SREBP Inhibitor, PF429242, Suppresses Proliferation of
Prostate Cancer Cell Lines

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Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Physiology
University of Saskatchewan
Saskatoon
By
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ABSTRACT

According to experimental and epidemiological evidence cholesterol may play an important role in development and promotion of prostate cancer. Sterol regulatory element-binding proteins (SREBP; SREBP-1 and SREBP-2) are key transcription factors controlling lipogenesis via the regulation of genes related to biosynthesis of fatty acid and cholesterol. Since the over-expression of SREBPs has been associated with aggressive features of human prostate cancer, the goal of present study was to evaluate the anti tumor activity of a novel SREBP inhibitor, PF429242 for the first time.

PF429242 suppressed cell proliferation in both androgen sensitive LNCaP and androgen insensitive C4-2 prostate cancer cells. The inhibitory effect of PF429242 (mean±SEM) was comparable to the similar compound, Fatostatin (mean±SEM), which was studied earlier on prostate cancer. The observed IC50 was closely similar between the two cell lines (IC50 9.6µM in LNCaP, IC50 9.8µM in C4-2). However, when the inhibitory effects of PF429242 compared to Paclitaxel which is the currently the rational drug for clinical management of prostate cancer, a significant difference was observed. Further, PF429242 suppressed n-SREBP activation followed by decreased FAS expression in both androgen sensitive and insensitive cells. Also, caspase3 activation increased in both cell lines following treatment. The pharmacological effects of PF429242 are attributed to the inhibition S1P in SREBP pathway. The findings from the present study provide the impetus to examine whether S1P inhibitor, PF429242, would serve as a potential additive agent in suppressing the early stages of hormone dependent/Independent prostate cancer.
AKNOWLEDGMENT

This thesis represents the culmination of my work as a Master’s Candidate in the Department of Pharmacology at the University of Saskatchewan. Over the course of my graduate studies, I have had the opportunity to learn the fundamentals of scientific inquiry, perform various lab techniques, and gain professional development.

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DEDICATION

I dedicate this thesis to my Family: Mom, Dad and Brother, as a small symbol of my gratitude for their unconditional love, insurmountable encouragement and unwavering support, which allowed me to overcome my biggest challenges and pursue my most passionate dreams.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACL</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen Deprivation Therapy</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BHLH</td>
<td>basic helix–loop–helix</td>
</tr>
<tr>
<td>CAB</td>
<td>Combined Androgen Blockade</td>
</tr>
<tr>
<td>CCHFV</td>
<td>Crimean-Congo Hemorrhagic Fever</td>
</tr>
<tr>
<td>COPII</td>
<td>Coat protein</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital Rectal Exam</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropine Releasing Hormone</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HMGCOA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
</tr>
<tr>
<td>HSP</td>
<td>Heatshock protein</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin Growth Factor 1</td>
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<tr>
<td>Insig</td>
<td>Insulin induced gene</td>
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<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LASV</td>
<td>Lassa Virus</td>
</tr>
<tr>
<td>M</td>
<td>Metastasis</td>
</tr>
<tr>
<td>N</td>
<td>Node</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PF</td>
<td>PF429242</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>R18</td>
<td>R1881</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
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<tr>
<td>SCD 1</td>
<td>Stearoyl CoA desaturase 1</td>
</tr>
<tr>
<td>S1P</td>
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<tr>
<td>S2P</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding proteins</td>
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<tr>
<td>TNM</td>
<td>Tumor/Node/Metastasis</td>
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<tr>
<td>T</td>
<td>Tumor</td>
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<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
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CHAPTER 1: INTRODUCTION

1.1. Epidemiology of Prostate Cancer

Prostate cancer is placed as the second most common cancer in men [1]. The clinical presentation of prostate cancer can vary from a microscopic, well-differentiated tumor to an aggressive cancer with invasion and metastasis. Prevalence of prostate cancer differ over 50-fold depending on the nationality and ethnicity [2]. These variations are due to the increased frequency of prostate biopsies performed in asymptomatic men because of an elevated prostate specific antigen (PSA) level. There are several risk factors in prostate cancer explained as the followings:

1.2. Risk Factors

1.2.1. AGE—there is a strong relationship between age and prostate cancer. The review of autopsy studies in multiple countries revealed a dramatic increase in prevalence of prostate cancer in older men[3]: According to statistics, 2 to 8 percent of men are diagnosed with prostate cancer between age 20 to 30 years old, 9 to 31 percent between 31 and 40 years old, 3 to 43 percent between 41 and 50 years old, 5 to 46 percent between 51 and 60 years old, 14 to 70 percent between 61 and 70 years old, 31 to 83 percent between 71 and 80 years old and 40 to 73 percent between 81 and 90 years old.

The variability between reports may be due to differences in techniques employed to diagnose prostate cancer incidence as well as geographic differences due to environmental or ethnic factors.

1.2.2. ETHNICITY — Prostate cancer is more common in black than white or Hispanic men, probably due to dietary and/or genetic factors. In addition to higher incidence rates, the onset of disease is at earlier age in African-American compared to other groups [4].

1.2.3. GENETIC FACTORS—several studies indicate the presence of a genetic component contributing in the pathogenesis of prostate cancer. However, identifying the specific genes has been
challenging due to inconsistency of data among different studies. Best known cancer-associated genes are BRCA2 and BRCA1. The risk of prostate cancer is approximately two fold higher in men with one or more affected first-degree relatives (brother, father) [5-8]. Based on case-control studies men with two or three affected first-degree relatives had a 5- and 11-fold increased risk of prostate cancer[9]. Also, early age of onset in a family member increases the risk.

BRCA2 and BRCA1 — the presence of BRCA1 or BRCA2 mutations increases the risk of prostate cancer. BRCA1 located on chromosome 17q21 and BRCA2 located on chromosome 13q are tumor suppressor genes that maintain the genome integrity by involving in the repair of double-strand DNA breaks and regulate the strand invasion recombinase required for homologous recombination of double strand DNA breaks respectively [10]. Mutations in BRCA1 and BRCA2 increase genomic instability and tumorigenesis which mainly increase the risk of breast and ovary cancer. It has been suggested that intact BRCA1 inhibits ligand-independent transcriptional activation of the estrogen receptor alpha leading to altered hormonal regulation of mammary and ovarian epithelial proliferation [11]. Over a thousand different mutations throughout the entire coding region of BRCA1 and BRCA2 have been reported. However, some mutations are more common in certain geographical or ethnic background [12]. BRCA1 mutations have also increased the risk of cervical, uterus, pancreas, gastric and prostate cancer [13, 14] as well as elevated risk of gallbladder, bile duct, pancreas, stomach cancer in BRCA2 mutations [15, 16]. BRCA2 might increase the risk of prostate cancer five to sevenfold, while the increase rate is unclear in BRCA1 mutations [17].

1.2.4. DIET— there are several comprehensive reviews on the association between intake of various nutrients and the risk of prostate cancer. Animal Fat- A high animal fat diet is considered to be an important factor in the development of prostate cancer [18-20]. A few number of studies suggest that intake of large amounts of alpha-linoleic acid (ALA) and low amounts of linoleic acid appear to increase the risk of prostate cancer; this combination is mostly common in red meat and some dairy products. One study has suggested that flaxseed supplementation could be associated with biological alteration and is
protective against prostate cancer, although further studies are required to support the data [21]. A low vegetable diet may be another risk factor for the development of prostate cancer [22, 23].

Soy intake — Phytoestrogens (flavones, isoflavones, lignans) are naturally occurring plant compounds that have estrogen-like activity. Genistein and daidzein, the predominant isoflavones in human nutrition, are derived mainly from soybeans and other legumes. Soy intake has been considered to decrease prostate cancer probability due to both estrogen like activity and the possibility of inhibiting 5-α reductase enzyme activity [24, 25].

Alcohol — According to a meta-analysis in 2001 which was based upon 235 studies that included over 117,000 cases, there is no consistent relationship between alcohol intake and prostate cancer [26]. This issue was subsequently addressed in the prospective Prostate Cancer Prevention Trial. There was no association between alcohol consumption and prostate cancer risk in 10,660 men with no or moderate alcohol intake (0 to <50 g/day). However, among the 260 men consuming ≥50 g/day (2.4 percent of the entire population), the relative risk of high-grade prostate cancer was much higher (95% CI 1.3-3.1) [27].

