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Discovery of a Novel CCR5 Antagonist as an Effective Therapeutic Agent for Prostate Cancer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

by

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List of Abbreviations

%	percent
°C	degrees Celsius
μM	micromolar
Å	Angstroms
AAH	atypical adenomatous hyperplasia
AP-1	activatory protein 1
AR	androgen receptor
BPH	benign prostatic hyperplasia
CCL5	CC chemokine ligand 5
CCR5	CC chemokine receptor 5
CDCl ₃	deuterated chloroform
CI	chronic inflammation
CO ₂	carbon dioxide
COX-2	cyclooxygenase enzyme 2
CXCL12	CXC chemokine ligand 12
CXCL16	CXC chemokine ligand 16
CXCL8	CXC chemokine ligand 8
CXCR4	CXC chemokine receptor 4
DAG	Diacylglycerol
DC	dendritic cells
DHT	dihydroxytestosterone
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DU-145	dura mater derived prostate cancer cell line
ECM	extracellular matrix
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor
EGF-R	epidermal growth factor receptor
EL2	extracellular loop 2
EP	endocrine-paracrine cell
FBS	fetal bovine serum
FGF-R	fibroblast growth factor receptor
g	grams
G-CSF	granulocyte-colony stimulating factor
GOLD	genetic optimization for ligand binding
GPCR	G protein coupled receptor
HIV	human immunodeficiency disorder
HPV	human papillomavirus
Ic ₅₀	half maximal inhibitory concentration
IFN	interferon
IGF	insulin-like growth factor

IGFB	insulin-like growth factor binding protein
IGF-I	insulin-like growth factor type 1
IGFR	insulin-like growth factor receptor
IGFR	insulin-like growth factor receptor
IL	interleukin
IP3	inositol-1,4,5-triphosphate
IR	infrared
ITS	insulin, transferrin, selenium
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LC50	half maximal lethal concentration
LNCaP	lymph node derived prostate cancer cell line
m	multiplet
M12	metastatic prostate cancer cell line
MeOH	methanol
MIF	mitosis initiation factor
MIP	mitosis initiation factor
mL	milliliters
mmol	millimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleotidic acid
NAD:	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
nm	nanometers
nM	nanomolar
NMR	nuclear magnetic resonance
p53	tumor protein 53
P69	non-neoplastic prostate epithelial cell line P
PBS	phosphate buffer solution
PC-3	bone derived prostate cancer cell line
PCA	prostate cancer
PIA	proliferative inflammatory atrophy
PIN	prostate intraepithelial neoplasia
PIP2	phosphatidylinositol-4,5-bisphosphate
PKC	Protein Kinase C
PLC	phospholipase C
PSA	prostatic specific antigen
PYK2	protein tyrosine kinase 2
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Abstract

DISCOVERY OF A NOVEL CCR5 ANTAGONIST AS AN EFFECTIVE THERAPEUTIC AGENT FOR PROSTATE CANCER

By Tasrif Ahmed

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: Zendra Zehner, Professor, Department of Biochemistry

Previously, the CCR5 receptor was found to be a good target for treating prostate cancer (PCa). Dr. Yan Zhang's laboratory designed several CCR5 antagonists, which were screened for their inhibitory effect on the growth and invasion of the M12, DU145 and PC-3 PCa cell lines. Primary *in vitro* screening showed one compound (Drug 17) significantly inhibited the proliferation of PCa cells at 1 μ M concentration, with a half-maximal inhibitory concentration of 237.68 nM. Further *in vitro* assays including a proliferation, cytotoxicity and invasion assay confirmed the inhibitory effect of drug 17. The physiological effect of drug 17 was tested by the Ware laboratory *in vivo* by subcutaneous injection of M12 cells into male, athymic nude mice. Tumor growth was slowed in mice receiving injections of drug 17 compared to sham injected

controls. Thus, in vitro and in vivo assays suggest drug 17 might be an effective therapy to block PCa progression.

I. Background

Prostate Cancer

Prostate cancer (PCa) originates in the prostate gland, which is an accessory male reproductive organ. The prostate gland surrounds the urethra that carries urine and semen and secretes its products into the urethra. A few symptoms of PCa are urinary hesitancy, urinary retention, pain with urination, pain with ejaculation, and pain with bowel movement [1]. According to the American Cancer Society's Cancer Statistics for the year 2009, PCa will be the most diagnosed cancer in males. It is also the second most lethal cancer in men after lung cancer [2]. Since 1991, many major chronic disease rates have decreased substantially but the rate of death from cancer only decreased by 16%, although this rate varies among various ethnic groups and genders [2]. The cancer death rate is higher in men than women in every ethnic group. African Americans exhibit the highest cancer death rate whereas Asian American and Pacific Islanders have the lowest [3]. Several therapies are available for prostate cancer, but they are only beneficial at the early stage, with no significant effect after metastasis [4]. PCa can metastasize to several sites, but bone metastasis is fatal [5]. In the past, chronic inflammation has been linked to many types of cancers, but recent investigation showed that there is also a relationship between prostate cancer and inflammation. Significant up-regulation of various inflammatory chemokines and receptors in prostate cancer compared to benign prostatic hyperplasia (BPH) has been noted.

Classification of Prostate Cancer and Other Prostate Abnormalities

PCa is classified as a slow progressing adenocarcinoma (cancer of the glandular tissue). The glands of the prostate produce about 25-30% of the semen volume consisting of acid phosphatase, citric acid, fibrinolysin, prostate specific antigen (PSA), proteolytic enzymes, and zinc. This makes the semen slightly alkaline to neutralize the acidity from the vaginal tract and therefore, increase the sperm lifespan [6]. The prostate consists of glandular tissue and mesenchymal stroma. It has tubulo-acinar glands that open into the prostatic urethra. The glands are divided into zones: central, transitional and peripheral. The peripheral zone makes up 70% of the prostate where PCa is thought to originate [7]. The prostate epithelium consists of three different types of cells: secretory luminal, basal, and endocrine paracrine (EP) cells. Secretory luminal cells are the major phenotype in normal prostate epithelium and are highly proliferative. Their differentiation and development is controlled by the stroma through androgen secretion. Secretory cells express the androgen receptor (AR) and require a continuous supply of androgens for growth; however, basal cells are androgen-independent. The stroma and the luminal epithelium cells act in a paracrine fashion to support the development and differentiation of each other by releasing various factors [8]. The AR expression level is higher during prostate hypertrophy compared to the normal prostate. For this reason, androgen suppression is a widely used first line of defense against prostate cancer. However, this may lead to the selection of androgen-independent cancer cells [9].

There are several different pathways by which cells can become androgen independent, such as the hypersensitivity, promiscuous, outlaw, bypass or lurker cell pathway [9]. In the hypersensitivity pathway the AR expression is up-regulated or AR is more sensitive for the ligand to compensate for the low levels of androgen. In the promiscuous pathway, the AR loses

its specificity and binds other molecules. In the outlaw pathway, receptor tyrosine kinases are activated so the AR is activated through phosphorylation by protein kinase B (AKT) or mitogen-activated protein kinase (MAPK) such that androgen binding is obviated. In the bypass pathway, other parallel survival pathways are activated, which involve anti-apoptotic proteins that no longer require the AR pathway. Lastly, in the lurker cell pathway androgen independent cells are selected for by androgen depression therapy [9].

Beside PCa, several other types of abnormalities occur in different zones of the prostate gland such as BPH, atypical adenomatous hyperplasia (AAH), proliferative inflammatory atrophy (PIA) and prostate intraepithelial neoplasia (PIN). BPH and AAH occur in the transitional zone while PIA and PIN occur in the peripheral zone [10]. BPH is very common in men as they age and usually is non-symptomatic and non-cancerous [11,12]. What causes the prostate cells to become cancerous is unknown, but the role of chronic inflammation is evident from previous studies.

Diagnosis of Prostate Cancer

The median age of diagnosis for PCa is 68 years [13]. There are several available ways to diagnose localized PCa such as blood PSA levels, digital rectal examination, and Gleason score of biopsied material. One study suggests that a “Gleason score higher than 6, PSA levels higher than 40 ng/mL, and white skin color are independent markers of poor prognosis” [14]. There is a controversy over whether the PSA level should be used for diagnosing PCa, because it often leads to over detection and patient anxiety from false-positives. However, another study suggests that having more information is better than having no information at all, and thus, PSA levels should be used together with other diagnostic techniques to diagnose PCa [15].

Treatments for Prostate Cancer

Various treatments are available for PCa including active surveillance, radical prostatectomy, radiation therapy, chemotherapy, and hormonal therapy. The optimal therapy varies for each person and physicians are encouraged to discuss all available options with the patient. The available hormonal therapy uses diethylstilbestrol or luteinizing hormone-releasing hormone to block the cells' growth response to the AR by reducing the release of testosterone from the testicles. However, not all PCa cells grow in response to AR so it can lead to selection of cells that are AR independent [16]. A person can also reduce the risk of PCa by maintaining their diet and other lifestyle changes. Vitamin A, retinoid, several B vitamins, vitamin C, vitamin D, and vitamin E act as anti-oxidants to help against PCa [17]. All the available treatments are only beneficial at early stages of PCa prior to metastasis. Therefore, there is a high demand for a treatment that will stop the metastasis and invasion of PCa cells.

The Role of Chronic Inflammation in Prostate Cancer

The role of chronic inflammation in various cancers is well known. Inflammation is the body's natural mechanism to fight foreign pathogens, heal damage, and respond to irritants. Inflammation is usually self-limiting and does not cause any harm to the body; however, sometimes the control is lost and inflammation becomes chronic leading to various diseases such as cancer, Crohn's disease, Blau syndrome, etc. [11]. What sets the fire by initiating inflammation in the prostate is unknown, but infectious agents increase the risk of PCa [18]. According to Lucia et al, inflammation can cause carcinogenesis by either releasing cytokines or growth factors that favor tumor growth, inducing the cyclooxygenase-2 enzyme (COX-2), or by the production of reactive oxygen (ROS) and reactive nitrogen species (RNS). ROS and RNS

can cause DNA damage and mutagenesis. Macrophages at the inflammatory site also release other factors that cause angiogenesis and stromal remodeling. The presence of an inflammatory tumor microenvironment to promote tumor growth was first hypothesized by Virchow in 1863. Chronic inflammation at the site of the tumor releases various factors that promote the survival and growth of the tumor. The cells of the immune system include macrophages and leukocytes, which release a wide spectrum of chemicals such as cytokines, interleukins (IL's), and growth factors that mediate inflammation. The receptors for growth factors are found both on the immune cells as well as on the tumor cells. All of these chemicals together create a tumor microenvironment and can cause carcinogenesis, tumor growth, and tumor progression [19].

Role of CCR5 and CCL5 in Prostate Cancer

Chemotactic Chemokine Receptor 5 (CCR5) and Chemotactic Chemokine Ligand 5 (CCL5) play an important role in the immune response and in cancer. CCR5 is a co-receptor for HIV entry and many anti-HIV drugs are designed as antagonists for CCR5. Chemokines and their receptors are used by tumor cells to promote angiogenesis, tumor growth, survival and metastasis [20]. Konig et al. compared the mRNA level of various chemokines and chemokine receptors in PCa versus BPH and found that CCL5 and CCR5 were both expressed at higher levels in PCa. Other factors were also up-regulated in PCa such as IL-8, MMP-9, MMP-2, and CXCR-3 [21]. Robinson et al correlated CCR5 and CCL5 with breast cancer disease progression and showed that a CCR5 antagonist inhibits breast tumor growth in the presence of CCL5 [22]. Vaday et al used the TAK-779, a receptor antagonist of CCR5 in the presence of CCL5 to test the effect of the antagonist on prostate cell growth and invasion. They compared the migration index and growth of DU-145 and PC-3 cells with CCL5 alone or CCL5 plus varying concentrations of TAK-779. Increasing the concentration of TAK-779 decreased the migration index and reduced

growth of DU-145 and PC-3 cells indicating that TAK-779 must affect the metastatic ability of these cells by opposing the effect of CCL5. These results suggest the importance of designing new, more effective antagonists of CCR5.

The Role of Chemokines in Prostate Cancer

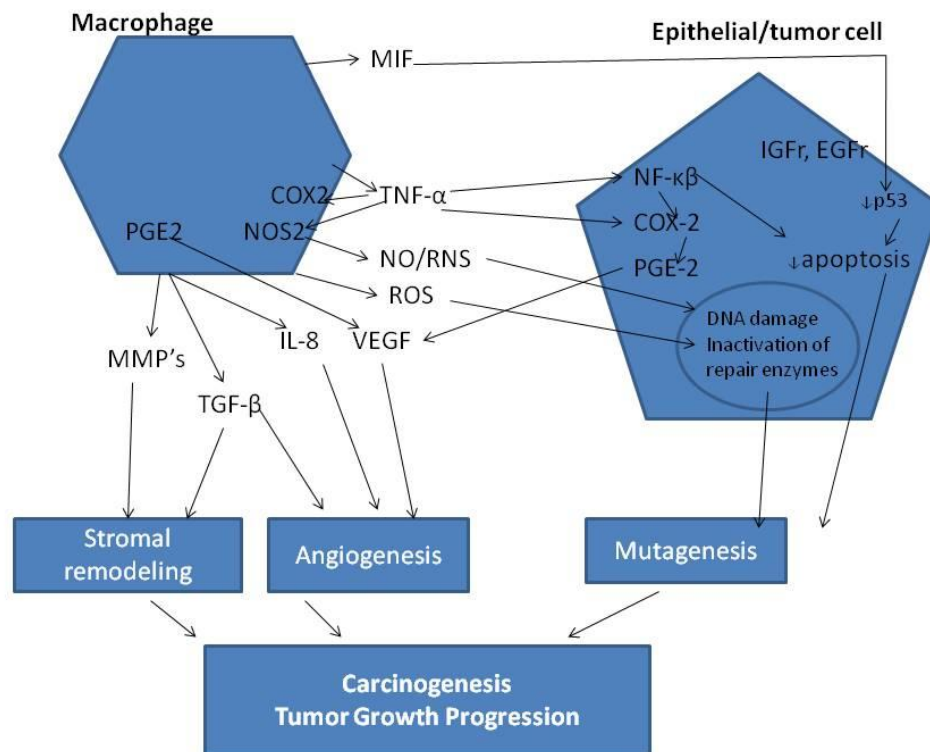
Chemokines and their receptors are used by immune cells such as macrophages and T cells. Chemokines are small soluble molecules that attract various types of cells such as leukocytes to induce inflammation. However, since chronic inflammation has been linked to tumor and cancer, chemokines can also play an important role in creating a microenvironment to enhance tumor survival. Receptors for chemokines can be found on target cells which by binding these soluble ligands promote numerous downstream events. Among these chemokines and chemokine receptors, CCR5 and CCL5 play a vital role in PCa by promoting angiogenesis, growth, survival, and invasion [20]. Figure 1 diagrams the basic chemokine pathway in cancer [19].

Tumor Microenvironment

Numerous studies confirm that human and murine tumor cells express chemokines and chemokine receptors, which are commonly involved in the autocrine pathway supporting the growth and survival of tumor cells. Figure 1 shows the possible role of macrophages in the tumor microenvironment in the promotion of tumor growth progression and carcinogenesis. How the inflammatory response initiates is not known, but once the fire starts, macrophages at the site of the tumor release various chemokines like mitosis initiation factor (MIF) which promotes cell division. They also induce the COX-2 enzyme that produces prostaglandins, which promotes MMP's, TGF- β , IL-8, and vascular endothelial growth factor (VEGF). Macrophages also release

Figure 1. Inflammation in Cancer. Macrophages in the tumor microenvironment produce factors such as MMP, TGF- β , VEGF, RNS, ROS, TNF- α , and interleukins. These factors cause stromal remodeling and angiogenesis at the tumor site. They also cause mutagenesis of the tumor epithelial cells and down-regulate apoptotic pathways, leading to mutagenesis. The end result of stromal remodeling, angiogenesis, and mutagenesis is carcinogenesis and tumor growth progression. Adopted from Lucia et al [19].

