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA) unless specified otherwise. Image quantification software (ImageJ, US National Institute of Health, Bethesda, MD, USA) was used to semi-quantitatively determine protein expression levels, relative to β-actin.

Immunocytochemisty Analysis

LNCaP and PC3 cells were trypsinized and seeded in triplicate at $2x10^5$ cells per well in a 24-well cell culture plate on round coverslips (Thermo Fisher Fisher#100411-9), and left to adhere overnight. After 24hrs cells were treated with capsaicin (10μM), and/or irradiated with 6 Gy (with vehicle alone). At 24hrs post-radiation and/or capsaicin treatment, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. After washing with 200 ul of TBS-TX 3 times, cells were blocked in 100ul PBS with 0.1% Triton, 1% BSA, and 1% goat serum at RT for 1 hour. Then blocking buffer was removed and cells were incubated with anti-phospho histone H2AX (ser139) antibody (Millipore) in blocking buffer over-night at 4^oC. The wells were then washed with 200ul of PBS 3 times and incubated with Alexafluor 488 labeled goat-anti-mouse secondary antibody (Invitrogen) at room temperature in the dark for 1 hour. Wells were washed with 200ul of PBS and nuclei stained with 0.5ug/ml DAPI in PBS for 10 min in the dark. After washing 3 times with 200ul PBS, wells were covered with mounting medium and coverslips. All images were processed identically and analyzed using the Zeiss Imager M1 microscope with Stereo Investigator software (MicroBrightField, Williston, VT) at 20X or 100X magnifications.

Effect of capsaicin and radiation on prostate cancer cells *in vivo*

Ethical approval for the *in vivo* component of this work was obtained from the University of Toronto Animal Research Ethics Board and all work was conducted in accordance with established guidelines and protocols approved by the Canadian Council on Animal Care (CCAC). Six-week old male nu/nu athymic nude mice (Harlan Laboratories Mississauga, ON,

Canada) were used in this study. 1 X 10^6 LNCaP cells resuspended in 100µL Matrigel solution (BD Biosciences, CA, USA) were inoculated subcutaneously unilaterally into the flank of each mouse. Mice were monitored each day for tumour growth. When tumours achieved an average volume of 100mm^3 (considered as day 1) mice were randomized into four treatment groups; control (n=15), capsaicin (n=15), radiation alone (n=15) or capsaicin and radiation (n=15). Capsaicin was administered orally by gavage on alternating days over a period of two weeks at a dose of 5mg/kg body weight. Capsaicin in ethanol (0.2M) was diluted fresh in saline solution prior to administration. Mice randomized to RT received one 6Gy fraction of RT (Faxitron machine) in sterile cages under general anesthesia (GA) (Dai, DeSano et al. 2009) , following the third administration of capsaicin or saline. Control animals were mock irradiated (See Figure 35). Mice were weighed thrice weekly with simultaneous tumour measurement. Mice with tumours exceeding 17mm diameter were culled in accordance with CACC guidelines. Serum samples were obtained by saphenous vein puncture during the treatment period. At experiment termination (day 54) serum samples were collected by direct cardiac puncture. Liver samples were also obtained at the termination of the study and fixed for histological analysis by a pathologist for analyzing any toxicities associated with the study. (See Figure 36)

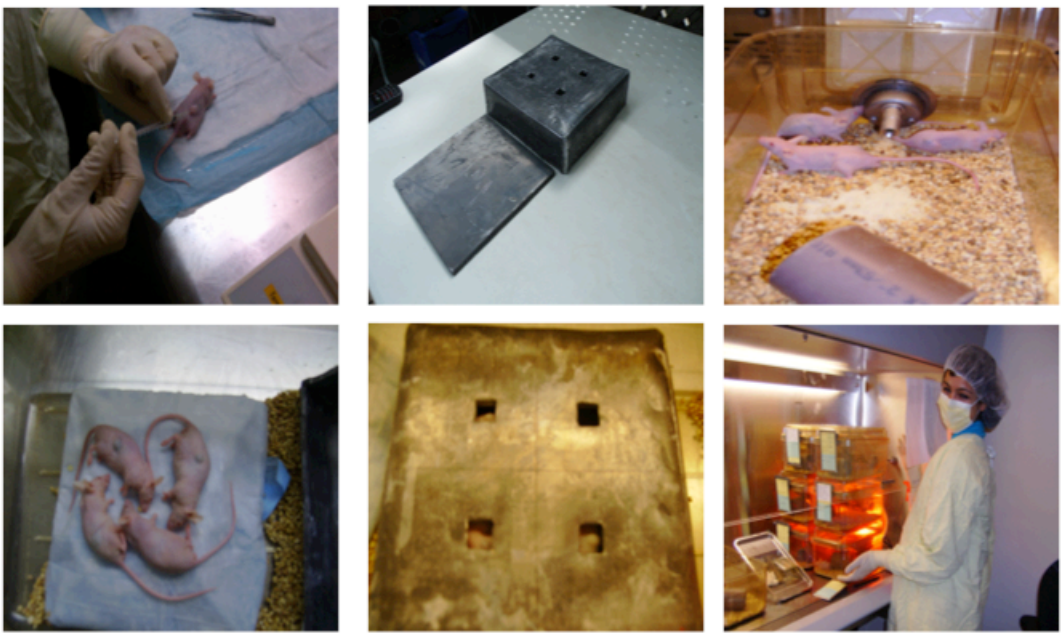


Figure 35: Protocol for Inoculation and Irradiation of Mice. *At 6 weeks of age, nude mice are inoculated with 1 X 10^6 LNCaP cells (resuspended in 100µL Matrigel solution) in the right flank using a 1mm syringe. Once tumours have reached 100mm^3, mice can begin treatment. A custom lead box was prepared for radiation of only xenograft tumours (second image). To irradiate mice, mice were anesthetised in their cages, and then gently positioned at the bottom of the lead shield. The top of the lead shield was then placed on the mice so that only their tumours were exposed to radiation. Radiation (6Gy) was administered in a sterile environment. After irradiation, mice were brought back to their rooms where they were monitored until they woke up from their anaesthetic.*

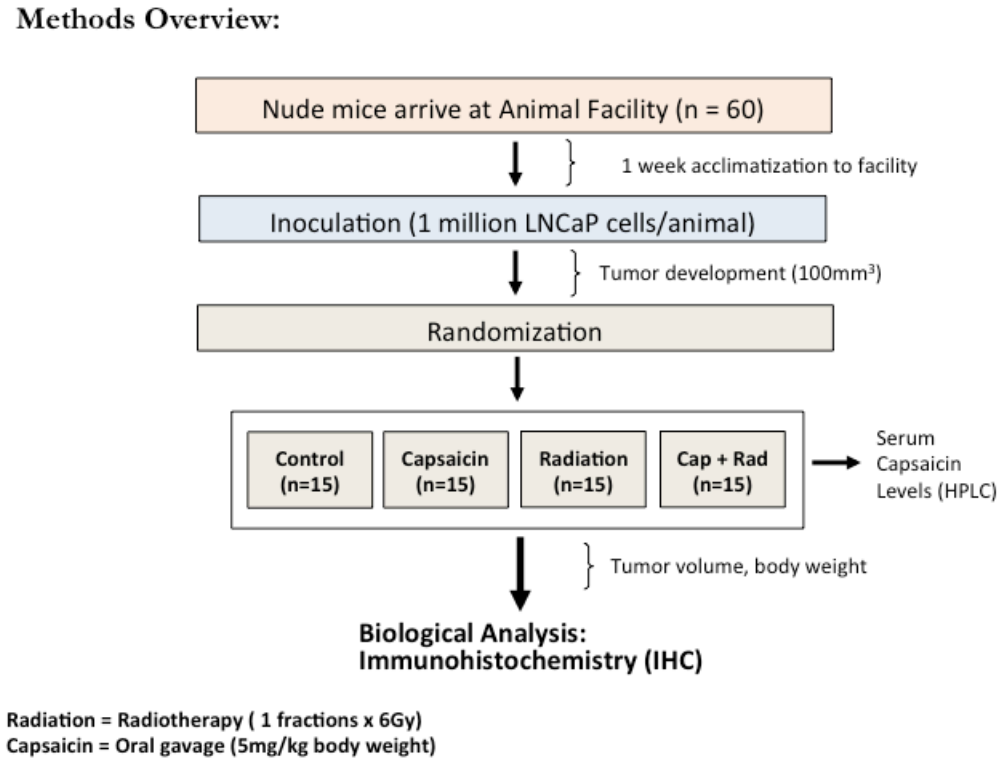


Figure 36: Capsaicin and Radiation Xenograft Experiment Timeline. *Sixty nude mice were randomized and left to acclimatize for one week after their arrival at the animal facility. Once acclimatized, mice were inoculated with LNCaP cells and left to grow. After the xenograft reached approximately 100mm^3, mice were randomized and treated with either capsaicin (5mg/kg body weight) or vehicle control for a period of two weeks on alternating days. One week into capsaicin treatment, mice were irradiated (6Gy) or mock irradiated (controls). Tumour volume and body weight was monitored thrice weekly throughout the duration of the study. At the termination of the study, sera and tumours were collected and analyzed appropriately.*

Serum extraction and liquid chromatography-mass spectrometry (LC-MS) for capsaicin analysis

Twenty microliters of serum from each sample of the *in vivo* studies was thawed and transferred to individual Eppendorf tubes. 5µL of 1µg/ml deuterated testosterone (d3T, C/D/N Isotopes) was

then added, followed by 50 µL of acetonitrile after which samples were vortexed for 5-10 sec and centrifuged for 5 min at 20,000 g to sediment precipitated protein. The clarified supernatant was transferred to LC vials for analysis. Standards were prepared in 50% methanol and QC samples with capsaicin spiked blank mouse serum were prepared similarly to the standards. Optima grade (Fisher) solvents and 18 MΩ water (Millipore) were used for sample preparation and subsequent LC-MS analysis.

Analysis was carried out with an Acquity UPLC coupled in series with a Quattro Premier (Waters). A 100 mm BEH C18, 1.7µ column (Waters) was used for separations with a 40-85% acetonitrile (ACN) gradient for 0.2-3 min, ramped 0.5 min to 98% ACN for flushing (2.5min), followed by a 2 min re-equilibration for an 8 minute run length (0.1% formic acid present throughout). All MS data was collected in ES+ at unit resolution with the following instrument parameters: capillary, 3.0 kV; extractor and RF lens, 3V and 0V; source and desolvation temperatures, 120 ºC and 300 ºC; desolvation and cone gas (N_2), 1000 L/hr and 50 L/hr; collision gas (Ar), 0.15 ml/min (6.2e-3 m bar). Compounds were detected using multiple reaction monitoring (MRM) with m/z 306>137 and 306>182 for capsaicin and m/z 292>97 for d3T (22V/17V, 22V/11V and 32V/21V cone/collision volt combinations used respectively for the three transitions) with 0.1 sec dwell each. Retention times (RT) for d3T and capsaicin were 2.35 and 2.8 min, respectively.

Quanlynx (Waters) was used for analysis of data using peak area ratios of capsaicin/d3T for calibration and quantification. Calibration standards ranged from 0.002 to 1µg/ml (6 points) with R2 >0.99 and all % deviation from nominal < 15% above 0.01µg/ml (omitting 1µg/ml due to curvature). Comparison of spiked serum with QC samples indicated little to no matrix interference with extraction efficiencies of 95%.

A peak with RT at 1.9 min was also observed in the capsaicin MRM's only in the serum from mice with capsaicin in diet, likely a capsaicin metabolite. Characterization of this potential metabolite was not possible however; a parent m/z could not be observed due to the low serum levels of both capsaicin and the metabolite.

Immunohistochemical Analysis

Immunohistochemical analysis was performed as previously described (Venkateswaran, Klotz et al. 2009). Briefly, deparaffinized sections were incubated with the primary antibody [anti-p27^{Kip1} rabbit polyclonal anti- body (Santa Cruz Biotechnology) diluted 1:100 (200 μg/mL) in PBS; Ki67 rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 (200 μg/mL) in PBS; and anti-γH2AX (Cell Signaling), phosphor-NFκB p65 (Ser 536) monoclonal antibody (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; NFκB p65 (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; IκBα (L35A5) (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS; phospho-IκBα (Ser 32) (Cell Signaling) diluted 1:100 (200 μg/mL) in PBS respectively, overnight at 4°C. Slides were then reacted with biotin-labeled anti-rabbit IgG/anti-mouse IgG and incubated with preformed avidin-biotin peroxidase complex (Vector Laboratories). Metal-enhanced diaminobenzidine substrate (Vector Laboratories) was added and sections were counterstained with hematoxylin and eosin.

Statistics

All *in vitro* clonogenic experiments were analyzed using GraphPad Prism. Between group variations for all *in vitro* experiments were assessed using Student's t-testing. Analyses of the *in vivo* results were performed using either Student's t-testing or repeated measures ANOVA techniques. Statistical analysis was performed using SAS software, version 8 (SAS Institute Inc., Cary, NC, USA).

5.4 Results

5.4.1 Capsaicin treatment reduces colony formation in prostate cancer cells

The colony forming assay was used to test the impact of capsaicin on the proxy proliferative rate of various prostate cancer cells: LNCaP (AR positive, p53 wild-type), PC3 (AR negative, p53 negative) PC3AR2 (PC3 cells transfected with full length androgen receptor, AR positive, p53 negative), and DU145 (AR negative, p53 mutated). In each of the cell lines tested, increasing concentrations of capsaicin treatment significantly reduced the proxy proliferation rate of the cancer cells. In the LNCaP cell line, cells were treated with 1, 10, and 20µM of capsaicin, which resulted in a 29%, 72%, and 92% reduction in colony formation, respectively. Treating the PC3AR2 cell line with these same concentrations of capsaicin resulted in a 30%, 83%, and 99% reduction in colony formation, a similar dose-response to LNCaP cells. The PC3 cell line was treated with 1, 10, and 50µM of capsaicin and had 11%, 35%, and 85% reduction in colony formation, respectively. The DU145 cell line was treated with 50, 100, and 200µM of capsaicin, a significantly higher dose compared to the other cell lines, and resulted in a 25%, 75%, and 99% reduction in colony formation. The concentrations of capsaicin were selected to determine the IC50 concentration of capsaicin for each cell line (Figure 37).

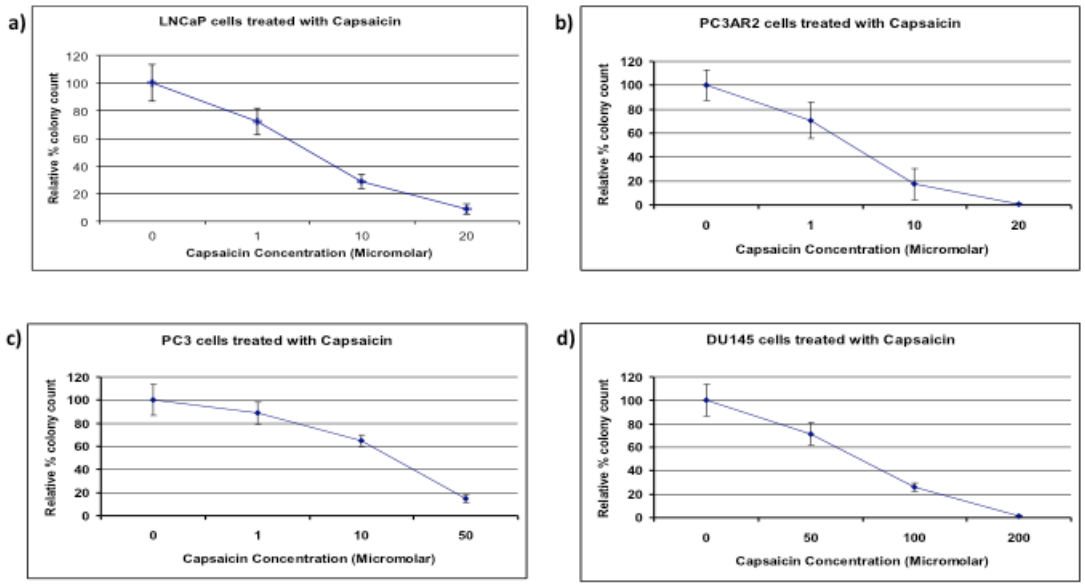
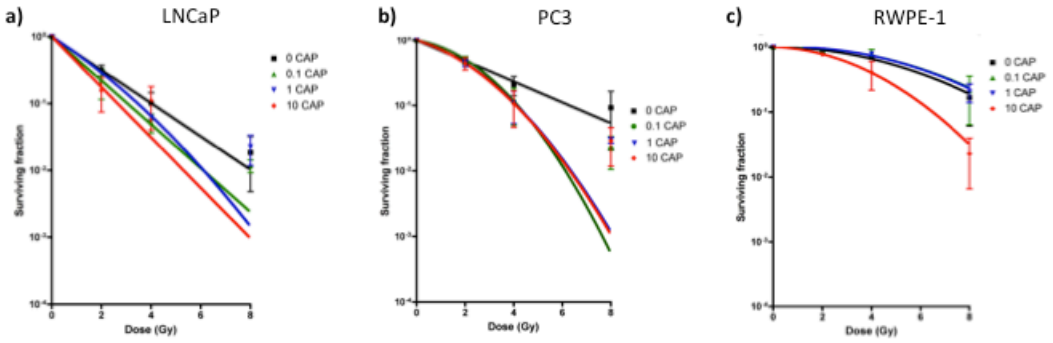


Figure 37: Capsaicin reduced the colony formation of Prostate Cancer cells in a dose-dependent manner. *To determine the effect of capsaicin on colony formation, we performed colony formation assays on a) LNCaP, b) PC3 c) PC3AR2 and d) DU145 prostate cancer cell lines with increasing concentrations of capsaicin (0-250µM). Each assay was carried out a minimum of three times for each cell line.*

5.4.2 Capsaicin treatment sensitizes prostate cancer cells to radiation

In order to assess the sensitizing effect of capsaicin on radiation, we generated radiation survival curves for LNCaP, PC3, and RWPE-1 cells using 0.1, 1.0, and 10 µM of capsaicin, respectively (See Figure 38). The RWPE-1 epithelial cell line was assessed to compare the sensitizing effect on malignant and non-malignant cells. Curves were normalized for cell kill with capsaicin alone. As depicted in Figure 38, PC3 cells were sensitized by capsaicin at 0.1 µM (SER = 1.2, SD = 0.195), 1 µM (SER = 1.2, SD = 0.099) and 10 µM (SER = 1.2, p = 0.283). LNCaP cells had a similar sensitization effect at the 0.1 µM (SER = 1.2, SD = 0.075), 1 µM (SER = 1.2, SD = 0.027) and 10 µM (SER = 1.2, SD = 0.022). These results suggest that there is not a dose-dependent response with increasing concentrations of capsaicin. Interestingly, RWPE-1 cells did not respond in the same manner, at concentrations of 0.1 µM (SER = 0.9, SD = 0.11) and 1 µM (SER = 0.9, SD = 0.33) capsaicin had no effect, while at 10 µM, capsaicin sensitized cells to RT (SER = 1.3, SD = 0.149). Since we did not see a dose-dependent response with increasing doses of capsaicin, we decided to perform subsequent mechanistic studies using only one concentration of capsaicin, which was found to be effective (10 µM).



[CAP]	LNCaP	PC3	RWPE-1
0.1 uM	SER = 1.2 (SD = 0.195)	SER = 1.1 (SD = 0.099)	SER = 0.9 (SD = 0.283)
1 uM	SER = 1.1 (SD = 0.075)	SER = 1.2 (SD = 0.027)	SER = 0.9 (SD = 0.022)
10 uM	SER = 1.2 (SD = 0.011)	SER = 1.2 (SD = 0.033)	SER = 1.3 (SD = 0. 149)

Figure 38: Low doses of capsaicin sensitize prostate cancer cells to radiation. *Survival curves for were generated using various concentrations of capsaicin (0.1, 1.0, and 10.0 µM) in a) LNCaP, b) PC3 and c) Normal Prostate Epithelial cell lines (RWPE-1). The sensitizer enhancement ratio (SER) was calculated using Area Under Curve (AUC) DMSO Control / Area Under Curve with Capsaicin, with corresponding standard deviation (SD). Each of the following experiments was performed a minimum of three times for each cell line.*

Immunocytochemical analysis revealed that treatment of LNCaP cells and PC3 cells with capsaicin and radiation increases the expression of the DNA damage marker, phospho-H2AX as demonstrated in Figure 39. Morphological changes to the nuclei of these cells also reflected an increase in cell death in the treatment groups.

