Identification of Small Molecules that Inhibit Prostate Cancer Cell Proliferation

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IDENTIFICATION OF SMALL MOLECULES THAT INHIBIT PROSTATE CANCER CELL PROLIFERATION

by

RAINEL ZELAYA

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Biomedical Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Ratna Chakrabarti
Abstract

Prostate cancer is the second most often diagnosed cancer and internationally the sixth foremost cause of cancer death in males, as of 2011. Within the United States it is the most common form of cancer in men with 186,000 new cases and with an overall 28,600 deaths in 2008, and it is the second leading kind of cancer-related death in men. The widespread threat that prostate cancer poses against men across the globe cannot be understated, and its initiation and progression must be understood in order to truly comprehend its implicated risks and possible forms of treatment.

As its name implies, prostate cancer is a form of cancer that develops in the prostate gland located in the male reproductive system. Its progress starts when standard semen-secreting prostate gland cells mutate into cancer cells. Although its developments may start at the prostate gland, cancer cells may metastasize to other parts of the body through circulation systems such as the lymph nodes. The main sites of metastasis for prostate cancer include the adrenal gland, the bones, the liver and the lungs.

Although there are treatments available for prostate cancer, there is no definitive cure. The primary goal of this project was to find an alternative form of treatment, which is what will be necessary to combat this cancer.
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Introduction

**Events with Implications on Prostate Cancer Initiation**

One of the most important prostate cancer risk factors is increasing age, and several studies have linked aging to changes in overall gene expression, such as those included in inflammation, oxidative stress, and cellular senescence.

There is support that indicates a link between chronic inflammation and prostate cancer carcinogenesis, and expression of particular chemokines has been known as a predictor of biochemical disease recurrence in human prostate cancer (Begley, Monteleon et al. 2005). An example of this was shown by the administration of the heterocyclic amine PhIP in rodents, which caused chronic inflammation and promoted prostatic hyperplasia and prostatic intraepithelial neoplasia (PIN), which is a premalignant stage that is closely associated with cancer (Elkahwaji, Zhong et al. 2007). Also, the well-studied gene GSTP1 is known to detoxify reactive carcinogens such as PhIP, and it has been shown to be silenced as a result of DNA methylation in various prostate cancers (Nakayama, Gonzalgo et al. 2004).

Oxidative stress and its potential in causing DNA damage are also significant factors that may contribute to prostate carcinogenesis. This stress comes from a disproportion of reactive oxygen species and enzymes that regulate their levels (Shen and Abate-Shen 2010). Studies have shown a reduction in antioxidant enzymes in human PIN and prostate cancer along with an increase in the oxidized DNA adduct 8-oxy-dG (Bostwick, Alexander et al. 2000). The NKX3.1 gene is often downregulated in the initial stages of prostate cancer, and this may contribute to
DNA damage due to oxidative stress, since loss of function of the Nkx3.1 homeobox in rodent prostate has been shown to lead to a decrease in expression of oxidative damage response genes and amplified levels of 8-oxy-dG (Ouyang, DeWeese et al. 2005).

Cellular senescence has also been studied in relation to prostate carcinogenesis and its transition from a latent stage to the more aggressive clinical stage. Cellular senescence refers to a type of cell cycle arrest in which cells maintain their viability but remain in a nonproliferative state even in the presence of mitogenic signals. Studies have shown that cellular senescence may contribute as a form of tumor suppression that thwarts the progression to a malignant state. For instance, complete inactivation of the tumor suppressor gene known as Pten results in prostatic intraepithelial neoplasia lesions that exhibit a senescence phenotype (Chen, Trotman et al. 2005). This phenotype may be bypassed by the inactivation of the p53 tumor suppression gene, which indicates that extra oncogenic occurrences are most likely necessary to overcome senescence (Chen, Trotman et al. 2005).

**Molecular Basis for Prostate Cancer Development**

Several molecular mechanisms play a potential role in the initiation or advancement of prostate cancer, some of which have already been mentioned. The upregulation of the human oncogene MYC has been known to be amplified in some advanced forms of prostate tumors, and more recently the MYC protein has been shown to be overexpressed in PIN lesions and in several carcinomas, which suggests that elevated levels of nuclear MYC may be a significant oncogenic incident for initiation of prostate cancer (Gurel, Iwata et al. 2008).

MiRNA expression in relation to the progression of prostate cancer has also been studied, and in addition to regulating normal cell functions miRNAs have been found to play a role in
regulating pathogenic cancer processes. For instance, miRNAs have been shown to be involved in precise regulation in the expression of target genes such as the previously mentioned Pten, which has been revealed to be negatively regulated by the miRNA-106b-25 (Poliseno, Salmena et al. 2010). Another example is the miRNA-101, which has been found to inhibit the expression and function of EZH2, a histone methyltransferase associated with aggressive tumors that has been found to play a role in the epigenetic silencing of target genes and in the regulation of cancer cell metastasis (Varambally, Cao et al. 2008).

The alteration of intracellular signaling pathways in prostate cancer cell progression has also been observed. Simultaneous activation of the signaling pathways known as Akt/mTOR and Erk (p42/44) MAPK stimulates tumor progression and castration resistance in prostate cancer cell lines and mouse models (Kinkade, Castillo-Martin et al. 2008). Simultaneous inhibition of each has been shown to hinder castration-resistant prostate cancer in genetically engineered mice, suggesting the possibly important role that these pathways play together (Kinkade, Castillo-Martin et al. 2008).

