# ROLE OF AKT IN INFLAMMATORY BREAST CANCER

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Arts in Biological Sciences with Distinction

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## ABSTRACT

Inflammatory Breast Cancer (IBC) is a rare and aggressive form of breast cancer with a rapid progression and lower survival rates than non-IBC types. Research aimed at understanding the role of various enzymes involved in controlling cell growth identified the serine/threonine kinase Akt as a major factor involved in cell growth and proliferation. Previous published studies have shown that Akt activation may be increased in IBC cell line SUM-149 relative to non-IBC cell line MDA-231. Building on these studies, it was hypothesized that Akt inhibition using Perifosine may be an effective strategy for reducing proliferation and invasion of breast cancer cells. MTT assay revealed that SUM-149 cells proliferated more than MDA-231 cells and Akt inhibition preferentially reduced IBC cell proliferation relative to non-IBC proliferation. Immunoblot analysis revealed that SUM-149 and MDA-231 cells expressed equal amounts of Akt 1 and Akt 3 isoforms but SUM-149 cells expressed lower amount of Akt 2 than MDA-231 cells. Attempts to further examine the role of individual Akt isoforms, especially Akt 3, on proliferation of IBC cells by siRNA knockdown of individual Akt isoforms to determine whether specific inhibition of an isoform reduces IBC cell proliferation proved to be unsuccessful. Combination therapies (Perifosine in combination with the PDGF inhibitor Crenolanib) were explored in order to limit drug toxicities through possible use of lower concentrations of each drug. Total Akt was immunoprecipitated in SUM-149 cells and activation was assessed using Akt-phospho-ser473. The experiment revealed that Perifosine treatment

completely inhibited Akt activation while Crenolanib did not inhibit Akt activation. MTT assays conducted to assess proliferation revealed that Perifosine treatment alone was superior to inhibition of IBC cell proliferation compared to Crenolanib alone or in combination. Scratch wound assays were also conducted to assess role of Akt inhibition in migration in both IBC and non-IBC cells. In both cell lines, Perifosine did not appear to inhibit migration while Crenolanib, working through a different pathway, was successful in inhibiting migration. These results suggest that IBC cells utilize different pathways for proliferation and migration and Akt inhibition may be an effective strategy for inhibition of proliferation alone.

# Chapter 1

# INTRODUCTION

## 1.1 Inflammatory Breast Cancer

Inflammatory Breast Cancer (IBC) is a rare and aggressive type of breast cancer accounting for only 5% of all breast cancer types and characterized by a rapid disease progression (Giordano et al. 2003). Dermal lymphatic vessels in breast tissue are invaded by malignant cells causing blockage of lymph vessels resulting in swelling and redness characteristic of IBC (Kleer et al. 2000). The defining characteristic of normal breast cancer, a palpable lump, is absent in IBC, so diagnosis of disease occurs only when significant changes in breast appearance manifest. Clinical symptoms of IBC include inflammation of the breast, edema and erythema (redness of skin) of the breast (Giordano et al. 2003). Most patients with IBC can be clinically diagnosed due to presence of metastasis to axillary or supraclavicular lymph nodes (Robertson et al. 2010). IBC is characterized pathologically by the presence of dermal lymphatic invasion but this pathology is not necessary for clinical diagnosis (Thapaliya and Karlin 2009). IBC also exhibits angiogenic and invasive properties resulting in infiltration of lymph vessels and formation of tumor emboli, which can lead to development of metastasis (Kleer et al. 2000).

IBC is frequently misdiagnosed as bacterial infection, congestive heart failure, primary breast lymphoma or post radiation dermatitis (Robertson et al. 2010). This problem is exacerbated due to the absence of a definitive system used to identify IBC as pathological and clinical definitions of the disease can vary and even contradict one

another (Thapaliya and Karlin 2009). Several diagnostic techniques are employed for imaging of breast cancer but not all the tools available (mammography, ultra sound and PET/CT scans) are successful at detecting IBC. MRI is currently the best imaging modality for diagnosing IBC as it can detect edema and changes in skin thickness (Robertson et al. 2010). Given its aggressive nature and difficulty in diagnosis, more research is needed to determine specific characteristics of IBC in order to develop treatment strategies tailored for this disease.

Diagnosis of IBC is complicated since the molecular etiology of IBC differs from that of other breast cancers. Enhanced migratory ability of cells leading to invasion is the precursor to later metastasis of many cancers. Epithelial cells are tightly linked to one another by cell-cell junctions, which are formed by binding of an e-cadherin transmembrane protein to a catenin (Morales and Alpaugh 2009). The presence of these tight junctions prevents cells from migrating and confers polarity (apical and basal surfaces) to epithelial cells (Guarino 2007). Decreased cellular adhesion is associated with loss of e-cadherin and is considered the initial step in cancer progression (Morales and Alpaugh 2009). Epithelial cells gradually lose cell polarity and organization due to loss of e-cadherin and acquire more mesenchymal cell characteristics in a process called epithelial-mesenchymal transition or EMT (Guarino 2007). Mesenchymal cells lack the adhesion junctions characteristic of epithelial cells allowing cells to be more motile. However, in IBC, despite its aggressive and invasive nature, e-cadherin loss is not reported, rather, an increase in e-cadherin expression is observed (Morales and Alpaugh 2009). It is thought that overexpression of e-cadherin in IBC is essential for tumor cell adhesions and subsequent formation of tumor emboli (Woodward and Cristofanilli 2009).

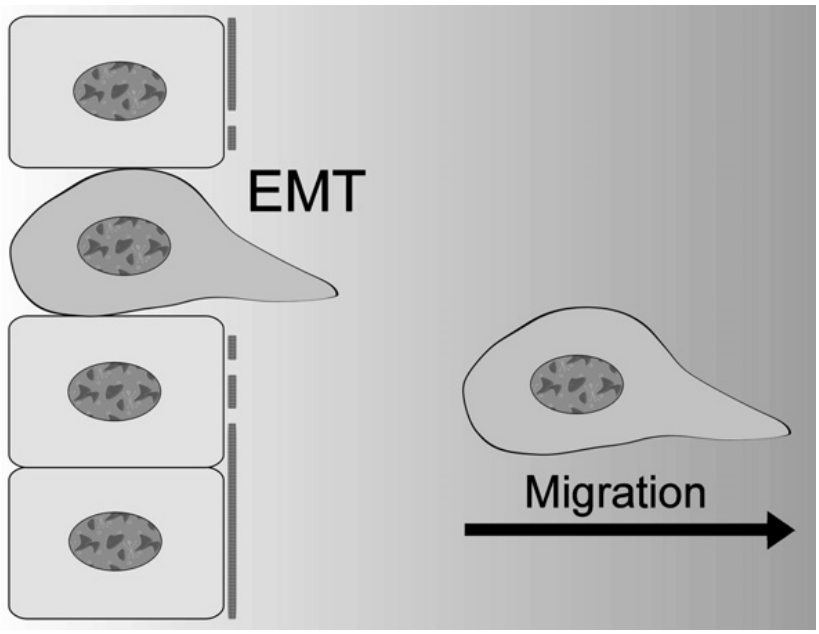


**Figure 1. Epithelial-mesenchymal transition.** Epithelial cells gradually lose adhesion and become more motile (Guarino 2007).

Cellular migration is mediated by extracellular factors, which cause intracellular changes leading to cytoskeletal reorganization. This reorganization is mediated by Rho proteins, small guanosine triphosphatases responsible for contractile motion of actin filaments and for regulating many cellular activities such as proliferation, migration and survival (Rosenthal et al. 2010). A migrating cell establishes a protrusion followed by actin-myosin contractions to move the cell forward and to retract the cell rear into the cell body (Guarino 2007). The contractile and retractile motions of the cell are regulated by Rho proteins, which are known to be overexpressed in many cancers (van Golen 2010). In particular, RhoC has been identified as promoting the migratory ability of cells and as being associated with IGF-I signaling in IBC (van Golen 2010). Rho GTPase participates in numerous

downstream signaling events to mediate some of the cellular metabolic activities mentioned above. One pathway of particular importance is the Akt/PKB signaling pathway (discussed in detail below), which is active in IBC (Unger and van Golen 2010). The main goal of the present thesis is to understand the mechanism by which Akt affects IBC growth and metastasis. It has been reported that an increase in Akt activity is correlated with an increase in phosphorylation of RhoC GTPase but it is unknown how Akt phosphorylation affects RhoC function (Unger and van Golen 2010).

### 1.1.1 Disease Stages and Survival Statistics

IBC is diagnosed at a younger age (52 years) than other breast cancer types (57 years) and is characterized by progression along three stages: Stage IIIB/C and Stage IV (American Cancer Society). Because of the rapid progression and harder diagnosis of IBC, women are commonly diagnosed further along in the disease progression, leading to a survival rate of 40% in five years, a far lower statistic than the 87% survival rate reported for other breast cancer types (American Cancer Society). Based on data from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database, median survival for patients diagnosed with Stage III IBC is 57 months and for those diagnosed with Stage IV IBC, it is 21 months.

**Table 1. Stages and survival statistics of Inflammatory Breast Cancer (American Cancer Society).**

| Stage | Description | Median Survival |
|---|---|---|
| Stage IIIB | Cancer has advanced locally to surrounding tissues of breast | 57 months |
| Stage IIIC | Cancer has spread to lymph nodes within breast and surrounding areas | |
| Stage IV | Cancer has spread to distant organs (metastasis) | 21 months |

### 1.1.2 Differential Biological Characteristics of IBC and Non-IBC tumors

IBC and non-IBC exhibit differential expression of prognostic markers that allow the two diseases to be distinguished from one another. Identification of these molecular characteristics are important for developing better treatment strategies that are unique for IBC to improve patient outcomes. Though both non-IBC and IBC patients typically exhibit negative estrogen receptor (ER) and progesterone receptor (PR) expression, IBC patients have a lower percentage of ER+ and PR+ (Radunsky et al. 2005). IBC patients have 44% cystolic ER+ levels compared to 64% in non-IBC patients and 30% PG+ in IBC compared to 51% PG+ in non-IBC (Radunsky et al. 2005). Both IBC and non-IBC cell lines express vascular endothelial growth factor (VEGF), which is important for vascularization and angiogenesis but IBC cells exhibit higher levels of VEGF and preferentially exhibit VEGF-D, which is postulated to be involved in lymphotactic processes (Kleer et al. 2000).

In addition, e-cadherin, necessary for cellular adhesion, is over-expressed in IBC cell lines compared to non-IBC cell lines, which was unexpected as downregulation of e-cadherin expression is typically associated with metastasis (Hoffmeyer et al. 2005). In IBC cell line SUM-149, e-cadherin overexpression is a

contributing factor to tumor emboli formation, which can aid in invasion via passive metastatic mechanism (Hoffmeyer et al. 2005).

Several genes have been identified that exhibit differential expression in IBC and non-IBC cell lines. In particular, loss of WISP3 (also known as lost in inflammatory breast cancer or LIBC) and overexpression of RhoC GTPase in IBC may contribute to the aggressive behavior associated with the disease (van Golen 1999). RhoC was overexpressed in 90% of IBC compared to 38% in non-IBC tumors and is a potential marker of a proliferative tumor due to its role in regulating actin cytoskeleton and cellular proliferation (van Golen 1999, Lehman and Van Laere 2012). Similarly, WISP3 was lost in 80% of IBC compared to 21% of non-IBC tumors (Woodward and Cristofanilli 2009). It is a member of the insulin-like growth factor binding protein (IGFBP) related family and is hypothesized to be a tumor suppressor gene and loss of this gene's expression in IBC is thought to contribute to proliferation (van Golen 1999).

### 1.1.3 Treatment

Given the low survival rates for patients with IBC, exploring and designing viable treatment options is essential to improving disease prognosis. Three main treatment modalities are employed in treating IBC: chemotherapy (systemic approach), surgery and radiation therapy (Robertson et al. 2010). Surgical treatment alone, typically mastectomy, has not proven to be effective in curtailing disease progression and in some cases, serves to worsen outcomes (Thapaliya and Karlin 2009). Local radiation therapy or a combination of radiation and surgery also had detrimental effects with regard to long-term survival rates (Giordano et al. 2003). Due to the rarity of IBC, there is a lack of adequate data from randomized-control trials on

chemotherapy treatments alone (Robertson et al. 2010). However, data from clinical trials indicates that chemotherapy in addition to local radiation therapy with or without mastectomy increased survival rates (Robertson et al. 2010). The current approach to treating IBC is initial administration of chemotherapy (neoadjuvant therapy) and assessing prognosis followed by surgery or radiation therapy as response to primary chemotherapy predicts higher survival rate (Giordano et al. 2003). A mastectomy with axillary node dissection (ALND) is considered an appropriate surgical follow-up procedure following response to neoadjuvant chemotherapy (Robertson et al. 2010). It is a work in progress to determine optimal combination of treatment modalities that will be the most effective strategy to combat IBC. Akt inhibitor and PDGF inhibitor, alone and in combination, have been evaluated to determine initial effects on IBC growth and metastasis *in vivo*.

### 1.2 Molecular Signaling Pathway

Given the effectiveness and prevalence of chemotherapy, more research is focused on determining which drugs can better halt the progression of inflammatory cell growth. Central to the creation of drugs is the identification of the role of various enzymes in controlling cell growth. Of particular importance is the serine/threonine kinase Akt or protein kinase B (PKB), which has been identified as a major factor in regulating cell proliferation (Kumar et al. 2001). Three isoforms of Akt are known to exist: Akt1 has been shown to promote cell survival by blocking pro-apoptotic proteins in IBC cell line compared to other breast cancer cell lines; Akt 2 has been determined to have a pro-invasive effect in IBC cells, which is absent in other breast cancer cell types; and the role of Akt 3 has not yet been conclusively determined (Unger and van Golen 2009). The three isoforms differ in tissue distribution and

expression levels but exhibit highly conserved PH and protein kinase domains as well as carboxy-terminal regions (Kumar et al. 2001). Akt is a downstream effector of phosphatidyl inositol 3-kinase (PI3K) and upon growth factor stimulation, the PI3K pathway is activated resulting in the formation of $PIP_3$ (phosphatidylinositol (3,4,5)-triphosphate), which binds to the PH domain and recruits Akt to the plasma membrane (Kumar et al. 2001). Akt recruitment to the membrane allows for its phosphorylation and activation at one of two residues: threonine-308 (Thr308) by Pdk1 or serine-473 (Ser473) by the mTOR complex (Unger and van Golen 2009, Castaneda et al. 2010). After activation, Akt relocates from the plasma membrane and migrates inward and phosphorylates multiple other substrates that are important for controlling cellular metabolic activities (Castaneda et al. 2010).

