UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
DOCTORAL SCHOOL OF MEDICINE

PhD THESIS

MOLECULAR THERAPY IN GLIOBLASTOMA BY SELECTIVE TYROSINE KINASE RECEPTOR INHIBITION

ABSTRACT

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STATE OF KNOWLEDGE

Glioblastoma is the most aggressive primary malignant tumor in adults, with a very low survival rate and a limited prognosis, despite the numerous diagnostic and therapeutic advances. Over the past decades efforts were made into understanding the complex biology of these tumors, focusing on the detection of new biomarkers that could allow early detection and the development of therapeutic strategies.

New treatment methods for glioblastoma are currently investigated in the field of medical research. Due to the tumors high capacity to invade neighboring tissues, adjuvant therapies are recommended in malignant gliomas. Recent studies suggest that the genetic fingerprint of each patient will be a keypoint in developing more efficient personalized molecular therapies.

The most common genetic alterations in malignant glioma are EGFR mutations. Another novel therapeutic approach is angiogenesis. Two other receptors frequently used as therapeutic targets in glioblastoma are VEGFR and PDGFR. Recent studies have suggested that ELTD1, a receptor involved in angiogenesis, has an important role in the progression and development of glioblastoma multiforme.

PERSONAL CONTRIBUTION

In this study, we took into consideration the necessity to emphasize more specific biomarkers for malignant glioma, which could make diagnosis and possible treatment options much easier. Simultaneously, we tried blocking tyrosine kinases receptors using targeted molecular therapy and silencing the genes responsible for the receptors which are considered more specific for malignant glioma.

The study achieved the following objectives:

Objective 1 - Study of distribution of tumors correlated with histological type, age, sex, urban-rural origin and geographical distribution per regions

In Romania, there are no statistical studies available at this time recording the incidence of brain tumors. This is the main reason why our study aims to determine the distribution of brain tumors during the 2006-2012 period, correlating age and gender, using data from the tumor registry of the Department of Neurosurgery at Bagdasar

Arseni Hospital Bucharest, for the 262 patients who were included in a Brain Tumor Bank (BTB).

Objective 2 – The study of the survival rates of patients with recurrent malignant glioma after immunotherapeutic treatment with dendritic cells, compared to the therapeutic combination of bevacizumab and irinotecan.

The recommended treatment for recurrent malignant glioma consists of bevacizumab plus irinotecan. Vaccination with dendritic cells has been reported in literature to be an innovative, effective therapeutic alternative for the treatment of recurrent malignant glioma. In this study, we deemed it necessary to statistically compare the survival after both types of treatment, which are fundamentally different in terms of management.

Objective 3 – The study of the effect of inhibiting tyrosine kinases receptors on GB cell culture viability *in vitro*.

The physiological role of tyrosine kinase receptors is to regulate cell proliferation. These protein receptors and their intracellular signaling pathways, are frequently genetically or epigenetically altered in cancer cells, thus confering an advantage in proliferation for the tumor cells. Alongside VEGFR and PDGFR, recent studies suggest that the ELTD1 receptor is also involved in tumor angiogenesis. Tyrosine kinase inhibitors have been extensively studied over the past few years, considered as a niche molecular targeted therapy in the treatment of brain tumors.

This objective aims to analyze the role of tyrosine kinase receptor as therapeutic targets in the treatment of high grade glioma.

Objective 4 - Evaluation of the dose - response type synergistic action of the chemical agent TMZ and the inhibitor AG556 *in vitro*.

The administration of combined therapy in brain tumors is a standard practice with promising results. The alkylating agent TMZ is used to treat both low and high grade brain tumors. It is a known fact that glioblastoma is remarkably resistant to treatment with TMZ, several molecules of the tyrosine kinase receptor family are suspected to be interfering with its action. This suggests that combined approaches involving inactivation TKRs and TMZ could be a possible future therapeutic direction for this malignancy. In this study, we aimed to evaluate the effects of TMZ treatment in combination with AG556, an EGFR inhibitor on the glioblastoma cells.

MATERIALS AND METHODS.

This chapter presents the materials used in research and their origins. Thus, reference is made to the cell culture medium, the cell growth determination kit MTT, fetal bovine serum (FBS), antibiotics, trypsin, phosphate-buffered saline (PBS) used. Also, the inhibitors used were SU1498, AG1433, AG556 and the agent TMZ.

For the implementation of Objective. 1 it was necessary to use the information stored within the tumor registry of the Department of Neurosurgery at Bagdasar Arseni Hospital in Bucharest. The indicators followed were incidence rates for the main brain tumors in the last 5 years and their relationship with age and gender.

In order to implement Objective. 2 an epidemiological study was considered in order to systematically evaluate the previous research in a specific area, which provides a more accurate estimate of the treatment effect or a risk factor for certain diseases compared to individual studies.

