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Targeting Cancer Stem Cells in Recurrent Lung Cancer

By

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Submitted in partial fulfillment of the requirements for Graduation summa cum laude
and
for Graduation with Honors from the Department of Biology

University of Louisville

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ABSTRACT:

Lung cancer is the leading cause of cancer mortality in the United States. The development of drug resistance with cisplatin or platinum/taxane combination chemotherapies has led patients to suffer from recurrent lung cancer. These chemotherapy treatments target cancer cells but leave behind cancer stem cells (CSCs). CSCs are stem-like tumor cells that have the potential to differentiate and proliferate. This allows the cancer to relapse even after initial elimination of the tumor. Investigations were performed on a secondary metabolite from the Myrothecium fungus family called Verrucarin J (VJ) to see its effects in lung cancer cell lines. VJ significantly inhibits cell proliferation in lung cancer cell lines A549 and H1793. The IC$_{50}$ of VJ was approximately 10 nM in A549 after 48 h of treatment and 20 nM in H1793 after 72 h of treatment. Treatment of the A549 cell line with VJ induced apoptosis and reduced expression levels of key markers and genes found in cancer stem cells, such as ALDH1, Notch1, HEY1, CD 133, Oct 4, β-catenin, WNT1, TCF-4, and LGR5, in a dose-dependent manner. The mechanism that VJ targets the lung cancer cells and CSCs is currently being investigated by considering the WNT canonical signaling pathway. Treatment of the GSK-3 inhibitor (CHIR99021) in A549 cell lines increased expression levels of specific canonical WNT signaling pathway genes including β-catenin, Cyclin D1, LGR5, c-Myc and TCF-4. Preliminary results of the combination treatment of VJ and GSK-3 inhibitor in A549 cell line indicate that VJ potentially targets the WNT canonical signaling pathway. However, further experiments are needed to confirm where exactly VJ is targeting that pathway. From these experiments, Verrucarin J has the potential application to be a novel chemotherapeutic drug.
INTRODUCTION:

Lung cancer is the leading cause of cancer mortality in the United States for both males and females [1]. It is estimated that there will be 222,500 new cases of lung cancer in 2017 [1]. Two major types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common type making up 85% of lung cancer cases [2]. SCLC is the more aggressive form that constitutes 15% of the lung cancer cases. There have been substantial improvements in the treatment of lung cancer throughout the years. Surgery and radiation have shown to be effective in dealing with localized/regionalized earlier stages of lung cancer. Administration of chemotherapeutic drugs like cisplatin along with pacitaxel/docetaxel are deemed effective initially. However, more progress is needed to be made for patients with advanced stages of lung cancer.

The use of chemotherapy is effective in some patients, but there are a select view who don’t receive promising results. The 5-year survivability rates of lung cancer remain low at an average of 18% [1]. A big contributor to recurrent lung cancer and low survivability rates is drug resistance to chemotherapeutic drugs such as cisplatin [5-7]. Cisplatin is a widely used anti-cancer drug that forms platinum complexes, which causes the formation of intrastrand and interstrand DNA cross links [2,6]. This prevents lung cancer cells from proliferating, leading to DNA damage and eventually cell death [2]. A potential mechanism of drug resistance toward cisplatin includes increased DNA repair capacity with the excision repair cross-complementation group 1 (ercc1) and breast cancer susceptibility gene 1 (BRCA1) genes [2]. These genes play crucial roles in chemoresistance.
Studies have shown the potential root to this drug resistance comes from cancer stem cell (CSCs). CSCs are a small subpopulation of stem-like tumor cell that can differentiate and proliferate [3]. They are known to be resistant to chemotherapeutic drugs and radiation, which allows for the reoccurrence of the cancer and tumor progression. It is believed that CSCs are surviving the current treatments due to their activation of anti-apoptotic pathways, increased telomere length, increased membrane transporter activity and the CSCs’ abilities to metastasize [4]. Current treatments are killing the cancer cells but leaving behind the cancer stem cells [Fig. 1]. Therefore, there is a dire need to develop an innovative way to target not only lung cancer cells, but also cancer stem cells [Fig.1].

