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Original Article

Temozolomide and targeted therapy against epidermal growth factor receptor in glioma

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Abstract: Gliomas represent a heterogeneous group of diseases difficult to diagnose and even more difficult to treat. Intrinsic or acquired resistance to Temozolomide (TMZ) treatment is a common feature of both low- and high-grade glioma and is the major reason for therapeutic failure. The aim of this study is to improve the response to TMZ by blocking the Epithelial Growth Factor Receptor (EGFR) activity in glioma cells. GB1B and AC1B glioma cell lines used in this study were low passage cultures established from fresh tissues obtained from consented glioma patients undergoing surgery. Cell viability was quantified by hemocytometer cell counting, using trypan blue. Interactions between TMZ and EGFR inhibitor were classified by the Multiplicative Model. We found that TMZ treatment and inhibition of EGFR activity by AG556 suppressed tumor growth in glioblastoma and astrocytoma cells, but their co-administration induced additive and synergistic cell death in astrocytoma, whereas the treatment failed to elicit synergistic cytotoxicity in glioblastoma cells.

Keywords: EGFR, temozolomide, glioblastoma, combined therapy, synergy

Introduction

The combination of procarbazine, lomustine and vincristine (PCV) is the most commonly used chemotherapy in brain cancer [1]. The alkylating agent TMZ is another therapeutic approach used for the treatment of both low and high grade astrocytic tumors, largely replacing the PCV treatment, as a result of its oral administration and minor side effects [2, 3]. Addition of TMZ to surgery and radiotherapy acts as the standard treatment of high grade glioma. However, the enthusiasm for TMZ treatment decreased successively since it became clearly that the TMZ treatment provides survival benefits only for a subgroup of patients that have an altered O6-methylguanine-DNA methyltransferase (*MGMT)* [4]. Intrinsic or acquired resistance to TMZ is also critical points in treatment efficacy. In addition to MGMT, many other molecules that regulate tumor growth and survival have been suggested to interfere with brain tumour cells response to TMZ [5]. Several growth factor receptor family members are overexpressed or overactivated in glioma, playing an important role in treatment resistance [6]. EGFR dysfunction is considered one of the most common causes of TMZ therapy failure in glioma patients. EGFR has constantly been in the spotlight as a target for brain tumour therapy, yielding various results. In the case of GMBs, overexpression of the wild- and mutant-type of EGFR is a striking trademark. Around 36-40% of GMBs present EGFR gene amplification [7, 8] while in anaplastic astrocytomas only 3% of receptor alterations were reported [9, 10]. EGFR protein overexpression, without subsequent gene amplification, has been observed in both high and low grade gliomas [11, 12]. A certain distinct feature of glioblastomas is the presence of mutant variants of EGFR with the most notable of the group being EGFRvIII (Δ

EGFR), a mutation created by the deletion of exons 2-7 from the gene encoding the wild-type of receptor. This mutation has been encountered in approximately one third of glioblastomas [13-15] and has been linked to both high tumoral activity [16] and drop in patient life expectancy [17, 18]. Recent efforts to improve the glioma patients' outcome have yielded several approaches used to overcome glioma cells resistance to TMZ therapy, such as correction of TMZ doses and administration procedure, elimination of brain tumour stem cells, combining TMZ with other therapeutic regimes that target malignant cell survival pathways [19]. In this paper, we aim to determine the effect of EGFR inactivation on glioma cells response to TMZ treatment.

Material and methods

Cell line establishment

Fresh tumor tissue segment was minced using a sterile blade in a petri dish and cultured in DMEM/Nutrient Mixture F-12 Ham media, supplemented with 10% FBS, 2 mM L-glutamine and antibiotic (100 IU/mL penicillin and 100 IU/mL streptomycin). The cell mixture was passed through a cell strainer to obtain a single cell suspension. Cells were washed twice with PBS and seeded in 6 well plates [20]. Cell suspension wad then was transferred to T175 culture flasks and then passaged 2-3 times.

Cell culture

The cells were cultured in MEM containing 10% FBS, 2 mM glutamine and antibiotic (100 IU/mL penicillin and 100 IU/mL streptomycin) and 1% insulin-transferrin-selenium. The cells were grown in tissue culture flasks maintained in a 95% air/5% carbon dioxide atmosphere at 37°C in a humidified incubator. Cell culture has been amplified 2 to 3 passages from the initial biological material and then has been preserved at passage 3. All patients provided signed consent forms indicating that they agreed to donate the tissue for the research study propose, when they were hospitalized.

Cell treatment and proliferation assay

For experimental propose, cells were seeded in 12-well culture plates (2-5×10^4 cells/well) and treated with various concentrations of AG556 (1 μL, 5 μL and 10 μL), TMZ (1 μM and 5 μM) or combination of them. The drugs were administered as a single dose in the first day, the cells were incubated for 24 h, gently washed and then further incubated in drug-free medium for up to 15 days. The control cells were continuously incubated in standard medium. Appropriate control groups with diluents only were included. During incubation time, treated and control cells were isolated every day and a uniform cell suspension was counted (one well/day) in a Bürker hemocytometer, using trypan blue. Treatments were compared with untreated control. Cell viability was calculated as the ratio of number living cells to number of total cells. Each experiment was repeated five times.

