METHIONINE RESTRICTION INHIBITS NON-SMALL CELL LUNG CANCER GROWTH BY TARGETING THE BETA-CATENIN PATHWAY

Katherine Hobson

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ABSTRACT

METHIONINE RESTRICTION INHIBITS NON-SMALL CELL LUNG CANCER GROWTH BY TARGETING THE BETA-CATENIN PATHWAY

by

Katherine A. Hobson

Background: Lung cancer is the leading cause of cancer related death for both men and women. Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer with a 15% five-year survival rate. Current treatment options have serious side effects creating the need for alternative treatments. Methionine restriction (MR) has shown anti-tumor effects on various cancer cells, but the mechanisms involved in NSCLC is unclear. The purpose of this study is to determine the anti-tumor effects of MR on NSCLC cells through the beta-catenin pathway.

Objective: The purpose of this study is to determine the anti-tumor effects of MR on NSCLC cells through the beta-catenin pathway.

Methods: Human NSCLC cell lines, A549 and H520 were obtained from ATCC and treated in the presence of normal or MR media (95% methionine restriction). After 48 hours of incubation, cell viability was determined by the alamar blue assay and a clonogenic assay was performed separately. A549 and H520 were treated for 24, 48, and 72 hours and cultured for harvest. Cell cycle was analyzed by measuring the DNA content of each cells determined using flow cytometry and western blot was performed using the antibodies β-actin, β-catenin, phospho β-catenin, and PARP. In order to investigate the potential molecular mechanism of MR on NSCLC cell, a human phospho-kinase array was performed.
**Results:** MR significantly inhibits the cell proliferation in A549 and H520 cells after 48 hours. MR induces cell cycle arrest in G1 compared with the control after 24 hours of treatment. Protein expressions of PARP and phospho β-catenin are reduced in response to MR. The protein kinase array indicates MR exerts its anti-cancer effects by reducing phosphorylation of beta-catenin.

**Conclusion:** Our results show MR has an inhibitory effect on the cell proliferation and colony formation of A549 and H520 cancer cell lines. Cell cycle arrest and reduced phosphorylated β-catenin provides insight into how methionine metabolism inhibits lung cancer development and progression. Further in vivo studies are needed in order to testify the efficacy of MR as a cancer prevention approach for NSCLC.
METHIONINE RESTRICTION INHIBITS NON-SMALL CELL LUNG CANCER GROWTH BY TARGETING THE BETA-CATENIN PATHWAY

by
Katherine A. Hobson

A Thesis

Presented in Partial Fulfillment of Requirements for the Degree of

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# TABLE OF CONTENTS

List of Tables ..................................................................................................................... iv
Abbreviations ..................................................................................................................... v

Chapter

I. INTRODUCTION .............................................................................................................. 1
   Lung Cancer Prevalence and Treatments ................................................................. 1
   Methionine Overview ............................................................................................... 2
   Wnt Signaling Pathway ............................................................................................. 5

II. LITERATURE REVIEW .................................................................................................. 7
   Tumors’ Methionine Dependence ........................................................................... 7
   Methionine Restriction ............................................................................................... 7
      Breast Cancer ........................................................................................................... 7
      Prostate Cancer ........................................................................................................ 8
      Colon Cancer .......................................................................................................... 9
      Non-small Cell Lung Cancer .................................................................................. 10
      Phase I Clinical Trials ............................................................................................ 10

III. METHODS .................................................................................................................. 12

IV. RESULTS ..................................................................................................................... 16

