Preclinical and Phase I studies of Phenoxodiol: A translational approach for the development of a novel isoflavone for the treatment of prostate cancer

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Abstract

Isoflavones are phytoestrogens that have pleiotropic effects in a wide variety of cancer cell lines. Many of these biological effects involve key components of signal transduction pathways within cancer cells, including prostate cancer cells. Epidemiological studies have raised the hypothesis that isoflavones may play an important part in the prevention and modulation of prostate cancer growth. Randomized Phase III trials of isoflavones in prostate cancer prevention are lacking, and the best evidence (albeit modest) for this concept is provided by case - control studies. On the other hand, *in vitro* data are much more convincing than clinical studies in regard to the potential anticancer activity of a number of isoflavones. As a result of this body of work, flavopiridol, genistein, and phenoxodiol are now being developed as potential treatments for cancer. In addition, their activity in combination with cytotoxics or radiotherapy warrants further investigation.

The clinical pharmacology of isoflavones demonstrates that this group of compounds are orally bioavailable, and have a complex pharmacokinetic profile. In general, the absorption and distribution of these compounds is extensive, and only a small proportion of the many metabolites generated in the body have been studied in any detail. The bowel microbiological environment appears to be important in determining both absorption and the mix of metabolites generated, though pharmacogenomic mechanisms have not been ruled out. Genistein is the prototypical isoflavone that has been best studied. Its half-life is short, and the drug is extensively conjugated. Most of the product appears in the urine within hours of ingestion. There is some evidence to suggest that the prostate can concentrate isoflavones, possibly up to ten times the

concentration found in the plasma. A number of studies have attempted to show the ability of isoflavone preparations to slow the development or progression of prostate cancer, often by measuring PSA changes over time, but almost universally, the studies have been grossly underpowered and are suboptimal with regard to design.

The Akt pathway, or more accurately, network, has assumed increasing importance with the understanding that it represents a key role in cancer cell survival and proliferation. Intense efforts to target proteins and enzymes within this pathway with highly selective compounds have led to the development of diverse agents now in Phase I – III clinical trials. Moreover, the notion that exploitation of multiple “druggable” targets simultaneously or in the appropriate drug sequence may provide better anti-tumour effects than single drugs holds promise that chemoresistance may be overcome, at least in part. Chapter 2 also reviews important aspects of the Akt network and why isoflavones and in particular phenoxodiol, might affect this pathway, with a particular focus on prostate cancer biology.

Phenoxodiol is a novel isoflav-3-ene, currently undergoing clinical trials, which has broad *in vitro* activity against a number of human cancer cell lines. Phenoxodiol alone inhibited the human prostate cancer cell lines DU145 and PC3 in a dose and time - dependent manner with IC50s of 8 ± 1 μM and 38 ± 9 μM, respectively. The combination of phenoxodiol and cisplatin was synergistic in DU145 cells, and additive in PC3 cells, as assessed by the Chou–Talalay method. Carboplatin was also synergistic in combination with phenoxodiol in DU145 cells. The activity of the phenoxodiol and cisplatin combination was confirmed *in vivo* using a DU145 xenograft model in nude mice. Pharmacokinetic data from these mice suggest that the mechanism of synergy

may occur through a pharmacodynamic mechanism. An intracellular cisplatin accumulation assay showed a 35 % ($p < 0.05$) increase in uptake of cisplatin when it was combined in a ratio of 1 μM cisplatin: 5 μM phenoxodiol, resulting in a 300 % ($p < 0.05$) increase in DNA adducts. Western blot experiments show, for the first time, that phenoxodiol inhibits both phospho-Akt and mTOR function in DU145 cells, and phospho-Akt in PC3 cells. Taken together, our results suggest that phenoxodiol has interesting signal transduction inhibitory properties that make combination therapy with cisplatin or carboplatin appealing.

The pharmacokinetics of phenoxodiol was studied in patients with advanced cancer following single administrations of an intravenous bolus and during a continuous intravenous infusion in a first-in-human clinical trial. Three men with prostate cancer and three women with breast cancer received an intravenous bolus injection of phenoxodiol at 5mg/kg and plasma was sampled for 12 hours for the measurement of free and total (free plus conjugated) phenoxodiol levels. On a separate occasion, five of the same patients received a continuous intravenous infusion of phenoxodiol at 2mg/kg/h for up to 5 hours and plasma was sampled as above. Phenoxodiol was measured using gradient HPLC with ultraviolet detection. Following bolus injection, free and total phenoxodiol appeared to follow first order pharmacokinetics. The elimination half-lives for free and total phenoxodiol were 0.67 ± 0.53 h and 3.19 ± 1.93 h respectively, while the total plasma clearance rates were 2.48 ± 2.33 L/h and 0.15 ± 0.08 L/h, respectively. The respective apparent volumes of distribution were 1.55 ± 0.69 L/kg and 0.64 ± 0.51 L/kg. During continuous intravenous infusion, free levels of phenoxodiol accumulated rapidly to reach a mean concentration at steady state of 0.79 ±

0.14 μg/ml after 0.87 ± 0.18 h. The apparent accumulation half life of free phenoxodiol was 0.17 ± 0.04 h while the plasma clearance during continuous infusion was 1.29 ± 0.23 L/h. Total phenoxodiol accumulated at a slower rate during continuous infusion and in two of the subjects did not reach steady state during the period of the infusion. Phenoxodiol has a short plasma half-life, particularly in the free form, leading to a rapid attainment of steady state levels during continuous intravenous infusion.

In a Phase I study of phenoxodiol given intravenously weekly to patients with advanced cancer, we wished to define the maximum tolerated dose (MTD), toxicity, and pharmacokinetics of this novel isoflavone. Twenty-one patients with advanced cancers were treated with weekly phenoxodiol at escalating dose levels with 1-4 patients at each dose cohort. Plasma sampling was undertaken to characterize the pharmacokinetic (PK) profile of the compound. Toxicity was minimal, with asymptomatic Grade 3 lymphocytopenia occurring in nine patients. Nine patients developed Grade 1 nausea, six patients developed Grade 1 increases in alkaline phosphatase, and six patients developed Grade 1 increases in transaminases. Two patients experienced hypersensitivity reactions. The MTD was not reached. Most patients had progressive disease on treatment but eight completed 12 weeks and two completed 24 weeks of treatment. The best response was stable disease of 6 months duration. The plasma half-life (T1/2), clearance (Cl), and volume of distribution (V_D) were 304 (± 91) minutes, 82 (± 19) mL/min and 32663 (± 7199) mL respectively, for total phenoxodiol. Phenoxodiol is well tolerated and can be given safely as an intravenous infusion over 1-2 hours at a dose of at least 30 mg/kg.

We also conducted a dose escalation Phase I trial to determine the safety and bioavailability of a novel formulation of oral phenoxodiol combined with either cisplatin (cis) or carboplatin (carbo). Patient eligibility included at least one prior systemic therapy, and adequate bone marrow, renal and hepatic function. Cisplatin (50 mg/m^2 iv on days 2 and 9) or carboplatin (AUC=5 iv on day 2) was given in a 21-day cycle. Phenoxodiol was given orally three times daily from days 1-10. Cohorts of 6 patients (3 cisplatin, 3 carboplatin in each) were treated at each of 50 mg, 100 mg, 400 mg and 800 mg phenoxodiol dose levels. Pharmacokinetic assessments were made before or during cycle 1. Twenty five patients were recruited. Dose limiting toxicity (DLT) was reached at the 800 mg phenoxodiol dose level. Grade 4 toxicity occurred in two patients at this dose level: exacerbation of airways disease, and ventricular tachycardia (prior implantable defibrillator for ventricular tachycardia). Grade 3 non-hematologic toxicity included fatigue, nausea/vomiting, diarrhea, hypotension, pain, raised ALP, raised ALT/AST, raised GGT, and hyperglycemia. Hematological toxicity was modest (Grade 3 at worst). Best responses included one patient with a complete response (CR based on CA125 marker criteria), three minor tumour marker responses not enough to qualify for partial response (PR), 11 patients with stable disease (SD), 10 patients with progressive disease (PD), and three who were not assessable for response. Mean maximum concentration (Cmax), area under the curve (AUC), and T1/2 for total phenoxodiol was 18.4 μg/mL, 1637 hr*μg/mL, and 7.2h respectively, for an intravenous dose of 400 mg. Bioavailability of oral phenoxodiol was estimated to be 17.5%. The combination of carboplatin or cisplatin and oral phenoxodiol is well tolerated. The dose limiting toxicity is 800 mg oral phenoxodiol three times daily when given together with cisplatin or carboplatin in these schedules.

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I am very grateful for the advice, encouragement, and help from many individuals, without which this work would not have been possible.

I was introduced to phenoxodiol very early in the drug development process by Professors Laurie Howes (LH) and Graham Kelly (GK), which of course gave me a priceless chance to study a new compound. I would like to acknowledge some helpful early discussions with Dr. David Brown and Professor Alan Husband, which led to me being appointed as a consultant for Novogen Pty Ltd, Sydney, for 2002, and which I am declaring here as a potential conflict of interest. My special thanks to Novogen Pty Ltd, Sydney, for funding for the Phase I clinical studies and some of the preclinical work, which could not have otherwise been performed.

My thanks to Professors Beng Chong and Laurie Howes for initially taking me on as a mature age PhD student, which I am sure must have presented its challenges! I am also grateful to Professors John Kearsley and Pam Russell for taking over supervision well after the course of my studies had been set, and for their grace and helpfulness in guiding me through the latter parts of my thesis. Professor Howes kindly agreed to continue my supervision from afar, even though he moved to Queensland some time ago, and also continues to give me very practical advice and help.

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Statement

The following table outlines an estimate of my efforts for this thesis, with credit due to others where I have not personally carried out work. Responsibilities set out in the table are based on those required for authorship by the International Committee of Medical Journal Editors.

	Concepts and protocol design and writing	Experiments or recruitment of patients	Collation of data and analysis	Writing of draft and final report	Submission for publication and correspondence (responsibility for work)
Chapter 1	100%	N/A	N/A	100%	N/A
Chapter 2	100%	N/A	100%	5% JK 5% PR 5% LH 85% PDS	100%
Chapter 3	100%	70% RM 10% PG 5% SL 15% PDS	50% RM 50% PDS	60% RM 40% PDS	100%
Chapter 4	20% GK 80% LH	70% LH 30% PDS	90% LH 10% PDS	70% LH 30% PDS	100%
Chapter 5	100%	60% PDS 25% ML 15% WL	15% SP 85% PDS	5% LH 95% PDS	100%
Chapter 6	100%	75% PDS 20% ML 5% WL	25% CC 75% PDS	5% LH 95% PDS	100%
Chapter 7	100%	N/A	N/A	100%	N/A

ORIGINALITY STATEMENT

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Signed ……..

Date ……..

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANC	neutrophil count
APTT	activated partial thromboplastin time
AR	androgen receptor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
AUC_{0-t}	area under the plasma concentration versus time curve measured to the last plasma sample
AUC_{0-inf}	area under the plasma concentration versus time curve extrapolated to infinity
^{o}C	degrees Celsius
carbo	carboplatin
cav-1	caveolin-1
CBP	CREB-binding protein
c-FLIP	cellular FLICE inhibitory proteins
CI	combination index (Chou-Talalay analyses)
CI	confidence interval
cis	cisplatin
Cl, C_L	clearance
Cmax	maximum concentration
CNS	central nervous system
C_0	concentration at time zero
CR	complete response
C_{ss}	concentration at steady-state
CTC	Common Toxicity Criteria (National Cancer Institute)
DLT	dose limiting toxicity
DNA	deoxyribonucleic acid
ECOG	Eastern Cooperative Oncology Group (performance status)
eIF4E	eukaryotic initiation factor 4E
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERK	extracellular regulated kinase
FAK	focal adhesion kinase
FBC	full blood count
FGF	fibroblast growth factor
FLIP	FLICE inhibitory protein
FOXO	Forkhead Box Class O
FSH	follicle stimulating hormone
GAP	GTPase – activating protein
GFR	glomerular filtration rate
GGT	gamma-glutamyl transferase
Hb	hemoglobin
HIF1α	hypoxia inducible factor alpha

h, hr	hours
HPLC	high performance liquid chromatography
IC50	inhibitory concentration at the 50% level
ICP-MS	inductively coupled plasma mass spectrometry
IGF-1R	insulin – like growth factor 1 receptor
IKKβ	I kappaB kinase β
iv	intravenous
K_{el}	elimination rate constant
KPS	Karnofsky performance status
LH	leutenising hormone
LKB1	tumour suppressor gene product responsible for Peutz-Jeghers syndrome
mg, g, kg	milligram, gram, kilogram
min	minutes
mRNA	messenger ribonucleic acid
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
N/A	not applicable
NF1	Neurofibromin 1
NFκB	NF kappa B
NSCLC	non – small cell lung cancer
NV06	phenoxodiol
p70S6K	p70 ribosomal S6 kinase
PAGE	polyacrylamide gel electrophoresis
PART-1	prostate androgen regulated transcript 1
PBS	phosphate buffered saline
PD	progressive disease
PDGF	platelet- derived growth factor
PDK1	3-phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3 – kinase
PI3,4-P2	phosphatidylinositol 3,4-bisphosphate
PI3,4,5-P3	phosphatidylinositol 3,4,5-trisphosphate
PK	pharmacokinetics
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PP1, PP2A	serine / threonine protein phosphatase classes
PR	partial response
PSA	prostate specific antigen
PT	prothrombin time
PTEN	Phosphatase and Tensin homologue deleted on chromosome Ten
RT-PCR	reverse transcriptase polymerase chain reaction
S-1-P	sphingosine 1 phosphate
s	seconds
SCID	subacute combined immunodeficiency
SD	stable disease
SD	standard deviation
SDS	sodium dodecyl sulfate
Ser473	Serine at the 473 position

SEM	standard error of the mean
SGK	serum and glucocorticoid inducible kinase
SREBP1	Sterol regulatory element binding transcription factor
T1/2	half-life
TGFβ	transforming growth factor beta
Thr308	threonine at the 308 position
Tmax	time to reach maximum concentration
tNOX	tumor-specific NADH oxidase
TSC1, 2	tuberous sclerosis complex 1, 2
μL, mL, L	microlitre, millilitre, litre
μM, mM, M	micromolar, millimolar, molar
Vd, V_D	volume of distribution
VEGF	vascular endothelial growth factor
VHL	Von Hippel Lindau
WCC/WBC	white cell count / white blood count
XIAP	x linked inhibitor of apoptosis

List of Publications

Journal articles

1. de Souza P, Liauw W, Links M, Pirabhahar S, Kelly G, and Howes LG. Phase I and pharmacokinetic study of weekly NV06 (Phenoxodiol™), a novel isoflav-3-ene, in patients with advanced cancer. *Cancer Chemotherapy & Pharmacology*. 2006, 58(4):427-433. (IF 2.57)
2. de Souza PL, Russell PJ, Kearsley JH. Role of PI3K and Akt biology in prostate cancer. *Current Cancer Drug Targets* 2009, 9(2): 163-175 (IF 5.39)
3. McPherson RAC, Galettis PT, de Souza PL. Enhancement of the activity of phenoxodiol by cisplatin in prostate cancer cells. *British Journal of Cancer* 2009, 100:649 – 655. (IF 4.64)
4. Howes JB, de Souza PL, West L,Huang LJ, Howes LG. Pharmacokinetics of phenoxodiol, a novel isoflavone derivative with anticancer activity, following intravenous administration to patients with advanced cancer. Submitted for publication.
5. de Souza PL, Chan C, Links M, Galettis P, Howes LG. Phase I and bioavailability study of oral phenoxodiol in combination with either cisplatin or carboplatin in patients with advanced cancer. Submitted for publication.
6. de Souza PL, Russell PJ, Kearsley JH, Howes LG. Clinical Pharmacology of isoflavones and its relevance for the potential treatment of prostate cancer. Submitted for publication.

Conference poster presentations

1) Liauw W, Links M, Pirabhahar S, Husband A, Kelly G, and de Souza P. Phase I trial of weekly NV-06 (Phenoxodiol), a novel isoflavonoid, in patients with advanced cancer. Accepted for presentation at AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, Miami, Florida, October 29 – November 2, 2001 (abstract).
2) Liauw WS, Links M, Pirabhahar S, Kelly G, and de Souza P. Phase I and pharmacokinetic study of weekly NV-06 (PhenoxodiolTM), a novel isoflav-3-ene, in patients with advanced cancer. *Proc. Am. Soc. Clin. Oncol*., Orlando, 2002, #1883 (abstract).
3) McPherson R, Johnson L, de Souza P. In vitro synergy between phenoxodiol, a novel synthetic phenolic compound, and cisplatin. Presented at Australian Health and Medical Research annual Meeting, Melbourne, 25-29 November 2002. (abstract)
4) McPherson RAC, Galettis P, and de Souza PL. In vitro and in vivo combination studies with phenoxodiol, a synthetic isoflav-3-ene, show synergy with cisplatin. 8th World Conference on Clinical Pharmacology and Therapeutics, Brisbane, August, 2004 (abstract)

1. Introduction and Aims

1.1 Background

1.1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed internal malignancy in men. For those with localized disease who choose either surgery or radiation as primary therapy, a significant proportion will relapse some years later with rising prostate specific antigen (PSA) levels. At that stage, androgen deprivation usually allows control of the disease for some time, but eventually a castrate-resistant state develops, at which point median survival shortens to around 12-15 months. So far, docetaxel is the only agent that has been shown to improve median survival of patients with castrate-resistant prostate cancer, albeit by a modest 3 months (Petrylak, Tangen et al. 2004; Tannock, de Wit et al. 2004). Hence there is a clear need to find new treatments that prolong survival without unduly increasing the side effect burden. On the other hand, if the right treatment was available, it could be argued that primary prevention of prostate cancer may be an even more efficacious and cost-effective approach to the problem. For this reason, a number of compounds, including phytoestrogens, have been put forward as suitable candidates for the prevention of prostate cancer, based largely on their relatively non-toxic safety profile.

1.1.2 Isoflavones

Isoflavones are phytoestrogens that have pleiotropic effects in a wide range of cancer cell lines. Many of these biological effects involve key components of signal

transduction pathways within cancer cells, including prostate cancer cells. Epidemiological studies have raised the suggestion that isoflavones may thus play an important part in the prevention and modulation of prostate cancer cell growth. However, there are no randomized Phase III trials testing the utility of isoflavones in prostate cancer, and the best evidence for this concept is provided by Level III (case control) studies. *In vitro* data provide a more convincing argument for the growth inhibitory effect of a range of isoflavone analogues on cancer cell lines, which has led to the development of genistein and flavopiridol, for example, in the clinic as potential treatments for cancer. In addition, their potential activity in combination with cytotoxics or radiotherapy warrants further investigation.

1.1.3 The Akt pathway and its relevance for prostate cancer and isoflavones

The Akt pathway, or more accurately, network, has assumed increasing importance with the understanding that it represents a key role in cancer cell survival and proliferation. Intense efforts to target proteins and enzymes within this pathway with highly selective compounds have led to the development of diverse agents now in Phase I – III clinical trials. Moreover, the notion that exploitation of multiple “druggable” targets simultaneously or in the appropriate sequence may provide better anti-tumor effects than single drugs holds promise that chemoresistance may be overcome, at least in part. This thesis reviews important aspects of the Akt network, with a particular focus on prostate cancer biology.

1.2 Structure and flow of thesis

As the title of the thesis suggests, this work is based on developing a compound for the potential treatment of prostate cancer. The translational aspect, by definition, crosses the boundary between, and marries together, both the preclinical and basic science, and early clinical studies. As a result, this thesis is laid out in a manner similar to a way an Investigator's Brochure for a new compound might be written: literature review followed by preclinical work, followed in turn by clinical studies. In reality, questions that arose before the compound was tested in humans were answered in the Phase I studies (pharmacokinetics), but this raised questions in turn which were answered by going back to the preclinical setting (the relevance of achievable concentrations, and therefore, whether the compound might be better paired with another chemotherapeutic drug). This lead to the synergy studies with cisplatin and carboplatin, and in turn, back to clinical studies with another Phase I clinical trial to investigate the safety and tolerability of such a combination. The iteration (and translation between preclinical and clinical work) thus closely parallels the drug development paradigm, and the way new compounds are commonly developed for the clinic. Although phenoxodiol is now in Phase III clinical trials for ovarian cancer, it is hoped that funding will extend to prostate cancer studies in the future.

2. Literature Review

2.1.1 Clinical pharmacology of isoflavones and its relevance for the potential treatment of prostate cancer

2.1.2 Introduction

Given the large body of epidemiological data noting the low incidence of prostate cancer in Asian men in comparison to the incidence in Western countries, it has been widely hypothesized that a variety of phytochemicals, and specifically isoflavones, could be used as chemopreventative agents for this disease. Although many epidemiological studies are supportive of the potential benefit of isoflavones for the prevention of prostate cancer, there are also a significant proportion of negative studies that suggest a cautious approach to widescale use is probably appropriate (Sirtori *et al*, 2005; Bosetti *et al*, 2006; Ward *et al*, 2008).

Although much of the literature is based on the premise that isoflavones may be potentially useful in the chemoprevention of prostate cancer (see Adlercreutz 2002; Castle and Thrasher 2002; Chan *et al*, 2005; Messina *et al*, 2006; Von Low *et al*, 2007 and many others for reviews), this review will focus on the pharmacology of isoflavones and their potential use as treatment for established prostate cancer. Only a summary of the extensive epidemiological and biological data for isoflavones is intended.

2.1.3 Isoflavones

2.1.3.1 Source and contents

Fruits and vegetables, and some herbs, contain phytochemicals that include a group of compounds that have potential for the chemoprevention of a number of diseases. These naturally occurring phytochemicals can be essentially divided into flavonoids and lignans (Adlercreutz, 2002). Foods such as soy and soy products, rye bread, and red clover are particularly rich sources of flavonoids. Flavonoids can be further classified into flavones, flavonols, isoflavones, flavonones, catechins, anthocyanins, and chalcones. Of these, the isoflavones are by far the most extensively studied. They have weak estrogenic activity, and can interfere with intracellular steroid metabolism (Miksicek 1993; Collins *et al*, 1997; Adlercreutz 2002). Soybeans are a rich source of isoflavones, but also contain lipids and linoleic acid and α-linolenic acid (Jian, 2009). Isoflavone content ranges from 0.3 mg/g protein to 5.5 mg/g protein depending on the preparation (eg. dry isolate compared to soy flour) (Franke *et al*, 1995; Anderson *et al*, 1999). Isoflavone intake in Asian countries is approximately 50 mg daily, about ten times more than in Western countries (Messina *et al*, 2006b). The main isoflavones derived from most soy foods are (in order of proportion) genistein, daidzein and glycitein, but there are also many intermediates and metabolites produced in humans (particularly of daidzein), some of which have demonstrated anticancer activity.

2.1.3.2 Chemistry

The basic structure of isoflavones involves two benzene rings connected by a pyrone ring (Figure 1 A). Four chemical forms of the three main isoflavones exist: glycosides (genistin, daidzin, glycitin), aglycones (genistein, daidzein, glycitein), acetylglucosides, and malonylglucosides (Jian, 2009). Formononetin and Biochanin A (found in some legumes) are precursors of genistein (Figure 1B) and daidzein (Figure 1C), respectively.

Isoflavones generally exist as glycosides, but these are not well absorbed through the gut due to their hydrophilic nature (Hur *et al*, 2000), though there is some debate about whether this is true (Zubik & Meydani, 2003). Isoflavones are further metabolized in the body to many different intermediates including equol, which is perhaps the best studied metabolite of daidzein. Phenoxodiol (Figure 1D) is an intermediate in the metabolism of daidzein to equol (Joannou *et al*, 1995).

Figure 1A: Isoflavone general chemical structure

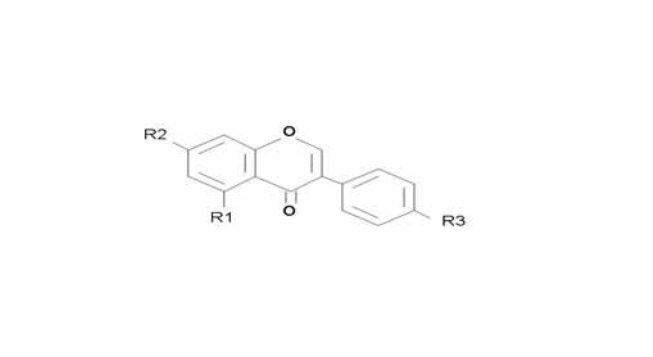


Figure 1B: Chemical structure of genistein

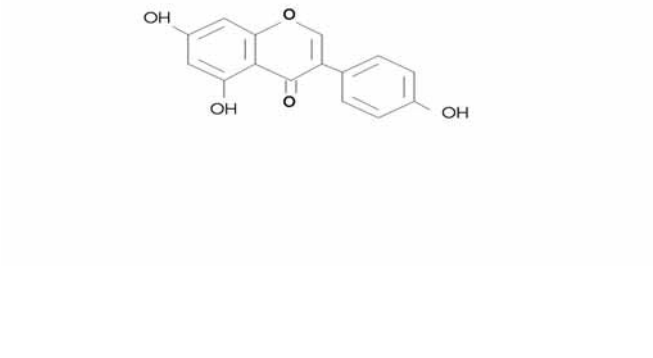


Figure 1C: Chemical structure of daidzein

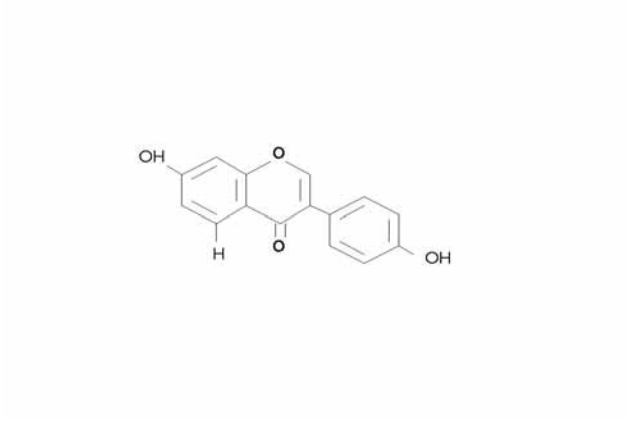
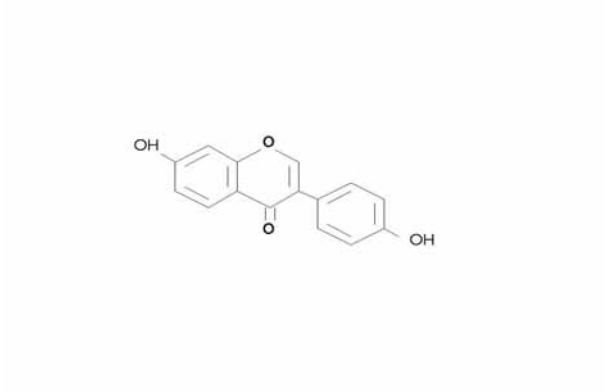


Figure 1D: Chemical structure of phenoxodiol



2.1.3.3 Absorption and distribution

After ingestion, the absorption of isoflavones across the gut membrane requires hydrolysis and the action of β-glucosidase provided by gut bacteria, which facilitates the conversion of glycosides to aglycones (Hur *et al*, 2000; Setchell *et al*, 2002; Yuan *et al*, 2007; Zubik & Meydani, 2003), which are then absorbed into the bloodstream. Some entererohepatic circulation ensues however, so that the remaining glycosylated, sulfated and glucuronidated forms, in addition to excretion of aglycones from the small intestine, reach the colon and are available for further metabolism by gut bacteria (Yuan *et al*, 2007; Zubik & Meydani, 2003). It is this highly variable gut microbacterial environment

between individuals that has led to the explanation for the variability in absorption of isoflavones found in many studies, and therefore highly variable results. However, little is known about this aspect of isoflavone bioavailability. Interestingly, β-glucosidase has been shown to be induced by a high soy diet (Wiseman *et al*, 2004), suggesting that changing the diet could change gut microflora, and in turn improve absorption of isoflavones.

Recent literature has focused on equol as a possible mediator of isoflavone benefits. It has been shown for example, that the ability to produce equol, or equol per se, is inversely related to the incidence of prostate cancer (Akaza *et al*, 2004), and that consumption of isoflavones by Western men can increase their ability to produce equol (Hedlund *et al*, 2005). Equol itself, though a metabolite of daidzein, is up to ten times more potent in inhibiting prostate cancer cell growth (Hedlund *et al*, 2003). It is also a more potent estrogen receptor (ER) binder and anti-oxidant than its parent, possibly explaining its mechanism of action (Wiseman, 2000). It is estimated that only 30-50% of the population are equol producers (Atkinson *et al*, 2005).

2.1.4 Anti-cancer properties of isoflavones

2.1.4.1 In vitro data

The similarity of various isoflavone structures (Figure 1A-D) might lead to the prediction that biological properties are also very similar. However, whilst there appears to be considerable overlap, there are also some material differences. For example, genistein has been shown to bind up to 4% of ERα and 87% of ERβ relative to estrogen (Kuiper *et al*, 1998), but daidzein has less potency in binding ERβ. Further, equol has

tighter binding to estrogen receptors than its parent, and more potency for inhibiting cancer cell growth (Hedlund *et al*, 2003).

Many potential mechanisms of action have been found for isoflavones in a variety of i*n vitro* model systems. Table 1 lists some of the biological properties of isoflavones, and more specifically, genistein, in prostate cancer cells. Genistein inhibits the growth of prostate cancer cells (Peterson & Barnes, 1993), and causes apoptosis (Kyle *et al*, 1997). However, there may be a biphasic effect in that low doses promote cell growth while higher doses inhibit growth (Zhang *et al*, 2003b); whether this is a real phenomenon or a limitation of the growth inhibition assay remains to be confirmed. While isoflavone-induced downregulation of Bcl-2 in prostate cancer cells has not been shown, Bcl-x(L), another anti-apoptotic protein, is downregulated by genistein in PC3 prostate cancer cells, abrogating its resistance to apoptosis (Li *et al*, 2001). Inhibition of proteasome activity in LNCaP prostate cancer cells is accompanied by accumulation of the cyclin-dependent kinase inhibitor, p27(Kip1), inhibitor of nuclear factor-kappa B, NFκB (I kappa B-alpha, IκBα), and the pro-apoptotic protein Bax (Kazi *et al*, 2003). Further, genistein reduces NFκB binding in LNCaP and PC3 cells (Davis *et al*, 1999) and inhibits phosphorylation of the inhibitory protein IκBα, thereby blocking the nuclear translocation of NFκB. In essence, genistein promotes the function of NFκB regulators in keeping NFκB in check, and stunting its role in apoptosis resistance and control of cell growth and differentiation (Banerjee *et al*, 2008). Since NFκB is a target of Akt signaling (Ozes *et al*, 1999; Romashkova and Makarov 1999), it is not difficult to postulate that this potential mechanism for the action of genistein is an important one, and could have a number of far-reaching effects on downstream effectors, given a key role for NFκB in linking signal transduction and transcription. Indeed, Li and Sarkar (Li

& Sarkar, 2002) showed that phosphorylation of Akt was directly inhibited by genistein in PC3 cells.

Table 1. Selected molecular targets of isoflavones in prostate cancer cells. To date, all but three (caspaes, XIAP, sphingosine kinase) of the targets listed here have been documented in prostate cancer cell lines.

	Isoflavone	**References**
Signal transduction		
Protein tyrosine kinase	Inhibition of EGF tyrosine kinase activation by genistein	(Akiyama *et al*, 1987)
PTEN	Expression is induced by genistein and daidzein in PC3 and LNCaP	(Cao *et al*, 2006b)
Akt	Inhibited by genistein	(Li and Sarkar 2002c; Bemis *et al*, 2004; Park *et al*, 2005; El Touny and Banerjee 2007)
mTOR	Inhibited by genistein	(Tepper *et al*, 2007)
MAPK	MAPK inhibited by genistein	(Huang *et al*, 2005; Xu and Bergan 2006)
ERK1/2	Inhibited by genistein; induced by isoflavones	(Agarwal 2000; Bhatia and Agarwal 2001; Wang *et al*, 2004; Wang *et al*, 2006; Clubbs and Bomser 2007); (Gopalakrishnan *et al*, 2006)
JNK	Activation by genistein	(Lazarevic *et al*, 2008)
NFκB	Inhibited by genistein and soy isoflavones	(Davis *et al*, 1999; Li and Sarkar 2002; Raffoul *et al*, 2007b; Singh-Gupta *et al*, 2009)
IGF-1/R	Inhibition by genistein	(Wang *et al*, 2003a; Takahashi *et al*, 2006b)
Stat	Activated by genistein	(Pinski *et al*, 2006)
TGFβ	TGFβ inhibited by genistein	(Xu and Bergan 2006)
Apoptosis		
Mdm2	Downregulated by genistein	(Li *et al*, 2005b)
Bax	Increased by genistein	(Kazi *et al*, 2003)
Bcl-x(L)	Downregulated by genistein	(Li *et al*, 2001)
XIAP	Inhibited by phenoxodiol in ovarian cancer cells and melanoma cells	(Kamsteeg *et al*, 2003; Sapi *et al*, 2004; Alvero *et al*, 2006; Herst *et al*, 2007; Kluger *et al*, 2007)
Caspases	Activated and caspase inhibition overcome by phenoxodiol in HN12 cells; Caspase mediation by genistein induced apoptosis	(Aguero *et al*, 2005; Choueiri *et al*, 2006b); (Kumi-Diaka and Butler 2000; Kumi-Diaka *et al* 2000)
Other targets		
HIF1α	Inhibition by genistein in PC3 cancer cells	(Singh-Gupta *et al*, 2009)
VEGF	Downregulated by genistein	(Li and Sarkar 2002; Cao

		et al, 2006; Guo *et al*, 2007)
ERβ	Antagonism and partial agonism by genistein; expression reduced by genistein	(Kuiper *et al*, 1998; Pike *et al*, 1999; Booth *et al*, 2006; Cao *et al*, 2006; Wang *et al*, 2006)
5α reductase	Inhibited by genistein	(Evans *et al*, 1995)
PART-1	Prostate androgen regulated transcript 1 inhibited by genistein and daidzein	(Yu *et al*, 2003)
AR	Transcriptionally downregulated by genistein	(Davis *et al*, 2000; Takahashi *et al*, 2006; Tepper *et al*, 2007)
PSA	mRNA expression and secretion reduced by genistein	(Davis *et al*, 2000; Rice *et al*, 2007)
Cyclins	Cyclin B downregulated by genistein	(Davis *et al*, 1998)
Myt-1	Upregulated by genistein	(Touny and Banerjee 2006)
Wee-1	Phosphorylation reduced by genistein	(Touny and Banerjee 2006)
$p21^{WAF1}$	Upregulated by genistein	(Davis *et al*, 1998; Lian *et al*, 1998)
P27(Kip1)	Increased by genistein	(Bhatia and Agarwal 2001; Kazi *et al*, 2003; Rice *et al*, 2007)
MMP	MMP2 and 9 downregulated by genistein; MMP2 expression inhibited by phenoxodiol	(Li and Sarkar 2002; Huang *et al*, 2005; Kumi-Diaka *et al*, 2006; Xu and Bergan 2006); (Gamble *et al*, 2006)
COX-2	mRNA and protein expression reduced by genistein in LNCaP and PC3	(Swami *et al*, 2008)
Sphingosine Kinase	Inhibited by phenoxodiol in endothelial cells	(Gamble *et al*, 2006)
TNOX	Inhibited by phenoxodiol	(De Luca *et al*, 2005; Davies and Bozzo 2006)
Focal adhesion kinase	Activity reduced by genistein	(Kyle *et al*, 1997)
Hypermethylation	Reversal of DNA methyltransferase activity by genistein	(Fang *et al*, 2005; Fang *et al*, 2007)
Urokinase plasminogen activator	Inhibited by genistein	(Skogseth *et al*, 2005)

Early studies found that genistein was a specific inhibitor of protein tyrosine kinases (Akiyama *et al*, 1987), which are known to be upregulated in many different cancer cell types. Control of tyrosine kinase phosphorylation may therefore result in increased apoptosis or inhibited cell growth. For example, reduction of focal adhesion kinase

(FAK) activity just prior to apoptosis induced by genistein has been described (Kyle *et al*, 1997), though others have noted transient activation of the FAK: beta–1–integrin complex (Bergan *et al*, 1996; Liu *et al*, 2000) by genistein, consistent with its theoretical ability to reduce metastases. These effects were independent of genistein's known estrogenic activity (Kyle *et al*, 1997). Other studies (Agarwal *et al*, 2000) noted that DU145 growth inhibition was associated with reduction in the activity of ERK1/2 and various cyclin dependent kinases, but whether doses of the polyphenol extract studied (10 – 100 μg/mL) are clinically relevant is questionable. Nevertheless, confirmation of these data with EGCG (flavonol, a major component of green tea), genistein, and silymarin (flavonone, derived from milk thistle) in DU145 prostate cancer cells suggests significant interaction with a number of important cell signaling pathways (Agarwal, 2000).

Axanova (Axanova *et al*, 2005) recently showed that the tumor-specific NADH oxidase (tNOX) inhibitors capsaicin, EGCG, capsibiol T, and phenoxodiol could inhibit the growth of LNCaP, though the effect was attenuated when co-cultured in the presence of osteoblasts. TNOX is thought to be expressed in cancer tissue, but not normal cells, and this differential has been proposed as the basis for its selective activity (Yagiz *et al*, 2007).

A range of other potential mechanisms for the growth-inhibiting effects of isoflavones has been described, including inhibition of NFκB activation (Davis *et al*, 1999), inhibition of cyclin-dependent kinase (Shen *et al*, 2000), induction of glutathione peroxidase leading to downregulation of the expression of a range of genes (Suzuki *et al*, 2002), reduction of androgen receptor transcription (Gao *et al*, 2004), and down

regulation of prostate androgen-regulated transcript-1 (PART-1) gene expression (Yu *et al*, 2003).

Effects on cell cycle regulation appear to be an important contributor to genistein's mechanism of action. Genistein induces G2/M arrest in PC3 and LNCaP cells (Davis *et al*, 1998), possibly due to downregulation of Cyclin B. However, kinases upstream of Cyclin B also appear to be affected, particularly increased levels of Myt-1 and reduced phosphorylation of Wee-1 (Touny and Banerjee, 2006).

Interestingly, genistein appears to increase the binding of STAT3 to the telomerase reverse transcriptase promoter in the TRAMP model, at least at physiological concentrations (Chau *et al*, 2007), which would ultimately be expected to promote the growth of prostate cancer cells.

2.1.4.2 *In vivo* data

Soy protein or Biochanin A administration has been shown to inhibit growth and increase apoptosis in LNCaP prostate cancer cell xenografts in athymic nude mice (Bylund *et al*, 2000; Rice *et al*, 2002). Dietary genistein supplementation has been reported to reduce the incidence of poorly differentiated adenocarcinoma in a transgenic strain of mice in which prostate cancer occurs almost universally after 28-30 weeks of age (Mentor-Marcel *et al*, 2001). Further, survival in these mice is improved by genistein, possibly through suppression of osteopontin expression (Mentor-Marcel *et al*, 2005). Taking a different approach, others have shown that the administration of soy protein or genistein reduces the incidence of prostate cancer induced by the chemical carcinogens methyl nitrosourea (NMU) or 3,2-dimethyl-4-aminobiphenyl (DMAB) in

rats (Pollard and Luckert 1997; Onozawa *et al*, 1999; Kato *et al*, 2000; Wang *et al*, 2002; McCormick *et al*, 2007). A soy protein isolate / isoflavone diet can also reduce the spontaneous recurrence of prostate cancer in Lombard-Wistar rats (a strain genetically prone to prostate cancer) from 30% to 3% (Pollard & Wolter, 2000). Traditional xenograft experiments with LNCaP cells in subacute combined immunodeficiency (SCID) mice support the growth inhibitory effect of an isoflavone - enriched diet (Aronson *et al*, 1999), though in these experiments, strict caloric control may have also played a part as the best data were achieved in mice on a low fat + soy protein + isoflavone extract diet. Mechanistic studies suggest that dietary genistein downregulates the expression of both androgen and estrogen receptors in the ventrolateral rat prostate, a factor which could potentially explain the epidemiological differences in prostate cancer between populations with low and high phytoestrogens in the diet (Fritz *et al*, 2002). Short-term studies of soy in the adult male neoDES mouse model do not show evidence of anti-estrogenic action (Makela *et al*, 1995a) but could impact prostate development in neonatal mice (Makela *et al*, 1995b). Yet other studies suggest that relatively short-term, high dose phytoestrogens (containing 600µg isoflavones) can reduce mice bodyweight, prostate size, and androgen levels without affecting gonadotrophin function (Weber *et al*, 2001). In elegant dose - response studies in subcutaneously and orthotopically implanted tumours in mice, Zhou and colleagues (Zhou, Gugger *et al*, 1999; Zhou, Yu *et al*, 2002) demonstrated clear reduction of tumour growth with a variety of isoflavone preparations, in association with reduced IGF-1, testosterone, and microvessel density whereas p53 expression and apoptosis were increased. However, these changes were not consistent, and depended on the type of isoflavone preparation, suggesting that multiple mechanisms were in play.