Drug interaction analysis

The interaction (I) between LHAE and TMZ or DOXO was classified by the Multiplicative Method as previously reported. I values were counted according to the levels of cell death fraction affected by each agent individually and combination of them. The I was calculated to determine additive ($I_{1,2} = I_1 + I_2$), synergistic ($I_{1,2} > I_1 + I_2$) and sub-additive ($I_{1,2} < I_1 + I_2$) response.

Statistical analysis

Each study was replicated in at least three independent experiments. The statistical processing was performed by numerical characterization of the data, involving the average behavior for numerical variables and the distributions of various parameters. Analysis of variance (ANOVA) was used to analyze the significance of differences between study groups. The levels of significance for comparisons between samples were analyzed by Student's t-test. $P < 0.05$ values were considered statistically significant. All data are represented as mean ± standard deviation (SD).

Results

The effect of temozolomide on glioma cells

TMZ is an oral second generation imidazotetrazinone prodrug that undergoes a chemical change under physiological state to the alkylating drug, commonly used for GB treatment. DNA methylation is believed to be its major mechanism of action in cancer cells [21]. The drug is known to alkylate DNA, causing malignant cells to die.

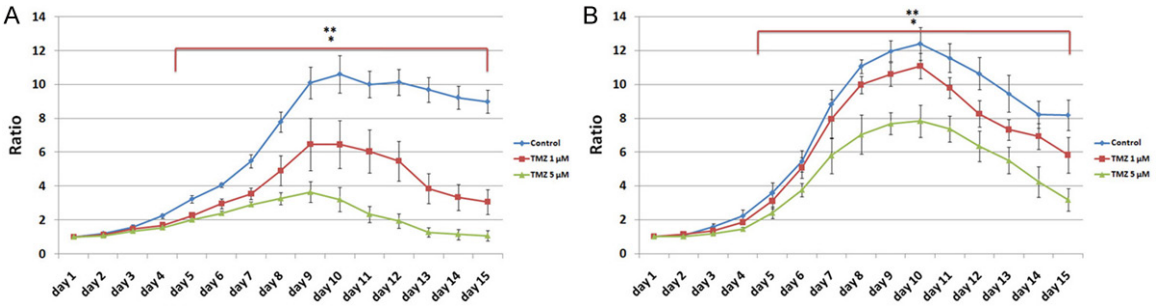

Figure 1. TMZ effect on viability of GB1B cells (A) and AC1B cells (B). Notes: Results are expressed as ratio of number of living cells to number of total cells. Appropriate control groups with diluents only were included. Data are mean and standard error of five separate experiments.

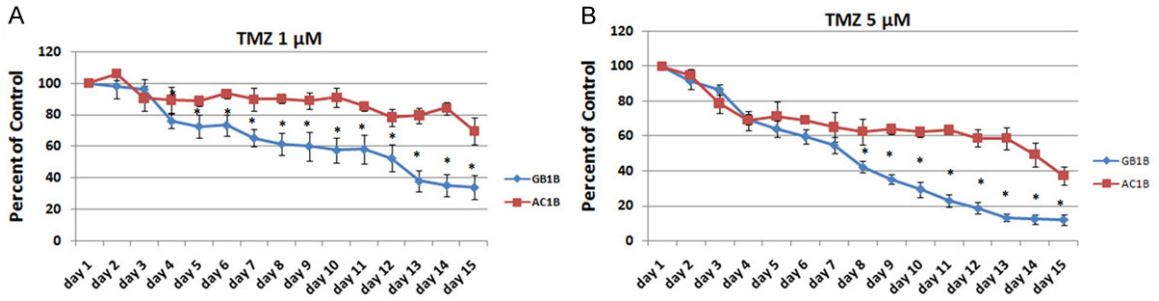

Figure 2. Comparison between the cytotoxic effects of the treatment with 1 μM TMZ (A) and 5 μM TMZ (B) on malignant glioma cells and low grade glioma cells. Notes: Results are expressed as percent of control. Data are mean and standard error of five separate experiments.

Here, we analyzed the effect of TMZ on brain tumor cultures GB1B and AC1A (**Figure 1**). For experimental purposes, growing brain tumor cells (GB1B and AC1B) were treated with 1 μM or 5 μM of TMZ for 24 h, the cells were then washed free of drug and incubated continuously up to 15 days. The number of the cells was counted every day. The cytotoxic effect of the chemotherapeutic agent is shown in **Figure 1**. As seen in Figure, TMZ inhibited the growth of GB1B (**Figure 1A**) and AC1B (**Figure 1B**) cells in time and dose dependent manner. The treatment with 1 μM TMZ induced significant ($P \leq 0.05$) inhibition in GB1B cell viability ratio from day five and increased over the time. The GB1B viability ratio decreased with about 1 at five and seven days after the treatment with 1 μM TMZ, 3 at eight and nine days, about 4 at eleven and twelve days and with circa 6 at thirteen, fourteen and seventeen days, compared to untreated control cells. The treatment with 5 μM of TMZ induced 0.7 inhibition in GB1B cell viability ratio four days after the treatment, about 1 at day five, about 2 at day six, 3 at day seven, about 5 at day eight and about 7, nine and ten days after the treatment. The inhibition of growth ratio was about 8 eleven days after the treatment and remained constant up to the end of the experiment (**Figure 1A**). In AC1B cells, 1 μM of TMZ treatment did not induce significant decrease in cell viability ratio, the first five days of treatment ($P \geq 0.05$). The cell growth ratio was reduced by 0.4, six days after the treatment with 1 μM of TMZ. Cell viability ratio showed a decrease with about 1 for the next 7-10 days interval, compared to untreated cells. During the next five days (11-15 days), the viability ratio decrease by circa 2 in 1 μM TMZ treated cells versus untreated cells (**Figure 1B**). The cytotoxic effect induced by the treatment with TMZ was more marked and in GB1B cells than in AC1B cells.

