Aurora Kinase, EZH2, and BET Inhibitor Drug Synergy in Glioblastoma Multiforme

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ABSTRACT: Glioblastoma (GBM) is the most common primary brain cancer with an incidence rate of 3.21 per 100,000 people. Even with standard-of-care treatment, the median survival time is 15 months. Therefore, new, more effective therapies are sought. In recent years, interest in combination therapies has grown because of the ineffectiveness of many mono-therapies. In particular, combination therapies present the possibility of synergistic drug-drug interactions, which allow for promising clinical implementation. This investigation aims to create novel compound combinations of Aurora kinase, EZH2, and BET inhibitors to determine whether pharmacotherapies such as these can effectively treat GBM in vitro as an immunotherapy by interacting synergistically with one another. With five different combinations within two separate cell lines (LN229 and GBM22) of an Aurora kinase inhibitor drug (alisertib), EZH2 inhibitor drug (tazemetostat), and BET inhibitor drug (UM-002), the combination therapy of alisertib and UM-002 tested in vitro in LN229 cells displayed the most positive and synergistic results. Compared to the other combinations tested, the inhibition percentages were at least 34% greater for the alisertib and UM-002 combination, indicating promising drug-drug interactions between Aurora kinase and BET inhibitors which cause the GBM cell survival to decrease significantly. This illustrates the possibility of highly effective immunotherapy for this malignant disease with poor prognosis and survival.

KEYWORDS: Biology; Cancer Biology; Neuroscience; Glioblastoma Multiforme; Drug Synergy.

Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive types of brain tumor.¹ It is categorized under the more common glioma, a primary brain tumor classified according to its particular cell of origin; among these are astrocytic tumors, oligodendrogliomas, ependymomas, and mixed gliomas, which are all tumors related to the brain’s central nervous system.² Indeed, GBM is the most common primary malignant brain tumor as it accounts for approximately 60% of all brain tumors in adults.³⁴ It is classified as a grade IV glioma and grade IV astrocytoma.¹ As the cancer invades adjacent tissue, distant organs are generally unaffected. GBM usually occurs in the cerebral hemispheres, especially in the frontal and temporal lobes.⁵ Although modern therapies continue to evolve for GBM (current standard treatment is surgery, radiation, and chemotherapy with temozolomide), due to its poor prognosis, patients are expected to have a median survival rate of 15 months post-diagnosis.⁴

GBM is highly resistant to several forms of therapeutic intervention, mainly due to its incredible complexity.¹ GBM is multiform grossly, microscopically, and genetically: the etiology of GBM consists of numerous regions of necrosis and local or systemic hemorrhage; microscopically, regions of pseudopalisading necrosis, pleomorphic cells and nuclei, and microvascular proliferation can be seen; genetically, GBM consists of several deletions, amplifications, and point mutations, which lead to the activation of abnormal cell production.⁶⁻⁸ Additionally, GBM shows intratumor genetic heterogeneity with subclones within the tumor cell population.⁹ It has been estimated that p53-deficient and cultured neoplastic cells that occur with GBM could have mutations in any gene at a rate of 1 in 1,000 cells.¹⁰ Assuming this is correct for GBM (in vivo), a tumor of 1 billion cells could harbor as many as 1 million cells with mutations in any given gene.¹

Nevertheless, current therapies for GBM include surgery (where a neurosurgeon works towards manually resecting the GBM), radiation therapy (use of high energy beams to kill cancer cells), chemotherapy (use of drugs), tumor treating fields therapy (use of an electric field to disrupt cancer cells from multiplying), targeted drug therapy (focus on abnormalities), and clinical trials (where patients can take part in studies leading to advanced treatment).² These standard treatments have been somewhat effective to the extent that GBM patients live around 2-4 more years. However, this regimen is not highly curative (not every tumor cell is killed), hence increasing the need for a significantly effective treatment strategy.¹¹

