Perphenazine and Prochlorperazine Decrease Glioblastoma U87-MG Cells Migration and Invasion: Analysis of the ABCB1 and ABCG2 Transporters, E-Cadherin, α-Tubulin, and Integrins (α3, α5, and β1) Levels.

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Research Article

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Abstract

Purpose**:** Glioblastoma multiforme is the most frequent malignant brain tumor as well as one of the most lethal and untreatable human tumors with a very poor survival rate. Thus, novel and effective strategies of treatment are required. Integrins play a crucial role in the regulation of cellular adhesion and invasion. Moreover, integrins and alpha-tubulin are very important in cell migration, while E-cadherin plays the main role in tumor metastasis. Their ability to penetrate the BBB and signs of intracerebral activity are very important in glioblastoma therapy. ABC transporters ABCB1 and ABCG2, which are localized in the brain endothelial capillaries of BBB, play a crucial role in the development of multidrug resistance, and are modulated by phenothiazine derivatives.

Methods: The impact on the motility of human glioblastoma U87-MG was evaluated with a wound healing assay; cellular migration, and invasion by the transwell assay, while ABCB1, ABCG2, E-cadherin, α-tubulin, and integrins content was determined with the Western blot.

Results: This study explores the effect of perphenazine and prochlorperazine on ABCB1, ABCG2, E-cadherin, α-tubulin, and integrins (α3, α5, and β1) amount as well as on migration and invasion ability of human glioblastoma (U87-MG) cells. The results suggest that perphenazine and prochlorperazine modulate multidrug resistance proteins (they decrease ABCB1 and increase ABCG2), E-cadherin, α-tubulin, and integrins amount as well as impair migration and invasion of the U87-MG cell line.

Conclusions: The decrease of migration and invasion ability after phenothiazine derivatives treatment due to the increase of ABCG2 and E-cadherin as well as the decrease of α-tubulin, and integrins amounts can support the hypothesis that perphenazine and prochlorperazine have the anticancer effect on human glioblastoma U87-MG cells.

Introduction

Glioblastoma multiforme (GBM) is the most frequent malignant brain tumor leading to 225,000 deaths per year, which translates into 30% of all central nervous system tumors (CNST), 45% of malignant CNST as well as 80% of primary malignant CNST (Alphandéry et al. 2018). Although the global GBM incidence rate is less than 10 per 100,000 people, the survival rate after diagnosis is only 14-15 months, which makes it a crucial public health issue (Hanif et al. 2017). The primary treatment for glioblastoma is surgery (maximal safe resection) followed by radiotherapy and chemotherapy using temozolomide (TMZ), which increases patient survival up to 18 months (Alphandéry et al. 2018).

During glioblastoma therapy one of the goals is to alter EGFR/PI3K/PTEN/NF1/RAS, TP53/MDM2/MDM4/p14ARF, RB1/CDK4/p16INK4A/CDKN2B, and IDH1/IDH2 pathways to limit the development and growth of the tumor. Drug therapy may also inhibit DNA repair mechanisms, tumor invasion, vascular endothelial growth factor (VEGF), dopamine receptors, epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR)α (Bai et al. 2011, Kast et al. 2014, Wick et al. 2011).

The key role in the regulation of cellular adhesion, migration, and invasion is played by integrins, which as cell surface receptors activate also intracellular signaling proteins (Masoumi et al. 2021, Wick et al. 2011). Moreover, integrins have a role in metastasis and angiogenesis of various tumors, which makes the integrin inhibitors potentially useful in glioblastoma therapy (Wick et al. 2011). The up-regulation of such integrins as α6β4, α5β1, αvβ6, αvβ3, αvβ5, and α7 is related to poor patient prognosis in different tumors, including glioblastoma (Masoumi et al. 2021). Furthermore, α3β1, α5β1, α9β1, and β8 integrins affect migration and/or invasion of glioblastoma cells (Ellert-Miklaszewska et al. 2020, Malric et al. 2017). Alpha-tubulin, by controlling dynamics of focal adhesion for lamellipodial extension after the tubulin acetylation, also influences cellular migration (Lee et al. 2018). E-cadherin epithelial cell adhesion protein has the main role in tumor metastasis (Na et al. 2020) and is a negative regulator of cellular invasion, including glioblastoma. Thus, this study explores the impact of phenothiazine derivatives (perphenazine, prochlorperazine) on migration and invasion of glioblastoma by the analysis of E-cadherin, α-tubulin, and integrins (α3, α5, and β1) level. The delivery of drugs during therapy of intracranial tumors is problematic due to parameters that need to be taken into account, such as intratumor pressure, blood supply to the tumor, the state of blood-brain-barrier (Bai et al. 2011). Drugs used in the treatment of newly diagnosed or recurrent glioblastoma should be effective in the brain-blood-barrier (BBB) penetration or exhibit the signs of intracerebral activity (Wick et al. 2018). Perphenazine and prochlorperazine used in this study penetrate BBB (Bartek and Hondy 2014). The influence of those drugs on the level of ATP-binding cassette drug efflux transporters, i.e. ATP-binding cassette subfamily B member 1 (ABCB1, also called P-gp or MDR1) and ATP-binding cassette subfamily G member 2 (ABCG2, also called BCRP – breast cancer resistance protein), was analyzed. Those transporters are responsible for moving biologically important substrates (amino acids, cholesterol) across the cell membranes, and for impeding the penetration of the blood-brain barrier by many chemotherapeutic agents, actively transporting them back into the bloodstream (Balça-Silva et al. 2018).

Materials And Methods

Cell culture and reagents

The human glioblastoma cells U87-MG were obtained from the Sigma Aldrich (USA) and cultured in DMEM basal medium supplemented with FBS (10%), neomycin (10μg/mL), amphotericin B (0.25μg/mL), and penicillin G (100 U/mL) at 37^{o}C in 5% CO_2. Perphenazine, prochlorperazine dimaleate, bacitracin, elacridar, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), amphotericin B, and penicillin G were purchased from Sigma-Aldrich Inc. (USA). Neomycin sulfate was obtained from Amara (Poland). Trypsin/EDTA 0.25%/0.02% in PBS, Fetal bovine serum (FBS) EU Professional heat-inactivated and growth medium DMEM with 4.5g/L Glucose, L-glutamine, and 3.7g/L NaHCO3 were obtained from PAN Biotech GmbH (Germany). Geltrex LDEV-Free reduced growth factor basement membrane matrix without Phenol Red was obtained from Gibco (USA). Methanol, acetic acid, and crystal violet were obtained from POCH S.A. (Poland). Buffered formalin was obtained from Chempur (Poland).

Western blot analysis of ABCB1 and ABCG2

The ABCB1, ABCG2 protein, and β-actin amounts were determined by Western blotting according to the slightly modified method described earlier (Otręba et al. 2019). The negative control was elacridar (5.0 μM in growth medium with 0.5% DMSO) and it was compared to DMSO control (growth medium with 0.5% DMSO). Cells were treated with various concentrations of perphenazine, prochlorperazine, elacridar, or medium for 24h. Elacridar was used as an inhibitor of the ABCB1 transporter.

After cell lysis in ice-cold Pierce RIPA buffer (Thermo Fischer Scientific, USA) and a Halt Protease Inhibitor (Thermo Fischer Scientific, USA) and protein concentrations analysis by Pierce BCA Protein Assay Kit (Thermo Fischer Scientific, USA) samples were stored at -80°C. Proteins were separated on 6% SDS-PAGE along with color pre-stained protein standard 11-245 kDa (New England BioLabs, USA) and transferred onto nitrocellulose membranes (Thermo Scientific, USA) using a semi-dry Trans-Blot Turbo Transfer System (Bio-Rad., USA). Then the membranes were blocked for 1 hour at room temperature in a blocking buffer.

