Original Paper

Effect of VEGFR, PDGFR and PI3K/mTOR Targeting in Glioblastoma

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ABSTRACT: Resistance to targeted therapy is a well known obstacle in cancer therapy. The cross-talk between several growth factor receptors generates redundancy in their intracellular pathways that usually mediates resistance to receptor targeted therapy. Simultaneous inactivation of two or more growth factor receptors has been suggested to prevent the cross-talk between their signaling pathways and to better eliminate malignant cells. Here we found that targeted therapy against these receptors induced moderate cell death in glioblastoma cells. More important, dual PDGFR and VEGFR inactivation induced more pronounceable cell death compared to inactivation of each receptor alone but failed to induce synergistic cell death in glioblastoma. PI3K/mTOR dual targeting has been identified as an efficient therapeutic approach in several malignant diseases, including glioblastoma. Therefore, we also investigated the PI3K/mTOR pathways inhibition effect in glioblastoma cells. Our results showed that inactivation of PI3K/mTOR pathways were more efficient than PDGFR or VEGFR single targeting or their dual inhibition.

KEYWORDS: *VEGFR, PDGFR, glioblastoma*

Introduction

Glioblastomas (GBs) are the most common malignant intracranial diseases with high incidence in older pacients [1]. Even if, in the recent years, various therapeutic approaches have been developed for GB treatments, this lethal disease remains the main clinical challenge in neuro-oncology [2]. Currently, GB treatment is focused on a combination of surgery, radiotherapy, and chemotherapy. Chemotherapeutic agents like temozolomide [3] and irinotecan were suggested to improve overall survival and quality of life in patients gloma patients, although based on cellular resistance to these drugs and presence of O-6-methylguanine-DNA methyltransferase (MGMT) [4], a large number of patients do not benefit from this treatment. Nowadays, there is a concern in developing novel chemotherapeutic drugs that target multiple molecular pathways in GB cells [5].

Increased expression and activation of angiogenic tyrosine kinase receptors (RTKs) occurs frequently in human brain cancers and mediates various ways that stimulate tumor growth [6].

The main angiogenic factor involved in endothelial cell proliferation and blood brain barrier permeability is vascular endothelial growth factor (VEGF), known to be secreted in high level by glioblastomas [7]. Bevacizumab (Avastin), a VEGF-A antibody was approved by Food and Drug Administration (FDA) as therapeutic agent for recurrent GB mainly used as monotherapy. However, bevacizumab in combination with other chemotherapeutically agents such as temozolomide, was reported to develop a variety of resistance mechanisms in malignant cells [8]. A number of recent clinical trials showed a poorly outcome for newly diagnosed GB pacients treated with bevacizumab and standard therapy (radiotherapy and temozolomide) [9]. Two clinical trials (phase II and phase III) using another VEGFR inhibitor, cediranib were completed at the end of year 2014. The analysis of these clinical trials showed that pacients diagnosed with recurrent GB received cediranib and lomustine in monotherapy, or a combination of them. The results also shows that there is no considerable benefit in treatment efficacy with cediranib either alone or combined, when compare with lomustine monotherapy; furthermore the combination of cediranib and lomustine seems to increase the frequency of hematological toxicities [8].

Platelet-derived growth factor (PDGF) family is also implicated in tumor growth and angiogenesis [8, 10]. From all existing PDGF ligands, many studies revealed that in low grade glioma PDGF-AA are more expressed, while in high grade glioma PDGF-BB expression was more abundant [11]. Despite of numerous small molecule inhibitors that are used in clinical trials, such as Sunitinib, Imatinib, Sorafenib, only few of the drugs are in phase III developmental stages and Avastin is approved for treatment of recurrent GB [8].

Acting as intermediate signaling molecules phosphatidylinositol 3-kinases (PI3Ks) are involved, in cell cycle, apoptosis and angiogenesis, playing an important role in PI3K/AKT/mTOR (PI3K/protein kinase B/mammalian target of rapamycin) signaling pathway. Abnormal activation of PI3K/AKT/mTOR pathway stimulates oncogenesis and tumor microvascular development. Furthermore, dysregulation of PI3K pathway signaling is a well known cause of resistance to current therapies in glioblastoma, a serious problem, given the extremely high frequency of glioblastoma patients (almost 88%) who have an elevated PI3K/AKT/mTOR signaling pathway [12-14].

