8-2011

CHANGES IN EXPRESSION OF AKT PATHWAY PROTEINS FOLLOWING TREATMENT WITH rG3 IN VITRO

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CHANGES IN EXPRESSION OF AKT PATHWAY PROTEINS FOLLOWING TREATMENT WITH rG3 IN VITRO

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

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by
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August 2011

Accepted by:
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ABSTRACT

To assess changes in AKT pathway signaling, a recombinant protein of the G3 domain of rat laminin-5 (rG3) that specifically binds the alpha subunit of integrins α6β1 and α6β4 expressed on cancer cells (e.g., MDA-MB-231) was produced. This recombinant protein is believed to interrupt the intracellular signaling events of the AKT pathway, causing a decrease in proliferation and survival of cells after treatment. Viability assays confirmed an apoptotic effect of rG3 on cells in a dose-dependent manner. However, data from gene expression studies of Caspase-9, GRB10, and CDKNIB proved non-conclusive that rG3 is acting upon gene expression, leading to the further investigation of the AKT pathway and proteins involved in this signaling cascade. P53 and phosphorylation of AKT, NFkB/p65, and IKKαβ were evaluated after treatment with rG3 at 0, 3, 6, 9 and 12 hours. Results show significant differences in protein expression for these proteins in cells treated with rG3 compared to untreated cells. Significantly higher levels of AKT and phosphorylated AKT were seen in untreated cells, indicating the inhibitory effect the rG3 protein has on this pathway. Both IKKαβ and the phosphorylated IKKβ catalytic subunit were expressed at a significantly higher level in untreated cells, as were the levels of phosphorylated nuclear NFκB. These results also indicate an inhibition of downstream proteins of the AKT cell survival pathway with rG3 treatment. Cytosolic NFκB, however, was expressed at significantly higher level in cells treated with rG3 when compared with untreated cells because the majority of this protein in actively proliferating untreated cells is in the phosphorylated form. The greatest change was seen in expression of the pro-apoptotic protein, p53. In treated cells, this

protein was expressed at higher levels than in the untreated cells, especially at 9 hours after treatment, indicating the large impact rG3 treatment has on the AKT pathway and its ability to significantly reduce cell viability through specific signaling events.

DEDICATION

I would like to dedicate this work to my advisor, mentor, and friend, Dr. Thomas R. Scott. It is because of his support that I have had this research experience and I cannot thank him enough for everything he has done for me, even with all of the other responsibilities he holds as Dean of CAFLS. I would also like to dedicate my work to Dr. Heather Walker, who has provided support, knowledge, friendship, encouragement, and optimism when I needed it most. Her character keeps our lab running smoothly and my success as a scientist and student is largely due to the two wonderful mentors I have been blessed with during my time at Clemson.

ACKNOWLEDGMENTS

In addition to Dr. Scott and Dr. Walker, I would like to thank the other members of my committee, Dr. Tamara McNealy and Dr. Charles Rice. Their enthusiasm and support guided my research in a successful direction and I am very grateful they took the time to help me through this experience. I would also like to thank Dr. Marcy Owens for her extensive help, teaching, and technical support in the lab.

I would not have gained so much research experience prior to my graduate experience if it had not been for Dr. Jonn Foulk allowing me to spend summers, and eventually years, working for him at the USDA Cotton Quality Research Station. My love of research came in part from the experience in data analysis and problem solving I gained from this job, and he has gone above and beyond to help me in any way he can.

Of course, I couldn't have made it without the help and support of my wonderful network of family and friends. Thank you to my family for being excellent role models, supporting me, providing me with home-cooked meals, pushing me to succeed. To Amy Anderson, for being there through long nights in lab, gallons of Einstein's coffee, and both commiseration and celebration, depending on the week. To my group of wonderful friends, especially Joanna Ward, who provided me with shoulders and laughs when I needed both. To Blake Elliott, for his encouragement, patience, and multitude of practical advice. All of you have helped shape me into the person I am, and I share my success with you.

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CHAPTER ONE

LITERATURE REVIEW

The mammary gland is an organ of specific tissue organization that depends on cell-cell adhesion, interactions with the extracellular matrix, and interactions with the intracellular scaffolding of the cell. An important part of the tissue is the basement membrane, which is a sheet-like extracellular matrix that covers the basal portion of the tissue and provides mechanical stability, barriers between different cell types, and contributes to cell differentiation, survival, and migration (Sasaki et al., 2004). Various proteins of this basement membrane promote cell adhesion and migration, including laminins. Laminins interact with specific integrin receptors on the cell surface to regulate various functions of the cell, including tumor invasion. Integrins are heterodimeric transmembrane receptors found at focal adhesions and serve to link the extracellular matrix intracellularly as well as with signaling enzymes that direct the survival, growth, and migration of cells. Integrins, laminins, and the basement membrane are important elements involved in cancer cell invasion. Invasion is known as the penetration of tumor cells into adjacent tissues, which is a characteristic of malignant tumors (Mercurio and Rabinovitz, 2001). By exploiting the functions of integrins, laminins, and intracellular signaling cascade proteins, cancer cells can invade and proliferate in the mammary tissue. The PI3K/AKT signaling pathway is activated via the integrin-laminin association, and in cancer cells, abnormal activity of this pathway is observed.

Laminins

Laminins are trimeric proteins found primarily in the basal lamina. Each laminin molecule forms the common cruciform structure with three chains, α, β, and γ, linked by

disulfide bonds. Five α, three β, and three γ chains have been identified (Kariya et al., 2003). Various combinations of these chains have yield at least fifteen different known isoforms of laminin. The chains intersect to form a structure that can bind to other molecules, with the shorter chains binding to other laminin molecules to form sheets and the longer chains binding to cells and providing an anchor to the membrane. All laminin α-chains contain a globular region at their carboxyl-terminal region that consists of five sub-domains (G1-5), while the β and γ chains have globular domains at the amino terminal regions of the chain. One specific laminin isoform, laminin-5, has unique properties from the other isoforms (Kariya et al., 2003). It consists of α3Aβ3γ2 chains and has been found to promote adhesion, migration, and scattering of cells in culture more vigorously than laminin-1, laminin-2, laminin-4, vitronectin, and fibronectin (Miyazaki et al., 1993). Laminin-5 regulates the adhesion of the epithelium to the underlying connective tissue and keeps it stable by interacting with the integrin cell surface receptors. It also differs from other laminin forms in its subcellular localization and its cellular receptors. It is a large component of anchoring filaments and plays a major role is assembly of the basement membrane (Kunneken et al., 2003). Within the five sub-domains of the C-terminal of this laminin, the G3 domain has been proven to be essential for the unique biological activity of laminin-5 (Kim et al., 2001). Deletion of the G3 domain in recombinant forms of laminin-5 leads to a drastic loss of cell adhesion and motility, proving that this domain has an indispensible function in the activity of this laminin (Hirosaki et al, 2000). Small changes in the structure of the G3 domain also modulate the intracellular signals induced by laminin-5, suggesting that the signaling of laminin-5 specifically relies on the G3 domain (Kariya et al., 2003). Previous studies

have shown the G3 domain to contain at least two sites that regulate cell adhesion and motility in different ways, and activities of laminin-5 are largely dependent on these two sites (Kariya et al., 2003). Laminin-5 has been found localized in the epithelial-stromal interface of many invasive tumors, suggesting that this protein serves as a ligand for migratory carcinoma cells. In malignant transformation, the integrity of types of collagen and laminin-5 is often lost (Lohi, 2001). The integrins α6β1, α6β4, and α3β1 are known receptors for laminin-5, and have been found on invasive carcinoma cell membranes (Lohi, 2001).

Integrins

Adhesion to the extracellular matrix is also mediated by integrins, a class of heterodimeric transmembrane receptors that control many properties of the cell, including adhesion, migration, and cell-cycle progression through their signaling capabilities (Schatzmann et al., 2003). Integrin heterodimers consist of α and β subunits that are noncovalently linked. The subunits can form a minimum of 23 different heterodimeric receptors with the majority serving as receptors for proteins of the extracellular matrix (Nuzhat et al., 2005). The composition of each heterodimer determines specificity to an individual ligand. Integrins and laminins form a network of cell signaling molecules that allow the cell to respond to changes in its external environment or within the cell by increasing or decreasing the integrin affinity for the ligand (Nuzhat et al., 2005). Integrins interact with the extracellular matrix in clusters known as focal adhesions. Two enzymes that associate with the focal adhesions of integrins are focal adhesion kinase (FAK) and integrin-linked kinase (ILK). These enzymes are essential to modulate cell and extracellular matrix interactions by associating with other proteins in cell signaling

pathways (Nuzhat et al., 2005). Integrins have been linked to metastasis in numerous studies, some specifically linking the α6 subunit in human breast cancer cells with tumorigenicity (Mercurio and Rabinovitz, 2001). Integrins also promote survival of the cell via activation of phosphatidylinositol 3-kinase (PI3K), integrin-linked kinase (ILK), extracellular regulated kinase (Erk), and c-Jun N-terminal kinase (JNK). The PI3K/AKT pathway was considered in this research.

The link between integrins, laminins, and invasion is observed in the three steps of the invasion process. Tumor cells must attach to the basement membrane, degrade the membrane, and migrate into the stroma (Mercurio and Rabinovitz, 2001). Integrins are essential for invasion due to their association with the extracellular matrix as well as their control over signaling pathways in the cell that influence proliferation and growth. As mentioned, the α6β4 integrin is a known receptor for laminin-5 and has been linked to invasion of tumors and promotion of carcinoma migration (Mercurio and Rabinovitz, 2001). The feature of the α6β4 integrin that increases its ability to perform these activities is the distinctness in the cytoplasmic domain of the β4 subunit. It is 1000 amino acids in size and is also different in structure from any other integrin subunit (Mercurio and Rabinovitz, 2001). Studies have proved a mechanistic involvement of this subunit in activation of PI3K/AKT in invasive cancer cells.

PI3K/AKT Signaling and Apoptosis

The link between α6β4 integrin based progression of breast carcinoma invasion and cell signal transduction is the PI3K/AKT cell signaling pathway (Osaki et al., 2004). This integrin can activate phosphatidylinositol-3-kinase (PI3K), which is essential for the translocation of Akt to the plasma membrane where it is phosphorylated. Phosphorylated

AKT then phosphorylates a range of proteins that are fundamental in cell growth and survival as well as in some cases, apoptosis (Osaki et al., 2004). In cancer cells, the α6β4 integrin stimulates the PI3K pathway to a greater level than other integrins (Osaki et al., 2004). Figure 1 shows the PI3K/AKT pathway and its activation by integrin and laminin.

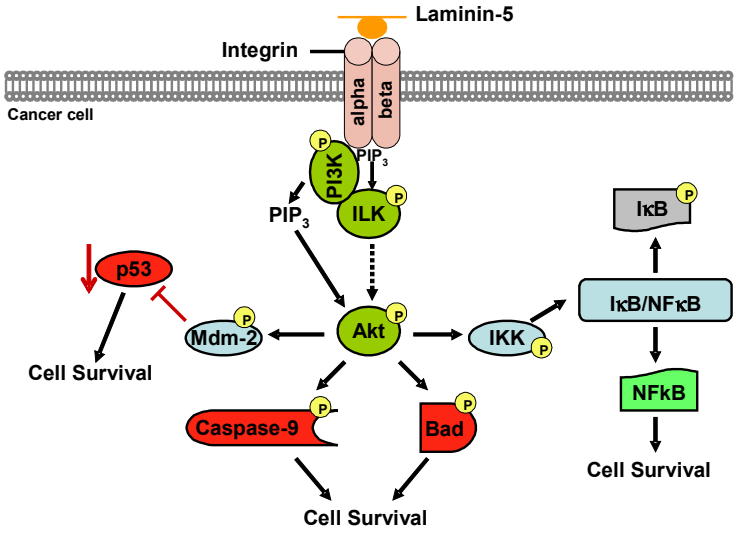


Figure 1. Activation of Akt Pathway (Turner et al., 2008)

PI3K is a lipid kinase signal transduction enzyme that is involved in cellular functions such as growth, differentiation, proliferation, apoptosis, motility, and survival, all of which play important roles in cancer. It is a heterodimeric molecule composed of regulatory and catalytic subunits. PI3K can be dephosphorylated by the phosphatase and tensin homolog (PTEN) tumor suppressor protein, which serves as a negative regulator of the PI3K pathway (Vivanco and Sawyers, 2002). The PI3K group has the ability to phosphorylate the inositol ring 3'OH group in inositol phospholipids to generate the

second messenger phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3). This second messenger interacts with Akt (also known as protein kinase B, or PKB), a serine/threonine kinase, and serves to translocate it from the cytoplasm to the inner membrane, where it is then phosphorylated at two key residues and activated by phosphoinositidependent kinase (PDK) 1 and PDK 2 (Osaki et al., 2004). AKT is a serine/threonine kinase with an amino-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a regulatory domain at the carboxy-terminal. The AKT family includes three members, AKT1-3, and each member requires both phosphorylation on the Thr308 and Ser472 residues as well as translocation to the plasma membrane to be activated. PIP_3 on the membrane associates with the PH domain of AKT and phosphorylates the molecule (Vivanco and Sawyers, 2002).

