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In vitro evaluation of Axitinib and Sorafenib treatment in glioblastoma cell viability and morphology

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Abstract

The formation, proliferation, and evolution of glioblastoma (GB) are significantly influenced by pathological angiogenesis. This is supported by several growth factor receptors, such as the vascular endothelial growth factor receptor (VEGFR). In this experiment, we examined how the *Food and Drug Administration* (FDA) approved VEGFR blockers Sorafenib and Axitinib affect the viability of GB cells *in vitro*. Cells were cultivated in 96-well culture plates for the experiments, afterwards Sorafenib and Axitinib were administered at doses ranging from 0.3 μM to 80 μM. 2,5-Diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess the impact of VEGFR inhibition on high-grade glioma (HGG) cell lines. To observe the morphological changes in cell shape, we used a 10× magnification microscopy. Our results showed that both Axitinib and Sorafenib retarded GB1B culture proliferation in a dose- and time-dependent manner in comparison to control cohorts that had not received any treatment. The half maximal inhibitory concentration (IC_{50}) value for Axitinib was 3.5839 μM after three days of drug administration and 2.2133 μM after seven days of drug administration. The IC_{50} value for Sorafenib was 3.5152 μM after three days of drug administration and 1.6846 μM after seven days of drug administration. After the treatment with Axitinib or Sorafenib, very few cells became rounded and detached from the support, others remained adherent to the culture substrate, but acquired a larger, flatter shape. Our results indicate that VEGFR might serve as a key target in the treatment of GB. Although it is known that *in vitro* some drugs block the VEGFR more potently, clinical evidence is required to show whether this actually translates to better clinical outcomes.

Keywords: Axitinib, Sorafenib, high-grade glioma, treatment, targeted therapy.

Introduction

Because of their clinical significance, angiogenic biomarkers are potential therapeutic candidates for glioblastoma (GB), the most fatal malignant brain tumor with an average lifespan of fewer than 15 months. Angiogenesis is mediated by complex interactions between many pro- and anti-angiogenic molecules. Several receptor tyrosine kinases (RTKs) are known to be associated with tumor angiogenesis: vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), latrophilin and seven transmembrane domain-containing protein 1 receptor (ELTD1) and TEK receptor kinase (TIE2) [1–5]. Recently, a new angiogenic receptor, ELTD1, was reported by several research groups to be involved in both physiological and pathological angiogenesis [6, 7]. ELTD1 plays an important role in tumor angiogenesis. ELTD1 was found to be expressed in human glioma, its expression begins significantly higher in high-grade gliomas (HGGs) compared to low-grade gliomas (LGGs) [8]. Several classical and non-classical proangiogenic factors were described in recent years, their role in tumor angiogenesis progression is still under debate in the scientific community. Nine classical proangiogenic factors [*i.e.*, VEGF, PDGF, FGF-2, platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP), angiopoietins (Ang), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), tumor necrosis factor (TNF) and interleukin-6 (IL-6)] and three non-classical proangiogenic factors [*i.e.*, stem cell factor (SCF), tryptase and chymase] were reported in the literature [9–12].

The most frequent mechanisms that drive and maintain tumor angiogenesis are described to be concurrently: (*i*) increased VEGF, acidic and basic FGF, IL-8 and IL-6, hypoxia-inducible factor-1 alpha (HIF-1α) and Ang, together with (*ii*) downregulation of thrombospondins, angiostatin, endostatin and interferons [13–16]. Proangiogenic growth factors (GFs) activate surface RTKs as well as other cell membrane receptors such as integrins. In tumor angiogenesis, VEGF is upregulated by hypoxia and a variety of other GFs [17]. The interaction between HIFs and RTKs has also been reported to be important for expansion of the new blood vessel formation [18, 19]. In this frame, VEGF (also called VEGF-A), as a crucial molecule controlling the development and microarchitecture of angiogenic vessel networks, being at the top of the list [20]. Several anti-angiogenic drugs work by binding directly to VEGF, blocking it from binding to the VEGFR. Over the last decade, five

different VEGF/VEGFR targeted agents were approved by the *Food and Drug Administration* (FDA) for the treatment of cancer: Axitinib, Sorafenib, Sunitinib, Pazopanib and Bevacizumab.

