Targeting Protein Arginine Methytransferase 5 (PRMT5) Overexpression by Use of Small Molecule PRMT5 Inhibitors in Glioblastoma Multiforme (GBM)

Honors Research Thesis

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ABSTRACT

High grade astrocytomas (grade III and IV tumors) are the most common and aggressive brain tumors that carry a bleak prognosis with an average survival of less than 15 months despite multimodal therapy. Therefore, there is a great need for new therapies to target grade III and grade IV (glioblastoma multiforme, GBM) astrocytomas. Recent studies have shown epigenetic regulation of chromatin plays a key role in cell growth, differentiation, and survival. Chromatin remodeling enzymes like histone deactylase (HDAC), DNA methyltransferase, and protein arginine methyltransferase 5 (PRMT5) are involved in silencing tumor suppressor gene (TSG) expression and contribute towards cellular transformation. PRMT5 contributes towards transcriptional inhibition of several regulatory genes by symmetric dimethylation of arginine residues on histone proteins (histone 4 arginine residue 3 (H4R3) and H3R8) and works more efficiently when associated with other co-repressor enzymes. Our lab has shown that epigenetic processes driven by over expression of PRMT5 are important in regulation of oncogenic pathways operative in GBM. Our studies have shown that the amount of PRMT5 over expression inversely correlates with the survival of GBM patients and correlates directly with proliferation of these cancerous astrocytes. Our lab developed small RNA molecules (siRNA) that inhibit PRMT5 expression which leads to loss of symmetric dimethyl H4R3 and transcriptional de-repression and translation of tumor suppressor and immune modulatory genes. PRMT5 knockdown in GBM cells by this siRNA leads GBM cells to undergo cell cycle arrest, apoptosis, inhibition of cell migration, and sensitization to the anti-tumor effects of temazolomide, a drug used in therapy for GBM patients. Because therapy using siRNA is still in the experimental stage, there was a need to look for small molecule compounds that could inhibit

PRMT5 in a similar way. Using a computational modeling system, our lab identified several compounds that looked promising. Two compounds were identified as selective inhibitors of PRMT5 activity (CMP5 and BLL54). Both compounds were able to selectively inhibit the methylation of histone 4 arginine 3. This knockdown led GBM cells to undergo cell cycle arrest and apoptosis. When used in combination with other inhibitors, we saw de-repression of transcriptional activity and restoration of protein expression of the chemokine CXCL10 which was identified as a potential target of PRMT5. Both compounds were able to induce these effects, but BLL54 showed to be more potent. These findings identify these small molecule PRMT5 inhibitors as a new potential drug regimen for patients afflicted with this cancer.

INTRODUCTION

1.1 Biology of Astrocytomas

Gliomas are the most common form of tumor that affects the human brain. Astrocytomas, the most common type of glioma, is a specific type that affects the star-shaped glial cells called astrocytes. Astrocytes are the most common type of cell in the brain, causing this to me the most frequently diagnosed brain cancer. Astrocytomas are classified into four different grades (I, II, III, IV), according to the World Health Organization (WHO). Grade II, III, and IV astrocytomas involve diffuse zones of infiltration that can cause compression, invasion, and destruction of normal brain tissue. [(1)]

High grade astrocytomas (grade III and IV or glioblastoma multiforme, GBM) are incurable, aggressive malignancies that account for more than 14,000 new cases each year just in the United States. [(2)] Despite using many treatments, the median survival is less than 15 months despite aggressive therapy. [(3)] At this point, surgery is the only curative option, however, the invasive nature of these tumors makes cure extremely rare. The addition of radiation, chemotherapy, and the newly-FDA-approved agent temozolomide, add minimal benefit to duration of remission and survival. Discovery of effective therapies has been limited due to the complex pathogenesis of this disease. There have been several genome-wide studies that have demonstrated that GBM has a remarkable degree of heterogeneity when it comes to genetic mutation, gene expression, and epigenetic modifications. [(4-6)] This heterogeneity has made it difficult to find any sort of selective agent to fight this disease. Thus, the discovery of new agents to treat this disease is highly desirable.

1.2 Genetics of GBM

In general, at the molecular level, cancer is caused by mutations in DNA, which causes abnormal cell proliferation. Today, the science world is beginning to understand that there is a mechanism beyond the genetics stored in a nucleotide sequence that can affect the development of cancer. This new area, called epigenetics, includes modifications that can alter gene expression without changing the DNA sequence. This has shed more light on the complex disease of cancer and given more clues on how cancer could develop.

Recent studies have shown epigenetic regulation of chromatin plays a key role in cell growth, differentiation, and survival of patients diagnosed with various cancers. Examples of this chromatin remodeling would include DNA methylation and histone modification. Chromatin remodeling enzymes like histone deacetylase (HDAC), DNA methyltransferase, and protein arginine methyltransferase 5 (PRMT5) are involved in silencing tumor suppressor gene (TSG) expression and contribute towards cellular transformation (Figure 1). The SWI/SNF complexes are a group of proteins that work to remodel the way DNA is packaged, often aiding in transcription activation; however, in combination with PRMT5, it leads to repression of important anticancer genes. The PRMT5 enzyme is a type II (symmetric) arginine methyltransferase that utilizes the donor molecule S-adenosyl-L-methionine to catalyze the transfer of a methyl group to two of three guanidino nitrogen atoms within the arginine molecule. PRMT5 drives the formation of both symmetric dimethylarginine (S2Me) residues to affect a wide range of key biologic functions at the level of chromatin to control transcription and other regulatory processes. [7-13]