Coffee — Increased consumption of coffee appears to decrease the risk of metastatic prostate cancer (defined as fatal or metastatic) [28]. According to a prospective analysis of almost 48000 men from the health professionals, the decrease in risk of lethal prostate cancer was inversely proportional to increased coffee consumption for those drinking six or more cups of coffee per day, and a decreased risk was present after controlling for other known prostate cancer risk factors. The inverse relationship appeared to be related to coffee components other than caffeine. A similar level of protection was observed for those drinking regular and decaffeinated coffee.

1.2.5. HORMONE LEVEL AND OBESITY — Serum concentrations of androgens and insulin-like growth factor-1 (IGF-1) have been studied as possible risk factors for prostate cancer.

Insulin and insulin-like growth factor — based on multiple studies analyzing the relationship between insulin and insulin-like growth factor (IGF) and the subsequent development of prostate cancer, higher
serum levels of insulin and insulin like growth factor could be associated with increased risk of prostate cancer. It has been suggested that this association was mostly observed with low grade prostate cancer rather than high grade prostate cancer [29].

Obesity — there are conflicting results on the association between the incidence of prostate cancer and obesity as assessed by body mass index (BMI) [30]. The most extensive data came from a 2006 meta-analysis which included data from over 68,000 men with prostate cancer [31]. Overall, there was a statistically significant increase in prostate cancer with increased BMI. The relationship between obesity and Prostate cancer aggressiveness has been subsequently observed in multiple additional studies [32-35] which is more pronounced in African Americans compared to whites.

1.2.6. PHYSICAL ACTIVITY — based on the data linking BMI and prostate cancer aggressiveness, regular physical activity might be beneficial in preventing prostate cancer. However, there is uncertainty regarding exercise protecting against the development or progression of prostate cancer. Some (but not all) of the beneficial effects of exercise in older men could be related to sun exposure while exercising outdoors. According to a cohort study, men with higher levels of physical activity had higher circulating levels of 25-hydroxyvitamin D [36]. However, while both vigorous and non vigorous exercise were associated with higher vitamin D concentrations, only vigorous exercise was associated with a lower risk of advanced prostate cancer.

1.3. ROLE OF STATINS — multiple observational studies have suggested that use of statins may affect the overall risk of cancer and of specific cancers. However, based on meta-analyses of randomized trials, there is no consistency between the effects of statins on cancer incidence of cancer mortality.

Several large studies have specifically focused on the impact of statin use and the risk of prostate cancer. Results of these analyses suggest that statin use reduces the risk of advanced, clinically significant disease, although there are conflicting data regarding the effect of statins on overall incidence of prostate cancer [37]. A meta-analysis that included data from six randomized trials and 13 observational studies
found no relationship between statin use and the incidence of prostate cancer either in six randomized trials or 13 observational studies [38].

The variable results by two large studies are as the followings:

- The Health Professionals Follow-up Study evaluated 2579 cases of prostate cancer, which included 316 cases with regionally invasive, metastatic, or fatal disease. There was no association between statin use and the overall risk of prostate cancer. While, multivariate analysis found a significant decrease in the risk of advanced disease after controlling for known risk factors. Moreover, decreased risk was more pronounced when the duration of statin use was considered.

- In a retrospective study of more than 55,000 men taking either a statin or an antihypertensive medication, there was a statistically significant 31 percent reduction in the incidence of prostate cancer compared to men on an antihypertensive medication [39]. The risk reduction was more pronounced on those with high-grade prostate cancer compared to those with low-grade lesions.

Some, but not all [40], observational studies have raised the possibility that use of statins may decrease overall risk of cancer and of specific cancers.

On the other hand, meta-analyses of randomized trials have consistently shown no effect of statins on cancer incidence of cancer mortality[41]:

- A meta-analysis of 26 randomized trials of statins involving 169,138 patients found no increased risk of cancer death after a median follow-up of 4.9 years [42].

- Another meta-analysis reviewed 26 randomized trials of statins involving 86,936 patients, with a mean follow-up of at least one year; there was no effect of statins on cancer incidence or cancer death [43]. There was no effect of various statin subtypes (i.e., hydrophilic, lipophilic, natural, synthetic) statin on different type of cancers.
• Overall, there is no convincing data regarding statin effects on risk of cancer [44].

1.4. CLINICAL PRESENTATION — with the introduction of widespread screening using serum PSA, prostate cancer is often diagnosed while asymptomatic [45, 46]. However, screening with serum PSA is controversial, since many of the prostate cancers diagnosed in this manner may never be clinically significant.

In patients who are not diagnosed by PSA screening, prostate cancer typically is detected initially either by digital rectal examination or following genitourinary symptoms.

1.5. TNM STAGING SYSTEM — the tumor, node, metastasis (TNM) system is generally used for staging prostate cancer and provides important information that can guide initial therapy. This system is supported by both the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC)

Tumor (T) — Anatomic staging of the primary tumor (T) is carried out both clinically and pathologically:

• T1 lesions are neither palpable nor detectable by imaging.

• T2 lesions are palpable but appear to be confined to the prostate gland.

• T3 lesions extend through the prostatic capsule.

• T4 tumors are fixed to or invade adjacent structures.

The T1 and T2 categories are subdivided based upon the extent of prostate gland involvement.

Although clinical staging is necessary for planning initial treatment, both digital rectal examination and imaging procedures may significantly underestimate the extent of disease.

Node (N) — both clinical and pathologic staging define regional lymph nodes as not assessed (NX), negative (N0), or positive (N1).
Metastasis (M) — Patients are classified as either not having distant metastasis (M0) or distant metastasis present (M1). Patients with metastases are subclassified based upon the site(s) of disease.

Anatomic stage/prognostic groups — The 2010 TNM system specifies that the Gleason score be used to assess tumor grade. In addition, the 2010 TNM system has incorporated the pretreatment serum PSA level along with the Gleason score into anatomic stage/prognostic groups [47].

1.6. ANDROGENS IN PROSTATE CANCER

Androgens stimulate both normal and cancerous prostate cell growth. Charles Huggins first established the critical role of androgens in prostate cancer growth in 1941. These findings led to androgen deprivation therapy (ADT) as the primary treatment for patients with advanced prostate cancer [48].

Although ADT is palliative and not curative [49], it can normalize serum levels of prostate specific antigen (PSA) in over 90 percent of patients and can produce objective tumor responses in 80 to 90 percent. This antitumor activity can improve quality of life by reducing bone pain as well as the rates of complications, such as pathologic fracture, spinal cord compression, and urethral obstruction [48]. It remains unclear whether or not overall survival is prolonged [50]. The duration of response to ADT for patients with metastatic disease is highly variable, and unfortunately most prostate cancer patients eventually experience disease progression despite treatment [51, 52]. Patients who have progressed while on ADT are considered to have castration-resistant disease. Secondary hormone manipulations often produce clinical benefit after progression on ADT, although the duration of such effects is usually limited. Patients who progress despite secondary hormone treatment are said to have androgen-independent disease.

1.7. THERAPUTIC ENDPOINTS
Outcomes that have been used in trials to establish the role of hormonal therapy in men with advanced prostate cancer include overall survival, measurable tumor response, changes in serum PSA, skeletal-related events, and quality of life. Complicating the interpretation of results, many studies conducted prior to the routine use of serum PSA testing in screening and monitoring of disease do not reflect typical contemporary patient populations or current practice patterns.

The prolonged natural history of advanced prostate cancer, its occurrence in older men who often have substantial co-morbidity, and the heterogeneity of disease between patients complicate the use of overall survival as an endpoint in assessing response to treatment.

The standard classifications of complete response, partial response, stable disease, and progressive disease are inadequate to evaluate response in most men with metastatic prostate cancer. Measurable disease is present in only 10 to 20 percent of patients [53]. Bone metastases are the most common site of disease, and bone involvement is difficult to measure objectively. Bone scan interpretation is variable, and there is a long healing time when lesions do respond to treatment.