Proteins were detected by incubation with primary antibodies: MDR1/ABCB1 (E1Y7B) Rabbit mAb, ABCG2 Rabbit Ab, and β-actin Rabbit Ab (Cell Signaling Technology, USA) at 1:1000 dilution in blocking buffer overnight at 4°C. β-actin was used as an internal control protein for loading normalization of the quantification analysis. The membranes were washed with TBST solution and then incubated with secondary Peroxidase antibody (goat anti-rabbit IgG whole molecule) diluted at 1:2500 (Sigma Aldrich, USA) at room temperature according to the manufacturers' instructions. Immunoreactive bands were visualized using a Pierce ECL Western Blotting Substrate (Thermo Fischer Scientific, USA) for ABG2 and β-actin visualization as well as Clarity Max Western ECL Substrate (Bio-Rad, USA) for ABCB1 visualization following the manufacturer’s protocol. The signals were detected with ChemiDoc MP (Bio-Rad, USA) and expressed as the percentage of the controls.

Wound healing assay

The assay was performed according to the method described previously (Otręba et al. 2019) with a slight modification. In brief, some $1x10^6$ U87-MG were incubated with supplemented growth medium for 24h to approximately 80–90% confluence on a 35mm plate (Sarstedt, Germany). Then the wound area was generated by scratching cells with a sterile 200 μl pipette tip. The used medium was carefully aspirated with cell debris, and fresh medium, perphenazine, or prochlorperazine solutions were added. The wound area was photographed at 0, 3, 6, 9, 12, and 24 hours after scratching with the use an inverted microscope Nikon TS100F (Nikon Corporation, Japan) equipped with a Canon EOS 450D digital camera (Canon Inc, Japan). The scratch areas were measured at each time point using ImageJ 1.51j8 software (National Institute of Health, USA) with the MRI wound healing tool plugin (Montpellier RIO Imaging, France) (Kabała-Dzik et al. 2017). The wound closure was calculated using the following formula (Grada et al. 2017):

$$Woundclosure(\%) = \left[\frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}}\right] \times 100\%$$

$A_{t=0h}$ is the area of the wound measured at time t_0, immediately after the scratch

$A_{t=\Delta h}$ is the area of the wound measured h hours after the scratch

The rate of cell migration after 24h was calculated using the formula [11]:

$$Rate of Cell migration(nm/h) = \frac{\mathrm{Wi} - \mathrm{Wf}}{\mathrm{t}}$$

Wi is the initial wound width [nm]

Wf is the final wound width [nm]

t is the time duration [hours]

Transwell chemotaxis and invasion assay

The transwell migration and invasion assay was performed according to a slightly modified method described by Bernhart et al. (2013), Limame et al. (2012) and the Corning assay protocol for cell migration, chemotaxis, and invasion was used. In the migration assay, we used Sarsted TC-inserts with 8 µm pore diameter and 11 µm polyethylene terephthalate membrane thickness (Sarstedt, Germany) as well as 24 well culture plates (Sarstedt, Germany). In the case of the invasion assay, the insert membrane was covered by 45 µl of the Geltrex diluted at 1:1 v/v in medium with 1% FBS. Inserts with Geltrex were kept for 45 minutes at 37^{o}C before the use. Then 25,000 U87-MG cells were seeded into inserts for 48h in 100 µl of starvation medium (medium with 1% FBS) or a starvation medium containing bacitracin (2.5 mM), perphenazine (0.5 µM), or prochlorperazine (0.5 µM). Bacitracin was used as an inhibitor of U87-MG cell migration and invasion (Li et al. 2016). The lower compartment was filled with 600 µl of the normal growth medium (medium with 10% FBS), starvation medium, as well as normal growth or starvation medium containing perphenazine (0.5 µM), and prochlorperazine (0.5 µM). After 48h of incubation at 37°C, the medium was aspirated from the upper surface of the membrane, cells were washed in PBS and fixed in 2% buffered formalin for 20 minutes. After fixation the inserts were washed in PBS and incubated with methanol for 20 minutes. The cells were next washed in PBS and stained with 0.1% crystal violet for 10 minutes. Next, the inserts were washed in PBS until the water ran clear, and non-migrated cells from the upper part of the insert were removed with a cotton swab. Finally, the insert was put into a 24 well plate filled with 700 µl of 10% acetic acid for 30 minutes to wash out the crystal violet. Then 200 µl of each sample was transferred into a 96-well plate (Sarstedt, Germany) and absorbance was measured at λ = 590nm using the microplate reader UVM-340 (Biogenet, Poland).

As a part of the procedure, we made a standard curve using the inserts without Geltrex as a control to calculate total invasion according to the Corning assay protocol of cell migration, chemotaxis, and invasion. The standard curve was constructed according to the above description, but with the use of a different number of cells: 0, 500, 1000, 2500, 5000, 1000, 15000, 20000, 25000, 30000, 35000, 40000, 45000, and 50000 cells. Moreover, the standard curve was determined in two different manners using

invasion from the normal growth medium to the normal growth medium, and from the starvation medium to the starvation medium.

The results were shown as % of migrated and/or invaded cells after calculation using the standard curve equation. Relative cell migration and invasion were normalized by the subtraction of negative control (results of cells which migrated/invaded from the starvation medium to the starvation medium was called random migration) from results of cells which migrated/invaded from the starvation medium with 1% FBS to the normal growth medium with 10% FBS.

Western blot analysis of E-cadherin, α-tubulin, integrin α3, integrin α5, and integrin β1

The E-cadherin, α-tubulin, Integrin α3, Integrin α5, Integrin β1, and β-actin amounts were determined by Western blotting according to the method described in 2.2 with a slight modification. The positive control of E-cadherin, α-tubulin levels was bacitracin (1.25, 2.5, and 5.0 μM in growth medium) and it was compared to the control (growth medium). The obtained proteins were separated on 10% SDS-PAGE and visualized using a Pierce ECL Western Blotting Substrate (Thermo Fischer Scientific, USA). Proteins were detected by incubation with primary antibodies: E-Cadherin (4A2) Mouse mAb, α-tubulin Rabbit Ab, Integrin α5 Rabbit Ab, Integrin β1 (D2E5), β-actin Rabbit Ab (Cell Signaling Technology, USA), and Anti-Integrin α3 Rabbit Ab (St. John's Laboratory, USA) at 1:1000 dilution in blocking buffer overnight at 4°C. β-actin was used as an internal control protein for loading normalization of the quantification analysis. The used secondary Peroxidase antibodies were Anti-Rabbit IgG and Anti-Mouse IgG (Sigma Aldrich, USA). Proteins were expressed as the percentage of the controls.

Statistical analysis

In migration experiments, mean values of at least three separate experiments (n=3) performed in triplicate ± standard error of the mean (S.E.M) were calculated. In the Western blot analysis, mean values of at least three separate experiments (n=3) ± standard deviation (SD) were calculated. Statistical analysis was performed with one-way ANOVA with Dunnett's multiple comparison test and two-way ANOVA (the influence of cell line and time or drug concentration) followed by the Tukey post-hoc test using GraphPad Prism 8 software. The significance level was established at the value of $p<0.05$ (*) or $p<0.01$ (**).

