

Syracuse University

SURFACE

Syracuse University Honors Program Capstone Projects Syracuse University Honors Program Capstone Projects

June 2016

Effect of Rapamycin as an Inhibitor of the mTOR Cell Cycle Entry Complex on the Selective Lysis of Human Leukemia Cells Lines in Vitro Using 20 kHz Pulsed Low-Frequency Ultrasound

Yasmine Hemida

Follow this and additional works at: https://surface.syr.edu/honors_capstone



Part of the [Biotechnology Commons](#)

Recommended Citation

Hemida, Yasmine, "Effect of Rapamycin as an Inhibitor of the mTOR Cell Cycle Entry Complex on the Selective Lysis of Human Leukemia Cells Lines in Vitro Using 20 kHz Pulsed Low-Frequency Ultrasound" (2016). *Syracuse University Honors Program Capstone Projects*. 942.

https://surface.syr.edu/honors_capstone/942

This Honors Capstone Project is brought to you for free and open access by the Syracuse University Honors Program Capstone Projects at SURFACE. It has been accepted for inclusion in Syracuse University Honors Program Capstone Projects by an authorized administrator of SURFACE. For more information, please contact surface@syr.edu.

Effect of Rapamycin as an Inhibitor of the mTOR Cell Cycle Entry Complex on the Selective Lysis of Human Leukemia Cells Lines in Vitro Using 20 kHz Pulsed Low-Frequency Ultrasound

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

Yasmine Hemida

Candidate for Bachelor of Science Degree
and Renée Crown University Honors
May 2016

Honors Capstone Project in Biotechnology

Capstone Project Advisor: _____
Thomas Fondy, Professor

Capstone Project Reader: _____
Surabhi Raina, Assistant Professor

Honors Director: _____
Stephen Kuusisto, Director

© Yasmine Hemida; May 2, 2016

Abstract

It has been shown that the mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and proliferation, is aberrant in many hematological malignancies. Rapamycin inhibits mTOR signaling which regulates cell growth and cell cycle progression. This study sought to determine the effects of mTOR inhibitors on the cell sizes of normal and neoplastic cells and to determine ultrasonic sensitivity of normal and neoplastic cells treated with mTOR inhibitors. The effects of rapamycin (rapa), an extensively studied natural product that affects cell cycle entry by inhibiting mTORC1, and its analogs temsirolimus (tems) and everolimus (eve), were examined on leukemic cell lines (U937: Human monocytic leukemia, THP-1: Human acute monocytic lymphoma, K562: Human chronic myelogenous leukemia, and MOLT-4, Human acute lymphoblastic leukemia) in combination with 20kHz ultrasound. Each cell line was treated for 48 hours with each analog before being exposed to ultrasound. By using low frequency ultrasound of 5 pulses (0.6 seconds of ultrasound and 0.4 seconds without ultrasound) at 20 kHz and 30% amplitude (60W), we determined that as the concentrations of Rapa, Eve, and Tems (2, 20, and 50nM) increased, there is usually the most sonic damage observed in U937, MOLT-4, K562, and THP-1 cells. The most preferentially damaged cell line is K562 by all of the analogs and with all the concentrations used. In addition, larger cells were more susceptible to ultrasonic damage than were smaller cells. Further, there are no significant differences among the three analogs in the potentiation of ultrasonic damage. This preliminary therapeutic approach involving the use of ultra-sound in combination with mTOR inhibitors might eventually provide an improved approach to the treatment of leukemia and other hematological malignancies in a clinical setting.

Key words: *Low frequency ultrasound, mTOR, Rapamycin, Everolimus, Temsirolimus, Human Leukemia*

Executive Summary

Common treatments for cancers include radiation, chemotherapy, immunotherapy, hematopoietic stem cell transplants, gene therapy, and host response modification using hormones, cytokines, and other bio-therapeutic agents. These alternative modalities are used alone or in combination. I propose to add treatment with continuous or pulsed low frequency ultra-sound as an additional cancer treatment modality. Low-frequency ultrasound as a physically based cancer treatment can circumvent chemically based drug-resistance and can be controlled and finely modulated from outside of the patient. Sirolimus, also known as Rapamycin, and analogs of Rapamycin, Everolimus and Temsirolimus, are used to treat human leukemia cell lines in vitro altering the size, proliferative capacity, and viability of the treated cells. We determine the ability of low-frequency ultrasound to produce damage to leukemia cells treated with Rapamycin or analogs in comparison with the effects of low frequency ultra-sound on untreated and treated leukemia cells. In this work we attempt to determine the effects of Rapamycin (Rapa), Temsirolimus (Tems) and Everolimus (Eve) on human leukemic cell lines (U937: monocytic leukemia, THP-1: acute monocytic lymphoma, K562: chronic myelogenous leukemia, and MOLT-4, an acute lymphoblastic leukemia) non-sonicated or sonicated with low frequency ultrasound for 5 pulses (0.6 seconds of ultrasound and 0.4 seconds without ultrasound) at 20kHz and 30% amplitude (60W). The cells were treated with each chemotherapeutic agent at 2 nM, 20 nM, Or 50 nM for 48 hours prior to sonication to explore whether preferential damage is dependent on concentration and duration of treatment prior to sonication.

Table of Contents

Abstract.....	iii
Executive Summary.....	iv
Preface.....	vi
Acknowledgements	vii
Advice to Future Honors Students	viii
Chapter 1: Introduction	1
Chapter 2: Literature Search.....	2
Chapter 3: Inquiry.....	6
Chapter 4: Materials and Methods	8
Cell Culture.....	8
Drugs.....	8
Ultrasound apparatus and intensity measurement.....	8
Measurement of Cell Survival.....	10
Statistical Analysis.....	10
Chapter 5: Results.....	11
Chapter 6: Discussion.....	15
Chapter 7: Additional Work.....	17
Chapter 8: Conclusion.....	20
References.....	21

Preface

As a student, I am constantly looking for ways to acquire the knowledge and skills in order to help people. I have a strong passion to become a physician and alleviate human suffering because I hope to someday bring smiles to my patients with a simple caring touch of understanding. Ever since I was little, I knew I wanted to give back to the medical field as a doctor because they are the ones that saved my mom's life from breast cancer. As my mom continues to battle breast cancer, her fight continues to have a huge impact on my life and influences my decision to do cancer research at Syracuse University.

For my undergraduate education, I have specifically chosen biotechnology over standard biology degrees because of its extra emphasis on learning about public policy, management and engineering. My other motive to pursuing my biotechnology major is essentially because of the applied concept behind it. It truly helps one to take theories and put them into practical use. Along with my biotechnology major, I have chosen to pursue Arabic and Middle Eastern studies because of my Egyptian descent.

At Syracuse University, I was granted the opportunity to do extracurricular cancer research with Dr. Thomas Fondy and his team. We are examining the use of ultrasound irradiation and chemotherapeutic agents as potential treatment modalities to produce a substantial synergistic effect amongst leukemic cell lines (U937: Human monocytic leukemia, THP-1: Human acute monocytic lymphoma, K562: Human chronic myelogenous leukemia, and MOLT-4, Human acute lymphoblastic leukemia).

After my undergraduate education I wish to continue to acquire the knowledge and skills in medical school in order to help people. I look forward to seizing this opportunity of a lifetime.

Acknowledgements

I wish to express my deepest gratitude to my research mentor, Dr. Thomas P. Fondy at Syracuse University for his time, kindness, expertise and professional guidance in helping me through this project's completion. Thank you to SUNY Upstate Medical University for providing human blood cells for experiments. I also thank the Renee Crown Honors Staff at Syracuse University and to my Biotechnology, Biology, and Chemistry professors as well as the Arabic department whom all have helped mentor, teach, guide and encourage me through my studies at Syracuse. Thank you to the Syracuse University Athletic Department and Syracuse Women's Rowing team for sponsoring my studies. A special thanks to my family and friends for their unconditional support and encouragement during the toughest of times. I thank all of those who are not listed above, but have helped me during my time as a Renee Crown Honors student at Syracuse University. I have most certainly cherished the time being here and the experience has been priceless.