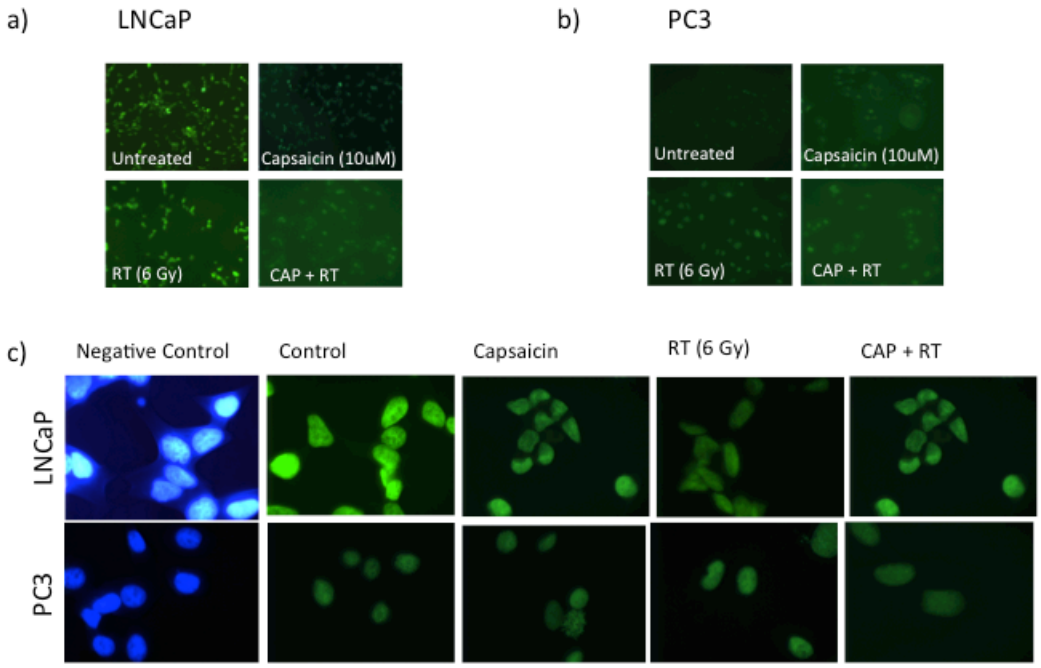


Figure 39: Capsaicin and radiation increase yH2AX expression in LNCaP and PC3 Cells. *a) Immunocytochemistry was performed on LNCaP and PC3 cells to determine the expression of H2AX after treatment with capsaicin (10uM), a single 6Gy dose of radiation (RT), or a combination of capsaicin and radiation (CAP + RT). Cells were counterstained with DAPI, and viewed at a 20X magnification (a,b), or 100X magnification (c).*

5.4.3 Capsaicin treatment alters the cell cycle distribution in LNCaP cells

To determine whether alterations in cell cycle contributed to the sensitization effect of radiation, LNCaP cells were treated with capsaicin (10 μM) and radiation, collected and fixed, and assessed for cell cycle alterations at increasing time points (10 minutes, 6 hours, 24 hours, 48 hours, and 72 hours) (Figure 40 and Table 3). We observed a significant reduction in the percentage of cells in the S-phase of the cell cycle when cells were treated with capsaicin and/or radiation compared to their respective controls at 24, 48, and 72 hours ($p < 0.05$). However, we did not see a significant difference in the percentage of cells in the S-phase after radiation alone when compared to cells that were treated with capsaicin followed by radiation. This data suggests that the inhibition of the cell cycle is not the primary mechanism mediating the sensitization effects of capsaicin.

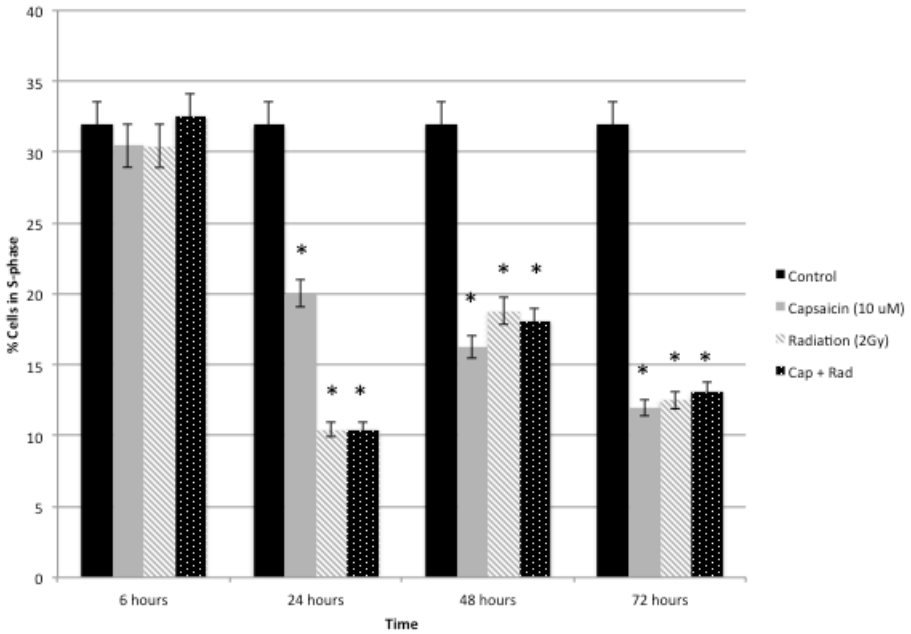


Figure 40: Capsaicin and Radiation Reduce the Percentage of Cells in the S-phase of the cell cycle maximally at 24 hours. *LNCaP cells treated with capsaicin and/or radiation were treated, labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI), and fixed at 6, 24, 48, or 72 hour timepoints, and then subsequently analyzed by flow cytometry to determine the percentage of cells in the S-phase of the cell cycle. Error bars represent the standard deviation (SD). All experiments were carried out in triplicate. The * symbol represents significance (p<0.001) relative to control.*

	CAP	RT	CAP + RT
6 hours	G0/G1: - 1.4 % S: - 1.5 % G2/M: + 2.9 %	G0/G1: - 8.4 % S: - 1.6 % G2/M: + 10%	G0/G1: - 7.4 % S: + 0.5 % G2/M: + 6.9 %
24 hours	G0/G1: +13.0% S: - 12.0% G2/M: - 1.1%	G0/G1: +24.8% S: - 21.6% G2/M: - 3.8%	G0/G1: +24.4 % S: - 21.6% G2/M: - 2.9%
48 hours	G0/G1: +17.3 S: - 15.8% G2/M: - 1.5%	G0/G1: + 11.8% S: - 13.2% G2/M: + 1.5%	G0/G1: + 13.5 S: - 13.9% G2/M: + 0.4%
72 hours	G0/G1: +23.3% S: - 20.0% G2/M: - 3.3%	G0/G1: +20.0% S: -19.5% G2/M: -0.8%	G0/G1: +20.2% S: - 18.1% G2/M: -1.3%

Table 3: Capsaicin and Radiation alter cell cycle distribution in LNCaP Cells. *LNCaP cells treated with capsaicin (CAP) and/or radiation (RT) were treated, labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI), and fixed at 6, 24, 48, or 72 hour timepoints, and then subsequently analyzed by flow cytometry to determine the distribution of cells in various phases of the cell cycle, relative to control.*

5.4.4 Capsaicin treatment suppresses NFκB expression in LNCaP cells by Western blot analysis

To better understand the mechanisms of radiosensitization after capsaicin treatment, we examined the NFκB pathway by Western Blot analysis. Analysis of the lysates after treatment revealed a reduction in phosphorylated NFκB (pNFκB) expression after capsaicin treatment alone or in combination with radiation. However, the levels of pNFκB expression increased in lysates that were treated with radiation alone, suggesting that capsaicin suppressed the

upregulation of pNF**κ**B following radiation exposure (Figure 41). Representative Western blots of pNF**κ**B and NF**κ**B are depicted in Figure 41, supporting the suppressive effects of capsaicin on the NF**κ**B pathway. Correspondingly, Western blot analysis of LNCaP cells treated with capsaicin and/or radiation demonstrated an up-regulation of the tumour suppressor proteins p21 and p27 ^{Kip1} in a time-dependent manner consistently across all treatment groups.

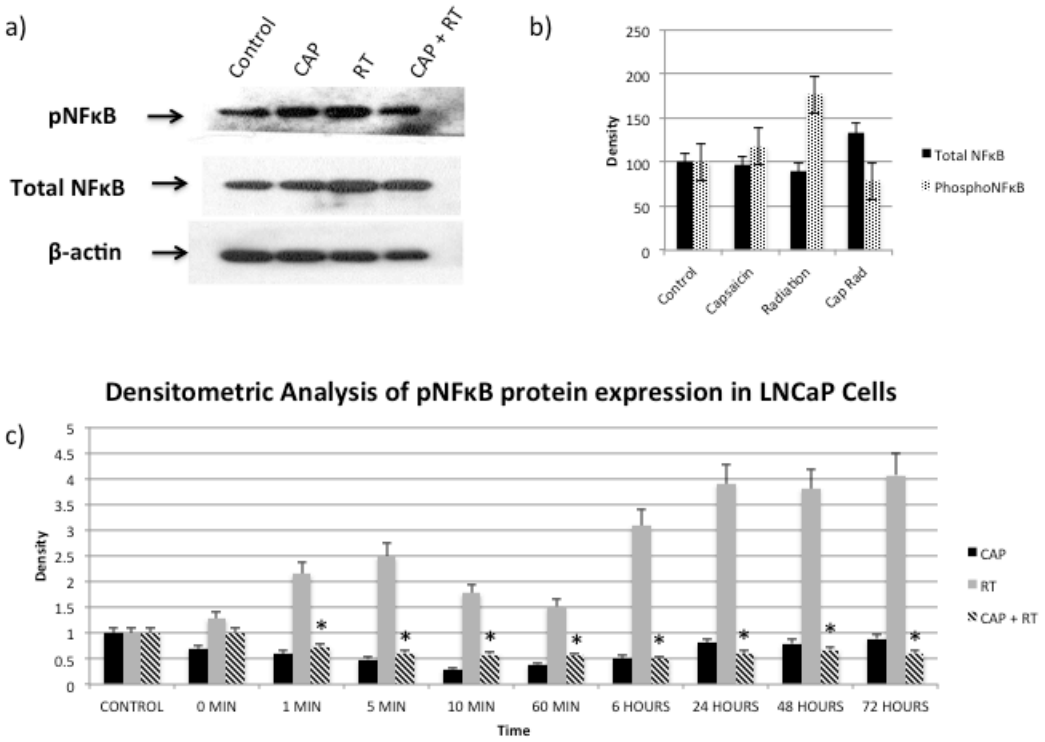


Figure 41: Capsaicin suppresses radiation-induced pNFκB activity in LNCaP Cells. *Using Western Blot we examined the change in total and phosho-NFκB expression over time. a) Western blot reveals suppression of radiation-induced pNFκB expression compared to control at 24 hours. b) Densitometry quantification of pNFκB and NFκB blots 24 hour timepoint. C) Western blot densitometry reveals that capsaicin (10μM) supresses the NFκB expression in LNCaP Cells treated with radiation (2Gy) at increasing timepoints (0 minutes – 72 hours). All Western blotting was carried out a minimum of three times to obtain accurate biological replicates. The * symbol represents significance (p<0.001) relative to radiation alone.*

5.4.5 Capsaicin treatment sensitizes cells to radiation independent of the vanilloid receptor, TRP-V1

A number of studies have suggested that the transient receptor potential-vanilloid 1 receptor (TRP-V1)—also commonly referred to as the capsaicin receptor—may be involved in mediating some of the anti-proliferative effects of capsaicin (Ziglioli, Frattini et al. 2009). To understand whether TRP-V1 was involved in mediating the radio-sensitization effects on PC3 cells, we used one of the most commonly used pharmaceutical inhibitors of TRP-V1, capsazepine (CZP). To assess whether CZP could reverse the inhibitory effect of capsaicin on colony formation, clonogenic assess were treated concurrently capsaicin and CZP. Results of these experiments demonstrated that the inhibition of the TRP-V1 receptors by CZP did not reverse the effects of capsaicin as depicted by no alteration on colony formation (Figure 42). Furthermore, mechanistic studies analyzing NFκB signaling by Western Blot analysis revealed that treating PC3 cells with capsaicin and CZP does not reverse the phosphorylated NFκB (pNFκB) suppression induced by capsaicin. Unexpectedly, CZP treatment exerted independent effects on pNFκB inhibition, which prevented us from determining the role of TRP-V1 receptor in sensitizing cells to radiation (Dickenson and Dray 1991, Brandelius, Yudina et al. 2011, Tsagogiorgas, Wedel et al. 2012). Taken together, we found that treating PC3 cells with the TRP-V1 inhibitor, CZP, did not reverse the sensitizing effects of capsaicin, suggesting that capsaicin is not sensitizing cells predominately through TRP-V1 signalling. Furthermore, we have found that CZP alters clonogenic survival and exerts effects on NFκB signaling independent of capsaicin.

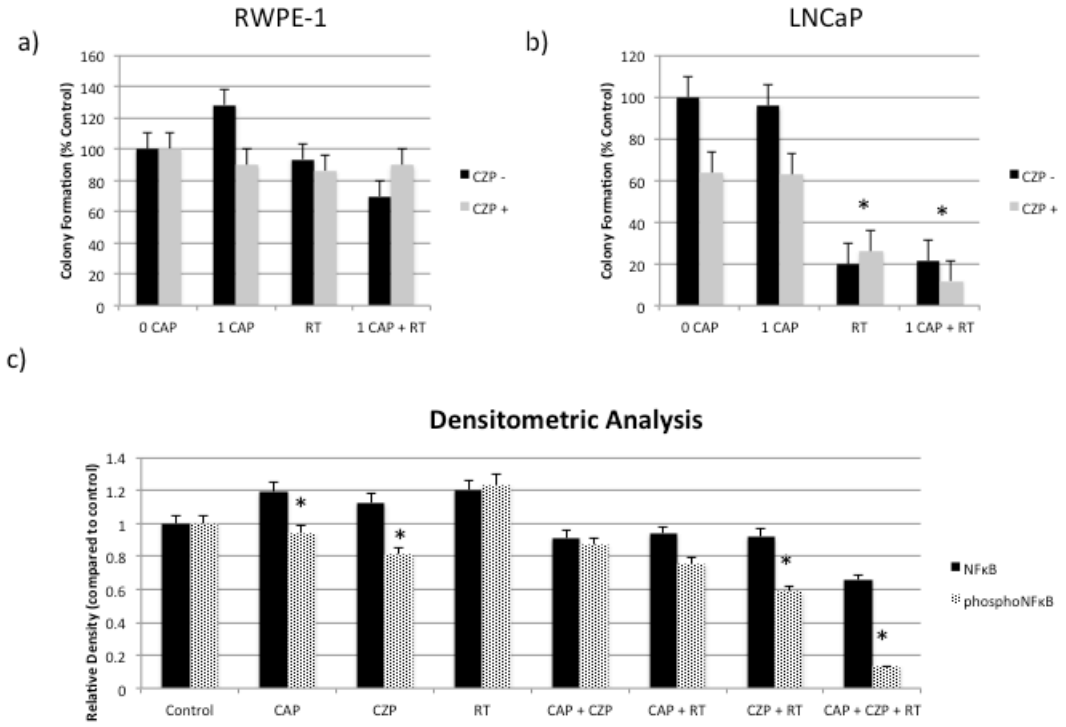


Figure 42: Capsaicin does not sensitize cells predominately through the TRP-V1 receptor. *a) Colony formation assays in the RWPE-1 and LNCaP cell lines treated with 1uM of capsaicin (CAP) and/or 2Gy of radiation (RT) with and without the TRP-V1 inhibitor, capsazepine (CZP). RWPE-1 cells (a) show no significant changes in colony formation when treated with CAP and RT in the presence or absence of CZP. LNCaP cells (b) show a reduction in colony formation when treated with CAP and RT. These effects were enhanced, but not reversed, by CZP. Mechanistic studies examining the expression of pNFκB and NFκB by Western blot analysis revealed that LNCaP cells treated with CAP, RT, or CZP showed a reduction in NFκB activity, suggesting that the TRP-V1 receptor may not be fully responsible for the sensitization effects of capsaicin on prostate cancer cells. All clonogenic assays and Western blot experiments were replicated a minimum of three times. The * symbol represents significance ($p<0.05$) relative to control.*

5.4.6 Capsaicin and radiation treatment reduced tumour growth in a mouse xenograft model

We tested the radiosensitizing effect of capsaicin *in vivo* by using the LNCaP xenograft model (described above) (Figure 43). Tumours were measured thrice weekly during the duration of the study. We observed a significant difference in the overall tumour growth rates between each of the groups overtime ($p<0.001$). Mice in the capsaicin treatment group ($p<0.001$), radiation group, and capsaicin and radiation group had a significantly slower tumour growth rate compared to the control group ($p<0.001$ and $p<0.001$, respectively). Furthermore, the capsaicin and radiation group had a significantly slower growth rate than either the capsaicin alone group ($p<0.001$) or radiation alone group ($p<0.03$), suggesting that capsaicin might be potentiating the effect of radiation by sensitizing the tumour cells. Growth delay calculations depict a 'greater than additive' effect for the capsaicin and radiation group compared to either treatment alone. Mice had no significant change in body weight or food consumption (data not shown). Capsaicin was well tolerated with no liver toxicities as assessed histologically. Two mice experienced mild to moderate inflammation of the esophagus/upper stomach area. Interim serum analyses revealed the presence of capsaicin and an associated metabolite in the treatment groups one hour after capsaicin administration.

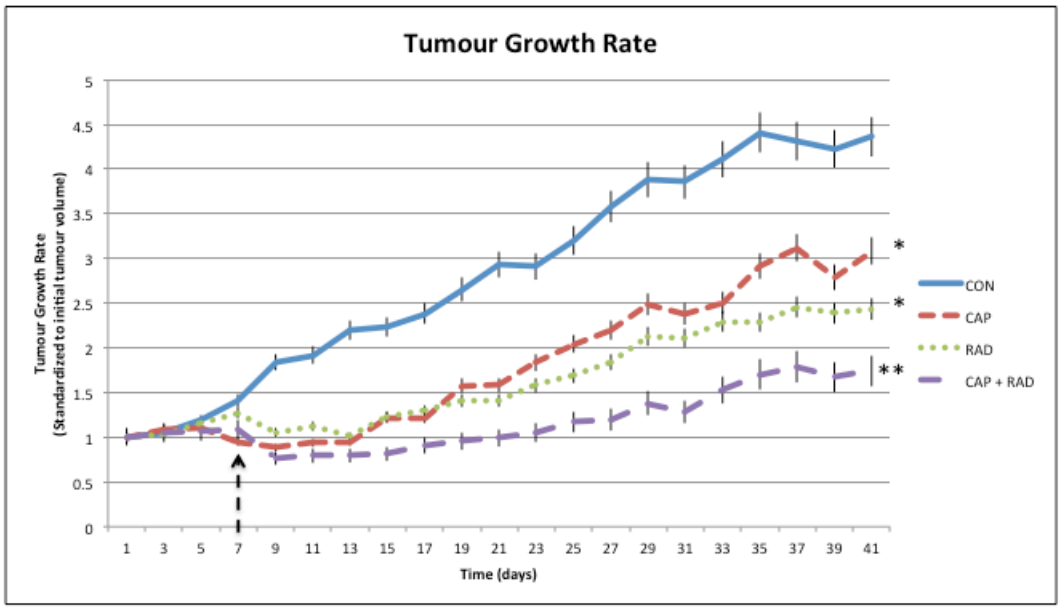


Figure 43: Capsaicin and Radiation significantly reduce tumour growth rate. *Variation in tumour volume (mm3) over time in four different treatment groups; Control (vehicle alone), Capsaicin (5mg/kg body weight), Radiation (6 Gy) or Capsaicin and Radiation in combination (indicated by the broken arrow). Capsaicin was administered on Days 12-24, and animals were irradiated (6 Gy) on Day 16. Tumour growth was monitored over time. Error bars represent standard deviation (SD). The * symbol denotes significance (p<0.001) * relative to control. ** denotes significance (p<0.05) relative to radiation alone.*

5.4.7 Tumours of mice treated with capsaicin and radiation display markers of persistent DNA damage (γH2AX), reduction in proliferative marker, Ki67, and NFκB

At the termination of the study, tumours were extracted from the mice and processed for pathological analyses (Figure 44). Immunohistochemical staining for Ki67 of prostate cancer tumours showed a reduction in the levels of Ki67 expression in all of the treatment groups. Next, we studied if there was any occurrence of DNA damage as a consequence of the treatment using the DNA damage marker, γH2AX. I demonstrated the presence of positively stained nuclei only in the capsaicin and radiation combination group, suggesting sustained and persistent DNA

damage in this treatment group. The *in vitro* studies suggested that capsaicin mediated its radio-sensitizing effects through the inhibition of radiation-induced NF**κ**B expression. To determine whether capsaicin altered the expression of NF**κ**B expression *in vivo*, prostate tumours were stained for NF**κ**B expression. Expression levels were positive in the control group, high in the radiation group, and weak in both the capsaicin group and the capsaicin and radiation group, recapitulating the effects observed *in vitro*.