![Figure 1. The Targeting of Cancer Stem Cells.](image)

Cancer stem cells are “stem-like” tumor cells that have the potential to differentiate, self-renew, and proliferate. The most accepted theory about cancer stem cells is that they originate from normal stem cells, since they express the key stem cell markers.
This project is focused on identifying an anti-cancer compound that can effectively target cancer cells, as well as cancer stem cells in lung cancer. This led to examining the anti-cancer activity of a purified fungus compound called Verrucarin J (VJ). VJ is a cytotoxic secondary metabolite that comes from the *Myrothecium* fungus [4]. The molecular formula of this compound is $\text{C}_{27}\text{H}_{32}\text{O}_8$. Its anti-cancer activity is unknown. However, after previous experiments to see its effectiveness in targeting ovarian cancer cells and cancer stem cells, VJ has the potential to be developed into a novel chemotherapeutic drug [4]. The specific aims to be addressed in this project include determining if VJ can target lung cancer cells/ CSCs and to define the potential self-renewal mechanisms which Verrucarin J targets lung cancer cells and CSCs. Verrucarin J inhibits cell proliferation in lung cancer cells and CSCs by inducing apoptosis, targeting the canonical WNT signaling pathway and reducing the expression levels of key stem cell markers and genes.

**MATERIALS AND METHODS**

**Cell Proliferation Assay using MTT Reagent**

Cell proliferation assay with MTT reagent was conducted to measure the cell proliferation rate and the reduction in cell viability in treated lung cancer cells. Cell lines A549 and H1793 were used. These cell lines are non-small cell lung cancer cells, specifically adenocarcinoma cells. The cells lines were received from Dr. Mariusz Ratajczak’s lab. Preparation of the compound Verrucarin J (N18282 AnalytiCon) involved dissolving the compound in DMSO (dimethyl sulfoxide) and making a 10 mM stock solution. The A549 and H1793 cells were rinsed with phosphate-buffered saline
(Sigma), trypsinized, and plated into 96-well plates (in a final volume of 100 μl per well). Cells were grown in Roswell Park Memorial Institute (RPMI) medium (Sigma) modified with 10% FBS (Fetal Bovine Serum). FBS was added to assist in growth and proliferation of the cells. After 24 h in an incubator at 37°C, the medium was replaced with the RPMI medium containing 5% FBS.

Cells were treated in triplicates with VJ at concentrations 0, 1, 2, 5, 10, 20 and 50 nM. There were three different treatment periods: 24, 48 and 72 h. After each set period, the media of that section of cells were replaced with 100 μl of 5% RPMI media and 20 μl of MTT reagent (CellTiter96 System, Promega) in each well. After the administration of MTT reagent, cells were incubated at 37°C for approximately 30 min to 1 h. Cells that are functioning with active mitochondria will react to the yellow MTT reagent to produce formazan, a purple product. Cells that are dead or do not have functioning mitochondria will not react to the reagent. Cell proliferation was assessed through color development using ELISA reader at 490 nm to take the absorbance readings [4].

**Apoptosis assay using flow cytometry for Annexin V**

Apoptosis assay was conducted to see if VJ induce apoptosis in lung cancer cells and to determine at what percentage. A549 cell line was plated into T-150 flasks using 10% RPMI media (Sigma). After 24 h of incubation at 37°C, cells were treated with VJ at concentrations 0, 5, 10, 20, 50 nM for 24 h and were harvested. Cells were collected by centrifuging at 1,500 rpm for 5 min and resuspended in Annexin V binding buffer (FITC Annexin V Apoptosis Detection Kit, BD Pharmingen) according to
supplier’s instructions. FITC (fluorescein isothiocyanate) is a fluorescence marker conjugated to the Annexin V. To ensure that cells were not clumped and were single cells, they were passed through nylon mesh and diluted to a final concentration of 10×10^6 cells/ml. The 100 μL of cell suspension (1×10^6 cells) was used for each assay. For each concentration of VJ, an independent control was used. To each sample except controls, 2 μL of Annexin V from the kit was added, and was incubated for 15 min at room temperature in the dark. After completion of incubation, 400 μL of binding buffer was added and immediately analyzed by Fluorescence- Activated Cell Sorting (FACS) analysis using the FACSCalibur (BD BioSciences). The stained and unstained cells were analyzed using FlowJo software [4].

