12-2013

# Investigating Potential Bioactive Compounds from Rhodococcus and Their Effects on MCF7 Breast Cancer Cells

Megan N. Crabtree
*East Tennessee State University*

# Investigating Potential Bioactive Compounds from *Rhodococcus* and Their Effects on MCF7 Breast Cancer Cells

_____

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

_____

by

Megan Crabtree

December 2013

_____

Dr. Allan Forsman, Chair

Dr. Bert Lampson

Dr. Victoria Palau

Keywords: *Rhodococcus,* MCF7, MTT assay, breast cancer

ABSTRACT

Investigating Potential Bioactive Compounds from *Rhodococcus* and Their Effects on MCF7 Breast Cancer Cells

by

Megan Crabtree

Many drugs used in the treatment of various cancers are derived from or influenced by compounds from nature. The soil bacterium *Rhodococcus* is of interest because of its identified secondary metabolic pathways and the production of novel natural antibiotics from several strains. In this study a solid agar extraction method was used to collect compounds from strains of *Rhodococcus.* These bacterial compound extracts were then tested using a MTT assay in order to evaluate their effectiveness in augmenting MCF7 breast cancer cell death. The results of two way ANOVA analyses revealed 18 compound extracts from 15 strains of *Rhodococcus* that showed significant p-values when assayed with MCF7 breast cancer cells but nonsignificant interaction p-values when assayed with the healthy cell control. These results prompt further identification of specific compounds present in the bacterial extract that caused cell death as well as a mechanism of interaction with the breast cancer cells.

ACKNOWLEDGEMENTS

I would first like to thank my advisor and committee chair, Dr. Allan Forsman. You have introduced me to an exciting area of research and took the time to guide and to teach me through this whole process. I cannot thank you enough for your patience and encouragement during my time here in this graduate program. I would like to further extend my thanks to my committee members , Dr. Bert Lampson and Dr. Victoria Palau, for providing insightful suggestions throughout my process. Additionally I would like to thank the Department of Health Sciences and Dr. Ranjan Chakraborty for welcoming me as a researcher. Also, I would like to thank the GK-12 Science First fellowship program for the chance to work with many talented teachers, students, and faculty at East Tennessee State University and North Side Elementary School.

I am especially thankful to my family for all of their love and support. To my husband Shane, thank you for being there every day and encouraging me to work hard. To my parents Jay and Leann and my siblings, Ryan, Kyle, and Emily, thank you for always supporting me and inspiring me to continue my education. I would not have been able to branch out in search of new adventures without you guys. To my fellow classmates in the Biomedical Sciences program, Megan Carr, Ada Young, Brittany Moore, and Veronica Fergoso, you guys welcomed me to Tennessee and made me feel at home. Thank you for the guidance, advice, and laughs along the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1

# INTRODUCTION

## Cancer at a Glance

In recent years cancer has ranked number 2 among the leading causes of death in the United States. It is estimated that in 2012 alone there were 577,000 deaths from this disease along with 1.6 million new cases of cancer diagnosed in the United States. The occurrence of this disease in both men and women across the racial divides makes it a cause for concern in the global community. Improvements in cancer treatments across the spectrum of cancers have aided in increasing the 5-year survival rate from 49% to 67% over a 30-year period (American Cancer Society, 2012). Improvements in cancer treatments not only stem from a more in-depth understanding of cancer cell behavior at the cellular level and organismal level but also from improved treatment technologies.

Today breast cancer is the most frequently diagnosed cancer among women and ranks second in the leading cause of cancer death in this gender (American Cancer Society, 2012). Depending on factors such as cancer cell characteristics, size, stage, and patient preference, the current treatments for breast cancer include lumpectomy, mastectomy, hormone therapy, and a combination of radiation and chemotherapy drugs. These treatments have increased the 10-year survival rate of breast cancer patients to 83% (American Cancer Society, 2012). While it has been demonstrated that the current treatment plans that involve chemotherapy drugs are effective, these chemicals are often physically taxing to the patient and damaging to the healthy tissues of the body. The compounds that have emerged from nature have been shown to be successful in the treatment of many diseases and conditions without all of the side effects

that are associated with traditional pharmaceuticals. A more in depth look into compounds isolated from nature could be the key to more specific and successful treatment of cancer that is less taxing to the body.

## History of Cancer Treatment

Many drugs used today to treat a wide variety of human diseases and conditions are derived from compounds that have been isolated from nature. In the past 20 years there have been great strides seen in the area of biotechnology. This has given scientists the ability to synthesize compounds. This opportunity has shifted the focus from isolating natural products to creating synthetic compounds in hopes of designing more efficient drugs. This shift brought great hope and possibilities in the beginning stages of its exploration; however, there has not been the large increase in drug productivity that was first hoped.

A 25-year study investigated the trend in the production of new active substances (NASs), also referred to as New Chemical Entities (NCEs). Initially there was a sharp increase in the number of synthesized drugs that had been approved for clinical testing. As time progressed it was seen that the number of synthesized drugs approved for clinical trials had decreased by a large margin (Newman and Cragg 2007). This same 25-year study evaluated all the drugs used for the treatment of all diseases in every country from every source. It was found that only 30% could be classified as purely synthetic in origin. The other 70% of the drugs examined where either a natural product or had some basis in a natural product such as a natural mimic, nature derived pharmacophore, or natural product with a synthetic modification (Newman and Cragg 2007). With all of the advancements in synthesis technology as well as a

better understanding on how chemical entities impact the cell under disease conditions, a large number of successful chemical products still have a natural basis. In this same study by Newman and Cragg, a closer look was given to those drugs on the market that are specifically anticancer. Out of the 175 drugs in this category that are available to Japan and the Western World, only 24% can be classified as purely synthetic. The other 76% of the anticancer drugs are natural products or have some natural product influence (Newman and Cragg 2007). These percentages demonstrate the vast influence that nature has on providing effective compounds to help treat a wide range of diseases and has led many scientists to reconsider the exploration of natural products (Zhu et al. 2011).

## Natural Compounds Inhibiting the mTOR Pathway

Depending on the cancer cell type and stage of progression, cancerous cells can present one or multiple types of mutations. These mutations can occur in regulatory pathways and lead to uncontrolled proliferation, angiogenesis, and evasion of cell death, which is a modification of the cellular response that promotes cell survival instead of cell death when the cell receives stress stimuli. Recently scientists have explored the use of kinase inhibitors to correct or bypass the mutations in regulatory pathways. Kinases are enzymes responsible for adding a phosphate group to another protein. This can cause the phosphorylated protein to undergo a conformational change. This conformational change can result in activation of an enzyme or series of enzymes within a pathway that promote essential cell processes such as metabolism, protein synthesis, and proliferation.

An important regulator within the cell is mTOR, a serine/threonine kinase. This protein is responsible for regulating cell functions such as cell growth, transcription, translation, metabolism, energy balance, and cell survival. It works by acting on signals carrying information such as nutrient levels, hormones, various growth factors, and other cell stresses and activating or inhibiting the appropriate downstream pathways (Watanabe et al. 2011). For example, a receptor on the surface of the cell binds to a growth factor and activates a signaling cascade. This signal gets passed through a series of enzymes to mTOR. From here mTOR is responsible for phosphorylating transcription factors and translational regulators that promote cell growth and survival while at the same time inhibiting enzymes that would result in cell death.

Given its important role in regulating cell death and cell survival, the mTOR pathway has been a target of some recent anticancer drugs with varying amounts of success. Sirolimus, also known as rapamycin is a kinase inhibitor that is derived from a soil sample collected from Ester Island, Rapa Nui. It was identified as a macrolide antibiotic produced by the bacterium *Streptomyces hygroscopicus* (Vezina et al. 1975). Upon discovery and extraction using organic solvents, rapamycin was initially used as an antifungal against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. These fungi can cause oral and genital infections, fungal meningitis, and invasive lung infections in patients who have suppressed immune systems (Vignot et al. 2004). Rapamycin is currently used as an immunosuppressant in patients who undergo organ transplantation because of its effectiveness in prohibiting T-lymphocyte activation and proliferation.

Along with immunosuppressive properties, it was also discovered that rapamycin displayed antiproliferative characteristics. The National Institutes of Cancer (NIC) screened 60

tumor cell lines and found that there was rapamycin sensitivity in doses under 2000 ng/ml in leukemia, ovarian, breast, central nervous system, and small cell lung cancer cell lines (Vignot et al. 2004). After success in clinical trials, Rapamycin was approved in 1999 by the FDA for use in anticancer treatment (Zhu et al. 2011). Investigations continue in creating different analogues of rapamycin to more specifically target different types of cancer based on their defining characteristics and behaviors. Two successful analogues of rapamycin currently on the market are Everolimus and Temsirolimus, which are both used to impede the growth and spread of advanced renal cell carcinoma in cases of tumor resistance to other anticancer drugs. Both of these drugs have been successful in extending the survival of the patient for a number of months to years (Zhu et al. 2011).

In recent years scientists have taken a closer look at the effects that rapamycin has on breast cancer. In this study breast cancer cell lines MDA-MB-231, T47D, MCF-7, and SK-BR3 were examined. These cell lines were selected based on different cell characteristics (Table 1).

Table 1: Breast cancer cell lines and their receptor characteristics. ER-Estrogen receptor. erbB2-an epidermal growth factor receptor.

| **Cell Line** | **Characteristics** | **Rapamycin Sensitivity** |
|---|---|---|
| MDA-MB-231 | ER-Negative<br>Does not overexpress erbB2 | no |
| T47D | ER-Positive | yes |
| MCF-7 | ER-Positive | yes |
| SKBR3 | ER- Negative<br>erbB2-overexpressing | yes |

It was found that the cell lines T47D, MCF-7, and SKBR3 showed sensitivity to rapamycin while MDA-MB-231 showed resistance (Table 1). This study gave insight into the type of cell profile or receptors that need to be present in order for rapamycin to be effective in halting cancer progression (Chang et al. 2004).

## The Use of Steroids in Anticancer Treatment

Steroids are characterized by their origin and their chemical structure. They are fat soluble molecules that originate from a plant or animal source and have a steroid nucleus that contains three 6-member carbon rings and one 5-member carbon ring. The human body has the ability to synthesize and secrete steroids into the bloodstream. These are referred to as steroid hormones and are responsible for regulating bodily functions that include the antiinflammatory response, sexual maturation, and fetal development during pregnancy. Through a multistep process called sterodogenesis, cholesterol is converted to progestins, then to androgens, and lastly into estrogens. The regulation of this process is essential in ensuring the body has a correct response to stimuli (Gupta et al. 2013).

Some cancer cell types have been shown to be stimulated by certain steroid hormones. Estrogen has proven to be a major player in promoting proliferation in some hormone sensitive breast, uterine, ovarian, prostate, and endometrial cancers (Gupta et al. 2013). Estrogen receptors in breast tissue are located on the cellular membrane and in the cytoplasm. These receptors have 2 isoforms, ERα and ERβ. Both isoforms are ligand-regulated transcription factors that transmit hormone signals to cause various responses in the cell. In normal breast tissue, ERα and ERβ bind estradiol causing a signaling cascade that controls proliferation and

differentiation of the cell. These 2 isoforms are present at equally low levels in normal breast tissue; however, in some type of breast cancer ERα is overexpressed. This overexpression stimulates the hormone sensitive cells to excessively proliferate in response to estrogen (Renoir et al. 2013). Estrogens have a direct effect on cell proliferation in certain hormone sensitive cancers. This has prompted researchers to focus their treatment efforts on disrupting steroidogenesis with aromatase inhibitors and disrupting the ability for estrogen to bind to its receptors. Some successful steroid drugs on the market have a natural origin or natural influence. Further exploration of sources of steroid production such as Rhodococcus could provide even more success in this cancer treatment approach (Renoir et al. 2013).

For their use as anticancer agents, steroids have been chemically modified to act as cytotoxic and cytostatic agents. The use of antiestrogens, a cytostatic approach in hormone sensitive breast cancer, has shown some success in arresting tumor development. Antiestrogens compete with endogenous estrogens for binding with estrogen receptors. When the antiestrogen binds to the receptor, it does not initiate the signaling cascade that eventually leads to cell proliferation. This prevents the estrogen sensitive breast cancer cells from dividing in the presence of estrogen (Gupta et al. 2013).