In this study, we analyzed the effect of PDGFR and VEGFR inactivation alone, their combined inhibition and PI3K/mTOR dual targeting in glioblastoma cells *in vitro.*

Material and methods

Reagents and chemicals

Dulbeccos Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 Ham, Dimethyl Sulfoxide (DMSO), Ethylenediaminetetraacetic Acid (EDTA), Cell Growth Determination Kit MTT based [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma-Aldrich (Germany), Fetal Bovine Serum (FBS), Penicillin/Streptomycin antibiotics, Trypsin, Phosphate-buffered saline (PBS) were obtained from Gibco by Life Technologies™, Cell Lysis Buffer was provided by Invitrogen, Life Tehnologies, USA.

SU1498 (VEGFR2 selective inhibitor) and Dactolisib (dual PI3K/mTOR inhibitor) were acquired from Santa Cruz Biotechnologies, AG1433 (PDGFR-β and a weak inhibitor of angiogenesis and VEGFR2) were obtained from Sigma-Aldrich.

Cell culture and treatment

Low passage tumor cell lines, used in our study, were established from tumor tissue obtained from patients diagnosed with glioblastoma at the "Bagdasar–Arseni" Emergency Hospital, Bucharest, Romania. The tumor cell lines were established following to standard procedures [15, 16].

The cells were cultured in DMEM/Nutrient Mixture F-12 Ham, in the presence of 10% FBS, 2 mM Glutamine and antibiotics combination (100 IU/ml Penicillin and 100 mg/ml Streptomycin). The tumor cells were maintained at 37^0C and 95% air with 5% CO_2 atmosphere in a humidified incubator (CO_2 Incubator Innova CO-170) in 25 cm^2 tissue culture flasks. The culture cell lines were transferred one time per week.

In our study, cells were seeded 96-well culture plates at concentration of 3×10^3 cells/well in DMEM and treated with different concentrations of small molecules inhibitors tyrosine kinases, SU1498 (0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM), AG1433 (0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM), with various concentrations of dual pathways inhibitor Dactolisib (0.1nM, 1 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM) or combination of them for 48 or 72 hours. Appropriate control groups with culture medium and 0.01% DMSO were included. Each of the three experiments was performed in triplicate.

MTT cell proliferation assay

To investigate the antiproliferative effect of the treatment we used MTT assay (Sigma Aldrich). This method can be explain by the properties of metabolically active cells to cleavage the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals. Target cells were seeded on 96-well plates at concentrations of 3000 cells/well in 200 μl DMEM/Nutrient Mixture F-12 Ham, in the presence of 10% FBS, 2 mM Glutamine and antibiotics combination (100 IU/ml Penicillin and 100 mg/ml Streptomycin). The tumor cells were maintained at 37^0C and humidified atmosphere overnight. After 48 and 72 hours of incubation, the culture medium was replaced with medium supplemented with tyrosine kinase inhibitors: SU1498 (0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM), AG1433 (0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM), or with various concentrations of dual pathways inhibitor Dactolisib (0.1 nM, 1

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nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM) in triplicate wells. After incubation time with the named inhibitors, 10 µl of MTT reagent was added to each well and incubated for 4 hours at 37^0C and than 50 µl of solubilization reagent was added. Appropriate control groups with culture medium or 0.01% of DMSO, also blank controls were included. Optical density was measured using a microplate reader (StarFax-2100 Awareness Technology Inc) at 570 nm differential wavelength of 492 nm and 630 nm.

IC25 and IC50 determination

In order to determine the inhibitory concentration for the used drugs that kills 25% or 50% of GB10B cells (IC25 and IC50 values) the applied formulas were: IC25 = [(75-X)/(Y-X)] x (W-Z) + Z, whither X is first percent inhibition that is less than 75%, Y represents the first percent inhibition that is higher or equal to 75%, Z and W are the concentrations of inhibitors that corresponds to X% and respectively Y% inhibition; respectively IC50 = [(50-X)/(Y-X)] x (W-Z) + Z, whither X is first percent inhibition that is less than 50%, Y represents the first percent inhibition that is higher or equal to 50%, Z and W are the concentrations of inhibitors that corresponds to X% and respectively Y% inhibition.