**Current Prostate Cancer Treatments**

Various forms of treatment are available to treat prostate cancer and current cancer treatment takes into consideration the age and expected lifespan of the patient, the stage and the grade of cancer, the patient’s health conditions, the probability that the chosen treatment will cure the cancer, the possible side effects of the specific form of treatment, and the overall opinions of the patient and doctor of the need for treatment. Frequently, before any forms of treatment are administered, physicians may suggest taking a period of watchful waiting, during which the progression of the prostate cancer is regularly checked. If a prostatectomy is not a
viable form of treatment (which may be due to the development of a late stage form of cancer that has spread to other areas), other current treatment that may be used include chemotherapy, radiation therapy, and hormone therapy.

**Chemotherapy**

Chemotherapy treatments involve the injection or oral consumption of anti-cancer drugs and as a result they are generally utilized in cases that involve metastatic spread of cancer through the bloodstream. One of the primary drugs used in chemotherapy is Docetaxel, a taxane (a drug that comes from a plant of the genus *Taxus*) which interferes with cancer cell division. The mechanism of action used by docetaxel involves inhibition of mitotic cell division between metaphase and anaphase through the suppression of microtubule assembly and disassembly (halting cancer cell division), and an accumulation of microtubules inside cells which may result in the initiation of apoptosis (Lyseng-Williamson and Fenton 2005). Docetaxel has been shown to slow metastatic cancer progression and studies have shown significant survival benefit from this drug in androgen-independent metastatic prostate cancer (Tannock, de Wit et al. 2004).

Nevertheless, many adverse effects have been associated with docetaxel treatment, such as its cytotoxicity to all dividing cells in the body. Side effects include cytotoxicity to the hair follicles and the bones marrow, and even hematological effects such as neutropenia, anemia, and thrombocytopenia, which can make patients more prone to infections and overall weakness and fatigue (Lyseng-Williamson and Fenton 2005).

Cabazitaxel, also a microtubule inhibitor and a taxane, is often a drug used for treatment after docetaxel. Just like docetaxel, it has also shown to cause common adverse effects such as anemia, leukopenia, neutropenia, thrombocytopenia, diarrhea, fatigue, nausea, vomiting and
constipation (Abidi 2013).

Docetaxel and cabazitaxel have been more recently utilized for prostate cancer treatment, and mitoxanthrone is generally used as a second line of treatment since it does not offer the improved survival and disease free period as these latest forms of treatment. Mitoxanthrone is a type II inhibitor of topoisomerase and disrupts DNA synthesis and repair (Mazerski, Martelli et al. 1998). A study showed a palliative response when treated along with prednisone against hormone-resistant prostate cancers in 23 of 80 randomized patients, and an improvement in quality-of-life scales and a decrease in serum prostate-specific antigen level, which is often increased in patients with prostate cancer (Tannock, de Wit et al. 2004). Mitoxanthrone only serves as a drug whose treatment can result in palliative responses and not as a definitive cure, and the main goal of its treatment is to improve the quality of life of the patient. In addition to this, mitoxanthrone treatment may cause adverse effects with varying severity such as cardiomyopathy, nausea, vomiting, hair loss, immunosuppression and heart damage, which may result from the fact that it may affect both cancerous and healthy cells in the body (Fox 2006).

**Radiation Therapy**

Radiation therapy for prostate cancer involves treatment of cancer cells with high energy rays such as x-rays to kill them or to reduce the size of tumors. Two different types of radiation methods may be used, External beam radiation therapy (EBRT), radiation which comes from outside of the body, or internal radiation from radioactive materials inserted into the tumor, which is also known as brachytherapy.

Standard EBRT is not frequently used currently due to its substandard level of accuracy compared to newer techniques, which has been known to cause undesired exposure of nearby
tissues to radiation. EBRT is also only able to be primarily used in more localized stages of prostate cancer, and even in these cases has been shown to result in transitional cell carcinoma of the bladder, which is more aggressive and its diagnosis may occur later due to the fact that some radiation oncologists believe that the hematuria occurring after this treatment is considered to be common (Suriano, Altobelli et al. 2013). Some patients treated for localized forms of prostate cancer may end up having invasive bladder cancer that requires surgical treatment (Suriano, Altobelli et al. 2013). Many other adverse effects have been associated with EBRT patients, such as bowel problems, fatigue, bladder problems, urinary incontinence, erection dysfunction, urethral stricture, and lymphedema. Brachytherapy is a more common form of radiation treatment due to its fewer serious side effects, and its more specific localization, which focuses radiation exposure to the prostate rather and limits other nearby tissue exposure (Zaorsky, Doyle et al. 2013). A 2007 study observing patient quality life showed brachytherapy as a more favorable form of treatment, as opposed to treatments such as EBRT and prostatectomy (Buron, Le Vu et al. 2007). Brachytherapy as a form of treatment is only useful for localized forms of prostate cancer, since it requires the insertion of tiny radioactive particles into a tumor site, and therefore would not be useful for treating more advanced forms of prostate cancer involving metastasis, which may have been diagnosed too late.