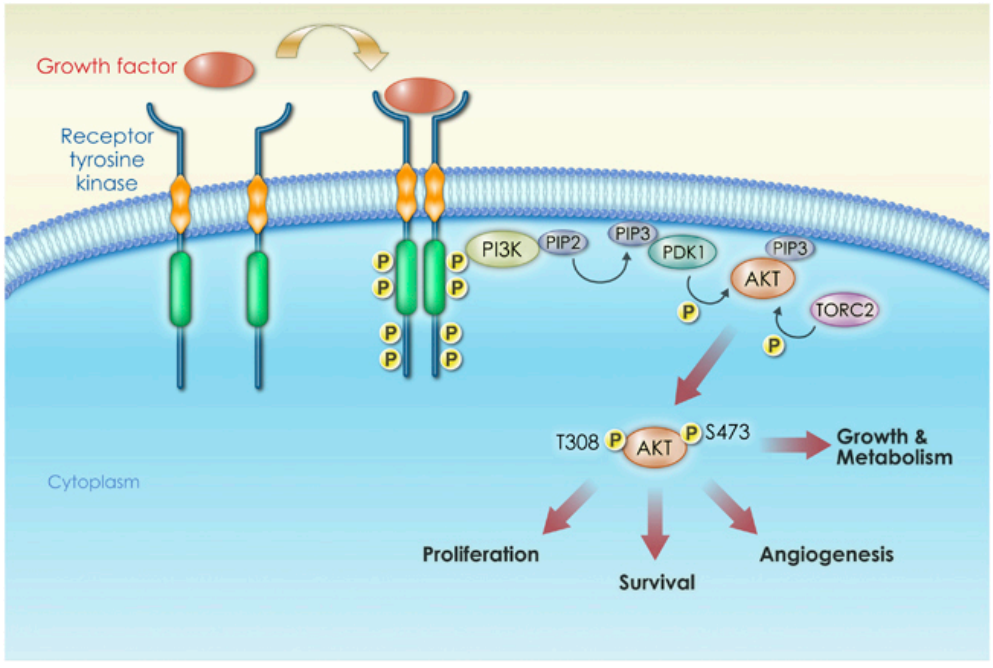


**Figure 2**. **PI3K/Akt pathway.** Growth factor stimulation causes PI3K binding to receptor tyrosine kinase and subsequent activation of Akt pathway (Garcia-Escheverria and Sellers 2008).

Abnormal activation of the PI3K/Akt pathway resulting in tumors can be due to a myriad of factors in the pathway: overexpression of receptor tyrosine kinases resulting in amplification of PI3K activation, loss of lipid phosphatase and tumor suppressor PTEN and overexpression of Akt downstream (Garcia-Escheverria and Sellers 2009). Therefore, inhibiting the PI3K/Akt pathway at the appropriate step in the pathway can potentially inhibit proliferation of cancerous cells. To assess the impact of Akt inhibition on efficacy of IBC, Akt inhibitor Perifosine (discussed below) was chosen.

### 1.3 Akt Inhibitor Perifosine

Since inhibition of the Akt pathway has been identified as a potential approach to reduce inflammatory breast cancer proliferation, I worked with Akt inhibitor Perifosine to determine whether Perifosine treatment of IBC and non-IBC cell lines would have differential effects on proliferation and migration. Perifosine is an alkylphosphocholine, which can be administered orally and is known to affect the activation of Akt (Garcia-Escheverria and Sellers 2008). The mechanism of action is unknown but it appears to regulate the activity of cell surface receptors and obstruct Akt localization to the membrane (Leighl et al. 2007, Garcia-Escheverria and Sellers 2008). While Perifosine has shown some promising results in clinical trials, the side-effects (fatigue, vomiting and diarrhea) associated with treatment leave much to be desired (Leighl et al. 2007). Some data exist that suggest that administration of Perifosine alone is associated with high toxicity making the use of combination drug therapies attractive as lower concentrations of the drugs can be administered, theoretically reducing the negative effects (Unger and van Golen 2009).

## 1.4 PDGFR Inhibitor Crenolanib

Crenolanib, a platelet-derived growth factor receptor (PDGFR), has demonstrated some efficacy in non-IBC cells and was identified as a potential drug that could work in combination with Perifosine to better inhibit IBC cell proliferation. Crenolanib typically binds to the active conformation of the PDGF receptor, which is a receptor tyrosine kinase with two similar isoforms, α and β, that homo- or heterodimerize upon stimulation with PDGFs as seen in Figure 3 (Joglekar-Javadekar 2013, Andrae 2008).

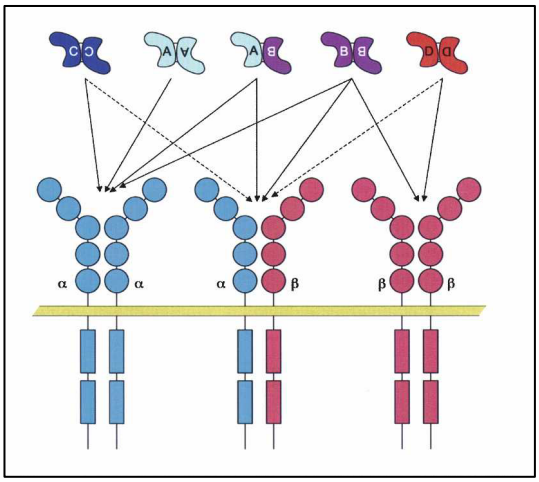


**Figure 3. PDGF-PDGFR interactions.** PDGFR α and β form dimeric subunits upon stimulation with ligand (Andrae 2008).

Dimerization of the receptors allows autophosphorylation of the tyrosine residues and initiates downstream signaling (Andrae 2008). Various signaling pathways are activated leading to diverse cellular responses. Ras-ERK and PI3K signaling pathways are particularly important as both pathways are involved in

stimulation of cell growth (Andrae 2008). The Ras-ERK pathway is also important for cellular migration and differentiation while the Akt pathway promotes inhibition of apoptosis (Andrae 2008).

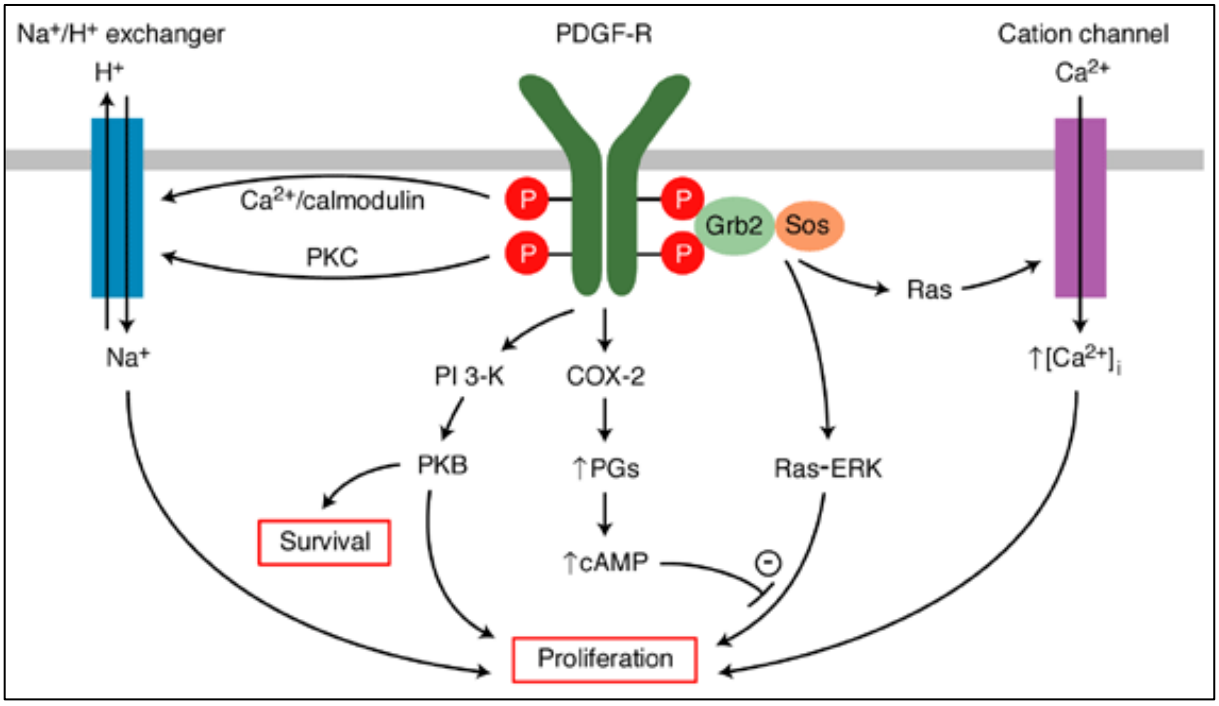


**Figure 4. PDGF/PDGFR signaling pathway.** This pathway is instrumental in cell survival and proliferation (Hui 2003).

Blocking PDGFR signaling is another way of reducing cellular proliferation and various drugs have been developed to block PDGFR signaling by inhibiting its kinase activity. Crenolanib binds to the active conformation and selectively inhibits phosphorylation of PDGFR α and β (Joglekar-Javadekar 2013). I studied the efficacy of Crenolanib (obtained from Arog Pharmaceuticals) in inhibiting proliferation and

migration of IBC cells to explore the role of receptor tyrosine kinase inhibitors as an effective treatment approach for IBC. The effects of Crenolanib alone were compared with parallel Perifosine treatments to determine which drug was a better inhibitor of IBC proliferation and migration. In addition, the two drugs were employed in combination at a high and low concentration to determine whether combination treatment was more successful in reducing IBC proliferation and migration. Combination treatment was also used in non-IBC cells to assess migration rates and to compare with IBC cell response.

### 1.5 IBC Cell Line SUM-149 and non-IBC Cell Line MDA-MB-231

Human cancer cell lines isolated from primary tumors serve as models for studying cancer development and to assess the effects of pharmacological agents on cell proliferation and survival. Though there are some limitations associated with the use of cell lines as they can have differing characteristics from the primary tumor cells, much care has been exercised in identifying appropriate cancer cell lines that are clinically relevant (Barnabas 2012).

SUM-149 cells were derived from invasive ductal carcinoma and are triple negative for ER, PR and HER-2 expression, similar to IBC, which is negative for ER and PR expression as well (Barnabas 2012, Thapaliya and Karlin 2009). SUM-149 cells can induce formation of tumors in nude mice post injection and exhibit a mixed morphology of epithelial, rounded and spindle cells (Barnabas 2012). In addition, the SUM-149 cell line is also associated with loss of expression of the LIBC gene and characterized by overexpression of RhoC GTPase (van Golen 1999).

The MDA-MB-231 adenocarcinoma cell line is epithelial in origin and was isolated from a pleural effusion of a breast cancer patient (Chavez et al. 2010). MDA-

MB-231 is also characterized as a TNBC (triple-negative breast cancer) cell line and is capable of producing tumors in nude mice (Chavez et al. 2010, Cailleau et al. 1978). MDA-MB-231 cells exhibit a spindle-shaped phenotype (Cailleau et al. 1978).

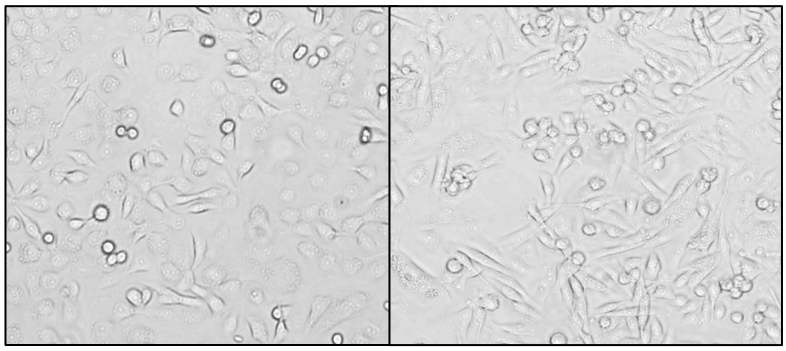

**Figure 5. Morphology of cancer cell lines.** IBC cell line SUM-149 (left) and non-IBC cell line MDA-MB-231 (right).

## 1.6 Hypothesis

We hypothesized that Akt inhibition may be an effective strategy for reducing proliferation and invasion of inflammatory breast cancer cells.

Aim 1: To determine the effect of Akt inhibition on cell proliferation in IBC vs. non-IBC using the SUM-149 and MDA-MB-231 cell lines as models

Aim 2: To assess the role of activation of individual Akt isoforms in proliferation of IBC cell line SUM-149

Aim 3: To compare the effect of Akt and PDGF inhibitors, alone and in combination, on migration of IBC and non-IBC cells

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Tissue Culture

Cell lines used were non-IBC cell line MDA-MB-231 and IBC cell line SUM-149. The MDA-MB-231 cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/L glucose, L-glutamine and sodium pyruvate and supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin and 750 µg of insulin. SUM-149 cells were maintained in Ham's F-12 medium containing L-glutamine and supplemented with 5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% antibiotic/antimycotic solution, 1% ITS cocktail (insulin, transferrin, selenium) and 0.1% hydrocortisone stock solution (1 mg/mL). Both cell lines were grown in an incubator maintained at 5% $CO_2$ with an internal temperature of 37°C. Cells were grown to confluence and typically split 1:10 into new flasks. Cells were washed gently with 1x phosphate-buffered saline (PBS) and treated with Trypsin EDTA to detach cells. Fresh cells previously frozen and stored in liquid nitrogen were used after cells were split more than 10 times.

