The Effect of 4-Methylthio-2-Oxobutyric Acid Analogs and CtBP siRNA on Cancer Cell Viability

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

Meghan Cockerill

April 29, 2010

APPROVED:

Steven Grossman, M.D., Ph.D.
Cancer Biology
UMASS Medical School
Major Advisor

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor
The two primary differences between normal epithelial cells and cancerous cells are the ability of cancerous cells to metastasize and avoid apoptosis. The drug 4-methylthio-2-oxobutyric acid (MTOB) has been shown to interact with the CtBP transcription repressor causing the induction of apoptosis, as well as a reduction in migration of cells. The focus of this MQP was to explore the most efficacious use of MTOB through the study of structural analogs, as well as combination therapy with CtBP siRNA. While structural analogs seemed to be less effective than similar doses of MTOB, using siRNA against CtBP2 in combination with low doses of MTOB proved to significantly lower cell growth rate and increase apoptosis. Immunoblotting analysis also demonstrated a sizeable decrease in expression of the CtBP2 protein with the combination MTOB/siRNA treatment.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>4</td>
</tr>
<tr>
<td>Background</td>
<td>5</td>
</tr>
<tr>
<td>Project Purpose</td>
<td>12</td>
</tr>
<tr>
<td>Methods</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>18</td>
</tr>
<tr>
<td>Discussion</td>
<td>27</td>
</tr>
<tr>
<td>Bibliography</td>
<td>30</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First and foremost, I would like to extend a tremendous thank you to Steven Grossman (MD/PhD) at the University of Massachusetts Medical School Cancer Biology Department for the generous use of his laboratory, and for all his help and guidance throughout this project. I would also like to thank Michael Straza who provided the direction and focus for this project, and who also provided me with countless protocols, incredible guidance, and who also gave me a great deal of insight and theory into this project. Without his help and guidance this experience would not have been possible. Additional thanks goes out to all the Grossman Lab personnel, expressly Dan Parker for his assistance with protocols and reagents, Seema Paliwal (PhD) for her insight, assistance, and for the use of her siRNA, and Roman Kulikov (PhD) for his insight and the use of his cell lines. Lastly, I must deeply thank Professor Dave Adams (PhD) as both my major and project advisor for all his assistance and wisdom throughout the years. My project and my education at WPI would not have been as rewarding without him.
BACKGROUND

Current Treatments for Cancer and Their Downfalls

The most common current cancer therapies available are toxins that kill cancer cells. In most cases these toxins target cancer cells only slightly more than normal cells, and in some cases don’t really target cancer cells at all, meaning that normal cells are also affected. This is the most prominent downfall to cancer therapies and the reason that cancer continues to be such a potent killer. Most chemotherapies work by targeting rapidly proliferating cells, such as cancer cells, however the human body contains a great number of other cells that grow quickly such as blood cells forming in bone marrow, cells in the digestive tract, reproductive organ cells, and hair follicles (Sadanandam, et al., 2010). These effects on normal cells sometimes explain lethal side effects of chemotherapy.

Chemotherapy is not the only method to treat cancer. Today there are a variety of drugs on the market that have less harsh side effects than chemotherapy. While they are often used in conjunction with chemo, they are also primarily effective in only a small handful of neoplasms. Despite all forms of cancer having some common traits, each type of cancer has certain differentiating factors such as the expression of specific marker proteins, or the inactivation of tumor supressors such as p53 (Sadanandam, et al., 2010). Often different types of neoplasms have very different signal transduction pathways that are involved in growth stimulation, therefore the most effective cancer therapies are those which target proteins that are involved in most if not all types of cancer.
C-Terminal Binding Proteins and Their Role in Cancer

C-terminal binding proteins (CtBP) are transcriptional factors that are an important part of the normal cell’s ability to selectively allow for cell migration while avoiding apoptosis. While these functions are necessary to a small extent in normal cells, it is these primary mechanisms that make cancer so dangerous. It has been found that in particular that CtBP2 is highly upregulated in a large number of cancer types. This protein is localized to the nucleus of a cell and acts as a transcriptional corepressor of several genes in apoptotic, anti-migratory, and anti-cellular proliferation pathways. (Figure-1). Due to its ability to allow many of the critical traits of cancer to flourish, it is a prime target for a therapeutic agent.

