

**Gene Expression Changes in Prostate Cells Upon Exposure to
Environmental Anti-Androgenic Pesticide Vinclozolin**

By:

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This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and
Molecular Medicine

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Environmental Anti-Androgenic Pesticide Vinclozolin**

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Abstract

Vinclozolin (VCZ), an antiandrogenic fungicide, is an endocrine disrupting chemical that is known to possess high affinity for the androgen receptor (AR) and modulate expression of critical androgen-dependant genes in the prostate. In this study, viability and expression of AR, NKX3.1 and CYP3A4 genes were measured in androgen-sensitive prostate cells LNCaP after exposure to VCZ and VCZ treated with S9 microsomes in a time and dose dependent manner. NKX3.1 is an androgen regulated gene that plays a vital role in prostate development. CYP3A4 is involved in xenobiotic metabolism. VCZ decreased the viability at high doses after 48 hours which was slightly mitigated by treatment with S9 metabolites. Expression of NKX3.1 and CYP3A4 was upregulated while an initial downregulation of AR was observed. NKX3.1 upregulation corroborates with possibility of antiandrogens to act as androgens in LNCaP. The results illustrate that VCZ can interfere with the expression of critical prostate genes.

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List of Abbreviations

°C	Degrees Celsius
μM	Micro Molar
ANOVA	Analysis of variance
AR	Androgen Receptor
ARA	Androgen Receptor-Associated
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-Related
BPH	Benign Prostate Hyperplasia
CAR	Constitutive Androstane Receptor
CBP	CREB-Binding Protein
cDNA	complementary DNA
CO ₂	Carbon Dioxide
CYP	Cytochrome P ₄₅₀
DAPI	4',6-diamidino-2-phenylindole
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DES	Diethylstilbestrol

DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
DMWM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDC	Endocrine Disrupting Chemicals
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
HDAC1	Histone Deacetylase 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KCl	Potassium chloride
K _i	Binding Affinity
LH	Luteinizing Hormone
LNCaP	Lymph Node Carcinoma of the Prostate
M1	2-(3,5-dichlorophenyl)-carboxymoyl)-2-methyl-3-butenic acid
M2	3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide
MgCl ₂	Magnesium Chloride

mM	Milli Molar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger Ribonucleic Acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NKX3.1	NK3 Transcription Factor Related Locus 1
nM	Nano Molar
NOEL	No Observable Effect Level
PBS	Phosphate Buffered Saline Solution
PCa	Prostate Cancer
PCB	Polychlorinated Biphenyl
PI3K	Phosphatidylinositol 3-Kinases
PIN	Prostatic Intraepithelial Neoplasia
PXR	pregnane X receptor
qRT-PCR	Quantitative Real Time-Polymer Chain Reaction
RNA	Ribonucleic Acid

RXR	Retinoid X Receptor
S9	Liver Microsomes, Supernatant Fraction
SRC-1	Steroid Receptor Coactivator-1
TBE	Tris/Borate/ Ethylenediaminetetraacetic acid
TRPM-2	Testosterone-Repressed Prostatic Message
VCZ	Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione)

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1. Introduction

1.1 Endocrine Disrupting Chemicals (EDCs)

The advances in modern manufacturing and industrial development within the second half of the 20th century has brought an abundance of synthetic chemicals into market that are wildly used in production of an array of consumer and agriculture products (Soto and Sonnenschein, 2010); these include pesticides used on common food crops, resins and plasticizers used in manufacturing of plastics, and a number of other chemicals used in trade and commerce. Some of these chemicals, although intended for beneficial purposes, may have potential adverse effects. These chemicals are generally toxic to the environment and ecology while others do not appear to be toxic at current levels of usage but possess properties similar to natural hormones and are, therefore, hormonally active at very low levels. These chemicals are identified as endocrine disrupting chemicals (EDCs). EDCs are commonly used in commerce and trade and household products. The toxicity of these chemicals is under scrutiny for their toxicological profile is still emerging.

During the last half of the 20th century, the use of these chemicals has found its way into aquatic and wildlife species through leaching in soil and water from agriculture runoffs and industrial effluents (Munkittrick, 2001; Yin et al., 2002). Little is known on the adverse effects produced by these chemicals in the flora and the fauna in the environment. However, recent observations in mammal and birds have indicated behavioural abnormalities, and reproductive and developmental defects in the wildlife

(Cooper and Kavlock, 1997; Patisaul and Adewale, 2009). High levels of pesticides, persistent organic chemicals such as polychlorinated biphenyl (PCB) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) are thought to have played a role in altered development of wildlife species that includes abnormal thyroid function, demasculinization/feminization and reduced offspring survival of fish, birds and mammals (Crisp et al., 1998; Vos et al., 2000). In humans, a decrease in sperm count and quality has been noticed and the rate of these changes cannot be explained by genetic factors alone and environmental factors need to be considered (Carlsen et al., 1992; Merzenich et al., 2010).

Chemicals that are capable of interfering with the endocrine system and referred to as endocrine disrupting chemicals were first reported by Theo Colborn and her colleagues in the early 1990s to define environmental compounds and pollutants that disrupt endocrine development in wildlife and animals, including humans (Colborn et al., 1993). A succinct definition of EDCs is given by the United States Environmental Protection Agency which defines EDCs as exogenous compounds capable of “interfering with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (United States Environmental Protection Agency, 2011). Synthesized hormones are the key chemical messengers involved in regulation of the endocrine system. Endocrine glands release hormones into the bloodstream where they act on target tissues to induce specific functions that may range from developmental, behavioural, metabolic or reproductive processes. The network of glands of the endocrine

system allows for communication between different organs and this constant communication is necessary for proper homeostasis and development of the organs.

EDCs can affect the system in different ways at various sites depending on the nature of the compound and its toxicity and persistence. Normal steroid hormones of the endocrine system are synthesized from blood cholesterol and absorbed into the appropriate glands (Miller, 1988); EDCs may alter the synthesis of steroid hormones (Hirsch et al., 1987) or alter their transport and clearance by binding to steroid binding proteins that normally have an affinity for endogenous hormones thereby increasing the level of steroid hormones available for the cell (Danzo, 1997). Although these mechanisms are important, the most common and well-studied method of EDC action is through direct interaction with steroid hormone receptors such as the androgen receptor (AR) or estrogen receptor (ER). Through *in vitro* hormone binding assays, it has been shown that some EDCs are capable of binding to steroid receptors and mimicking natural ligands or inhibiting natural ligands from interacting with the receptor thereby acting as an agonist or antagonist, respectively (Kelce et al., 1995; Kelce et al., 1994; White et al., 1994). More recently, it has been shown that, through activation or inhibition of steroid receptors, EDCs are capable of altering molecular events via changes in gene expression profile (Bonefeld Jorgensen et al., 1997; Singleton et al., 2006).

1.2 Endocrine Disruptors and Effects on Prostate

Because of their potential to act like natural hormones and ability to deviate or amplify normal hormonal effects, EDCs have been linked to developmental and reproductive

abnormalities and hormone related cancers such as breast, testis, and prostate. The prostate is an exocrine gland of the male reproductive system located below the bladder and wrapped around the urethra. The anatomy of the prostate varies among species; within dogs, humans and primates, the prostatic ducts radiate peripherally to completely surround the urethra but in rats and mice the ducts are organized in distinct lobes located in particular positions around, but not encircling, the urethra (Cunha et al., 1987; Timms et al., 1994). The prostate functions to produce and secrete chemicals and compounds such as citric acid, zinc, polyamines, and a variety proteins and proteases that constitute the seminal fluid and help provide optimal conditions for sperm survival and mobility (Luke and Coffey, 1994). Steroid hormones, particularly androgens, are essential to prostate endocrinology and play a critical role in the gland's development, maturation, maintenance and function.

The prostate is considered to be extremely sensitive to the effects of EDCs and there is reasonable evidence to suggest EDCs may play a role in etiology of diseases such as benign prostate hyperplasia (BPH) and prostate cancer (PCa) (Prins, 2008). Although no direct link between environmental EDCs and PCa in humans have been found, chronic or intermittent exposures to pesticides has been suggested as a possible factor for higher prostate cancer rates observed in farmers (Van Maele-Fabry and Willems, 2003). This is supported by two epidemiological studies. A study by Alavanja et al (2003) with approximately 90,000 individuals within the United States revealed a significant increase in prostate cancer diagnosis among individuals with no familial history of the disease and exposure to chlorinated pesticides and methyl bromide (Alavanja et al, 2003). These

researchers have also reported of significant elevation of prostate cancer diagnosis among individuals with family history of PCa suggesting gene-environment interaction (Alavanja et al., 2003). Similarly, a significant association between the number of prostate cancer related deaths and the number of acres sprayed with herbicides by Canadian farmers has been reported (Morrison et al., 1993).

Estrogenic compounds such as estradiol have been reported to decrease AR expression in prostatic cells, and retard growth and epithelial cell differentiation *in utero*, suggesting epithelial cells of the prostate are possible targets for EDCs (Prins and Birch, 1995). Fetal exposure to low doses of diethylstilbestrol (DES) and postnatal exposure to PCBs have shown to increase prostate weight and to effect histological alterations in the ventral prostate, respectively (Sager et al., 1987; Welshons et al., 1999). These studies further demonstrate the sensitivity of the prostate to EDCs and insinuate that exposure to pesticides that have estrogenic or androgenic activity may impact the developmental and maturation of the prostate gland and prostate diseases.

1.3 Vinclozolin

Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione) (VCZ) is a dicarboximide fungicide widely used to protect food crops such as fruits, vegetables, and wine grapes in the United States and Europe. Dicarboximide fungicides have been extensively used to control gray mold and brown rot caused by *Botrytis cinerea* and *Monilinia* spp (Gullino and Garibaldi, 1986; Jeffers, 1991). These fungicides commonly

work by inhibiting the biosynthesis of the steroid, ergosterol, found in fungal cell membranes and induce distortion, bursting and lysis of cells. Biotransformation of VCZ by mammalian, plant and bacterial systems produces several metabolites including the two most potent metabolites, 2-(3,5-dichlorophenyl)-carboxymoyl)-2-methyl-3-butenic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) (Szeto et al., 1989c; Kelce et al., 1995; Sierra-Santoyo et al., 2004); these two metabolites have strong affinity for the androgen receptor and act as potent inhibitors of AR activity relative to VCZ, which itself has little affinity for AR (Kelce et al., 1994). The metabolites of VCZ (M1 and M2) have previously been found in soils, plants and animals exposed to VCZ, and have the potential to displace from treatment sites to non-target areas by runoff and leaching (Steege and Garber, 2009). Low level traces of VCZ have been found in the Mediterranean Sea and the Southern Dead Sea basin (El-Shahat et al., 2003; Readman et al., 1997). The U.S. Environmental Protection Agency has established a chronic reference dose for VCZ at 0.012 mg/kg/day and it is estimated that the exposure of the U.S. population is not greater than 16% of the reference dose (BASF, 1997; USEPA, 1997). Occupational workers in close contact with pesticides have a higher risk for exposure; workers in greenhouses have an estimated dermal exposure of 2.5 mg/day which is higher than the E.P.A permitted value (Nilsson and Papantoni, 1996). The affinity of VCZ and its metabolites for the AR, the activity and effects of VCZ *in vivo* and *in vitro*, and the persistence of VCZ in the environment, have been under scrutiny for their potential toxicological outcomes.

1.3.1 Toxicity

Animal toxicity to VCZ has been well established. Studies have shown that the no observable effect level (NOEL), a measure of exposure at which the chemical is tolerable and has no significant adverse effects, is around 9 mg/kg/day for a 3 month exposure period and 2.5 mg/kg/day for a 12 month exposure period in beagles (Hofmann and Munk, 1997). Other observed effects included increased adrenal cortical weight in females, and slight increase in testicular weight and prostate atrophy in males.

In vivo experiments in rats have indicated developmental and reproductive toxicity with signs of carcinogenicity after chronic exposure. Three month exposure in male rats followed by varying durations of recovery periods showed statistically significant decrease in weights of epididymis, seminal vesicles and prostate accessory glands; testicular interstitial cell tumors, prostate gland adenomas and reduction in sensitivity to testosterone was also noticed (Mellert et al., 1995). Similar conditions including epididymal and testicular granulomas, and atrophic seminal vesicles and ventral prostate glands were seen in one year male rats after perinatal exposure to 100 mg/kg/day VCZ (Gray et al., 1993). Exposure of female rats during pregnancy resulted in urogenital malformations, delayed onset of puberty with peripubertal growth and development of accessory sex organs leading to, at 1000 ppm, increased levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and dihydrotestosterone (DHT) reproductive hormones in blood of male rats (Gray et al., 1994; Gray et al., 1999; Matsuura et al., 2005; Monosson et al., 1999). Oral exposure of VCZ at doses as low as 1

mg/kg/day during gestation also showed abnormal effects on reproductive organs including reduced prostate, testis and seminal vesicle weights, and reduced sperm counts (Elzeinova et al., 2008). VCZ did not induce chromosomal aberrations in lymphocytes, affect rate of micronuclei, produce clastogenic or aneugenic activities in polychromatic erythrocytes, or showed oxidative DNA damage in liver suggesting lack of genotoxic potential (Hrelia et al., 1996; Kevekordes et al., 1996; Lodovici et al., 1997).