## 2.2 Cell Counting

MDA-MB-231 and SUM-149 cells grown in 6-well dishes were washed gently with 1x PBS and treated with Trypsin EDTA to detach cells. Media was added to each well and cells were collected in 15-ml tubes. A small volume (less than 50 µl) of cells was pipetted into a 1.5-ml eppendorf tube and diluted in equal amount of Trypan blue

dye, which selectively stains dead cells blue. 12 µl of this mixture was placed on a hemocytometer under a microscope and cells visible in all four quadrants were counted and counts were averaged. Cell concentration (cells/ml) was determined by multiplying the average number of cells counted by the dilution factor and $10^4$ (volume of the quadrants).

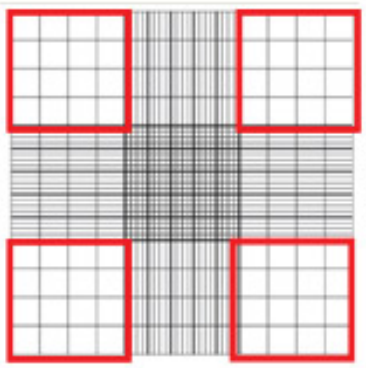

**Figure 6. Hemacytometer grid.** Cells visible in each of the red squares are counted and averaged (*Human Immunology Portal*).

### 2.3 Proliferation Assay

Proliferation of SUM-149 and MDA-MB-231 cells was assessed using Methylthiazol Tetrazolium (MTT) assay, a colorimetric assay used to determine cell number from absorbance readings. Yellow tetrazolium salts are reduced by active cells resulting in insoluble purple formazan, which is solubilized and analyzed by spectrophotometry. Cells were plated in 96-well dishes in minimum replicates of three in 200 µl of media. Following overnight incubation, cells were treated with inhibitor(s). MTT assays were performed at 48-hour intervals typically over a 5- or 7-day period. Old media was aspirated and replaced with fresh media with additives

every two days. Media was aspirated and 50 µl of MTT reagent along with 150 µl of media with serum was added to each well. The plate was incubated at 37°C for 3 hours. Media in each well was aspirated and replaced with 100 µl of DMSO and pipetted to dissolve the purple precipitate formed in the wells. Absorbance readings were recorded at 570 nm.

A separate dish was maintained to generate a standard curve. Cells were plated at varying concentrations ranging from 0 to 100,000 cells/well (without inhibitor treatment) and incubated overnight. Absorbance was recorded the next day and readings were plotted against known cell concentrations to derive an equation to estimate cell number from absorbance readings. The equation was utilized to analyze absorbance data from the treated cells and convert those readings into cell numbers.

### 2.4 Protein Detection by Western Blot

MDA-MB-231 and SUM-149 cells grown in 6-well dishes were lysed in 250 µl of 1x lysis buffer containing Tris-HCl, EDTA, Triton X-100 and protease inhibitor. Cells were scraped on ice using a sterile cell scraper and collected into 1.5-ml eppendorfs and placed on a rotator at 4°C for 20 minutes. Lysates were then stored at -20°C until samples were run. Frozen samples were thawed on ice, diluted in sample buffer and boiled at 95°C for 5 minutes before being centrifuged at 13.2 rpm for a minute. Protein concentration was determined using BCA Assay kit (Pierce Thermo Scientific) and normalized so equal amount of protein was loaded into each well. Protein samples (containing 12-50 µg of protein) were loaded and resolved on a 7% or 10% gradient gel in a 135-min run at 120V and transferred to nitrocellulose membrane in an 80-min run at 120V. Membranes were then blocked in 5% milk for at least an hour to prevent non-specific binding and incubated in primary antibody (diluted in 3%

milk) at 4°C overnight. After a series of three 10-min TBST washes, the membranes were incubated in appropriate HRP-conjugated secondary antibody (diluted in 1% milk) at room temperature for an hour followed by another series of three 10-min TBST washes. Membranes were then developed using chemiluminescent detection methods.

Akt activation was assessed by immunoblotting with phospho-specific antibody to ser473 residue of Akt and incubated in anti-rabbit HRP-conjugated secondary IgG (Table 2). The presence of specific Akt isoforms (Akt 1, 2 or 3) was detected by immunoblotting with isoform-specific antibody to Akt and incubated in anti-mouse (Akt 1) or anti-rabbit (Akt 2 and Akt 3) HRP-conjugated secondary IgG (Table 2).

### 2.5 Immunoprecipitation

SUM-149 cell lysates were collected the same way as described in the previous section and pre-cleared with Protein G Agarose beads and incubated on ice for an hour. Washed Protein G beads were also coated with 2 µg of Normal Rabbit IgG (Table 2) or primary antibody of interest and incubated with rocking at 4°C for 4 hours. After preclearance, the beads were spun down at 2500 rpm for 3 min at 4°C. The antibody-coated beads were also spun down at 2500 rpm for 3 min at 4°C and the supernatant was discarded. The precleared lysate was added to the antibody-coated beads followed by overnight incubation on a shaker at 4°C. Samples were then washed three times in lysis buffer with 1x protease inhibitor prior to suspension in sample buffer. Samples were kept at room temperature for 5 minutes before they were heated, centrifuged and loaded onto gel the same way as described above.

SUM-149 cells post inhibitor treatment with Perifosine or Crenolanib or untreated control were immunoprecipitated with Akt total antibody to isolate the protein of interest. Akt phosphorylation was assessed by immunoblotting with phospho-ser473 Akt-specific antibody. The blot was re-probed with Akt total antibody to assess whether equal amounts of protein were loaded in each well.

### 2.6 Antibodies

A variety of antibodies were used to assess protein expression.

**Table 2. Antibodies used for western blot (WB) and immunoprecipitation (IP).**

| Antibody | Provider | MW (kDa) | WB | IP |
|---|---|---|---|---|
| Akt-Phospho473 | Cell Signaling Technology | 60 | 1:500 | |
| Akt 1 | Cell Signaling Technology | 60 | 1:1000 | |
| Akt 3 | Cell Signaling Technology | 60 | 1:1000 | |
| Pan Actin | Cell Signaling Technology | 45 | 1:1000 | |
| Total Akt | Cell Signaling Technology | 60 | 1:1000 | 1:50 |
| Normal Rabbit IgG | Santa Cruz Biotechnology | | | 2 µg/ml |
| Anti-rabbit Secondary IgG (HRP-conjugated) | Cell Signaling Technology | | 1:2000 | |
| Anti-mouse Secondary IgG (HRP-conjugated) | Cell Signaling Technology | | 1:2000 | |

### 2.7 Transfection

siRNA knockdown of Akt isoforms was conducted by transfecting SUM-149 cells with siRNA (Santa Cruz Biotechnology) at a final concentration of 25 nM or 40 nM using TransIT-siQuest Reagent. SUM-149 cells were plated in 6-well dishes overnight and grown to 80% confluency prior to transfection. TransIT-siQuest

Reagent was added to Opti-MEM Reduced-Serum Medium and appropriate siRNA stock solution (scrambled control, Akt 1, Akt 2 or Akt 3) and incubated at room temperature for 20 minutes to allow formation of complexes. The TransIT-siQuest Reagent:siRNA complexes were added drop-wise to each well and rocked gently to ensure equal distribution of the complexes. Cells were collected 24 hours after transfection and proliferation was assessed by MTT assay at various days post-transfection. Western blot analyses were also conducted to verify protein knockdown.

### 2.8 Scratch Wound Assay to Assess Cell Migration

SUM-149 and MDA-231 cells were plated in sterile 6-well dishes at concentrations of $0.7 \times 10^6$ cells/well suspended in 2 mL media and incubated at 37°C over night. The media was aspirated and fresh media containing appropriate additives (Perifosine, Crenolanib or a combination of both at various concentrations) was added. Cells were incubated for 13 hours before a scratch was performed down the center of each well with a sterile 1000-µL pipette tip. Pictures of each treatment condition were taken immediately following the scratch (0 hour), 4 hours, 8 hours and 24 hours post scratch to assess whether treatment inhibited migration. ImageJ software was used to calculate the diameter of the scratches. For each treatment condition, average diameter at each of the time points was compared to the average diameter at 0 hour to determine the migration rate of the cells.

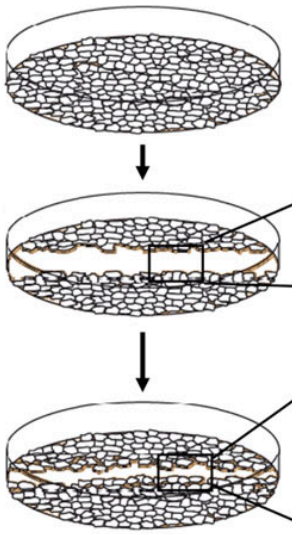

**Figure 7.** **Scratch wound assay.** Cells were grown to confluency in 6-well dish before surface was scratched with a pipette tip. Pictures were taken at various time points to assess migration rate (Moreno-Bueno 2009).

# Chapter 3

# RESULTS

## 3.1 Inhibition of Akt Activity Preferentially Reduces IBC Cell (SUM-149) Proliferation Relative to non-IBC Cell (MDA-MB-231) Proliferation

To determine whether inhibition of Akt activity would have an effect on proliferation in the IBC and non-IBC cell lines, proliferation assays were conducted over a seven-day period. Cells were plated at a concentration of 2000 cells/well in a 96 well plate and treated with varying concentrations of Perifosine (Akt inhibitor). IBC cells SUM149 have an increased sensitivity to inhibition of proliferation at lower concentrations of Perifosine than non-IBC cells MDA-MB-231 (Figure 8).

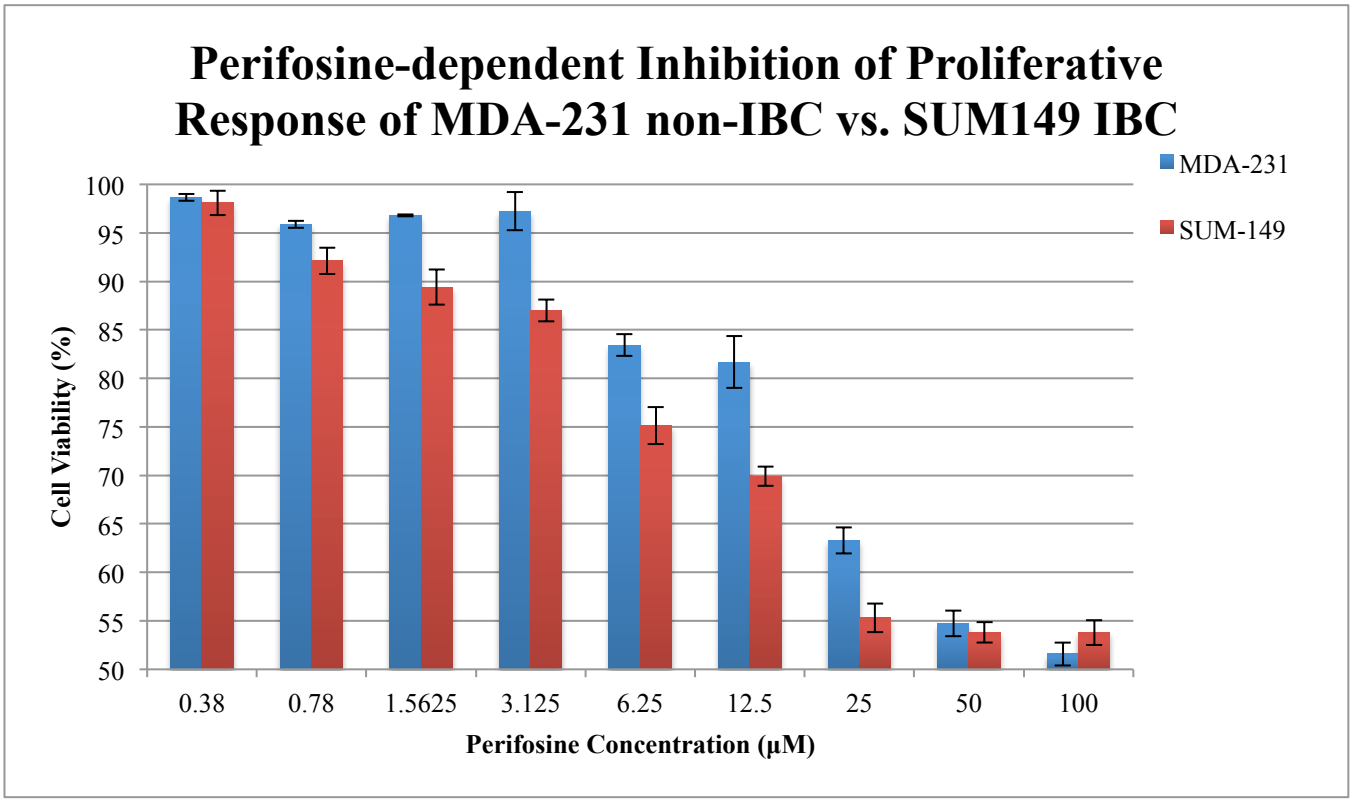

**Figure 8. Dose response curve of non-IBC and IBC cells post treatment with Perifosine.** MTT assay conducted on Day 3 to assess differences in proliferation between the two cell lines. IBC cells SUM-149 have an increased sensitivity to inhibition of proliferation at lower concentrations of Perifosine than non-IBC cells (MDA-231).

In addition, SUM-149 cells also respond earlier to Perifosine treatment than MDA-MB-231 cells (Figure 9a, b).

