# CHARACTERIZING THE ROLE OF DEPTOR IN THE MTOR PATHWAY IN CELLULAR PROTEIN HOMEOSTASIS

An Undergraduate Research Scholars Thesis

by

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# ABSTRACT

Characterizing the Role of DEPTOR in the mTOR Pathway in Cellular Protein Homeostasis

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Understanding the dynamic balance between protein synthesis and degradation is critical in characterizing skeletal muscle growth, maintenance, or wasting. Over 160,000 people in the United States suffer from Cachexia, a condition characterized by rapid muscle wasting, and other conditions associated with muscular atrophy affect millions. Previous research has linked muscle cell growth with the mTOR {mammalian target of rapamycin} anabolic pathway; and has also shown that degradation or upregulation of DEPTOR {DEP-domain containing mTOR-interacting protein}, our protein of interest, has a direct impact on mTOR activation. Both mTOR and DEPTOR are vital for the overall health of the cell, but the interaction between these two proteins is often disrupted, favoring either muscle growth or muscle wasting. What is unclear is if this interaction is a consequence of underlying features of the cell, or a direct culprit**.** The hypothesis of the proposed study is that the rate of growth and degradation of skeletal muscle cells is a direct result of the DEPTOR/mTOR interaction. We will investigate growth and wasting of cultured skeletal muscle cells by altering the interaction between mTOR and DEPTOR via pharmacological intervention. This study will have implications on how we combat anabolically aggressive cancers, muscular dystrophy and atrophy.

# DEDICATION

This thesis is dedicated to my beloved father who passed on after battling cancer in 2017- Isaac Anjorin, my loving mum– Grace Anjorin, supporting engineer and Brother – Michael Anjorin; fearless sister – Gloria Anjorin who has chosen to embark on a journey to become a nurse practitioner, and supporting Johnson family for their unwavering support throughout this academic journey.

# ACKNOWLEDGMENTS

I will like to start by thanking Jesus Christ, my personal Lord and Savior for his grace and mercy during this part of my academic journey.

Special thanks to my advisor and mentor Dr. Fluckey for believing in me and supporting me during this project. I will also like to extend my gratitude to all members of the Fluckey Lab especially Ph.D. candidates Selina Uranga, Collin O'Reilly, Will Deaver, Patrick Ryan, Jessica Cardin who are all contributors to this project.

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I will also like to extend my gratitude to my friends, working colleagues, and role-model faculty members in the College of Biomedical Sciences & Veterinary Medicine; Dr. Maria Esteve-Gassent, Dr. Ken Turner, Dr. Keely Young and staff for making my time as a teaching assistant a great experience at Texas A&M University.

Finally, a big thank you to everyone that participated in my journey thus far; if it wasn't for you all, I wouldn't have made it this far!

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# NOMENCLATURE

| | |
|---|---|
| AKT | Protein Kinase B |
| AMPK | AMP dependent Kinase |
| ATG4B | Autophagin-1 |
| BTrCp | Beta-Transducin repeat-Containing protein |
| CK1alpha | Casein Kinase 1-alpha |
| DEPTOR | Domain-Containing mTOR-interacting Protein |
| EGF | Epidermal-like Growth Factor |
| GF | Growth Factor |
| IGF | Insulin-like Growth Factor |
| MAPK | Mitogen-Activated Protein Kinase |
| MLN4924 | Millennial Compound-4924 |
| mLST8 | Mammalian Lethal with SEC13 protein 8 |
| MTOR | Mechanistic Target of Rapamycin |
| NSC-185-058 | N-2-Pyridinyl-2-Pyridinecarbothioamide |
| PI3K | Phosphatidylinositol 3-kinase |
| S6K1 | Ribosomal protein S6 kinase beta-1 |
| TSC | Tuberous Sclerosis Complex |
| 4EBp1 | Eukaryotic translation initiation factor 4E-Binding protein 1 |

# CHAPTER I

# INTRODUCTION

## Project Significance

Millions of people globally suffer from muscular dystrophies and atrophies either due to lack of exercise, genetics, malnutrition, or illnesses. For example, Cachexia is an illness characterized by severe muscle loss. It is also a major clinical concern for physicians because muscle wasting is often associated as a prognostic parameter for cancer, and have a negative impact on heart muscle cells- cardiomyocytes (6). Physicians are not the only ones concerned about muscular atrophy. Researchers from NASA are also very concerned, publishing a report in 2017 stating astronauts lose an average of 20% or more skeletal muscle mass during spaceflights that last more than five days (4), causing extreme discomfort. The good news is muscle mass can be regained by dietary therapy, and muscle strength by exercise. However, this will always be a major concern especially for longer space missions. mTOR {mammalian target of rapamycin} is a protein signaling kinase that regulates cell growth, and many other vital cellular functions. Alternatively, while mTOR hyperactivation in the cell typically promotes the growth of cell, an uncontrolled mTOR hyperactivation leads to cancer cell proliferation/metastases in a number of cancer cell types and a number of metabolic abnormalities in skeletal muscle. Many studies have confirmed that the interaction between mTOR and the gene coding protein- DEPTOR {DEP-domain containing mTOR-interacting protein} has a profound influence on mTOR activity in the cell (2). During an anabolic event in the cell, upstream signals lead to the activation of mTOR, which then leads to protein synthesis, and the anabolism in the cell. Once this upstream signal reach mTOR, a major step in full activation is mTOR 'tagging' DEPTOR for degradation via a

mTOR dependent degradative pathway, which allows for mTOR to be fully activated when its binding partner has been destroyed. More will be discussed regarding the mTOR anabolic pathway, but till date, it is not known if the silencing of upstream mTOR signaling is sufficient to restore normal protein homeostasis, or is the rescuing/restoring DEPTOR by preventing mTOR from 'tagging' it for degradation the central regulator of this pathway.