**Expression of genes using real-time PCR**

Real-time PCR (Polymerase Chain Reaction) was conducted to see the effect of VJ on the gene expression of key CSC markers in lung cancer cells. To test this, A549 cells were plated in three T-150 flasks, containing 10% RPMI media. After 24 h of plating, the media was replaced with 5% RPMI media and the cells were treated with VJ at concentrations 0, 10, and 20 nM. After 48 h of treatment, cells were harvested and rinsed twice with PBS. The total RNA from each sample was purified following the instructions from the RNeasy Mini Kit (Qiagen) and quantitated using NanoDrop. Then first strand cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) for each of the samples. The first cDNA prepared from each sample was subjected to gene amplification in real-time PCR.
Preparation of the real-time PCR involved making a master mix for each selected gene. This master mix included RNA-free water (Sigma), the forward and reverse primers of the selected genes and FastStart Universal SYBR Green Master with ROX (Sigma). Volumes for each component on the master mix were determined by the supplier’s instructions in the SYBR Green Master. The specific primers used for each gene were the reverse and forward of Wnt1, β-Catenin, TCF-4, Notch1, Hey1, LGR5, ALDH1, Oct 4. and CD 133. The sequences of the primers are listed in Table 1. Then 18 µL of the master mix were added to each well in the PCR plate (ThermoFisher). 2 µL of the 1st strand cDNA from the 3 samples were added to the respective the wells. Each sample was tested in triplicates. The plate was then sealed and put in the centrifuge for 2 minutes at a speed of 1000-1500 rpm. Then the plate was inserted in a real-time PCR detection system (Applied Biosystems StepOnePlus™ Real-Time PCR System). The experiment ran for 45 cycles. The Cycle Threshold (CT) values of each of the genes were normalized with the CT values for GAPDH used as an internal control [4].

A549 cells were also treated with a GSK-3 inhibitor (CHIR99021 Sigma). This inhibitor blocks the function of glycogen synthase kinase 3 and increases self-renewal of stem cells [10]. The A549 cells were plated in five T-150 flasks, containing RPMI media with 10% FBS. After 24 h of plating, the media was replaced with RPMI media with 5%FBS. A 5 mM stock solution was prepared for the GSK-3 inhibitor. The cells were then treated with the inhibitor at concentrations 0, 1, 3 and 5 µM. After 24 h of treatment, the cells from each of the 5 samples were harvested. RNA extraction for each of the treated samples occurred using the RNeasy Mini Kit. 1st strand cDNA was made for each of the samples. Real-Time PCR was conducted as described previously
and the specific primers used for each gene were the reverse and forward of β-Catenin, TCF-4, c-Myc, Cyclin D1 and LGR5. The sequences of the primers are listed in Table 1.

Then the treatment of A549 cells also included the use of both VJ and GSK-3 inhibitor. The A549 cells were plated in six T-150 flasks, containing RPMI media with 10% FBS. After 24 h of plating, the media were replaced with RPMI media with 5% FBS. One of the flasks was labeled as the control, only containing A549 cells treated with DMSO. Then 4 flasks were treated with the GSK-3 Inhibitor at concentrations 1 and 3 µM. Then 3 hours later, 2 of those 4 (1 and 3 µM) flasks were treated with VJ at a concentration 10 nM. Then the 6th flask only received the treatment of VJ at 10 nM. The 6 samples were labeled as such: control (0 nM), 10 nM (VJ), 1 µM (GSK-3), 3 µM (GSK-3), 10 nM (VJ)+ 1 µM (GSK-3), and 10 nM (VJ)+ 3 µM (GSK-3). After 24 h of treatment, RNA extraction occurred, 1st strand cDNA was made and real-time PCR was conducted as described previously. The specific primers used for each gene were the reverse and forward of TCF-4, Oct 4, Cyclin D1 and ALDH1. The sequences of these primers are listed in Table 1.