Antiestrogens became an area of interest following the initial success of tamoxifen for treating breast cancer. Tamoxifen emerged as a treatment in the early 1970s and still remains an effective choice for treating receptor positive breast cancer in women who are both premenopausal and postmenopausal. This synthetic drug works through a competitive inhibitor method where it binds in the place of estrogen and does not stimulate cellular growth. While this drug has proven successful, resistance is always a factor when dealing with an endocrine

treatment. There has been resistance to the tamoxifen in later stages of breast cancer as well as in breast cancers that overexpress the ErbB-2 gene, which makes up 15%-20% of all breast cancers diagnosed (Clemons et al. 2002).

To combat cancer cell drug resistance further research has been conducted to investigate the binding pocket of the estrogen receptors. In understanding the structure of the receptor, a more specified competitive inhibitor can be designed. It was seen that a modification at position 7α or 11β is effective in promoting antiestrogenic activity. This led to the discovery of nine potential steroidal estrogens, all of which have shown success and promise in clinical trials (Table 2).

Table 2: Modified steroids that have shown potential as an antiestrogen treatment in breast cancer cells that are hormone sensitive (Gupta, Kumar, and Negi, 2013).

| | |
|---|---|
| 1. | SR16137 (153) |
| 2. | SR16234 (154) |
| 3. | RU3941 (155) 11β-Amidoalkoxyphenyl estradiol (159) |
| 4. | RU51625 (156) |
| 5. | ICI 164,384 (157) |
| 6. | 11β-Perfluorenated fulvestrant (158) |
| 7. | 11β-Amidoalkoxyphenyl estradiol (159) |
| 8. | 11β-(4-Pentafluorinated alkylsulphonylpentayloxyphenyl estradiol (160) |
| 9. | Fulvestrant (161) |

Fulvestrant (161), marketed under the name “Faslodex”, is currently in clinical use against breast cancers that are resistant to tamoxifen. To date it remains the only steroidal antiestrogen that down regulates ERα and does not have estrogenic activity. This means that it does not mimic or block the actions of naturally occurring estrogen in the body. Fulvestrtant works by binding to the estrogen receptors and inhibiting the receptors from dimerizing and becoming active transcription factors. In addition to deactivation, the binding of fulvestrant to

the estrogen receptors speeds up the degradation of the estrogen receptors thus decreasing the number of them in the cell. In this case tumor cells that are estrogen dependent and thrive by using estrogen as a growth factor die off because they are not getting the excess amount of growth signaling that promotes cell division and growth. This competitive binding does not cause all of the estrogen receptors to undergo degredation, thus allowing the naturally occurring estrogen to function on the remaining receptors in the cell. The other promising steroidal antiestrogens are SR 16137 (153) and SR 16234 (154), which act as a competitive inhibitor similar to tamoxifen (Gupta et al. 2013).

## Polar Compounds as Anticancer Agents

Histones play a large role in gene expression in the cell. Histone acetylation by histone acetyltransferases (HAT) cause the condensed chromatin to relax. When the DNA is relaxed, it provides the opportunity for the exposed genes to be transcribed. The reverse process, histone deacetylation by histone deacetylases (HDAC), removes the acetyl group from the histone and causes DNA condensation. This is a highly regulated process and disruption of HAT and HDAC is associated with cancer (Schmudde et al. 2010).

It is understood that in addition to genetic mutations epi-genetic changes to the regulation of HAT and HDAC can alter cell phenotype. Regulation of gene expression has been introduced as another method of cancer treatment. HDAC inhibitors (HDIs) are molecules that interfere with the function of histone deacetlyase (Richon 2006; Richon et al. 2009). They work by binding to the catalytic domain of histone deacetlyase and preventing it from removing the acetyl group from the histone protein. This leaves the DNA in a relaxed state and more

transcriptionally active (Schmudde et al. 2010). By altering the transcription of about 22% of genes, HDIs have shown to be effective in causing cell cycle arrest, terminal differentiation, or apoptosis in transformed cells.

After small polar molecule screens in the 1970s through 1990s, several molecules emmerged from screens of bishydroxamic acids that showed inhibitory potential. These molecules lead to the development of vorinostat (also known as suberoylanilide hydroxamic acid or SAHA). Vorinostat is a synthetic, second generation polar-planar compound. It is a potent HDI and acts by biding to the catalytic domain of the histone deacetylase. In addition vorinostat increases the acetylation of nonhistone related transcription factors such as p53, HIF-1α, and EF2 and also acetylation of cytoplasmic proteins α-tubulin, cortactin, and HSP90 (Richon et al. 2009). This change in acetylation pattern in the cell has been shown to induce differentiation, arrest cell growth, and induce apoptosis in transformed cells at a low dose that does not prove to be toxic to normal cells (Richon 2006). It was approved by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma in October 2006 (Hymes, 2010; Schmudde et al. 2010).

## Nature Derived Histone Deacetylase Inhibitor

The only other approved HDI currently on the market for treatment of T cell lymphoma is romidepsin (Lu et al. 2012). This drug got FDA approval in November 2009 and also goes by the name Istodax. Romidepsin is a cyclic peptide antibiotic that was isolated from the bacterium *Chromobacterium violaceum*. Similar to vorinostat, romidepsin inhibits histone deacetylase and alters gene expression in the cell. This has been shown to not only increase

histone acetylation but also increase p21 and cyclin E and decrease Cyclin D1, which leads to cell cycle arrest at both the G1 and G2/M phases (Hymes, 2010).

Romidepsin and its success in treating T cell lymphoma has promoted further exploration as to its effect on other cancers. In preclinical models, romidepsin has been shown to induce apoptosis of lung, breast, and melanoma cancers. (Hymes, 2010). Combination studies with romidepsin and cisplatin have shown promise in clinical trials by causing DNA damage induced apoptosis in ovarian cancer (Wilson et al. 2012). This drug is also in phase II clinical trials with patients who have recurrent or metastatic head and neck cancer and has shown mild success in stopping tumor progression (Haigentz et al. 2012).

## Bioactive Compounds from Bacterial Families

In an expansive phylogenetic study the origins of many successful and commonly used natural drug-like structures were analyzed. From this study the top 10 phylogenic families that have produced the highest number of drugs approved or currently in clinical trials were identified. The family *Actinomycetales* contains 9 genera that have been known to produce compounds with drug activity (Zhu et al. 2011). Presently, these 9 genera have been studied and shown to be successful at producing drug-like compounds; however, there has been little focus on the other species within this family and their role in creating drug-like compounds. Close examination of the structure of the nature-derived approved and clinical trial drugs in use today reveals that they are composed of several dozen molecular scaffolds, or core structures. These molecular scaffolds are created by enzymes that are partly encoded in specific secondary metabolite gene clusters in a selected group of species. It is not known if these bioactive

compounds are present within select species or scattered within the phylogenetic tree; however, evidence from the drug productive families to date suggests that they are clustered (Zhu et al. 2011). Exploring species that are closely related to species that are known to produce useful compounds may be a good strategy of bioprospecting. This strategy may reveal new natural product compounds that could potentially be of great importance to the medical community.

In looking at the bacterial phylogenetic tree for the order *Actinomycetales*, the family *Nocardiaceae*, which includes *Rhodococcus,* is related to the family *Corynebacteriaceae*. In 2000 the family *Corynebacteriaceae* was identified as a drug-producing family. Due to this discovery it has quickly become an focus of bioprospecting research (Zhu et al. 2011). Given the observation that drug producing families tend to cluster in the phylogenetic tree rather that scatter, the family *Nocardiaceae,* more specifically the genus *Rhodococcus,* would be a good area of focus in hopes of investigating potential bioactive compounds.

## The Genus Rhodococcus

The bacterium *Rhodococcus*, a genus of aerobic, gram positive bacteria, is a member of the order *Actinomycetales*. This genus displays a wide range of different phenotypes and inhabits many environments. It has been isolated from a variety of locations including sea level and Alpine soils and deep sea and coastal sediments (de Carvalho et al. 2005). This order, which has shown great success in producing drug-like products, includes *Streptomyces*, a major antibiotic producer widely used in medicine today (McLeod et al. 2006). *Rhodococcus* is best known for its impact on industry in the areas of fossil fuel desulfurization and the production of

acrylamide and acrylic acid. *Rhodoccucus* is also known for bioactive steroid production, which makes it a target for further exploration in its use in a medical setting.

## Genome Sequencing of Rhodococcus

A better understanding of the genus *Rhodococcus* came to light when the genome of strain RHA1 was sequenced and examined (McLeod et al. 2006). RHA1 is a strain of *Rhodococcus* that has been isolated from soil and is best known for its ability to break down polychlorinated biphenyls (PCBs). This strain is also noted for its ability to use aromatic compounds for its source of carbon. The genome consists of 9,702,737 base pairs compiling 4 linear DNAs, 1 chomosome and 3 plasmids. It has also been observed that the DNA contains a high percentage of guanine and cytosine content.

Sequencing of this strain revealed that *Rhodococcus* has a diverse secondary metabolism. This discovery has led to bioactive compound discovery, thus opening the door to the potential of this bacterium to influence other areas of science. In examining the genome it was discovered that RHA1 contains 9,145 predicted protein coding sequences. Based on current knowledge 38.4% of these sequences encode proteins of unknown function (McLeod et al. 2006). When the secondary metabolism of RHA1 was investigated, it was found that the genome encodes for 24 nonribosomal peptide synthatases and 7 polyketide synthases. Other *Actinomycetes* also possess these secondary metabolic genes, all of which are involved in cell signaling, pigment production, siderophores, and antibiotic production. This finding of a relatively large number of potential secondary metabolic genes is of interest because no rhodococcal secondary metabolites had been reported up to this point (McLeod et al. 2006).

Upon further investigation of the genome, it was discovered that RHA1 contains an opp-encoded oligopeptide transporter, which is a known regulator of secondary metabolic genes (McLeod et al. 2006). These unidentified synthases and transporters show promise in uncovering more applications for this genus of bacteria.

## Bioactivity from Compounds Isolated from Rhodococcus

Until the published work of McLeod, *Rhodococcus* was not widely investigated for the potential production of secondary metabolites. Shortly after the complete sequencing of the RHA1 genome, an in-depth look at the genome resulted in the discovery of 2 antimicrobial peptides from *Rhodococcus josti* K01-B0171. These compounds were isolated from a soil sample taken from Yunnan Province, China. Two cyclic peptide structures demonstrated a lariate or "lasso" type structure and were named lariatins A and B. Both Lariatin A and B were unable to inhibit the growth of Gram negative strains; however, they were both able to inhibit the growth of Gram positive *Mycobacterium smegmatis.* In addition it was found that lariatin A was able to inhibit the growth of *Mycobacterium tuberculosis* (Iwatsuki et al. 2007).

A lab in Japan isolated and identified a new antibiotic from a strain of Rhodococcus. Aurachin RE that emerged from the strain R. erythropolis JCM 6824. It is a quinolone antibiotic that has a similar structure to aurachin C antibiotics that had previously been identified from *Stigmatella aurantiaca*. Compared to aurachin C, aurachin RE has a wider scope of antibiotic activity against Gram positive activity (Kitagawa and Tamura 2008).

Further investigation was done in a lab in Florida focusing on the potential of Rhodococcus. The investigation involved horizontal gene transfer and the production of

antibiotics by *Rhodococcus*. In this study *Rhodococcus* was cocultured with *Streptomycete padanus*, which is known to produce the antibiotic actinomycin. This coculturing resulted in the production of 2 antibiotics, Rhodostreptomycin A and Rhodostreptomycin B by the strain of *Rhodococcus*. These results indicate that gene transfer occurred between these 2 cocultured bacteria that resulted in the production of a new antibiotic (Kurosawa et al. 2008).

## Rhodococcus in the Medical Setting

It is already known that Rhodococci have an exceptional ability to degrade hydrophobic natural compounds as seen by their success in the area of fossil fuel desulfurization. This success can be because their large genome size and the typical appearance of large linear and circular plasmids allow for the presence of many catabolic enzymes and secondary metabolites (Ludmila et al. 2009). *Rhodococci* produce oxygenases, enzymes that are able to oxidize compounds. However, the potential of secondary metabolic compounds has not been explored for their use in a medical setting.