The Muscle Biology Lab at Texas A&M University, under the direction of Dr. Jim Fluckey, has a strong interest in muscle protein homeostasis under a variety of conditions including; advancing age, disuse, overload, and cancer. Our group has used the pharmacological administration of upstream mTOR inhibitors, called rapalogs, in cancer cells, to determine the impact of mTOR activation on cellular proliferation and growth. The implementation of these rapalogs resulted in a statistically significant suppression of proliferation and cellular protein synthesis, likely due to a reduced mTOR activation and subsequent anabolic signaling (1,30). During this pharmacological intervention experiments, it was discovered that when mTOR is suppressed, DEPTOR is significantly upregulated in cancer cells. This discovery further supports DEPTOR's involvement in mTOR activation (1). However, at this time, it cannot be determined if the reduction of anabolism in these cells was due to the upstream silencing of mTOR or the rescue of DEPTOR in the cells. It should also be noted that the use of rapalogs, although promising, has only marginally (albeit statistically) been successful in the treatment of anabolically aggressive cancers. This could potentially be caused by an observed inability to adequately express DEPTOR protein. Therefore, our underlying hypothesis is that the interaction between DEPTOR and mTOR dictates cellular protein homeostasis, regardless of upstream cellular influences over mTOR. This is also consistent with observations from our lab that

indicate that cellular protein homeostasis is restored *only* when DEPTOR expression has been recovered in the cell.

**The MTOR Anabolic Pathway**

MTOR is a well-known protein kinase that regulates many cellular functions such as cell growth, proliferation, differentiation, autophagy, apoptosis and recently important roles in conferring immunity (16,25).

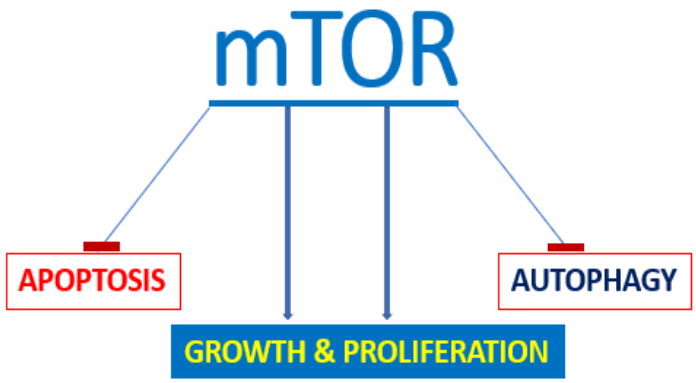



Figure 1. General mTOR Signaling Pathway.

In recent years, the MTOR protein kinase has been more referenced in clinical scenarios due to its significant role in promoting tumor/ cancer growth and metastasis (25).
MTOR catalyzes the phosphorylation of many regulatory proteins that lead to protein synthesis via a series of signal transduction (25). Here are a few important proteins that will be discussed in greater detail which are regulated by MTOR activity: p70S6K1, 4E-Bp1, AMPK, and DEPTOR to mention a few.

MTOR has been biochemically characterized to have two complex major pathways; MTORC1 complex and, MTORC2 complexes. Although both MTOR complexes have a commonly shared genetic component known as mLST8(Mammalian Lethal with SEC13 protein 8) (25). The MTORC1 and MTORC2 complexes are intricately intertwined with regulatory

proteins that sometimes function to counteract each other's activity. To briefly summarize these individual complexes, it is important to note that the function of these complexes is majorly intertwined with very specific yet different activation processes. Furthermore, it is important to note that there are still many unknown factors and information regarding this pathway yet to be discovered.

*The MTORC1 complex* is well established for its role in catalyzing the phosphorylation of protein kinases responsible for protein synthesis that result in cell growth and proliferation. (Sabatini) Thus, this complex is responsible for the phosphorylation of RAPTOR, AKT substrate (PRAS40), and DEPTOR — our protein of interest. In addition, the MTORC1 complex can be activated through three different mechanisms; growth factor (GF), cellular energy ratio, and amino acids (16).

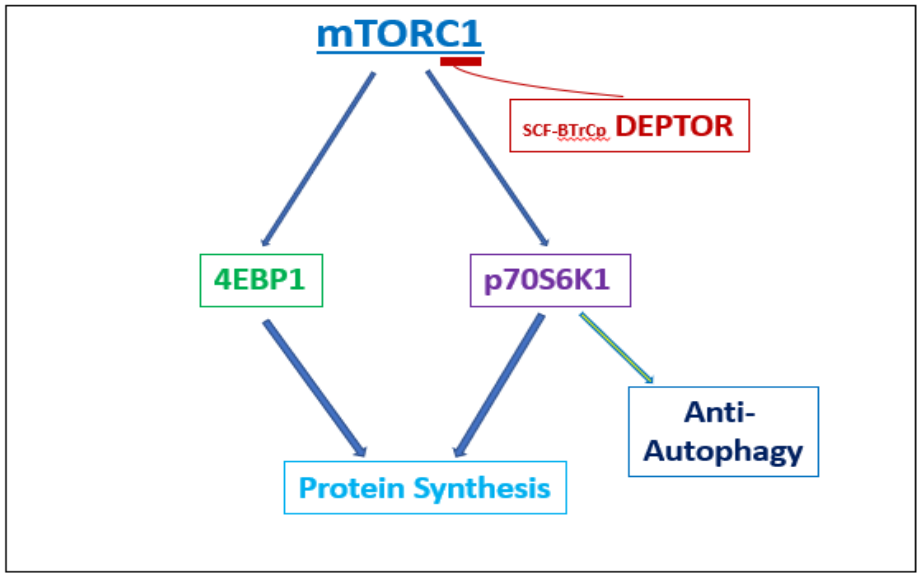



Figure 2. mTORC1/ DEPTOR Signaling

*The MTORC2 Complex* has more implications in actin polarization and endocytosis (16). However, this complex pathway has been hypothesized to have multiple mechanisms due to activation/phosphorylation cites at multiple locations.