The antiandrogenic property of the compound leads to interactions with the androgen receptor in the prostate and alterations of androgen receptor regulated genes is thought to be the primary mode of action through which the observed side effects are produced (Kavlock and Cummings, 2005; Kelce et al., 1997). No evidence of adverse effects to VCZ exposure has been demonstrated in humans but conservation of the androgen receptor among species suggests similar antiandrogenic effects may be plausible (Kavlock and Cummings, 2005).

1.3.2 Metabolism

There is limited information regarding biotransformation and distribution of VCZ. Majority of studies have focused on two main metabolites, M1 and M2, due to their increased potential to act as AR antagonist compared to the parent compound.

Sierra-Santoyo et. al (2008) conducted one of the few studies analyzing the pharmacokinetics of VCZ and reported that after an acute oral dose administration of 100 mg/kg in Long-Evans rats, VCZ was readily absorbed due to its lipophilic nature (half-life of absorption of 0.66 h); the elimination half-life of the parent compound was 3.6 h

while metabolites M2, the compound with the greatest affinity for AR, and M1 had elimination half-lives of 18.8 h and 3.3 h, respectively (Sierra-Santoyo et al., 2008).

The distribution of VCZ and its metabolites in serum and tissue varies with time as seen in Table 1 (Sierra-Santoyo et al., 2008). Due to the rapid absorption and subsequent transformation by hydrolysis, enzymatic and oxidative processes, the concentration of VCZ decreases in all tissues from 2 h to 8 h (except in the prostate and fat where it is 1.8 and 3.7 fold higher at 8 h, respectively) and, with the exception of M5, low levels of metabolites are observed in tissues with high AR levels (testis, prostate and brain). M5 and its glucuronide conjugates are the major metabolites found in serum and tissues after treatment with rat liver slices (Bursztyka et al., 2008; Sierra-Santoyo et al., 2008). Higher levels of metabolites are present in the liver and kidney, tissues with the greatest capacity to metabolize xenobiotics. Presence of metabolites in tissues suggests that they are products of enzymatic reactions in the tissue or they are taken up from the serum (Sierra-Santoyo et al., 2008).

In aqueous solution and serum, VCZ is partially converted into its main metabolites M1 and M2 by reversible non-enzymatic hydrolysis and loss of CO₂ following hydrolysis, respectively (Bursztyka et al., 2008; Sierra-Santoyo et al., 2004; Szeto et al., 1989a).

Table 1. Distribution of VCZ and its metabolites in serum and organs of male Long-Evans rats after an acute oral administration of 100 mg/kg. Table reproduced with permission from Sierra-Santoyo et al., 2008.

Metabolite	Time (h)	Concentration						
		Serum	Liver	Kidney	Brain	Testes	Prostate	Fat
Vinclozolin	2	16.9 ± 4.0	48.19 ± 16.64	32.86 ± 8.90	9.05 ± 0.83	7.36 ± 0.70	8.64 ± 0.95	49.71 ± 9.63
	8	8.7 ± 2.5	26.87 ± 7.43	19.42 ± 4.74	6.19 ± 1.98	4.83 ± 1.28	15.62 ± 7.59	182.77 ± 22.28
M1	2	17.5 ± 1.8	4.74 ± 0.88	9.76 ± 5.04	2.25 ± 0.25	1.03 ± 0.27	1.91 ± 0.28	3.70 ± 0.22
	8	42.5 ± 9.8	11.30 ± 1.16	16.76 ± 2.68	0.82 ± 0.14	1.60 ± 0.40	1.67 ± 0.19	1.86 ± 0.27
M2	2	0.1 ± 0.1	0.19 ± 0.06	ND	ND	ND	ND	0.18 ± 0.03
	8	0.1 ± 0.1	0.26 ± 0.07	ND	ND	ND	ND	1.09 ± 0.11
M3	2	0.3 ± 0.2	0.70 ± 0.08	0.67 ± 0.41	0.41 ± 0.14	ND	0.24 ± 0.02	0.60 ± 0.07
	8	0.5 ± 0.3	1.15 ± 0.27	0.61 ± 0.07	0.11 ± 0.01	ND	ND	1.75 ± 0.74
M5	2	57.7 ± 2.1	57.74 ± 8.48	47.57 ± 16.58	20.20 ± 2.36	12.60 ± 1.30	16.04 ± 1.50	5.29 ± 0.81
	8	75.3 ± 10.9	70.98 ± 12.63	57.22 ± 7.84	36.68 ± 6.30	26.24 ± 4.02	25.90 ± 6.88	6.72 ± 1.15
M7	2	1.8 ± 0.4	2.87 ± 0.33	6.37 ± 2.87	2.43 ± 0.26	1.43 ± 0.23	2.56 ± 0.43	1.63 ± 0.27
	8	0.8 ± 0.2	1.66 ± 0.25	2.41 ± 0.19	0.99 ± 0.15	1.26 ± 0.21	1.22 ± 0.27	0.56 ± 0.05
M8	2	27.5 ± 8.9	12.18 ± 2.46	9.87 ± 3.62	0.48 ± 0.07	1.45 ± 0.23	1.35 ± 0.39	0.61 ± 0.11
	8	16.9 ± 1.3	12.05 ± 4.41	4.43 ± 0.97	ND	1.09 ± 0.16	1.04 ± 0.22	0.17 ± 0.04
M9	2	9.2 ± 1.0	3.29 ± 0.79	1.81 ± 0.62	0.12 ± 0.05	0.29 ± 0.03	0.30 ± 0.15	ND
	8	5.3 ± 1.8	1.27 ± 0.13	0.63 ± 0.24	ND	0.39 ± 0.07	0.28 ± 0.05	ND

VCZ in DMEM media at 37°C without any treatment shows 90 % transformation of VCZ into M1 and M2 after 2 hours with M1 being excessively favoured (Bursztyka et al., 2008). Similar results are observed *in vivo* and the presence of these primary metabolites may also be attributed to the initial uptake of VCZ by tissues and their release into the blood following non-enzymatic hydrolysis (Bursztyka et al., 2008; Sierra-Santoyo et al., 2008; Sierra-Santoyo et al., 2004; Szeto et al., 1989b). The serum levels of VCZ, M1 and M2 detected in rats have been well below the binding affinity, K_i , for the AR with maximum M1 at 44 % of K_i and VCZ and M2 at 3 % of K_i ; this may suggest that M1 is the main metabolite interacting with the AR although the maximum M2 detected in serum could still be antagonistic (Sierra-Santoyo et al., 2008). It is possible for M1 to recycle into VCZ but this only occurs at significantly high pH (Szeto et al., 1989b).

A proposed scheme of VCZ transformation in rats is presented in Figure 1 (Sierra-Santoyo et al., 2011). The enzymatic phase-I step includes an epoxide hydrolase reaction of the vinyl group to form metabolite M4 which is further transformed by opening of the 2,4-oxazolidinedione ring by the addition of water to give an unstable intermediate, M7, and a decarboxylation step from M7 gives the final metabolite M5 (Bursztyka et al., 2008; Sierra-Santoyo et al., 2011). It has been suggested that M1, produced by non-enzymatic hydrolysis of VCZ, is dihydroxylated on the ethylene group to produce the M6 metabolite which is transformed into M5 via ring closure to M4 (Bursztyka et al., 2008) but incubations with rat liver microsomes did not produce any evidence of M1 biotransformation (Sierra-Santoyo et al., 2011). M2 is mainly metabolized into M5 by enzymatic epoxidation and also into minor levels of M3, an unimportant metabolite

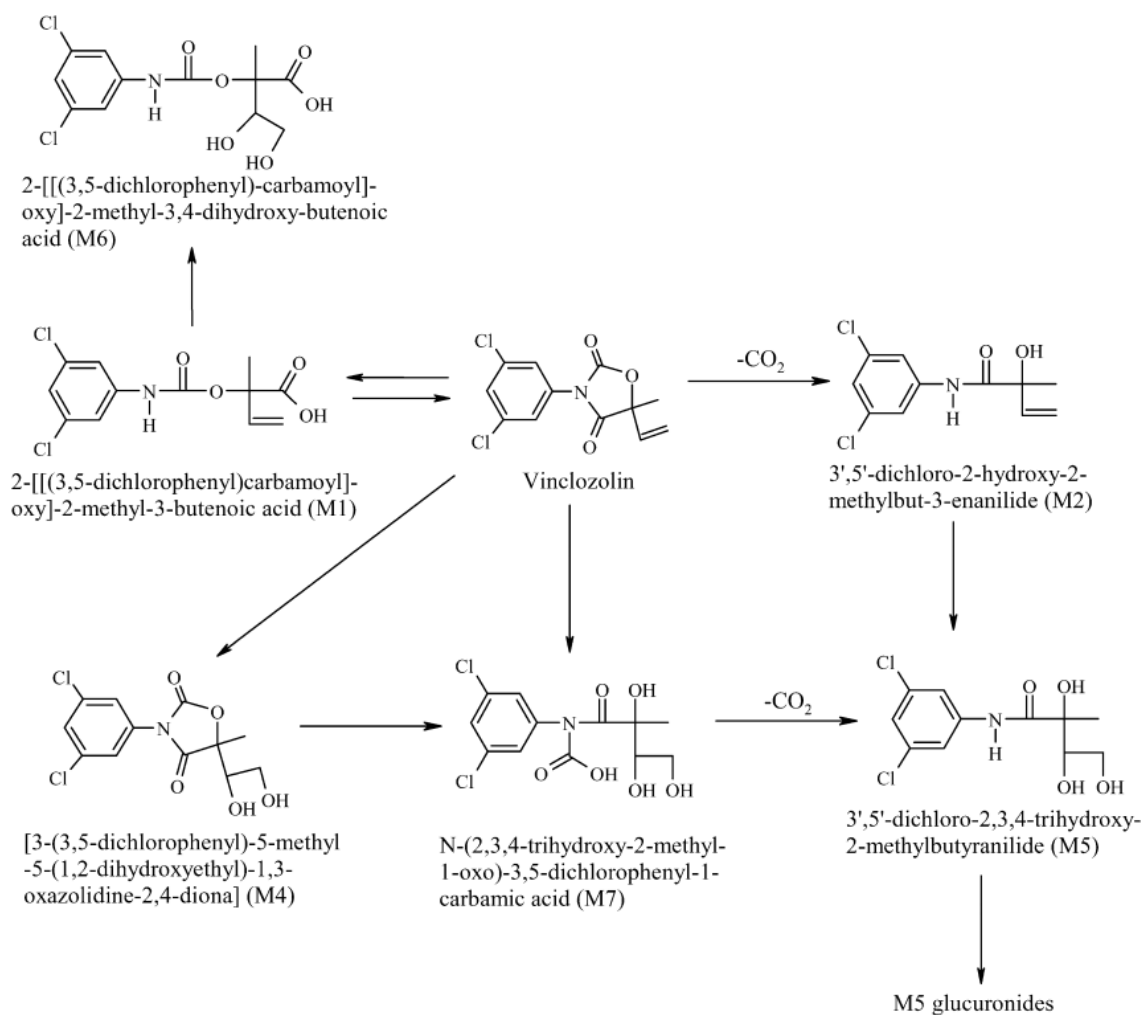


Figure 1. Proposed in vivo biotransformation of VCZ in rats. Figure reproduced with permission from Sierra-Santoyo et al., 2008.

possibly present due to acidic conditions during analysis (Pothuluri et al., 2000; Sierra-Santoyo et al., 2004). More studies are needed to clarify M1 and M2 biotransformation (Sierra-Santoyo et al., 2011). Phase II glucuronide conjugates of M5 is the main metabolite of VCZ found in urine of rats (Bursztyka et al., 2008; Sierra-Santoyo et al., 2008). Maximum solubility of VCZ in dimethyl sulfoxide (DMSO) added to DMEM media was found to be 250 μ M (Bursztyka et al., 2008).

1.3.3 Mode of Action

The most commonly discussed mode of action for VCZ induced effects is through the AR pathway.

Androgen receptor pathway

AR signalling plays a fundamental role in prostate gland growth, differentiation and development, and in early and late stage prostate cancer (Bluemn and Nelson, 2012; Dehm and Tindall, 2005). The AR pathway is depicted in Figure 2.