Figure-1: The Role of CtBP2 in Cancer Formation. Transcription factor CtBP is known to block the expression of apoptotic genes (increasing cell survival) (diagram center) and block gp15 and p16 cell cycle arrest proteins, leading to cell proliferation (diagram right), while stimulating MDM2 to block tumor suppressor p53 (diagram left). CtBP2 acts as a transcriptional corepressor of BH3 apoptotic genes, as well as various tumor suppressors such as p15^{INK4a}, p16^{INK4b}. This diagram also shows the activation of MDM2, an inhibitor of p53 dependant apoptosis, by CtBP2 (Paliwal, et al. 2007).
CtBP targets several groups of genes, many of which were crucial to this project, including genes directly related to cell proliferation and apoptosis. CtBP2 regulates apoptosis in large part by repressing the expression of pro-apoptotic BH3 genes. These include genes such as Bik, Puma, and NOXA, which are known to powerfully regulate the induction of apoptosis. CtBP2 inhibits these genes allowing for the increase in cell survival for many cells that would normally undergo apoptosis. (Chinnadurai, 2009) This is one of the many ways in which CtBP assists in the survival of cancer cells. Additionally, CtBP is an activator of MDM2, which inhibits p53, a vital protein for apoptosis in many cells, but furthermore CtBP directly inhibits p53 independent apoptosis as well (Paliwal, et. al 2006). CtBP therefore effectively inhibits a number of important apoptotic pathways, making the inhibition of CtBP a promising new area for cancer therapy research.

Uncontrolled cellular proliferation is a crucial component of cancerous cells. The genes p16^{INK4a} and p15^{INK4b} directly affect proliferation of cells by causing G1 phase cell cycle arrest, making them cell cycle inhibitors. In many forms of cancer, CtBP inhibits these genes, allowing for unregulated proliferation (Kovi, et. al 2010). By inhibiting these genes, CtBP allows for an increase in cellular mitosis, which allows for rampant growth for cancer cells.

**4-Methylthio-2-Oxobutyric Acid (MTOB) and its Interaction with CtBP2**

The compound 4-methylthio-2-oxobutyric acid (MTOB) is naturally occurring in the human body. In particular, it is the penultimate compound in the methionine-salvage pathway (Tang et al., 2006). In addition to its involvement in that pathway however, the Grossman Lab at the University of Massachusetts Medical School discovered that MTOB is able to bind to CtBP2 via the dehydrogenase domain causing the repression of its activity (Straza, Kovi, Paliwal,
Messina, Trench, & Grossman, submitted). Previous studies by this lab have shown that MTOB alone is able to induce apoptosis in vitro and also inhibit cell migration and proliferation. The suppression of CtBP2, and the absence of toxicity, makes MTOB a wonderful lead compound for therapeutic research.

**Analog Compounds of MTOB**

The method of most pharmaceutical companies use to take a lead drug from a lead to a product is to select the most pharmacologically effective analog of the drug through a process called high-throughput screening. This was similar to the purpose of this project, on a much smaller scale. By examining structural analogs of MTOB it may be possible to find a chemical with a similar or better ability to bind CtBP2, and additionally lower the compound’s expense for production. The major issues with the use of MTOB as a therapeutic agent primarily reside in its expense, and the high dosages at which it is most effective.

This project focused on using two different analogous compounds to test their efficacy in comparison to MTOB. These compounds are 4-methylthio-2-hydroxybutyric acid (MTHB) and L-phenyllactate (Figure-2). These compounds vary only slightly from MTOB. Where MTOB has a ketone adjacent to the carboxyl group (alpha ketoacid), MTHB has a hydroxyl group (Summers, et al., 1998). In the case of phenyllactate, a phenyl ring replaces the thioester at the end of the carbon chain (Collier, Butler, & Mitch, 1980).
MTHB is also involved in the methionine-salvage pathway in the body, and therefore also makes a great potential MTOB-like drug due to its similarity to MTOB and its apparent lack of toxicity. In solid form it is stored with a calcium salt where there are two molecules of MTHB for every one of CaCl$_2$ (Tang, Kadariya, Murphy, & Kruger, 2006). L-phenyllactate by itself has been only minimally studied, but one study showed phenyllactate to increase growth in normal and germ-free rats eating a phenylalanine-free diet (Collier, Butler, & Mitch, 1980). Both of these compounds were studied alongside MTOB to compare their ability to suppress CtBP2 by way of studying cell proliferation and viability.