V. DISCUSSION AND CONCLUSIONS ......................................................................... 22

REFERENCES ....................................................................................................................
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Salvage and Recycling Pathways for Methionine Metabolism</td>
<td>51</td>
</tr>
<tr>
<td>2. Wnt/β-catenin Signaling Pathways Regulating Target Gene Transcription</td>
<td>52</td>
</tr>
<tr>
<td>3. Cell Proliferation in A549 and H520 Cells with 48h Control and MR Treatment</td>
<td>53</td>
</tr>
<tr>
<td>4. Colony Formation in A549 and H520 Cells with 24h and 48 Control and MR Treatment</td>
<td>54</td>
</tr>
<tr>
<td>5. Cell Cycle Data for A549 and H520 from Flow Cytometry</td>
<td>56</td>
</tr>
<tr>
<td>6. Results from Human Phosphokinase Array for H520 with 48h Control and MR Treatment</td>
<td>51</td>
</tr>
<tr>
<td>7. Expression of Proteins by Western Blot after MR Treatment in A549 and H520 Cells</td>
<td>51</td>
</tr>
<tr>
<td>8. Salvage and Recycling Pathways for Methionine Metabolism</td>
<td>51</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

NSCLC  Non-small Cell Lung Cancer
SCLC  25-hydroxyvitamin D
EGFR  Epidermal Growth Factor Receptor
ALK  Anaplastic Lymphoma Kinase
GSH  Glutathione
DFMO  Difluoromethylornithine
SAMe  S-adenosyl-methionine
MR  Methionine Restriction
SAH  S-adenosylhomocysteine
MS  Methionine Synthase
MAT  Methionine adenosyltransferase
THF  Tetrahydrofolate
MTA  Methylthioadenosine
MTAP  Methylthioadenosine Phosphorylase
MTOB  4-Methylethio-2-oxobutanoic acid
TNBC  Triple-negative Breast Cancer
FAK  Focal Adhesion Kinase
AIF  Apoptosis Inducing Factor
TRAMP  Transgenic Adenocarcinoma of the Mouse Prostate
ACF  Aberrant Crypt Foci
AOM  Azoxyemethane
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<thead>
<tr>
<th>Abbr</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP Ribose Polymerase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
</tr>
</tbody>
</table>
CHAPTER I
METHIONINE RESTRICTION INHIBITS NON-SMALL CELL LUNG CANCER GROWTH BY TARGETING THE BETA-CATENIN PATHWAY

INTRODUCTION

Lung Cancer Prevalence and Treatment Options

Lung cancer is the leading cause of cancer-related death in both men and women.\(^1\) The American Cancer Society estimates for 2018 that there would be 234,030 new lung cancer cases and 154,050 deaths from lung cancer in US.\(^1\) There are two main types of lung cancer, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for 85% of all lung cancer and includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma subtypes.\(^2\) Survival rates for NSCLC vary greatly depending on the stage and location of the tumor.\(^3\) Approximately 70% of patients have a locally advanced or metastatic diagnosis leading to a 15% five year-survival rate in NSCLC.\(^3,4\)

Traditional treatment options for NSCLC include surgical resection of the lungs, chemotherapy, and radiation therapy.\(^4\) Our growing understanding of cancer biology suggests patient-specific treatments including target therapy and immunotherapy. Target therapy reduces tumor cell growth by blocking essential oncogenic pathways via monoclonal antibodies or molecular inhibitors.\(^5\) The common targeting compounds used in NSCLC are biological inhibitors that aim at these mutated oncogenic proteins such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK).\(^6\)
Overexpressions of these proteins lead to cancer proliferation, anti-apoptosis, angiogenesis, and escape from the immunity. Target therapy could be achieved by the blockage of the protein activation site. Immunotherapy utilizes monoclonal antibodies as immune checkpoint inhibitors to boost an immune response against the cancer cells. Although these therapies have saved many lives, there is still a need for anti-cancer agents that are highly effective and minimize side effects as well as drug resistance. Historically, nutritional supplements have been used to lower the side effects of chemotherapies (i.e. nausea, weight loss), but research supporting the use of nutritional compounds as anti-cancer agents is progressing.