Selection of Primers for real-time PCR

Primer selection was determined after review of literature on key stem cell markers found in lung cancer stem cells and important genes in the WNT Canonical signaling pathway. Particularly, ALDH1 (aldehyde dehydrogenase 1) and CD 133 are popular cancer stem cell marker. ALDH1 is known for its regulation of cell differentiation and gene expression. Further research has shown ALDH1 positive cells express stem-like characteristics, making it a key marker for CSCs in lung cancer [4]. Oct 4 is key
stem cell marker that highly expressed in stem cells and plays a significant role in the maintenance and proliferation of stem cells [14]. The primers were purchased from Sigma and their sequences are listed in Table 1.

Table 1. Primers sequences for various genes in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (Forward)</th>
<th>Anti-Sense (Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNT1</td>
<td>CTCTCTTCTTCCCCCTTTGTC</td>
<td>AACTCGTGCGCTCTGTATCC</td>
</tr>
<tr>
<td>β-catenin</td>
<td>TGGATGGCCTGCCTCCAGGTGAC</td>
<td>ACCAGCCCCTCGAGCCC</td>
</tr>
<tr>
<td>TCF-4</td>
<td>TATGCTCCATCGCAAGCACTG</td>
<td>TGGATGCAGGCTACAGTAGCTG</td>
</tr>
<tr>
<td>Notch1</td>
<td>TCAGCGGGATCCACTGTGAG</td>
<td>ACACAGGCAGGTGAACGAGTTG</td>
</tr>
<tr>
<td>HEY1</td>
<td>TGGATCACCTGAAAAATGCTG</td>
<td>TTGTTGAGATGCGAAACCAG</td>
</tr>
<tr>
<td>LGR5</td>
<td>GCAAACCTACGTCTGGACAA</td>
<td>TGGATCTGGAGCTGGTAAAG</td>
</tr>
<tr>
<td>ALDH1</td>
<td>GCACGCCAGACTTACCTGTC</td>
<td>CCACTCACTGAATCATGCCA</td>
</tr>
<tr>
<td>Oct-4</td>
<td>CGCTGGCTTATAGAAGGT</td>
<td>ACAGGTGTCATAAGAATGGATA</td>
</tr>
<tr>
<td>CD133</td>
<td>AGTGGCATCGTGAACACTG</td>
<td>CTCCGAATCCATTCCGACGATAGTA</td>
</tr>
<tr>
<td>c-Myc</td>
<td>GGACGACGAGACCTTCATCAA</td>
<td>CCAGCTTCTCTGAGAGAGCTT</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>GCTCTGGCTTCTGTGGCGAGCGC</td>
<td>TCGGTGATAGTGACACACAGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGATGACATCAAGAAGGTG</td>
<td>TCCTGGAGGCGCATGTGGGCC</td>
</tr>
</tbody>
</table>
Statistical analysis

Student’s t-test was performed to calculate the statistical differences between the control and the treated groups. P ≤ 0.05 was considered statistically significant while p ≤ 0.001 was considered highly significant. The error bars represent the standard deviation of two to three independent experiments [4].

RESULTS

Verrucarin J inhibits cell proliferation in A549 and H1793 Cell lines

The efficiency of VJ to target lung cancer cells was tested by performing cell proliferation assays using a MTT reagent. This experiment involved two different lung cancer cell lines, A549 and H1793. Cells were plated into 96-well plates and after 24 h and were treated with VJ at concentrations 0,1, 2, 5, 10, 20, and 50 nM. Cell proliferation was assayed using MTT assays as described previously in the materials and methods section. Treatment of cells with VJ showed a significant dose and time dependent inhibition of cell proliferation [Fig. 2]. IC₅₀ values for VJ after treatment of cells for 48 h were found to be approximately 10 nM for A549 cell line and 20 nM for H1793 cell lin. These results indicate that VJ is a highly potent anti-cancer compound.