Akt regulates cell cycle progression, survival, and cell migration through phosphorylation of downstream proteins including cyclin-dependent kinase inhibitor B (CDKN1B)/P27 and pro-apoptotic and apoptotic factors Caspase 9, p53, and BAD (Schley et al., 2005). GRB10 is also suggested as a downstream target in this pathway as a co-activator of AKT, helping translocate it to the membrane. AKT also activates NFκB, an important transcription factor for cell growth and survival, through the activation of the IκB kinase. IKK will phosphorylate the inhibitor of kappa B protein (IκB) that will release NFκB and allow it to localize to the nucleus, where it can induce transcription of many genes including anti-apoptotic genes (Merav et al., 2005).

PI3K first became of interest in the mid 1980's in the cancer research field when its activity was linked to viral oncogene transformation. The regulatory subunit of PI3K, p85, associates with many tyrosine kinases through the interaction of its SH2 domain

with the kinase phosphotyrosine residues. The regulatory subunits of PI3Ks are encoded with α, β, or γ genes, which can be alternatively spliced, whereas the catalytic subunit is composed of α, β, or δ genes. After PI3K activation, PIP_3 phosphatases are activated. The phosphatase that is most obviously involved in oncogenesis is PTEN, and it functions to convert PIP_3 back to PIP_2. It has been proven that the PIP_3 phosphatase activity is responsible for PTEN and its tumor suppression activities (Vivanco and Sawyers, 2002).

Further downstream, several important molecules are affected by AKT. Apoptosis normally controls excessive proliferation via AKT and the phosphorylation of components of the apoptotic pathway including BAD and Caspase-9, as well as indirectly affecting two other cell death regulators, NFκB (through IKK activation) and the tumor suppressor p53. p53 is a pro-apoptotic molecule normally degraded by the p53 binding protein, or ubiquitin ligase mouse double minute homolog 2 (MDM2) when it is phosphorylated by AKT. AKT is also a survival kinase that regulates the cell cycle by preventing degredation of cyclins and allowing them to accumulate (Igor and Sawyers, 2002).

NFκB is a heterodimer composed of p50 and p65 subunits (Madrid et al., 2000). It can activate gene expression and regulate genes that control cell proliferation, survival, and transformation with the presence of transactivating domains in the 120 amino acids in the C-terminal of the p65 protein, also known as RelA. In normal cells of the mammary epithelium, NFκB proteins are located in the cytoplasm where they are sequestered with inhibitory IκB proteins until these proteins are phosphorylated and can dissociate from NFκB (Romieu-Mourez et al., 2001). The IKK complex is composed of three subunits, α, β, and γ. IKKα and IKKβ have a high degree of similarity and are both

catalytic subunits, whereas the γ subunit is regulatory. IKKβ, however, mediates most phosphorylation of IκB proteins and is responsible for most of the activation of signal transduction pathways leading to NFκB activation and has been shown to be involved in apoptosis to a greater extent than IKKα (Hacker and Karin, 2006). Once the IKK complex is phosphorylated by AKT, IκB proteins of the complex are targeted for degredation by the 26S proteasome, a large protein complex that degrades proteins by destroying their peptide bonds. NF-κB is then free to regulate transcription in the nucleus. Optimal induction of NF-κB target genes involves phosphorylation of certain NF-κB proteins, specifically p65 (Viator, et al., 2005). It has also been shown that NF-κB is an important mediator in signaling pathways of apoptosis, and also that NF-κB blocks apoptosis in several different tissues. Inactivating this protein makes the cells more susceptible to agents that induce apoptosis (Wang et al, 1998). However, it has been shown that the induction of p53 can activate NFκB and the two proteins function in concert to induce apoptosis. Although NFκB is essential in cell growth and transcription of genes for survival, it also is suggested to be essential in p53-mediated apoptosis (Ryan et al., 2000). Evidence suggests a pro-apoptotic role for dimers of NFκB, indicating that the protein can be an activator or an inhibitor of apoptosis depending on the levels of RelA and another subfamily of NFκB, c-Rel (Dolcet et al., 2005). Studies show that attenuation of normal NFκB by treatments that inhibit this protein induce apoptosis (Mun-Ock et al, 2008). It has been observed that inhibition or loss of NFκB activity can repeal p53-mediated apoptosis, indicating that NFκB is both essential in cell growth and death (Ryan et al., 2000). In most cases, NFκB is not directly changed, but is held active by mutation of upstream components, such as AKT (Grivennikov et al., 2010).

p53 is a tumor suppressor protein that is critical in cell cycle regulation and also apoptosis when necessary. In unstressed cells, p53 is negatively regulated by the p53-binding protein MDM2, which blocks the apoptotic properties of p53 and also targets it for ubiquitination (Igor and Sawyers, 2002). This is part of a negative feedback loop, because MDM2 is also a target of p53 for transcription. MDM2 is phosphorylated by AKT and can translocate into the nucleus, enhancing p53 degredation further.

The interactions between the PI3K/AKT pathway and the p53 apoptosis pathway also are present downstream and include the target of p53, cell cycle regulator protein $p21^{Cip1/WAF1}$. This protein is stabilized when the AKT pathway is activated, similar to the inhibition seen when AKT phosphorylates the cell cycle inhibitor $p27^{Kip1}$ (CDKN1B) at the threonine 157 site resulting in retention of the protein in the cytoplasm and inhibition of this protein that if transcribed can inhibit DNA synthesis (Chang et al., 2003). $p27^{Kip1}$ ensures an orderly progression of the cell cycle, and studies have shown that overexpression of this protein in cancer cells results in halting of the cell cycle and apoptosis (Katayose et al., 1997). AKT is linked in this way to cell cycle regulation, which is important in the progression of cancer (Franke et al., 2003).

PI3K/AKT and Cancer

Survival, proliferation, and growth are all effects of AKT that can contribute to cancer if they are not controlled, and there is a strong link between PI3K/AKT deregulation and aberrations in many types of cancer. It has also been shown that the α6β4 integrin relies on the PI3K pathway for breast carcinoma survival by associating with a receptor on the surface of the cells. The α6β4 integrin is required for PI3K activation, invasion, and subsequent AKT signaling to promote the survival of the cells

(Mercurio et al., 2001). The stimulation of cell survival through the AKT pathway by α6β4 integrins is only apparent in cells with inactive or mutated p53 (Mercurio et al., 2001). The extensive list of proteins in the AKT pathway contains many in which mutations at the DNA level have been reported in tumors (Igor and Sawyers, 2002). Samuels et al have reported gene mutations and amplification in the gene encoding the P110 catalytic subunit of PI3K in breast cancer, as well as the regulatory subunit of PI3K, p85. Amplification in the gene encoding for AKT2 has been reported in breast cancers, as well as overexpression in mRNA from AKT3 (Osaki et al., 2004). An over-active AKT pathway is seen *in vivo* for many cancer cell types, including breast and ovarian cancer, and linked with poor prognosis (Osaki et al., 2004). Much of the evidence for the involvement of the AKT pathway in cancer stems from the involvement of the PTEN tumor suppressor gene. Loss of PTEN function has been seen in many cancers and leads to the overexpression of AKT and exploitation of this pathway by cancer cells for excessive growth (Chang et al., 2003). Downstream protein expression is altered in cancer as well. Most of the primary human and rodent breast tumor tissue samples show high levels of nuclear NFκB (Romieu-Mourez et al., 2001), and many cancers have been found to have a defect in the ability to produce p53 or a mutation in the gene encoding for this protein (Ryan et al., 2000). Abnormal signals for growth, proliferation, and apoptosis from the Pi3K/AKT pathway have been linked to malignant transformation (Chang et al., 2003) and make the pathway and apoptosis a target for treatment and study by the recombinant protein, rG3, in this research.

CHAPTER TWO

MATERIALS AND METHODS

Recombinant G3 Expression and Purification

The G3 domain of the rat Laminin-5 α3 chain was generated through polymerase chain reaction (PCR) amplification from a plasmid containing a length of the G3 domain. The G3 cDNA was cloned into a vector including an ampicillin resistance gene to allow for selection of the plasmid and an N-terminal 6X-histidine tag. ArcticExpress™ cells were used for expression of recombinant G3 (rG3). Once cells were induced for protein expression and harvested, they were prepared for separation by SDS-PAGE to confirm rG3 expression. Once soluble rG3 expression was confirmed, a culture was prepared to yield a larger volume of the recombinant protein for purification. A protein concentration of the product was determined by performing a Bio-Rad Bradford Protein Assay, and the fraction was applied to a G50 Sephadex column for size exclusion separation. As previously demonstrated, the molecular weight of rG3 including the histidine tag is ~27 kDa (Turner, 2005). To determine which fractions contained the purified protein, an OD reading was taken, and to confirm the identity of the expressed soluble protein as rG3, a Western Immunoblot was performed using an anti-polyHistidine monoclonal antibody.

Cell Culture

MDA-MB-231 cells are a metastatic breast cancer cell line obtained from a 51-year old female patient, derived from a pleural effusion removed on October 17, 1974. This invasive cell line displays an epithelial-like morphology and forms mammary fat pad tumors in nude mice. These cells have lost expression of both estrogen and progesterone receptors (Brinkley, BR et al., 1980). MDA-MB-231 human breast cancer

cells were grown in 75 cm^2 tissue culture flasks and maintained at 37ºC with 5% CO_2. The cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) with 4.0 mM L-glutamine and 4500 mg/L glucose (HyClone, Logan, Utah). The culture medium was supplemented with 10% bovine growth serum, penicillin G (100 units/ml)/streptomycin sulfate (100 µg/ml) and sodium pyruvate (0.11 µg/ml). Cells were routinely passaged using a PBS wash (2X), addition of 400uL cell-stripper (Mediatech, Manassas, Virginia) and incubation for 8 minutes at 37ºC in a humidified incubator (5% CO_2). They were then spun down at 820 rpm for approximately 10 minutes and resuspended in fresh culture medium before being allotted to new flasks. A viable cell count was performed using trypan blue on a hemacytometer.

Real-Time PCR

To observe changes in gene expression of treated and untreated cells, MDA-MB-231 cells were plated at 3 x 10^6 cells/well in a Falcon 6-well tissue culture plate (Falcon, Franklin Lanes, NJ) in a 2 ml volume of DMEM (HyClone, Logan, Utah) without antibiotic in duplicate. rG3 solution or corresponding control buffer was added to treatment wells for a final concentration of 175 µg/ml due to the higher concentration of cells needed for this procedure as compared to the MTT assay and following the microarray assays completed previously. Treatment and control cells were incubated overnight at 37ºC in a humidified incubator (5% CO_2). Cells were collected at 0, 12, 24, 36, 48, and 60 hours after treatment using a PBS wash (2X), addition of 8 ml cell-stripper and incubation for 8 minutes at 37ºC in a humidified incubator (5% CO_2). The cells were then pelleted at 14,000 x *g* in a bench-top micro-centrifuge for five minutes. Cells were suspended in 100µl RNAlater® (Qiagen, Valencia, California) until RNA isolation was

performed. RNA was isolated using RNeasy kit (Qiagen, Valencia, California) according at manufacturer's instructions. An eppendorf Mastercycler® realplex (Eppendorf, Hamburg, Germany) 96-well plate reader was used for PCR analysis. Standard curves with housekeeping gene primers were optimized and beta actin was chosen at a concentration of 75 ng/ml and a temperature of 56ºC. RNA from each duplicate time point was added at a concentration of 10 ng/well on a 96-well, 25 µl twintec-skirted plate along with Master Mix containing 2X Buffer, Rt Enhancer, Primers, and Verso Enzyme mix from a Verso SYBR 1-step QRT + Rox kit (Thermo Scientific, Waltham, Massachusetts). All PCR reactions were performed in triplicate at 56ºC with CDKN1B GRB10, and Caspase-9 forward and reverse primer sets (all from Integrated DNA Technologies, Coralville, Iowa) along with the housekeeper (beta actin, Integrated DNA Technologies) also in triplicate. Genes of interest were chosen based on preliminary changes of expression from a microassay done previously. See Table A1 for primer sequences. Fold change was calculated using Pfafl's Equation as follows:

$\frac{(1+E)^{(treated\ CT-control\ CT)}}{(1+E)^{(treated\ CT-control\ CT)}}$ Where the numerator is for the gene of interest at a certain time point and the denominator is the control with E = efficiency of standard curve.

