STUDYING THE EFFECT OF OGX-225, A NOVEL ANTISENSE THERAPY, ON PROSTATE CANCER CELLS

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ABSTRACT

Prostate cancer (PCa) is the most common malignancy diagnosed in Canadian men. If the disease is not detected early the patients would present with metastatic disease. Metastatic PCa represents a challenge for treatment. The primary treatment at this stage is androgen ablation (castration). However, metastatic PCa invariably progress to a castration-resistant form, which does not respond to androgen ablation. This form is a lethal form of the disease, and there are several strategies that have been described to treat this form of cancer. The Insulin-like Growth Factor (IGF) system plays a pivotal role in prostate development and castration-resistant progression. It is tightly regulated by seven high affinity Insulin-like Growth Factor Binding Proteins (IGFBP 1-7). IGFBP-2 and IGFBP-5 are over-expressed in PCa and play a role in castration-resistant progression, while IGFBP-3 is downregulated and inhibits growth of PCa cells.

OGX-225 is a novel second- generation antisense drug (ASO) that targets both IGFBP-2 and IGFBP-5. M. Muramaki et al, at the prostate center in Vancouver, showed in their unpublished data that it inhibits the growth PCa cells both in vitro and in vivo, and that it downregulates IGFBP-2 and IGFBP-5.

In my thesis, I showed the inability to rescue PCa cells exposed to OGX-225 by adding back IGFBP-5, indicating that the action of OGX-225 is irreversible and targets several targets in addition to IGFBP-2 and IGFBP-5. I also showed the growth inhibitory effect of IGFBP-3 through a mechanism other than apoptosis, while not having an additive effect on PCa cells when combined with OGX-225. This thesis reports the multi-targeting ability of OGX-225 resulting in the inability to rescue PCa

cells exposed to it, and gives some insight into the mechanism of action of IGFBP-3 and its effect when combined with OGX-225.

TABLE OF CONTENTS

ABSTRACT .... **ii**
TABLE OF CONTENTS .... **iv**
LIST OF TABLES .... **vi**
LIST OF FIGURES .... **v**
ACKNOWLEDGEMENTS .... **viii**
DEDICATIONS .... **ix**
1 INTRODUCTION **1**
1.1 Introduction 1
1.2 The Prostate 1
1.3 Prostate Cancer (PCa) 3
1.4 IGF Axis 5
1.4.1 IGFs and IGF Receptors 9
1.4.2 IGFBPs 11
1.4.2.1 IGFBP-1 12
1.4.2.2 IGFBP-2 13
1.4.2.3 IGFBP-3 14
1.4.2.4 IGFBP-4 15
1.4.2.5 IGFBP-5 16
1.4.2.6 IGFBP-6 17
1.4.2.7 IGFBP-7 18
1.4.3 IGFBP Proteases 18
1.4.4 IGFBP-rPs 19
1.4.5 IGF Axis and Androgen Receptor in Prostate Cancer 20
1.5 Antitumor Activity of OGX-225, A Novel ASO Inhibitor of IGFBP-2 and IGFBP-5 in Prostate Cancer Models 21
1.6 Specific experimental objectives 23
2 MATERIALS AND METHODS **25**
2.1 Prostate Cancer Cell Lines and Reagents 25
2.2 MTS Assay 26

2.3 Technique for Testing the Ability to Rescue PC3 Cells Exposed to OGX-225.27
2.4 Technique for Testing the Combined Effect of OGX-225 and Recombinant IGFBP-3 on PC3 Cells.. 28
2.5 Technique for Testing the Combined Effect of OGX-225 and Recombinant IGFBP-3 on PC3 Cells, Using Flowcytometry……....................................... 28
3 RESULTS ………………….. 30
3.1 Inability to Rescue PC3 Cells Exposed to OGX-225 30
3.2 IGFBP-3 Does Not Have An Additive Effect on PC3 Cells When Combined with OGX-225 34
3.3 IGFBP3 Growth Inhibitory Effect on PC3 Cells is Not Apoptotic ………..…… 36
4 DISCUSSION AND CONCLUSIONS.. 38
4.1 Discussion 38
4.2 Conclusions 46
BIBLIOGRAPHY 47

LIST OF TABLES

Table 1: Homologous targets to OGX-225……………………………..……..……….42

LIST OF FIGURES

Figure 1: The Insulin-like Growth Factor (IGF) receptor activation and down-stream signalling……………..……….…………….………………..…………7

Figure 2: Rescuing PC3 cells after being exposed to OGX-225 using 350ng/ml recombinant IGFBP-5. ……………..……………………………32

Figure 3: Comparing The cytotoxic Effect of OGX-225 and Recombinant IGFBP-3 on PC3 cells………………………..………………………… 35

Figure 4: Comparing The Apoptotic Effect of OGX-225 and Recombinant IGFBP-3 on PC3 cells………………………..…………………..………37

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DEDICATIONS

To my mother Saadiyah, and to my wife Atlal.

1 INTRODUCTION

1.1 Introduction

Prostate cancer (PCa) is the third leading cause of death in Canadian men(1). When it presents late it represents a great challenge for doctors to treat. Castration-resistant prostate cancer (CRPC) is a terminal and lethal form of the disease, and different therapeutic strategies have been proposed to treat this form of cancer. OGX-225 is a novel antisense oligodeoxynucleotide (ASO) that is designed to target IGFBP-2 and IGFBP-5, and delays the progression of CRPC.

1.2 The Prostate

The prostate is an exocrine gland of the male reproductive system. It is situated at the base of the bladder and is part of the male sex accessory tissues along with the seminal vesicles, ampullae, and bulbourethral glands(2, 3). It weighs normally between 20-30 grams. The prostate functions to secrete parts of the seminal fluid. It secretes many biologic substances in the ejaculate such as fructose, citric acid, spermine, prostaglandins, zinc, proteins, and specific enzymes such as immunoglobulins, proteases, esterases, and phosphatases. The prostate also contains smooth muscles that help expel the semen during ejaculation. Embryologically, five epithelial buds form in a paired manner on the posterior side of the urogenital sinus on both sides of the verumontanum, and they then invade the mesenchyme to form the prostate(4).

The prostate is composed of epithelial and stromal components. The stroma of the prostate contains smooth muscle cells, fibroblasts, and endothelial cells

embedded in an extracellular matrix. It supports the epithelial cells and regulates their growth and differentiation(5). Andromedins, such as Insulin-like Growth Factor (IGF) and Fibroblast Growth Factors (FGFs), play a major role in normal prostatic growth and differentiation(6, 7). They are secreted by the stromal cells and mediate the androgen action on epithelial cells. Transforming Growth Factor-beta 1 (TGF-beta 1) inhibition of epithelial cells is opposed by the stimulatory action of IGF and FGFs to maintain the equilibrium of epithelial cell numbers(8). The epithelium forms the glandular acini, which is separated from the stroma by a basement membrane. The epithelium is composed of three major cell types: secretory epithelial, basal, and neuroendocrine cells. The most common type is the secretory epithelial cell, which is terminally differentiated and is easily distinguished by their morphology and abundant secretory granules and enzymes that stain abundantly with the Prostate Specific Antigen (PSA) and acid phosphatase. They are also rich in keratins(2). The basal cells are smaller and less abundant, being less than 10% of the number of epithelial cells. They are less differentiated and are almost devoid of secretory granules(9). The neuroendocrine cells of the prostate regulate the secretory epithelial cells and are composed of three types, with the major type containing both serotonin and thyroid stimulating hormone. The two minor cell types contain calcitonin and somatostatin(10).

1.3 Prostate Cancer (PCa)

PCa is the most frequently diagnosed cancer in Canadian men (except for nonmelanotic skin cancer, which is rarely fatal)(1). Adenocarcinoma is the most common form of PCa representing > 95% of this cancer. Other rare types of PCa includes (Rhabdomyosarcoma, leiomyosarcoma, lymphoma, and transitional cell carcinoma)(11). It arises from the glandular epithelial cells, usually from the peripheral zone of the prostate(12, 13). Most of the patients with PCa are asymptomatic until the disease is advanced. Therefore, screening for PCa becomes a necessity to detect the disease at an early stage. Screening is done through Digital Rectal Examination (DRE) to detect masses or nodules, and through measuring the serum level of the PSA, which is usually elevated in PCa. The disease is confirmed and graded by histologic analysis of prostate biopsies, using Gleason score, which predicts the prognosis, based on the histologic features of the PCa. The higher the Gleason score, the more aggressive the tumor(11). In localized PCa, a big challenge for the clinician is determining which patients should be offered conservative treatment and which patients should be offered the definitive management. This is especially true for the older population, as the definitive treatment options can carry morbidity to the patient. Generally, the conservative treatment should be offered to patients with life expectancy of less than 10 years, while definitive management is recommended if Gleason score of 4 or 5 is present, more than two biopsy cores are involved, or more than 50% of a biopsy core is involved(11). The definitive treatment options for localized PCa include surgical (radical prostatectomy), radiotherapy, or other

