

# Reversing glioma malignancy: a new look at the role of antidepressant drugs as adjuvant therapy for glioblastoma multiforme

Anna M. Bielecka-Wajdman<sup>1</sup> · Marta Lesiak<sup>2</sup> · Tomasz Ludyga<sup>1</sup> · Aleksander Sieron<sup>2</sup> · Ewa Obuchowicz<sup>1</sup>

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## Abstract

**Purpose** The role of glioma stem cells (GSCs) in cancer progression is currently debated; however, it is hypothesised that this subpopulation is partially responsible for therapeutic resistance observed in glioblastoma multiforme (GBM). Recent studies have shown that the current treatments not only fail to eliminate the GSC population but even promote GSCs through reprogramming of glioma non-stem cells to stem cells. Since the standard GBM treatment often requires supplementation with adjuvant drugs such as antidepressants, their role in the regulation of the heterogeneous nature of GSCs needs evaluation.

**Methods** We examined the effects of imipramine, amitriptyline, fluoxetine, mirtazapine, agomelatine, escitalopram, and temozolomide on the phenotypic signature (CD44,

Ki67, Nestin, Sox1, and Sox2 expression) of GSCs isolated from a human T98G cell line. These drugs were examined in several models of hypoxia (1% oxygen, 2.5% oxygen, and a hypoxia-reoxygenation model) as compared to the standard laboratory conditions (20% oxygen).

**Results** We report that antidepressant drugs, particularly imipramine and amitriptyline, modulate plasticity, silence the GSC profile, and partially reverse the malignant phenotype of GBM. Moreover, we observed that, in contrast to temozolomide, these tricyclic antidepressants stimulated viability and mitochondrial activity in normal human astrocytes.

**Conclusion** The ability of phenotype switching from GSC to non-GSC as stimulated by antidepressants (primarily imipramine and amitriptyline) sheds new light on the heterogeneous nature of GSC, as well as the role of antidepressants in adjuvant GBM therapy.

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✉ Anna M. Bielecka-Wajdman  
amb2@vp.pl

Marta Lesiak  
mlesiak@sum.edu.pl

Tomasz Ludyga  
ludyga.tomasz@gmail.com

Aleksander Sieron  
asieron@sum.edu.pl

Ewa Obuchowicz  
eobuchowicz@sum.edu.pl

<sup>1</sup> Department of Pharmacology, School of Medicine, Medical University of Silesia, Medyków 18, Katowice, Poland

<sup>2</sup> Department of Biology and Genetics, School of Medicine, Medical University of Silesia, Medyków 18, Katowice, Poland

**Keywords** Glioma malignancy · Glioma stem cells · Adjuvant therapy in glioma · Antidepressant drugs in glioma therapy

## Introduction

The discovery and isolation of cancer stem cells (CSC) were a critical finding in the fight against aggressive neoplasms, including *glioblastoma multiforme* (GBM)—the most invasive human brain tumour. Understanding the nature of CSCs is key for the design of an effective anti-GBM treatment. To date, relevant research has not adequately addressed questions concerning CSC origins or their role in maintaining the phenotype of malignant tumours [1].

The CSC hypothesis postulates the existence of a distinct population of small cells within tumour masses that

display the following properties: self-renewal, de novo tumour reconstruction after xenograft transplantation, pluripotency, neurosphere formation, proliferation, invasion, angiogenesis, and differentiation into different cell lineages [2–4]. According to the Brain Stem Cell Theory, all tumour cells have CSC potential [5]. Some divergences concern the identification of CSCs in primary and secondary gliomas and their strong phenotypic plasticity.

