Investigating the Role of IGF-1 Receptor in Glioma Cell
Survival, Migration and Proliferation

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Dedication and Acknowledgements

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Abstract

Glioblastoma (GB) is the most common primary brain tumor, distinctive by its aggressive, highly invasive, angiogenic and necrotic presentation. The Insulin-like growth factor (IGF) pathway plays an important role in cancer cell proliferation, survival and migration. This study was initiated to investigate the role of the IGF-1 receptor in glioma cell survival, migration and proliferation. We tested glioma cells’ response to IGF1 receptor inhibition and whether the response is dependent on the endogenous levels of pIGF1R β (phosphorylated IGF receptor). We used a small molecule inhibitor of IGF1R, Tyrphostin AG1024, to test for dose-dependent apoptosis and for sensitization to the combination treatment with temozolomide, an oral alkylating agent used for the treatment of Grade IV astrocytoma. We also observed that glioma cell migration and proliferation may depend on the endogenous level of pIGF1R β. Because IGF1R is widely expressed in healthy and malignant cells, development of therapeutic uses for IGF1R-inhibitors will require defining additional genomic or proteomic characteristics. This would confer differential vulnerability between tumor and normal cells. Further investigation is needed to determine
the molecular predictors of a glioma cell’s response to IGF1R inhibition.
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**Introduction**

Glioblastoma (GB) is the most common primary brain tumor, distinctive by its invasive, angiogenic, highly cellular and necrotic nature. GB is classified as Grade IV astrocytoma and is known as extremely malignant, extensively proliferative and usually becomes colossal in size before the patient develops symptoms. Epidemiologically, GBs are the most common primary brain tumors in the adult age group. GB accounts for approximately 50% of all primary malignant brain tumors (12).

Symptoms and morbidity of GB come from its location, progression and mass effect on the surrounding tissues. The overall prognosis for GB patients is poor, despite major improvements in neuroimaging, surgery and radiation therapy. Supportive care is the major mode of treatment available today. Recent studies in combining treatment options such as surgery, radiation and temozolomide demonstrated improvement in patient survival. However, median survival in patients with GB with this combination therapy is still less than 15 months (2). The variability with which patients with the same grade GBM tumor respond to the treatment has been a focus of multiple investigations.
Insulin Growth Factors (IGFs) are known to be important growth factors in many types of tumors. (13) Substantial evidence indicates that IGF-1 and its endogenous receptor function are important factors responsible for growth, proliferation and survival of neuroblastoma cells (11). Nowadays, IGF-1R is being widely investigated as a possible treatment target, however potential problems with specific drug design posed by abundance of IGF-1R in normal tissues and its close homology to the insulin receptor still interfere with the acceptance of the treatment.

IGF-1 Receptor is composed of two α and two β tetramers held together by disulfide bonds. Its ligand binds to cysteine-rich α-subunit and leads to signal transmission through the transmembrane domain on β-subunit leading its conformational change and stimulation of tyrosine kinase activity(3) This in fact leads to activation of multiple pathways involved in suppression of apoptosis, cell proliferation, migration and lead to overall increase in survival (1).

The focus of this study is to gain insight into how IGF1R signaling drives glioma cell survival, migration and proliferation in order to predict whether or not a patient would respond to IGF1R targeted therapies. Since the binding of the ligand induces
autophosphorylation of the IGF1 Receptor β chain into pIGF1R and thereby stimulates activation of intracellular pathways (3), the overall hypothesis of this project is that the response to IGF1R inhibition is dependent on endogenous levels of pIGF1R β. The IGF1 R inhibitor, Tyrphostin AG 1024 was used in this experiment based on its effects of specifically targeting IGF-1 receptor by inhibiting phosphorylation of the beta-subunit and therefore down regulating tyrosine kinase activity.(4)
Research Materials and Methods

a. IGF1R Antibody

   The IGF-1 receptor Inhibitor, Tyrphostin AG 1024 was purchased from CalBiochem.

b. Cell lines and culture

   Human GB cell lines SF767, SNB19, T98G, U118, U251 and U87 were maintained in RPMI medium at 37°C in a humidified incubator with 5% CO2. Cell lines were stored at the Brain Tumor Laboratory, TGen, Phoenix, AZ.

c. IGF-1R Inhibitor treatment

   The effect of Tyrphostin AG 1024 on cell proliferation, migration and survival in vitro were examined following application of the inhibitor to the known number of cells for various periods of time. Endogenous levels of phosphorylated alpha, beta and total IGF-1R were determined in all 6 cell lines prior to inhibitor application using Western blot technique. Results were not normalized for density.