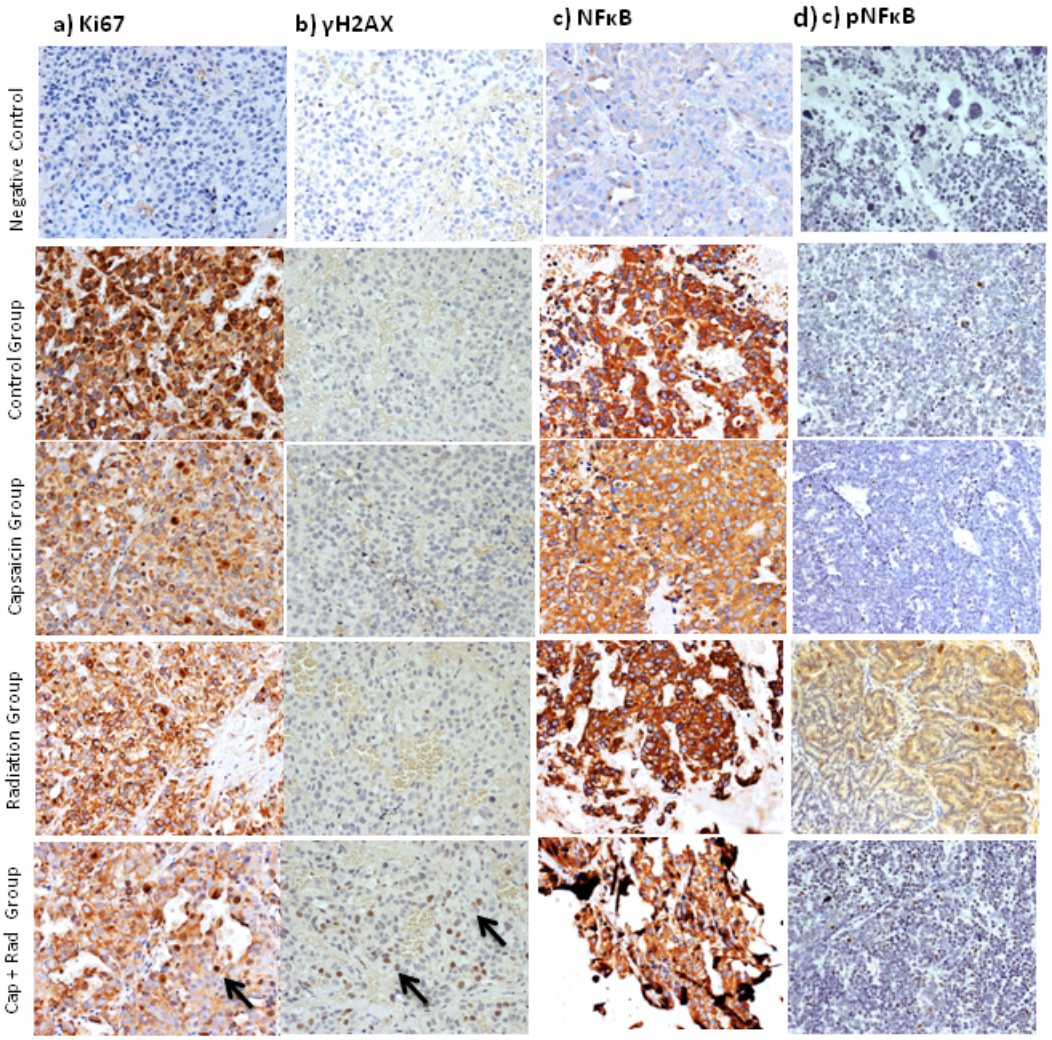


Figure 44: Immunohistochemical analysis of LNCaP xenograft tumours reveal that capsaicin and radiation alter the expression of Ki67, γH2AX, and NFκB. *LNCaP xenograft tumours were fixed and stained with a) Ki67, expressed as Ki67 index control group (KI:55), capsaicin group (KI: 7.5), radiation group (KI: 32.5), capsaicin and radiation group (KI: 5); b) of γH2AX expression control group (negative), capsaicin group (negative), radiation group (negative), capsaicin and radiation group (positive); c) NFκB expression control group (diffusely positive), capsaicin group (weak), radiation group (highly expressed), capsaicin and radiation group (weak); d) pNFκB expression control group (diffusely negative), capsaicin group (negative), radiation group (diffusely positive), capsaicin and radiation group (negative); The asterisk (*)*

and double asterisk (**) symbol represents significance (p<0.001) relative to control or radiation alone, respectively.

Enhancing the effect of radiotherapy through the use of successful radio-sensitizing agents is a promising strategy for the treatment and management of prostate cancer (Girdhani, Bhosle et al. 2005, Palacios, Miyake et al. 2013).

5.5 Discussion

In the current study, we have demonstrated that capsaicin leads to preferential radio-sensitization of prostate cancer cells compared to non-malignant epithelial cells through the suppression of the NFκB signalling. The *in vivo* analyses revealed that the oral administration of physiologically relevant doses of capsaicin, when combined with radiation, is well tolerated, significantly reduces the tumour growth rate, and correspondingly alters NFκB and γH2AX expression in LNCaP xenograft tumours. These findings provide pre-clinical evidence supporting capsaicin as a novel radio-sensitizing agent for prostate cancer.

Targeting the androgen signaling pathway has been one approach to enhancing the effect of radiation for prostate cancer (Comstock, Augello et al. 2013). The 5-alpha-reductase inhibitors, dutasteride and finesteride, have been intensely investigated for their chemopreventive effects and can be used as an adjunct to radiotherapy (Musquera, Fleshner et al. 2008). To understand whether capsaicin altered the effects of radiation in cells with variable androgen dependence, we generated survival curves for both the androgen-sensitive LNCaP cell line, as well as the androgen-insensitive PC3 cell line. Results of these studies indicated that although capsaicin alone was more effective at reducing the colony formation in androgen-sensitive cell lines, as previously reported (Venier, Colquhoun et al. 2012), the sensitizing enhancement ratio (SER) was altered independent of androgen status. This suggests that the radio-sensitizing effect of capsaicin is not mediated via the androgen signaling pathway *in vitro*. We did not observe a dose-dependent effect, showing similarity in the sensitizing effects in both PC3 and LNCaP cells with capsaicin concentrations at 1.0μM and 10.0μM. It is possible that I would have seen a dose-

dependent effect with treatment at higher concentrations of capsaicin, however, these concentrations are not deemed to be physiologically relevant.

A number of agents that target cell cycle regulation have been investigated as potential radio-sensitizing agents (Ding, Zhang et al. 2013, Palacios, Miyake et al. 2013). As radiotherapy induces cell cycle arrest predominately in the G2/M phase of the cell cycle, allowing for an increase in the percentage of cells in the G1-phase and a reduction in the percentage of cells in the S-phase, targeting alternate phases of the cell cycle has been used to sensitize tumours to radiation (Ding, Honda et al. 2003). Since capsaicin inhibits cell cycle through G1/S phase inhibition, we anticipated that cell cycle inhibition might be responsible for the radio-sensitizing effects of capsaicin. However, my results suggest that although capsaicin and radiation caused significant reduction in the percentage of cells in the S-phase of the cell cycle, this effect was not significantly greater than with either treatment modality alone. This suggests that similar mechanisms may be affecting cells after treatment with radiation or capsaicin. Protein analyses of the tumour suppressor proteins p21 and p27^{Kip1} demonstrated up-regulation in a time-dependent manner, when treated with either capsaicin or radiation alone. However, the combination did not demonstrate a 'greater than additive' effect, indicating that capsaicin does not enhance the effect of radiation primarily through cell cycle inhibition.

The nuclear transcription factor kappa-B (NFκB) signalling pathway is upregulated following radiation in prostate cancer cells which promotes cell survival and proliferation (Kimura, Bowen et al. 1999, Pajonk, Pajonk et al. 1999, Li and Stark 2002, Ahmed and Li 2008). Such responses have been attributed to clonogenic survival and resistance; hence, targeting this pathway has been proposed to enhance the effect of radiation by combating NFκB-induced resistance (Li and Stark 2002). Several studies have found that impairing NFκB activation, through the inhibition of TNF activation or IκB phosphorylation, significantly enhances the lethality of radiation (Royds, Dower et al. 1998, Rayet and Gélinas 1999, Ding, Honda et al. 2003, Ahmed and Li 2008, Mohamed and McFadden 2009, Cachaço, Carvalho et al. 2010, Tafani, Pucci et al. 2013). Mori et al. have shown capsaicin to down regulate NFκB activity through IKBα degradation (Mori, Lehmann et al. 2006). These studies reveal that capsaicin inhibits radiation-induced NFκB expression in a time-dependent manner. Furthermore, at the 24 hour time point, LNCaP cells treated with capsaicin and radiation have a reduction in a number of proteins involved in the NFκB signalling pathway (data not shown). This indicates that NFκB signalling plays a role in

mediating the radio-sensitizing response induced by capsaicin. In the *in vivo* studies using the LNCaP xenograft model, we observed similar results as radiation-induced NFκB expression was suppressed by capsaicin administration and resulted in sustained DNA damage, marked by the presence of phosphorylated H2AX, at the termination of the study. Hence, it is likely that inhibiting NFκB may act to inhibit cell survival factors and to enhance DNA damage repair, and thus increase cell death and tumour control, which suggests that the inhibition of NFκB is responsible for radio-sensitizing prostate cancer cells. Several reports have shown that inhibition of NFκB by natural compounds can sensitize prostate cancer cells to radiation (Singh and Khar 2006, Luqman, Meena et al. 2011).

The vanilloid receptor, TRP-V1, belongs to the family of transient receptor potential (TRP) cation-selective receptors that modulate the concentration of intracellular calcium upon activation by capsaicin or other stimuli (i.e. heat, acidity, etc.) (Stein, Santos et al. 2004, Van Haute, De Ridder et al. 2010). Few studies have reported that TRP-V1 mediates the anti-carcinogenic potential of capsaicin and this mechanism still remains unclear (Sanchez, Sanchez et al. 2005, Ziglioli, Frattini et al. 2009). To understand whether capsaicin acts to enhance the effect of radiation through TRP-V1 activation, we used the TRP-V1 inhibitor, capsazepine (CZP), and found that it did not reverse the radio-sensitizing effects of capsaicin. Interestingly, capsazepine independently altered a number of proteins in the NFκB pathway, suggesting that capsazepine may have TRP-V1 independent effects, which has been established previously (Dickenson and Dray 1991, Brandelius, Yudina et al. 2011, Tsagogiorgas, Wedel et al. 2012). Therefore, we were unable to fully understand the role of the TRP-V1 receptor in terms of its radio-sensitizing potential in prostate cancer cells. It is also likely that capsazepine may alter other receptors in the TRP-V1 family (Yamamura, Ugawa et al. 2004, Zhang and Barritt 2004). Further detailed analyses with more specific TRP-V1 inhibitors or knock-out models are necessary to understand the how the TRP-V1 receptor affects the radio-sensitizing effect of capsaicin in prostate cancer.

To study the effects of capsaicin and radiation *in vivo,* an LNCaP xenograft model was used. Results of this study revealed that oral administration of capsaicin administered three times a week over a two-week period significantly reduced the tumour growth rate, prolonged the tumour growth delay, decreased the proliferative index, weakened NFκB expression, and induced sustained DNA damage, marked by the presence of phosphorylated form of H2AX.

Sanchez et al. have reported that capsaicin administered subcutaneously can reduce the growth rate of PC3 xenograft tumour (Sánchez, Sánchez et al. 2006).

The bioavailability of capsaicin in mice is not well documented and limited clinical studies have found that consumption of chili peppers can result in serum concentrations of capsaicin in the nanomolar range (Govindarajan and Sathyanarayana 1991, Al Othman, Ahmed et al. 2011). In this study, mice had detectable levels of capsaicin in the blood, with no indication of toxicity, after the oral administration of 5mg/kg b.w. of capsaicin, suggesting that capsaicin is bioavailable and well tolerated. Furthermore, mice administered capsaicin alone also experienced a significant reduction in the growth rate and the proliferative index of LNCaP tumours, and when combined with one 6Gy fraction of radiation, tumours demonstrated a further reduction in tumour growth rate and growth delay. Immunohistochemical studies of these tumours revealed the presence of DNA damage only in tumours treated with both capsaicin and radiation, indicating sustained DNA damage, as tumours were collected nearly four weeks after treatment at the termination of the study (Dai, DeSano et al. 2009, Scully and Xie 2013). Similar to the *in vitro* mechanistic studies, *in vivo* tumours treated with capsaicin and radiation resulted in the suppression of radiation-induced NFκB expression, suggesting that this mechanism may be driving radio-sensitizing effects of capsaicin.

In summary, we have revealed for the first time, that capsaicin enhances the effect of radiation in pre-clinical model systems with no significant toxicities on normal tissue. Furthermore, at low doses capsaicin treatment differentially affects cancerous and normal epithelial cells through the suppression of NFκB activity *in vitro*. The *in vivo* analyses revealed that oral administration of capsaicin is well tolerated and significantly reduced the tumour growth rate, proliferative index, and NFκB expression, resulting in induced and sustained DNA damage. Based on the present findings, it would be important to conduct clinical trials to assess the radio-sensitizing effects of capsaicin and determine whether it can be used as an adjunct to current radiotherapy regimens.

5.6 Supplementary Experimental Work Evaluating the Role of the Transient Receptor Potential (TRP) Proteins and Capsaicin in Prostate Cancer

The Transient Receptor Potential – Vanilloid Receptor-1 (TRP-V1) ion channel is often referred to as the capsaicin receptor. It has six trans-membrane segments (similar to the voltage-gated potassium channels), as well as both an amino (N) and carboxyl (C) terminal, which resides in the intracellular compartment as depicted in Figure 45 (Julius 2013, Zheng 2013). The TRP-V1 receptor can be activated by capsaicin and a number of other stimuli such as heat (>45°C), noxious chemicals, and acidity (protons).

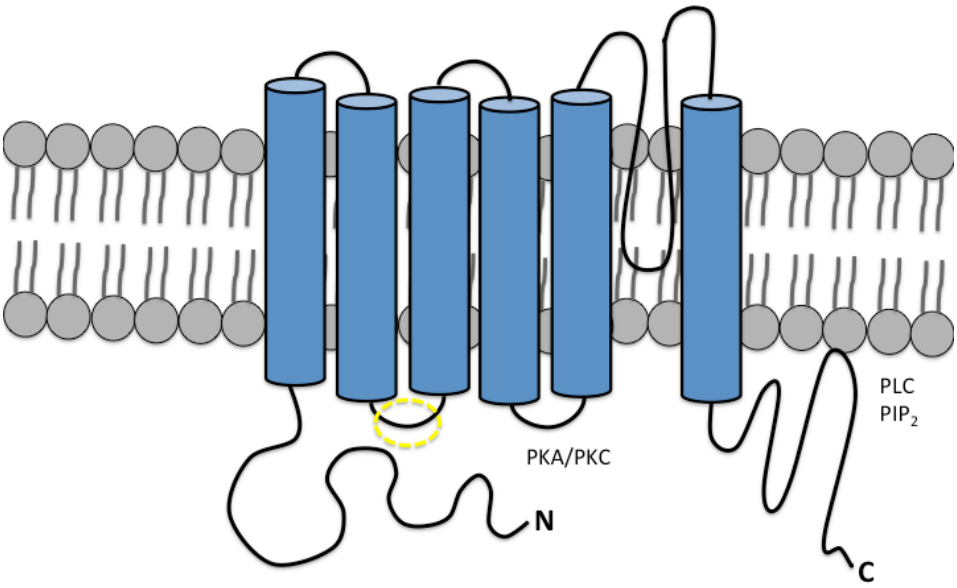


Figure 45: The Transient Receptor Potential – Vanilloid Receptor-1 *comprises six trans-membrane segments (similar to the voltage-gated potassium channels), as well as both an amino (N) and carboxyl (C) terminal, which resides in the intracellular compartment. Capsaicin*

binding occurs between the second and third trans-membrane segment as depicted by the yellow circle.

Capsaicin binds the TRP-V1 receptor by penetrating through the cell membrane and binding to the cytoplasmic loop intervening between the second and third trans-membrane (TM2 and TM3) regions in the vanilloid binding pocket, where the vanillyl aliphatic side chain is proposed to interact with aromatic and/or other hydrophobic residues (Jordt and Julius 2002, Julius 2013). Upon activation, TRP-V1 mediates the translocation of important signalling molecules (mainly calcium) across the plasma membrane, which can alter enzymatic activity and initiate various processes (e.g. endocytosis and exocytosis) within the cell (Julius 2013). This process can mediate a number of biological processes including calcium regulation, neuronal growth cone guidance, keratinocyte development, sensory transduction, fertilization, and cell survival (Jordt and Julius 2002, Zheng 2013).

Historically, capsaicin has been used pharmaceutically as a topical ointment for pain management, as the TRP-V1 ion channels are located predominately on sensory neurons (Tominaga and Caterina 2004). Capsaicin (applied topically) penetrates through the membrane to activate the TRP-V1 receptors located on the sensory neurons. This causes the release of Substance P, which desensitizes the neuron and thereby reduces pain sensation. It is important to note that the TRP-V1 receptor is also activated by a number of other stimulants (e.g. acidity and heat) and can be sensitized when other pathways are activated, namely phospholipid C (PLC) and phosphatidyl-inositol bisphosphate (PIP_2) (Julius 2013, Zheng 2013).

The role of capsaicin on activation of the TRP-V1 receptor in prostate cancer remains unclear (Ziglioli, Frattini et al. 2009). It has been reported that the TRP-V1 receptor is expressed in prostate cancer tissue, corresponding to increases in grade and stage of disease (Czifra, Varga et al. 2009). Although *in vitro* studies have reported that the TRP-V1 receptors are functional in prostate cancer cells and can induce an increase in intracellular calcium upon activation, these studies have not yet been validated and replicated using *in vivo* models (Sanchez, Sanchez et al. 2005, Czifra, Varga et al. 2009). In the following supplementary studies, we set out to confirm whether TRP-V1 receptors were present on prostate cancer cells and whether they were functional using established agonists and antagonists.

To understand whether the TRP-V1 receptors were present on LNCaP cells, we performed immunocytochemistry on fixed cells using anti-TRP-V1 antibodies for staining (as previously described). Results from this study indicated that the TRP-V1 receptor was present on the LNCaP cells in both the cytoplasmic and nuclear regions (See Figure 46).

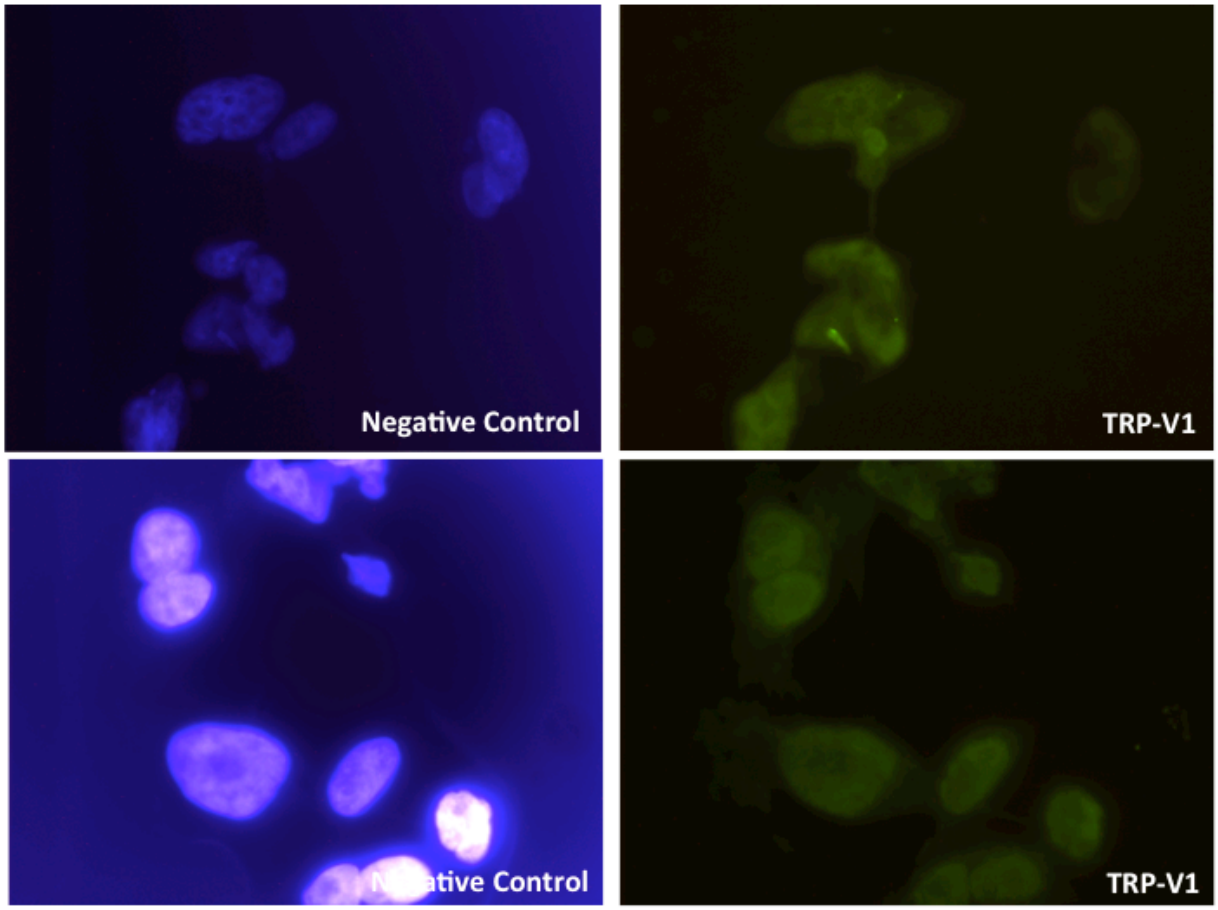


Figure 46: TRP-V1 is present on LNCaP prostate cancer cells in both the nuclear and cytoplasmic compartments. *LNCaP cells were fixed and stained for TRP-V1. Images were processed identically and analyzed using the Zeiss Imager M1 microscope with Stereo Investigator software (MicroBrightField, Williston, VT) at 100X magnifications.*

Once we confirmed expression of TRP-V1 receptors in LNCaP cells, we set out to determine whether the TRP-V1 receptor played a role in mediating the anti-proliferative effects of

capsaicin. To evaluate this, functional studies were conducted using commercially available TRP-V1 agonists and antagoinsts, Resiniferatoxin and Capsazepine (Santa Cruz), respectively.