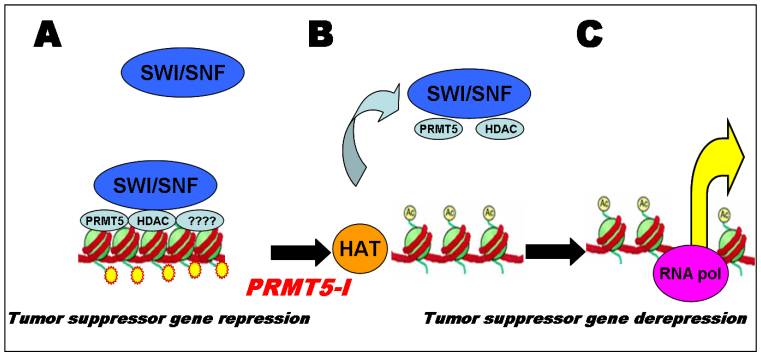


Figure 1. Epigenetic repression of anticancer genes and rationale for targeting PRMT5. (A) Both activating and repressive SWI/SNF complexes co-exist in the cellular proteome. Hypoacetylation of histones H3 and H4 promotes PRMT5-driven methylation of arginine residues resulting in condensed nucleosomes and repression of target genes. (B) Following treatment with PRMT5 inhibitors, enzymes promoting gene expression are able to access chromatin and (C) restore expression of regulatory genes. [14]

1.3 Recent Studies

Our lab found that PRMT5 is over expressed in GBM cell lines and in primary GBM tumors [15]. We found that inhibition of PRMT5 with siRNA molecules led to loss of symmetric dimethylation of the third arginine residue on the tail of histone 4 (H4R3, S2Me-H4R3) in GBM cells that correlated with restoration of critical regulatory pathways affecting GBM cell growth and survival. When PRMT5 catalyzes the epigenetic modification of H4 to S2Me-H4R3, this leads to nucleosomal compression and tightening of chromatin structure, preventing transcriptional machinery from docking onto the DNA and accessing gene promoters. The outcome is global gene repression (which is a common feature of cancer). Because of PRMT5 activity knockdown, many PRMT5 target genes became re-expressed leading to apoptosis and sensitivity to other anti-tumor drugs. However, at this point, treatment with siRNAs is still in the experimental stage, so we wanted to look for a small molecule inhibitor that could have the same effect.

Using a computational model, created by Dr. Chenglong Li, in the College of Pharmacy, and Dr. Baiocchi, we discovered a new class of drugs that selectively targets and inhibits

PRMT5. The crystal structure of homologous PRMT enzymes were used as a template to construct a human PRMT5 3-D model because the human PRMT5 crystal structure was unknown. Rat PRMT1 crystal structure was aligned with Hu PRMT5. The 3D model was assembled with modeler software and is seen in Figure 2. The red circle indicates the active site of the molecule and in Figure 2B we see that active site zoomed in. The active sites of crystal rat PRMT1 (left) and human model PRMT5 (right). The green molecule is the S-adenosyl-L-methionine (SAM) cofactor, the methyl donor, and below that, an arginine peptide. Next, SAM and arginine were docked into the PRMT5 model and showed arrangement similar to Rat PRMT1. Next the PRMT5 model was used to screen 10,000 small molecules and the top hits, with the lowest binding energy, were theorized to be effective PRMT5 inhibitors. [23]

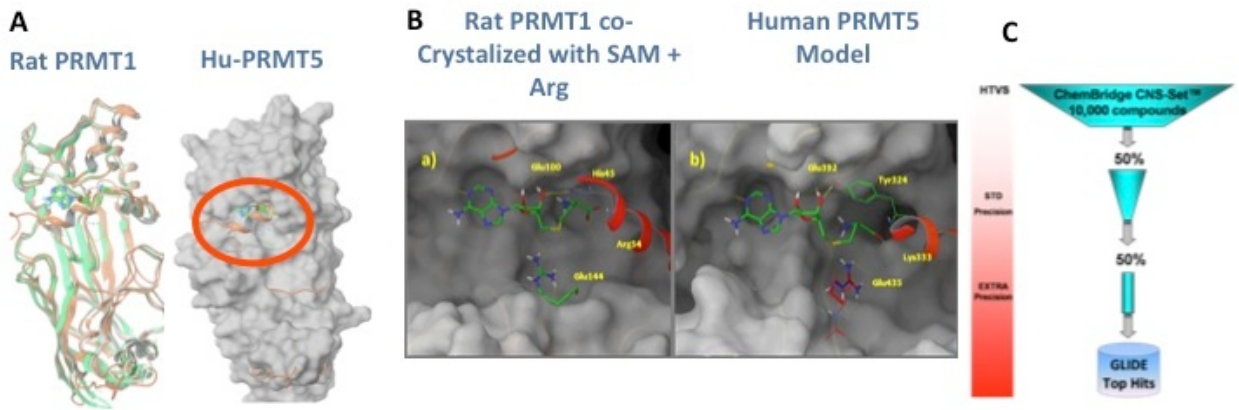


Figure 2. Drug development of PRMT5 inhibitors. 2A) The known rat PRMT1 molecule was aligned with human PRMT5 to synthesize what the crystal structure of human PRMT5 might look like. The red circle indicates the active site of this molecule. 2B) The active site of rat PRMT1 and the human PRMT5 model are shown here. 2C) 10,000 small molecule compounds were docked into the active site to block the transfer of the methyl groups from the methyl donor SAM to the arginine molecule.

This was the first type II PRMT enzyme inhibitor of its kind. Unlike siRNAs, these small

molecule inhibitors would be practical in creating a new anti-tumor drug because these small molecule compounds are more easily delivered and absorbed.

1.4 Study Rationale and Hypothesis

We hypothesized that generation of novel small molecule inhibitors of PRMT5 would allow for rapid development of agents capable of targeting this newly discovered oncogenic pathway and improve strategies to treat patients with GBM. It was hypothesized that with the PRMT5 inhibitory activity of these small molecule compounds, chromatin repression of anti-tumor genes could be reversed and transcription of these genes would be restored. This work would allow us to address the use of these agents to evaluate anti-tumor activity *in vitro* and will aid in testing its uses in preclinical animal models of human GBM. The promising compounds will enhance our efforts to develop an experimental therapeutic program in GBM. This work can have a significant impact on cancer research because of the severity of this type of cancer and the lack of reasonable treatment options for patients diagnosed with GBM.