forms of minimally invasive therapies such as brachytherapy. It has not been determined yet if one of these options is superior to the others. However, when PCa cells metastasize to lymph nodes, bone, or other organs, the treatment plan also changes as a more systemic approach is needed(11). The first line of treatment for these patients would typically be androgen withdrawal as PCa is a hormonally sensitive tumor. This was demonstrated through the pioneering work of Huggins and Hodges in the early 1940s(14). Charles Huggins was awarded the noble prize for Medicine for his pioneering work in 1966. Androgens enable growth in PCa through stimulation of cellular proliferation and inhibition of apoptosis(15). Androgen withdrawal can be achieved through surgical castration (bilateral orchiectomy), or medical castration (Gonadotropin-releasing hormone GnRH agonists). Patients usually respond to the hormonal treatment, and will be in remission for an average of 24 months(16). PCa will then exhibit a transformation from Androgen Dependent (AD) to castration-resistant phenotype, which will worsen the prognosis, as the median survival then is 8 to 12 months(15). This phenotype represents a clinical dilemma. Several agents were previously used to treat CRPC, including mitoxantrone, which has shown modest subjective benefits with otherwise minimal evidence of objective antitumor activity. It was mostly beneficial for palliative purposes(17). Docetaxel is a newer cytotoxic agent that induces apoptosis through p53 independent mechanisms, possibly by inhibiting microtubule depolymerization(18). It has become the agent of choice as of 2004 on the basis of a large phase III randomized trial, TAX 327, which demonstrated its superiority to the past standard, mitoxantrone and

prednisone, in terms of survival prolongation(19). These benefits, while clinically significant, remains modest, and there is an increased need for newer therapeutic modalities or second-line agents for patients progressing after docetaxel therapy. For this reason, several new agents have been developed to combat CRPC. OGX-011, an antisense oligonucleotide targeting clusterin, was found to enhance the effects of the cytotoxic chemotherapy in docetaxel-refractory cells(20). On the other hand in clinical trials, Erlotinib, a new small-molecule Tyrosine Kinase Inhibitor (TKI) targeting Epidermal Growth Factor (EGF) receptor, known to be overexpressed in PCa, was found to have insignificant clinical benefits in patients with CRPC(21). Abiraterone is even a newer drug that inhibits CYP17, which is a key in androgen and estrogen synthesis, and has clinically significant benefits in CRPC in terms of PSA level and Circulating Tumor Cells (CTC) counts, further confirming the fact that CRPC remains hormone driven(22). Etoposide inhibits androgen/AR-mediated cell growth in PCa cells(23). All these and other new therapeutic modalities are promising new frontiers in managing CRPC.

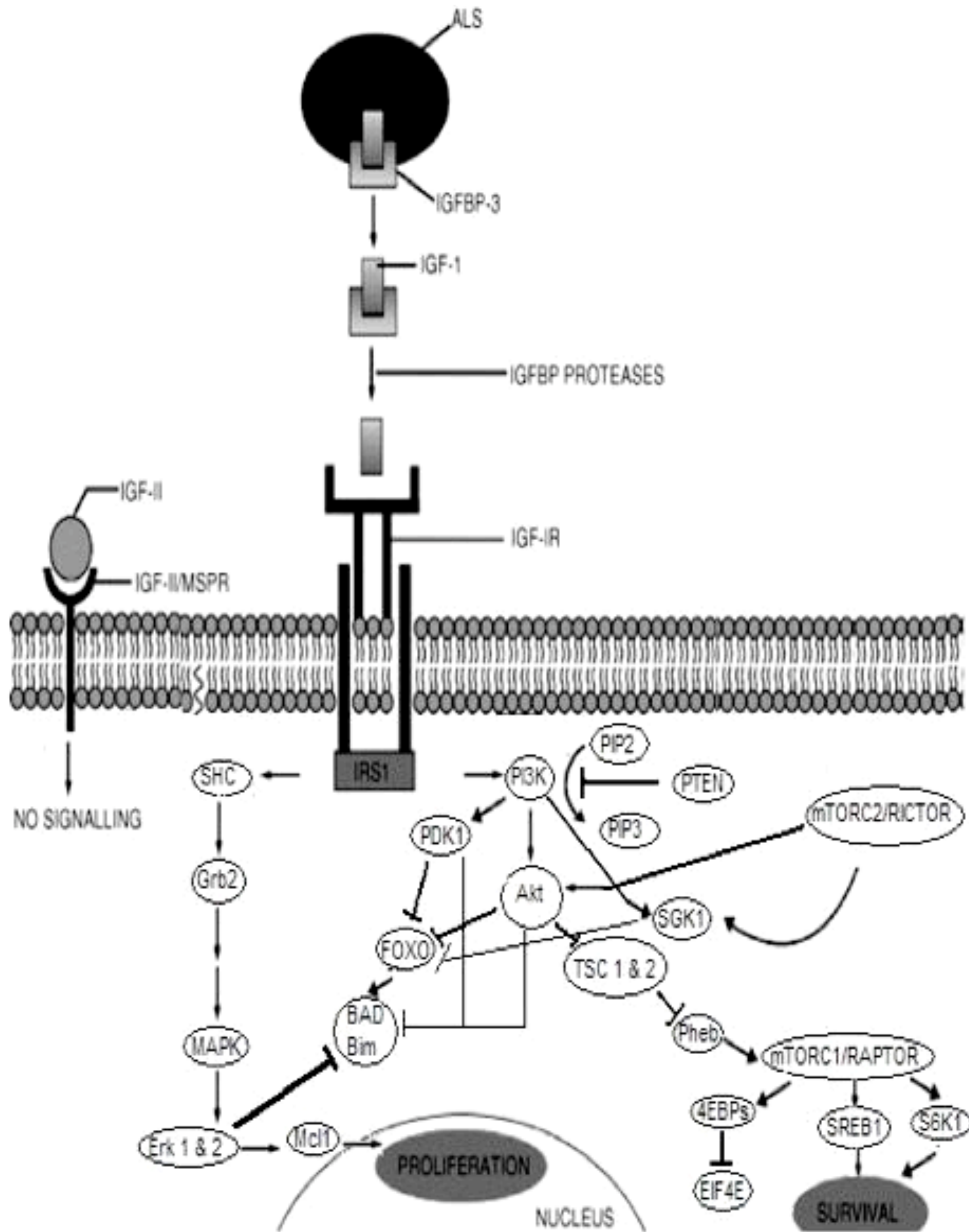
1.4 IGF Axis

Androgens are the primary growth factors for PCa. They act indirectly through AR-positive stromal cells resulting in their release of paracrine growth factors, stimulating epithelial proliferation and survival(24, 25). Other non-androgenic growth factors were also found to play a role in PCa growth regulation through different mechanisms, such as activation of AR in CRPC, or activation of different

signal transduction pathways as in the andromedins (FGF, EGF, and IGF) signalling pathways(26). Since the late 1990s, the IGF family was found to play a major role in different malignancies including PCa(27, 28). It was also found that it has a major mitogenic and anti-apoptotic effect on PCa cell lines(29). The IGF family consists of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), seven high affinity IGF binding proteins (IGFBPs 1-7), a large group of IGFBP proteases, and low affinity IGFBP-related peptides (IGFBP-rPs)(29) (Fig.1).

IGF-I is a mitogen for a variety of cells through stimulation of DNA synthesis and by increasing the level of Cyclin D1, which accelerates the progression of cells from G1 to S phase(7). It also stimulates the expression of Bcl and suppresses Bax, which results in increasing the Bcl/Bax ratio resulting in the blocking of apoptosis(30). By binding to a Tyrosine Kinase (TK) receptor, IGF activates the TK receptor at the cell surface and activate downstream signal cascades (PI3K/AKT cascade) to increase PCa cell growth and proliferation(31). It is also implicated in tumor angiogenesis by increasing vascular endothelial growth factor (VEGF)(32) and in cell migration(33). IGF can mediate growth in CRPC through signalling pathways independent of androgens, and therefore new strategies are being pursued through the development of small molecule and humanized antibody inhibitors of the IGF-1R and antisense inhibition of IGFBP expression for treatment of CRPC.

Fig. 1: The Insulin-like Growth Factor (IGF) receptor activation and downstream signalling.



Legend to Fig. 1: The Insulin-like Growth Factor (IGF) receptor activation and downstream signalling.

IGF-IR binds preferentially to, and activates the IGF-IR, which is a tyrosine kinase (TK) receptor. This triggers down-stream signalling pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK) through adaptor proteins, which through ERK1 and 2 mediates cellular proliferation by downregulating Bim and BAD and upregulating Mcl-1. TK also activates PI3K (a process amplified in PTEN depleted cells) resulting in activation of PDK1 and Akt, which inhibits Bim and BAD directly or indirectly (via inhibiting FOXO). Akt also activates mTORC1/RAPTOR indirectly through inhibiting the inhibitor proteins TSC1 and 2, thus promoting cell survival. mTORC2/RICTOR also promotes survival by activating Akt and SGK1 (an inhibitor of FOXO). The availability of IGF-I to its receptor is influenced by IGF-binding proteins (IGFBPs) and IGFBP proteases. IGF-IIR has no TK activity, and it reduces IGF-I availability to IGF-IR. (Modified from Gennigens 2005(29), Grant 2008(34), and Laplante 2009(35)).