Immunotherapy, a treatment in which the immune system is manipulated to attack tumor cells and minimizes adverse effects, has been on the rise as a possible advancement in the treatment of GBM.¹² Most immunotherapies are tested through clinical trials. By participating in these clinical trials, immunotherapies can lead to advances in treating GBM. Current clinical trials for GBM predominantly focus on peptide vaccines, adoptive T-cell therapy, oncolytic virotherapy, dendritic cell vaccines, and checkpoint inhibitors.¹³ As monotherapies prove ineffective; an alternative treatment strategy consists of combination therapies: a treatment modality that combines two or more therapeutic agents.¹⁵ Combination therapies present the
possibility of positive or even synergistic drug–drug interactions, which could conclude with clinical implementation.¹⁶

Aurora kinases, a family of serine and threonine kinases, regulate centriole and microtubule function and play an essential role in maintaining normal mitosis and regulated meiosis.¹⁶ Overexpression or gene amplification of Aurora kinases leads to aneuploidy — the state of abnormal chromosome numbers that deviate from a multiple of haploid complement — thereby leading to cancer. In past decades, a series of Aurora kinase inhibitors (AKIs) developed have successfully repressed the progression and growth of many cancers both in vivo and in vitro, suggesting that Aurora kinases could be a novel therapeutic medium for the treatment of GBM.¹⁷ Alisertib, an AKI drug, has been extensively characterized using in vivo and in vitro preclinical models. It has been shown to display antiproliferative activity in various human tumor cell lines, including glioblastoma.¹⁸

The enhancer of the zeste homolog 2 (EZH2) gene provides instructions for making an enzyme modifying histone proteins. It has been implicated in oncogenesis as the catalytic methyltransferase within the PRC2 protein. This gene is overexpressed in cancers and is correlated with a lower survival rate of GBM. Pharmacological inhibition of EZH2 activity eventually leads to a reduction in tumorigenicity in GBM.¹⁹ Tazemetostat, an EZH2 inhibitor drug, has previously been shown to reduce viability in GBM cell lines. Furthermore, it is the first Food & Drug Administration-approved EZH2 inhibitor drug, suggesting it has undergone extensive trials to test its efficacy and safety for patients.²⁰

The bromodomain and extra-terminal (BET) proteins act as epigenetic readers with broad specificity on transcriptional activation, which includes the recruitment of positive transcription elongation factor and control of RNA polymerase II transcriptional activity. Bromodomain-containing protein 4 (BRD4) is a target in multiple cancers (in GBM, the inhibition or depletion of BRD4 reduces the expression of oncogenes).²¹ Small molecule BET inhibitors reduce the growth of GBM and other brain tumors by competing with BET–histone interaction (reduced transcription of oncogenes essential for GBM cell interaction). There are multiple BET inhibitors, yet very few are brain penetrant; BET Inhibitor JQ1 is widely used in research and is brain penetrant, yet it is not clinically used because of its short half-life. BET Inhibitor; MK-8628 was used in a clinical study but was terminated when it was found to have no significant effect on GBM. BET Inhibitor; UM-002 is modified to increase potency (more than JQ1 or MK-8628) in reducing GBM cell proliferation in vitro.²²

To test each compound combination, synergy matrix screens were carried out. The standard plating and drugging procedure used for cell culture was followed. There are many ways to measure cell viability, including MTT Proliferation Assay. The MTT assay measured cellular metabolic activity as an indicator of cell proliferation, viability, and cytotoxicity. Instead of counting each individual cell, this colorimetric assay is based on the reduction of a yellow tetrazolium salt (expressed as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals through metabolically active cells. Since the viable cells contain NAD(P) H-dependent oxidoreductase enzymes, the MTT is reduced to formazan. A solubilization solution is used to dissolve the insoluble formazan crystals, and the resulting colored solution is quantified by measuring absorbance at 500–600 nanometers with a cell plate reader. The darker or more purple the solution, the greater the number of viable, metabolically active cells.²³

Small molecule inhibitors each feature the ability to inhibit GBM proliferation individually, yet are of limited effectiveness against this highly aggressive brain tumor. This investigation aims to test novel compound combinations of these inhibitors to determine whether pharmacotherapies such as these, by interacting synergistically with one another, can more effectively treat GBM in vitro.

