

AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE ANTINEOPLASTIC EFFECTS OF THE
SYNTHETIC RETINOID ST1926 AND ITS UNDERLYING
MOLECULAR MECHANISM ON PROSTATE CANCER

by
HOUDA HASSAN SAMMAN

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biochemistry and Molecular Genetics
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
April 2015

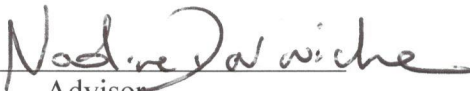
AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE ANTINEOPLASTIC EFFECTS OF THE
SYNTHETIC RETINOID ST1926 AND ITS UNDERLYING
MOLECULAR MECHANISM ON PROSTATE CANCER

by
HOUDA HASSAN SAMMAN

Approved by:

Dr. Nadine Darwiche, Professor
Department of Biochemistry and Molecular Genetics



Advisor

Dr. Wassim Abou-Kheir, Assistant Professor
Department of Anatomy, Cell Biology and Physiological
Sciences



Co-Advisor

Dr. Firas Kobaissy, Assistant Professor
Department of Biochemistry and Molecular Genetics



Member of Committee

Dr. Mazen Kurban, Associate Professor
Department of Dermatology
Department of Biochemistry and Molecular Genetics



Member of Committee

Date of thesis defense: April 27, 2015

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: SAMMAN HOUDA HASSAN
Last First Middle

Master's Thesis Master's Project Doctoral Dissertation

I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

I authorize the American University of Beirut, **three years after the date of submitting my thesis, dissertation, or project**, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

houde
samman

MAY 6, 2015

Signature

Date

ACKNOWLEDGEMENTS

Though the following thesis is an individual work, I could never have reached the heights or explored the depth without the help, guidance, support, and efforts of a lot of people.

First of all, I would like to thank my mentor and supporter Dr. Nadine Darwiche for teaching me the qualities of being a scientist. Her enthusiasm and unlimited passion have been major driving forces through my graduate career at AUB. She was always available for my questions, and she was positive and gave generously of her time and vast knowledge.

My co-advisor Dr. Wassim Abou-Kheir, I am hugely indebted to you for showing me always interest in my research, and for giving me your precious and kind advice regarding the topic of my research.

I would like also to thank my committee members, Dr. Firas Kobaissy and Dr. Mazen Kurban for being an excellent guide throughout. Your lectures have been really tempting and helpful.

A very special thank you goes to Dr. Darwiche's lab members, Leeanna, Melody, Patrick, Rana, and Zainab for their friendly attitude and support they have lent me over all these years. Dr. Wassim's lab members, thank you for your help throughout my thesis.

Thanks also go out to my parents and my brothers who keep praying for me and giving me their moral and financial support in order to finish this study.

I would like to express my special thanks to my beloved and supportive fiancé who is always by my side when times I needed him most and helped me a lot in finishing this study.

Above all, greatest appreciation to the Almighty God who has given me the health and strength to finish this work, and without his graces and blessings, I would never have been reached into this level.

Thanks a lot for everyone.

AN ABSTRACT OF THE THESIS OF

Houda Hassan Samman for Master of Science
Major: Biochemistry

Title: Investigating the Antineoplastic Effects of the Synthetic Retinoid ST1926 and its Underlying Molecular Mechanism on Prostate Cancer

Prostate cancer, the most common non-cutaneous cancer among men worldwide, remains the second leading cause of death among male cancer patients. Despite its curability with castration, radiation and hormonal therapy, some patients develop metastatic disease and die. Initially, prostate cancer cell growth depends on androgens and androgen receptor (AR) signaling, however it often becomes androgen-independent, a state called castration-resistant prostate cancer (CRPC). This later form of prostate cancer is lethal and metastasizes to secondary distant organs. The limited therapeutic options, in addition to acquired-resistance to existing and new anti-androgen and anti-AR drugs, have paved the way for a new generation of treatments for CRPC.

Retinoids are vitamin A derivatives and synthetic analogs, which regulate crucial biological processes such as cellular proliferation, apoptosis, and differentiation. Therefore, retinoids have been used successfully in the treatment and prevention of solid and liquid tumors. However, the use of natural retinoids as anti-cancer agents is restricted by their toxicity and acquired resistance. As a result, synthetic retinoids were developed with reduced toxicity and increased specificity, namely the atypical adamantyl retinoid ST1926 which has increased bioavailability and reduced toxicity.

We were interested in testing the anti-tumor properties and mechanism of action of ST1926 in prostate cancer using well-characterized human prostate cancer lines, namely DU145 and PC3. We have shown, using 2D conventional models, a significant reduction in cell viability and inhibition of proliferation in both cell lines upon treatment with pharmacologically achievable micromolar (μM) concentrations of ST1926. ST1926-induced cell death was through apoptosis, as it was evident by the accumulation of treated cells in the pre- G_1 region of the cell cycle, TUNEL positivity, and PARP cleavage. Moreover, 1 μM ST1926 induced a major upregulation in the expression levels of the sensitive marker for DNA double-strand breaks, γH2AX , in both cell lines, clearly indicating that ST1926 is a potent inducer of early DNA damage. Importantly, 1 μM ST1926 treatment was able to inhibit cell migration and invasion which are crucial steps in the metastatic process. Many studies have suggested that resistance to chemotherapeutics and cancer relapse is due to the presence of cancer stem cells (CSCs). Therefore, we tested the ability of ST1926 in targeting the CSC population in prostate cancer cells using the 3D sphere-formation assay. Interestingly, our results showed a significant reduction in sphere formation ability at 0.1 μM of ST1926 which was sufficient to eradicate the self-renewal ability of highly resistant CSCs.

In conclusion, our studies indicate a potential therapeutic use of ST1926 in prostate cancer and the need to test this synthetic retinoid in pre-clinical prostate cancer models.

CONTENTS

	Page
ACKNOWLEDGEMENTS	v
ABSTRACT	vi
LIST OF ILLUSTRATIONS	x
LIST OF ABBREVIATIONS	xii
Chapter	
I. INTRODUCTION	
A. Cancer Overview	1
B. The Prostate Gland	2
C. Prostate Cancer and its Significance	6
D. Treatments for Prostate Cancer Patients	8
E. Retinoids	9
1. Overview	9
2. Retinoids Mechanism of Action	10
3. Natural <i>versus</i> Synthetic Retinoids	11
4. Synthetic Retinoids	11
F. ST1926: A Potent Analogue of CD437 with Promising Anti-tumor Activities	12
1. Overview	12
2. ST1926 Mechanism of Action	14
3. Retinoids in Prostate Cancer Treatment	14

G. Aim of the Study.....	15
--------------------------	----

II. MATERIALS AND METHODS

A. Cell Culture.....	17
1. Human Prostate Cancer DU145 Cell Line.....	17
2. Human Prostate Cancer PC3 Cell Line.....	18
3. Cell Growth and Treatment.....	18
4. Preparation of ST1926.....	19
B. Cell Growth Assay/MTT.....	19
C. Cell Viability / Trypan Blue Exclusion Method.....	20
D. Microscopic Imaging.....	21
E. Cell Cycle Analysis by Flow Cytometry.....	21
F. TUNEL Assay.....	21
G. Trans-well Invasion Assay.....	22
H. Wound Healing Assay.....	23
I. Western Blotting.....	23
J. Sphere Formation Assay.....	24
K. Statistical Analysis.....	25

III. RESULTS

A. Effects of ST1926 <i>versus</i> ATRA at Various Concentrations on Prostate Cancer Cell Proliferation <i>in vitro</i> using MTT Assay.....	26
B. ST1926 Reduces Prostate Cancer Cell Viability.....	28
C. ST1926 Induces an Accumulation of Prostate Cancer Cells in pre-G ₁ and G ₀ /G ₁ , and Induces an S Phase Arrest.....	29
D. ST1926 Induces Apoptosis in Prostate Cancer Cells.....	30

E. Modulation of Caspase Activity by ST1926 in Prostate Cancer Cells	34
F. ST1926 Exerts its Anti-tumor Activities Through p53-Independent Pathway.....	35
G. ST1926 Promotes Early DNA Damage	36
H. ST1926 Inhibits Prostate Cancer Cell Invasion	37
I. ST1926 Reduces the Migration Ability of Prostate Cancer Cells.....	39
J. The Effect of ST1926 in Targeting an Enriched Population of Prostate Cancer Stem/Progenitor Cells using 3D Spheres Formation Assay.....	42
 IV. DISCUSSION	 46
 REFERENCES	 51

ILLUSTRATIONS

Figure	Page
1. Normal prostate anatomy.....	3
2. Cellular components of the human prostate gland.....	3
3. Zonal anatomy of the prostate: the three glandular zones of the prostate and the anterior fibromuscular stroma.....	5
4. The basic chemical structure of the retinoid family.....	9
5. Chemical structures of CD437 and ST1926.....	13
6. The effect of various concentrations of ATRA and ST1926 on the proliferative activity of prostate cancer cells using MTT assay.....	27
7. The effect of various concentrations of ST1926 on the viability of prostate cancer cells using trypan blue exclusion method.....	28
8. ST1926 inhibits cell proliferation of prostate cancer cells as reflected by confluency changes.....	29
9. ST1926 treatment induces an accumulation of prostate cancer cells in the pre-G ₁ region and G ₀ /G ₁ , and induces an S phase arrest.....	31
10. ST1926 induces apoptosis in DU145 cells.....	32
11. ST1926 induces apoptosis in PC3 cells.....	33
12. ST1926 treatment of prostate cancer cells induces PARP cleavage.....	34
13. ST1926 induces p53-independent apoptosis in DU145 (mutated <i>p53</i>) and PC3 (<i>P53</i> -null) cells.....	35
14. ST1926 is a potent inducer of early DNA damage.....	36
15. ST1926 reduces the invasive potential of human prostate cancer cells.....	38
16. ST1926 reduces the migration potential of DU145 cells.....	40

17. ST1926 reduces the migration potential of PC3 cells.....	41
18. The effect of ST1926 on sphere-forming ability of DU145 cells.....	43
19. ST1926 treatment inhibits the self-renewal capacity of prostate cancer stem/progenitor cells enriched from DU145 cells.....	44
20. Representative images of the data shown in Figure 19.....	45

ABBREVIATIONS

AR	androgen receptor
ATRA	all- <i>trans</i> retinoic acid
9c-RA	9- <i>cis</i> retinoic acid
CD437	6-[3-(1-adamantyl)-4-hydroxyphenyl] -2-naphthalene carboxylic acid
CRPC	castration-resistant prostate cancer
CSC	cancer stem cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSB	double-strand break
EDTA	ethylene-diamineteraacetic acid
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hour
H&E	Hematoxylin and Eosin
HPR	N-(4-hydroxyphenyl) retinamide
JNK	Jun N-terminal kinase
K	cytokeratin
μM	micromolar
MAP	mitogen-activated protein
mn	minute

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PI	propidium iodide
PSA	prostate specific antigen
RAR	retinoic acid receptor
RARE	retinoic acid response element
RXR	retinoid X receptor
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFU	sphere-formation unit
ST1926	E-4-(4'-hydroxy-3'-adamantyl biphenyl-4-yl) acrylic acid
TUNEL	dUTP nick end labeling

CHAPTER I

INTRODUCTION

A. Cancer Overview

Cancer is one of the leading causes of morbidity and mortality worldwide. According to the National Cancer Institute in the United States, it is estimated that by the year 2050, the global burden of cancer will increase from an estimate of 1.3 million in 2000 to almost 3 million new cases, mostly because of the aging of the population (Hayat 2007). Despite the great improvement in understanding the molecular basis of cancer and the advancements in cancer detection and treatment, mortality is still high and there is no major cure.

Cancer refers to a large group of genetic diseases that infiltrate and destroy any normal body tissue, and share a common phenotype: abnormal cell growth and proliferation (Balmain 2001). During the multistep development of human tumor, six hallmarks of cancer are acquired including sustaining proliferative signaling, evading growth suppressors, resisting cell death, limitless replicative potential, sustained angiogenesis, and activating tissue invasion and metastasis. In addition, an increasing body of research has added two additional hallmarks of cancer; one that involves the capability to modify cellular metabolism in order to support neoplastic proliferation, and the second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells (Hanahan 2011). Normally, growth of human cells is strictly controlled by a gene network that regulates cell proliferation, cell death and differentiation, each of which has to be overcome in turn (Hanahan 2011). When cancer develops, alterations in these regulatory

genes occur over time, usually one after the other (McCormick 1999). The first mutation that occurs will provide a selective growth advantage to the cell. The developing tumor then acquires mutations in oncogenes, tumor suppressors or stability genes (for example, DNA repair genes), which eventually lead to a continuous cellular division and creation of a mass of cells that forms the primary tumor (Pozo 2007). These cancer causing genetic mutations could be inherited, or can also arise during a person's lifetime. Aside from genetic mutations, disruption of epigenetic processes results in deregulated gene expression and leads to cancer progression. Epigenetic deregulations include DNA methylation, histone modifications, chromatin structure, and non-coding RNAs (Schneider-Stock 2012). Furthermore, other cancers can be caused by external agents such as radiation, chemical damage or viral infection or result from random errors that occur during DNA replication (Danaei 2005).

B. The Prostate Gland

The human prostate gland is part of the male reproductive system which makes the fluid component of the semen. It is located underneath the bladder, surrounding part of the urethra, and has the size of the walnut (Figure 1). The indicative unit of the prostate is composed of epithelial and stromal components (Figure 2), in addition to the neuroendocrine cells, non-epithelial fixed macrophages, and intra-acinar lymphocytes (Chatterjee 2003). The epithelium consists mainly of secretory columnar epithelial and basal epithelial cells. Secretory columnar cells are arranged into a single cell layer, lining the epithelium. They synthesize prostatic proteins such as prostate specific antigens (PSA) and prostate specific phosphatase and secrete them into the ductal lumen (Barron 2012).

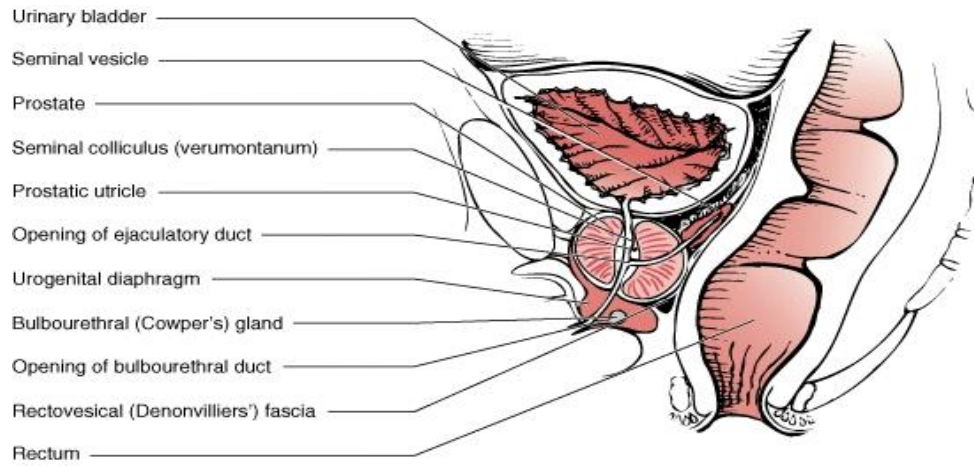


Figure 1. Normal prostate anatomy. Adopted from (Hong 2010)

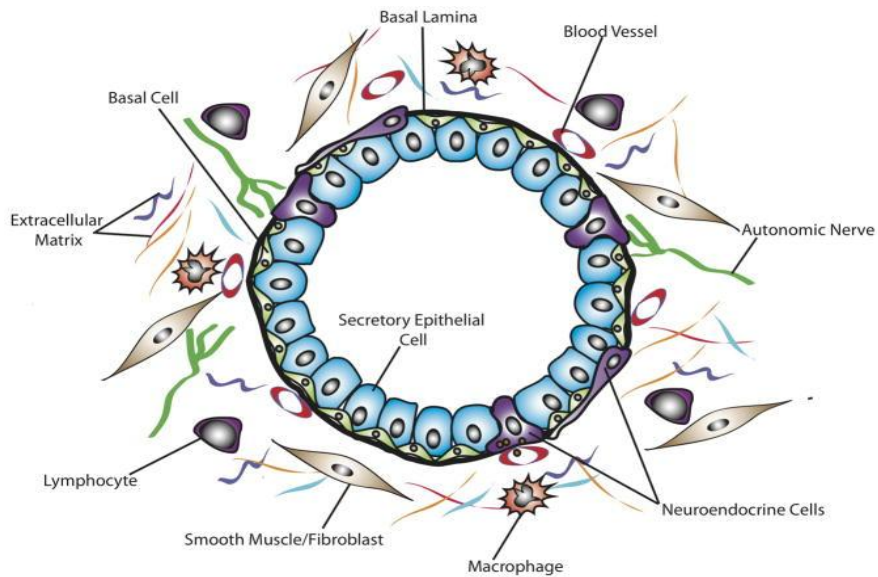


Figure 2. Cellular components of the human prostate gland. Adopted from (Barron 2012).

