THE MODULATION OF ANDROGEN ACTION IN PROSTATE CANCER BY EXOGENOUS CHEMICALS, EFFLUX TRANSPORTER P–GLYCOPROTEIN AND Y-BOX BINDING PROTEIN-1

by

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Pathology and Laboratory Medicine

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Abstract

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer deaths in Canada. Human prostate carcinomas are often androgen-dependent and respond to androgen withdrawal therapy by temporary regression, followed by androgen-independent recurrence. These well-established features of prostate cancer strongly suggest that androgens play a major role in prostate carcinogenesis.

The overall goal of the research described in this thesis is to gain a more comprehensive understanding of the mechanisms modulating androgen action in prostate cancer cells. This focused on the influence of exogenous chemicals, the efflux transporter P-glycoprotein and the multi-functional transcription factor Y-box Binding Protein-1. Epidemiological evidence, based on agricultural occupational exposures, instigated experimental work that showed pesticides have the potential to mimic or antagonize hormone action, in many cases through androgen receptor binding and interfering with transcriptional activity, thus are capable of disrupting the male hormone-signaling pathway. Furthermore, in prostate cancer cells, androgen responsiveness is modulated by Pgp activity and expression. The biological consequences of increased Pgp expression are decreased androgen accumulation and a corresponding decrease in androgen-regulated transcriptional activity and prostate-specific antigen gene expression. Experimental evidence further supports the hypothesis that early in prostate cancer progression, increased YB-1 expression increases Pgp activity, which consequently lowers androgen levels in prostate tumour cells. Suppression of androgen levels may activate cell survival pathways and lead to an adaptive survival advantage of androgen-independent prostate cancer cells following androgen ablation therapy.

Understanding the complex molecular mechanisms by which prostate cancer cells can control androgen function, and how prostate cancer cells evade apoptotic death, provides a paradigm to explain the relationship between androgen action and chemotherapeutic
resistance. As a result, the final objective investigated the effects of YB-1 knockdown using antisense oligonucleotides *in vitro*, and *in vivo* after androgen withdrawal in human LNCaP prostate cancer tumour xenografts, with the aim of suppressing cellular proliferation and increasing chemosensitivity. Intratumoral injection of 2'-(methoxy)ethyl ribose-modified YB-1 ASO and paclitaxel incorporated into a biodegradable, controlled-release formulation in castrated mice delayed AI progression. These results suggest that YB-1 may be a promising target for the treatment of prostate cancer based on a strategy of inhibiting cellular proliferation. Our understanding of the molecular links between androgen action, tumorigenesis, apoptosis, and drug resistance provides the foundation for a new era of targeted cancer therapy.
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List of Abbreviations

2,4-D 2,4-Dichlorophenoxy Acetic Acid
2,4-DB 2,4-Dichlorophenoxy-Butyric Acid
2'-MOE 2'-O-(methoxy) ethyl
ABC ATP-Binding Cassette
ACTH Adrenocorticotropic Hormone
AD Androgen-Dependent
AhR Aryl Hydrocarbon Receptor
AI Androgen-Independent
ANOVA Analysis of Variance
AP-1 Activator Protein-1
AR Androgen Receptor
ARE Androgen Response Element
AR-LBD Androgen Receptor-Ligand Binding Domain
ARR3-Luc Androgen-Responsive Luciferase Reporter Gene Construct
AS Androgen Sensitive
ASO Antisense Oligonucleotide
ATP Adenosine Triphosphate
BC British Columbia
BCCA British Columbia Cancer Agency
Bcl-2 B Cell Lymphoma-2
BPH Benign Prostatic Hyperplasia
BRT Beam Radiation Therapy
BSA Bovine Serum Albumin
CBP CREB Binding Protein
CPM Counts Per Minute
CSD Cold Shock Domain
Da Dalton
DCC Dextran-Coated Charcoal
DCM Dichloromethane
dCx days Post-Castration
DDT Dichloro-Diphenyl-Trichloroethane
DBD DNA-Binding Domain
DES Diethylstilbesterol
DHEA Dihydroepiandrosterone
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>e1F4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Grp78</td>
<td>Glucose-Related Protein</td>
</tr>
<tr>
<td>GTA</td>
<td>General Transcriptional Apparatus</td>
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<td>Gud-Luc1.1</td>
<td>Dioxin-responsive Luciferase Reporter Gene Construct</td>
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<td>HAP</td>
<td>Hydroxylapatite</td>
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<tr>
<td>hAR</td>
<td>Human Androgen Receptor</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HPLC</td>
<td>High-Pressure Liquid Chromatography</td>
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<td>Heat Shock Proteins</td>
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<td>HSP27</td>
<td>Heat Shock Protein-27</td>
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<tr>
<td>IAS</td>
<td>Intermittent Androgen Suppression</td>
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<td>IGEPAL</td>
<td>Octylphenoxo Poly(EthyleneOxy) Ethanol</td>
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<td>Interleukin-6</td>
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<td>ILK</td>
<td>Integrin-Linked Kinase</td>
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<td>JEM</td>
<td>Job-Exposure Matrix</td>
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<td>LBD</td>
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<td>LH</td>
<td>Leutenizing Hormone</td>
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<tr>
<td>LHRH</td>
<td>Leutenizing Hormone Releasing Hormone</td>
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<tr>
<td>MCPA</td>
<td>4-Chloro-2-Methyl-Phenoxy-Acetic Acid</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-Drug Resistance</td>
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<td>MDR-1</td>
<td>Multi-Drug Resistance Gene-1</td>
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<tr>
<td>MDR-NF-1</td>
<td>Multi-Drug Resistance Nuclear Factor-1</td>
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<td>MMP-2</td>
<td>Matrix Metalloproteinase-2</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MRP</td>
<td>Multi-drug Resistance Protein</td>
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<tr>
<td>MSY-1</td>
<td>Murine YB-1 Protein</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NCIC</td>
<td>National Cancer Institute of Canada</td>
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<tr>
<td>NBD</td>
<td>Nucleotide Binding Domain</td>
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<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
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<td>NF-Y</td>
<td>Nuclear Factor-Y</td>
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<td>NHT</td>
<td>Neo-Adjuvant Hormone Therapy</td>
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<tr>
<td>NSEP-1</td>
<td>Nuclease Sensitive Element Binding Protein-1</td>
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<tr>
<td>PAP</td>
<td>Prostate Acid Phosphatase</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated Biphenyl</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PreCx</td>
<td>Pre-Castration</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase Tensin Homologue</td>
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<tr>
<td>PTP-1B</td>
<td>Protein Tyrosine Phosphatase 1B</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Luminescence Units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SBP</td>
<td>Steroid Binding Protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SHBG</td>
<td>Steroid Hormone-Binding Globulin</td>
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<tr>
<td>SP-1</td>
<td>Stimulating Protein-1</td>
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<td>SRC-1</td>
<td>Steroid Receptor Co-Activator-1</td>
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<tr>
<td>SRC-3</td>
<td>Steroid Receptor Co-Activator-3</td>
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<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-Dioxin.</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
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<td>YB-1</td>
<td>Y-box Binding Protein-1</td>
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Acknowledgments

On the eve of completing my Ph.D. studies, ready to complete and open another chapter in my life, it seems like a fitting time to reflect on the past five years. My journey and interest in the field of cancer research began rather serendipitously, shaped by two influences in my life. The first was a keen fascination in biology, shaped in a large part by summers spent on the West Coast, and accompanying my grandmother to her genetics lab at the University of Victoria, Biology Department. Over 60 years ago, before the discovery of DNA, she graduated with a Masters in Agriculture at UBC. The smell of biology is something I always remember, pouring over drawers of preserved insects, making fruit fly food, trips to the animal room and greenhouse adventures.

The other guiding influence was my admiration for Terry Fox, the young man who despite his leg amputation decided to raise cancer awareness in Canada by running from coast to coast. My aunt Leslie Scrivener, a reporter with the Toronto Star, followed Terry’s Run for Hope across the country and wrote a book about his journey – Terry Fox: his story. Through the commitment and determination of my own athletic pursuits, I have come to understand that Terry embodied an extreme will and grace. By trying to better himself and accomplish his own goals, he had a profound impact on the world around him. He touched the hearts of many Canadians and my hope is that his spirit lives on in all those who are involved with cancer – patients, survivors, family members, clinicians and researchers such as us. In humans, the incidence of cancer rises exponentially in the final decades of life, culminating in a lifetime risk of 1 in 2 for men and 1 in 3 for women (American Cancer Society, Cancer Facts and Figures 2000). No other disease has such a profound impact on our society, both socially and economically. Thanks to the legacy of Terry Fox, there has been a dramatic improvement in the treatment and survival of sarcomas, childhood cancers, and leukemia. 30 years later, if Terry were diagnosed today, he would likely be spared his leg amputation, and his chances of survival would have increased from 15% to over 80%. Clearly, cancer research makes a difference. I am
proud to be part of such a dedicated group of researchers who are constantly working towards the goal of eradicating cancer.

There have been many accomplishments, joys and frustrations, and the world has changed profoundly in the past half decade. The publication of the human genome in both Science and Nature in February 2001 is perhaps one of the greatest achievements of humankind. To many, putting a human on the moon or routine transplant surgery can be considered other profound achievements, however the potential impact of the knowledge of the human genome on the future of disease and medicine is incredible. The power of now unraveling our genetic blueprint is astounding, but largely unappreciated by the general public. It struck me how the world, society and culture, and science are so integrated. A few months ago I attended a keynote lecture by Dr. Leroy Hood, the head of the Institute of Systems Biology near Seattle. His brilliance and predictions for our future left a lasting impression. He predicts that in 15 years time we will be able to sequence the entire genome of a person in less than one hour, diagnose many diseases with simple blood tests, and treat diseases with personalized medicine. During his lecture he spoke of the integration of many fields - science, engineering, and medicine into a complex field known as systems biology. His knowledge and predictions drew amazement from the audience. The ability to monitor the workings of a single cell in real time on a glass slide designed with nanotechnology, gene therapies designed “while you wait” in a doctor’s office, and the common use of DNA identification techniques. His speech made little mention of ethical or moral issues of science, and got me thinking about the path our society and cultural values are sending us into future.

Cancer research makes a positive contribution to society and I wish to thank all those wonderful and dedicated people that I have been fortunate enough to work with over the past five years. My supervisor, Dr. Colleen Nelson, is a woman of intense determination, drive, and intellect. She looks at the positive side of any result with enthusiasm, and has given me the independence to develop my research path and direction to help put meaning into sometimes
obscure and contradictory results. Her advice has been invaluable. Emma Guns has been an unbelievable positive influence and given me endless direction and responsibility. I feel like an honorary member of her group, and feel privileged to have made a contribution to her lab. Simon Cowell has become a wonderful friend both in and out of the lab and I really value all the experiences we have shared over the years, both academic and athletic. Jody Ralph has a contagious smile and laughter that makes her a great friend and she has provided me great support and advice throughout our graduate studies. Thank you to my supervisory committee members: Dr. Stelvio Bandiera, Dr. Emma Guns, Dr. Wan Lam, Dr. Colleen Nelson, and Dr. Paul Rennie for their direction and guidance during my graduate studies in the Department of Pathology and Laboratory Medicine. A special thanks to Dr. Pepita Gimenez-Bonafe, the post-doc who I worked most closely with on my project, she is woman who has a passion for science and her cooperation and guidance was unparallel. Thank you to all the wonderful people at the Prostate Centre who make the lab a special workplace and who are always there to give a helping hand or offer advice. This work is dedicated to my fellow graduate student, colleague and friend Wesley Sydor, who tragically left us all. We will all remember you and hope you have found eternal peace.

Finally, I would like to give the greatest thanks to my wonderful family and friends for their endless and unconditional love, support and encouragement. Live strong.
Co-Authorship Statement

Under the mentorship of my Ph.D. supervisor Dr. Colleen Nelson, the research presented in this Ph.D. thesis was undertaken in collaboration with many talented researchers. As detailed in the authorship list following the chapter titles, those individuals played an important role in the research presented in those respective publications and chapters. Further specific contribution details can be found preceding the *Introduction* section of each chapter.
CHAPTER 1. LITERATURE REVIEW, HYPOTHESES AND OBJECTIVES

Cancer is a broad term describing a plethora of conditions characterized by abnormal and uncontrolled cellular proliferation. In Canada, cancer is the leading cause of premature death and morbidity (NCIC, 2005). Cancerous tumours are pathologically variable and heterogeneous; however, all share the ability to proliferate beyond the constraints limiting growth in normal tissue (Hanahan and Weinberg, 2000). There are many diverse causes of cancer, which may include genetic predisposition, dietary and environmental influences, infectious agents, and ageing (Hsing and Devesa, 2001). The massive challenge is to identify and understand the complex molecular anatomy and biology of the pivotal steps in tumour progression, identifying how tumour cells differ from normal cells and how those differences can be exploited to develop effective therapies.

This Ph.D. dissertation represents a broad project that began as a study of endocrine disruption in the context of prostate cancer, first examining the influences of exogenous agents, specifically pesticides, on steroid receptor binding and transcriptional activity interference. Further research examined the deregulation of cellular androgen action in prostate cancer cells, revealing the novel finding that the cellular transport and accumulation of dihydrotestosterone (DHT) is dependent on the expression of functional androgen receptor (AR) and is modulated in part by cell membrane efflux transporter Permeability-glycoprotein, which is commonly known as P-glycoprotein (Pgp).

The project further focused on studying Y-box binding protein-1 (YB-1), shown in the literature to be directly involved in multi-drug resistance (MDR1) gene activation in a variety of cancers in response to genotoxic stress (Kohno et al., 2003). Results show that in prostate cancer, YB-1 expression is markedly increased during benign to malignant transformation, and further following androgen ablation. It is hypothesized that increased YB-1 expression leads to decreased androgen-regulated gene expression, in part through a Pgp-mediated mechanism.

Finally, to bring clinical relevance to the project, targeted suppression of YB-1
expression *in vitro* and *in vivo* using antisense oligonucleotides, possibly leading to decreased cellular growth and/or taxane chemosensitivity, was examined in PC-3 cells and a murine LNCaP prostate tumour xenograft model. The results suggest that YB-1 may be a promising target for treatment of prostate cancer based on a strategy of inhibition of cellular proliferation.

### 1.1 Prostate Cancer

#### 1.1.1 Prostate Biology and Function

Anatomically, the human prostate gland is located between the base of the bladder and the rectum, completely surrounding the proximal urethra. It is a single alobular structure with central, peripheral and transitional zones, and is roughly the size and shape of a walnut, weighing approximately 20 grams (Cunha et al., 2004). The prostate is a hormone-target organ whose differentiation, development, growth and function are hormone-regulated, primarily by androgens – or male sex hormones (Cunha et al., 2004; Hayward and Cunha, 2000; Huggins et al., 1941; Labrie et al., 1993). The prostate undergoes dramatic morphologic changes at puberty as it acquires the ability to produce seminal fluid in response to stimulation with androgens. After puberty, the normal function of the prostate is to produce a protein- and organic solute-rich fluid that contributes approximately 30-50% of the total seminal fluid secretions. Prostatic secretions are poorly characterized; however, recent proteomic studies have been aimed at identifying proteins necessary for sperm viability, and better biomarkers of prostate cancer (Fung et al., 2004; Starita-Geribaldi et al., 2001). Future comparative studies of seminal fluid obtained from normal patients and patients diagnosed with prostate cancer may identify a set of protein biomarkers that offer improved diagnostic and/or prognostic sensitivity and specificity (Fung et al., 2004).

The prostate is made up of epithelial glands and a fibromuscular stroma as characterized in Figure 1.1. The glandular epithelium, where prostate adenocarcinoma commonly develops, has three types of cells: basal; luminal secretory; and neuroendocrine, in two defined
Figure 1.1 Representation of a Prostate Cross-Section with Epithelial Cell Layers.

The prostate is made up of epithelial glands and a fibromuscular stroma. The glandular epithelium has three main cell types: basal; luminal secretory; and neuroendocrine. Intermediate "amplifying" cells are characterized by their state of differentiation, migration, and proliferation and express combinations of markers, such as keratins, common to both the basal and luminal epithelial layers. The prostate epithelium is variable in androgen-sensitivity, with androgen-independent basal stem cells, and androgen-dependent luminal secretory cells (Modified from Long et al., 2005).
compartments (Garraway et al., 2003). It remains unclear how the prostate epithelia differentiates to basal, neuroendocrine and luminal secretory cells. The basal compartment contains basal cells, which possess high proliferative activity and have been shown to be the stem cells of the prostate (Bonkhoff and Remberger, 1996). They secrete components of the basement membrane and are thought to be responsible for generating the secretory epithelial cells. The basal cells are also androgen-independent, and lack expression of the AR. In contrast to the basal cells, the luminal compartment harbours the terminally differentiated secretory cells, which secrete components of prostatic fluid, express the AR and secrete prostate specific antigen (PSA) in an androgen-dependent manner. Thus, the normal prostate epithelium is composed of different cell types that have varying androgen-sensitivity, including androgen-independent basal stem cells, androgen-dependent luminal secretory cells, and androgen-independent but androgen-sensitive transitional “amplifying” intermediate cells (Isaacs, 1999), as shown in Figure 1.1. According to Isaacs (1999), the androgen milieu of the normal prostate determines the normal progression of basal stem cell to secretory cell to allow for a balanced “steady-state” in which neither prostatic regression nor continuous glandular overgrowth occurs (Isaacs, 1999). However, there is still significant debate about which specific cell type gives rise to prostate cancer. One theory that Isaacs and Coffey (1989) and Arnold and Isaacs (2002) have proposed postulates that prostate cancer originates from the luminal secretory cell compartment because of the expression of AR, PSA, and prostate acid phosphatase (PAP) in adenocarcinoma cells. However, most androgen-independent prostate cancer also express basal cell characteristics, such as the expression of B cell lymphoma-2 (bcl-2) proto-oncogene, which contributes to their apoptotic-resistant phenotype (McDonnell et al., 1992). Long et al. (2005) propose that prostate cancer arises from the basal layer and that androgen-dependent tumours consist of heterogeneous cell types that may include androgen-independent cells. Alternatively, intermediate cells may develop into prostate cancer cells due to their state of cell proliferation, migration, and differentiation (van Leenders et al., 2002).
Neuroendocrine cells are also found in the basal compartment and their functions are poorly understood. It has been hypothesized that increased prevalence combined with the low proliferation rate of neuroendocrine cells in hormone-refractory prostate cancer may allow them to survive treatment with most chemotherapeutic agents, as well as endocrine and radiation treatments (Debes and Tindall, 2004). In addition, they may secrete neuropeptides that can increase the proliferation of neighbouring cancer cells, thereby promoting progression of hormone-refractory prostate cancer (Hansson and Abrahamsson, 2001).

The stroma is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves, and some infiltrating cells, such as mast cells and lymphocytes. Some stromal cells are androgen-responsive and produce growth factors that act in a paracrine fashion on the epithelial cells. Stromal-epithelial crosstalk is an important regulator of the growth, development, and hormonal responses of the prostate (Cunha et al., 2004; Kurita et al., 2001). Thus, the normal prostate is inherently heterogeneous in its sensitivity to androgens.

1.1.2 Androgen Sensitivity and Progression to Androgen-Independence
Substantial epidemiological data supports a critical role for androgens in prostate cancer development in the context of steroid hormone signaling (Platz and Giovannucci, 2004; Soronen et al., 2004). Prostate cancer development is absent in men with marked androgen deficiency, such as eunuchs, or men with markedly reduced androgenicity of the prostate. For example, those with the absence of 5α-reductase enzyme activity in whom the prostate remains a vestigial organ (Ross et al., 2003; Wu and Gu, 1991). Pioneering studies by Huggins and Hodges showed as far back as the 1940's that nearly all prostate cancer is androgen-dependent at the time of diagnosis, and androgen deprivation has since been the cornerstone of therapy for prostate cancer (Huggins et al., 1941; original paper reprinted in Huggins and Hodges, 2002). Androgen ablation typically results in an initial reduction of tumour volume, but unlike normal prostate epithelial cells, malignant cells survive and adapt to the androgen-deprived environment as shown in Figure 1.2 (Craft et al., 1999; Isaacs, 1999; Rennie and Nelson,
Figure 1.2 Androgen-Independent Prostate Cancer Progression.

Tumour burden in prostate cancer is monitored by serum PSA, and decreases dramatically with androgen ablation. During this androgen-dependent (AD) phase of disease the tumour still requires androgens for growth and survival; therefore, removal of androgens leads to apoptosis of cancer cells and initial tumour regression. For reasons not fully understood, a small population of prostate cancer cells switch to an androgen-independent (AI) state in which cells bypass the growth signals required for AD growth, and proliferate uncontrollably. As a consequence, androgen-independent recurrence is characterized by a dramatic increase in both tumour burden and serum PSA levels.
These cells ultimately create the androgen-independent (AI) phenotype that is characterized in part by the up-regulation of genes that initially require androgens for expression, such as PSA, but become constitutively re-expressed in the absence of androgens (Gregory et al., 1998). Historically, two main hypotheses attempt to explain the acquisition of androgen-independence – the clonal selection hypothesis and the adaptation hypothesis (Isaacs and Kyprianou, 1987). Clonal selection involves both the selective survival and outgrowth of pre-existing androgen-independent cells within the tumour by androgen ablation. Adaptation, on the other hand, suggests that androgen-independence may be an intrinsic, but dormant, property of some prostate cancer cells involving adaptive changes in gene expression patterns that are activated in response to androgen ablation (Bruchovsky et al., 1990; Isaacs and Coffey, 1981; van Weerden, 1991). These general theories have more recently been superseded largely by additional concepts related to the androgen receptor. Several mechanisms may lead to androgen-independent adaptation in prostate cancer cells, including increased androgen receptor sensitivity to low serum androgen levels, androgen receptor mutations leading to aberrant activation, androgen receptor-related protein-protein interactions and co-regulators, effectual circumvention of normal ligand-steroid hormone receptor activation by growth factors such as insulin-like growth factor (IGF), and facilitation of proliferation and apoptotic inhibition by the activation of anti-apototic signaling pathways, involving proteins such as bcl-2 or clusterin (Buchanan et al., 2001; Colombel et al., 1993; Koivisto et al., 1997; Miyake et al., 2000). These mechanisms share an important prerequisite: Prostate cancers can be characterized as heterogeneous tumours, comprised of various subpopulations of cells that respond differently to androgen withdrawal therapy (So et al., 2005). Furthermore, it is very likely that other gene expression pathways are activated and contribute to the progression phenotype leading to invasion, metastasis, and drug resistance. Consequently, tumour progression is a highly dynamic process involving the interplay between a plethora of complex
molecular mechanisms, ultimately allowing cells to escape from normal regulatory controls. As a result, androgen-independent progression is the main obstacle to improving the survival and quality of life in patients with advanced disease, emphasizing the need for novel therapeutic strategies targeting the molecular basis of the resistance of prostate cancer to both androgen withdrawal and chemotherapy.

1.1.3 Prostate Cancer Epidemiology

Prostate cancer is commonly a disease of the elderly; around the world, three-quarters of cases occur in men aged 65 years and older (Quinn and Babb, 2002). In Canadian men, prostate cancer will continue to be the leading form of diagnosed cancer with approximately 20,500 new cases of prostate cancer diagnoses expected in 2005, representing an annual age-adjusted incidence of 133 per 100,000 men (NCIC, 2005). Over their lifetimes, 1 in 8 men will develop prostate cancer, but only 1 in 26 will die from it. Approximately 4,300 men are predicted to succumb to the disease in 2005 (NCIC, 2005). The incidence of prostate cancer has increased rapidly during the last 15 years, partly due to the widespread use of PSA measurements as a routine diagnostic test (Hankey et al., 1999). The incidence of prostate cancer shows strong age, race, and geographical dependence. Very few cases of prostate cancer occur in men younger than 50 years old, but rates then increase very steeply with age. Racial variation in prostate cancer incidence and mortality rates in the US is pronounced with African-American men having the highest prostate cancer incidence and mortality rates among any racial or ethnic group (Platz and Giovannucci, 2004). The incidence and mortality rates in African-American men are 1.6 and 2.3 times those for whites, respectively (Platz and Giovannucci, 2004). The recorded incidence of prostate cancer varies enormously around the world. The rate in the USA is more than twice that of Sweden and Australia, over three times the rate of the UK and elsewhere in Europe, and 10 times the level in the Far East in countries such as Singapore, Japan, India and China. (Quinn and Babb, 2002). Mortality in Singapore, Japan, India and China was lower than in other Western nations and consistent with the pattern in
incidence (Quinn and Babb, 2002). Due to the ageing demographic, prostate cancer continues to affect lives and account for a substantial number of deaths in British Columbia, with approximately 3,200 predicted diagnoses in 2005, and 580 predicted deaths (NCIC, 2005). In spite of the high incidence and mortality rate of this malignancy, the etiology of prostate cancer remains poorly understood.

The relative contributions of and relationships between multiple etiologic factors associated with prostate cancer are complex and unclear. Environmental factors are clearly shown in migration studies; for example, large increases in prostate cancer risk in Japanese men occur when they move to the United States (Shimizu et al., 1991). Other etiologic factors include ageing, race, hormonal influences, diet (both inductive and preventative factors), and lifestyle factors (Hsing and Devesa, 2001). Although age and race are clearly important, well-documented positive and negative risk factors also include dietary influences. Red meat consumption and high fat diets correlate with elevated risk; antioxidants such as vitamin E, lycopene, selenium, cesium are associated with lowered risk; and hormone levels are most likely critical factors as well (Alavanja et al., 2003; Heinonen et al., 1998; Yoshizawa et al., 1998). Finally, during the last two decades, evidence for genetic risk factors has accumulated. Positive family history is one of the strongest risk factors. An individual in North America with a first-degree relative who is affected has a two-fold increased risk of developing prostate cancer (Bruner et al., 2003). Most studies suggest that genetic factors are clearly involved in about 5-10% of prostate cancer cases (Carter et al., 1992; Ostrander and Stanford, 2000; Schaid et al., 1998). However, a twin study indicated that up to 40% of prostate cancers might be influenced by inherited effects (Lichtenstein et al., 2000). Regardless of the specific percentage of genetically determined cases, the interplay between genetic factors, endogenous hormones and environmental factors is likely to be important in the pathogenesis of prostate cancer (Bosland, 2000; Ekman et al., 1999; Pentyala et al., 2000; Platz and Giovannucci, 2004). Results presented in Chapter 2 of this thesis suggest that the interplay between mechanisms involved
in gene transcription and exogenous environmental chemicals, specifically pesticides, could
induce abnormal gene activation and expression in prostate cancer cells, potentially leading to
increased prostate cancer risk in occupationally-exposed workers.

1.1.4 History and Diagnosis

The course of prostate cancer is highly variable and difficult to predict. The prognosis of a
man with prostate cancer depends on the stage of the cancer and the age at which the disease
is diagnosed. The history of prostate cancer is incompletely understood, partly because early
stages of prostate cancer may go undetected for many years. It has long been recognized that
prostate cancer may exist in men not clinically diagnosed (Carter et al., 1990; Quinn and Babb,
2002; Schröder, 1995). Although autopsy studies of older men frequently identify prostate
cancer as an incidental finding, studies in younger men report a surprising incidence of prostate
cancer (Sakr et al., 1993; Sakr et al., 1994). In 152 men in California, all less than 50 yrs old,
who died of other causes, 34% of those 40-49 yrs old and 27% of those 30-39 yrs old had
microscopic evidence of cancer in their prostate glands (Sakr et al., 1993). The history of
localized prostate cancer is generally one of slow local progression with late development of
lymph node and distal metastases, indicating that cancer cells have escaped the prostate
capsule, consequently indicating a poor prognosis. Most carcinomas arise in the peripheral
zone of the prostate gland, where also the earliest detectable precursor lesion of prostate
cancer, prostatic intraepithelial neoplasia (PIN) is found. Most of the remaining cancers are
found in the periurethral region termed the transition zone (Marker et al., 2003). Curiously, the
virtually ubiquitous process of benign prostate hyperplasia (BPH) originates in the transition
zone of the prostate (Marker et al., 2003).

Clinically, prostate cancer is diagnosed upon histological evaluation of needle biopsy
samples of prostate tissue, taken because of an abnormal physical examination, an elevated
serum PSA, or both. The majority of all prostate carcinomas are typical adenocarcinomas,
which are commonly divided into different tumour Gleason grades (Gleason, 1992). The
average 5-year survival of patients with clinically detected prostate cancer is largely dependent on the stage of the tumour at the time of diagnosis and varies from approximately 87% for localized, early-stage, low grade disease, to approximately 25% for patients with advanced disease (NCIC, 2005; Quinn and Babb, 2002). Regrettably, approximately a third of prostate cancer patients present with advanced disease (Klotz, 2000). Fortunately, with early clinical diagnosis and treatment, the five-year relative survival has been reported to be 89% for men between the ages of 55 and 74 (NCIC, 2002).

PSA was introduced as a screening tool in the mid 1980’s and is primarily responsible for the two-fold increase in incidence rates observed between 1986 and 1992 (McDavid et al., 2004), as well as the substantial decrease in the percentage of cases diagnosed annually with disseminated disease (Catalona et al., 1991). PSA is a serine protease with a chymotrypsin-like substrate specificity, which is normally secreted by the prostate in large amounts into the seminal plasma, with only small amounts entering into the bloodstream. Serum PSA is currently the most useful marker of response and prognosis in patients treated with androgen ablation. PSA levels greater than 4μg/L after six months of androgen ablation are associated with a median survival of 10 months compared with 42 months with a serum PSA below 4μg/L (Miller et al., 1992). Furthermore, a rising PSA is the earliest sign of progression, preceding clinical signs of recurrence by 6-12 months (So et al., 2003).

Current diagnostic approaches have their limitations, resulting in both false-negatives and false-positives. PSA results are limited by poor tumour specificity: one in three cases of prostate cancer are associated with a normal PSA value (Thompson et al., 2003), while genetic polymorphisms can increase basal levels of PSA in individuals without prostate cancer (Cramer et al., 2003). Therefore, extensive research using DNA microarrays and proteomics is focused on developing new biological markers, and identifying susceptibility genes and somatic genetic changes associated with prostate cancer (Ahram et al., 2002). These may include members of the kallikrein family of serine proteases other than PSA, insulin-like growth factor-1 (IGF-1) and
IGF binding protein-3 (IGFBP-3), IL-6, and transforming growth factor β1 (Watson and Schalken, 2004). In addition, Chapter 4 presents data suggesting that the cellular localization of YB-1 may have diagnostic potential in prostate cancer, and may be a marker of early tumour development.

1.2 Prostate Cancer Therapy

1.2.1 Early Stage Prostate Cancer

Early stage prostate cancer is typically treated with either surgical removal or internal or external localized radiotherapy, or in some cases is just followed without treatment ("watchful waiting"). Surgery or radiation can be very successful at curing early stage prostate cancer when the tumour is confined to the gland (Diaz and Patterson, 2004). Radical prostate cancer therapy, or treatment with the intention to cure, can be achieved by two means: surgery or radiation therapy (RT). Radical prostatectomy, the surgical approach, has become the routine procedure, and improved operation techniques have led to a marked decrease in the intraoperative and postoperative mortality and morbidity (Walsh et al., 1994). The operation is performed mostly on younger patients with active sexual functions, and aims to preserve neurovascular bundles and the capacity for penile erection (Walsh et al., 1983). The most common long-term adverse effects of radical surgery are impotence and incontinence (Catalona et al., 1999; Walsh et al., 1994).

RT is applied as a curative mode externally or internally (brachytherapy) with radioactive seed implants. The results of external RT reported in some studies are as good as radical prostatectomy (Martinez et al., 2000; Shipley et al., 1999). For patients with locally advanced disease, or for patients too old for surgery, external RT is recommended, with or without adjuvant therapy (Akakura et al., 1999a and 1999b; Stromberg et al., 1997). On the other hand, brachytherapy is feasible for small tumours (D’Amico and Coleman, 1996). Especially for “low risk disease” patients, brachytherapy gives as good a prognosis as radical prostatectomy or external RT (D’Amico et al., 1998). The most common adverse side effects of
RT are irritation of the urinary bladder and rectum, enteritis, incontinence and impotence (Shipley et al., 1994). Other less commonly used radical treatment forms include ultrasound-guided percutaneous cryoablation (Bahn et al., 1995) and high intensity focused ultrasound (Beerlage et al., 1999).

### 1.2.2 Advanced Prostate Cancer

Androgen withdrawal remains the only effective form of systemic therapy for patients with advanced disease; that is, men whose cancer has spread outside the prostate gland. In addition, up to 80% of patients demonstrate objective responses to continuous androgen ablation, but the median length of response is only 18-24 months (Denis et al., 1993). Unfortunately, progression to androgen-independence ultimately occurs in the majority of these cases and the average range of overall survival is only 23 to 37 months (Hurtado-Coll et al., 2002). Recently, Petrylak et al. (2004) and Tannock et al. (2004) are the first to report studies of the treatment benefits with the use of the cytotoxic agent docetaxel, increasing the survival of patients with metastatic androgen-independent prostate cancer.

Huggins and Hodges pioneered the use of androgen ablation in prostate cancer using surgical orchiectomy (testicular removal), which subsequently became the “gold standard” and earned them a Nobel Prize for Medicine in 1941 (original paper reprinted in Huggins and Hodges, 2002). Until recently, androgen withdrawal therapy was restricted to a choice between bilateral orchiectomy and the administration of estrogen in the form of diethylstilbestrol (DES / Honvol®) (Beck et al., 1978; So et al., 2003). Over the past two decades, the most common agents used for androgen ablation are leutenizing-hormone releasing hormone (LHRH) agonists, such as leuprolide acetate, which result in down-regulation of LHRH receptors in the pituitary gland, and those blocking the peripheral effects of androgens (Gleave et al., 1999). Steroidal and non-steroidal anti-androgens have been used alone or in various combinations to achieve total medical castration (Sarosdy, 1999). This prevents residual androgens from activating AR-mediated gene expression pathways in the prostate (Feldman and Feldman,
Antiandrogens approved for use in Canada include the steroidal agent, cyproterone acetate (Androcur®), and non-steroidal agents, such as bicalutamide (Casodex®), flutamide (Euflex®), and nilutamide (Anadron®). The mechanisms of action for the two classes of AR antagonists differ. While steroidal antagonists compete with androgens for binding to the hydrophobic ligand-binding pocket of AR to prevent cognate ligands from binding, non-steroidal agents encourage assembly of transcriptionally inactive AR protein complexes in the nucleus (Masiello et al., 2002).

Unfortunately, there are limitations to the continuous use of androgen ablation therapy. Treatment can result in numerous side effects, including hot flashes, loss of libido, impotence, and general fatigue (Blackledge et al., 1996). Bone demineralization, anemia, lipid disorders and muscle wasting have been reported with long-term chemical castration (So et al., 2003).