MATERIALS AND METHODS

2.1 Cell Culture

The human GBM cell lines were provided by Dr. E. Antonio Chiocca. Human GBM cells were maintained in DMEM growth medium, from Life Technologies (Grand Island, NY), supplemented with 10% heat-inactivated FBS and 100 units/mL of penicillin and streptomycin, and cultured at 37°C with 5% CO_2.

2.2 Antibodies and Reagents

β-actin, PRMT5 specific antibodies and the anti-rabbit antibody linked to horseradish peroxidase were purchased from Cell Signaling Technologies (Danvers, MA). CXCL10 (IP-10) polyclonal antibodies were from Abcam (Cambridge, MA). Symmetric-H4R3 polyclonal antibody was purchased from Abcam (Cambridge, MA). FITC-labeled goat anti-rabbit antibody and DAPI were from Sigma (St. Louis, MO). Annexin V and propidium iodide (PI) were from BD (Franklin Lakes, NJ). MTS reagent was from Promega (Madison, WI). The CXCL10 ELISA kit was from R&D Systems (Minneapolis, MN).

2.3 MTS Assays

Human GBM cells were plated at a density of 2,000 cells/well in 96-well microplates. The next day, cells were treated with CMP5, BLL54, or DMSO control for 24, 48, and 72 hours. At the end of culture, a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS, 5 mg/mL] (Promega, Madison, WI) and an electron coupling reagent (phenazine methosulfate, PMS) were mixed and 20 μl of the mixture was added to each well, and plates were placed at 37°C for 3 hours. The number of viable cells was quantified using the CellTiter 96 AQ_{ueous} cell proliferation assay

(Promega), absorbance was measured at 570 nm using a multiwell spectrophotometer (Emax; Molecular Devices, Sunnyvale, CA).

2.4 Cell Cycle Analysis

Cells were plated at 200,000 cells/well in 6-well plates. The next day, cells were treated with CMP5, BLL54, or DMSO control for 24 and 48 hours. Conditions were collected using 0.25% Trypsin-EDTA and washed twice with sample buffer [1 g of dextrose in (1000 mL) Ca^{++} Mg^{++} free PBS]. The cells were then fixed in 75% EtOH [added dropwise] at 4°C overnight. Before analysis, cells were stained with propidium iodide staining solution [sample buffer, RNase A (1 mg/ml), propidium iodide (5 μg/ml)] for 30 minutes at room temperature. Analyses were performed with a Beckman flow cytometer.

2.5 Apoptosis Analysis

Cells were plated at 200,000 cells/well in 6-well plates. The next day, cells were treated with CMP5, BLL54, or DMSO control for 24 and 48 hours. The treated cells were collected using 0.25% Trypsin-EDTA and labeled using Annexin-V and propidium iodide (PI) as recommended by the manufacturer (BD Biosciences, San Jose, CA). Annexin-V and PI positive cells were measured using a Beckman flow cytometer. All experiments were repeated three times.

2.6 Western Blot Analysis

Cells were collected (by scraping) and lysed with lysis buffer containing protease inhibitors and phosphatase inhibitors. Whole cell lysates were collected following centrifugation, and aliquots containing equal amounts of protein from samples were resolved by SDS-PAGE (using 14% gel), transferred onto a polyvinylidene difluoride (PVDF) membrane, and dipped in 100% methanol and allowed to dry. The membranes were probed with primary antibodies specific for PRMT5, sym-H4R3, asm-H4R3, CXCL10 (IP-10), or β-actin overnight at 4°C in 5% nonfat

milk. After four washes with TBS-T buffer, membranes were incubated with appropriate horseradish peroxidase-linked secondary antibodies. Protein signals were detected with an enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent Substrate or SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL).

2.7 PCR and Real-time quantitative RT-PCR

Total RNA was prepared from GBM cells treated with CMP5, BLL54, TSA, 5-AZA, or a DMSO control using TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The yield and quality of RNA was evaluated by measuring its absorbance at A260/A280 using a NanoDrop spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE). The cDNA was prepared with the MMLV Reverse Transcription Kit (Invitrogen) following the manufacturer's recommendations. Real-time PCR was performed using a TaqMan 2 × Universal PCR Master Mix kit on an Applied Biosystems 7900HT Fast Sequence Detection System. The 10 μl PCR reaction included 1 μl RT product, 1×TaqMan Universal PCR Master Mix, 0.2 μM TaqMan probe, 0.5 μM forward primer and 0.5 μM reverse primer or 20 × TaqMan primers and probe (Applied Biosystems/Life Technologies, Grand Island, NY). The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Copy numbers were normalized to GAPDH control amplification.

2.8 Immunofluorescence

Cells were plated at 200,000 cells/well in 6-well plates. The next day, cells were treated with CMP5, BLL54, or DMSO control for 24 and 48 hours. The treated cells were collected using 0.25% Trypsin-EDTA and cells were collected following centrifugation, and aliquots containing equal amounts of cells were fixed with Cytofix/Cytoperm solution for 20 minutes on ice. After

washing with 1X Perm/Wash Buffer twice, the cells were blocked with 10% goat serum in PBS for 1 hour at room temperature, then incubated with primary antibody (1 µL/100 µL of 1X Perm/Wash) overnight at 4°C. The cells were washed three times with 1x Perm/Wash buffer and incubated with FITC-conjugated anti-rabbit secondary antibody (2 µL/100 µL 1X Perm/Wash) for 2 hours at room temperature. The cells were washed twice with PBS and 2 µL of DRAQ-5 was added prior to microscopy. Images were visualized and recorded using an Olympus Flowview 1000 Laser Scanning Confocal microscope.