Many small-molecule RTK inhibitors (RTKIs) have also been used to block angiogenesis in malignant tumors. Sorafenib (Nexavar®, Bayer), a multi-targeted RTKI, has been *FDA* approved as a single agent in advanced renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC) [21]. Another multi-targeted RTKI, Sunitinib (Sutent®, Pfizer), is also *FDA* approved for gastrointestinal stromal tumors and advanced RCC [22].

Axitinib is a more potent VEGFR inhibitor, selectively inhibiting VEGFRs 1, 2, and 3 tyrosine kinase activity. The drug has been approved for second line treatment of advanced RCC, showing antitumor activity both as a single-agent and in combination with other therapeutic approaches [23, 24].

VEGFR is strongly expressed in GB and progression from LGGs to HGGs is also mediated by this surface receptor [25]. Thus, VEGFR inactivation represents the most promising line of attack in GB. In May 2009, *FDA* approved Bevacizumab (Avastin®), a humanized anti-VEGF monoclonal antibody, for recurrent GB in United States. Although many clinical studies have shown the drug's effectiveness as a single treatment or in combination with other therapeutic modalities in recurrent GB, the *European Medicines Agency* (EMA) denied this drug, due to a lack of evidence. The use of Bevacizumab drug in human GB is controversial, the effect of the drug on GB tumor size, recurrence, and vascularization is still unclear [26]. Several RTKIs have also been under investigation in the setting of recurrent HGG. Cediranib, Sunitinib, Pazopanib, Vandetanib, and Sorafenib are just some of the multi-kinase VEGFR inhibitors that were evaluated in GB [27, 28]. Yet, several phase III trials demonstrated relatively modest advantages when anti-angiogenic RTKIs were compared with conventional chemotherapy, despite the efficacy seen in clinical trials with several such RTKIs [29, 30].

These findings raise concerns about the impact of anti-angiogenesis medications on the tumor uptake of other chemotherapeutic drugs and the overall anti-tumor effect of combination therapy. Thus, the dominant role of VEGF system in the angiogenesis process, makes it the most attractive target in disrupting tumor angiogenesis and in this study, we investigated how VEGFR suppression affects GB cells viability *in vitro*. By utilizing the *FDA*-approved VEGFR inhibitors Sorafenib and Axitinib, we examined the impact of receptor inhibition on GB cells viability *in vitro* [31–34].

Materials and Methods

Drugs and reagents

Sorafenib and Axitinib drugs were purchased from Redox Life Tech. Dulbecco's Modified Eagle Medium (DMEM)/nutrient mixture F-12 Ham, with L-glutamine and sodium bicarbonate, without 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sterile (MEM, D8062-500 mL, Sigma), Fetal Bovine Serum (FBS, F7524-500 mL, Sigma), Trypsin–Ethylenediaminetetraacetic acid (EDTA) solution 10X (T4174-100 mL, Sigma) and Cell Proliferation Kit I [2,5-Diphenyl-2H-tetrazolium bromide (MTT), RO11465007001, Roche Diagnostics] were supplied by Redox Life Tech.

Cell line establishment

The Bagdasar–Arseni Emergency Hospital in Bucharest, Romania provided us with surplus biological material from brain tumors that enabled our Laboratory to develop the GB cell line (GB1B), according to standard procedures [35, 36].

By signing the consent paperwork during their hospital stay, all patients agreed to donate their tissue for research purposes. In summary, fresh tumor tissues were diced in a Petri dish with an aseptic blade, and then combined with 0.5 mg/mL pronase, 0.25 mg/mL collagenase IV, and 0.4 mg/mL deoxyribonuclease (DNase). The specimens were placed in Hank's buffered saline solution and kept in a shaking incubator for 30 minutes at 37ºC, and subsequently another 30 minutes at 4ºC. Moreover, the cell mixture was passed through a cell filter to create a suspension of just one cell. Phosphate-buffered saline (PBS) was used to clean the cells twice before they were seeded in six-well plates. Ultimately, a cell suspension was placed into tissue culture flasks and passaged 2–3 times. In this experiment, the same passage cells were used.

