

**PRECLINICAL STUDIES OF 3',4',5'-TRIMETHOXYFLAVONOL, A PUTATIVE
AGENT FOR THE CHEMOPREVENTION AND THE MANAGEMENT OF
PROSTATE CANCER**

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By

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ABSTRACT

Flavonoids have shown much promise for the chemoprevention of PCa but their poor bioavailability is thought to hinder their chemopreventive efficacy *in vivo*. However, methoxylation of the flavonoid scaffold could improve bioavailability and efficacy. 3',4',5'-trimethoxyflavonol (TMFol) was identified as the most potent growth-inhibitory agent against the PCa cell lines tested. TMFol was 5-15 times more growth inhibitory than fisetin and quercetin, two widely studied flavonols.

TMFol caused a G₂/M arrest in androgen-dependent cells (LNCaP and TRAMP C2) whereas S phase arrest in the androgen-independent cells (PC-3). TMFol induced more apoptosis in the androgen-dependent cells than the androgen-independent one. TMFol inhibited the expression and activity of the AR and also repressed the mRNA levels of both AR and PSA. TMFol also modulated a number of key apoptotic proteins. *In vitro* data suggests that TMFol may modulate similar proteins to that of quercetin and fisetin, however, exerting its activity at much lower concentrations. Pharmacokinetic data revealed that TMFol levels achievable in the prostate tissue were higher than the concentrations required *in vitro* to exert pharmacological activity.

Nude mice were administered either control diet or diet supplemented with 0.2% w/w TMFol one week prior to cell inoculation. TRAMP C2 cells were implanted into the right flanks of the mice and tumours, once established, were measured twice a week. TMFol significantly reduced the size and weight of the tumour versus control ($p < 0.05$). Equimolar concentrations of quercetin and fisetin in the same model failed to exert efficacy. The expression of p27, bax and survivin which were significantly altered *in vitro* were also significantly changed following TMFol intervention. These results further our understanding of the *in vitro* and *in vivo* pharmacology and cancer preventative activity of TMFol and provide evidence that TMFol may be investigated in preference to quercetin or fisetin for the management of PCa.

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DEDICATION

To my wife and best friend, Hanan.

Thank you for the support and the patience throughout my PhD journey, I would not have done all that without your support and selflessness

To my kids, Waad, Sanad, Wajad and Majed

You give me a reason to love life and to work hard in all that I do

CONTENTS

Abstract

Acknowledgement

Dedication

CHAPTER 1: INTRODUCTION

1.1 Prostate cancer	1
1.1.1 Epidemiology of PCa	2
1.1.2 Risk factors	3
1.1.2.1 Age	3
1.1.2.2 Ethnicity and genetic factors	6
1.1.2.2.1 Race	6
1.1.2.2.2 Family history	6
1.1.2.2.3 Diet, lifestyle and environment	7
1.1.2.2.4 Steroid hormones.	8
1.1.3 Prostate Cancer Pathology	8
1.1.3.1 Clinical manifestations of prostate cancer and their management	11
1.1.3.2 Metastatic prostate cancer	14
1.1.4 Androgen receptor and prostate cancer	14
1.1.4.1 The androgen receptor cycle	17
1.1.4.2 Androgen receptor cross-talk with cell signalling pathways	17
1.1.4.3 Androgen dependent and non androgen dependent PCa	18
1.1.4.3.1 Hypersensitivity pathways	19

1.1.4.3.2 Promiscuous pathway	19
1.1.4.3.3 The outlaw pathway	20
1.2 Chemoprevention of prostate cancer	20
1.2.1 General concepts	20
1.2.2 Opportunities for chemoprevention in prostate cancer	21
1.2.3 Cell signalling targets for PCa chemoprevention	23
1.2.3.1 Androgen receptor and receptor tyrosine kinase signalling	23
1.2.4 Chemopreventive agents in prostate cancer	26
1.2.4.1 Finasteride and Dutasteride	26
1.2.4.2 Selenium and Vitamin E	29
1.2.4.3 Vitamin D	30
1.2.4.4 COX-2 inhibitors	31
1.3 Preclinical models of prostate cancer	32
1.3.1 Prostate cancer cell lines	32
1.3.2 Animal models	34
1.3.2.1 Rat models of PCa	34
1.3.2.2 Canine models of prostate cancer	34
1.3.2.3 Mouse models of prostate cancer	35
1.3.2.3.1 TRAMP mouse model	35
1.3.2.3.2 PTEN mouse model	36
1.3.2.3.3 Xenograft models of prostate cancer	37
1.4 Flavonoids and cancer	37
1.4.1 Flavonols and prostate cancer	40

1.4.1.1 Mechanisms of chemoprvention activity of flavonols in PCa	40
1.4.1.1.1 Alteration of redox state	40
1.4.1.1.2 Interference with cell cycle	41
1.4.1.1.3 Induction of apoptosis	44
1.4.1.1.4 Inhibition of androgen receptor pathway	47
1.4.1.2 Pharmacokinetics of flavonoids	48
1.4.1.2.1 Methoxylated versus hydroxylated flavonoids	50
1.5 Aims and objectives	53
CHAPTER 2: MATERIALS AND METHODS	
2.1 Materials	55
2.1.1 Chemicals and instruments	55
2.1.2 Antibodies against proteins	55
2.2 Buffers	57
2.3 Methods	60
2.3.1 Cell culture	60
2.3.2 Maintenance of cell lines	60
2.3.3 Cell passaging	60
2.3.4 Cell treatment and drug solvents	61
2.3.5 Cell proliferatory assay	62
2.3.5.1 Coulter counter method	62
2.3.5.2 ATP based luminescence assay	62
2.3.6 Cell cycle assessment	63
2.3.7 Annexin V apoptosis assay	64

2.3.8 Protein analysis and western blot	65
2.3.8.1 Preparation of the whole cell lysate and tissue lysate	65
2.3.8.2 Bradford protein assay	66
2.3.8.3 SDS-PAGE and western blotting	67
2.3.9 Intracellular and extracellular PSA levels in LNCaP	68
2.3.9.1 Intracellular PSA content	68
2.3.9.2 Media PSA immunoassay	69
2.3.10 Detection of apoptosis proteins using Human apoptosis array kit	69
2.3.11 Androgen receptor stability	70
2.3.12 Reverse transcription-polymerase chain reaction (RT-PCR) to determine mRNA levels of androgen receptor, PSA and GAPDH	70
2.3.13 Agarose gel electrophoreses	71
2.3.14 Animal experiment for pharmacokinetics	72
2.3.14.1 Single oral dose study	72
2.3.14.2 Long term dietary feeding	72
2.3.14.3 Sample preparation	73
2.3.14.4 Sample extraction	73
2.3.14.5 Calibration curves	74
2.3.15 Chromatographic conditions	74
2.3.16 <i>In vitro</i> generation of TMFol metabolites	75
2.3.16.1 Demethylation reactions	75
2.3.16.2 Glucuronidation reaction	75
2.3.16.3 Sulphation reaction	76
2.3.17 Metabolites identification using Liquid Chromatography-Mass Spectrometry (LC-MS)	76

2.3.18 TMFol intercellular content	77
2.3.19 Xenograft efficacy study	77
2.3.19.1 Animals and TMFol, quercetin and fisetin dosing	77
2.3.19.2 Intervention in TRAMP C2 tumour bearing athymic (nude) mice	77
2.3.19.3 Immunohistochemical analysis of TRAMP C2 tumours	78
2.3.20 Statistical analysis	79

**CHAPTER 3: EFFECT OF TMFol ON PROSTATE CANCER CELL
GROWTH- INVESTIGATION INTO THE POTENTIAL
MECHANISM OF ACTION**

3.1 Introduction	81
3.2 Effect of hydroxylated and methylated flavonoids on cell proliferation In androgen dependent and androgen independent prostate cancer cells	82
3.3 Effects of TMFol, quercetin and fisetin on the proliferation of LNCaP, PC-3 and TRAMP C2 and IC₅₀s at 72 and 144 h	87
3.4. Effect of TMFol on cell cycle distribution in LNCaP, PC-3 and TRAMP C2 cells	97
3.5. Effect of TMFol on apoptosis in LNCaP, PC-3 and TRAMP C2 using annexin V	101
3.6. Investigating the effect of TMFol on the androgen receptor signalling pathway	109
3.6.1 Effect of TMFol, fisetin and quercetin on androgen receptor expression in LNCaP cells	109
3.6.2 Effect of TMFol, on androgen receptor expression in TRAMP C2 cells	112
3.6.3 Effect of TMFol, fisetin and quercetin on androgen receptor expression in LNCaP cells in the presence of mibolerone	114

3.6.4 Effect of TMFol on PSA expression in LNCaP cells in presence or absence of mibolerone	117
3.6.5 Effect of TMFol on mRNA levels of androgen receptor and PSA	120
3.6.6 Effect of TMFol on the turnover of androgen receptor and PSA	122
3.7 Effect of TMFol on apoptosis related proteins	124
3.7.1 Effect of TMFol on a panel of apoptosis proteins	124
3.7.2 Effects of TMFol, quercetin and fisetin on the expression of p53	126
3.7.3 Effect of TMFol on the expression of the apoptotic proteins, bax and surviving, in TRAMP C2 cells	129
3.8 Effect of TMFol on p21 and p27 expression	131
3.8.1 Effect of TMFol, quercetin and fisetin on the expression of p21	131
3.8.2 Effect of TMFol on the expression of p27	134
3.9 Discussion	3.9

CHAPTER 4: METABOLIC AND BIO-DISTRIBUTION STUDIES OF TMFOL

4.1 Introduction	147
4.2 Plasma and tissue levels of TMFol following a single intra-gastric dose	148
4.3. Metabolism of TMFol in vitro and in vivo	154
4.3.1 In vitro generation of TMFol metabolites	155
4.3.2 Identification and characterisation of In vivo tissue metabolites	157
4.3.3 Identification of TMFol metabolites generated <i>in vivo</i> using liquid chromatography mass spectrometry (LC-MS)	160
4.3.4 Identification of TMFol metabolites in urine	164
4.3.5 Metabolism of TMFol by prostate cancer cell lines	166

4.4 Intracellular TMFol uptake by prostate cancer cell lines	168
4.4.1 Intracellular uptake of TMFol by LNCaP prostate cancer cells	168
4.4.2 Intracellular uptake of TMFol by PC-3 prostate cancer cells	171
4.5 Discussion	174
CHAPTER 5: <i>IN VIVO</i> EFFICACY OF TMFOL IN PROSTATE CANCER XENOGRAFTS	
5.1 Introduction	181
5.2 Effect of TMFol, quercetin and fisetin on TRAMP C2 prostate cell growth in nude mice	182
5.3 Modulation of the apoptotic proteins, bax, survivin and p27, by TMFol, quercetin and fisetin in tumour tissue	185
5.4 Effect of TMFol on Ki-67, cleaved caspase-3 and AR expression in paraffin-embedded xenograft tumour tissue using immunohistochemical staining	188
5.5 TMFol levels measureable in the prostate and tumour tissue following chronic dosing	190
5.6 Discussion	193
CHAPTER 6: THE FINAL DISCUSSION	198
CHAPTER 7: APPENDIX	205
7.1 List of abbreviations	206
7.2 Publications	210

REFERENCES**LIST OF FIGURES****CHAPTER 1**

- 1.1 Graphical representation of the average number of new cases of PCa and age-specific incidence rates for the UK between 2006-2008
- 1.2 Anatomy of the prostate
- 1.3 Diagrammatic illustration of the Gleason grading system
- 1.4 Prostate cancer stages and the treatment options
- 1.5 Summary of the essential steps of metastasis
- 1.6 The margin of chemoprevention opportunity in Prostate cancer
- 1.7 The possible potential targets for PCa chemoprevention
- 1.8 Potential PCa chemoprevention targets of AR and RTK pathways
- 1.9 Classification of common flavonoids
- 1.10 Schematic representation of the cell cycle
- 1.11 Extrinsic and intrinsic apoptosis pathways

CHAPTER 3

- 3.1 Effect of apigenin, tricin, PMF and compounds (1-12), (5, 10 and 20 μM) on the growth of LNCaP cells at 72 h (A) and 144 h (B)
- 3.2 Effect of apigenin, tricin, PMF and compounds (1-12), (5, 10 and 20 μM) on the growth of PC-3 cells at 72 h (A) and 144 h (B)

- 3.3 Effect of apigenin, tricetin, PMF and compounds (1-11), (5, 10 and 20 μM) on the growth of DU145 cells at 72 h (A) and 144 h (B)
- 3.4 Growth inhibitory bar charts of TMFol (0-20 μM) in LNCaP cells at 72 (A) and 144 (B) h
- 3.5 Growth inhibitory bar charts of TMFol (0-20 μM) in PC-3 cells at 72 (A) and 144 (B) h
- 3.6 Growth inhibitory bar charts of TMFol (0-20 μM) in TRAMP C2 cells at 72 (A) and 144 (B) h
- 3.7 Growth inhibitory bar charts of quercetin (0-40 μM) in LNCaP cells at 72 (A) and 144 (B) h
- 3.8 Growth inhibitory bar charts of quercetin (0-40 μM) in PC-3 cells at 72 (A) and 144 (B) h
- 3.9 Growth inhibitory bar charts of Fisetin (0-40 μM) in LNCaP cells at 72 (A) and 144 (B) h
- 3.10 Growth inhibitory bar charts of Fisetin (0-40 μM) in PC-3 cells at 72 (A) and 144 (B) h
- 3.11 The cell cycle distribution of LNCaP cells after treatment with TMFol (1-20 μM) or vehicle control (0) for 48 h (A) and 72 h (B)
- 3.12 The cell cycle distribution of PC-3 cells after treatment with TMFol (1-20 μM) or vehicle control (0) for 48 h (A) and 72 h (B)
- 3.13 The cell cycle distribution of TRAMP C2 cells after treatment with TMFol (1-20 μM) or vehicle control (0) for 48 h (A) and 72 h (B)
- 3.14 Representative fluorescence scattergrams of LNCaP cells analysed by flow cytometry after incubation with TMFol (1-20 μM) or vehicle control for 72 and 96 h
- 3.15 The proportion of live, necrotic and apoptotic LNCaP cells after incubation with TMFol (1-20 μM) or vehicle control for 72 h (A) or 96 h (B).

- 3.16 Representative fluorescence scattergrams of PC-3 cells analysed by flow cytometry after incubation with TMFol (1-20 μ M) or vehicle control for 72 and 96 h
- 3.17 The proportion of live, necrotic and apoptotic PC-3 cells after incubation with TMFol (1-20 μ M) or vehicle control for 72 h (A) or 96 h (B)
- 3.18 Representative fluorescence scattergrams of TRAMP C2 cells analysed by flow cytometry after incubation with TMFol (1-20 μ M) or vehicle control for 72 and 96 h
- 3.19 The proportion of live, necrotic and apoptotic TRAMP C2 cells after incubation with TMFol (1-20 μ M) or vehicle control for 72 h (A) or 96 h (B)
- 3.20 Effect of TMFol on AR protein expression in LNCaP cells, after (A) 24, (B) 48 and (C) 72 h
- 3.21 Effect of quercetin (A, B) and fisetin (C, D) on AR protein expression in LNCaP cells at 48 h (A, C) and at 72 h (B, D)
- 3.22 Effect of TMFol on AR protein expression in TRAMP C2 cells grown in standard media conditions (A and B) and in 10% v/vFCS media (C and D), at 48 h (A, C) and at 72 h (B, D)
- 3.23 Effect of TMFol on AR protein expression in LNCaP cells in presence of mibolerone, after (A) 24, (B) 48 and (C) 72 h
- 3.24 Effect of quercetin (A) and fisetin (B) on AR protein expression in LNCaP cells in the presence of 1 nM mibolerone
- 3.25 Effect of TMFol on PSA protein expression in LNCaP cells grown in absence (A and B) and in presence (C and D) of mibolerone, at 48 h (A, C) and at 72 h (B, D)

- 3.26 Effect of TMFol on secreted PSA levels from LNCaP cells grown in absence (A and B) and in presence (C and D) of mibolerone, at 48 h (A, C) and at 72 h (B, D)
- 3.27 Effect of TMFol on mRNA levels of AR (A, B) and PSA (C, D) at 48 h (A, C) and 72 h (B, D) in LNCaP cells
- 3.28 Effect of TMFol on protein stability of AR (A) and PSA (B) in LNCaP cells over time after blocking the protein synthesis using cycloheximide
- 3.29 Effect of 72 h incubation of TMFol on apoptosis related proteins in LNCaP cells using a human apoptosis array kit
- 3.30 The effect of TMFol on p53 protein levels, in LNCaP cells at 48 (A) and 72 h (B)
- 3.31 Effects of quercetin (A, B) and fisetin (C, D) on p53 protein expression in LNCaP cells, at 48 h (A, C) and at 72 h (B, D)
- 3.32 Effect of TMFol on bax (A, B) and survivin (C, D) protein expression in TRAMP C2 cells, at 48 h (A, C) and at 72 h (B, D)
- 3.33 Effect of TMFol on p21 protein expression in LNCaP cells (A, B) and PC-3 cells (C, D), at 48 (A, C) and 72 h (B, D)
- 3.34 Effect of quercetin (A, B) and fisetin (C, D) on p21 protein expression in LNCaP cells, at 48 h (A, C) and at 72 h (B, D)
- 3.35 Effect of TMFol on p27 protein expression in LNCaP cells grown in 10% v/v FCS media (A, B) or in 10% v/v FCS media supplemented with 1nM mibolerone (C, D), for 48 h (A, C) and for 72 h (B, D)

CHAPTER 4

- 4.1 HPLC fluorescence chromatograms of blank (black solid line) and 20 minutes tissue samples (A) Liver, (B), Kidney, (C), Prostate, (D) Heart, (E) Lung and (F) Small intestinal mucosa from mice that received 240mg/kg ig TMFol

- 4.2 Concentration of TMFol in (ng/g) tissue from liver (A), kidney (B), prostate (C), heart (D), lung (E) and small intestine (F) of male mice after receiving a single intragastric TMFol dose of 240 mg/kg
- 4.3 HPLC analysis of incubation extracts of mouse liver microsome or cytosol was incubated with TMFol in the presence of either UDPGA or PAPS to generate glucuronides (A) or sulphate (B) metabolites respectively
- 4.4 Identification of TMFol metabolites in liver
- 4.5 Identification of TMFol metabolites in intestinal mucosa
- 4.6 LC-MS extracted ion chromatography chromatograms of intestinal scrape extracts from mouse after 360 minutes post single dose of 240mg/kg TMFol
- 4.7 Metabolism of TMFol in the mouse. Chemical structures of the possible TMFol metabolites in mice intestinal mucosa extracts
- 4.8 Identification of TMFol metabolites in urine
- 4.9 Identification of TMFol metabolites produced by PCa cells in cell culture media
- 4.10 Levels of intracellular TMFol in LNCaP cells over time after incubation with 10 μ M TMFol
- 4.11 Representative HPLC fluorescence chromatograms of LNCaP cells extracts after incubation with (i) DMSO or 10 μ M TMFol for (ii) 15, (iii) 30, (iv) 60, (v) 90, (vi) 480 and (vii) 1440 h
- 4.12 Levels of intracellular TMFol in PC-3 cells over time after incubation with 10 μ M TMFol. Intracellular TMFol content is displayed in ng/million cells
- 4.13 Representative HPLC fluorescence chromatograms of PC-3 cells extracts after incubation with (i) DMSO or 10 μ M TMFol for (ii) 30, (iii) 60, (iv) 90, (v) 180, (vi) 480 minutes, (vii) 24, (viii) 48 and (ix) 72 h

CHAPTER 5

- 5.1 The effect of dietary TMFol on tumour growth in nude mice
- 5.2 The effect of dietary quercetin and fisetin on tumour growth in nude mice
- 5.3 Effect of TMFol on the expression of bax, survivin and p27 in TRAMP C2 tumour xenografts in nude mice
- 5.4 Effect of quercetin or fisetin on the expression of bax, survivin and p27 in TRAMP C2 tumour xenografts in nude mice
- 5.5 The effect of dietary TMFol on the expression of Ki-67 in TRAMP prostate cancer xenograft of male nude mice
- 5.6 The effect of dietary TMFol on the expression of caspase-3 cleaved in TRAMP prostate cancer xenograft of male nude mice
- 5.7 The effect of dietary TMFol on the expression of AR in TRAMP prostate cancer xenograft of male nude mice
- 5.8 HPLC analysis of TMFol in tumours and prostate of mice treated with 0.2% w/w TMFol in the diet

LIST OF TABLES

CHAPTER 1

- 1.1 Localized prostate cancer risk stratification depending on Gleason scoring and suggested treatments
- 1.2 Prostate cancer cell lines used in chemoprevention research

CHAPTER 2

- 2.1 Chemical structures of apigenin, tricetin, PMF and compounds 1-12.
- 2.2 Antibody conditions and dilutions

CHAPTER 3

- 3.1 IC₅₀ values \pm SD for TMFol, quercetin and fisetin in LNCaP, PC-3

and TRAMP C2 cells at 72 and 144 h

3.2 The secreted PSA levels (ng PSA/million cells) from LNCaP cells after 48 and 72 h incubation in the presence or absence of the AR agonist, mibolerone

CHAPTER 4

4.1 Relative extraction efficiencies for TMFol from murine plasma, tissues and human PCa cells

4.2 Pharmacokinetics of TMFol in the tissues of mice after a single oral dose (240 mg/kg)

4.3 Result of mass spectrometric screening of TMFol metabolites

CHAPTER ONE

Introduction

1.1 Prostate cancer

Prostate cancer (PCa) is a hormone related male genitourinary cancer. The prostate gland is fundamental for male sexual and reproductive functions as its secretions are necessary for sperm viability and motility. In addition, it is also important in the control of the urination process (Litvinov et al., 2003). As with most malignancies, PCa is thought to arise through the interaction between environmental and genetic factors which finally lead, in a stepwise progression, to cancer. Much remains unclear regarding prostatic carcinogenesis but intensive research is directed at the identification of new genetic and environmental risk factors (Wagner et al., 2007, Shand and Gelmann, 2006b).

1.1.1 Epidemiology of PCa

Published evidence suggests that the incidence of PCa may vary geographically, i.e. it occurs in various populations at different rates (Hsing and Devesa, 2001). According to the British National Health Service (NHS) Prostate Cancer Risk Management Statistics (<http://info.cancerresearchuk.org/cancerstats/types/prostate/>), the picture of PCa in the UK is as follows: PCa is the most common cancer among British men and accounts for about one fourth of the total new male cancer patients diagnosed annually. In the year 2008, 37,000 PCa cases were registered, with 10,168 deaths reported in the same year.

During the late 1980s the prostate specific antigen test (PSA) was introduced, which can detect PCa in the asymptomatic stage. As a result the incidence rates of PCa increased dramatically in the early 1990s, then declined again (Chen et al., 2003, Potosky et al., 1995). Despite the large number of new PCa cases and high morbidity, the causes and the exact aetiology of PCa are still not completely understood. Many studies have been undertaken to investigate and predict the risk factors for PCa, including familial history, lifestyle, body weight and steroid hormones.

1.1.2 Risk factors

1.1.2.1 Age

Age is widely considered to be the most significant risk factor for PCa (Kabalin et al., 1989, Sakr et al., 1996). It is generally accepted that PCa incidence is directly proportional to age. According to NHS figures the life time risk of PCa among UK men is 1 in 14. Furthermore, more than 75% of diagnosed PCa cases were in men over the age of 65 years (Figure 1.1) (<http://www.cancerscreening.nhs.uk/prostate/statistics.html>). Some western countries figures also reflect the UK statistics. Eighty percent of PCa cases in American men occurred in those over the age of 65 (Parkin et al., 1999), its prevalence increases with age greater than any other cancers in the USA (Leibovitz et al., 2004). Moreover, the National Cancer Institute's Surveillance, Epidemiology and End Results [SEER] program revealed that in the period 1996-2000 the incidence of PCa in American men under the age of 65 was 56.8 per 100,000, compared to a much higher incidence in men over 65, with 974.7 cases (Ries et al., 2003, Hsing and Chokkalingam, 2006).

Some hypotheses have been suggested to help explain this phenomenon. It is thought that with aging there is an increase in DNA damage, including deoxyguanosine malondialdehyde (dG-MDA) DNA adducts (Draper et al., 1995) and in the frequency of DNA strand breaks in all body tissues (He and Yasumoto, 1994, Stadtman, 1992). Damaged DNA if escaped repair can result in mutations, which may contribute to cancer initiation and progress (Bostwick et al., 2004a). It is believed these changes are produced because of the accumulation of reactive oxygen species (ROS) and free radicals, which are produced during normal metabolic processes and have the ability to react with DNA and other vital organs of the cell (Fleshner and Klotz, 1998, Leadon, 1990). Furthermore the capability of repairing damaged DNA is retarded by aging (Vijg, 2008, Sauvaigo et al., 2010). It is widely thought aging results in an imbalance in prooxidant-antioxidant statues of the cell (Warner, 1994). This hypothesis was supported by observations such as; (i) The cellular oxidative damage was retarded by the overexpression of antioxidative enzymes and also the maximum lifespan was extended in *Drosophila melanogaster* , (ii) a inverse

relation was found between the species longevity and the rates of mitochondrial production of the superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide. (iii) the restriction of caloric intake was found to extend the maximum life span in mammals, and decreasing the age associated changes, in addition to lowering the steady state levels of oxidative stress and damage (Sohal and Weindruch, 1996).

The decline of the immune system is also considered an important factor in the increased incidence of PCa in the elderly, since cancer cells are often produced in the human body but the immune system can detect them, treat them as foreign bodies and consequently, eradicate them (Markiewicz and Gajewski, 1999, Vesely et al., 2011). This notion is widely accepted since the term “immune surveillance” against cancer was coined by Thomas and Burnet (Thomas, 1959, Burnet, 1967). As the body gets older, the immune system gets weaker (immunosenescence), including the cellular immunity which might be the most affected (Yung, 2000, Ginaldi et al., 1999a, Ginaldi et al., 1999b), therefore older men are more prone to PCa.

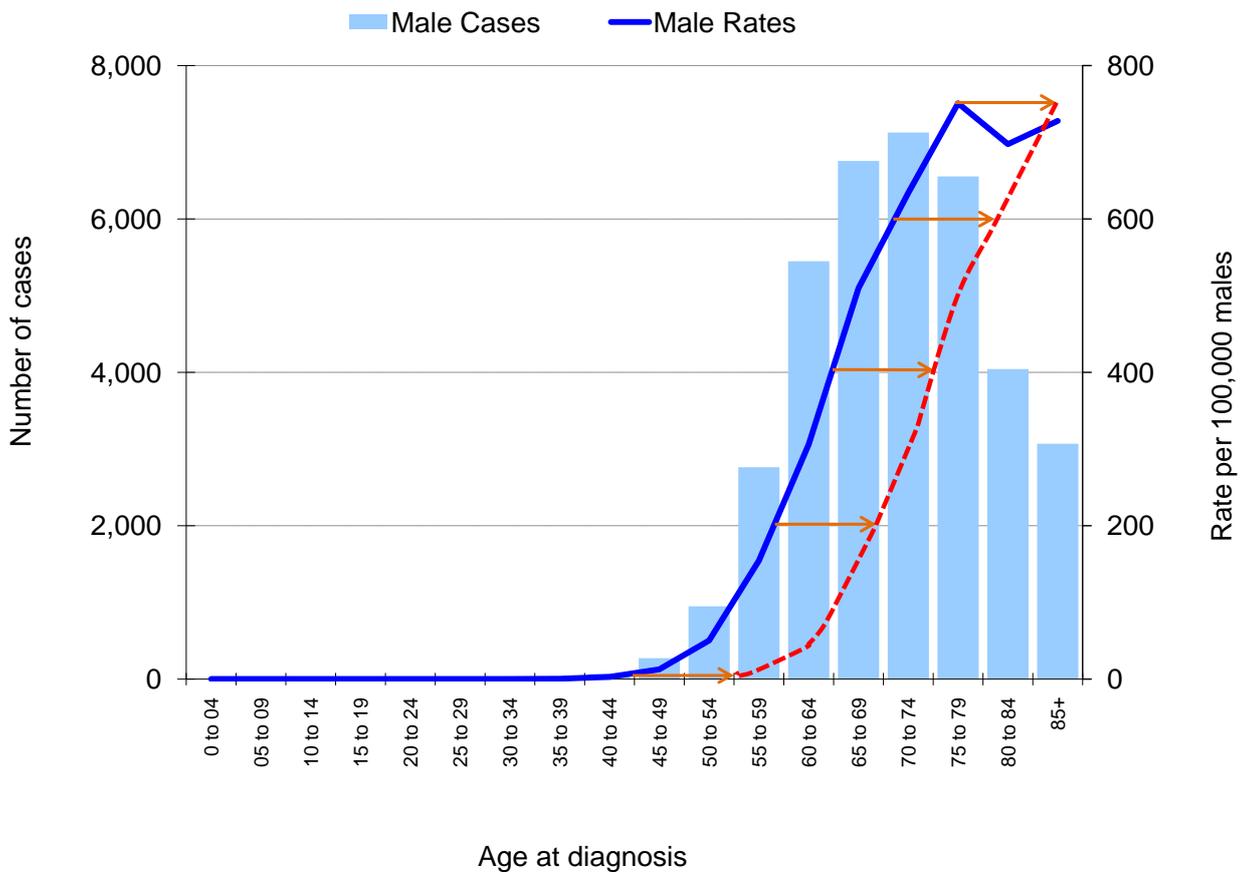


Figure 1.1 Graphical representation of the average number of new cases of PCa and age-specific incidence rates for the UK between 2006-2008. Blue curve represents the actual incidence rates seen while the dotted red curve is a predicted incidence rate if the use of chemopreventive agents could delay cancer development by approximately 10 years. This highlights that the number of new cases, if a chemoprevention strategy was successful, could almost be halved assuming life expectancy of around 75 years of age. Figure was adapted from <http://info.cancerresearchuk.org/cancerstats/types/prostate/incidence/#>

1.1.2.2 Ethnicity and genetic factors

1.1.2.2.1 Race

Although the incidence of PCa is geographically variable, the role that ethnicity plays in the disease is still not fully understood. It was noticed that the incidence and mortality of the disease among black Americans is higher than in their white American counterparts. Notably, the incidence of PCa is 1.5 times higher in black Americans (Littrup, 1997, Bostwick et al., 2004a). Moreover, the mortality rate from PCa is twice as high in African-American men (Powell, 2007). Many studies have been performed to elicit an explanation for this variation. For example, biopsy screening of 500 men between 2003-2005 indicated that African Americans with a positive biopsy were significantly younger than the Caucasian American (Swords et al., 2010). These high rates are not specific to Afro Americans but are also higher among African migrants to other regions and countries, such as Europe, Jamaica and Brazil. Therefore, a common genetic susceptibility factor might be contributing to this higher incidence and mortality (Antopoulos, 2001, Glover et al., 1998). Many other hypotheses have been proposed to decipher the cause, such as lifestyle factors, including diet and levels of vitamin D, as the ability of black people to produce UV induced-vitamin D3 is less than in other races (Schwartz and Hulka, 1990). Another explanation could be that the levels of 5 α -reductase are also higher in African people (Makridakis et al., 1997); this enzyme is responsible for the conversion of testosterone to the more potent analogue dihydrotestosterone (DHT), which is linked to the progression of PCa. (Gann et al., 1996, Comstock et al., 1993, Shaneyfelt et al., 2000, Thompson et al., 2003a).

1.1.2.2.2. Family history

The theory that genetics and family history might have an influential role in PCa incidence was based on the findings of many case-control, cohort, twin and family-based studies (Schaid, 2004). It has been confirmed that there is a functional role of heredity in PCa (Shand and Gelmann, 2006a, Negri et al., 2005). Men who have a first degree relative that has had PCa are 2-3 times more susceptible to PCa compared to men with no family history (Johns and

Houlston, 2003, Colloca and Venturino, 2011). It was also found that PCa risk in monozygotic twins is double that in dizygotic twins (Verkasalo et al., 1999). Moreover a number of studies have found an increased risk of prostate cancer among men who have a female relative with breast cancer (Cerhan et al., 1999, Rodriguez et al., 1998), however, some studies have been contradictory to these (Kalish et al., 2000, Andrieu et al., 1991) and therefore this link remains inconclusive.

1.1.2.2.3. Diet, lifestyle and environment

Significant variations in PCa incidence rates among countries exist but this difference disappears as people migrate from countries with low risk to one with a higher risk. Therefore, it is conceivable that lifestyle and the environment might contribute to these variations (Wigle et al., 2008, Donn and Muir, 1985). Furthermore, intensive lifestyle changes may have an effect on PCa progression at some stages (Ornish et al., 2005). Recent studies have described links between obesity and the risk of aggressive PCa. Obesity was related to an amplified risk of metastasis and death from prostate cancer in middle aged men (Gong et al., 2007). It was also found that consumption of a western-type diet rich in fat and cholesterol by TRAMP mice, a preclinical model of prostate carcinogenesis, increases prostate tumour incidence and burden (Llaverias et al., 2010). One explanation for the increased rates among obese men is the intrinsic bias in the detection of PCa, since overweight men have larger prostate size and lower PSA levels, leading to delay in the diagnosis of the disease making the disease more fatal in this population of men. In addition, high blood levels of some mediators such as, insulin, estradiol, free leptin, and IGF-1 and low levels of others such as adiponectin and testosterone might contribute to the severity of the disease in this high risk group (Buschemeyer and Freedland, 2007). A positive correlation was also found between cancer and diets high in animal fat (Hayes et al., 1999) from different sources such as red meat (Kolonel, 1996) and dairy product (Gao et al., 2005, Raimondi et al., 2010). However, calcium which is available in dairy products may also be associated with prostate cancer incidence and progress (Butler et al., 2010). Many studies have been carried out to investigate the effects of different diet

compositions on prostate cancer. Other environmental and lifestyle related factors have been considered as having a possible role in prostate cancer development including smoking (Hickey et al., 2001, Malila et al., 2006, Zu and Giovannucci, 2009, Rizos et al., 2010), alcohol consumption (Sesso et al., 2001, Platz et al., 2004, Baglietto et al., 2006), physical activity (Wannamethee et al., 2001, Sung et al., 1999) and occupation (Bostwick et al., 2004a), but the results have been conflicting.

1.1.2.2.4. Steroid hormones

Since prostate carcinogenesis is mostly androgen sensitive, and can respond to hormonal therapy before it become insensitive, it was proposed that anti-androgens might have a chemopreventive benefit (Bosland, 2000). Studies have also been performed to determine the influence of endocrine function on PCa, but the results were conflicting; some found a relationship between endogenous androgen levels and PCa (Gann et al., 1996, Comstock et al., 1993, Shaneyfelt et al., 2000, Thompson et al., 2003a) whilst, others didn't support this notion (Eaton et al., 1999, Stattin et al., 2004). Generally epidemiological studies haven't found convincing evidence for a role of endogenous androgens in prostate carcinogenesis (Wigle et al., 2008).

1.1.3 Prostate Cancer Pathology

The prostate gland is an oval shaped structure, which lies below the bladder and in front of the rectum. It is divided histologically or functionally into three zones (Figure 1.2); the central zone, the transitional zone representing only ~5% of the prostate volume, which consists mainly of two small lobes and the peripheral zone which accounts for about 70% of the prostatic volume (Oesterling et al., 1997). The prostate is comprised of tubuloalveolar glands, lined with epithelial compartments consisting of secretory epithelial cells under which are located the basal cells. The stromal compartment is composed mainly of smooth muscle and connective tissue (Oesterling et al., 1997). It is widely believed that Prostatic Intraepithelial Neoplasia (PIN) is the putative precursor of most prostate adenocarcinomas (Bostwick and Brawer, 1987). PIN initiates within the epithelium of the prostate ducts, ductules and acini, and is

characterized by cellular changes such as nucleolar and nuclear enlargement (Bostwick, 2000, Graham et al., 1992). There are four common architectural patterns of PIN, including tufting, flat, cribriform and micropapillary, it is not known if there is any clinical significance associated with these differences (Bostwick, 2000). PIN can be either high or low grade and has been linked to prostate cancer based on morphological, morphometric, immunohistochemical, genetic and molecular evidence (McNeal et al., 1995, Qian et al., 1997).

The Gleason score is the most commonly used method for grading localized prostatic adenocarcinoma and it has been shown to have considerable diagnostic use (Gleason, 1992). The Gleason system depends on architectural classification of the tumour growth, according to five histological grades. If the tumour tissue closely resembles normal tissue, in that it is uniform, well formed small glands (nearly normal pattern), then it is graded 1, conversely, the highest level (grade 5) is when the tumour consists of a diffuse sheet-like arrangement of cancer cells and the infiltration of single isolated cells can be detected in the stroma (absence of glandular form) as shown in Figure 1.3. Finally, the two most prominent grades are added together to determine the final score, between 2-10 (Pan et al., 2000). Gleason scoring process is usually undertaken on specimens of prostate biopsy or samples of prostate tissue after prostatectomy. Prostate biopsy includes about 12 samples, each sample scored separately and the grades combined to produce the Gleason score (<http://www.hadit.com/forums/index.php?/topic/39619-prostate-biopsy-and-the-gleason-score-what-you-should-know/>).

Gleason scoring is used as a method for stratifying the risk of PCa, and therefore assists in determining the appropriate treatment. For example, the national collaborating centre for cancer has designed guidelines for the diagnosis and treatment of localised PCa based on the Gleason scoring system Table 1.1.

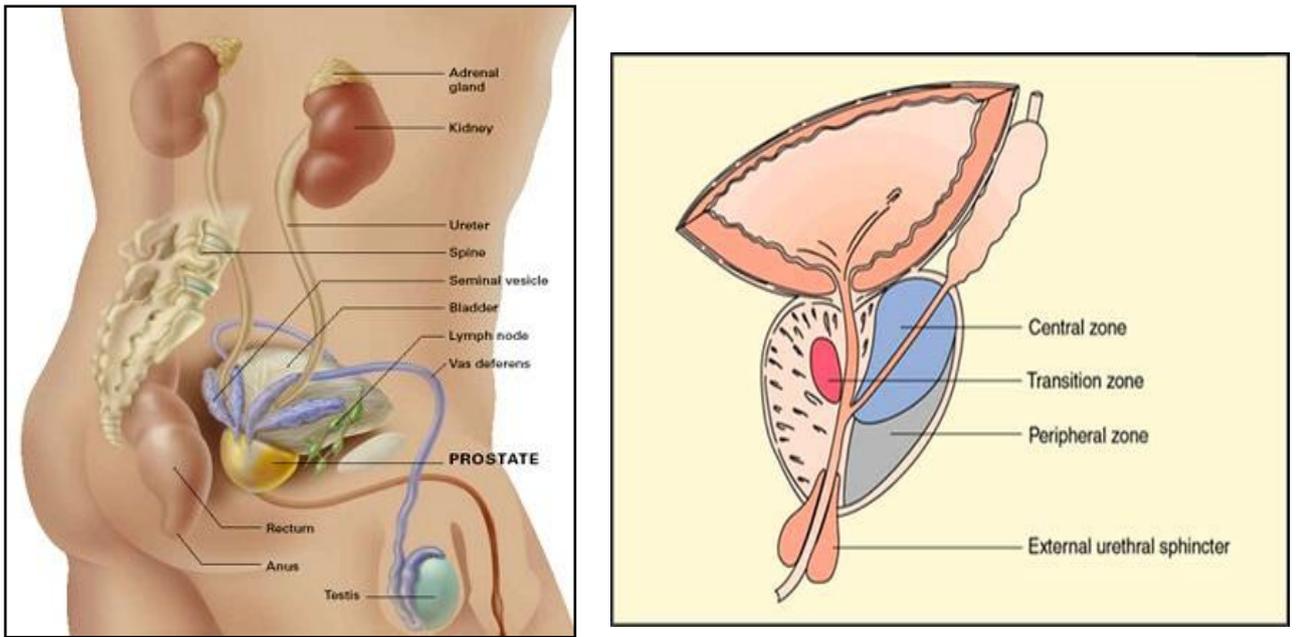


Figure 1.2. Anatomy of the prostate. Diagram was taken from <http://www.fesupanallah-dizisi.com/alternative-medicine/prostate-cancer-treatment-what-is-best/> and http://www.cancertreatment-wecareindia.com/prostate_cancer/images/prostate1.jpg

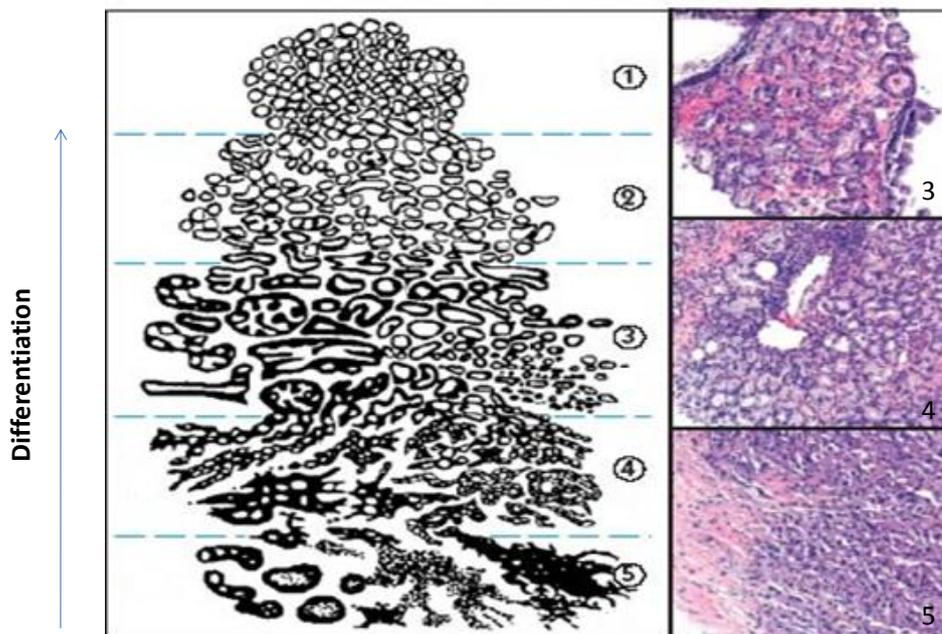


Figure.1.3. Diagrammatic illustration of the Gleason grading system. Stages from 1 to 5 representing the histological changes in prostate tissue. Grade 1 tumour tissue mimics the normal prostate tissue, well differentiated small glands. Grade 5 poorly differentiated cells in diffuse sheet-like arrangement Diagram was adopted from Prostate cancer research Institute (PCRI) website;

Table 1.1 Localized prostate cancer risk stratification depending on Gleason scoring and suggested treatments.

Gleason score	Prostate cancer risk	Recommended treatment	Treatment option
≤ 6	Low risk	Active surveillance	Watchful waiting / brachytherapy / radical prostatectomy / radical radiotherapy
7	Intermediate risk	Radical prostatectomy / Radical radiotherapy	Watchful waiting / Active surveillance / brachytherapy
8-10	High risk	Radical prostatectomy / Radical radiotherapy	Watchful waiting

The content of the table was summarized from; Prostate cancer: diagnosis and treatment full guidelines, a publication of the National Collaborating Centre for Cancer, 2008,(<http://www.nice.org.uk/nicemedia/live/11924/39687/39687.pdf>).

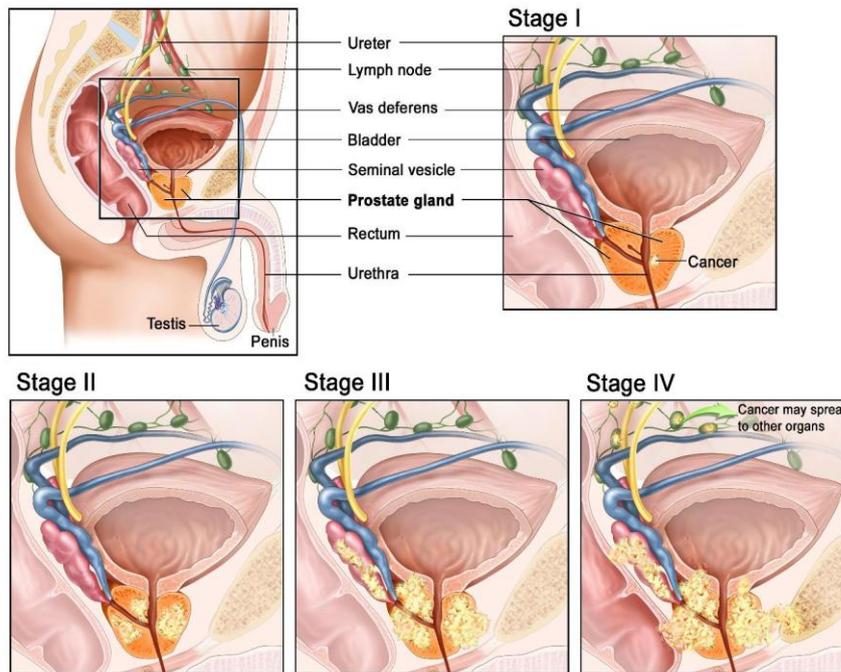
1.1.3.1 Clinical manifestations of prostate cancer and their management

The general manifestation of PCa includes many histological and pathological changes. One of the most common features is the level of Prostatic Specific Antigen (PSA), which is increased in PCa. PSA is a protease belonging to the Kallikrein family and is normally secreted by prostate cells into the bloodstream at very low levels. However, during development of PCa the morphological and cytological changes lead to the breakdown of cellular structures, such as the basement membranes that separate epithelial cells from the blood vessels, ultimately leading to an increase in the level of PSA secreted into blood. Therefore, blood PSA assessment is routinely used as a diagnostic marker for PCa. Other findings such as an abnormal digital rectal examination can also

indicate PCa, and is used alongside PSA screening to make a clinical diagnosis. Additional symptoms commonly present concomitantly with PCa are hematuria, incontinence and urinary retention. PCa can eventually metastasise and spread to the rest of the body and is accompanied by such symptoms as loss of appetite leading to weight loss, skeletal pain due to bone metastasis, oedema in the legs, and neurological problems due to metastasis to the spine (Theodorescu, 2001).

PCa staging are procedures by which the spread of cancer beyond prostate is categorized. Two common schemes are followed for staging PCa. The most commonly used staging system in the UK and the world is the American Joint Committee on Cancer (AJCC) TNM System. It evaluates the size of the primary tumour category (T) and its spread into the regional lymph nodes (N), also it describes the metastasis to distant body organs (M) category. T, N and M categories are scored depending on the progress of the tumour into the evaluated organs. The staging results can be classified into four stages (I-IV) similar to many other cancers (Sobin, 2009). Another less common staging system in use called Whitmore-Jewett system, it uses Latin letters (A-D) for the overall stages instead of Roman numerals (Held-Warmkessel, 2006).

The current treatment options in the UK (<http://cancerhelp.cancerresearchuk.org/type/prostate-cancer/treatment/types/treatment-options-for-prostate-cancer>) for men with PCa on diagnosis are outlined in Figure 1.4 and include “watchful waiting” (active surveillance), (Drachenberg, 2000), androgen ablation therapy (Singh and Agarwal, 2006, Sommer and Haendler, 2003, Isaacs and Isaacs, 1994, Oh and Oh, 2002), radical prostatectomy (Bill-Axelson et al., 2005), radiotherapy, brachytherapy, or cryotherapy (Fournier et al., 2004, Krupski et al., 2000).



Stage	Clinical findings	Treatment options
Stage I	The cancer is localized in prostate only, cannot be recognized by digital rectal examination and imaging.	Treatment could be, watchful waiting, radical prostatectomy, radiation therapy.
Stage II	Cancer is more developed at this stage although it has not spread to other organs.	Radical prostatectomy with pelvic lymphadenectomy radiation therapy after surgery, watchful waiting.
Stage III	Cancer starts to spread during this stage to surrounding tissues, such as seminal vesicles.	External beam radiation therapy. Hormone therapy. Radical prostatectomy with pelvic lymphadenectomy. Palliative therapy to relieve the symptoms. Watchful waiting, chemotherapy. steroids
Stage IV	Characterized by clear metastasis to the nearby tissues such as lymph nodes, bladder, rectum and bones and distant organs such as liver, and lungs.	Hormone therapy. Radiation therapy or transurethral resection of the prostate as a palliative therapy. Watchful waiting. Chemotherapy. steroids

Figure 1.4. Prostate cancer stages and the treatment options. *Diagram was adapted and the information in the table was summarised from (<http://cancerhelp.cancerresearchuk.org/type/prostate-cancer/treatment/types/treatment-options-for-prostate-cancer>) and cancer Information Japan website (<http://www.cancer.gov/cancertopics/pdq/>).*

Other strategies for the management of PCa are currently under investigation and include immune therapy (Simons et al., 1999, Isaacs, 2005), gene therapy (Rodriguez et al., 1997, DeWeese et al., 2001) as well as chemoprevention (Pomerantz and Kantoff, 2007).

1.1.3.2 Metastatic prostate cancer

Metastasis is the way by which malignant cells can move from the primary tumour to a more distant site via the circulatory system and eventually produce a secondary malignancy. The basic processes of metastasis are described in Figure 1.5 (Eastham, 2005). Metastatic PCa is an incurable disease and very few treatment options are available at this stage of the cancer progression. Hormone therapy is one approach and involves the removal, blockade or addition of hormones to treat the cancer or alternatively chemotherapy. Both approaches come with side-effects and only marginally improve survival rates.

1.1.4 Androgen receptor and prostate cancer

The androgen receptor (AR) is a member of the nuclear receptor super family, which plays a pivotal role in the male reproductive system. In addition, in females it is involved in some functions like folliculogenesis (Donath et al., 1997) and breast development (Liao and Dickson, 2002). It is expressed as two isoforms, isoform A (80 kDa) and isoform B (110 kDa) with the latter being most predominant (Gao and McPhaul, 1998, Liegibel et al., 2003). Isoform A was found to lack the ability to stimulate cell proliferation and probably has a role in regulating the activation of isoform B (Liegibel et al., 2003). AR is a ligand-dependent receptor stimulated by androgens. It is a transcription factor that controls the expression of a number of androgen responsive genes (Zhou, 2010). AR is important for prostate development function and it also contributes to prostate carcinogenesis (Berry et al., 2008). At advanced stages PCa can turn into an androgen independent form, in which tumour growth becomes independent of androgen and androgen withdrawal does not retard tumour growth.

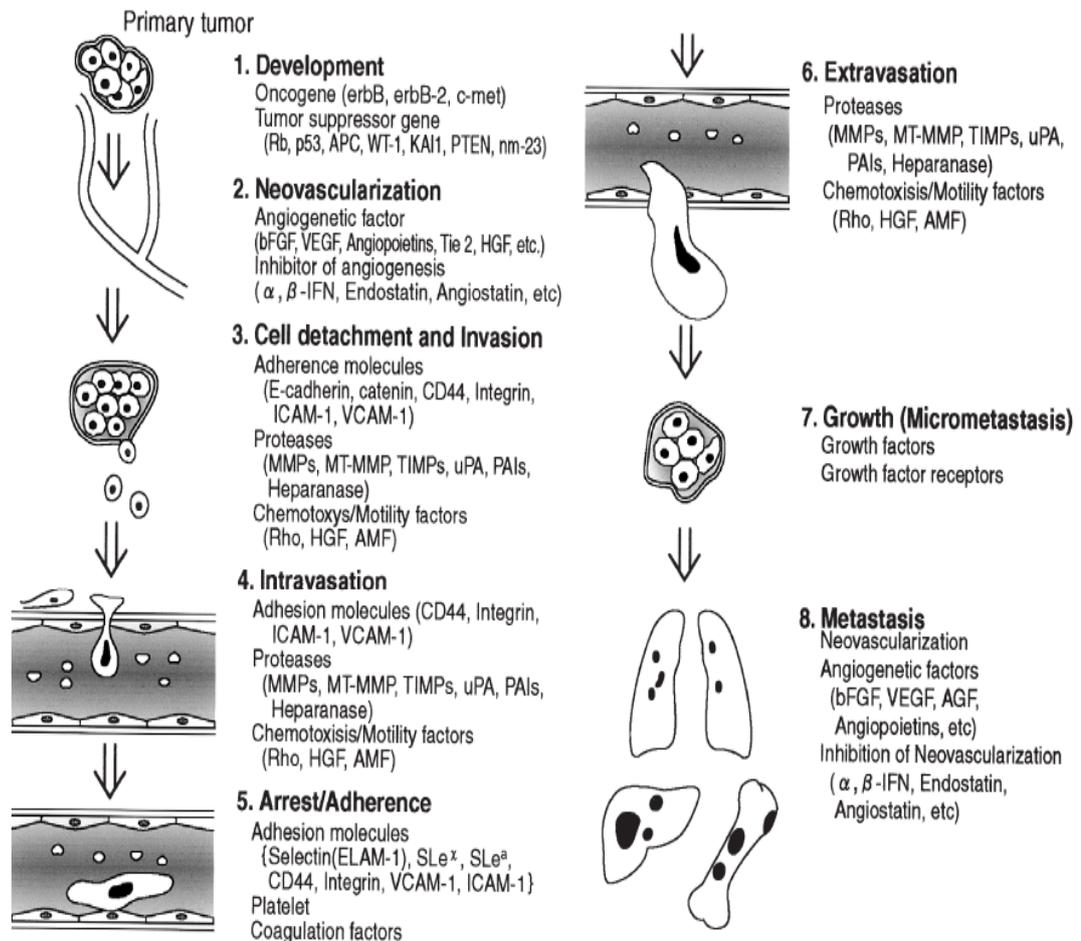


Figure 1.5 Summary of the essential steps of metastasis. Diagram was taken from (Eastham, 2005).

Expression of functional AR has been detected in almost all stages of the disease from as early as PIN to metastatic forms of PCa (Ostling et al., 2011). It is widely accepted that the AR is involved in the progression of androgen dependent to androgen independent forms of PCa (Koochekpour, 2010). The use of antiandrogens for the treatment of PCa was coined after Huggins and Hodges (Huggins and Hodges, 1941) reported that low testosterone levels following castration caused repression of acid phosphatase levels in localized and metastatic prostate cancer patients (Glina et al., 2010). Prostate acid phosphates test was introduced in 1940s and 1950s as a serum prostate tumour marker and it was in use until the introduction of the PSA serum test

(Taira et al., 2007), Accordingly, extensive work has been undertaken suggesting that higher testosterone levels contribute to PCa and therefore using anti-androgens in the management of locally advanced or systemic PCa would be an effective treatment (Sharifi et al., 2005). Androgen ablation therapy is designed to suppress the production of male hormones by using AR blockers such as bicalutamide and/or by using GnRH agonists such as goserelin (Zoladex) and leuprolide (Scherr et al., 2003).

The PSA gene is an AR target and its expression is regulated by androgens (Young et al., 1991). As such it is frequently used to determine the level of AR activity and also used as a marker of progression of prostate cancer (Han et al., 2001). Although evidence supporting the use of serum PSA levels as a screening method for early detection of PCa is not strong enough, as it is not a sufficiently sensitive test (Alacreu et al., 2008). However, it is still considered the most effective tool currently available for the detection of early PCa (Tosoian and Loeb, 2010). Studies have shown normal PSA levels in some patients with advanced PCa and elevated levels have also been found in other conditions including benign prostatic hyperplasia (Alacreu et al., 2008). Despite its name, PSA is not exclusive to the prostate and is also found in other tissues of the body including breast and ovarian tissue, although the levels detected in the prostate are typically 10,000 times higher (Diamandis, 1995). Some anticancer agents that interfere with AR expression have also been shown to reduce PSA expression (Lee et al., 2006).

Male androgens include testosterone and its derivative 5 α -dihydrotestosterone (DHT). Testosterone is produced mainly in the testes, and to a lesser extent in the adrenal gland. In the prostate, testosterone undergoes an irreversible conversion through the action of 5 α -reductase to DHT. Both testosterone and DHT bind and stimulate the AR but DHT has a greater affinity, therefore, the AR produces higher transcriptional activity when it binds DHT (Hsing et al., 2008). The use of 5 α -reductase inhibitors (i.e. finasteride and dutasteride) has shown proof-of-principle that chemoprevention of prostate cancer is a possibility in humans (Bonovas et al., 2008, Crawford et al., 2010, Chaudhary and Turner, 2010).

1.1.4.1 The androgen receptor cycle

The AR is located in the cytoplasm of cells and associates with chaperones and other proteins such as cytoskeletal and heat shock proteins (HSPs) (Bennett et al., 2010) . After an androgen ligand binds to the AR it results in conformational changes in the receptor (Liao et al., 2003), which leads to the dissociation of the receptor from HSPs and subsequent binding to co-regulators such as importin- α and filamin-A. This step helps in AR dimerisation and nuclear targeting (Cutress et al., 2008, Schaufele et al., 2005). Ligand binding to the AR also promotes phosphorylation, which is thought to protect the AR from proteolytic degradation and is necessary for AR genomic activity (Blok et al., 1998). AR movement to the nucleolus, triggered by ligand binding, is followed by binding of the receptor to tissue specific AR elements (AREs) (van Royen et al., 2007), which leads to the recruitment of co-activators, thus triggering the general transcription machinery and transcription of androgen-dependent genes such as PSA and probasin (Heinlein and Chang, 2002). Loss of ligand binding to the AR in the nucleolus leads to activation of nuclear export signals which induces the transition of AR to the cytoplasm. Once in the cytoplasm AR can be targeted for proteasomal degradation by ubiquitylation by E3 ubiquitin ligase (Gaughan et al., 2005). Agents that can interfere with this process and therefore prevent translocation of the AR to the nucleus to initiate transcription of response genes would be ideal candidates for prostate cancer prevention.

1.1.4.2 Androgen receptor cross-talk with cell signalling pathways

The AR is capable of crosstalk with other signalling pathways to promote PCa growth. It is able to induce the phosphorylation of phosphoinositide 3-kinase (PI3K) and therefore activate the downstream effector Akt (Baron et al., 2004). PI3K/Akt is a major pathway for cell growth and survival in PCa and other malignancies (Cantley and Neel, 1999). The AR has been shown to interact with and activate tyrosine kinase receptors (Migliaccio et al., 2000). This association and activation activates mitogen activated protein kinase (MAPK) in PCa cells (Unni et al., 2004), which induces cell proliferation, differentiation and migration (Thomas and Brugge, 1997, Schlessinger, 2000). Furthermore,

androgen can upregulate epidermal growth factor receptor (EGFR) (Torrington et al., 2003) and subsequently activate MAPK (Peterziel et al., 1999) through an AR-dependent pathway.

The AR can also be a downstream target for various signalling pathways. Akt has been shown to upregulate AR levels in PCa (Ha et al., 2011). It is widely thought that the AR is a downstream kinase substrate for many tyrosine kinase receptors (RTK) such as HER2 and G-protein coupled receptor, as both were shown to activate AR in the absence of androgen (Manin et al., 2002). Additionally, insulin like growth factor-1 (IGF1), which is important for tumour growth (Capoluongo, 2011), was found to increase AR transactivation in the absence of androgen (Culig, 2004). Therefore, the cross talk between AR and these pathways could initiate an autocrine positive feedback loop, as ERK, Akt and PI3K can phosphorylate and activate AR without the need for androgen binding (Bennett et al., 2010). Alternatively, it has been suggested that prostate epithelial cell proliferation induced by androgens is indirectly controlled by other mediators such as EGF (Leotoing et al., 2007).

1.1.4.3 Androgen dependent and non androgen dependent PCa

Castration or androgen ablation is standard therapy for prostate cancer, as it reduces the effects of AR stimulation. Initially it provides a significant response and is considered the most effective palliative technique against advanced and/or metastatic PCa (Koochekpour, 2010). Unfortunately, however, in the majority of patients, this approach becomes ineffective and the disease progresses to a more aggressive form, called hormone independent prostate cancer (HIPC) (Palmberg et al., 1999, Gittes, 1991). It is widely accepted that the AR has a major function in the development of HIPC. Even though androgen levels are low as a result of androgen ablation, the AR is still active in hormone independent tumours and they can be stimulated by other ligands to initiate transcription of target genes to promote PCa growth.

1.1.4.3.1 Hypersensitivity pathways

Hypersensitivity of AR to androgens is one mechanism by which PCa advances to HIPC. AR becomes highly sensitive to low levels of androgens. AR gene amplification has been proposed as a mechanism via which HIPC can develop, as gene amplification and upregulation of AR were found to be significantly higher in hormone independent tumours (Linja et al., 2001, Visakorpi et al., 1995, Koivisto et al., 1997). AR mutation is another proposed mechanism that could increase the sensitivity of AR to androgens or even its activation by other steroids (Mellado et al., 2009). AR gain of function mutations are more frequent than loss of function mutations (Gottlieb et al., 2004). Generally, the occurrence of AR mutations is rare in untreated localized PCa. For example, alterations have been reported to be present < 2% of Caucasian patients. However, mutations occur at higher frequency in hormone independent and metastatic PCa tumours (Gottlieb et al., 2004, Linja and Visakorpi, 2004). The enzymes responsible for androgen synthesis have been demonstrate to be upregulated in hormone resistant PCa tumours (Titus et al., 2005, Stanbrough et al., 2006), as was the enzyme 5- α -reductase, which converts testosterone to DHT, and is thought to be important for increasing the sensitivity of ARs (Thomas et al., 2008).

1.1.4.3.2. Promiscuous pathway

The advancing of PCa to HIPC through the promiscuous pathway takes place when the AR becomes activated by ligands other than androgens as a result of AR mutations (Dutt and Gao, 2009). These AR mutations are usually missense in the AR gene and result in a lack of specificity of the receptor for ligands. As a consequence, compounds with similar structures to androgens such as other steroids and even anti-androgens can bind to the AR (Feldman and Feldman, 2001). In LNCaP human PCa cells, mutations in the ligand binding domain were identified to be the cause of steroid binding and activation by anti-androgens (Veldscholte et al., 1990). Such agonistic activity exerted by anti-androgens in hormone resistant tumours can give an explanation for the anti-androgen withdrawal response reported in about 30% of patients (Mellado et al., 2009).

Androgen withdrawal syndrome is a clinical phenomenon in which a group of patients benefit from the discontinuation of antiandrogens or hormonal therapy, manifested in improvement of the clinical situation and decreasing of the serum PSA levels of the patient (Paul and Breul, 2000)

1.1.4.3.3 The outlaw pathway

The term outlaw receptor has been referred to a hormone receptor which can be activated without the need for ligand binding (McGuire et al., 1991). In this situation, growth factor pathways can activate the AR in the ablation of androgens. Growth factors such as IGF-I and EGF are able to activate the AR in the absence of androgens and such activation is able to induce AR target genes. For example, IGF-I led to an up to five-fold increase in PSA secretion in LNCaP cells. However, these effects were completely blocked by the AR antagonist bicalutamide (Culig et al., 1994). Activation of certain kinases, such as PI3K/Akt, could be a possible mechanism of the outlaw AR. Many studies in PCa cell lines have shown that activation of these kinase pathways through their upstream tyrosine kinase receptors leads to the activation of AR response in conditions of very low androgen levels (Yuan and Balk, 2009).

Current research strategies of many laboratories are aimed at identifying an agent(s) that can interfere with any of these potential mechanisms that activate the AR and consequently its target genes. If successful, the progression of PCa could be delayed or alternatively the disease prevented.

1.2 Chemoprevention of prostate cancer

1.2.1 General concepts

Cancer chemoprevention is described as a process to prevent arrest or reverse carcinogenesis using diet-derived or synthetic agents (Tsao et al., 2004). Cancer is a multistep disease, that takes place over many years, even decades, and as such provides an excellent opportunity for chemopreventive intervention (Greenwald, 2002). The aim of cancer chemoprevention research is to develop and evaluate new agents that could have the ability to modulate specific molecular and cellular targets. (Lippman and Hong, 2002), in order to inhibit one

or more stages of cancer development (Kelloff et al., 1999). The ideal chemopreventive agent should satisfy a number of specific criteria: it should have high efficacy, with minimal side effects and no toxicity. It must also be formulated in an easily administered form and be of low cost, since chemopreventive agents are intended to be used over prolonged periods (Gupta, 2007).

