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Erin Lawrence

Belmont University, erin.lawrence@pop.belmont.edu

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Selinexor Hyperactivates Akt and Induces Its Nuclear Accumulation

Erin Lawrence

A Senior Honors Thesis project submitted to the Honors Program
in partial fulfillment of the requirements for the degree

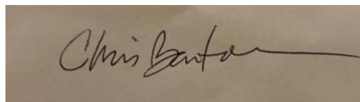
Bachelor of Science in Biology

Belmont University Honors Program

2020

__Marilyn E. Thompson Odom__ Date _4-17-20____

Dr. Marilyn Odom, Thesis Director

_________________ Date ____4-19-20______

Dr. Chris Barton, Committee Member

_________ ________ Date __4-17-20________

Dr. Jennifer Thomas, Committee Member

Accepted for the Honors Council and Honors Program:

_________________________ Date ___________

Dr. Bonnie Smith Whitehouse, Director

The Honors Program

I. Introduction/Literature Review

According to the National Cancer Institute, breast cancer is the most commonly occurring cancer in the United States. The estimated number of new cases of breast cancer in 2019 was 268,600 with approximately 41,760 deaths.[1] Although breast cancer accounts for ~15.2% of all cancers, only 6.9% of cancer deaths are due to breast cancer.[1] This low percentage of deaths relative to the number of cases is reflected in the five-year survival rate from 2009-2015 of 89.9%.[1] Although the survival percentage is high, it varies with factors such as race/ethnicity and type of breast cancer.

Breast Cancer

Most breast cancers occur within the ducts of the breast. These ducts, which are composed of epithelial cells, form the channels through which milk made within the mammary gland travels to the nipple. Breast cancer can also occur within the lobes of the gland. These two types of cancer are referred to as ductal or lobular carcinomas, respectively[2], and this represents one way in which breast cancer can be classified. Breast cancer can also be classified into one of five molecular subtypes.[3] The subtypes are based on the presence or absence of estrogen and/or progesterone receptors and the expression of Her2 (human epidermal growth factor receptor 2) (Table 1). Each receptor is key to normal cellular proliferation and therefore, can serve as therapeutic targets for the treatment of breast cancer.[4] Luminal A and B subtypes are similar, but are distinguished based on the levels of Ki67, which helps regulate the rate of cellular proliferation. Luminal A cancers express low levels, whereas luminal B cancers express high levels of

Ki67. The work in this thesis focuses on triple negative breast cancer (TNBC) cells,[3] which lack estrogen and progesterone receptors, and Her2.

Subtype	ER	PR	HER2	Ki67
Luminal A	+	+	-	+
Luminal B	+	+	-/+	++
TNBC	-	-	-	-
HER2 enriched	-	-	+	-
Normal-like	+	+	-	-

Table 1: Receptor Expression in Breast Cancer Subtypes Expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 in each of the five different molecular subtypes of breast cancer.[3] (TNBC, triple negative breast cancer)

Triple Negative Breast Cancer

Of the five types of breast cancer, triple negative breast cancer is the most aggressive. It accounts for 10% of all breast cancer diagnoses and has a 5-year survival rate of 76.5%.[5] In contrast, other subtypes have survival rates of 83.4-94%.[5] Treatment options for TNBC are fewer than those for other subtypes. With the expression of ER, receptor antagonists can be used, and drugs such as Herceptin® and lapatinib are available to treat HER-2-positive cancers.[6] These targeted therapies are not options for the treatment of TNBC.

Nuclear-Cytoplasmic Shuttling

The movement of certain proteins between the nucleus and cytoplasm of cells allows their function to be localization dependent.[7] Proteins less than 40 kDa can freely diffuse through nuclear pores. Those with a molecular weight greater than 40 kDa are shuttled in and out of the nucleus by specific carrier proteins, or karyopherins. Importins are a type of karyopherin involved in the movement of protein and RNA from the cytoplasm to the nucleus. These transporters recognize specific sequences within the proteins termed nuclear localization sequences (NLS) that allow interaction and translocation at the expense of Ran-GTP. Karyopherins involved in nuclear export of proteins are referred to as exportins. There are many exportin proteins, but exportin 1 (XPO1, also known as chromosome region maintenance 1 or CRM1) binds to a leucine rich nuclear export sequence (NES) within target proteins to move them out of the nucleus and into the cytoplasm. This, too, requires energy generated by GTP hydrolysis (Figure 1).[7]