Injecting a note of caution, Cohen (Cohen *et al*, 2003) found that a soy protein isolate in combination with linoleic acid (LA) enhanced the growth of androgen-independent R-3327-AT-1 rat prostate tumor cells inoculated ectopically into male Copenhagen rats. However, another *in vivo* study suggested that LA could enhance the effect of androgen ablation on inducing apoptosis (McEntee *et al*, 2008), and a clinical study showed that serum linoleic acid levels were associated with a reduced risk of prostate cancer (Chavarro *et al*, 2007), suggesting the role of LA is far from clear. It is possible that specific isomers of linoleic acid, cis9, trans11 (c9, t11-LA) or trans10, cis12-CLA (t10, c12-LA) may have quite varying cellular effects including tumour growth promotion or inhibition (see Kelley *et al*, 2007 for review).

2.1.5 Combinations of isoflavones and other drugs

By definition, since many preclinical studies involve unspecified soy isoflavones or unspecified dose combinations of soy protein (eg. genistein / daidzein / glycitein), it is not possible to conclude whether true synergistic growth inhibition occurs when other drugs are used in combination. Studies in which purified genistein or any other isoflavone are used as single agents are therefore easier to interpret for possible synergy, since the contribution of each agent to cell growth inhibition can be calculated. Indeed, this is necessary for determining synergy, additivity, or antagonism when more than one compound is combined under the Chou-Talalay scheme (Chou and Talalay, 1984). With this caveat in mind, it is possible to find *in vitro* data that support the notion of synergistic combination therapy with isoflavones in the treatment of various cancer cell lines, including prostate cancer. Synergy or enhanced activity has been reported in prostate cancer cell lines using genistein in combination with paclitaxel (Ping *et al*,

2008), radiation (Raffoul *et al*, 2007), calcitriol (Swami *et al*, 2005), perifosine (Vinall *et al*, 2007), docetaxel (Burich *et al*, 2008), bicalutamide (Burich *et al*, 2008), beta – lapachone (Kumi-Diaka 2002; Kumi-Diaka *et al*, 2004), and thearubigin (Sakamoto, 2000). However, conjugated linoleic acid and a soy protein isolate were found to increase the growth of prostate cancer cell lines in a mouse model (Cohen *et al*, 2003). Phenoxodiol in combination with cisplatin has been shown to be synergistic against DU145 cells and probably additive in PC3 cells (McPherson *et al*, 2009).

In vivo combination therapy of soy isoflavones and radiation for prostate cancer has also been investigated, with favourable effects on the control of the disease (Wang *et al*, 2006; Raffoul *et al*, 2007). Interestingly, pure genistein increased the incidence of para-aortic nodal metastases in this orthotopic mouse model. Nevertheless, the notion that isoflavones may be best used clinically in drug combinations is appealing, and warrants further investigation. The advantage of this approach is that the benefit: risk ratio may increase if compounds being combined are more effective (synergistic) without necessarily being more toxic, particularly if they are known to be well tolerated as single agents.

2.1.6 Epidemiology of isoflavones in prostate cancer

The potential anticancer activity of isoflavones and their metabolites on prostate cancer cells *in vitro* was first noted nearly 20 years ago (Adlercreutz 1990; Peterson and Barnes 1993; Naik *et al*, 1994), and continues to provide the rationale for their use in the prevention of prostate cancer (Rokhlin and Cohen 1995; Santibanez *et al*, 1997; Geller *et al*, 1998; Hempstock *et al*, 1998; Onozawa *et al*, 1998; Davis *et al*, 1999; Kumi-

Diaka *et al*, 2000; Mitchell *et al*, 2000; Shen *et al*, 2000; Kumi-Diaka 2002; Suzuki *et al*, 2002; Hedlund *et al*, 2003; Gao *et al*, 2004). A number of epidemiological studies have reported an inverse association between isoflavone consumption and the risk of prostate cancer (Jacobsen *et al*, 1998; Strom *et al*, 1999; Lee *et al*, 2003; Ozasa *et al*, 2004; Ozasa *et al*, 2005; Kurahashi *et al*, 2007; Kurahashi *et al*, 2008). In common with each other, these studies have been largely based on dietary questionnaires, which of course have limitations in terms of properly defining isoflavone exposure. Attempts to improve the quality of these studies by using nested case-control designs, prospectively followed cohorts (particularly in relatively homogenous genetic populations such as Chinese and Japanese men) and correlation with serum phytoestrogen levels (eg. genistein, diadzein, equol) have generally supported and strengthened the conclusion of a dose-response effect. Although the degree of statistical significance varies between studies, some of these data are impressive: a prospective cohort study in the United States involving over 12,000 Seventh Day Adventist men found that the consumption of soy milk more than once per day was associated with a striking 70% reduction in the risk of prostate cancer after approximately 20 years of follow up, despite extensive adjustment for potential confounding factors (Jacobsen *et al*, 1998). Four other studies demonstrate that dose-dependent serum isoflavone levels are associated with a reduction in the risk of prostate cancer (Lee *et al*, 2003; Ozasa *et al*, 2004; Kurahashi *et al*, 2007; Nagata *et al*, 2007). As a corollary, a study of a cohort of 2000 Chinese men showed a strong inverse association between isoflavone intake and the presence of lower urinary tract symptoms (Wong *et al*, 2007), after exclusion of patients with previous bladder surgery or prostate cancer. In contrast, one study found a positive association between dietary intake of the non-isoflavone phytoestrogens campesterol and stigmasterol and prostate cancer, and no significant relationship between genistein and prostate cancer

(Strom *et al*, 1999). However, this was a small study (n=83 cases, 107 controls), unlikely to provide sufficient statistical power. Further, the study was performed in a Caucasian population in which the dose of ingested isoflavones was likely to be low. Akaza showed for example, that the mean serum concentration of daidzein and genistein in American men was around 3 ng/mL and 2 ng/mL respectively, compared to a Japanese and Korean population, where the corresponding concentrations were 10 times higher for daidzein and 20 times higher for genistein (Akaza *et al*, 2004). By contrast, Adlercreutz (Adlercreutz *et al*, 1993) found that plasma levels of genistein reached a geometric mean of 0.276 μM (=74 ng/mL) following a soy rich diet in Japanese men. Perhaps not surprisingly, low baseline urinary isoflavone levels in Western men (daidzein, genistein, enterolactone, equol) do not correlate with the risk of localized prostate cancer progression (Venkitaraman *et al*, 2008). A very recent meta – analysis of the epidemiological literature provides strong support for the potential protective effect of isoflavones on the development of prostate cancer (Yan & Spitznagel, 2009).

The pleiotropic biological effects of isoflavones seem to occur at widely varying doses, raising the question of whether these concentrations are actually achievable in humans, and whether the biological effects are relevant. Since concentrations vary widely during the day after intermittent consumption and between individuals, it is very difficult to conclude what might be “relevant” concentrations, and whether complex *in vivo* conditions might reasonably compare with controlled laboratory conditions. At least some of these effects, however, can occur at concentrations that are found in the prostate (Hedlund *et al*, 2003; Hedlund *et al*, 2006). Lower genistein concentrations in the prostate were also found in Austrian men with prostate cancer compared to benign

prostatic hypertrophy (Brossner *et al*, 2004), suggesting an inverse association. Other case-controlled studies support the concept of a protective effect of soy foods for prostate cancer (Jacobsen *et al*, 1998; Heald *et al*, 2007), possibly because the prostate contains higher concentrations of isoflavones than the corresponding plasma (Hedlund *et al*, 2005; Rannikko *et al*, 2006b; Gardner *et al*, 2009).

2.1.7 Clinical Pharmacology of Isoflavones

2.1.7.1 Pharmacokinetics

Pharmacokinetic studies of isoflavones are limited, and most involve only single dose designs. Isoflavones are generally rapidly absorbed from the gut, and aglycones appear in the bile and plasma within two hours (Franke *et al*, 1995; Setchell, Brown *et al*, 2001; Richelle *et al*, 2002; Atkinson *et al*, 2005). A second peak concentration at 4-8h after ingestion suggests enterohepatic circulation. Approximately 48h after ingestion, plasma levels are no longer detectable after a single dose. Peak plasma concentrations (Cmax) for aglycones occurred at 4-7h, whereas the corresponding time for glycosides was 8-11h, suggesting that the rate limiting step for absorption was initial hydrolysis of the isoflavone (Setchell *et al*, 2001; Zubik & Meydani, 2003). In a formal carbon-labeled isoflavone study, peak plasma genistein concentrations occurred earlier than those of daidzein (Setchell *et al*, 2003), with Cmax observed at 5.5h and 7.4h, respectively. Pharmacokinetic parameters were not linear when higher doses were given, suggesting that uptake was rate-limiting and saturable. The mean volume of distribution corrected for bioavailability (Vd/F), clearance rate, and half-life of [13C]-daidzein were 336.25 L, 30.09 L/h, and 7.75h, respectively; the corresponding values for [13C]-genistein were 258.76 L, 21.85 L/h, and 7.77h (Setchell *et al*, 2003). A study of long term administration of red clover (a total of approximately 80 mg isoflavones daily)

suggested that once-a-day administration was sufficient to give concentrations of genistein and daidzein consistent with that found in patients on a high isoflavone diet (Howes *et al*, 2002).

In a recent well-conducted study, pure daidzein in its aglycone and glycoside forms were given in a single oral dose of 1 mg/kg to 7 volunteer men in a randomized, double-blind fashion (Rufer *et al*, 2008). The study showed that bioavailability of the glycoside form was 3-6 times higher than that of the aglycone form, based on data from the Cmax, AUC, and recovery of isoflavone in the urine. Half-life was measured at 6.4h for the glycoside, and 8.9h for the aglycone. Cmax for the glycoside and aglycone forms were 2.5 μM and 0.4 μM, respectively. The data also lent credence to the idea of the importance of gut microflora generation of metabolites, given the lag in their appearance in the blood, and their later recovery in the urine. Inter-individual variability in pharmacokinetic parameters was very high for these metabolites, up to 320 fold for dihydrodaidzein, which clearly could not be explained by differences in pharmacogenomic factors, due to the randomized crossover design.

2.1.7.2 Dose escalation studies

In a dose-escalation, single dose study of one of two isoflavone preparations in men (Busby *et al*, 2002), other than mild increases in lipoprotein lipase and hypophosphatemia, miminal toxicity was noted although one patient did develop NCICTC Grade 2 neutropenia at the highest dose level. The preparations contained different ratios of genistein: daidzein: glycitein (90: 10: 1 compared to 43: 21: 2) but interestingly, Cmax and AUC were consistently higher for the 43: 21: 2 formulation, dose for dose. This implies that absorption of isoflavones could vary depending on the

proportion of proteins presented in the preparation. Doses of 1, 2, 4, 8, or 16 mg/kg isoflavone were administered, so that the highest absolute dose (1520 mg) was given to a 95kg man, far in excess of many other trials. Cmax for total genistein and total daidzein were 27.5 µM and 16.9 µM, respectively, at the 16 mg/kg dose level. Bioavailability estimates ranged from 27% to 49% for genistein, and 25% to 56% for daidzein. The same group reported a similarly designed single dose study in 24 healthy postmenopausal women (Bloedon *et al*, 2002) in which doses of up to 16 mg/kg were explored. Toxicity again was minimal, with only 4 mild (Grade 1) adverse reactions suspected to be possibly related to drug: pedal edema (n=2), nausea (n=1), and breast tenderness (n=1). On average, a 7% fall in systolic and diastolic blood pressure was noted within 24h, as well as a decrease in neutrophil count over the 30 days of observation. While there was no statistical difference between the two formulations, changes seen with the 43: 21: 2 formulation seemed to be generally of lesser degree. In three patients in whom biomarkers were studied, Akt – 1 activity was increased at 3h post - dosing, but protein tyrosine phosphorylation was reduced, consistent with one of the proposed mechanisms of action for genistein. No change in the rate of lymphocyte apoptosis was noted. The half-lives for free genistein, free daidzein, and free glycitein were estimated to be 3.8h, 7.7h, and 3.4h, respectively, similar to the results obtained in men. The terminal half-lives for total genistein and daidzein (10.1h and 10.8h respectively) in women were not different from those found in men (9.2h and 8.2h, respectively). Much shorter corresponding half-lives of around 3-6h for genistein and 3-6h for daidzein were reported earlier by others (King & Bursill, 1998; Lu & Anderson, 1998), though these were much smaller studies. Cmax for total genistein and daidzein (28.2 µM and 8.9 µM, respectively) were also similar to those found in men (Bloedon *et al*, 2002). These data are broadly in line with those found by Takimoto (Takimoto *et al*,

2003), who studied two different preparations of unconjugated soy isoflavones (PTI G-2535 containing 43% genistein and PTI G-4660 containing 90% genistein) in a single dose study of escalating doses from 2 mg/kg to 8 mg/kg. Cmax for total genistein and half -life for PTI G-2535 and PTI G-4660 were 4.3 µM and 16.3 µM, and 15h and 22h, respectively. Bioavailability was again estimated from urinary excretion of isoflavones, but seemed to be higher in women than previously noted in men (Bloedon *et al*, 2002). Since there was no intravenous formulation in all of these studies to compare with AUC achieved with oral administration, bioavailability can only be estimated. Certainly, the use of higher dose cohorts in these pharmacokinetic studies appears to give rise to plasma concentrations of isoflavones that are commensurate with *in vitro* anti-cancer activity.

2.1.8 Potential mechanisms of action in the clinical setting

The clinical mechanism for the proposed protective effect of isoflavones for prostate cancer is unknown. Some authors have suggested an endocrine mechanism, for example, the weak anti-estrogenic activity of isoflavones, whereas others have suggested something other than isoflavones from the diet may carry more importance (for example lignans or resveratrol, or other flavones including metabolites). One provocative study randomised 40 men post-prostatectomy to a low fat / high isoflavone diet (Li *et al*, 2008). After 4 years of follow up, lower 6 month IGF-1 concentrations were found in the treatment group; further, sera from treated patients were able to reduce LNCaP growth by 20%. A favourable suppressive effect for soy has been found for localized prostate cancer, but not for advanced disease (Kurahashi *et al*, 2007), suggesting that one reason why studies have not consistently shown benefit for

isoflavones is that prostate cancer is a heterogenous disease, and therefore may respond differently depending on the underlying biology. A pharmacogenomic mechanism has also been proposed by the work of Akaza and others (Akaza *et al*, 2002; Akaza *et al*, 2004) . In their study, 141 prostate cancer patients and 112 control subjects provided blood for the estimation of serum concentrations of genistein, daidzein, and equol. In exploratory analyses, 25% of patients with poorly differentiated prostate cancers were found to be daidzein metabolisers, in contrast to 44% of those with well or moderately differentiated cancers. In another study, serum and plasma equol were found to correlate with the TT polymorphism in the CYP19 gene (associated with sex hormones) but did not correlate with the CT or CC polymorphism (Low *et al*, 2005). Thus the reduction in risk of prostate cancer may be positively associated with a greater ability to produce equol from other isoflavones. A recent nested case-control study would seem to support this notion (Kurahashi *et al*, 2008). "Nutrigenomic" factors may therefore play an important part in predicting those who might benefit from phytoestrogen supplementation (Steiner *et al*, 2008). Certainly, the ability of more potent metabolites of various phytoestrogens to mediate tumour suppression have by no means been excluded (Xiang *et al*, 2002). Another study suggested that the prostate has the ability to concentrate isoflavones relative to plasma levels (Rannikko *et al*, 2006), even after short term administration, which could also help to explain their mechanism of action.

2.1.9 Effect of isoflavone administration on prostate specific antigen (PSA) levels

Showing material differences in favour of isoflavones in a prostate cancer prevention trial would ideally involve a prospective, randomized, placebo-controlled trial, most likely requiring tens of thousands of men, and at least 15 years of follow up. In the

absence of such a trial, attempts have been made to investigate the effect of isoflavone administration in men with established prostate cancer (Table 2). The lack of standardization of endpoints (eg. PSA, sex hormone changes) and other design features across these trials makes any interpretation difficult, if not impossible. One open – label study of a genistein-rich supplementation diet over a 6 month period found no convincing evidence of suppression of PSA levels in 62 men (deVere White *et al*, 2004). Another randomized, placebo-controlled trial in 76 men with early prostate cancer found that PSA levels after 12 weeks of therapy with genistein did not significantly differ between the active and placebo groups (Kumar *et al*, 2004), although the authors suggested that surrogate measures were being affected by treatment. In a study of 20 men with rising PSA levels after local therapy, soy milk three times per day for 12 months was associated with a slowing of the rate of rise of PSA relative to the previous rate of rise (Pendleton *et al*, 2008). There are other trials that support the idea that isoflavones, even given over relatively short periods of time, can possibly slow the rate of rise of PSA, though no statistically significant conclusions can be drawn (Hussain *et al*, 2003; Dalais *et al*, 2004; Maskarinec *et al*, 2006) due to the small size of these studies.

Table 2. Clinical studies of isoflavone administration in patients with prostate cancer

Intervention/Diet	Design	Outcome	Reference
ISP (high dose vs low dose genistein and daidzein)	n=34. Randomised crossover trial. 6 week intervention	Reduced cholesterol but no change in PSA	(Urban *et al*, 2001)
Single dose formulations of genistein, daidzein, glycitein	Pharmacokinetic study	Mean elimination half – lives of genistein was 3.2h and daidzein was 4.2h	(Busby *et al*, 2002)
160mg daily of red clover isoflavone preparation (genistein, daidzein,	n=38, pilot study of treatment prior to prostatectomy. 18 treated vs 18 untreated patients. Non – randomized	Apoptosis in treated patient specimens significantly higher than controls. No changes in PSA, testosterone.	(Jarred *et al*, 2002)

formonetin, biochanin A)	and non – blinded study		
Soy isoflavone preparation for 3 – 6 months	n=41, Pilot study in 3 groups (watchful waiting, rising PSA after local therapy, hormone insensitive)	Reduction in rate of rise of PSA in whole group. Serum genistein concentrations increased from 0.11 to 0.65µM and daidzein from 0.11 to 0.51µM. PSA stabilization in 83% of hormone sensitive group and 35% hormone insensitive patients	(Hussain *et al*, 2003)
Single doses of two soy isoflavone preparations	n=13. Phase I dose escalation with genistein at 2, 4, 8mg/kg	Cmax between 4.3 and 16.3µM, half – life between 15 and 22h.	(Takimoto *et al*, 2003)
Approximately 300mg or 600mg genistein and daidzein in soy formulation	n=20. Phase I multiple dose, orally over 84 days	31% reduction in dehydroepiandrosterone. Possibly slowing of PSA rise (non – significant)	(Fischer *et al*, 2004)
Soy vs Soy + linseed vs wheat in bread diet	n=29, Pilot study. randomised comparison prior to prostatectomy	-12% and 24% change in PSA and free/total ratio respectively. In favour of phytoestrogen activity.	(Dalais *et al*, 2004)
Genistein – rich extract for 6 months	n=62, range of rising PSA states including post – prostatectomy, off cycle during intermittent hormones, surveillance	One patient had PSA decline >50% and 8 patients had PSA decline <50%.	(deVere White *et al*, 2004)
60mg soy isoflavone preparation	n=59 evaluable patients who completed 12 weeks treatment. Gleason grade 6 or less	Reduction of testosterone in 61% of treatment group vs 33% of controls. PSA stabilization in 69% of treatment group vs 55% controls.	(Kumar *et al*, 2004)
Supplement of soy, isoflavones, lycopene, silymarin, antioxidants	n=46 (intent to treat). Randomised, double blind, crossover analysis. 10 week treatment periods separated by 4 week washout	Statistically significant reduction in slope of PSA induced by treatment. Increase in PSA doubling time from 445 to 1150 days (2.6 fold) with supplement	(Schroder *et al*, 2005)
240mg clover phytoestrogens daily for 2 weeks prior to prostatectomy	n=20, pilot study, placebo controlled	Non – significant decline in testosterone levels, but compensated rise in LH levels	(Rannikko *et al*, 2006)
High or low soy diet for 3 months	n=24, randomized crossover to alternative diet after 1 month washout	Decline of PSA (not significant) of 14% while on high soy diet	(Maskarinec *et al*, 2006)
Lycopene with or without soy isoflavones for 6 months	n=71, includes hormone sensitive and resistant patients.	95% of patients in lycopene group and 67% of patients in the combined group achieved PSA stabilization.	(Vaishampayan *et al*, 2007)

Soy protein isolate diet (high or low dose isoflavones)	Randomised trial. n=58. Randomised study, 6 months duration	Significant reduction of androgen receptor expression in prostates of men taking high dose isoflavones	(Hamilton-Reeves *et al*, 2007)
80mg daily, purified isoflavones	n=50 men with prostate cancer Gleason grad 6 or less completed treatment. Randomised, placebo controlled, double blind	No changes in sex hormones or PSA over 12 weeks	(Kumar *et al*, 2007)
Soy milk 3 times daily for 12 months	N=20, open label study observing rate of PSA rise after local therapy	Regression modeling showed slowing of the rate of PSA rise, from 56% per year to 20% per year while on study	(Pendleton *et al*, 2008)
Soy isoflavone supplement for 2 - 4 weeks prior to prostatectomy	N=25 (12 placebo, 13 soy). Randomised, double blind, placebo controlled	Tissue COX-2 mRNA expression were reduced by soy isoflavones. Statistically significant correlation between isoflavone levels and p21 mRNA expression in the treatment group.	(Swami *et al*, 2008)

Clinical trials in normal men (Jenkins *et al*, 2003; Adams *et al*, 2004) and in elderly men with elevated PSA levels (Urban *et al*, 2001) found that dietary soy supplementation at doses sufficient to alter plasma lipid levels did not significantly reduce PSA levels. This would imply that the underlying rate of PSA secretion was not changed by isoflavones, and a direct effect on prostate cancer cells may exist. Interestingly, there is some *in vitro* evidence to suggest that genistein reduces PSA mRNA expression and secretion, but also inhibits prostate cancer cell growth independently of its effects on PSA secretion (Davis *et al*, 2000). The implication from this study is that genistein has effects independent of the androgen responsiveness of the cell line. On the other hand, Habito and others showed that there may have been minor sex hormone changes when a meat diet was swapped for a tofu diet (Habito *et al*, 2000). In an exploratory study of over 600 British men, no correlation was found between soy milk consumption and changes in sex hormone levels (Allen *et al*, 2001). For many

studies published to date, however, drawbacks include short term isoflavone administration, highly varying isoflavone preparations and combinations, small patient numbers, and possibly insufficient doses.

The question of dose was addressed by a Phase I and pharmacokinetic study in which up to 600mg genistein was administred daily to men with prostate cancer (Fischer *et al*, 2004). Serum dehydroepiandrosterone fell by by 31.7% (P = 0.0004) at the end of the study, and estrogenic side effects were encountered (hot flashes, breast changes), but essentially no statistically significant PSA changes were noted. In another Phase I study of genistein - enriched isoflavone extracts, Takimoto (Takimoto *et al*, 2003) showed that peak plasma concentrations reached between 4.3 and 16.3 uM at doses up to 8 mg/kg orally (equivalent to 560mg for a 70kg person), commensurate with *in vitro* activity. From these studies, it would seem that relatively high doses of isoflavones, higher than used in many published studies, are required to ensure plasma concentrations sufficient for activity as predicted from preclinical work.

2.1.10 Phenoxodiol

Phenoxodiol is a novel isoflavone derived from equol (Brown *et al*, 2005). Unlike in many other studies on isoflavones noted above, this compound has been purified and studied as a single agent. Another notable difference is the study population (advanced cancer patients) and its repeated dose (compared to single dose) design in the early Phase I studies (Choueiri *et al*, 2006a; de Souza *et al*, 2006). When administered in a continuous infusion schedule over 7 days, steady state concentrations of phenoxodiol reached 60 µg/mL at the 27 mg/kg/24h dose cohort (Choueiri *et al*, 2006), higher than

that required for *in vitro* activity. The mean time to steady state concentration was 53h, and the mean accumulation half-life was 10.6h. No objective tumour responses were noted with this schedule, although one patient with metastatic renal cancer had stable disease for 6 months. Toxicity was generally mild, with only two patients experiencing Grade III toxicity (emesis, rash). In another Phase I study, repeated weekly infusions of phenoxodiol administered over 1-2h was again well tolerated, with Grade III lymphopenia noted in 9 patients (de Souza *et al*, 2006). Two patients completed 24 weeks of treatment, including one patient with advanced renal cancer who had stable disease over this period. The mean half – life of total phenoxodiol was 5h, whereas Cmax at the highest dose cohort (30 mg/kg) reached a mean of 88.7µg/ml (de Souza *et al*, 2006). Other studies of phenoxodiol have been reported only in abstract form at present, including two other Phase I studies, and a Phase I/II study in patients with hormone refractory cancer (Kelly, 2004). In this study, doses of 20 mg to 800 mg three times a day in 5 dose cohorts were studied in a 21 out of 28 day schedule. No objective responses were noted, although 37% of the 24 patients enrolled had stable disease.

2.1.11 Conclusions

Ultimately, relatively low patient numbers and the methodological limitations of case-controlled studies reduce the clinical impact of these studies. To date, there have been no published prospective, randomized clinical trials with sufficient statistical power to assess whether isoflavone administration can reduce the development or delay the progression of prostate cancer.

Much evidence suggests that isoflavones have growth modulating effects on cancer cells that may be divergent in nature and which may result from a wide range of mechanisms. Suppression or facilitation of cancer cell growth may occur depending upon the specific isoflavone, combination of isoflavones present in the preparation, the concentration (or dose) of isoflavones, bioavailability, and host factors such as individual variation in absorption and the ability to form equol (pharmacogenomics). To complicate matters, the design of many clinical trials to date has been less than ideal, with small patient numbers, lack of randomization, and short duration amongst some of the issues that cloud interpretation of results. Of course, it is entirely possible to conclude that despite the wealth of preclinical literature supporting its use, isoflavones may simply be insufficiently effective in inhibiting or treating prostate cancer "in the real world". Interesting *in vitro* studies suggest that genistein may have biphasic properties with low doses stimulating cancer growth and higher doses retarding growth (see Magee & Rowland, 2004 for review). This observation could form a basis for the provocative reverse hypothesis that low doses of phytoestrogens, such as those found in Western diets, might promote prostate cancer growth, whereas higher doses are protective in Asian men, thereby potentially explaining the disparity of prostate cancer incidence in Asian and non-Asian countries noted in many epidemiological studies.

Although there is generally good preclinical data to suggest that isoflavones may be of benefit in the prevention or treatment of prostate cancer, clinical trials of the effects of isoflavone administration in prostate cancer have so far been disappointing. Under the current circumstances it would be prudent to guard against any public health recommendations about the value of increasing dietary isoflavone intake or the use of commercial isoflavone supplements for the prevention of cancer. There may be a role

for the use of isoflavone supplementation for the treatment or prevention of particular cancers in specific populations such as prostatic cancer in older men, but these have yet to be adequately defined. There is also a potential role for isoflavones to be used in combination with other treatments or radiation therapy in certain circumstances, but this requires further evaluation. Efforts to study purified compounds such as phenoxodiol and flavopridol in the traditional drug development approach (Phase I, II, III) should be encouraged in view of the high quality of evidence obtained clinically. Although isoflavones such as phenoxodiol show promising activity in the clinic, it is still too early to tell whether the drug will prove effective for the treatment of prostate cancer. In the event that it is only modestly active, it can still be developed for use as preventive therapy, but this will require a much longer timeframe, as well as very large studies that may be beyond the scope of pharmaceutical companies. In view of the inconsistency of clinical data, and the lack of Level I or II evidence, isoflavones cannot yet be recommended for the prevention or treatment of prostate cancer.

2.2 Role of the Akt pathway in prostate cancer

2.2.1 Introduction

Intracellular pathways utilized by cancer cells have become the focus of intense research over the past couple of decades. Mutations in two key pathways, the Ras and phosphatidylinositol 3 – kinase (PI3K) pathways, are activated in many cancers. These pathways act in a complex network of lipids, proteins and enzymes that coordinate multiple cellular processes to regulate cell growth and survival (Nicholson and Anderson, 2002; Shaw and Cantley, 2006). However, cell signaling is not straightforward, and there is a significant interaction between these, as well as other pathways. PI3K is activated by both Ras and receptor tyrosine kinases (Shaw and Cantley, 2006), and functions as an important conduit through which lipid second messengers and other substrates are activated.

Akt was initially identified as an oncogene from a T cell lymphoma (Staal and Hartley, 1988) and was later shown to have pleckstrin homology (PH) and serine / threonine kinase domains. It is also referred to as Protein Kinase B (PKB) because its catalytic domain is related to Protein Kinase A (PKA) and Protein Kinase C (PKC) family members. The kinase activity of Akt is stimulated by a variety of signals including growth factors, cytokines, integrin engagement, and T cell receptor activation. Activation of Akt leads to phosphorylation and regulation of a large number of substrates involved in numerous cellular processes including cell survival, cell growth, cell differentiation, cell cycle progression, cell proliferation and cellular metabolism.

Akt has a central role in a number of cell signaling pathways that are important in cancer cells. Upstream components of the Akt pathway include PI3K, Phosphatase and Tensin homologue deleted on chromosome Ten (PTEN), Neurofibromin 1 (NF1), and the tumour suppressor gene product responsible for Peutz-Jeghers syndrome (LKB1). Downstream components include tuberous sclerosis complex 2 (TSC2), Forkhead Box Class O (FOXO), and eukaryotic initiation factor 4E (eIF4E) (Altomare and Testa, 2005). Some of these components can be considered as activators (Akt, eIF4E, and both the catalytic p110 and regulatory p85 subunits of Akt) or tumour suppressors (PTEN, FOXO, LKB1, TSC1/TSC2, NF1, and Von Hippel Lindau or VHL).

Recent studies show that the PI3K -Akt pathway is a major survival signaling pathway in many cancers (see Blume-Jensen and Hunter, 2001; Testa and Bellacosa, 2001; Cantley, 2002; Nicholson and Anderson, 2002; Vivanco and Sawyers 2002; Paez and Sellers, 2003; Shaw and Cantley, 2006 for reviews). It has been estimated that up to 30% of all cancers harbour a mutation of a component in this pathway (Luo *et al*, 2003) and a higher percentage of activated Akt is found in many cancers (Altomare and Testa, 2005), so it arguably represents one of the most important signal transduction pathway in cancer cells, particularly in the way it links multiple other pathways.

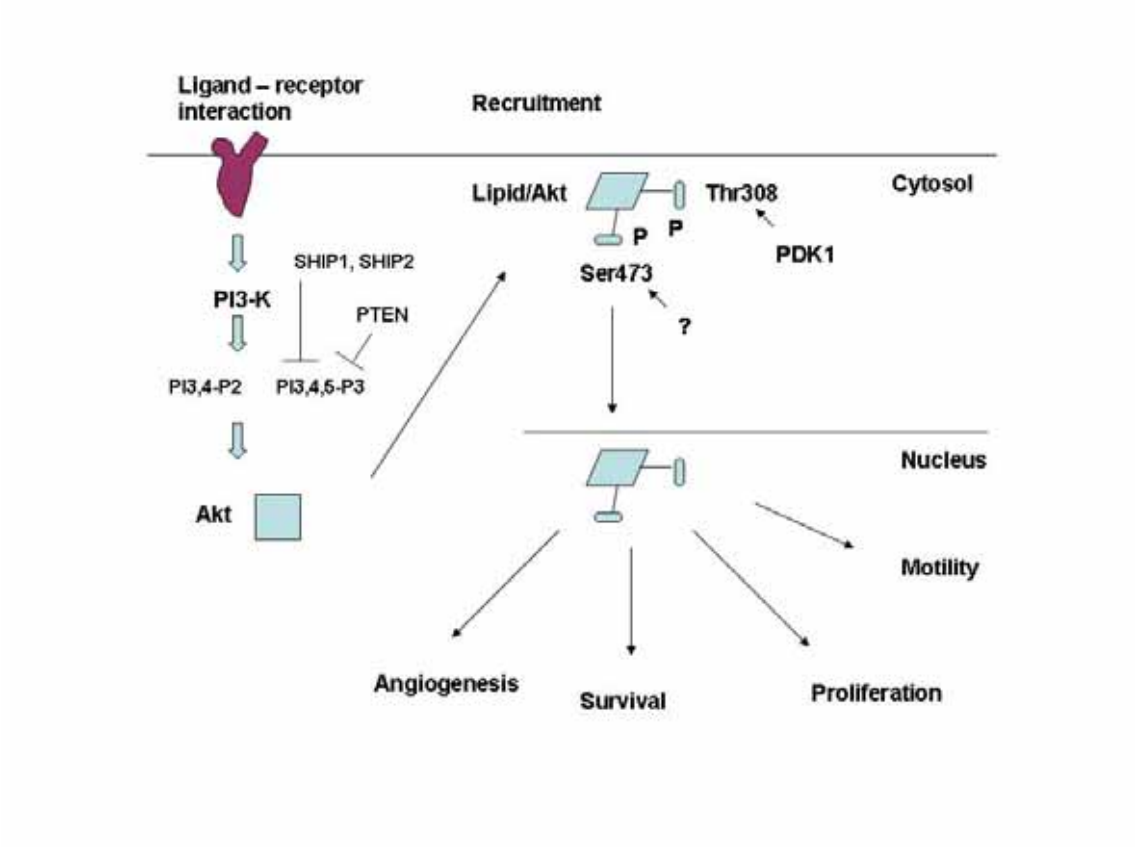
2.2.2 Regulation of PI3K

The importance of PI3K was highlighted by the discovery of amplified genes encoding its p110 α catalytic subunit and Akt2 in ovarian, breast and pancreatic cancer (Luo *et al*, 2003). Moreover, mutations in the p85α regulatory subunit of PI3K were found in primary ovarian and colon tumours (Bader *et al*, 2005). The most frequent mutations of

the p110 α catalytic subunit show gain of function (Samuels *et al*, 2005). This results in the generation of the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5-P3), and ultimately the activation of Akt. The most important regulator of PI3K, however, is the status of the phosphatase and tensin homolog (PTEN).

PTEN is a phosphatase that converts PI3,4,5-P3 to PI4,5-P2 by removal of the D3 phosphate which dampens the stimulatory effects of PI3K on the Akt signaling cascade. The SHIP phosphatases (SHIP 1 and SHIP 2) also dephosphorylate PI3,4,5-P3, but convert it to phosphatidylinositol 3,4-bisphosphate (PI3,4-P2), which can still function as a second messenger to recruit PH-domain-containing proteins, including Akt. PTEN, however, is the primary negative regulator of the PI3-K-Akt pathway (Wu *et al*, 1998; Cantley and Neel, 1999; Dahia, 2000; Di Cristofano and Pandolfi, 2000; Leslie and Downes, 2002) (Figure 2). In normal mammalian cells, the levels of the second messengers PI3,4,5-P3 and PI3,4-P2 are controlled by PI3K regulation as well as several other PI3,4,5-P3 / PI3,4-P2 phosphatases.

Figure 2. Activation of Akt. Binding of ligands such as insulin, EGF, PDGF, FGF to tyrosine kinase receptors leads to activation of PI3K followed by binding of Akt by lipid second messengers. This induces a conformational change and relocation of Akt from the cytosol to the cell membrane, where it is phosphorylated at Thr308 (by PDK1) and Ser473 (by an unknown mechanism, shown here with "?"). Activated Akt translocates to the nucleus, leading to downstream effects such as cell survival, angiogenesis, cell motility, and proliferation. PTEN dephosphorylates PI3,4,5-P3, and is the dominant negative regulator of Akt.



The importance of PTEN is highlighted by the discovery of phakomatoses (neurocutaneous syndromes) such as Cowden's disease and Bannayan Zonana syndrome, due to dominantly inherited germline mutations in PTEN that allow the growth of multiple hamartomas. Sporadic mutations of PTEN are also found in some cancers such as breast, ovarian and colon as well as glioblastoma (Cantley and Neel, 1999).

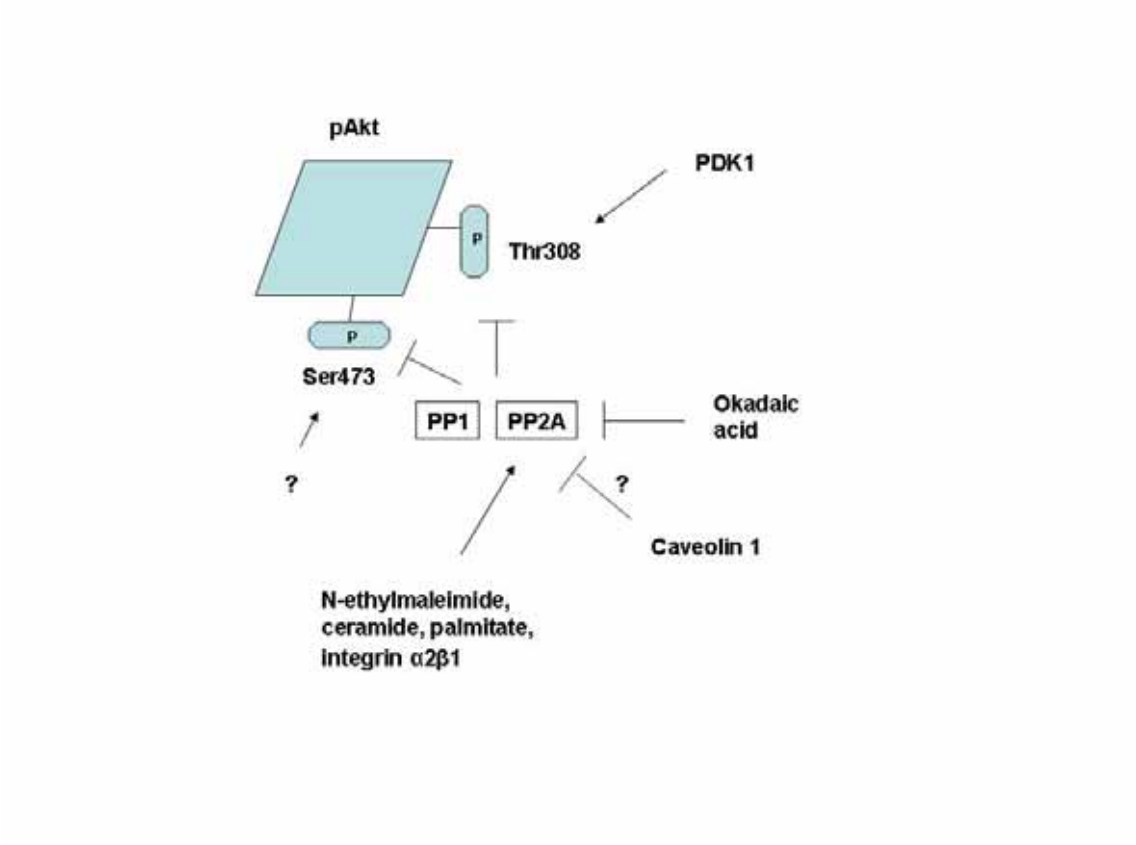
2.2.3 Regulation of Akt

Akt has an N - terminal domain, a catalytic domain, and a C-terminal regulatory domain. In humans, there are 3 highly homologous members Akt1, Akt2, and Akt3 (Nicholson and Anderson, 2002). Whether all 3 isoforms are important in human

cancers is controversial; all appear to be expressed in normal tissue / cancer pairs when examined by RT-PCR (Zinda *et al*, 2001), though there are some conflicting studies suggesting differing influences of each isoform (Barnett *et al*, 2005; Le Page *et al*, 2006; Maroulakou *et al*, 2007). In unstimulated cells, Akt mainly resides in the cytosol. After activation of PI3-K by tyrosine kinase receptor or other cell surface receptor binding to ligands such as insulin, platelet- derived growth factor (PDGF), epidermal growth factor (EGF), or fibroblast growth factors (FGFs), the resulting second messenger phospholipids, PI3,4,5-P3 or PI3,4-P2, recruit Akt to the cell membrane, and bind to its N - terminal domain. This leads to a conformational change of Akt, making it favourable for phosphorylation at Thr308 by 3-phosphoinositide-dependent kinase (PDK1) and phosphorylation at Ser473 by TORC2 (see below). Ser473 phosphorylation is required for full activation of Akt, which then dissociates from the membrane and travels to the nucleus and other subcellular components to regulate functions such as proliferation, survival, motility and angiogenesis (Figure 2) (Testa and Bellacosa, 2001; Paez and Sellers, 2003; Brazil *et al*, 2004). PHLPP is a recently - described phosphatase that specifically dephosphorylates Ser473 of Akt (Gao *et al*, 2005).

