# Two faces of Amitriptyline in an *in vitro* study on C6 glioma cells: The effects of Amitriptyline and its combination with Temozolomide and radiation

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**Abstract.** Failures in treating glioma have made it necessary to search for new therapies, especially those that have immunological properties. Amitriptyline (AMI) is not only used in the treatment of major depression but is also effective in the therapy for neuropathic and chronic cancer pain. Previous data have indicated anticancer and anti-inflammatory effects of AMI. However, at present, its effect on glioma cells in combination with the standard treatment, namely temozolomide (TEMO, the first-line cytostatic) and radiotherapy, has not been investigated. It would also be interesting to determine whether the mechanism of the immunomodulatory action of AMI is associated with its effect on the programmed death ligand-1 (PD-L1) expression, a key target for anticancer therapies. In the present study, the effect of AMI or its concomitant use with AMI and TEMO and/or radiation (10 Gy) on the viability, mortality (MTT, Trypan blue), proliferation (BrdU), colony forming (microscopic analysis) of C6 glioma cells and PD-L1 expression (enzyme immunoassay) was investigated. Although AMI induced the anticancer effects, it attenuated the effects of radiation. In radiated cell cultures, the combination of AMI and TEMO provoked the formation of larger glioma cell colonies and reversed the cellular effects of radiation. Moreover, AMI suppressed the expression of PD-L1 in cells that had been exposed to or had not been exposed to radiation, whereas radiation enhanced its expression. Because AMI exhibited promising anticancer properties including an interesting, previously unknown immunomodulatory effect, it appears that its potential therapeutic should be verified in an *in vivo* study.

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*Abbreviations:* AMI, amitriptyline; BrdU, bromodeoxyuridine; GSCs, glioma stem cells; IL, interleukin; TEMO, temozolomide

*Key words:* supportive therapy in glioma, immunomodulatory/anticancer effects of AMI, drug repositioning in glioma

## Introduction

Gliomas (grade IV) are the most malignant tumors and still remain a lethal brain cancer with a short overall survival (OS) time. Standard treatments, including surgical resection, chemotherapy with temozolomide (TEMO), radiotherapy, and new therapeutic strategies that appeared to be promising did not prolong patients' lives nor did they reduce cancer recurrence rates. Genetic, epigenetic, environmental heterogeneity and cell plasticity, which are specific to each patient, are responsible for the resistance to treatment of gliomas (1). Therefore, there is a great effort to propose new therapeutic lines or to design new drugs for glioma therapy. Immunotherapeutic strategies are emerging as a promising avenue that might offer new hope for the treatment of gliomas.

Research that has been conducted in recent years has shed a light on a new direction related to an attractive strategy called ‘drug repurposing’, which includes broadening the clinical indications of some drugs to use in therapy for other diseases (2,3). Among the current candidates for glioma adjuvant therapy are antidepressants, which have been used in other clinical treatments for numerous years. Their potential anticancer properties, which are beyond their antidepressant effect, have been revealed. Their well-understood safety profile and cellular effects, including as an anti-inflammatory/immunomodulatory for glial cells, antioxidant activity, influence on neural plasticity and transcriptional factors, receptor action, and modulatory effect on trophic factors were assessed. Additionally, the possibility of them reaching a high concentration in the brain due to their ability to cross through the blood brain barrier, which is occasionally impossible for

typical anticancer drugs, renders antidepressants an attractive tool in the fight against brain cancer (3-7).

Furthermore, depression is a common psychological disorder among patients with glioma (8); therefore, finding antidepressants with well-documented antitumor effects would be a promising adjuvant strategy for treating glioma. However, this issue requires further investigation because the results of the experimental studies that have been conducted in various models are contradictory. Some results have also suggested that antidepressants may also promote cancer progression (9).

Amitriptyline (AMI), a tricyclic antidepressant, appears to be particularly interesting due to its high neurobiological activity and effectiveness in therapy not only for depression but also for chronic pain and neuropathic pain in oncology patients (10). However, at present, it cannot be clearly stated whether AMI inhibits or supports the progress of cancer. The majority of preclinical *in vitro* studies have been focused on the effects of AMI alone on glioma cells, whereas, in clinics, patients with glioma are subjected to both polypharmacotherapy and integrated treatment. Therefore, experimental models should reflect not only the hypoxia that is typical for the glioma environment, but the impact of any interactions between the therapeutic strategies on glioma cells should also be studied.

Gliomas are immunologically 'cold' brain cancers that activate various mechanisms in order to escape from immune surveillance (11). The pathway that is mediated by programmed cell death ligand 1 (PD-L1) and programmed death receptor 1 (PD-1) includes the major negative regulatory molecules at the immune checkpoint axis and the main players in immunotherapy (12). PD-L1 is also a marker of the activation of the natural killer cells that infiltrate head and neck cancers (13). Its expression is found in 85% of newly diagnosed and 73% of patients with recurrent glioma. Moreover, it has also been shown that PD-L1 is even more overexpressed in certain patients with glioma compared with the others (14). PD-L1 acts like a pro-cancer factor, stimulates cellular proliferation, and later tumor progression. Its overexpression makes the prognosis worse, and as a result, correlates with a short OS time (15). Therefore, inhibiting the PD-L1 pathway could have significant therapeutic value and has been hailed as a promising therapeutic strategy to activate antitumor immunity.