Doubling time and growth rate

A concentration of $2x10^5$ GB10B glioblastoma cells were seeded and cultured in DMEM/ Nutrient Mixture F-12 Ham into 6-well plate for 72 hours. During the experiment, the medium was replaced daily. Trypsinized cells were used for counting living cells in a Bürker hemocytometer using trypan blue. DT was determined using an available algorithm [17, 18].

Statistical analysis

The statistical analysis was expressed as mean ± standard deviation (±SD) and statistical comparison was expressed using Student t-test. Statistically significant was considered for a p-value <0.05. All experiments were performed in triplicate.

Results

Growth Rate and Doubling Time

In this experiment a low-passage GB cell line (GB10B) was used. The cell line was obtained from fresh samples of brain tumor tissue, cultured in standard conditions and frozen after the third passage. Growth rate (GR) and doubling time (DT) were assessed for cell line used in this study. The growth patterns of cell line are showed in Fig.1. GB10B cells proliferate with a GR of 0.2117 and a DT of 3.27 days.

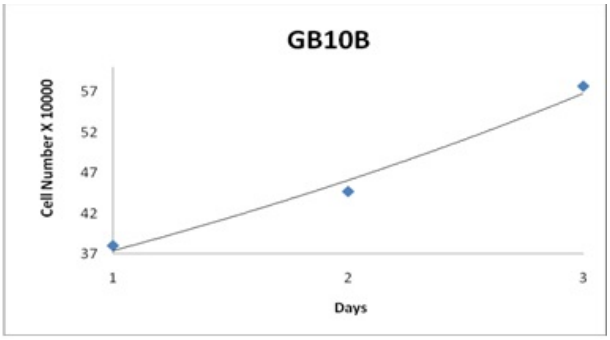

Fig.1. Glioblastoma cell growth. Growth Rate and Doubling Time.

The effect of tyrphostins AG1433 and SU1498 on GB10B cell line

To determine the cytotoxicity of SU1498 and AG1433 on GB cells, we determined a dose-response curve by treating GB10B cell lines with increasing concentrations of drugs for 48 and 72 hours (Fig.2 and 3). Using these values, we were able to determine IC25 and IC50 for each drug.