Advice to Future Honors Students

Be prepared to grow as an individual no matter what. You will hit those turning points throughout your undergraduate career that persuade you to constantly question your inner principles. But, this is all part of the process of growing up. You will grow to become a stronger and smarter individual, then when you first arrived to college. Enjoy the process and I wish you all the best. P.s. - Remember to try new things and stick with the ones you love!

Chapter 1: Introduction

The first discovery of leukemia was independently observed by researchers John Hughes Bennett and Rudolph Virchow (Piller, 2008). Leukemia occurs worldwide and represents about 5 percent of all cancer. The *Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute* lists leukemia as the seventh leading cause of cancer death in the United States. The number of deaths was 6.9 per 100,000 men and women per year based on 2009-2013 deaths. Furthermore, leukemia has the capability to strike anyone, at any time, at any age. In 2016 the *National Cancer Institute* estimated there will be 60,140 new cases of leukemia in the United States and an estimated 24,400 people will die from this disease. Leukemia is slightly more common in men than women with a male/female ratio to be about 1.7:1.

Leukemia is cancer of the blood cells, denoting a group of malignant disorders in the blood-forming cells. The bone marrow (where blood cells are made) malfunctions and produces abnormal white (leukemic) cells, which damage the function of other essential blood cells (Piller, 2008). In the marrow, at least 200 billion red cells, 10 billion white cells, and over 400 billion platelets are produced in the marrow each day (Piller, 2008). In normal conditions, circulating blood has approximately 1,000 red cells to each white cell (Piller, 2008). However, in leukemia, normal production of blood cells fails. White blood cells reproduce abnormally and create poorly developed cells or immature blasts. These leukemic cells overpopulate the bone marrow, enter the bloodstream and lymph system, and infiltrate organs and glands, causing them to enlarge and malfunction. The bone marrow becomes unable to produce sufficient levels of red cells and platelets; consequently, the balance of the blood cell population is seriously disturbed, and the body's defenses based on white blood cells and platelets are rendered ineffective.

Chapter 2: Literature Search

The mammalian target of rapamycin (mTOR) has received considerable attention as a potential target for cancer chemotherapy, especially in endocrine therapy in breast cancer. According to the *National Cancer Institute's online dictionary (2012)*, endocrine therapy is referred to as hormonal therapy which focuses on slowing or stopping the growth of a hormone-sensitive tumor by blocking the body's ability to produce hormones such as estrogen and progesterone, which may promote the growth of some breast cancers. Previous research has indicated estrogen-induced breast tumor cells proliferation require mTOR signaling (Boulay *et al*, 2005). As such, most of the research involving the mTOR pathway has been directed toward inhibiting the mTOR pathway as a key oncoprotein. mTOR as a member of the phosphatidylinositol 3-kinase related kinase protein (PIKK) family, is a serine/threonine protein kinase which regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription, and is also a constituent of the PI3K/AKT/mTOR (PI3K: phosphatidylinositol-4, 5-bisphosphate 3-kinase; AKT: protein kinase B) pathway (Guertin and Sabatini, 2007). Further, mTOR serves as part of an evolutionarily conserved signaling pathway that controls the cell cycle in response to changing nutrient levels (Nakamura, 2014). Dysregulation of the mTOR pathways has been linked to human diseases such as diabetes, obesity, depression, and certain cancers (Nakamura, 2014).

The rapamycin actions are regulated through specific inhibition of the mTOR protein kinase. Inhibition of the mTOR pathways prevents proliferation of normal cells, but not the proliferation of cancer cells due to their elevated expression of the mTOR protein. Further, a central component for development and homeostasis for cancer involve the formation of new blood vessels, or angiogenesis (Veikkola and Alitalo, 1999). These blood vessels supply growth

to the tumor, and the chemicals released by the tumor engage other host cells near the tumor causing the cancer to spread.

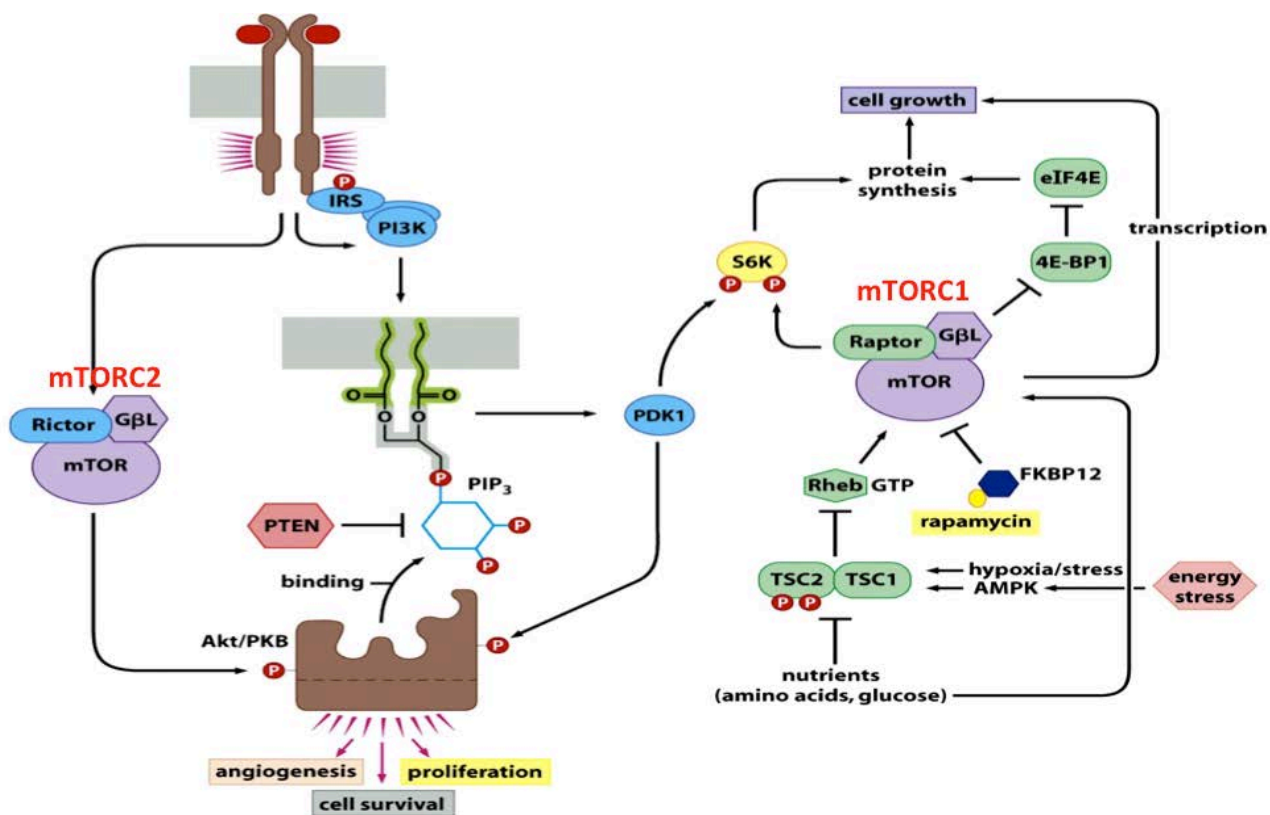


Figure 16-44a The Biology of Cancer (© Garland Science 2007)

Figure 1: mTORC1 and mTORC2 Pathway. mTORC1 consists of the mTOR, Raptor, and GβL. mTORC1 acts on cell size. mTORC2 consists of mTOR, Rictor, and GβL. mTORC2 acts on cell proliferation (number). Lower concentrations of rapamycin and its analogs can inhibit only mTORC1. Higher concentrations of rapamycin and its analogs can inhibit both mTORC1 and mTORC2