1.4.1 IGFs and IGF Receptors

IGF-I and IGF-II are single chain polypeptides(36). They share 62% homology between them. Their A and B regions also share a homology to proinsulin. IGF-I gene is located on the long arm of chromosome 12 (12q23.2), while IGF-II is located on the short arm of chromosome 11 (11p15.5), at 1.4 kilobases (kb) downstream from the insulin gene(37). IGF-I and II act as both endocrine hormones and as tissue growth factors. IGF-I is a 70 amino acid molecule that is synthesized primarily in the liver and bone marrow(38), and is regulated primarily by the Growth Hormone (GH)(39). Its liver production is increased by physical exercise and smoking(40), while decreased by low calorie intake(41). IGF-I is increased gradually after birth, peaks at puberty, and decline afterwards(42). It plays an important role in organogenesis and promotes cellular differentiation of several mesothelial cells, such as myocytes, osteoblasts, and chondrocytes(36). It also promotes antiapoptotic and mitogenic functions on tumor cells that secrete IGF-I(43). IGF-II is a 67 amino acid molecule, which is synthesized primarily in the liver, and is present in much higher plasma levels than IGF-I(44). It is instrumental in embryonic and fetal growth and development(45), and is believed to have an antiapoptotic effect(46). Its expression is suppressed by maternal imprinting and regulated by 3 IGF-II mRNA Binding Proteins (IMPs 1-3)(47).

Both IGF-IR and IGF-IIR are glycoproteins that are located on the cell membrane(48-50). They differ completely in their structure and function. IGF-IR

is a tetramer composed of two identical α subunits (each subunit weighs 130 kDA) and two identical β subunits (each subunit weighs 90 kDa), which are encoded by a single allele and are generated by proteolytic processing(48-51). The holomeric IGF-IR weighs 320 kDa(52). It has a 60% homology with insulin receptor (IR). IGFs and insulin cross-binds with each other's receptors, but with a weaker affinity than with each one's own receptor(53, 54). The IGF-IR and IR can hybridize to form a heterodimer composed of one α and one β subunits of IGF-IR and one α and one β subunits of IR. This hybrid receptor can bind to IGF-I and II and Insulin. However, it binds IGF-I with higher affinity than insulin. It also activates MAPK/PI3K signalling pathway(55, 56).

IGF-IIR is a monomer that weighs 250 kDa(50, 57-59). It has three binding sites, one for IGF-II, and two for proteins that contains mannose-6-phosphate (M6P) including renin, proliferin, thyroglobulin, and TGF-β(60). The expression of IGF-IR is regulated by steroid hormones and different growth factors(50, 51). Since high levels of IGF-I suppress IGF-IR, then IGF-I has a negative feedback effect on the receptor. Expression of IGF-IR is stimulated by other growth factors such as basic FGF, PDGF, and EGF. It is also stimulated by estrogens, glucocorticoids, growth hormone, FSH, luteinizing hormone, and TSH. It was reported that increased IGF-IR expression is associated with castration-resistant antiapoptotic and promitotic IGF signaling in PCa disease progression(61). IGF-IR is inhibited by tumor suppressor gene products such as the wild type of p53 protein and Wilm's Tumor-1 (WT-1) protein(62-65). Nutrition also affects IGF-IR levels(41, 66, 67).

Binding of IGFs to IGF-IR activates its intrinsic TK activity, which triggers a cascade of reactions involved in signal transduction pathways. Two major signal transduction pathways have been identified for IGF-IR: the Ras, Raf, mitogen-activated protein kinase (MAPK) pathway, and the other pathway involves phosphoinositol-3-kinase pathway(48, 60). Activation of IGF-IR is necessary to mediate the actions of IGF, and IGF-IR is involved in cell transformation. In vitro studies have shown that by eliminating IGF-IR gene, by suppressing its expression, or by inhibiting its function, cell transformation can be abolished(68).

IGF-IIR has no known intracellular catalytic or adaptor signalling capacity, and selectively binds to IGF-II(57). Since binding of IGF-II to IGF-IIR results in the degradation of IGF-II, IGF-IIR can work as an antagonist to IGF-II, decreasing its biological activity(57). Therefore, IGF-IIR has been considered a tumor suppressor molecule. Another feature that makes IGF-IIR efficient as an antagonist to IGF-II is its unusually enhanced biological ability to antagonize IGF-II. The extracellular domain of IGF-IIR dissociates after proteolytic degradation of the receptor upon binding IGF-II, and this extracellular part binds IGF-II in the blood resulting in its further degradation(69-74).

1.4.2 IGFBPs

Approximately, 99% of IGF-I is circulating in the bound form with the IGFBPs. There are seven IGF binding proteins known so far (IGFBP 1-7). All IGFBPs bind preferentially to IGF-I(75, 76), except IGFBP-6, which binds preferentially to IGF-II(77). IGFBPs consist of three domains of approximately equal length(78-80).

The N-terminal and C-terminal domains are highly conserved and cysteine-rich, and are joined by a variable linker domain. There are intra-domain disulfide bonds within the N or C domains but there is no inter-domain disulfide bond(79). The N and C domains are both required for high-affinity IGF binding, because N and C domain fragments bind IGFs with lower affinities than full-length IGFBPs(78, 79, 81-85).

IGFBPs bind IGFs and modulate IGF effects by regulating ligand availability to the IGF-IR. By binding 99% of IGF-I, the IGFBPs create a slow release circulating pool, which control the free IGF level available for ligand interaction. This tight control of the free IGF is instrumental for enhancing the function of IGF by preventing the downregulation of its receptor from ligand overexposure(86).

1.4.2.1 IGFBP-1

IGFBP-1 is a 30kDa protein produced in the liver and kidney. Its gene is located on the short arm of chromosome 7 (7p12.3). It binds to IGF-I and IGF-II with equal affinity (KD 0.1 nM)(87-90). It inhibits metabolic as well as the growth-promoting function of IGF-I(91). The N- and C-terminal portions are highly conserved across the IGFBP family and are responsible for IGF binding(92). Phosphorylation of IGFBP-1 enhances its binding affinity to IGF-I by six- to eightfolds, thus enhancing also its ability to inhibit it(93). IGFBP-1 is associated with increased extracellular matrix production and kidney hypertrophy. It is upregulated by insulin deficiency and downregulated by insulin and steroids(76, 94).

1.4.2.2 IGFBP-2

The gene for IGFBP-2 is located on the long arm of chromosome 2 (2q35)(95), encoding for a 31kDa protein(96). The concentrations of IGFBP-2 are age-dependent, with high level in infancy and older age adults, but lower levels in young adults(97). The concentration of IGFBP-2 in the seminal fluid (10,000ng/ml) is higher than any other IGFBP in any biological fluid(98). IGFBP-2 is produced by the prostate and the central nervous system (CNS), where it is the major IGFBP in the cerebrospinal fluid (CSF), and was found to be elevated in certain CNS tumors. It was also found to be markedly over-expressed in a number of other malignancies, such as colon, breast, and prostate cancers(99, 100). IGFBP-2 immunohistochemical staining levels are higher in high Gleason grade cancers compared with low-grade cancers or benign epithelial cells(101), and with recurrent castration-resistant tumors than with untreated hormone naïve cells(102).

IGFBP-2 has been shown to function as an enhancer of IGF-I function in several cell lines(103, 104), and it participates in CNS development(76). The C-domain of IGFBP-2 has an RGD motif that binds integrins, leading to altered cell migration(105). IGFBP-2 may associate with the cell surface via glycosaminoglycans, and presumably proteoglycans if high concentrations of IGF-I or -II are present(106, 107). The transfection of IGFBP-2 in human epidermoid carcinoma was found to increase tumorigenisis(108). All these findings suggest that changes in IGF/IGBP-2 signaling may activate alternate

growth factor pathway involved in castration-resistant progression. Although the exact mechanism by which IGFBP-2 works is not clearly defined, it has been suggested that it works via phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin pathway(109, 110). IGFBP-2 is upregulated by reduced insulin levels and reduced protein diet, and is downregulated by rapid proliferation, growth factors, and TGF-β(76).

1.4.2.3 IGFBP-3

The IGFBP-3 gene is located on the short arm of chromosome 7 (7p12.3)(95, 111). It is produced primarily in the liver, while also produced by prostate epithelial cells, osteoblasts, Kupffer cells, and bone tissue. It has three glycosylation sites, and resides in the circulation in different glycosylated forms ranging from (40-44kDa)(97). The non-glycosylated form of IGFBP-3 weighs 29kDa(112). Serum levels of IGFBP-3 and IGF-I vary with age. They start low at birth, then increase over childhood, peaks at puberty, and then start to decline afterwards(112, 113). 75 to 90% of IGF-I in the serum is complexed with IGFBP-3 in a ternary complex composed of IGFBP-3, Acid-Labile Subunit (ALS), and IGF. ALS is produced in the liver as a direct effect of GH. It stabilizes the IGF-IGFBP-3 complex, prevents IGF-I extravasation to the extravascular compartment, and prolongs its half-life(114).

IGFBP-3 is a potent anti-proliferative protein, which induces apoptosis and inhibits proliferation in breast and prostate cancer cells(115-117). Blocking IGFBP-3 synthesis or action inhibits the anti-proliferative activity of transforming

growth factor (TGF)-β, retinoic acid, tumor necrosis factor-α and p53(115, 118-123), while IGFBP-3 inhibitory signals require an intact (TGF)-β signaling pathway(124). Decreased expression and plasma levels of IGFBP-3 are associated with a number of malignancies, including hepatocellular carcinoma(125), non-small cell lung carcinomas(126), PCa(127-129), and Ewing tumors(130, 131). It was initially thought that IGFBP-3 works by binding IGF-I and IGF-II and prevent their attachment to their receptors, thus inhibiting their action. However, it is believed now that IGFBP-3 also acts via IGF-independent mechanism(117, 132, 133). The exact mechanism of action is not well understood, but it is proposed that it attach to an unidentified cell surface receptor(134, 135) and activate signal transduction pathways(75) or internalization(136).