**Hormone Therapy**

Various types of hormone therapies exist, and most involve direct or indirect deprivation of the androgens dihydrotestosterone (DHT) and testosterone, which stimulate the growth of prostate cancer cells. Several methods may be used in androgen deprivation therapy, such as orchiectomy (removal of testicles to prevent androgen production), luteinizing hormone-
releasing hormone (LHRH) agonists and antagonists (which chemically reduce the level of androgen production by the testicles), and anti-androgens, which block the androgen receptors on prostate cells. A major problem with hormone therapy is the lack of consensus regarding the best time to begin and to cease treatment. There are several opinions and current studies regarding the advantages and disadvantages of early deprivation therapy treatment versus delayed treatment, and continuous versus intermittent treatment, and definitive answers regarding the most effective methods are yet to be seen (Trendel 2013).

Unfortunately, many hormone dependent cancers such as prostate cancer become refractory after one to three years and continue to grow even with androgen deprivation. This type of development is known as castration-resistant prostate cancer due to the cancer cells’ lack of response to the surgical or chemical reduction of androgen. Once this occurs, chemotherapeutic drugs such as docetaxel may be provided as treatment.

Since each form of hormone therapy affects the action of androgen on its receptor and ends in a similar result, through surgical or chemical removal of the source of androgen or by the direct blocking of its receptor, many adverse side effects are shared as a result of the reduction of testosterone or DHT. Changes in the level of androgen may also include changes in estrogen levels, and side effects may include a reduced libido, impotence, the shrinking of the testicles and penis and gynecomastasia. More serious side effects that stem from androgen deprivation include anemia, osteoporosis, loss of muscle mass, fatigue, increase cholesterol, decreased mental sharpness, and depression (Miwa, Mizokami et al. 2013).
Potential Forms of Treatment

**Antimitotics**

Many different types of inhibitors have been and are currently being studied to treat prostate cancer. As previously mentioned, docetaxel is a chemotherapeutic drug and an inhibitor of mitosis that works by binding to microtubules, subsequently preventing cell division. A major focus of chemotherapeutics has recently been placed on antimitotic drugs such as docetaxel due to the fact that cancer is a disease of unrestrained mitosis. However, instead of focusing on microtubules as the major targets to halt mitosis, recent research has focused on targeting proteins that play a major role in mitotic events in order to prevent possible binding to microtubules of non-tumorigenic cells. These target proteins include kinases and kinesins.

**Aurora Kinase Inhibition**

The serine/threonine kinases known as Aurora have been known to have three types: Aurora A, Aurora B, and Aurora C. Aurora A plays a role in mitotic spindle assembly during the early stages of mitosis, and its inhibition can lead to mitotic arrest and eventually apoptosis as a result of faulty spindle morphology. Aurora B and Aurora C play a role in later stages of mitosis and especially in cytokinesis, and inhibition of Aurora B can lead to the prevention of cytokinesis, polyploidy and then apoptosis.

This family of kinases has been shown to be overexpressed in cancer cells, and have therefore been targeted in recent developments (Wissing, van Diest et al. 2013). One of the first aurora kinase inhibitors to enter clinical trials was VX680, which has been shown to inhibit each of these three kinases in vitro with IC$_{50}$ values ranging from 15 to 130 nM (Jeet, Russell et al. 2012). Even with its promising results, its clinical trials were suspended due to toxicity values.
Another inhibitor under study is AZD1152, an inhibitor of Aurora B that is currently undergoing phase 2 clinical trials, and so far has been shown to be reasonably well tolerated for the treatment of diffuse large B-cell lymphoma, although side effects such as stomatitis and suppression of the bone marrow have been observed (Jeet, Russell et al. 2012). Another promising candidate in current drug developments is MLN8237. MLN8237 is an Aurora A kinase inhibitor that is also presently undergoing phase 2 clinical trials, and it has been reported to have high specificity and potency as an inhibitor of Aurora A, with an IC$_{50}$ value of 1 nM (Jeet, Russell et al. 2012).

There are many more aurora kinase inhibitors that have or are currently undergoing clinical trials, and aurora kinases appear to be very promising as targets for the disruption of mitosis.

**Polo-Like Kinase Inhibition**

Polo kinases (PLKs) have also been targeted in recent anticancer drug developments. These are a family of serine/threonine kinases that are involved in the generation of spindle poleward pulling forces and cytokinesis, and PLK1, one of the four members of this family, has been shown to be elevated in many tumors (Harrison, Holen et al. 2009). One promising distinction in regards to these kinases as possible targets is that the inhibition of PLK1 has been shown to lead to a G2/M arrest and apoptosis (Warner, Gray et al. 2006). In addition, while PLK1 depletion has been shown to be harmful to cancer cells, normal cells have displayed little to no cytotoxicity in response to this treatment, thus making it a desirable target in antimitotic cancer research (Liu, Lei et al. 2006).

One promising compound in polo-like kinase inhibition is BI2536, a highly specific inhibitor of PLK1. A phase 1 clinical trial with 21 patients with advanced solid tumors showed
the maximum dosage of this compound to be 60 mg, which was determined based on dose-limiting toxicities (Harrison, Holen et al. 2009). The most commonly reported side effects included fatigue, leukopenia, constipation, nausea, mucosal inflammation, anorexia, and alopecia. This drug is currently undergoing phase 2 trials involving metastatic or relapsed non-small cell lung cancer.