Figure 1. Inflammation in Cancer.



TNF- α that binds to its receptor on target cells and causes various downstream events including both apoptotic death and survival [19]. TNF- α can activate NF- κ B and promote cell survival and proliferation. It can also activate MAPK pathways and caspases to promote apoptosis. IL-8, VEGF, and TGF- β released from the macrophage promote angiogenesis of tumor tissue to provide more blood and nutrients to the tumor site. MMP's cleave extracellular matrix to help tumor cells metastasize. Macrophages produce ROS and RNS, which cause DNA damage and inactivation of repair enzymes of tumor cells, causing mutagenesis. Thus, the net effect of the chemokines is to promote carcinogenesis [19].

Chemokines and Chemokine Receptors

Chemokines and their receptors are grouped into four families. Chemokines are classified on the position of cysteines within the primary amino acid sequence. In the C family there is only one cysteine [16]. In CC chemokines the cysteines are adjacent, in CXC and CX₃C there is one or three non-cysteine residues between the cysteines, respectively. Chemokines guide cells as a chemoattractant by setting up a chemical concentration gradient for chemotaxis (movement of cells towards a concentration gradient).

Chemokine receptors are also classified in groups based on what chemokines they bind [16]. Chemokine receptors are G-protein-coupled receptors (GPCRs), which can bind many different ligands. Upon binding of the ligand, chemokine receptors act on G-proteins to activate their function inside the target cell [16]. Chemokines induce the inflammatory response by attracting leukocytes to the tumor site and induce tumor growth. According to the counter invasion model, chemokines are secreted from tumor cells to attract leukocytes, which degrade the extracellular matrix during entry into the site of tumor, which then allows the tumor cells to enter the blood vessels and migrate to remote sites [23].

Role of MMP in Metastasis of Prostate Cancer

Chemokines play a dual role. Not only do they attract leukocytes and elicit the immune response to take care of infections or tissue damage, but they also act in tumor survival and metastasis. There are a few key steps that tumor cells must follow to metastasize, but the exact mechanism is not clearly defined. In order to metastasize, the tumor cell must detach and degrade extracellular matrix, enter the blood or lymphatic vessel and then traffic to the remote tissue [16]. MMP's are very important in the metastasis process because they are responsible for degrading the extracellular matrix, specifically the type IV and V collagens. MMP's are first secreted as inactive proproteins which are activated by the cleavage of other proteinases. There is no direct link between MMP-9 level and CCL5 in PCa; however, CCL5 and MMP-9 were both up-regulated in PCa compared to BPH [21]. Experiments in breast cancer confirmed that CCL5 expression up-regulates MMP-9 and furthers disease progression [16]. Vaday et al. showed that CCR5 antagonists for CCL5 reduced the invasive and metastatic ability of PCa cells; therefore, CCL5 must promote metastasis in PCa [20]. This result suggests that CCL5 is involved in the production of MMP-9, which results in the metastasis of PCa cells.

Chemokines such as CXCL12 and its receptor CXCR4 have been shown to be involved in breast cancer metastasis. Antibody for the receptor blocked the metastatic ability of the breast cancer cells [16]. The chemokines possibly setup a chemical gradient to attract cancer cells, displaying the appropriate receptors. Once these cells have reached the target site, a secondary tumor develops with the help of similar chemokines found in the tumor environment at the metastatic site [16].

CCL5 Function

CCL5 contains 91 amino acids and is part of the CC family of chemokines usually expressed in T-cells with increased expression following antigen stimulation. CCL5 is one of the HIV-suppressive factors produced by CD8-positive T cells and is involved in the basic immune response against viruses. CCR5 is a co-receptor for HIV-entry into cells and CCL5 acts as an inhibitor of HIV interacting with CCR5. Macrophages use CCL5 to clear viruses and in CCL5-deficient mice, viral clearance is delayed [24]. CCL5 exerts its effect by binding to CCR5 on neighboring cells and activating the ERK, MAPK, and AKT pathways in a paracrine fashion. CCL5 also binds CCR1 and CCR3 [24]. Previous studies suggest that CCL5 levels are directly correlated with tumorigenicity and invasiveness of cancer cells [20].

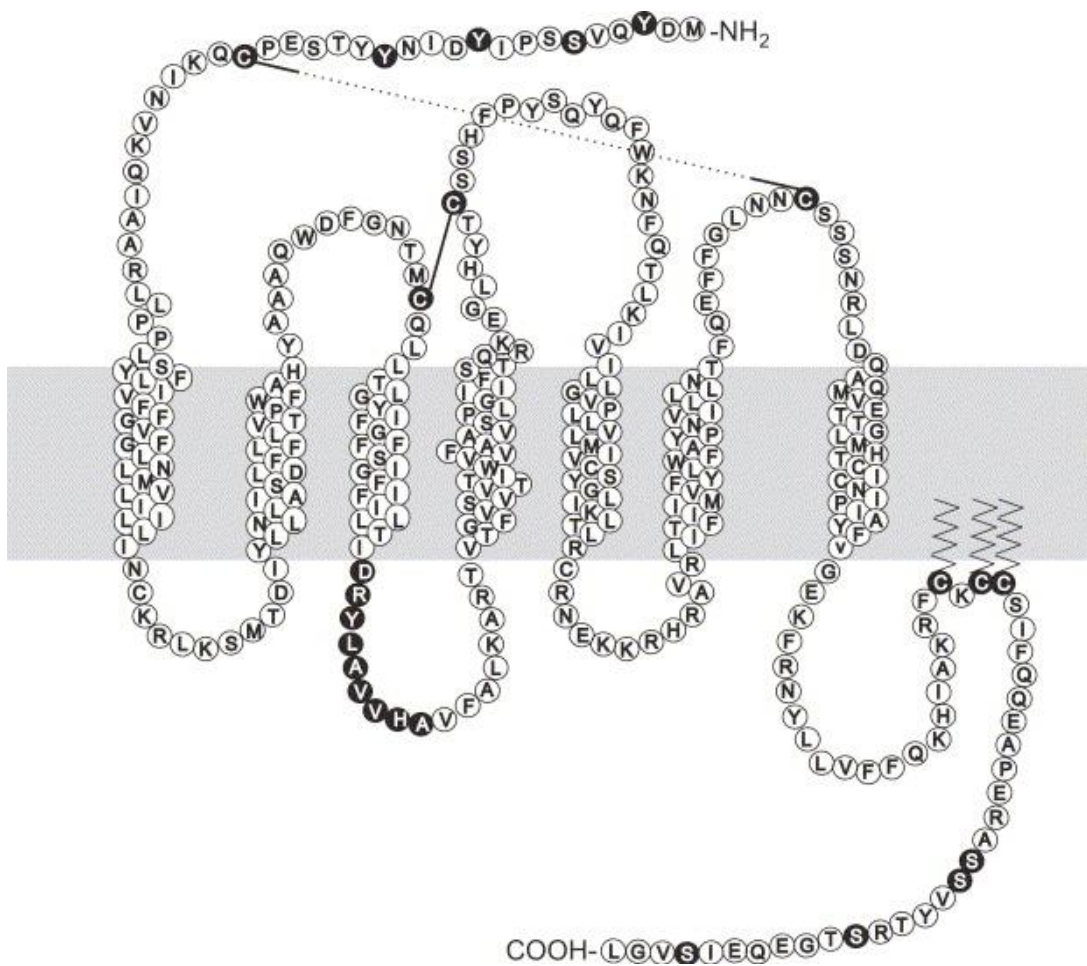
CCR5 Structure and Signaling Pathway

CCR5 is a GPCR chemokine receptor belonging to the rhodopsin-like family. It has 7 transmembrane segments (TMS), 3 intracellular loops, 3 extracellular loops, an extracellular N-terminus, an intracellular C-terminus, and is located on chromosome 3p21 in humans [16]. CCR5 is preferentially expressed on Th1 cells, monocytes, macrophages, and immature dendritic cells [16]. It is also a co-receptor of HIV-1 entry binding to the HIV-1 envelope glycoprotein gp120 along with CD4. A mutation in CCR5 called CCR5 Δ 32 (containing a 32 base pair deletion) leads to resistance against HIV [16]. CCR5 is “posttranslationally modified by o-linked glycosylation and sulfation of N-terminal tyrosines” [25]. Rhodopsin-like family members have highly conserved amino acids and a disulfide bridge that connects the first and second extracellular loops and many have palmitoylated cysteines in the carboxy-terminal tail. CCR5 has 4 cysteine residues in its extracellular region and a conserved DRYLA amino acid sequence in the second intracellular loop to bind G protein (see Figure 2) [16].

Figure 2. The Two Dimensional Structure of CCR5 with Palmitoylation Sites and Two Disulfide Bonds.

The 2-D structure of CCR5 is shown with 7 transmembrane α -helical segments with extracellular N-terminal and intracellular C-terminus. The top side is extracellular whereas the bottom is intracellular. CCR5 consists of 3 intracellular and 3 extracellular loops. The palmitoylated cysteines are shown with zig-zag lines. The extracellular cysteines form disulfide bonds depicted as solid and dashed lines. Adopted from Dr. Yan Zhang's unpublished grant proposal.

Figure 2. The Two Dimensional Structure of CCR5 with Palmitoylation Sites and Two Disulfide Bonds.

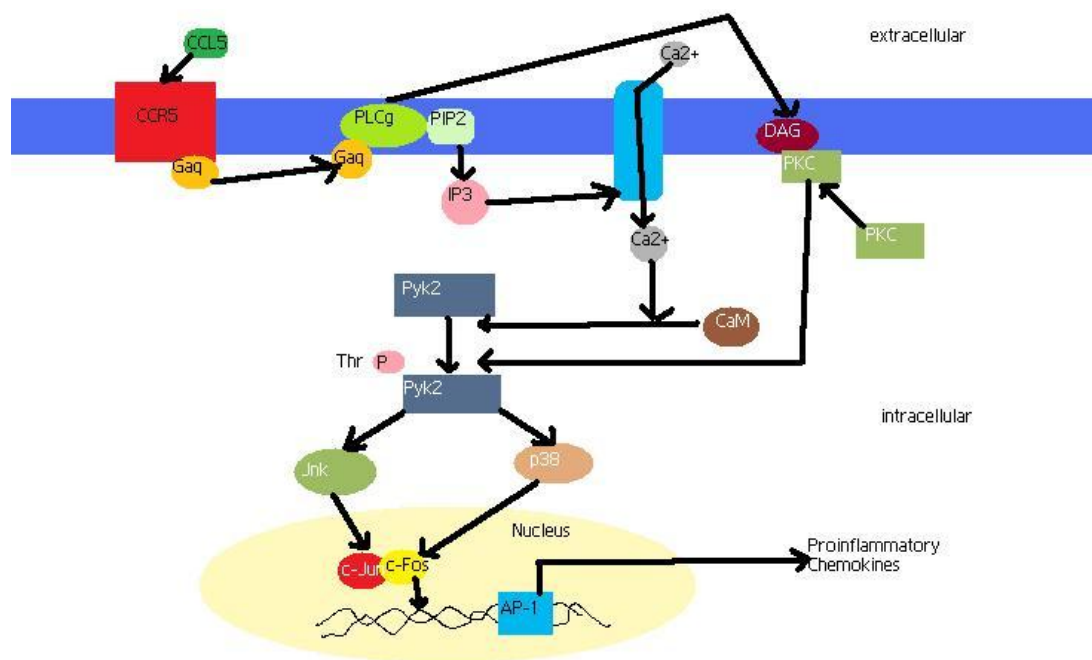


CCR5 binds CCL5 and Macrophage Inflammatory Protein (MIP- α and MIP- β). MIP- α and β are now known as CCL3 and CCL4 respectively [16, 25]. CCR5 is involved in migration, chemotaxis, cellular shape changes, metastasis, degranulation, and ROS production [26]. Figure 3 outlines the chemokine/chemokine receptor pathway for CCL5 and CCR5 [27]. CCR5 is associated with a G-protein and upon ligand binding CCR5 exerts its effect on the cell through G-protein subunits. G-proteins are trimeric proteins composed of α , β , and γ subunits. $G\alpha$ dissociates from $G\beta\gamma$ upon binding GTP and re-associates with $G\beta\gamma$ upon hydrolysis of GTP to GDP. Ligand binding promotes the exchange of GDP for GTP on $G\alpha$ and causes the dissociation of $G\alpha$ from $G\beta\gamma$. Subsequently $G\beta\gamma$ binds phospholipase C γ (PLC- γ) which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). PIP2 increases intracellular Ca^{2+} ion concentration by opening the Ca^{2+} channel while DAG activates the protein kinase C (PKC) enzyme. Ca^{2+} forms a complex with calmodulin and together with PKC activates protein tyrosine kinase 2 (PYK2) through phosphorylation. PYK2 activates c-Jun N-terminal kinase (JNK) and p38, which then activates c-Jun and c-Fos respectfully [27]. C-Jun and c-Fos together form the activator protein (AP-1), a transcription factor, which regulates the gene expression of various molecules such as cytokines and growth factors. These factors induce chemoattraction, inflammation, tumor growth, and carcinogenesis [28]. There is no clear evidence linking CCL5 and production of MMP's but studies in breast cancer showed that up-regulation of MMP-9 increased with CCL5 expression allowing the tumor cells to degrade ECM and migrate to the blood vessel [16]. Therefore, inhibiting CCL5/CCR5 interaction could be an important mechanism to block the production of proinflammatory signals that sustain chronic inflammation at the tumor site and also promote tumor growth and metastasis.

Figure 3. The CCR5 and CCL5 Pathway in Macrophages.

The signaling pathway of CCL5 and CCR5 is depicted. The arrows represent the binding or activation of each factor in the signal transduction pathway. The blue solid rectangle represents the plasma membrane, top is extracellular side and bottom is intracellular side. The end result is the expression of various gene products in the nucleus by the binding of CCL5 to CCR5. Binding of CCL5 leads to activation of the $G\alpha$ subunit, which dissociates from $G\beta$ and $G\gamma$. $G\alpha$ activates $PLC\gamma$, which converts PIP2 to IP3 and DAG. IP3 acts on Ca^{2+} channel to cause entrance of Ca^{2+} ions into the cell. DAG activates PKC enzyme. Ca^{2+} forms a complex with calmodulin (CaM) and acts on Pyk2 along with PKC to activate Pyk2 through phosphorylation. Pyk2 activates Jnk and p38, which activates the c-Jun and c-Fos transcription factors in the nucleus. Together they form the early response transcription factor AP-1. AP-1 leads to the expression of proinflammatory chemokines [27, 28]. Adopted from Chemokine Signaling pathway at <http://www4.appliedbiosystems.com/tools/pathway/>

Figure 3. The CCR5 and CCL5 Pathway in Macrophages.