IGFBP-3 is upregulated by IGF-I, IGF-II, TGF-β, 1α,25-dihydroxy Vitamin D, and retinoic acid, and is downregulated by glucocorticoids (e.g. Prostate Specific Antigen PSA, Cathepsin D, and dexamethasone)(76).

1.4.2.4 IGFBP-4

The gene for IGFBP-4 is located on the long arm of chromosome 17 (17q21.2)(95), and it is produced in the liver, bone, central nervous system, and prostate. Its translated protein has a molecular weight of 24 kDa. It is upregulated by parathyroid hormone, GH, and 1α,25-dihydroxy vitamin D, while it is downregulated by IGF-II, and glucocorticoids(76). Several cancer cell-lines express IGFBP-4, including PCa(137). IGFBP-4 binds to IGF-I and acts as an

antagonist both in vitro and in vivo, by sequestering it and making it unavailable to its receptor(112, 138). It also delays the onset of tumor formation and enhances apoptosis in a malignant prostate tumor cell lines(139). Antisense inhibition of IGFBP-4 provides a growth advantage of cells in response to IGF-I(140). Colony formation of PCa is strongly inhibited in animals with IGFBP-4 transfected cells(139). IGFBP-4 also acts in an IGF-I independent fashion. It inhibits human ovarian steroidogenesis(141), and blocks the differentiation in skeletal myoblasts(142, 143). IGFBP-4 mRNA expression is not necessarily correlated with the protein level, which carries the functional aspects of IGFBP-4(137).

1.4.2.5 IGFBP-5

The gene for IGFBP-5 is located on the long arm of chromosome 2 (2q35), next to IGFBP-2(95). It weighs 29kDa(97), and can be found in several glycosylated forms ranging from 29 to 32kDa. Like IGFBP-3, IGFBP-5 levels decline with age, starting after puberty(144). It forms also a trimeric complex with IGFs and ALS.

IGFBP-5 is the most abundant IGFBP in the bone. It binds strongly to bone cells due to high affinity for hydroxyapatite(112), thus it sequesters IGF-I in bone matrix. It also binds to endothelial cell monolayers, and is found in high concentrations in the Extracellular Medium (ECM). The affinity of IGFBP-5 to IGF-I when bound to ECM is decreased by 7 to 12 folds when compared with intact IGFBP-5 in solution(145), but this form of IGFBP-5 has a prolonged half-

life(146). IGFBP-5 enhances IGF-I action especially in osteoblasts(146). It is upregulated by diabetic conditions, and downregulated by Follicle stimulating hormone(76).

IGFBP-5 is expressed in the PCa cell-line PC3(147). In situ hybridization and immunohistochemical studies have shown the overexpression of IGFBP-5 in malignant prostatic epithelial cells. However, in situ hybridization showed that IGFBP-5 mRNA is restricted to stromal cells, suggesting that IGFBP-5 is released by stromal cells to associate with epithelial cells through internalization to serve as a route for intercellular communication(148). Several studies have shown the growth enhancing effect of IGFBP-5 on PCa cells(149). For example, when the PCa cell line LNCaP was transfected with IGFBP-5, a faster growth was observed when compared to vector-transfected cells. Apoptosis was induced by androgen withdrawal in both transfected and untransfected cells, but only transfected cells were rescued when IGF-I was introduced(149). This suggests that IGFBP-5 enhance the anti-apoptotic effect of IGF-I.

1.4.2.6 IGFBP-6

The gene for IGFBP-6 is located on the long arm of chromosome 12 (12q13.13)(95). It has a molecular weight of 34 kDa, and is produced in the ovary and prostate(76). It is the only IGFBP that binds to IGF-II with higher affinity than IGF-I(150-152).

The major function of IGFBP-6 appears to be regulation of IGF-II function by inhibiting its action while having minimal effect on IGF-I function(153). It is

upregulated by IGF-II, growth factors, bone morphogenetic proteins, and downregulated by differentiation inducing drugs, and dexamethasone(76).

1.4.2.7 IGFBP-7

The IGFBP-7 gene is located on the long arm of chromosome 4 (4q12). It encodes a 277 amino acid protein, which binds IGF-I. It is found in normal leptomeningeal and senescent mammary epithelial cells(154). It has been suggested to play a role in the growth-regulatory pathways of meningiomas and breast cancer(155).

1.4.3 IGFBP Proteases

IGFBP proteases act to hydrolyze IGFBPs. Their categories include kallikreins(156), cathepsins(157), and matrix metalloproteinases proteases (MMPs)(158). Kallikrein-like serine proteases that cleave IGFBP-3 include PSA, gamma-nerve growth factor, and plasmin(159). In fact, plasmin degrades multiple IGFBPs(159). Thrombin, another serine protease, cleaves IGFBP-5 at physiological doses(160). Transmembrane protease, serine 2 (TMPRSS2), is a putative serine protease to IGFBP-3, and is overexpressed in normal and malignant prostatic epithelial cells(161). TMPRSS2-ERG fusion gene was also found to be overexpressed in PCa(162).

Cathepsins are intracellular proteases that are activated under acidic conditions, which might be relevant to certain physiologic and pathologic processes, like tumor infiltration. Under these conditions, hydrogen ions are

released creating acidic environment for extracellular cathepsins to work, and through interaction with the IGF system might affect cellular growth rate(163).

MMPs are a group of peptide hydrolases that degrade collagen and proteoglycans during tissue remodeling. They need metal cations for their action, and their function is impaired by metal chelators and direct inhibitors(164).

IGFBP proteases cleave IGFBPs into fragments that have lower affinity for IGF, increasing the bioavailability of free IGF to the cell receptors(165). IGFBP proteases also have an important autocrine/paracrine growth regulatory rate and they are important for several physiological processes, such as ovarian follicular growth and atresia(166). They also play an important role in certain malignant processes, such as the autocrine IGF axis in PCa(167).

1.4.4 IGFBP-rPs

IGFBP-rPs (IGFBP-related peptides) is a recently recognized group of proteins that bind to IGFs with low affinity(168). They exhibit significant structural homology to IGFBPs(169). IGFBP-rP1 mRNA is downregulated in several tumor cell lines, and are highly expressed in senescent mammary epithelial cells, suggesting that IGFBP-rP1 has a role in growth suppression(78). Introduction of IGFBP-rP1 retrovirally into prostate cell lines has resulted in growth suppression. IGFBP-rP2 is involved in atherosclerosis, fibrotic disorders (e.g. scleroderma)(78), and childhood acute lymphoblastic leukemia (ALL)(170). IGFBP-rP3 is a target for Wilm’s Tumor (WT-1) regulation, indicating it is involved in kidney morphogenesis and Wilm’s Tumor(171). IGFBP-rP4 has been shown to

promote adhesions of fibroblasts and epithelial cells, and to induce chemotaxis of fibroblasts(172). IGFBP-rP5 mRNA and protein expression is found to be elevated in osteoarthritis. Other IGFBP-rPs have low affinity for IGFs, and their biological functions are not clear(173).

1.4.5 IGF Axis and Androgen Receptor in Prostate Cancer

Most PCa patients progress to CRPC after androgen ablation. Several mechanisms have been described or hypothesized to be responsible for castration-resistant transformation(174, 175). They include mutation or amplification of the androgen receptor (AR) to increase sensitivity to androgens, mutations of AR to allow AR activation by other steroids and anti-androgens, coactivators to increase the sensitivity of the AR, enhanced AR signaling through activation of AR by peptide growth factors and cytokines, and bypass of AR signaling(15, 176).

Several interactions have been reported between IGF-I and AR. Androgens increase IGF-IR levels in prostate epithelial cells, IGF-IR signaling alters AR phosphorylation, IGF-IR signaling effects translocation of AR to the nucleus, and inhibition of IGF-IR in conjunction with castration(177).

1.5 Antitumor Activity of OGX-225, A Novel ASO Inhibitor of IGFBP-2 and IGFBP-5 in Prostate Cancer Models

If PCa is not detected early, it will metastasize to lymph nodes and bone. The treatment will need to be androgen ablation through medical or surgical castration, and usually PCa will respond initially to the hormonal treatment. But eventually PCa will become castration-resistant. There are different mechanisms proposed for the castration-resistant disease that were mentioned earlier. Many genes are found to be upregulated in PCa, including Bcl-2, Bcl-X, clusterin, Hsp-27, IGFBP-2, and IGFBP-5, and they were reported to confer resistance to pro-apoptotic stimuli(178-180). Experimental evidence strongly suggests the involvement of the IGF system and subsequent activation of downstream kinase signaling in the castration-resistant progression(181, 182). IGFBP-2 and IGFBP-5 levels are correlated while IGFBP-3 is inversely associated with poor prognosis(101). It is clear that IGFBP-3 and IGFBP-4 antagonize the IGF system and enhance the sensitivity to apoptosis(183). However, IGFBP-2 and IGFBP-5 are increased after androgen ablation in CRPC. This suggests that IGFBP-2 and IGFBP-5 play a role in CRPC progression(184, 185). This has made IGFBP-2 and IGFBP-5 a potential target for therapeutics aiming to potentiate the effect of hormonal therapy and prevent the progression to CRPC.

