Aug 6th, 9:00 AM - 12:00 PM

Characterizing and inhibiting two pathways activated in Glioblastoma Multiforme

Andrea Jydstrup
Nevada Cancer Institute

Sheri L. Holmen
Nevada Cancer Institute

Andrea Jydstrup
Mentor - Sheri Holmen

Despite major improvements in imaging, radiation, and surgery, the prognosis for patients with Glioblastoma multiforme (GBM) remains clinically challenging. New treatment strategies are badly needed to reduce the mortality and morbidity associated with this disease. The resistance of these tumors to conventional treatments makes GBM patients ideal candidates for molecularly targeted therapies and several agents are currently being developed(1). Because GBM is genetically heterogeneous, combination therapies or the use of multikinase inhibitors are more likely to achieve the greatest therapeutic benefit(2,3). However, genes that can be productively targeted for effective therapies in patients remain to be identified. The overall objective of this project was to better understand the signaling pathways driving cell survival so that new targets can be identified in gliomas. These studies will lead to an increased understanding of the proteins that are altered in this disease and should provide promising opportunities to develop better treatment strategies based on specific molecular targets.

Two parallel pathways, which are both activated in GBM, converge on downstream survival signaling cascades. Studies have demonstrated that blocking only one pathway often leads to a transient response (e.g., delayed time to progression), but tumors eventually progress(4). More effective therapies are likely to be those that inhibit more than one target or pathway(5). Targeting anti-apoptotic Bcl-2 proteins in combination with RAS/MAPK or AKT/mTOR inhibition is a rationale approach.

To determine if inhibiting both the RAS/MAPK and AKT/mTOR pathways in combination results in increased apoptosis in glioma cells, I compared the level of apoptosis in cells treated with each inhibitor alone and in combination. Treatment of glioma cells with a MEK inhibitor in combination with a PI(3)K inhibitor has not previously been reported and therefore represents a new approach in the field. We already know that just inhibiting RAS/MAPK or AKT/mTOR alone results in cell cycle arrest but not death. I tested the effect on cell death when combining the inhibitors of both pathways, and saw an increase in cell death. I determined the growth inhibitory and apoptotic sensitivity of several human glioma cell lines to inhibition of both RAS/MAPK and AKT/mTOR pathways. Due to the heterogeneous nature of GBM, I predicted and saw that these cell lines display varying levels of sensitivity to MEK/PI(3)K inhibition. These differences can then be used in the future to further define the mechanism(s) by which the AKT and MAPK pathways mediate survival signaling in glioma cells.

Characterizing and Inhibiting Two Pathways Activated in Glioblastoma Multiforme

Andrea Jydstrup PI: Sheri L. Holmen, PhD.
Nevada Cancer Institute, Las Vegas, NV

INTRODUCTION

Despite major improvements in imaging, radiation, and surgery, the prognosis for patients with Glioblastoma multiforme (GBM) remains clinically challenging. New treatment strategies are badly needed to reduce the mortality and morbidity associated with this disease. The resistance of these tumors to conventional treatments makes GBM patients ideal candidates for molecularly targeted therapies and several agents are currently being developed(1). Because GBM is genetically heterogeneous, combination therapies or the use of multikinase inhibitors are more likely to achieve the greatest therapeutic benefit(2,3). However, genes that can be productively targeted for effective therapies in patients remain to be identified. The overall objective of this project was to better understand the signaling pathways driving cell survival so that new targets can be identified in gliomas. These studies will lead to an increased understanding of the proteins that are altered in this disease and should provide promising opportunities to develop better treatment strategies based on specific molecular targets.