RESULTS

3.1 PRMT5 is over expressed in human GBM cell lines

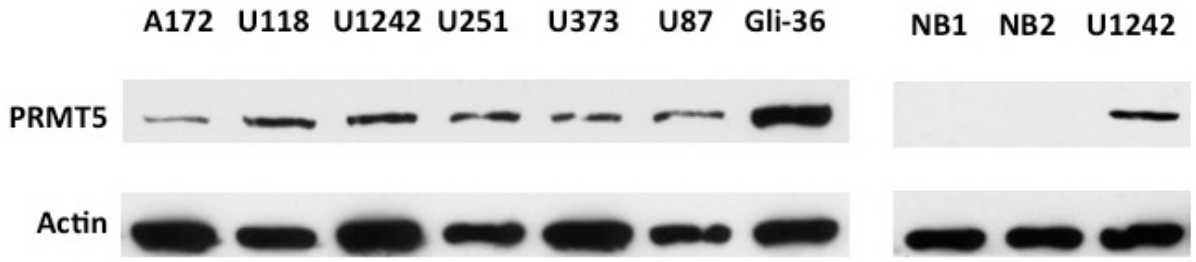


Figure 3. PRMT5 is over expressed in human GBM cell lines. Lysates from seven GBM cell lines and two normal brain cell lines were harvested and PRMT5 was detected using a PRMT5 antibody. Actin was used as a loading control.

Seven different primary GBM cell lines were shown, through western blot, to over-express PRMT5 (Figure 3). This figure also shows that normal human astrocytes and normal brain (NB1 and NB2) did not show any PRMT5 expression. Because of this, GBM cells were identified to be a good target for these new PRMT5 inhibitor compounds. These small molecule inhibitors were created to knockdown the function of PRMT5, so they would not affect these normal brain cells that do not express PRMT5. Cell lines U251 and GLI-36 were chosen to conduct the majority of experiments with. U251 is a wild-type GBM cell line that is generally more resistant to drug treatment and GLI-36 is typically more sensitive, but since it expresses a higher amount of PRMT5, we wanted to test the effects of the new inhibitors.

3.2 CMP5 is able to silence expression and activity of PRMT5

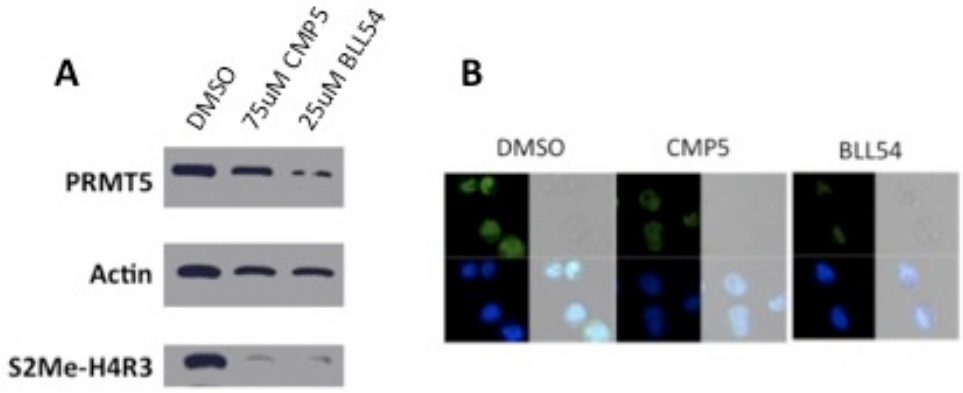


Figure 4. CMP5 and BLL54 reduced H4R3 symmetric dimethyl arginine (S2Me-H4R3). U251 was treated with DMSO alone, 75uM CMP5, or 25uM BLL54; cells were incubated with antibodies specific for S2Me-H4R3, and immunofluorescence microscopy was used to evaluate H4R3 methylation. CMP5 was shown to be able to interfere with maintenance of S2Me-H4R3 and BLL54 was able to do so to at an even lower concentration.

Since we found that expression of PRMT5 correlated with proliferation rate of GBM cells and prognosis of GBM patients (not shown), we sought to determine the consequences of PRMT5 inhibition *in vitro* with the new inhibitors. Because PRMT5 methylates histone 4 arginine residue 3 (H4R3), we looked at this as our target for whether or not these inhibitors were working. If we saw a decrease in the methylation of H4R3, then we would know that the function of PRMT5 was being inhibited even if the amount of protein itself was not decreasing. In Figure 4A, a western blot shows a decrease in S2Me-H4R3 with an increasing amount of the small molecule inhibitor compound 5 (CMP5). This shows that CMP5 is able to inhibit the transfer of the two methyl groups onto the histone 4 protein. Figure 4B shows this inhibition with confocal microscopy. The immunofluorescence detects the amount of symmetric di-methylation of histone 4 arginine 3 and we see this methylation decreasing after treatment with

CMP5 and BLL54. Referring back to Figure 1 in the introduction, this could allow de-repression of the chromatin and potentially re-allow transcription of key regulatory genes. Because these PRMT5 inhibitors act by inhibition of the function of PRMT5 and not by getting rid of the protein, the decrease of PRMT5 expression after treatment might be due to cell stress although it is unclear. Perhaps PRMT5 can regulate its own expression.