Cell culture

DMEM containing 10% FBS, 2 mM L-glutamine and antibiotics (100 IU/mL Penicillin, 100 IU/mL Streptomycin) were used to develop the cell line cultures. The cells were maintained in tissue-culture flasks and kept in a humidified incubator at 37ºC with a 95% air/5% carbon dioxide (CO_2) environment. From the original biological material, the cell cultures were amplified 2–3 passages, and the third passage was preserved. When the confluence hit 30–50%, experiments were started. For our study, cells seeded in monolayers in 96-well culture plates ($1–10\times10^3$ cells/well), under the same environmental conditions as during the amplification phase, were treated with varying concentrations of Axitinib or Sorafenib (0.3; 0.6; 1.25; 2.5; 5; 10; 20; 40; and 80 μM), for three days and seven days, respectively. We included adequate control groups that contained solely diluents and blanks.

Cell proliferation assay

Cellular proliferation was quantified using the MTT assay, which relies on the ability of cells with active metabolism to cleave yellow tetrazolium salt into purple formazan crystals. Cells at a confluence of 1×10^3 cells per well/200 μL medium were seeded in 96-well plates, incubated for eight hours, and then exposed to different doses of SU1498 (an inhibitor of VEGFR2) alone or in combination with radiation. The cells were incubated for three and seven days, after which 10 μL of the MTT labeling agent was added to each individual well. The cells were incubated for four hours, at 37°C, after which they were solubilized, and the optical density (OD) was measured at 595 nm. The percentage of cells in the control group used to measure cell viability. To observe the morphological changes in cell shape, we used a 10× magnification phase contrast microscopy.

IC_{50}

The cells were exposed to increasing Axitinib or Sorafenib doses (0.3 μM; 0.6 μM; 1.25 μM; 2.5 μM; 5 μM; 10 μM; 20 μM; 40 μM; and 80 μM) for three and seven days and cell viability was determined by MTT. The free online Quest Graph™ IC_{50} Calculator offered by AAT Bioquest was used to solve the equation to estimate the inhibitory

dosage that results in the death of 50% of cells (IC_{50}). The Hill coefficient in the equation is negative for promotion activity and positive for the inhibition effect.

Statistical analysis

Using the Student's *t*-test, mean values were statistically compared. Differences were deemed statistically significant if their *p*-value was less than 0.05.

Results

The cytotoxic effect of Axitinib on GB cells

Axitinib (AG-013736), an oral tyrosine kinase inhibitor (TKI), exhibits promising anticancer efficacy in several other advanced malignancies, including GB [33, 37]. In this section, we have analyzed how VEGFR inhibition *via* Axitinib induced cell death in GB cells. For this reason, we used a GB cell line (GB1B) established in our Laboratory. The cells were cultivated in regular DMEM supplemented with 10% FBS, 2 mM of L-glutamine and a mix of two antibiotics (100 IU/mL Penicillin, 100 IU/mL Streptomycin). In the experimental setting, Axitinib was used to treat cells that were seeded in 96-well culture plates (0.5–1–3×10^3 cells/well, at doses ranging from 0.3 μM to 80 μM. The proliferation of the GB cells was measured after three and seven days. The GB1B cell line experienced the following notable effects after three days of exposure to increasing Axitinib concentrations: the treatment with 1.25 μM reduced GB1B cell survival by 8%, 2.5 μM Axitinib treatment produced 25% cell death in GB1B cells, 5 μM drug treatment reduced cell viability by 47%, 10 μM treatment decreased cell viability by 51%, 20 μM treatment produced 63% cell death, 40 μM drug treatment reduced cell survival by 66%, and proliferation was decreased by 73% with 80 μM treatment (Figure 1A). Higher cytotoxicity was seen in GB1B cells after extended exposure to a 7-day treatment with an increasing Axitinib concentration. The treatment with 1.25 μM drug impaired cell survival by 25%, 2.5 μM drug treatment reduced cell viability by 42%, 5 μM drug treatment reduced cell viability by 53%, 10 μM drug treatment decreased cell viability by 72%, 20 μM drug treatment reduced cell viability by 92%, 40 μM drug treatment produced a 93% reduction in cell viability, and 80 μM produced a 97% reduction in proliferation (Figure 2A). Axitinib's IC_{50} value was 3.5839 μM after three days of treatment (Figure 1B) and 2.2133 μM after a 7-day course of treatment (Figure 2B). The cells that survived three and seven days of Axitinib treatment did not undergo a marked change in cell shape or size, as can be seen in Figure 3 (A–C). Assessment of phase contrast microscopy (10× magnification) on cells treated with Axitinib showed very few alterations in cell shape (rounded cell) and detachment from cell substrate (Figure 3, A–C).