## Present Work

In the present study we examine the cytotoxicity of polar compounds that have been extracted from various strains of *Rhodococcus* on the MCF7 breast cancer cell line. The cytotoxicity, or the loss of viability of a cancer cell by an external source was determined using an MTT assay. Based on the phylogeny of the genus *Rhodococcus* and its relation to other drug-producing families in the order *Actinomycetales,* we hypothesize that *Rhodococcus* may produce compounds that will show activity against the MCF7 breast cancer cell line.

# CHAPTER 2

# MATERIALS AND METHODS

## Bacterial Strains

Sixty isolated strains of *Rhodococcus* were used in this study. These bacterial isolates were cultivated from soil samples from multiple sites in Eastern Tennessee as well as several sites abroad (Table 3). Each strain was purified and catalogued by previous graduate students Tom Barber (Barber 2010) and Ralitsa Borisova (Borisova 2011).

Table 3: Isolated bacterial strains from the genus *Rhodococcus* collected and purified from various soil samples.

| | | | |
|---|---|---|---|
| WFDE08 | NPDEM14 | NPDE012 | CODE08 |
| MTDE09 | WFDEM2 | WLDET3 | CBRMT10 |
| MTM306 | MTM3W5.2 | NP3T8.1 | APDE02 |
| NFDEM13 | KCHXC3 | MTM3T17 | WFDEM3 |
| MTM3W13 | NPM311 | MTM3W12.1a | CBM3T2 |
| CBM3W9 | CBRMT7 | MTM310 | MTM3T5 |
| BTHXC2 | MTM304 | CODET15 | EMHX01 |
| A2M306 | BTHXC10 | CBM3T2 | ACM3C8 |
| ACM3W1 | BTHXC8 | DSM301 | AZM3R1 |
| NPDE011 | CODET15 | WLDE08 | WDM310A |
| BTHXC10 | WDM3P2 | ACM3C1 | WFIEW1 |
| WFDEM6 | SCM3011 | MTM3015 | WFDEM6 |
| ACM3W7 | MTM309a | ACM3W7 | WDM318 |
| MTM304 | MTM3T20 | CBRMT7 | ACM3W5 |
| NPM311 | WDM310a | WDEW | MTDE09 |

## Growth Conditions

All strains were grown using Rich Media (RM) broth and agar. RM was prepared using the following components:

**Rich Media (RM) agar**

| | |
|---|---|
| 1) Distilled H2O | 500 milliliters(mL) |
| 2) Dextrose | 5 grams(g) |
| 3) Nutrient Broth | 4 g |
| 4) Yeast Extract | 0.25 g |
| 5) Bacto Agar | 7.5 g |

**Rich Media (RM) broth**

| | |
|---|---|
| 1) Distilled H2O | 500 milliliters(mL) |
| 2) Dextrose | 5 grams(g) |
| 3) Nutrient Broth | 4 g |
| 4) Yeast Extract | 0.25 g |

For agar media 30ml of the autoclaved RM was poured into 100 mm petri dishes. The agar was allowed to cool and solidify at room temperature followed by storage at 4°C until use. For RM broth media 100ml of the autoclaved broth was transferred into five 150ml sterile bottles and stored at room temperature until use.

## Seed Cultures and Agar Plates

Seed cultures of each strain were prepared and used for inoculation of agar plates for compound extraction. A small amount of bacterial culture for each strain was taken from RM storage slants using a sterile loop. The bacteria were placed in a test tube containing 2.5 mL of RM broth. Each tube was placed in a water bath at 30°C for 24 hours or until the broth reached an appropriate level of turbidity to indicate bacterial growth.

The bacteria grown in the broth was then used to inoculate the agar plates. For each strain 100µL of the seed culture was transferred to a 100mm diameter RM agar plate and spread over the entire surface of the agar using a sterile cotton swab. Each plate was then wrapped in parafilm to prevent desiccation and left to grow at room temperature on the laboratory bench top for 2-4 weeks.

## Compound Agar Extraction

An agar extraction method was used to collect any polar compounds that were being made and released by the soil isolates (derived from Carr, 2010). After growth for 2-4 weeks, each agar plate was divided in half. Each half was placed into a 100mL beaker and the agar was chopped into small pieces. Two beakers for each strain underwent extraction; one with 100% methanol and the other with 100% ethyl acetate. In each case 50mL of the solvent was added to the designated beaker with the chopped up agar and left covered for 24 hours in a fume hood. After soaking for 24 hours, the methanol extract and the ethyl acetate extracts were poured into separate 100mL labeled beakers. These beakers containing the extracts were left uncovered in a designated corner of the fume hood to evaporate for 24 to 72 hours. The agar

that remained in the first set of beakers was then treated with another 50mL of either methanol or ethyl acetate, covered, and left to soak for another 24 hours. After this, the extracts were combined with the previous extracts in their respective beakers and left for 1-4 days in the fume hood for complete evaporation (Figure 1a). Each of the extracts was then redissolved in 1.5mL of 100% methanol and transferred to a 1.5mL eppendorf tube and stored at 4°C until use (Figure 1b).

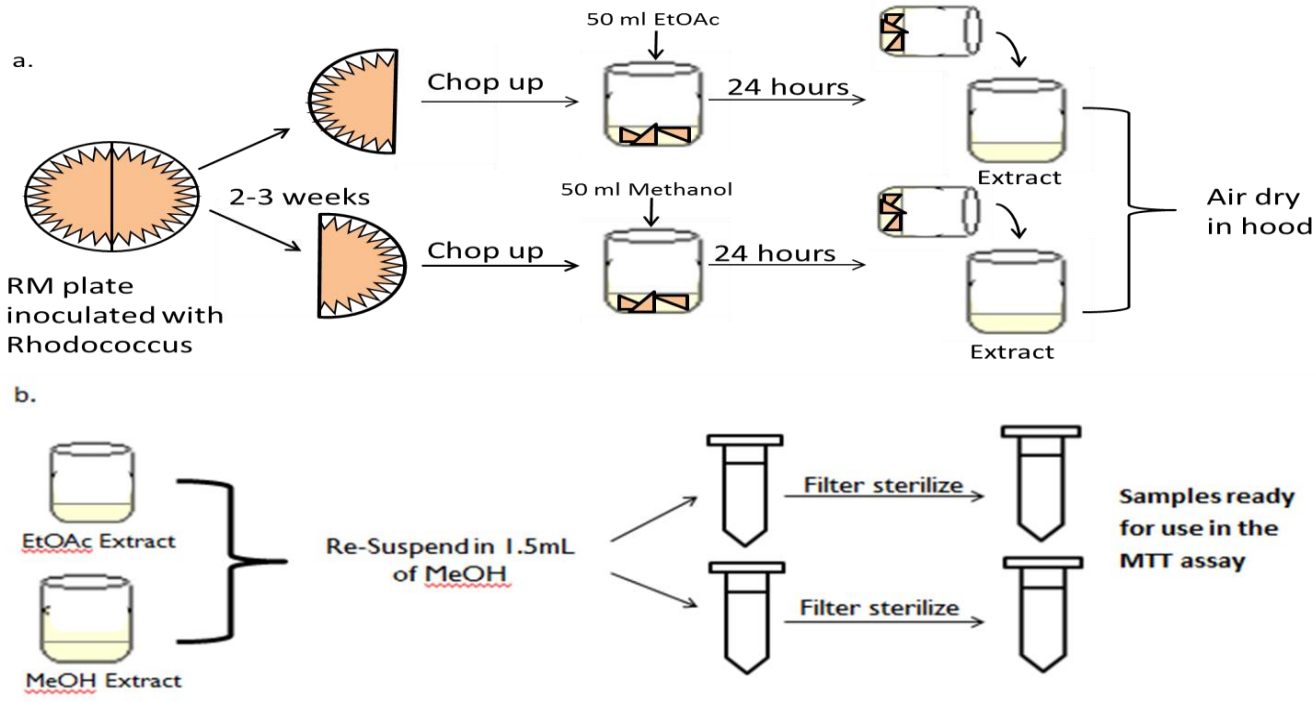


Figure 1: a. Extraction from agar cultures (adapted from Borisova 2011) b. Resuspension of extract in methanol and sterilization in preparation for the MTT assay.

## Cancer Cell Lines

The cell lines used in these studies were MCF7, a Human Breast Adenocarcinoma (cat # HTB-22, ATCC, Manassas, Virginia) and LM929. The MCF7 were the experimental cell line that was originally harvested from the mammary gland of a 69 year old Caucasian female. The

LM929 cells are from a macrophage cell line and were generously donated by Stephen Chapes (Kansas State University, Manhattan, Kansas). The LM929 cells represent healthy tissue and were selected as a cellular control.

## Cell Preparation and Treatment

The LM929 cell line was maintained by culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). No antibiotics were added to the culture media. Passage of these cells was executed using the following procedure. Cellular debris and used media was suctioned out of the 25$cm^3$ culture flask (Fisher Sci, Pittsburgh, Pennsylvania) and 2 ml of Trypsin (1X) was added to the flask. LM929 cells are adherent cells and form cellular adhesions that allow them to attach to the bottom of the flask. The addition of Trypsin promotes the breaking of the cellular adhesions and causes release of the cells into the solution in order for cell harvesting and transfer. The Trypsin was left in the flask with the LM929 cells for 3-5 minutes while the flask remained in the sterile hood. Cells were then rinsed from the flask using 4 ml of DMEM supplemented with 10% FBS. The collected suspension with a total volume of 6 ml (cells, 2 ml Trypsin, 4 ml DMEM media) was transferred to a 15 ml centrifuge tube (Fisher Sci, Pittsburgh, Pennsylvania) and centrifuged (1200 rpm, 5 min, 6°C). After centrifugation, the supernatant was removed and disposed of. The cellular pellet was resuspended in 6 ml of DMEM supplemented with 10% FBS. A fraction of this suspension was added to the culture flask and brought up to a final volume of 6 ml with DMEM supplemented with 10% FBS and returned to the incubator (37°C, 5% $CO_2$).

An alternate procedure was used to maintain the MCF7 cell line. DMEM supplemented with 10% FBS was used in the same quantities as described with the LM929 cell line. MCF7 cells were collected by suctioning out the media in the 25 $cm^3$ flask and adding 2 ml of Trypsin. The flask was then placed in the incubator (37°C, 5% $CO_2$) for 25-30 minutes. MCF7 cells form cell adhesions to the bottom of the flask and to their neighboring cells. These adhesions make the cells appear very clumpy and, when compared to those of the LM929 are stronger and cause the cell to adhere more strongly to the flask and its neighboring cells. Therefore, a longer incubation period with the trypsin is needed to cause the MCF7 cells to release from the bottom of the flask and separate from their neighboring cells. After incubation the cells in the flask were rinsed with 4 ml of DMEM supplemented with 10% FBS and the cell suspension (cells, 2 ml Trypsin, 4 ml DMEM media) collected and transferred to a 15 ml centrifuge tube (Fisher Sci. Pittsburgh, Pennsylvania). The tube was centrifuged at 450 rpm for 5 minutes at 6°C. The supernatant was then removed and the cell pellet was resuspended in 6 ml of DMEM supplemented with 10% FBS. A fraction of this suspension was added to the 25 $cm^3$ culture flask and brought up to a final volume of 6 ml with DMEM supplemented with 10% FBS and returned to the incubator (37°C, 5% CO2).

## MTT Assay

An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay that is used to examine cell viability. This assay is based on a cellular reduction reaction that takes place in the mitochondria of living cells (Figure 2). MTT is a positively charged tetrazolium salt that is a pale yellow color. It is taken up by the cell and

enters into the mitochondrion. Within the mitochondrion MTT is acted on by the enzyme mitochondrial reductase and converted to formazan, a purple colored crystal. Once the MTT is converted to formazan crystals, it cannot pass through or be transported across the plasma membrane and begins to accumulate within the cell. After a fixed incubation period, a solubilization buffer is added. This buffer lyses the cells and dissolves any formazan crystals that were formed. If there were living cells in the test wells, there would be mitochondrial reductase activity promoting MTT conversion to formazan crystals. In this case the addition of the solubilization buffer would result in the solution in the test well turning a purple color. If there were no living cells present in the test well, there would be no active mitochondrial reductase enzyme to convert MTT to formazan crystals. This would result in a test well that has no purple color. Absorbencies for each of the test wells are measured with a plate reader. Varying shades of purple color that are measured in each well are proportional to the number of living cells in that well. This means that lighter purple color indicates fewer living cells and dark purple color indicates more living cells. The MTT assay is used as a quantitative method to allow cell viability to be determined through a colormetric, or color changing reaction which is directly proportional to the activity of the mitochondrion.