Therapeutic ultrasound is an emerging field with many medical applications. In previous clinical studies, ultrasound was used to provide the ability to localize the deposition of acoustic energy within the body, which can cause tissue necrosis (cell death) and hemostasis (interruption of blood flow). Ultrasound is a mechanical wave with periodic vibrations of particles in a continuous, elastic medium at frequencies equal to or greater than 20 kHz. In liquids, its velocity of about 1000–1600 m/s translates into the wavelength range from micrometers to centimeters

(Rosenthal et al., 2004). Furthermore, cavitation occurs when applying high intensity ultrasound to liquids. A sinusoidal pressure is superimposed on the constant ambient pressure in generating cavitation. Cavitation is a continual process of gas bubble nucleation, growth, and collapse as a result of intense ultrasonic waves. During the negative pressure cycle, the liquid is pulled apart at sites containing a gaseous impurity, which is known as “weak spots” in the fluid (Brennen, 1995). The number of bubbles produced during this rarefaction cycle is proportional to the density of such weak spots present in the fluid (Brennen, 1995). Bubble collapse in liquids results in an immense concentration of energy from the conversion of kinetic energy of liquid motion into heating of the contents of the bubble (Suslick *et al*, 1999). It is this energy that disrupts the cell membrane of cells to cause cell rupture and lysis.

Indirect sonication is used predominantly to avoid cross-contamination, aerosolization, and foaming of the sample (Hielscher, 1999). Indirect sonication describes coupling of ultrasound waves via ultrasonic bath through the test tube’s wall into the sample liquid (Hielscher, 1999). The ultrasonic energy is transmitted from the horn, up through the water and into a vessel or multiple sample tubes (Heilscher, 1999). As indirect sonication of the ultrasonic energy is transmitted via the wall of the sample tube or beaker into the medium, the ultrasonic intensity that is finally coupled into the sample liquid is quite low (Hielscher, 1999). The two variables on the cell sonicator are frequency and amplitude. Higher ultrasonic frequencies lead to the production of smaller cavitation bubbles as compared with lower frequencies. Size correlates with the energy required to produce a cavitation bubble and its energy released. In other words, more energy is required to produce larger cavitation bubbles. At 20 kHz ultrasound frequency, the bubbles generated are relatively large. The collapse produced results in strong shockwaves which can be later used for mechanical shearing applications such as emulsification (Leong *et al*,

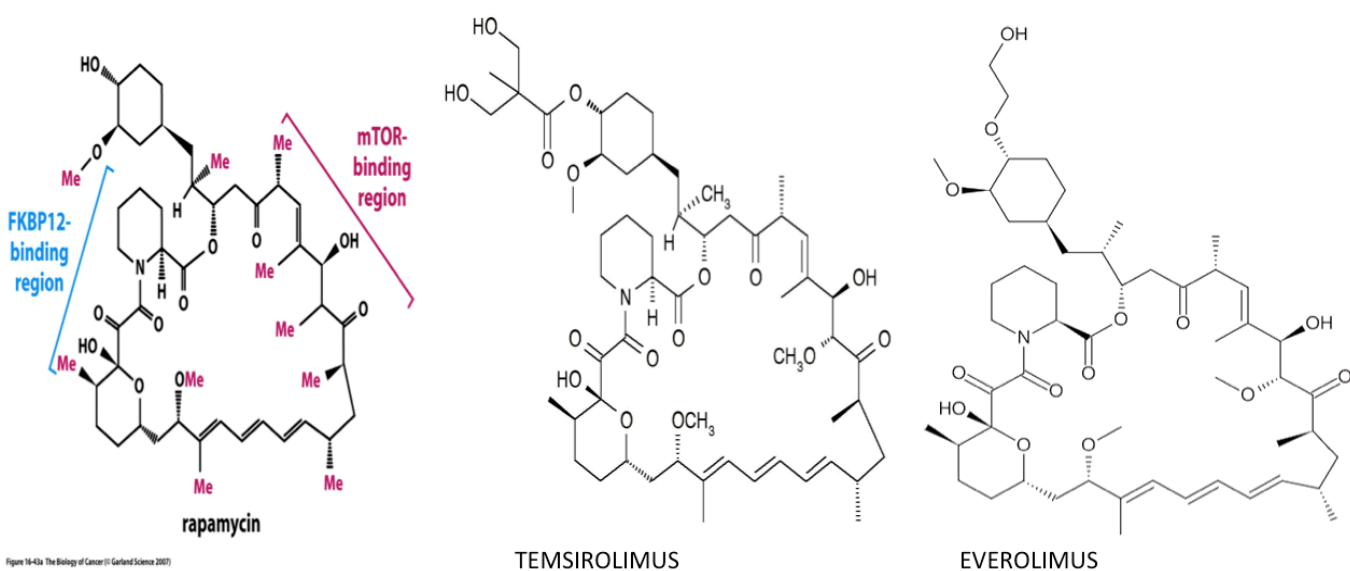
2009). Increasing ultrasonic power results increases the quantity of cavitation bubbles but has no effect on bubble size (Fuchs, 2011). Thus, increasing ultrasonic frequency, while delivering the same ultrasonic power, results in a larger number of smaller cavitation bubbles (Fuchs, 2011). Overall, this is a quite attractive feature from a clinical standpoint and has led to considerable experimentation of ultrasound for medical purposes.

Chapter 3: Inquiry

This study could provide a new approach to management of cancer. Time after time, patients that are diagnosed with cancer are left to be fighting the disease with harsh treatment modalities in order to remove the cancer. Using ultrasound irradiation will provide an alternate method. In my research, I am striving to enhance the effects of ultrasonic irradiation by using anticancer drugs, Rapamycin (Rapa) and its analogs – Temsirolimus (Tems) and Everolimus (Eve) to treat leukemic cell lines. The leukemic cells lines used for this work are: U937 (Human monocytic leukemia), THP-1, (Human acute monocytic lymphoma), K562 (Human chronic myelogenous leukemia), and MOLT-4 (Human acute lymphoblastic leukemia). I proposed that by regulating the concentration of rapamycin and its analogs, the cell susceptibility of cancer cells to ultrasonic damage may be enhanced. In order to confirm this hypothesis, conclusive evidence needs to be collected before the role of ultrasound irradiation and rapamycin's anticancer effect can be evaluated.

Questions to assess in my research:

- What effect does rapamycin have on cell proliferation?
- Do mTOR inhibitors preferentially damage cells in combination with low-frequency ultrasound?
- What are the effects of rapamycin alone or in combination with low-frequency ultrasound on cell viability?
- Which of the three mTOR inhibitors causes the greatest damage in combination with ultrasound?
- Is the sonic damage concentration dependent?



Rapamycin and its analogs

Image retrieved by *Google Images* by searching the respective name of compound

Chapter 4: Materials Methods

Cell Culture

Human myelomonocytic lymphoma U937 cells (ATCC® CRL- 1593.2) were placed at 5.2×10^4 viable cells/ml in 20 % heat-inactivated fetal bovine serum (FBS) in Iscove's medium without glutamine, with the following added: 200 units/ml penicillin, 200 µg/ml streptomycin, 100 µg/ml gentamicin sulfate, 40 µM glutamine (50 µl of 2 mM glutamine per 5 ml medium), and 50 µl of amphotericin B (2.5 µg/ml concentration) per 5 ml of medium. K562, Molt-4, and THP1 human leukemia (ATCC® CCL-243, CRL-1582, and TIB-202) were cultured under the same conditions. These cell lines were retrieved from the State University of New York Upstate Medical University (Syracuse, NY, USA). The cells were incubated in 5 % CO₂ in a humidified chamber at 37 °C. Viability was assessed by 0.4 % trypan blue stain in isotonic saline, followed by cell counting and sizing using a Z2 Beckman-Coulter® Particle Count and Size Analyzer (Beckman Coulter Inc., Brea, CA, USA), along with a Bio-Rad® TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Extent of multinucleation after treatment with rapamycin with Wright stain.