1.2.2 Opportunities for chemoprevention in prostate cancer

Prostate cancer is an attractive cancer type for chemopreventive intervention. Development of sporadic clinical PCa takes place over 30 - 40 years and involves genetic and biochemical changes that can be targeted by agents for prevention (Lieberman et al., 2001). The development of high grade prostatic intraepithelial neoplasia (HGPIN) may take 20-30 years, whilst progression to cancer may take a further 3-15 years (Bostwick, 1992). Due to this long latency period there is a potential window of opportunity for intervention with chemopreventive agents to inhibit, delay or reverse prostatic carcinogenesis, as illustrated in Figure 1.6.

Many studies have found evidence of lesions such as atypical adenomatous hyperplasia and proliferative inflammatory atrophy in human prostate peripheral zones that could progress to low-grade or even to high grade PIN, (Lieberman, 2002) both of which, are considered early indicators of PCa (Bostwick, 1996). Many epidemiological studies have found an association between diet and PCa development and it is recommended that by avoiding certain dietary components and high consumption of others a two thirds reduction in PCa could be achieved (Singh and Agarwal, 2006, Clinton and Giovannucci, 1998, Abdulla and Gruber, 2000).

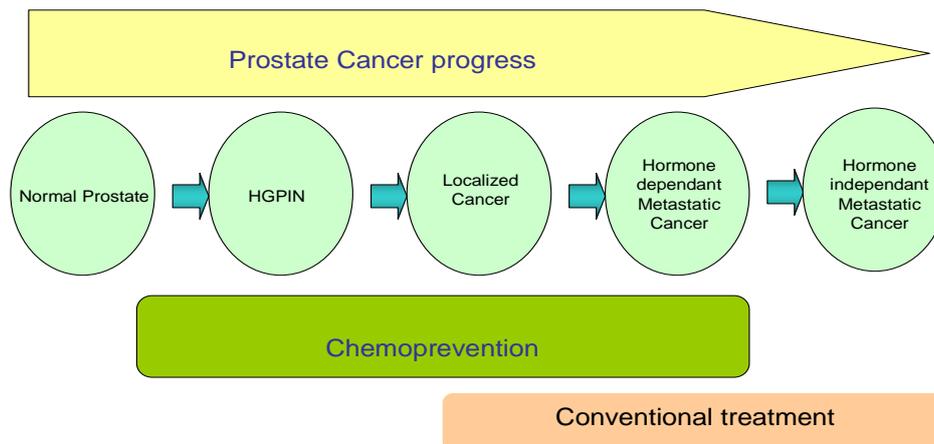


Figure 1.6 The margin of chemoprevention opportunity in Prostate cancer

PCa chemoprevention could be very beneficial for men at high risk of PCa. It is crucial to identify the target population, which might include people at risk of PCa, recurrence or progression and classify them suitably (Silberstein and Parsons, 2010) Many risk factors that contribute to PCa have been identified. Chronic inflammation and prostatitis were described as a possible risk factor for PCa (Hamid et al., 2011, Nelson et al., 2004). Age is another potential risk factor of PCa. In general 75% of the PCa cases diagnosed are over the age of 65; therefore, any success in delaying the disease could improve quality of life for those people (Syed et al., 2007). Other factors such as race and family history are also considered as PCa risk factors (section 1.1.2).

As PCa is a disease of the older person generally a delay of 10-15 years in its onset could be as good as a cure. Therefore, development of an agent that could exert this activity may have a huge impact, not only to the patient personally, but also cost implications to the NHS as many of the complications associated with metastatic PCa could be eliminated.

1.2.3 Cell signalling targets for PCa chemoprevention

An understanding of the mechanisms of action of new chemopreventive agents is critical before they can be clinically developed (Singh and Agarwal, 2006). Multiple components of numerous signalling pathways have been identified to be altered during the progression of PCa and therefore, agents that can modulate these targets could be important in PCa cancer chemoprevention (Sarkar et al., 2010). Figure 1.7 summarises some potentially important chemoprevention targets.

1.2.3.1 Androgen receptor and receptor tyrosine kinase signalling

Androgens are important for the maintenance of prostate function and they also contribute to PCa development. Remarkably, many studies have shown that PCa depends on AR signalling even when the tumour reaches hormone independence. Therefore, targeting the AR signalling pathway could be an attractive approach for chemopreventive intervention.

Receptor tyrosine kinases (RTK) such as EGFR and insulin like growth factor receptor type-1 (IGF-1R) are essential in conducting the signals from extracellular ligands to cytoplasmic effectors. RTK activation induces many pathways that maintain cell cycle progression, apoptosis and cell migration (Ramsay and Leung, 2009). Activation of the RTK pathway is a major factor for PCa development and progression (Lorenzo et al., 2003). Remarkably, EGFRs were found to be overexpressed in the human prostatic tissue of various stages of cancer development (Myers et al., 1994, Mendelsohn and Baselga, 2000, Di Lorenzo et al., 2002, Dong et al., 2010). Cross talk between the AR and RTK pathway has been identified. Furthermore, RTK pathways themselves regulate the activation of AR (Yuan and Balk, 2009), therefore, RTK inhibitors that can modulate downstream signal transduction are putative agents for PCa chemoprevention and therapy (Gan et al., 2010). Possible chemopreventive targets of the AR-EGFR pathways are displayed in Figure 1.8.

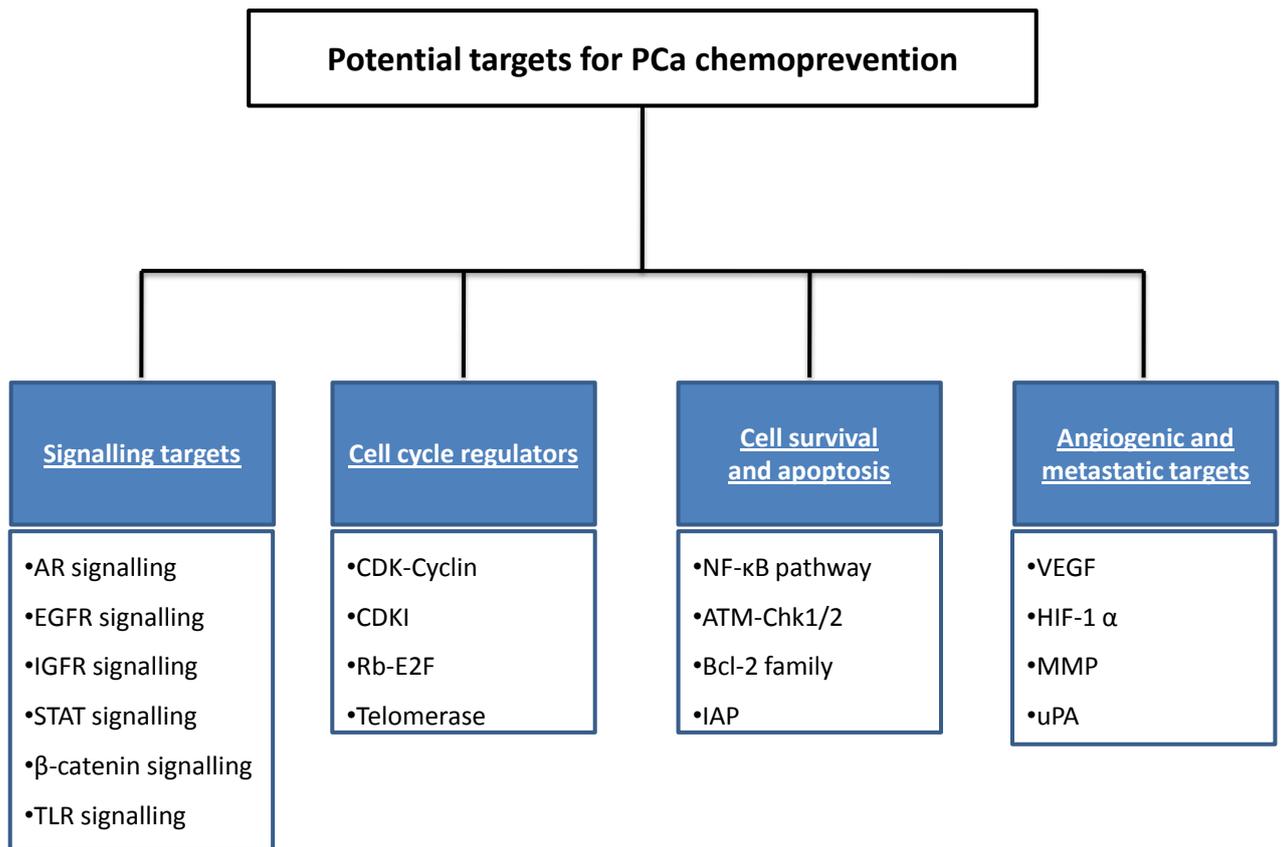


Figure 1.7 The possible potential targets for PCa chemoprevention.
 Legands: ATM, ataxia telangiectasia-mutated; Bcl-2, B-cell CLL/lymphoma-2; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; Chk, checkpoint kinase; E2F, E2-promoter binding factor; IGFR, insulin-like growth factor receptor; HIF, hypoxia-inducible factor; IAP, inhibitor of apoptosis protein; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; Rb, retinoblastoma; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor. *Figure was modified from (Singh and Agarwal, 2006).*

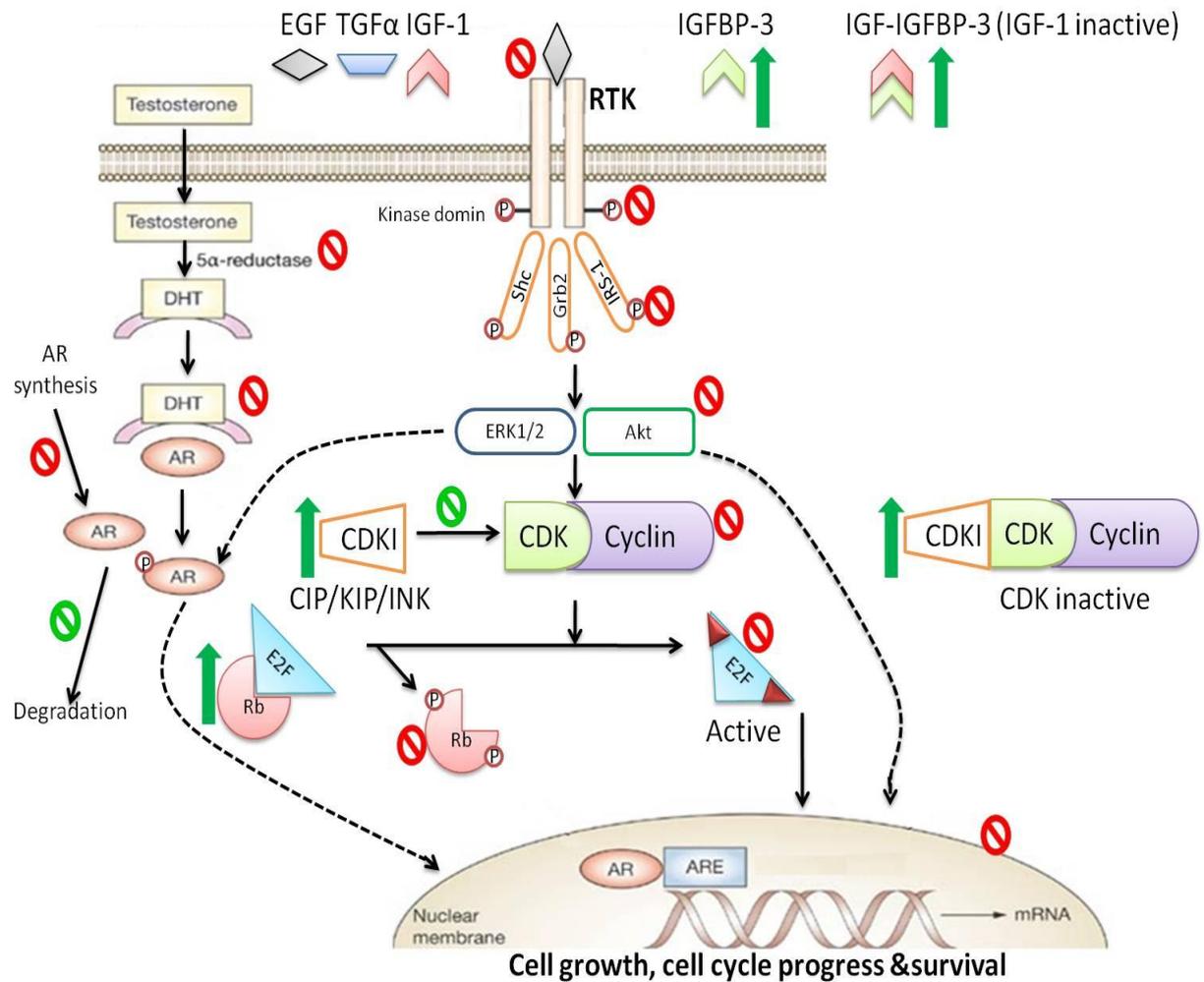


Figure 1.8 Potential PCa chemoprevention targets of AR and RTK pathways. The figure shows the interaction sites of compounds which are tested for PCa chemoprevention. The dotted line shows how AKT and ERK1/2 could activate the AR or use other pathways to induce proliferation and cell survival in addition to the CDK-cyclin pathway. Green arrows indicate increase in target protein expression by chemopreventive agents. Red no entry signs indicate inhibition and the green indicate activation by chemopreventive agents. *Figure was compiled from information in references (Lattouf et al., 2006, Singh and Agarwal, 2006).*

1.2.4 Chemopreventive agents in prostate cancer

Data from epidemiological studies have identified associations between PCa incidence and various risk factors including diet, lifestyle, geography, race and family history. So, many studies have been performed or are being undertaken to find the impact of these factors on PCa. Recent advances in the understanding of prostate cancer aetiology and molecular mechanism have helped in identifying new targets for chemoprevention that might lead to the design and synthesis of new agents that are able to interfere with prostate cancer progression and metastasis. Therefore, chemoprevention trials seem to be a very promising approach. The following are some compounds that have been studied extensively to date for their chemopreventive activity in prostate cancer.

1.2.4.1 Finasteride and Dutasteride

Finasteride and Dutasteride are inhibitors of 5- α -reductase; finasteride is a selective isoenzyme-2 inhibitor whereas dutasteride is a non selective inhibitor (Klein, 2006). The role of 5- α -reductase is to convert circulating testosterone to a more potent analogue, dihydrotestosterone, which is needed to maintain proliferation of the prostatic epithelium (Brawley et al., 1994). Generally, 5- α -reductase inhibitors reduce the risk of PCa incidence (Wilt et al., 2010, Strome and Andriole, 2010). However, in December 2010 the US food and drug administration (FDA) recommended against the use of 5 α -reductase inhibitors for prostate cancer chemoprevention because of the increased likelihood of high-grade tumours (<http://www.medscape.com/viewarticle/733490>). Important to note is that; finasteride was approved by the FDA in 1992 for the treatment of BPH and in 1997 for the treatment of male pattern baldness (Colli and Amling, 2009).

The Prostate Cancer Prevention Trial (PCPT) was started in 1993 and planned to finish in 2004 but it was stopped in 2003 because of clear finding of reduction in PCa incidence (<http://www.cancer.gov/clinicaltrials/noteworthy-trials/pcpt/Page1>). PCPT investigated the effects of finasteride on PCa

incidence in healthy 18,882 men aged 55 or older for 7 years, (Hamdy and Rouprek, 2008). The participants were selected on the basis of a PSA level of 3 ng/ml or lower and a normal digital rectal examination and the primary end point was PCa (Chaudhary and Turner, 2010). The results were controversial; finasteride resulted in a 24.5% reduction in PCa incidence compared to the control group (Colli and Amling, 2009). However, the finasteride group experienced sexual side effects and in those that developed PCa a higher Gleason score was more prevalent compared to the non treated controls. Interestingly, urinary symptoms, prostatitis and urinary tract infection were more frequent among the control group (Thompson et al., 2003b, Thorpe et al., 2007).

Over-expression of the isoenzyme-1 5- α -reductase has been reported in some human prostate tumours (Thomas et al., 2003). Therefore, it may be that blocking this enzyme could have a beneficial effect in PCa chemoprevention. In support of this, use of dutasteride, which non-selectively inhibits 5- α -reductases, in the treatment of BPH patients resulted in a slight decrease in PCa risk (Roehrborn et al., 2002).

A multinational trial called Reduction by Dutasteride of Prostate Cancer Events (REDUCE) was conducted in the USA and the EU to evaluate whether dutasteride decrease the risk of biopsy detectable prostate cancer (Andriole et al., 2004). Over 8,000 men were recruited to the study, which lasted 4 years and ended in 2008. Participants were eligible if they were considered at high risk of PCa. The recruitment age was 50 to 75 years old; participants had PSA serum levels of 2.5 to 10 ng/ml and their age was 50 to 60 years or participants over 60 must had PSA of 3.0 to 10 ng at the time of recruitment. Histological biopsies were taken at 24 and 48 months and were considered as primary endpoints if cancer was detected (Andriole et al., 2004). Dutasteride resulted in a reduction in PCa incidence and improved the complications of benign prostatic hyperplasia (Andriole et al., 2010). Over the course of the study, 19.9% of the dutasteride group developed PCa compared to 25.1% in the placebo control group. Gleason scores for tumours from each group were statistically comparable (Andriole et al., 2010, Chaudhary and Turner, 2010).

However a non significant increase in tumours of grades 8-10 was occurred in the dutasteride group during years 3 and 4 (Parnes et al., 2010) .

It is widely thought the increase in the high grade tumors in the finasteride arm was biased. Some reasons have been suggested to be contributed to the biases; Inhibitors of 5- α -reductase and other androgen deprivation agents can result in histological changes in prostate tumours such as, chromatin condensation, nuclear and nucleolar shrinkage and clearing of the cytoplasm. Therefore in treated men tumours would have smaller nuclear and nucleolar diameters and higher Gleason grade in comparison to the controls (Bostwick et al., 2004b). The reduction in prostate volume produced by finasteride in the treatment group could have led to an increase in the tumour sampling (a biopsy of same needle cores from a smaller prostate). Therefore, that would raise the possibility of the detection of high grade tumours (Kulkarni et al., 2006). During the PCPT the abnormal digital rectal examination (DRE) or levels of serum PSA of 4 or more were considered as the threshold for prostate biopsy (Goodman et al., 2004). Since finasteride increases the sensitivity of DRE and PSA tumour markers in treated men (Thompson et al., 2007, Thompson et al., 2006). Then the higher performance of these two tests might have led to the elevated number of high grade tumours in the finasteride group (Jayachandran and Freedland, 2008). Importantly, PCPT data were reanalysed after adjusting for biopsy sampling, including factors such as; race, age, family history, baseline prostate-specific antigen level. The results showed reduction in risk of Gleason scores (4-7) in the finasteride group and no significant effect on the risk of Gleason scores of 2, 3, or 8-10 (Kaplan et al., 2009). Regarding the noticed increase of high grade cancer (Gleason scores 8-10) in the dutasteride arm of REDUCE during years 3 and 4, it was assumed that; if the excess number of men in control group who had Gleason grades 5-7 during year 1 and 2 were not excluded, the treatment groups would have had less or similar 8-10 Gleason scores during years 3 and 4 (Andriole et al., 2010).

1.2.4.2 Selenium and Vitamin E

Selenium is an essential trace element that occurs in organic and non organic forms, with the presence of selenium in food being related to the local soil content. Some epidemiological studies have found evidence of a protective effect of selenium against different types of cancer (Klein, 2006). Epidemiological evidence, supporting the suggestion that selenium could prevent PCa incidence, such evidence include *in vitro* data and cohort, case control, and randomized control trials (Klein, 2004). The secondary analysis of effect of selenium on carcinoma of the skin in a randomized controlled trial for incidence and mortality of PCa, pointed to a protective effect of selenium in PCa (Clark et al., 1996). It is thought the anticancer effect of selenium is through multiple mechanisms exerted by its active metabolite, methyl selenol (Ip, 1998). Some of the mechanisms proposed, include; as an anti oxidant, protector against DNA damage of selenium anti cancer effect (Jayachandran and Freedland, 2008) and apoptosis inducer. For example; dietary selenium supplementation increased the epithelial cell apoptosis and reduced DNA damage in canine prostate (Waters et al., 2003). Selenium also potentiates the effect of tumour necrosis factor-related apoptosis inducing ligand (TRAIL) in LNCaP and DU-145 human prostate cancer cell lines; TRAIL is a cytotoxic agent that induces apoptosis in many cancer cells (Yamaguchi et al., 2005). Another proposed mechanism for the anticancer effects of selenium is by inhibiting the angiogenesis through reducing tumour-induced neovascularisation (Jiang et al., 2004).

Vitamin E is a lipid soluble vitamin with antioxidant activity, so it can counteract the effects of reactive oxygen species that induce DNA damage, which may initiate cancer. The primary analysis of a large scale trial for the effect of long term supplementation of vitamin E (α -tocopherol) and β -carotene on the incidence and mortality of PCa in male smoker, showed a substantial reduction in PCa incidence and mortality by vitamin E. (Heinonen et al., 1998). Vitamin E was found to inhibit prostate cancer cell growth and modulate AR/PSA signalling in LNCaP cells (Israel et al., 1995), and to induced a G₁ cell cycle arrest (Ni et al., 2003). Importantly, vitamin E doses greater than 400IU/day

have been associated with heart failure and all-cause mortality rates (Miller et al., 2005a, Miller et al., 2005b).

The National Cancer Institute sponsored a large selenium and vitamin E cancer prevention trial (SELECT) to test the efficacy of selenium and vitamin E. The trial included 35, 533 men aged over 50, and it contained four groups: selenium, vitamin E, selenium and vitamin E and control with doses of selenium at 200 µg/day and/or vitamin E at 400 IU/day (Lippman et al., 2005). The study was intended to last 7-12 years, however, it was terminated early due to lack of efficacy, and also there was a non significant 13% increase in PCa incidence in vitamin E group. The primary end point of this study was the clinical incidence of PCa. SELECT's initial results showed selenium and vitamin E alone or in combination could not prevent PCa incidence in the tested population (Lippman et al., 2009, Chaudhary and Turner, 2010). It is thought that selenium supplementation is only effective in people who are deficient in selenium that might explain the lack of efficacy in SELECT. A study continued for 4.5 years and recruited 1,312 participants who were living in low selenium soil levels area, showed a decrease in PCa risk by 63% in selenium arm, in addition, there was a decline in the risk of lung and colorectal cancers (Clark et al., 1998, Clark et al., 1996, Platz, 2010)

1.2.4.3 Vitamin D

Many epidemiological studies have highlighted the potential of vitamin D as a cancer chemopreventive agent. Afro-Americans, whose skin is opaque to UV light, which is essential for vitamin D synthesis, have a higher incidence rate of PCa compared to Caucasians. Japanese food, which is rich in vitamin D, has been proposed as the reason for the low PCa incidence in Japan. The high incidence and mortality rates of PCa among older men is also thought to be linked to vitamin D since deficiency is more common among elderly people (Klein, 2006). Vitamin D was found to inhibit the growth and induce the differentiation of cultured human prostate cancer cells (Peehl et al., 2003). It was indicated that vitamin D has an anti-proliferative effect on LNCaP prostate cancer cells, due to the induction of cell cycle arrest (Krishnan et al., 2003a). A

small clinical trial showed that vitamin D can decrease the rate of PSA rise in PCa patients (Krishnan et al., 2003b). Calcitriol, the active metabolite of vitamin D, has been shown to reduce the expression of genes responsible for the metabolism of prostaglandins, known mediators of cancer growth in primary prostatic epithelial cells and human prostate cancer cell lines. Calcitriol also inhibited COX-2 expression, which is the key enzyme in prostaglandin synthesis (Moreno et al., 2005). Although the biological evidence for the protective effects of vitamin D against PCa appears convincing, an inconsistent correlation between circulating levels of vitamin D derivatives and PCa incidence has been reported (Giovannucci, 2005).

Vitamin D toxicity is a major concern in clinical trials as it may lead to hypercalcemia if taken at high doses (Schwartz, 2009). Hypercalciuria was considered as the reason for avoiding doses higher than 2.5 µg/day (Gross et al., 1998). Most of the vitamin D clinical trials for the management of PCa were undertaken using calcitriol, the natural vitamin D receptor ligand and several dosing approaches were applied to avoid unwanted side effects (Jensen et al., 2010). In general, vitamin D could interfere with the PCa biology, but the clinical data has not confirmed a link for between vitamin D and PCa. (Barnett and Beer, 2011).

1.2.4.4 COX-2 inhibitors

Inflammation and BPH are commonly associated with PCa incidence and as a result cyclooxygenase enzyme (COX) inhibitors have been investigated as potential agents for chemoprevention of this malignancy (Hamid et al., 2011). Furthermore, non steroidal anti-inflammatory drugs (NSAIDs) which are COX inhibitors have been shown to reduce the incidence of colorectal cancer (Colli and Amling, 2009). Generally, over-expression of COX-2 was found to correlate with a decrease in apoptosis and an increase in tumour invasion and angiogenesis in various human cancers (Harris, 2009, Pruthi et al., 2003). The results of studies on the role of COX-2 in PCa are controversial due to inconsistent findings. The results of some NSAID epidemiological studies showed no protective role (Zha et al., 2001, Wang et al., 2008b), while others

describe reductions in PCa risk with NSAID use (Habel et al., 2002, Roberts et al., 2002). COX-2 is highly expressed in proliferative inflammatory atrophy and chronically inflamed prostatic areas, but very weakly expressed in PCa (Wang et al., 2005, Zha et al., 2001) suggesting COX-2 inhibitors may only be beneficial in precancer for this specific tissue. The effects of celecoxib and nimesulide (COX-2 selective inhibitors) were investigated in PCa cells *in vitro* at the gene level and revealed reduced expression of androgen-inducible genes and decreased AR protein expression. The drugs also reversed AR mediated activation of PSA (Pan et al., 2003). The cardiovascular side effects of the COX-2 inhibitors have limited their use in chemoprevention clinical trials, as highlighted by the rofecoxib PCa prevention trial which was prematurely aborted as a result of concerns over its cardiovascular toxicity (Klein, 2006).

Interestingly, evaluation of the long term use of aspirin (non selective COX inhibitor) on PCa risk was undertaken in 51,529 health professionals aged between 40-75 years over a duration of 18 years (between 1988-2006). The study revealed that two or more adult strength (325 mg/tablet) aspirin tablets per week could significantly reduce the overall risk of PCa by 10%, whilst taking more than 6 tablets resulted in a up to 28% reduction in the risk of high-grade and lethal prostate cancer (Dhillon et al., 2011).

1.3 Preclinical models of prostate cancer

Preclinical models are essential tools for studying the activity, efficacy, bioavailability and toxicity of putative chemopreventive agents.

1.3.1 Prostate cancer cell lines

Preclinical cell models of prostate cancer currently in use are commonly derived from mice, rats, canines and humans. There have been numerous PCa cell lines used for PCa research, however, the LNCaP (Horoszewicz et al., 1980, Horoszewicz et al., 1983), PC-3 (Kaighn et al., 1979) and DU145 (Stone et al., 1978) are still the most commonly employed. Table 1.2, shows the 12 most cited human PCa cells in PubMed (Pienta et al., 2008).

Table 1.2 Prostate cancer cell lines used in chemoprevention research

Species	Model	Source	AR status	PSA expression	p53 status
Canine	Ace-1	Cell line from primary adenocarcinoma	-ve	--	--
Rat	Dunning	Spontaneous from primary adenocarcinoma	Multiple sublines	Multiple sublines	Multiple sublines
Mouse	TRAMP C2	Cell line from primary adenocarcinoma	+ve	-ve	--
Human	PC-3	Cell line from bone metastasis	-ve	-ve	-ve
	LNCaP	Cell line from lymph node metastasis	Mutated AR	+ve	WT
	DU145	Cell line from dural metastasis	-ve	-ve	Mutated
	CWR22rv1	Gleason 9 TURP	Mutated AR	+ve	Mutated
	LuCaP 23	Lymph node metastasis	+ve	+ve	--
	LuCaP 35	Cell line from lymph node metastasis	WT	+ve	--
	MDA PCa 2a	Cell line from bone metastasis	Mutated AR	+ve	WT
	VCaP	Cell line from bone metastasis	WT	+ve	Mutated
	LAPC-4	Cell line from lymph node metastasis	+ve	+ve	Mutated
	PC-82	Primary prostate, cribriform pattern	+ve	--	--
	PreC	Non-immortalized prostate epithelial cells	-ve	-ve	--
RWPE	Immortalized prostate epithelial cells	+ve	+ve	--	

Legends; (-ve) non expressing, (+ve) expressing, (WT) wild type, and (--) no information. *Table was adapted from (Pienta et al., 2008, Foster et al., 1997).*

1.3.2 Animal models

The use of animal models is a fundamental part of preclinical PCa chemopreventive research. Unlike humans, in most animals and primates PCa does not develop spontaneously, with some exceptions, including dogs and Dunning rats (Rosol et al., 2003). PCa progression involves many stages and therefore, the best animal model would be the one that mimics these same changes that occur in the human disease.

1.3.2.1 Rat models of PCa

Some strains of rats have been reported to develop spontaneous PCa following long latency periods (Sharma and Schreiber-Agus, 1999). The Dunning rat model (Dunning, 1963) is the most commonly used rat model in PCa chemoprevention (Lamb and Zhang, 2005). The original Dunning R-3327 tumour was a spontaneous prostatic adenocarcinoma in the inbred Copenhagen rat, which was then transplanted into the syngenic Copenhagen and (Copenhagen X Fischer) F1 hybrid. The Dunning R-3327 tumour resembles human PCa histologically in the ultrastructure (Lubaroff et al., 1980) and it is a well differentiated slow growing tumour with limited metastatic ability (Navone et al., 1998).

1.3.2.2 Canine models of prostate cancer

Dogs are one of the few species that develop PCa spontaneously with a median age at diagnosis of 10 years (Waters et al., 1998). Like in humans, PIN in dogs is frequently seen among elderly male dogs. The development of PIN could make this model relevant for investigating potential agents for prostate cancer prevention (Aquilina et al., 1998). However, the tumour of dog prostatic carcinoma is androgen independent and does not express a functional AR (Lamb and Zhang, 2005). Such a discrepancy to the human disease limits the use of this model in PCa chemoprevention. Nonetheless, it could be a better model for investigating bone metastasis as the animals spontaneously develop bone metastasis (Navone et al., 1998).

1.3.2.3 Mouse models of prostate cancer

There are many differences between the anatomy of the mouse and human prostate (Abate-Shen and Shen, 2002). In addition, mice do not develop spontaneous prostate carcinoma (Shappell et al., 2004). There are also other physical differences such as size, weight and lifespan. Despite all these differences, mice are still a commonly used model for PCa research. Some advantages such as cancer can be initiated by genetic manipulation make mouse an attractive cancer models (Valkenburg and Williams, 2011) The human and mouse genome is > 95% identical, and many gene alterations that have been contributed in cancers are similar (Maser et al., 2007, de Jong and Maina, 2010). Human tumours or cancer cells can also be transplanted into immunosuppressed mice and grown. This then allows the investigation of novel agents for cancer prevention and therapy. Mice are also cheap to maintain, quick to breed and also easy to handle (Valkenburg and Williams, 2011).

The following are some of the most commonly used mouse models for PCa chemoprevention research;

1.3.2.3.1 TRAMP mouse model

Transgenic adenocarcinoma of the mouse prostate (TRAMP) is considered one of the most suitable models developed for investigating PCa to date (Jeet et al., 2010). The TRAMP mouse is a viral oncogene model, in which PCa is produced from the prostate expression of SV40 early gene. It employs the rat probasin promoter to develop the expression of SV40 T antigen (Tag) which interferes with the function of p53 and retinoblastoma (Rb) (Greenberg et al., 1995). Carcinogenesis in the TRAMP mouse is characterized by the expression of the viral gene, fibroblast growth factor receptor (FGFR) and IGFR (Kaplan-Lefko et al., 2003). The pattern of disease progression in this model mimics the human disease, including low grade then high grade PIN, followed by the development of adenocarcinoma and metastasis. TRAMP mice also display an androgen independency phenotype over time (Jeet et al., 2010). Therefore, the tumour can be studied for long durations to assess this change in hormone dependency

(Gingrich et al., 1996). Tumour incidence has been reported in all TRAMP animals, some of which express mutated AR like humans. Furthermore, after castration, comparable to human tumours the TRAMP tumour becomes androgen independent (Han et al., 2001). By 8 weeks of age, TRAMP mice develop PIN in 100% of males, and between 16-32 weeks metastatic PCa develops at distant sites including the lymph nodes, lung, liver and spinal cord (Kaplan-Lefko et al., 2003, Gingrich et al., 1996). As the tumour develops spontaneously there is no need to use chemicals to initiate cancer. This is an advantage as it means there will be no interactions with any potential agent used that could interfere or mask compound efficacy. Disadvantages of the TRAMP model include the logistics and expense of maintaining a breeding colony and generating large numbers of animals of the same age and sex to include in efficacy studies. Also, until studies are terminated the tumour is very difficult to assess, therefore, monitoring tumour progression over time is very challenging (Teicher, 2006). The model could be improved with the development of better imaging systems that allow tumours to be monitored from the same animal over the duration of an efficacy study. The TRAMP model has been extensively used in chemoprevention studies and has shown promising results with agents (Bosland, 1999, Jeet et al., 2010) such as tea components (Saleem et al., 2003) and dietary genistein (Mentor-Marcel et al., 2005).

1.3.2.3.2 PTEN mouse model

The *PTEN* gene is often mutated or lost in PCa. The gene encodes a phosphatase enzyme that is responsible for the dephosphorylation of phosphatidylinositol-3-phosphate, which in turn down regulates Akt. Therefore, *PTEN* functions as a tumour suppressor gene (Cantley and Neel, 1999, Suzuki et al., 1998). *PTEN* has also been linked to Akt-independent mechanisms (Freeman et al., 2003). The PTEN mouse is a knock out model and develops PIN between 8-10 months of age, and often does not progress beyond this point. However, if the PTEN knockout is combined with the knockout of *p27*, then this results in an invasive adenocarcinoma in the prostate and metastasis to other tissues (Di Cristofano et al., 2001). Homozygous deletion of prostate *PTEN* in mice leads to 100% formation of PIN after 6 weeks of age, with the

development of invasive adenocarcinoma after 12 weeks. Apparently, 50% of mice develop metastasis in the lymph nodes or lung by the age of 29 weeks (Klein, 2005). Generally, such a model could be useful for study of chemopreventive agents, however the long development and progression period makes this model less attractive and very costly.

1.3.2.3.3 Xenograft models of prostate cancer

Xenografts are *in vivo* models which include the use of different host mice and sources of tissues (Navone et al., 1998). These models are produced by transplanting human or animal prostate tissue or cells into the animal, commonly an immune-deficient mouse. Therefore, human derived prostate tumours can be grown *in vivo* enabling tumour progression and behaviour to be studied after administration of a chemopreventive agent (Marques et al., 2006). The advantage of this model is the fact human cells can be used that are easily transplanted into the mice with reproducible results. The experiments are generally very quick (< 2 months), therefore, are much cheaper than the transgenic models. Disadvantages include the fact that the model does not display the natural development of human PCa (Teicher, 2006) and therefore is not ideal to study chemopreventive agents that are intended to be given over long periods of time before cancer has developed. However, xenograft mouse models are commonly used as models of efficacy of PCa chemoprevention (Hu et al., 2008, Ma et al., 2004b, Shukla and Gupta, 2006, Shukla et al., 2005), as a method to investigate potential mechanisms of actions and identify agents that may be of potential use in transgenic models.

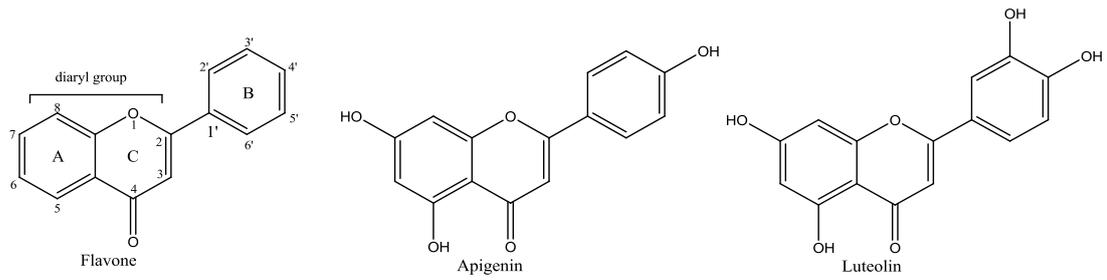
1.4 Flavonoids and cancer

Flavonoids are a group of phenolic compounds possessing a common diaryl nucleus as shown in Figure 1.9. Flavonoids are naturally occurring in plants and are mostly responsible for the colour of various fruits. There are more than 4000 known flavonoids, which have been identified in plant foods such as soy, apple, tea, red wine and onions (Rosenberg Zand et al., 2002).

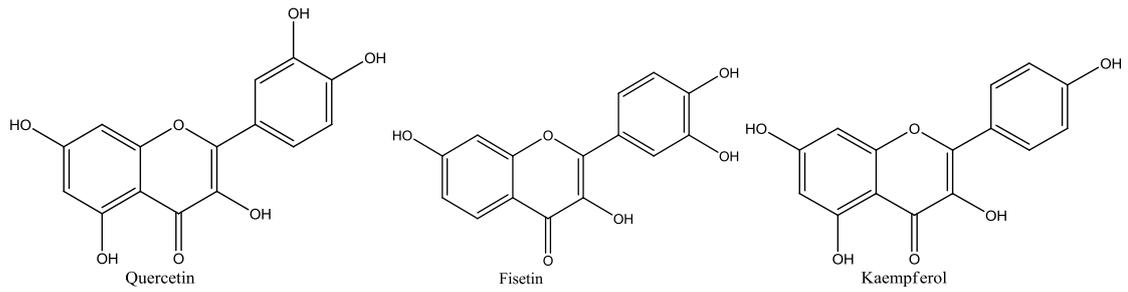
The available information about the role of dietary flavonoids in cancer is not comprehensive enough for reasons such as the difficulty of determining the quantities of flavonoids in food and the presence of more than one flavonoid in the same food source. However, numerous epidemiological and animal studies have provided evidence to suggest they may possess anticarcinogenic properties (Kuo, 1997).

Apigenin, a polyhydroxyflavone, repressed the growth of 22Rv1 human prostatic carcinoma xenografts in athymic nude mice (Shukla and Gupta, 2008) and feeding studies in TRAMP mice demonstrated a significant reduction in the volume of prostate tumours (Shukla et al., 2007). Fisetin, another widely studied flavonol, also reduced tumour volume significantly in the 22Rv1 human prostatic carcinoma xenograft model (Khan et al., 2008b) .

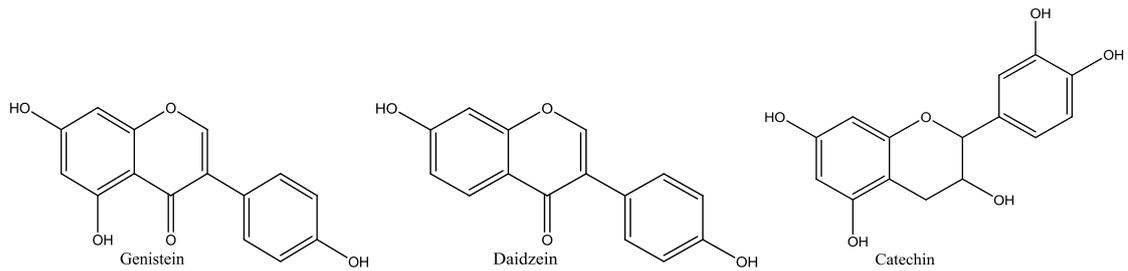
The importance of flavonoids as potential PCa chemopreventive agents was initially highlighted by the findings of epidemiological studies, which linked the incidence of PCa to dietary flavonoid intake. Benign prostatic hyperplasia (BPH), which is thought to contribute to PCa incidence, was found to be less common in south Asian men in comparison to their Western counterparts, which was attributed to them eating a diet richer in flavonoids (Ekman, 1989). Furthermore, it is widely thought that soya based food rich in flavonoids could retard the progress of PCa, as it was shown that in age matched men the incidence of PCa was eight times higher in western men than in Japanese men, and 30 times greater than in Chinese counterparts (Dhom, 1991), two countries where high levels of soya based foods are consumed. Therefore, a comprehensive meta-analysis of the consumption of soya-based food on PCa risk was performed and results suggest soy consumption could decrease the risk of PCa (Hwang et al., 2009). Variations in the PCa incidence rates between Western and Asian men was suggested not to be genetically or racially determined, but due to variations in diet and lifestyle (Brown et al., 2005). This was further highlighted by the fact that Asian migrants moving to North America developed PCa at a similar rate to Caucasian Americans after one or two generations (Dunn, 1975, Kolonel et al., 1985).



Flavones

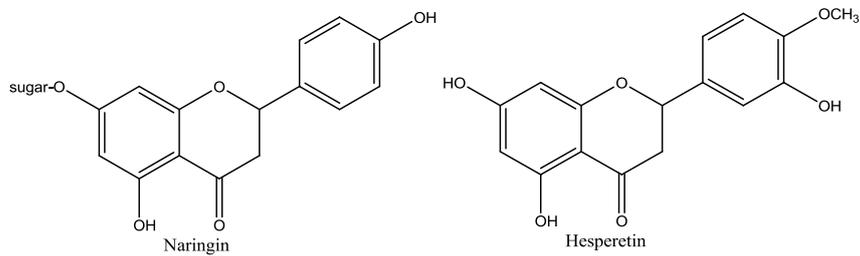


Flavonols



Isoflavones

Flavanols



Flavanones

Figure 1.9 Classification of common flavonoids (Kuo, 1997).

1.4.1 Flavonols and prostate cancer

Flavonols are a subgroup of flavonoids and some members of this group have been extensively studied for their cancer chemopreventive properties. The most commonly studied members of this group are quercetin, fisetin, and kaempferol. It is widely thought quercetin could exert many health benefits (reviewed in (Jan et al., 2010)). Quercetin has shown considerable activity against various cancers, and many cancer chemopreventive properties of quercetin have been found (reviewed by (Lamson and Brignall, 2000)). In regard to prostate cancer specifically, quercetin has shown antiproliferative activity in numerous PCa cell lines (Aalinkeel et al., 2008, Huynh et al., 2003). Furthermore, quercetin showed efficacy in *in vivo* models of PCa by the inhibition of tumour growth in a PC-3 (Asea et al., 2001) and a CWR22Rv1 cancer cell xenograft model (Ma et al., 2004b). Fisetin is another flavonol which has demonstrated chemopreventive activity in both *in vitro* and in *in vivo* models of PCa. It exerted antiproliferative activity in PCa cell lines (Haddad et al., 2010, Haddad et al., 2006, Khan et al., 2008a) and significantly reduced tumor growth in CWR22Rv1 PCa xenografts (Khan et al., 2008b). The less commonly studied member kaempferol has also been shown to inhibit the growth of PCa cells (Brusselmans et al., 2005, Gopalakrishnan et al., 2006).

1.4.1.1 Mechanisms of chemoprevention activity of flavonols in PCa

1.4.1.1.1 Alteration of redox state

Reactive oxygen species (ROS) can produce oxidative damage to the cellular and the genetic components of the cell, which could lead to the development of cancer (Waris and Ahsan, 2006). ROS could be a result of metabolic processes or the natural defence mechanism and inflammation (Halliwell, 1994). Due to their chemical nature as powerful antioxidant agents, flavonoids including flavonols, have the ability to scavenge and reduce the production of free radicals, which may constitute a potential chemopreventive mechanism. Quercetin has been shown to reduce ROS production in human hepatoma cell line (HepG2) (Alia et al., 2006).

1.4.1.1.2 Interference with cell cycle

Cellular proliferation is achieved through cell cycle progression. It is a process which includes DNA duplication and segregation into two cells by cell division. The replication of DNA occurs in the S phase which is preceded by a gap called G₁ phase in which the preparation for DNA synthesis takes place (Figure 1.10). However, cells in G₁ can also enter G₀ phase in which cells are non proliferative. S phase is followed by another gap called G₂ during which the cell gets prepared for mitosis (M) (Norbury and Nurse, 1992). During M phase the chromosomes separate to form two identical cells.

The progress from phase to phase is controlled by various cellular proteins, mainly a group called cyclin-dependent kinases (CDKs). These kinases are activated by binding to specific cyclins, which enables them to perform various phosphorylation reactions on target proteins, and therefore, trigger downstream processes that lead to progress of the cell cycle. Various CDK have been identified, which include CDK4, CDK6 and CDK2 that act during G₁ phase, CDK2 during S phase and CDK1, which regulates G₂ and M phases (Morgan, 1995). Cyclin D (D1, D2 and D3) binds to CDK4 and CDK6. The CDK-cyclin D complexes regulate the transitions from G₁ to S phase. The kinase activity of these complexes leads to phosphorylation of the retinoblastoma protein (Rb), which causes it to dissociate from the transcription factor E2F. The activated E2F induces the transcription of downstream genes which are responsible for the G₁-S phases transition. During G₁ cyclin E binds to CDK2 and the resulting kinase regulates the progress to S phase (Ohtsubo et al., 1995). The kinase resulting from the binding of cyclin A and CDK2 is necessary for S phase progression (Walker and Maller, 1991). Progress from late G₂ and early M phase is controlled by the kinase as a result of cyclin A binding to CDK1. Regulation of mitosis is controlled by the complex of cyclin B and CDK1. Importantly, p53 can bind to the cyclin B1/CDK1 complex and decrease its kinase activity (Ababneh et al., 2001).

The kinase activity of the CDK is regulated by a group of proteins called cyclin dependent kinase inhibitors (CKI) which can bind to CDK or to CDK/cyclin

complex leading to a decrease in their activity. CDKI are divided into two families, the INK4 family which includes p15, p16, p18 and p19 and these can activate G_1 by binding to CDK4 and CDK6. The other family is the Cip/Kip, which consist of p21, p27 and p57; proteins of this group bind to the CDK/cyclin complex.

The Cip/Kip proteins play an important role in retarding cell proliferation as a response to cellular stress, development or differentiation (Sherr and Roberts, 1999). However, each member can regulate a specific function differentiating it from the rest of the members. For example, p21 is a target gene for p53 and is involved in G_1 and G_2 cell cycle arrest induced by DNA damage, mediated by p53 (Eldeiry et al., 1993). While p27 expression and activation can be induced by transforming growth factor β (TGF- β), which leads to growth inhibition (Reynisdottir et al., 1995).

Check points are a mechanism by which cells are checked for DNA integrity and the formation of spindle and spindle pole. These check points control the transition of cells from phase to phase, hence defective cells will not progress to the next phase (Nakanishi, 2001). Importantly, it has been reported that cell cycle check points are deregulated in many cancers (Pommier and Kohn, 2003).

Flavonols are reported to induce cell cycle arrest in PCa cells. Quercetin and fisetin were both found to induce G_2/M arrest in LNCaP and PC-3 PCa cells (Shenouda et al., 2004, Haddad et al., 2006, Vijayababu et al., 2005). In addition, kaempferol, was shown to arrest PC-3 cells in the G_2/M phase (Knowles et al., 2000) and exerted similar effects in the AT6.3 rat prostate cancer cell line (Wang et al., 2003). Other studies are contradictory and have shown quercetin and fisetin to arrest LNCaP cells in G_1 phase and in PC-3 cells quercetin was found to cause cell cycle arrest at the S phase (Knowles et al., 2000).

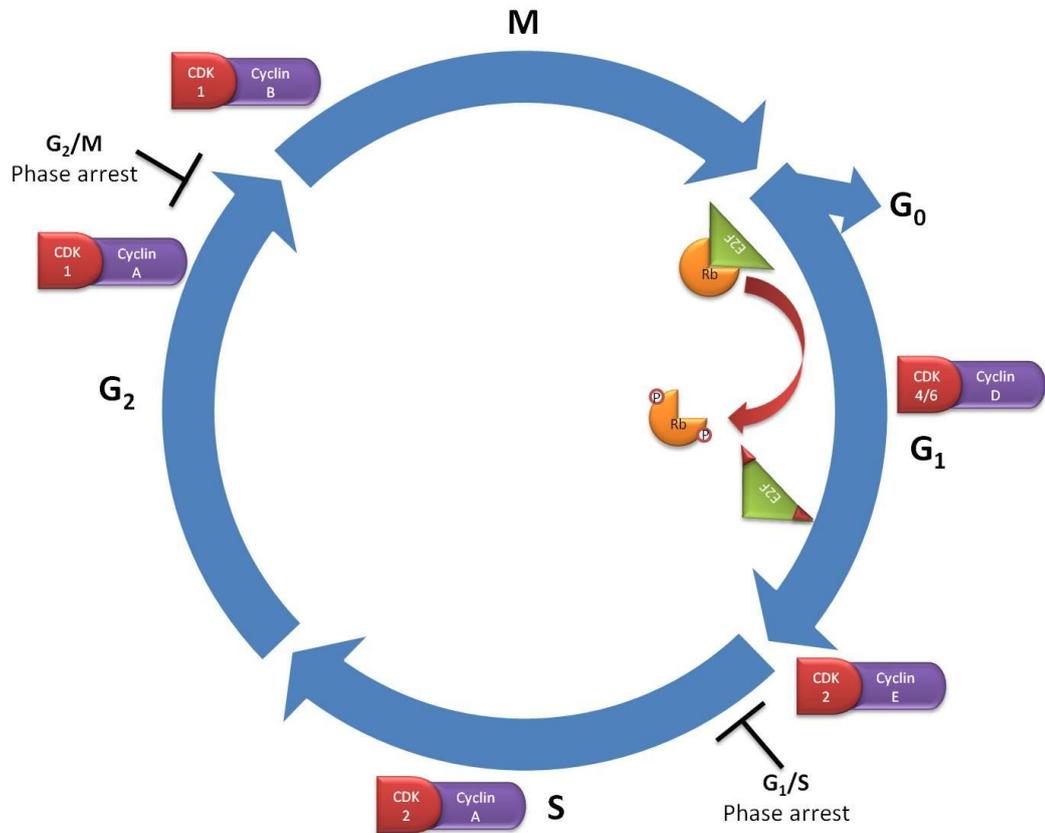


Figure 1.10 Schematic representation of the cell cycle. Cells either enter G₁ to start another cell cycle or enter the non proliferative G₀ phase. Progression of the cell cycle from phase to phase is controlled by the binding of cell phase specific cyclins to CDKs. The formed complexes lead to the activation of downstream targets that result in progression through the cell cycle.

1.4.1.1.3 Induction of apoptosis

The control over cell multiplicity is a very organised process, mediated by various molecular mechanisms. Programmed cell death or apoptosis, is a mechanism by which the body can dispose of cells and maintain a balance between cell proliferation and death, therefore, no uncontrolled cell growth occurs. However, any disturbance in this balance in favour of cell proliferation might lead to neoplasia (Blank and Shiloh, 2007). Induction of apoptosis is considered as a main mechanism of cancer chemoprevention (D'Agostini et al., 2005). Apoptosis is a complex self destruction process characterized by morphological hallmarks, including cell shrinking, membrane blebbing, chromatic condensation, nuclear DNA fragmentation, and the formation of membrane-enclosed apoptotic bodies. The process is ended by cell engulfment by macrophages or the surrounding tissue in order to decrease the local inflammatory response (Savill and Fadok, 2000).

Apoptosis includes various biochemical and molecular features. Two apoptosis pathways have been described, the intrinsic and the extrinsic pathway as illustrated in Figure 1.11.

The intrinsic or mitochondrial-mediated pathway is activated by factors such as DNA damage, the deprivation of growth factors, hypoxia or oxidative stress. Mitochondrial-mediated apoptosis involves the activation of Bcl-2 family pro-apoptotic proteins. The Bcl-2 family includes pro-apoptotic and anti-apoptotic proteins localized on the outer side of the mitochondrial membrane or in the cytosol, hence they balance the release of cytochrome C through the regulation of membrane potential (Kluck et al., 1997). The anti-apoptotic sub-family includes Bcl-2, Bcl-xl, Mcl-1, Bcl-w and A1/Bfl1 (Leibowitz and Yu, 2010), while the pro- apoptotic members include others such as Bax, Bad, and Bid (Iannolo et al., 2008). The Bcl-2 family proteins were found to be deregulated in various cancers (Adams and Cory, 2007). The function of pro-apoptotic proteins is to induce the release of the mitochondrial cytochrome C, which leads to the formation of apoptosomes by the association with Apaf-1 and pro-caspase-9 (Hengartner, 2000). Various polypeptides such as, second mitochondrial

activator of caspases (Smac/Diablo), Apoptosis-inducing factor (AIF), Endo G and HTrA2/Omi are released concomitantly with cytochrome C from the intermembrane space of mitochondria. These polypeptides play an important role in the regulation of apoptosis. For example, Smac/Diablo and HTrA2/Omi activate certain caspases by neutralizing the effects of the inhibitor of apoptosis proteins (IAP_s). Activated caspase-9 can cleave procaspase-3, resulting in apoptosis (Ekert et al., 2001) (Figure 1.11). Other factors such as release of calcium from endoplasmic reticulum can also induce apoptosis by activating caspases and endonucleases (Gurumurthy et al., 2001). Caspases are activated by cleavage into proteases. In cells caspase-3 is the most predominant and is responsible for most of the apoptotic effects. However, caspase-3 action is supported by caspases-6 and -7. These caspases function in the degradation of various cellular substrates which results in the apoptotic phenotype (Zimmermann et al., 2001).

The initiation of the extrinsic pathway is caused by the activation of transmembrane death receptors such as Fas, DR3, DR4, DR5 and tumour necrosis factor (TNF). Stimulation of these receptors leads to its clustering and the formation of a death inducing signalling complex. A molecule of this complex called Fas-associated death domain (FADD) induces the activation of caspase-8 which activates the downstream pathway of apoptosis through other caspases, such as caspase-3 and -7 (Figure 1.11) (Khan et al., 2010).

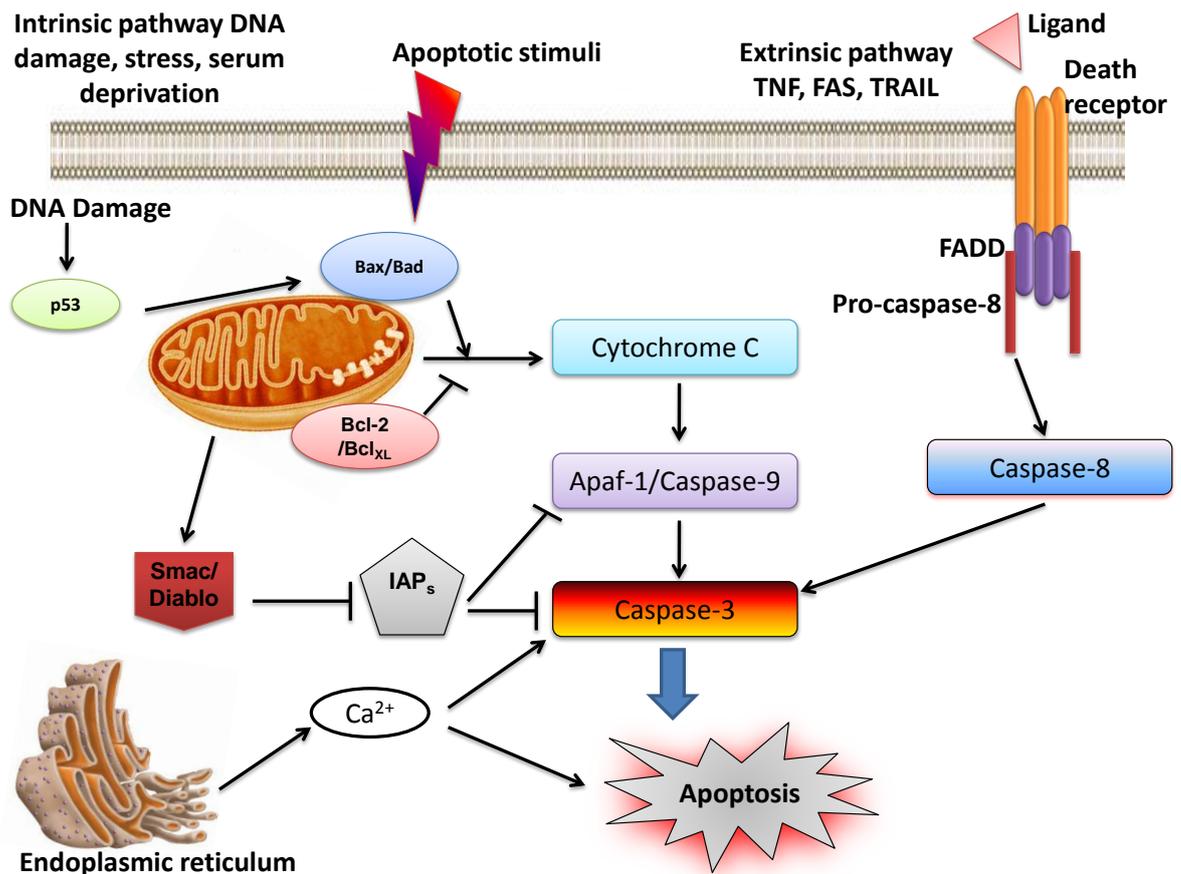


Figure 1.11 Extrinsic and intrinsic apoptosis pathways. The intrinsic (mitochondrial) pathway can be activated by factors such as DNA damage, growth factor deprivation or oxidative stress. Consequently, it activates the pro-apoptotic proteins such as, Bax and Bad and inhibits the anti-apoptotic proteins such as Bcl-2 and Bcl_{XL}. This process leads to the release of mitochondrial cytochrome C and membrane polypeptides such as Smac/Diablo. Cytochrome C binds to APaf-1 and pro-caspase 9 which leads to the activation of caspase-9 and consequently the cleavage of caspase-3, which results in apoptosis. Smac/Diablo role is in neutralizing the effects of (IAPs). The extrinsic apoptosis pathway is initiated by the stimulation of death receptor by ligands such as Tumor necrosis factor (TNF), Fas or TNF-related apoptosis-inducing ligand (TRAIL). Death receptor stimulation leads to the formation of Fas-associated death domain (FADD) that activates caspase-8 leading to the cleavage of caspase-3. Increasing the free intracellular Ca²⁺ could also directly activate caspase-3.

Flavonols have been shown to induce apoptosis in PCa cells (Khan et al., 2010). Quercetin induced apoptosis in LNCaP androgen dependent PCa cells (Aalinkeel et al., 2008, Lee et al., 2008) and androgen independent PC-3 (Aalinkeel et al., 2008, Kim et al., 2008, Vijayababu et al., 2006b, Senthilkumar et al., 2010) and DU145 PCa cells (Kim et al., 2008, Kim and Lee, 2007). Fisetin was also found to induce apoptosis in androgen dependent LNCaP (Khan et al., 2008a) and androgen independent PC-3 PCa cells (Haddad et al., 2010). Kaempferol was reported to inhibit the antiapoptotic activity of IGF-1 in AT6.3 rat PCa cells (Wang et al., 2003).

Various apoptosis sub-pathways and mediators have contributed to the apoptotic effects of flavonols in PCa. Quercetin induced apoptosis in LNCaP and PC-3 cells was accompanied by the activation of caspase-3, -8 and -9 (Aalinkeel et al., 2008, Lee et al., 2008, Senthilkumar et al., 2010). Quercetin also decreased the expression of survivin the IAP_s member in PC-3 and DU145 cells, and induced cleavage of caspase-3 and -8 (Kim and Lee, 2007). The bcl-xl: bax ratio was decreased by quercetin treatment in LNCaP cells, leading to the increase in the release of cytochrome C and cleavage of pro-caspase-3, -8 and -9 (Lee et al., 2008). Furthermore, in PC-3 cells, quercetin increased the expression of cytochrome C, Bax and Bad mRNA levels, while decreasing the levels of Bcl-xl proteins (Senthilkumar et al., 2010, Vijayababu et al., 2006a, Vijayababu et al., 2005). Fisetin induced apoptosis in LNCaP cells, which resulted in a decrease in the Bcl-xl :Bax ratio, and the upregulation of Bak, Bad and Bid expression. Fisetin also increased levels of cytosolic cytochrome C which led to the inhibition of IAPs by the increase in Smac/Diablo mediators. Furthermore, it induced the activation of caspase-3, -8 and -9 (Khan et al., 2008a). Fisetin was found to induce apoptosis in PC-3 by the alteration in expression of many apoptosis genes (Haddad et al., 2010).

1.4.1.1.4 Inhibition of androgen receptor pathway

The AR plays a fundamental role in PCa progression and flavonols have been shown to interfere with its function. In LNCaP cells quercetin repressed the function of AR (Yuan et al., 2006, Yuan et al., 2004). It also reduced the

expression of AR protein and inhibited the secretion of PSA and was shown to inhibit the AR and PSA genes at the transcription level (Xing et al., 2001, Yuan et al., 2005, Yuan et al., 2010). Fisetin has also been reported to decrease the expression of AR protein and was shown to compete with androgens for binding to AR. This in turn led to a decrease in the activity and stability of the AR, as well as an increase in its degradation (Khan et al., 2008b). All these factors can result in cancer cell growth inhibition.

Quercetin, fisetin and kaempferol were reported to inhibit the 5 alpha-reductase enzyme which is responsible for the conversion of testosterone to dihydrotestosterone (DHT), (Hiipakka et al., 2002) so to some extent may behave similar to finasteride and dutasteride.

1.4.1.2 Pharmacokinetics of flavonoids

Many flavonoids have been demonstrated to have important anti-proliferative properties *in vitro*, but studies *in vivo* do not always correlate, as a result of poor bioavailability, which can generally be explained by their lower absorption and/or rapid metabolism (Scalbert and Williamson, 2000, Walle, 2004, Gao and Hu, 2010). Flavonoid absorption through the gastrointestinal tract (GIT) is generally poor and depends on their lipid solubility (Birt et al., 2001). Nonetheless, it has been reported that flavonoids can be absorbed through various transporter systems (Passamonti et al., 2009). When dietary flavonoids are administered orally to a human or animal typically only a small amount reaches the systemic circulation, partially due to the fact that these compounds are mostly heavily hydroxylated and/or present as glycosides, which probably decreases their absorption (Walle, 2007a). A sugar moiety linked to a flavonoid could be a determinant of its absorption (Graefe et al., 2001), as some studies have shown flavonoid glycosides can be absorbed through the GIT (Griffith.La and Barrow, 1972). For example, the absorption of quercetin glycoside in human through the GIT has been reported, and furthermore the rate of the quercetin glycoside absorption was higher than that of the aglycone (Hollman et al., 1995). Most of the flavonoids present in plants are in the glycoside form (Rupprich and Kindl, 1978, Kudou et al., 1991). Polyphenol-O-glycosides

undergo hydrolysis through the action of hydrolase and glycosidase from the intestine and bacteria, to produce more lipophilic aglycones and metabolites, which normally improves their absorption relative to the parent glycosidic compound (Hur et al., 2000). Since the transport rate is mainly affected by lipophilicity, the absorption of methoxylated flavonoids might be higher than their non-methoxylated counterparts. Non absorbed flavonoids in the small intestine undergo substantial structure modification in the colon by the action of colonic flora, such as hydrolysis of glycosides or degradation of flavonoids to simple phenolic acids (Aura et al., 2005, Kuhnau, 1976).

After a flavonoid is absorbed by the small intestine it can undergo structural modifications in the enterocytes (small intestine cells) or the liver which is mainly by conjugation (Felgines et al., 2005). The common conjugation processes in the GIT are glucuronidation, sulphation and methylation. In the liver flavonoids can undergo further Phase I or Phase II metabolism, and conjugated products can be re-secreted in the intestinal lumen by means of enterohepatic circulation (Prasain and Barnes, 2007, Crozier et al., 2010).

Conjugation mainly occurs on the free hydroxyl groups of flavonoid's, therefore, heavily hydroxylated flavonoids could be more susceptible to conjugation than their methoxylated counter parts (Walle, 2007b). The glucuronidation process is undertaken by the action of the endoplasmic reticulum bound enzyme Uridine-5'-diphosphate Glucuronosyltransferases (UGTs), which catalyses the addition of UDP-glucuronic acid to the hydroxylated flavonoids (Spencer et al., 1999).