The comparison between the cytotoxic effect induced by TMZ on GB1B and AC1B is shown in **Figure 2**. The difference between the cytotoxic effect induced by 1 μM TMZ was observed 4 days after the treatment (13%) and was further increased with prolonged exposure: 16% after 5 days, 20% after 6 days, 24% after 7 days,

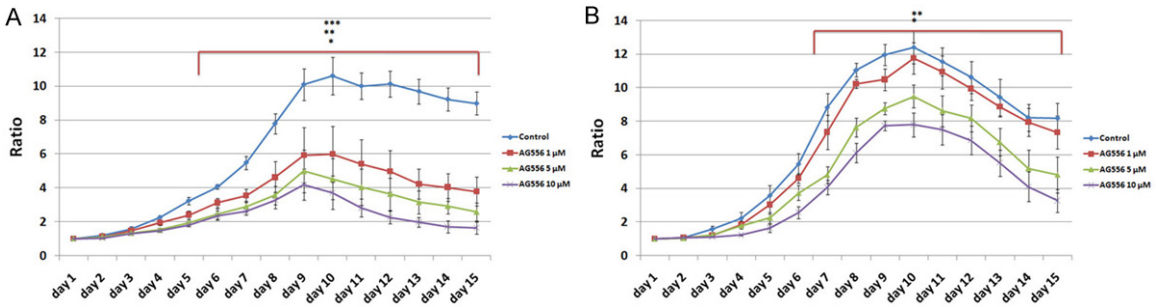

Figure 3. AG556 effect on viability of GB1B cells (A) and AC1B cells (B). Notes: Results are expressed as ratio of number of living cells to number of total cells. Appropriate control groups with diluents only were included. Data are mean and standard error of five separate experiments.

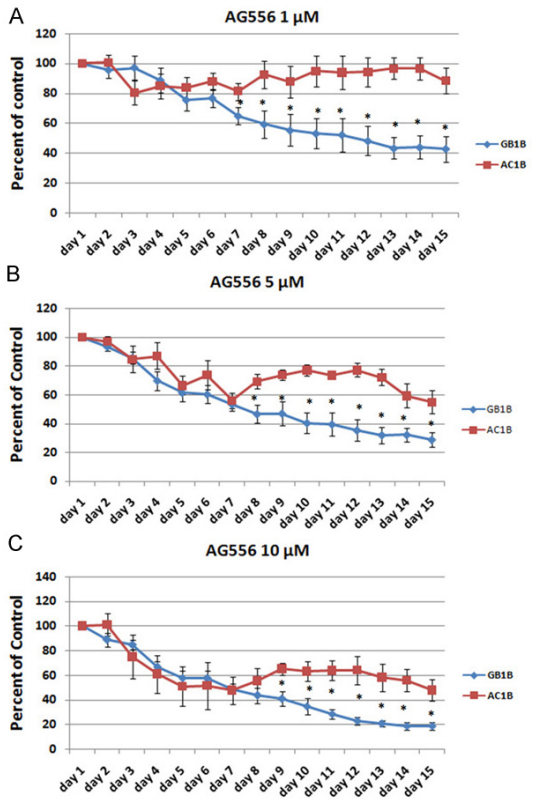

Figure 4. Comparison between the cytotoxic effects of the treatment with 1 μM AG556 (A), 5 μM AG556 (B) on and 10 μM AG556 (C), GB1B and AC1B cell lines. Notes: Results are expressed as percent of control. Data are mean and standard error of five separate experiments.

28% after 8 and 9 days, 33% after 10 days, 27% after 11 days, 25% after 12 days, 41% after 13 days, 48% after 14 days and 35% after 15 days (**Figure 2A**).

When the cells were treated with 5 μM TMZ, difference in cell killing efficiency between GB1B and AC1B cells was: 7% after 5 days, 10% after 6 and 7 days, 28% after 8 days, 33% after 9 days, 40% after 10 and 11 days, 46% after 12 days, 37% after 13 days, 25% after 14 days and 25% after 15 days (**Figure 2B**).

The effect of EGFR inhibition on glioma cells

EGFR inhibition on gliomas has been extensively researched both in preclinical and clinical environments, in monotherapy or in tandem with other therapies such as chemotherapy or radiotherapy [22-28]. We investigated the effects of EGFR inactivation on the viability of GB1B and AC1B glioma cells (**Figure 3**). Both cell lines were subjected to specific EGFR-inhibition with AG556 (1, 5 and 10 μM) for 24 h and then washed free of drug and incubated continuously up to 15 days. The cytotoxicity of the inhibitor was assayed by daily cell quantification, as described in Material and Method section. As seen in **Figure 3**, AG556 inhibited cell growth in a time and dose dependent manner. 1 μM of AG556 induced a modest cytotoxic effect in both cell lines. In the GB1B glioblastoma line, 1 μM of AG556 determined a decrease in cell viability ratio by approximately 1 at day 6, 3 at day 8 and over 5 at days 12, 13, 14 and 15, respectively, in comparison to the untreated cells (**Figure 3A**). Five μM of AG556 produced a drop in cell proliferation ratio by almost a half at day 7, around 5 at day 11 with a widening gap in cell growth ratio between the treated and untreated GB1B cell lines, towards the end of the experiment (**Figure 1A**). Ten μM AG556 induced a decrease in cell viability ratio in the GB1B cell line by almost 2 at day five, 6 at day nine, over 7 at the eleventh day and this level was maintained until the last day of treatment

