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mTOR in Cell Signaling and Size Enlargement as a Target for the Chemico-Physical Therapy of Cancer

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

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05/2010

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Abstract

mTOR is a kinase protein meaning it phosphorylates target proteins affecting their cell signaling properties[1]. The drug Rapamycin, analogs of Rapamycin, and cell signaling proteins that interact with mTOR control the activities mediated by mTOR[1]. mTOR is located in the cytoplasm at a convergent point of many signaling pathways that regulate a multiplicity of cellular processes including metabolism that precede cell enlargement (cell "growth"), cell proliferation (cell division), and angiogenesis[1,2]. Cells with mTOR inappropriately activated can proceed with cell enlargement and cell proliferation in the absence of normal cell signaling[2]. Rapamycin and Rapamycin analogs can inhibit mTOR and prevent cell enlargement that precedes cell proliferation[3,4].

We wanted to know if there is a concentration of Rapamycin that will inhibit cell enlargement and proliferation of normal human hematopoietic stem cells (HSC) under conditions that will still permit enlargement and proliferation of human U937 leukemia cells. We performed experiments where we treated HSC and U937 cells with Rapamycin. We compared the results of these experiments to see whether there is a dose response difference to Rapamycin between the two cell types.

We found that cell size of both HSC and U937 leukemia cells was affected to comparable levels by Rapamycin at low nanomolar concentrations. However, Rapamycin appeared to have a startling differential effect on cell proliferation of HSC as compared to U937 cells. HSC proliferated very slowly or not at all in the presence of low nM concentrations of Rapamycin. U937 cells on the other hand were able to proliferate more strongly even at very high concentrations of Rapamycin. Rapamycin inhibited the rate of cell proliferation to some extent but it did not prevent the U937 cells from completing cell division and increasing in number.

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I would like to thank my friends and family for all the support.

Introduction

The mammalian target of Rapamycin (mTOR) is a kinase protein that regulates many essential processes[1]. Cells with mTOR inappropriately "turned on" proceed with cell division often when they are not supposed to[2]. As Figure 1 and Table 1 show, mTOR has been linked with many cancers, including breast cancer, colon cancer, prostate cancer, lymphoma, and melanoma[2].

Figure 1. Cancers in which mTOR is abnormally activated.

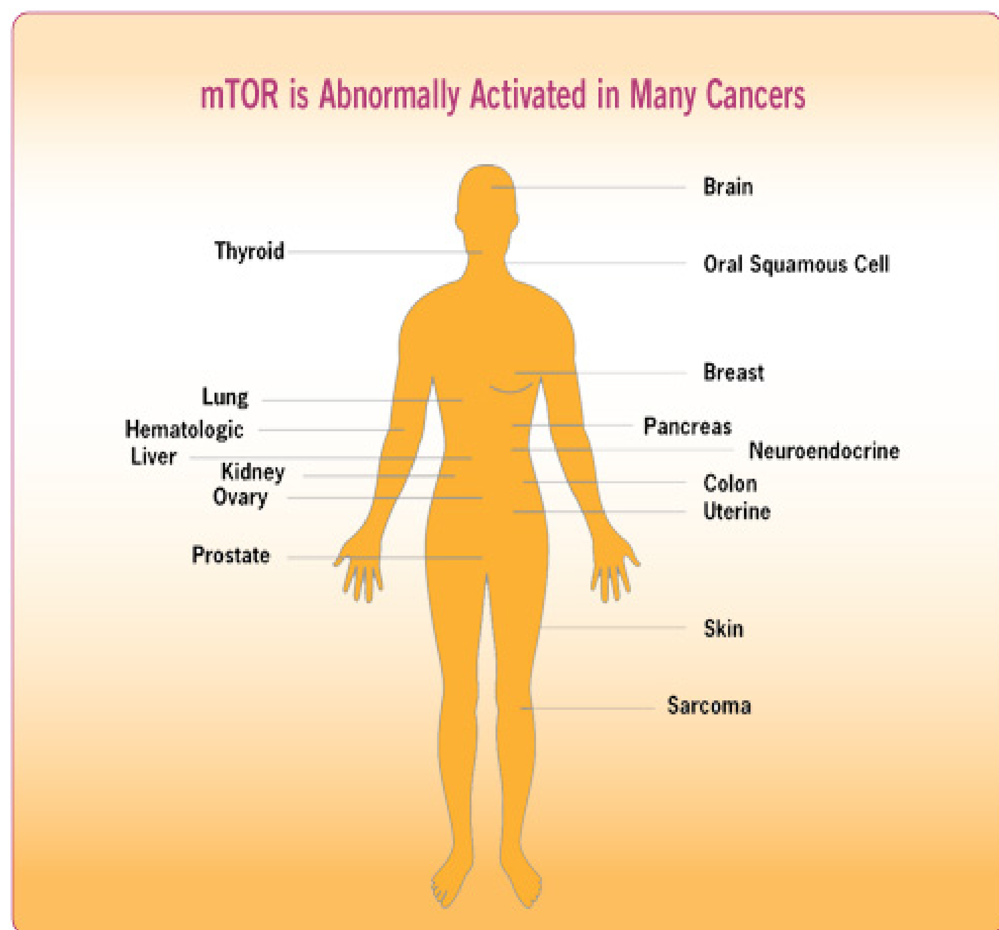

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Table 1. The table lists mutations in upstream proteins that may lead to inappropriate activation of mTOR. Representative cancer types are listed.

Selected Mutations That May Abnormally Activate mTOR in Cancer

Target	Type of Protein	Genetic Aberration	Representative Tumors
EGF/ EGFR [1]	Growth factor/ receptor	Amplification, mutation	Colorectal, lung, gastric, pancreas, liver, neuroendocrine, others
HER2 [2]	Growth factor receptor	↑ Expression	Breast
ER [3]	Hormone receptor	↑ Expression	Breast, endometrial
IGF-1/ IGF-1R [4,5,20-25]	Growth factor/ receptor	↑ Expression	Neuroendocrine, renal cell, breast, lung, prostate, others
PTEN [6]	Lipid phosphatase	Silencing, allele loss	Glioma, endometrial, prostate, melanoma, breast, thyroid, neuroendocrine
PI3KCA [7]	Serine-threonine kinase	Mutations	Colorectal, breast, lung, brain
TSC1 [8,9]	TSC complex protein	Mutation	Bladder, renal cell
TSC2 [9,10]	TSC complex protein	Allele loss	Neuroendocrine, renal cell
LKB1 [11,12]	Serine-threonine kinase	Mutation, silencing	Colorectal, lung, breast
K-Ras [13]	GTP-binding kinase	Mutation	Colorectal, pancreas, lung, melanoma
Bcr-Abl [14]	Tyrosine kinase	Translocation	CML, ALL
TGF [15]	Growth factor	↑ Expression	Renal cell

EGF/EGFR = epidermal growth factor/epidermal growth factor receptor; ER = estrogen receptor; IGF-1/IGF-1R = insulin-like growth factor 1/ insulin-like growth factor 1 receptor; TSC1 = tuberous sclerosis complex 1 (hamartin); TSC2 = tuberous sclerosis complex 2 (tuberin); TGF = transforming growth factor; CML = chronic myeloid leukemia; ALL = acute lymphocytic leukemia

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mTOR is a protein kinase located in the cytosol at a converging point of numerous signaling pathways[1,2]. mTOR integrates the various signals and determines the appropriate cell response[1,2].

Since mTOR is a converging point of many signaling pathways, inhibiting mTOR may affect crucial cell processes such as cell proliferation, angiogenesis, or cell metabolism[2]. Abnormal expression of mTOR can lead to abnormal regulation of these crucial processes[2]. Inhibition of abnormal mTOR expression may provide an approach to cancer therapy. Inhibition of mTOR may enhance the effect of other therapeutic agents or methods, and indeed, Rapamycin and analogs of Rapamycin have been examined for anti-neoplastic efficacy[3,4]. In our laboratory on the other hand, we are proposing to exploit the over-expression of mTOR in leukemia cells as a novel approach to leukemia therapy rather than to inhibit mTOR activity.

mTOR controls cell proliferation (cell division) by regulating the production of cyclin D1[2]. Cyclin D1 is involved in the regulation of cell passage through the G_1-S cell-cycle checkpoint[2]. This regulation is performed via the activation of cyclin-dependent kinases 4 and 6 by cyclin D1[2]. Many cancers show overexpression of cyclin D1[2].

Angiogenesis, an essential process that provides nascent tumor cells with a blood supply, requires the production of many factors such as vascular endothelial growth factor (VEGF)[2]. mTOR controls the production of HIF1-α, also known as hypoxia-inducible factor[2]. It is the HIF1-α which activates the angiogenesis

factors[2,5]. In many cancers, HIF1-α is present at abnormal levels[2]. This can be due to overproduction or failure to degrade HIF1-α factors[2].

mTOR also regulates cell metabolism[1,2,5]. When nutrient levels are high, mTOR activates protein synthesis[1,2,5]. Likewise, when nutrient levels are low, mTOR inactivation leads to down regulation of protein synthesis and cell growth arrest[1,2,5]. Many cancers grow inappropriately due to having mTOR "switched on" upregulating protein synthesis[2].

Our laboratory is particularly interested in exploiting inherent or chemotherapeutically-induced differences in cell size between normal cells and cancer cells as a possible approach to the physical destruction of enlarged cells. Rapamycin and analogs of Rapamycin can control cell size by inhibiting cell growth by specifically inhibiting mTOR[2,5]. Thus Rapamycin and Rapamycin analogs are antagonistic to cytoskeletal-directed anticancer chemotherapeutic agents such as Vincristine, Taxol, and Cytochalasin B that can cause cancer cells to become greatly enlarged. Cancer cells with abnormal expression of mTOR could become significantly enlarged under conditions where normal cells remain small because their mTOR expression is correct. Thus, a combination of cytoskeletal abnormalities along with errors in mTOR signaling may amplify the size differences between normal and cancer cells.

We wanted to see if there is a dose-response effect of Rapamycin on cell size and proliferation of human stem cells (HSC) in culture and U937 leukemia cells in culture. We would like to know the conditions under which Rapamycin inhibits cell enlargement and proliferation of HSC more strongly than it does of

U937 leukemia cells. Moreover, we would like to know whether any concentration dependent effects are present.

We performed experiments where we treated HSC and U937 cells with Rapamycin. We found that HSC and U937 cells are affected in terms of cell enlargement, although there may be a slight dose response difference between the two cell types. However, Rapamycin appears to have a startling effect on cell proliferation. As expected, HSC fail to proliferate in the presence of Rapamycin. U937 cells, on the other hand, proliferate strongly in the presence of Rapamycin. This differential characteristic may be further examined for sensitivity to physical destruction and/or interaction with cytoskeletal-directed chemotherapeutic agents.