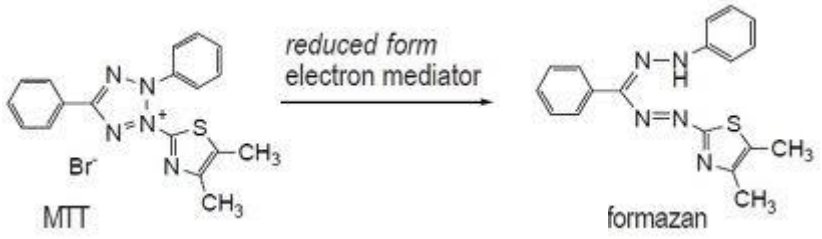


Figure 2: Conversion of MTT to formazan in the mitochondria (Stockert et al, 2012)

In preparation for the MTT assay cells were collected from a 25 $cm^3$ flask of the appropriate cell line and transferred to a Corning 75 $cm^3$ flask (Fisher Sci, Pittsburgh, Pennsylvania) in order to increase cell yield. These larger flasks were maintained using the same procedures as mentioned above corresponding to their cell line. Once the fraction of cells was added to the 75 $cm^3$ flask, DMEM supplemented with 10% FBS was added to a final volume of 15 ml. Cells were grown until confluency was achieved before an assay was performed.

Cells from the MCF7 and LM929 cell lines were collected from confluent 75 $cm^3$ flasks using the procedures mentioned above with the only modifications being the use of 5mL of trypsin in order to break cell adhesions and 6mL of DMEM supplemented with 10% FBS to rinse the flask. The incubation times and centrifugation parameters were all kept consistent for all flasks of each cell line. After cells were centrifuged the supernatant was removed and the cell pellet was resuspended with 10mL of DMEM supplemented with 10% FBS. A cell count was then preformed using a hemocytometer and a new cell suspension of $5 \times 10^4$ cells/mL was made. This new cell suspension was used to seed all the wells of the 96 well cell culture plates used for the MTT assay so that there were $5 \times 10^4$ cells per well.

Each well of the 96 well cell culture plate was seeded with 100µl of DMEM supplemented with 10% FBS. An additional 70µl of DMEM supplemented with 10% FBS was added to the first 3 wells of each of the rows corresponding to a sample. Next, 30µl of filter sterilized sample compounds was added to the first 3 wells of a row. The compounds were then serially diluted in triplicate across one row of the cell culture plate by removing 100µl from each of the first 3 wells and pipetting it into the following triplicate of wells in that row using a

multichannel pipette for accuracy. The dilution was carried out through the last triplicate of the row and the remaining 100µl from the last 3 wells after dilution was discarded. 100µl of the 5 x $10^4$ cell dilution was then added to each of the wells. The final volume of each well was 200µl (Figure 3). Each set of assay cell culture plates was run with a serial dilution in triplicate of HCl across 2 rows in order to establish a percent killing curve. Additional controls run across one row of wells in triplicate included cells in media only control, a 15% methanol control, a 15% blank methanol RM agar plate extraction control and a 15% blank ethyl actetate-methanol RM agar plate extraction control.

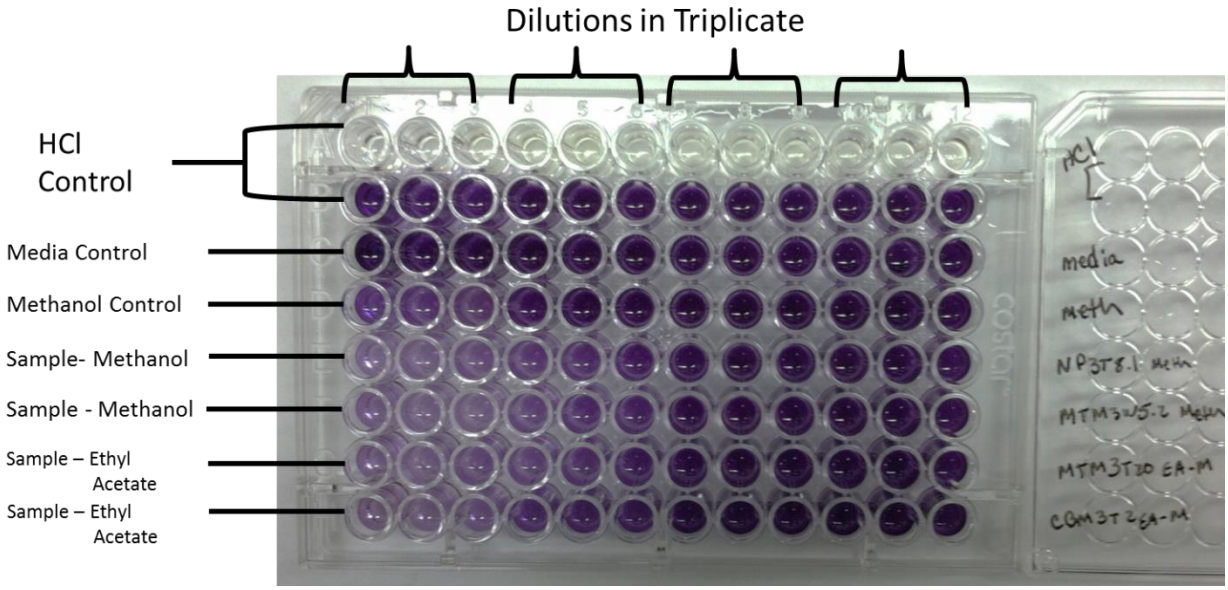


Figure 3: MTT assay cell culture plate set up

All cell culture plates were placed in the incubator (37°C, 5% $CO_2$) for 72 hours. These conditions promoted cell growth and proliferation while giving the cells enough time to propagate so that any effect the compound had on cell growth could be observed. After this incubation period, the plates were removed from the incubator and 20µl of MTT reagent was

added to each well. The plates were then placed back into the incubator for an additional 4 hours to allow the MTT to be taken up by any living cells and converted to formazan crystals by the enzyme mitochondrial reductase. After this incubation period, the cell culture plates were removed from the incubator and the growth media in each well was suctioned off and discarded, taking care not to suction off the cells that were still adherent to the bottom of the wells. Then, 200µl of Iso-PBS, a solubilization buffer, was added to each of the wells in order to dissolve any formazan crystals that were present in the wells. Each plate was then wrapped tightly with plastic wrap and left overnight on the bench top at room temperature and read by the plate reader the following day. This was to ensure that the Iso-PBS solubilization buffer had sufficient time to dissolve all the formazan crystals that were formed in order to get an accurate absorbency reading of each test well.

The cell culture plates were read using at plate reader at 620nm. After the plates were read and the absorbencies recorded, the average absorbency of each triplicate was calculated. The percent kill (% kill) and percent inhibition of each of the compounds and controls was determined by comparing the average absorbency of the sample triplicate to the average absorbency of each of the controls using the following formulas where Max = the acid control, Ctl = the media control, A = mean absorbance value of treated wells, and B = mean absorbency values of the control wells.

$$\% \text{ Kill} = (\text{Exp} - \text{Ctl})/(\text{Max}-\text{Ctl}) \times 100$$

$$\% \text{ Inhibition} = (1-A/B) \times 100\%$$

The absorbencies of each of the compounds were evaluated using a two-way ANOVA. Being that each treatment was done in triplicate it was important to select a test that considered the spread of the data within each treatment and was able to compare the means of each treatment. The two-way ANOVA statistical test was chosen because it is a more robust statistical analysis that is able to determine if there is a statistically significant difference between the means of the treatments relative to the spread of the observations for each concentration treatment.

# CHAPTER 3

# RESULTS

## MTT Assay Analysis

A two-way ANOVA was performed for each one of the compound extracts in order to assess if there was a significant variation between the mean absorbency reading at 620nm of the triplicates undergoing treatment and the means of the control triplicates. Each of the compound extracts was compared to a media control and to a methanol control that was conducted on the same set of assay plates in order to control for factors such as barometric pressure, humidity, and cell passage number. The methanol compound extracts were then compared to a blank-methanol control while the ethyl acetate extracts were compared to a blank-ethyl acetate methanol control. The hydrochloric acid control (HCl) was not used to compare the treatments because it demonstrated an all or nothing cell survival response and a cell survival curve could not be determined.

A nonsignificant two-way ANOVA, meaning the p-value for interaction is above the level of significance ($\alpha > 0.05$), indicates that the effect of concentration is similar for the treatment and the control. The interaction plot for a compound and a control that was not significant would show the control and the treatment with the compound extract behaving in a similar pattern with respect to concentration (Figure 4).

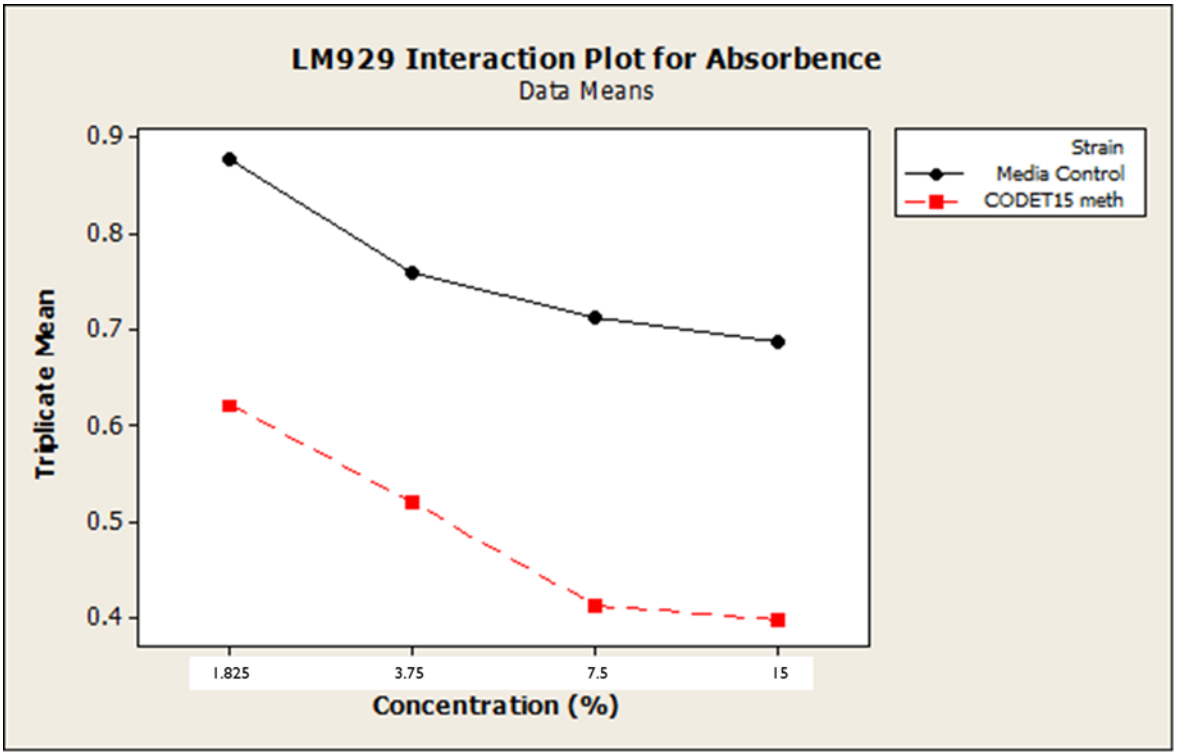


Figure 4: Non-significant interaction plot with LM929 cells between the media control and strain CODET15 meth. Both the media control and CODET15 meth show a decrease in the triplicate mean with an increase in concentration.

A significant two-way ANOVA, meaning the p-value for interaction is below the level of significance ($\alpha < 0.05$), indicates that the effect of concentration is different for the treatment and the control. The interaction plot for a compound and a control that was significant would show the control and the compound extract behaving differently with respect to the concentration (Figure 5).