**siRNA Technology and its Applications**

Another area of interest for this project was to examine the use of CtBP2 specific siRNA treatment in combination with lower concentrations of MTOB. The technology behind using...
small interfering RNA to silence gene expression at the translational level has only existed for a relatively short amount of time. SiRNA transfection is a process by which small segments of RNA designed to conjugate to specific target mRNAs within the cell are inserted in order to silence the target gene (Dykxhoorn, Novina, & Sharp, 2003). This is done through the use of micro RNA fragments that can conjugate to the target mRNA (Figure-3). Once these miRNA fragments conjugate, the now dsRNA is targeted by an RNA-induced silencing complex, or RISC, for degradation. Through this process of targeted conjugation and degradation a knockdown of the target gene is accomplished (Long, et al., 2010).

Figure-3. siRNA Treatment. The above graphic shows the process through which siRNA silences targeted gene expression at the translational level (Dykxhoorn, et al. 2003).

This siRNA technology has proven effective at silencing genes that code for the expression of proteins that assist in the growth and metastasis of cancer cells. Using a siRNA
specific to the gene that codes for CtBP2 should allow for a decrease in the expression of CtBP2, promoting apoptosis and decreasing cell proliferation. While there is currently no in vivo delivery methods that would allow for the use of siRNA technology as a therapeutic agent in mouse or human models, that topic is being researched (Sorensen & Sioud, 2010). Using this technology in conjunction with MTOB treatment could prove to be a very effective and targeted cancer therapeutic.
PROJECT PURPOSE

The need for a targeted and effective cancer drug with limited toxicity to healthy cells is evident. While MTOB is a promising cancer treatment that has shown promise against a variety of cancer cells \textit{in vitro}, it also presents difficulties due to its high cost at effective doses. The purpose of this Major Qualifying Project was to take this therapeutic agent, and explore the use of its various structural analogs, in combination with siRNA treatments against a known tumor-inducing transcription factor CtBP2, to increase the efficacy of the drug. The hypothesis tested in this project was that an MTOB analog, or a combination treatment including a CtBP2-specific siRNA knockdown, could work as effectively or more effectively than MTOB alone. It was also the intention to find methods that could reduce the effective dosage of MTOB to reduce the overall cost of treatment.
METHODS

Cell Culture

Cell Lines

The two cell lines used for the combination siRNA and MTOB treatments were H1299 and U20s cells. H1299 cells were originally isolated from a non-small cell lung carcinoma metastasized to a lymph node, and these cells are p53 negative. U2OS cells were originally isolated from an osteosarcoma, and are p53 positive. For the colony assays done to test the comparative efficacy of MTOB analogs, the HCT116 -/- cell line (HCT-/-) was also used. HCT -/- cells are a line of human colorectal carcinoma cells which have a targeted deletion of both p53 alleles, making them p53 negative. Each of these cell lines was originally provided by Mike Straza, and later H1299 and U20S cells were provided by Roman Kulikov (PhD).

Cell Subculture and Plating

For the H1299 and U20S cell lines, high glucose DMEM media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (Pen Strep) was used for culture inside of BD falcon T25 culture flasks. For HCT-/- cells, McCoy’s Media supplemented with 10% FBS and 1% Pen Strep was used for culture inside of T25 flasks as well. When subculturing, after the media was aspirated off the cells, each flask was washed with Phosphate Buffered Saline (PBS), and then 0.5 ml of trypsin for was incubated with the cells for 3-5 minutes to remove the adherent cells. 5.0 ml of medium was used to deactivate the trypsin, and then the proper dilution ratio was used to either plate or re-culture cells into flasks.
Cell Treatments

A 40 mM stock of each of the compounds used was created in each of the media used for each cell line. For MTOB, 62 mg of MTOB was added per 10 mL of each media. In the case of MTHB, 136 mg of the compound was used for 10 ml of media, 66 mg was used for Phenyl Lactate, and 59 mg was used for CaCl₂. Each solution was then mixed until dissolved, and filtered into a new 15ml falcon tube. The resulting 40 mM solution was stored at 4°C for use for up to 7 days. The media was then diluted in a separate 15ml falcon tube for each dilution needed, and 2 ml of the proper concentration was added to the corresponding well for each assay.