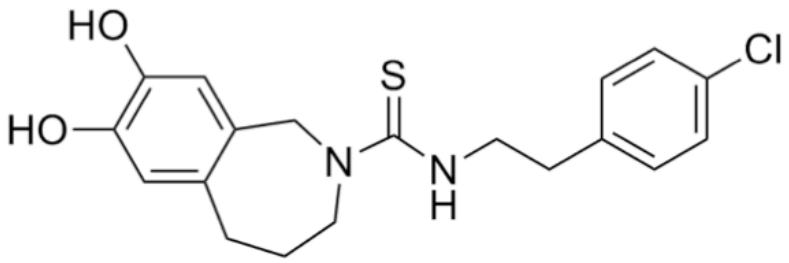


Figure 47: Chemical Structure of TRP-V1 antagonist, Capsazepine.

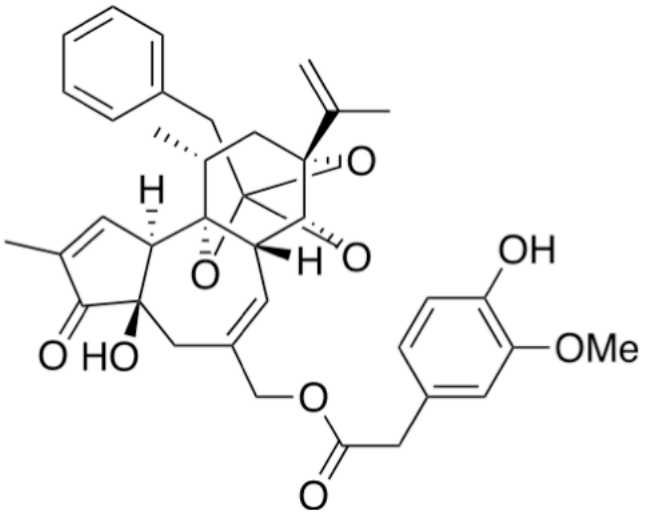


Figure 48: Chemical Structure of the TRP-V1 Agonist, Resinfertoxin.

To understand whether the activation of the TRP-V1 receptor can inhibit proliferation, we assessed the antiproliferative effects of the commercially available ultra-potent TRP-V1 activator, Resinferatoxin (RFX) (which is derived from the Euphorbia cactus), using the MTS cell proliferation assay. We found that RFX was able to significantly reduce the proliferation of both LNCaP and PC3 cells (Figure 47 and 48). The proliferation of LNCaP cells was significantly reduced at concentrations ranging from 20μM to 100μM, whereas PC3 cells were inhibited at concentrations greater than 100μM (as depicted in Figure 49 and 50). This suggests

that the activation of the TRP-V1 receptor can induce significant proliferative changes. Nonetheless, it is also plausible that at such high concentrations, RFX may induce anti-proliferative effects through other pathways.

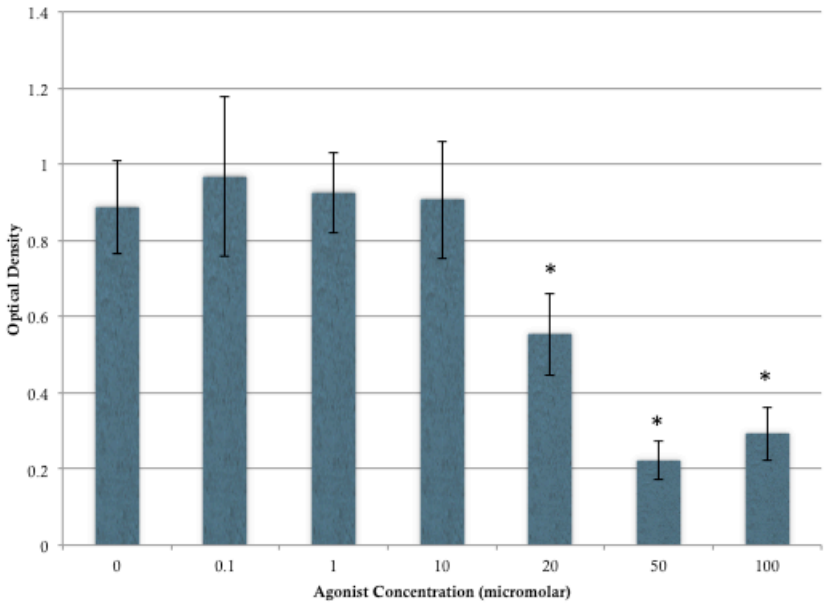


Figure 49: Resinferatoxin (RFX) Reduces the proliferation of LNCaP Cells in a Dose-Dependent Manner. *MTS Proliferation studies demonstrate that RFX reduces the proliferation of LNCaP cells at concentrations ranging from 20µM -100µM. The * symbol denotes statistical significance (P < 0.001).*

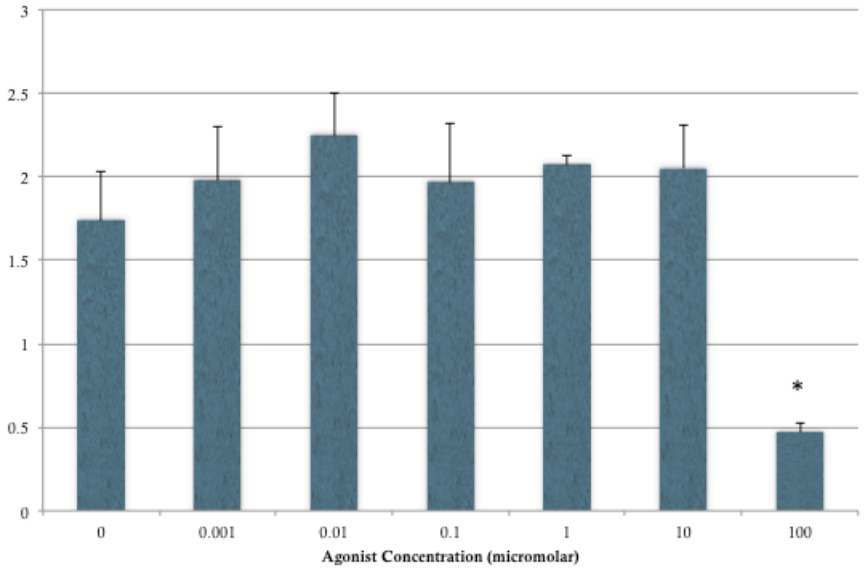


Figure 50: Resinferatoxin Reduces the proliferation of PC3 Cells at high concentrations. *MTS Proliferation studies demonstrate that RFX reduces the proliferation of PC3 cells at a concentration of 100µM. The * symbol denotes statistical significance (P < 0.001).*

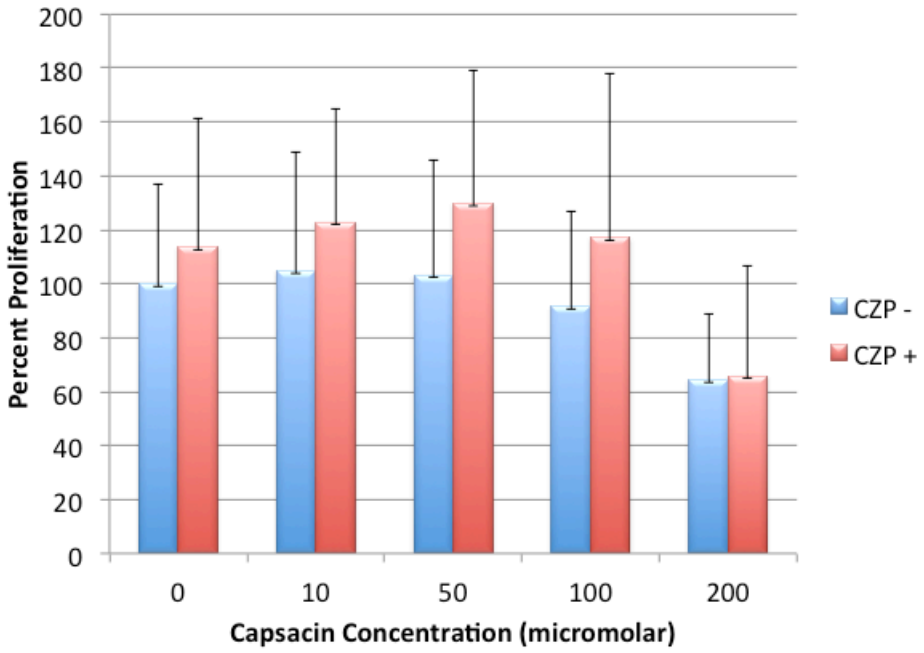


Figure 51: Capsazepine (CZP) does not reduce the anti-proliferative effects of capsaicin at high doses in PC3 Cells. *Using the MTS Cell Proliferation Assay, PC3 cells were treated concurrently with increasing doses of capsaicin with or without CZP. Results suggest that CZP was unable to reverse the anti-proliferative effects of capsaicin in a significant manner in PC3 cells.*

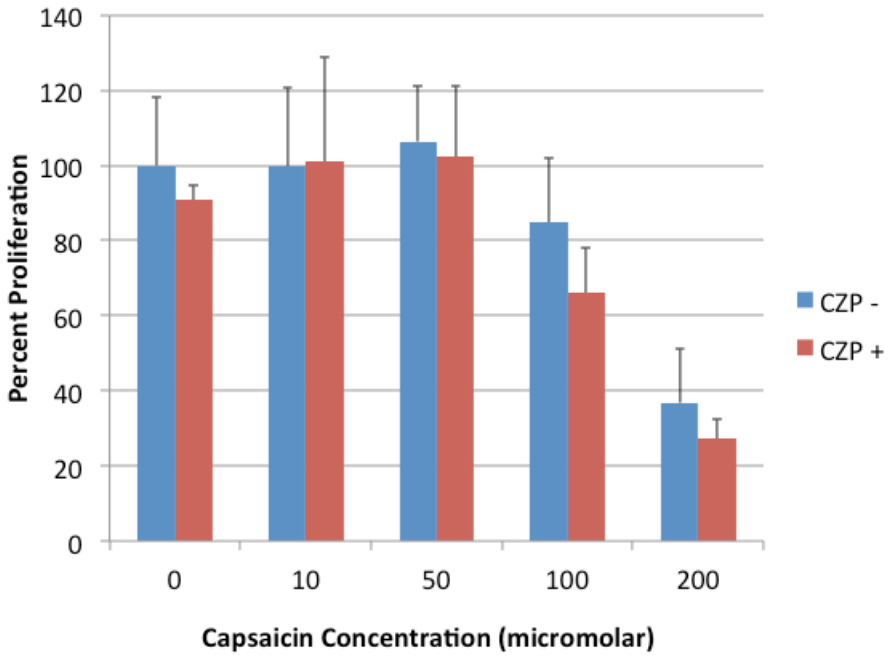


Figure 52: Capsazepine does not reduce the anti-proliferative effects of capsaicin at high doses in LNCaP Cells. *Using the MTS Cell Proliferation Assay, LNCaP cells were treated concurrently with increasing doses of capsaicin with or without CZP. Results suggest that CZP was unable to reverse the anti-proliferative effects of capsaicin in a significant manner in LNCaP cells.*

Since TRP-V1 activation inhibited proliferation, we then went on to determine whether inhibiting the TRP-V1 receptor using capsazepine (CZP) could inhibit the anti-proliferative effects of capsaicin in prostate cancer cells. Here, as demonstrated in Figure 51 and 52, we found that in both LNCaP and PC3 cell lines CZP could not reverse the anti-proliferative activity of capsaicin, suggesting that TRP-V1 was not the predominate mechanism causing the inhibition of proliferation in these cell lines.

5.7 Caveats and Pitfalls

In chapter 5, *Capsaicin: A novel radio-sensitizing agent for prostate cancer*, capsaicin sensitized prostate cancer cells to radiation *in vitro* and *in vivo*. The following points outline some caveats and pitfalls of the study and mitigation strategies to overcome them in the future. Since the radiation sensitization properties of capsaicin became apparent at low concentrations, using a wider capsaicin concentration range help to determine if the radiosensitization effect is dose-dependent. Furthermore, using cell lines from different malignancies (e.g. breast cancer cells), one could gain information on whether the radiation sensitizing properties of capsaicin extend to other tumour types.

Mechanistic studies examining the NF**κ**B signaling pathway are very interesting and informative, however this data could be further developed. Analyzing the mRNA levels using RT-PCR techniques could further validate my Western blot findings. In addition, inhibiting the various aspects of the NF**κ**B signaling pathway using commercially available inhibitors would add further credence to the mechanism of sensitization.

Cells were treated concurrently with capsaicin and radiotherapy *in vitro*. Whether or not the sensitizing effect of capsaicin varies with different timing of administering these two treatment modalities is not clear. Studies treating cells with capsaicin before or after radiation treatment would aid in optimizing therapeutic options.

Serum concentrations of capsaicin were not detailed in this study. More in-depth analysis on the bioavailability and pharmacokinetics of capsaicin *in vivo* could have been more intensely investigated in the serum of xenograft mice. To better understand the role of TRP-V1 receptor in the radio-sensitizing effects of capsaicin *in vivo*, additional groups using the TRP-V1 antagonist, capsazepine, or other more specific antagonists could have been included in the xenograft study. More detailed experiments examining the role of TRP-V1 could have been carried out to validate agonist and antagonist studies. These studies are outlined in the future directions section.

CHAPTER SIX

DISCUSSION AND CONCLUSIONS

6.1 Discussion and Conclusions

In this thesis we have provided evidence supporting the hypothesis that capsaicin, the active compound in chili peppers, exhibits chemopreventive and therapeutic effects on prostate cancer. Initially, we established that treating various prostate cancer cell lines with capsaicin causes a reduction in proliferation and an increase in apoptosis. These effects were not found in non-malignant prostate stromal cells. Combinatorial studies utilizing lycopene, the potent anti-oxidant found in a number of fruits and vegetables, revealed that these anti-cancer effects were significantly enhanced in prostate cancer cells, and were mediated through the upregulation of tumour-suppressor proteins, the inhibition of cell cycling, the induction of apoptosis through mitochondrial destabilization, and activation of the caspase-3 cascade (detailed in Figure 6).

Based on these *in vitro* findings, we then went on to investigate the effects of capsaicin in a transgenic mouse model predisposed to developing prostate cancer. We found that animals treated with capsaicin demonstrated reduced overall disease grade, and significantly reduced metastases. These effects were mediated through a number of processes, including the up-regulation of tumours suppressor proteins (i.e. p21 and $p27^{Kip1}$), the inhibition of the invasion and migration potential, and possibly the inhibition of the neuroendocrine differentiation process. Moreover, long-term oral administration was safe and well tolerated, supporting its role as a safe and effective anti-cancer agent.

In Chapter 5, the therapeutic value of capsaicin as a radiosensitizing agent was investigated. Here, we demonstrated that capsaicin enhances the effect of radiation *in vitro* and *in vivo*. Prostate cancer cells treated with capsaicin had a greater response to radiation, compared to either therapy alone. Furthermore, low doses of capsaicin treatment were shown to differentially affect cancerous and normal epithelial cells through the suppression of NF**κ**B signaling, a survival and resistance promoting pathway commonly upregulated by radiation (as depicted in

Figure 53). In a xenograft mouse model, oral administration of capsaicin was well tolerated and significantly reduced the tumour growth rate, proliferative index, and NFκB expression, and induced sustained DNA damage, validating the therapeutic potential of capsaicin as a radio-sensitizing agent for prostate cancer.

We strongly believe that the novel evidence presented in this thesis will further the understanding of the chemopreventive and therapeutic effects of dietary agents on prostate cancer, as well as the pathophysiology of the disease as a whole. This information will provide a solid foundation and framework for future clinical trials that will improve the way in which prostate cancer is managed and treated, thus reducing the morbidity and mortality of the disease.

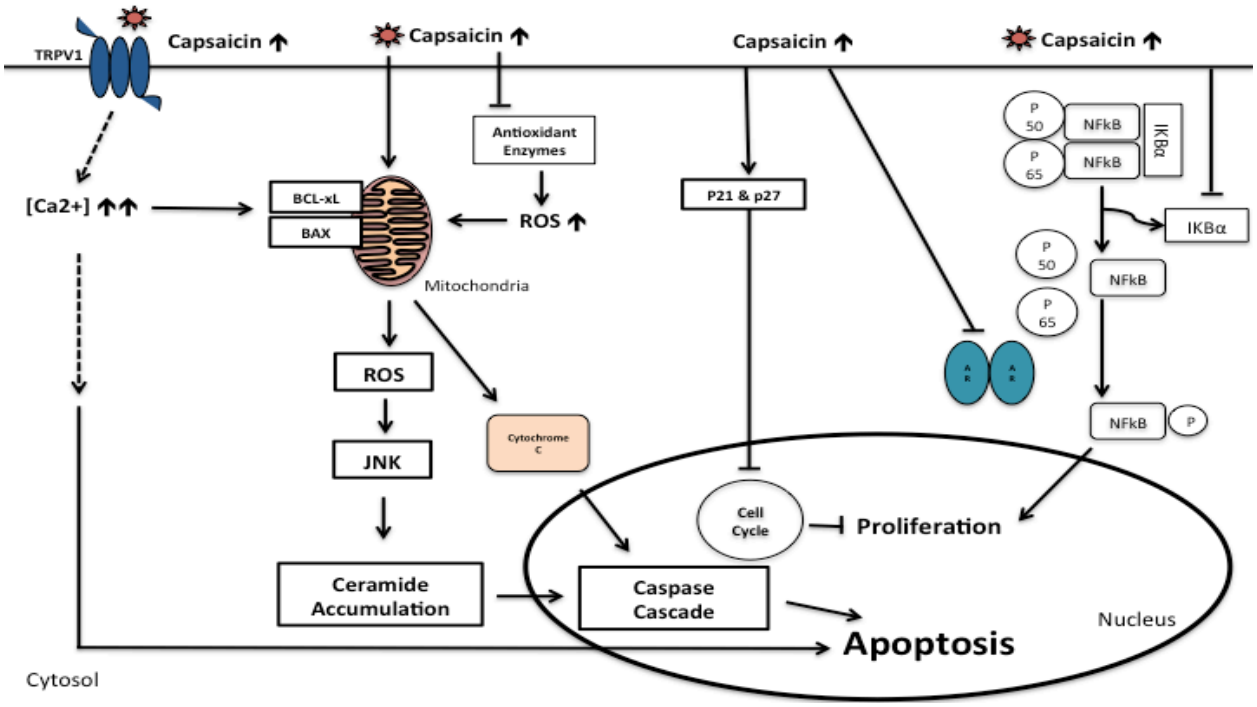


Figure 53: Summary the Potential Mechanisms Exerted by Capsaicin on Prostate Cancer.

CHAPTER SEVEN
FUTURE DIRECTIONS

7.1 Future Directions

Throughout the course of my studies, a number of questions arose that we were unable to investigate in great detail. Fortunately, we were able to conduct some preliminary studies and/or develop hypotheses. In the following section, some of the areas of interest that have emerged from this thesis are outlined.

1. Explore the role of TRP-V1 and TRP-V6 in prostate cancer.

a) What is the detailed mechanism of TRP-V1 in prostate cancer?