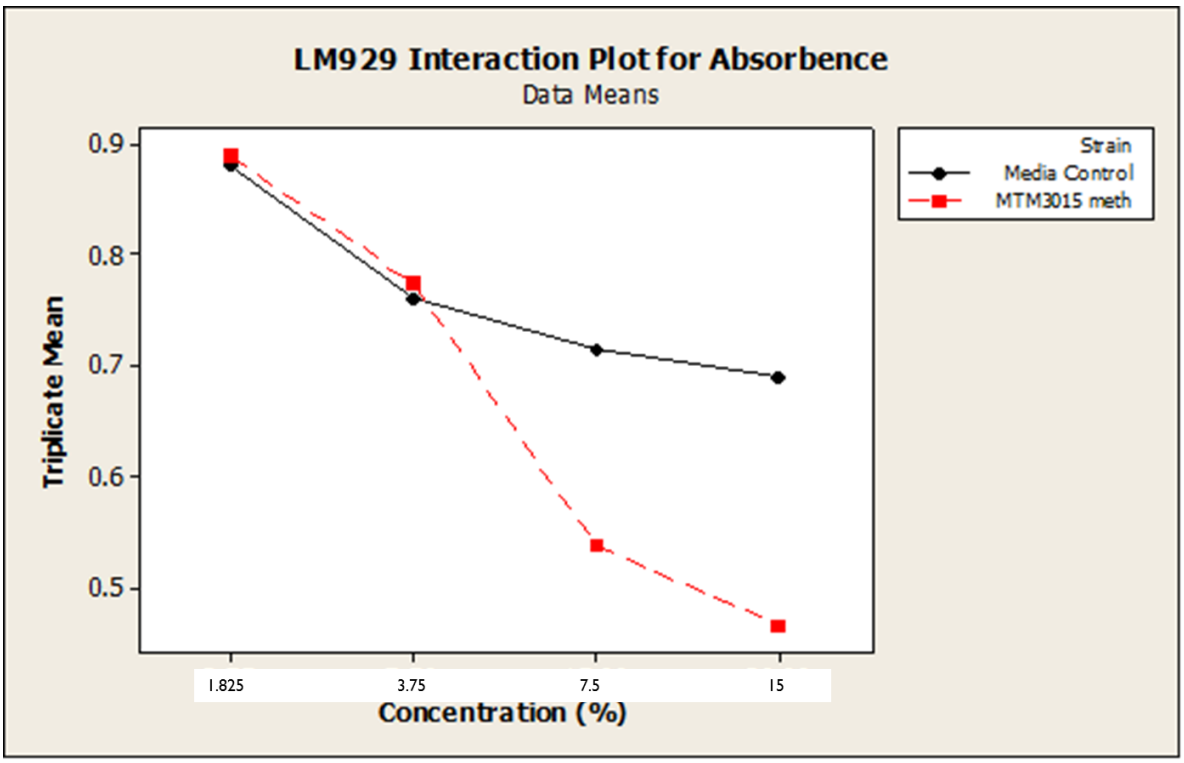


Figure 5: Significant interaction plot with LM929 cells between the media control and strain MTM3015 meth. Both the media control and MTM3015 meth show a decrease in the triplicate mean with an increase in concentration, however MTM3015 showed a significant decrease in mean absorbency at the 7.5% and 15% concentrations.

## MCF7 MTT Assays

All 3 controls had a significant interaction p-values when compared to the media control (Figure 6). As the concentration of the control treatment increased, the absorbency decreased.

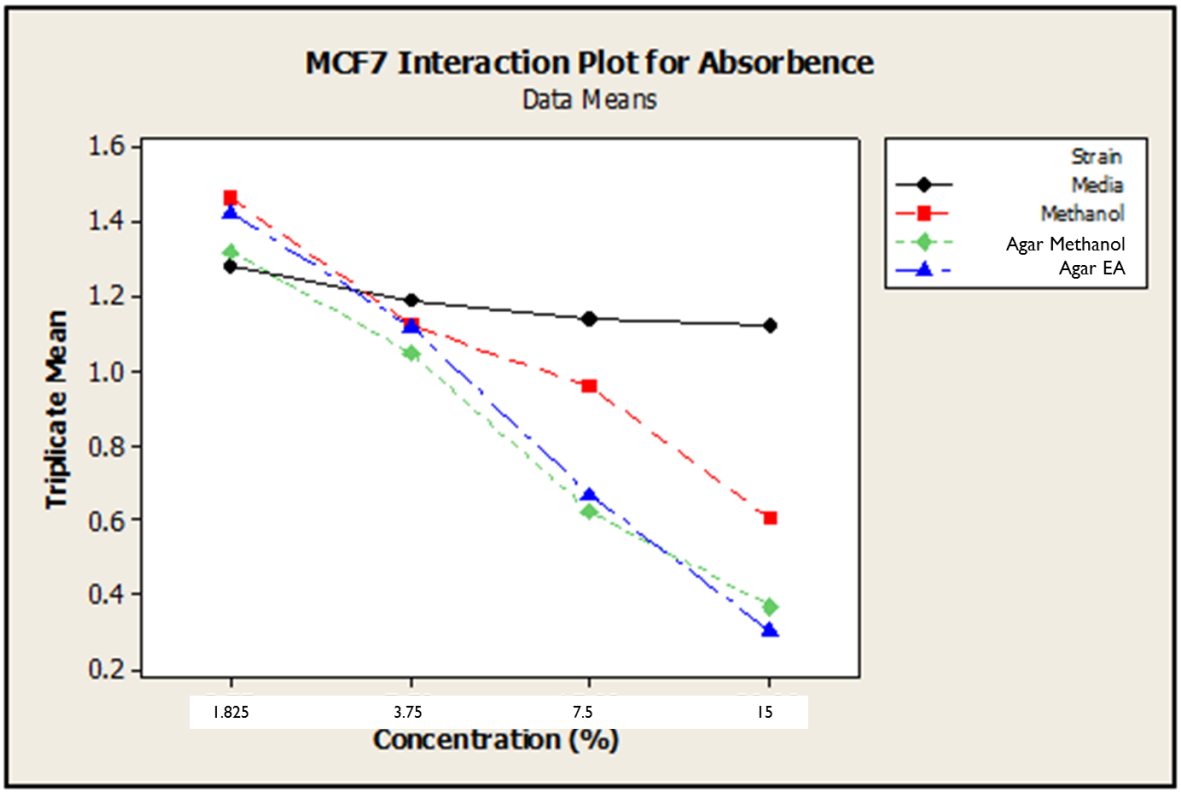


Figure 6: The interaction plot for MCF7 cells assayed on plates 1-5. The methanol control, BLANK methanol control and the BLANK EA-M controls showed a significant interaction ($p < .05$).

In comparing all 60 compound extracts, as well as the methanol, blank-methanol, and blank ethyl acetate-methanol controls to the media control in the assays using MCF7 cells, it was seen that all 60 showed an interaction p-value that was significant ($\alpha < 0.05$). This means that the addition of each of the compound extracts to their respective wells caused there to be a significant difference in the mean absorbency of the treated triplicates when compared to the mean absorbencies of the media control triplicates. In looking at the individual interaction plots it was seen that the biggest variation in mean absorbency reading was seen at the 15% concentration and the 7.5% concentration (Figure 7). This indicates that at higher

concentrations of compound extract, there were fewer living cells as indicated by the low absorbency reading.

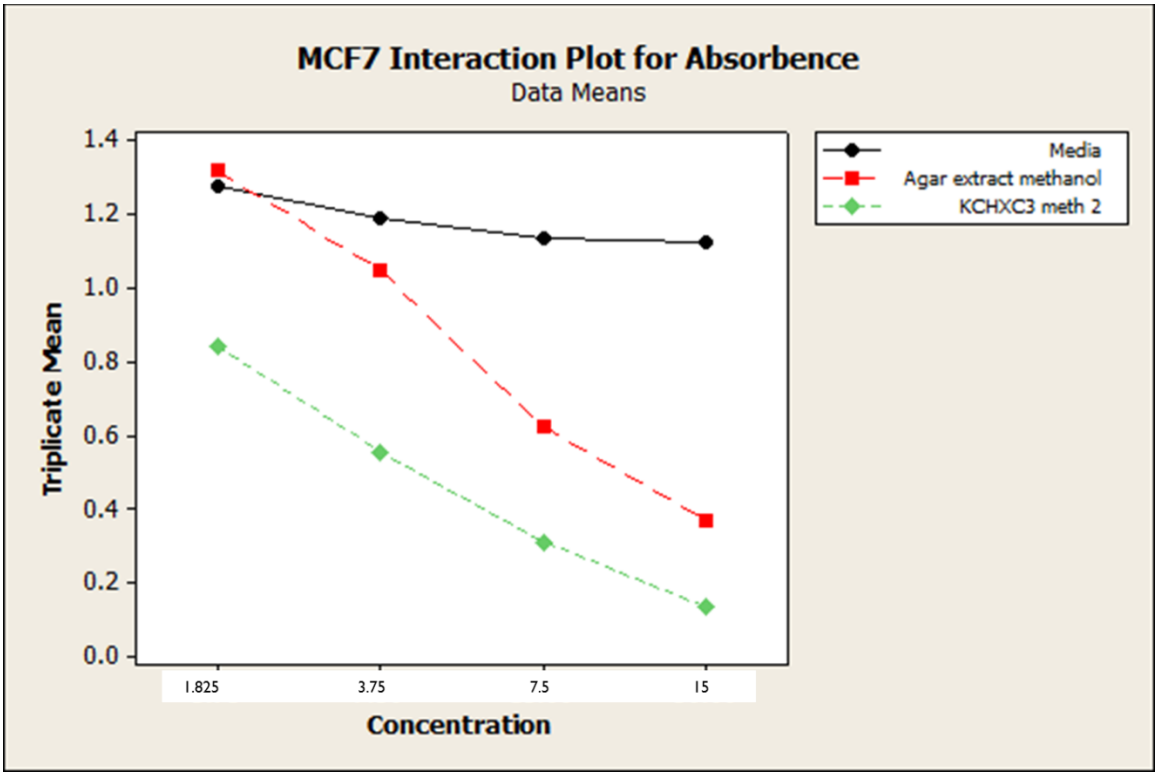


Figure 7: The interaction plot for strain KCHXC3 with MCF7 cells showing a significant interaction when compared to the media and agar controls ($p < .05$).

In comparing 60 compound extracts to the methanol control, 36 of them showed an interaction p-value that was significant. Eighteen of these compounds extracts were methanol and the other 18 where ethyl acetate-methanol compound extracts (Table 4).

Table 4: a. Methanol compound extracts that showed significant interaction p-values when compared to the methanol control. b. Ethyl Acetate-Methanol compound extracts that showed significant interaction p-values when compared to the methanol control.

a.

| | | |
|---|---|---|
| WLDET3 meth | AZM3R1 meth | WDM3P2 meth |
| DSM301 meth | ACM3W7 meth | CODET15 meth |
| MTM3015 meth | NPDE011 meth | ACM3W7 meth |
| WFIEW1 meth | MTM309a meth | WDM310A meth |
| SCM3011 meth | WDM318 meth | WFDEM6 meth |
| CBRMT7 meth | ACM3W5 meth | WDM310a meth |

b.

| | | |
|---|---|---|
| NFDEM13 EA-M | MTM3W12.1a EA-M | WDM3P2 EA-M |
| DSM301 EA-M | BTHXC10 EA-M | AZM3R1 EA-M |
| WFDEM6 EA-M | NPDE011 EA-M | ACM3W7 EA-M |
| WLDE08 EA-M | MTM309a EA-M | WDM310A EA-M |
| ACM3W7 EA-M | WFIEW1 EA-M | WDM318 EA-M |
| ACM3C1 EA-M | WFDEM6 EA-M | MTM3T20 EA-M |

The blank-methanol control was used to account for any component in the agar that may have an effect on the proliferation of the MCF7 breast cancer cells. The blank-methanol rich media (RM) plates did not contain bacteria and were extracted using the same methods as the RM plates with bacteria. There were 11 methanol compound extracts that had a significant p-value when compared to the blank methanol control (Table 5).

Table 5. Compounds extracts that showed a significant interaction p-value when compared in a two-way ANOVA to the blank-methanol control.

| | |
|---|---|
| WFDE08 meth | NPDEM14 meth |
| CODE08 meth | WFDEM2 meth |
| WLDET3 meth | CBRMT10 meth |
| MTM3W5.2 meth | NFDEM13 meth |
| KCHXC3 meth 1 | KCHXC3 meth 2 |
| MTM3T17 meth | |

For the bacterial strains that were extracted using ethyl acetate and methanol, a blank ethyl acetate-methanol (EA-M) control was used. All the strains that were extracted with ethyl acetate and reconstituted in methanol were compared to this control in order to assess if there was a component of the agar that was causing a variation in the absorbency reading or the variation was caused by something in the compound extract. There were 24 ethyl acetate-methanol compounds extracts that showed significant interaction p-values when compared to the blank EA-M control (Table 6).