The modular domains of the AR are similar to other nuclear receptors; it includes an transcriptional regulatory N-terminal domain, a zinc-finger DNA binding domain, a hinge region and C-terminal ligand binding domain. Prior to ligand binding, the AR is retained in the cytoplasm bound to heat shock protein chaperone molecules such as Hsp90 and 70 (Pratt and Toft, 1997). Natural lipophilic ligands such as testosterone are able to diffuse into target cells and act upon the receptor. Typically testosterone is reduced to a more potent hormone, DHT, by the 5 α -reductase enzyme. DHT has 2-3 times greater binding

affinity for the androgen receptor than testosterone leading to a more stabilized ligand-receptor complex and a higher amplification of the androgen signalling. Upon binding to the ligand, the AR changes conformation to release the heat shock proteins and is translocated into the nucleus. Within the nucleus, dimerization of the ligand bound AR occurs (Wong et al., 1993) and the homodimer binds to specific androgen response elements within the promoter and enhancer regions. The binding of ARs to the DNA recruits coregulatory proteins such as AR coactivators ARA70, 55 and 54, and other

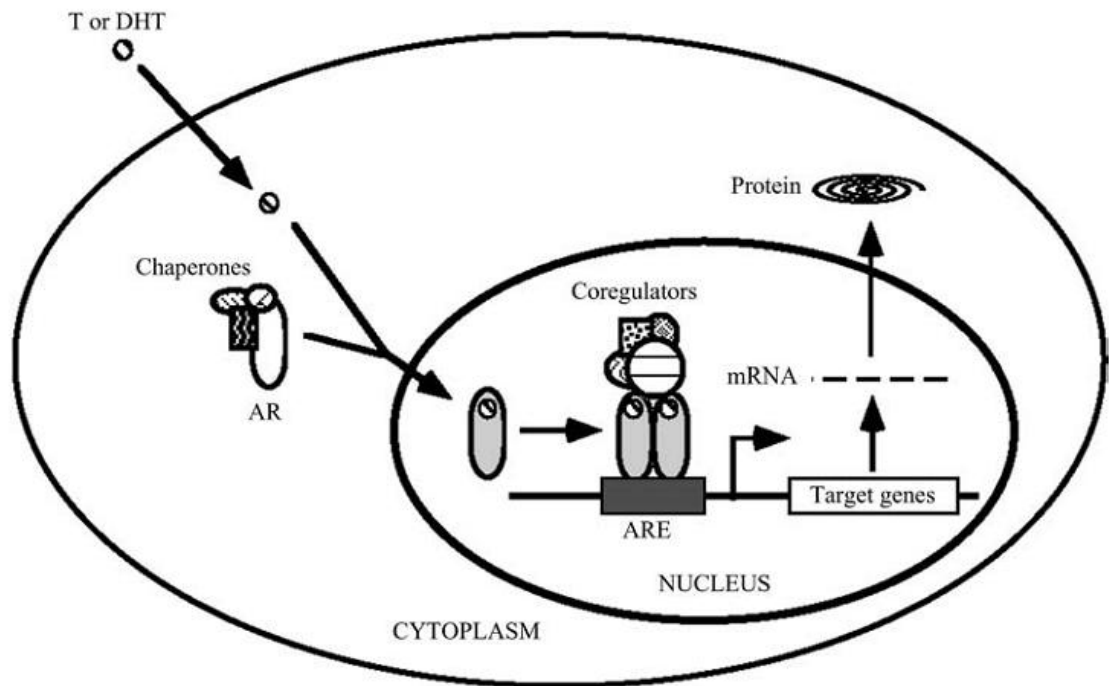


Figure 2. Normal function of the androgen receptor signalling activated by androgens.

Figure reproduced with permission from Lee H.-J. and Chang C., 2008.

coactivators that have histone acetyltransferase activity such as SRC-1 and CBP (Glass et al., 1997). This transcriptionally active structure allows for RNA polymerase activity and transcription of the androgen regulated gene.

Vinclozolin and the Androgen Receptor

It has been shown through *in vitro* competition-binding assays that VCZ and its metabolites bind competitively to rat and human AR thereby antagonizing the binding of natural hormones, such as DHT, and inhibiting AR binding to androgen receptor response elements (ARE) (Kelce et al., 1994; Molina-Molina et al., 2006; Nellemann et al., 2003; Wong et al., 1995). Although VCZ itself is a weak AR antagonist, its two primary metabolites, M1 and M2, have much stronger antagonism for the AR (K_i values of 92 μ M and 9.7 μ M, respectively) (Kelce et al., 1994). M2 has been observed to be 50-fold more potent inhibitor than M1 and only 2-fold less potent than hydroxyflutamide, an antiandrogen drug used to treat prostate cancer (Wong et al., 1995). VCZ metabolite antagonists inhibit AR-dependant process such as induction of testosterone-repressed prostatic message (TRPM-2) and repression of testosterone-induced prostatic message (prostatein subunit C3) in rats (Kelce et al., 1997).

Structurally, the prominent VCZ metabolites, M1 and M2, have similarities to the pure AR antagonist drug flutamide and its metabolite, hydroxyflutamide (Kelce et al., 1995). Responses *in vivo* to VCZ show similar phenotypic outcomes as flutamide such as disruption of sex differentiation after exposure of pregnant rats, decrease in seminal and prostate weights, decrease in immunohistochemical staining of the AR in the epididymal

nuclei, and alterations in serum reproductive hormone levels (Gray et al., 1994; Kelce et al., 1997; Nellemann et al., 2003; Yu et al., 2004).

Although commonly regarded as an AR antagonist, in some studies, it has been shown that VCZ may act as an AR *agonist* depending on the availability of natural androgens and their binding affinity and concentration (Körner et al., 2004; Wilson et al., 2002; Wong et al., 1995). It should also be noted that, *in vitro*, the complexity of studying antiandrogenicity of VCZ in prostate is increased due to mutations in the AR of prostate cancer cell lines. Antiandrogens cyproterone acetate and hydroxyflutamide have shown to act as agonists in the prostate cancer LNCaP cell line (Veldscholte et al., 1992; Wilding et al., 1989); this is commonly explained to be due to a T877A mutation in the AR of LNCaP that allows the ligand binding domain to be agonized by a wider range of hormones and antiandrogens (Veldscholte et al., 1992).

1.4 Expression of Genes Involved in Prostate Development and Disease

1.4.1 NK3 transcription factor related locus 1 (NKX3.1)

Expression of prostate-specific homeobox gene *NK3 transcription factor related locus 1* (NKX3.1) is thought to play an important role in prostate development and differentiation (Prescott et al., 1998). Murine NKX3.1 is expressed in the early prostatic epithelium within emerging prostatic buds and during all states of prostate differentiation (Bhatia-Gaur et al., 1999); the prostatic epithelial buds are involved in ductal outgrowth and branching into the mesenchyme during the first three weeks of postnatal development (Sugimura et al., 1986; Timms et al., 1994). Although its role is only partially known, it

is possible that NKX3.1 is involved in reciprocal signalling between the epithelial and mesenchyme during early prostate development (Bhatia-Gaur et al., 1999). NKX3.1 may have a protective role against agents that cause DNA damage by activating ATM and ATR, members of the phosphoinositide 3-kinase (PI3K) family signalling cascade responsible for activating cell's response to DNA damage (Bowen and Gelmann, 2010), and by also up-regulating antioxidant enzymes while down-regulating pro-oxidants thereby protecting the genome from oxidative stress (Ouyang et al., 2005). Multiple tissue specimens showed that NKX3.1 expression is limited to the prostate with very low levels of detection in the testis (Prescott et al., 1998).

There is considerable evidence to suggest a prospective link between NKX3.1 expression and prostate carcinogenesis. NKX3.1 maps to the minimal region of human chromosome 8p21 (He et al., 1997; Swalwell et al., 2002; Voeller et al., 1997), the region to most frequently undergo loss of heterozygosity in prostatic intraepithelial neoplasia and prostate cancer (Bova et al., 1993; Emmert-Buck et al., 1995; Vocke et al., 1996), and is commonly regarded to function as a haplo-insufficient tumor suppressor. Histological studies of NKX3.1 mutant mice shows development of prostatic intraepithelial neoplasia (PIN) and dysplasia (Bhatia-Gaur et al., 1999; Kim et al., 2002; Tanaka et al., 2000) that resemble human PIN by the age of 1 or 2 years (Kim et al., 2002). Although loss-of-function of NKX3.1 results in development of PIN, a presumed precursor of prostate cancer, the progression to invasive carcinoma was not seen; however, promotion of neoplastic progression by serial transplantation of recombinant prostatic epithelium from NKX3.1 mutant mice with embryonic urogenital sinus mesenchyme show abnormal

growth patterns and poor differentiation characteristic of neoplastic progression (Kim et al., 2002).

Human prostate cancer samples were reported to show reduced expression of NKX3.1 protein levels which inversely correlated with Gleason score (Bethel et al., 2006) and tumour progression (Bowen et al., 2000). A decrease in NKX3.1 protein levels is thought to play a role in onset of prostate carcinogenesis (Bethel et al., 2006). Although a reduction, but not complete loss, in the expression of protein levels of NKX3.1 was observed in prostate cancer patients (Asatiani et al., 2005), expression of messenger RNA (mRNA) levels appeared to be inversely associated with protein levels in prostate cancer. Xu et al. (2000) reported that NKX3.1 mRNA was overexpressed in 31% of prostatic adenocarcinoma specimens, decreased in 21% of the samples, and no change was observed in 48% of the samples, and overexpression correlated with metastatic disease (Xu et al., 2000). Concordance between protein and mRNA levels was only seen within prostate atrophy lesions where 4 of 6 samples with increased or normal NKX3.1 mRNA levels had decreased protein accumulation (Bethel et al., 2006). Benign prostate hyperplasia tissue samples in humans were reported to show higher gene expression of NKX3.1 compared to normal prostate tissues (Irer et al., 2009). This suggests that post transcriptional alterations and other mechanisms may play a major role in NKX3.1 protein down regulation within atrophic and neoplastic prostate epithelial cells (Bethel et al., 2006; Guan et al., 2008). Lack of mutation in the coding region of NKX3.1 suggests that it may function in a different manner than classical tumour suppressor genes (Prescott et al., 1998; Voeller et al., 1997).

Expression of NKX3.1 mRNA is rapidly upregulated in the presence of androgenic compounds such as synthetic androgens R1881 or mibolerone and naturally present hormone DHT (He et al., 1997; Possner et al., 2008; Prescott et al., 1998; Xu et al., 2000) in a time- and dose-dependent manner (Prescott et al., 1998). Nuclear run-off assays in LNCaP prostate cell line show that androgens directly affect the rate of synthesis of new NKX3.1 transcripts by two-fold without the need for other post-transcriptional mechanisms, and this increase does not depend on intermediary proteins that androgens may express or changes in half-life of NKX3.1 transcripts (Prescott et al., 1998). Significant correlation between AR expression and NKX3.1 expression in epithelial cells of human prostate tissue and rapid induction of NKX3.1 also suggests AR involvement in regulation of NKX3.1 expression (Xu et al., 2000). Reduction of androgen levels in luminal epithelium of adult mouse prostate through castration decreased mRNA levels of NKX3.1 by 10-fold in 24 hours (Bieberich et al., 1996). Three androgen-responsive regions have been identified within the 3'UTR of NKX3.1 that function co-operatively to allow for binding of the activated AR in order to mediate the regulation of NKX3.1 (Thomas et al., 2010).

1.4.2 Androgen Receptor (AR)

As previously mentioned, the expression of AR and its signalling is critical for development and functioning of the prostate, and plays a central role in prostate carcinogenesis. PCa and hypertrophy does not occur in the absence of androgens, and

men who are unable to produce testosterone or DHT, the natural ligands that activate the AR, by either castration before puberty or by being 5 α -reductase deficient, do not develop PCa (Wu and Gu, 1991). Low serum testosterone levels and approximately 60% of castration-resistant prostate cancers show a genomic amplification and/or overexpression of the AR (Schatzl et al., 2002; Taylor et al., 2010). Although elevation of AR expression is important in PCa development, the heterogeneity of human PCa, such as observance of epigenetic silencing in some cases or potential decrease in protein stability leading to reduced AR expression, suggests that increased AR expression is not generally associated with PCa initiation (Lin et al., 2002; Sasaki et al., 2002). Nonetheless, endocrinological treatment of PCa generally involves modulating the activity of AR by limiting the availability of circulating testicular androgens through castration or by administering of anti-androgens to prevent the binding of natural androgens such as testosterone or dihydrotestosterone.

It is possible that EDCs may alter the expression of AR and affect responsiveness to androgens during early fetal life (Anway and Skinner, 2006). Interaction between VCZ and AR pathway has been described in the mode of action.

1.4.3 Cytochrome P₄₅₀ 3A4 (CYP3A4)

The cytochromes P450 constitute a diverse group of heme-containing mono-oxygenase enzymes that play a major role in detoxification and clearance of xenobiotics and toxins, and biosynthesis and breakdown of endogenous compounds such as steroid hormones,

cholesterol and lipids. Although the major xenobiotic metabolizing families of cytochrome P₄₅₀ (CYP) enzymes are primarily found in liver hepatocytes, expression of CYP enzymes has also been identified in many extra-hepatic tissues such as lung, kidney, brain and prostate (Finnstrom et al., 2001; Gonzalez and Lee, 1996).

Cytochrome P450 genes are divided among many families of which the CYP3A subfamily contains four members: CYP3A4, CYP3A5, CYP3A7 and CYP3A43. The CYP3A subfamily of enzymes are the most abundant of all human CYP isoforms and have an extremely broad substrate specificity for drugs from all major drug classes including calcium channel blockers, HIV protease inhibitors, chemotherapeutic agents, and steroids (Wrighton et al., 2000). Extensive inter-individual variations in CYP3A expression contributes greatly to the bioavailability of CYP3A substrates and their metabolites (Lamba et al., 2002); steroids such as estrogens are targets for CYP3A metabolism and variations of CYP3A expression may influence circulating estrogen levels and estrogen-mediated carcinogenicity such as breast cancer (Huang et al., 1998).