Sulphation of flavonoids is undertaken by sulphotransferase (SULT) enzymes, which catalyse the addition of a sulphate group from phosphoadenosine-phosphosulphate to a flavonoid's hydroxyl group in a process that mainly occurs in the liver (Piskula and Terao, 1998).

Flavonoids that possess a catechol (diphenolic) moiety such as quercetin and fisetin can undergo methylation, via the action of Catechol-O-methyltransferase (COMT). COMT catalyses the transfer of a methyl group from

adenosylmethionine to flavonoids; this enzyme is widely distributed in liver and kidney tissues (Tilgmann and Ulmanen, 1996).

After absorption, flavonoids are delivered to various tissues by means of the systemic circulation. However, it has been reported that flavonoids can bind to plasma proteins (Bolli et al., 2010) or red blood cells (Fiorani et al., 2003) and as a consequence, the free fraction of flavonoids is reduced which in turn reduces their bioavailability (Leon, 2005). The availability of flavonoid to target tissue is crucial in order to exert any pharmacological action. Cellular uptake of flavonoids can take place. Therefore, these flavonoids could interfere with intracellular mechanistic targets and undergo intracellular metabolic conversion as well (Spencer et al., 2003).

The purpose of biotransformation and the conjugation processes is to convert foreign compounds and toxicants to a more water soluble form in order for excretion. Conjugated flavonoids are mainly excreted into the bile and urine (Williamson et al., 2000).

1.4.1.2.1 Methoxylated versus hydroxylated flavonoids

Most of the common naturally occurring flavonoids are hydroxylated, while methylated flavonoids are mostly synthetic, although there are some that occur naturally, for instance a number of citrus flavonoids e.g. nobiletin and tangeretin (Mizuno et al., 1991).

The presence of free hydroxyl groups facilitates the intestinal and hepatic conjugation and as a consequence leads to more rapid excretion (Otake et al., 2002). Quercetin and fisetin have shown poor bioavailability, explained by the presence of their hydroxyl groups which decreases their absorption and makes them prone to conjugation (Landis-Piwowar et al., 2008). Therefore, methylation of hydroxylated flavonoids has been suggested to improve the absorption and increase the metabolic stability of these molecules (Wen and Walle, 2006a, Walle, 2007b).

Methylation can improve the entry of flavonoids through the cell membrane and accordingly increases their ability to exert effects on intracellular targets. Principally, many cancer chemopreventive mechanisms depend on the interference of the agent with intracellular targets (Spencer et al., 2004, Ramos, 2008). This can be illustrated by 4'-O-methyl quercetin which showed higher cellular uptake than its quercetin aglycone (Spencer et al., 2003). In addition, masking the hydroxyl groups of quercetin by a pivaloxymethyl moiety increased its cellular uptake and its intracellular concentration in HCT116 colon cancer cells (Kim et al., 2010).

It is widely thought that the anticarcinogenic potency of flavonoids is partly due to their antioxidant properties and therefore to the presence of free hydroxyl groups. As a result, the anticarcinogenic activity of methylated flavonoids has not been well studied. However, methylated flavonoids have shown antiproliferative activity against prostate cancer cells (Pouget et al., 2001) and inhibitory effects on carcinogen activating enzymes (Alvarez-Gonzalez et al., 2011). 5,7-Dimethoxyflavone showed a high ability to inhibit benzo[a] pyran induced DNA binding and also decreased the CYP1A1 protein expression and activity in Hep G2 cells (Wen et al., 2005). Benzo[a] pyran is converted to a carcinogen after bioactivation by cytochrome P450 1A1 (CYP 1A1) (Alvarez-Gonzalez et al., 2011).

A comparison between methylated and non methylated flavonoids was performed (Walle, 2007b, Walle, 2007a, Walle et al., 2007a) using apigenin and chrysin (hydroxylated flavonoids) and their methoxylated analogues 5,7-dimethoxyflavone (5,7-DMF) and 5,7,4' trimethoxyflavone (5,7,4' TMF) These studies highlighted many advantages for methylated flavonoids over non-methylated counterparts. The pharmacokinetic advantages were higher GIT absorption and metabolic stability, while advantages related to their antiproliferative potency revealed that the methoxylated analogues were 8-10 times more effective.

A study was undertaken in our laboratory to determine the effect of O-methylation on the chemopreventive activity of flavones in colorectal cancer

models, *Apc^{Min}* mice and *in vitro* in murine adenoma cells derived from these mice. The rank order of activity of these compounds was 3', 4', 5', 5, 7-pentamethoxyflavone (PMF) > tricetin (4', 5, 7-trihydroxy-3', 5'-dimethoxyflavone) > apigenin (4', 5, 7-trihydroxyflavone), suggesting methylation of hydroxylated flavones could improve the chemopreventive activity in gastrointestinal cancers (Cai et al., 2009). This has not been determined for other cancer types.

1.5 Aims and objectives

The work in this thesis was designed to identify and then investigate the most potent antiproliferative compound (TMFol) from a panel of hydroxylated and methoxylated flavonoids, using an *in vitro* screen conducted in PCa cell lines.

The overall aim was to perform the preclinical evaluation of TMFol, generating pharmacokinetic and pharmacodynamic data, which might assist in the decision making as to whether TMFol should be developed further for PCa chemoprevention.

The specific objectives to fulfil the overall aim were:

- i. To compare the *in vitro* anti-proliferative activity of TMFol to other previously studied members of hydroxylated flavonols i.e. quercetin and fisetin, in PCa cell lines, and identify the potential mechanisms by which TMFol inhibits PCa cell proliferation.
- ii. To investigate the pharmacokinetic characteristics of TMFol *in vivo* and *in vitro* through:
 - Determining plasma and tissue levels of TMFol in mice after single dosing and continuous TMFol administration.
 - Performing metabolism studies of TMFol in order to identify *in vitro* and *in vivo* metabolites using HPLC and LC-MS techniques.
- iii. To explore the *in vivo* chemoprevention efficacy of TMFol in the TRAMP C2 xenograft model of PCa and compare these to quercetin and fisetin.
 - Determine the *in vivo* mechanisms that might explain the anticarcinogenic activity of TMFol

CHAPTER TWO
Materials and methods

2 Material and methods

2.1 Materials

2.1.1 Chemicals and instruments

Unless otherwise mentioned, all chemicals and solvents for general laboratory consumption were purchased from Fisher Scientific (Loughborough, UK) and Sigma (Poole, UK) respectively, cell culture reagents and media were supplied by GIBCO-Invitrogen (UK), tissue culture plastic ware was from Appleton Woods, (Birmingham, UK).

Antibodies against proteins

Antibody for	Supplier	Catalogue number
Androgen receptor	Santa Cruz Biotechnology	816
Anti-goat HRP linked secondary antibody	Santa Cruz Biotechnology	2020
Anti-mouse HRP linked secondary antibody	Pierce- Thermo Fisher Scientific	1858413
Anti-rabbit HRP linked secondary antibody	Pierce- Thermo Fisher Scientific	1858415
Bax	R&D systems	AF820
Caspase-3-cleaved	Cell signalling	9661s
CDC2	Cell signalling	9112s
Cyclin B1	Abcam	76017
Ki67	Leica Microsystems	NCL-Ki67p
p21	Dako	M7202
p27	Cell signalling	2552s
p53	Cell signalling	2524s
Phospho-CDC2	Cell signalling	9111s
PSA	Dako	A0562
Survivin	Cell signalling	2808s
β -Actin	Santa Cruz Biotechnology	1616

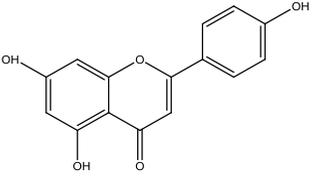
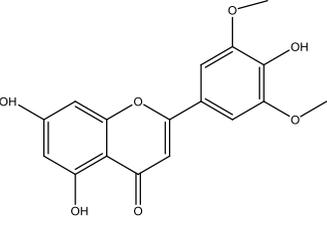
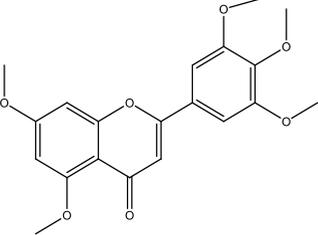
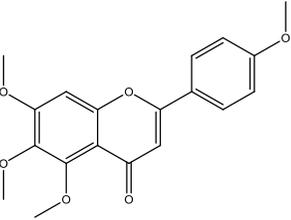
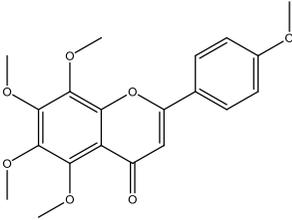
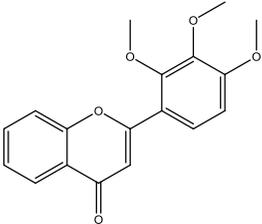
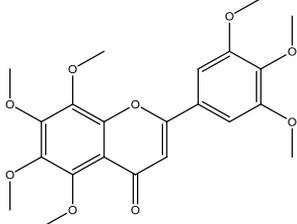
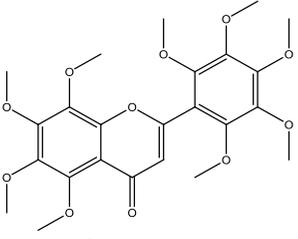
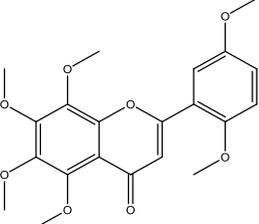
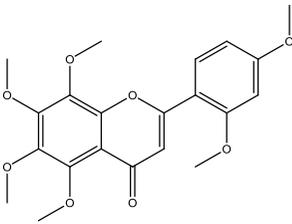
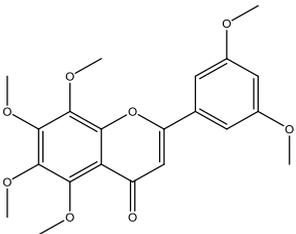
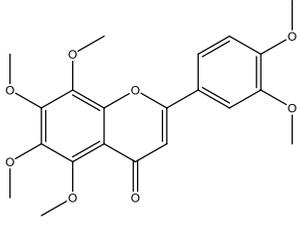
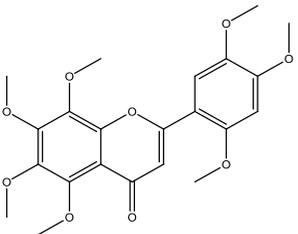
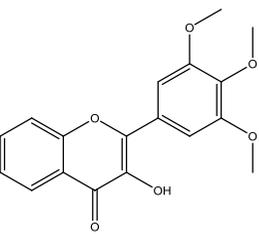
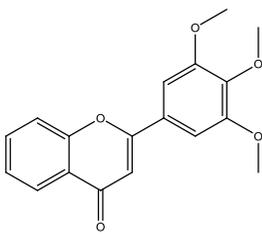
 <p>Apigenin 4',5,7-Trihydroxyflavone C₁₅H₁₀O₅ (MW 270)</p>	 <p>Tricin 3',5'-Dimethoxy-4',5,7-trihydroxyflavone C₁₇H₁₄O₇ (MW 330)</p>	 <p>PMF 3',4',5',5,7-Pentamethoxyflavone C₂₀H₂₀O₇ (MW 372)</p>	 <p>Compound 1 4',5,6,7-Tetramethoxyflavone C₁₉H₁₈O₆ (MW 342)</p>	 <p>Compound 2 4',5,6,7,8-Pentamethoxyflavone C₂₀H₂₀O₇ (MW 372)</p>
 <p>Compound 3 2',3',4'-Trimethoxyflavone C₁₈H₁₆O₅ (MW 312)</p>	 <p>Compound 4 3',4',5',5,6,7,8-Heptamethoxyflavone C₂₂H₂₄O₉ (MW 432)</p>	 <p>Compound 5 2',3',4',5',6',5,6,7,8-Nonamethoxyflavone C₂₄H₂₈O₁₁ (MW 492)</p>	 <p>Compound 6 2',5',5,6,7,8-Hexamethoxyflavone C₂₁H₂₂O₈ (MW 402)</p>	 <p>Compound 7 2',4',5,6,7,8-Hexamethoxyflavone C₂₁H₂₂O₈ (MW 402)</p>
 <p>Compound 8 3',5',5,6,7,8-Hexamethoxyflavone C₂₁H₂₂O₈ (MW 402)</p>	 <p>Compound 9 3',4',5,6,7,8-Hexamethoxyflavone C₂₁H₂₂O₈ (MW 402)</p>	 <p>Compound 10 2',4',5',5,6,7,8-Heptomethoxyflavone C₂₂H₂₄O₉ (MW 432)</p>	 <p>Compound 11 3',4',5'-Trimethoxyflavonol C₁₈H₁₆O₆ (MW 328)</p>	 <p>Compound 12 3',4',5'-Trimethoxyflavone C₁₈H₁₆O₅ (MW 312)</p>

Table 2.1. Chemical structures of apigenin, tricin, PMF and compounds (1-12).

2.2 Buffers

Agarose gel (2.0% w/v) composition

Sea Plaque Agarose	3 g
0.5X TBE buffer	150 ml
Ethidium bromide	5 μ l

The agarose (Invitrogen Life Technology, Paisley, UK) and TBE buffer mixture was microwaved with intermittent swirling to ensure complete dispersion of agarose. Ethidium bromide (Invitrogen Life Technology, Paisley, UK) was added prior to the gel being poured into the moulding chamber of the tank. Immediately afterwards, 15 or 20 well combs were inserted into the gel and then it was allowed to set.

Annexin binding buffer

4X Annexin buffer concentrate (Bender Med Systems, Vienna, Austria) was diluted to a 1X working stock, which consisted of: 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂.

Citrate buffer

The citrate buffer consisted of 10 mM citric acid in distilled water. The pH was adjusted to 6.0 using 2.0 M sodium hydroxide solution.

Neutral buffered formalin (10% w/v)

The 10% neutral buffered formalin solution was made up of 4.0 g sodium dihydrogen phosphate monohydrate (NaH₂PO₄.H₂O), 6.5 g anhydrous disodium hydrogen phosphate (Na₂HPO₄), 100.0 ml formaldehyde 37% w/v and 900 ml distilled water.

Phosphate buffered saline

Phosphate buffered saline was prepared with:

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Di-sodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Distilled water up to	1000 ml.

Polyacrylamide running gel (recipe for two gels)

The amount of water and 30% w/v acrylamide (National Diagnostics, Fradley, UK) varied depending on the percentage of gel required:

10% w/v- 8.0 ml water and 6.8 ml 30% w/v acrylamide.

12% w/v- 6.8 ml water and 8.0 ml 30% w/v acrylamide.

15% w/v- 4.6 ml water and 10.0 ml 30% w/v acrylamide.

A fixed volume (5 ml) of resolving buffer (4X) (National Diagnostics, Fradley, UK) was added. Polymerization of polyacrylamide was induced by the addition of 200 μ l 10% w/v ammonium persulphate and 15 μ l TEMED.

Polyacrylamide stacking gel (recipe for two gels)

The stacking gel consisted of 1.7 ml 30% w/v acrylamide, 1.25 ml stacking buffer (National Diagnostics, Fradley, UK) and 6.8 ml water. Finally, addition of 15 μ l TEMED and 100 μ l 10% w/v ammonium persulphate induced polymerization. Ten or 15 well combs were inserted before the gel had set.

Potassium phosphate buffer

Phosphate buffer was prepared by mixing 198 ml 0.1M monobasic potassium phosphate KH_2PO_4 and 802 ml 0.1M dibasic potassium phosphate K_2HPO_4 to make 1litre (pH 7.4).

Resolving buffer (4X)

Resolving buffer (National Diagnostics, Fradley, UK) was supplied as 4X ready for use stock which consisted of 1.5 M Tris-HCl, 0.4% w/v SDS.

Running buffer

Running buffer (National Diagnostics, Fradley, UK) was supplied as a 10X concentrate, which was diluted to a 1X working stock in distilled water. The buffer consisted of 25 mM Tris, 192 mM glycine and 0.1% w/v SDS.

Sample buffer Laemmli (2X) for western blot

Sample buffer contained 10% w/v of 2-mercaptoethanol, 4% w/v SDS, 20% w/v glycerol, 0.004 % w/v bromphenol blue and 0.125 M Tris HCl. Sample buffer was mixed (1:1 v/v) with the protein lysate sample prior to use.

Stacking buffer

Stacking buffer (National Diagnostics, Fradley, UK) consisted of 0.5 M Tris-HCl and 0.4% w/v SDS, pH 6.8.

Transfer buffer

Transfer buffer (National Diagnostics, Fradley, UK) was supplied as a 10X concentrate, which was diluted to a 1X working stock in distilled water. The buffer consisted of 25 mM Tris base, 192 mM glycine and 20% v/v methanol.

2.3 Methods

2.3.1 Cell culture

The AR receptor expressing PCa cell lines used in this project were, human LNCaP cells which were established from a metastatic prostatic adenocarcinoma of the lymph node (Horoszewicz et al., 1983), and TRAMP C2 murine prostatic adenocarcinoma cells, that were established from the prostate of a C57BL/6 male TRAMP mouse (Foster et al., 1997). While the non androgen dependent cells were, PC-3 cells, which were established from a human prostatic adenocarcinoma bone metastases (Kaighn et al., 1979) and DU145, human prostate carcinoma cell line, that were derived from a prostatic adenocarcinoma metastasized to the brain (Stone et al., 1978). All the above mentioned cell lines were sourced from either the American Type Culture Collection (ATCC) or the European Collection of Cell Cultures (ECACC).

2.3.2 Maintenance of cell lines

Cell lines were cultured as a monolayer under incubation conditions of 100% humidity, 5% v/vCO₂ and 37°C. LNCaP, PC-3 and DU145 were cultured in RPMI1640 media containing L-glutamine (Invitrogen, Paisley, UK) and 10% w/v foetal calf serum without sodium pyruvate (Invitrogen, Paisley, UK), as sodium pyruvate could interfere with reactive oxygen species (Giandomenico et al., 1997). The TRAMP C2 cell line was maintained in DMEM high glucose (4500 mg/L) with L-glutamine and no sodium pyruvate (Sigma Aldrich, Poole, UK) fortified with 5% v/v foetal calf serum, 5% v/v Nu serum IV (BD Biosciences, California, USA) , 5 µg/ml insulin and 10⁻⁸ M dihydrotestosterone (Foster et al., 1997).

2.3.3 Cell passaging

Cells were passaged not more than 30 times after restoration from liquid nitrogen frozen stocks, to reduce the possibility of genetic and/or phenotypic alterations. Passaging of cells was performed at approximately 80-90% confluency. The procedure of cell passaging was started by aspiration of media from flasks, then cells were washed with warm (37°C) PBS twice, followed by

addition of 1X trypsin EDTA (Invitrogen, Paisley, UK). The trypsinized cells were incubated for not more than 5 minutes at 37°C and gentle agitation was applied to assist the detachment of cells. Complete detachment of cells was checked under the microscope and was immediately followed by the addition of a volume of FCS containing media in order to neutralise the effects of trypsin on the cells. Then the cell suspension was centrifuged at 1300 rpm for 3 min and re-suspended in fresh media. A 100 µl aliquot of the cell suspension was mixed with 9.9 ml isoton and counted on a coulter particle size counter. Approximately 5×10^5 cells were added to a new flask containing culture media to maintain each cell line. Cells were passaged approximately twice a week.

2.3.4 Cell treatment and drug solvents

Tricin, apigenin (Apin Chemicals Ltd, Abingdon, UK), 3',4',5',5,7-pentamethoxyflavone (PMF) (synthesized by Dr. R. Britton, Department of Cancer Studies and Molecular Medicine, University of Leicester), 3',4',5'-trimethoxyflavone (compound 12) (OTAVA, Kyiv, Ukraine), and compounds 1-11 (NCI Repository) (for names and chemical structures refer to Table 2.1), were prepared at concentrations of 20 mM in DMSO. Quercetin and fisetin stocks were prepared in 40 mM stocks in DMSO. Mibolerone (Steraloids Inc, New Port, USA) was dissolved in ethanol and prepared as a 1 µM stock. Stocks of 10 µM DHT were prepared in ethanol. The final concentration of DMSO or ethanol in the treatment medium was always below 0.2% v/v. Vehicle controls for each experiment received DMSO and/or ethanol alone in a v/v concentration identical to the treatment groups.

Mibolerone treatment: LNCaP cells were grown in RPMI 1640 media containing 10% v/v FCS for 48 h the media was replaced with RPMI 1640 media containing 5% v/v FCS and 1 nM mibolerone along with (0-20 µM) TMFol, or (0-100 µM) quercetin or fisetin. Cells were incubated for 24, 48 or 72 h and then counted and harvested for western blot analysis or for mRNA analysis as detailed in sections 2.3.8.3 and 2.3.12.

2.3.5 Cell proliferation assay

Cell proliferation assays were performed using two methods. The first method counted the raw cell number, whilst the second approach, the ATPLite assay, measured luminescence generated when ATP reacts with the assay substrate. The two methods were compared and similar results were achieved using both methods.

2.3.5.1 Coulter counter method

LNCaP, PC-3 or DU-145 cells were seeded in 24 well plates at a density of 5×10^3 cells/well in 2 ml RPMI 1640, while TRAMP C2 cells were seeded at a density of 3×10^3 cells/well in 2 ml DMEM media. Cells were incubated for 24 h, except LNCaP cells, which were incubated for 48 h, at that time the media was replaced with media containing drug treatments (0-20 μ M) for all compounds in Table 2.1 or 0-40 μ M for quercetin and fisetin, with each treatment performed in triplicate wells. Treated cells were incubated for 72 or 144 h, before counting. Briefly, media was aspirated using a vacuum pump, followed by immediate washing of each well twice with 1 ml PBS. After washing each well received 0.5 ml of 1X trypsin EDTA. The trypsin EDTA was neutralized by adding 0.5 ml of media. The well content was transferred into coulter counter cups containing 9 ml isoton, and the cell suspension was then counted using the Coulter counter. Cell numbers were calculated as % vehicle control and plotted against drug concentration. The linear line equation was used to calculate IC_{50} values for each agent. Each experiment was repeated in triplicate using cells of different passages.

2.3.5.2 ATP based luminescence assay

The principle of the ATPLite assay is based on measuring the emitted light produced when ATP molecules react with D-Luciferin in the presence of the Luciferase enzyme. The emitted light is proportional to the number of ATP molecules. ATP plays a crucial role in the cellular energy exchange processes, it acts as a donor of energy. It is present in living cells that are metabolically active and all living cells need ATP to stay alive. Therefore ATP levels are

proportional to the metabolically active cells and could be used as a tool for functional integrity of the cell (Kangas et al., 1984, Crouch et al., 1993, Cree and Andreotti, 1997). Cell lysis solution is used to release the intracellular ATP, this solution also contains reagents that irreversibly block endogenous ATPases to prevent the degradation of ATP molecules (Kangas et al., 1984, Cree and Andreotti, 1997).

The ATPLite assay kit (PerkinElmer, Massachusetts, USA) was used to assess cell proliferation. The Supplier's instructions were followed to perform the assay. LNCaP, PC-3 or DU145 cells were prepared in stock suspensions of 750 cells/50 μ l. Cells were seeded in 96 well PerkinElmer viewplates at a density of 750 cells per well. Plates were transferred to the incubator and 24 h post plating received drug treatments at final concentrations of 0, 5, 10 or 20 μ M. Treatments were prepared in triplicate wells. This assay was used to test the following compounds: tricin, apigenin, PMF and compounds 1-12, at 72 and 144 h after treatment. The luminescence generated by cells was measured using a Fluostar Optima luminescence plate reader (BMG LabTECH, Aylesbury, UK.). The average intensity of luminescence of each treatment was expressed as a percentage of vehicle control. Each experiment was repeated in triplicate using a different cell passage, to determine the means \pm SD.

2.3.6 Cell cycle assessment

The theory of cell cycle analysis using flow cytometry is based on staining the nuclei of intact cells with a fluorescent dye. The intensity of staining is proportional to the amount of DNA. Upon excitation in a flow cytometer using UV light or laser beams, the emitted fluorescent signal reflects the cell's DNA content. Cells in the G₂/M phase have double the content of cells that are in G₀-G₁ phase, while cells in S phase mainly contain DNA amounts somewhere in between. Using mathematical models and computer software the percentage of cells in each phase can be calculated. As the content of DNA in G₂ and M phase are similar, it is not possible to obtain separate values for each phase so these are expressed as being in G₂/M phase. (Darzynkiewicz and Juan, 2001, Nunez, 2001).

Cells were seeded in 90 mm Petri dishes, at densities between 2×10^5 (TRAMP C2) and 5×10^5 (LNCaP and PC-3) cells per dish in 6 ml media. Cells were incubated for 24 h, except LNCaP cells which were left for 48 h to adhere. Media was aspirated and replaced with 6 ml media containing TMFol treatment (0, 1, 5, 10 or 20 μM). Cells were incubated for 48 or 72 h. Media was aspirated, washed twice with PBS, and cells were then detached with 1 ml 1X trypsin. After detachment of cells, 1 ml of media was added and the content was transferred into a centrifuge tube. Cells were centrifuged at 1500 rpm for 5 min. Cell pellets were resuspended and washed in PBS. The suspension was centrifuged and the supernatant discarded. The resulting pellet was suspended in 2 ml of ice cold 70% v/v ethanol. Samples were stored at 4-8 °C for not longer than one week prior to FACs analysis. Prior to analysis samples were centrifuged for 10 min at 1500 rpm, and the supernatant discarded. The pellet was suspended in 800 μl PBS, 100 μl Rnase A (1mg/ml) and 100 μl propidium iodide (PI, 50 $\mu\text{g}/\text{ml}$). The suspensions were incubated in the fridge overnight. Samples were analysed for DNA content using BD FACScan Flow cytometer (BD Biosciences, California, USA) and cell cycle profiles were generated using ModFit software.

2.3.7 Annexin V apoptosis assay

When cells undergo apoptosis a number of morphological and biochemical changes occur. During cell apoptosis the translocation of phosphatidyl serine (PS) takes place. PS in healthy cells is located on the cytosolic side of cell membranes, but it is externalized in apoptotic cells. Annexin V in the presence of calcium can bind to PS. This important phenomenon is employed to differentiate between apoptotic and living cells (Liu et al., 2009). Apoptosis can be detected quantitatively by flow cytometry after incubation of cells with Annexin V labelled with fluorescein isothiocyanate (FITC). Although PS is externalised in both apoptotic and necrotic cell, the two can be differentiated as the latter loses cell membrane integrity and as a result the cell's DNA and contents becomes exposed, so upon addition of PI it binds immediately to the necrotic cell's DNA. Apoptotic cells are FITC⁺/PI⁻, necrotic cells are FITC⁺/PI⁺, and live cells are FITC⁻/PI⁻ (Vermes et al., 1995).

Cells were seeded in 6 well plates, at densities of 2×10^4 cells/well for TRAMP C2 and 5×10^4 cells/well for LNCaP and PC-3 in 4 ml media. Cells were incubated for 24 h, except for LNCaP cells which were incubated for 48 h in order to adhere. Media was replaced with fresh media containing TMFol (0 - 20 μ M). Plates were incubated for 72 or 96 h. Before harvesting the cells the media containing the floating cells was collected and retained. The adherent cells were washed twice with warm PBS before 0.5 ml of 1X trypsin EDTA was added. Cells were left in the incubator to detach. Then 0.5 ml of fresh media was added immediately, and these cells were added to the floating cells collected earlier. The cell suspension was centrifuged for 5 min at 1500 rpm and the resulting cell pellet was resuspended in 10 ml fresh media and incubated at 37 °C for 20 min. Cell suspensions were centrifuged and pellets resuspended in 1 ml of 1X annexin binding buffer and 10 μ l of annexin V-FITC conjugate (BenderMedSystems, Vienna, Austria) and left for 10 min on ice. Then 10 μ l (20 μ g/ml) PI was added one min before performing flow cytometry analysis using BD FACsAria (BD Biosciences, California, USA). Data was expressed as percentage of cells that were either apoptotic, necrotic or live using FACsDiva Software.

2.3.8 Protein analysis and western blot

2.3.8.1 Preparation of the whole cell lysate and tissue lysate

Cells were seeded into 75 cm² flasks at a density of 8×10^5 for LNCaP, 6×10^5 for PC-3 and 4×10^5 for TRAMP C2, in 10 ml media. Cells were incubated for 24 h, except LNCaP cells which were left for 48 h to adhere. Cells were treated with TMFol, quercetin or fisetin. Concentrations were 0, 1, 5, 10 and 20 μ M for TMFol and 0, 20, 40 and 60 μ M for quercetin and fisetin. Vehicle control flasks received DMSO alone. The amount of DMSO was equal in all controls and treatments. Cells were incubated for 24-72 h before harvesting.

To harvest cells, media was aspirated and cells were washed twice with warm PBS, before 1.5 ml of 1X trypsin EDTA was added to each flask to detach cells. After cells had detached 3.5 ml media was added immediately to each flask to neutralize trypsin, a 100 μ l aliquot of the suspension was added to 9.9 ml isoton

and counted using a coulter counter. Cells were then centrifuged at 1500 rpm for 3 min. The supernatant was discarded and the resulting pellets were washed twice with 1 ml PBS, with each wash being followed by centrifugation at 13000 rpm for 3 min. The resulting pellets were stored at -80°C before cell lysates were prepared.

Cells were lysed on ice by adding 35 μl lysis buffer per million cells, fortified with Phos.Stop inhibitor cocktail (Complete Lysis-M buffer, Roche, Diagnostics, Mannheim, Germany). Cells were mixed vigorously by pipetting up and down, before tubes were transferred to a rotary mixer at 4°C for 30 min. Cells were vortexed briefly, then centrifuged at 13000 rpm for 20 min at 4°C . The supernatants were transferred to clean Eppendorf tubes and stored at -80°C .

Tumour tissues for Western blot were weighed and then cut into small pieces. One part tissue was added to 4 volumes lysis buffer (w:v) and homogenised using a Ystral X 10/20 homogeniser (Ballrechten-Dottingen, Germany). Homogenates were transferred to a rotary mixer for 30 min at 4°C , followed by brief vortexing, and then centrifuged at 13000 rpm for 20 min at 4°C . Resulting supernatants were transferred to fresh tubes and kept at -80°C .

2.3.8.2 Bradford protein assay

A protein assay was undertaken for all cell and tissue lysates to determine protein concentration, thus allowing equal protein amounts for each sample to be loaded onto protein gels. Protein concentrations in each sample were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, California, USA). A sample of 1 μl of cell or tissue lysate was added to 999 μl distilled water and vortexed briefly then 200 μl of the solution was replaced with 200 μl of Bio-Rad protein assay reagent (Bio-Rad Laboratories, California, USA). The resulting mixture was vortexed and kept at RT for 5 min. The absorption of each sample was measured at 595 nm using a UV spectrophotometer (Gene Quant Pro Spectrophotometer, Amersham Biosciences, UK). Protein concentrations were calculated using a pre-prepared standard curve, which had been prepared using known protein standards (0-20

µg/ml) of bovine serum albumin (BSA) in water. Concentrations were plotted against absorbance, and this standard curve was used to calculate protein concentration from cell or tissue lysates.

2.3.8.3 SDS-PAGE and western blotting

Cell or tissue lysates were loaded onto (8 - 15% w/v) PAGE gels in protein amounts of (30 - 80 µg/well). The percentage of acrylamide in gel was chosen depending on the size of the protein of interest. Samples were mixed with an equal volume of 2X sample Laemmli buffer (Sigma Aldrich, Poole, UK), and boiled at 100 °C for 5 min. The lysates were loaded onto the gel, which included one lane of a coloured protein size marker. Gels were resolved in running buffer and proteins were separated by electrophoresis at 100 V. Proteins were transferred onto nitrocellulose membrane of pore size 0.2 µM (Whatman, Dassel, Germany) for either 2 h at 100 V or overnight at 30 V at 4 °C. Membranes were then blocked in 5% w/v Marvel non fat milk in 0.05% v/v Tweens-PBS (T-PBS) for 2 h. Membranes were then incubated with a primary antibody against the protein of interest, either for 2 h at RT or overnight at 4 °C on a rocking platform. All antibodies were diluted in 5% w/v non fat milk in T-PBS; for antibody dilutions and conditions refer to Table 2.2. After washing (5x5 mins in T-PBS) membranes were incubated with a corresponding secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at RT. Membranes were washed (5x5 mins in T-PBS) to remove any excess antibody then incubated in ECL solution (Biological Industries, Isreal) for 1 min, before exposing to ECL hyperfilm (Amersham Biosciences, Buckinghamshire, UK) for 0.5-20 min, depending on the antibody. Films were developed using an Agfa Curix 60 developer (AGFA Gevaert N.V, Germany). To ensure the samples were equally loaded, membranes were reprobed for the expression of β-actin. Protein bands on ECL hyperfilm were captured using an imaging device (Gene Genius Bio Imaging system, Syngene, USA) with Gene Snap software installed. Band densities were measured using Gene Tool software from Syngene (Version, 3.03.030). Protein band densities on the same membrane were normalised to β-actin.

Table 2.2 Antibody conditions and dilutions

Target protein	Primary antibody dilution	Secondary antibody	Secondary antibody dilution
Androgen receptor	1:1000	Goat anti-rabbit-HRP	1:2000
Bax	1:1000	Goat anti-rabbit-HRP	1:2000
cdc2	1:1000	Goat anti-rabbit-HRP	1:2000
Cyclin B1	1:500	Goat anti-rabbit-HRP	1:2000
p21	1:1000	Goat anti-rabbit-HRP	1:2000
p27	1:1000	Goat anti-rabbit-HRP	1:2000
p53	1:1000	Goat anti-mouse-HRP	1:2000
PARP-1	1:500	Goat anti-mouse-HRP	1:2000
Phospho-cdc2	1:1000	Goat anti-rabbit-HRP	1:2000
PSA	1:2000	Goat anti-rabbit-HRP	1:2000
Survivin	1:2000	Goat anti-rabbit-HRP	1:2000
β -Actin	1:10000	Donkey anti-goat-HRP	1:2000

2.3.9 Intracellular and extracellular PSA levels in LNCaP cells

2.3.9.1 Intracellular PSA content

Intracellular levels of PSA were determined using western blotting. LNCaP cells were seeded at a density of 1 million cells/75 cm² flask in 10 ml RPMI 1640 phenol red free media. Cells were incubated for 48 h, after which media was replaced with 10 ml media containing TMFol (0 - 20 μ M) or media containing 1 nM mibolerone and TMFol (0 - 20 μ M). All flasks received equal volumes of vehicle solvent: DMSO for TMFol and ethanol for mibolerone. After 48 or 72 h aliquots of media (1ml) were removed and stored at -80°C. Cells were harvested, counted and lysates derived from the cells were analysed by western blotting as described in section 2.3.8.

2.3.9.2 Media PSA immunoassay

The analysis of PSA in media was carried out using a human KLK3/PSA Immunoassay kit (R&D Systems, Abingdon, UK), which employs a quantitative sandwich enzyme immunoassay technique. The assay was performed according to the manufacturer's instructions. The optical density of samples and standard solutions was measured at 450 nm using a Fluostar Optim luminescence plate reader (BMG LabTECH Ltd, Aylesbury, UK). PSA standards were prepared in duplicate, and their averages used to plot a standard curve, which was used to calculate the concentration of PSA in media. Values for treatment samples were calculated and expressed as a percentage of vehicle control.

2.3.10 Detection of apoptosis proteins using human apoptosis array kit.

A human apoptosis array kit (R&D systems, Abingdon, UK) was used to detect the expression of 35 apoptosis regulatory proteins, from the same sample lysate on one nitrocellulose membrane. The assay was performed according to the manufacturer's instructions. Briefly, cell lysates had been prepared from LNCaP cells treated with either vehicle or TMFol (10 or 20 μ M). Cells were lysed as advised by the manufacture using the supplied lysis buffer. Protein content was determined using the Bio-Rad protein assay as described earlier (section 2.3.8). An aliquot (500 μ g) of each cell lysate were incubated with each single membrane overnight. After washing off any unbound proteins, the membrane was incubated in a cocktail of biotinylated detection antibodies. Membranes were washed again before membranes were incubated with a streptavidin-HRP reagent. Membranes were then washed once more before chemiluminescent detection reagents were added and the membranes developed onto hyperfilm.

Spot densities were measured and normalised to the intensity of the internal controls on each membrane. Treatments were then compared and expressed as a percentage of the vehicle control.

2.3.11 Androgen receptor stability

Cycloheximide (Calbiochem, California, USA), a translation inhibitor was used to inhibit the synthesis of the AR in LNCaP cells and determine its stability following TMFol treatment. LNCaP cells were seeded in 75 cm² flasks at 8x10⁵ per flask and incubated for 48 h in order to adhere to the flasks. Flasks were pre-incubated for 30 min with 40 µM cycloheximide, before being treated with either vehicle solvent, 10 or 20 µM TMFol for 0, 2, 4, 6, 8, 16, 24, 48 and 72 h. Cells were then lysed and analysed for AR expression using western blot analysis (section 2.3.8). The band density for each treatment and time point was expressed as a percentage to the vehicle at time 0. Data was expressed as the mean ± SD of three independent experiments and was plotted as percentage of the time 0 control against time.

2.3.12 Reverse transcription-polymerase chain reaction (RT-PCR) to determine mRNA levels of androgen receptor, PSA and GAPDH.

LNCaP cells were seeded and treated with TMFol (0 - 20 µM). Cells were harvested at 48 and 72 h. Cell pellets were kept frozen at -80 °C until RNA extraction was performed.

RNA extraction on all samples was carried out using a Qiagen RNeasy kit (QIAGEN, Hilden, Germany). The extraction procedure was performed according to the manufacturer's protocol. RNA content of each sample was quantified using a Nanodrop ND-1000 spectrophotometer (Lab-Tech, Ringmer, UK) at 260 nm. Purity of the RNA was checked using the 260/280 nm (A_{260}/A_{280}) ratio. All the extracted RNA samples had ratios greater than 2.0. It is widely accepted that RNA samples with ratios higher than 1.8 are acceptable (Wilfinger et al., 1997). Samples were stored at -80 °C until use.

The reverse transcription reaction was performed using 1 µg of RNA from each sample using the QuantiTect reverse transcription kit (QIAGEN, Hilden, Germany). RNA reverse transcription was carried out according to the manufacturer's protocol. The produced cDNA was stored at -20 °C until use.

The PCR reaction was performed using a GeneAmp PCR System 9700 (AB Applied Biosystems, California, USA). Amplification of cDNA was performed using 5 µl of each sample, in the presence of 36 µl of RNA-DNA free water, 5 µl of 10X Vibuffer S, 1 µl of 10mM dNTP (Vivantis, California, USA), 1 µl of 100 pmol/µl of each of the forward and the reverse primers. Then 1 µl of Taq DNA (Vivantis, California, USA) polymerase enzyme was added just prior to running the reaction.

Primer sequences and PCR conditions were as follows: for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (230 bp); 5'-TCA AGA AGG TGG TGA AGC AG-3' (forward) and 5'-CTT ACT CCT TGG AGG CCA TG-3' (reverse) (95 °C 30 s, 57 °C 1 min, 72 °C 1 min, 18 cycles); For AR (590 bp) 5'-ATG GAA GTG CAG TTA GGG-3' (forward) and 5'-CAG GAT GTC TTT AAG GTC AGC-3' (reverse) (95 °C 30 s, 57 °C 1 min, 72 °C 1.5 min, 32 cycles); for PSA (710 bp) 5'-GAT GAC TCC AGC CAC GAC CT-3' (forward) and 5'-CAC AGA CAC CCC ATC CTA TC-3' (reverse), 95 °C 30 s, 57 °C 1 min, 72 °C 1.5 min, 22 cycles) (Wang et al., 2008a, Hsieh et al., 2002). All the primers were purchased from Sigma Aldrich (Poole, UK).

2.3.13 Agarose gel electrophoreses

Agarose gel electrophoresis was used to separate the generated cDNA-PCR product. A double comb 15 or 20 well agarose gel was prepared as described in section 2.2. PCR product samples were loaded on the gel after mixing 12 µl of sample with 1.2 µl 10X loading buffer. All samples were loaded onto the same gel along with 5 µl of DNA size marker in order to determine correct PCR product band size. The gel was then run at 100 V for 1.5 h using 1X TBE as running buffer.

DNA bands were visualized and captured using an imaging device (Gene Genius Bio Imaging system, Syngene, USA) installed with Gene Snap Software. Band densities were measured using Gene Tool software from Syngene (version, 3.03.030). DNA band densities were then normalised to GAPDH for equal loading.

2.3.14 Animal experiment for pharmacokinetics

2.3.14.1 Single oral dose study

All animal studies were performed under the animal project licence PPL 80/2167, awarded to the University of Leicester by the UK Home Office. Protocols of all animal studies were reviewed and approved by the University of Leicester Ethical Committee for Animal Experimentation. Animal house facility conditions were as follows: 20-30 °C temperature, 40% - 60% humidity and 12 hours dark/light cycle. Animals were kept at least one week after purchase for acclimatization. Male C57BL/6J mice were used for the TMFol pharmacokinetic study and were purchased from Charles River (Margate, UK).

For the single dose TMFol study, animals were randomly divided before the experiment into 7 groups each containing 4 mice. Animals received a single intra-gastric dose of 240 mg/kg TMFol suspended in 0.5% w/v methylcellulose at 24 mg/ml. Animals were sacrificed (4 per group) at 5, 20, 40, 60, 120, 360 and 1440 min after dosing. Animals were culled under general anaesthesia (halothane), blood was collected directly by cardiac puncture into lithium heparin tubes. The following tissues, heart, liver, kidney, prostate, lung and small intestine mucosa were harvested and immediately stored in liquid nitrogen and then transferred to –80 °C until analysis.

2.3.14.2 Long term dietary feeding

For chronic TMFol intervention in the diet, 10 animals were randomly divided into control or treatment groups. AIN93G diet was administered to control animals while treated mice received AIN93G diet containing 0.2% (w/w) TMFol. After 3 weeks all the animals were culled.

Animals were culled under general anaesthesia (halothane), blood was collected directly by cardiac puncture into lithium heparin tubes. Heart, liver, kidney, prostate and lung tissue as well as small intestine mucosa were harvested and immediately stored in liquid nitrogen and then transferred to –80 °C until analysis.

2.3.14.3 Sample preparation

Blood samples were collected by cardiac puncture and stored in heparin tubes on ice until transportation to the laboratory where they were centrifuged at 3000 rpm for 20 min. The plasma was collected and stored at - 80 °C.

Tissue samples were mixed with PBS (1:2 w/v), then homogenised using a Ystral X 10/20 homogeniser (Ballrechten-Dottingen, Germany). Tissue homogenate was stored at -80 °C until analysis.

2.3.14.4 Sample extraction

Frozen samples of plasma and tissues were extracted using a validated method for TMFol (Britton et al., 2009) Samples were thawed to room temperature. Plasma aliquots of (100 µl) were mixed with PBS (700 µl) and 2% w/v formic acid (200 µl). Samples were loaded onto a Waters Oasis HLB Extraction Cartridge (Waters Corporation, Milford, USA), which were equilibrated and pre-conditioned with methanol and water (1 ml each). The cartridge was subsequently washed with 1ml each of 5% v/v MeOH in 2 % v/v CH₃COOH, 5% v/v MeOH in 2% w/vNH₄OH and 5% v/v MeOH in 0.1 M NH₄⁺CH₃COO⁻ buffer (pH 5.1). The analyte was eluted with 1 ml of acetone containing 0.1M CH₃COOH. The eluate was evaporated to dryness (under nitrogen) and reconstituted in 100 µl of mobile phase. An aliquot (5 µl) of the supernatant was injected onto the HPLC column as described in section 2.3.15. In the case of the tissue samples and cells, 1 part tissue was placed into 2 volumes PBS (w:v) and homogenized (Ystral X 10/20 homogeniser, Ballrechten-Dottingen, Germany). An aliquot of the homogenate (100 µl) or cell pellet was mixed with two volumes of acetone containing 0.1M CH₃COOH, to precipitate protein and extract the analyte. After vortexing (2 x 60 secs) and centrifugation (13,000 rpm, 20 min) the supernatant was evaporated to dryness (under nitrogen) and reconstituted in 100 µl of mobile phase. An aliquot (5 µl) of the supernatant was injected onto the HPLC column (section 2.3.15) In case of media an aliquot of (100 µl) was mixed with one volume of acetone containing 0.1M CH₃COOH, after vortexing (2x60 secs) and centrifugation (13,000 rpm, 20 min). An aliquot (5 µl) of the supernatant was injected onto the HPLC column. TMFol

concentrations in the calibration standards ranged from 50 to 2,500 ng/ml for plasma and 0.05 to 40 µg/ml for tissue and cell samples. In the case of murine plasma samples TMFol levels detected were at or below the limit of quantification, therefore, 50 µl of sample rather than 5 µl was injected onto the column (Britton et al., 2009).

2.3.14.5 Calibration curves

Calibration curves of TMFol concentrations were prepared spanning concentrations of analyte anticipated in mouse plasma, tissues and cells following either single oral dose or dietary administration of 0.2% w/w TMFol. Calibration curves were linear within the range investigated (50 – 2500 ng/ml for plasma and 0.05 – 40 µg/ml for tissues and cells). The correlation coefficients (r) were > 0.99 for all tissue and plasma calibration curves. A standard curve for un-extracted TMFol was also performed in mobile phase. Extraction efficiencies could therefore be calculated for plasma and each tissue.

2.3.15 Chromatographic conditions

HPLC analysis was performed using a method developed and validated for the determination of TMFol in murine plasma and tissues (Britton et al., 2009). The HPLC instrumentation consisted of a Varian Prostar HPLC unit (Varian, UK), including a 410 Varian autosampler, a Varian ProStar 230 solvent delivery system and a Pro-Star 363 fluorescence detector. A Gemini C₁₈ column (4.6 mm x 150 mm, 3 µm, Phenomenex, UK) was used with an isocratic mobile phase, run at flow rate of 0.75 ml/min with 69% v/v methanol in 0.1 M NH₄⁺CH₃COO⁻ buffer (pH 5.1) for analysis of the parent compound in plasma and tissues. A gradient mobile phase was used for studying the metabolites in plasma and tissues and also for determination of intercellular TMFol content, which consisted of A; aqueous ammonium acetate (pH 5.1, 0.1 M) and B; methanol. HPLC conditions were: methanol percentage 50% B at the start, increasing to 65% B at 18 min, 70% B at 25 min, and 90% B at 28 min, followed by a decrease to 50% at 35 min.

2.3.16 *In vitro* generation of TMFol metabolites

Generation of TMFol metabolites *in vitro* was performed using mouse liver S9 fractions (Wu and McKown, 2004, Nielsen et al., 1998). Mixed gender mouse liver S9 (microsomal and cytosolic) fractions were obtained from an in-house stock, which had been previously prepared and stored at -80 °C.

2.3.16.1 Demethylation reactions

For demethylation reactions TMFol (80µM) as a substrate was pre incubated at 37 °C with the S9 fraction (microsomal) (2mg/ml) for 5 min. The reaction was initiated by adding 25 µl NADPH regenerating system solution A (NADP⁺ and glucose-6-phosphate) and 5 µl NADPH regenerating system solution B (glucose-6-phosphate dehydrogenase) (BD Biosciences, San Jose, USA), distilled water was added to make final volume of 500 µl. All the samples were incubated at 37 °C for 2 h and stopped by adding 0.4 ml ice cold methanol, then centrifuged at 13,000 rpm for 10 min. Aliquots of 5 µl of the resulted supernatants were injected onto the HPLC column for HPLC analysis.

2.3.16.2 Glucuronidation reactions

Glucuronidation of TMFol was performed with and without prior demethylation. TMFol (80 µM) was pre incubated at 37 °C with S9 fraction (microsomal) (2mg/ml) for 5 min. The reaction was started with the addition of 40 µl UDPGA cofactors A (BD Biosciences, San Jose, USA) which consisted of 25 mM uridine-5-diphospho-glucuronic acid. and 100 µl of UDPGA cofactors B (BD Biosciences, San Jose, USA) which consisted of:50 mM tris-HCl, 8 mM MgCl₂ and 25 µg alamethicin. For demethylation glucuronidation experiments a NADPH regeneration system was added to the mixture, in addition to the UDPGA reagents. All the samples were incubated at 37 °C for 2 h and stopped by adding 0.4 ml ice cold methanol. Samples were then centrifuged at 13,000 rpm for 10 min. Aliquots (5 µl) of the resulting supernatants were injected onto the HPLC column for analysis as described in section 2.3.15.

2.3.16.3 Sulphation reaction

For TMFol sulphation reactions, mouse S9 fractions (cytosolic) (2mg/ml) were incubated for 5 min at 37 °C with 3-phosphoadenosine-5'-phosphosulphate (PAPS) (200 µM) in the presence of 3 mM mercaptoethanol in 0.1M KH₂PO₄-K₂HPO₄ buffer (pH 7.4) The reaction was initiated by adding 80 µM TMFol. The reaction was performed with and without the demethylation reaction. 0.1 M Potassium phosphate buffer (KH₂PO₄-K₂HPO₄) was used to make up the reaction volume to 500 µl. All the samples were incubated at 37 °C for 2 h and stopped by adding 0.4 ml ice cold methanol. Samples were then centrifuged at 13,000 rpm for 10 min. Aliquots (5 µl) of the resulting supernatants were injected onto the HPLC column for analysis. As described in section 2.3.15.

2.3.17 Metabolite identification using Liquid Chromatography-Mass Spectrometry (LC-MS)

Mouse intestinal samples were extracted and used for the TMFol metabolite identification experiments. A mass spectrometer (Waters Quattro Ultima Platinum triple stage, Waters Ltd, Hertfordshire, UK) coupled to a Waters Alliance 2690 HPLC was used for analysis. The mobile phase consisted of two solvents: A, 0.1% v/v acetic acid and B, methanol, at a flow rate of 0.5 ml/min through a Gemini C₁₈ column (4.6 mm x 150 mm, 3 µm, Phenomenex, UK). A gradient method was used to separate the metabolites, and the conditions were as follows: at the start the mobile phase was 50% A and 50% B. Phase B was increased to 65% after 18 min, 70% after 25 min and then 90% by 28 min and finally at 35 min it was returned back to 50%. Samples of 50 µl were injected onto the column for each LC-MS run. Samples were subject to positive ion electrospray analysis and a scan over the range m/z 200-900 was performed over 35 min to detect any possible metabolites. The detected metabolites were individually analysed using extracted ion chromatography, so an ion chromatogram was generated for each metabolite. Mass spectra were acquired by subtracting the background from the summed spectra under the chromatographic peak of interest.

2.3.18 TMFol intercellular content

Cells were seeded into large flasks at a density of 5×10^6 cells in 25 ml phenol red free 1640 RPMI media. Cells were left to adhere for either 24 h (PC-3) or 48 h (LNCaP). One flask was treated with fresh media alone while the remaining flasks were treated with media containing 20 μ M TMFol. Flasks were incubated and cells harvested after 15, 30, 60, 90 and 480 min and, 24, 48 and 72 h, respectively. Cells were counted when harvested in order to calculate the concentration of TMFol per million cells. Cell pellets were resuspended and centrifuged three times in 1 ml PBS in order to remove any extracellular TMFol. Each pellet was spiked with 1 μ l internal standard, 2 mM, 2',5',5,6,7,8-Hexamethoxyflavone. Intracellular TMFol was extracted from the pellets by adding 200 μ l 0.1 M acetic acid in acetone. The mixture was vortexed for one min and centrifuged at 13000 rpm for 20 min. The supernatants were mixed (1:1) (v:v) with mobile phase and an aliquot of 30 μ l was injected on to a HPLC column and analysed using the method described in section 2.3.15.

2.3.19 Xenograft efficacy study

2.3.19.1 Animals and TMFol, quercetin and fisetin dosing

Male MF-1 outbred nude mice (30-40 g body weight) were supplied by Harlan UK. For housing conditions and licensing refer to section 2.3.14.1. The animals were grouped and identified by ear punching. For the TMFol study, 15 animals were designated as controls and 15 as treatment, for quercetin and fisetin, each group consisted of 5 animals (control, quercetin and fisetin). One week prior to tumour inoculation with 5×10^6 TRAMP C2 cells, animals were started on either control AIN93G diet or treatment consisting of AIN93G diet fortified with 0.2% w/w w/w TMFol, or equimolar concentrations of quercetin (0.184% w/w) or fisetin (0.174% w/w). The dose of TMFol was based on the previously undertaken pharmacokinetic study (Section 2.3.14).

2.3.19.2 Intervention in TRAMP C2 tumour bearing athymic (nude) mice

TRAMP C2 prostate cancer cells were suspended in a mixture of matrigel and serum free media (1:1) (v:v), at a concentration of 5×10^7 cells/ml. A 100 μ l

suspension volume was injected (s.c) into the right flank of each animal, under light halothane inhalation anaesthesia. Animal weight was assessed weekly and tumour volume was measured twice a week using digital callipers. Tumour volume was calculated from the equation: $volume = length \times width^2 / 2$, where length is the larger diameter and width is the smaller tumour diameter (Tomayko and Reynolds, 1989). Animals were culled after 7-8 weeks by which point tumour size had not exceeded 17 mm³ in length, which is the maximum limit of our U.K. project licence. Tumours were harvested and immediately divided into three portions: half the tumour was fixed in 10% w/v formalin and used for immunohistochemical analysis, the other two quarters were snap frozen in nitrogen then stored at -80 °C until used for HPLC and western blot analysis. Western blot analysis was performed as described in section 2.3.8 and HPLC analysis of tumour TMFol content was as described in section 2.3.14.

2.3.19.3 Immunohistochemical analysis of TRAMP C2 tumours

Tumours were prepared in the form of paraffin embedded sections (4 µM) on polysine coated slides in the Department of Pathology, University of Leicester. The slides were de-waxed by dry heat at 65 °C for 20 min. Tissue sections were then rehydrated by immersing in xylene, followed by a series of graded alcohol washes, and then finally water. Antigen retrieval was carried out by microwaving the sections at 1300 Watts for 20 min soaked in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by soaking the slides in 3% v/v H₂O₂ for 10 min. Non specific protein binding was blocked using a protein blocking reagent. Sections were incubated with an antibody against the protein of interest overnight at 4°C. Antibodies were diluted in a solution of 3% w/v bovine serum albumin and 1% v/v Triton X in PBS. Antibody conditions were as follows: Ki-67 rabbit polyclonal (1:800), cleaved-caspase-3 rabbit polyclonal (1:50) and AR rabbit polyclonal (1:700). After overnight incubation with the primary antibody, sections were washed thoroughly and incubated for 30 min with rabbit on rodent HRP-polymer detection reagent containing a mouse super background blocker reagent (A.Menarini, Berkshire, UK). Sections were then incubated with a chromogen labelling system diaminobenzidine (DAB)(Dako, Denmark) for 5 min. After washing, sections were counterstained with Mayer's

Haematoxylin (Vector, California, USA). Sections were then dehydrated back through graded alcohol and finally xylene. Slides were mounted using DPX mountant and visualized using a microscope (Leitz Orthoplan microscope, Leica DC 300 camera, magnification, X40, Leitz, Solms, Germany). For each slide 10 random microscope fields were captured. Slides were scored for Ki-67 and cleaved caspase-3 by counting all the positive cells and all the negative cells in each field for each slide. The mean (\pm SD) number of positively stained cells as a percentage of the overall total was then calculated. For the AR, scoring advice was taken from Dr. Peter Greaves (Expert pathologist). The scoring was performed by two independent observers, and staining of the slides was assessed visually using a scale of 0-4: 0-negative, 1-weak, 2-moderate, 3-strong and 4-very strong. The averages of controls and treatments were summed and expressed out of 4 as mean \pm SD.

2.3.20 Statistical analysis

Data were statistically assessed after undertaking the normality test. A one-way ANOVA test was undertaken to compare between groups, using Excel (Microsoft office 2007) and SPSS (v16.0) software, followed by *Post hoc* analysis (Bonferroni or Dunnett) to test the significance between vehicle control and treatments. If data were not normally distributed, non parametric statistical (Mann–Whitney) analysis was used instead. Student *t-test* was used when only one treatment group was compared to control group. Data were considered statistically significant if *p* values were < 0.05 .

CHAPTER 3

Effect of TMFol on prostate cancer cell growth- Investigation into the potential mechanism of action

3.1 Introduction

Uncontrolled cell growth is a major feature of cancer cells. Assessment of anti-proliferative activity in PCa cell lines is a widely used approach for testing the potential value of chemopreventive agents against prostate cancer (Gupta, 2004). Many flavonoids have shown anti-proliferative activity against prostate cancer cell lines (Kampa et al., 2007). Quercetin and fisetin are members of the flavonoid subgroup called flavonols, to which TMFol belongs, and they have been shown to possess antiproliferative activity against PCa cells (Aalinkeel et al., 2008, Khan et al., 2008a) and chemopreventive activity in PCa xenograft models (Khan et al., 2008b, Ma et al., 2004b). It has been suggested that the inclusion of methoxy moieties in the flavonoid structure instead of hydroxy groups could improve their bioavailability and growth inhibitory potency (Walle, 2007a). Cell cycle arrest, induction of apoptosis and inhibition of AR pathways are all important mechanisms by which flavonoids could exert chemopreventive activity in PCa (Singh and Agarwal, 2006). The AR is important in the development of normal prostate but also in PCa, it has also been implicated in hormone refractory prostate cancer (HRPC) where the AR and target genes are still active. Therefore, interference with the AR pathway, either by blocking AR activities, or by decreasing AR protein expression, may provide a means of HRPC, PCa prevention and management (Dehm and Tindall, 2007). The AR has also been shown to be involved in processes such as cell cycle arrest and the induction of apoptosis, two mechanisms that are considered important for chemopreventive intervention (Balk and Knudsen, 2008, Lin et al., 2006).

The work in this chapter investigates the anti proliferative activity of a series of hydroxylated and methylated flavonoids in androgen dependent (LNCaP) and androgen independent (PC-3 and DU145) PCa cell lines. The most potent agent, as determined by IC_{50} value was then explored further. The lead compound (TMFol – a methoxylated flavonol) was compared *vis-à-vis* quercetin and fisetin in most of the subsequent mechanistic studies, as these have been the most widely studied flavonols for prostate cancer to date.

3.2 Effect of hydroxylated and methylated flavonoids on cell proliferation in androgen dependent and androgen independent prostate cancer cells.

The anti proliferative activities of a panel of hydroxylated and methoxylated flavones were investigated in the androgen dependent (LNCaP), and androgen independent (PC-3 and DU145), PCa cell lines at 72 and 144 h time points. This screening included, apigenin (a hydroxylated flavone), tricetin (a hydroxylated-methoxylated flavone) and compounds 1-12 and PMF (methoxylated flavones), except compound 11 which is a flavonol (for chemical names and structures see Table 2. 1). The aim of the screening was to compare the activity of the methoxylated and the hydroxylated flavones in PCa cell lines. Based on observations in our laboratory that insertion of methoxy instead of hydroxyl groups into the flavone scaffold may improve chemopreventive activity in colorectal cancer models (Cai et al., 2009), we wished to explore whether such actions could be translated to other cancer types, most notably PCa.

Cells were grown in the presence of flavonoids (5, 10 or 20 μM) or DMSO (control) for 72 or 144 h after which they were counted. Cell numbers were expressed as a percentage relative to the vehicle control. Figures 3.1, 3.2 and 3.3 show the effects of the test agents on the growth of LNCaP, PC-3 and DU145 cells respectively.

As can be seen in Figure 3.1 (A) apigenin and compounds 2, 4, 6, 7, 9, 11 and 12 showed significant antiproliferative activity after 72 h in LNCaP cells at a concentration of 20 μM . Compounds 6 and 11 had significant growth inhibitory effects at all tested concentrations (5, 10, and 20 μM), although the activity of compound 6 was not dose dependent. The same trend was observed at the 144 h time point (Figure 3.1 B), with only compounds 11 and 12 exhibiting dose dependency. In the PC-3 cell line all compounds showed a dose dependent decrease in cell proliferation (Figure 3.2). The only exception to this was PMF where the effects at 10 and 20 μM were comparable. This can in part be explained by the compound precipitating out of solution at the highest concentration, as needle shape crystals were observed at the bottom of the

wells. However, such crystals had not been observed in the stock solution (20 μM) which was prepared in DMSO. These effects on growth were observed at both time points but greater inhibition was observed at 144 h (Figure 3.2 B). The effect of the compounds on the DU145 cell line with regard to proliferation was minimal, with only apigenin and TMFol reducing cell numbers below 50% of the control value (Figure 3.3). For both of these compounds, at both time points, these effects on growth were dose-dependent.

