BIRC6 AS A NOVEL THERAPEUTIC TARGET IN ADVANCED PROSTATE CANCER: CLINICAL RELEVANCE, DEVELOPMENT OF POTENTIAL THERAPEUTIC AGENTS & PRECLINICAL DRUG EFFICACY

by

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Abstract

The lack of effective therapy for advanced prostate cancer (PCa) remains a major unmet clinical need. Recently approved therapeutics, such as enzalutamide (ENZ), have only delayed the inevitable progression of castration-resistant PCa (CRPC), as resistance will typically emerge following treatment. Although increased apoptosis-resisting ability of cancer cells represents a fundamental mechanism for the onset of treatment resistance, no relevant agents have yet been developed. Preliminary work in our laboratory has revealed an association between elevated expression of BIRC6, an Inhibitor of Apoptosis (IAP) protein, and advanced PCa.

The overall objective of this doctoral study is to investigate the roles of BIRC6 in advanced PCa, and to assess the therapeutic efficacy of a novel anti-BIRC6 agent. Firstly, I evaluated the clinical relevance of BIRC6 using patients’ PCa specimens, and the functional importance of BIRC6 using cell line-based PCa models. A significant correlation was found between elevated BIRC6 protein expression in clinical PCa and poor patient prognostic factors. Functional assays validated the importance of BIRC6 in PCa cell proliferation and apoptosis suppression.

Next, I designed BIRC6-based, dual IAP-targeting antisense oligonucleotides (dASOs) to inhibit BIRC6 and an additional IAP. Two dASOs, 6w2 and 6w5 targeting BIRC6+cIAP1 and BIRC6+survivin, showed substantial inhibition of CRPC cell proliferation in vitro and in vivo. Functional studies showed that both dASOs significantly induced apoptosis, cell cycle arrest and suppression of NFκB activation in CRPC cells.

Finally, I assessed the growth-inhibitory efficacy of dASO-6w2 in ENZ-resistant CRPC, which has become an increasingly prominent problem in the clinic. The efficacy of dASO-6w2 was studied using both ENZ-resistant PCa cell lines and a clinically relevant, transplantable
patient-derived xenograft PCa tissue model, designated LTL-313BR, which exhibits primary ENZ resistance. Importantly, I showed that treatment with dASO-6w2 markedly suppressed the growth of LTL-313BR xenografts. The dASO-6w2 was also found to increase tumour apoptosis and inhibit the expression of several pro-survival genes that were up-regulated in the LTL-313BR line.

In conclusion, this doctoral study has established the clinical relevance and functional importance of BIRC6 in advanced PCa, and has also presented new BIRC6-targeting agents that markedly suppress the growth of advanced PCa.
Preface

Tumor tissues were obtained from patients through a protocol approved by the Clinical Research Ethics Board of the University of British Columbia (UBC) and the BC Cancer Agency (BCCA). All patients signed a consent form approved by the Ethics Board (UBC Ethics Board #: H12-03428 and H04-60131). Animal care and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under the approval of the Animal Care Committee of the University of British Columbia (protocol #: A11-0275 and A15-0152).


A version of Chapter 2 has been published. Low CG*, [Luk IS]*, Lin D, Fazli L, Yang K, Xu Y, Gleave M, Gout PW and Wang Y. BIRC6 protein, an inhibitor of apoptosis: role in survival of human prostate cancer cells. PLoS One. 2013; 8(2):e55837. (* Co-first author). Low CG and I are the lead investigators of this study. Low CG and I wrote the manuscript, conducted experiments, data analysis and interpreted the results. Experiments in Figure 2.4A, 2.5B and 2.6A in this thesis were conducted by Low CG. Lin D and Fazli L analyzed and scored IHC staining. Gout PW provided critical review of the manuscript. Gleave ME and Wang Y provided funding support. Wang Y supervised the study.

A version of Chapter 3 has been published. [Luk SU], Xue H, Cheng H, Lin D, Gout PW, Fazli L, Collins CC, Gleave ME and Wang Y. The BIRC6 gene as a novel target for therapy of prostate cancer: dual targeting of inhibitors of apoptosis. Oncotarget. 2014; 5(16):6896-6908. I am the lead investigator of this work. I wrote the manuscript, conducted experiments, data
analysis and interpreted the results. Xue H and Cheng H assisted in vivo experiments. Lin D, Gout PW, Fazli L, Gleave ME and Wang Y’s roles are the same as above.

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Portion of Chapter 3 and 4 have been used in U.S.A and Canada patent applications.

Title: Dual-targeting Antisense Oligonucleotides as Apoptotic Inhibitor Therapeutic Compositions and Methods for Their Use in the Treatment of Cancer. Canada No. 2,897,389 (Filed July 2015). U.S. Patent Application Serial No. 15/211,857 (Filed July 2016). Applicants’ affiliation: University of British Columbia; and British Columbia Cancer Agency. I am one of the inventors and conducted experiments, data analysis and interpreted the results as described above.
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List of Abbreviations

ADT: Androgen deprivation therapy
AML: Acute myeloid leukemia
AR: Androgen receptor
ASO: Antisense oligonucleotides
BCA assay: Bicinchoninic acid assay
BCL2: B-cell lymphoma 2-encoded protein
BIR: Baculoviral IAP Repeat
BIRC6: Baculoviral IAP repeat-containing 6
BPH: Benign prostatic hyperplasia
CNV: Copy number variations
CRPC: Castration-resistant prostate cancer
DAPI: 4’,6-diamidino-2-phenylindole nuclear stain
DRE: Digital rectal exam
ECM: Extracellular matrix
ENZ: Enzalutamide
FBS: Fetal bovine serum
FDA: Food and Drug Administration
FDR: False discovery rate
FGF: Fibroblast growth factor
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GATA2: GATA binding protein 2
Gen 2.5: Generation 2.5
GEO: Gene expression omnibus

GPCR: G-protein-coupled receptor

IGF: Insulin-like growth factor

IHC: Immunohistochemistry

IP: Intraperitoneal

IV: Intravenous

LHRH: Luteinizing hormone-releasing hormone

MSKCC: Memorial Sloan Kettering Cancer Centre

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MYC: Avian myelocytomatosis viral oncogene

MSigDB: Molecular Signature Database

NCBI: National Center for Biotechnology Information

NCI: National Cancer Institute of the United States

NEPC: Neuroendocrine prostate cancer

NFƙB: Nuclear factor NF-kappa-B

NKX3.1: NK3 homeobox 1

NOD-SCID: Non-obese diabetic/severe combined immuno-deficient

NSG: NOD-scid IL2Rgamma(null)

PBS: Phosphate buffered saline

PCa: Prostate cancer

qRT-PCR: Quantitative real-time polymerase chain reaction
PDX: Patient-derived xenograft

PI: Propidium iodide

PIA: Proliferative Inflammatory Atrophy

PIN: Prostatic intra-epithelial neoplasia

PSA: Prostate-specific antigen

PTEN: Phosphatase and tensin homolog

RPMI: Roswell Park Memorial Institute

SC: Subcutaneous

SDS: Sodium dodecyl sulfate

siRNA: Small interfering RNA

TMA: Tissue microarray

TMPRSS-ERG: Transmembrane protease, serine 2 - ETS-related gene
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Dedication

To Zion Wu,

Thanks for your amazing care and prayers that have sustained me through the hardest times.
Chapter 1: Introduction

1.1 Prostate Cancer

1.1.1 Overview

Prostate cancer (PCa) represents a significant global health problem. According to statistics from 2012, PCa accounted for over 1.1 million estimated new cases and 307,500 estimated deaths worldwide [1]. Furthermore, this disease has remained consistently as the most common non-cutaneous cancer and the second leading cause of cancer-related deaths for males in the Western world. In Canada and the United States, 1 in 8 men will be diagnosed with PCa at some point during their lives. Although early stage PCa is readily treatable with localized therapies, around 30% of these patients will progress to a more advanced disease state [2]. As a result, these patients whose tumours relapse after systemic therapies [e.g. androgen deprivation therapy (ADT) and chemotherapy] will face a dismal prognosis. The high incidence and morbidity of PCa also results in considerable socio-economic burdens. As such, research in improving the management of this disease holds great potential at both the individual and societal level.

Currently, there are two major well-recognized challenges pertaining to the management of PCa [3, 4]; these involve the clinical differentiation between aggressive and indolent PCa, and the lack of effective therapeutic agents or treatment strategies for advanced PCa (i.e. castration-resistant prostate cancer, CRPC). Currently, no curative treatments are available for patients with metastatic castration-resistant prostate cancer (mCRPC), and the historical median survival for mCRPC is less than two years [5]. Despite the advent of new FDA-approved agents (e.g., Enzalutamide, Abiraterone acetate and Cabazitaxel) that have resulted in incremental improvements towards overall survival, mCRPC remains an incurable and substantial obstacle in the management of this disease.
1.1.2 The Prostate

The prostate is the largest accessory sex gland in the male reproductive system; in adults, the prostate is approximately the size of a walnut. It is located between the bladder and penis, in front of the rectum and encircling the urethra. Histologically, the prostate is composed of 30-50 tubuloalveolar glands (parenchyma) that are surrounded by dense fibromuscular stroma [6]. These tubuloalveolar glands are lined with pseudostratified columnar epithelium, comprised of mainly columnar and cuboidal cells. Present at the epithelium, but less frequently, are also basal and neuroendocrine cells [7]. The prostate gland produces a fluid that, in combination with sperm and fluids from the seminal vesicles and the bulbourethral gland, makes up semen. This prostate secretion also contains various enzymes that increase the mobility of sperm, such as prostate-specific antigen (PSA). These proteins are secreted from the luminal cells located at the epithelium into the lumen of the gland. During ejaculation, the smooth muscle contracts and leads to expulsion of prostatic fluid.

Anatomically, the human prostate can be divided into 3 zones; these include the central, peripheral, and transitional zone, which all differ histologically. The peripheral zone is the largest region, and represents 70% of the total mass of the prostate. The peripheral zone is also the region from which adenocarcinoma and post-inflammatory atrophy commonly arise [8-10]. Next, the central zone accounts for approximately 25% of the prostate, but has a lesser likelihood of developing carcinoma. Finally, the transitional zone is the smallest region of the prostate, and comprises 5-10% of the total mass. The transitional zone is prone to the development of benign prostatic hyperplasia (BPH), a non-malignant overgrowth that causes urethra compression, particularly in older men [10, 11].
The development of the prostate begins at embryogenesis, and reaches maturity following puberty. The prostate gland is initially formed from epithelial budding at the urogenital sinus, which originates from the endoderm [12]. Early prostatic development is initiated by interactions between the urogenital epithelium and the urogenital mesenchyme mediated by androgen signalling [13]. Tissue recombination experiments (urogenital epithelium and urogenital mesenchyme recombined in kidney capsules of mice) by Cunha’s group have demonstrated that androgens initially act on the mesenchyme for prostate induction and growth. Later on in development, androgen signalling is required in the epithelium for the secretory function of differentiated cell types [13, 14]. Therefore, androgen is essential for the development, growth, and maintenance of the prostate gland; as such, androgen deprivation by castration would result in intensive prostatic cell death [15]. Notably, tissue recombination experiments have suggested that aberrant growth factor signalling from stromal components may play an integral role in cancer progression [16, 17]. However, the specific signals that mediate these events remain yet to be identified.

1.1.3 Carcinogenesis and Disease Progression

Prostate cancer (PCa), also known as prostate carcinoma, is a cancer derived from prostatic epithelial cells. The vast majority of prostatic cancers are acinar adenocarcinomas, originating from the luminal secretory cells of the prostatic epithelium [18]. Non-acinar carcinoma variants account for approximately 5–10% of prostatic carcinomas, including neuroendocrine prostate cancer (NEPC) [19]. PCa is a multifocal disease; multiple tumour foci with differing dysplasia and genetic aberrations are commonly found within a prostate [20-23], thus suggesting an independent clonal origin [23, 24]. Among other cancer types, prostate cancer is a comparatively slow developing disease. It is believed that prostatic neoplasms are initiated at a relatively early
age and remain indolent [3]. For example, prostate cancer foci have been detected in specimens from healthy men in their 30s [25, 26].

PCa is highly heterogeneous, with no single causative agent or mechanism responsible for carcinogenesis. Three risk factors that have been established to be associated with PCa are age, race, and family history [27]. Advanced age is by far the most important risk factor for PCa. It is believed that this relationship between prostate cancer and advanced age likely reflects the interplay between certain environmental, physiological, and molecular influences—with the normal consequences of aging presumably intensifying the impact of these influences [3]. Some of the more established processes that have been implicated in prostate carcinogenesis include inflammation; oxidative stress and DNA damage; and interrelated genetic and epigenetic aberrations [3]. The prostate gland is also prone to inflammation, which may be caused by infections, physical trauma, hormonal variations, or dietary factors such as the consumption of charred meats. Consequently, these insults can trigger the onset of epithelial cell regeneration to replace damaged cells; this condition is manifested morphologically as Proliferative Inflammatory Atrophy (PIA) [28]. PIA has been proposed to induce prostatic intraepithelial neoplasia (PIN), a well-accepted precursor for early prostate cancer [29-31]. Characterized as a neoplastic transformation of the lining epithelium of prostatic ducts and acini, PIN is typically confined within the epithelium (i.e. intraepithelial). It is identified histologically by hyperplasia of luminal epithelium, reduction in basal cells, and abnormal cellular morphology. High-grade PIN displays an elevation of cellular proliferation markers, and is also believed to be the immediate precursor of early invasive adenocarcinoma [30, 32].

Although prostatic adenocarcinoma is very common in men at an older age (in autopsy studies, it is found in 80% of men >80 years old), only a small proportion will result in
clinically-significant disease [33]. This more aggressive subset of PCa can cause urinary problems and readily invades regional tissues, such as pelvic lymph nodes and seminal vesicles. Some patients may also develop distant metastases in various bones (90% of metastasis) [34], which can result in severe bone pain. Metastasis to distant lymph nodes, lungs, the liver, and the brain can also frequently occur [2]. Even though the relative 5-year survival rates for local stage (confined in prostate) and regional stage PCa (affecting regional tissues but not distant sites) are nearly 100%, survival rates can drop dramatically down to ~30% when distant metastases are present [35].

PCa can also advance to become castration-resistant prostate cancer (CRPC), which is the final stage of the disease. CRPC (previously known as hormone-refractory PCa or androgen-independent PCa) is a recurrent disease that develops following androgen deprivation therapy (ADT). ADT exerts its therapeutic action by effectively inhibiting PCa via the androgen-dependent nature of PCa cells. However, all PCa will inevitably relapse in about 2-3 years [36, 37]. The median overall survival of CRPC patients ranges between 16-18 months [38], with no curative treatments currently available.

Neuroendocrine prostate cancers (NEPC) represent a highly aggressive subtype of PCa, with the median overall survival ranging between only 10-13 months [39]. Although pure *de novo* NEPC comprise only 0.3% to 1% of all PCa [19], NEPC loci can be found in up to 25% of CRPC patients [40]. Distinct from adenocarcinoma, NEPC cells do not express androgen receptors and thus do not rely on androgen for survival. There is mounting evidence that support the assertion that the transformation from adenocarcinoma to NEPC is promoted by androgen-deprivation therapy, and may subsequently arise as a mechanism of resistance [41-43].
Therefore, the introduction of novel, highly potent androgen receptor–targeted therapies in the clinic may consequently increase the incidence of NEPC [44].

1.1.4 Molecular Aberration in Prostate Cancers

The advent of high throughput and low-cost profiling technology has enabled the genomic and transcriptomic analysis of an increasing number of clinical samples [45]. Numerous large genomic and transcriptomic studies in PCa have identified many recurrent genetic aberrations involved in PCa initiation and progression [4]. Collectively, these studies have demonstrated that the AR represents a central driver of PCa. Over half (57%) of primary prostate cancers possess a gene-fusion involving the fusion of a 5’ untranslated region of an androgen receptor-regulated gene (\textit{TMPRSS2} in 90% cases) with the coding sequence of a ETS transcription factor family member (e.g., \textit{ERG} or \textit{ETV1}) [46]. The non-ETS fusion (ETS negative) PCa is frequently associated with elevated \textit{SPINK1} gene expression [47], \textit{SPOP} mutations, \textit{FOXA1} mutations [48], \textit{CHD1} deletion [49], and activation mutations in the \textit{RAF} family [50]. In particular, \textit{SPOP} and \textit{FOXA1} mutant tumours have been reported to have the highest levels of AR-induced transcripts [51]. The loss or mutation of \textit{TP53} and \textit{PTEN} are among the most common genetic events in PCa, occurring more frequently in ETS-positive cancers [48, 52]. Other genetic events that occur most frequently include 8q24 amplification (\textit{MYC}) [53], \textit{PTEN} loss [54], \textit{NKKX3.1} loss [55], and \textit{TP53} loss [56]. Integrated pathway analysis has revealed that the AR pathway (including several known AR coactivators and corepressors) is altered in 56% of primary PCa, despite no AR gene alterations (outlier expression or somatic mutation) being detected [56]. Notably, the AR pathway has been found to be altered in 100% of metastatic PCa cases, while the AR gene was activated in 58% of metastatic cases. Three other commonly altered pathways in both primary
and metastatic PCa include PI3K, RAS/RAF, and RB signalling, occurring in 42/100%, 43/90% and 34/74% of primary/metastatic cases, respectively [56].

1.1.5 Clinical Management

The clinical management of PCa involves the detection, diagnosis, and treatment of the disease. Generally, early stage localized PCa will typically present as mostly asymptomatic. Symptoms only become apparent when PCa becomes invasive and metastatic, whereupon patients will experience frequent and painful urination, weight loss, fatigue, and pelvic or lower back pain [57]. PCa is first suspected with positive results from prostate-specific antigen (PSA) testing and a digital rectal exam (DRE). During a DRE, urologists will determine the presence of a palpable tumor, in addition to the degree of the local extension of the tumour, as PCa will commonly arise at the peripheral zone of the prostate which lies immediately in front of the rectum. For PSA testing, a continuous increase in the PSA level indicates a greater likelihood of PCa [58]. There exists some controversy over the use of PSA testing for prostate cancer screening, due to the likelihood of false positive results from non-cancerous conditions, such as from BPH and inflammation, and also to a tendency to over-treat indolent diseases that otherwise may never have caused symptoms within a patient’s lifetime. It is estimated that there could be a 15-year (or more) lag-time between the initial detection of PSA elevation until the onset of clinically manifested PCa [59]. Currently, the BC Cancer Agency and the Vancouver Prostate Centre have recommended PSA screening for men of 50 years or older, with an estimated life expectancy of more than 10 years [60]. Aside from diagnosis, PSA is also routinely used to monitor disease progression before or after treatment. Notably, recurrent PSA levels, following systemic therapies (e.g., hormone deprivation, chemotherapies), are an indicator of disease recurrence and will require further attention for clinical management.
1.1.5.1 Diagnosis and Staging

Once PCa has been suspected from PSA testing and a DRE, patients will be subjected to a needle biopsy for definitive diagnosis based on histopathological verification by a pathologist. Histopathological assessment could confirm the presence of malignant tissue, if present, and its degree of aggressiveness. The latter is evaluated according to the Gleason grading system, based on the tissue histology and extent of differentiation. The Gleason grade ranges from 1 to 5, where grade 1 signifies a well-differentiated architecture and grade 5 represents a poorly differentiated tumour. The final Gleason score is the sum of two most prominent histological grades [61]. A Gleason score of 6 or less refers to a low-grade cancer, whereas a Gleason score of 8-10 refers to a high-grade aggressive disease with poor patient prognosis [62]. Although the Gleason score serves as a powerful indicator of PCa aggressiveness and prognosis, the clinical status of the disease is often assessed by the TNM staging system. TNM defines the tumor size (T, 1-4), the presence of regional lymph nodes metastases (N, 0-1), and the presence of distant metastases (M, 0-1). More specifically, T1 indicates a clinically inapparent tumour, not palpable or visible by imaging; T2: tumour confined within the prostate; T3: tumour extends through the prostatic capsule; T4: tumour invades adjacent structures other than the seminal vesicles, such as the external sphincter, rectum and/or pelvic wall [63]. The patients are then advised on their treatment strategies based on a risk assessment that takes into consideration the Gleason score, clinical TNM stage, PSA level, and the positive biopsies, in addition to other factors, such as patient age and physical condition [4].
1.1.5.2 Treatments

Guidelines for PCa clinical management have been established in various countries and regions, reflecting the availability of therapeutic options (Canadian Urology Association, American Urology Association, European Association of Urology, etc). Although the recommendations for patient stratification may vary, all of these guidelines provide the same general recommendation for typical disease stages, including localized, metastatic, or castration-resistant prostate cancer (CRPC).