In the present study, the influence of AMI alone or its co-administration with TEMO (the first-line cytostatic in therapy of glioma) on rat C6 glioma cells that had or had not been exposed to radiation for the first time were investigated. The viability and proliferation of cells and PD-L1 expression were assessed. Moreover, morphological changes in the cell cultures were determined using microscopic analyses.

## Materials and methods

*Model in vitro*. Experiments have been conducted on a C6 glioma cell line that is considered to be a safe and popular glioma model in the literature providing a favorable simulation of glioblastoma multiforme in human. C6 is a glial cell line that was isolated from the brain of a rat with glioma. The glial tumor was induced by N-nitrosomethyl urea after a series of alternate culture and animal passages. Genetically, C6 cells overexpress the same genes that are expressed in human gliomas. This cell line can be used in neuroscience and toxicology research.

C6 rat glioma cells (cat. no. 92090409; MilliporeSigma) were cultured in DMEM with 10% FBS (cat. no. SLM-241) as a monolayer in cell culture bottles and hypoxia conditions (2.5% oxygen, 5% $CO_2$; Hypoxylab, AnimaLab). After achieving 90% confluence, glioma cells were trypsinized and passaged. On the second day, the medium was replenished with a fresh portion containing AMI [Amitriptyline hydrochloride, powder, ≥98% (TLC; cat. no. A8404-10G; MilliporeSigma); 10 $\mu$M and/or TEMO (99% HPLC, powder, cat. no. T2577-25 mg; 1 mM MilliporeSigma. The cells were exposed to the studied drugs for 72 h. In order to compare the effect of radiation and its interaction with the studied drugs, C6 glioma cells were exposed to radiation [single exposure, 10 Gy; Glioma (rad)] or were not exposed (glioma (non-rad). Next, functional tests (viability, mortality, cell proliferation) and microscopy observations of glioma cells (morphology and colony forming) were conducted. Finally, the PD-L1 expression was determined in each experimental group.

*Radiation*. The samples were irradiated with 6 MV energy and a dose rate of 600 on a Clinac 600 CD accelerator from Varian Medical Systems. The Clinac accelerator is a type of linear accelerator used in radiotherapy to treat lesions, both cancer and non-cancerous conditions, by delivering precise doses of ionizing radiation directly to the tumor or affected area. These devices utilize high-energy X-rays, and the Clinac 600 CD is equipped with a multileaf collimator to shape the radiation beam, allowing for more precise targeting and minimizing the radiation dose to healthy tissue. All of the measurements were taken in triplicate.

*Viability/mortality tests*. To estimate the percentage of live and dead glioma cells in each experimental group, an MTT test [as previously described (16)] and an analysis using Trypan blue dye in EVE (Cell automated counter; NanoEntek) were performed. Each test for each experimental group was conducted three times. The average results are presented in Fig. 1A and B.

*Cell proliferation*. The intensity of cell division was evaluated using an immunoenzymatic assay kit based on a determination of the bromodeoxyuridine (BrdU) level (Abcam), which is incorporated into the DNA of dividing cells (cell density was $3x10^4$ cells/ml). The determination was conducted according to the manufacturer's instructions. Absorbance was measured in a Multiscan RC microplate reader at 450 nm (Thermo Labsystems). (Fig. 1C). All of the measurements were received in triplicate.

*PD-L1 expression*. The expression of PD-L1 was measured using a commercially available immunoenzymatic assay (cat. no. E1629 Ra; ACRO Biosystems) according to the manufacturer's protocol. The optical density (OD) values were measured using an automated microplate reader (Multiscan RC microplate reader (Labsystems) with a wavelength of 450 nm as the reference wavelength. All of the measurements were received in triplicate.