**Methionine Overview**

Methionine is a sulfur-containing, essential amino acid that plays a key role in cancer development through glutathione formation, polyamine synthesis, and DNA methylation. Glutathione (GSH) is a tripeptide antioxidant known for being elevated in relation to proliferative responses from both normal and malignant cells. In the trans-sulfuration pathway, GSH is synthesized after the formation of cystathionine from homocysteine, which is then converted to cysteine leading to the formation of GSH. GSH is a marker for oxidative stress and found at low levels during inflammatory states such as in degenerative diseases and many types of cancer. Moreover, methionine is the precursor for two polyamines, spermidine and spermine, which are critical for protein synthesis, growth, and cellular proliferation. Previous studies have shown that elevated levels of polyamines are associated with increased tumor growth. Inhibitors of polyamine metabolism, such as alpha-difluoromethylornithine (DFMO), lead to a reduction in
polyamine production, disruption of the cell cycle and DNA synthesis in cancer cells.\textsuperscript{16,17} Methionine plays a crucial role in DNA methylation through formation of S-adenosyl-methionine (SAMe), the universal methyl donor responsible for normal and cancerous cells’ functions such as DNA methylation, oxidative stress, gene regulation, and cell differentiation.\textsuperscript{18}

Due to the many functions of methionine, methionine restriction (MR) has been widely studied \textit{in vivo} and \textit{in vitro} for extended lifespan, increased insulin sensitivity and decreased adiposity, inflammation, and oxidative stress.\textsuperscript{19-23} The metabolic benefits of MR could potentially have an anti-cancer effect since cancer diagnoses have been linked to common metabolic factors such as obesity and insulin resistance.\textsuperscript{24}

Many studies have shown that MR exhibits anti-cancer affects in various cell culture and animal models.\textsuperscript{25} Although the specific mechanisms are not clear, the pathways involved in methionine metabolism are well understood. The difference of methionine metabolism between the normal and cancer cells has been hypothesized as the basis for MR’s anti-cancer effect.\textsuperscript{26} Once methionine is metabolized from dietary protein, it is activated by ATP to produce SAMe which is then converted to S-adenosylhomocysteine (SAH). Subsequently, SAH is hydrolyzed by SAH hydrolase to form homocysteine which can then be converted to L-methionine with the addition of two co-factors, vitamin B12 (cobalamin) and 5-methyl THF. The major enzyme responsible for methionine regeneration is methionine synthase (MS). MS expression is low in many cancer cells, thus leading to one possible mechanism for the methionine dependency of tumors.\textsuperscript{27} Meanwhile, normal mammalian cells are able to recycle homocysteine to form methionine independent of exogenous methionine.\textsuperscript{26}
Figure 1: Salvage and recycling pathways for methionine metabolism. Dietary methionine is converted to SAMe which can be utilized in the two pathways. In the recycling pathway (right), SAMe is converted to homocysteine and methionine; methionine synthase, Vitamin B12, and 5-methyltetrahydrofolate are involved in the conversions. In the salvage pathway (left), the enzyme MTAP converts MTA to methylthioribose 1-PO4 after the two polyamines, spermine and spermidine, are formed. Methylthioribose 1-PO4 is converted to methylthioribulose which produces MTOB, eventually yielding methionine as the end product.

Further evidence for methionine dependency in tumors begins with the decarboxylation of SAMe to enable the synthesis of the two polyamines spermidine and spermine, byproducts of methylthioadenosine (MTA). Furthermore, phosphorylation of MTA by methylthiasdenosine phosphorylase (MTAP) yields adenosine and 5-methylthioribose-1-phosphate, which lead to the formation of L-methionine. This alternate pathway for methionine synthesis is dependent on MTAP, which is found to be minimally expressed in tumor cells.
Wnt Signaling Pathway

Wnt signaling pathway is involved in tumor initiation and progression.\textsuperscript{29,30} Beta-catenin, as a ligand, is a subunit of the cadherin protein complex and acts as an intracellular signal transducer in the Wnt signaling pathway.\textsuperscript{31} The Wnt/beta-catenin pathway has been linked to lung adenocarcinoma metastasis and beta-catenin is highly expressed in a subset of NSCLCs.\textsuperscript{32} Beta-catenin could potentially be linked to methionine due to Wnt’s protein structure which includes 23 conserved cysteine residues.\textsuperscript{33} Due to beta-catenin’s involvement in cancer progression and development, nutraceuticals targeting beta-catenin could be of benefit for cancer prevention.