1.7.1. INITIAL HORMONAL THERAPY — as mentioned above, androgen plays the key role in both growth and malignant transformation of prostate tissue; 90 to 95 percent of circulating androgen is produced in testes, while the rest is produced by adrenal gland. Based on this rationale, preventing the production of androgens or blocking the action is the therapeutic approach in prostate cancer. Androgen deprivation therapy (ADP) could be achieved with surgical or medical castration. Surgical castration is used less frequently, but decreases serum testosterone level rapidly. A different number of agents have been used in medical approach of therapy; gonadotropin releasing hormone agonists (GnRH) (eg, leuprolide, goserline, buserlin, triptorelin) that are commonly used suppress luteinizing hormone (LH) production and therefore the synthesis of testicular androgens [54], GnRH antagonists such as Degarelix, bind to the GnRH receptors on pituitary gonadotropin-producing cells and rapidly suppress serum testosterone level [55]. Estrogens suppress pituitary luteinizing hormone release by inhibiting the release
of GnRH from hypothalamus and lowering the serum testosterone level within one to two weeks [56]. Antiandrogens (flutamide, bicalutamide) bind to androgen receptors and competitively block binding of dihydrotestosterone (DHT) and testosterone to the androgen receptor [57]. Ketoconazole is an antifungal agent that blocks androgen synthesis in the adrenal gland by inhibiting the cytochrome P450 enzyme and has a direct cytotoxic effect on prostate cancer cells in vitro [58, 59].

Combined androgen blockade (CAB), which uses a GnRH agonist along with an antiandrogen, has also been used as a way to provide maximal depletion of testosterone activity.

The management of patients who either present with metastatic disease or who relapse after their initial treatment for localized disease generally uses androgen deprivation therapy. Secondary hormonal therapies and chemotherapy often provide an additional period of disease control. However, most men with metastatic prostate cancer eventually develop castrate-resistant disease that cannot be controlled by systemic chemotherapy.

Experimental research suggests that activation of de novo lipogenesis [60] and cholesterologenesis [61, 62] induces prostate cancer cell proliferation and promotes cancer development and progression. Therefore, pharmacologic intervention, blocking fatty acid and cholesterol anabolisms, could potentially be a novel therapy for malignant prostate cancer.
CHAPTER 2: SREBPS

Studies on regulation of cholesterol synthesis have led to identification of a unique family of membrane bound transcription factors called sterol regulatory element binding proteins (SREBPs). These transcription factors bind to the sterol regulatory element DNA sequence TCACNCCAC. Mammalian SREBPs are basic helix–loop–helix leucine (bHLH) zipper transcription factors that transcriptionally activate genes involved in fatty acid and cholesterol biosynthesis and homeostasis. Precursor SREBPs are synthesized as endoplasmic reticulum (ER) membrane-bound forms. Through sequentially proteolytic cleavage by site-1 (S1P) and site-2 (S2P) proteases, the N-terminus of SREBPs translocates into the nucleus and trigger the expression of target genes having sterol regulatory elements (SRE), cis-acting elements in their 50-flanking promoter regions [63]. SREBPs have a structure similar to E-box-binding helix-loop-helix (HLH) proteins. However, in contrast to E-box-binding HLH proteins, an arginine residue is replaced with tyrosine making them capable of recognizing StREs and thereby regulating membrane biosynthesis. Three isoforms of SREBPs have been identified, including SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1 is encoded by SREBF1 gene. This gene is located on chromosome 17. Two transcript variants encoding different isoforms have been found for this gene, which are SREBP-1a and -1c (ADD-1). SREBP-1c regulates genes required for glucose metabolism and fatty acid and lipid production and its expression is regulated by insulin. SREBP-1a regulates genes related to lipid and cholesterol production and its activity is regulated by sterol levels in the cell [64].

SREBP-2 is a protein in human that is encoded by SREBF2 gene which is particularly involved in the transcriptional control of cholesterol anabolism.

Over-expression of SREBP-1 has been found in human prostate cancer tissues and LNCaP xenograft tumor tissues during androgen refractory/castration-resistant progression. Furthermore, SREBP-1 promoted cell viability and castration-resistant progression via alterations of lipogenesis, oxidative stress, and androgen receptor (AR) expression in prostate cancer cells [65].
Transcription of genes responsible for fatty acid and cholesterol synthesis and LDL receptor are controlled by SREBPs. Up to now, more than 30 genes are known for transcription and regulation of fatty acid, cholesterol, triglyceride and phospholipids [66]. The activity of each SREBP isoform is mediated by sterol and SCAP (SREBP cleavage activating protein) [67]. In sterol depleted cells, as shown in the picture 1, SCAP binds to cholesterol in ER membrane and makes some conformational changes to promote cholesterol binding to a protein called Insig, which prevents interaction of SCAP with COPII vesicle formation proteins Sar 1, Sec23 and Sec24. SCAP then escorts SREBP to Golgi where site 1(S1P) and site 2 (S2P) proteases release the N terminal transcription factor domain from the membrane [68]. Then the SREBP is transported to the nucleus and activates transcription by binding to SRE sequence in the promoters of target genes that results in increased synthesis and uptake of cholesterol [69]. This major role of SREBP is highlighted by other signaling pathways such as nuclear hormone receptors RXR and LXR that function to up-regulate SREBP-1c in response to cholesterol overloading to increase the supply of unsaturated fatty acids needed for esterification and storage of cholesterol. Liver converts excess carbohydrate to fatty acids to make triglycerides. Insulin also increases fatty acid synthesis in response to excess carbohydrate which is mediated by SREBP-1c [70]. Recent studies have also shown that SREBP-1c may contribute to regulation of glucose uptake and synthesis [71]. In case of glucose excess in hepatocytes, SREBP-1c induces the expression of glucokinase to provide glucose utilization and suppresses phosphoenolpyruvate carboxykinase to promote gluconeogenesis [72].
Picture 2.2: SREBP pathway: SREBP is activated by proteolytic cleavage. SREBP precursors stay in the ER membrane while they are tightly attached to SCAP and a protein of INSIG family. Under the appropriate conditions, SCAP dissociates from INSIG and escorts the SREBP precursors from the ER to the Golgi apparatus, where, two proteases, S1P and S2P cleave the precursor protein, releasing the mature form of SREBP into the cytoplasm. The mature form then migrates to the nucleus, to activate the genes promoter involved in cholesterol uptake or in cholesterol synthesis. SREBP processing can be controlled by the cellular sterol content [73]. (With a written permission from the copy right owner)
2.1. SREBPs: Novel therapeutic targets:

Statins have been used as a lipid lowering drugs in patients with metabolic diseases like dyslipidemia as both primary and secondary prevention of cardiovascular disease. Other than their use in metabolic disease and cardiovascular disease, there are some potential therapeutic effects for statins. Post-hoc analysis of clinical trials has shown some benefits of statin use in both patients with normal renal function and moderate renal insufficiency [74, 75]. There is some evidence that statins could benefit venous thromboembolism [76-78]. There have been conflicting results regarding statin effects on increasing bone formation, volume and density in older patients with the risk of osteoporotic fractures [79-83]. There are also some evidence regarding statins benefits in hypertension [84], heart failure [85] and reducing the risk of cholecystectomy for patients with gallstone [86, 87]. Animal studies suggested better outcome with statin use in sepsis [88, 89]; initial observational studies showed that prior statin therapy might decrease the rate of mortality from sepsis [90, 91].

Some observational studies have shown that statin use could decrease the overall rate of cancer in general as well as some specific types of cancers [92, 93]. However, there is no convincing data that statins increase or decrease the rate of cancer [94-97].

2.2. SREBP INHIBITION IN PROSTATE CANCER: Previous reports revealed that SREBP-2 activity was increased in prostate cancer cells and targeting this activity altered cell membrane cholesterol content and inhibited signaling transduction mediated by cholesterol-rich lipid rafts [98]. These discoveries provide a rationale for blocking SREBPs in an attempt to control the metabolic and signaling pathways that regulate and promote prostate cancer progression. Fatostatin, a nonsteroidarylthiazole derivative, was originally developed from a chemical library to inhibit insulin-induced adipogenesis [65] and decreased the amounts of fatty acid, triglyceride and low-density lipoprotein and reduced body weight by perturbing the nuclear translocation of SREBP in obese mice with low cytotoxicity [99]. Fatostatin (4-[4-(4-methylphenyl)-1,3-thiazol-2-yl]-2-propylpyridine hydrobromide) has been shown to have the potential
efficacy in arresting glioma cells proliferation. It has also been reported that fatostatin, a novel anti-prostate tumor agent that suppresses cell proliferation, tumorigenesis, and progression and induces apoptotic death in prostate cancer cells in vitro and in vivo by blocking SREBP activation and inhibiting fatty acid and cholesterol biosynthesis as well as AR signaling.