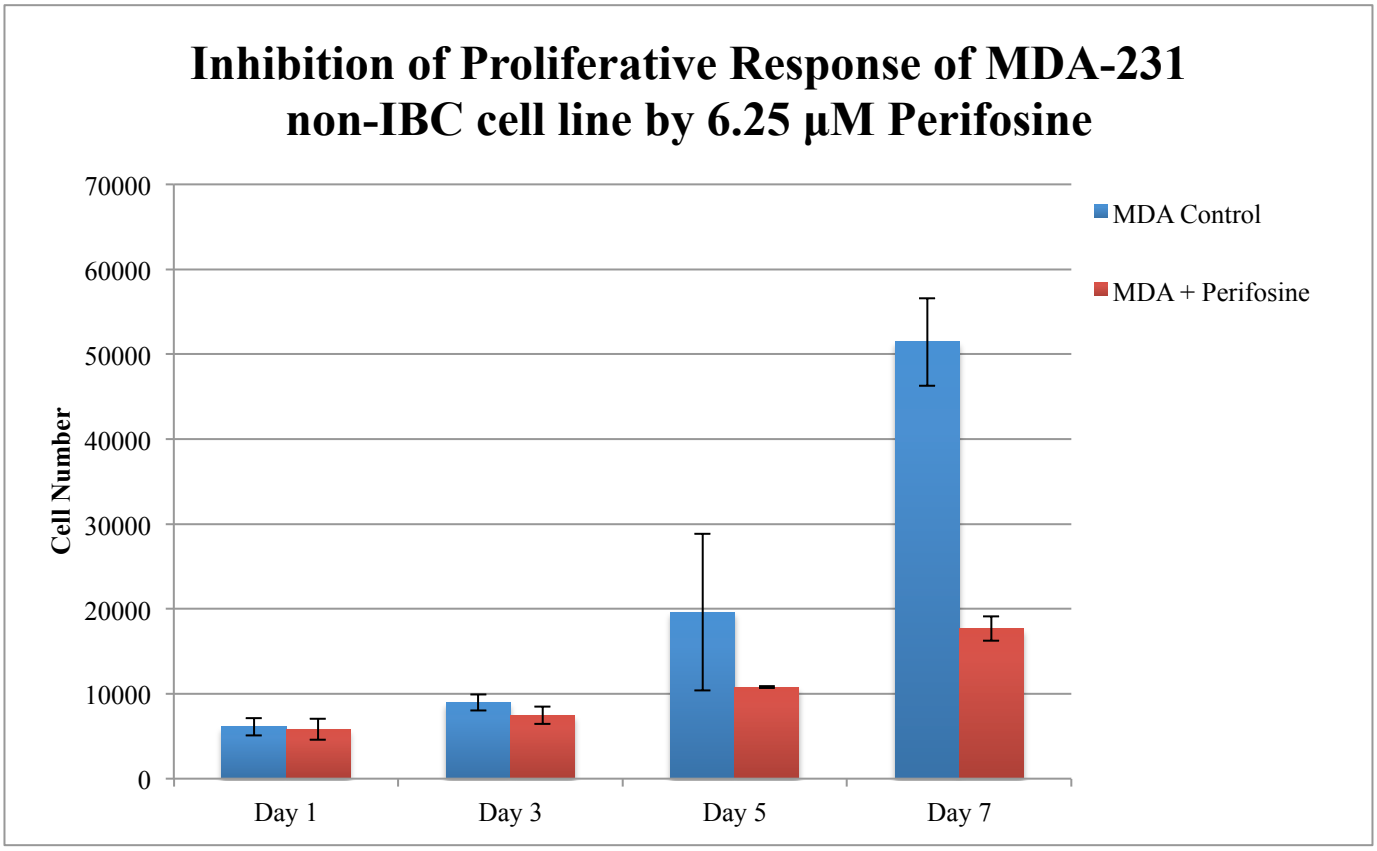


**Figure 9a. Growth-response curve of non-IBC cell line MDA-231 post treatment with 6.25 µM of Perifosine.** Proliferation was assessed over a 7-day interval. Treatment with Perifosine dramatically reduced proliferation of MDA-231 cells by day 7.

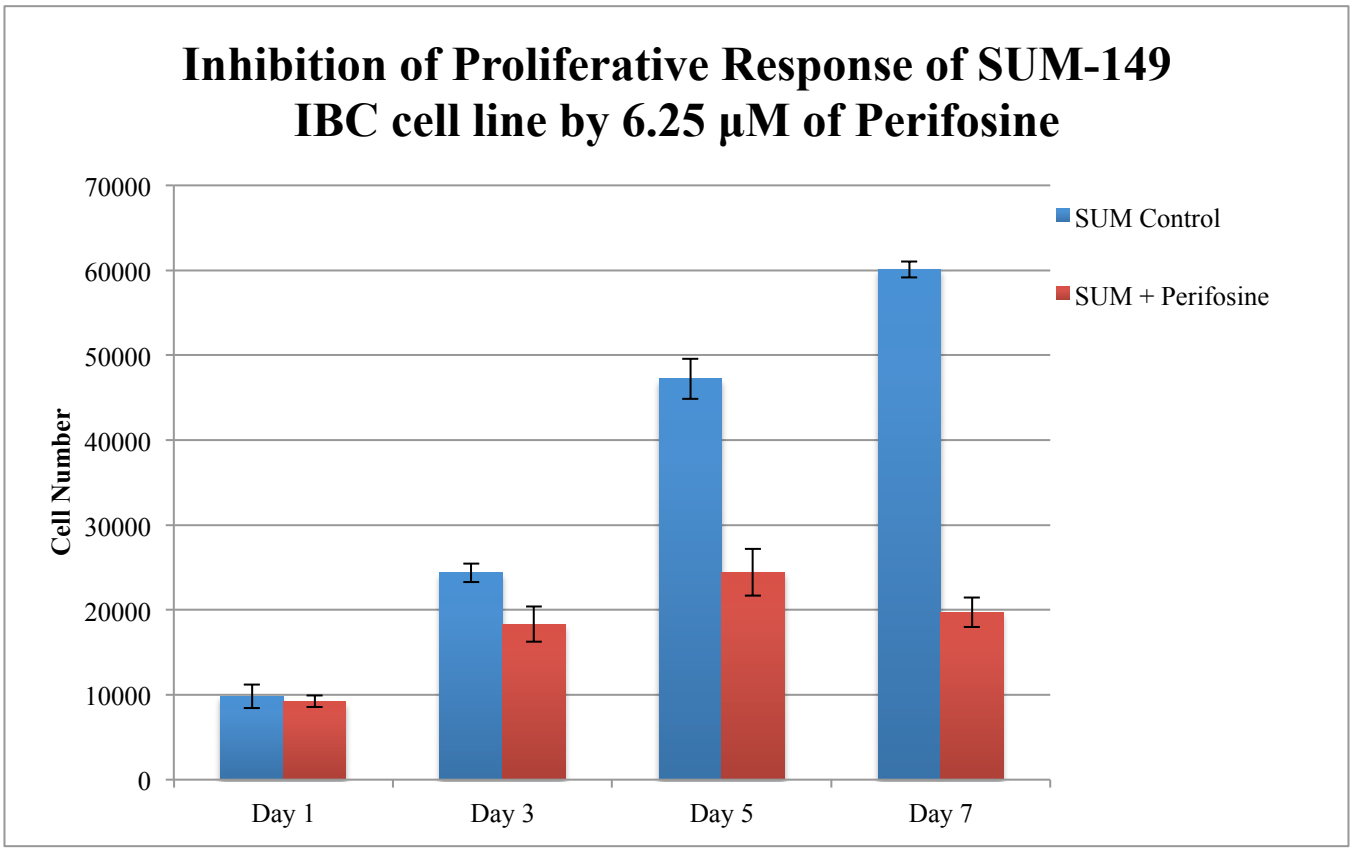


**Figure 9b. Growth-response curve of IBC cell line SUM149 after treatment with 6.25 µM of Perifosine.** Proliferation was assessed over a 7-day interval. Treatment with Perifosine dramatically reduced proliferation of SUM149 cells by day 5.

### 3.2 Assessment of Akt Isoforms and Akt Phosphorylation Expression Levels in SUM-149 and MDA-231 Cell Lines

It was suspected that different Akt isoforms would be expressed differently between the IBC and non-IBC cell lines. Presence of specific Akt isoforms was detected by conducting Western blot analyses on untreated MDA-231 and SUM-149 cell lines. Figure 10 reveals that all three Akt isoforms (Akt 1, 2, 3) are expressed in

both cell lines but expression levels vary. Akt 1 and Akt 3 appear to be equal in both cell lines while Akt 2 expression level was down-regulated in untreated SUM-149 cells compared to MDA-231 cells (Figure 10). GAPDH was used as a loading control to ensure the wells were loaded equally.

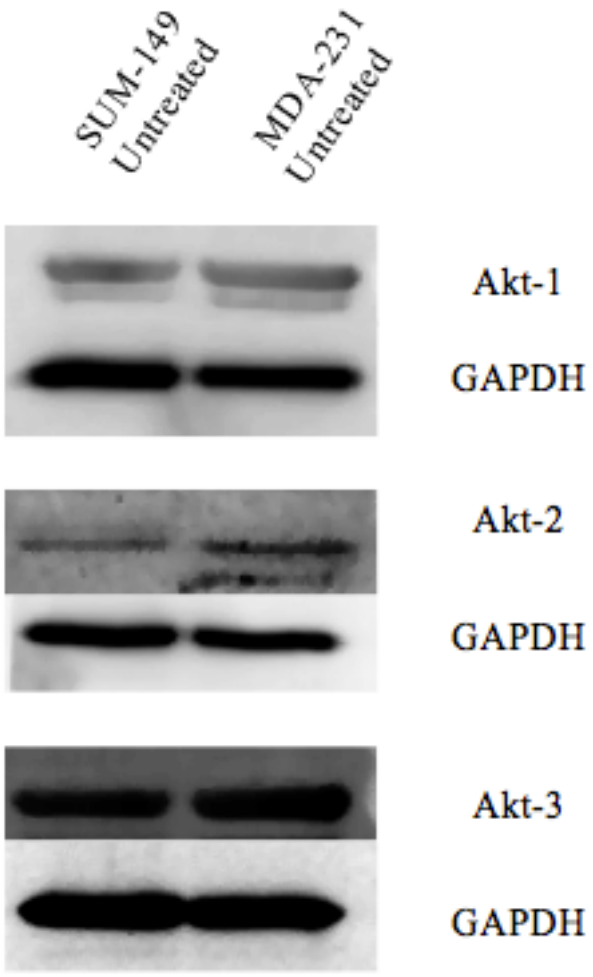


**Figure 10. Western blots on untreated IBC and non-IBC cell lines.** Akt 1 and Akt 3 were expressed similarly in both cell lines while Akt 2 was notably deregulated in SUM-149 cells.

Though the role of individual isoforms needs to be assessed to truly understand the role Akt plays in IBC cell proliferation, Akt 3 was chosen to be evaluated first since Akt 2 expression appears to be down-regulated in SUM-149 and Akt 1 expression was relatively weak (in both cell lines) compared to Akt 3 expression. Given the equal expression of Akt 3 in both cell lines, Akt 3 expression post Perifosine treatment was assessed to see what effect, if any, Perifosine had on Akt 3 specific expression. The effect of Perifosine treatment on Akt phosphorylation in IBC and non-IBC cell lines were also assessed (Figure 11). Western blot analyses on IBC and non-IBC cells lines were conducted after 2-day treatment with Perifosine. Akt 3 is down-regulated in both cell lines post Perifosine treatment but appears to be completely inhibited in IBC cells. Akt activation was also revealed to be generally higher in IBC cell line than non-IBC cell line. Perifosine treatment served to inhibit Akt phosphorylation in both cell lines. For both cell lines, approximately equal amounts of protein was loaded though slightly lower amount of protein may have been loaded in the SUM-149 Perifosine treated cells when assessing Akt phosphorylation (Figure 11).

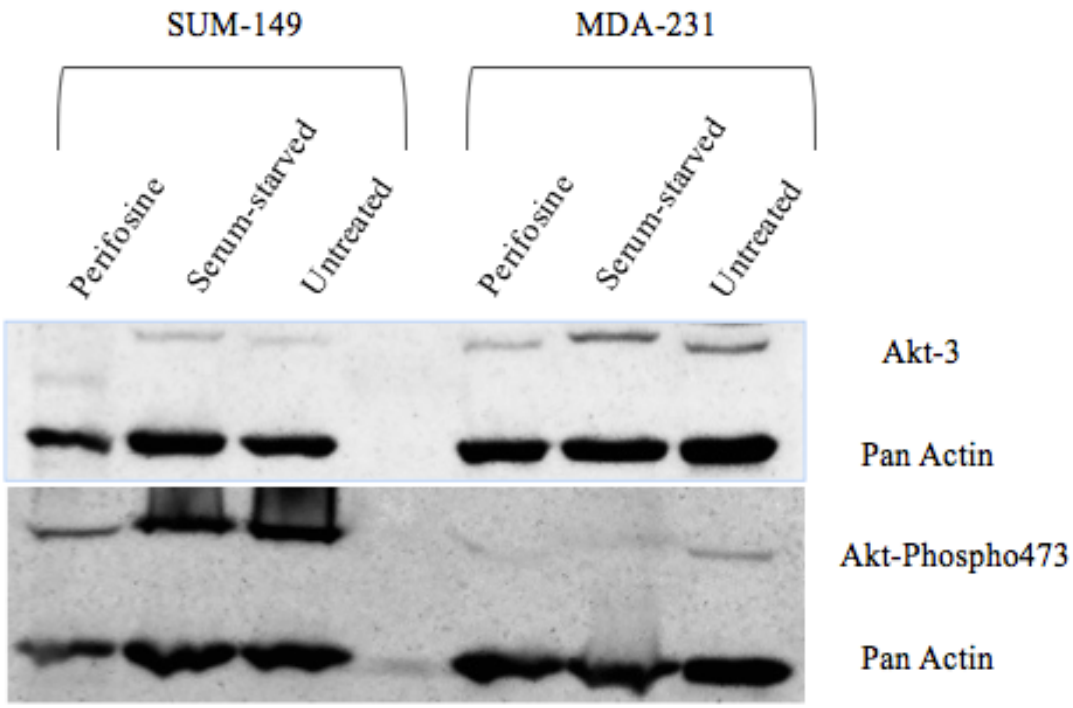


**Figure 11. Western blot analyses on SUM149 and MDA-MB-231 cell lines.** SUM149 and MDA-231 cells express less Akt 3 expression post Akt inhibitor treatment. Perifosine treatment inhibits Akt activation in both cell lines.