3.3 Consequences of PRMT5 silencing

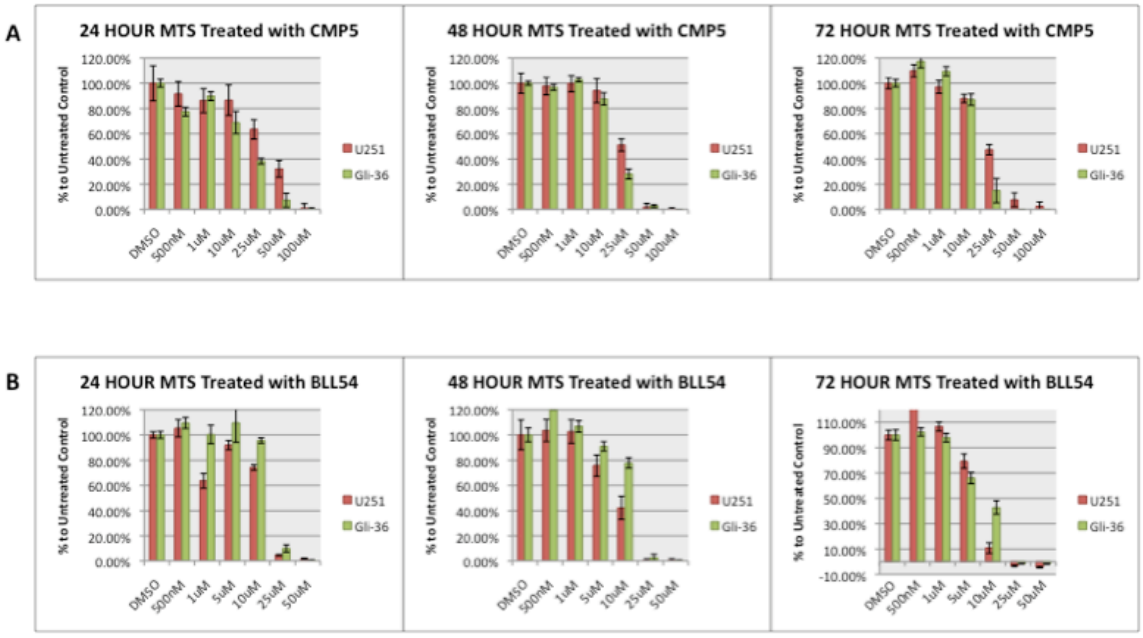


Figure 5A. PRMT5 silencing prevented proliferation of human GBM cell lines. U251 and GLI-36 cells were treated with DMSO alone or treated with CMP5. Proliferation of cells after 24, 48, and 72 hours were evaluated by MTS assay; CMP5 was shown to prevent proliferation of human GBM cell lines U251 and GLI-36.
Figure 5B. BLL54 showed inhibition of cell proliferation in U251 and GLI-36. This compound is more potent than CMP5 and has the ability to be effective at lower doses.

We next examined whether PRMT5 knockdown affected cell proliferation. U251 and GLI-36 cells were plated in a 96-well plate, treated with CMP5 or DMSO control, and cell proliferation was measured by MTS assay. As shown in Figure 5A, CMP5 elicited a significant

cell growth inhibition in both U251 and GLI-36 after the cells were treated with CMP5 for 24, 48, and 72 hours.

Figure 5B shows the proliferation assay with BLL54, a compound derived from CMP5. This compound was hypothesized to be more selective and more potent than CMP5, and we see similar cell growth inhibition with use of a lower concentration of drug. These graphs show that cell proliferation decreases as time increases showing that the drug was still having an effect even after 72 hours.

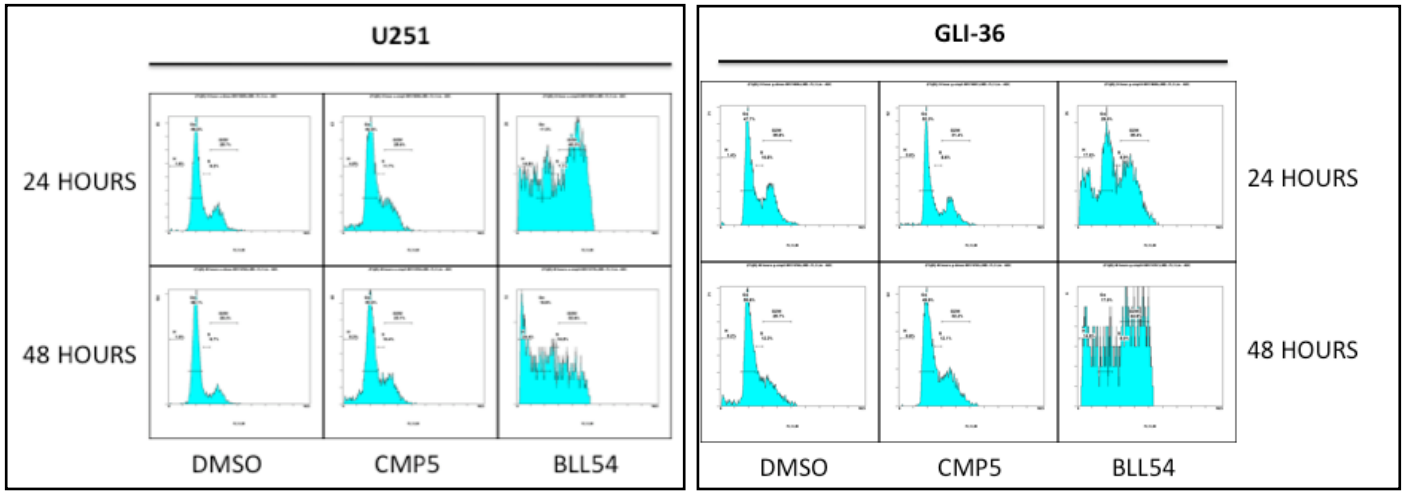
3.4 PRMT5 inhibition promotes cell cycle arrest

As shown in Figures 6A and 6B, after 48 hours, PRMT5 inhibition by BLL54 led to a decrease in the percentage of U251 cells in G1/G0 (68.1% in DMSO and 19.9% in BLL54 treatment). PRMT5 inhibition led to a time dependent increased accumulation of cells in S phase (6.7% in DMSO and 10.5% in BLL54 treatment) and G2/M (20.2% in DMSO and 32.6% in BLL54 treatment) phases of the cell cycle. In GLI-36 cells, after 48 hours the percent of cells in G1/G0 decreased (50.6% in DMSO and 17.0% in BLL54 treatment) and the percent of cells in S phase (12.3% in DMSO and 8.0% in BLL54 treatment) seemed to stay relatively the same although decreasing slightly. The percent of cells in G2/M (25.7% in DMSO and 44.9% in BLL54 treatment) dramatically increased, along with percent of cells that were hypodiploid (apoptotic). These results demonstrate that PRMT5 inhibition by BLL54 promotes cell cycle arrest and leads to hypodiploid nuclear content consistent with programmed cell death.