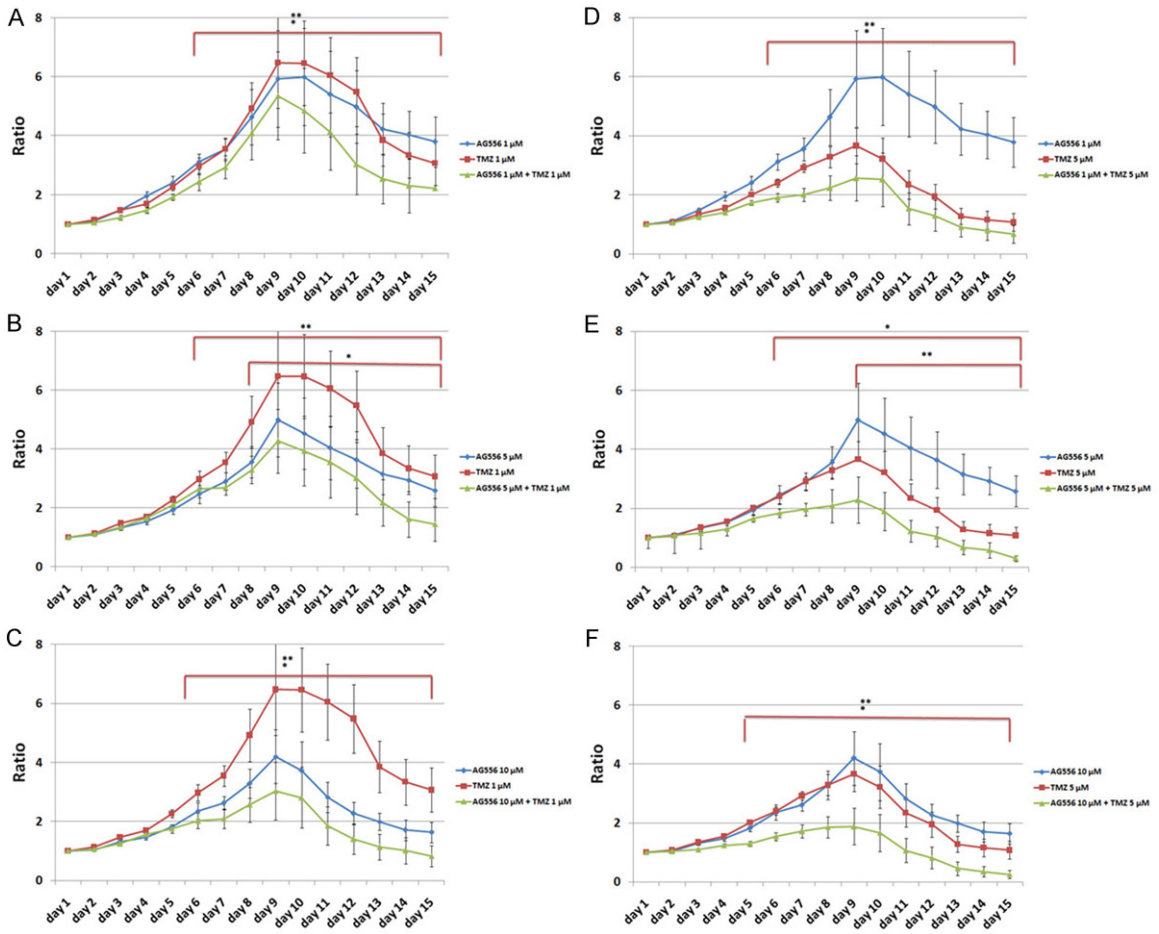

Figure 5. The effect of combined treatment with TMZ and AG556 on glioblastoma cells. Notes: Results are expressed as ratio of number of living cells to number of total cells. Appropriate control groups with diluents only were included. Data are mean and standard error of five separate experiments.

(**Figure 1A**). We then applied the same treatment to the AC1B cell line (**Figure 3B**). The treatment with 1 μM AG556 did not alter AC1B cells. The difference in viability ratio between the AC1B cells treated with 5 and 10 μM the untreated cells was constantly at around 0.5 up until the seventh day when it scored a value of 1, after which it remained relatively constant till the end of the study. For 5 μM of AG556, cell growth inhibition was approximately 1 at five days, 4 at seven days from where it dropped to around 2 for the following days of the study. The cytotoxic effect of the 10 μM AG556 was more pronounced inducing a decrease in cell viability of the treated cell line versus the control cell line by 2 during the fifth day, 4.8 during the eight day and around 4 for the rest of the days of the study.