The secretory epithelial cells are characterized by the expression of androgen receptors (ARs), cytokeratins (K) 8 and K18, CD57, and PSA, and they require continuous direct androgenic stimulation to maintain structural and functional viability (Barron 2012). When the androgen levels get below a threshold, in the case of surgical or chemical castration, the secretory cells will undergo apoptosis, causing glandular involution. The basal epithelial cells express K5, K14, and CD44. Moreover, basal cells are thought to represent the stem cells of the prostate gland, these cells remain after castration since most of them do not possess ARs (Barron 2012). Although the survival of the basal cells does not depend on androgen, but they require, under normal physiological conditions, androgens for proliferation and differentiation into secretory cells. Under normal physiological conditions, these basal/stem cells are stimulated by androgens to undergo normal proliferation and differentiation, however, with accumulated mutations, these cells are removed by apoptosis and a steady state balance is maintained between cell proliferation and apoptosis (Scott D 2013).

McNeal and his colleagues have studied the normal and pathological anatomy of the prostate and defined a concept of anatomical zones, rather than lobes. There are four distinct anatomical zones in the prostate gland, all of which are derived from different embryonic origins (McNeal 1981) (Figure 3). The peripheral zone is mesodermal in origin and contains the majority (70%) of the glandular tissue in a normal prostate. Approximately 70–80% of prostatic cancers originate from this zone and it is the most zone susceptible to inflammation. The central zone surrounds the ejaculatory ducts and contains about 25% of the glandular tissue of the prostate gland. It accounts for roughly 2.5% of prostate cancers although these cancers tend to be more aggressive and more likely to invade the seminal vesicles. The

transition zone which is responsible for the disease of benign prostatic hyperplasia, is endodermal in origin and is responsible for 5% of the prostate volume. Lastly, the anterior fibromuscular stroma or the periurethral zone, which accounts for less than 5% of the prostate tissue and it may undergo pathological growth that causes increased urethral compression and further retention of urine in the bladder (McNeal 1981).

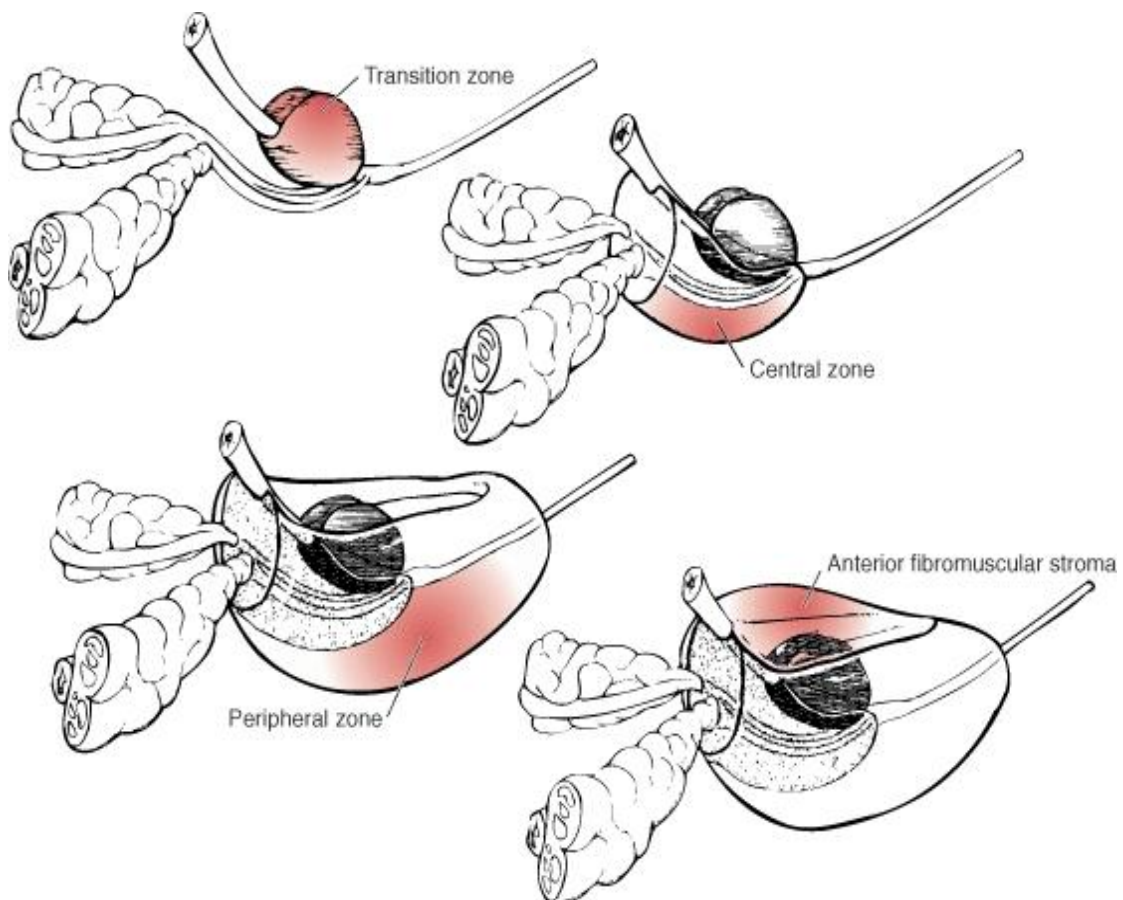


Figure 3. Zonal anatomy of the prostate: the three glandular zones of the prostate and the anterior fibromuscular stroma. Adopted from (Hong 2010).

C. Prostate Cancer and its Significance

Prostate cancer is the most leading cause of mortality and morbidity among men worldwide (Siegel 2015). However, according to the World Health Organization, mortality from prostate cancer is relatively low in Middle Eastern-Asian countries. This difference may be related with the consumption of local-grown plants and herbal products. Prostate cancer is a very heterogeneous disease with a long natural history in most of the cases. Most prostate cancer tumors are relatively slow-growing of minor clinical significance, while about 10% develop to aggressive disease in a short time with poor prognosis (Roudier 2003). Although this finding suggests that prostate cancer follows an indolent course, however, in 2015, about 220,800 American men will be diagnosed with prostate cancer, and 27,540 men will die from this disease (Siegel 2015). Remarkably, prostate cancer has a high incidence/mortality ratio of approximately 8, making it distinct from any other major cancer; this ratio is approximately 5.7 in breast cancer, 1.9 in colon cancer, 1.4 in lung cancer and 1.2 in pancreatic cancer (Siegel 2015).

The course of prostate cancer progression is best categorized as a series based on the extent of the disease, from androgen-dependent prostate cancer to castration-resistant prostate cancer (CRPC). A study of Huggins and Hodges presented the close relationship of androgens with prostate cancer growth, and accordingly, androgen-deprivation therapy (castration treatment) became the aim of treatment (Huggins 2002). The goal of this treatment is to interfere with the androgen signaling that is critical to the growth of androgen-dependent prostate cancer cells. This can be achieved by several approaches including the use of agonists to suppress the pituitary gland's need for testosterone, or the use of

antagonists to stop the production of testosterone in the testes and in the adrenal gland. Alternatively, anti-androgens can be used to inhibit the action of testosterone that has already been secreted into the androgen-dependent prostate cancer cells. Initial responses to castration treatment were relatively favorable with a decrease in the level of the serum marker PSA up to 80%. However, after one to three years, cancer cells become refractory and continue to grow despite the hormonal treatment. In this case, the prostate cancer will progress into a castration-insensitive phase, known as CRPC (Harris 2009). Resistance mechanism can be divided into six groups: increased expression of enzymes involved in steroidogenesis, increased expression of ARs, AR gene mutations and altered ligand specificity, downstream signaling receptor for androgens, bypass pathways, and the presence of stem cells that can maintain tumor growth by surviving under androgen-deprived conditions (Amaral 2012). The median survival for patients with localized prostate cancer is more than 5 years, compared to 1 to 3 years for patients with CRPC. This later form of prostate cancer is lethal and progresses and metastasizes to secondary distant organs most commonly to the bones, which occurs in as many as 90% of the patients with advanced prostate cancer (Carlin 2000). Prostate cancer can further metastasize to lymph nodes, liver, and lungs. Emerging evidence indicates that somatic tumors, including prostate cancer, contain a small subset of stem-like cells, called cancer stem cells (CSCs), with capacities for self-renewal, differentiation and initiation of new tumors (Zuo 2015). Identification of these prostate CSCs has provided a new insight into prostate carcinogenesis. Currently available therapeutic treatment like chemotherapy, radiotherapy, and hormonal treatment cannot effectively kill the highly resistant CSCs population, which subsequently lead to uncontrolled

growth, relapse and then metastasis of prostate cancer (Wu 2015). Therefore, it is crucial and urgent to develop a direct treatment targeting prostate cancer cells including CSCs.

D. Treatments for Prostate Cancer Patients

Patients with prostate cancer have different treatment options such as chemotherapy, radiotherapy, secondary hormonal therapies, vaccine-based immune therapy, and novel targeted therapies. High dose (150 mg daily) of bicalutamide (Casodex), an oral non-steroidal anti-androgen, results in more than 50% PSA reduction in 20% to 45% of patients (Suzuki 2008, Kassouf 2003). A synthetic estrogen, diethylstilboestrol, and other estrogens, suppress the hypothalamic-pituitary gonadal axis and reduce more than 50% the total PSA in 26% to 66% of patients with CRPC (Kim 2011). As for chemotherapy, docetaxel is the only drug approved that has been shown to prolong survival among men with CRPC. Besides, cabazitaxel is used in case of docetaxel-refractory CRPC (Sartor 2011). Additionally abiraterone and enzalutamide are indicated for treatment of metastatic CRPC. Abiraterone is indicated for use in combination with prednisone, and it was approved by the Food and Drug Administration in the United States in April 2011 after an expedited six-month review (Monneret 2013). Enzalutamide (marketed as Xtandi and formerly known as MDV3100) is an androgen receptor antagonist drug, and has reported up to 90% PSA reduction in 25% of treated patients compared to 1% in the placebo arm ($P < 0.001$) (El-Amm 2013, Quintela 2015). Focal external beam radiation therapy is also considered as a possible treatment for men with CRPC (Boyer 2014). However, the survival benefit of these conventional drugs in CRPC is still uncertain and they fail to achieve the satisfactory effect. Moreover, cancer cells

may acquire hormone and drug resistance characteristics under these treatments. Therefore, developing novel therapeutics and examining new alternative approaches is needed in treating and managing prostate cancer.

E. Retinoids

1. Overview

Retinoids are natural vitamin A metabolites or synthetic analogs with vitamin A activities. Natural retinoids consist of four joined isoprenoid units and a cyclohexenyl ring (Figure 4) and they originate from the diet either as provitamin A carotenoids (β -carotene) or as preformed vitamin A (retinol). In the body, retinols are converted into all-*trans* retinoic acid (ATRA) and its isoforms (Alizadeh 2014). Retinoids are involved in many crucial biological processes such as embryonic development and organogenesis, as well as later in adult life in crucial processes such as vision, reproduction, cell growth arrest, apoptosis, differentiation, and immune response (Alizadeh 2014). Moreover, retinoids have been investigated widely for the prevention and treatment of many cancer types, including prostate cancer. Several lines of evidence indicated that retinoids inhibited prostate cancer growth and suppressed the development of prostate carcinogenesis (Pasquali 2006).

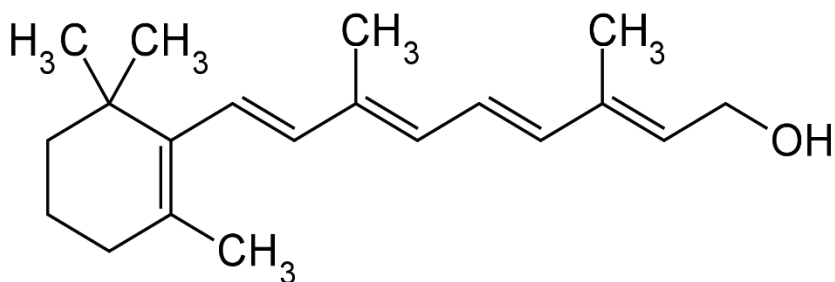


Figure 4. The basic chemical structure of the retinoid family.

2. Retinoids Mechanism of Action

ATRA functions through binding to two distinct classes of receptors which belong to the steroid/thyroid hormone nuclear receptors family: retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each of which have three different isoforms – α , β and γ . Differential splicing and promoter usage produce the different retinoid receptor isoforms (Mary Ann Asson 2014). RARs are activated by both ATRA and 9-*cis* retinoic acid (9*c*-RA), while RXRs are activated only by 9*c*-RA. Once they bind with their ligands, RARs and RXRs form heterodimers and function as ligand-dependent transcription factors to trigger their downstream effectors by binding to DNA specific elements called retinoic acid response elements (RAREs), located in the promoter region of their target genes (Mary Ann Asson 2014). This model of binding of RARs or RXRs to RAREs is called the retinoic acid classical pathway which upon activation triggers cellular differentiation, cell cycle arrest, and subsequently apoptosis (Tang 2011). Alternatively, RXRs can form heterodimers with other receptors such as estrogen receptors α , AP-1 receptor, peroxisome proliferator-activated receptors, and many others. When RXRs heterodimerize with these later receptors, they regulate their partner receptor's pathway, and this is referred as the nonclassical pathway (Tang 2011).

Interestingly, RAR β is well known for its tumor-suppressive effects in epithelial cells and its expression causes RA-dependent and –independent growth arrest and apoptosis (Alvarez 2007). However, RAR β expression is lost by mutational events or is epigenetically silenced early in carcinogenesis in many solid cancers, enhancing the development of novel treatment strategies in ATRA- resistant cancer cells (Connolly 2013).

3. *Natural Versus Synthetic Retinoids*

Natural retinoids derivatives include β -carotene, 9-cRA, retinol, retinal, and the most abundant metabolite ATRA (Theodosiou 2010). ATRA causes cell cycle arrest at the G₁ phase, inhibits cellular proliferation, and induces cell death (Siddikuzzaman 2011).

However, ATRA has several limitations since the duration of complete remission induced in treated patients is brief (3-6 months) and relapse is often associated with acquired resistance to ATRA-mediated differentiation. Drug resistance may be due to attenuation or mutations in retinoid receptor signaling pathway. Moreover, retinoids administration is associated with severe toxic effects including teratogenicity, an increase in serum triglycerides, mucocutaneous cytotoxicity, headache, and bone toxicity (Theodosiou 2010). To overcome these limitations, pharmaceutical companies have developed a series of synthetic retinoids with similar biological activities, but different structures and chemical properties. Synthetic retinoids are more specific and less toxic than natural retinoids. The promising synthetic retinoid ST1926, is active by oral administration on both solid tumors and hematological malignancies (Cincinelli 2003), and is in Phase I clinical trials (Sala 2009).

4. *Synthetic Retinoids*

Synthetic or atypical retinoids have shown promise in cancer treatment, namely in prostate cancer (Pasquali 2006). Although the exact mechanism of action of the synthetic retinoids is not fully understood, their anti-proliferative and apoptotic effects are attributed to their ability to induce both receptor-dependent and -independent mechanism of action. The most promising synthetic retinoids with anti-neoplastic activities are N-(4-hydroxyphenyl)

retinamide (4HPR) (Bernard 1992), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) (Fontana 2002), and ST1926; an analogue of CD437.

HPR, also called fenretinide has been shown to have anti-proliferative effect through both receptor-dependent and -independent pathways (Delia 1993). Since then, HPR is one of the promising clinically tested synthetic retinoids. Replacing the carboxyl end of ATRA with an N-4-hydroxyphenyl group in HPR has increased its efficacy as a chemopreventive agent as well as reduced its toxicity. HPR induces its apoptotic effects by generating reactive oxygen species and lipid second messengers (Bushue 2010). Moreover, it induces cell death and a major loss of mitochondrial membrane potential in several ATRA-resistant cancer cell lines including breast carcinoma, prostate adenocarcinoma, cervical carcinoma, small-cell lung cancer and leukemias (Fontana 2002).

CD437 also exerts its effect through retinoid receptor-dependent and -independent pathways (Parrella 2006). It binds selectively to RAR β and RAR γ , and minimally to RAR α (Bernard 1992). CD437-induced apoptosis activates Jun N-terminal kinase (JNK), which is required for maximal induction of apoptosis and membrane depolarization (Bushue 2010). Furthermore, CD437 induces cell cycle arrest at different stages depending on the cancer cell type (Kabbout 2004, Zhang 2000).

F. ST1926: A Potent Analogue of CD437 with Promising Anti-tumor Activities

1. Overview

ST1926 or E-4-(4'-hydroxy-3'-adamantyl biphenyl-4-yl) acrylic acid is synthesized in a three-step sequence through using CD437 as a reference compound, and replacing the naphthalene ring in CD437 by a styrene moiety in ST1926 (Figure 5) (Cincinelli 2003). HPR

and CD437 have a narrow window between therapeutic and toxic doses and unfavorable pharmacokinetic profiles (Garattini 2004), for these reasons, the synthetic retinoid, ST1926, was developed with increased specificity and bioavailability. ST1926 with its anti-tumor role, has a broad spectrum of activities in a large panel of cancer cell lines (Cincinelli 2003). As mentioned earlier, ST1926 is effective in cancer cell lines that are resistant to ATRA, and was tested in Phase I clinical trials in patients with ovarian cancer (Sala 2009). Moreover, ST1926 is effective orally at pharmacologically achievable micromolar (μM) concentrations (Fratelli 2013); while sparing normal cells (El Hajj 2014).