\textbf{Figure 2:} Wnt/\beta\textsuperscript{-}catenin signaling pathways regulating target gene transcription.\textsuperscript{30} When the Wnt pathway is “off” (A), the protein attached to the cell membrane are inactive. This allows the destruction complex comprised of CK-1, GSK-3Beta, APC, and AXIN to bind to \beta\textsuperscript{-}catenin, which is phosphorylated and then degraded. When the Wnt pathway is “on” (B), Wnt binds to the frizzled (Fzd) receptor, which leads to the binding of the destruction complex to the cell membrane. \beta\textsuperscript{-}catenin is free in the cytosol and travels to the nucleus to activate Wnt target genes by binding to TCF/LEF.
The purpose of this study is to determine whether MR inhibits NSCLC growth by targeting the beta-catenin pathway.

**Specific Aim 1**: To measure the effects of MR on NSCLC cell proliferation and cell cycle.

*Research Hypothesis 1A*: MR induces cell cycle arresting and inhibits cell proliferation in NSCLC.

*Null Hypothesis 1A*: MR produces insignificant change in cell cycle and proliferation of NSCLC.

**Specific Aim 2**: To determine whether MR decreases expression of β-catenin.

*Research Hypothesis 2A*: MR decreases expression of the β-catenin pathway in NSCLC.

*Null Hypothesis 2A*: MR does not affect the expression of the β-catenin pathway in NSCLC.
CHAPTER II
LITERATURE REVIEW

Tumors’ Methionine Dependence

Previous research demonstrating the methionine dependence of tumors was conducted on rats fed iso-caloric diets lacking specific amino acids. The groups lacking methionine, valine, and isoleucine showed a reduction in Walker tumors. A follow-up study showed normal cells can withstand MR by the addition of folic acid (cyanocobalamin-rich media) while malignant cells’ growth is impaired. These results supported the hypothesis that malignant cells lack the ability to recycle homocysteine for methionine, unlike normal cells.

Methionine Restriction

Breast Cancer

To understand the effects of MR in breast cancer, mice were injected with MCF10A breast cancer cells were fed either MR or control diets for 12 weeks. Results for the MR group showed reduced body weight and adiposity. In addition, cell proliferation and tumor size were decreased with enhanced apoptosis. Reduction of insulin and IGF1, both linked to tumor growth, were the rationale for the anti-cancer effect of MR. A study involving both human triple-negative breast cancer (TNBC) cell lines, MDA-MB-231 and HS 578T
cells, and xenograft TNBC in BALB/c mice showed reduction in cell viability, migration, and invasiveness of cancer cells grown in MR media compared to the control group. The results were linked to the phosphorylation of focal adhesion kinase (FAK), a cell adhesion protein involved in cancer metastasis upon the methionine deprivation. An alternative method for decreasing TNBC with MR was shown in conjunction with TRAIL-R2 agonists, TNF-related apoptosis-inducing ligand receptor-2. Human TNBC cell lines cultured in methionine-free media showed increased sensitivity to TRAIL-R2 agonist treatment. Additionally, a study conducted in nude mice showed that methionine-free diets led to tumor growth inhibition in refractory SCLC6 (a small-cell lung cancer), SNB19 (a glioma), and TC71-MA (a colon cancer). MR caused the greatest inhibition (71%) when used in SCLC6 that was pretreated with doxorubicin (a chemotherapeutic agent). These results show the synergistic effects of MR to treat drug-refractory tumors.