Studies report that, in gliomas, CD133 positive and CD133 negative cells can be detected, suggesting that CSC formation may be regulated by genetic/epigenetic factors, as well as metabolic, microenvironmental, and niche factors that are not yet fully understood. Due to the presence of glioma stem cells in various oxygen tumour microenvironments, regulation of the metabolic processes in these niches has a substantial effect on the GSC phenotype [6, 7]. Recently, it has been shown that GSCs can effectively mimic endothelial cells in vascular mimicry and organise vessel-like structures that allow blood flow. Moreover, the role of CSCs in the mechanisms of radiochemoresistance is debated. It is believed that primary CSCs demonstrate high resistance to treatment from the outset; however, there are reports showing that the standard treatments (temozolomide and radiotherapy) of GBM can promote reprogramming of non-CSCs to CSCs and, in effect, increase the CSC fraction within the tumour [8, 9].

A more concise understanding of GSC biology is essential for the improvement GBM treatment. It has been suggested that adjuvant drugs used to relieve the side effects of radio/chemotherapy, while improving the patient's quality of life, may be responsible for the promotion of GSCs with malignant phenotypes [10–13].

Upwards of 90% of patients with GBM are suffering from depressive disorders and neurological disturbances. Depression is not only a consequence of the diagnosis, tumour size/grade, or past psychiatric history, but is often also a consequence of the standard treatment: surgery, radio/chemotherapy (temozolomide), and steroid application [14, 15]. In this clinical situation, oncologists commonly prescribe antidepressant drugs.

In addition to their antidepressant effects, these drugs are prescribed to cancer patients to treat chronic and neuropathic pain, anxiety disorders, anorexia, migraines, and circadian rhythm disorders [16–18]. The results of in vitro and in vivo studies suggest that antidepressant drugs, through their influence on immune system function, cytochrome P450 activity, intracellular signalling pathways, and mitochondrial bioenergetics, may not be neutral with regard to cancer progress and therapy. Moreover, it has been demonstrated that the same antidepressants can either promote or inhibit tumour growth and modulate the cytotoxic effects of anticancer drugs and their accumulation in cancer cells [19–21]. Despite these

**Table 1** Characterization of antidepressant drugs used in the study

Drug	Action
Imipramine	A tricyclic drug used occasionally in the treatment of major depression coexisting with panic disorders, a reference drug used in many in vitro and in vivo studies [23]
Amitriptyline	A TCA drug used to treat various forms of depression, neuropathic pain during cancer therapy and migraines, the most common antidepressant drug supporting oncology treatment [24]
Fluoxetine	A selective serotonin reuptake inhibitor (SSRI), used for the treatment of depressive, appetite, panic and obsessive–compulsive disorders [25]
Escitalopram	An SSRI, effective in the treatment of anxiety and panic disorders; it is also used for the prevention of depression in head and throat cancer patients [26]
Mirtazapine	An atypical drug. In addition to its antidepressant effects, it is known for stimulating appetite and increasing weight gain [27]
Agomelatine	A melatonin receptor agonist, used in the treatment of major depression and sleep disorders. Agomelatine also presents anxiolytic properties and resynchronises the circadian rhythm [28]
Temozolomide	An alkylating agent of DNA in the O6-guanine, N7-guanine and N3-adenine N-7 position. It is used for the treatment of anaplastic astrocytoma and GBM [29]

controversial reports about the effects of antidepressants as applied in the treatment of cancer patients, this problem is marginalised in clinical practice.

The present study focuses on the influence of antidepressant drugs (imipramine, amitriptyline, fluoxetine, mirtazapine, agomelatine, and escitalopram (Table 1) or temozolomide on the phenotypic signature of the GSC population in several models of hypoxia using a human T98G cell line. As GSCs are not self-autonomous units but are under specific microenvironmental control (niche) [22], to reconstruct the oxygen conditions in the intratumour space, experiments were conducted in complex models of hypoxia (1% oxygen, 2.5% oxygen, or a hypoxia-reoxygenation model). As a control, the standard laboratory conditions of 20% oxygen were used. In these models, the effects of antidepressants or temozolomide on the quantity and expression of stem cells markers CD44, Ki67, Nestin, Sox1, and Sox2 (Table 2) in a human T98G GBM cell line were investigated.