The cytotoxic effect of Sorafenib on GB cells

In a study by Jakubowicz-Gil *et al.*, it has been reported that after Sorafenib treatment, autophagy was most frequently seen in T98G cells [38]. After three days of treatment with Sorafenib, the percentage of viable cells decreased as follows: 9% for a drug concentration of 1.25 μM, 25% for a drug concentration of 2.5 μM, 41% for a drug concentration of 5 μM, 59% for a drug concentration of 10 μM, 60% for a drug concentration of 20 μM, 61% for a drug concentration of 40 μM, and 80% for a drug concentration of 80 μM (Figure 4A). After a 7-day course of Sorafenib therapy, cell viability was significantly reduced, resulting in 46% cell death after a 1.25 μM treatment course, 52% cell death after a 2.5 μM treatment course, 60% cell death after a 5 μM treatment course, 80% cell death after a 10 μM treatment course, 91% cell death after a 20 μM treatment course, 93% cell death after a 40 μM treatment course, and 90% cell death after a 80 μM treatment course (Figure 5A). Sorafenib's IC_{50} value was 3.5152 μM after a 3-day course of treatment (Figure 4B) and 1.6846 μM after a 7-day course of treatment (Figure 5B). Overall, our findings show that GB express VEGFR and might constitute a significant target in GB treatment. For most of these anchorage-dependent cells there were no changes in cell shape or size treated after Sorafenib treatment for three or seven days (Figure 6, A–C).

Comparison of cytotoxicity induced of Axitinib and Sorafenib by equimolar doses on GB cells

Axitinib was superior to Sorafenib (Nexavar®) and was approved for use in advanced RCC [39]. As seen in Figure 7A, Axitinib treatment provided to be cytotoxic for GB cells at very low concentrations, starting from 0.3 μM, while Sorafenib at concentrations of 0.3 μM and 0.6 μM induced cell proliferation in GB cells, becoming cytotoxic from a concentration of 1.25 μM. The cytotoxicity produced by Axitinib was approximately 26% higher than that produced by Sorafenib at concentrations of 0.3 μM and by 13% higher at concentrations of 0.6 μM. A very large difference in cytotoxicity was also recorded at the concentration of 5 μM, Axitinib producing 15% stronger cytotoxic effect than Sorafenib in GB1B cells. In general, the treatment with Axitinib was much more effective than the one with Sorafenib, at three days treatment. On the contrary, after seven days of treatment, Sorafenib treatment was superior to Axitinib treatment (Figure 7B). Except for the maximum concentration used in the study (80 μM), where the cytotoxic effect of the two drugs was comparable, at all other concentrations used, the cytotoxic effect of Sorafenib was 8–10% stronger than the cytotoxic effect produced by Axitinib (Figure 7B).

Discussions

In GB, VEGF is the most prevalent and significant mediator of angiogenesis. The use of VEGF system inhibition for the treatment of HGGs is becoming more and more popular. Several VEGFR inhibitors are being researched for HGGs. These drugs were investigated in a variety of clinical trials, with the results advancing our understanding of antiangiogenic therapy as a whole [5, 40, 41].

The recombinant humanized monoclonal antibody Bevacizumab (Avastin®) has also been investigated in both recurrent and newly diagnosed GB. Bevacizumab has shown significant activity in recurrent GBs, resulting in the *FDA* approved in 2009 [42, 43]. The combined treatment with Bevacizumab and Irinotecan for malignant gliomas treatment was also used in several clinical trials, and the results were promising [26]. Cancer immunotherapy has also raised the interest of the scientific community in recent years. In a meta-analysis study, it was found that dendritic cell (DC) therapy improves overall survival and progression-free survival (PFS) of HGG patients, both recurrent and newly diagnosed. Viral therapy also slightly improved overall survival but PFS was similar to the control arms [26, 44].