Phosphorylation of Akt can also be controlled by serine / threonine / protein phosphatases (Figure 3). PP1 and PP2A are two major classes of serine / threonine protein with the capacity to dephosphorylate substrates, including Akt, that are involved in many different cellular processes including oxygen metabolism, cell cycle regulation, protein synthesis, RNA splicing, and signal transduction. The activation of PP2A by N-ethylmaleimide, ceramide, palmitate or integrin α2β1 inhibits Akt phosphorylation. Conversely, the inhibition of PP2A by okadaic acid increases Akt function (Cazzolli *et al*, 2001; Teruel *et al*, 2001; Ivaska *et al*, 2002; Yellaturu *et al*, 2002).

Figure 3. Phosphorylation of Akt. Phosphorylation of Akt at Thr308 (by PDK1) and Ser473 (by an unknown mechanism, shown here with "?"). Both PP1 and PP2A are serine/threonine phosphatases that dephosphorylate Akt. N-ethylmaleimide, ceramide, palmitate or integrin α2β1 can activate PP2A, thereby inhibiting Akt phosphorylation. Maintenance of Akt in phosphorylated form has been shown with Okadaic acid, which inhibits PP2A, as well as Caveolin 1, which interacts with PP2A but it is not clear whether it directly inhibits PP2A (shown here with "?).



Caveolin-1 (cav-1) expression is elevated in a number of cancers, including prostate cancer (Yang *et al*, 1998; Tso *et al*, 2000; Davidson *et al*, 2001; Fine *et al*, 2001; Goh *et al*, 2001; Rajjayabun *et al*, 2001; Tahir *et al*, 2001; Ito *et al*, 2002; Kato *et al*, 2002). Caveolae are invaginations of the cell membrane involved in critical cellular processes including regulation and transportation of lipids, endocytosis, and signal transduction (see Sternberg and Schmid, 1999; Schroeder *et al*, 2001; Ikonen *et al*, 2004 for reviews). Li and others (Li *et al*, 2003) found that Caveolin-1 participated in the maintenance of Akt by keeping it in the phosphorylated (active) form. LNCaP prostate

cancer cells transfected with the *cav-1* gene show higher activities of Akt and are more resistant to apoptosis than *cav-1* negative vector control LNCaP cells (Li *et al*, 2003). PP1 and PP2A interact with cav-1, leading to inhibition of PP1 and PP2A activity thereby favouring the maintenance of Akt phosphorylation (Figure 3). It appears that both PP1 and PP2A can dephosphorylate Akt, but PP2A may be the predominant phosphatase (Li *et al*, 2003). Furthermore, caveolin-1 can function in an autocrine manner to stimulate LNCaP cell growth (Tahir *et al*, 2001).

2.2.4 Downstream regulation of Akt function

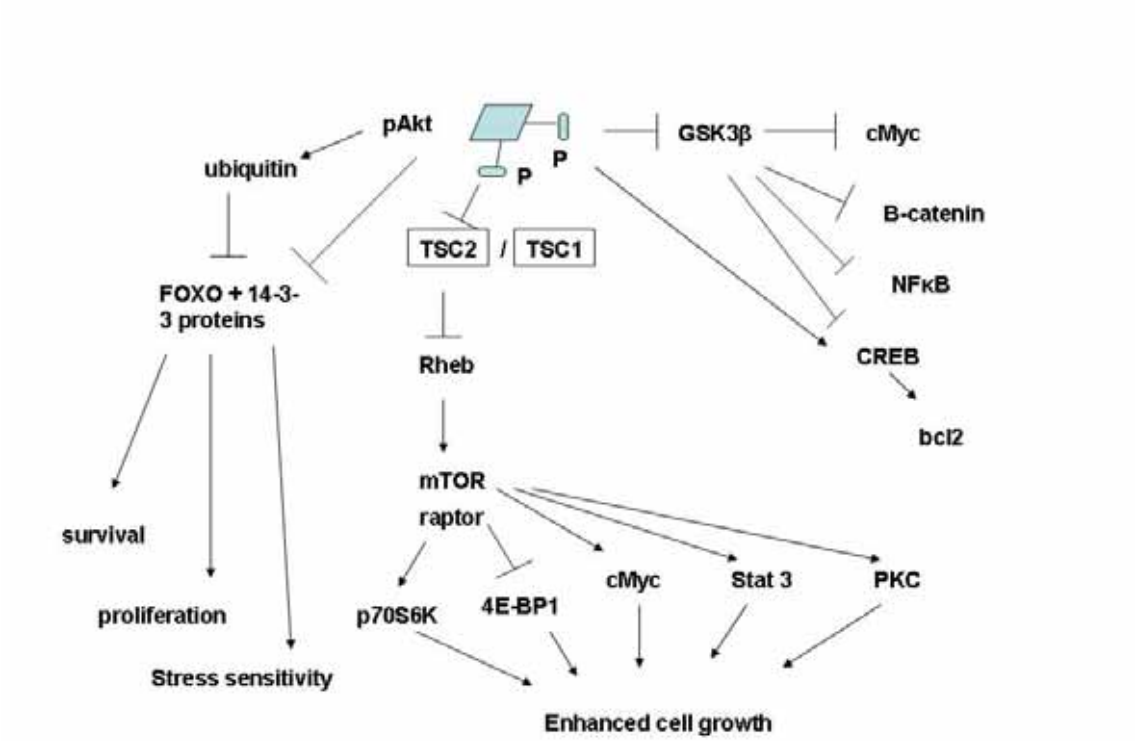
One of the major roles of Akt is promotion of cell survival by targeting molecules regulating pro-survival genes. Akt plays a key role in the anti-apoptotic cascade by regulation of a variety of downstream targets, as described below.

2.2.4.1 The FOXO subfamily

FOXO transcription factors belong to the Forkhead family of proteins which have a common DNA-binding domain called the “forkhead box” (Kaestner *et al*, 2000). There are 19 subgroups (FOX for “Forkhead Box A to S”), of which the FOXO subgroup is of most interest in cancer (Greer and Brunet, 2005). FOXO1 (also known as FKHR), FOXO3 / FKHRL1, and FOXO4 / AFX are expressed abundantly in adipose tissue, brain, and heart, respectively. These FOXO transcription factors are direct substrates of Akt (Greer and Brunet, 2005). Following cell stimulation by growth factors or insulin, PI3K, and in turn, Akt and the related SGK (serum and glucocorticoid inducible kinase) are activated. Phosphorylation of FOXO proteins by Akt triggers their relocalisation from the nucleus to the cytoplasm, chaperoned by 14-3-3 proteins (Brunet *et al*, 1999;

Brunet *et al*, 2002). This inhibits FOXO – dependent transcription, and allows cell proliferation, stress sensitivity, and cell survival (Figure 4).

Figure 4. Downstream pathways of Akt. Phosphorylated Akt results in multiple downstream events, some of which are depicted here. Antagonism of FOXO transcription factors, as well as enhancing their degradation by ubiquitin promotes cell proliferation, increased stress sensitivity, and cell survival. Inhibition of the TSC2/TSC1 complex allows the accumulation of mTOR, which in turn promotes enhanced cell growth through p70S6K and inhibition of 4E-BP1. Other effects include the regulation of GSK3β, which in turn regulates a number of tumorigenic proteins, thereby permitting cell growth and also increased apoptotic resistance.



Within the cytoplasm, FOXO1 and FOXO3 transcription factors are degraded by ubiquitin - dependent mechanisms regulated by Akt (Plas and Thompson, 2003), whereas FOXO3 is also phosphorylated by I kappaB kinase β (IKKβ). IKKβ causes degradation of FOXO factors (Hu *et al*, 2004), and is known to activate the transcription

factor NF- κB through the phosphorylation and subsequent degradation of IκB, itself an inhibitor of NF- κB (reviewed in Karin *et al*, 2002). To add to the complexity of this regulation, Akt can phosphorylate IκB kinase, leading to its degradation, leaving NF-κB free to translocate to the nucleus and activate its target genes (Romashkova and Makarov, 1999). NF-κB transcription factor is an important promoter of cell survival in response to several apoptotic stimuli.

Activation of the Akt/SGK pathway inhibits the function of FOXO factors, so it is not surprising that many functions of FOXO factors oppose those of Akt/SGK (Greer and Brunet, 2005). These include cell cycle arrest in G1/S by upregulation of cell cycle inhibitors p27 and p21 as well as repressing cell cycle activators (cyclin D1/D2), promotion of DNA repair through upregulation of target genes such as GADD45, detoxification of reactive oxygen species, promotion of cellular differentiation, mediation of apoptosis via genes such as BIM, a pro-apoptotic BH3 family member, and promotion of gluconeogenesis (reviewed in Greer and Brunet, 2005). Although the functions of different FOXO factors are likely to overlap, the complex interaction and overall thrust of these roles supports the notion that this family of proteins act as tumour suppressors.

2.2.4.2 TORC1 and TORC 2

Akt directly inhibits tuberous sclerosis 2 (TSC2) function by phosphorylation (Dan *et al*, 2002). TSC2 is a GTPase – activating protein (GAP) that, in association with TSC1, inactivates the small G protein Rheb (Garami *et al*, 2003; Inoki *et al*, 2003; Zhang *et al*, 2003a). Thus, Akt activation allows Rheb to accumulate in a GTP - bound state that, in turn, activates the kinase activity of the mammalian target of rapamycin (mTOR).

mTOR is a serine / threonine kinase that complexes with the raptor adaptor protein (mTOR:raptor or mTORC1) that regulates cell growth by activating p70 ribosomal S6 kinase (p70S6K or RSK), which promotes the translation of mRNA with 5′ polypyrimidine tracts. mTOR:raptor also inhibits 4E-BP1, which can act as a translational inhibitor of some types of mRNAs (Vivanco and Sawyers, 2002). In addition, mTOR regulates transcription of c-Myc and activation of Stat 3, PKCα, and PKCδ, all involved in tumorigenesis (Schmelzle and Hall, 2000; Blume-Jensen and Hunter, 2001). mTORC1 is inhibited by rapamycin and its analogues, but mTORC2, the second mTOR complex, is rapamycin insensitive. mTORC2 is required for phosphorylation of the ser473 site of Akt, but whether inhibition of this target is important for cancer therapeutics remains to be seen (Sarbassov *et al*, 2005). TSC1 and TSC2 association can be markedly impaired by extracellular regulated kinase (ERK), reducing its ability to inhibit mTOR signaling (Ma *et al*, 2005), and placing the Ras / MAPK pathway upstream of the mTORC1 complex. Akt activation via a number of upstream events thus profoundly influences mTOR signaling, which ultimately enhances cell growth (Figure 4).

2.2.4.3 Other downstream targets

CREB is a group of transcription factors that modulate the expression of genes containing promoters with cyclic AMP response elements. Activation of CREB by Akt phosphorylation recruits the coactivator CREB – binding protein (CBP) (Chrivia *et al*, 1993) and promotes transcription of pro-survival genes such as Bcl-2 (Wilson *et al*, 1996).

GSK3β is a kinase involved in glycogen synthesis, but is also a regulator for many cell functions, including cell cycle elements such as c-Myc, cyclin D and cyclin E. It also regulates transcription factors such as β-catenin, CREB, NF-κB, GLI, Notch, Snail and Sterol regulatory element binding transcription factor (SREBP1). Some of these in turn are implicated and overexpressed in certain types of cancers. Phosphorylation and inactivation of GSK by Akt favours increased activity of these downstream kinases and proteins that affect survival through multiple mechanisms (Grimes and Jope, 2001; Shaw and Cantley, 2006) (Figure 4). Akt has been reported to upregulate the expression of insulin-like growth factor I receptor (IGF-IR), leading to enhanced invasiveness and increased survival of pancreatic cancer cells (Tanno *et al*, 2001).

Akt can also influence the activity of the tumour suppressor protein p53, through binding of Mdm2. Although Mdm2 is regulated by p53, it can also regulate p53 and promote its degradation. Phosphorylation of Mdm2 by Akt promotes its translocation to the nucleus where it binds to p53 and inhibits its ability to regulate cell cycle (Mayo and Donner, 2001). Moreover, mTOR can promote translation of Mdm2, and inhibition of either mTOR or Mdm2 is sufficient to block cell survival by growth factor receptors such as Met (Moumen *et al*, 2007). PTEN appears to protect p53 function (Mayo *et al*, 2002), possibly by controlling Mdm2 promoter activity (Chang *et al*, 2004).

Overexpression of nuclear receptor coactivator, SRC-3, is found in prostate cancer tissues relative to normal tissue levels (Gnanapragasam *et al*, 2001). SRC-3 stimulates cell proliferation, cell size, increases invasiveness, and suppresses apoptosis in prostate cancer cells (Zhou *et al*, 2003) through induction of Akt expression and its activation.

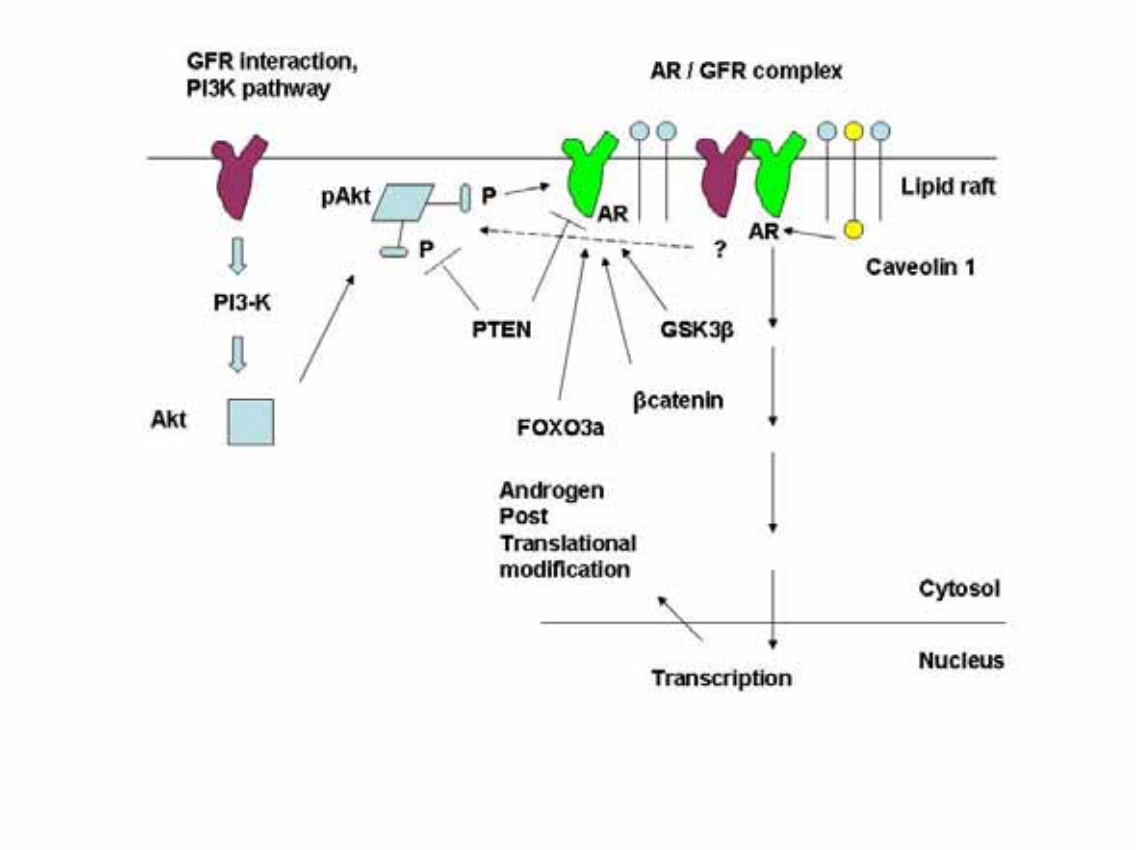
SRC-3 null mutant mice have lower Akt levels and activity compared to their wild – type litter mates, supporting the observed link between SRC and Akt (Zhou *et al*, 2003).

2.2.5 Akt and the Androgen receptor

Akt can interact directly (Wen *et al*, 2000) or indirectly (Chesire *et al*, 2002; Salas *et al*, 2004) to interfere with the androgen receptor (AR) pathway at multiple levels upstream and downstream of Akt (Reddy *et al*, 2006; Wang *et al*, 2007). There is also good evidence that androgens can promote Akt activity independent of PI3K, possibly through the formation of an extranuclear complex within lipid rafts in the cell membrane (Cinar *et al*, 2007). Li and others (Li *et al*, 2003) found that caveolin-1 (cav-1) induced phosphorylation of the androgen receptor led to nuclear translocation in cav-1 negative LNCaP cells at both low and physiological levels of testosterone. More evidence linking the Akt pathway and androgen receptor regulation was provided by Lin (Lin *et al*, 2004) who found that PTEN repressed AR in a dose - dependent manner in LNCaP, PC3, and DU145 prostate cancer cells. In low passage numbers in LNCaP, PTEN regulates AR function in a PI3K/Akt independent manner, but in a PI3K/Akt dependent manner in high passage numbers. Others showed that the PI3K / Akt pathway is required for basal and dihydrotestosterone-induced AR protein expression (Manin *et al*, 2002). Akt can phosphorylate AR and suppress binding of AR coactivators (Lin *et al*, 2001). Furthermore, FOXO3a has been shown to induce AR expression in LNCaP cells (Yang *et al*, 2005). Interestingly, blocking PI3K by LY294002 induces G1 arrest, but introducing siRNA to inhibit AR function leads to dramatic cell death, providing a model of AR / PI3K / Akt cooperation in the control of LNCaP survival (Yang *et al*, 2005). Notably, the close cooperation between Akt and AR pathways occurs in other

prostate cancer cell lines (Vinall *et al*, 2007), and can interact synergistically to promote carcinogenesis and override the effect of androgen ablation (Xin *et al*, 2006). Akt may indeed provide an important reactionary means of prostate cancer cell survival in the face of androgen deprivation (Mikhailova *et al*, 2008). The complex nature of Akt regulated AR function, and in turn, an AR independent Akt mediated "escape" pathway through crosstalk with other pathways is reviewed in detail elsewhere (Wang *et al*, 2007). In PTEN deficient cells, AR function may also play an enabling role to prevent feedback inhibition of mTOR through expression of proteins enhancing nutrient availability (Xu *et al*, 2006). The potential clinical relevance of the AR and Akt pathways has also been investigated in an immunohistochemical study in Japanese men, where a positive correlation between Akt, pAkt, AR expression and high grade cancer was noted (Shimizu *et al*, 2007). Taken together, there is strong evidence for the importance of AR and Akt pathway cooperation in the progression of prostate cancer in both androgen dependent and independent model systems (Figure 5).

Figure 5. Interaction between Akt and the androgen receptor. The complex interaction between the androgen receptor (AR) and Akt pathways are not fully understood. PI3K – dependent (via Growth Factor Receptor, GFR) and independent (via AR / GFR associated complex within the lipid raft) mechanisms (shown here with "?") for influencing AR expression and function have been proposed. Akt can phosphorylate AR directly, but other elements of the Akt pathway (eg. FOXO3a, GSK3β, βcatenin) could also be important. Post-translational modification of AR induced activity may also influence cellular signaling.



2.2.6 Akt and cellular metabolism

Signal transduction cascades mediate the metabolism of tissues such as liver, skeletal muscle, and adipose tissue in response to insulin (Plas & Thompson, 2005). Akt is responsible for transducing these signals triggered by insulin receptor binding to metabolic and transcriptional programs within the cell (Burgering and Coffer, 1995; Brazil *et al*, 2004). Activated Akt can cause increased transcription and plasma membrane localization of GLUT1, the principle glucose transporter in most cell types (Barnes *et al*, 2005). Interestingly, transgenic mice with deficient Akt function show impaired glucose tolerance due to defective insulin secretion (Bernal-Mizrachi *et al*, 2004). This finding is consistent with the observation that Akt promotes the shift towards aerobic glycolysis in glioblastoma and leukemia cell lines (Elstrom *et al*, 2004),

which occurs without a corresponding increase in oxidative phosphorylation. Presumably, the increase in glycolysis induced by Akt is necessary to supply nutrients for mitochondrial processes caused by cell stress, such as that induced by growth factor withdrawal (Plas *et al*, 2001). It would appear that activation of the Akt pathway is a prominent feature of many cancers, whereas defects in the pathway contribute to Type II diabetes (Cantley, 2002). Some of the control of cellular metabolism by Akt may be mediated by FOXO factors (Plas & Thompson, 2005).

2.2.7 Akt and apoptosis

A number of observations suggest that Akt actively promotes cell survival by reducing the impact of the apoptotic machinery. BAD is a pro-apoptotic member of the Bcl-2 family of proteins that promotes cell death by forming a nonfunctional heterodimer with the pro-survival molecule Bcl-X(L). Phosphorylation of BAD by Akt prevents its association with Bcl-X(L), thereby allowing Bcl-X(L) to function and promote cell survival (Datta *et al*, 1997). Further, Akt inhibits the conformational change of other pro-apoptotic members of Bcl-2 family proteins, thus preventing the disruption of mitochondrial membranes and the activation of caspase 3 (Yamaguchi and Wang, 2001). Finally, phosphorylation of caspase 9 by Akt abrogates cytochrome c - induced proteolytic processing of procaspase 9, which prevents activation of downstream effector caspases (Cardone *et al*, 1998), and ultimately reduces apoptosis.

2.2.8 Akt, cell cycle and angiogenesis

Akt also affects key components of the tumour cell environment, including extracellular matrix and angiogenic factors. Stimulation of the secretion of matrix metalloproteinase 9 has been described (Thant *et al*, 2000) which can lead to increased tumour cell invasiveness. In addition, Akt is known to mediate vascular endothelial growth factor (VEGF) stimulated endothelial cell proliferation (Chan *et al*, 2002).

The tumour suppressor $p21^{WAF1}$ is a p53 target gene that acts as a cell cycle inhibitor when prompted to respond following induction of p53 in response to stress signals such as DNA damage. Phosphorylation of $p21^{WAF1}$ by Akt prevents its nuclear localization, effectively quarantining it from its target cyclin – dependent kinase genes, and leads to abrogation of cell cycle arrest (Zhou & Hung, 2002). In a similar manner, $p27^{Kip1}$ phosphorylation by Akt traps the molecule in the cytoplasm allowing its sequestration by the 14.3.3 scaffolding protein, thereby preventing its cell cycle inhibiting function (Viglietto *et al*, 2002). The Akt – mediated reduction of free $p27^{Kip1}$ protein is associated with accelerated growth of prostate xenografts (Graff *et al*, 2000).

Activation of Akt can also enhance tumour angiogenesis. Loss of PTEN in prostate cancer is associated with increased microvessel density in prostate cancer tissue, consistent with promotion of angiogenesis (Giri & Ittmann, 1999). Re-introduction of PTEN into PC3 and LNCaP cells causes decreased VEGF secretion (Koul *et al*, 2002). Akt increases hypoxia inducible factor 1 alpha (HIF-1α) and its transcriptional targets such as VEGF. Further, Akt can promote angiogenesis by stimulating production of nitric oxide within endothelial cells. It achieves this through phosphorylation of endothelial nitric oxide synthase (eNOS), which is responsible for maintaining systemic blood pressure, vascular remodeling, and angiogenesis (Dimmeler *et al*, 1999; Fulton *et*

al, 1999). Arsham (Arsham *et al*, 2004), however, found that transfection of Akt into a hepatoma cell line lacking in HIF-1α is still able to induce VEGF secretion and tumour vascularization, suggesting that not all angiogenic effects of Akt are mediated by HIF-1α.

2.2.9 Dysregulation of the PI3-Akt pathway

Whether Akt is constitutively active in many cancers due to amplification of the gene or amplification / mutation of Akt regulator genes is controversial (Testa and Bellacosa, 2001; Vivanco and Sawyers, 2002; Luo *et al*, 2003). An earlier micro-array study suggested that *Akt* gene amplification can occur in prostate cancer (Edwards *et al*, 2003) as well as in other cancers including breast (Bellacosa *et al*, 1995) and ovarian cancer (Cheng *et al*, 1992). However, a more recent publication suggests that amplification of Akt genes in any malignancy is a rare event (Altomare & Testa, 2005). Rather, elevated Akt expression and activity are associated with poor prognosis in human prostate and breast cancer (Nakatani *et al*, 1999; Sun *et al*, 2001; Edwards *et al*, 2003; Liao *et al*, 2003).

Amplification of genes controlling components of the signal transduction pathway upstream of Akt has also been described. *PI3K* amplification has been reported for prostate (Edwards *et al*, 2003) as well as for other cancers. Taking the pathway further, several protein tyrosine kinases upstream of PI3K have been found to be overexpressed in prostate cancer. These include FGFR1, which is overexpressed in localized prostate cancer and amplified in hormone – resistant prostate cancer (Edwards *et al*, 2003), as

well as Erb2 (HER2) (Edwards *et al*, 2006) and IGF1-R, which are overexpressed in prostate cancer and other cancers.

An important observation in recent years is the association of the functional loss of PTEN with many types of cancer (Cantley and Neel ,1999; Di Cristofano and Pandolfi, 2000), including prostate cancer (Deocampo *et al*, 2003). In prostate cancer, 3 mechanisms have been identified for loss of PTEN: chromosome deletion or loss of heterozygozity (Rubin *et al*, 2000; Dong, 2001; Latini *et al*, 2001), somatic mutations (Feilotter *et al*, 1998; Gray *et al*, 1998; Pesche *et al*, 1998) and epigenetic abnormalities (Rennie and Nelson, 1998; Whang *et al*, 1998). Key prostate cancer cell lines such as LNCaP also show loss of PTEN function. The PTEN tumour suppressor gene maps to 10q23.3 where loss of heterozygosity has been detected in up to 49% of matched normal prostate and prostate cancers (Feilotter *et al*, 1998). Immunohistochemical studies show complete loss of PTEN in 15-20% of clinically localized prostate cancers, with another proportion showing reduction in expression (Li *et al*, 2005a). Loss of PTEN is associated with hormone refractory (Wang *et al*, 2003b) as well as advanced stage disease (McMenamin *et al*, 1999; Yang *et al*, 2002).

Overexpression of caveolin-1 is common in prostate cancer (Yang *et al*, 1998; Goh *et al*, 2001). As previously shown by Li et al (Li *et al*, 2003), caveolin-1 is linked to Akt by inhibition of PP1 and PP2A, phosphatases which would otherwise regulate Akt. This leaves Akt in an activated form.

2.2.10 Clinical biomarkers of Akt activity in prostate cancer

Increased expression or phosphorylation of Akt is linked to the development of androgen-independent disease, and is also predictive of a more aggressive course of prostate cancer in patients. Use of microarray (Ayala *et al*, 2004) has shown that high levels of phosphorylated Akt correlated with a higher probability of recurrence of prostate cancer. The relationship holds even within the group of patients with Gleason grade 6-7 disease. Of interest, the presence of high levels of phosphorylated Akt in non-neoplastic tissue is also predictive of recurrence. The cytoplasmic or nuclear localization of Akt isoforms may also have a bearing on patient outcome (Le Page *et al*, 2006). Increased phospho-Akt, alone or together with decreased phospho-ERK, is predictive of PSA failure in patients up to 5 years post-prostatectomy (Kreisberg *et al*, 2004), and also in a subset of patients that recur locally in the setting of androgen - independent disease (Assikis *et al*, 2004). Amplification of MAPK and PI3K / Akt pathway genes are associated with the development of clinical hormonal resistance (Edwards *et al*, 2003). Complete loss of PTEN in prostate cancer tissue has been proposed as an early marker for aggressive prostate cancer (Schmitz, Grignard et al. 2007), an observation supported by a number of preclinical studies (Nakatani *et al*, 1999; Ghosh *et al*, 2005; Gao *et al*, 2006; Shi *et al*, 2006; Li *et al*, 2007). However, biomarker studies may be subject to chance and bias, because many are retrospective studies on available tissues, and therefore tend to be conducted in selected populations. Prospective assessment of the clinical validity of phospho-Akt overexpression as a prognostic or predictive factor in clinical studies is awaited.

2.2.11 Akt and autophagy

Autophagy ("self-digestion") is a well-regulated process whereby cells that are stressed (through lack of nutrition or by drugs or other means) induce lysosomal degradation of cytoplasmic organelles. Although described as both a means of cell death as well as cell survival, it appears that this process is linked to but not synonymous with apoptosis, and allows cells to survive by shutting down general protein translation, though the ultimate effect is to halt tumour growth (Jin *et al*, 2007; Levine, 2007). Inhibition of the Akt and mTOR pathway results not only in increased apoptosis, but also in increased autophagy. The exact mechanisms for entering either program are not clear, but appear to closely involve mTOR. Recent reviews suggest that Akt and mTOR are central linking components of different pathways involving cell survival as well as cell metabolism. This centrality allows it to play critical roles in how a cell responds to threats to both survival and metabolic stress (Arico *et al*, 2001; Takeuchi *et al*, 2005; Cao *et al*, 2006b; Lavieu *et al*, 2006; Aoki *et al*. 2007; Gossner *et al*, 2007).

2.2.12 The PI3K – Akt pathway as a target for treatment

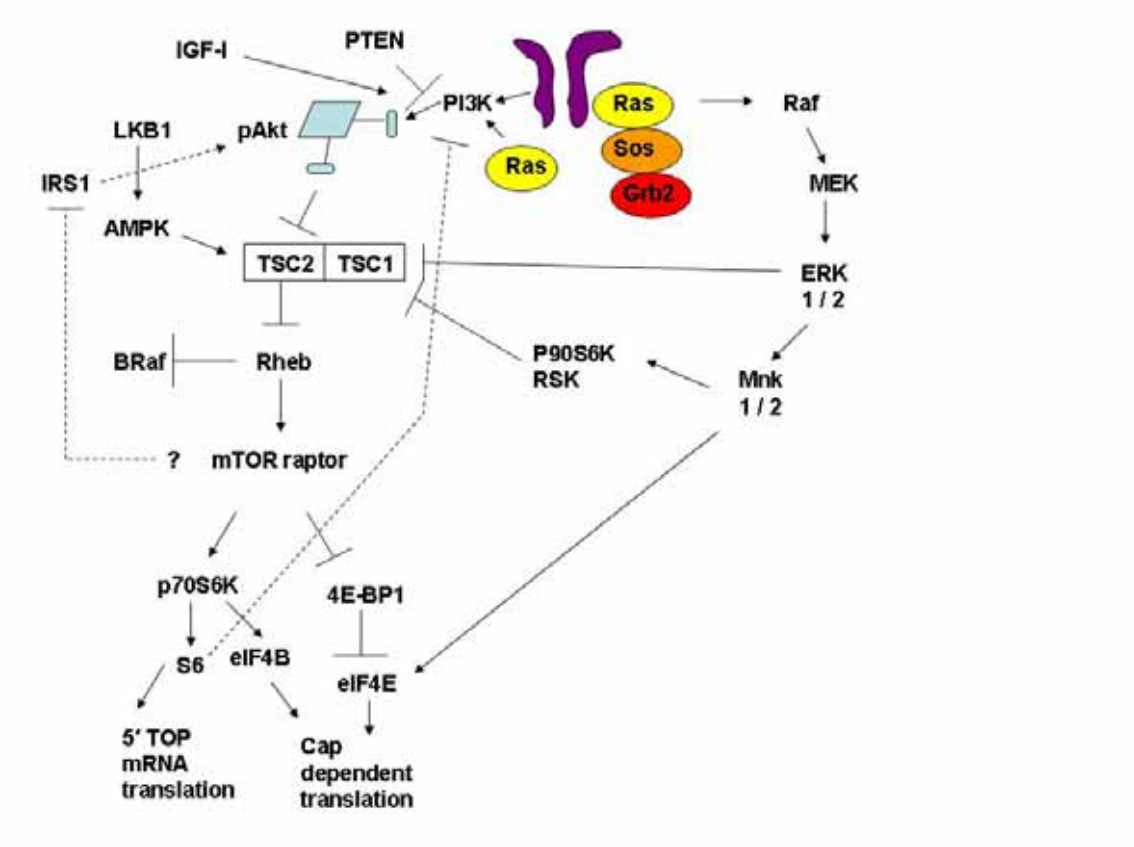
Wortmanin and LY290042 have been used to inhibit the p110 catalytic subunit of PI3K *in vitro*, but have not been tested in the clinic. LY290042 can also inhibit other p110 isoforms and possibly other kinases. A more specific approach is to inhibit the phosphotyrosine binding domain in the p85 subunit of PI3K, thereby preventing activation of PI3K. Isoliquiritigenin (a flavonoid) for example, can inhibit the recruitment of the p85 subunit of PI3K to ErbB3, thereby inhibiting Akt in DU45 prostate cancer cells (Jung *et al*, 2006).

Blocking the binding of Akt to PI-3,4,5-P3 or PI-3,4-P2 is appealing, as it would prevent Akt recruitment to the cell membrane and subsequent activation by PDK1.

Other targets upstream of Akt include caveolin-1. An antibody to caveolin-1 has been shown to inhibit tumour growth and metastases *in vivo* (Tahir *et al*, 2001).

Targeting mTOR has been the focus of intense research over the past few years. Rapamycin analogues such as temsirolimus, AP23573 and everolimus are now in Phase II and III clinical trials for cancer (Easton and Houghton, 2004; Easton and Houghton, 2006). All have been shown to have significant growth inhibitory properties *in vitro* and *in vivo* against a broad array of cancer types (Dudkin *et al*, 2001; Neshat *et al*, 2001; Podsypanina *et al*, 2001; Yu *et al*, 2001). However, these compounds are considered cytostatic, even though significant tumour shrinkage is seen in a small minority of patients. Presumably, the cross-talk and redundancy between the Akt and Ras pathways, which intersect in a number of ways, renders some cancers relatively resistant to single drug insults (Figure 6).

Figure 6. Interaction of Akt and Ras pathways. Inhibition of mTOR can lead to feedback stimulation of phosphorylated Akt mediated by PI3K. The exact mechanism for this is not clear, but appears to be via S6K. In addition, mTOR inhibition leads to increased IRS1 expression (mechanism unknown, shown here with "?"), which in turn stimulates Akt. The details of both pathways are unclear. Activated Ras, through ERK and RSK, can interfere with mTORC1 function, leading to increased Rheb accumulation. Other examples of cross – talk and feedback inhibition have been described, adding to the complexity of Akt pathway regulation.



Feedback stimulation of PI3K / Akt induced by mTOR inhibitors is a source of concern clinically (Sun *et al*, 2005), and may be a means by which the tumour escapes control of chronically administered agents (O'Reilly *et al*, 2006). The mechanism for feedback inhibition may involve PI3K, as administration of LY294002, a PI3K inhibitor, abolishes the increase in phospho-Akt by mTOR inhibition (Sun *et al*, 2005). Perhaps the best use of PI3K/Akt pathway inhibitors, therefore, is by targeting multiple signal transduction pathways or different parts of the same pathway by combining compounds that produce synergistic growth inhibition or apoptosis. This approach has been taken by a number of authors (Boulay *et al*, 2005; Takeuchi *et al*, 2005; Tanaka *et al*, 2005). Hypothetically, not only might this approach afford better efficacy in the treatment of prostate cancer, but the need to use each agent at full dose (and therefore the risks for side effects) is potentially alleviated. There is also good evidence that the Akt pathway

plays an important part in chemoresistance, at least *in vitro*, in a variety of cancers including prostate cancer, so inhibition of this pathway is an attractive approach (Beuvink *et al*, 2005; Engelman *et al*, 2007; Kim *et al*, 2007; Takeuchi *et al*, 2005; Tanaka *et al*, 2000; Tanaka *et al*, 2005).

2.2.13 Akt and isoflavones in prostate cancer

Isoflavones are plant-derived compounds that have been proposed for use as treatment and prevention of prostate cancer (Sarkar and Li, 2002; Hussain *et al*, 2003; Kelly and Husband, 2003; Brown *et al*, 2005; Pollard and Suckow, 2006; Sarkar *et al*, 2006). Recently, the potential of isoflavones to inhibit important components of the PI3K / Akt pathway has been examined. Genistein and daidzein can induce PTEN in prostate cancer cell lines (Cao *et al*, 2006), while genistein polysaccharide inhibits prostate cancer cell lines in a dose-dependent manner, suppresses mTOR signaling and reduces AR signaling (Tepper *et al*, 2007), and slows the growth of LNCaP *in vivo* (Bemis *et al*, 2004). Genistein has also been shown to inhibit VEGF signaling via reduction of HIFα accumulation in PC3 and HUVEC cells (Guo *et al*, 2006). Interestingly, genistein has been shown to reduce phosphorylated Akt and induce autophagy, at least in ovarian cancer cells (Gossner *et al*, 2007). The dual activity of isoflavones on both the Akt and AR pathways make them attractive therapeutic agents for prostate cancer (Tepper *et al*, 2007). Degeulin, another flavonoid, has been reported to inhibit Akt as well as NFκB, and endothelial cell migration (Dell'Eva *et al*, 2007). Phenoxodiol, an analogue of genistein, also appears to inhibit Akt function via inhibition of FLICE inhibitory protein (FLIP) (Kamsteeg *et al*, 2003). Like other flavonoids, however, phenoxodiol may have effects on other signal transduction pathways (Alvero *et al*, 2006; Yu *et al*, 2006).

Isoflavones readily lend themselves to combination studies in view of their relatively low toxicity and pleiotropic effects on cancer cells, including the apparent ability to overcome chemoresistance (Sapi *et al*, 2004).

2.2.14 Perspective

Better understanding of the signal transduction machinery of cancers has already led to the introduction of specific inhibitors for clinical practice. The success of angiogenesis inhibitors as single agents in renal cancer for example (Escudier *et al*, 2007; Motzer *et al*, 2007) implies the relative "addiction" of these cancer cells to the HIF1α and VEGF pathways, which is perhaps not surprising given the key role of the Von – Hippel Lindau mutation in over 50% of sporadic renal cell cancers. Cancers that have redundant pathways, however, or complex cross-talk between pathways, may be more resistant to attempts to block single targets (Shaw and Cantley, 2006; Wang *et al*, 2007). This may be one explanation for the relative chemoresistance of prostate cancer to treatments, despite the progress that has been made in terms of greater understanding of prostate cancer biology in recent years. Combination therapy targeting of the Akt and associated pathways may therefore provide novel and logical ways to improve treatment efficacy for prostate cancer. Another issue is the potential conflict between studies in cell lines and studies of the molecular pathology of prostate cancer. Improved techniques and methods over time can seem to reduce the impact of findings from earlier (particularly *in vitro*) studies, and well-worked signal transduction hypotheses can later prove to be unimportant or unfounded in pathological specimens. Further, although knowledge regarding Akt signaling has been drawn from many sources including yeast studies, the significance of much of the pathway is yet to be confirmed

in prostate cancer. Given the limitations of both the *in vitro* and molecular pathological approaches, neither may be valid models for the spectrum of prostate cancer disease, the molecular basis of which still remains poorly understood. In order to improve this state of affairs, we would propose that more prospective biomarker and translational sub-studies as part of clinical trials of novel agents and combinations are required.

3. Aims

Our hypotheses were:

1) That phenoxodiol has preclinical activity against prostate cancer cells

2) That phenoxodiol has synergistic activity in combination with either cisplatin or carboplatin

3) That phenoxodiol inhibits phospho-Akt and mTOR

4) That phenoxodiol has a short half-life in humans

5) That weekly intravenous phenoxodiol is well tolerated

6) That oral phenoxodiol would be bioavailable in concentrations that match *in vitro* activity

7) That the combination of oral phenoxodiol and intravenous cisplatin or carboplatin has acceptable toxicity

Therefore, the aims of this thesis were to:

1) Review the literature regarding isoflavones, the Akt pathway, and their relevance to prostate cancer

2) Investigate the preclinical activity of phenoxodiol in regard to its ability to to inhibit the growth of prostate cancer cell lines:

- perform growth inhibition assays with phenoxodiol alone and in combination with other drugs, particularly cisplatin and carboplatin
- investigate potential drug combinations of phenoxodiol that could be taken into clinical trials
- investigate the potential mechanism of synergy between phenoxodiol and cisplatin, particularly in regard to the Akt pathway

3) Perform a first-in-man clinical trial of phenoxodiol given intravenously to determine pharmacokinetic characteristics of the compound

4) Perform a Phase I and pharmacokinetic clinical trial of intravenous phenoxodiol administered weekly to patients with advanced cancer

5) Perform a Phase I and biovailability study of oral phenoxodiol given in combination with either cisplatin or carboplatin to patients with advanced cancer.

4. Preclinical activity of phenoxodiol alone and in combination with cisplatin in prostate cancer cell lines

4.1 Introduction

Hormone refractory prostate cancer (HRPC) carries a poor prognosis, with an expected median survival of around 12 months in symptomatic patients. Recently, docetaxel in combination with either prednisone or estramustine has been shown to improve survival when compared to the combination of mitoxantrone and prednisone in Phase III trials (Petrylak *et al*, 2004; Tannock *et al*, 2004). In these trials, median survival in the mitoxantrone/prednisone control arm was only 16 months. Given that many men are unsuitable for chemotherapy, either because they are elderly or have comorbidities, there is still a pressing need to evaluate better tolerated and more effective compounds and combinations for the treatment of this disease.