Patients with localized, low-risk PCa (Gleason score of 6 or less) are recommended for active surveillance, where patients are monitored with PSA testing, repeat biopsies, and imaging for signs of aggressive disease. Active surveillance can avoid the use of unnecessary treatment procedures that are associated with harmful side effects, as men with low-risk PCa have an excellent prognosis. Large studies have indicated that most men with low-risk PCa may avoid direct treatment, with a 1% risk of death from PCa at 10 years [64, 65].

Patients with high-risk (Gleason score >6) PCa require intervention involving localized therapies, such as surgery (radical prostatectomy) and radiation therapy (external beam radiotherapy or brachytherapy). Both surgery and radiation therapy are standard local treatments and considered as curative. Radical prostatectomy involves the surgical removal of the prostate, in addition to adjacent tissue, including the seminal vesicle [66]. This can be done via open or laparoscopic surgery by small incision. External beam radiotherapy is used in combination with long term (at least 6 months) androgen deprivation therapy (ADT), where the use of adjuvant ADT can significantly prolong overall survival [67, 68]. The 5-year survival rate for localized or regional prostate cancer is nearly 100% [35].
Although the surgery and radiation therapy may be considered curative, 20-30% of these cases will progress to more advanced stages within 5 years [2]. First indicated by rising PSA levels, these patients will develop metastatic PCa, persisting from treatment with local therapies. ADT, which involves the suppression of testicular androgens by medical or surgical castration, is the current mainstay of treatment for metastatic PCa. Medical castration involves the continuous administration of luteinizing hormone-releasing hormone (LHRH) agonists or antagonists. Despite an excellent initial response, in approximately 2 to 3 years, most patients will succumb to the castration-resistant form of the disease, otherwise known as CRPC [36].

Patients who develop progressive disease following castration and exhibit a rising PSA level, but with no radiological evidence of metastases, is referred to as M0 CRPC. However, no standard treatment recommendation is currently available for M0 CRPC. Men with progressing M1 CRPC have several treatment options, following recent FDA approval of new anti-androgens. Abiraterone acetate (Zytiga) with prednisone and enzalutamide (Xtandi) were both first-line therapies for men with asymptomatic or minimally symptomatic, chemotherapy-naive M1 CRPC (approved in 2012 and 2014 based on the COU-302 [69] and PREVAIL trials [70] respectively). Abiraterone inhibits androgen synthesis by the adrenal glands, testes, and the prostate tumour, by blocking CYP17, a critical enzyme in testosterone biosynthesis [71]. Enzalutamide, previously known as MDV-3100, is a second generation AR antagonist and an AR signalling inhibitor with no agonist activity [72]. Sipuleucel-T (Provenge), a form of autologous cellular immunotherapy, was approved for patients with asymptomatic mCRPC in the United States in 2010 [73]; however, the therapy is not currently widely used due to its high treatment cost.
Men with symptomatic mCRPC are recommended for treatment with docetaxel (first line chemotherapy), a taxane-based microtubule-stabilizing agent that was shown to improve overall survival, disease control, and symptom palliation in mCRPC [74, 75]. The bone-targeting agent, Radium-223, is recommended for mCRPC with symptomatic bone metastases and no known visceral metastasis [76-78] after FDA approval in 2013 based on the ALSYMPCA trial [79].

Patients who have progressed after docetaxel use are recommended for treatment with abiraterone, enzalutamide, or cabazitaxel [5, 77], all of which have demonstrated significant improvements on overall survival in COU-AA-301 [80], AFFIRM [81], and TROPIC [82] trials, respectively. Cabazitaxel is a second generation taxane-based chemotherapeutic, designed to be effective against docetaxel-resistant tumours. Studies have found taxanes to inhibit the microtubule disassembly that impairs mitosis, as well as tubulin-dependent androgen receptor nuclear shuttling [83]. Cabazitaxel has been determined to be superior in resisting drug efflux, due to its poor affinity for P-glycoprotein, a drug efflux pump [84].

Although these recently approved agents have shown incremental improvements towards the overall survival of mCRPC patents, none of these drugs are curative. Additionally, incidences of resistance towards these agents, occurring shortly after treatment, have been increasingly reported. Clearly, the establishment of more effective therapeutic targets and drugs is of critical importance for improved disease management and patient survival.

1.1.6 Mechanism of Treatment Resistance

When cancer progresses beyond the primary or regional sites, the disease prognosis can worsen significantly due to the tendency of tumours to develop resistance to treatments. Even if treatments such as androgen deprivation therapies, androgen axis targeting agents, or chemotherapies have shown good initial efficacies, the development of acquired resistance or de
*novo* resistance in tumours will eventually render these treatments ineffective. Therefore, an improved understanding of the mechanism underlying treatment resistance is crucial for the development of new strategies to suppress aggressive cancers or delay their recurrence.

Resistance towards next-generation androgen axis-targeting agents, such as abiraterone and enzalutamide, has emerged as a major clinical problem. Clinical studies have revealed that approximately 1/4 of all patients demonstrated primary resistance to ENZ (progressed within 3 months) and furthermore, that all patients with an initial response eventually progressed by 24 months [81]. As for abiraterone, approximately 1/3 of all patients had demonstrated primary resistance, with all patients eventually progressed by 15 months [80].

Several major mechanisms underlying the development of resistance to androgen axis-targeting agents and ADT, have been identified. These include pathways that reactivate AR signalling (ligand-dependent or ligand-independent), a pro-survival pathway independent of AR, and extrinsic microenvironmental influences [85-87]. The reactivation of AR, independent of androgen, involves modification of AR via gene amplification [88, 89], gain of function mutations [90], and truncation and splice variants [91, 92]. AR amplification was detected in the plasma cell-free DNA of 53% of patients progressing on enzalutamide [89]. The AR splice variant 7 (AR v7), which lacks the ligand-binding domain (LBD), was found in 39% and 19% of patients treated with enzalutamide and abiraterone, respectively [91]. Without the LBD, AR v7 becomes constitutively active in the absence of androgen. In addition, a F876L mutation within the LBD has been reported to turn enzalutamide into an agonist of AR *in vitro* [90]; this observation was detected in plasma cell-free DNA of CRPC patients [89, 93]. Other known ligand-independent mechanisms include an increase in AR coactivator recruitment, such as FKBP51 [94]; activation through the glucocorticoid receptor [95]; and outlaw pathways
involving the activation of AR signalling via cross-talk with pathways such as PI3K/Akt [96, 97] and NF-κB [98, 99]. On the other hand, as observed in CRPC patients, the androgen-dependent reactivation of AR signalling involves the continuous increase in testicular, adrenal, and intratumoural androgen synthesis. Accordingly, abiraterone acetate is a CYP17 inhibitor that has been developed to target this axis, and showed promising clinical activity. Regardless, treatment resistance still developed and may be attributed to an increase in substrates of the ‘backdoor’ pathway of DHT synthesis, such as pregnenolone [100]. Furthermore, intracrine androgens and AKR1C3 activation have been reported to confer resistance towards enzalutamide in a cell line model [101].

Relatively less-studied is the treatment resistance mediated by AR-independent mechanisms, the most prominent example being neuroendocrine transdifferentiation after castration [102-105]. Castration-resistant NEPC cells are AR negative and do not require androgen for survival or growth. Consequently, these resistant tumours would not respond to treatment with androgen axis-targeting agents, and subsequently represented a rising clinical problem. One of the key mechanisms exacerbating androgen-independent growth is the inhibition of apoptosis as a method for bypassing the need for AR or its ligand [106]. A number of aberrations within the apoptotic mechanism have been reported for CRPC [107], and include the upregulation of the anti-apoptosis regulator Bcl-2 [108-110], Inhibitor of Apoptosis (IAP) protein family member, survivin [111], and BIRC6 [112]. Notably, overexpression of survivin mediates the development of resistance to antiandrogens [111], and BIRC6 expression has been found to be elevated in CRPC [112].
1.1.7 Experimental Models

Both the study of disease progression and the development of therapeutic agents rely heavily upon the use of experimental models. The establishment of PCa models, such as cell lines, is a very challenging process, and the number of available cell lines is hence very limited [113]. To date, the majority of PCa research has been conducted using LNCaP, C4-2, PC-3, and DU145 cell lines [114]. The LNCaP line was originally derived from a prostate cancer lymph node metastasis, and its castration-resistant subline, C4-2, was developed via serial transplantation in castrated mice following ADT [115]. Although both LNCaP and C4-2 express AR, only LNCaP exhibits androgen-responsive growth [115]. In addition, a few enzalutamide-resistant LNCaP sublines have been developed, including MR49F and MR42D, which allow study of the enzalutamide resistance mechanism [96]. In contrast to LNCaP and C4-2, the PC-3 and DU145 lines were both derived from PCa bone and brain metastases. Both PC-3 and DU145 do not express AR or PSA, and both are therefore both androgen-independent. These two cell lines are representative of more aggressive PCa models, as they grow efficiently and are highly metastatic in immunocompromised mice.

Animal models of PCa are essential for studies of its growth, pathophysiology and evaluation of potential anticancer agents. Models that have been used include spontaneous murine PCa models, such as the transgenic adenocarcinoma of mouse prostate (TRAMP) as induced by the constitutive expression of SV40 T antigen [116], and the PTEN knock-out mice model [117]. Both models allow study of the development of the disease and also contain an intact immune system. However, the resulting tumours developed by these models have been found to contain major discrepancies from its human counterpart. This limitation therefore renders such models to be sub-optimal for preclinical studies [132]. In order to circumvent the
problem arising from the difference in species, human cell line xenograft models are frequently used. All four of the aforementioned major cell lines can be grafted into nude (athymic) or non-obese severe combined immuno-deficient (NOD-SCID) mice subcutaneously (s.c.), orthotopically, or at subrenal capsule (SRC) sites [118]. The SRC site has demonstrated the highest successful engraftment rate (tumour take rate) due to its high degree of vascularization [119]. This high engraftment rate therefore permits the survival and growth of low grade and slow growing cancers, including primary prostate cancer [120]. Although cell line xenograft models provide valuable tools for the study of human PCa in vivo, they are highly homogeneous and lack the tumor heterogeneity, tissue architecture and particular genetic aberrations seen in clinical cancer specimens [121]. More importantly, these models harbor a very low predictability for anti-cancer drug responses, as illustrated by a National Cancer Institute retrospective study comparing cell line xenograft models with phase II clinical results [122].

Recent technological advances have resulted in the successful transplantation of patients’ tumour tissue into mice, thus permitting the development of patient-derived xenograft (PDX) cancer models. The use of PDX models, so far, appears to have largely increased clinical relevance and preclinical predictability. The development and application of PDX models will be further discussed in section 1.4.

1.2 Inhibitor of Apoptosis Protein (IAP) Family and BIRC6

1.2.1 Apoptosis

Apoptosis is a tightly regulated process of programmed cell death; it is characterized by morphological changes such as blebbing, fragmentation of the nucleus, cell shrinkage, and the disintegration into apoptotic bodies [123]. Apoptosis is an essential component of various
physiological processes, including normal cell turnover, tissue homeostasis, embryonic development, functioning of the immune system, and hormone-dependent atrophy [124]. However, deregulation of apoptosis, can lead to many pathological conditions, such as neurodegenerative diseases, autoimmune disorders, and cancers. The ability to resist apoptosis is one of the six hallmarks of cancer, as defined by the landmark papers by Hanahan and Weinberg [125, 126]. Evading cell death is a fundamental property for cancer cells to develop, metastasize, and resist various anti-cancer treatments.

At the cellular level, apoptosis is under tight molecular control, which involves (1) initial triggers from internal or external death signals [127], (2) activation of the proteolytic cleavage cascade (caspases activation) and degradation of the cellular architecture [128], and finally, (3) fragmentation into apoptotic bodies and recognition by immune cells for phagocytosis, or digestion by neighboring cells [129]. Apoptosis can be activated via the (1) extrinsic (death-receptor-mediated) pathway, (2) intrinsic (mitochondrial) pathway, and (3) Granzyme B pathway. All three pathways are mediated by a family of cysteine proteases called caspases, which are cysteine proteases that cleave after an aspartate residue of the substrates [130]. Caspases are originally present within cells as inactive procaspases; once activated by cleavage, caspases can often activate other procaspases, thus allowing for the initiation of a protease cascade.

The extrinsic pathway is initiated by the binding of a ligand to the transmembrane death receptors of the tumor necrosis factor (TNF) receptor gene superfamily, such as TNFR1 or FasR. The binding of FasL to FasR or TNF-α to TNFR1, will lead to the trimerization of receptors and the subsequent recruitment of specific adaptor proteins, such as the Fas-associated death domain (FADD) protein to FasL/FasR and TNF receptor-associated death domain (TRADD) to TNF-
α/TNFR1 [124]. Following along the FasR route, FADD will then associate with procaspase-8 via the dimerization of the death effector domain. At this point, a death-inducing signalling complex (DISC) is formed, which results in the autocatalytic activation of procaspase-8 [131].

Following the TNFR1 route, apoptosis is mediated via two sequential signalling complexes. The initial plasma membrane bound complex (complex I) is comprised of TNFR1, TRADD, RIP1, TRAF2, cIAP1, and cIAP2, and rapidly signals the activation of nuclear factor κB (NF-κB). In the second step, both TRADD and RIP1 associate with FADD and caspase-8, forming a cytoplasmic complex (complex II) that facilitates caspase-8 activation and cell death. Complex II then mediates apoptosis in situations where the initial signal (via complex I, NF-κB) fails to be activated [132]. Activated caspase-8 can directly cleave effector caspases-3 and -7, or it can cleave BID, which in certain cell types will translocate into the mitochondria to stimulate mitochondrial-mediated apoptosis [133].

The intrinsic (mitochondrial) pathway is initiated by the detection of a diverse array of intracellular stresses by the BH3-only proteins in the B-cell lymphoma-2 (Bcl2) family [134]. Some of these stimuli include DNA damage, free radicals, hypoxia, hyperthermia, radiation, viral infections, accumulation of misfolded proteins, and the absence of growth factors, hormones, or cytokines. Once a crucial threshold has been reached, BH-3 proteins will overcome the inhibitory effect of the anti-apoptotic B-cell lymphoma-2 (BCL-2) family members and induce the oligomerization of the pro-apoptotic BCL-2-family members BAX and BAK in the mitochondrial outer membranes [127]. BAK–BAX oligomers form channels that permit the efflux of intermembrane space proteins into the cytosol, such as cytochrome c, Smac/DIABLO, and HtrA2/Omi [135]. Cytochrome c, in conjunction with Apaf-1 and procaspase-9, form a large complex apoptosome in which caspase-9 becomes activated [136]. Active caspase-9 will then
propagate a proteolytic cascade of further caspase activation [127]. Smac can bind and inhibit IAP family proteins, including XIAP, cIAP1, cIAP2, survivin [137], ML-IAP [138], and BIRC6 [139].

The granzyme/perforin pathway is an alternative cell death pathway that is mediated exclusively by cytotoxic T cells. Secreted proteases granzyme A or B enter the cell through a perforin channel in the plasma membrane. Granzyme B involves the activation of procaspase-10 and the mitochondrial pathway [124]. The extrinsic, intrinsic, and granzyme B pathways converge on the same execution pathway that is mediated by the activation and cleavage of caspase-3, thus resulting in DNA fragmentation, degradation of cytoskeletal proteins, etc.

1.2.2 Inhibitors of Apoptosis Proteins Family and Functions

The Inhibitors of Apoptosis Proteins (IAP) form a family of functionally and structurally related proteins that all have a major role in cell death, in addition to regulatory roles in immunity, inflammation, cell cycle, and migration [140]. The human IAP family consists of 8 members, which include NAIP (BIRC1) [141], c-IAP1 (BIRC2), c-IAP2 (BIRC3) [142], XIAP (BIRC4) [143], survivin (BIRC5) [144], Apollon/Bruce (BIRC6) [145], ML-IAP (BIRC7 or livin) [146], and ILP-2 (BIRC8) [147] (Fig. 1.1). The proteins of this family are characterized by the presence of 1 to 3 baculovirus IAP repeats (BIR), a 70 amino acids zinc-binding motif that mediates protein-protein interactions. These interactions include the binding with caspases, which accounts for the direct effect of apoptosis inhibition [148-150]. NAIP, c-IAP1, cIAP2, and XIAP all have 3 BIR domains, whereas ILP-2, ML-IAP, survivin, and BIRC6 all have only 1 BIR domain. Several IAPs, including cIAP1, cIAP2, XIAP, ML-IAP, and ILP-2 also possess a RING (Really Interesting New Gene) finger domain, which are defined by the presence of seven
cysteines and one histidine that are able to coordinate two zinc atoms. The RING domain also has ubiquitin protease ligase (E3) activity, and is responsible for the auto-ubiquitination and degradation of IAPs following an apoptotic stimulus [151]. Both cIAP1 and cIAP2 possess a caspase-recruitment (CARD) domain within the linker region between the BIR and the RING domains. The CARD domain was shown to prevent cIAP1 auto-ubiquitination and degradation [152]. Uniquely, BIRC6 contains an ubiquitin-conjugating (UBC) domain that has chimeric E2/E3 ligase activity [139]. The UBC domain facilitates the proteosomal degradation of various proteins, including pro-apoptotic proteins p53, caspases-9 [153], Smac [139], and the mitotic regulator cyclin A [154].

IAPs are first known to demonstrate anti-apoptotic ability through antagonizing caspases, the final executors of apoptosis. XIAP is the most effective inhibitor of caspases of the IAP family; it directly binds to and inhibits caspase-3, -7 and -9 [155, 156]. Other IAPs such as cIAP1, cIAP2, survivin and BIRC6 are less efficient in caspases inhibition [153, 157-159]. However, they demonstrate significant anti-apoptosis properties. For instance, cIAP1 and cIAP2 primarily counteract apoptosis by promoting activation of the TNFR1 death receptor complex I (pro-survival) and inhibiting complex II (pro-apoptotic) at the extrinsic apoptotic pathway [160]. Survivin is reported to inhibit apoptosis via cooperative interactions with other IAPs, e.g., by stabilizing XIAP [161, 162], whereas BIRC6 is known for its role as an antagonist for both the precursor and mature forms of Smac and caspase-9 [163], and in the protection of cells from both intrinsic and extrinsic apoptosis pathways [164].

IAPs have recently emerged as broader regulators of cellular homeostasis, with functions extending beyond apoptosis inhibition [165]. Recent studies have shown IAPs to also modulate inflammatory signalling and immunity, mitogenic kinase signalling, proliferation and mitosis, as
well as tissue invasion and metastasis [166]. Most notably, cIAP1 and cIAP2 both control Ub-dependent signalling events that are essential for the activation of NF-κB signalling, which in turn drives the expression of genes important for inflammation, immunity, cell migration, and cell survival [166]. In particular, cIAP1 and cIAP2 polyubiquitinate components in TNFR complex I (including RIP1 and TRADD) subsequently activate IKK-mediated degradation of IκB and therefore release NF-κB into the nucleus [160]. cIAPs also mediate the ubiquitination of TNFR-associated factor 3 (TRAF3), leading to its proteosomal degradation and subsequently releases the brake for MAPK activation [167]. Notably, cIAP1 was found to be important for the maintenance of vasculature integrity at the physiological level via inhibition of caspase-8-mediated apoptosis in endothelial cells [168].

XIAP has been shown to form a complex with survivin that activates NF-κB, which in turn leads to increased fibronectin gene expression, signalling by beta1 integrins, and activation of cell motility kinases FAK and Src [169]. XIAP was also reported to regulate Akt activation [170], TGF-beta signalling [171], and TNFR2-mediated p38 and JNK activation [172, 173].

Survivin plays essential roles in regulating mitosis [174], the cellular stress response [175, 176], and the developmental pathways of gene expression [177]. Not typically expressed in normal, terminally-differentiated adult tissues, survivin has been found to be expressed in a large proportion of common human cancers [144]. With regards to its role in mitosis, survivin associates with microtubules of the mitotic spindle. Therefore, it can serve as a mitotic spindle checkpoint; a disruption to survivin-microtubule interactions can result in the loss of anti-apoptotic function and increased caspase-3 activity [174]. Notably, survivin expression can be induced by androgen (DHT) via the IGFR-1/AKT-dependent mechanism [111][178-181]. As a
result, overexpression of survivin can induce androgen-independent growth of androgen-dependent cells [111].
Figure 1.1 Schematic representation of the human IAP family of proteins (Adapted from Srinivasula and Ashwell 2008 Mol Cell [165])
1.2.3 Baculoviral IAP Repeat-Containing Protein 6 (BIRC6)

BIRC6 is an unusual member of the IAP family. The BIRC6 gene (BRUCE/APOLLON) encodes a 528 kDa protein in mammals, which consists of a single N-terminal BIR domain and a C-terminal ubiquitin-conjugating (UBC) domain; the latter has chimeric E2/E3 ubiquitin ligase activity as well as anti-apoptotic activity [139]. Through the BIR domain, the BIRC6 protein is able to bind to active caspases, including caspases-3, 6, 7, and 9. These interactions have been shown to underlie the ability of BIRC6 to inhibit the caspase cascade, and ultimately apoptosis [139]. Through the UBC domain, BIRC6 is able to facilitate the proteasomal degradation of pro-apoptotic proteins, including caspase-9 [153], SMAC/DIABLO [91, 163], and HTRA2/OMI [139, 182]. In contrast with other IAPs, BIRC6 has been shown to have a cytoprotective role, essential for the survival of mammalian cells [153, 163]. BIRC6 is also known for its essential role in regulating cytokinesis, the final event of cell division [183]. More recently, BIRC6 has been shown to be a novel regulator of mitotic cyclin A degradation, a critical process for the completion of mitosis [154]. Collectively, these studies have served to further support the key role of BIRC6 during cell proliferation. The dual roles of BIRC6 during the processes of cell death and division seem to resemble those of survivin, and thereby render it a promising target for the therapy of a variety of cancers [184].