*A Potential Alternative MTOR Pathway*

MTORC1 complex facilitates protein synthesis and regulates anabolism of a cell. However, mTOR might also be responsible for intentional dual-activation to regulate protein synthesis in kinases, which favor its activation and inhibit proteins that alter its activation either via feedback or a noncanonical pathway. According to a randomized study on the mTOR anabolic pathway, mTORC1 phosphorylates a series of Ubiquitin Ligases to inhibit SKP2 ubiquitination leading to specific degradative pathway of some proteins (16, 25,26).

This mTORC1 activity feature is no surprise to researchers that study the dynamic protein kinase, as many hypothesize the necessity of mTOR to potentially have multiple alternative pathways. This systematic mechanism is brought up in this manuscript due to our underlying hypothesis, and on-going research that indicates that mTOR tags specific proteins leading to their degradation. In our case, our protein of interest is DEPTOR, and this protein is degraded by a mTOR dependent BTrCp (Beta-Transducin repeat-Containing protein) activation.

**DEPTOR: Our Protein of Interest**

DEPTOR is an intriguing protein that functionally inhibits mTOR (25). Generally, DEPTOR is in abundance and acts as a tumor suppressant. Ergo, in its abundance, it can act to inhibit mTOR dependent cellular activities that lead to protein synthesis, and ultimately reduced anabolism in the cell (25). This is consistent with our understanding that mTOR is highly activated in cancer cells while DEPTOR activity is relatively low. We embrace the hypothesis that mTOR activity is highly involved in the degradation pathway of DEPTOR. Many factors can influence the degradation of DEPTOR; however, the mTOR dependent BTrCp activation stands out. BTrCp is an important gene coding protein and member of the F-Box protein family.

In addition, it is one of the four subunits of a ubiquitin protein ligase known as SCF and is often referred to as SCF-BTrCp ubiquitin ligase in scientific literature (27,28).

This complex functions by ubiquitinating via phosphorylation. Furthermore, in a study done on mitogens (28), a mTOR dependent activation of S6K1 and RSK1 suggests that phosphorylation of these proteins activates CK1-Alpha. CK1-Alpha (Casein Kinase 1-alpha) is a protein that functions in the activation of the BTrCp complex, which leads to the ubiquitin proteasomal pathway degradation of DEPTOR (28). This phenomenon becomes very important when we are discussing our pharmacologically inhibitory approach in this study (28).

**Specific Aim**

My underlying hypothesis is that the rate of growth and degradation of skeletal muscle cells is a direct result of the DEPTOR/mTOR interaction, regardless of upstream cellular influences on mTOR. Thus, this study is geared towards investigating growth and wasting of cultured skeletal muscle cells by altering the interaction between mTOR and DEPTOR via pharmacological intervention.

**Pharmacological Inhibitors**

*NSC-185058*

The NSC 185058 specific isotype of NSC compound discovered from a diverse library of NSC compounds is considered to be an anti-autophagic drug. Autophagy is often referred to as a necessary intracellular degradative pathway important to maintain cellular homeostasis (19). Everything in life requires balance; thus, to cause growth or retardation there are specific cell signal transductions that either lead to protein synthesis, or lead to protein degradation. Furthermore, autophagy can be induced in response to cellular environmental changes like, cell stress, or starvation (19). Consequently, mTOR is a negative regulator of autophagy (22).

The NSC 185058 compound functions by inhibiting ATG4B (Autophagin-1) (5,6). ATG4B is a specific cysteine protease molecule that induces autophagy (8,9).

The major autophagic protein responsible for this activity is LC3B (microtubule-associated protein 1 light chain 3B). This protein is strongly associated with continuous tumor growth in anabolically aggressive pancreatic, and breast carcinomas (17).

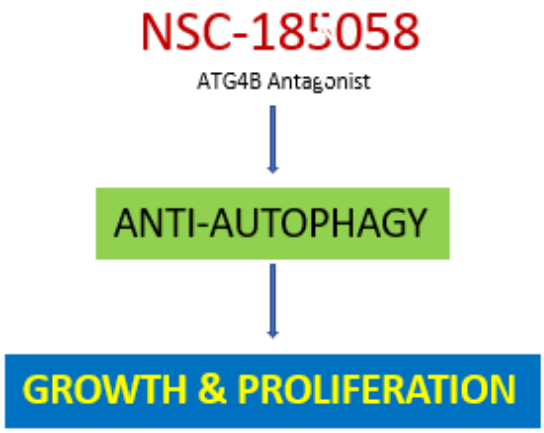



Figure 3. General NSC-185058 Functional Pathway

However, like many novel molecules being studied, its expression and direct mechanism in link with the mTOR complex is not yet fully understood (9). Autophagy is a key pathway for cell survival during starvation; however, this becomes a major problem with cancer treatments because autophagy can promote cell survival in tumors during chemotherapy or nutrient depletion (20).

*MLN-4924*

MLN 4924 works by inhibiting NAE (NEEDD8 Activating enzyme), which inactivates CRL (Cullin-RING E3 ubiquitin Ligases), blocking cullin neddylation (23). Simplistically, the MLN drug induces accumulation of substrates from CRL. In addition, one of the most dominant

substrates which accumulate in a cellular system as a result of this drug, is DEPTOR in cancer cells (23).