## Materials and methods

### Cell culture

The human GBM cell line T98G (Sigma-Aldrich, St Louis, MO, USA), a normal human astrocytes line (NHA, Lonza,

**Table 2** Characterization of markers used in the study

Marker	Characterisation
CD44	A protein involved in many pathological and physiological processes. In cancer, CD44 is responsible for growth promotion and cell migration. The CD44 expression correlates not only with the degree of malignancy but also with the density of malignant cells in tumour-affected regions [30]
Nestin	First described as a neural stem/progenitor cell marker, Nestin plays an important role in cellular processes including stemness, migration and cell cycle regulation. Its expression in GBM cells is correlated with self-renewal properties, the degree of malignancy, poor survival and infiltration into surrounding tissues [31]
Ki67	A proliferation marker of all tumours. The expression of Ki67 is determined in G1, S, G2 and M phase cell cycles and is associated with the degree of invasion. It is also recognised as a prognostic predictor of survival and tumour recurrence. Moreover, approximately 20% of GBM tumours have a mutation in the Ki67 protein [32]
Sox1, Sox2	Transcriptional factors involved in regulating neural precursor cells, as well as in CNS development. Sox1 plays a role in determination and differentiation. The expression of Sox1 is detected in GBM but not in all tumour samples. Sox2 is overexpressed in all brain tumours (including high-grade glioma) and is believed to regulate stem cell self-renewal. Sox2 can reprogramme differentiated cells into pluripotent cells [33]

Switzerland), and the human U87 astrocytoma line (Sigma-Aldrich, St Louis, MO, USA) were utilized in these studies. Cellular media, gentamicin, and foetal bovine serum (FBS) were purchased from Gibco-BRL (USA). The following antidepressant drugs were used: imipramine, amitriptyline, fluoxetine, escitalopram, agomelatine, mirtazapine, and the cytostatic drug, temozolomide (Sigma-Aldrich, St Louis, MO, USA). Plasticware for cell culture (monolayer and spheres) was purchased from Nunc (USA), Falcon (Lexington, TN, USA), and Eppendorf (Germany).

### Study design and experimental oxygen models

Since intratumour glioma microenvironments are unstable and characterised by areas of differing oxygenation levels [33], we applied hypoxic in vitro models (1% oxygen, 2.5% oxygen, or a hypoxia-reoxygenation model) aimed to recreate the variations in oxygen levels observed in gliomas. The hypoxia-reoxygenation model (1% oxygen followed by 3% oxygen) reflects a specific niche in the intratumour. The standard laboratory conditions (20% oxygen) were used to compare our data with the results of other studies. This oxygen model, however, creates delusive conditions for cancer cells that do not apply in vivo, even though this method is commonly used in in vitro studies.

Cell density was evaluated using the following cell counters before the experiments were performed: Eve (Life Technologies, Grand Islands, NY, USA), Scepter (Millipore, Germany), and the Muse flow cytometer (Millipore, Germany). Our experiments were performed at a cell density of  $1.9 \times 10^5$  cells per 1 ml medium. Glioma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% gentamicin, 1 g/L D-glucose, L-glutamine, 25 mM HEPES, and pyruvate, which was replenished every 3 days. Throughout the duration of the experiments, the cultures

were maintained in hypoxia or standard conditions as described above. When cells reached 90% confluence, the cultures were trypsinized and passaged. On the second day following trypsinization, the cells were washed and fresh medium was added containing the following antidepressants: imipramine, amitriptyline, fluoxetine, mirtazapine, escitalopram or agomelatine at a concentration of 10  $\mu$ M, or 1 mM temozolomide. The cultures were exposed to drugs for 24 h in the various oxygen conditions. In the hypoxia-reoxygenation model, the cultures were maintained in an atmosphere containing 1% oxygen for 12 h and then in 3% oxygen for the remaining 12 h.