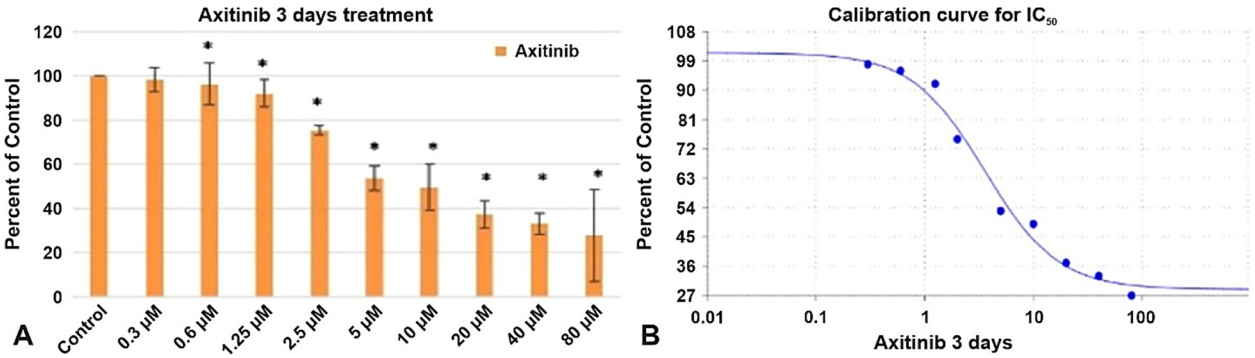

Figure 1 – ***The effect of Axitinib on GB1B proliferation (A) and the calibration curve for IC_{50} (B) at three days of treatment. Results are expressed as percentage of control. Data represents the mean and standard error of three separate experiments. Error bars are the mean ± SD for each drug concentration, representing the linear model fit to the data. *Represents significant difference from control (p<0.05). GB1B: Glioblastoma cell line; IC_{50}: Half maximal inhibitory concentration; SD: Standard deviation.***

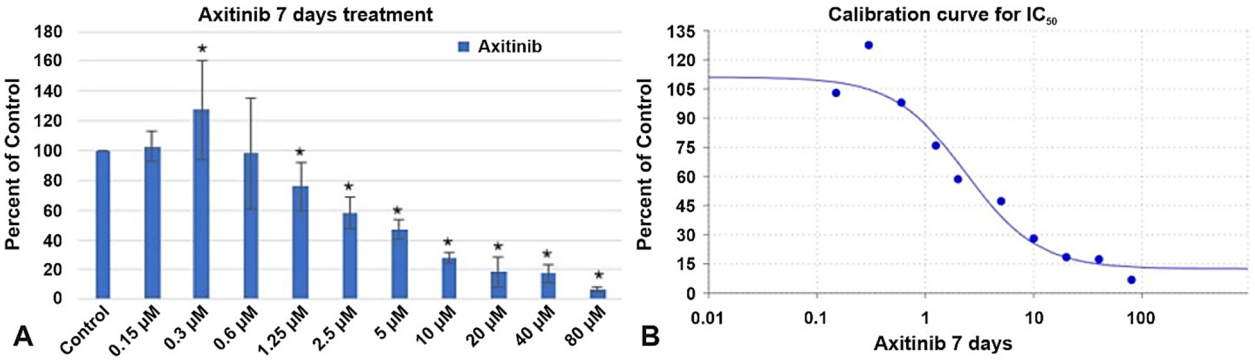

Figure 2 – ***The effect of Axitinib on GB1B proliferation (A) and the calibration curve for IC_{50} (B) at seven days of treatment. Results are expressed as percentage of control. Data represents the mean and standard error of three separate experiments. Error bars are the mean ± SD for each drug concentration, representing the linear model fit to the data. *Represents significant difference from control (p<0.05).***