Colony Assays and Cell Staining

The cell lines U20S and HCT -/- were used for this assay. The assay was performed by plating 100,000 cells/well into each well of a 6-well plate. The cells were then allowed to adhere and grow for 24 hours. These cells were then treated in duplicate with varying doses of either MTOB, MTHB, L-phenyl lactate, or 2 ml of compound-free media for the control. Because MTHB is stored in CaCl₂, there are two molecules of MTHB for every molecule of CaCl₂ meaning that the concentrations used actually indicate double that concentration of MTHB. After 72 hours of compound treatment, the medium was aspirated, and 2 ml of fresh compound-free medium was added to the wells. After 4 days of recovery, the cells were fixed to the 6-well plates in order to be stained.

For fixing the cells to the plates, the medium was first aspirated off the cells, and they were then washed with 2 ml of cold PBS for each well. 2 ml of a solution of 70% methanol and 30% glacial acetic acid was then added to each well, and plates were placed at -20°C for 15-20 minutes. The fixing solution was then removed from the wells, and the wells were allowed to dry
completely before staining. Giemsa stain was added at a 1:5 dilution with diH$_2$O from the commercially available stock solution at a volume of 2 ml per well. The stain was allowed to stay on the wells for 5-10 minutes before being rinsed repeatedly, submerging the 6-well plate into water. The plates were then stored at room temperature. Photographs of stained wells were taken by a 10-megapixel camera.

**siRNA Transfection**

The siRNA transfections were performed by first incubating 4 µl of oligofectamine per single well reaction with 24 µl of MEM serum-free medium for 10 minutes at room temperature in a culture hood. After this period, a combination of 3 µl of either control (scramble) siRNA or CtBP2 specific siRNA, and 97 µl of MEM serum free medium was added to the oligofectamine reaction mix, and allowed to incubate for an additional 30 minutes. After this incubation period, the full 128 µl reaction was added drop wise to 2 ml of the appropriate medium in each of the wells to be treated with siRNA, while gently swirling the media in the wells.

**Combination siRNA and MTOB Treatments**

H1299 and U20S cells were both used with this assay. The cells were plated in 6-well plates at noted concentrations (either 30,000 or 15,000 cells per well) and allowed to grow for 24 hours. These plates were then transfected with either control (scramble) siRNA, or CTBP2 siRNA using the protocol discussed above. The siRNA treatment was taken off the cells by aspirating the media at either 12 or 24 hours, which is noted in the results. After aspirating the siRNA media, the cells received either fresh media for the controls or siRNA alone, or they were treated with medium containing a specific concentration of MTOB, most often at 1mM. The cells
were left to grow with the treated media with MTOB or the control media for 72 hours, at which point the viability assay was run using Trypan Blue (protocol below). Figure-4 shows a representation of the plating process with an image of each of the compounds and controls used in the assay.

![Design of the Six Well Plate Layout for the Combination of siRNA and MTOB Treatments.](image)

**Figure 4.** Design of the Six Well Plate Layout for the Combination of siRNA and MTOB Treatments.

**Viability Assays with Trypan Blue**

Cell viability was determined using a Trypan blue assay on the cells collected from each well after 72 hours of treatment. The supernatant was removed and collected from each well, and the wells were washed with a very small quantity of trypsin to deactivate the cells. After deactivation, the cells were trypsinized, and each well was scraped to remove all the adherent cells. 0.5 ml of PBS was used to wash the wells and ensure all the cells were collected. An aliquot of the cell suspension diluted 1:1 with Trypan blue was placed into a hemocytometer to count live and dead cells. Each well was counted with three separate counts. The remaining unstained cells were lysed using 1 ml of lysis buffer. These lysates were used in to perform
western blots. Prior to being tested for viability, the plates were photographed at 200x magnification to illustrate the differences between the wells.