All experiments were conducted in two types of CO<sub>2</sub> incubators. In the standard CO<sub>2</sub> incubator (NuAire, USA), we cultured cells in the standard laboratory conditions (5% CO<sub>2</sub>, 20% oxygen, and 97% humidity). The second incubator (New Brunswick Galaxy 48R, Germany) was utilized for hypoxic experiments.

### Mitochondrial activity and cell viability assay (MTT)

Examination of NADPH-dependent oxidoreductase to convert tetrazolium dye (MTT) in T98G, U87, and NHA cell lines when exposed to antidepressants or temozolomide (described in Sect. 2.2) was carried out in 96 well plates according to a previously described method [34, 35].

### Characterisation of the GSC phenotype isolated from T98G cells by flow cytometry

To determine the influence of temozolomide- or antidepressant-based treatment on the GSC phenotype, T98G cells were cultured as spheres in the varied oxygen conditions. Cells were exposed to either temozolomide (1 mM) or one of the following antidepressant drugs: imipramine, amitriptyline, fluoxetine, mirtazapine, escitalopram, or agomelatine (10  $\mu$ M). The phenotypes of cells

were estimated using fluorescence through the use of the FACS Aria I flow cytometer (Becton–Dickinson, USA). Approximately  $5 \times 10^6$  cells were recovered from the culture dish using Accutase Cell Detachment Solution (Becton–Dickinson). Single cell suspensions were washed with phosphate-buffered saline (PBS), centrifuged ( $300 \times g$ , 10 min), resuspended in Cytofix Fixation Buffer (Becton–Dickinson), and incubated at room temperature for 20 min in the dark. Cells were then washed twice in PBS, centrifuged ( $1110 \times g$ , 10 min), and permeabilised using Phosflow Perm Buffer III (Becton–Dickinson). The cells were resuspended in  $1 \times$  PBS supplemented with 1% foetal calf serum (FCS) to a final cell density of  $1 \times 10^6$  cells/200  $\mu$ L. Fluorochrome-conjugated antibodies against CD44, Sox1, Sox2, Nestin, and Ki67 from the Human Neural Lineage Analysis kit (Becton–Dickinson) were added to the cell suspension, and samples were incubated at room temperature for 30 min in the dark. Following staining, excess antibody was washed off using 2 mL of  $1 \times$  PBS. The cell suspension was centrifuged ( $1110 \times g$ , 5 min) and resuspended in 400  $\mu$ L  $1 \times$  PBS prior to analysis with the FACS Aria flow cytometer.

## Results

### Mitochondrial activity and cell viability of T98G, U87, and NHA cells exposed to temozolomide and antidepressant drugs

Results from the MTT assay showed that both human glioma lines (T98G, U87) presented similar sensitivity to antidepressant drugs and temozolomide in all oxygen conditions. The cytotoxic effect of temozolomide, as well as inhibition of mitochondrial activity, was enhanced with increased oxygenation. Temozolomide reduced glioma cell viability by 75% (T98G) and 78% (U87) as compared to the control.

Fluoxetine, mirtazapine, escitalopram, and agomelatine did not significantly alter cell viability of either glioma lines in all oxygen models. By contrast, imipramine and amitriptyline inhibited mitochondrial activity at a rate dependent on the oxygen content in the atmosphere (from 6% in hypoxia, 11% in average hypoxia, and 19% in hypoxia-reoxygenation to 26% (imipramine) and 39% (amitriptyline) in 20% oxygen). Moreover, all antidepressant drugs increased the mitochondrial activity of NHA. The strongest pro-survival effect was observed after NHA exposure to imipramine or amitriptyline in the hypoxia-reoxygenation model (18% as compared to the control) and in the standard laboratory conditions (26% compared to the control). However, temozolomide strongly reduced viable NHA cells in all oxygen models compared to untreated

