Synergistic Potential of ALP-Temozolomide Drug Combination:

PI3K signaling and AR activity in U87-EGFP Cells

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ABSTRACT: Glioblastoma (GBM) is the most common brain cancer and is a devastating disease. One widely used chemotherapy drugs against GBM is Temozolomide (TMZ), although it is not universally effective and resistance can develop. Sex differences exist in both GBM and in TMZ efficacy. This study evaluates the synergy of the previously untested combination of TMZ and Alpelisib (ALP), a selective PI3Ka inhibitor to determine the potential synergistic effects on the U87-EGFP GBM cells. Synergy of the TMZ/ALP combination is assessed and the role of PI3K signaling in these drugs' mechanisms examined, both with and without testosterone treatment. Preliminary experiments were conducted to determine single-treatment cell viability dose response to TMZ, ALP, and testosterone using CCK-8 assay; next, cell viability at varying concentration combinations of TMZ and ALP, with and without a single dose of testosterone, were determined. Flow cytometry was used to examine PI3K pathway signaling in the same treatment groups. The viability analysis results demonstrate synergy of the TMZ and ALP combination, synergy that is enhanced in high testosterone conditions. Additionally, ALP and TMZ both were effective at countering the pro-cancer effects of testosterone. PI3K pathway activity was not significantly impacted under any treatment conditions, indicating that TMZ, ALP, and testosterone's mechanisms of action are not mediated by PI3K pathway activity. These results provide evidence for the promise of the combination of TMZ and PI3K inhibition as a treatment strategy, especially for males, and suggest that alternative mechanisms for the impact of these three drugs on GBM.

Introduction

GBM (GBM) is one of the deadliest brain cancers and has no known cure.[1] The median survival time after diagnosis is 15 months, and less than 5% of patients survive after 5 years of

onset.[1] GBM is very prevalent among brain cancer patients, making up 50% of all gliomas and 15% of all brain cancer.[1] GBM also affects more men than women with a 4 to 3 ratio. Known treatments for GBM are surgical removal of the tumor or chemotherapy/radiation therapy. Surgical removal of tumors, although successful, may not completely remove all the cancer cells, resulting in a high chance of recurrence. Chemotherapy/radiation therapy is also limited due to the blood-brain barrier, although research is ongoing. Due to its severity and prevalence, GBM is one of the most heavily researched cancers, with many seeking to determine what makes GBM so deadly and how best to treat it.

TMZ is considered one of the first lines of defense against GBMs. The drug can be taken orally since it is able to cross the blood-brain barrier to reach brain cancer cells.[2] It can also be used concurrently with radiation therapy or by itself. TMZ kills by modifying the DNA of GBM cells; it is an alkylating agent that methylates single DNA strands at specific sites, usually the N7 position of guanine, O3 position of adenine and O6 position of guanine.[2] These mutations lead to cell cycle arrest and apoptosis of GBM cells. However, many patients develop a resistance to the drug, limiting treatment efficacy. Over 50% of GBM patients that use TMZ do not respond to treatment.[2] Resistance develops mainly through methylguanine methyltransferase (MGMT) and glioma stem cells, both of which are highly studies. MGMT, a DNA repair protein, is thought to be the highest contributor to TMZ resistance, as it is able to fix the mutations caused by TMZ, rendering the treatment ineffective. Glioma stem cells are a special type of cells that is especially resistant to treatment and leads to tumor recurrence. With new research, additional resistance pathways have been

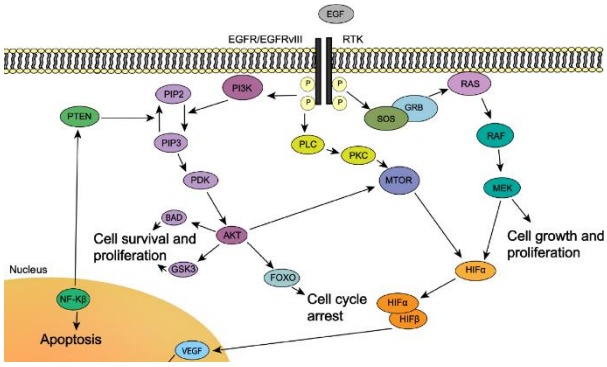


Figure 1.[2] *Pathways for TMZ resistance*

identified, including the PI3K pathway, which contributes to the carcinogenesis, proliferation, invasion, and metastasis of tumor cells.[3] We chose a well known PI3K inhibitor, ALP, to use in conjunction with TMZ. ALP has shown to be an effective PI3K inhibitor in breast cancer patients leading to favorable outcomes. It has also shown to directly target the PI3Ka pathway in U87 cells.[5] Currently, there exists a gap in knowledge in the literature, as no study examines the combination of a selective PI3Ka inhibitor, like ALP, and TMZ in GBM cells.[4]