Suggested Pathway

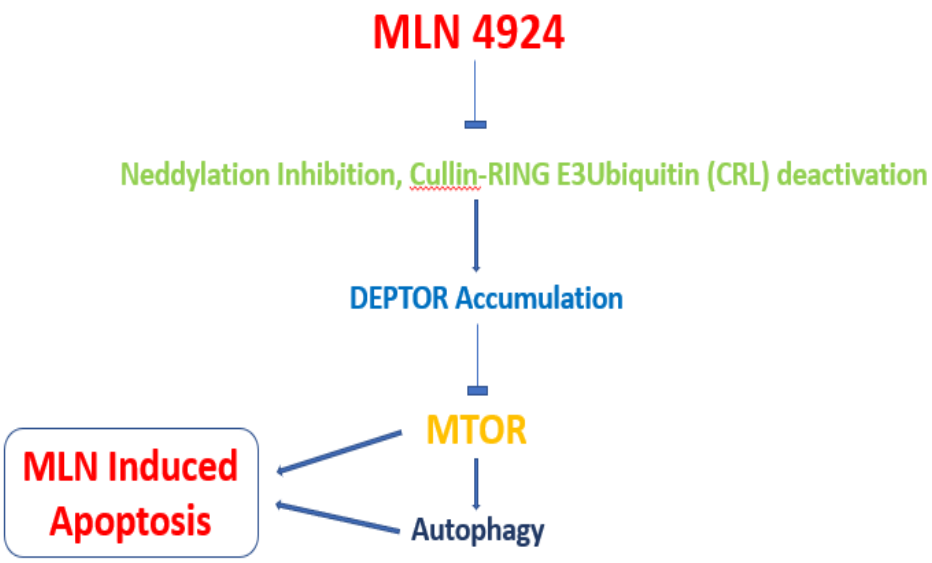



Figure 4. MLN4924 Signaling Pathway

Furthermore, DEPTOR is necessary for MLN induced apoptosis of a cell (23). However, there is no reported statistical data that suggests that DEPTOR alone is enough to mitigate this response. Moreover, since it has been established that the accumulation of DEPTOR causes retardation in tumor growth via the MLN induced apoptosis mechanism, this pharmacological interventive drug still supports our underlying hypothesis.

As things get more intriguing with our result on muscle cells, it is important to note that the cellular biochemistry and cell density of muscle cells and cancer cells are very different and could be a factor when comparing result outcomes.

# CHAPTER II

# METHODOLOGY

## Cell Culture

L6 myoblast were grown with growth media (DMEM-Dulbecco Modified Eagle Medium) (Corning, Mediatech INC., Manassas, VA) composed of 20% Fetal Bovine Serum(FBS) from (VWR International, Randor, PA), all pressure-filtered aseptically with 0.2Um filters. Cells were cultured and maintained in 6-well sterile plates and 10cm cell culture plates (Corning Inc., Corning, NY) with 10ml of growth media. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 until 60-70% confluency.

Cells at 60-70% confluency were differentiated to myotubes with a prepared differentiation media (DMEM- Dulbecco Modified Eagle Medium W/O Sodium pyruvate) (Corning, Mediatech INC., Manassas, VA) composed of 10% Horse Serum (VWR International, Randor, PA) and 1% Penicillin/Streptomycin antibiotic from (BioVision, Milpitas, CA) all pressure-filtered aseptically with 0.2Um filters. Pharmacological Inhibitory drugs for mTOR/DEPTOR to induce alterations on gene expression and protein synthesis was assessed and approved by compliance entities at Texas A&M University. The NSC 185-058 drug was applied at concentrations of 0.5Um and 1.0Um during a 24hour exposure period with DMSO (Dimethyl sulfoxide) controls. The MLN 4924 was applied at concentrations of 0.5Um and 1.0Um during a 24hour exposure period with DMSO (Dimethyl sulfoxide) controls. Both independent cell samples were harvested using cell scrapers (VWR International, Randor, PA) in 1.5ml of cold Phosphate Buffer Solution (PBS) (VWR International, Randor, PA) into 2ml microcentrifuge tubes (VWR International, Randor, PA), decanted using a centrifuge at 1000rpm

for 5minutes, supernatant were discarded and cell pellets were snap frozen in liquid nitrogen and transferred to storage at -80°C for future analysis. Cells were thawed, and then lysed in Norris Buffer kit (VWR International, Randor, PA) for protein extraction. Cytosolic protein components were separated from membrane proteins with intense centrifugation of 14000rpm for 30mins, supernatants were kept for immunoblotting.