Results

The effect of perphenazine and prochlorperazine on ABCB1 and ABCG2 content in glioblastoma (U87-MG)

ABCB1 and ABCG2 proteins analyses were performed with the Western blot after a 24h-treatment of glioblastoma cells under different concentrations of perphenazine, prochlorperazine, and elacridar (as a negative control) (Fig. 1A). The full-length immunoblots with the molecular mass marker are shown in Supplementary Figure S1, and the Western blot raw data are in Supplementary Files S1 and S2.

Elacridar significantly decreased the ABCB1 level by 45.7% in comparison to DMSO control (Fig. 1B). Perphenazine only in the concentration of 0.1 μM significantly reduced the ABCB1 amount by 30.9%, and increased ABCG2 amount by 29.4% and 50.7% in 0.1 μM and 1.0 μM concentrations, respectively (Fig. 1C). A similar situation was observed in the case of prochlorperazine: significant reduction of the ABCB1 amount by 30.9% only in the concentration of 0.1 μM, and a significant increase of ABCG2 amount in the concentration of 0.1 μM and 1.0 μM by 34.9% and 140.2% respectively, when compared with its control group (Fig. 1D).

Perphenazine and prochlorperazine impact on wound closure and rate of cell migration

The effect of perphenazine and prochlorperazine on wound closure and the rate of cell migration is presented in Figure 2A-C. The raw wound healing assay data represented in these figures are in Supplementary Files S3 and S4.

Figure 2A shows original photos of wound healing after a given period of time, i.e. 0, 3, 6, 9, 12, and 24 hours. In all the cases the calculations showed an increase in wound closure (Fig. 2B). After 6, 9, 12 and 24 hours of treatment with 1.0 uM prochlorperazine, the wound closure in human glioblastoma cell cultures increased from 24.6–62.7% in comparison to t_0. For the control and perphenazine (1.0 uM), after 3 to 24 hours of incubation, significant stimulation of wound closure and reduction of total wound area from 22.0–74.7% as well as from 27.0–69.3% were observed, respectively, in comparison to t_0 group (Fig. 2B). The significant difference between the effect of perphenazine and prochlorperazine on wound closure was observed only after 6h. The analysis of the rate of cell migration showed a significant difference between the control and prochlorperazine after 24h-incubation. The calculated rate of cell migration for the control, perphenazine, and prochlorperazine were 21613.24±969.53, 19489.18±1134.90, and 17045.01±1567.25 nm/h, respectively.

Perphenazine and prochlorperazine impact on migration and invasion determined with the transwell assay

The raw transwell migration and invasion assay data presented in Figures 3 and 4 are included in Supplementary Files S4, S5, and S6. As long as invasion from the growth medium with 1% FBS to the growth medium with 1% FBS, in 45000 cells and 50000 cells samples is concerned, we observed a high decrease in the cell amount, thus we finished constructing the standard curve at 40000 cells when it was still linear. The standard curves are presented in Fig. 3.

The transwell invasion assay showed a significant decrease in invasion by 22.91, 11.31, and 12.19% for bacitracin (2.5mM), perphenazine (0.5 μM), and prochlorperazine (0.5 μM) in comparison to the control, respectively (Fig. 4A). The analysis of internal control showed that 2.71% of cells invaded randomly. Moreover, perphenazine and prochlorperazine significantly increased the percentage of invaded cells by 5.65 and, 6.85% respectively, in comparison to the control (Fig. 4C).

For the transwell migration assay, only perphenazine (0.5 μM), and prochlorperazine (0.5 μM) significantly decreased the percentage of migrated cells by 13.49 and 8.15%, respectively, in comparison to the control (Fig. 4B). The observed decrease of U87-MG cell migration caused by bacitracin was not significant. The level of random migration was 1.97%. Moreover, perphenazine and prochlorperazine significantly increased the percentage of invasion by 6.80 and 10.30% respectively, in comparison to the control (Fig. 4C).

The effect of perphenazine and prochlorperazine on E-cadherin, α-tubulin, integrin α3, integrin α5, and integrin β1 content in glioblastoma (U87-MG)

E-cadherin, α-tubulin, and integrins (α3, α5, and β1) levels analyzed with Western blot after a 24h-treatment of glioblastoma cells with different concentrations of perphenazine, prochlorperazine, and bacitracin (as the negative control) are presented in Fig. 5A. The full-length immunoblots with the molecular mass marker are shown in Supplementary Figure S2. The Western blot raw data represented in this figure are included in Supplementary File S1.

The Western blot analysis of E-cadherin showed a significant increase of the protein amount by 45.3% and 32.8% after treating U87-MG cells with perphenazine in the concentration of 0.25 and 0.5 μM, respectively (Fig. 5B). Prochlorperazine in the concentration of 0.25 μM also significantly increased the level of E-cadherin by 31.8%, while incubation of the cells with prochlorperazine in the concentration of 1.0 μM caused a decrease of E-cadherin amount by 23.9% (Fig. 5C). Bacitracin, which was used as an inhibitor of cellular migration, significantly increased the E-cadherin amount by 31.7% only in the concentration of 1.25 mM (Fig. 5D).

The analysis of α-tubulin showed a significant decrease of 54.3% and 65.7% in U87-MG cells with perphenazine in the concentration of 0.5 and 1.0 μM, respectively (Fig. 5B). In the case of prochlorperazine, the significant increase of α-tubulin by 27.5% was observed with perphenazine in the concentration of 0.25 μM, while a significant decrease by 60.2% was observed in the concentration of 1.0 μM (Fig. 5C). Bacitracin, which was used as an inhibitor of cellular migration, significantly increased the α-tubulin amount by 48.7% only in the concentration of 1.25 mM (Fig. 5D).

The analysis of integrins (α3, α5, and β1) showed a significant decrease of α3 integrin by 34.2% and 27.1% after incubation of U87-MG cells with perphenazine in the concentration of 0.25 and 1.0 μM, respectively. Moreover, a significant decrease of β1 integrin by 47.8% was also observed after incubation of U87-MG cells with perphenazine in the concentration of 1.0 μM. The analysis of α5 integrin showed that perphenazine did not significantly decrease the level of α5 integrin (Fig. 5B). In the case of prochlorperazine, only its concertation of 1.0 μM significantly decreased α3 and β1 integrins by 53.1 and 38.1%, respectively. The analysis of α5 integrin showed that prochlorperazine also did not significantly decrease the level of α5 integrin (Fig. 5C). Moreover, bacitracin also did not significantly influence the level of all analyzed integrins (Fig. 5D).

Discussion

In glioblastoma therapy, many factors should be taken into consideration. These factors include therapy goals (regulation of invasion as well as dopamine receptors, VEGF, EGFR, and PDGFR suppression), the ability of drugs to penetrate the brain-blood-barrier, or intracerebral activity since some chemotherapeutic drugs are unable to cross the BBB. The small populations of glioblastoma cells can survive the therapy despite surgery, radiation therapy, or chemotherapy because of their ability to invade the surrounding brain tissue at any stage of tumor progression (Zhao et al. 2019). Thus, the current study focused on the impact of phenothiazine derivatives (perphenazine and prochlorperazine) on migration, invasion, and the ABC transporters levels in human glioblastoma U87-MG cells.