In Chapter 5 (supplementary information), it was established that the TRP-V1 receptor is present on prostate cancer cells, but that it is not primarily responsible for mediating the anti-proliferative effects of capsaicin. While these studies were informative, they did not clearly elucidate the role of TRP-V1 in capsaicin-mediated anticancer effects. Examining the TRP-V1 receptor in more detail using more specific targeting techniques *in vitro* (i.e. siRNA) and *in vivo* in the presence of capsaicin (low and high concentrations) would provide better insight as to how TRP-V1 is implicated in the anti-cancer effects of prostate cancer.

b) What is the role of TRP-V6 in prostate cancer?

The TRP-V6 calcium channel belongs to the same family as TRP-V1, however it acts as a calcium channel and not a receptor, enabling the passive influx of calcium into the intracellular space of the cell (Fixemer, Wissenbach et al. 2003). It is expressed predominately in the kidneys, placenta, and pancreas and is mainly involved in maintaining calcium homeostasis, affecting intestinal absorption, renal excretion, and bone metabolism (Wissenbach, Niemeyer et al. 2004).

Interestingly, several studies have reported that the expression of TRP-V6 mRNA is low or undetectable in healthy and benign prostate tissue, whereas TRP-V6 is highly expressed in prostate cancer tissue, with its expression correlating with increasing tumour grade (Peng, Zhuang et al. 2001, Fixemer, Wissenbach et al. 2003, Wissenbach, Niemeyer et al. 2004). The latter suggests that TRP-V6 may serve as a prognostic factor and potential therapeutic target for prostate cancer.

Preliminary immunocytochemistry studies revealed that in addition to TRP-V1, LNCaP prostate cancer cells express TRP-V6. Furthermore, capsaicin treatment (10uM) of LNCaP cells appears to increase the expression of both TRP-V1 and TRP-V6 as depicted in Figures 54-56.

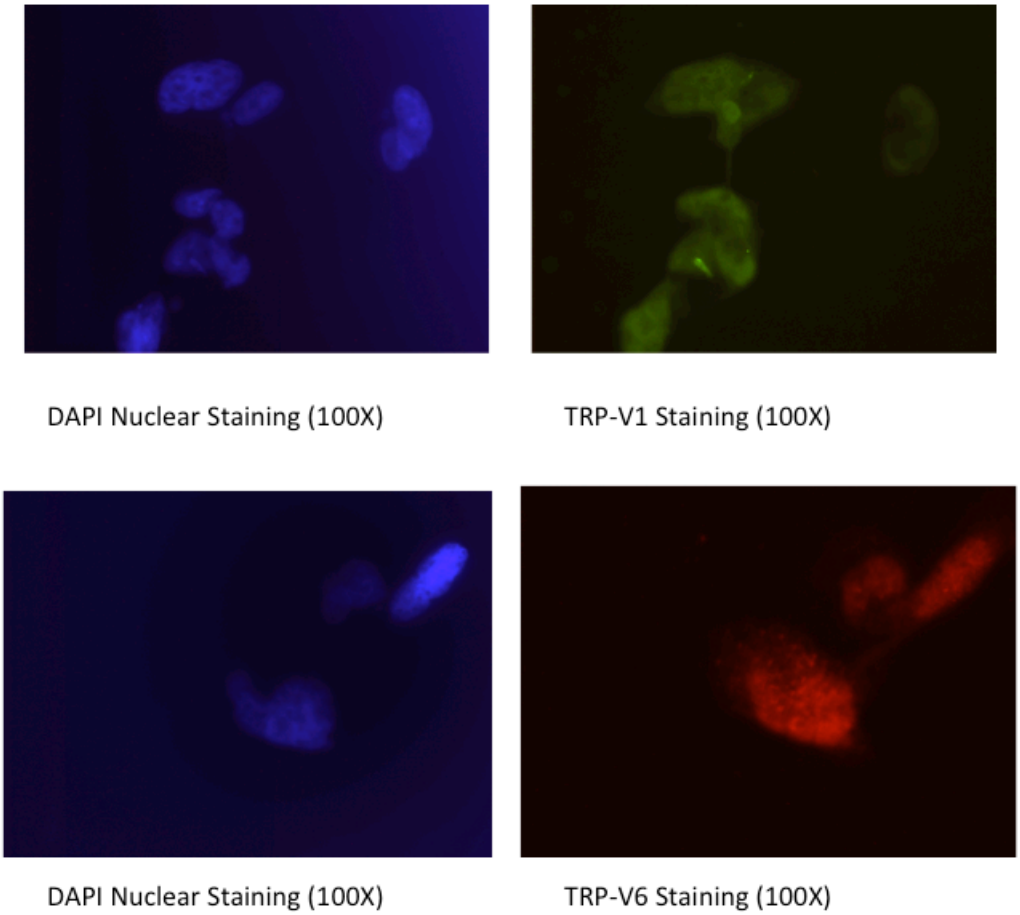


Figure 54: Immunocytochemistry images of untreated LNCaP cells stained for TRP-V6 and TRP-V1. *The TRP-V6 calcium channel is depicted in red, the TRP-V1 receptor in green, nuclear stain by DAPI is depicted on the left in blue.*

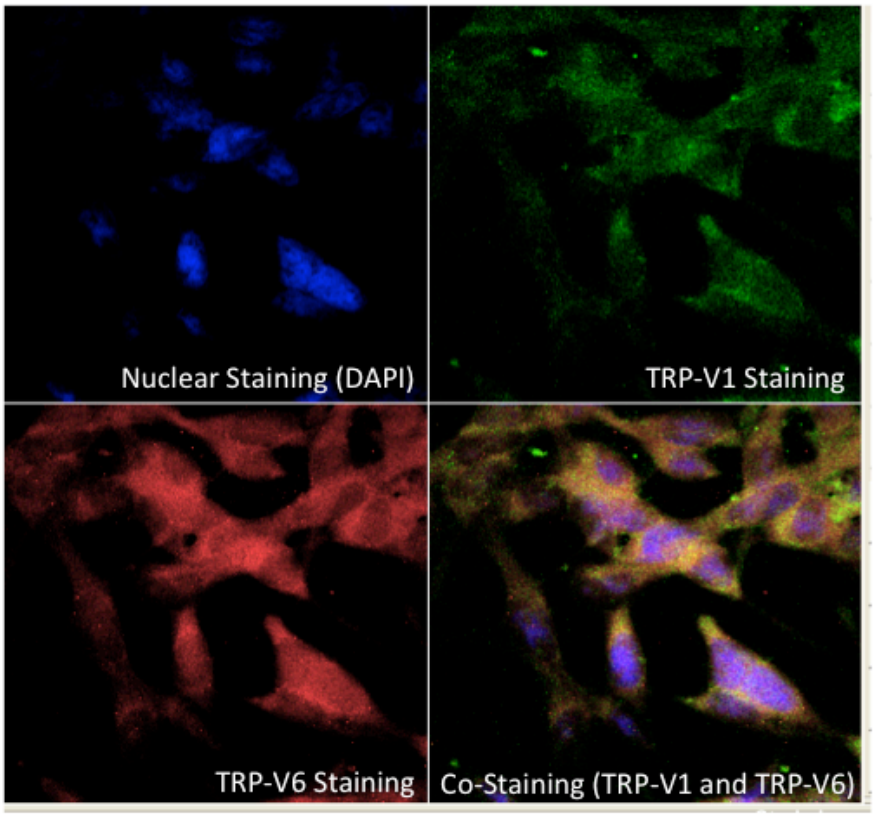


Figure 55: Immunocytochemistry Images of LNCaP Cells treated with capsaicin (10uM). *Blue images represents the cell nuclei, the green represents TRP-V1 expression, and red represents TRP-V6 expression. Overlapping images are expressed in the bottom, right panel. All images were taken at 63 X magnification lens.*

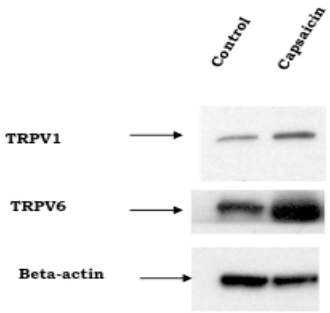


Figure 56: Western Blot analysis reveals an increase in TRP-V1 and TRP-V6 expression in LNCaP cells following 24-hour treatment with capsaicin (10µM).

Although these correlative studies are exciting, the function of TRP-V6 in prostate cancer is not fully understood. Limited studies have shown that TRP-V6 can control prostate cancer cell proliferation via calcium-dependent mechanisms (Lehen'kyi, Flourakis et al. 2007, Prevarskaya, Flourakis et al. 2007). However, how capsaicin is interacting with this calcium channel requires further investigation.

Time course experiments have revealed that the expression of TRP-V6 is increased in a time-dependent manner in prostate cancer cells when they are treated with capsaicin (as well as radiotherapy), suggesting that this increase may be occurring as an adaptive cellular response (Figure 57). Analyzing how and why this is occurring using knock-out or up-regulation techniques that target TRP-V6 could provide insight into the anti-cancer effects of capsaicin.

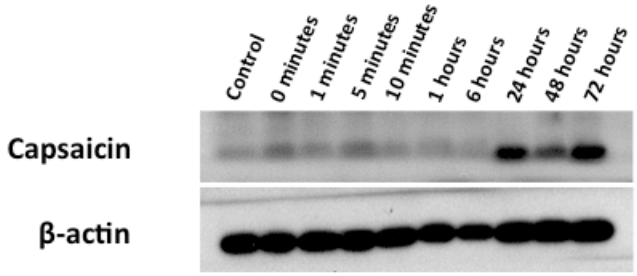


Figure 57: TRP-V6 expression is increased in a time-dependent manner in prostate cancer cells.

Maintaining calcium homeostasis is important for normal cell growth and proliferation. Perturbing intracellular calcium levels could lead to different survival outcomes. Low calcium levels lead to growth inhibition and potentially cell death, high levels of calcium lead to an increase in cell proliferation, and very high levels of calcium can lead to intracellular stress and apoptosis (Berridge, Bootman et al. 1998). The tight regulation of calcium makes it very difficult to study calcium channels and receptors. For example, a calcium channel knock-out experiment may reduce proliferation independent of agents that may affect the channel, or chelating calcium may cause intracellular stress making a cancer cell more susceptible to cell death by a minor insult or stressor. Hence, developing techniques or experiments that can isolate the effect of a receptor, and examine the role of calcium channels and receptors, is important for a greater understanding of the effect of capsaicin on TRP calcium channels.

Based on the preliminary studies, we hypothesized that treating cells with capsaicin causes an alteration in calcium levels, causing the up-regulation of the TRP-V6 calcium channel as an adaptive response. A schematic representation of this hypothesis is depicted in Figure 58. To test this hypothesis, strategies that monitor the intracellular calcium levels pre- and post-capsaicin treatment over time, while examining the expression levels of TRP-V6, would be necessary. It is critical that studies of this nature are carefully designed and conducted, as calcium homeostasis is critical for cell growth and survival.

If this hypothesis were validated, the inhibition of TRP-V6 would prime cells resistant to capsaicin and/or radiation (i.e. cells with high levels of TRP-V6) to undergo apoptosis more easily. In other words, it would sensitize cells to capsaicin (and/or radiation) at lower concentrations.

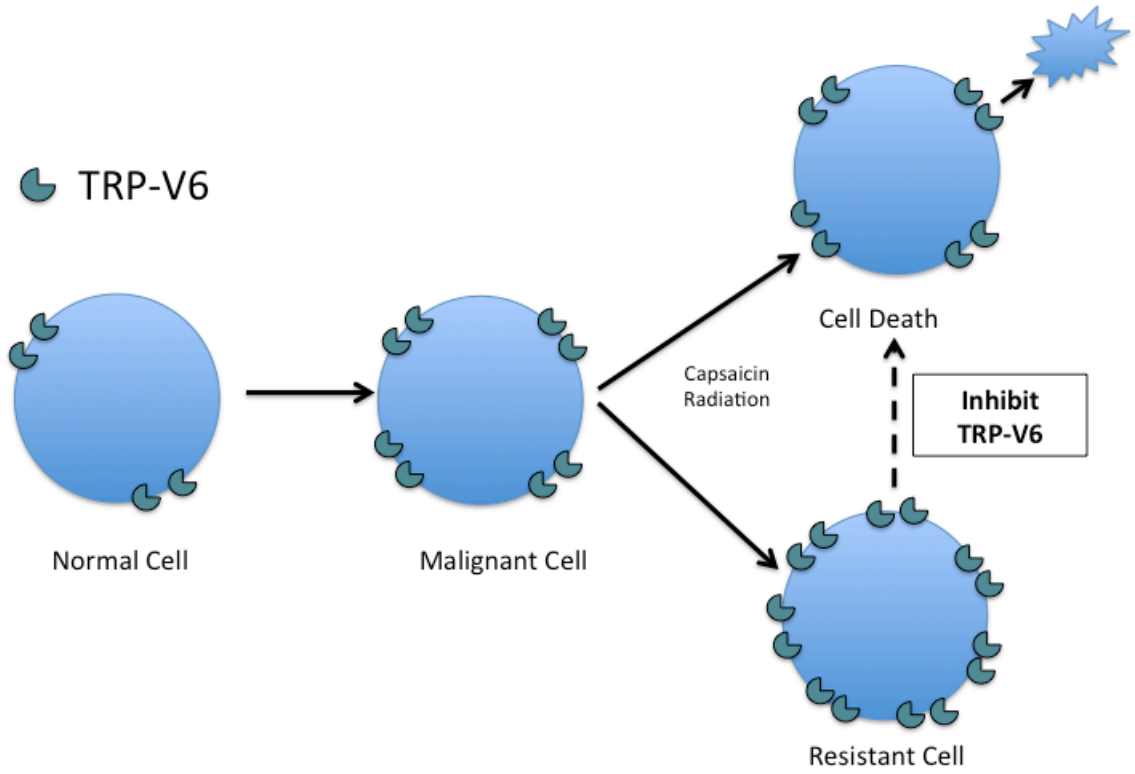


Figure 58: Hypothesized role of TRP-V6 in prostate cancer.

c) Do TRP-V1 and TRP-V6 work in a co-ordinated fashion?

In these studies we have found that low concentrations of capsaicin increase the expression of TRP-V1 and TRP-V6, and high concentrations of capsaicin cause a significant increase in TRP-V6 corresponding to reduction in proliferation and an increase in apoptosis (as depicted in Figure 59).

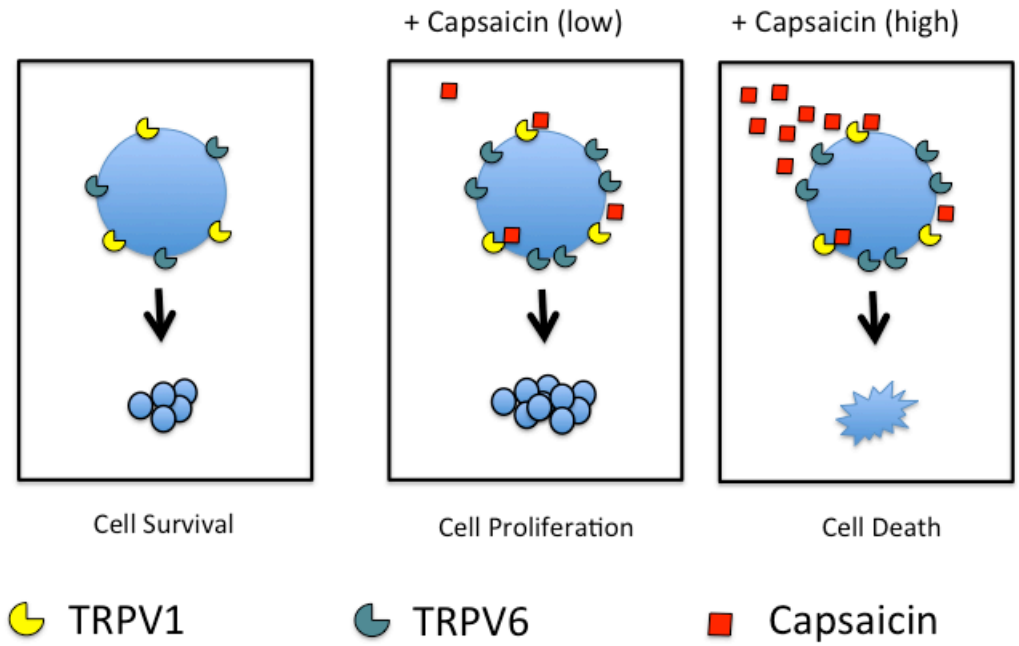


Figure 59: Schematic representation of the effect of capsaicin on prostate cancer cells.

Based on these studies, we can hypothesize that both the TRP-V1 and TRP-V6 receptor are working in a coordinated fashion to allow capsaicin to exert effects on prostate cancer cells.

Further studies examining the role of capsaicin and TRP-V1 and TRP-V6 are necessary to develop a better understanding of these mechanisms. This can be studied by generating stable

cell lines with knock-out or transfected TRP-V1 and TRP-V6 proteins (using the Plasmid DNA Transfection Protocol), and monitoring alterations in intracellular calcium levels in the presence and absence of capsaicin, for example by using flow cytometry (Screen QuestTM Fluo-8 NW Calcium Assay Kit, BioQuest, Inc.) [13]. Based on these findings, *in vivo* studies using TRP-V1/TRP-V6 negative mice would beneficial to validate these hypotheses.

2. Perform detailed mechanistic studies on capsaicin in various types of cancer

The various mechanisms of action that capsaicin exerts on prostate cancer is highlighted throughout this thesis. We have shown that capsaicin can induce apoptosis by destabilizing the mitochondrial membrane and activating caspase cascade; inhibit proliferation by inhibiting cell cycle and deregulating tumour suppressor proteins and oncogenes; sensitize cells and tumours to radiation by inhibiting the NFκB pathway; and prevent the invasion and migration capacity of prostate cancer cells and tumours. The interconnectivity between these mechanisms is not well established so more detailed studies are necessary to better understand how these pathways are related and able to coordinate the anti-cancer effects of capsaicin. Furthermore, other cellular processes may be involved, as discussed hereafter.

a) What is the role of endoplasmic reticulum stress pathway in the anti-prostate cancer effects of capsaicin?

Preliminary studies examining the endoplasmic reticulum (ER) stress pathway revealed that a number of proteins implicated in this pathway, specifically Inositol-requiring protein 1 alpha (IRE1-α) and protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), were up regulated with capsaicin treatment, correlating to a reduction in proliferation. This suggests that this pathway may also be involved in capsaicin-mediated anti-cancer effects. More detailed studies using ER stress inhibitors or knock-out experiments would be useful to determine how the ER Stress pathway is involved in the anti-cancer effects of capsaicin.

b) Can capsaicin induce the endoplasmic reticulum stress pathway in prostate cancer?

In chapter 4, we outlined the chemopreventive and anti-metastatic properties of capsaicin in the TRAMP Model. These studies were very useful for understanding the chemopreventive effects of capsaicin. However, we did not investigate a detailed mechanismfor how these effects are mediated. Future studies examining the anti-metastatic properties of capsaicin could add value to support the existing studies.

c) Can capsaicin inhibit the neuroendocrine differentiation process in prostate cancer?

Early inhibition of the neuroendocrine differentiation process was also outlined in the Chapter 4. As the origin of neuroendocrine cells remains unclear, more research examining this pathway is necessary to identify how capsaicin may be altering this process (Conteduca, Aieta et al. 2014).

In summary, capsaicin has been shown to interact with a number of pathways involved in proliferation, differentiation, migration, and apoptosis. Understanding the interplay of all these pathways requires more detailed studies. Future studies using large-scale microarray technique and ingenuity pathway analysis may be a useful approach for developing a visual representation of the cross-talk between these pathways, as well as aid in identifying novel pathways by which capsaicin may be exerting its downstream effects.

3. Translating the chemopreventive effects of capsaicin into clinical trials

To date, there have not been any published clinical trials examining the anti-cancer effects of capsaicin on prostate cancer or other tumours. In an unpublished retrospective study, we attempted to examine the effect of intermittent capsaicin use on prostate cancer progression. We identified 20 prostate cancer patients who had intermittently consumed capsaicin supplements and studied their electronic personal records for the use/duration of capsaicin consumption and PCa progression markers (PSA and increase in Gleason Score). Our goal was to determine whether there was a correlation between their prostate cancer status and capsaicin consumption. In brief, four patients were excluded due to limited recorded information. The majority of the patients had rising PSA post-surgery (75%), three patients (18.8%) experienced a rising PSA post-ADT, and one patient (6.2%) was enrolled in the active surveillance program.

Unfortunately, we were unable to reach a preliminary conclusion regarding the benefit of capsaicin due to the small sample size, the lack of a control group, and inconistent dosing and reporting between physicians. In general however, capsaicin supplementation was well tolerated and not associated with any adverse effects.