We then analyzed the difference in cytotoxicity determined by the EGFR-targeting inhibitor between the two cell lines. For the 1 μM AG556 dose, the difference in cell proliferation between the GB1B and AC1B cell lines was not significant during the first six days (**Figure 4A**). Seven days after the treatment with 1 μM AG556 resulted in 11% higher cytotoxicity in GB1B cells compared to AC1B cells. From the sixth day till the end of the treatment cell inhibition became increasingly higher in the GB1B line, the difference in drug cytotoxicity was approximately 40% at day eight, over 50% in the thirteenth and fourteenth days and 45% during the last day of the treatment. The cytotoxicity induced by the treatment with 5 μM AG556 was more pronounced in the GB1B cell line compared to AC1B cell line, the difference in treatment induced effect was significant since the eighth day (about 40%), increased until the twelfth day when it reached a value of 41% and then the difference in drug effect decreased very little for the following 3 days

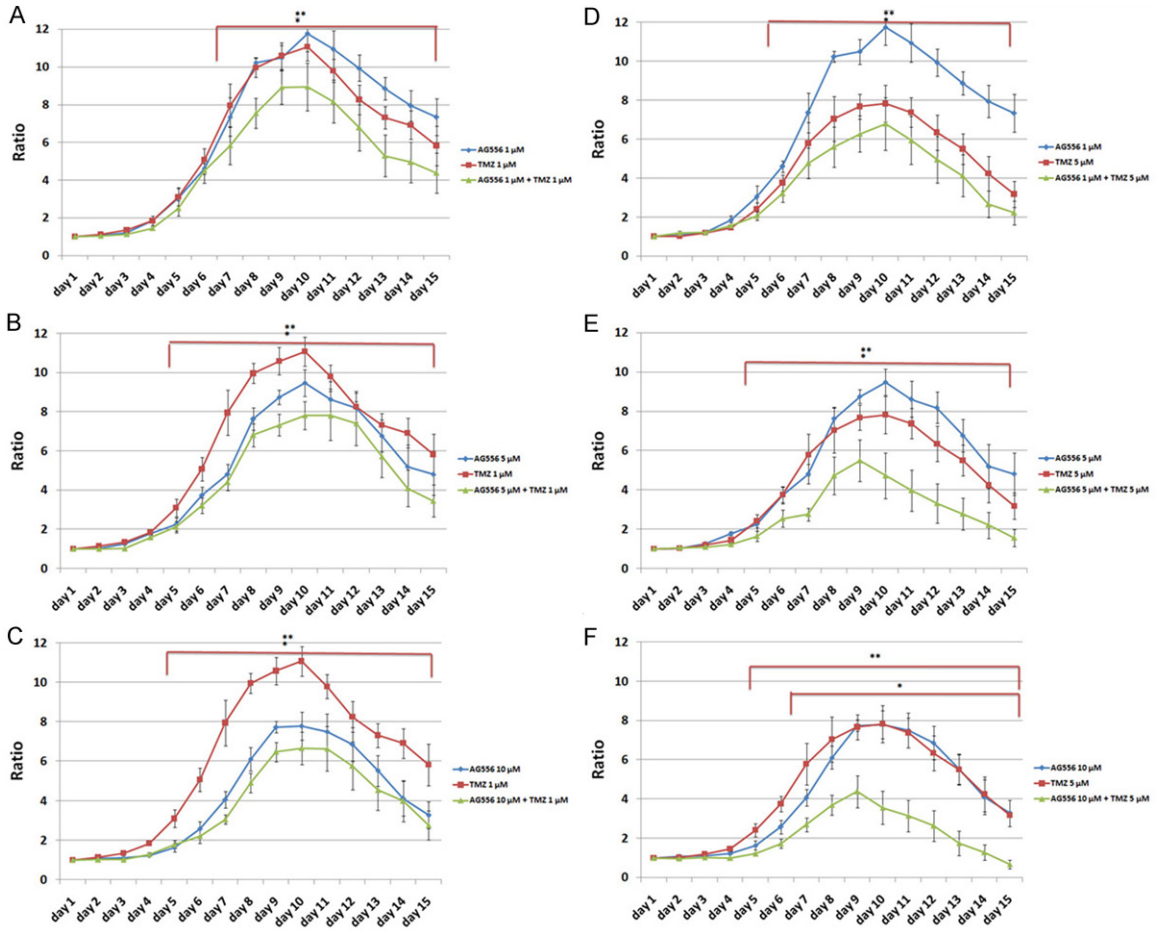

Figure 6. The effect of combined treatment with TMZ and AG556 on low grade glioma cells. Notes: Results are expressed as ratio of number of living cells to numbe r of total cells. Appropriate control groups with diluents only were included. Data are mean and standard error of five separate experiments.

(**Figure 4B**). No significant difference in efficacy between the GB1B and AC1B cell lines was registered in the seven-day interval of treatment with AG556 10 μM (**Figure 4C**). Prolonged exposure determined a difference of 24% in cell death of by the ninth day, peaking during the twelfth day with 41%, after which it reached 37% during the thirteenth day, 36% during the fourteenth and 29% on the last day of the study.

In conclusion, our results showed that GB1B cell line was notably more sensitive to EGFR inhibition than AC1B cell line.