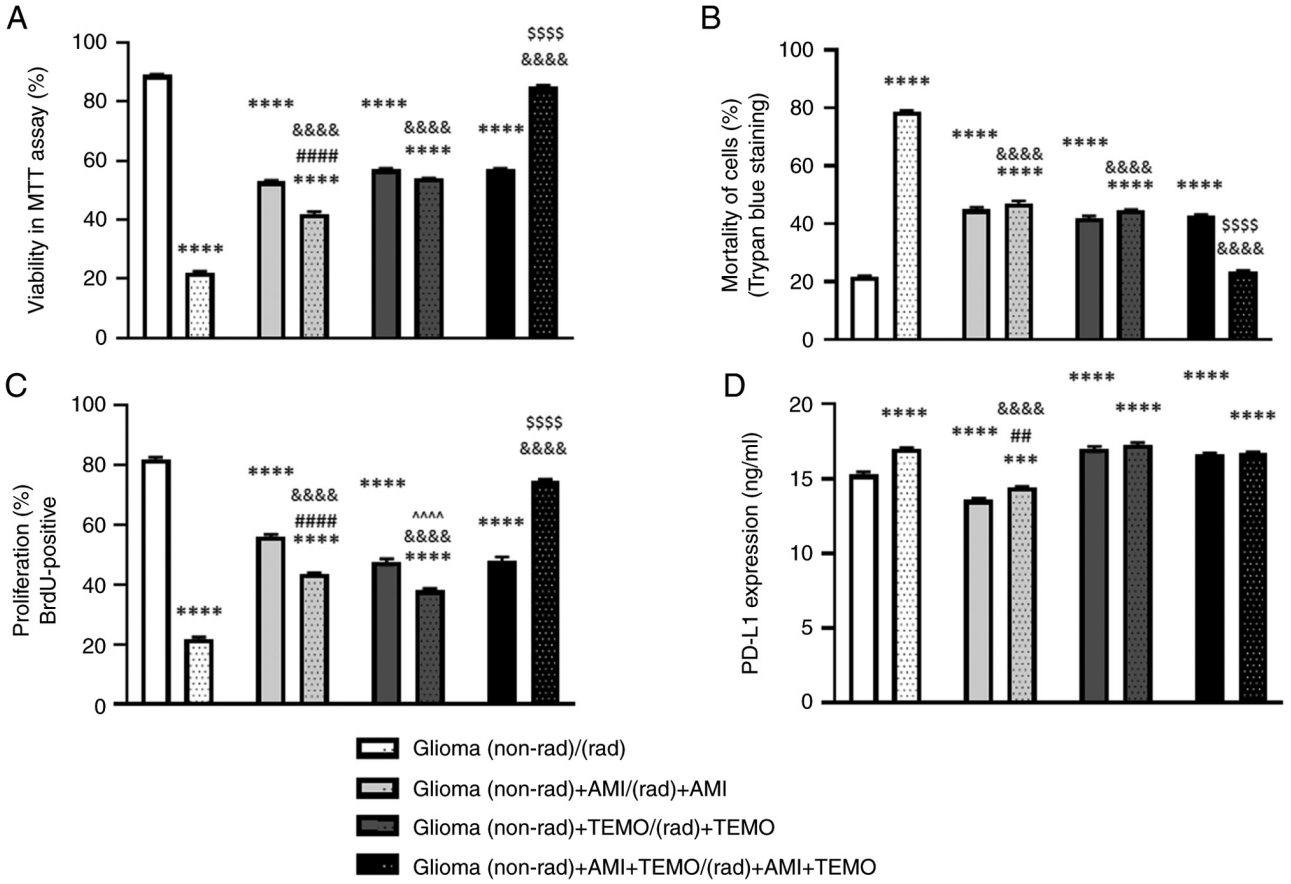


Figure 1. Effects of AMI (10 $\mu$M) and /or TEMO (1 mM) (or radiation) administration into culture medium on (A) viability of cells - MTT assay (%); (B) mortality of the cells analyzed in EVE cell counter (%); (C) proliferation of the cells (BrdU positive) (%), (D) PD-L1 expression (ng/ml). Experiments were conducted on C6 rat glioma cells. Data are presented as mean ± SEM from three independent experiments (n=9 per group). The results were analyzed using two-way ANOVA followed by post hoc Bonferroni's test. $^{***}$P<0.001 vs. Glioma (non-rad); $^{****}$P<0.0001 vs. Glioma (non-rad); $^{\&\&\&\&}$P<0.0001 vs. Glioma (rad); $^{\#\#}$P<0.01 and $^{\#\#\#\#}$P<0.0001 vs. Glioma (non-rad) + AMI; $^{\wedge\wedge\wedge\wedge}$P<0.0001 vs. Glioma (non-rad) + AMI, $^{\$\$\$\$}$P<0.0001 vs. Glioma (non-rad) + AMI + TEMO. AMI, amitriptyline; TEMO, temozolomide.

*Microscopy observations*. Daily observations of the growth dynamics, morphology and dispersion of glioma cells of C6 lineage that had been exposed to AMI and/or TEMO and /or radiation were performed using a JuLi Stage (NanoEntek) microscope. The image analysis was performed using a camera software (Image software NIS Element), which is an integrated part of an Eclipse TS-100 microscope and ImageJ software (Fiji version, 10.1038/nmeth.2019). Colony formation assessment (minimum number of cells forming a colony was 50) was performed using crystal violet staining (in concentration 0.25% in 25% methanol, at room temperature for 20 min).Each experimental group was analyzed in 10 fields of view. Representative pictures are presented in Fig. 2.

*Statistical analysis*. The normality of the distribution of the data was assessed using the Shapiro-Wilk test ($\alpha$=0.05). The homogeneity of the variance was calculated using the Levene's test with the $\eta$ 2 effect type ($\alpha$=0.05). If the obligatory assumption was established, the significance of the differences between the groups was evaluated using a two-way ANOVA (factor 1: radiation; factor 2: the studied drugs) followed by a post hoc Bonferroni's test. The data are expressed as the mean ± SEM. P<0.05 was considered to indicate a statistical significance. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc.; Dotmatics).