Methods

Cells were cultured in complete medium (45 mL Dulbecco’s Modified Eagle Medium, 5 mL Fetal Bovine Serum, and 1 mL Pen/Strep). Once cells were sufficiently confluent within a 15mL cell culture flask, the complete media was suctioned. Cells attached to the flask’s surface were separated with 5 mL of trypsin, which was added to the bottom of the flask for approximately 4 minutes and neutralized with 5 mL of complete media, ensuring the neutralized trypsin reached every part of the bottom of the flask. The cells were resuspended and counted with a hemocytometer and trypan blue to determine the volume of complete media needed to plate 5,000 cells per well. Cells and complete media were plated at 5000 cells/well in each of 64 wells, in an 8 x 8 pattern, for four 96-well plates and placed in a cell incubator.

After 24 hours, the complete media was removed from each of the wells, careful not to suction many cells. 200 µL of fresh complete media was added to all the wells. Cells were then treated with each drug in 7 1:4 serial dilutions, starting at 10 µM with a final Dimethyl Sulfoxide (DMSO) concentration of 0.25 - 0.5%, depending on the solubility of the solid drug in a DMSO and Phosphate Buffer Saline (PBS) stock solution. The positive control or vehicle was 25% DMSO. 10 µM Velcade was the negative control. Drugged cells were incubated for 72 hours.

10X stock solution of MTT was diluted with sterile 1X PBS to create the MTT working solution. Media was removed from cells. 100 µL of MTT working solution was added to each well. Cells were incubated for 1 hour. At the end of the incubation time, 100 µL of solubilization solution was added. The cells were kept at room temperature for another hour to allow the converted dye to dissolve completely. With a cell plate reader, the absorbance of the converted dye was measured at 570 nm and a reference wavelength of 650 nm. Each plate was measured without the cover to avoid condensation or smudge on the cover affecting the reading.

It was essential to take proper safety precautions during this experiment. All the experiments were conducted in a research laboratory with rules and safety precautions. Proper PPE equipment was also used throughout the investigation.
Results and Discussion

The optical density or transmission of light values of each well (produced as an Excel spreadsheet by the cell plate reader) were analyzed using the SynergyFinder software, which produced graphs depicting inhibition and synergy levels for each drug combination. A positive result indicates the inhibition of proliferation, and a negative result indicates the enhancement of proliferation. A synergy score can be expressed as the average excess response due to drug interactions. A synergistic effect occurs when the sum of the effect is more than the two individual chemical effects combined. An additive effect occurs when the sum of the effect equals the two individual chemical effects combined. Hence, a synergistic effect is ideal since more drug-drug interactions would be occurring that would allow a greater positive effect on cell viability.

Alisertib and Tazemetostat in LN229:

Figure 1: Alisertib shows positive results on the percentage inhibition of LN229 cells.

Figure 2: Tazemetostat shows negative results on the percentage inhibition of LN229 cells.

Figure 3: Alisertib and tazemetostat combination shows slightly positive effects on LN229 cell.

Figure 4: Alisertib and tazemetostat combination shows a slight positive effect on the percentage inhibition of LN229 cells.

Figure 5: Alisertib and tazemetostat combination shows no significant synergy in LN229 cells.

The alisertib and tazemetostat combination in LN229 displayed moderate results. As seen in Figure 1, there was a greater percentage of inhibition at higher doses of alisertib. Figure 2 suggests that higher doses of tazemetostat induced a negative inhibition response. The combination of alisertib and tazemetostat moderately reduced cell viability, as seen in Figure 3 and increased inhibition by an average of 16%, as seen in Figure 4. Furthermore, based on the low mean and high p-value displayed in Figure 5, there was no synergistic effect between the alisertib and tazemetostat combination in the LN229 cells.

Figure 6: UM-002 shows positive results on the percentage inhibition of LN229 cells.

Figure 7: Tazemetostat shows negative results on the percentage inhibition of LN229 cells.