   i. Effect of IGF-1R inhibition on cell survival

   After incubation in serum free media for 24 hours, appr. 800,000 cells were subjected to various levels of the Tyrphostin AG1024 with DMSO
control for a period of 1 hour (12.5 µM, 25 µM and 50 µM). Levels of p-IGF-1R-beta and phosphorylated AKT were determined by Western blot using the appropriate antibodies to observe inhibition and cell survival.

ii. **Effect of IGF-1R inhibition on cell migration**

Appr. 400,000 cells were incubated for 24 hours in serum free media and then subjected to various concentrations of the inhibitor (12.5 µM, 25 µM, 50 µM) with vehicle control (0 µM) for a period of 24 and 48 hrs. Drug treatment with serum was repeated at 24 hours for continuous drug effect. Migration rate was calculated manually.

iii. **Effects of IGF-1R inhibition on cell proliferation**

Known number (appr. 1000 per well) from each cell line were subjected to various concentration of the inhibitor (0 µM, 12.5 µM, 25 µM and 50 µM) and inhibitor with temozolomide (TMZ, TMZ + 12.5 µM AG1024, TMZ + 25 µM AG 1024, TMZ + 50 µM AG 1024) to observe cell proliferation and the effect of the sensitization to TMZ. Cells treated with IGF-1R antibody were used as control for the TMZ treatment group (at concentration of 1.5 microg/mL). Cell number was determined at 24 and 48 hrs using CellTiter-Glo Luminescent Cell Viability Assay. Results were normalized to the curve plate.
d. Western blot assay

After each treatment, cells from each cell lines were lysed with lysis buffer (consisted of SDS buffer, protease inhibitor along with phosphatase inhibitor, NaOV and PMSF) and sonicated. Equal amounts of proteins were analyzed on SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membranes and analyzed by specific antibodies. Proteins were detected via incubation with appropriate secondary antibodies. To determine endogenous levels of phosphorylated IGF-1R and the effects of treatment, p-IGF-1R antibody were used. LC3B antibody, marker for autophagy, which is a catabolic process for the autophagosomic-lysosomal degradation, was used to determine the effects of the treatment on the cell survival along with PARP antibody, which is the marker for the apoptosis. Tubulin was used as a control for loading; samples were not normalized for band density.

e. Statistical analysis

The data was presented as the means +/- SD with t-test used for statistical comparison. P < 0.05 was accepted as statistically significant.
Results

1. The endogenous levels of phosphorylated IGF-1R in 6 glioma cell lines

   This experiment was designed to determine the endogenous levels of phosphorylated alpha, beta, and total IGF-1R in all 6 cell lines. The proteins were detected using human p-IGF-1R and total IGF-1R antibody. Figure 1 below depicts the results. Western blot showed that SF767, U118, and T98G glioma cells had significantly higher endogenous levels of p-IGF1R-β than remaining SNB19, U251, and U87 glioma cells. Levels of p-IGF1R-α and total IGF1R were less variable among the 6 glioma cell lines. Samples were not normalized for band density.
Figure 1. Protein levels of phospho-IGF1R-β vary in 6 glioma cell lines.
2. Effect of IGF-1R inhibition

This experiment was designed to determine the effects of IGF-1R inhibitor, Tyrphostin AG 1024 at various concentrations on cell survival. For this purpose, U118 glioma cells were treated with Tyrphostin AG1024 at concentrations of 12.5 µM, 25 µM and 50 µM for a period of 30 minutes and 1 hour. Proteins from each sample were collected and Western blot showed reduced levels of pIGF1R and increased levels of pAKT with the Tyrphostin AG1024 treatment. Data suggested that Tyrphostin AG1024 had effect on pIGF1R at all concentrations, while showing the highest effect at 25 µM in 1 hour of treatment. pAKT, marker of growth-factor associated cell survival, was elevated compared to not treated cells across all the treated samples and showed the highest levels with the increased concentration and increased duration of the treatment (50 µM at 1 hour being the highest). Samples were not normalized for density.
Figure 2. Decreasing levels of phospho-IGF-1R-beta and increasing levels of pAKT in U118 glioma cells when treated with Tyrphostin AG1024.
To determine cell survival, U118 cells were treated with Tyrphostin AG 1024 at various concentrations of 12.5 µM, 25 µM and 50 µM for a period of 30 minutes. Proteins from each sample were collected and Western blot showed that autophagy was induced with the drug treatment in dose dependent manner by looking at LC3B-II levels, marker for autophagy. These results suggest that more autophagy was induced at the higher dose of the inhibitor. Samples were not normalized for band density.
Figure 3. Autophagy (LC3B-II) induced apoptosis in U118 glioma cells following the treatment with Tyrphostin AG 1024 IGF1R inhibitor for 30 minutes.
Based on these results we further investigated the effect of sensitization to temozolomide (TMZ) treatment at optimal concentration of 25 µM of AG 1024 IGF-1R inhibitor on survival of cells. For this purpose levels of p-IGF1R beta were determined in SNB19 glioma cells following treatment with 25µm AG1024 inhibitor and TMZ+25µm AG1024 inhibitor with TMZ and DMSO as controls (Figure 4). The results depicted in images below show possible effect on sensitization of cells to TMZ, hence p-IGF1R beta levels were decreased in cells treated with additional TMZ. Samples were not normalized to density.
Figure 4. Levels of phospho-IGF-1R-beta decrease with IGF-1R inhibitor treatment and further decrease with the addition of TMZ to the treatment in SNB19 glioma cells suggesting sensitization.
3. Effect of IGF-1R inhibition on cell migration