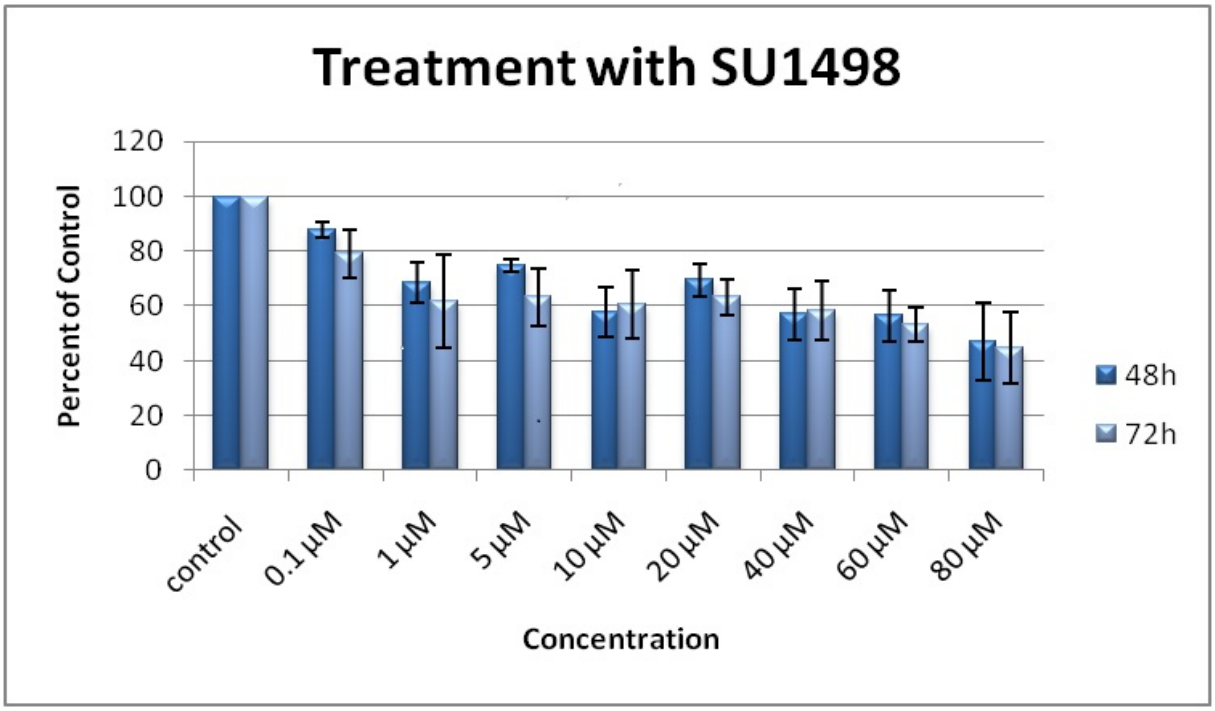

Figure 2. The effect of thyrphostin SU1498 on GB10B cell proliferation. The results obtained over the three days exposure of GB10B cells to SU1498 are expressed as percent of control and the values are mean and standard deviation of three separate experiments.

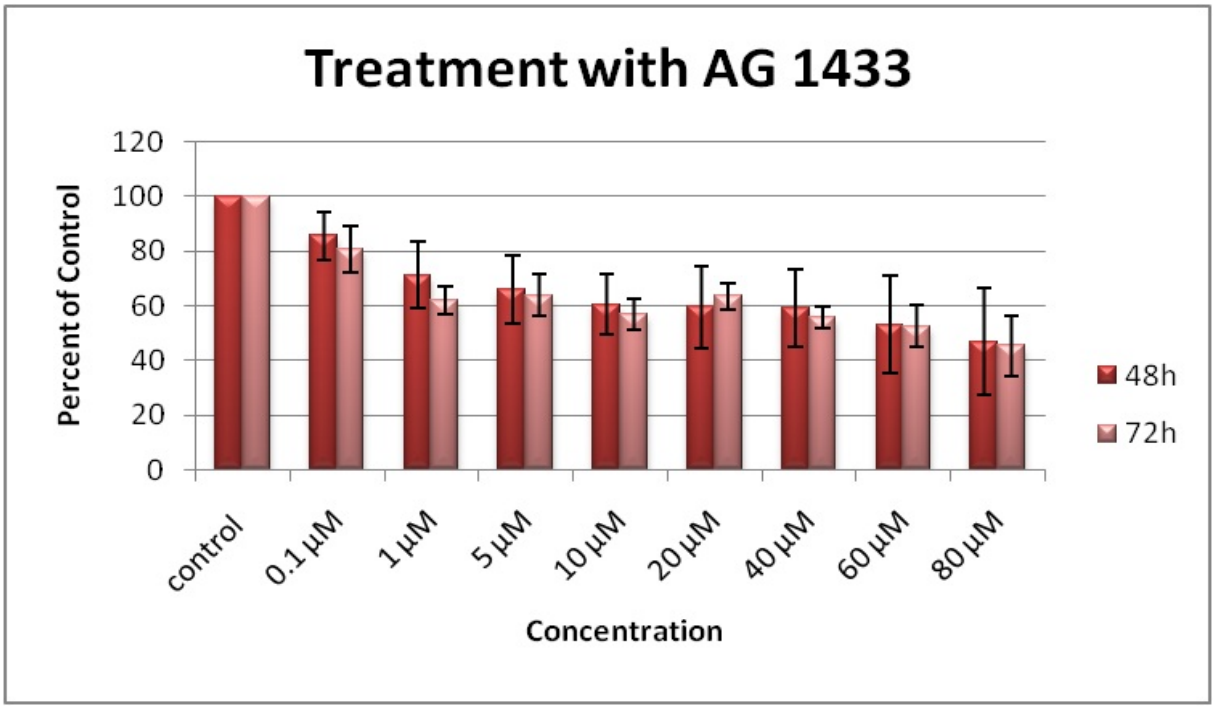

Fig.3. The effects of thyrphostin AG1433 on GB10B cell proliferation. The results obtained over the three days exposure of GB10B cells to AG1433 are expressed as percent of control and the values are mean and standard deviation of three separate experiments.

The treatment with 0.1 µM SU1498 for 48 or 72 hours had a minor cytotoxic effect on GB10B cells, as illustrated in figure 2. Increasing concentration to 1, 5, 10, 20, 40, 60 and 80 µM SU1498 determined a continuous decrease of cell viability by 21.41%. Inhibitory concentrations, IC25 and IC50, were calculated using the formulas described above, and they were 0.3171 µM and 67.5431 µM, respectively.