**Western Blotting**

Using a Bradford Assay, protein concentrations from the lysates mentioned above were determined. These concentrations were used to normalize the amount of protein loaded into each well of a 4-12% polyacrylamide gel. Transfer was performed to a nitrocellulose membrane. After being washed overnight in 5% milk, the membranes were probed with CtBP2 and GAPDH as a primary antibody, and subsequently washed with a mouse secondary antibody and developed with enhanced chemiluminescence reagents.
RESULTS

The overall purpose of this project was to find a more effective method of treating cancer cells through either siRNA treatments, and/or treatments with analog compounds of MTOB. This study was conducted by performing a variety of viability-based assays, such as the trypan blue assay and colony assay.

Viability Assays Using siRNA and MTOB on U20S Cells

Figure 5 shows the effect of CtBP2 siRNA alone and in combination with 1 mM MTOB. This image was taken at 200X magnification, and shows a typical grouping of cells in each of the 6-well plates. For all the U20S plates used for this assay the siRNA treatment remained on the cells for 24 hr, and the MTOB for 72 hr. The microscopy indicated that the combined treatment inhibited cell division best.

Figure 5. CtBP2 siRNA Combination Treatment with MTOB in U20S Cells. The above images show the Untreated (A), 1 mM MTOB (D), Control siRNA (B), Control siRNA with 1 mM MTOB (E), CtBP2 siRNA (C), CtBP2 siRNA with 1 mM MTOB (F). These wells were plated at 30,000 cells/well, treated with siRNA for 24 hours, then treated with 1mM MTOB for 72 hours.
**Figure-6** shows the normalized data for each repeat of this U2OS viability assay. It clearly shows the combined treatment works best, both on cancer cell viability (percentage of cells that are living versus those that are dead), and on cell numbers (number of cells in each well compared to the control).

![U2OS Viability](image)

**Figure 6. Normalized Viability Data for U2OS Cell Assays.** The above graph shows the viability of U20S cells treated with CtBP2 or control siRNA with or without MTOB for 24 hours, as quantified by a trypan blue viability assay. The percentage of live versus dead cells is shown, in addition to the percentage of cell numbers compared to the control. The histobars represent the mean of 3 experiments. Error bars denote standard error.

Whole cell lysates were prepared from the U2OS cells collected at the end of the viability assay for immunoblot analysis to determine expression of the CtBP2 protein to see if protein expression differed from the CtBP2 siRNA-treated cells, and those treated with CtBP2 siRNA in combination with MTOB. **Figure-7** is a gel image chosen in which the cells were treated with 2 mM MTOB, but this image was quite similar to all other immunoblots run using this cell line. The primary antibodies for this assy included CtBP2, and GAPDH as a positive control. Both
were developed with a mouse secondary antibody. The data indicate that the CtBP2 signal is lowest in the cells treated with both MTOB and CtBP2 siRNA.

![Western Blot of CtBP2 Protein Expression](image)

**Figure 7. Western Blot of CtBP2 Protein Expression.** The above images show the total cellular levels of CtBP2 and GAPDH in U20S cells treated with various combinations of CtBP2 siRNA and 2 mM MTOB. The Western Blot was probed for CtBP2 protein, and for GAPDH as a positive control.

**Viability Assays Using siRNA and MTOB on H1299 Cells**

The most notable difference between the H1299 assays and the ones performed on U20S cells was the amount of time the siRNA treatment was performed on the cells. Because oligofectamine, used in the siRNA transfection, has some cell toxicity, a change in the protocol was made to allow for a shorter incubation time. This allowed for the analysis of the effect of oligofectamine on the cells, as well as a comparison of efficacy of the siRNA when the delivery time is shortened. **Figure 8** shows the effect of the various treatments via a microscopy images taken at 200X magnification. This image is a good representation of what other plates that underwent the same protocol looked like after the 72 hour compound treatment period. As with
the p53-positive U2OS cells, the p53-negative H1299 cells showed the least cell numbers in the combined MTOB/siRNA treatment.