Figure 8: UM-002 and tazemetostat combination shows slightly positive effects on LN229 cell viability.
The alisertib and UM-002 combination in LN229 displayed the most positive results. Figures 11 and 12 show that both alisertib and UM-002 demonstrated greater inhibition at higher doses. Figures 13 and 14 suggest that cell viability significantly decreased, and inhibition increased by 51% and 39% with the alisertib and UM-002 combination. In addition, Figure 15 indicates that, compared to the previous assays, there is some synergy between alisertib and UM-002 in LN229 cells given the positive mean value, but the p-value is not significant.

The UM-002 and tazemetostat combination in LN229 displayed more positive results than the alisertib and tazemetostat combination in LN229. From Figure 6, as UM-002 doses increased, there was a greater percentage of cell inhibition. Figure 7 suggests the same dose response with negative inhibition of tazemetostat, as seen in Figure 2. The combination of UM-002 and tazemetostat decreased cell viability slightly more and increased inhibition by 12% than the previous combination results, as seen in Figures 8 and 9 compared to Figures 3 and 4. Despite positive results, Figure 10 suggests no significant synergy between UM-002 and tazemetostat in LN229 cells based on the low mean value and high p-value.

The alisertib and UM-002 combination in LN229 displayed the most positive results. Figures 11 and 12 show that both alisertib and UM-002 demonstrated greater inhibition at higher doses. Figures 13 and 14 suggest that cell viability significantly decreased, and inhibition increased by 51% and 39% with the alisertib and UM-002 combination. In addition, Figure 15 indicates that, compared to the previous assays, there is some synergy between alisertib and UM-002 in LN229 cells given the positive mean value, but the p-value is not significant.
The alisertib and tazemetostat combination in GBM22 displayed results similar to that in LN229 cells. Figures 16 and 17 suggest that alisertib induced a positive inhibition and tazemetostat induced a slightly positive inhibition response, with only 5% inhibition. Figures 18 and 19 show that this combination leads to a slight decrease in cell viability and positive inhibition. Figure 20 indicates no significant synergistic effects of the alisertib and tazemetostat combination in GBM22 cells.

Figure 17: Tazemetostat shows slightly positive results on the percentage inhibition of GBM22 cells.

Figure 18: Alisertib and tazemetostat combination shows slightly positive effects on GBM22 cell viability.

Figure 19: Alisertib and tazemetostat combination shows a slight positive effect on the percentage inhibition of GBM22 cells.

Figure 20: Alisertib and tazemetostat combination shows no significant synergy in GBM22 cells.

The alisertib and tazemetostat combination in GBM22 displayed results similar to that in LN229 cells. Figures 16 and 17 suggest that alisertib induced a positive inhibition and tazemetostat induced a slightly positive inhibition response, with only 5% inhibition. Figures 18 and 19 show that this combination leads to a slight decrease in cell viability and positive inhibition. Figure 20 indicates no significant synergistic effects of the alisertib and tazemetostat combination in GBM22 cells.

Figure 21: UM-002 shows positive results on the percentage inhibition of GBM22 cells.

Figure 22: Tazemetostat shows slightly positive results on the percentage inhibition of GBM22 cells.

Figure 23: UM-002 and tazemetostat combination shows slightly positive effects on GBM22 cell viability.

Figure 24: UM-002 and tazemetostat combination positively affect the percentage inhibition of GBM22 cells.

Figure 25: UM-002 and tazemetostat combination shows no significant synergy in GBM22 cells.
The UM-002 and tazemetostat combination in the GBM22 cell line also yielded similar results as the same combination in LN229. Again, Figures 21 and 22 show that the UM-002 demonstrated positive inhibition while the tazemetostat displayed negative inhibition. The combination of these drugs also decreased cell viability and led to a moderate increase in percentage inhibition, as seen in Figures 23 and 24. However, there was no significant synergy between the UM-002 and tazemetostat combination in GBM22, as displayed in Figure 25.