Drugs

Rapamycin (Sigma-Aldrich Corp., St. Louis, MO, USA) was prepared in 40 µM stock solutions using 95 % EtOH (Sigma-Aldrich Corp.). Rapamycin analogs everolimus and temsirolimus were prepared using the same conditions. Cell size, viability, and proliferation rates were determined by the cell counters. Vehicle controls of 95 % EtOH were tested in parallel with the rapamycin-treated cells.

Ultrasound apparatus and intensity measurement

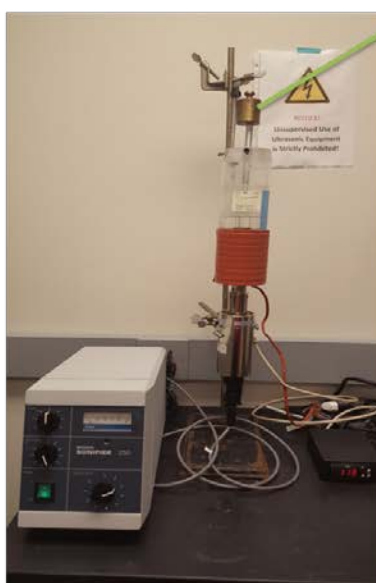
For the sonication, I will be using a 20 kHz Branson Sonifier 250 (60mm diameter cup) and 24mm Mylar Bottom Glass Bottle. The ultrasound apparatus has a 2 cm horn tuned to 3 cm

of degassed water above the horn. There was 30% amplitude (60 W). Water was heated to about 37 °C between each sonication.

Untreated U937, THP-1, K562, MOLT-4; Rapa (2nM, 20nM, 50nM); Eve (2nM, 20nM, 50nM); Tems (2nM, 20nM, 50nM) control counts are taken twice duplicate on TC20 and Z2. Then, sonicate 3ml of untreated U937, THP-1, K562, MOLT-4; Rapa (2nM, 20nM, 50nM); Eve (2nM, 20nM, 50nM); Tems (2nM, 20nM, 50nM) cells in glass vial. The next step is to take cell count on TC20 and Z2 after sonication (have 2 counts per sample). The following step is to take untreated U937, THP-1, K562, MOLT-4; Rapa (2nM, 20nM, 50nM); Eve (2nM, 20nM, 50nM); Tems (2nM, 20nM, 50nM) control count on TC20 and Z2 after all sonication is done. TC20 used a 1:1 dilution untreated U937, THP-1, K562, MOLT-4; Rapa (2nM, 20nM, 50nM); Eve (2nM, 20nM, 50nM); Tems (2nM, 20nM, 50nM) cells : Trypan Blue. The Z2 used 1:201 dilution untreated U937, THP-1, K562, MOLT-4; Rapa (2nM, 20nM, 50nM); Eve (2nM, 20nM, 50nM); Tems (2nM, 20nM, 50nM) cells : Isoton



20 kHz Branson Sonifier 250



Sonication apparatus



Measurement of cell survival

A BioRad TC20 automated hemocytometer was used to determine cell viability using trypan blue dye exclusion by viable cells. A Beckman Z2 Coulter Counter was used to determine total cell numbers and size distribution.

Statistical Analysis

All of the data are presented as the mean \pm SD. The differences between groups were assessed with Student's *t* test at a 95% confidence interval; $P < 0.05$ was considered to be significant. The assessment of synergy was performed by two-way factorial ANOVA.

Chapter 5: Results

All three mTOR inhibitors decrease the mean cell diameter of U937 monocytic leukemia cells. The various concentrations of inhibitors (green, blue, and black lines) are shifted to the left relative to the untreated control (red line) (Figure 6). As you increase the concentration of mTOR inhibitors it produces less viable cells even though it does not increase sonic sensitivity (Figure 7). Sonication of the U937 monocytic leukemia cells reduced the cell number (Figure 6). This reduction becomes greater as the mean cell diameter increases (Figure 6). Sonicated U937 monocytic leukemia treated with 20 nM everolimus showed the least survivability compared with the untreated unsonicated control cells (Figure 3, 4 & 5). Further, there was no significant difference between analogs on the survivability of each leukemia cell line (Figure 3, 4 & 5). In figure 6, the temsirolimus unsonicated graph demonstrated a higher peak for unsonicated 20 nM, which could mean there was higher cell density to begin with prior to adding the 20 nM temsirolimus. Also, in figure 6, higher left peaks in all of the sonicated graphs indicate debris that is caused by sonication. As the cell membrane is lysed particles of the membrane are released.

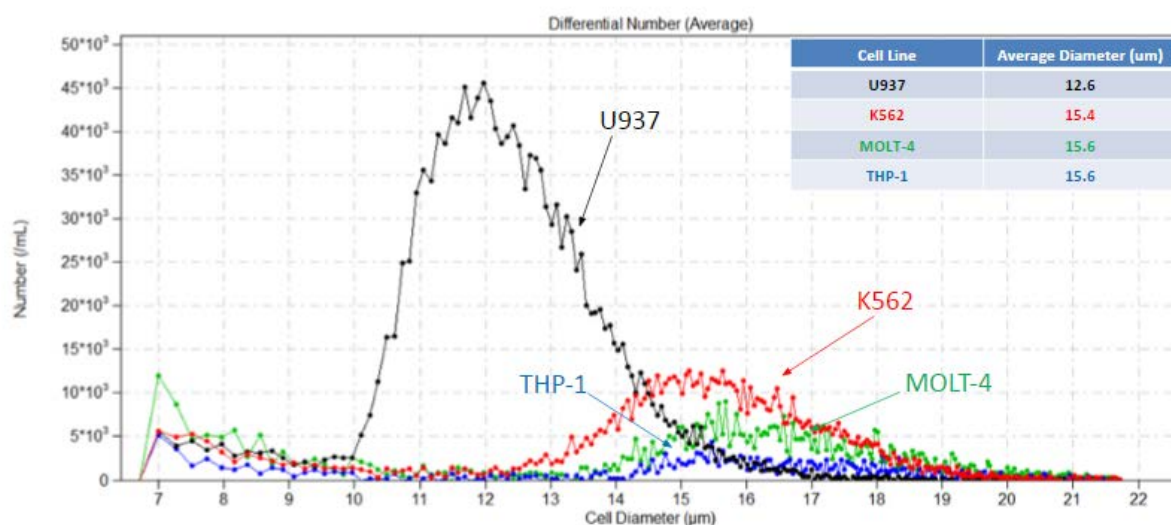


Figure 2: Average Cell Diameters of U937, K562, MOLT-4, & THP-1 unsonicated and untreated taken by Z2 Beckman-Coulter® Particle Count and Size Analyzer