In order to implement Objective. 3 we studied the effect of AG14433 and SU1498 on the GB8B glioblastoma cell line, and the effect of AG556 and TMZ on the GB1B cell line.

For the implementation of Objective. 4, we used the glioblastoma cell line GB1B in order to determine the synergistic action of the AG556 + TMZ combination.

RESULTS AND DISCUSSIONS

- Meta-analysis study

After processing data from literature based on the eligibility criterias, seven studies that have described patients who received bevacizumab plus irinotecan and 7 studies that described patients receiving immunotherapy with dendritic cells remained. A total of 381 patients were included in our systematic study of which 302 (79.26%) received bevacizumab plus irinotecan and 79 (20.74%) were vaccinated with dendritic cells. The study included 243 men and 138 women.

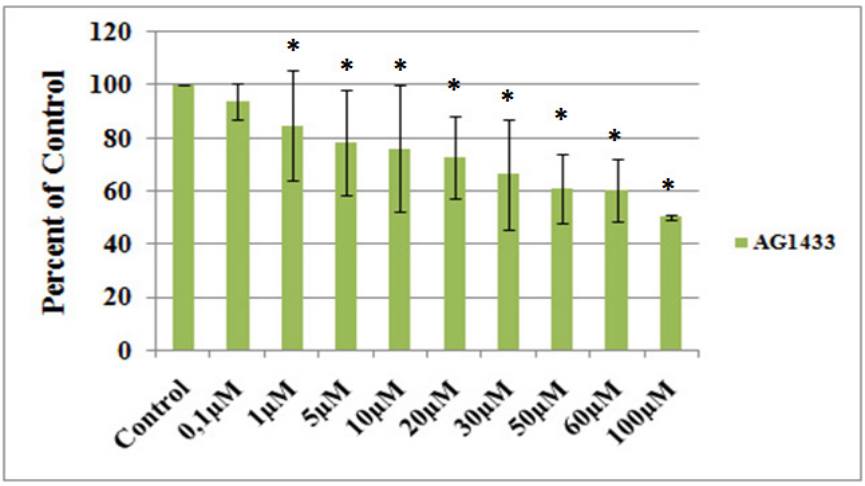
Regarding the outcome of treatment, the mean reported MoS was 7.5 (95% CI 4.84-10.16) months for patients receiving bevacizumab and irinotecan. For the patients who received vaccination with dendritic cells, the mean reported MoS was 17.9 (95% CI; 11.34-24.46) months. In comparison, studies using bevacizumab and irinotecan

reported a survival benefit of -0.02 ± 2.00, while the reported benefit in survival was -0.01 ± 4.54 for the group that used dendritic cell immunotherapy. As a general aspect, we observed that, compared to the protocol based on bevacizumab and irinotecan, vaccination with dendritic cells had no statistically significant effect on OS (P = 0.535) and did not improve the statistical increase in weighted survival gain (P = 0.620) for patients with malignant gliomas.

-The effect of PDGFR inactivation on glioblastoma cell cultures

To analyze the effect of inhibiting PDGFR we used a glioblastoma cell line (GB8B). The tumor cells were exposed to concentrations of 0.1, 1, 5, 10, 20, 30, 50, 60, 100 μM AG1433. The effect of the inhibitor on cell viability was investigated after a 72h interval.

AG1433 treatment produced 15% cytotoxicity in the GB8B cells beginning with the concentration of 1 μM. The concentrations of 5, 10 and 20 μM AG1433 caused the death of about 25% of the cells. Higher concentrations of AG1433 (50 and 60 μM) induced the death of 40% cells and the maximum concentration of 100 μM AG1433 induced the death of more than 50% of GB8B cells.

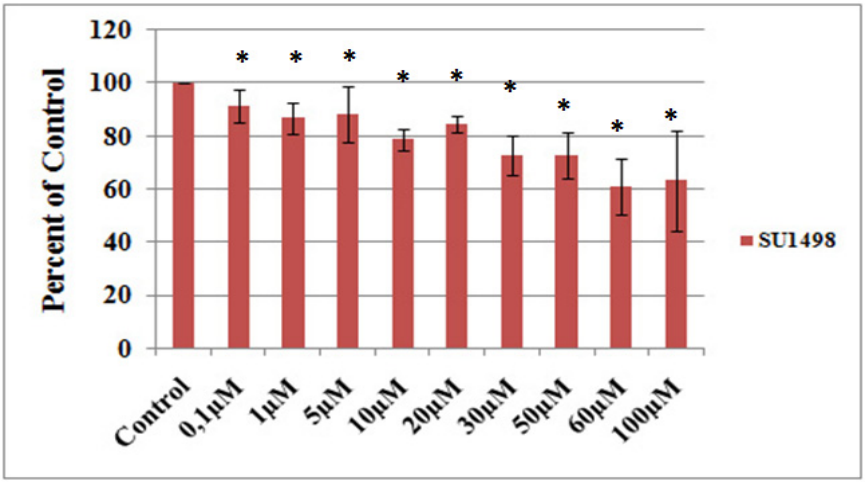


The effect of PDGFR inactivation on glioblastoma cell cultures

-The effect of VEGFR inactivation on glioblastoma cell cultures.