Epidemiological studies suggest an inverse relationship between isoflavone consumption and the risk of prostate cancer (Jacobsen *et al*, 1998; Strom *et al*, 1999). A significant (70%) reduction of the risk of prostate cancer was associated with the consumption of soy milk in a cohort of over 12,000 Seventh Day Adventist men (Jacobsen *et al*, 1998). While there are, as yet, no randomized trials of isoflavones in treatment or prevention of prostate cancer, there is strong *in vitro* evidence of the activity of a variety of isoflavones on hormone sensitive and insensitive prostate cancer cell lines (Hempstock *et al*, 1998; Mitchell *et al*, 2000; Hedlund *et al*, 2003) and *in vivo*

in rats (Risbridger *et al*, 2001). Isoflavones appear to have pleiotropic effects on prostate cancer cells, including an ability to exert hormonal influences.

Phenoxodiol is a synthetic isoflav-3-ene metabolite that is a natural intermediate (dehydroequol, 7,4'-dihydroxyisoflav-3-ene) in the metabolism of daidzein to equol (Joannou *et al*, 1995). It is cytotoxic to a variety of cancer cell lines *in vitro* (Novogen Research Pty Ltd 2000; Mor *et al*, 2006) and *in vivo* in rats (Constantinou *et al*, 2003; Mor *et al*, 2006). It may be capable of re-sensitising platinum and taxane resistant ovarian cancer cells *in vitro* (Kamsteeg *et al*, 2003; Sapi *et al*, 2004; Kluger *et al*, 2007) and in humans (Rutherford *et al*, 2004) and appears to have antiangiogenic (Gamble *et al*, 2006) and anti-inflammatory properties (Widyarini *et al*, 2001). It has improved bioavailability when compared to genistein (Kelly and Husband, 2003) and low toxicity in clinical trials (Joshua *et al*, 2003; Choueiri *et al*, 2006; de Souza *et al*, 2006).

Phenoxodiol appears to create a 'pro-death' environment in cancer cells by activating the caspase cascade through up-regulation of pro-apoptotic Bax (Alvero *et al*, 2006). It inhibits cellular FLICE-inhibitory proteins (c-FLIP), thereby activating the FAS apoptotic pathway, and causes down-regulation and cleavage of X-linked inhibitor of apoptosis (XIAP) (Kamsteeg *et al*, 2003; Kluger *et al*, 2007), leading to further activation of the caspases (Straszewski-Chavez *et al*, 2004). Phenoxodiol also causes cell cycle arrest at G1 due to loss of cdk2 activity by p53 independent induction of the cdk inhibitor $p21^{WAF1/CIP1}$ (Aguero *et al*, 2005). Cell cycle interference occurs through inhibition of topoisomerase II (Constantinou & Husband, 2002). The molecular target for growth inhibition by phenoxodiol is unclear, but may involve tumour-associated NADH oxidase (tNOX) (Yagiz *et al*, 2007). Given the ability of genistein to inhibit Akt

(Li and Sarkar, 2002c; Bemis et al, 2004; Park et al, 2005; El Touny and Banerjee 2007), we hypothesized that phenoxodiol might also be able to inhibit phosphorylation of Akt and mTOR.

Our aim was to determine the *in vitro* growth-inhibitory ability of phenoxodiol against prostate cancer cells, to identify whether cisplatin could enhance these abilities, and investigate whether intracellular cisplatin uptake was altered by phenoxodiol. We also hypothesized that the degree of inhibition of Akt or mTOR could possibly explain any synergistic action noted between cisplatin or carboplatin and phenoxodiol.

4.2 Material and Methods

4.2.1 Drugs and Chemicals

Phenoxodiol was supplied by Novogen Research Pty Limited (Sydney, Australia). Cisplatin and carboplatin were purchased from Sigma Chemicals (St Louis, Mo, USA). Cell culture reagents and consumables were obtained from Sigma Chemicals (Perth, Australia) or Invitrogen (Melbourne, Australia). All other chemicals not otherwise specified were of the highest grade and purchased from local suppliers.

4.2.2 Cell culture

Growth of the human androgen-independent prostate cancer cell lines DU145 and PC3, as well as HepG2 (hepatoma) and 786-0 (renal), was maintained in an atmosphere of 5% CO_2 at 37^0C. DU145 cells were cultured in MEM Eagle media, while PC3 cells were maintained in high glucose RPMI-1640 media. All media were supplemented with

fetal calf serum (10 % v/v), 10 mM Hepes, 1.5 g/L sodium bicarbonate and penicillin-streptomycin-glutamine (1 % v/v). DU145 and PC3 cells were from the American Tissue Culture Collection (ATCC) originally and were obtained from Professor David Morris, (Department of Surgery, St George Hospital, Sydney, Australia).

4.2.3 Growth inhibition experiments

Cells were plated in 96-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at 3,000 cells per well in culture medium and incubated for 24 hours. Drug stock solutions were diluted in culture medium and added (100 μL per well) in triplicate to achieve final concentrations ranging from 0.1–10 μM phenoxodiol, 0.001–100 μM cisplatin, and 1 – 100 μM carboplatin. Drugs were investigated for their activity as single agents, and in various combinations and concentrations. After incubation for 72 h, cell viability was measured by the sulforhodamine B (SRB) assay as previously described (Skehan *et al*, 1990) . Growth inhibition was expressed as % control (media alone, no drugs) and quantitated by IC50 values. All experiments were repeated in triplicate; results are shown as mean ± standard error of the mean (± SEM). Schedule dependency of the phenoxodiol and cisplatin combination was also investigated by exposing cells simultaneously to both drugs for 72 hours or sequentially to phenoxodiol first for 2 or 24 hours followed by the addition of cisplatin for a further 48 hours, or vice versa.

4.2.4 Chou – Talalay analysis for synergy

Methods for assessing synergy were used as previously described (de Souza *et al*, 1997). Briefly, Calcusyn (version 2.0), a Windows® - based computer program

automating the multiple drug effect analysis of Chou and Talalay, based on the median - effect principle (Chou and Talalay, 1984), was used to calculate combined drug effects. The combination index (CI) equation $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$ was then used to determine synergy, additivity or antagonism. Data are expressed as CI ± SEM from multiple experiments. Combination indices of <1, =1 and >1 represent synergy, additivity or antagonism respectively. Mutually non-exclusive CIs are shown because this does not assume knowledge of the mechanism of action of the drugs in combination.

4.2.5 Whole cell platinum accumulation and DNA binding studies

DU145 cells were treated with cisplatin alone and in combination (1 and 10 μM) with phenoxodiol at 5 μM for 24 hours, harvested by trypsin, then washed twice with ice cold PBS. Samples for whole cell analysis of platinum accumulation were lysed with 500 μL of water and analysed for total protein content by the biochinchonic acid protein assay kit (Sigma). Samples for measurement of platinum DNA binding were lysed in 100 mM Tris HCl, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl with 100 μg proteinase K per mL added immediately before use. DNA was precipitated with ice cold isopropanol and the sample digested in 500 μL TE (10 mM Tris HCl pH 7.5, 0.1 mM EDTA,) and shaken overnight at 37 °C. A total DNA measure was performed by analysis at 260/280 OD. Experiments were repeated in triplicate. Samples were diluted 1:5 with 0.1 % nitric acid and analysed for platinum content by ICP-MS as described below. Platinum measurements were standardized as relevant to per mg protein and per μg DNA before comparisons between treatment groups were made.

4.2.6 In vivo experiments

Male Nu-Nu Balb/c mice (15-20 g) were obtained from the Animal Resource Centre (Perth, Australia), housed in sterile filter-topped micro-isolation cages and maintained on sterile water and a sterile isoflavone-free diet (Gordon's Speciality Stockfeeds, Australia) ad libitum. Mice were monitored until a bodyweight of 20-25g was reached. Xenografts were established by the dual subcutaneous injection of 1 x 10^6 DU145 cells in serum - free MEM and Matrigel (1:1) on each hind flank under inhalation anaesthesia (induced with 5% isofluorane / 95% oxygen and maintained with 1-2% isofluorane). The xenografts were left to grow until approximately 20 mm^2 in size before mice were randomised into four treatment groups (at least n=6 per group): vehicle only, cisplatin (1 $mg.kg^{-1}$), phenoxodiol (5 $mg.kg^{-1}$), and cisplatin plus phenoxodiol (0.5 $mg.kg^{-1}$, 2.5 $mg.kg^{-1}$ respectively). Cisplatin was prepared fresh each day, dissolved in 0.9% saline (0.1 mg/mL) whilst stock phenoxodiol was prepared in 20% hydroxypropyl b-cyclodextrin (HPBCD) 5 mg/mL) solubilised at 120°C. Phenoxodiol was diluted daily in 0.9% saline. Drugs were given via the intraperitoneal route daily on days 1-5 and 8-12. Doses of cisplatin and phenoxodiol were chosen to give plasma concentrations that would approximate the concentrations used in *in vitro* experiments from literature (Litterst and Magin, 1988) and bioavailability data supplied by Novogen. Xenograft diameters were measured daily (except on weekends) with vernier callipers and volumes calculated by the formula Volume = Length x $Width^2/2$. Toxicity of the drugs was determined by inspection of mice and bodyweight analysis.

4.2.7 Derivation of drug doses for in vivo experiments

Doses were halved for both drugs in the cisplatin / phenoxodiol group in order to allow the demonstration of synergy, if present, as previously described (de Souza *et al*, 1997). Thus, if the combination proved to be as, or more effective than either agent alone at double the dose of each agent in the combination, we could conclude that the combination showed additive, or synergistic activity, respectively. The evidence for this decision is based on the Chou – Talalay equation:

$CI = (Dcomb)1 / (Dalone)1 + (Dcomb)2 / (Dalone)2 + \alpha(Dcomb)1\ (Dcomb)2 / (Dalone)1\ (Dalone)2$

where (Dalone)1 is the dose of drug 1 alone required for a given effect (fa),

(Dcomb)1 is the dose of drug 1 in the combination required for a given effect (fa),

(Dalone)2 is the dose of drug 2 alone required for a given effect (fa),

(Dcomb)2 is the dose of drug 2 in the combination required for a given effect (fa),

CI = combination index, a measure of the degree of synergy,

and $\alpha = 0$ if the effects of the two drugs are mutually exclusive.

Let (Dalone)1 = some concentration p,

(Dalone)2 = some concentration q,

(Dcomb)1 = 0.5p,

(Dcomb)2 = 0.5q,

Then $CI = 0.5p / p + 0.5q / 5 + \alpha(0.5p)(0.5q) / pq$

$= 0.5pq / pq + 0.5pq / pq + 0.25pq / pq$

If the term $\alpha = 0$, which is likely given the different mechanisms of action of cisplatin and phenoxodiol, then CI = 1, the definition of additivity. The advantage of this method is that it is dose - independent, and does not rely on the arbitrary definition of time to recurrence / regrowth of tumours *in vivo*, for which there is no mathematical definition

of synergy. As a result, only four treatment arms are required (vehicle control, Drug A single agent control, Drug B single agent control, 0.5xDrug A + 0.5xDrug B in combination) for *in vivo* studies to test synergy.

4.2.8 Whole tissue platinum analysis

Total platinum in the plasma, kidney and tumours was analysed by inductively coupled plasma mass spectrometry (ICP-MS) as previously described (Screnci *et al*, 1998). Tissues were prepared by nitric acid digestion overnight and for 2 hours at 90°C in a closed container. Sample volume was adjusted to 10 mL with MilliQ and the platinum content analysed. Plasma (50 μL) was diluted in 1.2 mL lysis buffer before analysis by ICP-MS. Calibration was against a platinum standard curve from 0.1-10000 $ng.mL^{-1}$. Individual weights of tumours and kidneys were also recorded.

4.2.9 Pharmacokinetics

Upon completion of the study, mice were killed at specified time points after cisplatin dosing by cervical dislocation for pharmacokinetic analysis. Cardiac puncture was performed immediately and blood was centrifuged at 2000g for 5 mins and the plasma supernatant stored at -80^0C for cisplatin concentration measurements. At the same specified time points, the xenograft, liver and kidney tissue were also removed, washed in PBS (pH 7.6) and stored at -80^0C. The data were analysed using 2-way repeated measures ANOVA performed using STATVIEW version V (Abacus Concepts, Inc., Berkley, CA., USA). Fishers Post-hoc tests were performed on all significant data. Pharmacokinetic parameters were determined using non-compartmental analysis. Total

platinum adjusted for tissue weight was plotted against time of sample. Cmax (maximum platinum concentration) and Tmax (time of maximum platinum concentration) were measured and the area under the concentration versus time curve (AUC) was calculated by the trapezoid rule (GraphPad Prism v3.00 for Windows, San Diego, CA, USA).

4.2.10 Western blot experiments

PC3 and DU145 cells were grown in medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL of streptomycin at a constant temperature in a humidified atmosphere (37^0C, 5% CO_2) as previously described. Phenoxodiol, cisplatin and carboplatin were prepared fresh for each experiment. Anti-β actin and horseradish peroxidase (HRP)-linked anti-goat antibody were purchased from Santa Cruz Biotechnology (CA, USA), whereas anti-phospho-Akt, anti-phospho-ERK1/2, anti-S6, anti-XIAP, and HRP-linked anti-Rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology (MA, USA). Cells were allowed to reach 70% confluence, followed by drug treatment in a serum-free medium for another 24 hours. Cells were then washed with cold PBS buffer and lysed in buffer containing 50m M Tris-HCI (ph7.5), 150 mM NaCI (150 mM), EGTA (1 mM), EDTA (2 mM), 2.5%(V/V) glycerol, NaF (10 mM), 0.2%(V/V) Triton X-100, 0.3% (V/V) NP40, aprotinin (5 μg/mL), Leupeptin (10 μg/mL), pepstatin (10 μg/mL), activated NaVO3 (2 mM) and PMSF (1 mM). Lysates were sonicated in an ice water bath for 20 seconds; the supernatant containing protein was separated from the insoluble material by centrifugation at 10,000 rpm for 10 minutes at 40^0C. The total protein in each sample was then quantified using the Bicinchoninic Acid Kit (Sigma-Aldrich, Sydney) by

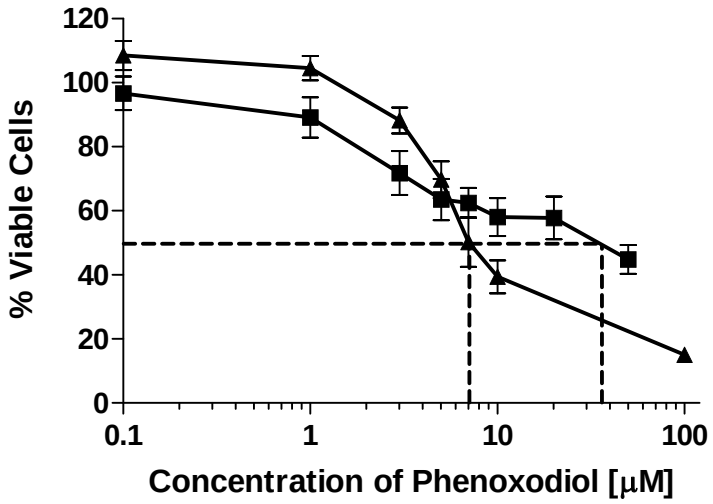
following the manufacurer's protocol. Samples containing equal amounts of protein were mixed with loading buffer and boiled for 5 minutes. Proteins were then separated by SDS-PAGE, and the proteins on the gels were transferred to PVDF-Membrane (Amersham Biosciences, Sydney) and incubated with different antibodies. After incubation with HRP-conjugated secondary antibodies, the primary antibody binding was visualized with Western LightningTM Chemiluminescence (PerkinElmer, Melbourne). A semi-quantitative estimate of density was obtained by scanning the blot intensity and comparing it with control (GEL DOC 2000, Bio-Rad, Sydney).

4.3 Results

4.3.1 Growth inhibition by phenoxodiol

Phenoxodiol was active against DU145 and PC3 cells, with IC50s of 8 ± 1 μM and 38 ± 9 μM, respectively (Figure 7). Cell growth inhibition of both cell lines was dependent on both duration of exposure and concentration of phenoxodiol.

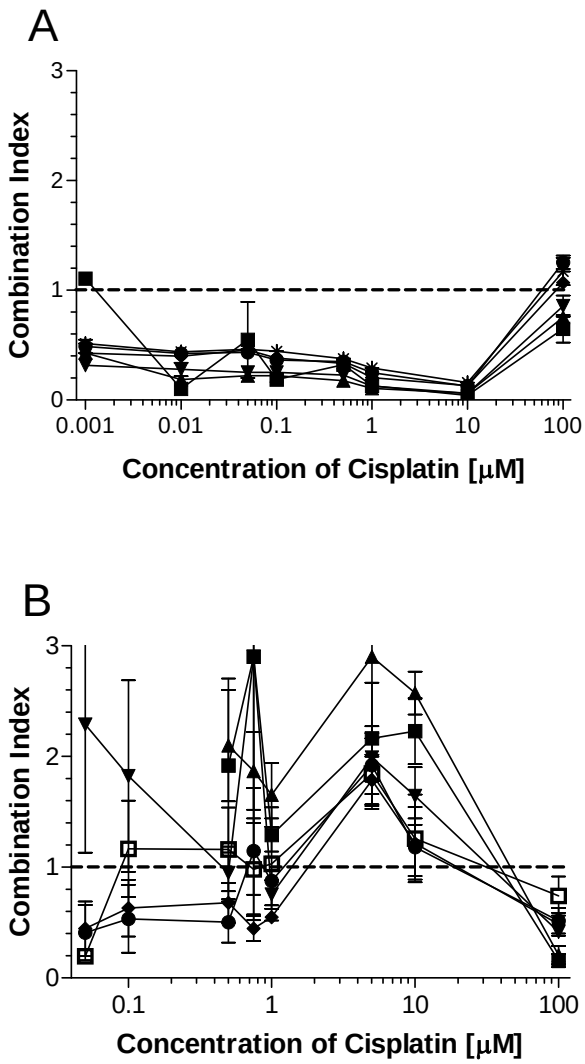
Figure 7. Cell viability curve following 72 hour exposure to 0.1 – 100 μM Phenoxodiol in ■ PC3 and ▲ DU145 cells. Dashed line marks IC50.

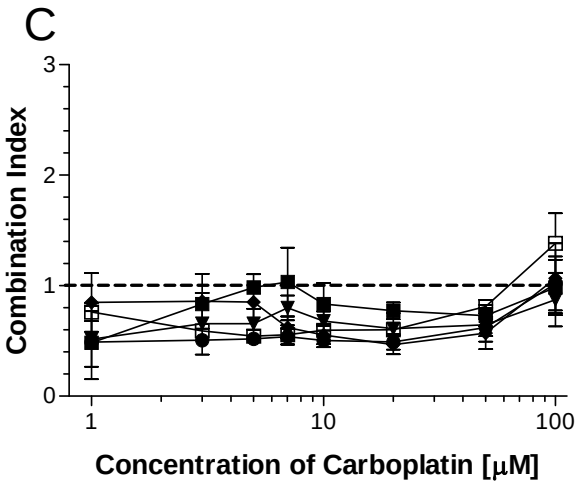


4.3.2 Synergy analysis by the Chou – Talalay method

Cisplatin and phenoxodiol generally showed synergistic activity against DU145 cells (Figure 8A) with combination indices less than 1.0. Although there were some combination ratios that suggested antagonism, these occurred at the extremes of the dose-response curves and are therefore subject to error and the limitations of the experimental technique. Phenoxodiol and cisplatin combined in PC3 cells to produce a mixed (synergistic, additive, antagonistic) effect that overall suggested addtivity at best (Figure 8B). Synergy was also noted with carboplatin at all concentrations of phenoxodiol 0.1 μM – 20 μM against DU145 cells (Figure 8C). Mostly synergistic interactions were found in other (Hepatoma HepG2 and Renal 786-0) cell lines (data not shown).

Figure 8. Combination Indices (CI, mutually non-exclusive) for cisplatin and phenoxodiol in (A) DU145 cells and (B) PC3 prostate cancer cells, and (C) carboplatin and phenoxodiol in DU145 cells. CI<1 denotes synergism, CI=1 additivity, and CI>1 antagonism. Combination indices > 3 are not shown. Phenoxodiol concentrations in (A), (B), and (C) are as follows: ■ 0.1 μM , ▲ 1 μM, ▼ 3 μM, ◆ 5 μM, ● 7 μM, ∗ 10 μM, □ 20 μM. All cells were exposed to simultaneous drug combinations for 72 hours. Horizontal dashed line highlights CI = 1.



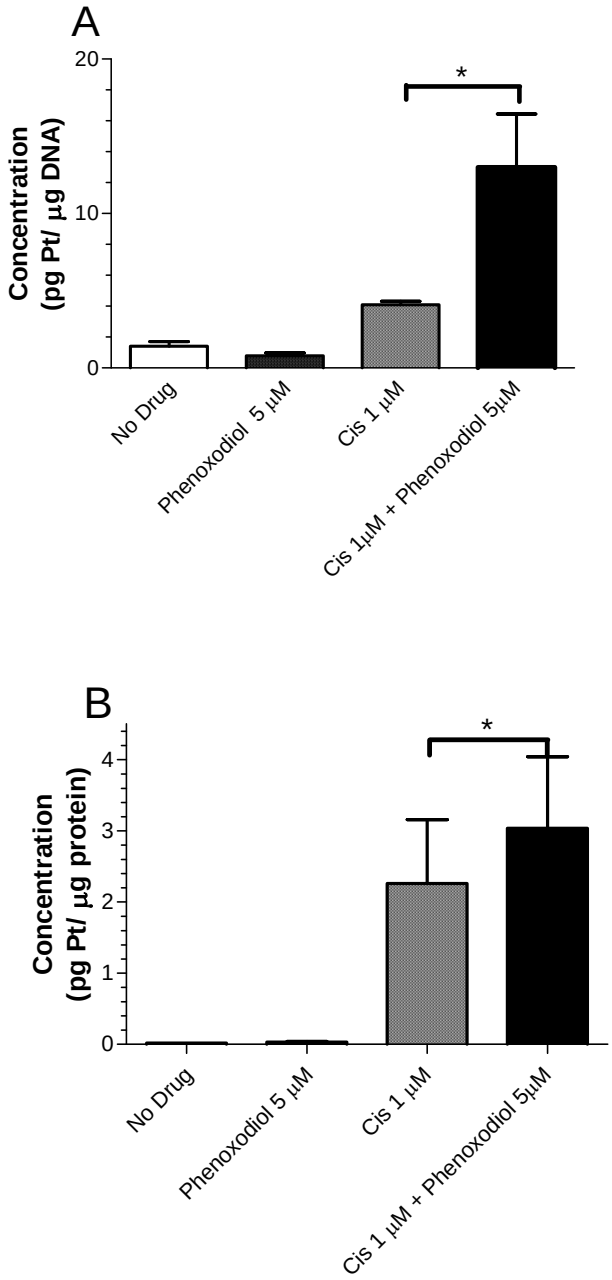


Sequence of administration of the drugs was important. A 24 hour delay between the administration of cisplatin followed by phenoxodiol significantly decreased the synergism measured ($p < 0.05$), resulting in an antagonistic combination (average CI 1.8 ± 0.5 vs. 0.8 ± 0.15). A 2 hour delay did not affect synergism between phenoxodiol and cisplatin nor did administering phenoxodiol 24 hours prior to cisplatin (Data not shown).

4.3.3 Cisplatinum whole cell accumulation and DNA binding

A greater than 300 % increase ($p < 0.05$) in platinum binding to DNA was measured in DU145 cells following treatment with 1 μM cisplatin and 5 μM phenoxodiol compared to 1 μM cisplatin alone (Figure 9A). A 35 % increase ($p < 0.05$) in the whole cell accumulation of platinum was measured in DU145 cells at the same concentrations (Figure 9B). No significant difference in whole cell accumulation of platinum or DNA platinum binding was seen with 10 μM cisplatin after the addition of phenoxodiol.

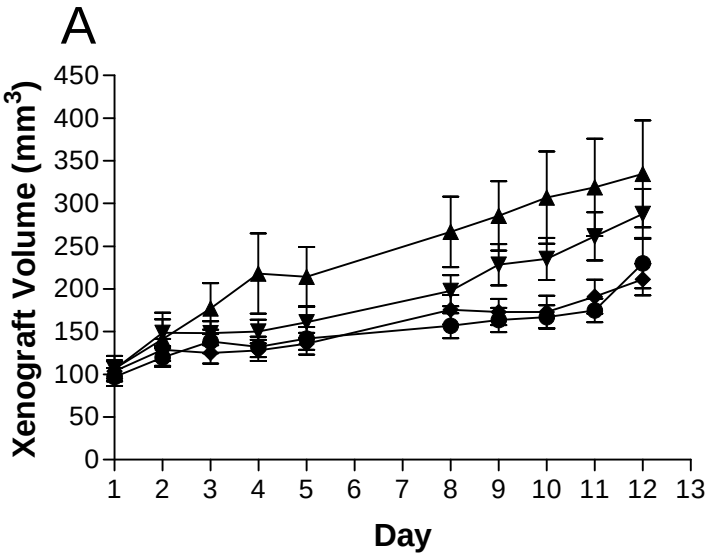
Figure 9. Comparison of (A) cisplatin DNA adducts, measured as the concentration of platinum bound to the DNA (pg platinum.μg DNA^{-1}) and (B) whole cell platinum accumulation, recorded as the measure of platinum present in the whole cell sample (pg platinum.μg $protein^{-1}$) following exposure to no drug (control), phenoxodiol (5 μM), cisplatin (Cis) (1 μM) or cisplatin (Cis) (1 μM) in combination with phenoxodiol (5 μM). Data shown is average level ± SEM, * $p < 0.05$.

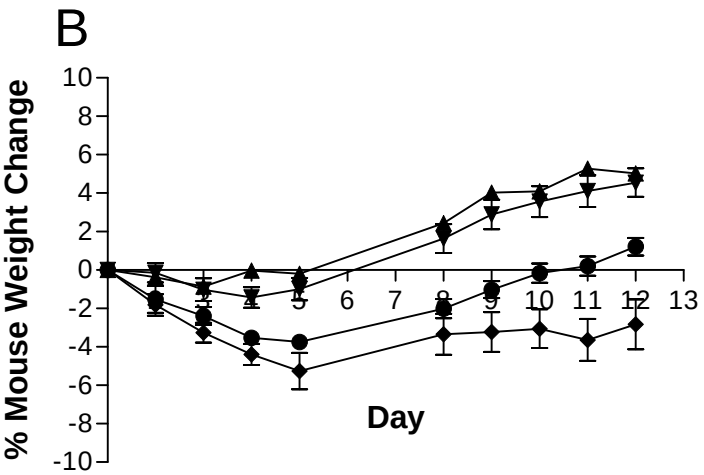


4.3.4 Xenograft studies

Both cisplatin (1 $mg.kg^{-1}$) alone and the combination of phenoxodiol (2.5 $mg.kg^{-1}$) and cisplatin (0.5 $mg.kg^{-1}$) inhibited ($p<0.01$) the rate of absolute xenograft growth compared to vehicle control (Figure 10A). There was no statistically significant difference between cisplatin alone and the combination arms. Toxicity of cisplatin was monitored by recording weight loss. A statistically significant average 3.4 ± 0.3 % loss of weight was recorded for mice treated with cisplatin alone ($p < 0.01$), compared to other arms (Figure 10B). Mice in the phenoxodiol and cisplatin group had less weight loss ($p < 0.01$) compared to the cisplatin alone group. No mice were removed from treatment groups because of excessive weight loss (>10% bodyweight). There was no difference between mice in the vehicle control and phenoxodiol groups, where weight gain was observed in the mice.

Figure 10. DU 145 xenograft tumor volumes (mm^3) (A) and nude mice % bodyweight changes (B) recorded over 12 day treatment period for the four treatment groups: ▲ control, ▼ phenoxodiol alone (5 $mg.kg^{-1}$), ◆ cisplatin alone (1 $mg.kg^{-1}$), and ● the combination of cisplatin (0.5 $mg.kg^{-1}$) and phenoxodiol (2.5 $mg.kg^{-1}$). ** denotes a statistically significant difference ($p<0.01$) for the cisplatin arm and the combination arm compared to control; only statistically significant differences are shown (See Results).



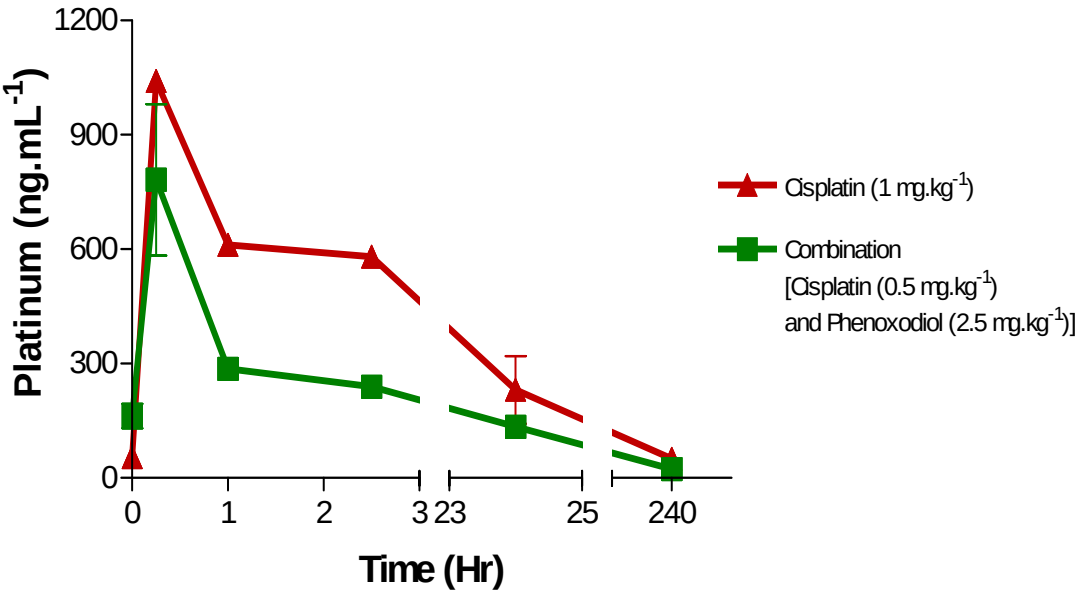


4.3.5 Cisplatin pharmacokinetics

Phenoxodiol did not appear to affect cisplatin plasma pharmacokinetics. Tmax was rapid and occurred at 25 minutes for both cisplatin alone and the cisplatin-phenoxodiol combination. The Cmax for the combination of cisplatin (0.5 $mg.mL^{-1}$) with phenoxodiol (2.5 $mg.mL^{-1}$) was 782 ± 115 $ng.mL^{-1}$ (Figure 11). This was about 75% of the Cmax of cisplatin alone at twice the concentration (1 $mg.mL^{-1}$) (1041 ± 21 $ng.mL^{-1}$).

The plasma AUC of cisplatin dosed at 0.5 mg.mL^{-1} was approximately half that of the 1 mg.mL^{-1} group (21950 ng.mL^{-1}*hr at 0.5 mg.mL^{-1} in combination with phenoxodiol versus 40740 ng.mL^{-1}*hr at 1 mg.mL^{-1} alone).

Figure 11. Nude Mouse plasma cisplatin concentration-time curves for ◆ cisplatin alone (1 mg.kg^{-1}) and ● the combination of cisplatin (0.5 mg.kg^{-1}) and phenoxodiol (2.5 mg.kg^{-1}). Halving the cisplatin dose results in approximately half the area under the concentration x time curve (AUC).



In kidney and tumor tissue there was also no apparent difference in circulating levels of free platinum with measured area under the concentration x time curve (AUC) for cisplatin at 0.5 mg.mL^{-1} in combination with phenoxodiol approximately half the AUC of cisplatin 1 mg.mL^{-1} alone (2914 concentration*time and 4294 concentration*time for the kidney and 812.4 concentration*time and 1861 concentration*time for the tumour respectively, data not shown).

4.3.6 Western blot experiments

Figure 12 shows that phenoxodiol alone does not reduce p-Akt at 6 or 24hrs treatment in DU145 cells, but cisplatin inhibits p-Akt at 24hrs. Phospho-Akt was markedly reduced by the combination of cisplatin and phenoxodiol. Phospho-ERK 1/2 expression was unaffected by both drugs.

Figure 12. Inhibition of p-Akt. DU145 cells were treated with 50 μM phenoxodiol (NV06) alone for 6 or 24hrs and 50 μM cisplatin alone for 6 or 24hrs, or the combination for the same durations. The first lane represents untreated controls. Beta actin was used as the loading control.

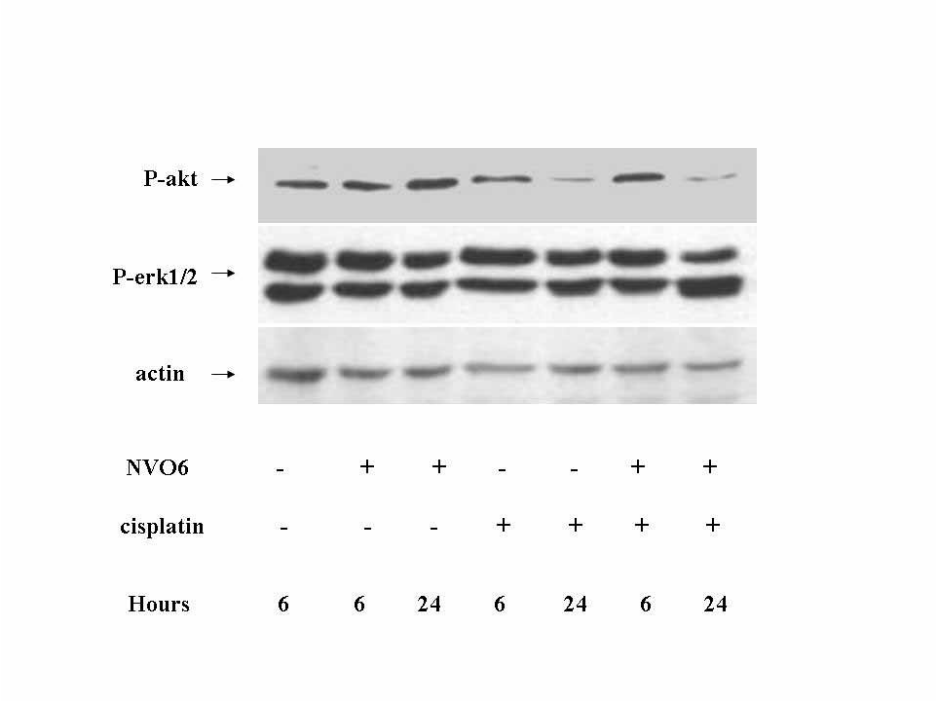


Figure 13. Inhibition of c-FLIP. DU145 cells were treated with phenoxodiol (NV06) alone, or cisplatin alone, or carboplatin alone, at the concentrations specified. When platinum and phenoxodiol were combined, only 20 μM concentrations of both drugs were used. The first lane represents untreated controls (Ctrl), with a density of 1. Beta actin was used as the loading control.

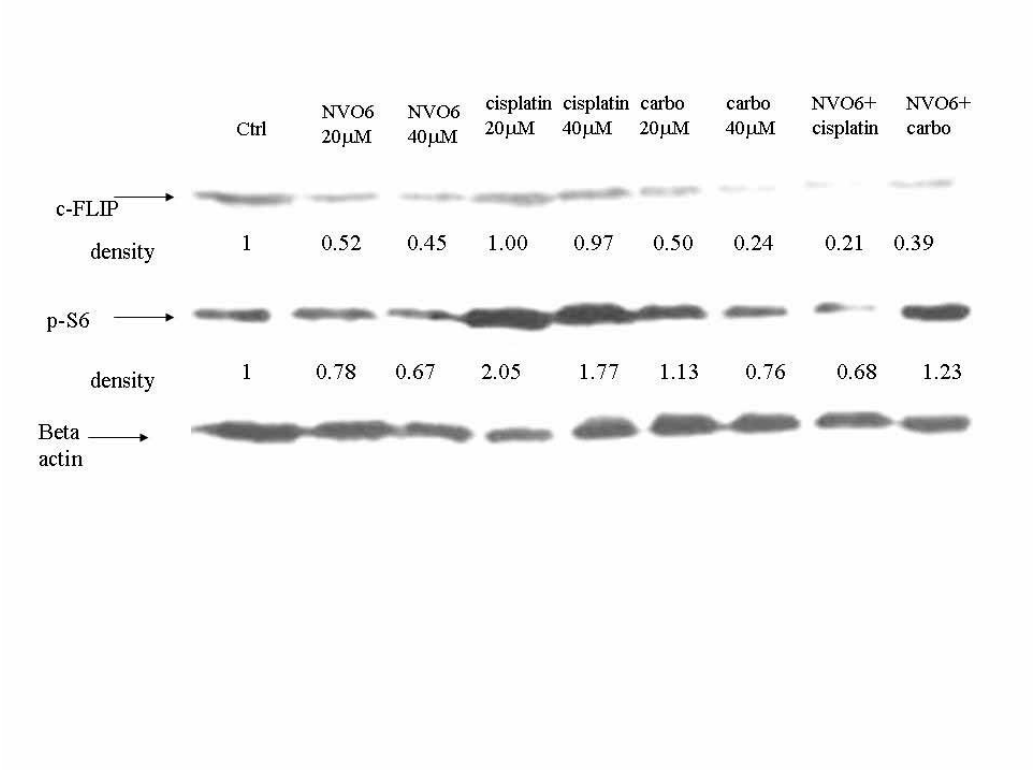
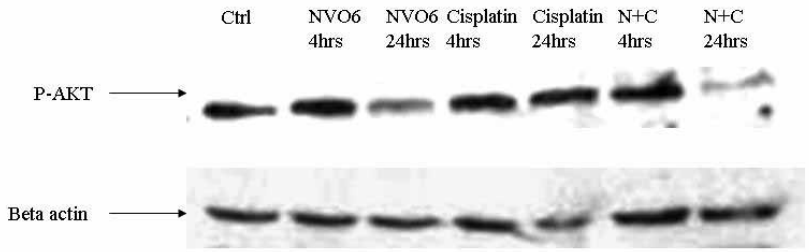


Figure 13 shows that c-FLIP is inhibited by both phenoxodiol and carboplatin in DU145 cells, by approximately 48-55% and 50-76%, respectively, depending on the concentrations used. Cisplatin does not appear to affect c-FLIP expression. Phospho – S6, used as a surrogate for mTOR inhibition, was reduced by phenoxodiol at both 20 μM and 40 μM, whereas carboplatin only reduced p-S6 at 40 μM. The combination of phenoxodiol and either cisplatin or carboplatin markedly reduced C-FLIP expression, but had relatively little effect on p-S6, except for the combination of cisplatin and phenoxodiol, which appeared to reduce p-S6 expression significantly.

Figure 14. Inhibition of pAkt. PC3 cells were treated with 50 μM phenoxodiol (NV06) alone or 50 μM cisplatin (C) alone, or the combination, for the durations specified. The first lane represents untreated controls. Beta actin was used as the loading control.



Similar findings were noted for PC3 cells (Figure 14), in that p-Akt was reduced by the combination of phenoxodiol and cisplatin, though the changes were less marked than for DU145 cells. Phospho-ERK 1/2 expression was unaffected by phenoxodiol or cisplatin in PC3 cells (data not shown).

4.4 Discussion

Phenoxodiol has promising *in vitro* activity against the prostate cancer cell lines DU145 and PC3, with DU145 being more sensitive than PC3. Complete inhibition of DU145 was achieved whereas only 60% inhibition of PC3 could be achieved at the maximum possible solubility of phenoxodiol. Differences in sensitivity may be due to phenotypic or genetic differences between the cell lines but may also be dependent on the ability of

phenoxodiol to enhance apoptosis induction. For example, PC3 cells express more Bcl-2 than DU145 cells (Skjoth and Issinger, 2006), and this may explain the relative resistance to treatment.

Our data show that the combination of phenoxodiol / cisplatin is highly synergistic in inhibiting growth of DU145 cells *in vitro* (Figure 8A). Chou-Talalay analysis of the combination shows a degree of synergy consistent with that noted when other isoflavones are combined with cisplatin (Bible and Kaufmann, 1997; Khoshyomn *et al*, 2000), suggesting that this may be a class effect. Phenoxodiol was also synergistic with carboplatin, perhaps not surprisingly, given its similar mechanism of action to cisplatin. In our hands, synergism between phenoxodiol and cisplatin is schedule-dependent with the optimal schedule appearing to be the co-administration of phenoxodiol and cisplatin, or at least phenoxodiol prior to cisplatin. This is consistent with the finding that cisplatin reduces cyclin D1 (Skjoth and Issinger, 2006), thereby inhibiting cell cycle progression and reducing the number of actively dividing cells available for phenoxodiol-induced apoptosis.