The main problem in the conservative regimen of hormonal treatment is that the cancerous tissue may become increasingly less sensitive to therapy resulting in disease relapse. The concept of intermittent androgen suppression (IAS) is based on the hypothesis that the maintenance of apoptotic potential by successive rounds of androgen withdrawal and replacement might delay androgen-independent tumour progression. There are several studies concerning intermittent hormonal therapy (Crook et al., 1999; Goldenberg et al., 1995; Hurtado-Coll et al., 2002; Klotz et al., 1986). A recent clinical study of patients undergoing IAS suggests the average time for onset of androgen-independent disease is extended to 48 months following initial treatment (Pether et al., 2003).

1.2.3 Androgen-Independent Prostate Cancer

Regardless of treatment modality, disease progression to androgen-independence seems inevitable. Treating androgen-independent prostate cancer with systemic therapies has proved difficult; however, recent advances in combined chemotherapy regimes and the development of novel therapies may make it possible to modulate the adaptive changes in gene expression that characterize or mediate progression to androgen-independence and enhance cytotoxic
chemosensitivity (Diaz and Patterson, 2004). Taxane-based chemotherapy regimens appear to be a promising step in the evolution of prostate cancer treatment. Taxanes act by disrupting the microtubular network that is essential for mitotic and interphase cellular functions in prostate cancer cells. They promote the assembly of tubulin into stable microtubules and inhibit their disassembly, causing cell-cycle arrest and eventual apoptosis (Pienta, 2001). Many clinical studies have recently investigated the dosing regimens and the choice of which agents to combine with taxanes, but a clear consensus has not yet been established. Docetaxel has been successfully paired with both estramustine and calcitriol (Diaz and Patterson, 2004). For the first time, an increase in median survival has been reported in Phase II studies enrolling metastatic androgen-independent prostate cancer patients for treatment with chemotherapy. Two randomized studies – TAX327 and SWOG S9916 – have reported the first Phase III randomized data available for survival comparisons showing significantly increased survival for docetaxel-based regimens over other cytotoxic agents (Petrylak et al., 2004; Tannock et al., 2004).

Further studies that combine compounds including angiogenesis inhibitors, small molecules, and antisense targets with docetaxel, are underway at several institutions. These new therapeutic strategies emphasize molecular mechanisms. For example, Gleave and Miyake (2005) have identified clusterin as an anti-apoptotic protein upregulated in an adaptive survival manner by androgen withdrawal and chemotherapy, that confers resistance to various cell triggers, including hormone treatment, radiotherapy, and chemotherapy. Phase II clinical trials are currently underway using clusterin-targeted antisense oligonucleotides to enhance cell death, following treatment with androgen ablation and docetaxel (Gleave and Miyake, 2005). In another example, Phase I clinical trials with G3139, a bcl-2 antisense oligonucleotide whose antitumor effects in preclinical models are enhanced when combined with taxane-based chemotherapy, report positive safety and biologic activity when given with paclitaxel or docetaxel for the treatment of progressive prostate tumours (Morris et al., 2005). Finally, Chapter 5 presents preclinical in vitro and in vivo data suggesting that targeted knockdown of
YB-1 using antisense oligonucleotides may decrease cell growth and prolong progression to androgen-independence.

1.3 Androgen Action and Endocrine Disruption in the Prostate

1.3.1 Endocrine Systems

Endocrine systems have evolved in multi-cellular organisms as vital communication networks designed specifically to orchestrate dynamic physiological changes necessary for reproduction and survival in response to stimulation from our environment. Hormones are the transmission signals the endocrine system utilizes and are produced in a wide range of tissues, including the pituitary, thymus, thyroid, parathyroid, and adrenal glands, as well as the pancreas, testes, and ovaries. Hormone signaling to target tissue occurs through four modes of delivery: endocrine, neuroendocrine, paracrine, and autocrine. In endocrine transport, a hormone is produced and released which travels through the blood stream to its target tissue. Neuroendocrine cells can also produce a hormone that travels to the target cell via the bloodstream. Neuroendocrine hormones such as norepinephrine, inhibin, and oxytocin are released by neurons and act on target tissues throughout the body. Paracrine interactions occur locally, and the hormone is secreted to a neighbouring cell via gap junctions or close contact. Finally, autocrine action occurs when a cell produces a hormone that then acts on the same cell. Steroid hormones elicit their actions at very low concentrations, typically in the range of $10^{-7}$ to $10^{-12}$ g/L. Effective endocrine regulation requires tight temporal control of individual hormones in order to elicit the appropriate response in the desired time frame (Shan et al., 1997). As discussed in this thesis, steroid hormones, specifically androgens, and the androgen-signaling axis are the principal regulators of the development, function, and growth of the prostate gland. Due to the critical role of androgens in prostate cancer development and progression, further elucidation of the molecular mechanisms governing androgen action may allow us to more accurately predict how modulation of androgen action may affect normal prostate development and function, and/or possibly encourage the progression of a malignant phenotype.
1.3.2 Androgen-Receptor Mediated Action

Androgens are steroid hormones that carry out their function through the androgen receptor. The AR is a member of the nuclear hormone receptor superfamily, which is a large family of hormone-regulated transcription factors. The AR belongs to a family of steroid receptors along with the glucocorticoid, progesterone, and mineralocorticoid receptors. These Class I steroid receptors share similar domain structures and mechanisms of action. The mechanism of AR action is discussed in detail in Section 1.5.5. Members of this superfamily share a common tripartite structure with a highly conserved central DNA-binding domain (DBD), which separates the receptor amino (NH$_2$) terminus from the carboxy (COOH) terminus. The COOH terminus, linked to to DBD by a hinge region, harbours the ligand-binding domain (LBD) and the ligand-dependent transcriptional activation function (Beato et al., 1995). After binding to androgen, AR is able to recruit general transcription factors to its target gene promoters. In addition to its direct interaction with several factors of the general transcriptional machinery, it has become clear that associating proteins termed coactivators (such as Steroid Receptor Co-Activator-1 (SRC-1), p300/ CREB binding protein (CBP) and ARA70) and corepressors (such as calreticulin or cyclin D1) modulate the transcriptional activity of AR (McKenna et al., 1999). Both types of coregulators are necessary for efficient modulation of AR target gene transcription.

1.3.3 Androgen Biosynthesis and Metabolism

The differentiation and maturation of the male reproductive organs, maintenance of spermatogenesis and the development of male secondary sex characteristics are induced by androgens (Hsing et al., 2002). In men, androgens are formed primarily in the testes and the adrenal gland, but peripheral tissues such as the prostate and skin are also responsible for producing small amounts of androgens. Testosterone biosynthesis begins with the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme (P450cc). Pregnenolone is a precursor for multiple steroids including testosterone, estrogen, progesterone, cortisol, and aldosterone. Figure 1.3 is a simplified diagram of steroid synthesis,
Figure 1.3 An Overview of Steroid Hormone Biosynthesis.
Multiple steroid hormones, including testosterone, aldosterone, cortisol, estradiol and progesterone are derived from cholesterol. The enzymes involved are (1) the cholesterol side chain cleavage enzyme; (2) steroid C(17) hydroxylase; (3) steroid C(17), C(20) lyase; (4) steroid C(21) hydroxylase; (5) steroid 11β-hydroxylase; (6) steroid C(18) hydroxylase; (7) 18-hydroxysteroid oxidase; and (8) aromatase. (Reproduced from Voet, 1990, Chapter 23, p.657).
including testosterone. Approximately 90% of the androgens produced by the Leydig cells in the testes are secreted as testosterone, the principal androgen in circulation. The remainder is secreted by the adrenal cortex as dehydroepiandrosterone (DHEA), which can be converted to testosterone in other tissues (So et al., 2003). The production of testosterone by the testes is regulated by a negative feedback stimulated by luteinizing hormone (LH) produced by the pituitary gland, and LHRH via the gonad-hypothalamus-pituitary axis (Figure 1.4).

In men, the prostate is a major site of non-testicular production of DHT, which is derived primarily from testosterone. Sex hormone-binding globulin (SHBG) is the major binding protein for sex steroids, including testosterone, in the circulation, thereby regulating the availability of free testosterone to the hormone-responsive prostate cells. In blood, roughly 44% of testosterone is bound with high affinity to SHBG, 54% is bound with low affinity to albumin, and only 1–2% of testosterone exists in a free (unbound) state (Bruchovsky and Wilson, 1968), which equates to a serum concentration of 1nM (Partin and Rodriguez, 2002). More recently, SHBG has also been shown to function as part of a novel steroid-signaling system that is independent of the classical intracellular steroid receptors that are ligand-activated transcription factors. Kahn et al. (2002) report that SHBG mediates androgen signaling at the cell membrane by way of cAMP, which in turn triggers downstream signaling and initiates genomic effects through activation of promoters containing cAMP-responsive elements (Kahn et al., 2002; Rosner et al., 1999).

Within the prostate, testosterone is converted irreversibly to DHT by 5α-reductase type 2, as shown in Figure 1.5. In humans, two 5α-reductase isoenzymes have been identified. The type 1 enzyme is expressed mostly in skin and hair, whereas the type 2 enzyme is localized primarily in androgen target tissue, including prostate, epididymus, and seminal vesicles (Thigpen et al., 1993). DHT, the primary nuclear androgen and the most potent ligand for the androgen receptor, is found at higher concentrations than testosterone in prostate tissue. The
Figure 1.4 Androgen-Signaling Axis.

A simplified representation of the endocrine pathways leading to the synthesis, release, and action of androgens in the prostate. In the hypothalamus, LHRH is released and travels to the pituitary where it interacts with LHRH receptors (LHRH-Rs). This interaction stimulates the release of LH, adrenocorticotropic hormone (ACTH), and prolactin. LH that is released by the pituitary binds to LH receptors (LH-R) in the testes, inducing production of testosterone, which is synthesized from the precursor, cholesterol. Testosterone enters prostate cells, and is converted to DHT by 5α-reductase. DHT binds tightly to AR, dimerizes, and the complex translocates to the nucleus where it activates transcription of genes that regulate cell growth and survival. Increased testosterone levels can also decrease LHRH and LH production through negative-feedback loops, thereby maintaining serum testosterone at physiological levels. The adrenal gland can also produce androstenedione that subsequently acts on the prostate (Adapted from Partin and Rodriguez, 2002).
Figure 1.5 Conversion of Testosterone to DHT.

The 5α-reductase reaction involved in the conversion of testosterone to DHT is irreversible.

Both isoenzymes 1 and 2 can perform this conversion (Adapted from Soronen et al., 2004).
concentration of DHT in blood serum is only one tenth that of testosterone, the concentration of DHT in prostatic tissue is several times higher than that of testosterone, suggesting that DHT levels in tissue are important in prostate development and tumourigenesis (Deslypere et al., 1992). About 25% of the DHT in the circulation is secreted by the testes, while most (65–75%) arises from conversion of testosterone in peripheral tissue, in a reaction catalyzed by the enzyme 5α-reductase from circulating inactive androgens, such as androstenedione, DHEA, and DHEA sulfate. DHT is much more potent than testosterone in promoting growth of the prostate; however, testosterone is better in regulating its differentiation (So et al., 2003).

1.3.4 Mechanisms of Androgen Action and Androgen-Independent Prostate Cancer

Androgens elicit their effects via AR signaling thereby modulating prostate differentiation, development, growth and function as shown in Figure 1.6. Consequently, androgens have the ability to regulate the expression of hundreds of target genes (Nelson, 2002). The best-characterized androgen-responsive genes are PSA, kallikrein-2, kallikrein-3, prostatic acid phosphatase, sex-limited protein, and probasin. Throughout the development of the normal prostate and of prostate cancer, cell survival depends primarily on the androgen receptor. This steroid receptor is bound to heat-shock proteins in the cytoplasm of prostate cells. The active androgen dihydrotestosterone, formed from the testicular androgen testosterone, binds to the AR, thereby dissociating it from heat-shock proteins and allowing it to be translocated into the nucleus. It is known that although the prostate AR can bind both testosterone and DHT, the affinity of the AR for testosterone is only 33% of that for DHT (Rennie and Bruchovsky, 1972). In the nucleus, the androgen receptor dimerizes, binds to androgen-response elements in the DNA, ultimately activating the transcription of genes involved in cellular growth, survival and proliferation. Since androgens are essential to the survival of prostate cells, a major question is how prostate cells survive after androgen ablation therapy. Recent research suggests that the androgen receptor plays a critical role in the development of androgen-independent prostate
Androgen action within the prostate is defined by both the concentration of DHT and several other factors, including the level of androgen receptor, androgen receptor coactivators, and growth factors. In the blood, testosterone is bound to albumin and sex hormone binding globulin (SHBG). Testosterone dissociates from SHBG as free testosterone and passively diffuses across the cell membrane into the prostate cell. The enzyme 5α-reductase converts testosterone to the more potent physiological ligand DHT. Heat-shock proteins (HSP) dissociate from the androgen receptor (AR) and allow binding of DHT and subsequent receptor phosphorylation. The AR then dimerizes, and translocates into the nucleus where it binds to androgen-response elements in the promoter regions of target genes (Brinkman et al., 1999). The AR complex binds specific co-activators (such as SRC-1, p300/CBP and ARA70) and corepressors (such as calreticulin or cyclin D1) that facilitate or prevent, respectively, the interaction of AR with the general transcription apparatus (GTA). Biological responses, including growth, survival and the production of PSA, are a result of the activation (or repression) of target genes. Androgens are also able to influence potential transcription-independent actions (Adapted from Feldman and Feldman, 2001).
cancer (Hoffman and Sommer, 2005). Chen et al. (2004) showed that the androgen receptor gene is the only gene that is consistently upregulated during tumour progression in different experimental models of androgen-independent disease (Chen et al., 2004).

The mechanisms of the development of androgen-independent prostate cancer are commonly divided into two distinct pathways in the literature – those involving the androgen receptor and those that bypass the receptor. These pathways are not mutually exclusive and frequently co-exist (Debes and Tindall, 2004). The pathways involving androgen-receptor mediated survival of prostate cancer cells include amplification or mutations of the receptor, deregulation of growth factors or cytokines, and alteration of coactivators and/or co-repressors (Feldman and Feldman, 2001). Thus, a receptor that is normally activated specifically by dihydrotestosterone can, if mutated, respond to other steroids as well as to antiandrogens. In addition, there can be an alteration of the function or expression of androgen-receptor coactivators and/or co-repressors that facilitate a “promiscuous” activation of the receptor. Experimental data have shown that in the absence of androgen, non-androgenic hormones, such as estradiol, vitamin D, and insulin-like growth factors in combination with AR can trigger androgenic action (Culig et al., 1996; Gnanapragasam et al., 2000). Androgen-independent prostate cancer cells may also use survival pathways that aim to bypass the androgen receptor. These include neuroendocrine differentiation of prostate cancer cells and deregulation of apoptotic genes. Neuroendocrine cells are more prevalent in androgen-independent prostate cancer and they are very resistant to most chemotherapeutic agents, as well as endocrine and radiation treatment (Debes and Tindall, 2004). In addition, deregulation of apoptotic genes can occur through loss of tumour-suppressor gene PTEN (phosphatase and tensin homologue), which in turn increases Akt activity and blocks apoptosis (Debes and Tindall, 2004). Akt activation frees bcl-2, allowing it to increase cell survival. Overexpression of bcl-2 has been shown to be involved in progression to androgen-independent prostate cancer progression (Gleave et al., 2003). Indeed, many other oncogenes and tumour-suppressor genes may also
be involved in allowing malignant cells to develop alternative signaling pathways to subvert therapeutic attempts to control cell growth by androgen ablation.

**1.3.5 Disruption of Endocrine Action**

Over the past four decades, there has been steadily growing awareness and concern from the scientific world, the media, and other interested parties that a wide variety of hormonally active environmental contaminants may exert adverse physiologic effects on the reproductive fitness and health of many species by disrupting normal endocrine function. An endocrine disruptor can be described as an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Fisher, 2004).

While chemicals may receive routine toxicity screening during development prior to marketing, endocrine activity is not specifically assessed. Concurrently, there has been a steady increase in deleterious health effects thought to be associated with environmental endocrine disruption, including disordered fetal development, impaired reproductive capability and behaviour, and excessive cell proliferation-promoting carcinogenesis (Safe, 2004). While some of the increase can be attributed to improved detection, it is speculated that increases in endocrine-sensitive cancers, birth defects, endometriosis, and immune disorders, may be attributed in part to the effects of environmental contaminants on endocrine systems (Brucker-Davis et al., 2003; Colborn et al., 1993; Foster, 2003; Guillette et al., 1995; Safe, 2004; Skakkebaek, 2002; Weidner et al., 1998). These contaminants include many complex molecules, such as pesticides and aromatic hydrocarbons, as well as relatively simple molecules such as nonylphenol, which are ubiquitous and persistent in the environment. Sex hormone-regulated tissues, such as the prostate, are particularly vulnerable and sensitive to endocrine disruption (Gill, 1988; Sato et al., 1997).

It is apparent from research with synthetic therapeutic steroid agonists and antagonists
that the ligand-binding pocket of steroid receptors has the ability to bind to molecules that are structurally distinct from their cognate physiological ligands, albeit with different binding affinities and activities (Blair et al., 2000; Fisher, 2004; Waller et al., 1996). While a specific endogenous steroid hormone elicits the appropriate physiological response upon binding to its receptor, exogenous ligands may bind to the receptor resulting in a high level (agonist) or a low level (weak agonist) of gene expression, or may block the expression of a gene (antagonist). For example, agonistic binding of anti-androgens, such as Casodex or flutamide to the AR, is thought to induce a conformational change in the receptor, differing from that of agonist binding, thereby altering its ability to activate transcription (Martin et al., 1988). Hormone antagonists may bind to the receptor and promote DNA binding but fail to initiate transcription, or prevent DNA binding and transcriptional activation (Truss et al., 1994). Sensitivity to hormone levels change within the individual over the course of their lifetime. During periods of low levels of endogenous hormones, such as fetal development and early childhood, an endocrine disrupter that is normally a weak agonist, may act as a strong agonist. Conversely, the same compound may act antagonistically during reproductive years when high levels of endogenous hormones are present in a tightly regulated fashion necessary for appropriate physiological function (Bigsby et al., 1999). Whereas chemical exposure may be transient, some of the effects are irreversible (Gray and Kelce, 1996). The mechanisms of action of endocrine disrupters within an androgen target cell are presented in Figure 1.7.

It is evident that the endocrine system is an extremely complex one in which many hormones interact in order to make all facets of reproduction possible. It is precisely because of the highly complex nature of hormonal systems that there are a large number of points at which disruption can occur. Moreover, endocrine disrupters can attack various sites simultaneously. Although natural steroid hormones function by binding to specific receptor sites, synthetic environmental hormonally active compounds can interfere with the hormonal system in a number of ways (Bigsby et al., 1999; Colborn et al., 1993; Fisher, 2004; Gray et al., 1999). They
Figure 1.7 The Potential Mechanisms of Endocrine Disruptor Action in an Androgen-Responsive Target Cell can occur by affecting: (1) competition for the ligand-binding domain; (2) conformational change of AR; (3) nuclear translocation; (4) DNA binding and transcriptional activation (Adapted from Sultan et al., 2001).
may competitively bind to specific receptor sites, and evoke a response that mimics endogenous hormones. Inappropriately bound ligands may increase the metabolism of the hormone receptor, which orphans natural hormones (Breinholt et al., 2000; Pink and Jordan, 1996). In addition, the number of receptor sites may decrease when a hormone is present in excess. Alternatively, endocrine disruptors may alter endogenous hormone synthesis or metabolism upsetting the normal hormonal homeostasis within an organism (Badawi et al., 2000; Fisher, 2004; Kester et al., 2000). Two key examples of this are the ability of certain phthalate esters to disrupt testosterone synthesis, and the ability of certain endocrine disrupting compounds to inhibit the bio-inactivation of estradiol by inhibiting the sulfotransferase enzymes (Fisher, 2004). Although steroid hormones evoke their final actions by binding to nuclear receptors, regulation of steroid hormone transport to target tissues is controlled in part by steroid binding proteins (SBPs) that bind to steroid hormones with low but specific affinity, then transport hormones to the appropriate tissues. Improper binding of SBPs to endocrine disrupters may result in altered levels of exogenous compounds within cells. Alternatively, exogenous ligands may bind to SBPs to displace native hormones, and effectively increase the concentrations of endogenous hormones (Danzo 1997; Dechaud et al., 1999). Finally, tissue localized membrane expression of the multixenobiotic transport protein Pgp suggests its role in both hormone secretion and xenobiotic clearance (Bain and LeBlanc, 1996). Hormonal interference can especially be detrimental during development in utero, pre-puberty, and at puberty, where acute exposure to endocrine disruptors may have a permanent consequence on tissue differentiation as was demonstrated by transplacental exposure to DES on fetal development (Bisby et al., 1999).

Interference of steroid receptor action may also occur through the indirect mechanism of activating other transcriptional factors that compete for the same limiting co-activator proteins (Kamei et al., 1996; Kumar and Perdew, 1999; Sheppard et al., 1998). For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can transcriptionally activate the aryl hydrocarbon receptor
(AhR) and activation of AhR-driven transcription in LNCaP prostate cancer cells can competitively inhibit AR-driven transcription (Jana et al., 1999). It has been postulated that this occurs because both receptor systems compete for common co-activators, such as p300/CBP, ARA70, or SRC-1, and thus stimulation of one system may repress the other (Kamei et al., 1996).

The classical view that steroids act only through the binding of the steroid to a high affinity steroid receptor has been challenged. There is increasing literature regarding rapid, non-genomic actions of steroid hormones (Simoncini and Genazzani, 2003). These rapid effects do not rely on gene transcription or protein synthesis, but rather support steroid-induced modulation of cytoplasmic and cell membrane bound regulatory proteins, and intracellular signaling cascades which have been shown to involve mitogen activated protein kinases, phosphotidylinositol-3-OH kinase or tyrosine kinases (Fisher, 2004). Therefore, the action of steroid hormones upon target cells and organs is regulated by a complex interplay of genomic and non-genomic signaling, which is required to maintain both cellular and bodily homeostasis (Fisher, 2004).

1.3.6 Pesticides Possess Endocrine Disrupting Activity

A wide variety of synthetic chemicals are found in the environment as a consequence of modern manufacturing and agricultural productivity efforts. The general population is exposed to these chemicals primarily through dietary channels, and because of their stability and hydrophobicity, these chemicals accumulate in lipids and adipose tissue and their concentration increases as they progress up the food chain. Exposure to endocrine disrupting pesticides and organochlorine industrial chemicals and their byproducts during critical stages of life, including developmental periods, can produce permanent alterations of hormonal systems. At such times, even a single, relatively low dose of a hormone-disrupting substance, even below normal toxicity levels, can result in irreparable damage. Further, an important theme that emerges from many studies is the particular susceptibility of the developing organism to exposures of
hormonally active substances at levels that have minor, transient, or no impact in adults. Low-level developmental exposures to substances that modulate endocrine activity can have lifelong impacts if the exposure occurs during a period of unique vulnerability. Several cases of clear-cut, cause-and-effect relationships have been documented between environmental exposures and adverse reproductive outcomes in fish, birds, and mammals (Ankley and Giesy, 1998; Colborn and Clement, 1992). In addition, there have been numerous cases where a significant correlation has been established between environmental chemical exposure and impaired reproduction, including abnormalities found in reproductive organs or sexual behaviour of birds, marine mammals, panthers, alligators, fish and snails (Begley and Glick, 1994; Raloff, 1994). Human reproduction has been affected by chemicals of this nature as well, with reported effects ranging from declining sperm counts (Carlsen et al., 1992) to an increased female / male sex ratio (Davis et al., 1998). Finally, increasingly frequent human cancers over the past few decades include those of the breast, ovaries, testes and prostate, all tissues that are sensitive to steroid hormones (Reis et al., 1994). In the case of prostate cancer, it has been shown that occupational PCB exposure increases the risk of prostate cancer (Robinson et al., 1999).

Steroid receptors such as androgen, estrogen, and progesterone receptors are transcription factors that regulate gene expression via ligand dependent DNA-binding (Quigley et al., 1995) and cross-talk with other transcription factors (Peterziel et al., 1999; Picard, 2003). Unfortunately, certain environmental chemicals are able to interact with steroid receptors. An interplay between the multiple factors involved in gene transcription and environmental contaminants with the potential to disrupt normal endocrine function could induce abnormal gene activation and expression (Kelce et al., 1998; Wolff and Toniolo, 1995). Androgen actions mediated by the AR play an essential role in the development and differentiation of the male embryo and spermatogenesis initiation and maintenance (Bentvelsen et al., 1994). Activation of the AR by various polyaromatic hydrocarbons has been shown to have detrimental effects on the development and function of the male reproductive system (Gray et al., 2001). In addition,
many studies have reported the interference of environmental contaminants, such as herbicides, pesticides, and fungicides, with AR function (Gray et al., 1996; Gray et al., 1999; Kelce et al., 1995; Kojima et al., 2004; Lemaire et al., 2004; Monosson et al., 1999; Ostby et al., 1999; Sultan et al., 2001; Tamura et al., 2003).

### 1.3.7 Endocrine Disruption and Mechanisms of Hormone Transport

Organisms, including humans, have evolved defense mechanisms to protect themselves from toxic xenobiotics. The multidrug transporter Pgp, encoded by the multidrug resistance gene, is a transmembrane efflux pump shown to regulate the cellular efflux of a wide variety of chemicals, including both natural substances such as steroids, and xenobiotics, such as environmental contaminants and chemotherapeutic agents (Bain et al., 1997; Bain and LeBlanc, 1996; Ernest and Bello-Reuss, 1998; Kim and Benet, 2004; Lanning et al., 1996; Orlowski and Garrigos, 1999). Increased Pgp expression has been widely reported in normal and malignant tissues upon xenobiotic exposure. Pgp is predominantly located on apical membranes of epithelia, on the luminal surface of the small intestine, colon, capillary endothelial cells of the brain and on kidney proximal tubules, indicating its role as a natural detoxification system (van de Vrie et al., 1998). A limited number of pesticides have been directly investigated for their effects on Pgp expression and activity (Abu-Qare et al., 2003). Studies with MDR1a knockout mice further support this protective function, showing that an absence of Pgp expression in brain capillary endothelium cells causes increased brain intoxication to the pesticide ivermectin (Schinkel, 1999). Furthermore, Pgp expression in the testis and placenta can protect these ‘sanctuaries’ from cytotoxins (Cordon-Cardo et al., 1990). Other environmentally persistent endocrine disruptors, such as PCBs, dioxin, and other pesticides have yet to be evaluated for their effects on Pgp. Although Pgp is often thought of as a cellular detoxification pump, many studies investigating the normal physiological function of Pgp suggest steroid hormones could be natural Pgp substrates (Barnes et al., 1996; Kim and Benet, 2004; Naito et al., 1989; Ueda et al., 1992; van Kalken et al., 1993). Taken together, these results indicate that xenobiotics may...
affect the efflux of natural steroids through a Pgp mediated mechanism by possibly influencing both Pgp expression and activity. In male reproductive organs, such as the prostate, disruption of steroid hormone signaling and hormonal levels could have detrimental effects on tissue differentiation, development, and homeostasis, leading to fertility problems and endocrine-related cancer.

1.4 P-glycoprotein and Multi-Drug Resistance

It is well established that Pgp plays an important role in multidrug resistance (MDR). Intrinsic or acquired resistance of cancer cells to a wide variety of structurally and functionally unrelated drugs characterizes the phenomenon MDR (Schinkel and Jonker, 2003). Most importantly, MDR is a major problem in the chemotherapeutic treatment of cancer (Stavrovskaya, 2000). The efficacy of anticancer drugs is diminished because Pgp actively effluxes them out of the cell, thereby preventing their interaction with intracellular targets (Kartner et al., 1983). The characterization of MDR and the reversal of MDR are two major areas of cancer research with the objective of improving patient response to chemotherapeutic treatment. Juliano and Ling (1976) were the first to detect Pgp and initially thought it played a role in modulating cellular permeability. Since then, Pgp has been implicated in MDR by acting as an energy-dependent drug efflux pump, and consequently has been extensively molecularly and clinically characterized (Ambudkar et al., 1999; Leith et al., 1999; Ng et al., 1998; Taylor et al., 2001; Trock et al., 1997).

1.4.1 Structure

P-glycoprotein belongs to the adenosine triphosphate (ATP) binding cassette (ABC) superfamily of transporter proteins, subfamily B (Germann and Chambers, 1998). ABC transporters are ubiquitous with over 300 family members identified in all known organisms from bacteria to mammals. Forty-eight different ABC transporter proteins (grouped into seven subfamilies ranging from A-G) have been defined in the human genome based on sequence homology. ABC transporter proteins are known to perform transport functions across both
intracellular and/or plasma membranes, which depends on their intracellular location. Some examples of the transport functions include uptake of nutrients, the transport of ions and peptides, the extrusion of noxious compounds, the secretion of toxins, or cell signaling. The most striking property of ABC transporter proteins is they can transport an incredibly diverse range of compounds, which do not share obvious structural characteristics. Interestingly, many of these compounds are natural in origin, derived from plants, bacteria, fungi, and sponges, or minor variants of natural products, including sugars, amino acids, cholesterol, steroids, phospholipids, peptides, proteins, toxins, antibiotics, and xenobiotics (Higgins, 1992). Other members of the ABC transporter superfamily include multidrug resistance related proteins (MRP1-MRP7), MXR/BCRP (mitoxantrone resistance/breast cancer resistance protein) and the cystic fibrosis transmembrane regulator (Dean and Allikmets, 2001). The ability to confer MDR is common to many members of the ABC superfamily of transporter proteins.

As shown in Figure 1.8, Pgp is a 170 kDa membrane protein of about 1280 amino acids and 12-segments which localize predominantly to the plasma membrane of the cell and are N-glycosylated. As an ABC transporter, Pgp is an integral membrane protein consisting of two homologous halves each containing six putative transmembrane α-helical domains and a large intracytoplasmic loop encoding an energy-coupling ATP-binding site. This topology; however, remains controversial and has been challenged by alternative topologies (Jones and George, 1998; Skach et al., 1993).

1.4.2 P-glycoprotein Gene Family

There is a strong body of research in the MDR family of genes that encode for drug resistance. Extensive studies have isolated and characterized the genes responsible for Pgp expression in normal tissues and overexpression in MDR cancer cell lines. Three classes of mammalian Pgps are shown in Table 1.1. Only two classes, I and III, can confer the MDR phenotype. Of the two human genes, primarily MDR1 confers drug resistance (Ueda et al., 1987), whereas out of the three rodent genes products, two have the MDR phenotype. The
Figure 1.8 Putative Two-Dimensional Topology of Human MDR1 Pgp Based on Predicted Structure and Amino Acid Sequence Homology.

Pgp consists of two transmembrane domains, each containing 6 transmembrane segments, and the two nucleotide-binding domains (NBDs) are ATP-binding sites shown as grey circles. N-linked glycosylation sites are represented by branches at the first extracellular loop (Adapted from Germann, 1993).
<table>
<thead>
<tr>
<th>Species</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>MDR1/ABCB1</td>
<td></td>
<td>MDR3/ABCB4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse</td>
<td>mdr1a/Abcb1a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>mdr1b/Abcb1b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>mdr2/Abcb4</td>
</tr>
<tr>
<td>Rat</td>
<td>mdr1a/Abcb1a</td>
<td>mdr1b/Abcb1b</td>
<td>mdr2/Abcb4</td>
</tr>
</tbody>
</table>

Table 1.1 Mammalian Multidrug Resistance Genes - Nomenclature and Classification.

Two systems of nomenclature are commonly used in the literature. Hsu et al. (1989) introduced the first system of nomenclature. A new system of nomenclature for ATP-binding cassette (ABC) transporter genes that adopts the "ABC" prefix is now the official nomenclature. Class I and II genes convey the MDR phenotype; class III genes encode a Pgp functioning as a phosphatidylcholine translocase. Rat mdr genes are designated pgp1, pgp2 and pgp3 in some studies. Notes: (a) MDR3 is also known as MDR2; (b) mdr1a is also known as mdr3; (c) mdr1b is also known as mdr1.
murine mdr1b (mdr1, pgp2) cDNA was cloned and confers MDR when transfected into drug sensitive cell lines (Gros et al., 1986), whereas the second MDR conveying gene, mdr1a (mdr3, pgp1), was subsequently cloned based on its high homology (Devault and Gros, 1990). Both genes have overlapping but distinct transport properties (Devault and Gros, 1990). Silverman et al. (1991) published the cloned mdr1b gene of rats, whereas the rat mdr1a gene was more recently cloned (Hooiveld et al., 2001). The mammalian Pgp multigene families are clustered in tandem on a single chromosome, human chromosome 7, mouse chromosome 5, and rat chromosome 4.

1.4.3 Biochemistry

Pgp mediated transport is an active process dependent on ATP hydrolysis. The coordinate functioning of the molecule requires the interaction of the two halves of Pgp, specifically the interaction of the two ATP binding sites (Ambudkar et al., 1999; Sharom, 1997). During the transport of one molecule of substrate, hydrolysis of two molecules of ATP occurs such that one is involved directly in a conformational change in the transmembrane domains that results in translocation of the drugs out of the cell, while the second seems to be necessary to restore the transporter to its original high affinity state for substrates (Sauna et al., 2001; Sauna and Ambudkar, 2000). Substrate transport is dependent on the interaction between ATP binding sites and the drug-binding pocket. Substrates interact with different overlapping regions of a common drug-binding pocket that is large enough to accommodate more than one compound (Loo et al., 2003a; Sharom, 1997). It has been hypothesized that drug binding may occur through a substrate-induced fit mechanism; the conformation of transmembrane segments is changed upon Pgp binding to particular substrate (Loo et al., 2003b).

A large diversity of compounds, which do not share obvious structural characteristics, can be actively effluxed by Pgp across the cell membrane generating a drug concentration gradient. Interestingly, many Pgp substrates are cytotoxic compounds of natural origin or minor variants of natural compounds that are used extensively as cancer chemotherapeutics, such as Vinca
alkaloids, taxanes, and anthracyclines. As a consequence of the promiscuous nature of Pgp, known substrates also include a diverse variety of medical and synthetic compounds, for example, antibiotics (rifampicin), antimicrobial agents (colchicines), antiepileptics (phenytoin), hormones (dexamethasone), calcium channel blockers (verapamil), HIV protease inhibitors (indinavir), pesticides (ivermectin), antidepressants (amitriptyline), immunosuppressants (cyclosporine A), digitoxin, and DNA intercalators (ethidium bromide) (Chen et al., 1986; Endicott and Ling, 1989; Schinkel, 1997; Stouch and Gudmundsson, 2002). Transported compounds have diverse chemical structures, and much research has tried to define common properties of a typical substrate (Ueda et al., 1997). Physicochemical properties are currently the main determinants of whether a particular compound is transported or not, with hydrophobicity, three-dimensional structure and surface charge distribution all being possible factors which influence interaction with Pgp. The only common structural denominator identified so far is that all transported Pgp substrates are hydrophobic and amphipathic (containing spatially separated hydrophobic and hydrophilic moieties) in nature, possibly related to the mechanism of drug translocation by Pgp (Ueda et al., 1997). One theory suggests that the amphipathic nature of substrates may allow them to insert in one hemileaflet of the lipid bilayer (Higgins, 1992). Transported compounds can range in molecular mass from 250-2000 Da (Ford and Hait, 1990; Germann, 1996; Sharom et al., 1999). How Pgp manages biochemically to recognize and transport such a wide diversity of chemical structures remains an area of intense research, highly debated but as yet, unresolved.