Prostate cancer incidence has been linked to a Western diet, which includes high levels of red meat, saturated fat, and dairy products. The molecular basis for these epidemiological relationships is obscure; however, an unknown constituent of dietary fat has been suggested to be a potential contributor to a progression mechanism [100, 101]. Cholesterol, a neutral lipid that is a prominent component of the Western diet, contributes to the unique biophysical properties of the lipid raft microdomain and is mechanistically important for signal transduction by raft proteins. Disruption of lipid rafts by dispersion or extraction of membrane cholesterol results in inhibition of raft-dependent signaling events [102-104]. Cholesterol is synthesized by tumor cells but also enters cells from the circulation by LDL receptor-mediated endocytosis [105]. Feedback regulation of cholesterol absorption and synthesis is lost in prostate and other types of cancer cells, resulting in up-regulation of the cholesterol synthesis (mevalonate) pathway and increases in LDL receptor expression [106].

High cholesterol content of benign prostatic hyperplasia tissues was reported as early as 1942 [107]. Subsequent studies of prostate tissues also found increases in cholesterol content that correlated with the presence of malignancy [108]. Further studies identified abnormalities in lipid homeostasis as the underlying cause of cholesterol accumulation in prostate, resulting in massive accumulation of cholesteryl esters and a marked increase in free cholesterol levels. Normally, the prostate contains high cholesterol levels, even in comparison to the liver, perhaps reflecting the fact that the prostate is an androgen target organ. Androgens stimulate lipogenesis in human prostate cancer cells directly by increasing transcription of genes such as fatty acid synthase (FAS) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase [109]. Increased levels of FAS are associated with oncogenesis and increased levels of fatty acids have been suggested to alter signaling processes at the plasma membrane [110]. Lipid metabolism is
a major target of androgenic signaling mechanisms and is strictly controlled by androgens in the normal adult prostate. Recent attempts to identify genes under transcriptional control of the androgen receptor revealed more than 300 androgen-responsive transcripts, the majority of which encode proteins involved in lipid metabolism [111]. The androgen receptor recruits a cohort of transcription factors including SREBPs, that drives expression of the enzymes involved in cholesterol metabolism [112]. These sterol response element binding proteins (SREBPs) consist of the three related transcription factors, SREBP-1a, SREBP-1c and SREBP-2, which are critical regulators of androgen-regulated lipogenesis. Whereas SREBP-1c is primarily responsible for the transcription of fatty acid biosynthesis genes, including FAS, SREBP-2 predominantly regulates mevalonic acid (cholesterol synthesis) pathway genes such as farnesyl diphosphate synthase and HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol synthesis. The strictly coordinated expression and feedback regulation by this family of transcription factors is frequently lost in prostate cancer. After challenge with androgen, LNCaP cells, a human prostate cancer cell line, accumulate cholesterol as a result of SREBP activation. The observed changes in gene expression that contribute to the malignant phenotype of prostate cancer cells, were recently investigated and the picture that emerges from these studies indicates that dysregulation of SREBPs represents a critical adaptive response to cell proliferation and survival. Both the genes for SREBP-1 and 2 are constitutively up-regulated in LNCaP xenograft tumors in vivo [113]. Interestingly, SREBP-2 protein expression in these xenografts increased more than four-fold during progression to androgen independence, whereas SREBP-1 protein levels did not change appreciably. SREBP-2 expression also does not show sterol feedback regulation in prostate cancer cells [114], which lends support to the idea that SREBP-2 dysregulation is particularly critical for prostate cancer, leading to elevated cholesterol levels in prostate tissue. The increased expression of many genes involved in lipid metabolism suggests a role for cholesterol synthesis in prostate cancer, but the origin of the uncontrolled activation of SREBPs has been poorly understood. Recent observations suggest that the serine-threonine kinase, Akt (protein kinase B) recruits transcription factors of the SREBP family to induce expression of lipogenic enzymes. Activation of Akt has recently been demonstrated to induce synthesis of SREBP-1
and 2 in human retinal pigment epithelium and osteosarcoma cells [115]. This finding may also be relevant for advanced prostate cancer in which SREBP-1 has been found to show increased activation [116] and phosphoinositide-3-kinase (PI3K)/Akt signaling is constitutively activated [117]. The significance of elevated cholesterol levels in prostate cancer is not fully understood. Increased steady-state cholesterol levels may reflect the high demand for membrane biosynthesis of proliferating cells; however, this is unlikely to be the case in prostate cancer because of the low mitotic index characteristic of this malignancy. Is there any reason to believe that cholesterol accumulation, up-regulation of cholesterol synthesis or loss of lipid homeostasis is functionally relevant in the promotion of malignant growth? In the following sections, we focus on the question of how cholesterol participates in cell signaling and may contribute to tumor cell survival.

**Prostate cancer and lowering cholesterol by Inhibitors of HMG-CoA reductase (often referred to as ‘statins’)** - Statins are cholesterol-lowering drugs widely used for the prevention of cardiovascular disease. Statins have also been proposed as potential chemo preventive agents for human cancers [118]. The potential connection between statin use and cancer incidence was initially established in studies that were conducted to monitor the safety of these compounds with long-term use. Statin-associated reductions in the risk of colon, prostate and other cancer types have been reported in several large population based studies. A retrospective, case-control study on statin use found a 47% reduction in risk of colorectal cancer [119]. The largest case-control study performed to date [120], with more than 2000 incident cases and a follow-up of 7–10 years, demonstrated a greater than 20% reduction in prostate cancer incidence. Another study [121] found that users of HMG-CoA reductase inhibitors were 28% less likely to develop cancer. Despite these apparent clinical responses, a recent meta analysis of 26 randomized controlled trials concluded that statins have no discernible effect on cancer incidence and death from cancer at multiple sites [122].

According to a study by Zhuang L et al., simvastatin, a cholesterol synthesis inhibitor lowered cholesterol content of lipid rafts in prostate cancer cell lines both in vivo and in vitro lipid rafts that are cholesterol
and sphingolipid enriched microdomains regulate phosphorylation originated from membrane bound proteins. Based on their results, cancer cells are dependent on cholesterol as a signal transduction mediator regulating Akt pathway for their survival. Results showed that the HMG-CoA reductase inhibitory effect of simvastatin on lowering endogenous cholesterol alters cell survival and signal transduction in androgen sensitive LNCaP cell line. Moreover, simvastatin induced apoptosis consistent with another study that reported increased caspase7 activation, apoptosis mediator; in prostate cancer cell line with lovastatin [123]. All the results reported to be due to alterations in membrane cholesterol [124].

Our rationale was based on the facts that PF429242 has been previously used to reduce the expression levels of hepatic SREBP target genes and to lower rates of cholesterol and fatty acid synthesis in mice. PF-429242 is also a potent antiviral agent against prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) and LASV in cultured cells. PF-429242 efficiently prevented the processing of GPC from the LCMV and LASV. This compound is expected to be active against Crimean-Congo Haemorrhagic Fever Virus (CCHFV) [125].
CHAPTER 3: HYPOTHESIS AND OBJECTIVES

In this study we investigated PF429242 effects on two different prostate cancer cell lines. We hypothesized that PF-429242, a potent inhibitor of S1P, inhibits proliferation prostate cancer cells by inhibiting the activation of SREBP1 and suppressing its down-stream genes.

We therefore proposed the following research objectives:

1- Investigate the effects of SREBP inhibitors (PF429242 and Fatostatin) on both androgen sensitive and insensitive prostate cancer cell line and determine PF429242 IC50 values.

2- Compare anti-proliferative effects of PF429242 and Fatostatin with each other and with paclitaxel separately.

3- Investigate the potential effects of PF429242 on SREBP1 down-stream gene(s).
CHAPTER 4: MATERIAL AND METHOD

4-1. Cell lines and culture conditions:

The human prostate cancer LNCaP and C4-2 cells were cultured in T Medium. LNCaP cell line was purchased from ATCC as well as obtained as a generous gift from Dr. Leland Chung’s laboratory (Cedar Saini Hospital, LA, USA). C4-2 cell line was generously provided as a gift by Dr. Kishor Wasan’s research laboratory (UBC, Vancouver). All cell lines were grown in medium with 10% FBS, 100 U/mL of penicillin and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

4-2. COMPOUNDS AND REAGENTS:

Fatostatin (4-{4-(4-methylphenyl)-1,3-thiazol-2-yl}-2-propylpyridinehydrobromide), PF429242 with chemical Name: 4-{[(Diethylamino)methyl]-N-[2-(2-methoxyphenyl)ethyl]-N-(3R)-3-pyrrolidinylbenzamide and Paclitaxel were all purchased from Sigma Aldrich (Mississauga, Canada).