Prostate cancer cell lines

Immortalized prostate cancer cell lines are excellent *in vitro* models to study PCa. They allow the study of anti-proliferative, anti-invasive, and anti-metastasis factors [12]. In this project, three immortalized PCa cell lines have been used to study the effect of CCR5 antagonists on prostate tumor growth and metastasis. These tumor cell lines do not share any genetic background and two are derived from metastatic tumor sites.

a. PC-3 Cell line

The PC-3 cell line was isolated from a human PCa metastasis to the lumbar vertebra. PC-3 cells are poorly differentiated and do not express PSA. The PC-3 cells are androgen independent and therefore do not respond to positive estrogen therapy [29]. PC-3 displays anchorage independent growth and expresses high levels of TGF- α , TGF- β , epidermal growth factor (EGF), insulin-like growth factor (IGF) and their receptors. These growth factors and their receptors might form an autocrine loop resulting in autonomous growth for PC-3 cells [30].

b. DU-145 Cell line

The DU-145 cell line was isolated from a metastatic central nervous system (CNS) tumor. They produce a high amount of TGF- β , EGF, IGF-1, TGF- α and TGF receptor. Like PC-3, they also potentially exhibit an autocrine growth loop. DU-145 cells are also androgen independent and do not respond to positive estrogen therapy [30].

c. M12 Cell line

The M12 cell line was derived from the parental P69SV40T cell line [31, 32]. P69SV40T was derived by immortalizing cells with a construct expressing the SV40 large-T antigen. P69SV40T cells were injected subcutaneously into male athymic nude mice where tumors formed after 9 months. Tumors were retrieved; M1929 cells were isolated and re-injected into

mice for a total of two cycles yielding M2182s and finally the M12 subline. The later subline was found to be invasive as their orthotropic tumors readily metastasized to the diaphragm and lungs. Moreover, M12 cells retain their tumorigenicity and metastatic capacity during *in vitro* culture as well, as measured by re-injection of cultured M12 cells into male athymic nude mice, which results in tumor growth requiring euthanasia. After euthanasia, M12 cells were found to metastasized to lung and diaphragm [31].

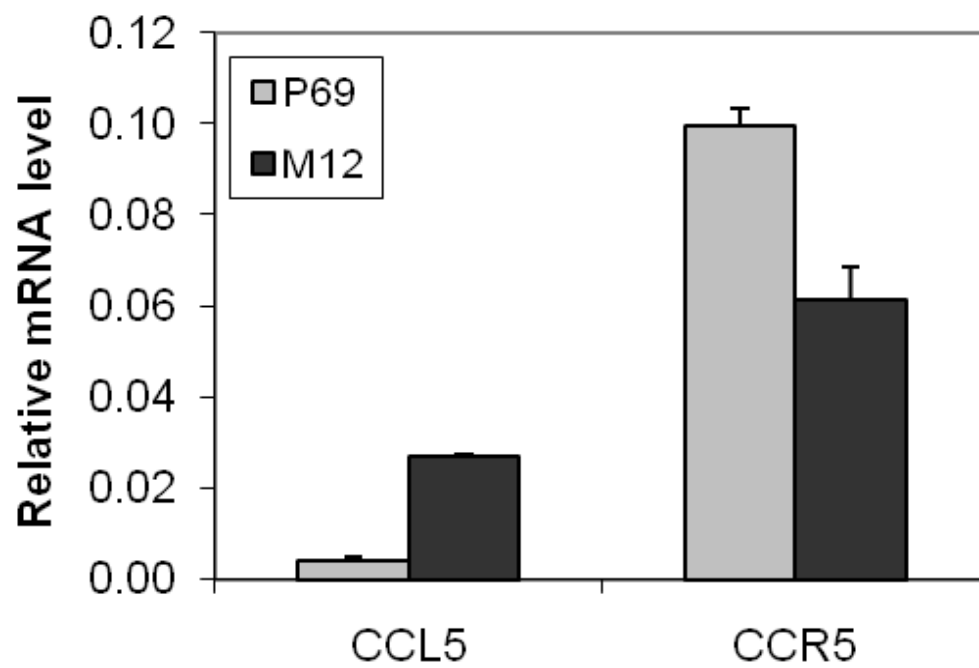
Dr. Xueping Zhang's (Department of Biochemistry, Virginia Commonwealth University, Richmond, VA) unpublished study compared the level of CCL5 and CCR5 mRNA between P69 and M12 cells by qRT-PCR (see Figure 4). P69 is the non-metastatic and non-tumorigenic parental cell line for the M12 subline. The M12 subline is highly tumorigenic and metastatic. The CCL5 mRNA level was highly upregulated in the M12 subline. However, the CCR5 level was lower in M12 than P69. It was proposed that this lower level of CCR5 was sufficient to bind enhanced levels of CCL5 and cause the up-regulation of downstream pro-inflammatory pathways. This result suggested that the level of CCL5 might be directly correlated with the invasiveness of PCa cells.

Figure 4. M12 Cells Express More CCL5 mRNA than Parental P69 Cells.

qRT-PCR measured the level of CCL5 and CCR5 mRNA in M12 and its parental P69 cell line.

The bars represent the standard error. P69, the parental cell line of M12, is less tumorigenic than M12. Adopted from Dr. Xueping Zhang's unpublished research.

Figure 4. M12 Cells Express More CCL5 mRNA than Parental P69.



Anti-Prostate Cancer Drug Design

The interaction between CCL5 and CCR5 was determined to be a good target for the design of an anti-PCa drug because CCL5 binding to CCR5 promotes the production of numerous chemokines that induce inflammation, tumor growth, and invasion [27]. Vaday et al has shown that a known CCR5 antagonist called TAK-779 significantly reduces the growth and invasiveness of PCa cells in the presence of CCL5 [20]. Based on this result, we hypothesized that CCR5 antagonists would be effective as an anti-prostate cancer agent. Using molecular modeling approaches several novel putative CCR5 antagonists were designed by Dr Yan Zhang (Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA), which have a high affinity for the CCR5 receptor. The structure of currently known CCR5 ligands including that part which interacts with the CCR5 binding site (the pharmacophore), was used to design CCR5 antagonists [23]. CCR5 is a GPCR whose crystal structure is unknown. Due to this fact, homology modeling is commonly used to study GPCR's. CCR5 belongs to the rhodopsin family of GPCR's and the closest GPCR with a known crystal structure is bovine rhodopsin. Therefore, the bovine rhodopsin crystal structure was used along with the pharmacophores of the known CCR5 ligands to characterize the binding of CCR5 antagonists. By this approach, several novel compounds were designed. Energy minimization and molecular dynamic simulation were used to select the optimal compounds. Based on the binding interface of the known ligands, Maraviroc and Vicriviroc to CCR5, a molecular scaffold was constructed [Dr. Yan Zhang's Unpublished Data].

This scaffold, along with the structure of the ligand binding pocket, was analyzed to design a basic structure of the novel compounds (drugs) to be tested as CCR5 antagonists [23]. The basic structure of such a drug includes a tri-substituted phenyl ring as a spacer which

connects amino and amide groups. There is a hydrogen bond acceptor on the phenyl ring. Different substitutions (R1, R2, and R3) were tried on both ends of the basic molecule.

II. Thesis Objectives

The purpose of this project was to evaluate the effect of the newly synthesized putative CCR5 antagonists in the presence of CCL5 on the tumorigenicity and invasiveness of PCa cell lines. The binding of CCL5 to CCR5 activates downstream signals that promote tumor growth and invasion. An effective CCR5 antagonist is expected to block this interaction between CCR5 and CCL5, stop the production of those downstream signals, and therefore, inhibit tumor growth and invasion. Three sets of drugs for a total of 76 drugs were tested for their effectiveness as CCR5 antagonists on M12, DU145, and PC-3 cell lines. The initial goal was to use a simple screening assay such as proliferation and cytotoxicity assays to test the effectiveness of all the drugs. A shortened list of lead drugs would then be subjected to further testing *in vitro* by a more rigorous assay such as the invasion assay. The responsibility for the primary assay was divided between Dr. Xueping Zhang and myself as follows: I would conduct the proliferation and cytotoxicity assays of the drugs on the M12 cell line whereas Dr. Zhang would conduct similar assays on the PC3 and DU145 cell lines. I would subsequently test the most effective compounds from these assays via the invasion assay for M12, PC3, and DU-145 cell lines. The most effective compounds would then be tested on subcutaneous tumor growth in male, athymic nude mice *in vivo* by Dr. Joy Ware (Department of Pathology, Virginia Commonwealth University, Richmond, VA). The objective was to find the most effective antagonist for CCR5 among the list of 76 drugs (see Table 1), starting with *in vitro* assays to assess cell proliferation and invasive potential and finalizing with *in vivo* tumor assays.

Table 1: List of the 76 Drugs That were Tested in *in vitro* Assays.

Set #	# of Drugs	Drugs
1	24	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25
2	24	27, 29, 30, 35, 36, 37, 38, 39, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69
3	28	21, 26, 28, 31, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162

III. Materials and Methods

Cell Media

M12 cells were cultured in RPMI 1640 medium (GIBCO Invitrogen, 21870) with 5% FBS (Invitrogen, S11150), 1% L-glutamine (Invitrogen, 25030), 0.1% ITS (insulin, 5ug/mL; transferrin, 5ug/mL; and selenium, 5ng/mL), and 0.1% gentamicin 10mg/mL (Gibco 15710). PC3 and DU145 cells were cultured in RPMI media containing 10% FBS.

2D Cell Culture

PCa cell lines M12, DU145 and PC3 were obtained in frozen aliquots from Dr. Xueping Zhang. Tubes of the frozen cells were quickly thawed in a water bath at 37°C, resuspended in 10 mL of culture media, centrifuged at 1700RPM for five minutes, and the supernatant removed by aspiration. The pellet was resuspended in 10mL fresh media and plated on 100mm x 20 mm plastic tissue culture dishes (Falcon, 353003). The cultures were incubated at 37°C in 5% CO₂. Cultures were split after 80-90% confluency and the media was changed every 48 hours or as needed.

To split the culture, the old media was first aspirated and 2 mL of trypsin-EDTA (Gibco Invitrogen, lot #39808) was added to the dish. After a few min when the cells had started to detach from the plate, 5mL of media was added to loosen the cells and cells plus media were transferred to a 50 mL centrifuge tube. Following centrifugation at 1700RPM for 5 min, the supernatant was removed; the cell pellet was resuspended with new media, and plated on a new dish at a split of 1:4.

Cell Counting

Cells were removed from the culture by adding trypsin/EDTA and washed with media containing serum. Cells plus media were transferred to a plastic tube in a total volume of 500uL. The cells were counted with an automated cell counter called Beckman Coulter (Vi-Cell XR Cell Viability Analyzer), which differentiates viable cells from dead cells using Trypan blue. If a cell is viable, it does not absorb trypan blue because of the high selectivity of the plasma membrane of live cells. However, trypan blue can pass through the membrane of dead cells and therefore it is absorbed. Thus, dead cells appear blue because of trypan blue absorbance and live cells appear clear because of the lack of trypan blue absorbance. The cell count was used to determine the correct number of cells needed for the cell proliferation or invasion assay. Cell counting was also used in the cytotoxicity assay to determine the number of cells alive after drug addition.

Cell Proliferation Assay

The cell counting method was used to calculate the volume of the media plus cell solution needed to plate 3000 cells per well in a 96-well plate. The media/cell solution was diluted with media to a total well volume of 100 uL for each well. To ensure the cells are plated evenly, 50 uL of media only was first plated and then 50 uL of the properly diluted cell/media solution was added to each well and gently mixed by pipetting. The plate was placed in the incubator for 24 hours at 37°C in 5% CO₂. After 24 hours, the media was changed and different drugs were added at varying concentrations to some wells while control wells received only media. Initially cell growth was measured every 24 hours after drug addition; however, it was experimentally determined that there was little effect on the growth of these cells before 72 hours. Subsequently, 72 hours became the only time point measured for each drug. After 72 hours, the serum media was aspirated and 100 uL of serum-free media (RPMI 1640 and 0.1%

gentamicin) was added to each well plus 10 uL of the WST-1 cell proliferation reagent (10% of the media amount). After 1 hour of incubation, the absorbance of each well was measured using a Microplate Bio-Kinetics Reader (BIO-TEK instrument) at 450nm, subtracting the background at 650nm. Each cell line was normalized to its control average. WST-1 is a slightly red solution which is cleaved to formazan, a dark red product, by the mitochondrial dehydrogenase enzyme. The amount of formazan formed is directly related to the activity of the mitochondrial dehydrogenase, which is correlated to the metabolic rate of active cells in the culture. A higher absorbance is thought to be proportional to a higher metabolic rate in direct correlation to an expected higher number of active cells, hence this assay is proposed to measure cell proliferation (company manual).

Cytotoxicity Assay

A cell count was done to calculate the volume of the cell solution required to plate 20,000 cells per well in a 24 well plate. The cells are diluted with media to a final volume of 1 mL per well. The plate was then incubated for 24 hours after which the media was changed. Drugs were added to the experimental wells at 1 uM concentration whereas media only was added to the control wells. After 72 hours, the cells were washed with 300 uL of PBS per well. To each well, 300 uL of Trypsin/EDTA was added to detach the cells from the plate and 300 uL of media was added. From this solution, 500 uL was transferred to a plastic tube for cell count. The cell count for the wells with drugs was compared to the control wells to determine the reduction in cell growth of cells caused by the addition of drugs. This procedure is referred to as a cytotoxicity assay since the effect of drug addition of cell growth is directly measured.

Invasion Assay

The invasiveness of cells was determined by a Transwell filter (Coastar, Cambridge, MA, USA) assay with an insert of 12.0 μm pore size. Filters were coated with 30 μL of 1:10 ice-cold Matrigel (BD Biosciences) in media with 10% FBS. Following incubation at 37°C for 30 minutes a thin film of polymerized matrigel coated the filters. Cells were detached from culture using Cell Stripper (Cellgro, 25-056-CI), washed with serum-free media, and pelleted. The pellet was resuspended with serum-free media at a concentration of 5×10^5 cells/mL and 250 μL of this cell suspension (1.25×10^5 cells) was added to the top chamber of the well above the matrigel-coated insert. For DU145 and PC-3 cell line, 1.5×10^5 cells were plated per insert. For drug testing, the desired concentration of drug was added to the top chamber together with the cells. To the bottom chamber, 1 mL of 20% FBS media was added containing 20 ng/mL EGF and 5ng/mL of CCL5 as a chemoattractant to enhance cell migration. The inserts containing the M12 cells were incubated for 16 hours, while the inserts containing DU145 and PC3 cells were incubated for 21 hours before monitoring invasion. The ideal time point and density of cells to use were determined by repeating the invasion assay at several time points with varying cell density. Following incubation, both upper and lower chambers were aspirated. Cells were fixed by the addition of 1 mL of 0.1% glutaraldehyde in PBS to the bottom chamber for 20 minutes at room temperature. The bottom chamber was aspirated and the filter was stained with 0.1% crystal violet in PBS for 30 minutes at room temperature. The filter was then rinsed with deionized water, cut, and placed on a glass microscope slide, with the side facing the bottom of the chamber down. The non-migratory cells that faced the upper chamber of the filter were scrapped off using a Q-tip. The filter on the glass slide was mounted using Permount and the number of migrated cells in 10 random microscopic fields were counted at 40x magnification

and expressed as a percentage of the control. The results represented the mean \pm S.E. of the percentage of invaded cells relative to the control.

Mathematical Analysis and Statistical Calculation

All results were analyzed via the Microsoft Excel program. Averages were calculated and the results were plotted as graphs. The standard error was calculated by using the following equation: **Standard error = Standard deviation / \sqrt{n}** , where n is the sample size (3 – 7) and standard deviation was calculated by the excel function.