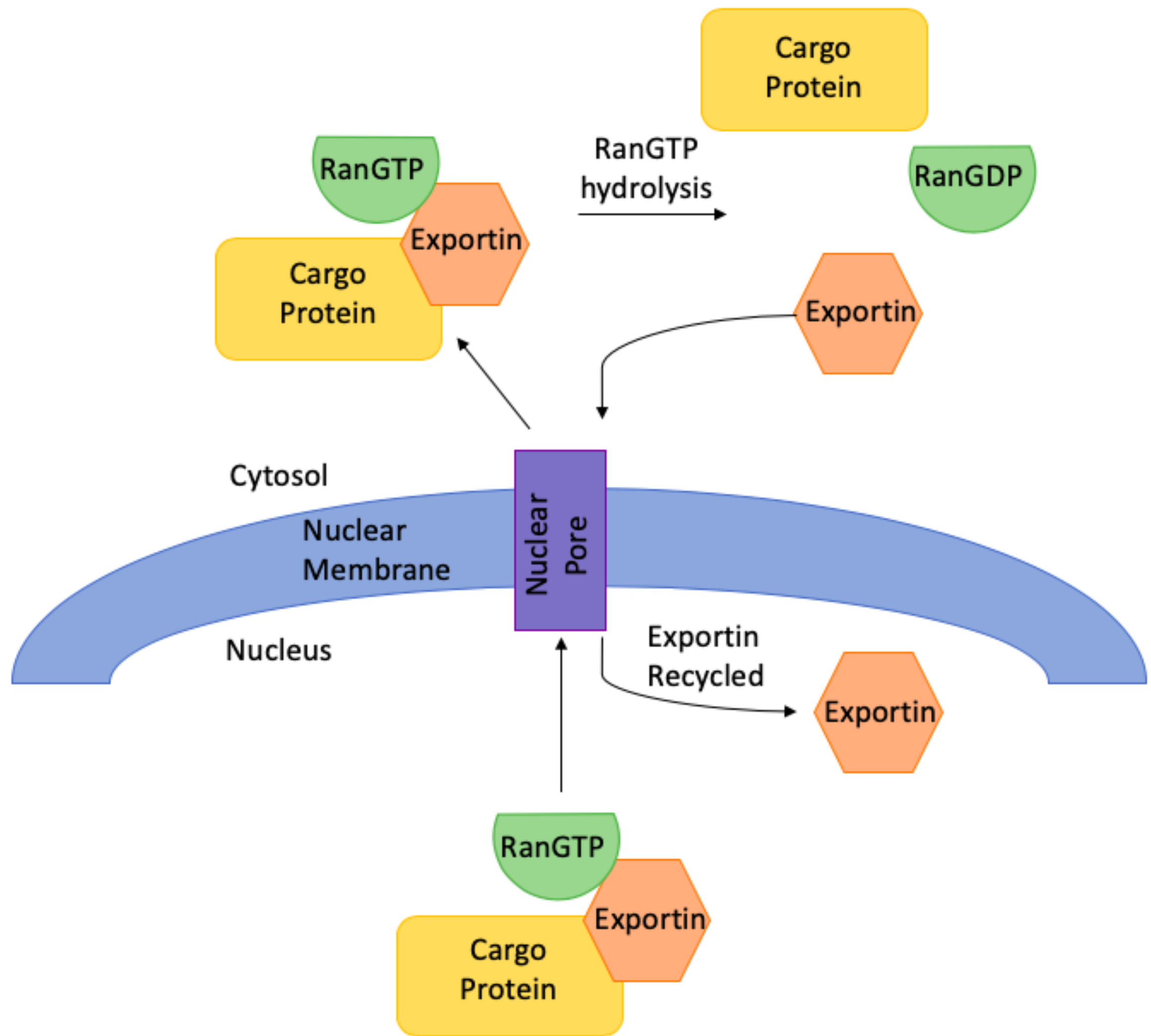


Figure 1: Nuclear-cytoplasmic shuttling Before nuclear export can occur, a complex of the exportin, cargo protein, and a Ran-GTP molecule must be formed. Once the complex is exported to the cytosol, Ran-GTP is hydrolyzed. This hydrolyzation provides energy for the exportin, cargo protein, and Ran-GTP complex to dissociate from one another. The exported protein remains in the cytosol and the exportin re-enters the nucleus.

The relevance of protein nuclear export to cancer is tied to the observations that a number of proteins, including some that regulate the cell cycle, undergo nuclear import and export as a means to regulate their function.[8] Inhibition of nuclear export can result in dysregulation of the cell cycle. For example, inhibition of nuclear export of cyclin D1, a protein vital to cell cycle progression, results in tumor growth in immune compromised mice.[9] p53 is a protein that is activated in the nucleus when there is DNA damage or a signal indicating the presence of oncogenic properties.[7] Activated p53 stimulates apoptosis and is exported once it has done its job. This export, and the export of many other tumor suppressors, is carried out by XPO1, and many cancer cells overexpress XPO1. In a non-cancerous cell, tumor suppressors are well-regulated by XPO1 to prevent them from being activated in the nucleus when it is not necessary. When XPO1 is overexpressed, it is constantly shuttling tumor suppressors out of the nucleus. This promotes tumor formation and growth and can prevent apoptosis from occurring.[7] The ability to inhibit XPO1 activity could return the level of regulation in cancerous cells to normal levels.

Selinexor, A Nuclear Export Inhibitor

Since XPO1 is upregulated in several types of cancer, it has become an attractive therapeutic target, as pharmacological inhibition of nuclear export could serve to treat patients. This concept has been proven effective in treating multiple myeloma, a cancer of the bone marrow. Selinexor, a nuclear export inhibitor, received FDA approval for the treatment of this cancer in 2019.[10]

Selinexor is a first in class SINE (selective inhibitor of nuclear export) drug that has been shown to induce apoptosis in triple negative breast cancer cells *in vitro.*[11] Specifically, selinexor inhibits XPO1 by blocking its binding site for protein transport. Studies indicate that selinexor treatment resulted in increased XPO1 mRNA expression, suggesting that the cells were trying to compensate for reduced nuclear export by synthesizing more XPO1.[1]

The successful outcomes from clinical trials resulted in an expedited approval process for selinexor. Currently there are 21 ongoing clinical trials and nine trials have been completed using the drug. For acute myeloid leukemia (AML), diffuse large B cell lymphoma (DLBCL), multiple myeloma (MM), and soft tissue sarcoma, selinexor has been given an “orphan drug status” in the United States. This means that a single company has the rights to try to develop a cure for a certain disease while receiving benefits such as tax reductions for certain clinical testing. In patients with breast cancer, selinexor has been shown to have anticancer effects when combined with chemotherapy.[10]

Selinexor is not the first nuclear export inhibitor designed. Leptomycin B was first used in clinical trials in 1996.[12] However, it was found to be too toxic to be used therapeutically. Karyopharm Therapeutics has developed a series of compounds that are structurally similar to leptomycin B. These compounds, designated with the prefix KPT, were shown to inhibit nuclear export, with KPT-330 being the most efficacious. KPT-330 is selinexor.[10]

PI3K/Akt

The PI3K/Akt pathway is frequently dysregulated in cancers and can contribute to disease progression.[13] Akt is a cell survival kinase. It has the ability to phosphorylate a number of proteins to result in inhibition of apoptosis and changes in cell cycle progression. Under normal conditions, Akt is activated when a growth factor binds to its receptor, which has tyrosine kinase activity associated with it. Through adapter proteins, phosphatidylinositol 3-kinase (PI3K) is activated and phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to produce phosphatidylinositol 3,4,5-triphosphate (PIP3) on the plasma membrane. PIP3 recruits proteins that contain a pleckstrin homology (PH) domain to the plasma membrane. This includes Akt and pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1). PDK1 and Akt associate at PIP3 on the membrane, and Akt is phosphorylated by PDK1 at threonine 308 (T308), which partially activates Akt. Akt is further activated by phosphorylation by mTORC2 (mammalian target of rapamycin complex 2) at serine 473 (S473). Activated Akt promotes cell growth, survival, and proliferation[13] (Figure 2).

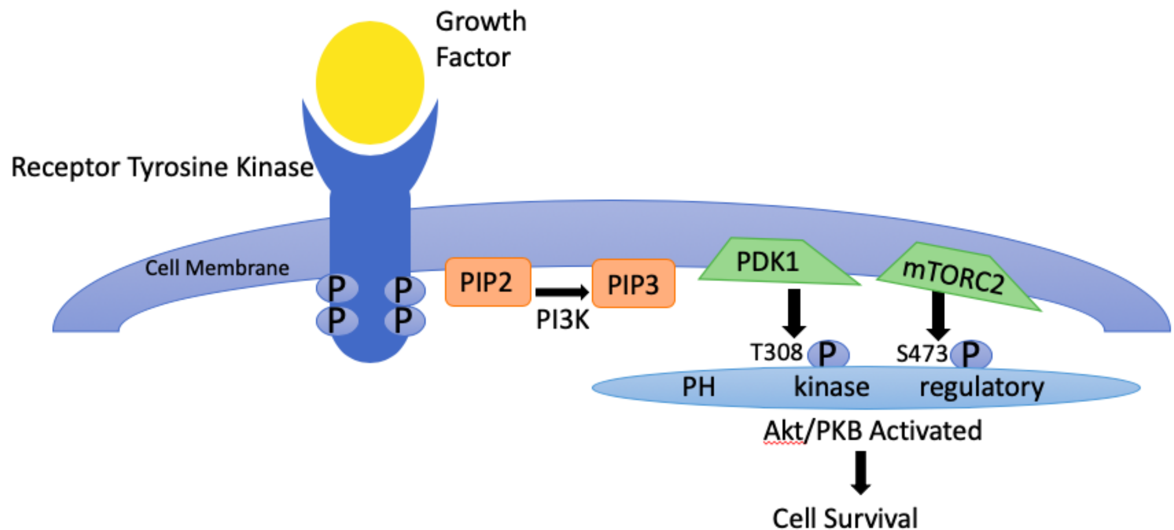


Figure 2: Akt activation pathway Activation of PIP3 occurs when growth factor binds to its tyrosine kinase receptor, initiating a cascade that leads to the activation of Akt.