Induction of CYP450s is primarily regulated through the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), both of which serve important roles in xenobiotic sensing and act as master regulators of phase I and phase II metabolizing enzymes (Waxman, 1999). Activation of PXR and CAR induces the major detoxification enzymes CYP3A4 and CYP2B10, respectively (Honkakoski and Negishi, 2000; Waxman, 1999). The ligand-binding pocket of PXR is larger than other nuclear receptors and allows for greater promiscuity of target compounds, many of which are

exogenous and identified as endocrine disrupting chemicals (Watkins et al., 2001; Watkins et al., 2003). When activated, PXR forms a heterodimer with retinoid X receptor (RXR) in the nucleus and this complex binds to xenobiotic response elements of PXR target genes (Blumberg and Evans, 1998).

The major target gene of PXR, CYP3A4, is constitutively expressed in cancerous and normal human prostate tissue, and facilitates the metabolism of drugs, dietary compounds, mutagens and steroids such as androgens testosterone and dihydrotestosterone thereby affecting their bioavailability (Finnstrom et al., 2001; Lawson and Kolar, 2002; Sundin et al., 1987; Waxman et al., 1991). Decreased expression of PXR, and consequently CYP3A4, has been associated with increased Gleason score and poor prognosis of PCa (Fujimura et al., 2009; Fujimura et al., 2012). It has been suggested that increased androgen clearance by PXR-activated pathway may be a therapeutic target for PCa (Zhang et al., 2010).

VCZ is a known inducer of various CYP enzymes; in Swiss Albino CD1 mice, it appeared to preferentially induce CYP3A (in liver and kidney) and CYP2E1 (in liver) although a complex pattern of induction and suppression was seen among other phase I metabolizing enzymes including CYP2B1 and CYP1A1 in males and females (Hrelia et al., 1996). Similar patterns of CYP450 isozymes induction by VCZ were found in hepatic tissues of male Sprague-Dawley rats and bobwhite quail with 8.2 and 3.7 fold increase of CYP3A, respectively (Ronis et al., 1994). Northern blot analysis revealed that

VCZ regulates CYP3A and CYP2E1 expression at the mRNA level in hepatic tissues of CD1 mice (Hrelia et al., 1996).

Exposure to EDCs and activation of PXR, and subsequently metabolizing enzymes such as CYP3A4, may interfere with steroid hormone metabolism in the liver and prostate and therefore, increase prostate cancer risk (Prins, 2008). It has previously been shown that *in vivo* inhibition of estrogen sulfotransferase by environmentally relevant chemicals such as polychlorinated biphenols and polyhalogenated aromatic hydrocarbons including bisphenol A are capable of elevating bioavailable estrogen and potentially inducing estrogenic activity in various target organs (Kester et al., 2000; Kester et al., 2002). Although VCZ by itself only weakly antagonizes the AR, induction of aromatase and other CYP450s by VCZ may further influence its androgenicity *in vivo* (Sanderson and Van Den Berg, 2003).

1.5 Aims and Objectives

VCZ is a known EDC that is widely used as a pesticide on food crops. Previous experiments have shown that VCZ has a strong affinity towards the AR and may play a role in altered development of the prostate and induce prostate disease. Till date, no studies have looked at the effects of VCZ on the human prostate and majority of research has been focused on *in vivo* studies in rats and mice. The aim of this research is to investigate how exposure to VCZ and its metabolites affect the viability of human prostate cell line, LNCaP, in a time- and dose-dependent manner and how VCZ and its

metabolites alter the expression of the androgen receptor, androgen regulated and xenobiotic metabolizing enzyme genes that may be involved in initiation or progression of human prostate abnormalities or disease such as PCa. LNCaP is an androgen-sensitive cell line established from a metastatic lesion of human prostatic adenocarcinoma. It is one of the most widely used cell line in PCa research and one of the only established PCa cell lines that is androgen responsive and expresses the androgen receptor. Due to the mutation in the AR and the tumorigenic properties of the LNCaP cell line, it is important to note that the potential of VCZ to induce PCa development cannot be discussed with confidence – instead, the results will mainly demonstrate if VCZ has the capacity to further influence the progression of PCa.

Objectives

1. To examine time-dose dependant effects of Vinclozolin on viability of LNCaP by using MTT assay.
2. To study the effects of Vinclozolin on expression of androgen receptor, androgen regulated gene, NKX3.1, and xenobiotic metabolizing enzyme gene, CYP3A4, involved in prostate cancer by determining gene expression using qRT-PCR technique.
3. To study the effects of Vinclozolin metabolites by treatment with S9 microsomes on cell viability and gene expression as mentioned in objectives 1 and 2 using similar approaches mentioned above.

Significance

There is considerable interest in endocrine disrupting chemicals and their potential role in hormonal diseases. As certain environmental agents may mimic natural hormones, it is important to assess this biological-environmental relationship. Pesticides are known to interact with hormonally active glands such as the prostate although there is limited information on the incurred molecular and genetic changes. This study will attempt to expand the current understanding of how Vinclozolin, a commonly used dicarboximide fungicide, and its metabolites can alter the expression of critical genes commonly dysregulated in prostate cancer. We hope to establish if exposure to pesticides such as Vinclozolin can further promote the progression of prostate cancer.

2. Materials and Methods

2.1 Chemicals and Reagents

The complete cell culture medium was RPMI-1640 (Wisent, St-Bruno, QC) supplemented with 10% fetal bovine serum (FBS; Sigma, Oakville, ON), 1.0 mM sodium pyruvate (Wisent) and 1% antibiotic-antimycotic solution (Sigma). The exposure medium contained phenol red free RPMI-1640 (Wisent) supplemented with 5% charcoal-stripped FBS (Wisent), 1.0 mM sodium pyruvate (Wisent), 1% L-Glutamine (Wisent) and 1% antibiotic-antimycotic solution (Sigma). 40mM stock Vinclozolin (analytical grade; Sigma) was prepared in DMSO. 1.2 mM of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Eugene, OR) was prepared in phosphate buffered saline solution (PBS; Sigma). Aurum Total RNA Extraction Mini Kit, iScript cDNA synthesis kit and SsoFast EvaGreen Supermix were obtained from Bio-Rad (Mississauga, ON).

2.2 Cell Culture

Human epithelial prostate carcinoma cell line LNCaP isolated from lymph node metastasis (CRL-1740, ATCC) were grown in RPMI-1640 complete cell culture medium at 37°C in a humidified 5% CO₂ atmosphere. Medium was changed every 2-3 days and cells were passaged at 80-90% confluency. Cell sub-culturing was done in 100 mm tissue culture dishes with initial seeding of 750,000 cells per dish in 10mL media. 80-90% confluency would be reached in approximately 4 days.

2.3 Objective 1: Cell Viability Assay

LNCaP cells were seeded in the exposure medium at 3×10^5 cells/well in 24-well clear tissue culture assay plates. Cells were allowed to grow for 48 hours before treatment with VCZ test compound. After 48 hours, the exposure medium was replaced with a range of VCZ or S9 treated VCZ concentrations (200 μ M, 100 μ M, 10 μ M, 1 μ M, 0.1 μ M, 0.01 μ M) in 0.5% DMSO. The cells were incubated for 6, 12, 24, 48, 72 and 96 hours and media was replaced with the test compound at 48h; for exposures with S9, only up to 48 hours was tested due to increased toxicity of S9. At the end of each time point, media from each well was aspirated and 500 μ L of 1.2 mM MTT-PBS solution in RPMI-1640 phenol red free media was added to each well. The plate was incubated for 4 hours at 37°C and 500 μ L of 10% SDS-0.01M HCl was added at the end of the incubation time. The plate was placed on a shaker at 37°C overnight in order to dissolve the formazan crystals. Absorbance was recorded at 570 nm wavelength.

2.4 Objective 2: Gene Expression

Approximately 1×10^6 LNCaP cells were seeded in the exposure medium into 100 mm cell culture dishes and grown for approximately 48 hours until 70-80% confluency was reached. After 48 hours the medium in each plate was replaced with the exposure medium containing VCZ or VCZ treated with S9 at concentrations of 100 μ M, 1 μ M and 0.01 μ M dissolved in 0.5% DMSO. Exposures were conducted for 6, 12, 24 and 48 hours. Co-exposure with 10 nM DHT (Sigma) or 1 μ M flutamide (Sigma) and VCZ was

also conducted for a 24 hour time point to determine if expression of NKX3.1 is potentially being mediated through the androgen receptor. After the end of each exposure time points the cells were trypsinized with 0.05% trypsin/0.53 mM EDTA (Wisent), washed with PBS and stored as cell pellets in -80 °C prior to RNA extraction.

RNA isolation was carried out using the Aurum Total RNA Mini Kit following manufacturer's instructions. Quantity and quality of RNA was measured using optical density ratio 260/280 and 260/230 on a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA). It was ascertained that ratios 260/280 and 260/230 were greater than 1.8 for all samples. RNA integrity was checked by running a 1% denaturing agarose gel electrophoresis in 1X MOPS buffer. 1 µg RNA mixed with 1X formaldehyde loading dye and 10 µg/mL ethidium bromide was heated at 65-70°C for 15 minutes prior to loading the gel. The gels were visualized for identification of the 28S and 18S bands in order to verify the integrity of the RNA.

Reverse transcription of RNA was carried out using the iScript cDNA Synthesis Kit following manufacturer's instructions. Semi-quantative real-time PCR was performed on a iCycler iQ real-time PCR detection system (Bio-Rad). The qRT-PCR mixture contained 7.5 µL SsoFast EvaGreen Supermix, 1.5 µL forward primer (5 µM), 1.5 µL reverse primer (5 µM) and 4.5 µL cDNA diluted in nuclease free water. The PCR conditions were: 95.0°C for 3:00 and 40 cycles of 95.0°C for 0:30, 61.0°C for 0:30, 72.0°C for 0:30. After amplification, melt-curve analysis was performed to ensure a single product was

amplified. Selected products were run on a 1% TBE agarose gel to validate the amplification of only a single product.

Primers for the qRT-PCR reactions were designed using Primer-BLAST (NCBI) and QuantPrime. The primer sequences to amplify the mRNA of interest were as follows:

NKX3.1, F: ACCCAGACAGCCTGTTTCTATCC R: AGATTGGAGCAGGGTTTGTATGC; AR, F: AGGTCCTAGCCCCTGGCTGC R: TCAGGGGAGGCCAGGAAGGC; CYP3A4, F: GTTGGCGTGGGGCCTTTGTCA R: AACAAATGGGCAAAGTCACAGTGGAT; Actin, F: GGGCATGGGTCAGAAGGAT R: GTGGCCATCTCTTGCTCGA. BLAST (NCBI) search was performed for each primer to confirm specificity and limit cross-reactivity with other potential target sequences. Primer and amplicon quality were checked with online software NetPrimer (Premier Biosoft International) and mfold in order to screen for hairpins, primer-dimers and secondary structures in the amplicon that may reduce efficiency. Average expression stability value for housekeeping gene was calculated using GeNorm in order to ensure that the stability value is below the default cut-off limit.

2.5 Objective 3: *In vitro* Treatments with Metabolic Activation System

Isolated S9 microsomes from animal livers are often used to metabolize compounds during screening of drugs and chemicals. S9 microsomes are derived from homogenized livers of animals such as rat and contain many of the common phase I and phase II metabolizing enzymes produced by the liver. Since exogenous compounds ingested

orally are initially metabolized by passing through the liver before reaching systemic circulation, S9 microsomes provide an *in vitro* method of screening the effects of liver produced metabolites.

For treatment with metabolic activation system S9, the chemical with S9 mixture was first incubated overnight before exposure to cells in order to minimize toxicity. The S9 mixture contained 10 μ L Aroclor 1254 induced rat S9 (1 % v/v), 870 μ L of cell culture media and 105 μ L of S9 cofactor which contained 50 μ L of 20 mM HEPES pH 7.2, 2.5 μ L of 500 mM $MgCl_2$, 2.5 μ L of 3300 mM KCl, 25 μ L of 50 mM Glucose-6-Phosphate and 25 μ L of 40 mM NADP. 10 μ L of the chemical in DMSO was added to the S9 mixture with the final concentration of the chemical being 10x the required final concentration for exposure to cells. The final 1 mL mixture was incubated and shaken in a 37 °C waterbath overnight for approximately 12 hours and 9 mL of cell culture media was added to this mixture the following morning in order to create the final treatment media. The cells were incubated in the treatment media for 6, 12, 24 and 48 hours (longer timepoints were not performed due to increase in cytotoxicity with S9). After end of each exposure time period, cell viability or gene expression analyses were performed as described earlier.