Another polo-like kinase inhibitor is ON01910.Na, an ATP noncompetitive inhibitor of PLK1 that hinders ability of PLK to bind substrates. This compound is currently undergoing Phase 3 studies involving the treatment of metastatic pancreatic adenocarcinoma (Harrison, Holen et al. 2009).

**Kinesin Spindle Protein Inhibition**

Kinesin spindle proteins are motor proteins that play a role in bipolar spindle formation and centrosome separation. It has been shown that expression of kinesin spindle proteins is elevated in tumor cells, and inhibition of these proteins has displayed mitotic arrest due to the formation of a monopolar spindle (Harrison, Holen et al. 2009). Ispinesib is a compound that inhibits kinesin spindle protein ATPase via uncompetitive inhibition with ATP and ADP. It has shown activity in patients with metastatic breast cancer in phase 2 clinical trials, but has not shown activity in other cancers such as ovarian and colorectal cancers (Harrison, Holen et al. 2009).

Other compounds that practice kinesin spindle protein inhibition and are currently undergoing trials are SB-743921 and GSK-923295. SB-743921 has shown even more potency than ispinesib and is currently undergoing phase 1 and 2 trials in non-Hodgkin’s lymphoma.

**Drug Discovery: An Observation of Natural Compounds**

Numerous possible forms of treatment for diseases such as cancers have been discovered through synthetic combinatorial chemistry. Recently, however, many drug discovery programs have been focusing on natural compounds in their research efforts. Some of the main problems that have been associated with the utilization of naturally-derived compounds in drug discovery and have therefore retarded its process involve difficult screening procedures, some of which include the presence of impure samples which contain mixtures rather than a single compound and the possibility of product degradation. Efforts with pure combinatorial synthetic chemistry have also met with some failure in the last two decades with high throughput screening. This process has resulted in the production of millions of molecules via random generation of compound libraries that have not effectively met the requirements of the drug discovery industry.

These main problems associated with working purely with naturally-derived compounds or synthetically-derived compounds have been under study. One recent manner of overcoming these problems, which has already been applied by several research groups, is to look into utilizing natural compound structures as starting points in the development of drug-like libraries. This approach allows drug discovery programs to utilize the advantages that come from each of these methods while avoiding the complications that usually come with their isolated execution.

One complication in the implementation of this combined approach has been translating the inherent complexity of natural products into a manageable form of chemistry. One manner of overcoming this unique dilemma has been to investigate simple structural elements that
commonly occur in natural products. The BioCore has been identified by the company ASINEX as one of these elements, and its structure consists of two heterocycles, one aromatic and one saturated, that are linked by a carbon-carbon bond or a carbon-carbon linear spacer fragment (Kombarov, Altieri et al. 2010). The concept of the BioCore was obtained from a statistical analysis of known drugs and natural products and it may solve some of the complications that come from designing small molecule scaffolds from complex natural products (Kombarov, Altieri et al. 2010).
Methods

Cell Culture

Three types of cell lines were utilized for this study: PC-3 cells, BPH-1 cells, and NIH-3T3 cells. PC-3 cells are an aggressive prostate cancer cell line that have high metastatic potential. BPH-1 cells are benign prostatic hyperplasia cells, which are immortalized cells that are not metastatic, and NIH-3T3 cells are standard fibroblast cells. PC-3 cells were maintained in HAM media (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 1% antibiotic-antimitotic, and 1% L-Glutamine. BPH-1 cells were maintained in phenol red-free Dulbecco’s Modified Eagle’s Medium media (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 1% antibiotic-antimitotic, and 1% L-Glutamine. NIH-3T3 cells were maintained in phenol red-free Dulbecco’s Modified Eagle’s Medium media (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 1% antibiotic-antimitotic, and 1% L-Glutamine. All cells were maintained in a 5% CO₂ humidified incubator at 37°C.

Cell Seeding

The three cell lines that were previously mentioned were seeded in 384-well plates. Approximately 500 PC-3 cells were seeded per well, approximately 500 BPH cells were seeded per well, and approximately 200 3T3 cells were seeded per well. All cell lines were maintained in 20 cm tissue culture plates, and cells were washed with 3 mL of 1X PBS, then 3 mL of 1X trypsin was added to each tissue culture plate and maintained at room temperature for
approximately 30 seconds, then trypsin was removed and the tissue culture plate was incubated at 37°C with 5% CO₂ for approximately 10 minutes. All cells were then resuspended in their respective media, and 20 uL of this cell-media mixture was added to each well in a 384-well plate.

**Compound Dilution Preparation**

The compounds used in this experiment were obtained from a library that included 511 ASINEX compounds whose structures are derived from the concept of the BioCore. Each compound was resuspended in DMSO solvent in a glass vial at a concentration of 10mM and stored at -80°C. For each day of treatment of cells with these compounds, each compound was thawed at room temperature, vortexed for 2 minutes, and added to the cell line’s respective media in a microcentrifuge tube, which was then also vortexed for 2 minutes.