Akt function is regulated by phosphatase and tensin homolog (PTEN), which regulates PIP3 by dephosphorylating it and returning it to PIP2. Loss of PTEN function results in an increase in PIP3 and a constitutively activated Akt pathway. In addition, studies have shown that when PTEN function is lost, breast cancer cells are more sensitive to PI3K inhibitors.[13]

Akt function is also dependent upon the subcellular localization of the protein. Akt translocates to the nucleus after being phosphorylated at the plasma membrane.[14] Although the mechanism of Akt nuclear import is not fully understood[15], Akt contains a leucine-rich, leptomycin B sensitive, nuclear export sequence suggestive of nuclear export occurring via XPO1.[14]

While Akt's normal function aids in cellular survival, under certain conditions, these functions can trigger apoptosis via reactive oxygen species (ROS) production. This Akt-mediated increase in ROS has been demonstrated in glioblastoma and ovarian cancer cell lines.[16] These studies showed that the increase in ROS triggered cell death via apoptosis.

Reactive Oxygen Species

Reactive oxygen species are oxygen radicals, such as hydroxyl radicals or hydrogen peroxide (OH, H_2O_2), produced by normal cellular reactions. They are important in many essential processes, such as immune defense mechanisms, but they can also be volatile and toxic.[17] While ROS are necessary, when their levels in the cell become too high, oxidative stress occurs. This means that DNA, RNA, proteins, and lipids, all essential molecules for cellular survival, can be damaged, which has implications in overall health.[19] ROS have both tumor suppressing and activating properties, which some chemotherapies utilize to create toxicity to kill cancer cells.[18]

The PI3K/Akt pathway is frequently hyperactivated in cancer and this can result in an increase in cellular ROS levels. This occurs via an increase in normal metabolic processes that are activated by Akt and normally result in acceptable levels of ROS production. However, many cancer cells already have higher level of ROS in comparison to normal cells. Therefore, when more ROS are produced, the levels can become toxic and trigger apoptosis.[16]

Statement of Research Problem

Shuttling of proteins between the nucleus and cytoplasm can be an important regulatory process in the development of cancer and therefore a key therapeutic target. The nuclear export inhibitor selinexor, marketed as XPOVIO®, has been demonstrated to be effective in the treatment of certain cancers. However, the full mechanism by which this occurs is not known and very little is understood about its effects on triple negative breast cancer. The working hypothesis of this thesis states that selinexor inhibits the nuclear export of Akt, resulting in increased reactive oxygen species (ROS) and cell death of TNBC cell lines.

II. Materials and Methods

BT-549 Cells

The primary focus of this research was to investigate the effects of selinexor in triple negative breast cancer cells. Several triple negative breast cancer cell lines are available for research purposes. For this project, BT-549 cells were used. BT-549 cells are triple negative cells derived from a 72-year-old Caucasian female diagnosed with ductal carcinoma.[20] The cells have a homozygous deletion for PTEN, an inhibitor of the PI3K/Akt pathway. Therefore, Akt should be constitutively active in these cells.

Cell Culture

BT-549 cells were purchased from American Type Culture Collection. They were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were grown in a humidified incubator at 37°C in 5% carbon dioxide.

Akt Activity Assays

Activity assays were performed using kits from Cell Signaling Technology or Abcam. Cells were plated at a concentration of 5×10^6 cells in six 100 mm dishes. Approximately 20 hours post plating, three plates were treated with 1 μM selinexor (Selleck Chemical) and three were treated with corresponding volumes of dimethyl sulfoxide (DMSO), the vehicle for selinexor, to serve as the negative control. The cells were harvested 21 hours later by first rinsing them in cold phosphate-buffered saline (PBS) followed by incubation in 0.5 mL cold Cell Lysis Buffer for 5 minutes. Cells were

scraped off plates and transferred to microcentrifuge tubes to be sonicated on ice. The cells were microcentrifuged for 10 minutes at 4ºC at 14,000 x g and the supernatant was removed. This cell lysate, containing proteins, was stored at -80ºC, to be later used for protein analysis.

The protein concentration for each sample was determined by BioRad Protein Assay Dye Reagent Concentrate. Akt was immunoprecipitated from 200 µg of protein by incubating overnight at 4ºC with immobilized phospho-Akt primary antibody conjugated to beads. The immunoprecipitated Akt was isolated and incubated with glycogen synthase kinase-3 (GSK-3) protein, for 30 minutes at 30ºC. GSK-3 is a known substrate of Akt and its phosphorylation by the immunoprecipitated Akt indicates activation of the kinase. The samples were then subjected to SDS-PAGE gel electrophoresis, and the proteins were transferred to a membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). After blocking the membrane in blocking buffer (tris-buffered saline [TBST] with 5% nonfat dried milk), it was incubated in a phospho-GSK 3 (Ser473) rabbit monoclonal antibody at a 1:1000 dilution in 10mL blocking buffer overnight at 4ºC. The following day, the membrane was rinsed three times for 10 minutes each before being incubated with a horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) at a 1:10,000 dilution in blocking buffer. The membrane was rinsed again three times for ten minutes each and developed using the SuperSignal West Pico PLUS Chemiluminescent Substrate Kit from Thermo Scientific.

Western Blots

Cells were plated at a concentration of 5 x 10^6 cells in six 100 mm dishes. Approximately 20 hours post plating, three plates were treated with 1 μM selinexor and three were treated with DMSO, as a control. The cells were harvested 21 hours later by first rinsing them in cold phosphate-buffered saline (PBS) followed by scraping in 1 mL cold PBS per plate. The cells were collected into 15 mL conical tubes. The cells were centrifuged at 500xg for 5 minutes at 4ºC, and the supernatant was decanted. Depending on the size of the pellet, 35 μL- 50 μL of RIPA with Halt Phosphatase Inhibitor (Thermo Scientific) and Protease Inhibitor Cocktail (Sigma) (final dilution of 1:100) was added and the pellet resuspended before being transferred to a 1.5 mL microcentrifuge tube. The tubes were vortexed for ~10 seconds before being placed on ice for 30 minutes. After the 30-minute period, DNA was sheared by using a 1mL syringe and a 24-gauge needle before being centrifuged at 18,000 x g at 4ºC for 15 minutes. A protein assay using BioRad Protein Assay Dye Reagent Concentrate was performed on the supernatant (cell lysate) to determine protein concentration.

Western blots were performed to detect proteins by subjecting cell lysates to SDS-PAGE gel electrophoresis, and the proteins were transferred to a membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). After blocking the membrane in blocking buffer (tris-buffered saline [TBST] with 5% nonfat dried milk), it was incubated in a primary antibody at a 1:1000 dilution in 10mL blocking buffer overnight at 4ºC. The following day, it was washed three times for ten minutes each time before being incubated with a secondary antibody at a 1:10,000 dilution in blocking buffer. The

membrane was washed again and developed using the SuperSignal West Pico PLUS Chemiluminescent Substrate Kit from Thermo Scientific.

In order to ensure changes in Akt activity were not due to differences in Akt protein levels, a total Akt Western blot was performed on both the vehicle (DMSO) and selinexor-treated cell lysates. To probe for Akt, a pan antibody for Akt was used to incubate the membrane. Western blots were also performed to analyze changes in the levels of phosphorylated Akt as a second way of demonstrating Akt activity. This was performed using a phospho (Thr308)-Akt rabbit polyclonal antibody to specifically analyze Akt phosphorylation at threonine 308.

