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THE EFFECT OF TRP CHANNEL INHIBITORS ON PROSTATE CANCER CELL (PC3) MIGRATION AND APOPTOSIS

by

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A THESIS

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DEDICATION

I would like to dedicate this to my mom, Patricia Young, for being there for me every step of the way. She has always been there throughout my whole life and I really appreciate her love and support. If it wasn't for her I would not know where I would be in life.

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Thank you

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ABSTRACT

Tumor cell metastasis to distant parts of the body is a process that is still poorly understood. Many people who develop cancer do not die from the primary tumor, but instead die from metastatic tumor growth. Primary tumors are often easily removed from the body, although cancer cells that are not visible to the naked eye may already have metastasized through blood circulation. In prostate cancer, one of the prominent areas of metastasis is to bone. Since Transient Receptor Potential Vanilloid (TRPV) calcium ion channels are expressed in all bone cancer cell lines, the expression of TRPV channels in prostate cancer cells may be involved in prostate cancer cell migration through survival within the bone. We hypothesize that TRPV channel activity promotes migration and cellular survival of tumor cells within bone; therefore, inhibition of TRPV channels with ruthenium red will decrease prostate cancer cell migration and cellular survival. Our data indicate that broad inhibition of the TRPV channels with Ruthenium Red decreases the amount of migration, and this is likely through the regulation of MAPK activity. The activation of MAPK leads to migration of cells. In contrast, TRPV channel inhibition has no effect on the Akt pathway, which is responsible for cellular survival when activated. In addition, the insulin-like growth factor (IGF) pathway, highly expressed in both bone and prostate cancer cells, may modulate TRPV expression and/or activity.

TABLE OF CONTENTS

LIST OF FIGURES

Pages

ABBREVIATIONS

Akt: Protein Kinase B

BAD: Proapoptotic member of Bcl-2 family

BCL-X: B- cell lymphoma extra large

BCL-2: B-cell lymphoma 2

Ca^{2+}: Calcium

CREB: cAMP response element-binding protein

FKHR: Forkhead in rhabdomyoarcoma,

HCC: Hepatocellular Carcinoma Cells

ICC: Immunocytochemistry

IGF-1: Insulin-like Growth Factor 1

IGF-2: Insulin-like Growth Factor 2

IGF-IR: IGF Receptor

MAP Kinase: ERK, MEK, or p44/42 MAP Kinase

PI3 Kinase: Phosphoinositide 3-kinase

PC3: Prostate cancer cell line

Protein Kinase B: Akt.

RR: Ruthenium Red

TRP: Transient Receptor Potential

TRPV1: Transient Receptor Potential Vanilloid 1

TRPV2: Transient Receptor Potential Vanilloid 2

TRPV6: Transient Receptor Potential Vanilloid 6

TRPM8: Transient Receptor Melastatin 8

CHAPTER 1: INTRODUCTION

Prostate cancer is the second leading cause of death for men in the United States; therefore, research in prostate cancer is very important (Siegel, R., et. al., 2014). Prostate cancer is a major health concern for African American men because they have the highest incidence rate, at 220 per 100,000 population, compared to other races. Hispanics have the second highest incidence rate at 138.6 per 100,000 population. Certain areas such as the District of Columbia have higher incidence rates of prostate cancer, which reflect differences in PSA testing prevalence as well as racial distribution compared to Arizona (Siegel, R., et. al., 2014). Incidence rates of prostate cancer are higher among agricultural workers due to exposure to many chemicals, sunlight, viruses, and pesticides (Koutros, S., et al., 1997). Research shows the overall cancer incidence rates decrease if the rate of smoking decreases and physical activity increases (Siegel, R., et. al., 2014).

It is estimated that in 2014, there will be 233,000 new cases of prostate cancer, which makes up 27 percent of all cancer cases (Siegel, R., et. al., 2014). It is also estimated that 29,480 prostate cancer related deaths will occur in 2014 (Siegel, R., et. al., 2014). That makes ten percent of all cancer related deaths (Siegel, R., et. al., 2014). Prostate cancer is rare in Asia, Africa, and also Latin America, showing that environmental factors play a role in prostate cancer formation (Pan, J., et. al., 2014).

Survival rates of prostate cancer depend on the stage of the cancer at the time of diagnosis. Stages include cancer that is localized, regional, or distant. Localized cancer, or cancer that has remained in the tissue it originated, is referred to as stage one cancer. The five-year survival rate at the localized stage is 100 percent. Unfortunately, the 5-year survival rate for all cancers in African Americans are lower compared to Caucasians. African Americans are also less likely to be diagnosed with cancer at the localized stage. This is unfortunate due to most successful treatments of localized cancer (Siegel, R., et. al., 2014). Regional cancer, or cancer that has spread to regional lymph nodes, also has a 100 percent five-year survival rate. Tumors that have spread to organs far away from primary cancer is known as distant cancer.. Men with metastatic prostate cancer have only a 28 percent five year-survival rate. Eighty-one percent of patients are diagnosed with localized cancer, 12 percent with regional cancer, and four percent of people with prostate cancer are diagnosed with metastatic cancer (Siegel, R., et. al., 2014).

There are different age groups that usually have different percentages of new cases each year. Men who are over the age of 60 usually have the highest risk of developing prostate cancer compared to any other group (Siegel, R., et. al., 2014).

CHAPTER 2: LITERATURE REVIEW

The prostate is a gland that is located in front of the rectum and below the bladder in men, and is responsible for coating sperm with semen. Hormones, such as testosterone, cause the prostate to begin growing before the male is born and continue to grow until the male becomes an adult. As long as there are normal levels of male hormones present, the prostate gland remains normal in size.

The prostate gland consists of different tissue types, including fibro-muscular tissue, exocrine gland tissue, smooth muscle, dense irregular connective tissue, and glandular tissue. Three types of epithelial cells are basal, TA/I, and apical/luminal (Bidaux, G., et. al., 2007). Fibro-muscular tissue consists of smooth muscle tissue and forms the outermost layer of the prostate as well as the tissue that is around the urethra. The prostate also consists of dense irregular connective tissue, which consists of collagen fibers. Prostate cancer arises in the glandular tissue that is androgen dependent and is known as adenocarcinoma (Hägglöf, C., et. al., 2010). Prostate carcinoma occurs primarily in the peripheral zone of the prostate. Once the prostate cancer differentiates, the cancer can become androgen independent, meaning it does not rely on the presence of androgens to grow and divide. The cancer can change enough to spread to distant organs of the body by

receiving cues migrating to better areas for survival (Bidaux, G., et. al., 2007). During metastasis cells invade the blood vessels, and other organs. Invasion includes migration of cells through extracellular matrix, migration to surrounding stromal cells, intravasation to blood capillaries, survival in circulation, extravasation, colonization, and proliferation to distant tissue.

Based on the cancer's ability to metastasize to different organs of the body, there is a Gleason scoring system created so that it could be understood how likely cancer is able to spread to a different region of the body (Figure 1) (Humphrey, P., 2004). According to the Gleason scoring system, stage one of prostate cancer is less likely to spread to distant organs, and as the stages of prostate cancer increase, the likelihood of prostate cancer metastasizing increases (Lepor, H., et. al., 2014). One prominent area that prostate cancer tends to metastasize to are the bones (Humphrey, P., 2004).

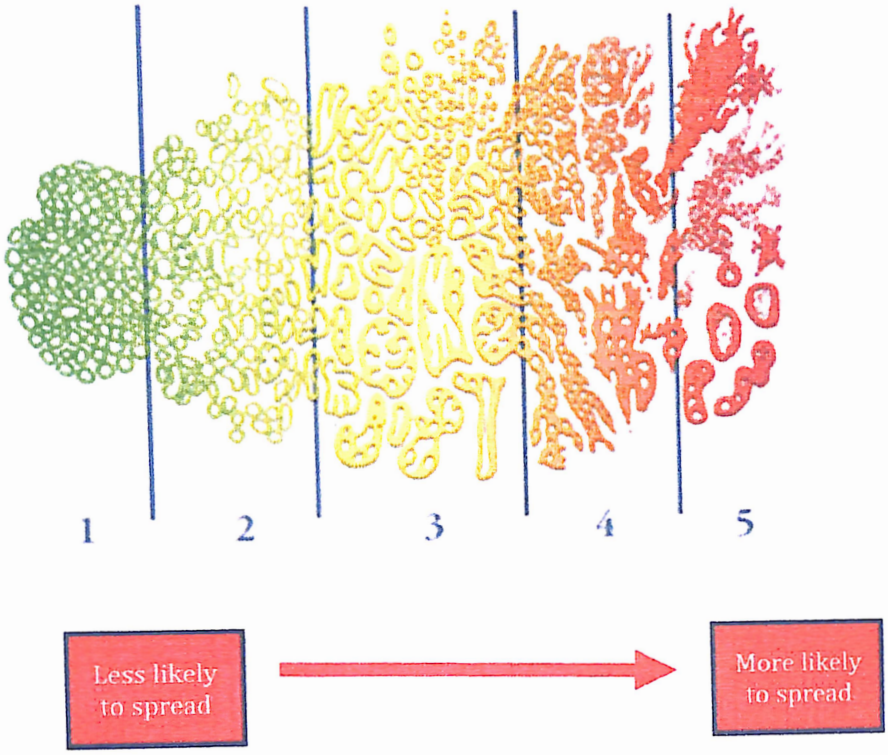


Figure 1: The different stages of prostate cancer that correlates with the Gleason score. The Gleason scoring system is used to measure how aggressive prostate cancer is. At stage one, the cancer is not very aggressive and displays low possibility of prostate cancer metastasis. As the stages of aggressiveness increases, the likeliness of cancer metastasizing increases. This figure is from Stjohnprovidence.org (Providence Prostate Cancer Evaluation, 2014).

Once prostate cancer becomes metastatic, it is able to invade the blood vessels and is then able to travel to distant organs including the bone (Brodland, W., et. al., 2012). Prostate cancer metastases to bone occur in several typical sites, such as the spine, ribs, pelvis, skull, and the upper bones of the arms and legs (Figure 2) (Buga, S., et. al., 2011). Prostatic adenocarcinomas are able to produce osteoblastic metastasis. Osteoblasts are responsible for laying down new bone. PC3 cell-induced osteoblast proliferation may be caused by different growth factors. If they do not differentiate to osteocytes, they will

continue to proliferate uncontrollably (Figure 3) (Perkel, V., et al., 1990). Calcium signaling in osteoblasts is still being studied extensively. Osteoblasts do in fact express the TRP family Ca^{2+} channels, and calcium waves are greater in differentiated osteoblasts due to other families of voltage-gated ion channels (Lieben, L., et. al., 2012). Ca^{2+} signaling is also involved in osteoblast differentiation. Ca^{2+} signaling in chondrocytes include the expression of several calcium channels including TRPV4. Chondrocytes secrete a matrix of cartilage and become embedded in it. In order to activate the Ca^{2+}/calmodulin signaling cascade that promotes chondrocyte differentiation, calcium influx via TRPV4 is required (Lieben, L.,et. al., 2012).

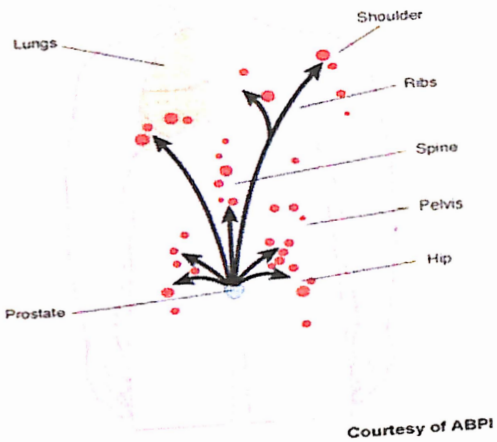


Figure 2: Prostate cancer metastasis to the bones. This occurs after cancer becomes aggressive. Prostate cancer usually comes in contact with multiple organs of the body, but tend to be more attracted to the bones than anywhere else. This is due to the bones' nutrient rich area red bone marrow. This figure is from drugdevelopmenttechnology.com (Zytiga (arbiraterone acetate)-Treatment of Metastatic Castration-Resistant Prostate Cancer (mCRPC), United States of America, 2014).

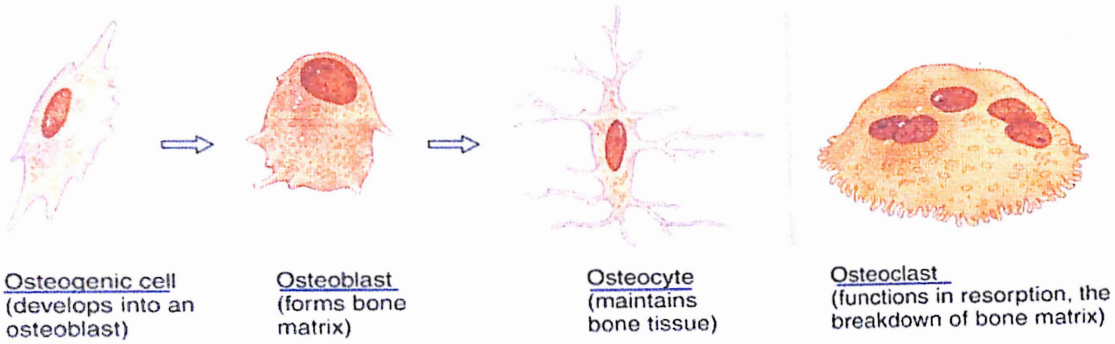


Figure 3: Osteoblasts differentiation. Osteoblasts are able to form bone matrix and can differentiate into osteocytes that maintain the bone tissue. Osteocytes are then able to differentiate into osteoclasts that function in resorption and breakdown of the bone matrix This figure is from quizlet.com (Skeletal System, 2014).

Once cancerous cells reach the bone, they enter the bone tissue through selective adhesion and extravasation across the endothelial cell layer lining the bone marrow sinusoids and gain access to the bone matrix (Figure 4) (Vinik, A., 2008). Prior to this, primary malignant neoplasms form followed by new blood vessel formation (Brodland, W., et. al., 2012). Cells receive cues such as signaling molecules that result in invasion of blood vessels to metastasize to distant organs of the body. The Paget seed and soil theory states that the interactions between tumors and stromal cells help metastasis occur (Fidler, I., 2003). These interactions can be through growth factors, cytokines, chemokines, and signaling molecules that play a role in survival and growth of tumor cells (Hung, T., et. al., 2011). The interactions cause cells spreading to distant areas of the body to be more attracted to certain areas based on their ability to survive there. An example is when prostate cancer metastasizes it normally is attracted to the bone over other organs due to

the bones nutrient rich area that will support prostate cancers survival there (Rahim, F., et al., 2014).

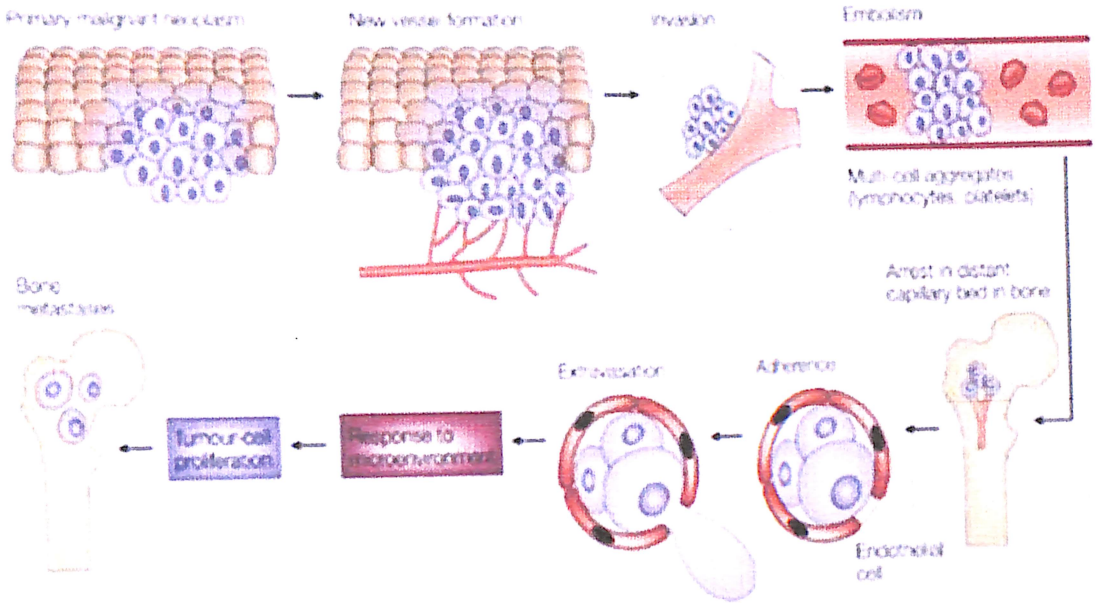


Figure 4: Bone Metastasis Mechanism. This figure shows how cancer develops in the primary region and changes so that it is able to metastasize to other organs in the body. The cancer cells are able to invade blood vessels and move to the bone tissue where it is able to reside there and grow and divide. People first believed that cancer cells that have invaded the blood vessels and travel through circulation were able to reside in the first set of organs it came in contact with. They later found that this was not true. The seed and paget theory explains how cancer cells actually spread to areas where they can best survive in based on the conditions of the organ. This figure is from lib.znate.ru (Carcinoid Bone metastasis-osteolytic and osteoblastic, 2012).

When cancer cells spread to the bone, they disrupt that balance causing cancer or metastatic niche (Roato, L., et. al., 2008). In bone, there is marrow tissue that include both red and yellow marrow. The yellow marrow contain fat cells and the red marrow, which is the common metastasis site. This site also contains hematopoietic stem cells. It makes sense

for prostate cancer cells to metastasize to the bone, due to its unique biological properties including staying, surviving, and proliferating circulatory cells (Rahim, F., et. al., 2014). The seed and soil theory explains the metastasis of prostate cancer because the prostate tumor cells are able to metastasize to the bone due to the conditions in the bone that support prostate cancer cells growth and survival (Vinik, A., 2008). Although bone metastases are treatable, they are not curable, and there is a lot of associated pain.

Pain is commonly due to the tumor pressing against the spinal cord or the nerves in the bone (Morita, K., et. al., 2014). Although it is uncommon, bone pain may also be caused by fractures that are the result of weakened bones due to the invasion of cancer. This may cause bone to be broken down and reabsorbed resulting in bone fractures in men. Men who go through prostate cancer metastasis may develop osteoporosis, which also makes it easier for the person to develop fractures. Men who go through hormone therapy also have an increased risk of developing osteoporosis.

The bone is a huge source of calcium and one mechanism that may be responsible for prostate cancer's attraction to the bone are Transient Receptor Potential Vanilloid (TRPV) channels. TRPV channels are ion channels that are calcium ion channels which are mainly located on the plasma membrane of most cell types (Figure 5) (Lehen'kyi, V., et.al., 2011). These channels are normally activated by osmotic pressure, cold, heat, stress, pain, and even vision in normal cells. Organs that express TRP channels include, lungs, eyes, the brain and the spinal cord, as well as the bone, and other various organs. Recently, TRP channels involvement in cancer progression has been studied. Four channels of

interest involving cell migration as well as cellular survival in cancer cells are TRPV2, TRPV4, TRPV6, and TRPM8 (Haute, C., et. al., 2010).

TRPV2 expression is higher in metastatic cancer patients than in others with tumors (Figure 5). Previous research has shown that TRPV2 is not involved in growth, but it is involved in the migration as well as cellular survival (Haute, C., et. al., 2010). Prostate cancer cells are able to migrate to other parts of the body via the TRPV2 channel when the prostate is stimulated by lysophospholipids (LPI) and lysophosphatidylcholine (LPC) (Monet, M., et. al., 2009). When the channel TRPV2 is activated by LPI and LPC, which are endogenous lysophospholipids that induce a calcium influx in TRPV2 channels, migration of cancer cells is increased (Monet, M., et. al., 2009). TRPV4 is involved in cell migration, but there is not much information readily available on TRPV4's involvement in prostate cancer progression.

TRPV6 has also played a role in cell proliferation and is correlated with the Gleason score (Figure 5). In early stages of prostate cancer, TRPV6 is not expressed in prostate cancer cells, but as prostate cancer progresses, TRPV6 expression increases. In Lymph Node Carcinoma of the Prostate (LNCaP) cells, TRPV6 silencing assays were conducted which led to a decrease in cell viability (Lehen'kyi, V., et al., 2007). LNCaPs are prostate cancer cells that have metastasized to the lymph nodes. TRPV6 in LNCaP cells are suggested to play a role in LNCaP proliferation which is mediated by Ca^{2+} entering the cell. Its involvement also includes activating NFAT or nuclear factor of activated T cells. In this case TRPV6 has increased survival of cells as well as played a role in avoiding

apoptosis. If this is true, using TRPV inhibitors on prostate cancer cell lines may prove that TRPV are important in prostate cancer migration to other areas of the body such as the bone (Lehen'kyi, V., et al., 2007).

TRPM8 is another TRP channel that is involved in cancer cells (Figure 5). This channel belongs to the melastatin subfamily of TRP channels. TRPM8 is a calcium ion channel that is upregulated in prostate cancer cells. (Zang, L., et. al., 2006). TRPM8 is expressed in prostate cancer at high levels and in low levels in normal prostate. (Zang, L., et. al., 2006). TRPM8 is also responsible for cellular migration in PC3 cells (Valero, M., et. al., 2012.).