**Compound Treatment**

20uL of compound-media solution was added to each well in a 384 well plate for a total volume of 40uL in each well for each cell line after treatment. Each plate was incubated for 72 hours.

**MTS Assay and Absorbance Readings**

In addition to qualitative microscopy analysis, inhibition of cells was quantified via an MTS assay after the 72 hour time point. The MTS assay is a cell viability assay that allows for the measurement of the relative number of living cells in culture. MTS is a tetrazolium salt that is converted into formazan by dehydrogenase enzymes found in metabolically active cells. The amount of formazan product measured at a 490nm absorbance is directly proportional to the
number of living cells in culture. In addition to 490nm readings, background absorbance readings were also collected at 620nm and subtracted from the initial 490nm values to obtain values that are not affected from chemical interference from other compounds that may be present in the media. 8uL of MTS was added to each well in this experiment, in relation to the amount of total media in each well which was 40uL. After MTS assay dye was added, for each plate treated in this experiment, the cells were incubated for four hours. Then each plate was scanned for absorbance values by using a multifunctional plate reader (Biotek) and then values were compared to the DMSO control values in order to detect inhibition.

**Data Analysis**

511 compounds were initially screened at 10µM in triplicate using PC-3, BPH, and 3T3 cells. Those compounds with which we observed high percentage of inhibition of PC-3 cells and lower percentages of inhibition of the other cell lines were tested at again at 10µM and at lower concentrations, specifically at 5µM, 2.5µM, 1.25µM, and 0.625µM. For each cell line, the average of the triplicate 490nm-620nm triplicate absorbance values at the initial screening of 10µM were calculated and divided by the DMSO-only treated cells to produce a ratio to control. The controls included live cells with the same total amount of media, DMSO-only treated cells, and dead cells treated with 50% tween. The absorbance values for these controls were also included in the ratio over the vehicle (DMSO-only treated cells). These ratios were calculated and displayed in graphical form.

These ratios were also organized into a visualized heat map through the use of Gene Cluster 3.0. This clustering program provides methods to determine similarities between different values (such as Euclidean distance, which was utilized in this experiment) and it
provides multiple hierarchical clustering options (such as complete linkage clustering, which was utilized in this experiment) (de Hoon et al., 2004). In order to create a visualized heat map, the ratios were processed in log space (in log base 2 for simplicity). This means, for example, that any 2-fold changes up or down from an initial data point are symmetric about 0. The data was then normalized and the Euclidean distance and complete linkage clustering methods were taken into account. The Euclidean distance takes the magnitude of changes in inhibition levels into account and complete linkage clustering makes the distance between two items \( a \) and \( b \) the maximum of all pairwise distances between items contained in \( a \) and \( b \). This analysis was then visualized as a heat map with Java Treeview (Page, 1996).

Percent inhibition is a value demonstrating the percentage of cells that were inhibited per well. The percentage of inhibition was calculated for each compound and for each cell line. This was obtained by utilizing the values from the 490nm-620nm absorbances that were read in triplicate per compound. To calculate percent inhibition per well, the absorbance reading from each compound-treated well was subtracted from the average absorbance reading for DMSO-only treated cells from the respective cell line from triplicate values in each plate. This value was then divided by a second value, which was the average absorbance calculated for the dead cell wells in triplicate from the same cell line subtracted from the same average absorbance reading for DMSO-only treated cells. This total value was then multiplied by 100 to obtain the final percent inhibition.

After percent inhibition was calculated for each compound out of the 511 compounds that were screened, the values were sorted based on the highest inhibition percentage values obtained from treated PC-3 cells, since one of the primary goals of this procedure was to acquire possible
inhibitors of aggressive prostate cancer cells by utilizing PC-3 cells as a model. There were two tiers of compounds that were sought after in the initial screening in order to conduct further tests: those with high levels of inhibition of PC-3, low levels of inhibition of BPH, and low levels of inhibition of NIH-3T3 cells, and those with high levels of inhibition of PC-3, high levels of inhibition of BPH-1, and low levels of inhibition of NIH-3T3 cells. The former tier was the most favorable because it would resemble a compound with the ability to inhibit aggressive prostate cancer cells while maintaining viable BPH-1 (unaggressive cancer cells) and 3T3 cells (which in this case model normal cells of the human body). The second tier of compounds would resemble an inhibitor of both aggressive and unaggressive prostate cancer, and thus would not be as highly sought after due to this decreased specificity of inhibition.

Nine compounds were placed in one of these tiers out of the initial screening at 10µM. Four were placed in the first tier and five in the second tier. These nine compounds were tested again at the original concentration of 10µM, and at four lower levels of concentration: 5µM, 2.5µM, 1.25µM, and 0.625µM.