## Results

*Viability of the cells (%) that were analyzed in the MTT assay (mitochondrial activity)*. The alterations in the viability of the cells (%) are presented in Fig. 1A. The results of the two-way ANOVA revealed a significant interaction between the two analyzed factors (factor 1: radiation; factor 2: studied drugs). Differences between the groups of cells that had been treated with the drugs and the untreated cells were strongly significant ($F_{(3,64)}$=892.9; P<0.0001). The same statistical pattern was observed between the groups that had been exposed to radiation or those that had not been exposed ($F_{(1,64)}$=1621.0; P<0.0001). Drug administration (AMI, TEMO and combined AMI + TEMO) significantly reduced the viability of the cells in the cultures that had not been exposed to radiation [decrease of ~36, 32 and 32%, respectively, vs. Glioma (non-rad) P<0.0001; post hoc Bonferroni]. The radiation was stronger than the drugs and decreased the viability of cells (~67%, P<0.0001; post hoc Bonferroni). The AMI effect [a decrease in the viability of the cells by ~36% vs. Glioma (non-rad)] was potentiated in the radiated cells [decrease ~11% vs. Glioma (non-rad) + AMI,

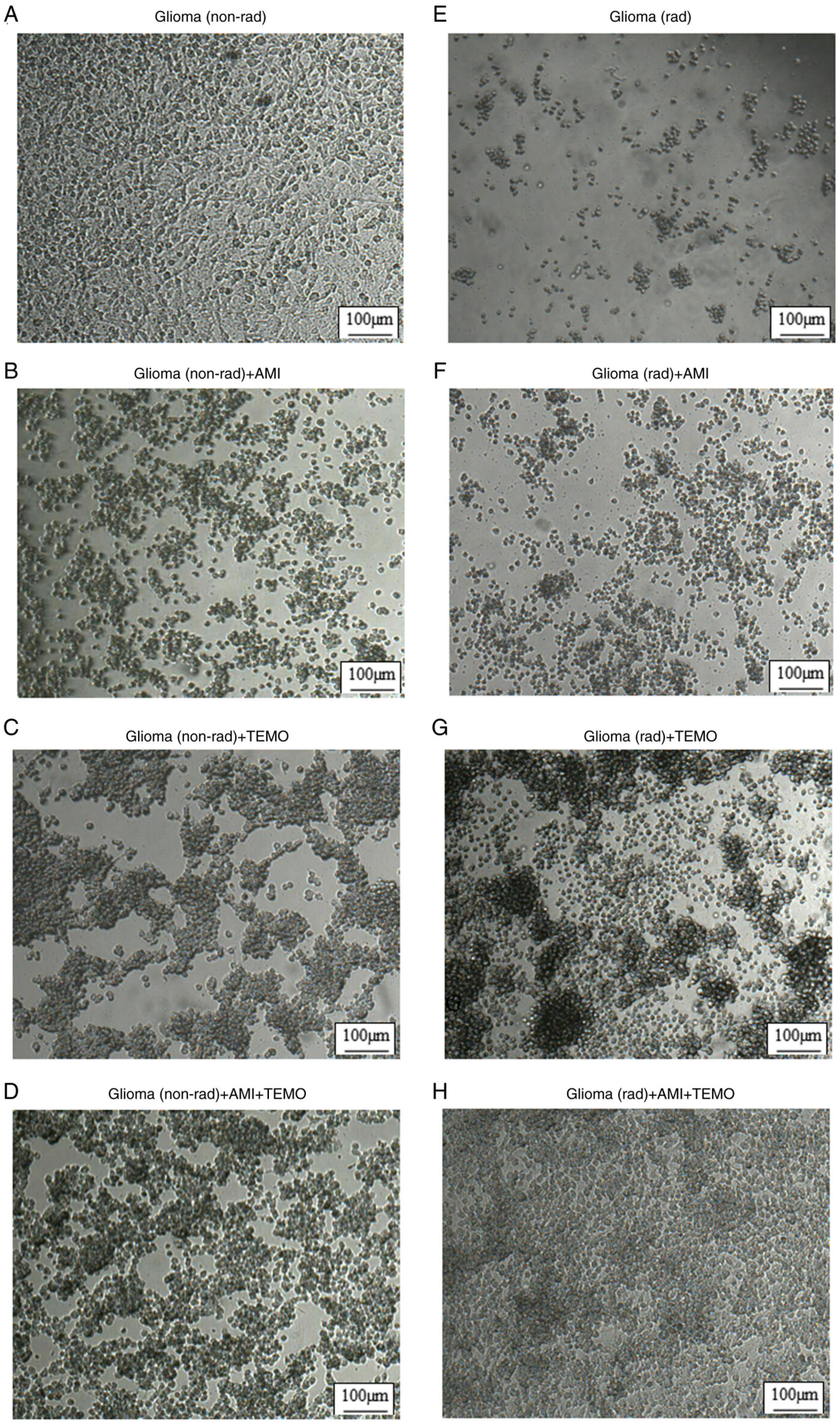


Figure 2. Representative pictures C6 glioma cells: (A) untreated; (B) exposed to 72 h of amitriptyline (10 $\mu$M); (C) TEMO; (D) AMI + TEMO combination; (E-H) or radiation. Observations were performed using JuliStage (NanoEntek) in a bright field (9 views for each group; magnification, x40).