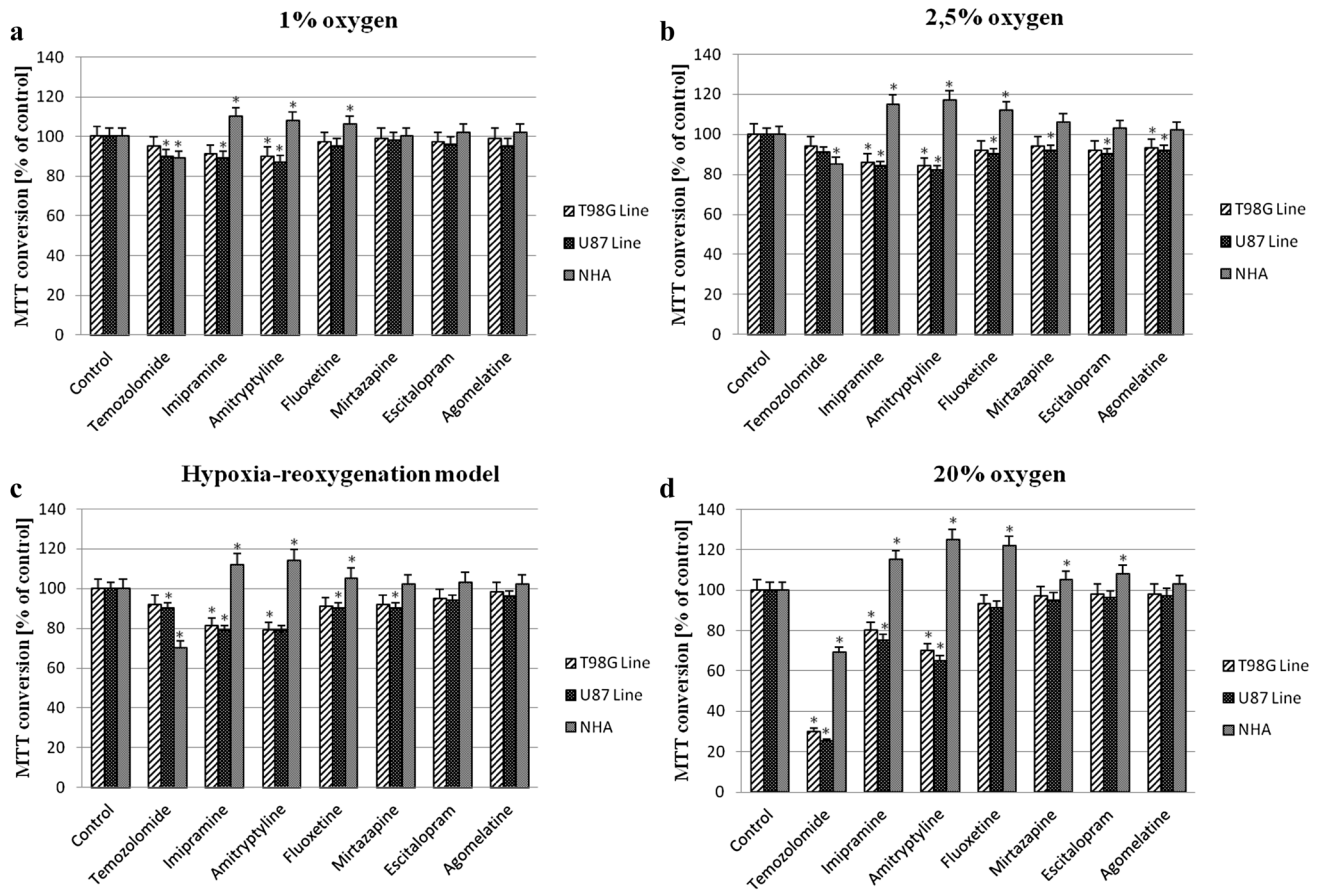
astrocytes (15% in hypoxia, 17% in 2.5% oxygen, and 26% in both the hypoxia-reoxygenation model and the standard laboratory conditions) (Fig. 1a–d).