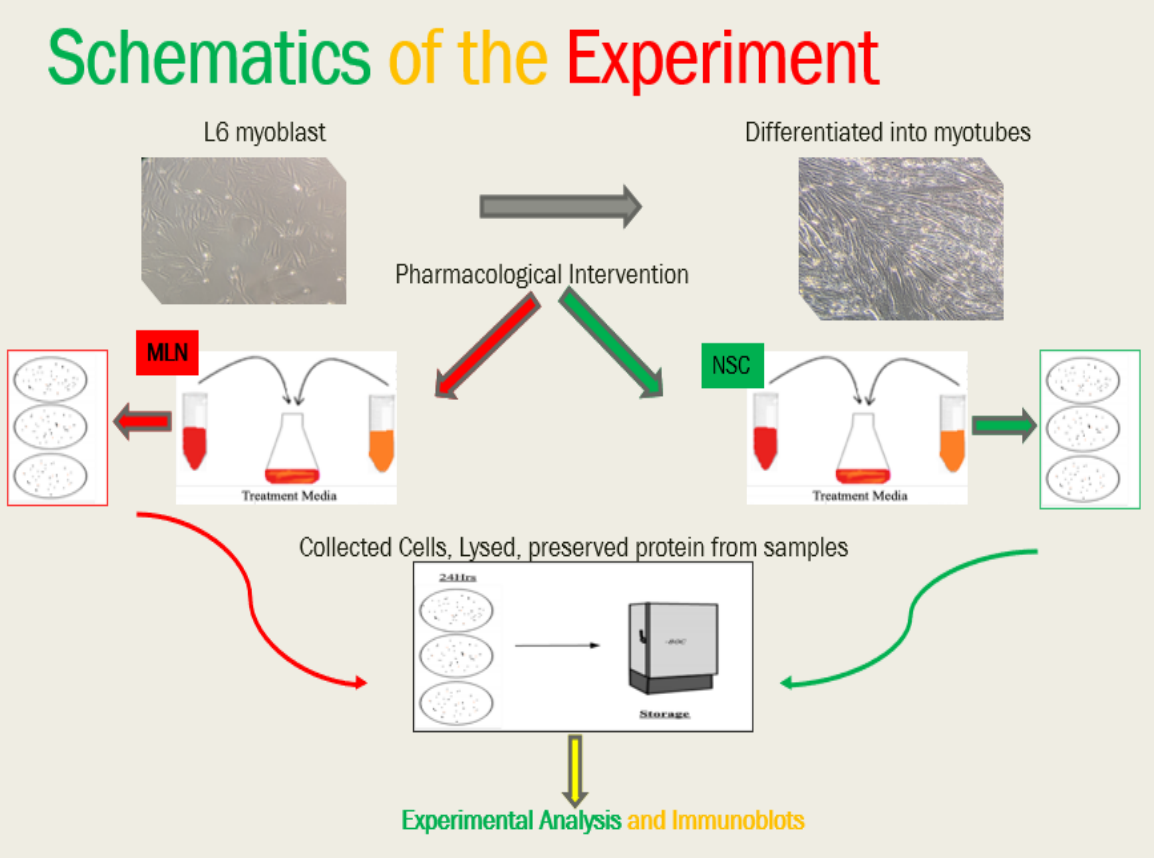


Figure 5. Experimental Schematic

**BCA Assay**

Bicinchoninic Acid Assay Kit (VWR International, Randor, PA) was used to investigate protein concentration of various sample sets. BCA reagents were used in a 96 well plate format (description shown below). Cytosolic cell component was diluted 1:12 with Norris Buffer prior to procedure. All samples were standardized using standard samples of different concentration ranging from 0.00mg/ml to 2.0mg/ml and done in triplicates.

Table.1 BCA Set-Up for protein concentration analysis on NSC-185058

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Standard 1 | Standard 1 | Standard 1 | Sample A | Sample A | Sample A | Control 1 | Control 1 | Control 1 |
| Standard 2 | Standard 2 | Standard 2 | Sample B | Sample B | Sample B | Control 2 | Control 2 | Control 2 |
| Standard 3 | Standard 3 | Standard 3 | Sample C | Sample C | Sample C | Control 3 | Control 3 | Control 3 |
| Standard 4 | Standard 4 | Standard 4 | Sample D | Sample D | Sample D | Control 4 | Control 4 | Control 4 |
| Standard 5 | Standard 5 | Standard 5 | Sample E | Sample E | Sample E | Control 5 | Control 5 | Control 5 |
| Standard 6 | Standard 6 | Standard 6 | Sample F | Sample F | Sample F | | | |
| Standard 7 | Standard 7 | Standard 7 | Sample G | Sample G | Sample G | | | |
| Standard 8 | Standard 8 | Standard 8 | Sample H | Sample H | Sample H | | | |

Table. 2 BCA Set-Up for protein concentration analysis on MLN4924

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Standard 1 | Standard 1 | Standard 1 | Sample A | Sample A | Sample A | Control 1 | Control 1 | Control 1 |
| Standard 2 | Standard 2 | Standard 2 | Sample B | Sample B | Sample B | Control 2 | Control 2 | Control 2 |
| Standard 3 | Standard 3 | Standard 3 | Sample C | Sample C | Sample C | Control 3 | Control 3 | Control 3 |
| Standard 4 | Standard 4 | Standard 4 | Sample D | Sample D | Sample D | Control 4 | Control 4 | Control 4 |
| Standard 5 | Standard 5 | Standard 5 | Sample E | Sample E | Sample E | Control 5 | Control 5 | Control 5 |
| Standard 6 | Standard 6 | Standard 6 | Sample F | Sample F | Sample F | | | |
| Standard 7 | Standard 7 | Standard 7 | Sample G | Sample G | Sample G | | | |
| Standard 8 | Standard 8 | Standard 8 | Sample H | Sample H | Sample H | | | |

**Western Blots**

Western Blotting was done to observe and characterize expression, and activation levels of different independent proteins within the MTOR signaling pathway, both canonical and non-canonical pathways.

The following primary antibodies were applied at a 1:1000 antibody/buffer ratio: DEPTOR 4243 (Cell Signaling #11816), p70S6K1 (Cell Signaling #2114), phospho-AKT(Cell Signaling #2114), AKT (Cell Signaling #2114), 4EBp1 (Cell Signaling #); and secondary antibodies applied at variant antibody/buffer ratios as deemed necessary per protein.
Cell contents were homogenized and applied to nitrocellulose gels and compared in conjunction with molecular weight ladder from (Lonza #193837) to verify molecular size. Gels were transferred to PVDF (Polyvinylidene difluoride) blotting membrane and set at 350mA for

50minutes. All bands on membranes were normalized by Ponceau S staining prior to imaging as precaution. Membranes were imaged using an advanced SP imaging system.

# CHAPTER III

# RESULTS

## Cell Morphology

*NSC-185058*

Image results show an increase in myotube growth and proliferation post 24hour application of the NSC185058 drug at both 0.5Um, 1.0Um concentrations. Size discrepancy is suggested based on only visual observations, and not myotubular diameter, or cross-sectional area.