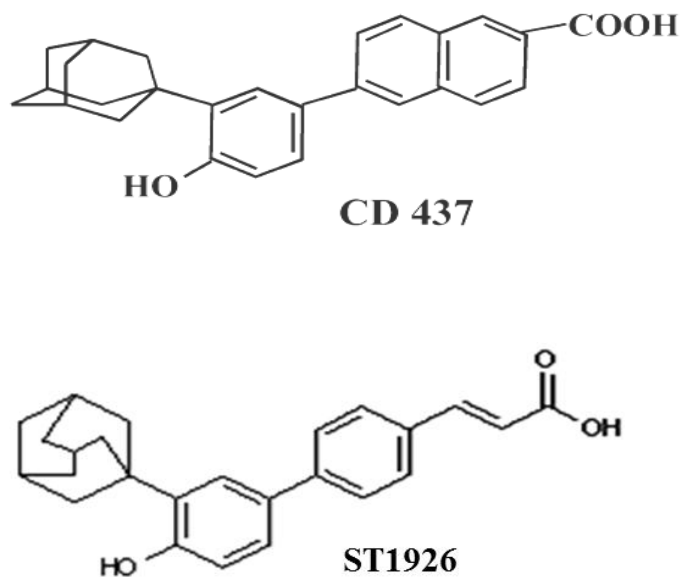


Figure 5. Chemical structures of CD437 and ST1926.

2. *ST1926 Mechanism of Action*

Earlier studies have shown that ST1926 binds to RAR γ receptors with low affinity, but other studies have demonstrated that it functions independently of RAR γ (Parrella 2006). ST1926 is a promising drug which appears more apoptotic than CD437 in several cancer cell lines (Parrella 2006, El Hajj 2014, Nasr 2014). ST1926 causes cell cycle arrest and apoptosis in ATRA-resistant cancer cells through retinoid receptor-dependent or -independent pathway (Valli 2008). ST1926 mediates its apoptotic effects through inducing massive genotoxic stress and DNA damage (Valli 2008). Moreover, it modulates intracellular calcium homeostasis by increasing the cytosolic level of calcium that is directly related to apoptosis (Garattini 2004). Additionally, ST1926 has multiple mechanism of actions including activation of the mitogen-activated protein (MAP) kinases p38 and JNK, release of cytochrome c in the cytosol and subsequent activation of the caspase proteolytic cascade (Di Francesco 2007). Moreover, ST192 induces its anti-proliferative effects through p53-independent mechanisms (Cincinelli 2003). It induces phase-specific DNA double-strand breaks (DSBs) and cell death through mitochondrial pathways of apoptosis, and causes G₁/S and G₂/M cell cycle arrest prior to apoptosis induction (Pisano 2004, Parrella 2006, Valli 2008).

3. *Retinoids in Prostate Cancer Treatment*

Natural retinoids, such as ATRA, are shown to be beneficial treatment of patients with early stage organ-confined low grade prostate cancer (Huss 2004). ATRA was able to slow proliferation of the prostate tumor cells, induce apoptosis, and block the emergence of the neuroendocrine phenotype (Huss 2004). Furthermore, ATRA is used as an

immunomodulating agent to arrest the activity of prostate cancer tumor-associated macrophages. It inhibits the cancer cell-stimulated proliferation of the pro-tumoral macrophages, and restores their cytotoxicity capacity towards prostate cancer cells (Tsagozis 2014). Many studies have shown that retinoids inhibited effectively the growth of prostate cancer cells *in vitro*, and suppressed the development of prostate carcinogenesis (Pasquali 2006). Other clinical trials have demonstrated that retinoids showed better activities when combined with other anti-cancer agents such as interferon- γ and paclitaxel. Moreover, CD437 was found to inhibit growth and induce apoptosis of both androgen-dependent and -independent human prostate cancer cells (Pasquali 2006). Keedwell and his colleagues have shown that five days exposure to the high-affinity pan-RAR antagonist, AGN194310, inhibited the growth of several prostate cancer cell lines, with half-maximal inhibition 250 nM (Keedwell 2004). The growth of prostate cancer cells was also repressed more than that of normal cells when RAR β with RAR γ , but not RAR α alone, were antagonized (Keedwell 2004).

However, there is no research about the effect of the synthetic retinoid ST1926 in specifically prostate cancer, thus the aim of this study is to detect the anti-tumor impact of ST1926 in 2D and 3D prostate cancer models.

G. Aim of the Study

Previous work in our laboratory has demonstrated the anti-cancer effects of ST1926 on several cancer cell lines, including adult T-cell leukemia, chronic myeloid leukemia and colon cancer cells (El Hajj 2014, Nasr 2014, and unpublished data). Using 2D and 3D *in vitro* models of prostate cancer, we aim in this study to investigate the effects of ST1926

on the proliferation, viability, cell cycle progression, cell death mechanism, invasion and migration ability using human prostate cancer cell lines that are grown in a conventional 2D monolayer model. Furthermore, this study will focus on an advanced 3D assay (sphere-formation assay) to investigate the effect of the synthetic retinoid ST1926 in targeting an enriched population of prostate cancer stem/progenitor cells. To date, there is no drug combination that showed enhanced clinical outcomes in patients with prostate cancer and especially with advanced stages. This work will hopefully support a potential therapeutic role of ST1926 in prostate cancer, alone or in combination treatment.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

1. *Human Prostate Cancer DU145 Cell Line*

DU145 is one of the “classical” human prostate cancer cell lines that was derived from a tumor mass excised from the brain of a 69-year-old man with prostate cancer (Sobel 2005). DU145 cells are epithelial and they are the first cell line to be established in tissue culture with a population doubling time around 34 hours (Stone 1978). They demonstrate characteristics of expressing low levels of prostatic acid phosphatase and very little or no expression of ARs, PSA, and human glandular kallikrein 1. Karyotypic analysis reveals an aneuploid human karyotype with a range of 55 to 63 chromosomes per cell, with many chromosomal aberrations (18 aberrations per diploid cell), such as complex chromosomal rearrangements and a high degree of karyotypic instability. Because this range of chromosome number is less than three times the haploid chromosome count, DU145 is most probably derived from a hypotriploid stem line (Beheshti 2001, Sobel 2005). Three distinctive marker chromosomes are identified: a translocation Y chromosome, metacentric minute chromosomes, and three large acrocentric chromosomes (Stone 1978). However, most early-stage prostate cancer tumors show a normal diploid karyotype with the most common chromosomal alterations affecting chromosome 8 (Beheshti 2001).

2. Human Prostate Cancer PC3 Cell Line

PC3 cells were the first reported human adenocarcinoma cell line in literature in 1979, and were derived from a 62-year-old man from his lumbar vertebral metastasis (Kaighn 1979). PC3 are epithelial cells, and androgen-independent representing the CRPCs. Moreover, PC3 cells show highly aggressive behavior with a doubling time of 33 hours (Atala 2012). PC3 cells are 100% aneuploidal; hypotriploid with more karyotypic abnormalities than DU145 cells with approximately 34 aberrations per diploid cell. Every chromosome in this cell line has either structural or numerical abnormalities with chromosomal loss being more prevalent than chromosomal gain (Beheshti 2000).

3. Cell Growth and Treatment

DU145 and PC3 cells were cultured in RPMI-1640 AQ medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), 1% of penicillin-streptomycin (Sigma, USA), 1% non-essential amino acids (Sigma, USA) and incubated at 37°C in a humidified incubator (95% air, 5% CO₂) to grow. Typically, the media over the cells was replenished every 2-3 days. When cells reached 70 to 80% confluency, they were washed twice with calcium free- phosphate buffered saline (PBS) then were trypsinized with trypsin- ethylene-diamineteraacetic acid (EDTA) for two minutes at 37°C (Sigma, USA). Trypsin effect is then inhibited by a ratio of 1:1 complete media. Cells were then centrifuged for 5 minute at 900 rpm to obtain a pellet, which was resuspended and transferred into new 75 cm² tissue culture flasks for maintenance and expanded before an experiment. For starting any experiment, cell number was calculated using a hemocytometer according to the following formula: cells/ml = average number of cells x dilution factor x volume of

suspension $\times 10^4$. Cells were counted using trypan blue dye exclusion using 0.4% trypan blue solution.

4. Preparation of ST1926

ST1926 was obtained from Biogen Institute (Ariano Irpino, Italy) and was reconstituted in 0.1% dimethylsulfoxide (DMSO) at a concentration of 1×10^{-2} M, aliquoted, stored at -80°C , and used up to six months. For use in experimentation, an aliquot of stock ST1926 (1×10^{-2} M) was either applied directly to cells, or serially diluted in 0.1% DMSO.

B. Cell Growth Assay/MTT

Anti-proliferative effect of ST1926 was measured *in vitro* by using the MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay. DU145 and PC3 cells were plated in 100 μl complete medium in 96-well culture plates at the density of 5000 cells per well. Cells were incubated overnight in the incubator then treated in triplicates with 0.1% DMSO or various ST1926 concentrations diluted in 100 μl complete media for 24, 48 and 72 hours. ST1926 concentrations used ranged from 0.01-3 μM . For each time point, cells were washed with PBS, fresh serum-free medium was added, and 10 μl of 5 mg/ml (in 1x PBS) MTT reagent was added to each well and incubated at 37°C for 4 hours. In this step, metabolically active/viable cells had the ability to convert the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals due to the high levels of NADH and NADPH, which is a measure of mitochondrial metabolic activity. One hundred μl of solubilization solution was then added into each well to dissolve the formazan crystals and stop the reaction.

Finally, after overnight incubation, the reduced MTT optical density (OD) was measured at a wavelength of 595 nm using an ELISA reader (Multiskan Ex). The percentage cell viability was expressed as percentage growth relative to DMSO (control wells) and treated wells at indicated concentrations, and are derived from the mean of triplicates wells, and represented as mean \pm SEM.

C. Cell Viability/Trypan Blue Exclusion Method

A simple method of measuring cell viability in cell culture is to count viable cells using the trypan blue dye exclusion method. The technique is based on the disruption of the cell membrane that distinguishes non-viable from viable cells. The disrupted membranes of non-viable cells allow the trypan blue dye to be taken up by the cell, and thus viable cells can be visualized under microscope as white cells compared to the blue-colored non-viable cells. Cells were seeded in triplicate in 6-well plates at a density of 40,000 cells/500 μ l per well and were grown in RPMI media in the absence or presence of ST1926. Supernatants containing the dead cells were collected and attached live cells were harvested by trypsin/ EDTA and added to the supernatant. Cell pellet was resuspended in 100 μ l media then 50 μ l of cell suspension was mixed with 50 μ l of trypan blue (1.4 mM trypan blue, 154 mM NaCl, 500 mM EDTA, pH=8). Cells were counted on the four corner chambers of a hemocytometer by the previously mentioned formula.

D. Microscopic Imaging

DU145 and PC3 cells were treated with 1 μ M ST1926 for 48 hours and representative bright field images were acquired using Zeiss axiovert light microscope. Control cells were treated with 0.1% DMSO.

E. Cell Cycle Analysis by Flow Cytometry

DU145 and PC3 cells were seeded into petri dishes at 600,000cells/well which were incubated overnight prior to drug treatment for 24 and 48 hours. Cells were then harvested, washed twice with PBS, centrifuged at 1500 rpm for 5 minute at 4°C, resuspended in 1 ml of cold PBS, fixed in 4 ml of cold absolute ethanol and then stored at -20°C until staining and analysis. Fixed cells were then treated for 1 hour with 200 μ g/ml DNase-free RNase A, stained with 1 mg/ml propidium iodide (PI) (Sigma, USA) and incubated for 10 minutes in the dark in a flow tube (BD Flacon). Fluorescence of PI, a measure of DNA content in a cell population, was done using flow cytometry (FACScan, Becton Dickinson). A total of 10,000 gated events were acquired in order to assess the proportions of cells of different stages of the cell cycle.

F. TUNEL Assay

The TUNEL assay is a measure of apoptosis through detection of DNA DSBs (a late apoptotic event). DNA strand breaks contain free 3'-OH termini which may be conjugated to dUTP-fluorescein through the enzymatic action of terminal deoxynucleotidyl transferase and fluorescein fluorescence can be detected by flow cytometry. In brief, cells were seeded

(600,000 cells/well) and treated with 0.1% DMSO for control or 1 μ M ST1926. Two extra control wells, one for positive and one for negative controls, were prepared to be used in the experiment. At the indicated time point, cells were washed with 1% BSA in 1X PBS, and then fixed with 4% formaldehyde for 30 minutes at room temperature. Subsequently, cells were then washed with 1X PBS and incubated with 100 μ l of permeabilization solution (0.1% triton X-100 in 0.1% sodium- citrate) on ice for 2 min. Only the positive control cells were then incubated in 20 μ l of 1mg/ml DNase for 30 min at room temperature, then washed twice with 1X PBS. Other samples were washed once with 1X PBS, and pellets were re-suspended and incubated for one hour at 37⁰ C in an incubator in the dark in TUNEL reagents: 50 μ l of labeling solution for negative control, and 50 μ l of TUNEL reaction mixture for the other samples (TUNEL reaction mixture: 50 μ l enzyme + 450 μ l labeling solution). Cells were then washed twice with 1X PBS, re-suspended in 1 ml PBS, and transferred into polystyrene falcon round bottom tubes for flow cytometry analysis (with the excitation wavelength set at 470–490 nm and the emission wavelength at 505 nm).

G. Trans-well Invasion Assay

Using 24-well plate and 24-well inserts (pore size 8 μ m; Falcon) each insert was freshly coated with 1:10 dilution of MatrigelTM (BD Bioscience) in cold PBS, put inside the well, and then was air-dried overnight before starting the invasion assay. Next day, 250,000 cells were seeded in a serum-free medium with or without treatment in the top chamber onto the MatrigelTM-coated membrane, and a medium supplemented with 5% FBS was used as a chemo-attractant in the lower chamber. Cells were allowed to migrate through the membrane coated with MatrigelTM at 37°C in a 5% CO₂ incubator for 48 hours. Non-

migratory cells in the upper chamber were gently scraped off with a cotton-tip applicator. Invading cells on the lower surface of the membrane were fixed and stained with Hematoxylin and Eosin (H&E). After staining, the total number of invading cells was counted under the light microscope ($\times 10$ objective) from 6 consecutive fields for each well. Cell invasion was expressed as percentage of invaded cells in the absence and presence of the treatment.

H. Wound Healing Assay

For wound healing, or scratch assay, cells were seeded in a six-well plate using the same concentrations, and incubated until they reached 80% to 90% confluence. A uniform scratch was made down the center of the well using a 200 μ l micropipette tip, and the plates will be then washed twice with PBS to remove the detached cells. Remaining cells were cultured in complete media with or without treatment. Microscopic photos were subsequently taken at 0, 24, and 48 hours. The distance traveled by the cells into the wounded area was computed from the closure of the wounds, and expressed as percentage of the wound closure upon treatment compared to the control condition.

I. Western Blotting

Proteins were extracted from cells with Laemmli sample buffer (Biorad). Equal amounts of each extract were electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then transferred into nitrocellulose membranes. Membranes were blocked for 1.5 hours with fat free milk 5%. After washing, membranes were blotted with primary antibodies overnight at 4°C. The next day, membranes were then washed and

blotted with corresponding secondary antibodies for 2 hours and developed prior to adding Luminol reagent (Santa Cruz Biotechnology). The following antibodies were used: p53, PARP, γ H2AX, and membranes were probed with anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for assessment of equal protein loading. Antibodies were purchased from Cell Signaling and Santa Cruz Biotechnology.

J. Spheres Formation Assay

DU145 cells were able to form spheroids in a non-adherent culture, suggesting the presence of cancer stem-like cells within this cell line. Other terms used for cancer stem-like cells are cancer-initiating cells or cancer-repopulating cells (Taylor 2010). Using 24-well plates, 1,000 cells/well were suspended in cold MatrigelTM/serum free RPMI-1640 (1:1) in a total volume of 50 μ l. DU145 cells were seeded uniformly in a circular manner around the bottom rim of the well and allowed to solidify in the incubator at 37°C for one hour. Subsequently, 0.5 ml of RPMI-1640 2% FBS media treated with ST1926 or with DMSO were added gently in the middle of each well. Spheres were replenished with warm media as in the original seeding every other day. DU145 spheres were counted after 10 to 13 days. To propagate spheres, the medium was aspirated and MatrigelTM was digested with 0.5 ml dispase solution 1 mg/ml (Invitrogen, Carlsbad, CA), dissolved in RPMI-1640 incomplete medium for 60 minutes at 37°C. Spheres were then collected, incubated in 1 ml warm Trypsin/ EDTA at 37°C for 5 minutes, and then passed through a 27,25 and 20-gauge syringes three times. DU145 cells were counted by a hemocytometer and re-seeded as before. Spheres were propagated for at least 5 generations and the sphere-formation unit (SFU) was calculated for each generation as follows: SFU = (number of spheres

formed/number of cells plated) x100. Results were represented as a percentage of the SFU of the treated spheres compared to the untreated ones.

K. Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013. Data presented are the means \pm SEM of three assays as noted in the figure legends. The significance of the data was analyzed using a Student's t test, and statistical significance was reported when the P-value was < 0.05 (*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$).

CHAPTER III

RESULTS

A. Effects of ST1926 *versus* ATRA at various concentrations on prostate cancer cell proliferation *in vitro* using MTT assay

First, we sought to determine the anti-proliferative effect of the synthetic retinoid ST1926 *versus* the natural active derivative of vitamin A, ATRA. Using MTT assay, the proliferation activity of human prostate cancer cell lines, DU145 and PC3, was not significantly altered in the presence of different concentrations of ATRA at different time points even at supra-pharmacological 10 μ M concentrations (Figure 6A and 6B). On the contrary, the proliferation activity was found to be significantly altered at pharmacologically achievable μ M concentrations in the presence of ST1926 in a time- and dose-dependent manner (Figure 6C and 6D). Interestingly, sub-pharmacological concentration of ST1926 as low as 0.5 μ M was able to inhibit cell proliferation by approximately 50% at 48 hours, strongly demonstrating that ST1926 is more potent and has a specific mode of action compared to ATRA.