MTT Viability Assay

To assay for cell viability after varying treatments with rG3, triplicate MTT (3-[4,5-Dimethylthiozol –2-yl] –2, 5-diphemyltetrazolium bromide) assays were performed with purified rG3 and controls, including untreated cells and blank wells with no cells present. Cells were collected as previously described and plated at 1 x 10^4 per well in 100 µl of antibiotic-free medium and the plate was incubated overnight at 37ºC in a humidified incubator (5% CO_2). rG3 was diluted in culture medium to concentrations of

4, 6, and 8 µg/ml. Cells were either treated with the different concentrations of rG3 or with 100 µl of culture medium for a total of 200 µl per well except in background wells which contained 100 µl of culture medium only and no cells. The background wells allow the plate reader to calculate the normalized the OD of wells containing cells. The treated and untreated cells were then incubated for 20 hours at 37°C in a humidified incubator (5% CO_2). 20 µl of MTT (Sigma-Aldrich Co., St. Louis) was added to all wells and incubated for 4 hours. At the end of the 4 hr incubation, the culture supernatant was removed from the wells and 150 µl of dimethylsulfoxide (DMSO) were added to solubilize cells. The plate was shaken for 15 min and absorbance values for the MTT reaction were recorded using a BioTek plate reader at dual wavelengths of 570/650 nm. Optical Density (OD) values were used to create a stimulation index (SI), which was calculated using the ratio of treatment OD ÷ control OD.

Western Blotting

To determine any effects of rG3 treatment on signaling in cells, proteins of the AKT pathway were observed at different time points in MDA-MB-231 cells. The proteins of interest were AKT, p53, IKK-beta/alpha, and NFkB (p65), with beta-actin as a housekeeping protein. Antibodies used were ordered from Cell Signaling Technology (Boston, Massachusetts). The cells were stripped and counted as previously stated and plated at 3 x 10^5 cells/ml in a Falcon 6-well tissue culture plate (Falcon, Franklin Lanes, NJ). The cells were allowed to adhere overnight at 37°C in a humidified incubator (5% CO_2). The following day, treated cells received 40 µg/ml of rG3, while untreated cells received a corresponding volume of medium. This concentration differs from RT-PCR concentrations due to the lesser amount of cells used, but all rG3 came from the same

stock. Following a 0, 3, 6, 9, or 12 hr treatment, cells were washed with PBS and lysed with 100 µl of 1X SDS sample buffer by incubating 5 minutes on ice. The cells were scraped and transferred to a microcentrifuge tube and pelleted with a 10 minute spin in a microcentrifuge at 10,000 rpm. To reduce sample viscosity, the cells were vigorously pipetted up and down to shear DNA before centrifugation. The supernatant was removed and transferred to a separate microcentrifuge tube and frozen until needed. In preparation for protein separation by SDS-PAGE, a sample was boiled for 5 minutes with an equal volume of Laemmli Buffer with 2-mercaptoethanol added. Treated and untreated lysate samples at different time points were loaded onto a 10-20% Ready Gel Precast Gel (Bio-Rad Laboratories, Inc., Hercules, CA) together with a biotinylated protein ladder (Cell Signaling Technology, Inc.). The gel was run in the Ready Gel System (Bio-Rad Laboratories, Inc., Hercules, CA) at 200 V for 30 minutes. Meanwhile, a sheet of Immuno-Blot™ PVDF Membrane and two pieces of 14 x 11 cm filter pads (Bio-Rad Laboratories, Inc., Hercules, CA) were soaked in chilled Western transfer buffer for at least 30 minutes. Following electrophoresis, the gel was washed in cold Western transfer buffer for 30 min. The filter pads, gel, and PVDF membrane were arranged in a layered fashion, and protein transfer was completed at 100 V for 45 minutes using a Trans-Blot Semi-Dry Transfer Cell. Non-specific proteins were blocked with a 30 minute 10% Blotto incubation at room temperature on a shaker, followed by alternating washes with PBS and TBS. An overnight primary antibody incubation at 4^{o}C was performed with the rabbit antibodies phospho-Akt (Ser 473), Akt (pan; C67E7), beta actin (13E5), p53 (7F5), phospho-IKK-alpha/beta (Ser176/180), IKK-beta, NFκB (p65), and phospho-NFκB (p65) at dilutions according to manufacturer's instructions (Cell Signaling Technology, Inc.).

Following primary antibody incubation, a 15 min wash with PBS/Tween 20 was performed to remove unbound antibody. Next, a secondary anti-rabbit IgG HRP-linked antibody was added for 1 hr at RT. Protein detection was obtained through the use of the HR Peroxidase Visualization Kit (Vector Laboratories, Burlingame, California).

Western Blot Densitometry Analysis

To show quantitative changes in protein expression seen in the Western Blots, the relative densities of bands for each protein were calculated using ImageJ software from the National Institute of Health (Bethesda, Maryland). Western images (.jpeg) were imported into the software and converted to grayscale. Bands were selected manually and plotted by their relative densities, with higher peaks representing darker bands. The peaks were manually closed off and the area under each peak was measured and converted to a percentage density of all peaks for all bands of the image. Then, the percent value was divided by the untreated control percent density value to produce a relative density value for each band, with a value of 1 for the control, less than 1 for lower expression of the protein, and greater than 1 for higher expression of the protein.

Statistical Analysis

Analysis of Variance (ANOVA) was performed on all data collected from viability and Western Blot densitometry assays using the Statistical Analysis System (SAS, Research Triangle Park, NC). The general linear model (GLM) procedure was used for ANOVA with a p-value of 0.01.

CHAPTER THREE

RESULTS

Real-Time PCR

Genes of interest were chosen based on a previous microarray that suggested potential changes of expression in CDKN1B, GRB10, and Caspase-9 when MDM-MB-231 cells were treated with rG3. Primers for these genes were used with sequences as seen in Table A1 in the Appendix.

After analyzing several housekeeping genes to use as a control, beta actin was chosen because of the high efficiency and R^2 seen in the RT-PCR standard curve. This gene is often chosen as a control because of its consistent expression in cells. The standard curve for beta actin is shown in Figure A2 in the Appendix.

To observe changes in gene expression of human breast cancer cells following rG3 treatment, RNA was isolated from treated and untreated cells and RT-PCR was performed in triplicate. Fold change of each gene of interest was calculated based on the Ct values by using Pfafl's formula. There were no significant effects of rG3 on gene expression, and all RT-PCR results can be found in the Appendix. Fold changes for CDKN1B can be seen in Figure A3. Three separate RT-PCR trials were performed on RNA collected at each time point (0, 12, 24, and 36 hours) after treatment, which can be seen on the horizontal axis of Figure A3. There were very small changes in expression seen for two of the three trials, and an unusually large increase in expression seen in Trial Two at 12 and 36 hours after rG3 treatment. GRB10 and Caspase-9, however, had marginal changes in expression after treatment with rG3, none of which rose above a 4-fold change. These slight changes for GRB10 and Caspase-9 can be seen in Figure A4

and Figure A5, respectively. The variation in CDKN1B expression could be due to its role as a cyclic gene and varies depending on which cycle stage the cells were in at the time. It was surprising that greater trends were not seen in the expression of GRB10 and Caspase-9, especially since Caspase-9 is involved in the apoptotic pathway and has been observed in previous research (Turner and Borick, 2008) to be affected by rG3 treatment. After re-evaluation, it was decided to investigate the trends in expression of signaling components of the PI3K/AKT pathway at the protein level rather than the gene levels.

MTT Viability Assay

Since such small changes in gene expression were seen from the RT-PCR data, three replicate 24-hour MTT viability assays were performed with 0, 4, 6, and 8 μg/ml concentrations of rG3 to confirm that the rG3 was in fact affecting the viability of the cells. As shown in Figure 2, rG3 exhibited an inhibitory effect on the cells. With a treatment level of both 6 and 8 μg/ml, the stimulation indices (SI) of the cells were significantly reduced from the control, and reduction can be seen beginning at concentrations of 4 μg/ml. The cells show incremental inhibition by treatment with rG3 after only 24 hours of continuous treatment.

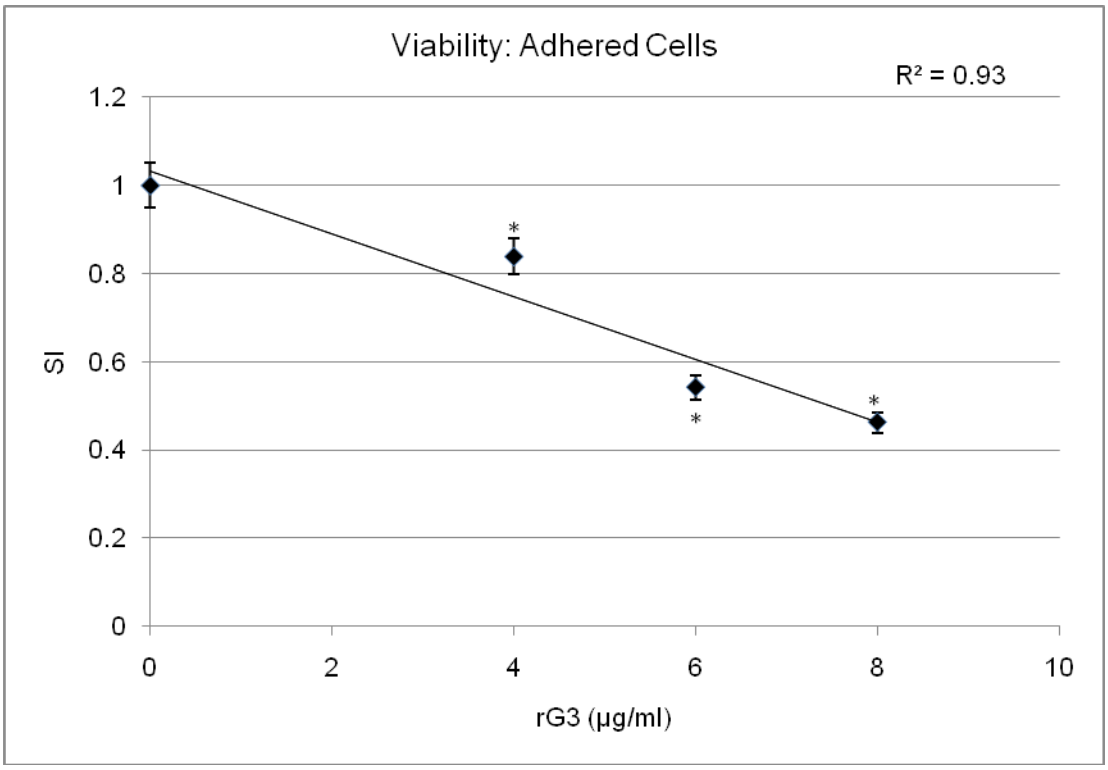


Figure 2. Viability assays were performed with the MDA-MB-231 breast cancer cell line with varying concentrations of rG3. Optical density (OD) of dissolved MTT was recorded at dual wavelengths of 570/650 nm. Stimulation index (SI) is the calculated ratio of treatment OD ÷ control OD. *P ≤ 0.01 for differences between treatments and from control.

Western Blotting and Densitometry Analysis

Since it could not be confirmed that rG3 was affecting gene expression, but a definite decrease in viability due to rG3 treatment of the cells was seen with the MTT assays, treated and untreated cells were analyzed by Western Blots to determine how signaling components of the PI3K/AKT pathway were affected by the rG3 treatment. The pathway proteins chosen were phospho-AKT (Ser 473), AKT (pan; C67E7), beta actin control (13E5), p53 (7F5), phospho-IKKαβ (Ser176/180), IKKβ, NFκB (p65), and phospho-NFκB (p65).

All original Western Blot images from which density of bands were analyzed can be found in the Appendix. For the beta actin housekeeping protein, in Figure A6, bands of a relatively constant intensity can be seen, which is confirmed in the densitometry analysis (Figure 3) with a constant relative density around 1 and no differences between treated and untreated cells with the exception of a small difference 3 hours after treatment and incubation.