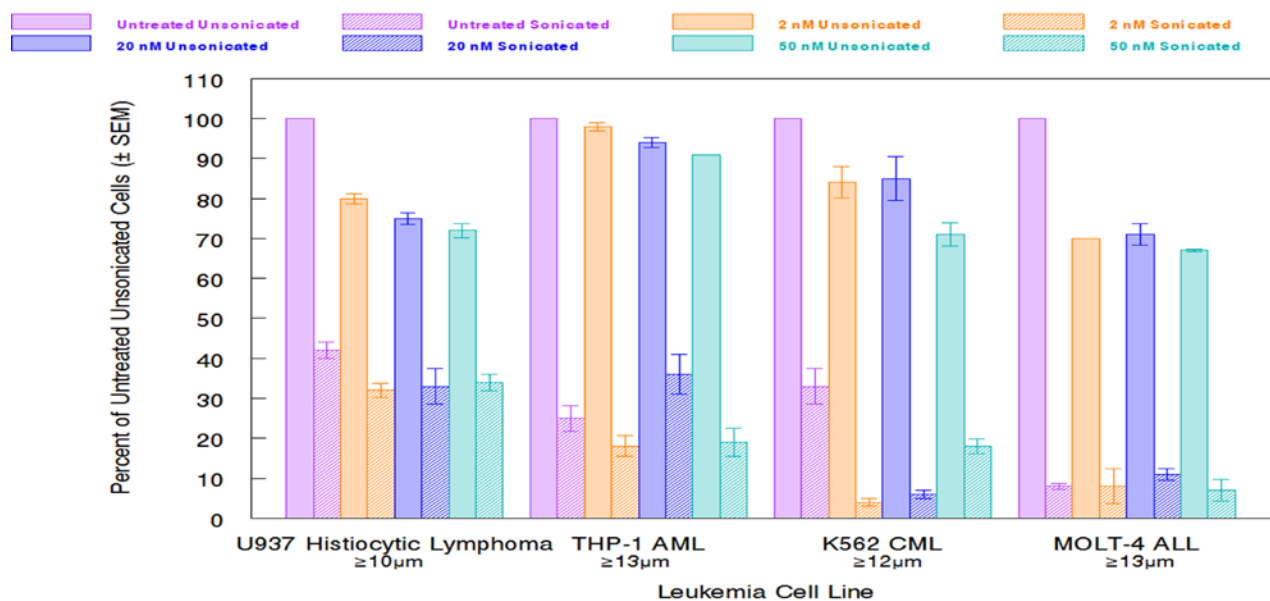


Figure 3: Effect of 2, 20, and 50 nM of Rapamycin on the inhibition of proliferation and on sonic sensitivity to U937 (AML), THP-1 (AML), K562 (CML), and MOLT-4 (ALL) Human leukemia cell lines. Numbers calculated by Beckman Coulter Z2 Counter using a 20 kHz System at 30% Amplitude, 5 x 0.6 sec pulses with 0.4 sec spacing, glass vials, n=4.

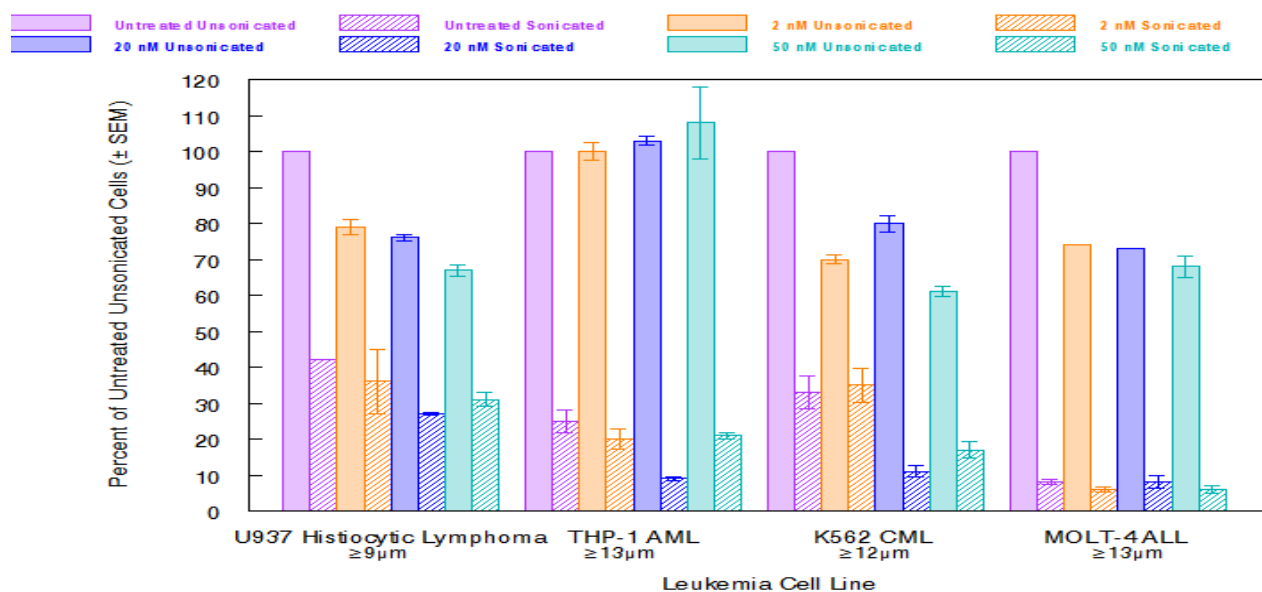


Figure 4: Effect of 2, 20, and 50 nM of Everolimus on the inhibition of proliferation and on sonic sensitivity to U937 (AML), THP-1 (AML), K562 (CML), and MOLT-4 (ALL) Human leukemia cell lines. Numbers calculated by Beckman Coulter Z2 Counter using a 20 kHz System at 30% Amplitude, 5 x 0.6 sec pulses with 0.4 sec spacing, glass vials, n=4.

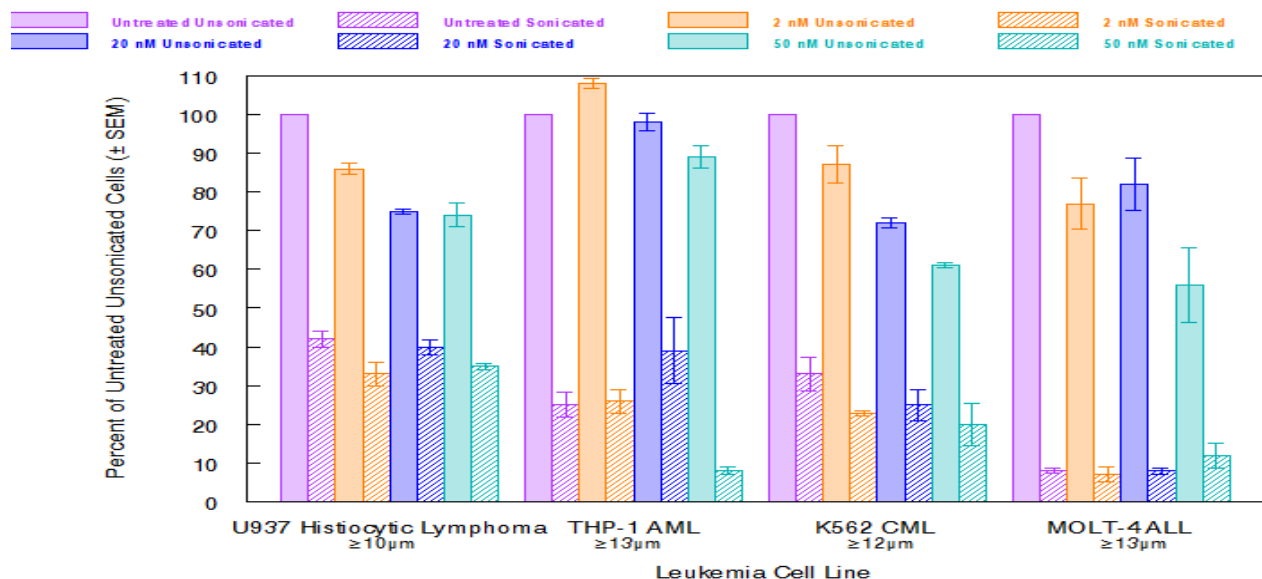


Figure 5: Effect of 2, 20, and 50 nM of Temozolomide on the inhibition of proliferation and on sonic sensitivity to U937 (AML), THP-1 (AML), K562 (CML), and MOLT-4 (ALL) Human leukemia cell lines. Numbers calculated by Beckman Coulter Z2 Counter using a 20 kHz System at 30% Amplitude, 5 x 0.6 sec pulses with 0.4 sec spacing, glass vials, n=4.