Literature overview

The focus of this literature overview is on the mTOR protein kinase and its effects on cell size and the metabolic cycle. I will not elaborate on angiogenesis or cell proliferation more than I have already. For definitions of the various molecules mentioned please refer to Appendix A.

Discovery

In the 1970s, the bacterial strain *Streptomyces hygroscopicus* was shown to produce a strong antifungal metabolite[5]. *S. hygroscopicus* is native to Easter Island[5]. This macrocyclic lactone metabolite was named Rapamycin after the

place of its discovery[1,5]. In the local language Easter Island is known as Rapa Nui[5].

Figure 2. Structure of Rapamycin showing the FKBP12-binding region and the mTOR-binding region.

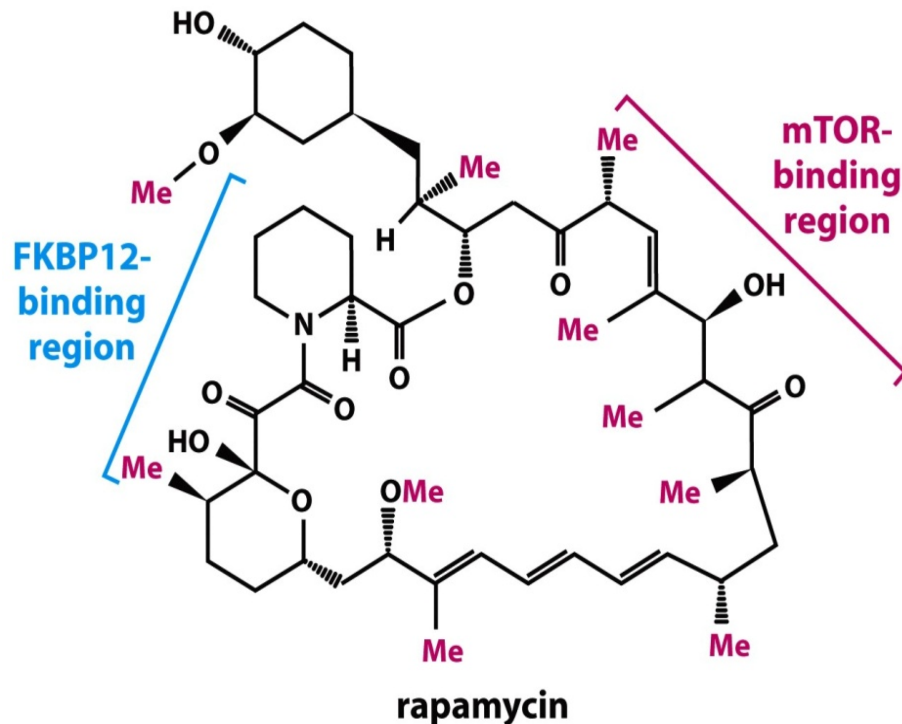


Figure 16-43a The Biology of Cancer (© Garland Science 2007)

Rapamycin (Figure 2) suppresses immune response and cell proliferation[6]. Through gene knockout mutations the target of Rapamycin (mTOR) was identified in yeast[5]. Mutations mTOR1-1 and mTOR2-1 allowed the yeast *Saccharomyces cerevisiae* to be resistant to Rapamycin[5]. It was also shown that peptidyl-prolyl cis/trans isomerase FKBP12 is an essential intracellular co-factor for Rapamycin effectiveness[5,6]. Rapamycin forms a complex with FKBP12 that together binds and inhibits mTOR[5].

Structure

mTOR is highly conserved across eukaryotic species[5]. Every eukaryote genome examined so far: yeast, algae, plants, flies and mammals among many, all contain the mTOR gene[5]. Yeast have been found to contain 2 mTOR genes[5]. Higher organisms contain only 1 gene[5].

mTOR is a large protein (about 280 kDa)[5,6]. About 40%–60% of the mTOR primary sequence is identical to the phosphatidylinositol kinase-related kinase (PIKK) family of proteins[5]. At the carboxy-terminus end of mTOR the PIKK family have a serine/threonine protein kinase domain[5,6]. This domain shares features of the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks) and PI4Ks[5,6].

The amino-terminus of mTOR contains the FKBP12-Rapamycin binding domain (FRB) which is the binding site of Rapamycin (Figure 3)[5]. Mutations in this domain yield mTOR proteins that no longer respond to inhibition by FKBP12-Rapamycin complex[5]. Other selective mutations have uncovered many domains of protein-protein interaction such as the C-terminal FAT and FATC domains that are found in all PIKKs[5]. Another indication of protein-protein interaction is the presence of HEAT sequences found at the amino terminus of mTOR[5]. These HEAT sequences may extend and form helices offering regions for protein-protein interaction[5].

mTOR forms two distinct complexes (Figure 4)[5,7]. mTOR complex 1 (mTORC1) is sensitive to Rapamycin[1,5]. This complex dictates how the cell grows[1,5]. mTOR complex 2 (mTORC2) is Rapamycin insensitive[1,5]. It dictates the

actin cytoskeleton organization[1,5]. As such it controls cell motility, morphology, position, polarization and location. mTORC2 is poorly understood[5]. The complexes are distinct in the associated proteins they bind[5,7]. Both are multimers, likely dimers[5,7].

Figure 3. The binding of FKBP12-Rapamycin complex to the FRB domain of mTOR.

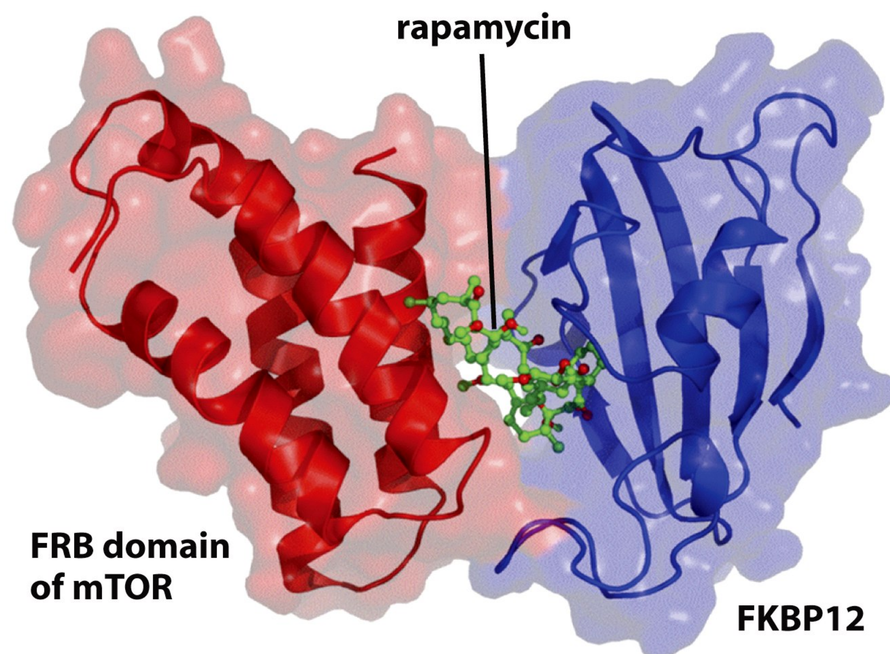
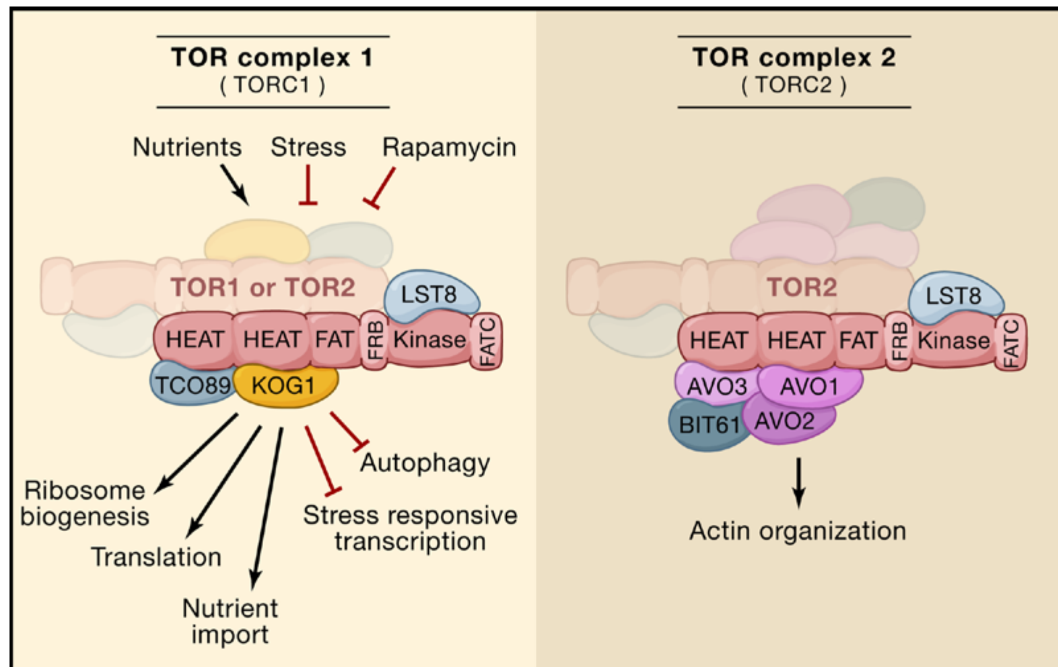


Figure 4. The structure of the two distinct mTOR complexes and their respective functions.



Wullschleger, S., Loewith, R., Hall, M.N. (2006). TOR Signaling in Growth and Metabolism. Cell *124*. 471-484.

Early Development

Experiments which involve the deletions of the mTOR gene in *Caenorhabditis elegans* or in *Drosophila melanogaster* result in embryos that show developmental arrests similar to starved larvae[5]. This indicates that mTOR is important in the early stages of development where cell growth and proliferation are of utmost importance[5]. For example, Bateman and McNeill have shown that overexpression of mTOR in *Drosophila* accelerates differentiation[5]. Likewise, underexpression of mTOR in *Drosophila* leads to slower differentiation[5].

Experiments performed on mice also indicate the essentiality of mTOR function[5]. Mouse embryos with missing mTOR show developmental problems and often fail to survive because the defective embryos suffer from amino acid deprivation[5]. When mouse cells are treated with Rapamycin, they also fail to proliferate[5].