1.4.4 Transport Models

The wide variety of chemical structures transported by Pgp has led to the development of many different models proposing the mechanism used by Pgp to transport substrates. All models agree that the translocation of substrates from the cell by Pgp is ATP-dependent (Gottesman and Pastan, 1988; Sharom, 1997; Stein, 1997), but the precise transport mechanism is still a matter of strong debate. The more popular models propose a direct-pump
mechanism (Gottesman and Pastan, 1993; Higgins and Gottesman, 1992), although alternative indirect-pump models have also been developed (Roepe, 1995; Zhu, 1999).

The majority of experimental data as depicted in Figure 1.9 favours direct transport models (Ambudkar et al., 1999; Gottesman et al., 1996; Gottesman and Pastan, 1993; Sauna et al., 2001; Sharom, 1997). These hypotheses propose that pharmacological agents passively diffuse down a concentration gradient through the cell membrane, because they are hydrophobic. Pgp subsequently extrudes drugs through the lipid bilayer directly from the cytoplasm, or from the inner leaflet of the lipid bilayer even before they enter the cytoplasm (Eytan, 2005; Eytan and Kuchel, 1999; Gottesman and Pastan, 1993; Shapiro and Ling, 1997; Sharom, 1997). Besides the binding affinity for Pgp, the lipid solubility of the substrate and the rate of partitioning within the bilayer also determine whether a substrate is efficiently transported (Begley, 2004; Seelig and Landwojtowicz, 2000). The possibility of Pgp extracting substrates from the cytoplasmic, extracellular, or both leaflets of the membrane remains to be elucidated.

According to the ‘hydrophobic vacuum cleaner’ hypothesis proposed by Raviv et al. (1990), Pgp extracts drugs in a nonselective fashion from both leaflets of the membrane directly to the extracellular environment (Gottesman and Pastan, 1993). The ‘flippase model’ alternatively suggests that Pgp carries its substrate from the inner leaflet to the outer leaflet (Higgins and Gottesman, 1992; Shapiro et al., 1997; Shapiro and Ling, 1997; Sharom, 1997) whereupon the substrate is effluxed to the extracellular environment. Finally, as there are many different substrates, the possibility cannot be excluded that there is more than one mechanism with which Pgp transports drugs, depending on the particular substrate (Germann and Chambers, 1998).

1.4.5 Pgp Modulators

Pgp chemosensitizers or modulators can reverse Pgp-mediated MDR resulting in decreased drug efflux and increased cellular drug accumulation (Van Zuylen et al., 2000). The ability to reverse MDR in cancer patients is of intense interest; however, has only
Figure 1.9 Models of Substrate Efflux by P-glycoprotein.

Substrates enter the membrane before they interact with Pgp. (Left) Pgp might recognize substrates in the cytosol and expel them directly to the extracellular medium in an ATP-dependent manner ('pump efflux or vacuum cleaner model'). (Right) Alternatively, the substrate and lipid membrane interact, and the substrate intercalated into the inner leaflet is flipped into the outer leaflet by Pgp and subsequently released into the extracellular medium ('flippase efflux model'). Membrane release from the inner leaflet to the cytosol should be a slow process for effective functioning of Pgp (Adapted from Johnstone et al., 2000a).
had limited success due to dose-limiting toxicities, poor Pgp selectivity, altered pharmacokinetics and biodistribution of co-administered anticancer drugs, and adverse side effects. According to Litman et al. (2001), there are likely as many Pgp modulators as there are "true" Pgp substrates, in particular because of the difficulty in discriminating between the two. The complex interaction of Pgp with its various substrates is such that, for instance, substrates can modulate Pgp transport of other substrates, while at the same time they are themselves transported. Inhibitors with a strong affinity for Pgp usually seem to be substrates, and may be transported at a low rate. They may have such a high affinity for Pgp that their off-rate is too low to detect. Alternatively, they may enter the membrane faster than Pgp can pump them, occupying binding sites (Barecki-Roach et al., 2003).

1.4.6 Pgp Distribution in Non-malignant Tissue

Pgp is expressed in various non-malignant human and rodent tissues, besides its well-characterized expression in MDR tumour cells (Stavrovskaya, 2000). Northern blot (Fojo et al., 1987), RT-PCR, and immunohistochemical studies using different antibodies (Cordon-Cardo et al., 1990; Bradley et al., 1990; Sugawara et al., 1988; Thiebaut et al., 1987; Thiebaut et al., 1989) have shown that Pgp is differentially expressed among tissues of various species (Stavrovskaya, 2000). High expression has been discovered in the intestine, adrenal gland, pregnant uterus and placenta where its normal physiological functions include detoxification and transport of lipophilic molecules. Significant levels of Pgp have also been shown in the brain, spinal cord, liver, kidney, heart, testes, lung and spleen (Endicott and Ling, 1989).

Very high levels of Pgp have interestingly been located in the adrenal gland of humans, mice and hamster (Sugawara et al., 1988; Thiebaut et al., 1987). Observations in hamsters indicate that high level of adrenal expression is limited to males, suggesting that Pgp may be involved in transport of sex-specific adrenal hormones (Bradley et al., 1990). With the exception of the homogenous distribution at adrenal cortical cell membranes, Pgp is mainly expressed at the luminal membrane of epithelial or endothelial cells forming physiological barriers like at the
secretory surface of intestinal mucosa and renal proximal tubular cells (Thiebaut et al., 1987), and is highly expressed at blood-tissue barriers of the brain, testis, and placenta (Fromm, 2004; Thiebaut et al., 1989). This localization suggests that Pgp is primarily involved in the extrusion of substrates from the epithelial cell layer into the adjacent luminal space.

Tumours that arise from tissues that normally express Pgp may be intrinsically resistant to chemotherapy, whereas tumours that are initially responsive may develop MDR during chemotherapy by overexpression of Pgp (Stavrovskaya, 2000). Gene transfer experiments involving MDR cDNA under control of different eukaryotic promoters have shown that increased expression of Pgp confers MDR to cultured cells sensitive to chemotherapeutic agents (Ueda et al., 1987). In cancer, Pgp overexpression may be the result of an adaptive reaction to chemotherapy that occurs at the transcriptional level through the MDR-1 gene promoter (Kohno et al., 1989).

1.4.7 P-glycoprotein Expression in the Prostate

Pgp expression has been assessed in the normal urothelial tract and prostate. Pgp is not expressed in the normal urothelium, but there are reported low levels of expression within the prostate (Cordon-Cardo et al., 1990; Giménez-Bonafé et al., 2004, Thiebaut et al., 1987). Studies by Kawai et al. (2000) confirmed that not only normal prostate epithelial cells, but also most of the prostate cancer cells expressed at least the MDR1 Pgp isoform (Kawai et al., 2000).

In contrast to many other malignancies, there has been very little work on Pgp expression and prostate cancer. At present there have been very limited immunohistochemical studies of Pgp expression in prostate cancer. In these studies, patients with metastatic disease expressed high levels of Pgp and patients with localized disease expressed low levels or did not express Pgp (Bhangal et al., 2000; Izbicka et al., 1998;). Using immunohistochemistry, Fojo et al. (1987) detected low levels of MDR1 mRNA in normal prostate and van der Valk et al. (1990) reported Pgp expression in prostate epithelial cells. Goldstein et al. (1989) looked at two prostate carcinomas by a RNA slot blot method and could not detect MDR1 expression in either
specimen. Siegsmund et al. (1997) looked at 35 benign and malignant prostate samples of different origin using RT-PCR and found MDR1 mRNA in 31.

High levels of Pgp expression are associated with the emergence of drug resistance in many malignancies and are thought to correlate with a reduced response to chemotherapy, although a clear functional role of Pgp in advanced prostate cancer remains to be established (Bhangal et al., 2000). Although prostate cancer is initially treated with hormonal therapy, chemotherapy is administered at relapse and leads to an improved quality of life if given in conjunction with steroids (Moore and Tannock, 1996). In breast cancer, which in many ways parallels prostate cancer, there is a considerable body of evidence that suggests treatment with hormonal agents or chemotherapy is associated with an increased likelihood of expression of MDR1. Those patients whose tumours expressed MDR1 were three times as likely to fail to respond to chemotherapy as those women with low levels of MDR1 expression (Trock et al., 1997). It is still not clear whether response to hormonal treatment or any other clinical parameter is related to Pgp expression in prostate cancer; however, increased expression of Pgp may have a role in both drug resistance and androgen-independent progression of advanced prostate cancer. Van Brussel et al. (2001) demonstrated that the emergence or up-regulation of several multidrug resistance-related proteins was also associated with a significantly higher histological grade, although in the prostate cancer samples analyzed they were unable to show Pgp expression. It remains possible that a variety of multidrug resistance-related proteins, not only Pgp, may provide prostate cancer cells with protective mechanisms, resulting in more progressive and multidrug resistant tumours.

1.4.8 Regulation of Pgp Expression

Regulation of Pgp expression is complex and remains incompletely understood, although substantial advances have been made in recent years in understanding the mechanisms governing Pgp induction. The human MDR1 promoter region contains a number of putative recognition sites for transcription factors, including activator protein-1 (AP-1), stimulating
protein-1 (SP-1), nuclear factor-Y (NF-Y), and CCAAT-enhancer binding protein-β (Labialle et al., 2002). Cancerous tumours are known to increase their Pgp expression when treated with chemotherapeutic drugs, and this increase may be due to selection of Pgp-expressing cells or due to an increase in mRNA translation (Gottesman and Pastan, 1993). Further, it is known that several stressors, such as oxidative stress, increase Pgp expression (Felix and Barrand, 2002). The MDR1 gene may also be activated after exogenous simulation from cytotoxic drugs and/or carcinogens (Kohno et al., 1989; Mickley et al., 1989). This activation appears to be mediated through nuclear transcription factors, including YB-1, where studies have shown that it has a role in transcriptional activation of the MDR1 gene in the presence of environmental or genotoxic stress (Asakuno et al., 1994; Ohga et al., 1998). It seems plausible that upstream regulators of Pgp expression such as YB-1 may play a large role in adaptational responses to stress, and this thesis offers further insight into this relationship.

Other results also indicate that steroids may be able to alter Pgp expression (Demeule et al., 1999; Kim and Benet, 2004; Piekarz et al., 1993; Zhao et al., 1993). The MDR1 gene promoter is not known to have any androgen-responsive elements, although regulation of Pgp expression through protein-protein interactions of androgen receptors and other transcription factors might be possible. Another mechanism of increased MDR1 expression is the frequent amplification of the MDR1 gene (Riordan et al., 1985). This gene amplification can be regulated by the methylation of the promoter. DNA methylation has been shown to be a plausible regulator of gene expression since there is an inverse correlation between methylation and transcription in both normal and malignant cells (Laird and Jaenisch, 1994). These findings suggest that increased expression of the MDR1 gene that leads to overexpression of Pgp and an MDR phenotype in human cancers is induced through localization of transcription factors such as YB-1 or by gene amplification through methylation of the MDR1 promoter.

Knowledge of the transcriptional regulation of the MDR1 gene is far from being complete. However, the previously mentioned studies bring new insights into the links, on the
one hand, between xenobiotics and the physiological modulation of MDR1 and, on the other, between oncogenes and the pathological expression of MDR1 in tumours.

1.4.9 The Role of Pgp in Normal Physiological Processes

It has been postulated that the specific tissue distribution and subcellular localization of Pgp, combined with its substrate transport characteristics, strongly suggest that it plays an important role in limiting cellular absorption and distribution of exogenous toxins and in increasing excretion of these xenobiotics or metabolites (Stavrovskaya, 2000). Not surprisingly, the majority of research has focused on the protective function of Pgp, categorizing the plethora of substances transported, and understanding the precise mechanisms of Pgp structure and function. In depth investigation of the normal physiological role of Pgp has been made possible by the generation of mdr1a, mdrb, or mdr1a/mdrb gene knockout mice (Schinkel, 1997; Sparreboom et al., 1997). All three types of knockout mice appear to have normal growth, development, viability, life span and fertility and do not show gross anatomical or histological abnormalities; they are completely normal as long as they are not challenged with drugs. Analysis to date has shown that Pgp is not vital to basic liver, kidney, or intestinal function, or to functions of the brain, adrenal gland, ovaries, or uterus during pregnancy. Unfortunately, no studies in knockout mice have examined Pgp function in the prostate. Severe problems with pharmacological handling appear in Pgp knockout mice when treated with drugs that are Pgp substrates. For example, mice with an inactivated mdr1a gene have no detectable Pgp in gut epithelium and brain capillaries, and consequently increased oral uptake, decreased clearance, shifts in excretion route and enhanced uptake in fetuses of various potentially harmful or therapeutic compounds have been demonstrated in these knockouts (Smit et al., 1999; Sparreboom et al., 1997).

Various other physiological functions of Pgp, in addition to its protective role, have been proposed to provide an explanation for the unusual basal ATP hydrolysis in the absence of any endogenous substrate. Pgp might be involved in transport of cytokines or translocation of
phospholipids (Chong et al., 1993), and recently it was suggested to actively translocate cholesterol to the outer cell membrane (Garrigues et al., 2002). In addition, Pgp has been implicated in the etiology of Alzheimer’s disease as it may be involved in elimination of β-amyloid from the brain (Lam et al., 2001). Closely related to later observations in Chapter 5, it has been proposed that Pgp may participate in programmed cell death (Johnstone et al., 2000b). A role in steroid transport; however, is the most frequently suggested function, which may explain its expression and possible role in the protection or homeostasis in steroid hormone-associated tissues.

1.4.10 Pgp as an Anti-apoptotic Protein

There is little doubt that Pgp plays a pivotal role as a MDR conferring efflux pump. New evidence suggests that in addition, Pgp may act as a general anti-apoptotic protein to increase the threshold for cell death (Johnstone et al., 1999; Johnstone et al., 2000b; Ruefli and Johnstone, 2003; Scotto and Johnstone, 2001). Effectively, the cells are protected at two levels, firstly by decreasing the amount of toxins that accumulate in the cell, and secondly by blocking apoptotic pathways induced by toxins and cellular stress. For example, MDR cells were found to be cross-resistant to a number of stimuli, including radiation, rather than exclusively chemotherapeutic drugs (Gottesman and Pastan, 1993). Recent studies provide evidence that Pgp can act as a general anti-apoptotic molecule to inhibit caspase-dependent cell death, and further suggests that Pgp may inhibit caspase activation (Ruefli and Johnstone, 2003; Scotto and Johnstone, 2001).

1.5 P-glycoprotein and Androgens

1.5.1 The Interaction between Pgp and Steroids

Central to the main hypothesis presented in this thesis, relatively little attention and research has been given to factors, such as the possible interaction between Pgp and steroids, which may influence the intracellular concentration of steroids. The current view suggests that steroids, by virtue of their highly lipophilic nature and small size, readily diffuse across plasma
membranes. An energy-dependent transport mechanism within the membrane, resulting in reduced accumulation of steroid molecules by mammalian cells was first proposed in 1968 (Gross et al., 1968). It was observed that the transport process only affected some of the steroids tested, such as cortisol, dexamethasone, and prednisolone. In addition, testosterone and estradiol were shown to interfere with the extrusion mechanism (Gross et al., 1970). Thus, this publication was the first report of carrier-mediated transport of a steroid molecule by mammalian cells.

Many studies have demonstrated the importance that steroids play in physiological functions; however there has been a long held dogma that steroids move freely into and out of the cell by simple diffusion. Steroid hormones affect every organ in the mammalian body with a wide variety of functions from modulation of nuclear transcription and protein synthesis to cellular signal transduction, thus triggering genomic events that are responsible for physiological responses (Wehling, 1997). In vitro studies by Johnson et al. (1984) were among the first to examine the interactions between mouse mdrl Pgp and glucocorticoids. In addition, high levels of Pgp expression in the adrenal cortex and placenta gave rise to speculation that the major physiological function of Pgp may involve steroid transport (Fojo et al., 1987; Holcberg et al., 2003; Thiebaut et al., 1987). This idea was further strengthened when it was discovered that Pgp could transport the corticosteroid hormones cortisol, corticosterone, and aldosterone, and the glucocorticoid dexamethasone (Ueda et al, 1992; Van Kalken et al, 1993; Wolf and Horwitz, 1992). On the basis of steroid structure, it was proposed that steroid interaction with Pgp depends on the hydroxyl groups present in positions 11, 16, and 17 of the glucocorticoid (Bourgeois et al., 1993). Interestingly, this was also the first suggestion of the possible transport of androgens by mouse P-glycoprotein due to the inhibitory effect of testosterone on vinblastine binding. In an extensive study of interactions of Pgp and steroid hormones using MDR human colon carcinoma cells, Barnes and colleagues were the first to suggest that a wide range of steroids, including the androgens, testosterone and DHT, were reduced to an extent that
correlated well with their relative hydrophobicity (Barnes et al., 1996). More recently, studies in mice, deficient for both mdr1a and mdr1b Pgp, report that penetration of endogenous steroid hormones corticosterone, cortisol, aldosterone and progesterone into the brain is enhanced (Uhr et al., 2002). Taken together, these studies show that interaction of steroids with Pgp comprises a spectrum – at one end, steroids like dexamethasone and cortisol are mainly Pgp substrates, and in opposition, steroids such as progesterone may mainly inhibit Pgp. The distinction between Pgp substrates and inhibitors is based on their respective Pgp binding and transport behavior. While steroid Pgp inhibitors bind avidly to Pgp, the steroid is not transported out of the cell, in comparison to the Pgp-mediated transport of other steroid substrates. This paradoxical phenomenon may be explained by the high lipophicity of Pgp inhibitors, such as progesterone, resulting in a fast partitioning into the plasma membrane leaflets (Eytan et al., 1996). The high rate of back diffusion fully counteracts the Pgp mediated outward flipping of progesterone and no drug gradient can be established (Eytan et al., 1996; Sharom, 1997). It strongly competes for Pgp mediated transport and due to its rapid insertion into the plasma membrane, progesterone overwhelms the Pgp transport machinery, which leads to the inability of Pgp to transport other substrates.

A recent article by Li et al. (2004) examining the structure-function relationships between Pgp and steroids shows that steroid hydrophobicity plays an more important role in the binding affinity to Pgp of inhibitors than of substrates. Steroids with stronger inhibitory capacities, such as pregnanediol and progesterone have intense hydrophobicity making their binding with Pgp too strong for them to leave or to be pumped out by Pgp. However, steroid compounds with weak inhibitory effects, such as aldosterone, corticosterone, and dexamethasone are less hydrophobic (Li et al., 2004).

1.5.2 The Hypothesized Role of Pgp on Androgen Transport and Prostate Cancer

Steroid efflux by drug transporters may be a more generally occurring phenomenon than
is currently appreciated (Kralli and Yamamoto, 1996; Thompson, 1995). The previously discussed in vitro studies combined with the presence of Pgp in hormone-sensitive cells has led to suggestions that Pgp has a physiological role in steroid secretion. The importance of Pgp as a steroid transporter is questioned; however, by the generation of Pgp knockout mice, and the observation that these mice show no gross physiological disturbances, including steroid transport (Schinkel et al., 1997). Pgp may be involved in steroid transport in a more subtle way by protecting the plasma membranes of steroid-producing cells from the toxic effects of high steroid concentrations under the conditions of stress induced activity (Ambudkar et al., 1999; Van Kalken et al., 1993). A protective role of Pgp would also be more consistent with its property to transport drugs out of the plasma membrane. Alternatively, Pgp may play a role in the regulation of steroid exposure of hormone responsive cells like prostate cells. A role of Pgp in the development of androgen-independent prostate cancer in "protecting" the cancer cell against steroids has therefore been postulated. Subtle changes in intraprostatic hormone levels due to ageing or during benign hyperplasia may act to signal the increased expression of Pgp in prostate cells as an adaptational stress response. Together with other genetic alterations, this may act as an early trigger encouraging the upregulation of other hormone-independent growth pathways and consequently aberrant cellular growth. Changes in the cellular androgen levels after androgen ablation may further act to affect cellular Pgp expression. The resultant increase of Pgp expression in androgen-independent prostate cancer progression and ability of Pgp to transport several androgens suggests that Pgp may play an important role in modulation of androgen access to androgen receptors and transcriptional machinery, thus affecting androgen action. This hypothesis formed the basis of research described in this thesis.

Prostate cancer cell growth depends on the ratio of proliferating cells to those dying. Androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. Prostate cancer depends on a critical level of androgenic stimulation for growth and survival. In this context, one major focus of this thesis is the observation that the physiological
androgen DHT appears to be a Pgp substrate in prostate cancer cells. This observation has further led to the hypothesis that in Pgp overexpressing cells, which are present heterogeneously in prostate cancer samples (Kawai et al., 2000), cellular DHT levels may be reduced and facilitate adaptation to androgen withdrawal prior to castration. In addition, since the multifunctional transcription factor YB-1 is directly involved in MDR1 gene activation in response to genotoxic stress, coordinated upregulation of YB-1 and Pgp may be a positive selection factor in the development of drug resistance in prostate cancer. Ultimately, characterizing the role of YB-1 and Pgp, together with elucidation of the molecular mechanisms of drug-steroid interactions in prostate cancer progression, may contribute to the development of novel prognostic markers and therapeutic approaches for the treatment of androgen-independent prostate cancer.
1.6 The Y-box Protein Family and Y-box Binding Protein-1

1.6.1 Y-box Proteins

Y-box proteins are members of the cold shock domain (CSD) protein family, thought to play a role in a wide variety of environmental stress reactions, and are evolutionarily highly conserved from bacteria to mammals (Kohno et al., 2003). Prokaryotic Y-box proteins were initially characterized as major cold-shock proteins because they allow bacteria to adapt to growth at low temperatures due to strongly enhanced synthesis by decreasing temperatures (Gualerzi et al., 2003). Y-box proteins are multifunctional regulators of gene expression in both eukaryotic and prokaryotic organisms, interact with DNA and RNA to control the transcriptional and translational expression of specific genes and are thought to play a role in cellular proliferation or transformation (Ladomery and Sommerville, 1995). First identified in 1987, Y-box proteins were isolated by binding to a DNA probe containing the Y-box sequence (5'-TTCTGATTGGTTAA-3') (Dorn et al., 1987). It has been demonstrated that Y-box proteins can exert their control over gene expression by binding a large range of nucleic acids; specifically, to double and single-stranded DNA and RNA, as well as damaged DNA and RNA (Koike et al., 1997; Matsumoto and Wolffe, 1998). Kloks et al. (2002) describes the domain structure of Y-box proteins which are generally composed on three domains: an amino-terminal alanine-proline-rich region (N-terminal), a centrally located CSD, and a carboxyl-terminal (C-terminal) region characterized by four alternating clusters of basic and acidic amino acids, each of which are about 30 amino acids in length, called a B/A (basic/acidic) repeat (Kloks et al., 2002). Figure 1.10 is a schematic representation of the domain structure of Y-box proteins. The function of the N-terminal domain is unclear; however, it seems to be critical for transcriptional regulation. Interestingly, p53 has been shown to bind to the N-terminal region (Okamoto et al., 2000). The CSD facilitates binding to nucleic acids. It forms an anti-parallel β-barrel, enfolding the nucleic acid like a chaperone protein (Kloks et al., 2002). The C-terminal domain facilitates RNA binding and is involved in protein-protein interactions (Bouvet et al., 1995).
Figure 1.10 Y-box Protein Domain Structure.

The Y-box proteins have three functional domains: the variable N-terminal domain, the cold-shock domain (CSD) and the C-terminal domain. The C-terminal domain consists of acidic (A) and basic (B) blocks of about 30 amino acid residues each. The central CSD has a sequence identity which is highly conserved within the family of Y-box proteins. The function of the N-terminal domain is not well understood (Adapted from Kloks et al., 2002).
Sakura et al. (1988) first identified two novel prokaryotic DNA-binding proteins, known as DNA-binding protein A (dbpA) and dbpB, as a result of developing a simple method for isolating DNA clones encoding DNA-binding proteins which bind with epidermal growth factor-1 receptor enhancer and c-erbB-2 promoters. Subsequently in eukaryotes, dbpB has become most commonly known as YB-1, but depending on the species, is also known as p50, murine YB-1 protein (MSY-1), Nuclease Sensitive Element Binding Protein-1 (NSEP-1), and Multi-Drug Resistance Nuclear Factor-1 (MDR-NF-1). The human YB-1 gene spans approximately 19 kilobase pairs of genomic DNA containing 8 exons, and is located on chromosome 1p34 (Makino et al., 1996; Toh et al., 1998). YB-1 mRNA is approximately 1.5kb long which encodes a 43kDa protein (324 amino acids) (Kohno et al., 2003). Human YB-1 was initially identified as a transcription factor that binds to the Y-box sequence appearing in the major histocompatibility complex (MHC) class II promoter (Didier et al., 1988). A closely related Y-box-binding gene identified as dbpC (contrin) was cloned in 1999 and is germ-cell-specific (Kudo et al., 1995; Tekur et al., 1999). Regarding the domain structure, the CSD is similar in dbpA, YB-1 and contrin in its ability to bind DNA and RNA, and the amino acid sequences are highly homologous (Kohno et al., 2003). In all vertebrates studied, the CSD of YB-1 proteins are 100% identical, and are 45% identical to the *E. coli* CspA bacterial protein (Bader et al., 2003). The C-terminal domain of YB-1 may also contribute to DNA/RNA binding and may also be a docking site for other proteins that interact with YB-1 (Sommerville and Ladomery, 1996; Wolffe, 1994). For example, *in vitro* studies measuring translational activities attribute an inhibitory activity to this domain; the mechanism by which it interferes with translation remains unclear.

1.6.2 Pleiotropic Functions of YB-1

YB-1 has a multitude of biological roles in both normal and malignant cells. Multifunctional YB-1 binds to DNA and RNA, and has been shown to regulate gene expression at both the transcriptional and translational levels (Matsumoto and Wolffe, 1998; Sommerville. 1999). YB-1 has been established to play a critical role in cellular proliferation and drug
resistance by functioning as a transcription factor, regulating the expression of genes by binding to promoters with a Y-box (5'- CTGATTG -3') motif (Swamynathan et al., 1998). As a transcription factor, YB-1 can either positively or negatively regulate gene expression depending on the cellular context. YB-1 can regulate transcriptional control in three ways: by directly binding to the Y-box and related sequences or directly binding to the Y-box in combination with other transcription factors; interacting with transcription factors, thereby functioning as a co-activator or co-repressor; and finally, by binding the single-stranded region of the promoter either enhancing or blocking the binding of transcription factors to the DNA (Kohno, et al., 2003). Examples of genes that are transcriptionally activated by YB-1 are MDR-1, matrix metalloproteinase 2 (MMP-2), epidermal growth factor, thymidine kinase, DNA topoisomerase II, DNA polymerase, protein tyrosine phosphatase 1B (PTP-1B), proliferating cell nuclear antigen (PCNA) (Gu et al., 2001; Fukada and Tonks, 2003; Ladomery and Sommerville, 1995; Mertens et al., 1997; Ohga et al., 1998; Shibao et al., 1999; Wolff, 1994). Genes that are transcriptionally repressed by YB-1 include MHC class II genes, glucose-related protein (grp78), collagen α1 and collagen α2 (Didier et al., 1988; Higashi et al., 2003; Li et al., 1997; Norman et al., 2001; Ting et al., 1994).

In addition, YB-1 also shows pleiotropic modulation of not only transcription, but is also responsible for the induction of DNA damage repair systems (Ise et al., 1999; Marenstein et al., 2001; Okamoto et al., 2000), DNA replication (Holm et al., 2002), the modification of chromatin (Hasegawa et al., 1991) and translational events. While in the cytoplasm, YB-1 participates in alternative splicing of mRNA (Chansky et al., 2001), as well as serving as a main mRNA packaging protein, being involved as a chaperone in mRNA transport, modulating mRNA turnover and regulating protein synthesis (Evdokimova et al., 2001; Skabkin et al., 2004; Skabkina et al., 2005). YB-1 binds double-stranded DNA with low-affinity; it has much higher affinities for single-stranded RNA (Izumi et al., 2001; MacDonald, 1995; Mertens et al., 1997). The association with RNA is not sequence-specific, but sites with a high G content are preferred.
(Zasedateleva et al., 2002). YB-1 is the predominant protein component of messenger ribonucleoprotein particles and has a dose-dependent effect on translation; low concentrations promote the formation of RNA duplexes, while high concentrations cause unwinding of double-stranded forms thereby inhibiting translation (Skabkin et al., 2001). It has been proposed that because of the known deregulation or aberrant expression of several distinct components of the translation apparatus in human malignancy, perhaps the deregulation translational control of YB-1 plays a role in tumour progression (Fukuda et al., 2004). Nekrasov et al. (2003) showed that YB-1 can turn off the interaction of mRNA with the mRNA cap binding protein, eukaryotic translation initiation factor 4E (eIF4E), thereby affecting the translation of the 5'-untranslated (UTR) regions which play an key role in the regulation of gene expression. If the UTRs of mRNAs encoding important proteins are disrupted, such as heat shock proteins known to protect cells against cellular stresses, including heat shock, viral infection, or exposure to oxidative free radicals, then increased YB-1 expression could indirectly result in the cells inability to positively respond to adverse extracellular stimuli or environmental stress (Nekrasov et al., 2003). To understand the integrated functions of YB-1, Figure 1.11 indicates how the pleiotropic functions of YB-1 are expressed via a diverse range of molecular interactions.

1.6.3 YB-1 and Cancer

The cascades of gene expression initiated by YB-1 have been linked to invasion in cancer (Levenson et al., 2000; Ise et al., 1999; Shibao et al., 1999). In the last few years, there has been rapid progress in understanding the biochemical, physiological and pathological roles of YB-1 in cancer research, in the hope of exploiting it as a potential novel therapeutic target and prognosticator. The immunohistochemical analyses performed in clinical studies have identified increased cellular expression of YB-1 protein in breast cancer (Bargou et al., 1997; Janz et al., 2002), ovarian cancer (Kamura et al., 1999; Yahata et al., 2002), non-small cell lung cancer (Shibahara et al., 2001), bone osteosarcoma (Oda et al., 1998), colon cancer (Shibao et al., 1999), synovial sarcomas (Oda et al., 2003), and thyroid cancer (Ito et al., 2003). Subsequently,
Figure 1.11 Multiple Functions of YB-1 in Various Cellular Processes.

YB-1 performs a diverse range of roles in both normal and malignant cells – including multiple drug resistance and cellular proliferation (Adapted from Kohno et al., 2003).
our research has also shown increased YB-1 protein expression during androgen-independent prostate cancer progression. Ohga et al. (1996) and Koike et al. (1997) were the first to suggest that cellular YB-1 protein is localized mainly in the cytoplasm but is translocated into the nucleus when cells are treated with ultra-violet (UV) radiation or DNA-damaging chemotherapeutic agents, such as cisplatin and mitomycin C. Subsequently, Ohga et al. (1998) first demonstrated the direct involvement of YB-1 in the activation of MDR-1. YB-1 levels directly alter the genotoxic stress-induced activation of the MDR-1 promoter with an increase in the mRNA expression of MDR-1 and its protein product Pgp (Ohga et al., 1998). Since YB-1 is known to play a large role in limiting the chemosensitivity of cancer cells by coordinately increasing the expression of Pgp, many studies have further examined the clinical implications of Pgp expression and YB-1 nuclear translocation. Immunohistochemical studies of clinical tissue specimens show an association between YB-1 and Pgp in breast cancer (Bargou et al., 1997; Huang et al., 2005; Janz et al., 2002; Saji et al., 2003), ovarian cancer (Huang et al., 2004; Kamura et al., 1999), synovial sarcoma (Oda et al., 2003), and osteosarcoma (Oda et al., 1998). Conversely, Shibao et al. (1999) showed no association between YB-1 and Pgp in colon cancer. Therefore it remains unclear how early in neoplastic evolution YB-1 expression predicts drug resistance and/or poor prognosis. For instance, in normal breast tissue YB-1 is not expressed, but becomes highly expressed in tumours (Bargou et al., 1997; Rubenstein et al., 2002), and nuclear YB-1 was associated with increased risk of cancer relapse, as well as decreased overall survival in women with breast cancer (Janz et al., 2002). Further, YB-1 expression was specifically associated with Pgp overexpression in synovial sarcoma, opposed to three other ABC transporters: MRP-1, MRP-2, and MRP-3 (Oda et al., 2003). In lung and synovial sarcoma, nuclear YB-1 expression significantly correlated with tumour size, degree of invasion, lymph node metastasis and poor prognosis (Oda et al., 2003; Shibahara et al., 2001). These studies suggest YB-1 is directly involved in the transcriptional regulation of Pgp, and appears to have
prognostic and predictive significance in human malignancies; however, this is incompletely understood. As described in Chapter 4, our research suggests that YB-1 may be an important factor in the diagnosis of prostate cancer progression, and may correlate closely with upregulation of Pgp, perhaps indicative of a progression to an androgen-independent and more chemoresistant phenotype.

1.6.4 The Oncogenic Protein YB-1

Major questions remain unanswered regarding the network of interactions between YB-1 and signaling pathways that modulate cell growth and survival. Recent research suggests that YB-1 may play an important role in controlling cell survival by co-ordinately regulating the expression of cell growth-associated and apoptosis-associated genes. In general, YB-1 is expressed at high levels during development and proliferation of cancer cells, and particularly interesting are studies showing that YB-1 is linked to growth-associated gene expression (Grant and Deeley, 1993; Ladomery and Sommerville, 1995). In fact, reduction of YB-1 expression using antisense RNA expression produced severe inhibition of growth (Kohno et al., 2003). YB-1 may play a key role in controlling the survival of a cell by affecting multiple cellular pathways. For example, the induction of cyclin A and B mRNA by YB-1 is reported to stimulate cancer cell proliferation (Jurchott et al., 2003). In addition, multiple E-boxes and GC-boxes on the YB-1 gene promoter have been shown to bind the proto-oncogene c-Myc, leading to the transactivation of the YB-1 gene promoter, suggesting cellular proliferation may be controlled by the level of expression of YB-1 (Kohno et al., 2003). This results in transactivation of YB-1 gene through interaction with p73, a relative of p53 (Uramoto et al., 2002). Both c-Myc and p73 activate YB-1 transcription and may regulate important biological processes via their effect on YB-1 gene expression (Uramoto et al., 2002). Also, YB-1 has also been shown to be a transcriptional repressor of fas, which encodes a protein that can increase the apoptotic susceptibility of a cell (Lasham et al., 2000). High levels of YB-1 could activate the expression of growth-associated genes at the same time as repressing the expression of the apoptosis-
associated gene fas, sending the cell down a survival and growth-directed pathway. Conversely, when levels of YB-1 are low, there would be little expression of growth-associated genes, but fas expression, and therefore apoptotic susceptibility would be increased (Lasham et al., 2000).