Figure 8. Microscopy of Various Treatments on H1299 Cells. The above images show: Untreated (A), 1 mM MTOB (D), Control siRNA (B), Control siRNA with 1 mM MTOB (E), CtBP2 siRNA (C), and CtBP2 siRNA with 1mM MTOB (F). These wells were plated at 30,000 cells/well, treated with siRNA for 12 hours, then treated with 1mM MTOB for 72 hours.

The H1299 viability data is quantified in Figure 9. As with the U2OS cells, the least number of cells and lowest tumor viability is observed for the combined treatment.
H1299 lysates were used to run Western Blots to determine expression of the CtBP2 protein to see if the CtBP2 levels differed with the various treatments (Figure-10). The primary antibodies for this gel included CtBP2, and GAPDH as a positive control. Both were developed with a mouse secondary antibody. As was observed for the U2OS cells, the weakest CtBP2 band occurred with the combined treatment.

**Figure 9. Normalized Viability Data for H1299 Cells.** The above graph shows the viability of H1299 cells treated with CtBP2siRNA for 12 hours as quantified by a trypan blue viability assay. The percentage of live versus dead cells is shown in addition to the percentage of cell numbers compared to the control. Histobars denote the mean of 3 experiments. Error bars denote standard error.
Colony Assays Using U2OS Cells Treated with Analog MTHB

The U2OS colony assays were plated with 10,000 cells/well for each of the 6-well plates. After the 72-hour compound treatment, and 4-day cell recovery period, the cells were fixed and stained. Figure 11 shows a photograph of one of the duplicate sets of treatments from a 6-well plate treated with two concentrations (4 mM and 10 mM) of MTOB, to serve as a control to compare with the analog MTHB.
Figure 12 shows a photograph of one of the duplicate sets of treatments from a 6-well plate treated with two concentrations (4 mM and 8 mM) of analog MTHB. Due to the CaCl₂ in the solid stored form of MTHB, the negative control includes 4 mM CaCl₂ which is the amount of CaCl₂ present in the 8 mM MTHB treatment.

![Figure 12. Colony Assay of U2OS with MTHB Treatment. The wells above are from a colony assay with 72-hour treatments of control media with 4 mM CaCl₂, 4 mM MTHB, and 8 mM MTHB, left to right, respectively.](image)

Colony Assays Using HCT -/- Cells Treated with MTHB and Phenyllactate

The HCT -/- colony assays were plated with 100,000 cells/well for each of the 6-well plates. After the 72-hour compound treatment, and 4-day cell recovery period, the cells were fixed and stained. Figure 13 shows a photograph of one of the duplicate sets of treatments from a 6-well plate treated with decreasing concentrations (4 mM and 1 mM) of MTOB, to serve as a control to compare against the analog compounds MTHB and Phenyllactate.
Figure 13. Colony Assay of HCT Cells with MTOB Treatment. The wells above are from a colony assay with 72-hour treatments of control media, 4 mM MTOB, and 1 mM MTOB, left to right, respectively.

Figure 14 shows a photograph of the fixed and stained MTHB-treated plates in the same increasing concentrations of 4 and 8 mM, as the previous figure. The control used here was the same 4 mM CaCl$_2$ used in the U20S cell line.

Figure 14. Colony Assay of HCT Cells with MTHB Treatment. The wells above are from a colony assay with 72-hour treatments of control media with 4 mM CaCl$_2$, 4 mM MTHB, and 8 mM MTHB, left to right, respectively.
**Figure-15** shows the effect of Phenyllactate on HCT -/- cells using increasing concentrations (4 and 10 mM). The control on this experiment was media with no compounds added, as with the MTOB treatments.