Cell growth

In order to maintain homeostasis cells need to respond to nutrients appropriately. When nutrients are abundant cells usually respond by increasing their metabolic rate[5]. When mTOR is activated, cells show very high rates of nutrient transport, ribosome biogenesis, and protein synthesis[1,5].

Conversely, when nutrients are lacking cells will downregulate metabolic rates to better conserve energy[5]. Studies of Rapamycin treated yeast have shown that when mTOR is inhibited, translation is downregulated; macroautophagy and other stress-response systems are initiated[1,5].

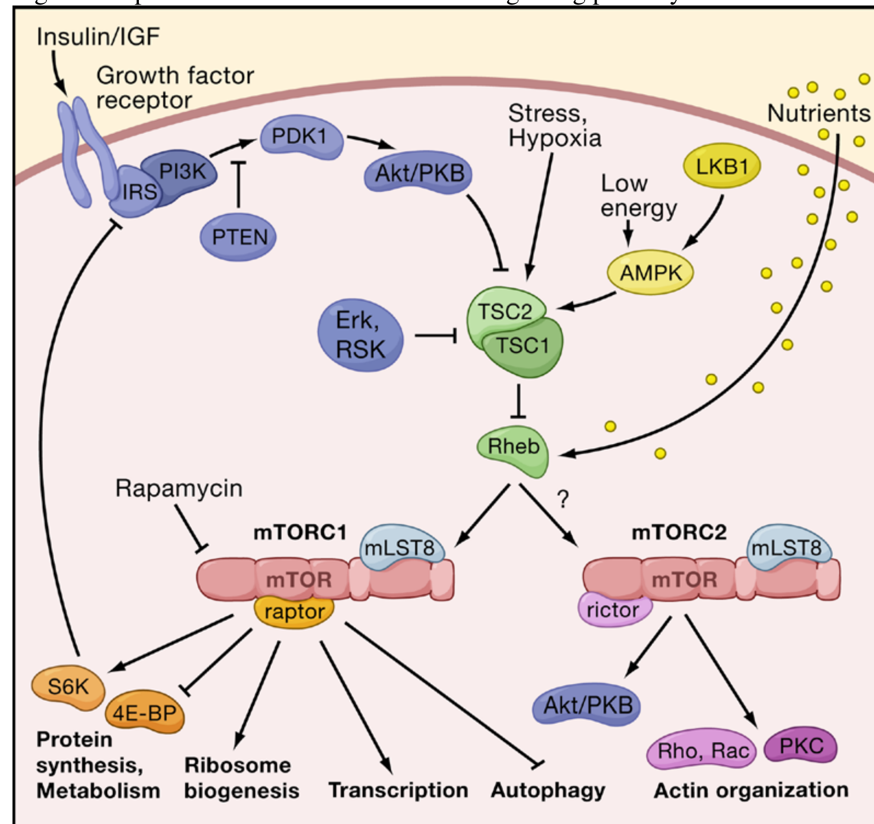
Moreover, studies in yeast have also shown that mTOR is not only responsible for how much the cell grows, but also where it grows[1,5]. mTOR2, not mTOR1, regulates the polarization of actin skeleton[1,5,7]. In yeast that reproduce by budding, mTOR2 allows actin to polarize toward the bud causing protein synthesis and sequestration to be localized in the bud[5]. This function of mTOR2 is insensitive to the effects of Rapamycin[5]. The effect of mTOR2 on actin organization is conserved across organisms[5].

Cell Growth Regulation Upstream

Growth Factors

mTOR is important in regulation of cell growth because it integrates the various signals that the cell experiences such as the presence or absence of growth factors (GF), nutrients, energy or stress[1,5]. When growth factors are present, mTOR will respond via the PI3K pathway[5]. For example, Figure 5 shows how insulin (a GF) stimulates the pathway. Upon its binding to the receptor, the insulin receptor substrate (IRS) is phosphorylated which in turn attracts PI3K[5]. PI3K-IRS complex then converts phosphatidylinositol-4,5-phosphate (PIP2) into phosphatidylinositol-3,4,5-phosphate (PIP3)[5]. Phosphatase and tensin homolog (PTEN), the enzyme that acts as a tumor suppressor, may antagonize the accumulation of PIP3[5].

Figure 5. Upstream and downstream mTOR signaling pathways.



Wullschleger, S., Loewith, R., Hall, M.N. (2006). TOR Signaling in Growth and Metabolism. Cell *124*. 471-484.

Subsequently, PIP3 will recruit PDK1 and Akt to the membrane and activate them by phosphorylation[5]. mTOR recognizes the PI3K pathway through the tuberous sclerosis proteins TSC1 and TSC2[5]. TSC1 is a hamartin and TSC2 is tuberin[5]. Both act as a heterodimers that antagonize mTOR[5]. When insulin is present, activated Akt phosphorylates TSC2, thereby rendering it inactive and unable to form the heterodimer[1,5].

TSC2 is a GAP (GTP-ase activating protein) for the Rheb molecule, which is a GTPase[5]. There is no consensus on whether Rheb directly binds to the mTOR kinase domain; however, there is agreement that Rheb induces a conformational change in mTORC1, leading to the activation of mTORC1[5]

Nutrients

mTOR1 is regulated by amino acids, particularly the amino acid leucine[5]. When leucine is absent mTOR effectors S6K1 and 4E-BP1 are dephosphorylated[5]. When leucine is re-added the dephosphorylation is reversed[5].

One way this regulation might come about is through the interaction of amino acids with the TSC1-TSC2 complex[5].The presence of amino acids has been suggested to either inhibit the TSC1-TSC2 complex or to stimulate the Rheb molecule[5]. When TSC-1 is inactivated the cell does not respond properly to the presence of amino acids[5]. Other studies suggest that amino acids affect the interaction and binding of Rheb and mTOR[5]. More recent studies have implicated another molecule, hVPS34, a class III PI3K that signals to mTOR independently of the TSC-Rheb complex[5]. The mechanism is not clear[5].

Energy

The cell gauges the levels of energy based on the levels of AMP-activated protein kinase (AMPK)[1]. Cell growth requires a lot of energy to synthesize proteins at a high rate. Low levels of energy are characterized by a high AMP/ATP ratio that activates AMPK[1,5]. When activated, AMPK inhibits protein synthesis and activates ATP synthesis[1,5]. AMPK directly phosphorylates TSC2, enhancing its activity[5]. Experiments done using the AMPK analog called AICAR have shown mTORC-1 inhibition of S6K1 and 4E-BP1[5].

Stress

Cells respond to stress, such as hypoxia (low oxygen) by downregulating processes that require a lot of energy, such as cell growth[5]. When a cell experiences hypoxia it downregulates mTOR and protein synthesis is inhibited[1]. Experiments performed on *Drosophila* and mammalian cells have shown that cells recognize hypoxia through the REDD1 and REDD2 proteins[5]. When the cell experience hypoxia, HIF1 stimulates the production of REDD molecules[5]. REDD molecules act downstream of Akt but upstream of TSC[5]. REDD inhibits mTOR1[5]. Since hypoxia leads to eventual ATP depletion, it is suggested that the AMPK pathway and REDD pathway are interrelated[5]. The mechanism is still unknown[5].

Cells experience other stresses[5]. When p53 senses DNA damage it signals to mTOR via the AMPK-TSC2 signaling pathway[5]. Other stresses include the presence of reducing agents in the environment[5]. Reducing agents have been suggested to inhibit mTOR via a redox sensor in the FAT domain of mTOR[5].

Cell Growth Regulation Downstream

Ribosome Biogenesis

The creation of ribosomes is a very energy-consuming process[5]. It is essential for a cell to have a ribosome control mechanism that responds to the changes of cellular energy levels[5]. Studies have shown that mTOR controls the synthesis of ribosomes by controlling the transcription of RNA polymerase I-dependent rRNA genes, RNA Polymerase II dependent ribosomal protein genes,

RNA Polymerase III dependent tRNA genes and also by controlling 35S rRNA[5]. In humans and mice, mTOR controls ribosome synthesis by inhibiting these genes[5]. mTOR has been shown to control transcription by forming complexes with transcription initiation proteins, such as Pol I associated TIF1A[5]. Cells treated with Rapamycin showed inactivation of TIF1A and prevention of transcription[5]. FHL1 Pol II associated transcription factor has been shown to be essential for regulation of Pol II-dependent RP gene expression[5].

Transcription/Translation

Translation is regulated by S6K1 and 4E-BP, both of which are regulated by mTOR[5]. mTOR activates S6K1 through phosphorylation at Thr389[5]. The second phosphorylation comes from PDK1[5]. It is unknown how S6K1 augments translation[5]. mTOR 1 phosphorylation has been shown to be important in the nuclear localization of several transcription factors[5].

Actin

mTOR2 complex controls the polarization of actin molecules[1,5]. mTOR signals to actin through Rho1 GTPase[5]. Rho1 then signals to PKC1 which in turn activates the MAP kinase pathway that reorganizes the actin cytoskeleton[5]. How TOR signals to Rho1 is not understood[5]

Rosner *et al*. show that the effects on cell cycle and cell size by mTORC1 and mTORC2 appear to be separate[7]. However, mTORC2 appears to not only

control actin reorganization but also regulates cell size and cell cycle via Akt, TSC2 and Rheb cell signaling[7]. This is independent of mTORC1 signaling[7].

Macroautophagy

Macroautophagy occurs when cells that are without nutrition for a prolonged time begin to degrade cytoplasmic contents to maintain energy levels[1]. ATG1 is a kinase that activates macroautophagy[5]. mTOR has been implicated in yeast and higher organisms in controlling this process[5]. Yeast studies have shown that TOR inactivates a protein kinase ATG1[5]. When mTOR is inactivated, macrophagy is initiated and the cells begin this catabolic process[5].

Metabolism

Metabolic processes such as amino acid synthesis, glucose homeostasis and fat metabolism are controlled by mTOR as well[5]. Rapamycin treatment inhibits adipogenesis, which is poorly understood[5]. However, the mechanism seems to involve mTOR and S6K1 since mTOR signals to S6K1[5]. S6K1 mutants inhibit fat accumulation[5]. Moreover, flies with inactivated mTOR show fat deficiency[5].

Materials

Fetal Bovine Serum, Atlanta Biologicals, premium, triple 0.1 μm filtered.

StemSpan SFEM: Serum Medium for Expansion and Culture of Hematopoietic Cells, Stem Cell Technologies.

Amphotericin B (Fungisone) 0.250 μg/ml, Sigma.