Verrucarin J induces apoptosis in A549 cell line

Apoptosis or programmed cell death is a process that regulates cellular development. Cancer cells often have missregulated apoptotic pathways that allows for uncontrolled cell proliferation to take place [12]. It is important for anti-cancer drugs to induce apoptosis in cancer cells to stop the progression of the tumors. To understand
how VJ inhibited the cell proliferation of lung cancer cells, apoptosis assays were performed to observe if VJ induces apoptosis. A549 cells were treated with various concentrations of VJ for 24 h. After treatment, cells were washed with PBS, stained with Annexin V antibody and analyzed by FACSCalibur as described previously in the materials and methods section. The results from the two experiments showed an average of 7.24%, 9.01%, 19.95%, 20.95%, and 22.05% of FITC labeled apoptotic cells relative to non-apoptotic cells when treated with VJ at concentrations 0, 5, 10, 20, and 50 nM, respectively. These results suggest that VJ induces apoptosis in the A549 cell line a dose-dependent manner [4].
Figure 2. The Effect of Verrucarin J on Cell Viability in A549 and H1793 Cell Lines

Lung cancer cells were treated with various concentrations of Verrucarin J. The graphs show the percentage of cell viability from 3 experiments per time period: 24, 48, and 72 h. The mean ± S.D (standard deviation) were plotted using Graph Pad. Asterisks (*, **) indicate significant (p ≤ 0.05) and very significant (p ≤ 0.001). From the 48 h time period, the IC\textsubscript{50} for A549 cell lines was determined to be 10 nM and found to be significant when compared to control. For the 72 h time period, the IC\textsubscript{50} for H1793 cell lines was determined to be approximately 20 nM.
Figure 3. The Effect on Verrucarin J in inducing apoptosis
To test induction of apoptosis, we analyzed the A549 cell line by flow cytometry using the VJ concentrations of 0, 5, 10, 20 and 50 nM concentrations of Verrucarin J. Cells were analyzed after 24 h of treatment. A) FAC Analysis: The graphs shown are from two independent experiments. The percentages of FITC in the treated samples from experiment one are 7.58%, 10.1%, 21.4%, 22.4% and 23.3% respectively. The percentages of FITC in the treated samples from experiment two are 6.91%, 7.92%, 18.5%, 19.5%, and 20.8% respectively. The blue section represents the apoptotic cells. B) Quantitative analysis: the graph shows the mean ± S.D. (standard deviation) of two experiments, plotted using GraphPad. The asterisk (*) indicates significance (p ≤ 0.05).
Verrucarin J inhibits the expression of CSCs markers and genes

Notch and Wnt pathways are two prominent self-renewal pathways. Specific genes were selected from these pathways due to their pivotal role in cellular development and survival. Dysregulation of the Notch pathway promotes tumorigenesis and increases upregulation of genes such as Notch1 and HEY1 [13]. The Wnt pathway is also known for promoting oncogenesis especially when there is upregulation of β-catenin and TCF-4 [14]. Results showed a significant, dose-dependent suppression of WNT1, β-catenin, TCF-4, Notch1, HEY1, LGR5, ALDH1, Oct4 and CD133 genes in A549 cells when treated with VJ [Fig. 4]. This decrease in expression by VJ at the RNA level suggests VJ has the ability to target cancer stem cells.

GSK-3 Inhibitor increases expression of key genes in WNT Canonical Signaling Pathway

GSK-3 (glycogen synthase kinase 3) regulates transcription and cell proliferation in the WNT canonical pathway [11]. A GSK-3 inhibitor was used to inhibit the functionality of GSK-3 in the lung cancer cells to increase the production of WNT target genes. Various concentrations of the GSK-3 inhibitor were used to treat the A549 cell line (0, 1, 3, and 5 µM). Results as shown in Figure 5 indicated an increased, dose-dependent expression of β-catenin, TCF-4, c-Myc, Cyclin D1 and LGR5 in A549 cells when treated with GSK-3 inhibitor. Cyclin D1, c-Myc, and β-catenin showed significant differences in concentrations at 3 and 5 µM. LGR5 showed a significant difference from the control in the 3 µM. These results suggest that the GSK-3 inhibitor is increasing the expression of key genes in the WNT canonical signaling pathway.
Verrucarin J inhibits the WNT Canonical Signaling Pathway