1.2.3.1 BIRC6 and Cancer

Elevated expression of BIRC6 has been found in clinical specimens of a variety of cancers, including colorectal cancer [185], neuroblastoma [186], melanoma [164], non-small cell lung cancer [187], childhood de novo acute myeloid leukemia [188], ovarian cancer [189], and hepatocellular carcinoma [190]. Furthermore, BIRC6 silencing has been shown to sensitize cancer cells to various anticancer agents, including DNA damaging agents such as: camptothecin.
in glioma [191]; 5-fluorouracil in fibrosarcoma, cervical and breast cancer cells [192]; and oxaliplatin and cisplatin in non-small cell lung cancer, glioma [187, 191], and colonospheres [193]. Kinase inhibitors include: MEK inhibitor, a BRAFV600E-specific inhibitor in melanoma cells [164]; Sorafenib in hepatoma [190]; and apoptosis inducer TRAIL in melanoma [164].

1.2.4 IAP Family as Therapeutic Targets in Cancers

Elevated expression of several members of the IAP family has been found in cancers. For instance, survivin is highly expressed across various cancer types, but not in adult differentiated tissue [194]. Expression of XIAP, cIAP1, cIAP2, ML-IAP, and BIRC6 is frequently elevated in various cancers [146, 195-199]. In prostate cancer, elevated expression has been reported for XIAP, survivin, cIAP1, cIAP2 and BIRC6 [112, 200].

An increasing number of IAP-targeting agents are currently evaluated in the clinic for treatment of various cancers. One major class of IAP inhibitor are the Smac-mimicking IAP antagonists, or Smac mimetics that target cIAP1, cIAP2, XIAP, and ML-IAP. These small molecules contain the IAP-binding motif (Ala-Val-Pro-Ile) that are present in Smac, which effectively block IAP–caspase interactions and sensitize cancer cells to pro-apoptotic stimuli [137, 201]. There are currently 5 different Smac mimetics undergoing phase I and II clinical evaluations, including LCL161 (Novartis, phase I, II), GDC-0917/CUDC-427 (Genetech, phase I), TL32711/birinapant (Tetralogic Pharm, phase II), AT-406/Debio1143 (phase I), and HGS1029 (phase I) [202]. The inhibitors developed against survivin are currently under clinical trials, and include the small molecule YM155 (Astellas, phase II [203]) and antisense oligonucleotide (ASO) LY2181308 (Eli Lilly, phase II). An XIAP-targeting ASO, AEG35156 (Aegera), is also under clinical development (phase II) [107, 155]. To date, no inhibitor of BIRC6 has been reported in clinical or preclinical studies.
1.3 Antisense Oligonucleotides

1.3.1 Overview

Natural antisense transcripts were first described in prokaryotes, where they were found to downregulate the expression of complementary sense transcripts via Watson-Crick hybridization [204]. These antisense transcripts were later determined to be widely present within the eukaryotic world, inhibiting sense transcripts that encode proteins involved in extremely diverse biological functions, such as control of proliferation, development, structure, hormonal response, viral replication, etc [205]. It is estimated that 5-10% of the human genome may possess an antisense transcript [206], thereby suggesting that antisense modulation of gene expression may be a common regulatory mechanism within human cells.

Antisense oligonucleotides (ASOs) are synthetic polymers of chemically modified deoxyribonucleotides that containing sequence designed to be complementary to the sense sequence of target mRNAs. ASOs can be used as biochemical tools for basic scientific studies, and also be utilized as therapeutic agents against various diseases by specifically reducing gene or protein expression levels. The first demonstration of the concept of an ASO as a therapeutic agent was by Stephenson and Zamecnik in 1978 [207]. They reported the synthesis of a 13 nucleotide oligodeoxyribonucleotide that was complementary to a sequence of the Rous Sarcoma virus genome; its implementation demonstrated effective inhibition of viral RNA translation [207]. Since the early 1990s, substantial progress has been made in the development of antisense technology as new pharmaceutical agents have been discovered [208]. In particular, chemical modifications of ASOs, such as the replacement of the phosphodiester backbone with a phosphorothioate (PS) backbone (first generation ASO), and sugar modification at the 2’-position [including 2’-O-methoxyethyl (MOE) at the 3’ and 5’ ends of ASO (gapmer) (second
generation ASO]), have both greatly enhanced their nuclease resistance (i.e. stability) and RNA binding affinity, respectively [208]. A second generation 2’-MOE gapmer ASO has since entered clinical trials for multiple indications and demonstrated good activities and safety profiles.

Newer generation ASO, such as the sugar modified constrained-ethyl (cEt) ASO (Gen 2.5) (STAT3 [209, 210]), locked-nucleic acid (LNA) [211], and sugar – phosphate modified morpholino [212] are also under active development for clinical use, and are expected to show superior efficacies than second generation ASOs.

1.3.2 Mechanism of Action

Several mechanisms have been reported to explain the inhibitory action of ASOs, in terms of a target gene/protein. The most well-known mechanism involves the formation of an mRNA–ASO duplex (through Watson–Crick binding), which leads to RNase-H-mediated cleavage of the target mRNA in the RNA-DNA hybrid [213, 214]. Other mechanisms include the inhibition of mRNA splicing, the inhibition of mRNA maturation via the prevention of 5′-cap formation and polyadenylation, and the inhibition of ribosomal read-through during translation [215].

As ASOs target the RNA located within the nucleus and/or cytoplasm, they therefore must be delivered into target cells and cross the plasma membrane. A systemic application of ASOs is administered through parenteral injection, either via intravenous (IV) infusion, intraperitoneal (IP), or subcutaneous (SC) injection. Following systemic administration, ASOs are bound to plasma proteins (≥85%) from across all species [216, 217], with the greatest binding affinity for albumin. PS-ASO then rapidly transfers from the blood into tissues, a process that occurs within minutes to hours [218, 219]; this rapid transmission into cells is predominantly facilitated by endocytotic uptake. Once inside the intracellular space, ASOs exhibit long half-
lives (2–4 weeks) and prolonged activity, in suppressing or altering the expression of their target RNA [220].

1.3.3 Use of Antisense Oligonucleotides as Therapeutic Agents

Antisense oligonucleotides (ASOs) were first approved by the FDA for clinical use in 1998. Fomivirsen (Vitravene) is a first-generation ASO, and is used for treatment of cytomegalovirus retinitis (by Ionis Pharmaceuticals, previously known as Isis Pharmaceuticals) [221]. However, despite substantial efforts since the approval of Fomivirsen, no ASO-based drugs successfully passed phase III trials until 2013. In 2013, Mipomersen (a second-generation ASO that inhibits the apolipoprotein B100) was approved for homozygous familial hypercholesterolaemia [222, 223]. The slow initial development of ASO therapeutics can be attributed to hurdles regarding ASO stability, affinity, and off-target toxicity. However, with recent advances in chemical modification, the latest generation ASOs have acquired significant improvements in all three aspects [224]. Currently, there are approximately 70 clinical trials underway for the evaluation of ASOs targeting various cancers and neurodegenerative diseases [225]. ASOs targeting Bcl-2, Clusterin (CLU), Hsp27, STAT3, Raf-1, AR, and XIAP are being tested for treatment of various cancers.

Custirsen (OGX-011), a second generation ASO targeting CLU, was the first mCRPC-targeting ASO to enter phase III clinical trials (SYNERGY, NCT01188187). Results from phase I & II demonstrated Custirsen to be well tolerated in combination with docetaxel, and over half of the patients exhibited a ≥ 50% decline in PSA levels [226]. However, ultimately Custirsen did not meet the primary endpoint dictating a statistically significant improvement in overall survival for men with mCRPC. Two more phase III clinical trials for Custirsen (in combination with cabazitaxel/prednisone as a second-line chemotherapy for mCRPC (AFFINITY, NCT01578655)
and stage IV non-small cell lung cancer (NCT01630733) are currently ongoing. In addition, Apatorsen (OGX-427), a second generation ASO targeting Heat Shock Protein 27, is also under seven phase II evaluations, e.g., for CRPC [227, 228]. In 2014, AZD5312, a generation 2.5 ASO targeting AR, has entered a phase I clinical trial to determine the maximum tolerated dose in men (NCT02144051). Finally, since 2012, another generation 2.5 ASO IONIS-STAT3Rx targeting STAT3 is currently undergoing phase I/II evaluation in patients with advanced cancers (NCT01563302).

1.4 Patient-Derived Xenograft (PDX) Cancer Models

Successful development of novel cancer therapeutics is often hampered by discrepancies between drug efficacies, obtained in preclinical studies, and patient outcomes in clinical trials. These inconsistencies can be attributed to a lack of clinical relevance of the cancer models used for preclinical testing [121]. Recently, there is an increasing interest in the development and use of patient-derived xenograft (PDX) cancer models in various areas of cancer research, including drug efficacy testing, identification of new therapeutic targets and biomarkers, and elucidation of mechanisms underlying disease progression and treatment resistance, etc. [229]. PDX models are produced via grafting of fresh cancer tissue specimens into immunodeficient mice, subcutaneously, orthotopically (into the type of organ from which the cancer was derived), or under the kidney capsules. Fresh cancer tissues from the patients’ tumours, obtained following surgery or from biopsies, are cut into small pieces for grafting. Through serial transplantation of developing tumours transplantable tumour tissue lines can be established.

Transplantable PDX cancer tissue models, in particular those developed under kidney capsules, are well recognized to have a more accurate predictive ability, and hence greater
clinical relevance, than the commonly used cell line-based models. In contrast to the latter, the PDX models retain histopathological properties of the original cancers, such as tumour heterogeneity and tumour-stroma architecture, both of which are key factors in the progression of patients’ tumours [229]. Even during serial transplantation, PDX tissues retain a high degree of genomic, epigenetic, and gene expression patterns of the original tumours [230-234]. Therefore, this feature adds further confidence towards the assertion that PDX tumour tissue lines harbour a high degree of resemblance to patient tumours, in terms of disease progression and response to treatments [235]. For instance, colorectal PDX models responded to the anti-EGFR antibody cetuximab with rates and extents analogous to those observed in the clinic [236]. PDX models and patients exhibited comparable responses to treatment of pancreatic cancers with gemcitabine [237]. In addition, selected drugs that were effective in PDX models produced similar responsiveness (resistance and sensitivity) for patients with advanced refractory cancers [238]. These highly clinically relevant models could therefore be used as a platform for personalized or precision medicine, a rapidly developing area in translational cancer research [239, 240]. Besides its application in predicting drug response, the PDX system can also be an excellent resource for biomedical discovery, which include the identification of novel regulators in disease development [241, 242], new biomarkers, and therapeutic targets [243-245]). Previous work from our laboratory has used PDX models, generated from prostate cancer biopsies or prostatectomy samples, for a number of basic science discoveries. These include the novel metastasis master regulator gene GATA2 [242]; the epigenetic regulator CBX2 for advanced PCa [246]; the PEG10 gene, which has a crucial role in promoting NEPC progression [247]; various miRNAs as biomarkers for metastatic PCa [248]; as well as long non-coding RNA, SNORA55, as a novel biomarker and therapeutic target for PCa [249, 250].
However, certain factors have been recognized to limit full application of PDX. One major limitation is the lack of key immune components in the immunocompromised host. The strains that are most commonly used for PDX hosts are NOD-scid and NOD-scid IL2Rgamma (null) (NSG) mice. NOD-SCID mice lack mature T and B cells [251], whereas NSG mice lack mature T cells, B cells, and natural killer cells [252]. As a result, the interactions that occur within patients’ tumours, e.g., between cancer cells and immune cells/secreted cytokines, are not captured. Accordingly, the effect of immunotherapies on tumour growth can also not be fully assessed. Furthermore, the technical difficulties in the development and maintenance of stable PDX lines also represent a major hurdle in the widespread application of PDX system. For example, patient-derived tumour tissue is known to be more difficult to maintain and grow in mouse hosts than traditional cell lines. This is particularly true for less aggressive tumours or slow-growing cancer types, in which the “take-rate” can range from 62–89% in pancreatic and colorectal cancers to 13–27% in breast cancers [229, 253]. Prostate cancer has one of the lowest take rate of all cancer types [254]. Moreover, for the common research laboratory, the limited availability of obtaining clinical samples also limits the use of PDX cancer model in basic and translational research.

Given the great potential of PDX models in biomedical research, a number of large institutions have focused on the development of large PDX repositories. In early 2016, the US National Cancer Institute (NCI) announced the replacement of the traditional NCI-60 cancer cell lines panel with PDX models for preclinical drug testing [255]. The NCI PDX repository was launched in the spring of 2016, and has since made 75 PDX PDX models available for cancer research institutions worldwide. Meanwhile, a number of other research institutions have also reported and made their PDX collections available commercially. EuroPDX, a consortium from
16 European institutions, have reported 1500 established PDX models (majority of colorectal, pancreatic and gynecological cancers) and has become one of the largest PDX collections within the field [229]. Additionally, the Switzerland-based pharmaceutical giant, Novartis, has also reported the use of 1000 PDX models, of various organs of cancer origin, in a drug screening study [256]. Another well-known group with large PDX models includes Jackson Laboratory (450 models) (https://www.jax.org/jax-mice-and-services/in-vivo-pharmacology/oncology-services/pdx-tumors).

1.4.1 In Vivo Patient-Derived Xenograft (PDX) Prostate Cancer Tissue Models

At the Living Tumour Laboratory (LTL) of the BC Cancer Agency, we have established 'high-fidelity' transplantable prostate tumour xenograft lines by grafting and transplanting patients’ tumour tissue into NOD-SCID mice at the renal graft site [118, 119, 121]. The LTL prostate cancer repository encompasses models that include primary prostate adenocarcinoma, CRPC, enzalutamide-resistant CRPC, and NEPC (www.livingtumourlab.ca). These stable LTL PDX lines represent an excellent platform for drug efficacy testing, notably for advanced and currently incurable prostate cancer subtypes.

1.5 Thesis Theme and Rationale

The lack of effective therapy for advanced prostate cancer remains as one of the major unmet clinical needs. The recent approvals of more potent androgen axis-targeting agents have shown encouraging results in delaying disease progression in CRPC patients; however, resistance towards these agents occurs shortly after treatment. The increasing ability of cancer cells to resist apoptosis represents a fundamental mechanism of treatment resistance; yet, no related agents have been developed for targeting such recurring diseases.
Preliminary work by Low in our laboratory has revealed an association between BIRC6 and advanced PCa samples. BIRC6 was found to be increasingly expressed in Gleason score 6 to 8 PCa and CRPC clinical specimens [257]. Moreover, accumulating evidence supports the roles of BIRC6 in disease progression and treatment resistance in various cancers. Collectively, this evidence suggests that BIRC6 may play functional roles in PCA progression, including anti-apoptosis and treatment resistance, and may represent a potential therapeutic target in advanced, highly treatment-resistant PCa. Therefore, the current study first sought to more thoroughly validate the clinical association of BIRC6 with PCa using clinical samples and investigate the functional roles of BIRC6 in *in vitro* models. Next, we designed a new anti-BIRC6 agent, an antisense oligonucleotide, and evaluated the potential anti-cancer effect using cell line-based *in vitro* and *in vivo* models. Finally, based on the initial activity, we further explored the therapeutic potential of the anti-BIRC6 agent in enzalutamide-resistant, castration-resistant PCa using a highly clinically relevant PDX model developed in our laboratory.

The overall goal of this study is to evaluate the potential of targeting BIRC6 in advanced prostate cancer and assess the therapeutic efficacy of a novel anti-BIRC6 antisense oligonucleotide using a high fidelity PDX PCa model.

### 1.6 Hypotheses and Specific Aims

The main hypotheses of the study are as follows: (1) BIRC6 plays functional role in promoting survival of advanced PCa cells, and (2) Targeting tumor BIRC6 expression can suppress advanced PCa growth.
Main objective: To study the roles of BIRC6, an inhibitor of apoptosis protein, in advanced PCa and to assess the therapeutic efficacy of a novel anti-BIRC6 agent developed for treating the disease.

Specific Aims:

Specific aim 1. To investigate the clinical relevance and functional roles of BIRC6 in PCa

Specific aim 2. To develop anti-BIRC6 ASOs as a new therapeutic agent and validate the targeting effect in PCa cell line-based models

Specific aim 3. To evaluate the preclinical therapeutic efficacy of anti-BIRC6 ASO using patient-derived xenograft PCa models
Chapter 2: Clinical Relevance and Functional Roles of BIRC6 in Prostate Cancer

2.1 Introduction

Prostate cancers (PCa) initially present as androgen-dependent tumors, susceptible to growth arrest/apoptosis induced by androgen deprivation therapy. Although initially effective, androgen deprivation frequently results in the development of castration-resistant prostate cancer (CRPC), an advanced disease state that is highly resistant to existing therapies. Consequently, castration resistance commonly marks the end stage of PCa and as such, is a major obstacle in disease management [258]. The development of CRPC is characteristically associated with marked increases in resistance to apoptosis, a major death pathway that is commonly exploited by drug action [259, 260]. Apoptosis resistance results from the up-regulation of anti-apoptotic genes and their products, and is thought to be a key contributor in the development of resistance to castration and anti-cancer treatments. Therefore, elucidating the role of anti-apoptotic proteins in the progression of PCa will likely lead to improvements in the treatment of refractory disease.

The Inhibitors of Apoptosis Protein (IAP) family has been previously reported to play a key role in establishing apoptosis resistance in many cancers, and is characterized by the presence of one to three Baculoviral IAP Repeat (BIR) domains. IAPs have been demonstrated to bind to and inhibit a variety of pro-apoptotic factors, thereby effectively suppressing apoptosis induced by a wide range of effectors, including chemotherapeutics and irradiation [194]. The BIR domain is essential for the interaction of IAPs with pro-apoptotic factors, including caspases. A family of cysteine aspartic acid-specific proteases, caspases is present in a pro-form that, once activated via cleavage, is responsible for the degradation of death substrates (such as
poly-ADP-ribose polymerase (PARP)) that subsequently trigger apoptosis. Cleaved caspase-3 and cleaved PARP can be readily detected by Western blot analysis and are commonly used as markers for apoptosis [196].

The BIRC6 protein is, at 528 kDa, an unusually large member of the IAP family. It is comprised of an N-terminal BIR domain and a C-terminal ubiquitin-conjugating (UBC) domain; the latter has chimeric E2/E3 ubiquitin ligase activity and anti-apoptotic activity [139]. Via the BIR domain, BIRC6 is capable of binding to and inhibiting active caspases, including caspases-3, 6, 7, and 9. These interactions have been demonstrated to underlie the ability of BIRC6 to inhibit the caspase cascade and ultimately apoptosis [139]. Through the UBC domain, BIRC6 facilitates the proteasomal degradation of pro-apoptotic proteins caspase-9 [153], SMAC/DIABLO [153, 163], and HTRA2/OMI [139, 182]. BIRC6 is also a critical regulator of cytokinesis [183] and mitosis [154], and therefore plays an important role in cell proliferation.

Recent evidence corroborates the widespread role of BIRC6 in conferring apoptosis resistance to cancer cells, as indicated by in vitro studies with cells from gliomas [191], lung cancers [187], cervical cancers [192], fibrosarcomas [153, 192], osteosarcomas [183], breast cancers [192, 261], and colon cancers [193]. In breast and lung cancer cells, apoptosis triggered by the loss of BIRC6 expression has been shown to involve p53 stabilization [261, 262]. BIRC6 expression in clinical cancer samples has also been observed for colorectal cancer [185] and childhood de novo acute myeloid leukemia (AML) [188]. Within AML, elevated expression of BIRC6 mRNA was associated with an unfavourable response to chemotherapy and poor relapse-free survival rates [188]. An earlier study by Low first reported the role of BIRC6 in prostate cancer, and found BIRC6 expression to be significantly elevated in the malignant tissues of clinical specimens (Gleason 6–8 cancers and castration resistance) and PCa cell lines [257].
In this present study, we aimed to establish the role(s) of BIRC6 in the progression and survival of PCa. First, we sought to examine the frequency of genetic alterations in the BIRC6 gene by using large, publicly available PCa clinical cohorts (primary PCa, metastatic CRPC, and NEPC). Additionally, we determined the presence of elevated BIRC6 protein expression by using a clinical cohort of the Vancouver Prostate Centre, with a particular focus on correlation with clinical parameters, including T stages, lymph node metastasis, and PSA recurrence. Following clinical validation, we studied the functional roles of BIRC6 in PCa cell proliferation and survival by silencing BIRC6 gene expression. Finally, the status of BIRC6 in PCa cells in response to cell death induced by chemotherapeutic agents was also assessed.