To ensure equal loading of the samples, the membranes from the total Akt and phospho-Akt Westerns were stripped and reprobed for actin. The membranes were rewet in methanol using the capillary technique. To strip the primary antibody from the membrane, the membrane was washed six times in deionized water by shaking for 30 seconds each time before being incubated at room temperature in 0.1M HCl for 5 minutes with shaking. Next, the membrane was incubated in blocking buffer on the shaker at room temperature for 1 hour before being rinsed in 1x TBST. For actin reprobing of total Akt Westerns, 3% BSA blocking buffer (0.3g bovine serum albumin, 10 mL 1x TBST) was used, while for actin reprobing of phospho-Akt Westerns, 5% milk blocking buffer (TBST with 5% dried milk) was used. The membrane was then incubated on the shaker at room temperature for 30 minutes in a 1:1500 dilution of a monoclonal antibody against ß-actin. The membranes were washed three times for 10 minutes each time before being incubated for 45 minutes in the secondary antibody (peroxidase conjugated donkey anti-mouse IgG) at a 1:15,000 dilution in blocking buffer. After washing again, membranes

were developed using the SuperSignal West Pico PLUS Chemiluminescent Substrate Kit from Thermo Scientific. Equal signals between lanes demonstrated equal loading between samples.

Indirect Immunofluorescence

To determine if selinexor caused nuclear accumulation of Akt, indirect immunofluorescence was performed. BT-549 cells were plated at 750,000 cells per plate on 35 mm MatTek dishes. After approximately 48 hours, cells were treated with 1 µM selinexor or equal volume DMSO and harvested 21 hours later. Cells were washed in 1 mL filtered PBS and fixed in 2% paraformaldehyde. Cells were washed twice for five minutes each in 1 mL 0.1M cold tris-glycine buffer. Cells were again washed with filtered PBS for five minutes. Following incubation in blocking buffer (5% normal donkey serum, 1% BSA, 0.1% Triton-X 100 in PBS) for 30 minutes, the primary Akt (pan) antibody at a 1:400 dilution in blocking buffer was added. Plates were incubated overnight at 4ºC in a moistened environment. The next day, the plates were washed three times in filtered PBS for 5 minutes each. Cells were then incubated in a Cy™3 donkey anti-rabbit IgG diluted at 1:1500 in blocking buffer in the dark at room temperature for 45 minutes. The cells were washed in filtered PBS three times for five minutes each. Cells were then incubated in 4',6-diamidino-2-phenylindole (DAPI) at a 1:1500 dilution in filtered PBS for two minutes. The cells were washed again in filtered PBS and then rinsed in DI water before Aqua Poly Mount was added to the plate. Cells were viewed under a Nikon Eclipse TI-5 fluorescence microscope. Cy3 was excited at 550 nm and DAPI at 358 nm. Cells were imaged with NIS Elements BR4.6 imaging software.

ROS Assays

To determine whether ROS were increased in response to selinexor, ROS assays were performed. BT-549 cells were analyzed using the Cellular ROS Assay Kit (Red) from Abcam. This procedure consisted of several steps. First, BT-549 cells were plated at specified concentrations. The cells were then treated with 100 µL ROS Red Working Solution and incubated at 37ºC for 1 hour. Next, the cells were treated with 20 µL of selinexor at 1µM or 10 µM concentrations to induce ROS or corresponding volumes of DMSO. One set of wells was treated only with 10 µL 1x PBS. Fluorescence changes were monitored at 520 nm over varying time periods using the Bio-Tek Synergy 2 microplate reader and Gen 5 Microplate Data Collection and Analysis Software. If the amount of ROS increased, the fluorescence intensity measured also increased.

Statistical Analysis

Data were quantified using LiCor Image Studio Lite software and analyzed using GraphPad Prism Software. Data were evaluated using a two-tailed, paired student T-test. Significance was set at less than 0.05.

III. Results

Selinexor Activates Akt

Akt is considered a cell survival kinase and it is generally thought that cell death can result from decreases in its activity.[21] To determine whether selinexor influenced Akt activity levels in TNBC cells, Akt activity assays were performed to assess the levels of active Akt in BT-549 cells treated with DMSO vehicle or with selinexor. Relative Akt activity levels were determined by evaluating the phosphorylation of the Akt substrate GSK-3 by performing Western blot analysis (Figure 3).

Under vehicle conditions, the levels of Akt activity were low to undetectable. In contrast, the activity levels in cells treated with selinexor were higher than those in vehicle-treated cells. In order to obtain a visible signal for the vehicle-treated samples, the selinexor-treated samples' band intensities were overexposed. Therefore, over the course of the three independent experiments, the data were not quantifiable. However, these results suggest that selinexor activates Akt in BT-549 cells.

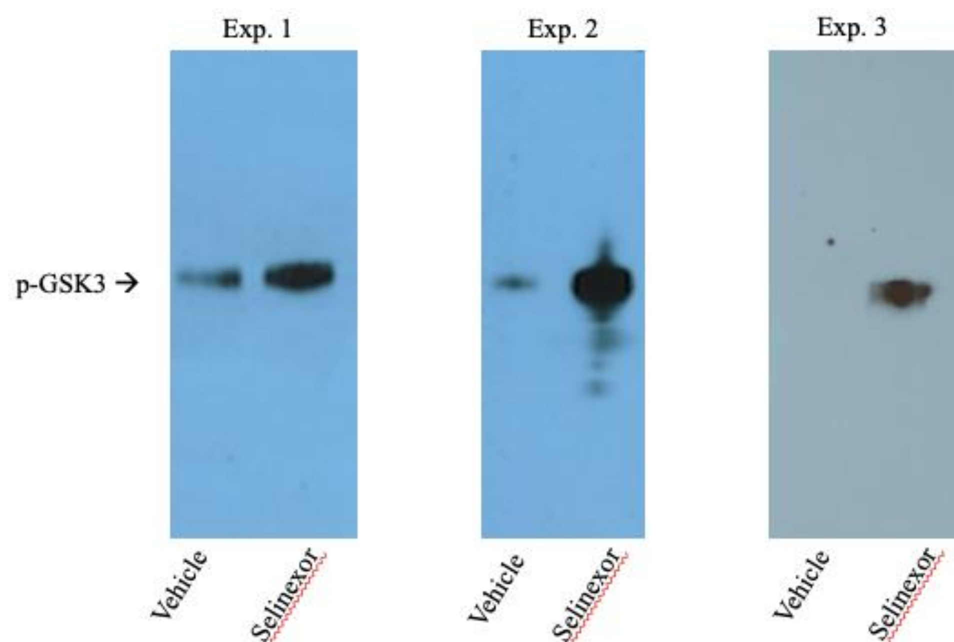


Figure 3: Akt Activity Western Blots Immunoprecipitated Akt was incubated with GSK3, a substrate of Akt. Phosphorylated GSK3 is a measure of Akt activity. The results show three independent experiments.

Selinexor Does Not Change Akt Protein Levels

To determine if Akt protein levels changed in response to selinexor, Western blotting was performed on vehicle-treated and selinexor-treated cells (Figure 4). Probing with a pan-Akt antibody (Figure 4A), the blot revealed no apparent difference in protein levels between the two samples. In order to ensure equal protein loading onto the gel, the membranes were stripped of primary antibody with 0.1 M HCl and reprobed for actin. There appeared to be no difference in the actin levels between the two samples (Figure 4A). Densitometric analyses of bands revealed a slight increase in the Akt/actin ratio in selinexor-treated cells; however, the increase was not statistically significant (p=0.353)

(Figure 4B). Overall, these data imply that the selinexor-induced increases in Akt activity were not due to elevated Akt protein levels but were reflections of increases in the activity of the enzyme already expressed.