Prostate Cancer

The results of MR in cancer cells has been shown to vary depending on the cell lines. In prostate cancer cell lines PC3 and DU145, cell proliferation was measured after cells were cultured in normal and methionine-free media. While cell cycle arrest in G0/G1 was observed in both cell lines due to MR, MR-induced apoptosis was only demonstrated in PC3 cells. Overall, anti-proliferative effects were observed in both PC3 and DU145. In these cell lines, MR appeared to be FAK and ERK dependent. In a subsequent study, MR treated cells showed cell cycle arrest, predominantly in G2/M phase, and apoptosis leading to an accumulation of cyclin-dependent kinase-inhibitors. PC3 and
DU145 were, however, differentially modulated with MR inducing caspase-dependent and caspase-independent apoptosis of PC3, while Raf, Bcl2 family proteins, and apoptosis inducing factor (AIF) were modulated in DU145 cells. Another study conducted on prostate cancer involved an animal model using MR treatment in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice. Researchers found that prostatic intraepithelial neoplasia lesions were reduced by ~50% in the MR group compared to the control, supporting the possibility of using MR as a treatment option for prostate cancer.

**Colon Carcinogenesis**

To determine the relationship between MR and colon carcinogenesis, the formation of aberrant crypt foci (ACF), biomarkers of colon cancer, were measured in rats treated with the carcinogenic compound azoxymethane (AOM). F344 rats were given AOM (15mg/kg body weight) before, during and after being fed control (0.85% methionine) or methionine-restricted (0.15% methionine) diets. MR greatly inhibited ACF formation in the post-AOM induced group showing the benefits of MR during post-initiation phases of colon carcinogenesis. A recent *in vitro* study using the BALB/c 3T3 cell transformation assay analyzed the effect of MR and methionine supplementation on malignant colonic cells. Results showed that an increase in the concentration of methionine by the same percentage as restriction (40%) did not have an effect on the malignant cellular transformation while MR showed a significantly lower rate.
Non-small Cell Lung Cancer

To date, MR in NSCLC cell lines has not been conducted, but analysis of gene expression involved in the methionine pathways have been assessed. Paired A549 cells were used to decipher the vulnerability of naturally occurring MTAP-positive and -negative cell lines to various treatments, including methionine depletion. This study established MTAP deficiency directly correlates to the reaction of cancer cells to methionine depletion, but did not postulate the molecular mechanism and did not investigate whether MR, rather than methionine depletion, would produce similar responses.

Clinical Trials

As preclinical data has shown efficacy of MR in various models, clinical trials have been conducted to assess the safety of MR, as well as the proposed anti-tumor effect in conjunction with traditional treatment. A Phase I clinical trial conducted on 8 patients with metastatic tumors were fed amino-acid modified medical food that restricted methionine to 2mg/kg/day. Despite protein intake remaining at the target of 0.6-0.8g of protein per day, one side effect was a weight loss of 0.5% per week. Overall, MR resulted in reduced serum methionine levels (58% decline) which proved to be safe and tolerable, but anti-cancer affects were not measured. To assess the optimal duration required for lowering plasma methionine levels, another Phase I clinical trial was performed on patients suffering from metastatic melanoma and recurrent glioma. Patients were given synthetic methionine-free solution in place of all protein before and after four cycles of 2 week chloroethylnitrosourea (chemotherapy) treatments. This study showed that methionine-free
diets for even 1 day in conjunction with chemotherapy, can significantly reduce plasma methionine levels while remaining tolerable for cancer patients. Future clinical trials are needed to examine the efficacy of MR alone and in combination with current treatment options for optimal cancer treatment.