Cell counting kit, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) was purchased from Sigma Aldrich (Mississauga, Canada). Metribolone (also known as methyltrienolone or R1881) a potent non-aromatizable androgen was purchased from PerkinElmer.

4.3. CELL PROLIFERATION ASSAY:

Prostate cancer cells were seeded on 96-well plates in triplicate and treated with vehicle or Fatostatin, PF29242, Paclitaxel for 72 hours. Cell proliferation and viability were determined by Cell Counting Kit-8 (CCK-8) that allows convenient assays using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bio reduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 solution is added directly to the cells, no pre-mixing of components is required. WST-8 is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of
formazan produced is directly proportional to the number of living cells. Since the CCK-8 solution is very stable and it has little cytotoxicity, a longer incubation, such as 24 to 48 hours, is possible.

Cell Counting Kit-8 allows sensitive colorimetric assays for the determination of the number of viable cells in the proliferation and cytotoxicity assays. The detection sensitivity is higher than any other tetrazolium salts such as MTT, XTT or MTS.

4.4. WESTERN BLOT ANALYSIS

Cultured tumor cells were washed with cold phosphate buffered saline (PBS) and rocked gently while kept on ice. PBS then discarded and RIPA buffer consisted of 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, and PH 8.0 added to each flask. A cell scraper used to dislodge cells and cells transferred into micro centrifuge tubes while homogenized by passing through a 23 guage needle in and out of a 3 ml syringe 6 times. The homogenized lysates were kept on ice for 20 minutes, and then centrifuged at 1500 RPM at 4°C for 5 minutes. Supernatant or protein mix then transferred to a fresh tube and stored at -80°C for later protein analysis. Following protein analysis, gel electrophoresis, membrane transfer, membrane blocking and incubation with primary and secondary antibody performed. At the end visualization in the dark room performed.

Primary antibodies against SREBP-1 (sc-8984), FASN (sc-48357), ß-actin (sc-47778; Santa Cruz Biotechnology), SREBP-2 (ab72856 and ab112046; Abcam), and caspase 3 (Santa Cruz) were used. Briefly, for each experimental condition 20-30 μg of the cell homogenates was separated with SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane, and probed with the target antibodies. For sequential reprobing of the same blot, the membrane was first stripped of the initial primary and secondary antibodies and then subjected to immunoblotting with another target antibody. Blots were developed using enhanced chemiluminescence detection (Amersham Biosciences). Band
intensities were quantified using NIH ImageJ software and normalized to the quantity of β-actin in each sample lane. Western blot analysis was performed using Mini-PROTEAN system (Bio-Rad).
CHAPTER 5: DATA ANALYSIS

The data was entered into GraphPad Prism® and various statistical tests were used, as indicated, to determine statistical significance. All results are shown as mean ± SEM. One way analysis of variance (ANOVA) and post-hoc Dunnett's multiple comparison tests were used. Results considered statistically significant when $p \leq 0.5$ reported.
CHAPTER 6: RESULTS

6.1. Determination of the IC50 value of PF429242 compound in C4-2 cancer cell line

To evaluate the effects of PF429242 on SREBP inhibition, C4-2 cells were seeded in 96well plates and incubated in 37 °C degree, CO2 level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with PF429242 between 0-50µM. n=24, triplicate experiments. The value of IC50 was calculated for PF429242 in C4-2 cells. IC50 calculated as 9.8µM in C4-2 cells. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle)
**Figure 6.1.** The value of IC50 for a 48 hour treatment was calculated and is shown in the histogram. PF429242 inhibited cell proliferation in a dose dependent manner (0-50µM). P value<0.05 represented a significant difference between PF429242 (10-50µM) and control group. IC50 calculated as 9.8µM in C4-2 cells. No statistically significant difference observed between concentrations 10 µM to 50 µM (P>0.05).
6.2. Metribolone (R1881) enhanced proliferation of LNCaP tumor cells.

Since LNCaP prostate cancer cell line is androgen-sensitive and needs androgen to maintain its proliferative condition. To evaluate this effect LNCaP cancer cells were seeded in 96well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 0.1 nM Metribolone (methyltrienolone or R1881) a potent non-aromatizable androgen to the culture media and incubated for 48 hours. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle). As shown in Fig. 6.2, the presence of the androgen enhanced cellular proliferation 35.5±2.5% when compared with androgen deprived LNCaP cells indicating androgen sensitivity of this tumor cell line.

![Cell Viability](image)

**Figure 6.2.** Treatment of androgen sensitive prostate cancer cells with Metribolone (R1881) increased cell proliferation (Pvalue<0.05). The presence of the androgen enhanced cellular proliferation 35.5±2.5% (Pvalue<0.05) when compared with androgen deprived LNCAP cells indicating androgen sensitivity of this tumor cell line.
6.3. Determination of the IC50 value of PF429242 compound in LNCaP prostate cancer cell line

To evaluate the effects of PF429242 on SREBP inhibition, LNCaP cancer cells were seeded in 96well plates and incubated in 37 °C degree, CO2 level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with PF429242 between 0-50µM. n=24, triplicate experiments. The value of IC50 was calculated for PF429242 in LNCaP cells. IC50 calculated as 9.6µM in LNCaP cancer cells. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle). All the experiments were performed in the presence of R1881 0.1nM.
Figure 6.3. The value of IC50 for a 48 hour treatment was calculated and is shown in the histogram. PF429242 inhibited cell proliferation in a dose dependent manner (0-50µM). P value<0.05 represented a significant difference between PF429242 (10-50µM) and control group. IC50 calculated as 9.6µM in LNCaP cells. No statistically significant difference observed between concentrations 10 µM to 50 µM (P>0.05).
6.4. Inhibition of SREBP activation by two mechanistically different, PF429242 and Fatostatin agents which suppressed cell proliferation in C4-2 tumor cell line

To investigate the anti-proliferative effects of PF429242, we seeded androgen insensitive C4-2 cancer cells in 96well plates and incubated in 37 °C degree CO2 level 5% for 24 hours to reach 70% confluency. After 24 hours medium was refreshed and cells were treated with 10μM/L of Fatostatin or PF429242 for 48 hours. Following 48 hours incubation, 1 μL WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) was added to each well and plates were incubated for another 2 hours. Water-soluble formazan dye was produced upon bio reduction in the presence of an electron carrier, 1-Methoxy PMS. The amount of formazan produced is directly proportional to living cells. Cell viability then was determined by plate reader.

According to the results PF429242 inhibited cell proliferation in C4-2 cells after 48 hours of treatment. Bars demonstrate mean±SEM of triplicate experiments (n=24 treated with vehicle). Cell proliferation was significantly decreased in concentration of 10μM/L. Cell proliferation inhibition compared to a similar drug Fatostatin which was previously studied; no significant difference observed between Fatostain 10μM/L and PF429242 10μM/L. PF429242 reduced cell viability 20%±3.21% compared with Fatostatin that reduced cell viability 26%±3.21% (P<0.05). No statistically difference observed between the anti-proliferative effect of two compounds (P>0.05). The combination of compound used to investigate the additive effects; cell viability reduced by 33%±3.21% which was not significantly different from each compound (P>0.05).
Figure 6.4. PF429242 inhibits cell proliferation of C4-2 prostate cancer cell in vitro. The values are represented as percentage of viable cells where the vehicle treated cells were regarded as 100%. P value<0.05 represents significant difference between PF429242 10µM, Fatostatin 10µM and control group. PF429242 reduced cell viability 20%±3.2%. Fatostatin reduced cell viability 26%±3.2% (P<0.05). The combination of PF429242 10 µM and Fatostatin 10 µM reduced cell viability 33%±3.2% (P>0.05) that was not statistically significant compared to each compound.
6.5. Comparison of anti-proliferative effect of Paclitaxel with PF429242 compound in C4-2 cancer cells

To investigate the anti-proliferative effects of PF429242, we seeded androgen insensitive C4-2 cancer cells in 96well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM/L of Paclitaxel and PF429242 for 48 hours. Following 48 hours 1 µL WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl)-2H-tetrazolium, monosodium salt) added to each well and plates were incubated for another 2 hours. Water-soluble formazan dye was produced upon bio reduction in the presence of an electron carrier, 1-Methoxy PMS. The amount of formazan produced is directly proportional to living cells. Cell viability then was determined by plate reader.