In addition, studies have shown the mutual interaction of YB-1 and the tumour-suppressor p53 reciprocally modulates the DNA binding function of each protein (Lasham et al., 2003). This mutual interaction also modulates the expression of human genes containing p53 or YB-1 binding sites, suggesting that modulation of expression levels of these genes can induce cell cycle arrest or apoptosis under genotoxic stress through cell cycle checkpoints, to ensure genomic stability. The tumour suppressor p53 regulates a similar cluster of genes, but unlike YB-1, it represses the expression of growth and resistance genes, including MMP-2, EGFR, and MDR1, and promotes the expression of pro-apoptotic genes, such as fas. However, as p53 regulates these genes to protect against tumour development, Lasham et al. (2003) show evidence to support the function of YB-1 as a dominant-negative regulator of p53.

Finally, YB-1 may play an important role in the excessive proliferation and reduced susceptibility to apoptosis of cancer cells because of its ability to modulate the activity of several major signal transduction pathways. For example, inactivation or loss of tumour suppressors such as p53 (Okamoto et al., 2000), inhibition of the phosphoinositide 3-kinase (PI3K) pathway (Bader et al. 2003; Bader and Vogt, 2005) and activation of anti-apoptotic molecules such as Akt and nuclear factor-κB (NF-κB) (Raj et al. 1996; Sutherland et al., 2005), all may contribute to apoptotic resistance and prostate cancer progression. In a recent publication, Sutherland et al. (2005) report the association of YB-1 and the activation of the serine/threonine kinase Akt signal transduction pathway in breast cancer cells. Their data show that phosphorylated Akt is able to bind YB-1 and induce phosphorylation on the CSD domain, suggesting this interaction is a principal mediator of the nuclear translocation of YB-1. In the nucleus, YB-1 acts as an oncogenic protein, promoting cellular growth and proliferation, as well as mediating drug resistance; therefore, they further suggest the possibility of using YB-1 a novel target for cancer
therapy. Applying this idea to prostate cancer, progression from normal prostate epithelium to prostatic intraepithelial carcinoma may be associated with elevated Akt phosphorylation (Malik et al., 2002). Activation of the Akt pathway can suppress the normal apoptotic response, undermine cell cycle control and selectively enhance the production of key growth and survival factors, and therefore may be a particularly potent signal in prostate cancer progression. In prostate cancer cells, growth factors and hormones, such as integrin-linked kinase (ILK) and steroid receptor coactivator 3 (SRC-3), DHT, estradiol and testosterone may activate the serine/threonine kinase Akt that protects cells from death by phosphorylating downstream targets (Castoria et al., 2004; Edwards et al., 2005; Kang et al., 2004; Zhou et al., 2003). Thus a small molecule-based therapeutic approach targeting YB-1 and the Akt pathway may have positive therapeutic effects. Parallel to this idea, results of this thesis show that YB-1 is upregulated in prostate cancer cells and therefore, as a regulator of cell growth-/cell death-associated genes, may play a pivotal role in the development or maintenance of a cancerous state. The results presented in Chapter 5 suggest that by downregulating YB-1 expression, decreased cellular growth and increased sensitivity to chemotherapeutics may be achieved, thus delaying progression to androgen-independence. We have taken the approach of targeted knockdown of YB-1 expression, thereby impacting multiple oncogenic factors, with the goal of identifying YB-1 as a novel molecular target for prostate cancer therapy.
1.7 Scope of Thesis

1.7.1 Hypotheses

Pharmaceuticals and other xenobiotics influence androgen action by mechanisms affecting cellular androgen transport and accumulation through modulation by Pgp, which in part is regulated by transcription factor YB-1.

Targeted YB-1 knockdown using antisense oligonucleotides suppresses tumour growth and enhances taxane chemosensitivity, thereby delaying androgen-independent prostate cancer progression.

1.7.2 Rationale and Specific Objectives

The aim of the studies described in this thesis is to examine the interaction of androgens and the efflux transporter Pgp, overly expressed in prostate cancer cells, as a possible novel mechanism by which Pgp-expressing prostate cancer cells exhibit aberrant androgen receptor signaling and androgen responsiveness. Research presented in this thesis further suggests that targeted down-regulation of YB-1 expression using antisense oligonucleotides, and the consequential effects downstream on Pgp expression and function, may modulate androgen action, cellular growth, and enhance taxane chemosensitivity, thus delaying androgen-independent prostate cancer progression. These data suggest that YB-1 is a promising target for new therapeutic approaches to attenuate multi-drug resistance and inhibit cellular proliferation, and identify YB-1 antisense oligonucleotides as a potentially new therapeutic strategy to enhance taxane chemosensitivity in AI prostate cancer. Finally, results further demonstrate proof of principle for a site-directed, injectable, controlled-release formulation of antisense oligonucleotides and paclitaxel for some localized prostate tumours.
The first objective was to determine the mechanism by which endocrine disruptors interfere with androgen receptor action and androgen transport, and perform a detailed investigation of selected endocrine disruptors for androgen and aryl hydrocarbon related activity in vitro (Chapter 2).

The second objective was to determine the physiological impact of the upregulation of Pgp in prostate cancer progression, with an emphasis on steroid transport and drug resistance. This was aimed at determining whether DHT is a substrate of Pgp, and assessing the functional and physiological consequences of modulation of cellular androgen levels (Chapter 3).

Since previous studies had identified the MDR1 gene, which encodes Pgp, as a downstream target of YB-1 transcriptional control, the third objective was to determine the role of YB-1 in prostate cancer progression. Immunohistochemistry specifically addressed the occurrence and localization of YB-1 expression in clinical samples and coordinate regulation of downstream targets such as Pgp during tumour progression. We further hypothesized that increased YB-1 and Pgp levels decrease androgen accumulation in prostate cancer cells, leading to suppression of androgen-regulated gene expression (Chapter 4). Observing the increased expression of both YB-1 and Pgp in prostate tumour cells, we hypothesized that YB-1 may be an appropriate target for suppressing cellular growth and/or enhancing the effectiveness of chemotherapeutic treatment. We targeted YB-1 expression using antisense oligonucleotides, thereby downregulating YB-1 in prostate cancer cells (Chapter 5).

Finally, to bring clinical relevance to the project, the final objective was to target the expression of YB-1 using antisense oligonucleotides in the LNCaP prostate tumour xenograft model, thereby downregulating YB-1 expression, suppressing tumour growth and PSA expression, and possibly enhancing chemosensitivity to paclitaxel, resulting in delayed progression to androgen-independence (Chapter 5). Therefore, we hypothesize that the role YB-1 plays in regulating of Pgp expression and function, modulating androgen action, and influencing cellular growth, may make it a valid therapeutic target for advanced prostate cancer.
1.8 Bibliography


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CHAPTER 2. AGRICULTURAL PESTICIDES INTERFERE WITH ANDROGEN INDUCED TRANSCRIPTIONAL ACTIVATION AND HORMONE BINDING

Dr. Jody Ralph was responsible for the completion of the AhR transactivation gene reporter assays and consulted during the data analysis. Dr. Nhu Le and Dr. Emma Guns were valuable collaborators during this work. I was responsible for the completion all of the other experimental work and data analysis. Dr. Simon Cowell assisted in compiling results and manuscript editing.

2.1 Introduction

In Canada and in the United States prostate cancer is the most common life-threatening cancer in men (Hsing et al., 2000; NCIC, 2005). These two countries have the highest incidence rates for prostate cancer in the world (Parkin et al., 1992), and the two populations with highest rates include Black Americans in the United States (Coleman et al., 1993; Parkin et al., 1992) and men in BC, Canada (Band et al., 1993). The epidemiology of prostate cancer has been the subject of several recent reviews (Bostwick, 2004; Gann, 2002; Platz and Giovannucci, 2004) despite the common occurrence of this tumour, its etiology remains largely unknown; age, family history, Black American ethnicity, hormonal and sexual factors, and a high consumption of animal fat and red meat, are the most consistent risk factors reported (Botswick, 2004; Giovannucci, 1995; Kolonel, 1996; Nomura and Kolonel, 1991; Pienta and Esper, 1993; Whittemore et al., 1995). A large number of diverse occupations have also been suggested to be associated with an increased risk for prostatic cancer including administrative, managerial, professional, health and clerical occupations, mechanics, welders, policemen and farmers, as well as workers in the metal, paint and rubber industries (Bosland, 1988; Giovannucci, 1995; Nomura and Kolonel, 1991; Zeegers et al., 2004). Numerous studies have also shown that agricultural workers have elevated rates of some cancers and other diseases (Settimi et al.,

1A version of this chapter has been submitted for publication.
2003; Wood et al., 2002). These studies show farmers with consistently elevated rates of leukemia, non-Hodgkin's lymphoma, multiple myeloma, soft tissue sarcoma, and cancers of the lip, stomach, brain, and prostate.

A consistent relationship with prostate cancer has been found for farmers (Acquavella et al., 1998; Blair et al., 1985; Blair and Hoar Zahm, 1995; Bosland, 1988; Keller-Bryne et al., 1997; Nomura and Kolonel, 1991; Pearce and Reif, 1990; Pienta and Esper, 1993; Van der Gulden and Vogelzang, 1996; Zeegers et al., 2004). A case-control study of 1516 prostate cancer patients in BC (Band et al., 1999) identified farming occupations with elevated risks, with important confounding factors being accounted for. Exposure to pesticides, insecticides, fertilizers, herbicides and other agricultural chemicals has been suggested as a potential risk factor (Aronson et al., 1996; Parent and Siemiatycki, 2001; Siemiatycki, 1991). However, the impact of agricultural chemicals on cancer incidence has not been established due to the lack of information on specific exposures in epidemiologic data collected to date. Analyses have mainly been based on occupation or industry titles which potentially may entail a host of different chemical exposures. This type of analysis prevents identification of specific chemical risk factors and may produce biased risk estimates (Blair and Hoar Zahm, 1995). Thus, there is a need for better assessment of chemical exposure in epidemiologic studies.

To characterize BC agricultural exposures more fully, and to facilitate epidemiological impact assessments, a job-exposure matrix (JEM) was created containing exposure assessments from 1950 to 1998 (Wood et al., 2002). The 'exposure' axis of the completed matrix included 290 chemical, biological and physical agents. Approximately 180 of these agents were pesticides including 68 insecticidal, 39 fungicidal and 53 herbicidal active ingredients. The remaining 110 non-pesticide exposure agents include all other identified agricultural products including cleaning and maintenance chemicals as well as physical and biological agents. The job title axis, more aptly named 'type of work' includes a combination of 8 regions, 45 crop or animal commodities, 6 job titles and 36 different tasks.
Following a detailed epidemiological analysis correlating agricultural occupational exposure to increased risk of prostate cancer (Le et al., unpublished data), a list of 27 compounds was compiled and these compounds were selected for further biological analysis based on their corresponding odds ratios. This list included pesticides showing significantly increased risk, a few with elevated but not significant risk, as well as two with no elevated risk. The pesticides in each risk group are identified in Table 2.1. In addition, due partly to solubility and availability issues, a final list of 27 compounds was tested, although other compounds showing significantly increased risk based on higher odds ratios were identified.

The prostate, whose hormonal sensitivity makes it vulnerable to endocrine disruptor compounds, is considered one of the most susceptible sites in the male reproductive endocrine system (Crisp et al., 1988). An endocrine disruptor, through androgen receptor (AR) and aryl hydrocarbon receptor (AhR) related activities, can alter the synthesis of steroid hormones (Hirsh et al., 1987), or alter the transport and clearance of hormones by binding to steroid binding proteins in the serum. Environmental hormones are also known to alter steroid hormone receptor activation indirectly by down-regulating the receptor or decreasing the sensitivity of the receptor to the ligand (Sikka and Naz, 1999). However, the most common known mechanism of endocrine disruption is through direct hormone receptor binding by the contaminant or its metabolites. By mimicking the natural ligand or inhibiting the natural ligand from binding to the receptor, a compound can act as a steroid hormone agonist or antagonist, respectively. This has been shown through in vitro hormone binding and transcriptional assays that demonstrated the ability of many environmental contaminants to interact at the molecular level with one or more steroid hormone receptors (Kelce et al., 1994; Mueller and Kim, 1978; White et al., 1994). The prototypical AhR ligand is the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), although AhR is also known to bind a structurally diverse group of ligands (Denison and Heath-
Furthermore, TCDD and steroid hormones (17β-estradiol and testosterone) have been shown to negatively regulate each other in transient transfection reporter assays in MCF-7 and LNCaP cells (Jana et al., 2000). Studies to date on AhR antagonism of the AR system have focused on the extremely potent ligand of AhR, TCDD. However, AhR may interact with variety of environmental contaminants and their effects on androgenicity have not been fully assessed. Several fungicides and pesticides have demonstrated endocrine disruption properties. Ralph et al. (2003) have recently completed an extensive study of the hexachlorobenzene fungicide to show the androgen receptor action in vitro (Ralph et al., 2003).

The fungicide vinclozolin has also been shown to alter sex differentiation in male rats (Gray et al., 1994). Pesticides with endocrine disrupting properties include DDT, dieldrin and toxaphene (Cheek et al., 1998; Sohoni and Sumpter, 1998; Soto et al., 1994; Sumpter, 1998). The DDT metabolite, p, p'-DDE has been shown to bind androgen receptor and act as an antagonist in rodents and reptiles at concentrations similar to that in the environment (Kelce et al., 1995).

Steroid receptors such as androgen, estrogen, progesterone, glucocorticoid and aryl hydrocarbon receptors are transcription factors that regulate gene expression via two organic modes: ligand-dependent DNA binding (Tsai and O'Malley, 1994; Quigley et al., 1995) and crosstalk with other transcription factors (Culig et al., 1998; Kallio et al., 1995; Meyer et al., 2004). A complex interplay between the multiple factors involved in gene transcription and environmental contaminants with the potential to disrupt normal endocrine function could induce abnormal gene activation and expression (Kelce et al., 1998; Wolff and Toniolo, 1995). This in vitro study examines the endocrine disruption properties of agricultural chemicals associated with increased risk of prostate cancer. Specifically, the hypothesis tested is that pesticides are able to interfere with both AR and AhR-mediated transcription, which may in part be due to direct binding to the AR. To screen for AR and AhR-mediated endocrine disruption, we use two highly sensitive tissue-culture-based reporter gene assay systems designed to detect compounds, which may affect hormonal action of AR, and assess the activity of AhR-mediated
transcription using a comparable dioxin-responsive reporter system. Each compound was
tested in vitro to assess the endocrine-like effects, specifically agonist or antagonist activity of
AR and AhR using the respective tissue culture-based reporter systems. To determine
interference with AR-mediated transcription, LNCaP cells were transfected with AR, an
androgen-responsive promoter linked to Luciferase (ARR3-Luc) and an internal control plasmid
(pRLTK) to monitor transfection efficiency and general toxicity. Following 48-hour incubation
with a range of test compound concentrations in the presence of DHT, Luciferase activity was
quantified and normalized to the internal reporter. To monitor AhR activity, LNCaP cells were
transfected with AhR and a xenobiotic-responsive promoter linked to a firefly luciferase reporter
gene (GudLuc 1.1). The amount of luciferase produced was proportional to the degree of
transcriptional activity induced by the ligand-bound receptor, thus this approach allowed us to
assess the AR and AhR activities.

The objective of this research was to identify agricultural chemicals associated with an
increased risk of developing prostate cancer, and specifically characterize the nature of their
biological properties focusing on endocrine disruption.

2.2 Materials and Methods

Chemicals. Twenty-seven pesticides identified by epidemiological analysis including 2,4-D (2,4-
dichlorophenoxy acetic acid), 2,4-DB (2,4-dichlorophenoxy butyric acid), captan, carbaryl,
chloromethionat, copper oxychloride, copper sulfate, diazinon, dichlone, dichlorvos, dicofol,
dimethoate, dinoseb, dodine, endosulfan, ethephon, ethion, ferbam, malathion, mancozeb,
maneb, MCPA (4-chloro-2-methylphenoxy acetic acid), metiram, o,p'-DDT (dichloro-diphenyl-
trichloroethane), p,p'-DDT, paraquat, and simazine (Table 2.2) were made by AccuStandard
Inc. (New Haven, CT, USA), had a purity of 95-100%, and were purchased from
Chromatographic Specialties (Ontario, Canada). TCDD was also obtained from
Chromatographic Specialties. Compounds were dissolved in absolute methanol or ether:methanol for a 0.1M stock for cell culture and serial dilutions were prepared in absolute
methanol. Dilutions of dodine were dissolved in dH₂O and serial dilutions were prepared in absolute methanol. For the cell culture-based luciferase reporter assay, the compounds were further diluted in 10% DCC stripped RPMI to produce final working concentrations of 0.1nM, 1nM, 10nM, 100nM, 1μM, 10μM and 100μM. DHT (Sigma-Aldrich, St. Louis, MO) and dexamethasone (Sigma-Aldrich, St. Louis, MO) were dissolved in absolute ethanol for 1mg/mL stocks and further diluted in 20% ethanol to produce final working serial dilutions. The final solvent concentration in the culture medium did not exceed 0.2%, and this concentration did not affect cell viability. All chemicals were stored at -20°C and warmed to room temperature immediately before use. The acceptable daily intake values were taken from the EXtension TOXicology NETwork website at http://extoxnet.orst.edu and represent the daily exposure level which during lifetime appears to be without appreciable risk. The values are set by the U.S. Environmental Protection Agency and are obtained by dividing the NOEL (no observable effects level) obtained in a chronic feeding study by a safety factor.

**Plasmids.** The full-length rat AR plasmid construct was prepared as described previously (Rennie et al., 1993). We transiently transfected the AR (Rennie et al., 1993) into the prostate cell line because LNCaP cells express a mutated AR (Sack et al., 2001). The AhR construct was prepared as described previously by Dr. Jody Ralph (Ema et al., 1994). One of the reporters is pARR3-Luc containing three tandem repeats of -244 to -96 of the 5' flanking region of the rat probasin gene (Snoek et al., 1996). The GudLuc 1.1 plasmid was a gift from Dr. M. Denison (University of California, Davis) and contains the firefly luciferase gene under the control of a portion of the upstream promoter region of the CYP1A1 gene containing four dioxin response elements (Garrison et al., 1996). The pRLTK vector contains a thymidine kinase promoter upstream of the modified cDNA encoding Renilla luciferase from the sea pansy *Renilla reniformis* (Promega, Madison, Wisconsin). The pRLTK is co-transfected as a transfection control and was constitutively expressed. All plasmid DNA was propagated in JM109 *E. Coli* and was prepared using QIAGEN Maxiprep Kit (QIAGEN, Mississauga, ON).
**Cell Culture Transfections.** The test compounds were dissolved to produce stock solutions and then further diluted in 10% DCC stripped RPMI to produce final working concentrations of 0.1nM, 1nM, 10nM, 100nM 1μM, 10μM and 100μM and a final solvent concentration of 0.2%. LNCaP prostate cancer cells were cultured in RPMI 1640 defined medium (GibcoBRL, Burlington, ON) supplemented with 5% Dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) at a density of 6 x 10⁴ cells/well in 24 well cell culture plates (Costar, Corning NY) and incubated overnight under standard conditions (37°C, 5% CO₂). For routine maintenance, LNCaP cells were grown in RPMI 1640 defined medium (GibcoBRL, Burlington, ON) supplemented with 5% fetal bovine serum (FBS) under standard conditions, and passaged every week by trypanblueization with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt solution (GibcoBRL, Burlington, ON). Plasmids were transiently transfected into the cells using LIPOFECTIN Reagent (Invitrogen, Burlington, ON). Each plate received 1.2 μg of the AR or AhR plasmid, 0.8 μg of pARR₃-Luc or GudLuc, and 0.01 μg of pRLTK in serum-free RPMI. The cells were incubated overnight and then compounds were added in 10% DCC-FBS RPMI. Cells were harvested 48 h later in PBS with 1mM EDTA and microcentrifuged at 3000 rpm at 4°C for 4 min. Supernatant was removed and 20μl Passive Lysis Buffer (Promega, Madison, WI) was added. Cells remained on ice for 15 min and were then gently vortexed and frozen at -80°C until analysis.

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and the EG&G Berthold Microplate Luminometer LB 96V (Berthold Technologies, Bad Wildbad, Germany).

**Recombinant Androgen Receptor Ligand Binding.** Recombinant rat thioredoxin-fused AR ligand binding domain (Trx-ARLBD) was obtained from PanVera (Madison, USA) and diluted in Binding Buffer (50 mM Tris pH 7.5, 10% glycerol, 0.8 M NaCl, 1 mg/ml BSA and 2 mM dithiothreitol). A stock of assay mix was prepared by combining 20 nM ^3^H-R1881 (NEN Life Science Products, Inc., Boston, MA) and 2% EtOH in Binding Buffer. The range of compound...
concentrations tested was added to the diluted Trx-ARLBD and assay mix. Following overnight incubation at 4°C, 50% Hydroxylapatite (HAP) slurry was added (Calbiochem Fast Flow Hydroxylapatite in 10 mM Tris pH 8.0 and 1 mM EDTA). The HAP pellets were incubated on ice for 10 minutes and then washed three times with Wash Buffer (40 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA and 1 mM EGTA). The HAP pellet was resuspended in ethanol and transferred to a scintillation vial containing Scintiverse scintillation fluid. Scintillation counting was completed using the Beckman LS 6500 scintillation counter. To measure non-specific binding, two tubes were also prepared containing solvent, Binding Buffer and assay mix. A scintillation vial containing the protein/ assay mix, EtOH and 50% HAP slurry was counted and this number represented the total count.

Calculation:

\[
\text{pmole bound } ^3\text{H-R1881 per mg protein} = (\text{CPM sample-CPM non-specific binding}) \times (\text{protein conc. in mg/ml})/(\text{Total CPM}) \times ( ^3\text{H-R1881 concentration in nM})
\]

Statistical analysis. The average of three experimental determinations, with three replicates each, was carried out for each experimental group. Firefly luciferase values were normalized for transfection efficiency using activity of Renilla luciferase. Values are expressed as a percentage of the appropriate solvent control and presented as mean ± standard error of the mean (SEM). Statistical significance (p≤0.05) among the various parameters assessed was established by Analysis of Variance (ANOVA). Upon demonstration of statistical significance, the Dunnett’s Multiple Comparison Test indicated which groups were significantly different from the control group.

2.3 Results

Dose-dependent response of 5α-DHT in AR transcriptional reporter assay, and antagonistic response of TCDD-mediated AhR activity by 5α-DHT in AhR assays.

Figure 2.1A shows the dose-dependent transactivation of AR occurs at very low
hormone concentrations. Increasing concentrations of DHT, a positive control, induced rising firefly luciferase production (p<0.01) in LNCaP cells transfected with the androgen-responsive ARR3-Luc reporter plasmid and the full-length rat AR. The maximal AR transactivation activity was achieved at 1μM, exhibiting over 3500% control increase in androgen-responsive transactivation activity compared to that of the control solvent (0nM). In the absence of exogenously expressed AR, minimal levels of luciferase activity are detected; therefore the transcriptional responses reported throughout these studies represent the activity of the exogenous wild-type AR introduced into LNCaP cells by transfection. From these dose-response curves, a half-maximal response dose of 0.01nM DHT was selected for further experiments that examine both agonistic and antagonistic biological responses. As previously demonstrated by Jana et al. (1999), Figure 2.1B shows that DHT antagonized TCDD-mediated AhR activity (p<0.01) in a dose-dependent manner. Significant antagonistic activity was observed between 10nM and 1μM. Following this standardization of our assay, the impact of synthetic AR and AhR ligands were assessed.

Androgenic and anti-androgenic effects of the pesticides

Figures 2.2-2.4 show the results for the 27 compounds screened for their ability to act as either androgen agonists or antagonists in vitro. Many compounds exhibit weak to strong antagonistic activity at higher concentrations, and Table 2.2 summarizes the results, together with the AhR activity and AR-LBD binding assay results. As shown in Table 2.2, 17 of the 27 pesticides were found to antagonize AR activity in the presence of DHT (< 100% control luciferase activity). The most potent antagonistic effects were observed with captan, carbaryl, copper oxychloride, copper sulfate, dichlone, dicofol, dodine, endosulfan, ethion, ferbam, maneb, o,p'-DDT, and p,p'-DDT, at concentrations between 1μM to 100μM (Figures 2.2-2.4). It is important to note that these upper treatment concentrations are still environmentally relevant. Endosulfan, ethion, o,p'-DDT, and p,p'-DDT have been previously shown to act as AR antagonists. Androgenic effects of the pesticides were measured as their ability to enhance AR transcriptional activity in the
presence of DHT (> 100% control luciferase activity). Figure 2.4 shows that only 2,4-DB and MCPA exhibited a more than 2-fold increase in androgenic transcriptional activity compared to control. Several pesticides identified in the case-control study indicate their ability to act as partial androgen agonists (> 100% control luciferase activity at low concentrations and <100% control luciferase at high concentrations) including chinomethionat, dichlorvos, ethephon, and MCPA (Figures 2.2-2.4). These four compounds exhibit weak to strong antagonistic activity at higher concentrations and weakly agonistic activity at lower concentrations. It may be possible that these compounds interact with the endogenous hormone and gene transcriptional machinery, enhancing activity at lower concentrations, but inhibiting normal transcriptional activation at higher concentrations. Although examining the synergistic activity of these compounds is beyond the scope of this study, it is interesting to consider the combined effects on AR activity of a number of weak androgen antagonists, perhaps simulating chronic environmental exposure.

**Agonistic and antagonistic effects of pesticides through the AhR**

*In vitro* AhR activation studies demonstrated the ability of many compounds to alter AhR activity. Figure 2.1B demonstrates the dose-dependent AhR activity of the dioxin-responsive reporter GudLuc with DHT, both alone and in the presence of half-maximal TCDD (10nM). DHT has no effect on AhR activity in the absence of TCDD, whereas in the presence of 10nM TCDD, it acts as an AhR antagonist between 10nM-1μM DHT. Figures 2.5-2.7 show that 12 of the 27 compounds showed antagonistic, agonistic, or partial agonistic effects, also summarized in Table 2.2. 2,4-DB, carbaryl, diazinon, dichlone, and ferbam exhibited weak to strong agonist effects in the absence of TCDD on dioxin-responsive reporter activity. Partial agonistic effects were seen with four compounds, chinomethionat, dicofol, ethephon, and o,p'-DDT, acting as agonists at low concentrations and having no effect at higher concentrations, with the exception of the significant antagonistic effect of chinomethionat at 100μM. Antagonistic compounds included dinoseb, dodine, and mane which showed 50% or greater reduction in AhR
transcriptional activity at 10μM-100μM.

**Combined AR and AhR activity of the pesticides**

Table 2.2 summarizes the 11 pesticides exhibiting dual activities as AR agonists or antagonists and AhR agonists or antagonists. Among these pesticides, many types of pesticides were predominant including organochlorine and organophosphorus pesticides, and carbamate insecticides and dithiocarbamate fungicides. Carbaryl, dichlone, ferbam, and o,p'-DDT were shown to have AR antagonistic and AhR agonistic effects, thus demonstrating the ability of several pesticides to impact androgen and aryl hydrocarbon activity, respectively, by possibly interfering with common mechanisms like influencing receptor expression, competing for common co-activators, or as discussed below, direct binding to AR. Three pesticides dinoseb, dodine and manebo showed AR and AhR antagonistic activity, while 2,4-DB acted as an AR and AhR agonist, and chinomethionat and ethephon showed partial agonist effects on both receptors, acting as an agonist at low concentrations and as an antagonist at high levels.

**Anti-androgenic action mediated through the ligand binding to AR**

To determine if increases or decreases in androgenic or aryl hydrocarbon activity were due to interference with androgen binding to AR by pesticides, a direct AR ligand-binding assay was employed. To directly assess if pesticides displace androgen bound to AR we used a biochemical assay utilizing purified AR-LBD bound to $^3$H-R1881. As expected, DHT and R1881 effectively displaced $^3$H-R1881 (Figure 2.8) in a dose-dependent fashion, whereas increasing concentrations of dexamethasone failed to displace $^3$H-R1881 from AR-LBD. The anti-androgen Casodex also clearly binds to AR, albeit with lower affinity, displacing bound $^3$H-R1881 at between 10-100μM. This purified ligand-binding assay system clearly demonstrated that eleven of the 27 pesticides tested could interfere with androgen bound to AR in a manner that parallels the antagonistic activity of pesticides on AR transcriptional activity. Some of the compounds show weak binding characteristics, with copper sulfate, dodine, dinoseb, ferbam, and malathion having the most pronounced effect on displacement of bound $^3$H-R1881 (Figures 2.9-2.11). It
was difficult to establish whether the ability of individual pesticides to displace DHT from AR-LBD mirrored the potency of AR antagonism of the pesticides. For example, copper sulfate, dodine, dinoseb, ferbam and malathion all showed strong displacement and AR antagonistic activity.

Although no apparent structure-binding patterns can be elucidated by examining the pesticides that showed ligand binding to AR, it appears that because AR is not highly promiscuous for all pesticides studied, perhaps the structural conformation combined with the functional group substitution pattern may be responsible for explaining the ability of a particular pesticide to effectively bind AR. For example, as previously reported, there is a potential correlation between chlorine substitution, AR binding and reduction in AR transcriptional activity, as seen with o,p'-DDT and p,p'-DDT. Most interestingly, dinoseb and dodine showed strong AR-binding, and reduced activity of both AR and AhR, suggesting that the presence of nitrogen containing side chains may play a role in combined AR and AhR activity through interference with AR-binding. Taken together, these novel data strongly suggest that specific pesticides are able to directly bind to AR-LBD and affect the transcriptional activity of both AR and AhR.
2.4 Discussion

The risk of prostate cancer in farmers in the agriculture industry, which has been reviewed recently has been associated with lifestyle factors and with exposure to pesticides, insecticides, herbicides, and other agricultural chemicals (Band et al., 1999a, 1999b; Keller-Byrne et al., 1997; Mills and Yang, 2003; Morrison et al., 1993; Siemiatycki, 1991; Van der Gulden et al., 1996; Van Maele-Fabry and Willems, 2003). To our knowledge, this is the first study that has linked epidemiological evidence from agricultural pesticide exposure associated with an increased risk of developing prostate cancer, and specifically characterized the nature of their biological properties in prostate carcinogenesis focusing on endocrine disruption. The prostate is highly sensitive to androgens and requires precise hormonal control to regulate its growth and function. The development of the androgen-sensitive prostate may be altered in utero in the presence of low levels of androgens, as well as during puberty when a surge of circulating androgen in serum initiates prostate growth and development. Furthermore, early alteration of normal prostate development may have implications on prostate growth in later life.

Prostate cells express both AR and AhR and cross-talk between the two pathways may affect the function of the prostate cell and organ development (Jana et al., 1999). Pesticides have been documented to interfere with the activities of ER, AR and AhR both in vitro and in vivo (Wormke et al., 2000a, 2000b). Suppression of AR action may occur via several mechanisms including competition with AhR for co-regulator proteins (Misiti et al., 1998). We hypothesize that this may occur by both receptor systems competing for mutual co-activators, which are limiting in the nucleus, such as CREB binding protein (CBP) or Steroid Receptor Co-activator 1 (SRC-1) and thus stimulation of one system may repress the other (Aarnisalo et al., 1998, Fronsdal et al., 1998, Kumar and Perdew 1999). Studies to date on AhR antagonism of the AR system have focused on the extremely potent ligand of AhR, TCDD. However, we show that AhR interacts with a wide spectrum of structurally diverse pesticides, and this is the first time their androgenicity has been assessed.
Our interest has focused on the ability of a specific group of pesticides to interact with the AR and AhR receptor, and to determine if pesticides can affect the transcriptional activity of AR and AhR, we employed a reporter-based assay using transient transfection into a human prostate cancer cell line, LNCaP. LNCaP cells express only low levels of endogenous AR and AhR (Cleutjens et al., 1997). In this transcriptional assay system we have transiently transfected a luciferase reporter, ARR₃Luc or GudLuc 1.1, in combination with an AR or AhR construct. The reporter construct, ARR₃Luc or GudLuc 1.1, responds to AR or AhR in the presence of DHT or TCDD, respectively, in a ligand concentration-dependent manner. In the absence of exogenously expressed AR or AhR minimal levels of luciferase are detected in LNCaP cells under the conditions used, therefore the transcriptional response reported throughout these studies represents the activity of the exogenous wild type AR and AhR introduced into the LNCaP cells.

The biological effects of a compound on endocrine disruption can occur either directly by its binding to a particular hormone receptor or indirectly by altering the availability of steroid hormones, competition for common co-regulatory proteins, as well as other speculative mechanisms. Therefore, to elucidate a possible mechanism for endocrine disruption of our pesticides of interest, we performed competitive ligand binding assays using a purified AR-LBD, ^3H-R1881, and increasing concentrations of pesticides. It has been demonstrated in previous studies that the ligand-binding pocket of steroid receptors has an unexpected amount of latitude in binding specificity (Blair et al., 2000; Waller et al., 1996). Our results clearly showed that a number of structurally diverse pesticides could bind AR, however binding to the AR cannot be predicted from the overall structure of the compound.

We were able to classify the pesticides tested into six groups according to their chemical structure: organochlorines, carbamate fungicides, phenoxy herbicides, inorganic copper fungicides, organophosphorus pesticides, and other pesticides (includes fungicides, herbicides, and insecticides). Previous studies have identified organochlorine-type pesticides as endocrine
disruptor candidates because of their widespread agricultural use and bioaccumulation in the ecosystem because of their highly lipophilic properties (Kutz et al., 1997; Simonich and Hites, 1995). Moreover, the anti-androgenic properties of the organochlorine pesticides, DDT isomers, dicofol, endosulfan, and ethion, have already been reported (Andersen et al., 2002; Golden et al., 1998; Kelce et al., 1995; Kojima et al., 2004; Maness et al., 1998), and in the present study we have confirmed these results, and identified o,p'-DDT and dicofol as partial AhR agonists. Organochlorines may exert their anti-androgenic effects through AR-ligand binding as we have demonstrated with o,p'-DDT and p,p'-DDT, however this mechanism is not unique because dicofol, endosulfan, and ethion failed to displace the synthetic androgen ligand from its AR-binding site.

Some carbamate fungicides have been previously shown to have AR anti-androgenic activity (Andersen et al., 2002; Kojima et al., 2004) and our studies further this evidence, showing carbaryl, and the dithiocarbamate fungicides ferbam, mancozeb, and maneb, all reduce AR transcriptional activity, with the exception of metiram. Carbaryl and ferbam showed AR antagonistic and AhR agonistic activity, suggesting that by possibly interfering with common mechanisms like influencing receptor expression, competing for common co-activators, or in the case of ferbam, direct binding to AR.

Use of and exposure to chlorophenoxy herbicides, including 2,4-D, 2,4-DB, and MCPA is widespread. Studies have previously shown that exposure to chlorophenoxy herbicides leads to increased prostate cancer mortality (Schreinemachers, 2000), damage to sperm (Lerda et al., 1991) and chlorophenoxy herbicides can be detected in the seminal fluid of herbicide users (Arbuckle et al., 1999). We are the first to report the strong AR agonistic activity of 2,4-DB and MCPA, the AR antagonistic activity of 2,4-D, and the AhR agonistic activity of 2,4-DB. No binding to the AR was shown indicating that perhaps the AR and AhR agonistic activity of these compounds may occur through increased receptor expression.