The treatment of GB10B cell line with PDGFR inhibitor, AG1433, showed an almost linear effect on cell viability: minimum

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concentration of inhibitor (0.1 µM) led to decrease of cell viability to 85.63% and 80.76% at 48 and 72 hours, respectively (Figure 3), while the maximum dosage of receptor reduced cell survival to of 46.9% and 45.4% at 48 and 72 hours. The IC25 and IC50 values for AG1433 were 1.7649 µM and 67.3710 µM.

The effect of combination therapy on GB10B cell line

A combined therapy with tyrphostin inhibitors (SU1498 and AG1433) were tested using a concentration of 5 µM and 10 µM of both SU1498 and AG1433 for 48 and 72 hours (Fig.4).

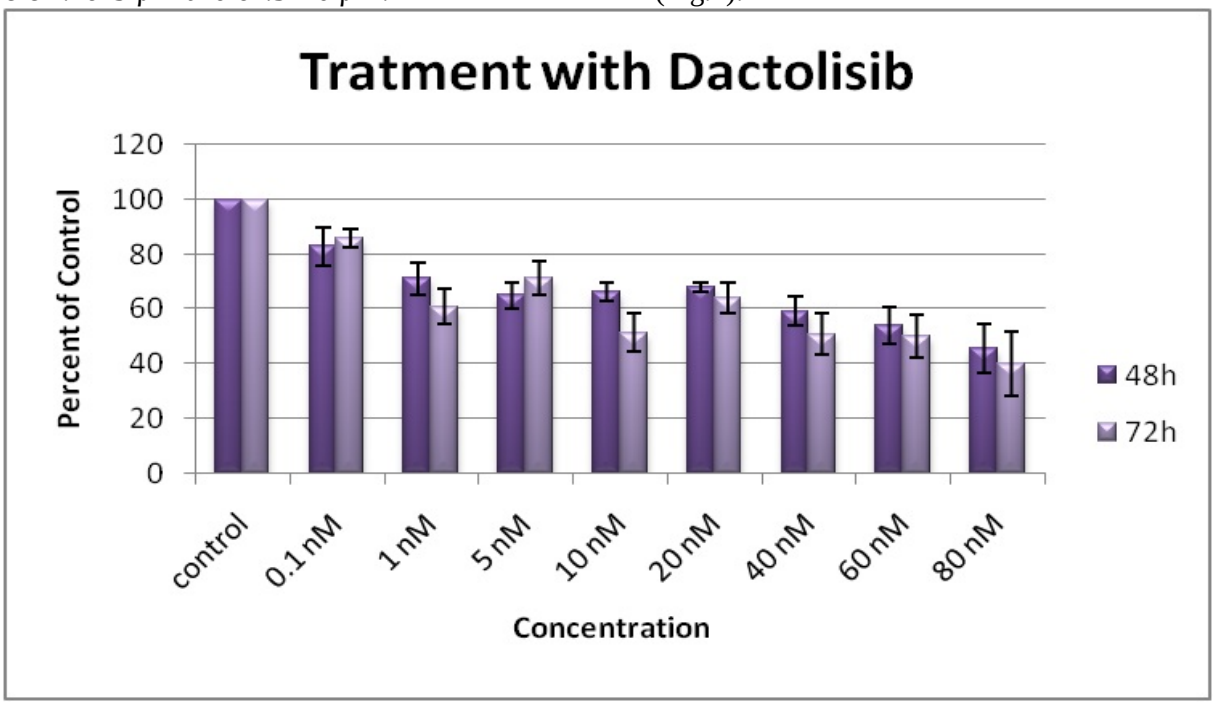

Fig.4. Effects of combination therapy on GB10B cell proliferation. The results obtained over the three days exposure of GB10B cells to thyrphostins AG1433 and SU1498 are expressed as percent of control and the values are mean and standard deviation of three separate experiments.

The combined treatment with 5 µM AG1433 and the similar dose of SU1498, reduced GB10B cell survival by 33.4% at 48 hours and 41.8% at 72 hours. Increasing the dosage of VEGFR inhibitor to 10 µM the GB10B cells induced 32.54% and 40.49% for 48 and 72 hours. The combination of 10 µM of AG1433 plus 5 µM SU1498 determined a decrease in cell proliferation by 49.54% and 55.5%, while increasing both tyrphostin inhibitors concentration to 10 µM showed a decrease of cell survival with 57.8% and 63% for 48 and 72 hours.