### 3.3 siRNA Knockdown of Akt Isoforms

Since Perifosine was shown to down-regulate Akt 3 in SUM149, Akt 3 was considered to potentially play a role in reducing IBC cell proliferation; however, the other two Akt isoforms were also examined to assess their potential roles in cell proliferation. IBC cell line SUM149 was transfected with siRNA obtained from Santa Cruz Biotechnology against each of the specific isoforms (and scrambled control) for

24 hours at a concentration of 25nM to determine whether knockdown of a specific isoform would be effective in inhibiting proliferation. Cells were plated at a concentration of 2000 cells/well in 200 μl of media in a 96-well dish in replicates of three. MTT assays were conducted on various days to assess cell viability after transfection. Effectiveness of the siRNA against the isoforms was verified by western blot analyses of the appropriate antibody at various days. Cells were plated at the equivalent concentration in a 6-well dish for western blot analyses.

### 3.3.1 Cell Viability Post-Transfection

SUM149 cells were transfected with scrambled control siRNA and siRNA against each of the isoforms for 24 hours prior to MTT assay over a 3-day interval. No major differences were found in cell viability among the different treatment conditions post-transfection though cells transfected with isoform-specific siRNA exhibited slight reduction in cell viability by day 3 (Figure 12).

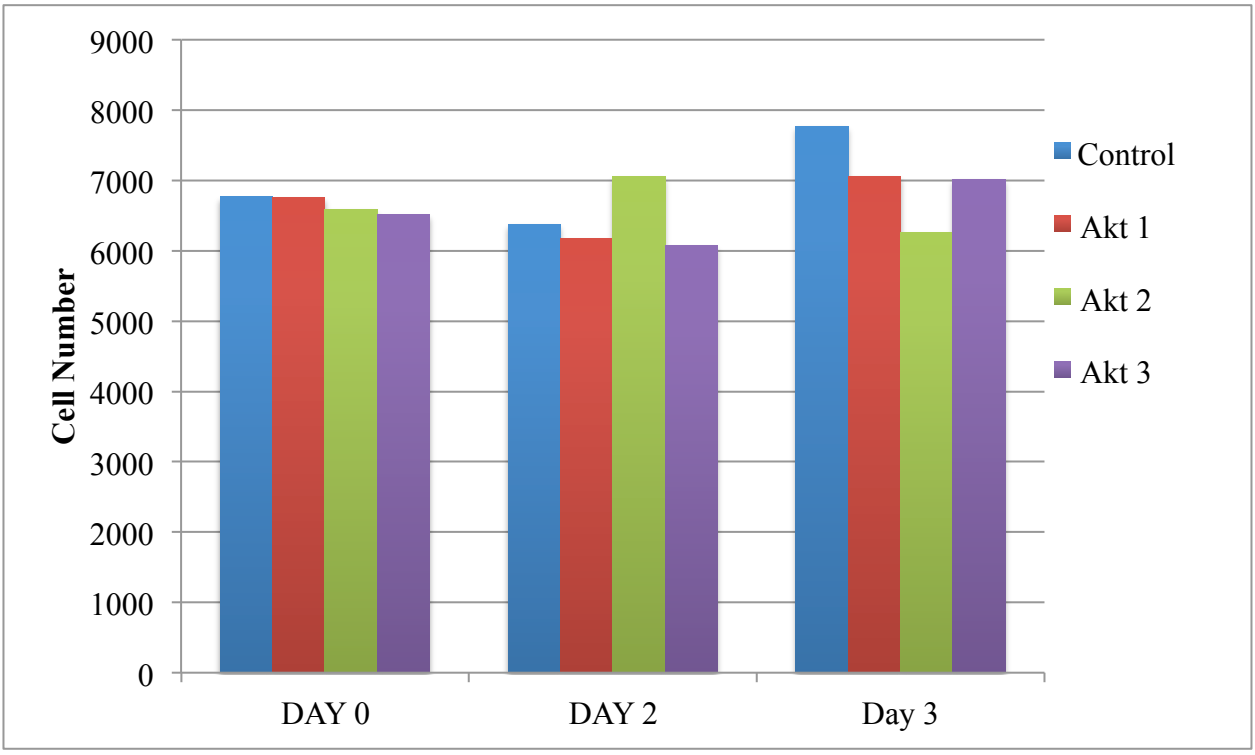


**Figure 12. Assessing cell viability post transfection.** SUM-149 cells transected with isoform-specific siRNA (Akt 1, Akt 2, Akt 3) did not differ significantly from the scrambled control treated cells.

The time course was extended to determine whether there was a difference in cell viability over a prolonged period (Figure 13). siRNA knockdown against Akt 3 revealed a significant difference compared to the other treatment conditions on day 2 and was most likely an anomaly due to experimental error. By day 6, there was reduced viability for the Akt 3 knockdown cells.

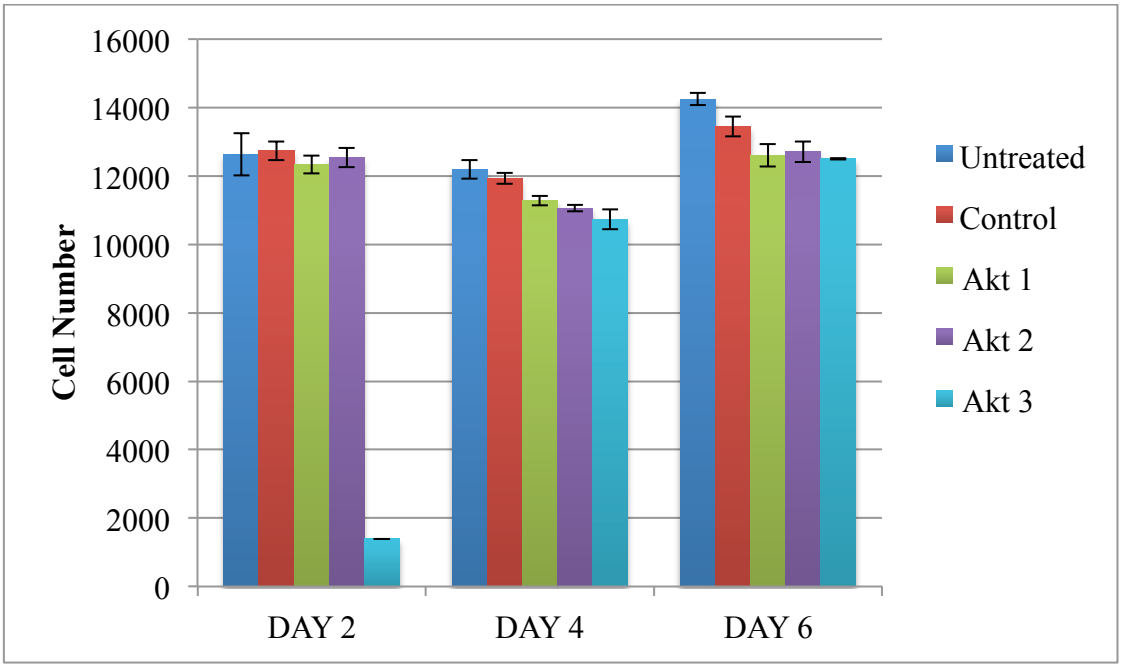


**Figure 13. Assessing cell viability post transfection.** SUM-149 cells transfected with isoform-specific siRNA against Akt 1 and Akt 2 along with scrambled control did not differ significantly from the untreated cells initially. Isoform- specific siRNA transfected cells did exhibit reduced cell viability by day 6. (*n = 2)

### 3.3.2 Verification of siRNA Effectiveness

Western blot analyses were conducted to verify the effectiveness of the siRNA transfection. Cells were plated with the intention of collecting over a 6-day interval to match the MTT data but cells for days 5 and 6 were contaminated so data displayed concludes on day 4. Anti-Akt 1 and anti-Akt 3 antibodies were used to determine effectiveness of the respective siRNA. The scrambled control transfected cells were expected to exhibit consistent levels of protein expression over the 4-day interval while the cells transfected with Akt 1 or Akt 3 specific siRNA were expected to decrease in protein expression over time. Akt 1 knockdown was not successful as there was no decrease in Akt 1 expression for the knockdown treatments over time.

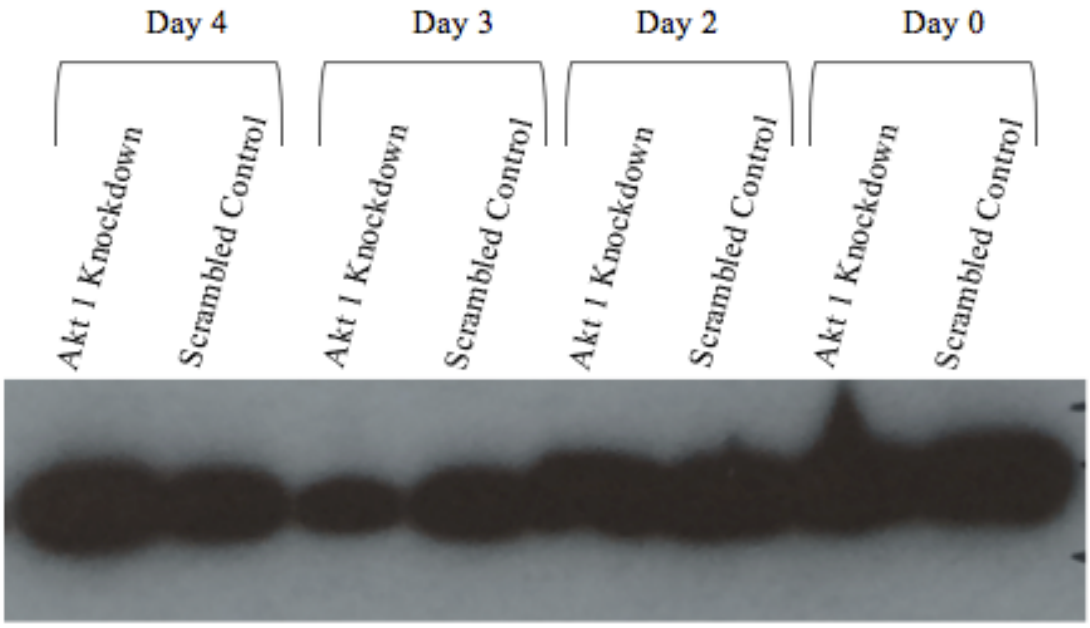


**Figure 14. Western blot for Akt 1.** Akt 1 knockdown and scrambled control SUM149 cells were probed using anti-Akt 1 antibody. Akt 1 knockdown was unsuccessful.

Akt 3 knockdown was also not successful though Akt 3 protein expression decreased in the Akt 3 knockdown cells over the 4-day interval (Figure 15). However, Akt 3 protein expression in the scrambled control transfected cells also decreased over the interval, which should not have occurred. Therefore, the results indicate that the transfection was not efficiently conducted.

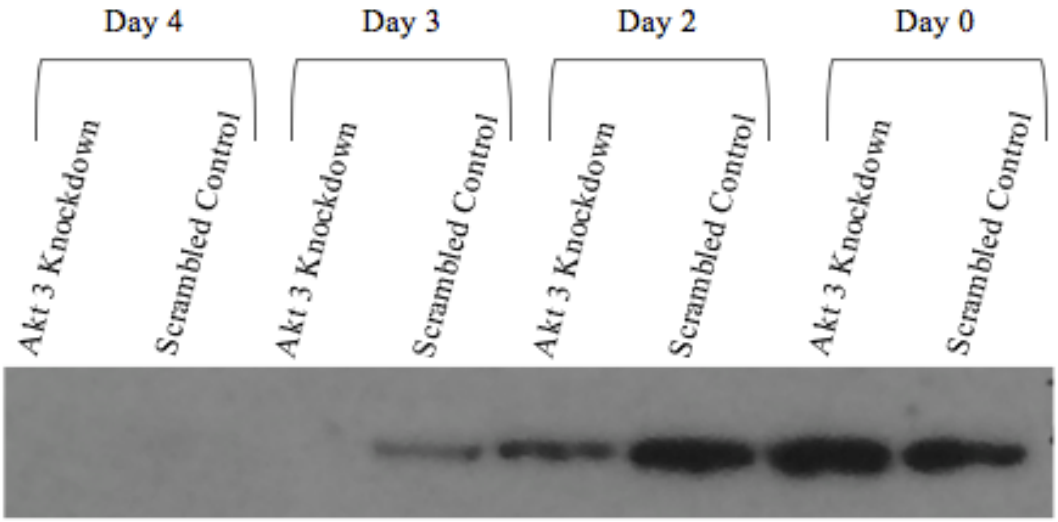


**Figure 15. Western blot for Akt 3.** Akt 3 knockdown and scrambled control SUM149 cells were probed using anti-Akt 3 antibody. Akt 3 knockdown was unsuccessful.

### 3.3.3 siRNA Knockdown of Akt 3

Since attempting to transfect all three Akt isoforms was not successful, one isoform (Akt 3) was selected to improve transfection efficiency. Consultation with the Santa Cruz Biotechnology troubleshooting section yielded a number of possible ways to increase transfection efficiency. One suggestion was to increase transfection time but as a 24 hour interval has been sufficient previously for knockdown efficiency at the mRNA level, the time interval for quantifying efficiency at the protein level was increased instead (4 days to 6 days). In addition, cells were plated at a concentration of 200,000 cells/well in 2mL of media in a 6-well dish. siRNA transfection concentration was also increased from 25nM to 40nM.

Cell viability post-transfection was assessed after staining with Trypan blue dye and counting on a hemocytometer. There were no significant differences in cell

viability between the scrambled control and Akt 3 knockdown cells though both treatment conditions began to show reduced cell viability after day 4 (Figure 16).