U937 – Human Acute Monocytic Leukemia

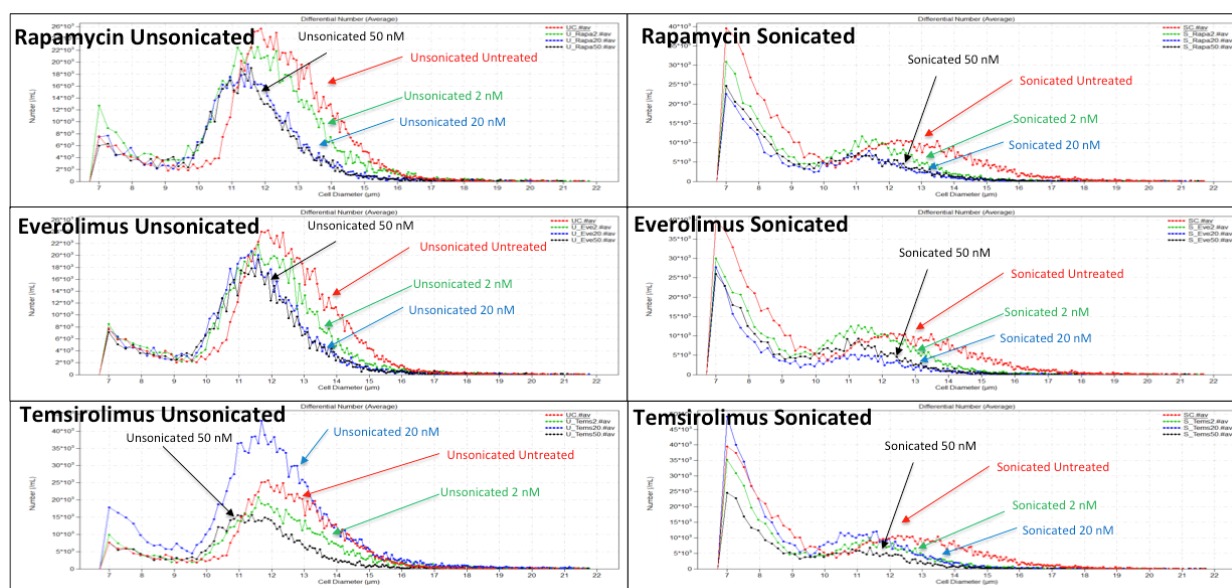


Figure 6: Cell size distribution calculated by Beckman Coulter Z2 Counter for U937 monocytic leukemia treated with 2, 20, and 50 nM of Rapamycin, Everolimus, and Temozolomimus. A&B- Unsonicated and sonicated Rapamycin treated, respectively. C&D- Unsonicated and sonicated everolimus- treated respectively. E&F- Unsonicated and sonicated temsirolimus-treated, respectively. Red-Untreated control. Green- 2nM. Blue- 20 nM. Black- 50 nM

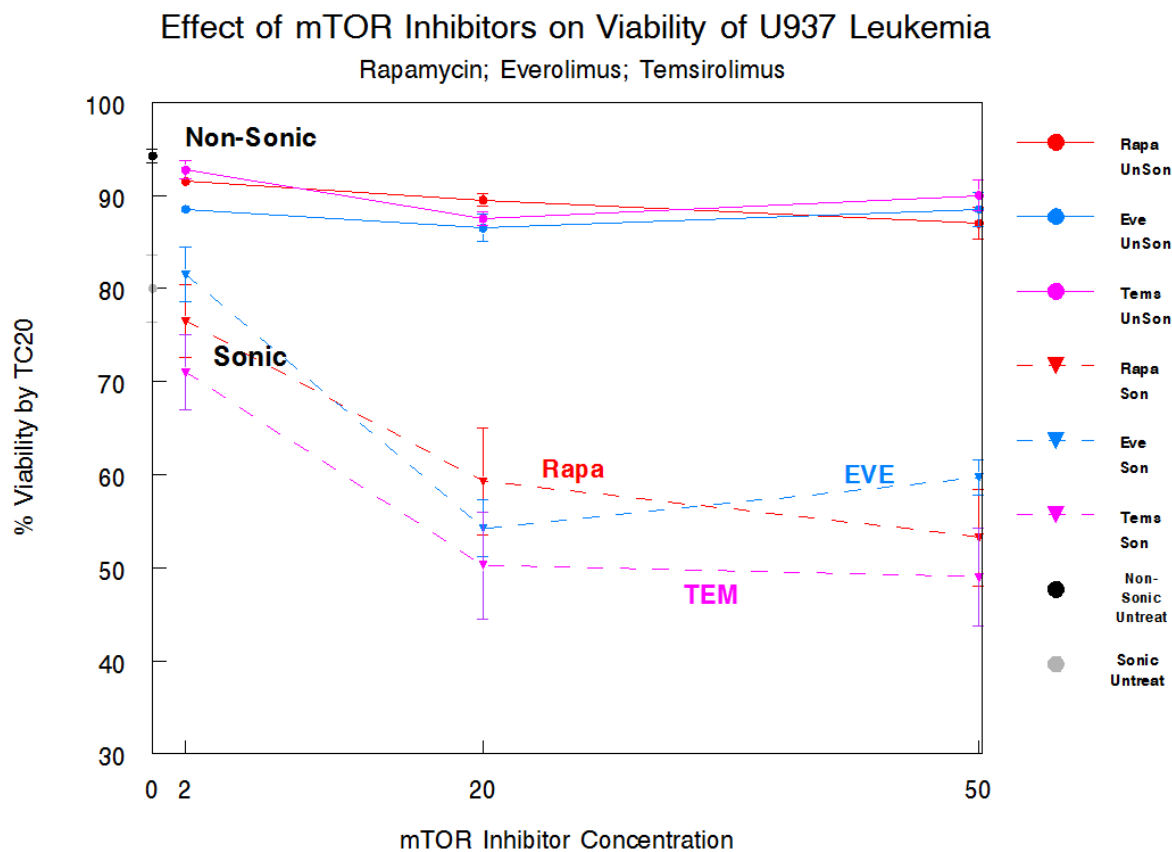
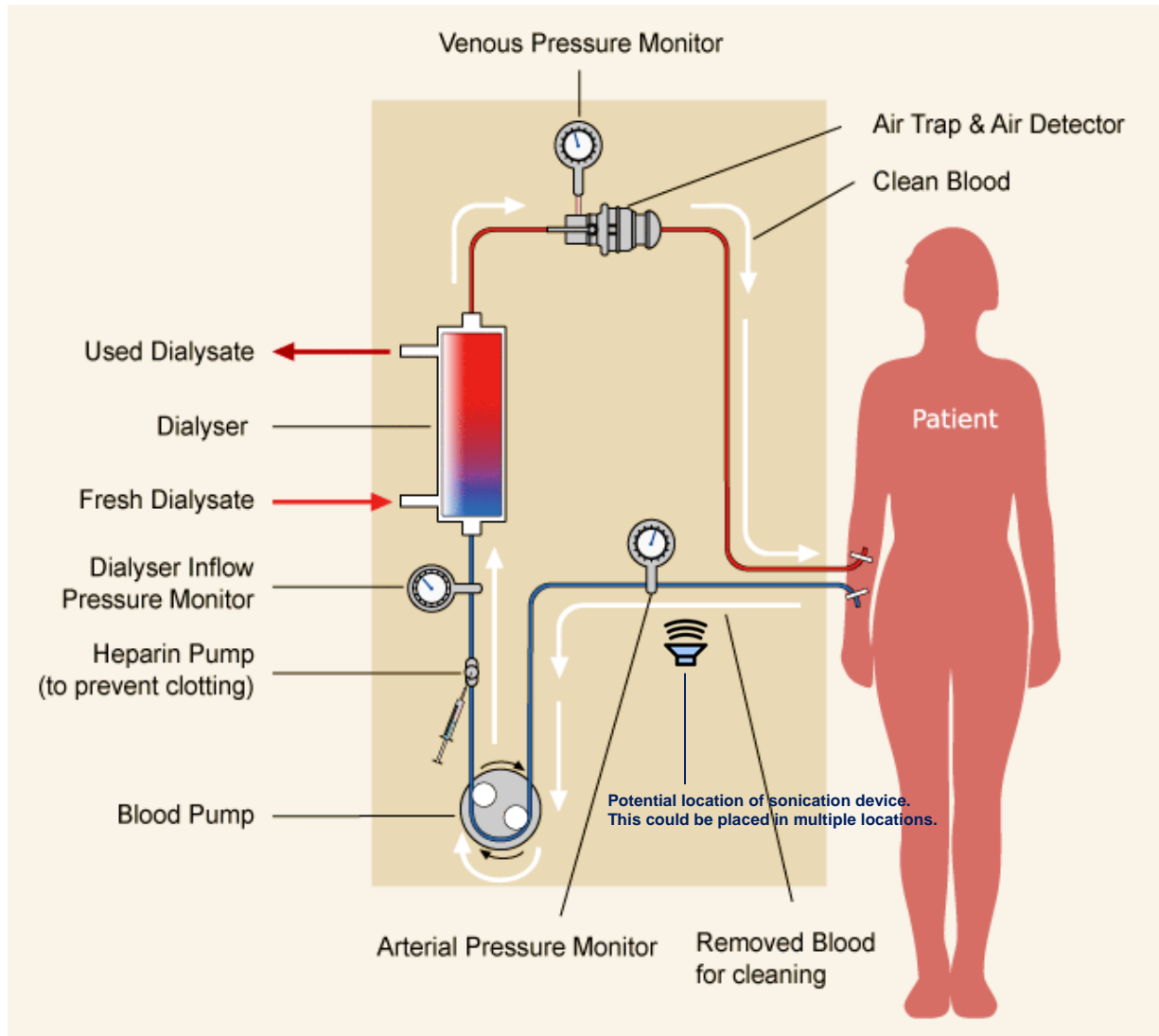