To analyze the effect of VEGFR inhibition we used the same glioblastoma cell line (GB8B). The tumor cells were exposed to concentrations of 0.1, 1, 5, 10, 20, 30, 50, 60, 100 μM SU1498. The effect of the inhibitor on cell viability was investigated after a 72 hours span.

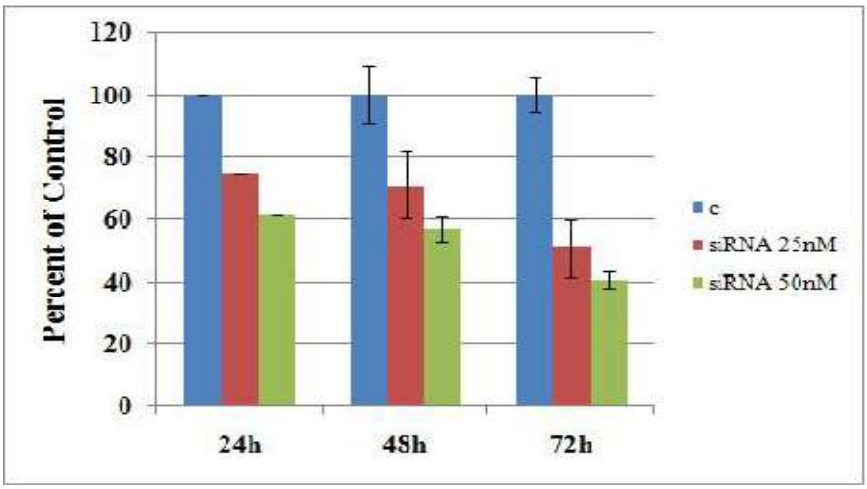
The results show that the treatment with 0.1 μM SU1498 induced a 10% cell cytotoxicity. The 1-20 μM concentrations of SU1498 1-20 caused a 15-20% decrease in viability, treatment with 30 and 40 μM SU1498 decreased cell viability by 29%, and doses of 60 and 100 μM SU1498 caused the death of 60% of the tumor cells after 72 hours of treatment.



The effect of VEGFR inactivation on glioblastoma cell cultures

- The effect of ELTD1 underexpression

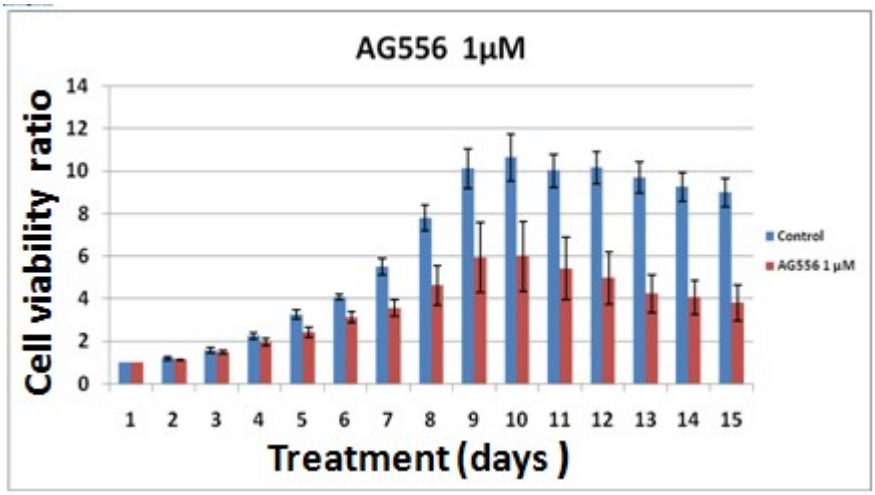
Blocking the ELTD1 receptor function affects the birth of new blood vessels and their development, reducing tumor growth and improving survival. ELTD1 inhibition in GB8B glioblastoma cells was performed by transfection with siRNA. Cells were transfected with siRNA-ELTD1 (25 and 50 nM) and incubated for 24, 48 and 72 hours. A significant decrease in cell survival after 72 hours of treatment was observed, for both doses. The dose of 25 nM ELTD1 siRNA induced the death of about 25% of the cells at 24 and 48 hours and had a cytotoxic effect of 50% at 72 hours. 50 nM of ELTD1 siRNA had cytotoxicity of 40% at 24 and 48 hours and resulted in the death of 60% of the cells at 72 hours.