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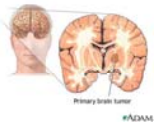
BACKGROUND

Glioma Grade and Prognosis



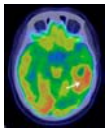
Grade 1: Pilocytic astrocytomas, Curable by surgery
Very distinct from the other grades

Grade 2: Survival can be as long as 10-15 years

Grade 3: Anaplastic astrocytomas, 2-3 year survival

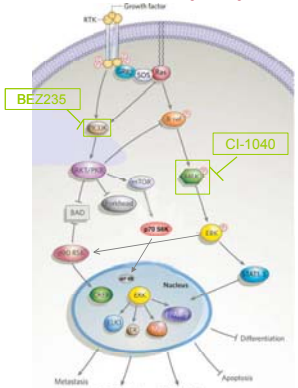
Grade 4: Glioblastoma multiforme (GBM)
GBMs make up ~50% of all primary brain tumors
~21,800 new cases per year*
Average survival is ~12 months
~13,070 deaths per year

*CA Cancer J Clin 58:71-96 (2008)



Positron Emission Tomography (PET) Scan of GBM Tumor
www.mayoclinic.org/images/pet-tumor-bdy.jpg

AKT/mTOR and RAS/MAPK Signaling Cascades Active in GBM



The PI(3)K/mTOR and RAS/MAPK pathways are both active in GBM and engage in "cross-talk" via positive and negative regulation that makes it very difficult to inhibit both pathways simultaneously.

Modified from: Sebolt-Leopold, J. S. & English, J. M. Mechanisms of drug inhibition of signalling molecules. Nature 441, 457-62 (2006).

METHODS

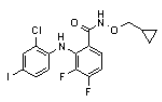
A high-throughput system (SuperArray CASE ELISA) was used to determine the optimal concentrations of inhibitors to decrease phosphorylation in the two signaling cascades after a 48 hour (BEZ235) and 72 hour (CI-1040) treatment period.

Western Blots were used to verify the inhibition of phosphorylated proteins in the pathway (P-ERK, P-AKT, P-p70s6K, and P-MEK) and reprobed for total protein and α-tubulin. Flow cytometry was used to determine cell cycle arrest and apoptosis.

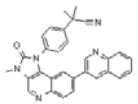
The two inhibitors used were CI-1040 (Pfizer), which is a 2nd generation MEK inhibitor, and BEZ235 (Novartis), which is a Class I phosphatidylinositol-3-kinase (PI3K) tyrosine kinase inhibitor.

Inhibitors were used alone and in combination to compare levels of phosphorylation and apoptosis in the six human GBM cell lines SF-268, SF-295, SF-538, SNB19, SNB75, and U251.

Inhibitors used:
CI-1040 and BEZ 235



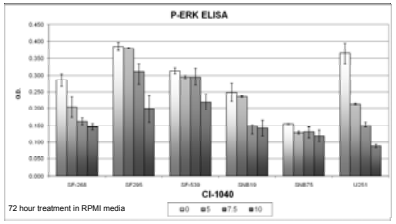
CI-1040
Noncompetitive inhibitor of MEK1



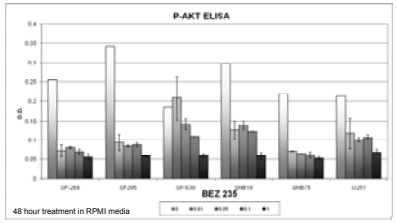
BEZ 235
Class I PI(3)K inhibitor

RESULTS

For the CASE ELISA, a colorimetric determination system is used to determine the relative amount of phosphorylated protein in cells. The lower the O.D., the lower the amount of phosphorylated protein, and therefore the pathways are less active and the cells are less able to proliferate.

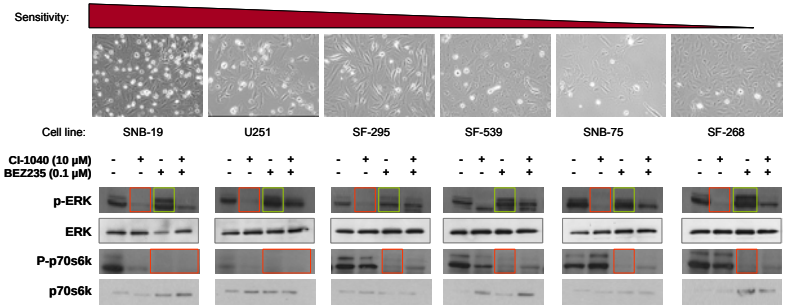


Dose curve for CI-1040- determination of the relative concentration required to inhibit phosphorylation of ERK (MAPK pathway). The dose curve shows that 10 μM appears to be the best concentration of CI-1040 to inhibit phosphorylation of proteins downstream of MEK in the RAS/MAPK signaling cascade.