Figure 7: Effect of mTOR inhibitors (rapamycin, everolimus, temsirolimus) on the viability of U937 human acute monocytic leukemia

Chapter 6: Discussion

mTOR inhibitors appear to have a dose dependent effect on the inhibition of proliferation on U937, THP-1, K562, and MOLT-4 leukemia cell lines. Treatment with pulsed low frequency 20 kHz ultrasound further decreases cell number by inducing cell lysis. 2nM temsirolimus caused the most cell lysis in U937 cells and THP-1 cells, whereas 2nM everolimus caused the cell lysis in K562 cells. Viability of unsonicated U937 does not change as mTOR inhibitor concentration is increased from 2nM to 50nM. Effects of mTOR inhibitors on the inhibition of proliferation of leukemia cells can be combined with the effects of 20 kHz pulsed low frequency ultrasounds on cell lysis to decrease the total number of U937, THP-1, K562, and MOLT-4 cells. U937 acute monocytic leukemia cells that remain after sonication become increasingly less viable as the concentration of mTOR inhibitors are increased. MOLT-4 cells appear to be significantly susceptible to ultrasonic cell lysis whereas U937 cells appear to be least sensitive. mTOR inhibitors do not significantly change the sonic sensitivity in any of the examined cell lines.

For future research, it would be beneficial to introduce the combination of cytochalasin B, a microfilament directed agent, in combination with rapamycin. According to previous clinical studies, cytochalasin B is known to produce enlarged and multinucleated cells (Trendowski *et al*, 2015). Therefore, this could potentially increase susceptibility to ultrasonic damage. Furthermore, *in vivo* work with mice would be useful in determining how a live animal tolerates ultrasound. For humans, the sonication of leukemia cells can be administered via hemodialysis as indicated in the image below.



For extracorporeal blood sonication, the patient's blood is to be pumped outside of the body to provide an opportunity for leukemia cells to be sonicated.

Image retrieved from <http://mountbakerkidneycenter.org/treatment/hemodialysis/>

Chapter 7: Additional Work Completed

Concomitant Effects of mTOR inhibitors (2 nM Rapamycin) with cytoskeletal-direct agent (2 μ m Cytochalasin B) on U937 cells before and after Pulsed Sonication with 20 kHz System (60 W)

Stemming off of the previous research, I had the time to assess the addition of cytochalasin B (CB) and its affect with rapamycin. I wanted to assess how much more damage is caused after sonication to the treated cells? I am using U937 human monocytic leukemia cell lines because previous research has indicated that this cell line reveals the most reliable data. In lab we have already seen the effect of rapamycin and CB on cell size of U937 cells without sonication (Trendowski *et al*, 2015).

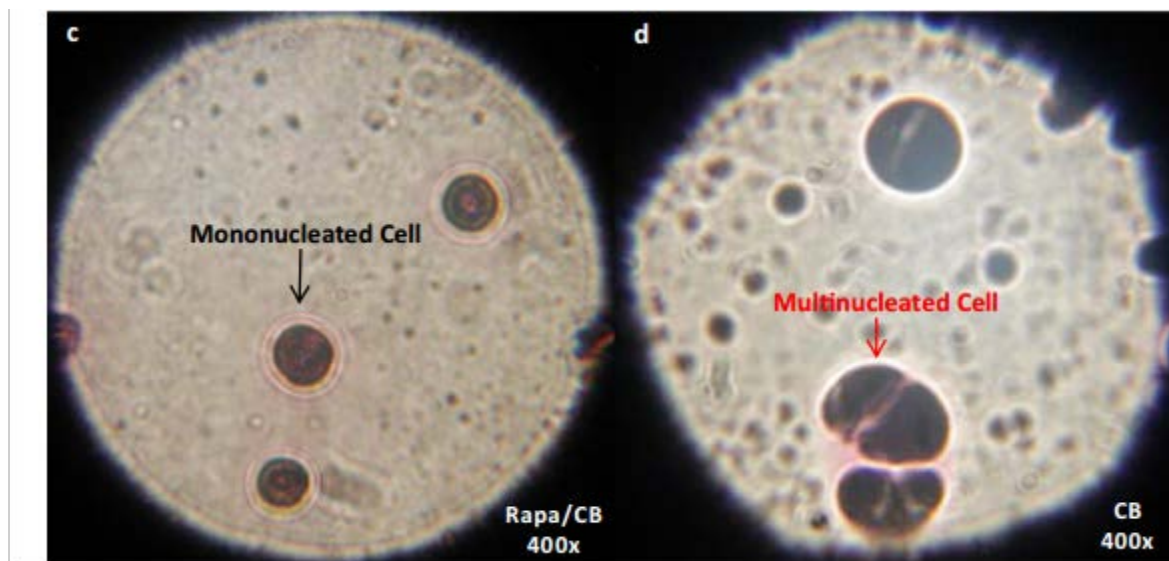


Figure 4. Effects of rapamycin and cytoskeletal-directed agents on cell size of U937 human monocytic leukemia cells (Trendowski *et al*, 2015, p. 1169).

The procedures protocols were set up the same way as the previous experiment.

Control counts of untreated U937 cells; Rapa (2 nM), Cytochalasin B (CB) (2 μ M), 2 nM Rapa + 2 μ M CB were taken in a duplicate manner on TC20 and Z2. 2 μ M CB-treated U937 cells and 2 nM rapamycin treated cells were treated for 48 hours. Then, sonicate 3ml of untreated U937 Rapa (2 nM), Cytochalasin B (CB) (2 μ M), 2 nM Rapa + 2 μ M CB, cells in glass vial. Cell count was taken on TC20 and Z2 after sonication, 2 counts per sample. Untreated U937; Rapa (2 nM), Cytochalasin B (CB) (2 μ M), 2 nM Rapa + 2 μ M CB control count on TC20 and Z2 was also taken after all sonication was done. The TC20 used 1:1 dilution untreated U937; Rapa (2 nM), Cytochalasin B (CB) (2 μ M), 2 nM Rapa + 2 μ M CB: Trypan Blue. Z2 used 1:201 dilutions untreated U937; Rapa (2 nM), Cytochalasin B (CB) (2 μ M), 2 nM Rapa + 2 μ M CB: Isoton.