2.6 Calculations and Statistical Analysis

The relative cell viability was calculated as a percentage of absorbance from treated cells to control cells. qRT-PCR results were calculated using the $2^{-\Delta\Delta C_t}$ method wherein the amount of target is presented as a fold change normalized to the endogenous reference

and relative to the control. The ΔC_t value was determined by subtracting the average reference C_t value from the average target C_t value, and the $\Delta\Delta C_t$ was calculated by taking the difference of the experimental control ΔC_t from the ΔC_t of treated samples.

Statistical significance was tested with an independent two-tailed Student's *t*-test and analysis of variance (ANOVA). The differences were considered to be significant if $P < 0.05$.

3. Results

3.1 Cell Viability Assays

LNCaP cells were exposed to various concentrations (0.01 μ M – 200 μ M) of VCZ and VCZ treated with S9 microsomes over a 96 and 48 hour time period, respectively. The cell viability of LNCaP cells in response to treatment with VCZ and its metabolites was assessed using MTT assay. It is important to note that the MTT assay measures the capacity of the cell to reduce the water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase enzymes in healthy, living cells, and is therefore an indirect measurement of viability. A decrease in the colorimetric absorbance cannot be used to distinguish between cytostatic and cytotoxic effects (Carmichael et al., 1987).

Little change in viability was noticed at concentrations of 10 μ M and below. By 48 hours the viability had decreased to 71% and 64% of control, and 51% and 31% of control by 72 hours at 100 μ M and 200 μ M, respectively (Figure 3A&C). The reduced viability coincided with elongated cell morphology and the sparse distribution of the cells at 100 μ M and 200 μ M (Figure 4); this may be attributed to the cytotoxic effects at these concentrations.

After incubating VCZ with S9 microsomal fraction overnight prior to exposure, a slight increase in viability was seen relative to exposure without S9 microsome treatment (Figure 3B&D). By 48 hours the difference in viability was significant at the higher

concentrations. At this timepoint, 200 μ M and 100 μ M had a viability of approximately 78% and 88% after treatment with S9 while treatment without S9 showed decreased viability of 64% and 71% at the same respective concentrations. Due to the toxicity of the S9 mix, longer exposures with S9 treated VCZ were not conducted.

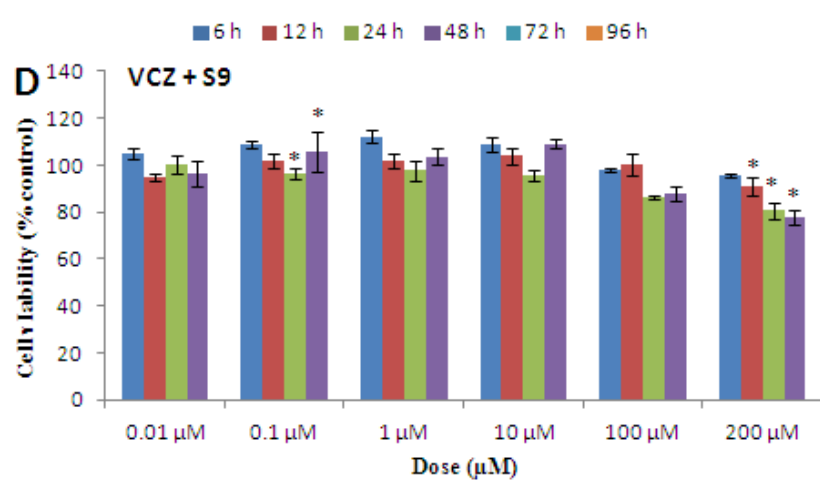
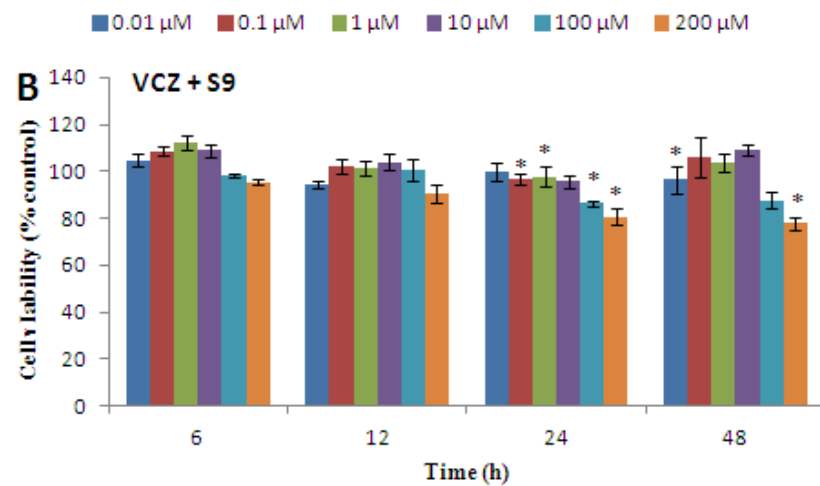
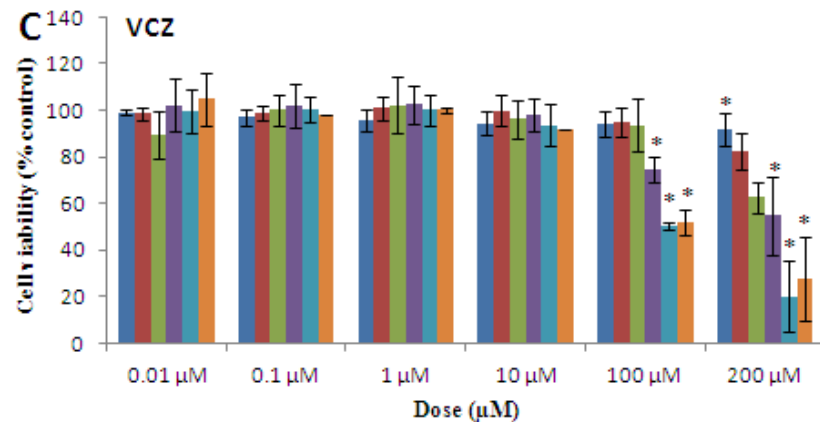
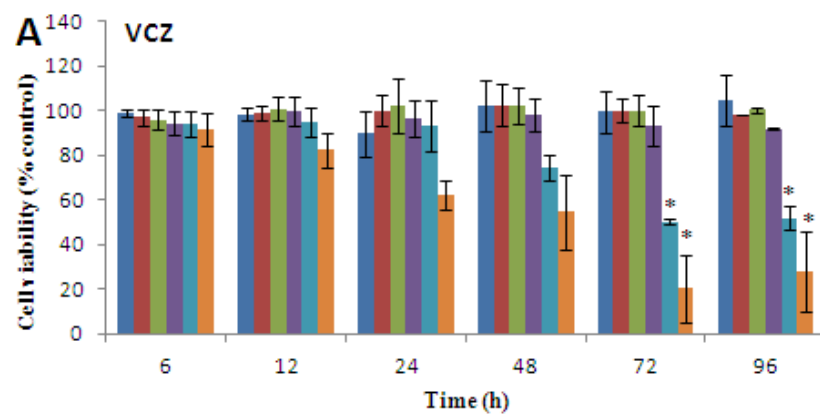


Figure 3. Time-dependent (A, B) and dose-dependent (C, D) MTT assay for determination of cell viability on LNCaP cells using VCZ and VCZ treated with S9 microsomes. Results are shown as the mean of three independent measurements containing three technical replicates each, plus or minus the standard error of the mean (S.E). Statistical significance was determined by performing a Student t-test between the 6 h time point and the remaining time points at each dose for time-dependent analysis and between the control and doses at each time point for dose-dependent analysis. Note: * in figure corresponds to $p < 0.05$. See Appendix I for p-values of significant points.

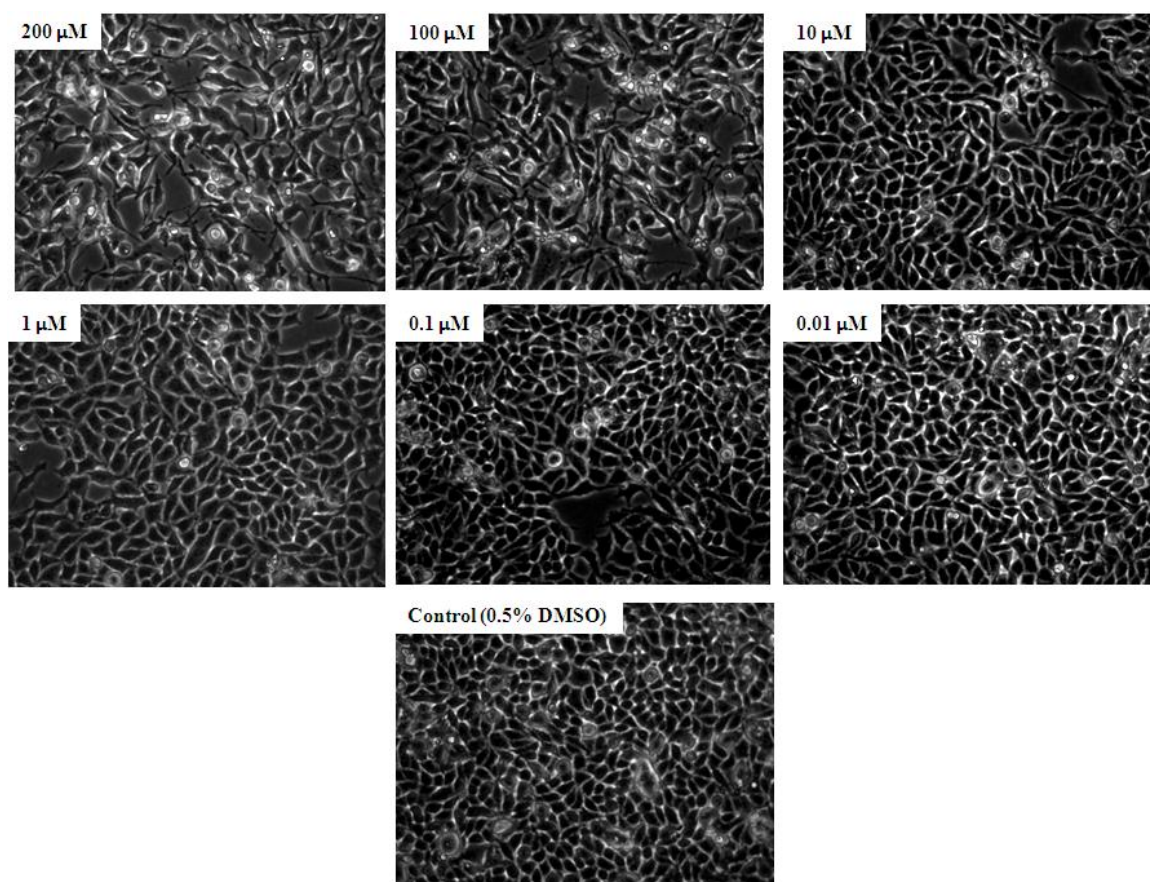


Figure 4. LNCaP cells treated with 200, 100, 10, 1, 0.1 and 0.01 μM VCZ after a 48 hour exposure and viewed under a inverted light microscope at 100X magnification. Higher concentrations of the compound show indications of changes in cell morphology.

3.2 Gene Expression Changes After Exposure to VCZ

VCZ, through its main two metabolites M1 and M2, is known to interact with the AR and regulate the expression of downstream genes. NKX3.1, a gene known to be androgen regulated, showed no significant changes in expression until 24 hours where dose-dependent up-regulation was observed with fold changes of 1.87 and 1.59 at 100 μ M and 1 μ M, respectively (Figure 5A&D). No considerable changes in expression were seen at 6 and 12 hours for NKX3.1. At 48 hours, expression of NKX3.1 had started to gradually decrease at a concentration of 100 μ M and a slight, insignificant, decrease was also noticed at 1 μ M. At 10 nM, no change in expression was seen except a slight up-regulation at 48 hours.

The AR gene showed an initial, non-significant, down-regulation at 6 and 12 hours at all concentrations (Figure 5B&E). This effect was mitigated by 24 hours and 48 hours at concentrations of 1 μ M and 10 nM. 100 μ M showed an increase in expression relative to control at 24 hours that appeared to diminish by 48 hours.

Gene, CYP3A4, which is heavily involved in xenobiotic metabolism and activated by Vinclozolin, showed the greatest increase in expression at 24 hours similar to NKX3.1; fold changes of 2.61, 4.74 and 2.84 were seen at 100 μ M, 1 μ M and 10 nM concentrations, respectively (Figure 5C&F). Surprisingly, at 100 μ M, the highest concentration of exposure, the expression of CYP3A4 remained the lowest at all time

points. By 48 hours, expression had decreased below a 2-fold increase at all concentrations.