Table 6: Compound extracts that showed significant interaction p-values when compared in a two-way ANOVA to the blank EA-M control.

| | | | |
|---|---|---|---|
| WFDE08 EA-M | NPDEM14 EA-M | NPDE012 EA-M | CODE08 EA-M |
| MTDE09 EA-M | WFDEM2 EA-M | WLDET3 EA-M | CBRMT10 EA-M |
| MTM306 EA-M | MTM3W5.2 EA-M | NP3T8.1 EA-M | APDE02 EA-M |
| NFDEM13 EA-M | KCHXC3 washed EA-M | KCHXC3 EA-M 1 | KCHXC3 EA-M 2 |
| MTM3T17 EA-M | BTHXC8 EA-M | CODET15 EA-M | WFIEW1 EA-M |
| SCM3011 EA-M | ACM3W7 EA-M | WDM318 EA-M | MTM3T20 EA-M |

All compound extracts that showed interaction with any of the controls were noted and used for comparison to the interaction with LM929 cells (Table 7).

Table 7: Summary of all the extracts that showed significant interaction p-values in a two way ANOVA when compared to a methanol, blank methanol, and blank ethyl acetate-methanol control for the MTT assays conducted with MCF7 cells.

| Strain | Methanol | Blank Meth | Blank EA-M |
|---|---|---|---|
| WFDE08 meth | no | yes | |
| WFDE08 EA-M | no | | yes |
| NPDEM14 meth | no | yes | |
| NPDEM14 EA-M | no | | yes |
| NPDE012 EA-M | no | | yes |
| CODE08 meth | no | yes | |
| CODE08 EA-M | no | | yes |
| MTDE09 EA-M | no | | yes |
| WFDEM2 meth | no | yes | |
| WFDEM2 EA-M | no | | yes |
| WLDET3 meth | yes | yes | |
| WLDET3 EA-M | no | | yes |
| CBRMT10 meth | no | yes | |
| CBRMT10 EA-M | no | | yes |
| MTM306 EA-M | no | | yes |
| MTM3W5.2 meth | no | yes | |
| MTM3W5.2 EA-M | no | | yes |
| NP3T8.1 EA-M | no | | yes |
| APDE02 EA-M | no | | yes |
| NFDEM13 meth | no | yes | |
| NFDEM13 EA-M | yes | | yes |
| KCHXC3 washed EA-M | no | | yes |
| KCHXC3 meth 1 | no | yes | |
| KCHXC3 EA-M 1 | no | | yes |
| KCHXC3 meth 2 | no | yes | |
| KCHXC3 EA-M 2 | no | | yes |
| MTM3T17 meth | no | yes | |
| MTM3T17 EA-M | no | | yes |
| MTM3W12.1a EA-M | yes | | no |
| BTHXC8 EA-M | no | | yes |
| DSM301 meth | yes | no | |
| DSM301 EA-M | yes | | no |

| Strain | Methanol | Blank Meth | Blank EA-M |
|---|---|---|---|
| AZM3R1 meth | yes | no | |
| AZM3R1 EA-M | yes | | no |
| NPDE011 meth | yes | no | |
| NPDE011 EA-M | yes | | no |
| CODET15 meth | yes | no | |
| CODET15 EA-M | no | | yes |
| WLDE08 EA-M | yes | | no |
| WDM310A meth | yes | no | |
| WDM310A EA-M | yes | | no |
| BTHXC10 EA-M | yes | | no |
| WDM3P2 meth | yes | no | |
| WDM3P2 EA-M | yes | | no |
| ACM3C1 EA-M | yes | | no |
| WFIEW1 meth | yes | no | |
| WFIEW1 EA-M | yes | | yes |
| WFDEM6 meth | yes | no | |
| WFDEM6 EA-M | yes | | no |
| SCM3011 meth | yes | no | |
| SCM3011 EA-M | no | | yes |
| MTM3015 meth | yes | no | |
| WFDEM6 EA-M | yes | | no |
| ACM3W7 meth | yes | no | |
| ACM3W7 EA-M | yes | | no |
| MTM309a meth | yes | no | |
| MTM309a EA-M | yes | | no |
| ACM3W7 meth | yes | no | |
| ACM3W7 EA-M | yes | | yes |
| WDM318 meth | yes | no | |
| WDM318 EA-M | yes | | yes |
| MTM3T20 EA-M | yes | | yes |
| CBRMT7 meth | yes | no | |
| ACM3W5 meth | yes | no | |
| WDM310a meth | yes | no | |

## LM929 MTT Assays

It is important to use the LM929 cell line in this investigation. This cell line acts as a healthy cell control. In the scope of cancer treatment, the ideal goal is to eradicate the cancerous cells while leaving the healthy cells unharmed. While it is important to assay for the effect of potential compounds against cancerous cells, it is also important that the compounds be assayed with healthy cells to assess their effects. A strong candidate for further investigation would be a compound that showed significant interaction when assayed with MCF7 cells but nonsignificant interaction when assayed with LM929 cells.

All 60 compound extracts, as well as the blank-methanol and blank-ethyl acetate-methanol controls, were assayed using LM929 cells and compared in a two-way ANOVA to media and methanol controls. When compared to the media control, the methanol control did not show a significant interaction p-value; however, both the blank-methanol and blank EA-M controls did show a significant interaction p-value (Figure 8). Again a significant P-value indicates that that the test component in the well is behaving differently than the control to which it is being compared.

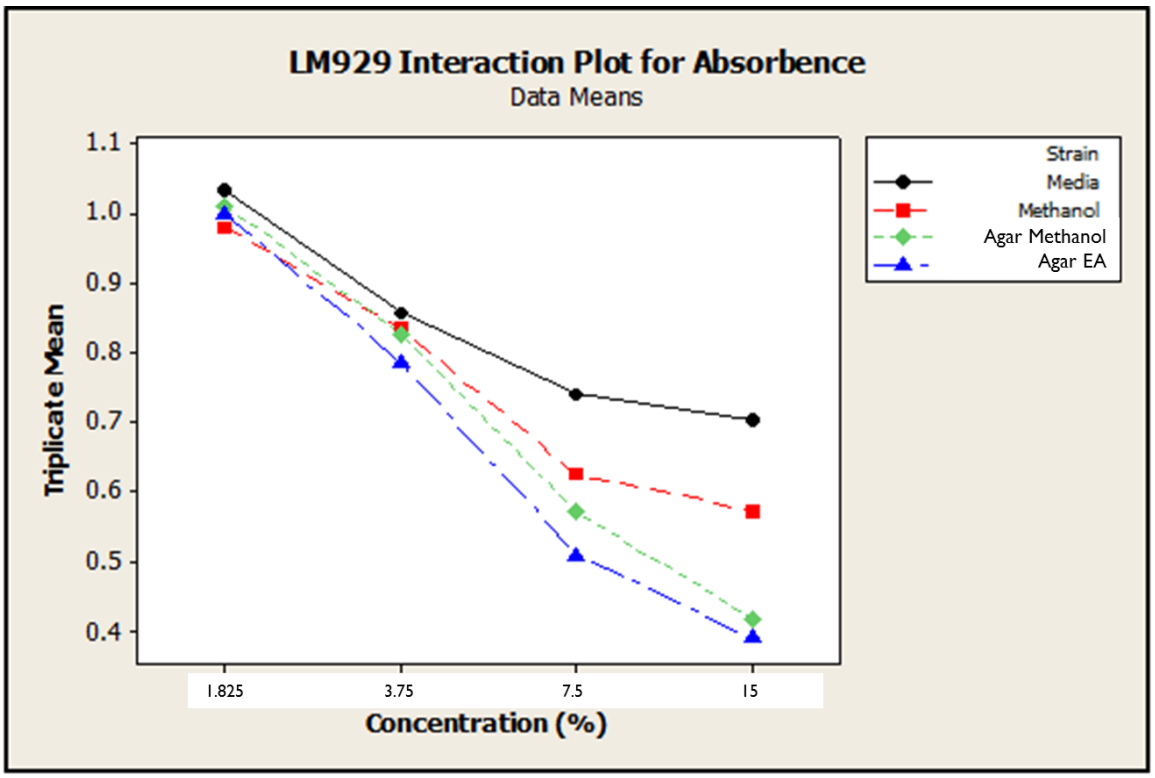

Figure 8: The interaction plot for LM929 cells assayed on plates 1-4. The BLANK methanol control and the BLANK EA-M controls showed a significant interaction while the methanol control did not show a significant interaction p-value.

There were 16 methanol compound extracts and 21 EA-M compound extracts that showed significant interaction p-values in a two-way ANOVA when compared to the media control (Table 8).

Table 8: a. Methanol compound extracts that showed significant interaction p-values when compared to the media control in a two-way ANOVA. b. EA-M compound extracts that showed significant interaction p-values when compared to the media control in a two-way ANOVA.

a.

| | |
|---|---|
| CBRMT10 meth | NP3T8.1 meth |
| ACM3C8 meth | ACM3W1 meth |
| BTHXC2 meth | MTM3010 meth |
| MTM304 meth | CODET15 meth |
| MTM3W12.1a meth | KCHXC3 washed meth |
| DSM301 meth | AZM3R1 meth |
| MTM3015 meth | WFDEM6 meth |
| ACM3W7 meth | MTM309a meth |

b.

| | |
|---|---|
| KCHXC3 EAM | MTM3W12.1a EAM |
| AZM306 EAM | BTHXC10 EAM |
| ACM3C8 EAM | ACM3W1 EAM |
| BTHXC8 EAM | BTHXC2 EAM |
| MTM3010 EAM | CODET5 EAM |
| EMHX01 EAM | KCHXC3 EAM 2 |
| MTM3W13 EAM | NPM311 EAM |
| KCHXC3 washed EAM | DSM301 EAM |
| AZM3R1 EAM | NPDE011 EAM |
| MTM3015 EAM | MTM309a EAM |
| MTM304 EAM | |

There were 8 methanol compound extracts and 7 EA-M compound extracts that had significant interaction p-values against the methanol control (Table 9).

Table 9: a. Methanol compound extracts that showed significant interaction p-values when compared with a methanol control in a two-way ANOVA. b. EA-M compound extracts that showed significant interaction p-values when compared with a methanol control in a two-way ANOVA.

a.

| | |
|---|---|
| CBRMT10 meth | ACM3C8 meth |
| MTM3010 meth | MTM304 meth |
| CODET15 meth | MTM3T17 meth |
| MTM3W12.1a meth | KCHXC3 washed meth |

b.

| | |
|---|---|
| NFDEM13 EAM | KCHXC3 EAM |
| AZM306 EAM | BTHXC2 EAM |
| MTM3010 EAM | EMHX01 EAM |
| KCHXC3 washed EAM | |

A blank-methanol control was used in the two-way ANOVA analysis for those compound extracts that were extracted with methanol. There were 23 methanol compound extracts that showed significant interaction p-value (Table 10a). A blank EA-M control was used in the two-way ANOVA analysis for those compound extracts that were extracted with ethyl acetate and reconstituted in methanol. There were 23 EA-M compound extracts that showed significant interaction p-values (Table 10b).

Table 10: a. Methanol compound extracts that showed significant interaction p-values when compared to a blank methanol control. b. Ethyl acetate-methanol compound extracts that showed significant interaction p-values when compared to a blank EA-M control.

a.

| | | |
|---|---|---|
| WFDE08 meth | NPDEM14 meth | MTDE09 meth |
| WFDEM2 meth | MTM306 meth | MTM3W5.2 meth |
| MTM3W12.1 a meth | ACM3C8 meth | MTM310 meth |
| MTM3010 meth | CODET15 meth | MTM3T17 meth |
| KCHXC3 washed meth | KCHXC3 meth 1 | CODET15 meth |
| WLDE08 meth | ACM3C1 meth | WFIEW1 meth |
| SCM3011 meth | ACM3W7 meth | NPM311 meth |
| WDM310a meth | MTDDE09 meth | |

b.

| | | |
|---|---|---|
| WFDE08 EAM | MTDE09 EAM | MTM306 EAM |
| NFDEM13 EAM | KCHXC3 EAM | AZM306 EAM |
| CBM3T2 EAM | CBMT7 EAM | MTM310 EAM |
| BTHXC2 EAM | MTM3010 EAM | KCHXC3 EAM 2 |
| NPM311 EAM | KCHXC3 washed EAM | KCHXC3 EA- 1 |
| CODET15 EAM | WLDE08 EAM | WDM3P2 EAM |
| ACM3C1 EAM | WFDEM6 EAM | SCM3011 EAM |
| ACM3W7 EAM | WDM318 EAM | |

A summary of all the compound extracts and their activity with LM929 cells were noted to be used for a comparison (Table 11).