P<0.0001, post hoc Bonferroni)]. Radiation did not change the TEMO effect. By contrast, treatment with AMI or TEMO attenuated the effect of radiation vs. Glioma (rad), P<0.0001; post hoc Bonferroni) and the co-administration of AMI + TEMO even reversed its effect. In the radiated cultures that had been treated with AMI and TEMO, the viability of the cells was comparable to the viability of Glioma (non-rad) cells.

*Mortality of the cells (%) analyzed in EVE cell counter*. The alterations in the mortality of the cells (%) are presented in Fig. 1B. The results of the two-way ANOVA revealed a significant interaction between the two analyzed factors (factor 1: radiation; factor 2: Studied drugs). The differences between the untreated and treated cell cultures were significant ($F_{(3,64)}$=311.6, P<0.0001). The impact of radiation was also relevant ($F_{(1,64)}$=677.4, P<0.0001). Drug administration (AMI, TEMO and combined AMI + TEMO) caused a significantly higher mortality of the cells than was observed in the untreated cell cultures [increase by ~23, 20 and 21%, respectively, vs. Glioma (non-rad) P<0.0001; post hoc Bonferroni]. The mortality of the cells (%) was definitely higher in the cultures that had been exposed only to radiation [increase by ~57% vs. Glioma (non-rad), P<0.0001; post hoc Bonferroni]. Treatment with AMI or TEMO attenuated the radiation effect vs. Glioma (rad) (P<0.0001; post hoc Bonferroni) and the combined treatment with both drugs, AMI + TEMO, caused the most remarkable effect. In this group, the mortality of the cells was similar as in Glioma (non-rad).

*Proliferation of cells (BrdU positive %)*. The differences in the proliferation level (%) are presented in Fig. 1C. The results of the two-way ANOVA indicated a significant interaction between the two analyzed factors (factor 1: radiation; factor 2: Studied drugs). The influence of drug administration was strongly significant ($F_{(3,64)}$=187.1; P<0.0001). The same statistical pattern was observed between the groups that had been exposed to or had not been exposed to radiation ($F_{(1,64)}$=619.3; P<0.0001). Treatment with AMI, TEMO and the co-treatment with AMI + TEMO reduced the proliferation of cells (decrease of ~26, 34 and 34%, respectively). The effect of radiation was stronger (decrease of ~60% vs. Glioma (non-rad); P<0.0001, post hoc Bonferroni). Moreover, radiation enhanced the effect of AMI or TEMO. Each drug reduced the proliferation of cells that had been exposed to radiation more than the proliferation of the cells that had not been exposed to radiation [by ~12% vs. Glioma (non-rad) + AMI; by ~10% vs. Glioma (non-rad) + TEMO; P<0.0001; post hoc Bonferroni)]. However, when used together, both drugs attenuated the effect of radiation vs. Glioma (rad; P<0.0001; post hoc Bonferroni). In the cultures that had been exposed to radiation and AMI+TEMO, an increased proliferation of cells was even observed [by ~35% vs. Glioma (non-rad) + AMI + TEMO; P<0.0001; post hoc Bonferroni].

*PDL-1 expression*. The alterations in the PDL-1 expression (ng/ml) are presented in Fig. 1D. The results of the two-way ANOVA revealed a significant interaction between the two analyzed factors (factor 1: radiation; factor 2: Studied drugs). There were significant effects of the treatment ($F_{(3,64)}$=213.1; P<0.0001) and radiation ($F_{(1,64)}$=60.94, P<0.0001). The cells that had not been exposed to radiation and that had been exposed to AMI demonstrated a decrease in the PDL-1 expression (~12%; P<0.0001; post hoc Bonferroni), but treatment with TEMO or co-treatment with AMI + TEMO caused an increase in the PDL-1 expression [~11 and 9%, respectively, vs. Glioma (non-rad); P<0.0001; post hoc Bonferroni]. The radiation also increased the PDL-1 expression (by ~11%; P<0.0001; post hoc Bonferroni). In the radiated cultures that had been exposed to AMI, the PDL-1 expression was still lower than in the non-radiated glioma cultures (~6%; P<0.01; post hoc Bonferroni) and were also lower than in the radiated cell cultures (by 11%; P<0.0001; post hoc Bonferroni). Radiation did not change the effect of TEMO and the co-treatment with AMI + TEMO.

*Microscopy observations*. The influence of one of the following factors: AMI, TEMO, or radiation and their interactions on the C6 glioma cells were not only assessed in the cell analysis but the morphological changes in the cell cultures were also evaluated during a microscopic analysis (the representative pictures of the non-radiated cultures are presented in Fig. 2A-D and the radiated cultures in Fig. 2F-H. In the control (non-radiated and untreated) group, the C6 cells created a dense network with tight connections (full confluence) (A). Exposure to radiation at a single dose of 10 Gy induced a strong mortality after 72 h (E). AMI caused a stronger cytotoxic effect on C6 glioma cells (B) than TEMO (C) and AMI + TEMO (D). TEMO (C) and AMI + TEMO (D) had a more intense formation of larger colonies than AMI alone (B). The radiated cultures that had been exposed to AMI (F) were characterized by a lower number of live cells than the non-radiated cultures. By contrast, the application of TEMO (G) or AMI + TEMO (H) did not induce such an effect. These cultures exhibited a full confluence. TEMO (G) and AMI + TEMO (H) promoted the formation of larger colonies. The microscopic analysis for each group was performed in a bright field in 9 views using JuliStage (NanoEntek; magnification, x40).