Penicillin-Streptomycin: 10,000 units penicillin and 10 mg streptomycin per ml in 0.9% NaCl, Sigma.

L-Glutamine: 200 mM, Sigma.

Gentamycin Sulfate: 10 mg/ml, BioWhittaker.

StemSpan Cytokine Cocktails, 100X (Flt-3, SCF, IL-3, IL-6) Stem Cell Technologies.

Iscove's Modified Dulbecco Medium with $NaHCO_3$ and 25 nM Hepes. No glutamine, thioglycerol, BME, BioWhittaker.

Rapamycin: Sigma.

Methods

<u>Experiment 1</u>

The U937 Cells: The R1A Line Day 8 plateau-phase cells were checked for viability. They were sub-cultured to 5 x 10E4 Cells/ml in Iscove's Modified Dulbecco's Medium with 20% FBS, 1% FGZ (Amphotericin B) (2.5 μg/ml), 1% Gln (2 mM), 2% Pen-Strep (200 units penicillin, 200 μg streptomycin per ml), 0.5% Gentamycin (200 μg/ml) in 5% CO_2 at 37°C. They were grown for 24 hours. Cells were counted before seeding and checked for viability. The cells were seeded 500 μl into 1 ml wells in a 48-well plate. Rapamycin 40 μM in EtOH was given to final concentrations of 800, 400, 200 (10 μl, 5μl, 2.5 μl). A 5 μM Rapamycin Stock Solution in EtOH (10 μl, 5 μl, 2.5 μl) was used to give 100, 50,

and 25 nM Rapamycin. Control wells were either untreated or treated with 1.9% EtOH. Cells were Coulter counted at days 1 and 4, at every micron size from 8µm to 22µm.

Human Stem Cells: Were revived on March 11, 2009, from cells that were stored at the temperature of -86°C on November 17, 2006. They were grown for one week in 20% FBS Iscove's, 1% FGZ, 1% Gln, 1% Stem Span from Stem Cell Technologies. Before seeding cells were counted with a hemocytometer and checked for viability using trypan blue. The cells were seeded in a volume of 500 µl into 1 ml wells of 48-well plate. Rapamycin treatment was same as for U937 cells. The cells were Coulter counted at days 1, 4, 8, and 9. They were counted at every micron size from 8µm to 18µm.

Experiment 2

U937 Cells: The R1A Line Day 6 late log-phase cells were checked for viability. They were sub-cultured to 5 x 10E4 Cells/ml in 20% FBS Iscove's, 1% FGZ, 1% Gln. They were grown for 24 hours. Rapamycin solutions were prepared by making 4 µM Rapamycin in 95% EtOH using 40 µM Rapamycin Solution. At the day of treatment there were 1.3 X 10E5 Cells/ml in 4 ml flasks. There were 8 flasks total. Six flasks (40, 20, 10, 5, 2, 1 nM) were treated with 40, 20, 10, 5, 2, and 1µl of 4 µM Rapamycin, respectively. Control cells were either untreated or treated with 0.95% EtOH. Cells were Coulter counted at days 1 and 4, at every micron size from 8µm to 22µm.

Three days later Rapamycin was removed from flask. Rapamycin-free medium was added (medium dilution is 1:100 after addition of 5 ml medium to 50 µl residual medium and cells). Growth was monitored at 3, 6, 9, and 21 hours after Rapamycin-removal.

Experiment 3

U937 Cells: R1A Line Day 4.5 log-phase cells were checked for viability. They were diluted 1:4 to ~5.5 x 10E4 Cells/ml in 20% FBS Iscove's, 1% Gln, 1% FGZ. One ml of cells was inserted into the 1st well in 8 well series of 48 well plate. Subsequent 7 wells received 0.5 ml of cells. Well 1 received 8 µl of 4 µM Rapamycin in 95% EtOH. Serial transfer of 500 µl from 1st well through 7th well gave 32, 16, 8, 4, 2, 1, 0.5 nM Rapamycin concentrations. The last well received no transfer (untreated/control U937 cells). Second to last well (0.5 nM) had 1.0 ml cells and not 500 µl. Cells were counted with Coulter Counter after 72 hours.

Stem Cells: Cells were freshly prepared by Dr. Prabal Banergee, Upstate Medical School, June 26, 2009. They were grown in Stem Cell Expansion Medium + 1% FGZ + 1% Gln + 5% FBS for four days and checked for viability. Cells were diluted 1:10 with Stem Cell Expansion Medium + 5% FBS + 1% Gln, 1% FGZ to ~ 5 x 10E4 Viable Cells/ml. Three days later they were Rapamycin-treated in 7 wells as for U937 cells. Rapamycin treatment was the same as for U937 cells. U937 and Stem Cells were counted with a Coulter Counter after 72 hours.

Results/Discussion

Experiment 1

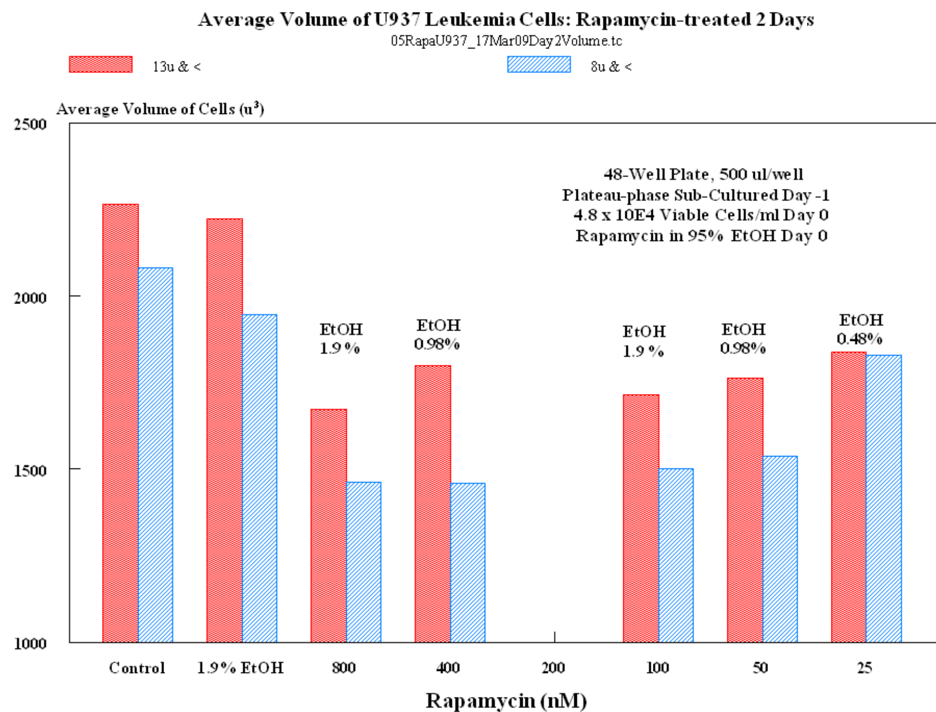
We wanted to determine the dose-response effect of Rapamycin on HSC and U937 human leukemia cells over the Rapamycin range from 800 nM to 25 nM. Table 2 lists the effect of Rapamycin treatment on cell size (average cell volume) of HSC and U937 leukemia cells.

Table 2. The effect of 800 to 25 nM Rapamycin concentrations on cell size inhibition of U937 leukemia cells and HSC.

Rapamycin	U937 Avg. Volume		HSC Avg Volume	
Conc (nM)	**8u & <**	**% inhibition**	**8u & <**	**% inhibition**
0	2083		900	
800 (1.9% EtOH)	1462	30	872	3.1
400 (0.98% EtoH)	1458	30	715	21
200 (0.48% EtOH)	N/A	N/A	645	28
100 (1.9% EtOH)	1500	28	682	24
50 (0.98% EtoH)	1536	24	619	31
25 (0.48% EtOH)	1829	12	698	24

Figure 6a shows the effect of 800 nM and 400 nM Rapamycin on the size distribution of early log U937 cells 2 days after Rapamycin treatment. 200 nM results are not included due to an experimental error.

Figure 6a. The effect of Rapamycin on average cell volume of U937 cells two days after treatment. Red bars represent average volume of cells 13μm and higher. Blue bars represent the average volume of cells 8μm and higher.

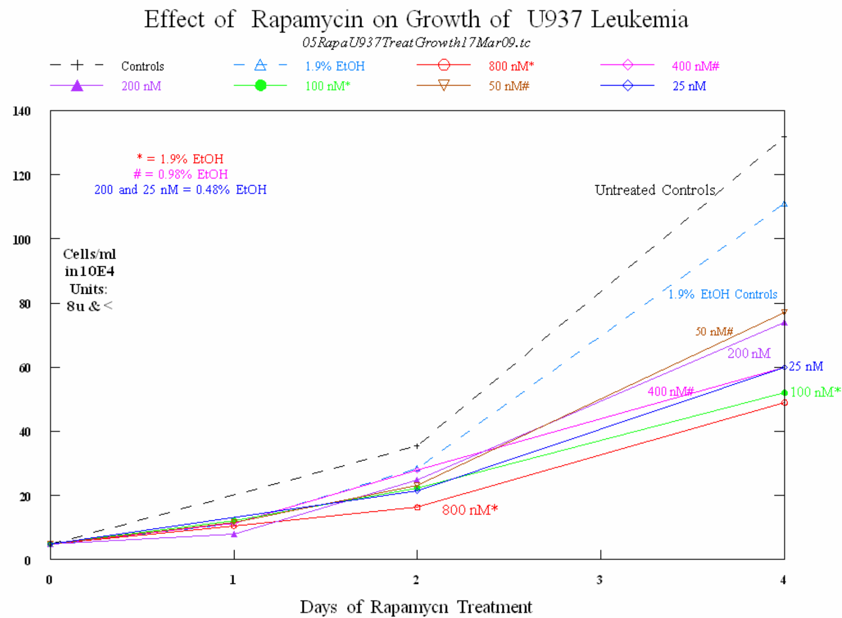


Average cell volume of the U937 cells decreased. When compared with control cells, 800 and 400 nM Rapamycin concentrations decreased average cell volume by 30%. The 100 nM concentration inhibited cell size by 28%. The 50 nM Rapamycin inhibited cell size by 24%. The 25 nM Rapamycin concentration inhibited cell size by 12%. EtOH treated cells showed slight size inhibition, about 6.5%.

Table 2 and Figure 6a both show the predicted response of human leukemia cells to Rapamycin in terms of cell size. All bar graphs in Figure 6a show cell size inhibition. At this point, it appeared that lower Rapamycin concentrations affected cell size less than did the higher Rapamycin concentrations.