The effect of PI3K/mTOR signaling pathways inhibition on GB10B cell line

The GB10B cells were exposed to an inhibitor for the common PI3K/mTOR signaling pathway, Dactolisib. The drug was added at a concentration range of 0.1 - 80 nM for 48 and 72 hours and the cell viability was evaluated with MTT assay.

The drug induced cytotoxic in a time and dose-dependent manner in GB10B cells (Fig.5), decreasing cell survival by 54.3% and 60% for 48 and 72 hours. In order to evaluate the concentration of Dactolisib that induces 25% and 50% cell death in GB10B cells, we determined IC25 and IC50 values. They were found to be 3.7519 nM and 60.2776 nM, respectively.

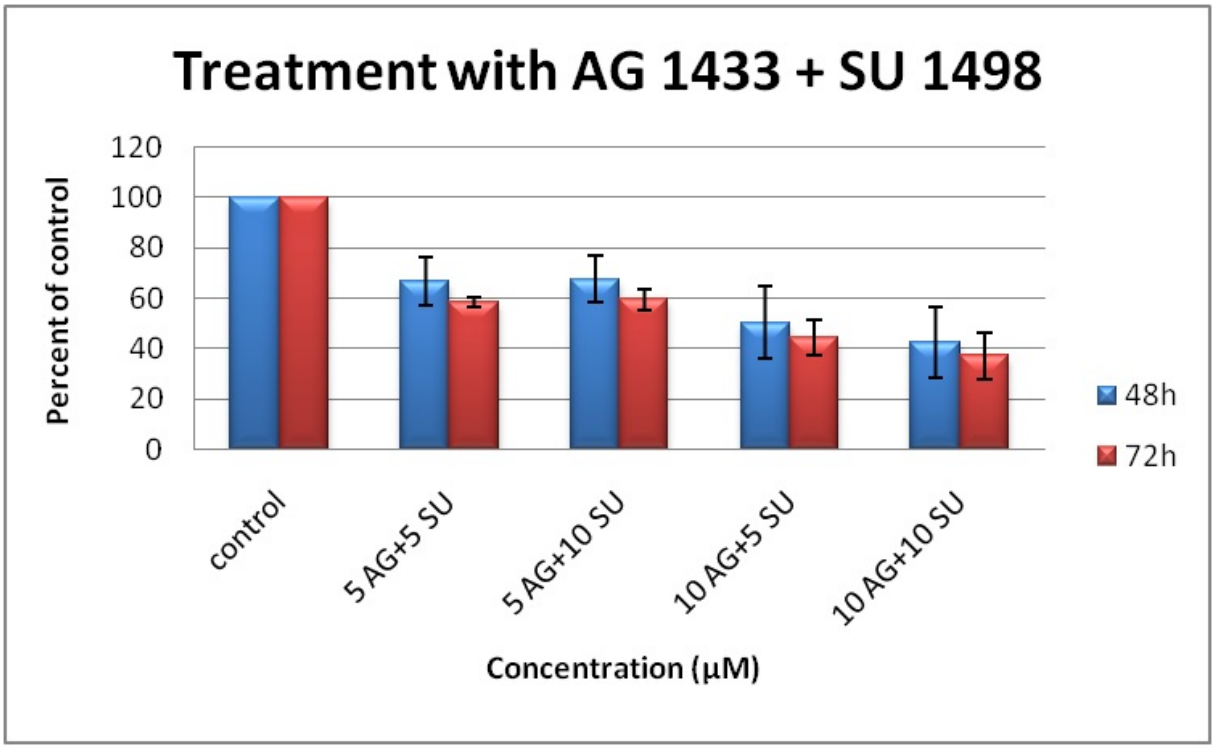

Figure 5. The effects of signaling pathways inhibitor Dactolisib on GB10B cell proliferation. The results obtained over the three days exposure of GB10B cells to Dactolisib are expressed as percent of control and the values are mean and standard deviation of three separate experiments.

