Potentiation of Alisertib by Cabozantinib in Glioblastoma Cells

Andrew J. Hey[1], Cory T. Zumbar[1], Muge Sak[1,2], Norman L. Lehman, M.D., Ph.D[1,2]
[1]Department of Pathology and Laboratory Medicine, [2]Department of Biochemistry and Molecular Genetics
University of Louisville School of Medicine, Louisville, KY 40202

Abstract

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. With current treatments, GBM has a high rate of recurrence and a five-year mortality rate of 90%. Refractory GBM may require use of a novel combination of antineoplastic drugs. Previous studies have shown that alisertib, a potent selective aurora A kinase inhibitor, can inhibit the growth of GBM cells *in vitro* and *in vivo*. Alisertib was well tolerated in clinical trials as a single agent, making it a good candidate to be used in combination therapies for GBM. VEGFR inhibition is currently in clinical trials for GBM. This study assesses the ability of alisertib to potentiate the effects of the VEGFR2 inhibitor cabozantinib in GBM cell lines. We used annexin V binding assays, and proliferation assays and western blotting under normoxic and hypoxic conditions to analyze the effects of alisertib on potentiating the antineoplastic capabilities of cabozantinib in U87 and U251 GBM cells. In annexin V binding assays, alisertib potentiated the apoptotic effect of cabozantinib in U87 cells. Western blotting confirmed the presence of cleaved PARP, a protein indicative of apoptosis, in U251 cells treated with alisertib, cabozantinib, and alisertib+cabozantinib, but not in untreated cells. Under hypoxia, U251 cells treated with cabozantinib and alisertib+cabozantinib expressed the highest levels of cleaved caspase-3, indicating a higher rate of apoptosis. Potentiation of cabozantinib by alisertib, however, was not seen in U251 cells in proliferation assays under normoxic or hypoxic conditions. In fact, cabozantinib was less effective at inhibiting U251 cellular proliferation during hypoxia. In western blotting, greater VEGFR2 expression was seen in all hypoxia samples in comparison to their normoxia counterparts, which may explain the decreased sensitivity of U251 cells to cabozantinib under hypoxic conditions. These preliminary results do not support a synergistic effect between cabozantinib and alisertib in U251 cells. Further studies utilizing other cell lines and tumor models are needed to determine if the drug combination may be useful for treating GBM.

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults.[1] With current treatments, GBM has a high rate of recurrence and a five-year mortality rate of 90%.[2] Refractory GBM may require use of a novel combination of antineoplastic drugs. Previous studies have shown that alisertib, a potent selective aurora A kinase inhibitor, can inhibit the growth of GBM cells *in vitro* and *in vivo*.[3,4] Alisertib was well tolerated in clinical trials as a single agent, making it a good candidate to be used in combination therapies for GBM. VEGFR inhibitors are currently in clinical trials for GBM. This study assesses the ability of alisertib to potentiate the effects of the VEGFR2 inhibitor cabozantinib in GBM cell lines.

Methods

Annexin V Binding Assay

Cells were seeded at 1.25x10^5 cells/mL in 6-well plates for 24 hours. Cells were then treated with 500 nM alisertib, 10 μM cabozantinib, or 500 nM alisertib+10 μM cabozantinib for 24 hours. An annexin V binding assay was conducted using Alexa Fluor 594 annexin V conjugate (Invitrogen A13203) according to the manufacturer's instructions. To measure cell apoptosis, we used a Countess II FL cell counter (AMQAF1000) with a Texas Red light cue that was used to measure the percent of annexin V positive cells. Cells were seeding in triplicate for each drug treatment, and two readings were taken for each sample.

Proliferation Assay under Normoxic and Hypoxic Conditions

Cells were seeded at 1.25x10^5 cells/mL in 6-well plates, and treated with 500 nM alisertib, 10 μM cabozantinib, or 500 nM alisertib+10 μM cabozantinib. The samples were then incubated in normoxia (standard conditions: 5% CO_2/air) or hypoxia (2% O_2 and 5% CO_2) for 6 hours. Cell survival was then measured with a Countess II FL cell counter.

Western Blotting

10 μg of protein was loaded into a 10% acrylamide gels. Gel electrophoresis was conducted for 90 minutes at 100 volts constant. Transfer took place over night. Primary antibodies were incubated for 90 minutes at room temperature (HIF1-a, Novus Biologicals, NB100-105, 1:500; cleaved PARP, Cell Signaling Technology, 9541, 1:1000; VEGF, Abcam, ab1316, 1:1000; B-actin, Sigma Aldrich, a2228, 1:10,000; VEGFR2, Cell Signaling Technology, D5B1, 1:1000; cleaved caspase-3, Cell Signaling Technology, 9661, 1:1000), while HRP-conjugated secondary antibodies were incubated for 60 minutes (anti-rabbit IgG, Cell Signaling Technology, 7074, 1:2000; anti-mouse IgG, Cell Signaling Technology, 7076, 1:2000).