2.2 Materials and Methods

2.2.1 Materials

Chemicals, solvents and solutions were obtained from Sigma-Aldrich, Oakville, ON, Canada, unless otherwise indicated.

2.2.2 Bioinformatic Database Analysis

The cBIO Cancer Genomics Portal (www.http://cbioportal.org) was used to assess the genomic alterations (mutations and copy number variations) affecting the BIRC6 locus in PCa. Eight large, publicly available independent PCa cohorts, with mutation and CNV datasets, were included; the details have been summarized in Table 2.1. The results pertaining to BIRC6 gene alteration frequencies in the cohorts were generated by online cBIO portal query searches.
2.2.3 Cell Lines

The PCa cell line LNCaP was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 media supplemented with FBS, in a humidified incubator at 37°C and 5% CO2. To determine the effect of chemotherapy-induced apoptosis on BIRC6 expression in LNCaP cells, 6x10^5 cells were seeded in 6-well plates, incubated overnight, and finally incubated with doxorubicin (1 or 0.5 µg/ml) for 24 hours (or as indicated).

2.2.4 Tissue Microarray (TMA) Construction and Immunohistochemistry (IHC)

Prostate specimens (consisting of 60 benign prostate samples, 137 primary tumours with no lymph node metastasis, 30 primary tumours with lymph node metastasis, 65 neo-adjuvant treated primary tumours, and 67 CRPCs) were obtained from the Vancouver Prostate Centre Tissue Bank, following written informed patients’ consent and institutional study approval. All samples were obtained through radical prostatectomy, except for the CRPC samples, which were obtained through transurethral resection of prostate (TURP). TMAs were constructed as previously described [263]. Immunohistochemical staining, using rabbit polyclonal antibody against BIRC6 (NB110-40730, Novus Biological, 1:50), was conducted with a Ventana autostainer (model Discover XT; Ventana Medical System, Tucson, AZ) using an enzyme-labelled biotin-streptavidin system and a solvent-resistant DAB Map kit (Ventana). BIRC6 staining of the tissues was evaluated and scored by pathologist Dr. Ladan Fazli (Vancouver Prostate Centre). 0 designates no staining of any tumour cells; 1 designates a faint or focal, questionably-present stain; 2 designates a stain of convincing intensity in a minority of cells; and 3 designates a stain of convincing intensity in a majority of cells.
2.2.5 siRNA Transfections

Custom siRNAs, synthesized by Dharmacon (Lafayette, CO) and known to target BIRC6, had the following sequences: siRNA-2, sense, 5’-CUC-AGG-AGA-GUA-CUG-CUC-AdTdT-3’ [183]. Non-targeting siRNA (siGENOME Non-Targeting Smartpool; Dharmacon) was used as the control. To examine the effect of the siRNAs on BIRC6 protein expression, LNCaP cells were plated in 6-well plates, in antibiotic-free RPMI-1640 medium supplemented with fetal bovine serum (10%). After 24 hr, the cells were transfected with 100 nM siRNA in lipofectamine 2000 reagent (Invitrogen; Burlington, ON), as per the manufacturer’s instructions. Vehicle control and non-targeting siRNAs were also applied to the replicate cell cultures.

2.2.6 Western Blotting

Cell lysates were prepared using cell lysis buffer (1% NP-40, 0.5% sodium deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche, Nutley, NJ). For detection of BIRC6 (528 kDa), 10 μg of whole cell lysate was resolved in a 5% SDS-polyacrylamide gel and electrotransferred overnight to a PVDF membrane in tris (25 mM), glycine (191.5 mM), methanol (10%), and SDS (0.05%) buffer at 40V and 4°C. Membranes were probed for BIRC6 using a rabbit polyclonal anti-BIRC6 antibody (1:500; Novus Biologicals). For detection of PARP and caspase-3 protein expression, 5–15 μg of whole cell lysate was run on 10 or 12.5% SDS-polyacrylamide gels, and following protein transfer, membranes were probed using rabbit anti-PARP and anti-caspase-3 antibodies (1:1000; Cell Signalling; Beverly, MA). Actin or vinculin were used as loading controls, and detected on membranes using rabbit anti-actin polyclonal antibody (1:2000; Sigma-Aldrich) and mouse anti-vinculin antibody (1:3000; Sigma-Aldrich).
2.2.7 Annexin V Assay

Apoptosis was measured by fluorescence-activated cell sorter (FACS) analysis, using annexin-V conjugated with fluorescein isothiocyanate (annexin-V-FITC) (BD Biosciences PharMingen) to stain for early apoptosis and propidium iodide (PI) for late apoptosis, as per the manufacturer’s protocol. Cells were cultured in RPMI-10% FBS medium and subsequently treated with BIRC6 siRNA. Forty-eight hours following treatment with siRNA, the cells were harvested, washed with cold PBS, and resuspended in 1X Binding Buffer (BD PharMingen, San Diego, CA) at a concentration of 1 x 10^6 cells/ml. Next, cell suspensions (100 µl; 1 x 10^5 cells cells) were transferred into new tubes, alongside the addition of 5 mL Annexin V–FITC and 5 µl PI aliquots. Finally, the cells were incubated in the dark for 15 min at 21°C. Annexin–FITC fluorescence was measured in the FL1 channel (using a 530/30 band pass filter) and PI in FL3 channel (660/20 BP band pass filter). Ten thousand events were collected, and apoptosis was assessed by counting the percentage of AnnexinV-positive cells. Data are shown as means ± SD of triplicate cultures.

2.2.8 MTT Cell Viability Assay

Twenty-five thousand cells were seeded per well, of a 24-well dish, and transfected with siRNA-2. At 0, 24, 48, and 72 hr following transfection, 50 µl of MTT (5 mg/ml) was added to each well and cultures were incubated in a humidified incubator at 37°C and 5% CO₂ for 4 hr. Five hundred µl of 20% SDS solution was then added to each well and incubated overnight at room temperature, in the absence of light. Samples (100 µl) were then transferred to 96-well plates, and absorbance was measured at 570 nm.
2.3 Results

2.3.1 Analysis of Genetic Alterations in the BIRC6 Gene in Multiple Prostate Cancer Clinical Cohorts

The presence of genetic alterations in the BIRC6 gene in prostate cancer, including mutations and copy number variations (CNV), were examined using publicly available clinical cohorts from the cBIO cancer genomics portal. Eight large independent prostate cancer cohorts, including mutation and CNV datasets, were available. Four out of the eight studies were comprised of mostly primary prostate cancers, including MSKCC 2010 [56], Broad/Cornell 2012 [48], Broad/Cornell 2013 [264], and TCGA 2015[265], whereas the remaining four studies focused primarily on metastatic CRPC or NEPC, including Michigan 2012 [266], SU2C/PCF Dream Team [267], Fred Hutchinson 2016 [268], and Trento/Cornell/Broad 2016 [105] (see Table 2.1). Analyses from the cBIO portal database found that the majority of patients in the cohorts, from both primary prostate cancers and metastatic CRPCs, have low frequencies of BIRC6 alteration, with about 6% or less overall gene alterations. However, an exception was found for the Trento/Cornell/Broad 2016 cohort, which showed alterations in ~17% of samples (Fig. 2.1A). The Trento/Cornell/Broad 2016 dataset consists of 114 PCa clinical samples (from 77 patients), from either CRPC (63 samples) or castration-resistant NEPC subtypes (44 samples) [105], thereby composing the largest NEPC cohort to date. Further analyses showed that both NEPC and CRPC subsets to have similar frequencies of alteration (20% vs 14% respectively), with NEPC demonstrating higher BIRC6 amplification (Fig. 2.1B). The discrepancy between the Trento/Cornell/Broad 2016 cohort with other CRPC cohorts may have resulted from differing methods of CNV analysis (in the Trento cohort: putative copy-number alterations adjusted by ploidy and purity with CLONET), thus potentially complicating the current analysis. Therefore,
in order to check whether the CNV analyses in these cohorts are comparable, the CNV change in the AR gene for these 8 cohorts was examined as a reference. As shown in Figure 2.1C-D, the percentage of AR gene amplification in the Trento/Cornell/Broad 2016 dataset was found to be highly comparable to the other 4 CRPC cohorts, with 60.3% (CRPC subset in the Trento cohort) versus 44.7% to 54.4% in the Michigan 2012, SU2C/PCF Dream Team, and Fred Hutchinson 2016 cohorts. Given that between the CRPC cohorts there were no significant variations in CNV within the “reference gene”, the high percentage of BIRC6 gene amplification, as shown in the Trento/Cornell/Broad 2016 cohort, is likely indicative of a significant gain of BIRC6 for this group of CRPC/NEPC patients. Taken together, these results suggest that BIRC6 amplification (and its resultant elevated expression) may potentially favour PCa progression in advanced PCa.
<table>
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Table 2.1 Details of 8 prostate cancer clinical cohorts from cBIO cancer genomics portal
Figure 2.1. Analysis of gene alterations in prostate cancer clinical cohorts from the cBIO cancer genomics portal. 

A-B, alterations of BIRC6 gene. A, majority of clinical cohorts analysed have low frequencies of BIRC6 alteration, except the Trento/Cornell/Broad 2016 cohort, shown in (B). C-D, alterations of AR gene were examined as reference for CNV analyses.
2.3.2 Elevated BIRC6 Protein Expression is Associated with Poor Prognostic Factors in Prostate Cancer Patients

Next, we set out to investigate the protein expression levels of BIRC6 in PCa clinical samples from a Vancouver Prostate Centre cohort (177 cases). The associations between various clinical parameters of PCa, i.e. clinical T stage, PSA recurrence, lymph node metastasis, and capsule invasion, with respects to changes in BIRC6 protein expression were examined. Immunohistochemical staining of BIRC6 in PCa tissue arrays showed BIRC6 expression to be elevated in tumours of more advanced clinical stages, i.e. expression of BIRC6 was significantly higher for tumours in the T3-4 stages, compared to the T1-2 stages or the benign prostate (mean intensity: 1.91, 1.60, and 1.53, respectively; benign to T3-4, p = 0.003; T1-2 to T3-4, p = 0.006; Student’s t test) (Fig. 2.2A). Elevated BIRC6 expression also correlated positively with poor prognostic factors, such as PSA recurrence (Fig. 2.2B), lymph node metastasis (Fig. 2.2C), and prostatic capsule invasion (Fig. 2.2D) (p = 0.057, 0.029 and 0.025, respectively, Chi square test for trend); thus indicative of its association with more advanced prostate cancers.

The expression of survivin was also found to be elevated in PCa specimens (p = 0.004, benign to T3-4) and similarly to BIRC6, was correlated with the previously mentioned poor prognostic factors (p = 0.017, PSA recurrence; p = 0.028, capsule invasion; p = 0.006, lymph node metastasis) (Fig. 2.3). Elevated XIAP expression was also observed in PCas with poor prognostic factors; however, statistical significance was not reached. Finally, no correlation was seen for cIAP1 (Fig. 2.3). Taken together, the data indicate that BIRC6, similar to survivin, may play a role in PCa progression.
Figure 2.2. Elevated BIRC6 protein expression is associated with poor clinical parameters in clinical samples. A, correlation of IHC staining intensity of BIRC6 and clinical (T) stages of prostate cancer (mean staining intensity ± SEM) in Vancouver Prostate Centre tissue microarray clinical cohort. Statistical test: Student t test. B-D, correlation of BIRC6 IHC staining intensity with the absence and presence of poor prognostic factors, such as recurrence of PSA, lymph node metastasis and prostatic capsule invasion. The statistical significance of positive trends was determined by the Chi square test for trend.
Figure 2.3. Correlations between survivin, XIAP, and cIAP1 protein expressions with poor prognostic factors. Positive correlations between survivin expression with clinical (T) stages, recurrence of PSA, lymph node metastasis and prostatic capsule invasion were observed in Vancouver Prostate Centre clinical cohort. Statistical tests: T-stage correlation (Student’s t test); other clinical parameters (Chi square test for trend)
2.3.3 BIRC6 is Important in Survival and Inhibits Apoptosis in Prostate Cancer Cells

It was previously reported that a reduction in BIRC6 expression induces apoptosis, e.g. in breast cancer cells. We used the LNCaP cell line to study the effects of reducing BIRC6 expression on prostate cancer cell viability. Incubating LNCaP cells transfected with BIRC6-targeting siRNA-1 and -2 (Lanes 3, 4, 7, 8, 11, 12) resulted in substantial losses of BIRC6 protein, as compared to cells treated with lipofectamine only (Lanes 2, 6, 10), lipofectamine + non-targeting siRNA (Lanes 5, 9, 13), or no treatment (Lane 1) (Fig. 2.4A). This effect was apparent 30 hr following transfection, and became increasingly more prominent at 54 and 78 hr. It may be noted that the cells transfected with lipofectamine only, or with lipofectamine + non-targeting siRNA, showed a small increase in BIRC6 expression, presumably due to the vehicle. All subsequent knockdown experiments were conducted using siRNA-2.

Following transfection, the siRNA-2 transfected cultures showed a marked reduction in cell viability, relative to the non-targeting siRNA-treated cultures (Fig. 2.4B). Cell viability of siRNA-2 cultures was found to be considerably lower than that of the non-targeting siRNA-treated cultures at 78 and 102 hr. The effect of BIRC6 reduction was also studied in PC-3 PCa cells. Similar to LNCaP cells, siRNA-2 transfected PC-3 cells showed a significant decrease in cell viability, as compared to the non-targeting siRNA-treated cells 72 hr following transfection (Fig. 2.4C).

BIRC6 reduction induces apoptosis in LNCaP cells, as demonstrated by Annexin-V staining and immunoblotting. As shown in Fig. 2.5A, there was a significant increase in Annexin-V positive cells in siRNA-2 transfected cells (12.19%), compared to non-target siRNA (3.65%, p = 0.01) and lipofectamine treated cells (3.55%, p = 0.012). Consistent with the results of the Annexin-V assay, BIRC6-silenced cells showed marked changes in the expression of
apoptosis markers (Figure 2.5B). An increase in cleaved caspase-3, loss of full length PARP, and an increase in a cleaved PARP, were observed in comparison with LNCaP cells transfected with non-targeting siRNA (Lane 3, 4). A decrease in full length PARP was observed following the transfection of LNCaP cells with lipofectamine or non-targeting (control) siRNA (Lanes 2, 4); this might be the result of non-specific degradation of full length PARP, particularly in LNCaP cells. The loss of full length PARP in these controls was not associated with a corresponding increase in cleaved PARP, and was also not coupled to a significant increase in cleaved caspase-3, thus indicating that these controls did not lead to induction of apoptosis.
Figure 2.4. Reduction of BIRC6 expression decreases prostate cancer cell viability and proliferation. 

A. Treatment of LNCaP cells with siRNAs targeting BIRC6 leads to reduction of BIRC6 protein expression. siRNA-1 and siRNA-2 showed comparable BIRC6 inhibition.

B. Treatment of LNCaP cells with siRNA-2 targeting BIRC6 leads to reduced cell proliferation as shown by MTT assay. The relative cell viability in the siRNA-2 cultures was considerably lower than that in the non-targeting siRNA treated cultures.

C. Knock-down of BIRC6 in PC-3 cells by siRNA-2 also resulted in significant reduction of cell viability at 72 h after transfection by MTT assay. Western blot shows apparent decrease of BIRC6 expression at 72 h after siRNA transfection. Error bars depict SD.
Figure 2.5. Reduction of BIRC6 expression induces apoptosis in LNCaP cells. A, annexin V assay assessing apoptosis of LNCaP cells transfected with Lipofectamine (Lipo), non-targeting control siRNA (NT), or BIRC6 siRNA2. B, Western blotting of caspase-3 and PARP in LNCaP cells 96 hours after indicated treatment.
2.3.4 BIRC6 Reduction Precedes Cell Death Induced by a Chemotherapeutic Agent

Although it has been observed that BIRC6 reduction leads to increased apoptosis, little is known about the role of BIRC6 when cancer cells are undergoing apoptotic stress, such as induced by chemotherapy. Doxorubicin, a cytotoxic drug commonly used for the therapy of prostate and other cancers [269], is known to effectively induce apoptosis in cancer cells [270]. To investigate the effect of doxorubicin-induced apoptosis on BIRC6 expression in PCa cells, LNCaP cells were incubated for 24 hr with or without doxorubicin (1 µg/ml). As shown by Western blot analysis, treatment with doxorubicin resulted in a substantial reduction in BIRC6 expression (Fig. 2.6A). Additionally, there was also a reduction in the protein expression of full length PARP and an increase in cleaved PARP, which are both indicative of apoptosis. Treatment of LNCaP cells with doxorubicin demonstrated a dose- and time-dependent reduction of BIRC6 expression (Fig. 2.6B). Finally, to understand whether BIRC6 reduction was the cause or consequence of doxorubicin-induced apoptosis, temporal expression of both BIRC6 and PARP following doxorubicin treatment was studied. BIRC6 protein expression decreased continuously, beginning at 4 hours and continuing up until to 24 hours, following treatment with doxorubicin at 500 ng/ml. The reduction of BIRC6 was marked at 8 hours, yet PARP cleavage was not observed until 24 hours after treatment; this implies BIRC6 reduction to be an early response to doxorubicin, and not the consequence of doxorubicin-induced apoptosis (Fig. 2.6C).
Figure 2.6. BIRC6 reduction precedes cell death induced by doxorubicin. A, incubation (24 hr) of LNCaP cells with doxorubicin (1 µg/ml) leads to reduction of BIRC6 protein expression and apoptosis (PARP cleavage) as indicated by Western blot analysis. B, doxorubicin treatment was associated with BIRC6 reduction in a dose-dependent manner. C, doxorubicin induced BIRC6 reduction precedes apoptosis induction as indicated by PARP cleavage at 24 hr. Doxorubicin at 500 ng/ml equals to 920nM.
2.4 Discussion

Despite recent advances in PCa therapy, disease progression still remains unavoidable and treatment resistance persists as the major challenge in the management of the disease [107, 271]. It has now been well established that the treatment resistance of cancers is largely based on resistance to apoptosis. In particular, the upregulation of inhibitors of apoptosis proteins (IAP) is considered to be one of the major mechanisms by which cancer cells can evade cell death [107, 258]. In the present study, we have established the clinical significance of BIRC6 in PCa progression. First, the BIRC6 gene was found to be amplified or altered in 17% of cases, using the latest clinical cohorts of CRPC and NEPC (Fig. 2.1). More importantly, elevated BIRC6 protein expression is correlated with poorer prognoses for prostate cancer patients (Fig. 2.2). This is consistent with our previous study, which demonstrated upregulation of BIRC6 in Gleason 6-8 prostate cancers and CRPC [257]. In addition, similar correlations were found for survivin (Fig. 2.3), an IAP which has previously been implicated in PCa [200, 272, 273]. Although the genetic data from the cBIO portal did not show consistent increases in BIRC6 gene alteration among all CRPC and PCa cohorts, the IHC data provide strong evidence to support the role of BIRC6 in promoting PCa progression at the protein level.

Functional studies have shown BIRC6 to be important for the survival of PCa cells. Notably, the specific reduction of BIRC6 expression by siRNAs led to a marked inhibition of PCa cell viability (Fig. 2.4B-C) and an increase in apoptosis (Fig. 2.5). These results are consistent with reports regarding the critical role of BIRC6 in the survival of a variety of cancer cells [163, 183, 192, 261, 262]. Importantly, we have shown that a decrease in cell viability, as induced by BIRC6 reduction, was not confined solely to cells expressing wild-type p53; this is contrary to previous reports which have suggested that apoptosis resulting from BIRC6
knockdown in lung and breast cancer cells requires functional p53 [163, 262]. Both wild type p53 (LNCaP) and p53 null (PC-3) cells were demonstrated to be sensitive to BIRC6 siRNA-induced growth inhibition. This variation signifies that apoptosis induction by loss of BIRC6 can be facilitated by either p53- or non-p53-based mechanisms in different models. Therefore, further investigation is necessary to fully understand the mechanism that underlies apoptosis that results from BIRC6 reduction in cancer cells lacking functional p53.