CMP5 seemed to have less of an effect on nuclear content of the cell. After 48 hours in U251, treatment with CMP5 led to a decrease in the percentage of U251 cells in G1/G0 (68.1% in DMSO and 53.0% in CMP5 treatment). PRMT5 down-modulation led to a time dependent

slight increase of cells in S phase (6.7% in DMSO and 10.4% in CMP5 treatment) and G2/M (20.2% in DMSO and 23.7% in CMP5 treatment) phases of the cell cycle. In GLI-36 cells, after 48 hours the percent of cell cells in G1/G0 (50.6% in DMSO and 49.9% in CMP5 treatment) and the percent of cell in S phase (12.3% in DMSO and 12.1% in CMP5 treatment) seemed to stay the same. The percent of cells in G2/M (25.7% in DMSO and 32.2% in CMP5 treatment) slightly increased. These results might indicate that CMP5 is less effective than BLL54 at inducing cell cycle arrest by way of inhibiting PRMT5.

A



B

Figure 6A. DNA content analysis by flow cytometry in U251 cells. Cells were treated with DMSO control, CMP5, or BLL54; they were stained with propidium iodide, and analyzed by flow cytometry. CMP5 and BLL54 significantly increased the ratio of apoptosis, S phase, and G2/M, and decreased the ratio of G0/G1 across the cell lines and time points compared to the DMSO control

Figure 6B. DNA content analysis by flow cytometry in Gli-36 cells. Cell were treated the same as U251. The results show similar results to U251. BLL54 is more effective on cell cycle in both cell lines.

3.5 Inhibition of PRMT5 leads GBM cells to undergo apoptosis

Other studies have shown that silencing PRMT5 with siRNA can induce apoptosis in several different cancers [16]. To see if these new inhibitory compounds could produce a similar effect in GBM, these cell lines were treated with CMP5, BLL54, and DMSO control and incubated for 24 and 48 hours. Cell viability was assessed by annexin-V-FITC/PI staining and flow cytometry. As shown in Figure 7, PRMT5 inhibition by both CMP5 and BLL54 resulted in the induction of apoptosis in both lines compared to DMSO control.

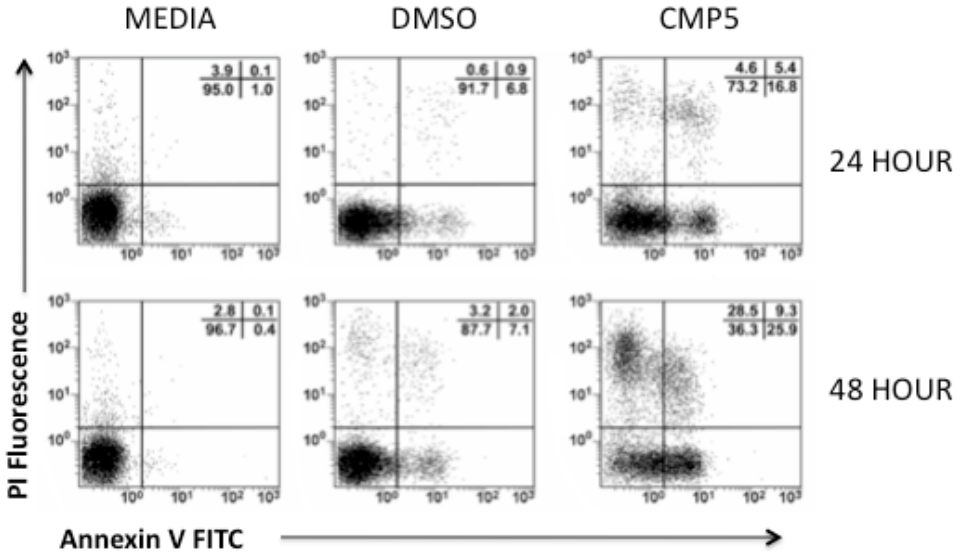


Figure 7. PRMT5 silencing promoted cell death of GBM cells. U251 cells were treated with media alone, DMSO control, CMP5, or BLL54. Cells were stained by Annexin V and PI (propidium iodide) after 24 and 48 hours, and cell death was assessed by flow cytometry.

3.6 PRMT5 inhibitors work in combination with other drugs

These next experiments were done with both CMP5 and BLL54 as well as other chromatin remodeling agent inhibitors. Referring back to Figure 1 in the introduction we see that histone deacetylases (HDAC) potentially play a role with PRMT5 to inhibit the histone acetlytransferase and other transciption factors from getting to the DNA. Because of this relationship, we decided to use an HDAC inhibitor, Trichostatin-A (TSA), in combination with the compounds. We also decided to try a hypomethylating agent (5-AZA) to combine with these drugs to see if we get a synergistic effect.

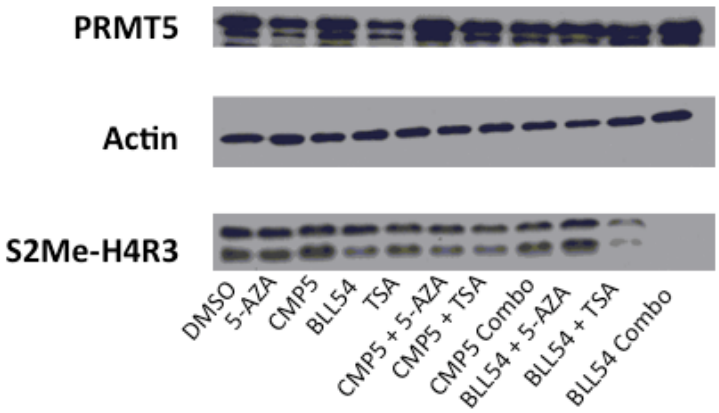


Figure 8. Symmetric di-methyl H4R3 (S2Me-H4R3) was reduced by BLL54 (small molecule inhibitors) and other anti-co-repressor drugs in human GBM cell lines. Human GBM cell line U251 was treated with DMSO alone, CMP5, BLL54, 5-AZA, HDAC, or a combination of the drugs. Symmetric di-methyl H4R3 was detected by Western blot.