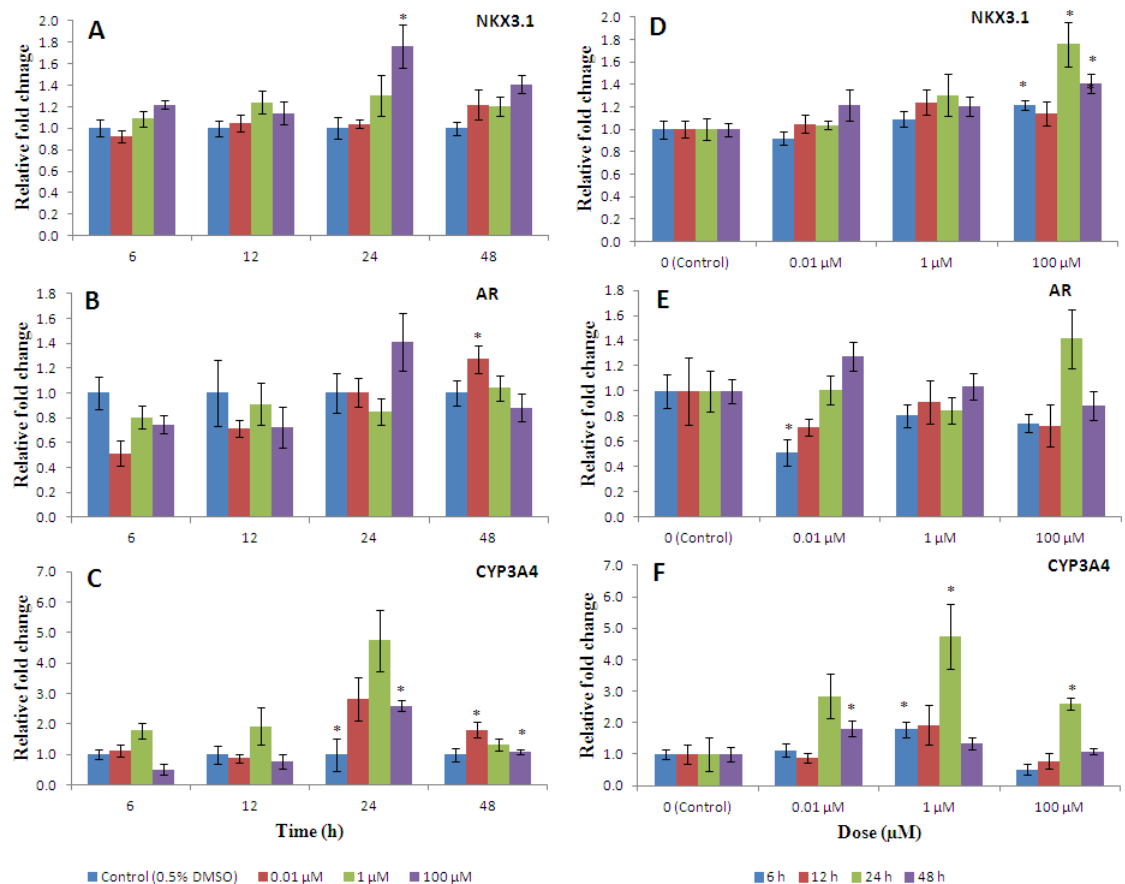


Figure 5. Time-dependent (A, B, C) and dose-dependent (D, E, F) gene expression changes of NKX3.1, AR, and CYP3A4 as measured by RT-PCR after VCZ treatment on LNCaP cells. Results are shown as the mean of three independent measurements plus or minus the S.E. Statistical significance was determined by performing a Student t-test between the 6 h time point and the remaining time points at each dose for time-dependent analysis and between the control and doses at each time point for dose-dependent analysis. Note: * in figure corresponds to $p < 0.05$. See Appendix II for p-values of significant points.

3.3 Treatments With Dihydrotestosterone and Flutamide

NKX3.1 expression after a 24 hour treatment and co-treatment with a known AR agonist, DHT, and known antagonist, flutamide, along with VCZ was tested to determine if expression is potentially being mediated through the AR (Figure 6).

DHT at 10 nM caused a slight upregulation of approximately 1.30 fold which is similar to the fold change seen by VCZ at 1 μ M. 10 μ M of flutamide, which has previously been used to downregulate NKX3.1 in LNCaP cells, decreased the expression to around 0.67 fold. Co-treatment with the two controls, DHT and flutamide, gave an fold change of approximately 1.07 which lies in between that of the single treatments; this value is slightly below the expression seen by DHT but above that of flutamide.

Co-treatment with the AR agonist, DHT, and the AR antagonist test compound, VCZ, showed a downregulation with 0.82 fold change which was in contrast to the upregulation seen by DHT and VCZ treatments alone. This potentially underlies the ability of compounds such as VCZ to act as agonists or antagonists depending on treatment conditions in LNCaP cells (Wong et al., 1995).

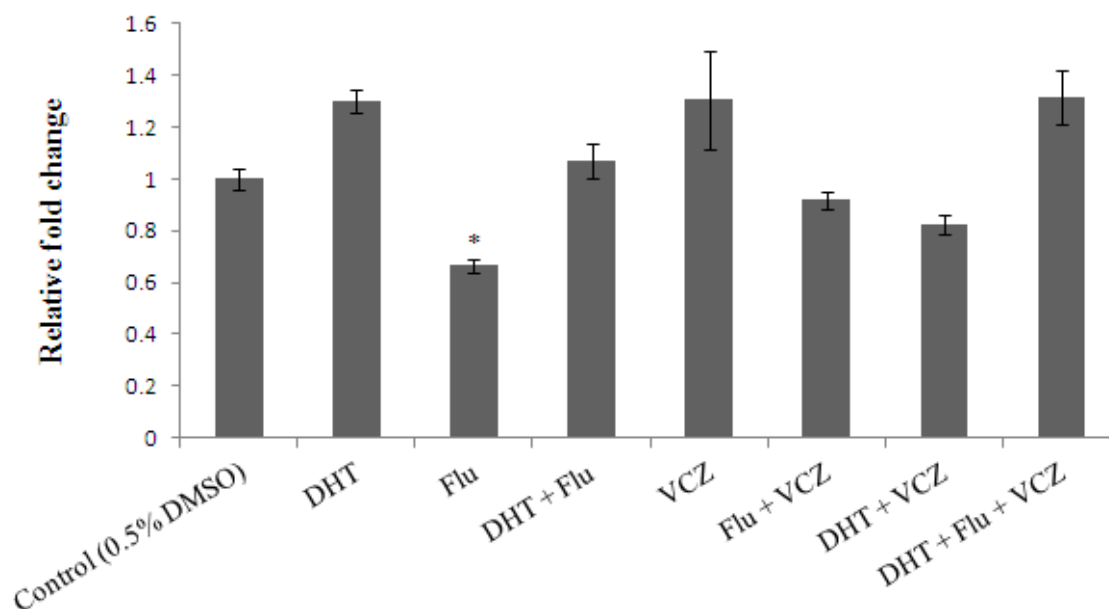


Figure 6. Expression of NKX3.1 after single and co-treatments with VCZ, flutamide (Flu) or dihydrotestosterone (DHT) on LNCaP cells for 24 hours. Concentrations of VCZ, Flu and DHT are 1 μ M, 10 μ M and 10 nM, respectively. Results are shown as the mean of three independent measurements plus or minus the S.E. Statistical significance was determined by performing a Student t-test between the control and treatments groups.

Note: * in figure corresponds to $p < 0.05$.

3.4 Gene Expression Changes After Exposure to VCZ Treated With S9 Microsomes

VCZ treatment without S9, showed very little difference in profile of NKX3.1 expression was seen at 1 μ M and 10 nM, although S9 treatment produced less significant results (Figure 7A&D). 1 μ M of S9 treated VCZ produced a stable increase in expression of 1.29 fold from 24 to 48 hours while an increase to 1.25 fold was seen at 48 hours with 10nM treatment. 100 μ M of S9 treated VCZ showed an inconsistent trend of an increase in expression at 12 and 48 hours but not at 24 hours.

The AR showed an overall decrease in expression that reached a minimum at 24 hours (Figure 7B&E). S9 treated VCZ at 10nM showed the most reduction in expression reaching 0.54 fold change compared to 0.71 and 0.82 fold change at 1 μ M and 100 μ M, respectively, at the 24 hour time point. Compared to non-S9 microsome treated samples, a similar down-regulation was seen but, in the presence of S9, the expression never increased above the control within the 48 hour period.

In contrast to NKX3.1 and AR, CYP3A4 showed slightly more varied expression between S9 microsome treated and non-treated VCZ (Figure 7C&F). Although expression was still upregulated, S9 treatment showed an overall reduced expression upto 24 hour time period. The order between the three different exposure concentrations was comparable to the non-S9 treated samples with the 100 μ M concentration showing the least expression. 1 μ M and 10 nM showed similar increase in expression reaching a maximum of 2.4 fold change at 48 hours.

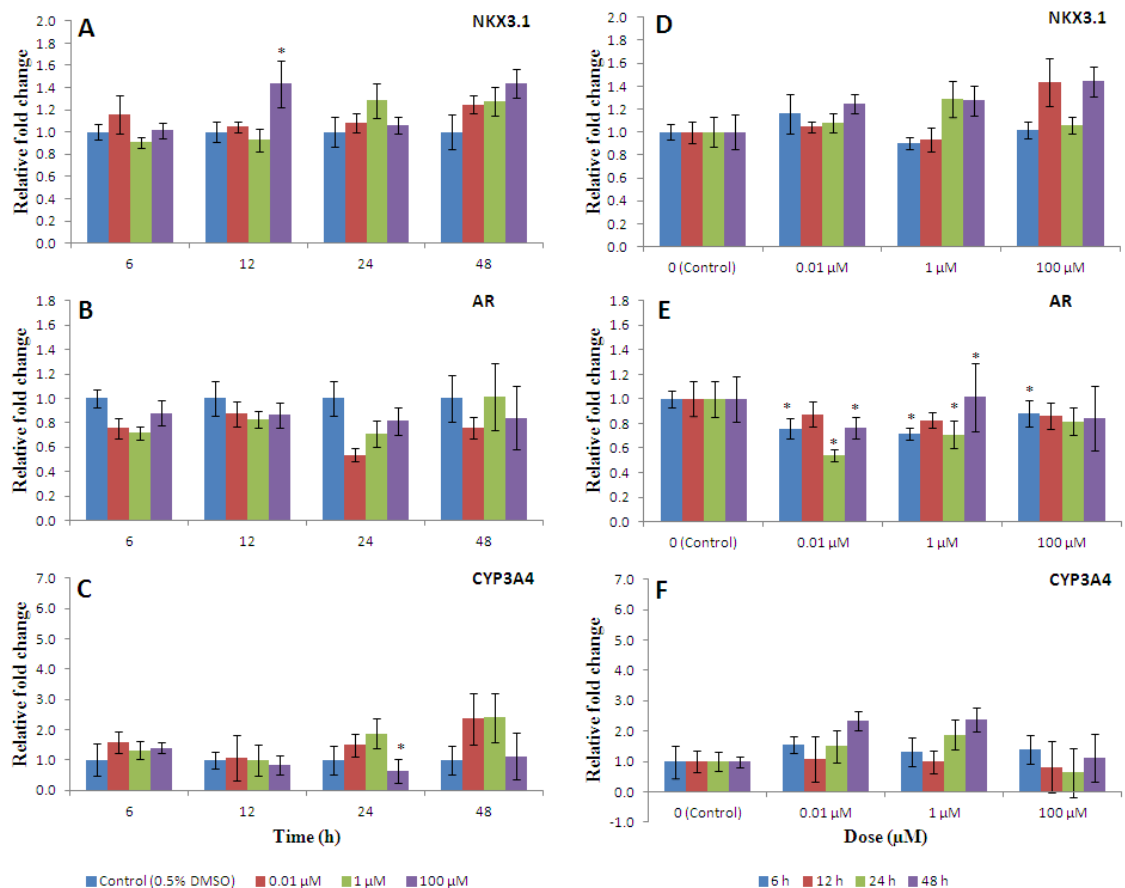


Figure 7. Time-dependent (A, B, C) and dose-dependent (D, E, F) gene expression changes of NKX3.1, AR, and CYP3A4 as measured by RT-PCR after treatment on LNCaP cells with S9-treated VCZ. Results are shown as the mean of three independent measurements plus or minus the S.E. Statistical significance was determined by performing a Student t-test ($p < 0.05$) between the 6 h time point and the remaining time points at each dose for time-dependent analysis and between the control and doses at each time point for dose-dependent analysis. Note: * in figure corresponds to $p < 0.05$. See Appendix II for p-values of significant points.

4. Discussion

4.1 Effects of exposure on Cell Viability

Vinclozolin, a non-steroidal anti-androgen, has known affinity for the human AR, and like other anti-androgens (Bologna et al., 1995; Lee et al., 2003; Olea et al., 1990), has the potential to elicit viability changes in the androgen sensitive cell-line, LNCaP.