The effect of combined inhibition on glioma cells

Glioblastoma is remarkably resistant to TMZ treatment, several molecules in the growth factor receptors (GFRs) signal transduction were reported to interfere with TMZ action, suggesting that combinational approaches involving TMZ and GFRs inactivation might be a possible future therapy direction for this high malignant disease. In this study, we assessed the effects of TMZ treatment in combination with AG556, an EGFR inhibitor, on glioma cells. We used low drug concentrations (1 and 5 μM TMZ combined with 1, 5 or 10 μM AG556) and short treatment exposure, to avoid the side effect induced by of the drugs. As seen in **Figure 5**, the combination between with 1 μM AG556 plus 1 μM of TMZ induced significant cell death in GB1B cells during the last 5 days after the treatment ($P \leq 0.05$). The combined treatment (1 μM AG556 plus 1 μM of TMZ) determined a drop in proliferation ratio by 1.5 when compared to TMZ treatment and by 1 when compared to single anti-EGFR therapy after ten days, by 1.7 in comparison to anti-EGFR treatment and by 1.3 when compared to TMZ treat-

Table 1. Analysis of interaction between Temozolomide and AG556 (a comparison of the predicted survival and the experimentally observed survival after combination treatment), in AC1B cells, according to the additive model

AG556 Conc (μM)	Temozolamide Conc (μM)	Time (Days)	Observed Survival	Predicted Survival
1	1	2	1	1.1**
		3	0.7	0.7
		4	0.7	0.7*
		5	0.7	0.7*
		6	1.1	0.8
		7	0.7	0.7*
		8	0.7	0.8**
		9	0.7	0.8**
		10	0.7	0.8**
		11	0.7	0.8**
		12	0.6	0.7**
		13	0.6	0.7**
		14	0.6	0.8**
		15	0.5	0.6**
	5	2	1.1	1
		3	0.8	0.6
		4	0.7	0.5
		5	0.6	0.6*
		6	0.6	0.6*
		7	0.5	0.5*
		8	0.5	0.6**
		9	0.5	0.6**
		10	0.5	0.6**
		11	0.5	0.6**
		12	0.5	0.6**
		13	0.4	0.5**
		14	0.3	0.5**
		15	0.3	0.3*
5	1	2	0.9	1**
		3	0.6	0.7**
		4	0.7	0.7*
		5	0.6	0.5
		6	0.6	0.6*
		7	0.5	0.5*
		8	0.6	0.6*
		9	0.6	0.6*
		10	0.6	0.7**
		11	0.7	0.6
		12	0.7	0.6
		13	0.6	0.6*
		14	0.5	0.5*
		15	0.4	0.4*
	5	2	1	0.8

ed cells after the thirteenth day (**Figure 5A**). The 5 μM AG556 and 1 μM TMZ combination reduced glioblastoma cells proliferation ratio by around 1.7 when compared to single TMZ treatment and by 0.3 when compared to single, anti-EGFR based therapy after eight days. At the twelfth day of the treatment, cell proliferation ratio was decreased by 2.5 when compared to TMZ treatment and by 0.6 in comparison to single AG556 treatment (**Figure 5B**). The simultaneous treatment of glioblastoma cells with 10 μM AG556 and 1 μM TMZ induced a more pronounced cell death in GB1B cells. Cell viability ratio was mildly decreased during the first days of the experiment. After the tenth day, cell proliferation ratio dropped by 1 when compared to AG556 therapy and by 3.8 versus single TMZ treatment, by 5 when compared to the TMZ treatment and 0.9 when compared to anti-EGFR treatment, after the twelfth day and remained constant until day fifteen (**Figure 5C**). Using 1 μM AG556 plus 5 μM TMZ, cellular viability ratio was decreased by up to 4 in comparison to anti-EGFR therapy and by approximately 1 in comparison to TMZ therapy, during the first ten days of the study. After the last day of the study, cellular proliferation dropped by 0.4 when compared to TMZ in monotherapy and by 3.1 in comparison to AG556 therapy (**Figure 5D**). Concomitant treatment of glioblastoma cells with 5 μM AG556 and 5 μM TMZ decreased the cell proliferation ratio by approximately 0.6 compared to both agents in monotherapy, at seventh day. After the eleventh day, cell proliferation dropped by 2.7 when compared to anti-EGFR therapy and by 1.3 in comparison with TMZ single treatment. At the end of the experiment, cell growth ratio was decreased by 0.7 compared to TMZ-based therapy and by 2.2 in comparison to AG556 therapy (**Figure 5E**). The combination using 10 μM of AG556 plus 5 μM TMZ determined a decrease in cell proliferation ratio by 0.5 when compared to both single therapies, after the sixth day and by 1.7 when compared to TMZ therapy and 2.4 in comparison to AG556 therapy, after the tenth. At the end of the study, cell growth ratio dropped by 0.8 when compared to TMZ therapy and by 1.4 when compared to anti-EGFR therapy (**Figure 5F**). We then applied the same combined treatment to the AC1B cell line in order to evaluate their cytotoxic effect as opposed to single therapies (**Figure 6**). By using 1 μM AG556 plus 1 μM TMZ treatment, we found that proliferation ratio was decreased by 0.5 after

		3	0.7	0.5
		4	0.5	0.4
		5	0.4	0.3
		6	0.5	0.3
		7	0.3	0.3*
		8	0.4	0.2
		9	0.5	0.1
		10	0.4	0.1
		11	0.3	0.1
		12	0.3	0.1
		13	0.3	0.1
		14	0.3	0.1
		15	0.2	0.1
10	1	2	1	1.1**
		3	0.6	0.6*
		4	0.6	0.5
		5	0.5	0.4
		6	0.4	0.4*
		7	0.3	0.4**
		8	0.4	0.5**
		9	0.5	0.5*
		10	0.5	0.6**
		11	0.6	0.6*
		12	0.5	0.5*
		13	0.5	0.5*
		14	0.5	0.5*
		15	0.3	0.3*
	5	2	0.9	0.8
		3	0.6	0.6*
		4	0.4	0.4*
		5	0.3	0.4**
		6	0.3	0.4**
		7	0.3	0.3*
		8	0.3	0.3*
		9	0.4	0.3
		10	0.3	0.2
		11	0.3	0.2
		12	0.2	0.1
		13	0.2	0.1
		14	0.2	0.1
		15	0.1	0.1*