Previous work has shown that PRMT5 associates with other co-repressor molecules like HDAC2 and DNA methyltransferase 3a (DNMT3a) [21-22]. Using lower doses of CMP5 and BLL54, plus the HDAC inhibitor (TSA) and hypo-methylating agent (5-AZA) led to similar results that were seen using the higher concentration of the compounds on their own (Figure 8). BLL54 showed a promising result in combination with the HDAC inhibitor and in the

combination of all three drugs. This experiment suggests the possibility of a future multi-drug treatment option targeting this protein and the promising effects of BLL54 on silencing PRMT5.

3.7 PRMT5 inhibitors work in combination with other drugs to enhance cell death

We saw that using these PRMT5 inhibitors lead to cell cycle arrest and apoptosis. When these PRMT5 inhibitors were used in combination with an HDAC inhibitor and a hypo-methylating agent, we saw a decrease in histone methylation, so we wanted to see if this produced a similar synergistic effect when looking at apoptosis. Concentrations of TSA (hypomethylating agent) and the PRMT5 inhibitor were used at concentration that had not shown to be effective on their own. After 24 hours, when using the PRMT5 inhibitor and TSA together, we see that many more cells have gone through programmed cell death than in either condition where these drugs were used individually, showing that there is synergistic effect in anti-tumor activity when multiple repressor molecules are inhibited at the same time.

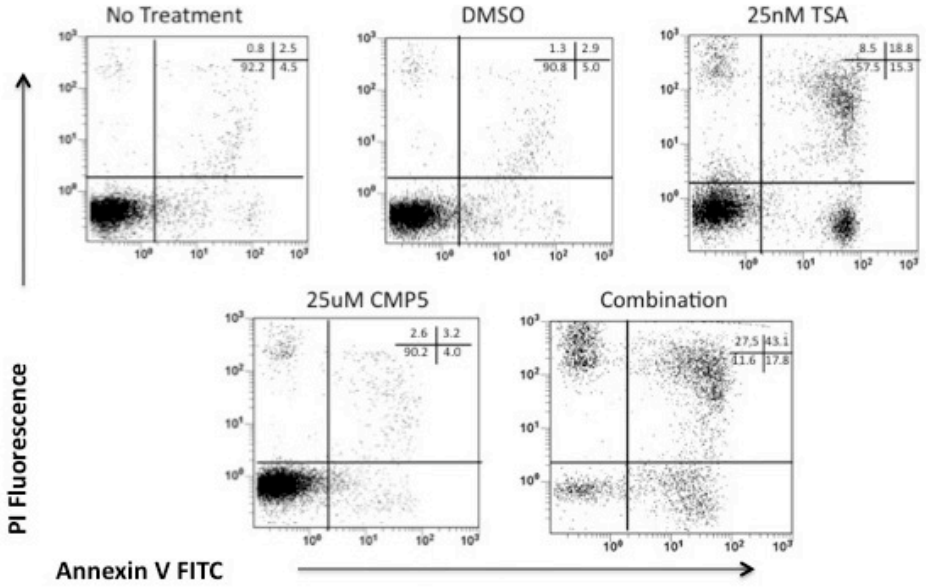


Figure 9. Enhanced cell death due to combination of drugs in GBM cells. U251 cells were treated with media alone, DMSO control, CMP5, TSA, or a combination of CMP5 and TSA. Cells were stained by Annexin V and PI (propidium iodide) after 24 and 48 hours, and cell death was assessed by flow cytometry.

3.8 Up-regulation of chemokines induced by PRMT5 and co-repressor silencing

In the past, our lab has observed the secretion of three chemokines (CCL5, CXCL11 and CXCL10) in culture medium after PRMT5 was silenced [15]. Chemokines are a small family of cytokines that conduct chemotaxis that help to regulate the trafficking and activation of leukocytes, especially dendritic cells (DCs), and B and T lymphocytes [17]. More and more evidence has demonstrated that chemokines might exert anti-tumor effects through local attraction and activation of tumor specific lymphocytes [18-20]. Even though the exact role of these chemokines is not fully understood, we hypothesized that the silencing of PRMT5 might help to increase anti-tumor immune responses through up-regulation of CCL5, CXCL10 and CXCL11. Therefore to look into this, CMP5, BLL54, and these drugs in combination with other anti-tumor drugs were used to see if they could up-regulate CXCL10 by silencing PRMT5.