IC$_{50}$, or half maximal inhibitory concentration, is a measurement used to measure the effectiveness of a compound’s inhibition ability. It indicates the minimum concentration that is needed for a compound to inhibit biological functions by half. The IC$_{50}$ values for each of these nine compounds was calculated by using their observed absorbance values at the five previously mentioned concentrations through the use Graphpad PRISM and were obtained by normalizing them to the controls. The lowest IC$_{50}$ values will be used to conduct further studies about the effectiveness of inhibition of these drugs.
The Lipinski Rule of Five was then utilized to determine the potential that these compounds have as potential oral drugs by utilizing the computer program MarvinSketch (Chemaxon). This method is used as a rule of thumb to determine if a compound can be used as an orally active drug in humans by analyzing the molecular structure of the compound (Lipinski, Lombardo et al. 2001). This method takes into account factors such as the number of hydrogen bond donors, the number of hydrogen bond acceptors, and the molecular mass of the compound.
Results

Initial 10µM Screening

The following graphs show the values obtained from the initial 10µM screening of 511 compounds over 72 hours. This was conducted by using the MTS assay. Each graph represents a plate with different absorbance average values which were normalized to the DMSO-only treated controls. In each graph, the red bars represent PC-3 cells, the blue bars represent BPH-1 cells, and the yellow bars represent NIH-3T3 cells. Each cell line has its own controls: live cells, dead cells, and vehicle (DMSO-only) treated cells.
Figure 1: Compounds 5a1 through 5c10

Figure 1 shows an initial screening of compounds 5a1 through 5c10 at 10µM with DMSO treated cells, live cells, and dead cells as controls.

In Figure 1, compound 5b7 was noted as a candidate for the next screening at lower concentrations due to its lower ratio of PC-3 over the DMSO-only treated PC-3 cells. BPH-1 and NIH-3T3 cells shared ratios similar to their live and vehicle controls.
Figure 2: Compounds 2a2 through 2d7 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 3: Compounds 2d9 through 2h1

Figure 3 shows an initial screening of compounds 2d9 through 2h1 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 4: Compounds 3a1 through 3d1

Figure 4 shows an initial screening of compounds 3a1 through 3d1 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
In Figure 4, 3a3 was ruled out as a candidate for the next screening due to its low 3T3 ratio in comparison to its live ratio, which suggests that this compound may be harmful to normal cells. 3c1 was noted as a candidate because of the proximity of its NIH-3T3 ratio to its live control, and its lower levels of BPH-1 and PC-3 ratios in relation to their live and vehicle controls.
Figure 5: Compounds 3d2 through 3g5

Figure 5 shows an initial screening of compounds 3d2 through 3g5 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 6: Compounds 1a1 through 1d10

Figure 6 shows an initial screening of compounds 1a1 through 1d10 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 7: Compounds 1d1 through 1h1

Figure 7 shows an initial screening of compounds 1d1 through 1h1 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
In Figure 7, compound 1e12 was also noted as a candidate for the next screening at lower concentrations due to its lower ratio of PC-3 over the DMSO-only treated PC-3 cells. NIH-3T3 cells shared a ratio similar to their live and vehicle controls, while BPH-1 cells had a ratio of approximately 0.6 in comparison to its live and vehicle ratios of 0.8 and 1, respectively. 1g6 was another candidate, since it showed ratios for PC-3 and BPH-1 cells (at approximately 0.3 and 0.3) that were much lower than their live and vehicle controls (at approximately 0.8 and 1).
Figure 8 shows an initial screening of compounds 4a1 through 4d1 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
In Figure 8, compound 4b3 was noted as a candidate for the next screening at lower concentrations due to its lower ratio of PC-3 over the DMSO-only treated PC-3 cells. BPH-1 and NIH-3T3 cells shared ratios similar to their live and vehicle controls.
Figure 9: Compounds 4d2 through 4g2

Figure 9 shows an initial screening of compounds 4d2 through 4g2 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 10: Compounds a1 through d3

Figure 10 shows an initial screening of compounds a1 through d3 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 11: Comounds d4 through g6

Figure 11 shows an initial screening of compounds d4 through g6 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 12: Compounds 101-3g6 through 1h12.

Figure 12 shows an initial screening of compounds 101-3g6 through 1h12 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
Figure 13: Compounds 3a2 through 4g7

Figure 13 shows an initial screening of compounds 3a2 through 4g7 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
In Figure 13, 3a6 was noted as a candidate for the next screening at lower concentrations due to the ratio value of PC-3, which was approximately 0.37, as opposed to the ratio obtained from the live cells, which was approximately 0.6. The BPH-1 ratio for this compound was approximately 0.49, as opposed to 0.68 for its live control. The NIH-3T3 ratio for this compound was close in value its live control.
Figure 14: Compounds 2a1 through 2d2

Figure 14 shows an initial screening of compounds 2a1 through 2d2 at 10µM with DMSO treated cells, live cells, and dead cells as controls.
In Figure 14, 2c3 was chosen as a candidate for the next screening as a result of its lower PC-3 ratio in relation to its live and vehicle controls. The BPH-1 ratio for this compound was also lower than its controls, while the NIH-3T3 ratio was higher than its controls at about 1.15.
Heat Map and Clustering