The Analysis of Variance (ANOVA) between the control wells and wells that received drug was calculated using the following website which provided the p-value:

<http://www.physics.csbsju.edu/stats/anova.html>

A p-value of less than 0.05 would indicate that there is 95% certainty that the values between the control group and the experimental drug group are not equal.

IV. Results

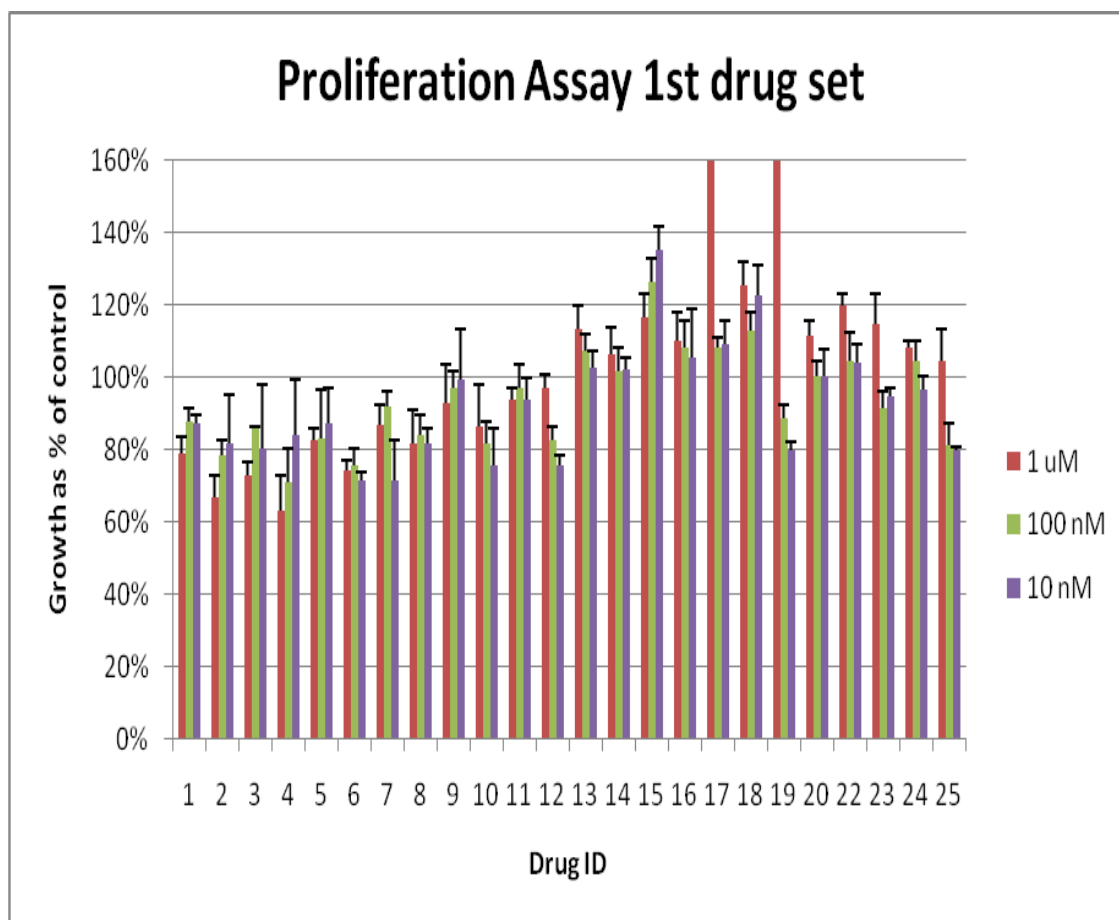
The Effect of the First Set of Drugs on Cell Proliferation

The effect of the 1st set of 24 drugs on M12 cell proliferation was measured using the WST-1 reagent as described in “Materials and Methods” [33]. This method measures the metabolic rate of cells as WST-1 is converted to the product formazan by mitochondrial dehydrogenase enzymes. The absorbance of formazan was measured on an ELISA plate reader at 450nm, subtracting the background absorbance at 650 nm. An increase in absorbance indicated a higher metabolic rate, which is proposed to correlate to increased proliferation of cells. An inhibition greater than 50% was set as the cut-off for a relevant drug effect on cell growth. The absorbance values of the experimental wells containing various drugs at different concentrations were compared to the control wells without drug. The result was expressed as the relative percentage of the average of the control wells (Figure 5). Drugs were added at a concentration of 1 μ M (red), 100 nM (green), and 10 nM (purple) done in triplicate compared to six control wells without any drug. The absorbance was measured 72 hours after drug addition. None of the 1st set of drugs showed a large inhibitory effect (more than 50%) on the proliferation of the M12 cells. Of all the drugs, drugs 2 and 4 showed some inhibitory effect that correlated with the drug dose being tested, i.e., inhibition was greater at 1 μ M than at 10nM. Drug 6 displayed growth inhibition but with little correlation to drug concentration. Several drugs (7, 10, 12, 19, 25) showed partial inhibition of growth, which was in opposition to drug dose, i.e., inhibition was greater at lower concentrations than higher.

Figure 5. First Set of Drugs Showed No Inhibitory Effect on Growth of M12 Cells.

The effect of the first set of 24 drugs at concentrations of 1 μ M, 100 nM, and 10 nM were measured on cell proliferation. Proliferation was monitored after 72 hours of drug addition by measuring WST-1 absorbance at 450 nm. There were six controls per microplate and four microplates were used for the assay. The bars represent the standard error.

Figure 5. First Set of Drugs Showed No Inhibitory Effect on Growth of M12 Cells.



To refine technical expertise, the proliferation assay for these first set of drugs was repeated as above with five control wells minus drug (Figure 6). A few members of the experimental groups (drugs 2, 6, and 10) were specifically highlighted (on the far right of Figure 6) to see if their effect in trial one was replicated in trial two. The result was very similar to the first trial. None of these drugs showed a large inhibitory effect, including the selected drugs 2, 6, and 10. Concurrently, Dr. Xueping Zhang conducted the proliferation assay of the same drugs at similar concentrations on DU-145 and PC-3 cells in triplicate with six control wells minus any drug. Unpublished data showed similar results as Figure 5 and 6: most of the first set of these drugs showed little inhibitory effect on the proliferation of the DU145 or PC-3 cells, with the exception of drug 17, 18, and 22.

To further verify these results, Dr. Xueping Zhang repeated the proliferation assay on a selected group of drugs which had shown some inhibitory effect (drugs 2, 6, and 10) on the previous proliferation assays and some that did not have any effect (drugs 17, 18, 22) at the same drug concentrations on M12, DU145 and PC-3 cells. Her unpublished data (see Figure 7) confirmed that none of these drugs inhibited the proliferation of M12 cells, but drugs 17 and 22 did significantly inhibit the proliferation of DU-145 cells (p-values < 0.05) at 1 μ M concentration (see Table 2). Furthermore, drugs 17 and 22 also significantly inhibited the proliferation of PC-3 cells at 1 μ M and 100 nM concentrations (p-values < 0.05). Drug 17 displayed the highest amount of inhibition (> 96% inhibition) for both DU145 and PC-3 cells.

The Effect of the Second Set of Drugs on Cell Proliferation

Dr. Xueping Zhang carried out the proliferation assay in triplicate on the 2nd set of 24 drugs at 1 μ M concentration on DU145 and PC-3 cells as above. Four control wells were used

Figure 6. Repeated Proliferation Assay Showed No Inhibitory Dffect for the First Set of Drugs.

The repeated cell proliferation assay in triplicate of the 1st set of drugs on M12 cells observed after 72 hours of drug addition at concentrations of 1 μ M, 100 nM, and 10 nM. Four microplates were used and each plate contained five control wells without any drug. Drug 2, 6, and 10 were highlighted to observe the reproducibility of the first proliferation assay. The bars represent the standard error.

Figure 6. Repeated Proliferation Assay Showed No Inhibitory Effect for the First Set of Drugs.

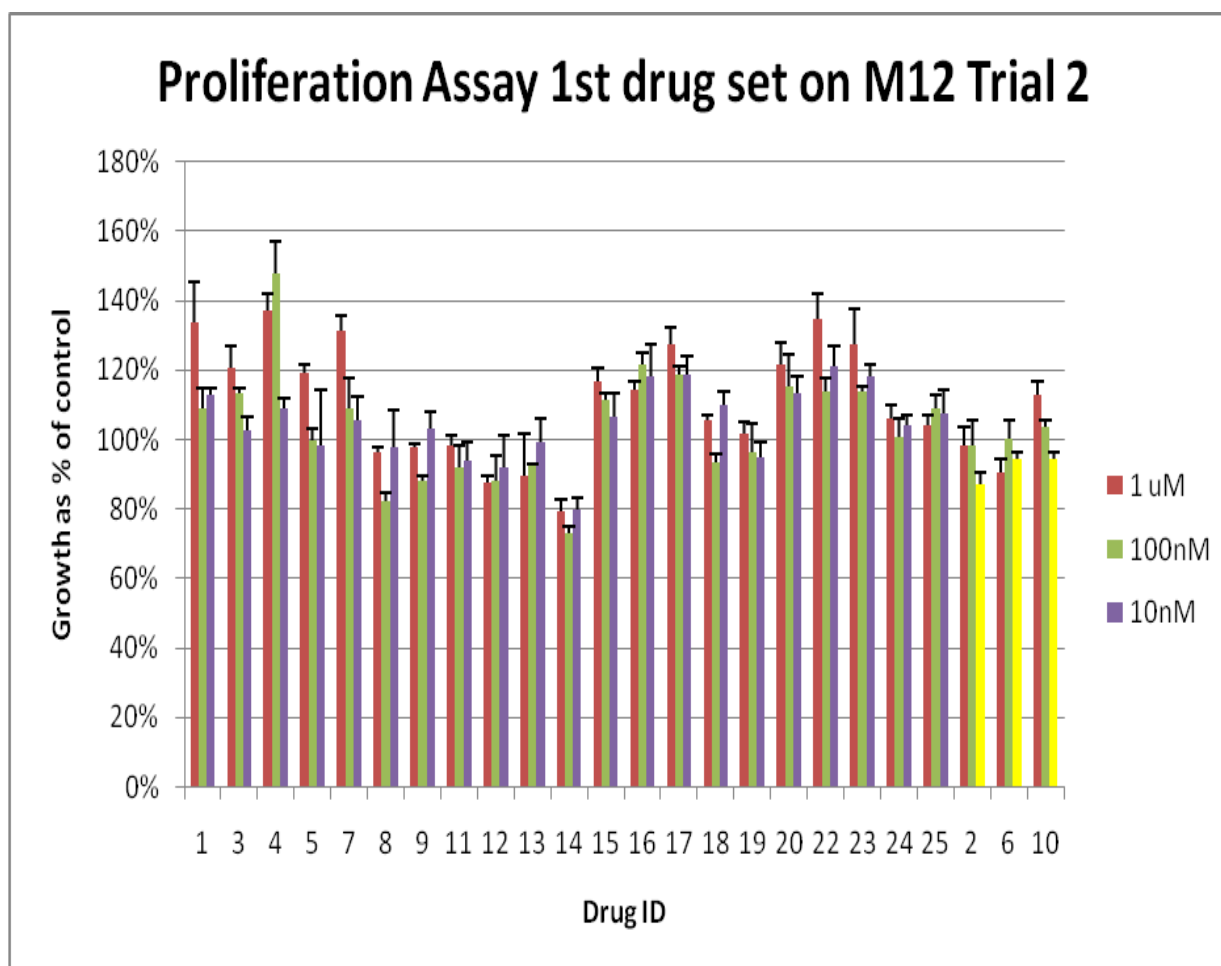


Figure 7. Drug 17 and Drug 22 Inhibit Proliferation of DU145 and PC3 Cells.

The proliferation of M12, DU145, and PC3 cells was observed after 72 hours of drug addition monitoring WST-1 absorbance at 450nm. Growth is expressed as the relative absorbance compared to the average of the control wells lacking drug. The drug concentrations of 1 μ M, 100 nM, and 10 nM were tested in triplicate with six control wells for each cell type. The bars represent the standard error. Drugs 17 and 22 showed significant inhibition of growth.

Figure 7. Drug 17 and Drug 22 Inhibit Proliferation of DU145 and PC3 Cells.

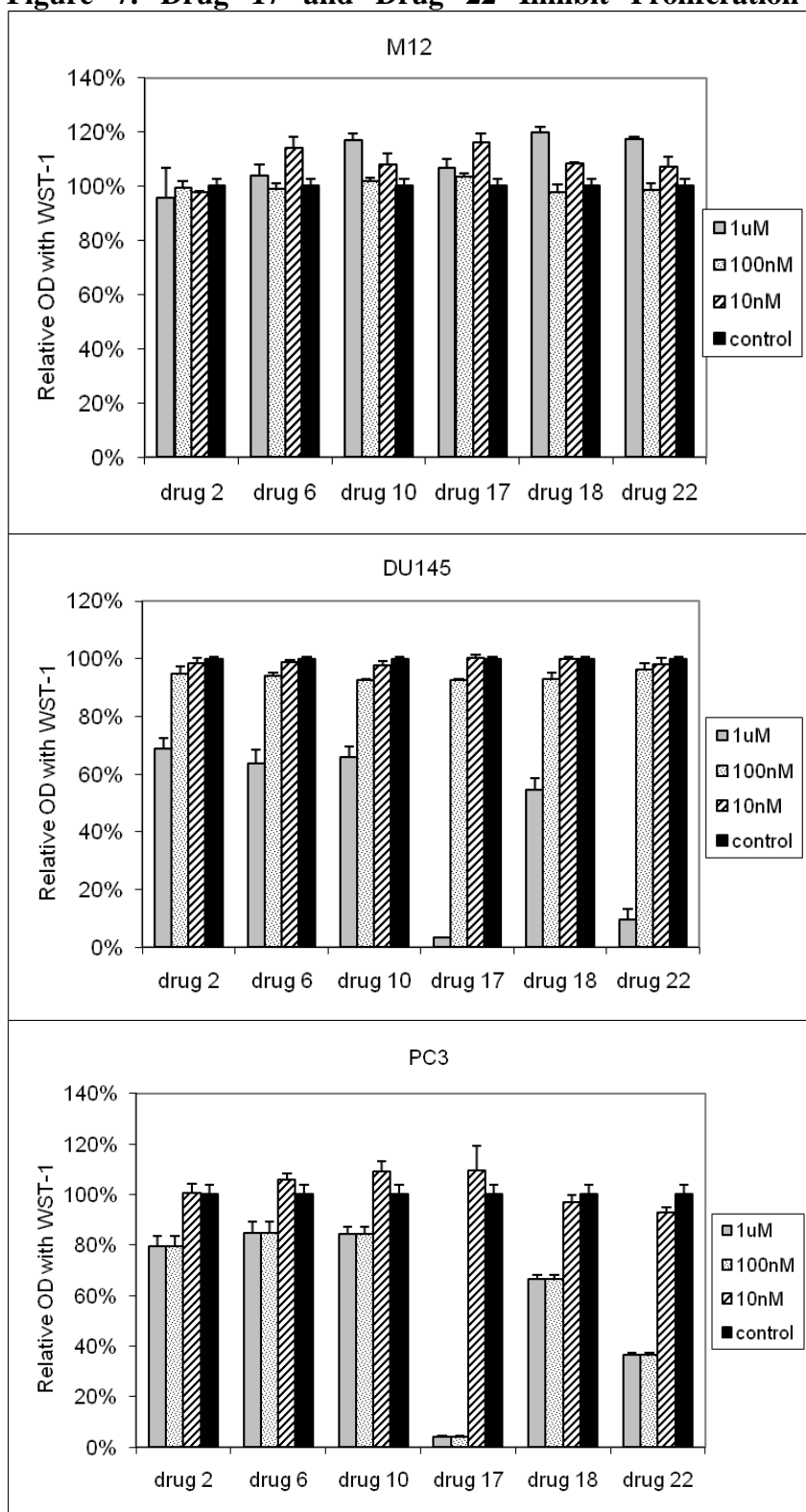


Table 2: Inhibitory Data of Drugs 17 and 22.