Table 11: Summary of all the extracts that showed significant interaction p-values in a two-way ANOVA when compared to a media, methanol, blank methanol, and blank ethyl acetate-methanol control for the MTT assays conducted with LM929 cells.

| Strain | Media | Methanol | Blank Meth | Blank EA-M |
|---|---|---|---|---|
| WFDE08 meth | no | no | yes | |
| WFDE08 EA-M | no | no | | yes |
| NPDEM14 meth | no | no | yes | |
| MTDE09 meth | no | no | yes | |
| MTDE09 EA-M | no | no | | yes |
| WFDEM2 meth | no | no | yes | |
| CBRMT10 meth | yes | yes | no | |
| MTM306 meth | no | no | yes | |
| MTM306 EA-M | no | no | | yes |
| MTM3W5.2 meth | no | no | yes | |
| NP3T8.1 meth | yes | no | no | |
| NFDEM13 EA-M | no | yes | | yes |
| KCHXC3 EA-M | yes | yes | | yes |
| MTM3W12.1 a meth | no | no | yes | |
| MTM3W12.1a EA-M | yes | no | | no |
| AZM306 EA-M | yes | yes | | yes |
| BTHXC10 EA-M | yes | no | | no |
| CBM3T2 EA-M | no | no | | yes |
| ACM3C8 meth | yes | yes | yes | |
| ACM3C8 EA-M | yes | no | | no |
| ACM3W1 meth | yes | no | no | |
| ACM3W1 EA-M | yes | no | | no |
| BTHXC8 EA-M | yes | no | | no |
| CBMT7 EA-M | no | no | | yes |
| MTM310 meth | no | no | yes | |
| MTM310 EA-M | no | no | | yes |
| BTHXC2 meth | yes | no | no | |
| BTHXC2 EA-M | yes | yes | | yes |
| MTM3010 meth | yes | yes | yes | |
| MTM3010 EA-M | yes | yes | | yes |
| MTM304 meth | yes | no | no | |
| CODET15 meth | yes | yes | yes | |
| CODET5 EA-M | yes | no | | no |
| EMHX01 EA-M | yes | yes | | no |
| KCHXC3 EA-M 2 | yes | no | | yes |
| MTM3T17 meth | no | yes | yes | |
| MTM3W13 EA-M | yes | no | | no |

| Strain | Media | Methanol | Blank Meth | Blank EA-M |
|---|---|---|---|---|
| NPM311 EA-M | yes | no | | yes |
| MTM3W12.1a meth | yes | yes | yes | |
| KCHXC3 washed meth | yes | yes | yes | |
| KCHXC3 washed EA-M | yes | yes | | yes |
| KCHXC3 meth 1 | no | no | yes | |
| KCHXC3 EA-M 1 | no | no | | yes |
| DSM301 meth | yes | no | no | |
| DSM301 EA-M | yes | no | | no |
| AZM3R1 meth | yes | no | no | |
| AZM3R1 EA-M | yes | no | | no |
| NPDE011 EA-M | yes | no | | no |
| CODET15 meth | no | no | yes | |
| CODET15 EA-M | no | no | | yes |
| WLDE08 meth | no | no | yes | |
| WLDE08 EA-M | no | no | | yes |
| WDM3P2 EA-M | no | no | | yes |
| ACM3C1 meth | no | no | yes | |
| ACM3C1 EA-M | no | no | | yes |
| WFIEW1 meth | no | no | yes | |
| WFDEM6 EA-M | no | no | | yes |
| SCM3011 meth | no | no | yes | |
| SCM3011 EA-M | no | no | | yes |
| MTM3015 meth | yes | no | no | |
| MTM3015 EA-M | yes | no | | no |
| WFDEM6 meth | yes | no | no | |
| ACM3W7 meth | yes | no | no | |
| MTM309a meth | yes | no | no | |
| MTM309a EA-M | yes | no | | no |
| ACM3W7 meth | no | no | yes | |
| ACM3W7 EA-M | no | no | | yes |
| WDM318 EA-M | no | no | | yes |
| MTM304 EA-M | yes | no | | no |
| NPM311 meth | no | no | yes | |
| WDM310a meth | no | no | yes | |
| WDEW3 meth | no | no | no | |
| MTDDE09 meth | no | no | yes | |

## Activity Comparison in the MCF7 and LM929 Cell Lines

An ideal candidate for further investigation would be a compound extract that showed growth inhibition or killing activity when assayed with the MCF7 cells but no activity when assayed with the LM929 cells. Evaluation based on the compound extract activity when compared to either the blank methanol or blank ethyl acetate-methanol control is used to evaluate potential strains of *Rhodococcus* that warrant further evaluation as to their compound properties in the cancer treatment setting. These both are reliable controls because they have been subject to the extraction procedure and they allow the bacterial strain compounds to be an isolated variable for testing.

Among the 60 *Rhodococcus* strains that were assayed and the 120 compound extracts, 18 compound extracts showed significant interaction p-values when compared to either the blank methanol control or the blank ethyl acetate-methanol control in the MTT assay with MCF7 cells (Table 12). These same compound extracts showed nonsignificant interaction p-values when compared to the blank methanol and blank ethyl acetate-methanol controls in the MTT assay with LM929 cells.

Table 12: All of the compound extracts that showed significant interaction p-values in a two-way ANOVA against the blank methanol or blank ethyl acetate methanol controls in a MCF7 MTT assay but showed no significant interaction p-values in a two-way ANOVA against these same controls in a LM929 MTT assay.

| | |
|---|---|
| NPDEM14 EA-M | NPDE012 EA-M |
| CODE08 meth | CODE08 EA-M |
| WFDEM2 EA-M | WLDET3 meth |
| WLDET3 EA-M | CBRMT10 meth |
| CBRMT10 EA-M | MTM3W5.2 EA-M |
| NP3T8.1 EA-M | APDE02 EA-M |
| NFDEM13 meth | KCHXC3 meth 2 |
| MTM3T17 EA-M | BTHXC8 EA-M |
| WFIEw1 EA-M | MTM3T20 EA-M |

## Washed RM Agar Plates and Their Effect on Activity

Washed rich media (RM) agar plates refers to removing the growing bacteria from the surface of the plate before extraction. Removal of the bacteria leaves only the compounds that were released in the agar to be extracted. This was done with the KCHXC3 *Rhodococcus* strain in order to see if there was a difference in the activity of the compound extract when the bacteria were extracted along with the agar and when the agar was extracted alone.

Methanol compound extracts were compared for the MCF7 and LM929 MTT assays using a two-way ANOVA (Table 12). The strain KCHXC3 was extracted on 2 different days in order to observe if there was a consistency in the activity of the compound extract. In looking at the MCF7 MTT assay results, it is seen that there is consistency with the activity of both the KCHXC3 methanol 1 and KCHXC3 methanol 2 compound extracts with both of them showing a significant interaction p-value when compared to the media and blank methanol controls but

no significant interaction when compared to the methanol control (Table 13a). When comparing this to the activity of KCHXC3 washed methanol compound extract there was a difference in activity when looking at the blank methanol control. This result could indicate that there is something being produced and not excreted by the bacteria that causes an inhibitory growth effect.

In the MTT assays with the LM929 cells, there was a difference in activity among the 2 KCHXC3 compound extracts (Table 13b). The difference is with their activity when compared to the blank methanol control. The KCHXC3 methanol 1 compound extract showed a significant interaction p-value when compared to the blank methanol control while the KCHXC3 methanol 2 compound extract did not show a significant p-value. The activity of the KCHXC3 washed methanol compound extract was different from both of the controls and showed a significant interaction p-value when compared to each one of the controls.

Table 13: a. Methanol compound extracts and their activity in a MTT assay with MCF7 against a media, methanol, and blank methanol control separately. b. Methanol compound extracts and their activity in a MTT assay with LM929 cells against a media, methanol, and blank methanol control. A yes result indicates that the compound extract had a significant interaction p-value when compared to the control individually.

| Strain | Media | Methanol | Blank Meth |
|---|---|---|---|
| KCHXC3 washed meth | yes | no | no |
| KCHXC3 meth 1 | yes | no | yes |
| KCHXC3 meth 2 | yes | no | yes |

a. MCF7 MTT Assay Activity

| Strain | Media | Methanol | Blank Meth |
|---|---|---|---|
| KCHXC3 washed meth | yes | yes | yes |
| KCHXC3 meth 1 | no | no | yes |
| KCHXC3 meth 2 | no | no | no |

b. LM929 MTT Activity

The ethyl acetate-methanol compound extracts for the strain KCHXC3 were also compared. With the MCF7 MTT assay, both the KCHXC3 compound extracts showed the same activity with significant interact p-values compared to the media and blank EA-M controls. The KCHXC3 washed EA-M compound extract also demonstrated the same activity pattern with significant interaction p-values compared to the media and blank methanol controls and no significance when compared to the methanol control (Table 14a).

There was a difference of activity in the 2 KCHXC3 compound extracts against the LM929 cells. The difference is with the media control. KCHXC3 EA-M 1 compound extract did not show a significant interaction p-value when compared to the media control while KCHXC3 EA-M 2 compound extract did show a significant interaction p-value (Table 14b). Both of the KCHXC3 EA-M compound extracts showed a different activity pattern when compared to the KCHXC3 washed EA-M extract. In this case the washed extracted showed a significant p-value when compared to all 3 controls.

Table 14: a. Ethyl acetate-methanol compound extracts and their activity in a MTT assay with LM929 against a media, methanol, and blank methanol control separately. b. Ethyl acetate-methanol compound extracts and their activity in a MTT assay with LM929 cells against a media, methanol, and blank methanol control. A yes result indicates that the compound extract had a significant interaction p-value when compared to the control individually.

| Strain | Media | Methanol | Blank EA-M |
|---|---|---|---|
| KCHXC3 washed EA-M | yes | no | yes |
| KCHXC3 EA-M 1 | yes | no | yes |
| KCHXC3 EA-M 2 | yes | no | yes |

a. MCF7 MTT Assay Activity

| Strain | Media | Methanol | Blank EA-M |
|---|---|---|---|
| KCHXC3 washed EA-M | yes | yes | yes |
| KCHXC3 EA-M 1 | no | no | yes |
| KCHXC3 EA-M 2 | yes | no | yes |

b. LM929 MTT Assay Activity

## Extraction Using a Minimal Media

For the MTT assays conducted with MCF7 cells, the blank methanol and blank EA-M extraction showed significant interaction p-values when compared to the media control in both the MTT assays with MCF7 and LM929 cells. These blank controls go through the same extraction process with the only difference being that they are not seeded with bacteria. This result indicates that there could be some component of the RM agar that is causing there to be an effect on the cells. Fiss minimal agar was used to assess if there was a component in the agar that was causing the activity with the MCF7 and LM929 cells in the MTT assays. Minimal media contains the essential components for bacteria to grow. Two separate Fiss agar plates from the same batch of media were extracted in order to test the activity against the 3 controls and the consistency of the activity. It was seen that there was no consistency in the pattern of activity to the controls with either the blank Fiss methanol or blank Fiss EA-M compound extracts (Table 15). In looking at the Blank Fiss methanol plates 1 and 2, it is seen that against the media control and the blank methanol control, plate 1 showed significance and plate 2 did not. An inconsistency in activity was also seen in the Blank Fiss ethyl acetate-methanol plates 1 and 2. In this case, plate 1 did not show significance against the media and Blank ethyl acetate-methanol control while plate 2 did show significance against these controls.