According to the results PF429242 inhibited cell proliferation in C4-2 cells after 48 hours of treatment. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle). Cell proliferation was significantly decreased in dose of 10µM/L. Suppression of cell proliferation compared to Paclitaxel which is the drug of choice in treatment of prostate cancer; significant difference observed between Paclitaxel 10µM and PF429242 10µM. PF429242 reduced cell viability 25%±2.88% compared with Paclitaxel that reduced cell viability 55%±2.88%.

The combination of PF429242 10µM and Paclitaxel 10µM used to investigate additive/synergistic effect of two compound together. Cell viability reduced 57%±2.88 which was not statistically significant compared with each compound individually (P>0.05)
Figure 6.5. PF429242 inhibits cell proliferation of C4-2 prostate cancer cell in vitro. The values are represented as percentage of viable cells where the vehicle treated cells were regarded as 100%. P value <0.05 represents significant difference between control and vehicle groups. PF429242 10µM reduced cell viability by 25%± 2.88% and Paclitaxel 10µM reduced cell viability 55%±2.88% (P<0.05). The combination of two compounds reduced cell viability 57%±2.88 (P>0.05), not statistically significant compared with each compound.
6.6. Inhibition of SREBP activation by two mechanistically different, PF429242 and Fatostatin agents which suppressed cell proliferation in LNCaP tumor cell line:

We investigated the anti proliferative effects of PF429242 in androgen sensitive LNCaP cells. Therefore, we seeded androgen sensitive LNCaP cancer cells in 96well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM/L of Fatostatin and PF429242 for 48 hours. Following 48 hours 1 µL WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) added to each well and plates were incubated for another 2 hours. Water-soluble formazan dye was produced upon bio reduction in the presence of an electron carrier, 1-Methoxy PMS. The amount of formazan produced is directly proportional to living cells. Cell viability then was determined by plate reader.

According to the results PF429242 inhibited cell proliferation in LNCaP cells after 48 hours of treatment. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle). Cell proliferation was significantly decreased in concentration of 10µM. Cell proliferation inhibition compared to the similar drug which was studied in the treatment of prostate cancer previously; no statistically significant difference observed between Fatostatin 10µM and PF429242 10µM (P>0.05). PF429242 reduced cell viability 37%±2.88% compared with control while, Fatostation reduced cell viability 33%±2.88% (P<0.05). Combination of two compounds reduced cell viability by 39%±2.88%, that was not statistically significant from each compound (P>0.05).

All experiments were performed with R1881 0.1nM.
Figure 6.6. PF429242 inhibits cell proliferation of C4-2 prostate cancer cell in vitro. The values are represented as percentage of viable cells where the vehicle treated cells were regarded as 100%. P value < 0.05 represents significant difference between PF429242 10µM, Fatostatin 10µM and control group. PF429242 reduced cell viability 26%±3.215%, Fatostatin reduced cell viability 20%±3.215 (P<0.05). Combination of both compounds reduced cell viability by 39%±2.88% (P>0.05), not statistically significant from each compound.
6.7. Comparison of anti-proliferative effect of Paclitaxel with PF429242 compound in LNCaP cells

We investigated the anti proliferative effects of PF429242 compared to Paclitaxel in androgen sensitive LNCaP cells; therefore, we seeded LNCaP cancer cells in 96well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM of Paclitaxel and PF429242 for 48 hours. Following 48 hours 1 µL WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) added to each well and plates were incubated for another 2 hours. Water-soluble formazan dye was produced upon bio reduction in the presence of an electron carrier, 1-Methoxy PMS. The amount of formazan produced is directly proportional to living cells. Cell viability then was determined by plate reader. According to the results PF429242 inhibited cell proliferation in LNCaP cells after 48 hours of treatment. Bars, mean±SEM of triplicate experiments (n=24 treated with vehicle). Cell proliferation was significantly decreased in concentration of 10µM. Cell proliferation inhibition compared to the Paclitaxel which is the drug of choice in treatment of prostate cancer; statistically significant difference observed between Paclitaxel 10µM and PF429242 10µM. Cell viability was reduced 37%±2.88% compared with Paclitaxel that reduced cell viability 55%±2.88% (P<0.05). The observed antiproliferative effect of Paclitaxel was statistically significant from PF429242 using the same concentration (P<0.05). Combination of two compounds reduced cell viability by 52%±3.52% (P>0.05) that was not statistically significant from each compound.
**Figure 6.7.** PF429242 inhibits cell proliferation of LNCaP prostate cancer cell in vitro. The values are represented as percentage of viable cells where the vehicle treated cells were regarded as 100%. P value <0.05 represents statistically significant between PF429242 10µM, Paclitaxel 10µM and the control group. PF429242 reduced cell viability 37%±2.88% and Paclitaxel reduced cell viability 55%±2.88% (P<0.05), both statistically significant from each other and control. Combination of both compounds reduced cell viability 52%±3.52%, not statistically significant from each compound (P>0.05)
6.8. Inhibition of SREBP 1 activation following treatment with PF429242 and Fatostatin in C4-2 cells and LNCaP cells

To investigate the pharmacological effects of PF429242, we treated both C4-2 and LNCaP cancer cells with 10µM PF429242 for 48 hours. We investigated the effects of PF429242 on SREBP1 expression. Both C4-2 and LNCaP cancer cells were seeded in 96 well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM PF429242 and incubated for 48hours. Following 48 hours of treatment 25 μg of cell homogenates were subjected to SDS-PAGE and immunoblotting for N-terminal fraction of SREBP1. Same membranes were stripped and reprobed for β-tubulin as loading marker protein. Results represent the mean±SEM of triplicate experiments. Both PF429242 and Fatostatin decreased n-SREBP1 expression in both C4-2 and LNCaP cancer cells.

A) PF429242 decreased band intensity 32%±2% (P<0.05) and Fatostatin decreased band intensity 35%±1.75% in C4-2 cells (P<0.05).

B) PF429242 decreased band intensity 70%±1.5% and Fatostatin decreased band intensity 68%±1.25% in LNCaP cells (P<0.05).
Figure 6.8. A  Treatment with 10μM PF429242 and Fatostatin 10μM decreased N terminal SREBP1 expression in C4-2 cells (P value<0.05). PF429242 decreased band intensity 32%±2% and Fatostatin decreased band intensity 35%±1.75% in C4-2 cells (P<0.05).
Figure 6.8.B. Treatment with 10µM PF429242 and Fatostatin 10µM decreased N terminal SREBP1 expression in LNCaP cells. PF429242 decreased band intensity 70%±1.5% and Fatostatin decreased band intensity 68%±1.5% in LNCaP cells (P<0.05).
6.9. Suppression of FAS expression following treatment with PF429242 in C4-2 cells

We investigated the effects of PF429242 on FAS expression. C4-2 cell were seeded in 96 well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM PF429242 and incubated for 48 hours. Following 48 hours of treatment, 25 μg of cell homogenates were subjected to SDS-PAGE and immunoblotting for FAS antibody. Same membranes were stripped and reprobed for β-tubulin as loading marker protein. Results represent the mean±SEM of triplicate experiments. PF429242 decreased FAS expression in C4-2 cells. PF429242 decreased band intensity 31.5%±1.5% in C4-2 cells (P<0.05).
**Figure 6.9.** PF429242 10µM decreased FAS expression in C4-2 cells (P <0.05). PF429242 decreased band intensity 31.5%±1.5% in C4-2 cells (P<0.05).
6.10. Suppression of FAS expression following treatment with PF429242 in LNCaP cells

We investigated the effects of PF429242 on FAS expression. LNCaP cells were seeded in 96 well plates. C4-2 cells and incubated in 37°C degree, CO2 level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with 10µM PF429242 and incubated for 48 hours. Following 48 hours of treatment, 25 g of cell homogenates were subjected to SDS-PAGE and immunoblotting for FAS antibody. Same membranes were stripped and reprobed for β-tubulin as loading marker protein. Results represent the mean±SEM of triplicate experiments. PF429242 decreased FAS expression in LNCaP cells. PF429242 decreased band intensity 31.5%±2.1% in LNCaP cells (P<0.05).
**Figure 6.**

PF429242 10μM decreased FAS expression in androgen sensitive LNCaP cells (P<0.05).