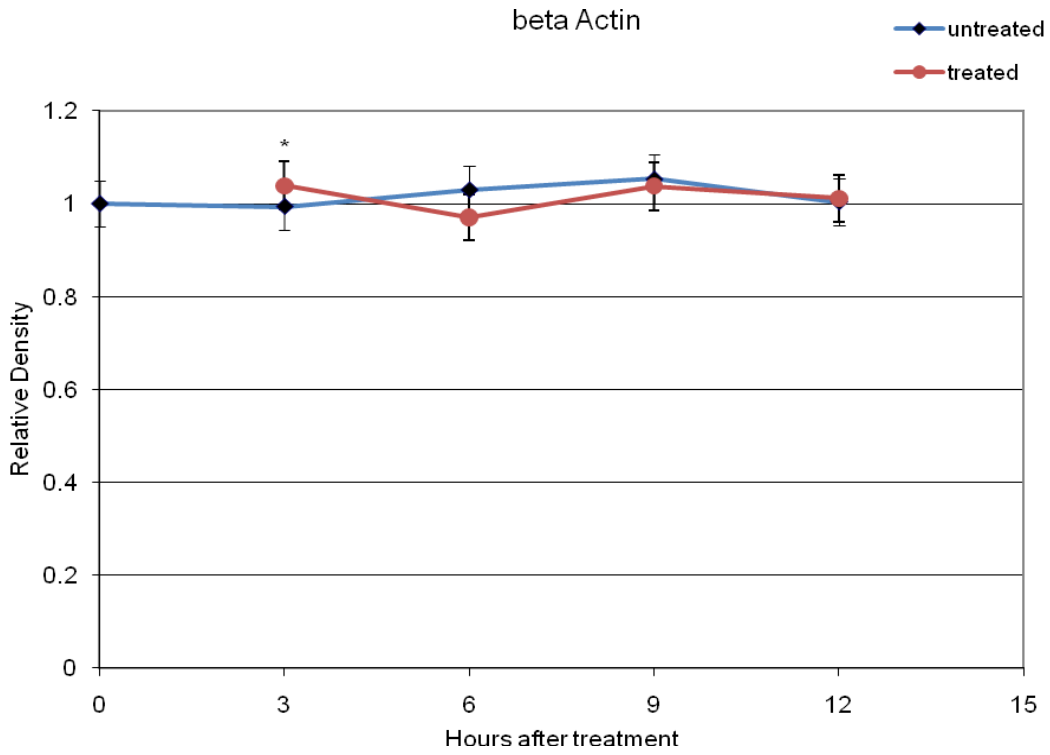


Figure 3. Beta Actin expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37ºC in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

In Figures A7 and A8, the band intensity is greater for both AKT and phosphorylated AKT, respectively, in untreated cells than cells treated with rG3. The relative density of both AKT and phosphorylated AKT is significantly lower in cells treated with rG3 compared to untreated cells. In Figures 4 and 5, the relative density of both AKT and phosphorylated AKT, respectively, increases with time in the untreated cells. In treated cells, the levels of AKT and phosphorylated AKT are lower after 12 hours of treatment when compared to 3 hours of treatment.

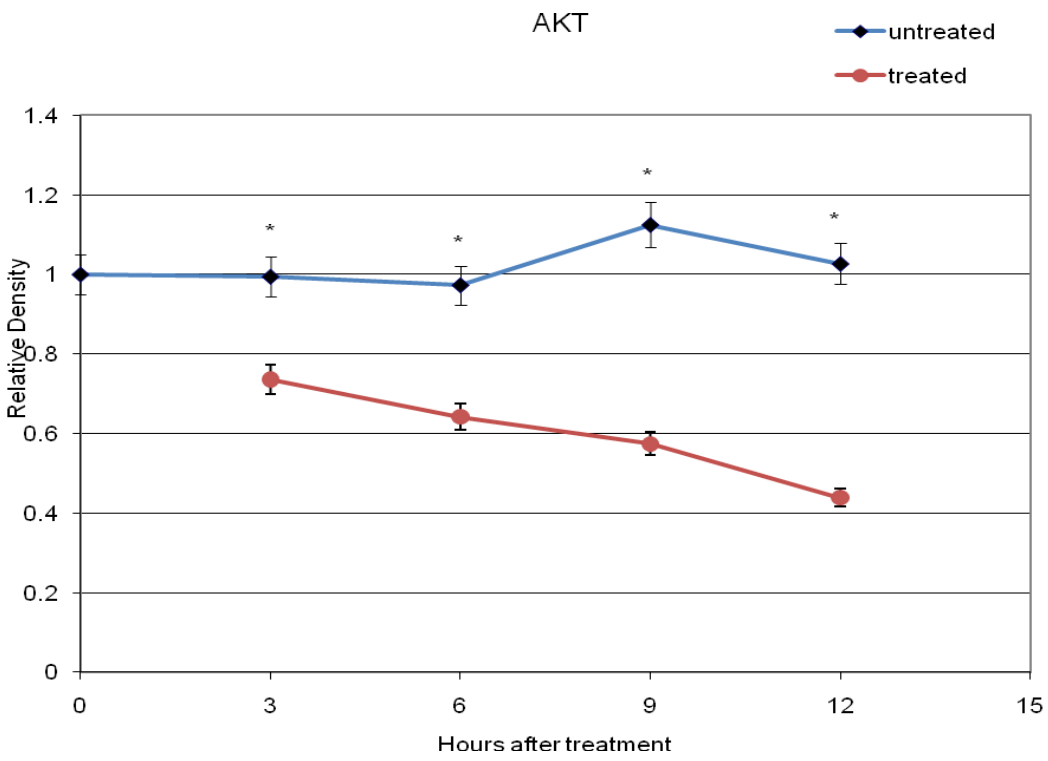


Figure 4. AKT expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37ºC in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

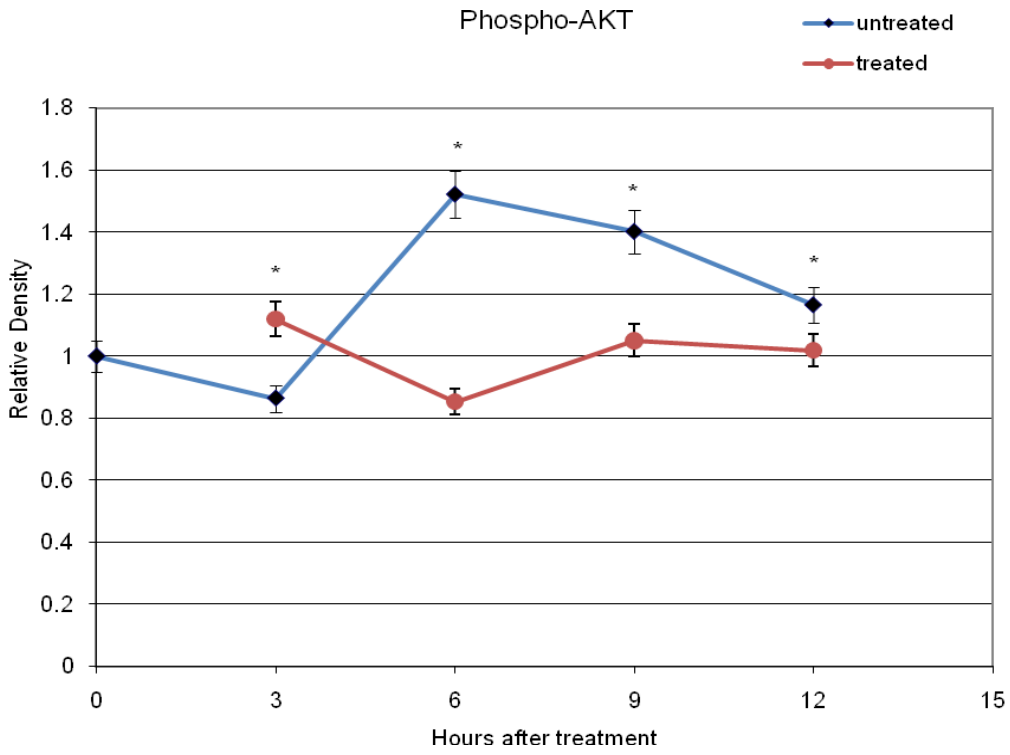


Figure 5. AKT expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37°C in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

In Figure A9**,** a discernable difference in band intensity of the p53 tumor suppressor protein between treated and untreated cells can be observed. The band intensity increases slightly in untreated cells beginning at the 9th hour after treatment, but is darker than the control in the treated cells beginning at only 3 hours after rG3 treatment. The intensity continues to develop up to 12 hours after treatment, with the most intense density at 9 and 12 hours. This is confirmed in Figure 6, which shows the relative density of bands as significantly greater in treated cells as compared to untreated and the greatest relative density of above 5 and above 25 at 9 and 12 hours after treatment, respectively. These results indicate that rG3 is affecting the activity of p53 via the PI3K/AKT signaling pathway.

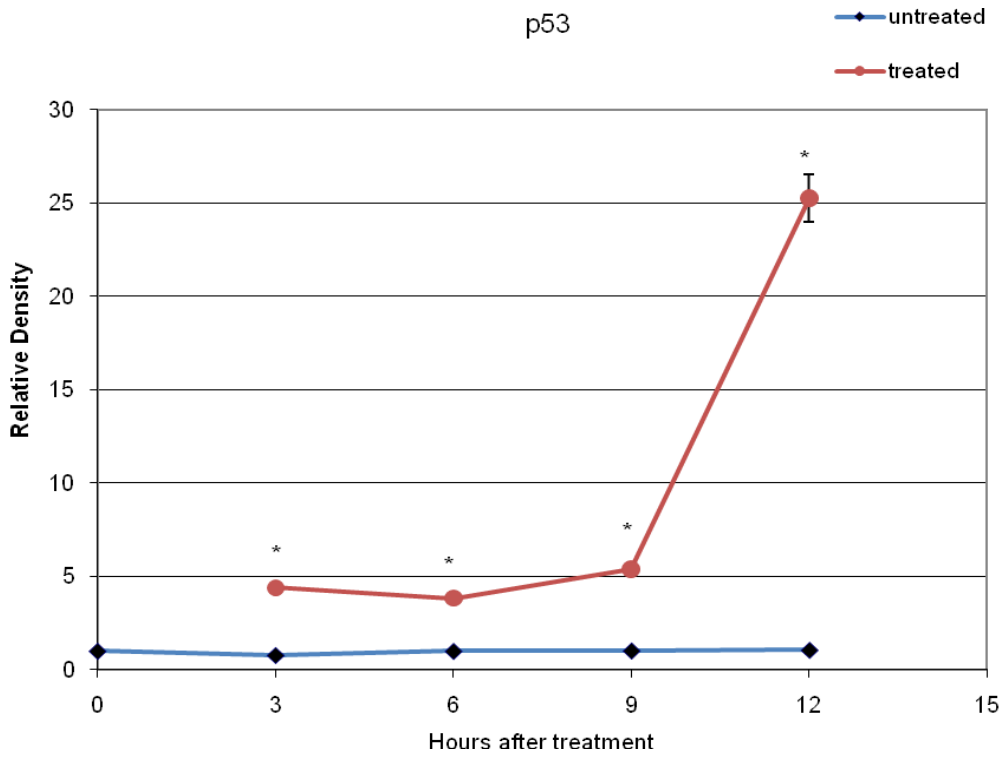


Figure 6. p53 expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37°C in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

In Figure A10, the differences in band intensities are not as easily observed as the p53 analysis. Referring to the densitometry quantification in Figure 7, the relative density of bands for phosphorylated IKKβ is significantly higher in the untreated cells as compared to the treated cells, and is also significantly different between time points, showing a gradual increase in expression of phospho-IKKβ while treated cells have very low levels.

Both the Western Blot in Figure A11 and the relative density plot in Figure 8 show an increase in IKKαβ in the untreated cells. The band intensity, and therefore IKKαβ expression, is significantly higher in the untreated cells than the treated cells. The relative density of treated cell expression is significantly lower at each increasing time point, indicating a time-dependent manner of inhibition from rG3.

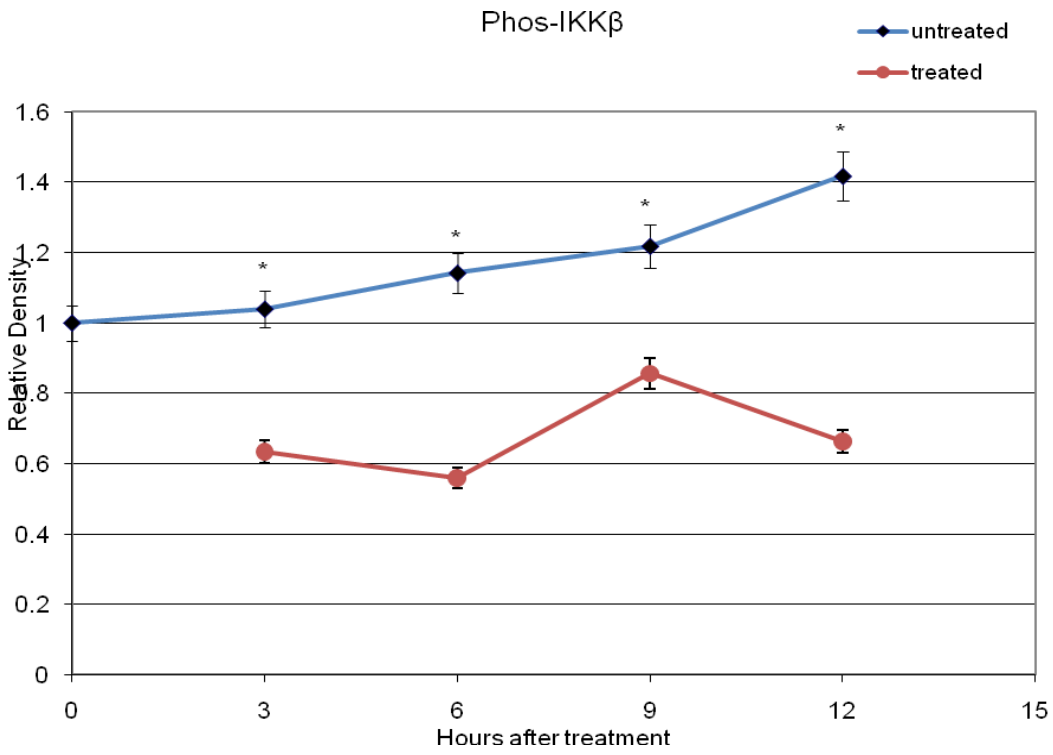


Figure 7. phospho-IKKβ expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37°C in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