The finding that treatment of LNCaP cells with doxorubicin results in a dramatic loss of BIRC6 expression (Fig. 2.6A-B) is consistent with a previous report, demonstrating that apoptosis induced by the topoisomerase inhibitors etoposide and camptothecin was associated with degradation of BIRC6 protein [163]. The authors concluded that degradation of BIRC6 appears to be a general event during the initiation of apoptosis [163]. In the present study, our finding that doxorubicin-induced BIRC6 decline precedes PARP cleavage (Fig. 2.6C) suggests that BIRC6 reduction may be a critical event, occurring prior to the execution of doxorubicin-induced apoptosis. In fact, BIRC6 was reported to be a critical cytoprotective regulator, a property not reported for other IAPs. The reduction of BIRC6 expression is thought to lower the threshold of apoptosis; therefore, it is likely that the apoptotic effect of doxorubicin and other chemotherapies, is based, at least in part, on reducted BIRC6 protein expression. However, the exact mechanisms underlying the reduction of BIRC6 expression by cytotoxic agents prior to apoptosis remain to be elucidated.

Targeting BIRC6, and hence promoting pro-apoptotic events, may be useful for sensitizing PCa cells to anti-cancer therapies. It is noteworthy that drugs targeting other IAP family members, e.g. XIAP and survivin, have shown promise for use as sensitizers in PCa therapy. For example, antisense inhibitors of XIAP have led to sensitization of CRPC cells to
cisplatin and TNF-related apoptosis-inducing ligand (TRAIL) [274]; treatment of PC-3 PCa xenografts with XIAP inhibitors (in combination with docetaxel) has resulted in sustained tumor regression [275]. In conclusion, results in this chapter provide substantial evidence that supporting BIRC6 as a potential novel therapeutic target for advanced PCa.
Chapter 3: Development of BIRC6-Based, Dual IAP-Targeting ASOs as Novel Therapeutic Agent for Prostate Cancer

3.1 Introduction

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related deaths for males in the Western world [276]. Prostate cancers are initially androgen-dependent, which allows for androgen deprivation therapy (ADT) to induce marked tumour regression. However, resistance to ADT will inevitably emerge and lead to castration-resistant prostate cancer (CRPC). The current standard of care for treating CRPC is systemic, docetaxel-based chemotherapy, which increases the overall survival of patients by about 2 months, as compared to mitoxantrone-based therapy [74, 75]. Recently, sipuleucel-T, cabazitaxel, abiraterone, MDV3100, and Radium-223 have shown a more prolonged overall survival benefit and have been approved by the FDA for treatment [277]. However, none of these drugs therapies are curative; at best, they incrementally improve overall survival. Clearly, establishment of more effective therapeutic targets and drugs, specifically of those targeting the molecular drivers of metastatic CRPC, is of critical importance for improved disease management and patient survival [278].

Apoptosis, a cell death-inducing process important for the regulation of cell numbers in normal tissues, can be triggered by a variety of extracellular and intracellular death signals. These involve the activation of caspases (intracellular cysteine proteases) that mediate the execution of apoptosis [279]. Human cancers are characterized by a resistance to apoptosis (intrinsic or acquired), which is considered to be a key factor underlying resistance to therapeutic intervention. As such, promising new strategies have been developed based on drug-induced
apoptosis [258]. Since the treatment resistance of CRPC is thought to be based on a heightened resistance to apoptosis, it may be therefore addressed by targeting anti-apoptotic genes and their products [107].

The Inhibitors of Apoptosis (IAP) form a family of functionally and structurally related proteins that all have a major role in the regulation of cell death. By binding to caspases, these proteins suppress apoptosis, acting as endogenous apoptosis inhibitors. The human IAP family consists of 8 members, characterized by the presence of 1 to 3 baculovirus inhibitor of apoptosis repeat (BIR) motifs that are involved in the binding of IAPs to caspases. There is increasing evidence that demonstrate the effect of IAPs on other cellular processes, such as ubiquitin-dependent signalling events. These events contribute towards the activation of nuclear factor κB (NFκB) transcription factors, which in turn drive the expression of genes important in various cellular processes, such as cell survival [166]. Subsequently, due to this ability to control cell death and their elevated expression in a variety of cancer cell types, IAP proteins are attractive targets for the development of novel anti-cancer treatments [280]. Four IAP members, i.e. XIAP, survivin, cIAP1, and cIAP2, have been reported to be up-regulated in PCa [200]; survivin in particular is promising as a potential therapeutic target for this disease [272, 273].

The BIRC6 gene (BRUCE/APOLLON) encodes a 528 kDa protein in mammals, consisting of a single N-terminal BIR domain and a C-terminal ubiquitin conjugating (UBC) domain; the latter has chimeric E2/E3 ubiquitin ligase activity, as well as anti-apoptotic activity [139]. The BIRC6 protein is able to bind to active caspases (including caspases-3, 6, 7, and 9) via the BIR domain, and such interactions have been shown to underlie its ability to inhibit the caspase cascade and ultimately apoptosis [139]. Through the UBC domain, BIRC6 facilitates the proteasomal degradation of pro-apoptotic proteins, including caspase-9 [153], SMAC/DIABLO.
Elevated expression of BIRC6 has been found in a variety of cancers, i.e. childhood de novo acute myeloid leukemia [188], colorectal cancer [185, 193], neuroblastoma [139, 186], melanoma [164], and non-small cell lung cancer [187]. Furthermore, BIRC6 has been implicated in the maintenance of resistance against cell death stimuli [191, 192]. In contrast to other IAPs, BIRC6 has been shown to have a cytoprotective role, which is essential for the survival of mammalian cells [153, 163]. Additionally, BIRC6 is also known for its essential role in regulating cytokinesis, the final event of cell division [183]. These dual roles of BIRC6 in regulating cell death and division processes resemble those of survivin, and thereby designate BIRC6 as a promising target for the therapy of a variety of cancers [184].

We recently showed elevated expression of BIRC6 in numerous prostate cancer cell lines and clinical specimens, and also found increased BIRC6 expression to be associated with Gleason score 6-8 prostate cancers and CRPC [112]. In the previous chapter, we used a larger cohort of clinical PCa samples and established a correlation between elevated BIRC6 expression and advanced PCa. In this chapter, we first examined a correlation between increased BIRC6 expression and expressions of other IAP members in PCa clinical samples. Next, we designed dual antisense oligonucleotides (dASOs) to simultaneously target BIRC6 and an additional IAP, in order to achieve maximal anti-tumour activity. Promising results have been found using in vitro and in vivo models.

### 3.2 Materials and Methods

#### 3.2.1 Materials

Chemicals, solvents and solutions were obtained from Sigma-Aldrich, Oakville, ON, Canada, unless otherwise indicated. Anti-BIRC6 (Novus Biologicals, #NB110-40730)
anti-survivin (71G4B7) (Cell Signalling, #2808) [49]; anti-XIAP (H-202) (#sc-11426, Santa Cruz Biotechnology, Santa Cruz, CA) [50]. cIAP-1/HIAP-2 antibody (R&D Systems #MAB8181) for IHC, anti-cIAP1 (D5G9) (#7065, Cell Signalling Technology, Danvers, MA) for Western Blotting. Unless otherwise indicated, the same antibodies were used for both immunohistochemistry and Western blotting.

3.2.2 Cell Lines

PC-3 human prostate cancer cell lines were obtained from the American Type Culture Collection (1991, ATCC), and C4-2 cells were kindly provided by Dr. L.W.K. Chung (1992, MD Anderson Cancer Center, Houston, TX). Both cell lines were maintained as monolayer cultures in RPMI-1640 (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). Prior to usage, the cells were determined to be mycoplasma free (Mycoplasma Detection Kit, Invitrogen # rep-pt2) and were not authenticated.

3.2.3 Tissue Microarray (TMA) Construction and Immunohistochemistry (IHC)

The method of TMA construction, IHC staining, and staining intensity scoring are described in the previous chapter (section 2.2.4). Rabbit polyclonal antibody against BIRC6 (NB110-40730, Novus Biological, 1:50), rabbit monoclonal antibody against Survivin (#2808, Cell Signalling, 1:50), monoclonal antibody against cIAP1 (MAB8181, R & D Systems, 1:200), and rabbit polyclonal antibody against XIAP (#sc- 11426, Santa Cruz, 1:25) were used. IAP family staining intensity was evaluated and scored by pathologist Dr. Ladan Fazli (Vancouver Prostate Centre), as mentioned previously.
3.2.4 Design and Validation of BIRC6-Based Dual IAP-Targeting Antisense Oligonucleotides (dASOs)

Dual IAP-targeting ASOs (dASOs) were designed as 20-mers with perfect complementarity to BIRC6 mRNA sections and with no more than 3 base mismatches to the second target mRNA (i.e. cIAP1 or survivin). Sequence alignment for each pair of targeted genes was performed using Clustalw (http://www.genome.jp/tools/clustalw/) and BLAST 2 Sequence in NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi), in order to identify sequences with highest complementarities. ASOs with full phosphorothioate-modified backbone were purchased (Eurofins MWG Operon). The dASO knock down efficacy of the six designed dASOs was checked using Western blot analysis, by determining target protein expression 48 hours after transfection. Two dASO candidates (6w2 and 6w5) were selected for further studies: dASO 6w2 (5’CTGCAGCATCATGTGGACT) and dASO 6w5 (5’CAGGTGAAACACTGGGACA).

Non-targeting control ASOs: scramble (Scrb) B control (5’CCTTCCCTGAAGGTTCCTCC), and mismatched (MM) control (5’CAGCAGCAGAGTATTTATCAT). Further information on dASO targeting regions and percentage of matched sequences to target mRNA are shown in Table 3.1.

3.2.5 siRNA and ASO Transfections

Small interfering RNAs (siRNAs) targeting cIAP1 (si-cIAP1, siGENOME SMARTpool human BIRC2), survivin (si-Surv, siGENOME SMARTpool human BIRC5), BIRC6 (si-BIRC6, 5’-GUU-UCA-AAG-CAGGAU-GAU-GdTdT-3’ [262]) and negative control (siCtrl) siRNAs were purchased from Dharmacon (Cat #M-004390-02-0005, M-003459-03-0005 and D001810-10-05, Chicago, IL). Cells were transfected with siRNA (2 nM for si-survivin and si-cIAP1, 10 nM for
si-BIRC6) or ASO (100-200 nM) for 72 hours using oligofectamin reagent (Invitrogen) as per the manufacturer’s instructions.

3.2.6 Western Blotting

Cell lysates were prepared using cell lysis buffer (1% NP-40, 0.5% sodium deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche, Nutley, NJ). For detection of BIRC6, 10 μg of whole cell lysate was resolved in 5% SDS-polyacrylamide gel and electrotransferred to a PVDF membrane in tris (25 mM), glycine (191.5 mM), methanol (10%), and SDS (0.05%) buffer at 40V and 4°C, overnight. Membranes were probed with anti-BIRC6 antibody (1:500; Novus Biologicals) at room temperature for 2.5 hours. For detection of cIAP1 and survivin, the lysate was resolved in 10% and 15% SDS polyacrylamide gel, respectively, and electrotransferred to a PVDF membrane in tris (25 mM), glycine (191.5 mM), and methanol (10%) buffer at 100V for 1 hour. Membranes were probed with anti-cIAP1 (1:500; Cell Signalling, #7065) and anti-survivin (1:500; Cell Signalling, #2808) antibodies at room temperature for 2.5 hours. Either actin or vinculin were used as loading controls and detected on membranes using rabbit anti-actin polyclonal antibody (1:2000; Sigma-Aldrich) or mouse anti-vinculin antibody (1:5000; Sigma-Aldrich).

3.2.7 Annexin V Assay

Apoptosis was detected by fluorescence-activated cell sorter (FACS) analysis with annexin-V conjugated with fluorescein isothiocyanate (Annexin-V-FITC) (Invitrogen) and propidium iodide (PI) staining, as previously described, per the manufacturer’s protocol [28]. Early apoptotic cells were identified as Annexin-V positive and PI negative. Results of triplicate experiments are presented as means ± SD.
3.2.8 MTS Cell Viability Assay

C4-2 (1 \times 10^5) or PC-3 cells (2.5 \times 10^4) were seeded onto 12- or 24-well culture plates and transfected the following day. MTS (Promega, Madison, MI) was added to wells at 0, 48, 72, and 96 hours after transfection and incubated at 37°C for 2 hours. Aliquots (100 μl) of the culture medium were then transferred onto a 96-well plate for measuring absorbance at OD490. Triplicate wells were tested per assay and each experiment was repeated twice.

3.2.9 Cell Proliferation Assay

PC-3 cells (5 \times 10^4) were seeded onto 12-well plates and transfected with ASOs the following day. Cell numbers were counted at 0, 48, 72, and 96 hours after transfection using a TC10™ Automated Cell Counter (Bio-rad Laboratories, Inc, Berkeley, CA). Triplicate wells were tested per assay and the experiment was repeated twice. Results are presented as percentage of untreated control values, mean ± S.D.

3.2.10 Cell Cycle Analysis

Cell cycle distribution was determined by flow cytometry of PI-stained cells as previously described [112]. Cells were fixed at 72 hours after transfection. The proportion of cells in G1, S, and G2-M phases of the cell cycle was determined using a FlowJo program (TreeStar Inc, Ashland, OR).

3.2.11 4,6-Diamidino-2-Phenylindole (DAPI) Staining.

PC-3 cells were seeded on cover slips in 12 well plates and transfected with ASOs the subsequent day. Following 72 hours of transfection, cells were washed twice with PBS and mounted on slides using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, CA). Cell morphology was examined using a fluorescent microscope (Carl Zeiss, Germany). Cells exhibiting fragmented nuclear bodies were characterized as undergoing apoptosis. A total
of 500 cells were counted in five randomly selected fields per sample, using a magnification of 400x.

3.2.12 NFκappa B Dual Luciferase Reporter Assay

PC-3 cells (7x10^3) were seeded onto 96-well plates and co-transfected the following day with 0.05 μg pGL4.32 [luc2P/NF-kB-RE/Hygro] (# E849A, Promega Corp., Madison, WI); 1 ng pRL-CMV (Renilla); and 100 nM dASOs, 10 nM si-BIRC6, or 2 nM si-cIAP1 using lipofectamine 2000, as per the manufacturer’s instructions. For induction of NFκB signalling, cells were incubated with 20 ng/ml TNFα at 37°C for 5 hours. Luciferase activity was assessed with a Dual-luciferase reporter assay system (#E1910, Promega) at 48 hours following transfection, and subsequently measured using a Tecan, Infinite 200Pro microplate reader (Tecan, Männedorf, Switzerland), as per the manufacturer’s instructions. Transfection efficiency was normalized to Renilla luciferase activity. Fold induction of NFκB signalling was calculated as the average of normalized relative light units of induced cells / average of normalized relative light units of non-induced cells. Triplicate wells were tested per assay and the experiment was duplicated.

3.2.13 Animal Studies

PC-3 cells (1 x 10^6) were mixed with matrigel and inoculated subcutaneously in the flanks of 6- to 8-weeks-old NOD-SCID mice under isoflurane anesthesia. When tumours reached a volume of 50-70 mm^3, the mice were randomized into 3 groups (n = 12 tumours per group): control ASO, dASO 6w2, and dASO 6w5. The ASOs were administrated via intraperitoneal injection, once daily for 15 consecutive days, at a dose of 10 mg/kg. Tumour volume was measured on day 0 and day 15 (i.e. last day of treatment), using the formula: volume = (width)^2 x length/2. Mice were euthanized on day 15 and the tumours were fixed in preparation for immunohistochemical
staining. The percentage of tumour growth represents the change in tumour volume, as measured on days 1 and 15. Viable tumour volume refers to the total tumour volume \( x \times (100\% - \% \text{necrotic area}) \), where \% necrotic area was determined by microscopic examination of the H&E stained sections. Scoring of BIRC6 was determined on a four-point scale, as mentioned above. Ki-67 positive cells were counted in 6-8 randomly selected fields (at 40x magnification) and results are presented as the percentage of cells with Ki-67 positive nuclei, as compared to the total number of cells.

### 3.2.14 Statistical Analyses

Comparisons between two groups were made using the Student t test. Analyses of the correlations between IAP members were performed using a Spearman nonparametric test. Analyses of the correlation between BIRC6 expression trend and various prognostic factors were carried out using the Chi-squared test for trend. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad), and results with a \( p < 0.05 \) were considered significant.

### 3.3 Results

#### 3.3.1 Positive Correlation Between Expressions of BIRC6 and Other IAP Members in Human Prostate Cancer

To establish whether there exists a correlation between increases in the expression of BIRC6 in PCa and those of other IAP members, the IHC expression profiles of BIRC6, XIAP, survivin, and cIAP1 in individual clinical prostate samples (including benign tissue, primary cancer, and CRPC) were analyzed for correlations by the Spearman’s rank correlation test using GraphPad 4 software. The Spearman \( r \) coefficients for the BIRC6 – survivin and BIRC6 – XIAP combinations were 0.3987 and 0.6025, respectively (\( p < 0.0001 \)). This indicates positive
correlations between BIRC6 and survivin, and between BIRC6 and XIAP. A weak, but significant, positive correlation was observed for the BIRC6 – cIAP1 combination, with a Spearman r coefficient of 0.194 (p = 0.0072). The positive correlations between the expressions of BIRC6 and the other IAPs were visualized by representative IHC stained images of matched patients’ samples (Fig. 3.1).
Figure 3.1. BIRC6 elevated expression is co-upregulated with other IAP members. Protein expressions of BIRC6, XIAP, survivin and cIAP1 in individual clinical prostate samples (including benign tissue, primary cancer and CRPC) were assessed by IHC staining. Correlations of expressions (staining scores) between BIRC6 and other IAP members were analysed by Spearman’s rank correlation test (see text). Representative IHC images of correlated expression levels between BIRC6 and survivin, XIAP and cIAP1 are shown. 20x magnification, scale bar, 100 μm.
3.3.2 Design of BIRC6-Based dASOs, 6w2 and 6w5, Targeting BIRC6 + cIAP1 and BIRC6 + Survivin

As BIRC2 (cIAP1), BIRC4 (XIAP), and BIRC5 (survivin) tended to be co-upregulated in PCa, simultaneous targeting of BIRC6 plus one of these three IAP members was more likely to produce superior anti-cancer effects than targeting of BIRC6 alone. Accordingly, dual-targeting antisense oligonucleotides (dASOs), specifically targeting combinations of BIRC6 with each of the other three IAPs, i.e. 6w2, 6w4, 6w5, were designed (see Table 3.1).
<table>
<thead>
<tr>
<th>dASO</th>
<th>Primary Target</th>
<th>% match</th>
<th>Secondary target</th>
<th>% match</th>
</tr>
</thead>
<tbody>
<tr>
<td>6w2</td>
<td>BIRC6 mRNA, nt 9299-9281</td>
<td>19/19 (100%)</td>
<td>cIAP1 transcript variant 2 mRNA, nt 955-937</td>
<td>18/19 (95%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cIAP1 transcript variant 1 mRNA, nt 678-660</td>
<td>18/19 (95%)</td>
</tr>
<tr>
<td>6w5</td>
<td>BIRC6 mRNA nt 12035-12017</td>
<td>19/19 (100%)</td>
<td>Survivin transcript variant 1-3 mRNA, Nt 282-300</td>
<td>16/19 (84%)</td>
</tr>
<tr>
<td>6w4</td>
<td>BIRC6 mRNA, nt 7910-7892</td>
<td>19/19 (100%)</td>
<td>XIAP transcript variant 1 mRNA, nt 6052-6037</td>
<td>16/16(100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XIAP transcript variant 2 mRNA, nt 6019-6004</td>
<td>16/16(100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XIAP transcript variant 3 mRNA, nt 5143-5128</td>
<td>16/16(100%)</td>
</tr>
</tbody>
</table>

Table 3.1 Dual-targeting antisense oligonucleotides (dASOs) candidates targeting IAP combinations and percentage of matched nucleotide sequences with reference transcript sequences from NCBI database.
3.3.3 Validation of Target Inhibition by 6w2 and 6w5 at mRNA and Protein Levels

The dASOs 6w2, 6w4, and 6w5, were tested for reduction of BIRC6 protein expression. As shown in Fig. 3.2, only 6w2 and 6w5 were found to markedly reduce BIRC6 protein levels in PCa cells. The effects of 6w2 and 6w5 on BIRC6, cIAP1, and survivin protein levels were subsequently tested by treating PC-3 and C4-2 cells with increasing doses of the corresponding dASO. As shown in Figures 3.2B and 3.2C, treatment with dASO 6w2 (100 and 200 nM) resulted in marked, dose-dependent reductions in both BIRC6 and cIAP1 protein expression. Similarly, dASO 6w5 (100 and 200 nM) led to marked reductions in both BIRC6 and survivin protein expressions. A time course experiment showed that treatment of PC-3 cells with dASOs 6w2 and 6w5 resulted in a marked reduction in BIRC6 protein expression following 48 hours of transfection, whereas reduction in cIAP1 and survivin protein expressions by these dASOs began at 72 hours after transfection (Fig. 3.2D).
Figure 3.2 Western blotting showing protein levels of BIRC6, cIAP1 and survivin in two CRPC cell lines treated with dASO 6w2, 6w4, and 6w5. A, dASOs 6w2, 6w4, and 6w5, were tested for reduction of BIRC6 protein expression. 6w2 and 6w5 were found to markedly reduce BIRC6 protein levels in PC-3 cells. B-C, PC-3 and C4-2 cells transfected with Mock or increasing dosages of scrambled ASO (Scrb), dASOs 6w2 and 6w5 for 72 hr. Dose-dependent reductions of target proteins were observed.
Figure 3.2 (continued). D, PC-3 cells treated with Mock, mismatched ASO (MM), dASOs 6w2 or 6w5 were assessed for target proteins expressions at 48, 72, 96 hr after transfection. Marked reductions in BIRC6 protein were observed at 48 hr after transfection. Reduction in cIAP1 and survivin expressions began at 72 hours after transfection.
3.3.4 BIRC6-Based ASOs Suppress Prostate Cancer Cell Proliferation

The anti-cancer effects obtained by single and dual targeting of IAPs were compared. At a comparable degree of silencing of BIRC6, cIAP1, and survivin, knockdown of each IAP alone by siRNA did not result in marked reduction in viable PC-3 cell numbers as compared to the mock control (27.8%, -20.8% and -17.7%, respectively). However, simultaneous silencing of BIRC6 + cIAP1 and BIRC6 + survivin by 6w2 and 6w5, respectively, led to marked reductions in the number of viable cells (49.1% and 59.8% of suppression, respectively, p < 0.001). Since different silencing methodologies were used, i.e. siRNA and ASO, these methodologies presumably work via different mechanisms [281]. As such, the viabilities of cells treated with either method were normalized using the cell viabilities obtained with the corresponding, non-targeting controls (Fig. 3.3A).