The inhibitory data for Drugs 17 and 22 is shown with their p-values from the ANOVA test. The gray boxes indicate that the particular drug caused no significant inhibition on the cells at that particular concentration.

Table 2: Inhibitory Data of Drugs 17 and 22.

Drug ID	Conc	DU145 inhib.	p-value	PC3 inhib.	p-value
17	1uM	97%±0%	0.0001	96%±0%	0.0001
17	100nM			96%±0%	0.017
22	1uM	90%±4%	0.0001	64%±1%	0.0001
22	100nM			64%±1%	0.0001

for each cell type. Unpublished data indicated no inhibitory effect by any of the drugs from the 2nd set; thus, the effect of this drug set on the M12 subline was not further tested.

The Effect of the Third Set of Drugs on Cell Proliferation

Dr. Xueping Zhang tested the effect of the 3rd set of 28 drugs on the cell proliferation of DU145 and PC-3 cells as above using three control wells for each cell type. Her unpublished data showed a moderate inhibitory effect on the proliferation of DU145 and PC-3 cells by drugs 46, 48, 50, 151, 157, and 160. Due to the promising results, I carried out the proliferation assay on the 3rd set of drugs at an 1 μ M concentration in triplicate on M12 cells. Each microplate contained six control wells. The relative absorbance values indicated a moderate inhibition of proliferation by a few of the same drugs that showed an inhibitory effect on DU145 and PC-3 cells (Drugs 46, 48, 50, 151, 157, and 160) (see Figure 8). However, none showed inhibition greater than 50%, our designated cut-off for relevant inhibition.

Since the same drugs showed some effect on growth, the proliferation assay was selectively repeated by Dr. Xueping Zhang on all three cell lines as above. Thus the effect of these drugs at a 1 μ M, 100 nM, and 10 nM concentration was assessed in triplicate on M12, DU145, and PC-3 cells (Figure 9). However, upon repeat none of the 3rd set of drugs displayed a large inhibitory effect on the proliferation of any of these cell lines.

The Effect of the First set of 24 drugs on Cytotoxicity

After testing the effect of all the drugs on cell proliferation only a few drugs from the 1st drug set were found to have a significant inhibitory effect (>50%) on the growth of the three

Figure 8. A Few Drugs from the Third Set of 28 Drugs Moderately Inhibit M12 Cell Proliferation. The proliferation assays of the 3rd set of drugs using WST-1 reagent. Drugs 46, 48, 50, 151, 157, and 160, which had showed a moderate inhibitory effect on PC3 and DU145 cells were highlighted here to see if the effect was repeated for M12 cells. The result is expressed as the relative absorbance of the experimental wells compared to the average of the control wells. The bars represent the standard error.

Figure 8. A Few Drugs from the Third Set of 28 Drugs Moderately Inhibit M12 Cell Proliferation.

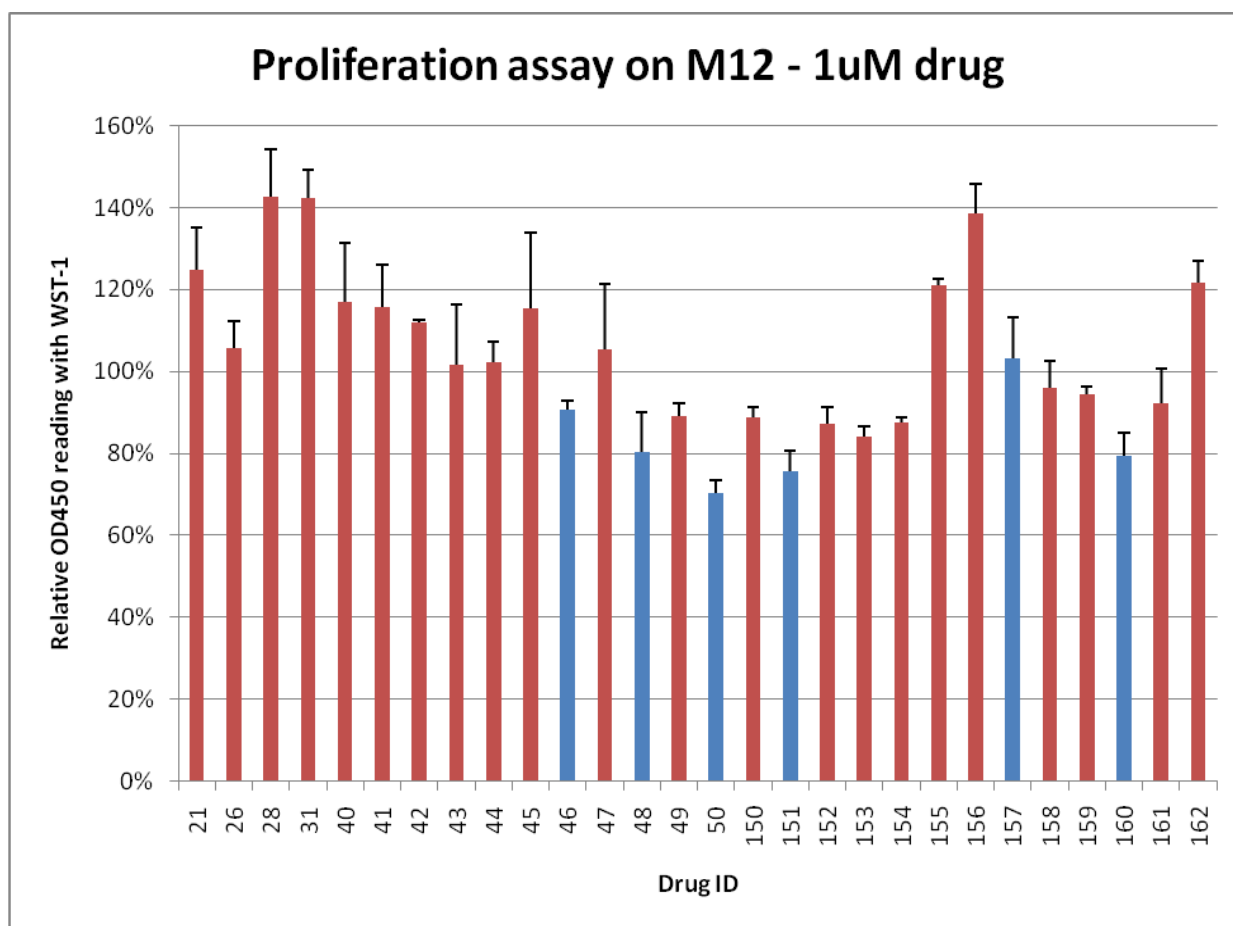
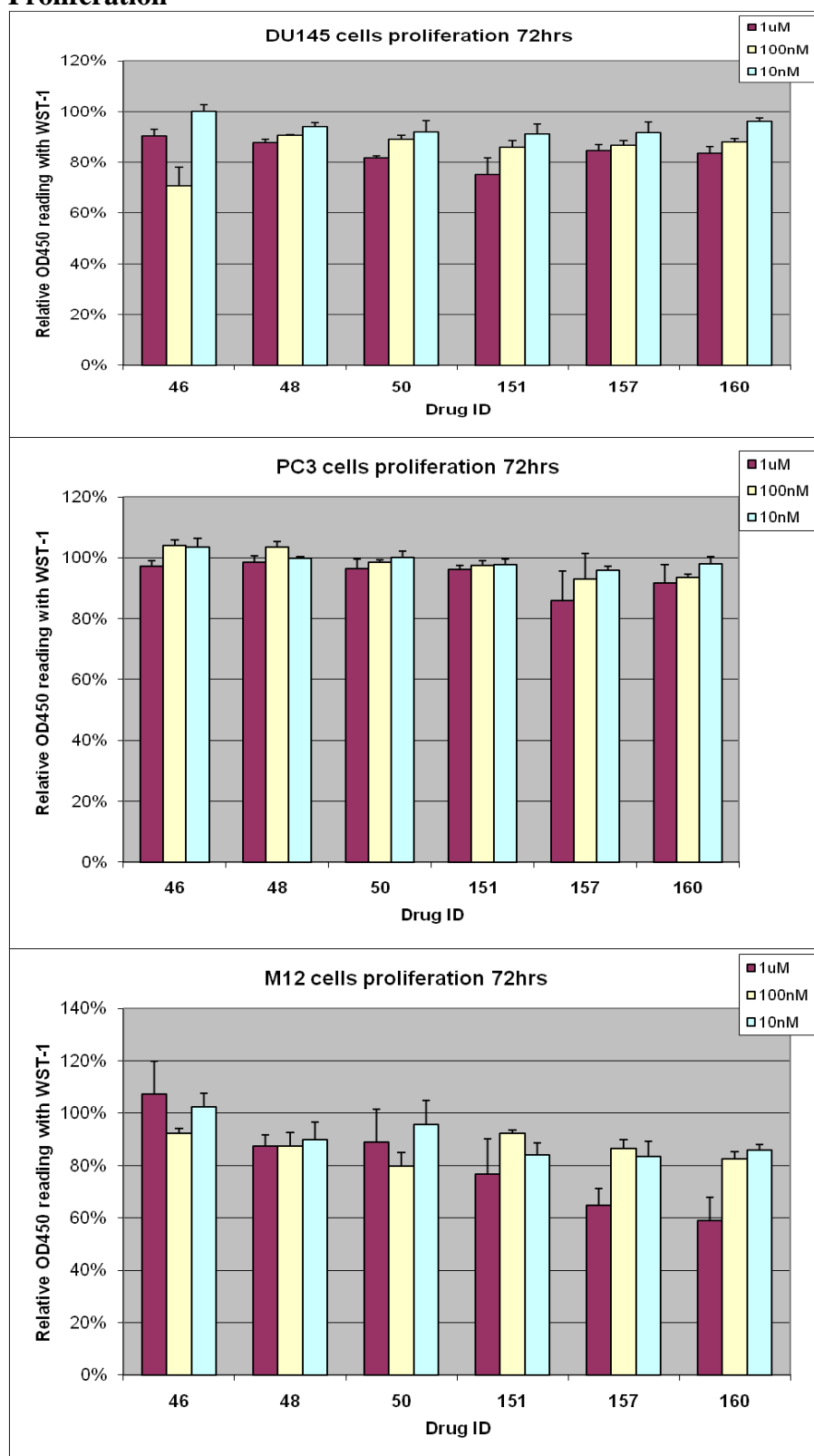


Figure 9. Selected Third Set of Drugs Showed No Inhibition of M12, DU145, or PC-3 Cell Proliferation. Six drugs that showed a moderate inhibitory effect on DU145, PC3, and M12 cells in previous proliferation assays were repeated in triplicate for all three cell lines at a 1 μ M, 100 nm, and 10 nm concentration by Dr. Xueping Zhang. WST-1 reagent was used for the assay, whose absorbance was measured at 450nm. The result is expressed as the relative absorbance of the experimental wells compared to the average absorbance of the control wells without any drug. The bars represent the standard error.

Figure 9. Selected Third Set of Drugs Showed No Inhibition of M12, DU145, or PC-3 Cell Proliferation



prostate cancer cell lines. Based on the results from repeated, similar proliferation assays, the 2nd and 3rd drug set were ruled out and only the 1st drug set revealed any drugs worth further testing.

It was proposed that the effect of these drugs on cell proliferation was worth analyzing by a second approach. The WST-1 reagent measures metabolic activity. A more direct measurement of growth would be to directly count the number of live versus dead cells. Thus, a cytotoxic assay was developed. This assay involves the direct counting of viable cells 72 hours after the addition of the drugs using trypan blue staining to distinguish dead cells from live cells. The cytotoxic effect of drugs from the 1st set was first tested on M12 cells, plating 20,000 cells per well. For the experimental wells a drug concentration of 1 μ M was used in triplicate, whereas 3 control wells received no drug. The result was expressed as the relative percentage of viable cells compared to the average of the control wells without any drug (see Figure 10). Several drugs showed inhibition of growth of M12 cells. Some drugs displayed high inhibitory effect while some did not. Drugs 1,2,6,7,8,9,13,15,16,20,22,24 showed more than 50% growth inhibition with p-values <0.05. To test the effect of these drugs on other cell types (DU145 and PC3 cells), the cytotoxicity assay was carried out by Dr. Xueping Zhang using a drug concentration of 1 μ M on 60,000 cells per well with 3 control wells (no drug). Her unpublished data (see Figure 11) showed that drug 17 yielded the highest percent of growth inhibition on both DU145 and PC-3 cells (31% and 41% respectively). Other drugs such as drug 3 yielded some inhibition for one cell type which was not confirmed in other prostate cell lines.

Hence drugs that showed marginal inhibition in only one cell line were not pursued at this time. Based on all these results, drug 17 was chosen to be the lead compound for further testing.

Figure 10. The Cytotoxicity Assay of the First Set of 24 Drugs on M12 cells.

The cytotoxic effect of the 1st set of drugs (1 μ M concentration) was measured on M12 cells (20,000 cells/well). The viable cells count was determined 72 hours after drug addition. The result was expressed as the relative number of live cells in the experimental wells compared to the average of the control wells that received no drug. The bars represent the standard error.

Figure 10. The Cytotoxicity Assay of the First Set of 24 Drugs on M12 Cells.