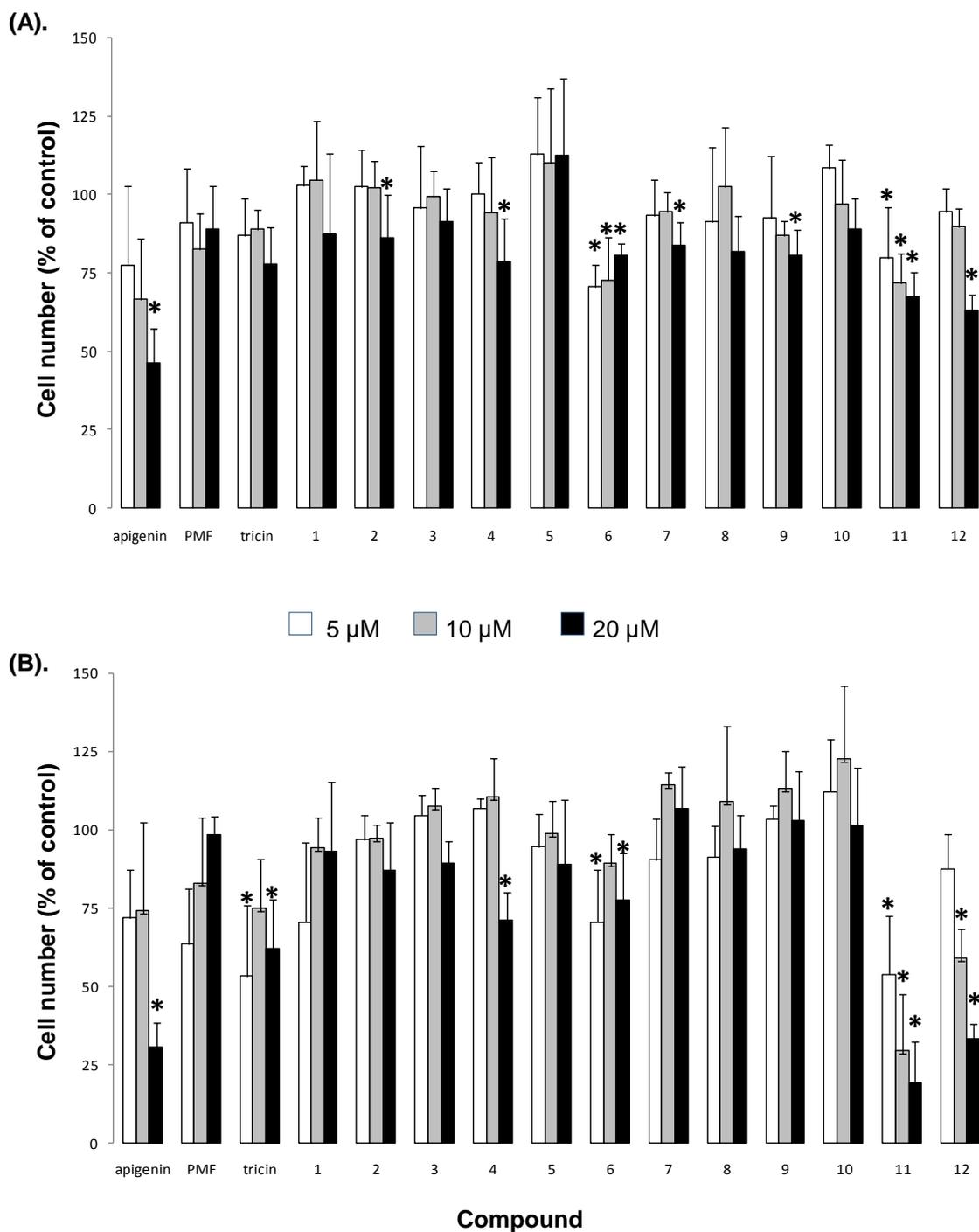


Figure 3.1 Effect of apigenin, tricin, PMF and compounds 1-12, (5, 10 and 20 μM) on the growth of LNCaP cells at 72 h (A) and 144 h (B). Growth assessment was performed as described in section 2.3.5. The values represent the percentage of cell number as compared to vehicle control and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

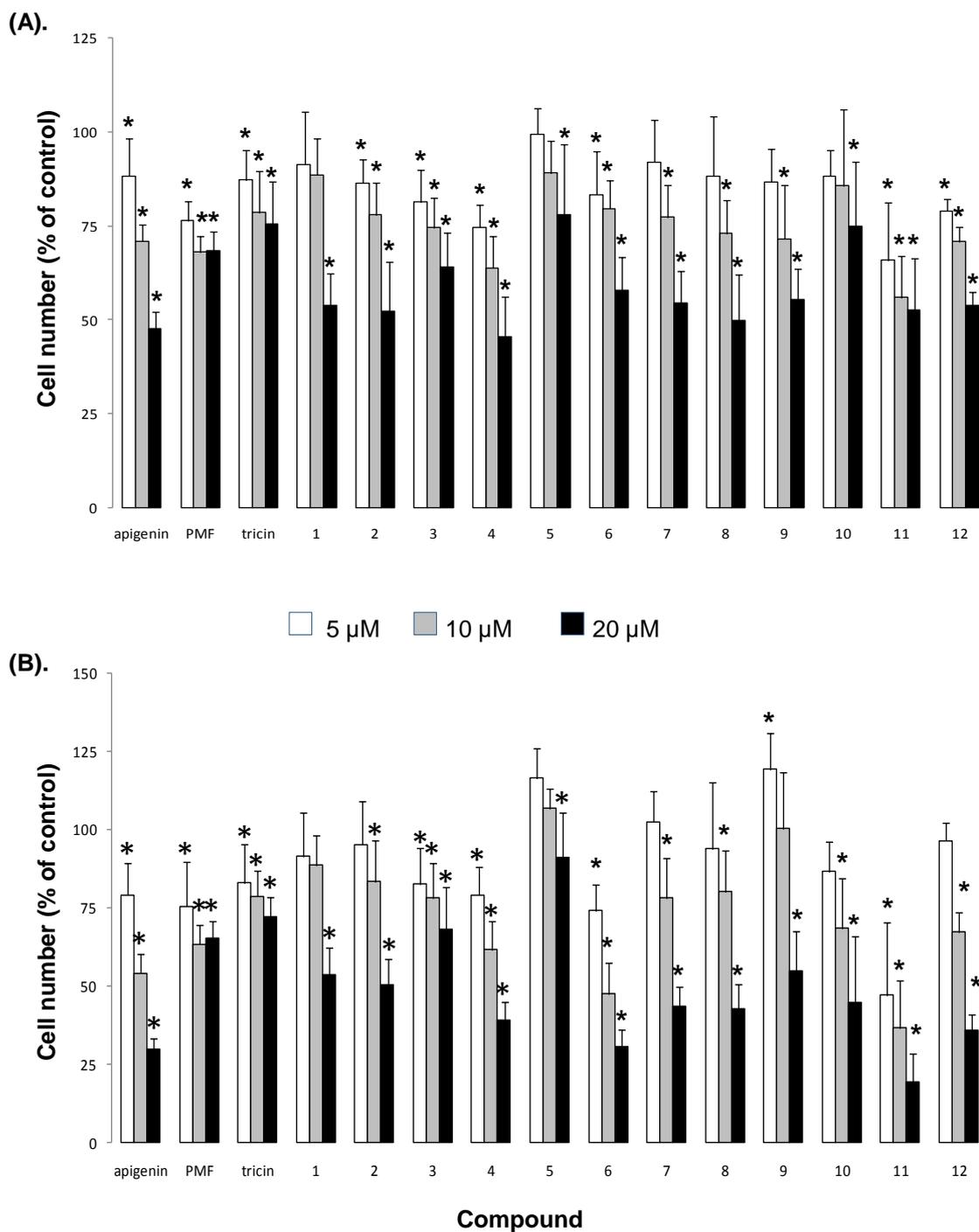


Figure 3.2 Effect of apigenin, tricin, PMF and compounds 1-12, (5, 10 and 20 μM) on the growth of PC-3 cells at 72 h (A) and 144 h (B). Growth assessment was performed as described in section 2.3.5. The values represent the percentage of cell number as compared to vehicle control and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

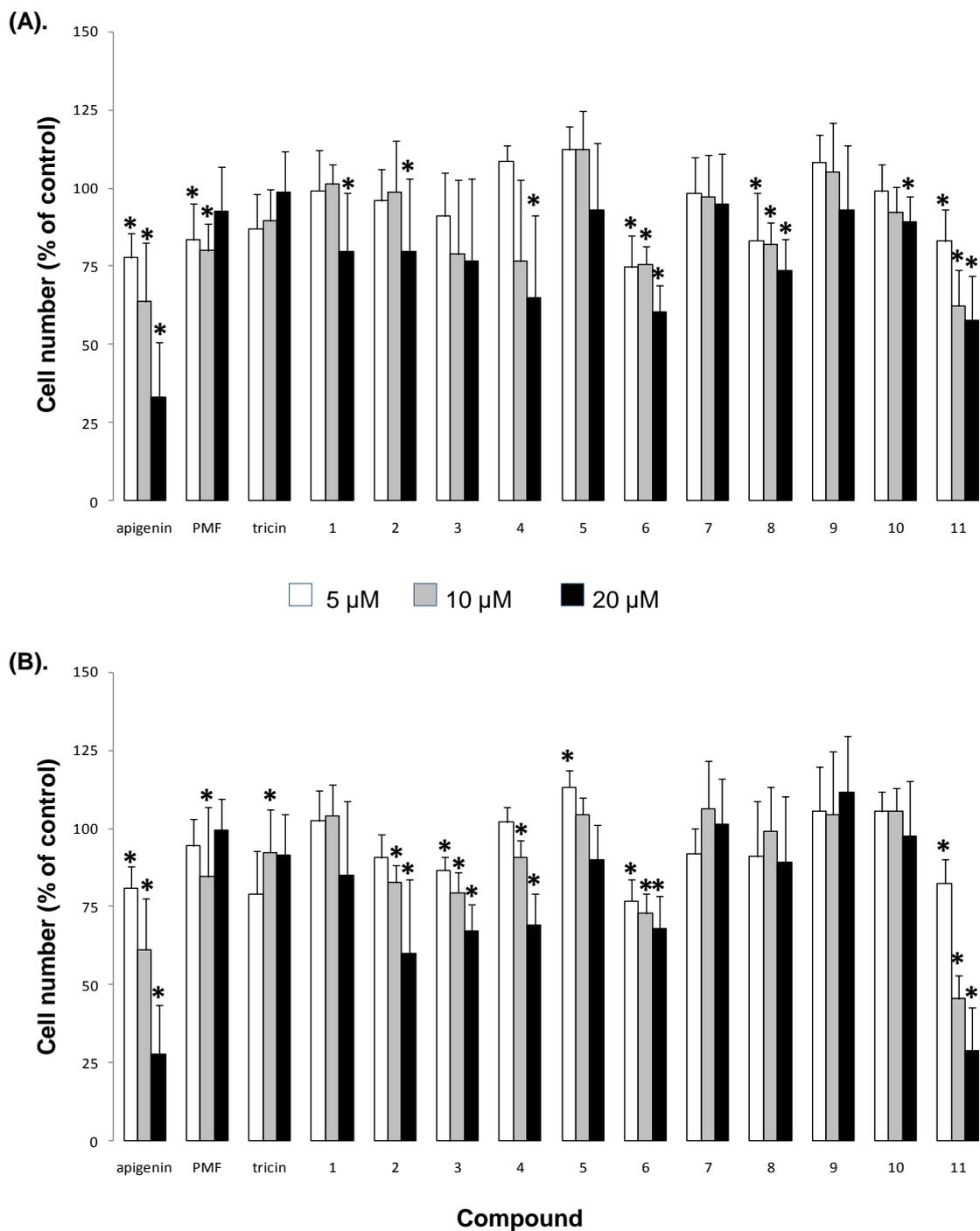


Figure 3.3 Effect of apigenin, triclin, PMF and compounds 1-11, (5, 10 and 20 μM) on the growth of DU145 cells at 72 h (A) and 144 h (B). Growth assessment was performed as described in section 2.3.5. The values represent the percentage of cell number as compared to vehicle control and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

3.3 Effects of TMFol, quercetin and fisetin on the proliferation of LNCaP, PC-3 and TRAMP C2 and the IC₅₀s at 72 and 144 h.

Following the preliminary drug screen compound 11 (3',4',5'-trimethoxyflavonol) or (TMFol) was identified as the most potent inhibitor of growth among the tested methoxylated compounds in the 3 cell lines. TMFol antiproliferative activity was comparable to apigenin with better dose-dependency effect than apigenin. The antiproliferative activity of TMFo has not been reported before. Therefore, it was selected as the lead compound for further investigation. More detailed growth inhibition studies were performed over a more extensive dose range so that accurate IC₅₀ values could be calculated. Since compound 11 is a flavonol but not a flavone so we had to compare it to members of the same class. Therefore, quercetin and fisetin were also included in these studies as these are the two most studied flavonols to date and were used for comparison purposes. DU145 was excluded in the next experiments as it resembles the late metastatic human PCa, as our focus was on the chemopreventive activity of TMFol. While TRAMP C2 was included in further TMFol testing as TMFol *in vivo* efficacy was to be tested in TRAMP C2 xenograft model. Therefore, the *in vivo* and *in vitro* TRAMP C2 data could be linked.

Figures 3.4, 3.5 and 3.6 show the effect of TMFol on the growth of LNCaP, PC-3 and TRAMP C2 PCa cells respectively, at 72 (A) and 144 h (B). Figures 3.7-3.10 show the growth inhibitory effect of quercetin and fisetin in the LNCaP and PC-3 cell lines respectively.

The results showed a dose-dependent growth inhibition for TMFol, quercetin and fisetin in all tested cell lines. TMFol showed higher potency than quercetin and fisetin in all three cell lines.

The same data represented as bar charts in Figures 3.4-3.10 were used to calculate the IC₅₀s at 72 and 144 h. Cell numbers were calculated as % vehicle control and plotted against drug concentration. The linear line equation was used to calculate IC₅₀ values for each agent. Data were represented here as bar

charts instead of the line graph of dose response curves because we wanted to clearly see the effect at lower doses.

For the LNCaP cell line at 72 h quercetin and fisetin have found to have IC_{50} values of 63.3 and 55.6 μM , whereas, TMFol had an IC_{50} value of 8.9 μM . For the 144 h time point the IC_{50} values were 5.6, 31.6, 39.7 μM for TMFol, quercetin and fisetin, respectively (Figures 3.4, 3.7 and 3.9).

In PC-3 cells, the IC_{50} values at 72 h were 9.4, 58.2 and 65.1 μM while at 144 h they were 4.1, 31.6 and 37.5 μM for TMFol, quercetin and fisetin respectively (Figures 3.5, 3.8 and 3.10).

For TRAMP C2 cells TMFol IC_{50} value was 1.7 μM at 144 hr (Figure 3.6) whereas quercetin and fisetin had values of 23.0 and 27.3 μM , respectively. All the IC_{50} values are summarized in Table 3.1.

Therefore, TMFol showed higher antiproliferative activity in the tested PCa cells with IC_{50} values 5-16 fold lower than that of quercetin and fisetin.

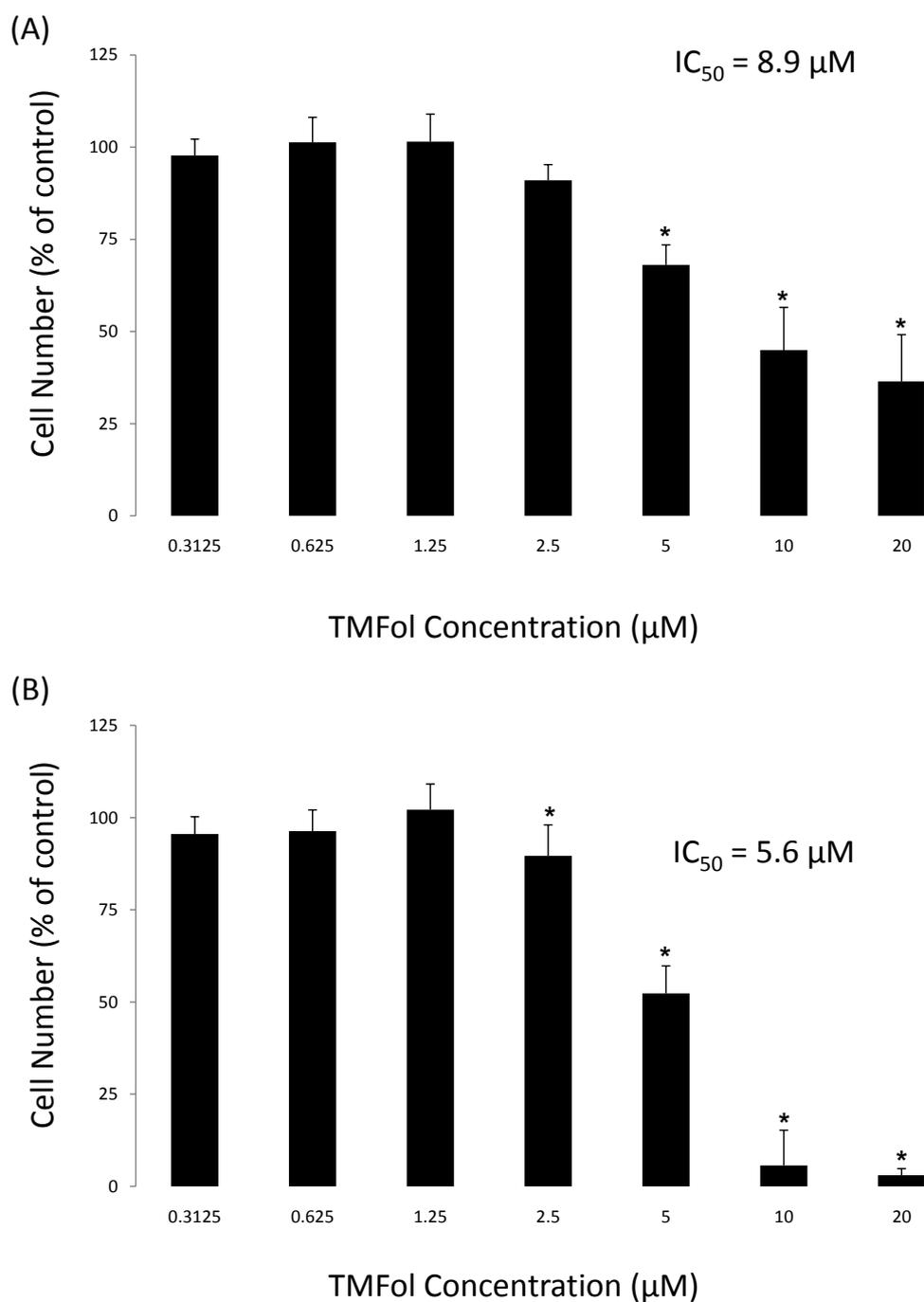


Figure 3.4. Growth inhibitory bar charts of TMFol (0-20 μM) in LNCaP cells at 72 (A) and 144 (B) h. Growth assessment and IC_{50} calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC_{50} . The values represent the percentage of cell number as compared to vehicle control expressed as the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

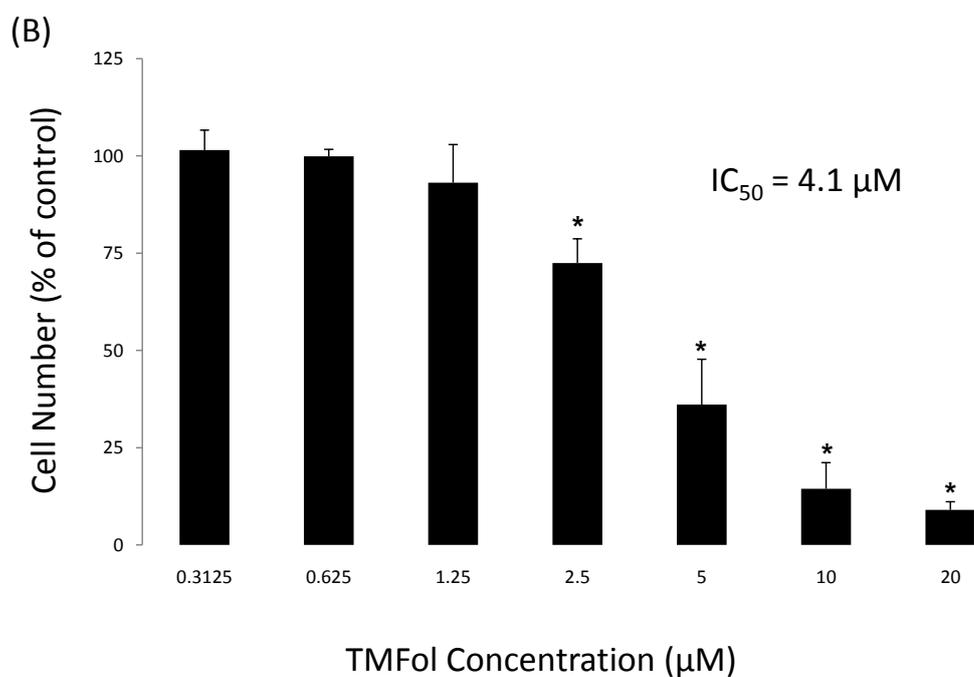
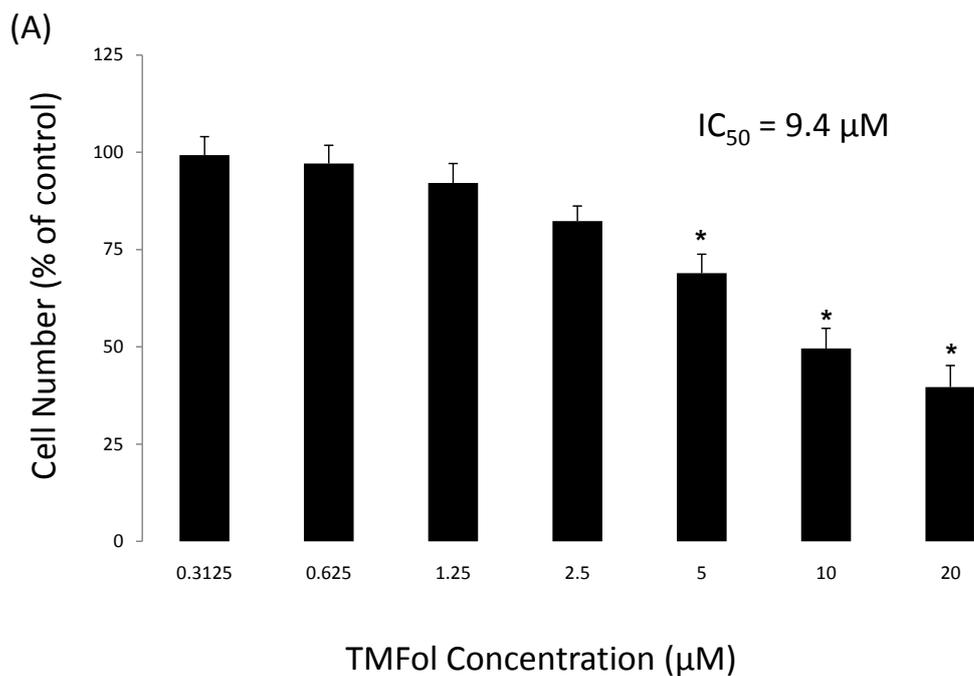


Figure 3.5 Growth inhibitory bar charts of TMFol (0-20 μM) in PC-3 cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

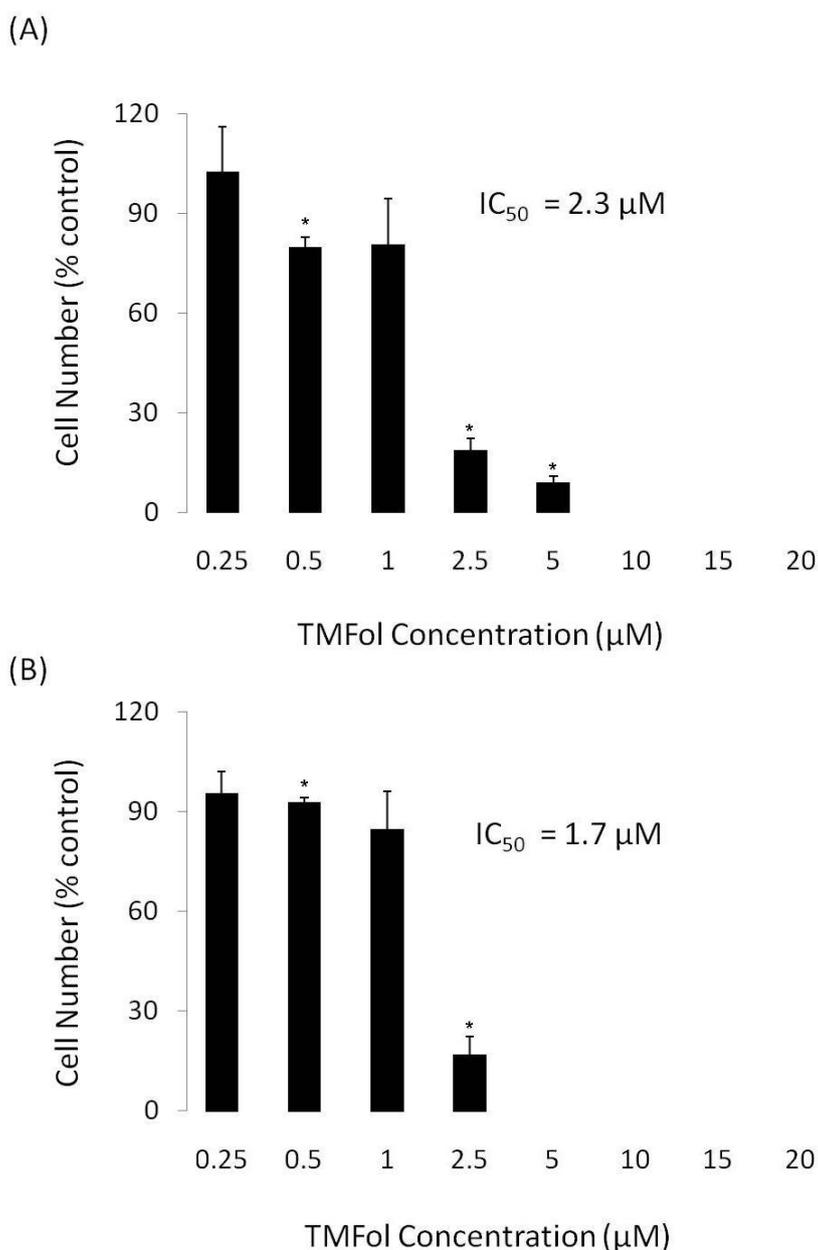


Figure 3.6 Growth inhibitory bar charts of TMFol (0-20 µM) in TRAMP C2 cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. At high doses the number of cells was very low, therefore they were not displayed in figures. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean ± SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

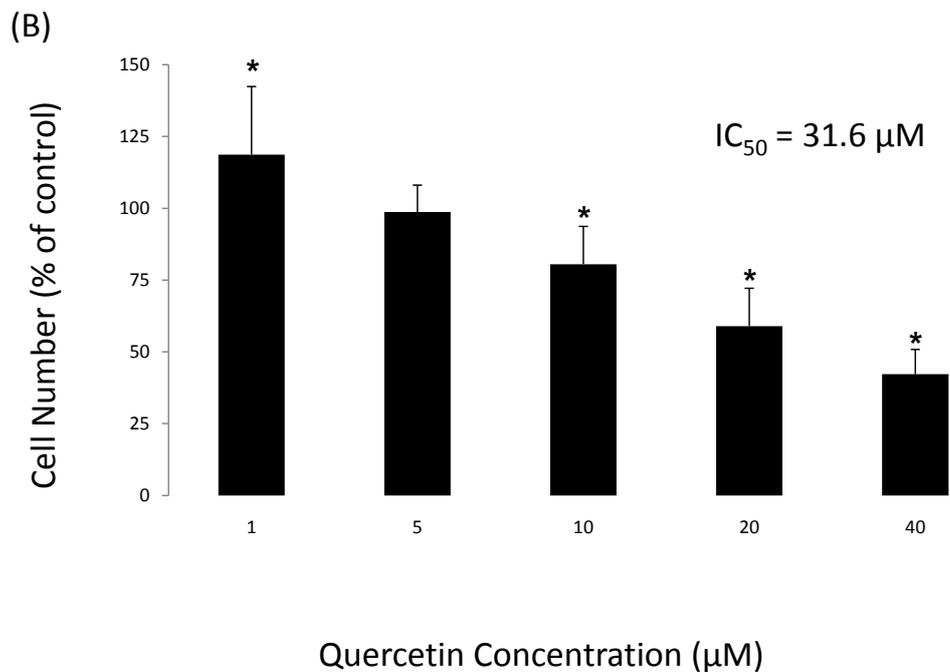
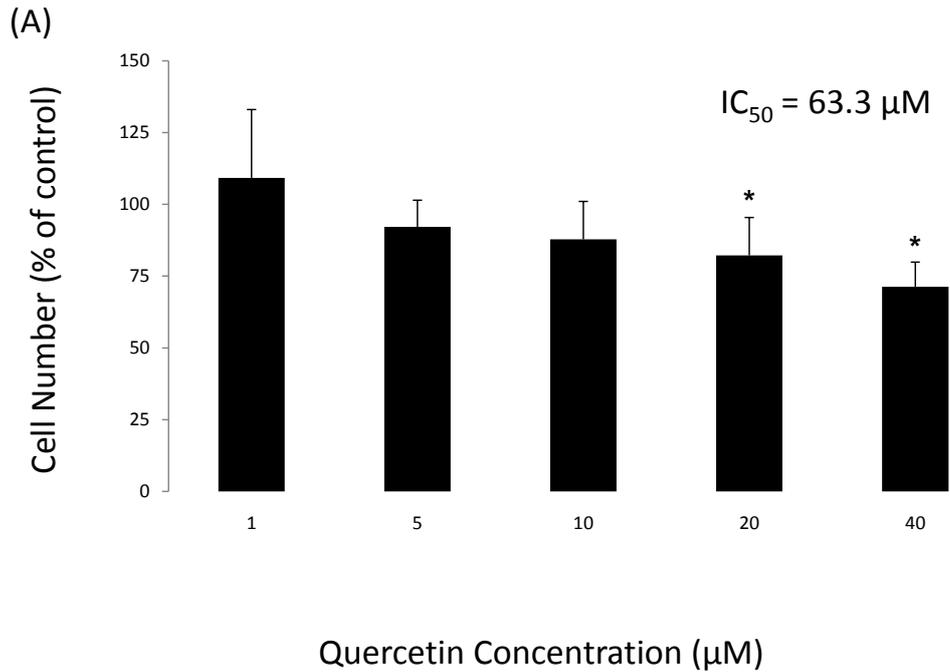


Figure 3.7 Growth inhibitory bar charts of quercetin (0-40 µM) in LNCaP cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean ± SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

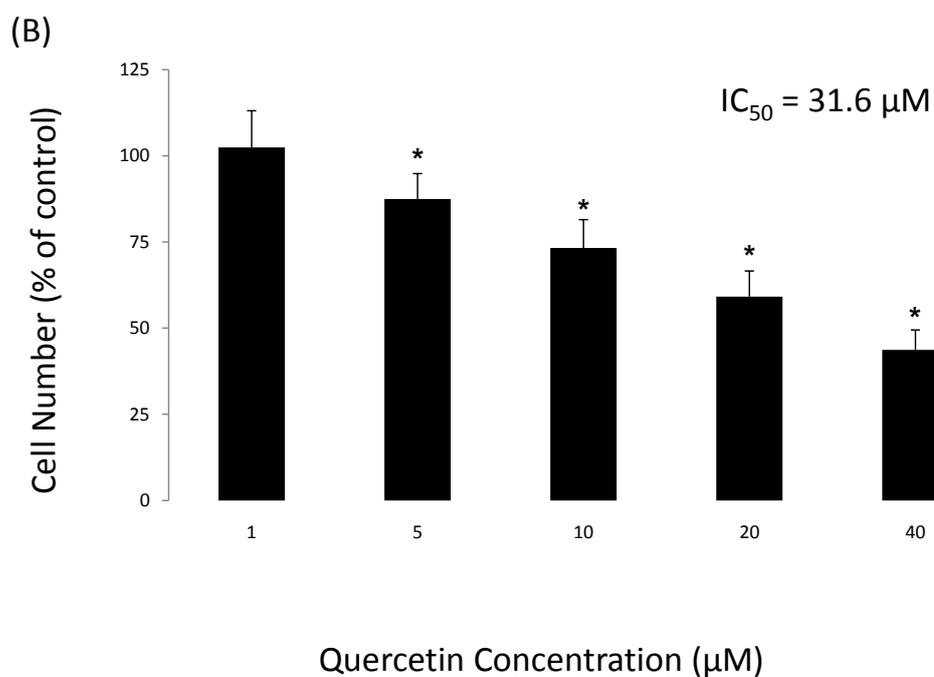
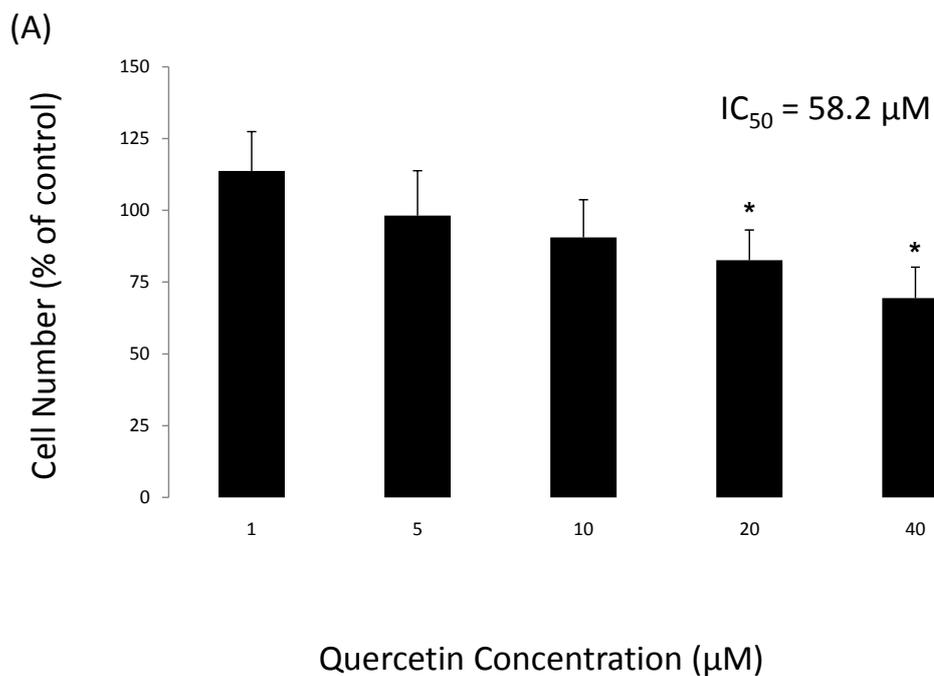


Figure 3.8 Growth inhibitory bar charts of quercetin (0-40 μM) in PC-3 cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

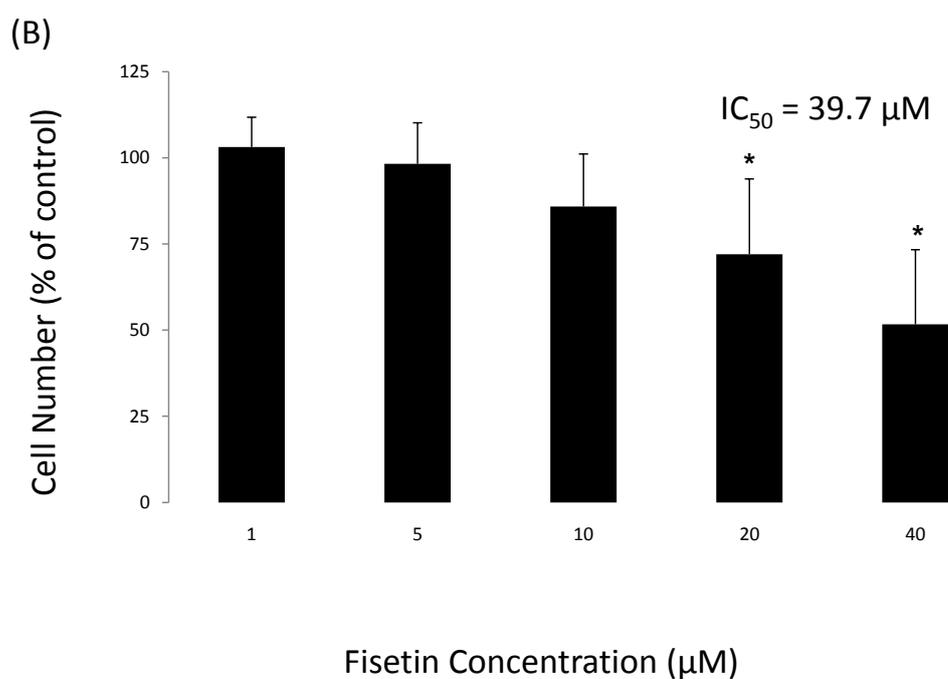
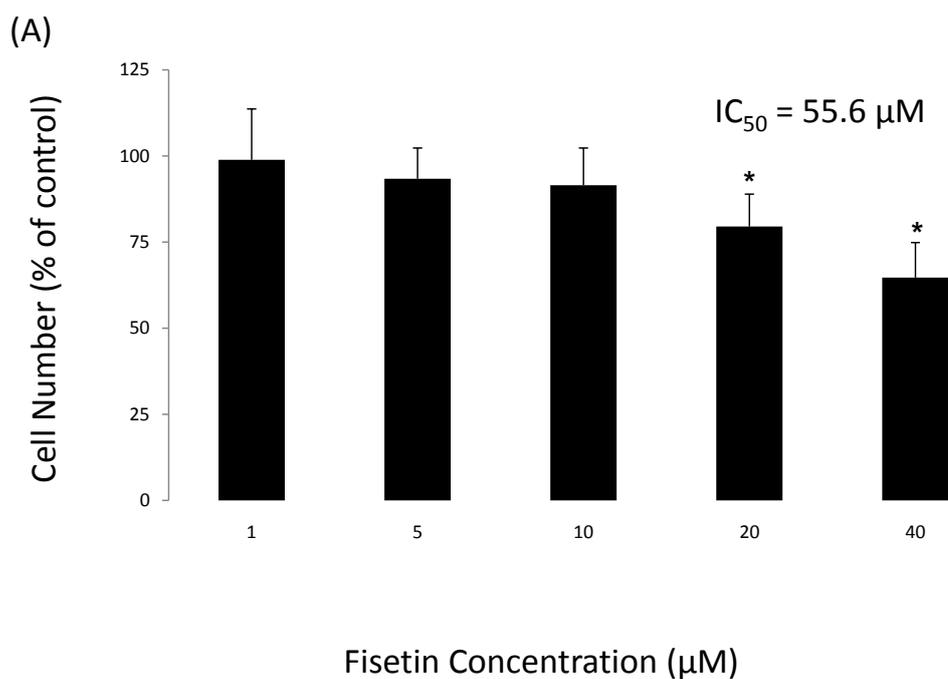


Figure 3.9 Growth inhibitory bar charts of Fisetin (0-40 μM) in LNCaP cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

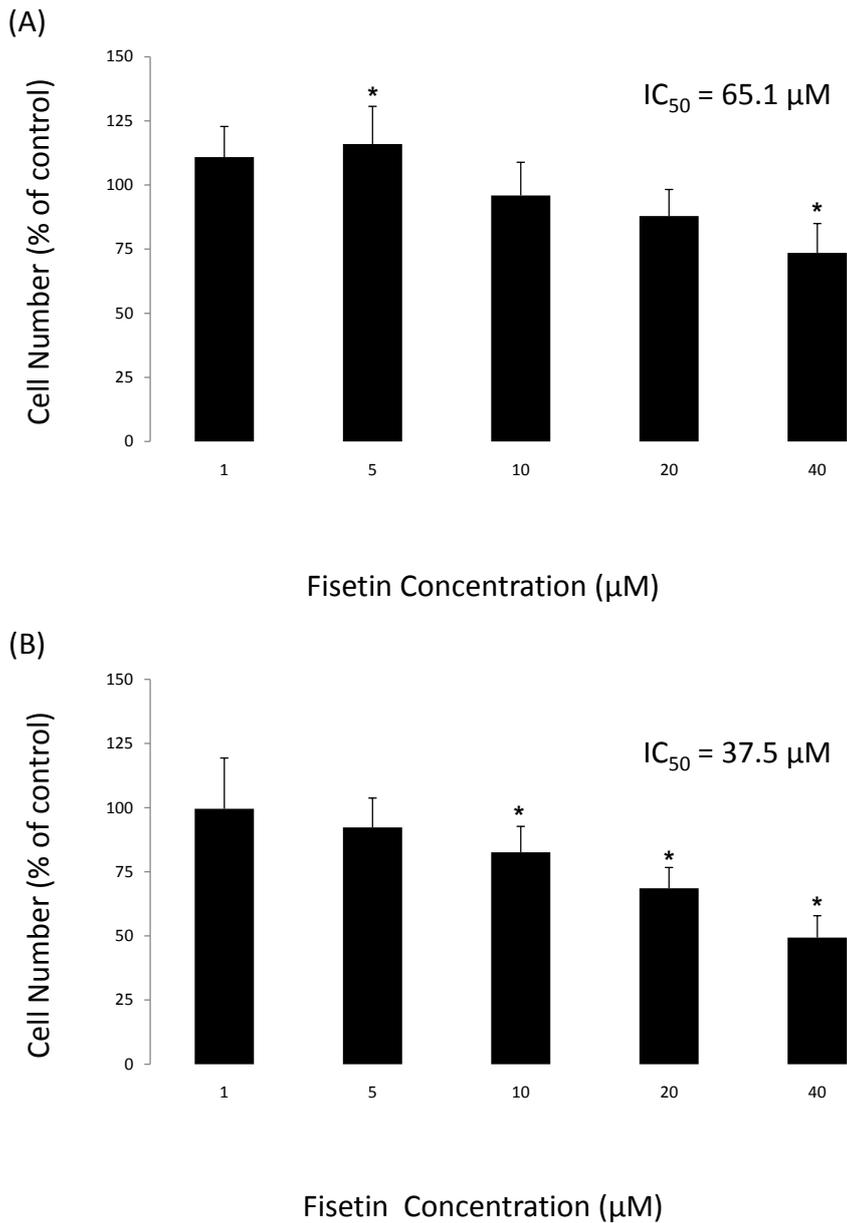


Figure 3.10 Growth inhibitory bar charts of Fisetin (0-40 µM) in PC-3 cells at 72 (A) and 144 (B) h. Growth assessment and IC₅₀ calculation were carried out as explained in section 2.3.5.1. The linear line equation of this bar chart was used to calculate IC₅₀. The values represent the percentage of cell number as compared to vehicle control expressed as the mean ± SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control value ($p < 0.05$).

Table 3.1 IC₅₀ values ± SD for TMFol, quercetin and fisetin in LNCaP, PC-3 and TRAMP C2 cells at 72 and 144 h.

	LNCaP		PC-3		TRAMP C2	
	72 h	144 h	72 h	144 h	72 h	144 h
TMFol	8.9 ± 0.9	5.6 ± 0.3	9.4 ± 1.1	4.1 ± 0.6	2.3 ± 0.4	1.7 ± 0.1
Quercetin	63.3 ± 4.3	31.6 ± 5.1	58.2 ± 2.7	31.6 ± 2.5	-	23.0 ± 2.0
Fisetin	55.6 ± 3.6	39.7 ± 2.4	65.1 ± 4.4	37.5 ± 2.2	-	27.3 ± 3.4

Quercetin and fisetin IC₅₀s at 72 h were not determined, as the reason behind TRAMP C2 *in vitro* testing is to link it to the *in vivo* xenograft study of TMFol, quercetin, fisetin. So short time points would not add much information.

The bar charts for the effect of quercetin and fisetin in TRAMP C2 were not included as only one time point was determined (144 h).

3.4 Effect of TMFol on cell cycle distribution in LNCaP, PC-3 and TRAMP C2 cells.

TMFol showed a potent anti-proliferative activity in prostate cancer cell lines (section 3.3). One explanation for the growth inhibition could be altered progression through the cell cycle. Therefore the effect of TMFol on the cell cycle was investigated. Other flavonoids such as quercetin and fisetin have been shown to induce cell cycle arrest in prostate cancer cell lines (Vijayababu et al., 2005, Khan et al., 2008a) so it is conceivable that TMFol may function mechanistically in a similar manner.

Cells were grown in the presence or absence of TMFol (0, 1, 5, 10 and 20 μM) for 48 or 72 h before cell cycle analysis was performed. The cell cycle can be divided into three distinct phases; G_1 , S and G_2/M . For each sample the number of cells as an overall percentage in each phase was plotted and then compared to control samples (Figures; 3.11, 3.12 and 3.13).

As shown in Figure 3.11 (A), there was no significant effect of TMFol on the cell cycle distribution of LNCaP cells after incubation for 48 h. However, at 72 h (Figure 3.11 (B)), the G_2/M population of the 10 and 20 μM TMFol treatments was significantly increased over that of the control. The effect of TMFol on the cell cycle profile of PC-3 cells is shown in Figure 3.12. At 48 and 72 h a dose dependent increase in the proportion of cells in S phase with TMFol treatment was observed. Noticeably, the increase in the S phase population was accompanied by a decrease predominantly in the G_1 population. However, it was only significant at the higher concentrations (10 and 20 μM) at 48h (A) and of 20 μM at 72 h (B). Cell cycle analysis of TRAMP C2 cells treated with TMFol is shown in Figure 3.13. TMFol induced a dose dependent increase in the G_2/M population with significant changes observed at 10 and 20 μM at 48 h (A) and 20 μM at 72 h (B). At 48 h there was a significant decrease in S phase at 20 μM while at 72 h the significant decrease was in the population of phase G_1 at 10 and 20 μM . Results of the TRAMP C2 (AR positive) cells are consistent with those observed with the human androgen sensitive cell line LNCaP.

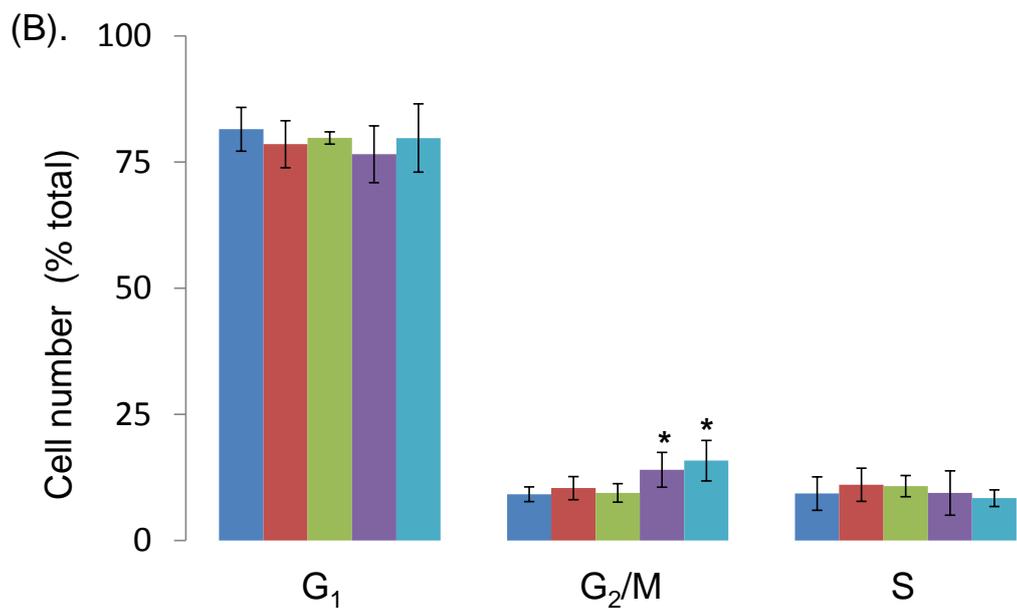
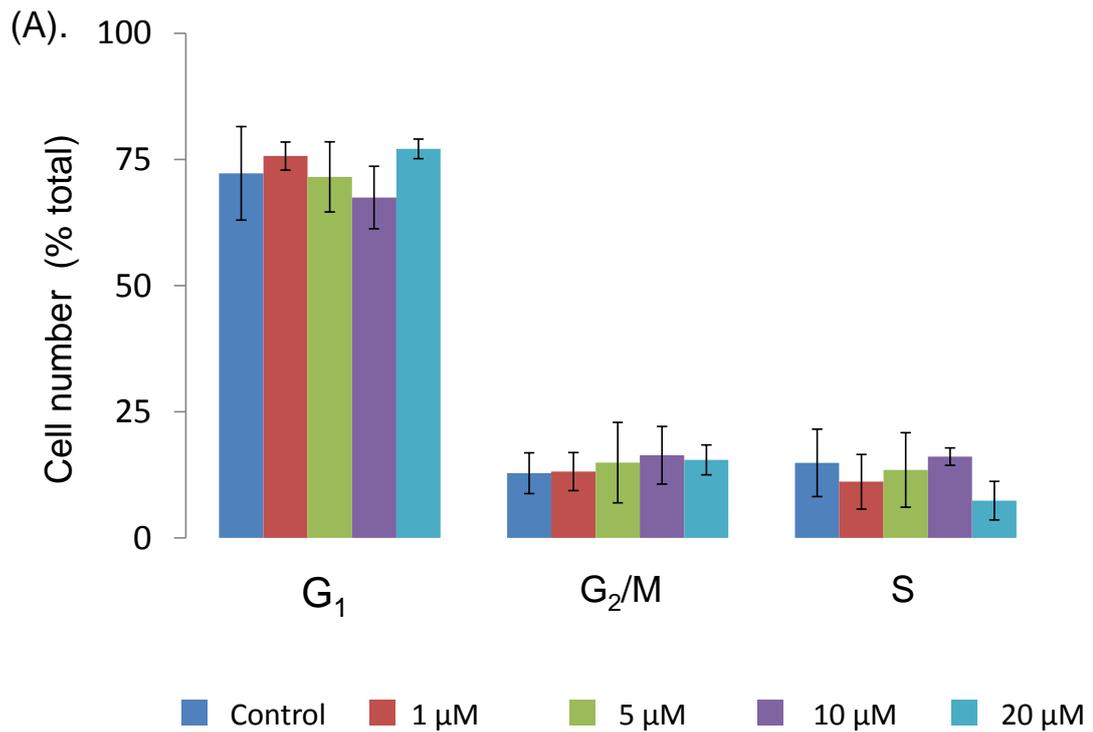


Figure 3.11 The cell cycle distribution of LNCaP cells after treatment with TMFol (1-20 μM) or vehicle control (0 μM) for 48 h (A) and 72 h (B). The values represent the percentage of cells in each phase of the cell cycle and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significantly different from vehicle control ($p < 0.05$).

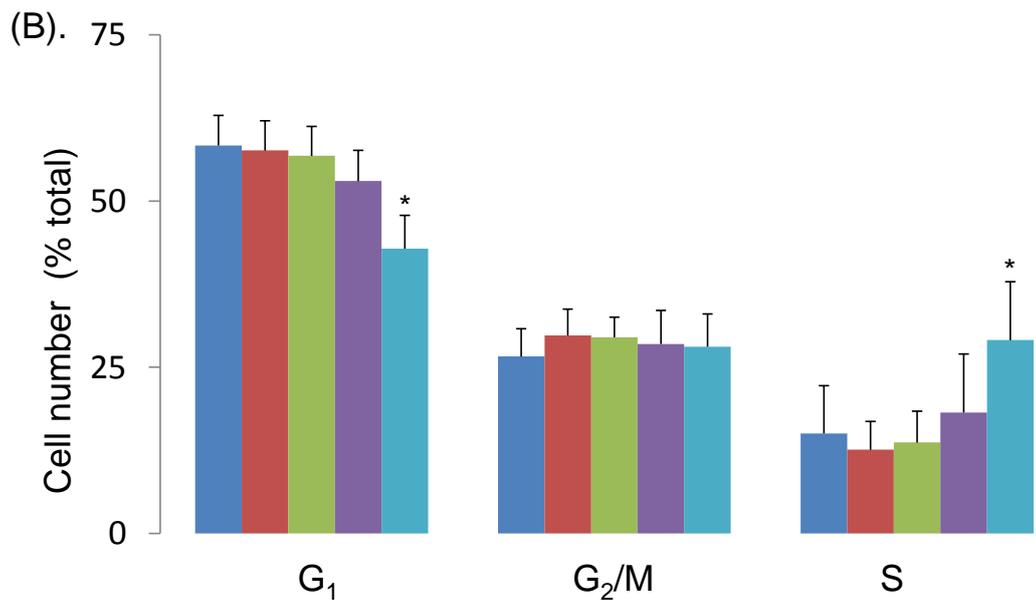
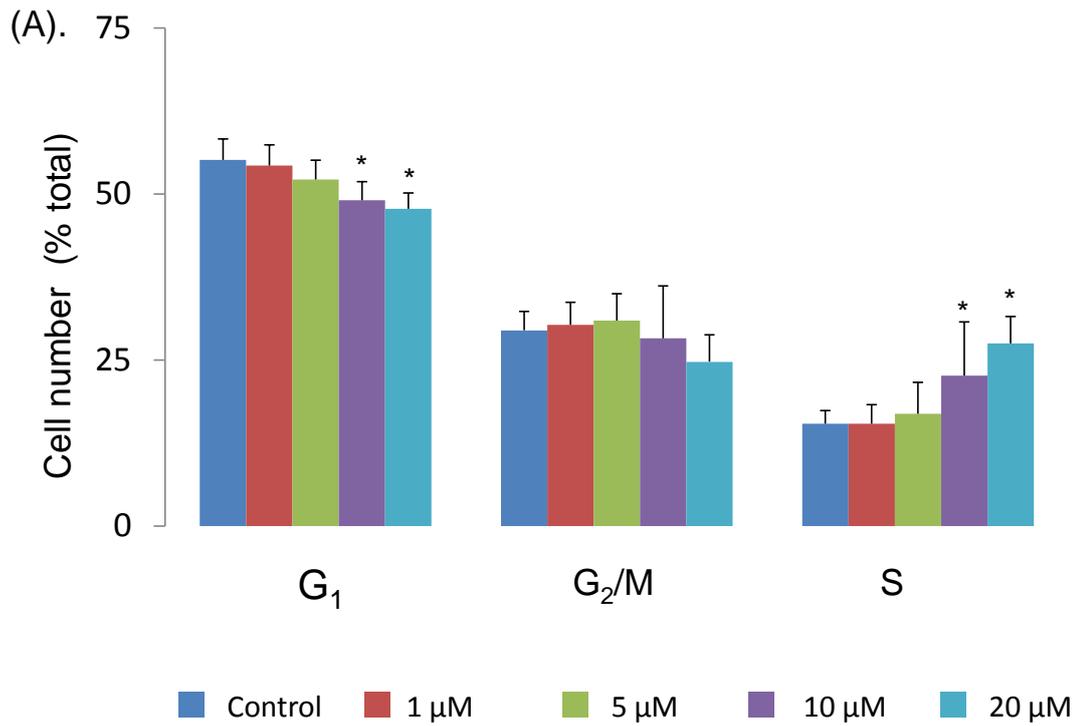


Figure 3.12 The cell cycle distribution of PC-3 cells after treatment with TMFol (1-20 μM) or vehicle control (0 μM) for 48 h (A) and 72 h (B). The values represent the percentage of cells in each phase of the cell cycle and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significantly different from vehicle control ($p < 0.05$).

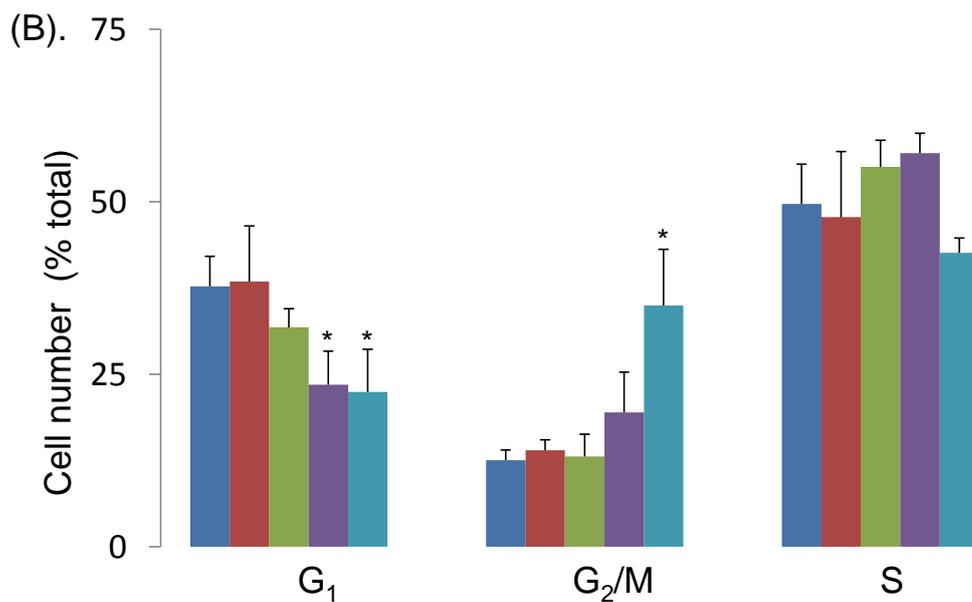
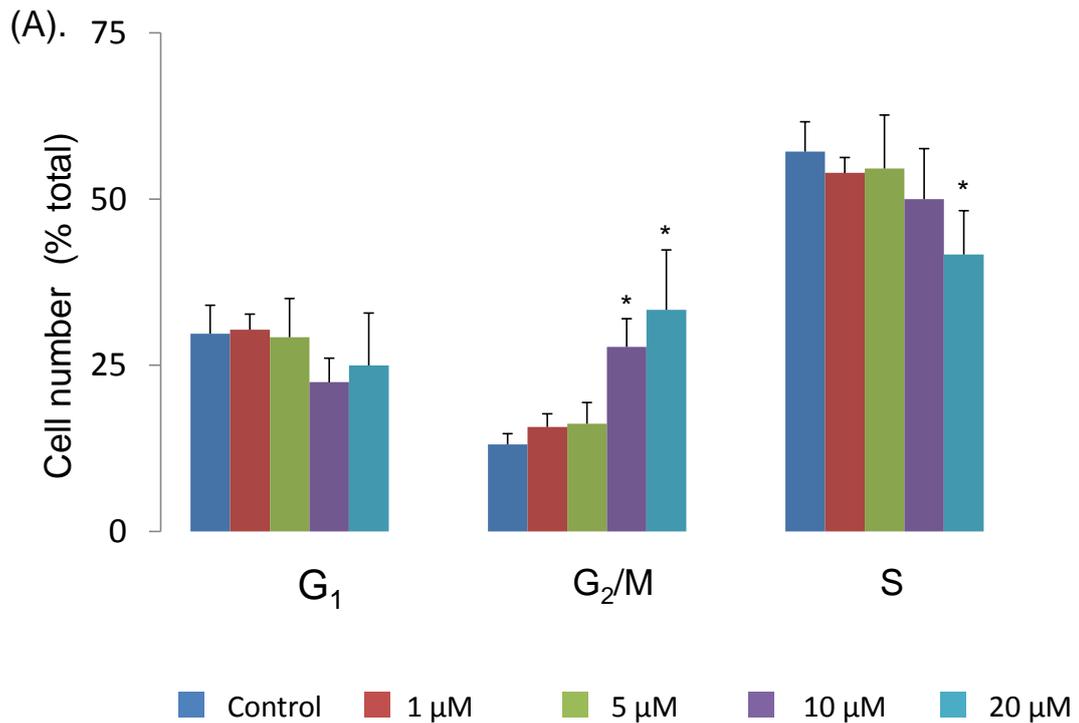


Figure 3.13 The cell cycle distribution of TRAMP C2 cells after treatment with TMFol (1-20 μM) or vehicle control (0 μM) for 48 h (A) and 72 h (B). The values represent the percentage of cells in each phase of the cell cycle and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significantly different from vehicle control ($p < 0.05$).

3.5 Effect of TMFol on apoptosis in LNCaP, PC-3 and TRAMP C2 using annexin V.

Flavonols including quercetin and fisetin have been shown to have an apoptotic effect in prostate cancer cell lines (Aalinkeel et al., 2008, Khan et al., 2008a). The finding that TMFol had potent growth-inhibitory effects in all of the cell lines prompted further experiments to investigate the mechanism. Growth inhibition could be a result of either cell cycle arrest and/or induction of apoptosis or necrosis. Therefore, the ability of TMFol to induce apoptosis was investigated in the LNCaP, PC-3 and TRAMP C2 cell lines.

Cells were incubated in the presence of TMFol (1-20 μ M) or with vehicle control for 72 or 96 h, and then with annexin V and PI before being analysed using FACS analysis (Section 2.3.7). Representative fluorescence scattergrams for each cell line are shown in Figures 3.14, 3.16 and 3.18. Graphical representation of these scattergrams is shown for each cell line in Figure 3.15, 3.17, and 3.19 respectively.

The proportion of live, apoptotic and necrotic cells following TMFol treatment was determined by quantification of cells within each quadrant. Each scattergram is divided into 4 quadrants, events in Q1-2 represent cell debris, Q2-2 represent the late apoptotic or necrotic cells, those in Q3-2 are the live population of cells, while Q4-2 are early apoptotic cells.

In LNCaP cells, TMFol induced apoptosis in a dose and time-dependent manner. This induction was found to be significant at 10 and 20 μ M (Figure 3.15). There was a significant increase in the necrotic population of 10 and 20 μ M at 96 h in concomitant with a decrease in the live population of 20 μ M.

The induction of apoptosis in the non-androgen dependent PC-3 cells was minimal with a significant increase observed at 20 μ M at 72 h only (Figure 3.17). TMFol (20 μ M) at 72h increased the apoptotic population in PC-3 (from 2.7% to 12.2%) while the increases in LNCaP was (from 3.7% to 19%). The increase at 96 h was (from 2.6% to 5.6%) in PC-3 and (from 2.5% to 29%) in LNCaP.

The apoptotic profile of TRAMP C2 cells after treatment with TMFol is shown in Figure 3.18. TMFol induced apoptosis in a dose dependent manner (Figure 3.19). Out of the three cell lines tested, the TRAMP C2 cells appear to be the most sensitive to TMFol induced apoptosis. TMFol (20 μ M) led to increase in the percentage of apoptotic population (from 2.5% to 36%) at 72 h and, (from 2% to 50%) at 96 h. The TRAMP C2 cells were also most sensitive to TMFol induced cell growth inhibition and cell cycle arrest. As induction of apoptosis was most sensitive in the two androgen sensitive cell lines, LNCaP and TRAMP C2, AR signalling was considered an important pathway to investigate as a potential mechanism of action of TMFol.

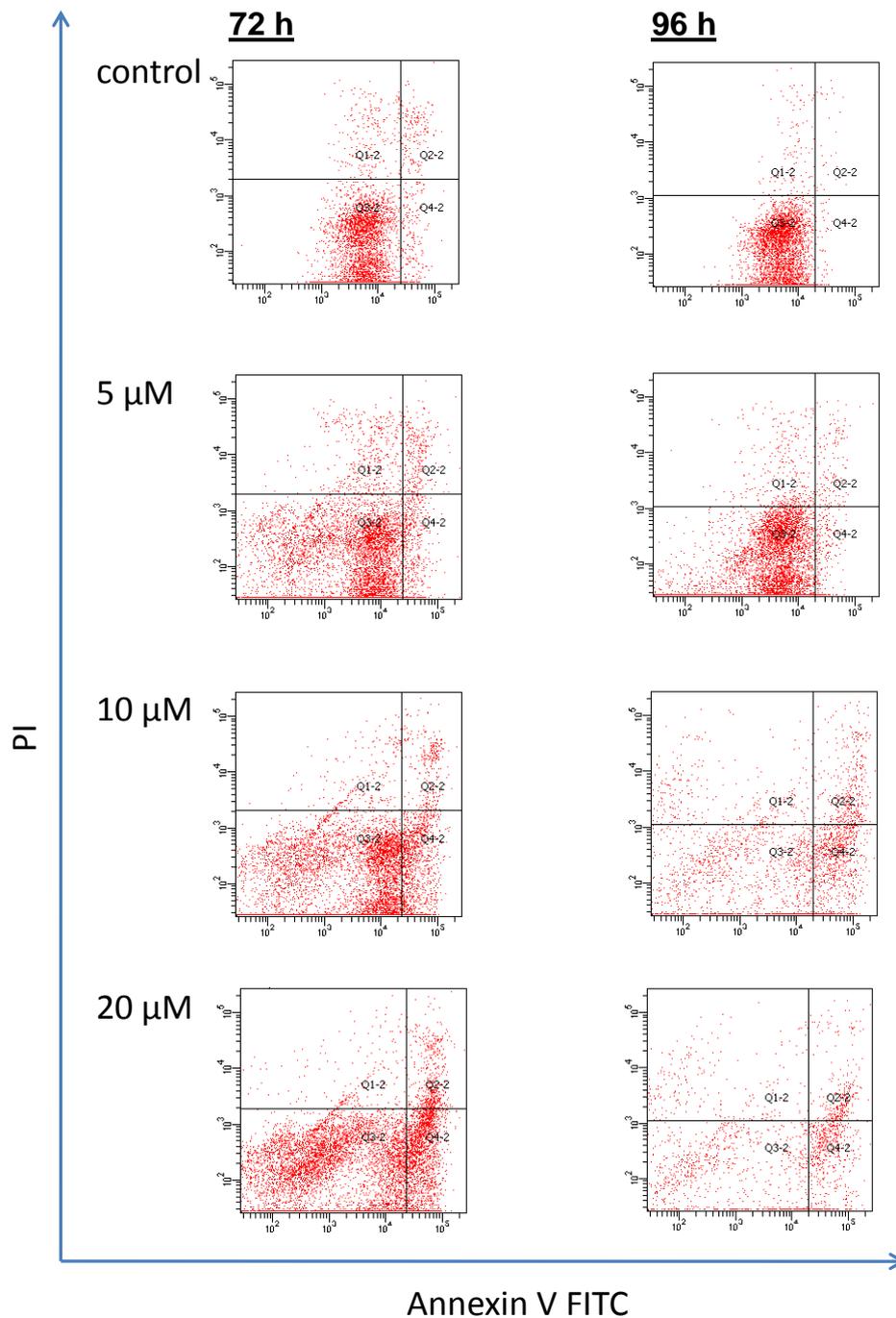


Figure 3.14 Representative fluorescence scattergrams of LNCaP cells analysed by flow cytometry after incubation with TMFol (1-20 μM) or vehicle control for 72 and 96 h. Cells were stained with annexin-FITC and PI as detailed in section 2.3.7. Live cells do not take up any of the stains, therefore are located in Q3-2, early apoptotic cells take up annexin-V only and appear in Q4-2, Late apoptotic or necrotic cells in Q2-2 stain with both dyes, while quadrant Q1-2 contains cell debris which stains with PI only.