## Discussion

Neuropathic pain and other complications, which are a consequence surgical resection, radiotherapy, tumor presence, or its recurrence, are a common problem in patients with cancer including glioma (17). Several drugs are used to relieve neuropathic pain, namely, some opioid analgesics, anticonvulsant drugs as well as some antidepressants, especially tricyclic antidepressants (AMI, nortriptyline; a metabolite of AMI and clomipramine) are recommended (18). It is known that AMI induces an analgesic effect quickly by modulating neuroinflammatory signaling and later produces an antidepressant effect. There are also data concerning its anticancer effects (19). Since not only neurons but also the astroglia cells that built a tumor mass are able to take up amitriptyline, the role of AMI as a potential adjuvant in glioma chemoradiotherapy was the research problem analyzed for this article.

In the present study, in order to mimic the polytherapy that is used in patients with glioma, AMI (a potential adjuvant drug) was administered in combination with TEMO and radiation, which to some extent imitates modern stereotactic radiotherapy. The effects of the drugs on glioma cells were

assessed after a single dose was administered into a culture medium for 72 h, which was when a full culture confluence that mimicked maximal tumor growth was achieved. The short duration of the experiments was due to the rapid cytotoxic effect that was observed. In the pilot study, it was observed that a single exposure of C6 glioma cells to radiation at 10 Gy for 72 h induced the death of 80% of the cells in the culture.

*AMI +/- radio effects*. AMI was used at a concentration of 10 $\mu$M because this level can be achieved in the brain during treatment. In the brain, the level of AMI is 13-16-fold higher than in serum (20), where it reaches a concentration from 0.15 to 0.7 $\mu$M (21,22). It was found that AMI induced significant anticancer effects on C6 glioma cells that had been exposed to or had not been exposed to radiation. AMI alone inhibited the viability, enhanced the mortality of glioma cells and also induced an antiproliferative effect. Moreover, its influence on the viability and proliferation of glioma cells was augmented by radiation however, it was weaker than the results of radiation alone.

The molecular mechanism of the interaction between AMI and radiation in our experimental model is undoubtedly complex. The observed differences in the response to AMI in the cultures that had or had not been exposed to radiation might be partially explained through the different time points of the DNA damage (23) or probably the influence of radiation and AMI on the cytokine network, especially IL-6. As was shown in another in vitro study (24), IL-6 is released spontaneously by glioma cells and stimulates an invasive tumor phenotype (high proliferation, viability of cells, migration). Moreover, radiation initiates the biological damage that is mediated by free radicals and acts on cytokine production at the transcriptional or translational level (25) in the acute phase of the reaction. In some *in vivo* and clinical studies, dose-and time-dependent effects of AMI on the pro-inflammatory cytokines were observed. AMI decreased the release of IL-6 in an acute and chronic model of inflammation (mouse sepsis model, inflammation reaction to the implant in mice, carrageenan-induced paw edema model, in animals with neuropathic pain, or in patients with major depression) (26-29). It can be assumed that decrease of IL-6 production might have significance for the reduced viability and proliferation of glioma cells that have been exposed to AMI and radiation compared with the AMI effect alone that was observed in the present experiments. This suggestion may be supported by observations by other authors that when AMI is used at concentrations between 33-100 $\mu$M, it induces an inhibitory effect on the III cancer respiratory chain while at lower doses, it induces a radioprotective effect in normal hippocampal cells (30). Although numerous promising agents are currently being developed to ameliorate the negative effects of radiotherapy, the range of doses at which AMI might interfere with the radioprotective mechanisms has not been assessed yet.

The unexpected lack of a synergistic effect with radiation and the stronger effect alone of imipramine (tricyclic antidepressant drug) was also observed by Royds *et al* (31) on prostate cancer cells. This effect was associated with blocking the voltage-gated potassium channels (EAG1), and as a result, increased lipid peroxidation, oxidative stress, and markedly altered cell integrity and lipid membrane permeability.

The present study revealed that AMI significantly reduced the expression of protein PD-L1 in glioma C6 cells for the first time, which expands knowledge about the complex mechanisms of action of this antidepressant including its anti-inflammatory action. Similar data were observed in an ovarian cancer model when a decrease in PDL-1 by AMI was associated with inhibiting serotonin, TGM2 (transglutaminase 2), and KYN/Indole signaling, which in effect can help overcome resistance and make immunotherapy more effective (32,33).