Based on pre-clinical evidence, we have begun a prospective study examining the effect of capsaicin supplementation on subjects with localized prostate cancer who have chosen to be managed by active surveillance or improve surgical outcome of patients undergoing radical prostatectomy. The details of this study are outlined in the CAPSAICIN Protocol, a copy of which can be found in the appendix.

Briefly, this is a Phase II, open label, single center study to evaluate the efficacy and safety of repeat oral dosing of capsaicin capsules twice daily for 6 months prior to a prostate biopsy in men on active surveillance for localized prostate cancer, as well as 6 weeks prior to radical prostatectomy (RP). One hundred men (sixty from active surveillance (AS) and forty patients scheduled to undergo radical prostatectomy) will be eligible for participation.

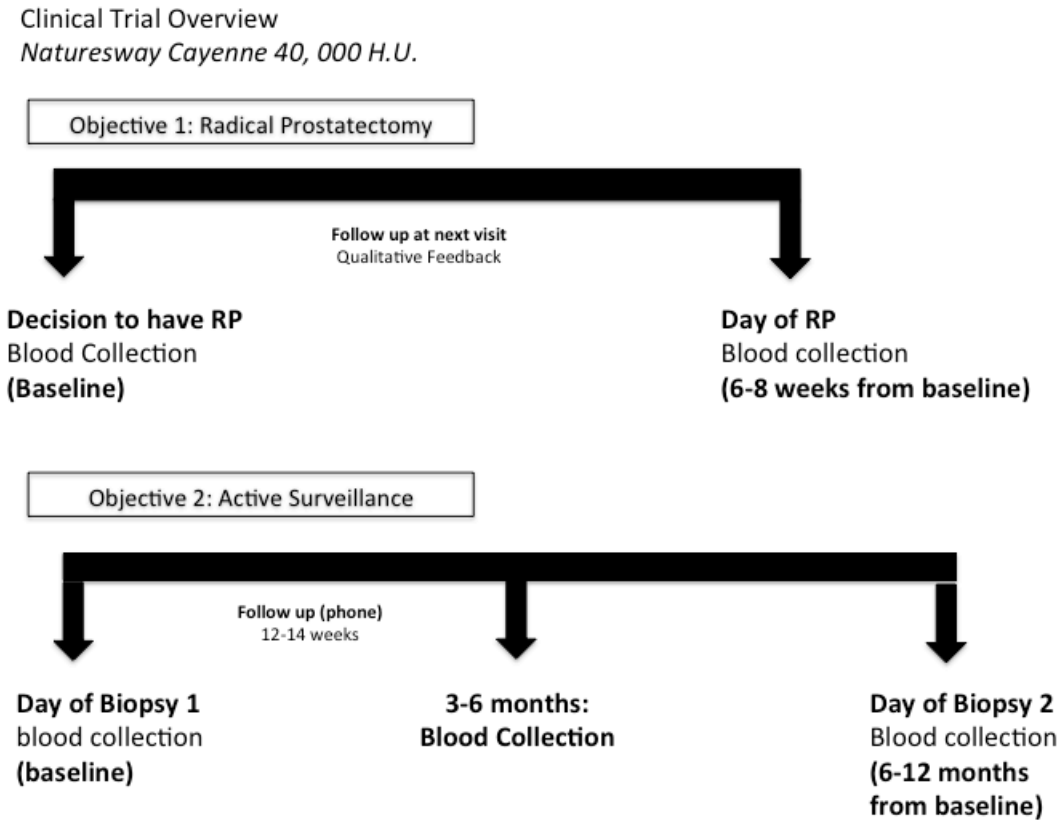


Figure 60: Schematic Representation of CAPSAICIN Trial evaluating the effect of capsaicin on prostate cancer patients.

The primary objective is to assess the effect of capsaicin therapy on the expression of Ki67 and p27 biomarkers in a post–treatment biopsy or prostate specimen from RP. Secondary objectives are to determine the effect of capsaicin therapy on (1) PSA kinetics in men on active surveillance; (2) Gleason grade and the presence of prostatic intraepithelial neoplasia (PIN) in post treatment biopsies; and (3) the expression of markers of apoptosis and cell cycle, and of TRP-V1 and TRP-V6. The primary and secondary outcome measures are detailed in Figure 61)

Based on the results of this study, we will be able to better understand the clinical impact of capsaicin supplementation on prostate cancer progression.

Outcome Measures:

1) Primary Outcome Measures – Biological changes:
   a) Ki67 & p27
   b) Proliferation changes, apoptosis, androgen signaling, TRP signaling & ER Stress.
   c) Progression
2) Secondary Outcome measures:
   a) PSA kinetics
   b) Tumor Size

TRPV Signaling	Proliferation	Apoptosis	ER Stress Pathway	Androgen Signaling
TRPV1	Ki67	Cleaved Caspase 3/ 8/ 9	ATF6/IRE1	AR
TRPV6	p27	Bcl-2; PARP	PERK/XBP1	PSA
	p21		GADD153/CHOP	

Figure 61: Primary and Secondary Outcome measures from CAPSAICIN Trial.

4. Assess the radio-sensitizing effect of capsaicin in clinical trials

Promising preclinical data outlined in Chapter 5 suggests that capsaicin demonstrates radio-sensitizing properties in prostate cancer cells. This novel finding is strong evidence for initiating clinical trials in prostate cancer patients undergoing radiotherapy. Results from the ongoing clinical trials evaluating the effect of capsaicin on prostate cancer patients enrolled in the Active Surveillance program or undergoing RP will support future clinical studies.

References

(NCI), N. C. I. (2012). "Prostate Cancer." from http://www.cancer.gov.

Ablin, R. J. (2005). "Lycopene: a word of caution." Am J Health Syst Pharm **62**(9): 899.

Adeniji, A. O., M. Chen and T. M. Penning (2013). "AKR1C3 as a target in castrate resistant prostate cancer." J Steroid Biochem Mol Biol **137**: 136-149.

Adhami, V. M., I. A. Siddiqui, N. Ahmad, S. Gupta and H. Mukhtar (2004). "Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer." Cancer Res **64**(23): 8715-8722.

Aggarwal, B. B. and S. Shishodia (2004). "Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning." Ann N Y Acad Sci **1030**: 434-441.

Aggarwal, B. B. and S. Shishodia (2006). "Molecular targets of dietary agents for prevention and therapy of cancer." Biochem Pharmacol **71**(10): 1397-1421.

Aggarwal, B. B., Y. Takada and O. V. Oommen (2004). "From chemoprevention to chemotherapy: common targets and common goals." Expert Opin Investig Drugs **13**(10): 1327-1338.

Ahmed, K. M. and J. J. Li (2008). "NF-kappa B-mediated adaptive resistance to ionizing radiation." Free Radic Biol Med **44**(1): 1-13.

Al Othman, Z. A., Y. B. Ahmed, M. A. Habila and A. A. Ghafar (2011). "Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography." Molecules **16**(10): 8919-8929.

Albini, A. and M. B. Sporn (2007). "The tumour microenvironment as a target for chemoprevention." Nat Rev Cancer **7**(2): 139-147.

American Cancer Society, I. (2014). "Prostate Cancer Statistics 2014 (c)." Retrieved 03/12/2014, 03/12/2014, from http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics.

Anandakumar, P., S. Kamaraj, S. Jagan, G. Ramakrishnan, S. Asokkumar, C. Naveenkumar, S. Raghunandhakumar and T. Devaki (2012). "Capsaicin inhibits benzo(a)pyrene-induced lung carcinogenesis in an in vivo mouse model." Inflamm Res **61**(11): 1169-1175.

Andriole, G. L., D. G. Bostwick, O. W. Brawley, L. G. Gomella, M. Marberger, F. Montorsi, C. A. Pettaway, T. L. Tammela, C. Teloken, D. J. Tindall, M. C. Somerville, T.

H. Wilson, I. L. Fowler, R. S. Rittmaster and R. S. Group (2010). "Effect of dutasteride on the risk of prostate cancer." N Engl J Med **362**(13): 1192-1202.

Ather, M. H., F. Abbas, N. Faruqui, M. Israr and S. Pervez (2008). "Correlation of three immunohistochemically detected markers of neuroendocrine differentiation with clinical predictors of disease progression in prostate cancer." BMC Urol **8**: 21.

Balkwill, F. and A. Mantovani (2001). "Inflammation and cancer: back to Virchow?" Lancet **357**(9255): 539-545.

Bernard, B. K., K. Ubukata, R. Mihara, Y. Sato and H. Nemoto (2010). "Studies of the toxicological potential of capsinoids, XI: pharmacokinetic and tissue distribution study of 14C-dihydrocapsiate and metabolites in rats." Int J Toxicol **29**(2 Suppl): 3S-14S.

Berridge, M. J., M. D. Bootman and P. Lipp (1998). "Calcium--a life and death signal." Nature **395**(6703): 645-648.

Bishop, F. L., A. Rea, H. Lewith, Y. K. Chan, J. Saville, P. Prescott, E. von Elm and G. T. Lewith (2011). "Complementary medicine use by men with prostate cancer: a systematic review of prevalence studies." Prostate Cancer Prostatic Dis **14**(1): 1-13.

Bode, A. M. and Z. Dong (2011). "The two faces of capsaicin." Cancer Res **71**(8): 2809-2814.

Brandelius, A., Y. Yudina, J. Calvén, L. Bjermer, M. Andersson, C. Persson and L. Uller (2011). "dsRNA-induced expression of thymic stromal lymphopoietin (TSLP) in asthmatic epithelial cells is inhibited by a small airway relaxant." Pulm Pharmacol Ther **24**(1): 59-66.

Bödding, M. (2007). "TRP proteins and cancer." Cell Signal **19**(3): 617-624.

Cachaço, A. S., T. Carvalho, A. C. Santos, C. Igreja, R. Fragoso, C. Osório, M. Ferreira, J. Serpa, S. Correia, P. Pinto-do-O and S. Dias (2010). "TNF-alpha regulates the effects of irradiation in the mouse bone marrow microenvironment." PLoS One **5**(2): e8980.

Cantley, L. C. and B. G. Neel (1999). "New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway." Proc Natl Acad Sci U S A **96**(8): 4240-4245.

Caprodossi, S., C. Amantini, M. Nabissi, M. B. Morelli, V. Farfariello, M. Santoni, A. Gismondi and G. Santoni (2011). "Capsaicin promotes a more aggressive gene expression phenotype and invasiveness in null-TRPV1 urothelial cancer cells." Carcinogenesis **32**(5): 686-694.

Catalona, W. J., J. E. Bailey-Wilson, N. J. Camp, S. J. Chanock, K. A. Cooney, D. F. Easton, R. A. Eeles, L. M. FitzGerald, M. L. Freedman, J. Gudmundsson, R. A. Kittles, E. H. Margulies, B. B. McGuire, E. A. Ostrander, T. R. Rebbeck, J. L. Stanford, S. N. Thibodeau, J. S. Witte and W. B. Isaacs (2011). "National Cancer Institute Prostate Cancer Genetics Workshop." Cancer Res **71**(10): 3442-3446.

Center, M. M., A. Jemal, J. Lortet-Tieulent, E. Ward, J. Ferlay, O. Brawley and F. Bray (2012). "International variation in prostate cancer incidence and mortality rates." Eur Urol **61**(6): 1079-1092.

Chaiyasit, K., W. Khovidhunkit and S. Wittayalertpanya (2009). "Pharmacokinetic and the effect of capsaicin in Capsicum frutescens on decreasing plasma glucose level." J Med Assoc Thai **92**(1): 108-113.

Chan, J. M., P. H. Gann and E. L. Giovannucci (2005). "Role of diet in prostate cancer development and progression." J Clin Oncol **23**(32): 8152-8160.

Chang, H. C., S. T. Chen, S. Y. Chien, S. J. Kuo, H. T. Tsai and D. R. Chen (2011). "Capsaicin may induce breast cancer cell death through apoptosis-inducing factor involving mitochondrial dysfunction." Hum Exp Toxicol **30**(10): 1657-1665.

Chen, J., G. D. Huang, S. R. Tan, J. Guo and Z. Q. Su (2013). "The preparation of capsaicin-chitosan microspheres (CCMS) enteric coated tablets." Int J Mol Sci **14**(12): 24305-24319.

Chuang, M. T., F. M. Ho, C. C. Wu, S. Y. Zhuang, S. Y. Lin, F. M. Suk and Y. C. Liang (2011). "15,16-Dihydrotanshinone I, a Compound of Salvia miltiorrhiza Bunge, Induces Apoptosis through Inducing Endoplasmic Reticular Stress in Human Prostate Carcinoma Cells." Evid Based Complement Alternat Med **2011**: 865435.

Clark, L. C., G. F. Combs, B. W. Turnbull, E. H. Slate, D. K. Chalker, J. Chow, L. S. Davis, R. A. Glover, G. F. Graham, E. G. Gross, A. Krongrad, J. L. Lesher, H. K. Park, B. B. Sanders, C. L. Smith and J. R. Taylor (1996). "Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group." JAMA **276**(24): 1957-1963.

Combs, G. F., L. C. Clark and B. W. Turnbull (1997). "Reduction of cancer mortality and incidence by selenium supplementation." Med Klin (Munich) **92 Suppl 3**: 42-45.

Comstock, C. E., M. A. Augello, J. F. Goodwin, R. de Leeuw, M. J. Schiewer, W. F. Ostrander, R. A. Burkhart, A. K. McClendon, P. A. McCue, E. J. Trabulsi, C. D. Lallas, L. G. Gomella, M. M. Centenera, J. R. Brody, L. M. Butler, W. D. Tilley and K. E. Knudsen (2013). "Targeting cell cycle and hormone receptor pathways in cancer." Oncogene **32**(48): 5481-5491.

Conte, C., A. Floridi, C. Aisa, M. Piroddi and F. Galli (2004). "Gamma-tocotrienol metabolism and antiproliferative effect in prostate cancer cells." Ann N Y Acad Sci **1031**: 391-394.

Conteduca, V., M. Aieta, D. Amadori and U. De Giorgi (2014). "Neuroendocrine differentiation in prostate cancer: Current and emerging therapy strategies." Crit Rev Oncol Hematol.

Craig, M., C. Ying and R. D. Loberg (2008). "Co-inoculation of prostate cancer cells with U937 enhances tumor growth and angiogenesis in vivo." J Cell Biochem **103**(1): 1-8.

Czifra, G., A. Varga, K. Nyeste, R. Marincsák, B. I. Tóth, I. Kovács, L. Kovács and T. Bíró (2009). "Increased expressions of cannabinoid receptor-1 and transient receptor potential vanilloid-1 in human prostate carcinoma." J Cancer Res Clin Oncol **135**(4): 507-514.

D'Andrea, G. M. (2005). "Use of antioxidants during chemotherapy and radiotherapy should be avoided." CA Cancer J Clin **55**(5): 319-321.

Dai, Y., J. T. DeSano, Y. Meng, Q. Ji, M. Ljungman, T. S. Lawrence and L. Xu (2009). "Celastrol potentiates radiotherapy by impairment of DNA damage processing in human prostate cancer." Int J Radiat Oncol Biol Phys **74**(4): 1217-1225.

Dehm, S. M. and D. J. Tindall (2006). "Molecular regulation of androgen action in prostate cancer." J Cell Biochem **99**(2): 333-344.

Dehm, S. M. and D. J. Tindall (2007). "Androgen receptor structural and functional elements: role and regulation in prostate cancer." Mol Endocrinol **21**(12): 2855-2863.

Devari, S., S. Jaglan, M. Kumar, R. Deshidi, S. Guru, S. Bhushan, M. Kushwaha, A. P. Gupta, S. G. Gandhi, J. P. Sharma, S. C. Taneja, R. A. Vishwakarma and B. A. Shah (2013). "Capsaicin production by Alternaria alternata, an endophytic fungus from Capsicum annum; LC-ESI-MS/MS analysis." Phytochemistry.

Dickenson, A. H. and A. Dray (1991). "Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception." Br J Pharmacol **104**(4): 1045-1049.

Ding, G. R., N. Honda, T. Nakahara, F. Tian, M. Yoshida, H. Hirose and J. Miyakoshi (2003). "Radiosensitization by inhibition of IkappaB-alpha phosphorylation in human glioma cells." Radiat Res **160**(2): 232-237.

Ding, M., E. Zhang, R. He and X. Wang (2013). "Newly developed strategies for improving sensitivity to radiation by targeting signal pathways in cancer therapy." Cancer Sci.

Díaz-Laviada, I. (2010). "Effect of capsaicin on prostate cancer cells." Future Oncol **6**(10): 1545-1550.

Epstein, J. I. (2010). "An update of the Gleason grading system." J Urol **183**(2): 433-440.

Ferreira, A. L., K. J. Yeum, C. Liu, D. Smith, N. I. Krinsky, X. D. Wang and R. M. Russell (2000). "Tissue distribution of lycopene in ferrets and rats after lycopene supplementation." J Nutr **130**(5): 1256-1260.

Fixemer, T., U. Wissenbach, V. Flockerzi and H. Bonkhoff (2003). "Expression of the Ca2+-selective cation channel TRPV6 in human prostate cancer: a novel prognostic marker for tumor progression." Oncogene **22**(49): 7858-7861.

Fleshner, N., W. R. Fair, R. Huryk and W. D. Heston (1999). "Vitamin E inhibits the high-fat diet promoted growth of established human prostate LNCaP tumors in nude mice." J Urol **161**(5): 1651-1654.

Fleshner, N. and A. R. Zlotta (2007). "Prostate cancer prevention: past, present, and future." Cancer **110**(9): 1889-1899.

Fraenkel, L., S. T. Bogardus, J. Concato and D. R. Wittink (2004). "Treatment options in knee osteoarthritis: the patient's perspective." Arch Intern Med **164**(12): 1299-1304.

Geng, H., B. L. Rademacher, J. Pittsenbarger, C. Y. Huang, C. T. Harvey, M. C. Lafortune, A. Myrthue, M. Garzotto, P. S. Nelson, T. M. Beer and D. Z. Qian (2010). "ID1 enhances docetaxel cytotoxicity in prostate cancer cells through inhibition of p21." Cancer Res **70**(8): 3239-3248.

Ghosh, A. K. and S. Basu (2012). "Tumor macrophages as a target for Capsaicin mediated immunotherapy." Cancer Lett **324**(1): 91-97.

Gingrich, J. R., R. J. Barrios, B. A. Foster and N. M. Greenberg (1999). "Pathologic progression of autochthonous prostate cancer in the TRAMP model." Prostate Cancer Prostatic Dis **2**(2): 70-75.

Gingrich, J. R., R. J. Barrios, M. W. Kattan, H. S. Nahm, M. J. Finegold and N. M. Greenberg (1997). "Androgen-independent prostate cancer progression in the TRAMP model." Cancer Res **57**(21): 4687-4691.

Gingrich, J. R., R. J. Barrios, R. A. Morton, B. F. Boyce, F. J. DeMayo, M. J. Finegold, R. Angelopoulou, J. M. Rosen and N. M. Greenberg (1996). "Metastatic prostate cancer in a transgenic mouse." Cancer Res **56**(18): 4096-4102.

Gingrich, J. R. and N. M. Greenberg (1996). "A transgenic mouse prostate cancer model." Toxicol Pathol **24**(4): 502-504.

Girdhani, S., S. M. Bhosle, S. A. Thulsidas, A. Kumar and K. P. Mishra (2005). "Potential of radiosensitizing agents in cancer chemo-radiotherapy." J Cancer Res Ther **1**(3): 129-131.

Gleason, D. F. (1992). "Histologic grading of prostate cancer: a perspective." Hum Pathol **23**(3): 273-279.

Gleave, M., J. T. Hsieh, C. A. Gao, A. C. von Eschenbach and L. W. Chung (1991). "Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts." Cancer Res **51**(14): 3753-3761.

Gonzalez, J. R., M. A. Laudano, T. R. McCann, J. M. McKiernan and M. C. Benson (2008). "A review of high-risk prostate cancer and the role of neo-adjuvant and adjuvant therapies." World J Urol **26**(5): 475-480.

Govindarajan, V. S. and M. N. Sathyanarayana (1991). "Capsicum--production, technology, chemistry, and quality. Part V. Impact on physiology, pharmacology, nutrition, and metabolism; structure, pungency, pain, and desensitization sequences." Crit Rev Food Sci Nutr **29**(6): 435-474.

Grabowska, M. M., D. J. Degraff, X. Yu, R. J. Jin, Z. Chen, A. D. Borowsky and R. J. Matusik (2014). "Mouse models of prostate cancer: picking the best model for the question." Cancer Metastasis Rev.

Greenberg, N. M. (1996). "Transgenic models for prostate cancer research." Urol Oncol **2**(4): 119-122.

Gropper, S., J. Smith and J. Groff (2005). Advanced Nutrition and Human Metabolism., Thomson Learning, Inc.

Grönberg, H., L. Damber and J. E. Damber (1994). "Studies of genetic factors in prostate cancer in a twin population." J Urol **152**(5 Pt 1): 1484-1487; discussion 1487-1489.