Statistical Analysis

The one-way ANOVA multiple comparisons statistical tests was used to test for potentiation and synergy of alisertib by cabozantinib in the proliferation assay under normoxic and hypoxic conditions.

Results

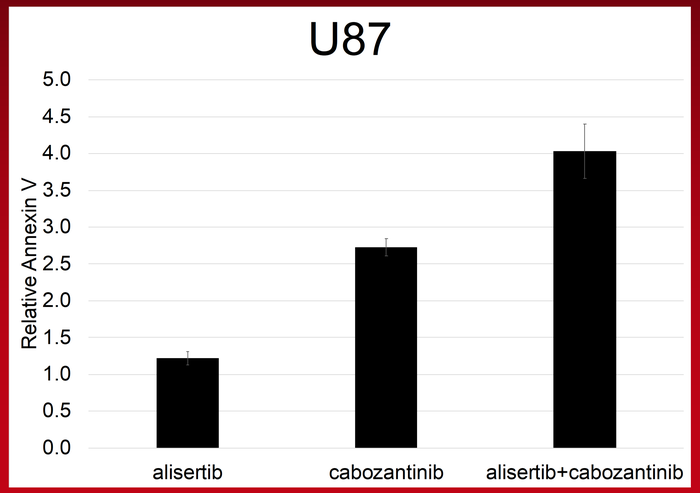

Figure 1: Annexin V binding assays were conducted in U87 cells to test for potentiation and synergy between alisertib and cabozantinib. U87 cells were treated for one day with 500 nM alisertib, 10 μM cabozantinib, and 500 nM alisertib+10 μM cabozantinib. Percent apoptosis was recorded with respect to untreated controls. n=2

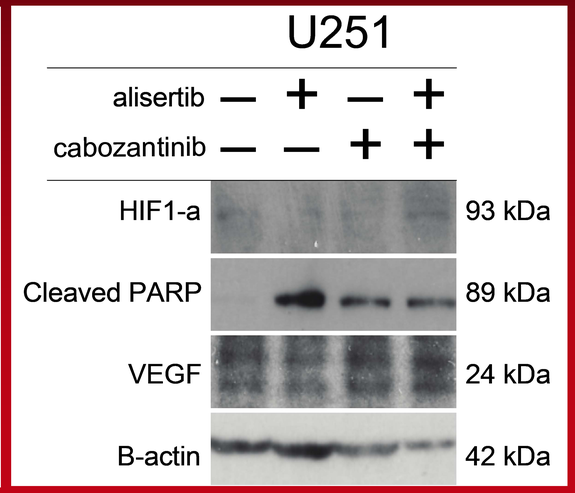

Figure 2: Western blot evaluation of cleaved PARP and VEGF expression in U251 cells after treatment. U251 cells were treated with with 200 nM alisertib, 13 μM cabozantinib, and 200 nM alisertib+13 μM cabozantinib. Administration of a drug is noted by a '+'.

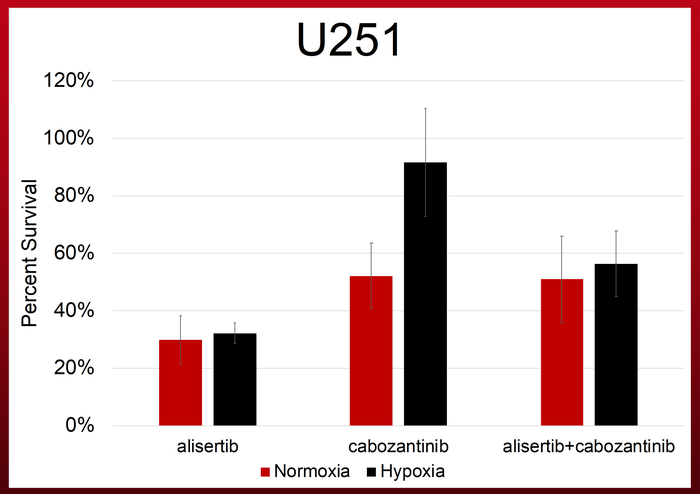

Figure 3: Proliferation assay under normoxic and hypoxic conditions was conducted in U251 cells to analyze how alisertib and cabozantinib functioned under differing levels of oxygen. U87 cells were treated for six hours with 500 nM alisertib, 10 μM cabozantinib, and 500 nM alisertib+10 μM cabozantinib, and were incubated in either normoxia or hypoxia chambers. n=1