Although TMZ is successful in decreasing the number of GBM cells, effectiveness varies between the sexes. Figure 2[6] illustrates TMZ effectiveness differences between males and females. Across the three dosages for the female samples, the distribution of tumor velocities across all samples decreases, indicating that TMZ is able to drive tumors to shrink. However, the same cannot be said for the male samples. The second dose of TMZ reduces the tumor velocity, but the third TMZ dosage does not result in a further decline in tumor velocity. By the last dose, the median tumor velocity for females is much lower than the median tumor velocity for males. It is possible that sex hormones may be driving these effects.

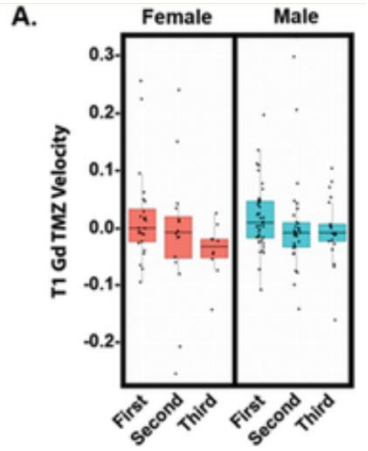


Figure 2. *TMZ sex differences*

ALP is mainly used in breast cancer treatment, and in these studies, ALP was effective in dealing with PI3K pathway mutations.[7] The sex differences across ALP treatment is unknown as men have historically been excluded from breast cancer clinical trials. However, one study examined ALP metabolism in four healthy male volunteers, with the study concluding that ALP may be a viable therapy since it was absorbed and cleared from the bodies of the male patients without causing safety concerns.[8]

This study aims to investigate the impact of TMZ, ALP, and testosterone on cell viability; the effect of testosterone on TMZ and ALP efficacy, and the synergy of ALP/TMZ under control and high-testosterone conditions. Additionally, we examine the role of the PI3K pathway in each drug and combination's mechanism of action. We gain a better understanding of the role and mechanism of testosterone in GBM and GBM treatments, and we assess the potential of TMZ/ALP as a therapeutic strategy. We also fill the gap in literature concerning sex differences in ALP in GBM. Given previous findings, it is hypothesized that TMZ and ALP will both be effective treatments and that testosterone will exhibit deleterious effects. ALP and testosterone are also expected to result in activation of the PI3K pathway. Testosterone is further expected to impact TMZ and ALP efficacy. As the PI3K pathway has been implicated in resistance to TMZ, the combination of TMZ and ALP is hypothesized to show synergy, and that the synergy will be enhanced when the cells are also treated with testosterone, as testosterone has been shown to inhibit TMZ efficacy potentially through PI3K activity. Accordingly, the first aim of the study is to assess drug and drug combination response and efficacy via cell viability assay, in order to determine individual and synergistic effects of the treatments. The second aim of the study is to assess PI3K activity via Muse kit flow cytometry, to determine the role of this pathway in the drug's effects.

Materials and Methods

Preliminary Experiment: Dose Range Determination

To determine the appropriate dose of TMZ, ALP, and Testosterone for subsequent combination experiments, a preliminary experiment determining cell viability of U87-EGFP in response to a range of doses for each drug was conducted. The range of concentrations was

determined based on literature IC50 values of TMZ and ALP and a typical dose range for testosterone treatments in U87-EGFP cells. Cell viability was assessed using a CCK-8 assay.