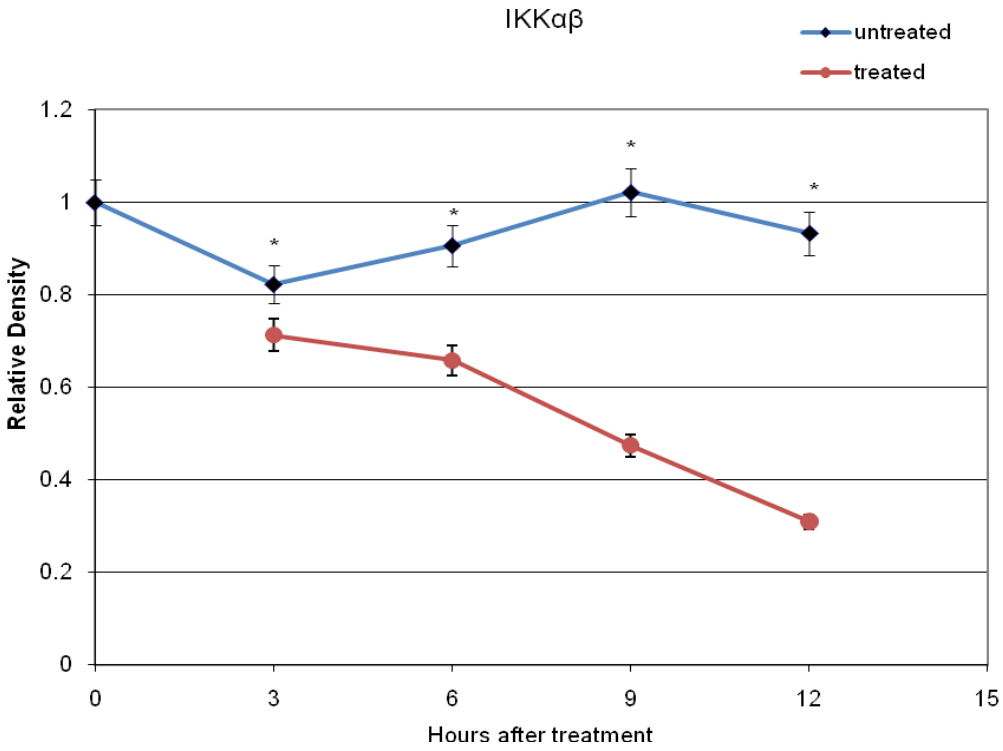


Figure 8. IKKαβ expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37°C in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

The Western Blot for NF-κB in Figure A12 shows increasing band intensity in untreated cells until 9 hours incubation time without rG3 and decreases slightly at 12 hours. The trends are confirmed in relative density plots of Figure 9. In treated cells, the amount of NFκB is significantly higher than in the control untreated cells. There is also a significant peak of expression compared to the other time points in both treated cells and untreated cells at 9 hours after treatment.

Figure A13 shows increasing band intensity in untreated cells for phosphorylated NFκB (Figure 10) with levels peaking at 6 and 9 hours incubation and decreasing at 12 hours. In the densitometry analysis, a significantly higher level of expression is seen in the untreated cells as compared to treated, with a significant peak at 6 hours after treatment.

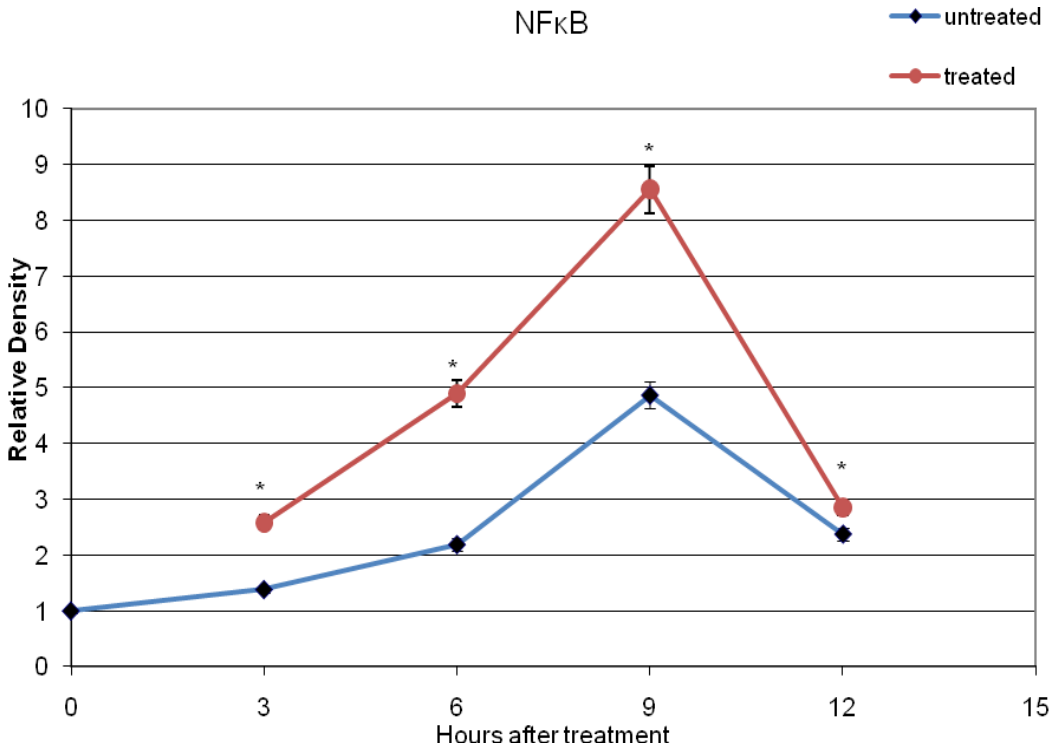


Figure 9. NFκB expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37°C in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

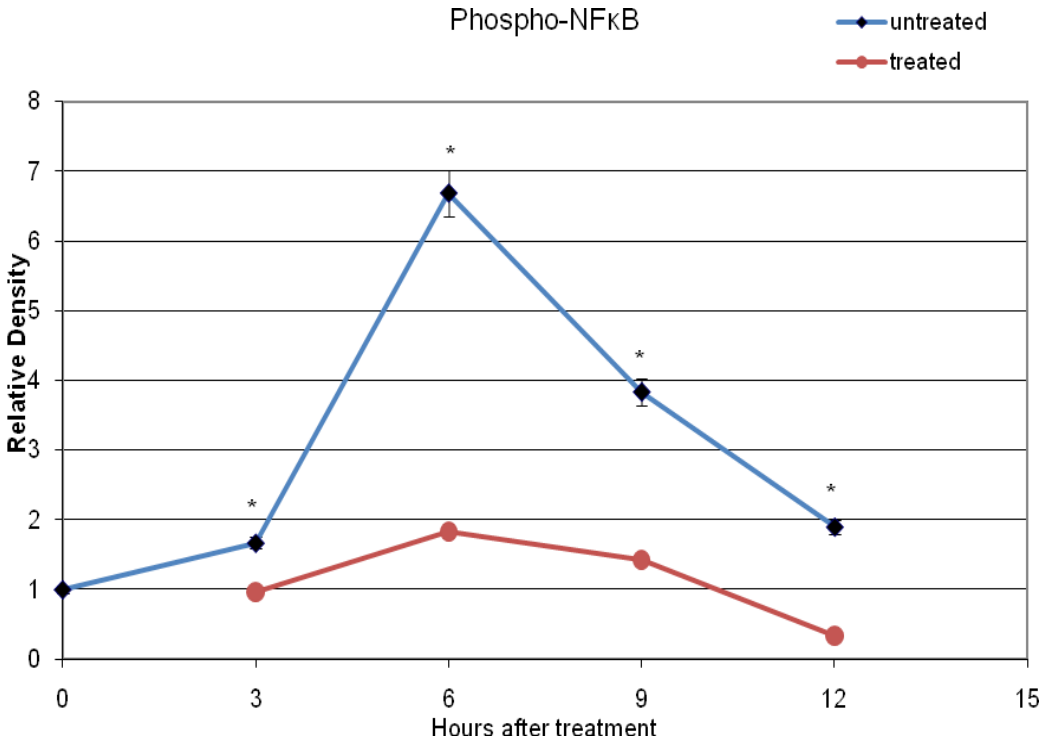


Figure 10. Phospho-NFκB expression in rG3 treated and untreated MDA-MB-231 breast cancer cells. Cells were allowed to adhere overnight at 37ºC in a humidified incubator and the following day, treated cells received 40 µg of rG3, while untreated cells received a corresponding volume of medium. Following a 0, 3, 6, 9, or 12 hr incubation, cells were lysed with 100 µl of 1X SDS sample buffer, run on a 10-20% Ready Gel Precast Gel, and analyzed with a Western Blot and ImageJ densitometry software. *$P \leq 0.01$ for differences in treated vs. untreated cells at each corresponding time point.

CHAPTER 4

DISCUSSION

One of the most important mechanisms regulating cell growth, survival, and apoptosis is the PI3K/AKT pathway mediated by the binding of integrins on the cell membrane to Laminin-5 in the ECM. Tumor cells can exploit this pathway by expressing certain integrins on their surface, leading to an increase in metastasis. Alterations in and overexpression of the PI3K/AKT pathway have been seen in many types of cancer, and inhibition of this pathway is implicated in potential cancer thereapy (Osaki et al., 2004).

Specifically, the α6β4 integrin expression increases tumor cell invasiveness, especially in breast carcinomas (Aiyer and Varner, 2005). It has been proven that the integrin subunits α3 and α6 bind to Laminin-5 G subdomains found at the C-terminal end of the α chain to increase metastasis and tumor growth (Hirosaki et al., 2000). In 2003, Kariya et al. provided evidence for the major role of the G3 subdomain of Laminin-5 in tumor development through a series of deletions and mutations in the human G3 domain. Previous research has proved rG3 binding to cancer cells is specific for the α integrin subunit, but fails to engage the β subunit necessary for signaling through cell survival pathways. It is also shown that the ligand binding specificity leads to reduced cell viability through pro-apoptotic signaling events (Turner, 2008).

To determine specifically which signaling events, proteins, and genes are involved in the altered survival pathway due to rG3 treatment, the breast cancer cell line MDA-MB-231 was selected for observation based on its high expression of α6β4 integrins. The recombinant G3 protein previously engineered (Turner, 2008) was used to assay signaling activity at both a genetic and protein level following treatment with

varying levels of rG3. Figure 11 shows hypothesized altered events of the cell survival signaling pathway with rG3 treatment (Turner, 2008). In theory, in treated cells, AKT, IKKαβ, GRB10, and NFκB, expression should go down, while p53, Caspase-9, and CDKN1B expression should increase.

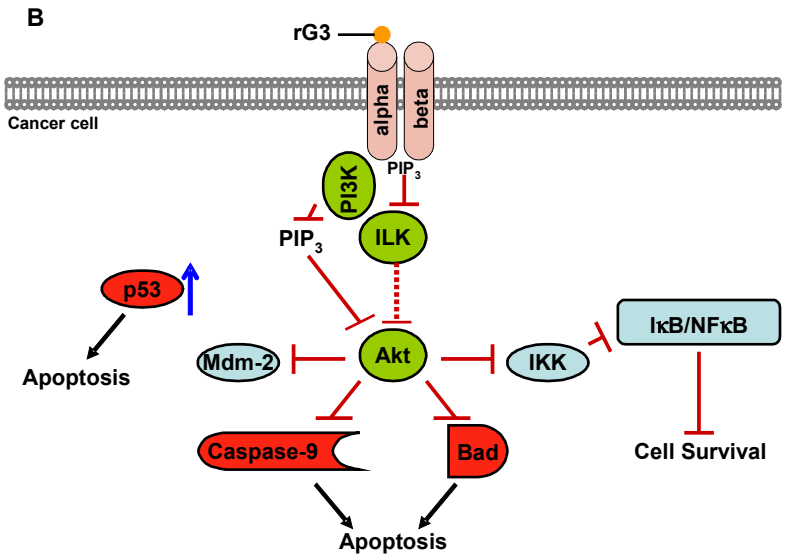


Figure 11. Inactivation of the Akt pathway following binding of rG3 to the alpha component of the integrin heterodimer leading to induction of apoptosis.