*Additive effect; **Synergistic effect.

the fifth day and by 2.5 after the eight day compared to both TMZ and AG556 single treatment. After the twelfth day, AC1B cell proliferation ratio was decreased by 1.5 when compared to single TMZ treatment and by 3.3 in comparison to AG556 monotherapy (**Figure 6A**). The therapeutic combination based on 5 μM AG556 and 1 μM of TMZ produced similar results. Cell growth ratio dropped by 3.5 in comparison to single, TMZ therapy and by 0.4 when compared to AG556 treatment after the sixth day. The proliferation ratio further decreased by 3.2 when compared to the TMZ treated AC1B cell line and by 1.7 in comparison to AG556 treatment, during the tenth day and by 2.3 compared to TMZ treatment and also 1.3 when compared to AG556 treated cell lines, at the end of the experiment (**Figure 6B**). The combination based on 10 μM AG556 and 1 μM TMZ determined a reduction the in the cellular proliferation ratio by 3.9 when compared to TMZ therapy and by 1 in comparison to anti-EGFR therapy after the seventh day. Cell growth ratio dropped by 3.9 in contrast to single, TMZ-based therapeutic approach and by 0.9 in comparison to single, anti-EGFR therapy at the eleventh day of the study. At the end of the study, cell survival ratio was down by 0.5 in comparison to AG556 treated cell lines and by 3 in contrast to those treated with TMZ (**Figure 6C**). The therapeutic approach based on 1 μM AG556 and 5 μM TMZ induced a drop in cell proliferation by 0.5 when compared to single TMZ therapy and by 1.4 in comparison to AG556 in monotherapy at day six of the study. Cell proliferation was down by 1 in contrast to TMZ therapy and by 5.1 in contrast to AG556 treated cells at the end of our study (**Figure 6D**). By using 5 μM of AG556 plus 5 μM of TMZ we observed a reduction in cellular growth by 0.6 when compared to monotherapy based on AG556 and by 0.8 when compared to TMZ therapy at day five. Proliferation dropped by 4.8 when compared to single AG556 therapy and by 3.1 when compared to therapy based solely on TMZ after the tenth day and after the fifteenth day, cell growth dropped by 3.3 as opposed to anti-EGFR therapy and by 1.6 in comparison to the TMZ-based approach (**Figure 6E**). The last combination we used in our study, consisting of 10 μM AG556 and 5 μM induced difference between single and combined treatment by 2 compared to single TMZ therapy and 0.8 during the sixth day. After the ninth day, cellular proliferation was down by 3.3 as opposed to both single therapeutic approaches. At the end of our study, the cell growth ratio decreased by 2.5 in contrast to TMZ-based therapy and by 2.5 in comparison to cell treated solely with AG556 (**Figure 6F**).

Table 2. Analysis of interaction between Temozolomide and AG556 (a comparison of the predicted survival and the experimentally observed survival after combination treatment), in GB1B cells, according to the additive model

AG556 CONC (μM)	Temozolamide Conc (μM)	Time (Days)	Observed Survival	Predicted Survival
1	1	2	0.9	0.9*
		3	0.8	0.8*
		4	0.7	0.6
		5	0.6	0.5
		6	0.6	0.5
		7	0.6	0.4
		8	0.5	0.2
		9	0.5	0.2
		10	0.5	0.2
		11	0.4	0.2
		12	0.3	0.2
		13	0.3	0.1
		14	0.3	0.1
		15	0.3	0.1
	5	2	0.9	0.8
		3	0.8	0.8*
		4	0.6	0.6*
		5	0.6	0.5
		6	0.5	0.5
		7	0.4	0.4
		8	0.3	0.2
		9	0.2	0.2*
		10	0.2	0.2*
		11	0.2	0.1
		12	0.1	0.1*
		13	0.1	0.1*
		14	0.1	0.1*
		15	0.1	0.1*
5	1	2	0.9	0.8
		3	0.8	0.6
		4	0.7	0.4
		5	0.7	0.3
		6	0.6	0.3
		7	0.5	0.2
		8	0.4	0.1
		9	0.4	0.1
		10	0.4	0.1
		11	0.4	0.1
		12	0.3	0.1
		13	0.3	0.03
		14	0.2	0.03
		15	0.2	0.03
	5	2	0.9	0.7

We then analyzed interactions between therapy combinations using the Multiplicative Model described under Materials and Method. In the GB1B cell line, 17 (20.24%) of the combined treatments were synergic, while 67 (79.76%) of them were sub-additive. None of the combinations between AG556 and TMZ proved to be synergic in comparison to the single therapeutic involving the said therapeutic agents. In the AC1B line, 29.8% of the combined therapeutic approaches proved to be synergic, 35.7% were additive and 34.52% were sub-additive.