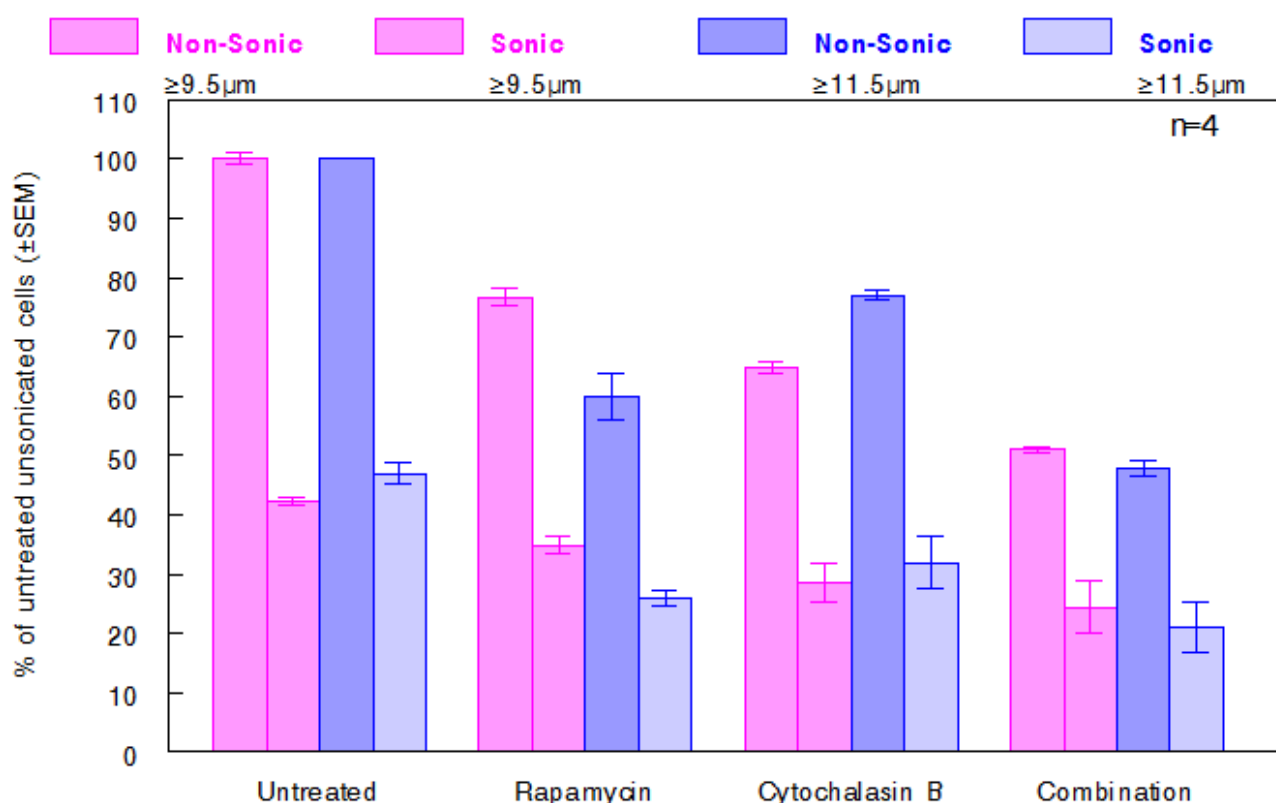


Figure 8: Effect of 2nM Rapamycin, 2 μ M cytochalasin B, or a combination on the percent survival of U937 human monocytic leukemia using the Beckman Coulter Z2 Counter in a 20 kHz system with 30% amplitude (60 W), glass vials and 5 x 0.6s of ultrasound and 0.4 seconds without ultrasound

Figure 8 demonstrates that sonicating U937 cells with combination of rapamycin and cytochalasin B produces the most damage to the cells survival. Cytochalasin B alone creates more cell lysis than Rapamycin alone. The potential for increasing synergy between rapamycin and cytochalasin B may be due to the inhibitory effects these agents have on the microfilaments located in the cell's cytoskeleton. As mentioned in my previous research , *in vivo* work with mice would be useful in determining how a live animal tolerates ultrasound with these chemotherapeutic agents.

Chapter 8: Conclusion

In this present study the sonic sensitivity increases as cell size increases. Introducing rapamycin to cytoskeletal-directed agents may induce a drug synergy effect to leukemia cells when low-frequency ultrasound is applied. Further characterization of the potential antineoplastic applications of mTOR inhibitors and cytoskeletal-directed agents in mammalian models is therefore crucial. The observations made by this research demonstrate a potentially novel therapeutic approach for leukemia, and hopefully for other cancers, which aberrantly express mTOR to elicit preferential damage.

References

- American Cancer Society (2015). Cancer Facts & Figures 2015. *American Cancer Society*. Accessed on September 13, 2015
<http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf>
- Boulay A, Rudloff J, Ye J, Zumstein-Mecker S, O'Reilly T, Evans DB, Chen S, Lane HA (2005) Dual inhibition of mTOR and estrogen receptor signaling *in vitro* induces cell death in models of breast cancer. *Clinical Cancer Research* 11(14): 5319-5328
- Brennen, CE (1995) Cavitation and bubble dynamics. *Oxford University Press*
- Fuchs, J (2011) Ultrasonics – Number and Size of Cavitation Bubbles. *Cleaning Technologies Group* Accessed on September 14th, 2014
<http://www.ctgclean.com/tech-blog/2011/12/ultrasonics-number-and-size-of-cavitation-bubbles/>
- Guertin DA, and Sabatini DM (2007) Defining the role of mTOR in cancer. *Cancer Cell* 12: 9–22
- Hielscher T (1999) Ultrasonic sonotrodes, flow cells & accessories. *Hielscher-Ultrasound Technology* Accessed on October 1st, 2014
<https://www.hielscher.com/ultrasonic-sonotrodes-flow-cells-accessories.htm>
- Leong TSH, TJ Wooster, Kentish SE, Ashokkumar M (2009) Minimising oil droplet size using ultrasonic emulsification. *Ultrason. Sonochem* 16 (6): 721-727
- Nakamura I (2014) Impairment of Innate Immune Responses in Cirrhotic Patients and Treatment by Branched-chain Amino Acids. *World J Gastroenterol* 20(23): 7298-305
- Oswald, N (2013) Breaking up is (Not That) Hard to Do: Sonication for Cell Lysis. *Bitesize Bio* Accessed on September 16th, 2014 <http://bitesizebio.com/10778/breaking-up-is-not-that-hard-to-do-sonication-for-cell-lysis/>
- Piller, GJ (2008) The Cambridge World History of Human Disease. *Cambridge University Press* 3(82): 843-848
- Rosenthal I, Sostaric JZ, Riesz P (2004) Sonodynamic Therapy – A Review of the Synergistic Effects of Drugs and Ultrasound. *Ultrasonics Sonochemistry* 11(6): 349-363
- Suslick KS, Didenko Y, Fang MM, Hyeon T, Kolbeck KJ, Mcnamara III WB, Mdeleni MM, Wong M (1999) Acoustic cavitation and its chemical consequences. *Philosophical Transactions of the Royal Society* 357: 335-353
- Trendowski M, Christen TD, Andonova AA, Narampanawe B, Thibaud A, Kusang T, Fondy TP (2015) Effects of mTOR inhibitors and cytoskeletal-directed agents alone and in combination against normal and neoplastic hematopoietic cells *in vitro*. *Investigational New Drugs* 33(6): 1162-1174
- Veikkola, T and Alital K (1999) VEGFs, receptors and angiogenesis. *Seminars in Cancer Biology* 9: 211–220
- Gao Z, Zheng J, Yang B, Wang Z, Fan H, Lv Y, Li H, Jia L, Cao W (2013) Sonodynamic therapy inhibits angiogenesis and tumor growth in a xenograft mouse mode. *Cancer Letters* 335 (1): 93-99