Figure 15: Heat Map of Initial Screening

Figure 15 shows a complete heat map visualization from clustering of the initial screening ratios that were calculated after normalization to the DMSO controls.
In Figure 15, the lightest spectrum of green coloring signals inhibition of cells, black coloring signals no inhibition or growth of cells, and the lightest spectrum of red coloring signals growth of cells.
Figure 16 shows a zoomed-in visualization of selections of the complete heat map with clusters of the initial screening with 511 compounds and the cell lines: PC-3, BPH-1, and NIH-3T3.
For Figure 16, green indicates inhibition of cells, black indicates no inhibition or growth of cells, and red indicates growth of cells. Nine compounds (labeled with a red box) were chosen for a second screening. Compounds 5b7, 1e12, 4b3, and d3 exhibited inhibition of PC-3 and no inhibition or growth of BPH-1 or NIH-3T3, and compounds 1g6, 3a6, 3c1, 2b2, 2c3 exhibited inhibition of PC-3 and BPH-1, and no inhibition or growth for NIH-3T3 cells.
**Selected Compound Testing at Lower Concentrations**

Table 1: Percent Inhibition

<table>
<thead>
<tr>
<th>Plate</th>
<th>PC-3</th>
<th>BPH-1</th>
<th>NIH-3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compound</td>
<td>% Inhibition</td>
<td>Compound</td>
</tr>
<tr>
<td>P1</td>
<td>5b7</td>
<td>84.4</td>
<td>5b7</td>
</tr>
<tr>
<td>P7</td>
<td>1e 12</td>
<td>83.8</td>
<td>1e 12</td>
</tr>
<tr>
<td>P7</td>
<td>1g6</td>
<td>81.0</td>
<td>1g6</td>
</tr>
<tr>
<td>P8</td>
<td>4b3</td>
<td>72.3</td>
<td>4b3</td>
</tr>
<tr>
<td>P13</td>
<td>3a6</td>
<td>71.0</td>
<td>3a6</td>
</tr>
<tr>
<td>P4</td>
<td>3c1</td>
<td>62.2</td>
<td>3c1</td>
</tr>
<tr>
<td>P2</td>
<td>2b2</td>
<td>61.9</td>
<td>2b2</td>
</tr>
<tr>
<td>P14</td>
<td>2c3</td>
<td>58.6</td>
<td>2c3</td>
</tr>
<tr>
<td>P10</td>
<td>d3</td>
<td>57.8</td>
<td>d3</td>
</tr>
</tbody>
</table>

Table 1 displays the percent inhibition that was calculated for all compounds and for each cell line from the initial screening.
Nine compounds were selected from 511 based on two categories. The first category, labeled in green, represents compounds that exhibit high percent inhibition for PC-3 cells, low to moderate percent inhibition for BPH-1 cells, and very low percent inhibition for NIH-3T3 cells. The second category, labeled in yellow, represents compounds that exhibit high percent inhibition for PC-3 cells, high percent inhibition for BPH-1 cells, and very low percent inhibition for NIH-3T3 cells.

The following graphs show the ratio to the DMSO-only treated control values for these 9 selected compounds at the screening of concentrations at 10µM, 5µM, 2.5µM, 1.25µM, and 625nM. This was performed twice with different cell populations each time.
Figure 17: Second Screening: 5b7 through 2b2, Trial 1

Figure 17 shows the first trial of the second screening of compounds 5b7 through 2b2 at 10µM, 5µM, 2.5µM, 1.25µM, and 625 nM.
After 9 compounds were selected based on the previously mentioned categories of inhibition, these 9 compounds were further tested at the concentrations of 10µM, 5µM, 2.5µM, 1.25µM, and 625nM to check for specificity. The cells were incubated for 72 hours. Figure 16 shows compounds 5b7 through 2b2, with data labels representing the ratio of actual absorbance to DMSO-only treated cells. This test was conducted twice from two different cell populations and this graph represents the first population, trial 1. Figure 18 shows the same compounds utilizing a second population of cells, trial 2. Figures 19 and 20 show compounds 2c3 and d3 with two different populations as well.
Figure 18: Second Screening: 5n7 through 2b2, Trial 2

Figure 18 shows the second trial of the second screening of compounds 5b7 through 2b2 at 10µM, 5µM, 2.5µM, 1.25µM, and 625 nM.
Figure 19: 2c3 and d3, Trial 1

Figure 19 shows the first trial of the second screening of compounds 2c3 and d3 at 10µM, 5µM, 2.5µM, 1.25µM, and 625 nM.
Figure 20: 2c3 and d3, Trial 2.

Figure 20 shows the second trial of the second screening of compounds 2c3 and d3 at 10µM, 5µM, 2.5µM, 1.25µM, and 625 nM.
IC$_{50}$ Analysis (µM)

Table 2: IC$_{50}$ Values

<table>
<thead>
<tr>
<th>Compound Label</th>
<th>5b7</th>
<th>1e12</th>
<th>1g6</th>
<th>4b3</th>
<th>3a6</th>
<th>3c1</th>
<th>2b2</th>
<th>2c3</th>
<th>1d3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ Trial 1</td>
<td>38.07</td>
<td>55.72</td>
<td>4.9</td>
<td>31.11</td>
<td>7.6</td>
<td>43.02</td>
<td>9.6</td>
<td>9.637</td>
<td>25.21</td>
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<tr>
<td>IC$_{50}$ Trial 2</td>
<td>36.02</td>
<td>22.91</td>
<td>10.5</td>
<td>21.3</td>
<td>10.3</td>
<td>29.52</td>
<td>9.5</td>
<td>8.666</td>
<td>59.04</td>
</tr>
<tr>
<td>Average IC$_{50}$</td>
<td>37.0</td>
<td>39.3</td>
<td>7.7</td>
<td>26.2</td>
<td>9.0</td>
<td>36.3</td>
<td>9.6</td>
<td>9.2</td>
<td>42.1</td>
</tr>
</tbody>
</table>