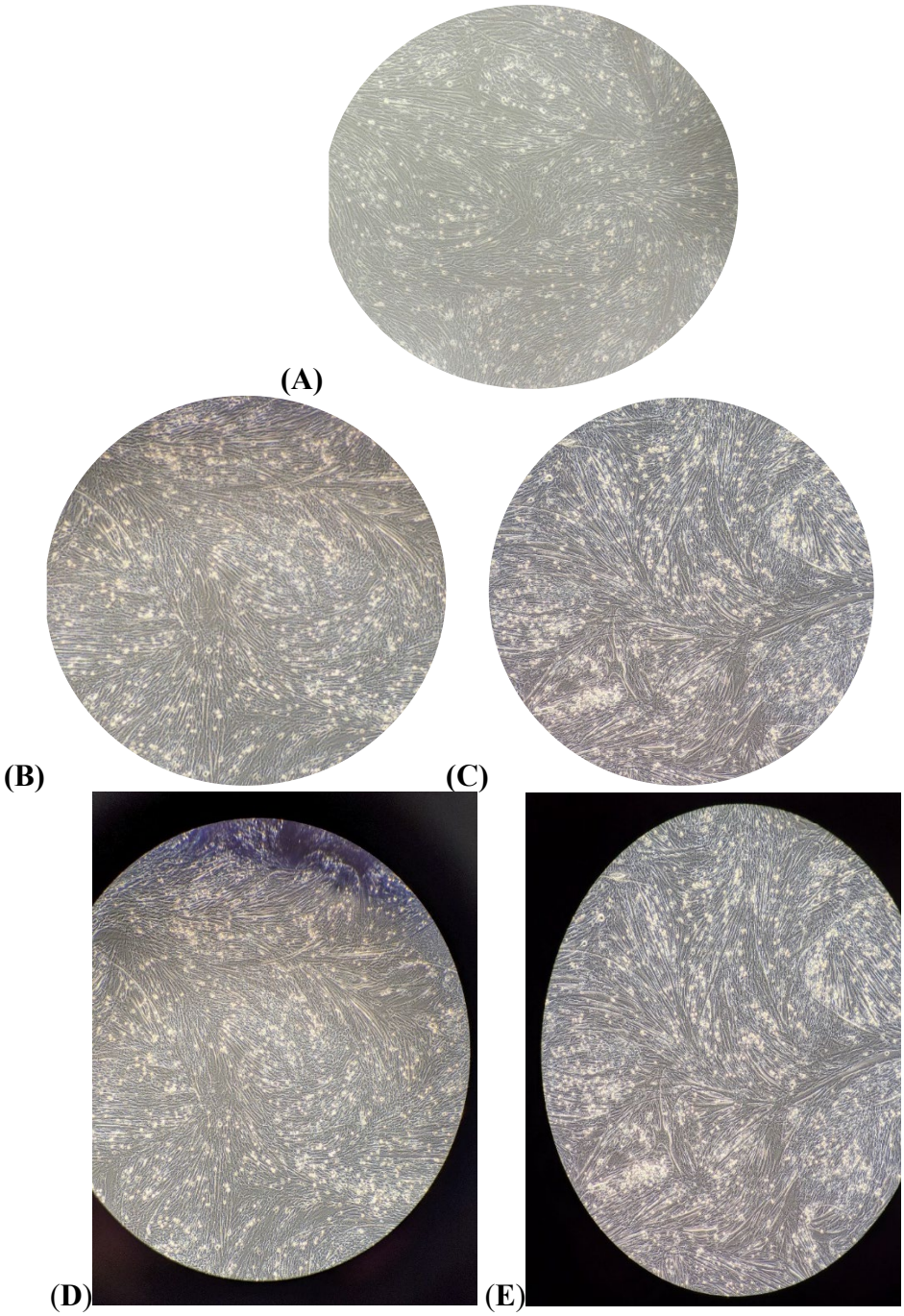


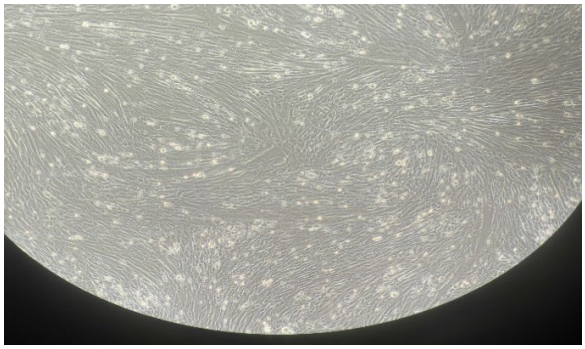

Figure 6. Pre & Post Cell Culture Images from Pharmacological Interventive NSC-185058. (A) Differentiated Myotubes, (B) 24hours exposure to the NSC185058 @ 0.5 Um, (C) 24hours exposure to the NSC185058 @ 1.0 Um, (D) 24hours exposure to the NSC185058 @ 0.5 Um with background, (E) 24hours exposure to the NSC185058 @ 1.0 Um with background.

**Cell Morphology**

*MLN-4924*

Image results show apoptotic/necrotic myotubes, and decrease in myotube growth and proliferation post 24hour application of the MLN-4924 drug at both 0.5Um, 1.0Um concentrations. Size discrepancy, and necrosis is suggested based on only visual observations, and not myotubular diameter, or apoptotic assay.

**(A)**



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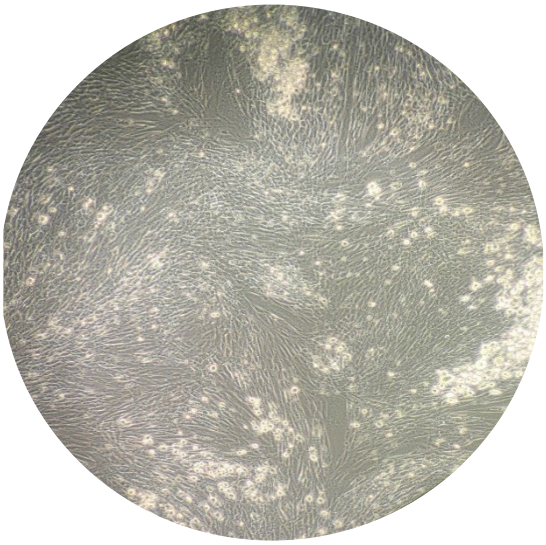

**(C)**

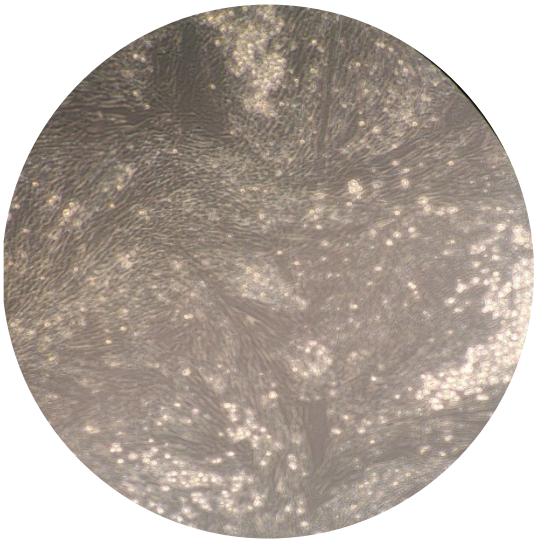

Figure 7. Pre & Post Cell Culture Images from Pharmacological Interventive MLN4924. (A) Differentiated Myotubes, (B) 24hours exposure to the MLN4924 @ 0.5 Um, (C) 24hours exposure to the MLN4924 @ 1.0 Um

**Protein Concentration**

Protein Concentration were determined via BCA analysis. Exact values, statistical data, and accuracy graphs are unavailable due to the unforeseen events surrounding the COVID-19 pandemic in spring 2020.