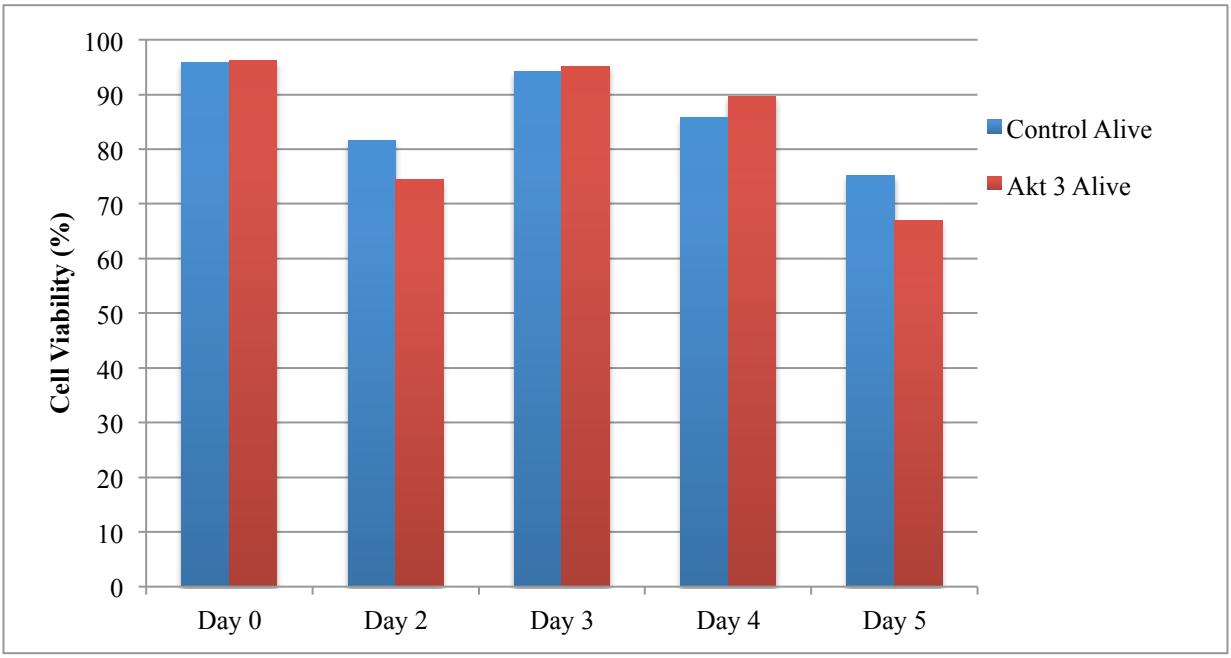


**Figure 16. Cell viability of SUM149 cells post-transfection.** No differences in cell viability between the two treatment conditions over the time interval.

siRNA efficiency was verified by conducting western blot analysis using anti-Akt 3 antibody and Pan Actin antibody (Figure 17). The transfection proved to be unsuccessful again. Though the Akt 3 protein expression decreased over time (except on day 2, where it was up-regulated) for the Akt 3 knockdown condition, the scrambled control condition also exhibited a similar pattern. Probing with Pan Actin revealed that it was not a loading problem as the wells were all loaded equally.

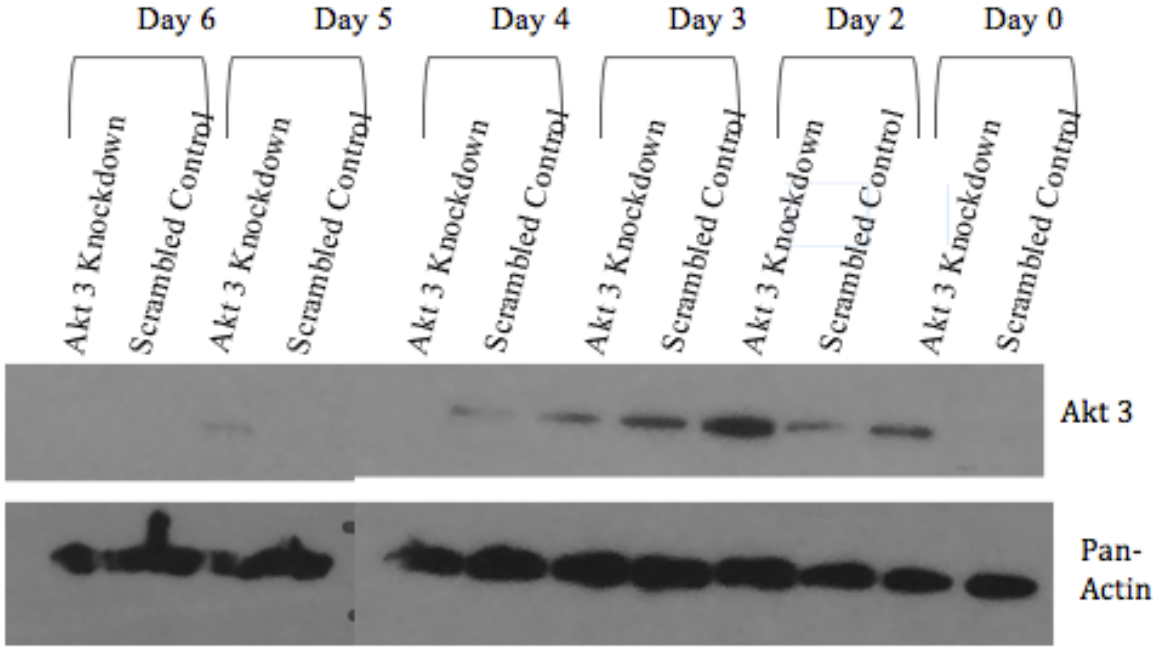


**Figure 17. Western blot for Akt 3 and Pan Actin.** Akt 3 knockdown was once again unsuccessful.

### 3.4 Perifosine in Combination with Crenolanib

Given the effectiveness of Perifosine in reducing cell proliferation in SUM149 cells, another inhibitor was examined to determine whether combination with Perifosine would yield better results. Combination of two inhibitors would be advantageous in therapeutics as lower concentrations of individual drugs could be potentially used in treating patients resulting in fewer side effects. PDGF-inhibitor Crenolanib was used to determine its role, if any, in inhibiting cell proliferation. Post treatment with Perifosine or Crenolanib, SUM149 cells were immunoprecipitated with

Akt total followed by immunoblotting with Akt-Phospho-473 and Akt total. The immunoblotting wells (left) were used as a control to determine whether the immunoprecipitation (right) worked appropriately. Both immunoblotting and immunoprecipitation wells demonstrated that Perifosine completely inhibited Akt activation while Crenolanib did not inhibit Akt activation to any degree.

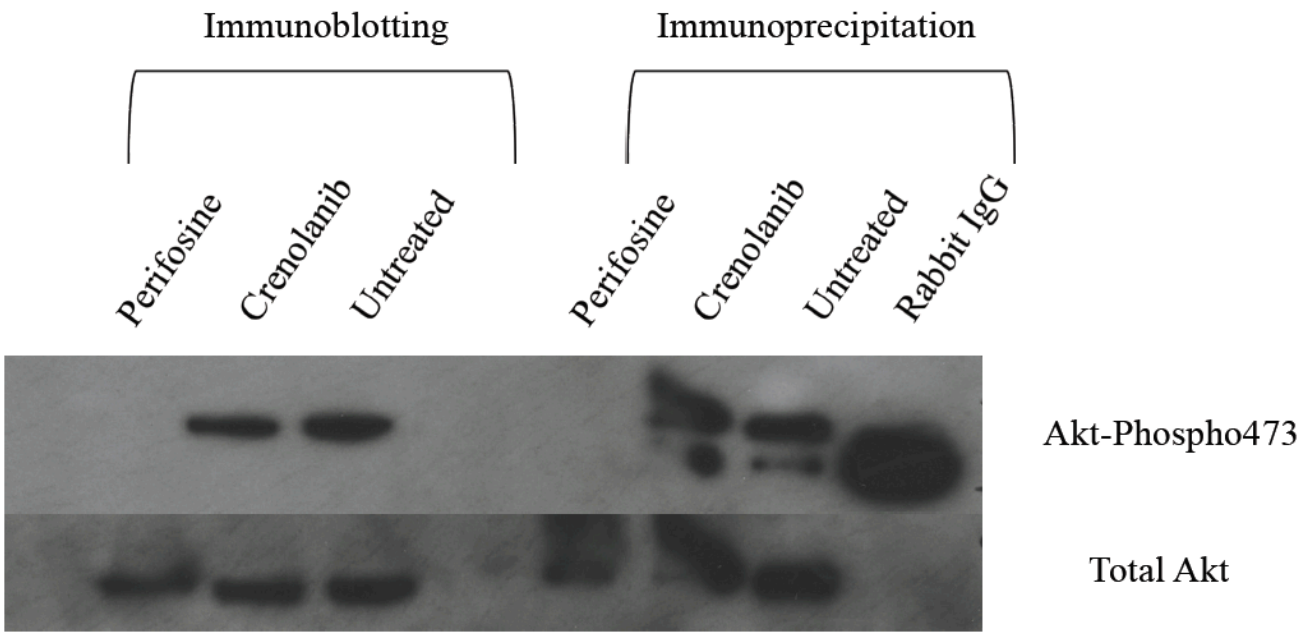


**Figure 18. Immunoprecipitation of Total Akt following inhibitor treatment.** Akt phosphorylation was clearly inhibited by Perifosine treatment. Total Akt was used as a loading control to demonstrate equal protein was loaded in each well.

### 3.4.1 Cell Viability Post Inhibitor Treatment

Perifosine was shown (as seen in Figures 8, 9b) to reduce IBC cell proliferation but the effect of Crenolanib on cell proliferation in comparison to

Perifosine and the combination of Perifosine and Crenolanib inhibitors were not known. Cells were plated at a concentration of 4000 cells/well in 200 µl of media in a 96-well dish in replicates of three. Cells were treated with Perifosine (12.5 µM), Crenolanib (2 µM), Perifosine and Crenolanib at high concentration (12.5 µM and 2 µM respectively) or low concentration (6.25 µM and 1 µM respectively) over 5 days. Figures 11 and 12 display the same data formatted differently for ease of accessibility. Treatment with Perifosine alone appears to reduce cell proliferation the most. Treatment with Perifosine worked the earliest though the other treatment conditions were also successful at inhibiting proliferation but take longer to achieve the same results. Crenolanib alone was the least effective treatment condition in reducing cell proliferation.

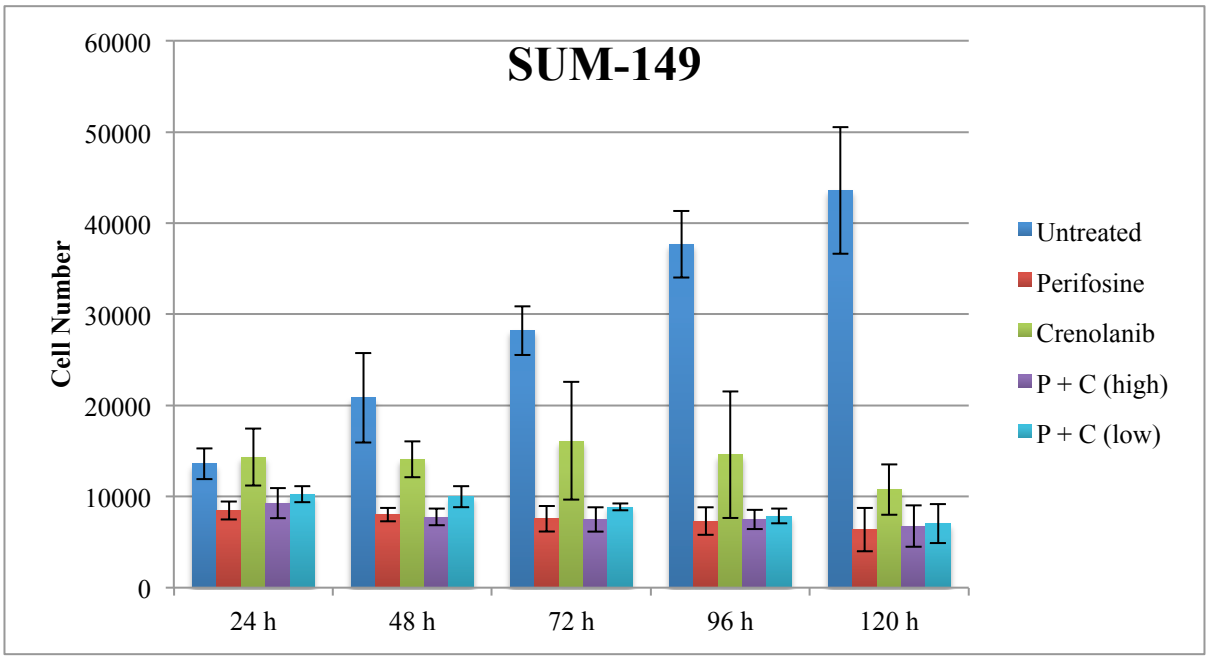

**Figure 19. SUM149 cells following treatment with various inhibitors.** P + C (high) refers to combination of the two inhibitors at a high concentration (12.5 µM Perifosine and 2 µM Crenolanib) and P + C (low) refers to combination of the two inhibitors at a low concentration (6.25 µM Perifosine and 1 µM Crenolanib).

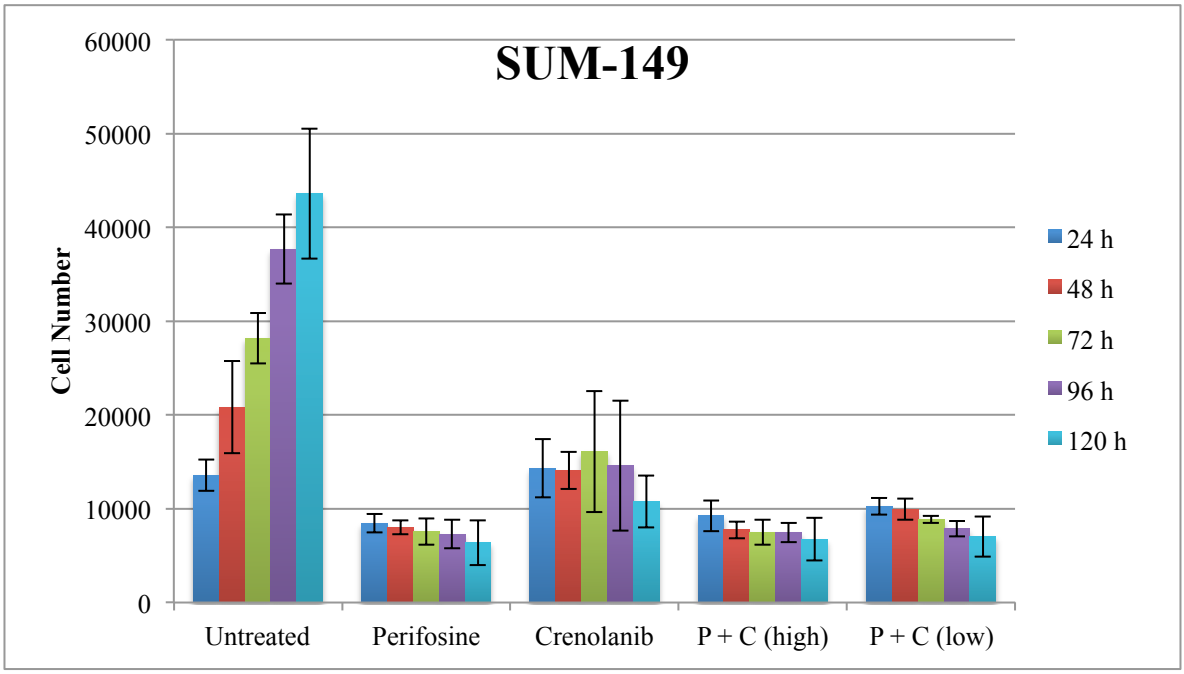


**Figure 20. SUM149 cells following treatment with various inhibitors.** P + C (high) refers to combination of the two inhibitors at a high concentration (12.5 µM Perifosine and 2 µM Crenolanib) and P + C (low) refers to combination of the two inhibitors at a low concentration (6.25 µM Perifosine and 1 µM Crenolanib).