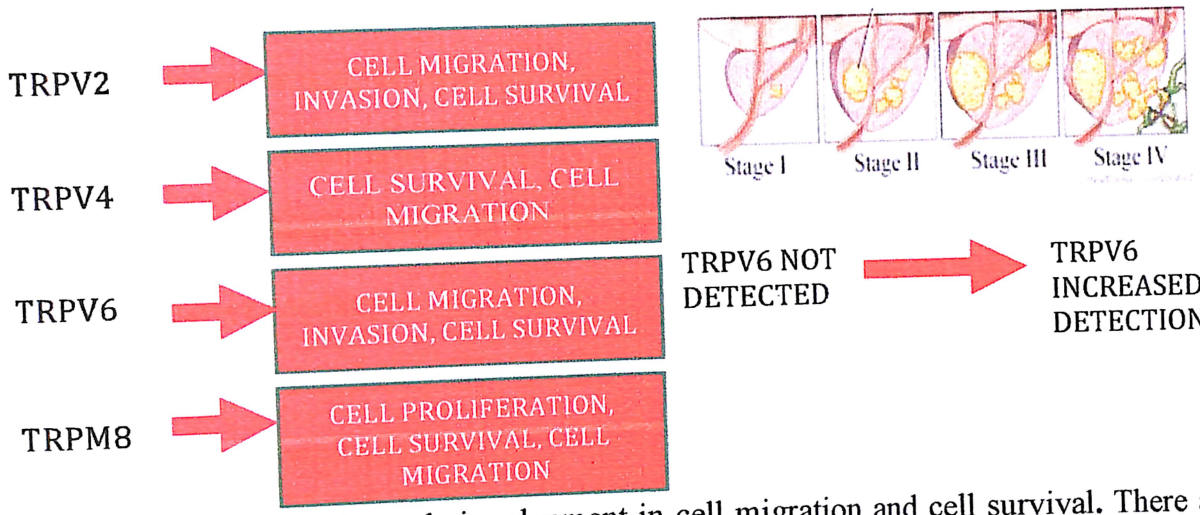


Figure 5: Four TRP channels involvement in cell migration and cell survival. There are four TRP channels of interest in this study due to their association with PC3 cells migratory abilities as well as their ability to survive. The four TRP channels are linked to cancers survival and migratory abilities. TRPV6, was the only found to correlate with the Gleason score system. In stage one of prostate cancer, TRPV6 is undetected, and as the stages of prostate cancer increases, TRPV6 detection increases as well. This figure is from Thermal imaging of the Southwest (New Hope for prostate cancer Detection, 2013).

Recent results show that insulin and IGF can influence ion channel function by phosphorylating and translocating receptors (Tian, Y., et. al., 2013) TRPV1 has increased responsiveness to heat, capsaicin, and pH when in the presence of IGF and insulin. TRPV1 is able to increase sensitivity and lower thresholds. Since TRP channels play a role in both bone and prostate cancer, we are interested to see if TRP channels are the link between prostate cancer metastasis to bone. TRP channels, also known as Ca^{2+} ion channels, have been expressed in bone and play a role in bone homeostasis. Calcium is important in bone formation and 99% of Ca^{2+} is stored in the bones. Osteoblasts, osteoclasts, chondrocytes, and nerve endings receive intracellular Ca^{2+} signaling. The intracellular signaling in each of these areas have been able to regulate multiple functions such as differentiation, signal transduction, and sensing of osmotic, mechanical, and pain stimuli (Lieben, L., 2012).

Studies on Arab populations show low levels of IGF-1 compared to Caucasians who have higher levels of IGF-1(Chan, J., K., et. al., 2013). There are also low levels of IGF-1 in Chinese populations. Both Chinese and Arab populations have low incidence rates of prostate cancer, suggesting that IGF levels may be associated with prostate cancer risks (Pan, J., et. al., 2014). Arab men who are diagnosed with prostate cancer display higher levels of IGF-1 than usual when diagnosed (Chan, J., K., et. al., 2013). The low incidence rates of prostate cancer in Arab populations may be linked to low IGF-1 expression levels. Men who had higher levels of IGF are at higher risk of developing cancer than men who had lower levels of IGF (Chan, J., K., et. al., 2013). Once the IGF receptor is activated by IGF-1, it allows Ca^{2+} to influx into the cell and activates the intracellular pathways

involving protein kinase B (Akt) as well as Mitogen Activated Protein Kinase (MAP kinase), also known as Extracellular Signal-Regulated Kinase (ERK). ERK as well as Akt, are also correlated in the Gleason scoring system because at stage one of prostate cancer, low levels are shown. As the stages of prostate cancer increase so does the expression levels of both Akt and ERK.

ERK is activated in a cellular pathway once Ras, Raf, and then MEK is activated through phosphorylation. ERK, also known as MAPK or mitogen activated protein kinase, plays a role in cell proliferation as well as angiogenesis (Wilhelm, S., et. al., 2004). When Ca^{2+} calcium enters the cell, the Ras-Raf-MEK-ERK pathway is activated. ERK is activated through phosphorylation and then is able to translocate into the nucleus. Once it translocates into the nucleus, ERK is able to act as a transcription factor by binding to DNA and causing cellular migration as well as proliferation. ERK plays a role in many tumor cells and is involved in invasion and metastasis in different cell lines as well such as hepatocellular carcinoma (HCC) cells (Chen, K., et. al., 2013). In other cells, such as nasopharyngeal carcinoma, cell growth, migration and invasion are inhibited by triggering VEGF-A/ERK signaling as well as DNA damage using iodine-125 seed (^{125}I Seed) (Tian, Y., et. al., 2013). For example, when IGF-IR is phosphorylated, the SHC adaptor protein moves to the receptor and is phosphorylated. SHC is then activated and binds to Growth Factor Receptor Bound Protein-2 (GRB2). The SOS protein is then recruited. Ras is activated and a cascade of phosphorylation begins. After Raf is activated, MEK1/2 or MAP

kinase kinase is then activated, and then ERK1/2, or extracellular signal regulated kinases, are activated (Weinberg, R., 2007).

Akt is part of the intracellular pathway that is activated by the IGF receptor after the IGF ligand, such as IGF-1 or IGF-2, binds to the IGF receptor. Akt is also known as Protein kinase B or PKB and is important in prevention of apoptosis when phosphorylated (Figure 6) (Kim, AH., et. al., 2001). Akt is part of the Phosphatidylinositol 3-Kinase (PI_3 kinase) pathway, which is involved in controlling glucose metabolism, protein synthesis, and proliferation (Diehl, N., et. al., 2013). A cell that undergoes cellular death does so by receiving multiple death signals or cues from the environment. Akt is a serine/threonine kinase that responds to growth factor stimulation and Ca^{2+} influx and mediates cell survival (Kim, AH., et. al., 2001).

When calcium enters the cell, PI3 Kinase is activated and then binds to Akt's PH domain. Once this occurs, Akt translocates to the plasma membrane and is then phosphorylated (Badve, S., et. al., 2010). Akt is then able to prevent the cell from going through apoptosis by regulating other downstream pathways (Weinberg, R., 2007). This is important because once Akt is activated, it is able to phosphorylate and inactivate several proteins, including BCL2 Antagonist of cell Death (BAD) that are able to cause the cell to go through apoptosis. When BAD is not phosphorylated, it is seen in the mitochondrial membrane where it is able to prevent BCL2 from stopping apoptosis by keeping the channel on the mitochondrial membrane open to let cytochrome c out (Weinberg, R., 2007). Once cytochrome c moves out of the mitochondria, it is able to aggregate with the

Apaf-1 molecule to form the seven-spoked apoptosome. Caspase-9 is then activated and connects with the Apaf-1 molecule which then activates caspase-3 and caspase-7. Once this occurs, other procaspases become active and death substrates are cleaved. This creates an apoptotic cell phenotype. When BAD is phosphorylated by Akt it is then unable to interact with BCL2. At this point BAD cannot keep the channels on the mitochondrial membrane open so that cytochrome c can move out of the mitochondria to promote apoptosis. Akt is also known to prevent the initiation of the caspase cascade by inactivating Caspase-9 by phosphorylation. Akt also prevents the activity of FOX03a (FKHRL1), FKHR, the forkhead transcription factor family by phosphorylating them. Akt decreases Fas-mediated apoptosis by diminishing the expression of the Fas ligand. Akt has also been known to increase the amount of anti-apoptotic proteins such as BCL2 and BCL-X. A phosphorylation substrate for Akt is mTOR (Weinberg, R., 2007).

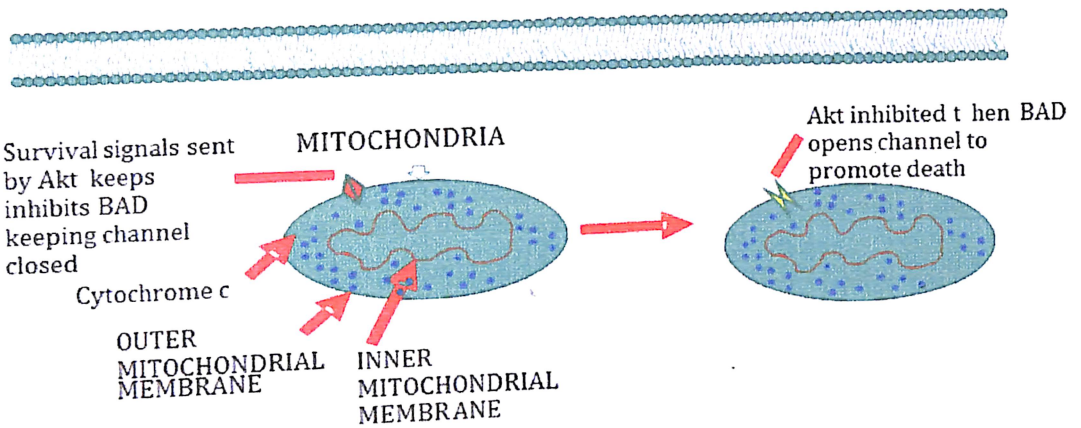


Figure 6: Akts involvement in apoptosis. Akt is essential in the cellular death process because it inhibits BAD when avoiding apoptosis. In normal cellular death, BAD is responsible for keeping the channel on the mitochondrial membrane open, so that it can allow cytochrome c to escape the mitochondria and start apoptosis. Unfortunately in cancer cells such as prostate, Akt is able to manipulate this by inhibiting BAD, and avoiding apoptosis.

The cell line we are interested in is PC3 cells, which have a high metastatic potential that have been isolated from the bone and the brain (Chung, LW., et. al., 1997) (Tai, S., et. al., 201. These cells were then able to metastasize to other areas of the body and became more aggressive (Tai, S., et. al., 2011). PC3 cells were originally isolated from the bone of a 62 year-old male (Chung, LW., et. al., 1997). The cell lines resemble small cell neuroendocrine carcinoma and are not able to respond to androgens, glucocorticoids, epidermal, or fibroblasts growth factors. Since, PC3 cells do not need the presence of androgen to grow and divide, this makes them androgen independent and highly metastatic compared to other cell lines, such as the LNCaP and DU145 prostate cancer cell lines (Tai, S., et. al., 2011).

The overall goal of this project is to investigate the role of TRP channel inhibition on Akt and ERK expression and localization in the cell (Figure 7). We are interested in this because TRP channels as well as Akt and ERK, are involved in cellular survival and cell migration. Previous work links activation of Akt as well as ERK to calcium influx into the cell, but fail to determine if the calcium influx inside of the cell affecting IGF is due to TRP channels. We aim to determine if inhibiting the TRP channels using Ruthenium Red, will affect Akt and ERK.

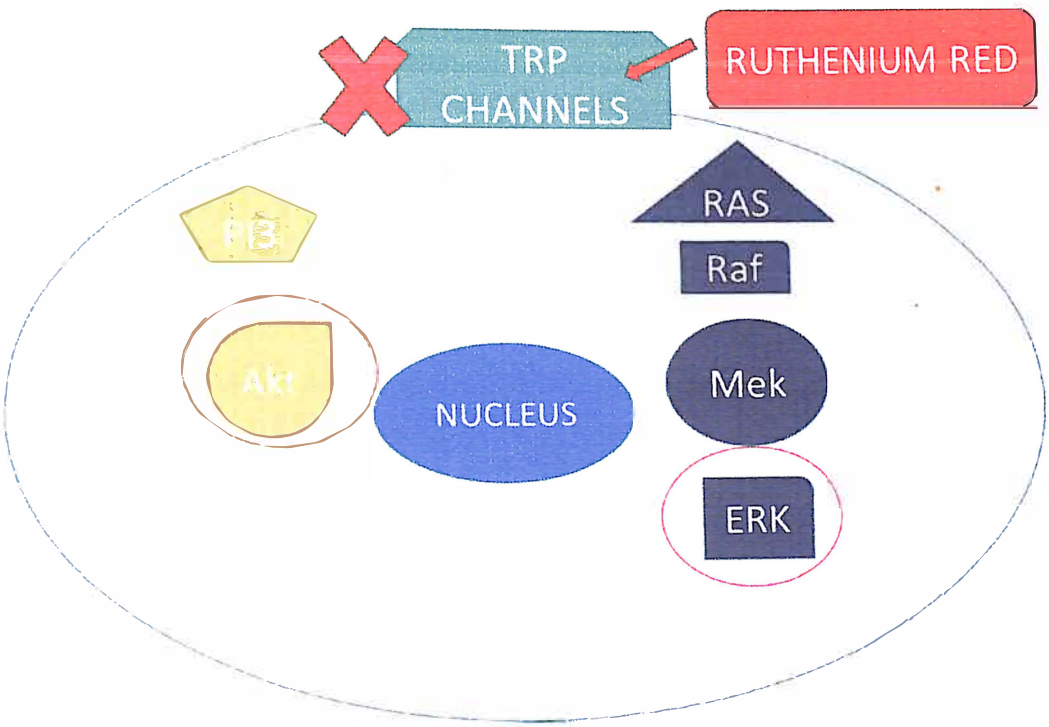


Figure 7: My working model. My working model shows which pathways I am interested in observing after treatment with Ruthenium Red. We believe that Akt and ERK (circled in red) will be affected due to our belief that TRP channels interact with ERK as well as Akt through calcium signaling.

MY HYPOTHESIS

My hypothesis is that TRP channel activity regulates prostate cancer cell migration, proliferation, and survival through modulation of the IGF intracellular signaling pathway.

We tested this hypothesis by:

1.) Determining if TRP channels are involved in proliferation, migration, and cellular survival.

2.) Determining if Akt and ERK expression levels are changed after Ruthenium Red treatment.

3.) Determining if Akt and ERK localization are changed after Ruthenium Red treatment.

4.) Determining if cells migration is slowed down after Ruthenium Red treatment.

5.) Determining if cells go through apoptosis after Ruthenium Red treatment.

CHAPTER 3: MATERIALS AND METHODS

Cell Culture

PC-3 cell lines were obtained from ATCC. PC-3 cells were grown in F12K (Kaighn's) medium with glutamine purchased from Gibco Life Technologies. In the medium, we add antibiotic-antimycotic, from Gibco Life Technologies, to prevent cells from becoming contaminated. Ten percent fetal bovine serum (FBS) was also added to the F12K medium for nourishment. Both the antibiotic antimycotic and the fetal bovine serum were from Life Technologies. During growth, PC3 cell lines were incubated at 37 degrees Celsius at a 5 percent CO_2 level. To ensure that cells remained healthy, the medium was changed every other day. When cells were ready to be passaged onto other flasks, Hanks Balance solution or PBS was used to rinse the cells. These solutions are used to wash cells before dissociation, transporting cells or tissue samples, diluting cells for counting, as well as preparing reagents. Afterwards trypsin (Thermo Scientific) was used to remove cells once they reached 80-90% confluence. Cells were then distributed evenly at a one to six or one to eight split onto another flask or petri-dish depending on the experiment to be conducted next.

Protein lysate collection and inhibitors:

Whole cell lysates were collected using Ripa Buffer and a protease and phosphatase inhibitor cocktail that were both purchased from Thermo Scientific. PC3 cells were also treated in F12K medium without FBS at 1 mM, 3 mM, 5 mM, 10 mM, 15 mM, and 20 mM. Ruthenium Red inhibitors from (Acros Organics) were diluted to a concentration of 291 mM. PC-3 lysates were collected with Ripa buffer from Thermo Scientific and a protease inhibitor cocktail from the same place. Lysates were labeled and stored in micro-centrifuge tubes at -20 degrees Celsius until further use.

Western blotting:

Protein assays were used to learn the total lysate protein concentration for whole and treated lysates. The reagent A, reagent B, and protein standards to test the protein concentration were from Biorad. PC-3 lysates were used on Any KD gels from Biorad. Approximately 30-40 μL of cell lysates (depending the protein assay) and sample buffer with β-mercaptoethanol from biorad were aliquoted and electrophoresed. All antibodies were incubated on nitrocellulose paper from Biorad. Gels were transferred using Biorads trans-blot turbo transfer starter system on the mixed molecular weight setting. A 5% nonfat dry milk solution was prepared with TBST, from Biorad, and used for blocking. Akt, phosphorylated Akt, p44/42 phosphorylated p44/42, and GAPDH were from Cell Signaling and incubated at 1:1000 dilution in 2-5% BSA from thermo scientific. Antibodies from Biorad were from rabbit and Biorads anti-rabbit secondary was used for incubation

on nitrocellulose. Antibodies were incubated at approximately 2-4 hours at room temperature and secondary antibodies were incubated for one hour. After transfer, blocking, primary antibody incubation, and secondary incubation the nitrocellulose was washed three times for five minutes with TBST. After secondary incubation, the AP conjugate substrate kit from Biorad was used to develop the image on the nitrocellulose. Afterwards, the blot was scanned onto the computer using an HP printer and densitometry using Image J was done.

Scratch wound healing migration assay:

PC3 cell lines were grown in F12k-media from Gibco also known as life technologies and passaged or split onto six well plates. They grew to approximately 90% confluence. Cells were then washed with PBS or HBSS and then serum starved overnight. After overnight serum starvation, a scratch was applied to the most confluent areas using a pipette tip. Ruthenium Red was then added at 1 mM, 3 mM, 5 mM,10 mM, and 20 mM concentrations. Right after the inhibitor was added, pictures were taken at zero hours. Cells were treated and monitored up to 24 hours after treatment, while incubated at 37 degrees Celsius. Pictures were taken with an Evos microscope at 0, 4, 8, 12, and 24 hours.

Immunocytochemistry:

Coverslips were sterilized using 90 percent ethanol overnight. Coverslips were placed in the six-well plates and left until they dried. Cells were then split from flasks and then equaled out in each well. Cells were then left to grow and once the cells reached confluency, they were serum starved overnight and then treated with Ruthenium Red for

24 hours. After 24 hours, cells were rinsed with PBS. Paraformaldehyde was then used to fix the cells. Cells were blocked for an hour in 10% goat serum from Acros. Cells were incubated overnight in phalloidin, which is important for outlining the cells, from Life Technologies at 1:500 dilution. Phalloidin had an excitation of 546 nm. Cells were washed with 1% goat serum three times for ten minutes and then incubated in Akt, phosho-Akt, ERK, phosphor-ERK, TRPV2, TRPV4, TRPV6, or TRPM8 for approximately 3 hours at room temperature. Cells were then rinsed again three times for ten minutes with 1% goat serum. Cells were then incubated in secondary for two hours at room temperature. The secondary has an excitation of 647 nm. Cells were washed again with 1% goat serum. The coverslips were then mounted to microscope slides with mounting medium containing DAPI (Life Technologies). Images were taken on a Leica confocal.

Apoptosis Assay/Caspase 3/7

Cells were grown in six well plates with coverslips added to each well. Once the cells reached confluency, they were serum starved overnight and then treated with Ruthenium Red for 24 hours. Thirty minutes before the cells reach their 24 hour mark, the Caspase 3/7 green from Life Technologies was added to each well at a 3µg/mL concentration. After 24 hours, 4% paraformeldehyde was used in each well for 20 minutes. Cells were then mounted with mounting medium onto microscope slides to be imaged later.

CHAPTER 4: RESULTS

Prostate Cancer cells (PC3) preferentially metastasize to bone, and once they form secondary tumors within the bone, osteoblastic/osteolytic mixed lesions form. In an effort to identify potential therapeutic targets, studies into the signaling mechanisms involved in bone metastasis have increased in recent years. TRP channels have been studied within components of bone, particularly chondrocytes and osteoblasts, given their role in calcium regulation. Therefore, we hypothesize that TRP channels are expressed within PC3 prostate carcinoma cells capable of metastasizing to bone, and that blocking these channels will prevent the migration of PC3 cells and affect their signaling and survival.

As a first step, TRP channel expression was analyzed in PC3 prostate carcinoma cells. TRPV2, TRPV4, TRPV6, and TRPM8 were chosen based upon previous studies indicating they play a role in cell migration and/or apoptosis (Figure 8). Whole cell lysates were collected and Western immunoblotting performed. Based upon our analysis, TRPV2, TRPV6, and TRPM8 are expressed in PC3 cells, whereas little TRPV4 was detected.