**Western Blots**

Due to the unforeseen events surrounding the COVID-19 pandemic in spring 2020, complete data on immunoblots was unavailable at the time of publication for this thesis.

*Specific targets proteins that were being studied.*

DEPTOR activity will be characterized to see if the pharmacological interventive drugs altered protein expression. Consequently, mTOR activity will be studied by analyzing the expression of proteins down-stream the anabolic pathway i.e. 4EBp1, P70S6K1, and AMPK. Furthermore, since P70S6K1 has also shown to have a major role in anti-autophagy, we will be comparing expression analysis with the effects of the NSC-185058 drug to see if activity was modulated, constant, or downregulated.

AMPK is another protein that is involved in the mTOR anabolic pathway that will be observed and analyzed due to the hypothesized effects of anti-autophagy mediated by the AMPK/MTOR Pathway (10).

# CHAPTER IV

# DISCUSSION

Due to the unforeseen events surrounding the COVID-19 pandemic in spring 2020, complete analytical data was unavailable at the time of publication in this thesis. In the absence of protein analysis data via immunoblotting, here are the potential outcomes of the study.

**Expectations**

Although it is suggested that the NSC-185058 has little to no effect on mTOR activity or P13K (5,6). Some recent studies hypothesize otherwise. In 2015, a study on microRNAs to downregulate ATG4B in prostate cancer cells, suggests the potential of ATG4B-induced autophagy through the AMPK/mTOR pathway (10). MicroRNAs are non-coding RNAs that regulate gene expression through epigenetics and translational repression. When AMPK is upregulated, mTOR is partially inhibited. This is relevant because prostate cancer is one of the most frequently diagnosed cancer in American men with a very high mortality rate (24). Additionally, mTOR inhibitors have been used to reduce the progression of Huntington's diseases in neuronal cells by inducing Atg4b (11). Thus, even though the exact mechanisms behind these pathways are still unclear, I would expect to see a downregulated AMPK with the application of the NSC-185058, which should ultimately have some form of inhibitory function on MTOR, putting the cell in an anabolic state. Similarly, I would expect 4EBp1, and S6K1 to have high activity, facilitate protein synthesis, and ultimately grow and proliferate the cells. Consequently, since the cell is in an anabolic state, we would also expect low activity of DEPTOR.

In opposition, we would expect the MLN drug to have an apoptotic effect on the myotubes as seen in the morphology above (Fig.7). It is important to note that MTOR is a negative regulator of autophagy; and clinical trials on human cancer cells with the MLN4924 drug have shown a dose dependent inhibition of the mTOR complex autophosphorylation (23). This reduces activity at multiple sites that impacts S6K1, 4E-Bp1, and AKT after a 24-hour exposure to the drug (23).

We applied the same approach on our L6 myotubes with different concentration to see if we could replicate and induce a dose dependent inhibition of mTOR as well, using a 0.5Um of MLN4924, 1.0Um of MLN4924, and DMSO (Dimethyl Sulfoxide), at respective concentrations as controls. With this systemic experimental set-up, I would expect a down-regulation of MTOR, and reduced activity of S6K1, and 4EBP1.

Consequently, as initially discussed with the MLN4924 drug, DEPTOR will be significantly upregulated, as it is one of the substrates accumulated as a result of MLN4924.

# CHAPTER V

# CONCLUSION

As a result of the unforeseen events with the COVID-19 pandemic in spring 2020, complete data was unavailable at the time of publication for this thesis project; ergo, it is difficult to conclude our hypothesis.

However, if my hypothesis were to be consistent with our observations from our lab at Texas A&M University which indicate that cellular protein homeostasis is restored only when DEPTOR expression has been recovered in the cell, there would be major implications and applications on how we study and combat anabolically aggressive cancers via similar pharmacological therapies.

Outcomes of this current study could also impact our understanding on protein homeostasis and shed light to new potential biochemical pathways. This project will ultimately provide insights to the possibility of using specific pharmacological strategies to promote or stifle cellular growth under a variety of conditions such as muscular dystrophy and atrophy. Furthermore, it could potentially mitigate the partial muscular atrophy astronauts experience during long space missions in the future!

# REFERENCES


1) Goodenough, C. G. (2019). MTOR: A MECHANISTIC TARGET OF MUSCLE AND CANCER CROSSTALK. The FASEB Journal.

2) Catena, V., & Fanciulli, M. (2017, January 13). DEPTOR: not only a mTOR inhibitor. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5237168/

3) Pópulo, H., Lopes, J. M., & Soares, P. (2012). The mTOR signaling pathway in human cancer. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3291999/

4) NASA INFORMATION, National Aeronautics and Space Administration. (2019). Muscle Atrophy. https://www.nasa.gov/pdf/64249main_ffs_factsheets_hbp_atrophy.pdf#targetText=It's%20a%20process%20called%20atrophy,lasting%20five%20to%2011%20days.&targetText=Astronauts%20on%20the%20International%20Space,the%20effects%20of%20muscle%20atrophy.

5) NSC 185058: CAS:39122-38-8 Probechem Biochemicals. (n.d.). Retrieved from http://www.probechem.com/products_NSC185058.aspx.