PF429242 decreased band intensity 31.5%±2.1% in LNCaP cells (P<0.05).
6.11. Caspase 3 activation following treatment with PF429242 in C4-2 cells

To understand the molecular mechanism of PF429242 for anti tumor activity, we investigated its potential effects on Caspase3 activity. C4-2 cancer cells were seeded in 96 well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with PF429242 10µM and incubated for 48 hours. Following 48 hours of treatment, 25 μg of cell homogenates were subjected to SDS-PAGE and immunoblotting for Caspase3 antibody. Same membranes were stripped and reprobed for β-tubulin as loading marker protein. Results represent the mean±SEM of triplicate experiments. PF429242 increased Caspase 3 activity in C4-2 cells. Band intensity was increased 42%±2.5% in C4-2 cancer cells (P<0.05).
Figure 6.11. PF429242 increased Caspase3 activity in C4-2 cells. Band intensity was increased 42%±2.5% in C4-2 cancer cells (P<0.05).
6.12. Caspase 3 activation following treatment with PF429242 in LNCaP cells

To understand the molecular mechanism of PF429242 for anti tumor activity, we investigated its potential effects on Caspase3 activity. LNCaP cancer cells were seeded in 96 well plates and incubated in 37 °C degree, CO₂ level 5% for 24 hours to reach 70% confluency. After 24 hours medium changed and cells were treated with PF429242 10µM and incubated for 48 hours. Following 48 hours of treatment, 25 g of cell homogenates were subjected to SDS-PAGE and immunoblotting for Caspase3 antibody. Same membranes were stripped and reprobed for β-tubulin as loading marker protein. Results represent the mean±SEM of triplicate experiments. PF429242 increased Caspase 3 activity in LNCaP cells. Band intensity was increased 35.5%±2.5% in LNCaP cancer cells.
Figure 6.12. PF429242 (10 μM) increased Caspase 3 activity in LNCaP cells. Band intensity was increased 35.5%±2.5% in LNCaP cancer cells (P<0.05).
CHAPTER 7: DISCUSSION

7.1. General Discussion

Cancer cells reprogram or take over the metabolic pathways to meet their abnormal high demands for uncontrolled proliferation and survival. Tumor cells thus need an abundant source of lipids for proliferation. Among different types of lipids phospholipids and cholesterol are the major components of cell membrane while fatty acid and triglycerides provide energy [126]. Moreover, cholesterol is a major lipid component of the plasma membrane microdomains (lipid rafts) which play a prominent role in cancer survival and metastasis[127, 128]. Lipids also are used as precursors to synthesize hormones and messengers that mediate signal transduction pathways [129, 130]. A body of evidence indicates that in malignant cells oncogenic signaling pathways regulate and alter fatty acid and cholesterol biosynthesis and tumor microenvironment, and metabolism-related transcription regulators are considered important contributors for the initiation and progression of tumors [131, 132]. Understanding the molecular mechanism for reprogramming lipogenesis and cholesterogenesis is the essential step in developing an effective therapeutic strategy against cancerous tumors including prostate cancer.

SREBPs are basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factors localized as immature forms in ER-membrane. They predominantly regulate expression of genes mostly involved in the biosynthesis of fatty acid, triglyceride, and cholesterol and cholesterol uptake by binding of their active 68 KDa fragment (nuclear form) to the SRE located in promoter region of these genes [133, 134]. SREBPs are regulated at the transcriptional and post-transcriptional levels by hormonal, nutritional and cellular signaling systems. ER cholesterol content is a regulatory feedback loop in controlling proteolytic activation of SREBP2. Dysregulation of these controlling steps has been linked to pathophysiology of variety of diseases including cancers [135-137].

SREBPs have been shown to promote growth, development, and progression of malignant tumors including prostate cancerous tumors. Accumulated research suggests that up-regulation of de novo lipid
synthesis is associated with prostate cancer malignancy [138]. Moreover, over-expression of SREBP-1 and its active form has been connected with prostate cancer development and lethal castration resistance [139]. All these evidence provide a rationale for targeting SREBPs transcriptional activity and their downstream metabolic pathways as a potential therapeutic approach for prostate cancer.

The expression of full-length and immature forms of transcription factors such as SREBPs are controlled by tightly regulated upstream mechanisms and appears to be very difficult to achieve by small molecules. However, interfering with SREBPs’ cytoplasmic maturation and nuclear translocation has been reported using small molecules and short bioactive peptide [140]. In the present study, a new SREBP blocker, PF429242, was tested for anti–prostate cancer efficacy in vitro by interfering with SREBP proteolytic maturation and nuclear translocation. We also compared PF429242 with a recently reported SREBP inhibiting small molecule fatostatin in both androgen sensitive (LNCaP) and insensitive (C4-2) prostate cancer cell lines.

According to recent studies, the small molecule PF429242 is reported to be a potent inhibitor of S1P. Hawkins et al showed the most direct evidence of S1P inhibition effect on SREBP mediation by western blotting of subcellular fraction of cells treated with PF429242. Using Chinese hamster ovary (CHO) cells, human embryonic kidney 293 cells as well as HepG2 cells, they demonstrated that PF429242 effectively inhibited activation of SREBPs and their downstream genes. Interestingly, PF420242 effectively inhibited both full-length and nuclear SREBP2 whereas exogenous sterol incubation only blocked appearance of n-SREBP2 and up-regulated expression of the immature transcription factor in cultured CHO cells. This effect of PF429242 on the abundance of both immature and mature SREBP is consistent with a previous observation by Yang et al on down-regulation of SREBP gene expression in S1P knockout mice.

According to this study, both SREBP1 and SREBP2 precursors and nuclear forms of SREBP are reduced accompanied by their reduced mRNA levels due to their regulation by their nuclear forms themselves through SREs in their promoters. The reduction of SREBP precursor levels occurred as a consequence of down-regulation of SREBP expression [141]. Hawkins et al showed 61% reduction of SREBP1 mRNA
level of HepG2 cells treated with 10μM PF429242 similar to S1P knockout mice. We made similar observations in both LNCaP and C4-2 prostate tumor cells treated with 10 μM of PF429242 and fatostatin.

Another study by Urata et al investigated the effects of S1P inhibition by PF429242 against Lassa virus (LASV). S1P plays the main role in processing of arenavirus glycoprotein (GP) precursor to generate the peripheral virion attachment protein and the fusion-active transmembrane protein GP2 that is a key factor in virus propagation and infectious progeny [142]. According to their study treatment of cultured cells with PF429242 prevented the processing of GPC from the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) and LASV in contrast to the recombinant forms of LCMV, whose GPC processing into GP1 and GP2 was mediated by furin, instead of S1P which were highly resistant to PF429242 treatment [143].

In this study we investigated the possible underlying molecular mechanism in which PF429242 suppresses proliferation of prostate cancer cell lines. After determining the IC50 value for the novel compound PF429242, the effect of compound on cell proliferation was investigated. The IC50 values of PF429242 for LNCaP and C4-2 tumor cell lines were determined as 9.6 and 9.8 μM, respectively. These values are almost doubled of the IC50 for the same molecule in HepG2 cells reported by Hawkins et al [144]. This difference can be attributed to the cell types and difference in assay end points. Although the IC50 values are similar in both androgen sensitive and insensitive prostate tumor cells, however, we were expecting more potent effects in LNCaP cell lines due to their dependency on AR and its downstream signaling pathway. The rationale for this expectation came from identification of sterol response element (SRE) at the promoter of AR gene. Our IC50 values are almost close to the IC50 values determined for fatostatin using the same tumor cell lines reported by Li et al. According to the results shown in table 7.1, 10μM concentration of PF429242 suppressed cell proliferation in both androgen sensitive and androgen insensitive cell lines (P<0.05). Results showed that PF429242 was able to suppress proliferation more in less invasive, androgen sensitive cell line. The effect of PF429242 compound compared to a similar
compound, Fatostatin, which was studied previously on prostate cancer. The anti-proliferative effect of both compounds using the same concentrations (10 µM) was not significantly different from each other (P>0.05). The combination of two compounds used to evaluate additive effect, no significant difference observed compared with anti-proliferative effect of each drug individually (P>0.05). Also, anti-proliferative effect of PF429242 compared with the rational treatment of prostate cancer, Paclitaxel; the anti-proliferative effect of paclitaxel 10 µM was significantly different with PF429242 10 µM (P>0.05), no synergistic/additive effect observed in concentrations of 10 µM when the two compound used together in treatment of prostate cancer cell lines (P>0.05). This is in contrast with a most recently published study in prostate cancer cell lines expressing null/mutant p53s. The study by Li et al. revealed that a combinational treatment with low concentrations of fatostatin (2.5 M) and another taxane agent, docetaxel (5.0 nM) resulted in synergistic effects on inhibition of cell growth and colony formation in metastatic prostate cancer cells with null and/or mutant P53s [145]. The controversy between our combinational therapy data with those from Li et al may originate from the differences in doses used in these studies. We used combination of optimal dose of these individual agents whereas suboptimal doses administered in the recently published study. In addition to difference in PCa cell types, our cell lines were neither mutated nor null for P53 protein.