We also checked the effect of Rapamycin on cell proliferation.

Figure 6b. Linear growth graph representing the effect of Rapamycin on cell proliferation of U937 leukemia cells.



As figure 6b shows, Rapamycin appears to have an effect on cell proliferation. The data at this point show no concentration-dependent effect of Rapamycin on cell proliferation since 800 nM and 100 nM cells were inhibited the most. This may be due to the different concentrations of EtOH that were used to dissolve Rapamycin. Proliferation was inhibited by about 50% to 75% but the cells still continued to proliferate. U937 cells can "grow through" the Rapamycin cell enlargement block.

Figure 6c. Exponential curve graph showing the effect of Rapamycin on cell proliferation of U937 leukemia cells.

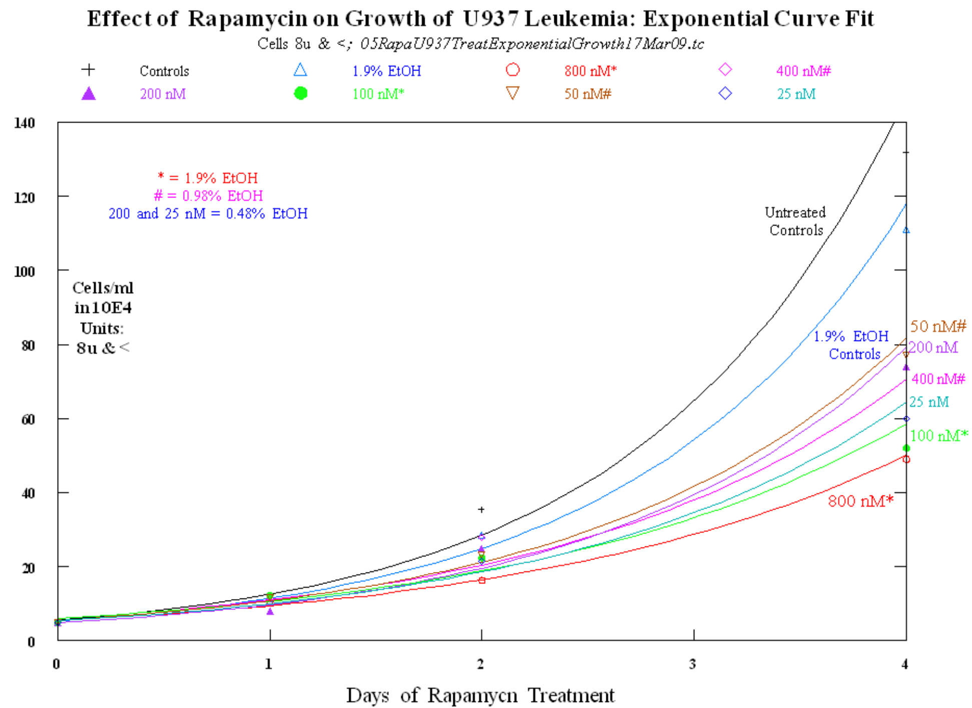


Figure 6c shows an exponential growth curve of the effect of Rapamycin on cell proliferation of the U937 cells. Again, there is no evidence of a dose dependent effect of Rapamycin on cell proliferation of U937 leukemia cells.

Next we wanted to see whether human stem cells show a similar trend, and we wanted to establish the dose response of HSC to Rapamycin.

Figure 6d. The effect of Rapamycin on the average volume of HSC two days after Rapamycin Treatment. The blue bars represent the average volume of cells 8µm or higher.

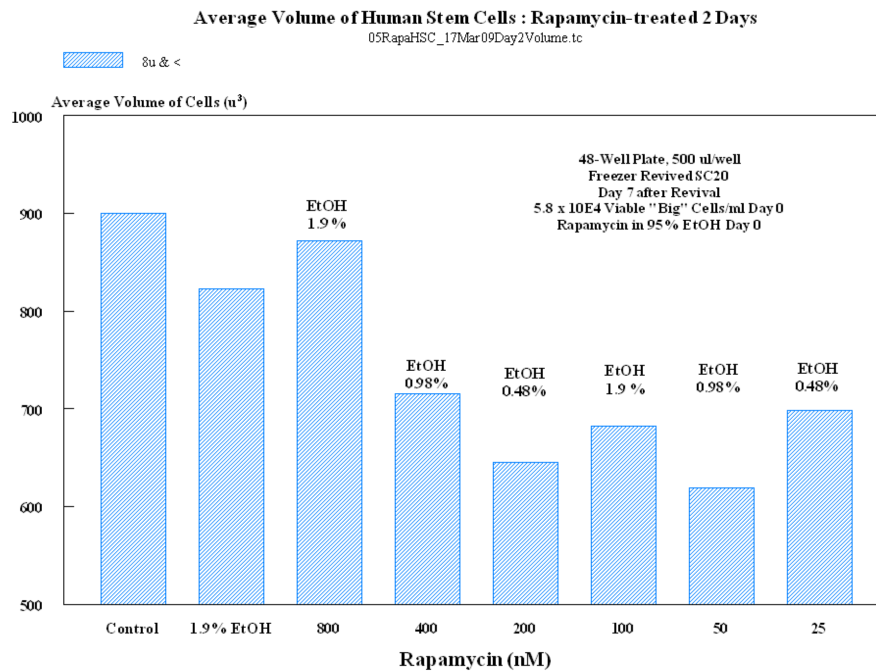
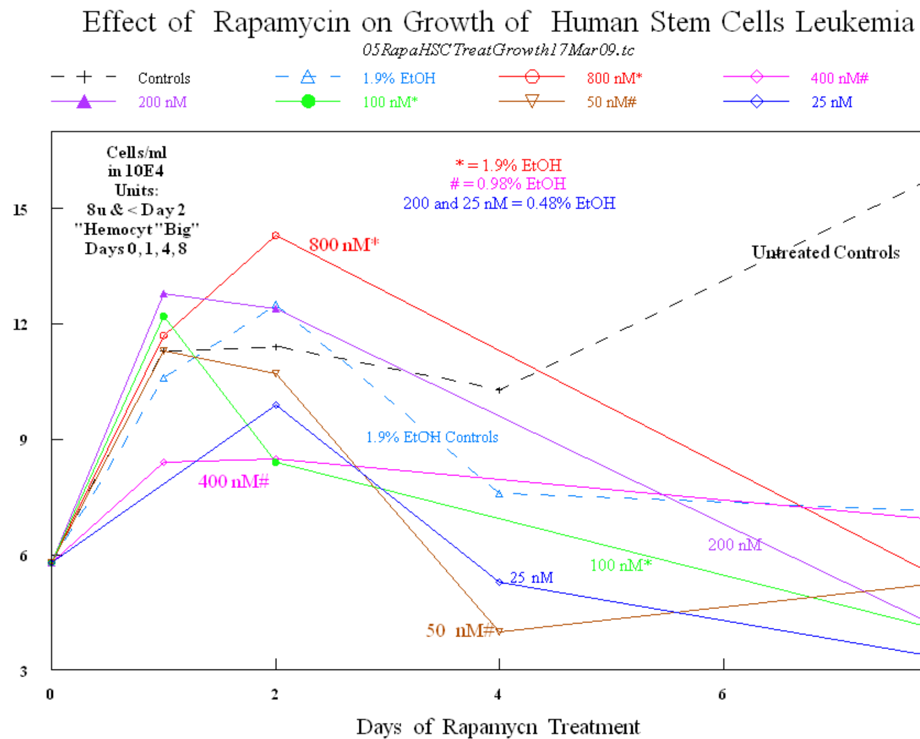


Figure 6d shows that HSC treated with Rapamycin become smaller. EtOH control cells had reduced cell volume by 8.5% as compared with non-EtOH control cells. As table 2 lists, the 800 nM concentration inhibited cell size by 3.1%, 400 nM concentration inhibited cell size by 21%, and 200 nM concentration inhibited cell size by 28%. Rapamycin at 200 nM actually reduced cell volume more than the 400 nM or 800 nM dose. It appears that 800 nM did not affect cell volume at all.

The 100 nM Rapamycin concentration inhibited cell size by 24%. The 50 nM concentration inhibited cell size by 31%. The 25 nM concentration inhibited cell size by 22%. Rapamycin at 50 nM reduced HSC cell volume more than any of the other Rapamycin concentrations did. There was no evidence of a dose dependent response of HSC to Rapamycin concentrations.

Figure 6e shows the effects of Rapamycin on cell proliferation.

Figure 6e. Effect of 800 nM to 25 nM Rapamycin on cell proliferation of HSC.



At day 2 of Rapamycin treatment, all Rapamycin concentrations permitted slight cell proliferation. However, cell proliferation was markedly reduced by day 8 of Rapamycin treatment. The cell number went down noticeably by day 8. This could be due to cell death or cell differentiation. Rapamycin removal experiment should be performed to establish toxicity of Rapamycin for both HSC and U937 cells.

From this experiment we concluded that 1.9% EtOH itself affects cell proliferation and cell volume slightly for both U937 human leukemia cells and for human stem cells. This may be important since we used EtOH as a Rapamycin

solvent. Moreover, we used different concentrations of EtOH. Future experiment should use Rapamycin dissolved in one constant concentration and the results should be compared to these results.

Stem cells are generally much smaller in diameters and average cell volumes than are U937 leukemia cells. Our experiment shows that this characteristic is maintained despite Rapamycin treatment. Both types of cells had inhibited cell size and lower cell volume when treated with Rapamycin. No apparent differential dose-response to Rapamycin between the HSC and U937 cells was observed. Rapamycin treated stem cells showed much lower cell proliferation, as expected. U937 cells on the other hand continued to proliferate in the presence of Rapamycin.

Stem cells showed an unusual response to very high doses of Rapamycin at 800 nM by failing to be inhibited in their loss of cell volume and by continuing to proliferate slightly. Moreover, stem cells used here are only 52% trypan blue viable, growing 8 days after freezer revival. Rapamycin effects should be determined on freshly-derived stem cells never frozen, and in full viable proliferation.