The activities of 6w2 and 6w5 were more closely examined in time course studies using cell proliferation/viability assays. Dual silencing of BIRC6 + cIAP1 by 6w2 in PC-3 cell cultures, effectively suppressed cell proliferation at 48, 72, and 115 hours by 77.0%, 82.4%, and 76.7%, respectively, as compared to Scrambled (Scrb) controls (p < 0.05). Similarly, silencing of BIRC6 + survivin by 6w5 resulted in 74.7%, 84.1%, and 78.5% growth inhibition, as compared to Scrb at the same time points (p < 0.05) (Fig. 3.3B). A consistent growth-inhibitory trend was also observed using C4-2 cells and viability assays. The growth suppressions obtained with 6w2, as compared to Scrb, at 48, 72, and 96 hours were 81.2% (p < 0.001), 91.1% (p < 0.01), and 99.9% (p < 0.01), respectively. Accordingly, growth suppressions obtained with 6w5, as compared to Scrb at 48, 72, and 96 hours were 54.0% (p < 0.05), 68.3% (p < 0.05), and 86.8% (p < 0.01), respectively (Fig. 3.3C). Reductions in BIRC6 protein expression were also observed in cells treated with 100 and 200 nM scrambled ASO, but to a lower extent than obtained with the
targeting ASOs. For further studies, PC-3 cells were selected due to their higher sensitivity to BIRC6 silencing.
Figure 3.3 Dual IAP-targeting ASOs knockdown BIRC6, cIAP1 or survivin proteins and lead to marked suppression of CRPC cell proliferation. A. Comparison of dual IAP targeting and single IAP-targeting. Cell viability of PC-3 cells transfected with dASOs 6w2, 6w5, or siRNA-targeting BIRC6, cIAP1 or survivin, was determined by MTS assay at 72 hr after transfection. Cell viabilities of ASO- and siRNA-treated cells were normalized with corresponding Scrb ASO and siRNA controls. siRNA (siBIRC6, sicIAP1, siSurvivin) were only specific for single IAP target; the sequences were different from dASO sequences. Error bars represent mean percentage cell viability ± SD. Western blotting of 3 IAPs showing comparable amounts of reduced protein expression obtained with dASO and siRNA single IAP-targeting. B. Proliferation of PC-3 cells transfected with mock, Scrb ASO, dASOs 6w2 and 6w5. Error bars represent mean cell number ± SD. C. MTS viability assay of C4-2 cells treated with dASOs.
3.3.5 BIRC6-Based dASOs Induce Apoptosis, Cell Cycle Arrest and Suppress NFκB Activation

To better understand the basis of growth inhibition by dASOs, apoptosis induction was the first to be investigated. PC-3 cells were incubated with dASOs 6w2 and 6w5 for 72 hours and then subjected to Annexin V and PI staining and FACS analysis to determine the amount of early apoptotic cells generated. FACS analysis showed that the treatment led to apoptosis of 11.3% and 16.6%, as obtained with 6w2 and 6w5, respectively, compared to 2.8% obtained with Scrβ ASO (p = 6.68 x 10^-5 and 0.047, respectively) (Fig. 3.4A, B). In addition, PC-3 cells treated with dASOs 6w2 and 6w5 for 72 hours were stained with DAPI; the numbers of fragmented nuclei (a key indicator of apoptosis) were determined with fluorescent microscopy. The percentage of cells containing apoptotic nuclei was 24.6% and 26.5% for 6w2- and 6w5-treated cells, respectively, in contrast to 0.64% for Scrβ ASO-treated cells (Fig. 3.4C, D). FACS analysis of PI-stained PC-3 cells showed that treatments with dASO 6w2 and 6w5 were associated with significant increases in the G2-M phase population [28.9% for 6w2 (p = 0.008) and 30.4% for 6w5 (p = 0.015)], as compared to the Scrβ control (14.4%) and mock control (14.8%) (Fig. 3.4E, F). Increases in the S phase population were also observed in both treated groups.

In view of a close link between IAPs and the NFκB pathway [282, 283], the effects of dASOs 6w2 and 6w5 on NFκB transactivation in PC-3 cells were examined using a dual luciferase reporter assay under TNFα-induced and non-induced conditions. The TNFα-induced NFκB activation was markedly suppressed in dASO 6w2-treated cells, compared to cells treated with Mock (97.0%, % suppression to mock, p = 0.003), whereas NFκB activation was 20.2% suppressed by Scrβ ASO, compared to Mock. A marked suppression of NFκB activation was also observed in dASO 6w5-treated cells (79.0%, % of suppression to mock, p = 0.011) (Fig.
3.4G). Furthermore, siRNA silencing of BIRC6 did not reduce TNFα-induced NFκB activation, in contrast to silencing of cIAP1 (p = 0.029 and 0.012 for Mock and siCtrl, respectively), indicating that the dASO induced inhibition of NFκB transactivation was not caused by the reduction of BIRC6 protein expression (Fig. 3.4H).

Taken together, the results demonstrate that growth suppression of dASO 6w2- and 6w5-treated PC-3 cells was associated with apoptosis induction, G2-M phase arrest, and repression of NFκB promoter activation, highlighting the multifaceted action of both dASOs.
Figure 3.4. dASOs 6w2 and 6w5 induce apoptosis, cell cycle arrest, and abolish NFκB signalling. A-B. annexin V assay of PC-3 cells treated with dASOs 6w2 and 6w5 for 72 hr. A, FACS plot showing cells under early apoptosis as identified by Annexin V +, propidium iodide (PI) -. B, mean percentage of early apoptotic cells from Annexin V assay. Error bars represent mean ± S.D. C, representative images of PC-3 cells stained with DAPI after 72 hr of dASO treatment; apoptotic cells were identified by fragmented nuclei. D, quantification of cells undergoing apoptosis: percentage of fragmented nuclei. E, cell cycle distribution of PC-3 cells treated with ASOs for 72 hr as determined by PI staining. F, percentage of cells at the G2-M phase. G-H, NFκB transcription activation was examined using a NFκB dual luciferase reporter assay. PC-3 cells were co-transfected with dASOs, NFκB-responsive firefly luciferase and Renilla luciferase plasmid. Luciferase activity was measured at 48 hr after transfection with prior induction by TNF-α treatment.
3.3.6 BIRC6-Based dASOs Suppress PC-3 CRPC Xenograft Growth In Vivo

The therapeutic potential of the dASOs was examined in vivo. NOD-SCID mice carrying subcutaneous PC-3 xenografts were treated daily for 15 days with dASOs 6w2, 6w5, or mismatched (MM) ASO (10 mg/kg). Tumour volumes were determined at the end of the treatment; there was no significant difference in total volume between tumours in the control and treatment groups (Fig. 3.5A). However, as demonstrated by H&E staining, tumours in the dASO-treated groups were found to contain a significantly higher percentage of tumour necrosis, as compared to the control group (46.67% ± 7.86 and 46.25% ± 8.17, % of necrotic area for 6w2 and 6w5, as compared to 19.33% ± 9.49 in the control; mean % of necrotic area ± S.E.M, Fig. 3.5B). To approximate the viable tumour volume, we used the following calculation: total tumour volume x (100% - % of necrotic area). As shown in Figure 3.5C, mice treated with dASOs 6w2 and 6w5 showed significantly lower viable tumour volumes, with percentage of viable tumour volume to control of 61.69% ± 9.30, p = 0.0139 and 58.56% ± 9.14, p = 0.0078, respectively.

The dASO-reduced tumour growth was associated with a significant decrease in intratumoural BIRC6 protein expression in both treatment groups, compared to the MM control (p = 0.026 for 6w2, p = 0.006 for 6w5) (Fig. 3.5D). However, no discernible reduction in the secondary target levels cIAP1 and survivin was detected via IHC staining, in the tumours under the current treatment regimen (data not shown). Ki-67 staining showed that the suppressed tumour growth was associated with a significant decrease in the number of proliferating cells in the 6w2-treated group (p = 0.045). Treatment with 6w5 was also associated with a reduction in the number of proliferating cells, although statistical significance was not reached (Fig. 3.5E, G). No significant increase in cleaved caspase-3 expression was observed in the dASO-treated
tumours at harvest (Fig. 3.5G). The treatment with dASOs did not induce host toxicity as the weights of the mice were not significantly affected during the course of the treatment (Fig. 3.5F); furthermore, the treated tumours looked pallid compared to the untreated tumours (data not shown). Taken together, the results indicate that treatment with dASOs 6w2 and 6w5 suppressed PC-3 tumour growth *in vivo* without major toxicity to the host.
Figure 3.5 Treatments with dASOs resulted in significant lower viable tumour volume without major host toxicity. A, tumour volumes at the end of treatment (day 15). NOD-SCID mice with established PC-3 subcutaneous xenografts were treated with control, 6w2 and 6w5 dASOs for 15 consecutive days and tumors were harvested at the end of treatment. Dash line refers to mean tumor volumes at day 0 (before treatment, 78 mm$^3$). B, percentage of tumor necrosis at harvest determined by H&E staining. C, percentage of viable tumour growth from day 0 to day 15 of treatment. Viable tumour volume refers to tumor without necrotic regions (see Materials and Methods). Error bars indicate mean ± SEM. D, BIRC6 IHC staining intensity of tumors of control ASO-6w2- and 6w5-treated groups at the end of treatment. E, percentage of Ki-67 positive cells as determined by IHC of 6w2- and 6w5-treated tumors at harvest. F, body weights of mice during 15-day treatment. No significant change was observed.
Figure 3.5 (continued) G. representative images of H&E, and IHC staining of BIRC6, cleaved caspases-3 and Ki-67 in control, ASO-6w2- and 6w5-treated PC-3 xenografts. 20 x magnification.
3.4 Discussion

Here, we report the first therapeutic agents developed to target BIRC6 in prostate cancers. dASOs 6w2 and 6w5 simultaneously target both BIRC6 and an additional secondary IAP target (cIAP1 or survivin). Both dASOs demonstrated more rapid knockdown of BIRC6 protein, than either cIAP1 or survivin in vitro (Fig. 3.3A). This suggests that the dASOs exhibit a more time-efficient knockdown of the primary target. The stability of the protein is another contributing factor that determines the length of time until protein reduction following dASO treatment. Since cIAP1 and survivin have relatively short half-lives, about 2.8 hours and 30 minutes, respectively [284, 285], their stability is not likely a contributing factor for the delay in protein reduction. Therefore, the action of the dASOs is likely to be the major explanation. Secondary targets are expected to be less effectively targeted than BIRC6, as a result of the presence of mismatched base pairs in the dASOs (Table 3.1).

The marked growth-inhibitory effects of the 6w2 and 6w5 dASOs on PC-3 and C4-2 cell proliferation (Fig. 3.3B, C), in addition to the growth of PC-3 xenografts (Fig. 3.5), indicate that these dASOs are potentially useful for treatment of advanced PCa, particularly since their use did not induce major host toxicity (Fig. 3.5F). It is worth noting that a substantial culture growth inhibition was obtained by treatment with either 6w2 or 6w5 alone (Fig. 3.3B, C). This is in contrast to growth inhibition reported for most IAP antagonists [286]. For instance, targeting cIAP1/2 and/or XIAP by Smac-mimetics alone did not induce cell death in most cancer cell lines, but rather only enhanced apoptosis and cell sensitivity to chemotherapeutics and radiation [166, 287-291]. Likewise, LY2181308, a survivin-targeting ASO (Eli Lilly), and AEG 35156, a XIAP-targeting ASO (Aegera Therapeutics), were shown to effectively induce apoptosis in vitro only when combined with gemcitabine (or paclitaxel) and TRAIL, respectively [13, 41]. This
highlights the distinctive growth-inhibitory effect that can be obtained by the BIRC6-based, dual IAP-targeting ASOs.

The growth-suppressive effects of the dASOs may be explained by the functional diversity of the primary and secondary IAP targets. BIRC6 has been shown to target pro-apoptotic molecules in the intrinsic apoptotic pathway. By contrast, cIAP1 exerts its anti-apoptotic activity primarily through NFκB-activated survival signalling through the extrinsic apoptotic pathway [155], which was not observed for BIRC6 (Fig. 3.4H). BIRC6 is functionally different from survivin in that it targets precursor and mature forms of caspases 9 and smac for ubiquitin-proteosomal degradation, without affecting effector caspases [163]. Survivin, on the other hand, binds to and suppresses the cleavage activities of activated effector caspases 3 and 7 [159]. Accordingly, dual targeting of BIRC6 and cIAP1 or survivin would more effectively induce cancer cell death, by acting simultaneously on mutually exclusive pathways.

Various mechanisms appear to play a role in the dASO-induced growth inhibition of the PCa cells. The increase in apoptosis, as observed in the PC-3 cell cultures, is fully expected in light of the reduction in IAP expressions (Fig. 3.4). Similarly, the accumulation of cells in the G2-M phase (Fig. 3.4E) is consistent with the roles reported for BIRC6 and survivin in cytokinesis [183, 292]. The suppression of NFκB activation (Fig. 3.4G) can be explained by the critical role of cIAP1 as an upstream regulator of NFκB [293], in addition to the regulatory role of survivin in NFκB activation [169].

Although both dASOs demonstrated substantial anti-cancer activity in vivo, the inhibition of secondary targets is not as obvious as was observed in vitro. This may be due to, firstly, insufficient sensitivity in the detection of protein knockdown (by IHC). Secondly, tumour cells with both BIRC6 and cIAP1/survivin silenced could have undergone apoptosis during the early
phase of treatment. As such, these silenced cells were likely not captured in the current detection window. Additionally, this may also explain the lack of increased apoptosis observed in vivo with cleaved-caspase 3 IHC staining (Fig. 3.5G).

The use of second-generation ASOs, with 2’-methoxyethyl modifications in their backbone, would greatly improve their treatment efficacy and knock-down efficiency, due to their higher tissue half-life and target affinity [294]. Further evaluation of the therapeutic efficacy of dual IAP-targeting ASOs, using patient-derived prostate cancer xenograft mouse models of various stages of prostate cancer [119], and in combination with other therapies, are warranted.

In conclusion, results in this chapter indicate that BIRC6-based dual-IAP targeting ASOs may represent novel therapeutic agents against advanced PCa.
Chapter 4: BIRC6-Targeting as Potential Therapy for Advanced, Enzalutamide-Resistant Prostate Cancer: Preclinical Efficacy from a Patient-Derived Xenograft Model

4.1 Introduction

Castration-resistant prostate cancer (CRPC) presents a major challenge in the clinical management of advanced PCa. As most forms of CRPC are still dependent on the androgen receptor (AR) for survival, the advent of new, powerful second generation AR antagonists, such as Enzalutamide (ENZ), has been beneficial for patients with metastatic CRPC [86]. ENZ significantly improves patient survival and has been approved for treating CRPC in post-docetaxel (2012) and pre-docetaxel settings (2014). However, treatment with ENZ is not curative and ENZ resistance in the clinic has been noted [86, 295]. One-fourth of patients showed primary resistance to ENZ (i.e. the presence of ENZ resistance in patients who never had been exposed to the drug) and progressed in 3 months, while all remaining patients eventually progressed by 24 months in spite of an initial positive response [81, 265]. Major mechanisms of acquired resistance to such AR inhibitors have been reported, including restored AR signalling, AR bypass signalling and complete AR independence [296]. However, other mechanisms that are not directly related to AR signalling, such as blockage of apoptosis, could also contribute to resistance of prostate cancers to increasingly powerful AR inhibitors. So far, not much attention has been given to this possibility.

The Inhibitor of Apoptosis Proteins (IAP) are a family of proteins that serve as endogenous inhibitors of programmed cell death by regulating the activity of caspases, the executors of apoptosis. The IAP family is characterized by the presence of Baculovirus IAP
Repeat (BIR) domains which bind and inhibit caspases. There are eight IAP members, namely BIRC1 (NAIP), BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC5 (survivin), BIRC6 (Apollon/BRUCE), BIRC7 (ML-IAP/LIVIN) and BIRC8 (ILP-2). Some IAPs such as cIAP1/2, XIAP and survivin, are well-known to enhance survival and treatment resistance of various types of cancer [160]. However, to date, no studies have reported a role for the IAP family in ENZ resistance of CRPC.

There is increasing evidence that BIRC6, a lesser-studied member of the IAP family, is also involved in promoting treatment resistance of a variety of cancers. BIRC6 is a large protein (528 kDa) with pleiotropic functions, including inhibition of apoptosis, cytoprotection and regulation of cytokinesis [183] and mitosis [154]. Its activity is upregulated in many cancers, including PCa, particularly CRPC [112], associated with poor patient prognosis [187-190, 297] and shown to enhance chemoresistance [164, 187, 190-193, 298]. In a previous study, we developed an antisense oligonucleotide (ASO), ASO-6w2, that specifically targets synthesis of BIRC6 and to a lesser extent cIAP1 (BIRC2), another IAP family member that is upregulated in prostate cancer [164]. BIRC6-targeting by ASO-6w2 was found to markedly inhibit the proliferation of CRPC cells in vitro and in vivo through induction of cell cycle arrest, apoptosis and suppression of NFκB signalling [164]. The results suggest that BIRC6 plays an important role in promoting survival of castration-resistant malignancies.

Development of effective PCa therapeutics has been hampered by a lack of clinically relevant experimental models of the disease. Traditional xenograft models based on human prostate cancer cell lines lack the tumour heterogeneity and the 3-dimensional architecture of the original cancer specimens from which the cell lines were derived. To overcome these deficiencies, we developed transplantable patient-derived xenograft (PDX) lines of prostate
cancer tissues at the Living Tumor Laboratory (LTL; www.livingtumorlab.com). These PDX lines, developed via implantation of patients’ cancer tissue specimens into NOD-SCID mice at the well-vascularised subrenal capsule graft site, retain the tumour heterogeneity and molecular characteristics of the original cancers. As such, these ‘high fidelity’ PDXs represent highly accurate preclinical model systems for therapeutic target identification and drug efficacy testing [119, 230].

In this chapter, we establish that the transplantable patient-derived CRPC tissue xenograft line, LTL-313BR [119], is ENZ-resistant and provides, together with its ENZ-sensitive, hormone-naïve parent line, LTL-313B, a novel in vivo model for studying the development of ENZ-resistant CRPC, as well as a role of IAPs in that process. Using the LTL-313B/LTL-313BR xenograft model and xenografts based on cultured, ENZ-sensitive and ENZ-resistant prostate cancer cell lines, we found that of the IAP family, BIRC6 was the top upregulated IAP member in both ENZ-resistant systems. We then investigated whether BIRC6 has a prosurvival role in ENZ-resistant cells and provides a potential target for therapy of ENZ-resistant CRPC.