Discussion

Current therapy approaches for GB treatment demonstrate efficacy but do not completely cure the patients. The TKRs, (such as EGFR, Met, IGF-1R, PDGFR, VEGFR etc.) and their intracellular signaling pathways are known to be used as molecular targets in GB treatment. However, the clinical responses to existent molecular targeted therapies are modest, mainly for recurrent GB [8]. Since 2007 there are studies that demonstrate the effect of RTKs inhibition on primary GB cells lines [19].

Due to their important role in angiogenesis and microvascular proliferation, VEGFR and its intracellular signaling pathway may represent an important target in GB treatment. Many modalities to block the action of VEGF system in tumour cells were reported: monoclonal antibodies, small-molecule tyrosine kinase, genes silencing, are some of them. The first developed drug, effective on VEGF, approved by FDA for glioma treatment, was bevacizumab in combination with other chemotherapeutic agents like irinotecan and carboplatinum [20, 21]. Whether we consider newly diagnosed gliomas or recurrent gliomas, bevacizumab exhibit promising successes in their treatment with significant improvement in progression-free survival for the first [22]. Tyrphostins, a class of inhibitors interfering with VEGFR activity, also showed cytotoxic properties in glioma cells. Of these, SU1498 was previously used *in vitro* experiments on a variety of cancers, such as prostate cancer [23], retinoblastoma [24], including brain tumours [25]. Here we found that SU1498 induced cytotoxicity in GB10B cells. The drug provided to be toxic at a low concentration, IC25 was achieved at 0.3 µM and IC50 at 67.5 µM, 3 days after the treatment.

Another growth factor receptor related to angiogenesis is PDGFR, [26]. A classic molecule used in leukemia treatment is imatinib, a small inhibitor of PDGFR [27], also evaluated for GB treatment in few clinical trials, with poor outcome [28]. We have previously shown that dual PDGFR and IGF-1R targeting inhibits proliferation and induces radiosensitivity in high-grade gliomas cells [29]. In this study we found that AG1433 treatment was cytotoxic on GB10B cells, IC25 and IC50 values were 1.8 and 67.4 µM, respectively.

High drug concentration (80 µM) of both AG1433 and SU1498 did not induce more that 60% toxicity in GB cells. It is possible that dose escalation may induce more cytotoxicity in GB

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cells but possible *in vivo* side effects may be expected.

To increase the efficacy of the drugs, we treated the cells with combination of AG1433 and SU1498 at low doses (IC25). The rationale for this combination was that the intracellular pathways emerging from tyrosine kinase receptors are redundant, thus, difficult to inactivate them by inhibition of one single receptor. Combination treatments with SU1498 and AG1433 at low concentrations (IC25) provide enhanced cytotoxicity, compared to treatment alone in GB cells. However, combination treatment with SU1498 and AG1433 did not resulted in a synergistic cell growth inhibition (data not shown).

Some drugs are targeting both tyrosine kinase receptors, VEGFR and PDGFR. Sorafenib (FDA approval in 2005) has a demonstrated efficacy in treating renal cell carcinoma and is currently tested in several phase I or phase II clinical trials as combined or single therapy for glioblastomas [30, 31]. Sunitinib was tested in a phase II clinical trial [32], but its effect on recurrent GB was not significant. Cediranib is also tested in some clinical trials, alone or in combination with lomustine, but the overall benefit was not eloquent [33].

Dactolisib (BEZ235), is an imidazoquinoline derivative, used as PI3K/mTOR dual inhibitor in different types of malignant cells. During last years, the treatment of solid tumors including for glioblastoma multiforme (GBM), was investigated in several clinical trial using Dactolisib, both alone and in combination with trastuzumab [34] or everolimus [35], providing suitable safety data, particularly for the last combination.

In this study, we also analysed the effect of Dactolisib, already known as promising drug in modern brain cancer treatment. [14]. Dactolisib anti-proliferative activity was shown against various tumour mouse xenograft models, including glioma model by inducing cell growth arrest in G1 phase cell cycle at nanomolar concentrations [13]. In accordance with previous reports, we also found that Dactolisib exhibit a increased cytotoxic effect with dose augmentation, showing a maximum effect for the maximal dosage.

Conclusions

Since the mortality through various types of brain cancers is currently very high, finding new approaches in order to cure the disease represents a challenge for many researchers worldwide. Our study revealed that using a combined therapy with PDGFR and VEGFR inhibitors or a dual targeting PI3K/mTOR signaling pathway provoked more cytotoxic effect in GB cells comparing to the single treatment.

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