OGX-225 is a novel antisense drug (AS) that targets IGFBP-2 and IGFBP-5(186). It was found to downregulate IGFBP-2 and IGFBP-5 and induce

apoptosis in high-grade human glioma cells(187). It also inhibited the in vitro and in vivo growth of breast cancer cells, which are overexpressing IGFBP-2(188).

Antisense olidodeoxynucleotides (ASOs) are chemically modified stretches of single-strand DNA complementary to mRNA regions of a target gene, which inhibit translation by forming DNA/RNA duplexes, thus reducing mRNA and protein levels of the target gene. Initially, ASO was rapidly degraded by cellular nucleases due to its phosphodiester backbone. However, second generation phosphothioate ASO modified at 5' and 3' ends modified by methoxy-ethoxy (MOE) residues at the 2'-α-position of the deoxyribose have enhanced RNA-binding affinity and prolonged the half-life of the second generation in comparison to the first(189, 190). It was recently reported in a phase I trial that OGX-011, a second-generation ASO targeting clusterin gene, was well tolerated and potently inhibited clusterin expression in PCa(191).

M. Muramaki, et al., from the prostate canter in Vancouver BC, have studied the therapeutic effects of OGX-225, on human PCa cells both in vivo and in vitro (unpublished data). In vitro, the results showed that the growth of both PC3 and LNCaP cells was inhibited by treatment with OGX-225 in a dose-dependent manner, compared to treatment with control oligodeoxynucleotide (ODN). Flowcytometric analysis indicated that OGX-225 resulted in a corresponding relative dose-dependent increase in fraction of cells in sub-G_0/G_1 phase of LNCaP and PC3 cells. In vivo, compared with control ODN, OGX-225 treatment resulted in the significant reduction of LNCaP and PC3 tumor volume and delayed tumor re-growth. In summary, M Muramaki experiments suggest that

OGX-225 inhibits PC3 and LNCaP cell growth (cytotoxic), and downregulates IGFBP-2 and IGFBP-5 (specific), both in vitro and in vivo.

The central hypothesis of my thesis is that since OGX-225 downregulates IGFBP-2 and IGFBP-5 in PCa leading to apoptosis, then adding back IGFBP-5 to OGX-225 treated PCa cells will rescue them. Also, that IGFBP-3 inhibits PCa cells growth through an apoptotic mechanism, and that combining IGFBP-3 with OGX-225 will have an additive effect.

1.6 Specific Experimental Objectives

CRPC represents a terminal stage in PCa development in which hormonal therapy no longer is effective, and different chemotherapeutic agents do not show a curative effect. Research is moving quickly to identify different cellular targets for therapies, to cure or prolong survival of patients with this disease. Different proteins were found to be upregulated in CRPC, including IGFBP-2 and IGFBP-5. This has made IGFBP-2 and IGFBP-5 interesting targets for therapeutics, as it is been suggested they may play a role in the castration-resistant progression. M. Muramaki et al experiments have demonstrated unequivocally that OGX-225 (a novel ASO targeting IGFBP-2 and -5) has a cytotoxic effect on PCa cells (PC3 and LNCaP) in a dose-dependent manner. In addition, it was shown that it does specifically downregulates IGFBP-2 and IGFBP-5, and that it inhibits PCa cell growth through induction of apoptosis.

My objectives during my one year of work at the prostate center at UBC Vancouver, was to determine the ability to rescue PC3 cells when exposed to

OGX-225, using recombinant IGFBP-5. Also, I was studying the effect of adding recombinant IGFBP-3 (a pro-apoptotic protein) to PC3 cells when used alone, and when added to OGX-225 treated cells, using MTS assay and flowcytometry.

2 MATERIALS AND METHODS

2.1 Prostate Cancer Cell Lines and Reagents

PC3 human PCa cells were obtained from American Type Culture Collection (ATCC---www.atcc.org) and maintained in DMEM supplemented with 10% FBS (Invitrogen Canada Inc. Burlington, Ontario). Frozen cells stored at liquid nitrogen (-196°C) were thawed rapidly in a 37°C water bath and immediately added to 9 ml of media with 5% FBS in a 10 cm plate. General reagents were from (Sigma-Aldrich, St. Louis, MO) or (Fisher Scientific, Ottawa, ON) unless otherwise indicated.

Cells were passaged at 70% confluency, and cultured in 10 cm^2 tissue culture plates (Nalge Nunc International, Rochester, NY) at 37°C and 5% CO_2. To passage cells, the media was aspirated and cells were washed once in 2 ml of phosphate buffered saline (PBS) and 1 ml of 1x Trypsin-EDTA [10% 10x Trypsin-EDTA (Invitrogen, Burlington, ON) and 90% Fetal Bovine Serum (FBS, Invitrogen, Burlington, ON)] was added to the plates and incubated for one to three minutes to loosen adherent cells. Cells were triturated from the plates with 5 ml of media and added to a larger volume of media for replating.

OGX-225 (Antisense Oligodeoxynucleotide ASO, OncoGenex Pharmaceuticals. Vancouver, BC. Canada). The sequence for OGX-225 is 5’-CAGCAGCCGCAGCCCGGCTC-3’. Recombinant IGFBP-5 (R&D Systems. Minneapolis, MN). Oligofectamine (Invitrogen, Burlington, ON) was used to

increase ASO uptake into cells in vitro. For transfection, the low-serum medium OPTI-MEM (Invitrogen, Burlington, ON) was used. Scramble B (ScrB) (control oligodeoxynucleotide) (Integrated DNA Technologies, Inc., Coralville, IA). The sequence for ScrB is 5’-CAGCAGCAGAGTATTTATCAT-3’.

2.2 MTS Assay

It is a calorimetric method used to detect viable cells in proliferation, cytotoxicity, or chemosensitivity assays. The reagent was a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. The electron coupling reagent PMS was maintained separately from the MTS and was added at the time of the MTS use to form a stable complex in the solution. The solution was added then to the medium. The MTS, an aqueous soluble formazan, was then metabolized by dehydrogenase enzymes found in metabolically active cells. In 1-4 hours, the absorbance of formazan crystals was read using 96-well plate at 490 nm. Absorbance was directly proportional to number of living cells in culture.

This method differs from MTT assay. Formazan crystals of MTS do not need solubilization in culture medium, whereas formazan crystals of MTT are insoluble in culture medium and they require SDS or DMSO for solubilization. This can be time efficient and cost effective. All MTS reagents were from (Promega, Madison, WI).

2.3 Technique for Testing the Ability to Rescue PC3 Cells Exposed to OGX-225

PC3 cells were plated at 40% confluence in 24-well plates. They were allowed to grow in DMEM + 10% FBS without antibiotics for 24 hours at 37°C in humidified 5% CO_2. ASOs [OGX-225 (Oncogenex) and ScrB (Integrated DNA Technologies, Inc)] dilutions were prepared at 50, 100, 250, and 500 nM concentrations.

The Oligofectamine Reagent (Invitrogen) was combined with OPTI-MEM. Incubation was allowed at room temperature for 10 minutes. The mixture of ASO dilution was combined with Oligofectamine reagent mixture and allowed for incubation for 20 minutes.

Media were removed from the plates and replaced with 500 µL of OPTI-MEM in each well. The above-mentioned final mixture was overlaid in each well. The test was done in duplicates. Then, the plates were incubated for 4 hours at 37°C in humidified 5% CO_2. 250 µL of DMEM + 15% FBS (Invitrogen) was then added to each well. The same procedure was then repeated on day two. When DMEM + 15% FBS (Invitrogen) was added however on day 2, half of the wells that were exposed to OGX-225 of all concentrations received 350 ng/mL of recombinant IGFBP-5 as well to test for rescue. 48 hours later, MTS assay was done to measure viable cells and thus level of cytotoxicity and rescue.

2.4 Technique for Testing the Combined Effect of OGX- 225 and Recombinant IGFBP-3 on PC3 Cells

The same reagents and transfection technique from the previous experiment was repeated here, however the test was done in quadriplicates and when DMEM + 15% FBS (Invitrogen) was added on day 2, half of the wells that were exposed to OGX-225 (OncoGenex) received 2000 ng/mL of recombinant IGFBP-3 (R&D Systems. Minneapolis, MN). Some wells that were not exposed to OGX-225 also received 2000 ng/mL of IGFBP-3 (R&D Systems) alone. This is to test for the sole effect of IGFBP-3 (R&D Systems) and the combined effect of OGX-225 (OncoGenex) and IGFBP-3 (R&D Systems), while other wells did not receive any reagent and were used as control. 48 hours later, MTS assay was done to measure viable cells and thus level of cytotoxicity.

2.5 Technique for Testing the Combined Effect of OGX-225 and Recombinant IGFBP-3 on PC3 Cells, Using Flowcytometry

In this experiment, using Flowcytometric Analysis (FACS) we analyzed the effect of OGX-225 (OncoGenex) on PC3 cell lines. We compared the effect of OGX-225 (OncoGenex) at a dose of 50 ng/ml to the effect of IGFBP-3 (R&D Systems) and assessed their combined effect. The effect was measured using apoptotic index and DNA content.