The genes GRB10, CDKN1B, and Caspase-9 were chosen for investigation using Real-Time PCR based on previous microarray data and their known involvement in cell survival and apoptotic pathways. The GRB10 gene encodes growth factor receptor bound protein 10 in humans, and has been suggested as a downstream target in the PI3K pathway. No direct effect of this protein has been observed on PI3K or AKT, but there is evidence that the expression level of GRB10 may influence AKT activity (Jahn et al., 2002). Studies have shown that GRB10 has a stimulatory effect on AKT activation when

it is overexpressed and that a complex is formed between GRB10 and AKT. This suggests that it is an adaptor protein that aids to relocalize AKT to the cell membrane, therefore acting as a positive regulator of the AKT pathway (Jahn et al., 2002). These findings suggest that when the cell undergoes apoptosis due to rG3 treatment, decreased expression of GRB10 will be observed because the cell is no longer using the AKT pathway for cell proliferation and growth, reducing the amount of AKT that is translocated to the membrane in a complex with GRB10.

The CDKN1B gene encodes the $p27^{Kip1}$ protein that is involved in regulating cyclins, or proteins forming complexes that guide cell cycle progression through multiple stages. It has been suggested that in cell surivival, AKT stimulates cell cycle progression by activating this cyclin dependent kinase inhibitor and causing it to be retained in the cytoplasm where it cannot produce growth-inhibitory effects (Chang et al., 2003).

Caspase-9 is a crucial mediator of apoptosis and is directly involved in the apoptotic signaling pathway. It is an initiator Caspase and its activation results in a cascade of additional Caspase activation eventually resulting in apoptosis (Salveson, 1998). Based on these conclusions, it would be expected that when MDM-MB-231 breast cancer cells are treated with rG3 and undergo apoptosis, gene expression of Caspase-9 would be upregulated, while GRB10 and CDKN1B gene expression would be down-regulated.

RT-PCR results show large inconsistency between the three trials and do not show a large enough fold change of gene expression for GRB10, CDKN1B, or Caspase-9 to conclude that rG3 is altering their expression. In trial 1, CDKN1B shows major upregulation, but this result could not be repeated in the other two trials. This is possibly

due to the fact that it is a cyclic regulator and its expression depends on what cell cycle stage the cells are in, which was not controlled for in this experiment. Caspase-9 was expected to have a much larger fold change due to the fact that it is a direct activator of the apoptotic pathway, and GRB10 showed very small changes in expression as well as inconsistency of expression when treated with rG3. Due to this disparity, and the small number of microarray replications from which these genes were chosen, it cannot be concluded that treatment with rG3 is activating the GRB10, CDKN1B, or Caspase-9 components of the PI3K/AKT pathway at the genetic level.

To check that the recombinant G3 protein used to treat the cells was functional, an MTT viability assay was performed in triplicate using varying concentrations of rG3. MDM-MB-231 cells were treated with 0, 4, 6, or 8 μg/ml of rG3 and incubated for 24 hours. After the cells were lysed and OD readings were obtained, a stimulation index could be calculated. The data exhibit a dose-dependent inhibitory effect of rG3 on the cells, with 6 and 8 μg/ml causing a significant reduction in the viability of cells as compared to the control. This confirms the inhibitory effect of rG3 on cell growth and survival despite the inconsistent RT-PCR gene expression data and deems it necessary to investigate the PI3K/AKT pathway involved at a protein expression level.

Based on the activity of the PI3K/AKT pathway and its downstream constituents, the proteins chosen for study were AKT (phosphorylated and nonphosphorylated forms), IKKαβ, phosphorylated IKKβ, NFκB (phosphorylated and nonphosphorylated forms), and p53, with beta Actin as a control. As mentioned, AKT is a serine/threonine kinase with both regulatory and catalytic domains. PIP_3 on the membrane associates with the PH domain of AKT and phosphorylates the molecule (Vivanco and Sawyers, 2002), allowing

Akt to regulated cell cycle progression, survival, and cell migration through phosphorylation of the downstream IKKαβ complex. Furthermore, activation of the IKKαβ complex targets IκB proteins sequestering NFκB in the cytoplasm for degredation and NFκB is then free to regulate transcription in the nucleus, regulating both growth and apoptosis (Ryan et al., 2000). Lastly, p53 is negatively regulated by the p53-binding protein MDM2. When MDM2 is phosphorylated by AKT, it enhances p53 degredation and inhibits the pro-apoptotic effect of the p53 protein (Igor and Sawyers, 2002). These components of the PI3K/AKT signaling pathway are involved in regulating cell growth and in some cases, initiating apoptosis. This leads to the assumption that cell signaling and protein expression would be altered when MDM-231-MB breast cancer cells were treated with the recombinant G3 protein that has been shown to reduce cell viability through this pathway (Turner, 2008). Cells were treated with 40 μg of rG3 or corresponding media volume, incubated at 37ºC, collected, and lysed at 3, 6, 9, and 12 hours after treatment.

Beta Actin protein levels are statistically the same in treated and untreated cells at 0-12 hours. Any variation in the levels may be explained by the suggestion that with longer Western Blot incubation times, beta Actin antibodies seem less able to pick up any differences in different levels of its target protein in MDA-MB-231 breast cancer cells and that the protein is not always constantly expressed with time (Dittmer, 2006). It is important to note, however, that the relative density for beta Actin at all time points in all cells is clustered around 1 and is much more constant than any of the other protein densities, so it is suitable for a control for these research purposes.

The relative density of both AKT and phosphorylated AKT is significantly lower in cells treated with rG3 compared to untreated cells, suggesting that rG3 treatment is affecting not only the cell's ability through PIP_3 to phosphorylate and activate AKT but also degrading the the enzyme entirely. The presence of both AKT and phosphorylated AKT increases with time in the untreated cells, which is due to an increase in survival and also likely to an increase in cell concentration with growth over time. In treated cells, the levels of AKT and phosphorylated AKT are lower after 12 hours of treatment when compared to 3 hours of treatment, showing a significant decrease in cell survival capability. These results coincide with current and numerous past studies that implicate activated AKT in regulation of cell cycle progression, promotion of survival, and increased cell migration (Schley et al., 2005; Vivanco and Sawyers, 2002). An active AKT has been detected in many cancers *in vivo*, including human breast cancer and unconstrained activation and expression of AKT has been shown to lead to the development of malignant tumors (Osaki et al., 2004). Recent studies also show phosphorylated AKT weakly expressed in normal breast tissue but abundant in invasive tumors (Crowell et al., 2007). Our results implicate less AKT activation and expression in cancer cells treated with rG3 and harmonize with the suggestion that inhibition of AKT and this pathway are possible therapeutic strategies for cancer (Osaki et al., 2004).

The next downstream protein of AKT investigated is the IKKαβ complex. When this classic IKK complex is phosphorylated by active AKT, the α and β subunits proceed to initiate NFκB transcription in the nucleus, promoting cell survival (Lee and Hung, 2008). IKKβ and IKKα have both been recently shown to function as oncogenic kinases, so it is important to note the higher expression of both the IKKαβ complex and

phosphorylated IKKβ in untreated cancer cells compared to cells treated with rG3. Treatment significantly reduces the expression of IKKαβ in cells while untreated cells hold a relatively constant level of the kinase complex. Phosphorylated IKKβ is present in significantly lower levels in cells treated with rG3 and its expression increases with time in untreated cells, suggesting an oncogenic role of this protein in actively growing and unregulated cancer cells. A recent study implicates IKKβ as an oncoprotein in breast cancer because of its ability to degrade forkhead box O3a (FOXO3a) that normally is a transcription factor that promotes apoptosis. It is also known that the β subunit phosphorylates the inhibitory kinase of NFκB, allowing NFκB to translocate into the nucleus and promote the expression of genes involved in angiogenesis, invasion, metastasis, and cell survival. IKKα also may possibly serve as a promoter of tumor metastasis by down regulating tumor suppressors (Lee and Hung, 2008). Similar studies also report a role in apoptosis for IKKαβ and the IκB complex and show that IκB knockdown in breast cancer cells inhibits their proliferation and survival and significantly arrest the cells in the G_0/G_1 phase (Qin and Cheng, 2010). Our data also support this theory, as a reduction of IKKαβ and active IKKβ is seen in cells with lessened survival due to rG3 treatment. The relationship between the IKK complexes and NFκB and the inhibition seen by rG3 treatment suggest that the NFκB transcription factor will also be affected.

The expression of the active form of NFκB, the transcription factor for cell survival and other processes activated by IKKαβ, is as expected for cells treated with rG3. Western Blots show a significantly higher expression of phosphorylated NFκB in untreated cells. The expression peaks at 6 hours, indicating the highest amount of

transcription at this point, with a decrease at 12 hours but expression levels still above the control. For regular, inactive NFκB, higher levels are seen in the treated cells as compared to the untreated cells. This could be because the majority of NFκ B in untreated, actively proliferating cancer cells is in the active, phosphorylated form while activation is inhibited in the treated cells resulting from the low amount of active AKT and IKKαβ present with inhibition of the survival pathway by rG3. The NFκB family of transcription factors regulates expression genes involved in cell survival and proliferation and can also promote metastasis and angiogenesis (Gilmore et al., 2002). Inhibition of NFκB activity has been shown to reverse the malignant phenotype, and is of interest for therapeutics and intervention (Gilmore et al., 2002).Normally, the NFκB complex is tightly regulated and held in the cytoplasm through the IKK complex, but when liberated, can bind to target gene enhancers to promote an invasive capability of malignant tumor cells. There are many studies that show a variety of human tumor cell lines, including breast cancer cells, expressing constitutively nuclear and active NFκB activity (Gilmore et al., 2002). This supports the low levels of inactive NFκB seen in untreated breast cancer cells by suggesting most of it is in the phosphorylated form actively transcribing genes in the nucleus.

Lastly, the largest trend in this research is seen in the expression of the tumor suppressor protein p53 in rG3 treated cancer cells. p53, in non-cancer cells, is continually produced and degraded immediately. The regulator of this protein, MDM2, is phosphorylated by AKT and can translocate into the nucleus, enhancing p53 degredation further (Igor and Sawyers, 2002). p53 mutations are the most common abnormality of human cancer and are seen in about 50% of all cancers, including breast cancer (Ziyaie et

al., 2000). It is also used as a diagnostic marker of breast cancer due to its prevalence in expression. Results of this study show very low levels of p53 expression in untreated, normally proliferating cancer cells. In treated cells, however, the levels of this protein are higher than the control cells and an extremely significant spike in p53 activity is observed beginning at only 9 hours after treatment. This increase in p53 activity suggests the cells are initiating apoptosis shortly after rG3 treatment. p53 induces apoptosis while AKT promotes cell survival, and previous studies have indicated cross-talk between AKT and p53 while influencing the apoptotic pathway in different ways (Wee and Aguda, 2006). A cell's decision to live or die greatly depends on the levels of each protein, so treatment with rG3 is influencing this decision by decreasing levels of active AKT and significantly increasing p53 levels.

This study suggests there are significant differences in expression of PI3K/AKT proteins of the signal transduction cascade in MDA-MB-231 cells treated with the recombinant protein, rG3. This cascade has been implicated and investigated extensively for its role in tumor invasion and oncogenic metamorphosis, and is a target for inhibition of anti-cancer drugs (Chang et al., 2003). Inhibition of this pathway is important to control uncontrolled growth and invasion of malignant cells into tissues and is also easier than to restore function to abnormal tumor suppressor proteins. Thus, antiapoptotic signals from the PI3K/AKT pathway are a focus of current cancer drug therapy research. There are already AKT inhibitors that have been discovered, as well as PI3K inhibitors. It is more effective to target the whole pathway rather than just an upstream protein because for example, tumors growing with a mutation in a downstream component such as p53 would not be inhibited by a drug solely targeting a single upstream protein (Hennessey, et

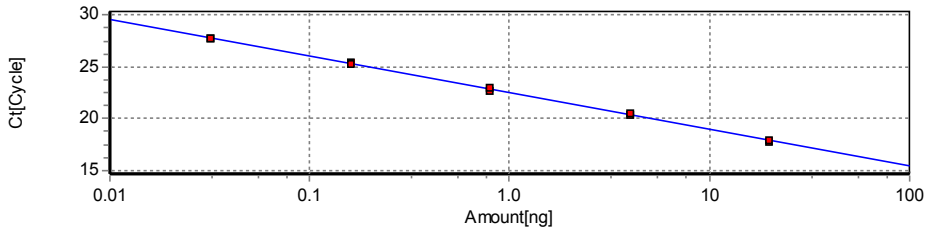
al., 2005). Most studies to date have been performed with inhibitory compounds that are virtually non-specific (Hennessey, et al., 2005), contrasting to the specificity and distinct binding properties of the recombinant G3 protein used in this study. Another benefit of the rG3 protein targeting integrin on the cell surface upstream of the entire PI3K/AKT pathway is that since this pathway does bifurcate and coalesce to such an extent, inhibition by rG3 is extremely global, and theoretically would be less vulnerable to the complexity of negative and positive feedback loops of the pathway. Therefore, the evidence presented here of rG3 treatment's significant effect on the PI3K/AKT pathway, including AKT, IKKαβ, NFκB, and p53, implicates a role for this recombinant protein in promotion of apoptosis in cancer cells and possibly therapy.