### 3.4.2 Western Blot Analyses Post Inhibitor Treatment

Western blot analyses were conducted to determine when Perifosine begins to inhibit Akt phosphorylation and to assess whether Crenolanib in combination with

Perifosine inhibited Akt activation. Cells were collected 1, 8 and 12 hours post treatment and probed with Akt-Phospho473 to assess Akt inhibition and with Pan Actin to ensure equal protein was loaded in each well. An hour following treatment did not reveal any differences in Akt phosphorylation in any of the treatment conditions. 8 hours post treatment revealed inhibition of Akt phosphorylation in Perifosine treated cells compared to untreated cells. 12 hours post treatment revealed inhibition of Akt phosphorylation in Perifosine and Crenolanib in combination with Perifosine at high and low concentrations. Pan Actin blots revealed equal protein loadings in all the wells (Figure 21). Therefore, differences observed in Akt phosphorylation were due to the specific treatment conditions.

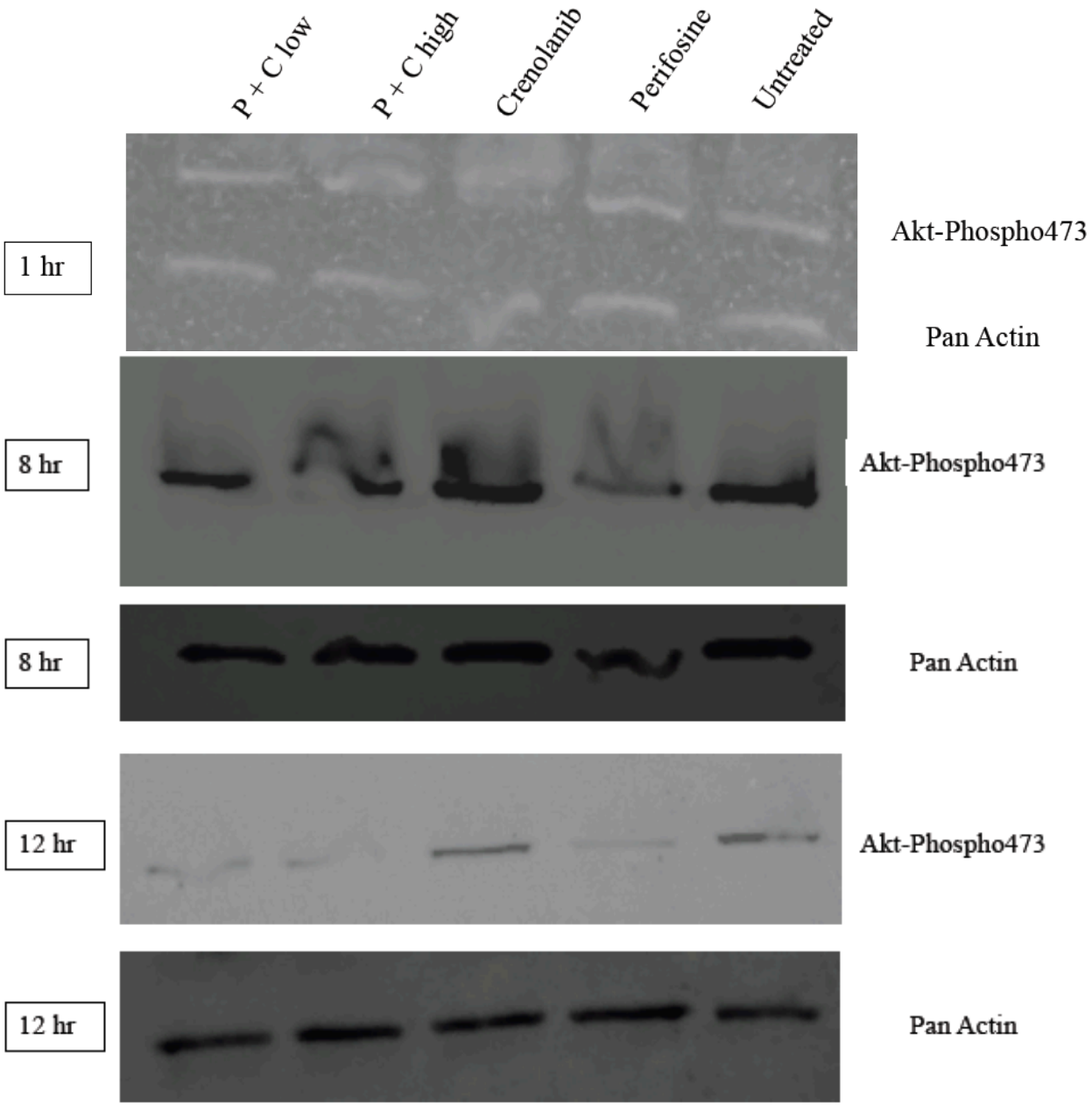


**Figure 21. Western blot analyses on SUM149 cells at different time points.** Perifosine (12.5 μM) inhibits Akt phosphorylation by 8 hours post treatment while Crenolanib (2 μM) in combination with Perifosine both at high (12.5 μM Perifosine and 2 μM Crenolanib) and low (6.25 μM Perifosine and 1 μM Crenolanib) concentrations inhibit Akt phosphorylation by 12 hours post treatment.

### 3.5 Migration in SUM-149 and MDA-231 Cells Post Inhibitor Treatment

Scratch wound assays on IBC and non-IBC cells post treatment with Perifosine, Crenolanib and combination of both inhibitors at high and low concentrations was assessed 0, 4, 8 and 24 hours after the initial 13-hour treatment with appropriate inhibitor(s).

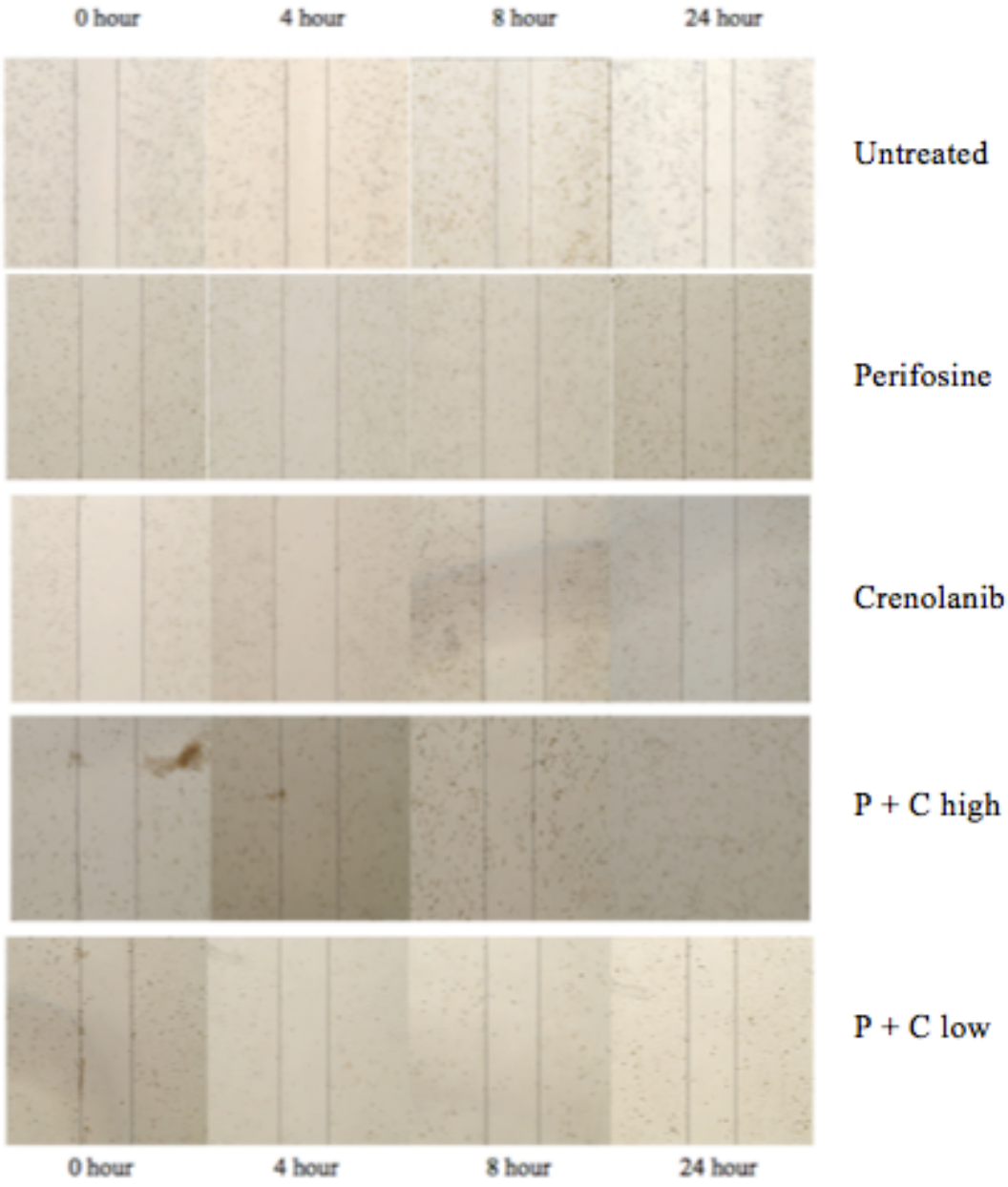


**Figure 22. Scratch wound assays of SUM-149 cells post inhibitor treatment over a 24-hour interval.** Perifosine was used at a concentration of 12.5 μM when used alone and at a high concentration in combination with Crenolanib. In the low concentration combination treatment, Perifosine was used at final concentration of 6.25 μM. Crenolanib when used alone and in the high concentration combination treatment was 2 μM and 1 μM in the low concentration combination condition.

Perifosine appeared to not play a role in inhibiting migration of SUM-149 cells as the migration rate in this condition paralleled untreated cells (Table 3, Figure 23). Perifosine in combination with Crenolanib at the high concentration proved to be the worst treatment condition for inhibiting migration, as in 24 hours, the wound was completely closed. Crenolanib alone was the best treatment condition in inhibiting migration of IBC cell lines.

**Table 3. Average migration rates in SUM-149 cells post treatment with various inhibitor(s).**

| | **Untreated** (%) | **Perifosine** (%) | **Crenolanib** (%) | **P + C high** (%) | **P + C low** (%) |
|---|---|---|---|---|---|
| **0 hour** | 0 | 0 | 0 | 0 | 0 |
| **4 hour** | 6.756223437 | 9.744891589 | 4.574299637 | 13.96069473 | 4.881688892 |
| **8 hour** | 16.43464469 | 23.14071852 | 7.050272816 | 31.11853084 | 8.889090125 |
| **24 hour** | 27.32372228 | 30.14436123 | 8.395565056 | 100 | 22.191676 |

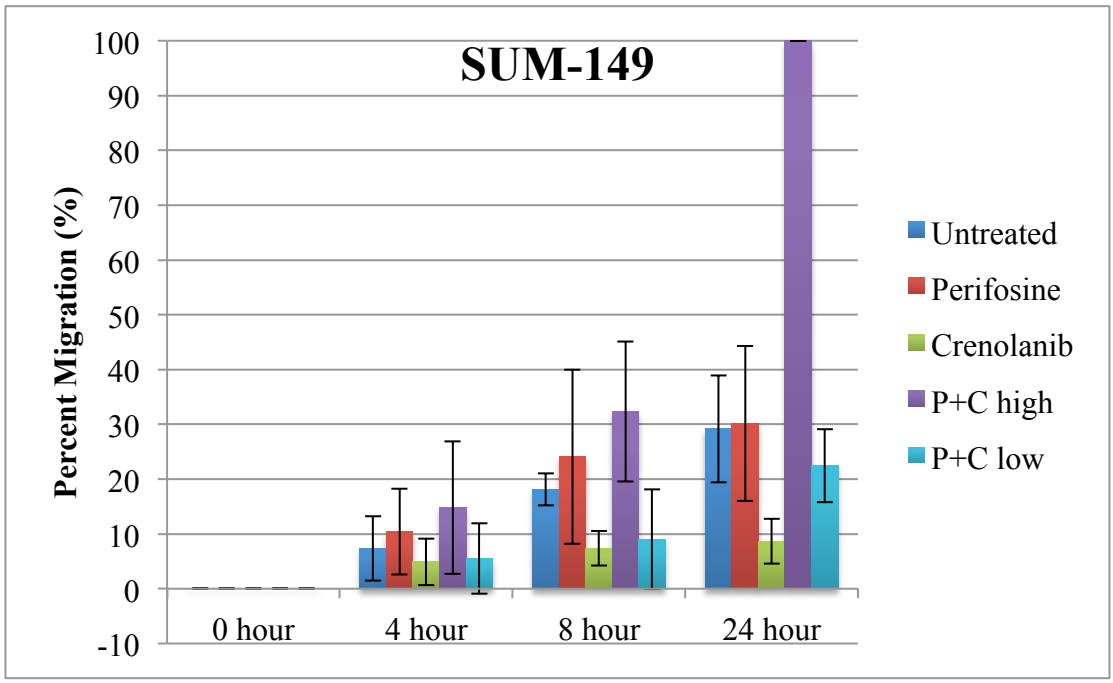


**Figure 23. Average migration rate in SUM149 cells.** Crenolanib treated cells were the best at inhibiting migration while combination of Perifosine and Crenolanib at a high concentration was the worst treatment condition for inhibiting migration.