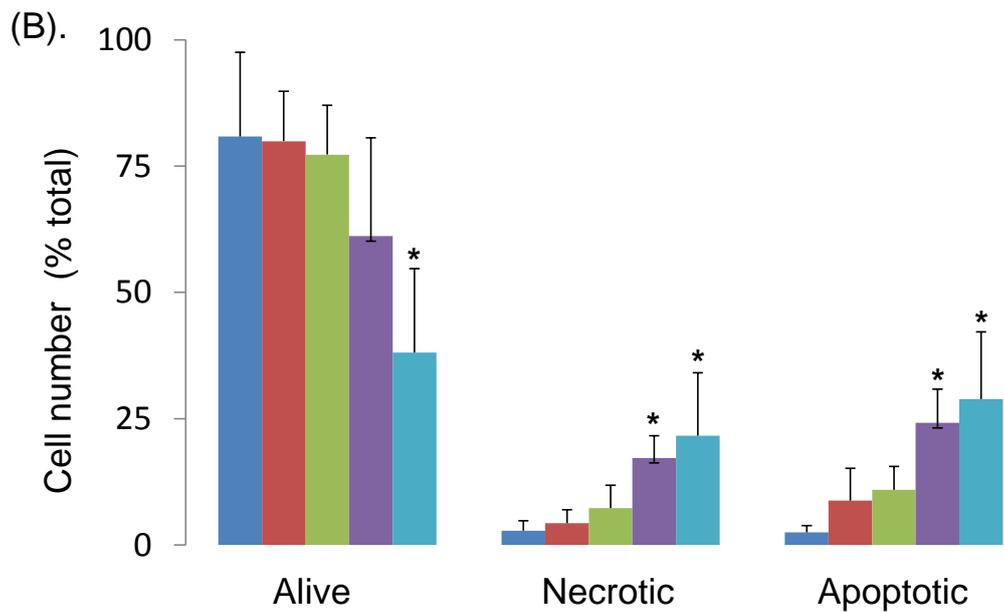
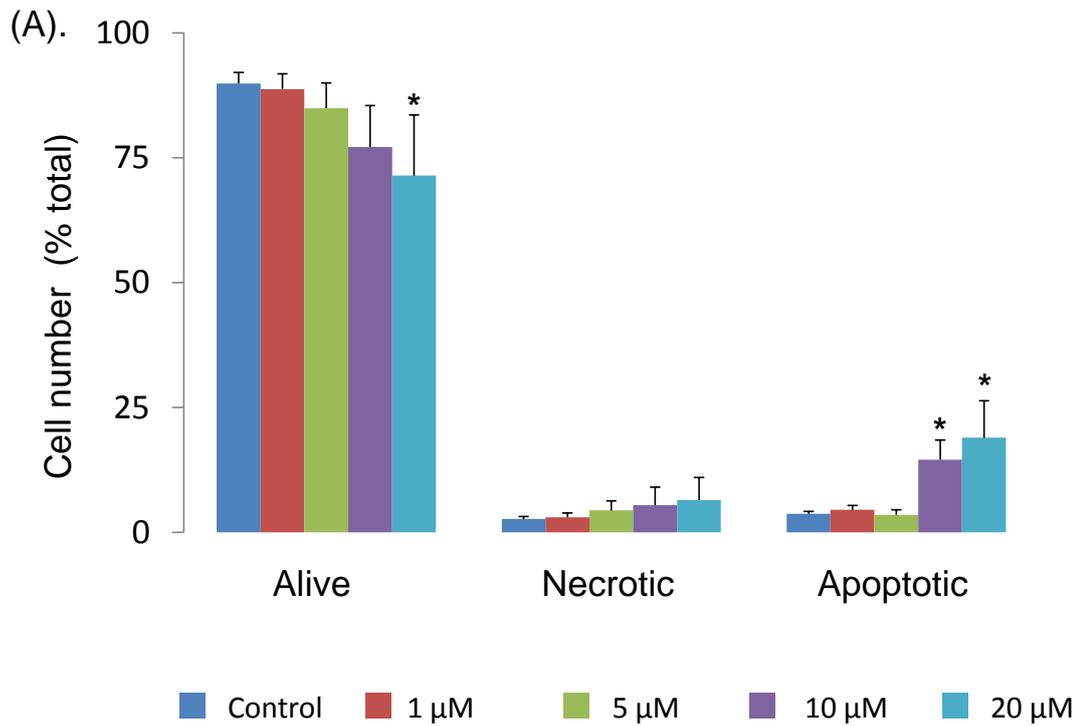


Figure 3.15 The proportion of live, necrotic and apoptotic LNCaP cells after incubation with TMFol (1-20 μ M) or vehicle control for 72 h (A) or 96 h (B). The values represent the number of cells that are live, necrotic or apoptotic as a percentage of the overall cell total and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significantly different from vehicle control ($p < 0.05$).

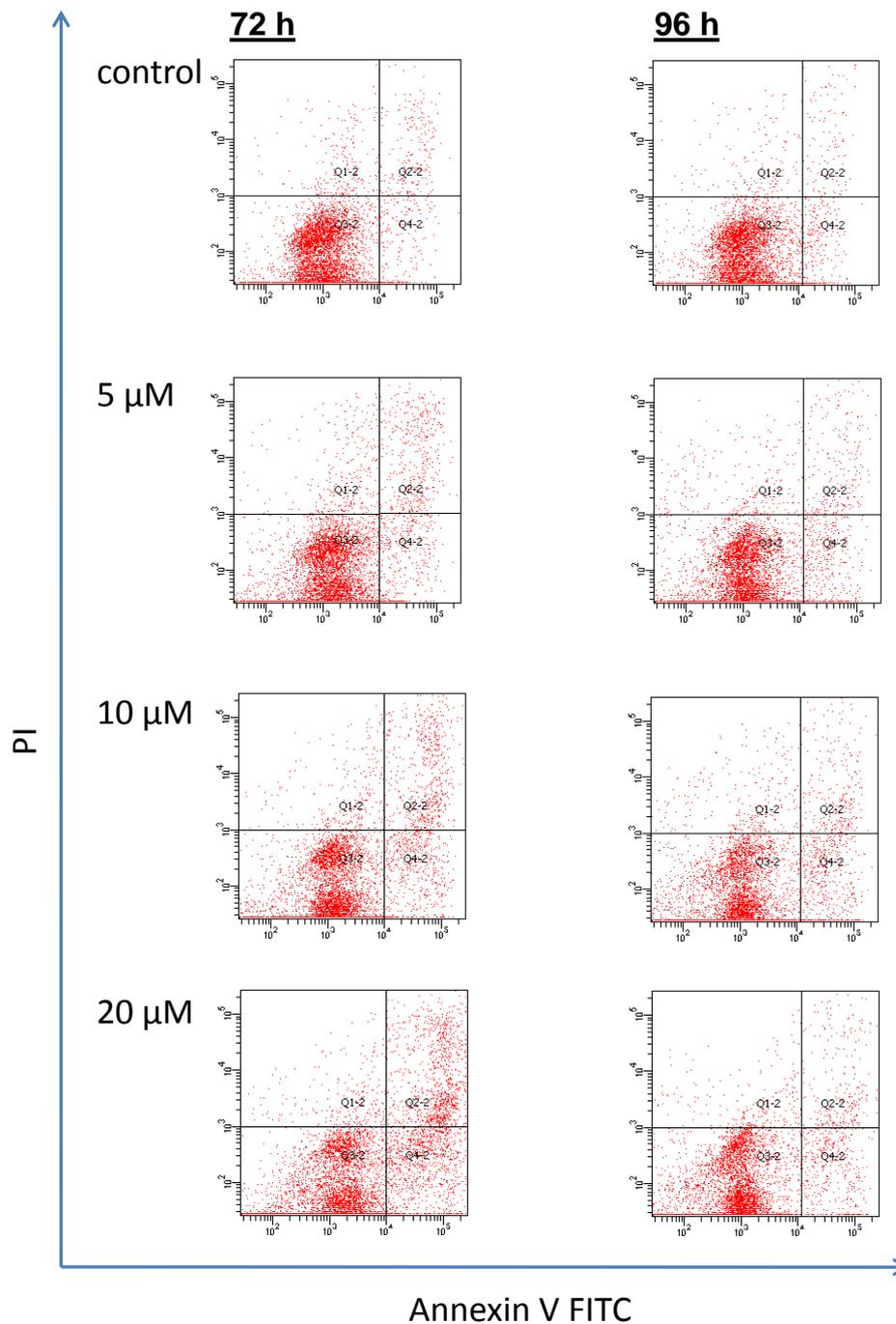


Figure 3.16 Representative fluorescence scattergrams of PC-3 cells analysed by flow cytometry after incubation with TMFol (1-20 μ M) or vehicle control for 72 and 96 h. Cells were stained with annexin-FITC and PI as detailed in section 2.3.7. Live cells do not take up any of the stains, therefore are located in Q3-2, early apoptotic cells take up annexin-V only and appear in Q4-2, Late apoptotic or necrotic cells in Q2-2 stain with both dyes, while quadrant Q1-2 contains cell debris which stains with PI only.

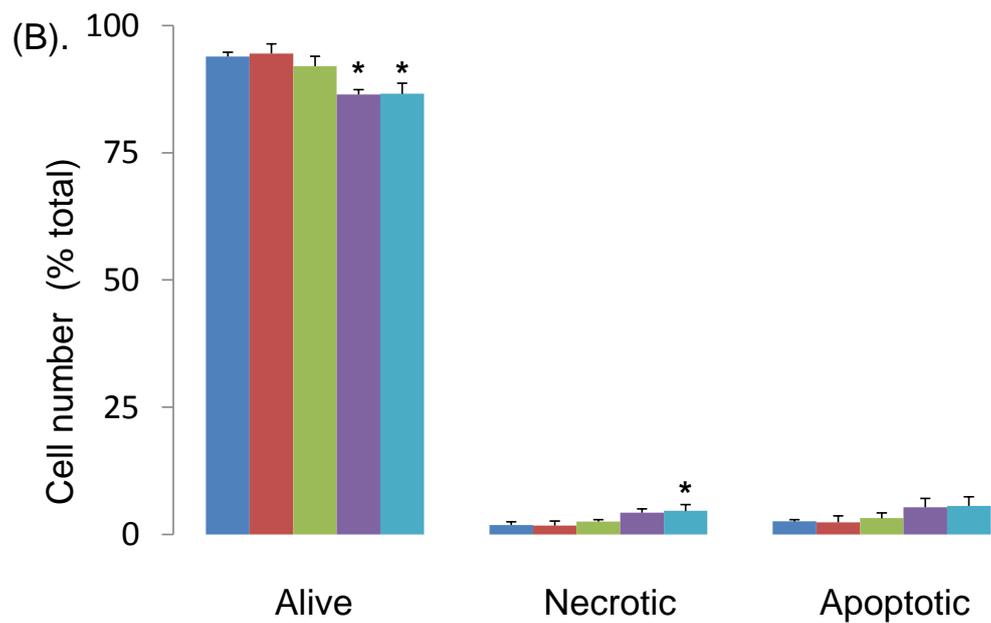
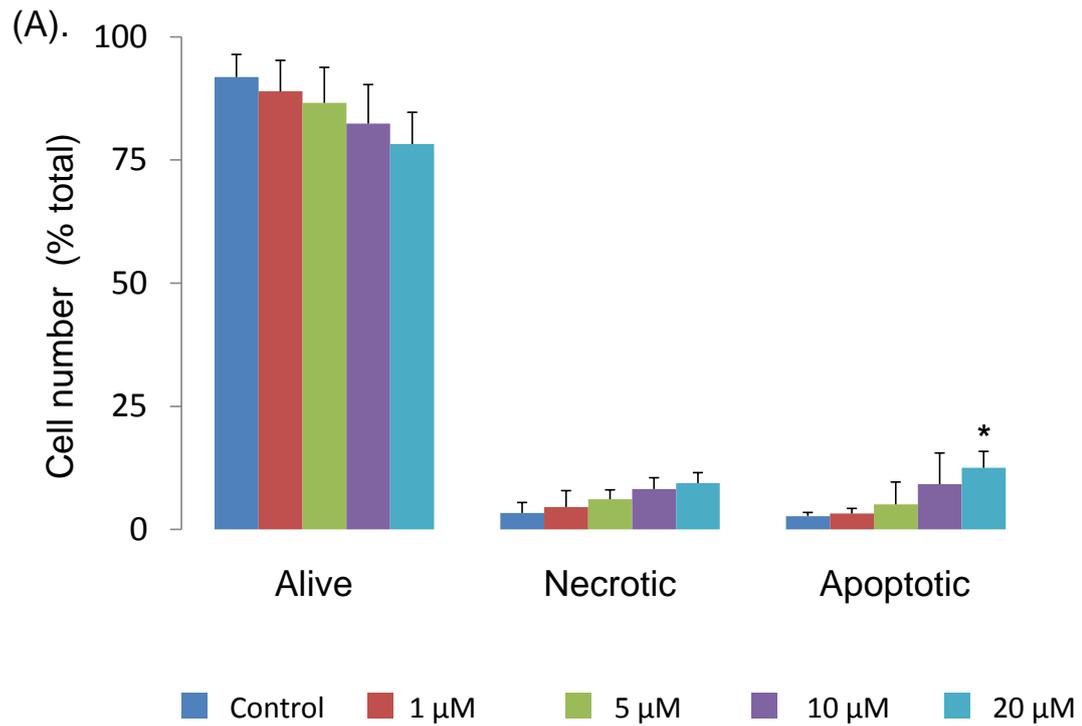


Figure 3.17 The proportion of Live, necrotic and apoptotic PC-3 cells after incubation with TMFol (1-20 μ M) or vehicle control for 72 h (A) or 96 h (B). The values represent the number of cells that are live, necrotic or apoptotic as a percentage of the overall cell total and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significantly different from vehicle control ($p < 0.05$).

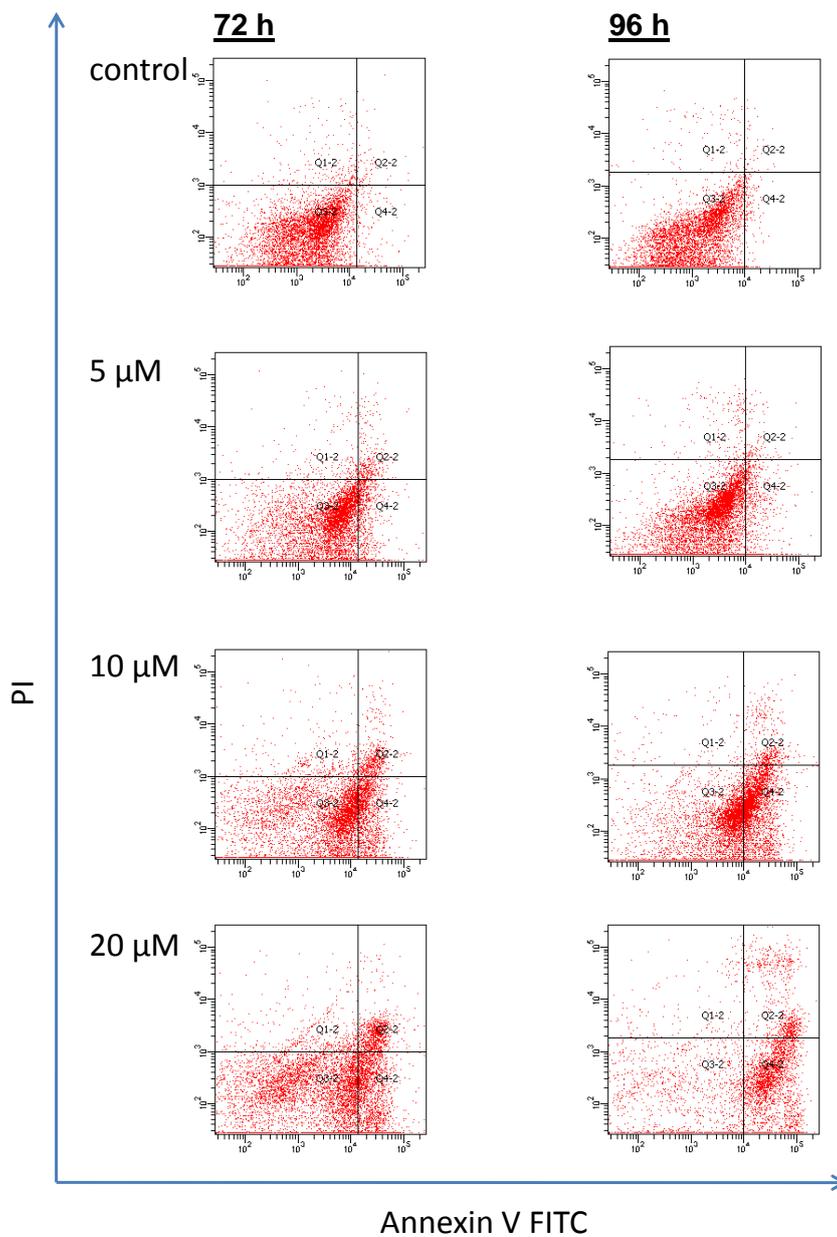


Figure 3.18 Representative fluorescence scattergrams of TRAMP C2 cells analysed by flow cytometry after incubation with TMFol (1-20 μM) or vehicle control for 72 and 96 h. Cells were stained with annexin-FITC and PI as detailed in section 2.3.7. Live cells do not take up any of the stains, therefore are located in Q3-2, early apoptotic cells take up annexin-V only and appear in Q4-2, Late apoptotic or necrotic cells in Q2-2 stain with both dyes, while quadrant Q1-2 contains cell debris which stains with PI only.

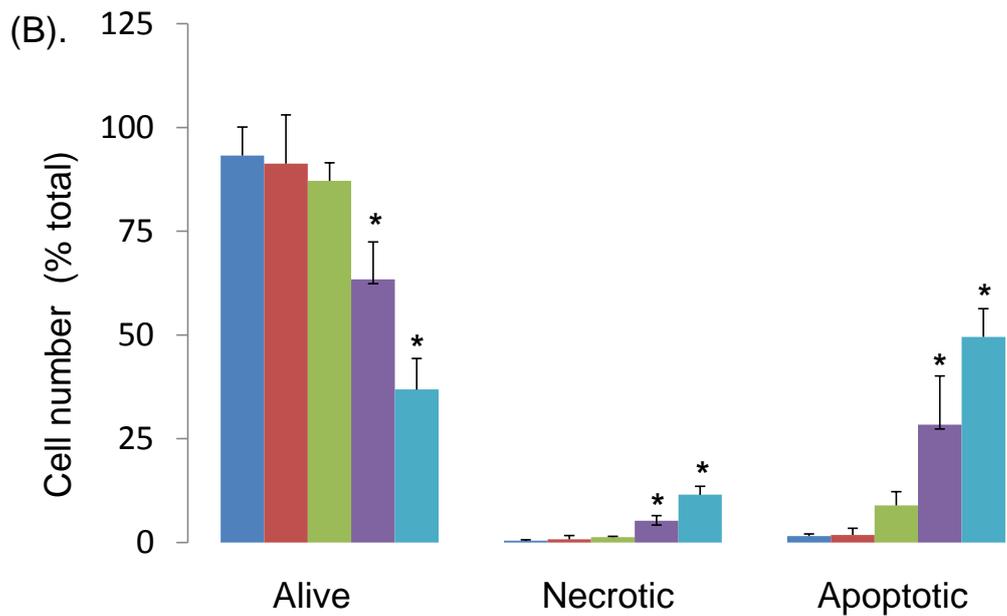
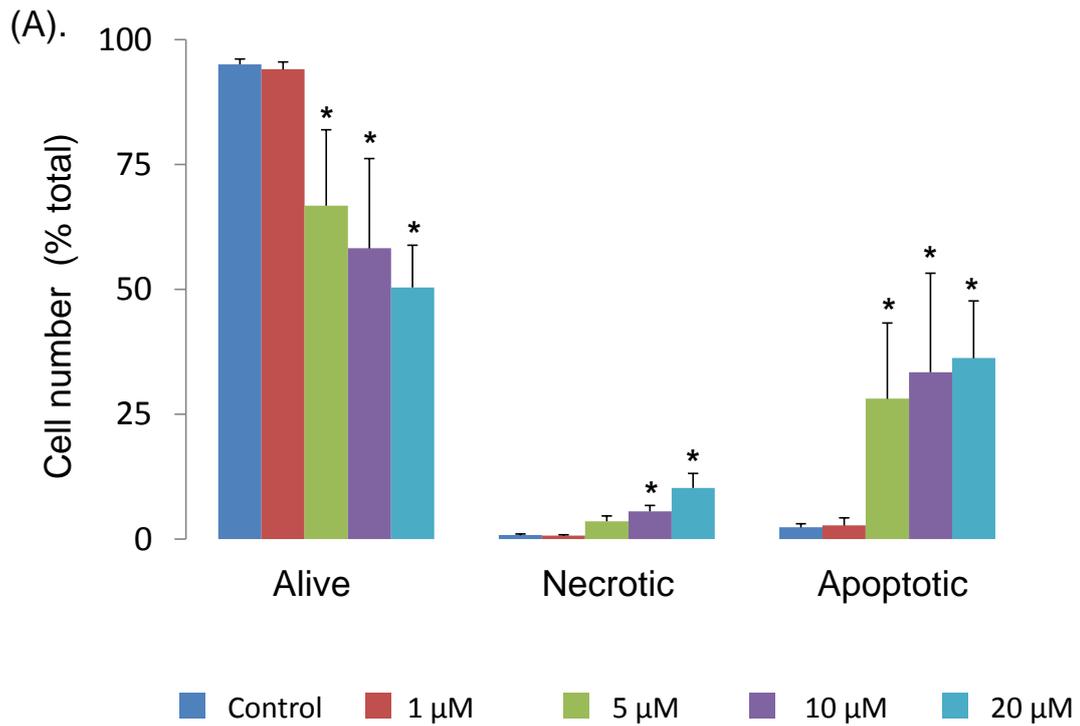


Figure 3.19 The proportion of live, necrotic and apoptotic TRAMP C2 cells after incubation with TMFol (1-20 μM) or vehicle control for 72 h (A) or 96 h (B). The values represent the number of cells that are live, necrotic or apoptotic as a percentage of the overall cell total and are the mean \pm SD of three separate experiments performed in triplicate. (*) indicates the data point is statistically significant from vehicle control ($p < 0.05$).

3.6 Investigating the effect of TMFol on the androgen receptor signalling pathway

3.6.1 Effect of TMFol, fisetin and quercetin on androgen receptor expression in LNCaP cells

Quercetin and fisetin have been shown to have antiproliferative activity against androgen dependent prostate cancer cell lines. This reduction was accompanied by a decrease in the expression of the AR, which suggests that AR downregulation could be one of the mechanisms by which flavonols inhibit proliferation of prostate cancer cells (Xing et al., 2001, Yuan et al., 2004, Khan et al., 2008b). AR expression was investigated using androgen receptor expressing cell lines, LNCaP and TRAMP C2. Cells were grown in the presence or absence of TMFol for 24, 48 and 72 h, and then the cellular levels of AR were determined as detailed in section 2.3.8.3. In order to compare TMFol with quercetin and fisetin, the effect of quercetin and fisetin on AR expression were also investigated.

TMFol resulted in a dose- and time-dependent decrease in AR protein levels in LNCaP cells at all tested time points. However, statistical significance was only observed with 20 μ M TMFol at 48 h and 10 and 20 μ M TMFol at 72 h (Figure 3.20).

The ability of the flavonols quercetin and fisetin to modulate the expression of AR was also studied in LNCaP cells at 48 and 72 h time points, using much higher concentration (20, 40 and 60 μ M) than TMFol. Both quercetin and fisetin resulted in a dose and time dependent decrease in AR protein levels, at concentrations of 40 and 60 μ M. The decrease in AR protein was found to be statistically significant at both time points examined at these two concentrations (Figure 3.21).

The results suggest that downregulation of AR protein expression may be a feature of the flavonol structure, at least for the three agents tested here. Of the three compounds TMFol was the most potent inhibitor of AR protein expression at concentrations 2-4 fold lower than quercetin or fisetin.

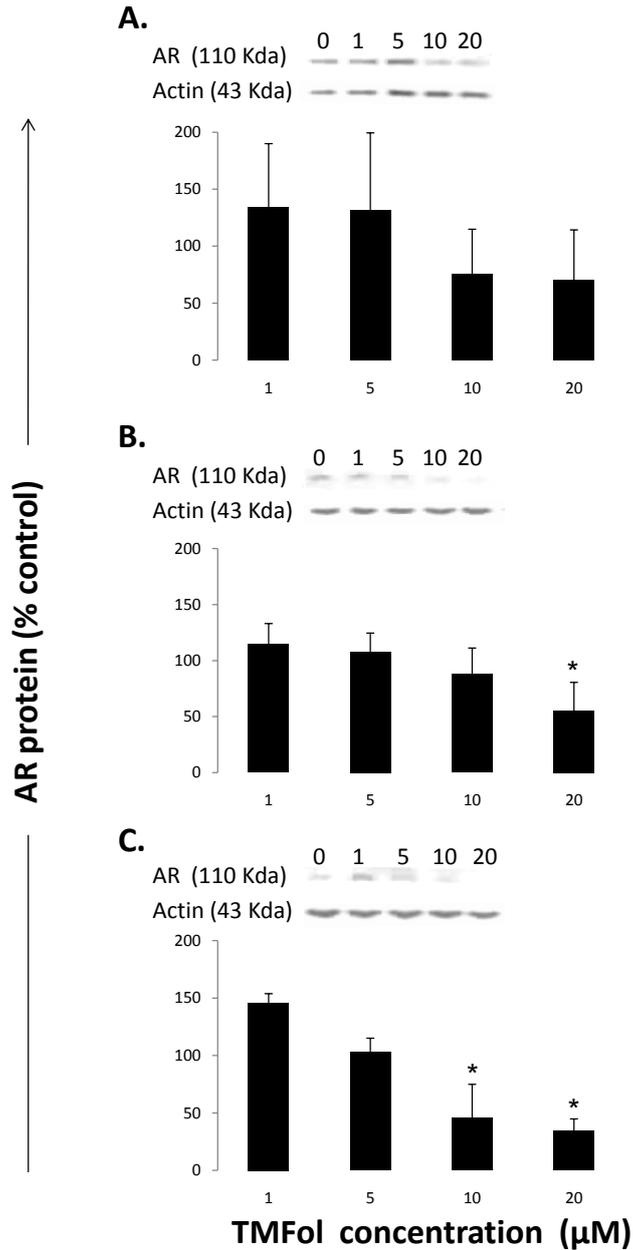


Figure 3.20. Effect of TMFol on AR protein expression in LNCaP cells, after (A) 24, (B) 48 and (C) 72 h. Cells were incubated with TMFol (1-20 μM) or vehicle control. Protein levels were measured by western blotting. The upper band indicates the AR protein while the lower band corresponds to actin, shown to demonstrate equal protein loading. The bar charts are the results of semi-quantitative densitometry analysis of band densities represented as a percentage of the control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

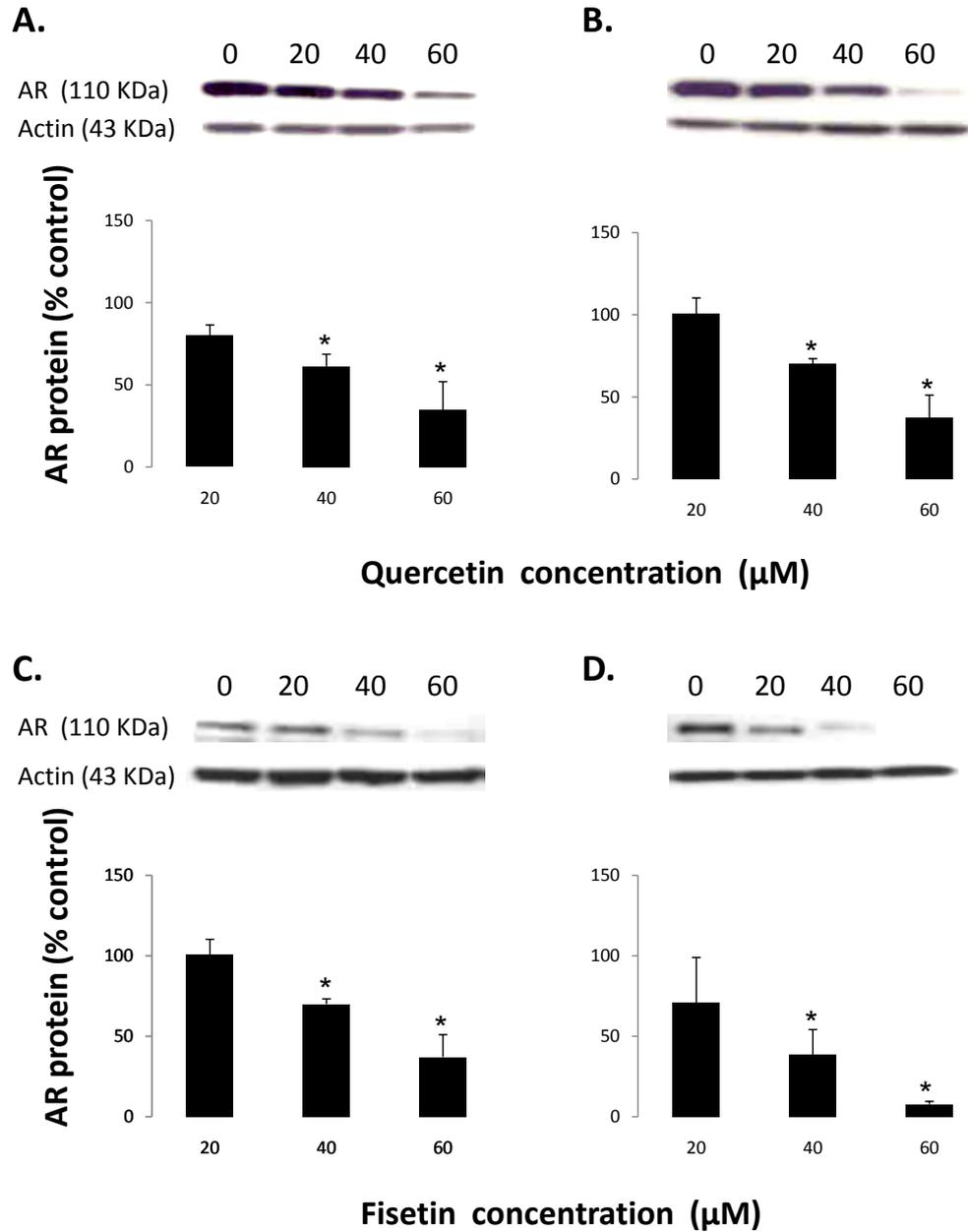


Figure 3.21 Effect of quercetin (A, B) and fisetin (C, D) on AR protein expression in LNCaP cells at 48 h (A, C) and at 72 h (B, D). Cells were incubated with quercetin and fisetin (20-60 μM) or vehicle control. Protein levels were measured by western blotting. The upper band indicates the AR protein while the lower band corresponds to actin, shown to demonstrate equal protein loading. The bar charts are the results of semi-quantitative densitometry analysis of band densities represented as a percentage of the control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.6.2 Effect of TMFol, on androgen receptor expression in TRAMP C2 cells.

TRAMP C2 are normally cultured in media containing 5 % v/v FCS, 5 % v/v Nu serum, insulin and DHT(Foster et al., 1997) (Section 2.3.2). Under these conditions TMFol had no effect on the protein expression of the AR (Figure 3.22 A, B). This result was not consistent with the effect of TMFol in human LNCaP cells where TMFol produced a significant decrease in the AR expression. What we expected was a decrease in AR expression in TRAMP C2 as a response to TMFol treatment. We thought the reason of these variations was the presence of high concentration of growth inducers (DHT, Nu serum and insulin) in growth media of TRAMP C2. Hence, we decided to grow TRAMP C2 cells in media contains (10% v/v FCS) only. Cells were grown for two weeks in 10% v/v FCS media before the actual experiment.

When cells were cultured in media containing only 10 % v/v FCS TMFol reduced AR protein in a time- and dose-dependent manner, with reduction was significant at 20 μ M for both time points and only at 10 μ M for 48 h (Figure 3.22 C, D).

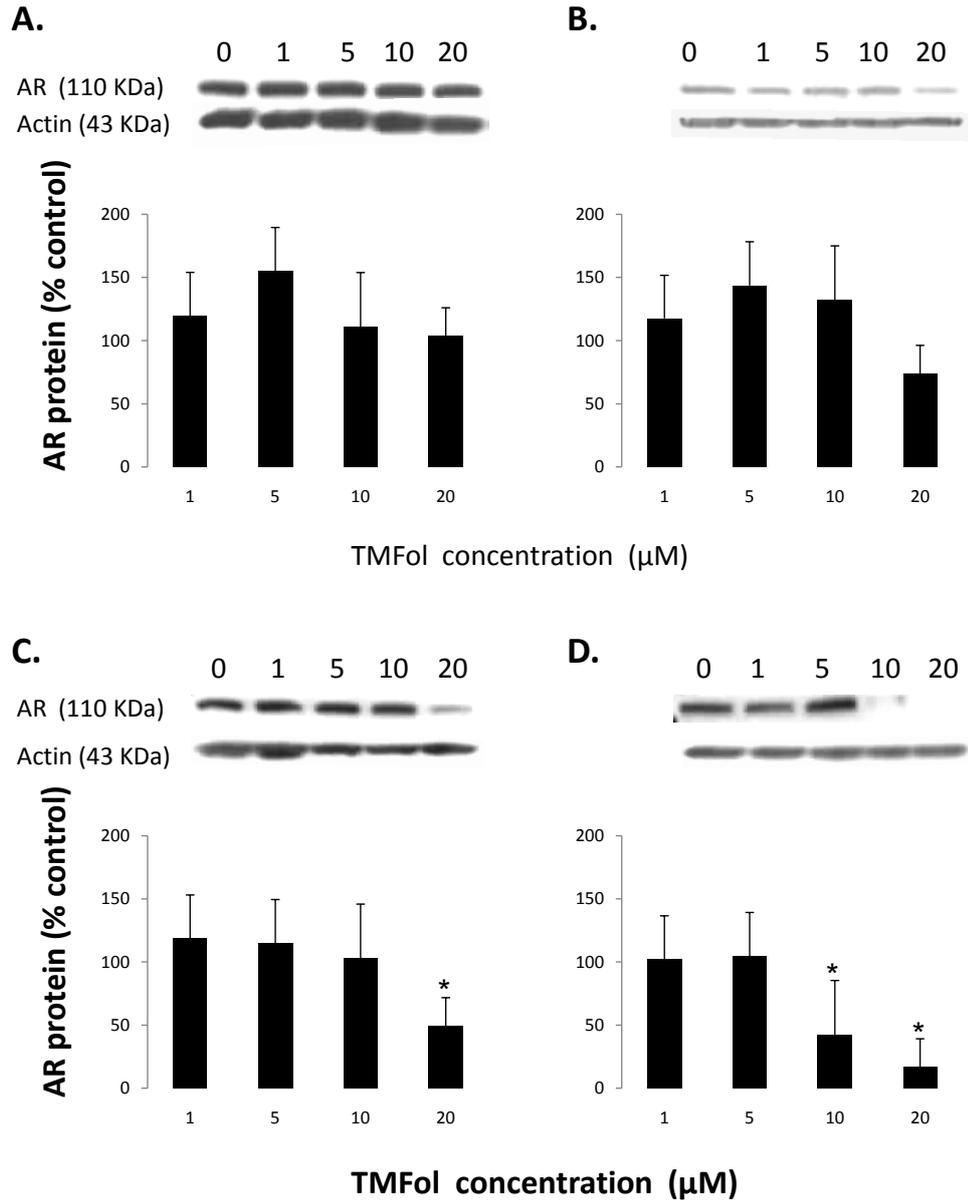


Figure 3.22 Effect of TMFol on AR protein expression in TRAMP C2 cells grown in standard media conditions (A and B) and in 10 % v/v FCS media (C and D), at 48 h (A, C) and at 72 h (B, D). Cells were incubated with TMFol (1-20 μM) or vehicle control. Protein levels were measured by western blotting. The upper band indicates the AR protein while the lower band corresponds to actin, shown to demonstrate equal protein loading. The bar charts are the results of semi-quantitative densitometry analysis of band densities represented as a percentage of the control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.6.3 Effect of TMFol, fisetin and quercetin on androgen receptor expression in LNCaP cells in the presence of mibolerone.

In media containing the AR agonist DHT, TMFol failed to reduce AR protein expression in TRAMP C2 cells (Figure 3.22). In contrast, quercetin has previously been found to decrease AR levels in LNCaP cells in the presence of the AR agonist mibolerone (Xing et al., 2001). Mibolerone is a potent synthetic AR agonist with higher affinity to AR than most other AR agonists such as DHT and R 1881, which is used in culture media because it resists the metabolism for longer periods (Murthy et al., 1986). As way of comparison, the effects of TMFol, quercetin and fisetin in the presence of mibolerone (1 nM) on AR expression were tested under the same conditions (5% v/v FCS and 1 nM mibolerone in media for 24h) as described in the literature for quercetin (Xing et al., 2001). For TMFol we tested the effect at 24, 48 and 72 h, as TRAMP C2 data was generated at 48 and 72h.

The effect of TMFol on the AR protein in the presence of mibolerone is displayed in Figure 3.23. At the earlier time points of 24 and 48 h (A and B), AR protein expression did not significantly change with any concentrations of agent. However, at 72 h a dramatic increase of more than 5-fold in AR protein levels was seen at concentrations of 10 and 20 μM (C), although this increase was only statistically significant at 20 μM .

LNCaP cells were treated with concentrations of 10, 50 and 100 μM quercetin or fisetin for 24 h. There was no significant effect of either flavonol on AR levels at a concentration of 10 μM , but at 50 and 100 μM both caused a dose-dependent decrease (Figure 3.24), with the most significant change exerted by quercetin (Figure 3.24 (A)). The different effects on AR protein expression with the three flavonols suggest that TMFol behaves differently to that of quercetin and fisetin in the presence of the AR agonist, mibolerone.

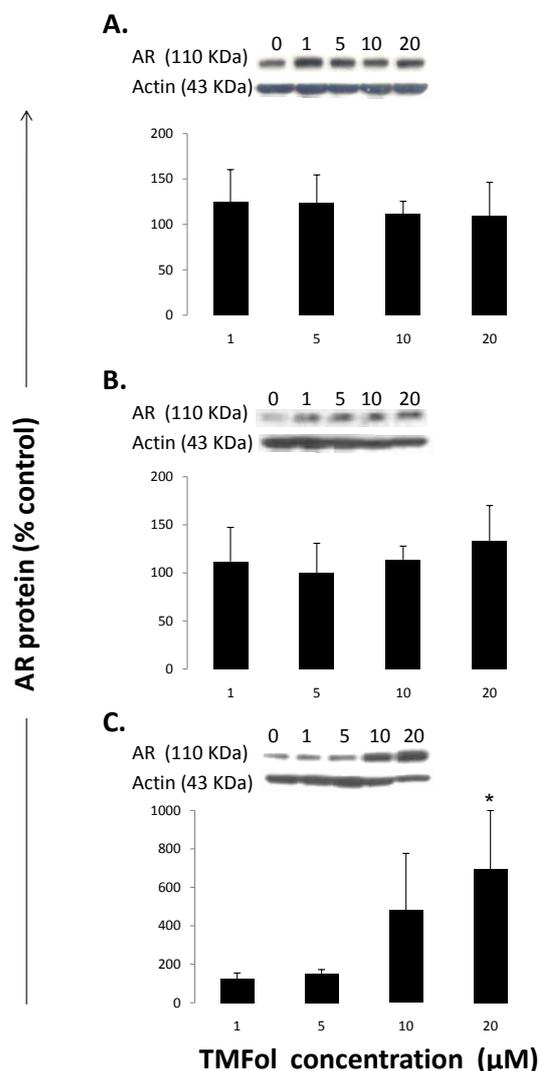


Figure 3.23 Effect of TMFol on AR protein expression in LNCaP cells in the presence of mibolerone, after (A) 24, (B) 48 and (C) 72 h. Cells were incubated with TMFol (1-20 μM) or vehicle control in media contained 5% v/v FCS and 1 nM mibolerone. AR protein levels were measured by western blot and normalised to actin as a loading control. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of the vehicle control. Values are the mean ± SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

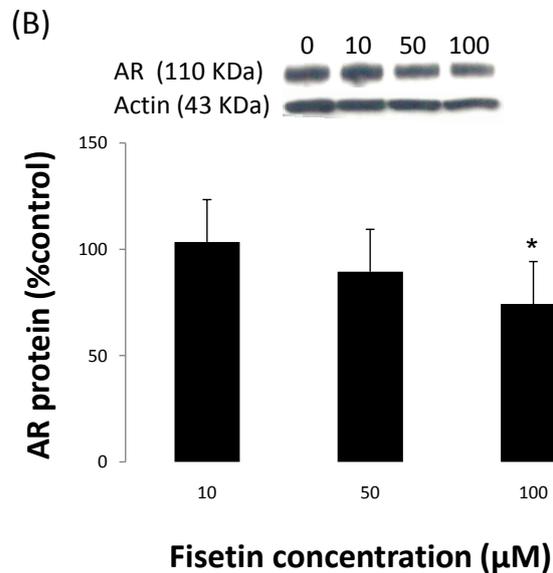
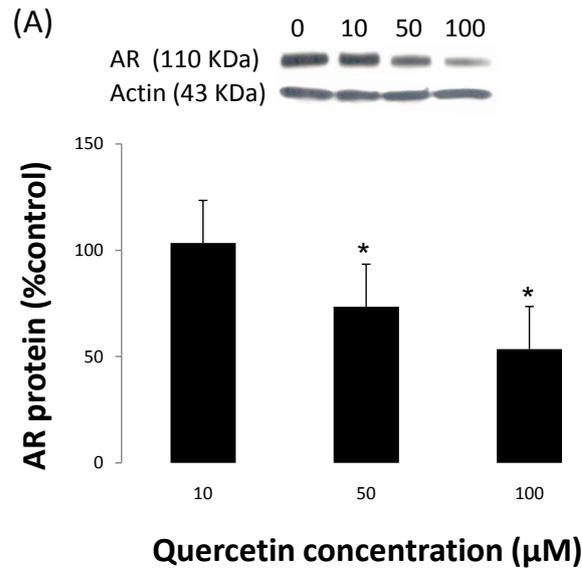


Figure 3.24 Effect of quercetin (A) and fisetin (B) on AR protein expression in LNCaP cells in the presence of 1 nM mibolerone. Cells were incubated with quercetin or fisetin (0-100 μM) or vehicle control in media contained 5 % v/v FCS and 1 nM mibolerone for 24 h. AR protein levels were measured by western blot and normalised to actin as a loading control. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of the vehicle control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.6.4 Effect of TMFol on PSA expression in LNCaP cells in presence or absence of mibolerone

PSA is target gene for the AR and a biomarker for PCa progress (Tosoian and Loeb, 2010). We wished to determine whether the change in AR protein expression observed with TMFol was accompanied by alterations in PSA. Therefore, the effect of TMFol (0-20 μ M) on the expression of PSA in cells and secreted levels of PSA in the culture media (Figures 3.25 and 3.26) of LNCaP cells was investigated.

TMFol significantly reduced cellular levels of PSA at 20 μ M although this was only observed at the 72 h time point. Furthermore, it was also only in the absence of mibolerone that these changes were observed.

TMFol reduced secreted PSA levels in a dose-related manner in both the presence and absence of mibolerone (Figure 3.26). Significant changes were observed at 20 μ M in the absence of mibolerone and close to significance in its presence at 72 h and significant at 48 h. The levels of PSA secreted into the culture media for vehicle control cells grown in the presence and absence of mibolerone are shown in Table 3.2. As can be seen, the levels of PSA increase with time and also with the addition of the AR agonist mibolerone, as expected.

Table 3.2 Levels of secreted PSA (ng PSA/million cells) from control LNCaP cells after 48 and 72 h incubation in the presence or absence of the AR agonist, mibolerone.

	- mibolerone	+ mibolerone
48 h	633 \pm 257	1069 \pm 229
72 h	789 \pm 243	2056 \pm 351

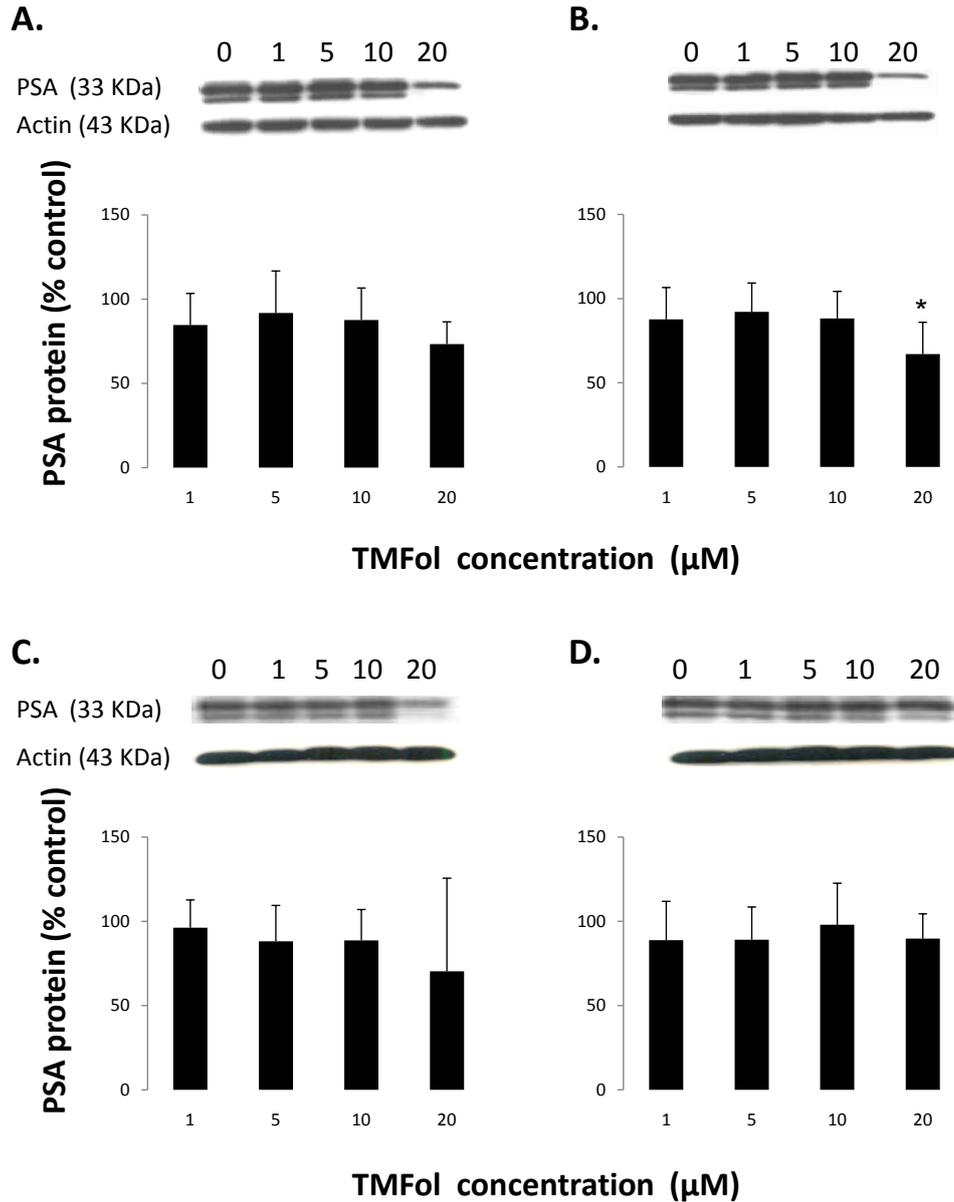


Figure 3.25 Effect of TMFol on PSA protein expression in LNCaP cells grown in absence (A and B) and in presence (C and D) of mibolerone, at 48 h (A, C) and at 72 h (B, D). Cells were incubated with TMFol (1-20 μ M) in the standard media (10% v/v FCS) or in media contains 1nM mibolerone and 5% v/v FCS. Cells also incubated for vehicle controls without TMFol. PSA protein levels were measured by western blot and normalised to actin as a loading control. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of the vehicle control. Values are the mean \pm SD of 3 separate experiments.

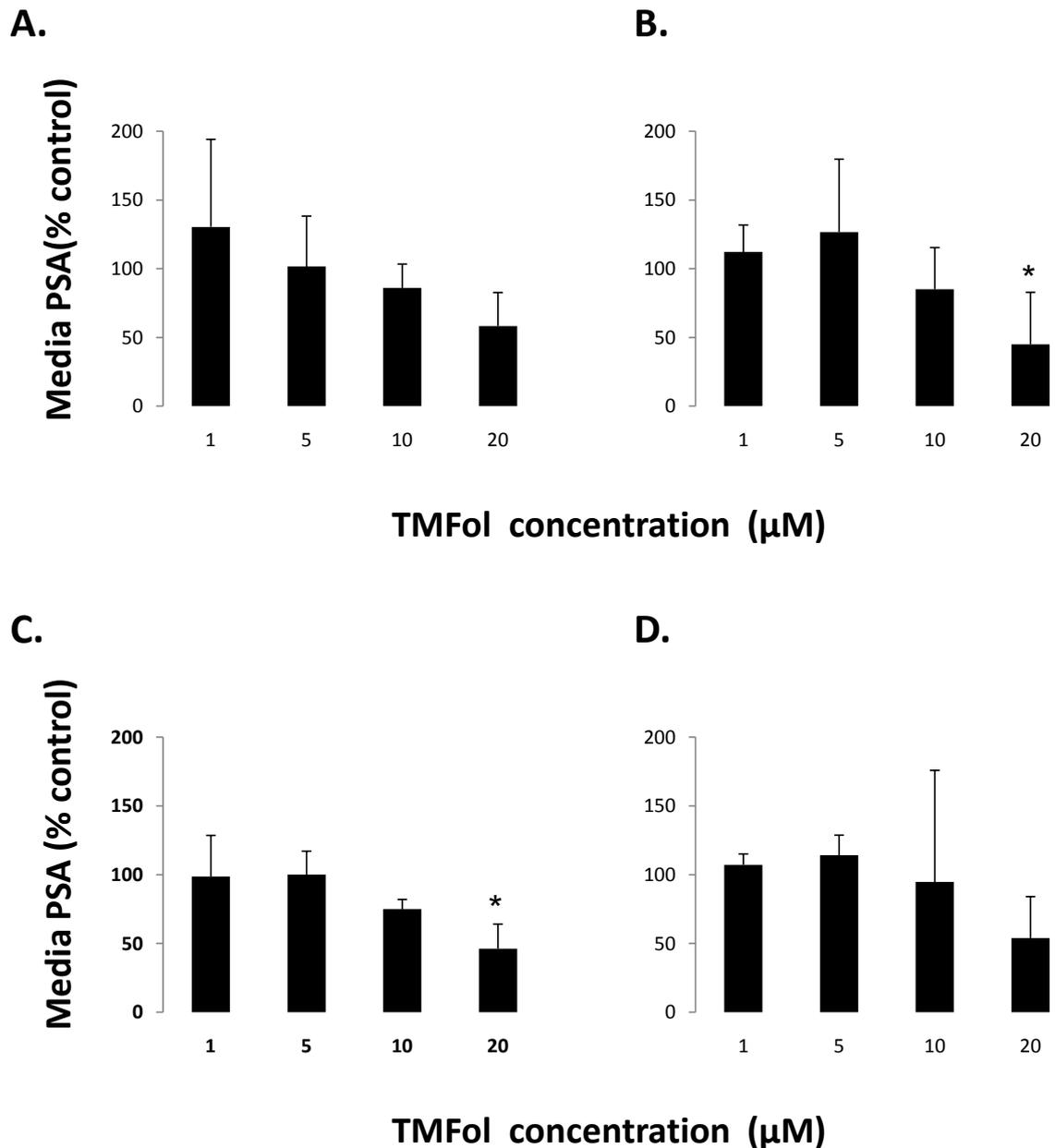


Figure 3.26 Effect of TMFol on secreted PSA levels from LNCaP cells grown in absence (A and B) and in presence (C and D) of mibolerone, at 48 h (A, C) and at 72 h (B, D). Cells were incubated with TMFol (1-20 μM) in the standard media (10% v/v FCS) or in media contains 1nM mibolerone and 5% v/v FCS. Cells also incubated with vehicle controls without TMFol. PSA levels were measured using a PSA Immunoassay kit. The bars represent the values of PSA as a percentage of vehicle control. Values are the mean ± SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.6.5 Effect of TMFol on mRNA levels of androgen receptor and PSA.

To determine whether the reduction observed in AR and PSA protein expression also occurred at the transcriptional level the expression of mRNA for AR and PSA was measured using reverse transcriptase-PCR.

Levels of mRNA of TMFol treated LNCaP cells were measured at 48 and 72 h (Figure 3.27). TMFol treatment resulted in a dose-dependent reduction in AR and PSA mRNA levels at 72h, though this decrease was only significant for 20 μ M., with about 1/2 levels of mRNA compared to the control.

This result is in general is consistent with AR and PSA proteins results, as the expression of these proteins was dependent to TMFol dose and the effect of TMFol is often pronounced at 20 μ M.

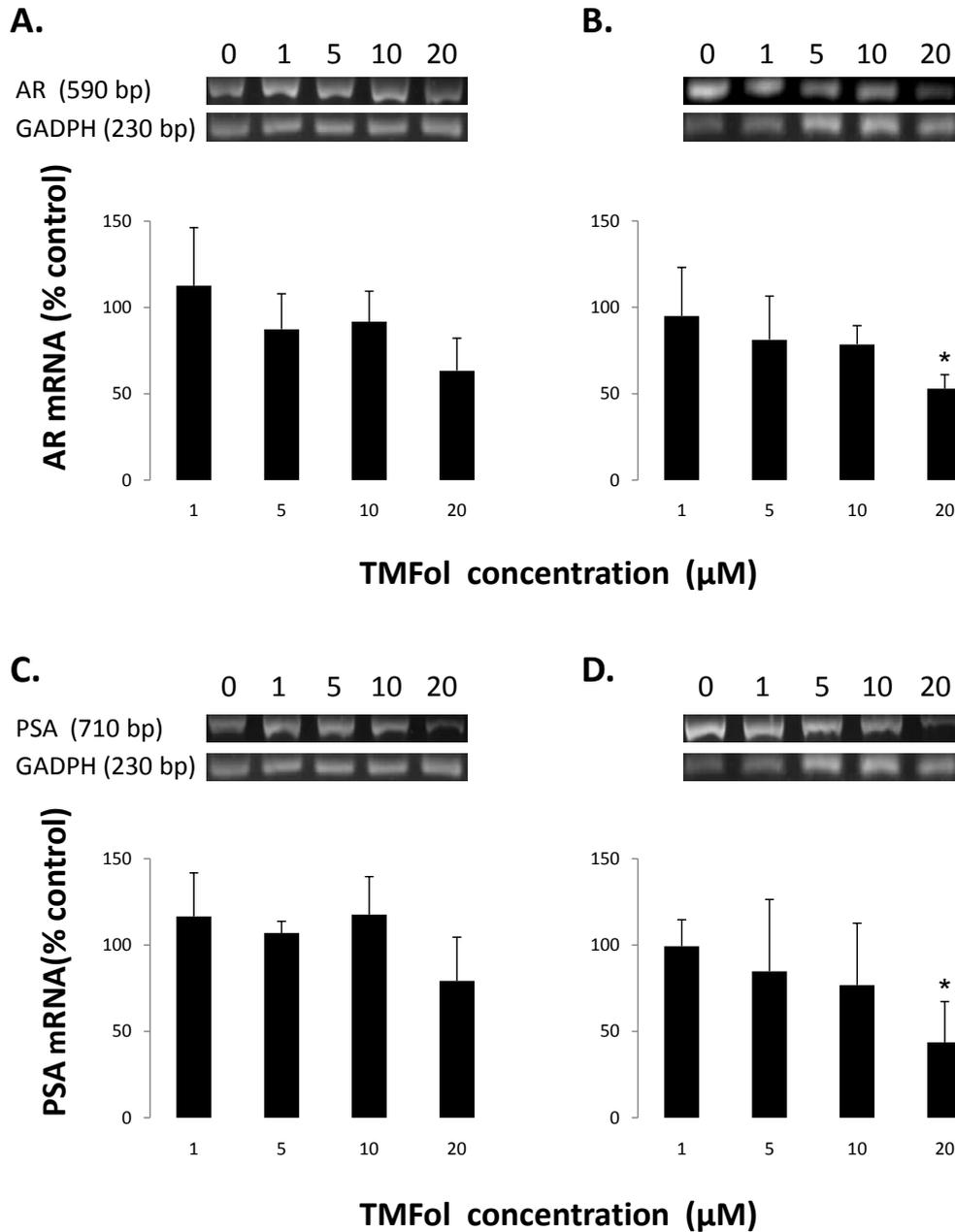


Figure 3.27 Effect of TMFol on mRNA levels of AR (A, B) and PSA (C, D) at 48 h (A, C) and 72 h (B, D) in LNCaP cells. Cells were incubated with TMFol (1-20 μM) or vehicle control. mRNA levels were determined by RT-PCR for AR or PSA and normalised to GAPDH. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean ± SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.6.6 Effect of TMFol on the turnover of androgen receptor and PSA

Fisetin has been reported to increase the rate of degradation of AR in LNCaP cells (Khan et al., 2008b). A change in rate of protein degradation could potentially result in a decrease in protein expression. Previously we studied the effect of TMFol on the transcription of AR and PSA. We wanted to test whether TMFol altered the rate of turnover of these proteins or the downregulation observed was only as a result of inhibition of the transcription.

Cycloheximide inhibits protein synthesis in eukaryotic cells (Baliga et al., 1969), we used it in investigating the effect of TMFol on AR protein degradation after stopping the synthesis of AR protein, as described in section 2.3.11 (Khan et al., 2008b).

Figure 3.28 shows that TMFol had no significant effect on the rate of degradation of AR (A) or PSA (B) over time, in comparison to the vehicle control. This suggests TMFol does not alter the stability of either of these proteins in the LNCaP cells and may function in an alternative fashion to fisetin in its ability to modulate the AR signalling pathway.

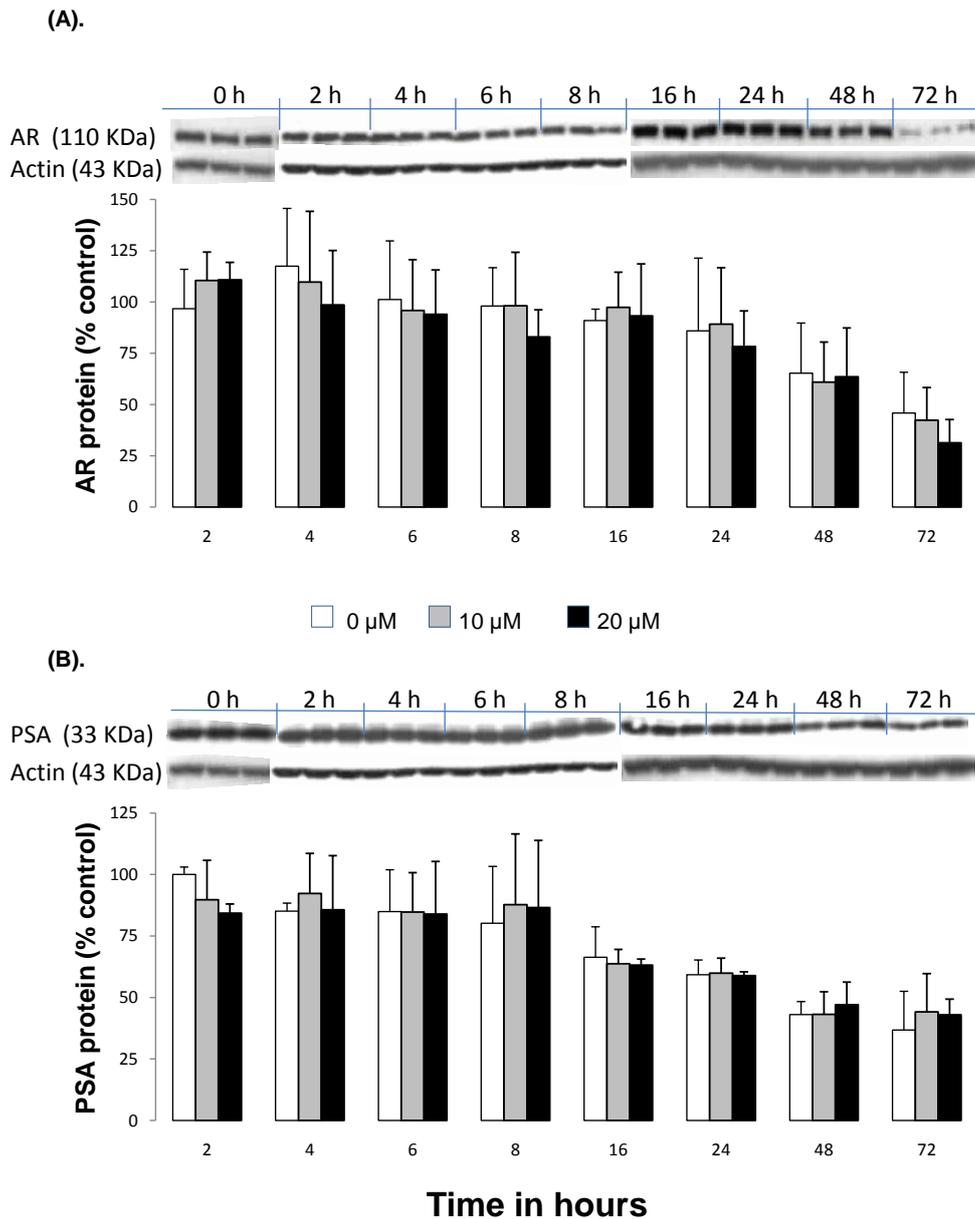


Figure 3.28 Effect of TMFol on protein stability of AR (A) and PSA (B) in LNCaP cells over time after blocking the protein synthesis using cycloheximide. Cells were incubated with TMFol (10 and 20 μM) or vehicle control in the presence of cycloheximide. AR and PSA protein levels were measured by western blot and normalised to actin. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of vehicle control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is statistically significantly different from vehicle control ($p < 0.05$).

3.7 Effect of TMFol on apoptosis related proteins

3.7.1 Effect of TMFol on a panel of apoptosis proteins

Apoptosis analysis performed by flow cytometry showed that TMFol induced apoptosis in the androgen dependent PCa cell lines LNCaP and TRAMP C2. At concentrations of 10 and 20 μM TMFol significantly increased the number of apoptotic cells. Therefore, these concentrations were used to investigate the effect of TMFol on apoptosis related proteins in LNCaP cells at 72h, using a human apoptosis array kit. The expression of the panel of apoptosis related proteins after treatment with 10 and 20 μM TMFol at 72 h can be seen in Figure 3.29

A number of proteins were found to be significantly altered following TMFol treatment. At 20 μM TMFol the proteins significantly up regulated were; bax, Hsp60, p21, p27 and phosph-p53 (S392) and those significantly decreased were bad, caspase and survivin. While at the concentration of 10 μM , TMFol failed to produce significant changes in any of the tested proteins.