### Phenotypic profile of GSCs isolated from T98G cell cultures exposed to antidepressants and temozolomide in oxygen conditions

Flow cytometry analysis revealed the remarkable plasticity of GSCs isolated from T98G cells. Both temozolomide and antidepressants affected the CD44/Ki67/Nestin/Sox1/Sox2 GSC phenotype, particularly in GSCs isolated from cultures maintained in a low oxygen atmosphere. The most significant alterations were detected in CD44 and Ki67 expression.

1. Hypoxia model (1% oxygen). Ki67 expression in the control group [untreated was higher (22%) than in the drug-treated cultures]. Temozolomide increased CD44 expression in control cultures from 50.5 to 63.5%. Both tricyclic antidepressant (TCA) drugs, imipramine and amitriptyline, significantly reduced CD44 expression to as low as 30.1% (after exposure to amitriptyline). Decreased levels of the following markers were detected: Nestin 2% (after imipramine exposure) and 7.5% (control); Sox1 0% (after imipramine exposure) and 12% (control); and Sox2 0.1% (after agomelatine exposure) and 8% (control) (Fig. 2a).
2. Average hypoxia model (2.5% oxygen). Imipramine and amitriptyline decreased the expression of the CD44 marker to 29% (imipramine) and 30% (amitriptyline) as compared to the 38% expression in the control group. Ki67 levels fell to 33% (imipramine) and 32% (amitriptyline) as opposed to the 47% expression observed in the control group. Interestingly, temozolomide elevated the level of the CD44 expression to 47% compared to 38% in the control. All antidepressants in the study decreased Sox1 and Sox2 expressions in GSCs to nearly 0%. In the control, Sox1 expression was 1%, while Sox2 expression was 3%; after temozolomide exposure, these levels increased to 4% (Sox1) and 1% (Sox2) (Fig. 2b).
3. Hypoxia-reoxygenation model (1% oxygen for 12 h followed by 3% oxygen for 12 h). The changes induced by temozolomide and antidepressants in the GSC phenotype profile (CD44, Ki67, Sox1, and Sox2) were similar those observed in the hypoxia model. Under these oxygen conditions, however, Nestin expression was significantly increased compared to other applied oxygen conditions. The Nestin + cells constituted 5% (after amitriptyline exposure) and 32% of cells (after temozolomide exposure) (Fig. 2c).
4. The standard laboratory conditions (20% oxygen). The expression of GSC markers was found to be markedly





**Fig. 1** MTT conversion. Cell viability in: T98G, U87 glioblastoma cell lines, and normal human astrocytes (NHA) after exposure to temozolomide (1 mM), imipramine, amitriptyline, fluoxetine, mirtazapine, escitalopram, and agomelatine (10  $\mu$ M). Cells were cultured in a different oxygen conditions: **a** 1% oxygen, **b** 2, 5% oxygen: an average oxygen concentration in intratumor environment, **c** hypoxia-reoxygenation model, and **d** standard laboratory conditions 20%

oxygen. Each bar represents the mean  $\pm$  SEM of at least three independent experiments. Values were analyzed by one-way ANOVA, followed by Tukey post hoc test,  $*p < 0.05$  vs. control-temozolomide, imipramine, amitriptyline, fluoxetine, escitalopram, mirtazapine, and agomelatine. The Bonferroni adjustment was applied for multiple comparisons. If the data were not normally distributed, then Kruskal–Wallis test followed by Mann–Whitney test was performed

different than GSC expression in cultures maintained in all tested hypoxic conditions. CD44, Sox1, and Sox2 were not detected in any experimental groups. We observed strong inhibition of Ki67 after exposure of GSCs to temozolomide (20% as compared to 87% Ki67+ cells in the control group). All antidepressants also decreased Ki67 expression in contrast to the control group. The strongest effects were induced by imipramine and amitriptyline, while Nestin was undetectable in GSCs (expression ranged between 0 and 1% after exposure to temozolomide).