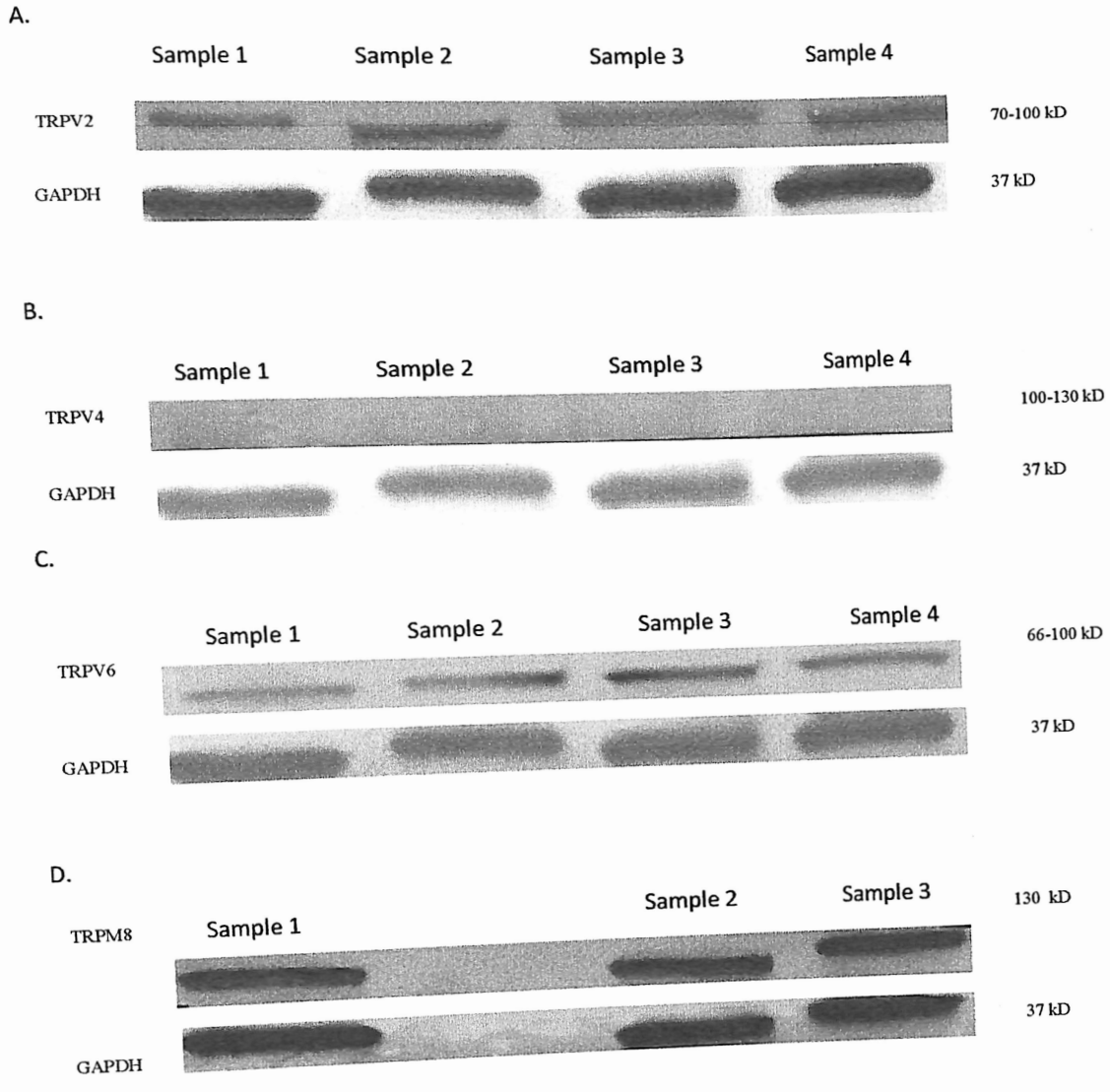


Figure 8: Western blot analysis using TRP channel antibodies specific for cell migration and cell survival. The represented blots show expression of TRP channels in PC3 whole cell lysates. Whole cell lysates samples were collected four times. All TRP channels were used at a 1:1000 concentration. TRPV2 was detected (A), TRPV4 was not detected (B), TRPV6 was detected (C), and TRPM8 was detected (D).

To confirm these results and investigate the localization of the TRP channels, immunocytochemistry was performed. Just as in the Western immunoblots, the expression of TRPV2, TRPV6, and TRPM8 was confirmed (Figure 9). Although TRPV4 was virtually undetectable through Western immunoblotting, a low level of expression was seen using immunocytochemistry. Expression of TRPV2, which is responsible for cellular migration, seems to be colocalized with actin in these cells. TRPV2, TRPV4, and TRPM8, however seem to be expressed intracellularly.

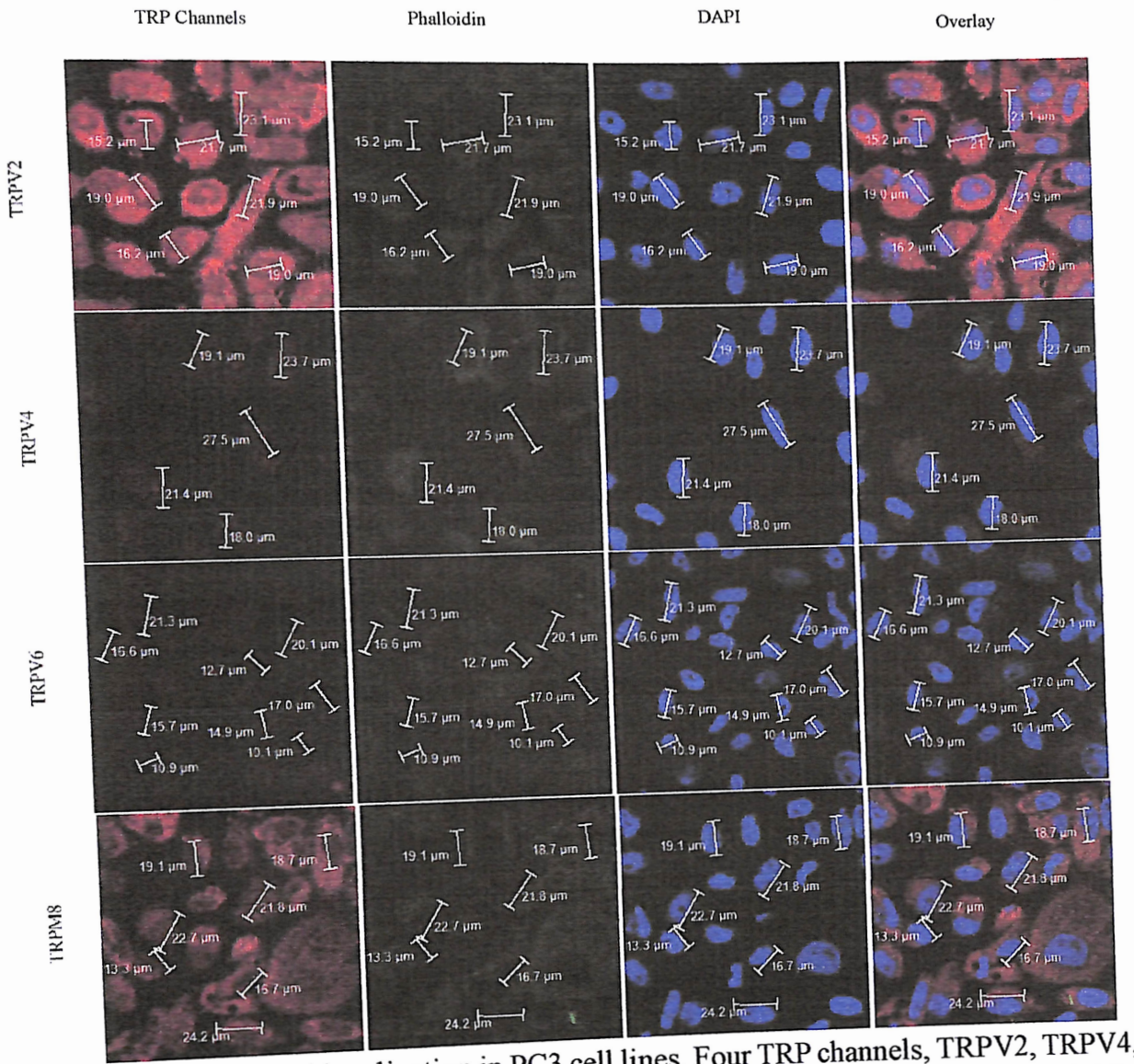


Figure 9: TRP channel localization in PC3 cell lines. Four TRP channels, TRPV2, TRPV4, TRPV6 and TPM8 were chosen for ICC. DAPI was used to stain the nucleus. Phalloidin was used to satin F-actin. TRP channels and phalloidin were incubated at a 1:500 concentration. White lines represent the nucleus of the cell, determining which cells were in the same plane as the next. Cells were imaged at the same. All pictures were imaged using the same intensity and exposure of a Leica 5000B Confocal Laser Scanning Microscope.

Due to expression of TRP channels in PC3 cell lines, and the correlation of those TRP channels to cellular migration, I decided to perform a scratch assay using Ruthenium Red treatment that inhibits these TRP channels (Figure 10). After treating the cells with Ruthenium Red, images were obtained using an EVOS microscope at zero, four, eight, twelve, and twenty-four hours. Cells were observed migrating into the wound area and analyzed using Image J. PC3 cells under normal conditions (plus serum) as well as minus serum, migrated into the wound area completely after 24 hours (Figure 11). PC3 cells began to move slower into the wound area as the concentration of Ruthenium Red increased. Cells were then treated with Ruthenium Red, and as the concentration of Ruthenium Red increased, cells did not migrate into the wound area as much as plus and minus serum (Figure 11 & 12). Even though cells migrated into the wound area after twenty-four hours at those concentrations of Ruthenium Red, cells were not as confluent as the untreated control. At 10 mM, the cells did not migrate completely into the wound area, and at 20 mM, the cells barely migrated at all (Figure 12). Statistical analysis shows that the p value using ANOVA, was less than 0.05, indicating that the cells migration into the wound area compared to minus serum showed a statistical significant difference each hour (Figure 13). I also observed the F value in ANOVA, and found that all values were above 1, also indicating a statistical significance in difference of migration into the wound area between treatments. Plus serum, minus serum, RR 1, RR3, RR5, RR10, RR 15, and RR 20 were compared at each hour. At 4 hours p is 0.005, 8 hours p is 0.002, 12 hours p is 0.00, and 24 hours p is 0.00.

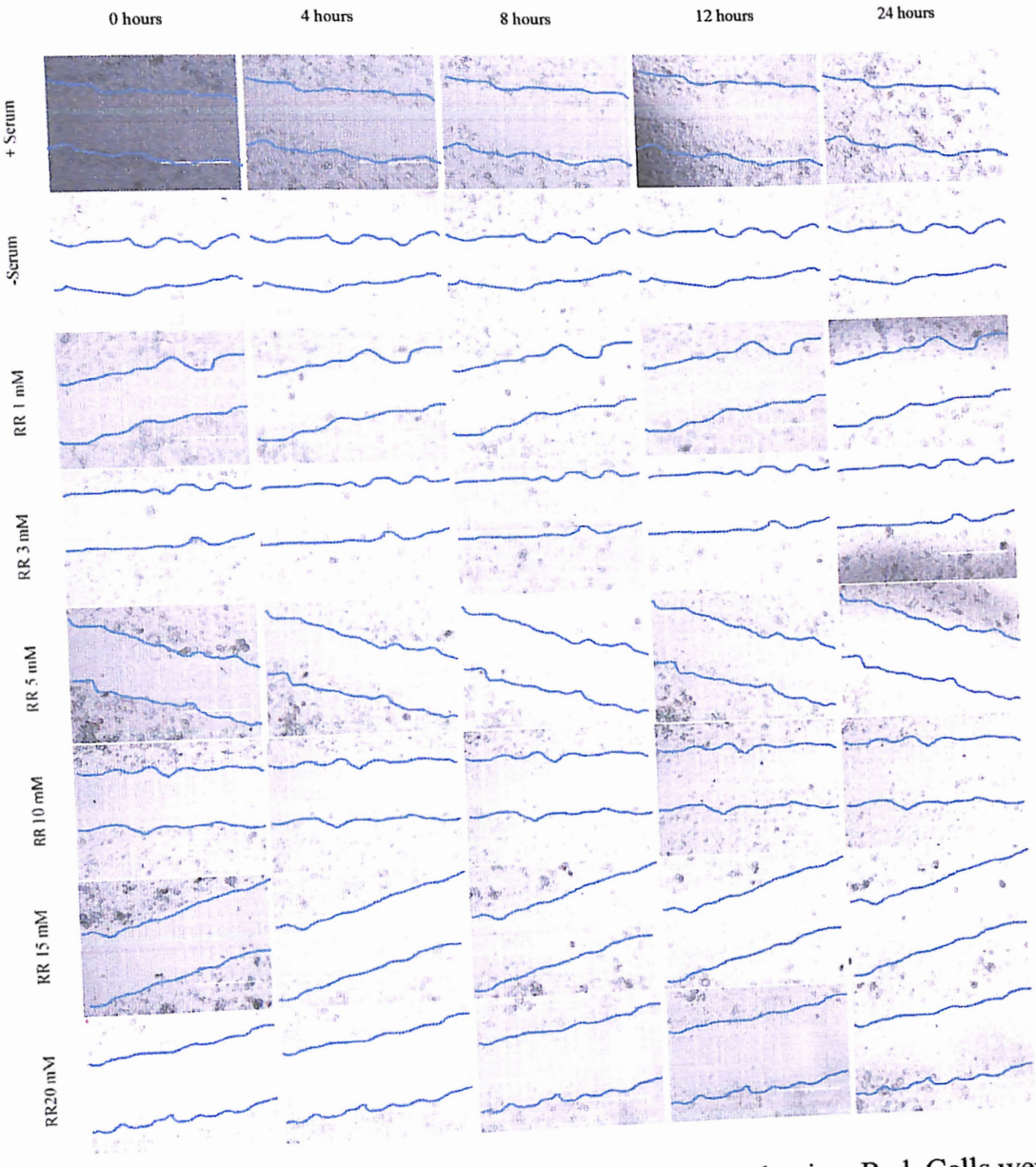


Figure 10: Scratch Assay of PC3 cells treated with Ruthenium Red. Cells were plated into six-well plates. They were grown to approximately 90 percent confluence and then serum starved overnight. Cells were then scratched with a 10 µL pippete tip and then Ruthenium Red treatments without serum 1 mM, 3 mM, 5 mM , 10 mM, 15 mM, 20 mM, were placed on the cells. Plus serum (contained FBS) was used as a control as well as minus serum (no FBS). Cells were then imaged using an EVOS microscope at 0, 4, 8. 12, and 24 hours.

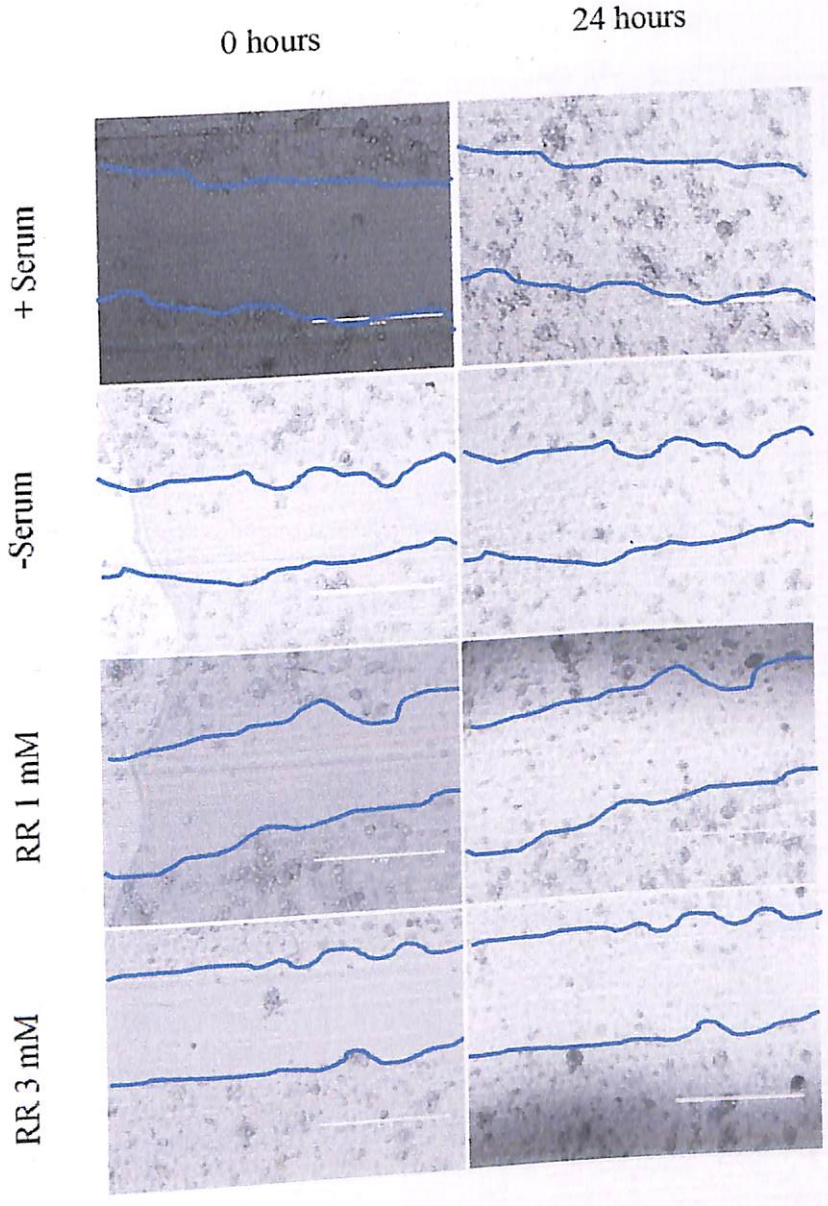


Figure 11: Closer view of scratch assay on PC3 cells treated with Ruthenium Red part 1. Lines drawn at zero hours and then over-layed at 24 hours to determine the difference of cell migration into the wound area at plus serum, minus serum. Cells were treated with Ruthenium Red at 1mM, and 3 mM with minus serum. Pictures were imaged using an EVOS Microscope.

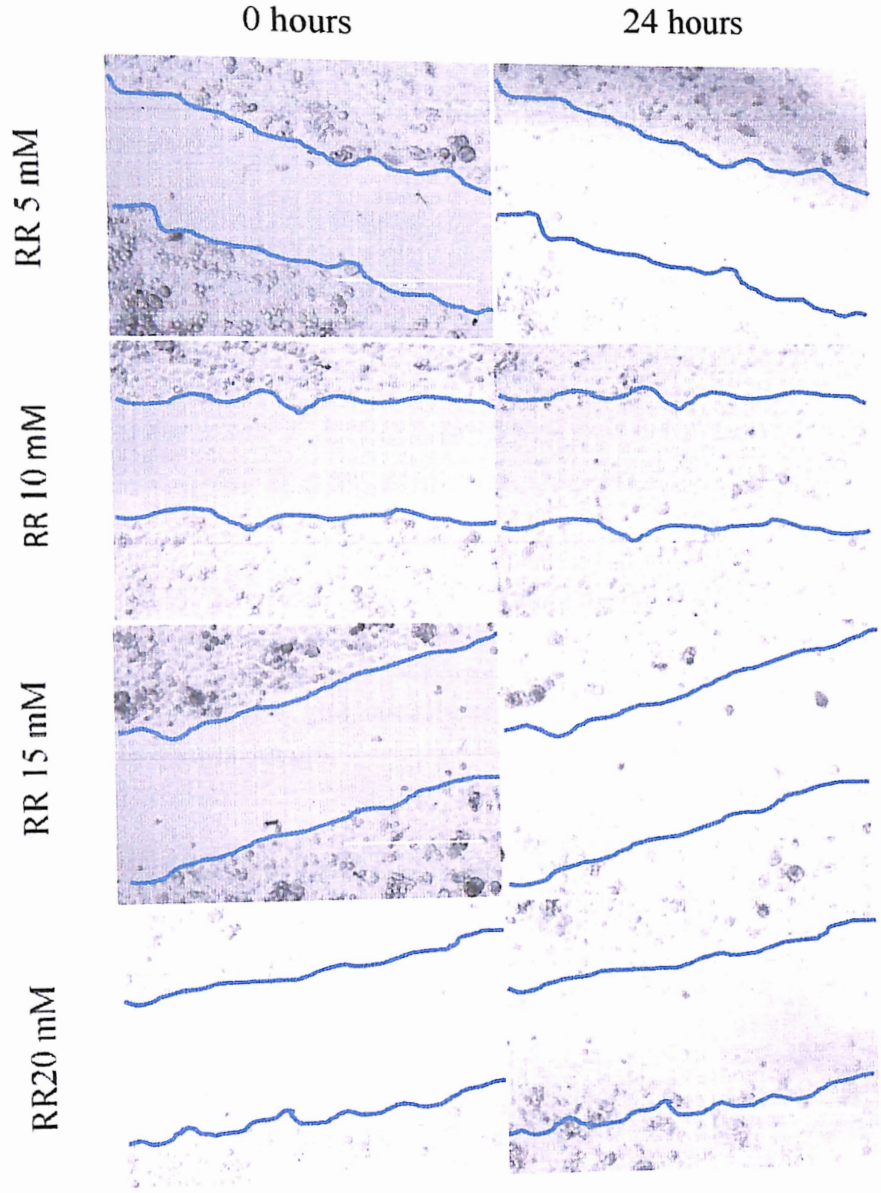


Figure 12: Closer view of scratch assay on PC3 cells treated with Ruthenium Red part 2. Lines drawn at zero hours and then over-layed at 24 hours to determine the difference of cell migration into the wound area at 5 mM, 10 mM, 15 Mm, and 20 mM. Pictures were imaged using an EVOS Microscope.

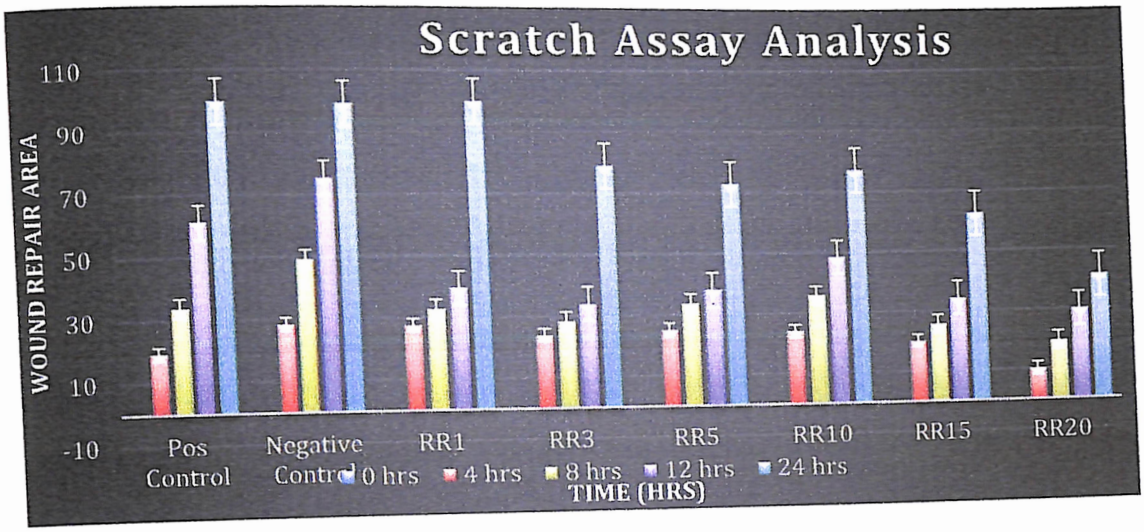
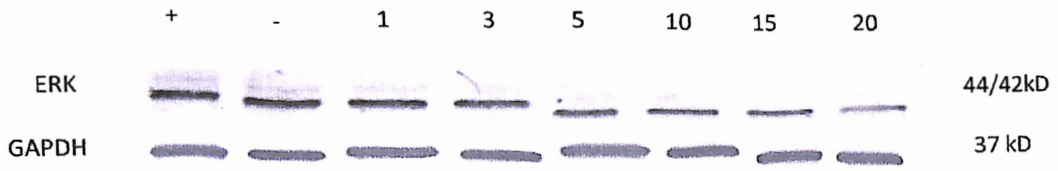


Figure 13: Statistical Analysis of Scratch Assay on PC3 cells treated with Ruthenium Red after 24 hours. ANOVA was used to compare each treatment to each other based on hours. At 4 hours, 8 hours, 12 hours, and 24 hours the p values were below 0.05. The F value was also above 1.00. There is a statistically significant difference in migration in the wound area compared to minus serum.