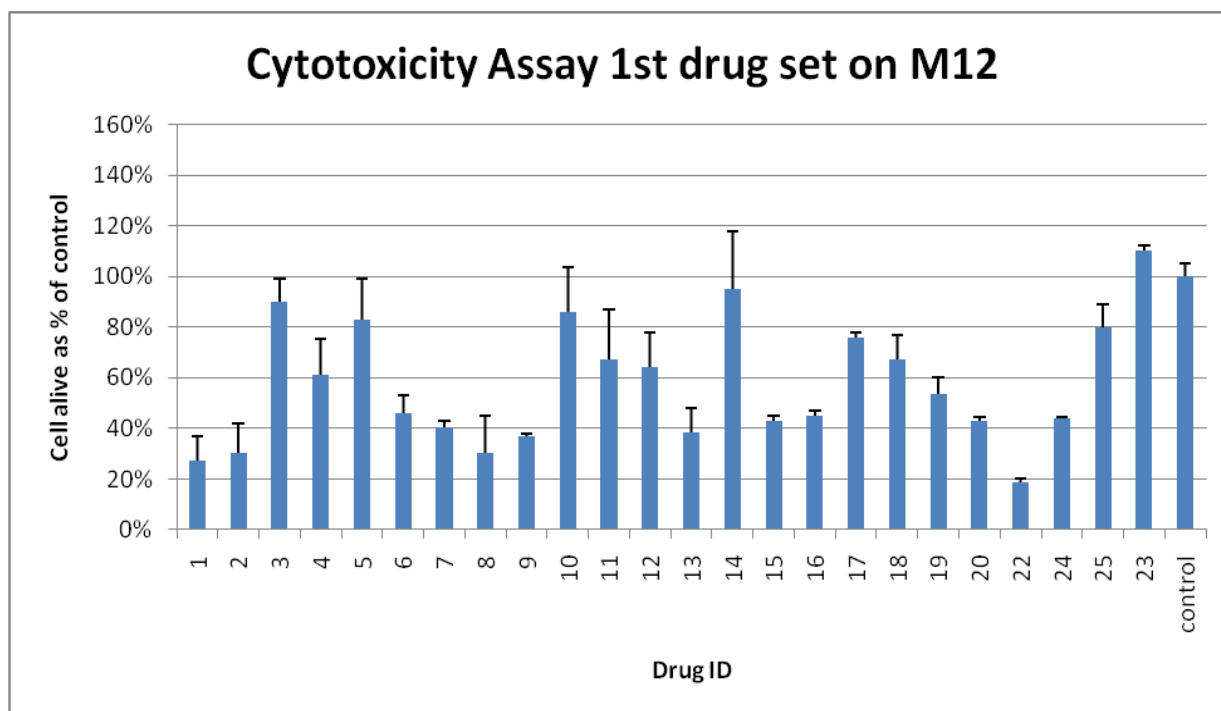
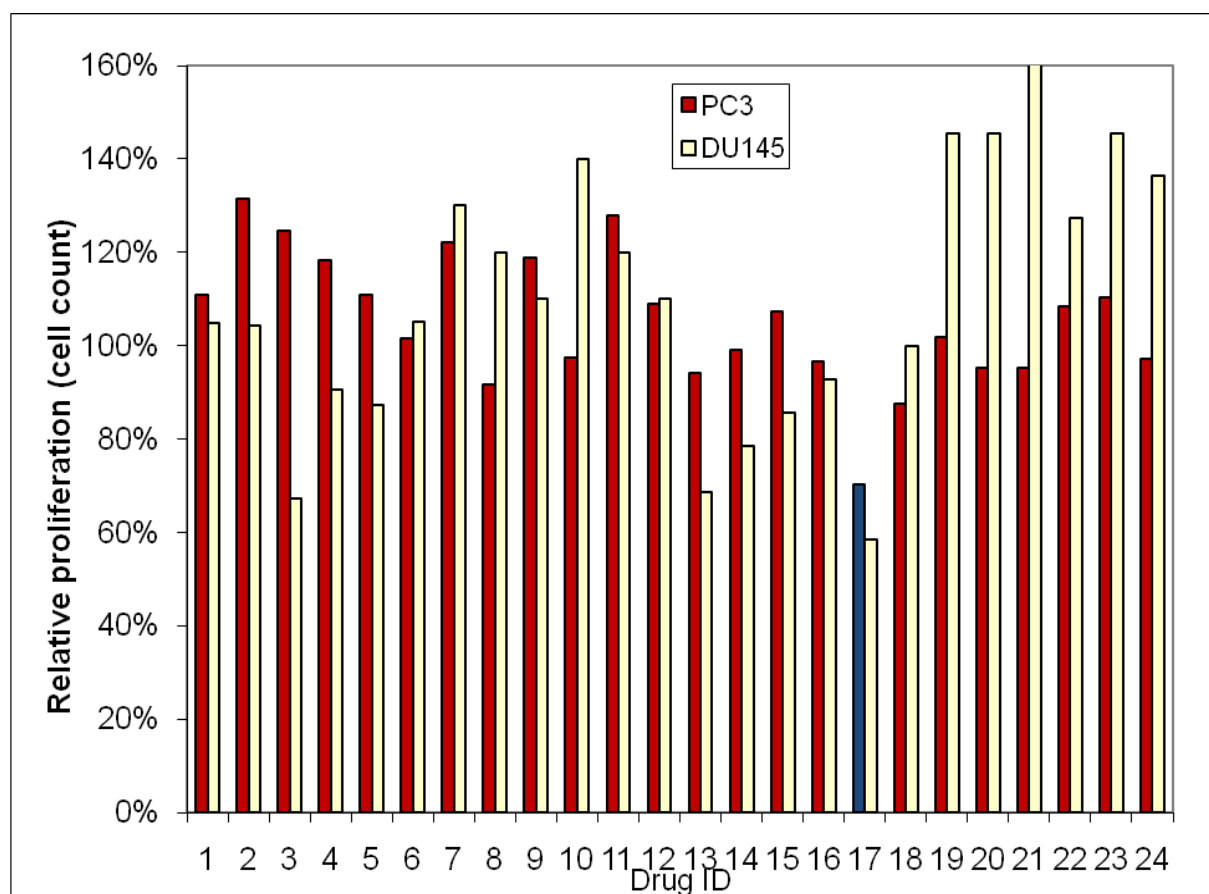


Figure 11. Drug 17 Caused the Highest Inhibition of DU145 and PC-3 Cells via the Cytotoxicity Assay. The cytotoxicity assay for the first set of drugs (at 1 μ M) on the DU145 (white bars) and PC-3 cells (red bars). The number of live cells was counted for each well, 72 hours after drug addition. The result was expressed as the relative cell count of the experimental wells compared to the average of the control wells. The data for drug 17 is recorded as blue (PC3) and white (DU145) since drug 17 yielded the greatest inhibition for both cell lines.

Figure 11. Drug 17 Caused the Highest Inhibition of DU145 and PC-3 Cells via the Cytotoxicity Assay



The Effect of Drug 17 on Proliferation

After selecting drug 17 as the lead compound, additional proliferation assays were done in triplicate for M12, DU145, and PC-3 cells using a 10 μ M drug concentration. The choice of a higher concentration was based on the fact that the lower concentration showed little inhibition in previous proliferation assays and perhaps a higher concentration was needed to inhibit growth of M12 cells. Drug 17 at a 10 μ M concentration significantly inhibited the proliferation of all three cell types (see Figure 12). Inhibition was the greatest for PC3 and DU145 at 94% and 90% respectively, but still, very significant for M12 cells at 71%, all with p-values equal to 0.0001.

The proliferation assay was repeated in triplicate for drug 17 on just M12 cells using a broader range of drug concentrations (10 μ M, 1 μ M, 100 nM, 10 nM, and 1 nM). Drug 17 significantly inhibited the proliferation of M12 cells at a concentration of 1 μ M and 10 μ M (see Figure 13). The level of inhibition was similar for both drug concentrations (78% \pm 1% and 77% \pm 2%, and p-values = 0.0001 for both concentrations). To find the half-maximal inhibitory concentration (IC₅₀) for drug 17 on M12 cells, the data was transformed into log scale of base 10 (see Figure 14). The log growth% and log concentration was plotted using Excel Curve Fitting software (see Figure 14). From this plot the IC₅₀ was found to be 237.68 nM. To determine the IC₅₀, the log of 50% was taken, which was equal to -0.301. The x-value for this y-value was found on Figure 14 to be 2.376. The inverse log of this value indicated that the concentration of drug 17 which yielded 50% inhibition of growth was 237.68 nM.

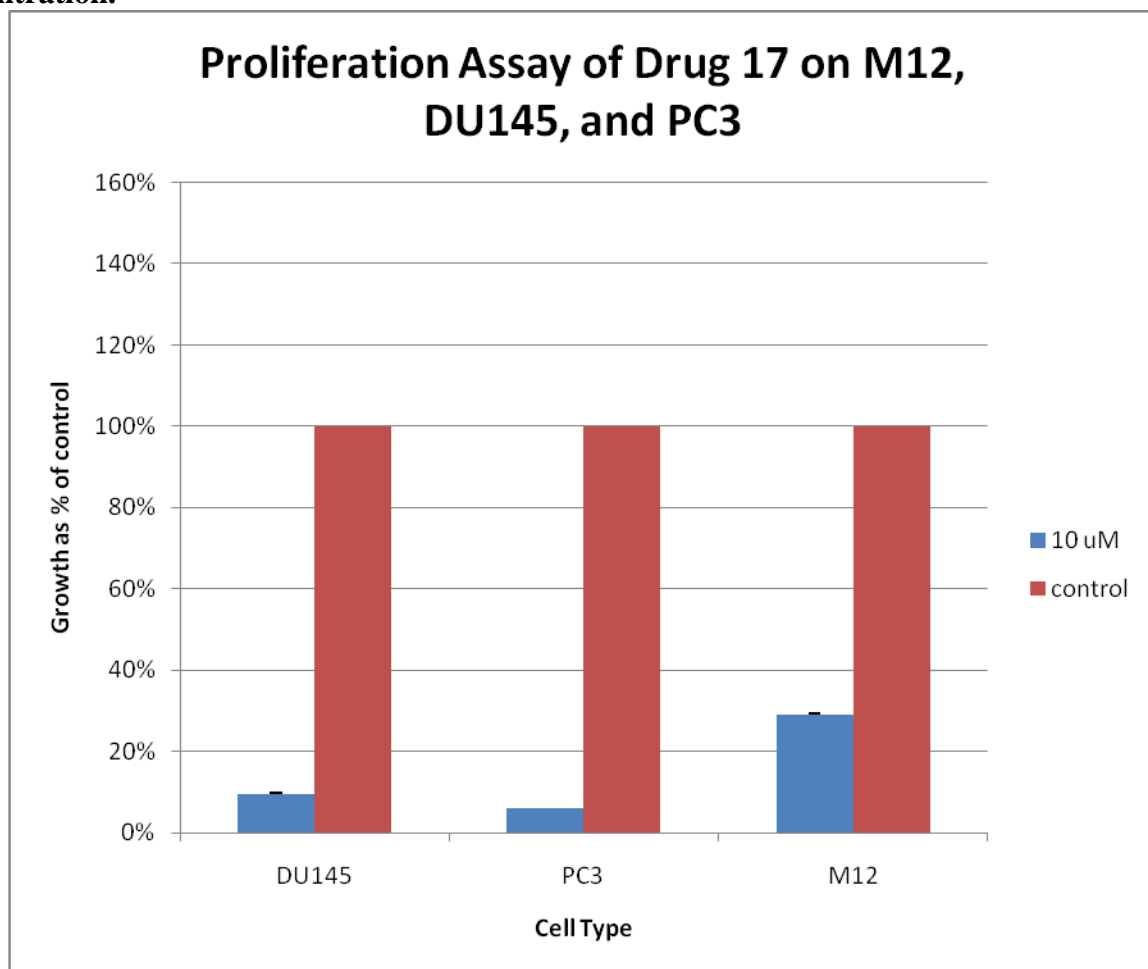
The Effect of Drug 17 on the Cytotoxicity of PCa Cells

The cytotoxicity assay was repeated on M12, DU145, and PC-3 cells for only drug 17 to determine if similar inhibitory results were as reproducible as the results from the proliferation assays (Figures 13 and 14). Drug 17 (at 1 μ M) was analyzed in triplicate with 3 control wells for

Figure 12. Drug 17 Inhibited Proliferation of DU145, PC3, and M12 cells at 10 μ M Concentration.

The proliferation assay of DU145, PC3 and M12 cells observed after 72 hours by measuring WST-1 absorbance at 450nm. Each cell type received drug 17 at 10 μ M. The assay was done in triplicate with six control wells minus drug. The growth was expressed as the percentage of the control, with bars representing the standard error and p-values = 0.0001.

Figure 12. Drug 17 Inhibited Proliferation of DU145, PC3, and M12 Cells at 10 μ M Concentration.

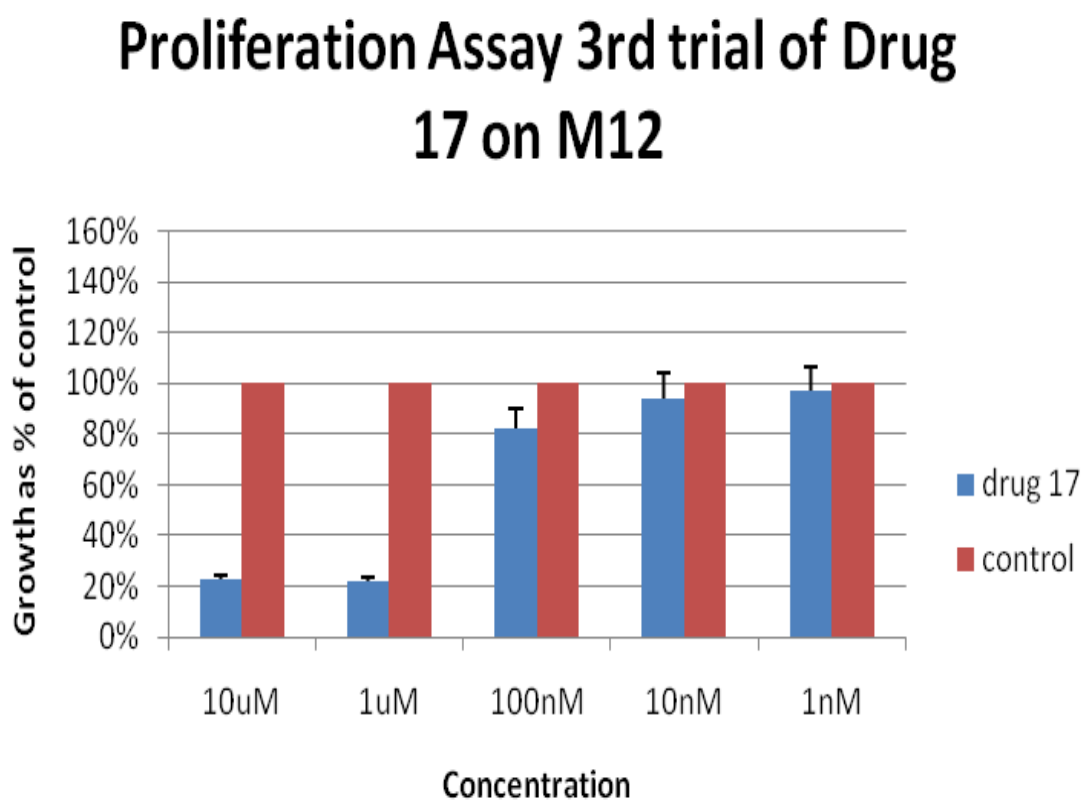


Cell Type	Inhibition by 10uM drug 17	p-value
DU145	90% \pm 0%	0.0001
PC-3	94% \pm 0%	0.0001
M12	71% \pm 0%	0.0001

Figure 13. Drug 17 Significantly Inhibited M12 Proliferation at 1 μ M.

The proliferation assay of M12 cells observed after 72 hours of drug 17 addition at concentrations of 10 μ M, 1 μ M, 100 nM, 10 nM and 1 nM by the measurement of WST-1 at 450nm. Each drug concentration was repeated in several wells with eight control wells. The bars represent the standard error.

Figure 13. Drug 17 Significantly Inhibited M12 Proliferation at 1 uM.