### Statistical analyses

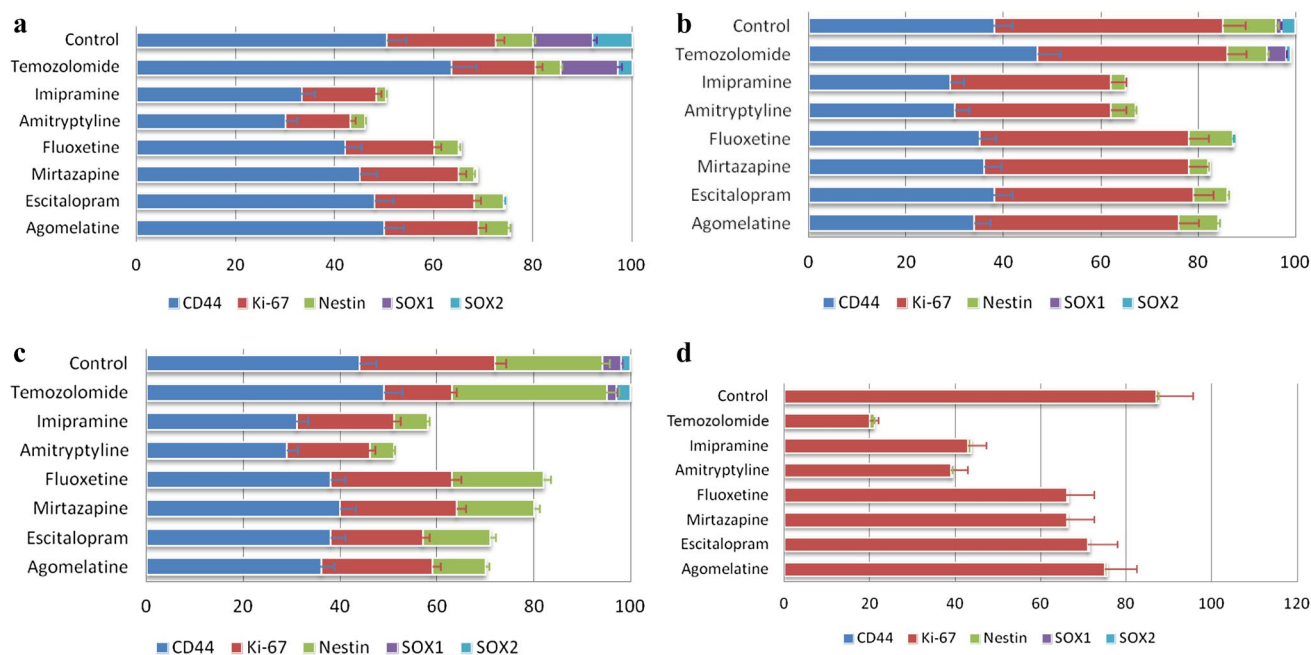
Statistical analysis was performed using one-way ANOVA followed by the post hoc Tukey test. Differences were considered statistically significant when  $p < 0.05$ . The results are presented as the standard error of the

mean (SEM). Statistical analysis was performed using the GraphPad Prism 7.01 software system (GraphPad Software Inc., San Diego, CA).

### Discussion

Based on previous studies, the ability of GBM to recur, despite maximal surgical resection, may be partially attributable to GSCs [36, 37]. As there is still minimal knowledge of GSC modulation in terms of plasticity and phenotypic interconversion (CD44, Nestin, Sox1, Sox2, and Ki67) induced by temozolomide and adjuvant drugs (imipramine, amitriptyline, fluoxetine, mirtazapine, agomelatine, and escitalopram), these interactions were addressed in the current study.