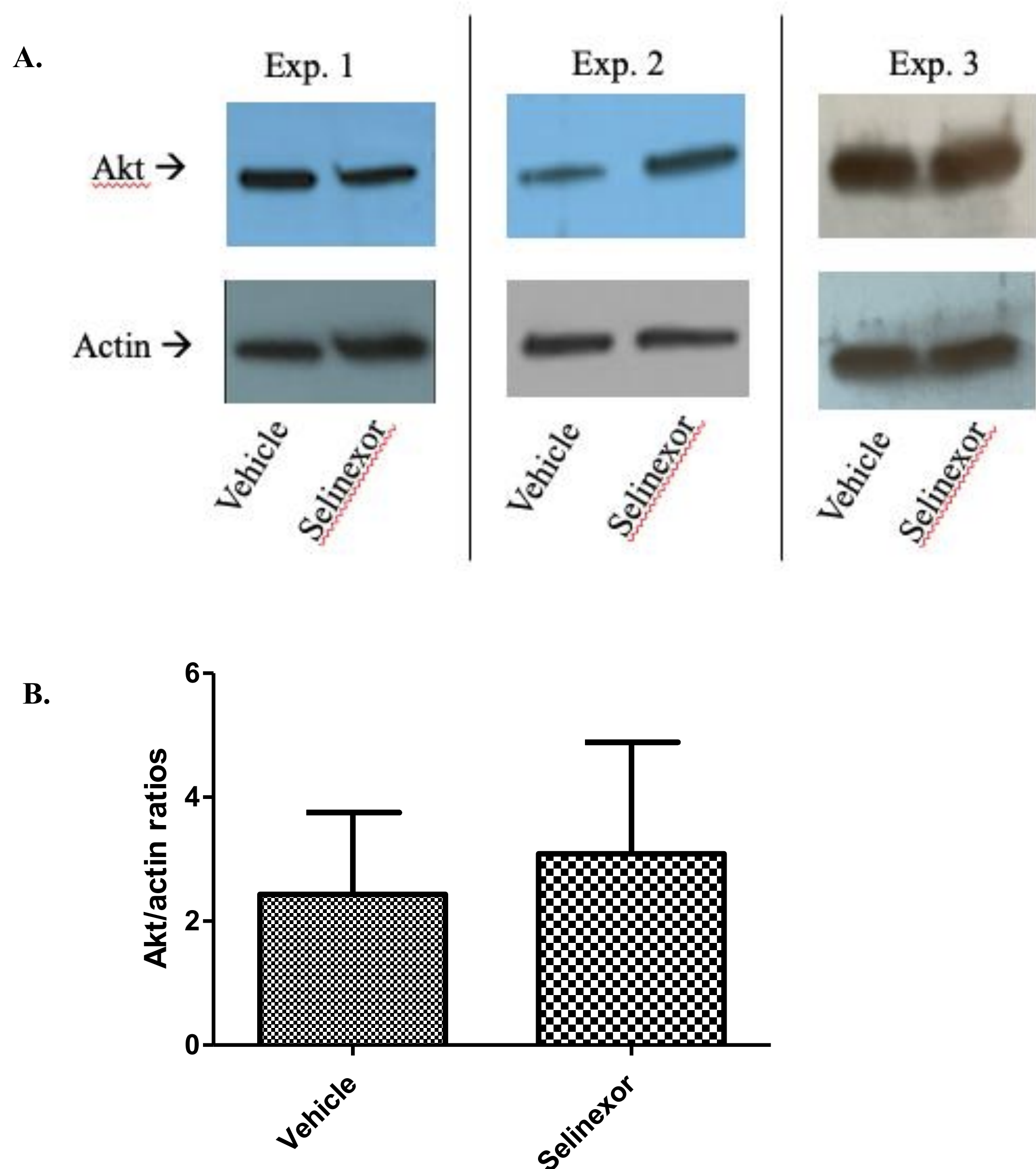


Figure 4: Total Akt Western Blots 10 µg (Experiments 1 and 2) or 25 µg (Experiment 3) of whole cell lysate was subjected to Western blot analysis. (A) Blots were probed with a pan Akt antibody. The membranes

were stripped and reprobed for actin. (B) Densitometric analysis of Akt expression. P = 0.3531. Data shown are representative of three independent experiments.

Selinexor Does Not Increase Akt T308 Phosphorylation

Initial activation of Akt occurs via phosphorylation at threonine 308, and for full activation, phosphorylation of serine 473 follows. To corroborate the results of the Akt activity assays, the levels of phospho-Akt were measured by Western blot using a phospho-threonine308-specific Akt antibody (Figure 5). Probing with this phospho-Akt antibody revealed that phosphorylation decreased or had no change upon treatment with selinexor (Figure 5A). Densitometric analyses of bands revealed a slight decrease in the phospho-Akt/actin ratio in selinexor-treated cells; however, the decrease was not statistically significant ($p=0.4671$) (Figure 5B). Overall, these data imply that the increase in Akt activity is not due to phosphorylation at threonine 308.