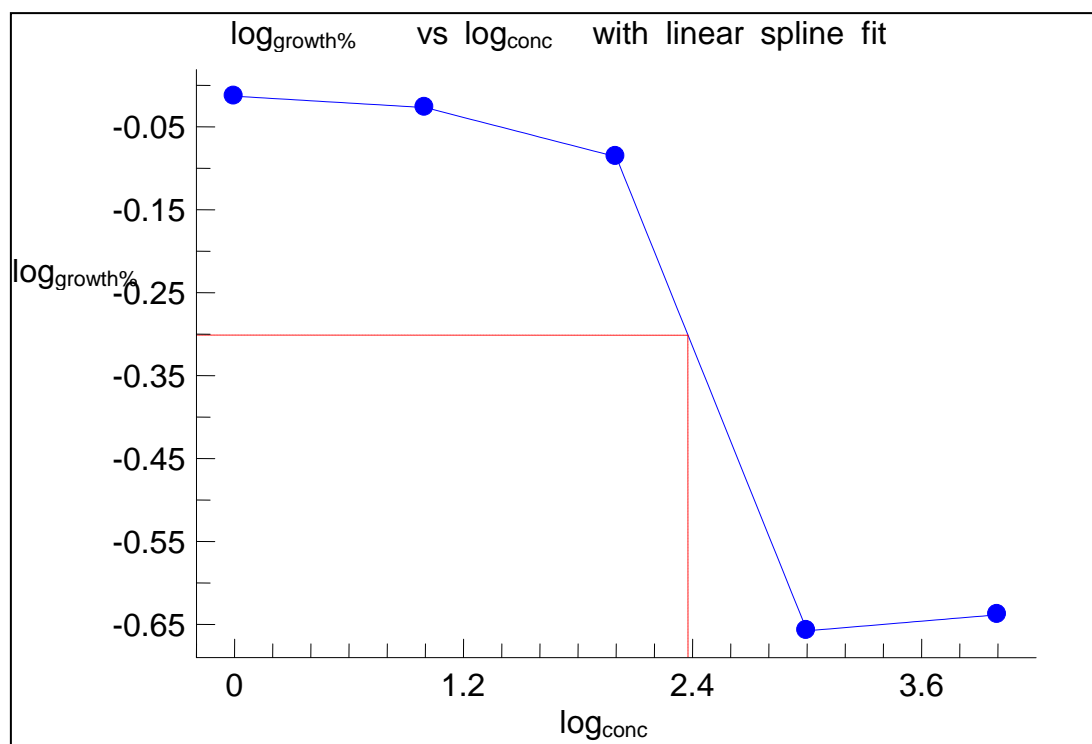


Drug 17 concentration	%inhibition	p-value
1μM	78%±1%	0.0001
10μM	77%±2%	0.0001

Figure 14. Calculation of the IC50 for Drug 17 Inhibition of M12 Cell Proliferation.

The data from Figure 13 was transformed into log values by taking the log based 10 of the percentage growth and concentration and plotting the data in $\log_{\text{growth\%}}$ vs $\log_{\text{concentration}}$ plot. The linear spline fit model was used on the Excel Curve Fitting software to fit the data. The intersection of the red lines indicate the coordinate where $\log_{\text{growth\%}} = -0.301$ or percentage growth = 50%. The y-value of the coordinate (2.376) indicates the $\log_{\text{concentration}}$ where percentage growth is 50%. Taking the inverse log shows the concentration = 237.68 nM, which is therefore the IC50.

Figure 14. Calculation of the IC50 for Drug 17 Inhibition of M12 Cell Proliferation.



each cell type. Drug 17 significantly inhibited the growth of all three cells types in the cytotoxicity assay (see Figure 15). Interestingly, the greatest inhibition of 97% was observed for the M12 cell line, but significant inhibition of 75% and 70% was observed for DU145 and PC3 respectively. It was evident that drug 17's inhibitory effect was repeated both in the cytotoxicity and proliferation assays, indicating drug 17 might be an important CCR5 antagonist to reduce the growth and proliferation of PCa cell lines.

The Effect of Drug 17 on *in vitro* Invasion

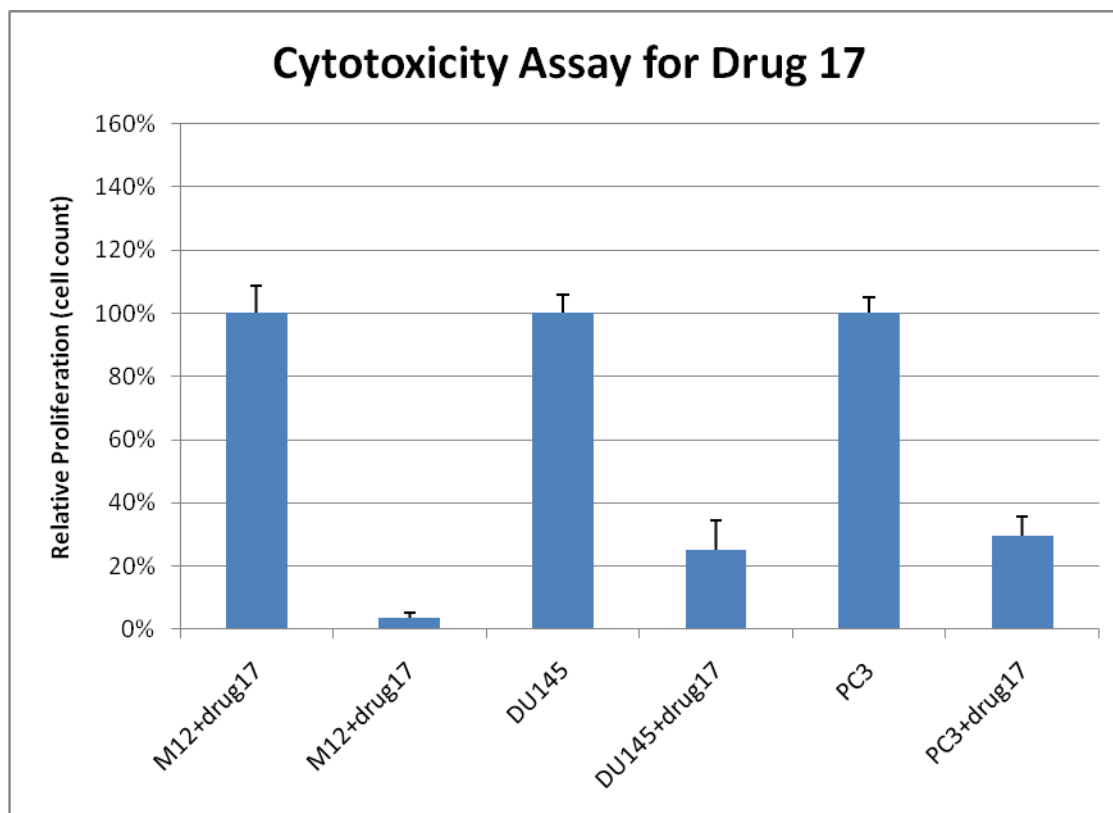
To date only the growth and proliferation of the PCa cells was assessed by the cytotoxicity and proliferation assays. The effect of drug 17 on invasion *in vitro* was next investigated. Since the invasion assay is considerably more expensive and time consuming, it is not an efficient way to screen a large number of drugs. Therefore, only the lead compound, drug 17, was assessed for its effect on cell invasion. By this method cells were plated on top of a gel of extracellular matrix components (transwell filters) and the ability of the cell to degrade the matrix components and move from the top to bottom chamber was measured as an index of the cell's invasive capabilities. For each cell type drug 17 was added to the experimental insert (at 1 μ M concentration), whereas the control insert received no drug. Due to the higher motility of M12 cells, these cells were allowed to invade for 16 hours, whereas 21 hours was found to be optimal for DU145 and PC-3 cells. Following invasion, cells were counted under the microscope and the number of cells that migrated to the bottom chamber were recorded (Figure 15).

Drug 17 significantly reduced the invasiveness of all three PCa cell lines, with the effect on M12 cells being the greatest at 75%, followed by PC-3 cells at 49% and DU145 cells at 41% (see Figure 15). Overall, the invasion of all cell types was greatly reduced by drug 17, indicating

Figure 15. Drug 17 Significantly Inhibits Growth in a Cytotoxicity Assay.

The number of viable M12, DU145, and PC-3 cells were counted after 72 hours of drug 17 addition at 1 μ M. Proliferation is expressed as a relative % of the control without drugs. Each concentration was repeated in triplicate for each cell type and there were three control wells. The bars represent the standard error. The result was summarized below the figure.

Figure 15. Drug 17 Significantly Inhibits Growth in a Cytotoxicity Assay



Cell type	Inhibition by 1μM Drug 17	P-value
M12	97%±2%	0.0004
DU145	75%±9%	0.0025
PC3	70%±6%	0.0008

that it not only inhibits the growth and proliferation of PCa cells, but also reduced their invasive ability. Since the inhibitory effect of drug 17 was reproduced for all three cell types in multiple *in vitro* assays, drug 17 may be an effective CCR5 antagonist. However, the effect of drug 17 remained to be tested *in vivo*.

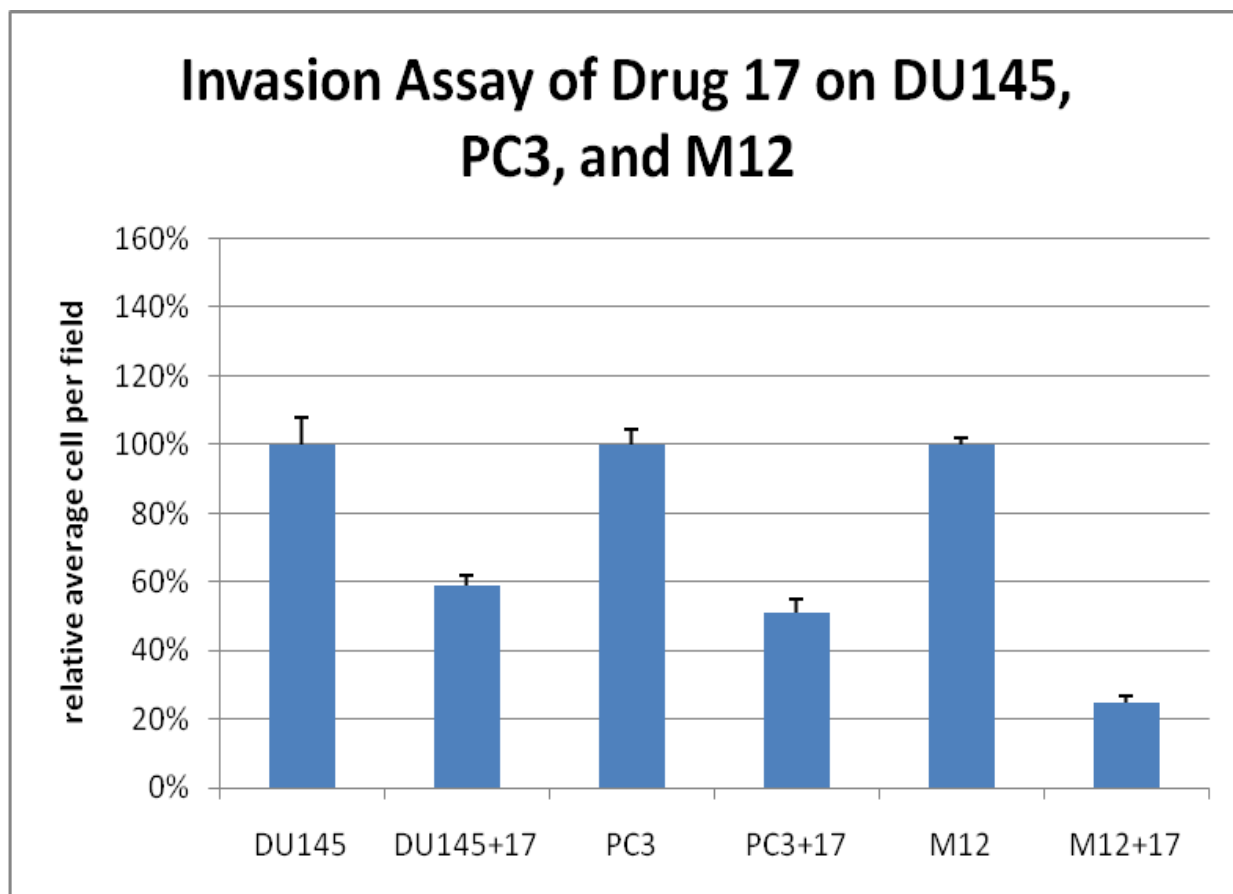
The Effect of Drug 17 on *in vivo* Tumor Growth

Dr. Joy Ware and Ms. Amanda Richardson (Department of Pathology, Virginia Commonwealth University, Richmond, VA) were responsible for testing the effect of drug 17 on tumor growth *in vivo*. M12 cells were subcutaneously injected into a group of male, athymic nude mice and tumors were allowed to form. Tumor growth was measured by a caliper where approximate tumor volume (mm^3) was calculated as: **(length x width²) /2**. Once tumors reached a predetermined optimal size of at least 50 mm^3 , mice were deemed ready for injection. Four experimental mice and three control mice were used. Both groups of mice were injected at 4 day intervals for a total of 4 injections. Tumor growth was monitored upto 8 days post the last injection. Control mice received no drug, only the carrier ddH₂O and 10% DMSO, while the experimental mice received 1 μM drug 17 resuspended in ddH₂O and 10% DMSO each. The unpublished data of Ware et al showed that the tumor volume was less for mice that received drug 17 compared to the control mice that did not receive any drug (see Figure 16). Comparing the average tumor size of the control and experimental groups indicated a significant difference in the tumor size between the two groups on the 3rd and 4th injection days (46% and 64% respectively, with p-values 0.0036 and 0.0052 respectively) (see Table 3). Each mouse started on the 1st injection day with a slightly different tumor size; the control group actually had smaller tumors at the beginning of the study. Over time, a difference in the tumor size started to emerge

Figure 16. Drug 17 Significantly Reduced Invasiveness of DU145, PC3 and M12 Cells.

DU145, PC3 and M12 cells were plated on top of Matrigel coated transwell inserts as mentioned in “Material and Methods”. Cells were counted from 10 random fields at 40x magnification and results were expressed as the relative average cell count compared to the control insert without any drug. Drug 17 was added at 1 μ M. The bars represent the standard error. The result was summarized below the figure.

Figure 16. Drug 17 Significantly Reduced Invasiveness of DU145, PC3 and M12 Cells.



Cell type	Inhibition of invasiveness by 1μM drug 17	p-value
DU145	41%±3%	0.0012
PC3	49%±4%	0.0001
M12	75%±2%	0.0001

Figure 17. Drug 17 reduces tumor growth *in vivo*.

M12 cells were injected subcutaneously into nude mice by Ware et al and tumors were allowed to form. Control mice received only ddH₂O and 10% DMSO at each injection, while the experimental mice received drug 17 at a 1 μ M concentration resuspended in ddH₂O and 10% DMSO. The drug was injected four times at four day intervals. Tumor volume was calculated prior to each injection. Mice were monitored upto 8 days post last injection except for mouse 9 which abruptly died after the 4th injection. In the graph, the doubled lines are control mice and the single lines are experimental mice.

Figure 17. Drug 17 reduces tumor growth *in vivo*.