In our study, inhibition of SREBPs by small molecule inhibitors, PF429242 and fatostatin triggered apoptotic pathway by activation the hub executioner “caspase-3” in prostate tumor cell lines. This is in agreement with previously published reports on fatostatin by Li et al [146, 147]. This is the first report demonstrating that PF429242 inhibits prostate tumor cell lines through caspase-3 dependent apoptotic mechanisms.
Table 7.1: Cell viability following treatment in prostate cancer cell lines

<table>
<thead>
<tr>
<th>Prostate Cancer cell line</th>
<th>LNCaP % of reduced viability after 48 hours mean±SEM n=24</th>
<th>C4-2 % of reduced viability after 48 hours mean±SEM n=24</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF429242 10µM</td>
<td>37%±2.88%</td>
<td>20%±3.21%</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Fatostatin 10 µM</td>
<td>33%±2.88%</td>
<td>26%±3.21%</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PF429242 10 µM +Fatostatin 10 µM</td>
<td>39%±2.88%</td>
<td>33%±3.21%</td>
</tr>
<tr>
<td></td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Paclitaxel 10 µM</td>
<td>55%±2.88%</td>
<td>55%±2.88%</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PF429242 10 µM +Paclitaxel 10 µM</td>
<td>52%±3.52%</td>
<td>57%±2.88%</td>
</tr>
<tr>
<td></td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>

Furthermore, the effect of PF429242 investigated on downstream SREBP1 genes. As shown in table 7.2, n-SREBP1 expression decreased in both androgen sensitive and androgen insensitive prostate cancer cell lines following treatment with PF429242 (P<0.05) as well as decreased FAS expression in both cell lines.
Based on the results, PF429242 significantly triggered caspase dependent apoptosis in both cell lines (P<0.05).

Table 7.2: Western blot analysis results

<table>
<thead>
<tr>
<th>Prostate Cancer cell line</th>
<th>LNCaP % of Band intensity mean±SEM n=3</th>
<th>C4-2 % of Band intensity mean±SEM n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hours treatment with</td>
<td>PF429242 10µM</td>
<td>PF429242 10µM</td>
</tr>
<tr>
<td>vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-SREBP1</td>
<td>70%±1.5% reduced</td>
<td>32%±2.0% reduced</td>
</tr>
<tr>
<td>FAS</td>
<td>31.5%±2.1% reduced</td>
<td>31.5±1.5% reduced</td>
</tr>
<tr>
<td>Caspase3</td>
<td>35.5%±2.5% Increased</td>
<td>42%±2.5% Increased</td>
</tr>
</tbody>
</table>

Fatty acid is an essential constituent of all biologic membrane lipids and an important substrate for energy storage and metabolism. ACL, FASN, and SCD-1 are three primary enzymes at rate-limiting steps for biosynthesis of long-chain fatty acid. Markedly increased ACL expression and activity have been reported
in cancer cells [148]. FASN is highly expressed and associated with poor prognosis in human carcinomas including prostate cancer and has been considered as a metabolic oncogene [149-151]. SCD-1 plays a critical role in the regulation of carcinogenesis, cancer cell proliferation, and programmed cell death [152].

7.2. Future directions:

In this study, we demonstrated that PF429242 inhibited expression of SREBP1 flag gene, FASN which subsequently would reduce intracellular levels of fatty acids in prostate cancer cells. The inhibitory mechanism of PF429242 is attributed to decreased SREBP-1 transcriptional activity, as SREBP-1 has been demonstrated to transcriptionally mediate expression of lipogenic genes including FASN [153]. The data suggest that PF429242 decreased the expression of cancer-associated lipogenic gene FASN and further reduced intracellular fatty acid, to inhibit prostate cancer growth in vitro. It would also be interesting to investigate potential roles of other lipogenic genes downstream of SREBP-1 including SCD1 as well as their lipid moieties as the end-products of this pathway in pathogenesis of prostate cancer. Although we did not investigate the potential effects of PF429242 on SREBP2, but based on its mechanism of action, it is plausible that PF429242 inhibits SREBP2 activation and cholesterol biogenesis as well. Cholesterol is one of the major components of lipid rafts, specialized microdomains of the plasma membrane that serve as organizing centers for the assembly of signaling molecules [154]. Therefore, rapidly proliferating cancer cells with highly activated signal transduction networks, such as prostate cancer cells, are likely to have an enhanced requirement for cholesterol. Besides, cholesterol is a metabolic precursor for biosynthesis of steroid hormones, including androgens. SREBP-2 is a master transcription regulator that up-regulates several key cholesterogenic genes, such as HMGCS1, HMGCR, and LDLR. According to previous studies HMGCR activity was increased in ex vivo LNCaP tumors during progression to a castration-resistant state in xenograft mouse models in previous studies [155, 156]. Expression of LDLR has been demonstrated to be elevated in prostate cancer cells compared with normal cells, which could be due to the lack of sterol feedback regulation controlled by SREBP-2 in
prostate cancer cells [157]. Blocking the intracellular cholesterol supply through inhibition of the LDLR pathway induced tumor cell death in vivo [158]. It is thus plausible to plan our future research goals to evaluate the effects of PF429242 on inhibition of SREBP2 and expand the study to in-vivo models.

Androgens exert their effects by interacting with AR to release heatshock proteins (HSPs) and allowing nuclear localization and DNA interaction. AR, a critical transcription, growth, and survival factor, plays a key role in regulation of androgen-responsive organs during normal development and maintenance as well as prostate cancer growth and lethal hormone refractory/castration-resistant progression [159, 160]. The AR signaling axis provides a promising targeted therapy for prostate cancer malignancy. Different strategies have been successfully attempted to target AR directly through gene transcription or translation or blocking the interaction between AR and its cofactors and their downstream functions in prostate cancer cells [161]. In addition, knocking down SREBP-1 leads to decreases in AR mRNA and protein expression and inhibited prostate cancer cell viability. Future studies will determine the efficacy of PF429242 in advanced prostate cancer as well as various cancer types.

S2P inhibitors such as Nelfinavir and 1,10-phenanthroline have been demonstrated to induce ER-stress and apoptosis in prostate cancer cells [162]. It is not known whether a combination of S1P inhibitor (PF429242) with a S2P inhibitor (Nelfinavir) would result in additive or synergistic anticancer effects.

There are several up-stream regulators of SREBP genes including LXR, RXR, and mammalian target of rapamycin complex 1 (mTORC1) which are potential targets for future research [163-165].

In summary, we provided evidence that targeting the SREBP1-dependent metabolic pathways by either fatostatin or a novel small molecule PF-429242 effectively inhibited cell proliferation and induced caspase-3 dependent apoptosis in LNCaP and C4-2 prostate cancer cell lines. In addition, we demonstrated that combinational therapy using either of these two small molecules (IC50 doses) with paclitaxel (IC50 dose) resulted in neither additive nor synergistic effects. It remains to be determined if suboptimal doses of these therapeutic agents would generate additive/synergistic effects. Collectively, our findings suggest
that PF-429242 can be a potential effective therapeutic measure against both androgen sensitive and insensitive prostate tumor cells.
CHAPTER 8: REFERENCES


Biochimie 86.11 (2004): 839-848.