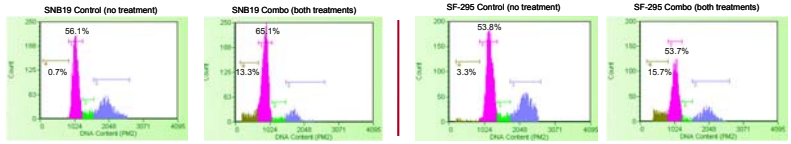


Dose curve for BEZ 235- determination of the relative concentration required to inhibit phosphorylation of AKT (PI3K pathway). The dose curve shows that 0.1 μM of BEZ235 inhibits phosphorylation of proteins downstream of PI3K in the AKT/mTOR signaling cascade in most of the cell lines. 0.1 μM was chosen over 1 μM because at a certain point, there is a risk of cytotoxicity unrelated to apoptosis.

RESULTS (cont)



Western blots were performed on cell lysates from cells treated with the indicated inhibitors for 72 hours, with the BEZ235 being refreshed every 24 hours. The results show that the CI-1040 inhibited p-ERK, while the BEZ235 compound inhibited p-p70s6k to varying degrees between the cell lines, as predicted (red). Interestingly, an increase in phosphorylated ERK can be seen when treated with the PI(3)K inhibitor (green), which corresponds to the negative regulation that AKT has on B-Raf, a protein upstream of ERK in the signaling cascade.



A flow cytometer (Guava) was used to determine cell apoptosis and G1 growth arrest to see if the inhibitors were killing the GBM tumor cells. Although there wasn't a large amount of apoptosis (sub G1= brown), there was a visible increase in cell death and cells present in the G1 phase (pink) when comparing the control cells and cells treated with the combination of drugs. The green area represents S phase and the blue peak represents G2/M phase in the cell cycle.

CONCLUSIONS

The main conclusion that can be drawn from these experiments is that the CI-1040 and BEZ235 compounds do inhibit their respective pathways, but there is something in the cell that is keeping the combination of them from inducing complete apoptosis. Different concentrations may need to be used in combination than the single agent studies indicated.

The data obtained also show significant "cross-talk" between the two pathways, which could also be affecting the combination's ability to work as effectively as expected. Specifically, an increase in P-ERK can be seen when the PI(3)K is inhibited, which may be enough for the cells to survive. This was apparent by both the Western blots and CASE ELISA studies (data not shown). The main objective now will be to find ways to stop the feedback between the two pathways so that complete cell death can be achieved.

FUTURE DIRECTIONS

Different concentrations of inhibitors can be tested to see if higher concentrations are necessary when combined. There are also newer generations of compounds being released that may hit their targets more effectively and be more stable in the cell than the ones tested here.

Other proteins known to be involved in apoptosis will be examined, like the anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-xl. If these can be inhibited, cell death may be achieved more fully.

siRNA will also be performed to completely knock out phosphorylated (activated) proteins. Some possibilities to target include all three isoforms of Raf (A,B, and C) and PI(3)K, thus disabling the pathways for cell survival.

ACKNOWLEDGMENTS

NVCI
Matthew VanBrocklin, PhD.
James Robinson, PhD.
Adam Guilbeault

This poster was made possible by NIH Grant Number P20 RR-016464 from the INBRE Program of the National Center for Research Resources.

REFERENCES

1. Kesari, S., Ramakrishna, N., Sauvageot, C., Stiles, C. D. & Wen, P. Y. Targeted molecular therapy of malignant gliomas. Curr Oncol Rep 8, 58-70 (2006).
2. Reardon, D. A. & Wen, P. Y. Therapeutic advances in the treatment of glioblastoma: rationale and potential role of targeted agents. Oncologist 11, 152-64 (2006).
3. Rich, J. N. et al. ZD6474, a novel tyrosine kinase inhibitor of vascular endothelial growth factor receptor and epidermal growth factor receptor, inhibits tumor growth of multiple nervous system tumors. Clin Cancer Res 11, 8145-57 (2005).
4. Galanis, E. et al. Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study. J Clin Oncol 23, 5294-304 (2005).
5. She, Q. B. et al. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 8, 287-97 (2005).