A decrease in the PD-L1 expression was observed in all of the cultures that had been exposed to or had not been exposed to radiation, but it was weaker in the radiated cultures. Immune checkpoints (PD-1/PD-L1) and inflammasomes, which are characterized by an enhanced production of pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF-α), are important for the development of pyroptosis in cancer cells (34). That is why these cytokines also appear to be an attractive additional target for future anticancer therapy. Unfortunately, in clinical studies, the inhibition of only one factor, for example, only PDL-1 or epidermal growth factor receptor has not yet produced the expected results in glioma treatment (35). AMI, in addition to its influence on the PDL-1 expression, induces pleiotropic effects. The anti-inflammatory properties of AMI have been proven in several studies. In the authors' previous *in vitro* experiments, it was shown that AMI inhibited the expression of subunit p65 of the NF-kappa B transcriptional factor, the secretion of lactic acid into the medium, integrated into the reactive oxygen species system, reduced the volume of swollen mitochondria, and silenced the expression of the markers that are characteristic for cancer cells in glioma (36). Moreover, in a previous study on LPS-stimulated primary mixed glial and microglial cultures, it was demonstrated that AMI and its metabolite nortriptyline inhibited the release of the proinflammatory cytokines, TNF-α and IL-1β (37).

The reported decrease in the PD-L1 expression in the cells treated with AMI may be important for its anti-glioma effects because other results have shown that glioma cells secrete PD-L1 into the peritumoral areas, particularly microglia, which contain highly expressed PD-1 (15). PD-L1 stimulates the PD-L1 receptors on microglial cells, which then promotes tumor growth and invasion. Recently, researchers have focused their attention on microglia as a potential and promising target for the future immunotherapy of glioma. Microglia, which are resident brain macrophages, play a key role in mediating the local immune response. Specific microglial phenotypes (M1, M2), which are characterized by their pro-or anti-inflammatory molecule expression, have different functions (38) and may promote or inhibit tumor growth. Although microglia cells determine the recruitment of immunological cells into the tumor microenvironment (TME), there are currently no methods for glioma therapy that target macrophage polarization. Therefore, AMI may be considered as a potential adjuvant drug in glioma therapy because of its anti-inflammatory impact on glia as well as some peripheral immunomodulatory properties. AMI suppresses interferon-γ production by the $Th_1$ cells, reduces T cell proliferation, and also has an influence on the T cell phenotype by reducing the frequency of the occurrence of $CD8^+CD45RA$, $CD27^+CD4^+$, and $CD27^+CD8^+$ in peripheral blood mononuclear cells (39).

Although the use of PD-1/PD-L1 inhibitors (Nivolumab, Pembolizumab, Cemiplimab, Atezolizumab, Avelumab and Durvalumab) have improved therapy in numerous types of cancer (lung, melanoma and prostate cancer) (38), no therapeutic benefits have been found in glioma. Despite the high expectations, in a large, randomized study III phase Checkmate 143 (NCT02617589), Nivolumab did not exhibit any significant benefits in glioma therapy (40). The available literature presents different, and occasionally, conflicting effects of the interactions between the PD-L1 inhibitors and the drugs that are used in the conventional therapy. Wang *et al* (41) demonstrated that the concomitant use of TEMO and a PD-L1 inhibitor strongly reduced tumor growth and improved the survival rate in a mouse model of glioma; however, in patients with a recurrent tumor, this therapy did not have such effects.

*TEMO +/- radio effects*. In the present study, TEMO (1 mM) affected the viability and proliferation of glioma cells that were similar to AMI but increased the expression of PD-L1 at a rate that was similar to radiation alone. Other *in vitro* studies also reported that TEMO induced the PD-L1 overexpression and researchers have suggested that the mechanism of this effect can be mediated by signal transducer and activator of transcription 3 signalization. The increase in the PD-L1 expression induced by radiation is in line with the observations of Xing *et al* (42), who proved that radiotherapy increased the PD-L1 expression that was detected in glioma specimens from 64 patients. Radiotherapy appears to increase the MHC1-mediated antigen presentation and tumor lymphocyte infiltration. These radiation effects appear to be partially modulated by an increased expression of interferon-Y19 (43). It has been hypothesized that in response to the aforementioned radiation effects, the expression of PD-L1 is higher in tumor cells in order to evade immune detection. Furthermore, an acquired glioma resistance to the PD-1 inhibitors can occur via mutations of the apelin receptor 20 and interferon-receptor-associated Janus kinase 1 (JAK1) 21 both of which lead to a decreased expression of interferon-Y as well as tumor infiltration by immune cells (44).

*AMI + TEMO +/- radio effects*. Surprisingly, the concomitant use of rad + AMI + TEMO has not shown synergistic anticancer effect, which was also confirmed by the restoration of the full confluence or colony formation that was observed during microscopic analysis. The radiated cell cultures that had been treated with both drugs were characterized by a significantly increased percentage of living cells, cell division, and a reduced mortality of cells compared with the radiated non-treated cultures, which means that concomitant use of AMI + TEMO reversed the effect of radiation alone. Although the molecular mechanism of this synergistic adverse effect requires further investigation, it may be hypothesized that GSCs could play an important role; on the one hand generated in response to TEMO/radiotherapy and on the other had initiate resistance to therapy (45). Moreover, it has been proven that in *in vitro* conditions, TEMO increased the HMGB1 expression that also promotes GSC phenotype (46). The measurement of the mitochondrial activity (MTT test) in the present experiments found that the sensitivity of this parameter to the studied combinations may also indicate the engagement of mitochondria in the mechanism of AMI action and the effects that were observed as a result of concomitant use of the AMI + TEMO interactions. The modulation of mitochondrial activity by AMI has already been described (47). These effects may at least partially explain the formation of larger cellular colonies in the radiated cultures treated with TEMO and, after they had been treated with the concomitant use of AMI + TEMO.