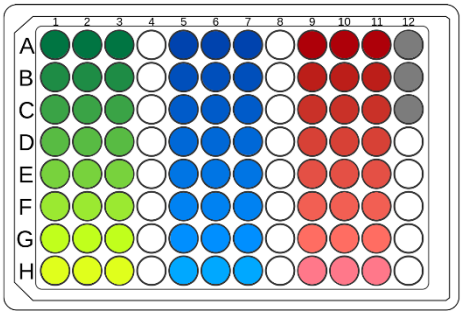


Figure 3. *Preliminary experiment plate map for TMZ (green), ALP (blue), and Testosterone (red) doses. No treatment controls (NTC) shown in grey. TMZ doses range from 1000uM (row A) to 7.8uM (row H), using a dilution factor of 2. ALP doses range from 100uM (row A) to 780nM (row H), using a dilution factor of 2. Testosterone doses range from 10uM (row A) to 0.61nM (row H), using a dilution factor of 4.*

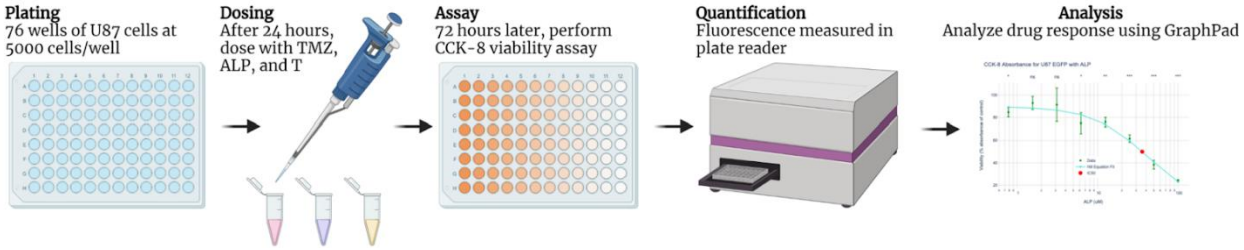


Figure 4*. Timeline of experimentation for the preliminary experiment. On Day 1, cells were plated in a 96-well according to the plate map shown in Figure 3 at a concentration of 5000 cells/well in 200ul of DMEM. On Day 2, the media was aspirated and cells were dosed with 200ul of drug diluted in DMEM, in accordance with the plate map in Figure 3. On Day 5, media was aspirated and cells were treated with 10% CCK-8 in DMEM. After 50 minutes of incubation, fluorescence was quantified in a plate reader for subsequent analysis.*

Aim 1: Cell Viability

Having determined dose response of TMZ and ALP, and the most effective Testosterone dose, cell viability in response to combination treatments was assessed with a CCK-8 assay.

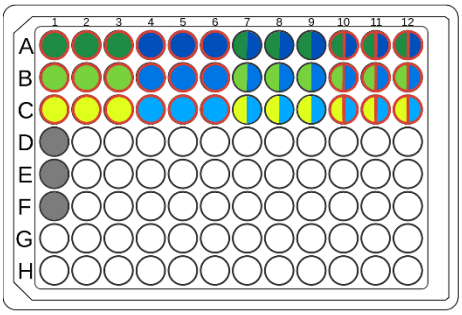


Figure 5*. Aim 1 plate map. NTC in grey, TMZ in green, ALP in blue, and Testosterone with a red outline. 13 total treatment groups: NTC (column 1/row D-F), TMZ (at IC50, IC30, and IC15) with Testosterone (columns 1-3), ALP (at IC50, IC30, and IC15) with Testosterone (columns 4-6), TMZ and ALP (at IC50, IC30, and IC15) (columns 7-9), and TMZ and ALP (at IC50, IC30, IC15) with Testosterone (columns 10-12).*

Doses of TMZ, ALP, and Testosterone were determined by the results of the preliminary experiment - the IC50, IC30, and IC15 of TMZ, the IC50, IC30, and IC15 of ALP, and the dose of Testosterone that resulted in greatest cell viability. Using the plate map from Figure 5, the

same workflow was followed in Aim 1 as the preliminary experiment (see Figure 4). Synergy of the TMZ/ALP combination, and its impact on the deleterious effects of Testosterone, was assessed.

Aim 2: PI3K Activity

In addition to measuring cell viability, the level of PI3K pathway activity in the different drug combinations was assessed using a Muse PI3K Activation Dual Detection Kit in order to examine the mechanism behind the drugs' activity.