We next wanted to test the effect of a TRP channel inhibitor, Ruthenium Red, on signaling, migration, and survival. We chose two signaling proteins to investigate: Akt, which is primarily involved in cell survival, and p44/p42 MAPK, which are primarily involved in cell proliferation and migration. ERK, like TRPV2, regulates cell migration. Since we are interested in slowing down or stopping cellular migration in PC3 cells, it was also necessary to test the expression level changes in ERK after treatment with Ruthenium Red. We also thought that if Akt levels were affected by treatment with Ruthenium Red, then ERK levels may be affected as well since Akt and ERK are both regulated by the IGF receptor.

After treating PC3 cells with Ruthenium Red, we tested the expression levels of ERK and observed that the expression levels of ERK decreased with an increase in concentration of Ruthenium Red (Figure 14). The levels of phospho-ERK were also tested, since the phosphorylation of ERK again indicates activation (Figure 15). Activation of phospho-ERK is typically associated with cell migration. We did not find detectable levels of phospho-Erk in our PC3 cells. Due to literature claiming that ERK played a role in cell migration in PC3 cells, we expected to find phopho-ERK in plus and minus serum lanes. This may indicate ERK's low role in PC3s migratory abilities.

A.



B.

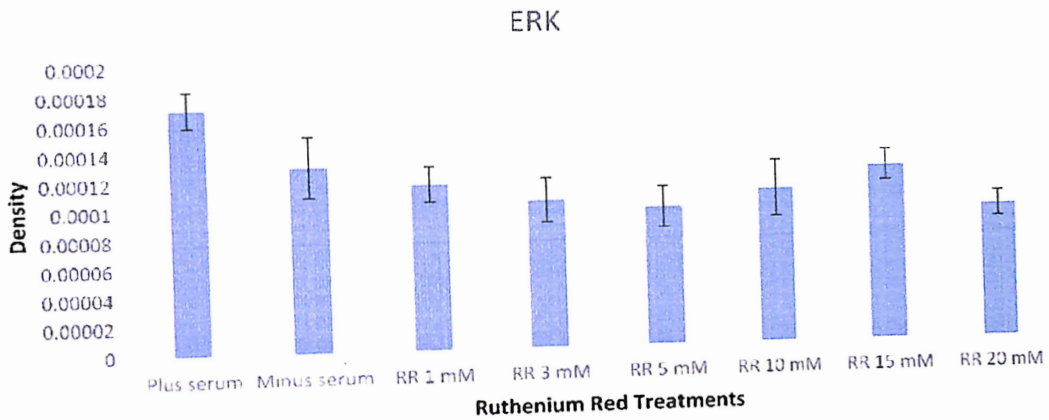


Figure 14: The expression of ERK in PC3 cells after treatment with Ruthenium Red. (A&B) ERK was treated with Ruthenium Red and the expression levels of ERK began to drop. A one way ANOVA was done and the P value indicated there was no significant difference compared to the minus serum $P>0.05$.

C.

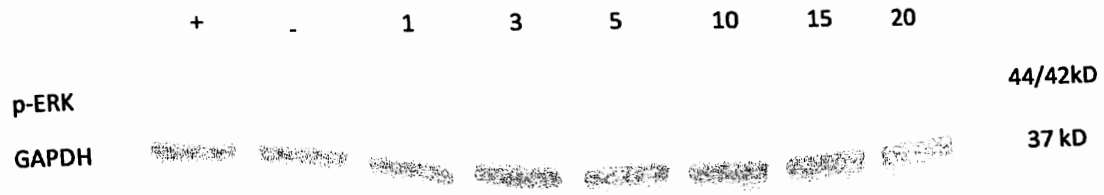


Figure 15: The expression of p-ERK in PC3 cells after treatment with Ruthenium Red. C) Phospho-ERK expression levels were tested and there was little to no expression detected. Statistical data was not taken due to no expression.

Since we observed the detection of ERK in PC3 cells, we were interested in the localization of ERK in PC3 cells. Cells were grown on coverslips and ICC was performed to observe localization of ERK in PC3 cells (Figure 16 & 17). Expression levels between different concentrations of treatment were not compared in these results. We were more interested in localization and we observed that as the concentration of Ruthenium Red increased, ERK seemed to become more co-localized with actin (Figure 18 & 19). Phalloidin was used in this study to tag F-actin in these cells. Image sizes were increased to get a better view of ERK localization in PC3 cells. Even though phosphorylated ERK was not detected in western blotting I thought it would still be interesting to see if it was detected in ICC (Figure 16 &17). I observed that phosphorylated ERK was detected in ICC at low concentrations indicating that ERK may not play a huge role in cell migration (Figure 20 & 21). I observed that localization in p-ERK seemed to be more intracellular (Figure 22 & 23). As the concentration of Ruthenium Red increases, p-ERK is co-localized with actin as well. ERK and p-ERK do not seem to translocate into the nucleus as expected.

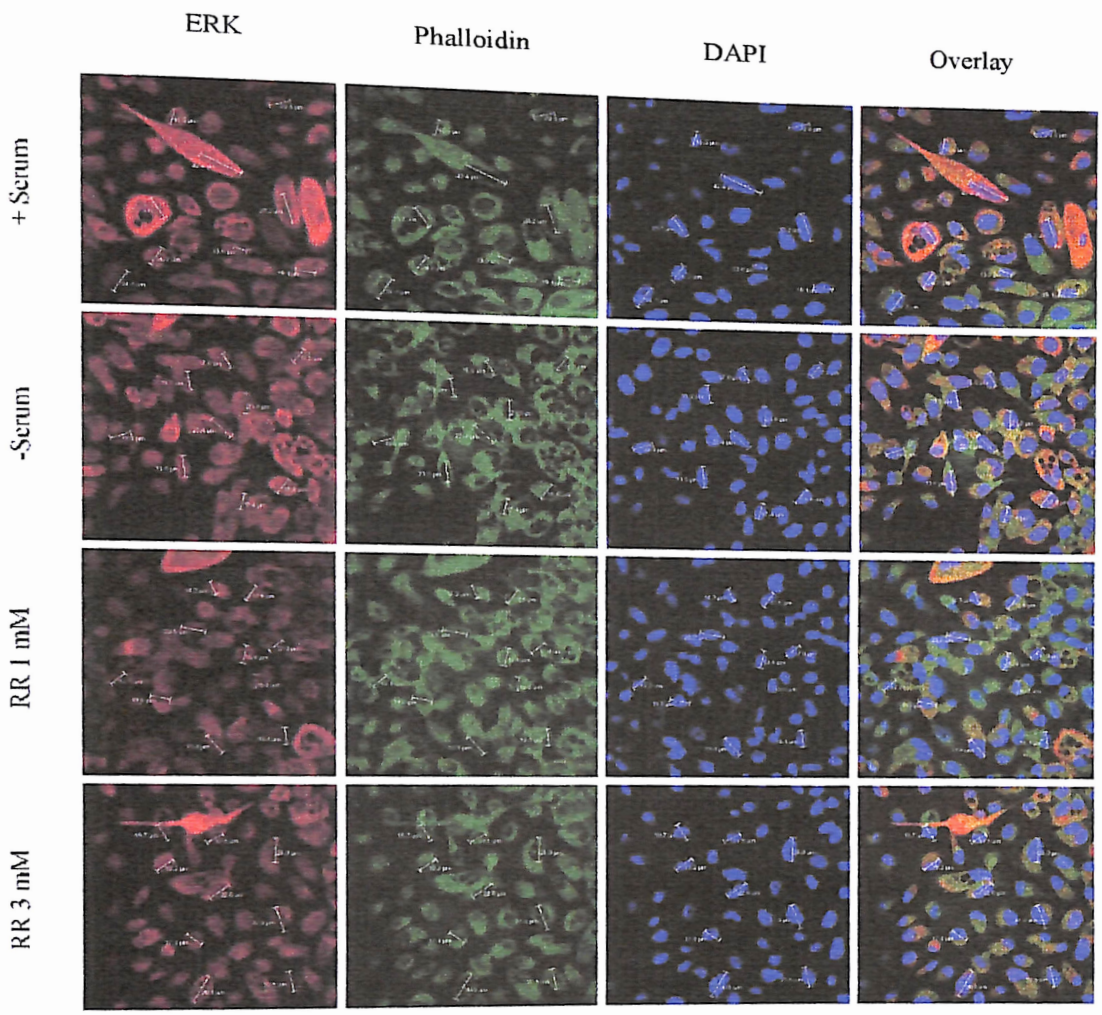


Figure 16: Localization of ERK in PC3 cells after treatment of Ruthenium Red part 1. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 1 mM and 3mM in serum, free medium. Cells were imaged with plus and minus serum controls. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

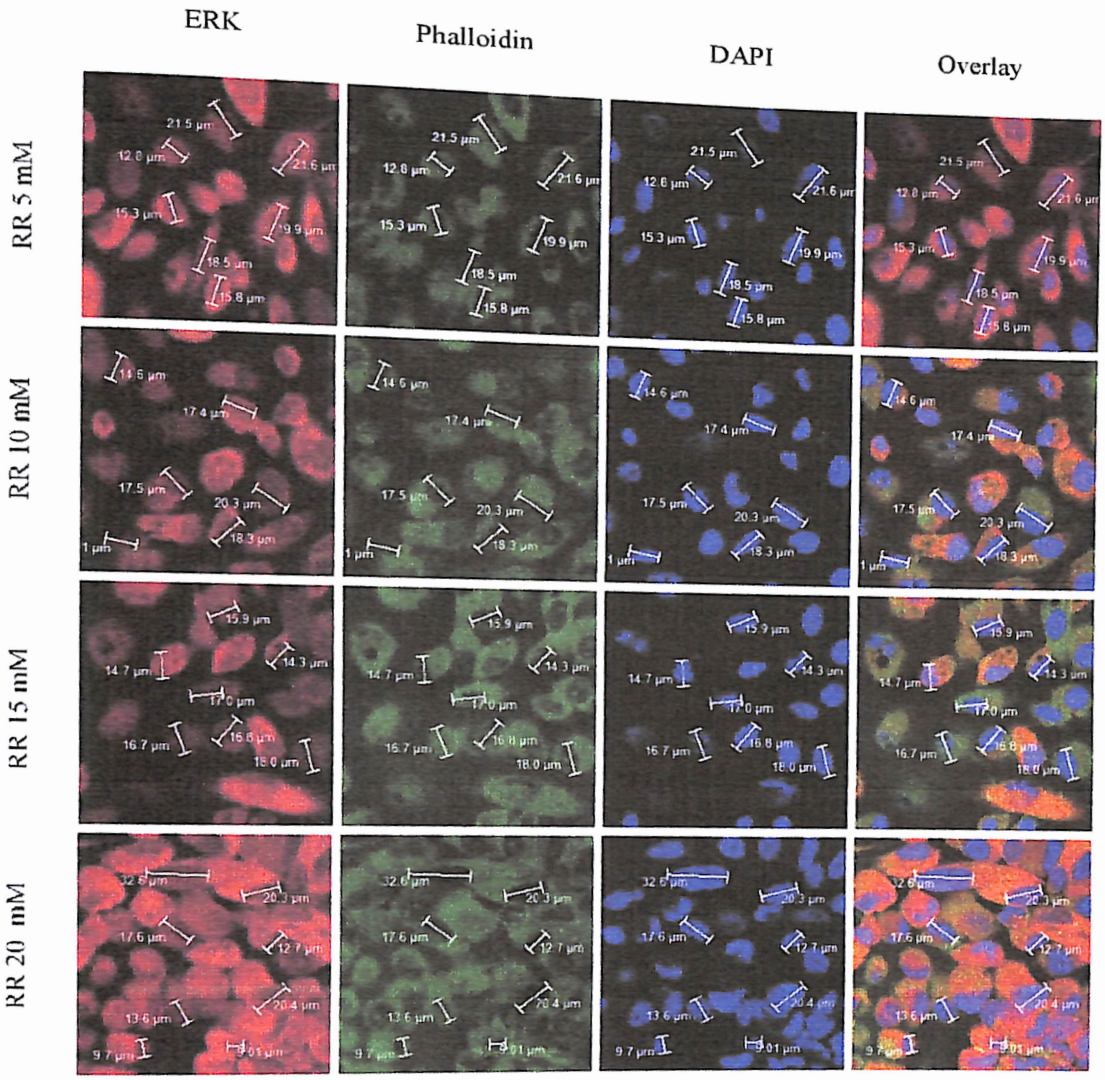


Figure 17: Localization of ERK in PC3 cells after treatment of Ruthenium Red part 2. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

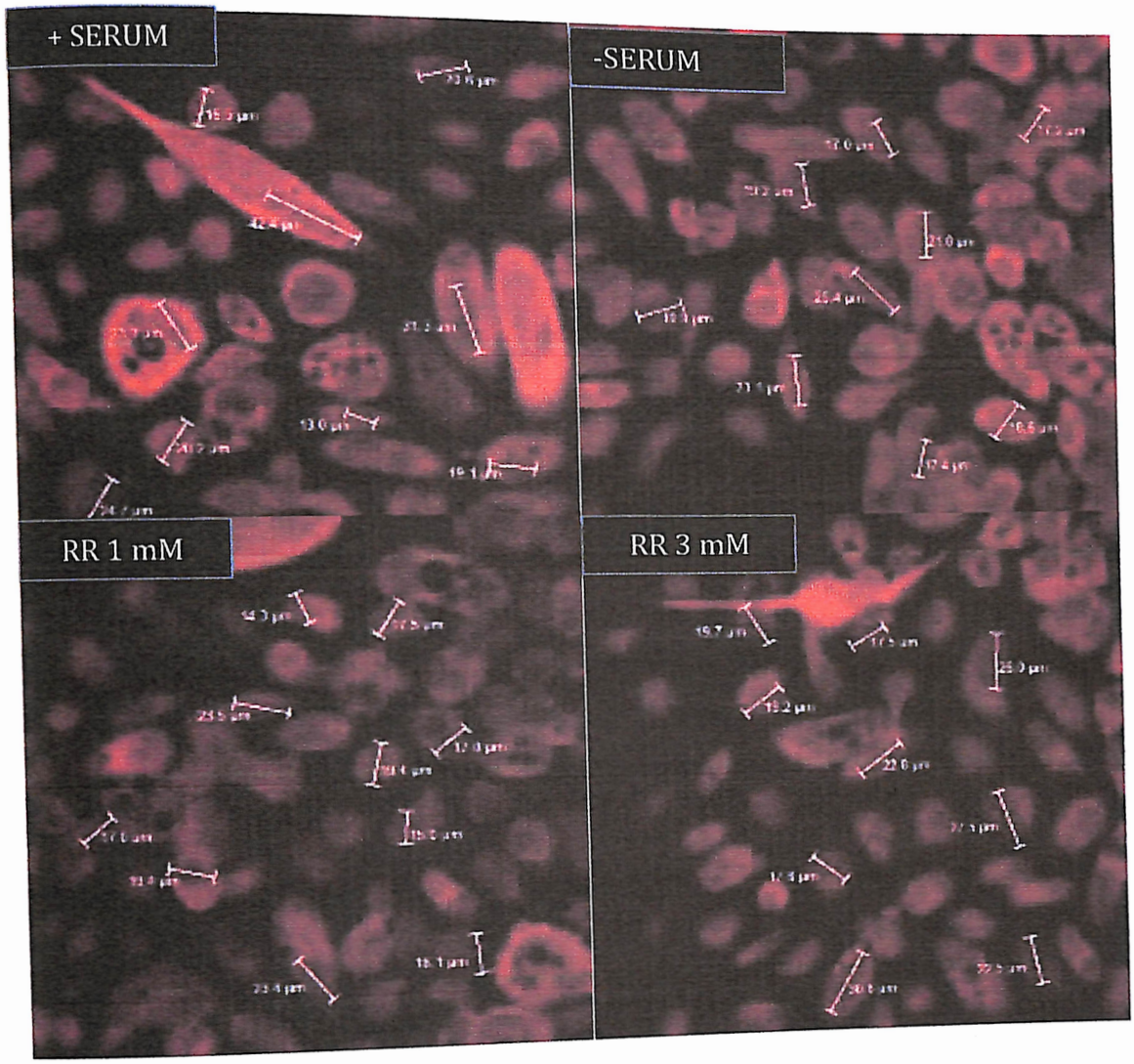


Figure 18: Closer view of ERK localization in PC3 cells treated with Ruthenium Red part 1. Cells were treated with Ruthenium Red at 1 mM and 3 mM in serum free medium. Cells were also imaged at plus and minus serum. Immunocytochemistry was performed to observe the localization of ERK after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

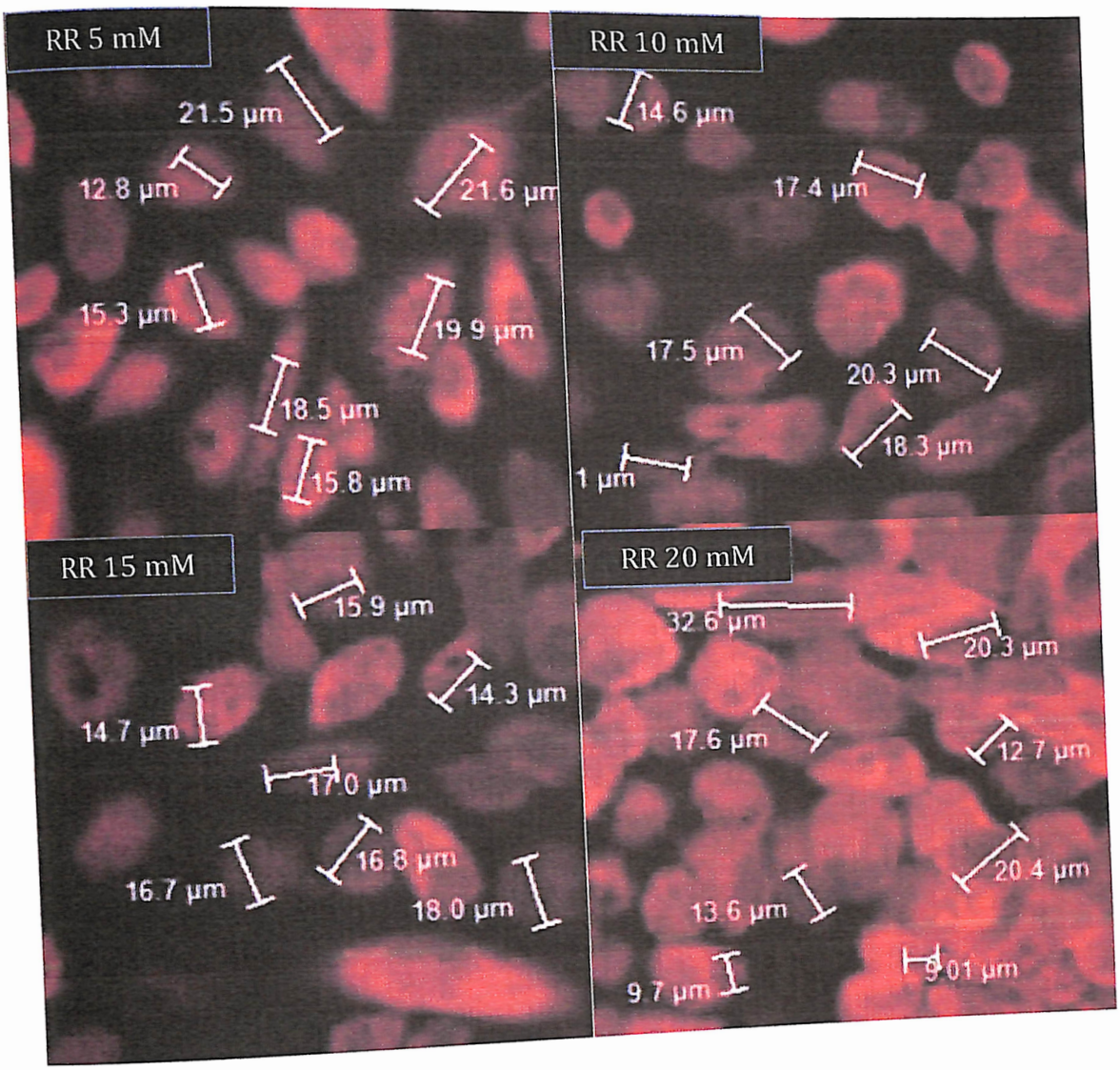


Figure 19: Closer view of ERK localization in PC3 cells treated with Ruthenium Red part 2. Cells were treated with Ruthenium Red at 5, 10, 15, and 20 in serum free medium. Cells were also imaged at plus and minus serum. Immunocytochemistry was performed to observe the localization of ERK after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