The inorganic pesticides, copper sulfate and copper oxychloride, are widely used as
fungicides and bactericides in agricultural fruit production, as algicides and for control of fungal rot, mildew, and decay of numerous crops, however their endocrine disrupting properties remain unknown. We found that copper sulfate and copper oxychloride decreased AR activity with no effect on AhR activity. Copper sulfate bound to AR suggesting it exhibits its potential antagonistic effects by direct competition with the endogenous androgen for AR-binding.

Organophosphorus pesticides are some of the most widely used pesticides used in Canada and other countries in both agriculture and pest control and research has linked relatively high levels of exposure to elevated prostate cancer risk (Mills and Yang, 2003). To date, studies have identified some organophosphorus pesticides as AR antagonists (Kojima et al., 2004; Sohoni et al., 2001; Tamura et al., 2001), however only the antagonistic activity of ethion on AR, examined in previous studies, was confirmed. In our assays, malathion also showed AR antagonistic activity with AR-binding. Diazinon and dichlorvos also bound AR, however dichlorvos acted as a partial AR agonist and diazinon showed AhR agonistic effects. Comparing and contrasting the chemical structures of these compounds may offer some insight into their AR-ligand binding behaviour, as it appears that perhaps the confirmation of the chemical structure based on the number and size of the side chains allows binding in the case of dichlorvos, diazinon, and malathion.

In the present study, 8 pesticides that could not be classified into the above groups were collectively grouped as "other pesticides." Most interestingly, dinoseb and dodine, although structurally unrelated, showed potent antagonistic activity via both the AR and AhR, and bound to AR at 100μM. This was the first time the endocrine-disrupting effects of these two compounds have been demonstrated. On the other hand, neither paraquat nor simazine showed any AR or AhR activity, similar to results demonstrated previously (Kojima et al., 2004). Therefore, these types of pesticides may exert hormonal activity through mechanisms independent of those associated with AR and AhR.

Most research on the effects of chemicals on biologic systems is conducted on one
chemical at a time. However, in reality people are exposed to mixtures, not single chemicals. Although various substances may have totally independent actions, in many cases two substances may act at the same site in ways that can be either additive or non-additive. More complex interactions may occur if two chemicals act at different but related targets. In the extreme case there may be synergistic effects, in which case the effects of two substances together are greater than the sum of either effect alone. Although epidemiological evidence grouped the pesticides in our study into three categories based on the significance of elevated prostate cancer risk, no definite correlation can be ascertained between risk and biological activity based on our data. Further analysis of the exposure information for individuals diagnosed with prostate cancer will help ascertain the characteristics of the pesticides that may contribute to prostate cancer formation and progression. The production and use of these compounds can be monitored and potentially reduced. Ultimately, the epidemiologic knowledge gained from understanding the impact of specific agricultural chemicals on prostate cancer and the better understanding of how these substances contribute to cancer development may lead to effective strategies for the prevention of occupationally related exposure.

We have demonstrated that pesticides may have the potential to mimic or antagonize hormone action, in many cases through binding to steroid receptors and/or interfering with transcriptional activity, thus they are capable of disrupting male hormone-signalling pathways. However, other indirect mechanisms of endocrine disruption may be also possible. There have been reports in the literature that the multidrug resistance efflux transporter Pgp may play a role in the cellular detoxification of pesticides in mammalian tissues. Thus, Pgp may play a physiological role in participating in the protection of human cells against xenobiotics, including pesticides, by active efflux of these compounds into bile, urine, or the intestinal lumen, and by preventing accumulation in critical organs such as the brain, testis, prostate, and other reproductive organs (Schinkel et al., 1997; van Tellingen, 2001). For example, expression of Pgp in the placenta has been shown to be a factor to protect fetuses against toxicity of the
pesticide ivermectin (Smit et al., 1999). Therefore, Pgp may be responsible for what could be defined as 'multi-pesticide resistance', a phenomenon that parallels multi-drug resistance in tumour cells.

As the function of Pgp in normal cells and the identity of its endogenous substrates remain unclear, other recent studies suggest that Pgp may interact with endogenous steroids. Previous studies suggest that Pgp can reduce the accumulation of a wide range of steroids, suggesting that cellular androgen levels could be significantly affected by this efflux transport mechanism. Only one previous study has examined the transport of androgens by multi-drug resistant cells, showing decreased androgen, both testosterone and dihydrotestosterone, accumulation in human colon carcinoma cells (Barnes et al., 1996). We hypothesized that this may in turn lead to decreased androgen-responsiveness in prostate cancer cells, leading to adaptation to an androgen-independent environment. Physiologically, decreases in androgen levels could lead to activation of androgen-independent gene pathways and/or reduction in the expression of androgen-responsive genes. Thus, an androgen-Pgp interaction may have a profound impact on both the genotype and phenotype of prostate cancer cells, and their response to chemical therapeutics. Three aspects of this possible androgen-Pgp interaction are examined in Chapter 3: (1), the transport of [3H]-DHT by Pgp; (2), the antagonism by pharmaceuticals on Pgp-mediated androgen efflux and/or cellular accumulation; and (3), the physiological consequences altered DHT transport and cellular accumulation on androgen-regulated gene expression in prostate cancer cells.
### Table 2.1. Pesticides tested for AR-LBD binding in the AR and AhR reporter assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type / Group of Pesticide</th>
<th>Acceptable Daily Intake (mg/kg b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dicofol</td>
<td>organochlorine acaricide</td>
<td>0.002</td>
</tr>
<tr>
<td>endosulfan</td>
<td>organochlorine insecticide</td>
<td>0.006</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>organochlorine insecticide</td>
<td>0.02</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>organochlorine insecticide</td>
<td>0.02</td>
</tr>
<tr>
<td>carbaryl</td>
<td>wide-spectrum carbamate insecticide</td>
<td>0.01</td>
</tr>
<tr>
<td>ferbam</td>
<td>iron dimethylthiocarbamate fungicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>mancozeb</td>
<td>polymeric dithiocarbamate fungicide</td>
<td>0.03</td>
</tr>
<tr>
<td>maneb</td>
<td>polymeric dithiocarbamate fungicide</td>
<td>0.03</td>
</tr>
<tr>
<td>metiram</td>
<td>polymeric dithiocarbamate fungicide</td>
<td>0.03</td>
</tr>
<tr>
<td>2,4-D</td>
<td>phenoxy acetic herbicide</td>
<td>0.3</td>
</tr>
<tr>
<td>2,4-DB</td>
<td>phenoxy butyric herbicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>MCPA</td>
<td>phenoxy acetic herbicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>copper oxychloride</td>
<td>broad-spectrum inorganic copper fungicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>copper sulfate</td>
<td>inorganic copper fungicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>dichlorvos</td>
<td>organophosphate insecticide</td>
<td>0.004</td>
</tr>
<tr>
<td>dimethoate</td>
<td>organophosphate insecticide</td>
<td>0.01</td>
</tr>
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<td>diazinon</td>
<td>pyrimidine organothiophosphate insecticide</td>
<td>0.002</td>
</tr>
<tr>
<td>ethion</td>
<td>aliphatic organophosphate pesticide</td>
<td>0.002</td>
</tr>
<tr>
<td>malathion</td>
<td>aliphatic organophosphate insecticide</td>
<td>0.02</td>
</tr>
<tr>
<td>captan</td>
<td>phthalimide fungicide</td>
<td>0.1</td>
</tr>
<tr>
<td>chinomethionat</td>
<td>quinoxaline fungicide and acaricide</td>
<td>0.003</td>
</tr>
<tr>
<td>dichlone</td>
<td>quinone fungicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>dinoseb</td>
<td>dinitrophenol herbicide</td>
<td>Not Available</td>
</tr>
<tr>
<td>dodine</td>
<td>aliphatic nitrogen fungicide</td>
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</tr>
<tr>
<td>ethephon</td>
<td>organic phosphorus plant growth regulator</td>
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</tr>
<tr>
<td>paraquat</td>
<td>quaternary ammonium herbicide</td>
<td>0.004</td>
</tr>
<tr>
<td>simazine</td>
<td>selective triazine herbicide</td>
<td>Not Available</td>
</tr>
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Table 2.2. Summary of the AR-LBD binding assay and *in vitro* results with AR and AhR.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Risk Group</th>
<th>Binds to AR</th>
<th>AR Activity (with DHT)</th>
<th>AhR Activity (no TCDD)</th>
</tr>
</thead>
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<tr>
<td>dicofol</td>
<td>0.85 c</td>
<td></td>
<td>*</td>
<td>1*</td>
</tr>
<tr>
<td>endosulfan</td>
<td>1.52 a</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>1.65 a</td>
<td>+*</td>
<td>*</td>
<td>1*</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>1.65 a</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>carbaryl</td>
<td>1.54 a</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ferbam</td>
<td>1.60 a</td>
<td>+*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>mancozeb</td>
<td>1.37 b</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>maneb</td>
<td>1.60 a</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>metiram</td>
<td>1.44 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>2.72 a</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>2,4-DB</td>
<td>1.77 a</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>MCPA</td>
<td>1.83 a</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>copper oxychloride</td>
<td>1.45 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>copper sulfate</td>
<td>1.39 b</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>dichlorvos</td>
<td>0.91 c</td>
<td>+*</td>
<td>*</td>
<td>1*</td>
</tr>
<tr>
<td>dimethoate</td>
<td>1.36 b</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>diazinon</td>
<td>1.43 b</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ethion</td>
<td>1.52 b</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>malathion</td>
<td>1.34 a</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>captan</td>
<td>1.56 a</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>chinomethionat</td>
<td>1.79 a</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>dichlone</td>
<td>1.79 a</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>dinoseb</td>
<td>1.77 a</td>
<td>+*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>dodine</td>
<td>1.83 a</td>
<td>+*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ethephon</td>
<td>1.49 b</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>paraquat</td>
<td>1.45 b</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>simazine</td>
<td>1.69 a</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Three pesticides were grouped into three categories based on their odds ratios: ^a^ pesticides showing significantly increased risk; ^b^ pesticides showing elevated, but not significant risk; ^c^ pesticides with no elevated risk. Symbol * indicates significant difference compared to the control for either AR or AhR activity (p<0.05). 1. Partial agonist (acts as agonist at low concentrations and as an antagonist at high levels).
Figure 2.1. Production of Luciferase in LNCaP Prostate Cancer Cells in Response to DHT Exposure. A. The androgen DHT is an AR agonist in LNCaP cells transfected with the androgen-responsive promoter ARR3Luc. B. DHT is an AhR antagonist in LNCaP cells transfected with the dioxin-responsive promoter GudLuc and incubated in the presence of half-maximal TCDD (10 nM). The symbol * indicates significantly different compared to the solvent control at 0nM (p<0.01).
Figure 2.2. Production of ARR$_3$Luc in LNCaP cells transfected with AR following incubation in the presence of pesticides and 0.1 nM DHT. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figure 2.3. Production of ARR3Luc in LNCaP cells transfected with AR following incubation in the presence of pesticides and 0.1 nM DHT. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figure 2.4. Production of ARR2Luc in LNCaP cells transfected with AR following incubation in the presence of pesticides and 0.1 nM DHT. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figures 2.5. Production of GudLuc in LNCaP cells transfected with AhR following incubation in the presence of pesticides. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figure 2.6. Production of GudLuc in LNCaP cells transfected with AhR following incubation in the presence of pesticides. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figure 2.7. Production of GudLuc in LNCaP cells transfected with AhR following incubation in the presence of pesticides. Values are expressed as a percent of the solvent control and presented as mean ± SEM. The symbol * indicates significantly different from the solvent control at 0nM (p<0.01).
Figure 2.8. Binding to the AR analyzed by displacement of tritiated androgen from the androgen receptor ligand binding domain. Values are expressed as a percent of the solvent control and presented as mean ± SEM. *Significantly different from the appropriate solvent control at 0μM (p<0.05).
Figure 2.9. Binding to the AR analyzed by displacement of tritiated androgen from the androgen receptor ligand binding domain. Values are expressed as a percent of the 0nM solvent control and presented as mean ± SEM. *Significantly different from the appropriate solvent control (p<0.05).
Figure 2.10. Binding to the AR analyzed by displacement of tritiated androgen from the androgen receptor ligand binding domain. Values are expressed as a percent of the 0nM solvent control and presented as mean ± SEM. *Significantly different from the appropriate solvent control (p<0.05).
Figure 2.11. Binding to the AR analyzed by displacement of tritiated androgen from the androgen receptor ligand binding domain. Values are expressed as a percent of the solvent control at 0nM and presented as mean ± SEM. *Significantly different from the appropriate solvent control at 0nM (p<0.05).
2.6 References


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Zeegers MP, Friesema IH, Goldbohm RA, van den Brandt PA. (2004). A prospective study of
CHAPTER 3. P-GLYCOPROTEIN INCREASES THE EFFLUX OF THE ANDROGEN DIHYDROTESTOSTERONE AND REDUCES ANDROGEN RESPONSIVE GENE ACTIVITY IN PROSTATE TUMOUR CELLS

The research presented in this chapter was performed with collaboration and valuable expertise in experimental design from Dr. Lawrence Mayer at the BC Cancer Agency and Dr. Emma Guns. Dr. Giménez-Bonafé was responsible for the performing the Northern blot experiments reported in this chapter.

3.1 Introduction

The fundamental mechanisms involved in the transport of steroid hormones through biological membranes, and their accumulation within target cells are still not completely understood. Many developmental, reproductive, and homeostatic functions are coordinated by nuclear steroid hormone receptors that upon binding to their cognate steroid activate the transcription of specific target genes (Evans, 1988). Delivery of the steroid to the target tissue is facilitated by serum binding proteins including steroid hormone-binding globulin (SHBG) (Rosner et al., 1999). The lipophilic nature of steroid hormones has led to the concept that the cell membrane plays a passive role in the transport of steroids (Giorgi and Stein, 1981). This current dogma fails to fully explain that tissue-specific homeostatic mechanisms involved in the influx, accumulation, nuclear localization, and efflux of steroid hormones require precise and differential regulation at a cellular level within target tissues, therefore a completely passive transport mechanism through the cell membrane seems overly simple. Emerging evidence supporting the transport of certain steroids by P-glycoprotein, is considerable, however not yet conclusive (Fojo et al., 1987; Gruol et al., 1999; Ueda et al., 1992).

1A version of this chapter has been published.
In the prostate, circulating testosterone enters the cells where it is converted by 5α-reductase to the more potent androgen, DHT, which regulates the growth, differentiation, and homeostasis of the gland (Bruchovsky and Wilson, 1968; Isaacs, 1996). Ultimately prostate secretory epithelial cells are dependent on the presence of androgens for survival, and will undergo apoptosis upon androgen withdrawal. In advanced prostate cancer, androgen ablation therapy is used routinely to remove or block androgen action. Androgen ablation is initially effective at inducing apoptosis and regression of prostate tumours, however some cells adapted to survive in the absence of androgens recur with an androgen independent phenotype (Isaacs, 1996). We have recently found that genes activated by YB-1, such as Pgp, are also increased during AI progression as presented in Chapter 4. This has lead to an exploration to understand the biological relevance of dysregulation and influence of Pgp on androgen transport and AR activity in prostate cancer cells.

Pgp belongs to a family of plasma membrane proteins encoded by the multi-drug resistance gene, MDR1. Pgp is a 170-kDa phosphorylated and glycosylated plasma membrane protein that belongs to the ABC superfamily of transport proteins (Juliano and Ling, 1976). The MDR1 gene, which is responsible for Pgp synthesis in humans, is well conserved in nature and appears to be involved in the transport of specific xenobiotics and the development of chemotherapeutic drug resistance in a variety of cancers (Fardel et al., 1996). Pgp expression and activity in prostate cancer has not been extensively studied. However, Pgp expression has been correlated with tumour grade and stage during prostate cancer progression (Bhangal et al., 2000). It is also known that Pgp is expressed at relatively high levels in androgen-independent PC-3 and DU-145 prostate carcinoma cells, while the androgen-sensitive LNCaP cell lines express a lower level of Pgp (Fojo et al., 1987; Theyer et al., 1993).

The majority of published data suggest that Pgp acts as a transmembrane pump to remove certain classes of xenobiotics from the cell. Research has largely focused on characterizing xenobiotics as substrates or inhibitors of the Pgp efflux mechanism and their
structure-function relationships (Seelig, 1998; Wacher et al., 1995). Chemicals transported by Pgp have diverse structures, with the primary similarities being hydrophobic amphipathic molecules (molecules having two sides with characteristically different properties) without a negative charge moiety and between 200-1800 Da. Pgp substrates include chemotherapeutic drugs, immunosuppressive drugs, steroids like aldosterone, hydrocortisone, cortisol, corticosterone and dexamethasone; HIV protease inhibitors, cardiac drugs, lipid lowering agents, dopamine antagonists, anti-diarrheal agents, anti-gout agents, antibiotics, anti-tuberculosis agents, anti-helminthic agents, and the fluorescent dye rhodamine-123 (Germann, 1996). Increased Pgp expression and/or activity functions to lower the intracellular concentration of Pgp substrates, thus creating a multi-drug resistant phenotype.

Pgp inhibitors include the immunosuppressant cyclosporin A and its non-immunosuppressive analogue PSC-833 (valspodar); the calcium channel blocker verapamil; the progesterone antagonist mifepristone (RU486); and cyclopropyldibenzosuberane LY335979 (Drach et al., 1996; Fardel et al., 1996; Huisman et al., 2000; Mayer et al., 1997; Sharom et al., 1999; Twentyman and Bleehen, 1991; Yumoto et al., 1999). The presence of Pgp in the adrenal glands and in the steroid-producing cells of the endometrium suggests it may also have a role in the export of steroids into the serum. Furthermore, it has been shown that Pgp expressing epithelial monolayers of cells are able to transport steroids and that some lymphoid cells expressing Pgp are resistant to the cytotoxic effects of steroids exhibited by these cells (Gottesman et al., 1996).

While Pgp is highly conserved throughout evolution, a complete catalogue of its endogenous substrates remains incomplete. Pgp is expressed in various normal tissues (Fojo et al., 1987), and among various physiological roles proposed it was suggested that some steroid hormones could be physiological substrates transported by Pgp (Pavek et al., 2002; Sugawara et al., 1988; Thiebaut et al., 1987). Consequently, Pgp might play a fundamental role in regulating cell differentiation, proliferation and survival in specific tissues (Johnstone et al.,
This has raised the possibility that Pgp may have a role in the regulation of homeostasis of various endogenous intracellular constituents, with steroid hormones being some of the key players in the regulation of cellular response and homeostasis in many endocrine sensitive tissues. In the context of hormone-dependent tissue such as the prostate, the relationship between steroid hormones and Pgp has not yet been addressed.

Data presented here that investigates the regulation of steroid transport and steady state androgen accumulation in prostate cancer cells, demonstrates that DHT efflux is increased by Pgp. Increased DHT efflux corresponds to decreased androgen-regulated transcriptional activity and gene expression. Furthermore, increased Pgp expression by exposure to aspirin likewise decreases androgen levels in prostate cancer cells. This evidence collectively suggests that Pgp affects the cellular transport and accumulation of DHT in a functionally and physiologically significant manner.

3.2 Materials and Methods

**Materials.** [1,2,4,5,6,7-\textsuperscript{3}H]-dihydrotestosterone (125Ci/mm mol, Amersham Pharmacia Biotech), [G-\textsuperscript{3}H]-vinblastine sulphate (9.40Ci/mm mol, Amersham Pharmacia Biotech), stock solutions of 5\textalpha;-dihydrotestosterone (Sigma), vinblastine sulphate (Sigma-Aldrich), Aspirin (Acetylsalicylic Acid) (Sigma-Aldrich), PSC-833 (provided by Dr. L. Mayer) and verapamil chloride (Sigma-Aldrich) were prepared in 95% ethanol.

**Plasmids.** The rat AR (Rennie et al., 1993) cloned into the mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA) was used for transfection studies as before (Nelson et al., 1999). The ARR\textsubscript{3}TK-luc reporter construct used contains three tandem repeats of -244 to -96 of the 5\textsuperscript{'}, flanking region of the rat probasin promoter linked to the pT81 vector which contains a thymidine kinase minimal promoter and the firefly luciferase gene (de Wet et al., 1985; Wood et al., 1984), as described previously (Snoek et al., 1998). The pRLTK-luc vector contains a thymidine kinase promoter upstream of the modified cDNA encoding Renilla luciferase (Promega). Pgp plasmid was initially obtained from the American Type Culture Collection
(pGEM-3Zf(-)Xba), the insert (full length of Pgp cDNA) was excised, and ligated into the polylinker region of the expression vector pcDNA 3.1. (Invitrogen). All plasmid DNA was propagated in JM109 E. coli and was prepared using QIAGEN Maxiprep Kit (QIAGEN, Mississauga, ON, Canada).

**Cell Lines.** HeLa cells expressing a stably transfected FLAG-tagged AR were obtained from Dr. M. Carey (Huang et al., 1999). HeLa and PC-3 cells were obtained from the American Type Culture Collection. HeLa cells are derived from cervical cancer, whereas PC-3 and LNCaP cells are prostate cancer cells. PC-3 cells are epithelial-like cells which grow adherently in monolayer and lack AR, consequently are androgen-insensitive. Conversely, LNCaP cells are androgen-responsive, possess a mutated AR and express PSA. LNCaP cells were established from a lymph node metastasis from a 50 year old man with prostate cancer. All these cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) for PC-3 and HeLa/HeLa-AR. LNCaP cells were obtained from the ATCC and maintained in RPMI 1640 (Life Technologies Inc.) supplemented with 5% FBS. Cells were maintained at 37°C in humidified air with 5% CO₂.

**Xenografts.** LNCaP tumours were propagated as previously reported (Gleave et al., 1992). Briefly, mice were subcutaneously inoculated with 2 x 10⁶ LNCaP cells and equal volumes of Matrigel. The tumours were excised before castration (Intact) and at specific time points after castration (dCx), flash frozen and stored at −80°C.

**In Vitro [³H]DHT Cellular Accumulation Assay.** All measurements of steroid accumulation in HeLa-AR, LNCaP, HeLa and PC-3 prostate tumour cells were carried out under standard conditions. Briefly, cells were seeded in 6 well cell culture plates at 3.0 x 10⁵ cells/well in 1mL media supplemented with 5% FBS and incubated for 24h. Media was aspirated, [³H]DHT was added (1.0nM final concentration) in 2mL media supplemented with Dextran-coated charcoal (DCC) stripped serum, and the plates were incubated and time-dependent accumulation was examined. After the incubation period (1-24 hr), the cells were promptly washed free of
hormone-containing media using PBS and harvested with PBS + 1mM EDTA. $[^3]H$DHT accumulation is expressed in moles of DHT/cell as an average of three replicates ± SEM.

**In Vitro $[^3]H$DHT cellular uptake with FLAG-AR immunoprecipitation assays.** HeLa FLAG-AR cells were seeded into 6 cm$^2$ plates at 6.0 x 10$^5$ cells/well in 4mL of complete media. After 24h at 37°C, the media was aspirated and 0.5 nM $[^3]H$DHT and a titration of DHT concentrations was added to media supplemented with DCC stripped serum. Cells were incubated for a further 24 h and washed with PBS and harvested by scraping in PBS + 1mM EDTA. Cytoplasm fractions (Schreiber et al., 1989) were prepared by resuspending cells in 400μl Buffer A containing 10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. 25μl 10% IGEPAL was then added and vortexed for 10 sec, then centrifuged for 30 sec at 9.3 g-force. The cytosol fraction was then removed. Western blot controls confirmed the cytosol fraction was free of AR. Nuclear extracts (Schreiber et al., 1989) were prepared and incubated for 6 h with 10μl of agarose beads conjugated with FLAG monoclonal antibodies (Sigma, USA) at 4°C. Beads were washed 2 times with Buffer D (Huang et al., 1999) containing 20mM HEPES pH 7.9, 20% glycerol, 0.3M KCl, 0.2mM EDTA, 0.05% IGEPAL, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, and then resuspended in Buffer D. Resuspensions of the FLAG-AR complex from nuclear extracts, and the cytoplasmic cell extracts were measured for levels of $[^3]H$DHT in a Beckman LS 6500 scintillation counter, and results were expressed as the average disintegrations per minute (DPM)/well of three replicates at each treatment over 24 h ± (standard error of the mean) SEM.

**In Vitro $[^3]H$DHT cellular accumulation with Pgp inhibitor PSC-833.** Cells were seeded as outlined previously. Growth media containing Pgp inhibitor was introduced (PSC-833 2μM final media concentration) and the treated cells were incubated for 2 h to ensure that Pgp-mediated action was sufficiently inhibited (Aszalos et al., 1999). $[^3]H$DHT (0.5nM final media concentration) was added in 5% FBS supplemented RPMI growth media for 24h. After the
incubation period, the cells were promptly washed free of hormone-containing media using PBS and harvested with PBS + 1mM EDTA. [³H]DHT accumulation is expressed as an average DPM/well of three replicates ± SEM over two experiments. Parallel experiments were performed in cells treated and untreated with Pgp inhibitor. As a control, cells at a density of 3 x 10⁵ cells/well were seeded into wells in both cell lines used, and protein concentrations after cell harvesting remained consistent between control and PSC-833 treated cells as determined by a BCA Protein Assay (Pierce, USA).

In experiments involving transient transfection, an expression plasmid encoding Pgp and/or AR was transfected into LNCaP cells with LIPOFECTIN (GibcoBRL) reagent in RPMI growth media and cells incubated for 24 h. PSC-833 treatment was administered in 5% FBS supplemented RPMI growth media for 2 h. 0.5nM [³H]DHT was added and the cells incubated for 12 h. After the incubation period, the cells were promptly washed free of hormone-containing media using PBS and harvested with PBS + 1mM EDTA. [³H]DHT accumulation is expressed as an average DPM/well of three replicates ± SEM over two experimental determinations. Experiments with [³H]-vinblastine (10nM final concentration), a known Pgp substrate, were performed as a positive control.

**Aspirin Treatment.** Aspirin was administered in 5% FBS supplemented RPMI growth media (2mM final concentration) for 72 h to 3.0 x 10⁵ cells LNCaP cells to induce Pgp over-expression (Rotem et al., 2000). Pgp over-expression in treated cells compared to control cells was confirmed by Western blot analysis with 5M Urea 10% SDS-PAGE gel. 0.5nM [³H]DHT was added and incubated for 24 h. Cells were harvested as outlined previously. [³H]DHT accumulation is expressed in DPM/well as an average of three replicates ± SEM over two experimental determinations.

**Protein extraction and Western Blots.** Total proteins were obtained homogenizing LNCaP cells and tumours in RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing protease inhibitor cocktail (Roche),

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and incubating the lysate at 4°C for 30 min. After centrifugation for 10 min at 3000 rpm to remove cell debris, the supernatant was again centrifuged at 29,000 rpm for 1 hr, the proteins were recovered in the pellet after resuspension in Laemmli buffer and quantified using a BCA Protein Assay (Pierce). Western blotting was performed according to the method of Harlow and Lane (Harlow and Lane, 1999). Pgp detection was performed using between 30-60 µg of total protein from LNCaP cells or LNCP tumour tissue. Proteins were resolved on 5M Urea / 10% SDS-PAGE gels and the proteins were transferred onto PDVF membranes (Immobilon™-P, MILLIPORE) at 400 mA for 1 h. Membranes were blocked during 2 to 3 h at 25°C in blocking buffer (5% dry nonfat milk in TBS; 1.37M sodium chloride, 200 mM Tris, pH 7.6) and incubated overnight at 4°C with mouse monoclonal C-219, C-494 or JSB-1 antibodies (ID Labs, Inc., London, Canada) at 1:1000 dilution in 1% bovine serum albumin (BSA) and 1% dry nonfat milk. After several washes with TBS-T (0.05% Tween-20 in TBS) blots were incubated with the secondary antibody, peroxidase linked anti-mouse immunoglobulin in 1% BSA-1% dry nonfat milk, for 45 min at 25°C and then washed again extensively with TBS-T. Mouse monoclonal anti-vinculin antibody (Sigma, USA) was used as a loading control (1:2000 dilution). In addition, to confirm levels of FLAG-tagged AR present in nuclear extracts of HeLa FLAG-AR cells, a mouse monoclonal antibody to human AR-DBD (Pharmingen) was used at 1:1000. For the quantification of AR protein expression in LNCaP cells, an anti-AR N-terminus rabbit antibody (Affinity BioReagents, Inc.) was used at 1:2000. Anti-mouse/rabbit secondary antibodies (Santa Cruz, USA) at 1:5000 were used. All Western blots were placed in ECL™ western blotting detection reagent (Amersham Pharmacia) for 2 min, followed by exposure to autoradiographic high sensitivity blue base film (Island Scientific, USA).

**cDNA probes and Northern blots.** Twenty µg of total RNA was separated by electrophoresis through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Biodyne® B membrane, Pall Corporation, East Hills, NY). Membranes were hybridized to a 32P-labelled human PSA cDNA probe using ULTRAhyb™ solution (Ambion) following the procedure
described by Ambion and performed by Dr. Giménez-Bonafé. Membranes were exposed for autoradiography. The same blots were hybridized with GAPDH3 probe to control for RNA integrity and quantification. PSA cDNA probe was created through the digestion of the plasmid pBluescript (American Type Culture Collection, ATCC) with Eco RI resulting in a 1.34kb probe. For quantification of mRNA expression levels, MS films were scanned on a Gel Doc 2000 gel documentation system (BioRad, Hercules, CA) and analyzed using Quantity One 4.2.1 software.

Cell Culture Transfections and Luciferase Assay. LNCaP cells were seeded at a density of $3.0 \times 10^5$ cells/well in 6 well cell culture plates and incubated for 24h. Plasmids were transiently transfected into LNCaP cells using LIPOFECTIN Reagent (Invitrogen). Each well received 0.25µg of AR, 0.25µg Pgp plasmid DNA, 0.01µg of ARR3TK-luc plasmid DNA, and 0.001µg of pRLTK-luc plasmid DNA. In experiments where the expression of Pgp was enhanced directly through transient transfection of an expression plasmid encoding Pgp, over-expression was confirmed by Western blot analysis with 10% SDS-PAGE / 5M Urea gels for transfected cells compared to control cells. The cells were transfected overnight and an equal volume fresh RPMI media was added supplemented with 10% FBS for a 2mL final volume. Cells were harvested 48 h later in PBS + 1mM EDTA, pelleted and resuspended in 0.1 ml Passive Lysis Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and a Berthold Microplate Luminometer LB 96V. Two experimental determinations, with three replicates each, were done for each transfection-hormone combination. Firefly luciferase values were normalized for transfection efficiency using activity of Renilla Luciferase. Values are presented as mean fold activity± (SEM).

Statistical Analysis. For statistical analysis, means were compared using a two-tailed Student's $t$ test. $P < 0.05$ was considered to be significant.
3.3 Results

Accumulation of DHT requires the presence of the androgen receptor. In order to examine the intracellular accumulation of the physiologically active androgen, DHT, a cellular uptake assay was performed using [³H]DHT in AR-deficient HeLa cells, and LNCaP and HeLa FLAG-AR cells that express AR at a level similar to human prostate epithelial cells (Figure 3.2) (Huang et al., 1999). Using these cell lines it was possible to directly assess the effect of AR on cellular steroid accumulation. In LNCaP and HeLa FLAG-AR cells, the accumulation of [³H]DHT in whole cell lysates was approximately 10-fold higher after 24 h than wild-type HeLa cells that do not express AR (Figure 3.2). The addition of unlabelled competitive DHT lead to a dose-dependent displacement of [³H]DHT. However, little change in intracellular [³H]DHT concentration was observed in wild-type HeLa cells with increasing concentrations of unlabelled DHT suggesting that the associated ³H-DHT observed with wild-type HeLa cells may be due to non-specific diffusion and not direct binding competition for the AR. Taken together, these observations suggest that the expression of a functional AR is responsible for the increase in internalization and/or retention of DHT within the cell, more than 10-fold that seen in the wild-type HeLa cells after incubation for 24 h.

The relationship of AR to the intracellular accumulation of DHT in LNCaP and HeLa FLAG-AR cells led to our examination of the time dependent accumulation of [³H]DHT. Figure 3.3 confirms that the presence of AR is required for enhanced DHT accumulation within the cell, and time dependent [³H]DHT accumulation is shown to increase to a maximum between 8-12 h in 3.0 x 10⁵ in LNCaP and HeLa FLAG-AR cells under the conditions used. Conversely, in HeLa cells that lack detectable AR expression, the accumulation of [³H]DHT was 10-fold less and did not increase over time.

Using HeLa cells stably transfected with a FLAG-tagged AR exposed to [³H]DHT it is possible to estimate the percent-bound [³H]DHT–AR complex by immunoprecipitation of the FLAG-AR complex from nuclear extracts. These experiments showed that, interestingly, only
approximately 12% of the \([^3H]DHT\) internalized by the cells appears bound to AR in the nucleus. The unbound \([^3H]DHT\) remains largely in the cytoplasmic fraction (Figure 3.4, A). This estimate is consistent with previous reports, which have estimated approximately \(1.9 \times 10^4 \pm 2.35 \times 10^3\) AR/cell nuclei (Callaway et al., 1982), therefore we would expect a total of \(1.1 \times 10^{10}\) AR molecules from \(6.0 \times 10^5\) cells. Our data suggest \(~3.75 \times 10^{10}\) \([^3H]DHT\) molecules bound to AR, whereas there is approximately 8-fold more \([^3H]DHT\) remaining in the cytoplasmic fraction. These observations suggest that DHT may be held within the cell by macromolecules other than AR, but that AR may be required in order for \([^3H]DHT\) to be efficiently internalized. To ensure that AR protein levels remained constant over the course of the experiment immunoprecipitated FLAG-AR was quantified by Western blot (Figure 3.4, B). These results show that AR protein levels were not altered with increasing DHT concentration and remained relatively consistent between extracts. Taken together, these results suggest a complex relationship between the androgen transport through cell membrane, androgen accumulation in the cell, and the androgen receptor.

**Expression of Pgp detected in LNCaP cells in vitro and in vivo in the LNCaP tumour model during AI prostate cancer progression.** To confirm Pgp expression in LNCaP cells, Western blot analysis was performed, as well as in the LNCaP tumour xenograft model during progression to androgen-independence (Figure 3.5 & 3.6). A panel of three anti-Pgp monoclonal antibodies (C-219, C-494, and JSB-1) that recognize different Pgp epitopes were used in the LNCaP tumour model as an initial antibody screen that had been previously characterized (Georges et al., 1990; Schinkel et al., 1991). Despite conflicting reports of Pgp expression in prostate cancer cell lines (Theyer et al., 1993), our results show that Pgp is expressed in LNCaP cells using the anti-Pgp antibody C-219 which recognizes both human MDR1 and MDR3 Pgp (Figure 3.6). A more biologically significant observation is that Pgp is highly upregulated following castration in the LNCaP tumour xenograft model during progression to androgen-independence (Figure 3.5). This indicates that the removal of...
androgens following castration initiates a cascade of events relating to androgen independent progression that includes the upregulation of Pgp. We propose that Pgp may play an important role in androgen independent tumour progression by diminishing the androgen accumulation in prostate tumour cells, thereby decreasing androgen receptor transcriptional activity, and possibly interfering with androgen induced prostate cell differentiation.