In this study, decrease in viability was seen in exposures at concentrations over 10 μ M at time points of 48 hours and greater. A previous study showed a similar decrease in viability to about 25 % of control at concentrations of 35 μ M and above in a 72 hour time period by MTS assay within the same cell line (Lorenzetti et al., 2010). No cytotoxic effects of VCZ and metabolites M1 and M2 were observed by MTT assay at concentrations between 0.01 and 10 μ M after a 24 hour exposure in prostate PC-3 derived PALM and liver HeLa derived HG5LN cell lines (Molina-Molina et al., 2006). These results suggest that the observed cell viability post treatments with VCZ are in concordance with previous studies. Although the proliferation of LNCaP is stimulated by androgens (Lee et al., 1995), the disruption in cell morphology at higher concentrations may suggest that the reduction in cell viability could be due to events other than cell growth inhibition by the antiandrogenic properties of VCZ. The effects of cytotoxicity may also be caused by induction of oxidative stress as previously indicated by an increase of glucose 6-phosphate in bovine lymphocyte cultures (Lioi et al., 1998) and an increase of malonaldehyde and free radical content, and decrease of glutathione levels in HepG2 cells (Radice et al., 1998) following treatment with VCZ.

Treatment with S9 liver microsomes showed a slight but observable increase in cell viability at the high doses. Similar inactivation of VCZ and reduction in cytotoxicity at high dose was observed after treatment with S9-mix in BALB/C-3T3 cells although inconsistent results were obtained at low doses (Perocco et al., 1993). Recently, it has been shown that VCZ is susceptible to oxidation by hepatic microsomal CYP-dependent monooxygenases and, except for the non-enzymatic hydrolysis product M1, is efficiently metabolized into the M5 metabolite by S9 liver microsomes (Sierra-Santoyo et al., 2011). It is possible that the metabolites, particularly M5, that are produced by S9 are less potent than the primary compound resulting in reduced cytotoxicity.

4.2 Effects of Exposure on Gene Expression

4.2.1 Expression of NKX3.1: NKX3.1 is an androgen-regulated prostate-specific homeobox gene that is positively modulated by androgens (Magee et al., 2003; Prescott et al., 1998). In this study, a small dose-dependent increase in expression that peaked at 24 hours was observed after treatment with the parent compound VCZ. As previously hypothesized, a stabilization or decrease in expression of NKX3.1 after reaching the peak could be due to feedback inhibition of transcription where newly synthesized proteins downregulate the NKX3.1 promoter (Prescott et al., 1998).

It is interesting to note that VCZ, an antiandrogen, caused an upregulation of the NKX3.1 gene. Although antiandrogens block AR transcription activation by destabilization of the AR and potentially disrupting promoter/enhancer binding or recruiting corepressors of androgen-regulated genes (Furutani et al., 2002; Kang et al., 2004; Shang et al., 2002), considerable evidence suggests that antiandrogens may act agonistically in a similar fashion to androgens in LNCaP cells. A widely reported T877A mutation in the AR of LNCaP and patients with metastatic prostate cancer is known to alter the ligand binding specificity of the mutated AR and allow for a wider range of substrates, including antiandrogens, to stimulate the transcription factor activity of the AR (Culig et al., 1999; Gaddipati et al., 1994; Gelmann, 2002; Grossmann et al., 2001; Taplin et al., 2003; Taplin and Balk, 2004; Veldscholte et al., 1992). Mutated AR exposed to antiandrogens have also shown to interfere with events early in the transactivation function that are similar to agonistic activity of androgens with wild-type AR; both antiandrogens and androgens acting on mutated and wild-type AR, respectively, induced detectable DNA-binding related immobilization, slowed-down the effective diffusion of the mobile AR fraction, and induced focal nuclear distribution of the ligand activated AR complex – all features of transactivation function that were not present with wild type AR treated with antiandrogens (Farla et al., 2005).

The aberrant signalling of the AR becomes more confounding in LNCaP cells when considering ligand concentrations and presence of multiple ligands. A previous study to measure these effects showed that in the absence of androgens, 0.2-1 μ M of VCZ M2 metabolite can strongly bind to the LNCaP AR and produce transcriptional response of

the expression vector while treatment with presence of androgens such as DHT inhibits this response (Wong et al., 1995). Similarly, within our study, treatment with VCZ caused a slight up-regulation of NKX3.1 that was down-regulated when co-treated with DHT. This may be due to mixed ligand dimers (ie. an agonist and antagonist bound to the AR dimer) producing antagonism through formation of incompatible conformation states for transcriptional activation (Wong et al., 1995). Similar results have been observed with other steroid hormone receptors such as the progesterone receptor where mixed ligand dimer alters the progesterone receptor affinity for the DNA (Allan et al., 1992; Garcia et al., 1992; Guiochon-Mantel et al., 1988; Meyer et al., 1990). Multiple mechanisms have been suggested to explain AR DNA binding inhibition by mixed ligand dimers including increased AR degradation by inappropriate receptor conformation or reduced AR stability, incompatibility of mixed ligand dimers to associate AR dimerization, and inability to release receptor associated proteins required for successive DNA binding (Kelce and Wilson, 1997). This suggests that in this study, VCZ, in the absence of androgens, may be producing agonistic gene activation of NKX3.1 through formation of same ligand AR dimers.

Treatment of LNCaP cells with VCZ at 1 μ M and 10 nM concentrations in the presence of S9 microsomes showed very little variation in expression of NKX3.1 compared to those cells treated with VCZ without the presence of S9. Possibilities for this outcome may include either incomplete metabolism of VCZ by the S9 microsomes or the metabolites formed, such as M5, may produce a similar response as the parent compound. Apart from the metabolites M1 and M2, which are produced without enzymatic

activation in solution such as tissue culture media (Bursztyka et al., 2008), no studies have been conducted to assess if the major metabolite M5 has the capacity to interact with AR. Furthermore studies need to address how metabolites of VCZ, other than M1 and M2, interact with steroid receptors and downstream gene activation or inhibition.

Although a thorough understanding of the role of NKX3.1 in prostate tumour development is still to be characterized several studies have indicated changes in expression of NKX3.1 in human prostate cancers and potential association with tumour progression (Aslan et al., 2006; Bowen et al., 2000; Xu et al., 2000). NKX3.1 has been reported to play a protective role from agents that cause DNA damage, to associate with HDAC1 and to stabilize the p53 tumour suppressor protein, and to activate the expression of insulin-like growth factor binding protein-3 to regulate cell growth (Bowen and Gelmann, 2010; Lei et al., 2006; Muhlbradt et al., 2009). Due to androgenic regulation of the NKX3.1 gene, VCZ is capable of modulating its expression and a reduction in the gene's expression may dispose the cells towards aberrant growth.

4.2.2 Expression of AR: Expression of the AR is important for normal prostate functioning and critical for prostate development and carcinogenesis. In this study, a transient, initial decrease in AR expression was observed. This response may be a result of autoregulation of the AR by the androgenic activity of VCZ. It has previously been reported that treatment with androgens is capable of reducing AR mRNA in rat tissues and LNCaP cells (Krongrad et al., 1991; Quarmby et al., 1990; Tan et al., 1988). It is

suggested that the negative autoregulation is primarily a result of reduced transcriptional initiation at the mRNA start site and post-transcription stabilization of the mRNA (Wolf et al., 1993). AR mRNA stability is hypothesized to be due to *trans*-acting androgen-regulated RNA binding proteins but the identity of these *trans*-acting factors on the AR mRNA are not well known (Yeap et al., 1999). More recently, it has been shown that NKX3.1 and AR form a signalling feedback loop within the prostate epithelium where NKX3.1 negatively regulates AR gene expression through a consensus binding site in the AR promoter (Lei et al., 2006). Lei *et al.*, 2006 speculate that this feedback loop is important for NKX3.1 to negatively regulate AR expression as well as AR-controlled signaling pathway, and may be necessary for both prostate development and cancer initiation.

Previous studies have shown no significant changes in AR expression in genital tubercles of male mice (Buckley et al., 2006) and a slight non-significant increase that is diminished by 24 hours in the testes of adult male rats (Kubota et al., 2003) after treatment with VCZ. Contrasts in AR expression between tissues may be due to the cell-specific differential regulation of AR gene expression (Yeap et al., 1999); prostate tissue and LNCaP cells have shown to consistently downregulate AR mRNA while Sertoli cells and peritubular myoid cells from rat testis showed no changes in AR gene expression after androgen treatment (Blok et al., 1992).

Treatment with S9 microsomes also showed a decrease in AR expression although this reduced expression appeared to persist for a longer time period than treatment without S9. The differences observed may be due to faster metabolism and excretion of the non-S9 treated compound. The increase in expression with time (more clearly visible in treatment without S9) may be a response to elevate AR expression back to basal levels. Further studies are needed to compare the rate of biotransformation and biological activity of VCZ and its metabolites in prostate cells.

4.2.3 Expression of CYP3A4: CYP3A4 is one of the most important xenobiotic metabolizing enzymes and has been shown to be active within the prostate. Treatment with VCZ showed a significant increase in expression peaking at 24 hours. VCZ is known to go through phase I metabolism which includes an oxidation reaction of the ethylene group catalyzed by CYP3A isoforms (Sierra-Santoyo et al., 2011). VCZ is capable of interacting with the CYP3A4 activating receptor, PXR, (Mikamo et al., 2003) and induce changes in expression of CYP3A at the mRNA level (Hrelia et al., 1996). The oxidation of VCZ by CYP3A4 most likely follows a common mechanism where binding of VCZ to the active site prompts the reduction of the ferric heme iron in the CYP protein by NADPH and this activated CYP intermediate along with the VCZ substrate and dioxygen favours the formation of the hydroxylated VCZ metabolite (Anzenbacher and Anzenbacherova, 2001; Guengerich, 1999). It should also be considered that induction of the catalytic cycle of cytochrome P450 enzymes can generate reactive oxygen species and promote oxidative stress (Parke, 1994; Perret and Pompon, 1998), and this phenomenon could potentially be playing a role in the increased oxidative stress markers

by VCZ such as lipid peroxidation, increased malonaldehyde and free radical content, and decreased glutathione levels observed within previous studies (Cabral and Cabral, 2000; Lioi et al., 1998; Radice et al., 1998). The decline in expression with time of CYP3A4 within this study could be due to complete metabolism of the VCZ compound and adaptive mechanisms by the cells.

S9 microsomal treatment showed slightly reduced expression of CYP3A4 when compared to treatment without metabolic activation. This effect may be due to prior metabolism of compound by S9; microsomal S9 mix activated by NADPH cofactor contains functioning phase I metabolizing enzymes including CYP3A4 and other cytochrome P450 isoforms (Clarke, 1998). Since VCZ metabolism is CYP-dependant and is efficiently transformed into its metabolites by liver microsomes (Sierra-Santoyo et al., 2011), it is possible that the observed reduction in expression of CYP3A4 could be due to the compound being pre-metabolized by the CYP-microsomal enzymes. It is unknown to what degree the metabolites of VCZ activate the PXR in order to induce expression of CYP3A4.

It is interesting to note that treatment with VCZ at the higher concentration (100 μ M) reduced expression of CYP3A4 relative to treatment at lower concentrations. This effect may be a result of reaching a cytotoxic threshold similar to the reduction in viability observed by MTT assay at higher concentrations. CYP3A4 is involved in defense against chemical stress and its reduction at high doses may be an indication of irreversible

cellular changes due to toxicity (Rosic et al., 2010). Likewise, silencing of CYP3A4 has shown to increase apoptosis and inhibit growth of MCF7, T47D, and MDA-MB-231 human breast cancer cell lines (Mitra et al., 2011). Within HEK293 human embryonic kidney cell line an inverse linear relationship was seen between CYP3A4 expression and nuclear DAPI condensation - a measure of cell apoptosis (Ghosh et al., 2011). Taken together, this suggests that high VCZ dosage can cause a reduction in CYP3A4 activity and potentially lead to cell death pathways or growth inhibition as witnessed by reduced viability in MTT assay.

An overall increase in CYP3A4 as observed within this study may have significant impact on testosterone metabolism within the prostate. CYP3A4 is involved in the oxidation of testosterone to 2 β -, 6 β -, or 15 β -hydroxytestosterone (Waxman et al., 1988; Waxman et al., 1991) which are biologically less active than testosterone or DHT. VCZ treatment has shown to facilitate testosterone metabolism and result in increased formation of CYP3A-dependent 6 β -hydroxytestosterone in male rat (Dai et al., 2001; Ronis et al., 1994). Therefore, induction of CYP3A isoforms by VCZ may reduce the concentration of bioavailable testosterone resulting in alterations of normal androgenic activity (Dai et al., 2001).

5. Conclusion and Future Considerations

The antiandrogenic fungicide, Vinclozolin, treated with or without S9 metabolites showed alterations in the expression of the androgen receptor gene, the androgen regulated gene, NKX3.1, and the xenobiotic metabolizing gene, CYP3A4, in the prostate cell line, LNCaP. An overall increase and a transitory decrease in expression of NKX3.1 and AR were observed, respectively. Co-exposure with DHT produced a decrease in expression of NKX3.1 supporting the potential for antagonists to act as agonists in LNCaP cells. AR and NKX3.1 are involved in multitude of roles within prostate cancer and modulation in their expression may further influence PCa progression. An increase in CYP3A4 expression induced by VCZ was also observed and this may play an important role in metabolism and availability of natural androgens within the prostate.