Previously, it was shown that perphenazine and prochlorperazine in the concentration of 0.5 and 1.0 μM reduced U87-MG cells viability by 32 and 54.5 as well as 30.5 and 56.3%, respectively after 24h-incubation (Otręba and Buszman 2018). In the present study, we observed a decrease in ABCB1 amount after 24h-incubation with perphenazine and prochlorperazine in the concentration of 1.0 μM. It is worth noting that ABCB1 also regulates cell proliferation and the knockdown of ABCB1 suppresses cell proliferation (Muriithi et al. 2020). Therefore, a similar cytotoxicity effect of perphenazine and prochlorperazine (1.0 μM) observed in the previous study by Otręba and Buszman (2018) can be explained now by the decrease in ABCB1 amount. Interestingly, in the case of perphenazine and prochlorperazine in the concentration of 0.1 μM, the observed significant decrease of ABCB1 amount was not caused by cell death or proliferation disturbances, since previous results of the WST-1 assay (Otręba and Buszman 2018) showed that perphenazine in the concentration of 0.1 μM did not affect U87-MG cells viability.

The main role of ABCB1 (P-gp) and ABCG2 (BCPR) transporters, localized in the brain endothelial capillaries (Balça-Silva et al. 2018), is related to multidrug resistance (Liu et al. 2016). The ABCG2 transporter protects tissues against deadly xenobiotic exposures by the contribution to the absorption, distribution, and elimination of the drugs and endogenous compounds (Gupta et al. 2020). Thus, high expression of ABCB1 and ABCG2 has been reported to be related with poor prognosis in certain glioblastomas (Liu et al. 2016). Lin et al. (2014) and Wijaya et al. (2017) noticed that the resistance to temozolomide (TMZ) treatment of glioblastoma could be related to the excretion of the drug from the brain by ABCB1 and ABCG2 transporters. Additionally, in 2017, Pan et al. (2017) measured ABCG2 protein level and gene expression in four different human malignant glioma cell lines (A172, U87, SHG-44, and U251). The Western blot analysis showed that U87 cells had the lowest ABCG2 amount among all the cell lines, whereas no significant differences were found in the mRNA expression levels of MRP1 and MDR1 in the four GBM cell lines. Interestingly, phenothiazine derivatives such as chlorpromazine (Abdallah et al. 2015, Wang et al. 2008, Wesołowska 2011), prochlorperazine (Wesołowska 2011), thioridazine (Spengler et al. 2016), and fluphenazine (Abdallah et al. 2015) impair drug efflux mediated by P-gp or BCPR. Inhibition properties of those drugs in the mentioned mechanism has their own significance due to the possibility of using phenothiazine derivatives in glioblastoma treatment.

We also evaluated the effect of perphenazine and prochlorperazine on wound closure, invasion and migration of human glioblastoma cell line, determined with the transwell assay, since migrating cells at the marginal zones of GMB tumors are less sensitive to apoptosis, leading in consequence to the frequent recurrences (Sarafian et al. 2009). The wound-healing assay showed a time-dependent increase in wound area closure. The significant differences were observed between the time t_0 and 3 hours only for the control and perphenazine ($p<0.01$), while statistically significant differences were recorded between t_0 and 6, 9, 12 and 24 hours for the control, perphenazine, and prochlorperazine ($p<0.01$). Moreover, stronger stimulation of U-87 MG migration after perphenazine (1.0 μM) treatment was observed after 6 hour-treatment in comparison to the use of prochlorperazine. The analysis of the rate of cell migration after 24h-incubation showed that the U-87 MG cells in perphenazine or prochlorperazine tended to migrate more slowly in comparison to the control. Interestingly, only in the case of prochlorperazine, the difference is statistically significant ($p<0.01$) and suggests that the cells migrate 1.3 times more slowly in prochlorperazine (1.0 μM) in comparison to the control.

Since regulation of invasion is an important target in glioblastoma therapy, by employing the transwell assay we analyzed migration and invasion of U-87 MG cells using the drug concentration which caused only an approximately 30% decrease in cell viability. The study showed that both of the analyzed drugs could decrease migration and invasion of the cells. It is worth observing that the analysis of internal control showed that perphenazine (0.5 μM) and prochlorperazine (0.5 μM) may be a chemoattractant for cellular invasion and migration but 5-6 times weaker and 3-4 times weaker than the growth medium with 10% FBS, respectively. This may also explain the fact that lower percentage of the cells invaded and/or migrated from the growth medium with 1% FBS and perphenazine or prochlorperazine to the growth medium with 10% FBS in comparison to the number of cells which migrated/invaded from the growth medium with 1% FBS to the growth medium with 10% FBS.

Our findings may be confirmed and explained by the results of other groups. In 2014, Kast et al. suggested that the migration of subventricular zone (SVZ) cells to glioblastoma as well as glioblastoma to SVZ was regulated by the D3 dopamine receptor (Kast et al. 2014), while in 2020 the same authors showed that perphenazine reduced migration of malignant or non-malignant SVZ cells to glioblastoma (Kast et al. 2020). Thus, the ability of perphenazine and prochlorperazine to decrease migration and invasion of U87-MG cells may be related to dopamine receptors activity. The dopamine receptor subtype 2 (DRD2) antagonists in combination with Epidermal Growth Factor Receptor (EGFR) inhibitors exhibit anti-glioblastoma activity since they impair cellular growth and survival by MEK/ERK signaling cascade. The mechanism is described in detail by Bartek and Hodny (2014). Caragher et al. (2019) showed that glioblastoma cells such as U251 human glioblastoma, patient-derived xenograft (PDX), and glioma specimens (GBM43, GBM12, GBM6, GBM5, and GBM39) could activate DRD2 due to dopamine generation. Agrawal et al. (2021) found that dopamine induced the formation of microglia extracellular traps in glioblastoma multiforme formed by monocytes, macrophages, eosinophils, basophils, and mast cells. Thus, the traps play a significant role in sterile neuroinflammation. Primary glioblastoma spheroids limit gliomas invasion (Aaberg-Jessen et al. 2013). According to Caragher et al. (2019), anti-glioma

chemotherapy may increase DRD2 protein expression, leading to 4 times higher increase in sphere formation capacity.

Weissenrieder et al. (2020) found that 7 days of incubation of U87-MG cells with thioridazine (0.1 μM) reduced spheroid formation and significantly reduced Sox2 expression. The authors also noticed clear spheroid formation effects at selective concentrations of D2R modulators (Weissenrieder et al. 2020). Furthermore, Bhat et al. (2020) used trifluoperazine (phenothiazine derivative) in an *in vivo* study to prevent the conversion of glioma cells into glioma-initiating cells, which led to prolongation of mouse survival. The authors noticed the loss of radiation-induced Nanog mRNA expression, GSK3 activation, and reduction in p-Akt, Sox2, and β-catenin levels. The therapy including trifluoperazine and a single dose of radiation reduced the number of glioma-initiating cells *in vivo*, which suggests that this kind of a therapy increases the efficacy of radiotherapy in glioblastoma treatment (Bhat et al. 2020).

Therefore, it is possible that the first generation of antipsychotics (perphenazine and prochlorperazine), which penetrate the blood-brain barrier (Bartek and Hondy 2014), as dopamine D2 receptors (D2R) antagonists (Kast 2020) block D2R protecting against DRD2 protein expression leading to the increase in glioblastoma invasion. Our findings confirmed that perphenazine and prochlorperazine reduced cellular invasion, and this hypothesis was confirmed by Liu et al. (2019), Arrillaga-Romany et al. (2020), and He et al. (2021).