Guns, E. S. and S. P. Cowell (2005). "Drug Insight: lycopene in the prevention and treatment of prostate cancer." Nat Clin Pract Urol **2**(1): 38-43.

Gunthorpe, M. J. and A. Szallasi (2008). "Peripheral TRPV1 receptors as targets for drug development: new molecules and mechanisms." Curr Pharm Des **14**(1): 32-41.

Gupta, S., V. M. Adhami, M. Subbarayan, G. T. MacLennan, J. S. Lewin, U. O. Hafeli, P. Fu and H. Mukhtar (2004). "Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model." Cancer Res **64**(9): 3334-3343.

Gupta, S., N. Ahmad, S. R. Marengo, G. T. MacLennan, N. M. Greenberg and H. Mukhtar (2000). "Chemoprevention of prostate carcinogenesis by alpha-difluoromethylornithine in TRAMP mice." Cancer Res **60**(18): 5125-5133.

Gupta, S. C., S. Prasad, S. Reuter, R. Kannappan, V. R. Yadav, J. Ravindran, P. S. Hema, M. M. Chaturvedi, M. Nair and B. B. Aggarwal (2010). "Modification of cysteine 179 of IkappaBalpha kinase by nimbolide leads to down-regulation of NF-kappaB-regulated cell survival and proliferative proteins and sensitization of tumor cells to chemotherapeutic agents." J Biol Chem **285**(46): 35406-35417.

Gwede, C. K., J. Pow-Sang, J. Seigne, R. Heysek, M. Helal, K. Shade, A. Cantor and P. B. Jacobsen (2005). "Treatment decision-making strategies and influences in patients with localized prostate carcinoma." Cancer **104**(7): 1381-1390.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hantz, H. L., L. F. Young and K. R. Martin (2005). "Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells." Exp Biol Med (Maywood) **230**(3): 171-179.

Hartley, T., B. Stevens, K. D. Ahuja and M. J. Ball (2013). "Development and experimental application of an HPLC procedure for the determination of capsaicin and dihydrocapsaicin in serum samples from human subjects." Indian J Clin Biochem **28**(4): 329-335.

Hatfield, D. L. and V. N. Gladyshev (2009). "The Outcome of Selenium and Vitamin E Cancer Prevention Trial (SELECT) reveals the need for better understanding of selenium biology." Mol Interv **9**(1): 18-21.

Hayes, J. H. and M. J. Barry (2014). "Screening for prostate cancer with the prostate-specific antigen test: a review of current evidence." JAMA **311**(11): 1143-1149.

Heidenreich, A., P. J. Bastian, J. Bellmunt, M. Bolla, S. Joniau, T. van der Kwast, M. Mason, V. Matveev, T. Wiegel, F. Zattoni, N. Mottet and E. A. o. Urology (2014). "EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer." Eur Urol **65**(2): 467-479.

Herzog, A., U. Siler, V. Spitzer, N. Seifert, A. Denelavas, P. B. Hunziker, W. Hunziker, R. Goralczyk and K. Wertz (2005). "Lycopene reduced gene expression of steroid targets and inflammatory markers in normal rat prostate." FASEB J **19**(2): 272-274.

Hidalgo, M., F. Amant, A. V. Biankin, E. Budinská, A. T. Byrne, C. Caldas, R. B. Clarke, S. de Jong, J. Jonkers, G. M. Mælandsmo, S. Roman-Roman, J. Seoane, L. Trusolino and A. Villanueva (2014). "Patient-derived xenograft models: an emerging platform for translational cancer research." Cancer Discov **4**(9): 998-1013.

Hurwitz, A. A., B. A. Foster, J. P. Allison, N. M. Greenberg and E. D. Kwon (2001). "The TRAMP mouse as a model for prostate cancer." Curr Protoc Immunol **Chapter 20**: Unit 20.25.

Huss, W. J., D. R. Gray, K. Tavakoli, M. E. Marmillion, L. E. Durham, M. A. Johnson, N. M. Greenberg and G. J. Smith (2007). "Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model." Neoplasia **9**(11): 938-950.

Ip, S. W., S. H. Lan, H. F. Lu, A. C. Huang, J. S. Yang, J. P. Lin, H. Y. Huang, J. C. Lien, C. C. Ho, C. F. Chiu, W. Wood and J. G. Chung (2012). "Capsaicin mediates apoptosis in human nasopharyngeal carcinoma NPC-TW 039 cells through mitochondrial depolarization and endoplasmic reticulum stress." Hum Exp Toxicol **31**(6): 539-549.

Irshad, S. and C. Abate-Shen (2013). "Modeling prostate cancer in mice: something old, something new, something premalignant, something metastatic." Cancer Metastasis Rev **32**(1-2): 109-122.

Ishak, M. B. and V. N. Giri (2011). "A systematic review of replication studies of prostate cancer susceptibility genetic variants in high-risk men originally identified from genome-wide association studies." Cancer Epidemiol Biomarkers Prev **20**(8): 1599-1610.

Ivanov, N. I., S. P. Cowell, P. Brown, P. S. Rennie, E. S. Guns and M. E. Cox (2007). "Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines." Clin Nutr **26**(2): 252-263.

Iwata, T., D. Schultz, J. Hicks, G. K. Hubbard, L. N. Mutton, T. L. Lotan, C. Bethel, M. T. Lotz, S. Yegnasubramanian, W. G. Nelson, C. V. Dang, M. Xu, U. Anele, C. M. Koh, C. J. Bieberich and A. M. De Marzo (2010). "MYC overexpression induces prostatic intraepithelial neoplasia and loss of Nkx3.1 in mouse luminal epithelial cells." PLoS One **5**(2): e9427.

Jankovic, B., D. A. Loblaw and R. Nam (2010). "Capsaicin may slow PSA doubling time: case report and literature review." Can Urol Assoc J **4**(1): E9-E11.

Jeet, V., P. J. Russell and A. Khatri (2010). "Modeling prostate cancer: a perspective on transgenic mouse models." Cancer Metastasis Rev **29**(1): 123-142.

Jerónimo, C., P. J. Bastian, A. Bjartell, G. M. Carbone, J. W. Catto, S. J. Clark, R. Henrique, W. G. Nelson and S. F. Shariat (2011). "Epigenetics in prostate cancer: biologic and clinical relevance." Eur Urol **60**(4): 753-766.

Jiang, Q., J. Wong, H. Fyrst, J. D. Saba and B. N. Ames (2004). "gamma-Tocopherol or combinations of vitamin E forms induce cell death in human prostate cancer cells by interrupting sphingolipid synthesis." Proc Natl Acad Sci U S A **101**(51): 17825-17830.

Johnson, J. J., H. H. Bailey and H. Mukhtar (2010). "Green tea polyphenols for prostate cancer chemoprevention: a translational perspective." Phytomedicine **17**(1): 3-13.

Jordt, S. E. and D. Julius (2002). "Molecular basis for species-specific sensitivity to "hot" chili peppers." Cell **108**(3): 421-430.

Julius, D. (2013). "TRP channels and pain." Annu Rev Cell Dev Biol **29**: 355-384.

Kanagaraj, P., M. R. Vijayababu, B. Ravisankar, J. Anbalagan, M. M. Aruldhas and J. Arunakaran (2007). "Effect of lycopene on insulin-like growth factor-I, IGF binding protein-3 and IGF type-I receptor in prostate cancer cells." J Cancer Res Clin Oncol **133**(6): 351-359.

Kasper, S., P. C. Sheppard, Y. Yan, N. Pettigrew, A. D. Borowsky, G. S. Prins, J. G. Dodd, M. L. Duckworth and R. J. Matusik (1998). "Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer." Lab Invest **78**(6): i-xv.

Khan, K. H., M. Blanco-Codesido and L. R. Molife (2014). "Cancer therapeutics: Targeting the apoptotic pathway." Crit Rev Oncol Hematol **90**(3): 200-219.

Khan, N., V. M. Adhami and H. Mukhtar (2010). "Apoptosis by dietary agents for prevention and treatment of prostate cancer." Endocr Relat Cancer **17**(1): R39-52.

Kim, J. Y., E. H. Kim, S. U. Kim, T. K. Kwon and K. S. Choi (2010). "Capsaicin sensitizes malignant glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and survivin downregulation." Carcinogenesis **31**(3): 367-375.

Kim, M. J., R. D. Cardiff, N. Desai, W. A. Banach-Petrosky, R. Parsons, M. M. Shen and C. Abate-Shen (2002). "Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis." Proc Natl Acad Sci U S A **99**(5): 2884-2889.

Kim, S. and A. Moon (2004). "Capsaicin-induced apoptosis of H-ras-transformed human breast epithelial cells is Rac-dependent via ROS generation." Arch Pharm Res **27**(8): 845-849.

Kim, S., M. Park, S. I. Yeom, Y. M. Kim, J. M. Lee, H. A. Lee, E. Seo, J. Choi, K. Cheong, K. T. Kim, K. Jung, G. W. Lee, S. K. Oh, C. Bae, S. B. Kim, H. Y. Lee, S. Y. Kim, M. S. Kim, B. C. Kang, Y. D. Jo, H. B. Yang, H. J. Jeong, W. H. Kang, J. K. Kwon, C. Shin, J. Y. Lim, J. H. Park, J. H. Huh, J. S. Kim, B. D. Kim, O. Cohen, I. Paran, M. C. Suh, S. B. Lee, Y. K. Kim, Y. Shin, S. J. Noh, J. Park, Y. S. Seo, S. Y. Kwon, H. A. Kim, J. M. Park, H. J. Kim, S. B. Choi, P. W. Bosland, G. Reeves, S. H. Jo, B. W. Lee, H. T. Cho, H. S. Choi, M. S. Lee, Y. Yu, Y. Do Choi, B. S. Park, A. van Deynze, H. Ashrafi, T. Hill, W. T. Kim, H. S. Pai, H. K. Ahn, I. Yeam, J. J. Giovannoni, J. K. Rose, I. Sørensen, S. J. Lee, R. W. Kim, I. Y. Choi, B. S. Choi, J. S. Lim, Y. H. Lee and D. Choi (2014). "Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species." Nat Genet **46**(3): 270-278.

Kimura, K., C. Bowen, S. Spiegel and E. P. Gelmann (1999). "Tumor necrosis factor-alpha sensitizes prostate cancer cells to gamma-irradiation-induced apoptosis." Cancer Res **59**(7): 1606-1614.

Klein, E. A. (2003). "Clinical models for testing chemopreventative agents in prostate cancer and overview of SELECT: the Selenium and Vitamin E Cancer Prevention Trial." Recent Results Cancer Res **163**: 212-225; discussion 264-216.

Klotz, L. (2013). "Active surveillance not only reduces morbidity, It saves lives." Oncology (Williston Park) **27**(6): 522, 593.

Klotz, L. (2013). "Active surveillance: current and future directions." Curr Opin Urol **23**(3): 237-238.

Klotz, L. (2013). "Prostate cancer overdiagnosis and overtreatment." Curr Opin Endocrinol Diabetes Obes **20**(3): 204-209.

Knuuttila, M., E. Yatkin, J. Kallio, S. Savolainen, T. D. Laajala, T. Aittokallio, R. Oksala, M. Häkkinen, P. Keski-Rahkonen, S. Auriola, M. Poutanen and S. Mäkelä (2014).

"Castration Induces Up-Regulation of Intratumoral Androgen Biosynthesis and Androgen Receptor Expression in an Orthotopic VCaP Human Prostate Cancer Xenograft Model." Am J Pathol.

Kodama, T., T. Masuyama, T. Kayahara, S. Tsubuku, T. Ohishi, B. M. Wagner and B. K. Bernard (2010). "Studies of the toxicological potential of capsinoids XIV: a 26-week gavage toxicity study of dihydrocapsiate in rats." Int J Toxicol **29**(2 Suppl): 27S-54S.

Kral, M., V. Rosinska, V. Student, M. Grepl, M. Hrabec and J. Bouchal (2011). "Genetic determinants of prostate cancer: a review." Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **155**(1): 3-9.

Kramer, B. S., K. L. Hagerty, S. Justman, M. R. Somerfield, P. C. Albertsen, W. J. Blot, H. Ballentine Carter, J. P. Costantino, J. I. Epstein, P. A. Godley, R. P. Harris, T. J. Wilt, J. Wittes, R. Zon, P. Schellhammer, A. S. o. C. O. H. S. Committee and A. U. A. P. G. Committee (2009). "Use of 5-alpha-reductase inhibitors for prostate cancer chemoprevention: American Society of Clinical Oncology/American Urological Association 2008 Clinical Practice Guideline." J Clin Oncol **27**(9): 1502-1516.

Krizanova, O., I. Steliarova, L. Csaderova, M. Pastorek and S. Hudecova (2014). "Capsaicin induces apoptosis in PC12 cells through ER stress." Oncol Rep **31**(2): 581-588.

Kryston, T. B., A. B. Georgiev, P. Pissis and A. G. Georgakilas (2011). "Role of oxidative stress and DNA damage in human carcinogenesis." Mutat Res **711**(1-2): 193-201.

Kuczyk, M., S. Machtens, K. Hradil, J. Schubach, W. Christian, R. Knüchel, J. Hartmann, C. Bokemeyer, U. Jonas and J. Serth (1999). "Predictive value of decreased p27Kip1 protein expression for the recurrence-free and long-term survival of prostate cancer patients." Br J Cancer **81**(6): 1052-1058.

Kuczyk, M. A., C. Bokemeyer, J. Hartmann, J. Schubach, C. Walter, S. Machtens, R. Knuchel, C. Kollmannsberger, U. Jonas and J. Serth (2001). "Predictive value of altered p27Kip1 and p21WAF/Cip1 protein expression for the clinical prognosis of patients with localized prostate cancer." Oncol Rep **8**(6): 1401-1407.

Lacy, J. M. and N. Kyprianou (2014). "A tale of two trials: The impact of 5α-reductase inhibition on prostate cancer (Review)." Oncol Lett **8**(4): 1391-1396.

Lam, E. T. and L. M. Glodé (2013). "Neoadjuvant and adjuvant hormonal and chemotherapy for prostate cancer." Hematol Oncol Clin North Am **27**(6): 1189-1204, viii.

Le, T. D., D. Jin, D. C. Jin, S. R. Rho, M. S. Kim, R. Yu and H. Yoo (2012). "Capsaicin-induced apoptosis of FaDu human pharyngeal squamous carcinoma cells." Yonsei Med J **53**(4): 834-841.

Ledesma, M. C., B. Jung-Hynes, T. L. Schmit, R. Kumar, H. Mukhtar and N. Ahmad (2011). "Selenium and vitamin E for prostate cancer: post-SELECT (Selenium and Vitamin E Cancer Prevention Trial) status." Mol Med **17**(1-2): 134-143.

Lee, C., D. M. Sutkowski, J. A. Sensibar, D. Zelner, I. Kim, I. Amsel, N. Shaw, G. S. Prins and J. M. Kozlowski (1995). "Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone." Endocrinology **136**(2): 796-803.

Lehen'kyi, V., M. Flourakis, R. Skryma and N. Prevarskaya (2007). "TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways." Oncogene **26**(52): 7380-7385.

Li, L. C., P. R. Carroll and R. Dahiya (2005). "Epigenetic changes in prostate cancer: implication for diagnosis and treatment." J Natl Cancer Inst **97**(2): 103-115.

Li, X. and G. R. Stark (2002). "NFkappaB-dependent signaling pathways." Exp Hematol **30**(4): 285-296.

Lin, S., J. Zhang, H. Chen, K. Chen, F. Lai, J. Luo, Z. Wang, H. Bu, R. Zhang, H. Li and H. Tong (2013). "Involvement of endoplasmic reticulum stress in capsaicin-induced apoptosis of human pancreatic cancer cells." Evid Based Complement Alternat Med **2013**: 629750.

Lippman, S. M., P. J. Goodman, E. A. Klein, H. L. Parnes, I. M. Thompson, A. R. Kristal, R. M. Santella, J. L. Probstfield, C. M. Moinpour, D. Albanes, P. R. Taylor, L. M. Minasian, A. Hoque, S. M. Thomas, J. J. Crowley, J. M. Gaziano, J. L. Stanford, E. D. Cook, N. E. Fleshner, M. M. Lieber, P. J. Walther, F. R. Khuri, D. D. Karp, G. G. Schwartz, L. G. Ford and C. A. Coltman (2005). "Designing the Selenium and Vitamin E Cancer Prevention Trial (SELECT)." J Natl Cancer Inst **97**(2): 94-102.

Lippman, S. M., E. A. Klein, P. J. Goodman, M. S. Lucia, I. M. Thompson, L. G. Ford, H. L. Parnes, L. M. Minasian, J. M. Gaziano, J. A. Hartline, J. K. Parsons, J. D. Bearden, E. D. Crawford, G. E. Goodman, J. Claudio, E. Winquist, E. D. Cook, D. D. Karp, P. Walther, M. M. Lieber, A. R. Kristal, A. K. Darke, K. B. Arnold, P. A. Ganz, R. M. Santella, D. Albanes, P. R. Taylor, J. L. Probstfield, T. J. Jagpal, J. J. Crowley, F. L. Meyskens, L. H. Baker and C. A. Coltman (2009). "Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT)." JAMA **301**(1): 39-51.

Liu, X., J. D. Allen, J. T. Arnold and M. R. Blackman (2008). "Lycopene inhibits IGF-I signal transduction and growth in normal prostate epithelial cells by decreasing DHT-modulated IGF-I production in co-cultured reactive stromal cells." Carcinogenesis **29**(4): 816-823.

Loblaw, D. A. and P. Cheung (2006). "External beam irradiation for localized prostate cancer--the promise of hypofractionation." Can J Urol **13 Suppl 1**: 62-66.

Lu, H. F., Y. L. Chen, J. S. Yang, Y. Y. Yang, J. Y. Liu, S. C. Hsu, K. C. Lai and J. G. Chung (2010). "Antitumor activity of capsaicin on human colon cancer cells in vitro and colo 205 tumor xenografts in vivo." J Agric Food Chem **58**(24): 12999-13005.

Luo, X. J., J. Peng and Y. J. Li (2011). "Recent advances in the study on capsaicinoids and capsinoids." Eur J Pharmacol **650**(1): 1-7.

Luqman, S., A. Meena, L. E. Marler, T. P. Kondratyuk and J. M. Pezzuto (2011). "Suppression of tumor necrosis factor-α-induced nuclear factor κB activation and aromatase activity by capsaicin and its analog capsazepine." J Med Food **14**(11): 1344-1351.

Luqman, S. and J. M. Pezzuto (2010). "NFkappaB: a promising target for natural products in cancer chemoprevention." Phytother Res **24**(7): 949-963.

Malagarie-Cazenave, S., N. Olea-Herrero, D. Vara and I. Díaz-Laviada (2009). "Capsaicin, a component of red peppers, induces expression of androgen receptor via PI3K and MAPK pathways in prostate LNCaP cells." FEBS Lett **583**(1): 141-147.

Malagarie-Cazenave, S., N. Olea-Herrero, D. Vara, C. Morell and I. Díaz-Laviada (2011). "The vanilloid capsaicin induces IL-6 secretion in prostate PC-3 cancer cells." Cytokine **54**(3): 330-337.

Masumori, N., T. Z. Thomas, P. Chaurand, T. Case, M. Paul, S. Kasper, R. M. Caprioli, T. Tsukamoto, S. B. Shappell and R. J. Matusik (2001). "A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential." Cancer Res **61**(5): 2239-2249.

Matikaine, M. P., E. Pukkala, J. Schleutker, T. L. Tammela, P. Koivisto, R. Sankila and O. P. Kallioniemi (2001). "Relatives of prostate cancer patients have an increased risk of prostate and stomach cancers: a population-based, cancer registry study in Finland." Cancer Causes Control **12**(3): 223-230.

Mohamed, M. R. and G. McFadden (2009). "NFkB inhibitors: strategies from poxviruses." Cell Cycle **8**(19): 3125-3132.

Moon, D. O., C. H. Kang, S. H. Kang, Y. H. Choi, J. W. Hyun, W. Y. Chang, H. K. Kang, Y. S. Koh, Y. H. Maeng, Y. R. Kim and G. Y. Kim (2012). "Capsaicin sensitizes TRAIL-induced apoptosis through Sp1-mediated DR5 up-regulation: involvement of Ca(2+) influx." Toxicol Appl Pharmacol **259**(1): 87-95.

Mori, A., S. Lehmann, J. O'Kelly, T. Kumagai, J. C. Desmond, M. Pervan, W. H. McBride, M. Kizaki and H. P. Koeffler (2006). "Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells." Cancer Res **66**(6): 3222-3229.

Musquera, M., N. E. Fleshner, A. Finelli and A. R. Zlotta (2008). "The REDUCE trial: chemoprevention in prostate cancer using a dual 5alpha-reductase inhibitor, dutasteride." Expert Rev Anticancer Ther **8**(7): 1073-1079.