Further work needs to be done in order to understand the biological activity of Vinclozolin metabolites on the androgen receptor within the prostate. As of now, there are no studies describing the effects of metabolites other than M1 and M2. Future experiments also need to investigate the role of Vinclozolin and its many metabolites on wild-type AR and the interactions between the AR and response elements. This will help establish the mechanistic differences between Vinclozolin and other androgens. It may also be beneficial to explore the changes in protein levels and relate these changes to alterations in mRNA expression levels of the target genes. The effect of Vinclozolin on other steroid receptors and target organs susceptible to endocrine regulation such as breast, ovarian and testis also needs to be studied in more detail. Furthermore, continued effort is required to understand the ill-health effects such as developmental and

transgenerational alterations posed by Vinclozolin and similar pesticides in relation to the observed cellular and molecular changes.

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Appendix I

Effect of VCZ without S9 treatment on viability of LNCaP cells following MTT Assay. Student t-test was performed on the raw absorbance values to determine statistical significance between the dose reading and the control reading at each time point **(A)** and between the time reading and 6 hour reading at each dose **(B)**. Significant p-values (<0.05) are listed.

	6 hours	12 hours	24 hours	48 hours	72 hours	96 hours
	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM
Control (C)	0.6377 $\pm$ 0.05057	0.6512 $\pm$ 0.07592	0.5790 $\pm$ 0.03839	0.7759 $\pm$ 0.05298	1.089 $\pm$ 0.07534	1.424 $\pm$ 0.1121
200 μ M	0.5853 $\pm$ 0.06277	0.5362 $\pm$ 0.08094	0.3590 $\pm$ 0.03704	0.4383 $\pm$ 0.05866	0.2837 $\pm$ 0.09065	0.4778 $\pm$ 0.1234
100 μ M	0.6052 $\pm$ 0.05770	0.6418 $\pm$ 0.07862	0.5772 $\pm$ 0.05217	0.5608 $\pm$ 0.02639	0.5883 $\pm$ 0.09249	0.7780 $\pm$ 0.1223
10 μ M	0.6030 $\pm$ 0.05665	0.6740 $\pm$ 0.07772	0.5815 $\pm$ 0.04684	0.7530 $\pm$ 0.03550	1.028 $\pm$ 0.09646	1.310 $\pm$ 0.1047
1 μ M	0.6148 $\pm$ 0.05748	0.6748 $\pm$ 0.07648	0.6268 $\pm$ 0.05805	0.7821 $\pm$ 0.03580	1.097 $\pm$ 0.09263	1.435 $\pm$ 0.1231
0.1 μ M	0.6217 $\pm$ 0.05630	0.6503 $\pm$ 0.07186	0.6008 $\pm$ 0.04260	0.7893 $\pm$ 0.03851	1.095 $\pm$ 0.09021	1.395 $\pm$ 0.1092
0.01 μ M	0.6337 $\pm$ 0.05330	0.6477 $\pm$ 0.07120	0.4918 $\pm$ 0.02611	0.7654 $\pm$ 0.03550	1.090 $\pm$ 0.09857	1.512 $\pm$ 0.1676

(A)	6 hours	12 hours	24 hours	48 hours	72 hours	96 hours
ANOVA F	0.1057	0.3861	4.346*	10.99*	12.87*	11.16*
p (C vs)	200uM = 0.0021			200uM = 0.0006 100uM = 0.0022	200uM < 0.0001 100uM = 0.0018	200uM = 0.0001 100uM = 0.0027

(B)	200uM	100uM	10uM	1uM	0.1uM	0.01uM
ANOVA F	5.844*	15.07*	0.2852	0.1381	0.1146	0.5250
p (6h vs)	72h = 0.0264 96h = 0.0419	72h = 0.0084 96h = 0.0165				

Effect of VCZ after S9 treatment on viability of LNCaP cells following MTT Assay. Student t-test was performed on the raw absorbance values to determine statistical significance between the dose reading and the control reading at each time point **(A)** and between the time reading and 6 hour reading at each dose **(B)**. Significant p-values (<0.05) are listed.

	6 hours	12 hours	24 hours	48 hours
	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM	mean(abs) $\pm$ SEM
Control (C)	0.4597 $\pm$ 0.02512	0.5453 $\pm$ 0.001764	0.4850 $\pm$ 0.001732	0.7070 $\pm$ 0.007937
200 μ M	0.4390 $\pm$ 0.006083	0.4950 $\pm$ 0.004509*	0.3910 $\pm$ 0.008622*	0.5503 $\pm$ 0.01767*
100 μ M	0.4510 $\pm$ 0.004509	0.5480 $\pm$ 0.009539	0.4187 $\pm$ 0.005812*	0.6207 $\pm$ 0.03017
10 μ M	0.5000 $\pm$ 0.008544	0.5670 $\pm$ 0.01002	0.4637 $\pm$ 0.01114	0.7710 $\pm$ 0.01637*
1 μ M	0.5160 $\pm$ 0.008544	0.5547 $\pm$ 0.01168	0.4737 $\pm$ 0.006566	0.7330 $\pm$ 0.009019
0.1 μ M	0.4997 $\pm$ 0.002603	0.5563 $\pm$ 0.01466	0.4683 $\pm$ 0.002963	0.7483 $\pm$ 0.01345
0.01 μ M	0.4820 $\pm$ 0.002517	0.5160 $\pm$ 0.01193	0.4857 $\pm$ 0.01014	0.6817 $\pm$ 0.01135

(A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	6.884*	6.429*	23.51*	21.87*
p (C vs)		200uM = 0.0005	200uM = 0.0004 0.1uM = 0.0004	200uM = 0.0013 0.01uM = 0.0245

(B)	200uM	100uM	10uM	1uM	0.1uM	0.01uM
ANOVA F	5.171*	2.312	3.223	4.755*	3.391	2.143
p (6h vs)	24h = 0.0299 48h = 0.0434	24h = 0.0164		24h = 0.0175	24h = 0.0010	48h = 0.0491

Appendix II

Effect of VCZ without S9 treatment on expression of NKX3.1, AR and CYP3A4 in LNCaP cells following qRT-PCR. Student t-test was performed to determine statistical significance between the dose reading and the control reading at each time point **(A)** and between the time reading and 6 hour reading at each dose **(B)**. Significant p-values (<0.05) are listed.

NKX3.1	6 hours	12 hours	24 hours	48 hours
	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM
Control (C)	1.08629 $\pm$ 0.08674	0.94528 $\pm$ 0.07085	0.93243 $\pm$ 0.09119	0.91225 $\pm$ 0.05401
100 μ M	1.32566 $\pm$ 0.04683*	1.07973 $\pm$ 0.1045	1.64203 $\pm$ 0.09202*	1.28476 $\pm$ 0.07489*
1 μ M	1.1818 $\pm$ 0.07552	1.17298 $\pm$ 0.1033	1.21701 $\pm$ 0.0832	1.09784 $\pm$ 0.07829
0.01 μ M	1 $\pm$ 0.06083	0.99129 $\pm$ 0.0764	0.96802 $\pm$ 0.03574	1.11018 $\pm$ 0.12577

NKX3.1 (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	4.074*	1.249	5.698*	3.041
p (C vs)	100uM = 0.0356		100uM = 0.0064	100uM = 0.0024

NKX3.1 (B)	C	100uM	1uM	0.01uM
ANOVA F	1.059	7.946*	0.3416	0.6023
p (6h vs)		24h = 0.0120		

AR	6 hours	12 hours	24 hours	48 hours
	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM
Control (C)	1.33675 $\pm$ 0.17733	1.09643 $\pm$ 0.29277	1 $\pm$ 0.16255	0.97686 $\pm$ 0.09655
100 μ M	0.99548 $\pm$ 0.09615	0.79404 $\pm$ 0.18344	1.41486 $\pm$ 0.15122	0.86243 $\pm$ 0.10946
1 μ M	1.07425 $\pm$ 0.12461	1 $\pm$ 0.18515	0.84866 $\pm$ 0.10642	1.01463 $\pm$ 0.10096
0.01 μ M	0.68796 $\pm$ 0.1388	0.78214 $\pm$ 0.07627	1.00589 $\pm$ 0.11611	1.24305 $\pm$ 0.11135

AR (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	3.781*	0.6053	2.253	2.321
p (C vs)	0.01uM = 0.0280			

AR (B)	C	100uM	1uM	0.01uM
ANOVA F	0.7081	3.985*	0.5170	4.819*
p (6h vs)				48h = 0.0206

CYP3A4	6 hours	12 hours	24 hours	48 hours
	mean(exp) ± SEM	mean(exp) ± SEM	mean(exp) ± SEM	mean(exp) ± SEM
Control (C)	0.69307 ± 0.1044	0.57422 ± 0.17518	0.28403 ± 0.06674	0.77911 ± 0.16829
100 µM	0.35647 ± 0.12493	0.44205 ± 0.143	0.7402 ± 0.05316*	0.85018 ± 0.07515
1 µM	1.2365 ± 0.18132*	1.10785 ± 0.35761	1.34685 ± 0.11969*	1.04642 ± 0.15372
0.01 µM	0.78146 ± 0.14122	0.50686 ± 0.08113	0.80542 ± 0.20185	1.41627 ± 0.20583*

CYP3A4 (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	6.633*	2.003	5.028*	3.262*
p (C vs)	1uM = 0.0266		100uM = 0.0177 1uM = 0.0088	0.01uM = 0.0088

CYP3A4 (B)	C	100uM	1uM	0.01uM
ANOVA F	2.511	4.985*	0.3623	5.376*
p (6h vs)	24h = 0.0080	24h = 0.0180 48h = 0.0069		48h = 0.0292

Effect of VCZ with S9 treatment on expression of NKX3.1, AR and CYP3A4 in LNCaP cells following qRT-PCR. Student t-test was performed to determine statistical significance between the dose reading and the control reading at each time point **(A)** and between the time reading and 6 hour reading at each dose **(B)**. Significant p-values (<0.05) are listed.

NKX3.1	6 hours	12 hours	24 hours	48 hours
	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM
Control (C)	1 $\pm$ 0.07031	0.91843 $\pm$ 0.08494	0.9681 $\pm$ 0.12858	0.82281 $\pm$ 0.12579
100 μ M	1.01665 $\pm$ 0.07055	1.31767 $\pm$ 0.09888	1.02952 $\pm$ 0.07113	1.18694 $\pm$ 0.10607
1 μ M	0.90609 $\pm$ 0.05204	0.85373 $\pm$ 0.09565	1.24596 $\pm$ 0.15145	1.04915 $\pm$ 0.10699
0.01 μ M	1.16211 $\pm$ 0.17296	0.96032 $\pm$ 0.04592	1.04485 $\pm$ 0.08422	1.02753 $\pm$ 0.06875

NKX3.1 (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	1.052	3.158	1.126	2.086
p (C vs)	No significant p-values			

NKX3.1 (B)	C	100uM	1uM	0.01uM
ANOVA F	0.5376	2.625	2.663	0.6428
p (6h vs)		12h = 0.0479		

AR	6 hours	12 hours	24 hours	48 hours
	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM	mean(exp) $\pm$ SEM
Control (C)	1 $\pm$ 0.06951	0.96867 $\pm$ 0.09735	1 $\pm$ 0.14567	1 $\pm$ 0.18756
100 μ M	0.88032 $\pm$ 0.10512*	0.94984 $\pm$ 0.0882	0.81756 $\pm$ 0.11211	0.84214 $\pm$ 0.26294
1 μ M	0.71717 $\pm$ 0.05039*	1 $\pm$ 0.11476	0.71072 $\pm$ 0.11102*	1.01454 $\pm$ 0.2771*
0.01 μ M	0.75838 $\pm$ 0.08227*	1.02311 $\pm$ 0.1011	0.539 $\pm$ 0.05073*	0.76345 $\pm$ 0.08925*

AR (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	9.074*	1.022	24.65*	12.21*
p (C vs)	100uM = 0.021 1uM = 0.009 0.01uM = 0.0018		1uM = 0.0031 0.01uM = 0.0181	1uM = 0.0059 0.01uM = 0.0006

AR (B)	C	100uM	1uM	0.01uM
ANOVA F	0.01388	0.1324	1.096	5.696*
p (6h vs)	No significant p-values			

CYP3A4	6 hours	12 hours	24 hours	48 hours
	mean(exp) ± SEM	mean(exp) ± SEM	mean(exp) ± SEM	mean(exp) ± SEM
Control (C)	0.75997 ± 0.40434	0.98486 ± 0.07369	0.69788 ± 0.12584	0.52177 ± 0.14288
100 µM	1.05717 ± 0.13278	0.81128 ± 0.11386	0.4387 ± 0.13624	0.58831 ± 0.1443
1 µM	1.00778 ± 0.23648	0.98036 ± 0.11509	1.30837 ± 0.20311	1.25 ± 0.11275
0.01 µM	1.19535 ± 0.26884	1.06515 ± 0.05152	1.04485 ± 0.11905	1.22954 ± 0.07499

CYP3A4 (A)	6 hours	12 hours	24 hours	48 hours
ANOVA F	0.4268	0.04664	1.568	1.047
p (C vs)	No significant p-values			

CYP3A4 (B)	C	100uM	1uM	0.01uM
ANOVA F	0.7132	4.162*	0.9028	0.3600
p (6h vs)		24h = 0.0174		