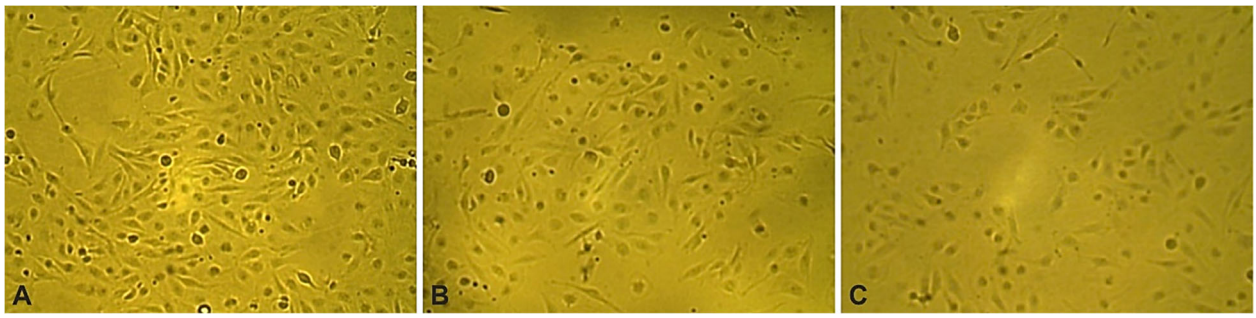

Figure 3 – ***The effect of Axitinib on GB1B cells viability and morphology. The cells were exposed to 10 µM Axitinib for three and seven days. Microscopy pictures were taken at initial culture day (A), three days (B) and seven days (C) after the treatment with 10 µM Axitinib (10× magnification).***

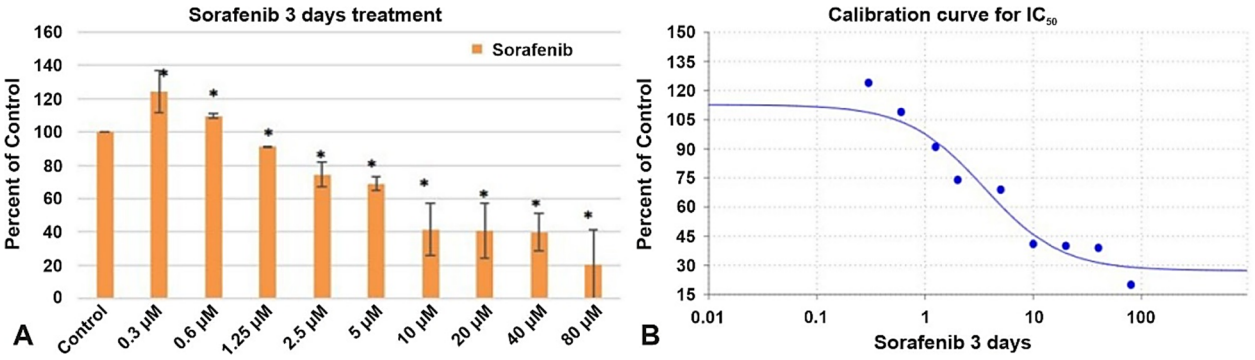

Figure 4 – ***The effect of Sorafenib on GB1B proliferation (A) and the calibration curve for IC_{50} (B) at three days of treatment. Results are expressed as percentage of control. Data represents the mean and standard error of three separate experiments. Error bars are the mean ± SD for each drug concentration, representing the linear model fit to the data. *Represents significant difference from control (p<0.05).***