**Specific Pgp inhibitors block Pgp mediated efflux of DHT.** Steroids can serve as substrates for Pgp efflux and/or inhibitors of drug transport (Gruol and Bourgeois, 1997). To determine whether DHT is a substrate for Pgp, cellular accumulation assays were performed with LNCaP and HeLa-AR cells and the synthetic Pgp inhibitor PSC-833 which blocks the Pgp-mediated efflux of Pgp substrates. Final media concentrations of 2 μM PSC-833 were used, and have previously been shown to maximally antagonize the effects of Pgp on substrate accumulation (Krishna and Mayer, 2000). LNCaP cells were chosen because they express Pgp and AR, albeit with slightly altered ligand specificity and reduced affinity for DHT (Issacs, 1996). In LNCaP cells, significant DHT accumulation was detected, however accumulation was lower in LNCaP cells than in HeLa FLAG-AR cells. In Figure 3.7, A, PSC-833 treatment increased the net cell content of [³H]DHT by approximately 50% in wild-type LNCaP cells after 10 h. Similar patterns of increased retention of [³H]DHT with PSC-833 treatment were observed with HeLa-AR cells, indicating that in both cell lines [³H]DHT accumulation is likely affected by the inhibition of the Pgp transport mechanism.

**DHT is a substrate for the Pgp mediated efflux in Pgp transiently transfected LNCaP prostate cancer cells.** Expression of Pgp was enhanced directly through transient transfection of an expression plasmid encoding Pgp (Figure 3.6). We chose to further examine whether DHT could serve as a substrate for Pgp efflux through a series of cellular [³H]DHT accumulation assays using wild-type and transiently transfected Pgp over-expressing LNCaP prostate tumour cells. Since DHT accumulation was observed with a mutated, yet functional AR in LNCaP cells, this led us to investigate whether transient transfection with AR DNA plasmid would enhance
DHT accumulation within the cell. When comparing [3H]DHT accumulation in AR-transfected vs. non-transfected LNCaP cells in Figure 3.7, B, we observed a 2-fold increase in [3H]DHT accumulation. We suggest that this difference could be due to an increased level of the AR, and the higher affinity binding of [3H]DHT to the wild type AR. Concentrations of 0.5nM [3H]DHT used in cellular [3H]DHT uptake assays were comparable to those used in previous in vitro studies, and have been correlated with physiological concentrations.

In addition, experiments were conducted to examine the difference in [3H]DHT accumulation after 12h between AR and Pgp DNA plasmid transfected cells as compared to only AR plasmid transfected LNCaP cells. Results in Figure 3.7, B and 3.8 show a 50% decrease of [3H]DHT in the whole cell lysates after 12 h with AR and Pgp over-expression treated with 0.5nM [3H]DHT. This further suggests Pgp transports DHT. Furthermore, in Figure 3.7, B, we demonstrate that this phenomenon can be significantly inhibited in all transfected cells by treatment with Pgp inhibitor PSC-833. [3H]DHT efflux was significantly inhibited in all transfected and non-transfected conditions suggesting Pgp is largely responsible for the efflux of DHT. Results in Figure 3.9 show that accumulation of [3H]-vinblastine, a known Pgp substrate and used as a positive control, decreased approximately 25% with Pgp transfection. This indicates that by overexpressing Pgp, [3H]-vinblastine efflux increased, as would be expected.

**Aspirin enhances Pgp mediated efflux of DHT.** A previous study has reported that prolonged incubation with aspirin enhances the ability of androgen-responsive prostate cancer cells to resist chemotherapeutic drug exposure by increasing Pgp expression (Rotem et al., 2000). Specifically, in LNCaP prostate tumour cells, the percentage of cells expressing Pgp tripled with dose-dependent aspirin treatment as measured by flow cytometry, and this aspirin-enhanced expression of Pgp returned to normal levels within 3 days following the removal of aspirin (Rotem et al., 2000). Incubating LNCaP cells in 2mM aspirin for 3 days, then measuring Pgp levels by Western blot analysis confirmed this finding. We observed a 75% increase in Pgp
expression after aspirin treatment (Figure 3.6). This interesting finding prompted us to examine whether aspirin treatment could lead to increased DHT efflux from LNCaP cells, thus supporting the hypothesis that Pgp can mediate DHT efflux and may result in interference between steroid hormone action and aspirin intake in target tissues. Following treatment of LNCaP cells with aspirin, we observed a 35-40% decrease in the retention of $[^3]$H]DHT within the aspirin treated cells, as compared to the non-treated cells (Figure 3.10). This suggests that Pgp may be involved in the efflux of DHT from the cell and can be modulated by common medications that may cause transient increases in Pgp expression.

**Over-expression of Pgp causes a suppression of androgen-regulated gene transcription and gene expression.** Pgp expression increases throughout prostate cancer tumour progression towards an androgen independent phenotype (Giménez-Bonafé, et al., 2004; Bhangal et al., 2000). In order to show the biological relevance of our results suggesting DHT transport is mediated by Pgp, we examined the effects of Pgp on androgen-regulated gene transcription using the ARR$_3$-tk-luc response elements in luciferase gene reporter assays. We hypothesized that by up-regulating Pgp and decreasing DHT accumulation in the cell, prostate cancer cells could become less responsive to androgen-regulated transcriptional activity. As shown in Figure 3.11, A, androgen-regulated transcriptional activity is significantly suppressed in the ARR$_3$TK-luc response element by Pgp over-expression, when compared to the empty vector control. As a control, Western blot analysis showed that Pgp expression was upregulated during transient transfection of LNCaP cells (Figure 3.11, B). Using verapamil to block Pgp activity increases androgen responsive transcriptional activity by approximately 2-fold (Figure 3.11, C) Transient transfection with empty vector controls without AR show that there was little androgen-mediated ARR$_3$TK-luc transcriptional response to hormone in the absence of exogenous receptor with increasing concentrations of DHT compared to transfection in the presence of AR DNA plasmid (Figure 3.11, D).
Pgp inhibitor verapamil increases androgen-regulated gene expression. Western blot analysis examining the effect of verapamil on the androgen-responsive gene PSA in LNCaP cells showed that if Pgp was inhibited, PSA expression increased over 25% (Figure 3.12, A). Androgen receptor expression remained constant between treatments (Figure 3.12, B). Verapamil was used as a Pgp inhibitor in these experiments due to the difficulty in obtaining additional PSC-833 from our previous sources.

Similarly, results of PSA mRNA expression in Northern blots showed that PSA expression increased with verapamil treatment (Figure 3.13). Combined together, these results suggest that Pgp can influence the expression of androgen-regulated gene by affecting cellular DHT accumulation and transport. This further provides insight into the physiological consequences of Pgp action on DHT efflux to endogenous androgen action in prostate cancer cells. Pgp has the potential to affect androgen transport and intracellular androgen accumulation, and thereby adversely affect androgen-regulated transcriptional activity and gene expression.
3.4 Discussion

The regulation of the complex interactions of steroid hormones with their target steroid receptors and a plethora of cellular components is of tremendous interest. The cellular response to steroids depends on a variety of factors including the permeability of cell membranes to steroids, and the intracellular interaction between steroids, receptors, and other macromolecules. Pgp is traditionally regarded as an ATP-dependent plasma membrane transporter in chemoresistant cells that is responsible for the efflux of various cytotoxic drugs with a wide-variety of chemical structures. However, Pgp also plays an important role in steroid secretion and we have demonstrated this role in terms of DHT transport. The potential biological consequences of increased Pgp expression during the progression of prostate cancer could therefore have a multi-dimensional role in prostate cancer beyond chemotherapeutic resistance.

In this context, we have found that DHT appears to be a Pgp substrate using [$^3$H]DHT in conjunction with the potent Pgp inhibitor PSC-833 and verapamil. In addition, our results show that the biological consequence of decreased DHT, mediated by Pgp, is a reduced activity of androgen-regulated gene transcription. This observation has led us to hypothesize that in Pgp over-expressing cells, which are present heterogeneously in prostate cancer samples (Kawai et al., 2000), DHT levels may be reduced in subpopulations of those cells and facilitate adaptation to androgen withdrawal prior to castration. Various investigators have speculated that aberrantly expressed growth factors replace the need for androgens during Al progression (Fox et al., 1993; Gil-Diez de Medina et al., 1998; Kaplan et al., al., 1996; Turner et al., 1996; Wang and Wong, 1998) therefore the increased efflux of DHT from prostate cancer cells may be an upstream step in pathways leading to preparing the tumour cell for growth factor activation. Overall, the results of our analysis provide strong support for the proposal that DHT is a substrate for Pgp and thereby modulates AR activity.
Steroids such as aldosterone, hydrocortisone, cortisol, corticosterone and dexamethasone have all been classified as Pgp substrates (Ueda et al., 1992), and on the other hand, progesterone and testosterone do not display significant Pgp transporting activity (Hamilton et al., 2001). Classes of steroids share general chemical structural characteristics, which have been shown to affect their ability to interact with Pgp. These include the presence of at least one planar aromatic ring, incorporation of hydroxyl groups on the steroid ring system at carbons 11, 16, 17, 21, and the number and strength of hydrogen bonds formed between the ligand and Pgp (Gruol et al., 1999; Gruol and Bourgeois, 1997; Hamilton et al., 2001). Previous studies demonstrated that the hydrophobicity of steroids is a strong determinant in their ability to affect Pgp transport (Barnes et al., 1996; Yang et al., 1989) and that carbonyl groups are prevalent in Pgp substrates (Seelig, 1998). Furthermore, it appears that subtle modifications in chemical structure largely affect the ability of steroids to interact and/or be transported by Pgp. Our results suggesting the Pgp efflux mechanism regulates DHT efflux are consistent with research showing hydroxylation at carbon 20 may be important for steroidal binding to this efflux transporter (Figure 3.1) (Hamilton et al., 2001).
Figure 3.1. The chemical structure of 5α-DHT, a novel Pgp substrate.

The active transport of DHT by Pgp may be particularly important for androgen regulation in the prostate and the development and progression of prostate cancer. Elevated Pgp in prostate tumour cells could decrease intracellular androgen levels prior to full androgen ablation providing an adaptive advantage for these cells that have been weaned off androgens. As the tumour cell becomes less dependent on hormonal homeostatic controls, further up-regulation of anti-apoptotic and cell-survival mechanisms may occur. Alternatively, during androgen ablation therapy, Pgp may affect accumulation of anti-androgens in the target tumour tissue that may adversely affect the effectiveness of therapy and further encourage AI progression.

In conclusion, our studies suggest that the cellular accumulation of DHT is AR-dependent and is modulated by the Pgp efflux mechanism across the cell membrane. An increase in Pgp expression or activity causes a decrease in DHT accumulation in the cell and decreases androgen-regulated cellular transcription. We propose that in early stage malignancy Pgp may reduce DHT in the cell and thereby up-regulate cell survival response. This is a double-edged sword because it not only encourages chemotherapeutic resistance, but also a decrease in androgenic response. Further studies of the role of Pgp in malignancy will contribute to our understanding of molecular mechanisms that underlie prostate tumour progression.
Figure 3.2. Cellular $[^3]$H]DHT accumulates in AR positive LNCaP and HeLa-FLAG-AR cells. Cellular $[^3]$H]DHT is also competitively displaced in the presence of increasing unlabelled DHT in LNCaP and HeLa-FLAG-AR cells. 10-fold less $[^3]$H]DHT accumulation is observed in the absence of androgen receptor in wild-type HeLa cells. Cellular $[^3]$H]DHT accumulation is expressed in moles of DHT/cell as an average of three replicates ± SEM. These observations suggest that the expression of a functional AR greatly increases the internalization and/or retention of DHT within the cell.
Figure 3.3. Time and AR dependent accumulation of 1nM $[^3]$H]DHT in LNCaP, HeLa FLAG-AR and wild-type HeLa cells. In HeLa and PC-3 cells with no detectable AR expression, the accumulation of cellular $[^3]$H]DHT was 10-fold less and did not increase over time. Cellular $[^3]$H]DHT accumulation is expressed as average moles of DHT/cell of three replicates at each time point over 24 h ± SEM.
Figure 3.4. A, Comparison of $[^3]$H]DHT bound to AR vs. "free" $[^3]$H]DHT in the cytoplasm in $6.0 \times 10^5$ HeLa FLAG-AR cells over increasing unlabelled DHT titration. These observations suggest that DHT may be held within the cell by macromolecules other than their cognate AR, but that AR may be still required in order for DHT to be internalized. $[^3]$H]DHT accumulation is expressed as the average DPM/well of three replicates at each treatment over 24 h ± SEM. B, Western blot analysis confirming levels of FLAG-tagged AR present in nuclear extracts of HeLa FLAG-AR cells. Results of the immunoprecipitated AR in nuclear extracts showed no change in AR protein expression levels as DHT concentrations were increased.
Figure 3.5. P-glycoprotein is upregulated following castration in the LNCaP tumour xenograft model during progression to androgen independence. Western blot analysis was performed using a 5M Urea 10% SDS-PAGE to detect Pgp expression with a panel of three anti-Pgp monoclonal antibodies (C-219, C-494, and JSB-1) that recognize different Pgp epitopes. As a loading control, mouse monoclonal anti-vinculin antibody was used.
Figure 3.6. Pgp expression in LNCaP cells increases after Pgp plasmid transfection and Aspirin treatment. Pgp protein levels were determined by Western blot using C-219 anti-Pgp antibody. Pgp expression after culturing LNCaP cells in 2mM Aspirin for 3 days (Lane 1), and after transient transfection with 0.25μg (Lane 3) and 0.5μg (Lane 2) of Pgp plasmid. Pgp expression in LNCaP cells cultured under normal conditions were used as a control (Lane 4). As a loading control, mouse monoclonal anti-vinculin antibody was used.
Figure 3.7. A, Pgp inhibitor PSC-833 (2μM) increases intracellular DHT accumulation in LNCaP and HeLa FLAG-AR cells showing that by inhibiting Pgp, [3H]DHT accumulates in AR expressing cells. Cells at a density of 3 x 10^5 cells/well were seeded into wells in both cell lines used. Cellular [3H]DHT accumulation is expressed as the average DPM/well of three replicates at each treatment ± SEM.

B, Pgp inhibitor PSC-833 (2μM) increases intracellular DHT accumulation in LNCaP cells in all transfection treatments. Transfection conditions were: 0.25μg AR plasmid DNA + 0.25μg empty pcDNA 3.1 plasmid; 0.25μg Pgp DNA plasmid + 0.25μg empty pcDNA 3.1 plasmid; and 0.25μg AR plasmid DNA + 0.25μg Pgp DNA plasmid. Cellular [3H]DHT accumulation is expressed as the average DPM/well of three replicates at each treatment ± SEM. *, PSC-833 Pgp inhibitor treatment values are significantly different from control (p<0.05, t-test).
Figure 3.8. [³H]DHT accumulation decreases in AR + Pgp transfected LNCaP cells compared with AR transfection alone. Transfection conditions were: 0.25μg AR DNA plasmid + 0.25μg empty pcDNA 3.1 plasmid; 0.25μg AR plasmid DNA + 0.25μg Pgp DNA plasmid and incubated for 24 h. [³H]DHT was added to the cells and incubated a further 10 h. Compared with AR + empty vector, results show a 50% decrease of [³H]DHT in the cell after 10 h with AR and Pgp over-expression. Thus, Pgp over-expression by transient transfection increases the efflux of intracellular [³H]DHT. Cellular [³H]DHT accumulation is expressed as the average DPM/well of three replicates at each treatment ± SEM. *, Pgp/AR transfected values are significantly different from empty vector/AR transfected control (p<0.05, t-test).
Figure 3.9. Pgp over-expression by transient transfection in LNCaP cells decreases the accumulation of intracellular $[^3]$HVinblastine, as a positive control. Transfection conditions were: 0.25µg empty pcDNA 3.1 plasmid or 0.25µg Pgp DNA plasmid and incubated for 24 h. $[^3]$HVinblastine was added to the cells and incubated a further 10h. Cellular $[^3]$HVinblastine accumulation is expressed as the average DPM/well of three replicates at each treatment ± SEM. *, Pgp transfected values are significantly different from empty vector control (p<0.05, t-test).
Figure 3.10. Aspirin (2mM) treatment in LNCaP cells decreases [³H]DHT accumulation. Therefore, by increasing Pgp expression, treatment with aspirin increases [³H]DHT efflux compared with untreated controls. Aspirin treatment was administered as described in Materials and Methods. Cellular [³H]DHT accumulation is expressed as the average DPM/well of three replicates at each treatment ± SEM. *, Aspirin treatment values are significantly different from no Aspirin control (p<0.05, t-test).
Figure 3.11. Pgp over-expression causes a suppression of androgen-regulated gene transcriptional activity in LNCaP prostate tumour cells. Transfection conditions were carried out as described in Materials and Methods. 

A, Androgen-regulated gene transcription is significantly suppressed in ARR3TK-luc response element by Pgp over-expression. Relative luciferase units represent firefly luciferase activity normalized relative to Renilla luciferase control. Data is expressed as fold activity of three replicates over 48 h ± SEM. *, Pgp transfected treatment values are significantly different from empty vector control (p<0.05, t-test).

B, Pgp expression is enhanced after transfection. Immunoblot probed with anti-C-219 Pgp antibody. 3 min exposure. Lane 1, 0.5µg Pgp plasmid; Lane 2, empty vector plasmid.
Figure 3.11  C, Reporter gene responsiveness to androgen in LNCaP cells transfected with +/- AR, and ARR3-Luc reporter plasmid with Verapamil treatment. Responsiveness increases over 2-fold when Pgp is inhibited with Verapamil. D. Reporter gene responsiveness to androgen in LNCaP cells transfected with +/- AR, and ARR3-Luc reporter plasmid as a positive control. There was no increase in transcriptional response to hormone in the absence of exogenous AR. Relative luciferase units represent firefly luciferase activity normalized relative to Renilla luciferase control, and adjusted firefly luciferase values were plotted relative to the maximal activity, which was set as 100%. Data is expressed as the % relative activity of three replicates over 48 h ± SEM.
Figure 3.12. Treatment with Pgp inhibitor verapamil increases PSA expression while AR expression remains unchanged in LNCaP prostate tumour cells. LNCaP cells were treated, cells lysed, lysates separated by SDS-PAGE, and immunoblotted with anti-AR or anti-PSA antibodies as outlined in Materials and Methods. **A,** Immunoblot analysis for AR - 40min exposure. **B,** Immunoblot analysis for PSA - 40 min exposure. Lane 1, Aspirin; Lane 2, Aspirin and Verapamil; Lane 3, DHT; Lane 4, DHT and Verapamil. Vinculin was used as a loading control.
Figure 3.13. Treatment with Pgp inhibitor Verapamil increases androgen-regulated PSA expression in LNCaP prostate tumour cells. LNCaP cells were treated, mRNA isolated, separated and probed as outlined in Materials and Methods. A, Northern blot – 30min exposure. Lanes 1 and 2, Aspirin and DHT; Lanes 3 and 4, Aspirin, DHT, and Verapamil; Lanes 5 and 6, Aspirin and DCC stripped serum only; Lanes 7 and 8, DHT; Lanes 9 and 10, DHT and Verapamil; Lanes 11 and 12, DCC stripped serum only. Duplicate lanes represent independent samples. B. MS films were scanned and PSA bands quantified by densitometry. The same blot was hybridized with GAPDH3 probe to control for RNA integrity and quantification.
3.6 References


CHAPTER 4. YB-1 IS UPREGULATED DURING PROSTATE CANCER TUMOUR PROGRESSION AND INCREASES P-GLYCOPEPTIDE ACTIVITY

This research in this chapter represents a collaboration by a number of graduate students and postdoctoral fellows at the Prostate Centre, with the majority of the experimental design and work, data analysis performed by Dr. Giménez-Bonafé and myself. Dr. Majid Akabari performed all the YB-1 and P-gp immunohistochemistry, pathological analysis and other consultations. I was responsible for the majority of the animal work, tissue preparation, in vitro assays, and manuscript preparation and editing.

4.1 Introduction

In early stage prostate cancer both surgery and radiation treatment can be curative. However, once the cancer has spread beyond the gland the primary form of treatment is androgen ablation, which induces apoptotic regression of malignant prostate cells as well as the benign secretory prostate epithelial cells (Buttyan et al., 1999; Colombel and Buttyan, 1995). Androgen ablation typically results initially in a reduction in tumour volume, but unlike normal prostate epithelial cells, some malignant cells survive, adapting to the androgen-deprived environment (Craft et al., 1999). These cells ultimately create the Al phenotype, which is characterized in part by the upregulation of genes that initially require androgens for expression but become constitutively re-expressed in the absence of androgens (Gregory et al., 1998). Likely other gene expression pathways are also activated that contribute to the Al phenotype to promote invasion, metastasis and drug resistance.

We have used the LNCaP human prostate tumour model both in vitro and in vivo to characterize changes in gene expression during Al progression. The LNCaP tumour model

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provides a highly reproducible biological system to study the effects of castration on prostate tumour gene expression and the subsequent progression to an Al phenotype as monitored by the production of PSA (Bladou et al., 1997; Gleave et al., 1992; Sato et al., 1996).

To identify changes in gene expression in the LNCaP tumour model cDNA expression arrays were used to analyze a series of LNCaP tumours ranging from pre-castration through progression to Al. One key gene found to be upregulated during tumour progression was YB-1, a transcription factor that binds to inverted CCAAT boxes present in the promoter region of a variety of genes, including MHC class II gene, EGF-R, PCNA, DNA polymerase α, topoisomerase IIα, MDR-1, and MMP-2 (Chernukhin et al., 2000; Diamond et al., 2000; Ise et al., 1999; Lasham et al., 2000; Levenson et al., 2000; Mertens et al., 1998; Raj et al., 1996; Shibao et al. 1999). The cascades of gene expression regulated by YB-1 have been linked to cell proliferation, genotoxic stress, drug resistance, and metastatic invasion (Ise et al., 1999; Levenson et al., 2000; Shibao et al., 1999).

YB-1 is directly involved in MDR-1 gene activation (Asakuno et al., 1994; Ohga et al., 1998) in response to genotoxic stress. MDR-1 gene encodes Pgp, a 170-kDa membrane-spanning cell surface protein of the ABC superfamily that functions in the efflux of a broad class of chemotherapeutics. Pgp is known to regulate the cellular efflux of a wide variety of chemicals, including both natural substances, such as some steroid hormones, and xenobiotics, including some pesticides and a range of pharmaceuticals including many chemotherapeutic agents (Bain et al., 1996; Bain and LeBlanc, 1996; Ernest and Bello-Reuss, 1998; Lanning et al., 1996; Orlowski and Garrigos, 1999). Pgp in humans is located on the secretory surfaces of a number of organs, playing a role in xenobiotic clearance (Horton et al., 1998). Increased Pgp expression has been reported in normal and malignant tissues upon exposure of xenobiotics. Pgp is also localized in hormone-producing and hormone-responsive organs such as the adrenal gland, the testes, and the placenta (Thiebaut et al., 1987) Steroid hormones such as cortisol (van Kalken, 1993), corticosterone (Wolf and Horowitz, 1992), and aldosterone (Ueda et al., 1992) have been
shown to be transported substrates for Pgp. Results in Chapter 3 suggest that Pgp functions to efflux androgens from prostate cancer cells and consequently decrease androgen-regulated gene expression.

In human breast cancers, osteosarcomas, and serous ovarian adenocarcinomas, nuclear localization of YB-1 is closely associated with increased Pgp levels (Bargou et al., 1997; Kamura et al., 1999; Oda et al., 1998). cDNA array hybridization of cell lines that exhibit a multidrug resistance phenotype also show consistent upregulation of YB-1 (Levenson et al., 2000). In human tumour samples, increased expression and nuclear accumulation of YB-1 has been found to be an independent indicator of poor prognosis in osteosarcomas, breast and ovarian cancers and is correlated with elevated Pgp and MMP-2 expression (Bargou et al., 1997; Kamura et al., 1999; Oda et al., 1998).

The results presented in Chapter 4 demonstrate that during prostate cancer progression YB-1 expression increases, and accumulates in the nucleus of malignant cells. Increased levels of YB-1 are also associated with increased Pgp expression. Increased YB-1 levels may decrease intracellular levels of androgens in prostate cells leading to down regulation of androgen regulated gene expression through a Pgp-mediated mechanism. Together these data suggest that YB-1 may facilitate the development of androgen independent progression in prostate cancer and may be of prognostic value in prostate cancer, as has been found in breast cancer, ovarian cancer, and osteosarcomas.

4.2 Materials and Methods

Xenografts. LNCaP tumours were propagated as previously reported (Gleave et al., 1992). Briefly, mice were subcutaneously inoculated by Mary Bowden with $1 \times 10^6$ LNCaP cells and equal volumes of Matrigel. The tumours were excised before castration (PreCx) and at specific time points after castration (dCx), placed in TRIzol Reagent (Life Technologies) or flash frozen and stored at -80°C.
**YB-1 Antibody.** Anti-YB-1 antibody was generated by John Cavanagh at the Prostate Centre at Vancouver General Hospital with synthetic peptides in the COOH-terminal domain (sequence CDGKETKAADPPAENS, residues 299-313 of the protein), and in the N-terminal domain (sequence CRSVGDGETVEFDVVEGEKG, residues 101-119), prepared by the Peptide Service Laboratory at UBC (British Columbia, Canada).

**RNA isolation.** Total RNA was isolated from LNCaP tumours and LNCaP cells using TRIzol Reagent (Life Technologies). Following the protocol from Life Technologies, the tumours were homogenized in 1 ml of TRIzol reagent per 0.1 mg of tissue using a Polytron power homogeneizer (Kinematica). Chloroform extraction was performed at a ratio of 0.2 ml per 1 ml of TRIzol, and samples were centrifuged at 12,000 x g for 15 min at 4°C. The RNA present in the aqueous phase was precipitated by mixing with isopropanol at a ratio of 0.5:1 of TRIzol originally added. Samples were incubated at -20°C for 30 min and pelleted. The pellet was washed with 70% ethanol. The ethanol was removed and the RNA pellet air-dried and reconstituted with diethylpyrocarbonate (DEPC) treated water. The RNA concentration was determined using either fluorimetry or standard A260/A280 ratio with a spectrophotometer.

**YB-1 Cloning.** YB-1 cDNA was obtained by RT-PCR amplification using Pfx (Life Technologies) and 1x enhancer, using the reverse primer 5'ATGAGCAGCGAGGCCGAGACCCAG 3', and the forward primer 5' TTACTCAGCCCCGCCCTGCTCA 3'. The cDNA was cloned into pBluescript, sequence verified, and subsequently subcloned into pRc/CMV, a mammalian expression vector to create pRc/CMV-YB-1. Bob Shukin and Dr. Pepita Giménez-Bonafé created the YB-1 DNA plasmid. All plasmid DNA was propagated in JM109 E. coli and was prepared using QIAGEN Maxiprep Kit (QIAGEN, Mississauga, ON, Canada).

**Cell culture transfections with ³H-vinblastine and ³H-DHT efflux assays.** LNCaP cells were cultured in RPMI 1640 defined medium supplemented with 5% fetal bovine serum (FBS) at a density of 3x10^5 cells/well in 6 well cell culture plates and incubated overnight under standard conditions (37°C, 5% CO₂). Plasmids (pRc/CMV or pRc/CMV-YB-1) were transiently transfected
into cells using LipofectAMINE Reagent (GibcoBRL), each well receiving 0.5 μg of plasmid, in a 2 ml final volume of RPMI. Cells were incubated during 20-24 h under standard conditions. Cells were incubated with 10 nM [³H]-vinblastine sulphate (9.40Ci/mmol, Amersham Pharmacia Biotech) or 1nM [1,2,4,5,6,7-³H(N)]-dihydrotestosterone (125Ci/mmol, Amersham Pharmacia Biotech) in a 2 ml final volume of RPMI supplemented with 5% Dextran-coated charcoal stripped FBS (DCC FBS), and incubated for 24 h under standard conditions. Cells were washed with PBS and harvested into scintillation vials from individual wells. The whole cells were measured for levels of [³H]-vinblastine sulphate or [³H]DHT in a Beckman LS 6500 scintillation counter, and values are presented as the average DPM/well of three replicates at each treatment ± standard error of the mean (SEM).

**Reporter gene assays.** Stock solutions of 5α-dihydrotestosterone (Sigma) were prepared in 95% ethanol and appropriate serial dilutions were prepared in 20% ethanol. Plasmids - The rat AR (Rennie et al., 1993) cloned into the mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA) was used for transfection studies as before (Nelson et al., 1999). The ARR₃tk-luc reporter construct used contains three tandem repeats of -244 to -96 of the 5' flanking region of the rat probasin promoter linked to pT81 vector which contains a thymidine kinase minimal reporter, and the firefly luciferase gene (de Wet et al., 1985; Wood et al., 1984), as described previously (Snoek et al., 1998). The pRL-TK vector contains a thymidine kinase promoter upstream of the modified cDNA encoding *Renilla* luciferase from the sea pansy *Renilla reniformis* (Promega).

For the reporter gene assays, LNCaP cells were cultured as previously mentioned. Plasmids were transiently transfected into the cells using LIPOFECTIN Reagent (GibcoBRL). Each well received 1.5 μg of AR, 0.25μg or 0.5μg YB-1, 0.01μg of pARR₃-tk-luc, and 0.001μg pRL-TK. The cells were incubated overnight and hormone was added in 5% DCC serum-stripped RPMI. To confirm YB-1 overexpression after transfection, LNCaP were cultured in 15cm plates and treated with control (pRc/CMV), 6μg and 9μg YB-1 (pRc/CMV-YB-1).
were incubated overnight and 1nM DHT was added in 5% DCC serum-stripped RPMI. For the transfection experiments, cells were harvested 48 h later in PBS with 1mM EDTA, pelleted and resuspended in 0.1 ml Passive Lysis Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and a Berthold Microplate Luminometer LB 96V. An average of at least two experimental determinations, with three replicates each, were done for each responsive element-hormone combination. Firefly luciferase values were normalized for transfection efficiency using activity of Renilla Luciferase. Values are presented as the mean percent relative luciferase activity of three replicates of each treatment ± standard error of the mean (SEM).

Northern blot and cDNA probes. Twenty micrograms of total RNA was separated by electrophoresis through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Biodyne® B membrane, Pall Corporation, East Hills, NY). Membranes were hybridized to a 32P-labelled human YB-1 or Pgp cDNA probe as required, using ULTRAhyb™ solution (Ambion) following the procedure described by Ambion. Membranes were exposed for autoradiography. The same blots were hybridized with GAPDH3 probe to control for RNA integrity and quantification. Two-tailed t test were performed to obtain p values.

YB-1 cDNA probe was created by Dr. Giménez-Bonafé using PCR to amplify a 406 bp band from YB-1 gene using the reverse primer 5'GCAGGCGAAGTTCCCACCTTACTACA3' and the forward primer 5'TTACTCAGCCCCGCCCCTGCTCA3'. Pgp cDNA probe was created through the digestion of the plasmid pHDR5A (American Type Culture Collection, ATCC) with Eco RI resulting in a 1.38 kb probe.

Tissue microarray slide preparation. Archival formalin-fixed paraffin-embedded LNCaP tumour specimens were obtained from 120 tumours excised from either intact nude mouse or 7, 14, 21, 28 and 35 days after castration. The specimens included 20 samples for PreCx, 7, 14, 21, 28 and 35 days after castration. For human prostate cancer Gleason grade arrays, a total of 400 tumours were arrayed: 34 benign; 70 Gleason grade 2; 235 Gleason grade 3; 34 Gleason
grade 4; and 27 Gleason grade 5. On the human prostate cancer tissue array of hormone naïve and neo-adjuvant hormone therapy (NHT) treated samples, 112 specimens from naïve, and 1-2, 3, 3-6, 8 month NHT and AI patients. These tissue arrays were produced by Dr. Majid Akbari. Two samples per tumour specimen were arrayed. Core tissue biopsy specimens (diameter, 0.6mm) were taken from the regions with less necrosis and hemorrhage of individual paraffin-embedded LNCaP tumours (donor blocks) and precisely arrayed into a new recipient paraffin block (35x25mm) with a tissue arrayer (Beecher Instrument, Silver Spring, MD). After the block construction was completed, 5 μm sections were cut with a microtome by use of an adhesive–coated tape sectioning system (Instrumedics, Hackensack, NJ) to support the adhesion of the array elements.

Immunohistochemistry

**YB-1 immunostaining:** Mounted tissue cores in the microarray slides were de-waxed and rehydrated. Sections were incubated with 10% BSA for an hour; then endogenous peroxidase activity been blocked with 3% H2O2 for 10 min. Antigen retrieval was performed using citrate buffer (pH 6) and steamer. Slides were incubated in a humidified chamber for 2 h at 37°C with a 1:1000 dilution in 1% BSA of YB-1 antibody. Slides were incubated for 15 min with Dako’s envision labeled polymer (Dako, Carpinteria, California, USA). Vector’s Nova Red (Vector Laboratories Ltd, Burlington, ON, Canada) was used for visualization before counterstaining with hematoxylin. Between the experimental steps, sections were washed 3 x 5 min with PBS (pH 7.5). Tissues were covered with mounting media (Permount, Fisher Scientific, Fair Lawn, NJ) and a cover slip.

Negative control slides were processed in an identical fashion to those above, with the substitution of 1% BSA for the primary antiserum. No color reactions were observed in negative control slides. Photomicrographs were taken through a Leica DMLS microscope coupled to a digital camera (Photometrics Cool SNAP, Roper Scientific, Inc., Glenwood, IL) and the corresponding computer software.
**Pgp Immunostaining**: For immunohistochemical staining, the Vector ABC Kit was used. All the steps before adding the primary antibody were the same as the above mentioned. The sections were probed with an antibody raised against the C-terminus intracellular domain of Pgp, C-494 (ID Labs, Inc., London, Canada), overnight at 4°C. C-494 antibody was used because it does not cross react with human MDR3 Pgp, unlike C-219 antibody. Sections were washed in PBS and incubated with anti-mouse biotinylated secondary antibody during 1 h, washed again, stained with Vector Novared during 12 min, and counterstained with hematoxylin during 12 min. Appropriate positive and negative controls were also run. For positive controls, sections of paraffin-embedded normal kidney were stained. For negative controls, the addition of the primary antibody was omitted.

**Scoring of YB-1 Staining.** Scoring has been calculated in the whole area of each core section of the tissue array (0.6 mm). The percentage of the cells that showed positive immuno-reactivity (in nucleus, cytoplasm or membrane) was counted as: number positive cells/number all tumour cells x100. The percentage of positive tumour cells was graded from 0-4 as follows: 0- none-9; 1- 11-33; 2- 34-66; 3- 67-89; 4- 90-100. (The specimen is considered positive if more than 10% of the cells are immuno-reactive). The intensity of the scoring was rated as follows: 0- negative; 1- weak; 2- moderate; 3- intense; 4- very strong. The final score was calculated as the percentage of positive cells x intensity rating. All comparisons of staining intensity and percentages were made at 400x magnification. Simultaneously expression of YB-1 protein was recorded as nucleus, cytoplasm and both. ANOVA was performed using a two-tailed t test. The pathologists Dr. Majid Akbari and Dr. Ladan Fazli evaluated the scoring.