Four 10 cm^2 dishes were prepared. PC3 cells were plated in each dish and were allowed to grow for 2 days to 60% confluence. Then, transfection with OGX-225 (OncoGenex) at a dose of 50 ng/ml was done in 2 of the dishes according to its protocol, which is a two-day transfection using oligofectamine (Invitrogen) in a reduced serum medium (OPTIMEM) with DMEM+10% FBS (Invitrogen) added after 4 hours of each transfection. One of the 2 dishes that received OGX-225 (OncoGenex) received 2000 ng/ml single dose of IGFBP-3 (R&D Systems). One of the 4 dishes received only the same dose of IGFBP-3 (R&D Systems), while another dish was used as a control. After 48 hours, cells floating in the medium were collected and the cells in the dishes were trypsinized and washed with PBS. Then they were fixed with 80% iced ethanol and kept on ice for 2 hours. The iced ethanol was washed with staining buffer. Then cells were stained with propidium iodide (PI) solution for 30 minutes at room temperature. Stained cells were analyzed for PI incorporation and relative DNA content with a BD FACSCanto™ II flow cytometer (BD Biosciences).

3 RESULTS

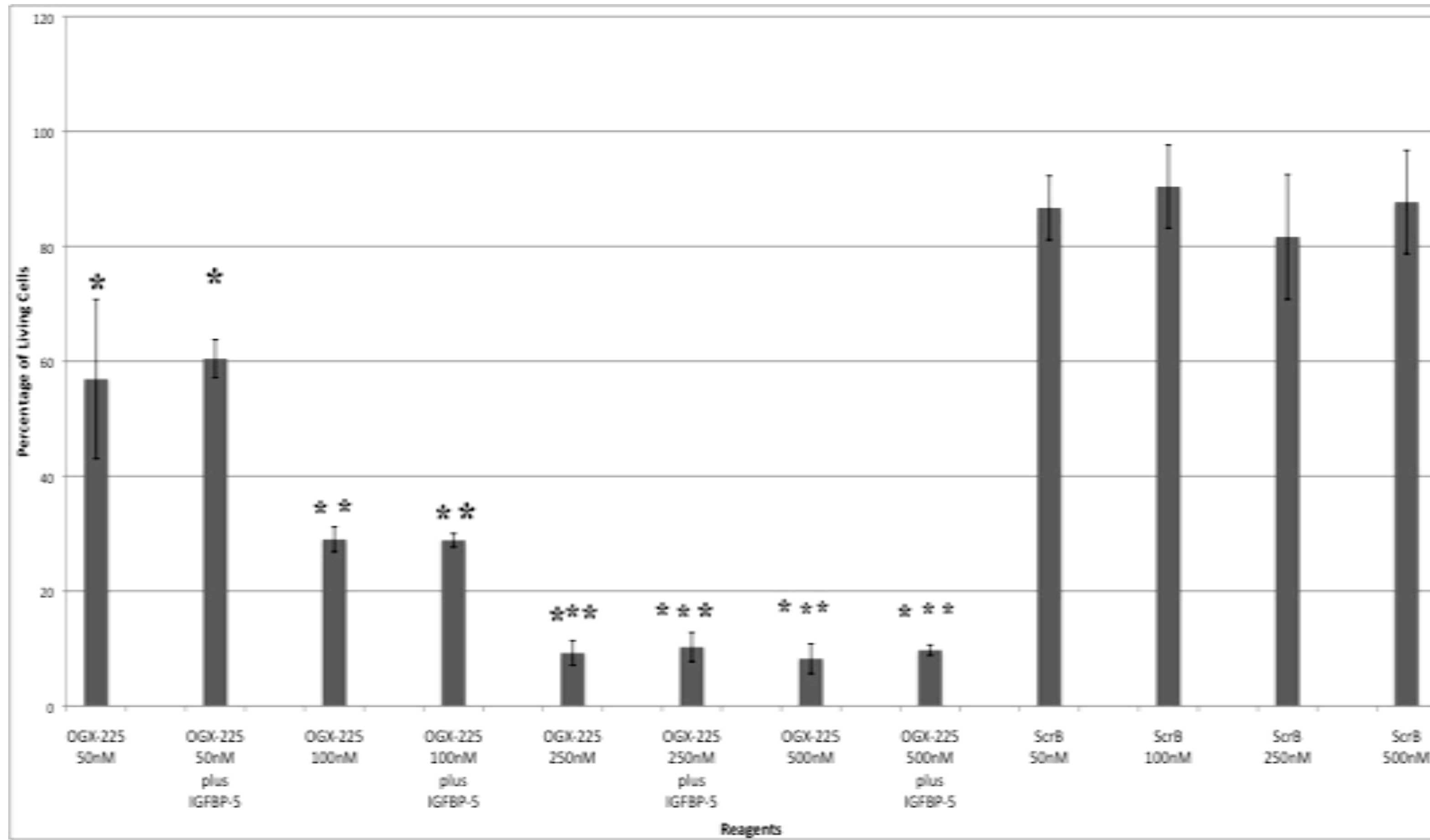
3.1 Inability to Rescue PC3 Cells Exposed to OGX-225

In order to test for the effect of OGX-225 on PC3 cells, a dose-response was measured at doses of 50, 100, 250, and 500 nM. The PC3 cells were treated daily with indicated concentrations of OGX-225 and control ODN daily for 2 days. After 72 hours incubation, the growth of PC3 cells was found to be inhibited by the treatment with OGX-225 in a dose-dependent manner compared to treatment with control ODN. The maximal toxicity was seen at 250 nM, and higher concentrations of OGX-225 did not increase toxicity. This might be explained by the fact that at 250 nM almost all the PC3 cells died and so further increases in the dose will not have a significant effect. Therefore, 250 nM might be the best maximum dose of OGX-225. ScrB had no obvious toxic effects at up to 500 nM. This experiment was modeled and designed on those previously designed and performed by Muramaki et al., but are yet unpublished. In his data he already showed dose and sequence-specific knockdown of IGFBP-2 and IGFBP-5.

This experiment was expanded to include testing whether PC3 cells exposed to OGX-225 can be rescued by adding back recombinant IGFBP-5. 350 ng/ml of recombinant IGFBP-5 was added to half the wells exposed to OGX-225 in each dose cohort. Using the MTS assay to test for cell viability there was no observed difference between cells that received recombinant IGFBP-5 and those that did not (Fig. 2), this indicates that the cellular events initiated by exposure to OGX-225 cannot be reversed by simply adding recombinant IGFBP-5.

The experiment was repeated (data not shown) to test for rescue of PC3 using higher concentration of IGFBP-5 (R&D Systems) (700 ng/mL), and recombinant IGFBP-2 (R&D Systems. Minneapolis, MN) (150, 360, and 700 ng/mL). All of these reagents did not show any rescue to PC3 cells when exposed to OGX-225. This probably indicates that OGX-225 action as a polyspecific agent is an irreversible one. These results were not shown because while I could see that adding those reagents clearly did not have an effect on the PC3 cells, the OGX-225 did not have a dose dependent effect either on PC3 cells Therefore, it was hard to conclude failure of rescue from this experiment.

Figure 2:



Legend to Fig. 2: Rescuing PC3 cells after being exposed to OGX-225 using 350 ng/ml recombinant IGFBP-5.

PC3 cells were exposed to different concentrations of OGX-225 and ScrB. MTS assay was done to detect viable PC3 cells after each treatment. 350 nM of recombinant IGFBP-5 was added to half the wells treated with OGX-225 on the second day of transfection to look for rescue. ScrB had no cytotoxic effects on PC3 cells. OGX-225 had a significant dose-dependent cytotoxic effect. Maximal effect was seen at 250 nM and further concentrations of OGX-225 above 250 nM did not increase toxicity. Recombinant IGFBP-5 did not rescue PC3 cells exposed to OGX-225. *Columns*, Percentage of living cells in duplicate analysis ($n = 3$), *bars*, SD. *, differ from control ($p < 0.0001$), **, differ from OGX-225 50nM ($p < 0.0001$), ***, differ from OGX-225 100 nM ($p < 0.0001$) by Student's t test.

3.2 IGFBP-3 Does Not Have An Additive Effect on PC3 Cells When Combined with OGX-225

IGFBP-3 has been shown to have a growth inhibitory effect in PCa cells(165) (Fig. 3). Rajah et al have shown that IGFBP-3 inhibits PC3 growth by inducing apoptosis(115). After demonstrating the growth inhibitory effect of OGX-225, we wanted to study the effect of combining OGX-225 with IGFBP-3 on the growth of PC3 cells, and whether they would have any additive effect. Using the above mentioned transfection technique; we divided the wells into four cohorts. One cohort received OGX-225, one received IGFBP-3, and one received a combined dose of IGFBP-3 and OGX-225, while one did not receive any agent. Recombinant IGFBP-3 was given at a dose of 2000 ng/ml because Rajah et al did their experiment using 500 ng/ml over 72hrs (Total of 1500 ng/ml). The MTS assay showed the expected growth inhibitory effect of OGX-225 and IGFBP-3. When they were combined however, there was no additive effect observed and it was similar to adding either one alone.