We hypothesize that phenoxodiol may interact with cisplatin in a number of ways to produce synergy in DU145 cells. DU145 cells overexpress c-FLIP, which may in part contribute to their chemoresistance (Hyer *et al*, 2002), and inhibition of c-FLIP by phenoxodiol has been shown in ovarian cancer cells (Kamsteeg *et al*, 2003). Here, we show that c-FLIP is inhibited by phenoxodiol and carboplatin (Figure 13) in DU145 cells, thereby reducing inherent chemoresistance, and this may be one explanation for the synergy observed in this cell line. On the other hand, we have also shown that one of the downstream targets of mTOR, p-S6, is reduced by phenoxodiol, suggesting that

phenoxodiol may also act as an mTOR inhibitor. Further, the combination of phenoxodiol and either cisplatin (in PC3 cells) or carboplatin (in DU145 cells) can inhibit phosphorylation of Akt (Figures 12 and 14). Since Akt and mTOR are key components of cancer cell signaling pathways that are important for cell growth and survival, it is possible that the combination may affect upstream and downstream targets in this pathway, ultimately causing apoptosis.

Other potential mechanisms for the synergy observed between phenoxodiol and cisplatin may exist. For instance, phenoxodiol also induces Bax transcription (Hutson *et al*, 2003), and could therefore potentiate the sensitivity of DU145 to cisplatin (Skjoth and Issinger, 2006) since transfection of Bax in DU145 cells (naturally deficient in Bax) has been shown to significantly enhance apoptosis to a variety of chemotherapy agents including cisplatin (Honda *et al*, 2002). Modulation of sphingosine-1-phosphate (S-1-P) by phenoxodiol (Choueiri *et al*, 2006b) may also directly alter cisplatin sensitivity. Finally, p38 induction and an intact PTEN/Akt pathway has been suggested as the mechanism for increased apoptosis in DU145 cells, relative to PC3, induced by cisplatin (Skjoth and Issinger, 2006).

The *in vivo* data are consistent with, though not strongly supportive of, our conclusions regarding the combination of phenoxodiol and cisplatin. Given that mice were treated with combination cisplatin / phenoxodiol at half-doses in the single agent control arms, we can conclude that the combination is additive if the tumour growth curves are matched, provided that they were better than the negative control (vehicle only) mice, as discussed in the methods. Indeed, though the combination was better than single agent phenoxodiol, it was no better than cisplatin alone, suggesting additivity in this xenograft

experiment. The findings in our nude mice studies also support the notion that reducing the dose of both drugs and relying on their synergistic activity can not only maintain growth inhibition, but reduce toxicity, as shown in the bodyweight curves. Further, this occurred at plasma concentrations approximately 15 times less than found in human studies (van Hennik *et al*, 1987).

Though there was no apparent pharmacokinetic interaction between phenoxodiol and cisplatin in our *in vivo* experiment, we hypothesized that a pharmacodynamic interaction could not be ruled out. Data from DNA binding assays seem to support this hypothesis. Phenoxodiol is synergistic with cisplatin in cisplatin sensitive cell lines but also re-sensitizes cisplatin- and carboplatin- resistant cell lines and tumors to the platinum agents (Mor *et al*, 2006). This also occurs with the isoflavone genistein as well as phenoxodiol's parent compound, daidzein (Gercel-Taylor *et al*, 2004). The mechanism of synergy between genistein and cisplatin is thought to be due to increased accumulation of cisplatin within tumour cells since cisplatin accumulation of up to 83 % occurs in sensitive ovarian cancer lines but only 43 % in resistant lines (Marveti and Andrews, 1996). Although cisplatin is not commonly used for the treatment of prostate cancer, we had previously determined that the combination of docetaxel and phenoxodiol was only additive (unpublished), and we wished to pursue the potentially synergistic combination. Further, platinums may be of renewed interest for this disease (Oh *et al*, 2007) in view of the development of satraplatin (Kelland, 2000; Petrylak, 2007; Wosikowski *et al*, 2007).

In conclusion, the combination of cisplatin or carboplatin with phenoxodiol has synergistic activity in DU145 prostate cancer cells, HepG2 hepatoma cells, and overall

probable additivity in 786-0 renal cancer cells and PC3 cells. The synergy seen between cisplatin and phenoxodiol in DU145 cells may possibly be explained by enhanced cisplatin accumulation by phenoxodiol *in vivo*. However, other mechanisms, such as our novel finding of inhibition of p-Akt and mTOR signalling by the phenoxodiol / cisplatin combination, may also play an important part. Given our promising preclinical findings, we initiated a Phase I study of the combination of either cisplatin or carboplatin together with phenoxodiol.

5. Pharmacokinetics of phenoxodiol following intravenous administration to patients with advanced cancer

5.1 Introduction

Phenoxodiol has been demonstrated to have anti-cancer activity in *in vitro* and animal studies (Constantinou and Husband, 2002; Constantinou *et al*, 2003; Kamsteeg *et al*, 2003). Phenoxodiol is a naturally occurring metabolite of genistein that is normally present in human plasma at very low concentrations through diet (Kamsteeg *et al*, 2003). The precise mechanism of action of phenoxodiol is not known, though it appears to have pleiotropic actions such as inhibition of tyrosine kinases, inhibition of topoisomerase II in a dose-dependent manner and inhibition of the X-linked inhibitor of apoptosis (XIAP) (Constantinou and Husband, 2002; Sapi *et al*, 2004; Alvero *et al*, 2006; Kluger *et al*, 2007).

The present study investigated the pharmacokinetics of phenoxodiol given as an intravenous bolus or a continuous intravenous infusion in a first-in-human study, to patients with advanced, incurable cancer.

5.2 Methods

A single center, open label study consisting of two parts was performed. Part 1 (bolus dose) involved the administration of a single intravenous injection of phenoxodiol over a five-minute period at a fixed dose of 5 mg/kg. Part 2 (continuous infusion) involved

an infusion of phenoxodiol administered to the same patients at a rate of 2 mg/kg per hour for a duration of up to ten times the average half-life determined in Part 1 of the study. The administration of phenoxodiol as a continuous infusion during Part 2 did not occur until analysis of the pharmacokinetic data from the bolus intravenous study during Part 1 had been performed. Doses of phenoxodiol were based on preclinical data that had shown no discernible toxicity, including histopathological studies of various tissues in mice, for bolus doses of up to 50 mg/kg.

5.2.1 Subjects

Six subjects with metastatic cancer were included in the study. The inclusion criteria were: male or female volunteers with metastatic disease from any solid tumour, age 18 to 70 years, normal hematological parameters and the provision of signed informed consent. Exclusion criteria were: diagnosis of leukemia or lymphoma, allergy to soy products, vegetarian diet or use of soy product more than three times a week, the use of sex steroids in the previous two months, antibiotic therapy within one month prior to the study period or at any time during the study, smoking of greater than 10 cigarettes/day, the presence of an active infection or other co-morbid disease that in the opinion of the investigator would have precluded the patient from participating in the study, a prognosis of at least three months survival, and the known presence of central nervous system metastases. The study was approved by the Ethics Committee of St George Hospital, Kogarah, NSW, Australia and patients had written, fully informed consent.

5.2.2 Study protocol

Prior to commencement of the drug administration studies a medical history, full physical examination and screening investigations for hematology and biochemistry were performed. The volunteers were then assessed for compliance with the inclusion and exclusion criteria.

5.2.2.1 Part 1

All patients were instructed to maintain a diet low in isoflavones for one week prior to and for the duration of the study and to arrive at the hospital on the treatment day at approximately 0800 hours having fasted and abstained from caffeine and alcohol for 24 hours. Blood samples were collected from an indwelling catheter placed in an antecubital vein. An initial sample of 40 ml was taken immediately prior to study medication administration. Fifteen ml of blood was collected 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0 and 12.0 hours after the administration of phenoxodiol. A standard, light, low fat meal was provided 4 hours after the administration of study medication. After 12 hours, the indwelling intravenous catheter was removed and the patient advised not to consume alcohol over the ensuing 24 hour period.

5.2.2.2 Part 2

The subjects were instructed to arrive at the hospital on the study treatment day at approximately 0800 hours having fasted and abstained from caffeine and alcohol for 24 hours. An indwelling intravenous catheter was inserted into an antecubital vein of one arm for the infusion of phenoxodiol and a second catheter was placed in the contralateral antecubital vein for the collection of blood samples. Fifteen ml of blood was collected at baseline and at 10 and 20 minutes following the start of infusion of phenoxodiol, then at 20-minute intervals up to five hours after the commencement of the infusion. A standard, light, low fat meal was provided 4 hours after the administration of study medication. Both indwelling catheters were removed six hours

after commencement of the infusion and the patient was then allowed to leave the hospital following a period of observation.

5.2.3 Safety assessments

5.2.3.1 Laboratory Safety Testing

The following tests were performed at baseline, 24h, and 48 h after the administration of phenoxodiol during Part 1 of the study, and at baseline, 6 h, 5-7 days and 12-14 days after the start of infusion of phenoxodiol during Part 2 of the study: hemoglobin, red cell count, white cell count including differential, platelets, hematocrit, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALKP) and urea and electrolytes.

5.2.3.2 Vital Signs and Other Safety Measurements

The patient's weight, height, blood pressure and heart rate were measured at baseline in both Part 1 and Part 2 of the study. In addition, blood pressure and heart rate was monitored when each plasma sample was collected following study medication administration.

5.2.4 Study medication administration

Phenoxodiol was manufactured by Novogen Laboratories, 140 Wicks Road, North Ryde NSW 2119, Australia, and was prepared as an intravenous injection of 100 mg in a 10 mL vial. Samples of the batches of vials used were tested for sterility and for the presence of pyrogens. In Part 1 of the study phenoxodiol was administered as a single bolus intravenous injection at a dose of 5 mg/kg diluted to a total volume of 50 mL in normal saline. The solution was infused over a five minute period using an infusion

pump. In Part 2 of the study phenoxodiol was infused at a rate of 2 mg/kg per hour. The solution was diluted to 500 mL in normal saline and infused using a pump at a constant rate of 100 mL/h.

5.2.5 Assay of phenoxodiol levels

Total phenoxodiol levels were measured in a 300 µL aliquot of plasma or urine which was mixed with 15 µL glucuronidase and incubated for 24 h at 24^{o}C prior to extraction. Free phenoxodiol levels were measured in a 300 µL aliquot of plasma or urine which was extracted without prior incubation with glucuronidase. After adding 10 µL of 2, 4, 4'– trihydroxydeoxy-benzoin (0.22 mg/mL) as an internal standard, the sample was extracted using 500 µL ethylacetate: hexane (6:4) solution. The sample–solvent mixture was centrifuged at 3000 rpm for 15 min and supernatant was collected and dried under vacuum. The extract was reconstituted in 100 µL 50% isopropanol in water. Five microlitres of the extracted sample was injected into the HPLC system. Standards of phenoxodiol were prepared in blank plasma or urine.

HPLC separation was carried out using an Alltech Alltima C18, 5 µm, 250 mm X 2.1mm column in a 40^{o}C oven. The gradient elution was performed at a flow rate of 0.2 mL/min from mobile phase A (25% acetonitrile, 74.95% water, 0.05% trifluoroacetic acid) to mobile phase B (99.95% acetonitrile, 0.05% trifluoroacetic acid). Phenoxodiol and the internal standard were detected by ultraviolet diode array detector at wavelength 335nm.

The calibration curve for the assay was linear in the range of 0.25 μg/mL to 10 μg/mL with a correlation coefficient (R^2)>0.994. The lower limit of quantitation (LOQ) for phenoxodiol was 0.25 μg/mL. Samples with too high a concentration were diluted with the same blank matrix before being analysed.

5.2.6 Pharmacokinetic analysis

The concentration at steady state (C_{ss}) and the time to reach steady state were taken as the actual values when the measured plasma phenoxodiol concentration appeared to have reached a maximum. The terminal elimination rate constant K_{el} was estimated using linear regression analysis using the final plasma sampling times over which the log of the plasma concentration versus time curve appeared to be straight. At least four plasma concentration time points were used for each estimate of concentration. The elimination half-life (T½) was to be calculated as 0.693/ K_{el}. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoid rule with the terminal phase (last measured plasma concentration time point to infinity) calculated as the final plasma concentration divided by K_{el}. The apparent volume of distribution (Vd) following acute administration in Part 1 of the study was calculated by extrapolating the log plasma concentration versus time line back to zero time to estimate the theoretical plasma concentration at zero time (C_0) and dividing C_0 by the dose. Clearance following acute administration was calculated as Vd multiplied by K_{el}.

Accumulation half-lives during continuous intravenous infusions of phenoxodiol were calculated as the time to achieve steady state plasma concentrations divided by 5.

Clearance (Cl) was calculated as the infusion rate of phenoxodiol divided by the plasma concentration at steady state (C_{ss}).

The pharmacokinetic analysis was performed with the assistance of the Excel software plug-in program pkf (Joel Usansky, PhD, Atul Desai, MS, and Diane Tang-Liu, PhD, Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA 92606, USA). The results are expressed as the mean values for the subject group along with standard deviations and ranges.

5.3 Results

5.3.1 Patients

Six patients entered and completed Part 1 of the study. Six weeks or more after the first phase of the study, five of these patients entered and completed Part 2 of the study. One patient completed Part 1 of the study but it was decided on the advice of the patient's oncologist not to include her in Part 2. The patients received the second administration of phenoxodiol in Part 2 of the study an average of 56 ± 21 days (range 41-98 days) following initial administration of the drug in Part 1 of the study. There were three males and three females enrolled in the study. The mean age of the patients was 61.2 ± 9.4 years, their mean weight was 75.3 ± 8.48 kg and their mean height was 169.2 ±10.0 cm. One patient smoked (5 cigarettes per day), three patients consumed between 10 and 30 g of alcohol per day and three patients did not regularly consume alcohol.

The three female patients had metastatic breast cancer and had a diagnosis of the primary malignancy 15, 7 and one year prior to the study. The three males had metastatic prostate cancer and two of these patients had a diagnosis of the primary malignancy 6 years prior to the study. The date of diagnosis of the primary malignancy for the remaining male was uncertain. The patients predominantly had bone metastases, although one male had a known hepatic metastasis. Five of these patients (3 of the males and 2 of the females) participated in Part 2 of the study (including the patient with a known hepatic metastasis). One male patient also had a history of laryngectomy for malignant disease. One patient had bilateral leg edema and hepatic enlargement on physical examination. Clinical examination findings in the remaining patients were unremarkable.

One patient had mild thrombocytopenia upon entry into the study but was included for analysis. Baseline hematology parameters were within normal limits for the remaining patients. Three patients had abnormal liver function tests at baseline. Two had serum alkaline phosphatase levels between three and five times the upper limit of normal, one had gamma glutamyl peptidase levels approximately nine times the upper limit of normal, and one had a gamma glutamyl peptidase level between one and two times the upper limit of normal. All six patients had normal renal function and normal serum electrolyte levels.

5.3.2 Pharmacokinetics

5.3.2.1 Part 1 – Bolus administration

Summaries of the pharmacokinetic data for free and total phenoxodiol from the first part of the study are presented in Tables 3 and 4. Plasma concentration versus time plots for

free and total phenoxodiol following the single bolus intravenous infusion of 5 mg/kg in part one of the study are presented in Figures 15 and 16.

Table 3**.** Pharmacokinetic parameters for free phenoxodiol following a single bolus dose of 5 mg/kg infused intravenously over 5 minutes. K_{el} is the elimination rate constant. T1/2 is the terminal half-life. AUC_{0-t} is the area under the plasma concentration versus time curve measured to the last plasma sample. AUC_{0-inf} is the area under the plasma concentration versus time curve extrapolated to infinity. Cl is the plasma clearance rate. Vd is the apparent volume of distribution. *The figures in parentheses are the estimated free phenoxodiol proportion for each patient, based on the calculation: free AUC_{0-inf} x100 / total AUC_{0-inf}.

Patient	**K_{el}**	**T1/2 (h)**	**AUC_{0-t} (μg.h/mL)**	**AUC_{0-inf} (μg.h/mL)**	**Cl (L/h)**	**Vd (L/kg)**
1	1.25	0.55	1.49	1.55 (4.1%)*	1.50	1.20
2	2.42	0.28	0.40	0.40 (1.2%)	7.01	2.90
3	1.59	0.43	1.04	1.05 (13.2%)	2.22	1.40
4	1.24	0.56	1.65	1.66 (10.3)	1.24	1.00
5	0.39	1.74	2.59	2.59 (11.3)	0.46	1.17
6	1.54	0.45	1.21	1.22 (11.8%)	2.46	1.60
Mean ± SD	1.41 ± 0.66	0.67 ± 0.53	1.40 ± 0.73	1.41 ± 0.73 (8.7%)	2.48 ± 2.33	1.55 ± 0.69

Table 4. Pharmacokinetic parameters for total phenoxodiol following a single bolus dose of 5 mg/kg infused intravenously over 5 minutes. Abbreviations are the same as for Table 3.

Patient	**K_{el}**	**T1/2 (h)**	**AUC_{0-t} (μg.h/mL)**	**AUC_{0-inf} (μg.h/mL)**	**Cl (L/h)**	**Vd (L/kg)**
1	0.21	3.33	27.10	37.76	0.06	0.31
2	0.25	2.71	33.47	34.64	0.05	0.21
3	0.37	1.85	7.45	7.93	0.29	0.80
4	0.29	2.32	14.19	16.13	0.15	0.50
5	0.35	1.98	21.12	22.83	0.15	0.45
6	0.09	6.98	10.37	10.37	0.16	1.60
Mean ± SD	0.26 ± 0.10	3.19 ± 1.93	18.93 ± 10.11	21.61 ± 12.45	0.15 ± 0.08	0.64 ± 0.51

Figure 15. Free phenoxodiol levels following the intravenous administration of 5 mg/kg of phenoxodiol over 5 minutes in Part 1 of the study.

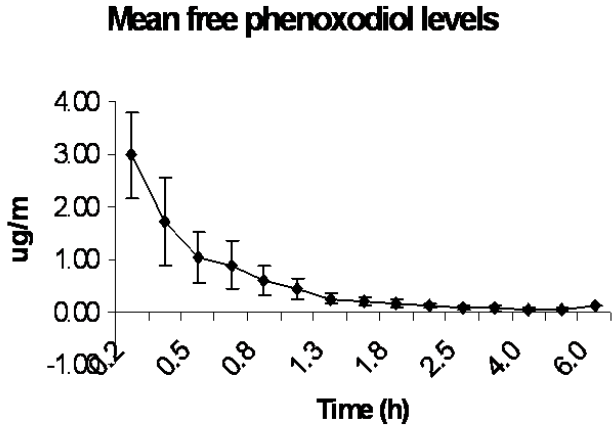
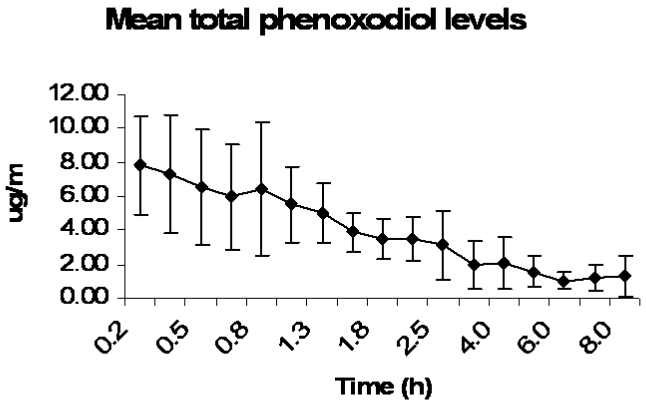


Figure 16. Total phenoxodiol levels following the intravenous administration of 5 mg/kg of phenoxodiol over 5 minutes in Part 1of the study.



Linear regression of log plasma concentration versus time plots indicated that phenoxodiol appeared to follow first order kinetics in all patients (that is, a single phase of elimination from plasma). This was the case for both free and total phenoxodiol. Total phenoxodiol had a longer plasma elimination half-life than free phenoxodiol, but both were relatively short (mean values 0.67 ± 0.53 h and 3.19 ± 1.93 h respectively). These short half-lives were associated with relatively high mean total plasma clearance rates of 2.48 ± 2.33 L/h and 0.15 ± 0.08 L/h respectively.

The apparent volumes of distribution at steady state for free and total phenoxodiol were relatively low (1.55 ± 0.69 L/kg and 0.64 ± 0.51 L/kg for free and total phenoxodiol respectively) and total phenoxodiol had a lower volume of distribution than free phenoxodiol. The area under the plasma concentration versus time curves for total phenoxodiol following the bolus intravenous dose in Part 1 of the study was approximately 15 times greater than for free phenoxodiol.

5.3.2.2 Part 2 – Prolonged intravenous infusion

Summaries of the pharmacokinetic data for free and total phenoxodiol from the 5 patients who completed the second phase of the study are presented in Tables 5 and 6. Plasma concentration versus time plots for free and total phenoxodiol following the continuous intravenous infusion of 2 mg/kg in phase two of the study are presented in Figures 17 and 18.

Table 5**.** Pharmacokinetic parameters for free phenoxodiol infused at a dose of 2 mg/kg/h in Part 2 of the study. C_{ss} is the plasma concentration at steady state. Time to steady state (ss) is the time taken to reach C_{ss}. Acc T1/2 is the accumulation half-life. Cl is the plasma clearance rate.

Patient	C_{ss} (μg/mL)	Time to ss (h)	Acc T1/2 (h)	Cl (L/h)
1	0.92	0.67	0.13	1.08
2	0.65	1.00	0.20	1.53
4	0.77	1.00	0.20	1.28
5	0.66	1.00	0.20	1.51
6	0.96	0.67	0.13	1.03
Mean ± SD	0.79 ± 0.14	0.87 ± 0.18	0.17 ± 0.04	1.29 ± 0.23

Table 6**.** Pharmacokinetic parameters for total phenoxodiol infused at a dose of 2 mg/kg/h in Part 2 of the study. Abbreviations are the same as for Table 3. * In patients 1 and 2 the infusion was not continued long enough to reach steady state and the values calculated are therefore underestimates of the true results.

Patient	C_{ss} (μg/mL)	Time to ss (h)	Acc T1/2 (h)	Cl (L/h)
1*	14.74	3.66	0.73	0.06
2*	32.31	4.00	0.80	0.03
4	13.33	2.00	0.40	0.07
5	14.80	2.00	0.40	0.07
6	12.04	2.33	0.47	0.08
Mean ± SD	17.45 ± 8.38	2.78 ± 0.96	0.56 ± 0.19	0.06 ± 0.01

Figure 17. Mean plasma concentrations of free phenoxodiol during continuous intravenous infusion in Part 2 of the study.

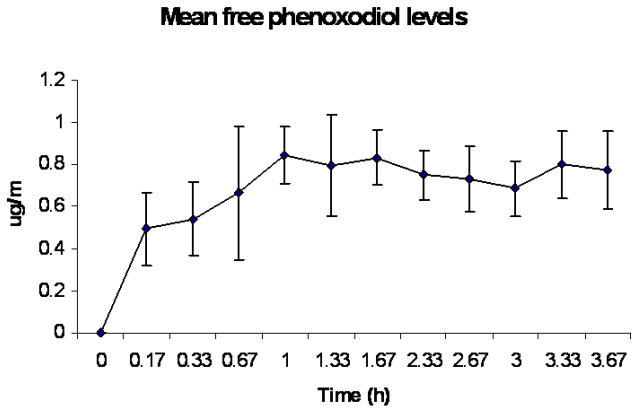
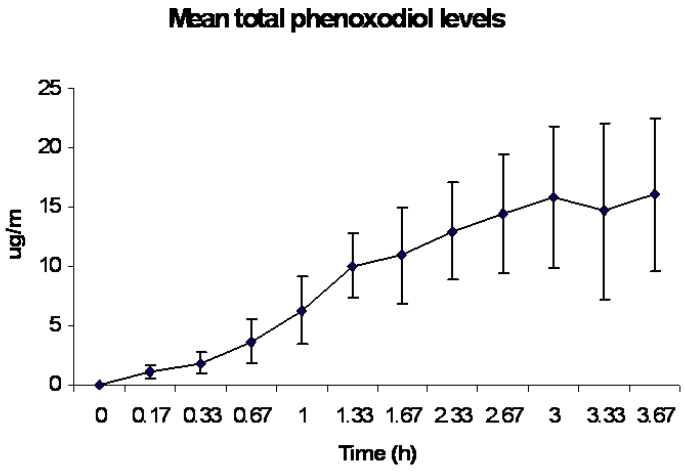


Figure 18. Mean plasma concentrations of total phenoxodiol during continuous intravenous infusion in Part 2 of the study.



Plasma levels of free phenoxodiol accumulated fairly rapidly with a mean accumulation half-life of 0.17 ± 0.04 hours. Plasma levels of total phenoxodiol accumulated at a relatively slower rate with a mean accumulation half-life of 2.78 ±0.96 hours. Steady state plasma levels of total phenoxodiol were more variable than steady state plasma levels of free phenoxodiol (Figures 17 and 18). The mean clearance rate for total phenoxodiol (0.06 ± 0.01 L/h) was lower than that of free phenoxodiol (1.29 ±0.23 L/h). In two of the patients (patient 1 and patient 2) the infusion did not appear to be continued long enough for total phenoxodiol to reach steady state plasma levels. The final plasma concentration time point in these patients was used to estimate C_{ss}, accumulation half-life and clearance. However, the values calculated are likely to have underestimated the true measurements.

5.3.3 Conjugation

The degree of phenoxodiol conjugation is estimated at 92%, based on calculation of the proportion of free phenoxodiol AUC: total phenoxodiol AUC (mean 8.7%) from bolus administration. For each individual subject, estimated proportion of free phenoxodiol from the bolus data correlates very well with total phenoxodiol clearance as measured during continuous infusion (data not shown).

5.3.4 Adverse events

Two patients reported five adverse events during the two studies. One patient experienced 3 serious adverse events: hepatic metastasis, leg edema and thrombocytopenia. All of these problems had been present prior to the study but had

increased in severity during the study. They were considered to be due to disease progression and unrelated to the study medication. This patient died 7 days after completing Part 2 of the study. His death was considered to be due to progression of malignant disease and unrelated to the study medication.

Another patient experienced two non-serious adverse events: groin pain and nausea. The groin pain occurred the day following the single dose of phenoxodiol in Part 1 of the study and was considered not to be related to the study medication. The episode of nausea occurred following the intravenous administration of phenoxodiol during Part 1 of the study, was mild in intensity and was considered to be possibly related to study medication. The episode of nausea did not require intervention and resolved spontaneously.

5.4 Discussion

This first-in-human study of phenoxodiol investigated the pharmacokinetics of the drug given as a single intravenous bolus injection and as a single episode of continuous intravenous infusion in the same patients. Part 1 of the study investigated the effects of an intravenous bolus of phenoxodiol at a standardized dose of 5 mg/kg. Mean plasma levels of free phenoxodiol declined rapidly from an initial value of around 3 µg/mL with an apparent half-life of 0.67 h. The log-plasma concentration plot for each patient appeared linear (data not presented) suggesting first order pharmacokinetics. The clearance of free phenoxodiol was relatively high (2.48 L/h) and the volume of distribution was 1.55 L/kg, indicating distribution of the drug into a body compartment approximately 2.3 times the extracellular fluid volume.

Plasma levels of total phenoxodiol declined from an initial concentration of approximately 8µg/mL with an apparent half-life of 3.19 h. Similar to free phenoxodiol, the log-plasma concentration plot for each patient appeared linear (data not presented) suggesting first-order pharmacokinetics. The clearance of total phenoxodiol (0.15 L/h) was considerably lower than for free phenoxodiol. In contrast, the volume of distribution of total phenoxodiol (0.64 L/kg) was smaller than for free phenoxodiol. Plasma concentrations of total phenoxodiol were more variable than for free phenoxodiol. The ratio of AUC (free phenoxodiol): AUC (total phenoxodiol) was 0.087, indicating that >90% of the drug following an intravenous bolus was present in the conjugated form. Furthermore, the initial plasma concentrations of total phenoxodiol were approximately 2.7 times the initial plasma concentrations of free phenoxodiol. These observations are most likely due to rapid conjugation of phenoxodiol in the liver and a slower clearance of conjugated phenoxodiol.

Part 2 of the study investigated the pharmacokinetics of a continuous infusion of phenoxodiol at a dose of 2 mg/kg. The duration of the infusion was planned to ensure that steady state levels of free pheonoxodiol were attained. The infusion was not continued long enough to ensure that steady state levels of total phenoxodiol were reached in all patients as it was considered that this would require a longer duration of study that would be potentially distressful for the patient volunteers. Plasma concentrations of free plasma phenoxodiol rose rapidly with an apparent accumulation half-life of 0.17 h. Although this appeared to be a little shorter than the half life estimated following the single intravenous bolus dose in Part 1 of the study, the results were more variable in Part 1 of the study. The plasma clearance calculated during the

continuous infusion (1.29 L/h) appeared a little lower than that calculated from Part 1 of the study, but the results were consistent with each other. Further, the degree of individual phenoxodiol conjugation estimated from the bolus part of the study correlated well with clearance measured in the continuous infusion part of the study. The mean plasma concentration of free phenoxodiol at steady state was approximately 0.79 µg/mL. This is in the range that has been demonstrated to have anti-cancer effects in *in vitro* studies.

Plasma concentrations of total phenoxodiol rose much more slowly than concentrations of free phenoxodiol during continuous infusion with an apparent half-life of accumulation of 0.65 h. This is shorter than the half-life of 3.19 h determined following a single intravenous bolus dose, but as two of the patients had not achieved steady state levels of total phenoxodiol the result calculated during the continuous infusion would have underestimated the correct value. Similarly, the plasma clearance calculated during the continuous infusion of phenoxodiol (0.06 L/h) would have underestimated the correct value and in part explained why it appeared lower than the value calculated following the single intravenous bolus injection.

The results of this study indicate that phenoxodiol has a short half-life when given intravenously, particularly for the free form. Administration by continuous infusion or by chronic oral administration may be the optimal modes of administration if it is considered that constant plasma levels are desirable for anti-cancer therapy.

The limitations of this study are that only a small number of patients with cancer were studied due to the fact that it was a first-in-human pharmacokinetic study of

phenoxodiol. In addition, the study examined only a limited dose range of phenoxodiol. It is not known whether the doses chosen were optimal nor whether the pharmacokinetics of phenoxodiol is truly linear. Furthermore, the effects of age and of renal or hepatic dysfunction on phenoxodiol pharmacokinetics are not known. Further studies are required to address these issues.

Subsequent to this study, other Phase I trials have investigated phenoxodiol given on a repeated dose schedule (Choueiri *et al*, 2006; de Souza *et al*, 2006). Phenoxodiol was prepared in a different (cyclodextrin) carrier in these studies, which may explain the different pharmacokinetic properties noted. In particular, clearance of phenoxodiol is higher (around 82 mL/min) and half-life is longer (around 300 mins) than in this study. Other than the different preparation, other possible explanations include the tight control of diet, caffeine and smoking in this study, whereas conditions were not stated in the other studies.

In summary, this study has demonstrated that free and total phenoxodiol have relatively short plasma half-lives when administered intravenously. The drug displays linear pharmacokinetics at the doses studied and has a relatively small volume of distribution.

6. Phase I and pharmacokinetic study of weekly phenoxodiol in patients with cancer.

6.1 Introduction

Phenoxodiol is a synthetic compound based on the diphenolic (*isoflavonoid*) ring structure. Recently, it was granted Fast Track status by the Federal Drug Administration, USA, (FDA) in its development as a chemo-sensitiser for platinums and taxanes for the treatment of recurrent ovarian cancer. The main mechanism of action of phenoxodiol appears to be related to Akt and FAS mediated signaling through inhibition of anti-apoptotic factors (Kamsteeg *et al*, 2003), resulting in either apoptosis or potential reversal of drug-mediated resistance. Other supplementary mechanisms of action involving down-regulation of sphingosine kinase or toposisomerase II are also possibly relevant (Constantinou & Husband, 2002). Phenoxodiol shows broad activity against human cancer cells, inducing mitotic arrest (G_1 phase of mitosis), terminal differentiation, and apoptosis; phenoxodiol also is a weak estrogen, and is an inhibitor of the androgen-associated enzymes 5-α-reductase and 17β-hydroxysteroid dehydrogenase (Novogen Research Pty Ltd, 2000). Recently, induction of $p21^{WAF1/CIF1}$ by phenoxodiol and subsequent specific cdk2 inhibition was shown for HN12 cell lines (Aguero *et al*, 2005).

We, and others, have previously shown that phenoxodiol has activity against a variety of human cancer cell lines. The concentration required to inhibit by 50% the growth of

PC3 and DU145 prostate cancer cells (IC50) *in vitro* was 3-5 µM (McPherson *et al*, 2009). The IC50 was 1.5, 1.5, 4.5, and 15.0 µM in HL60 leukemia, MCF7 breast, H460 NSCLC, and HT29 colon cancer cell lines, respectively (Novogen Research Pty Ltd, 2000). Phenoxodiol administered orally once daily for 18 days or intraperitoneally three times weekly for 21 days to athymic mice bearing xenografts of prostate cancer cells (DU145, PC3) or ovarian cancer cells (A2780) resulted in a significant inhibition of tumor growth (Novogen Research Pty Ltd, 2000; Kamsteeg *et al*, 2003). In mice, intravenously-administered phenoxodiol is subject to extensive conjugation (glucuronidation and sulfation) (Novogen Research Pty Ltd, 2000). As with steroidal hormones, bio-activation of the drug follows deconjugation by glucuronidases and sulfatases within target tissues. In rats, no toxicity by way of histological changes in a variety of organs, hematological, or serum biochemical abnormalities was associated with the intravenous dosage form. However, functional toxicity such as subdued behaviour, reduced activity, loss of balance, and head tremors, was noted at the highest doses. The maximum tolerated dose (MTD) by repeated (daily for 5 consecutive days) bolus injection in rats was 80 mg/kg (Novogen Research Pty Ltd, 2000). When phenoxodiol was delivered by continuous intravenous injection for 28 days, the MTD was determined to be 20 mg/kg/24-hr on the basis of reversible, moderate nephrotoxicity that was also observed in the vehicle (hydroxypropyl-β-cyclodextrin) control group.

Flavonoids, and in particular, flavopiridol (Senderowicz *et al*, 1998; Schwartz *et al*, 2002; Tan *et al*, 2002; Kouroukis *et al*, 2003), have attracted much interest recently, as they represent new agents active against specific cellular targets, targeting key signaling pathways such as cell cycle regulation that are critical to cell growth and survival.

Inhibition of Akt has also attracted much interest as Akt is a mediator of the effects of multiple genetic abnormalities including PTEN deletion, bcr-abl translocation, Her2-neu amplification and EGFR overexpression (Thompson & Thompson, 2004). Rapamycin is an inhibitor of the downstream effector of Akt, mTOR, and analogues are currently undergoing clinical trials.

On the basis of the preclinical properties of phenoxodiol, we performed a Phase I dose-escalation trial in patients with advanced cancer with the aim of determining a recommended Phase II dose based on a target concentration commensurate with active *in vitro* doses.

6.2 Patients and Methods

6.2.1 Patients

Patients were eligible for the study if they had refused standard therapy or had failed at least one standard systemic treatment regimen for their malignancy, were ≥18 years of age, had Eastern Cooperative Group (ECOG) Performance Status of 0-2, and a life expectancy of at least 3 months. Adequate organ function was required, including neutrophil count (ANC) $\geq 1.5 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L, Hemoglobin (Hb) ≥10.0 g/dL for men and ≥9.0g /dL for women, serum creatinine $\leq$ 0.12 mmol/L, and transaminases (AST/ALT) levels ≤5 times the upper limit of normal. Patients were ineligible if they had more than 25% of hemopoietic bone marrow previously irradiated, active infection, or active, untreated central nervous system (CNS) metastases. No investigational agent or chemotherapy was allowed within at least 3 weeks of study entry (6 weeks for nitrosureas or mitomycin C), and patients must have recovered from

previous side effects. All patients gave written informed consent, and the protocol was approved by the South Eastern Area Health Service Ethics Committee (St George Hospital). The trial was conducted in accordance with the Declaration of Helsinki (2000).

6.2.2 Dose escalation

Acute Dose Limiting Toxicity (DLT) within the first 4 weeks of treatment was defined as any of Grade 4 neutropenia lasting >7 days with or without colony stimulating factor support, febrile neutropenia, Grade 4 thrombocytopenia, Grade 2 or worse CNS neurotoxicity, or Grade 3/4 non-hematologic toxicity with the exception of alopecia, nausea or vomiting.

The starting dose of phenoxodiol, 5 mg/kg weekly as a 1-hour intravenous infusion, was one-tenth of the dose (50 mg/kg) that caused subdued behaviour in rats in preclinical studies (Novogen Research Pty Ltd, 2000). Previous preliminary pharmacokinetic data in patients with advanced malignancies had established that a single dose of 5 mg/kg had produced phenoxodiol concentrations that were compatible with *in vitro* activity, and mild nausea was the only reported adverse event (Howes JB *et al*, submitted for publication). In view of the potential for cumulative toxicity, however, lower doses were studied in single patient cohorts (1 mg/kg and 2.5 mg/kg) to observe toxicity for at least 6 weeks prior to the main starting dose level (5 mg/kg). For this and subsequent dose levels, a minimum of three patients was studied unless DLT was observed. If there was no DLT among the first three patients entered at a given dose level at the end of 3 weeks, three more patients were recruited for the next highest dose level. If a DLT was

observed in any of the three patients at any dose level, we planned to enter three more patients at the same dose level. If a second patient experienced DLT then dose escalation stopped. The MTD was defined as the dose level below the level that produced DLT in ≥33% of patients. The recommended Phase II dose was defined as either the MTD or a dose commensurate with maximum plasma concentrations above the IC50. The *in vitro* IC50 concentrations were thought to be more clinically relevant because pharmacokinetic assessments *in vivo* had been performed in single dose studies only in rats.

6.2.3 Drug administration

Phenoxodiol was supplied by Novogen Pty Ltd (Australia) in glass vials at a concentration of 15 mg/mL suspended in hydroxypropyl-β-cyclodextrin (HPBCD) in isotonic saline. The stock solution was diluted in normal saline to approximately 5 mg/mL and the resulting solution was administered via an infusion pump over 20 minutes through a peripheral intravenous cannula. At dose levels greater than 15 mg/kg, duration of infusion was increased to 2 hours to reduce pain at the site of infusion. Phenoxodiol was administered weekly, based on preclinical data in mice. We assigned a treatment period of 4 weeks as equivalent to one “cycle”, in order to assess acute toxicity within a reasonable time frame.

6.2.4 Pretreatment and follow-up investigations

Prior to the start of treatment, a medical history, physical examination, chest X-ray, ECG, vital signs, ECOG performance status, full blood count including differential,

serum biochemistry (electrolytes, urea, creatinine, liver function tests, calcium, phosphate, random blood sugar), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were performed. Males and females also had testosterone, FSH, LH, and estradiol, FSH, and LH measured, respectively. Laboratory testing was performed using standard techniques by the South Eastern Area Laboratory Service (SEALS). Glomerular Filtration Rate (GFR) was estimated using the Cockcroft–Gault method and by isotope scanning. A calculated GFR using the Cockcroft–Gault method was required at weeks 5 and 9 during treatment. Tumor size was assessed by radiological imaging. PSA or other tumor markers (where appropriate) were performed every week for the first cycle, then every 2 weeks thereafter. Male and female sex hormones (as for baseline) were performed monthly, along with fibrinogen, APTT and PT. ECOG performance status was assessed at the beginning of each cycle. For patients who had measurable disease, repeated radiological assessment of measurable and evaluable disease occurred at 6 weeks and 12 weeks during therapy. Patients who responded to treatment without toxicity at the end of 12 weeks were offered continuing treatment for a total of 24 weeks.

6.2.5 Toxicity assessment

Toxicity was graded using the NCI Common Toxicity Criteria, version 2.0. Toxicity assessment, physical examination and measurement of vital signs were carried out weekly in the first cycle, then once every cycle thereafter.

6.2.6 Pharmacokinetic studies

During and after the first infusion of phenoxodiol, plasma samples were taken at time 0 (0-5 minutes prior to infusion), at 20 minutes (just before termination of infusion), then at 30, 40, 60, 90, 120, 150, 180, 240 and 360 minutes for the measurement of free and conjugated phenoxodiol concentrations. Pharmacokinetic measurements were performed on day 1 of cycle 1. Plasma samples were centrifuged at the bedside and stored at -20^{0}C prior to analysis. Pharmacokinetic parameters were derived using compartmental methods with Win Nonlin statistical software (Pharsight corporation, NC, USA).