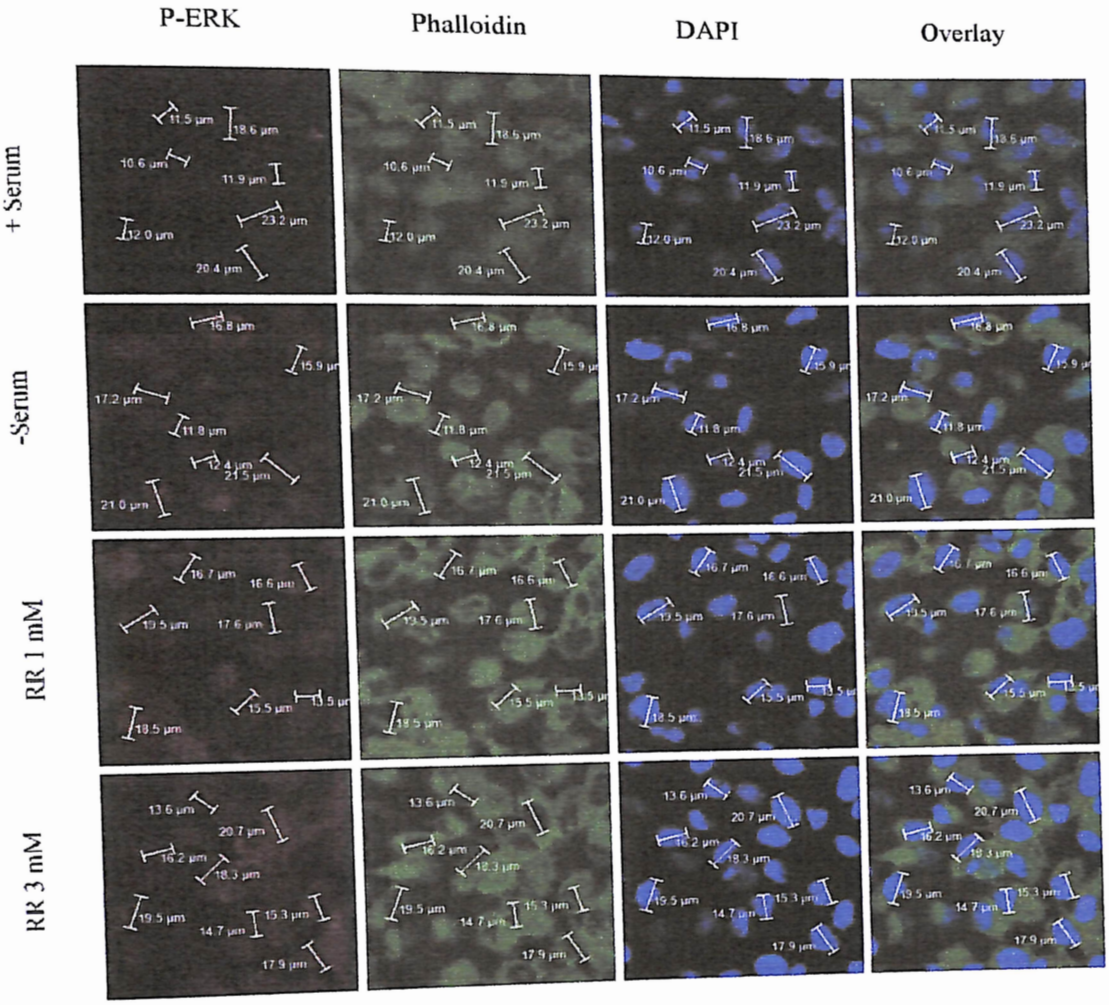


Figure 20: Localization of p-ERK in PC3 cells after treatment of Ruthenium Red part 1. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 1 mM and 3mM in serum free medium. Cells were imaged with plus and minus serum controls as well. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

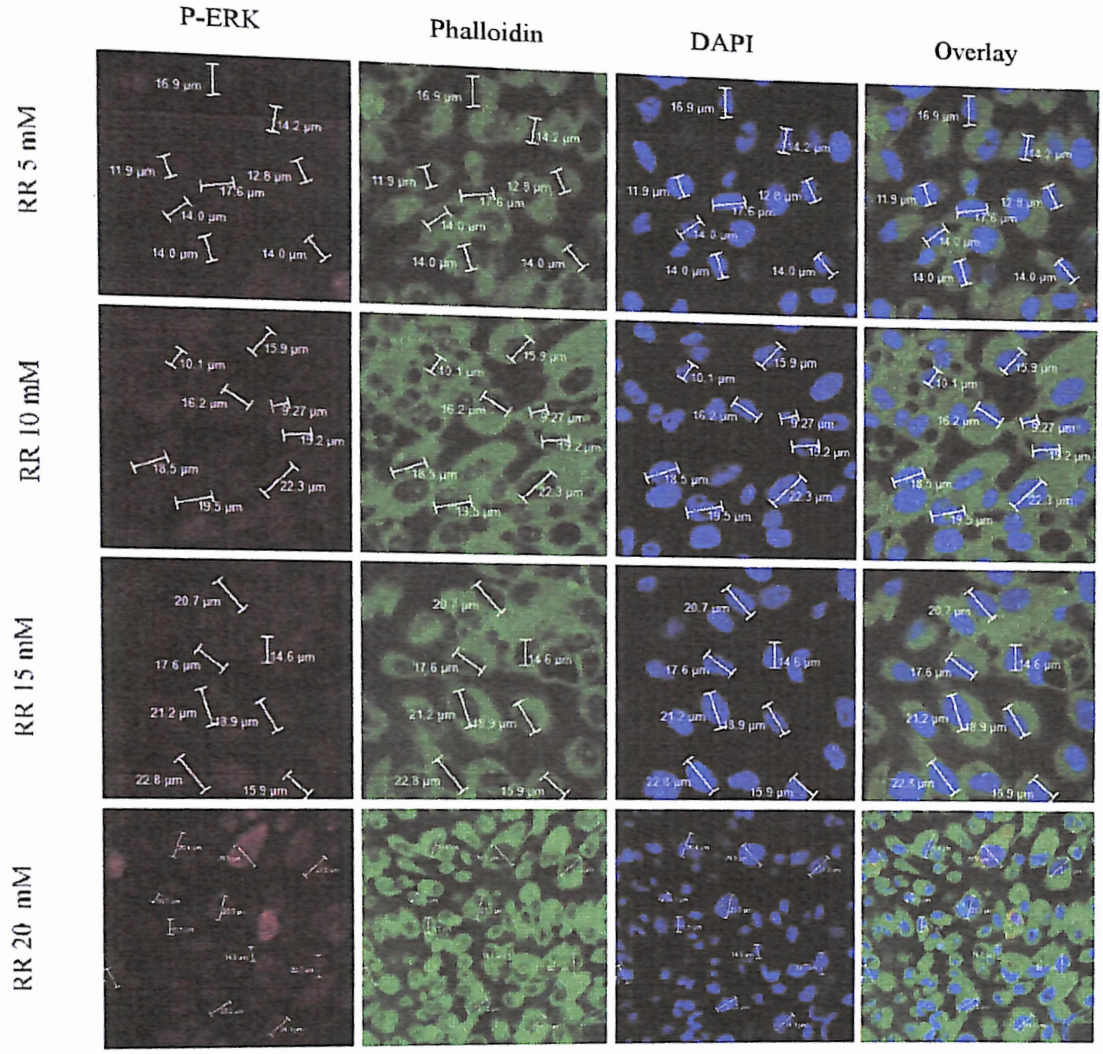


Figure 21: Localization of p-ERK in PC3 cells after treatment of Ruthenium Red part 2. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

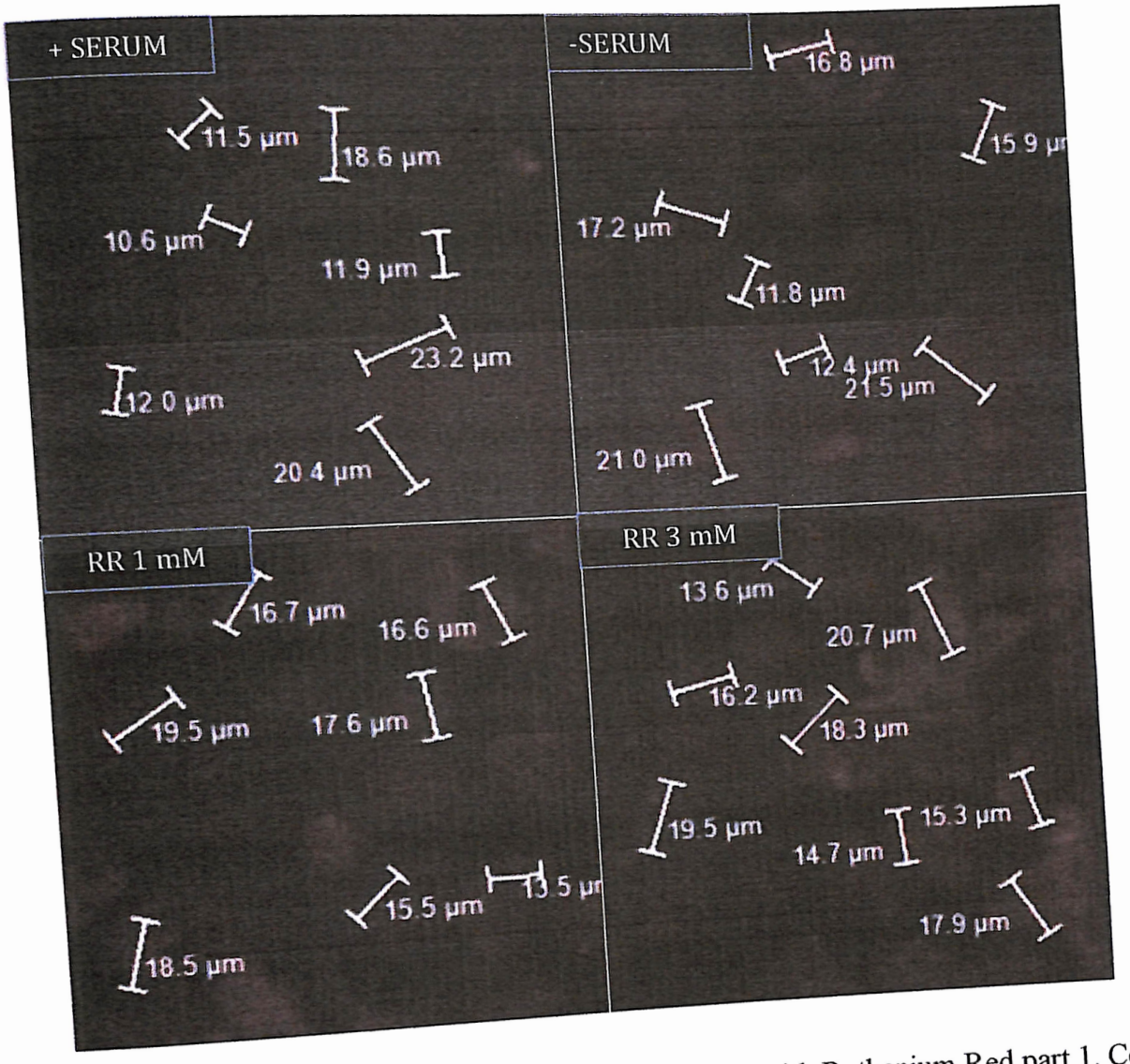


Figure 22: Closer view of ERK in PC3 cells treated with Ruthenium Red part 1. Cells were treated with Ruthenium Red at 1 mM and 3 mM in serum free medium. Cells were also imaged at plus and minus serum. Immunocytochemistry was performed to observe the localization of ERK after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

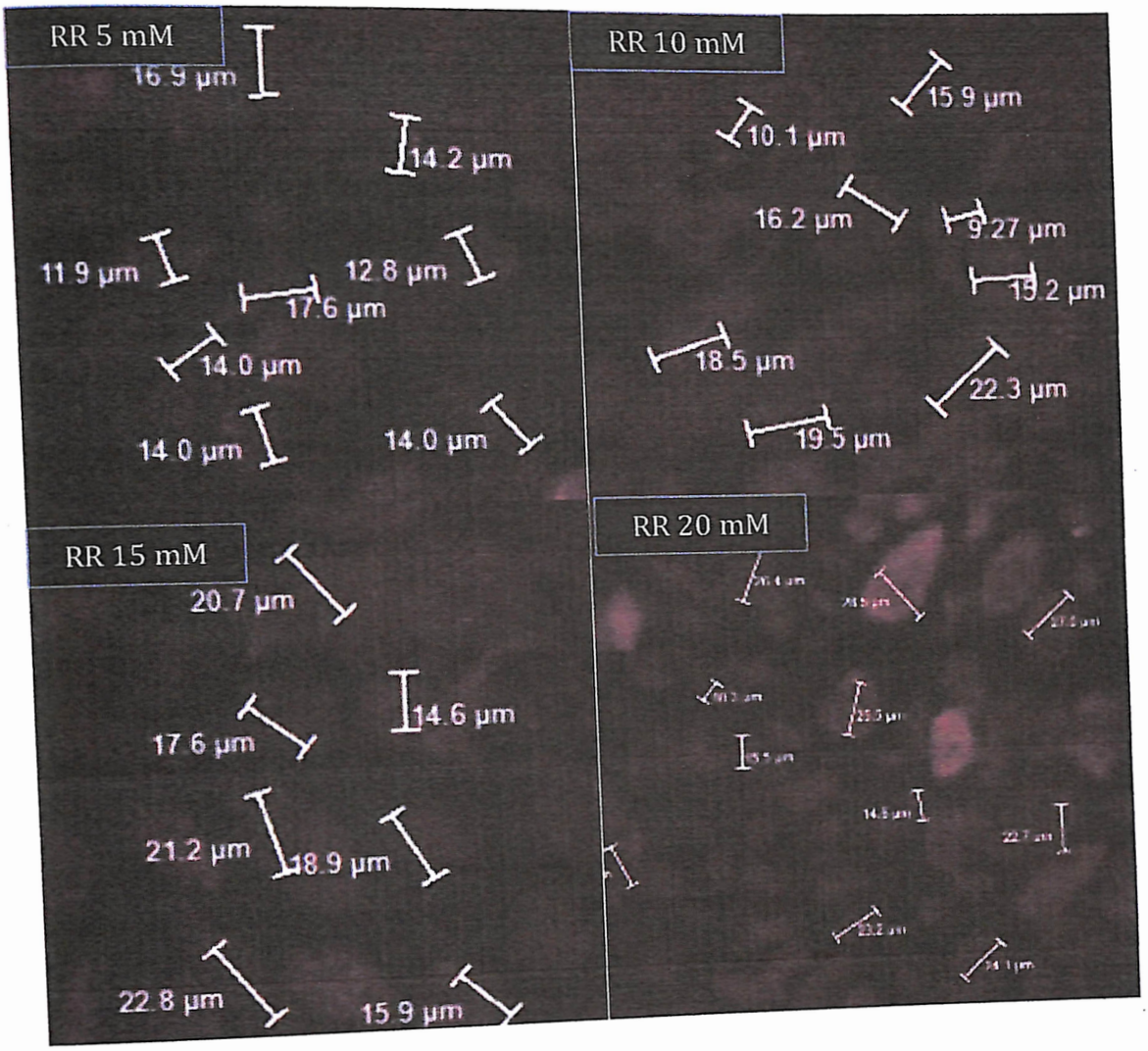
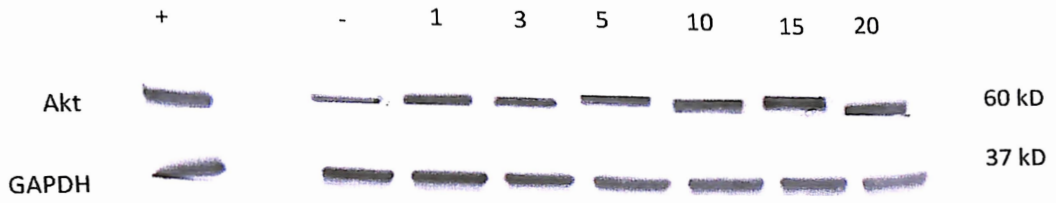


Figure 23: Closer view of p-ERK in PC3 cells treated with Ruthenium Red part 2. Cells were treated with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. Immunocytochemistry was performed to observe the localization of ERK after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

After going back to reanalyze my scratch assay, I noticed that in multiple repeats of data, at Ruthenium Red concentrations of 20 mM, the morphology of my cells begin to change suggesting that these cells were going through apoptosis, so we were curious to see if Akt was affected due to its role in apoptosis (Figure 24). As the concentration of Ruthenium Red increased, the expression level of Akt appeared to increase above 3 mM. Given the error bars, there isn't any difference between the concentrations above 3 mM. Since Akt is expressed in PC3 cells and activation of Akt requires phosphorylation, we next wanted to see if phosphorylation levels increased with an increase in concentration of Ruthenium Red. Activation of Akt through phosphorylation means that PC3s are able to avoid apoptosis. Like the Akt levels, with treatment of Ruthenium Red, the phosphorylated Akt seemed to increase in expression as well. Phosphorylated Akt then seemed to decrease again at 20 mM (Figure 25). After observing the changes we decided to perform statistical analysis to determine if expression level changes were statistically significant. I found that expression level changes after using ANOVA, were not statistically significant compared to minus serum. The p value was above 0.05 and the F value was above 1.00. We then were interested in observing localization of Akt to see if there were any changes.

A.



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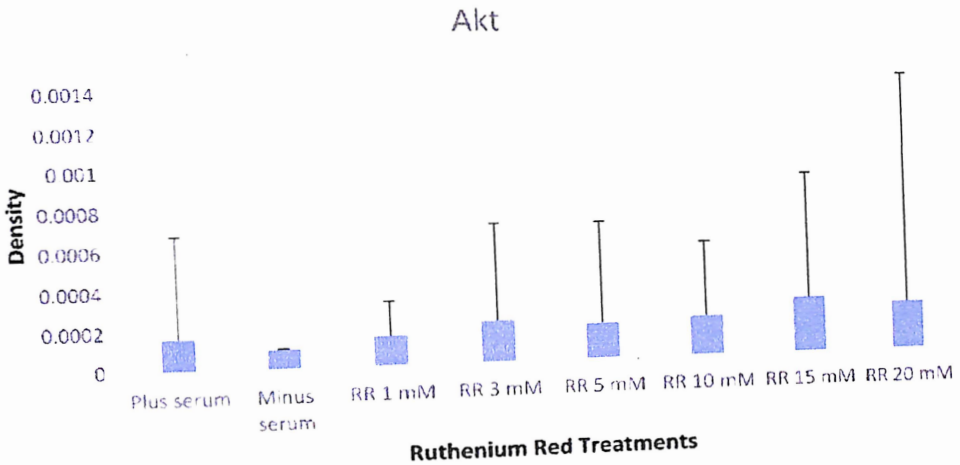
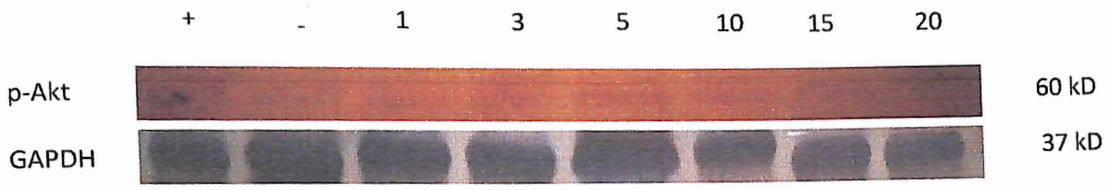


Figure 24: The expression of Akt after treatment with Ruthenium Red. (A&B) Cells were treated with Ruthenium Red and the expression levels of Akt were not significant. A one way ANOVA was done and the P value indicated there was no significant difference compared to the negative control. P value was more than 0.05.

C.



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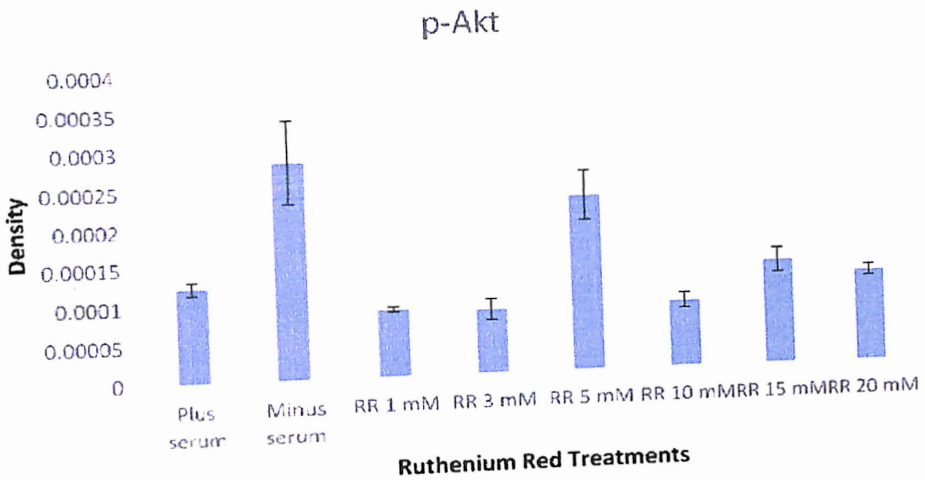

Figure 25: The expression of p-Akt after treatment with Ruthenium Red. (C&D) Cells were treated with Ruthenium Red and the expression levels of p-Akt were variable. A one way ANOVA was done and the P value indicated there was no significant difference compared to the control. P value was more than 0.05.

We were interested in observing the localization changes of Akt in PC3 cells after treating the cells with Ruthenium Red (Figure 26 & 27). Akt seemed to be co-localized with actin as well as localized in the cytosol. As the concentration of Ruthenium Red increased, I found that the co-localization of Akt with actin seemed to increase, and then

decrease again at Ruthenium Red 20 mM (Figure 28 & 29). We also observed the same effect on phosphorylated Akt (Figure 30 & 31). Although in western blotting there seemed to not be as much phosphorylated Akt expression, in ICC, there was a larger amount expressed. There seemed to be as much phosphorylated Akt as there is Akt indicating Akt does play a tremendous role in cellular survival (Figure 32 & 33). Due to the slight decrease in Akt at 20 mM, we came to the conclusion that the Ruthenium Red treatment was causing the cells to go through apoptosis, so we decided to use a caspase 3/7 apoptosis kit from Life Technologies. We chose this kit due to Akt's ability to keep caspase 3/7 from being activated. Due to the light decrease in Akt at 20 mM, we came to the conclusion that the Ruthenium Red treatment was causing the cells to go through apoptosis, so we decided to use a caspase 3/7 kit. We chose this kit due to Akt's ability to keep caspase 3/7 from being activated.