The effect of ELTD1 inhibition by transfection with siRNA

- The effect of EGFR inhibition on malignant glioma cells

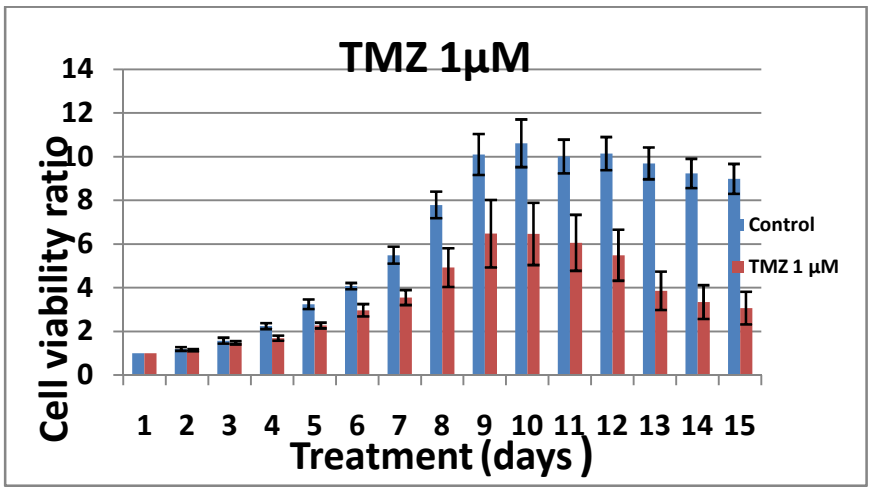
The current study investigated the effects of EGFR inactivation on viability in the GB1B brain cancer cell culture. The cells were subjected to EGFR specific inhibition with AG556 (1, 5 and 10 μM) for 24 hours and then the agent was removed by washing and the cells were then incubated continuously for up to 15 days. AG556 inhibited cell growth in a time and dose dependent manner. In the GB1B glioblastoma line, 1 μM of AG556 resulted in a decrease in the cell viability ratio of about 1 after day 6, 3 after the 8th day and over 5 after days 12, 13, 14 and 15 compared to untreated cells.



Treatment with 1 μM of AG556

- TMZ

We analyzed the effect of TMZ on glioblastoma tumor cells (GB1B line). GB1B tumor cells were treated with 1 μM or 5 μM of TMZ for 24 hours. Treatment with 1 μM TMZ induced significant inhibition ($P \leq 0.05$) in cell viability in the GB1B cell culture after the fifth day and increased with time. The viability rate for the GB1B cells decreased by about 1 from days five to seven after treatment with 1 μM TMZ, 3 from days eight and nine, with about 4 from days eleven to twelve and with approximately six during days thirteen, fourteen and seventeen in comparison to untreated cells.



Treatment with 1 μM TMZ

- Combined treatment with AG556 + TMZ

In this study, the effect of TMZ (1 and 5 μM) and AG556 treatment were assessed. The analysis of the interaction between the therapeutic combinations of the A556 and TMZ was carried out using the multiplicative model, as follows:

- additive effect occurs when $I_{1,2} = I_1 + I_2$;
- synergistic effect occurs when $I_{1,2} > I_1 + I_2$;
- antagonistic effect occurs when $I_{1,2} < I_1 + I_2$.

Following the evaluation of the interaction between the two molecules, we found out that none of the combinations produced a synergistic therapeutic effect compared to individual therapeutic approaches. Thus, our results show that in the GB1B cell line, 17 (20.24%) of the combined treatments presented an additive effect, while 67 (79.76%) of them had a subadditive result.

CONCLUSIONS

1. Patient age is an important factor in the incidence of brain tumors, the highest number of cases recorded was in the 55-65 years age group, followed by the 45-55 years age group.
2. In the study we conducted, we noticed a trend in the development of brain tumors at an earlier age than that reported in literature.
3. Gliosarcoma, meningiosarcomas and medulloblastomas were more common in men.
4. Schwannomas and meningiomas were more common in women.
5. Astrocytomas, hemangioblastomas, hemangiopericytomas and neurocytomas were evenly distributed between genders.
6. Compared to the bevacizumab + irinotecan treatment protocol, vaccination with dendritic cells did not show a significant benefit in the survival of patients with recurrent malignant glioma.
7. The cell viability assay performed after the inactivation of membrane receptors PDGFR, VEGFR and EGFR using the small molecule compounds AG1433, AG556 and SU1498 showed a moderate cytotoxic effects on GB cells.
8. Blocking the ELTD1 expression in glioblastoma cells by using siRNA, had proven to be more effective than inhibiting PDGFR, VEGFR and EGFR by using small molecule compounds.
9. The combined treatment of AG556 and TMZ produced additive effect in 20% of such combined treatments , while 80% of them resulted in a subadditive effect.

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