Liu et al. (2019) analyzed the combined effect of temozolomide and dopamine receptor inhibitors (haloperidol or risperidone) in glioblastoma therapy. The authors observed that inhibition of glioblastoma proliferation was more effective in comparison to monotherapy. It is possible since dopamine inhibitors can inhibit the extracellular signal-related kinase signaling pathway and block temozolomide-induced protective autophagy. Moreover, the authors noticed the increase of the levels of DNA damage marker (γH2AX) and expression of DRD2 transcripts in U251 glioma and glioblastoma stem cells (Liu et al. 2019). Interestingly, in 2020 Abbruzzese et al. designed a Phase II clinical trial involving the combination of chlorpromazine and temozolomide in glioblastoma treatment. The authors mentioned that chlorpromazine impacted glioblastoma multiforme growth and survival by the induction of cancer cell death, nuclear aberrations, autophagy as well as the inhibition of AKT/mTOR axis, glutamate receptors (AMPA, NMDA), and D2 dopamine receptors (Abbruzzese et al. 2020). Arrillaga-Romany et al. (2020) used a small-molecule DRD2 antagonist (ONC201) that penetrated the blood-brain barrier in the treatment of adult recurrent glioblastoma patients. ONC201 is well tolerated and induces biomarkers of pharmacodynamic signaling/apoptosis, which suggests that the DRD2 antagonist may be biologically active in a subset of glioblastoma patients (Arrillaga-Romany et al. 2020). A recent study (He et al. 2021) using patient-derived xenograft (PDX) glioblastoma models and 25 glioblastoma cell lines showed that EGFR and DRD2 expression anti-correlates in glioblastoma. Thus, low EGFR expression glioblastoma is most sensitive to DRD2 inhibition. Moreover, high EGFR expression is correlated with poor DRD2 expression in glioblastoma (He et al. 2021).

The observed strong effect of perphenazine and prochlorperazine on viability, migration, and invasion of human glioblastoma may be also related to ABCB1 and/or ABCG2 amount and BMP family protein BMP4. The Pim-1 protein in ABCB1 influence tumor cell growth by promoting cell cycle progression, cell migration, and protein translation as well as by the suppression of apoptosis (Brasó-Maristany et al. 2016). The overexpression of the serine/threonine protein kinase Pim-1 is often observed in different human malignancy tumors including glioblastoma multiforme (Herzog et al. 2015). Thus, the observed significant decrease in the ABCB1 level may explain a decrease in U87-MG migration. In the case of ABCG2, Liang et al. (2015) showed that in lung cancer ABCG2, localized also in the nucleus of glioblastoma multiforme (Bhatia et al. 2012), was involved in a transcription regulation of the E-cadherin–encoding gene (CDH1), which is a key cell-cell adhesion gene. The authors observed that the ABCG2 overexpression enhanced E-cadherin expression as well as increased nuclear ABCG2 expression (Liang et al. 2015). E-cadherin prevents the loss of cell-cell adhesion and cell junctions, which promotes cellular invasion and migration (Na et al. 2020). The relative expression of E-cadherin with the use of Western blot was shown in U87-MG cells by Zhang et al. (2015). Another possible mechanism leading to the increase of E-cadherin and suppression of glioblastoma cells was found by Zhao et al. (2019). The authors observed that BMP4 protein increased E-cadherin and claudin expression in human U251 and U87 cells through activation of SMAD signaling, which finally leads to the suppression of tumor cell invasion (Zhao et al. 2019). Therefore, a significant increase in ABCG2 level after perphenazine and prochlorperazine (0.1 and 1.0 μM) treatment of U87-MG cells, observed in our study, may explain the recorded increase in E-cadherin after perphenazine (0.25 and 0.5 μM) and prochlorperazine treatment (0.25 μM), which can lead finally to decrease in the migration and invasion of the analyzed glioblastoma cells. Our results confirm also that E-cadherin is a negative regulator of U87-MG migration since the decrease in E-cadherin level is accompanied by a decline in the cellular invasion. Although we cannot conclude that E-cadherin expression regulates invasion of other glioma cells based on these studies alone, our results do lend further support for this view.

Microtubules as dynamic tubular polymers of α- and β-tubulin provide structural integrity, promote migration, transport of molecules, vesicles, and organelles and play important role in cell division. This makes microtubule polymerization inhibitors as well as stabilizing and/or destabilizing agents a good target for the anticancer therapy (Calinescu et al. 2016). Zhou et al. (2020) found that sulforaphane-cysteine disrupted microtubules by ERK1/2 phosphorylation-mediated downregulation of α-tubulin and Stathmin-1 leading to the inhibition of U87-MG and U373-MG cells migration and invasion. The authors noticed also lower expressions of α-tubulin-mediated mitophagy-associated proteins (Zhou et al. 2020). This confirms our results since we observed a decrease in the alpha-tubulin level after perphenazine (1.0 μM) and prochlorperazine (0.5 and 1.0 μM) treatment of U87-MG cells accompanied by a decline in the U87-MG migration and invasion.

Nakada et al. (2013) found that the overexpression of α3 integrin in glioblastoma cells (U87-MG, SNB19, and U251) increased cellular migration and/or invasion via the extracellular signal-regulated kinase (ERK) 1/2 pathway, while the decrease of α3 integrin inhibits glioma invasion. The authors also observed that the invasion of U87-MG cells was stronger in α3 integrin overexpressing cells, which suggests that α3

integrin may be an invasion promotor (Nakada et al. 2013). This is in line with our results since we observed a decrease in α3 integrin level as well as inhibition of migration and invasion of U87-MG cells after 24h incubation with perphenazine (0.25 and 1.0 μM) and prochlorperazine (1.0 μM).

The α5β1 integrin is called the critical regulator of cell migration and invasion of many tumors including glioblastoma since it affects cytoskeleton rearrangement, cell adhesion, and the production of matrix metalloproteinase (MMP). The expression of α5β1 integrin is significantly higher in glioblastoma tissue than in normal brain tissue. The activation of the integrin stimulates migration, invasion, angiogenesis, and drug resistance of glioma cells. The stimulation of cellular invasion and metastasis is possible by the activation of the c-Met/FAK/ Src-dependent signaling pathway or regulation of the expression and activity of MMPs (Hou et al. 2020). Mallawaartchy et al. (2015) showed a high level of α5 integrin in U87-MG cells. The authors also identified 49 proteins connected with cell invasion. Moreover, the gene expression data of α5 integrin showed “prognostic significance in independent glioblastoma cohorts” (Mallawaartchy et al. 2015). Renner et al. (2016) found that α5β1 integrin also precipitated the aggressiveness of solid tumors. Thus, the high expression of the protein may decrease patient survival, which makes it an important factor in the therapy (Renner et al. 2016). Those observations are in line with our results. We observed a decrease in migration and invasion of U87-MG cells after treatment with perphenazine or prochlorperazine in the concentration of 0.5 μM and a non-significant decrease in the level of α5 and β1 integrins at the same concentration. This confirms that α3 integrin is more important than α5 and β1 integrins for the migration and invasion of U87-MG cells. In the case of perphenazine and prochlorperazine (1.0 μM), only the decrease in α5 integrin is non-significant, which suggests that the level of α3 and β1 integrins are important in the regulation of U87-MG migration and invasion.

In the future, we are planning to use PCR assay to confirm variations of the proteins as well as to use more human glioblastoma cell lines to get more generalized conclusions about the possibility of using phenothiazine derivatives in glioblastoma treatment. Since phenothiazine derivatives decrease viability, migration, and invasion of U87-MG glioblastoma next studies determining the type of cell death should be performed in near future.

Conclusion

In conclusion, we have found that perphenazine and prochlorperazine modulate multidrug resistance proteins (decrease in ABCB1 and cause an increase in ABCG2) amount, increase in E-cadherin level as well as a decrease in α-tubulin, and integrins (α3, α5, and β1) levels as well as inhibit migration and invasion of U87-MG cells. Our study has confirmed that the decrease of cellular migration and/or invasion is regulated by ABCB1 and ABCG2 proteins, as well as the decrease of cellular E-cadherin, α-tubulin, and integrins (α3, α5, and β1) levels. The level of the analyzed proteins corresponds to the decrease in cellular migration and/or invasion. Here presented data and previous results show that perphenazine and prochlorperazine exhibit the anticancer effects against U87-MG cells. These findings provide additional insights into a potential use of phenothiazine derivatives in the treatment of glioblastoma.