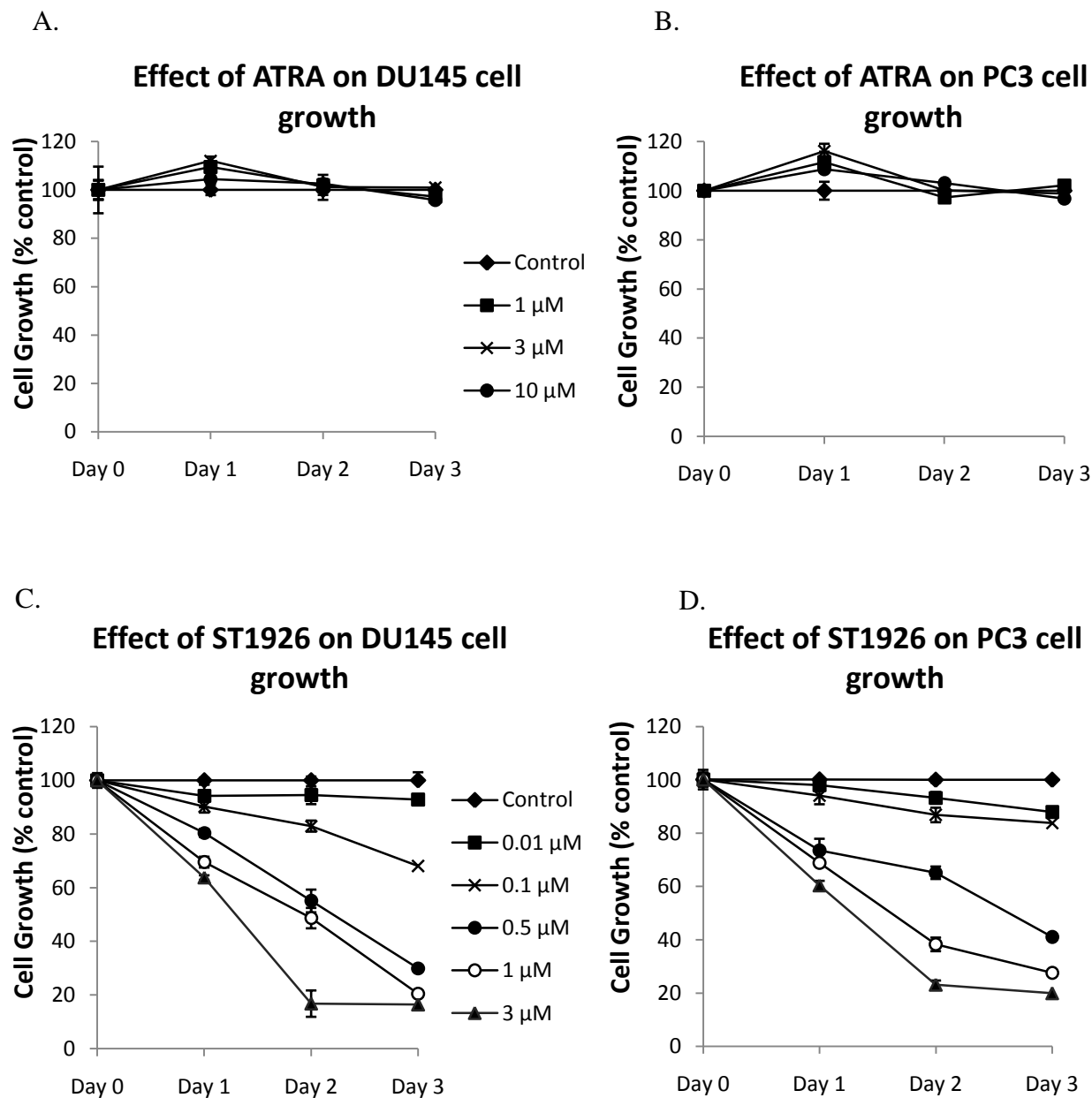


Figure 6. The effect of various concentrations of ATRA and ST1926 on the proliferative activity of prostate cancer cells using MTT assay. DU145 (A) and PC3 (B) cells are relatively resistant to various concentrations of ATRA. ST1926 treatment results in a concentration- and time-dependent growth inhibition in DU145 cells (C) and PC3 cells (D). Cell growth was assayed in triplicate wells with the MTT assay. Results are expressed as percentage of control (0.1% DMSO). Data represent an average of three independent experiments (\pm SEM).

B. ST1926 reduces prostate cancer cell viability

As treatment with ST1926 showed anti-proliferative activities on prostate cancer cells, we examined its effect on the cells' viability. Using trypan blue exclusion method, we showed that the viability of DU145 and PC3 cells was significantly altered in a time- and dose-dependent manner, similar to the results obtained with the MTT assay (Figure 7). Based on the results obtained from the MTT and trypan blue exclusion assays, we decided to use the pharmacologically achievable 1 μM concentration of ST1926 in subsequent experiments. Moreover, these results were compatible with confluency changes in culture where cells treated with 1 μM of ST1926 showed less growth and confluency, and a change in their morphology compared to control untreated cells (Figure 8).

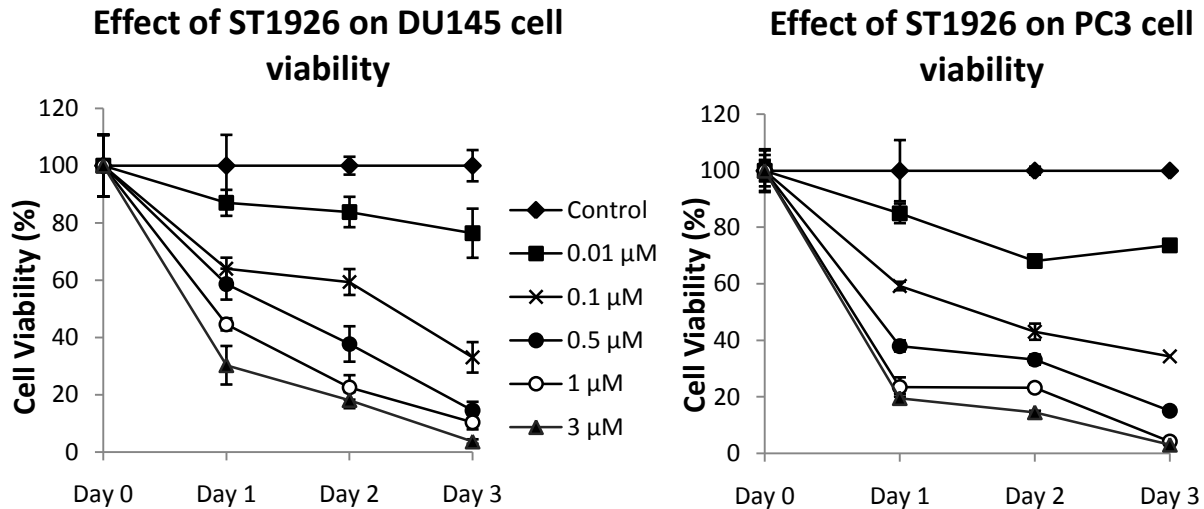


Figure 7. The effect of various concentrations of ST1926 on the viability of prostate cancer cells using trypan blue exclusion method. DU145 and PC3 cells were treated with the different indicated concentrations of ST1926 and time points. Cells were counted in triplicate measurements using the trypan blue exclusion method and results are expressed as percentage of control (0.1% DMSO). Data represent an average of three independent experiments (\pm SEM).

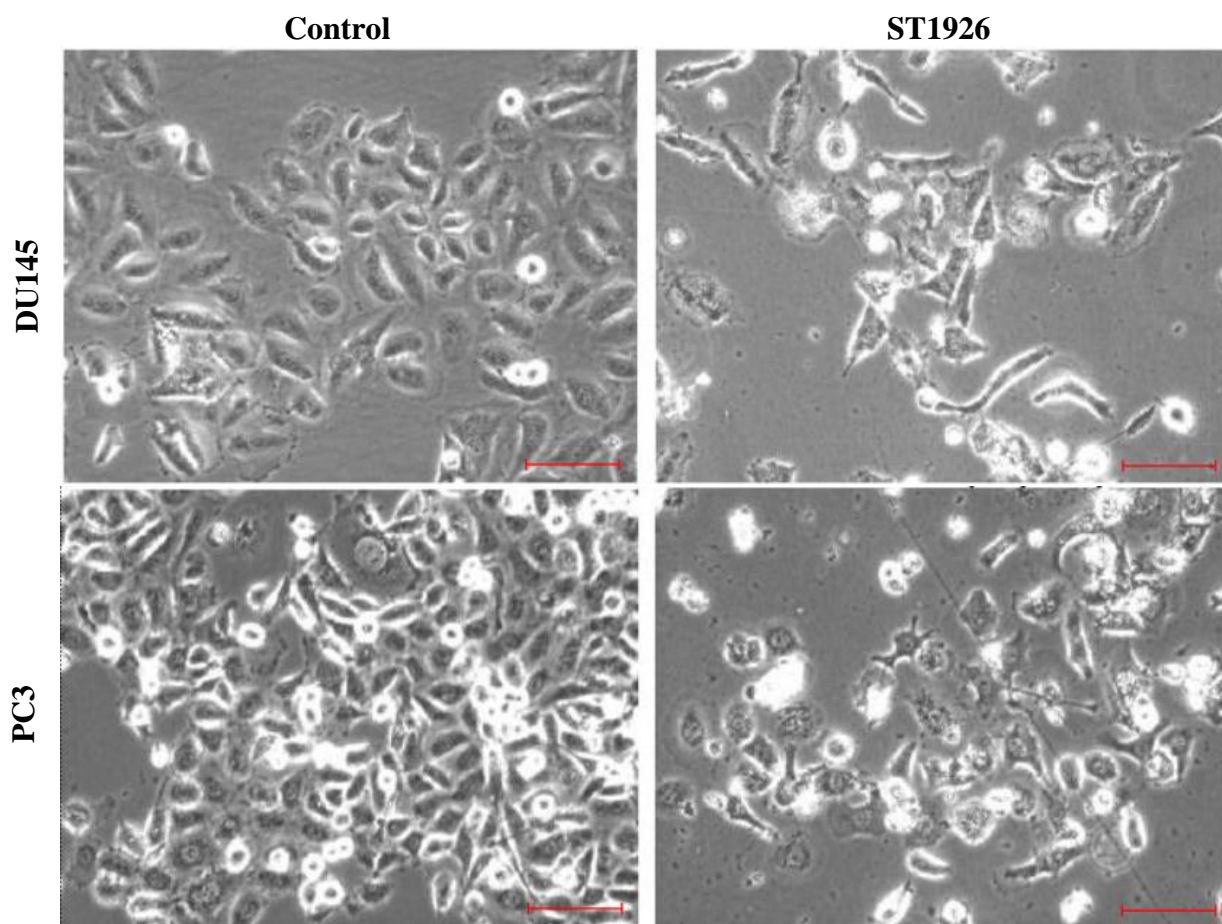


Figure 8. ST1926 inhibits cell proliferation in prostate cancer cells as reflected by confluency changes. DU145 and PC3 cells were treated with 1 μ M ST1926 for 48 hours and representative bright field images were acquired using Zeiss axiovert light microscope (x10). Control cells were treated with 0.1% DMSO. Scale bars represent 50 μ m.

C. ST1926 induces an accumulation of prostate cancer cells in pre-G₁ and G₀/G₁ and induces an S phase arrest

In order to investigate the mechanism of growth inhibition and cell death induced by ST1926 in prostate cancer cells, we analyzed the cell cycle distribution of DU145 and PC3 cells. We examined cellular DNA content distribution stained with propidium iodide by flow cytometry after treating cells with 1 μ M ST1926 for 24 and 48 hours (Figure 9). In DU145

cells, the percentage of cells in the pre-G₁ phase increased from 6% in the control to 12% upon treatment for 24 hours and reached 30% at 48 hours (Figure 9A). Similarly, in PC3 cells, the number of cells in the pre-G₁ phase increased from 5% in the control to 10% upon treatment for 24 hours and reached more than 30% at 48 hours (Figure 9B). In addition, there was G₀/G₁ cell cycle arrest, and an S phase arrest upon treating both cell lines with 1 μM ST1926 for 24 and 48 hours (Figure 9A and 9B). These results suggest a perturbation in the cell cycle, and an increase in the presumably pro-apoptotic region in the cell cycle in response to ST1926 treatment, that might explain the reduction of cell growth and viability.

D. ST1926 induces apoptosis in prostate cancer cells

We showed previously that ST1926 induced the accumulation of prostate cancer cells in the pre-G₁ phase which presumably represents apoptotic cells. To confirm apoptosis induction by treatment with ST1926, we performed the TUNEL assay that detects DNA cleavage through binding to the free 3'-OH ends.

DU145 and PC3 cells were treated with 1 μM ST1926. In DU145, the percentage of TUNEL-positive cells increased from 2% in the control to 18% upon treatment for 24 hours. Moreover, after 48 hours of treatment, the percentage of TUNEL- positive cells increased from 4% in the control to 40% in the treated cells (Figure 10). Similarly, in PC3 cells, ST1926-induced apoptosis was apparent after 72 hours of treatment, where the percentage of TUNEL- positive cells increased from 9% in the control to 30% in the treated cells (Figure 11).

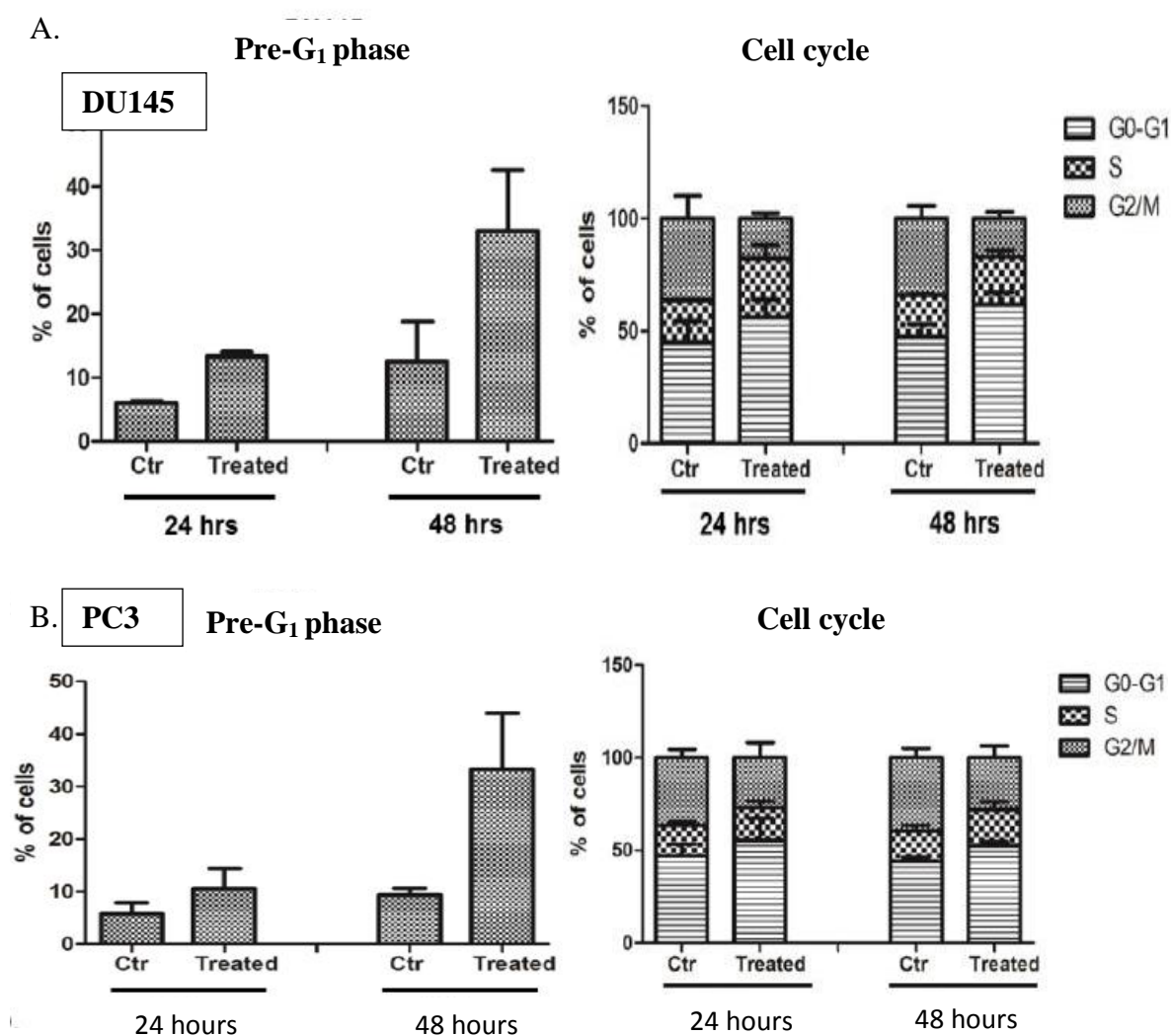


Figure 9. ST1926 treatment induces an accumulation of prostate cancer cells in the pre-G₁ region and G₀/G₁ and induces an S phase arrest. The distribution of phases of the cell cycle upon ST1926 treatment at 24 and 48 hours in DU145 cells (A) and PC3 cells (B). Data represent the average of three independent experiments (\pm SEM).