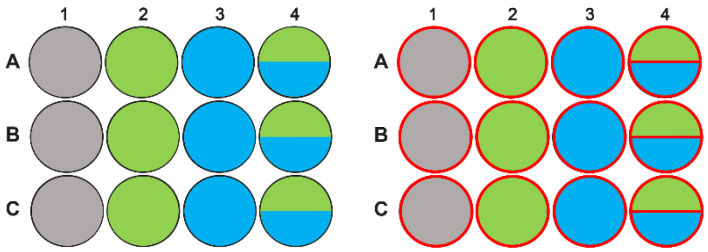


***Figure 6**. Plate map of the 2 12-wells that were used for Aim 2. The IC30 values of TMZ and ALP were used, with 2.5uM testosterone. TMZ is again shown in green; ALP in blue, Testosterone with a red outline, and NTC in grey.*

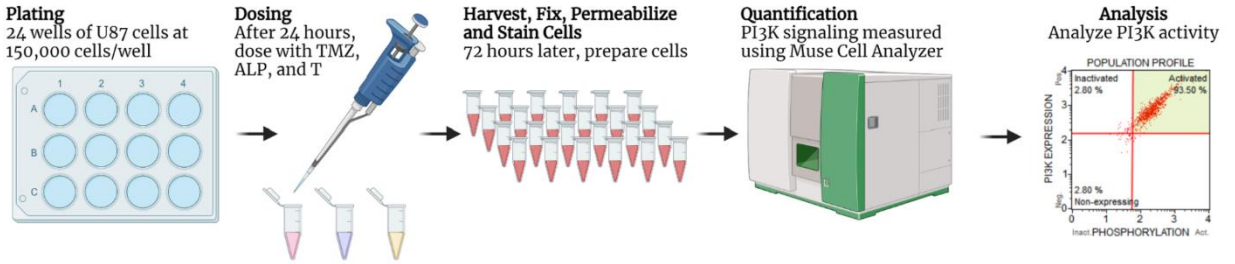
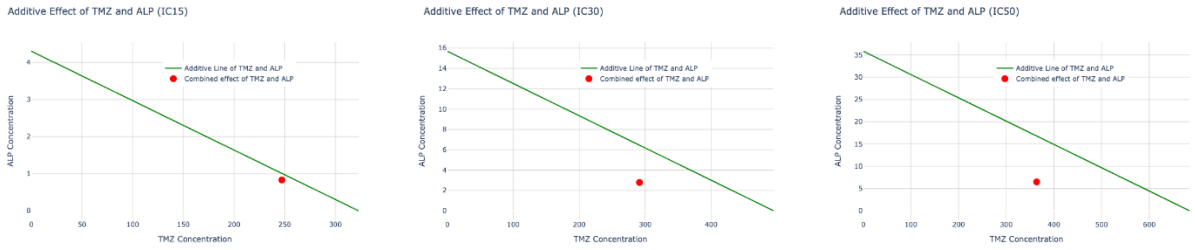


***Figure 7**. Timeline of experimentation for Aim 2. On Day 1, U87-EGFP cells were plated in two 12-wells (for a total of 24 wells) at a density of 150,000 cells/well in 1.5ml DMEM. On Day 2, cells were dosed with TMZ, ALP, and Testosterone in accordance with the plate map in Figure 6. On Day 5, cells were collected and spun down, then fixed, permeabilized, and stained according to the Muse Activation Kit protocol. Quantification and analysis were performed in the Muse Cell Analyzer.*