4.2 Materials and Methods

4.2.1 Materials

Chemicals, solvents and solutions were obtained from Sigma-Aldrich, Oakville, ON, Canada, unless otherwise indicated. Six-to-eight-week old NOD/SCID IL2 receptor gamma chain null (NSG) mice were bred by the BC Cancer Research Centre Animal Resource Centre, Vancouver, Canada. ASOs 6w2 and Scramble control (Scrb) with full phosphorothioate-modified backbone were purchased from Eurofins MWG Operon. ASO-6w2 has perfect complementary matches to BIRC6 mRNA sections and contains 1 base mismatch to BIRC2 (cIAP1) mRNA. The DNA
sequences of ASO-6w2 and Scrb have been reported [299]. Anti-BIRC6 (NB110-40730, Novus Biological, Littleton, CO) was used for immunohistochemical (IHC) staining and Western blotting. Anti-Cleaved Caspase 3 (#9664; Cell Signalling Technology, Danvers, MA) was used for IHC staining.

4.2.2 Immunohistochemistry

Staining and scoring of BIRC6 protein was performed as previously reported [299]. For cleaved caspase 3 staining, images of 3-5 representative fields at 400 x magnification were taken per tumour and cells counted to determine the number of positively stained cells per field.

4.2.3 Cell Culture

Human ENZ-resistant, castration-resistant MR49F prostate cancer cells (obtained from Dr. Amina Zoubeidi, Vancouver Prostate Centre, in October 2015) were maintained in RPMI-1640/5% FBS medium, supplemented with 10 μM ENZ. MR49F cells were authenticated using short tandem repeat profile analysis at the Genetics Resources Core Facility at John Hopkins (Baltimore, MD) in January 2013 [210].

4.2.4 Western Blotting

Western blotting of BIRC6 and actin was performed as previously described [299].

4.2.5 Xenografts

The transplantable, hormone-naïve, patient-derived prostate cancer tissue xenograft line, LTL-313B, was maintained in male NSG mice, using serial subrenal capsule transplantations as previously described [119, 120]. Its castration-resistant subline, LTL-313BR, was maintained in castrated mice. The LTL-313BR line was derived from the hormone-naïve LTL-313B line by castration of LTL-313B tumour-bearing NSG mice and propagation of tumours recurring after relapse of the LTL-313B tumours (Fig. 4.1). The LTL-313BR line is AR-positive, PSA-positive,
has a PTEN copy deletion and contains the TMPRSS2-ERG fusion [119]. The original cancer specimen had been obtained with the patient’s signed consent following a protocol approved by the Clinical Research Ethics Board of the University of British Columbia and the BC Cancer Agency [119]. Cell line-based xenografts V16D (ENZ-sensitive CRPC), MR42D and MR49F (ENZ-resistant CRPC) were generated and maintained as previously described [300, 301].

4.2.6 Treatments with Enzalutamide and ASOs

Treatment with ENZ: Mice bearing subrenal capsule-grafted LTL-313B or LTL-313BR tissues were randomized for treatment when the volumes of the grafts reached approximately 250 mm$^3$. Mice were treated with ENZ (10 mg/kg) or vehicle for 4 weeks (n = 10; 5 days on and 2 days off). Tumour volumes were measured at the end of the treatments. Treatments with ASOs: Mice bearing LTL-313BR tumours were randomized into Scrb or ASO-6w2 groups (n = 30) for a 21-day treatment. A 30 mg/kg loading dose on day 1 was followed by a daily maintenance intraperitoneal dose of 15 mg/kg. Tumours were harvested 1 week after the end of the treatment for IHC analysis or RNA extraction. Serum PSA levels were determined using a Cobas total-PSA kit and Cobas e411 analyzer (Roche Diagnostics, Switzerland).

4.2.7 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated and qRT-PCR was performed as previously described [242]. The primer sequences used are presented in Table 4.1. All qPCR primers are human specific and do not cross react with mouse transcripts as confirmed by NCBI BLAST search.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>BIRC6 F</td>
<td>5’-CAATGGAAGCAGTACCAGTGTT-3’</td>
</tr>
<tr>
<td>BIRC6 R</td>
<td>5’-ATCCAAACCCACCATGAAAGT-3’</td>
</tr>
<tr>
<td>AR wt F</td>
<td>5’-TCTTGTCTGTCTTGGAAATGT-3’</td>
</tr>
<tr>
<td>AR wt R</td>
<td>5’-AAGCCTCTCTCTTCTCTCTGTA-3’</td>
</tr>
<tr>
<td>TMPRSS2 F</td>
<td>5’-GTGAAAGCGGGTGAGGAG-3’</td>
</tr>
<tr>
<td>TMPRSS2 R</td>
<td>5’-CTGTGCGGGATAGGGGTTTT-3’</td>
</tr>
<tr>
<td>TNFRSF11A F</td>
<td>5’-TCCTCCACGGACAAATGCAG-3’</td>
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<tr>
<td>TNFRSF11A R</td>
<td>5’-CAAACCGCATCGGATTTTCTCT-3’</td>
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<tr>
<td>BCL2 F</td>
<td>5’-GAACTGGGGGAGGATTGTGG-3’</td>
</tr>
<tr>
<td>BCL2 R</td>
<td>5’-GCAGGCGATGTTGACTTCAC-3’</td>
</tr>
<tr>
<td>NRP1 F</td>
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</tr>
<tr>
<td>NRP1 R</td>
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</tr>
<tr>
<td>IGFBP5 F</td>
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</tr>
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<tr>
<td>GAPDH F</td>
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<td>GAPDH R</td>
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<tr>
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</tr>
<tr>
<td>K-alpha 1 F</td>
<td>5’-GAGGTTGGTGTGGATTCTGT-3’</td>
</tr>
<tr>
<td>K-alpha 1 R</td>
<td>5’-AGCTGAAATTCTGGAGCAT-3’</td>
</tr>
</tbody>
</table>

Table 4.1. qPCR primer sequences used
4.2.8 Gene Expression Profiling and RNA Sequencing

Gene expression profiling of Scrb- and ASO-6w2-treated LTL-313BR xenografts was performed using four replicates. The quality of the RNA samples was checked with the Agilent 2100 Bioanalyzer and NanoDrop ND-2000 UV-VIS spectrophotometer. Only samples with A260/280 OD values between 1.8 - 2.0, an A260/A230 OD value of 2.0 and RNA Integrity Number (RIN) \( \geq 8.0 \) were used for one-color labelling using Agilent’s One-Colour Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling v6.0 (Agilent Technologies, Santa Clara, CA). Total RNA (100 ng) was used to generate cyanine-3-labeled cRNA. cRNAs were hybridized on Agilent SurePrint G3 Human GE 8x60K Microarray (AMDID 028004). Arrays were scanned with an Agilent DNA Microarray Scanner at a 3 \( \mu \)m scan resolution and data were processed with Agilent Feature Extraction 11.0.1.1. Processed signals were quantile normalized using Agilent GeneSpring 12.0. The data have been deposited in NCBI’s Gene Expression Omnibus [302] and are accessible through GEO Series accession number GSE77516 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77516). Transcriptome sequencing (RNA-seq) of PDX models (LTL-313B and LTL-313BR) and cell line-based xenograft models (V16D, MR42D and MR49F) was performed as previously described [119, 210].

4.2.9 Pathway Enrichment Analysis

Top-1000 significantly differentially expressed genes with log2 fold-change >1.5, identified in gene expression profiling of ASO-6w2-treated (n = 4) versus Scrb-treated xenografts (n = 4), were analyzed for gene set enrichment against gene sets of pathways present in the Molecular Signature Database (MSigDB) v5.0 [303]. A Fisher's exact test based gene set enrichment analysis was used. A cut-off threshold of false discovery rate (FDR) \( \leq 0.05 \) was used to obtain significantly enriched pathways.
4.2.10 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad). The Student’s t test was used unless otherwise indicated. Results with a p < 0.05 were considered significant.

4.3 Results

4.3.1 A Patient-Derived Prostate Cancer Tissue Xenograft Model for Studying the Development of ENZ-Resistant CRPC

In search of a clinically relevant in vivo model for studying development of ENZ-resistant CRPC, we tested a number of our transplantable, patient-derived prostate cancer tissue xenograft lines for ENZ sensitivity. Included were the LTL-313B line, a hormone-naïve prostatic adenocarcinoma PDX line, and its CRPC subline, LTL-313BR, developed from the LTL-313B line via host castration and propagation of recurrent tumours (Fig. 4.1A) [119]. Groups of randomized mice, bearing tumours of these lines under the renal capsules, were treated for 4 weeks with ENZ (10 mg/kg) or vehicle. Whereas the growth of the LTL-313B parental line during the 4-week period was inhibited by ENZ (as compared to the control), the growth of the LTL-313BR CRPC subline was not (Fig. 4.1B). As such, the ENZ-sensitive, hormone-naïve LTL-313B line and its ENZ-resistant, castration-resistant LTL-313BR subline provide a PDX model for studying the development of ENZ-resistant CRPC. It may be noted that the ENZ insensitivity of the LTL-313BR line did not result from pre-exposure to ENZ, indicating that this line harbours primary resistance to ENZ.
Figure 4.1. A patient-derived xenograft model for ENZ-resistant CRPC. A. Development of patient-derived LTL-313B and LTL-313BR tumor tissue xenograft lines by serial subrenal capsule transplantation. The castration-resistant LTL-313BR subline was derived from the hormone-naive LTL-313B line. B. LTL-313BR showed primary enzalutamide resistance. Mice bearing LTL-313B or LTL-313BR tumors under the renal capsules were treated for 4 weeks with 10 mg/kg enzalutamide or vehicle (Veh). Tumor volume was measured at the end of the treatment. The LTL-313BR subline was resistant to enzalutamide treatment without prior exposure to the drug, whereas the parental LTL-313B remained sensitive. NS, not significant.
4.3.2  BIRC6 is the Highest Upregulated IAP in ENZ-Resistant CRPC

A study into a role for Inhibitor of Apoptosis Proteins in the development of ENZ-resistant PCa was initiated by determining their relative mRNA expressions in ENZ-sensitive versus ENZ-resistant xenografts. To this end we used the ENZ-resistant LTL-313BR xenograft line, in combination with the ENZ-sensitive, hormone-naïve LTL-313B parent line, as a model for development of primary ENZ resistance. In addition, we used a model of acquired ENZ resistance consisting of xenografts of cultured ENZ-resistant, castration-resistant MR42D and MR49F PCa cells versus ENZ-sensitive, castration-resistant LNCaP-V16D parental PCa cells [301]. The relative mRNA levels in the xenografts of all IAP family members were determined by transcriptomic sequencing.

As shown in Figure 4.2A, the transcript levels of cIAP1, cIAP2, XIAP and BIRC6 were upregulated in the ENZ-resistant MR42D and MR49F CRPC cell-based xenografts relative to the parental ENZ-sensitive V16D line. However, transcripts in the ENZ-resistant LTL-313BR cancer tissue xenografts, relative to the ENZ-sensitive LTL-313B xenografts, were only elevated in the case of survivin, BIRC6, and marginally for XIAP; the BIRC6 protein upregulation in the LTL-313BR xenografts was confirmed by immunohistochemical analysis (Fig. 4.2B). BIRC6 was among the top upregulated IAP members in both ENZ resistance models. Taken together, the results suggest that BIRC6 may be functionally important in promoting ENZ resistance (both primary and acquired resistance), and therefore renders it a desirable target over the other IAP members.
Figure 4.2 Expression level of IAP member, BIRC6, is elevated in models showing acquired and primary ENZ resistance. 

A. mRNA expressions of the IAP family members were determined in PDX models (LTL-313BR vs. LTL-313B) and LNCaP cell line–based xenografts models (enzalutamide-sensitive CRPC V16D vs. enzalutamide-resistant MR42D and MR49F) by transcriptomic sequencing. MR42D and MR49F are enzalutamide-resistant sublines developed after in vivo exposure to enzalutamide of parental V16D xenografts, that is, showing acquired enzalutamide resistance. 

B, BIRC6 protein expression determined by IHC in LTL-313B and LTL-313BR xenografts. Scale bar, 10 µm. Magnification, x 400. IHC scores 0, 1, 2, and 3 refer to negative, weak, mild, and strong staining intensities, respectively. ENZ-R, enzalutamide resistant.
4.3.3 BIRC6-Targeting ASO Suppresses Growth of ENZ-Resistant CRPC In Vitro and In Vivo

The effect of targeting BIRC6 by ASO-6w2 on ENZ-resistant CRPC growth was first examined in vitro using the ENZ-resistant cell line, MR49F (growing in vitro in the continuous presence of ENZ). The MR49F cell line was selected as it expresses functional ARs and PSA, thus resembling clinical ENZ-resistant cases; the MR42D line expresses AR but not PSA [301]. As shown in Figure 4.3A, down regulation of BIRC6 by ASO-6w2 was confirmed by Western blotting (No significant reduction in cIAP1 observed, data not shown). Treatment of MR49F cells with 100 nM ASO-6w2 resulted in growth suppression, while treatment with 200 nM ASO-6w2 led to marked growth suppression with loss of cells (Fig. 4.3A). The anti-cancer activity of ASO-6w2 was then studied in vivo using the LTL-313BR patient-derived CRPC tissue xenograft line as it has greater clinical relevance and precision in predicting patients’ responses than cell line-based xenografts. Mice bearing LTL-313BR tumours under the renal capsules were treated with Scrb ASO or ASO-6w2 daily for 21 days and tumours were harvested 1 week after the end of the treatment (Fig. 4.3B). As shown in Figure 4.3C, the treatment with ASO-6w2 led to marked inhibition of tumour growth, with a 37% reduction (p < 0.001) compared to the control group. As well, a substantially lower increase in serum PSA levels was observed, with a 39% reduction (p < 0.01; Fig. 4.3D) in the ASO-6w2-treated group. The anti-tumour effect of ASO-6w2 was associated with a significant increase in tumour apoptosis (Fig. 4.3E), but no major host toxicity was observed (Fig. 4.4). The inhibition of BIRC6 expression by ASO-6w2 in LTL-313BR xenografts was validated by IHC (Fig. 4.3F). Taken together, the results show that ASO-6w2 - as a single agent - can significantly inhibit the growth of ENZ-resistant CRPCs.
Figure 4.3 BIRC6-targeting ASO-6w2 suppressed growth of ENZ-resistant LTL-313BR xenografts and induced apoptosis. A, BIRC6-targeting ASO (ASO-6w2) effectively suppressed proliferation of enzalutamide-resistant MR49F cells in vitro in a dose-dependent manner. Decreased BIRC6 protein expression after ASO-6w2 treatment was confirmed by Western blotting. B, in vivo therapeutic potential of ASO-6w2 was examined in the enzalutamide-resistant PDX model LTL-313BR. Groups of mice bearing LTL-313BR xenografts were treated with Scr ASO or ASO-6w2 at 30 mg/kg on the first day, followed by 15 mg/kg for 20 days. Tumors were harvested and sera obtained for analysis 1 week after the end of the treatment. C, the ASO-6w2–treated group showed significantly smaller tumor volumes than the Scr ASO treated group. D, the increase in average serum PSA levels of the ASO-6w2–treated mice was also significantly lower than that of the Scr ASO control. Whisker, median/mean ± interquartile range.
Figure 4.3 (continued). E, representative images of cells stained by IHC for cleaved caspase-3. The ASO-6w2–treated group showed a significant increase in apoptosis, with a 2-fold increase in the number of cleaved caspase-3–positive cells. The numbers of positively stained cells were quantified in 3 to 5 fields per sample (magnification, x 400). Scale bar, 50 μm. Error bars, mean ± SD. F, suppression of BIRC6 expression by ASO-6w2 was confirmed by IHC staining. Scale bar, 10 μm. ENZ-R, enzalutamide resistant.
Figure 4.4. Mice weights during treatment of LTL-313BR xenografts with ASO-6w2.
4.3.4 BIRC6-Targeting Suppresses Pro-Survival Pathways that are Upregulated in the ENZ-Resistant LTL-313BR CRPC Tissue Xenograft Model

In comparing the expressions of genes of the ENZ-sensitive LTL-313B line with those of the ENZ-resistant LTL-313BR subline using RNA-seq, we noticed that in the LTL-313BR line genes were upregulated in pro-survival pathways, i.e., AR (AR pathway), IGFBP5 (IGF signalling) and BCL2, TNFRSF11A and NRP1 (NFkappa B pathway). Using qPCR we determined whether expression of these genes in LTL-313BR xenografts was affected by treatment with ASO-6w2. As shown in Figure 4.5, the treatment with ASO-6w2 led to a downward trend in AR and NRP1 gene expression and to significant down-regulations of TMPRSS2 (-44%, p < 0.01), IGFBP5 (-50%, p < 0.05), BCL2 (-64%, p < 0.05) and TNFRSF11A (-48%, p < 0.05).
Figure 4.5 ASO-6w2 suppressed expression of pro-survival genes up-regulated in ENZ-resistant tumours. The LTL-313BR enzalutamide-resistant CRPC xenograft line showed upregulated expression of prosurvival genes in AR, TMPRSS2 (AR pathway), IGFBP5 (IGF signaling), BCL2, TNFRSF11A, and NRP1 (NFkB pathway) compared with the parental, enzalutamide-sensitive LTL-313B line. Results are presented as mRNA reads relative to those of the LTL-313B line from transcriptomic sequencing expression data shown in stripped histograms. The expression of these genes in LTL-313BR were shown to be reduced upon treatment with ASO-6w2, including significant downregulations of TMPRSS2, IGFBP5, BCL2, TNFRSF11A, and a reduced expression trend for AR and NRP1. Relative mRNA expressions were determined by qRT-PCR; n = 7 per group. Error bars, mean ± SD. Ns, not significant.
4.3.5 Multiple Pathways Involved in ASO-6w2-Induced Growth Inhibition of ENZ-Resistant LTL-313BR Xenografts

To investigate pathways involved in the inhibitory effect of ASO-6w2 on LTL-313BR xenograft growth, differential gene expression profiling was performed. Genes (253) were selected from the top 1000 significantly differentially expressed genes with fold changes > 1.5. These genes were subjected to pathway enrichment analysis using the Molecular Signature Database. As shown in Table 4.2, G-protein-coupled receptor (GPCR) signalling and matrisome (extracellular matrix signalling) are among the top enriched canonical pathways. There was a general reduction in GPCR activation for ASO-6w2-treated tumours, including a reduction in expression of F2R, an upstream regulator of oncogenic pathways (Table 4.3). On the other hand, treatment with ASO-6w2 induced deregulation in matrisome (extracellular matrix or extracellular matrix-associated protein) pathways collectively leading to reduced cell proliferation/migration, as well as increased apoptosis (Table 4.4). In addition, treatment with ASO-6w2 led to significant upregulation of (i) genes responding to external stimulus, stress and wounding and (ii) membrane transporter genes, including genes encoding transporters of cations, anions, amino acids and water (Table 4.2).

Taken together, the data indicate that the anti-tumour activity of BIRC6-targeting ASO-6w2 involves suppression of multiple pathways, including those mediating mitogenesis, cell proliferation and tissue invasion.
<table>
<thead>
<tr>
<th>Functions</th>
<th>ASO-6w2 vs Scrb - Top Enriched genesets</th>
<th>Up/ down</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPCR signalling</td>
<td>Reactome signalling by GPCR</td>
<td>↓</td>
<td>8.06E-19</td>
</tr>
<tr>
<td></td>
<td>Reactome GPCR ligand binding</td>
<td>↓</td>
<td>3.63E-14</td>
</tr>
<tr>
<td></td>
<td>Reactome G alpha I signalling events</td>
<td>↓</td>
<td>4.96E-11</td>
</tr>
<tr>
<td>Matrisome (extracellular matrix)</td>
<td>Naba matrisome</td>
<td>↑↓</td>
<td>1.33E-15</td>
</tr>
<tr>
<td></td>
<td>Naba matrisome-associated</td>
<td>↑↓</td>
<td>3.08E-13</td>
</tr>
<tr>
<td></td>
<td>Receptor activity</td>
<td>↓</td>
<td>7.00E-11</td>
</tr>
<tr>
<td>Transmembrane transport</td>
<td>Establishment of localization</td>
<td>↑</td>
<td>3.08E-16</td>
</tr>
<tr>
<td></td>
<td>Transport</td>
<td>↑</td>
<td>1.35E-15</td>
</tr>
<tr>
<td></td>
<td>Reactome transmembrane transport of small molecules</td>
<td>↑</td>
<td>2.07E-12</td>
</tr>
<tr>
<td></td>
<td>Reactome SLC-mediated transmembrane transport</td>
<td>↑</td>
<td>1.34E-08</td>
</tr>
<tr>
<td>Response to external stimulus</td>
<td>Response to external stimulus</td>
<td>↑</td>
<td>4.10E-15</td>
</tr>
<tr>
<td></td>
<td>Response to wounding</td>
<td>↑</td>
<td>3.05E-13</td>
</tr>
<tr>
<td></td>
<td>Response to stress</td>
<td>↑</td>
<td>1.23E-10</td>
</tr>
</tbody>
</table>

Analysis was based on gene expression profiling in ASO-6w2- and Scrb-treated LTL-313BR xenografts (n = 4 per group; fold change >1.5). FDR, false discovery rate.