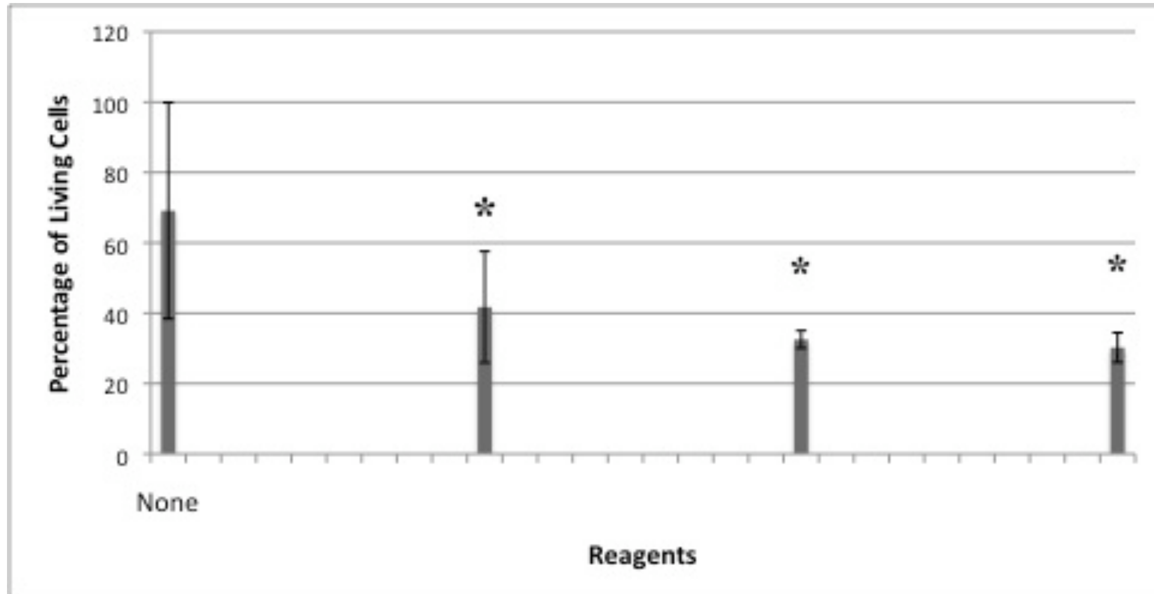


Fig. 3: Comparing The Cytotoxic Effect of OGX-225 and Recombinant IGFBP-3 on PC3 cells.

PC3 cells were exposed to OGX-225 and recombinant IGFBP-3. MTS assay was done to detect viable PC3 cells after each treatment. Recombinant IGFBP-3 (at 2000 ng/ml) had a similar cytotoxicity profile to 50 nM OGX-225 when compared to control. However, it does not have an additive cytotoxic effect when added with OGX-225. *Columns*, Percentage of living cells in quadruplicate analysis (n = 4), *bars*, SD. *, differs from No Treatment ($p < 0.05$) by Student's t test.

3.3 IGFBP3 Growth Inhibitory Effect on PC3 Cells is Not Apoptotic

After demonstrating the cytotoxic effect of adding IGFBP-3 to PC3 cells, we wanted to examine the cell cycle status of OGX-225 and IGFBP-3 treated PC3 cells for mitotic activity and relative DNA content. We had four cohorts in this experiment, similar to the previous experiment (IGFBP-3, OGX-225, combined OGX-225 and IGFBP-3, and untreated cells). The fraction of cells undergoing apoptosis (sub G0/G1 fraction) was significantly elevated for OGX-225 than for untreated cells. The apoptotic fraction (percentage of sub G0/G1 fraction) of IGFBP-3 was not different from the untreated cells. The apoptotic fraction of the cells exposed to the combined dose of OGX-225 and IGFBP-3 was not different from adding OGX-225 alone (Fig. 4). The possibilities are, IGFBP-3 did not work technically in this experiment or it might be reducing the PC3 count through a different mechanism by arresting the cell cycle, which is not in line with Rajah et al results.

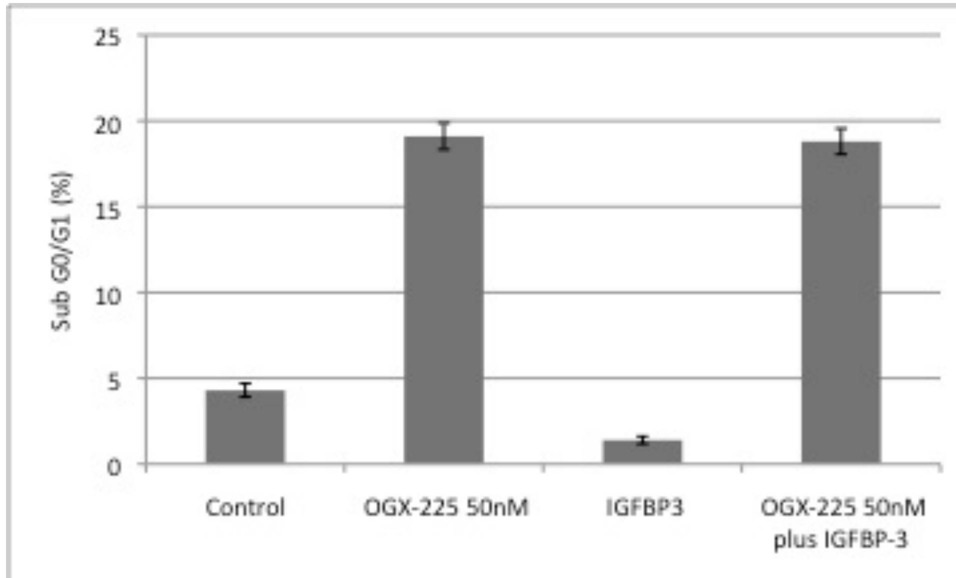


Fig. 4: Comparing The Apoptotic Effect of OGX-225 and Recombinant IGFBP-3 on PC3 cells.

PC3 cells were treated with OGX-225 and recombinant IGFBP-3. After 48 hours incubation, percentage of sub G0/G1 population was determined by flowcytometry. IGFBP-3 had similar apoptotic effect as control, while OGX-225 at 50 nM concentration had the expected apoptotic effect. The addition of IGFBP-3 to the PC3 cells exposed to OGX-225 did not have any increase in the apoptotic effect of OGX-225. *Columns*, percentage of sub G0/G1, *bars*, 95% confidence interval. The 95% confidence interval was calculated based on the relative number of events, as the test was done only once (n = 1).

4 DISCUSSION AND CONCLUSIONS

4.1 Discussion

PCa is the most commonly diagnosed malignancy in men. Initially, PCa is a hormonally sensitive malignancy, but eventually it progress to a castration-resistant form, which no longer responds to hormonal therapy, and chemotherapeutics do not cure the disease. The IGF family plays a pivotal role in prostate development and castration-resistant progression(181, 182). It is composed of two ligands, IGF-I and IGF-II, which regulate the mitotic and anti-apoptotic effects on PCa cell lines. Their action is mediated through two cell surface receptors. The IGF ligands are tightly regulated through 7 high-affinity binding proteins (IGFBP 1-7), a large group of proteases, and low affinity IGFBP-related peptides.

Several strategies have been used to disrupt IGF signaling(175). Growth hormone-releasing hormone (GHRH) antagonists inhibit the release of GH, and function to block IGF-I signaling(192). Neutralization of IGF ligands can inhibit the activation of their receptors. IGFBP-1 has been reported to inhibit IGF-I effects in an IGF-I-dependent manner to induce apoptosis in LNCaP cells(193). Another way to neutralize the ligands is to use antibodies against IGF-I and IGF-II(194). IGF-IR represents an attractive target for anticancer therapeutics(195). Several

approaches have been used to inhibit IGF-IR, including ASO(196), siRNA(197), and anti-IGF-IR-monoclonal antibodies(198).

Some IGFBPs enhance IGF function, while some IGFBPs act to antagonize IGF. Expression of IGFBPs differs in PCa, where IGFBP-2 and IGFBP-5 are found to be elevated while IGFBP-3 is below normal levels(101). The over-expression of IGFBP-2 and IGFBP-5 after castration represents an adaptive response to increased IGF-I activity and castration-resistant progression(104, 184). Forced over-expression of IGFBP-5 induces LNCaP proliferation, and a corresponding PI3K activation. PI3K inhibitor-induced apoptosis is inhibited by adding IGF-I, suggesting that high-levels of IGFBP-5 enhance the IGF-I anti-apoptotic effects.

IGFBP-2 is also involved in the progression of PCa, and is found to be elevated more in malignant prostatic disease than hyperplastic(199) and in higher Gleason grade tumor(101). It is also associated with higher risk of PSA recurrence after radical prostatectomy(200), and with castration-resistant progression(104). IGFBP-2 is found to be also elevated in LNCaP cells after androgen withdrawal in vitro, in LNCaP xenografts in vivo, and in the human disease using tissue microarray in untreated and hormonally-treated prostatectomy specimens(104). These data suggest that overexpression of IGFBP-2 and IGFBP-5 post-castration function to enhance IGF-I-mediated proliferation and cell survival.

Since both IGFBP-2 and IGFBP-5 are often co-expressed together in PCa progression, and IGFBP-5 is also expressed in the bone and play a role in bone metastases(201), they prove to be attractive targets to slow castration-resistant progression. Monospecific ASO against either IGFBP-2(104) or IGFBP-5(202) was found to delay the castration-resistant progression and attenuates IGF-I responsiveness. The homology between the genes encoding IGFBP-2 and IGFBP-5 allowed the design of bispecific ASO targeting both proteins. It is termed OGX-225 (OncoGenex, Vancouver, Canada).

M. Muramaki et al, have provided in their unpublished data the first preclinical proof-of-concept to support clinical trials with OGX-225. OGX-225 has potently suppressed both IGFBP-2 and IGFBP-5 in a dose- and sequence-specific fashion in LNCaP and PC3 cells, respectively. OGX-225 also inhibited the growth of LNCaP and PC3 cells both in vitro and in vivo, by increasing the apoptotic rates in a dose-dependent and sequence-specific manner. These findings provide the evidence that knocking down these targets with this drug can delay the castration-resistant progression.