APPENDICES

Appendix A

Table A1. RT-PCR Primer Sequences

Primer	Forward	Reverse
CDKN1B	5'-ACGAATGCGCAGGAATAAGGAAGC-3'	5'-TACGTTTGACGTCTTCTGAGGCCA-3'
GRB10	5'-AGACAACAGCTGCCTGGCTTCTAT-3'	5'-AAGCTTGTGACACTGCTCTAGGCT-3'
Caspase-9	5'-TGACTGCCAAGAAAATGGTG-3'	5'-CAGCTGGTCCCATTGAAGAT-3'
beta Actin	5'-AATGTGGCCGAGGACTTTGATTGC-3'	5'-AGGATGGCAAGGGACTTCCTGTAA-3'



Slope: -3.550
Y-Intercept: 22.51
Efficiency: 0.91
R^2: 0.999

Figure A2. Standard curve for beta Actin housekeeping primer at .075 µg/ml and a temperature of 56ºC.

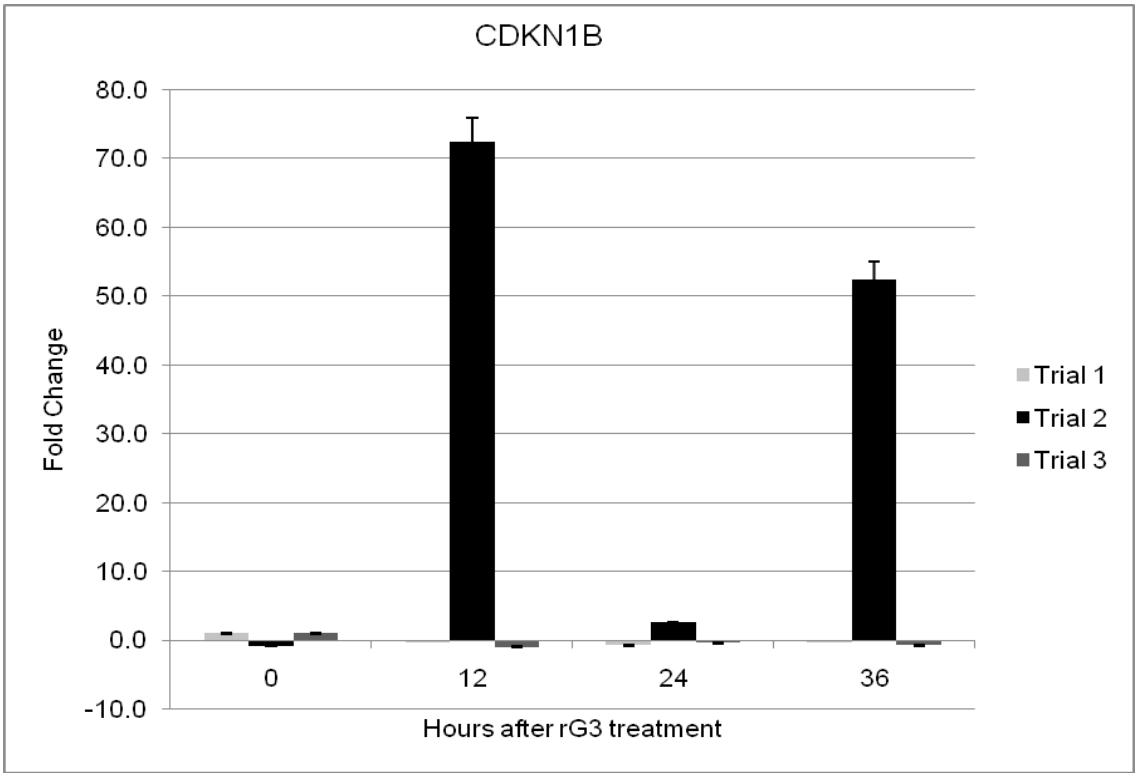


Figure A3. RT-PCR in triplicate with CDKN1B as the gene of interest was performed on RNA isolated from MDA-MB-231 breast cancer cells either treated with 175 µg/ml or none for control cells and collected 0, 12, 24, and hours after treatment. Fold change was calculated using Pfafl's Equation.

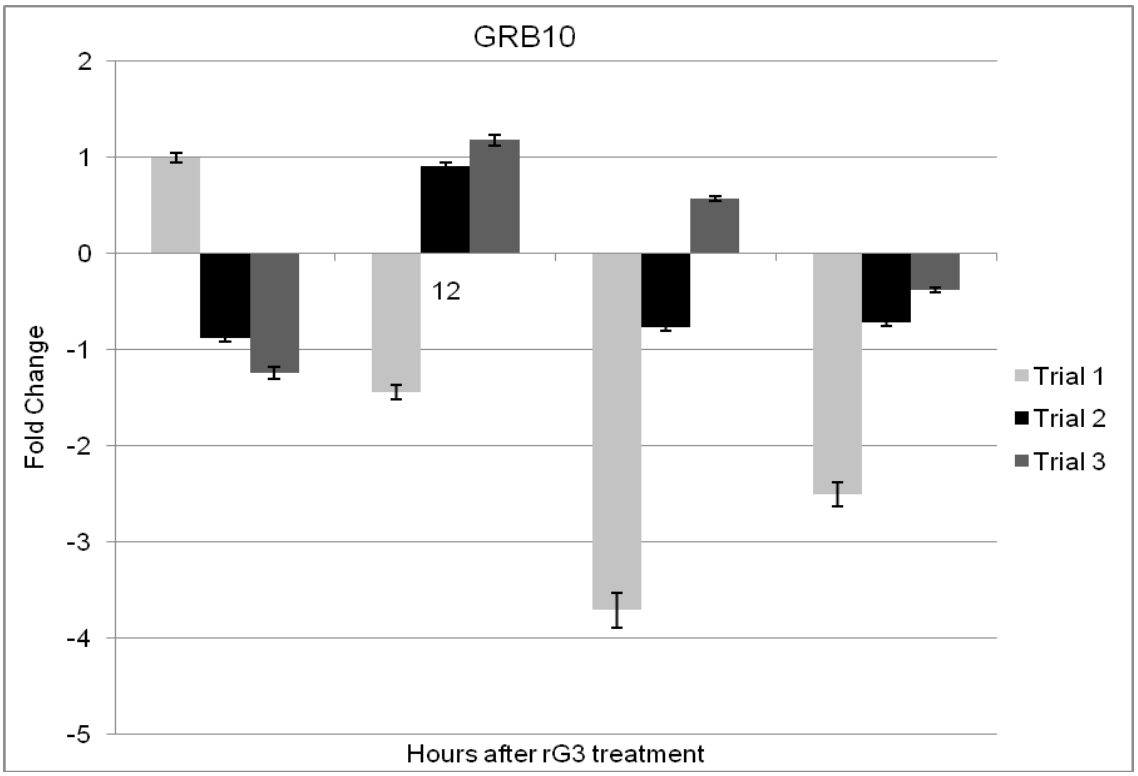


Figure A4. RT-PCR in triplicate with GRB10 as the gene of interest was performed on RNA isolated from MDA-MB-231 breast cancer cells either treated with 175 µg/ml or none for control cells and collected 0, 12, 24, and hours after treatment. Fold change was calculated using Pfafl's Equation.

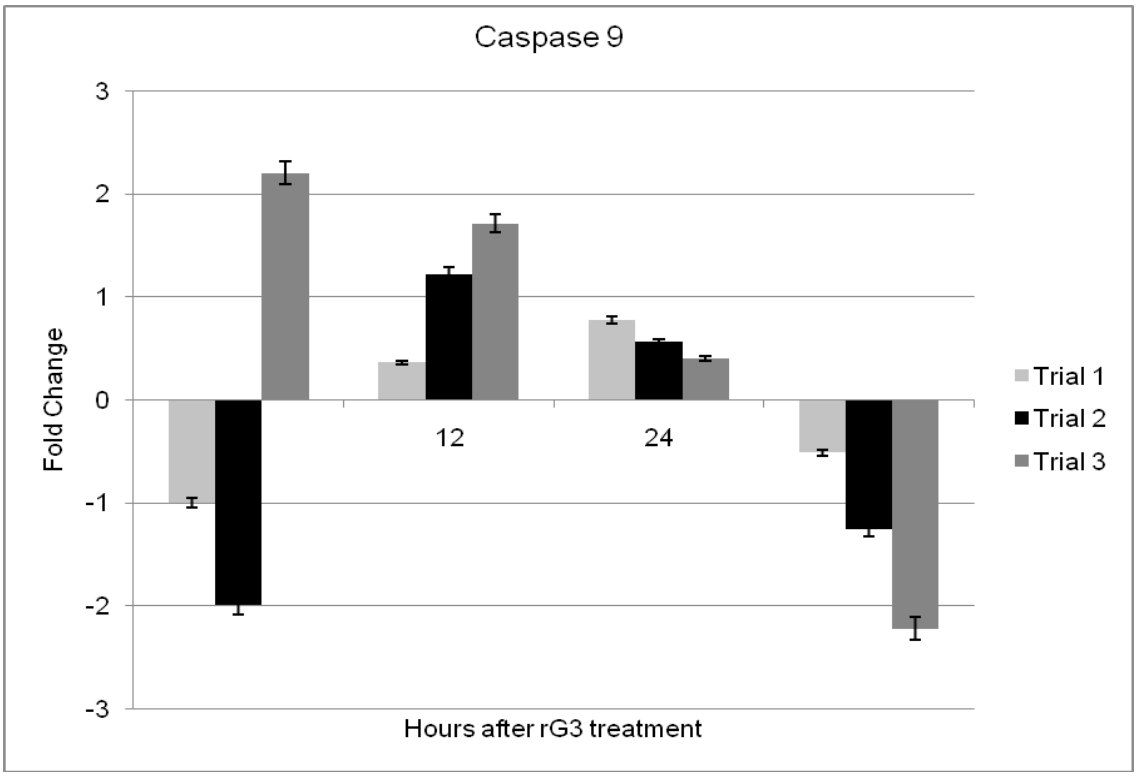


Figure A5. RT-PCR in triplicate with Caspase-9 as the gene of interest was performed on RNA isolated from MDA-MB-231 breast cancer cells either treated with 175 µg/ml or none for control cells and collected 0, 12, 24, and hours after treatment. Fold change was calculated using Pfafl's Equation.

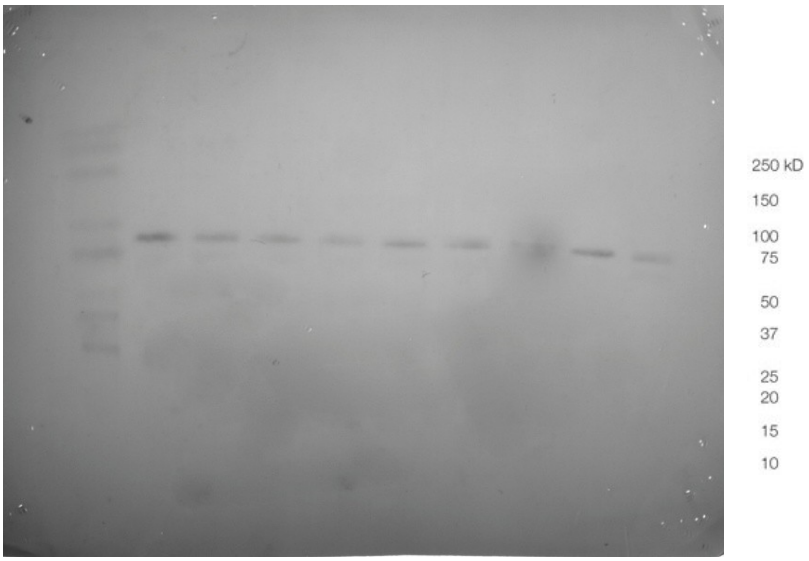


Figure A6. Western Immunoblot performed with a beta Actin (13E5) rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 10 represents the protein standard. Lanes 1-9 are as follows: 12 hours treated, 9 hours treated, 6 hours treated, 3 hours treated, 12 hours untreated, 9 hours untreated, 6 hours untreated, 3 hours untreated, 0 hours untreated.