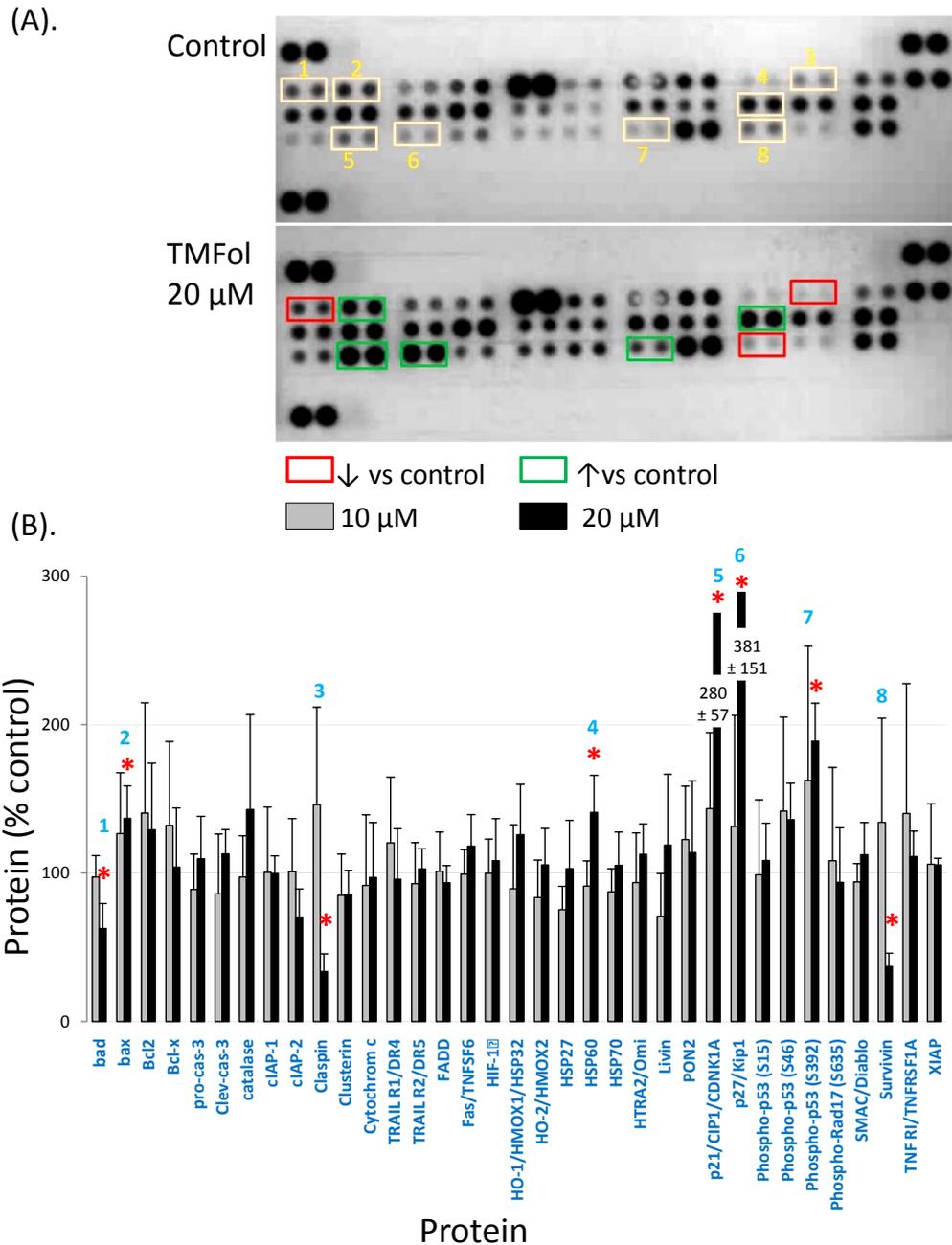


Figure 3.29 Effect of 72 h incubation of TMFol on apoptosis related proteins in LNCaP cells using a human apoptosis array kit. The upper panel (A) represents the dot blot protein expression of control and TMFol (20 μM) treated LNCaP cells on a panel of apoptosis markers. The lower panel (B) provides a graphical representation of the dot band intensity for TMFol (10 and 20 μM) in comparison to vehicle control when all data was normalised to the internal controls. Red rectangles indicate a significant decrease in protein in comparison to control, while green indicates significant increase. Orange rectangles numbered on the control blot highlight the proteins on the graph that were significantly altered. Values are the mean ± SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

3.7.2 Effects of TMFol, quercetin and fisetin on the expression of p53

As TMFol up-regulated the expression of the phosphorylated form of p53 in the apoptosis array kit and has previously been shown to modulate p53 in colorectal cancer models, *Apc^{Min}* mice and human-derived HCT116 adenocarcinoma-bearing nude mice (Howells et al., 2010), the ability of TMFol to modulate total p53 levels was investigated in LNCaP cells. Protein expression levels of p53 after TMFol treatment are shown in Figure 3.30. Effects of quercetin and fisetin on p53 were also investigated under the same conditions but using higher concentrations of 0-60 μM (Figure 3.31).

TMFol up-regulated the expression of p53 in a time- and dose-dependent fashion, however, a significant increase was only observed at 20 μM at the 72 h time point. This observation is consistent with data obtained using the apoptosis array kit, where the greatest changes to apoptotic proteins was observed with the 20 μM concentration of TMFol. These observations taken together could implicate p53 as being an important protein target for the induction of apoptosis in this cell line by TMFol. This data is in part consistent with the annexin V flow cytometry data, in which TMFol produced significant increase in apoptosis at concentration 10 and 20 μM . However p53 data was significant only at 20 μM , nonetheless at 72h TMFol (10 μM) resulted in ~1.5 fold increase in p53 expression.

Both quercetin and fisetin also increased p53 expression, however, only the increase induced by fisetin at 60 μM was significant. Nevertheless, quercetin resulted in a pronounced dose -dependent increase in the level of p53. To illustrate, the p53 levels were raised at 72 h up to ~ 5.5 and ~7.5 fold by 40 and 60 μM quercetin respectively. At 60 μM , fisetin produced ~3.5 and 4 fold significant increase of p53 levels at 48 and 72 h respectively, comparatively, 20 μM TMFol led to ~3 fold increase in the levels at 72 h. Hence, it seemed the 3 flavonols are able to induce p53 in LNCaP cells but at different concentrations. If we considered only the significant results, therefore, this would suggest that TMFol may be a more potent activator of p53 than quercetin and fisetin and therefore potentially a better inducer of apoptosis.

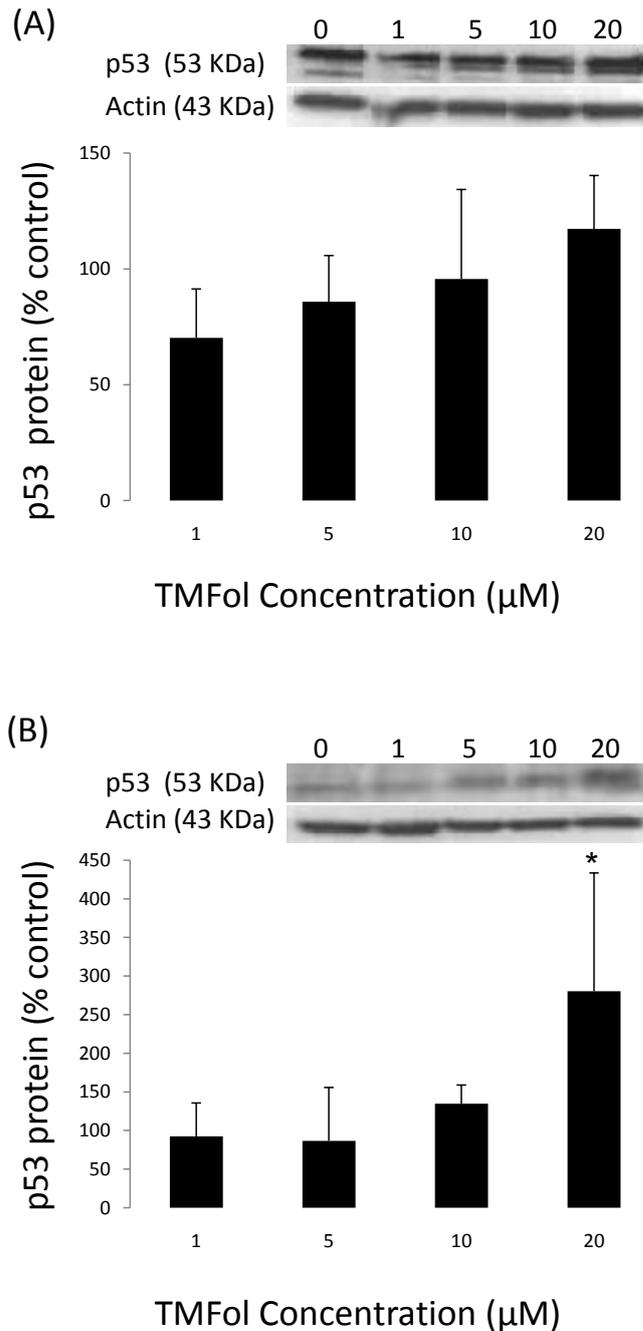


Figure 3.30 The effect of TMFol on p53 protein levels, in LNCaP cells at 48 (A) and 72 h (B). Cells were incubated with TMFol (1-20 μM) or vehicle control. Protein levels of p53 were measured by western blot and normalised to actin. The bar chart illustrates the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

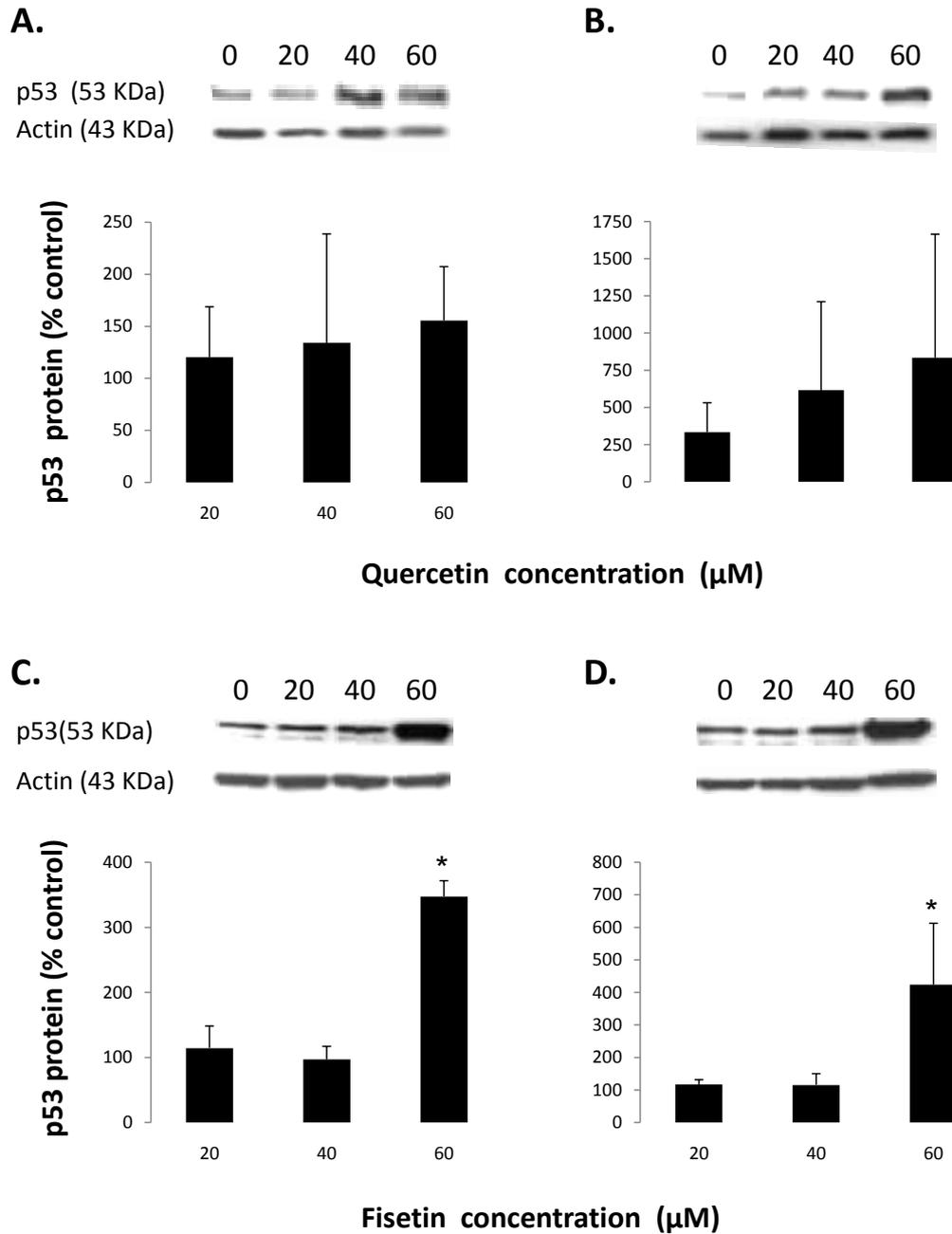


Figure 3.31 Effects of quercetin (A, B) and fisetin (C, D) on p53 protein expression in LNCaP cells, at 48 h (A, C) and at 72 h (B, D). Cells were incubated with quercetin or fisetin (20-60 μ M) or vehicle control. Protein levels of p53 were measured by western blot and normalised to actin. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

3.7.3 Effect of TMFol on the expression of the apoptotic proteins, bax and survivin, in TRAMP C2 cells.

The effect of TMFol on the expression of apoptotic proteins which are significantly altered in the human apoptosis array kit was further investigated. It was decided to examine the effect of TMFol on the expression of bax and survivin in the TRAMP C2 cell line, as they both are previously suggested as markers for apoptosis in PCa (Chia et al., 2000, Mackey et al., 1998, Scopa et al., 2001, Duffy et al., 2007, Zhang et al., 2010b). This cell line was selected as we wanted to investigate markers that we could also study *in vivo*. As we had encountered problems and had limited success with the LNCaP cell xenograft model we instead employed the TRAMP C2 cell xenograft model system to study the *in vivo* efficacy of TMFol (Chapter 5).

Figure 3.32 shows the effect of TMFol (1-20 μ M) on bax and survivin expression in TRAMP C2 cells at 48 and 72 h. Bax levels were significantly upregulated with TMFol at 20 μ M, TMFol produced a ~1.5 fold increase in the expression of bax at 48 h and ~2 fold at 72 h. This data is consistent with our previous observations in the LNCaP cells (Figure 3.29) where TMFol significantly induced bax expression. Surprisingly, at 72 h survivin levels were up-regulated in a dose- and time-dependent manner upon TMFol treatment, levels were raised up to ~2 and ~3 fold at 10 and 20 μ M respectively, (Figure 3.32 C and D). This data are in contrast to our previous finding in LNCaP cells (Figure 3.29) as TMFol (20 μ M) significantly reduced the expression of survivin by more than 2 fold.

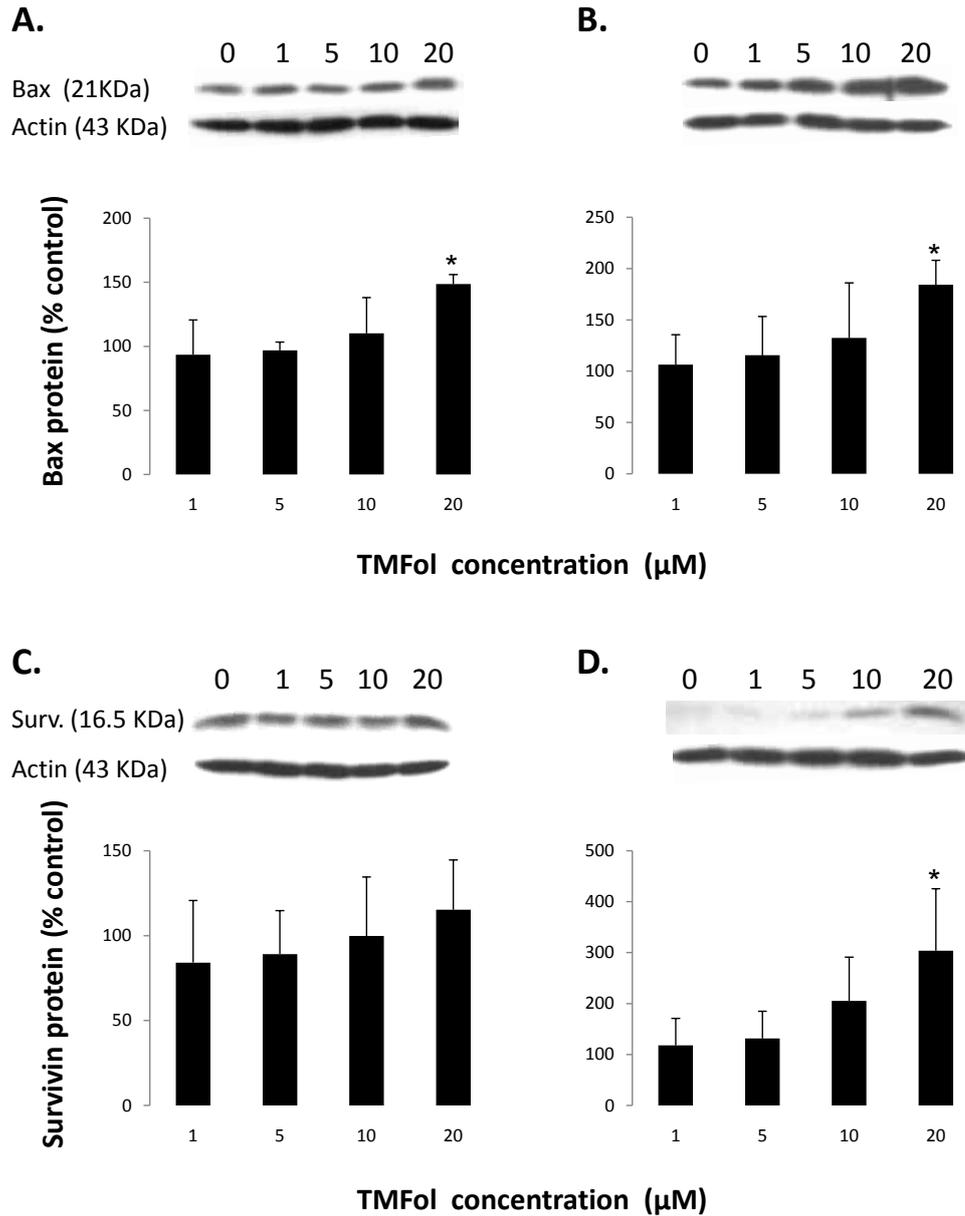


Figure 3.32 Effect of TMFol on bax (A, B) and survivin (C, D) protein expression in TRAMP C2 cells, at 48 h (A, C) and at 72 h (B, D). Cells were incubated with TMFol (1-20 μM) or vehicle control. Protein levels were measured by western blot and normalised to actin. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean ± SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

3.8 Effect of TMFol on p21 and p27 expression

3.8.1 Effect of TMFol, quercetin and fisetin on the expression of p21

From previous experiments we had shown that TMFol was capable of inducing cell cycle arrest, apoptosis and the ability to modulate the expression of p53. As p21 is an important cell cycle regulatory protein and a target gene of p53 that had also been shown to be up-regulated in the apoptosis array kit we investigated its expression in both a p53 wild-type (LNCaP) and a p53 null (PC-3) cell line. For TRAMP C2 cells we could not detect any p21 either *in vivo* or *in vivo* samples.

TMFol induced a dose-dependent increase in p21 expression in LNCaP cells, TMFol (20 μ M) resulted in a ~3 fold statistically significant increase observed at 72 h, the same concentration at 48 h produced an about ~2 fold increase in the levels but without reaching significance (Figure 3.33 A and B). This observation is consistent with data generated using the apoptosis array kit (Figure 3.29), where TMFol at the same concentration significantly induced the expression of p21 by ~3 fold in the same cells, additionally it consistent with the knowledge that p21 is a target gene of p53 (Eldeiry et al., 1993)

Basal expression of p21 in PC-3 cells was very low, possibly in part due to the p53 null status of the cell line and as such no significant effect was observed with TMFol treatment (Figure 3.33 C and D).

Expression of p21 in LNCaP cells following quercetin and fisetin treatment was very variable. Quercetin increased the expression of p21 at 72 h but this did not reach the level of significance (Figure 3.34 A and B). Interestingly, fisetin at both time points significantly decreased p21 protein expression at 72 h with a concentration of 60 μ M (Figure 3.34 C and D).

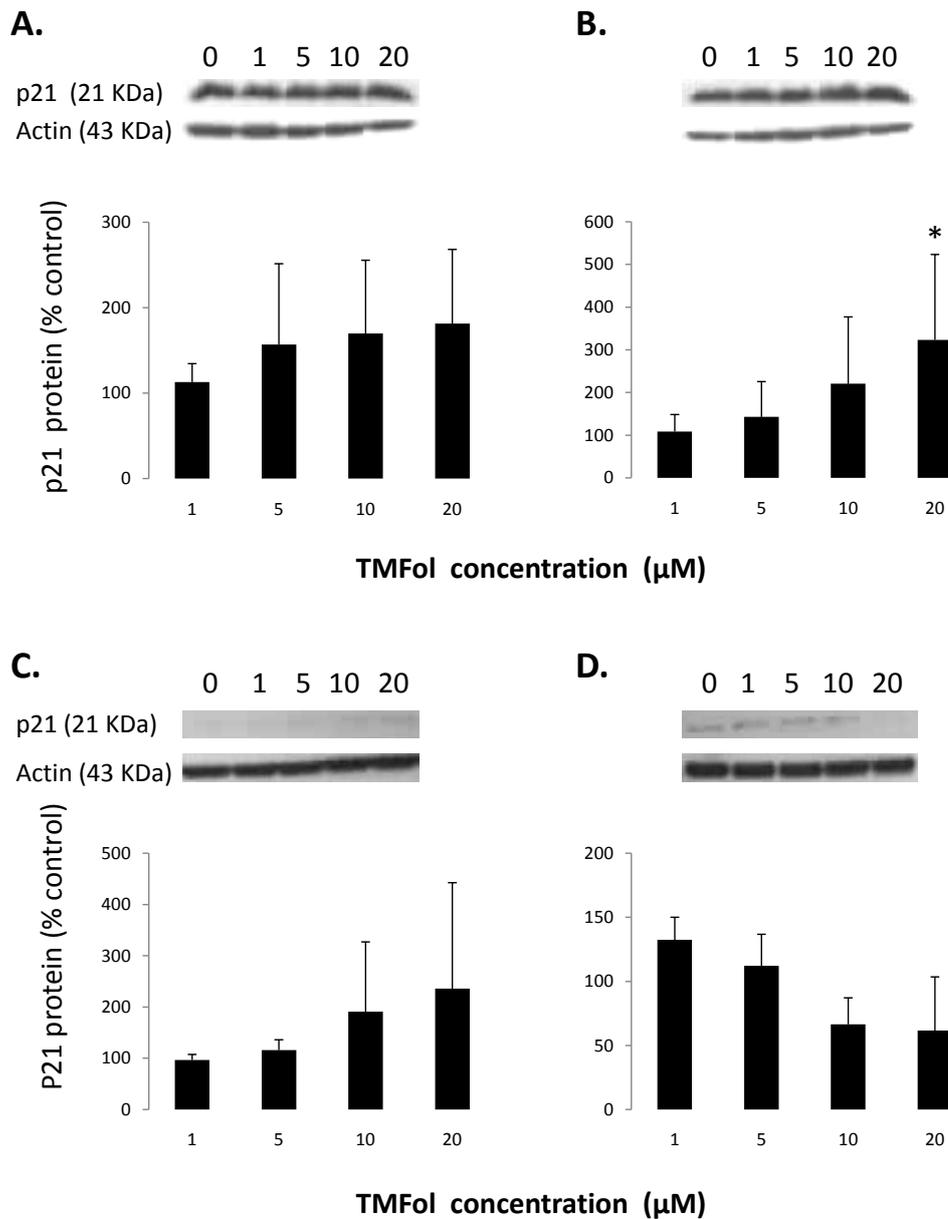


Figure 3.33 Effect of TMFol on p21 protein expression in LNCaP cells (A, B) and PC-3 cells (C, D), at 48 (A, C) and 72 h (B, D). Cells were incubated with TMFol (1-20 μM) or vehicle control. Protein levels were measured by western blot and normalised to actin. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean ± SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

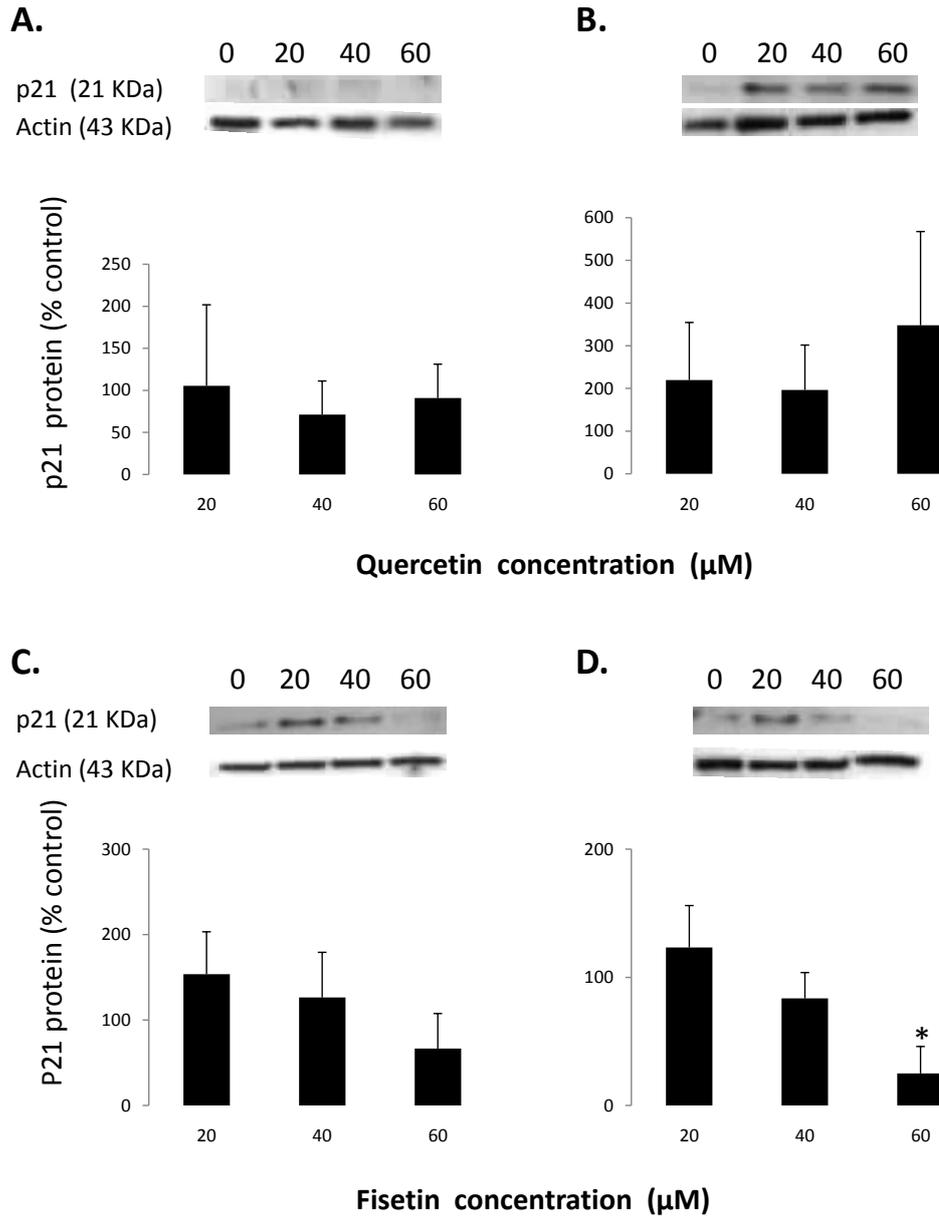


Figure 3.34 Effect of quercetin (A, B) and fisetin (C, D) on p21 protein expression in LNCaP cells, at 48 h (A, C) and at 72 h (B, D). Cells were incubated with quercetin or fisetin (20-60 μ M) or vehicle control. Protein levels were measured by western blot and normalised to actin. The bar charts illustrate the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the mean \pm SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

3.8.2 Effect of TMFol on the expression of p27.

The cell cycle regulatory protein p27 has been shown to be inversely correlated with prostate cancer progression (Macri and Loda, 1998). It was also found to be linked to the AR in that androgen depletion induced its expression (Balk and Knudsen, 2008). As we had shown previously that TMFol could induce cell cycle arrest and also modulate the expression of the AR, we investigated the effect of TMFol on p27 expression in LNCaP cells with and without androgen stimulation (Figure 3.35).

TMFol induced the expression of p27 at 20 μ M by ~1.5 and ~2 fold increase at 48 and 72 h respectively in the LNCaP cells in the absence of the androgen mibolerone (Figure 3.35 A and B). In the presence of mibolerone (Figure 3.35 C and D) TMFol had a profound increasing effect on p27 expression in a time- and dose-dependent manner. At 48 h, the fold increases were ~5, 20 and 45 produced by TMFol concentrations of 5, 10 and 20 μ M respectively. While at 72 h the fold increases were ~3, 14 and 150 respectively. Although, of the high levels of p27 produced by TMFol treatment in mibolerone group, the data was only statistically significant at 10 and 20 μ M TMFol of 72 h time point. This can be contributed to the high variability in spot densities observed with mibolerone group.

Androgens have been shown to increase the rate of degradation of p27 (Balk and Knudsen, 2008), which is clear from comparing the vehicle controls from the experiments with and without mibolerone. In this instance, TMFol could be antagonising the effects of mibolerone.

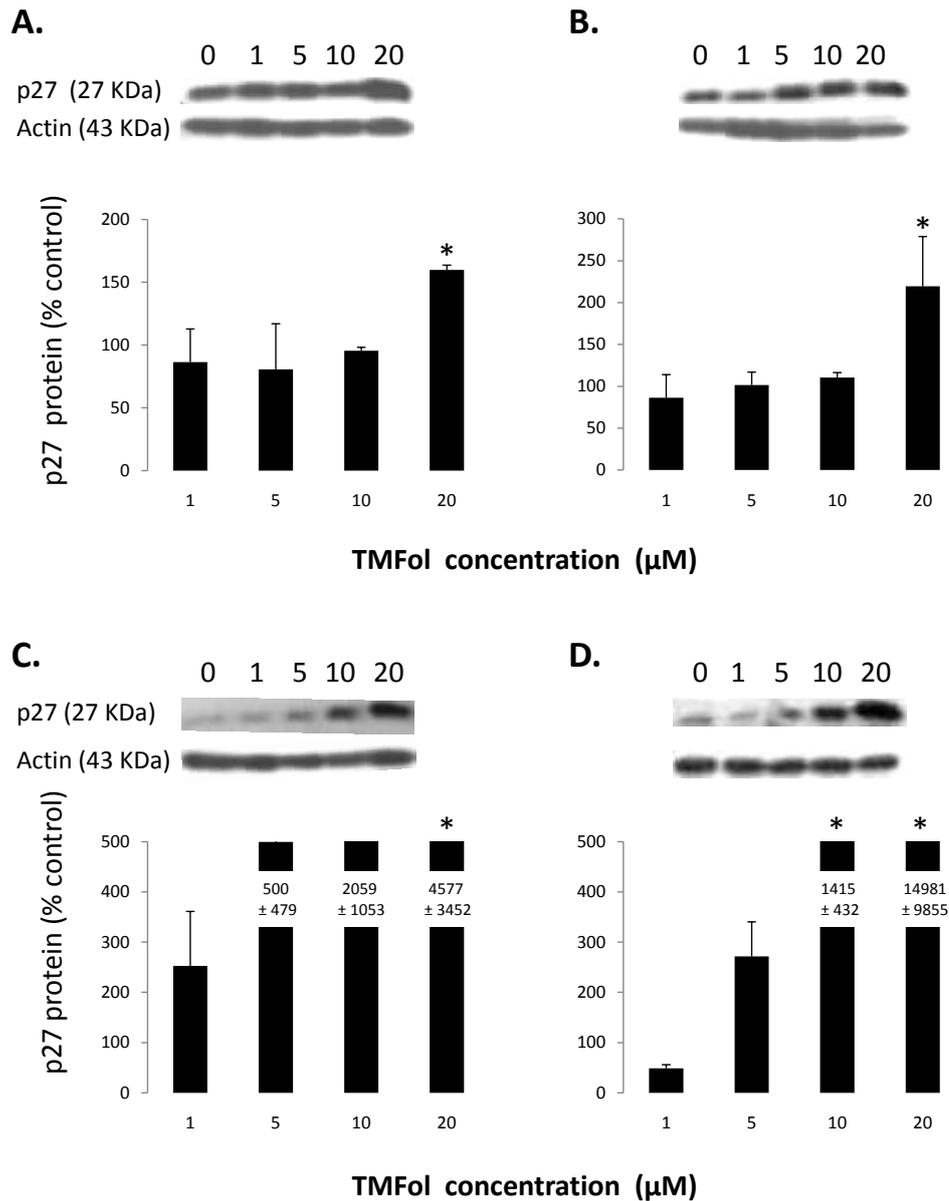


Figure 3.35 Effect of TMFol on p27 protein expression in LNCaP cells grown in 10% v/v FCS media (A, B) or in 10% v/v FCS media supplemented with 1nM mibolerone (C, D), for 48 h (A, C) and for 72 h (B, D). Cells were incubated with TMFol (1-20 μM) or vehicle control in the presence or absence of 1 nM mibolerone. p27 Protein levels were measured by western blot and normalised to actin. The bars are the results of semi-quantitative densitometry analysis of band densities represented as percentage of control. Values are the means \pm SD of 3 separate experiments. (*) indicates data is significantly different from vehicle control ($p < 0.05$).

3.9 Discussion

Based on observations in our laboratory that insertion of methoxy instead of hydroxyl groups into the flavone scaffold may improve chemopreventive activity in colorectal cancer (Cai et al., 2009), we wished to explore whether such actions could be translated to other cancer types, most notably prostate cancer (PCa). To that end we screened a panel of methoxylated flavonoids in 3 PCa cell lines (LNCaP, PC-3 and DU145). It was supposed the panel consisted of: a group of methoxylated flavones (compounds 1-12 and PMF) and a methoxylated-hydroxylated flavone (tricin) and a hydroxylated flavone (apigenin) (for chemical structure see table 2.1). Out of all the compounds tested one agent stood out as the most potent in the LNCaP, PC-3 and DU145 cells compared to the rest of series, therefore, it was selected as the lead compound for further investigation. Following further characterization of this compound, it was identified as a trimethoxyflavonol (3', 4', 5'-trimethoxyflavonol) or TMFol (a flavonol) but not a flavone as we thought before the screening. As a consequence, the biological activity of TMFol was subsequently compared to quercetin and fisetin, the most widely studied flavonols to date.

The literature evidence for the antiproliferative effects of hydroxylated versus methylated flavones is controversial, with some studies favouring the methylated agents (Walle et al., 2007b, Walle and Walle, 2007a, Walle, 2007a) and a few reports favouring the hydroxylated counterparts (Landis-Piwowar et al., 2008).

TMFol was found to be the most potent antiproliferative flavonoid among the 15 flavonoids tested. After TMFol, apigenin was the second most potent agent with regard to growth inhibition in all three cell lines. Apigenin, a trihydroxyflavone, had superior growth-inhibitory effects over tricetin and 3', 4', 5', 5, 7-pentamethoxyflavone (PMF), as well as the other methoxyflavones (Table 2.1) investigated as part of this compound screen. Unlike colorectal cancer, where the introduction of methoxy residues into the flavone scaffold imparted greater chemoprevention activity over the hydroxyl counterparts (Cai et al., 2009, Sale et al., 2009) the same effect was not observed for the prostate cancer cell lines

since apigenin was found to be the most potent out of all the flavones tested. The improved activity with degree of methoxylation may be cancer type specific as well as being dependent on the class of agent. Of the three cell lines used the PC-3 cells appeared to be the most sensitive to the growth-inhibitory effects of the compounds, followed by LNCaP and then finally the DU145 cell line.

In comparison to members of the same class (the flavonols) TMFol came up with IC₅₀ values of 4.1, 5.6 and 1.7 μ M in the PC-3, LNCaP and TRAMP C2 cell lines, respectively. These IC₅₀ values were 5.5 to 16-fold lower than those for quercetin or fisetin. Although, we showed improved activity of a methoxyflavonol (TMFol) over its hydroxyl counterparts as TMFol had superior growth-inhibitory activity to that of quercetin or fisetin. Further methoxy flavonols would need to be tested before definitive statements regarding these observations can be made. However, we can conclude that TMFol is the most potent agent identified from this compound screen and therefore warrants further investigation aimed at determining its mechanisms of action.

It is well documented in the literature that flavonols inhibit the growth of PCa cells by modulating the cell cycle (Haddad et al., 2010, Knowles et al., 2000). The effect of TMFol on the cell cycle was investigated to explain its growth inhibitory effect on PCa cells. TMFol induced cell cycle arrest in all the tested cell lines but at different phases. In androgen-dependent cancer cells (LNCaP and TRAMP C2) TMFol induced a G₂/M arrest, whereas TMFol caused S phase arrest in the androgen-independent PC-3 cells. LNCaP and TRAMP C2 cells both express p53, whereas PC-3 is p53 null (Polek et al., 2003). Therefore, p53 expression may be important in the observed G₂/M arrest. These results potentially identify two molecular targets of TMFol to suggest the differences observed in the cell lines used. These are the androgen receptor signalling pathway and the p53 signalling pathway for the LNCaP and TRAMP C2 cells. As PC-3 cells are null for both proteins (AR and p53) and TMFol induced growth-inhibitory effects in this cell line an alternative mechanism of action must be responsible.

Our results for TMFol on the cell cycle of prostate cancer cells are consistent with that reported for other flavonols. Fisetin and quercetin both arrested LNCaP cells at the G₂/M phases using concentrations between 20-35 μ M (Haddad et al., 2006, Shenouda et al., 2004), whereas PC-3 cells were arrested in the S phase with 100 μ M quercetin (Knowles et al., 2000) or again in the G₂/M phase with both quercetin and fisetin at 20-35 μ M (Haddad et al., 2006). These results suggest that TMFol may affect the cell cycle of prostate cancer cells in a similar manner to that of quercetin or fisetin, the difference being that TMFol can exert these effects at a much lower concentration (10 μ M compared to 20-100 μ M for quercetin and fisetin). This activity is consistent with the difference observed in potency between these three agents on cell growth inhibition.

Induction of apoptosis is another mechanism by which the growth inhibition with TMFol can be explained and as such was investigated in the LNCaP, TRAMP C2 and PC-3 cells. Flavonols have been found to induce apoptosis in PCa cell lines (Khan et al., 2008a, Aalinkeel et al., 2008). TMFol was able to induce a profound increase in the number of apoptotic cells in androgen receptor positive cells (LNCaP and TRAMP C2) with between a 6 and 25-fold increase in the extent of apoptosis at 20 μ M compared to solvent treated control incubations. Significant induction was observed in the TRAMP C2 cell line at concentrations as low as 5 μ M TMFol. In PC-3 cells, TMFol induced between 2 and 5 fold increase in the proportion of apoptotic cells, and was significant at 20 μ M. PC-3 cells are AR null and p53 null, whereas LNCaP and TRAMP C2 both express AR and have wild-type p53. Since the effect was much greater in AR and p53 expressing cells and the modulation of AR and p53 was linked to apoptosis (Alimirah et al., 2007, Coffey et al., 2002, Mirza et al., 2002), this could suggest that the AR and/or p53 pathways are important for the induction of apoptosis in the LNCaP and TRAMP C2 cells and are targets of TMFol. Fisetin has been shown to induce apoptosis in the LNCaP cells at concentrations between 10-60 μ M (Khan et al., 2008a) and quercetin induced apoptosis in the same cell line but at a higher concentration of 100 μ M (Lee et al., 2008). Similar to the effects on cell cycle, TMFol is able to induce apoptosis in prostate cancer cells

consistent with other flavonols. TMFol may be superior to quercetin and fisetin, as lower concentrations of TMFol were needed to exert these actions and these observations complement the potency of these agents in terms of growth-inhibitory effects described earlier.

The hypothesis was tested that TMFol modulates the expression of the AR in LNCaP and TRAMP C2 cells, which in turn induces cell cycle arrest and apoptosis. This potential mechanism of action may be of benefit in the clinical setting in preference to the use of androgen depletion therapy. Androgen depletion therapy, over time, can lead to mutation of the AR, and as such it can then be stimulated by other steroids or even androgen antagonists and the cancer can then develop once more (Culig et al., 2002). The ability of quercetin to down regulate AR expression in LNCaP cells was considered an attractive approach for the prevention and treatment of PCa (Xing et al., 2001, Khan et al., 2008b).

In comparison to quercetin and fisetin, TMFol showed an enhanced ability to downregulate AR expression in LNCaP cells. TMFol reduced AR protein levels by approximately 65% at 10 μ M while the same reduction by fisetin was achieved at 40 μ M. Quercetin at 40 μ M was only able to reduce AR expression by 30%. In the next chapter we show that the levels of TMFol detected in the prostate tissue of mice range from steady state levels of \sim 5 μ M to a maximal concentration of approximately 20 μ M after administration of a dose of 240 mg/kg. Therefore, it may be possible to achieve the concentrations of TMFol needed to reduce AR expression *in vitro* in prostate tissue of humans. However, this is unlikely to be the same in the cases of quercetin or fisetin as they are rapidly metabolised and excreted *in vivo* and are unlikely to reach the levels of 40 μ M in the target tissue using similar dose. However, this dose of TMFol might not be possible to administer in case of humans, also human body might deal with TMFol in different way as mouse. Therefore, all the variation between human and mouse must be taken in account.

PSA is a well validated target gene for AR and its presence and systemic concentration are used as a marker of prostate cancer progression (De Angelis et al., 2007). Consistent with the downregulation of AR protein expression, the inhibition of PSA protein expression was also observed with TMFol at 20 μ M. In the presence of mibolerone PSA protein levels were not reduced. Interestingly, when secreted levels of PSA were measured in the cell media in both the absence and presence of mibolerone a reduction was observed at 20 μ M relative to control incubations. Protein expression data for both the AR and PSA was complemented by the mRNA expression data. TMFol reduced the level of mRNA for both the AR and PSA at 20 μ M. Differences in the expression of the AR and PSA could be explained by the rate of degradation of these proteins in the presence of flavonols. It has been shown previously that fisetin can enhance the rate of AR degradation in LNCaP cells (Khan et al., 2008b). When these experiments were performed under the same conditions with TMFol no difference in rate of degradation was observed, which highlights a potential difference in the compounds mechanisms of action on the AR signalling pathway.

In summary, these studies on the AR signalling pathway have shown the ability of TMFol to downregulate AR and PSA protein expression in PCa cells through, in part, the inhibition of AR and/or PSA transcription. Modulation of the AR pathway could also be one of the mechanisms by which TMFol induced cell cycle arrest and apoptosis in the AR expressing cell lines. Our results also suggest that TMFol is a more potent inhibitor of the AR pathway at equimolar concentrations and therefore warrants further investigation in preference to either quercetin or fisetin.

The growth of the prostate and the development of prostate cancer are dependent on the AR. Hormone ablation therapy is one of the most common treatment options for PCa and it can reduce cancer growth by reducing the levels of circulating androgens and therefore prevent the activation of the AR (Isaacs and Isaacs, 2004). After prolonged treatment the tumour becomes hormone-refractory and the patient's cancer fails to respond to further hormone treatment (Potvin and Winqvist, 2008). Some of the many mechanisms that

lead to this event include increased AR protein expression, increased AR gene amplification and an increased number of AR mutations (Koivisto et al., 1996, Tomlins et al., 2006, Waltering et al., 2009). Therefore, a preventive approach that inhibits AR expression in preference to reducing androgens that stimulates its activation may be a more attractive treatment and reduce the unwanted drawbacks that are present with hormone ablation therapy.

The tumour suppressor p53 protein is involved in cell cycle arrest and apoptosis (Giono and Manfredi, 2006) and has also been shown to be a negative modulator of AR expression (Alimirah et al., 2007, Cronauer et al., 2004). Taken together with the significant effects of TMFol on cell cycle and apoptosis in AR and p53 expressing cells, that potentially suggest a role for p53 in the effects we observed in the LNCaP and TRAMP C2 cells. In addition, TMFol has been shown to be an activator of p53 in other cancer cell types (Howells et al., 2010). TMFol increased the protein expression of both phosphorylated p53 and total p53 in LNCaP cells.

In addition, TMFol positively modulated p21 protein in LNCaP cells. The p21 gene is a target gene of p53 and therefore that increase might be due to p53 (Eldeiry et al., 1993). In PC-3 cells, which are p53 null, TMFol failed to significantly increase p21 expression. This data suggests that the activation of p21 protein expression observed in the LNCaP cells was p53 dependent. The cyclin dependent kinase inhibitor p21 is also a powerful cell cycle modulator, and can induce cell cycle arrest at either the G₁ or G₂/M phase (Eldeiry et al., 1993). This suggests that the observed G₂/M cell cycle arrest observed with TMFol treatment in the LNCaP cells was via p21 upregulation. The published effects of quercetin and fisetin on protein levels of p53 and p21 in PCa cells are controversial and inconclusive (Haddad et al., 2005, Vijayababu et al., 2005, Kobayashi et al., 2002, Ma et al., 2004c, Khan et al., 2008a) (Nair et al., 2004, Park et al., 2008). Quercetin and fisetin both increased the expression of p53 in the LNCaP cells. Fisetin increased the expression significantly but only at 60 µM. Quercetin increased the expression in a dose-dependent manner but the increase did not reach significance. Consistent with p53 activation the expression of p21 was also increased with quercetin, with the most pronounced

effect observed with 60 μ M. This increase was not significant. Interestingly, fisetin significantly downregulated the expression of p21 in the LNCaP cells at 60 μ M. The decrease in the expression of p21 by fisetin could be a result of the AR down regulation observed. Fisetin reduced AR expression by more than 90 % at 60 μ M and taking into consideration that AR expression can be directly reflected in p21 expression (Alimirah et al., 2007, Lu et al., 1999) this could explain these observations.

In PC-3 cells the effect of TMFol on p21 expression is inconclusive. As the cell line is null for p53 the basal expression of p21 is also very low, therefore, any modulation by TMFol is difficult to detect. Because of the low expression it is unlikely the cell cycle arrest and growth arrest observed with TMFol can be attributed to the effects on p21. Furthermore, the role for p21 in prostate cancer is uncertain, with many researchers suggesting that p21 is more critical to the enhancement of PCa cell proliferation rather than cell cycle arrest (Balk and Knudsen, 2008). Therefore, more experiments are warranted to investigate the effects of TMFol on p21 expression and the importance of this on prostate cancer prevention and management.

The effects of TMFol on p27, another important member of the CDKI family, were studied in LNCaP cells. The apoptosis array kit had shown a significant induction of p27 expression following TMFol treatment and this was investigated further. The protein expression of p27 was measured in LNCaP cells in both the absence and presence of the androgen, mibolerone, as androgens have been found to increase the degradation rate of p27 (Balk and Knudsen, 2008). The p53 and AR pathways have also been reported to modulate the expression of p27. TMFol significantly increased the expression of p27 in the absence of mibolerone at 20 μ M. In the presence of mibolerone the increase in p27 expression was more pronounced with a significant 150-fold increase observed with 20 μ M TMFol. Consistent with previously published findings we observed that addition of the androgen reduced the expression of p27 expression in control samples, most likely through increased rate of degradation (Balk and Knudsen, 2008). As we have shown TMFol has the ability to activate p53 and

inhibit the AR pathway it is possible that the increase in protein expression of p27 is through the AR-p53 signalling pathways.

Furthermore, these findings are consistent with fisetin. Fisetin has been shown to induce G₂/M arrest (Haddad et al., 2005, Haddad et al., 2006), and downregulate the expression of G₂/M phase proteins at 25 µM (Haddad et al., 2010). In addition, fisetin also increased the expression of p27 in the LNCaP cell line between 20 and 60 µM (Khan et al., 2008a). The concentrations of fisetin required to exert activity on the cell cycle are comparable or higher than those observed for TMFol. As TMFol was ~7 fold superior in terms of growth inhibition in LNCaP in comparison to fisetin it is unlikely that effects on the cell cycle alone can explain this discrepancy, therefore, additional mechanisms could be responsible.

Evidence would suggest that p27 has a pivotal role in cell cycle progression (Guo et al., 1997). However, p27 has been proposed to have tumour suppressive activity as well (Shaffer et al., 2004). The expression of p27 is progressively downregulated in response to higher grade tumours, and this correlated with an increase in the aggressive behaviour of the PCa (Macri and Loda, 1998). Mutations of p27 in tumours are rare (Lee and Kim, 2009), so its expression is mainly regulated post transcriptionally. The protein expression in tumours is modulated by proteolysis and as such its re-expression does not require gene stimulation (Nickeleit et al., 2007). Therefore, based on these properties p27 could make an attractive target for cancer prevention and therapy. Literature evidence already exists to suggest that p27 could be a marker for PCa diagnosis and prognosis (Lloyd et al., 1999).

As part of the apoptosis array kit a number of other apoptotic proteins were significantly altered by TMFol treatment. The most significant of these were survivin and bax. Survivin has been associated with the inhibition of apoptosis (Dohi et al., 2004), regulation of the cell cycle (Altieri, 2010) and induction of angiogenesis (Tran et al., 2002). Survivin was also found to mediate the resistance of PCa cells to antiandrogen therapy (Zhang et al., 2005). Survivin is rarely expressed in normal tissue, but is over expressed in most malignant

tissues. These findings have highlighted the potential of survivin as a biomarker for disease as well as a novel target for cancer therapy (Duffy et al., 2007). In addition, survivin plays an important role in mitotic progression and cytokinesis (Li et al., 1999) and its protein expression is significantly higher in the G₂/M phase of the cell cycle (Li and Altieri, 1999). TMFol significantly down regulated the expression of survivin in LNCaP cells. Survivin expression has been suggested to be inversely related to wild-type p53 (Shao et al., 2010), and was found to be repressed by wild-type p53 at the mRNA level (Mirza et al., 2002). Therefore the downregulation of survivin may be linked to the up-regulation of p53. In addition, a positive correlation has been reported between AR activation and survivin expression. This is supported by the observation that blocking AR using flutamide reduced survivin expression in LNCaP cells (Zhang et al., 2005). Therefore, as TMFol has been shown to inhibit AR expression and activity one would expect the expression of survivin to be inhibited. Importantly quercetin was found to down regulate survivin gene and survivin protein expression in PCa cells at between 50 and 100 μ M (Kim et al., 2008). This data taken with our findings would suggest that TMFol is 2-5 fold more potent for the inhibition of survivin protein expression.

The pro-apoptotic member of the bcl-2 family bax was found to be up-regulated in LNCaP and TRAMP C2 cells after TMFol treatment. Bax expression has been associated with downregulation of the AR and activation of p53 protein. Androgens have been found to have anti-apoptotic effects in LNCaP cells, by the inhibition of bax mRNA levels (Coffey et al., 2002) and p53 has been reported to transactivate the bax gene and also activate it directly (Miyashita et al., 1994). Furthermore, the ability of p53 to induce apoptosis in the TRAMP model is partially dependent on bax up-regulation (Hernandez et al., 2003), therefore, as TMFol activates bax one would predict that apoptosis would be observed in the wild-type p53 expressing TRAMP C2 cells. The fact that the TRAMP C2 cell line was the most sensitive to TMFol-induced apoptosis supports this notion.

Quercetin was found to induce apoptosis in LNCaP cells through the activation of bax expression and the inhibition of bad protein phosphorylation and also

decreasing the Bcl-xL:bax ratio (Lee et al., 2008). Quercetin was shown to inhibit the expression of the AR (Xing et al., 2001) and upregulate p53 protein in LNCaP cells (Park et al., 2008) and upregulate the p53 gene in PCa cells (Nair et al., 2004). These observations are all consistent with the actions of TMFol and to some degree are expected as they are the same class of molecule. TMFol is able to exert these effects at much lower concentrations though and all the results taken together suggest the involvement of the AR and/or p53 pathway in the induction of apoptosis in LNCaP cells.

To conclude, methoxylated flavones do not appear to be superior in terms of antiproliferative activity over their hydroxylated counterparts in PCa cells. However the methoxylated flavonol TMFol showed superior growth inhibition to all other agents tested as well as in comparison to other hydroxylated flavonols, quercetin and fisetin.

On further investigation TMFol induced apoptosis and cell cycle arrest in PCa cells and modulated a number of the key regulatory proteins involved in these processes. Downregulation of AR protein expression and activity is possibly another mechanism by which TMFol exerts its growth-inhibitory effects. When TMFol was compared vis-à-vis quercetin and fisetin for its ability to modulate these proteins TMFol was the superior, since lower concentrations were needed to impart similar activity. As such, TMFol should be prioritised ahead of the other flavonols quercetin and fisetin, for potential development as an anti-prostate cancer agent. Studies to investigate its pharmacokinetics and biodistribution as well as its efficacy *in vivo* are fully warranted. In addition, the AR signalling pathway as well as the apoptotic proteins, p27, bax and survivin warrant further investigation *in vivo* as potential targets for TMFol activity.

CHAPTER FOUR

Metabolism and bio-distribution studies of TMFol

4.1 Introduction

Many flavonoids have been shown to possess anti-proliferative properties *in vitro* but studies *in vivo* do not always translate these observations. This to some extent can be explained by their poor bioavailability due to lower absorption and/or rapid metabolism (Walle, 2004, Scalbert and Williamson, 2000, Gao and Hu, 2010). Generally, flavonoids rely on passive diffusion for their absorption through the gastrointestinal tract (GIT). Therefore, the degree of lipophilicity could be an important factor governing this process. Consistent with this notion the absorption rate of methoxylated flavonoids is significantly higher than that of their hydroxylated counterparts (Prasain and Barnes, 2007). In addition, membrane transporters may also play a role in flavonoid absorption (Walle, 2009). Sodium-dependent glucose transporter (SGLT1) was found to be involved in the absorption of dietary flavonoid quercetin 4'-beta-glucoside (Walgren et al., 2000) and monocarboxylate transporter (MCT) was identified as a transporter for the tea flavonoid (-)epicatechin-3-gallate (ECG) (Vaidyanathan and Walle, 2003)

Absorption is not the only factor that contributes to the poor bioavailability of flavonoids. After uptake of a flavonoid by the lumen, it typically undergoes metabolism in the enterocytes. Three main metabolic reactions can then take place, glucuronidation, sulfation and/or methylation. After uptake, the parent flavonoid or its metabolised forms are delivered to the liver by the portal circulation. In the liver these species undergo further Phase I or Phase II metabolism. Conjugated products may then be re-secreted into the intestinal lumen by means of enterohepatic circulation (Prasain and Barnes, 2007, Crozier et al., 2010). Therefore, enterohepatic circulation could play a significant role in the bioavailability of flavonoids. For example, it was found that 70% of an orally administered dose of genistein to rats was absorbed from the GIT but as a result of enterohepatic circulation only 5% reached the general circulation (Sfakianos et al., 1997). In the colon flavonoids could also undergo ring fission by the action of colonic microflora which break them down into smaller molecules (Roowi et al., 2010).

Most known flavonoids are quite heavily hydroxylated, which decreases their lipophilicity and as a consequence reduces the absorption rate through the gastrointestinal tract and increases their rate of metabolism by conjugation. Methylation of flavonoids has been suggested to improve bioavailability and also their potential chemopreventive potency (Walle, 2007b, Walle, 2007a). In addition, methylation of the flavonoid scaffold could enhance the entry of the compound into cancer cells and therefore increase its ability to act on intracellular targets. Many cancer chemopreventive mechanisms rely on the interference with intracellular targets by the specific agent (Spencer et al., 2004, Ramos, 2008). In addition, accumulation of the compound in the intracellular compartment could retard its degradation and excretion (Walle, 2007b) and therefore improve its anticancer cellular activity.

TMFol showed a considerable anti-proliferative activity in PCa cell lines (chapter 3). If this activity could translate to *in vivo* systems, then TMFol could be developed as a promising agent for PCa chemoprevention and therapy.

Animal preclinical pharmacokinetic data is an essential piece of information for drug development. It is used to predict the pharmacokinetic properties and potential toxicity and pharmacological activity of the agent in man. It can also be used in determining the optimum dose and potential dosing regimen in future clinical trials.

If TMFol is to be developed clinically it is important to perform initial pharmacokinetic studies as well as gain an understanding of the metabolism of the agent *in vivo*. To that end we explored the plasma and tissue distribution and metabolism of TMFol after a single oral dose (240 mg/kg) in male mice.

4.2 Plasma and tissue levels of TMFol following a single intragastric dose

Male C57BL/6J mice (4 animals per group) received an intragastric bolus of TMFol at a dose of 240 mg/kg body weight suspended in 0.5 % w/v methylcellulose at a concentration of 24 mg/ml. TMFol levels were measured in plasma, liver, kidney, prostate, heart, lung and small intestine mucosa at 5, 20, 40, 60, 120, 360 and 1440 minutes after dosing using a validated HPLC assay

with fluorescence detection (Britton et al., 2009). The concentration of TMFol was calculated from a standard curve of the peak area ratio (PAR) of TMFol to internal standard (IS) against TMFol concentration (Section 2.3.14.5). 2',5',5,6,7,8-hexamethoxyflavone was used as an IS, since, its signal was comparable to that of TMFol and its retention time was suitably different such that it did not interfere with peaks of TMFol or any of its identified metabolites.

The relative extraction efficiencies for TMFol from plasma, tissues and prostate cancer cells are shown in Table 4.1. The extraction efficiencies of plasma and tissues were comparable to previously published values (Britton et al., 2009).

Representative HPLC chromatograms of extracts of tissue samples 20 min after dosing are shown in Figure 4.1. The peak for TMFol elutes at 8.2 min in all tissues and the IS elutes at 10.8 min. Plasma TMFol levels were below the limit of quantification (25.4 ng/ml), therefore, data not shown. The TMFol concentrations versus time profiles for tissues were plotted and are illustrated in Figure 4.2.

Table 4.1 Relative extraction efficiencies for TMFol from murine plasma, tissues and human PCa cells

Tissue	Extraction efficiency (mean ± SD (%))
Plasma	78.9± 12.4
Liver	97.3±11.5
Kidney	72.7±11.5
Lung	85.5±16.2
Heart	83.4±9.1
SI mucosa	90.7±20.6
Prostate	93.82±15.3
LNCaP cells	104.6±32
PC-3 cells	68.37±10

Standard curves were prepared by spiking plasma, tissues, or cells with TMFol standards and 1 µl (2mM) IS, curves were linear within the range investigated (50 – 2500 ng/ml for plasma and 0.05 – 40 µg/ml for tissues and cells). Data are the means ± SD of 3 separate experiments. The correlation coefficients (r) were > 0.99 for all tissues, cells and plasma standard curves. Recovery of TMFol from plasma, tissues and cells was assessed by comparison of the slope of the standard curve prepared in mobile phase with a calibration curve of the same concentrations extracted from plasma, tissue or cells samples [% extracted = (slope extracted/slope unextracted)×100].

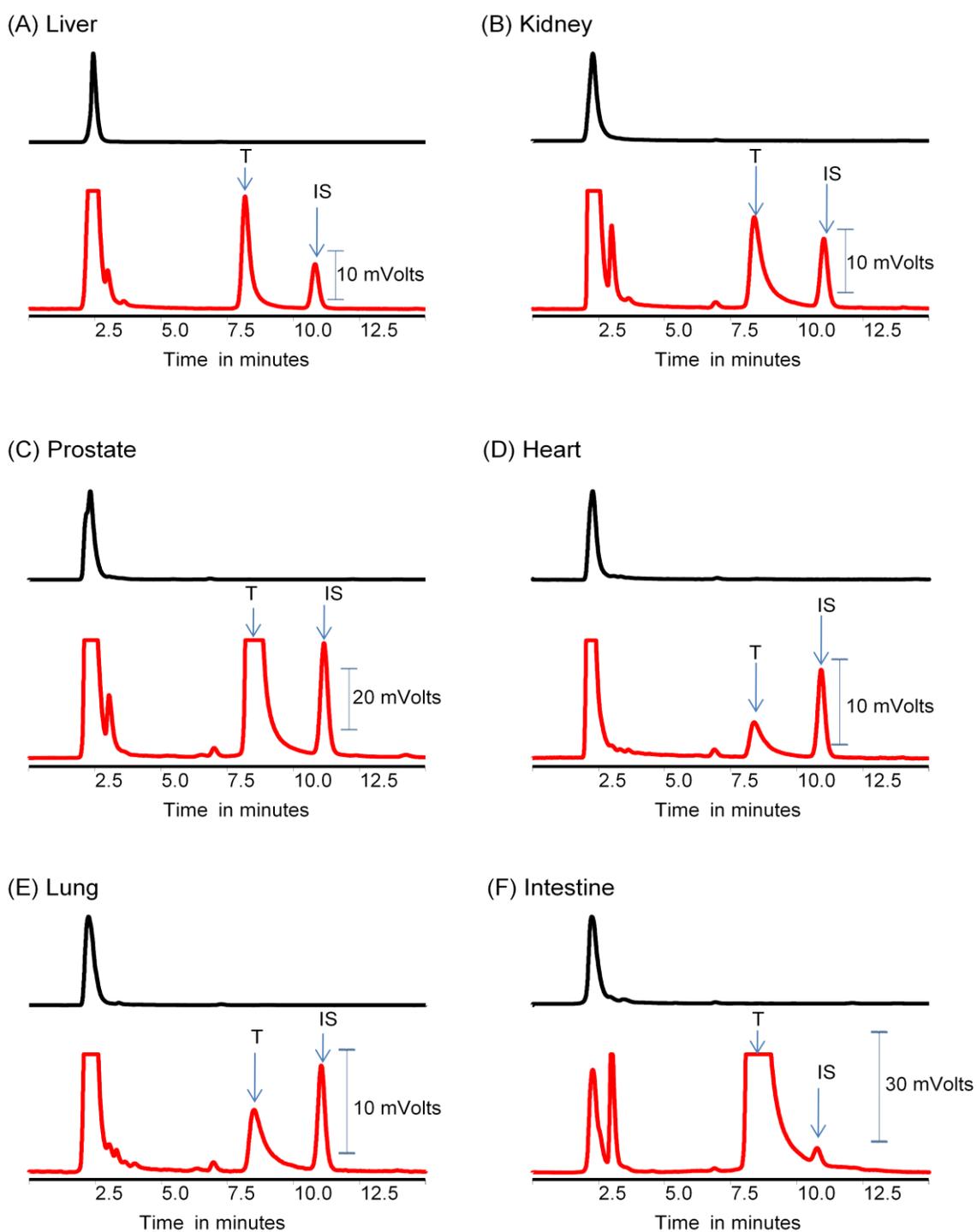


Figure 4.1 HPLC fluorescence chromatograms of control sample of taken from un treated animal (black solid line) and 20 minutes tissue samples (A) Liver, (B), Kidney, (C), Prostate, (D) Heart, (E) Lung and (F) Small intestinal mucosa from mice that received 240mg/kg ig TMFol (red solid lines). Peaks T and IS were characterised as TMFol and the internal standard respectively based on retention times of authentic standards.

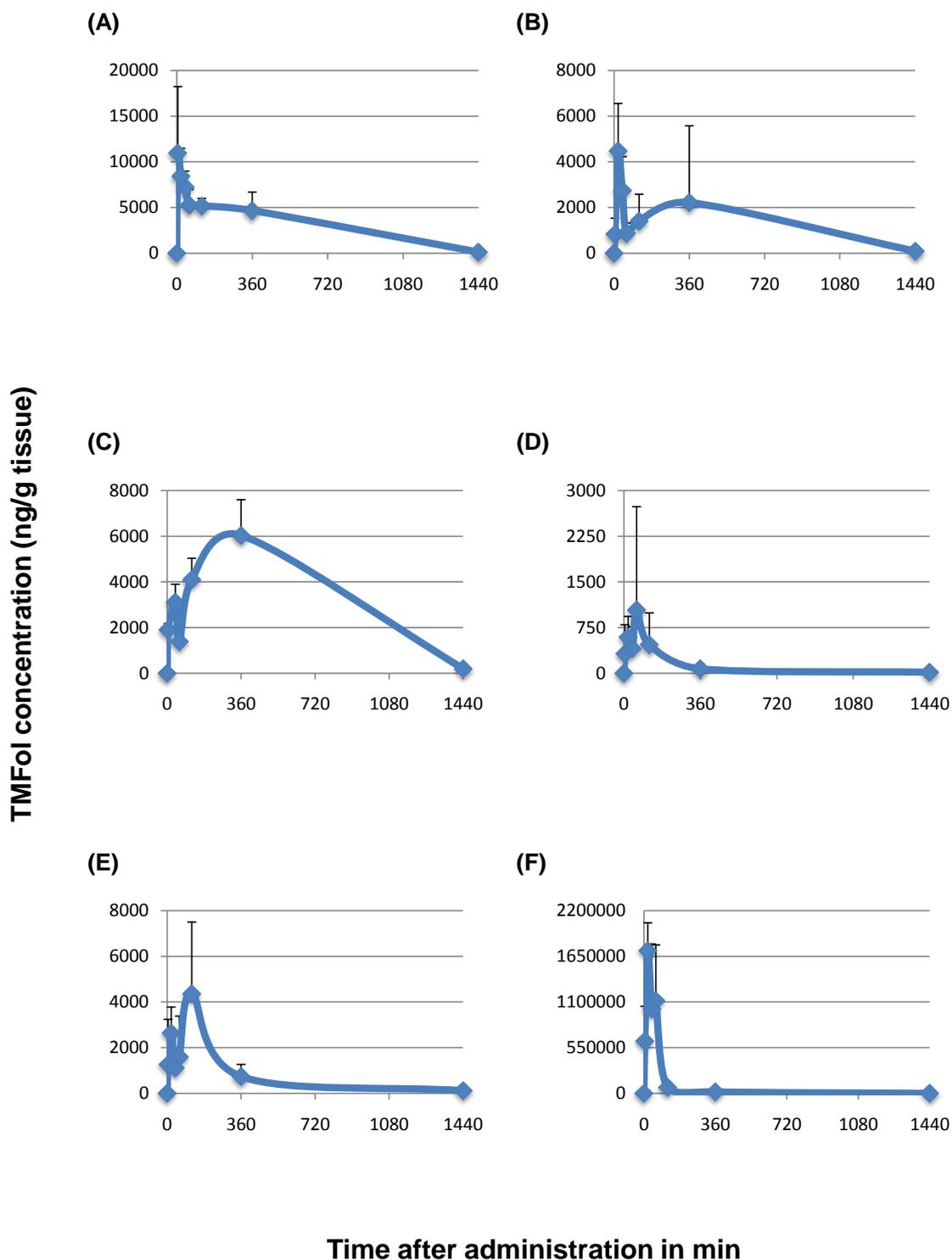


Figure 4.2 Concentration of TMFol (ng/g) over time in tissue from liver (A), kidney (B), prostate (C), heart (D), lung (E) and small intestine (F) of male mice after receiving a single intragastric TMFol dose of 240 mg/kg. Values are the mean \pm SD (n = 4).