Ethics approval

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

Declarations

Ethics approval

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

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Author contributions

MO, JS, AK-D, and ARz-S conceived and designed research. MO, JS, and ARz-S contributed reagents and/or analytical tools. MO conducted experiments, analyzed data, and wrote the manuscript. MO, JS, AK-D, and ARz-S edited the manuscript. MO, JS, AK-D, and ARz-S have read and approved the manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Conflicts of interest/competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files). The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

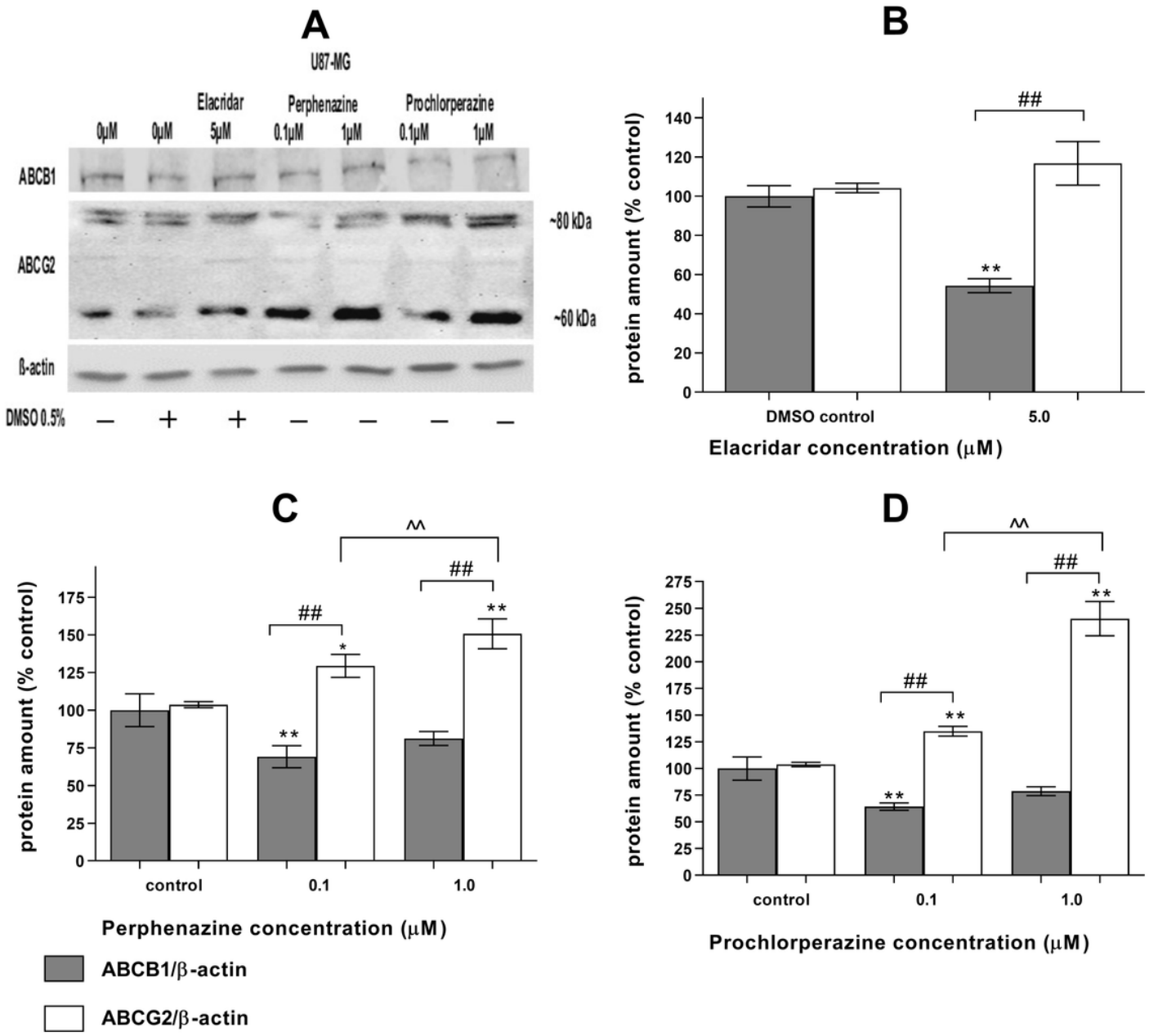

Figure 1

The Western blot analysis and a correlated graph of the relative amount of selected proteins including loading controls in U87-MG cells. (A) Blots of ABCB1, ABCG2, and β-actin as well as ABCB1 and ABCG2 relative amounts after (B) elacridar treatment, (C) 24h perphenazine treatment, (D) 24h prochlorperazine treatment, expressed as % of the control. Mean values ± SD from three independent experiments (n=3) are presented * p< 0.05, ** p < 0.01 vs the control samples or ABCB1 vs ABCG2 samples. The full-length immunoblots with the molecular mass marker are shown in Supplementary Figure S1. The raw Western blot data from this figure is included in Supplementary File S1.