For Table 2, IC$_{50}$ values were calculated with the observed absorbance values from the screening at 10µM, 5µM, 2.5µM, 1.25µM, and 625nM concentrations by using Graphpad Prism.
After the average IC$_{50}$ values were calculated, four compounds with low IC$_{50}$ values were observed: 1g6, 3a6, 2b2, and 2c3. Their respective IC$_{50}$ values were 7.7μM, 9.0μM, 9.6μM, and 9.2μM. These values indicate the minimum concentrations needed in order to obtain 50% inhibition of PC-3 cells.
Lipinski Analysis

Table 3: Lipinski Values

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Molecular Weight</th>
<th>clogp</th>
<th>H donors</th>
<th>H Acceptors</th>
<th>Total Polar Surface Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G06</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td>421.45</td>
<td>2.96</td>
<td>1</td>
<td>7</td>
<td>103.52</td>
</tr>
<tr>
<td>3A06</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td>380.44</td>
<td>4.43</td>
<td>1</td>
<td>6</td>
<td>64.03</td>
</tr>
<tr>
<td>2B02</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td>373.45</td>
<td>2.32</td>
<td>1</td>
<td>6</td>
<td>72.92</td>
</tr>
<tr>
<td>2C03</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td>442.51</td>
<td>3.85</td>
<td>1</td>
<td>5</td>
<td>80.49</td>
</tr>
</tbody>
</table>

Table 3 shows the structures of the four selected compounds with IC₅₀ values under 10μM that were drawn and the molecular weight, clogp, number of hydrogen donors and acceptors, and the total polar surface area were calculated to make sure that these compounds fell within the Lipinski Rules.
According to Lipinski’s rules, the molecular weight must remain below 500 daltons, the clogp must remain below 5, the number of hydrogen donors must remain below 5, the hydrogen bond acceptors must remain below 10, and the total polar surface area must remain below 140 squared angstroms. As shown in the table, each of these compounds follows Lipinski’s rules.
Discussion

After conducting an initial screening of 511 compounds derived from the ASINEX BioCore library, nine compounds were selected as candidates for a second screening at lower concentrations based on their percent inhibition of the three cell lines in order to study their potential as viable drugs against prostate cancer. Four of these nine compounds were selected based on their IC$_{50}$ values against PC-3 cells, and all four of this values were lower than 10µM. The four compounds, labeled 1g6, 3a6, 2b2, and 2c3, had respective average IC$_{50}$ values of 7.7µM, 9.0µM, 9.6µM, and 9.2µM. All four drugs followed Lipinski’s rules, which indicates that further studies may be performed due to the fact that they meet some of the guidelines necessary to become orally ingested.

Scaffolds and Previous Assays

Two of the four compounds selected from the second screening showed previous assays conducted in search of inhibitory effects in the PubChem BioAssay database. Compounds 3a6 and 2c3 showed no previously known scaffolds and no documented assays per PubChem, suggesting that further studies need to be performed to test for inhibitory effects against prostate cancer. Compound 2b2 was shown to be inactive against the strain H37rv of *Mycobacterium tuberculosis* (AID:1949 from the Southern Research Institute) and minimally active against two strains of *Plasmodium falciparum* (AID: 449707 from the European Bioinformatics Institute). Compound 1g6 was shown to be inactive against the CapD enzyme of *Bacillus anthracis*, which produces amide bonds with peptidoglycan cross-bridges in order to anchor capsular material
inside its cell wall envelope (AID: 492967 from the ICCB-Longwood/NSRB Screening Facility at Harvard Medical School). It was also shown to be inactive against human Hsp70, which are a family of heat shock proteins expressed that play a significant part in protein folding and in protection from cellular stress (AID: 583 from the Burnham Center for Chemical Genomics). Compound 1g6 was also shown to be inactive against HIV Rnase H in a cell-free assay. Despite this recorded lack of inhibitory effects, there have not been any known assays of any possible inhibitory effects compounds 2b2 and 1g6 have against prostate cancer or any other forms of cancer. Therefore further assays need to be performed to seek inhibition of prostate cancer and other cancers.

**Conclusion and Future Directions**

The compounds selected in this study may be used in the identification of inhibition of cellular processes for cell culture-based experiments. It would be important to repeat and optimize the initial and second screenings that were conducted in this experiment. After repetition and optimization, it would be useful to find the specific processes that are inhibited, and an evaluation must be completed to observe which process is plugged by each of these compounds. The compounds should be analyzed for any specific character, and knowledge of their mechanism of action can be obtained. The pharmacokinetics of these compounds must be studied, and an ADME (absorption, distribution, metabolism, and excretion) profile studied, since each characteristic influences the drug levels and kinetics of drug exposure to tissues and also affects the effectiveness and action of the compound as potential medication. In the future, animal testing may be conducted. If inhibition is still observed *in vivo*, the compounds may be
modified for an improved compound and the IC₅₀ values of the compounds may fall within a desired nanomolar range.
Works Cited