Experiment 2

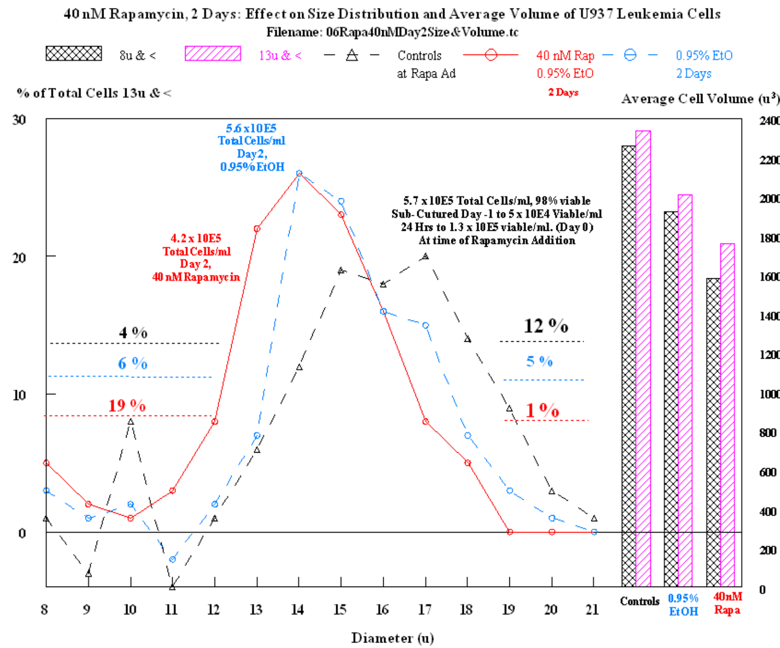
In this experiment we expanded on the previous experiment by lowering the Rapamycin concentration used. We wanted to see the effect of a 3-day treatment with Rapamycin on U937 leukemia cells over a concentration range from 40 nM to 1nM. We looked at cell size, cell proliferation and trypan blue viability. Table 3 shows the effect of Rapamycin on average cell volume of U937 cells. We also looked at the re-growth of leukemia cells after Rapamycin was removed.

Table 3. The effect of Rapamycin on average cell volume of U937 cells 2 days after treatment.

Rapamycin	U937 Avg. Volume	
Conc (nM)	**8u & <**	**% inhibition**
0	2048	
40 (0.95% EtOH)	1579	23
20 (0.5% EtOH)	1682	18
10 (0.25% EtOH)	1720	16
5 (0.12% EtOH)	1877	8.3
2 (0.05% EtOH)	1723	16
1 (0.025% EtOH)	2017	1.5

Figure 7a shows size-distribution of control cells, 0.95% EtOH treated cells and 40 nM Rapamycin treated cells at day 2.

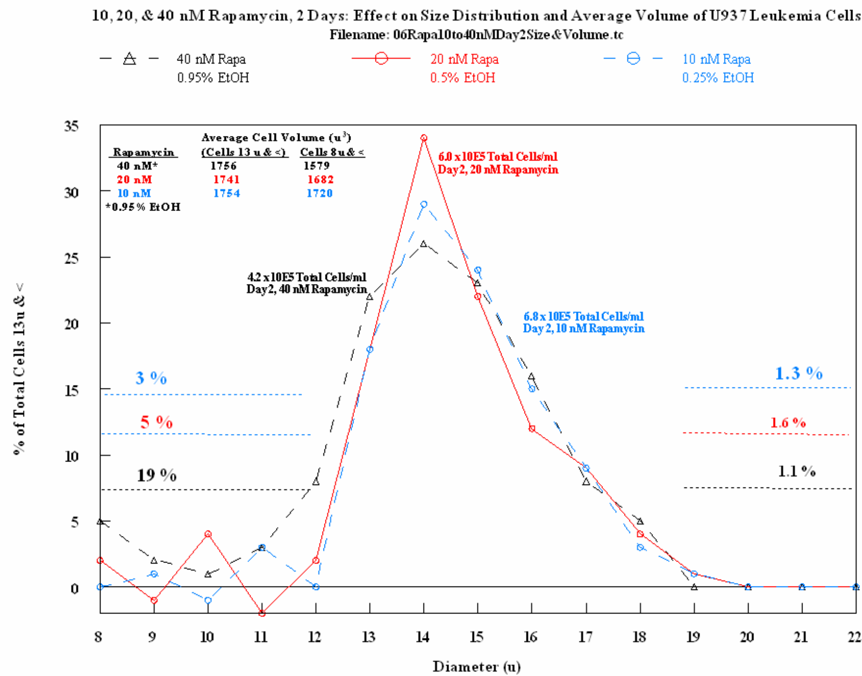
Figure 7a. Size-distribution of control cells, 0.95% EtOH treated cells and 40 nM Rapamycin treated cells at day 2.



The controls varied slightly at 2 days. The mid-log phase cells (at the beginning of Rapamycin treatment) got smaller in 2 days as they reached plateau-phase. EtOH treated cells when compared to control cells at day 0 had cell size inhibition of 15%. EtOH treated cells compared to day 2 control cells had 6.25% size inhibition. Subsequent cell size inhibition calculations use the Day 2 plateau-phase cells as control cells. The 40 nM Rapamycin treated U937 cells had 23% cell size inhibition.

Figure 7b shows the effect of 40 (repeated from above), 20 and 10 nM Rapamycin concentrations on U937 cells.

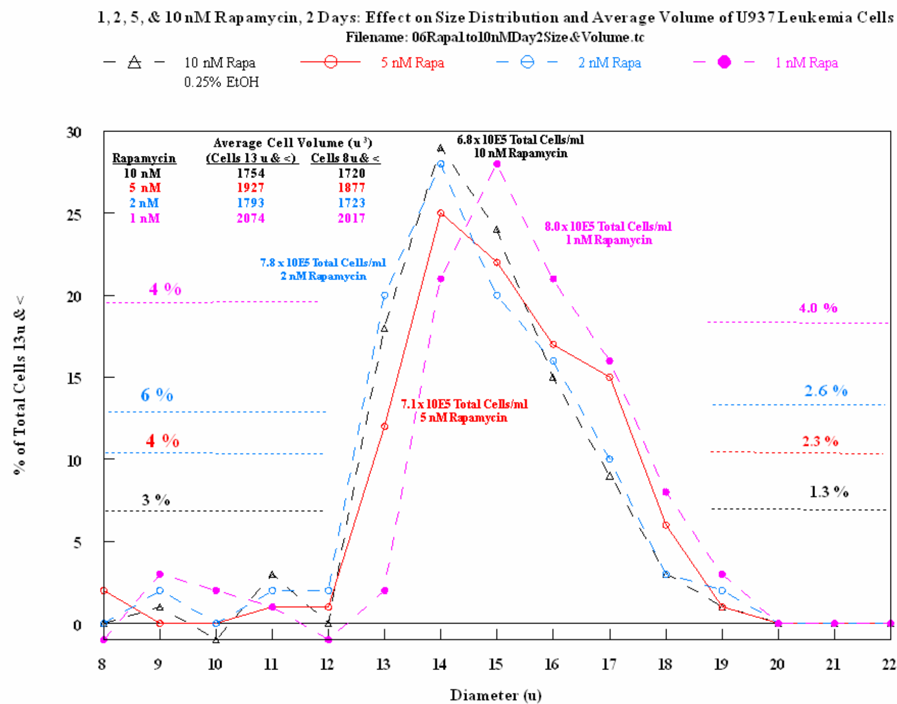
Figure 7b. The effect of Rapamycin 40, 20 and 10 nM concentrations on the cell size of U937 cells.



20 nM Rapamycin concentration showed 18% cell size inhibition. The 10 nM Rapamycin treatment showed 16% cell size inhibition.

Figure 7c shows Rapamycin concentration range of 10, 5, 2, and 1 nM at day 2 of treatment.

Figure 7c. The effect of Rapamycin concentration range of 10, 5, 2, and 1 nM at day two of treatment on the average cell volume of U937 leukemia cells.

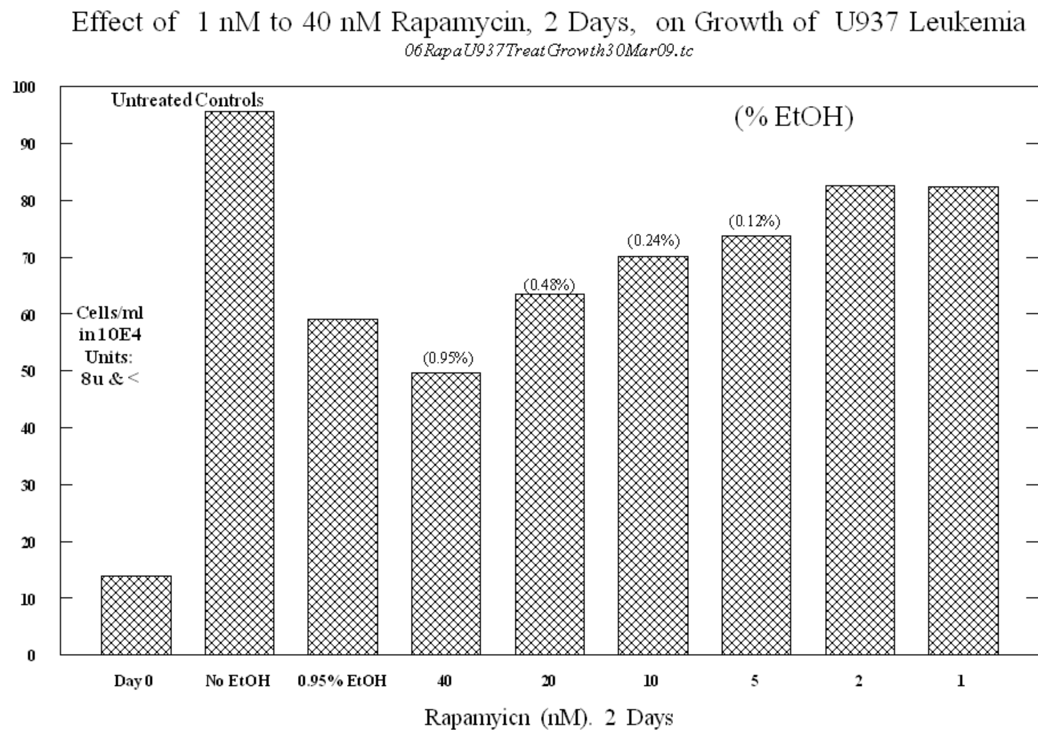


5 nM Rapamycin inhibited cell size by 8.3%. The 2 nM inhibited cell size by 16%, much more than the 5 nM dose. The 1 nM treatment inhibited cell size by 1.5%. It appears that somewhere between 1 nM to 5 nM Rapamycin begins to allow for cell enlargement.

The 2 nM concentration result is difficult to interpret because it might be due to experimental error. The 2 nM treatment should be repeated for both HSC and U937 cells to see whether Rapamycin affects cell size of HSC more strongly than of U937 cells. The 1 nM Rapamycin did not seem to affect the U937 cells.