6.2.7 Phenoxodiol assay methods

Plasma phenoxodiol assays were performed at Novogen Research Laboratories, Sydney, Australia. Free phenoxodiol was assayed using an HPLC-UV method and the total phenoxodiol was assayed using an LC-MS (ESI-MRM) method, following incubation with ß-glucuronidase. Both methods are validated but are not published. For the HPLC-UV method, the calibration curve was linear in the range of 0.25 µg/mL to 10 µg/mL with a correlation coefficient (R^2) >0.994. The lower limit of quantitation (LOQ) for phenoxodiol was 0.25 µg/mL. Recovery of phenoxodiol was >71% at low (0.25 µg/mL), medium (1 µg/mL) and high (10 µg/mL) concentrations. The intra-day accuracy was in the range of 86.2 to 107% and the inter-day accuracy was in the range of 94.5 to 107% at all concentrations. For the LC-MS method, the calibration curve was a polynomial curve in the range of 0.025 µg/mL to 20 µg/mL with correlation coefficient (R^2) >0.992. The lower limit of quantitation (LOQ) for phenoxodiol was 0.025 µg/mL by this assay. Recovery of phenoxodiol was >93% at low (0.063 µg/mL),

medium (5 µg/mL) and high (15 µg/mL) concentrations. The inter-day accuracy was in the range of 97 to 108% at all concentrations.

6.2.8 Statistical analysis

All results are expressed as the mean ± standard deviation (SD) except where the median and range are presented.

6.3 Results

6.3.1 General

Twenty–one patients were enrolled over a 15 month period. Patient characteristics are listed in Table 1. The median age was 61 (range 25-84) and there were 16 males. Most patients had performance status of ECOG 1-2, and there were a variety of cancers represented. All patients but two had received prior therapy (Table 7). A total of 185 infusions were administered with a median of 7 and range of 2 to 24 infusions.

Table 7. Patient characteristics. Abbreviations: NSCLC, non-small cell lung cancer; ECOG, Eastern Co-operative Oncology Group.

Characteristics	Number of Patients
Total	21
Age, years	
Median	61
Range	25-84
Gender	
Male	16
Female	5
Performance status, ECOG	
0	8
1	11
2	2
Tumor type	
Kidney	2
Prostate	8
Melanoma	1

Head and neck	3
Pancreas	3
Breast	1
NSCLC	2
Leiomyosarcoma	1
Number of previous treatments	
0	2
1	10
2	7
3	1
Baseline weight, kg	
Mean	73
Range	46-96

6.3.2 Dose escalation

The dose escalation scheme is summarized in Table 8. No DLT was seen during the first cycle. However, DLT was later encountered in two patients during cycle 2. Both were considered possibly related to the drug, but did not recur during cycle 3 despite continuation of treatment. The MTD was not reached by the 30 mg/kg dose level and the trial was terminated early because difficulty in dissolving higher concentrations of the drug precluded further dose escalation.

Table 8. Dose level cohorts and patient outcome.

Dose level	Dose (mg/kg)	Mean dose (mg/m^2) per cycle	No. of patients	No. of patients completing 12 weeks	No. of patients with DLT
-2	1	-	1	1/1	1
-1	2.5	-	1	0/1	0
0	5	197	3	1/3	1
1	10	413	3	1/3	0
2	15	564	3	3/3	0
3	20	767	4	1/4	0
4	25	902	3	0/3	0
5	30	1216	3	1/3	0

6.3.3 Hematologic toxicities

Lymphocytopenia was the most consistent hematologic toxicity noted (Tables 9 & 10), although this did not lead to any apparent clinical problems. Lymphocytopenia did not correlate with increasing dose levels, and did not correspond to increasing duration of therapy. Other hematologic toxicity was very mild, with fewer than 10% of patients experiencing Grade 2 or worse anemia, thrombocytopenia, or leucopenia.

Table 9. Overall worst NCI CTC toxicity (any toxicity, any grade, all cycles).

Dose cohort mg/kg	Grade 2	Grade 3	Toxicity
1			Hyperbilirubinemia
2.5	1/1		Nausea
5	1/3		ALP
10		2/3	Lymphocytopenia
15		3/3	Lymphocytopenia
20		1/3	Lymphocytopenia
25		1/3	Lymphocytopenia
30		2/3	Lymphocytopenia

Table 10. Worst NCI CTC hematologic toxicity per patient, all cycles.

Toxicity	Grade 1	Grade 2	Grade 3
Hemoglobin	6/21	1/21	
WBC	3/21	2/21	
Neutrophils	1/21	1/21	
Lymphocytes	1/21	9/21	9/21
Platelets	5/21		

6.3.4 Non-hematologic toxicities

One patient developed isolated, asymptomatic, Grade 2 hyperbilirubinemia, peaking during cycle 2 at the 1 mg/kg dose level of phenoxodiol. His pre-study bilirubin was slightly elevated and did not increase above 34 μmol/L, despite continued treatment

with phenoxodiol. After cessation of phenoxodiol, the patient's bilirubin levels remained slightly above normal. The only other DLT was the development of a Grade 2 mood disorder (depression) in one patient at the 5 mg/kg dose level during cycle 2 that responded promptly to an antidepressant. Two patients experienced hypersensitivity reactions after several infusions. The first discontinued treatment after sudden onset of flushing, back pain and a transient (48 hour) Grade 3 thrombocytopenia. The second was able to continue treatment with steroid and anti-histamine pre-medication. One death due to progressive disease occurred during the study. Other non–hematologic toxicities were mild (Table 11). GFR calculated by the Cockcroft–Gault formula did not change during the study.

Table 11. Worst NCI CTC non–hematologic toxicity per patient, all cycles.

Toxicity	Grade 1	Grade 2	Grade 3
Infections	1/21		
Fever (no infection)	2/21		
Nausea/vomiting	9/21	1/21	
Constipation			
Diarrhea	3/21		
Stomatitis			
Pulmonary	1/21		
Cardiac			
Hypotension	1/21		
Neurosensory	1/21		
Neuro-mood		1/21	
Neuro-headache	2/21		
Alopecia			
Skin	1/21		
Local site reaction	2/21		
Weight gain/loss	1/21		
Allergy	1/21		
Laboratory			
alkaline phosphatase	6/21	2/21	
Transaminases	6/21		
Creatinine	3/21		
Bilirubin			1/21
Proteinuria			
Hematuria			

6.3.5 Responses

Eighteen patients were evaluable for response. No objective responses were seen. Ten patients had stable disease of at least 6 weeks duration, and eight patients had progressive disease. Eight patients were able to complete 3 cycles of treatment (12 weeks), and two of these completed 24 weeks of treatment (including one patient with renal cell cancer).

6.3.6 Pharmacokinetics

Data from cycle 1 in 15 patients were used to determine pharmacokinetic parameters (Table 12); the remaining 3 patients had insufficient blood draws to estimate pharmacokinetic parameters. Only total phenoxodiol was analysed because "free" phenoxodiol concentrations were only just above the limit of quantitation, and are therefore considered unreliable (data not shown). Phenoxodiol reached a mean maximum concentration (Cmax) of 88.7 µg/mL (±25) (= 369 µM) in the highest dose cohort (30 mg/kg) at the end of the infusion. The mean volume of distribution (V_D) was 32663mL (± 7199). Phenoxodiol was rapidly eliminated with a mean clearance of 82 mL/min (± 19) and a mean half-life of 304 min (±91). The area under the concentration x time curve (AUC) for total phenoxodiol increased linearly with dose (Figure 19) and is described by the equation $y=5.08x + 2510$ ($R^2=0.69$). Figure 20 shows persistent levels of phenoxodiol at least 6 hours after a bolus dose for each patient, with a general trend towards higher concentrations in higher dose cohorts.

Figure 19. Dose (mg) plotted against AUC (min*µg/mL). Equation for line of best fit is described as y=5.08x + 2510 (R^2=0.69).

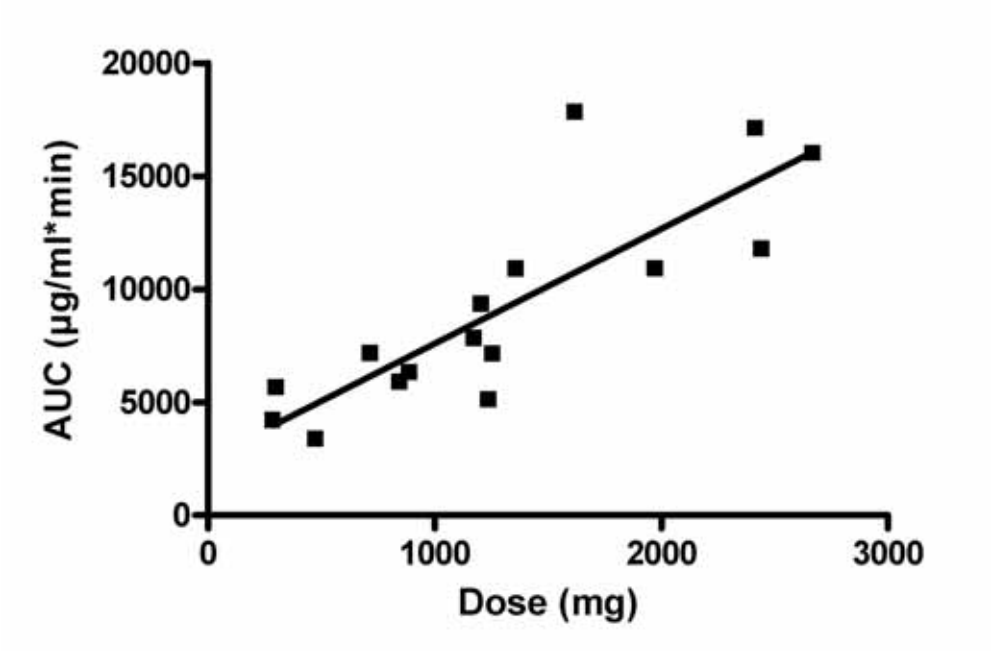


Figure 20. Total phenoxodiol (NV06) concentrations (µg/mL) expressed on a logarithmic scale, plotted over at least 6 hours for each of 16 patients.

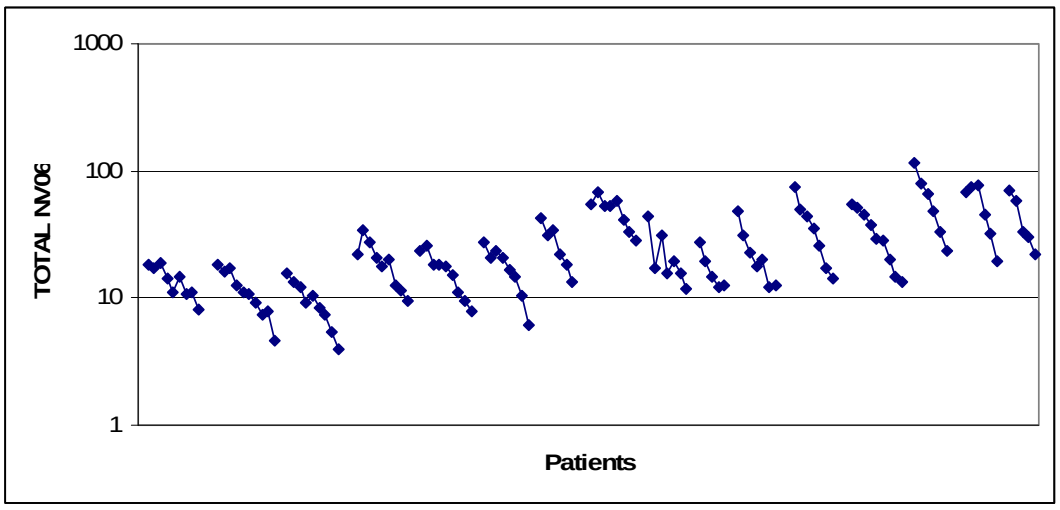


Table 12. Pharmacokinetic parameters for total phenoxodiol (Mean and SD for all figures).

Dose cohort (mg/kg)	N	Cmax (µg/mL)	AUC 0-∞ (min*µg/mL)	AUC	V_D (mL)	CL (mL/min)	T1/2 (min)
5	3	17.5 ± 1.6	7218 ± 2923	37	23621 ± 10027	58 ± 36	313 ± 79
10	3	29.4 ± 4.5	11316 ± 3363	40	36686 ± 8295	78 ± 31	366 ± 161
15	1	42.9	16266	43	33924	74	317
20	3	46.7 ± 20.2	21450 ± 12852	52	42505 ± 20219	76 ± 34	419 ± 204
25	3	59.5 ± 14.2	15256 ± 479	36	34323 ± 6731	98 ± 26	254 ± 89
30	2	88.7 ± 25	23039 ± 2249	28	24918 ± 1692	111 ± 19	156 ± 16
Mean					32663 ± 7199	82 ± 19	304 ± 91

6.4 Discussion

Our Phase I trial demonstrates that phenoxodiol was well tolerated in a weekly treatment schedule. Lymphocytopenia was the main hematologic toxicity seen, although low-grade neutropenia, anemia and thrombocytopenia occasionally occurred (Table 10). Only three patients developed Grade 1 diarrhea, whereas flavopiridol, a flavonoid currently under investigation, appears to be associated with dose limiting diarrhea (Schwartz *et al*, 2002; Tan *et al*, 2002). On the other hand, lymphocytopenia was relatively uncommon in the Phase I study of flavopiridol. No thromboembolic complications were observed in our study, in contrast to flavopiridol. Two patients in our study developed hypersensitivity reactions, one of whom also developed associated back pain. The cause of these reactions is unclear, but may be similar to the pro-inflammatory phenomena postulated for flavopiridol. Alternatively, they may be a side effect of the hydroxypropyl-β-cyclodextrin carrier, which is known to be a vascular

irritant, and can cause vacuolation of renal cells in rabbits (Irie *et al*, 1992) and distress and pulmonary edema in other species (Carpenter *et al*, 1995). One patient in our study had isolated hyperbilirubinemia, which we attributed to pre-existing Gilbert's syndrome on the basis of an elevated pre-treatment and post-treatment bilirubin. Hyperbilirubinemia had been previously reported with flavopiridol (Tan *et al*, 2002), so it is possible that the same proposed mechanism of a shared glucuronidation pathway is also relevant for phenoxodiol, at least for this patient. Overall, however, the toxicity profile of phenoxodiol appears to be quite different from that of flavopiridol. The MTD was not reached with this treatment schedule because of the difficulty in dissolving the drug at concentrations higher than 30 mg/kg and the practical limitations on the volume of drug able to be delivered.

Preclinical studies with phenoxodiol have established that the anti-tumor effect of the drug is proportional to its duration of contact with the tumor cells (Novogen Research Pty Ltd, 2000), an effect consistent with the known biochemical effects of phenoxodiol in down-regulating the phosphorylation of anti-apoptotic proteins (Kamsteeg *et al*, 2003). The pulsatile treatment regimen used in our study, therefore, was selected as a starting point for toxicity profiling rather than for efficacy, and, not surprisingly, we did not see any objective tumor responses with this weekly schedule. Nevertheless, several patients did show stable tumor marker levels during treatment, including one man with androgen independent prostate cancer who had failed two prior hormone regimens and who had rising PSA levels prior to treatment with phenoxodiol. Also, one patient with estrogen receptor positive breast cancer treated at the 20 mg/kg dose level, and who previously had been treated with three chemotherapy regimens including an

anthracycline-based regimen and docetaxel, was able to complete 12 weeks of treatment with stable CA15.3 levels.

Flavopiridol has been studied in Phase I trials by continuous infusion (Senderowicz *et al*, 1998) and by daily 1-hour infusions for 3 days (Tan *et al*, 2002). In the one hour infusion study, neutropenia was the DLT and peak concentrations achieved appeared to approximate peak concentrations in preclinical models associated with significant tumor regressions. When flavopiridol was combined with paclitaxel in a Phase I study, however (Schwartz *et al*, 2002), an increase in clearance between the 80 mg/m^2 and 94 mg/m^2 dose levels was seen. The authors ruled out an interaction with paclitaxel, and postulated that enterohepatic circulation of the compound was occurring, but did not find evidence of a post–infusion spike in concentration. In our study, evidence of such a spike with phenoxodiol was not found, though the dominant form of phenoxodiol is conjugated. This may be the more important and bioavailable form of phenoxodiol as "free" phenoxodiol concentrations were only just above the limits of detection (data not shown), and perhaps bound up with the cyclodextrin carrier. Experiments to clarify this possibility are underway. The low concentrations of free phenoxodiol and its high protein binding may also confound the relationship between the lack of responses and the apparently broad activity of relatively low *in vitro* phenoxodiol concentrations. Since relevant pharmacodynamic or surrogate markers were not available at the time of the study, we cannot categorically state that phenoxodiol at concentrations achieved by weekly intravenous bolus infusions is having a significant biological effect.

The clinical utility of phenoxodiol is most likely to lie with its effect on apoptosis and potential reversal of drug resistance. Subsequent results from a Phase Ib/IIa trial in

ovarian cancer have provided preliminary evidence of a striking reversal of paclitaxel resistance in women treated more intensely than a weekly schedule (Wilkinson, 2004). Further, *in vitro* evidence suggests that docetaxel resistance in ovarian cancer cell lines may be mediated by XIAP, and that this may be overcome by pretreatment with phenoxodiol (Sapi *et al*, 2004). The priority, therefore, is to achieve a biologically effective concentration over a sustained period of time rather than the maximum tolerated dose. The plasma concentrations achieved in this study are well above those required to achieve cytotoxicity *in vitro* and inhibit Akt (Kamsteeg *et al*, 2003; McPherson *et al*, 2009), though the duration of exposure is perhaps insufficient to mimic *in vitro* conditions over a 24hr period (Figure 20). Studies to better characterize its mechanism of action, to correlate pharmacokinetics with pharmacodynamic parameters of Akt inhibition and to explore its combination with standard cytotoxic agents are underway.

In conclusion, phenoxodiol is a novel isoflavone that is well tolerated in a weekly intravenous schedule. Its main side effects are mild nausea, asymptomatic lymphocytopenia, and mildly raised alkaline phosphatase levels. Concentrations required for cytotoxicity and inhibition of Akt can be achieved without significant toxicity.

7. Phase I and bioavailability study of oral phenoxodiol in combination with either cisplatin or carboplatin in patients with advanced cancer

7.1 Introduction

Phenoxodiol is currently being studied in a Phase III clinical trial in patients with ovarian cancer (see https:/clinicaltrials.gov). Like genistein, it has a number of potential mechanisms of action. Disruption of Akt-mediated activation of FAS signaling through inhibition of anti-apoptotic factors appears to be an important mechanism (Kamsteeg *et al*, 2003; Sapi *et al*, 2004), leading to reversal of drug-mediated resistance and ultimately apoptosis. Other mechanisms of action involving down-regulation of sphingosine kinase or toposisomerase II are also possibly relevant (Constantinou & Husband, 2002). Phenoxodiol also induces p21 $^{WAF1/CIF1}$ and subsequently cdk2 inhibition (Aguero *et al*, 2005), thereby promoting G1 arrest.

In vitro and *in vivo* studies in our laboratory show that the combination of phenoxodiol and cisplatin is synergistic in two prostate cancer cell lines (McPherson *et al*, 2009) and additive in a DU145 xenograft nude mouse model. We also performed a Phase I dose-escalation trial in patients with advanced cancer with an intravenous formulation of phenoxodiol (de Souza *et al*, 2006). The short half-life of around 5 hours of this formulation may explain the lack of activity of the drug noted in this once weekly intravenous schedule. An oral formulation could allow a more chronic dosing schedule,

and potentially superior exposure of cancer cells to compound. An essential part of the current study was therefore an oral bioavailability assessment of this oral phenoxodiol formulation. Our aim was to perform a Phase I trial of an oral formulation of phenoxodiol given in combination with either cisplatin or carboplatin. We wished to determine the Dose Limiting Toxicity (DLT) of the combination, determine the oral bioavailability of the oral formulation of phenoxodiol, and investigate the safety and tolerability of the combination regimen.

7.2 Patients and Methods

7.2.1 Patients

Eligibility criteria were similar to those of our previous Phase I single agent phenoxodiol trial (de Souza *et al*, 2006). These included patients who had refused standard therapy or who had failed at least one standard chemotherapy regimen for their advanced cancer, were ≥18 years of age, and had life expectancy of at least 3 months. Other criteria included neutrophil count (ANC) $\geq 1.5 \times 10^9$/L, WBC $\geq 3.0 \times 10^9$/L, platelets $\geq 100 \times 10^9$/L, Hb≥10.0 g/dL for men and ≥9.0 g/dL for women, serum creatinine ≤ 0.15 mmol/L together with calculated GFR >50 mL/min (Cockcroft-Gault method), and AST/ALT levels ≤5 times the upper limit of normal. Patients were ineligible if they had active infection, or active, untreated CNS metastases. No investigational agent or chemotherapy was allowed within at least 3 weeks of study entry (6 weeks for nitrosureas or mitomycin C), and patients must have recovered from previous side effects of prior chemotherapy. No breast cancer patients were allowed on this study, in view of the potential for the mildly estrogenic properties of phenoxodiol *in vitro*. Written informed consent was provided by all patients and the protocol was approved

by the South Eastern Area Health Service Ethics Committee (St George Hospital). The trial was conducted in accordance with Good Clinical Practice (GCP) and the Declaration of Helsinki (2000).

7.2.2 Dose escalation

The starting dose of oral phenoxodiol was 50 mg every 8 hours. For the initial and subsequent dose levels (Table 2), a minimum of three patients treated with carboplatin and three treated with cisplatin (a total of 6 per phenoxodiol dose level) were studied unless dose limiting toxicity (DLT) was observed. If there was no DLT among the first six patients entered at a given phenoxodiol dose level at the end of 3 weeks, six more patients were recruited for the next highest dose level, again split between carboplatin and cisplatin. If a DLT was observed in any of the three patients treated with either cisplatin or carboplatin at any phenoxodiol dose level, we planned to enter three more patients at the same dose level. If a second patient experienced DLT then dose escalation of oral phenoxodiol was stopped. The Maximum Tolerated Dose (MTD) was defined as the dose level below the level that produced DLT in ≥33% of patients. The recommended Phase II dose was defined as the MTD.

7.2.3 Dose Limiting Toxicity

Acute DLT was defined as any of the following: Grade 4 neutropenia lasting >5 days without fever; febrile neutropenia; Grade 3/4 thrombocytopenia with clinical evidence of bleeding; Grade 2 or worse non-hematologic toxicity or grade 3 / 4 hematologic toxicity that did not recover to Grade 1 or less within 21 days of halting treatment; or

Grade 3 / 4 non-hematologic toxicity with the exception of alopecia, nausea, vomiting, isolated hyperbilirubinemia or diarrhea not responding to standard agents.

7.2.4 Treatment schedule

Patients were assigned either cisplatin or carboplatin for the duration of their treatment at the discretion of their treating oncologist, without randomization or other systematic (eg. alternate) allocation. Cisplatin was given at a fixed dose of 50 mg/m^2 intravenously on days 2 and 9 of each 3 week cycle, and carboplatin was given at a fixed dose of AUC=5 intravenously on day 2 of each 3 week cycle. There was no dose escalation of either cisplatin or carboplatin, but doses could be reduced for toxicity. Given our preclinical synergy studies, oral phenoxodiol was given for the first 10 days followed by 11 days rest in each 21 day cycle, in order to allow for the possibility of enhanced toxicity with the combination schedule. Phenoxodiol was supplied by Novogen Pty Ltd (Sydney, Australia) in hydroxypropylmethylcellulose capsules containing 50 mg or 200 mg phenoxodiol.

7.2.5 Pretreatment and follow-up investigations

During the screening period the following were recorded: medical history, physical examination, chest X-ray, ECG, vital signs, Karnofsky performance status, full blood count including differential, serum biochemistry (electrolytes, urea, creatinine, liver function tests, calcium, phosphate, random blood sugar), and serum pregnancy test where relevant. Written informed consent was obtained. Laboratory testing was performed by the South Eastern Area Laboratory Service (SEALS). Glomerular

Filtration Rate (GFR) was estimated using the Cockcroft–Gault method at screening, then after every second cycle of treatment, and cisplatin or carboplatin dose was adjusted accordingly. Tumor size was assessed by CT scans at baseline and after cycles 3 and 6 of treatment in patients with measurable disease. Hematology (FBC) and serum biochemistry were performed weekly during cycle 1, then at mid-cycle and prior to day 1 treatment at each cycle thereafter. Urinalysis and tumor markers (where appropriate) were performed once per cycle. Karnofsky performance status was assessed at the beginning of each cycle.

7.2.6 Toxicity assessment

Toxicity was graded using the NCI Common Toxicity Criteria, version 2.0. Toxicity assessment, physical examination and vital signs were carried out on days 9 and 16 in the first cycle, then once every cycle thereafter.

7.2.7 Oral bioavailability and pharmacokinetic studies

During the two days preceding Day 1, Cycle 1 treatment, patients were randomized to either an oral or intravenous formulation of a single test dose of 1000mg phenoxodiol for pharmacokinetic (PK) measurements to assess oral bioavailability. For the first 14 patients, PK assessments were performed on consecutive days, based on our previous data that suggested a short half-life. Following the discovery that phenoxodiol was still detectable by start of the second day however, the protocol was amended to reduce the single test dose to 400mg and allow a 3 day gap between pharmacokinetic assessments. On both days, blood was drawn for PK measurements at time 0 (just before oral or IV

dose), then at 15, 30, 45, 60, 120, 180, 240, and 1440 minutes. Patients assigned carboplatin also had plasma samples drawn on Day 2, Cycle 1 at time 0 (just before observed oral phenoxodiol dose), then at 15, 30, 45, 60, 120, 180, 240, and 1440 minutes afterwards. The carboplatin infusion started around two hours after phenoxodiol and was administered over 1 hour. Patients assigned cisplatin had plasma samples drawn on Day 9, Cycle 1 at time 0 (just before observed oral phenoxodiol dose), and at 30, 50, 70, 90, 110, 130, 150, 180, 210, 240, and 1440 minutes afterwards. The cisplatin infusion commenced around 1 hour after the phenoxodiol dose, and was administered over 1 hour with the usual saline pre- and post-hyrdation. Once days with PK measurements had concluded, patients were asked to take phenoxodiol approximately every 8 hours daily from days 1-10 every cycle.

Plasma samples were centrifuged at the bedside and stored at -20^{0}C prior to analysis. Pharmacokinetic parameters were derived using non-compartmental methods with PK Solutions 2.0 (Summit Research Services, CO, USA). Oral bioavailability is defined as the ratio f = AUC (oral) / AUC (iv).

7.2.8 Phenoxodiol assay methods

Assays were performed at Novogen Research Laboratories, Sydney, Australia. The methodology was previously described (de Souza *et al*, 2006), and has been validated, but remains unpublished. In brief, free phenoxodiol was assayed using an HPLC-UV method and total phenoxodiol was assayed using an LC-MS (ESI-MRM) method, following incubation with ß-glucuronidase. The lower limit of quantitation (LOQ) for

free phenoxodiol was 0.25 µg/mL whereas the lower limit of quantitation (LOQ) for total phenoxodiol was 0.025 µg/mL.

7.2.9 Statistical analysis

All results are expressed as the mean ± standard deviation (SD) except where the median and range are presented.

7.3 Results

7.3.1 General

Twenty five patients were enrolled between April 2004 and July 2006. Three other patients had been enrolled on this study, but were replaced for the following reasons: death from pulmonary embolus after randomization but prior to treatment, diagnosis of brain metastases on staging CT scans after enrolment but prior to treatment, personal decision to withdraw after enrolment but prior to treatment (see below). Patient characteristics are listed in Table 13. The median age was 65 (range 48 - 79) and there were 16 males. Most patients had good Karnofsky performance status (90-100), and there were a variety of tumor histologies represented. All patients but three had received prior chemotherapy and just over half had failed at least 2 prior chemotherapy regimens, indicating a heavily pre-treated population (Table 13). A total of 99 cycles of phenoxodiol were administered and 10 patients completed all 6 planned cycles.

Table 13. Patient characteristics. Abbreviations: NSCLC, non-small cell lung cancer.

Characteristics	Number of Patients
Total	25
Age, years	
Median	65
Range	48-79
Gender	
Male	16
Female	9
Performance status, KPS	
100	12
90	7
80	4
70	2
Tumor type	
Ovary	6
Prostate	6
Renal	3
Melanoma	2
Leiomyosarcoma	2
Pancreas	2
Anal canal	1
Colorectal	1
NSCLC	1
Cholangiocarcinoma	1
Number of previous chemotherapy regimens	
0	3
1	9
2	5
3	3
≥ 4	5
Baseline weight, kg	
Mean	73.2
Range	52 - 112

7.3.2 Dose escalation

The dose escalation scheme is summarized in Table 14. No DLT was seen during the first 3 dose cohorts. DLT was encountered in two patients in cohort 4 (800 mg phenoxodiol dose level) during Cycle 1 (see below). Both recovered without sequelae, and were classified as serious adverse events. The recommended Phase II dose was defined as the dose level below the MTD: 400 mg oral phenoxodiol every 8 hours (Day

1-10) together with either intravenous cisplatin (50 mg/m^2 Day 2 and 9) or intravenous carboplatin (AUC=5 Day 2) in a 21 day cycle.

Table 14. Dose level cohorts and patient outcome. DLTs were ventricular tachycardia in a patient with previously implanted defibrillator for ventricular tachycardia, and exacerbation of emphysema in a patient with a longstanding history of emphysema.

Dose level	Phenoxodiol Dose	Mean dose (mg/m2) per cycle	No. of patients	No. of patients completing 6 cycles	No. of patients with DLT
1	50mg	26.8	6	2/6	0
2	100mg	56.9	6	2/6	0
3	400mg	217	6	3/6	0
4	800mg	446	7	3/7	2

7.3.3 Hematologic toxicities

As noted in our previous Phase I study (de Souza *et al*, 2006), lymphocytopenia was again a common adverse event in Cycle 1 as well as subsequent cycles (Tables 15 and 16). However, no complications occurred, and there were no episodes of infection. In keeping with the co-administration of carboplatin or cisplatin, anemia was also common, though usually mild (Tables 15 & 16). Lymphocytopenia occurred at all phenoxodiol dose levels. Other hematologic toxicity was mild, with fewer than 20% of patients experiencing Grade 2 or worse anemia, thrombocytopenia or leucopenia.

Table 15. Any toxicity, worst grade per patient during Cycle 1 (Denomimator n = 25). *One patient developed asymptomatic ventricular tachycardia, and *another patient was admitted with exacerbation of emphysema. Both were classified as serious adverse events, and as dose limiting toxicities.

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Laboratory				
Hemoglobin	7	2	1	
WBC	2	1		
Neutrophils	2			
Platelets	3			
Lymphocytes	5	9		
Creatinine	2			
Urea	1			
Alkaline phosphatase	5	1		
AST	3	1		
ALT	5			
GGT	6	2	2	
Albumin	2	4		
Hyperglycemia	16	1	1	
Hyperbilirubinemia	2			
Hyponatremia	3			
Hypokalemia	2		1	
Hypocalcemia	8	1		
Clinical				
Infections				
Fever (no infections)	2			
Anorexia	2	3		
Sweating	1			
Lethargy	7	3	2	
Nausea	6	1	1	
Loss of taste	2			
Vomiting	5			
Flatulence	1			
Reflux	2	1		
Constipation	6	4		
Diarrhea	4	2	1	
Pulmonary				1*
Cardiac				1*
Hypotension	1			
Hypertension	1		1	
Neurosensory	1			
Neuro-mood	2			
Neuro-headache	3			
Blurred vision	2			
Insomnia	1			
Skin rash	2			
Infusion site reaction	12			
Abdominal pain	4	1		
Hematuria	1			
Dysuria	1			

Table 16. Numbers of patients with Grade 2-4 hematologic toxicity, Cycles 2-6 (Denominator n = 25).

Toxicity	Grade 2	Grade 3	Grade 4
Haemoglobin	7/25		
WBC	3/25	2/25	
Neutrophils	3/25	3/25	
Lymphocytes	9/25	3/25	
Platelets	2/25	4/25	

7.3.4 Non-hematologic toxicities

Non-hematologic toxicities were varied (Table 16 for cycle 1 and Table 17 for cycles 2-6), but generally mild to moderate. Two serious adverse events and dose limiting toxicities were encountered, both at the 800 mg phenoxodiol dose level. One patient with metastatic hormone refractory prostate cancer developed asymptomatic ventricular tachycardia during Cycle 1, but he had a history of ventricular tachycardia 12 months prior to study entry and had been previously treated with an implantable defibrillator. We thought that the combination of phenoxodiol and cisplatin was possibly related to the adverse event. The other DLT, also in the 800 mg phenoxodiol dose cohort, was noted in a patient with metastatic anal carcinoma who was admitted to hospital for exacerbation of his emphysema. We assessed phenoxodiol and carboplatin as unlikely to be related to this serious adverse event. Three patients had been enrolled onto the study but were withdrawn for various reasons: one patient with metastatic hormone refractory prostate cancer withdrew his consent prior to starting treatment; another was diagnosed with brain metastases during screening investigations prior to the start of treatment and therefore became ineligible; the final patient died suddenly after signing the consent form from a pulmonary embolus, prior to screening investigations. None of these patients was counted as part of the study; they were not assessable for toxicity, and were replaced by others. Other non–hematologic toxicities were generally

infrequent (Table 17). GFR calculated by the Cockcroft–Gault formula fell by a mean of 22 mL/min in 8 patients, improved in 2 patients, whereas there was no change in 15 patients during the entire study.

Table 17. Numbers of patients with Grade 2-4 non–hematologic toxicity, Cycles 2-6 (Denominator n = 25).

Toxicity	Grade 2	Grade 3	Grade 4
Laboratory			
Creatinine / GFR	2		
Urea			
Alkaline phosphatase	1	1	
AST		1	
ALT			
GGT	4	2	
Albumin	7		
Hyperglycemia	8		
Hyperbilirubinemia			
Hyponatremia			
Hypokalemia		1	
Hypocalcemia	3	2	
Hypophosphatemia	2		
Hypomagnesemia		1	
Clinical			
Fever (no infections)	1		
Anorexia	2		
Lethargy	6	1	
Nausea	3		
Vomiting	2	1	
Reflux	1		
Constipation	4		
Diarrhea	1	2	
Hypotension	1	1	
Neuro-headache	2		
Skin rash	1		
Infusion site reaction	1		
Abdominal pain	2		
Ascites		2	
Back pain	3		

Of note were 18 patients who developed hyperglycemia (almost all Grade 1 only) during Cycle 1, of whom 7 had had prior elevated glucose levels at baseline. Hypocalcemia, hypoalbuminemia, lymphocytopenia, lethargy, and infusion site reactions were other relatively common adverse events, but most of these were mild,

and not unexpected with either carboplatin or cisplatin. One patient withdrew from the study after one cycle of treatment because of anxiety about possible side effects.

7.3.5 Responses

Three patients were unable to be evaluated because they had only one or part of one cycle of treatment. Seventeen patients had measurable disease, and five were evaluable for response by tumor marker only. Three patients, all with pre-treated ovarian cancer, had tumor marker responses to treatment. In one patient, CA125 normalised from 206 to 14 (complete response), whereas in another patient, CA125 fell 30% (from 153 to 105) over 6 cycles, and in the third, there was an initial rise from 699 to 838 after one cycle of treatment, but by the third cycle, this had steadily fallen to 483 (31% reduction from baseline). In one patient with metastatic hormone refractory prostate cancer, PSA fell from 1690 to 940 after the third cycle (45% reduction). Eleven patients had stable disease for a median of 10 weeks (range 9-21 weeks), and 10 patients had progressive disease on study.

7.3.6 Pharmacokinetics

Data from Cycle 1 in 9 patients was used to determine pharmacokinetic parameters (Table 18). Because persisting, but low, phenoxodiol concentrations could have complicated day 2 pharmacokinetic interpretation, no data from the first 14 patients will be considered here. Further, free phenoxodiol concentrations were inconsistently undetectable at varying time points for some patients after oral administration of phenoxodiol, making interpretation of pharmacokinetic data difficult. Only total

phenoxodiol concentrations are reported here unless otherwise stated. Phenoxodiol reached a mean maximum concentration (Cmax) of 2.5±0.8 µg/mL (= 10.5 µM) and 18.4±3.5 µg/mL (= 77 µM) for the free and total forms, respectively, after a single intravenous dose of 400 mg. Mean Cmax was 5.6±2.5 µg/mL total phenoxodiol (= 23.5 µM) after a single oral dose of 400 mg. The mean volume of distribution (V_D) was 15661mL (± 11189) for intravenous phenoxodiol, and 98990 mL (± 53130) for oral phenoxodiol. Phenoxodiol was rapidly eliminated with a mean clearance of 1637 mL/hr (±1321) and 2511 mL/hr (±1831) following intravenous and oral administration, respectively. Oral bioavailability of phenoxodiol is estimated to be 17.5% (f = 287 µg-hr/mL divided by 1637 µg-hr/mL, from Table 18). In general, very wide inter-patient variability in pharmacokinetic parameters was noted.

Table 18. Pharmacokinetic parameters for 9 patients randomised to a single dose of phenoxodiol 400 mg intravenously or orally on Day-7 and the alternate route administered on Day-4. (Mean ± SD for all figures).

Phenoxodiol	Cmax (µg/mL)	Tmax (hr)	AUC 0-∞ (hr*µg/mL)	V_D (mL)	CL (mL/hr)	T1/2 (hr)
Free (iv)	2.5 (±0.8)	1.5 (±0.6)	37.6 (±27.4)	18560 (±18130)	17889 (±14078)	0.6 (±0.3)
Total (iv)	18.4 (±3.5)	1.7 (±0.6)	1637 (±3941)	15661 (±11189)	1637 (±1321)	7.2 (±3.4)
Total (oral)	5.6 (±2.5)	5.7 (±7.4)	287 (±288)	98990 (±53130)	2511 (±1831)	22.6 (±11.1)

7.4 Discussion

Our Phase I trial demonstrates that oral phenoxodiol was well tolerated when combined with either cisplatin or carboplatin. Hyperglycemia was the main biochemical toxicity seen, although low-grade hypocalcemia and mildly abnormal transaminases also

occurred with reasonable frequency (Table 17). Infusion site reactions, constipation, nausea and lethargy were also fairly commonly noted, though generally mild and not unexpected with cisplatin or carboplatin. Two DLTs occurred, one of which was possibly related to study treatment and the other unlikely to be related to study treatment. Because one DLT occurred in the cisplatin group and the other in the carboplatin group, but both at the 800 mg phenoxodiol dose level, we considered expanding the cohorts to meet the definition of DLT for each platinum drug individually. However, given that 29/37 (78%) of all Grade 3 toxicities (Tables 15, 16, 17) also occurred at the 800 mg phenoxodiol dose level, we felt confident that true DLT had probably been reached without the need for further cohort expansion, and possibly exposing more patients to unnecessary toxicity. No hypersensitivity reactions were seen in this study, unlike our experience previously reported (de Souza *et al*, 2006).

We recently showed that phenoxodiol enhanced the activity of cisplatin in two human prostate cancer cell lines, DU145 and PC3 (McPherson *et al*, 2009). While most concentrations tested showed synergistic activity, some additivity and mild antagonism was also noted. In this study, we administered relatively high doses of oral phenoxodiol, (0.6 mg/kg to 15 mg/kg effectively, data not shown) so that mean concentrations of around 23 μM, commensurate with *in vitro* activity, were easily reached, even after only a single dose. Thus even though estimated bioavailabilty of oral phenoxodiol at 17.5% is not high, pharmacokinetic data from a 800 mg three times daily schedule suggests that steady state concentrations reach 20 – 37 μM, whereas lower doses of 100 mg three times a day produced concentrations up to 16 μM (data not shown). These data support our hypothesis that prolonged exposure through repeated oral dosing provides more favourable concentrations of phenoxodiol than a once a week intravenous schedule.

One of the issues with synergy studies however, is that toxicity may also be enhanced quite apart from tumour growth inhibition. This may explain the different toxicity profile encountered with the combination not entirely consistent with either enhanced phenoxodiol toxicity or platinum toxicity. For instance, although lymphocytopenia was commonly encountered in this study, consistent with our experience in the weekly intravenous schedule, the striking toxicity noted here was hyperglycemia. This was also a common toxicity noted in some mTOR studies (Hudes *et al*, 2007; Wolpin *et al*, 2009), and we hypothesize that our results could provide supporting clinical evidence that phenoxodiol and cisplatin inhibit the Akt / mTOR pathway. Our preclinical data (Chapter 4, Figures 12-14) provide evidence that is consistent with this hypothesis.