SF767 and SNB19 glioma cells lines were placed in a migration assay, left for a period of 24 hours, then treated with various concentrations of Tyrphostin AG1024 and TMZ and migration rate was measured 24 and 48 hours following the treatment. SF767 glioma cells with endogenously high levels of p-IGF1R-β, showed decreased cell migration after treatments with the inhibitor, with the most significant decrease at the dose of 25 μM. SNB19 glioma cells with endogenously low levels of p-IGF1R-β, showed insignificant change in migration rate suggesting that IGF1R inhibition depends on endogenous levels of p-IGF1R-β.
Figure 5. Glioma cell migration following the treatment with various concentrations of Tyrphostin AG1024 is dependent on endogenous levels of p-IGF1R-β.
4. Effects of IGF-1R inhibition on cell proliferation

This experiment was designed to determine the effects of IGF-1R inhibition on cell proliferation and sensitization to TMZ in cell lines with high (SF767) and low (SNB19) endogenous levels of p-IGF-1R-beta. SF767 and SNB19 glioma cells lines were seeded in a 96 well plate (100 µl/1000 cells per well), and then treated with various concentrations of Tyrphostin AG1024 and TMZ. Cells were incubated for a period of 2-4 hours and proliferation assay was performed using CellTiter-Glo Luminescent Cell Viability Assay. Percent cell survival was measured and graphed. SF767glioma cells with endogenously high levels of p-IGF1R-β and SNB19 glioma cells with endogenously low levels of p-IGF1R-β, both showed decreased cell proliferation after treatments with the inhibitor and the combination treatment, with the most significant decrease at the dose of 25 µM of Tyrphostin AG1024. Reduction in cell proliferation was observed in the dose dependent manner and enhanced reduction was observed in cells pretreated with TMZ.
Figure 6. SF767 glioma cell proliferation following the treatment with various concentrations of Tyrphostin AG1024 and TMZ showed possible sensitization to TMZ.
Figure 7. SNB19 glioma cell proliferation following the treatment with various concentrations of Tyrphostin AG1024 and TMZ showed possible sensitization to TMZ.
Discussion and Future Directions

Insulin-like growth factors and their receptors have been described to be involved in cancer pathophysiology and were found to be over expressed by many tumors. Moreover it has been shown that IGF-1R is universally expressed in various hematologic tumor cells, like multiple myeloma, lymphoma, leukemia, as well as solid tumor cells, such as breast, prostate, lung etc. (6) IGF1R inhibition has become a widespread target in attempts to arrest neoplastic processes and therefore to have a possible therapeutic implications in treating tumors including GB. (7)

In this study we investigated how IGF1R signaling effects glioma cell survival, migration and proliferation. Initially we found that protein levels of phospho-IGF1R-β vary in 6 glioma cell lines. SF767, U118 and T98G glioma cells had significantly higher endogenous levels of p-IGF1-R-beta than SNB19, U251 and U87 glioma cells (See Figure 1). We also found that the levels of p-IGF1-R alpha and total IGF1-R were less variable among the 6 glioma cell lines. Knowing that the binding of IGF-1 induces autophosphorylation of the IGF1 Receptor β chain and thereby stimulates activation of
intracellular pathways (3), we predicted that response to IGF1-R inhibition will vary based on the levels of the endogenous phospho-IGF1-R-beta proteins in glioma cell lines. Moreover, even though levels of IGF1-R were found to be over expressed in many tumors, including GBs, it seems that phospho-IGF1-R-beta expression varies. This finding may help in distinguishing tumors that will have better response to IGF1-R inhibition therapy.