The tissue level data were used to calculate the respective area under the curve, $AUC_{0-1440 \text{ min}}$, and other pharmacokinetic parameters: T_{max} , C_{max} and *half life* (Table 4.2). The plasma concentrations of TMFol were (25.4 ng/ml) below for all time points and therefore were excluded from this analysis.

TMFol was still detectable and could be quantified in all tissues at 24 hours post administration. Half life values ranged from 134 min for the small intestinal mucosa to 397 min for prostate tissue (Table 4.2). TMFol levels peaked after 5 min for liver, 20 min for kidney and small intestinal mucosa, 60 min for heart, 120 min for lung and 360 min for prostate. At these time points, the peak concentrations in ng/g and (nmol/g) were 10,969 ng/g (33.4 nmol/g), 4,479 ng/g (13.6 nmol/g), 3,160 ng/g (9.6 nmol/g), 1,040 ng/g (3.2 nmol/g), 6,034 ng/g (18.4 nmol/g), and 1,718,404 ng/g (5234 nmol/g) tissue in the liver, kidney, lung, heart, prostate and small intestinal mucosa, respectively. It is worth mentioning the levels of TMFol detected in tissues after two weeks continuous 0.2 %w/w feeding in diet (steady state levels) were 12223 ± 3169 ng/g (37.2 nmol/g), 1161 ± 829 ng/g (3.6 nmol/g), 295 ± 240 ng/g (0.9 nmol/g), 181 ± 219 ng/g (0.6 nmol/g) and 72086 ± 22200 ng/g (291 nmol/g) tissue in the liver, kidney, lung, heart and small intestinal mucosa, respectively (Britton et al., 2009). Steady state TMFol tissue concentrations were lower than the levels achieved following a single oral dose in all tissues except the liver.

Table 4.2 Pharmacokinetics of TMFol in the tissues of male C57BL/6J mice after a single oral dose (240 mg/kg).

	Liver	Kidney	Heart	Lung	Prostate	SI
T_{max} (min)	5	20	60	120	360	20
C_{max} (ng/g)	10969	4479	1040	4352	6034	1718404
Half-life (min)	236	334	288	351	397	134
AUC_{0-1440} ($\mu\text{g/g/min}$)	4510	1896	153	1138	4879	1220049

Data was calculated using pharmacokinetic file add-ins for Microsoft Excel software (2003). Data was calculated from the mean values (n=4) for each time point rather than individual data points, therefore there are no standard deviations for the dataset.

Abbreviations: AUC_{0-1440} , area under the concentration versus time curve; C_{max} , maximal tissue concentration; T_{max} , time point of maximal tissue concentration.

4.3 Metabolism of TMFol *in vitro* and *in vivo*

Metabolism is a natural biological process undertaken by the body in order to deactivate and facilitate the excretion of drugs and toxicants. Many possible products can be generated by the process of metabolism because of the presence of various metabolic enzymes. Therefore, studying the metabolism is an important part of understanding the bioavailability and pharmacokinetic profiles of new agents, such as TMFol.

4.3.1 In *vitro* generation of TMFol metabolites

In addition to parent TMFol peaks, a number of other peaks were present in the HPLC chromatograms, which could potentially be attributed to TMFol metabolites. Therefore, TMFol metabolites were generated *in vitro*, in order to act as reference standards to aid identification of the additional peaks in the mouse tissue samples. Glucuronide, sulphate and demethylation products were generated *in vitro* using liver S9 fractions (Section 2.3.16). TMFol glucuronide and sulphate species were generated before and after the process of TMFol demethylation of the 3', 4' and/or 5' positions.

The demethylation reactions of TMFol resulted in peaks at 19.4, 20.4 and 21.6 min (Figure 4.3 iv). These peaks potentially belonged to three or more demethylated TMFol species depending on which of the three methoxy residues on the TMFol molecule had been demethylated. Enzymatic glucuronidation of TMFol generated one peak, which eluted at 10.1 min (Figure 4.3 A (v)). This peak most probably belongs to TMFol-3-glucuronide as before demethylation TMFol has only one hydroxyl group available for conjugation. Glucuronidation of TMFol after it had undergone demethylation yielded an additional peak at 8.5 min (Figure 4.3 A (vi)), which can probably be attributed to the presence of one or more demethylated TMFol glucuronides at 3', 4' or 5' position. Sulphation of TMFol produced one peak at 17.1 min, which is probably TMFol-3-sulphate (Figure 4.3 B (v)). After the demethylation reactions three more peaks were detected at 10.1, 11.5 and 12.0 min, which could correspond to sulphates of demethylated TMFol at the 3', 4' and/or 5' positions. Interestingly, there was no peak at 17.1 min, which could suggest sulphation preferentially occurs at the positions that were previously methylated, as opposed to the 3-hydroxy position of TMFol.

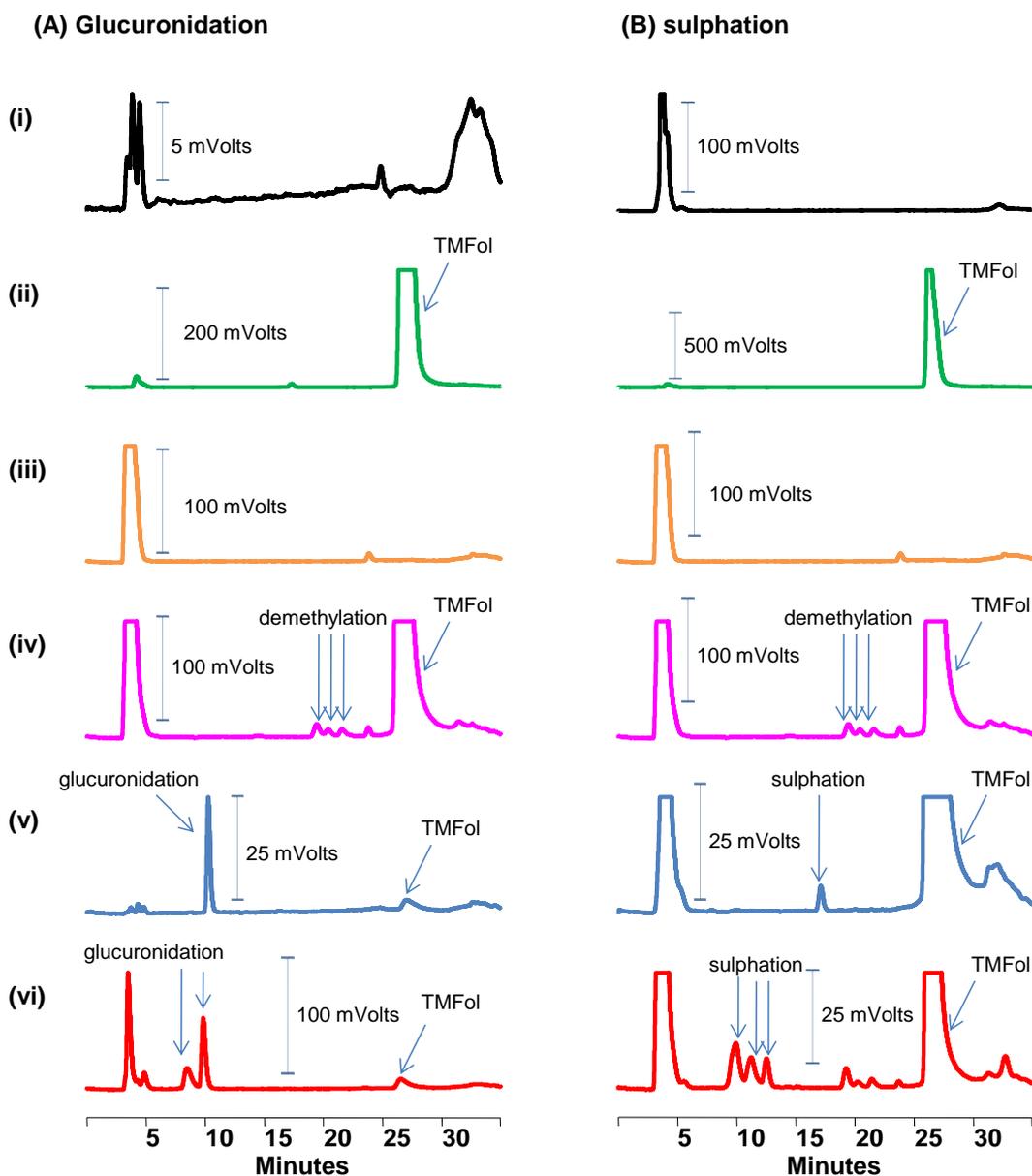


Figure 4.3 HPLC analysis of incubation extracts of mouse liver microsomes or cytosol was incubated with TMFol in the presence of either UDPGA or PAPS to generate glucuronides (A) or sulphate (B) metabolites respectively. To assess the metabolism of demethylated TMFol, NADPH regenerating system was also included in some incubations (i) Control incubations with microsomal or cytosolic enzymes only, (ii) TMFol with no enzymes, (iii) control demethylation incubation, (iv) TMFol demethylation, (v) glucuronidation and sulphation with no prior demethylation and (vi) glucuronidation and sulphation after prior demethylation reactions. For details of reaction condition see section 2.3.16.

4.3.2 Identification and characterisation of *in vivo* tissue metabolites

HPLC chromatograms of tissue samples from mice that received TMFol (240 mg/kg) as a single gavage dose and 0.2% w/w in the diet for two weeks, indicated some peaks in addition to the parent compound, which were thought to be TMFol metabolites. Figures 4.4 and 4.5 show the metabolite profile in mouse liver and small intestine respectively. The peaks present in liver and small intestine were compared to the corresponding peaks of TMFol metabolite standards, which were generated *in vitro* (Figure 4.3 etc). Both liver and intestine traces showed peaks at 8.5 and 10.1 min (Figure 4.4 and 4.5 (vii) and (viii)), which correspond to the TMFol-3-glucuronide and a demethylated TMFol glucuronide. In addition, the peak eluting at 10.1 min could also represent one of the TMFol sulphation products as the retention times are similar. One extra peak at about 17.4 min was present in the continuous feeding chromatogram for liver (Figure 4.4-(viii)), which corresponds to the TMFol-3-sulphation product.

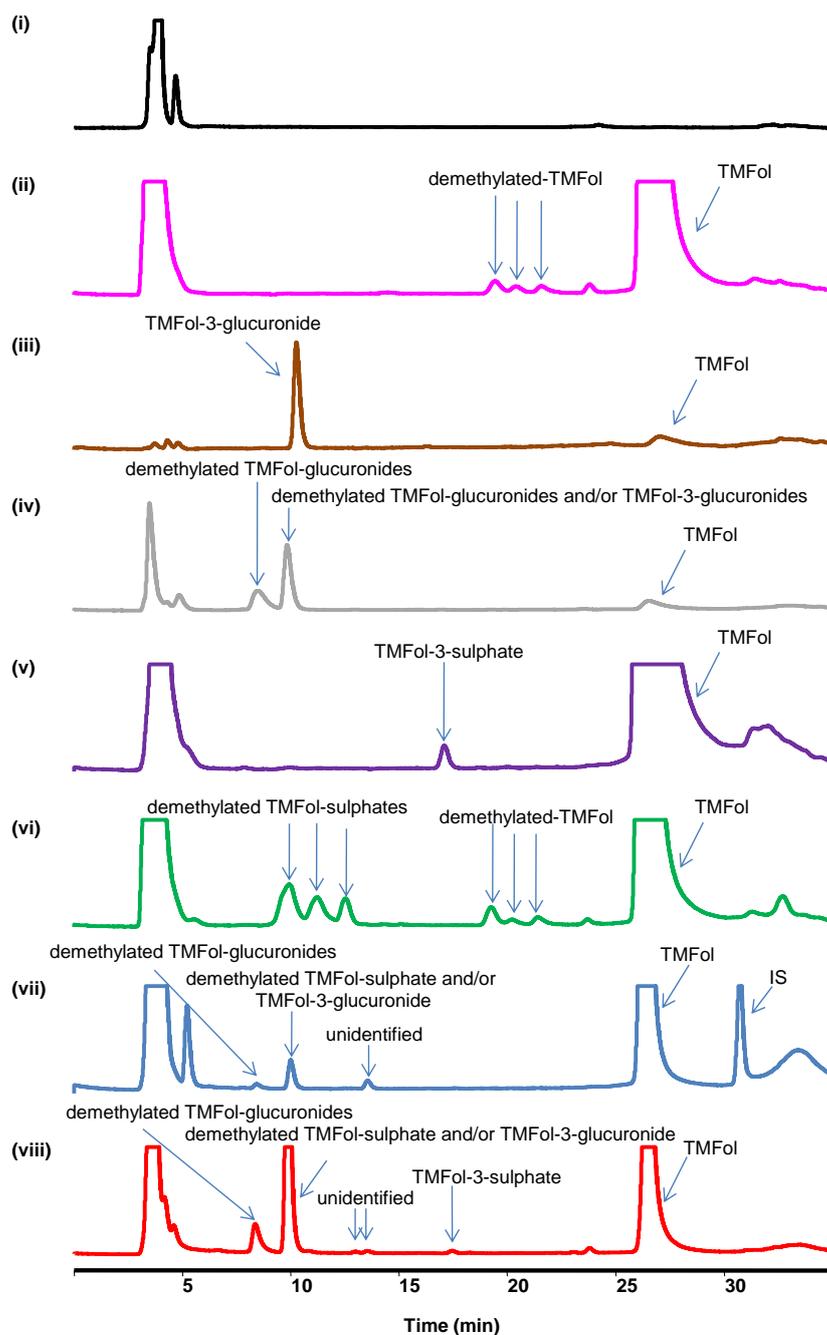


Figure 4.4 Identification of TMFol metabolites in mouse liver. Chromatogram (i) is for control liver from control. Chromatograms (ii), (iii), (iv), (v) and (vi) are *in vitro* generated products of TMFol demethylation, glucuronidation, glucuronidation after demethylation, sulphation and sulphation after demethylation respectively. HPLC trace (vii) shows the analysis of mouse liver 20 minutes after the animal received a single dose of 240 mg/kg TMFol, whilst (viii) depicts a liver sample following continuous feeding of 0.2 % w/w TMFol in the diet for 2 weeks.

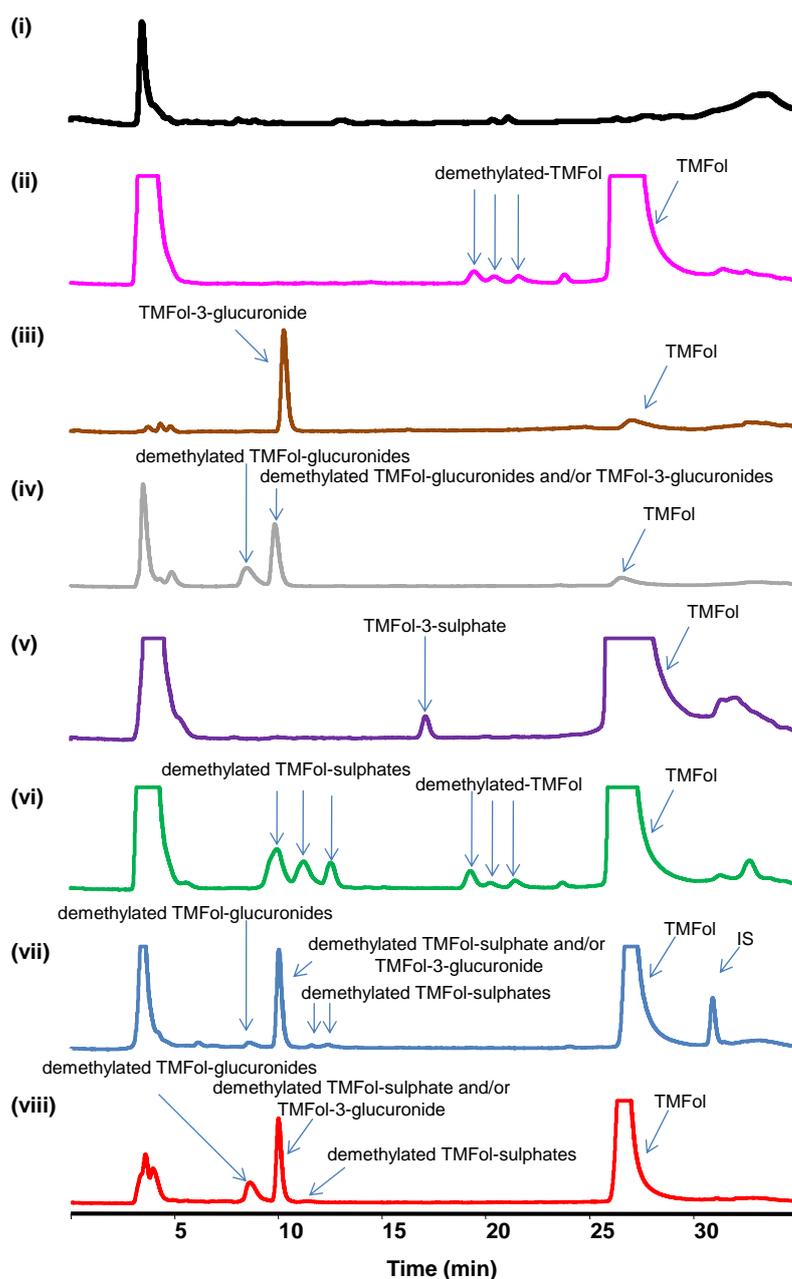


Figure 4.5 Identification of TMFol metabolites in intestinal mucosa. Chromatogram (i) is for intestinal mucosa from control. Chromatograms (ii), (iii), (iv), (v) and (vi) are *in vitro* generated products of TMFol demethylation, glucuronidation, glucuronidation after demethylation, sulphation and sulphation after demethylation respectively. HPLC trace (vii) shows the analysis of mouse intestinal mucosa 360 minutes after the animal received a single dose of 240 mg/kg TMFol, whilst (viii) depicts a small intestinal mucosa sample following continuous feeding of 0.2 % w/w TMFol in the diet for 2 weeks.

4.3.3 Identification of TMFol metabolites generated *in vivo* using liquid chromatography mass spectrometry (LC-MS)

LC-MS analysis was used to further characterise and identify potential TMFol metabolites detected in mouse tissue samples. Intestinal mucosa extracts from mice taken after 360 minutes of treatment with a single intra-gastric dose of TMFol (240 mg/kg) were investigated. Samples were injected onto the column for each LC-MS run, samples were subject to positive ion electrospray analysis and a scan over the range m/z 200-900 was performed over 35 min to detect any possible metabolites. The detected metabolites were individually analysed using extracted ion chromatography, so an ion chromatogram was generated for each metabolite. The detected metabolites are displayed in Table 4.3. The analysis of ion with retention time of 10.6 minute showed a $[M+H]^+$ ion at m/z 505 (Figure 4.6-(i)) which corresponds to the molecular weight of TMFol-3-glucuronide. The fragmented ion (about 95%) at m/z 329 probably equates to the TMFol molecule after loss of the glucuronide, smaller peaks at m/z 527 and m/z 543 are due to sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducted molecular ions respectively. The mass spectra of peaks with retention times of 7.86, 8.5 and 9.15 minutes (Figure 4.6-(ii)), all revealed predominant ions at 491 m/z , which is equal to the $[M+H]^+$ molecular ion of demethylated TMFol glucuronide. In addition fragments at m/z 315 corresponding to demethylated TMFol were identified. Therefore, the three analytes are monoglucuronides of demethylated TMFol, which obviously occurred at either carbon 3', 4', or 5'. Glucuronidation data before and after demethylation showed the presence of four different mono-glucuronide TMFol products in the intestinal scrapes. It suggests TMFol glucuronidation could occur at carbon 3 and after demethylation at carbon 3' or 4' or 5'.

Analysis of the peaks at 21.2 and 22.5 minutes, both show a molecular ion $[M+H]^+$ at m/z 315 (Figure 4.6-(iii)), which corresponds to the protonated demethylated TMFol molecule. The possible demethylation sites on TMFol are carbons at position 3', 4' or 5' and since LC-MS analysis may not necessarily resolve all isomers of demethylated TMFol, the results suggest that at least two

forms of demethylated TMFol were present in intestinal mucosa of mice treated with TMFol.

Table 4.3 Result of mass spectrometric screening of TMFol metabolites present in intestinal scrape extracts of mice 360 min after receiving TMFol (240mg/kg) as a single oral dose.

Metabolite	Retention time (min)	<i>m/z</i> (relative intensity %)
Demethylated TMFol	21.2	315 (100%)
	22.5	315 (100%)
TMFol glucuronide	10.6	505 (100), 329 (70%)
Demethylated TMFol glucuronide	7.9	491(100), 315
	8.5	315 (100), 491 (90%)
	9.2	491 (100), 315 (90%)

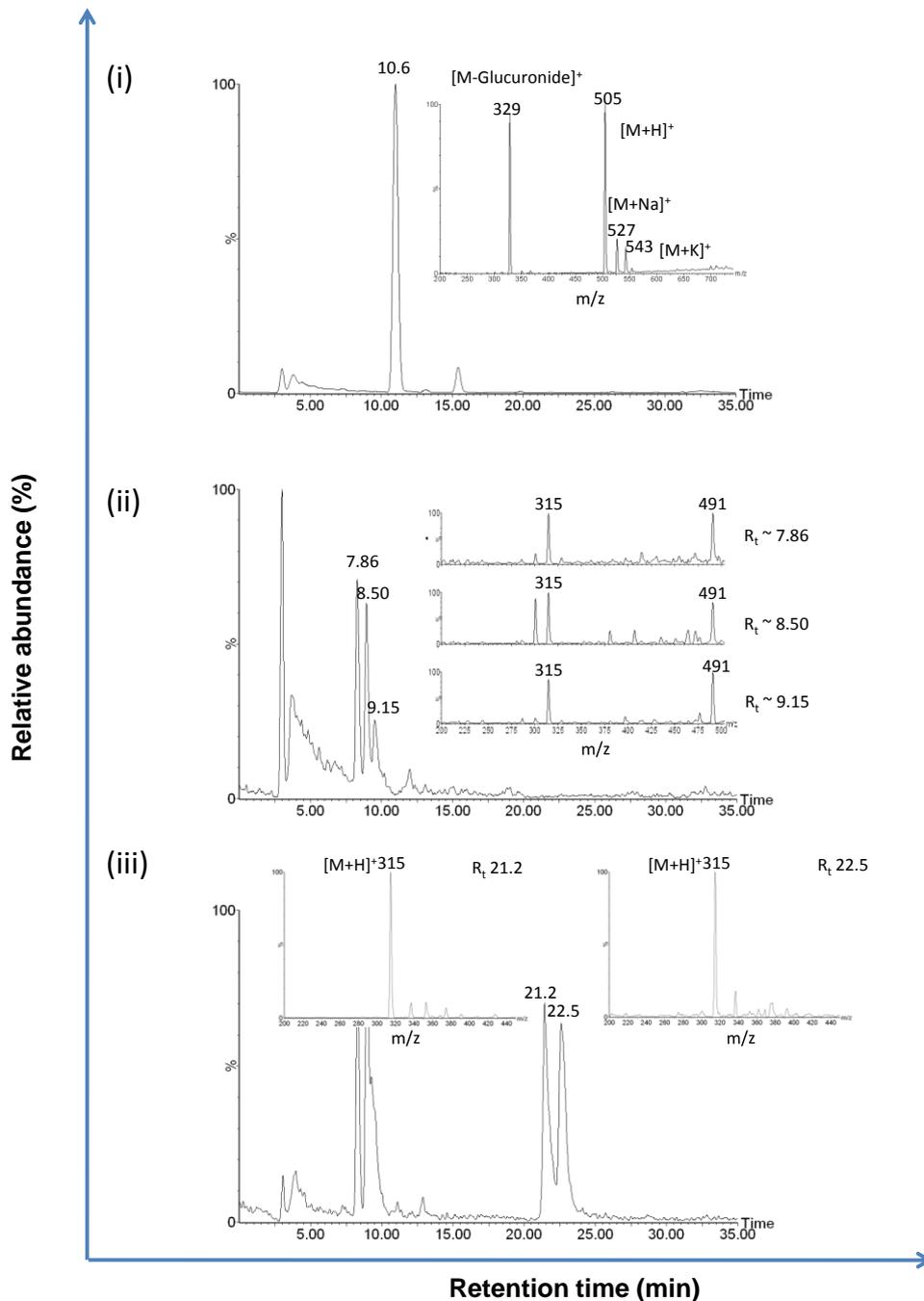


Figure 4.6 LC-MS extracted ion chromatograms of intestinal scrape extracts from a mouse taken 360 minutes after administration of a single TMFol dose (240 mg/kg). It shows the retention times, molecular ion peak and fragments peaks of TMFol metabolites. The mass spectra for each peak are shown as inserts within each figure. (i) chromatogram of TMFol-3-glucuronide (ii) is for demethylated TMFol glucuronide products (iii) shows the chromatogram of TMFol demethylation.

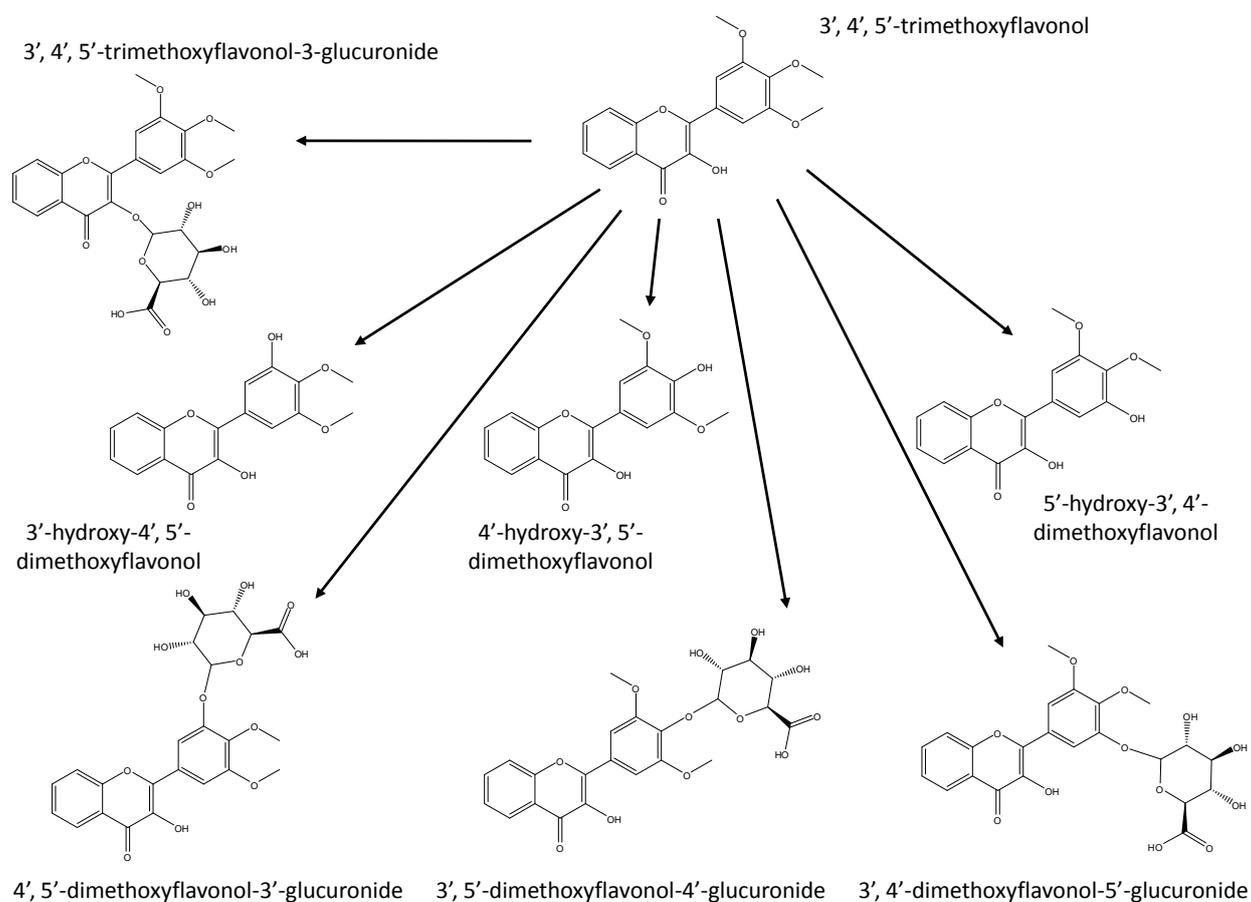


Figure 4.7 Metabolism of TMFol in the mouse. Chemical structures of the possible TMFol metabolites in mouse intestinal mucosa extracts 360 minutes after a single dose of TMFol (240 mg/kg). The TMFol-3-glucuronide was confirmed by mass spectrometry. The precise position of demethylation and demethylation together with glucuronidation cannot be ascertained based on the current results; synthetic standards and/or $^1\text{H-NMR}$ analysis would be needed for structural confirmation.

4.3.4 Identification of TMFol metabolites in urine

Urine was collected at either 1 or 24 hours post intra-gastric administration from mice administered TMFol (240 mg/kg). HPLC chromatograms were comparable for both time points, however, the TMFol peak was smaller at the later time point which is indicative of the normal decrease of agent concentration in the body over time. The peak at approximately 8.5 min correlates with the retention time for the demethylated TMFol glucuronide, which was generated *in vitro* (Figure 4.8). An additional peak was observed at 10.1 minutes which elutes with the same retention time as two possible TMFol metabolites: TMFol-3-glucuronide and a demethylated TMFol sulphate. The small peaks eluting at 26.5 minutes correspond to the parent compound, TMFol. An extra peak eluted at 12.5 minutes has not been identified. A peak with the same retention time was detected in the chromatograms of liver samples of single dose and continuous TMFol treatment (Figure 4.4). This could be referring to a TMFol metabolite we did not generate *in vitro* and was not contained in the intestine mucosa sample which was undergone LC-MS analysis.

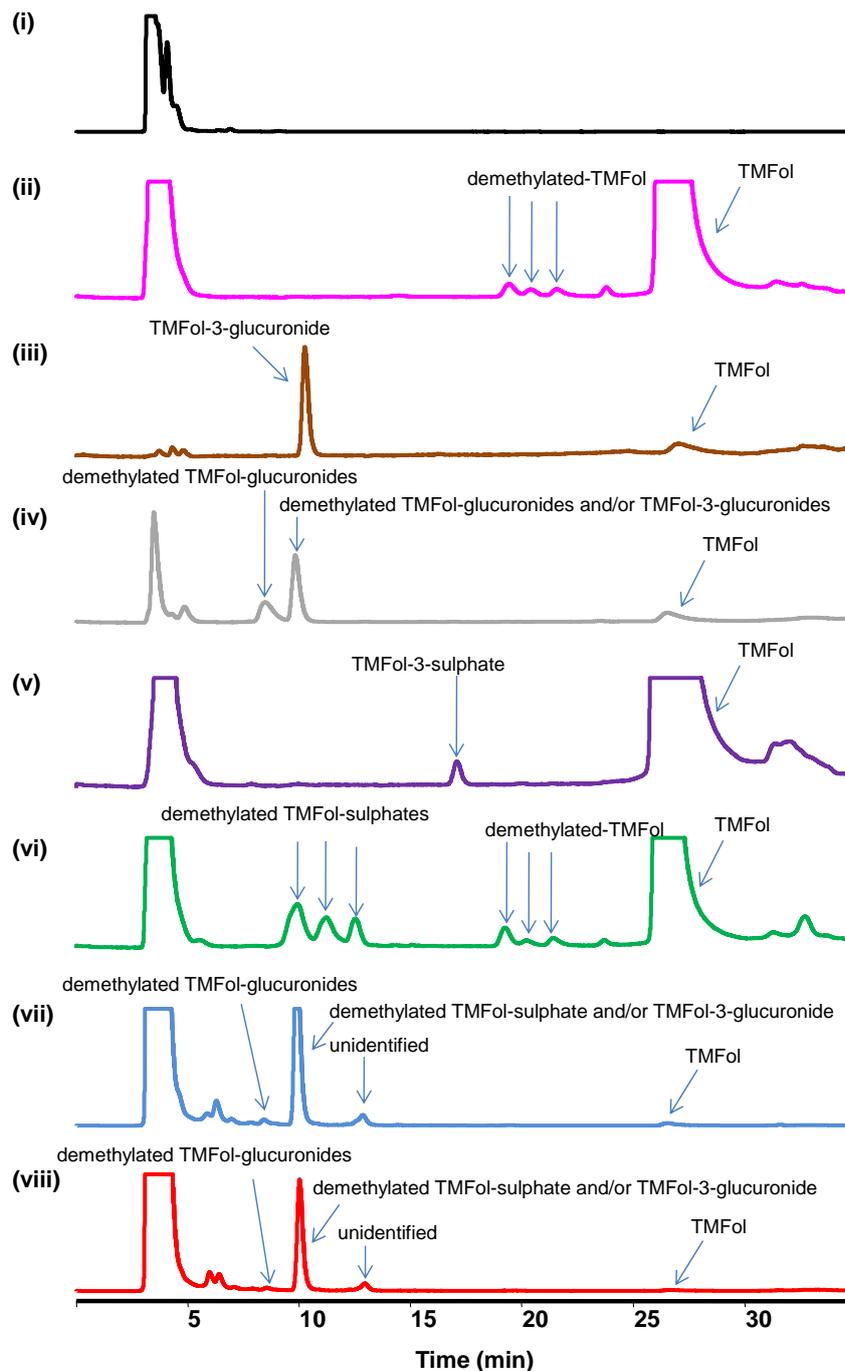


Figure 4.8 Identification of TMFol metabolites in urine. Chromatogram (i) is from control urine. Chromatograms (ii), (iii), (iv), (v) and (vi) are *in vitro* generated products of TMFol demethylation, glucuronidation, glucuronidation after demethylation, sulphation and sulphation after demethylation respectively. HPLC traces generated from mouse urine collected at 1 (vii) or 24 hrs (viii) post TMFol dosing (240 mg/kg).

4.3.5 Metabolism of TMFol by prostate cancer cell lines

Flavonoids have been shown to be taken up and metabolised by cells and undergo processing before being excreted. The rate of these processes are most probably cell type dependent as the rate of metabolism and excretion can vary between cells (Spencer et al., 2004).

Identifying the nature of TMFol metabolites in cells will improve understanding of the metabolic pathway by which PCa cells metabolise TMFol. It will also provide information regarding TMFol's intracellular metabolic stability.

Conditioned media was analysed for potential metabolites which could have been secreted by LNCaP cells after incubation with 10 μ M TMFol. HPLC chromatograms of media samples incubated with LNCaP cells for 8 and 24 hrs contained two peaks with retention times of 10.1 and 21.7 min, in addition to the TMFol and IS (Figure 4.9). The peak at 10.1min corresponds to TMFol-3-glucuronide and/or demethylated TMFol-sulphates, which was also identified in *in vivo* samples (Figures 4.4 and 4.5). Whilst the other peak of 21.7 min could be contributed to a demethylated product of TMFol,

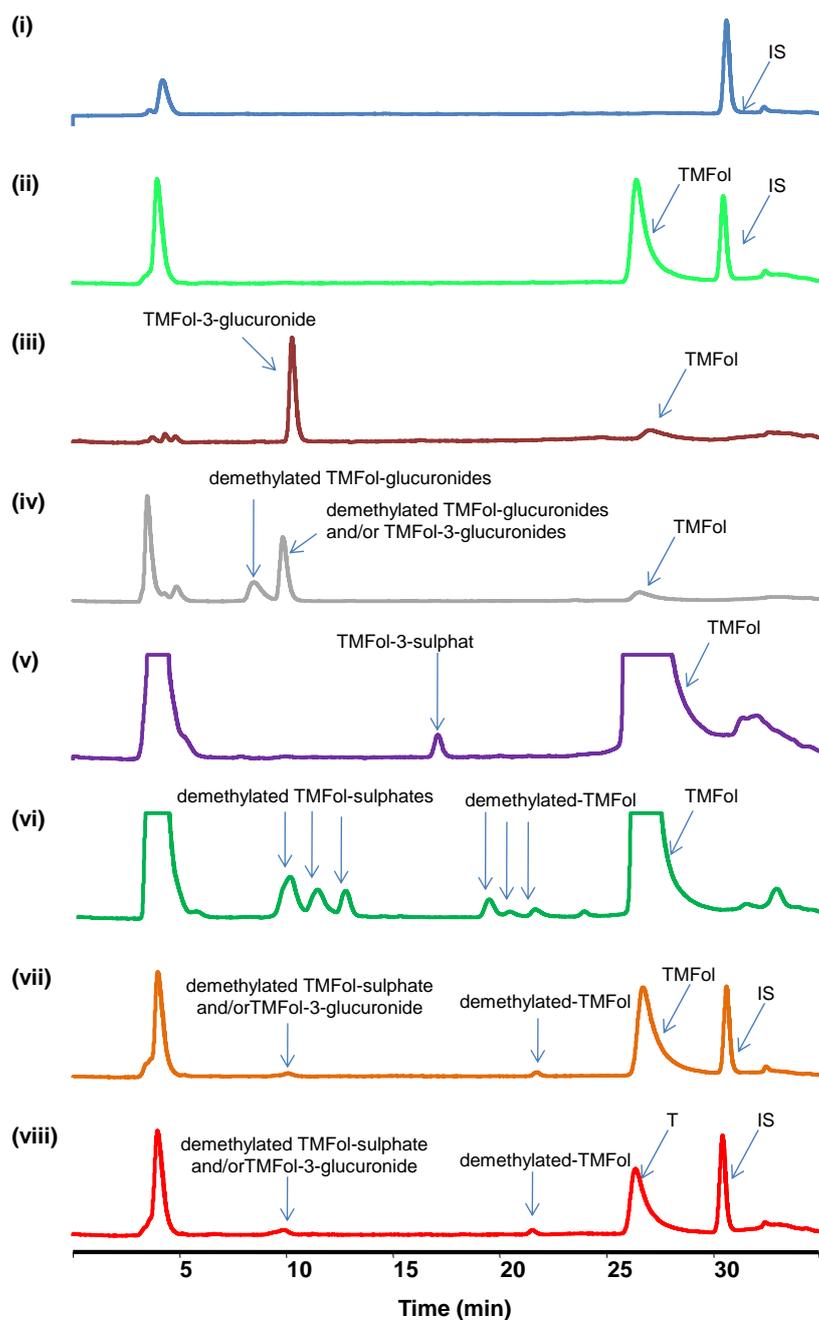


Figure 4.9 Identification of TMFol metabolites produced by PCa cells in cell culture media. Chromatogram (i) is from media from control incubations. Chromatograms (ii), (iii), (iv), (v) and (vi) are *in vitro* generated products of TMFol demethylation, glucuronidation, glucuronidation after demethylation, sulphation and sulphation after demethylation respectively. HPLC chromatograms generated from LNCaP media collected after incubation with 10 μ M TMFol for 8 h (vii) or 24 h (viii).

4.4 Intracellular TMFol uptake by prostate cancer cell lines

Literature evidence would suggest that methoxylation of the flavonoid structure can increase cancer chemoprevention potency, as a result of higher metabolic stability and improved cellular uptake (For review see (Walle, 2009)). Increasing the uptake and decreasing the excretion of an agent from cells could lead to accumulation, which would increase the length of time it is available for interaction with intracellular targets, as well as enhance the potency. We therefore investigated the intracellular levels of TMFol in PCa cells following treatment. For details see section 2.3.19.

4.4.1 Intracellular uptake of TMFol by LNCaP Prostate cancer cells

LNCaP cell lines were incubated with 10 μ M TMFol for 15, 30, 60, 90, 480 and 1440 minutes or with DMSO for 1440 min. Cellular TMFol content was measured by HPLC analysis. As shown in Figure 4.10 and 4.11, TMFol was detected in the intracellular compartment of LNCaP cells, which confirms TMFol has been taken up by them. There was an increase in cellular uptake over time, which suggests TMFol is taken up by LNCaP cells at a faster rate than the rate of excretion or metabolism. Figure 4.11 did not show any additional peaks could be contributed to the presence of metabolites inside or bounded to the cells. However, in media we could detect some metabolites (Figure 4.9). This could be explained as these metabolites tend to be rapidly excreted out of the cell due to the modification of their structure and by time accumulates in media. Therefore, their intracellular levels could be undetectable.

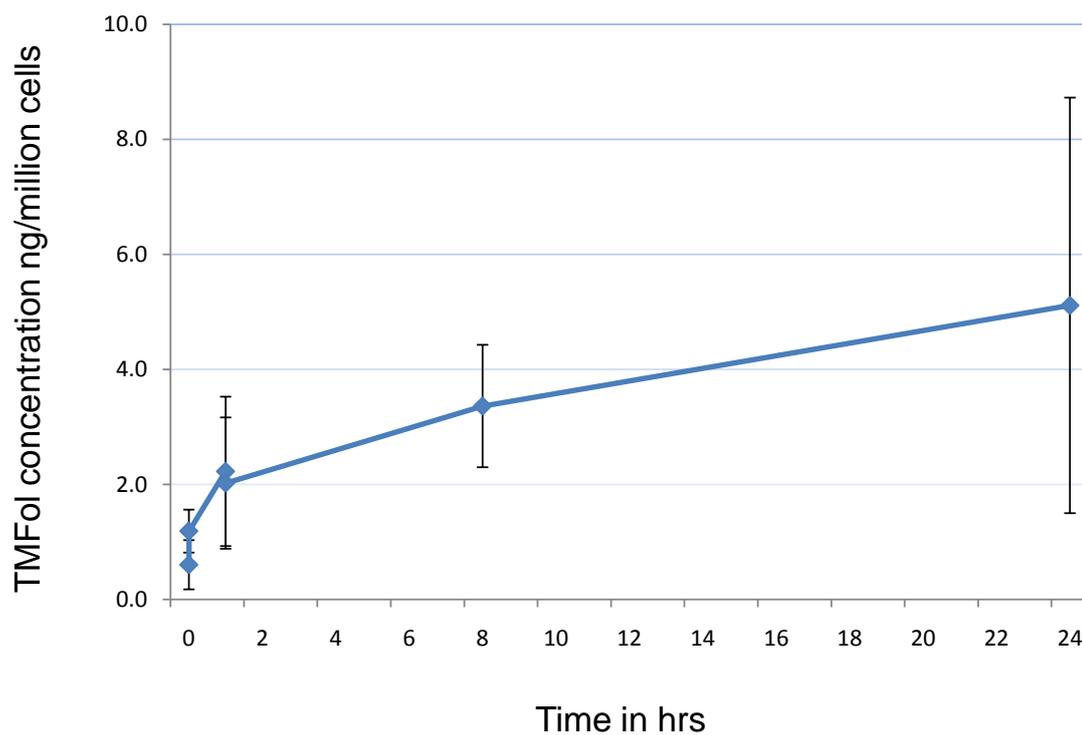


Figure 4.10 Levels of intracellular TMFol in LNCaP cells over time after incubation with 10 μ M TMFol. Intracellular TMFol content is displayed as ng/million cells. Data shown are the mean \pm SD (n=3).

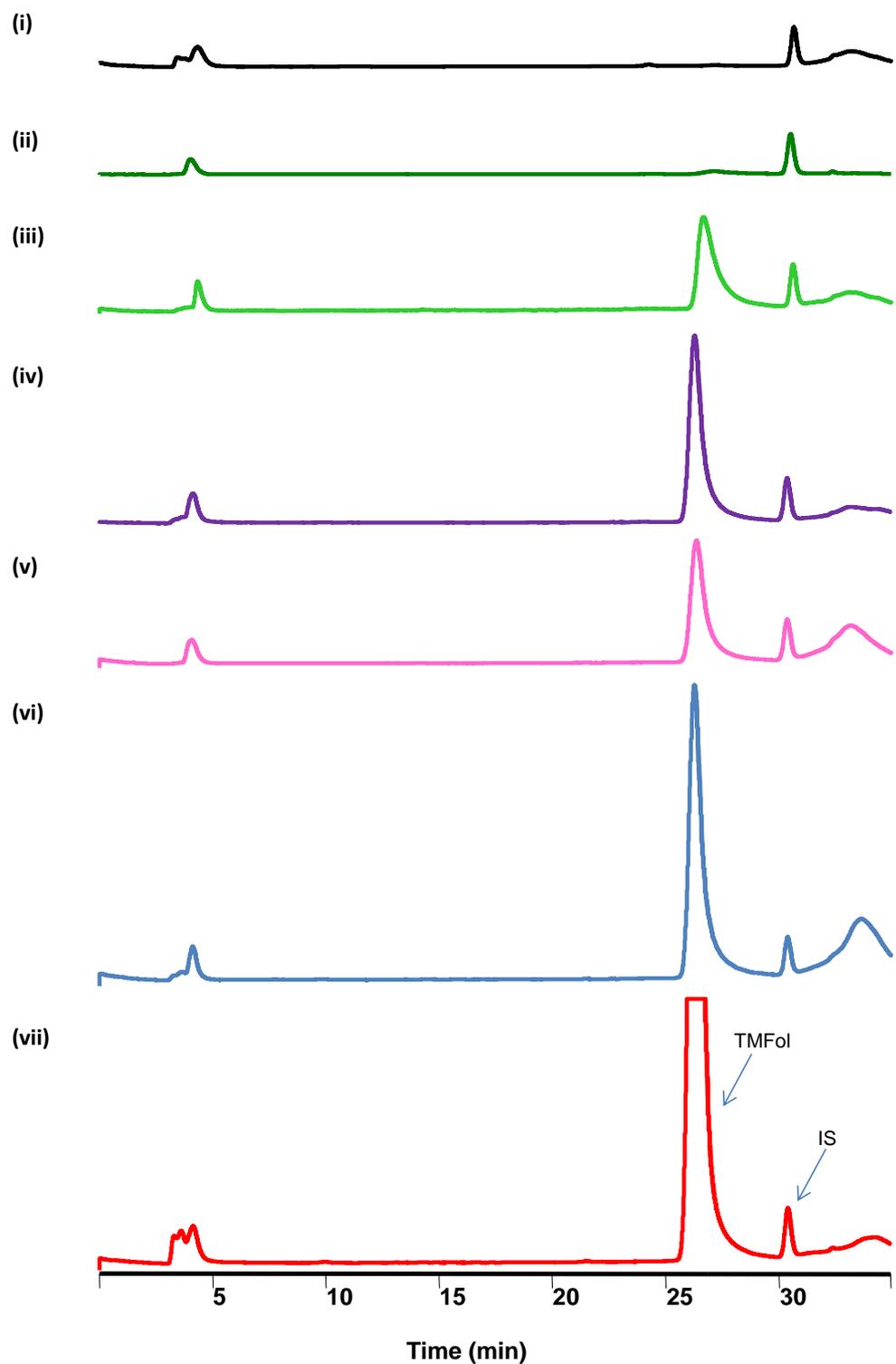


Figure 4.11 Representative HPLC fluorescence chromatograms of LNCaP cell extracts after incubation with (i) DMSO or 10 μ M TMFol for (ii) 15, (iii) 30, (iv) 60, (v) 90, (vi) 480 and (vii) 1440 min.

4.4.2 Intracellular uptake of TMFol by PC-3 Prostate cancer cells

As the human LNCaP prostate cancer cells were able to take-up and accumulate TMFol we investigated the accumulation of TMFol in the PC-3 cells to determine if they behaved in a similar manner to the LNCaP cells.

PC-3 cells were able to take-up and accumulate TMFol in a time-dependent manner. The concentration of cellular TMFol increased over 24 h before plateauing up to 72 h (Figures 4.12 and 4.13). The intracellular content of TMFol in the LNCaP cells was 7-fold higher than that of the PC-3 cells at the 24 h time point. An additional peak was detected in the chromatograms of PC-3 cells at 15.8 min, suggesting possible metabolism (Figures 4.13). This peak was not observed for the LNCaP cells and is not correlated to any TMFol-metabolite generated *in vitro*. Therefore, this could be for an unidentified TMFol-metabolic produced by PC-3 cells.

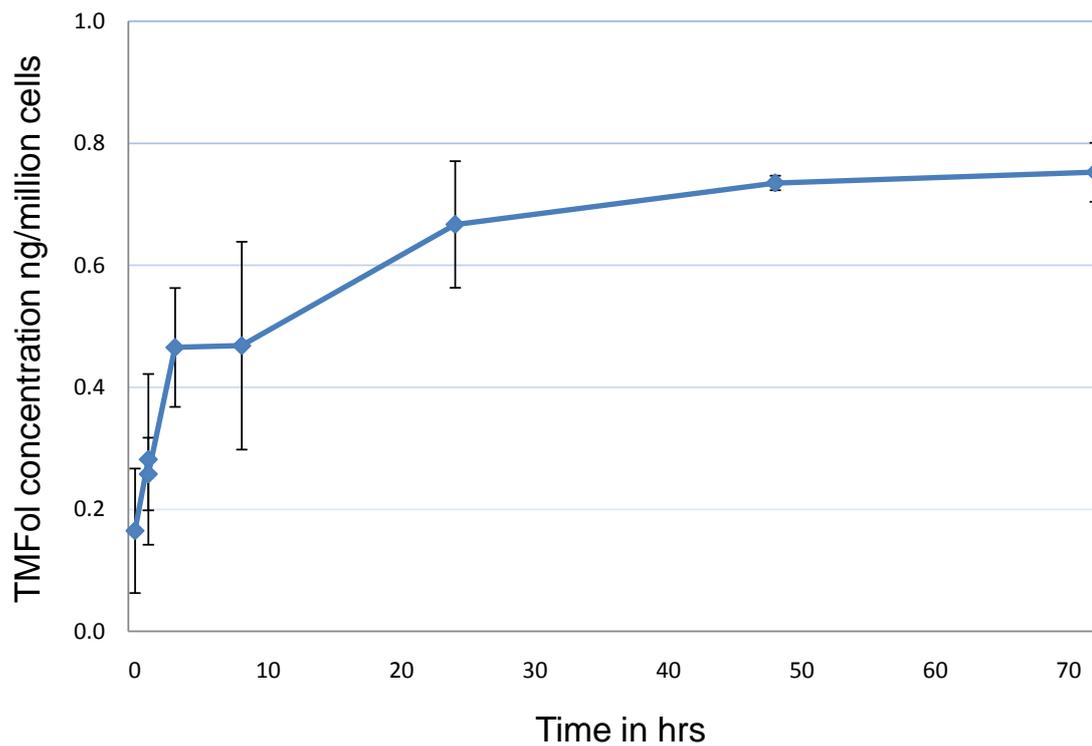


Figure 4.12 Levels of intracellular TMFol in PC-3 cells over time after incubation with 10 μ M TMFol. Intracellular TMFol content is displayed in ng/million cells. Data shown are the mean \pm SD (n=3).

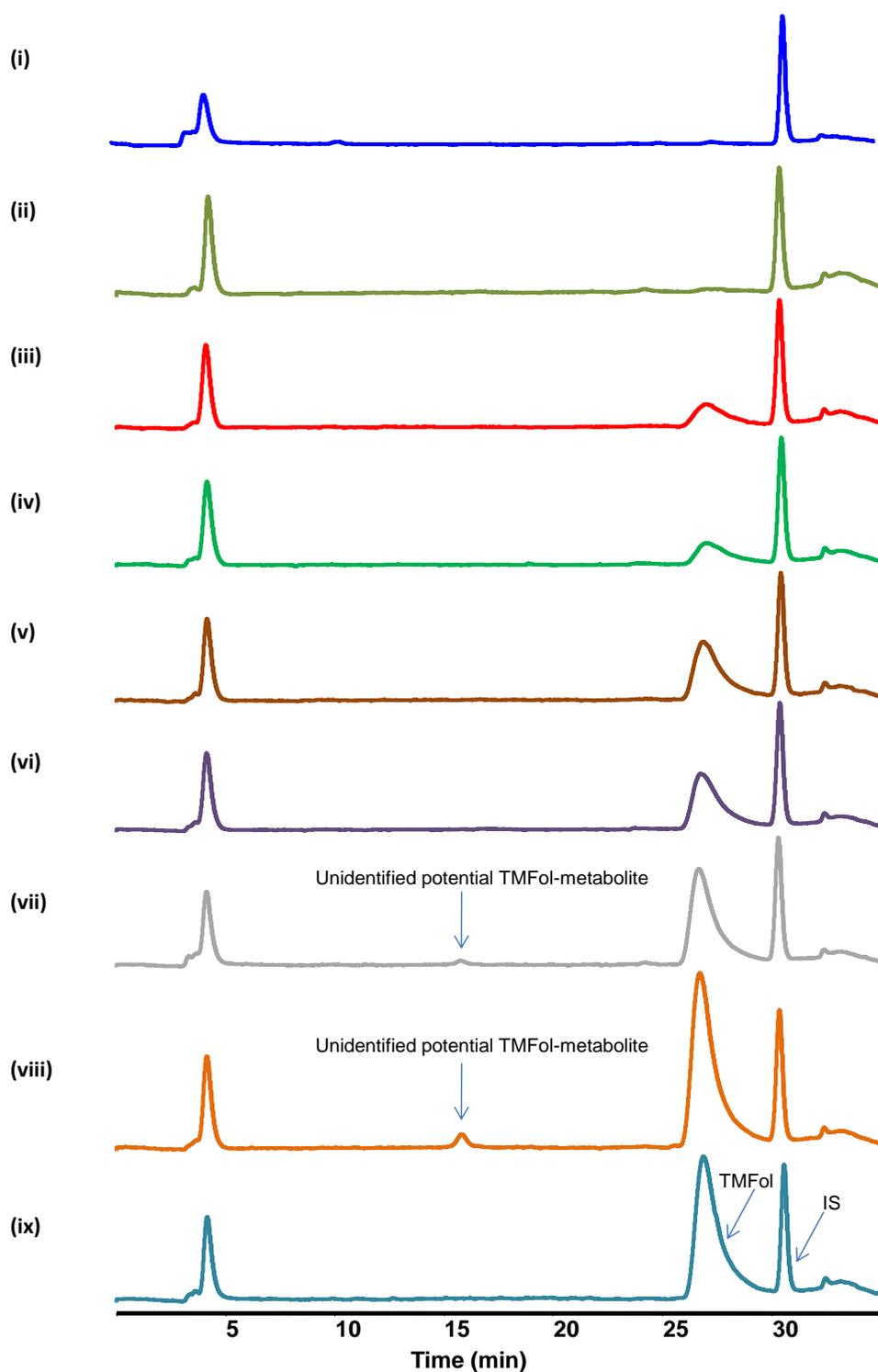


Figure 4.13 Representative HPLC fluorescence chromatograms of PC-3 cells extracts after incubation with (i) DMSO or 10 μM TMFol for (ii) 30, (iii) 60, (iv) 90, (v) 180, (vi) 480 minutes, (vii) 24, (viii) 48 and (ix) 72 h.

4.5 Discussion

Even though the *in vitro* pharmacological effects have been promising for many flavonoids (Walle, 2004), their poor bioavailability *in vivo* creates a serious obstacle for their clinical development. TMFol has shown significant antiproliferative activity in PCa cells *in vitro*, far superior to that of any other studied flavonol (Chapter 3). In order to correlate these *in vitro* findings to *in vivo* activity, it is important to determine levels that are achievable in plasma and tissue after oral dosing of mice. To that end we investigated the distribution of TMFol in the plasma and tissues of mice following a single oral dose of 240 mg/kg over a 24 h period. In addition, we examined the metabolism of TMFol using both *in vitro* and *in vivo* model systems to determine its metabolic stability.

Tissue levels of TMFol were determined in mice using a previously validated HPLC method (Britton et al., 2009). Plasma levels of free TMFol were at or below the limit of detection in all samples at all time points. Previously, it has been reported that blood cells are capable of taking up large amounts of flavonols and flavonoids, they associating with cell membranes, haemoglobin, other proteins and DNA. Therefore, it is suggested to analyse the whole blood instead of plasma to determine the levels of quercetin and resveratrol as up to 76% of the analyte, was found associated with the cellular fraction and had been unaccounted for when examining only plasma (Biasutto et al., 2010). It was also shown large amounts of quercetin can be taken up by RBCs by passive diffusion driven by binding to haemoglobin, suggesting these cells can act as an *in vivo* reservoir for flavonols (Fiorani et al., 2003). This, in part, could explain the absence of TMFol in the mouse plasma. The notion that flavonols have not great affinity for plasma has also been shown for quercetin whereby levels were not detectable in the plasma following oral administration (Erlund et al., 2000, Graefe et al., 2001, Yang et al., 2005, de Boer et al., 2005, Gugler et al., 1975). Fisetin was detected in the plasma following intravenous administration but declined rapidly with fisetin sulphates/glucuronides detectable almost instantaneously. When fisetin was given orally, fisetin was only transiently present with fisetin metabolites being predominant (Shia et al., 2009). This lack of detection of the hydroxylated flavonols, quercetin and fisetin,

could also be the results of rapid metabolism and excretion from the body (Reinboth et al., 2010, de Boer et al., 2005).

After continuous feeding of 0.2 % w/w quercetin in the diet for 6 weeks to mice, the steady state levels in the liver and lung of parent and conjugated forms were 0.65 and 0.67 nmol/g, respectively (Zhang et al., 2010a). In comparison, when 0.2% w/w TMFol was administered in the diet for 2 weeks it furnished concentrations of 37.2 and 0.9 nmol/g in the liver and lung respectively. Therefore, TMFol levels were higher in these two tissues compared to quercetin for a similar dose. This potentially suggests the improved absorption, reduced metabolism and/or decreased excretion, of TMFol *in vivo*.

To our knowledge no study has previously reported levels of quercetin or fisetin in the prostate tissue of mice following oral dosing. If we make the assumption that potential concentrations of quercetin and fisetin achieved in these tissues would not exceed those reported for other types of tissue such as the liver and lung, then we can conclude that levels of TMFol in the prostate are much superior. Interestingly, the parent form of TMFol was always the predominant peak in the HPLC chromatograms whereas for quercetin and fisetin the conjugated species are more predominant. This observation suggests superior metabolic stability of TMFol to its hydroxylated counterparts. This is in agreement with previously published studies investigating the metabolic stability of methoxylated flavonoids over their hydroxylated congeners. For example, after oral administration of chrysin and its methoxylated analogue 5,7-dimethylflavone (5,7-DMF), chrysin was barely detectable in rat tissue (liver, kidney and lung), whereas 5,7-DMF levels after one hour were 5-15 μM (Walle and Walle, 2007b).

In this project TMFol has been investigated in prostate cancer cell lines, and we aim to develop it as an agent for the management of prostate cancer. Therefore, determining levels of TMFol in the prostate tissues is critical. Prostate is considered a special organ in that it is widely thought it might possess a blood barrier (Leibovitz et al., 2004). However, some studies have shown the presence of flavonoids and polyphenols in prostate tissue after oral

administration, e.g. tea polyphenols (Henning et al., 2006) and daidzein (Murray et al., 2007). TMFol was detected in prostate tissue of mice and levels were above 5.6 nmol/g at all time points between 5 and 360 mins, with a C_{max} of 18.4 nmol/g at 360 mins. These concentrations of TMFol are theoretically above the IC_{50} values for cell proliferation of 4.1 and 5.6 μ M in PC-3 and LNCaP cells, respectively. As no additional time points were taken between 6 and 24 h, the 5.6 μ M level could have potentially been achieved for even longer than 6 hours. Furthermore, TMFol still could be detected in the prostate 24 h post dosing, highlighting its ability to avoid extensive metabolism and excretion.

TMFol prostate levels following chronic administration of TMFol (0.2% w/w in the diet) were 5.3 nmol/g, which is comparable to the IC_{50} values of TMFol in PCa cells. Additionally, chemopreventive agents are intended to be taken for extended periods of time, as such, even sub- IC_{50} concentrations achieved in the target tissue could be sufficient to render significant chemopreventive effects.

From our findings it would appear that TMFol is able to avoid rapid metabolism. Accordingly, it is conceivable that a regular dosing regimen could result in enhanced activity over time due to the accumulation of the agent in cancer cells in the target tissue. Literature would suggest that the intracellular presence of flavonoids may be essential for their chemopreventive activity (Spencer et al., 2003). TMFol was rapidly taken up by prostate cancer cells and accumulated over time. It has been reported that shielding the hydroxyl groups could be an approach to improve the uptake of flavonoid by cells. Masking the hydroxyl groups of quercetin by a pivaloxymethyl moiety was shown to increase its cellular uptake and intracellular levels in HCT116 cells colon cancer cells (Kim et al., 2010). Similarly, we have shown with TMFol that replacement of hydroxyl moieties with methoxy groups improves uptake and metabolic stability. TMFol concentrations detected in PC-3 cells were found to be 7 times lower than in LNCaP cells. TMFol exerted cell growth inhibition with similar IC_{50} values in both cell lines so this observation could suggest that the PC-3 cells are more sensitive to TMFol as a lower intracellular concentration imparts comparable activity. Alternatively, one or more of the mechanism by which TMFol acts in

PC-3 cells may involve modulating extracellular targets rather than only intracellular ones.

Parent TMFol was the predominant peak with very few metabolites detected in the media or cell samples. These findings are consistent with TMFol and metabolite tissue levels *in vivo* whereby TMFol was again the predominant species present. In contrast, the hydroxylated flavonol quercetin was shown to be taken up rapidly by cultured dermal fibroblasts cells, but then underwent extensive metabolism (Spencer et al., 2004). The quercetin analogue, 4'-O-methyl quercetin showed improved cellular uptake and significantly less intracellular biotransformation (Spencer et al., 2003).

TMFol levels in the intestinal mucosa were remarkably higher than any other tissue and levels peaked at 20 mins at 5234 nmol/g. Such levels would suggest TMFol may be particularly appropriate for the management of intestinal cancers. Accordingly, our laboratory has shown that TMFol significantly inhibited adenoma burden in the *Apc^{Min}* mouse model and reduced tumour volume in a human colorectal cancer cell xenograft model (Howells et al., 2010).

Gastrointestinal conjugation is reported to be the major contributor of poor bioavailability of hydroxylated flavonoids (Manach and Donovan, 2004, Manach et al., 2005, Williamson and Manach, 2005). Therefore, we investigated whether the additional peaks identified in the HPLC traces of intestinal mucosa samples from mice dosed with TMFol might be TMFol conjugated TMFol metabolites. TMFol is methoxylated at three positions on the B ring and has one hydroxyl group at the 3 position which is free to undergo conjugation. In order for additional conjugation products to be detected the methoxy residues must first undergo demethylation before subsequent conjugation. To that end conjugate products and demethylated conjugate products were synthesised *in vitro* and used as standards to identify additional metabolites peaks detected in tissue samples *in vivo*.