In our weekly phenoxodiol Phase I study we did not see any objective tumor responses, possibly because phenoxodiol was not present for sufficiently prolonged periods. However, one patient with androgen independent prostate cancer who had failed two prior hormone regimens did have stable PSA levels in that study, along with another man with metastatic renal cancer who had stable disease for 6 months. In the current study, one woman with ovarian cancer had a complete response according to CA125 criteria, but it is entirely possible that the platinum was the active agent, and not the combination with phenoxodiol. On the other hand, we did see minor activity in 3 other patients, including a man with metastatic hormone refractory prostate cancer who had a 45% reduction in PSA level after 3 cycles. Clearly, while this study cannot determine response rates, the early activity seen with this combination is of interest, given that phenoxodiol was given for only 10 days in a 21 day cycle.

Concentrations of free phenoxodiol were found to be low in the current study, contributing perhaps 10% of total phenoxodiol. This is consistent with extensive conjugation, a phenomenon previously described with genistein (Doerge *et al*, 2000; Bursztyka *et al*, 2008) and also noted in our first-in-human study (Chapter 5). In dose - escalation studies of isoflavones, pharmacokinetic parameters for free and total genistein and daidzein were similar, though a little longer than for phenoxodiol noted in this study (Bloedon *et al*, 2002; Busby *et al*, 2002; Setchell *et al*, 2003). Estimates of genistein and daidzein bioavailability in the literature have been hampered by the lack of data from comparative intravenous administration of the same compound. Nevertheless, using measurements of isoflavones and metabolites recovered in the urine, estimates of bioavailability have ranged between 20-50% (Setchell *et al*, 2003; Zubik and Meydani, 2003). As previously noted (Setchell *et al*, 2003), urine concentrations of isoflavones correlated poorly with serum concentrations, possibly because of the wide range of inter-individual variability in pharmacokinetic parameters, a feature of many studies. Our data would appear to be consistent with the literature.

In conclusion, phenoxodiol is a novel isoflavone that is well tolerated when given in combination with either intravenous cisplatin or carboplatin. The main adverse events include hyperglycemia, hypocalcemia, mild transaminase rises, as well as nausea, constipation, infusion site reactions and lethargy. Encouraging, though modest, activity is seen when phenoxodiol is administered orally for 10 days in a 21 day cycle together with either cisplatin or carboplatin.

8. General discussion and future directions

In the work presented in this thesis, we show that phenoxodiol is a novel isoflavone that has promising activity in prostate cancer. Phenoxodiol inhibits the growth of two standard human prostate cancer cell lines, DU145 and PC3, at concentrations that are clinically achievable. Given the observation that isoflavones can concentrate in the prostate gland (Hedlund *et al*, 2005; Rannikko *et al*, 2006b; Gardner *et al*, 2009), it would appear that more than sufficient phenoxodiol can be available to the prostate to act as a tumor suppressor. Further, phenoxodiol has synergistic or additive activity when used with cisplatin and carboplatin in DU145 and PC3 cells, though this association may not be as strong with other cell lines that were tested. In the nude mouse DU145 xenograft model, the combination of cisplatin and phenoxodiol inhibited growth of DU145 cells to the same extent as single agent cisplatin given at twice the dose, and with lower toxicity. The design of the experiment is a mathematically derived model to test synergy *in vivo*, and is based entirely on the Chou-Talalay mass action equation (Chapter 4). This result is important because it demonstrates that our data derived *in vitro* probably hold true *in vivo*, and because it demonstrates the proof of principle that doses of phenoxodiol and cisplatin can be reduced without loss of benefit in the first instance, potentially resulting in lower toxicity, at least in mice. Experiments to investigate the mechanism of synergy between phenoxodiol and cisplatin suggested that a pharmacodynamic, and not a pharmacokinetic, mechanism was in play, and that uptake of cisplatin into tumour tissue may have been enhanced. This resulted in markedly increased numbers of cisplatin–DNA adducts, a surrogate measure of cisplatin cytotoxicity that correlates with cell death. However, this pharmacological advantage may not have been the sole reason for synergy, as we also showed for the first time, that

phenoxodiol can inhibit phospho-Akt and mTOR signaling in prostate cancer cell lines. Although the results vary depending on the cell line, and also vary depending on whether phenoxodiol is used alone or in combination, we interpret the data as broadly supportive of suppression of the Akt / mTOR pathway particularly when cisplatin or carboplatin are used in combination with phenoxodiol. Further, inhibition of c-FLIP implies a pro-apoptotic mechanism for the combination. Since the Akt / mTOR pathway is arguably one of the key pathways in prostate cancer cell signaling and survival (Chapter 2), the combination of phenoxodiol and platinums could have a promising role in the treatment of prostate cancer.

Our first-in-man study of phenoxodiol pharmacokinetics (Chapter 5) showed that the compound had a short half-life, and that plasma levels achieved were commensurate with doses and concentrations used in the literature that produced a variety of biological effects including apoptosis of cancer cells. The drug appeared to be extensively conjugated, consistent with the pharmacokinetic literature on genistein and daidzein. There was little acute toxicity from a single dose given as a bolus or from a prolonged intravenous infusion.

Intravenous phenoxodiol given as an infusion over 1-2 hrs every week was well tolerated and associated with minimal toxicity (Chapter 6). The predominant toxicity was asymptomatic lymphocytopenia. One patient with renal cancer had stable disease of at least 6 months. Again, the pharmacokinetic data confirmed a short half-life, suggesting that a weekly intravenous schedule may not be the best way to optimize tumour exposure to the drug in subsequent clinical trials.

Oral bioavailability of phenoxodiol was estimated at 17.5% (Chapter 7), perhaps a little less that that noted with genistein and daidzein. Nevertheless, phenoxodiol was bioavailable at concentrations that were above that required for biological activity. Since the compound was given three times a day, we speculate that this schedule would be more favourable in terms of anticancer activity compared to the weekly intravenous schedule. In combination with cisplatin or carboplatin, oral phenoxodiol was also well tolerated, with the majority of toxicity attributable to the platinum drugs. Some modest activity was seen with the combination, particularly in ovarian cancer patients and a patient with castrate resistant prostate cancer. We cannot categorically state that any of the responses seen was due to phenoxodiol, as they could conceivably have happened with cisplatin or carboplatin alone, but the clinical circumstances in the patients who did respond would seem to suggest that the combination had some part to play.

In a sense, the work in this thesis raises more questions than it answers. First, while the pharmacological and pharmacokinetic properties of phenoxodiol appear to be similar to other isoflavones reported in the literature, there appear to be some material differences. The novel finding of mTOR and pAkt inhibition in DU145 and PC3 cells require further confirmation and investigation. In particular, it is important to determine whether phenoxodiol acts further upstream and downstream of Akt and mTOR. Even if other biological effects are found, it is puzzling why this compound might work at so many different sites in the cancer cell. One possible explanation is that the drug is acting at a very fundamental level. On the other hand, the variety of concentrations and doses used in the literature raises the question of whether some, if not many, of the proposed biological effects may not be relevant in the clinical situation. If, as the literature suggests, the prostate can concentrate isoflavones, then the question of dose - effect

correlations becomes less of an issue, since doses achieved in prostatic tissue would presumably be more than sufficient for treatment effects. Further work to develop these lines of investigation could include studies on absorption and distribution of phenoxodiol, including if possible, samples from the prostate gland. The question of microbacterial environment in the gut and colon and absorption of isoflavones is intriguing, and it would be important to know for example, whether oral phenoxodiol was subject to the same variation in absorption as for genistein and daidzein, and whether "equol producers" would have better or worse absorption and distribution than other patients. Finally, correlative studies with the extent of absorption and dose of oral phenoxodiol with outcome (tumor marker response, clinical response) and biological markers would also be of interest.

One limitation of our clinical studies is that we did not include biological samples that might speak to the potential mechanism of action of phenoxodiol in humans, at relevant and achievable concentrations. However, given the lack of knowledge about the drug at the time we instigated these studies, we did not wish to embark on a range of speculative experiments. Nevertheless, we think it is important that further translational work be undertaken in the development of the drug. In particular, its effects on other important signal transduction pathways including Ras / MAPK and the apoptotic cascade, would be of interest. At one level, if phenoxodiol "works" as a chemotherapy sensitiser, or put another way, is able to overcome inherent cancer cell resistance, then an important class of compounds will have been discovered. At another level however, in the context of our limited clinical studies and experience, and notwithstanding that Phase I studies are not designed to test for efficacy, phenoxodiol does not seem to have sufficient potency by itself. Ultimately, the compound may be best utilised in

combination with other drugs and chemotherapeutic agents. Hence further work on potential synergistic partner compounds is essential. One could conceive that its combination with many other targeted agents such as VEGF antagonists (eg. sunitinib, sorafenib, bevacizumab and many others) and signal transduction blockers (eg. c-met inhibitors, farnesyl transferase inhibitors) would be of interest. Given its promise for the treatment of prostate cancer as the underlying theme of this thesis, phenoxodiol should probably also be tested in combination with satraplatin, a platinum analogue already in development for the treatment of prostate cancer.

9. References

Adams KF, Chen C, Newton KM, Potter JD, Lampe JW (2004) Soy isoflavones do not modulate prostate-specific antigen concentrations in older men in a randomized controlled trial. *Cancer Epidemiol Biomarkers Prev* **13:** 644-8

Adlercreutz H (1990) Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scandinavian journal of clinical and laboratory investigation* **201:** 3-23

Adlercreutz H (2002) Phyto-oestrogens and cancer. *The Lancet Oncology* **3:** 364-73

Adlercreutz H, Markkanen H, Watanabe S (1993) Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* **342:** 1209-10

Agarwal C, Sharma Y, Agarwal R (2000) Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis. *Mol Carcinog* **28:** 129-38

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

Aguero MF, Facchinetti MM, Sheleg Z, Senderowicz AM (2005) Phenoxodiol, a novel isoflavone, induces G1 arrest by specific loss in cyclin-dependent kinase 2 activity by p53-independent induction of p21WAF1/CIP1. *Cancer research* **65:** 3364-73

Akaza H, Miyanaga N, Takashima N, Naito S, Hirao Y, Tsukamoto T, Fujioka T, Mori M, Kim WJ, Song JM, Pantuck AJ (2004) Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. *Jpn J Clin Oncol* **34:** 86-9

Akaza H, Miyanaga N, Takashima N, Naito S, Hirao Y, Tsukamoto T, Mori M (2002) Is daidzein non-metabolizer a high risk for prostate cancer? A case-controlled study of serum soybean isoflavone concentration. *Jpn J Clin Oncol* **32:** 296-300

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262:** 5592-5

Allen NE, Appleby PN, Davey GK, Key TJ (2001) Soy milk intake in relation to serum sex hormone levels in British men. *Nutr Cancer* **41:** 41-6

Altomare DA, Testa JR (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24:** 7455-64

Alvero AB, O'Malley D, Brown D, Kelly G, Garg M, Chen W, Rutherford T, Mor G (2006) Molecular mechanism of phenoxodiol-induced apoptosis in ovarian carcinoma cells. *Cancer* **106:** 599-608

Anderson JW, Smith BM, Washnock CS (1999) Cardiovascular and renal benefits of dry bean and soybean intake. *The American journal of clinical nutrition* **70:** 464S-474S

Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal B, Kondo Y (2007) Evidence That Curcumin Suppresses the Growth of Malignant Gliomas In Vitro and In Vivo Through Induction of Autophagy: Role of Akt and ERK Signaling Pathways. *Mol Pharmacol* **72:** 29-39.

Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E (2001) The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* **276:** 35243-6

Aronson WJ, Tymchuk CN, Elashoff RM, McBride WH, McLean C, Wang H, Heber D (1999) Decreased growth of human prostate LNCaP tumors in SCID mice fed a low-fat, soy protein diet with isoflavones. *Nutr Cancer* **35:** 130-6

Arsham AM, Plas DR, Thompson CB, Simon MC (2004) Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. *Cancer research* **64:** 3500-7

Assikis VJ, Do KA, Wen S, Wang X, Cho-Vega JH, Brisbay S, Lopez R, Logothetis CJ, Troncoso P, Papandreou CN, McDonnell TJ (2004) Clinical and biomarker correlates of androgen-independent, locally aggressive prostate cancer with limited metastatic potential. *Clin Cancer Res* **10:** 6770-8

Atkinson C, Frankenfeld CL, Lampe JW (2005) Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood)* **230:** 155-70

Axanova L, Morre DJ, Morre DM (2005) Growth of LNCaP cells in monoculture and coculture with osteoblasts and response to tNOX inhibitors. *Cancer letters* **225:** 35-40

Ayala G, Thompson T, Yang G, Frolov A, Li R, Scardino P, Ohori M, Wheeler T, Harper W (2004) High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res* **10:** 6572-8

Bader AG, Kang S, Zhao L, Vogt PK (2005) Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* **5:** 921-9

Banerjee S, Li Y, Wang Z, Sarkar FH (2008) Multi-targeted therapy of cancer by genistein. *Cancer letters* **269:** 226-42

Barnes K, McIntosh E, Whetton AD, Daley GQ, Bentley J, Baldwin SA (2005) Chronic myeloid leukaemia: an investigation into the role of Bcr-Abl-induced abnormalities in glucose transport regulation. *Oncogene* **24:** 3257-67

Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL, Malinowski J, McAvoy EM, Nahas DD, Robinson RG, Huber HE (2005) Identification and characterization of pleckstrin-homology-domain-

dependent and isoenzyme-specific Akt inhibitors. *The Biochemical journal* **385:** 399-408

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Benedetti Panici P, Mancuso S, Neri G, Testa JR (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *International journal of cancer* **64:** 280-5

Bemis DL, Capodice JL, Desai M, Buttyan R, Katz AE (2004) A concentrated aglycone isoflavone preparation (GCP) that demonstrates potent anti-prostate cancer activity in vitro and in vivo. *Clin Cancer Res* **10:** 5282-92

Bergan R, Kyle E, Nguyen P, Trepel J, Ingui C, Neckers L (1996) Genistein-stimulated adherence of prostate cancer cells is associated with the binding of focal adhesion kinase to beta-1-integrin. *Clinical & experimental metastasis* **14:** 389-98

Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA (2004) Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* **114:** 928-36

Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, O'Reilly T, Natt F, Hall J, Lane HA, Thomas G (2005) The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. *Cell* **120:** 747-59

Bhatia N, Agarwal R (2001) Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells. *Prostate* **46:** 98-107

Bible KC, Kaufmann SH (1997) Cytotoxic synergy between flavopiridol (NSC 649890, L86-8275) and various antineoplastic agents: the importance of sequence of administration. *Cancer research* **57:** 3375-80

Bloedon LT, Jeffcoat AR, Lopaczynski W, Schell MJ, Black TM, Dix KJ, Thomas BF, Albright C, Busby MG, Crowell JA, Zeisel SH (2002) Safety and pharmacokinetics of purified soy isoflavones: single-dose administration to postmenopausal women. *The American journal of clinical nutrition* **76:** 1126-37

Blume-Jensen P, Hunter T (2001) Oncogenic kinase signalling. *Nature* **411:** 355-65

Booth NL, Overk CR, Yao P, Burdette JE, Nikolic D, Chen SN, Bolton JL, van Breemen RB, Pauli GF, Farnsworth NR (2006) The chemical and biologic profile of a red clover (Trifolium pratense L.) phase II clinical extract. *Journal of alternative and complementary medicine (New York, NY)* **12:** 133-9

Bosetti C, Bravi F, Talamini R, Parpinel M, Gnagnarella P, Negri E, Montella M, Lagiou P, Franceschi S, La Vecchia C (2006) Flavonoids and prostate cancer risk: a study in Italy. *Nutr Cancer* **56:** 123-7

Boulay A, Rudloff J, Ye J, Zumstein-Mecker S, O'Reilly T, Evans DB, Chen S, Lane HA (2005) Dual inhibition of mTOR and estrogen receptor signaling in vitro induces cell death in models of breast cancer. *Clin Cancer Res* **11:** 5319-28

Brazil DP, Yang ZZ, Hemmings BA (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* **29:** 233-42

Brossner C, Petritsch K, Fink K, Auprich M, Madersbacher S, Adlercreutz H, Rehak P, Petritsch P (2004) Phytoestrogen tissue levels in benign prostatic hyperplasia and prostate cancer and their association with prostatic diseases. *Urology* **64:** 707-11

Brown DM, Kelly GE, Husband AJ (2005) Flavonoid compounds in maintenance of prostate health and prevention and treatment of cancer. *Molecular biotechnology* **30:** 253-70

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96:** 857-68

Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, Dalal SN, DeCaprio JA, Greenberg ME, Yaffe MB (2002) 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol* **156:** 817-28

Burgering BM, Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376:** 599-602

Burich RA, Holland WS, Vinall RL, Tepper C, White RW, Mack PC (2008) Genistein combined polysaccharide enhances activity of docetaxel, bicalutamide and Src kinase inhibition in androgen-dependent and independent prostate cancer cell lines. *BJU international* **102:** 1458-66

Bursztyka J, Perdu E, Tulliez J, Debrauwer L, Delous G, Canlet C, De Sousa G, Rahmani R, Benfenati E, Cravedi JP (2008) Comparison of genistein metabolism in rats and humans using liver microsomes and hepatocytes. *Food Chem Toxicol* **46:** 939-948

Busby MG, Jeffcoat AR, Bloedon LT, Koch MA, Black T, Dix KJ, Heizer WD, Thomas BF, Hill JM, Crowell JA, Zeisel SH (2002) Clinical characteristics and pharmacokinetics of purified soy isoflavones: single-dose administration to healthy men. *The American journal of clinical nutrition* **75:** 126-36

Bylund A, Zhang JX, Bergh A, Damber JE, Widmark A, Johansson A, Adlercreutz H, Aman P, Shepherd MJ, Hallmans G (2000) Rye bran and soy protein delay growth and increase apoptosis of human LNCaP prostate adenocarcinoma in nude mice. *Prostate* **42:** 304-14

Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science* **296:** 1655-7

Cantley LC, Neel BG (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 4240-5

Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, Lu B (2006a) Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer research* **66:** 10040-7

Cao F, Jin TY, Zhou YF (2006b) Inhibitory effect of isoflavones on prostate cancer cells and PTEN gene. *Biomed Environ Sci* **19:** 35-41

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282:** 1318-21

Carpenter TO, Gerloczy A, Pitha J (1995) Safety of parenteral hydroxypropyl beta-cyclodextrin. *Journal of pharmaceutical sciences* **84:** 222-5

Castle EP, Thrasher JB (2002) The role of soy phytoestrogens in prostate cancer. *The Urologic clinics of North America* **29:** 71-81, viii-ix

Cazzolli R, Carpenter L, Biden TJ, Schmitz-Peiffer C (2001) A role for protein phosphatase 2A-like activity, but not atypical protein kinase Czeta, in the inhibition of protein kinase B/Akt and glycogen synthesis by palmitate. *Diabetes* **50:** 2210-8

Chan J, Bayliss PE, Wood JM, Roberts TM (2002) Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell* **1:** 257-67

Chan JM, Gann PH, Giovannucci EL (2005) Role of diet in prostate cancer development and progression. *J Clin Oncol* **23:** 8152-60

Chang CJ, Freeman DJ, Wu H (2004) PTEN regulates Mdm2 expression through the P1 promoter. *J Biol Chem* **279:** 29841-8

Chau MN, El Touny LH, Jagadeesh S, Banerjee PP (2007) Physiologically achievable concentrations of genistein enhance telomerase activity in prostate cancer cells via the activation of STAT3. *Carcinogenesis* **28:** 2282-90

Chavarro JE, Stampfer MJ, Li H, Campos H, Kurth T, Ma J (2007) A prospective study of polyunsaturated fatty acid levels in blood and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* **16:** 1364-70

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proceedings of the National Academy of Sciences of the United States of America* **89:** 9267-71

Chesire DR, Ewing CM, Gage WR, Isaacs WB (2002) In vitro evidence for complex modes of nuclear beta-catenin signaling during prostate growth and tumorigenesis. *Oncogene* **21:** 2679-94

Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advanced Enzyme Regulation* **22:** 27 - 55

Choueiri TK, Mekhail T, Hutson TE, Ganapathi R, Kelly GE, Bukowski RM (2006a) Phase I trial of phenoxodiol delivered by continuous intravenous infusion in patients with solid cancer. *Ann Oncol* **17:** 860-5

Choueiri TK, Wesolowski R, Mekhail TM (2006b) Phenoxodiol: isoflavone analog with antineoplastic activity. *Current oncology reports* **8:** 104-7

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365:** 855-9

Cinar B, Mukhopadhyay NK, Meng G, Freeman MR (2007) Phosphoinositide 3-kinase-independent non-genomic signals transit from the androgen receptor to Akt1 in membrane raft microdomains. *J Biol Chem* **282:** 29584-93

Clubbs EA, Bomser JA (2007) Glycitein activates extracellular signal-regulated kinase via vascular endothelial growth factor receptor signaling in nontumorigenic (RWPE-1) prostate epithelial cells. *J Nutr Biochem* **18**(8): 525-32

Cohen LA, Zhao Z, Pittman B, Scimeca J (2003) Effect of soy protein isolate and conjugated linoleic acid on the growth of Dunning R-3327-AT-1 rat prostate tumors. *Prostate* **54:** 169-80

Collins BM, McLachlan JA, Arnold SF (1997) The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids* **62:** 365-72

Constantinou AI, Husband A (2002) Phenoxodiol (2H-1-benzopyran-7-0,1,3-(4-hydroxyphenyl)), a novel isoflavone derivative, inhibits DNA topoisomerase II by stabilizing the cleavable complex. *Anticancer research* **22:** 2581-5

Constantinou AI, Mehta R, Husband A (2003) Phenoxodiol, a novel isoflavone derivative, inhibits dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats. *Eur J Cancer* **39:** 1012-8

Dahia PL (2000) PTEN, a unique tumor suppressor gene. *Endocr Relat Cancer* **7:** 115-29

Dalais FS, Meliala A, Wattanapenpaiboon N, Frydenberg M, Suter DA, Thomson WK, Wahlqvist ML (2004) Effects of a diet rich in phytoestrogens on prostate-specific antigen and sex hormones in men diagnosed with prostate cancer. *Urology* **64:** 510-5

Dan HC, Sun M, Yang L, Feldman RI, Sui XM, Ou CC, Nellist M, Yeung RS, Halley DJ, Nicosia SV, Pledger WJ, Cheng JQ (2002) Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. *J Biol Chem* **277:** 35364-70

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:** 231-41

Davidson B, Nesland JM, Goldberg I, Kopolovic J, Gotlieb WH, Bryne M, Ben-Baruch G, Berner A, Reich R (2001) Caveolin-1 expression in advanced-stage ovarian carcinoma--a clinicopathologic study. *Gynecol Oncol* **81:** 166-71

Davies SL, Bozzo J (2006) Spotlight on tNOX: a tumor-selective target for cancer therapies. *Drug news & perspectives* **19:** 223-5

Davis JN, Kucuk O, Sarkar FH (1999) Genistein inhibits NF-kappa B activation in prostate cancer cells. *Nutr Cancer* **35:** 167-74

Davis JN, Muqim N, Bhuiyan M, Kucuk O, Pienta KJ, Sarkar FH (2000) Inhibition of prostate specific antigen expression by genistein in prostate cancer cells. *International journal of oncology* **16:** 1091-7

Davis JN, Singh B, Bhuiyan M, Sarkar FH (1998) Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer* **32:** 123-31

De Luca T, Morre DM, Zhao H, Morre DJ (2005) NAD+/NADH and/or CoQ/CoQH2 ratios from plasma membrane electron transport may determine ceramide and sphingosine-1-phosphate levels accompanying G1 arrest and apoptosis. *BioFactors (Oxford, England)* **25:** 43-60

de Souza PL, Castillo M, Myers CE (1997) Enhancement of paclitaxel activity against hormone-refractory prostate cancer cells in vitro and in vivo by quinacrine. *Br J Cancer* **75:** 1593-600

de Souza PL, Liauw W, Links M, Pirabhahar S, Kelly G, Howes LG (2006) Phase I and pharmacokinetic study of weekly NV06 (Phenoxodiol), a novel isoflav-3-ene, in patients with advanced cancer. *Cancer chemotherapy and pharmacology* **58:** 427-33

Dell'Eva R, Ambrosini C, Minghelli S, Noonan DM, Albini A, Ferrari N (2007) The Akt inhibitor deguelin, is an angiopreventive agent also acting on the NF-kappaB pathway. *Carcinogenesis* **28:** 404-13

Deocampo ND, Huang H, Tindall DJ (2003) The role of PTEN in the progression and survival of prostate cancer. *Minerva Endocrinol* **28:** 145-53

deVere White RW, Hackman RM, Soares SE, Beckett LA, Li Y, Sun B (2004) Effects of a genistein-rich extract on PSA levels in men with a history of prostate cancer. *Urology* **63:** 259-63

Di Cristofano A, Pandolfi PP (2000) The multiple roles of PTEN in tumor suppression. *Cell* **100:** 387-90

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399:** 601-5

Doerge DR, Chang HC, Churchwell MI, Holder CL (2000) Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry. *Drug Metab Dispos* **28:** 298-307

Dong JT (2001) Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev* **20:** 173-93

Dudkin L, Dilling MB, Cheshire PJ, Harwood FC, Hollingshead M, Arbuck SG, Travis R, Sausville EA, Houghton PJ (2001) Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. *Clin Cancer Res* **7:** 1758-64

Easton JB, Houghton PJ (2004) Therapeutic potential of target of rapamycin inhibitors. *Expert opinion on therapeutic targets* **8:** 551-64

Easton JB, Houghton PJ (2006) mTOR and cancer therapy. *Oncogene* **25:** 6436-46

Edwards J, Krishna NS, Witton CJ, Bartlett JM (2003) Gene amplifications associated with the development of hormone-resistant prostate cancer. *Clin Cancer Res* **9:** 5271-81

Edwards J, Traynor P, Munro AF, Pirret CF, Dunne B, Bartlett JM (2006) The role of HER1-HER4 and EGFRvIII in hormone-refractory prostate cancer. *Clin Cancer Res* **12:** 123-30

El Touny LH, Banerjee PP (2007) Akt GSK-3 pathway as a target in genistein-induced inhibition of TRAMP prostate cancer progression toward a poorly differentiated phenotype. *Carcinogenesis* **28:** 1710-7

Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer research* **64:** 3892-9

Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Janne PA (2007) MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling. *Science* **316(5827):**1039-43

Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM (2007) Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med* **356:** 125-34

Evans BA, Griffiths K, Morton MS (1995) Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. *The Journal of endocrinology* **147:** 295-302

Fang M, Chen D, Yang CS (2007) Dietary polyphenols may affect DNA methylation. *J Nutr* **137:** 223S-228S

Fang MZ, Chen D, Sun Y, Jin Z, Christman JK, Yang CS (2005) Reversal of hypermethylation and reactivation of p16INK4a, RARbeta, and MGMT genes by genistein and other isoflavones from soy. *Clin Cancer Res* **11:** 7033-41

Feilotter HE, Nagai MA, Boag AH, Eng C, Mulligan LM (1998) Analysis of PTEN and the 10q23 region in primary prostate carcinomas. *Oncogene* **16:** 1743-8

Fine SW, Lisanti MP, Galbiati F, Li M (2001) Elevated expression of caveolin-1 in adenocarcinoma of the colon. *Am J Clin Pathol* **115:** 719-24

Fischer L, Mahoney C, Jeffcoat AR, Koch MA, Thomas BE, Valentine JL, Stinchcombe T, Boan J, Crowell JA, Zeisel SH (2004) Clinical characteristics and pharmacokinetics of purified soy isoflavones: multiple-dose administration to men with prostate neoplasia. *Nutr Cancer* **48:** 160-70

Franke AA, Custer LJ, Cerna CM, Narala K (1995) Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY* **208:** 18-26

Fritz WA, Wang J, Eltoum IE, Lamartiniere CA (2002) Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate. *Mol Cell Endocrinol* **186:** 89-99

Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399:** 597-601

Gamble JR, Xia P, Hahn CN, Drew JJ, Drogemuller CJ, Brown D, Vadas MA (2006) Phenoxodiol, an experimental anticancer drug, shows potent antiangiogenic properties in addition to its antitumour effects. *International journal of cancer* **118:** 2412-20

Gao H, Ouyang X, Banach-Petrosky WA, Gerald WL, Shen MM, Abate-Shen C (2006) Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 14477-82

Gao S, Liu GZ, Wang Z (2004) Modulation of androgen receptor-dependent transcription by resveratrol and genistein in prostate cancer cells. *Prostate* **59:** 214-25

Gao T, Furnari F, Newton AC (2005) PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell* **18:** 13-24

Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G (2003) Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* **11:** 1457-66

Gardner CD, Oelrich B, Liu JP, Feldman D, Franke AA, Brooks JD (2009) Prostatic soy isoflavone concentrations exceed serum levels after dietary supplementation. *Prostate* **69**(7): 719-26

Geller J, Sionit L, Partido C, Li L, Tan X, Youngkin T, Nachtsheim D, Hoffman RM (1998) Genistein inhibits the growth of human-patient BPH and prostate cancer in histoculture. *Prostate* **34:** 75-9

Gercel-Taylor C, Feitelson AK, Taylor DD (2004) Inhibitory effect of genistein and daidzein on ovarian cancer cell growth. *Anticancer research* **24:** 795-800

Ghosh PM, Malik SN, Bedolla RG, Wang Y, Mikhailova M, Prihoda TJ, Troyer DA, Kreisberg JI (2005) Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. *Endocr Relat Cancer* **12:** 119-34

Giri D, Ittmann M (1999) Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma. *Hum Pathol* **30:** 419-24

Gnanapragasam VJ, Leung HY, Pulimood AS, Neal DE, Robson CN (2001) Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. *Br J Cancer* **85:** 1928-36

Goh M, Chen F, Paulsen MT, Yeager AM, Dyer ES, Ljungman M (2001) Phenylbutyrate attenuates the expression of Bcl-X(L), DNA-PK, caveolin-1, and VEGF in prostate cancer cells. *Neoplasia* **3:** 331-8

Gopalakrishnan A, Xu CJ, Nair SS, Chen C, Hebbar V, Kong AN (2006) Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. *Arch Pharm Res* **29:** 633-44

Gossner G, Choi M, Tan L, Fogoros S, Griffith KA, Kuenker M, Liu JR (2007) Genistein-induced apoptosis and autophagocytosis in ovarian cancer cells. *Gynecol Oncol* **105:** 23-30

Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, Paul JD, Hbaiu A, Goode RG, Sandusky GE, Vessella RL, Neubauer BL (2000) Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J Biol Chem* **275:** 24500-5

Gray IC, Stewart LM, Phillips SM, Hamilton JA, Gray NE, Watson GJ, Spurr NK, Snary D (1998) Mutation and expression analysis of the putative prostate tumour-suppressor gene PTEN. *Br J Cancer* **78:** 1296-300

Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* **24:** 7410-25

Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol* **65:** 391-426

Guo Y, Wang S, Hoot DR, Clinton SK (2007) Suppression of VEGF-mediated autocrine and paracrine interactions between prostate cancer cells and vascular endothelial cells by soy isoflavones. *J Nutr Biochem* **18:** 408-17

Habito RC, Montalto J, Leslie E, Ball MJ (2000) Effects of replacing meat with soyabean in the diet on sex hormone concentrations in healthy adult males. *Br J Nutr* **84:** 557-63

Hamilton-Reeves JM, Rebello SA, Thomas W, Slaton JW, Kurzer MS (2007) Isoflavone-rich soy protein isolate suppresses androgen receptor expression without altering estrogen receptor-beta expression or serum hormonal profiles in men at high risk of prostate cancer. *J Nutr* **137:** 1769-75

Heald CL, Ritchie MR, Bolton-Smith C, Morton MS, Alexander FE (2007) Phyto-oestrogens and risk of prostate cancer in Scottish men. *Br J Nutr* **98:** 388-96

Hedlund TE, Johannes WU, Miller GJ (2003) Soy isoflavonoid equol modulates the growth of benign and malignant prostatic epithelial cells in vitro. *Prostate* **54:** 68-78

Hedlund TE, Maroni PD, Ferucci PG, Dayton R, Barnes S, Jones K, Moore R, Ogden LG, Wahala K, Sackett HM, Gray KJ (2005) Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. *J Nutr* **135:** 1400-6

Hedlund TE, van Bokhoven A, Johannes WU, Nordeen SK, Ogden LG (2006) Prostatic fluid concentrations of isoflavonoids in soy consumers are sufficient to inhibit growth of benign and malignant prostatic epithelial cells in vitro. *Prostate* **66:** 557-66

Hempstock J, Kavanagh JP, George NJ (1998) Growth inhibition of prostate cell lines in vitro by phyto-oestrogens. *British journal of urology* **82:** 560-3

Herst PM, Petersen T, Jerram P, Baty J, Berridge MV (2007) The antiproliferative effects of phenoxodiol are associated with inhibition of plasma membrane electron transport in tumour cell lines and primary immune cells. *Biochem Pharmacol* **74:** 1587-95

Honda T, Kagawa S, Spurgers KB, Gjertsen BT, Roth JA, Fang B, Lowe SL, Norris JS, Meyn RE, McDonnell TJ (2002) A recombinant adenovirus expressing wild-type Bax induces apoptosis in prostate cancer cells independently of their Bcl-2 status and androgen sensitivity. *Cancer Biology and Therapeutics* **1:** 163-167

Howes J, Waring M, Huang L, Howes LG (2002) Long-term pharmacokinetics of an extract of isoflavones from red clover (Trifolium pratense). *Journal of alternative and complementary medicine (New York, NY)* **8:** 135-42

Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC (2004) IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **117:** 225-37

Huang X, Chen S, Xu L, Liu Y, Deb DK, Platanias LC, Bergan RC (2005) Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer research* **65:** 3470-8

Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J, McDermott D, Bodrogi I, Kovacevic Z, Lesovoy V, Schmidt-Wolf IG, Barbarash O, Gokmen E, O'Toole T, Lustgarten S, Moore L, Motzer RJ (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. *N Engl J Med* **356:** 2271-81

Hur HG, Lay JO, Jr., Beger RD, Freeman JP, Rafii F (2000) Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin. *Archives of microbiology* **174:** 422-8

Hussain M, Banerjee M, Sarkar FH, Djuric Z, Pollak MN, Doerge D, Fontana J, Chinni S, Davis J, Forman J, Wood DP, Kucuk O (2003) Soy isoflavones in the treatment of prostate cancer. *Nutr Cancer* **47:** 111-7

Hutson T, Plavney D, Mekhail T, Dreicer R, Budd GT, Peereboom D, Olencki T, Kelly G, Ganapathi R, Bukowski R (2003) A dose finding and pharmacokinetic study of the novel isoflavanoid phenoxodiol in patients with refractory malignancies. *Proceedings of the American Society for Clinical Oncology* **22:** Abstract 886

Hyer ML, Sudarshan S, Kim YA, Reed JC, Dong JY, Schwartz DA, Norris JS (2002) Downregulation of c-FLIP sensitizes DU145 prostate cancer cells to Fas-mediated apoptosis. *Cancer Biol Ther* **1:** 401 - 406

Ikonen E, Heino S, Lusa S (2004) Caveolins and membrane cholesterol. *Biochem Soc Trans* **32:** 121-3

Inoki K, Li Y, Xu T, Guan KL (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* **17:** 1829-34

Irie T, Fukunaga K, Garwood MK, Carpenter TO, Pitha J, Pitha J (1992) Hydroxypropylcyclodextrins in parenteral use. II: Effects on transport and disposition of lipids in rabbit and humans. *Journal of pharmaceutical sciences* **81:** 524-8

Ito Y, Yoshida H, Nakano K, Kobayashi K, Yokozawa T, Hirai K, Matsuzuka F, Matsuura N, Kakudo K, Kuma K, Miyauchi A (2002) Caveolin-1 overexpression is an early event in the progression of papillary carcinoma of the thyroid. *Br J Cancer* **86:** 912-6

Ivaska J, Nissinen L, Immonen N, Eriksson JE, Kahari VM, Heino J (2002) Integrin alpha 2 beta 1 promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 beta. *Molecular and cellular biology* **22:** 1352-9

Jacobsen BK, Knutsen SF, Fraser GE (1998) Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). *Cancer Causes Control* **9:** 553-7

Jarred RA, Keikha M, Dowling C, McPherson SJ, Clare AM, Husband AJ, Pedersen JS, Frydenberg M, Risbridger GP (2002) Induction of apoptosis in low to moderate-grade human prostate carcinoma by red clover-derived dietary isoflavones. *Cancer Epidemiol Biomarkers Prev* **11:** 1689-96

Jenkins DJ, Kendall CW, D'Costa MA, Jackson CJ, Vidgen E, Singer W, Silverman JA, Koumbridis G, Honey J, Rao AV, Fleshner N, Klotz L (2003) Soy consumption and phytoestrogens: effect on serum prostate specific antigen when blood lipids and oxidized low-density lipoprotein are reduced in hyperlipidemic men. *J Urol* **169:** 507-11

Jian L (2009) Soy, isoflavones, and prostate cancer. *Mol Nutr Food Res* **53:** 217-26

Jin S, DiPaola RS, Mathew R, White E (2007) Metabolic catastrophe as a means to cancer cell death. *J Cell Sci* **120:** 379-83

Joannou G, Kelly G, Reeder A, Waring M, Nelson C (1995) A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *Journal of Steroid Biochemistry and Molecular Biology* **54:** 167-184

Joshua AM, Ong S, Noney L, Millward M, Beale P, Clarke SJ, Beith J, Kelly G, Boyer MJ (2003) Phase 1 dose-escalation study of phenoxodiol in patients with advanced cancer. *Proceedings of the American Society for Clinical Oncology* **22:** Abstract 902

Jung JI, Chung E, Seon MR, Shin HK, Kim EJ, Lim SS, Chung WY, Park KK, Park JH (2006) Isoliquiritigenin (ISL) inhibits ErbB3 signaling in prostate cancer cells. *BioFactors (Oxford, England)* **28:** 159-68

Kaestner KH, Knochel W, Martinez DE (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* **14:** 142-6

Kamsteeg M, Rutherford T, Sapi E, Hanczaruk B, Shahabi S, Flick M, Brown D, Mor G (2003) Phenoxodiol--an isoflavone analog--induces apoptosis in chemoresistant ovarian cancer cells. *Oncogene* **22:** 2611-20

Karin M, Cao Y, Greten FR, Li ZW (2002) NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2:** 301-10

Kato K, Hida Y, Miyamoto M, Hashida H, Shinohara T, Itoh T, Okushiba S, Kondo S, Katoh H (2002) Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. *Cancer* **94:** 929-33

Kato K, Takahashi S, Cui L, Toda T, Suzuki S, Futakuchi M, Sugiura S, Shirai T (2000) Suppressive effects of dietary genistin and daidzin on rat prostate carcinogenesis. *Jpn J Cancer Res* **91:** 786-91

Kazi A, Daniel KG, Smith DM, Kumar NB, Dou QP (2003) Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein. *Biochem Pharmacol* **66:** 965-76

Kelland LR (2000) An update on satraplatin: the first orally available platinum anticancer drug. *Expert opinion on investigational drugs* **9:** 1373-82

Kelley NS, Hubbard NE, Erickson KL (2007) Conjugated linoleic acid isomers and cancer. *J Nutr* **137:** 2599-607

Kelly GE, Husband AJ (2003) Flavonoid compounds in the prevention and treatment of prostate cancer. *Methods in molecular medicine* **81:** 377-94

Khoshyomn S, Manske G, Lew S, Wald S, Penar P (2000) Synergistic actions of genistein and cisplatin on growth inhibition and cytotoxicity of human medulloblastoma cells. *Pediatric Neurosurgery* **33:** 123-131

Kim SH, Juhnn YS, Song YS (2007) Akt involvement in Paclitaxel chemoresistance of human ovarian cancer cells. *Ann N Y Acad Sci* **1095:** 82-9

King RA, Bursill DB (1998) Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *The American journal of clinical nutrition* **67:** 867-72

Kluger HM, McCarthy MM, Alvero AB, Sznol M, Ariyan S, Camp RL, Rimm DL, Mor G (2007) The X-linked inhibitor of apoptosis protein (XIAP) is up-regulated in metastatic melanoma, and XIAP cleavage by Phenoxodiol is associated with Carboplatin sensitization. *Journal of translational medicine* **5:** 6

Koul D, Shen R, Garyali A, Ke LD, Liu TJ, Yung WK (2002) MMAC/PTEN tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer. *International journal of oncology* **21:** 469-75

Kouroukis CT, Belch A, Crump M, Eisenhauer E, Gascoyne RD, Meyer R, Lohmann R, Lopez P, Powers J, Turner R, Connors JM (2003) Flavopiridol in untreated or relapsed mantle-cell lymphoma: results of a phase II study of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* **21:** 1740-5

Kreisberg JI, Malik SN, Prihoda TJ, Bedolla RG, Troyer DA, Kreisberg S, Ghosh PM (2004) Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer research* **64:** 5232-6

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139:** 4252-63

Kumar NB, Cantor A, Allen K, Riccardi D, Besterman-Dahan K, Seigne J, Helal M, Salup R, Pow-Sang J (2004) The specific role of isoflavones in reducing prostate cancer risk. *Prostate* **59:** 141-7