In my thesis, I have shown a replication of the dose-dependent cytotoxic effects of OGX-225 on PC3 cells in comparison to control ODN, which was observed in M. Muramaki et al experiments. In order to demonstrate that the cytotoxic effects of 225 are due to targeting the IGFBPs, I attempted a rescue experiment designed to replace the putative OGX-225 target IGFBP-5 with recombinant IGFBP-5. However, the PC3 cells exposed to OGX-225 could not be rescued by adding recombinant IGFBP-5 at 350 ng/ml concentration to them.

This might suggest that the initial apoptotic pathway triggered by OGX-225 is an irreversible one, and simply adding back growth factors might be too late to result in the survival of the exposed PCa cells. It also might suggest that OGX-225 has other sites of action, other than IGFBP-2 and IGFBP-5, due to possible sequence homology with other proteins or growth factors. A Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database revealed a homology of OGX-225 to several targets of different (sometimes opposing), or unknown functions (Table 1). As shown in table 1, while OGX-225 share a sequence homology with its intended targets IGFBP-2 and IGFBP-5 (enhancers of IGF-I function), it also share a sequence homology with TGF-β, IGFBP-3, and IGFBP-4 which are pro-apoptotic molecules. It also shows a sequence homology with TBX-2, which is implicated in the development of several tissues and has a possible role in several types of cancer including breast cancer and melanoma. However, its role in normal or malignant prostate has not been studied. Other homologous targets have either unknown function or their role in prostate has not been studied. These targets include PDE6D, SPEG, PRRT2, PIB5PA, GPR180, ARID1B, and ALX4. It is difficult, given the amount of the available information and the several sequence homologies with diverse targets, to determine the exact reason for the failure to rescue PCa cells exposed to OGX-225, and this remains an area that requires further research to be done.

IGFBP-3 plays a role as a growth inhibitory protein in PCa cells(165). Initially, it was thought that it functions only by sequestering IGF and preventing it from binding to its receptor. However, further data have suggested that it inhibits

growth through IGF-independent mechanism. R. Rajah et al, have shown that IGFBP-3 inhibits growth in PC3 cells by inducing apoptosis(115). Using MTS assay, I have demonstrated the growth inhibitory effect of IGFBP-3 on PC3 cells, it was comparable to adding OGX-225. However, adding IGFBP-3 in addition to OGX-225 did not have any additive effect. The fact that the growth inhibitory function of IGFBP-3 requires an intact TGF-β pathway(124), and OGX-225 downregulates TGF-β pathway, might explain the non-additive effect of IGFBP-3 on PC3 cells when combined with OGX-225.

Using flowcytometry, the apoptosis-inducing effect of OGX-225 on PC3 cells was again observed here. IGFBP-3 did not demonstrate any apoptotic effect in this experiment. The apoptotic effect -assessed by measuring the cells in sub-G_0/G_1 phase- of IGFBP-3 was similar to control, and when given in addition to OGX-225 to PC3 cells there was no increase in the apoptotic effect of OGX-225. This might suggest that recombinant IGFBP-3 did not work technically in this experiment, or that IGFBP-3 act through a different mechanism than apoptosis, possibly by arresting the cell cycle, which is not in line with R. Rajah et al results.

Table 1: Homologous targets to OGX-225

Name of Protein	Sequence Alignment	Accesion Number	Query coverage	Maximum Identity	Protein Function
Insulin-like growth factor binding protein 5 (IGFBP5)	Query 1 CAGCAGCCGCAGCCCGGCTC 20 |||||||||||||||||||| Sbjct 940 CAGCAGCCGCAGCCCGGCTC 921	NM_000599.2	100%	100%	Enhance IGF-I function(146)
Insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1	Query 1 CAGCAGCCGCAGCCCGGCTC 20 |||||||||||||||||||| Sbjct 341 CAGCAGCCGCAGCCCGGCTC 322	NM_001013398.1	100%	100%	Anti-proliferative protein(115)
Insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2	Query 1 CAGCAGCCGCAGCCCGGCTC 20 |||||||||||||||||||| Sbjct 341 CAGCAGCCGCAGCCCGGCTC 322	NM_000598.4	100%	100%	Anti-proliferative protein
Insulin-like growth factor binding protein 2, 36kDa (IGFBP2)	Query 1 CAGCAGCCGCAGCCCGGCTC 20 |||||||||||||||||||| Sbjct 389 CAGCAGCCGCAGCCCGGCTC 370	NM_000597.2	100%	100%	Enhance IGF-I function(103), enhance CNS development(76)
T-box 2 (TBX2)	Query 6 GCCGCAGCCCGGCTC 20 ||||||||||||||| Sbjct 1411 GCCGCAGCCCGGCTC 1425	NM_005994.3	100%	100%	Transcription factor in the development of several tissues, including kidney and mammary glands(203)

Name of Protein	Sequence Alignment	Accesion Number	Query coverage	Maximum Identity	Protein Function
Phosphodiesterase 6D, cGMP-specific, rod, delta (PDE6D)	Query 5 AGCCGCAGCCCGGCT 19 ||||||||||||||| Sbjct 72 AGCCGCAGCCCGGCT 58	NM_002601.2	90%	100%	Regulates the level of cGMP in vertebrate photoreceptor cells(204)
SPEG complex locus (SPEG)	Query 5 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 705 CAGCAGCCGCAGCCC 7042	NM_005876.4	90%	100%	Regulates the growth and differentiation of vascular smooth muscle cells(203)
Insulin-like growth factor binding protein 4 (IGFBP4)	Query 4 CAGCCGCAGCCCGGCTC 20 ||||||||||||||||| Sbjct 476 CAGCCGCAGCCCGGCTC 460	NM_001552.2	85%	100%	Anti-proliferative protein(114)
Transforming growth factor, beta 1 (TGFB1)	Query 1 CAGCAGCCGCAGCCCGG 17 ||||||||||||||||| Sbjct 894 CAGCAGCCGCAGCCCGG 878	NM_000660.3	85%	100%	Anti-proliferative protein(205)

Name of Protein	Sequence Alignment	Accesion Number	Query coverage	Maximum Identity	Protein Function
Proline-rich transmembrane protein 2 (PRRT2)	Query 1 CAGCAGCCGCAGCCCGG 17 ||||||||||||||||| Sbjct 189 CAGCAGCCGCAGCCCGG 173	NM_145239.2	0.85	100%	Unknown
Phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A (PIB5PA), transcript variant 1	Query 3 GCAGCCGCAGCCCGG 17 ||||||||||||||| Sbjct 1768 GCAGCCGCAGCCCGG 1782	NM_014422.2	75%	100%	Negative regulator of PI3-kinase signalling events that promote neurite elongation and survival(206)
G protein-coupled receptor 180 (GPR180)	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 99 CAGCAGCCGCAGCCC 85	NM_180989.4	75%	100%	May play a role in vascular remodelling(206)
Synaptojanin 2 (SYNJ2)	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 105 CAGCAGCCGCAGCCC 91	NM_003898.2	75%	100%	May be involved in different protein-protein interactions(207)
AT rich interactive domain 1B (SWI1-like) (ARID1B), transcript variant 3	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 178 CAGCAGCCGCAGCCC 1799	NM_175863.2	75%	100%	Associated with protein binding and transcription co-activator activity by chromatin-regulated transcription(208) and maintenance(208)

Name of Protein	Sequence Alignment	Accesion Number	Query coverage	Maximum Identity	Protein Function
AT rich interactive domain 1B (SWI1-like) (ARID1B), transcript variant 1	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 1785 CAGCAGCCGCAGCCC 1799	NM_017519.1	75%	100%	Associated with protein binding and transcription co-activator activity by chromatin-regulated transcription and maintenance
AT rich interactive domain 1B (SWI1-like) (ARID1B), transcript variant 2	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 1824 CAGCAGCCGCAGCCC 1838	NM_020732.2	75%	100%	Associated with protein binding and transcription co-activator activity by chromatin-regulated transcription and maintenance.
Aristaless-like homeobox 4 (ALX4)	Query 1 CAGCAGCCGCAGCCC 15 ||||||||||||||| Sbjct 432 CAGCAGCCGCAGCCC 446	NM_021926.2	75%	100%	Homeodomain protein expressed in the mesenchymal compartment of a number of developing tissues (209)

4.2 Conclusions

CRPC represents a lethal form of the disease. IGFBP-2 and IGFBP-5 are over-expressed in this form of cancer and are antiapoptotic, while IGFBP-3 is downregulated and has a growth inhibitory effect in PCa. OGX-225 is a second-generation ASO that targets both IGFBP-2 and IGFBP-5, and inhibits growth of PCa cells both in vitro and in vivo through apoptosis. Our data show inability to rescue PC3 cells exposed to IGFBP-5. Since OGX-225 affects different targets in PCa cells, affecting these targets in combination might be responsible for the cytotoxic effect of OX-225, and simply adding back IGFBP-5 would not rescue PCa cells from such a multi-targeting drug. Also, they demonstrate the growth inhibitory effect of IGFBP-3 on PC3 cells, however we showed that IGFBP-3 might not work through an apoptotic pathway, and it does not have an additive effect on PC3 cells when combined with OGX-225.

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