To determine the mechanism at which Verrucarin J targets lung cancer cells and cancer stem cells, a combination therapy was conducted to see how VJ works with the GSK-3 inhibitor in A549 cell line. The combination therapy entailed 6 different sample groups; control (0 nM), 10 nM of VJ, 1 µM of GSK-3 inhibitor, 3 µM of GSK-3 inhibitor, 10 nM of VJ plus 1 µM of GSK-3 inhibitor, and 10 nM of VJ plus 3 µM of GSK-3 inhibitor. The specific primers selected for this first experiment include ALDH1, TCF-4, OCT 4, and Cyclin D1. Referring to Figure 6, the preliminary results show an increased expression of ALDH1, TCF-4, OCT 4, and Cyclin D1 in 1 µM and 3 µM GSK-3 inhibitor treatment when compared to the control and the samples treated with 10 nM of VJ. After the administration of the two compounds, GSK-3 and VJ, the expression levels of these markers decreased compared to the inhibitor only samples [Fig. 6]. This suggests that VJ plays a role in targeting the WNT canonical signaling pathway as its effects are neutralized the GSK-3 inhibitor. However, more experiments are needed to further support these preliminary results.
Figure 4. RT-PCR Quantification of various genes

The graphs show the fold change of WNT1, β-catenin, TCF-4, Notch1, HEY1, LGR5, ALDH1, Oct-4 and CD133, respectively. Each graph represents two experiments using two different sets of first strand cDNA from the RNA samples of the treated A549 cells. The genes were normalized to GAPDH. The amplification level of each gene was quantified. The mean ± S.D were plotted using GraphPad. The asterisk (*) indicates significance (p ≤ 0.05). In WNT1, LGR5, Notch1, HEY1, CD133, ALDH1, and Oct-4 expressions on treatment with 10 nM and 20 nM were shown to be significant. For β-catenin and TCF-4, treatment with 20 nM was shown to be significant.
Figure 5. Treatment with GSK3 Inhibitor: RT-PCR Quantification
The graphs show the fold change of c-Myc, TCF-4, LGR5, β-catenin and Cyclin D1. Graph represents three experiments using two different sets of first strand cDNA from the RNA samples of the treated A549 cells. The genes were normalized to GAPDH. The amplification level of each gene was quantified using RT-PCR. The mean ± S.D were plotted using GraphPad. The asterisk (*) indicates significance (p ≤0.05). In the c-Myc, β-catenin, and Cyclin D1 expressions, treatment with 3 μM and 5 μM were shown to be significant. For LGR5, 3 μM was shown to be significant.
Figure 6. Preliminary Results: VJ inhibits target genes in WNT Signaling Pathway
The graphs represent one experiment using 1 set of first strand cDNA from the RNA samples of the treated A549 cells with the GSK-3 inhibitor and VJ. The genes were normalized to GAPDH. The amplification level of each gene was quantified. The genes represented include ALDH1, TCF-4, Oct-4, and Cyclin D1 respectively.
DISCUSSION

The current process of treating lung cancer has made significant strides over the years. However, the progression of this fight is still being inhibited by drug resistance and tumor reoccurrence. Patients are dying from this disease everyday as current treatments have failed them in eliminating the cancer. At the root of this is cancer stem cells. Tumor regression cannot occur due to current treatments not targeting the CSCs. Therefore, there needs to be continuous efforts made to develop innovative treatments that will effectively target lung cancer cells and cancer stem cells.

Previously, Dr. Kakar’s lab conducted a search to find a compound that could potentially be a solution to preventing recurrent lung cancer. The focus was to find a compound that not only has anti-cancer activity but the ability to effectively target cancer stem cells as well. This search led to the choice of investigating the effects of Verrucarin J, a secondary fungus metabolite. The compound is known to have cytotoxic abilities, but its anti-cancer activity remains to be determined [4]. To test its effectiveness in targeting lung cancer cells and CSCs, experiments showed how VJ reduced cell proliferation in lung cancer cell lines A549 and H1793 in a dose and time-dependent manner [Fig. 2]. Further tests were done to determine how VJ inhibited cell proliferation and reduced cell viability in these lung cancer cells. Through the induction of apoptosis, the increasing concentration of VJ increased the number of apoptotic cells. This provides more support in VJ’s anti-cancer capabilities [Fig.3].