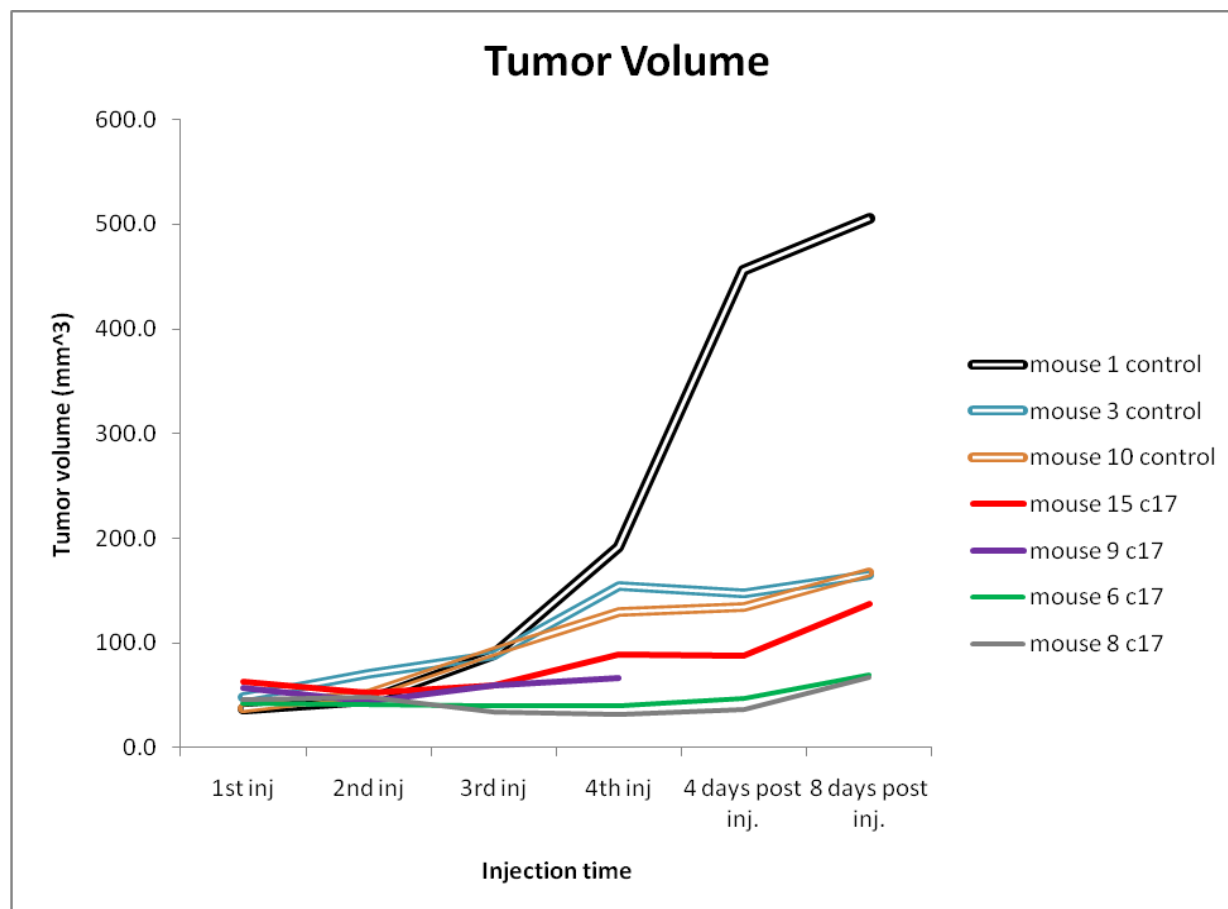


Table 3. Tumor Volume and Percent Inhibition of Tumor Size by Drug 17.

(A)The tumor volume at each day of injection, plus 4 and 8 days post last injection is listed for each mouse. (B) The percent inhibition in tumor volume caused by drug 17 was calculated by finding the percent difference between the average tumor sizes of control mice and experimental mice and dividing by the average tumor size of the control mice. P-values calculated between the two groups is listed. Unpublished data by Ware et al.

Table 3. Tumor volume and Percent Inhibition of Tumor Size by Drug 17.

A

Mouse id	1st inj	2nd inj	3rd inj	4th inj	4 days post inj.	8 days post inj.
mouse 9 c17	56.7	44.1	60.0	66.3		
mouse 15 c17	63.5	52.3	60.0	89.2	87.9	137.30
mouse 8 c17	46.4	48.4	33.6	32	37	67.5
mouse 6 c17	42.4	41.6	40.0	40	47.1	69.3
mouse 3 control	48.4	70.2	88.9	154.6	147.5	164.7
mouse 10 control	37.6	51.6	92.5	129.6	135	167.9
mouse 1 control	37.0	45.6	89.2	191.1	456.3	505.4

B

mouse group	1st inj	2nd inj	3rd inj	4th inj	4days post	8 days post
Experimental avg	52.3	46.6	48.4	56.9	57.3	91.4
Control avg	41.0	55.8	90.2	158.4	246.3	279.3
% inhibition	-27%	16%	46%	64%	77%	67%
p-value	0.14	0.23	0.0036	0.0052	0.15	0.18

where by the 2nd injection day, drug 17 caused a 16% reduction in tumor volume in the experimental group. The reduction in tumor volume increased on the 3rd injection day to 46% and by the 4th injection day was 64%. The highest reduction was achieved 4 days after the last injection (77%), which was reduced to 67% by 8 days.

Investigating the average tumor volume for each group in Table 2, it is evident that the tumor volume stayed almost constant for the experimental group that received drug 17. The average volume at the start is 52.3 mm³ and reached only 57.3 by 4 days after the last injection. However, for the control group, the tumor grew large, starting at 41.0 mm³ on the first injection day, and reaching 246.3 mm³ by day 4 after the last injection. Perhaps for the experimental group the tumor size did not increase because drug 17 inhibited tumor growth, but the tumor grew uncontrollably in the control group without drug 17. However, by 8 days after the last injection, the tumor size started to increase for the experimental group, reaching 91.4 mm³, perhaps because drug 17 was no longer being injected into the mice and the residual amount of drug 17 was being cleared from the body. The tumor also continued to grow for the control mice, reaching 279.3 mm³ by day 8 post injection. For the experimental group, once the injection of drug 17 was stopped, the inhibitory effect started to decrease (from 77% on 4 days post 4th injection, to 67% by 8 days post 4th injection). This suggested that the presence of drug 17 could stop the growth of the subcutaneous tumor in male nude athymic mice.

V. Discussion

In the discovery of a potent CCR5 antagonist that could function as a potent anti-prostate cancer drug, many *in vitro* assays were employed to screen 76 newly synthesized putative CCR5 antagonists. The journey started with a proliferation assay that after several trials suggested that drug 17 significantly inhibited the proliferation of M12, DU145, and PC-3 cells. This result was confirmed by a cytotoxicity assay. Since drug 17 was the only drug that showed a reproducible inhibitory effect by both assays, it was chosen as the lead compound for further studies. The computation of a full drug response curve yielded an IC₅₀ of 237.68 nM for drug 17 on M12 cells. The cytotoxicity assay for drug 17 was repeated yet again to re-confirm its inhibitory effect on all 3 PCa cell lines. In all cases the results from the cytotoxicity and proliferation assays agreed. Finally, an *in vitro* invasion assay confirmed drug 17's ability to block PCa cells from degrading and moving through the extracellular matrix to a bottom chamber in a transwell assay system. This result strengthened drug 17's candidacy as a good CCR5 antagonist with suitable anti-PCa properties, because not only did it inhibit the growth of PCa cells, but it also inhibited invasion. However, this was all *in vitro* findings and the story could be totally different *in vivo*. Therefore, the only way to truly validate drug 17's efficacy was to do an *in vivo* assay. Therefore, Ware et al subcutaneously injected M12 cells in two groups of mice. After tumor formation, one group received injection of drug 17 (1 μ M) whereas the other group received only the carrier. Their unpublished data showed that drug 17 stopped the growth of the tumor, but once injections were stopped this effect diminished over time probably due to the clearance of

the drug by the body. However, only a few mice were available for this study. An additional study with more mice needs to be done to obtain statistically significant results.

The first few attempts to investigate the effect of the novel CCR5 antagonist in proliferation assays produced no large inhibitory effect by any of the drugs. A few drugs showed small inhibition, but their results were not reproducible. The standard error for these first few assays was very high, because I was still perfecting the technical component of these assays. Concurrently, Dr. Xueping Zhang carried out proliferation assays on DU145 and PC3 cells and concluded that few of the drugs had any substantial effect on proliferation. She selected a few drugs that showed a small amount of inhibition or no inhibition and repeated the proliferation assay. This time drug 17 showed a significant inhibition of cell proliferation for DU145 and PC-3 cells. Drug 18 and 22 also showed some inhibitory effect, but drug 17's effect was much greater. She then tested the 2nd drug set on proliferation of M12 and PC3 cells and found none of the drugs inhibited proliferation. I decided to not focus on this drug set, since she did not see any effect. She went on to test the 3rd set of drugs on DU145 and PC-3 cells, and I tested M12 cells. Only a few drugs showed a small amount of inhibition at 1 μ M concentration. However, upon repetition, no significant inhibition was observed by any of these drugs.

At this point we were left with only the first set of drugs to be re-tested, because sets 2 and 3 did not produce any promising results. We decided to try a new method to test the effect of drugs on tumor cell growth. The former proliferation assay measures the conversion of the WST-1 reagent to formazan by mitochondrial dehydrogenase enzyme. Thus, this proliferation assay does not really count the number of viable cells, but relies on mitochondrial activity as an approximation of cell growth. If more metabolically active cells are present, more formazan product will be produced. This may or may not reflect a change in cell number and viability.

There are many factors that can effect the result of this assay, such as whether all the cells absorb the WST-1 reagent equally, how well it reacts with the mitochondrial dehydrogenase enzyme, whether more formazan product directly correlates to the number of cells or if larger cells with a higher number of active mitochondria can produce more formazan than others, etc. Thus, we wanted to develop an assay that could directly quantitate how many cells are killed by these drugs compared to the control. The cytotoxicity assay was developed to address this question. It does not use any enzymatic reaction to measure the number of cells, but rather direct counting of the number of viable versus stained dead cells in each well by the use of a microscope. It is therefore a more accurate measure of how many cells survive in the control versus the experimental wells dependent upon drug addition.

Another factor that reduced the accuracy of the proliferation assay was the fact that plating the cells in the small well of the 96-well micro plate is very difficult. It is near impossible to plate the cells evenly in such a small well. If the cells are not evenly plated then the results are not accurate. The technique was somewhat improved by first adding a small volume of media to the well followed by the addition of cells resuspended in media. By this approach the cells would not bounce off the plastic, but rather have a solvent to land on and spread out evenly. It was a lot easier to plate cells for the cytotoxicity assay because we used a 24 well plate where the volume was much greater. In this case, the cells were more evenly distributed in the wells. This increased the accuracy of the results for the cytotoxicity assay compared to the proliferation assay. However, it is important to note that the first implication of drug 17 as a possible lead compound actually came from the result of the proliferation assay of the selected 1st drug set done by Dr. Xueping Zhang. Why drug 17 did not show up on my assay is something worth considering? This may be due to my previous inexperience. I started the project with no previous experience

of cell culturing or any of the assays that were used for this project. It has been a learning curve throughout the entire project. Due to Dr. Xueping Zhang's years of experience with these methods, her results were more accurate from the start. Since I was still learning, my proliferation assays for the first set of drugs did not show any inhibitory effect by drug 17. Interestingly, Dr. Zhang's first assay also did not show any inhibition by any drug. However, when she repeated the assay the second time for a few selected drugs, the inhibitory effect for drug 17 became evident. Since she was more experienced with the assays, she tested the 2nd drug set first and concluded no drugs were effective. Based on her data, I decided to not spend time to test the second drug set on the M12 cell line. Dr. Zhang showed some drugs within set 3 had a marginal effect on DU145 and PC-3 cells. I confirmed a small amount of growth inhibition in my analysis of the M12 cells. However, when Dr. Zhang repeated the assay of these selected drugs on all three cell types, none of the drugs produced a large enough inhibitory effect to be considered lead compounds.

In my first try of the cytotoxicity assay on the M12 cells. I found several drugs from set 1 that inhibited cell growth, including drug 17 to a small extent. However, Dr. Zhang's cytotoxicity assay of the 1st drug set on DU145 and PC-3 cells confirmed only drug 17 to be effective in all 3 cell types. Analyzing all other data it was decided to focus only on drug 17, because its inhibitory effect was evident on both the cytotoxicity and the proliferation assays. The proliferation assay for drug 17 was repeated with a higher concentration on all three cell types because I did not see a significant effect by drug 17 in the previous assays with lower concentrations. Drug 17 at a 10 μ M concentration significantly inhibited the growth of all three cell lines in the proliferation assay. Assaying different drug concentrations on M12 showed that drug 17 significantly reduced the proliferation of M12 cells at 1 μ M and 10 μ M concentrations.

This is the first time drug 17's inhibitory effect was noted at the 1 μ M level. One reason might be due to my mastering a new technique. After several repeats; however, my results started to agree with the findings of Dr. Zhang. Subsequent cytotoxicity assays confirmed that drug 17 significantly inhibited the proliferation at 1 μ M concentration for all three cell types, increasing the confidence of these results.

A protocol for the invasion assay had not been developed in the lab necessitating several tries (at least 5) to deduce the correct number of cells and time to promote cell invasion. However, this careful study actually helped alleviate some of my problems encountered with the proliferation and cytotoxicity assays. The invasion assay is a better technique to measure the carcinogenic potential of PCa cells, because it measures the metastatic ability of the cells. Other assays measure the growth rate of the cells, which may be similar for tumor and normal cells. Many tumor cells are anti-apoptotic so they can outgrow the normal cells. A faster growth might not always indicate carcinogenicity. However, normal cells are not invasive and therefore, the invasion assay is a more accurate way to assess cell carcinogenicity. The *in vitro* invasion assay indicated a significant inhibition by drug 17 on the invasion of all three cell types. Altogether three completely different assay methods agreed that drug 17 is a potential CCR5 antagonist that reduced the growth and invasion of the PCa cells *in vitro*. More importantly, these results were confirmed *in vivo* by Dr. Joy Ware where the administration of drug 17 significantly slowed subcutaneous tumor growth. Once drug 17 administration was stopped, by day 8 tumor growth began to resume. However, these preliminary results need to be repeated before reaching the conclusion that compound 17 is a worthwhile drug of possible therapeutic value.

VI. Conclusion

PCa is a major problem for men of age 50 years or older. Current treatments are only effective at an early stage and once cancer cells progress to becoming metastatic, palliative care is the only option. The metastasis of prostate cancer cells to bone is fatal and demands a therapy that stops invasion and metastatic spread. CCL5 and CCR5 were found to be important in PCa cells. Previous studies have shown that CCL5 levels are directly correlated with high growth rate and invasiveness of PCa cells. CCL5 interacts with CCR5 to produce downstream proinflammatory factors that support the growth and invasion of PCa cells; therefore the interaction of CCL5 with CCR5 is an important therapeutic target for PCa [19]. Here, several novel putative CCR5 antagonists were designed based on the known ligand structures for CCR5. Their inhibitory effect was tested on M12, DU145, and PC-3 PCa cell lines by several *in vitro* assays measuring cell growth and invasion. Altogether these studies suggested drug 17 as a lead compound to significantly inhibit the growth and proliferation of PCa cells. The result was substantiated in *in vivo* studies in male, athymic nude mice. *In vivo* data confirmed that drug 17's inhibitory effect was not just evident *in vitro*, but also in *in vivo*. Based on several *in vitro* assays and *in vivo* confirmation, it can be concluded that drug 17 might be an important CCR5 antagonist, which has a strong potential as an effective therapeutic agent for PCa.

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List of References

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Vita

Tasrif Ahmed was born on August 20th, 1986 in Barisal, Bangladesh to Shamim Begum and Mohammed Zaher. He moved to the USA when he was twelve with his family. He has two younger brothers, Yaseen Ahmed and Mehraz Ahmed. Tasrif is currently engaged to Nur Sultana. He graduated from Southern High School, Durham, North Carolina. He then attended the University of North Carolina at Chapel Hill, North Carolina and received his Bachelor of Science degree in Biochemistry on May 2008. He is currently enrolled at Virginia Commonwealth University as a M.S. student in the Physiology Department since the fall of 2008.