*Figure 15. Colony Assay of HCT Cells with Phenyllactate Treatment.* The wells above are from a colony assay with 72 hour treatments of control media, 4 mM Phenyl Lactate, and 10 mM Phenyl Lactate, left to right, respectively.
DISCUSSION

Primary Conclusions

The data from this project shows that a combination treatment using CtBP2 siRNA and MTOB is markedly more effective than either treatment alone, and the particular analog compounds tested were not as efficacious as MTOB in the same doses. Figures 6 and 9 heavily support the primary hypothesis of the effect of a combination treatment providing incredibly promising results for enhancing the efficacy of MTOB in future experiments.

Additionally, the expression levels of CtBP2 as demonstrated by the western blot films in Figures 7 and 10 show a noticeable CtBP2 protein decrease in the CtBP2 siRNA lane, and surprisingly even more so in the combination CtBP2siRNA and MTOB lanes. While this expression decrease is not so striking in the H1299 blot due to uneven protein loading of the wells, it is certainly more convincing in Figure 7 with the U20S line.

This is a fascinating conclusion, as the research done on the interaction between MTOB and CtBP2 would not lead to a change in the expression of CtBP2, but rather just inhibit the protein’s ability to bind and affect the anti-migratory and apoptotic pathways in cells. While this could indicate a great number of things, and requires further investigation, a likely conclusion would be that in binding to CtBP2 (seemingly more so in the presence of CtBP2 siRNA in which there is less CtBP2 to bind, increasing the ratio of MTOB to CtBP2), MTOB in some way destabilized the protein causing degradation. If this were the case, it would make this treatment option even more effective as it would be combating not only the binding of CtBP2, but also the problem of its up-regulation.
The analog compounds in this study did not perform as effectively as desired. The process of finding effective and efficient pharmacological analogs of a lead compound is a very difficult process, and one that can take pharmaceutical companies years. The two used in this study were beneficial in their comparably low cost and similar structure, however when examining the colony assays, it is very clear that the amount of each analog needed to obtain equivalent cytotoxic effects is far greater than that of MTOB, making them poor agents for a deliverable drug. While MTOB and these analogs have a unique benefit in cancer therapy of being relatively harmless to normal cells, which is drastically different than most treatments such as chemotherapy, any drug in such large doses still presents problems with delivery and cost.

**Problems Faced and Questions Raised**

One of the most significant problems that arose throughout the project was the adjustment of the protocol for the siRNA transfections and treatments to combat oligofectamine’s toxicity to normal cells. While siRNA has had countless beneficial applications to the field of research, there is currently much discussion and research focused on viable delivery methods for this technology, as no current delivery method exists for *in vivo* experiments. One adjustment that was made to lower the toxicity was the amount of oligofectamine used, which was lowered from 6 µl to the 4 µl used for the experiments presented in this project. The other was the amount of time that the cells were exposed to the siRNA treatment. In Figures 6 and 9, there is a strong difference between the percentages of cells present in the control siRNA well compared to the control between U20S (treated for 24 hours) and H1299 (treated for 12). This would indicate a decreased toxicity effect by the oligofectamine, while the shortened exposure time does not appear to impact the potency of the
siRNA itself. However this lethality comparison cannot be limited to the difference in siRNA treatment time alone as they are two separate cell lines, which vary in their robustness.

Most of the other problems encountered throughout the project were limited to some normal trial and error. However, a particular issue that stands out stemmed from the effectiveness of the combined siRNA and CtBP2 experiment. As can be seen in Figures 5 and 8, the wells with the combination treatments were so impacted that the normal method of spinning down cells to form a pellet prior to performing the trypan blue assay was not possible. Because of this, the volume that the cells were suspended in was greater than desired, making for a greater degree of variance between trials due to lower cell numbers in the 10 µl samples. This did not pose a tremendous issue, as the effect was very similar in all trials, and the standard deviation was not too great.

**Future Experiments**

The Grossman lab has already discussed the possibility of injecting mice with the CtBP2 siRNA both alone and as a combination therapy with MTOB, though there is still a great deal of research needed before this combination therapy could be used in any human clinical testing. More analog compounds will be tested, in addition to testing some combination treatments with MTOB and analogs using a Trypan Viability Assay. More repeats of the experiments shown in this project will be conducted to enhance the quality of the data. There is of course more to be researched before MTOB can be brought to trials, however multiple studies have confirmed that it is a promising lead compound for anticancer therapy.