The effects of Perifosine and Crenolanib, alone and in combination, on migration were also assessed in non-IBC cell line, MDA-231 and compared with the results of IBC cells post inhibitor treatment.

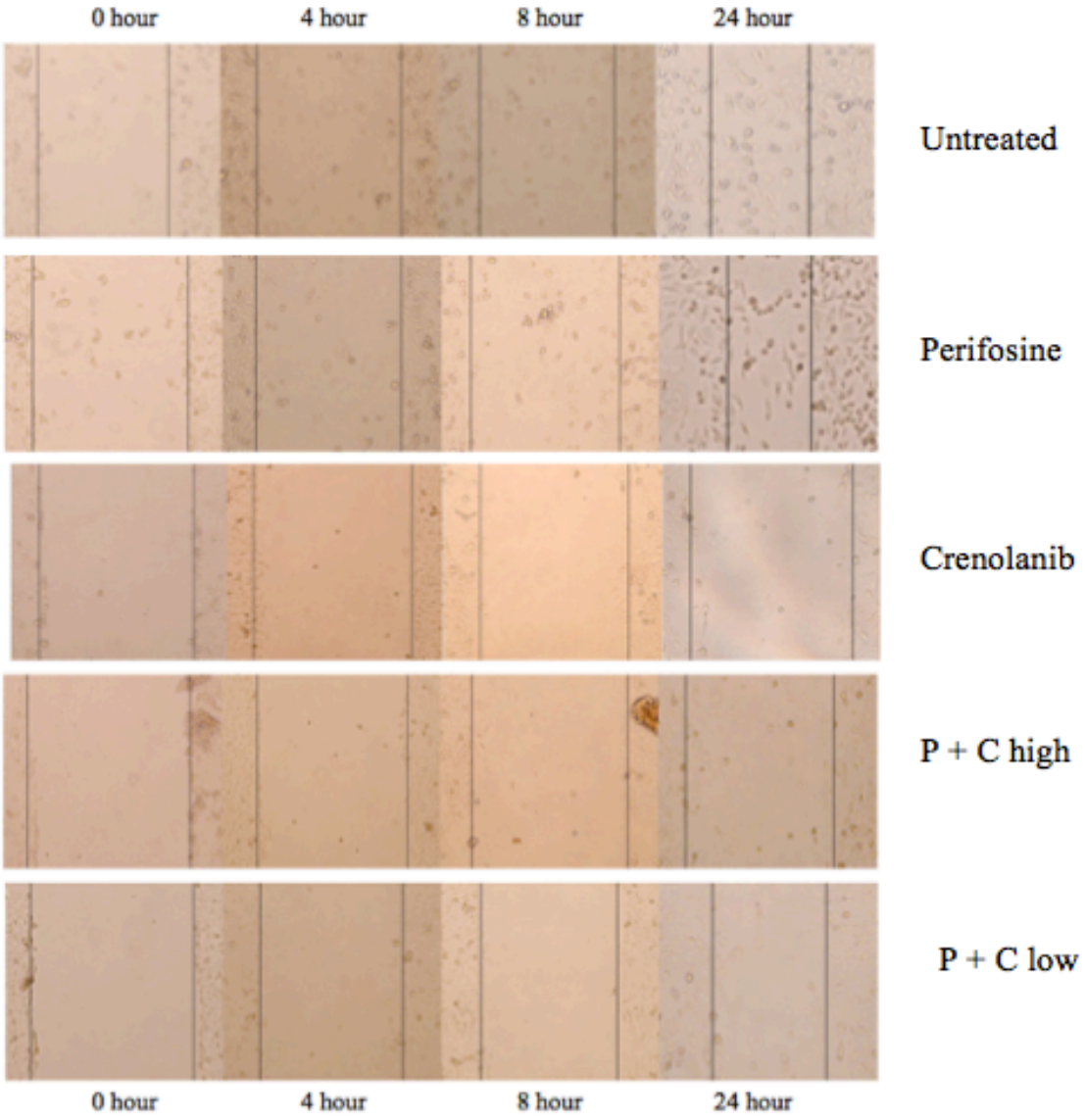


**Figure 24. Scratch wound assays of MDA-231 cells post inhibitor treatment over a 24-hour interval.** Perifosine was used at a concentration of 12.5 µM when used alone and at a high concentration in combination with Crenolanib. In the low concentration combination treatment, Perifosine was used at final concentration of 6.25 µM. Crenolanib when used alone and in the high concentration combination treatment was 2 µM and 1 µM in the low concentration combination condition.

Similar to SUM-149 cells, Perifosine alone did not appear to play a role in inhibiting migration while Crenolanib proved to be the best drug in inhibiting migration in MDA-231 cells. Perifosine and Crenolanib in combination at a high concentration was comparable with Crenolanib in inhibiting migration while the combination of the two drugs at a low concentration proved to be worse than the higher concentration at inhibiting migration though some inhibition did occur (Table 4, Figure 25).

**Table 4. Average migration rates in MDA-231 cells post treatment with various inhibitor(s)**

| | **Untreated** (%) | **Perifosine** (%) | **Crenolanib** (%) | **P + C high** (%) | **P + C low** (%) |
|---|---|---|---|---|---|
| **0 hour** | 0 | 0 | 0 | 0 | 0 |
| **4 hour** | 6.247367634 | 3.854457247 | 3.054684569 | 6.307970946 | 11.42678223 |
| **8 hour** | 16.1656986 | 15.15226859 | 6.787897601 | 13.65955291 | 25.18119713 |
| **24 hour** | 58.3094652 | 59.12053219 | 26.49853918 | 32.91057611 | 46.93195206 |

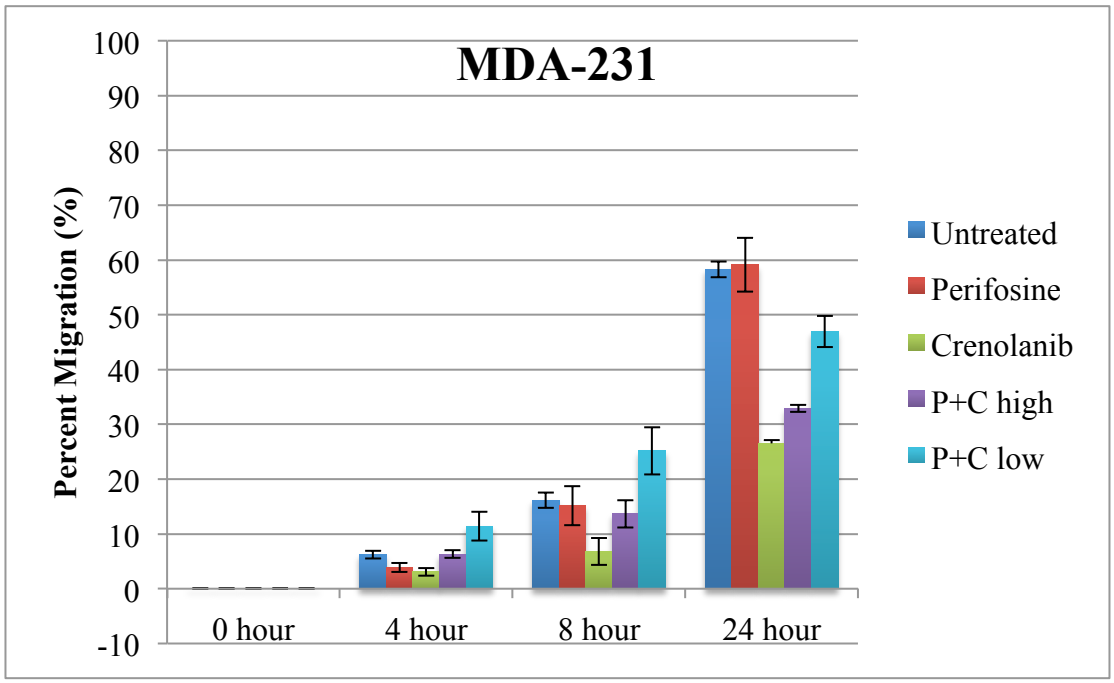


**Figure 25. Average migration rate in MDA-231 cells.** Crenolanib treated cells were the best at inhibiting migration while Perifosine alone was the worst treatment condition for inhibiting migration.

# Chapter 4

# DISCUSSION

## 4.1 Role of Akt in IBC Cell Proliferation

Akt inhibitor, Perifosine, preferentially reduced IBC cell proliferation over non-IBC cell proliferation as IBC SUM-149 cells responded earlier to Perifosine treatment as well as exhibited increased sensitivity to inhibition of proliferation at lower concentrations than non-IBC MDA-231 cells. Western blot analyses revealed that Akt phosphorylation was up-regulated in IBC cell line compared to non-IBC cell line and treatment with Perifosine inhibited Akt activation in both cell lines. Inherent greater Akt phosphorylation exhibited by SUM-149 cells could explain why Perifosine, an Akt inhibitor, worked better in SUM-149 cells compared to MDA-231 cells.

While it was demonstrated that Perifosine inhibits proliferation through the Akt pathway, Crenolanib does not appear to exert its effects through the Akt pathway. Though Crenolanib alone was less successful than Perifosine alone or in combination with Perifosine, it did inhibit IBC cell proliferation. However, this proliferation was due to a different mechanism as Western blot analyses conducted post Crenolanib treatment revealed that Crenolanib treatment did not inhibit Akt activation. It should be noted that Perifosine in combination with Crenolanib at both concentrations were better at inhibiting IBC cell proliferation than Crenolanib alone though Perifosine when used alone remained the best treatment in reducing IBC proliferation. It appears

that when used in combination, Perifosine exerts a greater role than Crenolanib in inhibition of IBC cell proliferation.

### 4.2 Role of Akt in IBC and non-IBC Cell Migration

The scratch wound assays demonstrated that Akt did not appear to play a role in regulating migration of IBC (and non-IBC cells) and further confirmed the notion that Crenolanib did not exert its effects through the Akt pathway. For both cell lines, Perifosine treatment alone did not result in significant differences in inhibition of migration compared to untreated condition. In the IBC cell line, Perifosine treated cells had an average migration rate of 30% compared to untreated cells, which had an average migration rate of 27%. Perifosine was no worse at inhibiting migration in non-IBC cell line MDA-231 as Perifosine treated cells exhibited an average migration rate of 59% compared to 58% in untreated cells. In addition, Crenolanib treatment was better at reducing migration of IBC and non-IBC cells. When Perifosine and Crenolanib were used in combination, they proved to have opposite effects in different cell lines. In IBC cell line, the combination of both drugs at a high concentration served to enhance migratory potential of the cells such that in 24 hours, the wound was completely healed. In contrast, the combination of both drugs at a high concentration was only slightly worse than Crenolanib at inhibiting migration of non-IBC cells. The lower concentration combination condition did somewhat inhibit migration in both cell lines compared to Perifosine treatment but it was worse than the higher concentration combination condition in non-IBC cells.

These results clearly demonstrate that the Akt inhibitor Perifosine does not halt migration in IBC and non-IBC cells, which suggests that Akt inhibition alone does not play a role in migration of IBC and non-IBC. Perifosine also appears to reverse the

inhibitory migratory effects of Crenolanib in IBC cells when used at a high concentration with Crenolanib. It has previously been shown that overexpression of RhoC GTPase in IBC cell lines is responsible for invasion (Lehman and Van Laere 2012). Inhibition of Akt activity, via pharmacological inhibitors or siRNA against Akt isoforms, has been correlated with decreased RhoC mediated invasion (Unger and van Golen 2010). Akt inhibitor Perifosine, while decreasing proliferation in both IBC and non-IBC cell lines, does not appear to work through the RhoC mechanism as migration was not significantly inhibited compared to the untreated condition. Any effect Perifosine has in inhibiting IBC growth *in vivo* remains restricted to inhibition of proliferation alone.

Since Crenolanib is effective at inhibiting migration of both cell lines while Akt inhibitor Perifosine alone does not affect migration rate of either cell line, it offers further evidence for Crenolanib exerting its effects via a different pathway. The success of Crenolanib in inhibiting migration in IBC and non-IBC cells suggests a closer examination of PDGF signaling in IBC and other cancers is needed. There is some evidence that increased PDGF signaling is involved in many cancers. PDGFRβ expression has been identified as being up-regulated in prostate cancer while inhibition of PDGFR has led to decreased cell migration in liver cancer (Heldin 2013). Though up-regulation of PDGFR has not been definitively identified in breast cancer or IBC, breast cancer cells are known to express PDGF and PDGFR in stromal cells (Heldin 2013). During epithelial-mesenchymal transition (EMT), PDGF expression can increase in tumor cells causing previously unresponsive cells to become increasingly sensitive to PDGF stimulation, which could lead to greater proliferation and migration (Heldin 2013). As such, it is important to identify PDGFR inhibitors

that could aid in reducing IBC proliferation and migration. Crenolanib has not yet been studied extensively though preliminary studies have reported limits on its efficacy due to vomiting and nausea experienced by patients (Lewis et al. 2009). There have been few combination studies done with Crenolanib but some success has been reported with use of Crenolanib in combination with axitinib (VEGFR inhibitor) and docetaxel when given to patients with advanced tumors (ranging from lung cancer to sarcoma to prostate cancer) in terms of disease stability and tolerability (Michael et al. 2010).

It should also be noted that the non-IBC cell line MDA-231 exhibited a higher migration rate under untreated conditions compared to the IBC SUM-149 cell line (58% in MDA-231 versus 27% in SUM-149), though IBC is more aggressive and has a higher probability of metastasis than other breast cancers (Kleer et al. 2000). Another study reported similar results where IBC cell line SUM-149 proved to be less invasive than non-IBC cell line SUM-102 (Hoffmeyer et al. 2005).

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