4.3 Results

**Gene expression changes in the LNCaP tumour model**

To investigate changes in gene expression during prostate cancer progression to androgen independence we initiated our studies using the LNCaP tumour model. The use of the LNCaP tumour model as a valid model for the *in vivo* study of the effects of androgen
ablation or chemotherapy in prostate cancer has been previously described (Gleave et al., 1992). Serum PSA levels are directly proportional to tumour volume in non-castrated mice, and both androgens and tumour volume are important co-determinants of circulating PSA levels. Immediately after castration, serum PSA levels decrease rapidly by 80%, without detectable castration induced tumor cell death or concomitant changes in tumour volume. These changes in PSA production in vivo reflect changes in androgen-regulated PSA gene expression. Approximately twenty-one days post castration serum PSA levels begin to increase in the absence of testicular androgens heralding progression to AI and surpass pre-castrate PSA levels by thirty-five days post castration. Paralleling these changes, after castration, tumour volume continues to increase in the absence of testicular androgens. Sustained increases in both PSA levels and tumour growth reflect the onset of AI regulation of PSA gene and AI tumour growth. The LNCaP prostate tumor model also permits the study of the efficacy of castration in combination with treatment of other apoptotic enhancing agents.

Nude mice were inoculated subcutaneously with LNCaP cells and serum PSA production was followed to monitor tumour growth in the intact animal and as a marker of progression to androgen independence following castration. During tumour progression, tumours were harvested pre-castration, and at four to seven day intervals post castration. Tumour sections were used to isolate RNA, proteins, or for formalin fixation for immunohistochemistry.

Changes in gene expression during tumour progression were identified with cDNA expression arrays probed created from RNA extracted from the LNCaP tumours harvested pre-castration and at various times post castration. Of particular focus was the upregulation of transcription factors known to initiate cascades of gene networks that may contribute to the AI phenotype. One of the transcription factors highlighted by the array data was YB-1. Analyses of array images from several series of tumours consistently demonstrated an increase greater than 3-5 fold of YB-1 mRNA expression from pre-castrated tumours to androgen independent tumours at 35 days post-castration.
To confirm upregulation of YB-1 gene expression, YB-1 cDNA probes were cloned and used to derive a more complete progression profile by Northern blot analysis using RNA extracts from LNCaP tumours harvested at several time points post castration. During the course of progression following castration YB-1 mRNA levels gradually increase approximately 5-fold at 35 days post castration (Figure 4.1A).

To investigate if YB-1 upregulation during prostate cancer progression leads to increased expression of downstream target genes we focused on Pgp, a transmembrane ABC transporter (Oda et al., 1998; Ohga et al., 1998). Elevated Pgp activity functions to efflux a broad class of cytotoxic drugs from cells and has been associated with the development of multidrug resistance in a large number of cancers. Northern blot analysis was performed using RNA of LNCaP tumours derived from pre- and post-castrate mice as above (Figure 4.1B). These results demonstrate that Pgp is also upregulated, approximately 4-fold, concomitant with YB-1 upregulation during LNCaP progression. Western blot analysis confirmed the upregulation of P-glycoprotein following castration (Figure 3.4).

Increases in YB-1 and Pgp expression are coordinately linking in prostate cancer progression. To further investigate the functional relevance and potential linkage of YB-1 and increased efflux of Pgp substrates, LNCaP cells were transfected with YB-1 and investigated for changes in the YB-1 expression and efflux activity. A well characterized Pgp substrate, vinblastine, was used to determine if transient overexpression of YB-1 in LNCaP cells leads to an increase in the efflux of vinblastine. LNCaP cells that overexpressed YB-1 were found to retain 40% less vinblastine than the control cells (Figure 4.2B). These results suggest that increased YB-1 levels result in increased efflux a known Pgp substrate potentially by a mechanism which involves Pgp-mediated efflux transport.
Over-expression of Pgp through YB-1 causes a suppression of androgen-regulated gene transcription

Experimental data presented in Chapter 3 shows that increased expression of Pgp also increases the efflux of DHT from prostate cells. To determine if overexpression of YB-1 could likewise cause increased efflux of DHT, we transiently transfected LNCaP cells with YB-1 expression constructs and monitored intracellular DHT accumulation. These data demonstrate that YB-1 overexpression decreases androgen levels in prostate cancer cells (Figure 4.2B). To determine if reduced androgen levels induced by YB-1 resulted in decreased androgen regulated gene activity we repeated the experiments in the presence of an androgen-regulated promoter linked to a luciferase reporter construct. Results indicated that YB-1 overexpression could markedly decrease androgen regulated gene activity in the LNCaP cells (Figures 4.2C-4.2D). To show increased YB-1 expression after transient transfection, Northern blot analysis was performed with a YB-1 mRNA probe (Figure 4.2E). These data taken together suggest that YB-1 upregulates Pgp, which in turn increases the efflux of chemotherapeutic substrates as well as physiological ligands such as DHT, and leads to decreased androgen regulated gene expression.

YB-1 protein expression in LNCaP tissues

To investigate YB-1 protein levels and subcellular localization, immunohistochemistry assays were performed in LNCaP tumours derived from mice prior to castration, and in LNCaP tumours derived from castrated mice (7, 14, 21, 28, and 35 days post castration). LNCaP tumour sections were analyzed for YB-1 using an antibody that recognizes a C-terminus epitope of YB-1. To examine a large number of tumour samples, a tissue array of LNCaP tumours was created and used to evaluate levels of expression and localization of YB-1 protein in tumours during progression to AI. A total of 120 specimens were observed for YB-1 immunostaining on the LNCaP tissue microarray. The mean intensity for 20 PreCx tumours was +1, and for 40 AI tumours (corresponding to 28 and 35 dCx) +2.3, illustrating that the intensity of YB-1
expression increased during AI progression (p<0.001), Figure 4.3. YB-1 intensity was found to increase in a linear manner up to day 14-post castration (R^2 = 0.78) and remained at a plateau throughout progression in the LNCaP model.

**YB-1 levels in human prostate tumours from clinical specimens**

YB-1 protein activity is regulated in part by translocation to the nucleus. It is known that YB-1 in normal tissues is primarily localized in the cytoplasm, and translocates to the nucleus upon genotoxic or other stress signals to regulate genes responsible for cell cycle, DNA repair, and cytotoxic drug efflux. In breast cancer and in osteosarcoma, YB-1 appears largely constitutively localized in the nucleus at advanced stages of cancer (Bargou et al., 1997; Oda et al., 1998). Koike et al. (1997) showed that YB-1 translocates to the nucleus when cells were treated with UV irradiation or anticancer agents. LNCaP tumours were isolated from a lymph node metastasis and represent relatively advanced prostate cancer, therefore it is possible that YB-1 levels may already be increased in LNCaP cells in comparison to benign prostate epithelium. The prognostic relevance of YB-1 in other tumour types and our observations in LNCaP tumours led us to investigate the subcellular localization of YB-1 in benign and malignant glands isolated from clinical prostate cancer samples of organ confined disease.

To determine whether YB-1 levels and localization correlate with Gleason grade progression in prostate cancer, YB-1 staining was evaluated in a tissue microarray of benign, and low (Gleason grade 2), intermediate (Gleason grade 3), and high grade (Gleason grade 4 or 5) prostate cancers. In the benign prostate acini, YB-1 is expressed at a low level and is primarily localized in the cytoplasm of prostatic secretory epithelial cells. Following transformation from benign to malignant, YB-1 expression levels are elevated and demonstrate an increased perinuclear and nuclear content. Prostate adenocarcinoma ranging from Gleason grade 2 to 5 is shown in Figure 4.4A. In Figure 4.5A, 'a' section, a representative section is shown illustrating benign prostate acini. In benign prostate glands YB-1 is localized in the cytoplasm, and the intensity of the staining is low when compared with malignant cells classified as Gleason grade
The staining for YB-1 significantly increases throughout malignant progression increasing with Gleason grade (p<0.001) (Figure 4.4A, 'b'-d' sections, Figure 4.4B). In androgen independent tumours YB-1 protein exhibits also staining located in the nucleus (Figure 4.5A, 'f' section).

To determine if translocation of YB-1 to the nucleus was in response to androgen ablation or exhibited specifically in the androgen independent phenotype we analyzed a tissue array prepared from radical prostatectomy specimens collected following androgen ablation by neo-adjuvant hormone therapy (NHT). Nuclear YB-1 staining was observed in prostate adenocarcinoma cells following NHT therapy (Figure 4.5A, 'b'-'e' sections). YB-1 increased its expression and after 3 months NHT treatment, YB-1 translocated to the nucleus. Staining of tissues obtained from transurethral resections of men with recurrent hormone refractory tumours (Al phenotype) also identified YB-1 levels in the nucleus (Figure 4.5A, 'f' section, Figure 4.5B).

To determine if Pgp was elevated during prostate tumour progression, sections were analyzed by immunohistochemistry for Pgp levels. An antibody that recognizes the C-terminus domain of Pgp was used (C-494). Figure 4.6 shows the localization of Pgp before and after NHT treatment as well as during LNCaP tumour progression. Pgp is low level expressed in the membrane of epithelial cells (Figure 4.6A, 'b'-'e' sections), whereas its expression increases after NHT, concomitant with YB-1 results, and is highly localized in the membrane surface of Al tumours (Figure 4.6A, 'f' section). Together with the Northern and Western results, the immunohistochemistry experiments realized in the LNCaP tumour series show Pgp increasing after castration and during the progression to Al (represented by the 35 dCx), Figure 4.6B, 'a'-'d' sections.

In summary, during prostate cancer progression YB-1 is upregulated at the RNA and protein levels. Increased YB-1 expression correlates with elevated Pgp in LNCaP tumours and in human prostate tumours similar to reports in other cancer types. Increased Pgp levels may be
one mechanism which leads to a decrease of intracellular DHT accumulation in LNCaP cells. Androgen-regulated gene expression is decreased by overexpression of YB-1 and Pgp in prostate cancer cells. Immunohistochemistry data from tissue microarrays from clinical prostate cancer samples demonstrated that YB-1 levels increased in expression with Gleason-grade progression in prostate cancer, and intensifies in nuclear staining following androgen withdrawal and during AI progression.

4.4 Discussion

Our data demonstrates that prostate cancer progression, like breast cancer, ovarian cancer and osteosarcoma, is associated with increased levels and nuclear translocation of YB-1 (Bargou et al., 1997; Kamura et al., 1999; Oda et al., 1998;). A recent publication (Janz et al., 2002) shows that in breast cancer, elevated levels of YB-1 are correlated with poor patient outcome, and 66% of patients with high YB-1 expression have relapsed within 5 years of receiving postoperative chemotherapy, whereas no relapse was found in patients with low YB-1 expression. These authors suggest that YB-1 expression has a prognostic and predictive significance in breast cancer. In vitro YB-1 has been demonstrated to directly regulate the MDR-1 promoter (Bargou et al., 1997; Kohno, 1997; Ohga et al., 1998). In colon carcinoma, YB-1 has also been shown to be involved in regulating the transcription of the Multidrug Resistance associated Protein-1 (MRP-1) gene (Stein et al., 2001), another ABC-transporter protein. In clinical samples, nuclear expression of YB-1 has been correlated with MDR-1 gene expression in breast cancer and osteosarcoma, and has been reported to be more highly concentrated in cisplatin-resistant cell lines than in their parental counterparts (Ohga et al., 1996). Ohga et al. (1996) have shown that YB-1 antisense could increase the sensitivity of human cancer cell lines to cisplatin. Kamura et al. (1999) suggested that nuclear localization of YB-1 might be a useful prognostic marker associated with MDR phenotype in breast cancer and osteosarcomas. In colon carcinoma cells, hyperthermia causes YB-1 translocation from the cytoplasm into the nucleus, and its nuclear translocation is associated with increased MDR-1 and MRP-1 gene
activity, reflected in increased transport activity (Stein et al., 2001). In non-small cell lung cancer nuclear expression of YB-1 may be considered as a novel marker of disease (Shibahara et al., 2001). Patients with nuclear YB-1 staining have a poorer prognosis than do those with cytoplasmic YB-1 staining.

Our results show that YB-1 becomes overexpressed during prostate cancer progression and translocates to the nucleus following androgen ablation and in the AI phenotype. Kawai et al. (2000) investigated the levels of Pgp in prostate cancer cell lines and found that Pgp activity is inversely correlated to chemosensitivity. MRP-1 can also confer cellular resistance to a variety of structurally unrelated cytotoxic drugs and both MDR-1 and MRP-1 have been implicated as resistance mediators in prostate cancer (Kawai et al., 2000; Van Brussel et al., 2001; Zalcberg et al., 2000). In the LNCaP tumour model, increased YB-1 expression correlates closely with upregulation of the MDR-1 gene product, Pgp. Advanced prostate cancer does not respond well to chemotherapeutic treatment in part due to the low proliferative index and an inherent lack of sensitivity to many anticancer drugs. Some tumours, including colon, kidney and liver cancer, are known to intrinsically express high levels of Pgp, and are consequently resistant to a variety of anti-cancer drugs. Other malignancies such as breast cancer and lymphoma are typically initially chemo-responsive, but acquire drug resistance after therapy through increased Pgp expression (Keith et al., 1990; Sanfilipo et al., 1991).

Our studies suggest that YB-1 may be functionally significant in prostate cancer progression and chemotherapeutic resistance by influencing upregulation of Pgp. A wide variety of Pgp substrates, including a number of steroid hormones and structurally-related pharmaceuticals, may be transported by Pgp mechanisms in prostate cancer cells. Therefore, elevated Pgp activity may affect therapeutic efficacy of particular anti-androgen based therapies in prostate cancer treatment. Furthermore, a number of commonly used over the counter medications, such as aspirin increase Pgp activity and have been shown in vitro to decrease intracellular
androgen levels in prostate cancer cells (Chapter 3). The impact of these potential drug-androgen interactions warrants further investigation.

Our results show that after androgen ablation, YB-1 is highly expressed and it translocates to the nucleus. In AI patient tumours, the tumours are largely unresponsive to chemotherapy, consistent with YB-1 nuclear accumulation and increased Pgp expression. Our data also shows that expression of YB-1 and its translocation from the cytoplasm to the nucleus increases in prostate cancer cells after NHT (Figure 4.5). In LNCaP cells, an upregulation of YB-1 increases lead to an increased efflux of the Pgp substrate vinblastine (Figure 4.2). Likewise we have found that overexpression of YB-1 increases efflux of the endogenous androgen DHT from prostate cancer cells resulting in decreased androgen gene regulation (Figure 4.2).

From these observations we hypothesize that upregulation of YB-1 in prostate cancer progression may result in decreased intracellular androgen accumulation and androgenic response in malignant prostate cells. In support of this hypothesis it is known that malignant prostate cells express lower levels of the androgen-regulated gene PSA in comparison to benign secretory epithelial cells although the mechanism has not been explored. In contrast to complete acute androgen ablation which induces apoptosis of prostate epithelial cells, gradually decreased intracellular androgen levels during prostate cancer progression may wean prostate cancer cells off androgen dependency and upregulate cell survival mechanisms. Prostate cancer cells adapted to lower intracellular androgen levels may therefore be more apt to survive upon androgen ablation and create the AI phenotype as further anti-apoptotic and proliferative pathways are upregulated. Understanding these complex relationships requires further investigation.
4.5 Figures

Figures

PreCx  4dCx  7dCx  13dCx  14dCx  21dCx  26dCx  27dCx  28dCx  35dCx

A

YB-1 mRNA

B

P-gp mRNA

C

GAPDH3

Figure 4.1. Northern blot analysis of YB-1 and Pgp during LNCaP tumour progression.

Total RNA from a tumour series (20 μg/lane) was loaded into a 1.2% formaldehyde/agarose gel. The membrane was exposed for 2 h (A) and overnight (B) on MR film. A, The mRNA for YB-1 increases over 400% after castration. B, During the course of progression there is an over 300% increase of Pgp mRNA post castration. C, GAPDH3 was used as a loading control and this membrane was exposed for 1 h. Membrane quantification was based on a 100% mRNA expression in pre-castration (PreCx) tumours.
Figure 4.2. The effect of YB-1 overexpression on DHT efflux and androgen-regulated transcriptional activity. A, LNCaP cells were transiently transfected with YB-1 (pRc/CMV-YB-1), and the plasmid control (pRc/CMV). Next, they were incubated with 10 nM of $^3$H-vinblastine (a Pgp substrate). After 24 h, the cells were harvested and whole cell lysates were counted on a liquid scintillation counter. The cells overexpressing YB-1 retain less $^3$H-vinblastine than the control cells, suggesting increased efflux activity of Pgp. B, YB-1 overexpression decreases
DHT levels in LNCaP cells. The same experiment as performed in A (above) was carried out but this time the cells were incubated with 1 nM $^3$H-DHT. C, Androgen-regulated reporter gene responsiveness to androgen in LNCaP cells transfected with YB-1, AR and ARR$_3$-Luc reporter plasmid. LNCaP cells transfected with control (pRc/CMV), 0.25µg, and 0.5µg YB-1 (pRc/CMV-YB-1) and exposed to a titration of DHT. D, Reporter gene responsiveness to androgen in LNCaP cells transfected with +/- AR, and ARR$_3$-Luc reporter plasmid as a positive control. E, Northern blot of YB-1 expression in LNCaP cells transfected with YB-1 plasmid. LNCaP cells transfected with control (pRc/CMV), 6µg and 9µg YB-1 (pRc/CMV-YB-1). For C and D, Relative luciferase units represent firefly luciferase activity normalized relative to Renilla luciferase control. Data expressed as the mean percent relative luciferase activity of three replicates over 48 hours ± SEM.
Figure 4.3. Immunolocalization of YB-1 in the LNCaP tumour model. Different sections of LNCaP tumours corresponding to pre-castrated and post-castrated mice were arrayed (see Material and Methods) and stained for YB-1 using an antibody that recognizes an epitope in the C-terminus of the protein. The degree of staining was quantified, +1 for low degree and +4 for high, and graphed. ANOVA: p<0.001.
Figure 4.4. YB-1 immunostaining in human prostate tumour samples. YB-1 increases increasing Gleason grade and accumulates in the nucleus after androgen withdrawal treatment. A, A selection of tumour sections from different stages of Gleason grade were arrayed and stained for YB-1. a- Gleason grade 2 tumour. b- Gleason grade 3 tumour. c- Gleason grade 4 tumour. d- Gleason grade 5 tumour. The presence of YB-1 is increased in the cytoplasm and translocates to the nucleus with grade progression. B, The degree of staining was quantified as above, and graphed. ANOVA: p<0.001. x400.
Figure 4.4. YB-1 immunostaining in human prostate tumour samples. YB-1 increases increasing Gleason grade and accumulates in the nucleus after androgen withdrawal treatment. 

B, The degree of staining was quantified as above, and graphed. a- Benign prostate b- Gleason grade 2 tumour; c- Gleason grade 3 tumour; d- Gleason grade 4 tumour; e- Gleason grade 5 tumour. The presence of YB-1 is increased in the cytoplasm and translocates to the nucleus with grade progression. ANOVA: p<0.001. x400.
Figure 4.5. YB-1 expression increases after NHT and YB-1 translocates to the nucleus. The transcription factor is present in the cytoplasm of benign prostate epithelial cells, as well as in non-treated tumours, and its expression increases after NHT treatment while YB-1 translocates to the nucleus. a- Benign prostate. b- Non-treated tumour. c- 1-2 months after NHT. d- 3 months after NHT. e- 3-6 months after NHT. f- Al tumour. Indicated by arrows is the localization of YB-1 in the nucleus. In the Al phenotype (f) YB-1 is localized in the nucleus. All images are displayed at x400 magnification.
Figure 4.5. Degree of Immunolocalization of YB-1 after NHT. B, YB-1 expression increases after NHT and YB-1 translocates to the nucleus. The degree of staining was quantified, +1 for low degree and +4 for high, and graphed. c- 1-2 months after NHT. d- 3 months after NHT. e- 3-6 months after NHT. f- Al tumour. Indicated by arrows is the localization of YB-1 in the nucleus. In the Al phenotype (f) YB-1 is localized in the nucleus. ANOVA: p<0.001.
Figure 4.6. P-glycoprotein is overexpressed during prostate cancer tumour progression to Al stage. A, Pgp accumulation before and after androgen ablation by neo-adjuvant hormone treatment (NHT). a- benign prostate. b- tumour before NHT. c- 1 month after NHT. d- 3 months after NHT. e- 8 months after NHT. f- AI tumour. Pgp is highly expressed in the membrane of AI
cells as well as in the cytoplasm (f). B, Pgp accumulation in the LNCaP tumour model. a- intact tumour (not castrated). b- 7 dCx. c- 21 dCx. d- 35 dCx. YB-1 increases its expression in the cellular membrane (see brown staining in the membrane surface) during the progression to the AI stage indicated by 35 dCx. All images displayed at x400 magnification.
4.6 References


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CHAPTER 5.  *IN VITRO AND IN VIVO INHIBITION OF PROSTATE CELL GROWTH USING ANTISENSE OLGONUCLEOTIDES TARGETING YB-1*  

In collaboration with the laboratory of Dr. Helen Burt in the UBC Department of Pharmaceutical Sciences and ISIS Pharmaceuticals (California), the data presented in this chapter is a result of *in vitro* experiments done together with Dr. Giménez-Bonafé, and *in vivo* experiments and data analysis conducted by myself. Helpful advice was provided by Dr. Gleave's laboratory staff. Dr. Giménez-Bonafé conducted the Northern blot analysis of LNCaP tumours. I was responsible for all the *in vitro* MTT assays and all data analysis. Wesley Sydor assisted with the animal experiments and Dr. John Jackson provided the *in vivo* polymeric paste. The publication was drafted and edited by myself with helpful input from all the authors.

5.1 **Introduction**

More than 25 years ago, Zamecnik and Stephenson (1978) first proposed using synthetic antisense oligonucleotide (ASO) analogs as a new class of rationally designed therapeutics capable of specifically inhibiting the synthesis of a chose target protein (Zellweger, et al., 2001). ASOs are chemically modified stretches of single-stranded DNA that are complementary to mRNA regions of a target gene, and they can effectively inhibit gene expression by forming RNA/DNA duplexes. Recent publications demonstrate the effects of several ASOs specifically targeted against genes involved in neoplastic progression both *in vitro* and *in vivo* as potential therapeutic agents (Gleave, et al., 1999; Leung, et al., 2001; Monia, et al., 1996; Miyake, et al., 2000; Rocchi, et al., 2004; Springate et al., 2005). Furthermore, many recent studies demonstrate that adjuvant treatment with antisense ASOs targeted against Bcl-2, clusterin, insulin-like growth factor binding protein 2, insulin-like growth factor binding protein 5, and hsp27 can significantly reduce mRNA levels and delay progression to androgen

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Two important factors influencing efficacy of an ASO are the affinity for targeted mRNA to bind with a high degree of specificity and its ability to resist degradation by intracellular nucleases. First-generation phosphodiester (P=O) ASO were highly unstable to nucleases, largely precluding their use to efficiently inhibit the expression of a targeted mRNA. In a first step to increase nuclease resistance, an equatorial oxygen atom in the phosphate backbone of P=O ASO was replaced by a sulfur atom. This phosphorothioate (P=S) modification provided considerable stability to both exo- and endonucleases (Wagner, 1994). However, each incorporation of a P=S generates a chiral center and reduces binding affinity for target mRNA. Furthermore, although P=S ASOs are less sensitive to nucleases, they will degrade in cells over time (McKay et al., 1996). To overcome these drawbacks, ongoing research has focused on backbone modifications that provide a more attractive pharmacological profile than P=S ASO. Among a number of different modifications at the 2'-sugar position, the 2'-O-(2-methoxy)ethyl (2'-MOE) incorporation was identified as enhancing both binding affinity and further resisting degradation by intracellular nucleases (Altmann et al., 1996). The 2'-MOE modification resulted in decreased binding affinity to RNase H, the principal nuclease that cleaves ASO-bound mRNA. This problem was overcome by the use of "gapped" ASO such that the 5' and 3' ends of the molecule contained 2'-MOE-modified sugar residues and the central portion of the ASO contained 2'-deoxy sugar residues that support RNase H activity (Monia et al., 1993; Baker et al., 1997). This chemical design is usually accompanied with a uniformly modified P=S backbone. The incorporation of 2'-MOE modifications into 20-mer P=S ASO showed a dramatic effect on the ability of the sequence to hybridize to a target mRNA as a result of the conformation of the sugar and the backbone. Furthermore, 2'-MOE incorporation into P=S ASO exhibited substantially increased resistance to intracellular nucleases, compared with conventional P=S ASO. Both increased hybridizing affinity toward the targeted mRNA and
enhanced resistance toward both serum and intracellular nucleases resulted in a 20-fold increase in activity of 2'-MOE-modified ASO (McKay et al., 1999). The enhanced potency of this new class of ASO did not lead to any decrease in specificity.

YB-1 has been characterized as a transcription factor and has been reported to induce numerous tumour-associated genes that promote drug resistance, tumour growth and conversely, repress pro-apoptotic genes. These include activation of the drug resistance and growth promoting genes, such as MDR-1, heat shock protein 70, annexin I, HER-2, epidermal growth factor receptor (EGFR or c-ErbB1), matrix metalloproteinase-2 (MMP-2), proliferating cell nuclear antigen (PCNA) and DNA topoisomerase II. Pro-apoptotic gene repression has been demonstrated with MHC Class II, fas ligand, grp78, and collagen α1 (Lasham et al., 2000; Mertens et al., 1998; Ohga et al., 1998; Shibao et al., 1998; Sakura et al., 1988). Elevated levels of YB-1 may facilitate tumour cell invasion and metastasis by the activation of MMP-2, and as a promoter of EGFR and mdrl gene expression, YB-1 might enhance cell growth and resistance to chemotherapeutic agents. Formation of heterodimeric complexes between YB-1 and other oncoproteins such as p53 (Lasham et al., 2003), AP-1 (Samuel et al., 2005), AP-2 (Yoshitomi et al., 1999), Smad3 (Higashi et al., 2003) and CTCF (Chernukhin et al., 2000) has been suggested as a mechanism in which YB-1 regulates gene expression. Using this mechanism, YB-1 may function as a dominant-negative regulator of oncogenes, encouraging tumour development when highly expressed (Lasham et al., 2000). In neoplasia, YB-1 is associated with multidrug resistance by inducing the MDR-1 gene (Ohga et al., 1998) and apoptosis (Kuwano et al., 2003; Kuwano et al., 2004; Swamynathan et al., 2002) and has been functionally linked to increased tumourigenicity and treatment resistance in breast (Holm et al., 2004; Saji et al., 2003) and colon (Ohga et al., 1996; Shibao et al., 1999) cancers.

Increased YB-1 expression during progression to AI suggests that YB-1 may confer resistance to androgen withdrawal by enhancing cell proliferation. Recent studies have shown that YB-1 can also inhibit the ability of p53, the tumour suppressor gene, and fas, a cell death
associated receptor, to cause apoptosis (Lasham et al., 2000; Okamoto et al., 2000; Zhang et al., 2003). Blockage of apoptosis is important in Al prostate cancer and is associated with the differential expression of cell survival genes like Bcl-2 and clusterin (Gleave et al., 2002), and YB-1 protein may interact with a DNA intron region of the clusterin gene (Nelson, unpublished data). This is significant because it suggests that YB-1 may be a key regulatory protein in the induction of the cellular stress response activating cell survival pathways. Several studies have reported that YB-1 is predominantly localized to the cytoplasm in both normal and tumour cells, but can translocate to the nucleus in response to environmental stresses, including DNA-damaging agents, UV irradiation, viral infection, and hypothermia (Stein et al., 2001). Nuclear localization of YB-1 protein has also been associated with poor patient prognosis in some types of human cancer, including lung cancer, synovial sarcoma (Gessner et al., 2004; Oda et al., 2003). YB-1 is a predictor of poor outcome and response to therapy in breast cancer (Saji et al., 2003), but has not been extensively studied in prostate cancer.

Direct local injection with therapeutic agents capable of inhibiting cellular proliferation is feasible due to the anatomical location of the prostate gland. For example, brachytherapy for localized prostate cancer is now routinely performed by transperineal placement of radioactive seed implants under transrectal ultrasound guidance. It is conceivable that the prostate gland could also be infiltrated with chemotherapeutics and ASO aimed at the selective inhibition of specific proteins and pathways using similar techniques. In previous studies, the cytotoxic effects of site-directed paclitaxel delivery using a variety of biodegradable polymeric paste formulations have been described (Jackson et al., 2000; Jackson et al., 2004; Springate et al., 2005). The controlled-release formulation can be easily injected via a 22-gauge needle and has been shown to be effective in inhibiting LNCaP tumour growth and PSA levels in mice bearing multiple non-metastatic tumours (Jackson et al., 2000; Springate et al., 2005). Similarly, the therapeutic efficacy of intratumoural delivery of ASOs against the cell survival gene clusterin in an injectable, biodegradable polymeric paste formulation was evaluated against subcutaneously
grown LNCaP tumours in mice. In this study, we report the therapeutic effects of intratumoural delivery of both paclitaxel and an ASO directed at the YB-1 target injected together in a novel formulation of biodegradable polymeric paste.

Accumulating evidence links rising YB-1 levels with AI and the development of chemotherapeutic resistance, marking YB-1 as a potential therapeutic target. However, the functional significance of changes in YB-1 associated with drug resistance and AI prostate cancer progression has not been investigated. ASOs, short synthetic stretches of chemically modified DNA capable of specifically hybridizing to the mRNA of a chosen cancer-relevant target gene. These agents promise enhanced specificity for malignant cells with a favorable side-effect profile due to well-defined and tailored modes of action. Phosphorothioate ASOs are stabilized to resist nuclease degradation and a new generation of 2'-MOE-modified phosphorothioate ASOs showed dramatically improved effects on hybridization with target mRNA and enhanced resistance to intracellular nucleases as a result of the sugar and the backbone modifications (Zellweger et al., 2001). The objective of Chapter 5 was to investigate the effect of YB-1 ASO on cellular growth and chemosensitivity in vitro, and in vivo delivered both systemically and by intratumoural injection of a novel controlled release polymeric paste formulation containing 2'-MOE-modified YB-1 ASOs and paclitaxel on serum PSA levels and LNCaP tumour progression in mice.

5.2 Materials and Methods

Animals and Cell Lines. Male 6-8 week old athymic nude mice (BALB/c strain) were purchased from Charles River Laboratory (Montreal, Quebec, Canada). LNCaP cells were maintained in RPMI 1640 (Invitrogen, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada) as described previously (39).

Chemotherapeutic Agents. Paclitaxel was purchased from Biolyse Pharma (St. Catharines, ON, Canada). Stock solutions of paclitaxel were prepared with sterile PBS to the required concentrations before each in vitro experiment. Dr. Helen M. Burt and Dr. John Jackson
Pharmaceutical Sciences, University of British Columbia (UBC), Vancouver, Canada) generously supplied the polymeric micellar paclitaxel and paclitaxel paste formulation used for \textit{in vivo} studies.

**Polymeric paste formulation.** Water soluble chitosan (protosan, FMC Biopolymer Drammen Norway), YB-1 or scramble oligonucleotide, paclitaxel (Hauser Chemical Company, Boulder, CO) and Polyethylene glycol 600 (PEG 600) (Union Carbide Danbury, CT) were blended together for 15 minutes in a weight ratio of 6%:3%:1%:90% respectively. The paste was sucked into 1 ml syringes and stored at 4\(^\circ\)C until use. Control paste contained PEG 600 only. This formulation is a viscous liquid at room temperature and was designed to be injected intratumourally in mice containing two LNCaP tumours, where it sets to a semi-solid implant within 1h at the tumour site (Springate et al., 2005).

**YB-1 ASO.** 2'-MOE-modified YB-1 and scramble oligonucleotides were kindly provided by Dr. Brett Monia (ISIS Pharmaceuticals (Carlsbad, CA) for the \textit{in vitro} and \textit{in vivo} studies. The 2'-MOE-modified YB-1 ASO sequence used was: No.3, 5'-CTTGTTCTCCTGCACCCTGG-3'. The 2'-MOE-modified control scramble oligonucleotide sequence used was 5'-CAGCGCTGACAACAGTTTCAT-3'.

**Treatment of Cells with Oligonucleotides.** Cells were plated at the density of \(8 \times 10^3\) cells per well in 96-well format or \(2 \times 10^5\) in 6-well format and treated one day later for 2 days with respective oligonucleotide. Oligofectamine (Invitrogen, Burlington, ON, Canada), a cationic lipid, was used to transfect the respective oligonucleotide into the cells. PC-3 cells were treated with various concentrations of YB-1 ASO or scramble oligonucleotide control after a pre-incubation for 20 min with 3mg/mL oligofectamine in serum-free Opti-MEM (Invitrogen, Burlington, ON, Canada). Four hours after the beginning of the incubation, the medium was replaced with standard culture medium as described above.

**Northern Blot Analysis.** Total RNA was isolated from LNCaP tumours using TRIzol Reagent (Invitrogen, Burlington, ON, Canada). Following the protocol from Life Technologies, the
tumours were homogenized in 1 ml of TRIzol reagent per 0.1 mg of tissue using a Polytron power homogeneizer (Kinematica). Chloroform extraction was performed at a ratio of 0.2 ml per 1 ml of TRIzol, and samples were centrifugated at 12,000xg for 15 min at 4°C. The RNA present in the aqueous phase was precipitated by mixing with isopropanol at a ratio of 0.5:1 of TRIzol originally added. Samples were incubated at -20°C for 30 min and pelleted. The pellet was washed with 70% ethanol. The ethanol was removed and the RNA pellet air-dried and reconstituted with diethylpyrocarbonate (DEPC) treated water. The RNA concentration was determined using either fluorimetry or standard A260:A280 ratio with a spectrophotometer. Twenty µg of total RNA was separated by electrophoresis through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Biodyne® B membrane, Pall Corporation, East Hills, NY). Membranes were hybridized to a 32P-labelled human YB-1 or Pgp cDNA probe as required, using ULTRAhyb™ solution (Ambion) following the procedure described by Ambion. Membranes were exposed for autoradiography. Densities of bands for YB-1 were normalized against GAPDH3 by densitometric analysis to control for RNA integrity and quantification.

YB-1 cDNA probe was obtained using PCR to amplify a 406 bp band from YB-1 gene using the reverse primer 5'GCAGGCGAAGTTCCCACCTTACTACA3' and the forward primer 5'TTACTCAGCCCCGCCCTGCTCA3'.