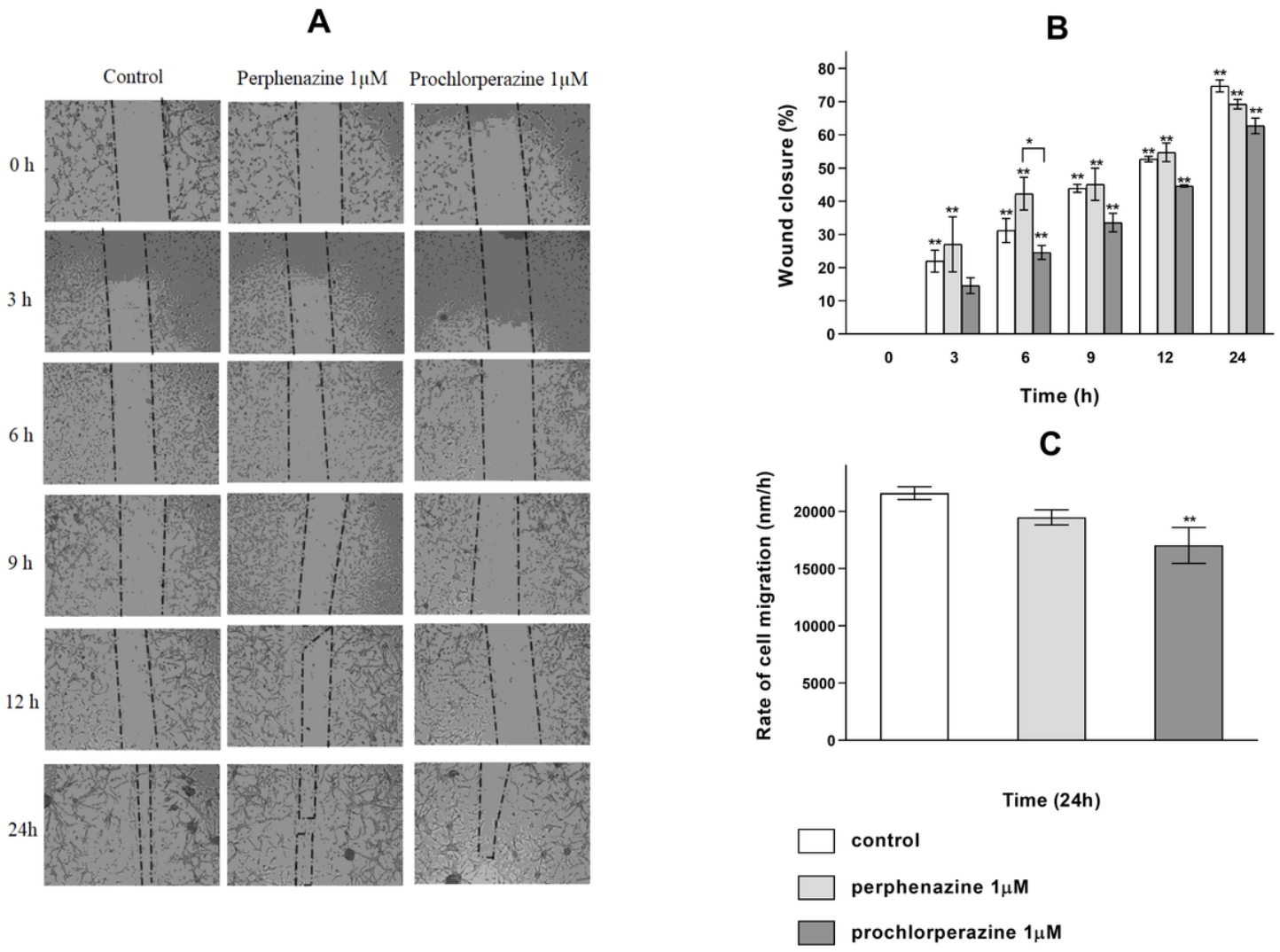

Figure 2

The impact of perphenazine and prochlorperazine on glioblastoma cell migration. In vitro, wound-healing assay U87-MG cells (A) were incubated for 24 h with perphenazine or prochlorperazine in the concentration of 1 μM. Cells were photographed after 0, 3, 6, 9, 12, and 24 hours after scratching by Nikon TF100 inverted microscope x4 magnification. Representative cell images from each group at the indicated time points are shown. Wound closure of U87-MG cells (B) is expressed as a percentage of time t0. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented * $p<0.05$ ** $p < 0.01$ vs the t0 group or between perphenazine and prochlorperazine at the same time point. The rate of cell migration of glioblastoma cells after 24h incubation (C) is expressed as nm per hour. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented ** $p < 0.01$ vs the prochlorperazine and the control.

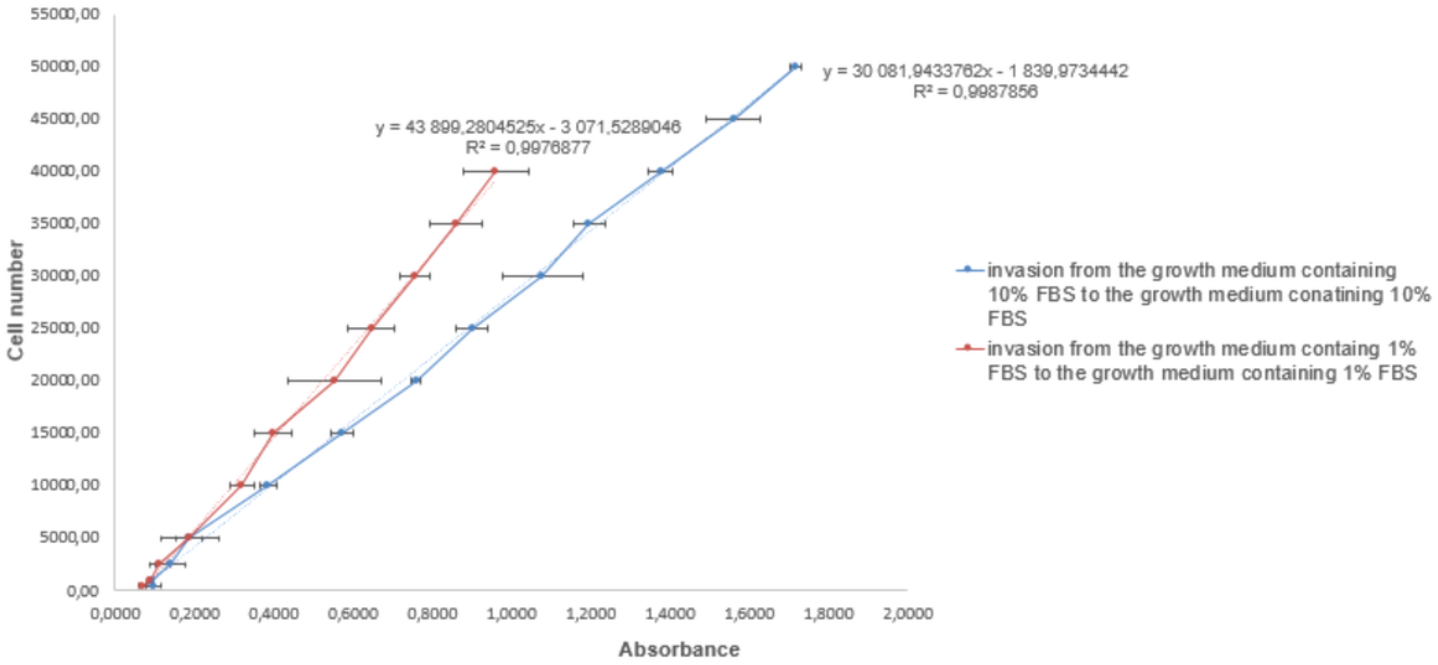

Figure 3

The standard curve invasion from the growth medium containing 1% FBS to the growth medium containing 10% FBS as well as the growth medium containing 1% FBS to the growth medium containing 1% FBS. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented.