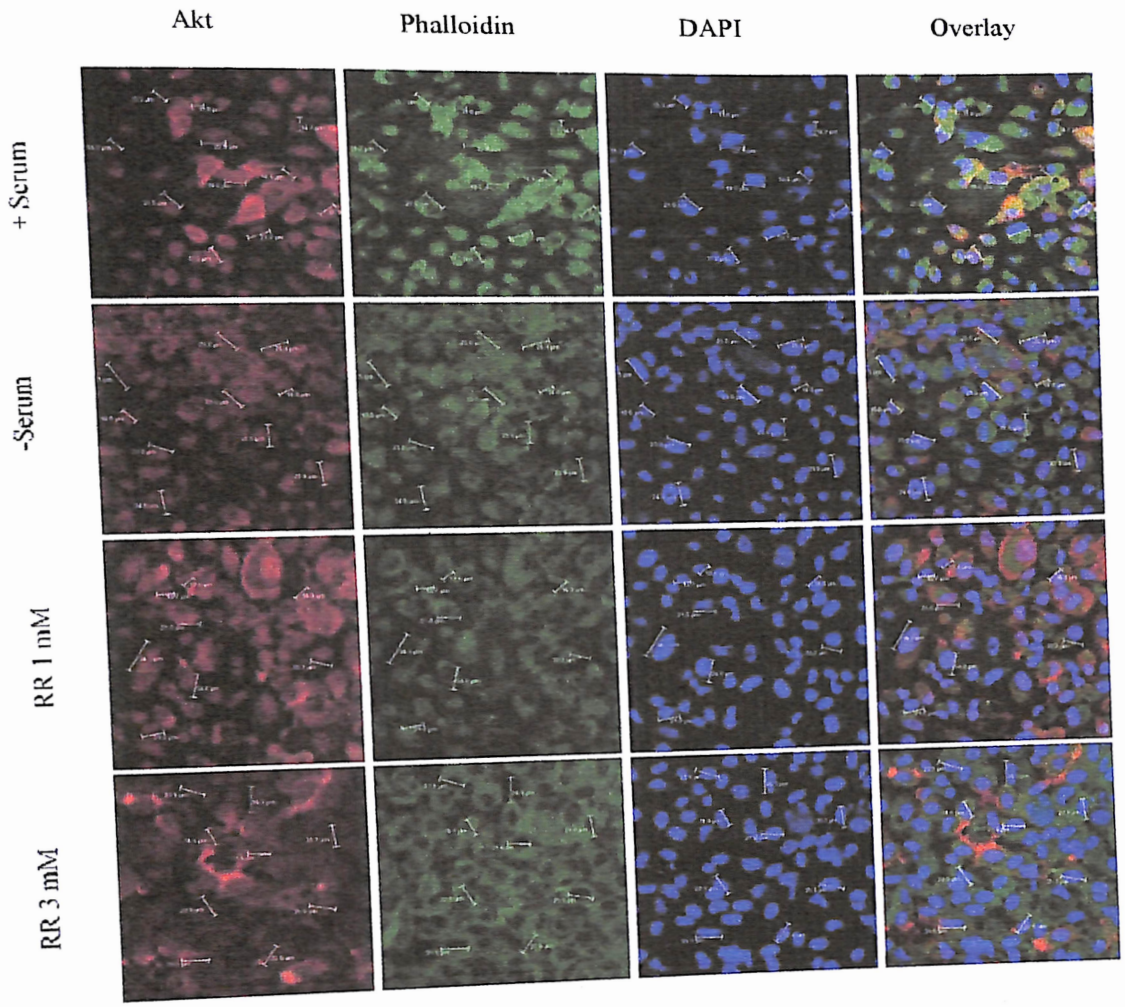

Figure 26: Localization of Akt in PC3 cells after treatment with Ruthenium Red part 1. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 1 mM and 3 mM in serum free medium. Cells were imaged with plus and minus serum controls as well. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

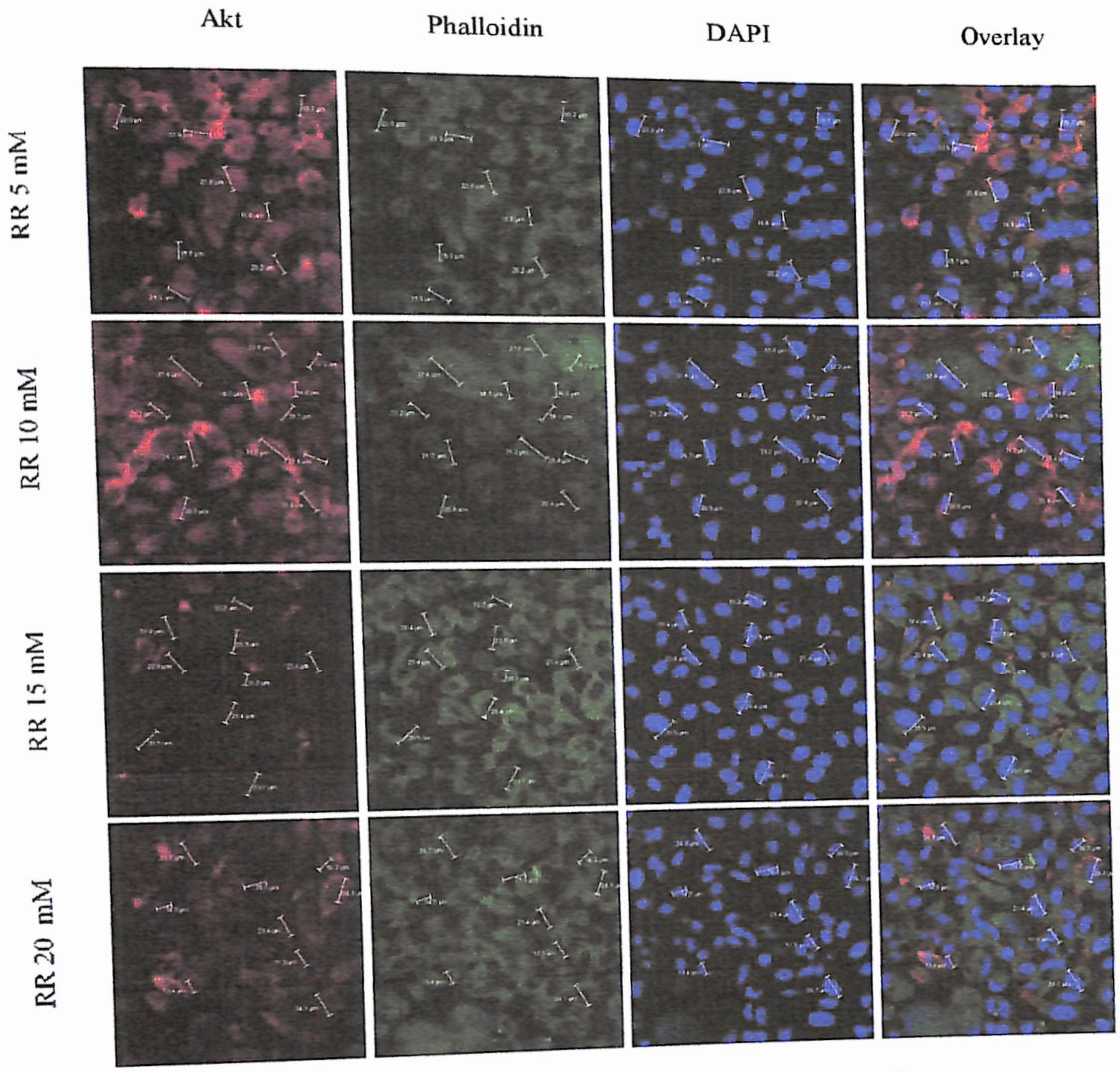

Figure 27: Localization of Akt in PC3 cells after treatment with Ruthenium Red part 2. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

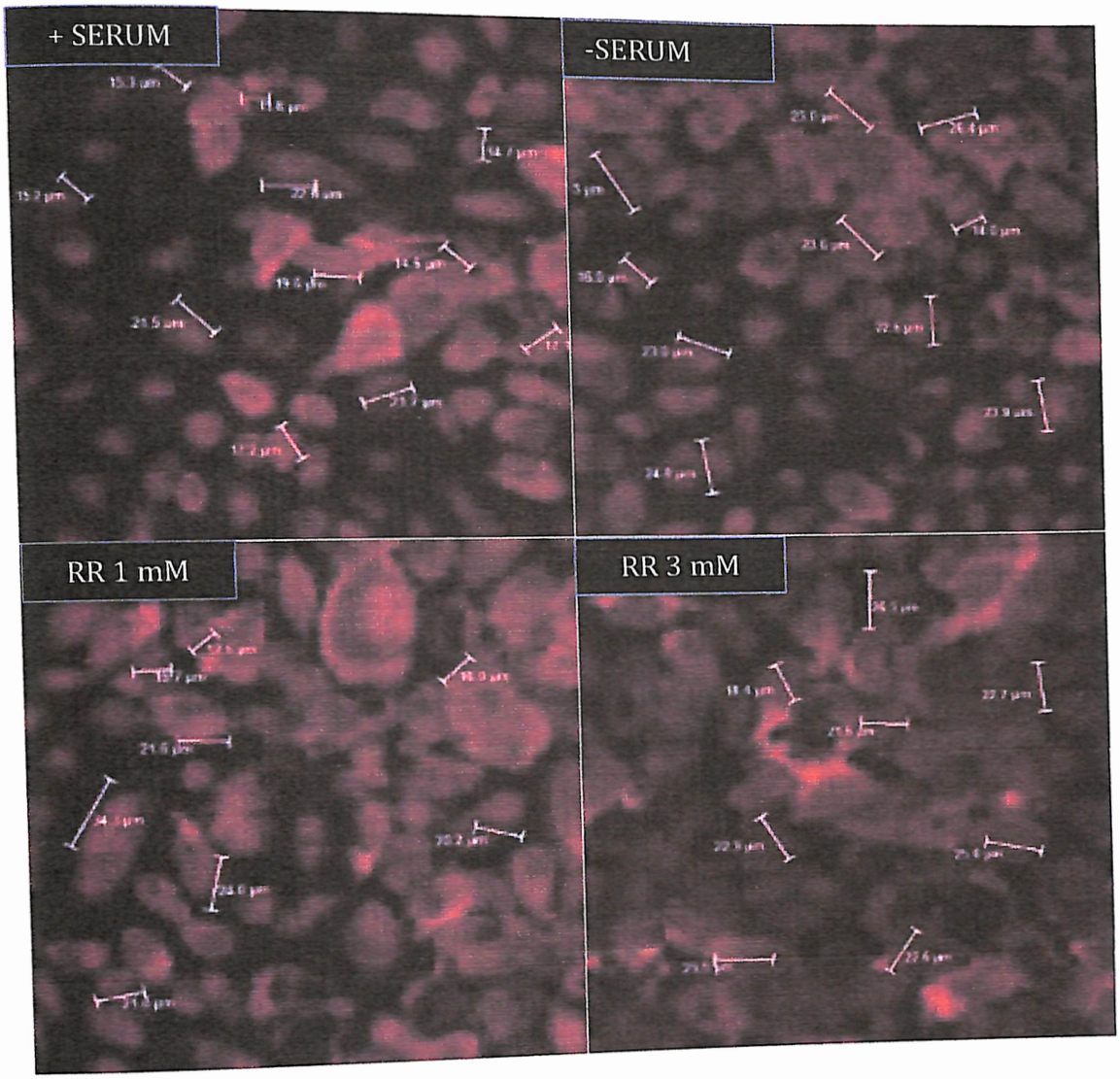

Figure 28: Closer view of Akt in PC3 cells treated with Ruthenium Red part 1. Cells were treated with Ruthenium Red at 1 mM and 3 mM in serum free medium. Cells were also imaged at plus and minus serum. Immunocytochemistry was performed to observe the localization of Akt after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

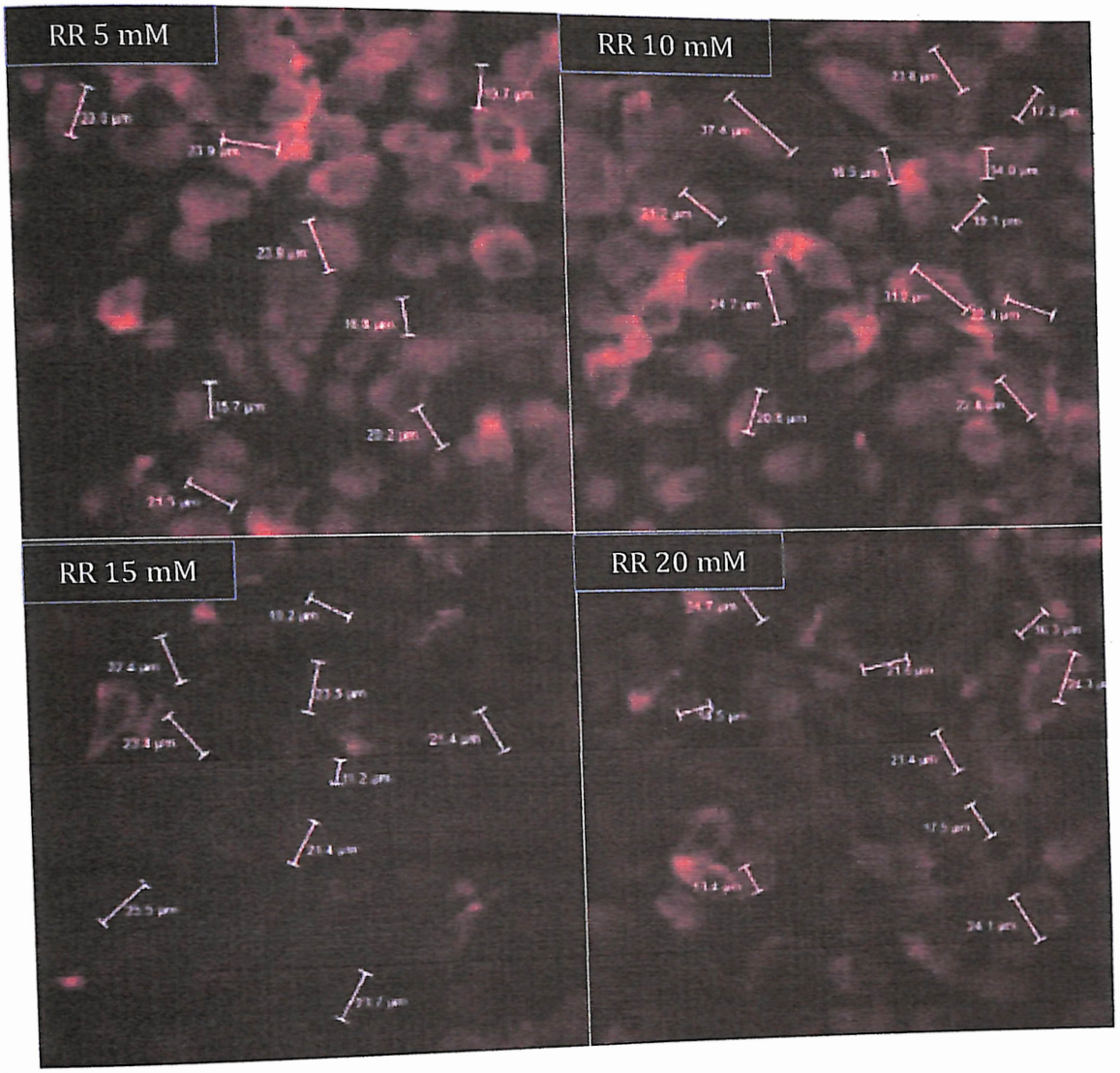

Figure 29: Closer view of Akt in PC3 cells treated with Ruthenium Red part 2. Cells were treated with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. Immunocytochemistry was performed to observe the localization of Akt after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

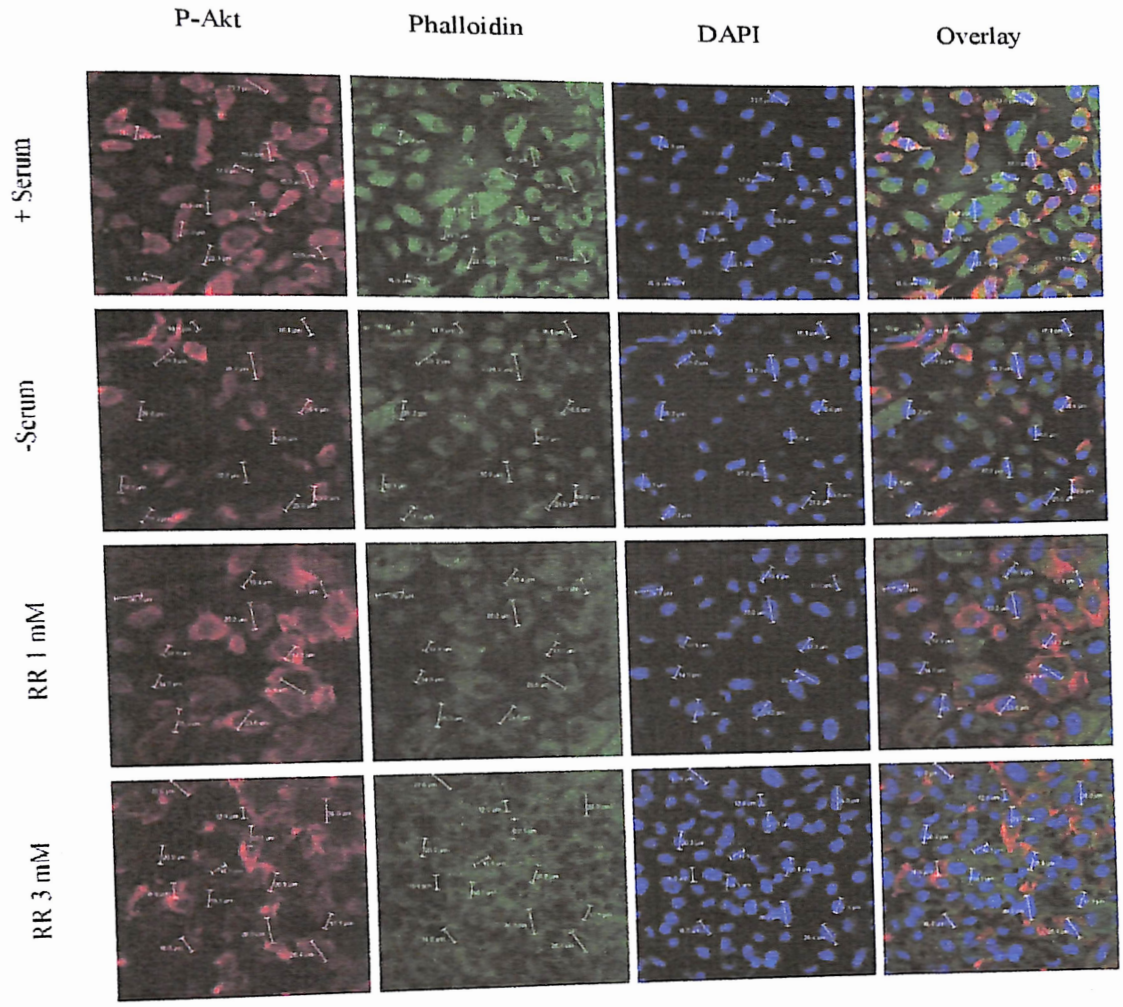

Figure 30: Localization of p-Akt in PC3 cells after treatment with Ruthenium Red part 1. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at a 1 mM and 3mM concentration in serum free medium. Cells were imaged with plus and minus serum controls as well. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using a Leica 5000B Confocal Laser Scanning Microscope.