Our investigation revealed that antidepressant drugs are able to silence the GSCs profile to a greater extent



**Fig. 2** Phenotype profile of GSC isolated from T98G cell cultures exposed to antidepressants and temozolomide in several oxygen conditions. Expression of CD44, Ki67, Nestin, and Sox1 and Sox2 markers in T98G cell culture after exposure to temozolomide (1 mM), imipramine, amitriptyline, fluoxetine, mirtazapine, escitalopram, and agomelatine (10  $\mu$ M). Cells were cultured different oxygen conditions: **a** 1% oxygen, **b** 2, 5% oxygen: an average oxygen concentration in intratumor environment, **c** hypoxia-reoxygenation model, and

**d** standard laboratory conditions 20% oxygen. Each bar represents the mean  $\pm$  SEM of at least three independent experiments. Values were analyzed by one-way ANOVA, followed by Tukey post hoc test,  $*p < 0.05$  control-temozolomide, imipramine, amitriptyline, fluoxetine, escitalopram, mirtazapine, and agomelatine. Correlation between markers expressed by glioma cancer cells was tested by calculating the correlation coefficient (Pearson's test)

than temozolomide, and the strongest effects were induced by imipramine and amitriptyline (tricyclic antidepressants). Antidepressants downregulated Sox1 and Sox2 expressions from the cell surface, and Nestin, Ki67, and CD44 expression were inhibited. This unique capacity of antidepressants to reverse the GSC phenotype from stemness to “non-stemness” or “less stemness” suggests the possibility of managing GBM malignancy using antidepressants. However, the mechanisms through which antidepressants modulate the GSC phenotype are complex and may be linked to microenvironmental conditions. Hypoxic niches, with the exception of the activation of specific factors and cellular signalling pathways (HIF, WNT, Notch, Shh, and BMP), promote increased cellular fractions exhibiting a stem phenotype [38]. In addition, they also induce dysfunction in cellular immune responses [modulation of T-cell responses, promotion of proinflammatory genes (RAGE, COX2, nf-kb expression, and upregulation of STAT3)] [39–41]. Antidepressant drugs, through their influence on various components of the immune system (balance between pro- and anti-inflammatory cytokines, B/NK/T cells) or reactive oxygen species (melatonin), can influence cancer immunity and GSC plasticity [42–44].

In the present study, we confirmed the critical role of the hypoxic microenvironment in the promotion of the cellular stemness profile. Our results shown that using a single oxygen model, particularly the standard laboratory conditions, in vitro investigations may provide a false perspective. For instance, on the basis of the results obtained under 20% oxygen conditions, it could be interpreted that temozolomide and TCA drugs (imipramine and amitriptyline) induce a robust downregulation of GSC marker expression. Specifically, after exposure to these drugs, only Ki67 expression was detected in the GSC population (T98G). If this was the case in clinical practice, chemoresistance should not take place, taking into account the suggested role of GSCs in multidrug resistance.

We also found that GBM cells not unexposed to drug and maintained in 1% oxygen presented a wide range of markers—CD44, Ki67, Nestin, Sox1, and Sox2. The expression of these markers was altered in response to increased oxygen concentration and following exposure to temozolomide or antidepressant drugs.

These studies emphasise that antidepressants, expressly imipramine and amitriptyline not only supported the elimination of glioma cells (T98G, U87) but also stimulated

viability of normal human astrocytes (by approximately 15–23%, respectively, in all oxygen conditions). In contrast, temozolomide inhibited the viability of astrocytes by almost 30% in the hypoxia-reoxygenation model and the standard laboratory conditions. This lack of specificity from temozolomide is a common problem observed in GMB patients, resulting in a number of side effect [45]. We anticipate that these investigational findings will aid in selecting the proper adjuvant drug for this patient cohort.

#### Compliance with ethical standards

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**Conflict of interest** All authors declare no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** No human participants were used in this study.

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