Table 4.2. Top enriched genesets in MSigDB All Canonical Pathways and GO biological process in ASO-6w2 versus Scrb-treated LTL-313BR tumours
<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Encoded protein function</th>
<th>Fold change</th>
<th>up/down</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPR3</td>
<td>Formyl Peptide Receptor 3</td>
<td>Mediates induction of neutrophil chemotaxis</td>
<td>2.46</td>
<td>↓</td>
<td>0.047</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Chemokine (C-X-C Motif) Receptor 1,</td>
<td>Receptor for interleukin 8, a neutrophil chemotactic factor</td>
<td>1.82</td>
<td>↓</td>
<td>0.013</td>
</tr>
<tr>
<td>RGS17</td>
<td>Regulator Of G-Protein Signalling 17</td>
<td>Inhibits signal transduction by driving G protein alpha subunits into their inactive GDP-bound form.</td>
<td>1.57</td>
<td>↓</td>
<td>0.033</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Chemokine (C-X3-C Motif) Ligand 1</td>
<td>Chemotactic for T-cells and monocytes</td>
<td>1.55</td>
<td>↑</td>
<td>0.022</td>
</tr>
<tr>
<td>F2R</td>
<td>Coagulation Factor II (Thrombin) Receptor/ PAR1</td>
<td>Regulates multiple kinase signalling pathways including PI3-K, Src family tyrosine kinases, JNK, Rho kinases, JAK2 and FAK</td>
<td>1.51</td>
<td>↓</td>
<td>0.033</td>
</tr>
<tr>
<td>GNAI1</td>
<td>G Protein, Alpha Inhibiting Activity Polypeptide 1</td>
<td>Inhibits alpha subunit of G-protein by inhibiting adenylate cyclase in response to beta-adrenergic stimuli</td>
<td>1.51</td>
<td>↑</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Table 4.3. Gene expression changes in GPCR signalling genesets associated with ASO-6w2 treatment of LTL-313BR xenografts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Encoded protein function</th>
<th>Fold change</th>
<th>up/down</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFC</td>
<td>Platelet-Derived Growth Factor C</td>
<td>Induces PDGF receptor activation and intracellular kinase activity, initiating intracellular signalling through the MAPK, PI3K and PKC gamma pathways.</td>
<td>2.58</td>
<td>↓</td>
<td>0.048</td>
</tr>
<tr>
<td>SEMA</td>
<td>Semaphorin 5A</td>
<td>Promotes angiogenesis by increasing endothelial cell proliferation and migration and inhibiting apoptosis.</td>
<td>1.53</td>
<td>↓</td>
<td>0.035</td>
</tr>
<tr>
<td>NELL1</td>
<td>Neural epidermal growth factor-like (NELL) 1</td>
<td>Multimodular extracellular glycoprotein that inhibits renal carcinoma cell migration</td>
<td>1.35</td>
<td>↑</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Table 4.4. Gene expression changes in matrisome genesets associated with ASO-6w2 treatment of LTL-313BR xenografts
4.4 Discussion

Enzalutamide (ENZ) is currently widely used in CRPC therapy. The development of ENZ resistance is therefore a major setback in the clinical management of late-stage PCa and novel therapeutic targets and more effective regimens are urgently needed [86, 295]. Using two distinct ENZ-resistant CRPC models, the present study has demonstrated that BIRC6, a member of the Inhibitor of Apoptosis Protein family, plays a key prosurvival role in the development of ENZ resistance of CRPCs. Thus BIRC6 expression was found to be elevated in ENZ-resistant CRPC cells relative to ENZ-sensitive parental cells (Fig. 4.2) and, importantly, ENZ-resistant CRPC cell proliferation and xenograft growth were markedly inhibited by specific, ASO-induced down-regulation of BIRC6 (Fig. 4.3). Furthermore, treatment of CRPC tumor-bearing mice with BIRC6-targeting ASO-6w2 did not lead to major host toxicity (Fig. 4.4). Taken together, the results indicate that BIRC6-targeting is a promising, new strategy for therapy of CRPCs harbouring ENZ resistance. It is likely that the efficacy of BIRC6-targeting ASOs can be further enhanced by incorporating 2’-methoxyethyl modifications and constrained ethyl chemistry (Gen 2.5) in their backbone.

For validation of BIRC6 as a potential therapeutic target for ENZ-resistant CRPC, the present study made use of the transplantable, ENZ-resistant LTL-313BR patient-derived CRPC tissue xenograft line that was developed in our laboratory (Fig. 4.1) [119]. Use of patient-derived cancer tissue xenograft lines, as distinct from cell line-based xenografts, is increasingly required in cancer research as there is a widespread push for ‘high-fidelity’ cancer models showing a closer link to the patients [255]. Use of PDX models instead of cell line-based models has also been advocated for studies of ENZ resistance [304]. So far, studies of ENZ resistance have employed cell line-based models [96, 101, 305, 306] and, to our knowledge, this is the first
report using a PDX CRPC tissue model for studying ENZ-resistant CRPC. The ENZ-resistant LTL-313BR line is AR-positive, PSA-positive, has a $PTEN$ copy deletion and contains the $\text{TMPRSS2-ERG}$ fusion [119], characteristics which reflect major features of CRPCs in the clinic. The LTL-313BR line was derived from the hormone-naïve LTL-313B line by castration of LTL-313B tumour-bearing NSG mice and propagation of tumours recurring after relapse of the LTL-313B tumours [119]. The LTL-313BR line shows high resistance to bicalutamide (unpublished data), anti-AR antisense-oligonucleotides [210] as well as primary resistance to ENZ. These features resemble those of a subset of CRPCs showing high, primary ENZ resistance. Thus, as observed in a recent ENZ phase II trial, 37% of patients in a 60-patient bone mCRPC cohort exhibited primary ENZ resistance [295]. The finding that ASO-6w2 as a single agent markedly inhibited LTL-313BR xenograft growth suggests that BIRC6 is a promising therapeutic target for CRPC patients showing primary ENZ resistance. Furthermore, the growth-inhibitory effect of ASO-6w2 was also observed in the case of acquired ENZ resistance exhibited by MR49F cells (Fig. 4.3A). This suggests that BIRC6 plays a fundamental role in promoting the survival of ENZ-resistant CRPCs showing either primary or acquired ENZ resistance.

Treatment of LTL-313BR xenografts with ASO-6w2 led to gene expression alterations in diverse biological signalling pathways. As indicated by pathway enrichment analysis, GPCR signalling at the plasma membrane and matrisome signalling at the extracellular matrix were the major pathways that were deregulated by treatment with ASO-6w2 (Table 4.2). Their deregulation would collectively lead to suppression of cancer cell proliferation. It is of particular interest that the treatment with ASO-6w2 led to inhibition of $F2R$ and $PDGFC$ genes, which encode proteins of the plasma membrane and extracellular matrix (Tables 4.3, 4.4). Inhibition of the expression of these genes can be expected to lead to effective blockage of downstream
activities of PI3K, MAPK, JNK and multiple kinase cascades and reduction of potential cross-talks of pathways. This would lead to growth inhibition. As the PI3K/Akt pathway is a prominent AR-independent pathway promoting resistance to androgen deprivation and anti-AR treatment [307, 308], ASO-6w2 may be effective in suppressing growth of ENZ-resistant cancers driven by this pathway. Furthermore, treatment with ASO-6w2 resulted in the down-regulation of pro-survival genes that showed elevated expression in the LTL-313BR xenograft line (Fig. 4.5). As ASO-6w2 was found to impede NFκB transactivation [299], it may inhibit AR signalling via suppression of NFκB [309].

It is not clear how BIRC6-targeting can induce the above gene expression changes. One could speculate that BIRC6 protein may regulate expression of relevant transcription factors and/or their upstream regulators with its chimeric E2/E3 UBC domain. Also, BIRC6 may, in a non-IAP function, facilitate interaction of certain regulatory proteins by acting as a scaffold structure [310]; down-regulation of BIRC6 would then lead to disruption of that interaction and changes in the expression of genes. Further mechanistic studies are needed to elucidate how BIRC6 targeting can lead to growth inhibition of ENZ-resistant CRPCs.

In summary, using the transplantable LTL-313BR xenograft line, a first PDX cancer tissue model for ENZ-resistant CRPCs, we have shown that BIRC6 plays an important prosurvival role in CRPCs exhibiting ENZ resistance and that growth of ENZ-resistant CRPCs can be inhibited by down-regulation of BIRC6 without inducing major host toxicity. BIRC6-targeting may hence represent a new option for clinical treatment of advanced, ENZ-resistant prostate cancer.
Chapter 5: Conclusions

5.1 Summary of Study and Findings

The lack of an effective treatment for advanced prostate cancer remains as a major unmet clinical need. Existing therapies for CRPC, such as androgen axis targeting agents and chemotherapies, only serve to delay the inevitable disease progression, as resistance towards these agents will typically occur shortly following treatment. The increased ability of cancer cells to resist apoptosis represents a fundamental mechanism for treatment resistance, and yet, no relevant agents have been developed.

The overall objective of this doctoral study is to investigate the roles of BIRC6, an inhibitor of apoptosis protein, in advanced PCa and additionally, to assess the therapeutic efficacy of a novel anti-BIRC6 agent. The main hypotheses are as follows: (1) BIRC6 plays a functional role in promoting the survival of advanced PCa cells, and (2) the targeting of tumour BIRC6 expression can suppress advanced PCa growth.

In chapter 2, I presented evidence to support the first hypothesis, in which BIRC6 is associated with advanced PCa and functionally important for PCa growth. Specifically, I first demonstrated a significant correlation between elevated BIRC6 expression (in clinical PCa specimens) and poor patient prognostic factors. Next, I showed that BIRC6 is important for PCa cell proliferation, using PCa cell line models. The subsequent silencing of BIRC6 expression by siRNA in LNCaP and PC-3 cells resulted in a marked reduction in cell proliferation, and was also associated with increased apoptosis. Moreover, I demonstrated that BIRC6 reduction occurs as an early event prior to the execution of apoptosis, upon treatment in PCa cells with doxorubicin. These results have thus prompted the development of an anti-BIRC6 agent for the targeting of PCa.
In chapter 3, I designed BIRC6-based dual IAP-targeting agents (dASOs) and then demonstrated that the two dASOs targeting BIRC6 + cIAP1 and BIRC6 + survivin, both showed substantial inhibition of CRPC cell proliferation *in vitro* and *in vivo* (addressing hypothesis 2). An initial analysis of IAP expression in clinical samples, using IHC, revealed that BIRC6 is co-upregulated alongside certain other IAP members. Based upon this observation, I then sought to design antisense nucleotide (ASO) sequences to simultaneously inhibit BIRC6 in addition to another co-upregulated IAP member. From the selection of all possible dual-targeting dASO sequences, dASO 6w2 (BIRC6 + cIAP1) and dASO 6w5 (BIRC6 + survivin) both showed superior growth inhibitory effects and target inhibitions. As such, these were subsequently selected for further investigation. *In vitro* functional studies found both dASOs to significantly induce apoptosis, cell cycle arrest, and suppression of NFκB activation in CRPC cell lines. More importantly, treatment with either dASO also led to significant reductions in the viable tumour volume *in vivo*, without development of major host toxicity. These results thereby indicate that BIRC6-based dual IAP-targeting ASOs may have the potential to serve as novel therapeutic agents against advanced PCa, including ENZ-resistant CRPC, now increasingly prominent in the clinics.

In chapter 4, in addressing hypothesis 2, I assessed the inhibitory efficacy of dASO in ENZ-resistant CRPC. In addition, I presented evidence that dASO markedly suppresses the growth of ENZ-resistant CRPC, in both cell line models *in vitro* and in the clinically-relevant, transplantable patient-derived xenograft (PDX) model *in vivo*. Specifically, I first examined a panel of transplantable PDX tumour lines (developed by our laboratory) and identified a CRPC model, LTL-313BR, which exhibits a high primary resistance towards ENZ. Next, I assessed the expression of BIRC6 and other IAP members in the LTL-313BR line, in comparison with its
parental ENZ-sensitive line LTL-313B; this was also done with the induced ENZ-resistant cell lines MR49F and MR42D, and its parental ENZ-sensitive cell line V16D. Of the eight IAPs examined, BIRC6 was found to be the only one with elevated expression in both primary and induced ENZ-resistant CRPC models. Next, I demonstrated that treatment with dASO-6w2 markedly suppressed the growth of LTL-313BR xenografts and also increased tumour cell apoptosis, without inducing major host toxicity. Finally, gene expression profiling and qPCR results revealed that dASO-6w2 affects multiple pathways in LTL-313BR tumours. Furthermore, these results also indicated that dASO-6w2 served to inhibit the expression of several pro-survival genes that were up-regulated in the LTL-313BR line. This demonstrates that the preclinical efficacy is consistent with the initial growth inhibitory effect, as was observed with siRNA silencing of BIRC6 in PCa cells (chapter 2) and treatment with dASO-6w2 and 6w5 in CRPC cell lines in vitro and in vivo (chapter 3).

5.2 Conclusions Regarding the Study Hypotheses

In conclusion, the findings regarding BIRC6 expression in clinical specimens and in vitro functional studies support the first hypothesis, in which BIRC6 is functionally important in promoting PCa growth and thus can be implicated in disease progression. Furthermore, the current study also designed and validated dual targeting ASOs to successfully target BIRC6 in conjunction with cIAP1 or survivin, in order to maximize the inhibitory activity. In cell line-based CRPC models, the dASOs showed significant inhibition of tumour growth, increased apoptosis, cell cycle arrest, and NFκB signalling inhibition. This supports the second hypothesis, which stated that targeting of BIRC6 would suppress PCa growth. Most importantly, in further support of the second hypothesis, a high-fidelity PDX model was used to demonstrate proof-of-
concept preclinical efficacy; a BIRC6-targeting dASO, 6w2, was shown to effectively suppress ENZ-resistant CRPC growth, without inducing major host toxicity.

5.3 **Strengths and Limitations**

Overall, this study has provided substantial evidence to support BIRC6 as a relevant therapeutic target for advanced CRPC. Beginning with the initial validation of the clinical relevance of BIRC6, in addition to its functional importance, this was followed by the design and validation of targeting agents, and eventual preclinical drug efficacy testing in multiple models. The results from the preclinical drug testing (using ENZ-resistant cell lines and PDX models), serve to further corroborate the initial hypothesis, which described a significant role of BIRC6 in promoting PCa.

This work made use of multiple PCa preclinical models in order to validate the anti-cancer effect of dASOs. With regard to PCa, it is well recognized that there is only a limited selection of relevant cell lines, a factor that is compounded by the caveat that most of these models do not reflect the genetic, pathological, and micro-environmental features of clinical cancer. Therefore, in order to increase the relevance and applicability of our preclinical efficacy testing, we have included the use of traditional cell lines, ENZ-resistant cell lines, and PDX transplantable tumour lines. The use of PDX models in PCa research is hampered by several factors; most notably, that PDX models are very challenging to develop. To my knowledge, our laboratory is so far the only group to have effectively established a panel of prostate cancer PDX tumour lines. As a result, this places us in a unique and advantageous position for drug efficacy testing, as the positive results derived from the use of PDX models thereby provide strong evidence to support the proposed efficacy in a clinical setting.
The development of an ASO to simultaneously target BIRC6 and cIAP1 or survivin is a major advancement in the development of IAP antagonists. Existing IAP antagonists either inhibit only one IAP (e.g. survivin, XIAP ASO or small molecule) or else a specific BIR domain present only in cIAP1, cIAP2, and XIAP (e.g. Smac mimetics). Since BIRC6 is structurally different from cIAPs and XIAP, they are not readily co-targeted. Furthermore, a BIRC6 antagonist is a new invention in and of itself. So far, there are no known BIRC6 inhibitors that have been developed, despite reports that have asserted the importance of BIRC6 in multiple cancers. One possible reason is that BIRC6 is a huge protein (528kDa), and hence its 3D structure has not been determined. Finally, the dASOs reported in this study represent new IAP targeting combinations that have not been previously assessed. For instance, dASO 6w2 suppresses both extrinsic (via cIAP1) and intrinsic (via BIRC6) apoptotic pathways, whereas dASO 6w5 inhibits BIRC6 + survivin, which are both major regulators of the cell cycle [154, 311] and cell division [183, 312].

On the other hand, there are a couple of major limitations to this study. Firstly, this study did not include a BIRC6 over-expression experiment to address the growth-promoting and anti-apoptotic effect on PCa. The importance of BIRC6 was only addressed in terms of loss-of-function studies. The reason for the absence of an over-expression study is due to the technical difficulties associated with over-expressing the large BIRC6 plasmid in PCa cells. Plasmids of full-length BIRC6 (> 15.7kbp) were obtained from Naito’s (U of Tokyo, Japan) and Jentsch’s (Max-Planck, Germany) groups and tested in PCa cells. BIRC6 over-expression was found to be unsuccessful after repeated rounds of testing. Although the lack of a gain-of-function experiment does render the overall BIRC6 functional study to be less complete, we have reasoned that the
absence of these data do not weaken the overall conclusion that BIRC6 is functionally important in PCa.

The second limitation of this study is the inability to rule out potential non-specific inhibitory effects associated with dASOs. One of the well-documented concerns of ASO therapeutics is the presence of non-specific effects [294]. In chapter 3, I presented that the dASO effectively inhibits PCa cells growth, in addition to the suppression of apoptosis and other cellular pathways. This effect was compared with scrambled (Scrb) ASO, which did not show major toxicity. Due to the differences between the sequences of Scrb and dASO, it may be argued that the inhibitory effect of dASOs could be the result of potential non-targeting effects associated with the sequences. To the best of my knowledge, this limitation is unable to be completely addressed, as it is impractical to examine the presence of an unknown non-specific effect. However, several considerations and measures were implemented in order to minimize the probability of a non-specific effect. For instance, by testing the dASO in various model systems, we have attempted to reduce the model-specific effect and thereby confirm that dose-dependent inhibitory effects are correlated with dose-dependent target inhibitions. Furthermore, the absence of sequence homology with other genes was also verified (via BLAST search). Given these measures, I believe that the inhibitory effects of dASOs, as seen in the tested models, are unlikely to be the consequence of non-specific ASO activity.

5.4 Overall Significance and Clinical Implications

The overall significance of this study has been the establishment of BIRC6 as a new molecular target for advanced PCa. Ample evidence has been provided to support that new BIRC6-targeting agents, dASO 6w2 and 6w5, can effectively suppress the growth of highly-resistant
CRPCs, including ENZ-resistant CRPC. This work may lead to the future clinical development of BIRC6-based dual IAP targeting ASOs, particularly for the treatment of advanced PCa patients. In addition, since BIRC6 and other IAP members have been found to be highly up-regulated in numerous other cancers, these two designed ASOs may be useful for therapy of cancer types other than prostate cancer.

In terms of biology, this is the first study to establish the role of BIRC6 in PCa. Firstly, by using a PCa clinical cohort, I have confirmed a significant clinical correlation between elevated BIRC6 and poor prognostic factors. Additionally, I have established that BIRC6 plays a significant role in promoting PCa proliferation and survival. These results will supplement the mounting evidence that supports the important cancer-promoting roles of BIRC6 in various cancer types.

5.5 Future Research Directions

Given the strong preclinical anticancer activity of the IAP-targeting dASOs that has been presented in this study, future directions will focus primarily on the preparation of the dASOs for clinical development. Accordingly, further studies on preclinical efficacy, dASO pharmacokinetics, and toxicology, will be tested in parallel. They will provide further indications as to whether the dASOs meet the safety and efficacy requirements for phase I clinical trials. Potential clinical developments will be based on the latest generation ASO, generation 2.5 ASO, instead of the first generation PS-ASO used in the present studies. New generation ASOs possess a significantly higher potency, target mRNA affinity, stability, and better safety profiles [313, 314].
To further evaluate its preclinical efficacy, the latest generation BIRC6-based dASO will be tested using a multiple PCa PDX panel developed by our laboratory. The Living Tumour Laboratory has established a large panel of PCa PDX covering the various forms of PCa observed in the clinics, including primary adenocarcinoma, CRPC, and neuroendocrine CRPC. The anti-tumour activity of the dASOs can therefore be assessed in all of these PCa subtypes, thereby providing indications for responders and non-responders. A pilot study of 7-10 PDX models will be tested in the first round of efficacy testing. In addition, the efficacy of combination treatment with existing treatment agents will be assessed. Combinations of dASOs with docetaxel, enzalutamide, and abiraterone can be examined in select PDX models, particularly in models that show a high resistance towards existing treatment in vivo. The goal of this combination study is to determine whether the dASOs can sensitize PCa tumours that are resistant to existing therapies, which may extend the overall patient survival in clinical trials.
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