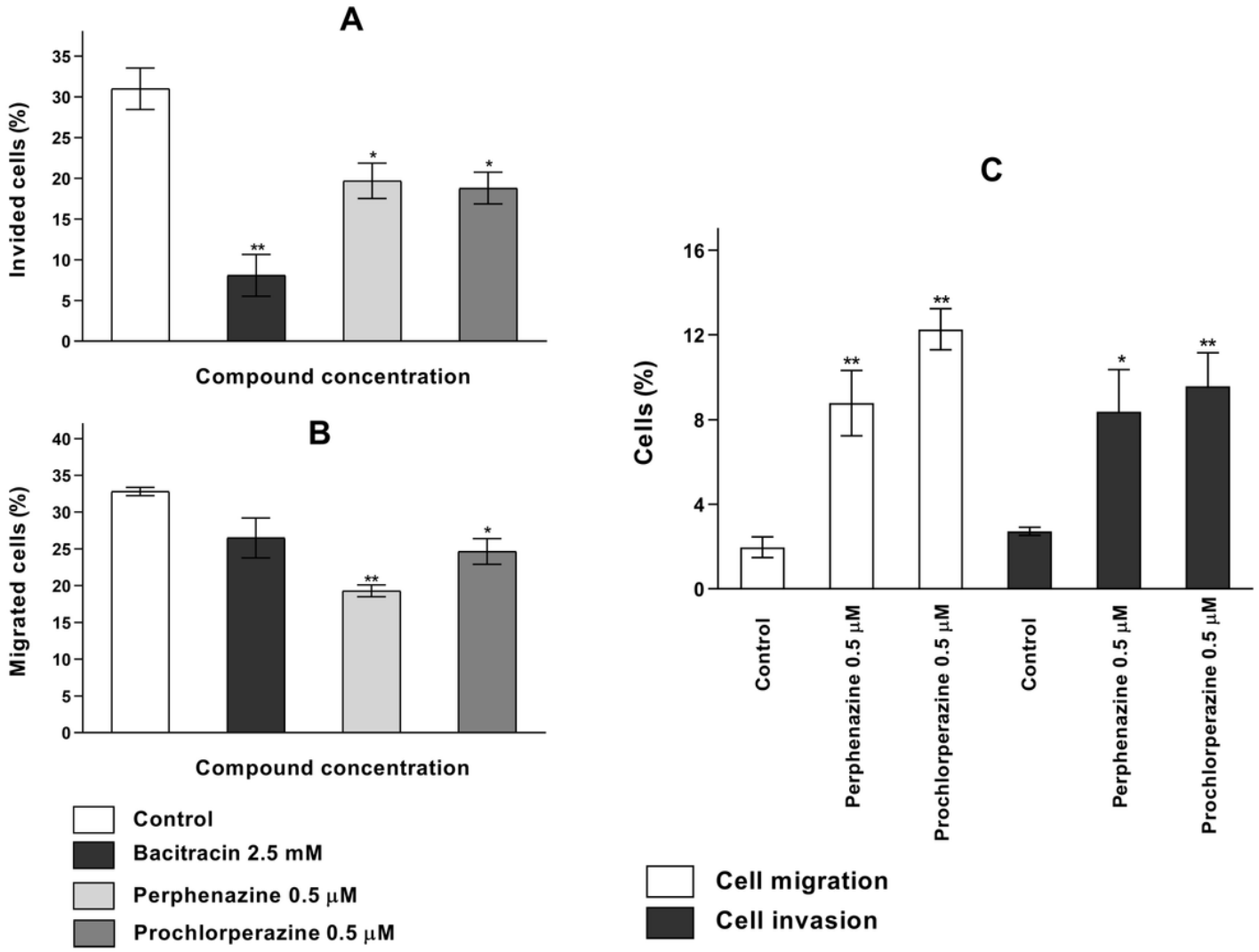

Figure 4

The impact of perphenazine and prochlorperazine on glioblastoma migration and invasion determined with the transwell assay. Transwell invasion assay of U87-MG cells (A) is expressed as a percentage of invaded cells. Transwell migration assay of U87-MG cells (B) is expressed as a percentage of migrated cells. Internal control of migrated and invaded cells (C) is expressed as a percentage of cells. Mean values ± SEM from three independent experiments (n=3) performed in triplicate are presented * $p<0.05$ ** $p < 0.01$ vs the control.

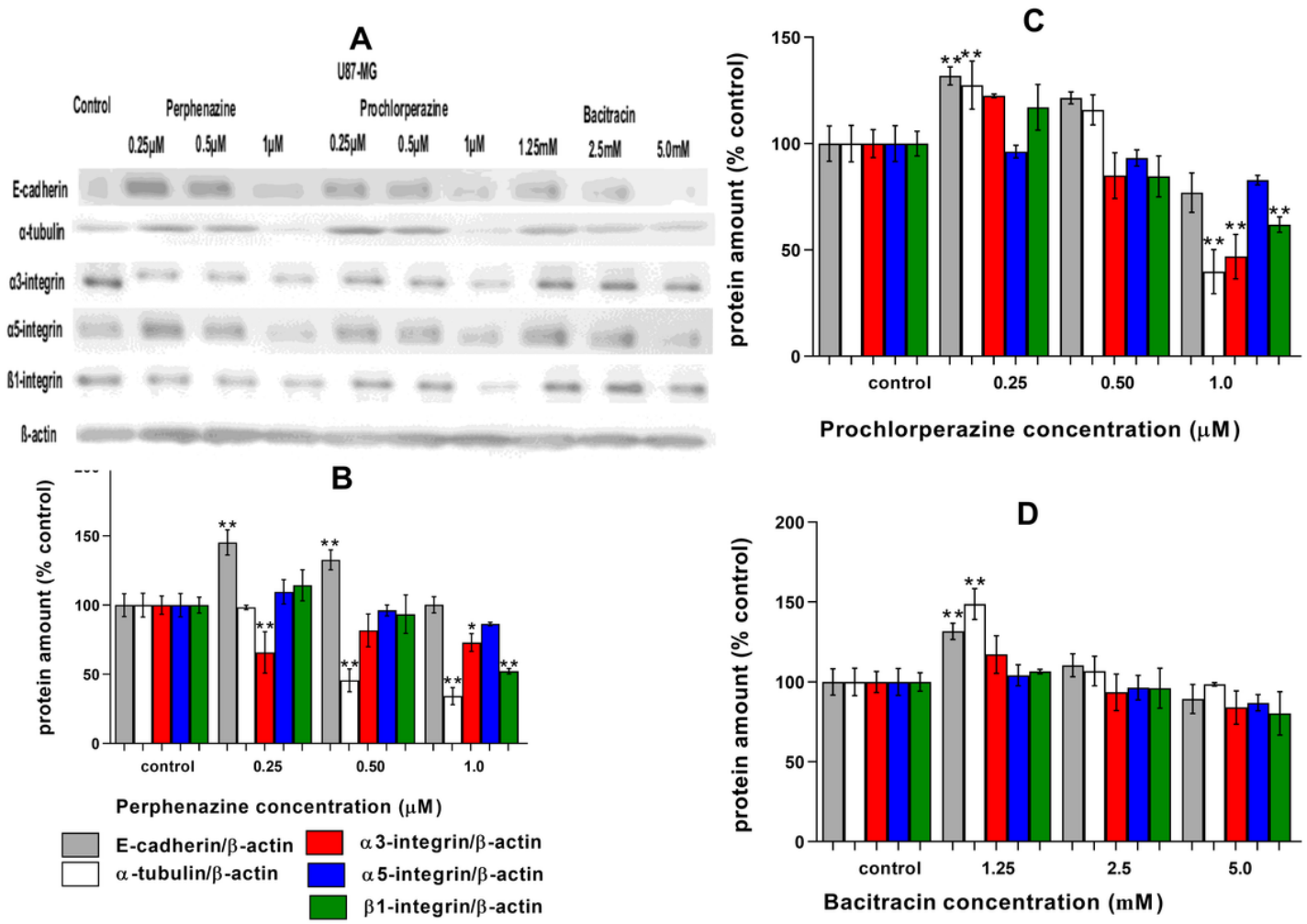

Figure 5

The Western blot analysis and a correlated graph of the relative amount of selected proteins including loading controls in U87-MG cells. (A) Blots of E-cadherin, α-tubulin, integrins (α3, α5, and β1), and β-actin as well as E-cadherin, α-tubulin, integrins (α3, α5, and β1) relative amounts after (B) 24h perphenazine treatment, (C) 24h prochlorperazine treatment, (D) 24h bacitracin treatment, are expressed as % of the control. Mean values ± SD from three independent experiments (n=3) are presented * $p < 0.05$, ** $p < 0.01$ vs the control samples. The full-length immunoblots with the molecular mass marker are shown in Supplementary Figure S2. The raw Western blot raw from this figure is included in Supplementary File S1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigureS1.docx
- SupplementaryFigureS2.docx
- SupplementaryFileS1Westernblotrawdata.xlsx

- SupplementaryFileS2statisticandgraphsforWBresults.pzf
- SupplementaryFileS3woundhealingassay.xlsx
- SupplementaryFileS4woundhealingtranswellassay.pzf
- SupplementaryFileS5callibrationcurveforinvasionassay.xlsx
- SupplementaryFileS6transwellmigrationandinvasion.xlsx