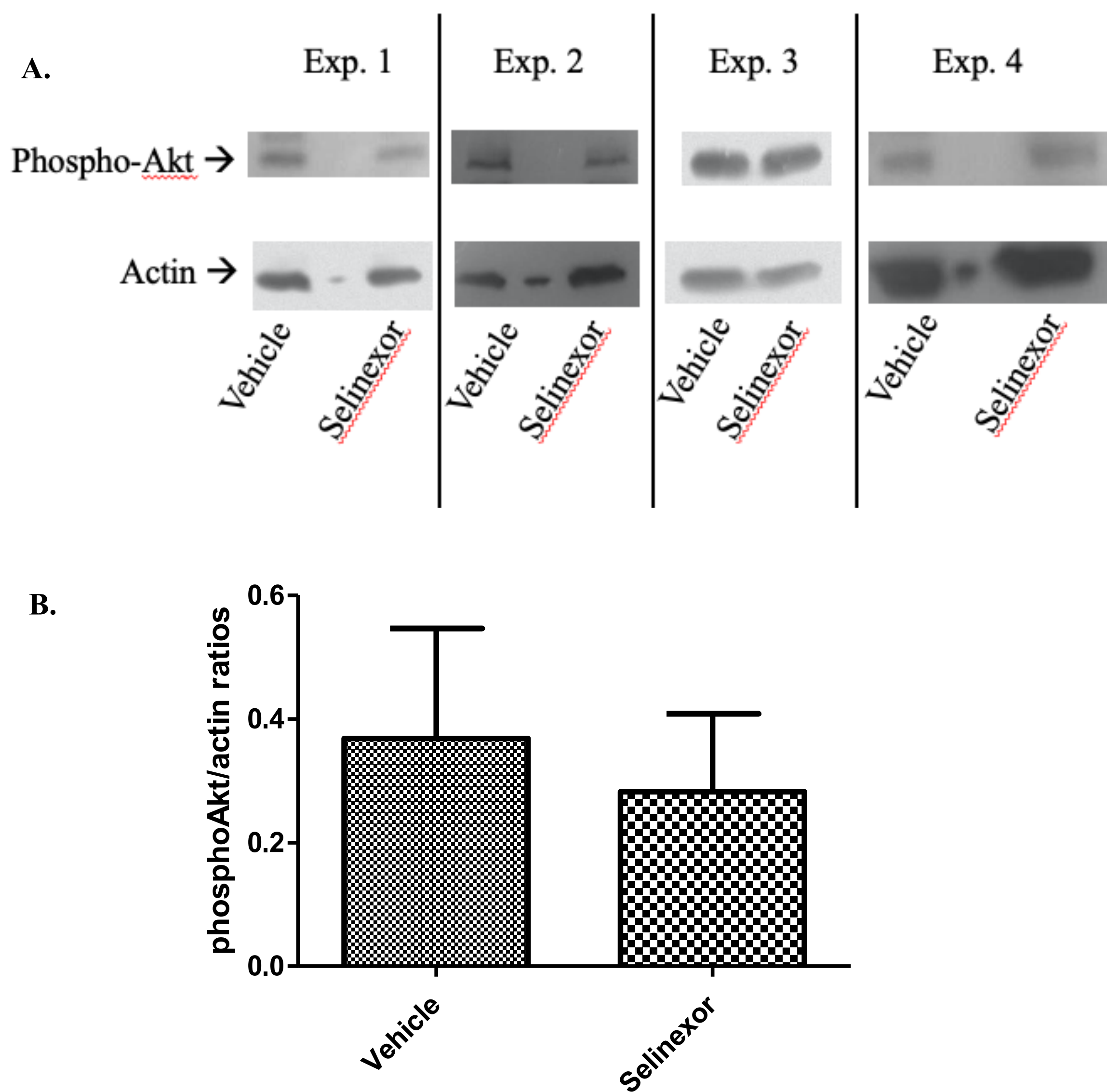


Figure 5: Phospho-Akt Western Blots (A) Blots probed with phospho-threonine 308-specific Akt antibody. The membrane was stripped and reprobed for actin. (B) Densitometric analysis of phosphorylated Akt at threonine 308. P = 0.4671. Data shown are representative of 4 independent experiments. Overflow of vehicle treated samples into lane 2 was observed upon sample loading and was included in the quantification for the vehicle sample.

Selinexor Localizes Akt to the Nucleus

Since selinexor inhibits nuclear export and Akt localizes to the nucleus in some cell lines[14], the effects of selinexor on the subcellular localization of Akt in BT-549 cells was evaluated. Indirect immunofluorescence was used to visualize the localization of Akt in cells under vehicle or treatment conditions (Figure 6). If nuclear export of Akt had been inhibited, the antibody would fluoresce red inside the nucleus more brightly in the selinexor-treated cells when compared to vehicle-treated cells. Under vehicle conditions, Akt was predominantly localized in the nucleus in a few cells and diffusely dispersed throughout the cell in the majority of them. However, when treated with selinexor, Akt predominantly localized to the nucleus. Based on overall observations, selinexor is able to trap Akt in the nucleus by inhibiting XPO1 activity (Figure 6).

Cells within each field were counted and categorized as having approximately equal staining intensity in the cytoplasm and nucleus or staining predominantly in nucleus or cytoplasm (Table 2). In the vehicle treated cells, the majority (45%) of the cells appeared to have Akt staining predominantly in the cytoplasm, with 35% of the cells having diffuse staining throughout the cell. Of the cells treated with selinexor, 80% appeared to have predominantly nuclear staining. These data suggest that selinexor causes nuclear accumulation of Akt.

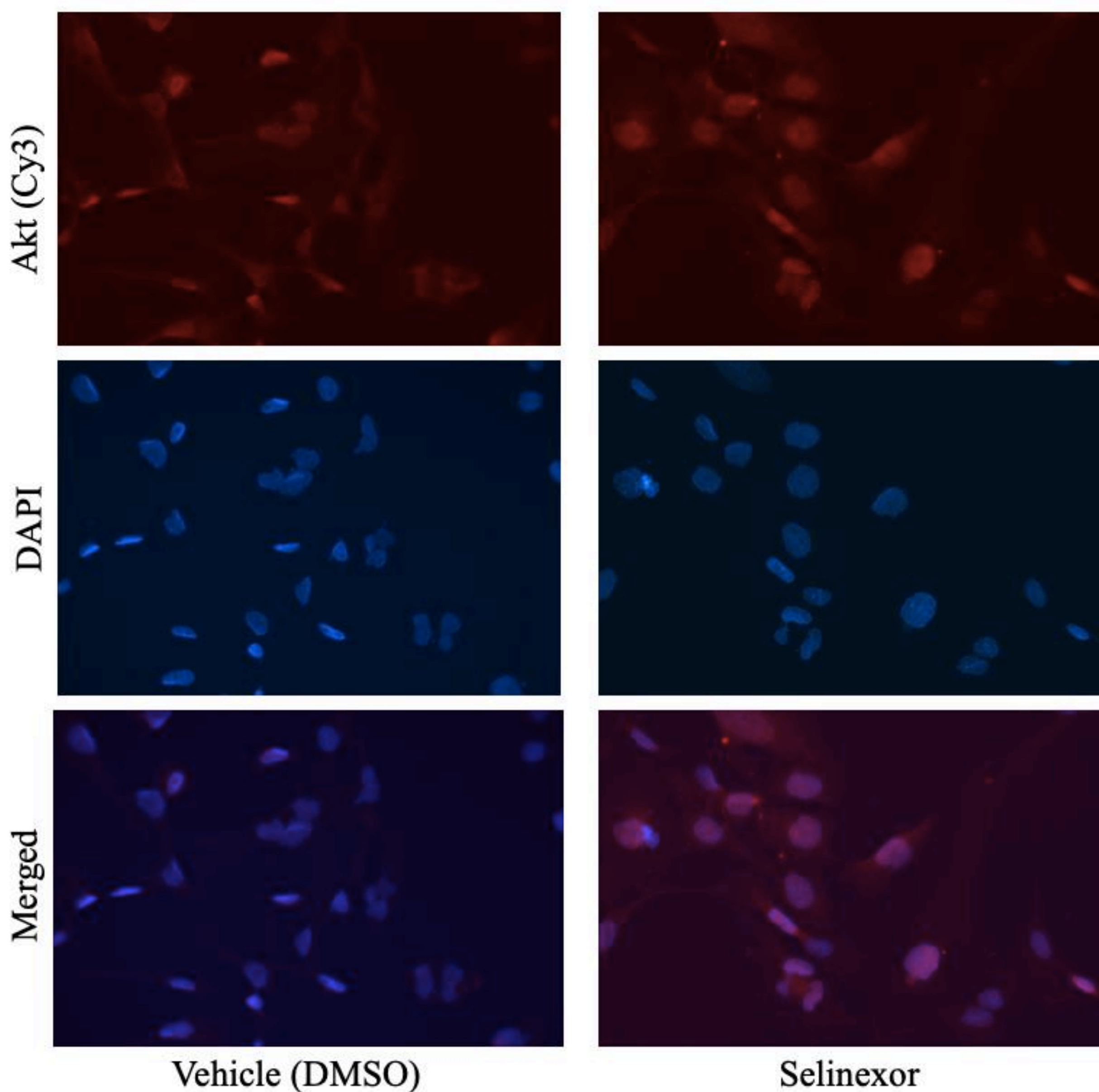


Figure 6: Indirect Immunofluorescence of Akt in BT-549 Cells. Akt was stained in red using a Cy3 antibody. Nuclei were stained in blue using DAPI. Areas of overlap between the Akt protein (red) and the nucleus (blue) appear as a purple color. Data shown are representative pictures of 2 independent experiments.