MR’s role in cancer initiation and progression *in vitro* and *in vivo*, as well as the safety and preliminary data from clinical trials, has been established in several types of cancer with the exception of NSCLC. This warrants the need for the proposed study that will aim to quantify and explain one of the possible mechanisms for MR in NSCLC.
CHAPTER III
METHODS

Cell Culture, Reagents, and Antibodies:

NSCLC cell lines A549 and H520 obtained from American Type Culture Collection (Rockville, MD) and were cultured in DMEM 1x containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin. During treatment periods, cells were counted and plated at appropriate concentrations. The cells used for the clonogenic assay, alamar blue assay, and H520 cells for western blot were either treated with either control media (DMEM 1x, 0.1% Bovine Serum Albumin (BSA), 1% Penicillin/Streptomycin (P/S), no glutamine) or MR media (DMEM 1x, 95% methionine-free media, 1% P/S, no glutamine). For analyzing cell cycle via flow cytometry, phosphor-kinase array, and A549 for western blot were treated with either control media (DMEM 1x, 0.1% BSA, 1% P/S, with glutamine) or MR media (DMEM 1x, 95% methionine-free media, 1% P/S, with glutamine). The difference between the two types of treatments is the glutamine that was either present or absent. The primary antibodies used during immunoblotting were rabbit beta-actin, beta-catenin, phosphorylated beta-catenin, and PARP (Cell Signaling). The secondary antibody used was anti-rabbit. Cell signaling lysis buffer was used for western blot and 70% isopropyl was used to fix cells for flow cytometry.
**Alamar Blue Assay**

A549 and H520 were cultured until 90% confluency and then cells were counted via Trypan blue staining. Cells were seeded in a 24-well plate at 3000 cells/well for A549 and 5000 cells/well for H520 using Control and MR media containing glutamine. Fluorescence was measured at day 0 and day 2.

**Cell Cycle Analysis**

A549 and H520 cells were seeded in 100 mm dishes and incubated overnight in 2% FBS DMEM 1x media. The plates were split into control and treatment groups and treated with the -glutamine media. Cells were collected at 0 h, 24 h, 48 h, and 72 h and fixed in ice-cold 70% ethanol. After centrifugation and 3x PBS washes, cells pellets were resuspended in PBS, propidium iodide, RNase, and incubated for 2 hours at room temperature. DNA content and cell cycle phases were analyzed via flow cytometry using the BD Fortessa. Cell cycle analysis was performed with FlowJo software.

**Clonogenic Assay**

Human A549 cell line were cultured and plated in 4, 100 mm dishes with two million cells per plate with. Control and MR media both containing glutamine was used. Cells were cultured for 24 and 48 hours and viable cells were re-plated on 100 mm dishes (5,000 cells per plate) and 6 well-plates (1,000 cells per plate). After a 14 day incubation period, colonies were fixed in methanol and stained with crystal violet for observation.
This assay was conducted a second time using both A549 and H520 seeding only in 6-well plates at 500 cells per well for A549 and 1000 cells per well for H520. This second assay was treated with the same control and MR media for 24 and 48 hours. Colonies were stained and observed after 7 days.

**Western Blot**

Cell lines A549 and H520 were cultured in control and MR media, then collected at 0h, 24h, 48h, and 72h. Cells were then suspended in lysis buffer and stored at -80°C. Protein concentrations were determined using the Bradford protein assay kit. 10% SDS-polyacrylamide gels were loaded with 4x laemmli loading buffer and cell lysates at appropriate concentrations. Gel electrophoresis was conducted and gels were transferred to nitrocellulose membranes via Bio-rad semi-dry transfer. Membranes were incubated in 5% non-fat dried milk in 1x TBST (TBS buffer and 0.1% Tween-20) for 30 minutes and then incubated with primary antibodies (2 mL of 5% non-fat dried milk/TBST) at 4°C. After overnight incubation, membranes were washed 3x with TBST and then incubated with secondary antibodies for 2 hours in 5% non-fat dried milk/TBST at room temperature. Signal strength was measured using the ChemiDoc imager by Bio-rad and quantification was performed in Imagej.