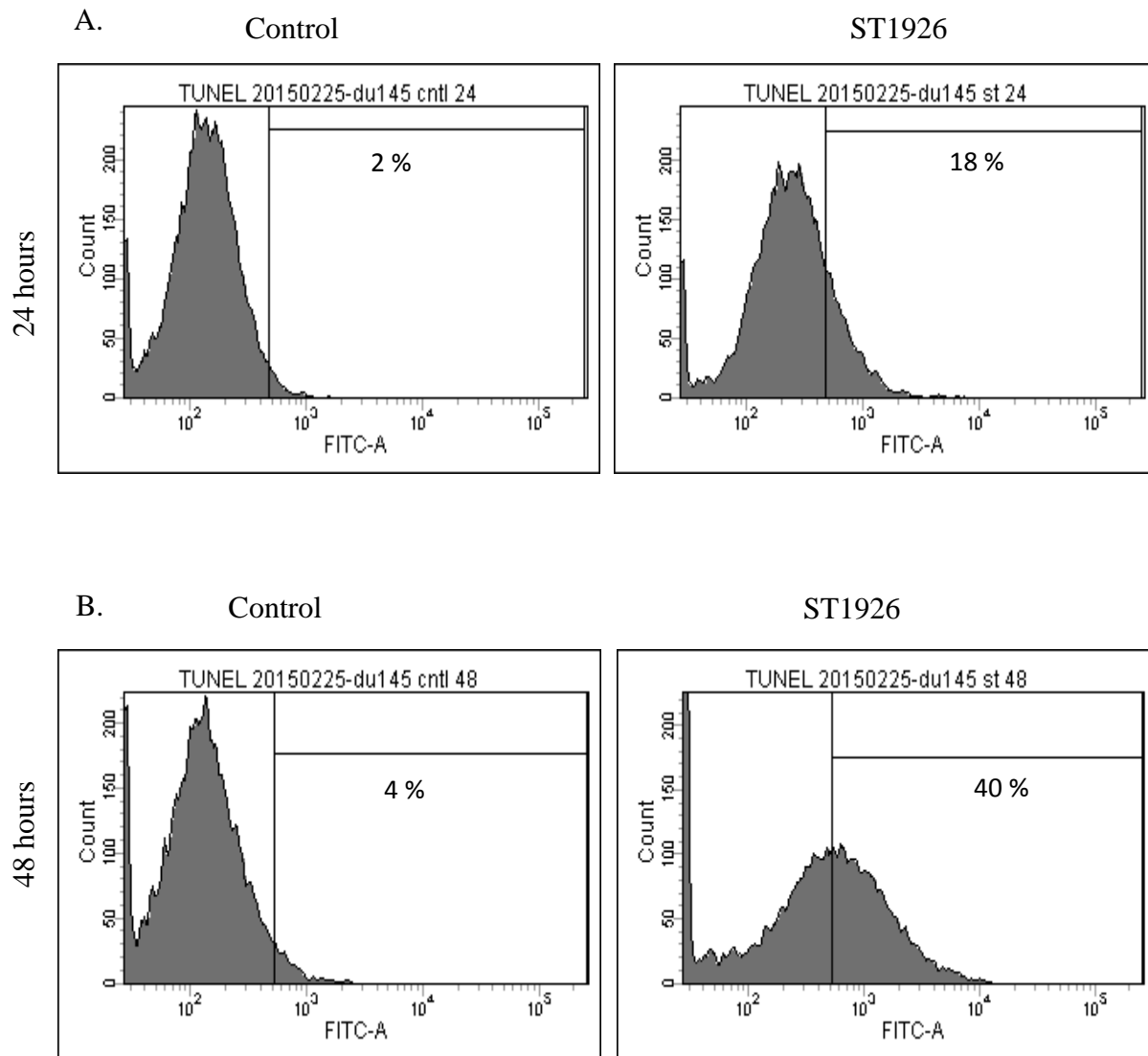


Figure 10. ST1926 induces apoptosis in DU145 cells. DU145 cells were treated with 1 μ M ST1926 for 24 hours (A) and 48 hours (B). Control cells were treated with 0.1% DMSO. TUNEL assay was performed as described in Materials and Methods.

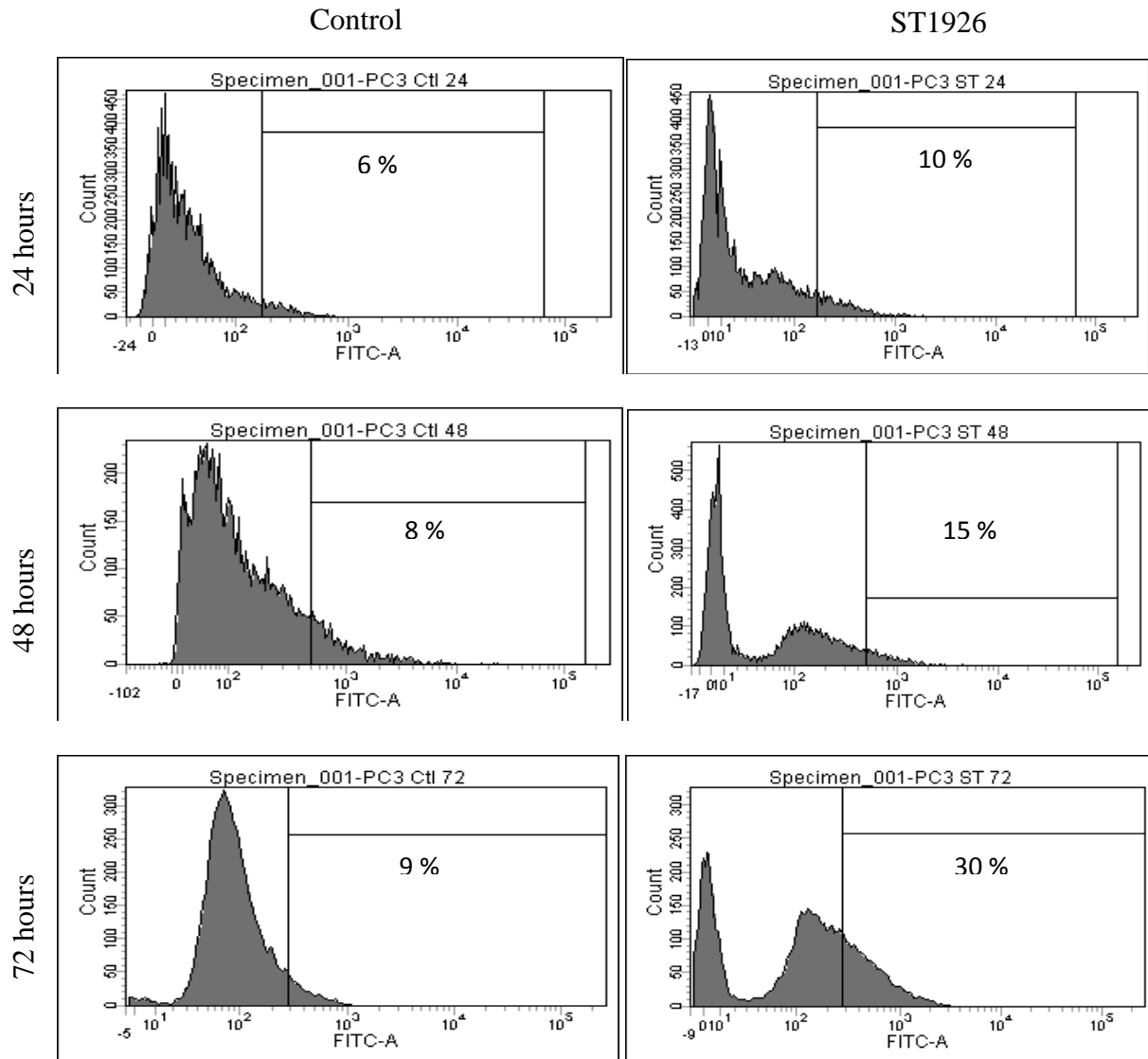


Figure 11. ST1926 induces apoptosis in PC3 cells. PC3 cells were treated with 1 μ M ST1926 for 24, 48, and 72 hours. Control cells were treated with 0.1% DMSO. TUNEL assay was performed as described in Materials and Methods.

E. Modulation of caspase activity by ST1926 in prostate cancer cells

In order to investigate whether ST1926-induced apoptosis in the prostate cancer cell lines is caspase-dependent, we studied the process of caspase activation and cleavage through immunoblotting techniques. DU145 and PC3 cells were treated with 1 μ M ST1926 up to 24 hours.

ST1926-induced apoptosis suggests caspase activation as it is evident by the cleavage of its substrate PARP (113 kD) into its death-associated fragment (89 kD) (Figure 12). PARP cleavage was detected as early as 24 hours post-treatment with 1 μ M ST1926.

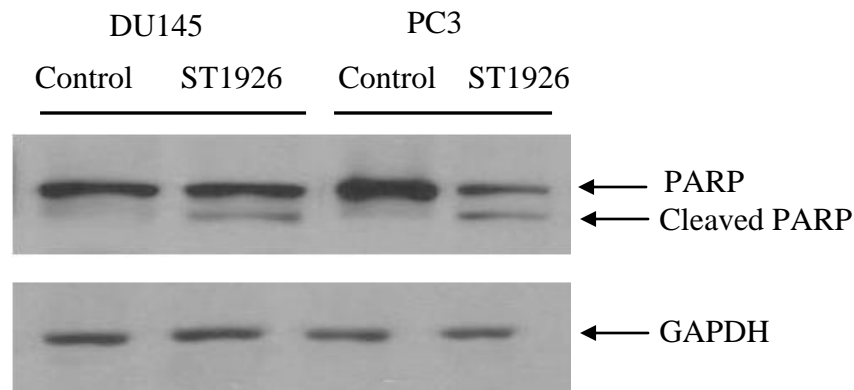


Figure 12. ST1926 treatment of prostate cancer cells induces PARP cleavage. DU145 and PC3 cells were treated with 1 μ M ST1926 for 24 hours. Whole SDS lysates (50 μ g/ml) were prepared and immunoblotted against PARP antibody. Similar trends were observed in two independent experiments.

F. ST1926 exerts its anti-tumor activities through p53-independent pathway

The tumor suppressor p53 is normally found at low levels, but when there is DNA damage, p53 levels increase and initiate anti-tumor events. This tumor suppressor protein binds to many target regulatory sites in the genome and initiates the expression of genes that halt cell cycle and induce cell death if damage is not repaired (Vogelstein 2000). Several studies have shown that ST1926 induces its anti-proliferative and cell death effects through p53-independent mechanisms (Cincinelli 2003), and we have proved this through immunoblotting technique of our ST1926-treated cells. DU145 and PC3 cells were treated with 1 μ M ST1926 for 24 and 48 hours. Results indicated that ST1926 induced p53-independent apoptosis in DU145 (mutated *p53*) and PC3 (*p53*-null) cells (Figure 13).

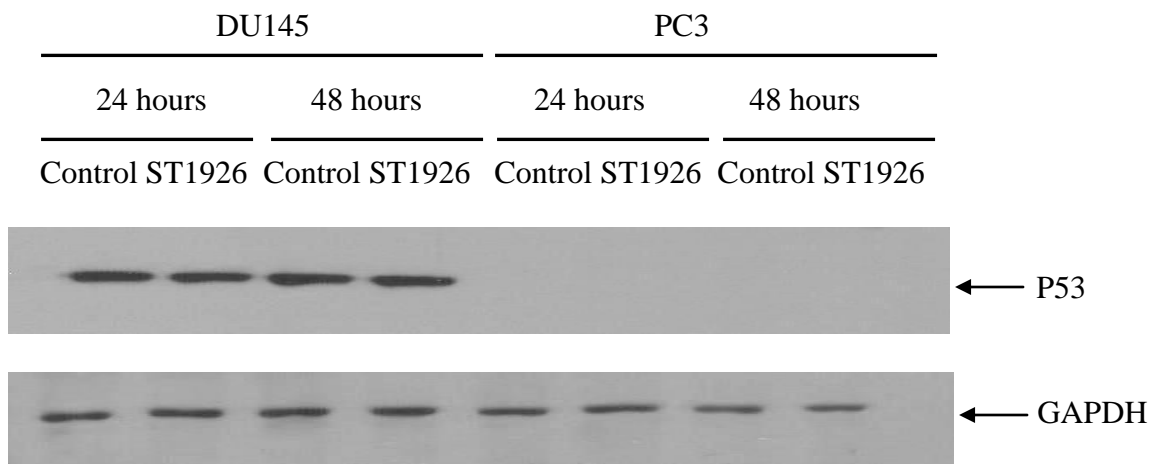


Figure 13. ST1926 induced p53-independent apoptosis in DU145 (mutated *p53*) and PC3 (*p53*-null) cells. All cells were treated with 1 μ M ST1926 for 24 and 48 hours. Whole SDS lysates (50 μ g/ml) were prepared and immunoblotted against p53 antibody. Similar trends were observed in three independent experiments.

G. ST1926 promotes early DNA damage

ST1926 has shown to induce early DNA damage in several types of tumor cells (Valli 2008). Determination of γ H2AX is the golden standard for DNA DSBs. Endogenous or exogenous DNA damage causes DSBs which are followed by phosphorylation of the histone, H2AX. This newly phosphorylated γ H2AX protein, is the first step in recruiting and localizing DNA repair proteins (Kuo 2008).

DU145 and PC3 cells were treated with 1 μ M ST1926 for 30 minutes, 2 hours, 6 hours, and 24 hours. Results showed that 1 μ M of ST1926 induced a major upregulation in the protein expression levels of the sensitive marker for DNA DSBs, γ H2AX, in both cell types as early as 2 hours, clearly indicating that ST1926 is a potent inducer of early DNA damage (Figure 14).

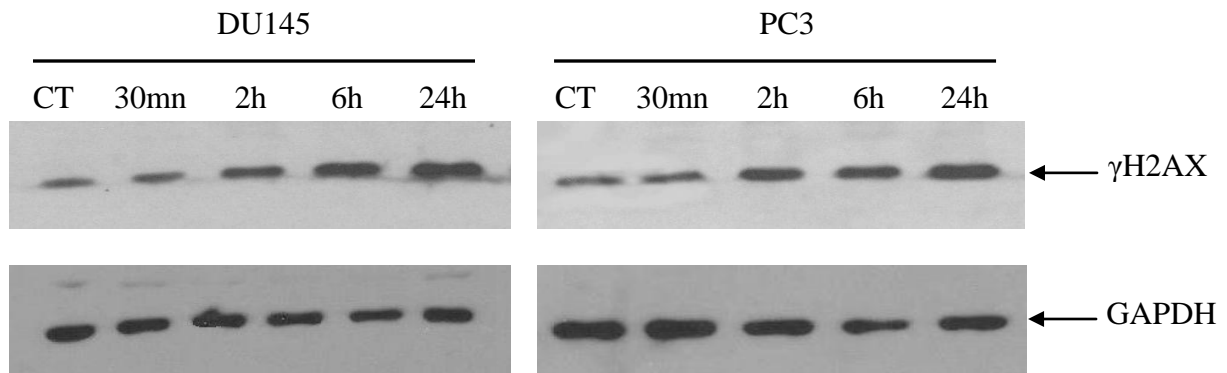
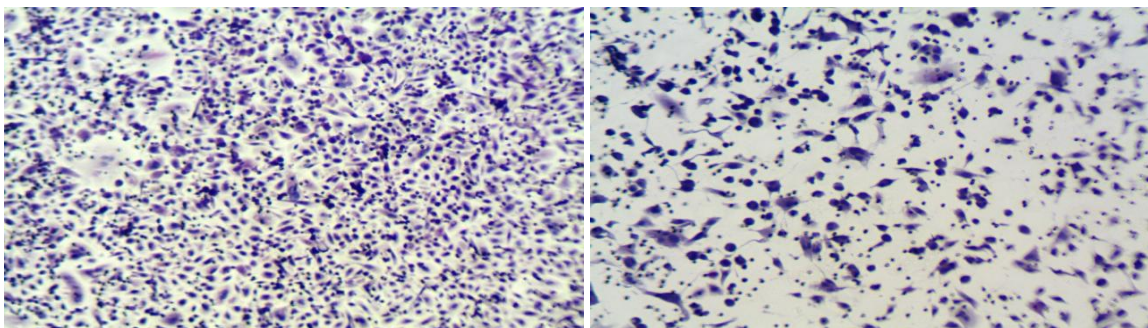


Figure 14. ST1926 is a potent inducer of early DNA damage. DU145 and PC3 cells were treated with 1 μ M ST1926 at the indicated time points. Whole SDS lysates (50 μ g/ml) were prepared and immunoblotted against γ H2AX antibody. Similar trends were observed in two independent experiments.

H. ST1926 inhibits prostate cancer cell invasion

Next, we investigated the effect of ST1926 on cell invasion, a property associated with progression to metastasis. We used the trans-well invasion assay as an *in vitro* model of invasion. In this assay, prostate cancer cells (DU145 and PC3) were plated on top of Matrigel-coated (representing an artificial extracellular matrix) 8 μm pored well (representing the space between endothelial cells). Under control conditions, cells sense by chemotaxis an attractant found in the lower chamber of the well, thus they begins to adhere to the Matrigel, secret enzymes to digest it and then migrate through the pores, where they are being trapped by a nitrocellulose paper. The invaded cells were counted in six random fields under the microscope at x10 magnification. Data in Figure 15 reveals that upon treatment with 1 μM ST1926 for 48 hours, invasion ability was shown to be massively inhibited to reach a value of less than three folds compared to the control conditions with 5% FBS chemo-attractant. This suggests that ST1926 has high inhibitory ability on tumor cell metastasis thus, tumorigenicity of prostate cancer cells.