6) Akin, D., Wang, S. K., Habibzadegah-Tari, P., Law, B., Ostrov, D., Li, M., … Dunn, W. A. (2014). A novel ATG4B antagonist inhibits autophagy and has a negative impact on osteosarcoma tumors. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4502682/. p.

7) Yang, Z., Wilkie-Grantham, R. P., & Teruki Yanagi, C.-W. S. (2015). Zhifen Yang. Retrieved from http://www.jbc.org/content/early/2015/09/16/jbc.M115.658088. p.

8) Maruyama, T., & Noda, N. N. (2017, September 13). Autophagy-regulating protease Atg4: structure, function, regulation and inhibition. Retrieved from https://www.nature.com/articles/ja2017104. p.

9) Wu, Y., Dai, X., Ni, Z., Yan, X., He, F., & Lian, J. (2017, October). The downregulation of ATG4B mediated by microRNA-34a/34c-5p suppresses rapamycin-induced autophagy. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5673697/. p.

10) Liao, Haiqiu, Xiao, Yang, Xiao, Yin, … Yonggang. (2016, January 1). Methylation-induced silencing of miR-34a enhances chemoresistance by directly upregulating ATG4B-induced autophagy through AMPK/mTOR pathway in prostate cancer. Retrieved from https://www.spandidos-publications.com. p.

11) Proenca, C. C., Stoehr, N., Bernhard, M., Seger, S., Genoud, C., Roscic, A., … Galimberti, I. (2013, July 8). Atg4b-dependent autophagic flux alleviates Huntington's disease progression. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3704647/. p. 2-3

12) Lin, X., Han, L., Weng, J., Wang, K., & Chen, T. (2018, November 30). Rapamycin inhibits proliferation and induces autophagy in human neuroblastoma cells. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6265625/. p.

13) Shi, W.-Y., Xiao, D., Wang, L., Dong, L.-H., Yan, Z.-X., Shen, Z.-X., … Zhao, W.-L. (2012, March 1). Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. Retrievedfromhttps://www.nature.com/articles/cddis2012. P.

14) Garza-Lombó, C., Schroder, A., Reyes-Reyes, E. M., & Franco, R. (2018, May 17). mTOR/AMPK signaling in the brain: Cell metabolism, proteostasis and survival. Retrieved from https://www.sciencedirect.com/science/article/abs/pii/S2468202017301134. p.

15) Zachari, M., & Ganley, I. G. (2017, December 12). The mammalian ULK1 complex and autophagy initiation. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5869855/. p.

16) Hua, H., Kong, Q., Zhang, H., Wang, J., Luo, T., & Jiang, Y. (2019, July 5). Targeting mTOR for cancer therapy. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6612215/#CR55. p.

17) Zhao, Y., Xiong, X., & Sun, Y. (2011, October 21). DEPTOR, an mTOR inhibitor, is a physiological substrate of SCF(βTrCP) E3 ubiquitin ligase and regulates survival and autophagy. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3216641/. p.

18) Catena, V., & Fanciulli, M. (2017, January 13). Deptor: not only a mTOR inhibitor. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5237168/. p.

19) Mizushima, N. & Komatsu, M. Autophagy: renovation of cells and tissues. *Cell* 147, 728–741, P 1-2 (2011).

20) Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 2008; 99:1813-9; PMID:18616529

21) Li M, Hou Y, Wang J, Chen X, Shao ZM, Yin XM. Kinetics comparisons of mammalian Atg4 homologues indicate selective preferences toward diverse Atg8 substrates. J Biol Chem 2011; 286:7327-38; PMID: 21177865; http://dx.doi.org/10.1074/jbc.M110.199059

22) Alers S, L€offler AS, Wesselborg S, Stork B. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. Mol Cell Biol 2012; 32:2-11; PMID:22025673; http://dx.doi.org/ 10.1128/MCB.06159-11.

23) Y Zhao1 X Xiong, L Jia1, and Y Sun (2012). Targeting Cullin-RING ligases by MLN4924 induces autophagy via modulating the HIF1-REDD1-TSC1-mTORC1-DEPTOR axis. doi:10.1038/cddis.2012.125

24) Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics, 2009. CA Cancer J Clin. 59:225–249. 2009. View Article : Google Scholar : PubMed/NCBI

25) Guertin, D. A., & Sabatini, D. M. (2007, July). Defining the role of mTOR in cancer. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17613433

26) Jiang, Y., Su, S., Zhang, Y., Qian, J., & Liu, P. (2019, May). Control of mTOR signaling by ubiquitin. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6621562/

27) BTRC beta-transducin repeat containing E3 ubiquitin protein ligase [Homo sapiens (human)] - Gene - NCBI. (n.d.). Retrieved from https://www.ncbi.nlm.nih.gov/gene/8945

28) Duan, S., Skaar, J. R., Kuchay, S., Toschi, A., Kanarek, N., Ben-Neriah, Y., & Pagano, M. (2011, October 21). mTOR generates an auto-amplification loop by triggering the βTrCP- and CK1α-dependent degradation of DEPTOR. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3212871/

29) Hermeking, H. (2007, November). p53 enters the microRNA world. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17996645

30) Ryan, P. J., Deaver, W., Koo, S., Touissaint, G., & Fluckey, J. D. (2019, April 1). Elucidating the Role of DEPTOR in Cultured Glioblastoma Multiforme Cancer Cells. Retrieved from https://www.fasebj.org/doi/10.1096/fasebj.2019.33.1_supplement.lb604

31) Dhanapal, R., Saraswathi, T., & Govind, R. N. (2011, September). Cancer cachexia. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3227249/

# APPENDIX

NASA Scientists curious to have insights on controlling muscle growth (4) to reduce Space Flight Induced Functional Atrophy for present and future astronauts.

The NSC185058 drug could mitigate a new perspective in anabolism constructs for competitive sport training like Body-building.

Cachexia is still an important clinical prognosis for cancer; reduction in muscle mass can be achieved in cancer patients if the NSC185058 is bioengineered to target heathy muscle cells only.

MLN4924 shows potential for drug therapy on glioblastoma cells (30).