Radiation triggers inter alia the depolarization of the mitochondrial membrane, induces the release of cytochrome c into the cytosol in response to apoptosis stimulus, and causes oxidative stress (37). However, a too low dose of radiation (single dose 10 Gy) could also escalate the repair processes and accelerate the repopulation despite a temporary therapeutic effect manifested by high mortality of C6 glioma cells, which in consequence could reverse the effect concomitant use of AMI + TEMO (48). Moreover, exposure to a single dose of radiation induces different effects than exposure to multiple doses. Tzadok *et al* (49) reported a dose-dependent inhibition of the viability of astrocytoma U87 cells as a result of using antidepressants (fluoxetine or sertraline) in increasing doses combined with radiation. However, the inclusion of TEMO into the studied combinations, namely, radiation + TEMO + antidepressant, did not intensify this inhibition. This unexpected drug interaction may be associated with a specificity of C6 glioma cell line in which cytochrome P450 activation has been discovered, which potentially alters drugs level, their effectiveness, and could lead to drug antagonism (50). Similarly, in another study, the concomitant use of fluoxetine + imatinib induced a stronger anticancer effect than their combination with radiation (51).

The last issue that could have affected the anticancer effect of the studied drug combinations is the sequence in which they were used. In clinical practice, in patients with glioma, after surgical resection, the cytostatic drug TEMO is used first followed by radiation (TEMO plays the role of a sensitizer on radiation) (52). In experimental *in vitro* models, following this order is not always possible within one passage.

The aforementioned data indicate that further research using other glioma cell lines and *in vivo* models are undoubtedly required to properly explain the mechanisms of the important interactions between radiation and potential anticancer drugs.

Finally, the lack of synergism between AMI + TEMO + radio can be explained by the fact that AMI is a modulator of autophagy acting as 'Trojan horse'. Depending on context, AMI in C6 cells can be an inducer or inhibitor of autophagy - the process that protects cells against oxidative and genotoxic stressors (in the initial stages, but in later phases it may contribute to cell death) (53). Other studies described that AMI may inhibit the efficacy of TEMO and radiotherapy, particularly in gliomas, by interfering with cell death pathways, potentially inducing autophagy, blocking proapoptotic signals (such as TRAIL-dependent apoptosis), and affecting DNA repair or cell cycle regulation. Although it may also exhibit complex, occasionally synergistic, effects depending on the context and cell type, often related to its effects on calcium, ceramide, and various receptors, ultimately complicating the efficacy of standard treatment (54,55).

Moreover, in C6 glioma cells, AMI may also have a direct 'postsynaptic' effect, increasing the availability of signaling molecules such as Gsα, potentially modulating cell survival

pathways. Indeed, AMI's effects on C6 cells span a broad network of non-dopaminergic receptors and intracellular pathways, creating unique drug interaction profiles that are currently being investigated for cancer therapy (56).

In conclusion, AMI is a drug that has a high biological activity and a multi-directional mechanism of action that induces dose- and time-dependent effects. Although this antidepressant has been used for numerous years, its pharmacological properties against cancers, and its position in cancer therapy have not yet been fully recognized.

The presented results of an *in vitro* study support other studies concerning the role of antidepressants as active players in the TME. The present study highlights some promising anticancer effects in C6 glioma cells cultures that were induced by AMI alone. The results suggest that the position of AMI in glioma therapy may be important especially for its novel mechanism, which is associated with decreasing expression of PDL-immune checkpoints. In the clinic, this immunomodulatory action might help overcome resistance, enhance immunotherapy, improve OS in patients with glioma, or open the door to new combination strategies (57,58). However, to achieve this effect, AMI should be probably used with TEMO and radiation in time intervals, which will require further analyses. At the same time, the current study presents a second 'face' and type of action of AMI on C6 glioma that depends on its being used alone or concomitantly with TEMO or radiation.

Although anticancer effects of AMI are interesting, there are certain limitations to the present study. An *in vitro* model does not reflect the immunological, hormonal and cross-talk interactions between a tumor and its niche, which is why it is difficult to uncritically extrapolate the obtained results to clinical practice. In *in vitro* experiments, only the direct effects of the tested drugs on cell cultures were examined but not the effects of their active metabolites. However, this does not change the fact that AMI appears to be a favorable candidate for drug repositioning strategy in further research of glioma. Finally, an estimation the cytotoxic/antiproliferative properties of AMI, its modulatory effect on the PD-L1 expression, and its influence on colonies should be verified in *in vivo* studies in order to precisely estimate its potential as an adjuvant therapy.

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**Availability of data and materials**

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Authors' contributions**

AMBW and EO conceptualized and supervised the study, prepared and revised the manuscript. AMBW performed the cell cultures. WM provided substantive support in the analysis of results. KS operated the radiotherapy machine and assisted during the radiation process. TC assisted in preparing the C6 cells for radiation and enabling cell irradiation. MG performed statistical analysis and the graphical presentation of the results. All authors read and approved the final version of the manuscript. All authors confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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