A. DU145



B. PC3

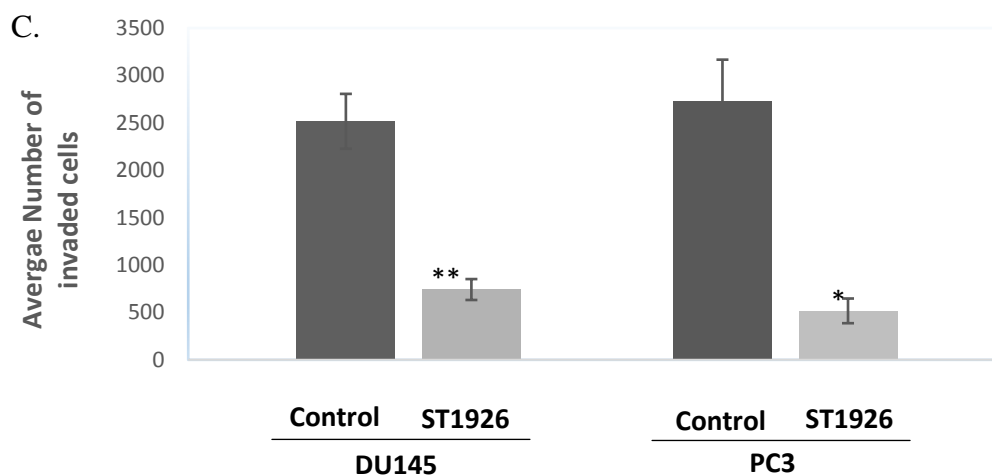
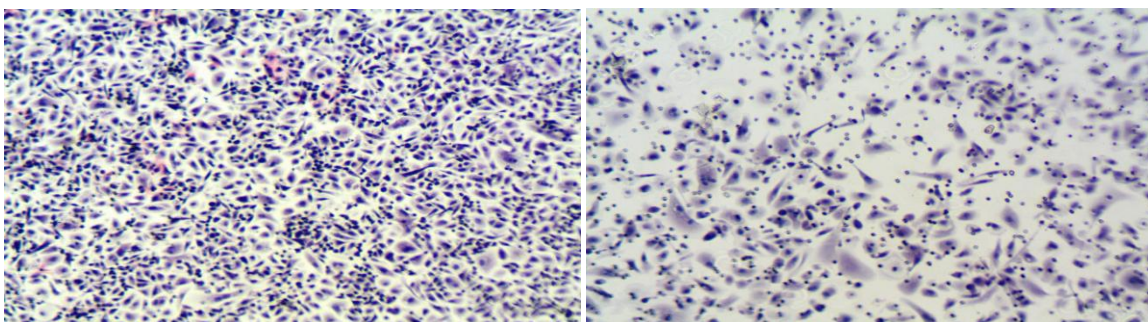


Figure 15. ST1926 reduces the invasive potential of human prostate cancer cells.

Representative images of Matrigel invasion assay. (A) DU145 and (B) PC3 cells were plated onto the Matrigel-coated membrane in the top chamber of the transwell and were treated with 1 μ M of ST1926 or 0.1% DMSO in the presence of 5% FBS in the lower chamber. Cells invaded to the lower chambered after 48 hours post-ST1926 treatment were fixed with methanol, stained with H&E and counted. (C) Data represent an average of three independent experiments of the percentage of invaded cells compared to the control cells treated with 0.1% DMSO. The data are reported as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$).

I. ST1926 reduces the migration ability of prostate cancer cells

Next, we investigated the effect of ST1926 on cell migration, another crucial step involved in the progression to metastasis. Using a wound-healing assay, treatment with 1 μM of ST1926 for 24 and 48 hours significantly suppressed cell migration ability of DU145 cells (Figure 16) and PC3 cells (Figure 17) compared to the control, whereby the wound was completely healed after 48 hours. This suggests that ST1926 displays a high inhibitory effect on prostate cancer cells migration, and potentially invasion.

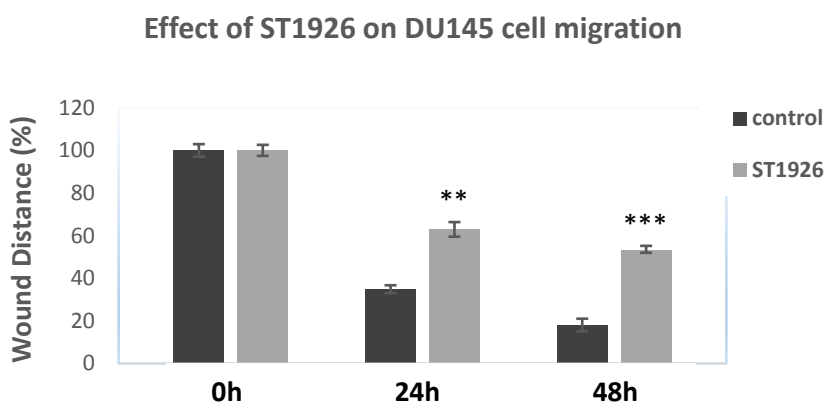
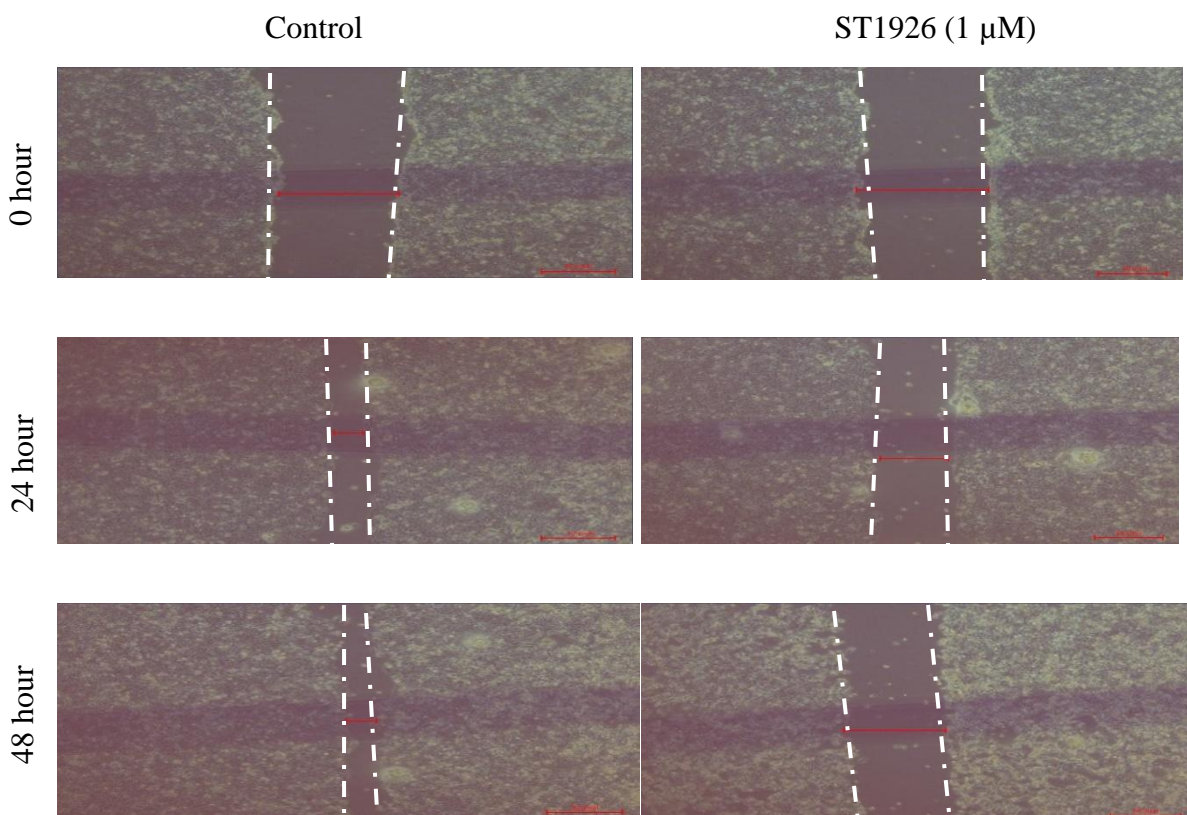


Figure 16. ST1926 reduces the migration potential of DU145 cells. After cells were allowed to reach 80% to 90% confluency, a scratch in DU145 cells was made using a tip. Cells were treated with 1 μ M ST1926 or 0.1% DMSO, and bright field images were taken at 0, 24, and 48 hours. Data represent an average of three independent experiments of the percentage of migrated cells compared to the control cells after 24 and 48 hours of ST1926 treatment. The data are reported as mean \pm SEM. (**, $P < 0.01$; *** $P < 0.001$).

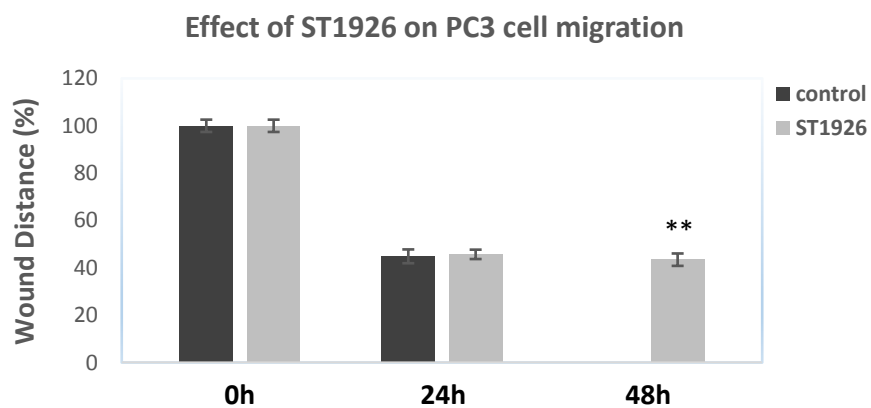
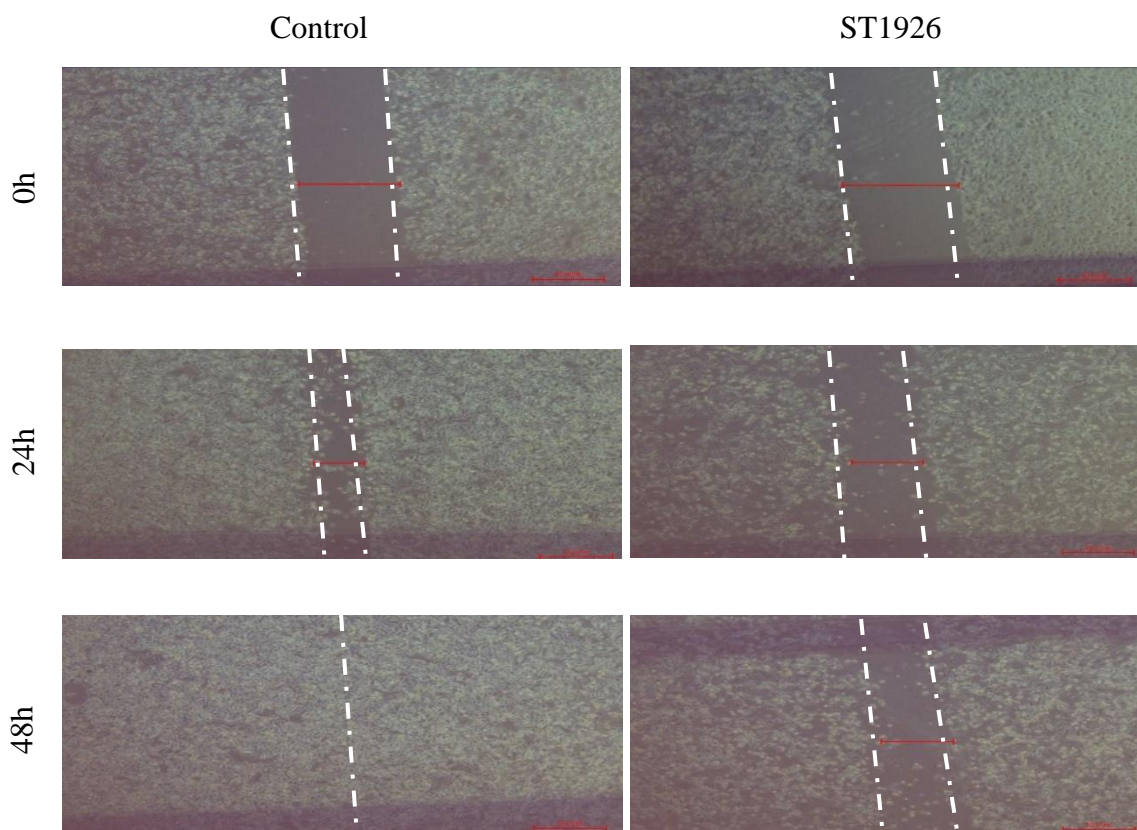


Figure 17. ST1926 reduces the migration potential of PC3 cells. After cells were allowed to reach 80% to 90% confluency, a scratch in DU145 cells was made using a tip. Cells were treated with 1 μ M ST1926 or 0.1% DMSO, and bright field images were taken at 0, 24, and 48 hours. Data represent an average of three independent experiments of the percentage of migrated cells compared to the control cells after 24 and 48 hours of ST1926 treatment. The data are reported as mean \pm SEM. (**, $P < 0.01$).

J. The effect of ST1926 in targeting an enriched population of prostate cancer stem/progenitor cells using 3D spheres formation assay

The ability to grow as non-adherent spheroids in sphere medium has been widely used to assess the self-renewal capability of CSCs and is one of the characteristics of prostate CSCs. To assess the ability of ST1926 in targeting prostate CSCs, prostate spheres (prostasphere) formation of DU145 cells was studied in the presence or absence of ST1926. As shown in (Figure 18), after culturing the cells for 11 days in non-adherent conditions, DU145 cells were able to form tumor spheres in untreated conditions. However, major reduction in prostaspheres formation was observed in wells treated with ST1926 at concentration as low as 0.1 μM .

To check the ability of DU145 cells to form and propagate heterogeneous prostaspheres, sphere forming assay was performed. In this assay, prostaspheres were generated utilizing 1000 single cells, namely generation 1 (G1). Cells from prostaspheres were dissociated into single units and seeded again to form spheres of generation 2 (G2). The assay was performed until generation 4 (G4). DU145 cells showed a decline in the percentage of SFUs upon passaging (Figure 19). These result suggest that a variable number of stem-like cells are found inside the cell line and are propagated over the generations. Furthermore, upon initial treatment with ST1926 (0.1 μM), SFU is steeply reduced approximately 70% as well as the size of spheres is diminished (Figure 20). In addition, when treated spheres are propagated once with 0.1 μM of ST1926, their SFUs are drastically lowered to 90% as compared to spheres treated in G1. These spheres were very small in size and were completely abolished, where no spheres were able to form. This suggests that treatment with ST1926 leads to extinguishing of main CSC characteristic which is the self-renewal ability. Interestingly,

whenever treated spheres are propagated to untreated conditions they were able to form spheres and this is depicted by an elevation in SFU, compared to treated conditions, observed in G2, G3, and G4 (Figure 19), and in the size of the spheres (Figure 20). Results demonstrated that whenever treated spheres are re-propagated to untreated conditions, they gradually returned to control stage if remained untreated.

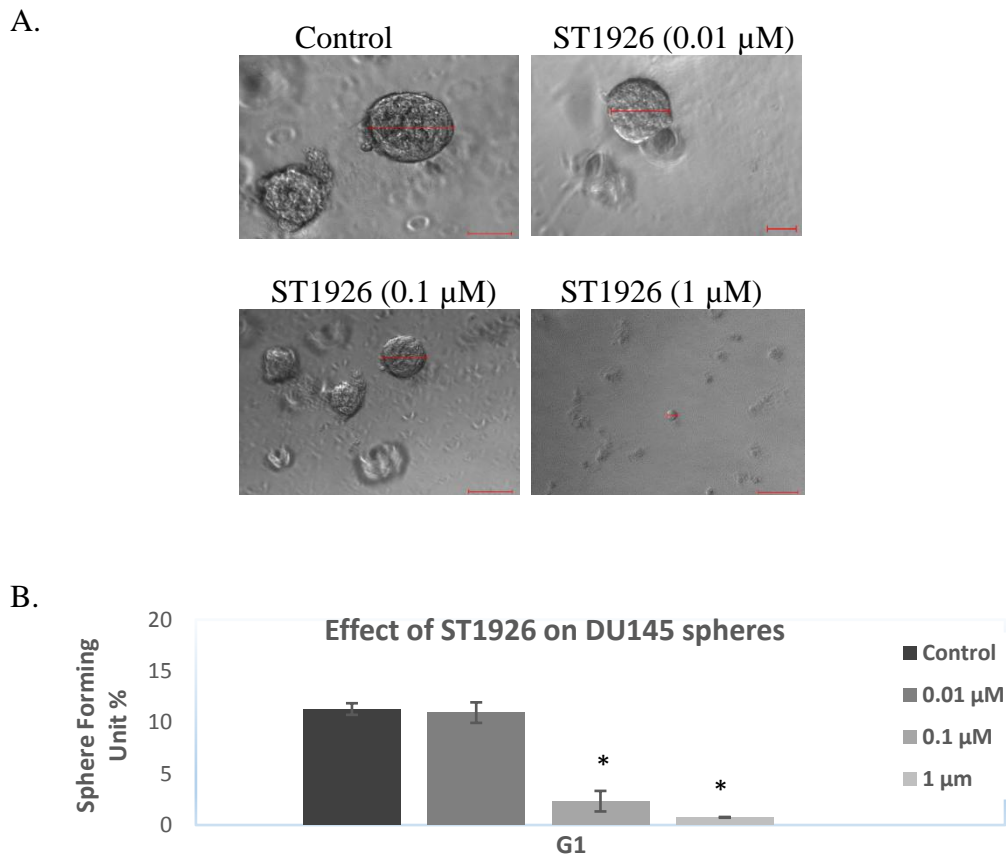


Figure 18. The effect of ST1926 on sphere-forming ability of DU145 cells.