**MTT Assay.** The in vitro growth inhibitory effects of 2'-MOE-modified YB-1 ASOs plus paclitaxel on PC-3 cells was compared using the MTT assay as previously described (Zellweger, et al., 2001). Briefly, 8 x 10³ cells were seeded in each well of a 96-well microtiter plate and allowed to attach overnight. Cells were then treated once daily with 200nM of 2'-MOE-modified YB-1 ASO, or 2'-MOE-modified scramble oligonucleotide control, respectively, for 2 days. Following ASO treatment, cells were treated with various concentrations of paclitaxel. After 48 h of incubation, 20 µL of 5 mg/mL MTT (Sigma Chemical Co., St. Louis, MO) in PBC was added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved in dimethyl sulfoxide. The optical density was determined with a microculture plate reader (Becton
Dickinson Labware, Mississauga, ON, Canada) at 540nm. Absorbance values were normalized to vehicle-treated cells to determine the percentage of survival. Each assay was performed in triplicate.

**Protein extraction.** Total proteins were obtained homogenizing LNCaP tumours in RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing protease inhibitor cocktail (Roche), and incubating the lysate at 4°C during 30 min. After a 5 min spin at maximum speed, the proteins were recovered in the supernatant and quantified using the BCA assay (Pierce).

**Western blot analysis.** For the detection of YB-1, 30mg of total proteins from LNCaP tumours were resolved on 10% SDS-PAGE gels containing 5M urea. The proteins were transferred onto PDVF membranes (Immobilon'-P, MILLIPORE) at 400 mA during 1 h. Membranes were blocked during 2 to 3 h at 25°C in blocking buffer (5% dry nonfat milk in TBS; 1.37M sodium chloride, 200 mM Tris, pH 7.6) and incubated at 25°C for 1.5 h with YB-1 antibody in 1% bovine serum albumin (BSA) and 1% dry nonfat milk. After several washes with TBS-T (0.05% Tween-20 in TBS) blots were incubated with the secondary antibody, peroxidase linked anti-mouse immunoglobulin in 1% BSA-1% dry nonfat milk, for 45 min at 25°C and then washed again extensively with TBS-T. Blots were placed in enhanced chemiluminescence (ECL) reagents (Pharmacia-Amersham) for 1 min, followed by exposure to autoradiographic MR film (Kodak). The antibody dilution used was as follows: 1:5000 for YB-1, and 1:10,000 for the secondary antibody. Anti-vinculin antibody (DAKO) was used to normalize the loading (1:2000 dilution).

**Inoculation of LNCaP Cells.** Approximately $1 \times 10^6$ human LNCaP cells were inoculated subcutaneously with 0.1mL of Matrigel (Becton Dickinson Labware, Mississauga, ON, Canada) via 27-gauge needle into the flank region of male 6-8 week old athymic nude mice under halothane anesthesia (5% induction, 1.5% maintenance concentration). Tumour volume measurements were performed twice weekly using calipers, and their volumes were calculated using the formula: length $\times$ width $\times$ height $\times$ 0.5236 (42). All animal procedures were performed
according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

**Determination of Serum PSA Levels.** Blood samples were obtained by tail vein incision of mice weekly as described previously (Gleave et al., 1992). Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2ng/mL (Abbott IMX, Montreal, PQ, Canada) according to the manufacturer’s protocol. Fifteen µL of mouse serum were diluted with 135µL of diluent to perform the assay; the lower limit of sensitivity in this murine model was 2ng/mL.

**In Vivo Treatment Protocols.** Six weeks after injection, LNCaP tumour volume reached between 500-600mm$^3$ and serum PSA levels were approximately 20-30 ng/mL, mice were anesthetized using halothane and castrated via abdominal approach (Sato et al., 1997).

In an initial experiment, mice were randomized into one of two arms for treatment with 2'-MOE-modified scramble oligonucleotide control or 2'-MOE-modified YB-1 ASO. Each treatment group consisted of at least 6 mice. After randomization, 12.5mg/kg of YB-1 ASO or scramble oligonucleotide control was injected intraperitoneally once daily into each mouse for the first five days beginning 4 days after castration, and three times per week up to 28 days beginning at day three post-castration. Tumour volume and serum PSA levels were measured as described above. Data points for both sets of experiments were expressed as average % tumour volume levels compared to Day 0 ± SEM.

In a second set of experiments, mice were randomized into one of three arms for intratumoural treatment with control paste, 2'-MOE-modified scramble oligonucleotide control plus paclitaxel paste, and 2'-MOE-modified YB-1 ASO plus paclitaxel paste. At day three post-castration, mice in all treatment groups were treated with a single intratumoural injection in a ratio of 1µL paste to 10mm$^3$ tumour of control paste or 3% ASO-loaded 1% paclitaxel-loaded paste. Tumour volume and serum PSA levels were measured as described above for a total of seven weeks.
**Statistical Analysis.** All of the results were expressed as the mean ± SEM. Statistical analysis was performed using Microsoft Excel software by a one-way ANOVA followed by a Student's t-test. *P*≤0.05 was considered significant (*).

**5.3 Results**

**Dose-dependent knockdown of YB-1 expression by ASO.**

It has been further hypothesized that YB-1 acts to control the expression and function of proteins involved in drug resistance and tumour growth (Kuwano et al., 2004). We hypothesized that YB-1 actively participates in the progression of the malignant phenotype and promotes the survival of prostate cancer cells. Specific targeting of YB-1 expression using ASO may act to suppress aberrant cellular growth and ultimately delay Al progression. Firstly, to evaluate the effects of sequence-specific and dose-dependent ASO treatment on YB-1 mRNA expression in LNCaP cells we conducted Northern blot analysis (Figure 5.1). As shown in Figure 5.1, YB-1 ASO decreased YB-1 mRNA levels in a dose-dependent manner, significant suppression was seen at 50nM, and YB-1 mRNA expression levels were almost absent with the 1000nM dose. In contrast, YB-1 mRNA expression was not affected with scramble control oligonucleotide after 2 days treatment at any of the used concentrations. Oligofectamine treatment alone was used as a comparison for non-specific mRNA suppressive effects of the ASO treatment.

**Effect of YB-1 ASO treatment on cell viability in vitro.**

The cascades of gene expression regulated by YB-1 have been linked to the functions of cell proliferation, genotoxic stress, and drug resistance. In particular, an increase in YB-1 expression has been reported to have an adverse effect on cancer cell viability and proliferation (Kohno et al., 2003). The MTT assay was optimized with PC-3 cells due to the low ability of LNCaP cells to attach to the tissue culture plates. These assays were first performed with LNCaP cells, but due to the cell loss during medium changes and the spontaneous detachment of the cells, PC-3 cells proved to be a much more ideal cell line for these experiments and allowed us to perform the MTT cell viability experiments by 96-well format with reproducibility.
and high-throughput. To determine whether the knockdown of YB-1 expression affects PC-3 cell growth, cells were treated for 2 days with 20, 100, and 200nM YB-1 ASO or scramble oligonucleotide. Cell viability was examined 2 days after treatment using the MTT assay (Figure 5.2). Figure 5.2 shows a dose-dependent reduction in PC-3 cell viability after treatment with up to 60% decreases in cell viability at the 200nM YB-1 ASO treatment compared with scramble control in the absence of paclitaxel.

Effect of YB-1 ASO on paclitaxel sensitivity in vitro.

Results in Figure 5.2 suggest that inhibition of YB-1 expression alone suppresses cell proliferation mechanisms. However, YB-1 over-expression in cancer cells functions as positive transcription factor to up-regulate the MDR-1 gene promoting drug resistance (Kuwano et al., 2004). To examine whether enhanced paclitaxel chemosensitivity can be achieved by the suppression of YB-1 expression in vitro, PC-3 cells were treated with 200nM YB-1 ASO or scramble oligonucleotide control. At the end of the treatment, PC-3 cells were incubated with different concentrations of paclitaxel. Cell viability was examined after 2 days using the MTT cell viability assay (Figure 5.3). As shown in Figure 5.3, ASO-induced knockdown of YB-1 significantly reduced PC-3 cell viability and decreased the IC₅₀ of paclitaxel from 4.0x10¹nM to 2.4x10²nM, a six-fold increase in sensitivity. In addition, at lower concentrations, for example 50nM, combined YB-1 ASO and paclitaxel treatment decreases cell viability from 85.7% to 46.7%, a 39% decrease. Significant single-agent activity makes detection of additive chemosensitivity difficult at high paclitaxel concentrations.

Systemic YB-1 ASO treatment inhibits LNCaP tumour progression and serum PSA levels in vivo.

Male athymic mice bearing LNCaP tumours were castrated and randomly selected for treatment with YB-1 ASO alone or scramble oligonucleotide control. Each experimental group consisted of at least 6 mice bearing similar mean tumour volumes at the beginning of treatment. Beginning 4 days after castration, 12.5mg/kg YB-1 ASO or scramble oligonucleotide control
were injected intraperitoneally once daily into each mouse for the first 7 days, and 3 times per week thereafter for 35 days. In mice treated with YB-1 ASO shown in Figure 5.4, tumour progression was significantly delayed as measured by tumour volume and serum PSA levels. By day 35 post castration, tumour volume were reduced by 33%, and PSA serum levels decreased 56% by day 32 post castration, in the YB-1 ASO treated compared to the scramble oligonucleotide control treated mice. In order to confirm the efficacy of YB-1 ASO treatment, Western blot analysis demonstrates approximately 40% downregulation in YB-1 protein levels with YB-1 ASO treatment in a selection of three tumours harvested at 35 days post castration from three different mice, compared with scramble oligonucleotide treatment control tumour protein levels (Figure 5.5). Pgp protein expression was also examined, however a smaller molecular weight band, opposed to the expected 170kDa Pgp band, was resolved by Western blot, indicating that the protein was possibly truncated during the homogenization process.

**Intratumoural YB-1 ASO enhances paclitaxel chemotherapy in vivo inhibiting LNCaP tumour progression and serum PSA Levels.**

After 6 weeks, male athymic mice bearing LNCaP tumours were castrated and randomly selected for treatment with polymeric paste alone, 3% YB-1 ASO plus 1% paclitaxel polymeric paste or 3% scramble oligonucleotide control plus 1% polymeric paste (Figure 5.6). Each experimental group consisted of at least 6 mice. Beginning 4 days after castration, Paste injections were calculated at a 1:10 ratio of paste to tumour volume (1cc paste : 10cc tumour) and injected intratumourally carefully "seeding" the paste throughout the tumour. As shown in Figure 5.6, YB-1 ASO treatment combined with 1% paclitaxel in a controlled release intratumoural polymeric paste significantly delayed time to AI progression as measured by decreased tumour growth and serum PSA levels after 21 days post castration. In Figure 5.6A, tumour growth after 49 days post castration with YB-1 ASO plus paclitaxel treated was reduced by 48% when compared to treatment with the unloaded polymeric paste, and decreased by 37% when compared to scramble oligonucleotide control plus paclitaxel treated mice. Similarly in
Figure 5.6B, after 49 days post castration, when compared with unloaded polymeric paste treatment, serum PSA levels decreased by 53% in mice treated with YB-1 ASO plus paclitaxel, and dropped 48% compared with scramble oligonucleotide control plus paclitaxel treatment.

No systemic toxicity characteristic of systemic paclitaxel such as weight loss, listlessness, or gait disturbance was observed in any animal during treatment. In some animals treated with paclitaxel-loaded paste, small red wounds could be seen close to the injection point. These wounds were probably caused by paclitaxel inhibition of wound healing at the injection site. This problem may have been exacerbated by animal scratching.

5.4 Discussion

Radical prostatectomy or irradiation remain the only curative treatment options for clinically localized prostate cancer, whereas until recently androgen ablation was the only effective form of systemic therapy with prolonged survival benefit for men with advanced hormone-naive disease. Resistance to chemotherapy and radiation is a major problem in the treatment of prostate cancer, and the development of androgen resistance represents the major obstacle to effective control of disseminated disease. Tannock et al. (2004) and Petrylak et al. (2004) are the first to report studies of the treatment benefits with the use of the cytotoxic agent docetaxel, increasing the survival of patients with androgen-refractory prostate cancer.

An improved understanding of the molecular pathogenesis of AI tumour progression must precede the development of new therapeutic strategies for prostate cancer patients. It is widely accepted that several distinct mechanisms regulate the balance between cellular proliferation and programmed cell death, which collectively determine the rates of tumour growth and tumour progression. YB-1 is a member of the highly conserved Y-box family of proteins, which regulate gene transcription by binding to either double- or single-stranded TAACC elements (the Y-box) contained within many eukaryotic organisms. The cascades of gene expression regulated by YB-1 have been linked to functions of cell proliferation, genotoxic stress, drug resistance, and metastatic invasion (Kohno et al., 2003). It has been shown to be
involved in a wide variety of cellular functions, including transcriptional and translational regulation, DNA repair, drug resistance and stress responses to extracellular signals. As a consequence, YB-1 is closely associated with cell proliferation and metastatic invasion. In a recent review, Kohno et al. (2003) reported data indicating that a reduction of YB-1 expression using ASOs inhibited cell growth. Similarly, we report that ASO-induced decreases in YB-1 expression significantly reduced androgen-independent prostate cancer cell viability and decreased the IC\textsubscript{50} of paclitaxel by 5-fold (Figure 5.3).

As highlighted in Chapter 4, YB-1 up-regulation and nuclear localization is associated with an androgen-independent phenotype in clinical prostate cancer samples. These results suggest that increases in YB-1 expression are paramount in the molecular pathogenesis of androgen-independent tumour progression, and that targeting YB-1 with new therapeutic strategies may significantly impact the survival of prostate cancer patients. The aim of Chapter 5 was to investigate the effect of either repeated systemic administration of YB-1 ASOs alone or a single intratumoural injection of YB-1 ASOs in combination with paclitaxel on LNCaP tumours grown subcutaneously in mice. Systemic administration of YB-1 monotherapy suppressed LNCaP tumour growth in this model (Figure 5.4A), suggesting that inhibition of YB-1 expression alone inhibits cell proliferation mechanisms. Similarly, the intratumoural injection of a polymeric paste formulation that acts as a controlled release drug delivery system for both paclitaxel and YB-1 ASOs was shown to significantly inhibit tumour growth and PSA expression in vivo (Figure 5.6).

We have previously described the use of a paclitaxel-loaded polymeric paste for the intratumoural treatment of LNCaP prostate tumours grown in mice (Jakcson et al., 2000; Springate et al., 2005). However, the formulation contained 10% (w/w) paclitaxel and although highly effective was associated with some inhibition of wound healing. Springate et al. (2005) have also demonstrated that a polymeric paste formulation containing just 1% (w/w) paclitaxel (or docetaxel) and microparticulate-bound clusterin ASOs released both these agents in a
controlled manner and inhibited PC-3 prostate tumour growth following intratumoural injection. In this study, a novel injectable formulation that contains ASOs and water-soluble chitosan suspended in a lipid polymer (PEG 600) containing 1% paclitaxel is described. This formulation allows for much higher loadings of both ASOs and chitosan than the previously used microparticulate system. When injected into an aqueous environment, such as tissue, water penetrates the formulation and dissolves both the chitosan and ASOs. The negatively charged ASOs then bind and precipitate the positively charged chitosan forming an insoluble hydrophobic waxy mass containing paclitaxel. This precipitated mass was shown to form an effective controlled release system for both paclitaxel and oligonucleotides in vitro (Jackson et al., unpublished data).

Almost 90% of men diagnosed with prostate cancer in North America present with localized disease. Therefore, these patients are particularly amenable to new treatment strategies using less invasive local treatments. Local treatments would be most appropriate for patients who are not candidates for radical therapy or for patients who develop a local recurrence after radical therapy to reduce the risk of local and/or metastatic progression of the prostate cancer. Paclitaxel, an anti-cancer taxoid that inhibits the depolymerization of microtubules, has been reported to inhibit human prostate cancer cell growth in vitro and in vivo. In addition, we have previously described the effective use of biocompatible, biodegradable polymeric pastes for the site-directed delivery of neoplastic agents such as paclitaxel. In this study, the polymeric paste formulation is a viscous liquid or paste at room temperature that may be successfully injected through a small-gauge needle and solidifies within 1h in vivo.

Collectively, the findings of this study support the hypothesis that inhibition of YB-1 can delay the progression of human prostate cancer cells to an androgen-independent phenotype. We have also provided pre-clinical efficacy data and proof of principle for a site-directed, injectable, controlled-release formulation of YB-1 ASO and paclitaxel as an effective form of treatment for localized prostate tumours. Although this study was performed using human
prostate tumour xenografts grown in mice, we believe that YB-1 is a rational target for biotherapy, and ASO combined with paclitaxel may offer a potentially effective form of treatment for patients with localized, early-stage tumours in combination with androgen ablation.
Figure 5.1. YB-1 ASO dose-dependent inhibition of YB-1 expression in LNCaP cells. LNCaP cells were treated daily with different concentrations of YB-1 ASO or scramble oligonucleotide control for 48 hours; total RNA was extracted from cultured cells, and YB-1 mRNA expression was analyzed by Northern blotting. Oligofectamine labeled cells represent YB-1 expression in LNCaP cells treated with oligofectamine vehicle control only. Membranes were hybridized with a $^{32}$P-labelled human YB-1 cDNA probe. 28S RNA was used as a loading control.
Figure 5.2. Effect of YB-1 ASO treatment cell viability in vitro. To compare the efficacy of YB-1 ASO on cell viability in vitro, PC-3 cells were treated for 2 days with 20, 100nM and 200nM YB-1 ASO sequence or scramble oligonucleotide control. After 48h of incubation, cell viability was determined by the MTT assay. Results are reported as % cell viability based on the scramble oligonucleotide control. Each data point represents the mean analysis of 8 samples ± SEM. Results were obtained over two experimental determinations.
Figure 5.3. Effect of combined YB-1 ASO and paclitaxel treatment on cell viability in vitro.

To compare the efficacy of YB-1 ASO sequence to enhance paclitaxel chemosensitivity in vitro, PC-3 cells were treated for 2 days with 200nM YB-1 ASO sequence, scramble oligonucleotide, or oligofectamine control. At the end of the treatment, cells were incubated with varying concentrations of paclitaxel. After 48h of incubation, cell viability was determined by the MTT assay. Results are reported as % cell viability relative to the cell viability at 0nM paclitaxel concentration, which was set as 100% for YB-1 ASO ISIS No.3, scramble oligonucleotide, and oligonucleotide control treatment. IC$_{50}$ values are reported for both YB-1 ASO or scramble oligonucleotide treated cells. Each data point represents the mean analysis of 8 samples ± SEM. Results were obtained over two experimental determinations.
Figure 5.4. Effect of systemic administration of YB-1 ASO after castration on inhibition of LNCaP tumour growth. A, Tumour volume was measured and calculated by the formula length \( \times \) width \( \times \) depth \( \times \) 0.5236. Data points were reported as an average % tumour volume of Day 0.
± SEM. Blood samples were obtained by tail vein incisions of mice before treatment and then once weekly after starting treatment. Data points were expressed as % PSA levels of Day 0 (Day of Castration) ± SEM.
Figure 5.5. YB-1 expression in LNCaP tumours harvested 35 days after castration after systemic administration. Western blot analysis was used to detect YB-1 protein levels in YB-1 ASO- and scramble oligonucleotide-treated mice. YB-1 protein was detected using anti-YB-1 antibody and anti-vinculin antibody was used as a loading control.
Figure 5.6. Effect of intratumoural injection of YB-1 ASO and paclitaxel-loaded polymeric paste on delay of AI tumour progression in the LNCaP tumour model. A, Tumour volume. Data points were reported as an average % tumour volume of Day 0 ± SEM. B, Blood samples were obtained by tail vein incisions of mice before treatment and then once weekly after starting treatment. Data points were expressed as % PSA levels of Day 0 (Day of Castration) ± SEM.
5.6 References


binding protein-5 helps accelerate progression to androgen-independence in the human prostate LNCaP tumour model through activation of phosphatidylinositol 3-kinase pathway. Endocrinology. 141:2257–65.


CHAPTER 6. GENERAL DISCUSSION AND PERSPECTIVES

In prostate cancer, both Pgp and YB-1 may play an important role in modulating androgen action and influencing cellular growth and proliferative events. Consequently, we hypothesize that these proteins have a role in aberrant androgen function and responsiveness in the development of advanced prostate cancer. The main hypothesis investigated proposes that pharmaceuticals and other xenobiotics may influence androgen action by mechanisms affecting cellular androgen transport and accumulation through modulation by Pgp, which in part is regulated by transcription factor YB-1. In prostate cancer, we further hypothesize that YB-1 expression is closely associated with both cellular proliferation and chemotherapeutic resistance. Although many studies have contributed a diverse array of knowledge with respect to our understanding the role of both YB-1 and Pgp in cancer, the evidence presented in this thesis uniquely characterizes the complex relationship between androgens, YB-1 and Pgp, and the molecular interaction between Pgp and androgens in the context of prostate cancer.

Epidemiological evidence, based on agricultural occupational exposures, instigated experimental studies that suggested pesticides may have the potential to mimic or antagonize hormone action, in many cases through binding to steroid receptors and interfering with transcriptional activity, thus are capable of disrupting the male hormone-signaling pathway (Chapter 2). From these studies, we hypothesized that many chemicals may have a profound effect of androgen accumulation in prostate cancer cells, which in part may be modulated by an active process which involves the expression of the efflux transporter Pgp. Although the current dogma exists that highly lipophilic and hydrophobic molecules, including androgens, pass freely through the cell membrane, our research has shown that the cell membrane-bound protein Pgp has the ability to efflux androgens (Chapter 3). P-glycoprotein, encoded by the MDR-1 gene, has been shown to be a highly promiscuous transporter, although structure-function relationships between Pgp and its
substrates are not well understood and highly debated. The physiological consequences of this active Pgp transport mechanism in the context of prostate cancer may be multifaceted, including effects on androgen responsive gene transcriptional regulation and multi-drug resistance. Through parallel research using cDNA microarrays which identified many potentially upregulated genes in prostate cancer progression our focus shifted to one important upstream regulator of MDR-1 expression, the multi-functional protein YB-1. Initially androgen-dependent tumours are treatable by androgen ablation, but become resistant to this approach when they become androgen-independent with time. Increased expression of both YB-1 and Pgp during prostate cancer progression in the LNCaP tumour model was confirmed by immunohistochemistry, Northern and Western blot analysis. Further, immunolocalization of YB-1 protein to the nucleus may occur during benign to malignant transformation and continue to accumulate during prostate cancer progression. In addition, in vitro transfection assays were used to suggest that transient expression of YB-1 results in reduced androgen accumulation and androgen-responsive gene activity, perhaps through a Pgp-mediated mechanism (Chapter 4). Observing the increased expression of both YB-1 and Pgp in prostate tumour cells, we hypothesized that YB-1 may be an appropriate target for enhancing the effectiveness of chemotherapeutic treatment and/or promoting an apoptotic tumour response. Using antisense oligonucleotides targeting YB-1 in vitro in prostate cancer cells, YB-1 mRNA expression was reduced in a sequence-specific and dose-dependent fashion. Further, YB-1 ASO treatment significantly decreased cell viability and increased chemosensitivity to paclitaxel. Using the in vivo LNCaP tumour model, systemic administration of YB-1 ASO decreased both tumour growth and serum PSA expression during androgen-independent prostate cancer progression. Intratumoral injection of polymeric paste containing YB-1 ASO and paclitaxel also suppressed tumour growth and PSA expression, although these effects may be due to YB-1 ASO alone delaying androgen-
independent progression, rather than due to YB-1 ASO and paclitaxel co-administration (Chapter 5).

The focus at the beginning of this research project was to screen a structurally diverse set of pesticides for their ability to influence AR- or AhR-mediated transcription of a firefly luciferase reporter gene using a highly sensitive in vitro reporter gene assay system. In addition, to determine if changes in androgen-responsive transcriptional activity were due to an interference mechanism involving displacement of androgen binding to the AR by pesticides, a AR-LBD ligand-binding assay was used. The results obtained using these methods showed that 23 pesticides are able to antagonize or agonize AR and AhR-mediated transcriptional activity, and with 10 pesticides data suggests this may be due to direct AR binding. Unfortunately, a structure-activity relationship was difficult to identify, however further in vitro testing using a larger representation of pesticides of each specific chemical classification may help to predict whether groups of structurally similar compounds have the ability to influence AR- or AhR-mediated activity. Additionally, prostate cells express both AR and AhR and cross talk between receptors may affect prostate differentiation, development, growth, and sensitivity to hormones. Therefore, in the future it would be interesting to examine the in vivo effects of pesticide exposure in a transgenic mouse model, which expresses an androgen-regulated prostate-specific chloramphenicol acetyl transferase reporter gene. This would allow a more physiologically relevant evaluation of potential deleterious effects on the prostate, including the ability to examine histological or morphological changes, as well as design multi-generational experiments. Another interesting experiment would be to use cDNA microarrays to evaluate a number of pesticides on gene expression patterns in treated and non-treated prostate cancer cells. It may be possible to draw parallels between specific groups of chemically similar pesticides on their ability to change specific gene expression patterns.
Other indirect mechanisms of endocrine disruption may be also possible. There have been reports in the literature that the multidrug resistance efflux transporter Pgp may play a role in the cellular detoxification of pesticides in mammalian tissues. Thus, Pgp may play a physiological role in participating in the protection of human cells against xenobiotics, including pesticides, by active efflux of these compounds into bile, urine, or the intestinal lumen, and by preventing accumulation in critical organs such as the brain, testis, prostate, and other reproductive organs (Schinkel et al., 1994; Tellingen et al., 2001). For example, expression of Pgp in the placenta has been shown to protect fetuses against toxicity of the pesticide ivermectin (Smit et al., 1999). Therefore, Pgp may be responsible for what could be defined as ‘multi-pesticide resistance’, a phenomenon that parallels multi-drug resistance in tumour cells. Previous studies suggest that Pgp can reduce the accumulation of a wide range of steroids, suggesting that cellular androgen levels could be significantly affected by this efflux transport mechanism. Further to this idea, cellular uptake and efflux assays would help determine the influence of pesticides on androgen accumulation and if pesticides have the ability to modulate androgen action through a Pgp-mediated mechanism or by directly influencing Pgp expression. Pesticides may also affect the expression of cytochrome P450 enzymes that are responsible for drug and steroid metabolism. Further experiments may examine the possibility that the intracellular concentration certain androgen metabolites may be altered in the presence of low levels of pesticide exposure, and consequently affect androgen-responsive gene expression. Experiments using high-pressure liquid chromatography of steroid metabolites upon isolation from treated cell lysates may help identify whether or not the relative concentrations of androgen metabolites are altered with pesticide exposure.

6.1 Effects of Dietary Constituents and Pharmaceuticals on Pgp Function

There has been a growing interest in the modulating effects of dietary constituents on Pgp function, and consequently on drug absorption. In the context of prostate cancer
treatment, dietary constituents such as lycopene, ginsenosides, and various herbal supplements have been shown to affect prostate cancer progression and treatment. Various flavonoids, particularly for flavonols, coumarins and other ingredients from natural fruits, vegetables, and herbs, have been found to modulate Pgp function, however no studies have examined Pgp or YB-1 expression levels. Steroid hormone levels may be affected by ingestion of various dietary constituents and therefore could potentially influence prostate cancer cells growth or androgen responsive gene activity via a Pgp-mediated mechanism. Given the co-ingestion with fruit and growing use of herbal extracts, due care should be taken in regard to the effects of these products on altering not only the pharmacokinetics of drugs, but also their effects on steroid hormone regulation and one the clinical consequences for disease outcome.

6.2 Contributions of Other ABC Transporters to Steroid Transport and Hormonal Response

Analysis to date has shown that Pgp is not vital to basic physiological functions and homeostasis in a variety of organs including the male reproductive tract. Unfortunately, no studies in knockout mice have examined Pgp function in the prostate. It may be possible that Pgp or other MRP transporter expression in the prostate is responsible for changes in the reproductive health of humans. Prostate secretions make up about 30-35% of semen (Hadley, 2000) and the fluid secreted by the prostate is rich in phospholipids and cholesterol, both shown to play a role in lipid dependence on Pgp transport (Pallares-Trujillo et al., 2000). Changes in ABC transporter expression or the interaction of these transporters with other exogenous agents in the prostate secretory epithelial cells may affect the balance of chemical compounds, including steroids, and reproductive quality of semen. Numerous studies indicate that male fertility may be in part due to the physiological effects of exogenous agents.
Although our studies have focused solely on the effects of Pgp on steroid transport, to fully comprehend the modulation of steroid activity, it will be necessary to further examine the influence of other members of the MRP family, including MRP1, MRP4 and MRP5. MRP1 has been shown to be overexpressed in advanced prostate cancer, and these studies indicate that flutamide and hydroxyflutamide but not dihydrotestosterone are transported by MRP1 (Grzywacz et al., 2003). MRP4 is highly expressed in the normal prostate and also overexpressed in MDR cell lines (Kool et al., 1997). Recently, it was reported that cyclic nucleotide efflux could be potently inhibited by progesterone and estramustine, a steroid-related compound used in the treatment of prostate cancer (Sundkvist et al., 2002). Other studies have shown steroid conjugates may be transported by MRP1, MRP4 and MRP5, raising the possibility androgens and anti-androgens might also be physiological substrates (Zelcer et al., 2003; Wielinga et al., 2003). It seems possible that modulation of both Pgp and members of the MRP subfamily may cause increased steroid transport and physiological response compared to modulation of just Pgp. Co-transfection assays using plasmid DNA encoding various members of the MRP family in Pgp overexpressing and wild-type prostate cancer cell lines examining androgen-responsive transcriptional activity or androgen efflux may help to elucidate the relative contribution of other efflux transporters compared with Pgp in prostate cancer.

6.3 YB-1 and Androgen-Responsiveness in Prostate Cancer

Our research suggests a complex mechanism by which YB-1 is involved as a main player in modulating androgen-responsiveness in prostate cancer progression (Figures 6.1 and 6.2). We hypothesize that YB-1 is upregulated early in prostate cancer development as a stress response, perhaps to environmental stimuli or in response to DNA damage. These early events result in the immediate translocation of YB-1 from the cytoplasm to the nuclei. We hypothesize that Pgp would then act to transport androgens out of prostate cancer cells, encouraging androgen independence. In the absence of androgens, YB-1 could conversely
act to regulate androgen responsive genes in a Y-box independent manner via interaction with other transcription factors (Figure 6.2). YB-1 protein may also bind to regions of DNA of androgen-regulated or anti-apoptotic genes, such as clusterin. In this way, YB-1 could be a master regulatory protein which influences the downstream transcription and translation of important cell survival pathways which encourage the progression of androgen independent prostate cancer. This requires further exploration. The nuclear localization of YB-1 may not only act as a transcription factor of various genes that are closely associated with DNA replication, cell proliferation, and drug resistance but also exert SOS signaling to protect cells or DNA integrity from genotoxic factors such as cisplatin, mitomycin C, and ionizing irradiation (Ohga et al., 1996; Ohga et al., 1998). To better understand the multifunctional effects of YB-1 on molecular pathogenesis and cell survival, it would also be interesting to examine the expression of other genes affected by YB-1 inhibition. Possible candidates include MMP-2, well known for its role in degradation of the extracellular matrix and contributing to metastatic invasion, as well as survival-associated genes such as NF-κB, and repressing death-associated genes such as fas. YB-1 may regulate the expression of a variety of genes in addition to the ones thus far identified that may impact the progressed phenotype of prostate cancer.

6.4 YB-1 as a Prognostic Marker in Androgen-Independent Prostate Cancer Progression

In view of the strong association between YB-1 expression and progression of prostate cancer, the important question remains whether YB-1 is an accurate prognostic marker for the disease. Co-expression of YB-1 and Pgp emerged as a promising relevant biomarker for unfavorable prognosis in ovarian cancer. (Huang et al., 2004). Nuclear expression of YB-1 has been associated with chemoresistance and poor prognosis of tumour patients. In lung cancer, nuclear expression of YB-1 seems to be an independent
Our research has shown that YB-1 is highly expressed and translocates into the nucleus during prostate cancer progression. Many questions remain regarding the specific mechanisms of YB-1 and gene regulation at the transcriptional and translational levels.

Figure 6.1 YB-1 expression and nuclear translocation during androgen-independent prostate cancer progression.

Tumour progression

benign malignant

Malignancy progression Late stage of malignancy
Consequences of YB-1 over-expression in prostate cancer cells

Extracellular stress (chemotherapeutics, UV, etc.)

Figure 6.2. Consequences of YB-1 over-expression in prostate cancer cells.

This figure summarizes the molecular interactions and physiological results of YB-1 overexpression. YB-1 acts to encourage drug resistance and increased androgen efflux. Decreased androgen-regulated gene activation may encourage upregulation of aberrant cell growth and metastatic pathways encouraging the development of androgen-independent prostate cancer progression.
prognostic marker (Gessner et al., 2004). Proliferative activity has been shown to be an independent prognostic marker for patients who have had radical prostatectomy (Bettencourt et al., 1996; Bubendorf et al., 1996) and also for hormonally treated patients (Ahlgren et al. 1999; Baretton et al., 1999). Thus, the measurement of YB-1 might be useful in detecting primary androgen-insensitive disease. Further studies examining YB-1 as a marker in predicting the presence of hormone-independent prostate cancer are thus warranted.

6.5 Additional Therapeutic Approaches - Integrated Molecular Approach

As numerous genes and cellular pathways are involved in the mechanism regulating AI progression, inactivation of a single target gene like YB-1 may likely be insufficient to adequately suppress tumour progression. It is likely that other cell survival pathways are activated in response to the down regulation of YB-1 because the cancer cell is highly adaptive in its stress response. Future research will track changes in gene expression by tumours over time to determine specific genes and expression patterns associated with the transition to androgen-independence. Exploration of additive or synergistic effects of blocking several anti-apoptotic genes simultaneously using antisense oligonucleotides in preclinical models will help guide the design of further clinical protocol (Miyake et al., 2001). Tumour progression is a highly dynamic process and cannot be attributed to singular genetic events, involving instead a perpetual accumulation of genetic alterations that allow escape from normal regulatory controls.

6.6 Future opportunities for prostate cancer diagnosis and treatment

Finally, our improved understanding of the molecular pathogenesis of androgen-independent tumour progression may lead to the development of novel diagnostic and therapeutic strategies for prostate cancer. Pgp and YB-1 may serve as useful diagnostic markers for prostate cancer disease progression and larger, more controlled clinical studies will be useful in determining their prognostic value. Additionally, results for this thesis show
the utility of antisense oligonucleotides for therapeutically modulating YB-1 expression. The androgen-dependent phase of advanced prostate cancer may be prolonged by inhibition of YB-1 expression and/or activity early in the course of treatment. In addition, our results link YB-1 to a general apoptotic mechanism, suggesting it may be an ideal target since YB-1 inhibition may suppress cell proliferation mechanisms and activate apoptotic pathways in prostate cancer cells. Many challenges lie ahead in translating research knowledge into effective patient therapies, with a multimodal and multidisciplinary approach being a prerequisite for success. It should be kept in mind that in the future development of prognostic and therapeutic potentials of Pgp and YB-1, they are only two in an ever-growing list of proteins whose aberrant expression contributes to androgen-independent prostate cancer progression. A better understanding of the interplay among various membrane transporters, androgen-dependent and androgen-independent apoptotic regulators, and their relative roles in prostate cancer tumourigenesis, uncontrolled growth, metastatic potential, and therapeutic resistance, will be essential to the translation of laboratory observations into the clinic.
6.7 References


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