**Statistical Analysis**

Data were analyzed using Student’s t-test to measure the difference between control and treatment groups for the Alamar blue assay. A p-value < 0.05 was considered to be statistically significant. The analysis was performed using Microsoft Excel.
CHAPTER IV

RESULTS

Figure 3 summarizes the results from the Alamar blue assay demonstrating the inhibitory effect of MR on both H520 and A549 cells’ proliferation. After 48 hours, there is a significant reduction of proliferation in the MR treated cells; \( p=.001 \) in H520 and \( p=.004 \) in A549.

**Figure 3.** Cell Proliferation in A549 and H520. A) Control and MR treatment (+ glutamine) after 48 hours. Day 0 had similar fluorescence with a \( p \)-value of .503. After 48 hours, the control had a significantly greater
proliferation rate than the MR treated cells (p=.004). B) Control and MR treatment (+ glutamine) after 48 hours. After 24 hours, H520 shows similar proliferation rates as A549, higher in control vs MR with a p-value of p=.001.

A second experiment aimed at measuring the proliferative response of cancer cells to MR was the clonogenic assay which is represented in Figure 4. The results showed a qualitative view of the colony size and quantity when treated with MR while compared to the control. In the 24 hour plates, there is a visual difference in colony formation compared to the MR plates. In the 48 hour treatment, there is not a clear difference between treatment and control groups. This assay was originally conducted in 100 mm dishes and was conducted a second time in 6-well plates with a 48 hr treatment period. The qualitative data demonstrate MR’s restrictive effect on both cells lines through decreased quantity of colonies and smaller formations.
Figure 4. A) Colony formation in A549 after 24h and 48h MR treatment (+glutamine). B) A549 and H520 with control and MR treatment (+glutamine) after 48 hour treatment.

While the inhibitory effect of MR treatment on A549 and H520 can be seen through cell proliferation and colony formations assays, the results from flow cytometry showing the variation within cell cycle phases are displayed in Figure 5. Cell cycle arrest in G1 phase was observed for A549 cells after 24 hour treatment of MR. For H520, cell cycle arrest occurred in G2 after 72 hours.
Figure 5. Cell cycle data from flow cytometry in both control and MR treated cells at 0, 24, 48, and 72 hours.

A) A549 shows cell cycle arrest in the G1 phase in the 24-hour MR treated cells. B) MR treatment in H520 shows cell cycle arrest after 72 hours in the G2 phase.

In order to identify a possible mechanism behind NSCLC’s response to MR, a Human Phospho-kinase array and immunoblotting confirmed β-Catenin’s involvement. The level of phosphorylated β-Catenin, along with Src and Yes proteins, was shown to be inhibited with MR treatment when compared to the control as shown in Figure 6. The phosphokinase array confirmed the expression of β-Catenin in NSCLC cell lines A549 and H520, and predicted that MR can inhibit phosphorylated β-Catenin.
Figure 6. Phosphokinase Array displays phosphorylation of β-Catenin, Yes, and Src show reduced expression when exposed to MR treatment for 48 hours in H520 cells (-glutamine) compared to control.

The results from Western Blot using β-actin as the loading control demonstrates that β-Catenin is highly expressed in both A549 and H520, but phosphorylated β-Catenin expression is decreased in A549 after 48 hours of MR. H520 showed similar results, but expression of phosphorylated β-Catenin is not decreased until after 72 hours.