Observation	Vehicle (# of cells)	Vehicle (%)	Selinexor (# of cells)	Selienxor (%)
Equal staining in cytoplasm and nucleus	70	35%	14	13%
Staining predominantly nuclear	29	15%	84	80%
Staining predominantly cytoplasmic	89	45%	3	3%
Staining too light to be detected	11	5%	4	4%
Total Number	199	100%	105	100%

Table 2: Localization of Intensity of Akt Staining in Vehicle and Selinexor Treated Cells Cells were counted and categorized by intensity of Cy3TM staining and treatments.

ROS Assays

Hyperactivation of Akt has been demonstrated to evoke the production of ROS.[21] The extent of activation of Akt in BT-549 cells treated with selinexor could be considered hyperactivation. To determine if the cells produce ROS in response to treatment with selinexor, ROS assays were performed. Four experiments were conducted using different strategies. Cells were plated at three different densities and treated with 2 different concentrations of selinexor. Fluorescence measurements were taken at different time intervals.

In experiment one, cells were plated at concentrations of 30,000 cells per well (Figure 7A) and 40,000 cells per well (Figure 7B). After one hour, absorbance measurements observed for the cells plated at 30,000 cells per well were between 0.08 and 0.12. The well that received no treatment had a higher reading than three of the remaining four treatments. After two hours, the readings were approximately 0.04, with some slight differences between treatments. At 21 hours, the measurement again dropped

slightly to be approximately 0.03. Twenty-four hours after treatment, two absorbance values increased, while three decreased. One of these values became a negative value. In the wells that contained 40,000 cells per well, the absorbance values after one hour were between 0.05 and 0.1, with the cells that received no treatment being the next to highest value. At 2 hours, all absorbance readings were below 0.05 and inconsistent, but the 10µM vehicle treatment value, which was the highest value at 1 hour, became negative. At 21 hours, all values were positive again and around 0.03-0.04. After 24 hours, all values were negative, with their absorbance values between -0.13-0.15.

In experiment two, cells were plated at 60,000 cells per well. At 19 hours and 21 hours, the highest absorbance values were for those cells that received no treatment. This value stayed steady at a value of 0.025 throughout all three timepoints. At 3 hours, the 10µM selinexor treatment had the highest absorbance value. This value decreased slightly at 19 hours and again at 21 hours, but it was still higher than the values for the other treatments. In this experiment, the 1µM vehicle treatment yielded a higher absorbance value than the 1µM selinexor treatment. However, the 1µM selinexor treatment yielded higher absorbance values than the 10 µM vehicle treatment at 3 hours, and these values were the same at both 19 and 21 hours.

The results from experiment three were also inconsistent. At 15 minutes, the absorbance values for 1 µM vehicle and 10 µM selinexor were very low, while the values for no treatment and 1 µM selinexor were between 0.2 and 0.3. After 30 minutes, the absorbance reading for 1 µM selinexor treatment were extremely high, at a value of around 0.45, while the other values were approximately 0.1 and lower. After an hour, the

value for 1µM selinexor decreased slightly, while the values for no treatment and 1µM vehicle treatment increased again.

Experiment four again shows inconsistent values. After 15 minutes, 1µM selinexor treatment yielded a higher absorbance value than any other treatment, but at both 30 minutes and 1 hour, 10 µM vehicle treatment yielded the highest absorbance values. While most values decreased slightly over time, the 10µM vehicle treatment values increased over time. In addition, the 10 µM selinexor treatment values never exceeded 0.05 and remained relatively constant.

When analyzing this data, there were no consistent trends in absorbance values when comparing between treatment options. Due to the negative and fluctuating absorbance readings, over time, no reliable conclusion could be drawn from the data. In spite of different experimental strategies, the data are insufficient to determine if selinexor impacts ROS levels in BT-549 cells (Figure 7).

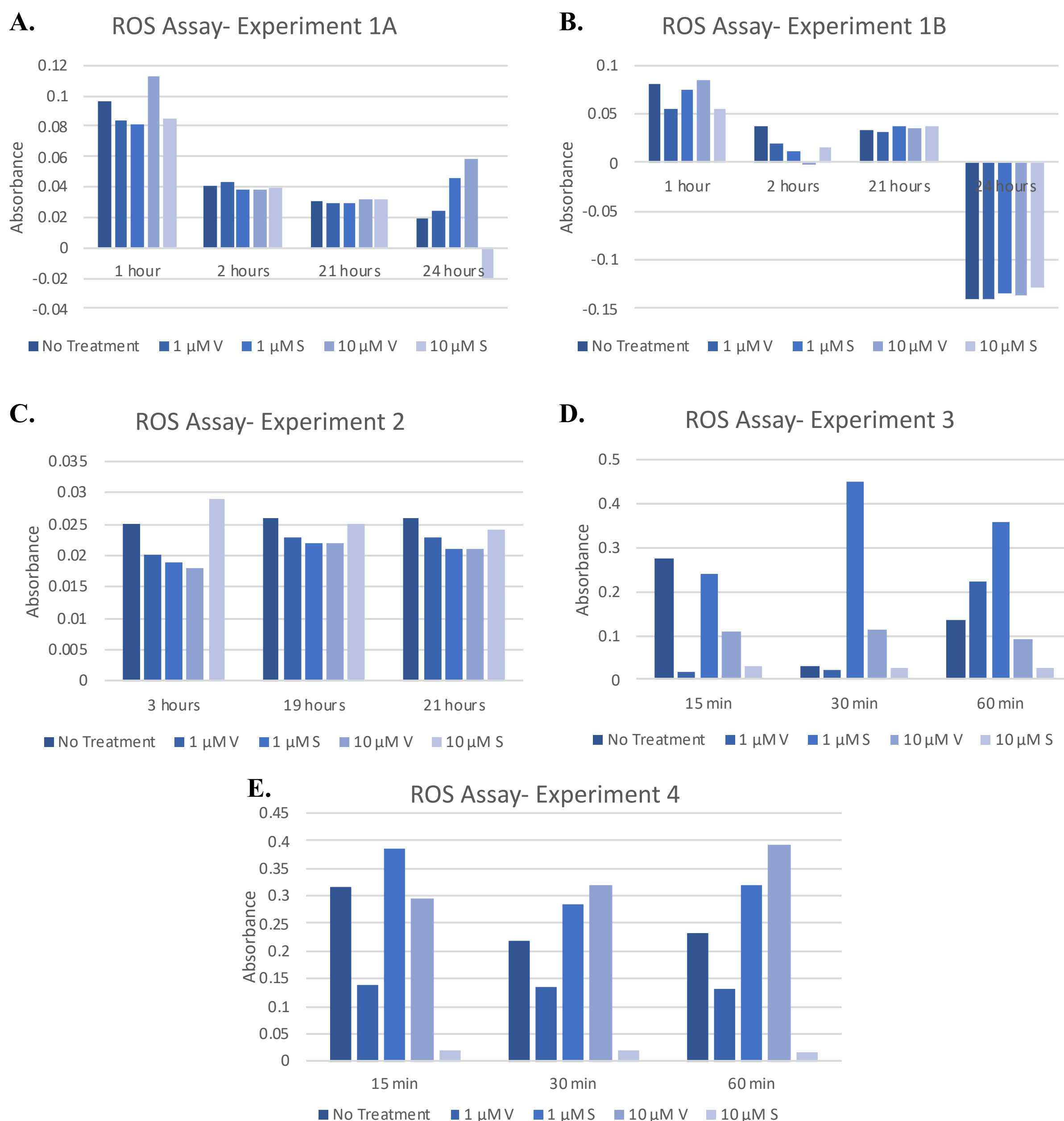


Figure 7: ROS Assay Absorbance Readings Cells were treated in a 90-well plate with 5 different treatments: no treatment, 1 μM DMSO, 1 μM selinexor, 10 μM DMSO, and 10 μM selinexor. (A) Cells were plated at 30,000 cells per well. (B) Cells were plated at 40,000 cells per well. (A-B) Absorbance readings were measured at 1 hour, 2 hours, 21 hours, and 24 hours. (C-E) Cells were plated at 60,000 cells per well. (C) Absorbance

readings were measured at 3 hours, 19 hours, and 21 hours. (D-E) Absorbance readings were measured at 15 minutes, 30 minutes, and 60 minutes. V, DMSO; S, Selinexor.