Figure 7d shows the effect of Rapamycin on cell proliferation of the U937 leukemia cells.

Figure 7d. The effect of 40 nM to 1 nM Rapamycin on cell proliferation of U937 cells two days after treatment.



Cell proliferation was inhibited at 40 nM to 1nM Rapamycin concentrations. Higher Rapamycin concentrations (40 nM) inhibited cell proliferation more strongly than did lower Rapamycin concentrations (1 nM). However, Rapamycin continued to permit cell proliferation. Rapamycin appears to not prevent U937 cells from proliferating.

After 3 days of treatment with 20 nM Rapamycin, removal of 99% of the Rapamycin (50 µl remaining after centrifugation and removal of Rapamycin-

medium, and suspension in 5 ml of medium) cells showed re-growth and increase in average volume between 9 and 21 hours after Rapamycin removal. Rapamycin appeared to have no toxicity on the treated cells.

We concluded that 0.95% EtOH affects cell proliferation and cell volume significantly, and should be taken into consideration when performing other experiments.

Rapamycin shrinks cell volume and inhibits proliferation at 40, 20, and 10 nM. It continues to have an effect at 5 nM and 2 nM concentrations. However, the effect of Rapamycin at concentrations 40 to 1 nM on cell size and proliferation is less strong than the effect of Rapamycin at concentrations 800 to 25 nM.

U937 cells can increase in size and can proliferate in the presence of 1 nM Rapamycin. It is important to determine the does-response limit of human stem cells and compare it to the U937 cells.

In addition, Rapamycin can be removed from cells treated with 20 nM Rapamycin and cells can begin to proliferate after 9 hours in Rapamycin-free medium (residual Rapamycin = 0.2 nM).

Experiment 3

In this experiment we compared the Rapamycin dose-response of HSC and U937 cells over the concentration range 32 nM to 0.5 nM. This time we used freshly derived human stem cells that we obtained from Dr. Prabal Banerjee, Upstate Medical School.

Stem cells used for Rapamycin treatment were Day 7 growth cells, 65% trypan-blue viable. They were grown in a multi-well plate with Rapamycin for 3 days from Day 7 to Day 10. In untreated control cells viability was 97% by day 10.

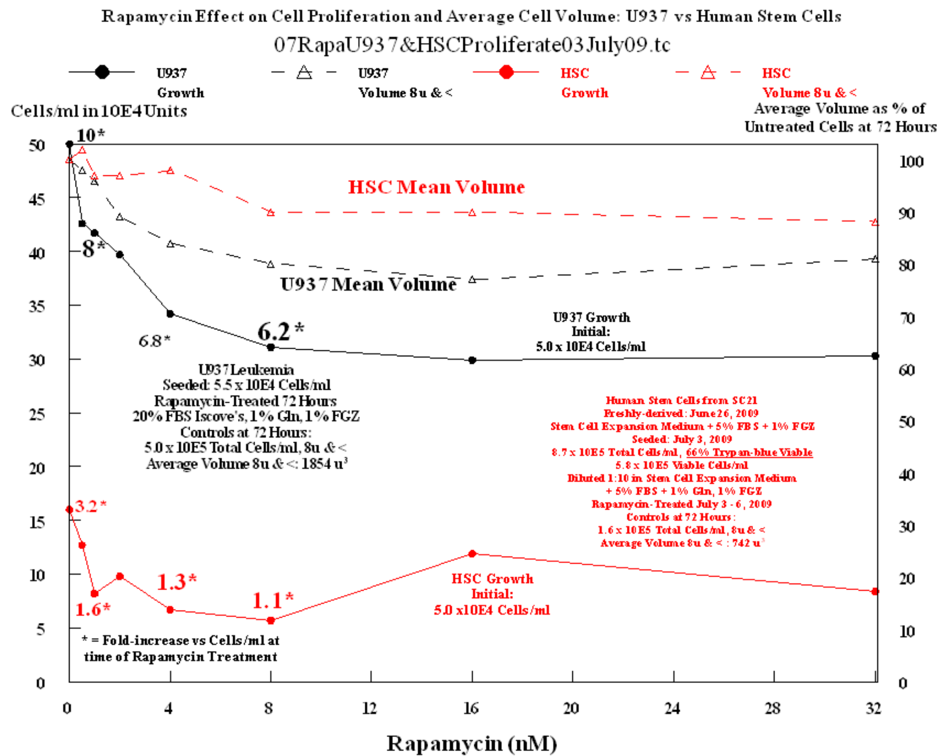
Table 4. The effect of Rapamycin on average cell volume and cell size inhibition of HSC and U937 cells.

	U937 Avg. Volume		HSC Avg Volume	
Conc (nM)	**8u & <**	**% inhibition**	**8u & <**	**% inhibition**
0	1800		742	
32	1465	19	653	12
16	1384	23	671	9.5
8	1443	20	668	10
4	1504	16	730	1.6
2	1609	11	723	2.6
1	1723	4.8	723	2.6
0.5	1759	2.3	760	-0.24

Table 4 shows the percent inhibition of Rapamycin on HSC and U937 cells on cell size. Both types of cells showed size inhibition; however, HSC cells were more strongly inhibited than were the U937 cells. The 0.5 nM concentration of Rapamycin had no effect on cell size.

Figure 8a shows the differential effect of Rapamycin on U937 cells and HSC in a graphical form.

Figure 8a. The effect of Rapamycin on cell proliferation and average cell volume of U937 cells and HSC cells.

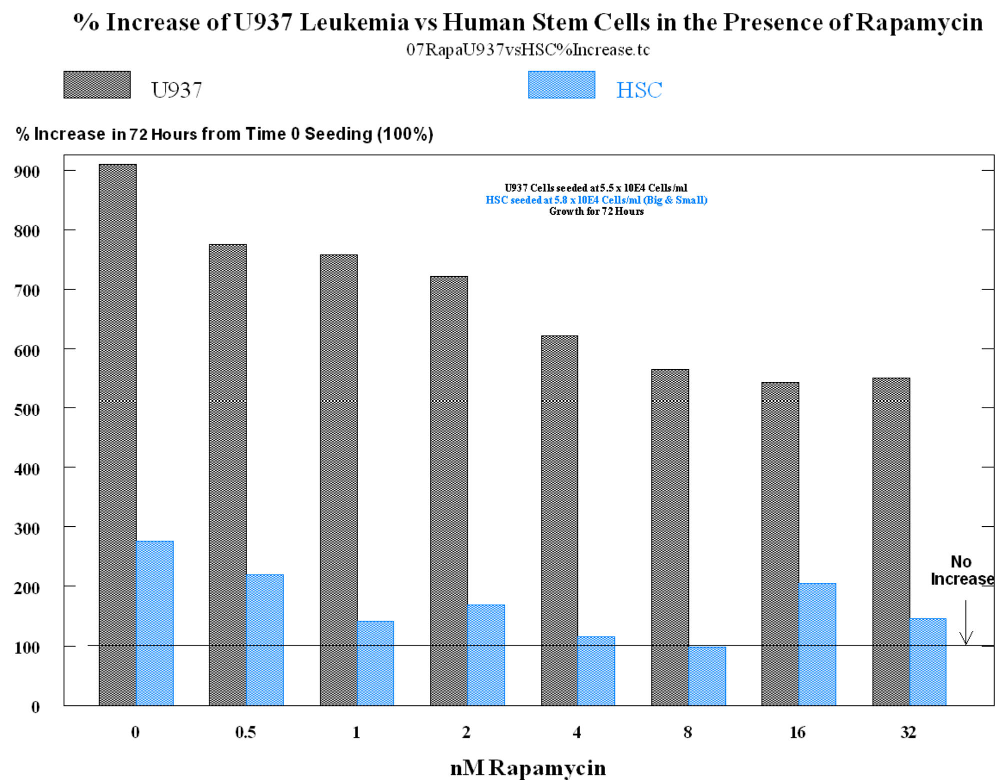


Rapamycin reduced cell volume for both U937 leukemia and HSC by about 10 to 20%. The differential dose response of U937 cells and HSC might exist in the range of 0 nM to 4 nM Rapamycin concentration. The data are inconclusive at this moment.

Cell proliferation on the other hand showed a startling result. Both figures 8a and 8b show cell growth and proliferation. As compared to a 10-fold increase of the

untreated cells, U937 cells were able to proliferate in the presence of 8 to 1 nM Rapamycin by 6- to 8 fold-.

Figure 8b. The effect of Rapamycin on cell proliferation and average cell volume of U937 leukemia cells and HSC



On the other hand, when compared to the 3-fold increase of untreated cells, HSC did not proliferate at all at 8 nM, and proliferated weakly at 2nM and 4 nM Rapamycin.

Leukemia cells were Rapamycin-treated in 20% FBS, whereas Stem Cells were treated in SCEM to allow for stem cell proliferation. If U937 leukemia cells proliferate in SCEM, Rapamycin treatment should be done in SCEM for both

Stem Cells and U937 cells to make sure that Rapamycin is not being bound by 20% FBS, reducing its effect on leukemia cells.

Rapamycin treatment should be repeated at concentrations from 8 nM, serially diluted to 4, 2, 1, 0.5, 0 25, 0.125 nM. Two sets of wells should be used for U937 and two for Stem Cells. Treatment should be in identical media allowing for growth of both stem cells and U937 cells.

Conclusions

In these laboratory experiments we wanted to establish whether there are conditions under which Rapamycin will affect HSC more strongly than U937 leukemia cells. We found that, in terms of cell enlargement, there may be a differential dose-response to Rapamycin between human leukemia cells and human stem cells to Rapamycin; however, the data are inconclusive and further analysis is required. Ultimately, we would like to see if there is a concentration of Rapamycin that will inhibit cell enlargement of normal cells while still permitting enlargement of leukemia cells by amplifying the size differences between normal and leukemia cells without additional treatments.

More startling is lack of effect of Rapamycin on cell proliferation of U937. As expected, HSC had inhibited cell proliferation. U937 cells, while inhibited slightly, continued to proliferate without increasing in size. This deserves further investigation.

Our laboratory is particularly interested in exploiting inherent or chemotherapeutically-induced differences in cell size between normal cells and cancer cells as a possible approach to the physical destruction of enlarged cells. Since Rapamycin and analogs of Rapamycin can control cell size by inhibiting cell growth, they are antagonistic to cytoskeletal-directed anticancer chemotherapeutic agents such as Vincristine, Taxol, and Cytochalasin B that can cause cancer cells to become greatly enlarged.