Figure 7. Expression of phosphorylated β-Catenin is decreased after MR treatment in H520 and A549 cells. In H520, 48- and 72-hour MR treatments increased the expression of cleaved-PARP.
CHAPTER V
DISCUSSION AND CONCLUSIONS

This study established preliminary data showing inhibitory effects of MR on cells’ proliferation. One of the hallmarks of cancer is the dysregulation of cell cycle progression\textsuperscript{49} which was demonstrated by the reduction of cell proliferation in A549 and H520 cells due to MR. In Figure 5. A, cell cycle arrest in the G1 phase could be related to the target genes, Cyclin D1 and Myc, being inhibited. Cyclin D1 and Myc are key regulators in the G1 phase and suppression of these genes could lead to a reduction in cell proliferation in A549 cells after 24 hours of MR treatment.\textsuperscript{50} These target genes are suppressed when the Wnt/\(\beta\)-Catenin pathway is “off” and \(\beta\)-Catenin is degraded.\textsuperscript{29} The dysregulation of Wnt signaling, potentially by MR, has been shown to cause mutations leading to carcinogenesis further supporting MR’s relationship to NSCLC and the Wnt/\(\beta\)-Catenin pathway.\textsuperscript{33} Cell cycle arrest could also be due to the reduction of polyamine synthesis since reductions have been shown to disrupt cell cycle and induce apoptosis.\textsuperscript{17} In this study, due to methionine’s role in the salvage pathway (Figure 1), MR could have reduced polyamine synthesis further leading to the G1 cell cycle arrest observed in A549 and G2 arrest in H520 cells. Previous studies confirm that MR can induce cell cycle arrest in difference phases based on the cell lines used.\textsuperscript{40}

Another key amino acid that played a major role in this study was glutamine. Glutamine is a key nutrient in cell proliferation for both cancerous and normal cells, and
glutamine metabolism has been shown to be altered during cancer progression. Due to glutamine’s crucial role in cell proliferation and cell cycle, the results from Figure 5 only demonstrate preliminary data and the results can only be substantiated through repetition of this experiment with cells grown in media containing glutamine.

The reduction of phosphorylated β-Catenin after MR treatment shown in Figure 6 and 7 demonstrate the potential for the Wnt signaling pathway to be turned “off” by MR. Additionally, the western blot results from Figure 7 show that 48- and 72- hour MR treatment in H520 increases the cleavage of PARP, which is an indicator of apoptosis in cancer cells. Western blot results also show that expression of total PARP, a marker of cell proliferation, is slightly inhibited in A549 cells after 72 hours of MR. While 48- and 72- hour MR treatments appear to yield the greatest inhibitory affect on NSCLC cells, the colony formation assay contradicted this by showing larger and higher quantities of A549 colonies after 24 hour treatment rather than 48 hours. The replicated experiment (Figure 4. B) aimed to confirm these results, but the 24 hours plates for both A549 and H520 were contaminated and unable to be analyzed. Due to these constraints, the optimal time for MR treatment in NSCLC is still unknown. Other in vitro studies conducted on various types of cancer have found a wide range of treatment times ranging from 24 hours of methionine deprivation or 6 days of methionine restriction.

Despite the intriguing results, the limitations of this study cause the findings to be inconclusive without further investigation. The lack of statistical power within this study was due to limited replication of studies caused by several laboratory constraints. During the course of this study, the identification of varying treatments led to a change in methodology which further limited replication. Since the realization of the lacking
glutamine in the control and treatment media, conducting cell proliferation, cell cycle, and immunoblotting with glutamine in all media would create stronger evidence towards MR and β-Catenin’s potential role in NSCLC because it would eliminate the confounding factor, glutamine. Additionally, future studies should aim to assess both cancerous and normal cells’ response to control and MR treatment to compare affects. Future studies could replicate the experiments detailed, distinguish between unclear data, and identify the optimal time and dosage for MR. Thus far, studies have used varying degrees of methionine restriction from complete deprivation\textsuperscript{37} to an 85\% reduction\textsuperscript{44}. Since MR has been shown to cause weight loss\textsuperscript{23}, an adverse effect for cancer patients\textsuperscript{53}, identifying the optimal concentration of MR is key. Conducting dose dependent studies \textit{in vitro} will lend stronger support for animal studies and clinical trials in order to test the efficacy of MR as a cancer preventative approach in NSCLC
REFERENCES