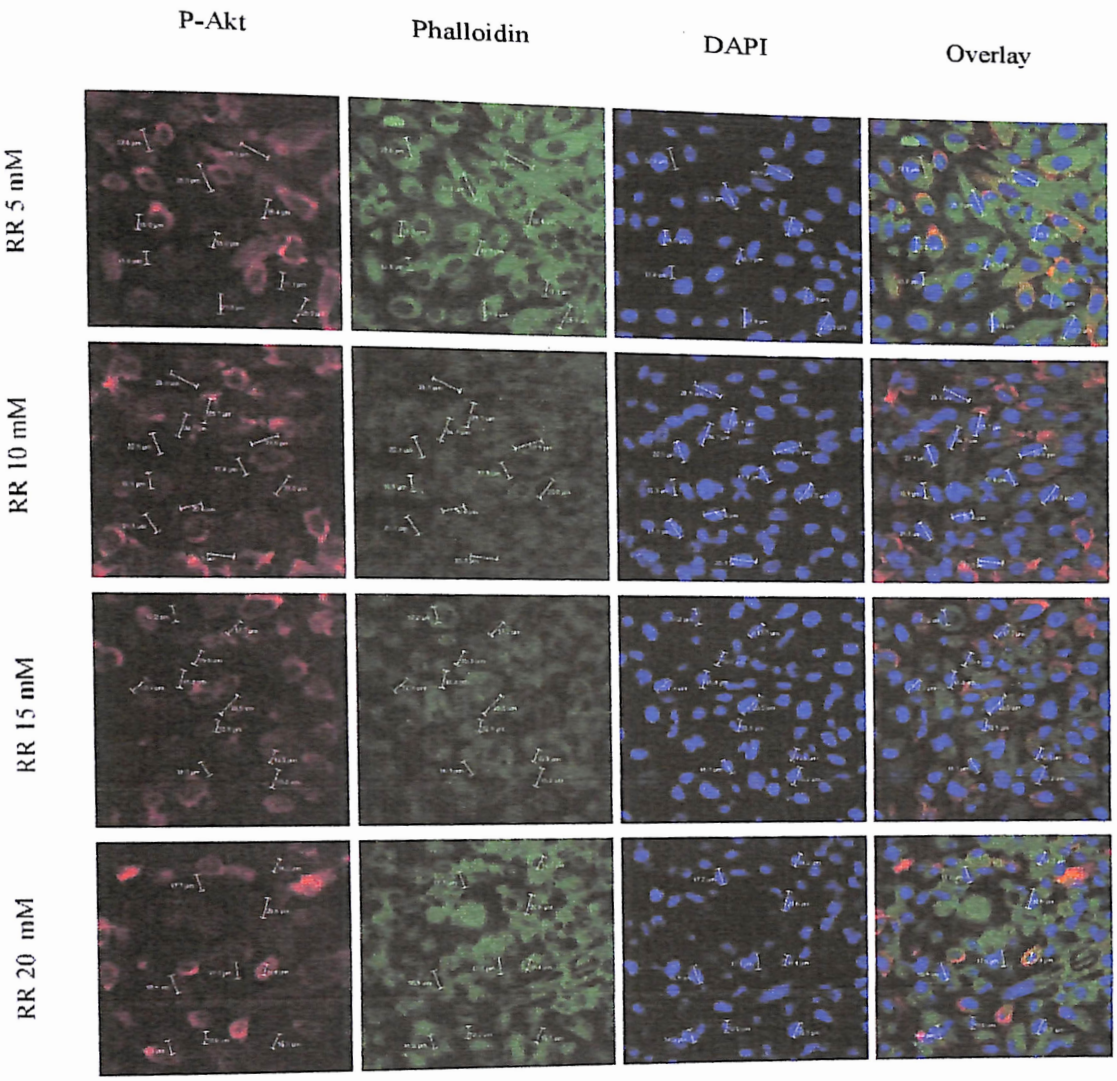

Figure 31: Localization of p-Akt in PC3 cells after treatment of Ruthenium Red part 2. Cells were plated on coverslips, then serum starved overnight. Cells were then treated for 24 hours with Ruthenium Red at 5 mM, 10 mM, 15 mM, and 20 mM concentrations in serum free medium. DAPI was used to stain the nucleus and phalloidin was used to stain F-actin. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

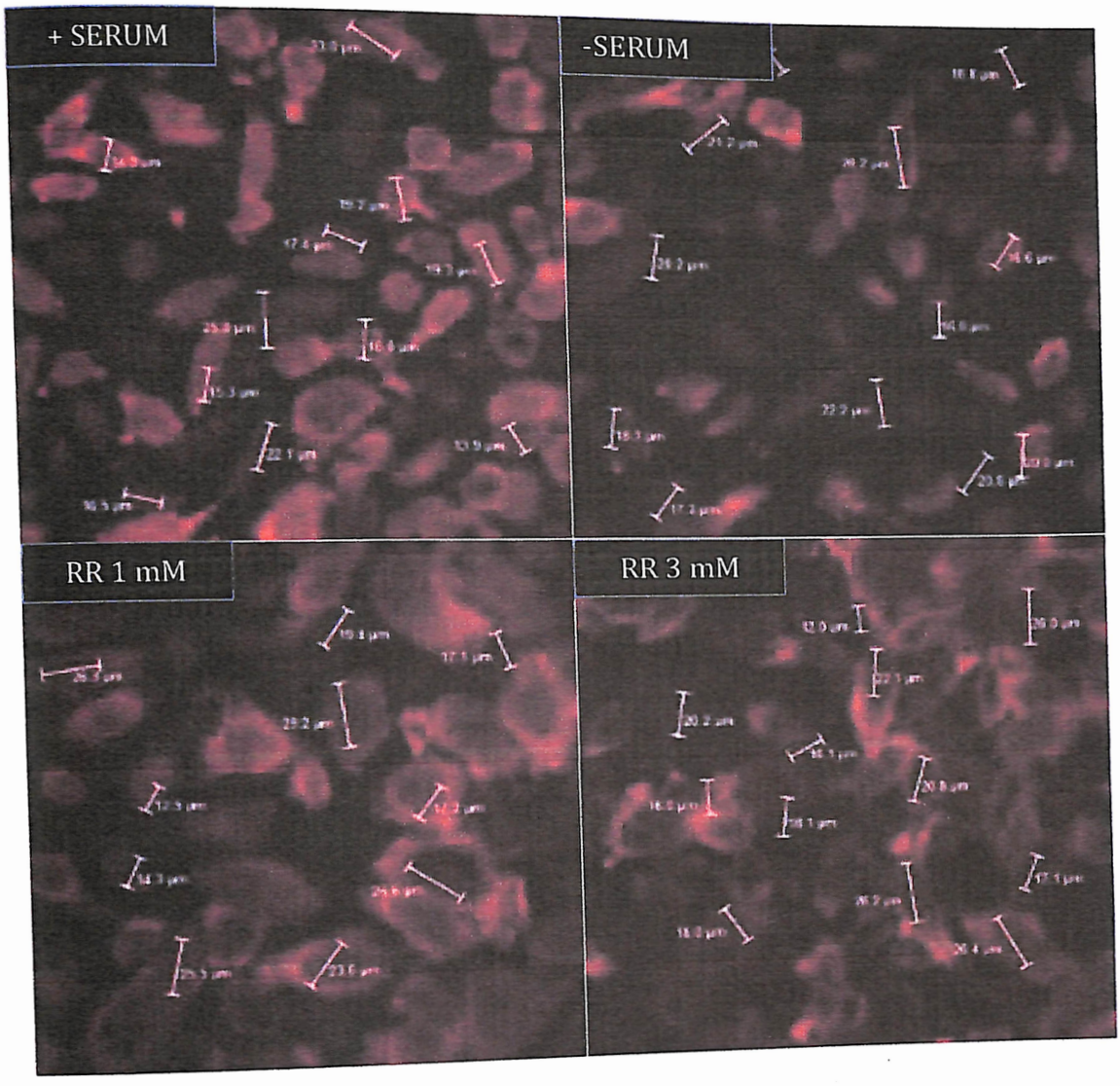

Figure 32: Closer view of p-Akt localization in PC3 cells treated with Ruthenium Red part 1. Cells were treated with Ruthenium Red at 1 mM and 3 mM in serum free medium. Cells were also imaged at plus and minus serum. Immunocytochemistry was performed to observe the localization of p-Akt after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

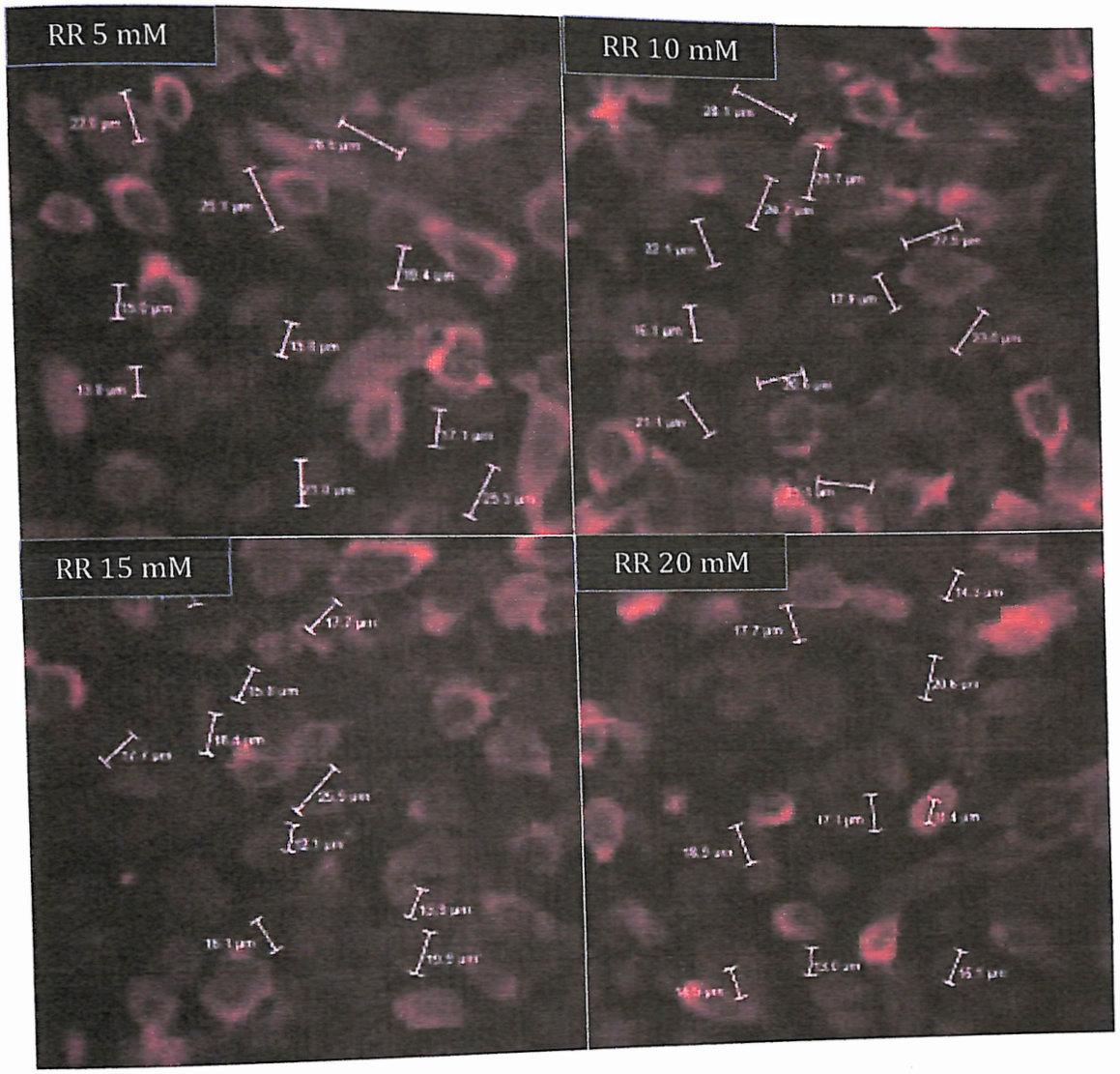

Figure 33: Closer view of p-Akt localization in PC3 cells treated with Ruthenium Red part 2. Cells were treated with Ruthenium Red at 5 mM, 10 mM, 15 mM and 20 mM in serum free medium. Immunocytochemistry was performed to observe the localization of p-Akt after treatment. Images were all taken using Leica 5000B Confocal Laser Scanning Microscope.

Due to the expression level changes of Akt, which regulates apoptosis, and the morphology changes displayed in the scratch assay, we hypothesized that the cells may be going through apoptosis. Therefore, we observed the expression level of caspase to determine if the cells were going through apoptosis (Figure 34 & 35). A half an hour before 24 hours were up, caspase 3/7 at a 3 mM concentration was added to the Ruthenium Red treated medium. This allowed the dye to bind to activated caspase-3 and caspase-7 recognition sequences in the nucleus. We observed that as the concentration of Ruthenium increased, more caspase was activated. Cells showed minimal fluorescence at plus serum, minus serum, 1 mM, and 3 mM, but were not going through apoptosis. An increase in fluorescence began at 5 mM and continued to increase as the concentration of Ruthenium Red increased. Cells at 5 mM, were in early apoptosis phase. After careful analysis of Ruthenium Red treatment at 20 mM, I noticed that cells were going through a process known as cellular blebbing. So, at 20 mM, cells were already dead.

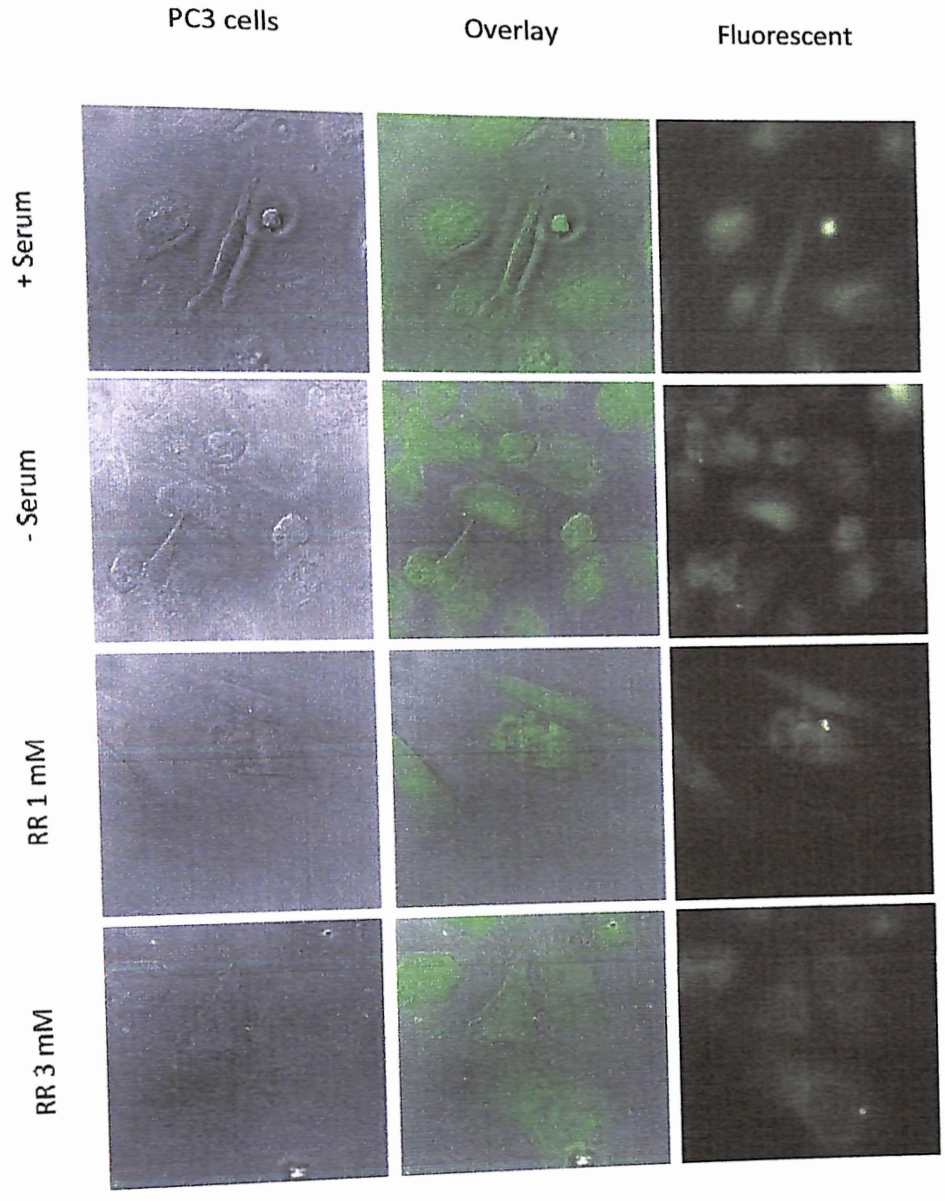

Figure 34: Caspase 3/7 apoptosis assay on PC3 cells determine cellular death part 1. Cells were grown on a cover slip. Ruthenium Red was used as treatment at 1 mM and 3 mM concentration without serum. Cells were also imaged using plus and minus serum. Caspase 3/7 was used at a 3 µM concentration on live cells and then fixed with paraformaldehyde. Cells were imaged using an Olympus IX71 Fluorescence and DIC Microscope. Cells were imaged with the same intensity.

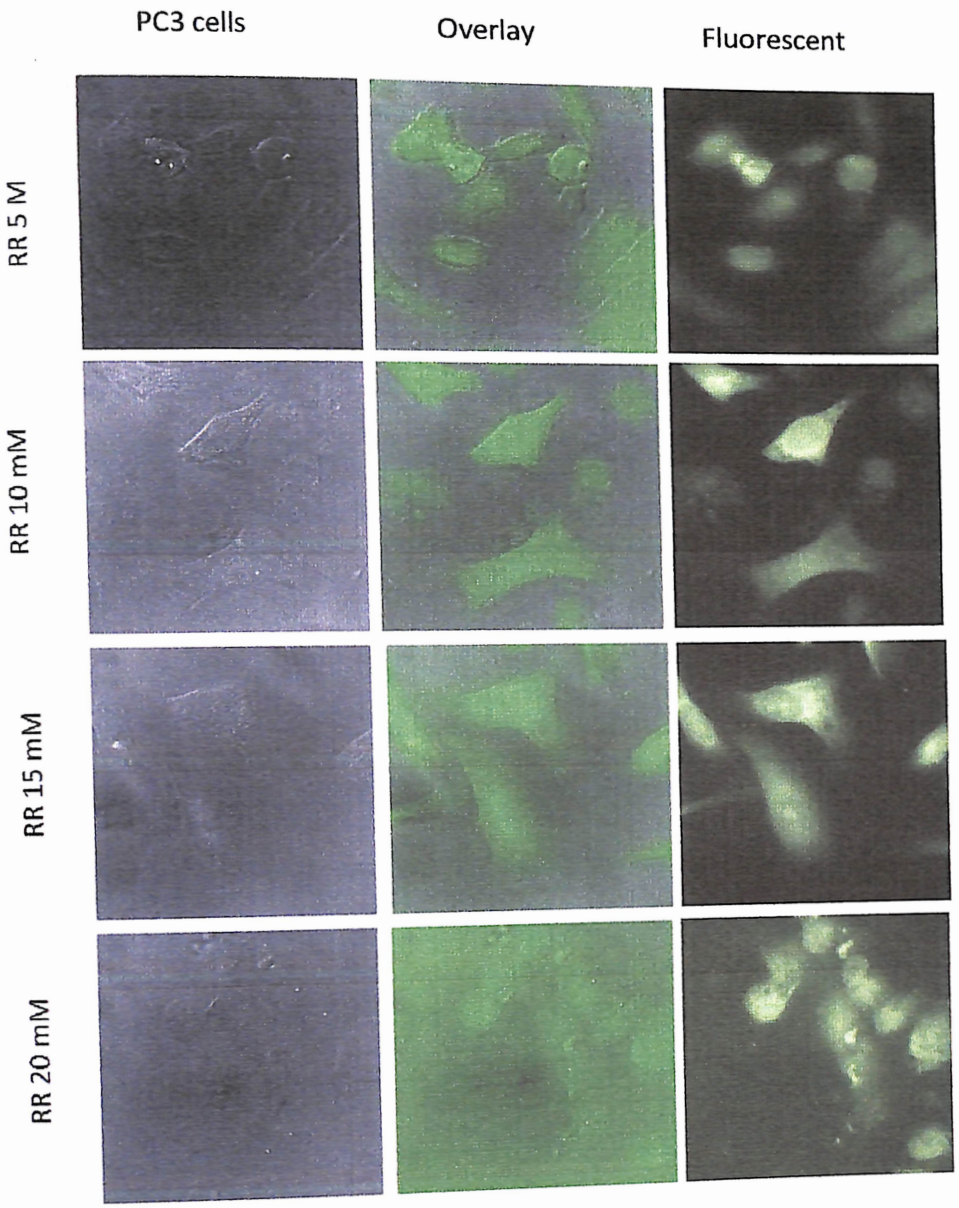

Figure 35: Caspase 3/7 apoptosis assay on PC3 cells determine cellular death part 2. Cells were grown on a cover slip. Ruthenium Red was used as treatment at 5 mM, 10 mM, 15 mM, and 20 mM concentration. Caspase 3/7 was used at a 3 µM concentration on live cells and then fixed with paraformaldehyde. Cells were imaged using an Olympus IX71 Fluorescence and DIC Microscope. Cells were imaged with the same intensity.

CHAPTER 5: DISCUSSION

Prostate cancer is commonly diagnosed and is the second leading cause of death in men in the United States. Most men receive radiation therapy as their treatment, as well as sometimes receiving a combination treatment of surgery. Unfortunately 30-40 percent of patients do not have success with radiotherapy, causing cancer cells to repair damages that are caused by radiation resulting in a more aggressive form of cancer. The cells also have the ability to adapt to and resist treatment (Skvortsova, I., 2008). Recently TRP channels have been suggested to play a role in prostate cancer progression. Current treatment using radiotherapy can result in prostate cancer to come back in a more aggressive form. Targeting TRP channels may become an alternative treatment for prostate cancer. TRP channels are described to be in the genitourinary tract or prostate. These channels are said to play a role in the normal physiology of the prostate as well as prostate cancer (Haute, C., 2010). Four TRP channels that are of interest due to their involvement of cellular survival, migration, as well as proliferation are TRPV2, TRPV4, TRPV6, and TRPM8.

TRPV2, which is the first channel of interest in our study, was shown to be expressed in my PC3 cells lines in western blotting (Figure 8). Its role in prostate cancer involves stimulation of cancer cell migration as well as cellular survival. TRPV2 is responsible for mediating the ability of prostate cancer cells to migrate and invade other

tissue in the body. This may occur by TRPV2s direct regulation of different proteins such as ERK. TRPV2 has been distinguished as a unique channel that is involved in castration-resistant PCa. TRPV2 is now viewed as a channel that is involved in development and progression of prostate cancer as opposed to before. This channel has been confined to prostate cancer cells with androgen resistance or cells that do not rely on androgens for survival such as PC3 cell lines that were taken from the bones. This indicates that the cells that are not under androgen control anymore may be using TRPV2 as a means to increase their oncogenic potential. In nude mice with silenced TRPV2, expressing PC3s tumors showed significant decrease, which shows its role in tumor survival (Monet, M., 2010).

For TRPV4, we saw little to no expression in PC3 cell lines (Figure 8). Thus, it is no surprise that there is a lack of published reports confirming TRPV4s role in prostate cancer. It was initially expected to be expressed in prostate cancer cells, due TRPV4 expression in bone cells and its role in migration (Pla, A., et. al. 2013). TRPV4 was thought to play a role in prostate cancer migration to bone. TRPV4 is responsible for normal bone remodeling by regulating Ca^{2+} influx. It is also responsible for intracellular Ca^{2+} signaling that is required for osteoclast maturation (Masuyama, R., et. al., 2008). TRPV4 is also expressed in breast tumor-derived EC and loss of TRPV4 in this tissue slows migration of breast cancer cells. TRPV4 in these breast cancer cell lines is important in cell migration (Pla, A., et. al. 2013).