We used Tyrphostin AG 1024 in this investigation based on its effect of specifically targeting IGF-1 receptor. First of all we found that levels of phospho-IGF-1R-beta decrease with Tyrphostin AG1024 treatment with the optimal reduction at 25 µM in 1 hour of treatment. This confirms the effects of the Tyrphostin AG1024 inhibitor on the phospho-IGF-1R-beta in glioma cells and since IGF1-R signaling involved in suppression of apoptosis, cell proliferation, migration, we predict that these processes are decreased with IGF1-R inhibition therapy. We also observed that the levels of pAKT, marker of growth-factor associated cell survival, was elevated compared to not treated cells across all the treated samples. Multiple studies in the past showed that IGF1-R inhibition induces a decreased expression of phospho-AKT and therefore induce apoptosis (4). We can explain our
finding by saying that sometimes cells in the process of apoptosis upregulate their cell survival mechanisms which lead to increase in proteins that are known markers for cell survival. Using LC3B-II as a marker for autophagy induced apoptosis, our investigation found that Tyrphostin AG 1024 IGF1R inhibitor therapy increased apoptosis. Autophagy is a catabolic process involving cell degradation via lysosomes. We observed that autophagy was induced with the drug treatment in dose dependent manner, suggesting that more autophagy was induced at the higher dose of the inhibitor (See Figure 3).

Recent studies proposed that sensitization to temozolomide can improve patient survival when treated with IGF1-R inhibitor (2). We found that levels of phospho-IGF-1R-beta decrease with IGF-1R inhibitor treatment and further decrease with the addition of TMZ to the treatment (See Figure 4). These findings confirm the proposed idea of cell sensitization by TMZ. Since SNB19 glioma cells have low initial endogenous levels of phospho-IGF-1R-beta, we can suggest that sensitization to TMZ might not be dependent on the initial phospho-IGF-1R-beta levels.

It has been shown previously that IGF1-R inhibition affects cell migration (8); we investigated possible effects of the endogenous levels
of p-IGF1R-β on cell migration with the inhibition therapy. We found that glioma cell migration following the treatment with various concentrations of Tyrphostin AG1024 is dependent on endogenous levels of p-IGF1R-β (See Figure 5). We observed that SF767 glioma cells with endogenously high levels of p-IGF1R-β, showed inhibition in cell migration after treatments with the inhibitor while SNB19 glioma cells with endogenously low levels of p-IGF1R-β, showed insignificant change in migration rate.

In further investigation, we observed dose dependent reduction in cell proliferation in glioma cell lines when treated with IGF1-R inhibitor in cells with high and low endogenous levels of p-IGF-1R-beta. Moreover the reduction in cell proliferation was enhanced when cells were pretreated with TMZ suggesting sensitization to TMZ (See Figure 6 and 7).

There are several key points of this investigation that need to be addressed. First of all, experiments in this study should be repeated with better conditions and data analysis should include standardization to better evaluate results. The concentration of the drug used should be compared to what is physiologically achieved in humans to better assess possibility of using this treatment clinically.
Another important key point that should be mentioned involves the limitation of using cell lines when conducting experimentations. On one hand, cell lines are extremely valuable in identifying aberrant genes and gene expression. On the other hand, one should keep in mind that the observation made in cell lines might not be occurring in patients and until the same finding can be obtained from surgical specimens, assumptions regarding possible clinical application of the treatment need to be made.

Based on findings of this investigation, we can propose future directions in testing glioma cell survival, proliferation and migration using neutralizing anti-human IGF1-R antibody and comparing it to the effects of small molecule inhibitor Tyrphostin AG1024 tested here. Moreover, one should take a closer look at the effects based on the initial endogenous levels of p-IGF1R-β in glioma cells. Also possible SNPs in IGF1R gene might be investigated in becoming possible predictors of poor prognosis to the anti-IGF1R therapy. Overall, based on aCGH, SNP and expression data for these 6 cell lines predict which xenografts will respond to the treatment with IGF1R inhibitors. These studies should assist in future clinical development of IGF-1 receptor antagonists for glioblastoma and other tumors.
Conclusion

The relevance of IGF1-R to cancer pathophysiology is now widely accepted and is investigated by multiple companies as a target in cancer treatment (12). Because it is so widely expressed, it is important to define certain subtype of the receptor which can act as a predictor in the response to the therapy. Moreover, it is important to investigate for possible biomarkers that can also be used as a predictor in the response to the therapy. Investigating response to various inhibitors as well as combination treatments in gliomas with various endogenous levels of the IGF1-R along with possible downstream signaling pathways can play a role in future therapeutics development.
References


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