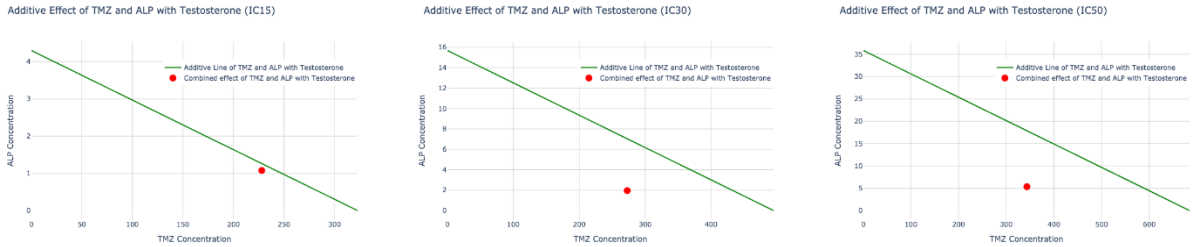
Results and Figures

Aim 1: Cell Viability



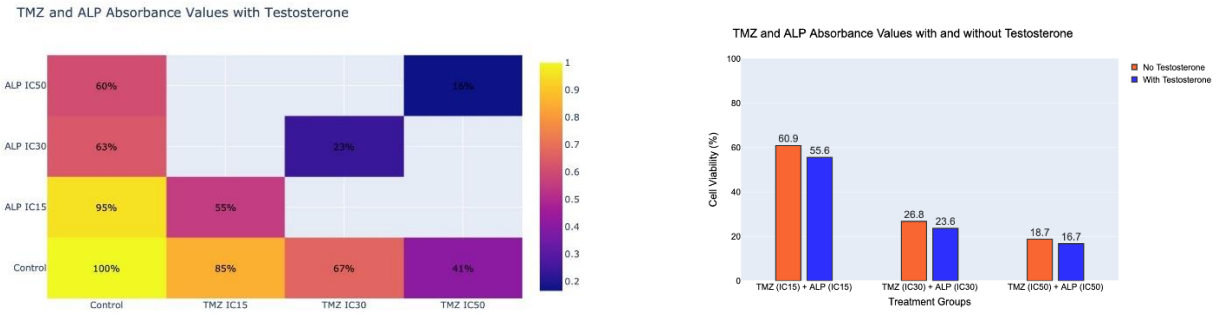
(a) (b) (c)

Figure 8. *Results of Combination Drug (TMZ + ALP) Study without Testosterone. (a) Isobologram Analysis with IC15; (b) Isobologram Analysis with IC30; (c) Isobologram Analysis with IC50*



(a) (b) (c)

Figure 9. *Results of Combination Drug (TMZ + ALP) Study with Testosterone. (a) Isobologram Analysis with IC15; (b) Isobologram Analysis with IC30; (c) Isobologram Analysis with IC50*



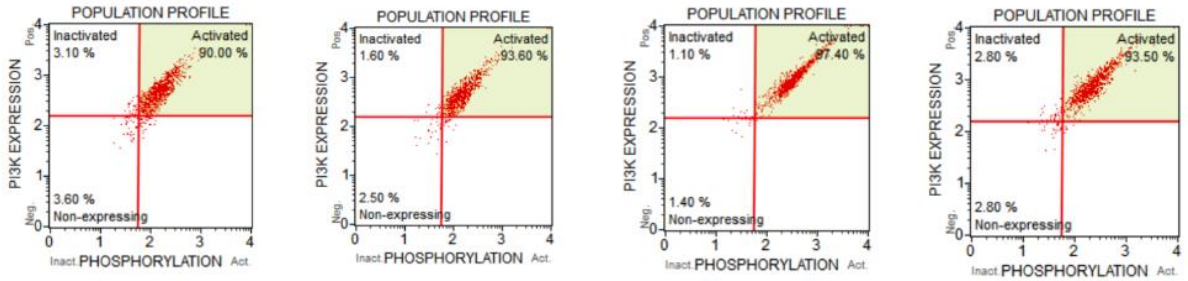
(a) (b)

Figure 10. *(a) Heatmap of Cell Viability at Different Drug Combinations; (b) Cell Viability of ALP, TMZ, and Combination Drugs at IC30 Concentrations Both With and Without Testosterone. Note no significant difference was found between With and Without Testosterone Groups Using Two-Way ANOVA (P-Value of 0.93).*

From Figures 8 and 9, one can see that synergy was present in all combination drug therapies whether Testosterone was present or not. This is indicated by the fact that the red dot,

representing the necessary concentration of each drug to create a combinational effect, is below the green line in each isobologram. Figure 10 includes more holistic figures to encapsulate all the relevant data collected throughout the aim and is dispersed throughout Figure 8 and 9.