As shown in Figure 4, the treatment of A549 cells with VJ results in a dose dependent suppression of various CSC markers expression levels in RNA. These markers include WNT1, β-catenin, TCF-4, Notch1, HEY1, LGR5, ALDH1, Oct-4.
CD 133. These specific genes were selected due to their involvement in identifying key cancer stem cells and their important roles in self-renewal signaling pathways. From these results, VJ has the potential to effectively target the CSCs.

After these promising results, an effort was made to determine which mechanism VJ targets the lung cancer cells and cancer stem cells. There is a possibility that there are multiple pathways that VJ could target, yet the concentration in this research was on the WNT canonical signaling pathway. A key factor of these cancer stem cells is their self-renewal capabilities. The WNT signaling pathway is associated with the progression and initiation of tumors and signals the cancer cells to proliferate [9,15]. Our previous experiments showed reduced expression of key genes such as WNT1, β-catenin and TCF-4 when treated with VJ. Therefore, making it appropriate to investigate this pathway further to see how and where exactly VJ targets this signaling pathway.
Referring to Figure 7, when the WNT ligand is absent, a complex forms and β-catenin is phosphorylated by CK1 (casein kinase 1) and GSK-3 (glycogen synthase kinase 3). GSK-3 is a key suppressor in this pathway [10,11]. Both GSK-3 and CK1 phosphorylate β-catenin and target it for degradation. This then prevents β-catenin from entering the nucleus, inhibiting self-renewal and proliferation of the cell [11]. When the WNT ligand is present, WNT binds to the receptors on Fz and LRP5/6 and prompts LRP/6 to be phosphorylated. With this complex formed, it allows for the stabilization of

Figure 7: WNT Canonical Signaling Pathway
This is a representation of the WNT/β-catenin signaling transduction. When WNT is absent, a complex forms and β-catenin is phosphorylated by CK1 and GSK-3. This then makes β-catenin targeted for degradation and not allowed to enter the nucleus. When WNT ligand is present, the receptor from Fz and LRP5/6 bind to WNT and lead to LRP/6 to be phosphorylated. With this complex formed, it allows for the stabilization of β-catenin. β-catenin travels into the nucleus where it activates gene transcription of WNT targeted genes.
β-catenin. β-catenin travels into the nucleus where it activates gene transcription of WNT targeted genes [8,14]. To initiate defining the mechanism, a GSK-3 inhibitor was used to block β-catenin from being targeted. This then allowed β-catenin to accumulate and enter the nucleus for transcription to be activated [Fig. 8]. Results from these experiments showed that increased concentration of the GSK-3 inhibitor, increased the expression levels of key WNT target genes, including c-Myc, TCF-4, β-catenin, Cyclin D1 and LGR5 [Fig. 5]. Steps were then taken to see how GSK-3 inhibitor and VJ would act together when used to treat the A549 cells.

![Figure 8: WNT Canonical Signaling Pathway with GSK-3 Inhibitor](image)

This is a representation of the WNT/β-catenin signaling transduction with an addition of a GSK-3 inhibitor. By blocking the function of GSK-3, the inhibitor allows for β-catenin to accumulate and travel into the nucleus where it activates gene transcription of WNT targeted genes.
The process of this combination therapy with these two compounds involved treating the A549 cells at different time points to prevent the compounds from canceling each other’s effects. Therefore, the inhibitor at concentrations of 1 µM and 3 µM were first administered to the cells following the VJ concentration of 10 nM (which was the previously determined IC\textsubscript{50} of VJ in the A549 cell line. In total, there are 6 groups: control, 10 nM of VJ, 1 µM of GSK-3 Inhibitor (CHIR99021), 3 µM GSK-3 Inhibitor (CHIR99021), 10 nM of VJ plus 1 µM of GSK-3 Inhibitor (CHIR99021), and 10 nM of VJ plus 3 µM GSK-3 Inhibitor (CHIR99021). After one experiment testing 4 genes (ALDH1, Oct-4, TCF-4, and Cyclin D1), the GSK-3 inhibitor is neutralizing the effects of VJ when used in combination together [Fig. 6]. However, furthering testing is needed to confirm these preliminary results. Tentatively, the results support a potential mechanism in how VJ targets these lung cancer cells and CSCs. This consistently adds further value in VJ being a potential chemotherapeutic drug.

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