Our data indicates that the main route of TMFol biotransformation in the mouse was glucuronidation. This is consistent with quercetin where the glucuronide product was the main metabolite produced in the gastrointestinal tract (Crespy et al., 1999). Glucuronidation of flavonoids has been considered the predominant route of metabolism due to the low intestinal epithelial sulfotransferase activity (Piskula and Terao, 1998). Mass spectrometric analysis confirmed the glucuronidation of TMFol. Both the TMFol-3-glucuronide and demethylated TMFol glucuronide were detected, however, the TMFol-3-glucuronide was the major metabolite. As the process of demethylation is uncommon in the intestine (Spencer, 2006) the presence of demethylated conjugates in the intestinal mucosa samples may be as a consequence of enterohepatic recirculation. Many flavonoids have been shown to be reabsorbed after enterohepatic circulation (Prasain and Barnes, 2007, Crozier et al., 2010). In the TMFol tissue concentration versus time curves of most tissues (Figure 4.2) there is the presence of two maximum peaks, one soon after administration and the second much later, consistent with enterohepatic recirculation (Xing et al., 2005).

Making the assumption that the extraction efficiencies and detection coefficient are identical for metabolites versus parent compound, one can conclude that TMFol is the predominant species detected which would support the notion that methoxylated compounds are less liable to metabolism versus their hydroxylated counter parts and could therefore impart superior chemopreventive activity.

In urine the predominant peaks detected were TMFol metabolites rather than parent compound. This may indicate the body's tendency to retain TMFol in its free form. In comparison hydroxylated flavonoids appear to undergo extensive conjugation and metabolism and are rapidly excreted in the urine (Walle, 2004, Manach and Donovan, 2004, Williamson and Manach, 2005).

In summary TMFol and its metabolites could be detected and quantified in a variety of murine tissues that may be a target of cancer chemopreventive or treatment interventions. Tissue levels in the small intestine, prostate, liver,

kidney and lung were all of the order of magnitude consistent with pharmacological activity *in vitro*. Glucuronidation, demethylation and demethylation followed by glucuronidation were the major metabolic pathways of TMFol in mice. TMFol has already been shown to be effective as an anticancer agent in the *Apc^{Min}* mouse model as well as a human colorectal cancer cell xenograft model (Howells et al., 2010). Therefore, in light of the pharmacokinetic findings presented in this chapter TMFol warrants further investigation for its use for the management of prostate cancer as well as for malignancies of the liver, kidney, lung and gastrointestinal tract.

CHAPTER FIVE

***In vivo* efficacy of TMFol in prostate cancer xenografts**

5.1 Introduction

The use of animal models to assess compound efficacy is an essential component of the drug development process for new anti-cancer drugs. *In vivo* models provide a greater understanding of how a new drug acts and undergoes metabolism in a biological system, thus allowing it to mimic the human body better than current *in vitro* model systems. With the knowledge of both pharmacologically active concentrations *in vitro* and target tissue levels *in vivo*, conclusions can be drawn regarding whether compounds will be active in efficacy models. These models also allow drugs to be administered in a way that is likely to mimic the human situation. Therefore, oral administration to animals can be compared to capsule consumption in humans, alternatively, intraperitoneal or intravenous dosing could also be used.

There is increasing evidence to suggest a positive correlation between efficacy in animal models and patient benefit in cancer prevention clinical trials, (Steele and Lubet, 2010). For example, difluoromethylornithine (DFMO) and sulindac combination has shown to prevent adenomas in the *Apc^{Min}* mouse (Ignatenko et al., 2008) the observation were also translated in human where low dose of DFMO and sulindac prevented sporadic colorectal adenomas (Meyskens et al., 2008). These observations provide valid justification for the use of such models to determine preclinical efficacy of new agents.

Although, some flavonoids have been shown to inhibit prostate tumour growth *in vivo* (Zhou et al., 2009, Miocinovic et al., 2005), the efficacy of orally flavonoids has not been achieved and has been linked to poor tissue bioavailability (Walle, 2009).

TMFol showed potent antiproliferative activity on prostate cancer cells *in vitro*. Furthermore, the tissue distribution study of TMFol identified its presence in considerable amounts in many tissues, including the prostate. Remarkably, the levels of TMFol found in the prostate were higher than the IC₅₀ concentrations in cells, suggesting TMFol may be efficacious *in vivo*. Due to these observations

TMFol was investigated in an *in vivo* efficacy model of PCa and compared to its hydroxyl counterparts quercetin and fisetin at equimolar doses.

5.2 Effect of TMFol, quercetin and fisetin on TRAMP C2 prostate cell growth in nude mice.

Nude mice were divided into 4 groups: control, 0.2% w/w TMFol, 0.184 % w/w quercetin and 0.174 % w/w fisetin. These doses of compound equate to equimolar concentrations of each flavonol making them comparable. Administration of the study diets began one week prior to cell inoculation. Cells were then inoculated at 5×10^6 per animal subcutaneously in 1:1 serum free media and matrigel.

After tumours had reached a measureable size, they were measured twice a week. TMFol caused a significant decrease in tumour volume as early as 19 days after inoculation (Figure 5.1). By study termination tumour volumes of the TMFol group were reduced by 42 % in comparison to control. In addition, TMFol significantly decreased the weight of the tumours by 15%. Murine body weight was not affected by TMFol. Fisetin and quercetin failed to decrease volume or weight of tumours significantly (Figure 5.2).

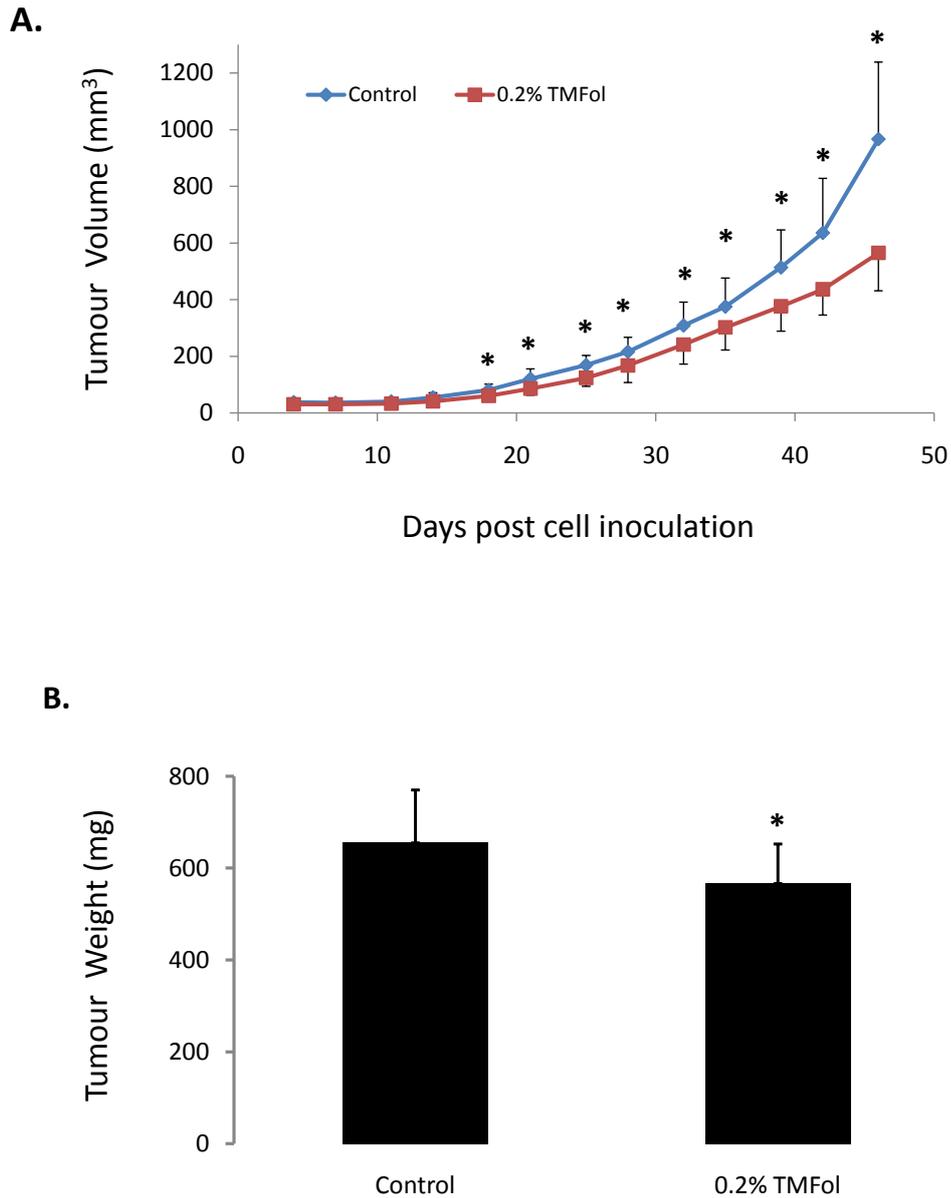


Figure 5.1 The effect of dietary TMFol on tumour growth in nude mice. Animals were divided into two equal groups (n=15), one week before inoculation, and were administered either 0.2 % w/w TMFol or standard diet (control). Tumour formation was initiated by inoculating mice flanks with 5×10^6 million cells. Tumour volumes were measure twice weekly. After 7 weeks, animals were scarified and the tumours were harvested and weighed. **(A)** Represents the volume of tumours over 7 weeks. Data points are the mean \pm SD of tumour volumes for either control or treatments. **(B)** Displays after harvesting tumour weight (mg) of the TMFol group in comparison to control. Student t-test was performed, (*) indicates data is statistically significantly different from control ($p < 0.05$).

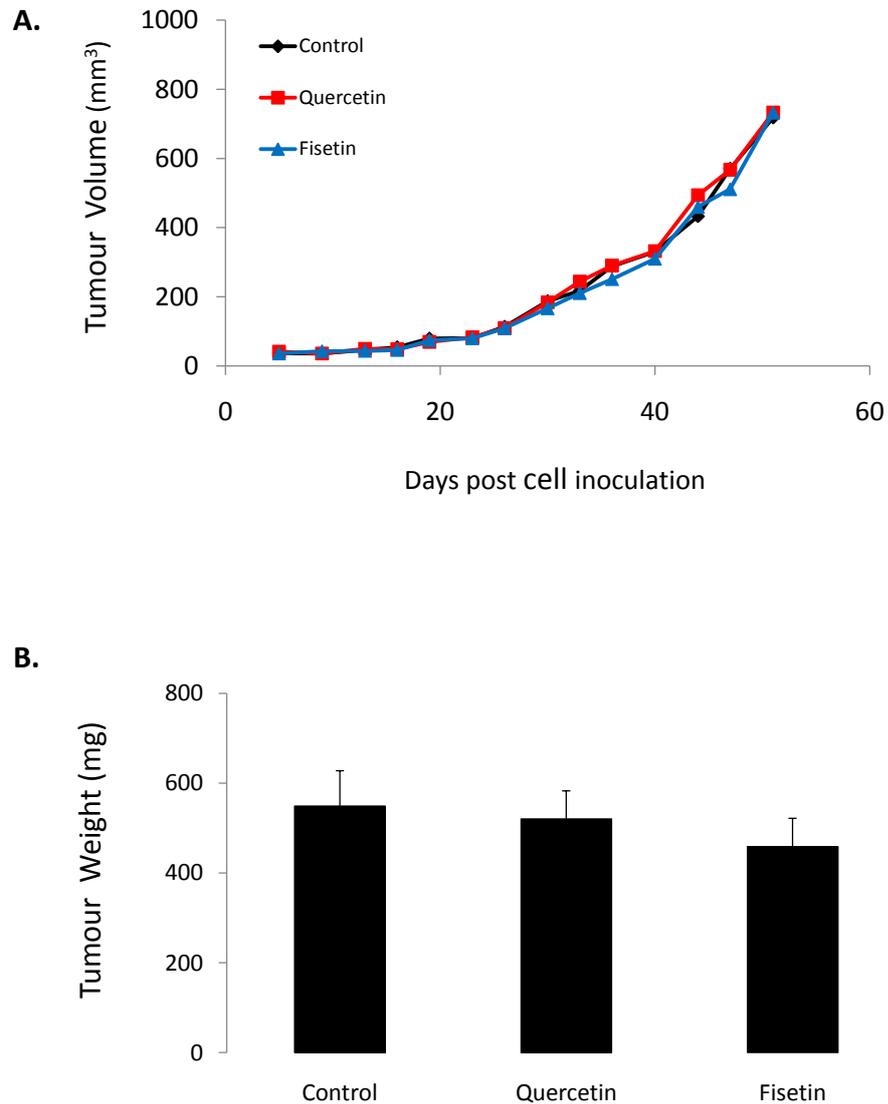


Figure 5.2 The effect of dietary quercetin and fisetin on tumour growth in nude mice. Animals were divided into three groups (n=5 per group), one week prior to cell inoculation mice started on diets containing either 0.184% w/w quercetin or 0.174% w/w fisetin or standard diet (control). Tumour formation was initiated by inoculating mice flanks with 5×10^6 cells suspended in 1:1 serum free media : matrigel. Tumour volumes were measure twice weekly. After 7 weeks animals were scarified and the tumours were harvested and weighed. **(A)** Represents the volume of tumours over 7 weeks. Data points are the mean \pm SD of tumour volumes for either control or treatments. **(B)** Displays the mean (\pm SD) after harvesting tumour weight (mg) of the quercetin or fisetin group in comparison to control.

5.3 Modulation of the apoptotic proteins, bax, survivin and p27, by TMFol, quercetin and fisetin in tumour tissue

TMFol induced cell cycle arrest and apoptosis and modulated a number of apoptosis related proteins when studied *in vitro*. Since TMFol also inhibited tumour growth *in vivo* these mechanisms were investigated in the xenograft tumour tissue as potential targets of TMFol.

The expression of Bax, survivin and p27 in the tumour xenograft tissue was determined in control and treated samples for TMFol (Figure 5.3), quercetin and fisetin (Figure 5.4). As can be seen in Figure 5.3, TMFol significantly increased the protein expression of bax and p27, and decreased the protein expression of survivin by ~40, 30 and 20% respectively in TRAMP C2 tumours compared to control tumours. Quercetin had no effect on bax expression, whereas fisetin caused a ~20% significant increase in the protein levels in tumour tissue (Figure 5.4 A). Quercetin significantly inhibited survivin expression by ~25%, in contrast fisetin had no effect (Figure 5.4 B). The levels of p27 were significantly decreased by both quercetin and fisetin by ~40 and 50% respectively (Figure 5.4 C). However, the effect was more pronounced with fisetin. These opposing effects of TMFol over quercetin and fisetin on p27 expression may highlight the importance of p27 as a potential mechanism by which TMFol exerted its efficacy in the *in vivo* model system.

The observed effects of TMFol on these proteins makes it desirable for the management of prostate cancer as it modulated the 3 markers (p27, bax and survivin) in favour of tumour inhibition, comparatively fisetin and quercetin each modulated one marker in favour and one marker against tumour inhibition.

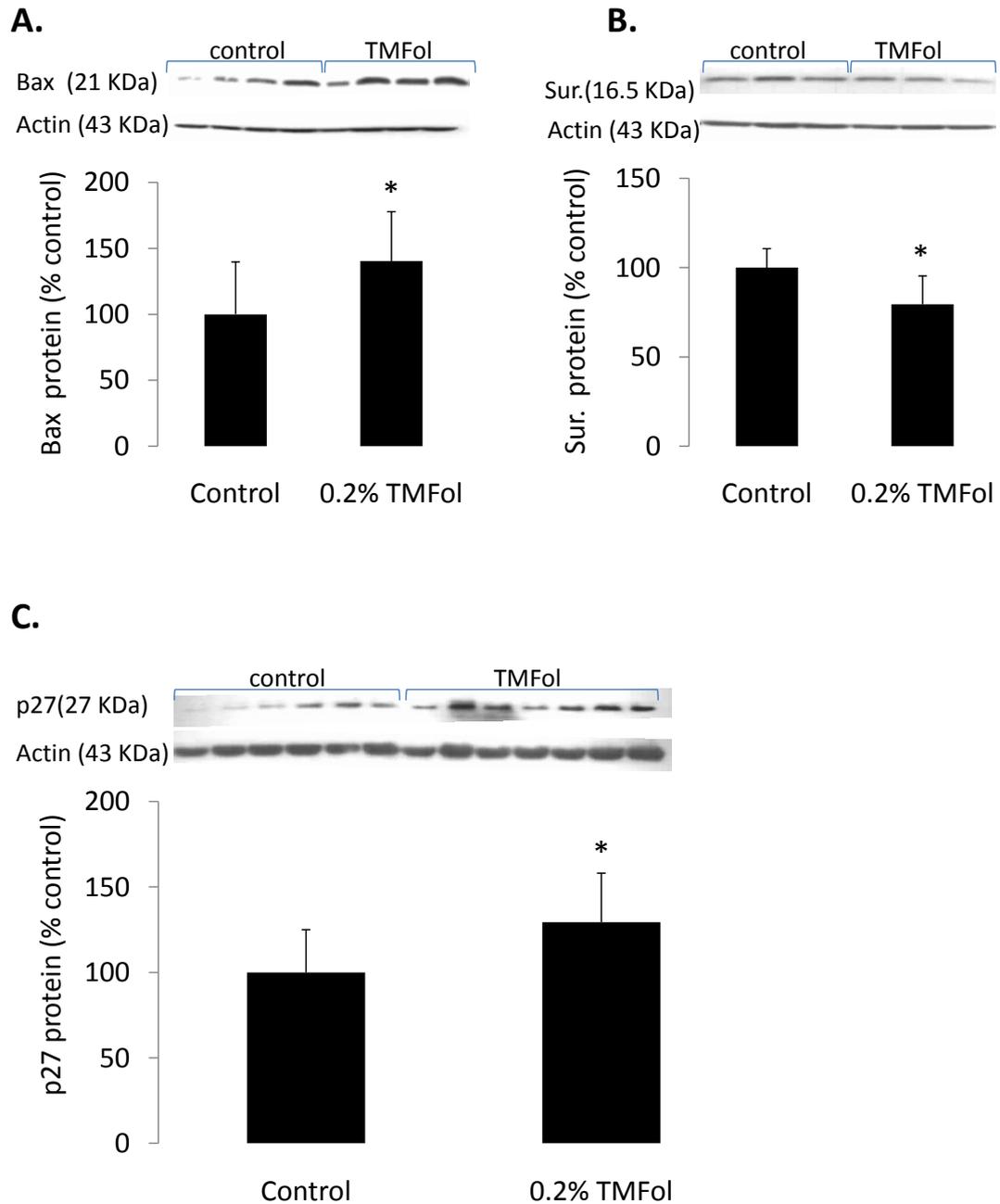


Figure 5.3 Effect of TMFol on the expression of bax, survivin and p27 in TRAMP C2 tumour xenografts in nude mice. Protein levels were measured after scarifying the animals which were received 0.2% w/w TMFol in diet or plain diet (controls) by western blotting of tumour xenograft tissue for bax (A), survivin (B) or p27 (C) and normalised to actin. The bar charts illustrate semi-quantitative densitometry analysis of bands represented as percentage of the control. Values are the mean \pm SD of 15 animals for the TMFol and control groups. (*) indicates data are significantly different from control ($p < 0.05$).

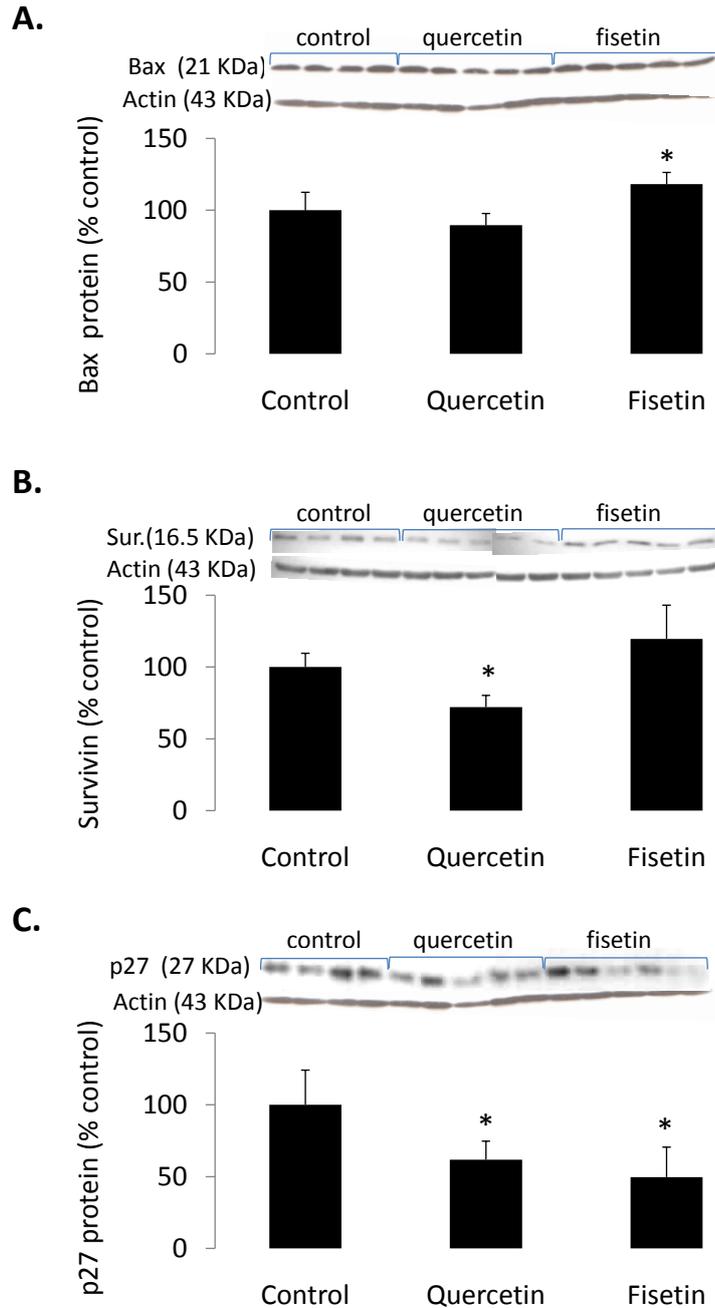


Figure 5.4 Effect of quercetin or fisetin on the expression of bax, survivin and p27 in TRAMP C2 tumour xenografts in nude mice. Protein levels were measured after sacrificing the animals which were received 0.184% w/w quercetin 0.174% w/w fisetin or plain diet (controls) by western blotting of tumour xenograft tissue for bax (A), survivin (B) or p27 (C) and normalised to actin. The bar charts illustrate semi-quantitative densitometry analysis of bands represented as percentage of the control. Values are the mean \pm SD of 5 animals for the TMFol and control groups. (*) indicates data are significantly different from control ($p < 0.05$).

5.4 Effect of TMFol on Ki-67, cleaved caspase-3 and AR expression in paraffin-embedded xenograft tumour tissue using immunohistochemical staining.

The nuclear protein Ki-67 is associated with cellular proliferation. It is linked to ribosomal RNA transcription (Bullwinkel et al., 2006). Ki-67 expression is a validated marker for cell proliferation (Scholzen and Gerdes, 2000). Therefore, the expression of Ki-67 was investigated in control and treated tumours, in order to assess TMFol's effect on tumour cell proliferation.

TMFol reduced the expression of Ki-67 by approximately 40% compared to the control group (Figure 5.5). This decrease in proliferation was consistent with the decrease in tumour volume and to some extent weight.

Western blot results of tissue from TMFol treated animals indicated changes to a number of apoptotic related proteins. For further verification the effect of TMFol on cleaved caspase-3 was determined

TMFol caused a significant ~2-fold increase in the number of cleaved caspase-3 stained cells in comparison to the control (Figure 5.6). This data complements the western blot data with bax, survivin and p27 that TMFol may, in part, exert efficacy by the induction of apoptosis.

In Chapter 3 the ability of TMFol to modulate the AR pathway was shown in PCa cells. Therefore, the expression of AR protein in xenograft tumours of treated and control animals were investigated. TMFol did not result in any significant change in the expression of AR in treated animals (Figure 5.7).

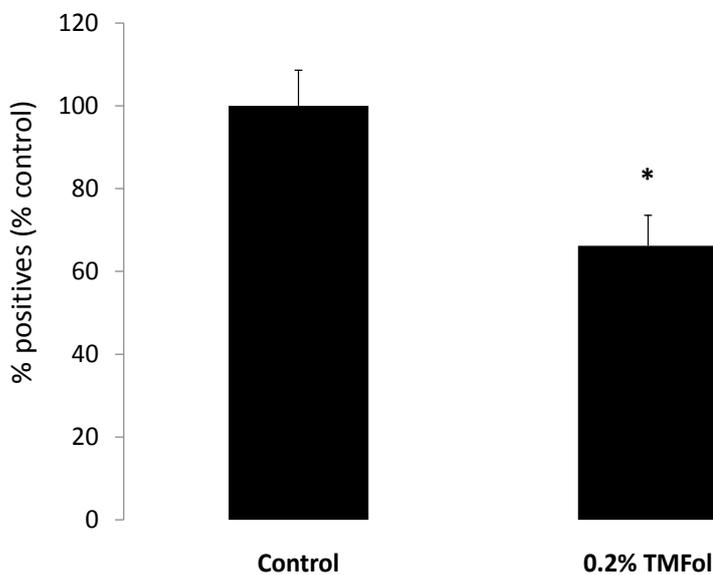
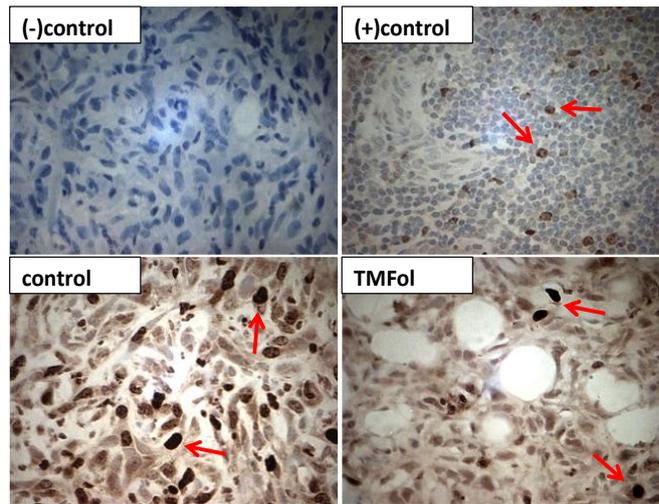


Figure 5.5 The effect of dietary TMFol on the expression of Ki-67 in TRAMP prostate cancer xenograft of male nude mice. Paraffin embedded sections of tumours from TMFol treated or vehicle control TRAMP C2 tumour bearing nude mice. Sections were immunohistochemically stained for Ki-67 (1:800). The bar charts represent the percentage of cells staining positive in the TMFol group versus those mice that received vehicle control only (n=15 animals per group). The photomicrographs are representative stained samples from the treated (TMFol) and vehicle (control) groups, in addition to a positive control (+ control) which is human tonsil tissue, and a negative control (- control), which is tumour tissue without antibody. Arrows point to positively stained cell. The percentage of positively stained cells was calculated from 10 random fields of view for each section. Bar charts illustrate the mean of positively stained cells for treatment samples as a percentage of the mean of controls \pm SD. (*) indicates data are significantly different to control ($p < 0.05$).

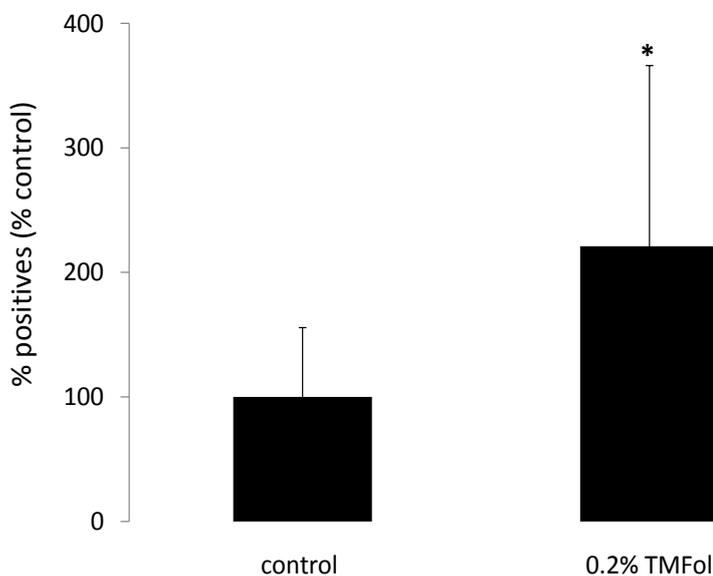
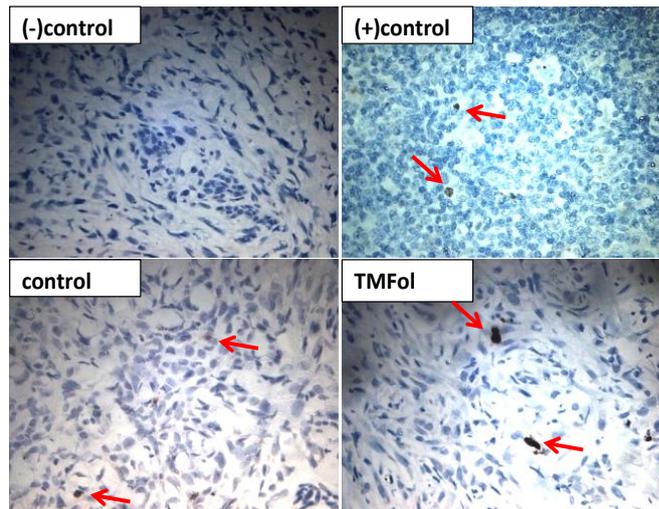


Figure 5.6 The effect of dietary TMFol on the expression of caspase-3 cleaved in TRAMP prostate cancer xenograft of male nude mice. Paraffin embedded sections of tumours from TMFol treated or vehicle control TRAMP C2 tumour bearing nude mice. Sections were immunohistochemically stained for cleaved caspase-3 (1:50). The bar charts represent the percentage of cells staining positive in the TMFol group versus those mice that received vehicle control only (n=15 animals per group). The photomicrographs are representative stained samples from the treated (TMFol) and vehicle (control) groups, in addition to a positive control (+ control) which is human tonsil tissue, and a negative control (- control), which is tumour tissue without antibody. Arrows point to positively stained cell. The percentage of positively stained cells was calculated from 10 random fields of view for each section. Bar charts illustrate the mean of positively stained cells for treatment samples as a percentage of the mean of controls \pm SD. (*) indicates data are significantly different to control ($p < 0.05$).

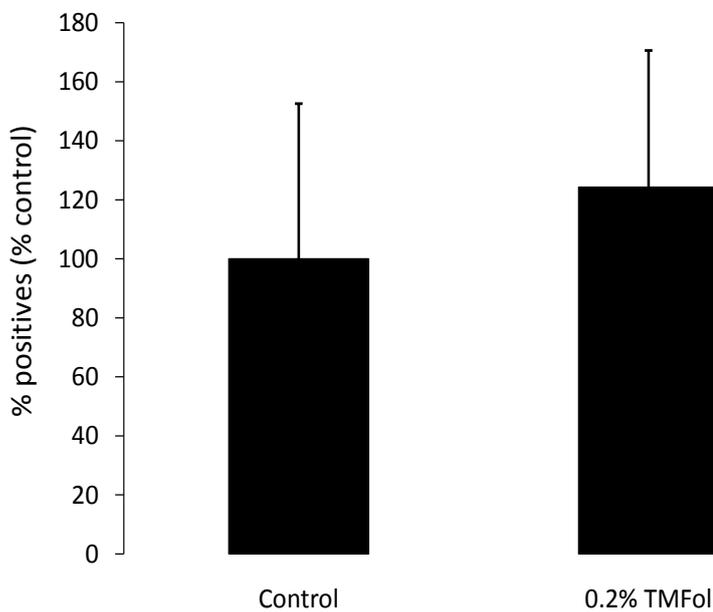
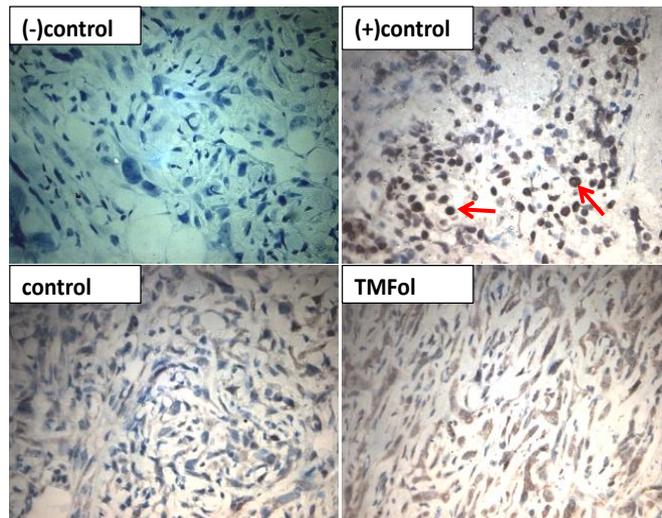


Figure 5.7 The effect of dietary TMFol on the expression of AR in TRAMP prostate cancer xenograft of male nude mice. Paraffin embedded sections of tumours from TMFol treated or vehicle control TRAMP C2 tumour bearing nude mice. Sections were immunohistochemically stained for AR (1:700). The bar charts represent the percentage of cells staining positive in the TMFol group versus those mice that received vehicle control only (n=15 animals per group). The photomicrographs are representative stained samples from the treated (TMFol) and vehicle (control) groups, in addition to a positive control (+ control) which is human prostate adenocarcinoma tissue, and a negative control (- control), which is tumour tissue without antibody. Arrows point to positively stained cell. The percentage of positively stained cells was calculated from 10 random fields of view for each section. Positive staining was assessed by giving score from 1-4. Bar charts illustrate the mean of positively stained cells for treatment samples as a percentage of the mean of controls \pm SD.

5.5 TMFol levels measureable in the prostate and tumour tissue following chronic dosing.

The delivery of cancer chemopreventive agents and anticancer drugs to tissue at concentrations that exert pharmacological activity is an essential requirement to impart efficacy. Many factors govern the availability and distribution of an agent in the target tissue. The levels of TMFol in the tumour and prostate tissue following chronic dosing were determined using HPLC analysis (Figure 5.8). TMFol levels were 0.92 ± 0.81 nmol/g in the xenograft tumour compared to 5.3 ± 2.7 nmol/g in the prostatic tissue of the same animals. This suggests that TMFol was approximately ~6-fold more abundant in the prostate tissue of these mice compared to the xenograft tumours. Nevertheless the relatively low levels of TMFol detected in the tumour still resulted in a 40% reduction in tumour volume. Parent TMFol appears to be the predominant peak in both HPLC traces suggesting that the compound is metabolically stable in both the prostate tissue and also the xenograft tumour.

As levels detected in the prostate were ~6-fold higher than that of the xenograft tumour it would be interesting to determine whether this would be reflected in enhanced efficacy in a transgenic model of PCa whereby the tumour develops in the target tissue, such as the TRAMP or Lady transgenic prostate cancer models (Nawijn et al., 2008)

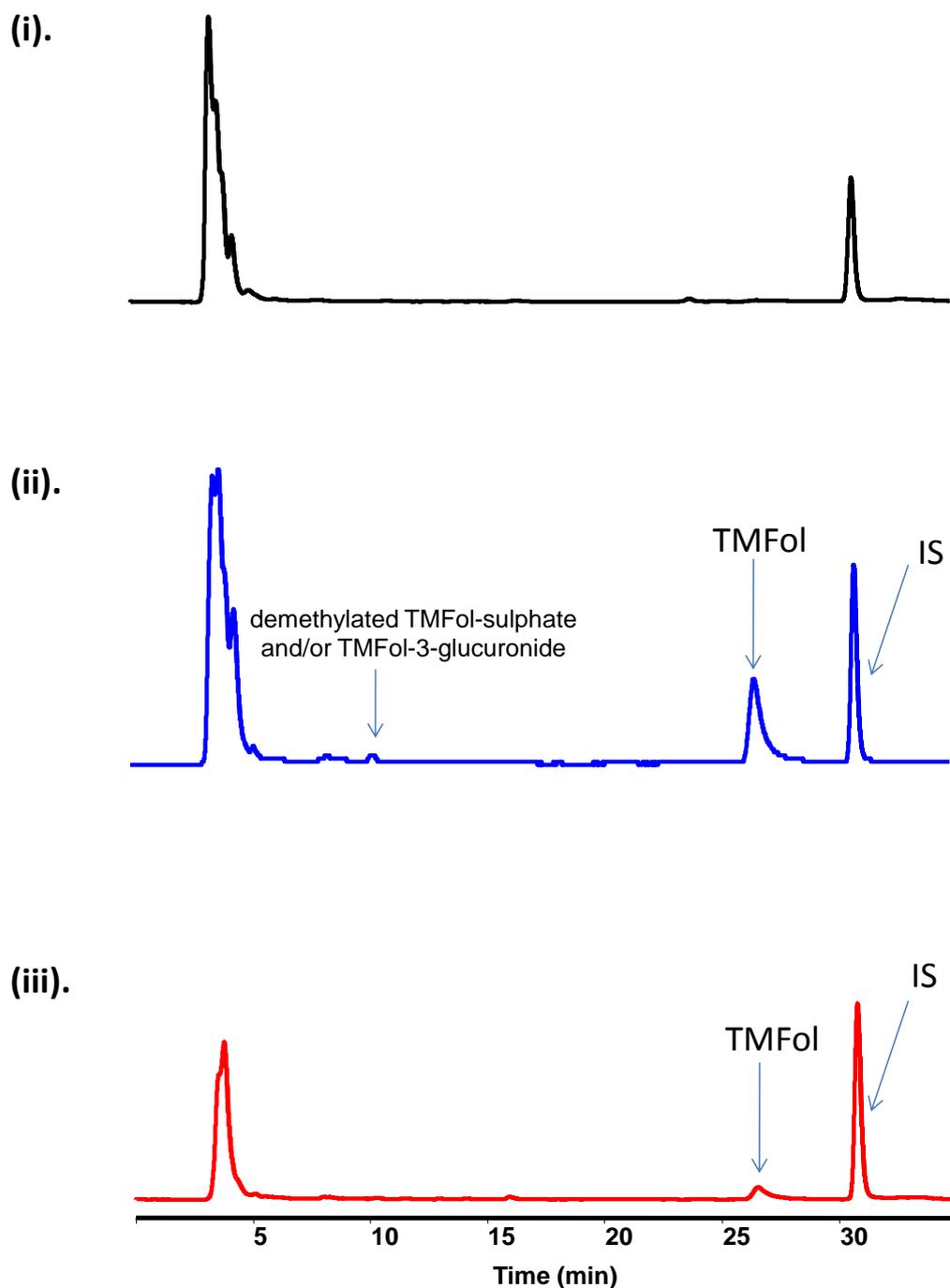


Figure 5.8 HPLC analysis of TMFol in tumours and prostate of mice treated with 0.2% w/w TMFol in the diet. Representative HPLC fluorescence chromatograms of (i) nude mouse prostate from the vehicle control group, (ii) prostate of a nude mouse that received 0.2% w/w TMFol in the diet and (iii) TRAMP C2 xenograft tumour of TMFol treated nude mouse. IS and TMFol arrows point to the peaks of internal standard (2',5',5,6,7,8-hexamethoxyflavone) and TMFol respectively.

5.6 Discussion

The objective of the work described in this chapter was to provide *in vivo* efficacy data for TMFol in comparison to quercetin and fisetin. It was also to investigate the potential mechanism of action of this agent in an *in vivo* model system.

Data generated in this study points to statistically significant inhibition of tumour growth resulting from oral TMFol consumption by mice. Comparatively, fisetin and quercetin at equimolar doses had no effect. This data is consistent with the *in vitro* data in which the antiproliferative potency of TMFol was more than 5-fold higher than fisetin and quercetin in PCa cells. Nonetheless, fisetin and quercetin have been found previously to inhibit tumour growth of CWR22Rv1 (Khan et al., 2008a) and PC-3 (Asea et al., 2001) PCa xenografts in nude mice, respectively. However, both were injected intraperitoneally as opposed to consumed in the diet. Therefore, the disparity is possibly due to a variation in the delivery of the flavonol to the tumour rather than potency alone. Hydroxylated flavonols including quercetin and fisetin are considered orally unstable as they potentially undergo extensive conjugation and metabolism in the GIT (Manach and Donovan, 2004, Walle, 2004). Perhaps as a consequence of this transformation, efficacious levels were not achieved in tumours tissue. This is partially true for TMFol where approximately 6-fold lower levels were achieved in the xenograft tumour as opposed to the prostate tissue itself. However, TMFol had a significant effect on tumour volume as early as 19 days post inoculation, and this effect continued until the end of the experiment. This may be as a consequence of accumulation in the tissue due to its improved stability *in vivo* and slower metabolism. This data supports the hypothesis tested, that methoxylated flavonols are superior to their hydroxylated counterparts as anti-prostate cancer agents. Methoxylation of flavonoids was previously found to improve *in vitro* and *in vivo* activity (Walle et al., 2007a, Walle, 2007a). For example, apigenin a hydroxylated flavone failed to decrease adenoma development in *Apc^{Min}* mouse, whereas the methoxylated flavones 5,7,3',4',5'-pentamethoxyflavone and tricetin did (Cai et al., 2009). Importantly, TMFol showed a significant ability to reduce tumour growth in two *in vivo* colorectal

preclinical models, the *APC^{Min}* mouse and HCT116 tumour xenografts (Howells et al., 2010), whereas, quercetin had no effect *in APC^{Min}* mouse model (Mahmoud et al., 2000).

HPLC analysis of tumour tissues taken from TMFol treated animals detected the presence of TMFol at an average concentration of 0.91 nmol/g of tumour. This confirms the ability of TMFol to reach the tumour in parent form, and therefore may highlight an advantage over other flavonols where their poor bioavailability has accounted for lack of *in vivo* efficacy (Gao and Hu, 2010). A recent study reported levels of 0.5 nmol/g of quercetin in pancreatic cancer xenograft tissues after consumption of 0.2% w/w in the diet (Zhang et al., 2010a) which is approximately half the value observed for TMFol in the TRAMP C2-derived tumours. As part of this study we did not investigate quercetin or fisetin levels in the tumour tissue. However, if similar levels were achieved in our model system as those described previously it is unlikely that we would have observed any efficacy with these agents as their IC_{50} values *in vitro* were 23 and 27 μ M in this cell line, respectively. This is not true for TMFol where the IC_{50} value was 1.7 μ M so detecting ~ 1 μ M (1 nmol/g = 1 μ M assuming 1 g of tissue equal to 1 ml media) in the tumour tissue *in vivo* we would have expected to detect some growth inhibition which is exactly what was observed.

Furthermore, TMFol prostate levels were ~ 6 -fold higher than the levels achieved in tumours so theoretically efficacy could be greater in a model system in which the tumour develops in the prostate itself such as the transgenic TRAMP mouse model.

When we investigated the potential mechanisms by which TMFol exerted efficacy we found that the levels of bax and p27 expression were elevated and survivin levels were decreased in TMFol treated tumours in comparison to controls. These results are consistent with *in vitro* data in the LNCaP cells, in which TMFol modulated these proteins in a similar manner. However *in vitro* this was at much higher concentrations of 10 and 20 μ M.

Up regulation of bax by TMFol could be indicative of the induction of apoptosis, and therefore, a mechanism by which tumour growth could be inhibited. Bax is used as a tumour progression marker for prostate cancer therapy (Mackey et al., 1998), so modulation of this protein could be an important marker for TMFol efficacy.

Survivin is a member of anti-apoptotic proteins (IAPs) that contribute to tumorigenesis. It is not expressed in most normal tissues but is highly expressed in tumour tissues and as a result, it is considered as a promising biomarker for tumours and cancer therapy. It was suggested survivin measurement might aid the early diagnosis of cancers such as bladder cancer, and also helps in determining the prognosis in multiple cancer types (Duffy et al., 2007) including prostate (Shariat et al., 2004) and predict response to diverse anti-cancer therapies (Duffy et al., 2007). The functional cellular roles of survivin include inhibition of apoptosis (Dohi et al., 2004), regulation of cell cycle (Altieri, 2010) and induction of angiogenesis (Tran et al., 2002). Survivin was also found to mediate the resistance to anti-androgen therapy in PCa cells (Zhang et al., 2005). Therefore, reduction in survivin levels is in contrast to all these tumourigenic effects and could be considered beneficial for the chemoprevention and management of the disease. Therefore, the ability of TMFol to down regulate survivin suggests it's potential as a putative agent for PCa management.

TMFol induced the expression of p27 in the xenograft tumours which is a potent cell cycle inhibitor and its expression has been considered to contribute to anticancer activity: p27 is a CDKI and a putative tumour suppressor gene, also it plays a role in cell differentiation and in the regulation of cell cycle, in addition it is promoter for apoptosis. Therefore, it has been postulated as a marker for diagnosis and prognosis of PCa and other malignancies (Lloyd et al., 1999, Tsihlias et al., 1998, Yang et al., 1998)

Although, fisetin and quercetin did not reduce tumour size, they were able to modulate bax and survivin expression. Bax levels were increased by fisetin and survivin levels decreased by quercetin. As no tumour reduction was observed

with either quercetin or fisetin it may be that these proteins are not essential for tumour growth in this particular model and not the main mechanism by which TMFol imparts efficacy. However, the effects of quercetin and fisetin on p27 expression are opposing to TMFol and this in part could explain the difference in efficacy observed between the three agents. This data contradicts to some extent the data observed *in vitro*, whereby quercetin and fisetin were both found to increase bax levels in PCa cells (Vijayababu et al., 2005, Lee et al., 2008, Khan et al., 2008a). In addition, quercetin also showed a decrease in the expression of survivin in PCa cells (Kim et al., 2008), and p27 expression was upregulated by fisetin in LNCaP PCa cells (Khan et al., 2008a). This disparity between *in vivo* and *in vitro* data could be a result of extensive drug metabolism leading to sub-efficacious concentrations achieved in the tumour tissue. It is important to note too that flavonols at lower concentration have been shown to induce proliferation in PCa cells (Dihal et al., 2006, Adlercreutz et al., 1995), an effect that we also observed (Figure 3.10 A).

The increase in cleaved caspase-3 in the TMFol treatment group could be indicative of apoptosis induction. Cleaved caspase-3 is a late downstream mediator of the mitochondrial-bax apoptosis pathway (Strasser et al., 2000). Bax contributes more to the early events of apoptosis and therefore does not necessarily prove apoptosis induction (Wei et al., 2001). This observation of increased caspase-3 cleavage is in agreement with the effects of TMFol in a colorectal cancer model. TMFol was found to induce the expression of cleaved caspase-3 in HCT116 adenocarcinoma mouse xenografts (Howells et al., 2010). Importantly, apoptosis is a desirable mechanistic approach in cancer chemoprevention and treatment as it resembles the normal processes of balancing the cell growth (Sun et al., 2004) so this highlights the potential advantage of TMFol for cancer chemoprevention and treatment.

Androgen receptor expression was not altered by TMFol treatment. TMFol reduced the expression of AR in PCa cells *in vitro* at concentrations higher than 5 μ M, but interestingly resulted in a marginal up-regulation at lower TMFol concentrations (Figure 3.20). Since tumour TMFol levels were found to be less than 1 μ M, this non significant increase in AR expression *in vivo* is consistent

with the *in vitro* observations. In addition, up regulation of AR at low concentrations has been described for other putative chemopreventive agents; prolonged treatment of finasteride was found to increase AR expression in LNCaP cells and benign prostatic hyperplasia tissues (Hsieh et al., 2011). Resveratrol, is a potential cancer chemopreventive agent, which occurs in grapes and red wine, and it is under clinical evaluation for various cancers (Bishayee, 2009, Patel, 2011). It was found to up regulate the expression of the AR by 160% in TRAMP mice when given in the diet at a dose of 625 mg/kg/day (Harper et al., 2007) and by 46% in SV-40 Tag rats (Harper et al., 2009). It was also found to increase the transactivation of AR at low concentrations in LNCaP cells (Gao et al., 2004). while it inhibited the growth at high concentrations in the same cell lines (Mitchell et al., 1999). These observations are consistent with results we found with TMFol in this study.

In summary, the data presented here is the first report of *in vivo* efficacy of TMFol in a model of PCa. TMFol significantly reduced the growth of tumours derived from TRAMP C2 cells in the nude mouse xenograft model. Comparatively, fisetin and quercetin failed to produce any reduction in growth. TMFol showed modulation of cleaved caspase-3, bax, survivin and p27 consistent with cell cycle and apoptosis induction. Of these, p27 may be the most important marker or target for TMFol efficacy. Xenograft tumour levels of TMFol, which resulted in the growth inhibition, are less than ~1/6 of the levels achieved in the prostate tissue itself. Additional studies in a transgenic model of PCa are therefore warranted with TMFol to elucidate its potential use for the management of the disease in humans.

CHAPTER SIX

The final discussion

PCa is a major health problem, and in 2008 was the second most common cause of cancer deaths among men and contributed to 12% of male deaths from cancer in the UK (<http://info.cancerresearchuk.org/cancerstats/types/prostate/mortality/#source1>). Accordingly, any significant reduction in PCa incidence would be of huge benefit to the general population. PCa is more prevalent among elderly men, with more than 75% of the diagnosed cases in men over the age 75. Therefore, any delay or reverse of the disease could lead to a significant decrease in the PCa incidence and the improvement in quality of life of these patients. Since the clinical development of PCa takes between 30-40 years (Lieberman et al., 2001). This provides a good pharmacological window of opportunity for chemopreventive intervention.

Even though it is widely accepted that PCa is ideally suited for a chemoprevention intervention strategy, 5- α reductase inhibitors (dutasteride and finasteride) are still the only agents which have proved successful (Strope and Andriole, 2010). Nonetheless, they come with their own problems with a number of disadvantages and side effects such as; reduction in sexual drive, erectile dysfunction, gynecomastia, and depression (Traish et al., 2011). In addition to their low potency in reducing PCa cancer development (5% of cases) (Strope and Andriole, 2010). Importantly, in December 2010 the US FDA recommended against prostate cancer chemoprevention using 5 α -reductase inhibitors because of the likelihood of incidence of high-grade tumours <http://www.medscape.com/viewarticle/733490>. Hence, research is driven by the need to find a more effective and less toxic PCa chemopreventive agent.

The most commonly used strategies for PCa drug treatment are the hormone deprivation and chemotherapy. Hormone deprivation therapy has been an essential approach for PCa treatment since it was coined by Huggins and Hodges (Huggins and Hodges, 1941). But by time it becomes ineffective and the cancer turns to the hormone independent form. The other approach is using chemotherapeutic agents for the treatment of metastatic disease which comes with their own side effects on various body systems. However, PCa is not

curable by any of the current options. Therefore, there is a need to find a drug with higher potency and less side effects than the available drugs for PCa.

Epidemiological studies have linked the intake of flavonoids to the reduction of PCa incidence (Griffiths et al., 1999). This impact could explain the low rates of PCa among south Asian men where flavonoids are highly consumed in the diet. There are a huge number of flavonoids that make up the human diet, therefore, the possibility of those that are regularly consumed exerting high toxicity in humans is low. Exerting little or no toxicity is an essential feature of any chemopreventive agent, which potentially highlights the attractiveness of this class of compounds.

The *in vitro* testing of flavonoids has shown important anti-proliferative properties but studies *in vivo* do not always reflect this activity, which could be due to the low absorption and/or rapid metabolism of these compounds (Walle, 2004, Scalbert and Williamson, 2000, Gao and Hu, 2010). Naturally occurring flavonoids predominantly consist of hydroxyl groups in their scaffold which is widely thought to be the main reason for their poor bioavailability and potency, as they can undergo rapid conjugation and excretion. Therefore, identification of methoxylated flavonoids with comparative or better activity may be one approach by which this class of agent may overcome these downfalls (Wen and Walle, 2006b, Wen and Walle, 2006a, Walle, 2007a).

TMFol was identified in this project through an initial *in vitro* antiproliferative activity screen of a panel of hydroxylated and methoxylated flavonoids and was found to be the most effective in both androgen dependent and independent PCa cells. Furthermore, in comparison to the most extensively studied flavonols, quercetin and fisetin, TMFol exhibited a 5-fold greater potency in terms of growth inhibition. TMFol significantly reduced the growth of TRAMP C2 PCa cell-derived xenograft tumors, whereas at equimolar doses fisetin and quercetin failed to show any significant inhibition. Interestingly, fisetin and quercetin have both been considered as putative cancer chemopreventive agents for PCa so the fact that TMFol is superior to both agents suggests the

potential advantage and therefore development opportunity of TMFol for this role.

Quercetin has been used in combination with the potential PCa chemopreventive agent finasteride and enhanced chemopreventive activity and eliminated unwanted side effects. The quercetin-finasteride combination enhanced the reduction in proliferation rate and wet prostate weight in rats compared to either agent alone (Ma et al., 2004a). With regard to fisetin, a patent for its use as a treatment for PCa has been submitted to the U.S.A Provisional Patent Application (<http://www.fags.org/patents/app/20100010078>). The application was based on *in vitro* and *in vivo* efficacy and mechanistic studies, whereby fisetin was shown to modulate components of both the cell cycle machinery and the apoptotic pathway (Khan et al., 2008a, Khan et al., 2008b, Haddad et al., 2006). Importantly, TMFol has been shown to regulate a number of these cell cycle and apoptotic proteins also modulated by fisetin, however, TMFol altered their expression at much lower concentrations and at levels achievable in target tissue *in vivo*. The accomplishment of quercetin and fisetin for clinical development is unlikely to be successful as it has been reported that both agents have mutagenic effects in the Ames test (Macgregor and Jurd, 1978, Stopper et al., 2005). As such TMFol was tested for its mutagenicity and in all systems tested proved negative (personal communication, Dr. Stewart Sale). This observation would make TMFol an attractive alternative over quercetin or fisetin for clinical development.

The pharmacokinetic studies with TMFol showed that levels that have pharmacological activities *in vitro* could be achieved in tissues of C57BL/6J mice. The prostate levels of TMFol were comparable to or higher than IC_{50} concentrations *in vitro* either after single or continuous administration. Furthermore, TMFol levels in mouse prostates were ~6-fold higher than the concentrations found in the xenograft tumors which induced significant inhibition of growth, suggesting the activity of TMFol may be even more pronounced in a transgenic model of PCa where the tumour develops in the target tissue itself.

By performing a series of metabolism studies, it could be concluded that TMFol underwent a degree of metabolism. However, the parent form of TMFol was generally the predominant species in tissues. This improved metabolic stability of TMFol may be advantageous over hydroxylated flavonols, such as quercetin and fisetin as it is more protected from rapid conjugation and excretion. Thus, it could exert pharmacological activity for longer periods of time in the target tissue and even to some extent accumulate.

Attempts were made to determine the mechanisms of action by which TMFol inhibited PCa cell growth. TMFol showed antiproliferative activity, induced cell cycle arrest and induced apoptosis in both androgen-dependent and androgen-independent PCa cells. The *In vivo* and *in vitro* investigation into some proteins which are contributed to cell cycle and apoptosis highlighted some pathways involved in TMFol's mechanism of action. TMFol effects on cell cycle and apoptosis could be linked to the down regulation of AR, PSA and survivin and up regulation of the CDKIs (p27 and p21), p53 and Bax. Most of these proteins have been widely accepted as biomarkers for tumour progression and cancer therapy as highlighted in the previous chapters. Quercetin and fisetin have both been found to modulate a number of these proteins similar to that of TMFol but at much higher concentrations that may not be achieved clinically. However, TMFol concentrations that modulated these proteins are potentially clinically achievable based on the pharmacokinetic study performed as part of this project and this again would suggest a reason to develop TMFol clinically in preference to either quercetin or fisetin.

As part of this project TMFol was compared *vis-à-vis* other compounds in the same class, flavonols. However, if it is to be developed clinically for the potential management of PCa it is interesting to compare it to other agents currently under investigation. To date the most widely used agents are those that interfere with AR signalling, a pathway shown to be modulated by TMFol. We showed that 48 hrs incubation of LNCaP cells with TMFol (20 μ M) resulted in a reduction of media PSA by 47%, which is comparable to the reduction (50%) caused by finasteride (25 μ M) (Wang et al., 1997), which has been shown to be of clinical benefit in PCa chemoprevention (Strope and Andriole, 2010, Sarvis

and Thompson, 2008). In addition, the reduction in PSA mRNA observed for TMFol was approximately 60% compared to 48% by finasteride at the 72hrs time point (Wang et al., 1997). The widely used androgen receptor blocker bicalutamide (100 μ M) resulted in a decrease in secreted PSA levels in the culture media by approximately 70% at 72 hrs (Masiello et al., 2002). TMFol resulted in a 55% reduction at concentration of 20 μ M. These comparisons of TMFol to other non flavonol compounds suggest that TMFol has comparable if not superior activity on the AR pathway *in vitro*, a pathway thought to be critical in PCa development. Future focus could be on comparing TMFol to other agents currently under preclinical evaluation for PCa prevention or therapy to observe how it compares.

This project has provided the groundwork for TMFol as a new agent for the management of PCa. It has highlighted the benefits of TMFol over other widely used flavonols, as well as other agents currently under preclinical and clinical evaluation. Additional preclinical studies, most notably acquiring toxicological data, are still needed for TMFol before it can realistically be developed clinically, initially for the treatment of PCa.

Although work in this project has tried to identify the mechanisms of action of TMFol, more work is still required to find definitive mechanisms and then identify those that are keys to its *in vivo* efficacy. Further studies employing a transgenic model of PCa are required to show efficacy in a model where the tumour develops in the target tissue as well as identify potential pathways or targets that TMFol can modulate. By incorporating analytical analysis into these studies the TMFol tissue levels can be then correlated to biomarker response and optimum dosing regimens determined ready for clinical trials.

In addition, toxicological investigations need to be performed before TMFol could enter clinical trials. Flavonoids are in general considered to have low toxicity as they make up a considerable component of the human diet. However, many dietary compounds have shown unpredictable toxicity upon clinical testing as they have been used in much higher doses than that normally consumed in the diet (Goodman, 2008). Moreover, TMFol is a synthetic

methoxylated analogue, and as such proper preclinical toxicity studies must be performed before any further clinical assessment can take place. Acute, sub-acute (sub-chronic) and chronic toxicity studies in *in vivo* models usually are part of this toxicity evaluation process. Work from our laboratory has shown that chronic dosing of TMFol 0.2% w/w in diet for up to 12 weeks has had no adverse effects on mice as measured by physical appearance, behaviour and weight loss. In addition tissues from these mice have undergone pathological analysis with no indications of toxicity.

Based on our pharmacokinetic data TMFol is distributed to various tissues at pharmacologically active levels, suggesting TMFol may be investigated for its potential benefit in other malignancy types such as liver, GIT, lung and kidney. Our laboratory has already shown in a preclinical model of colorectal cancer encouraging results (Howells et al., 2010) for TMFol in two separate *in vivo* model systems.

In conclusion, this project has identified TMFol as an exciting new agent that may have the potential to benefit cancer patients with a variety of malignancies, and as such warrants further preclinical investigation.

CHAPTER SEVEN

Appendix

7.1 List of abbreviations

AIF	apoptosis-inducing factor
ANOVA	analysis of variance
AR	androgen receptor
AREs	androgen receptor elements
ATCC	american type culture collection
AUC	area under the curve
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CAMs	cell adhesion molecules
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
COMT	catechol-o-methyltransferase
COMT	catechol-o-methyltransferase
COX	cyclooxygenase enzyme
DAB	diaminobenzidine
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECACC	european collection of cell cultures
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra cetic acid
EGFR	epidermal growth factor receptor

ERK	extracellular signal-regulated kinases
FACs	fluorescence-activated cell sorting
FCS	foetal calf serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GIT	gastrointestinal tract
HGPIN	high grade prostatic intraepithelial neoplasia
HIPC	hormone independent prostate cancer
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HRPC	hormone refractory prostate cancer
HSP	heat shock protein
i.g	intra-gastric
IAPs	anti apoptotic proteins
IC50	does which produces 50% reduction in the number of live cells
IGF-1	insulin-like growth factor 1
IS	internal standard
MAPK	mitogen-activated protein kinase
Mib	mibolerone
MS	mass spectra
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	british national health services

NSAIDs	non steroidal anti-inflammatory drugs
PAGE	polyacrylamide gel electrophoresis
PAR	peak area ratio
PARP	poly (adp-ribose) polymerase
PBS	phosphate buffer solution
PCa	prostate cancer
PDGF	platelet-derived growth factors
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIN	prostatic intraepithelial neoplasia
PMF	3',4',5',5,7-pentamethoxyflavone
PS	phosphotidyl serine
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma protein
ROS	reactive oxygen species
Rpm	revolutions per minute
RT	room temperature
RTK	tyrosine kinase receptor
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SGLT	sodium dependent glucose transporter

SULT	sulfotransferase
TBE	tris/borate/edta
TEMED	tetramethylethylenediamine
TEMED	n, n, n',n' tetramethyl-ethylenediamine
TGF- β	transforming growth factor β
TMFol	3',4',5'-trimethoxyflavonol
T-PBS	tween phosphate buffer solution
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAMP	transgenic adenocarcinoma of the mouse prostate
UDPGA	uridine 5'-diphosph-glucuronic acid
UGTs	uridine-5'-diphosphate glucuronosyltransferases
VEGF	vascular endothelial growth factor

7.2 publications and abstracts

Papers

Britton, R. G., Fong, I., Saad, S., Brown, K., Steward, W. P., Gescher, A., and Sale, S. (2009). Synthesis of the flavonoid 3',4',5'-trimethoxyflavonol and its determination in plasma and tissues of mice by HPLC with fluorescence detection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 877, 939-942

Abstracts

Shaban Saad, Donald Jones, Hong Cai, Robert Britton, Peter Greaves, Karen Brown, William Steward, Andreas Gescher and Stewart Sale. 3', 4', 5'-Trimethoxyflavonol (TMFol), a novel potential cancer chemopreventive agent. Tissue distribution in mice following a single oral dose. The National Cancer Research institute Cancer Conference, Oct 2009, Birmingham, UK.

Shaban Saad, Lynne Howells, Robert Britton, Ralf Schmid, William Steward, Andreas Gescher, Karen Brown and Stewart Sale. 3', 4', 5'-Trimethoxyflavonol (TMFol), a novel putative prostate cancer chemopreventive agent: *in vitro* and *in vivo* preclinical efficacy. The 50th Anniversary meeting of The British Association for Cancer Research, June 2010, Edinburgh, UK.

Shaban Saad, Lynne Howells, Robert G. Britton, William P. Steward¹, Andreas Gescher, Karen Brown and Stewart Sale. 3', 4', 5'-Trimethoxyflavonol (TMFol), a novel putative prostate cancer chemopreventive agent: In vitro and in vivo preclinical activity (2010). AACR International Conference on Frontiers in Cancer Prevention Research, Nov 2010, Philadelphia, USA.

Shaban Saad, Robert G Britton, Kuldeep Singh, William P Steward, Andreas Gescher, Karen Brown and Stewart Sale. Identification and characterisation of 3', 4', 5'-Trimethoxyflavonol (TMFol) as a novel prostate chemoprevention agent. The 2nd academic symposium of Libyan students of the UK, June 2009, Bradford, UK.

Shaban Saad, Donald J Jones, Hong Cai, Robert G. Britton, Peter Greaves, Karen Brown, William P. Steward, Andreas Gescher and Stewart Sale. 3', 4', 5'-Trimethoxyflavonol (TMFol), a novel potential cancer chemopreventive agent. Tissue distribution in mice following a single oral dose. The 2nd academic symposium of Libyan students of the UK, June 2009, Bradford, UK.

Shaban Saad, Lynne Howells, Robert Britton, Ralf Schmid, William Steward, Andreas Gescher, Karen Brown and Stewart Sale TMFol a novel prostate cancer chemopreventive agent: *in vivo* and *in vitro* efficacy study in prostate cancer models. The 3rd academic symposium of Libyan students of the UK, June 2010, Sheffield, UK

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