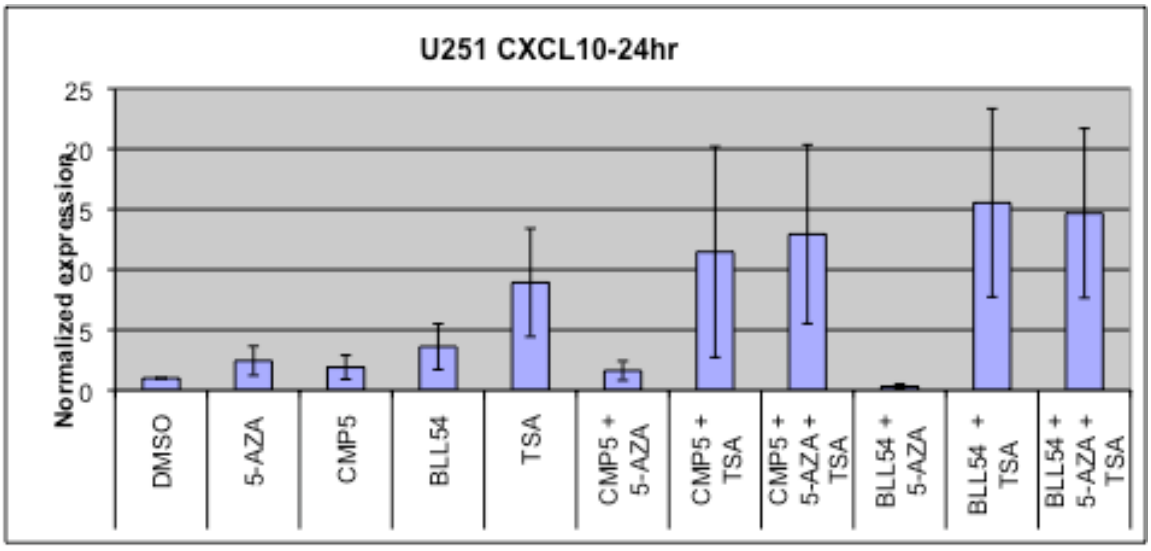


Figure 10. Transcription of CXCL10 (IP-10) is up-regulated by PRMT5 inhibitors. U251 was treated with DMSO control, CMP5, BLL54, TSA, and 5-AZA. RNA was extracted and cDNA was synthesized. Transcription of CXCL10 (IP-10) was detected by real-time PCR using specific primers.

In Figure 10 we see these results show some evidence of up-regulation of the chemokine CXCL10. The main conditions where we see this up-regulation of CXCL10 is with the use of TSA. From these data, it seems as though this histone deacetylase inhibitor has more of a role on up-regulation of this chemokine, although these data seem to show an additive effect with this addition of the compounds, especially BLL54. Since the introduction of this chemokine most likely plays a role in anti-tumor immune responses, it is exciting to see that these compounds were both able to produce an additive effect in this up-regulation effect alongside TSA. So far, all of these experiments have pointed to the capabilities of BLL54 to be a selective and potent PRMT5 inhibitor, especially in combination with other anti-GBM drugs.

DISCUSSION

Summary

This is the first time GBM cells have been treated by blocking the function of PRMT5 with small inhibitory compounds. The small molecule inhibitors, CMP5 and BLL54, proved to be effective at managing the activity of PRMT5. Use of these compounds to reduce PRMT5 activity led to reduced growth and invasiveness, cell cycle arrest, and apoptosis. Knockdown of PRMT5 led to the allowance of tumor suppressor gene transcription and the regeneration of a chemokine gene product that is capable of direct and indirect anti-tumor activity.

In addition, when CMP5 and BLL54 were used in combination with associated co-repressor molecule inhibitors (HDAC, 5-AZA), we saw an increased effect on knockdown of PRMT5 activity. The data suggests that these small molecule compounds have the potential to be a new therapy through inhibiting PRMT5 activity in the most common and aggressive CNS tumor.

CMP5 vs. BLL54

This project helped to narrow in on two compounds that showed inhibition of PRMT5 activity. The first round of compounds that were synthesized included CMP5. When CMP5 was tested with *in vitro* experiments, it was discovered that this was the most effective of the batch. After testing CMP5 in several experiments, we discovered that CMP5 was most effective at drug concentrations around 75μM-100μM. Even though CMP5 showed promising activity at this level, we wanted to look for a compound that could induce the same effects, but at a lower concentration.

BLL54 was synthesized after experimenting with CMP5. The structure of BLL54 was derived from the structure of CMP5 to make it more potent and specific. The goal of re-structuring this compound, to make it more potent, was to try to get the lowest concentration we could that still had an anti-tumor effect. We wanted the lowest concentration possible, so that, once this drug discovery project made it into the *in vivo* stage of testing, there would be minimal adverse effects and it would not be toxic to other cells in the body.

After testing BLL54 in the same cell lines that experiments were conducted with CMP5, we saw much more selective activity. Used by itself, BLL54 showed it was capable of repressing the symmetric di-methylation on histone 4 arginine 3 with a concentration of 25uM. When used with the HDAC inhibitor (TSA) and hypo-methylating agent (5-AZA), we saw effective concentrations of BLL54 in the nanomolar range. This concentration range is a much more reasonable dose level and gives us a promising look at what the future could hold for this compound. These tests show that there could be a possibility for a three-drug regimen that shows promising signs for the silencing of PRMT5 and treatment of GBM. BLL54 and CMP5 both showed a promising result in combination with the HDAC inhibitor and in the combination of all three drugs; however BLL54 showed to be the more potent and selective compound.

Future Projects

This research has paved the way for additional testing with these compounds in GBM and set up the stage to conduct an *in vivo* model. The next series of studies to be done would be a preclinical animal model using a xenograft mouse model. This would allow *in vivo* testing of this drug to get a better idea of how this drug may affect the entire central nervous system of an organism and hopefully lead to clinical trials in patients afflicted with GBM.

Significance

The completion of this project and the continuation of research on these small molecule inhibitors has huge implications for the future of this disease. High grade astrocytomas are incurable, aggressive malignancies. Therefore, the discovery of new agents to treat this disease is highly desirable. It was previously reported that PRMT5 over expression is an appealing therapeutic target for GBM and contributes towards the aggressive nature of this tumor. Therefore, PRMT5 could be used as a potential prognostic tool for patients with GBM to help identify patients who are at high risk. Our previous paper had also demonstrated that siRNAs had the ability to knockdown the function of PRMT5 effectively, but the use of siRNAs in disease treatment is still in early stages. [15] These compounds were able to inhibit this enzyme with greater potency and selectivity and in the future, we hope that they will promote the development of an experimental therapeutic program exploring this strategy. These promising compounds will enhance our efforts to develop a new drug for treatment of patients with GBM. Our work could potentially have a significant impact on cancer research because of the severity of this type of cancer and the lack of reasonable treatment options for patients diagnosed with GBM.

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