**Conclusion**

Experimental results suggest that novel combinations of aurora kinase, EZH2 and BET inhibitors display promise, suggesting the possibility of devising more effective immunotherapy for GBM. The alternate hypothesis was partially supported with one of the five combinations: there was evidence of some synergy between alisertib and UM-002. The other combinations did not suggest any synergistic effects due to the high p-values. While conducting the experiments, there may have been a random or systematic error source. For example, since the cell plate reader was utterly computerized, it was impossible to determine an inaccurate reading unless the MTT color did not correspond to the reading. Hence, the experimental design made it challenging to decide on any type of statistical error due to the methods with which the data was analyzed. Therefore, all the figures were derived from a website with only the p-value and no other statistical test.

As previously described, alisertib and UM-002 individually decreased cell viability and increased the percentage inhibition in LN229 and GBM22, supporting previous findings relating to these inhibitor drugs. However, especially in the LN229 cells, tazemetostat displayed a negative percentage inhibition, which suggests that the cells proliferate in response to being drugged with tazemetostat. This seemed to cause the drug combinations, including tazemetostat, not to display the expected positive and synergistic results. This is indeed a puzzling finding as previous studies have found tazemetostat to decrease cell survival. Although possible error cannot be ruled out as reasoning for this observation, it is doubtful since every replicate for every assay which tested tazemetostat in the LN229 cells displayed a negative inhibition of GBM cells.

Given that this contradicts previous findings relating to tazemetostat, it may be explained by the differences in experimental set-up and methodology. Namely, EZH2 inhibitors commonly take more than 72 hours to display full effects, so the tazemetostat might have shown more positive results if the cells were drugged for longer. Nevertheless, the results of this experiment suggest that the combination of aurora kinase and EZH2 inhibitors and EZH2 and BET inhibitors are not effective dual therapies for GBM.

This experiment had some limitations. Namely, there was a limited time of 10 weeks to complete these experiments. If there was more time, cells treated with tazemetostat could have been drugged for longer, such as 96 or 120 hours instead of 72 hours, to determine whether EZH2 inhibitors are effective in combination with other inhibitors. Also, the variety of alisertib and UM-002 in GBM22 could not be tested due to the limited time. Again, the random, systemic, and possible statistical error in this experiment could have been a factor in some of the results, which only displayed minimal synergistic effects in that the highest synergy score was 2 out of 20. Limiting any possible error could significantly aid in producing statistically significant results. Future directions for this experiment would focus on conducting additional assays to support and elaborate on the data found from this experiment. In addition to the LN229 and GBM22 cell lines, other primary GBM cell lines, such as GBM6, GBM10, and GBM39, could be tested since they have been shown to demonstrate positive effects.

Since tazemetostat displayed negative inhibition, other EZH2 inhibitors could be tested to determine whether EZH2 inhibitors are unsuitable for combination therapies. In addition to aurora kinase, BET, and EZH2 inhibitors, other inhibitors shown to reduce GBM cell proliferation, such as LIM kinase and HDAC inhibitors, could be tested.

Most importantly, among the combinations examined, the alisertib and UM-002 combination effectively reduced cell viability and displayed the most promising results overall. Compared to the other varieties tested, with an average of 68%, the GBM cell inhibition percentages were at least 34% greater for the alisertib and UM-002 combination (Figures 20-24), indicating that there are highly positive drug-drug interactions occurring, which cause the cancer cell survival to decrease significantly. Since this combination was tested in a cell line such as LN229, which is commonly used for in-vitro GBM experimentation, the ability of this combination to penetrate through the cells is very encouraging for the development of a dual aurora kinase and BET inhibitor therapy for GBM. Ideally, if tested in vivo, small doses of alisertib and UM-002 would be administered, and a much more pronounced effect (significant decrease of cancer cells due to drug-drug interactions) would occur, thereby minimizing side effects of these drugs while simultaneously more effectively eliminating as many GBM cells.

With glioblastoma as the most common primary brain cancer, highly effective treatments must be found to treat this disease. With compound therapies on the rise, the alisertib and UM-002 drug combination and, possibly, other Aurora kinase and BET inhibitor drug combinations could be promising therapy with highly efficacious results on GBM cell death.

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