Kumar NB, Krischer JP, Allen K, Riccardi D, Besterman-Dahan K, Salup R, Kang L, Xu P, Pow-Sang J (2007) A Phase II randomized, placebo-controlled clinical trial of purified isoflavones in modulating steroid hormones in men diagnosed with localized prostate cancer. *Nutr Cancer* **59:** 163-8

Kumi-Diaka J (2002) Chemosensitivity of human prostate cancer cells PC3 and LNCaP to genistein isoflavone and beta-lapachone. *Biology of the cell / under the auspices of the European Cell Biology Organization* **94:** 37-44

Kumi-Diaka J, Butler A (2000) Caspase-3 protease activation during the process of genistein-induced apoptosis in TM4 testicular cells. *Biology of the cell / under the auspices of the European Cell Biology Organization* **92:** 115-24

Kumi-Diaka J, Saddler-Shawnette S, Aller A, Brown J (2004) Potential mechanism of phytochemical-induced apoptosis in human prostate adenocarcinoma cells: Therapeutic synergy in genistein and beta-lapachone combination treatment. *Cancer cell international* **4:** 5

Kumi-Diaka J, Sanderson NA, Hall A (2000) The mediating role of caspase-3 protease in the intracellular mechanism of genistein-induced apoptosis in human prostatic carcinoma cell lines, DU145 and LNCaP. *Biology of the cell / under the auspices of the European Cell Biology Organization* **92:** 595-604

Kumi-Diaka JK, Hassanhi M, Merchant K, Horman V (2006) Influence of genistein isoflavone on matrix metalloproteinase-2 expression in prostate cancer cells. *J Med Food* **9:** 491-7

Kurahashi N, Iwasaki M, Inoue M, Sasazuki S, Tsugane S (2008) Plasma isoflavones and subsequent risk of prostate cancer in a nested case-control study: the Japan Public Health Center. *J Clin Oncol* **26:** 5923-9

Kurahashi N, Iwasaki M, Sasazuki S, Otani T, Inoue M, Tsugane S (2007) Soy product and isoflavone consumption in relation to prostate cancer in Japanese men. *Cancer Epidemiol Biomarkers Prev* **16:** 538-45

Kyle E, Neckers L, Takimoto C, Curt G, Bergan R (1997) Genistein-induced apoptosis of prostate cancer cells is preceded by a specific decrease in focal adhesion kinase activity. *Mol Pharmacol* **51:** 193-200

Latini JM, Rieger-Christ KM, Wang DS, Silverman ML, Libertino JA, Summerhayes IC (2001) Loss of heterozygosity and microsatellite instability at chromosomal sites 1Q and 10Q in morphologically distinct regions of late stage prostate lesions. *J Urol* **166:** 1931-6

Lavieu G, Scarlatti F, Sala G, Carpentier S, Levade T, Ghidoni R, Botti J, Codogno P (2006) Regulation of autophagy by sphingosine kinase 1 and its role in cell survival during nutrient starvation. *J Biol Chem* **281:** 8518-27

Lazarevic B, Karlsen SJ, Saatcioglu F (2008) Genistein differentially modulates androgen-responsive gene expression and activates JNK in LNCaP cells. *Oncol Rep* **19:** 1231-5

Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F (2006) Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. *Br J Cancer* **94:** 1906-12

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

Leslie NR, Downes CP (2002) PTEN: The down side of PI 3-kinase signalling. *Cell Signal* **14:** 285-95

Levine B (2007) Cell biology: autophagy and cancer. *Nature* **446:** 745-7

Li B, Sun A, Youn H, Hong Y, Terranova PF, Thrasher JB, Xu P, Spencer D (2007) Conditional Akt activation promotes androgen-independent progression of prostate cancer. *Carcinogenesis* **28:** 572-83

Li L, Ittmann MM, Ayala G, Tsai MJ, Amato RJ, Wheeler TM, Miles BJ, Kadmon D, Thompson TC (2005a) The emerging role of the PI3-K-Akt pathway in prostate cancer progression. *Prostate Cancer Prostatic Dis* **8:** 108-18

Li L, Ren CH, Tahir SA, Ren C, Thompson TC (2003) Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. *Molecular and cellular biology* **23:** 9389-404

Li M, Zhang Z, Hill DL, Chen X, Wang H, Zhang R (2005b) Genistein, a dietary isoflavone, down-regulates the MDM2 oncogene at both transcriptional and posttranslational levels. *Cancer research* **65:** 8200-8

Li X, Marani M, Mannucci R, Kinsey B, Andriani F, Nicoletti I, Denner L, Marcelli M (2001) Overexpression of BCL-X(L) underlies the molecular basis for resistance to staurosporine-induced apoptosis in PC-3 cells. *Cancer research* **61:** 1699-706

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

Li Y, Sarkar FH (2002b) Gene expression profiles of genistein-treated PC3 prostate cancer cells. *J Nutr* **132:** 3623-31

Li Y, Sarkar FH (2002c) Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via Akt signaling pathway. *Clin Cancer Res* **8:** 2369-77

Li Z, Aronson WJ, Arteaga JR, Hong K, Thames G, Henning SM, Liu W, Elashoff R, Ashley JM, Heber D (2008) Feasibility of a low-fat/high-fiber diet intervention with soy supplementation in prostate cancer patients after prostatectomy. *Eur J Clin Nutr* **62:** 526-36

Lian F, Bhuiyan M, Li YW, Wall N, Kraut M, Sarkar FH (1998) Genistein-induced G2-M arrest, p21WAF1 upregulation, and apoptosis in a non-small-cell lung cancer cell line. *Nutr Cancer* **31:** 184-91

Liao Y, Grobholz R, Abel U, Trojan L, Michel MS, Angel P, Mayer D (2003) Increase of AKT/PKB expression correlates with gleason pattern in human prostate cancer. *International journal of cancer* **107:** 676-80

Lin HK, Hu YC, Lee DK, Chang C (2004) Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. *Molecular endocrinology (Baltimore, Md* **18:** 2409-23

Lin HK, Yeh S, Kang HY, Chang C (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 7200-5

Litterst C, Magin R (1988) Alterations in Plasma Pharmacokinetics of Cisplatin in Tumour-Bearing Rats. *Cancer chemotherapy and pharmacology* **22:** 1-4

Liu Y, Kyle E, Lieberman R, Crowell J, Kellof G, Bergan RC (2000) Focal adhesion kinase (FAK) phosphorylation is not required for genistein-induced FAK-beta-1-integrin complex formation. *Clinical & experimental metastasis* **18:** 203-12

Low YL, Taylor JI, Grace PB, Dowsett M, Folkerd E, Doody D, Dunning AM, Scollen S, Mulligan AA, Welch AA, Luben RN, Khaw KT, Day NE, Wareham NJ, Bingham SA (2005) Polymorphisms in the CYP19 gene may affect the positive correlations between serum and urine phytoestrogen metabolites and plasma androgen concentrations in men. *J Nutr* **135:** 2680-6

Lu LJ, Anderson KE (1998) Sex and long-term soy diets affect the metabolism and excretion of soy isoflavones in humans. *The American journal of clinical nutrition* **68:** 1500S-1504S

Luo J, Manning BD, Cantley LC (2003) Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* **4:** 257-62

Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP (2005) Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121:** 179-93

Magee PJ, Rowland IR (2004) Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br J Nutr* **91:** 513-31

Makela S, Santti R, Salo L, McLachlan JA (1995a) Phytoestrogens are partial estrogen agonists in the adult male mouse. *Environmental health perspectives* **103 Suppl 7:** 123-7

Makela SI, Pylkkanen LH, Santti RS, Adlercreutz H (1995b) Dietary soybean may be antiestrogenic in male mice. *J Nutr* **125:** 437-45

Manin M, Baron S, Goossens K, Beaudoin C, Jean C, Veyssiere G, Verhoeven G, Morel L (2002) Androgen receptor expression is regulated by the phosphoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells. *The Biochemical journal* **366:** 729-36

Maroulakou IG, Oemler W, Naber SP, Tsichlis PN (2007) Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in

mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer research* **67:** 167-77

Marveti G, Andrews P (1996) Stimulation of cis-diamminedochloroplatinum(II) accumulation by modulation of passive permiability with genistein: an altered response in accumulation defective cells. *Clinical Cancer Research* **2:** 991-999

Maskarinec G, Morimoto Y, Hebshi S, Sharma S, Franke AA, Stanczyk FZ (2006) Serum prostate-specific antigen but not testosterone levels decrease in a randomized soy intervention among men. *Eur J Clin Nutr* **60:** 1423-9

Mayo LD, Dixon JE, Durden DL, Tonks NK, Donner DB (2002) PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. *J Biol Chem* **277:** 5484-9

Mayo LD, Donner DB (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 11598-603

McCormick DL, Johnson WD, Bosland MC, Lubet RA, Steele VE (2007) Chemoprevention of rat prostate carcinogenesis by soy isoflavones and by Bowman-Birk inhibitor. *Nutr Cancer* **57:** 184-93

McEntee MF, Ziegler C, Reel D, Tomer K, Shoieb A, Ray M, Li X, Neilsen N, Lih FB, O'Rourke D, Whelan J (2008) Dietary n-3 polyunsaturated fatty acids enhance hormone ablation therapy in androgen-dependent prostate cancer. *Am J Pathol* **173:** 229-41

McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR (1999) Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer research* **59:** 4291-6

McPherson RA, Galettis PT, de Souza PL (2009) Enhancement of the activity of phenoxodiol by cisplatin in prostate cancer cells. *Br J Cancer* **100:** 649-55

Mentor-Marcel R, Lamartiniere CA, Eltoum IA, Greenberg NM, Elgavish A (2005) Dietary genistein improves survival and reduces expression of osteopontin in the prostate of transgenic mice with prostatic adenocarcinoma (TRAMP). *J Nutr* **135:** 989-95

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

Messina M, Kucuk O, Lampe JW (2006a) An overview of the health effects of isoflavones with an emphasis on prostate cancer risk and prostate-specific antigen levels. *J AOAC Int* **89:** 1121-34

Messina M, Nagata C, Wu AH (2006b) Estimated Asian adult soy protein and isoflavone intakes. *Nutr Cancer* **55:** 1-12

Mikhailova M, Wang Y, Bedolla R, Lu XH, Kreisberg JI, Ghosh PM (2008) AKT regulates androgen receptor-dependent growth and PSA expression in prostate cancer. *Adv Exp Med Biol* **617:** 397-405

Miksicek RJ (1993) Commonly occurring plant flavonoids have estrogenic activity. *Mol Pharmacol* **44:** 37-43

Mitchell JH, Duthie SJ, Collins AR (2000) Effects of phytoestrogens on growth and DNA integrity in human prostate tumor cell lines: PC-3 and LNCaP. *Nutr Cancer* **38:** 223-8

Mor G, Fu HH, Alvero AB (2006) Phenoxodiol, a novel approach for the treatment of ovarian cancer. *Curr Opin Investig Drugs* **7:** 542-8

Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med* **356:** 115-24

Moumen A, Patane S, Porras A, Dono R, Maina F (2007) Met acts on Mdm2 via mTOR to signal cell survival during development. *Development* **134:** 1443-51

Nagata Y, Sonoda T, Mori M, Miyanaga N, Okumura K, Goto K, Naito S, Fujimoto K, Hirao Y, Takahashi A, Tsukamoto T, Akaza H (2007) Dietary Isoflavones May Protect against Prostate Cancer in Japanese Men. *J Nutr* **137:** 1974-1979

Naik HR, Lehr JE, Pienta KJ (1994) An in vitro and in vivo study of antitumor effects of genistein on hormone refractory prostate cancer. *Anticancer research* **14:** 2617-9

Nakatani K, Thompson DA, Barthel A, Sakaue H, Liu W, Weigel RJ, Roth RA (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* **274:** 21528-32

Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R, Frost P, Gibbons JJ, Wu H, Sawyers CL (2001) Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 10314-9

Nicholson KM, Anderson NG (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* **14:** 381-95

Novogen Research Pty Ltd (2000) Phenoxodiol Investigator's Brochure Vol. 4.0. pp 1 - 24. Sydney, Australia

O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer research* **66:** 1500-8

Oh WK, Tay MH, Huang J (2007) Is there a role for platinum chemotherapy in the treatment of patients with hormone-refractory prostate cancer? *Cancer* **109:** 477-86

Onozawa M, Fukuda K, Ohtani M, Akaza H, Sugimura T, Wakabayashi K (1998) Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP. *Jpn J Clin Oncol* **28:** 360-3

Onozawa M, Kawamori T, Baba M, Fukuda K, Toda T, Sato H, Ohtani M, Akaza H, Sugimura T, Wakabayashi K (1999) Effects of a soybean isoflavone mixture on carcinogenesis in prostate and seminal vesicles of F344 rats. *Jpn J Cancer Res* **90:** 393-8

Ozasa K, Nakao M, Watanabe Y, Hayashi K, Miki T, Mikami K, Mori M, Sakauchi F, Washio M, Ito Y, Suzuki K, Kubo T, Wakai K, Tamakoshi A (2005) Association of serum phytoestrogen concentration and dietary habits in a sample set of the JACC Study. *Journal of epidemiology / Japan Epidemiological Association* **15 Suppl 2:** S196-202

Ozasa K, Nakao M, Watanabe Y, Hayashi K, Miki T, Mikami K, Mori M, Sakauchi F, Washio M, Ito Y, Suzuki K, Wakai K, Tamakoshi A (2004) Serum phytoestrogens and prostate cancer risk in a nested case-control study among Japanese men. *Cancer Sci* **95:** 65-71

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB (1999) NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401:** 82-5

Paez J, Sellers WR (2003) PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res* **115:** 145-67

Park SS, Kim YN, Jeon YK, Kim YA, Kim JE, Kim H, Kim CW (2005) Genistein-induced apoptosis via Akt signaling pathway in anaplastic large-cell lymphoma. *Cancer chemotherapy and pharmacology* **56:** 271-8

Pendleton JM, Tan WW, Anai S, Chang M, Hou W, Shiverick KT, Rosser CJ (2008) Phase II trial of isoflavone in prostate-specific antigen recurrent prostate cancer after previous local therapy. *BMC Cancer* **8:** 132

Pesche S, Latil A, Muzeau F, Cussenot O, Fournier G, Longy M, Eng C, Lidereau R (1998) PTEN/MMAC1/TEP1 involvement in primary prostate cancers. *Oncogene* **16:** 2879-83

Peterson G, Barnes S (1993) Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **22:** 335-45

Petrylak DP (2007) New paradigms for advanced prostate cancer. *Reviews in urology* **9 Suppl 2:** S3-S12

Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr., Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* **351:** 1513-20

Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *The EMBO journal* **18:** 4608-18

Ping SY, Hour TC, Lin SR, Yu DS (2008) Taxol synergizes with antioxidants in inhibiting hormal refractory prostate cancer cell growth. *Urologic oncology* Sep 23 [Epub ahead of print]

Pinski J, Wang Q, Quek ML, Cole A, Cooc J, Danenberg K, Danenberg PV (2006) Genistein-induced neuroendocrine differentiation of prostate cancer cells. *Prostate* **66:** 1136-43

Plas DR, Talapatra S, Edinger AL, Rathmell JC, Thompson CB (2001) Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. *J Biol Chem* **276:** 12041-8

Plas DR, Thompson CB (2003) Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* **278:** 12361-6

Plas DR, Thompson CB (2005) Akt-dependent transformation: there is more to growth than just surviving. *Oncogene* **24:** 7435-42

Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J, Frost P, Dreisbach V, Blenis J, Gaciong Z, Fisher P, Sawyers C, Hedrick-Ellenson L, Parsons R (2001) An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 10320-5

Pollard M, Luckert PH (1997) Influence of isoflavones in soy protein isolates on development of induced prostate-related cancers in L-W rats. *Nutr Cancer* **28:** 41-5

Pollard M, Suckow MA (2006) Dietary prevention of hormone refractory prostate cancer in Lobund-Wistar rats: a review of studies in a relevant animal model. *Comp Med* **56:** 461-7

Pollard M, Wolter W (2000) Prevention of spontaneous prostate-related cancer in Lobund-Wistar rats by a soy protein isolate/isoflavone diet. *Prostate* **45:** 101-5

Raffoul JJ, Banerjee S, Che M, Knoll ZE, Doerge DR, Abrams J, Kucuk O, Sarkar FH, Hillman GG (2007a) Soy isoflavones enhance radiotherapy in a metastatic prostate cancer model. *International journal of cancer* **120:** 2491-8

Raffoul JJ, Banerjee S, Singh-Gupta V, Knoll ZE, Fite A, Zhang H, Abrams J, Sarkar FH, Hillman GG (2007b) Down-regulation of apurinic/apyrimidinic endonuclease 1/redox factor-1 expression by soy isoflavones enhances prostate cancer radiotherapy in vitro and in vivo. *Cancer research* **67:** 2141-9

Rajjayabun PH, Garg S, Durkan GC, Charlton R, Robinson MC, Mellon JK (2001) Caveolin-1 expression is associated with high-grade bladder cancer. *Urology* **58:** 811-4

Rannikko A, Petas A, Raivio T, Janne OA, Rannikko S, Adlercreutz H (2006a) The effects of short-term oral phytoestrogen supplementation on the hypothalamic-pituitary-testicular axis in prostate cancer patients. *Prostate* **66:** 1086-91

Rannikko A, Petas A, Rannikko S, Adlercreutz H (2006b) Plasma and prostate phytoestrogen concentrations in prostate cancer patients after oral phytoestogen supplementation. *Prostate* **66:** 82-7

Reddy GP, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, Sheng S (2006) Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *J Cell Biochem* **98:** 1408-23

Rennie PS, Nelson CC (1998) Epigenetic mechanisms for progression of prostate cancer. *Cancer Metastasis Rev* **17:** 401-9

Rice L, Handayani R, Cui Y, Medrano T, Samedi V, Baker H, Szabo NJ, Rosser CJ, Goodison S, Shiverick KT (2007) Soy isoflavones exert differential effects on androgen responsive genes in LNCaP human prostate cancer cells. *J Nutr* **137:** 964-72

Rice L, Samedi VG, Medrano TA, Sweeney CA, Baker HV, Stenstrom A, Furman J, Shiverick KT (2002) Mechanisms of the growth inhibitory effects of the isoflavonoid biochanin A on LNCaP cells and xenografts. *Prostate* **52:** 201-12

Richelle M, Pridmore-Merten S, Bodenstab S, Enslen M, Offord EA (2002) Hydrolysis of isoflavone glycosides to aglycones by beta-glycosidase does not alter plasma and urine isoflavone pharmacokinetics in postmenopausal women. *J Nutr* **132:** 2587-92

Risbridger G, Wang H, Frydenberg M, Husband A (2001) The in vivo effect of red clover diet on ventral prostate growth in adult mice. *Reproductive Fertility Development* **13:** 325-329

Rokhlin OW, Cohen MB (1995) Differential sensitivity of human prostatic cancer cell lines to the effects of protein kinase and phosphatase inhibitors. *Cancer letters* **98:** 103-10

Romashkova JA, Makarov SS (1999) NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401:** 86-90

Rubin MA, Gerstein A, Reid K, Bostwick DG, Cheng L, Parsons R, Papadopoulos N (2000) 10q23.3 loss of heterozygosity is higher in lymph node-positive (pT2-3,N+) versus lymph node-negative (pT2-3,N0) prostate cancer. *Hum Pathol* **31:** 504-8

Rufer CE, Bub A, Moseneder J, Winterhalter P, Sturtz M, Kulling SE (2008) Pharmacokinetics of the soybean isoflavone daidzein in its aglycone and glucoside form: a randomized, double-blind, crossover study. *The American journal of clinical nutrition* **87:** 1314-23

Rutherford T, O'Malley D, Makkenchery A, Baker L, Azodi M, Schwartz P, Mor G (2004) Phenoxodiol phase Ib/II study in patients with recurrent ovarian cancer that are resistant to ≥ second line chemotherapy. *Proceedings of the American Association for Cancer Research* **45:** Abstract 4457

Sakamoto K (2000) Synergistic effects of thearubigin and genistein on human prostate tumor cell (PC-3) growth via cell cycle arrest. *Cancer letters* **151:** 103-9

Salas TR, Kim J, Vakar-Lopez F, Sabichi AL, Troncoso P, Jenster G, Kikuchi A, Chen SY, Shemshedini L, Suraokar M, Logothetis CJ, DiGiovanni J, Lippman SM, Menter DG (2004) Glycogen synthase kinase-3 beta is involved in the phosphorylation and suppression of androgen receptor activity. *J Biol Chem* **279:** 19191-200

Samuels Y, Diaz LA, Jr., Schmidt-Kittler O, Cummins JM, Delong L, Cheong I, Rago C, Huso DL, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE (2005) Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* **7:** 561-73

Santibanez JF, Navarro A, Martinez J (1997) Genistein inhibits proliferation and in vitro invasive potential of human prostatic cancer cell lines. *Anticancer research* **17:** 1199-204

Sapi E, Alvero AB, Chen W, O'Malley D, Hao XY, Dwipoyono B, Garg M, Kamsteeg M, Rutherford T, Mor G (2004) Resistance of ovarian carcinoma cells to docetaxel is XIAP dependent and reversible by phenoxodiol. *Oncology research* **14:** 567-78

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307:** 1098-101

Sarkar FH, Adsule S, Padhye S, Kulkarni S, Li Y (2006) The role of genistein and synthetic derivatives of isoflavone in cancer prevention and therapy. *Mini Rev Med Chem* **6:** 401-7

Sarkar FH, Li Y (2002) Mechanisms of cancer chemoprevention by soy isoflavone genistein. *Cancer Metastasis Rev* **21:** 265-80

Schmelzle T, Hall MN (2000) TOR, a central controller of cell growth. *Cell* **103:** 253-62

Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, Nathan M, Giacchi S, Scheiden R, Kieffer N (2007) Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. *International journal of cancer* **120:** 1284-92

Schroder FH, Roobol MJ, Boeve ER, de Mutsert R, Zuijdgeest-van Leeuwen SD, Kersten I, Wildhagen MF, van Helvoort A (2005) Randomized, double-blind, placebo-controlled crossover study in men with prostate cancer and rising PSA: effectiveness of a dietary supplement. *Eur Urol* **48:** 922-30; discussion 930-1

Schroeder F, Gallegos AM, Atshaves BP, Storey SM, McIntosh AL, Petrescu AD, Huang H, Starodub O, Chao H, Yang H, Frolov A, Kier AB (2001) Recent advances in membrane microdomains: rafts, caveolae, and intracellular cholesterol trafficking. *Exp Biol Med (Maywood)* **226:** 873-90

Schwartz GK, O'Reilly E, Ilson D, Saltz L, Sharma S, Tong W, Maslak P, Stoltz M, Eden L, Perkins P, Endres S, Barazzoul J, Spriggs D, Kelsen D (2002) Phase I study of

the cyclin-dependent kinase inhibitor flavopiridol in combination with paclitaxel in patients with advanced solid tumors. *J Clin Oncol* **20:** 2157-70

Screnci D, Galettis P, Baguely B, McKeage M (1998) Optimization of an ICP-MS Assay for the Detection of Trace Levels of Platinum in Peripheral Nerves. *Atomic Spectroscopy* **19:** 172-175

Senderowicz AM, Headlee D, Stinson SF, Lush RM, Kalil N, Villalba L, Hill K, Steinberg SM, Figg WD, Tompkins A, Arbuck SG, Sausville EA (1998) Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasm. *J Clin Oncol* **16:** 2986-99

Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A, Heubi JE (2001) Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* **131:** 1362S-75S

Setchell KD, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AS, Heubi JE (2002) Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *The American journal of clinical nutrition* **76:** 447-53

Setchell KD, Faughnan MS, Avades T, Zimmer-Nechemias L, Brown NM, Wolfe BE, Brashear WT, Desai P, Oldfield MF, Botting NP, Cassidy A (2003) Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women. *The American journal of clinical nutrition* **77:** 411-9

Shaw RJ, Cantley LC (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* **441:** 424-30

Shen JC, Klein RD, Wei Q, Guan Y, Contois JH, Wang TT, Chang S, Hursting SD (2000) Low-dose genistein induces cyclin-dependent kinase inhibitors and G(1) cell-cycle arrest in human prostate cancer cells. *Mol Carcinog* **29:** 92-102

Shi Y, Chatterjee SJ, Brands FH, Shi SR, Pootrakul L, Taylor CR, Datar R, Cote RJ (2006) Role of coordinated molecular alterations in the development of androgen-

independent prostate cancer: an in vitro model that corroborates clinical observations. *BJU international* **97:** 170-8

Shimizu Y, Segawa T, Inoue T, Shiraishi T, Yoshida T, Toda Y, Yamada T, Kinukawa N, Terada N, Kobayashi T, Kinoshita H, Kamoto T, Nakamura E, Ogawa O (2007) Increased Akt and phosphorylated Akt expression are associated with malignant biological features of prostate cancer in Japanese men. *BJU international* **100:** 685-90

Singh-Gupta V, Zhang H, Banerjee S, Kong D, Raffoul JJ, Sarkar FH, Hillman GG (2009) Radiation-induced HIF-1alpha cell survival pathway is inhibited by soy isoflavones in prostate cancer cells. *International journal of cancer* **124:** 1675-84

Sirtori CR, Arnoldi A, Johnson SK (2005) Phytoestrogens: end of a tale? *Annals of medicine* **37:** 423-38

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren J, Bokesch H, Kenney S, Boyd M (1990) New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. *Journal of the National Cancer Institute* **82:** 1107-1112

Skjoth IH, Issinger OG (2006) Profiling of signaling molecules in four different human prostate carcinoma cell lines before and after induction of apoptosis. *International journal of oncology* **28:** 217-29

Skogseth H, Larsson E, Halgunset J (2005) Inhibitors of tyrosine kinase inhibit the production of urokinase plasminogen activator in human prostatic cancer cells. *Apmis* **113:** 332-9

Staal SP, Hartley JW (1988) Thymic lymphoma induction by the AKT8 murine retrovirus. *J Exp Med* **167:** 1259-64

Steiner C, Arnould S, Scalbert A, Manach C (2008) Isoflavones and the prevention of breast and prostate cancer: new perspectives opened by nutrigenomics. *Br J Nutr* **99 E Suppl 1:** ES78-108

Sternberg PW, Schmid SL (1999) Caveolin, cholesterol and Ras signalling. *Nat Cell Biol* **1:** E35-7

Straszewski-Chavez SL, Abrahams VM, Funai EF, Mor G (2004) X-linked inhibitor of apoptosis (XIAP) confers human trophoblast cell resistance to Fas-mediated apoptosis. *Molecular human reproduction* **10:** 33-41

Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC, Hursting SD (1999) Phytoestrogen intake and prostate cancer: a case-control study using a new database. *Nutr Cancer* **33:** 20-5

Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng JQ (2001) AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am J Pathol* **159:** 431-7

Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR (2005) Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer research* **65:** 7052-8

Suzuki K, Koike H, Matsui H, Ono Y, Hasumi M, Nakazato H, Okugi H, Sekine Y, Oki K, Ito K, Yamamoto T, Fukabori Y, Kurokawa K, Yamanaka H (2002) Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. *International journal of cancer* **99:** 846-52

Swami S, Krishnan AV, Moreno J, Bhattacharyya RS, Gardner C, Brooks JD, Peehl DM, Feldman D (2008) Inhibition of prostaglandin synthesis and actions by genistein in human prostate cancer cells and by soy isoflavones in prostate cancer patients. *International journal of cancer* **124:** 2050-2059

Swami S, Krishnan AV, Peehl DM, Feldman D (2005) Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D3 in DU145 human prostate cancer cells: role of the direct inhibition of CYP24 enzyme activity. *Mol Cell Endocrinol* **241:** 49-61

Tahir SA, Yang G, Ebara S, Timme TL, Satoh T, Li L, Goltsov A, Ittmann M, Morrisett JD, Thompson TC (2001) Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. *Cancer research* **61:** 3882-5

Takahashi Y, Hursting SD, Perkins SN, Wang TC, Wang TT (2006a) Genistein affects androgen-responsive genes through both androgen- and estrogen-induced signaling pathways. *Mol Carcinog* **45:** 18-25

Takahashi Y, Lavigne JA, Hursting SD, Chandramouli GV, Perkins SN, Kim YS, Wang TT (2006b) Molecular signatures of soy-derived phytochemicals in androgen-responsive prostate cancer cells: a comparison study using DNA microarray. *Mol Carcinog* **45:** 943-56

Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S (2005) Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. *Cancer research* **65:** 3336-46

Takimoto CH, Glover K, Huang X, Hayes SA, Gallot L, Quinn M, Jovanovic BD, Shapiro A, Hernandez L, Goetz A, Llorens V, Lieberman R, Crowell JA, Poisson BA, Bergan RC (2003) Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer. *Cancer Epidemiol Biomarkers Prev* **12:** 1213-21

Tan AR, Headlee D, Messmann R, Sausville EA, Arbuck SG, Murgo AJ, Melillo G, Zhai S, Figg WD, Swain SM, Senderowicz AM (2002) Phase I clinical and pharmacokinetic study of flavopiridol administered as a daily 1-hour infusion in patients with advanced neoplasms. *J Clin Oncol 20:* 4074-82

Tanaka M, Koul D, Davies MA, Liebert M, Steck PA, Grossman HB (2000) MMAC1/PTEN inhibits cell growth and induces chemosensitivity to doxorubicin in human bladder cancer cells. *Oncogene* **19:** 5406-12

Tanaka M, Rosser CJ, Grossman HB (2005) PTEN gene therapy induces growth inhibition and increases efficacy of chemotherapy in prostate cancer. *Cancer detection and prevention* **29:** 170-4

Tanno S, Tanno S, Mitsuuchi Y, Altomare DA, Xiao GH, Testa JR (2001) AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. *Cancer research* **61:** 589-93

Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* **351:** 1502-12

Tepper CG, Vinall RL, Wee CB, Xue L, Shi XB, Burich R, Mack PC, de Vere White RW (2007) GCP-mediated growth inhibition and apoptosis of prostate cancer cells via androgen receptor-dependent and -independent mechanisms. *Prostate* **67:** 521-35

Teruel T, Hernandez R, Lorenzo M (2001) Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* **50:** 2563-71

Testa JR, Bellacosa A (2001) AKT plays a central role in tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 10983-5

Thant AA, Nawa A, Kikkawa F, Ichigotani Y, Zhang Y, Sein TT, Amin AR, Hamaguchi M (2000) Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells. *Clinical & experimental metastasis* **18:** 423-8

Thompson JE, Thompson CB (2004) Putting the rap on Akt. *J Clin Oncol* **22**(20): 4217-26

Touny LH, Banerjee PP (2006) Identification of both Myt-1 and Wee-1 as necessary mediators of the p21-independent inactivation of the cdc-2/cyclin B1 complex and growth inhibition of TRAMP cancer cells by genistein. *Prostate* **66:** 1542-55

Tso CL, McBride WH, Sun J, Patel B, Tsui KH, Paik SH, Gitlitz B, Caliliw R, van Ophoven A, Wu L, deKernion J, Belldegrun A (2000) Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing

distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J* **6:** 220-33

Urban D, Irwin W, Kirk M, Markiewicz MA, Myers R, Smith M, Weiss H, Grizzle WE, Barnes S (2001) The effect of isolated soy protein on plasma biomarkers in elderly men with elevated serum prostate specific antigen. *J Urol* **165:** 294-300

Vaishampayan U, Hussain M, Banerjee M, Seren S, Sarkar FH, Fontana J, Forman JD, Cher ML, Powell I, Pontes JE, Kucuk O (2007) Lycopene and soy isoflavones in the treatment of prostate cancer. *Nutr Cancer* **59:** 1-7

van Hennik MB, van der Vijgh WJ, Klein I, Elferink F, Vermorken JB, Winograd B, Pinedo HM (1987) Comparative pharmacokinetics of cisplatin and three analogues in mice and humans. *Cancer research* **47:** 6297-301

Venkitaraman R, Thomas K, Grace P, Dearnaley D, Horwich A, Huddart R, Parker CC (2008) Baseline urinary phytoestrogen levels and the natural history of untreated, localised prostate cancer in a British population. *The International journal of biological markers* **23:** 192-7

Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nature medicine* **8:** 1136-44

Vinall RL, Hwa K, Ghosh P, Pan CX, Lara PN, Jr., de Vere White RW (2007) Combination treatment of prostate cancer cell lines with bioactive soy isoflavones and perifosine causes increased growth arrest and/or apoptosis. *Clin Cancer Res* **13:** 6204-16

Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* **2:** 489-501

Von Low EC, Perabo FG, Siener R, Muller SC (2007) Review. Facts and fiction of phytotherapy for prostate cancer: a critical assessment of preclinical and clinical data. *In Vivo* **21:** 189-204

Wang J, Eltoum IE, Lamartiniere CA (2002) Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. *Cancer letters* **186:** 11-8

Wang J, Eltoum IE, Lamartiniere CA (2004) Genistein alters growth factor signaling in transgenic prostate model (TRAMP). *Mol Cell Endocrinol* **219:** 171-80

Wang S, DeGroff VL, Clinton SK (2003a) Tomato and soy polyphenols reduce insulin-like growth factor-I-stimulated rat prostate cancer cell proliferation and apoptotic resistance in vitro via inhibition of intracellular signaling pathways involving tyrosine kinase. *J Nutr* **133:** 2367-76

Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS, Liu X, Wu H (2003b) Prostate-specific deletion of the murine PTEN tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* **4:** 209-21

Wang X, Clubbs EA, Bomser JA (2006a) Genistein modulates prostate epithelial cell proliferation via estrogen- and extracellular signal-regulated kinase-dependent pathways. *J Nutr Biochem* **17:** 204-10

Wang Y, Kreisberg JI, Ghosh PM (2007) Cross-talk between the androgen receptor and the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer. *Curr Cancer Drug Targets* **7:** 591-604

Wang Y, Raffoul JJ, Che M, Doerge DR, Joiner MC, Kucuk O, Sarkar FH, Hillman GG (2006b) Prostate cancer treatment is enhanced by genistein in vitro and in vivo in a syngeneic orthotopic tumor model. *Radiat Res* **166:** 73-80

Ward H, Chapelais G, Kuhnle GG, Luben R, Khaw KT, Bingham S (2008) Lack of prospective associations between plasma and urinary phytoestrogens and risk of prostate or colorectal cancer in the European Prospective into Cancer-Norfolk study. *Cancer Epidemiol Biomarkers Prev* **17:** 2891-4

Weber KS, Setchell KD, Stocco DM, Lephart ED (2001) Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. *The Journal of endocrinology* **170:** 591-9

Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH, Hung MC (2000) HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer research* **60:** 6841-5

Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL, Said JW, Isaacs WB, Sawyers CL (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 5246-50

Widyarini S, Spinks N, Husband A, Reeve V (2001) Isoflavanoid compounds from red clover (Trifolium pratense) protect from inflammation and suppression induced UV radiation. *Photochemical Photobiology* **74:** 465-470

Wilkinson E (2004) Phenoxodiol offers hope for ovarian cancer. *Lancet Oncol* **5**(4):201

Wilson BE, Mochon E, Boxer LM (1996) Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Molecular and cellular biology* **16:** 5546-56

Wiseman H (2000) The therapeutic potential of phytoestrogens. *Expert opinion on investigational drugs* **9:** 1829-40

Wiseman H, Casey K, Bowey EA, Duffy R, Davies M, Rowland IR, Lloyd AS, Murray A, Thompson R, Clarke DB (2004) Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults. *The American journal of clinical nutrition* **80:** 692-9

Wolpin BM, Hezel AF, Abrams T, Blaszkowsky LS, Meyerhardt JA, Chan JA, Enzinger PC, Allen B, Clark JW, Ryan DP, Fuchs CS (2009) Oral mTOR inhibitor

everolimus in patients with gemcitabine-refractory metastatic pancreatic cancer. *J Clin Oncol* **27:** 193-8

Wong SY, Lau WW, Leung PC, Leung JC, Woo J (2007) The association between isoflavone and lower urinary tract symptoms in elderly men. *Br J Nutr* **98:** 1237-42

Wosikowski K, Lamphere L, Unteregger G, Jung V, Kaplan F, Xu JP, Rattel B, Caligiuri M (2007) Preclinical antitumor activity of the oral platinum analog satraplatin. *Cancer chemotherapy and pharmacology* **60:** 589-600

Wu X, Senechal K, Neshat MS, Whang YE, Sawyers CL (1998) The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 15587-91

Xiang H, Schevzov G, Gunning P, Williams HM, Silink M (2002) A comparative study of growth-inhibitory effects of isoflavones and their metabolites on human breast and prostate cancer cell lines. *Nutr Cancer* **42:** 224-32

Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON (2006) Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 7789-94

Xu L, Bergan RC (2006) Genistein inhibits matrix metalloproteinase type 2 activation and prostate cancer cell invasion by blocking the transforming growth factor beta-mediated activation of mitogen-activated protein kinase-activated protein kinase 2-27-kDa heat shock protein pathway. *Mol Pharmacol* **70:** 869-77

Xu Y, Chen SY, Ross KN, Balk SP (2006) Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. *Cancer research* **66:** 7783-92

Yagiz K, Wu LY, Kuntz CP, James Morre D, Morre DM (2007) Mouse embryonic fibroblast cells from transgenic mice overexpressing tNOX exhibit an altered growth and drug response phenotype. *J Cell Biochem* **101:** 295-306

Yamaguchi H, Wang HG (2001) The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* **20:** 7779-86

Yan L, Spitznagel EL (2009) Soy consumption and prostate cancer risk in men: a revisit of a meta-analysis. *The American journal of clinical nutrition* **89**(4): 1155-63

Yang CP, Galbiati F, Volonte D, Horwitz SB, Lisanti MP (1998) Upregulation of caveolin-1 and caveolae organelles in Taxol-resistant A549 cells. *FEBS Lett* **439:** 368-72

Yang G, Ayala G, De Marzo A, Tian W, Frolov A, Wheeler TM, Thompson TC, Harper JW (2002) Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. *Clin Cancer Res* **8:** 3419-26

Yang L, Xie S, Jamaluddin MS, Altuwaijri S, Ni J, Kim E, Chen YT, Hu YC, Wang L, Chuang KH, Wu CT, Chang C (2005) Induction of androgen receptor expression by phosphatidylinositol 3-kinase/Akt downstream substrate, FOXO3a, and their roles in apoptosis of LNCaP prostate cancer cells. *J Biol Chem* **280:** 33558-65

Yellaturu CR, Bhanoori M, Neeli I, Rao GN (2002) N-Ethylmaleimide inhibits platelet-derived growth factor BB-stimulated Akt phosphorylation via activation of protein phosphatase 2A. *J Biol Chem* **277:** 40148-55

Yu F, Watts RN, Zhang XD, Borrow JM, Hersey P (2006) Involvement of BH3-only proapoptotic proteins in mitochondrial-dependent Phenoxodiol-induced apoptosis of human melanoma cells. *Anti-cancer drugs* **17:** 1151-61

Yu K, Toral-Barza L, Discafani C, Zhang WG, Skotnicki J, Frost P, Gibbons JJ (2001) mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocr Relat Cancer* **8:** 249-58

Yu L, Blackburn GL, Zhou JR (2003) Genistein and daidzein downregulate prostate androgen-regulated transcript-1 (PART-1) gene expression induced by dihydrotestosterone in human prostate LNCaP cancer cells. *J Nutr* **133:** 389-92

Yuan JP, Wang JH, Liu X (2007) Metabolism of dietary soy isoflavones to equol by human intestinal microflora--implications for health. *Mol Nutr Food Res* **51:** 765-81

Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D (2003a) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* **5:** 578-81

Zhang YC, Albrecht D, Bomser J, Schwartz SJ, Vodovotz Y (2003b) Isoflavone profile and biological activity of soy bread. *J Agric Food Chem* **51:** 7611-6

Zhou BP, Hung MC (2002) Novel targets of Akt, p21(Cipl/WAF1), and MDM2. *Semin Oncol* **29:** 62-70

Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ (2003) Role of the steroid receptor coactivator SRC-3 in cell growth. *Molecular and cellular biology* **23:** 7742-55

Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL, Clinton SK (1999) Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. *J Nutr* **129:** 1628-35

Zhou JR, Yu L, Zhong Y, Nassr RL, Franke AA, Gaston SM, Blackburn GL (2002) Inhibition of orthotopic growth and metastasis of androgen-sensitive human prostate tumors in mice by bioactive soybean components. *Prostate* **53:** 143-53

Zinda MJ, Johnson MA, Paul JD, Horn C, Konicek BW, Lu ZH, Sandusky G, Thomas JE, Neubauer BL, Lai MT, Graff JR (2001) AKT-1, -2, and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. *Clin Cancer Res* **7:** 2475-9

Zubik L, Meydani M (2003) Bioavailability of soybean isoflavones from aglycone and glucoside forms in American women. *The American journal of clinical nutrition* **77:** 1459-65