In PC3 cells, TRPV6 was expressed (Figure 8). TRPV6 is an epithelium TRP channel that is highly selective for calcium. The TRPV6 channel was not shown to be

expressed in normal prostate tissue, but has since been shown to be expressed in metastatic prostate cancer. The expression level has also been correlated with the Gleason score which makes it a stronger marker for of tumor progression and invasion into distant tissue. The role of TRPV6 in prostate cancer has been linked to proliferation, cellular survival, as well as cell migration (Haute, C., 2010) In fact, TRPV6, increases as the aggressiveness of the cancer increases, which is why it correlates with the Gleason score. Biopsies were obtained from those with prostate cancer to observe the amount of TRPV6 mRNA. This leads those to believe that TRPV6 can be used as a marker in prostate cancer to predict the clinical outcome. PC3 as well as LNCaPs also show increased expression of TRPV6 mRNA compared to benign tumors as well as normal cells. TRPV6 has not only been linked to cellular proliferation, but also plays a role in apoptosis resistance (Lehen'kyi, V., et. al., 2012).

The other TRP channel for which we observed expression in PC3 cell lines was TRPM8 (Figure 8). TRPM8 mRNA is expressed in the prostate epithelium. The expression levels of TRPM8 increases in cancerous cells as opposed to normal cells. It has also been reported that TRPM8 expression is higher in malignant prostate cancer cells compared to nonmalignant tissue (Haute, V., et. al., 2010). Although TRPM8 is expressed in prostate cancer, it is not clear what the exact role of TRPM8 is in cancer. Some believe that it plays a role in cell proliferation and others believe it plays a role in migration and cellular survival. They believe this because when using siRNA induced silencing of TRPM8 in LNCaPs, cells began to undergo apoptosis. This is an indication that LNCaPs are surviving

based on TRPM8, and it may be the same for PC3s (Haute, V., et. al., 2010). The expression of TRPM8 mRNA in malignant cells suggest it may have a role in cancer migration to distant organs. It is also expressed in early prostate tumors that are very well differentiated. Inhibition of TRPM8 channel reduces the ability of cells to proliferate (Valero, M., et. al., 2012). The most important fact about inhibiting TRPM8 is that it does not affect the non-tumor cells, indicating that TRPM8 is important for cancerous cells proliferation and not normal cell proliferation (Valero, M., et. al., 2012). As prostate cancer cells begin to become androgen independent, TRPM8 expression decreases (Valero, M., et. al., 2012).

Based on TRPV channels expression in PC3 cells, it was interesting to determine where the TRP channels were localized in PC3 cells.TRPV2 in my ICC results is co-localized with phalloidin of PC3 cell lines (Figure 9). In other studies, TRPV2 has been shown to be expressed in the plasma membrane as well as the early endosome in PC3s (Liberati, S., et. al., 2014). The activation of TRPV2, may be caused by growth factors. Once TRPV2 is activated, it causes PI_3 kinase to translocate to the plasma membrane. TRPV2 translocates to the plasma membrane from the intracellular compartments or the endosomes. This translocation results in death signals from downstream pathways such as Akt. TRPV2's activation as well as translocation to the plasma membrane, is dependent on calcium. This results in increased PC3 prostate tumor cell migration. The PI3,4-K pathway is involved in this (Liberati, S., et. al., 2014).

TRPV4 in my ICC results is barely expressed, but localization does not seem to be on the plasma membrane (Figure 9). There is no report in the literature about the

localization of TRPV4 in prostate cancer cells. This suggests that maybe TRPV4 hasn't been found by others to play a role in prostate cancer progression. TRPV6 localization in my ICC results does not seem to be on the plasma membrane but seems to be inside of the cell as well. However in literature, TRPV6 is a plasma membrane calcium channel (Abel, M., et. al., 2005). In my ICC results TRPM8 seems co-localized with actin as well as in the cytosol of the cell. There has also been some indication that TRPM8 is also expressed in the ER membrane and are regulated by the AR. TRPM8 is involved in prostate cells' secretory function as well as sperm motility and fertilization (Bidaux, G., et al., 2007).

Since TRP channels are expressed in my PC3 cell lines, we decided to use an inhibitor that targets all four channels of interest and perform a scratch assay. I feel that Ruthenium Red may be affecting other intracellular molecular processed within the cell due to the slower growth rate of PC3 cells into the wound area. Literature suggests that when receptors such as the IGF receptor are activated, calcium ion channels such as TRP channels are activated and calcium flows inside the cell (Tai, S., et. al., 2011. Calcium is then able to activate intracellular pathways such as ERK and Akt, which are also involved in cellular migration as well as cellular survival. I believe that the IGF receptor is able to control the influx of calcium within the cell using TRP channels, which controls migration and cellular survival through ERK and Akt. I believe that when these TRP channels are inhibited, Akt as well as ERK are affected (Tai, S., et. al., 2011).

The Ras/Raf/MEK/ERK pathway is expressed in human cancer including prostate cancer. An increased expression of ERK in prostate cancer is linked to advanced prostate

cancer (McCubrey, J., et. al., 2006). PC3 cells actually express lower levels of activated ERK compared to their expression of Akt. Since TRP channels are expressed in PC3 cells lines and the channels interact with IGF receptor, it was interesting to see what treating the cells with Ruthenium Red would do to the expression levels of Akt and ERK. Both Akt and ERK are part of the IGF intracellular pathway and both interact with calcium. Activation of Akt and ERK leads to cellular survival as well as proliferation and migration of PC3 cells. Since IGF, Akt, and ERK interact with calcium, we were curious to see if Ruthenium Red would have an effect on the expression levels of Akt and ERK. ERK expression levels in PC3s after treatment with Ruthenium Red begin to decrease. ERK expression also leads to prevention of apoptotic responses. The activation of the Ras-Raf-MEK-ERK pathway prevents apoptosis by phosphorylating anti-apoptotic Mcl-1 protein and pro-apoptotic Bim protein. Also, since both Bad and caspase-9 are phosphorylated by Akt, this indicates that Raf/MEK/ERK and PI3K/Akt pathways are carry out crosstalk which can prevent apoptosis (McCubrey, J., et. al., 2006).

ERK localization in PC3 cells was important to study (Figure 16-23). When calcium enters the cell, the Ras/Raf/MEK/ERK pathway is activated. ERK then translocates to the nucleus when activated, and acts as a transcription factor, activating the other factors that are involved in cellular migration. The other transcription factors that are activated are Ets-1, c-Myc, Elk-1, or NF-Kb. ERK activation can also lead to the phosphorylation of cytoplasmic or nuclear kinases such as MNK2, MPKAP-2, RSK, or MSK1 (Rodríguez-Berriguete, G.,et. al., 2012). The correlation between ERK and actin is

the migratory abilities that cells have from both. ERK is responsible for activating many factors involved in cellular migration. In order for cells to migrate the actin cytoskeleton has to undergo reorganization. This is triggered by stimuli such as growth factors and cell matrix adhesions. The signal from ERK also plays a role in this. ERK phosphorylates an F-actin cross-linking protein, called EPLIN, which controls actin organization and cell motility (Han, M., et. al., 2007). ERK in ICC results instead do not translocate into the nucleus when treated with Ruthenium Red, but begin to co-localize with actin (Figure 14) Phosphorylated ERK displayed the same results (Figure 15). This is important because it means that with treatment, ERK does not translocate into the nucleus to act as a transcription factor to promote cellular migration (Andreadi, C., et. al., 2012). Instead of translocating to the nucleus, my results with ERK in ICC seems to show co-localization with actin. ERK as well as actin are both important in cellular migration. Actin is involved in processes such as motility, migration, division, endocytosis, as well as gene expression due to actin polymerization and actin remodeling. Actin is regulated by many processes through actin-binding proteins. ERK is important in actin dynamics because it is required for the formation of stress fibers and proper assembly of focal adhesions. Focal adhesions are important sites of tight adhesions between the membrane as well as the cytoskeleton (Houle, F., et. al., 2003).

Akt as well as phos-Akt did show some change in expression levels by increasing with lower levels of treatment of Ruthenium Red and the then decreasing with higher levels of Ruthenium Red treatment (Figure 24-33). We wondered why Akt expression seemed to

increase and then decrease. We found that PC3 cells have the ability to up-regulate molecular mechanisms after treatment to induce or avoid survival. The result of this are cancer cells that are more aggressive than before (Skvortsova, I., et. al., 2008). Akt is important due to its involvement with calcium. Calcium has the ability to activated PI3 kinase, which then binds to the PH domain of Akt causing Akt to become phosphorylated in osteoblasts. The phosphorylation of Akt when it is available in osteoblasts and could very well give the same results for PC3 cells (Danciu, TE., et. al., 2003). After treatment of PC3s with Ruthenium Red, the localization of Akt seemed to change. In my ICC results, Akt as well as phosphorylated Akt seemed to be co-localized with actin and the cytosol of the cell. There are three isoforms of Akt, Akt1, Akt2, as well as Akt3. It has been suggested that the activation of PI_3K causes Akt to be translocated to the plasma membrane's inner leaflet (Santi, S., et. al. 2010). This is done by PI_3K phospholipid products binding to the PH domain of Akt. Once Akt translocates to the membrane, it is phosphorylated and activated by upstream kinases such as phophoinositide-dependent kinase-1. Akt is activated and then phosphorylates other cytosolic and nuclear substrates. These substrates are involved in promoting cellular survival, cell cycle progression, as well as regulation of cell growth (Gervais, G., et. al., 2006). There are other theories behind the location of Akt as well. New information tells us that Akt1 is found in the cytoplasm, Akt2 in the mitochondria with a small amount its isoform located in the golgi apparatus, and Akt3 in the nucleus (Santi, S., et. al. 2010).

In the scratch assay we noticed morphologic changes in the cells, so we did an apoptosis assay. Morphologic changes included shrinkage of the cells as well as fragments of apoptotic bodies (Figure 34 & 35) Cells treated at concentrations of Ruthenium Red above 5 mM began the apoptosis process. Caspases such as caspase-3 and 7 are able to act as effectors that are involved in the execution phase of cellular death. These effector caspases are activated by other upstream caspases that initiate apoptosis such as caspase-8 and 10. The noticeable morphology changes of cells going through apoptosis may be caused by effector caspases (Johnson, VL., et. al., 2000). Cells going through apoptosis usually begin to shrink and loose cell-to-cell contact. Next, the chromatin condenses to the periphery of the nucleus, followed by nuclear fragmentation, and DNA that is ingested. The cell then goes through blebbing, which is when small bulges of the cell surface occurs. Then cell fragmentation and phagocytosis by macrophages occurs (Coleman, ML., et al., 2001). Membrane blebbing began to occur with the Ruthenium Red treatment of 20 mM. It results from alterations of the microfilament organizations as a result to insult. During membrane blebbing, there are increased actin-myosin contractile forces, loss of focal adhesion, as well as new actin polymerization. This study shows that at 5 mM, cells were at early stages of apoptosis, and as the concentration increased, cells began dying off. At 20 mM, cells were at late stage of apoptosis. Ruthenium Red at higher concentrations in PC3 cell lines cause cellular death (Houle, F., et. al., 2003).

In conclusion, PC3 cells are a representative of advanced stage of prostate cancer, and TRPV6, Akt, as well as ERK correlate with the Gleason score. The Gleason score is

important in prostate cancer because it measure the aggressiveness in prostate cancer cell lines. As the aggressiveness of cancer increases TPV6, ERK, as well as Akt expression increases as well. We found that treating PC3 cells with Ruthenium Red affected the localization of Akt as well as ERK. Literature suggests that prostate cancer is able to up-regulate certain molecular processes, in order to avoid cellular death, which is probably why Akt seemed to slightly increase and then decrease. In other words, when prostate cancer is treated, to avoid apoptosis we believe that it is able to manipulate certain processes such as Akt by phosphorylation to avoid cellular apoptosis. This could be one reason why prostate cancer can come back more aggressive in patients who have gone through treatment. Akt did not seem to translocate above actin indicating that it was not being activated to prevent the cells from undergoing apoptosis as I increased the concentration of Ruthenium Red. ERK localization to the membrane was important as well due to it not localizing to the nucleus like we expected. This indicates that with Ruthenium Red treatment, migration may be prevented, since ERK would not be able to activate other factors that play a role in cellular migration through translocation into the nucleus. Although, I am not convinced that ERK plays a major role in migration due to lower levels of phosphorylated ERK in both western blotting and ICC. I do believe that it plays a minimal role. In the end the scratch assay also showed that migration of cells was affected, which could slow cells migratory abilities down. The apoptosis assay is important because after 24 hours of treatment it shows that the cells are able to go through apoptosis at higher concentrations. At this point Ruthenium Red could be a potential therapeutic treatment for

men with prostate cancer, since most treatments available result in a more aggressive from of prostate cancer once it comes back. Although Ruthenium Red seems like a potential treatment in the near future, due to TRP channels expression in normal cells such as the bones, nervous system like the brain and spinal cord, the muscle, eyes, and other organs, we are not sure how this inhibitor will affect other organs of the body. This research project does show promise, but warrants many future studies before looking in the direction of therapeutic studies.

CHAPTER 6: FUTURE DIRECTIONS

After testing the expression of cells with four TRP channels of interest, we came to the conclusion that since three of the channels are expressed in PC3 cells we would continue investigating those TRP channels. We are interested in seeing how each individual channel affects the migration as well as cellular survival of PC3 cells after treatment. To make the study more specific, we are interested in looking for inhibitors that inhibit individual TRP channels. TRPV2 which is expressed in PC3 cells, has a specific inhibitor of interest known as Tranilast. This inhibitor inhibits responses that are mediated by TRPV2. A known TRPV6 inhibitor is 2-APB, which inhibits Ca^{2+} signaling. TRPM8, also showed great expression in PC3s and in the future we would like to use a specific inhibitor known as TC-I 2000 or even AMTB hydrochloride. All of these inhibitors of interest affect the TRP channels individually and will be used in the future.

We also were able to see the localization of TRP channels in PC3 cells after performing ICC. Since we used Ruthenium Red in the other experiments and it caused localization differences in Akt as well as ERK, we are interested in seeing if localization of TRPV2, TRPV6, as well as TRPM8 after treating cells with Ruthenium Red is affected as well. We also would like to look further into seeing if these channels that are expressed in PC3 cell lines are actually active.

Since AKT and ERK expression levels were barely affected by Ruthenium Red treatment, we are interested in seeing if treating cells with the inhibitors for specific TRP channels also affect the expression levels of Akt and ERK. I would also like to see if the localization of both is affected by specific TRP channel inhibitors. There are other pathways in cells that are responsible for regulating cellular survival and cellular migration. Examples of proteins involved in cellular survival are AMP-activated protein kinase, lysophosphatidic acid, as wel as other proteinsExamples of proteins involved in migration are ubiquitin associated protein 2-like, amyloid precursor protein, as well as angiomotin.

Once the IGF receptor is activated, calcium enters the cell, activates PI_3K, and then Akt is phosphorylated at the plasma membrane. Calcium influx also activates the Ras/Raf/MEK/ERK pathway which causes ERK to translocate into the nucleus. Due to the IGF receptors involvement, I am interested in adding the IGF receptor to the study to see if the expression level as well as localization of IGF changes with treatment of Ruthenium Red. I am interested in seeing which TRP channels the IGF receptor is specifically cross talking with in PC3 cells by using those specific inhibitors mentioned earlier. I also would like to see what would happen to the TRP channel expression levels of interest, TRPV2, TRPV6, and TRPM8, after treating the cells with anti-IGF. Since IGF is responsible for the influx of calcium, it would also be interesting to determine if inhibiting the IGF receptor

is responsible for turning TRP channels on and off through calcium imaging. I am interested in the localization of the TRP channels after treating the cells with anti-IGF as well.

Since cells' migratory ability decreased after treatment with Ruthenium Red, it may be a good treatment to continue with. The concentrations of 1 mM and 3 mM do not cause PC3 cells to go through apoptosis, but 5 mM, 10 mM, 15 mM, as well as 20 mM are in different stages of apoptosis. Although the higher concentrations of Ruthenium Red cause cellular apoptosis, it could be a good treatment, but could also be too toxic for the cells. Future experimentation will be done to observe toxicity to the cells using Ruthenium Red. I am interested in using these concentrations in mouse models in the future. That future experiment will include injecting PC3 cells into the mice and then treating them with Ruthenium Red at those concentrations to see if the tumor size would decrease. If other inhibitors of TRP channels of interest show promise, I would like to use those in mouse models as well.

The scratch assay showed that the cell migrated less into the wound as treatment with Ruthenium Red increased. Although it showed that the Ruthenium Red treatment did slow down migration, I think that a better assay to use would be a migration assay using a membrane that makes it easy to count the cells that have migrated. I believe that this will

be a more accurate method then the traditional scratch assay. In future experiments using other inhibitors, I will use this assay over the scratch assay.

The bigger picture of this study is to see how using this inhibitor as well as other inhibitors targeting TRP channels will affect the tumor size as well as the body's' physiological pathways. This is important because the therapeutic treatments available today are successful in the beginning, but later cause cancer to come back in a more aggressive form. This is due to cells that are undifferentiated after treatment. The more aggressive form of cancer results in a death rate of 27% of patients (Rukstalis, D.,2002) Finding other treatments such as Ruthenium Red and other TRP channel inhibitors may be a better overall treatment for prostate cancer patients. Treatment of interests includes the ability of the inhibitors to cause cellular differentiation as well as cellular death if not too toxic for the cell. Ruthenium Red as well as other inhibitors that will be used in the future show promise as therapeutic treatment.

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EDUCATION

Delaware State University, Dover, DE 08/2012-Dec. 2014

Master of Science (expected December 2014)

Major: Biological Sciences

Studies focused in assisting in experimental design for a variety of research projects in cancer biology, follow protocols involving reagent preparation, assisted in analyzing data and manuscript preparation, and multi-tasking with multiple projects. Also participated in training new students in the lab with various experiments. Additional areas of focus within this degree included effective communication, and critically thinking of new ideas for projects. Major assignments consisted of writing technical documents and PowerPoint presentations on bench work performed during my graduate academic career.

Delaware State University, Dover, DE 08/2008-05/2012

Bachelor of Science

Major: Biological Sciences/Pre-professional

Studies focused in development and implementation of skills necessary to be successful in the field of Biological sciences. Training included learning techniques in lab, as well as attending large conferences to present research. It also included presenting research through power point to finish the degree.

POLYTECH, Woodside, DE May 2008

Medical Assisting (Technical Diploma)

Included learning how to take blood pressure, greet patients, answer the telephone, greet patients, update medical records, scheduling patients, taking medical history, explain procedures to patient, and assist physician during exams. Did clinical rounds at Kent General Hospital as well as OB/GYN office, and primary care physician office.

JOB EXPERIENCE

Dietary Aide

Milford Center (Genesis Healthcare) Milford, Delaware 2006-2011

Responsibilities included communicating food orders for residents to the cook, following resident diet restrictions, organizing the dining room, sterilizing surroundings, operating the dish machine, Mentoring and training new employees, helping with inventory, and other various tasks.

Supplemental Instructing/Tutoring

Delaware State University 2009-Present

Responsibilities included assisting students in learning various areas of biology. Communicating to students how to succeed in classes and filling in information that students could not comprehend. Responsibilities also led to mentoring students after tutoring experience and having a mentee student relationship.

RESEARCH EXPERIENCE

The effects of IGR IR inhibitors on the expression of Akt, IGF, and Map Kinase in prostate cancer cells (PC3), andThe effects of TRP inhibitor Ruthenium Red on the IGF pathway in prostate cancer cells (PC3)

Dr. Cynthia van Golen,
Delaware State University, Dover DE.
06/2011-08/2011

- Cell culture Techniques with Prostate cancer (PC3, LNCaP, C42, C42B), Bone cancer (RAW 264.7, MCT3-E1 subclone 4 Preosteoblasts, Osteoblasts SV40 large T antigen transfected osteoblast), and Neuroblastoma (IMR-32, SH, and SY5Y)
- Conduct research on the expression level changes of the IGF receptor, AKT, and ERK after treatment with Ruthenium Red using western blotting.
- Conducting research on the localization of AKT, ERK, and the IGF receptor after treatment with Ruthenium Red using immunocytochemistry
- Grew PC3 cell lines to conduct a scratch assay treated with Ruthenium Red

The expression of TRPV in prostate cancer cell lines LNCaPs and C42 with treatment of IGF and TRP inhibitors

Dr. Randall Duncan,
University of Delaware, Newark, DE. 06/2011-08/2011

- Conducted research using LNCaPs treated with Ruthenium Red and RN1734
- PCR conducted on LNCaPs treated with both inhibitors

- Scratch assay conducted on LNCaPs treated with Ruthenium Red and RN1734

The effects of IGF on prostate cancer growth and progression
Dr. Cynthia van Golen
Delaware State University, Dover, DE 06/2010-05/2011

- Tested the expression levels of AKT, and ERK on prostate cancer cells after treatment with anti-IGFIR through western blotting

Investigating Intra-protein Interactions in Cytohesin-2
Lorraine Santy,
Penn State University, Pennsylvania 06/2009-08/2009

- Studied the coiled coil domain of cytohesin-2
- Transfected bacteria with DNA and then Purified proteins to isolate protein of interest
- Performed western blotting and used coomasie blue staining

IGF in prostate cancer
Undergraduate research with Dr. Cynthia van Golen,
Delaware State University, Dover, DE. 01/ 2010-05/2010

- Learned tissue culture prostate cancer cells
- Studied LNCaPs using a cell death assay after treatment with anti-IGFIR

EXTRACURRICULAR ACTIVITIES

- Peer leader team leader (PLTL) for General biology — January 2011-May 2011
- Tutor for General Biology, Genetics, Cellular Biology, and Human Anatomy and Physiology, Human Biology — September 2011-Present

AWARDS

- DASH Scholar
- Cum Laude, B.S. Graduate
- Delaware State University Graduate Symposium Oral Presentation
- Dean's List

PERSONAL QUALITIES

- Excellent troubleshooting skills
- Loves to plan ahead
- Excellent people skills
- Excellent at training people
- Excellent communication skills
- Skilled in multi-tasking
- Able to adapt to any situation
- Can stand for long lengths of time
- Persistent
- Creative problem solver
- Excellent work ethic
- Energetic attitude
- Extremely productive
- Excellent organizational skills
- Goal-oriented
- Great at coming up with ideas
- Can handle heavy workloads
- Ready to learn anything