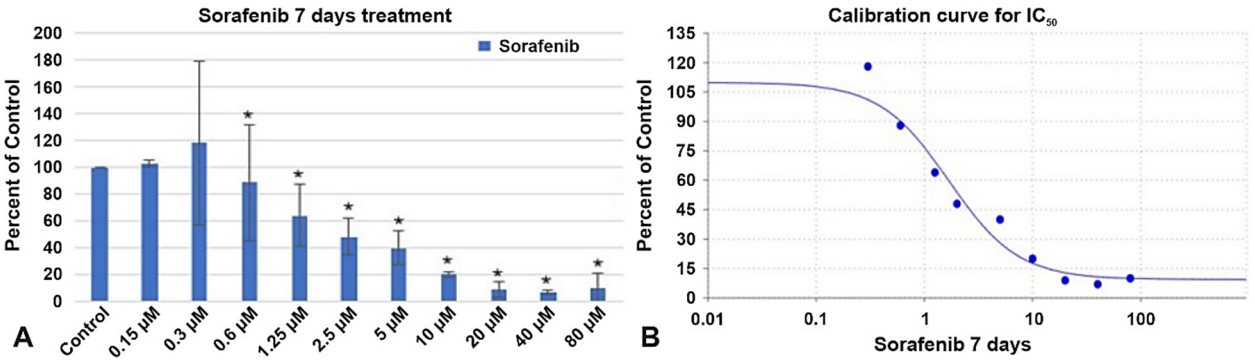

Figure 5 – ***The effect of Sorafenib on GB1B proliferation (A) and the calibration curve for IC_{50} (B) at seven days of treatment. Results are expressed as percentage of control. Data represents the mean and standard error of three separate experiments. Error bars are the mean ± SD for each drug concentration, representing the linear model fit to the data. *Represents significant difference from control ($p<0.05$).***

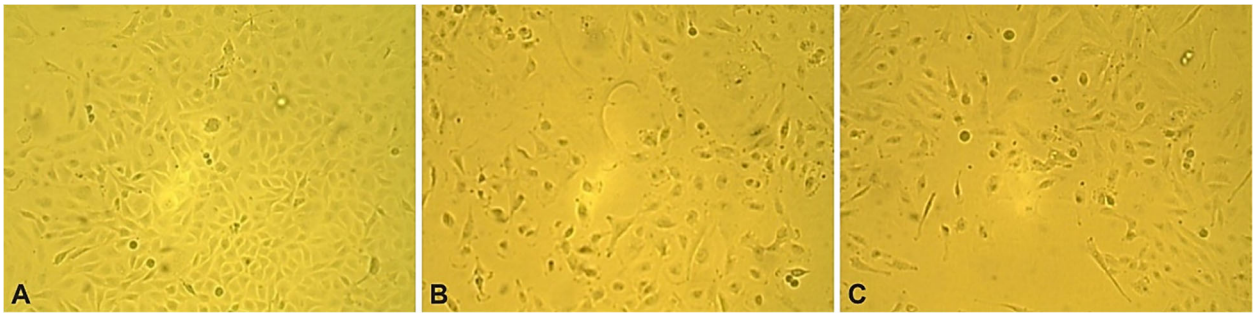

Figure 6 – ***The effect of Sorafenib on GB1B cells viability and morphology. The cells were exposed to 10 µM Sorafenib for three and seven days. Microscopy pictures were taken at initial culture day (A), three days (B) and seven days (C) after the treatment with 10 µM Sorafenib (10× magnification).***

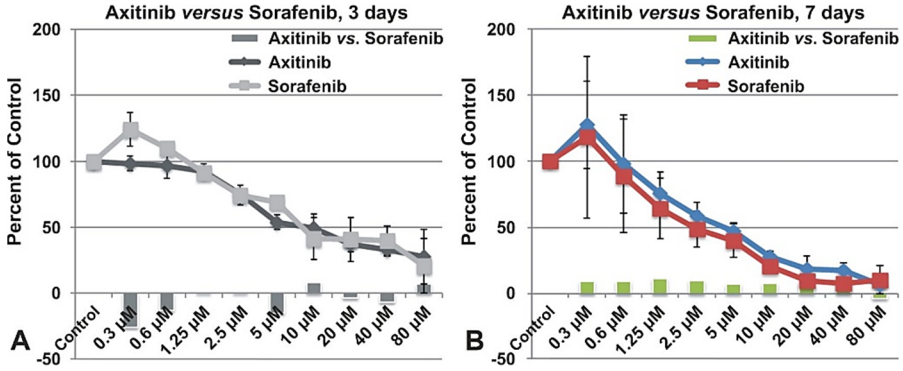

Figure 7 – ***Comparison of Axitinib and Sorafenib effect on GB1B proliferation, at three days (A) and seven days (B) of treatment. *Represents significant difference from control ($p<0.05$).***

Axitinib demonstrated anti-tumor activity in preclinical and clinical studies, for different types of cancer, including thyroid cancer, epithelial ovarian cancer, and nasopharyngeal cancer [45–47]. Other angiogenic inhibitors including Trebananib, Aflibercept, Nintedanib, Cediranib, Imatinib, are also in ongoing clinical trials, but Axitinib effects are different from those of earlier treatments due to its more potent and active inhibition of VEGFR [48, 49].