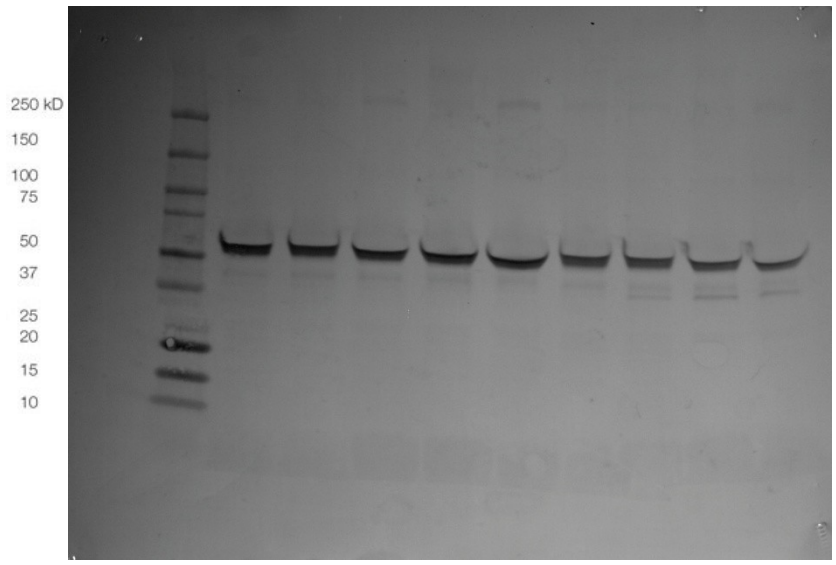


Figure A7. Western Immunoblot performed with am AKT rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 µg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 10 represents the protein standard. Lanes 1-9 are as follows: 12 hours treated, 9 hours treated, 6 hours treated, 3 hours treated, 12 hours untreated, 9 hours untreated, 6 hours untreated, 3 hours untreated, 0 hours untreated.

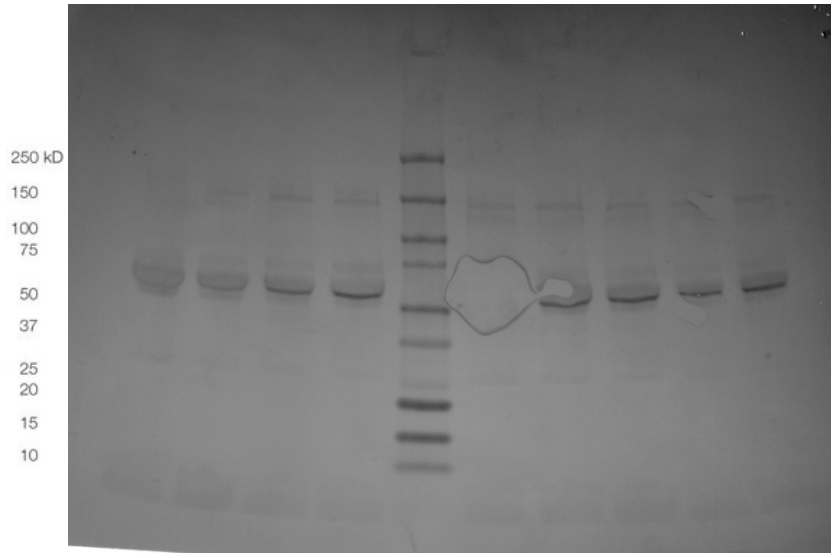


Figure A8. Western Immunoblot performed with a phospho-AKT rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 10 represents the protein standard. Lanes 1-9 are as follows: 12 hours treated, 9 hours treated, 6 hours treated, 3 hours treated, 12 hours untreated, 9 hours untreated, 6 hours untreated, 3 hours untreated, 0 hours untreated.

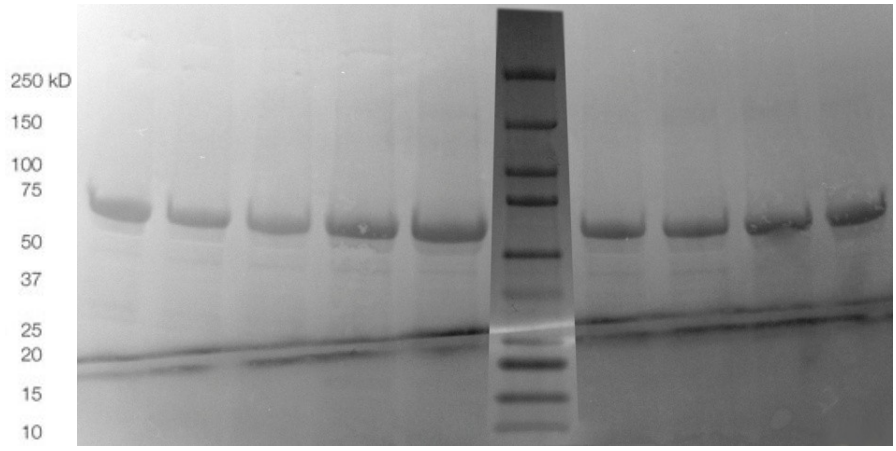


Figure A9. Western Immunoblot performed with a p53 (7F5) rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 6 represents the protein standard. Lane 1-10 are as follows: 0 hours untreated, 3 hours untreated, 6 hours untreated, 9 hours untreated, 12 hours untreated, standard, 3 hours treated, 6 hours treated, 9 hours treated, 12 hours treated.

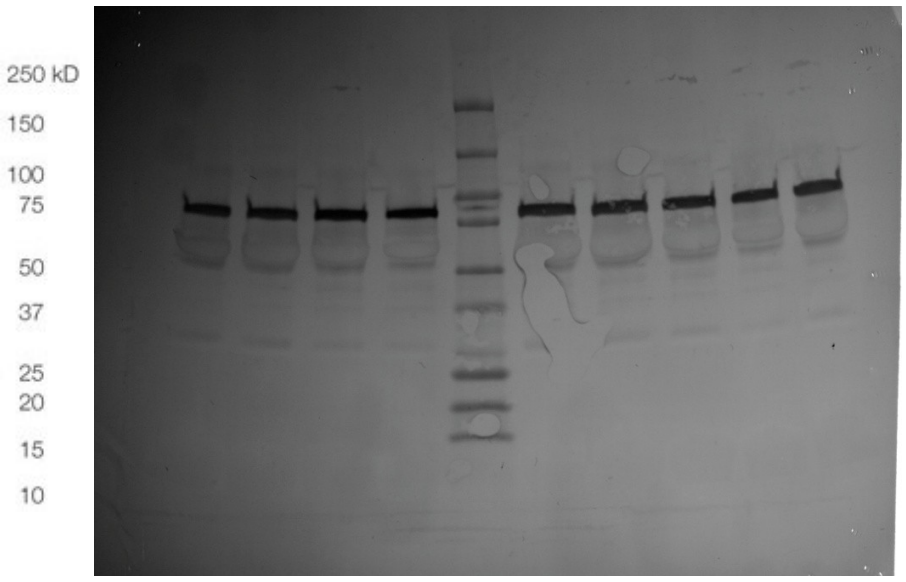


Figure A10. Western Immunoblot performed with a phospho-IKK-beta rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 5 represents the protein standard. Lane 1-10 are as follows: 3 hours treated, 6 hours treated, 9 hours treated, 12 hours treated, marker, 0 hours untreated, 3 hours untreated, 6 hours untreated, 9 hours untreated, 12 hours untreated.

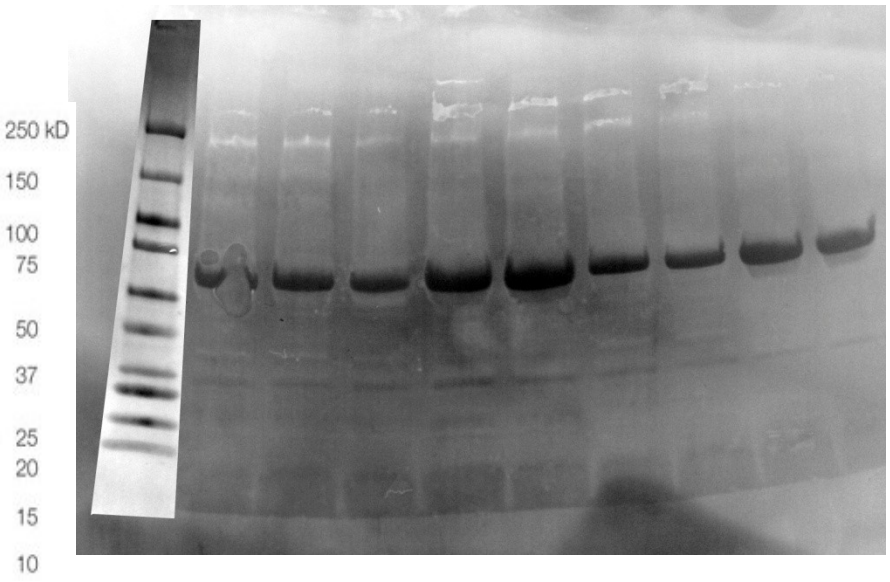


Figure A11. Western Immunoblot performed with an IKK-alpha/beta (Ser176/180) rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 1 represents the protein standard. Lane 2-10 are as follows: 0 hours untreated, 3 hours untreated, 6 hours untreated, 9 hours untreated, 12 hours untreated, 3 hours treated, 6 hours treated, 9 hours treated, 12 hours treated.

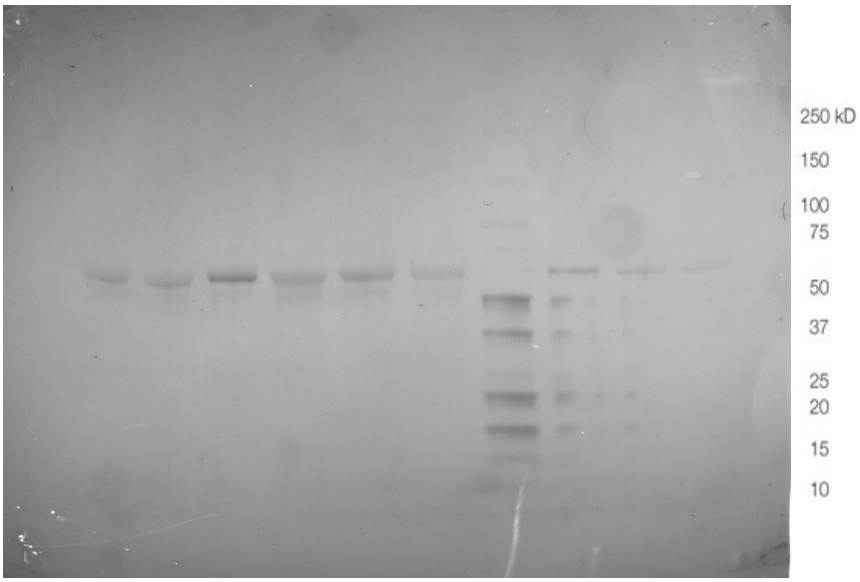


Figure A12. Western Immunoblot performed with an NF-κB (p65) rabbit mAb at a 1:2000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 7 represents the protein standard. Lanes 1-10: 12 hours treated, 12 hours untreated, 9 hours treated, 9 hours untreated, 6 hours treated, 6 hours untreated, marker, 3 hours treated, 3 hours untreated, 0 hours untreated.

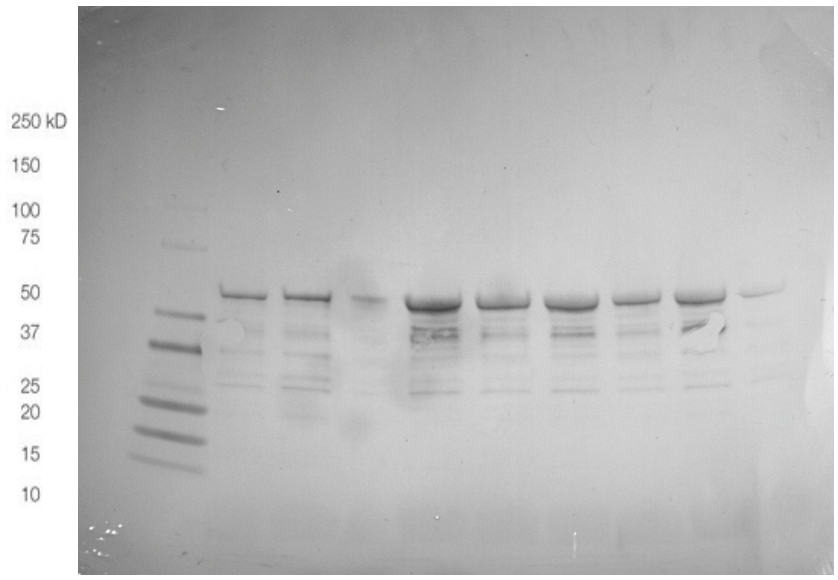


Figure A13. Western Immunoblot performed with a phospho-NF-κB (p65) rabbit mAb at a 1:1000 dilution. MDM-MB-231 breast cancer cells were treated with 175 μg/ml rG3 or media for control cells, and protein expression was observed at various hours after treatment. Lane 1 represents the protein standard. Lanes 2-10: 0 untreated, 3 untreated, 3 treated, 6 untreated, 6 treated, 9 untreated, 9 treated, 12 untreated, 12 treated.

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