IV. Discussion/Conclusion

Selinexor is currently being used in several clinical trials for cancer and has shown anti-cancer effects in combination with other chemotherapies. Clinical trials evaluating the use of selinexor in the treatment of triple negative breast cancer are limited, but *in vitro* studies show promising results in its ability to kill triple negative breast cancer cells. The mechanism by which this occurs, however, is not understood. The goal of this research was to determine the mechanism by which selinexor kills BT-549 triple negative breast cancer cells.

Several key observations were made in this research. First, this work has demonstrated that selinexor increases Akt activity. This was an unexpected finding. Since Akt is generally associated with cell survival, the levels were expected to decrease. In contrast, the levels were increased substantially. Hyperactivation of Akt has been previously published by Wang et al. when they reported on the effects of targeting Akt for potential therapy in diffuse large B-cell lymphoma.[22] This hyperactivation may be the mechanism utilized by selinexor in order to induce apoptosis in BT-549 cells.

Second, Akt phosphorylation is dependent on phosphorylation at T308. Therefore, it was expected that selinexor would increase the phosphorylation of Akt at this site since it induced Akt activity. The results did not indicate an increase in phosphorylation of T308, which is required for full activation and downstream phosphorylation of Akt. This is confounding as it is generally thought that this is the key to activation of the kinase. However, other kinases, such as protein tyrosine kinase 6 (PTK6), have been shown to activate Akt.[13] PTK6 is not expressed in normal mammary tissue, but is expressed in

many breast cancer cells. It phosphorylates Akt at tyrosines 315 and 326 and is localized to the nucleus.[23]

Third, cells treated with selinexor show increased nuclear localization of Akt. This indicates that inhibition of nuclear export in BT-549 cells results in nuclear retention of Akt. One important function of nuclear Akt is inhibition of forkhead box protein O1 (FOXO) transcription factors, which results in decreased expression of FOXO proteins. Since FOXO proteins are important for antioxidant production, decreased expression could impact the ability of the cells to respond to oxidative stress, such as that caused by hyperactivated Akt.

Lastly, the ROS assays gave inconclusive results, and need to be further investigated. Previous experiments in the Odom lab have shown that selinexor kills BT-549 cells by inducing apoptosis. An increase in ROS levels can cause oxidative stress and ROS toxicity to trigger apoptosis. Apoptosis can be induced by many factors, but studies have shown that cancer cells with constitutively activated Akt, such as BT-549 cells, are more sensitive to ROS-induced apoptosis.[16] Given this, it is still possible that levels of ROS are affected. Figure 8 demonstrates a model schematic of these events.

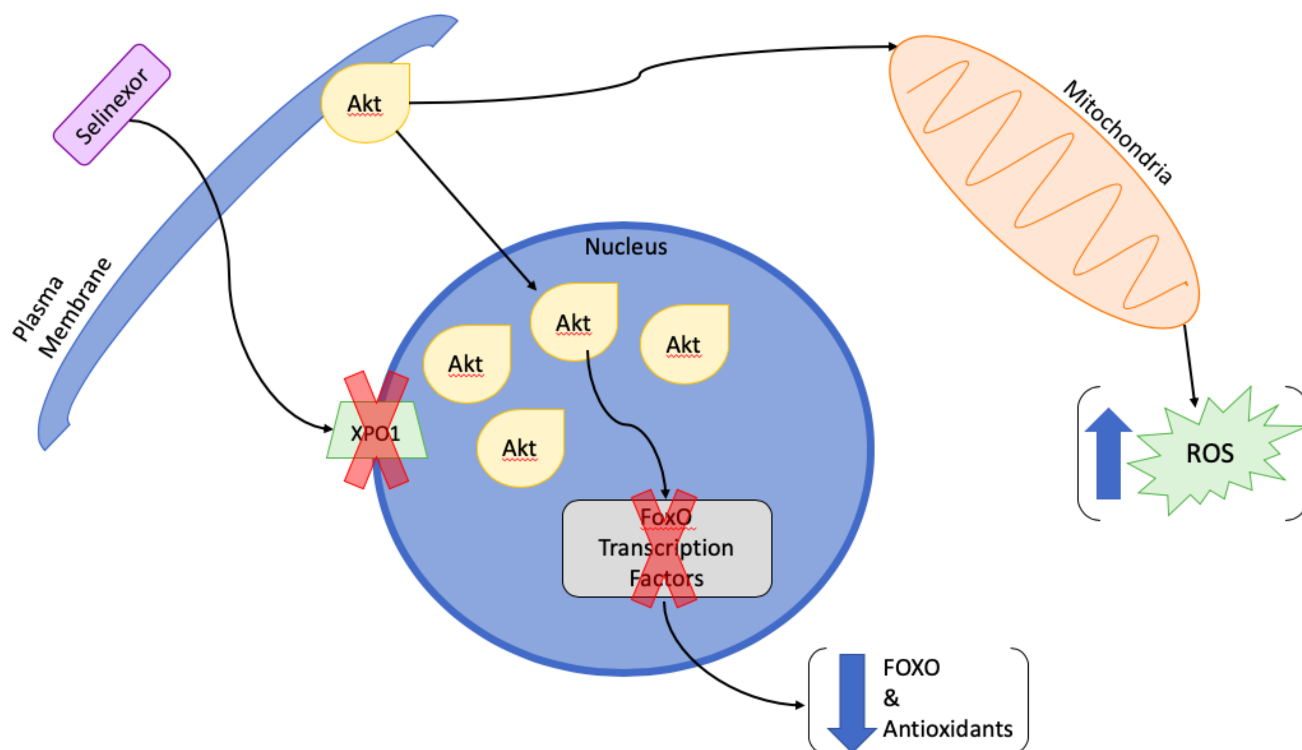


Figure 8: Proposed Method of Selinexor Induced BT-549 Cell Death

Selinexor inhibits the function of XPO1. Akt translocates to the nucleus and/or mitochondria after being activated at the plasma membrane. In the nucleus, Akt inhibits FOXO transcription factors and therefore decreases the amount of FOXO and antioxidants produced. At the mitochondria, Akt stimulates mitochondrial activity, resulting in an increase in the production of ROS.

In conclusion, the data reported here suggests that Akt is being sequestered into the nucleus and hyperactivated via an alternative mechanism of activation in BT-549 cells upon treatment with selinexor. Future experiments to investigate potential alternative methods for Akt activation are needed. In addition, ROS assays would need to

be repeated and cellular fractionation is needed to support the indirect immunofluorescence observations.

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