Table 15: MTT assay activity results based on a two-way ANOVA against a media, methanol, blank methanol, and blank EA-M controls

| Strain | Media | Methanol | Blank meth | Blank EA-M |
|---|---|---|---|---|
| Blank Fiss meth 1 | yes | no | yes | n/a |
| Blank Fiss meth 2 | no | no | no | n/a |
| Blank Fiss EA-M 1 | no | no | n/a | No |
| Blank Fiss EA-M 2 | yes | no | n/a | yes |

# CHAPTER 4

# DISCUSSION

The bacterium *Rhodococcus* is best known for its industrial success in the area of fossil fuel desulfurization. Following the sequencing of its genome in 2006, it was found that it contains 7 polyketide synthases and 24 nonribosomal peptide synthases (McLeod et al. 2006). The presence of these enzymes and the knowledge that they have been shown to produce antibiotics in other closely related bacteria has sparked interest in exploring Rhodococcus as a producer of inhibitory compounds.

Other related bacteria in the order Actinomycetales have produced compounds that have been used, or have influenced synthetic compounds that have been used in cancer treatment. Currently 9 genera in the order Actinomycetales have been studied and proved successful in producing drug-like products. The distribution of identified drug producing families on the bacterial phylogenetic tree suggest that the drug producing families tend to cluster. The family Nocardiaceae, which includes Rhodococcus, is related to the drug productive family Corynebacteriaceae that emerged in 2000 as a bioprospecting target (Zhu et al. 2011). Investigation into other related bacterial families could potentially lead to the discovery of useful bioactive compounds.

Based on these initial findings and previous success in this lab concerning the discovery of compounds that show effective bacterial growth inhibition, we hypothesized that *Rhodococcus* would be a bioprospecting target for inhibitory compounds against MCF7 breast cancer cells. Upon agar extraction screening using methanol and ethyl acetate, 18 compound extracts from 15 strains of Rhodococcus have shown significant activity against MCF7 breast

cancer cells while demonstrating no significant activity against the healthy cell control line of LM929 cells.

The results of this initial screening of these strains of *Rhodococcus* show promise in further investigation of this genus; however, a more in-depth investigation is necessary to determine the widespread potential of these compounds in the cancer treatment setting. It was seen that the blank methanol and blank ethyl acetate-methanol controls show significance when compared to the media control in both the MTT assays with MCF7 cells and LM929 cells. This indicates that there could be some component in the rich media agar that is causing this response. When the fIss media agar was tested, it was seen that each 1 of the 2 fiss agar extracts showed a different activity pattern. Further testing with this agar is suggested to confirm activity. If it shows no significant activity when compared to the media control, it would be a good candidate to grow and extract bacteria. If this is not the case, the use of another agar or agarose that does not have an effect on the growth of the cells would be an ideal choice.

Another possibility for growth media is nutrient broth. Allowing the bacteria to grow in liquid media with porous resin instead of on a solid surface could cause the production of these effective compounds. Changing from a solid surface to a liquid media form does have the potential to change bacteria expression and the compounds that the bacteria produce. This change in expression may be due to quorum sensing, a signaling system in bacteria that coordinates gene expression based on cell density (Martin et al. 2005). Changing to broth media could alter the identified activity of the compound extracts and may be a productive avenue for further bioactive compound production.

In looking at the dispersion of bioactive compounds there is an imbalance of distribution among the sets of assay plates. Out of the 5 sets of assay plates conducted for the MTT assays with MCF7 breast cancer cells, the majority of the significant compound extracts were found in the first set of plates. Because each of the compound extracts is compared to the 3 controls that are run on that set of plates it could be that there was an inaccuracy in the controls on the first set of plates. This would explain almost all of the compounds showing significant interaction p-values when compared to the blank methanol and blank ethyl acetate-methanol control. These compounds would need to be run again dispersed on different assay plates to confirm the positive activity.

The hypothesis for this investigation was that compound extracts from various strains of Rhodococcus would have some type of activity when grown with MCF7 breast cancer cells. More specifically we hypothesized that based on previous bioactivity discovered within the related bacterial families and the production of growth inhibiting compounds against bacteria from *Rhodococcus* strains isolated in the lab, there would be some compound or combination of compounds produced by this genus of bacteria that would have a inhibitory growth effect or killing effect on MCF7 breast cancer cells. Eighteen compounds that showed significant interaction p-values when assayed with the MCF7 breast cancer cells were identified. These compounds were also selected for future prospecting research because they showed a lack of activity against the LM929 healthy cell line. In this investigation we reject our null hypothesis that a compound produced by *Rhodococcus* does not cause an effect against MCF7 breast cancer cells.

## Future Direction of This Research

The 18 compound extracts that showed significant interaction ($p < 0.05$) when assayed with MCF7 cells and nonsignificant interaction p-values when assayed with LM929 cells require further investigation. The 15 strains of *Rhodococcus* that have shown activity represent potential leads for bioactivity. Purification and identification of the composition of these identified compound extracts could provide information about the type of compound or combination of compounds that are having an effect on the MCF7 cancer cells. Further expansion of these strains of bacteria that show activity can be done with the use of other growth media and cancer cell lines to see if they have an inhibitory or killing effect.

REFERENCES

American Cancer Society. Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012.

Barber,T. "Discovery and Characterization of an Antibiotic From the Soil Bacterium Bacillus." MA Thesis. East Tennessee State University, 2010.

Borisova R. “Isolation of a Rhodococcus Soil Bacterium that Produces a Strong Antibacterial Compound.” MA Thesis. East Tennessee State University, 2010.

Chang S, Miron P, Miron A, Iglehart J. "Rapamycin Inhibits Proliferation of Estrogen-Receptor-Positive Breast Cancer Cells." *Journal of Surgical Research* 138.1 (2007): 37-44.

Clemons M, Danson S, Howell, A. "Tamoxifen (‘Nolvadex’): A Review: Antitumour Treatment." *Cancer Treatment Reviews* 28.4 (2002): 165-80.

Galmarini D, Galmarini C, Galmarini F. ", Cancer Chemotherapy: A Critical Analysis of Its 60 Years of History." *Critical Reviews in Oncology/Hematology* 84.2 (2012): 181-99.

Carr G, Williams D, Diaz-Marrero A, Patrick B, Bottriell H, Balgi A, Donohue E, Roberge M, Anderson R. ". Bafilomycins Produced in Culture by Streptomyces Spp. Isolated from Marine Habitats Are Potent Inhibitors of Autophagy." *Journal of Natural Products* 73 (2010): 422-27

Carvalho C, Fonseca M. "The Remarkable Rhodococcus *erythropolis*." *Applied Microbiology and Biotechnology* 67.6 (2005): 715-26.

Guertin D, Sabatini D. "Defining the Role of mTOR in Cancer." *Cancer Cell*. (2007): 10-22.

Gupta A, Kumar B, Negi A. "Current Status on Development of Steroids as Anticancer Agents." *Journal of Steroid Biochemistry and Molecular Biology*. (2013)

Haigentz M, Kim M, Sarta C, Lin J, Keresztes R, Culliney B, Gaba A, Smith R, Shapiro G, Chirieac L, Mariadason J, Belbin T, Greally J, Wright J, Haddas R. "Phase II Trial of the Histone Deacetylase Inhibitor Romidepsin in Patients with Recurrent/metastatic Head and Neck Cancer." *Oral Oncology* 48.12 (2012): 1281-288.

Hymes K. "The Role of Histone Deacetylase Inhibitors in the Treatment of Patients With Cutaneous T-Cell Lymphoma." *Clinical Lymphoma, Myeloma & Leukemia* 10.2 (2010): 98-109.

Iwatsuki M, Uchida R, Takakusagi Y, Matsumoto A, Jiang C, Takahashi Y, Arai M, Kobayashi S, Matsumoto M, Inokoshi J, Tomoda H, Ōmura S. "Lariatins, Novel Anti-mycobacterial Peptides with a Lasso Structure, Produced by Rhodococcus *jostii* K01-B0171." *The Journal of Antibiotics* 60.6 (2007): 357-63.

Karpozilos, A. "The Treatment of Cancer in Greek Antiquity*1." European Journal of Cancer 40.14 (2004): 2033-040.

Kirosawa, K, Ghiviriga I, Sambandan TG, Lessard PA, Barbara JE, Rha C, Sinskey AJ. "Rhodostreptomycins, Antibiotics Biosynthesized Following Horizontal Gene Transfer from *Streptomyces padanus* to *Rhodococcus fascians." Journal of the American Chemical Society* 130 (2008): 1126-127.

Kitagawa W Tamura T. "A Quinoline Antibiotic from Rhodococcus erythropolis JCM 6824." *The Journal of Antibiotics* 61.11 (2008): 680-82.

Quinn L, Quinn A, Patel M, Semus S, Graves A, Bandyopadhyay D, Pope A, Thrall S. "Perspectives on the Discovery of Small-Molecule Modulators for Epigenetic Processes." *Journal of Biomolecular Screening* 17.5 (2012): 555-571.

Martin J, Casqueiro J, Liras P. "Secretion Systems for Secondary Metabolites: How Producer Cells Send out Messages of Intercellular Communication." *Current Opinion in Microbiology* 8.3 (2005): 282-93.

Martinkova L, Uhnakova B, Patek M, Nesvera J, Kren V. "Biodegradation Potential of the Genus Rhodococcus." *Environment International* 35.1 (2009): 162-77.

Newman D, Cragg G. “Natural Products as Sources of New Drugs Over the Last 25 Years.” Journal of Natural Products, 70.3 (2007): 461-477.

Renoir J, Marsaud V, Lazennec G. "Estrogen Receptor Signaling as a Target for Novel Breast Cancer Therapeutics." *Biochemical Pharmacology* 85.4 (2013): 449-65.

Richon V. "Cancer Biology: Mechanism of Antitumour Action of Vorinostat (suberoylanilide Hydroxamic Acid), a Novel Histone Deacetylase Inhibitor." *British Journal of Cancer* 95 (2006): S2-S6.

Richon V, Garcia-Vargas J, Hardwick J. "Development of Vorinostat: Current Applications and Future Perspectives for Cancer Therapy." *Cancer Letters* 280.2 (2009): 201-10.

Schmudde M, Friebe E, Sonnemann J, Beck J, Bröker B. "Histone Deacetylase Inhibitors Prevent Activation of Tumour-reactive NK Cells and T Cells but Do Not Interfere with Their Cytolytic Effector Functions." *Cancer Letters* 295.2 (2010): 173-81.

Stockert, J, Blazquez-Castro A, Canete M, Horobin R, Villanueva A. "MTT Assay for Cell Viability: Intracellular Localization of the Formazan Product Is in Lipid Droplets." *Acta Histochemica* 114.8 (2012): 785-96.

Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, Capaccioli S. "Natural Compounds for Cancer Treatment and Prevention." *Pharmacological Research* 59.6 (2009): 365-78.

Vezina C, Kudelski A, Sehgal S. "Rapamycin (AY-22,989), a New Antifungal Antibiotic Taxonomy of the Producing Streptomycete and Isolation of the Active Principle." *Journal of Antibiotics*. 28.10 (1975): 721-726.

Vignot S, Faivre S, Aguirre D, Raymond E. "mTOR-targeted Therapy of Cancer with Rapamycin Derivatives." *European Society for Medical Oncology*. 16. (2004): 525-537.

Watanabe R, Wei L, Huang J. "mTOR Signaling, Function, Novel Inhibitors, and Therapeutic Targets." *Journal of Nuclear Medicine*. 52.4 (2011): 497-500.

Wilson A, Lalani A, Wass E, Saskowski J, Khabele D. "Romidepsin (FK228) Combined with Cisplatin Stimulates DNA Damage-induced Cell Death in Ovarian Cancer." *Gynecologic Oncology* 127.3 (2012): 579-86.

Zhu F, Qin C, Tao L, Liu X, Shi Z, Ma X, Jia J, Tan Y, Cui C, Lin J, Tan C, Jiang Y, Chen Y. "Clustered Patterns of Species Origins of Nature-derived Drugs and Clues for Future Bioprospecting." *Proceedings of the National Academy of Sciences* 108.31 (2011): 12943-2948.

VITA

MEGAN CRABTREE

| | |
|---|---|
| Personal Data: | Date of Birth: January 3, 1989<br>Place of Birth: Joliet, Illinois<br>Marital Status: Married |
| Education: | New Paltz Public Schools, Ulster County, New York<br>B.S. Biology, State University of New York at Geneseo, Geneseo, New York, 2011<br>M.S Biology, East Tennessee State University, Johnson City, Tennessee, 2013 |
| Honors and Awards: | GK-12 National Science Foundation Fellow 2011-2013 |