Data already exists that suggests that Rapamycin can synergistically enhance or inhibit chemotherapy.[3,4] We would like to see whether Rapamycin concentration will permit the leukemia cells to be trapped in an enlarged state by cytoskeletal-directed agents that trap dividing cells in mitosis or at cytokinesis while normal cells remain out of the cell cycle and remain small. Moreover, cytoskeletal-directed agents prevent cells from proliferating. Since Rapamycin inhibited cell proliferation slightly while inhibiting HSC proliferation completely, the drug-drug interaction of Rapamycin and cytoskeletal-directed agents might be a potential subject of interest. Further experiments should determine the interaction between Rapamycin and its antagonist agents in both human leukemia cells and human stem cells.

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Appendix A

Glossary

VEGF – vascular endothelial growth factor; plays a role in angiogenesis by attracting and organizing vascular endothelial cells[2,8,9].

HIF1-α – hypoxia-inducible factor 1α; A transcription factor involved in angiogenesis, metabolism, proliferation, motility, adhesion, and survival[2,9,10].

FKBP12 – FK506 binding protein; protein folding chaperone[8,9,10].

FRB – FKBP12-Rapamycin binding domain[5].

PIKK – phosphatidylinositol kinase-related kinase family, protein family that phosphorylates other proteins to adjust their function[8,9].

PI3K – phosphatidylinositol 3-kinase; oncoprotein that phosphorylates lipids, resulting in their recruitment to the cell membrane.[2,5,8,9]

PI4K – phosphatidylinositol 4-kinase[5].

FAT and FATC – a conserved kinase domain on the C-terminus of PIKK, mediates protein-protein interaction and association[11].

HEAT sequences – amino acid sequences (varying from 30 to 60 residues) that appear in various proteins, including kinases; the motif consists of particular arrangement of A and B helices; thought to play a role in protein/protein interactions[12].

PIP2 – phosphatidylinositol-4,5-phosphate[5].

PIP3 – phosphatidylinositol-3,4,5-phosphate[5].

PTEN – phosphatase and tensin homolog[2].

PDK1 – 3-phosphoinositide-dependent protein kinase 1[9].

Akt – enzymes that are members of the serine/threonine-specific protein kinase family; involved in metabolism, proliferation and growth regulation[2,9,10].

TSC1 – Tuberous sclerosis protein 1, hamartin protein, tumor suppressor[2].

TSC2 – Tuberous sclerosis protein 2, tuberin, believed to be a tumor suppressor, stimulates
GTP-ases[2].

Rheb – Ras homolog enriched in brain, belongs to the Ras superfamily of GTP-ases, functions in cell proliferation[2].

S6K1 – S6 kinase 1, phosphorylates ribosomal 6S protein[9].

4E-BP1 – eukaryotic initiation factor 4E binding protein 1, initiate translation[9].

hVPS34 – a class III PI3K required for activation of p70 S6 kinase[5].

AICAR – 5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), an analog of AMP, is widely used as an activator of AMP-kinase (AMPK), a protein that regulates the responses of the cell to energy change[13].

REDD1 and REDD2 – hypoxia-inducible genes, suppress mTORC1[5,14].

Pol – polymerase[5].

TIF1A – tripartite motif-containing 24, also known as transcriptional intermediary factor 1 alpha, involved in regulation of transcription[8,10].

FHL1– four and a half LIM domains (Pol II associated transcription factor), involved in many cellular processes[8].

Rho1 – GTP-binding protein of the Rho subfamily of Ras-like proteins, involved in establishment of cell polarity

PKC1 – protein kinase C1, pertains to a family of serine/threonine protein kinases grouped by their activation mechanism[8].

MAP(K) – mitogen-activated protein kinase; are serine-threonine kinases that regulate a wide variety of cellular functions[8].

ATG1 – Autophagy-specific gene 1; involved in autophagy[8].

Capstone Summary

mTOR in Cell Signaling and Cell Size Enlargement as a Target for the Chemico-Physical Therapy of Cancer

In 1970, *Streptomyces hygroscopicus* bacteria were discovered in the soil samples from Easter Island.[5] *S. hygroscopicus* was found to produce a strong antifungal compound.[1] This compound was named Rapamycin after the place of its discovery.[5] In the local language, Easter Island is called Rapa Nui.[5]

At a later date the cellular protein target of Rapamycin was identified in mammalian cells.[5] The protein was named "mTOR" for mammalian target of Rapamycin.[1,5] mTOR is a kinase protein, meaning it adds a phosphate group to other proteins in order to adjust their function.[5] About 40%–60% of mTOR's primary amino acid sequence is identical to a larger family of kinases, the phosphatidylinositol kinase-related kinase (PIKK) family.[5]

mTOR is a large protein; it is about 280 kDa.[5] It is located in the cytoplasm at a convergent point of many signaling pathways, which allows mTOR to integrate the incoming signals and determine the appropriate response to these signals.[1,5]

mTOR is highly conserved across eukaryotic species.[5] In the cytosol, mTOR exists in two distinct complexes.[1,5,7] mTOR complex 1 binds specific proteins that are different from the proteins mTOR complex 2 binds.[1,5] mTOR complex 1 is responsible for determining cell's metabolism, growth, and proliferation (cell division).[1,5] It is sensitive to Rapamycin.[1,5] Recently it was discovered that mTOR complex 2 is responsible for actin reorganization.[1,5,7] Actin

is protein responsible for cell movement, shape and location. This complex is Rapamycin insensitive.[1,5,7]

Cells receive various signals such as growth factors (tell the cell when to grow), energy levels (AMP/ATP ratio), nutrient presence (presence of amino acids), and stress the cell experiences (for example, low oxygen levels).[1,5] mTOR integrates these signals and determines the appropriate response. mTOR regulates ribosome biogenesis (whether or not to form ribosomes), transcription/translation (what genes to activate and what genes to deactivate), actin assembly (to move or not to move), macroautophagy (in time of starvation to begin degrading components of self).[1,5]

mTOR has been linked with many cancers such as melanoma, lymphoma, breast cancer and others.[2] mTOR itself is not mutated in cancers usually.[2] It is all the incorrect signaling the cancer cell experiences that allows mTOR to determine an inappropriate response.[2] Cells with mTOR inappropriately activated can proceed with cell enlargement and cell proliferation in the absence of normal cell signaling.[2] Rapamycin inhibits mTOR, making it unable to perform its function.[1,5] For this reason, Rapamycin has been used in cancer therapy. For a cancer cell this means being unable to grow large and to divide.

In our laboratory we attempt to make cells larger either by synchronizing cell divisions to occur at the same time or by using therapeutic agents such as cytoskeleton-directed agents. Rapamycin is an agent that does the opposite, it makes cells smaller. We wanted to determine the effect of Rapamycin on human stem cells (normal cells) and human leukemia cells (cancer cells). Particularly, we

wanted to determine the concentration of Rapamycin that affects human stem cells (HSC) much more strongly than human leukemia cells (U937 cells). Ultimately we wanted to know if there is a concentration of Rapamycin that inhibits the cell size and cell proliferation of HSC but does not inhibit cell size and cell proliferation of U937 leukemia cells.

We performed experiments where we treated both HSC and U937 leukemia cells with various Rapamycin concentrations and observed the effects of Rapamycin on cell enlargement and cell proliferation. In experiment 1 we treated both HSC and U937 cells with Rapamycin concentrations of 800, 400, 200, 100, 50, and 25 nM. Both cell types showed cell size inhibition, meaning cells became smaller. At these concentrations there appeared to be no difference in the way Rapamycin affected HSC and U937 cells. HSC had cell size inhibited by 20% to 30%. U937 cells had cell size inhibited by 12% to 30%. However, the effect was not uniform for both cell types. For example, HSC cells were inhibited the most by 50 nM Rapamycin concentration while U937 cells were inhibited the most by 800 nM concentration.

Both cell types showed cell proliferation inhibition as well. Both cell types should have been proliferating very little in the presence of Rapamycin. Indeed, HSC proliferated much less than the U937 cells did. However, U937 cells proliferated a lot more than we expected. For U937 cells proliferation was not dose dependent either, meaning, cells treated with 50 and 200 nM proliferated more than had cells treated with 100 and 800 nM Rapamycin.

In experiment 2 we treated only the U937 leukemia cells. In the previous experiment we saw that 800 to 25 nM Rapamycin had inhibitory effect on the U937 cells. In this experiment we treated U937 cells with lower Rapamycin concentrations 40, 20, 10, 5, 2 and 1nM because we wanted to determine the concentration of Rapamycin that would have no effect on cell size. We found that concentrations as low as 1 and 2 nM had an inhibitory effect on cell size of the U937 cells.

Moreover, low concentrations of Rapamycin inhibited cell proliferation slightly. However, cells continued to proliferate despite the presence of Rapamycin. In experiment 2 we also removed Rapamycin a few days after treatment to see whether Rapamycin had any toxicity on cells growth. We found that there was no toxicity due to Rapamycin.

In experiment 3 we treated both HSC and U937 cells with Rapamycin concentrations 32, 16, 8, 4, 2, 1, and 0.5 nM and compared the effect of Rapamycin on cell size and cell proliferation of both cell types. In terms of cell size, we did not find much difference in the way HSC and U937 responded to Rapamycin. We found that even low Rapamycin concentrations had an effect on cell size even of both HSC and U937 cells. However, in terms of cell proliferation there was a distinct difference in how the cells responded to Rapamycin. HSC cells proliferated very little or not at all in the presence of Rapamycin. When compared to non treated cells, at 2 nM Rapamycin HSC cells increased by 2-fold while U937 cells increased by 8-fold. This difference in cell number increase is startling.

In summary, in terms of cell size we did not find a distinct difference between HSC and U937 cells. However, we found a startling difference in the way the cells responded to Rapamycin in terms of cell proliferation. Since Rapamycin and analogs of Rapamycin can control cell size by inhibiting cell growth, they are antagonistic to cytoskeletal-directed anticancer chemotherapeutic agents such as Vincristine, Taxol, and Cytochalasin B that can cause cancer cells to become greatly enlarged. In our laboratory we want to exploit inherent or chemotherapeutically-induced differences in cell size between normal cells and cancer cells in order to use outside physical force to preferentially destroy enlarged cells. In the future we would like to determine the effect of combination therapy of Rapamycin and cytoskeletal-directed agents. It is possible that HSC cells treated with Rapamycin and Vincristine, for example, will remain small while U937 cells will become preferentially big. These enlarged U937 cells would be subject to physical destruction while normal cells (HSC) are protected from the physical destruction by remaining small in size.