In the treatment of brain tumors, Axitinib has been very little studied. One clinical study demonstrated that Axitinib induced direct cytotoxic effect against several patient-derived GB stem cells and also extended survival in preclinical orthotopic GB models, when administered as systemic single agent [32]. The results from a randomized phase II trial [37], showed that in recurrent GB patients, Axitinib treatment resulted in improved response rate. In the present study, we also found that Axitinib induced cytotoxicity in a patient-derived GB cell line; Axitinib's IC_{50} value was 3.58 µM after three days of medical treatment and 2.21 µM after seven days of medical treatment. Axitinib treatment did not produce a marked change in cell shape or size. Assessment of phase contrast microscopy on cells treated with Axitinib showed very few alterations in cell shape (rounded cell) and detachment from cell substrate. Although the result from our and other research groups show that Axitinib is a potent anticancer drug *in vitro*, more clinical evidence is required to show whether this actually translates to better clinical outcomes.

Sorafenib, also known as Nexavar®, is a multikinase inhibitor that has been investigated in many solid tumors.

The medication has first been given clinical approval for the treatment of advanced RCC and HCC [50]. Nowadays, several clinical trials using Sorafenib are being conducted, including those for lung, thyroid, prostate, and breast cancer [51–54]. In GB patients, Sorafenib as monotherapy or in combination with Temozolomide showed only limited benefit

and considerable toxicity [55, 56]. When radiotherapy was added to Sorafenib and Temozolomide combination, same moderate outcome results were found, as compared to standard therapy alone, however the adverse effects were significantly increased [57]. Here, we found that Sorafenib was cytotoxic in GB1B cells, the IC_{50} value was 3.5152 μM after three days of medical treatment and 1.6846 μM after seven days of medical treatment. For most of these anchorage-dependent cells, there were no changes in cell shape or size after Sorafenib treatment for three or seven days.

In actuality, the *FDA* has authorized the use of four VEGFR TKIs: Sorafenib, Sunitinib, Pazopanib, and Axitinib; as well as one VEGF-direct antibody, Bevacizumab, for the management of metastatic RCC since 2005. A phase III trial was conducted evaluating the effectiveness of Axitinib with Sorafenib in individuals with metastatic RCC who have not had systemic treatment previously. According to this trial, Axitinib and Sorafenib showed a median PFS of 10.1 months and 6.5 months, respectively. Axitinib treatment resulted in a numerically longer PFS, compared to Sorafenib treatment, however this did not reach the required level of statistical significance, thus, the trial failure to increase PFS with Axitinib against Sorafenib for metastatic RCC was the final conclusion [58]. Our results showed that both Axitinib and Sorafenib retarded GB1B cell growth in terms of dose and duration in comparison with the untreated control groups. Axitinib treatment provided to be more cytotoxic on GB1B cells, at three days after the treatment, while Sorafenib treatment was superior to Axitinib treatment, at seven days after the treatment. For the majority of these GB1B anchorage-dependent cells, no significant change in shape or size could be observed after Axitinib or Sorafenib treatment. These results suggest that the treatment inhibits cell growth, rather than killing the cells. However, this observation is speculative, and the phenomenon must be studied in depth, in order to draw a conclusion.

Conclusions

GB treatment failure, as well as its progression and recurrence results from the fundamental nature of this disease, challenging the scientists to accelerate the study of the cellular mechanisms underlying tumor development, to provide better diagnostic methods and better therapeutic targets. Identifying biomarkers may increase the chance of GB patients' survival, since they can facilitate timely diagnosis and aid in the selection of a targeted treatment. Overall, our results point to VEGFR as a potential therapeutic target for the treatment of GB. Although it is known that some drugs block the VEGFR more potently *in vitro*, clinical evidence is required to show whether this actually translates to better clinical outcomes.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Alexandru Opriţa and Mihaela Amelia Dobrescu equally contributed to this study.

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