Discussion

Temozolomide, a drug with acceptable blood-brain barrier penetration, is now used to treat glioma patients [29]. This new chemotherapeutic agent was found to reach the cerebrospinal fluid, penetrating blood-brain barrier without hepatic metabolization [30]. For newly diagnosed glioblastoma, Temozolomide with radiation is given simultaneously, as a standard therapy [3]. *In vitro* treatment with TMZ, carried out in glioma monolayer [31, 32] and in spheroid three-dimensional cell cultures [33], showed that the drug induced time- and concentration-dependent inhibition of tumour cell growth. Similarly to other studies, we found that TMZ inhibits cell viability in human glioma cells *in vitro*. TMZ, administered alone as a single-dose treatment, induced a persistent cell death in grade II astrocytoma (AC1B cell line) and in glioblastoma (GB1B cell line), fifteen days after the treatment. We also found out that TMZ effect was more preeminent in glioblastoma than in low grade astrocytoma. Many molecularly targeted agents have also showed experimental and clinical evidence in glioma treatment. Tyrosine kinase inhibitors were intensively studied as a targeted therapy niche in the treatment of brain tumours, throughout the last years [6, 34]. Among them, EGFR inhibitors play a major role in the development of new medications in modern high grade glioma therapy, where EGFR activating mutations are currently used to select subsets patient for personalized therapy. EGFR amplifications occur in more than 50% of glioblastomas [35] while anaplastic astrocytomas harbor low frequency of oncogenic receptor amplifications [36]. However, EGFR alterations were suggested to play an important role in low-grade tumors progression to high-grade tumors, after a lengthy period of dormancy [37]. The benefit of EGFR targeted therapy in astrocytic tumours remains contro-

		3	0.8	0.6
		4	0.6	0.4
		5	0.5	0.3
		6	0.5	0.3
		7	0.4	0.2
		8	0.3	0.1
		9	0.2	0.1
		10	0.2	0.1
		11	0.1	0.04
		12	0.1	0.04
		13	0.1	0.02
		14	0.1	0.02
		15	0.04	0.02
10	1	2	0.9	0.8
		3	0.8	0.7
		2	0.7	0.5
		5	0.5	0.4
		6	0.4	0.4*
		7	0.4	0.3
		8	0.3	0.2
		9	0.3	0.1
		10	0.3	0.1
		11	0.2	0.1
		12	0.1	0.1
		13	0.1	0.03
		14	0.1	0.03
		15	0.1	0.03
	5	2	0.9	0.8
		3	0.7	0.7*
		2	0.6	0.5
		5	0.4	0.3
		6	0.4	0.3
		7	0.3	0.3*
		8	0.2	0.2*
		9	0.2	0.2
		10	0.2	0.1
		11	0.1	0.1*
		12	0.1	0.04
		13	0.05	0.02
		14	0.03	0.02
		15	0.02	0.02*

*Additive effect.

versial. Although EGFR gene amplifications are isolated in grade II astrocytoma, targeted EGFR therapy has been suggested [38]. The use of Erlotinib, lapatinib, gefitinib and other EGFR inhibitors, as single-agent therapies, in phase I or II clinical trials showed limited activity in glioblastoma patients and did not improve overall or progression-free survival [39-41]. In contrast, a phase II clinical trial demonstrated that in patients who acquire the EGFRvIII variant of the receptor, improved progression-free overall survival was achieved by EGFRvIII receptor-targeted vaccination [42]. In this study, AG556, an EGFR substrate-site competitor that belongs to the class of low molecular weight compounds, induced cell growth inhibition in GB1B and AC1B cells, but glioblastoma GB1B cells were significantly more sensitive to EGFR inhibition than low grade astrocytoma AC1B cells. The low sensitivity of AC1B cells to TMZ or AG556 may be due by the lack of gene alterations (e.g. MGMT and EGFR gene mutations) in low grade gliomas or the fact that tumours tend to be slow-growing. The growth factor receptor (GFR) targeted agents, on the other hand, act by mechanisms that interfere with TMZ action, suggesting that combinational approaches involving TMZ and GFRs inhibitors might be a possible future therapy direction for glioma patients. EGFR inhibition combined with TMZ and ionizing radiation have been reported to improve survival in newly diagnosed glioblastoma multiforme patients in a study conducted by Prados et al [19]. Low doses of TMZ treatment were reported to concert with EGFR inactivation to decrease cell growth in a panel of glioma cells *in vitro* [43]. In our study, we also used low doses of TMZ and EGFR inhibitor AG556 to study the effect of their combined treatment on glioma cell lines. Our results showed that both TMZ and EGFR-inactivation alone were efficient in inducing cell growth in glioblastoma cells, but their co-administration failed to elicit synergistic cytotoxicity. Unexpected, in AC1B cells that were minor sensitive to TMZ or AG556 single treatment, the action of the two drugs together generated synergistic or additive response in the most of the treatment combinations.

In summary, we found that both TMZ and AG556 treatment decreased GB1B and AC1B cells viability. Our results also indicated that TMZ or AG556 treatment alone was more efficient in inducing cytotoxicity in GB1B cells (**Table 1**) while the drugs concomitant administration was more potent in AC1B cell line (**Table 2**). These findings may help to improve the design of coming clinical trials for evaluating the effect of EGFR inactivation on TMZ sensitization in glioma patients.

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Disclosure of conflict of interest

None.

Abbreviations

EGFR, Epithelial Growth Factor Receptor; TMZ, Temozolomide; PCV, Procarbazine, Lomustine and Vincristine; MGMT-O^6, methylguanine-DNA methyltransferase.

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