Ni, J., X. Wen, J. Yao, H. C. Chang, Y. Yin, M. Zhang, S. Xie, M. Chen, B. Simons, P. Chang, A. di Sant'Agnese, E. M. Messing and S. Yeh (2005). "Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway." Cancer Res **65**(21): 9807-9816.

Ni, J. and S. Yeh (2007). "The roles of alpha-vitamin E and its analogues in prostate cancer." Vitam Horm **76**: 493-518.

Nomura, A. M., J. Lee, G. N. Stemmermann and G. F. Combs (2000). "Serum selenium and subsequent risk of prostate cancer." Cancer Epidemiol Biomarkers Prev **9**(9): 883-887.

Page, W. F., M. M. Braun, A. W. Partin, N. Caporaso and P. Walsh (1997). "Heredity and prostate cancer: a study of World War II veteran twins." Prostate **33**(4): 240-245.

Pajonk, F., K. Pajonk and W. H. McBride (1999). "Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells." J Natl Cancer Inst **91**(22): 1956-1960.

Palacios, D. A., M. Miyake and C. J. Rosser (2013). "Radiosensitization in prostate cancer: mechanisms and targets." BMC Urol **13**(1): 4.

Palmer, J., V. Venkateswaran, N. E. Fleshner, L. H. Klotz and M. E. Cox (2008). "The impact of diet and micronutrient supplements on the expression of neuroendocrine markers in murine Lady transgenic prostate." Prostate **68**(4): 345-353.

Peng, J. B., L. Zhuang, U. V. Berger, R. M. Adam, B. J. Williams, E. M. Brown, M. A. Hediger and M. R. Freeman (2001). "CaT1 expression correlates with tumor grade in prostate cancer." Biochem Biophys Res Commun **282**(3): 729-734.

Perry, L., R. Dickau, S. Zarrillo, I. Holst, D. M. Pearsall, D. R. Piperno, M. J. Berman, R. G. Cooke, K. Rademaker, A. J. Ranere, J. S. Raymond, D. H. Sandweiss, F. Scaramelli, K. Tarble and J. A. Zeidler (2007). "Starch fossils and the domestication and dispersal of chili peppers (Capsicum spp. L.) in the Americas." Science **315**(5814): 986-988.

Podsypanina, K., L. H. Ellenson, A. Nemes, J. Gu, M. Tamura, K. M. Yamada, C. Cordon-Cardo, G. Catoretti, P. E. Fisher and R. Parsons (1999). "Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems." Proc Natl Acad Sci U S A **96**(4): 1563-1568.

Pramanik, K. C., S. R. Boreddy and S. K. Srivastava (2011). "Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells." PLoS One **6**(5): e20151.

Prevarskaya, N., M. Flourakis, G. Bidaux, S. Thebault and R. Skryma (2007). "Differential role of TRP channels in prostate cancer." Biochem Soc Trans **35**(Pt 1): 133-135.

Prevarskaya, N., L. Zhang and G. Barritt (2007). "TRP channels in cancer." Biochim Biophys Acta **1772**(8): 937-946.

Rayet, B. and C. Gélinas (1999). "Aberrant rel/nfkb genes and activity in human cancer." Oncogene **18**(49): 6938-6947.

Reilly, C. A. and G. S. Yost (2006). "Metabolism of capsaicinoids by P450 enzymes: a review of recent findings on reaction mechanisms, bio-activation, and detoxification processes." Drug Metab Rev **38**(4): 685-706.

Rembrink, K., J. C. Romijn, T. H. van der Kwast, H. Rübben and F. H. Schröder (1997). "Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer." Prostate **31**(3): 168-174.

Rittmaster, R. S. (2011). "Chemoprevention of prostate cancer." Acta Oncol **50 Suppl 1**: 127-136.

Royds, J. A., S. K. Dower, E. E. Qwarnstrom and C. E. Lewis (1998). "Response of tumour cells to hypoxia: role of p53 and NFkB." Mol Pathol **51**(2): 55-61.

Ruotolo, R., L. Calani, F. Brighenti, A. Crozier, S. Ottonello and D. Del Rio (2014). "Glucuronidation does not suppress the estrogenic activity of quercetin in yeast and human breast cancer cell model systems." Arch Biochem Biophys.

Sanchez, M. G., A. M. Sanchez, B. Collado, S. Malagarie-Cazenave, N. Olea, M. J. Carmena, J. C. Prieto and I. Diaz-Laviada I (2005). "Expression of the transient receptor potential vanilloid 1 (TRPV1) in LNCaP and PC-3 prostate cancer cells and in human prostate tissue." Eur J Pharmacol **515**(1-3): 20-27.

Schmid, H. P., C. Fischer, D. S. Engeler, M. L. Bendhack and B. J. Schmitz-Dräger (2011). "Nutritional aspects of primary prostate cancer prevention." Recent Results Cancer Res **188**: 101-107.

Sciavolino, P. J., E. W. Abrams, L. Yang, L. P. Austenberg, M. M. Shen and C. Abate-Shen (1997). "Tissue-specific expression of murine Nkx3.1 in the male urogenital system." Dev Dyn **209**(1): 127-138.

Scott, E. N., A. J. Gescher, W. P. Steward and K. Brown (2009). "Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials." Cancer Prev Res (Phila) **2**(6): 525-530.

Scully, R. and A. Xie (2013). "Double strand break repair functions of histone H2AX." Mutat Res **750**(1-2): 5-14.

Shariff, A. H. and M. H. Ather (2006). "Neuroendocrine differentiation in prostate cancer." Urology **68**(1): 2-8.

Sharifi, N., J. L. Gulley and W. L. Dahut (2005). "Androgen deprivation therapy for prostate cancer." JAMA **294**(2): 238-244.

Shin, D. H., O. H. Kim, H. S. Jun and M. K. Kang (2008). "Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/Rac1 signal pathway." Exp Mol Med **40**(5): 486-494.

Shirai, Y., S. Ueno, A. Nakayama, K. Ikeuchi, K. Ubukata, R. Mihara and B. K. Bernard (2010). "Studies of the toxicological potential of capsinoids, XII: pharmacokinetic study of capsinoid-containing CH-19 Sweet extract in rats." Int J Toxicol **29**(2 Suppl): 15S-21S.

Shukla, S., N. Bhaskaran, M. A. Babcook, P. Fu, G. T. Maclennan and S. Gupta (2014). "Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoxO pathway." Carcinogenesis **35**(2): 452-460.

Shukla, S., G. T. MacLennan, C. A. Flask, P. Fu, A. Mishra, M. I. Resnick and S. Gupta (2007). "Blockade of beta-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice." Cancer Res **67**(14): 6925-6935.

Siegel, R., J. Ma, Z. Zou and A. Jemal (2014). "Cancer statistics, 2014." CA Cancer J Clin **64**(1): 9-29.

Singh, S. and A. Khar (2006). "Biological effects of curcumin and its role in cancer chemoprevention and therapy." Anticancer Agents Med Chem **6**(3): 259-270.

Smith, P. C., A. Hobisch, D. L. Lin, Z. Culig and E. T. Keller (2001). "Interleukin-6 and prostate cancer progression." Cytokine Growth Factor Rev **12**(1): 33-40.

Smith, P. C. and E. T. Keller (2001). "Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice." Prostate **48**(1): 47-53.

Sobel, R. E. and M. D. Sadar (2005). "Cell lines used in prostate cancer research: a compendium of old and new lines--part 1." J Urol **173**(2): 342-359.

Sobel, R. E. and M. D. Sadar (2005). "Cell lines used in prostate cancer research: a compendium of old and new lines--part 2." J Urol **173**(2): 360-372.

Stangelberger, A., M. Waldert and B. Djavan (2008). "Prostate cancer in elderly men." Rev Urol **10**(2): 111-119.

Stein, R. J., S. Santos, J. Nagatomi, Y. Hayashi, B. S. Minnery, M. Xavier, A. S. Patel, J. B. Nelson, W. J. Futrell, N. Yoshimura, M. B. Chancellor and F. De Miguel (2004). "Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract." J Urol **172**(3): 1175-1178.

Stephenson, R. A., C. P. Dinney, K. Gohji, N. G. Ordóñez, J. J. Killion and I. J. Fidler (1992). "Metastatic model for human prostate cancer using orthotopic implantation in nude mice." J Natl Cancer Inst **84**(12): 951-957.

Stolarczyk, M., J. P. Piwowarski, S. Granica, J. Stefańska, M. Naruszewicz and A. K. Kiss (2013). "Extracts from Epilobium sp. herbs, their components and gut microbiota

metabolites of Epilobium Ellagitannins, Urolithins, inhibit hormone-dependent prostate cancer cells-(LNCaP) proliferation and PSA secretion." Phytother Res **27**(12): 1842-1848.

Suzman, D. L. and E. S. Antonarakis (2014). "Castration-resistant prostate cancer: latest evidence and therapeutic implications." Ther Adv Med Oncol **6**(4): 167-179.

Suzuki, H., T. Ueda, T. Ichikawa and H. Ito (2003). "Androgen receptor involvement in the progression of prostate cancer." Endocr Relat Cancer **10**(2): 209-216.

Syed, D. N., Y. Suh, F. Afaq and H. Mukhtar (2008). "Dietary agents for chemoprevention of prostate cancer." Cancer Lett **265**(2): 167-176.

Sánchez, A. M., S. Malagarie-Cazenave, N. Olea, D. Vara, A. Chiloeches and I. Díaz-Laviada (2007). "Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation." Apoptosis **12**(11): 2013-2024.

Sánchez, A. M., J. Martínez-Botas, S. Malagarie-Cazenave, N. Olea, D. Vara, M. A. Lasunción and I. Díaz-Laviada (2008). "Induction of the endoplasmic reticulum stress protein GADD153/CHOP by capsaicin in prostate PC-3 cells: a microarray study." Biochem Biophys Res Commun **372**(4): 785-791.

Sánchez, A. M., M. G. Sánchez, S. Malagarie-Cazenave, N. Olea and I. Díaz-Laviada (2006). "Induction of apoptosis in prostate tumor PC-3 cells and inhibition of xenograft prostate tumor growth by the vanilloid capsaicin." Apoptosis **11**(1): 89-99.

Tabassum, A., R. G. Bristow and V. Venkateswaran (2010). "Ingestion of selenium and other antioxidants during prostate cancer radiotherapy: a good thing?" Cancer Treat Rev **36**(3): 230-234.

Tafani, M., B. Pucci, A. Russo, L. Schito, L. Pellegrini, G. A. Perrone, L. Villanova, L. Salvatori, L. Ravenna, E. Petrangeli and M. A. Russo (2013). "Modulators of HIF1α and NFkB in Cancer Treatment: Is it a Rational Approach for Controlling Malignant Progression?" Front Pharmacol **4**: 13.

Tang, Y., B. Parmakhtiar, A. R. Simoneau, J. Xie, J. Fruehauf, M. Lilly and X. Zi (2011). "Lycopene enhances docetaxel's effect in castration-resistant prostate cancer associated with insulin-like growth factor I receptor levels." Neoplasia **13**(2): 108-119.

Taylor, B. S., N. Schultz, H. Hieronymus, A. Gopalan, Y. Xiao, B. S. Carver, V. K. Arora, P. Kaushik, E. Cerami, B. Reva, Y. Antipin, N. Mitsiades, T. Landers, I. Dolgalev, J. E. Major, M. Wilson, N. D. Socci, A. E. Lash, A. Heguy, J. A. Eastham, H. I. Scher, V. E. Reuter, P. T. Scardino, C. Sander, C. L. Sawyers and W. L. Gerald (2010). "Integrative genomic profiling of human prostate cancer." Cancer Cell **18**(1): 11-22.

Thiele, R., E. Mueller-Seitz and M. Petz (2008). "Chili pepper fruits: presumed precursors of fatty acids characteristic for capsaicinoids." J Agric Food Chem **56**(11): 4219-4224.

Thoennissen, N. H., J. O'Kelly, D. Lu, G. B. Iwanski, D. T. La, S. Abbassi, A. Leiter, B. Karlan, R. Mehta and H. P. Koeffler (2010). "Capsaicin causes cell-cycle arrest and apoptosis in ER-positive and -negative breast cancer cells by modulating the EGFR/HER-2 pathway." Oncogene **29**(2): 285-296.

Thompson, I. M., P. J. Goodman, C. M. Tangen, M. S. Lucia, G. J. Miller, L. G. Ford, M. M. Lieber, R. D. Cespedes, J. N. Atkins, S. M. Lippman, S. M. Carlin, A. Ryan, C. M. Szczepanek, J. J. Crowley and C. A. Coltman (2003). "The influence of finasteride on the development of prostate cancer." N Engl J Med **349**(3): 215-224.

Thorat, M. A. and J. Cuzick (2013). "Role of aspirin in cancer prevention." Curr Oncol Rep **15**(6): 533-540.

Tominaga, M. and M. J. Caterina (2004). "Thermosensation and pain." J Neurobiol **61**(1): 3-12.

Tsagogiorgas, C., J. Wedel, M. Hottenrott, M. O. Schneider, U. Binzen, W. Greffrath, R. D. Treede, B. Theisinger, S. Theisinger, R. Waldherr, B. K. Krämer, M. Thiel, P. Schnuelle, B. A. Yard and S. Hoeger (2012). "N-octanoyl-dopamine is an agonist at the capsaicin receptor TRPV1 and mitigates ischemia-induced [corrected] acute kidney injury in rat." PLoS One **7**(8): e43525.

Tsai, Y. C. and A. M. Weissman (2010). "The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer." Genes Cancer **1**(7): 764-778.

van Breemen, R. B. and N. Pajkovic (2008). "Multitargeted therapy of cancer by lycopene." Cancer Lett **269**(2): 339-351.

Van der Meer, S., S. A. Löwik, W. H. Hirdes, R. M. Nijman, K. Van der Meer, J. E. Hoekstra-Weebers and M. H. Blanker (2012). "Prostate specific antigen testing policy worldwide varies greatly and seems not to be in accordance with guidelines: a systematic review." BMC Fam Pract **13**: 100.

Van Haute, C., D. De Ridder and B. Nilius (2010). "TRP channels in human prostate." ScientificWorldJournal **10**: 1597-1611.

Venier, N., A. J. Colquhoun, N. E. Fleshner, L. H. Klotz and V. Venkateswaran (2012). "**Lycopene enhances the anti-proliferative and pro-apoptotic effects of capsaicin in prostate cancer *in vitro***." Cancer Therapeutics and Research **1**(**2049-7962**).

Venier, N., L. H. Klotz, N. E. Fleshner and V. Venkateswaran (2012). Dietary Agents for Prostate Cancer Chemoprevention: An Overview. Current Cancer Therapy Reviews., Bentham Science. **6:** 308-316.

Venkateswaran, V., N. E. Fleshner and L. H. Klotz (2002). "Modulation of cell proliferation and cell cycle regulators by vitamin E in human prostate carcinoma cell lines." J Urol **168**(4 Pt 1): 1578-1582.

Venkateswaran, V., N. E. Fleshner and L. H. Klotz (2004). "Synergistic effect of vitamin E and selenium in human prostate cancer cell lines." Prostate Cancer Prostatic Dis **7**(1): 54-56.

Venkateswaran, V., N. E. Fleshner, L. M. Sugar and L. H. Klotz (2004). "Antioxidants block prostate cancer in lady transgenic mice." Cancer Res **64**(16): 5891-5896.

Venkateswaran, V. and L. H. Klotz (2010). "Diet and prostate cancer: mechanisms of action and implications for chemoprevention." Nat Rev Urol **7**(8): 442-453.

Venkateswaran, V., L. H. Klotz and N. E. Fleshner (2002). "Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines." Cancer Res **62**(9): 2540-2545.

Venkateswaran, V., L. H. Klotz, M. Ramani, L. M. Sugar, L. E. Jacob, R. K. Nam and N. E. Fleshner (2009). "A combination of micronutrients is beneficial in reducing the incidence of prostate cancer and increasing survival in the Lady transgenic model." Cancer Prev Res (Phila) **2**(5): 473-483.

Wahyuni, Y., A. R. Ballester, E. Sudarmonowati, R. J. Bino and A. G. Bovy (2013). "Secondary metabolites of Capsicum species and their importance in the human diet." J Nat Prod **76**(4): 783-793.

Wang, S., J. Gao, Q. Lei, N. Rozengurt, C. Pritchard, J. Jiao, G. V. Thomas, G. Li, P. Roy-Burman, P. S. Nelson, X. Liu and H. Wu (2003). "Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer." Cancer Cell **4**(3): 209-221.

Wei, F., J. Xu, L. Tang, J. Shao, Y. Wang, L. Chen and X. Guan (2012). "p27(Kip1) V109G polymorphism and cancer risk: a systematic review and meta-analysis." Cancer Biother Radiopharm **27**(10): 665-671.

Wetherill, Y. B., N. L. Fisher, A. Staubach, M. Danielsen, R. W. de Vere White and K. E. Knudsen (2005). "Xenoestrogen action in prostate cancer: pleiotropic effects dependent on androgen receptor status." Cancer Res **65**(1): 54-65.

Wissenbach, U., B. Niemeyer, N. Himmerkus, T. Fixemer, H. Bonkhoff and V. Flockerzi (2004). "TRPV6 and prostate cancer: cancer growth beyond the prostate correlates with increased TRPV6 Ca2+ channel expression." Biochem Biophys Res Commun **322**(4): 1359-1363.

Wu, X., S. Gong, P. Roy-Burman, P. Lee and Z. Culig (2013). "Current mouse and cell models in prostate cancer research." Endocr Relat Cancer **20**(4): R155-170.

Wu, Y., A. Starzinski-Powitz and S. W. Guo (2008). "Capsaicin inhibits proliferation of endometriotic cells in vitro." Gynecol Obstet Invest **66**(1): 59-62.

Wu, Y., H. Zhang, Y. Dong, Y. M. Park and C. Ip (2005). "Endoplasmic reticulum stress signal mediators are targets of selenium action." Cancer Res **65**(19): 9073-9079.

Wu, Y., K. Zu, M. A. Warren, P. K. Wallace and C. Ip (2006). "Delineating the mechanism by which selenium deactivates Akt in prostate cancer cells." Mol Cancer Ther **5**(2): 246-252.

Wutka, A., V. Palagani, S. Barat, X. Chen, M. El Khatib, J. Götze, H. Belahmer, S. Zender, P. Bozko, N. P. Malek and R. R. Plentz (2014). "Capsaicin treatment attenuates cholangiocarcinoma carcinogenesis." PLoS One **9**(4): e95605.

Xin, L., H. Ide, Y. Kim, P. Dubey and O. N. Witte (2003). "In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme." Proc Natl Acad Sci U S A **100 Suppl 1**: 11896-11903.

Yamamura, H., S. Ugawa, T. Ueda, M. Nagao and S. Shimada (2004). "Capsazepine is a novel activator of the delta subunit of the human epithelial Na+ channel." J Biol Chem **279**(43): 44483-44489.

Yoshimoto, M., J. C. Cutz, P. A. Nuin, A. M. Joshua, J. Bayani, A. J. Evans, M. Zielenska and J. A. Squire (2006). "Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias." Cancer Genet Cytogenet **169**(2): 128-137.

Yoshizawa, K., A. Ascherio, J. S. Morris, M. J. Stampfer, E. Giovannucci, C. K. Baskett, W. C. Willett and E. B. Rimm (2003). "Prospective study of selenium levels in toenails and risk of coronary heart disease in men." Am J Epidemiol **158**(9): 852-860.

Zhang, J. H., F. J. Lai, H. Chen, J. Luo, R. Y. Zhang, H. Q. Bu, Z. H. Wang, H. H. Lin and S. Z. Lin (2013). "Involvement of the phosphoinositide 3-kinase/Akt pathway in apoptosis induced by capsaicin in the human pancreatic cancer cell line PANC-1." Oncol Lett **5**(1): 43-48.

Zhang, L. and G. J. Barritt (2004). "Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells." Cancer Res **64**(22): 8365-8373.

Zhang, R., I. Humphreys, R. P. Sahu, Y. Shi and S. K. Srivastava (2008). "In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway." Apoptosis **13**(12): 1465-1478.

Zhao, R., N. Xiang, F. E. Domann and W. Zhong (2006). "Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells." Cancer Res **66**(4): 2296-2304.

Zheng, J. (2013). "Molecular mechanism of TRP channels." Compr Physiol **3**(1): 221-242.

Zhou, B. B. and S. J. Elledge (2000). "The DNA damage response: putting checkpoints in perspective." Nature **408**(6811): 433-439.

Ziglioli, F., A. Frattini, U. Maestroni, F. Dinale, M. Ciufifeda and P. Cortellini (2009). "Vanilloid-mediated apoptosis in prostate cancer cells through a TRPV-1 dependent and a TRPV-1-independent mechanism." Acta Biomed **80**(1): 13-20.

Zuniga, K. E., S. K. Clinton and J. W. Erdman (2013). "The interactions of dietary tomato powder and soy germ on prostate carcinogenesis in the TRAMP model." Cancer Prev Res (Phila) **6**(6): 548-557.

Appendices