(A) Representative images of DU145 prostaspheres. (B) Sphere-forming unit (SFUs) are shown with control or indicated ST1926 concentrations for 11 days. Generated spheres are referred to as G1 (Generation 1) spheres. SFU is calculated according to the following formula: $\text{SFU} = (\text{number of spheres counted} / \text{number of input cells}) * 100$. Data represent an average of three independent experiments. The data are reported as mean \pm SEM. (*, $P < 0.05$). Images were visualized by Carl Zeiss microscope at 10x magnification and analyzed by Carl Zeiss Zen 2012 image software. Scale bars represent 100 μm .

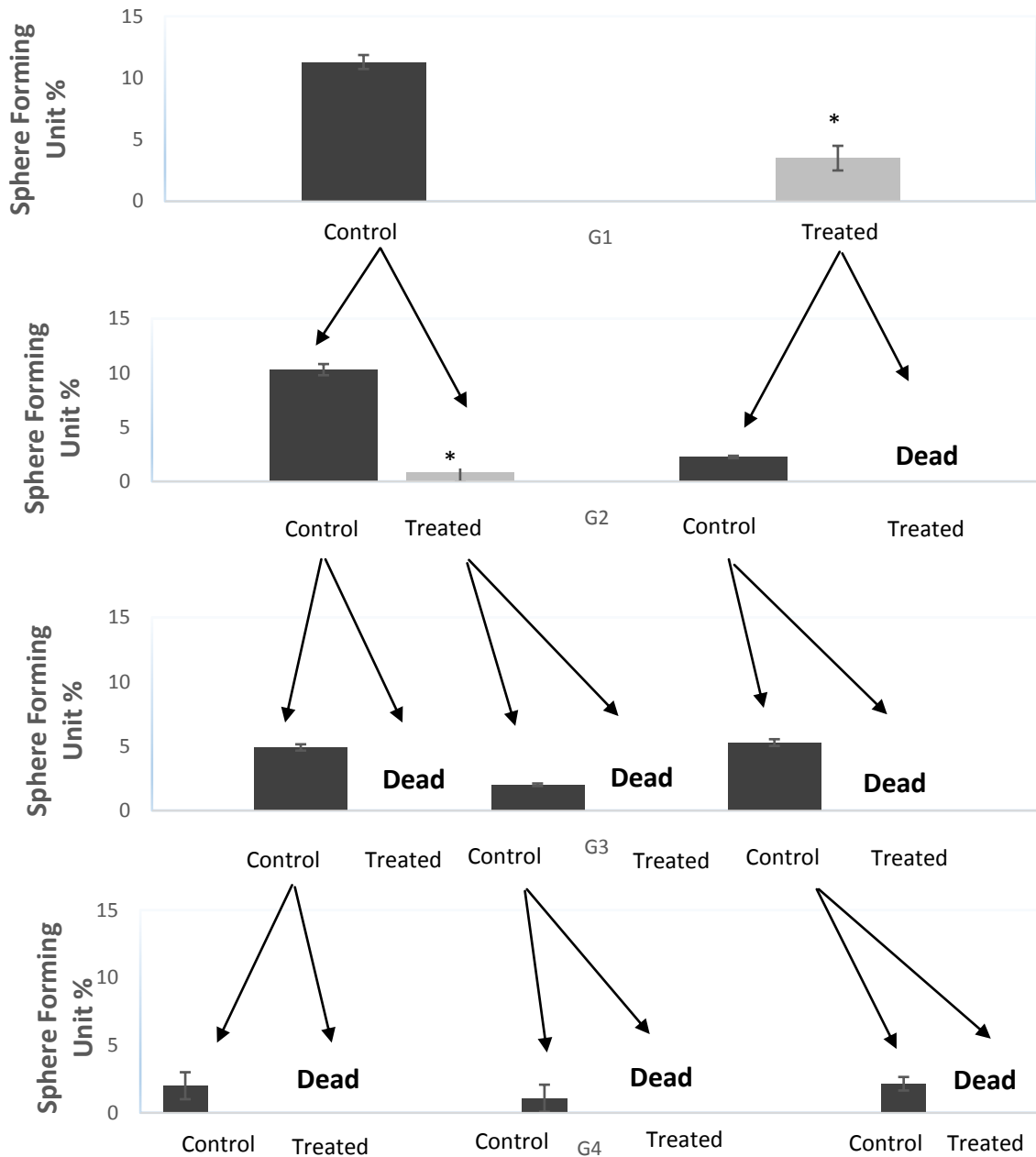


Figure 19. ST1926 treatment inhibits the self-renewal capacity of prostate cancer stem/progenitor cells enriched from DU145 cells. Prostate CSCs were enriched from DU145 cell line and treated with either ST1926 (0.1 μ M) or media (control) (G1). At each propagation, 1 μ M ST1926 treatment and media (control) were propagated in separate wells respectively. Spheres were propagated for 4 generations in duplicates of each condition. Sphere formation unit is counted and an average of three independent experiments. The data are reported as mean \pm SEM. (*, $P < 0.05$).

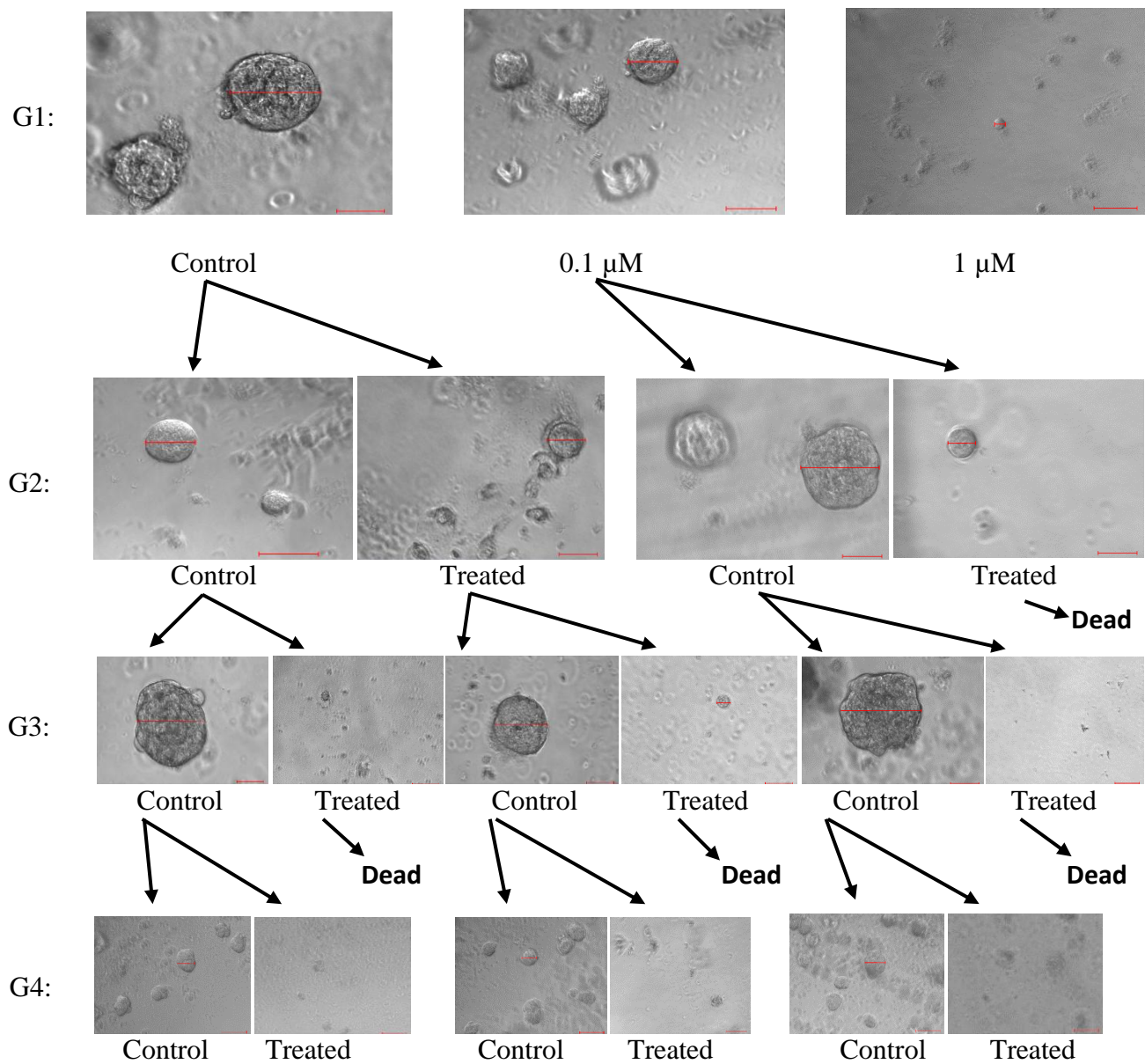


Figure 20. ST1926 treatment inhibits the self-renewal capacity of prostate CSCs enriched from DU145 cell line.

Representative images of the data shown in Figure 18. Scale bars represent 100 μm.

CHAPTER IV

DISCUSSION

Vitamin A and its derivatives (retinoids) exert a wide range of effects on embryonic development and organogenesis, as well as on cell growth, differentiation, and apoptosis (Alizadeh 2014). Interestingly, retinoids have been widely investigated as potent anti-cancer agents, particularly in leukemia and many solid cancers including prostate cancer, and are used clinically in the treatment of several cancers (Schenk 2014). However, cancer cells develop resistance to the natural retinoids due to attenuation or mutations in retinoid receptor signaling pathway (Tang 2011), necessitating the development of new retinoids. As a result, synthetic retinoids have been developed with more specific and less toxic mechanism of actions than natural retinoids (Fontana 2002). Although the exact mechanism of action of the synthetic retinoids is not fully understood, their anti-proliferative and apoptotic effects are attributed to their ability to induce both receptor-dependent and -independent mechanism of action. One of the promising synthetic retinoids with anti-neoplastic activities, increased bioavailability, and reduced toxicity is the novel adamantyl retinoid ST1926 (Cincinelli 2003, Sala 2009).

Based on the promising anti-neoplastic properties of ST1926, we were interested in studying its anti-tumor properties and mechanism of action in human prostate cancer cells. Prostate cancer is the most leading cause of mortality and morbidity among men worldwide (Siegel 2015). Prostate cancer progression is categorized as a series based on the extent of the disease, from androgen-dependent prostate cancer to CRPC. This later form of prostate

cancer is lethal, and occurs when patients develop resistance to treatments that interfere with androgen signaling, such as bicalutamide (Qian 2015). To the best of our knowledge, this is the first study to elucidate the detailed functions and signaling regulation mechanisms of ST192 in prostate cancer. Thus, the overall aim of this study was to investigate the anti-neoplastic effect of ST1926 and its underlying molecular mechanisms on prostate cancer.

In this study, cell proliferation assay showed that human prostate cancer cells (DU145 and PC3) were resistant to ATRA even at supra-pharmacological 10 μM concentrations. On the contrary, both prostate cancer cell lines were sensitive at μM pharmacologically achievable concentrations as shown by MTT and trypan blue cell viability assays. Therefore, we decided to use the pharmacologically achievable 1 μM ST1926 in investigating its mechanism of action in the subsequent experiments.

Cell cycle analysis revealed a significantly high percentage of cells accumulating in the presumably pro-apoptotic pre- G_1 phase in both tested cell lines with G_0/G_1 and S phase cell cycle arrest upon 1 μM ST1926 treatment. To confirm whether ST1926 is inducing apoptosis in prostate cancer cells, we tested for DNA fragmentation by TUNEL assay as this event is common during late apoptosis. The TUNEL assay showed that 1 μM ST1926 induced massive DNA fragmentation in DU145 and in PC3 cells. Caspases play a major role in apoptosis and our results show that ST1926 treatment of both prostate cancer cell lines result in PARP cleavage, suggesting a caspase-dependent mechanism of cell death. Additional studies should be performed using the pan-caspase inhibitor z-VAD, in order to confirm the involvement of caspases in ST1926-induced prostate cancer cell death. We have recently shown that apoptosis caused by ST1926 is partially caspase- dependent in ATL and in CML-treated cells (El Hajj 2014, Nasr 2014).

ST1926-induced apoptosis may be mediated through p53-dependent or -independent pathways (Cincinelli 2003, Valli 2008, El Hajj 2014). We have shown that ST1926-induced cell death is p53-independent in our tested prostate cancer cells as this drug induced apoptosis in DU145 cells with mutated *p53* (Bajgelman 2006), and in PC3 cells with null *p53* status (Scott 2003). Next, we tested for the effects of ST1926 on DNA damage, knowing that determination of γ H2AX is the golden standard for DNA DSBs. We showed that 1 μ M ST1926 induced a major early upregulation in the protein expression levels of γ H2AX in both cell lines; clearly emphasizing that ST1926 is a potent inducer of DNA damage that precedes cell death. Previous studies have demonstrated the involvement of DNA damage in ST1926-induced cell death, and that its loss is related to the development of ST1926 resistance in lung cancer (Zuco 2005) and in acute myloid leukemia cells (Fratelli 2013). Further studies should be performed to decipher the involvement of DNA damage response in ST1926 mode of action in prostate cancer cells.

The ability of cancer cells to metastasize to other organs is a life threatening stage of cancer. Thus, we tested for the effects of ST1926 on two major steps in cancer metastasis; invasion and migration. A significant decrease was observed in the ability of metastatic prostate cancer cells to invade the artificial basement membrane (Matrigel) and also its ability to migrate. This suggests that ST1926 has high inhibitory properties on metastasis thus, tumorigenicity of prostate cancer cells. It remains to be determined whether ST1926 inhibits metastasis in prostate cancer animal models and the underlying molecular mechanism.

One of the main reasons of a conventional chemotherapy failure is the presence of the quiescent or the slower dividing CSCs that are resistant to these treatments (Ni 2014). CSCs

can survive chemotherapy or any other conventional treatment by their ability to re-enter the cell cycle and to produce highly proliferative and rapidly-dividing progenitor cells and re-establish the tumor. Towards this end, we sought to determine the ability of ST1926 to target this sub-population of stem/progenitor cells in DU145 cells using a 3D sphere formation assay. Here, we showed that treatment with ST1926 at a concentration as low as 0.1 μM inhibited drastically SFU as well as sphere size. Furthermore, when treated spheres were propagated once to G2 with the same concentration of ST1926, their SFU were even more reduced than those in G1 and the spheres were almost completely abolished. This suggests that the therapeutic effect of ST1926 is in its ability to target the resistive CSCs' main characteristics which is the self-renewal ability, thus eradicating the whole tumor and preventing recurrence. It would be of interest to determine whether ST1926 treatment reduces the levels of prostate CSC markers namely Sox2, Oct4, Nanog, CD44, and CD166 (Tu 2012, Jiao 2012, Sharpe 2013). Furthermore, our results demonstrated that ST1926 treatment was partially reversible; whenever treated spheres were re-propagated to untreated conditions, they gradually returned to the control stage and began to form spheres, if remained untreated. This could be understandable, since CSCs are highly resistant and they always try to evade the therapy to keep proliferating again. A recent report showed that three rounds of treatment with LH4, a novel Lebanese plant extract from *Berberis Libanotica* Ehrh, was sufficient to reduce the spheres stem cell pool in DU145 cells (El-Merahbi 2014). Moreover, treatment with graphene oxide has been investigated as a therapeutic drug to selectively target and was shown to inhibit tumor-sphere formation in six independent cancer cell lines, including prostate CSCs (Fiorillo 2015). We suggest that the next step will be a combinatorial treatment of ST1926 with other drugs in order to prevent CSCs recurrence.

Ultimately, this might be a future treatment option in men with metastatic CRPC who relapsed after chemotherapy.

In conclusion, our study provides promising clues for the development of new therapeutic strategies for prostate cancer that involve the use of ST1926 and epigenetic modifiers (for example, vorinostat) as this was shown to be successful in enhancing radiation-induced cytotoxicity in DU145 cells, and showed synergy with zoledronic acid in inducing cell death in PC3 cells (Crea 2014). Our *in vitro* results clearly indicated that ST1926 significantly inhibited the key steps of tumorigenesis, including cell growth, proliferation, cell death, migration, invasion, and targeting CSCs population, in a time- and dose-dependent manner at pharmacologically achievable concentrations. Finally, future research must focus on better understanding and targeting of quiescent CSC populations. *In vivo* survival and efficacy studies should be performed to test for ST1926 effect in well-established prostate cancer animal models to determine whether to recommend ST1926 for human clinical trials.

REFERENCES

Alizadeh, F., Bolhassani, A., Khavari, A., Bathaie, S. Z., Naji, T., & Bidgoli, S. A. (2014). Retinoids and their biological effects against cancer. *International Immunopharmacology*, 18(1), 43-49.