Aim 2: PI3K Activity



(a) (b) (c) (d)

Figure 11. *PI3K Activation (a) Control; (b) Testosterone; (c) TMZ + ALP IC30 Combination; (d) TMZ + ALP IC30 Combination with Testosterone*

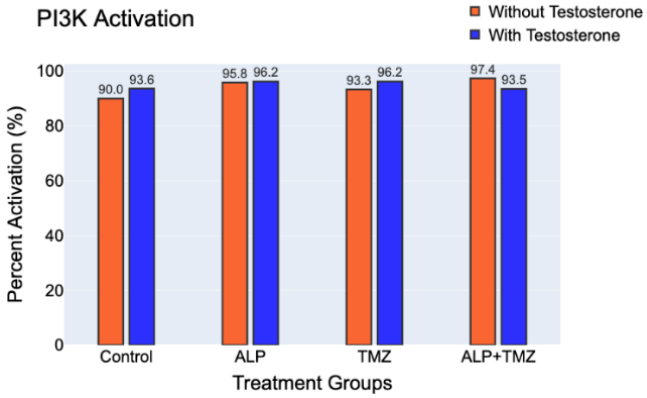


Figure 12. *PI3K Activation in Control, ALP, TMZ, and Combination at IC30 Concentrations of Each Both With and Without Testosterone*

Figures 11 and 12 show the activation of the PI3K pathway in the different treatment groups. It should be noted that for the most part, all treatment groups had similar amounts of activation in the PI3K pathway with minimal differences among each.

Discussion

As noted in the Aim 1 results and can be seen in the isobologram analyses, it is clear that a combinational use of ALP and TMZ creates a synergistic effect in U87-EGFP cells, both in the

presence of and lack of Testosterone. In other terms, both drugs can be used together and at lower concentrations to illicit a response that is of the same magnitude than using any one individual drug at a higher concentration. This finding does corroborate the hypothesis presented earlier in the paper that expects the presence of synergy to exist when using TMZ and ALP in combination.

What is interesting to note is the fact that the presence of Testosterone does not create a significant difference between treatment groups. This can be seen through visual analysis of Figure 10b and was further validated through the use of a two-way ANOVA which confirmed the lack of a significant difference, with a p-value of 0.93, between the treatment groups with Testosterone and the groups without. Now, this does reject the hypothesis stated earlier in this paper related to the effects of Testosterone on the treatment groups of the cell. Now, this rejection of the results can be due to a true lack in the presence of androgen effects within glioblastoma cells, specifically in the drug mechanism pathway, or it can be caused due to experimental limitations that are further elaborated upon in the latter part of this discussion.

Within the results of Aim 2, it is clearly seen that no treatment group, whether that be independent drug groups, combination drug groups, and presence of testosterone, caused large changes in the activation of the PI3K pathway in the treated cells. Now, this also rejects the aforementioned hypothesis that states the main mechanism of action is within the PI3K pathway. What this means is that the combinational drug therapy looked at in the previous aim is most likely acting through another mechanism, pathway, or secondary target that allows it to create a synergistic effect. This also would explain why there is no noticeable difference between treatment groups that were exposed to Testosterone vs. ones that weren’t.

Now, as mentioned before, the lack of PI3K pathway activation and effects due to the presence of Testosterone may be caused by experimental limitations. Some of these limitations include the duration of the study and the use traditional cell culture models rather than other cell models. These are the two main factors that create a stark difference between the experiment performed in lab vs. prior experiments that helped formed the initial background of this one. [19]

Once these experimental limitations are addressed in a new study, the results should be reevaluated to see if the observations seen here still hold. If they do, then a potential next step would be to look at other possible pathways that can be at play to explain the mechanism of action of these drug therapies. One possible pathway of interest can be the MAPK pathway, which is another commonly studied pathway in cancer cell lines.

Conclusion

Overall, what is seen is that ALP + TMZ is indeed a promising drug combination therapy that has synergistic effects. With that, it was observed that Testosterone did not seem to cause differences between treatment groups, and additionally, it was seen that the activation of the PI3K pathway was not altered among treatment groups as well. As such, with the given data, it seems that a promising combination drug therapy has been identified, but its mechanism of action still remains a mystery. By addressing the experimental limitations and next steps highlighted above, it will be ideal to better undersatnd the mechanism of action of these drugs to better understand how they work and interact within cells.

In light of all things, the work done here mainly focuses on gaining a better understanding of glioblastoma and finding potential ways to make treatment of this devastating disease more effective in hopes of increasing survival rates, decreasing chances for recurrence, and giving new hope to patients.

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