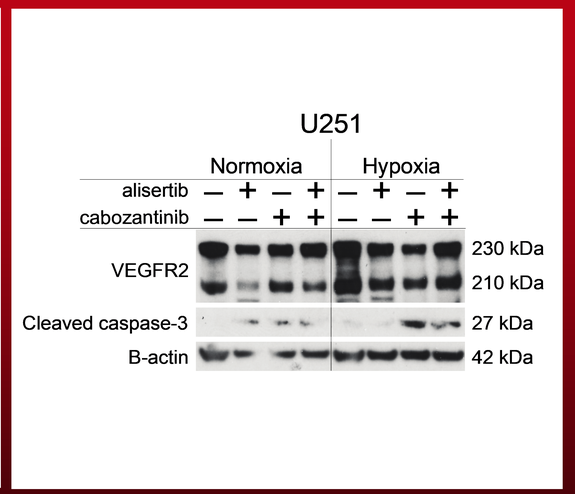

Figure 4: Western blot evaluation of VEGFR2 and Cleaved caspase-3 expression in U251 cells after treatment and incubation for six hours in normoxia or hypoxia. U251 cells were treated with with 250 nM alisertib, 25 μM cabozantinib, and 250 nM alisertib+25 μM cabozantinib. Administration of a drug is noted by a '+'.

In annexin V binding assays (**Figure 1**), alisertib potentiated the apoptotic effect of cabozantinib in U87 cells. Western blotting (**Figure 2**) confirmed the presence of cleaved PARP, a protein indicative of apoptosis, in U251 cells treated with alisertib, cabozantinib, and alisertib+cabozantinib, but not in untreated cells. In addition, U251 cells treated with cabozantinib and alisertib+cabozantinib displayed higher expressions of VEGF subtypes. Under hypoxia (**Figure 4**), U251 cells treated with cabozantinib and alisertib+cabozantinib expressed the highest levels of cleaved caspase-3, indicating a higher rate of apoptosis. Potentiation of cabozantinib by alisertib, however, was not seen in U251 cells in proliferation assays under normoxic or hypoxic conditions (**Figure 3**). This was calculated by one-way ANOVA (multiple comparisons) statistical test. In fact, cabozantinib was less effective at inhibiting U251 cellular proliferation during hypoxia. In western blotting (**Figure 4**), greater VEGFR2 expression was seen in hypoxia samples in comparison to their normoxia counterparts, which may explain the decreased sensitivity of U251 cells to cabozantinib under hypoxic conditions.

Conclusions

In U251 cell lines, the combination of alisertib and the VEGFR2 inhibitor cabozantinib was found to not be synergistic in a proliferation assay by one-way ANOVA (multiple comparisons). In U87 cells, however, the effects of alisertib appear to be potentiated by cabozantinib, as demonstrated by annexin V binding assay. Additional experiments and a statistical model are required to confirm these findings.

Future Directions

We will continue to study the effects of alisertib and cabozantinib in further GBM cell lines in additional apoptotic and proliferation models. Colony formation assays, caspase detection assays, and TUNEL assays can be used in the future to measure apoptosis of U87 and U251 GBM cells when treated with alisertib and cabozantinib. If future studies conclude if a synergy exists between alisertib and cabozantinib in treating GBM cell lines, we may conduct *in vivo* experiments with orthotopic xenograft GBM models.

References

1. American Brain Tumor Association. (2017). *Glioblastoma and Malignant Astrocytoma*. Chicago, IL.

2. Lau, D., Magill, S. T., & Aghi, M. K. (2014). Molecularly targeted therapies for recurrent glioblastoma: current and future targets. *Neurosurgical Focus*, *37*(6). doi: 10.3171/2014.9.FOCUS14519

3. Sak, M., Zumbar, C. T., King, P. D., Li, X., Mifsud, C. S., Usubalieva, A., … Lehman, N. L. (2019). Cytotoxic synergy between alisertib and carboplatin versus alisertib and irinotecan are inversely dependent on MGMT levels in glioblastoma cells. *Journal of Neuro-Oncology*, *143*(2), 231–240. doi: 10.1007/s11060-019-03164-5

4. Van Brocklyn, J. R., Wojton, J., Meisen, W. H., Kellough, D. A., Ecsedy, J. A., Kaur, B., & Lehman, N. L. (2014). Aurora-A Inhibition Offers a Novel Therapy Effective against Intracranial Glioblastoma. *Cancer Research*, *74*(19), 5364–5370. doi: 10.1158/0008-5472.CAN-14-0386

Acknowledgements

We would like to thank the R25 Cancer Education Program at the University of Louisville (National Cancer Institute R25 Grant: CA-134283) for funding this research project. Additional thanks to Charles Anderson for his technical assistance.