Alvarez, S., Germain, P., Alvarez, R., Rodriguez-Barrios, F., Gronemeyer, H., & de Lera, A. R. (2007). Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. *The International Journal of Biochemistry & Cell Biology*, 39(7-8), 1406-1415.

Amaral, T. M., Macedo, D., Fernandes, I., & Costa, L. (2012). Castration-resistant prostate cancer: Mechanisms, targets, and treatment. *Prostate Cancer*, 2012, 327253.

Atala, A. (2012). Re: PC3 is a cell line characteristic of prostatic small cell carcinoma. *The Journal of Urology*, 188(1), 325.

Bajgelman, M. C., & Strauss, B. E. (2006). The DU145 human prostate carcinoma cell line harbors a temperature-sensitive allele of p53. *The Prostate*, 66(13), 1455-1462.

Balmain, A. (2001). Cancer genetics: From boveri and mendel to microarrays. *Nature Reviews.Cancer*, 1(1), 77-82.

Barron, D. A., & Rowley, D. R. (2012). The reactive stroma microenvironment and prostate cancer progression. *Endocrine-Related Cancer*, 19(6), R187-204.

Beheshti, B., Karaskova, J., Park, P. C., Squire, J. A., & Beatty, B. G. (2000). Identification of a high frequency of chromosomal rearrangements in the centromeric regions of prostate cancer cell lines by sequential giemsa banding and spectral karyotyping. *Molecular Diagnosis: A Journal Devoted to the Understanding of Human Disease through the Clinical Application of Molecular Biology*, 5(1), 23-32.

Beheshti, B., Park, P. C., Sweet, J. M., Trachtenberg, J., Jewett, M. A., & Squire, J. A. (2001). Evidence of chromosomal instability in prostate cancer determined by spectral karyotyping (SKY) and interphase fish analysis. *Neoplasia (New York, N.Y.)*, 3(1), 62-69.

Bernard, B. A., Bernardon, J. M., Delescluse, C., Martin, B., Lenoir, M. C., Maignan, J., et al. (1992). Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor gamma. *Biochemical and Biophysical Research Communications*, 186(2), 977-983.

Boyer, M. J., Salama, J. K., & Lee, W. R. (2014). Palliative radiotherapy for prostate cancer. *Oncology (Williston Park, N.Y.)*, 28(4), 306-312.

Bushue, N., & Wan, Y. J. (2010). Retinoid pathway and cancer therapeutics. *Advanced Drug Delivery Reviews*, 62(13), 1285-1298.

Carlin, B. I., & Andriole, G. L. (2000). The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. *Cancer*, 88(12 Suppl), 2989-2994.

Chatterjee, B. (2003). The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. *Molecular and Cellular Biochemistry*, 253(1-2), 89-101.

Cincinelli, R., Dallavalle, S., Merlini, L., Penco, S., Pisano, C., Carminati, P., et al. (2003). A novel atypical retinoid endowed with proapoptotic and antitumor activity. *Journal of Medicinal Chemistry*, 46(6), 909-912.

Connolly, R. M., Nguyen, N. K., & Sukumar, S. (2013). Molecular pathways: Current role and future directions of the retinoic acid pathway in cancer prevention and treatment. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 19(7), 1651-1659.

Crea, F., Clermont, P. L., Mai, A., & Helgason, C. D. (2014). Histone modifications, stem cells and prostate cancer. *Current Pharmaceutical Design*, 20(11), 1687-1697.

Danaei, G., Vander Hoorn, S., Lopez, A. D., Murray, C. J., Ezzati, M., & Comparative Risk Assessment collaborating group (Cancers). (2005). Causes of cancer in the world: Comparative risk assessment of nine behavioural and environmental risk factors. *Lancet*, 366(9499), 1784-1793.

Delia, D., Aiello, A., Lombardi, L., Pelicci, P. G., Grignani, F., Grignani, F., et al. (1993). N-(4-hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. *Cancer Research*, 53(24), 6036-6041.

Di Francesco, A. M., Meco, D., Torella, A. R., Barone, G., D'Incalci, M., Pisano, C., et al. (2007). The novel atypical retinoid ST1926 is active in ATRA resistant neuroblastoma cells acting by a different mechanism. *Biochemical Pharmacology*, 73(5), 643-655.

Crea, F., Clermont, P. L., Mai, A., & Helgason, C. D. (2014). Histone modifications, stem cells and prostate cancer. *Current Pharmaceutical Design*, 20(11), 1687-1697.

El Hajj, H., Khalil, B., Ghandour, B., Nasr, R., Shahine, S., Ghantous, A., et al. (2014). Preclinical efficacy of the synthetic retinoid ST1926 for treating adult T-cell leukemia/lymphoma. *Blood*, 124(13), 2072-2080.

El-Amm, J., Patel, N., Freeman, A., & Aragon-Ching, J. B. (2013). Metastatic castration-resistant prostate cancer: Critical review of enzalutamide. *Clinical Medicine Insights.Oncology*, 7, 235-245.

El-Merahbi, R., Liu, Y. N., Eid, A., Daoud, G., Hosry, L., Monzer, A., et al. (2014). Berberis libanotica ehrenb extract shows anti-neoplastic effects on prostate cancer stem/progenitor cells. *PloS One*, 9(11), e112453.

Fiorillo, M., Verre, A. F., Iliut, M., Peiris-Pages, M., Ozsvari, B., Gandara, R., et al. (2015). Graphene oxide selectively targets cancer stem cells, across multiple tumor types: Implications for non-toxic cancer treatment, via "differentiation-based nano-therapy". *Oncotarget*, 6(6), 3553-3562.

Fontana, J. A., & Rishi, A. K. (2002). Classical and novel retinoids: Their targets in cancer therapy. *Leukemia*, 16(4), 463-472.

Fratelli, M., Fisher, J. N., Paroni, G., Di Francesco, A. M., Pierri, F., Pisano, C., et al. (2013). New insights into the molecular mechanisms underlying sensitivity/resistance to the atypical retinoid ST1926 in acute myeloid leukaemia cells: The role of histone H2A.Z, cAMP-dependent protein kinase A and the proteasome. *European Journal of Cancer (Oxford, England: 1990)*, 49(6), 1491-1500.

Garattini, E., & Terao, M. (2004). Atypical retinoids: An expanding series of anti-leukemia and anti-cancer agents endowed with selective apoptotic activity. *Journal of Chemotherapy* (Florence, Italy), 16 Suppl 4, 70-73.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, 144(5), 646-674.

Harris, W. P., Mostaghel, E. A., Nelson, P. S., & Montgomery, B. (2009). Androgen deprivation therapy: Progress in understanding mechanisms of resistance and optimizing androgen depletion. *Nature Clinical Practice.Urology*, 6(2), 76-85.

Hayat, M. J., Howlader, N., Reichman, M. E., & Edwards, B. K. (2007). Cancer statistics, trends, and multiple primary cancer analyses from the surveillance, epidemiology, and end results (SEER) program. *The Oncologist*, 12(1), 20-37.

Hayat, M. J., Howlader, N., Reichman, M. E., & Edwards, B. K. (2007). Cancer statistics, trends, and multiple primary cancer analyses from the surveillance, epidemiology, and end results (SEER) program. *The Oncologist*, 12(1), 20-37.

Hong, W. K. (2010). *Holland-frei cancer medicine* (8th edition). New York: PMPH USA, Ltd.

Huggins, C., & Hodges, C. V. (2002). Studies on prostatic cancer: I. the effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *The Journal of Urology*, 168(1), 9-12.

Huss, W. J., Lai, L., Barrios, R. J., Hirschi, K. K., & Greenberg, N. M. (2004). Retinoic acid slows progression and promotes apoptosis of spontaneous prostate cancer. *The Prostate*, 61(2), 142-152.

Jiao, J., Hindoyan, A., Wang, S., Tran, L. M., Goldstein, A. S., Lawson, D., et al. (2012). Identification of CD166 as a surface marker for enriching prostate stem/progenitor and cancer initiating cells. *PloS One*, 7(8), e42564.

Kabbout, M., Hatoum, A., Abou-Lteif, G., Chakroun, I., Homaidan, F. R., & Darwiche, N. (2004). Stage-specific effect of N-(4-hydroxyphenyl)retinamide on cell growth in squamous cell carcinogenesis. *Molecular Carcinogenesis*, 40(1), 12-23.

Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., & Jones, L. W. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Investigative Urology*, 17(1), 16-23.

Kassouf, W., Tanguay, S., & Aprikian, A. G. (2003). Nilutamide as second line hormone therapy for prostate cancer after androgen ablation fails. *The Journal of Urology*, 169(5), 1742-1744.

Keedwell, R. G., Zhao, Y., Hammond, L. A., Wen, K., Qin, S., Atangan, L. I., et al. (2004). An antagonist of retinoic acid receptors more effectively inhibits growth of human prostate cancer cells than normal prostate epithelium. *British Journal of Cancer*, 91(3), 580-588.

Kim, S. J., & Kim, S. I. (2011). Current treatment strategies for castration-resistant prostate cancer. *Korean Journal of Urology*, 52(3), 157-165.

Kuo, L. J., & Yang, L. X. (2008). Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo (Athens, Greece)*, 22(3), 305-309.

Mary Ann Asson-Batres, & Cecile Rochette-Egly. (2014). *The biochemistry of retinoic acid receptors I: Structure, activation, and function at the molecular level: Subcellular biochemistry 70*. DE: Springer Verlag.

McCormick, F. (1999). Signalling networks that cause cancer. *Trends in Cell Biology*, 9(12), M53-6.

McNeal, J. E. (1981). The zonal anatomy of the prostate. *The Prostate*, 2(1), 35-49.

Monneret, C. (2013). Four new drugs on the market: Abiraterone, belatacept, vandetanib and fidaxomicine. [Actualites therapeutiques: l'abiraterone, le belatacept, le vandetanib et la fidaxomicine] *Annales Pharmaceutiques Francaises*, 71(2), 95-103.

Nasr, R. R., Hmadi, R. A., El-Eit, R. M., Iskandarani, A. N., Jabbour, M. N., Zaatari, G. S., et al. (2014). ST1926, an orally active synthetic retinoid, induces apoptosis in chronic myeloid leukemia cells and prolongs survival in a murine model. *International Journal of Cancer*.*Journal International Du Cancer*.

Ni, J., Cozzi, P., Hao, J., Duan, W., Graham, P., Kearsley, J., et al. (2014). Cancer stem cells in prostate cancer chemoresistance. *Current Cancer Drug Targets*, 14(3), 225-240.

Parrella, E., Gianni, M., Fratelli, M., Barzago, M. M., Raska, I., Jr, Diomedede, L., et al. (2006). Antitumor activity of the retinoid-related molecules (E)-3-(4'-hydroxy-3'-adamantylbiphenyl-4-yl)acrylic acid (ST1926) and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) in F9 teratocarcinoma: Role of retinoic acid receptor gamma and retinoid-independent pathways. *Molecular Pharmacology*, 70(3), 909-924.

Pasquali, D., Rossi, V., Bellastella, G., Bellastella, A., & Sinisi, A. A. (2006). Natural and synthetic retinoids in prostate cancer. *Current Pharmaceutical Design*, 12(15), 1923-1929.

Pisano, C., Merlini, L., Penco, S., Carminati, P., & Zunino, F. (2004). Cellular and pharmacological bases of the antitumor activity of a novel adamantyl retinoid, ST1926. *Journal of Chemotherapy (Florence, Italy)*, 16 Suppl 4, 74-76.

Pozo, L., Sanchez-Carrillo, J. J., Martinez, A., Blanes, A., & Diaz-Cano, S. J. (2007). Differential kinetic features by tumour topography in cutaneous small-cell neuroendocrine (merkel cell) carcinomas. *Journal of the European Academy of Dermatology and Venereology : JEADV*, 21(9), 1220-1228.

Qian, S. B., Shen, H. B., Cao, Q. F., Zhang, L., Chen, Y. F., & Qi, J. (2015). Bicalutamide 150 mg as secondary hormonal therapy for castration-resistant prostate cancer. *International Urology and Nephrology*, 47(3), 479-484.

Quintela, M. L., Mateos, L. L., Estevez, S. V., Calvo, O. F., Herranz, U. A., Afonso, F. J., et al. (2015). Enzalutamide: A new prostate cancer targeted therapy against the androgen receptor. *Cancer Treatment Reviews*, 41(3), 247-253.

Roudier, M. P., True, L. D., Higano, C. S., Vesselle, H., Ellis, W., Lange, P., et al. (2003). Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. *Human Pathology*, 34(7), 646-653.

Sala, F., Zucchetti, M., Bagnati, R., D'Incalci, M., Pace, S., Capocasa, F., et al. (2009). Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of ST1926, a novel oral antitumor agent, adamantyl retinoid derivative, in plasma of patients in a phase I study. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences*, 877(27), 3118-3126.

Sartor, A. O. (2011). Progression of metastatic castrate-resistant prostate cancer: Impact of therapeutic intervention in the post-docetaxel space. *Journal of Hematology & Oncology*, 4, 18-8722-4-18.

Schenk, T., Stengel, S., & Zelent, A. (2014). Unlocking the potential of retinoic acid in anticancer therapy. *British Journal of Cancer*, 111(11), 2039-2045.

Schneider-Stock, R., Ghantous, A., Bajbouj, K., Saikali, M., & Darwiche, N. (2012). Epigenetic mechanisms of plant-derived anticancer drugs. *Frontiers in Bioscience (Landmark Edition)*, 17, 129-173.

Scott D. Cramer. (2013). *Stem cells and prostate cancer*. DE: Springer Verlag.

Scott, S. L., Earle, J. D., & Gumerlock, P. H. (2003). Functional p53 increases prostate cancer cell survival after exposure to fractionated doses of ionizing radiation. *Cancer Research*, 63(21), 7190-7196.

Sharpe, B., Beresford, M., Bowen, R., Mitchard, J., & Chalmers, A. D. (2013). Searching for prostate cancer stem cells: Markers and methods. *Stem Cell Reviews*, 9(5), 721-730.

Siddikuzzaman, Guruvayoorappan, C., & Berlin Grace, V. M. (2011). All trans retinoic acid and cancer. *Immunopharmacology and Immunotoxicology*, 33(2), 241-249.

Siegel, R. L., Miller, K. D., & Jemal, A. (2015). Cancer statistics, 2015. *CA: A Cancer Journal for Clinicians*, 65(1), 5-29.

Sobel, R. E., & Sadar, M. D. (2005). Cell lines used in prostate cancer research: A compendium of old and new lines--part 1. *The Journal of Urology*, 173(2), 342-359.

Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., & Paulson, D. F. (1978). Isolation of a human prostate carcinoma cell line (DU 145). *International Journal of Cancer*. Journal International Du Cancer, 21(3), 274-281.

Suzuki, H., Okihara, K., Miyake, H., Fujisawa, M., Miyoshi, S., Matsumoto, T., et al. (2008). Alternative nonsteroidal antiandrogen therapy for advanced prostate cancer that relapsed after initial maximum androgen blockade. *The Journal of Urology*, 180(3), 921-927.

Tang, X. H., & Gudas, L. J. (2011). Retinoids, retinoic acid receptors, and cancer. *Annual Review of Pathology*, 6, 345-364.

Taylor, R. A., Toivanen, R., & Risbridger, G. P. (2010). Stem cells in prostate cancer: Treating the root of the problem. *Endocrine-Related Cancer*, 17(4), R273-85.

Theodosiou, M., Laudet, V., & Schubert, M. (2010). From carrot to clinic: An overview of the retinoic acid signaling pathway. *Cellular and Molecular Life Sciences : CMLS*, 67(9), 1423-1445.

Tsagozis, P., Augsten, M., & Pisa, P. (2014). All trans-retinoic acid abrogates the pro-tumorigenic phenotype of prostate cancer tumor-associated macrophages. *International Immunopharmacology*, 23(1), 8-13.

Tu, S. M., & Lin, S. H. (2012). Prostate cancer stem cells. *Clinical Genitourinary Cancer*, 10(2), 69-76.

Valli, C., Paroni, G., Di Francesco, A. M., Riccardi, R., Tavecchio, M., Erba, E., et al. (2008). Atypical retinoids ST1926 and CD437 are S-phase-specific agents causing DNA double-strand breaks: Significance for the cytotoxic and antiproliferative activity. *Molecular Cancer Therapeutics*, 7(9), 2941-2954.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature*, 408(6810), 307-310.

Wu, G., & Wu, D. L. (2015). Prostate cancer stem cells: Advances in current research. *Zhonghua Nan Ke Xue = National Journal of Andrology*, 21(2), 170-174.

Zhang, Y., Rishi, A. K., Dawson, M. I., Tschang, R., Farhana, L., Boyanapalli, M., et al. (2000). S-phase arrest and apoptosis induced in normal mammary epithelial cells by a novel retinoid. *Cancer Research*, 60(7), 2025-2032.

Zuco, V., Zanchi, C., Lanzi, C., Beretta, G. L., Supino, R., Pisano, C., et al. (2005). Development of resistance to the atypical retinoid, ST1926, in the lung carcinoma cell line H460 is associated with reduced formation of DNA strand breaks and a defective DNA damage response. *Neoplasia (New York, N.Y.)*, 7(7), 667-677.

Zuo, J., Guo, Y., Peng, X., Tang, Y., Zhang, X., He, P., et al. (2015). Inhibitory action of pristimerin on hypoxia-mediated metastasis involves stem cell characteristics and EMT in PC-3 prostate cancer cells. *Oncology Reports*, 33(3), 1388-1394.